Pigment analysis of a chlorophyll *f*-containing cyanobacterium strain KC1 isolated from Lake Biwa

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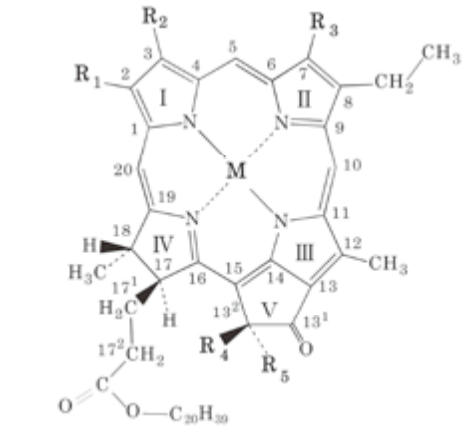
A unicellular cyanobacterium containing Chl *f* was isolated from Lake Biwa. A small amount of Chl *f* was detected only in the organism cultivated under near-infrared LED light. Chl *f′* and Phe *f* were not detected in this cyanobacterium, whereas Chl *a′* and Phe *a* were found at amounts to act as the primary electron donor of PS I and the primary electron acceptor in PS II, respectively. The results indicate that Chl *f* may function not as an electron transfer component but as antenna constituent.

Introduction

Chlorophylls are essential in plant photosynthesis. Chlorophyll *a* (Chl *a*, Fig. 1) and bacteriochlorophyll *a* (BChl *a*) are major chlorophylls in oxygenic and anoxygenic photosynthesis, respectively. The photosynthetic reaction centers (RCs) are classified into two types according to their electron acceptor chains. "Type I RCs" possess iron-sulfur centers, while "Type II RCs" two quinones in series, Q_A and Q_B (Fig. 2). Primary charge separation in the RCs is driven by a few structurally unique Chl and BChl derivatives, such as "prime-type" in the Type I RCs and "metal-free" chlorophylls in the Type II RCs, respectively.



	M	R_1	R_3	R_4	R_5	R_6
Chl *a*	Mg	CH_3	$CH=CH_2$	CH_3	H	$COOCH_3$
Chl *a′*	Mg	CH_3	$CH=CH_2$	CH_3	$COOCH_3$	H
Phe *a*	2H	CH_3	$CH=CH_2$	CH_3	H	$COOCH_3$
Chl *b*	Mg	CH_3	$CH=CH_2$	CHO	H	$COOCH_3$
Chl *d*	Mg	CH_3	CHO	CH_3	H	$COOCH_3$
Chl *d′*	Mg	CH_3	CHO	CH_3	$COOCH_3$	H
Chl *f*	Mg	CHO	$CH=CH_2$	CH_3	H	$COOCH_3$
Chl *f′*	Mg	CHO	$CH=CH_2$	CH_3	$COOCH_3$	H
Phe *f*	2H	CHO	$CH=CH_2$	CH_3	H	$COOCH_3$

Fig. 1 Molecular structure and carbon numbering of chlorophylls, according to the IUPAC numbering system.

In 1974, pheophytin *a* (Phe *a*, Fig. 1), a demetallated Chl *a*, was first postulated to be the primary electron acceptor in PS II (Fig. 2)(van Gorkom 1974), and the idea was experimentally confirmed in 1977 (Klimov *et al*. 1977a). In 1975, bacteriopheophytin (BPhe) *a* was found to function as the primary electron acceptor in the RC of purple bacteria (Fig. 2)(Parson *et al*. 1975; Rockley *et al*. 1975; Fajer *et al*. 1975; Kaufmann *et al*. 1975), and slightly later BPhe *b* was also found to perform the same function (Fig. 2)(Klimov *et al*. 1977b). In 1986, BPhe *a* was also found to function in green filamentous bacteria (Fig. 2) (Kirmaier *et al*. 1986; Shuvalov *et al*. 1986).

The 13^2-epimer of Chl *a*, Chl *a′* ("*a*-prime") (Fig. 1) was first reported in 1942 (Strain and Manning 1942), and it has been demonstrated to constitute P700 in 1988 (Fig. 2)(Kobayashi *et al*. 1988; Jordan *et al*. 2001). As shown in Fig. 2, it has been confirmed that P798 consists of BChl *g′* in the RC of heliobacteria in 1991 (Kobayashi *et al*. 1991), and that P840 consists of BChl *a′* in green sulfur bacteria in 1992(Kobayashi *et al*. 1992, 2000).

In 1943, Chl *d* (Fig. 1) was first reported as a minor pigment in several red macroalgae (Manning and Strain 1943). In 1996, a novel cyanobacterium, *Acaryochloris marina*, was isolated from colonial ascidians containing Chl *d* as the dominant chlorophyll (Miyashita *et al.* 1996; Ohashi *et al.* 2008). P740 in *A. marina* was found to be composed of Chl *d′* (Fig. 1) in 2001 (Akiyama *et al.* 2001). Note that the primary electron acceptors in *A. marina* are Chl *a* in PS I and Phe *a* in PS II, respectively (Fig. 2)(Akiyama *et al.* 2001, 2002, 2004).

It is of interest to turn one's attention to the primary electron acceptors in the Type I RCs. As illustrated in Fig. 2, they are all Chl *a*-derivatives even in the bacterial RCs; 8^1-OH-Chl *a* esterified

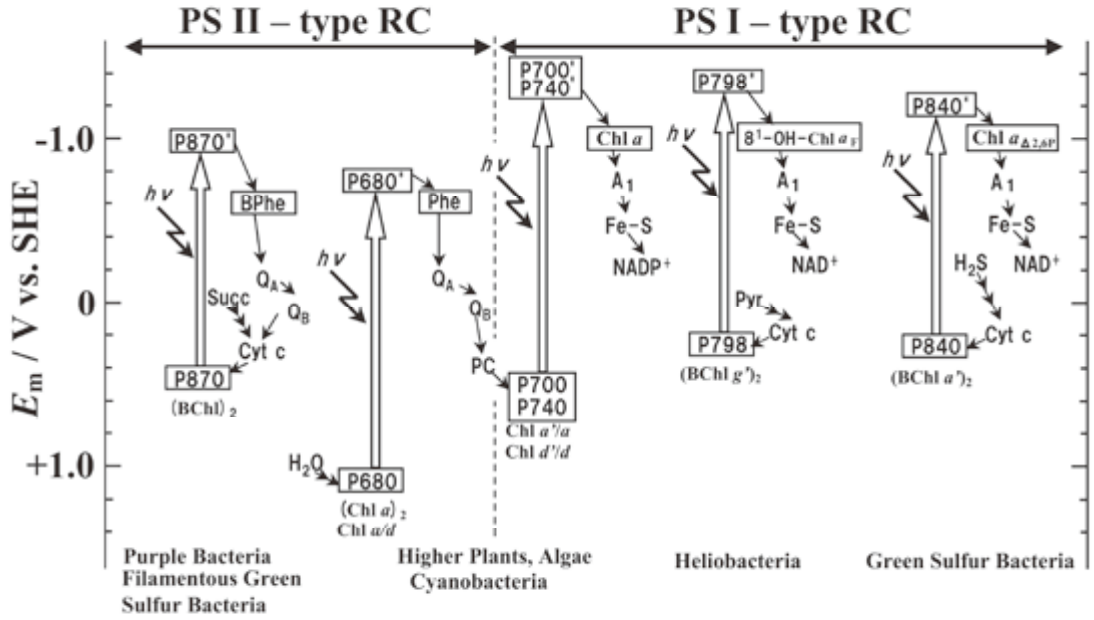


Fig. 2 Schematic comparison of photosynthetic electron transport in PS I-type RC and PS II-type RC. Components are placed according to their estimated or approximate midpoint potentials. The arrows indicate the direction of electron flow. For simplification, some primary electron donors, P970, P850 and P865 are omitted here: P970 and P850 are the primary electron donors of BChl *b* and Zn-BChl *a* containing purple bacteria, respectively; P865 is the primary electron donor of green filamentous bacteria.

with farnesol in heliobacteria (van de Meent *et al.* 1991) and Chl *a* esterified with Δ2,6-phytadienol in green sulfur bacteria (Kobayashi *et al.* 2000). In 2010, a red-shifted chlorophyll was discovered in a methanolic extract of Shark Bay stromatolites, and was named Chl *f* (Fig. 1)(Chen *et al.* 2010). In 2011, Chl *f* was also discovered in a unicellular cyanobacterium, strain KC1, isolated from Lake Biwa, and Chl *f* was detected only when cultivated under near-infrared(NIR) LED light (Ohkubo *et al.* 2011), and the amount of Chl *f* was very small. The function of Chl *f* has not been clarified to date.

In this paper, we report the results of pigment composition analysis of the Chl *f*-containing cyanobacterium strain KC1 by silica normal-phase HPLC. Neither Chl *f′* nor Phe *f* was detected, but Chl *a′* and Phe *a* were detected as minor pigments, like other cyanobacteria. The results indicate that Chl *f* may function not as an electron transfer component but as antenna constituent.

Materials and methods

Algal culture

Cells of the cyanobacterium strain KC1 were grown in BG-11 medium in a glass cell culture flask (1 L) at 297 K with continuous air-bubbling. Cells were incubated under continuous white fluorescent light (50 μmol photons/m^2/s) or near-infrared LED light (740 nm peak, EYELA, Tokyo). Cells at the early stationary phase were harvested by centrifugation. The cell pellet was stored in a refrigerator at 193 K until use.

Pigment preparation

Chl *f* was extracted with acetone/methanol (7/3, *v*/*v*) mixture at 277 K from the Chl *f*-containing cyanobacterium strain KC1. The extract was applied to a preparative-scale HPLC (Senshupak 5251-N, 250 mm x 20 mm i.d.) and eluted with hexane/ 2-propanol/methanol (100/2/0.4, *v*/*v*/*v*) at a flow rate of 7 mL min-1 at 277 K, as described elsewhere (Kobayashi *et al*. 1991). Chl *a* and Chl *b* were extracted from parsley (Petroselinum crispum Nym.), and Chl *d* from *A. marina*, which were then purified by the same method as for Chl *f*. Other authentic pigments, Chl *a′*, Chl *f′*, Phe *a* and Phe *f*, were prepared by epimerization and pheophytinization of Chl *a* and Chl *f* as described elsewhere (Watanabe *et al*. 1984).

Pigment analysis

Pigments were extracted from cell suspension (ca. 10 μL) by sonication in a ca. 300-fold volume of acetone/methanol (7/3, *v*/*v*) mixture for 2 min in the dark at room temperature. The extract was filtered and dried *in vacuo*. The whole procedure was completed within 5 min. The solid material thus obtained was immediately dissolved in 10 μL of chloroform, and injected into a silica HPLC column (YMC-pak SIL, 250 x 4.6 mm i.d.) cooled to 277 K in an ice-water bath. The pigments were eluted isocratically with degassed hexane/2-propanol/ methanol (100/0.7/0.2, *v*/*v*/*v*) at a flow rate of 0.9 mL min^{-1}, and were monitored with a JASCO UV-970 detector (λ = 670 nm) and a JASCO photodiode array detector MD-915 (λ = 300 - 800 nm) in series.

The molar ratios of Chl *a*/*a*′ were calculated directly from their HPLC peak area ratio with an absorbance detector (670 nm), because they have the same absorption spectra. The Chl *a*/Phe *a* ratio was calculated from their HPLC peak area ratio, which had been calibrated repeatedly by injecting a standard solution containing known amounts of authentic Chl *a* (>99.98 % in purity) and Phe *a* (99.90 %), based on the molar extinction coefficient of each pigment (Watanabe *et al*. 1984). The Chl *f*/*a*′ and Chl *f*/Phe *a* ratio were calculated from the corresponding HPLC peak area ratios by assuming that the Q_Y molar extinction coefficient of Chl *f* (>99.90 %) is the same as that of Chl *d* (French 1960).

Results and Discussion

Typical HPLC traces for acetone/methanol extracts from cells of the cyanobacterium strain KC1 cultivated under white fluorescent light and NIR LED light (λ = 740 nm) are shown in Figs. 3A and B, respectively. Large amounts of Chl *a*, as well as small amounts of Chl *a*′ and Phe *a*, were detected in both cells. Only the strain KC1 grown under NIR LED light showed the presence of Chl *f* as a minor pigment (Fig. 3B).

Absorption spectrum of Chl *f* purified from this cyanobacterium in methanol was shown in Fig. 4. As seen in Fig. 4, the spectrum has a red-shifted Q_Y transition (λ = 708.3 nm) compared to Chls *a* (665.8 nm), *b* (652.2 nm) and *d* (698.1 nm), and a blue-shifted Soret band (406.7 nm) compared to Chls *a* (432.5 nm), *b* (469.4 nm) and *d* (400.8 and 455.5 nm). The peak wavelengths of this pigment in methanol are almost the same as those of Chl *f* (λ = 406 nm and 706 nm) reported by Chen *et al*. (2010). However, the ratio of Soret/Q_Y-bands 0.9 was widely different from the reported value 1.9, most probably due to the absence of Phe *f* as an impurity in our Chl *f* sample. The identity of this pigment in the strain KC1 cultivated under NIR LED light was confirmed to be Chl *f* (Fig. 1) by means of HPLC, CD, mass and NMR spectroscopic analyses (data will be published elsewhere).

It is of interest to note that neither Phe *f* nor Chl *f*′ was detected in this cyanobacterium, even when it was grown under NIR LED light (Fig. 3B). This strongly suggests that, in the cyanobacterium strain KC1, Phe *a* and Chl *a*′ function, as in other cyanobacteria, as the primary electron acceptor in PS II and as the primary electron donor in PS I, respectively.

The molar ratios of Chl *a*/*a*′ in the cells of the strain KC1 grown under white fluorescent light and NIR LED light were almost the same, ca. 50 (Table 1), which is about a half of Chl *a*/*a*′ ≒ 120 in normal cyanobacteria grown under white fluorescent light (Kobayashi *et al.* 1988), and is slightly smaller than Chl *d*/*d*′ ≒ 70 (white fluorescent light) and much smaller than Chl *d*/*d*′ ≒ 140 (incandescent light) in *A. marina* (Akiyama *et al.* 2004). In contrast, the molar ratios of Chl *a*/Phe *a* showed a significant change; Chl *a*/Phe *a* = ca. 150 in the cells grown under white fluorescent light and ca. 60 under NIR LED light (Table 1); the former ratio is a little larger than that for normal cyanobacterium (ca.100), (Kobayashi *et al.*1988), and the latter is almost the same value, 50, observed in *A. marina* grown under both white fluorescent and incandescent light.

On the basis of Chl *a*′/PS I = 1/1 and Phe *a*/PS II = 2/1 (Kobayashi *et al.* 1988), stoichiometries of PS I/PS II are calculated to be 6.5 under white fluorescent light and 2.7 under NIR LED light (Table 1). Similar tendency was observed in *A. marina*; PS I/PS II = 1.4 under white fluorescent light and 0.82 under incandescent light (Table 1)(Akiyama *et al.* 2004). As seen in Table 1, similar stoichiometric changes were reported in *Synechocystis* PCC6714 (PS I/PS II = 3.02 and 1.16) (Fujita *et al*. 1997), *Synechocystis* PCC6803 (3.0 and 1.5)(Aizawa *et al*. 1992), and *Synechococcus* 6301 (3.7 and 1.4)(Manodori and Melis 1986).

Though the Chl *a*/PS I ratio are almost constant among the strain KC1, *Synechocystis* PCC6714, and *Synechococcus* 6301, in *A. marina* the corresponding stoichiometries of Chl *d*/PS I showed a significant change under different light conditions (Fig. 5A). In contrast, though the Chl *d*/PS II ratios are almost constant in *A. marina*, but the corresponding stoichiometries of Chl *a*/PS II changed

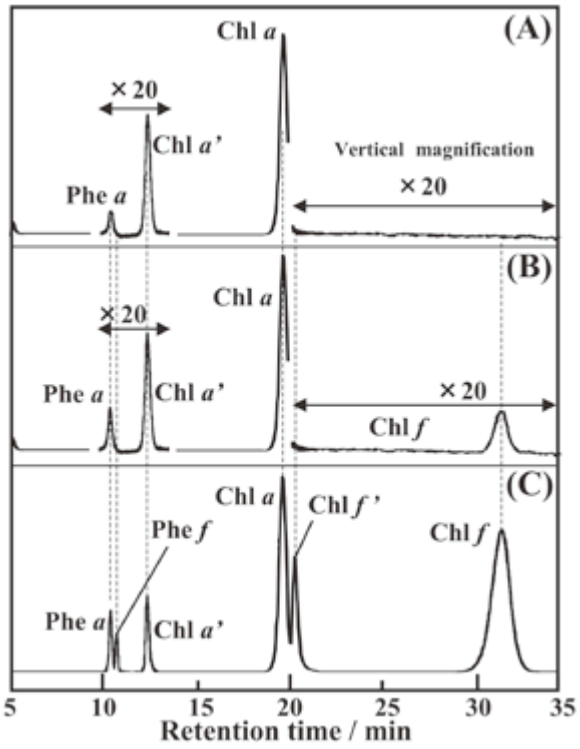


Fig. 3 HPLC elution profiles for acetone/methanol extracts of the cyanobacterium strain KC1 grown under (A) fluorescent light and (B) NIR LED light, and for (C) a mixture of authentic Phe *a*, Phe *f*, Chl *a*′, Chl *a*, Chl *f*′, Chl *f*. Detection wavelength is 670 nm.

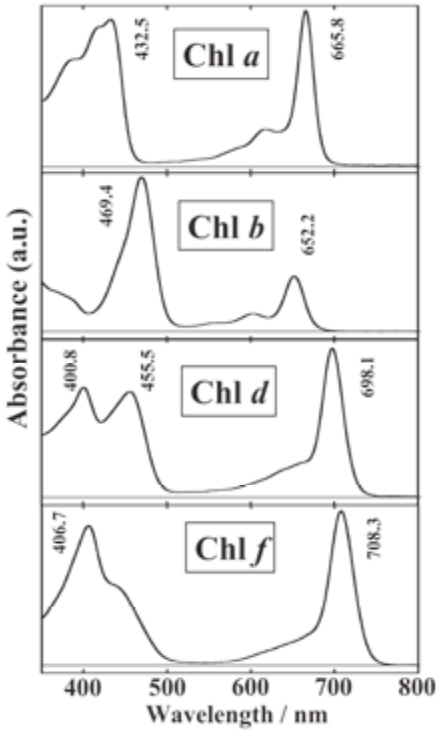


Fig. 4 Comparison of the absorption spectra of Chls *a*, *b*, *d* and *f* in methanol.

in the strain KC1, *Synechocystis* PCC6714, and *Synechococcus* 6301 (Fig. 5B). These results suggest that acclimation of *A. marina* to light regime is significantly different from that in the strain KC1, *Synechocystis* PCC6714, and *Synechococcus* 6301. The PS I/PS II stoichiometric change is smallest in *A. marina* and largest in the strain KC1 (Fig. 5C), indicating a high flexibility of the strain KC1 toward the light conditions during growth.

The function of Chl *f* in the strain KC1 is still unclear. Chl *f* biosynthesis was induced only when the strain KC1 was cultivated under NIR light, and Chl *f* was not induced under white fluorescent light even when NIR LED was used concurrently. Though the redox potential of Chl *f* is not yet been known, but the value is most probably similar to that of Chl *d* (Kobayashi *et al*. 2007), since one methyl moiety of Chl *a* is replaced by one formyl group in both Chl *d* and Chl *f* (see Fig. 1), and hence inductive effects of the substituent group on the macrocycle are estimated to be almost the same. Chl *f* may not function in the RC as an electron transfer component, but in the antenna as a light energy drain, because Chl *f* has the longest wavelength absorption in this cyanobacterium (Fig. 4). This hypothesis could be supported partly

Table 1. Comparison of PS I / PS II and pigment stoichiometries in the strain KC1, *A. marina*, *Synechocystis* and *Synechococcus*.

	cyanobacterium strain KC1		
	White fluorescent light	NIR LED light	Ref.
Chl *a* / Chl *a'*	47.6 ± 7.0 (n = 5)	46.3 ± 4.4 (n = 5)	this work
Chl *a* / Phe *a*	155 ± 32 (n = 5)	62.8 ± 5.3 (n = 5)	ibid.
Chl *a'* / Phe *a*	3.26 ± 0.52 (n = 5)	1.37 ± 0.17 (n = 5)	ibid.
Chl *f* / Chl *a'*	0 (n = 5)	2.46 ± 0.32 (n = 5)	ibid.
Chl *f* / Phe *a*	0 (n = 5)	3.27 ± 0.53 (n = 5)	ibid.
PS I / PS II*	6.51 ± 1.03 (n = 5)	2.71 ± 0.33 (n = 5)	ibid.
	Acariyochloris marina		
	White fluorescent light	Incandescent light	
Chl *d* / Chl *d'*	71.8 ± 10.8 (n = 7)	143 ± 7 (n = 30)	Akiyama *et al.* (2004)
Chl *d* / Phe *a*	48.6 ± 2.5 (n = 9)	57.7 ± 2.4 (n = 30)	ibid.
Chl *d'* / Phe *a*	0.70 ± 0.14 (n = 7)	0.41 ± 0.03 (n = 30)	ibid.
Chl *a* / Chl *d'*	2.65 ± 0.42 (n = 7)	5.14 ± 0.30 (n = 30)	ibid.
Chl *a* / Phe *a*	1.80 ± 0.07 (n = 9)	2.11 ± 0.09 (n = 30)	ibid.
PS I / PS II**	1.40 ± 0.28 (n = 7)	0.82 ± 0.06 (n = 30)	ibid.
ibid.	1.41	0.81	Boichenko *et al.* (2000)
	Synechocystis PCC6714		
	PSII light (550～670 nm)	PSI light (>660 nm)	
Chl *a* / Chl *a'***	150	171	Fujita *et al.* (1997)
Chl *a* / Phe *a***	227	99	ibid.
Chl *a'* / Phe *a***	1.51	0.58	ibid.
Chl *a* / PS I	150	171	ibid.
Chl *a* / PS II	453	198	ibid.
PS I / PS II	3.02	1.16	ibid.
	Synechocystis PCC6803		
	PSII light (550～670 nm)	PSI light (> 660nm)	
Chl *a'* / Phe *a***	1.5	0.75	Aizawa *et al.* (1992)
PS I / PS II	3	1.5	ibid.
	Synechococcus PCC6301		
	Yellow light	Red light	
Chl *a* / PS I	156	163	Manodori and Melis (1986)
Chl *a* / PS II	577	228	ibid.
PS I / PS II	3.7	1.4	ibid.

*Calculated from Chl *a'* / Phe *a* on the basis of Chl *a'* / PS I = 1 and Phe *a* / PS II = 2
**Calculated from Chl *d'* / Phe *a* on the basis of Chl *d'* / PS I = 1 and Phe *a* / PS II = 2
***Calculated from PS I / PS II and Chl *a* / PS II on the basis of Chl *a'* / PS I = 1 and Phe *a* / PS II = 2
****Calculated from PS I / PS II on the basis of Chl *a'* / PS I = 1 and Phe *a* / PS II = 2

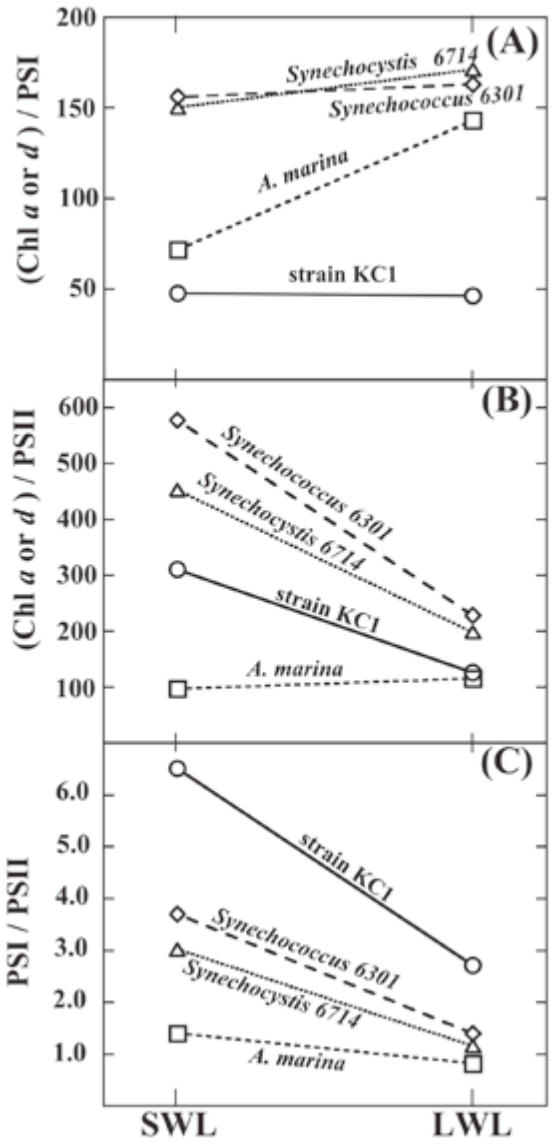


Fig. 5 Comparison of stoichiometries of (Chl *a* or *d*)/PS I, (Chl *a* or *d*)/PS II and PS I/PS II of (○) the strain KC1 (this work), (□) *A. marina* (Akiyama *et al*. 2004), (△) *Synechocystis* PCC6714 (Fujita *et al*. 1997), and (◇) *Synechococcus* 6301 (Manodori and Melis 1986) grown under short wavelength light (SWL: white fluorescent light for strain KC1 and *A. marina*, PS II light for *Synechocystis* PCC6714 and yellow light for *Synechococcus* 6301) and long wavelength light (LWL: NIR LED light for strain KC1, incandescent light for *A. marina*, PS I light for *Synechocystis* PCC6714, and red light for *Synechococcus* 6301).

by the observation of significantly strong and longer wavelength fluorescence derived from Chl *f* in the cyanobacterium.

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