Cover page

Title: Evolution of Green Plants Accompanied Changes in Light-Harvesting Systems

Running Title: Evolution of Photosystems in Green Plants

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Title: Evolution of Green Plants Accompanied Changes in Light-Harvesting Systems

Running Title: Evolution of Photosystems in Green Plants

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Abbreviations: CAO, chlorophyllide *a* oxygenase; CN, clear-native; DM, dodecyl maltoside; DVPchlide, divinyl protochlorophyllide *a*; GY, gigayears; LHC, light-harvesting complex; MOPS, 3-(N-morpholino)propanesulfonic acid; RACE, rapid amplification of cDNA ends.

Footnotes: The nucleotide sequences of full-length cDNAs of CAOs reported in this paper have been submitted to DDBJ under accession numbers BR001262 (*Klebsormidium flaccidum*), LC027965 (*Mesostigma viride*), LC027966 (*Nephroselmis pyriformis*), BR001263 (*Prasinoderma coloniale*), LC027968 (*Pseudoscourfieldia marina*), LC027967 (*Palmophyllum crassum*) and LC027969 (*Pyramimonas parkeae*).

Abstract:

Photosynthetic organisms have various pigments enabling them to adapt to various light environments. Green plants are divided into two groups: streptophytes and chlorophytes. Streptophytes include some freshwater green algae and land plants, while chlorophytes comprise the other freshwater green algae and seawater green algae. The environmental conditions driving the divergence of green plants into these two groups and the changes in photosynthetic properties accompanying their evolution remain unknown. Here, we separated the core antennas of photosystem I (PSI) and the peripheral antennas (LHCs) in green plants by green-native gel electrophoresis and determined their pigment compositions. Freshwater green algae and land plants have high chlorophyll *a*/*b* ratios, with most chlorophyll *b* existing in LHCs. In contrast, seawater green algae have low chlorophyll *a*/*b* ratios. In addition, chlorophyll *b* exists not only in LHCs but also in PSI core antennas in these organisms, a situation beneficial for survival in deep seawater, where blue-green light is the dominant light source.

Finally, low-energy chlorophyll (red chlorophyll) of PSI was detected in freshwater green algae and land plants, but not in seawater green algae. We thus conclude that the different level of chlorophyll *b* accumulation in core antennas and differences in photosystem I red chlorophyll between freshwater and seawater green algae are

evolutionary adaptations of these algae to their habitats, especially to high- or low-light environments.

Keywords: Evolution · Green algae · Land plants · Light adaptation · Photosystem.

[Introduction]

Photosynthesis, one of the most important biological processes, not only helps drive biological activity, but also contributes to maintenance of the global environment (Cavalier-Smith 2006). During their long evolutionary history, photosynthetic organisms have acquired various photosynthetic pigments (Grossman et al. 1995, Ballottari et al. 2012) that have contributed to the adaptation of their photosystems to various light conditions (Ruban 2015). The first photosynthetic organisms were anoxygenic photosynthetic bacteria that used bacteriochlorophylls (Gupta 2012). Cyanobacteria, which use chlorophylls and perform oxygenic photosynthesis, are proposed to have appeared approximately 2.7 gigayears (GY) ago (Gerard et al. 2009). Primary endosymbiosis gave rise to eukaryotic photosynthetic organisms, including glaucophytes, rhodophytes and green plants (Qiu et al. 2013). In addition to chlorophyll *a*, phycobilins are found in glaucophytes and rhodophytes and chlorophyll *b* is

distributed in green plants, where these compounds are used as peripheral antenna pigments (Neilson and Durnford 2010). Photosynthetic groups derived from secondary and tertiary endosymbiosis also have their own specific photosynthetic pigments; for example, fucoxanthin is distributed in heterokontophytes, whereas peridinin is present in dinophytes. In addition, chlorophyll *c* is found only in secondary and tertiary endosymbionts (Green 2011). Thus, the distribution of photosynthetic pigments among photosynthetic organisms is well correlated with their phylogenetic relationships. This phenomenon suggests that the acquisition of new photosynthetic pigments has been one of the factors driving the evolution of photosynthetic organisms.

Green plants, which include green algae and land plants and contain chlorophyll *b*, appeared approximately 1.2 GY ago (Becker 2013). Except for green plants, no primary endosymbionts use chlorophyll *b*. Recent studies have suggested that green plants diverged into two groups, chlorophytes and streptophytes, approximately 1.0 GY ago (Leliaert et al. 2012, Becker 2013). Prasinophytes, belonging to chlorophytes, initially evolved in seawater, whereas charophytes, belonging to streptophytes, evolved in freshwater (Becker and Marin 2009). The composition of the major photosynthetic pigments is almost the same among these green plants, but some minor pigments are differentially distributed (Latasa et al. 2004). For example, 8-divinyl

protochlorophyllide *a* (DVPchlide), an intermediate molecule of chlorophyll synthesis, is used as a photosynthetic pigment in some chlorophytes, including *Micromonas*, *Nephroselmis*, *Prasinococcus*, and *Ostreococcus*, but is not used in other chlorophyte and streptophyte organisms. In addition, prasinoxanthin is used as a photosynthetic pigment in some chlorophytes (Prasinophyceae). However, there is no known chlorophytes-specific pigments and streptophyte-specific pigments. This information suggests that the acquisition of a new photosynthetic pigment was not a driving force in the evolutionary division of green plants into chlorophytes and streptophytes.

It has been suggested that chlorophytes initially evolved in deep seawater (Leliaert et al. 2011), where light intensities are low and the available light spectrum is limited to the blue-green region. In contrast, streptophytes evolved in freshwater (Leliaert et al. 2011); in such an environment, photosynthetic organisms had to develop protection mechanisms against photo-oxidative damage to adapt their photosynthetic machineries to strong light environments. Although they have the same pigment composition, streptophytes and chlorophytes had to independently develop their specific light-harvesting systems for this purpose. In spite of these findings, our knowledge of light-harvesting systems among green plants is still limited. In addition, whether or not light-harvesting systems have been conserved during evolution has not been determined.

One such question concerns the level of conservation of the core antennas of photosystems among green plants. Although the photosystem I (PSI)-LHCI structure has been intensively studied and experimentally resolved in higher plants (Amunts et al. 2010), our knowledge of the core antennas in green algae is essentially based on experiments using a few model photosynthetic organisms such as *Chlamydomonas*.

To elucidate differences in light-harvesting systems of streptophytes and chlorophytes, we isolated light harvesting systems from green plants, separated these complexes by green-native gel electrophoresis, and examined their pigment compositions. We found that chlorophyll *b* in streptophytes is localized only in photosystem peripheral antennas, a situation indispensable for adaptation to high-light conditions. In contrast, we detected a large amount of chlorophyll *b* in both photosystem core antennas and peripheral antennas in seawater chlorophytes, especially in early-branching taxa; such a distribution would be beneficial for achieving efficient light-harvesting under deep seawater light environments. Finally, we considered these differences in chlorophyll *b* content and localization in the context of chlorophyte and streptophyte evolution.

[Results]

Phylogenetic relationships of representative green plants in this study

Current models of green algal evolution posit the early divergence of two discrete lineages: streptophytes and chlorophytes (Leliaert et al. 2012, Becker 2013) (Supplementary Fig. S1). Streptophytes comprise charophytes and land plants, whereas chlorophytes include prasinophytes and core chlorophytes. To compare their light-harvesting systems, in this study we used the following representative organisms: land plant *Arabidopsis thaliana*; charophytes *Klebsormidium flaccidum* and *Mesostigma viride*; prasinophytes *Micromonas pusilla*, *Nephroselmis pyriformis, Pyramimonas parkeae, Prasinoderma coloniale* and *Pseudoscourfieldia marina*; and core chlorophytes *Chlamydomonas reinhardtii*, *Chlorella variabilis* and *Bryopsis plumosa*. In addition, we used *Palmophyllum crassum*, which according to a previous report is probably an early branching chlorophyte (Zechman et al. 2010). All organisms except *Palmophyllum crassum* were cultured. Because a culture system has not been reported for *Palmophyllum*, we harvested this species from a natural habitat.

The consistent relationship of pigment composition and absorption spectra of green plants to their light environments

To examine the pigment composition of the studied organisms, all pigments were

extracted from cells (Supplementary Table S1). Chlorophylls *a* and *b*, violaxanthin, and α- or β-carotene were detected in all examined organisms. Lutein or dihydrolutein was detected in all examined organisms except for *Bryopsis*. Zeaxanthin, formed from violaxanthin in the xanthophyll cycle, was also detected in prasinophytes. Furthermore, we detected prasinoxanthin, siphonaxanthin and siphonaxanthin derivatives, which have only been reported in green algae. Prasinoxanthin, a major pigment of *Prasinoderma*, *Pseudoscourfieldia* and *Micromonas*, was not found in *Nephroselmis* and *Pyramimonas*, the other two prasinophytes. Siphonaxanthin was revealed to be a major pigment of *Mesostigma*, *Nephroselmis* and *Bryopsis*, thus indicating that siphonaxanthin is used regardless of light environment and phylogeny. Loroxanthin was found only in *Chlamydomonas*.

Although chlorophyll *b* was detected in all green plants, chlorophyll *a*/*b* ratios varied substantially among them (Table 1). Chlorophyll *a*/*b* ratios of the seawater chlorophytes were between 0.6 and 1.3, suggesting that a large amount of chlorophyll *b* is used for light harvesting. In particular, the earliest-branching chlorophyte, *Palmophyllum*, had an extremely low chlorophyll *a*/*b* ratio (0.64). In contrast, chlorophyll *a*/*b* ratios of streptophytes and freshwater chlorophytes (*Chlamydomonas* and *Chlorella*) between 2.1 and 3.4 were higher than seawater chlorophytes.

In addition to chlorophyll *a* and chlorophyll *b*, some green algae, as previously reported (Zapata 2006), were found to use DVPchlide (chlorophyll *c*-like) and chlorophyll *c*3-like (Chl *c* cs170) for light harvesting (Supplementary Table S1). All photosynthetic organisms can potentially synthesize DVPchlide because it is a precursor of chlorophyll, but the organisms that use it as a photosynthetic pigment are restricted to prasinophytes. The absorption spectrum of DVPchlide is similar to that of chlorophyll *c* and is suitable for the light conditions of their marine habitats (Garrido et al. 1995). Similarly, chlorophyll *c*3-like was found only in *Nephroselmis* and is suitable for this organism's deep seawater habitat. Interestingly, *Palmophyllum*—which lives in the deepest seawater habitats and had the lowest chlorophyll *a*/*b* ratio of green algae examined in this study—does not use DVPchlide and chlorophyll *c*3-like pigments as photosynthetic pigments. This finding is consistent with its phylogenetic position as the earliest-diverging lineage of chlorophytes or as a shared ancestor of streptophytes and chlorophytes (Zechman et al. 2010).

Light-harvesting apparatuses are evolutionarily adapted to environmental light conditions in which their organisms carry out photosynthesis and survive. We therefore monitored absorption spectra of cells to examine which light spectra can be efficiently used (Supplementary Fig. S2). In the case of filamentous *Bryopsis* cells, whose

absorption spectra are difficult to measure, we instead monitored the absorption of their thylakoid membranes (Supplementary Fig. S2). Seawater chlorophytes showed high absorbance in the blue-green region, while streptophytes and freshwater chlorophytes exhibited high absorbance in the red region.

In photosynthetic organisms living in clear oceans, the absorption spectrum of chlorophyll *b* is more suitable than that of chlorophyll *a*. Given the photosynthetically available light spectrum in seawater, the Soret band of chlorophylls is much more effective for light harvesting in seawater habitats compared with Qx and Qy bands. Because chlorophyll *b* has a higher Soret band absorption than chlorophyll *a*, chlorophyll *b* has more opportunity to harvest light energy than chlorophyll *a* in seawater habitats. In addition, the Soret band of chlorophyll *b* is shifted to longer wavelengths, which is a better fit to the light conditions of clear oceans (Sverdrup 1942, Stomp et al. 2007). With respect to harvesting of available light energy in seawater habitats, seawater chlorophytes with lower chlorophyll *a*/*b* ratios thus have an advantage over streptophytes and freshwater chlorophytes with higher chlorophyll *a*/*b* ratios.

Significant localization of chlorophyll *b* in LHCs among seawater chlorophytes as

revealed by green-native gel electrophoretic separation of pigment-protein complexes

The light-harvesting system of photosystems in green plants is divided into two parts, namely, peripheral and core antennas. The peripheral antennas of green plants are LHCs. The PSI core antenna consists of PsaA and PsaB proteins, while the photosystem II (PSII) core antenna is made up of PsbB (CP47) and PsbC (CP43). It should be noted that PsaA and PsaB not only constitute the core antenna of PSI, but also its reaction center, whereas the reaction center of PSII comprises PsbA (D1) and PsbD (D2). As is the case for PsbA/PsbD, PsaA and PsaB form heterodimers.

In green plants, the location of chlorophyll *b* has been predicted to be restricted to LHCs (Green and Durnford 1996). The high level of chlorophyll *b* observed in seawater chlorophytes thus suggests two hypotheses. The first hypothesis is that a large number of LHCs accumulate in those organisms. The second hypothesis is that chlorophyll *a*/*b* ratios of chlorophyte LHCs are lower than those in streptophytes and, especially, in freshwater core chlorophytes. To examine these hypotheses, we separated pigment-protein complexes in the green plants in this study using native-green gel electrophoresis. Because the PSI core and LHCII trimers were relatively stable against the dissociation effect of sodium dodecyl sulfate (SDS) under our experimental

conditions, we analyzed the pigment compositions of the PSI core (PsaA/PsaB heterodimer) and LHCII trimers. Because the PSII core was rather unstable under these experimental conditions and migrated very closely with LHC monomers—thus potentially heavily contaminating the PSII fraction—the pigment composition of the PSII core could not be determined.

Migration profiles of pigment-protein complexes on a green-native gel were almost identical among green plants, and the PSI core antennas and LHCII trimers could be resolved (Fig. 1). We first examined chlorophyll *a/b* ratios of LHCII trimers (Table 2). Chlorophyll *a/b* ratios of LHCII trimers were especially low in seawater chlorophytes, whereas ratios in streptophytes and freshwater chlorophytes were similar (Table 2). The lower chlorophyll *a/b* ratios of LHCII trimers in seawater chlorophytes were thus due, at least partially, to the lower chlorophyll *a*/*b* ratios of these organisms. We next examined chlorophyll *a/b* ratios of PSI core antennas (Table 2). Pigment compositions of PSI core antennas differed greatly among species. Chlorophyll *a*/*b* ratios of PSI core antennas in streptophytes and freshwater-adapted chlorophytes (*Chlamydomonas* and *Chlorella*) were greater than 15.0, which indicates that the core antenna chlorophylls of their PSI consisted almost entirely of chlorophyll *a*. In contrast, chlorophyll *a/b* ratios of PSI core antennas in seawater chlorophytes were between 2.0 and 6.7, indicating that

chlorophyll *b* is in fact a photosynthetic pigment of their PSI core antennas. Interestingly, the *Palmophyllum* PSI core antenna had an extremely low chlorophyll *a/b* ratio, thus demonstrating that more than 30% of total chlorophyll in the core antenna is chlorophyll *b*. These data suggest that the low chlorophyll *a*/*b* ratios of green algae in seawater habitats are due to the low chlorophyll *a*/*b* ratios of their LHCs and their PSI core antennas.

Low chlorophyll *a*/*b* ratios of purified PSI core antennas from *Nephroselmis*, *Prasinoderma* and *Pyramimonas* revealed by non-heated SDS-PAGE

Because LHCII trimers in seawater chlorophytes exhibited low chlorophyll *a*/*b* ratios, contamination of PSI core antennas with small quantities of LHCs could significantly decrease antenna chlorophyll *a*/*b* ratios. PSI core antennas separated by green-native gel electrophoresis may thus have been partially contaminated by LHCs. To estimate the extent of contamination, we first checked the purity of PSI core antennas and LHCII trimers. In particular, we screened for α- and β-carotenes, lutein and dihydrolutein, because distributions of β-carotene and lutein are reportedly different between PSI core antennas and LHCII trimers: β-carotene has been reported in the PSI core antenna (Amunts et al. 2010), while lutein is found in LHCII trimers (Liu et al. 2004). We

searched for these pigments in the PSI core antennas and LHCII trimers of all studied seawater chlorophytes other than *Micromonas*. We had previously determined that *Micromonas* PSI core antennas uncontaminated by LHCs contain significant levels of chlorophyll *b* (Kunugi et al. 2013). As a comparison, we also analyzed the PSI core antennas and LHCII trimers of *Arabidopsis* and *Chlamydomonas*. We found that α- and/or β- carotenes were present in all PSI core antennas but absent from LHCII trimers (Supplementary Table S2). In contrast, (dihydro)lutein was detected in all LHCII trimers except those of *Nephroselmis* and *Bryopsis* (Supplementary Table S2). Small levels of (dihydro)lutein were also detected in PSI core antennas of *Arabidopsis*, *Pseudoscourfieldia* and *Pyramimonas*, but these amounts, when normalized relative to total chlorophylls, were significantly lower than those in LHCII trimers (Supplementary Table S2). Although the separated PSI core antennas might have been slightly contaminated by LHCs, these data support our identification of the separated PSI core antennas and LHCII trimers.

To confirm the observed low chlorophyll *a*/*b* ratios in PSI core antennas among seawater chlorophytes by an alternative method, we next purified PSI core antennas from *Nephroselmis*, *Prasinoderma* and *Pyramimonas* using the non-heated Laemmli SDS-PAGE system (see Methods). After subjecting the non-heated protein samples to

SDS-PAGE, their PSI core antennas (heterodimers of PsaA/PsaB) still retained chlorophylls (Fig. 2). PSI core antenna identification was performed by immunoblot analysis with anti-PsaA/PsaB antibodies. No contamination of PSI core antennas by LHCs was detected in *Prasinoderma* and *Pyramimonas* according to immunoblot analysis using anti-Lhcb3 and anti-Lhca2 antibodies nor in *Nephroselmis* as assessed by two-dimensional SDS-PAGE and visualized by Coomassie Brilliant Blue staining (Fig. 2). We next determined the chlorophyll contents of their PSI core antennas and obtained chlorophyll *a*/*b* ratios of 2.8 for *Nephroselmis*, 2.8 for *Prasinoderma* and 0.5 for *Pyramimonas*. These data clearly demonstrate that the PSI core antennas of *Nephroselmis*, *Prasinoderma* and *Pyramimonas* possess significant proportions of chlorophyll *b*, consistent with the results of green-native gel analysis.

Light harvesting and energy transfer from chlorophyll *b* to chlorophyll *a* in the core PS complex

Pigment analysis of LHCII trimers and PSI core antennas revealed the presence of considerable amounts of chlorophyll *b* in both complexes in seawater chlorophytes. We next measured cell excitation and emission fluorescence spectra to examine whether light energy harvested by chlorophyll *b* molecules, especially in the PS core antenna

complex, is efficiently transferred to PS reaction centers. Figure 3 shows low-temperature emission spectra obtained when chlorophyll *b* was preferentially excited at 470 nm. No fluorescence emission from energetically-uncoupled chlorophyll *b* molecules was detected at the expected wavelength (around 660 nm) in any samples, including seawater chlorophytes, which indicates that chlorophyll *b* was completely incorporated into the photosystems (Fig. 3).

To confirm the transfer of energy from chlorophyll *b* to chlorophyll *a* in photosystem core antennas, we attempted to eliminate LHCs from photosystems using clear-native (CN)-PAGE. Native green gel electrophoresis generated energetically uncoupled chlorophylls in the photosystems during the experimental process, as a consequence of the denaturation effect of SDS. Because PSI-LHCI is more stable than PSII-LHCII, we tried to purify the PSII monomer by CN-PAGE using mild detergents. We selected *Nephroselmis* for the experiment because this species had the lowest chlorophyll *a*/*b* values except for uncultured *Palmophyllum*. In the CN-PAGE, the migration distance of PSI-LHCI was almost the same as that of the PSII dimer. To separate the PSII core complex (including the PSII core antenna without LHCs) from PSI-LHCI, generation of the PSII monomer was necessary. Prior to the CN-PAGE, we therefore solubilized thylakoid membranes from *Nephroselmis* with 4.0% β-dodecyl maltoside (β-DM) and

0.1% Triton X-100. CN-PAGE appeared to separate the PSII monomer and LHCs, and two-dimensional SDS-PAGE followed by visualization with silver staining confirmed that the monomeric PSII bands contained no LHCs (Supplementary Fig. S3). The chlorophyll *a*/*b* ratio of the monomeric PSII band was 7.74, indicating that chlorophyll *b* was incorporated into both PSI and PSII core antennas. The fluorescence emission and excitation spectra of the purified core antennas of PSII were then measured (Fig. 4). In addition to a peak at 437 nm, the excitation spectrum monitored at 688 nm contained peaks at 461 and 491 nm, indicating that chlorophyll *b* and carotenoids function as light-harvesting pigments in the PSII core antenna of *Nephroselmis*. These data demonstrate that the large amounts of chlorophyll *b* present in green algae in seawater habitats can contribute to the harvesting of light energy and can transfer this energy to chlorophyll *a* in both light-harvesting antennas and reaction centers. Notably, fluorescence from chlorophyll *b* was not observed, which indicates that all of the chlorophyll in the complex was energetically linked to chlorophyll *a*. We also measured the absorptance (1 − transmittance) of the separated PSII monomer (Fig. 4C). The absorptance spectrum was very consistent with the excitation spectrum (Fig. 4C), demonstrating the occurrence of efficient energy transfer from chlorophyll *b* to chlorophyll *a*.

Absence of red chlorophyll fluorescence emissions from PSI-LHCI of seawater chlorophytes

PSI contains chlorophyll, so-called red chlorophyll, that absorbs at lower energies than the primary electron donor of PSI that absorbs around 700 nm (Croce and van Amerongen 2013). The low-temperature fluorescence emission spectra of whole cells also revealed that *Arabidopsis* and some—but not all—green algae emitted long wavelength fluorescence from red chlorophyll of PSI (Fig. 3). Specifically, red chlorophyll fluorescence was observed from all streptophytes and freshwater core chlorophytes (*Chlamydomonas* and *Chlorella*), but not from seawater chlorophytes. It should be noted that the shoulder around 700 nm in the spectrum of seawater chlorophytes was likely emission from the primary electron donor and its adjacent chlorophyll molecules of PSI. To confirm the absence of red chlorophyll in PSI-LHCI, we measured the low-temperature fluorescence emission spectrum of the *Nephroselmis* PSI-LHCI separated by CN-PAGE (Supplementary Fig. 3). In contrast to emission from the primary donor and/or its adjacent chlorophyll molecules at 704 nm, red chlorophyll fluorescence emissions were not observed from PSI-LHCI. As judged by two-dimensional SDS-PAGE (Supplementary Fig. S3), an emission peak observed at

684 nm may have been due to slight contamination from the PSII dimer. Our data showed that the red chlorophyll fluorescence emission from PSI-LHCI is absent or at least very weak in seawater chlorophytes, which is consistent with a previous report that the red chlorophyll fluorescence emission from PSI-LHCI is absent in *Ostreococcus tauri* (Swingley et al. 2010).

Presence of a degron sequence regulating CAO levels in streptophyte algae

Chlorophyll *b* is synthesized from chlorophyllide *a* and/or chlorophyll *a* by CAO. Previous studies have revealed that chlorophyll *b* amounts in core antennas may be closely related in CAO molecular structure (Hirashima et al. 2006, Sakuraba et al. 2009, Kunugi et al. 2013). A degron sequence in the CAO regulatory domain has an essential role in regulation of chlorophyll *b* amounts in land plants (Sakuraba et al. 2009), but it is unclear whether this degron sequence is present in green algae. To study the evolutionary relationship between CAO amino acid sequences and the use of chlorophyll *b* in photosystems of green algae, we determined the full-length CAO sequences of the green algae used in this study. The catalytic domains of all CAO proteins except *Micromonas* CAO were found to have high sequence similarity (Supplementary Fig. S5). As we have reported previously, the *Micromonas* CAO protein

is split into two proteins, one containing a Rieske center and the other having a mononuclear iron-binding motif (Kunugi et al. 2013). The topology of a phylogenetic tree based on the CAO catalytic domain was consistent with known species relationships, indicating that CAO has been vertically inherited in green plants (Supplementary Fig. S5). However, the sequence corresponding to the degron sequence conserved among land plants was only found in *Klebsormidium*, suggesting that a degron with a function similar to the one in land plants has developed independently in streptophyte algae (Fig. 5).

[Discussion]

Chlorophyll *b* is one of the major chlorophyll pigments in the core antennas of photosystems of seawater chlorophytes

Light-harvesting complexes in green plants are divided into two groups; one group is the peripheral antenna complex (LHC) and the other consists of core antenna complexes such as PsbB and PsbC (the core antenna of PSII) and PsaA and PsaB (the core antenna of PSI). Chlorophyll *b* has been previously thought to be distributed only in the peripheral antenna complex, with the core antenna containing only chlorophyll *a* (Green and Durnford 1996). The crystal structure of the pea PSI-LHCI clearly supports this

idea (Amunts et al. 2010). The same is true for *Chlamydomonas* photosystems, which have been extensively studied. For example, only a trace amount of chlorophyll *b* (approximately 4.4% of total chlorophyll) is contained in the PSI core antenna in *Chlamydomonas reinhardtii* (Satoh et al. 2001). A single exception has recently been reported, *Micromonas*, whose CAO is split into two subunits and whose PSI core antenna has a significant level of chlorophyll *b* (Kunugi et al. 2013). However, this photosystem characteristic has been thought to be an exceptional evolutionary innovation because this CAO structure is unique to Mamiellales in Prasinophyta (Kunugi et al. 2013).

In this study, we examined the pigment composition of chlorophyll-protein complexes from 11 species of green plants. On the basis of pigment contents calculated from the results of green-native gel electrophoresis, we found that seawater chlorophytes accumulated chlorophyll *b* in core antennas at levels corresponding to 15–30% of total chlorophylls (Table 2). We cannot exclude the possibility that the green-native gel-separated PSI core antennas contained low amounts of LHCs. Slight contamination by LHCs could decrease chlorophyll *a*/*b* ratios of the separated PSI core antennas. Nevertheless, the experimental results presented here indicate that seawater chlorophytes use chlorophyll *b* as major pigments of the core antenna. First, the PSI

core antennas separated by green-native gel electrophoresis from freshwater chlorophytes and streptophytes had significantly higher chlorophyll *a*/*b* ratios than those from seawater chlorophytes (Table 2). These clear differences suggest that the amounts of chlorophyll *b* in PSI core antennas were different between seawater chlorophytes and the other green plants. In addition, LHC contamination of PSI core antennas had only limited effects on chlorophyll *a*/*b* ratios as judged by amounts of (dihydo)lutein relative to total chlorophylls (Table S2). Furthermore, PSI core antennas of *Nephroselmis*, *Prasinoderma* and *Pyramimonas* separated by non-heated SDS-PAGE also had low chlorophyll *a*/*b* ratios (Table 2). Chlorophyll *a*/*b* ratios of PSI core antennas separated by non-heated SDS-PAGE were lower than those separated by green-native gel electrophoresis. This result is likely due to the larger loss of chlorophylls from PSI core antennas during SDS-PAGE. Nevertheless, the presence of considerable quantities of chlorophyll *b* in all PSI core antennas separated by non-heated SDS-PAGE indicates that seawater chlorophytes use chlorophyll *b* as the major pigment in their PSI core antennas. Taking all of these data into account, we conclude that chlorophyll *b* is a major pigment of both peripheral and core antennas in seawater chlorophytes. Notably, our discovery is not the only case of a core antenna complex containing species other than chlorophyll *a*. Chlorophyll *d* is the major pigment of the core antenna in

Acaryochloris (Boichenko et al. 2000). On the basis of these data, we further speculate that photosystem core antennas can potentially bind various chlorophyll species that may have important roles in the evolutionary adaptation of photosynthetic organisms to various light environments.

Chlorophyll *b* in the core antennas of photosystems is advantageous for seawater chlorophytes. In seawater habitats, major challenges to photosynthetic organisms include low and blue-green light environments (Sverdrup 1942, Stomp et al. 2007), with blue-green light harvesting ability possibly a limiting factor in their survival. Chlorophyll *b* can harvest blue-green light more efficiently than other chlorophyll species. The presence of a considerable quantity of chlorophyll *b* in the core antenna would therefore help seawater chlorophytes adapt to seawater environments. Under high-light conditions, in contrast, chlorophyll *b* in the core antenna generates reactive oxygen species, thereby enhancing photodamage (Sakuraba et al. 2010). Although not a problem for seawater chlorophytes, this situation poses a serious challenge to streptophytes and any chlorophytes living in freshwater or terrestrial habitats. As shown in Table 2, these organisms must consequently exclude chlorophyll *b* from their core antennas.

The different level of chlorophyll *b* accumulation in core antennas is related to the molecular evolution of CAO in green plants

Land-plant CAOs consist of three domains: catalytic, regulatory and linker (Nagata et al. 2004). The catalytic domain is responsible for the conversion of chlorophyllide *a* and/or chlorophyll *a* to chlorophyll *b*, whereas the regulatory domain regulates CAO accumulation in response to cellular chlorophyll *b* levels (Yamasato et al. 2005). In a study using *Arabidopsis*, deletion of the CAO regulatory domain resulted in a low chlorophyll *a*/*b* ratio and considerable amounts of chlorophyll *b* in the core antenna (Sakuraba et al. 2010), thus demonstrating that this domain is indispensable for the regulation of chlorophyll *b* synthesis. With the exception of putative transit peptides, regulatory domains are highly conserved across land plants. In green algae, however, the degree of conservation has been unclear. In this study, we cloned *CAO* genes from various green plants and compared their amino acid sequences. The catalytic domains of all CAO proteins except for that of *Micromonas* were found to have high sequence similarity (Supplementary Fig. S5). We then compared the regulatory domains of green algae CAOs to those of land plants *Physcomitrella patens*, *Selaginella moellendorffii* and *Arabidopsis thaliana* (Fig. 5 and Supplementary Fig. S6). In contrast to the catalytic domain, the regulatory domain was found to vary drastically among green plants. First,

N-terminal sequences of *Micromonas, Pseudoscourfieldia* and *Prasinoderma* CAOs had no sequence homology with those of the other examined CAOs. Conversely, the N-terminal sequences of the other CAOs showed significant sequence homology with one another, thus indicating that a considerable portion of the regulatory domain originated in the common ancestor of streptophytes and chlorophytes (Fig. 5 and Supplementary Fig. S6). Given their phylogenetic positions, *Micromonas*, *Pseudoscourfieldia* and *Prasinoderma* lost the conserved sequences. Similarly, N-terminal sequences of *Palmophyllum* and *Nephroselmis* CAOs showed no significant similarity with the other CAOs except for the conserved sequence regions (Supplementary Fig. S6). Taking into account the low chlorophyll *a*/*b* ratios and substantial chlorophyll *b* levels in the core antennas, these data are consistent with the hypothesis that the structure of the CAO N-terminal sequence is important for regulation of chlorophyll *b* synthesis.

In contrast to the above organisms, *Pyramimonas* and core chlorophytes had N-terminal sequences that were highly similar to one another, but not significantly similar to land-plant CAOs (Fig. 5 and Supplementary Fig. S6). The length of the N-terminal sequence conserved among them is not directly related to chlorophyll *a*/*b* ratio (Tables 1 and 2). It is possible that unidentified motifs in their N-terminal

sequences may have a role for the post-transcriptional regulation of CAO accumulation. Further studies will be required to reveal the role of N-terminal sequences in these organisms.

On the other hand, we previously reported the existence of a degron sequence corresponding to amino acids 97–106 of AtCAO (Q^{97}DLLTIMILH^{106}) and essential for the post-transcriptional regulation of CAO (Sakuraba et al. 2009). This degron sequence is recognized by chloroplast Clp protease (Nakagawara et al. 2007), and the stability of a CAO protein possessing the sequence is negatively feedback-regulated by chlorophyll *b* (Sakuraba et al. 2009). Deletion of only the degron sequence resulted in a lower chlorophyll *a*/*b* ratio and considerable accumulation of chlorophyll *b* in the core antennas, suggesting that this sequence is indispensable for the regulation of core antenna chlorophyll *b* levels (Sakuraba et al. 2009). Interestingly, a degron sequence showing considerable similarity to the degron sequence of land-plant CAOs was found in *Klebsormidium*, suggesting that streptophyte algae have developed a degron that can function similarly to those of land plants (Fig. 5 and Supplementary Fig. S6). *Mesostigma*, which is part of an early-branching lineage of streptophytes, does not possess any sequences similar to identified degrons. Nevertheless, the molecular structure of the region in *Mesostigma* corresponding to the degron and its flanking

region in the other streptophyte CAOs was quite similar (Fig. 5), suggesting that the corresponding sequence in *Mesostigma* (EDTQRVVDLH) might have a similar degron function. Our data suggest that streptophytes have developed a unique degron sequence for regulation of chlorophyll *b* amounts in core antennas as an adaptation to high light, thereby contributing to their acclimation to freshwater habitats and furthering their colonization of land.

Red chlorophyll in PSI was not found in seawater chlorophytes

Similar to chlorophyll *b* in the core antenna, red chlorophyll in PSI was found in streptophytes and freshwater chlorophytes, but not in seawater chlorophytes. This distribution suggests that the existence of red chlorophyll depends on the habitat of the organism. The proposed roles of red chlorophyll are harvesting of near-infrared solar energy and photoprotection (Jensen et al. 2007, Croce and van Amerongen 2013). Because neither role is of importance in seawater chlorophytes, the absence of red chlorophyll is consistent with these organisms' habitats. These data suggest that the introduction of chlorophyll *b* into PS core antenna complexes and the loss of red chlorophyll is an adaptation by seawater green algae to light-limited environments.

Changes in the light-harvesting system may have greatly contributed to green plant evolution

The results uncovered in this study indicate that adaptation to high light or to deep seawater conditions may have been a critical factor in the divergence of streptophytes and chlorophytes from a common ancestor. This hypothesis is consistent with the finding that the photorespiratory pathway, indispensable to the survival of photosynthetic organisms, differs between these two groups (Becker 2013). Streptophytes have developed a "plant-type" photorespiratory pathway that uses peroxisomes for glycolate metabolism. This innovation enables these algae to convert high levels of glycolate with the requirement of additional energy (Stabenau and Winkler 2005), a suitable tradeoff in streptophytes under high-light environments. Our combined data reveal that streptophytes have developed a photosynthetic system that includes a chlorophyll *b*-free core antenna, red chlorophyll in PSI, and a plant-type photorespiratory pathway.

Although the diversification of green plants into two groups, streptophytes and chlorophytes, was an important event in green plant evolution, this event was not accompanied by the acquisition of a new photosynthetic pigment as observed in the evolution of other photosynthetic groups. Nevertheless, their light-harvesting systems

changed drastically with respect to light-harvesting properties and high-light resistance (Fig. 4). In Streptophyta, the photosystems became resistant to high-light conditions, a development that may have been partially responsible for the appearance of land plants. Consequently, changes in the light-harvesting system may have contributed greatly to the evolution of green plants.

[Materials and Methods]

Plant materials and growth conditions

Arabidopsis thaliana (Columbia ecotype) was grown in a chamber equipped with white fluorescent lamps under a 14-h photoperiod at a light intensity of 70 μmol photons m^{-2} s^{-1} at 25°C. The following algae were cultivated in the growth media indicated: *Chlamydomonas reinhardtii* (cc1931 [arg7]) in TAP medium with 200 mM arginine (Tanaka et al. 1998), *Chlorella variabilis* (NIES-2541) in CCA medium (Hoshina and Imamura 2009), *Micromonas pusilla* (CCMP1545) and *Nephroselmis pyriformis* (CCMP549) in L1 medium (Kunugi et al. 2013), *Pyramimonas parkeae* (NIES-254), *Pseudoscourfieldia marina* (NIES-1419) and *Prasinoderma coloniale* (NIES-2582) in f/2 medium as recommended by the Microbial Culture Collection at the National Institute for Environmental Studies (NIES Collection), and *Mesostigma viride*

(NIES-296) and *Klebsormidium flaccidum* (NIES-2285) in C medium as recommended by the NIES Collection. *Bryopsis plumosa* (Hudson) C. Agardh was collected from Muroran, Hokkaido, Japan and was grown in PES medium (Yamagishi et al. 2003). *Klebsormidium*, *Mesostigma*, *Chlorella*, and *Chlamydomonas* were each inoculated into 100 mL of liquid medium in 200-mL Erlenmeyer flasks and grown at 24.5°C under a 14-h photoperiod of 40 μmol photons m^{-2} s^{-1}. *Nephroselmis*, *Prasinoderma*, *Pseudomonas*, *Pyramimonas*, *Mesostigma*, and *Bryopsis* were inoculated and cultivated under the same conditions except with a light intensity of 15 μmol photons m^{-2} s^{-1}. *Palmophyllum crassum* was collected from a Takahama fishing port in Matsuyama, Ehime Prefecture, Japan.

Spectrophotometric measurements

Absorption spectra of leaves of *Arabidopsis*, thylakoid membranes of *Bryopsis* and cells of other green algae were recorded at room temperature with a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) equipped with an opal glass filter. Absorption of the PSII dimer of *Nephroselmis* was also recorded with the same spectrophotometer. The 77 K low-temperature fluorescence spectra of leaves of *Arabidopsis*, thylakoid membranes of *Bryopsis*, cells of other green algae and gel slices containing the

chlorophyll-protein complexes of those organisms were recorded with a fluorescence spectrophotometer (F-2500, Hitachi, Japan).

Isolation of thylakoid membranes

Arabidopsis thylakoid membranes were prepared from the leaves of 4-week-old plants. Thylakoid membranes of *Bryopsis* and *Palmophyllum* were prepared from logarithmic-phase cells. Leaves of *Arabidopsis* and cells of *Bryopsis* and *Palmophyllum* were homogenized using a glass homogenizer with 50 mM Tricine-NaOH (pH 8.0) at 4°C. The homogenate was immediately filtered through Miracloth (Merck, Darmstadt, Germany) and then centrifuged at 21,600 ×*g* for 2 min at 4°C. Thylakoid membranes of the other green microalgae were prepared from logarithmic-phase cells. The cells were harvested by centrifugation at 2,000 ×*g*. With the exception of *Chlorella*, cells of green microalgae were broken up with 0.5 mg glass beads (100 μm in diameter) in each growth medium at 4°C using a Mini-Bead Beater (Kunugi et al. 2013). *Chlorella* cells were frozen in liquid nitrogen and broken up with 0.5 mg glass beads (500 μm in diameter) in CCA medium at 4°C using the Mini-Bead Beater (Honjoh et al. 1995). After removal of beads and unbroken leaves and cells, supernatants were centrifuged at 21,600 ×*g* for 2 min at 4°C. The green

pellets were washed twice with 5 mM EDTA-2Na and used as thylakoid membranes.

Native-green gel electrophoresis

Thylakoid membranes of *Arabidopsis*, *Mesostigma*, *Klebsormidium, Nephroselmis*, *Prasinoderma*, *Pseudoscourfieldia*, *Pyramimonas*, *Micromonas* and *Bryopsis* were solubilized in 0.5% SDS at 4°C. Thylakoid membranes of *Palmophyllum* were solubilized in 1.0% SDS at 4°C, while those of *Chlorella* and *Chlamydomonas* were solubilized in 0.5% β-dodecyl maltoside at 4°C. The solubilized thylakoid membranes were centrifuged at 21,600 ×*g* for 5 min at 4°C, and the supernatants were applied to an 8% polyacrylamide disk gel (5 mm in diameter) containing 0.1% SDS. Electrophoresis was conducted according to a previous study (Kunugi et al. 2013) at 4°C for approximately 1.5 h at 0.5 mA/tube, with 0.1% SDS used as the upper and lower reservoir buffer.

SDS-PAGE using non-heated protein samples

The thylakoid membranes of *Nephroselmis*, *Prasinoderma* and *Pyramimonas* were solubilized in sample buffer (0.06 M Tris-HCl [pH 6.8], 1% [w/v] SDS and 10% [w/v] glycerol) at room temperature for 5 min. After centrifugation at 21,600 ×*g* for 5 min, the

supernatants were loaded onto a 10% polyacrylamide slab gel. SDS-PAGE was performed using the Laemmli buffer system. Anti-Lhca2 and anti-Lhcb3 antibodies were purchased from Agrisera (Vännäs, Sweden). Immunoblotting using anti-PsaA/B antibodies was performed as described previously (Takabayashi et al. 2011).

CN-PAGE and two-dimensional SDS-PAGE

CN-PAGE was performed as previously described (Järvi et al. 2011) with some modifications. Briefly, the thylakoid membrane proteins (corresponding to 6 μg chlorophyll) of *Nephroselmis* were suspended in ice-cold resuspension buffer A containing 50 mM imidazole-HCl (pH 7.0), 20% glycerol, 5 mM 6-aminocaproic acid and 1 mM EDTA-2Na and were then solubilized with 4% (w/v) β-DM and 0.1% Triton X-100 on ice for 5 min. The cathode buffer contained 0.05% β-DM and 0.05% sodium deoxycholate. For the second dimension using SDS-PAGE, the CN gel strips were soaked for 1 h at 37°C in a solution containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and run on a two-dimensional SDS-polyacrylamide gel (14% polyacrylamide and 6 M urea) using the Laemmli buffer system (Takabayashi et al. 2011). After the two-dimensional SDS-PAGE, the gels were stained with a Pierce Silver Stain kit for mass spectrometry (Thermo Fisher Scientific, USA) according to the

manufacturer's instructions.

Cloning of *CAO* cDNAs from green algae

Total RNA was isolated from *Mesostigma*, *Klebsormidium*, *Palmophyllum*, *Nephroselmis*, *Prasinoderma*, *Pseudoscourfieldia*, *Pyramimonas* and *Bryopsis* cells using a RNeasy Plant Mini kit (Qiagen, Germany). With the exception of *Palmophyllum* and *Bryopsis*, the corresponding cDNAs were synthesized from total RNA using a PrimeScript II 1st strand cDNA Synthesis kit (Takara, Japan). Messenger RNA of *Palmophyllum* was purified from total RNA using a Dynabeads mRNA purification kit (Life Technologies, USA). Complementary DNAs of *Palmophyllum* and *Bryopsis* were respectively synthesized from mRNA or total RNA using SuperScript III reverse transcriptase (Life Technologies, USA). The partial *CAO* genes of *Palmophyllum* and *Bryopsis* were isolated by degenerate PCR, with the degenerate primers (Supplementary Table S3) designed from sequences of *CAO* genes conserved across land plants and green algae. Partial or full-length CAO genes of the remaining organisms were obtained from GenBank under the following accession numbers: *Arabidopsis* (BT002075.1), *Chlamydomonas* (AB015139.1), *Chlorella* (XM_005843991.1), *Klebsormidium* (AB453278.1), *Micromonas* (XM_003058156.1 and XM_003060828.1), *Mesostigma*

(AB453277.1), *Nephroselmis* (AB453267.1), *Prasinoderma* (AB453271.1), *Pseudoscourfieldia* (AB453273.1) and *Pyramimonas* (AB453269.1). To obtain their full-length cDNA sequences, a GeneRacer Kit (Invitrogen, USA) using gene-specific primers (Supplementary Table S3) was used to perform 5′- and 3′-rapid amplification of cDNA ends (RACE) of *Mesostigma*, *Nephroselmis*, *Palmophyllum*, *Pseudoscourfieldia*, and *Pyramimonas CAO* genes as well as 5′- RACE of *CAO* genes of *Klebsormidium* and *Prasinoderma*. The manufacturer's protocol was followed in all procedures. Full-length cDNA of the *Bryopsis CAO* gene was isolated by the biotinylated cap-trapper method (Yamamoto et al. 2009).

Pigment determination

Pigments were extracted from *Arabidopsis* leaves by pulverization in acetone using a ShakeMaster grinding apparatus (BioMedical Science, Japan) and from cells of green algae by suspension in acetone. Pigments were extracted from native-green or SDS gel slices by crushing the slices in 50 mM MOPS-KOH (pH 7.0) at 4°C with a plastic pestle before addition of acetone. To extract pigments from CN-PAGE gel slices, the slices were crushed in 50 mM MOPS-KOH (pH 7.0) and 0.05% β-DM at 4°C with a plastic pestle before adding acetone. After centrifugation at 21,600 ×*g* for 5 min at 4°C, the

supernatants were loaded onto a Symmetry C8 column (4.6 × 150 mm; Waters, Japan). For chlorophyll *a* and chlorophyll *b* determination, supernatants were separated with an isocratic flow of solvent B (20% methanol, 60% acetonitrile and 20% acetone) for 6.5 min at a flow rate of 1.2 mL/min. Carotenoids were also separated according to this previously described method (Zapata et al. 2000). Elution profiles were monitored by measuring absorbance at 650 nm (for chlorophylls) and 450 nm (for carotenoids) using a diode array detector (SPD-M20A, Shimadzu, Japan), with the pigments identified according to their retention times and spectral patterns. Pigment quantification was performed using peak areas as previously described (Tanaka et al. 2010).

Multiple sequence alignment and phylogenetic analysis

Sequences of the catalytic domain of CAO proteins were aligned using M-Coffee (Moretti et al. 2007) with default settings. Regions with ambiguous alignments were manually truncated. The resulting alignment is shown in Fig. S5B. A phylogenetic tree was constructed in MEGA v6.1 (Tamura et al. 2013) using the maximum likelihood method under the LG substitution model (Le and Gascuel 2008) with discrete gamma distribution in eight categories and 2,000 bootstrap replicates (Supplementary Fig. S5A). Some gaps were eliminated by partial deletion with a site coverage cut-off of 80%. A

multiple sequence alignment of CAO N-terminal sequences was generated using Muscle in MEGA v6.1 with default settings and manual refinement (Fig. 5 and Supplementary Fig. S6).

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[Disclosures]

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Table 1 Chlorophyll *a*/*b* ratios in *Arabidopsis* leaves and green algae whole cells.

	Streptophytes			Freshwater chlorophytes		Seawater chlorophytes					
	Arab	*Kleb*	*Meso*	*Chlo*	*Chla*	*Palm*	*Neph*	*Pras*	*Pseu*	*Pyra*	*Micr*	*Bryo*
Chl *a*/*b* ratio	2.92	2.10	3.40	3.25	2.58	0.64	1.02	1.13	1.14	1.29	1.10	1.06

Arab, *Arabidopsis*; *Kleb*, *Klebsormidium*; *Meso*, *Mesostigma*; *Chlo*, *Chlorella*; *Chla*, *Chlamydomonas*, *Palm*, *Palmophyllum*; *Neph*, *Nephroselmis*, *Pra*, *Prasinoderma*; *Pseu*, *Pseudoscourfieldia*; *Pyra*, *Pyramimonas*; *Micr*, *Micromonas*; *Bryo*, *Bryopsis*.

Table 2 Chlorophyll *a*/*b* ratios of photosystem I (PSI) core antennas and LHCII trimers in *Arabidopsis* and green algae.

	Streptophytes	Freshwater	Seawater chlorophytes

		chlorophytes											
	Sample	*Arab*	*Kleb*	*Meso*	*Chlo*	*Chla*	*Palm*	*Neph*	*Pras*	*Pseu*	*Pyra*	*Micr*	*Bryo*
Chl *a*/*b*[*1]	PSI core	22.5	15	18.4	19.1	20.16	2.04	3.08	5.05	6.26	5.61	4.72	6.68
	LHCII	1.15	1.50	1.38	1.12	1.24	0.36	0.67	0.86	0.71	0.61	0.80	0.73
Chl *a*/*b*[*2]	PSI core							2.8	2.8		0.5		

Arab, *Arabidopsis*; *Kleb*, *Klebsormidium*; *Meso*, *Mesostigma*; *Chlo*, *Chlorella*; *Chla*, *Chlamydomonas*, *Palm*, *Palmophyllum*; *Neph*, *Nephroselmis*, *Pra*, *Prasinoderma*; *Pseu*, *Pseudoscourfieldia*; *Pyra*, *Pyramimonas*; *Micr*, *Micromonas*; *Bryo*, *Bryopsis*. PSI core antennas (heterodimers of PsaA/PsaB) and LHCII trimers (LHCII) were separated by native green gel electrophoresis. After pigment extraction from the corresponding bands, chlorophyll *a*/*b* ratios (*[1]) were determined. Alternatively, chlorophyll *a*/*b* ratios (*[2]) of the PSI core antennas were determined after purification by non-heated SDS-PAGE.

[Figure legends]

Fig. 1 Chlorophyll protein complexes separated by native green gel electrophoresis. Thylakoid membranes of Arabidopsis and other green algae were separated by native green gel electrophoresis. The red asterisk indicates the PSI core, whereas the blue closed circle indicates LHCII trimers.

Figure 2 Separation of PSI core antennas of *Prasinoderma*, *Pyramimonas* and *Nephroselmis* by non-heated SDS-PAGE. The PSI core antennas were purified by

SDS-PAGE and their chlorophyll contents were analyzed. (A) Separation of PSI core antennas of *Prasinoderma* (Pra) and *Pyramimonas* (Pyr) by non-heated SDS-PAGE. An asterisk indicates the green bands of PSI core antennas. (B) Immunoblot analysis using anti-PsaA/PsaB, anti-Lhca2 and anti-Lhcb3 antibodies. (C) Separation of the *Nephroselmis* PSI core antenna by non-heated SDS-PAGE. An asterisk indicates the green bands of the PSI core antenna. (D) Immunoblot analysis using anti-PsaA/PsaB. (E) Two-dimensional SDS-PAGE followed by Coomassie Brilliant Blue staining was performed using a 14% acrylamide gel containing 4M urea. The 4M urea was used for denaturation of PsaA and PsaB proteins.

Fig. 3 77-K fluorescence emission spectra of *Arabidopsis* leaves, *Bryopsis* thylakoid membranes and cells of other green algae. The excitation wavelength was set to 470 nm for preferential excitation of chlorophyll *b*.

Fig. 4 77-K fluorescence emission (A) and excitation (B) spectra and absorptance (1 − transmittance) (C) of the PSII core antenna (PSII monomer) electrophoretically purified from *Nephroselmis*. Excitation wavelengths were set to 440 nm (solid line) and 470 nm (dashed line). The emission peak was at 688 nm.

Fig. 5 Comparison of CAO amino acid sequences corresponding to the degron and its surrounding region in the regulatory domain of *Arabidopsis* CAO. CAO sequence regions in green plants corresponding to amino acids 90–141 of *Arabidopsis* CAO were compared. The degron sequence (Q^{97}DLLTIMILH^{106}) in *Arabidopsis* and corresponding sequences in streptophytes are indicated by a blue box. *Physcomitrella patens* and *Selaginella moellendorffii* are early-diverging land plants. Black shading indicates residues conserved in more than half of aligned sequences. *Prasinoderma*, *Pseudoscourfieldia* and *Micromonas* CAO sequences are not shown because no significant similarity was found between them and other CAO sequences.

Fig. 6 Molecular evolution of photosystems and CAO in green plants. The conserved region of the CAO regulatory domain was present in the common ancestor of green plants. As an adaptation to high-light environments, streptophytes have developed a degron sequence, excluded chlorophyll *b* from their core antennas, and acquired red chlorophylls in photosystem I (PSI). To efficiently harvest blue-green light energy, early-branching chlorophytes have incorporated large quantities of chlorophyll *b* into their core antennas. Their CAO N-terminal sequences have been substantially shortened

or altered. Seawater chlorophytes have incorporated substantial amounts of chlorophyll *b* into their core antennas, whereas freshwater chlorophytes have excluded this pigment from their core antennas and retained red chlorophyll in PSI.