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Review

Chlorophyll cycle regulates the construction and destruction of the light-harvesting complexes☆

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ABSTRACT

Chlorophyll *a* and chlorophyll *b* are the major constituents of the photosynthetic apparatus in land plants and green algae. Chlorophyll *a* is essential in photochemistry, while chlorophyll *b* is apparently dispensable for their photosynthesis. Instead, chlorophyll *b* is necessary for stabilizing the major light-harvesting chlorophyll-binding proteins. Chlorophyll *b* is synthesized from chlorophyll *a* and is catabolized after it is reconverted to chlorophyll *a*. This interconversion system between chlorophyll *a* and chlorophyll *b* refers to the chlorophyll cycle. The chlorophyll *b* levels are determined by the activity of the three enzymes participating in the chlorophyll cycle, namely, chlorophyllide *a* oxygenase, chlorophyll *b* reductase, and 7-hydroxymethyl-chlorophyll reductase. This article reviews the recent progress on the analysis of the chlorophyll cycle and its enzymes. In particular, we emphasize the impact of genetic modification of chlorophyll cycle enzymes on the construction and destruction of the photosynthetic machinery. These studies reveal that plants regulate the construction and destruction of a specific subset of light-harvesting complexes through the chlorophyll cycle. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

1. Introduction

There is remarkable variation in chlorophyll species in oxygenic photosynthetic organisms under aquatic environments (Fig. 1). Chlorophyll *a* (monovinylchlorophyll *a* or chlorophyll a_1) is common to nearly all oxygenic photosynthetic organisms. (It should be noted that some reaction center chlorophylls have the opposite configuration at C13[1] [1,2].) In rhodophytes, glaucophytes and most cyanobacteria, chlorophyll *a* is the only chlorophyll species functioning in the photosystems [3]. Marine cyanobacteria belonging to the *Prochlorococcus* genus mainly use 8-vinyl chlorophyll *a* (divinyl chlorophyll *a* or chlorophyll a_2), and 8-vinyl chlorophyll *b* [4]. Two other types of cyanobacteria, *Prochlorothrix hollandica* and *Prochloron dedemni* [5] produce chlorophyll *b* in addition to chlorophyll *a*. In another cyanobacterium *Acaryochloris marinus* [6], the major species of chlorophyll is chlorophyll *d*, in which a formyl group replaces the 3-vinyl group of chlorophyll *a*. A variety of algae contain chlorophyll c_1, c_2 and c_3 in which a phytol chain is absent and instead a free 7-acryl acid is conjugated (see Beale for review, [7]). Recently, the first new type of chlorophyll species has been discovered since sixty years, which is named chlorophyll *f* [8]. This chlorophyll species was found in stromatolite containing cyanobacteria and other microorganisms. A precursor of chlorophyll biosynthesis, divinyl-protochlorophyllide [9] has been also suggested to function as a photosynthetic pigment.

In contrast to photosynthetic organisms living in an aquatic environment, land plants have been conservative in terms of the evolution of chlorophyll species which make up their photosynthetic machinery. Specifically, land plants exclusively utilize chlorophyll *a* and chlorophyll *b*. (In this article, monovinyl chlorophyll *a* and chlorophyll *b* are referred to as chlorophyll *a* and chlorophyll *b*). Though other chlorophyll derivatives might have only transiently occurred during their evolutionary history, why did land plants evolve to utilize exclusively chlorophyll *a* and *b* species? The biological importance of chlorophyll *a* seems obvious: chlorophyll *a* is necessary for the photochemistry in oxygenic photosynthetic organisms, with the only known exception of *A. marinus* which utilizes both chlorophyll *d* and chlorophyll *a* for the photochemistry [10]. (For a detailed discussion which highlights the importance of chlorophyll *a* in photochemistry, see Björn et al. [11].) Now that we have stated the importance of chlorophyll *a* in photochemistry, it is important to consider why land plants would evolve to also specifically use chlorophyll *b*. Considering that nearly all land plants retain the genetic ability to synthesize both chlorophyll *a* and *b*, it would be reasonable to assume that chlorophyll *b* also plays an essential role in land plants. It is possible that the utilization of chlorophyll *b* in land plants provides an advantage by enabling them to harvest a wider range of light due to the different absorption spectrum of chlorophyll *b* from that of chlorophyll *a* (Fig. 2). Chlorophyll *b* has a strong absorption around 450 nm which is a region of light that chlorophyll *a* does not efficiently absorb. However, if we take into account that carotenoid species in land plants can absorb the blue light

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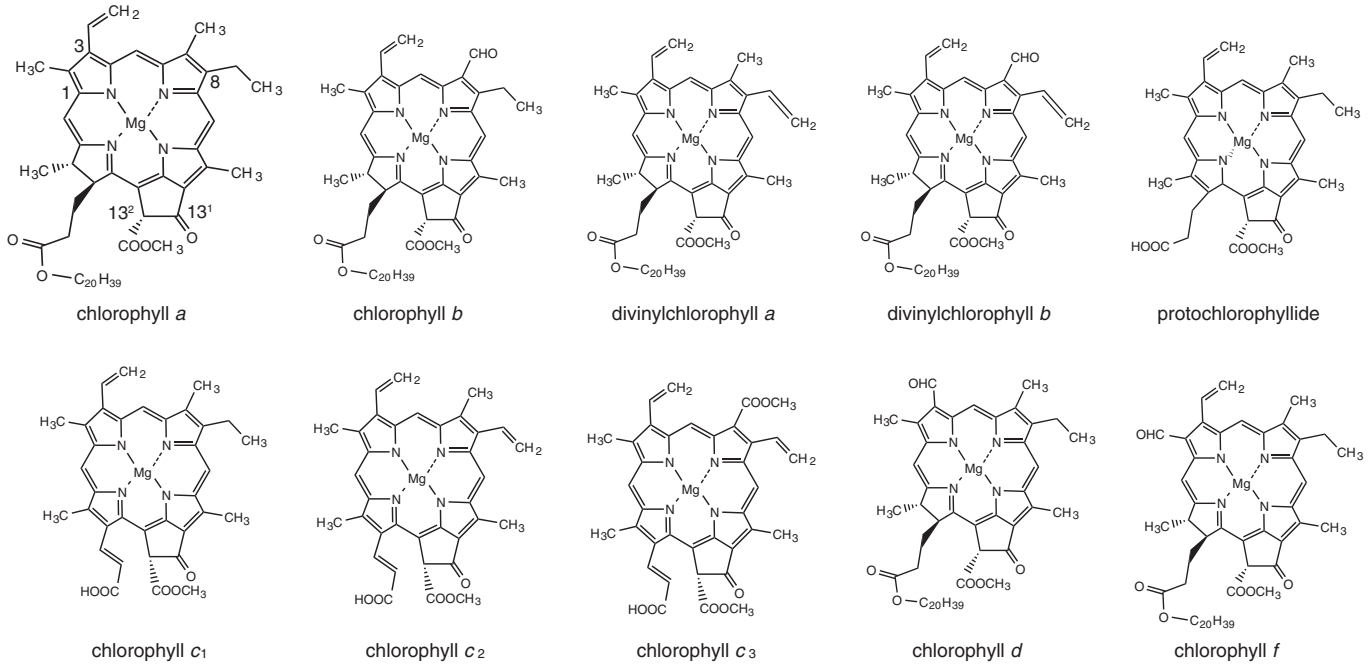

Fig. 1. Chlorophyll species found in oxygenic photosynthetic organisms. It should be noted that some reaction center chlorophylls have the opposite configuration at C13^1 [1,2]. Several carbon atoms are numbered on the structure of chlorophyll *a* according to the International Union of Pure and Applied Chemistry (IUPAC) numbering system.

region compensating for the absorption of chlorophyll *b*, this property of chlorophyll *b* does not sufficiently explain the necessity of plants to maintain chlorophyll *b* throughout the evolution of land plants. A more likely explanation for the need of chlorophyll *b* comes from the study of the structure of light-harvesting complex (LHC) proteins and from the studies with mutants and transgenic plants in which chlorophyll *b* metabolism was genetically modified. These studies suggested that the biosynthesis and breakdown of chlorophyll *b* is tightly linked with the turnover of LHC. This feature of chlorophyll *b* may be attributed to the chemical property of chlorophyll *b* which enables this pigment to bind more tightly to LHC than other chlorophyll species (see Hoober et al., [12] for extensive discussion on this aspect). Accordingly, the metabolism of chlorophyll *b* forms an essential part of light acclimation mechanisms in plants. In this article we provide an overview on the metabolism of chlorophyll *b* and its regulation. We then describe how chlorophyll *b* is related to the construction and destruction of LHC and thylakoid membranes.

2. Chlorophyll *b* biosynthesis

In photosynthetic eukaryotes, chlorophyll biosynthesis occurs within the chloroplast. Chlorophyll biosynthesis begins with the reduction of glutamyl-tRNA into glutamate-1-semialdehyde and is followed by eleven subsequent steps of enzymatic reactions leading to the formation of chlorophyllide *a*, which is an immediate precursor of chlorophyll *a* in the biosynthetic pathway [13] (Fig. 3). The attachment of a phytol side chain to this molecule by the action of chlorophyll synthase completes the biosynthesis of chlorophyll *a*. It has been proposed that chlorophyllide *a* is also an intermediate of chlorophyll *b* biosynthesis. Oster et al. [14] showed that chlorophyllide *a* oxygenase converts the 7-methyl group of chlorophyllide *a* into a formyl group in a two-step oxygenation reaction, which results in the formation of chlorophyllide *b*. Chlorophyllide *b* is then ligated with a phytol side chain to form chlorophyll *b* by chlorophyll synthase. The proposal for the biosynthetic pathway of chlorophyll *b* was based upon results from *in vivo* experiments in which CAO reacts with chlorophyllide *a*, but not with chlorophyll *a*. However, a possibility that CAO reacts with chlorophyll *a* *in vivo* cannot be excluded, since it is possible that chlorophyll *a* did not react with CAO in the *in-vitro* experiments mentioned above due to its hydrophobic nature. If chlorophyll *a* binds to certain proteins *in vivo* to which CAO is accessible, it is possible that CAO may be able to react with chlorophyll *a*. The conversion of chlorophyll *a* to chlorophyll *b* was observed when the de novo synthesis of chlorophyll was stopped under darkness [15–18]. Under the assumption that CAO does not react with chlorophyll *a*, without the de novo synthesis of chlorophyll, the chlorophyll *a* to *b* conversion should go through chlorophyllide *a* and chlorophyllide *b* (Fig. 3). In this route, chlorophyllase activity that detaches the phytol side chain from chlorophyll should be involved in the first step of the chlorophyll *a* to chlorophyll *b* conversion. However, recent findings did not support the presence of chlorophyllase in the chloroplast [19,20]. If chlorophyllase activity is not present in the chloroplast, it would be reasonable to assume that CAO is able to react with chlorophyll *a* and convert it to chlorophyll *b*. The presence of chlorophyllase activity is still under debate [21]. Further studies are necessary to clarify the precise route for the conversion of chlorophyll *a* to *b*.

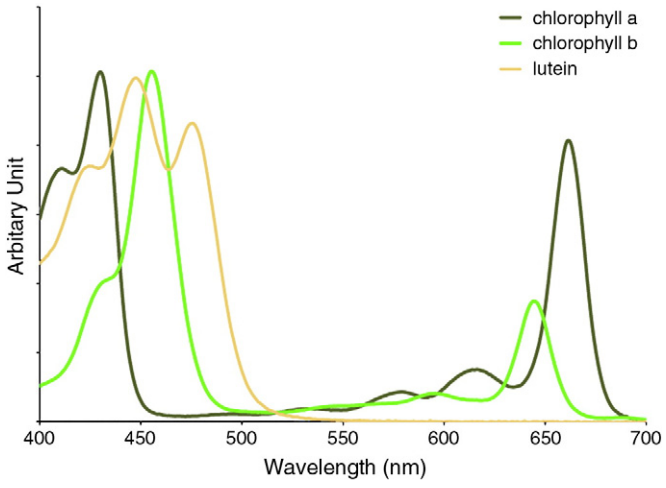

Fig. 2. Absorption spectra of chlorophyll *a*, chlorophyll *b* and lutein in acetone.

CAO is the only enzyme known to catalyze the conversion of chlorophyll(ide) *a* to chlorophyll(ide) *b*. All chlorophyll *b* biosynthesizing organisms including land plants, green algae and prochlorophytes (chlorophyll-*b* producing cyanobacteria) posses *CAO* genes or their homologues [22,23]. Insertion or deletion mutations in the *CAO* genes resulted in the complete loss of chlorophyll *b* in *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*, indicating that CAO is the only enzyme responsible for the formation of chlorophyll(ide) *b* in these organisms [14,24,25].

3. Chlorophyll *b*-to-chlorophyll *a* conversion

Chlorophyll *b* is capable of being reconverted to chlorophyll *a* within the chloroplast. The entire suite of inter-conversion reactions between chlorophyll *a* and chlorophyll *b* are referred to as the chlorophyll cycle (Fig. 4). In the reverse reactions from chlorophyll *b* to chlorophyll *a*, the 7-formyl group of chlorophyll *b* is first reduced to a hydroxyl group by the action of chlorophyll *b* reductase, which belongs to the short-chain dehydrogenase superfamily [26]. This intermediate compound is 7-hydroxymethyl chlorophyll *a* (HMChl), which is also an intermediate of the two-step reaction of CAO. The HMChl compound is then reduced to chlorophyll *b* by the action of an HMChl reductase (HCAR) enzyme that still remains to be identified.

Plants have two isoforms of chlorophyll *b* reductase, which are termed NYC1 and NOL, respectively [26]. The NYC1 isoform has three putative membrane-spanning domains, while NOL does not contain any predictable membrane-spanning domains [26]. In rice, NYC1 and NOL are proposed to function as a heterodimer. This model is based on the observation that rice mutants lacking either NYC1 or NOL are deficient in chlorophyll *b* reductase activity during leaf senescence [27]. Furthermore, the interaction of NYC1 and NOL was confirmed by an *in vitro* immunocoprecipitation experiment with recombinant NYC1 and NOL proteins [27]. Since the recombinant NOL enzyme has shown *in vitro* chlorophyll *b* reductase activity in the absence of NYC1, it is possible that NOL could function independently of NYC1 [26,28]. It is possible that the heterodimer formation of NYC1 and NOL is necessary only under specific developmental conditions such as leaf senescence.

4. Regulation of the chlorophyll cycle

The balance between chlorophyll *a* and chlorophyll *b* can be theoretically determined by the activity of both forward and backward reactions of the chlorophyll cycle. As described below, several lines of evidence have demonstrated the rate of the forward reaction appears to determine the chlorophyll *a* to *b* ratios under normal growth conditions in *Arabidopsis* (e.g. 23 °C, 100 μE m^{-2} s^{-1}). An elevation in the amount of CAO protein levels results in a drastic reduction in chlorophyll *a* to *b* ratios from approximately 3.0 to ≤1.0 (25% chlorophyll *b* to more than 50% chlorophyll *b* on the total chlorophyll basis) [29]. In contrast, the *Arabidopsis* mutant lacking both NYC1 and NOL shows chlorophyll *a* to *b* ratios that are similar to wild type plants during their vegetative phase [28]. These data indicate that neither NYC1 nor NOL contribute to the regulation of the chlorophyll *a* to *b* ratios under such conditions. At the present time, NYC1 and NOL are considered to play an essential role in the chlorophyll cycle only when chlorophyll is in the process of being actively degraded.

CAO activity is mainly regulated at the level of protein stability. When full-length *CAO* cDNA was overexpressed in *Arabidopsis*, chlorophyll *a* to *b* ratios were only slightly reduced to 2.7 (27% chlorophyll *b*) [30,31]. This minor reduction occurred despite the observation that *CAO* mRNA

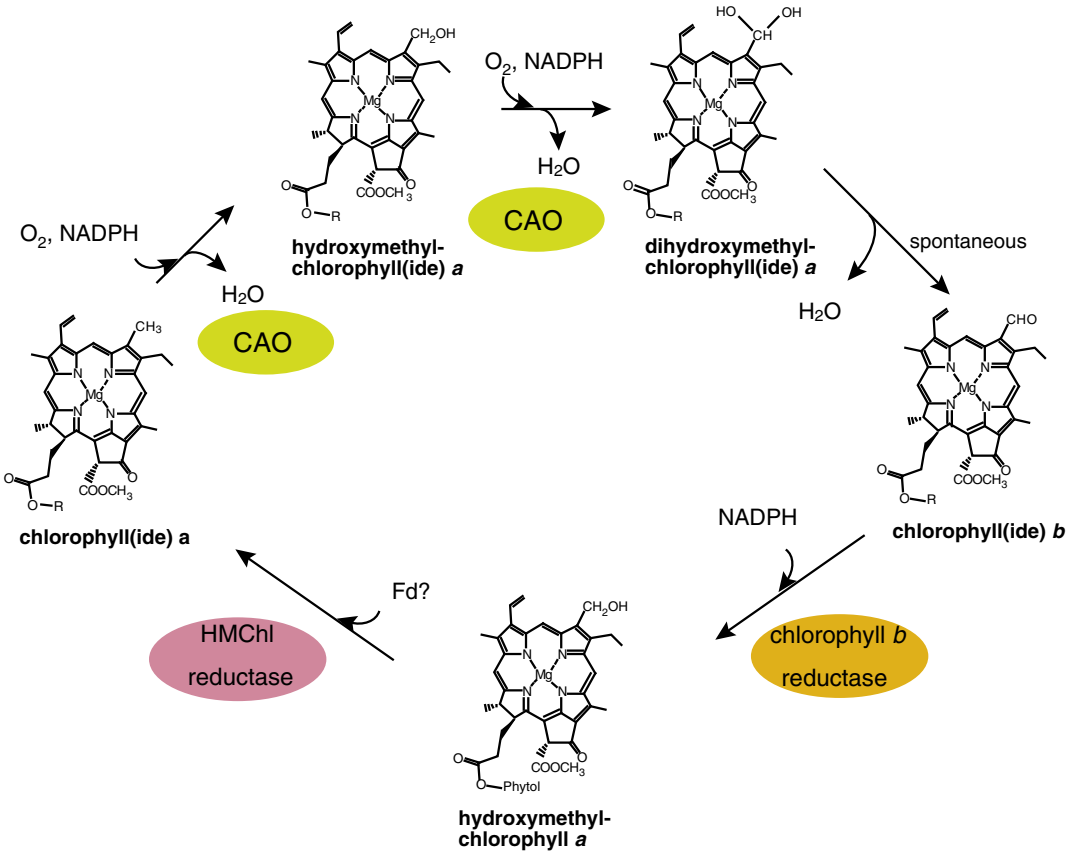

Fig. 3. Proposed biosynthetic route of the chlorophyll cycle. As described in the main text, the forward reaction of the chlorophyll cycle may occur with chlorophyll or chlorophyllide. The chlorophyll to chlorophyllide conversion and its reverse reaction are catalyzed by chlorophyllase and chlorophyll synthase, respectively, both of which reactions are not depicted in the diagram. R indicates either proton or phytol. Fd indicates ferredoxin.

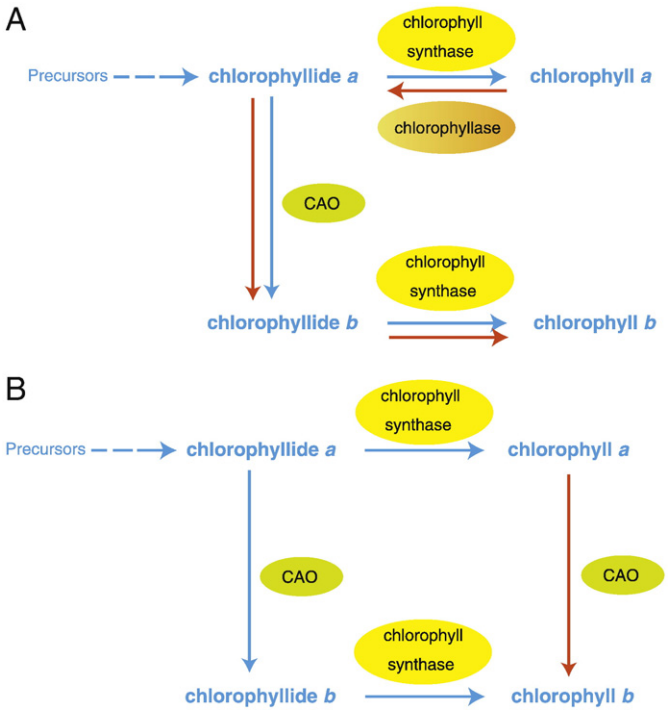

Fig. 4. Possible two routes for chlorophyll *a* to *b* conversion. In the route described in panel A, chlorophyll *a* is first dephytilated by chlorophyllase, and then converted to chlorophyll *b* by the action of CAO and chlorophyll synthase. In the other route described in panel B, chlorophyll *a* is directly converted to chlorophyll *b*.

levels in the overexpression plants increased approximately three-fold relative to wild type [31]. These results indicate that transcriptional control does not play a major role in the regulation of CAO activity and that the level of regulation must occur post-transcriptionally. Similarly, when the *CAO* gene is overexpressed in tobacco, only minor effects on the chlorophyll *a* to *b* ratios were observed [32]. In contrast, overexpression of a truncated *CAO* cDNA, which lacks the sequence encoding the N-terminal domain of CAO, resulted in a significant increase in CAO protein levels and chlorophyll *b* production [30]. These data clearly show that the N-terminal domain (referred to as the A domain) negatively regulates the accumulation of CAO protein (Fig. 5). Suppression of CAO protein levels by the A domain is dependent on the accumulation of chlorophyll *b*. When only the A domain was overexpressed in wild-type *Arabidopsis* plants, the A domain protein did not accumulate in the transgenic plants. In contrast, when the same construct was overexpressed in the *chlorina1* mutant, which contains a disruption in the endogenous *CAO* gene, the transgenic A domain protein accumulated to a significant level [30]. These experiments demonstrate that the A domain feedback-regulates accumulation of the CAO protein. It has been also shown that a specific ten amino acid sequence (QDLLTIMILH) which is located in the middle of the A domain is essential in this aforementioned A domain-induced degradation mechanism [33]. Attachment of this sequence to green fluorescent protein (GFP), which was specifically targeted to the chloroplast, resulted in destabilization of GFP. These data demonstrated that this sequence alone can function as a degron sequence for chloroplast proteases. Interestingly, this degron–GFP fusion was destabilized in both wild-type *Arabidopsis* and the CAO-deficient mutant. These results were in contrast to data obtained from the fusion protein consist of the GFP and the A domain, which was destabilized in wild type but stabilized in the *chlorina1* mutant [34]. Although these results show that the degron sequence of CAO is sufficient to induce proteolysis, the whole A domain, or at least another region of the A domain, is necessary for the chlorophyll *b*-dependent destabilization mechanism. It is hypothesized that the A domain "hides" the degron sequence from a specific protease system when there are insufficient amounts of chlorophyll *b*. At the present time, the detailed mode of interaction between chlorophyll *b* and the A domain of CAO remains unidentified. It is possible that chlorophyll *b* binds directly to the A domain as a means to alter the conformation of the A domain. Another possibility is that an additional unidentified protein mediates the presence of chlorophyll *b* to the A domain.

It is likely that the chloroplast Clp protease is involved in the destabilization of CAO. This hypothesis is supported by the observation that a mutant lacking the regulatory domain of Clp protease is partially impaired in CAO degradation [35]. Since Clp protease is involved in the degradation for a wide range of chloroplast proteins [36], it is tempting to speculate that other chloroplast proteins also possess similar degrons. Sakuraba et al. [33] identified a similar short sequence within the N-terminal region of CP47 that resembles the degron of CAO. Attachment of this degron-like sequence of CP47 to GFP moderately destabilized this fusion protein *in planta* [33]. As a result, it is likely that the degron-like

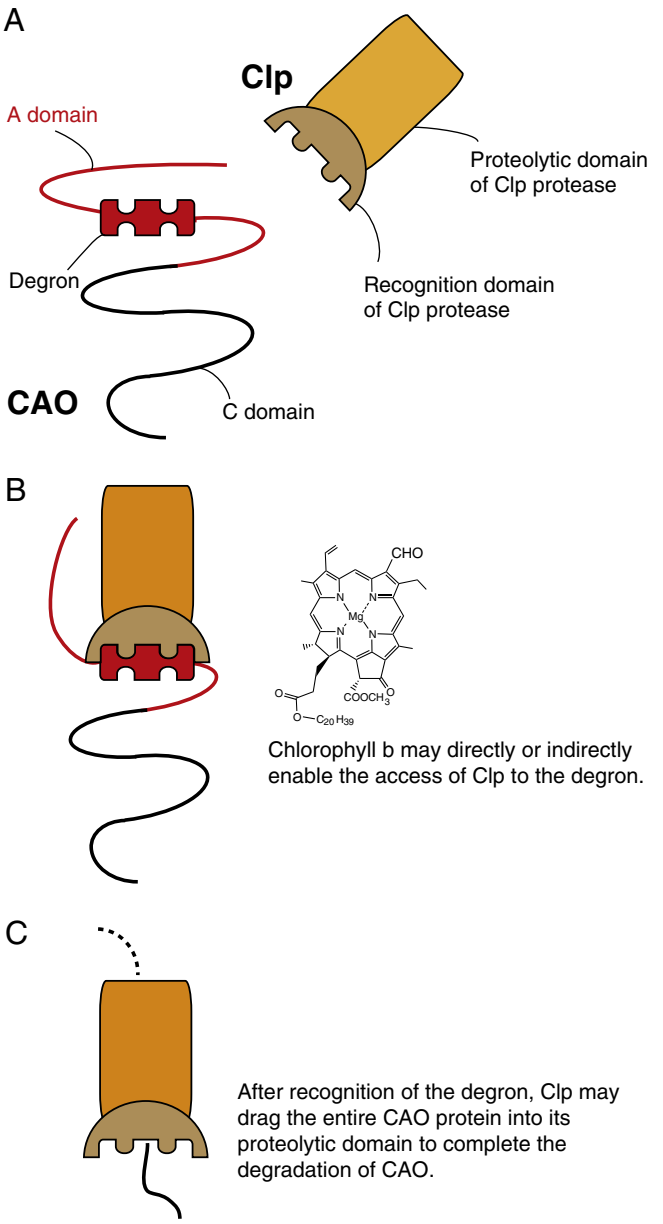

Fig. 5. A tentative model for chlorophyll *b*-dependent CAO degradation. A. In the absence of chlorophyll *b*, Clp may not be able to interact with the degron which is located in the middle of the A domain of CAO. B. Chlorophyll *b* directly or indirectly enables the access of Clp to the degron. C. After recognition of the degron, Clp drags the whole CAO protein into its proteolytic domain and digests CAO.

sequence of CP47 enables the rapid turnover of the CP47 protein. It should also be noted that the degron sequence of CAO is similar to the degron sequences for Clp proteases from *Salmonella typhimurium* and *Escherichia coli* [37]. Therefore, it is reasonable to hypothesize that chloroplasts inherited both Clp protease and its degron sequences from their ancestors and have adopted these proteolytic systems for specific regulation of the chlorophyll cycle.

Although the A domain is present in the CAO sequences of land plants, it is absent from cyanobacterial sequences, indicating that this sequence has been acquired after the evolution of photosynthetic eukaryotes [38]. It is important to note that the CAO sequences of green algae also contain N-terminal extensions, however none of these has been tested and confirmed to function as degrons. Thus, it is hypothesized that both land plants and possibly green algae may have acquired a sensitive feedback mechanism to regulate the chlorophyll *b* levels by incorporating degron sequences from other protein sequences after the evolution of photosynthetic eukaryotes.

In contrast to the forward reaction (chlorophyll *a* to *b*) of the chlorophyll cycle, little is known about the regulation of the backward reaction (chlorophyll *b* to *a*). Since the phenotype of the *Arabidopsis* and rice mutants lacking chlorophyll *b* reductase is only profound during leaf senescence [26,28], it is likely that chlorophyll *b* reductase activity is induced during leaf senescence. According to the public microarray database in the Nottingham Arabidopsis Stock Centre (http://affymetrix.arabidopsis.info/), the *Arabidopsis NYC1* gene is induced during leaf senescence while the *NOL* gene is generally expressed at constitutive levels. It is possible that the activity of the NYC1 isoform of chlorophyll *b* reductase is regulated at a transcriptional level, whereas the NOL isoform is regulated at a different level. Alternatively, NOL gene expression might be regulated at different stages or conditions. HCAR activity is induced upon senescence [39]. It seems plausible that the activities of chlorophyll *b* reductase and HCAR are coordinated in their regulation, otherwise, imbalance of those activities may lead to accumulation of the intermediate of the pathway (HMChl). Since the HMChl compound is a photo-sensitizer, it is deleterious to cells when it is accumulated. Further investigation on the chlorophyll breakdown pathway, which includes the identification of HCAR, would be a prerequisite to understand the regulatory mechanism of the backward reaction of the chlorophyll cycle.

5. Chlorophyll cycle and construction of the photosynthetic apparatus

The close link existing between the activity of the chlorophyll cycle and the construction/destruction of the photosynthetic apparatus was first indicated by the study of a barley chlorophyll *b*-less mutant (*chlorina f2*) which was isolated by Highkin [40]. Thornber and Highkin [41] found that this mutant lacked the majority of light-harvesting complexes (LHC). Although Bellemare et al. [42] observed that expression of *Lhc* genes and the import of the LHC apoproteins into the chloroplasts were not altered in the *chlorina f2* mutant, they determined that the turnover rates of several LHC proteins were increased. More specifically, Krol et al. [43] demonstrated that the accumulation of the most LHC subunits including Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb6, Lhca1, Lhca2, Lhca3 and Lhca4 was significantly reduced in this mutant (Table 1).

An exception to this was Lhcb5. This protein was barely affected in the *chlorina f2* mutant (Table 1). Bossman et al. [44] examined ten different alleles of *chlorina f2* and found that chlorophyll *b* levels correlated with LHC protein levels. In their analysis, Lhcb1 Lhcb6 and Lhca1 were the most prominently reduced among ten different LHC subunits. On the other hand, the Lhcb3, Lhcb5, Lhca1, Lhca2, and Lhca3 subunits were less affected. Tanaka and Tanaka [44] and Havaux et al. [45] obtained similar results with *Arabidopsis*, in which Lhcb1, Lhcb2, Lhcb3, and Lhcb6 were significantly reduced while Lhcb5 was not affected by chlorophyll *b* deficiency. The levels of other LHC subunits in *Arabidopsis* seem to be variable according to their growth conditions. It was shown that the major antenna complexes comprising Lhcb1, Lhcb2 and Lhcb3 were not formed in the chlorophyll *b* less mutants [46]. At the present time, it is not clear whether the other remaining LHC proteins form functional antenna complexes. Chlorophyll-*b* dependent accumulation of LHC proteins was observed in both dicotyledonous and monocotyledonous plants as it was described above, thus, the stabilization of LHC by chlorophyll *b* is likely a general rule in flowering plants. In contrast, a deficiency of chlorophyll *b* in green algae does not lead to a destabilization of LHC proteins. For example, in a chlorophyll *b*-less mutant strain of *C. reinhardtii*, the composition of LHC are similar to wild type [47]. For the future, it will be important to understand when and how plants acquired the chlorophyll *b*-dependent regulatory mechanism of LHC.

Studies on CAO-overexpressing plants further support the hypothesis that chlorophyll *b* biosynthesis regulates LHC levels. Specifically, an increase in the levels of chlorophyll *b* resulted in greater accumulation of the major LHC [29,31,48]. The effect of CAO overexpression on LHC levels is small under normal growth conditions, while its impact is more obvious under high-light conditions where LHC levels decrease in wild type [48]. Taken together, these studies demonstrate that there is a close correlation of the major LHC and chlorophyll *b* levels.

On the contrary, expression of genes encoding LHC proteins does not seem to correlate with their respective protein levels. Flachman and Kühlbrandt [49] and Flachman [50] reported that suppression of the *Lhcb1* gene via antisense *Lhcb1* cDNA expression in tobacco did not alter LHC protein levels, even though the *Lhcb1* mRNA level was reduced to less than 5% of the wild type level. Andersson et al. [51]

Table 1
Responses of the LHC protein levels to chlorophyll *b* deficiency in the barley *chlorina f2* mutant and the *Arabidopsis chlorina1* mutant. '—' denotes a trace amount, '+' denotes a reduced amount, and '++' denotes the wild type level. Light intensities of the growth conditions and the durations of illumination per day are indicated. n.d. means that the age of the plants were not clearly described in the reference.

Plant	Barley *chlorina f2*	Barley *chlorina f2*	*Arabidopsis chlorina1*	*Arabidopsis chlorina1*	*Arabidopsis chlorina1*
Light conditions for plant growth	200 μE/m^{-2} s^{-1}, (16 h)	75 μE/m^{-2} s^{-1}, (14 h)	100 μE/m^{-2} s^{-1}, (24 h)	1200 μE/m^{-2} s^{-1}, (24 h)	250 μE/m^{-2} s^{-1}, (8 h)
Age	2 w	1 w	3 w	3 w	n.d.
Lhcb1	−	−	−	−	−
Lhcb2	+	+	++	−	−
Lhcb3	+	+	++	−	−
Lhcb4	−	+	++	+	−
Lhcb5	++	++	++	++	++
Lhcb6	−	−	++	−	−
Lhca1	++	++	++	+	+
Lhca2	++	++	++	++	+
Lhca3	++	++	++	++	+
Lhca4	++	−	++	+	+
References	[43]	[44]	[48]	[48]	[46]

described a similar observation with one of their *Arabidopsis* antisense line in which both *Lhcb1* and *Lhcb2* mRNA levels were negligible but they retained detectable levels of Lhcb1 and Lhcb2 proteins. It can be hypothesized that plants synthesize an excess amount of *Lhcb* mRNAs and LHC apoproteins, but they degrade most (possibly more than 95% according to the data presented by Flachman [50]) of these mRNAs if they fail to form a proper conformation with appropriate pigments. While such a mechanism may appear to be a wasteful strategy for the plant, it is possible that this mechanism ensures that only LHC with chlorophyll pigments is properly bound and retained in thylakoid membranes. LHC with "vacant" pigment binding pockets, if present, may interfere with efficient energy transfer between pigments. Therefore, the removal of LHC apoproteins lacking chlorophyll *b* should be essential for plants to maintain photosynthetic efficiency. Taken together, it would be reasonable to hypothesize that chlorophyll *b* levels (at least partly) control the levels of LHC. It should be noted that the minimal level of LHC gene expression is a prerequisite for the synthesis of LHC protein. It was demonstrated in an *Arabidopsis* antisense-RNA expressing plant in which the *Lhcb1* and *Lhcb2* transcripts were reduced to almost undetectable levels [52]. In these plants, the Lhcb1 and Lhcb2 proteins were barely detectable [52].

The detailed mechanism of chlorophyll *b*-dependent accumulation of LHC remains unknown at this time. In one hypothesis, LHC does not form a proper conformation in the absence of chlorophyll *b*. Under these conditions, an LHC apoprotein will be recognized and degraded by specific proteases. Yang et al. [53] detected a specific protease activity using purified LHC as a substrate and found that such an activity is induced by the exposure of plants to higher light intensities. These findings are consistent with the observations with chlorophyll *b*-less mutants in which several LHC proteins were less stabilized and more degraded under strong illumination [48]. This presumable protease appears to specifically discriminate chlorophyll *b*-deficient LHC from those with chlorophyll *b*. Another line of evidence showing the effect of chlorophyll *b* on the LHC stability was obtained by an *in-vitro* import study. Kuttkat et al. [54] tested the stability of LHC apoprotein which was imported into the etioplast in the presence/absence of chlorophyll *a* or *b* derivatives. They determined that the chlorophyll *a* derivative was not required for the stable accumulation of LHC, but the absence of chlorophyll *b* led to destabilization of LHC apoprotein. A specific role of chlorophyll *b* was also inferred by a kinetic analysis in which LHC was reconstructed with chlorophyll *a* and *b in vitro* [55]. In this study, they demonstrated that chlorophyll *b* binds LHC more slowly but more tightly than chlorophyll *a*. Based on these results, the authors proposed that chlorophyll *a* and chlorophyll *b* sequentially bind to LHC apoproteins and it is the binding of chlorophyll *b* that finally stabilizes the conformation of LHC [55].

In another hypothesis, it has been proposed that destabilization of LHC occurs during the import of LHC apoprotein into the chloroplast in the absence of chlorophyll *b*-synthesizing activity [12]. In this hypothesis, it is postulated that chlorophyll *b* biosynthesis takes place while chlorophyll *a* is tentatively bound to LHC apoprotein during import. This hypothesis has been primarily based on observations with *C. reinhardtii*. It is possible that land plants and green algae have different mechanisms for the regulation of their LHC levels.

Since chlorophyll *b* biosynthesis controls the accumulation of LHC as described above, one might ask to what extent it is possible to increase the LHC protein contents on thylakoid membranes by enhancing chlorophyll *b* biosynthesis. In other words, how large could an LHC antenna become? One molecule of LHC protein typically binds 8 chlorophyll *a* and 6 chlorophyll *b* molecules [56], thus, if LHC dominates the photosynthetic machinery, and if chlorophyll *a* and *b* are bound stoichiometrically to LHC, the chlorophyll *a* to *b* ratio is theoretically reduced close to 1.33 (43% chlorophyll *b* in total chlorophyll). We have attempted to answer this question by overexpressing a cyanobacterial *CAO* gene (referred to as PhCAO) from *P. hollandica* in *Arabidopsis*. Since cyanobacterial CAO does not possess the regulatory A domain, its protein level could not be subjected to the plant-specific protease system within the chloroplast. Indeed, overexpression of *PhCAO* led to increased chlorophyll-*b* synthesizing activity in transgenic *Arabidopsis* plants, which resulted in an unusually low chlorophyll *a* to *b* ratio down to 0.8 (56% chlorophyll *b*; Hirashima et al. [29]). As discussed above, increased accumulation of LHC alone does not simply explain how such a low chlorophyll *a* to *b* ratio could arise. Therefore, it could be hypothesized that excessive chlorophyll *b* molecules are ectopically placed. Indeed, we found that chlorophyll *b* resides not only in LHC, but also in the subunits of the core antenna complexes including CP1, CPa (CP43 + CP47). Moreover, we observed reductions in chlorophyll *a* to *b* ratios of LHC from 1.3 (43% chlorophyll *b*) of wild type to 0.77 (56% chlorophyll *b*) of *PhCAO*-overexpression plants. These results indicate that there is certain limitation for the accumulation of LHC in thylakoid membranes and that excessive chlorophyll *b* accumulation leads to partial replacement of chlorophyll *a* with chlorophyll *b* in the subunits of the photosynthetic apparatus. As proposed by Horn et al. [55], if we assume that the difference in the binding kinetics of chlorophyll *a* and *b* is the mechanism which ensures proper assembly of chlorophylls and the LHC apoprotein, it is reasonable to consider that ectopic binding of chlorophyll *b* to the chlorophyll *a*-binding sites under excessive chlorophyll *b* accumulation could occur.

In Fig. 6, we summarized how chlorophyll *b* levels affect the composition of the photosynthetic apparatus. In this figure, the chlorophyll *b* levels are represented by the chlorophyll *a* to *b* ratios, which is the most common parameter used to indicate chlorophyll *b* levels. When the chlorophyll *b* level is zero, the accumulation of the major LHC will be minimum. As the chlorophyll *b* levels increase from 0 to the wild type level (approximately 3), the major LHC level will increase. When the levels of chlorophyll *b* are then increased over the wild type level, the major LHC level will continue to rise until a certain threshold level is reached (mostly likely until the chlorophyll *a* to *b* ratios decline to 2.6 or 2.7, which correspond to 27 or 28% chlorophyll *b*). Upon this further decrease in the chlorophyll *a* to *b* ratios, chlorophyll *b* will start to replace chlorophyll *a* in both the major LHC and the subunits of the core antenna complexes. The relationship of the chlorophyll *a* to *b* ratios to the LHC levels may represent the plasticity of pigment-binding proteins that are involved in photosynthesis and most likely represent its limitations as well.

It is notable that the transgenic plants with excessive chlorophyll *b* accumulation showed signs of damage from photo-oxidative stresses during their juvenile stage [34] or when they were grown under strong illumination [57]. Measurement of time-resolved fluorescence spectra indicated that the energy transfer between chlorophyll *a* molecules within PSII have been disturbed by the replacement of chlorophyll *a* with chlorophyll *b* [57]. In these plants, H_2O_2 is generated and this in turn results in alterations of global gene expression profiles and may ultimately lead to cell death. These studies show that the regulation of chlorophyll *b* biosynthesis through the A domain of CAO is essential for the light acclimation of plants.

6. Chlorophyll *b* degradation is necessary for the turnover of LHC

As described above, the binding of chlorophyll *b* to the LHC apoprotein makes LHC resistant to proteolysis, at least when the LHC apoprotein is actively synthesized. Due to this feature, it is interesting to consider if LHC associated with chlorophyll *b* is more resistant to proteolysis during leaf senescence, which is a point in plant development when the whole photosynthetic apparatus should be degraded. In other words, it would be intriguing to ask if the removal of chlorophyll *b* is necessary for LHC degradation even during leaf senescence. Kusaba et al. [26] reported that impairment in the chlorophyll *b* reduction led to LHC stabilization during leaf senescence in the rice mutant lacking chlorophyll *b* reductase. These results indicate that the conversion of chlorophyll *b* into chlorophyll *a* should precede the degradation of LHC

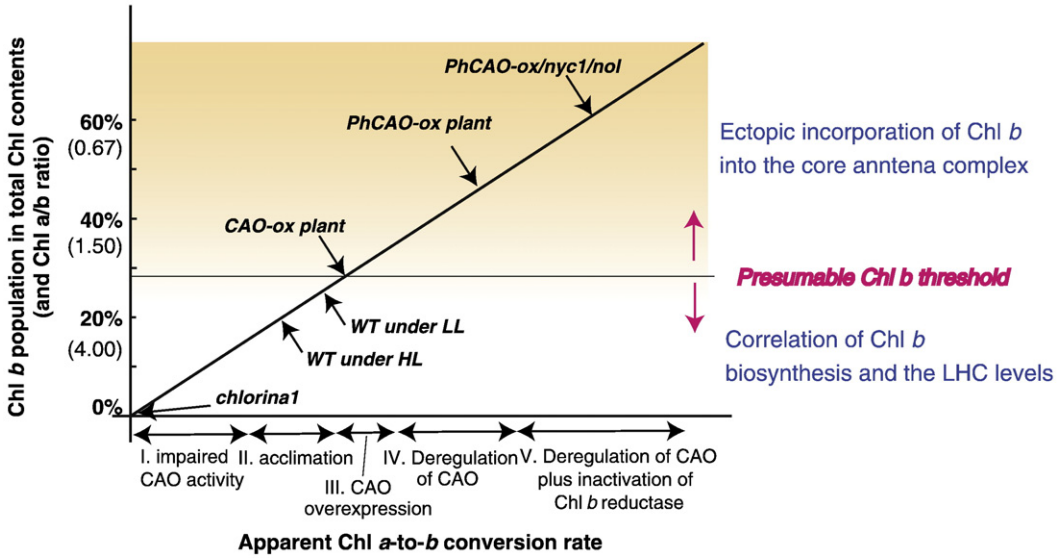

Fig. 6. Relationship between the apparent chlorophyll *a*-to-*b* conversion rate and its effects on the LHC levels and the ectopic incorporation into the core antenna complex. This graph was drawn based on the studies on the *chlorina1* mutant and various CAO-overexpressing transgenic plants as well as wild type (WT) plants grown under low-light (LL) or high-light "(HL)" conditions. It should be noted that the apparent chlorophyll *a*-to-*b* conversion rate shown on the X axis was deduced from the phenotypes of the plants we described in the main text. These values are not necessarily precise. The Y axis shows the proportion of chlorophyll *b* in the total chlorophyll contents. When CAO activity is impaired by genetic disorder (phase I on the X axis), the chlorophyll *a*/*b* ratio will be higher than 4 (less than 20% chlorophyll *b*), as it is represented by the *chlorina1* mutation. During light acclimation of plants between LL and HL, the CAO activity may change within a certain range (phase II), and it will typically result in the chlorophyll *a*/*b* ratios between 4.0 and 3.0 (20% to 25% chlorophyll *b*). Overexpression of the full-length CAO only lowers the chlorophyll *a*/*b* ratios down to 2.7 (27% chlorophyll *b*: phase III). In the transgenic plants overexpressing CAO protein that lacks the regulatory domain, chlorophyll *a*/*b* ratios decrease from 2.7 to 0.8 (27% to 56% chlorophyll *b*: phase IV). By crossing these plants with the mutants that lack chlorophyll *b* reductase (the *nyc1*/*nol* mutant), chlorophyll *a*/*b* ratios went further below 0.8 (56% chlorophyll *b*: unpublished results). We hypothesize that there is a threshold for chlorophyll *b* which can be incorporated to the "chlorophyll *b* binding" sites of LHCs. Below this threshold level, chlorophyll *b* will be ectopically incorporated into the core antenna complex or the chlorophyll *a* biding sites of LHCs.

during leaf senescence. This hypothesis implies that chlorophyll *b* reductase is able to act on chlorophyll *b* that is still assembled with LHC apoprotein. An *in-vitro* analysis showed that chlorophyll *b* reductase is able to react with chlorophyll *b* molecules on LHC [28]. Taken together, we could conclude that the initial reaction of LHC degradation during senescence is the reduction of chlorophyll *b* by chlorophyll *b* reductase, which is then followed by proteolysis.

Grana stacking of thylakoid membranes is a unique feature of thylakoid membranes in higher plants and in a group of algae, Charaphyta [58]. LHC has been implicated to play an essential role in grana stacking, as chlorophyll *b*-less mutants are known to have less stacked thylakoid membranes [46]. Standfuss et al. [59] proposed that the stromal membrane surface of the LHC forms a mixed pattern of positive and negative charges and that these surface characteristics may play an essential role in grana formation. The analysis of the mutants lacking chlorophyll *b* reductase provides evidence for the LHC-dependency of thylakoid formation. In wild type plants, LHC and thylakoid membrane disappear during leaf senescence. In contrast, thylakoid membranes in mutants lacking chlorophyll *b* reductase from both rice and *Arabidopsis*, were barely degraded during leaf senescence [26,28]. In these mutants, Lhcb5 and the core antennae complexes disappeared and only the other LHC proteins remained during leaf senescence. These observations demonstrate that the presence of LHC proteins is sufficient to retain grana stacking.

The studies of both Kuraba et al. and Horie et al. indicate that the protease system for LHC, with the exception of Lhcb5, is distinct from those for the core antenna complexes and Lhcb5. FtsH6 has been proposed to function as an LHC-specific protease. This hypothesis was based on the observation with an *Arabidopsis* mutant lacking FtsH6 in which the turnover of Lhcb3 was slightly retarded [60]. Considering the abundance of LHC within the chloroplast, it is likely that multiple proteases are responsible for the degradation of LHC. In this regard, the analysis of a single or a few protease mutants may not be sufficient to uncover the degradation mechanism of LHC. A combination of biochemical approaches and other strategies may be necessary to fully understand this mechanism.

7. Re-construction of the photosynthetic apparatus

As it was described above, synthesis of chlorophyll and chlorophyll-binding apoproteins should be tightly coordinated for the construction of the photosynthetic apparatus. Synchronization of gene expression of key genes at the transcriptional level provides a basal level of such coordination. Micro- and macroarray analyses show that expression profiles of genes encoding the key enzymes of the chlorophyll biosynthetic pathway are similar to those of Lhcb or Lhca genes [61], indicating a coordinated transcriptional regulation of those genes. However, it is apparent that transcriptional regulation only provides a rough coordination of chlorophyll biosynthesis to the construction of the photosynthetic apparatus. By carefully studying the construction of the photosynthetic apparatus during the greening stage of plants, researchers found that post-transcriptional regulation is essential in the coordination of chlorophyll and photosynthetic apoprotein synthesis. When angiosperms are germinated in darkness, they accumulate cytochrome b6/f complex and a chlorophyll precursor, protochlorophyllide *a*. However, they lack chlorophyll, the core complexes of PSI and PSII, and the peripheral antenna complexes [62,63]. Upon illumination, protochlorophyllide *a* is rapidly converted to chlorophyll *a* and the core complex proteins emerge earlier than the peripheral antenna complex and chlorophyll *b*. In case of barley seedlings, after 2 h of greening, the core complex proteins are detected and the synthesis of chlorophyll *b* and the accumulation of the peripheral antenna complexes follow after 4–6 h of illumination [64]. Electron transport from water to NADP can only be observed after 4 h of illumination [63]. This sequence of events during greening may reflect the fact that the peripheral antenna complexes are only functional with the core antenna complexes in terms of photosynthetic electron transport. Plants seem to have mechanisms to ensure that core antenna complexes are constructed prior to that of the peripheral antenna complexes in order to gain photosynthetic activity as early as possible. At the present time, these mechanisms are not understood in full detail but it could be hypothesized that plants are adopting the following three principles: 1) Chlorophyll *a* is preferentially associated with the core complex proteins. 2) Chlorophyll *a* molecules that fail to associate with the

core complex subsequently bind to the LHC apoproteins. Prior to or upon this assembling process, roughly half of the chlorophyll *a* molecules that fail to bind the core complex should be converted to chlorophyll *b*. 3) When further synthesis of the core complex proteins is necessary, the chlorophyll molecules that have already been bound to LHC can be recruited to the core complex. For this process, conversion of chlorophyll *b* to chlorophyll *a* is prerequisite. Existence of these "three principles" has been demonstrated by the following experiments. 1) When chlorophyll synthesis was boosted in barley seedlings by feeding the chlorophyll precursor 5-aminolevulinate, a substantial amount of chlorophyll *a* molecules were released from core complexes and larger amounts of chlorophyll *b* and LHC accumulated [16,65]. Likewise, when the synthesis of the core antenna complexes was repressed by an inhibitor of chloroplast protein synthesis, larger amounts of chlorophyll *b* and LHC accumulated [66]. These results indicate that synthesis of chlorophyll *b* and LHC respond to the amount of excessive chlorophyll *a* molecules. 2) When greening seedlings were transferred back darkness, conversion of chlorophyll *b* to chlorophyll *a* occurred. Consequently, this resulted in degradation of LHC and an increase in the core antenna complexes [18]. These experiments demonstrate that chlorophyll molecules can be re-distributed from LHC to the core antenna complexes and that the chlorophyll cycle plays a central role in this process.

A few important questions remain to be answered. For example: How does CAO sense the amounts of chlorophyll *a* that are not bound to the core antenna complexes in order to convert only half of them to chlorophyll *b*? How does chlorophyll *b* reductase initiate chlorophyll *b* to *a* conversion when further synthesis of the core complexes is necessary? Lastly, how does chlorophyll *b* reductase stop the conversion when the amount of the core complexes is sufficient? A further understanding of the reaction and regulatory mechanisms of the chlorophyll cycle is necessary to answer these questions. We hope that finding answers to these questions will enhance our understanding regarding how plants optimize their photosynthetic antenna systems.

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