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Chlorophyll *b* degradation by chlorophyll *b* reductase under high-light conditions

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Abstract

The light-harvesting chlorophyll *a/b*-binding protein complex of photosystem II (LHCII) is the main antenna complex of photosystem II. Plants change their LHCII content depending on the light environment. Under high-light conditions, the content of LHCII should decrease because over-excitation damages the photosystem. Chlorophyll *b* is indispensable for accumulating LHCII, and chlorophyll *b* degradation induces LHCII degradation. Chlorophyll *b* degradation is initiated by chlorophyll *b* reductase. In land plants, NON-YELLOW COLORING 1 (NYC1) and NYC1-Like (NOL) are isozymes of chlorophyll *b* reductase. We analyzed these mutants to determine their functions under high-light conditions. During high-light treatment, the chlorophyll *a/b* ratio was stable in the wild-type and *nol* plants, and the LHCII content decreased in wild-type plants. The chlorophyll *a/b* ratio decreased in the *nyc1* and *nyc1/nol* plants, and a substantial degree of LHCII was retained in *nyc1/nol* plants after the high-light treatment. These results demonstrate that NYC1 degrades the chlorophyll *b* on LHCII under high-light conditions, thus decreasing the LHCII content. After the high-light treatment, the maximum quantum efficiency of the photosystem II photochemistry was lower in *nyc1* and *nyc1/nol* plants than in wild-type and *nol* plants. A larger light-harvesting system would damage photosystem II in *nyc1* and *nyc1/nol* plants. The fluorescence spectroscopy of the leaves indicated that photosystem I was also damaged by the excess LHCII in *nyc1/nol* plants. These observations suggest that chlorophyll *b* degradation by NYC1 is the initial reaction for the optimization of the light-harvesting capacity under high-light conditions.

Keywords

chlorophyll *b* reductase; light-harvesting complex; high-light conditions; Arabidopsis

Introduction

Light energy is absorbed by chlorophyll or carotenoids, and the excited energy is transferred to the chlorophyll in the core complex of the photosystem. Photosynthetic organisms developed a peripheral light-harvesting complex because the core complex of the photosystem does not bind sufficient chlorophyll to efficiently drive photosynthesis. The core complex of the photosystem is conserved among oxygen-evolving photosynthetic organisms, whereas various peripheral light-harvesting complexes have evolved (Neilson and Durnford 2010). The diversity of these light-harvesting complexes is associated with the living environment. Photosystem I (PSI) and photosystem II (PSII) have peripheral light-harvesting complexes (LHCI, LHCII). LHCI and LHCII consist of several polypeptides (Dekker and Boekema 2005). Lhca1, 2, 3, and 4 constitute LHCI and are associated with the core complex as the monomeric form. Lhcb1, 2, and 3 occur in a trimeric form and constitute LHCII. CP29, CP26 and CP24 are also peripheral light-harvesting complexes of PSII that are encoded by *Lhcb4*, *Lhcb5* and *Lhcb6*, respectively. These complexes occur in a monomeric form.

The composition of the photosynthetic complex varies in response to the light environment. The content of LHCI proteins is not believed to be notably affected by light conditions. However, the LHCII level varies depending on the light intensity (Horton 2012; Ballottari et al. 2007). Under low-light conditions, variable amounts of LHCII organize PSII into large supercomplexes to capture light energy for efficient photosynthesis. Under high-light conditions, a proportion of the absorbed light energy is not used for photosynthesis. The over-excitation of the photosystem produces reactive oxygen species, which damage the photosystem. To prevent photodamage, plants decrease the LHCII content to reduce the light-harvesting capacity (Fristedt and Vener 2011).

Both chlorophyll *a* and *b* are related to the stability of LHCII. A chlorophyll *b*-less mutant does not accumulate LHCII. In this mutant, the LHCII apoprotein is synthesized in the cytosol and imported into the chloroplast (Nick et al. 2013). However, LHCII is degraded by proteases because of the absence of chlorophyll *b*. A reconstitution experiment using the recombinant LHCII apoprotein and purified chlorophyll demonstrates that chlorophyll *a* binds to the LHCII apoprotein without chlorophyll *b* and that chlorophyll *a* can occupy the chlorophyll *b* binding site (Horn et al. 2007). However, LHCII binding only chlorophyll *a* has never been isolated from plants, suggesting that the potential binding capacity of chlorophyll *a* to the chlorophyll *b* site is not actually achieved in vivo.

The crucial role of chlorophyll *b* in LHCII accumulation indicates that the LHCII level is regulated by chlorophyll *b* synthesis and degradation. The degradation of the LHCII apoprotein is not well understood. The proteolytic activity that is involved in LHCII degradation is elevated under high-light conditions (Yang et al. 1998), although the protease has not yet been identified. Chlorophyll *b* metabolism and the protease likely cooperate in LHCII degradation. While the studies

of protease as related to D1 degradation have promoted an understanding of PSII quality control (Kato and Sakamoto 2009), a detailed analysis of the LHCII protease remains to be revealed to advance the understanding of the adaptation of PSII to variations in the light environment.

5-Aminolevulinic acid is the first precursor, and chlorophyll *a* is the final product in the chlorophyll biosynthetic pathway. In land plants, a portion of chlorophyll *a* is oxidized to chlorophyll *b* by chlorophyllide *a* oxygenase (CAO) (Fig. 1). Chlorophyll *b* should be converted to chlorophyll *a* for degradation because chlorophyll *b* derivatives are not catalyzed in the subsequent steps of the chlorophyll degradation pathway (Hortensteiner et al. 1995). Chlorophyll *b* is reduced to 7-hydroxymethyl chlorophyll *a* by chlorophyll *b* reductase (CBR). Two CBR isozymes, NON-YELLOW COLORING 1 (NYC1) and NYC1-Like (NOL), are known in land plants (Kusaba et al. 2007). An NYC1-deficient mutant cannot degrade LHCII, suggesting that chlorophyll *b* degradation triggers LHCII degradation. NYC1 is expressed during dark-induced or natural senescence. Conversely, the NOL level is independent of the plant conditions (Sakuraba et al. 2013). These observations suggest that chlorophyll *b* degradation is controlled by NYC1 and that NOL is a housekeeping enzyme. 7-Hydroxymethyl chlorophyll *a* is successively reduced to chlorophyll *a* by 7-hydroxymethyl chlorophyll *a* reductase (HCAR) (Meguro et al. 2011; Shimoda et al. 2012). The regulation of the HCAR level or enzymatic activity is not known. This interconversion of chlorophyll *a* and chlorophyll *b* is called the chlorophyll cycle, which is believed to facilitate the regulation of the chlorophyll composition in a fluctuating light environment and throughout developmental stages. Recently, the analysis of a stay-green mutant demonstrates that the destabilization of a chlorophyll-protein complex participates in chlorophyll degradation (Christ and Hörttensteiner 2013; Yamatani et al. 2013; Huang et al. 2013). Chlorophyll *b* degradation might be regulated by certain destabilizing components in addition to CBR.

Three roles of chlorophyll *b* degradation in plant life have been suggested (Christ and Hörttensteiner 2013; Hörttensteiner 2006). (1) Chlorophyll *b* degradation promotes nutrient remobilization. LHCII is a very abundant protein in the chloroplast and is a large reservoir of nutrients. Preceding the degradation of LHCII, the chlorophyll *b* in LHCII should be degraded. Therefore, chlorophyll *b* degradation can affect the supply of nutrients from degraded LHCII for remobilization between source and sink organs. (2) Chlorophyll *b* degradation participates in the suppression of photodamage. When LHCII is not degraded properly, energy-transfer-uncoupled LHCII can produce harmful reactive oxygen species. Unregulated LHCII degradation is especially dangerous during senescence because a large amount of LHCII is degraded, and cell damage will disturb the recovery of the nutrients from the leaves. (3) Chlorophyll *b* degradation regulates the plant light-harvesting capacity. LHCII is the major light-harvesting complex in chloroplasts. Prior to reducing the level of LHCII, chlorophyll *b* should be degraded.

Although the phenotypes of CBR-deficient mutants during senescence have been studied

(Kusaba et al. 2007; Horie et al. 2009; Sato et al. 2009), the effect of the light conditions on the degradation activity of chlorophyll *b* has not yet been determined. In this report, we analyzed mutant plants that are defective in chlorophyll *b* degradation under a high-light treatment to elucidate the role of chlorophyll *b* degradation in the regulation of the light-harvesting system.

Methods

Plant growth conditions

Arabidopsis thaliana (Colombia ecotype) was used for experiments. The T-DNA insertion mutants lacking either AT4G13250 (SALK_091664) (NYC1) or AT5G04900 (167A10) (NOL) were obtained from the Arabidopsis Biological Resource Center and GABI-Kat, respectively. The mutants were crossed, and a double mutant (*cbr*) was obtained (Horie et al. 2009). The plants were cultivated for 4-5 weeks under controlled conditions (50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 25°C, 14 h light/10 h dark) and were then transferred to continuous high-light conditions (1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 25°C). Light was provided by a 400-W metal halide lamp (Mitsubishi). To analyze the changes in chlorophyll content and chlorophyll metabolism, plants were incubated for 4 days under high-light conditions. Fully expanded leaves were analyzed for the experiments.

Pigment analysis

Pigments were extracted from leaf discs (0.5 cm²) with 100% acetone and then separated and quantified by HPLC (Horie et al. 2009). The pigment content was normalized by the leaf area.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from leaf tissues using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The cDNA was synthesized using the PrimeScriptRT reagent kit with gDNA eraser (TaKaRa). Quantitative real-time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) and a MyiQ2 Two-Color Real-Time PCR Detection System (Bio-Rad). The data were obtained using the iQ5 Optical System software (Bio-Rad). The *ACT2* gene (5'-CGTACAACCGGTATTGTGCT-3', 5'-CAGTAAGGTCACGTCCAGCA-3') was used as an internal control to standardize the levels of *NYC1* (5'-TTGATGACCAAGGACGGGCGTTA-3', 5'-GCTTTGTAAGATGATGAAAGCGCA-3'), *NOL* (5'-GCCGAGAGGCAATGAATATGAT-3', 5'-CCATATGCAGCAAATCTGGGTGTT-3'), *HEMA1* (5'-AACAAAGAAGACAGGATGAG-3', 5'-AAACTGTGTGGATTCTCTGT-3'), *CHLH* (5'-GCTGGTCGTGACCCTAGAAC-3', 5'-TGCCAGCTTCTTCTCTGCC-3'), *CAO* (5'-TCTGTGGAGACATTCGCTG-3', 5'-AGTCTATACCGAACTCCGAGC-3') and *chalcone synthase (CHS)* (5'-AAGCGTCTCATGATGTACCA-3', 5'-ACTGAAAAGAGCCTGACCGA-3').

Fluorescence measurement

The maximum quantum efficiency of the PSII photochemistry (F_v/F_m) was measured with a PAM 2000 fluorometer (Walz). The plants were placed in the dark for 10 min prior to analysis. To measure the low-temperature fluorescence of the leaves, the leaves were placed into a glass tube and immediately frozen in liquid nitrogen. Fluorescence emission spectra were obtained at 77 K using a fluorescence spectrophotometer (F-2500, Hitachi). The wavelength of the blue excitation light was 440 nm. The excitation spectrum was measured by monitoring the fluorescence peaks of PSII and PSI at 688 nm and 737 nm, respectively. The subtracted fluorescence spectrum was measured after normalizing by the fluorescence at 650 nm and 688 nm, respectively.

Immunoblotting analysis

Each leaf disc (0.5 cm²) was homogenized in 100 µL of protein extraction buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 5% w/v sucrose, 5% w/w 2-mercaptoethanol). After centrifugation (22500 g, 5 min), 2 µL of the supernatant was subjected to SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Hybond-P, GE-Healthcare). All of the target proteins were detected using a primary antibody from Agrisera, except for CP1 (Horie et al. 2009). Anti-rabbit IgG or anti-chicken IgG linked to horseradish peroxidase (GE-Healthcare) was used as the secondary antibody. The primary antibodies were diluted with an immunoreaction enhancer solution (Can Get Signal, TOYOBO). The horseradish peroxidase activity was visualized using the Western Lightning Plus-ECL chemiluminescence detection kit (PerkinElmer). Chemiluminescence was detected and quantified using a LumiVision Analyzer (Aisin).

Results

Changes in the chlorophyll composition under high-light conditions

The chlorophyll *b* level changes depending on the light intensity (Schöttler and Toth 2014). Plants decrease chlorophyll *b* under high-light conditions, suggesting that chlorophyll *b* degradation activity is controlled by light intensity. The first step of chlorophyll *b* degradation is catalyzed by CBR. To understand the role of CBR in chlorophyll *b* degradation, CBR-deficient mutants were incubated under high-light conditions, and the chlorophyll composition was determined (Fig. 2). Wild-type (WT), *nyc1*-deficient, *nol*-deficient and *nyc1/nol*-deficient (*cbr*) Arabidopsis mutant plants were grown under a light-dark cycle for 4-5 weeks and then transferred to continuous high-light conditions. The chlorophyll content in the fully expanded leaves was analyzed during the 4 days of the high-light treatment. The high-light treatment induces starch accumulation and cell wall thickening. These phenotypes increase the cell weight; therefore, the chlorophyll content was normalized by the leaf area instead of the fresh weight. The total chlorophyll content decreased during the high-light treatment in all of the plants, and the rates of chlorophyll decrease were also

similar. The chlorophyll *a/b* ratio differed among the genotypes. WT and *nol* plants were characterized by stable chlorophyll *a/b* ratios during the high-light treatment. On the other hand, the chlorophyll *a/b* ratios of the *nyc1* and *cbr* plants decreased. These observations indicate that the WT and *nol* plants degrade both chlorophyll *a* and *b* at comparable rates under high-light conditions, whereas *nyc1* and *cbr* plants did not degrade chlorophyll *b* properly. This phenotype is identical to that of dark-induced senescent plants (Horie et al. 2009). When the *nyc1* and *cbr* plants are placed under dark conditions to induce chlorophyll degradation, chlorophyll *a* is degraded, whereas the chlorophyll *b* level remains stable. Under both the high-light and dark treatments, NYC1 is the major CBR, and NOL is the supporting enzyme in chlorophyll *b* degradation.

The chlorophyll content is regulated by the balance between chlorophyll biosynthesis and degradation; thus, the synthesis and degradation activities should be examined to evaluate the controls on chlorophyll accumulation. To examine the changes in chlorophyll biosynthesis activity, the transcriptional levels of chlorophyll biosynthetic genes (*HEMA1*, *CHLH*, *CAO*) were compared between WT and *cbr* plants (Supplementary Fig. S1). *HEMA1* and *CHLH* encode glutamyl-tRNA reductase and the H subunit of magnesium chelatase, respectively, and both of these enzymes catalyze regulatory steps of chlorophyll biosynthesis (Tanaka and Tanaka 2007). Under high-light conditions, the expression levels of the *HEMA1* and *CAO* genes decreased in a similar manner in WT and *cbr* plants. The *CHLH* levels also decreased. The levels in *cbr* plants were lower both before and after the 3-day high-light treatment than those of the WT plants. These observations suggest that the high level of chlorophyll *b* in the *cbr* mutant is not due to the high activity of chlorophyll *b* biosynthesis.

Expression of *NYC1* and *NOL* mRNA under high-light conditions

The NYC1 level is very low under standard conditions (Jia et al. 2015). The observation that the WT plants degraded chlorophyll *b* and *nyc1* plants did not degrade chlorophyll *b* under the high-light treatment suggests that NYC1 expresses under high-light conditions in WT plants. To examine the mRNA expression levels of *NYC1* and *NOL*, quantitative real-time PCR was performed using *ACT2* as the standard. The WT plants were exposed to high light for 1 and 3 days, and the expression levels were compared with the pre-exposure levels (Fig. 3). Both *NYC1* and *NOL* were induced by the high-light treatment, and the expression level of *NYC1* exceeded that of *NOL*. However, the induction levels of these two genes were lower than those of *CHS*, which encodes chalcone synthase and is the standard gene that is induced under high-light conditions (Müller-Xing et al. 2014). Mutation of the *NOL* gene did not affect the expression of the *NYC1* gene (Supplementary Fig. S2), suggesting that the low activity of chlorophyll *b* degradation in *nyc1* mutant might not be caused by the reduction in NOL activity.

The maximum quantum efficiency of the PSII photochemistry under the high-light treatment

When the WT plants were exposed to high light, both chlorophyll *a* and *b* were degraded. This reaction will reduce photodamage under conditions of excess light. To examine the damaging effect of chlorophyll degradation on the photosystem, the maximum quantum efficiency of the PSII photochemistry (Fv/Fm) was measured under high-light conditions (Fig. 4). The Fv/Fm values of all of the plants decreased within the first day; after which time, the value remained stable in WT plants. In *nol* plants, the Fv/Fm values decreased gradually. Significant reductions in the *nyc1* and *cbr* plants were observed in response to the high-light treatment. These observations suggest that chlorophyll *b* degradation by NYC1 is important for maintaining an active photosystem under high-light conditions.

Chlorophyll-protein levels under the high-light treatment

Chlorophyll-protein levels depend on the light intensity and are related to the chlorophyll *b* level (Tanaka and Tanaka 2005). To examine the effects of the light conditions and the chlorophyll *b* degradation activity on the chlorophyll-protein level, chlorophyll proteins were analyzed by immunoblotting (Fig. 5). To simplify the experimental conditions to understand the effect of chlorophyll *b* degradation on the chlorophyll-protein level, only WT plants and *cbr* plants were analyzed. The sample results were normalized by the leaf area in the same manner as performed for chlorophyll quantification. All of the proteins were determined to have almost identical profiles in three independently high-light treated plants. Two other profiles are shown in Supplementary Figure S3. The band intensities of these three samples were measured and quantified (Fig. 6).

Lhcb1, 2, and 3 form a trimeric complex and are known as the major LHCII. CP29 (Lhcb4), CP26 (Lhcb5), and CP24 (Lhcb6) are monomeric proteins and are known as the minor LHCII. The level of the major LHCII changes depending on the light conditions. In the WT plants, the levels of Lhcb1, 2, 3 and CP29 decreased during the high-light treatment. Compared to the WT plants, the *cbr* plants maintained a substantial level of these LHCII proteins. The CP26 and CP24 levels were similar in both of the genotypes and light treatments.

In both WT and *cbr* plants, the levels of phosphorylated Lhcb1 and 2 decreased after their transfer to high-light conditions and then gradually recovered. The temporal dephosphorylation of Lhcb is consistent with previous reports (Zer et al. 2002). The phosphorylation levels of Lhcb1 and Lhcb2 were higher in *cbr* plants exposed to the high-light treatment.

All of the LHCI proteins decreased under the high-light treatment. In contrast to the LHCII proteins, the LHCI protein level did not differ remarkably between the WT and *cbr* plants. These results are consistent with those previously reported for senescent plants (Horie et al. 2009). LHCI stability is not thus strictly dependent on the chlorophyll *b* content. The observation that the decrease in the level of LHCI is smaller than that of LHCII in the chlorophyll *b*-less mutant also supports the

idea that LHCI level is not markedly affected by the chlorophyll *b* content (Tanaka and Tanaka 2005).

The P700-chlorophyll *a*-protein complexes of PSI (CP1), D1 and CP43 bind only chlorophyll *a*. CP1 decreased during the high-light treatment, and the CP1 level was lower in the *cbr* plants after exposure to the high-light treatment. The levels of D1 and CP43 decreased in a similar manner in the WT and *cbr* plants. The reductions in D1 and CP43 indicate photodamage to PSII. The Fv/Fm values (Fig. 4) are not proportional to the D1 and CP43 contents. A number of damaged or nonfunctional (e.g., under repair) proteins may be detected by the immunoblotting analysis. The level of the RuBisCO large-subunit protein did not change significantly in any of the samples. All of the proteins were stained in a gel (Supplementary Fig. S4), and the band profiles did not contain any major differences.

Low temperature fluorescence analysis of PSI and PSII

The immunoblotting analysis results indicate that the photosystem components of the WT and *cbr* plants were affected by the high-light treatment. To examine the physiological responses of the photosystem, the low-temperature fluorescence of the leaves was determined after the high-light treatment. When the fluorescence peak of PSI (737 nm, 440 nm excitation) was normalized, the fluorescence peak of PSII (688 nm and 695 nm) of the *cbr* plants was lower than that of the WT plants (Fig. 7a), indicating that the *cbr* plants accumulated a lower amount of functional PSII than the WT plants. The difference between the PSII emission spectra of the WT and the *cbr* plants exhibited a peak at 684 nm (Fig. 7b). This peak is comparable to the fluorescence of LHCII (Caffarri et al. 2004). This strong fluorescence of LHCII in *cbr* plants supports the observation that the LHCII level in the *cbr* plants was high after exposure to the high-light treatment (Fig. 5).

To understand the excitation energy distribution for PSI, the excitation spectra of PSI were determined. The excitation peak corresponding to chlorophyll *b* (474 nm) was higher in the *cbr* plants than in the WT plants when the excitation spectra were normalized to 440 nm, corresponding to chlorophyll *a* (Fig. 7c). Considering the higher content of LHCII in the *nyc1* mutants, the increase in the contribution of chlorophyll *b* to PSI fluorescence might partially be attributed to the association of LHCII to the PSI complex. This hypothesis is supported by the finding that the level of LHCI did not differ between WT and *nyc1* plants. The level of CP1 in the *cbr* plants was lower than that in the WT plants after the high-light treatment (Fig. 5). Thus, CP1 would be disrupted by the excess light energy that is derived from the abundant light-harvesting system in the *cbr* plants.

Discussion

Regulation of the antenna size by chlorophyll metabolism

Plants change their antenna size depending on the light environment to optimize light harvesting

(Anderson and Andersson 1988). LHCII degradation under high-light conditions has long been a subject of research because LHCII is indispensable for photoacclimation. Although chlorophyll *b* degradation is believed to be related to LHCII content, it has not been examined in detail under various light conditions. To demonstrate the direct effect of chlorophyll *b* degradation on LHCII degradation under high-light conditions, we analyzed mutants that were defective in chlorophyll *b* degradation. We found that chlorophyll *b* degradation decreased the LHCII content and suppressed photodamage. These observations indicate that the chlorophyll metabolism regulates the light-harvesting capacity.

Feedback controls constitute one of the regulation mechanisms for chlorophyll synthesis (Masuda and Fujita 2008). Feedback regulation of chlorophyll *b* synthesis by CAO has also been reported (Yamasato et al. 2005). CAO is degraded in the presence of its product, chlorophyll *b*. The total chlorophyll synthetic activity and CAO activity are the major regulatory steps of the LHCII level. The degradation of chlorophyll *b* also participates in the regulation of the LHCII level. The effect of chlorophyll *b* degradation depends on the growth conditions and the developmental stage. Both natural and dark-induced senescence enhance NYC1 expression and promote chlorophyll *b* degradation. We found that NYC1 degraded chlorophyll *b* under high-light conditions (Fig. 2b). The level of chlorophyll *b* degradation was not significant compared to that degraded in senescence. The expression of mRNA was also not enhanced very much. An approximately 6-fold induction was observed under high-light conditions after 3 days (Fig. 3). When plants are placed in the dark for 3 days, the level of *NYC1* mRNA increases by over 10-fold (Sakuraba et al. 2013). During senescence, the entire plant is organized for reproduction, whereas the light conditions primarily affect photosynthesis. These differences in areas of impact are the reason for the differences in chlorophyll *b* degradation activity observed between senescence and high-light treatments. Although the levels of NYC1 induction differ, LHCII degradation is also controlled by NYC1 under high-light conditions.

LHCII consists of six homologous proteins, namely, Lhcb1, 2, 3, CP29, CP26 and CP24. These proteins have distinct functions (Dekker and Boekema 2005). In our study, the Lhcb1, 2, and 3 and CP29 contents decreased under the high-light treatment, whereas we did not observe a significant decrease in CP26 and CP24 in WT plants (Figs. 5 and 6). A 4-day treatment would not be sufficiently long to change all of the components of LHCII. A substantial decrease in the amount of all of the components of LHCII was detected in *cbr* plants after the high-light treatment. These observations indicate that the decreases in the components of LHCII depend on CBR. We cannot explain the difference between the stable and unstable LHCII components based on their physiological or structural properties. In contrast to the variation in LHCII, the LHCI level was independent of CBR. CBR might not play an important role in the downregulation of LHCI.

Defense mechanism to high-light conditions

When plants are exposed to high light, they reduce their antenna size to suppress photodamage. Changing the amount of the light-harvesting complex requires days to weeks because this process requires protein and pigment synthesis and degradation. As a short-term response to varying light intensities, the dissipation of excited energy suppresses photodamage, a portion of which is realized by LHCII (Horton 2012). Under high-light conditions, the acidification of the lumen induces aggregation or conformational changes in LHCII. These modifications enhance the photoprotective energy dissipation in LHCII (Ruban et al. 2012).

The phosphorylation/dephosphorylation of LHCII is also involved in decreasing photodamage by balancing excitation between PSI and PSII (Tikkanen and Aro 2012). Phosphorylated LHCII is detached from the PSII core complex and provides energy for PSI. The redox of the plastoquinone pool regulates the activities of the LHCII kinase STN7 and the phosphatase TAP38/PPH1. This regulation of LHCII phosphorylation is believed to function under low-light conditions (Horton 2012). The activity of STN7 is low under high-light conditions, and LHCII phosphorylation is not promoted by high light because LHCII is the energy-dissipation state and PSII excitation is not high. Furthermore, the accessibility of LHCII for STN7 might be limited under high-light conditions because high light decreases the interthylakoid distance. The LHCII in the stroma or at the interface between the stroma and grana is phosphorylated by STN7 (Horton 2012; Zer et al. 2002). We also found that, in both WT and *cbr* plants, Lhcb1 and Lhcb2 were dephosphorylated within the first day. Under our high-light conditions, the level of dephosphorylation was not significantly greater in *cbr* plants compared to that in WT plants, and a substantial amount of phosphorylated Lhcb1 and Lhcb2 was observed in *cbr* plants (Fig. 5). The LHCII complexes in the stroma thylakoid membrane can be phosphorylated (Dekker and Boekema 2005). If *cbr* plants reserve a large amount of LHCII in the stroma thylakoid, this LHCII will be preferentially phosphorylated.

Photodamage of PSI is caused by the reactive oxygen species that are produced at the acceptor side of PSI (Sonoike 2011). The electron flow from PSII is essential for the photodamage of PSI *in vivo*. The isolated PSI complex also suffers photodamage, although a considerably stronger light intensity is required compared to that of the *in vivo* experiments. Reactive oxygen species that are produced on antenna chlorophyll are thought to cause photodamage on isolated PSI in the absence of PSII activity. In our *in vivo* study, the CP1 level was lower in the *cbr* plants than in the WT plants after the high-light treatment (Fig. 5), suggesting that PSI photodamage was advanced in *cbr* plants. CP1 binds only chlorophyll *a*; thus, CBR would not directly control the CP1 level. The mechanism for the reduction in the CP1 level in *cbr* mutants is still unresolved in this study. One potential mechanism is that the CP1 levels are decreased to balance excitation between PSI and PSII (Pfalz et al. 2012). In *cbr* plants, PSII is damaged and the plastoquinone pool must be oxidized. Under these conditions, the PSI content should be reduced to maintain efficient photosynthetic electron transport.

Another possibility is that excess LHCII in *cbr* plants can transfer excited energy to and thus damage PSI. A significant amount of energy should be transferred from a chlorophyll *b*-rich antenna to PSI in vivo (Ballottari et al. 2007), and LHCII binds to PSI and transfers excitation energy after long-term acclimation (Wientjes et al. 2013). The results of the fluorescence analysis in this study are consistent with these reports (Fig. 6). Although the mechanism by which *cbr* plants enhance CP1 degradation remains unclear, our observations suggest that CBR protects both PSI and PSII from photodamage.

Functions of NYC1 and NOL

Land plants have two isozymes of CBR, NYC1 and NOL. NYC1 extends the N terminus and has a transmembrane domain. NOL activity has already been analyzed using recombinant proteins (Horie et al. 2009). In contrast, NYC1 activity has not been reported because of its insolubility in the expressing bacterium. Although an in vitro analysis of NYC1 has not been performed, high homology with NOL and the phenotype of the NYC1-deficient mutant ensure that NYC1 is a CBR.

In this study, we found that chlorophyll *b* is preferentially degraded by NYC1 under high-light conditions and that NOL is not the primary enzyme responsible for degradation. This result supports previous reports concerning the effects of senescence (Horie et al. 2009) and seed maturation (Nakajima et al. 2012) in Arabidopsis. The analysis of chlorophyll *b*-excess or deficient plants indicates a relationship between NYC1 accumulation and the chlorophyll *b* level (Jia et al. 2015). These reports and our present results suggest that NYC1 is the main enzyme responsible for the regulation of the level of chlorophyll *b* in Arabidopsis.

In the present study, the level of chlorophyll degradation in the *nol* mutants was similar to that of the WT plants under the high-light treatment. However, the activities of chlorophyll *b* degradation in the *nol* mutants of rice and Arabidopsis differ. In rice, NOL is indispensable in chlorophyll *b* degradation during senescence. In Arabidopsis, chlorophyll *b* degradation is not affected by the loss of NOL, although NOL retains high chlorophyll *b* reductase activity in vitro. To understand the regulation and processes of chlorophyll *b* degradation in Arabidopsis, the functions of NOL in the cell must be further clarified.

In this study, we found that NYC1 is the key protein in controlling the LHCII level under high-light conditions. The NYC1 and NOL properties in the chloroplast should be studied in detail to understand how plants optimize their light-harvesting system in response to the light environment.

Legends to Figures

Fig. 1 Chlorophyll cycle. The pigments in the chlorophyll cycle are shown. The dashed circles

indicate the reaction sites.

Fig. 2 Chlorophyll content under the high-light treatment. The plants were grown for 4-5 weeks at 25°C with a 14 h/10 h light-dark cycle at a light intensity of 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. A high-light treatment consisting of continuous light exposure (1,000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 25°C was then applied. Leaf discs from the fully expanded leaves were collected to extract chlorophyll. The chlorophyll content was normalized by the leaf area. The error bars represent the S.D. (n = 3). (a) Total chlorophyll content. (b) Chlorophyll *a/b* ratio. Asterisks indicate a significant difference at $P < 0.005$ (Student's *t*-test).

Fig. 3 Gene expression under the high-light treatment. The total mRNA was extracted from the leaves and reverse transcribed. The *NYCI*, *NOL* and *CHS* mRNA contents were quantified using specific primer sets. The mRNA levels in each sample were normalized by *ACT2*. *CHS* was used as the standard gene expressed under high-light conditions. The error bars represent the S.D. (n = 3).

Fig. 4 Maximum quantum efficiency of PSII photochemistry. The maximum quantum efficiency of the PSII photochemistry (Fv/Fm values) were measured every day of the high-light treatment. The error bars represent the S.D. (n = 11). Asterisks indicate a significant difference at $P < 0.005$ (Student's *t*-test).

Fig. 5 Immunoblotting analyses of the proteins from the WT and *cbr* leaves. The proteins were extracted from the leaf discs of the fully expanded leaves daily during the high-light treatment. The results were normalized by the leaf area. Each protein was detected using a specific primary antibody. Lhcb1-P, phosphorylated Lhcb1; Lhcb2-P, phosphorylated Lhcb2; RbcL, RuBisCO large subunit.

Fig. 6 Protein composition in WT and *cbr* plants under high-light conditions. The band intensities of the immunoblotting analyses presented in Figure 5 and Supplementary Figure S3 were quantified. The values are the signal strengths of each line relative to that of the WT prior to the high-light treatment. Error bars represent S.D. (n = 3).

Fig. 7 Spectra of the chlorophyll fluorescence at 77K. (a) The fluorescence spectra of the leaves from the WT and *cbr* plants were measured. The excitation wavelength was 440 nm. The fluorescence intensities were normalized by the PSI fluorescence (737 nm). (b) The subtracted fluorescence spectrum was measured. The chlorophyll fluorescence values that were measured in (a) were normalized by 650 nm and 688 nm corresponding to PSII and subtracted. (c) The excitation spectra of the leaves from the WT and *cbr* plants were measured by monitoring fluorescence at 737

nm (PSI). The fluorescence intensities were normalized with chlorophyll *a* fluorescence (440 nm).

Fig. S1 Transcriptional analysis of chlorophyll biosynthesis genes in leaves under the high-light treatment. The total mRNA was extracted from the leaves and reverse transcribed. The *HEMA1*, *CHLH*, *CAO* and *CHS* mRNA contents in the WT and in the *cbr* mutants were quantified using specific primer sets. The mRNA levels in each sample were normalized by *ACT2*. *CHS* was used as the standard gene expressed under high-light conditions. The error bars represent S.D. (n = 3).

Fig. S2 Transcriptional analysis of *CBR* genes in leaves under the high-light treatment. The total mRNA was extracted from the leaves and reverse transcribed. The *NYC1* and *NOL* mRNA contents in the WT, *nyc1* mutants and *nol* mutants were quantified using specific primer sets. The mRNA levels in each sample were normalized by *ACT2*. *CHS* was used as the standard gene expressed under high-light conditions. The error bars represent S.D. (n = 3).

Fig. S3 Immunoblotting analyses of the proteins from the WT and *cbr* leaves. The immunoblotting analyses were performed under the same conditions as described in Figure 5, using different plants. Lhcb1-P, phosphorylated Lhcb1; Lhcb2-P, phosphorylated Lhcb2; RbcL, RuBisCO large subunit.

Fig. S4 Gel staining profile of the extracted proteins. The proteins that were used for immunoblotting in Figure 5 were stained with Coomassie Brilliant Blue to detect all of the proteins.

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Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Lhcb1

CP26

Lhca1

CP1

Lhcb2

CP24

Lhca2

D1

Lhcb3

Lhcb1-P

Lhca3

CP43

CP29

Lhcb2-P

Lhca4

RbcL

Fig. 7

Supplemental information
Chlorophyll *b* degradation by chlorophyll *b* reductase under high-light conditions
Rei Sato, Hisashi Ito, Ayumi Tanaka

Fig. S1 Transcriptional analysis of chlorophyll biosynthesis genes in leaves under the high-light treatment.

The total mRNA was extracted from the leaves and reverse transcribed. The *HEMA1*, *CHLH*, *CAO* and *CHS* mRNA contents in the WT and in the *cbr* mutants were quantified using specific primer sets. The mRNA levels in each sample were normalized by *ACT2*. *CHS* was used as the standard gene expressed under high-light conditions. The error bars represent S.D. (n = 3)

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Fig. S2 Transcriptional analysis of *CBR* genes in leaves under the high-light treatment.

The total mRNA was extracted from the leaves and reverse transcribed. The *NYC1* and *NOL* mRNA contents in the WT, *nyc1* mutants and *nol* mutants were quantified using specific primer sets. The mRNA levels in each sample were normalized by *ACT2*. *CHS* was used as the standard gene expressed under high-light conditions. The error bars represent S.D. (n = 3).

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