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Participation of Chlorophyll *b* Reductase in the Initial Step of the Degradation of Light-harvesting Chlorophyll *a*/*b*-Protein Complexes in *Arabidopsis*[*]

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The light-harvesting chlorophyll *a*/*b*-protein complex of photosystem II (LHCII) is the most abundant membrane protein in green plants, and its degradation is a crucial process for the acclimation to high light conditions and for the recovery of nitrogen (N) and carbon (C) during senescence. However, the molecular mechanism of LHCII degradation is largely unknown. Here, we report that chlorophyll *b* reductase, which catalyzes the first step of chlorophyll *b* degradation, plays a central role in LHCII degradation. When the genes for chlorophyll *b* reductases NOL and NYC1 were disrupted in *Arabidopsis thaliana*, chlorophyll *b* and LHCII were not degraded during senescence, whereas other pigment complexes completely disappeared. When purified trimeric LHCII was incubated with recombinant chlorophyll *b* reductase (NOL), expressed in *Escherichia coli*, the chlorophyll *b* in LHCII was converted to 7-hydroxymethyl chlorophyll *a*. Accompanying this conversion, chlorophylls were released from LHCII apoproteins until all the chlorophyll molecules in LHCII dissociated from the complexes. Chlorophyll-depleted LHCII apoproteins did not dissociate into monomeric forms but remained in the trimeric form. Based on these results, we propose the novel hypothesis that chlorophyll *b* reductase catalyzes the initial step of LHCII degradation, and that trimeric LHCII is a substrate of LHCII degradation.

Photosynthesis is an indispensable process for plants to generate chemical energy for biological processes. Chlorophyll plays a central role in photosynthesis by harvesting light energy (1) and driving electron transfer (2). Chlorophyll exists as chlorophyll-protein complexes, which can be divided into two groups (3). One group consists of the core antenna complexes, which include CP43/CP47 of photosystem (PS)[2] II and P700-chlorophyll *a*-protein complexes of PSI (CP1). The composition and organization of these core antenna complexes is conserved in oxygenic phototrophs. The second group consists of peripheral antenna complexes, which harvest and transfer light energy to the core antenna complexes. Land plants and green algae contain light-harvesting complex II (LHCII) as a peripheral antenna complex (4, 5). LHCII is the most abundant pigment-protein complex, binding chlorophyll *a* and *b*, which account for >40% of the total chlorophyll (6, 7). In addition to its light-harvesting function, LHCII regulates energy distribution between PSI and PSII (6), and is involved in the dissipation of excess light energy (8). It is known that the amount of LHCII varies depending on developmental stages and changes in light environments (9). Taken together, it is considered that the formation and degradation of LHCII are important processes for the survival of plants (10).

LHCII formation has been extensively studied using mutants and transgenic plants of various species. These studies have clarified the close relationship between chlorophyll synthesis and the LHCII formation. LHCII does not accumulate in chlorophyll *b*-less mutants, probably because LHCII is not stabilized in the thylakoid membranes without chlorophyll *b* (11). In contrast, when chlorophyll *b* synthesis is accelerated by the overexpression of chlorophyllide *a* oxygenase LHCII increases (12), whereas other chlorophyll-protein complexes such as CP43 and CP1 remain constant (13). When 5-aminolevulinic acid, a precursor of chlorophyll synthesis, is fed to greening tissues, chlorophyll *b* accumulation is enhanced and LHCII increases (14). LHCII formation might be partly regulated by the expression of *Lhc* genes, because both *Lhc* mRNA and LHCII levels increase under low light conditions (15, 16), and the reduction of *Lhc* mRNA levels by antisense mRNA results in the decrease in LHCII (17). *In vitro* reconstitution experiments have clearly shown that the occupation of chlorophyll-binding sites by chlorophyll *b* is essential for complex stability (18, 19), which is consistent with *in vivo* experiments mentioned as above. *In vitro* experiments have also unraveled the binding of chlorophyll to the complexes in detail, using time-resolved fluorescence measurement (20).

In contrast to LHCII formation, its degradation processes are largely unknown. Degradation of LHCII occurs during the reorganization of photosystems and leaf senescence. When low light-grown plants are transferred to high light conditions, LHCII begins to degrade, which results in the formation of small antennae (21). Likewise when greening tissues are transferred to the dark, LHCII degrades and its chlorophyll is reused for the formation of core antenna complexes, resulting in an

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[2] The abbreviations used are: PS, photosystem; HPLC, high-performance liquid chromatography; CP1, P700-chlorophyll *a*-protein complexes of PSI; LHCII, light-harvesting chlorophyll *a*/*b*-protein complex of photosystem II.

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increase in the number of PSs of small antenna size (22). Furthermore, degradation of LHCII is an essential process of senescence, because LHCII comprises the most abundant membrane proteins (23), and their nitrogen reserve must be recovered and transported to sink organs. Although the molecular mechanism of LHCII degradation has not been fully elucidated, proteolytic activities against LHCII have been observed in thylakoid membranes. It has been reported that thylakoid membranes from intermittently illuminated bean leaves have high proteolytic activity against LHCII (24), which is consistent with the observation that the LHCII level is low in leaves greened under intermittent illumination. Proteolytic activity against LHCII has also been reported with the reduction in the antenna size of PSII upon acclimation of plants to high light intensities. This proteolytic activity is ATP-dependent, and the protease is thought to be a serine or cysteine protease but not clp or FtsH (25). Using a reversed genetic approach, the chloroplast-targeted protease FtsH6 was identified as being responsible for the degradation of LHC during senescence and acclimation to high light conditions (26). SppA protease is induced under high light conditions and may be involved in the light-induced degradation of LHCII (27). It has also been reported that the LHCII apoprotein degrades faster than the holoprotein, and the N-terminal domain of LHCII is found to be essential for the recognition of the protein by a protease system (28). Despite these studies, LHCII proteases have not been conclusively identified. Multiple proteases might participate in LHCII degradation, as is the case in other substrate proteins such as D1 (29, 30) and ssrA-tagged proteins (31).

Degradation of LHCII consists of two processes, one is the proteolytic degradation of the protein moiety, and the other is chlorophyll degradation. It is still unclear whether chlorophyll degradation precedes the degradation of the protein moiety or whether protein degradation is the first event. Chlorophyll *b* is a major pigment of LHCII. The conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a* is the first step of chlorophyll degradation (32, 33) and is catalyzed by chlorophyll *b* reductase (34). The *non-yellow coloring1* (*nyc1*) stay-green mutant was recently isolated (35). This mutant is defective in the *NYC1* gene encoding chlorophyll *b* reductase. In this mutant, degradation of chlorophyll *b* is suppressed, and LHCII is selectively retained during senescence. Phylogenetic analysis has revealed the presence of NYC1-like (NOL) protein as the most closely related protein to NYC1 in plants, and the *nol* mutant also exhibits the same phenotype as *nyc1* (36). These results suggest that chlorophyll *b* reductase participates in the initial step of LHCII degradation. We constructed an *in vitro* degradation system using isolated LHCII and recombinant chlorophyll *b* reductase to understand the molecular mechanism of LHCII degradation. Chlorophyll *b* in the LHCII trimer was converted to 7-hydroxymethyl chlorophyll *a* by chlorophyll *b* reductase. Concomitantly, chlorophyll absorbance maxima shifted to the blue, and all the chlorophyll molecules in LHCII were released. Based on these experiments, we propose an LHCII degradation mechanism.

EXPERIMENTAL PROCEDURES

Plant Materials and Growth Conditions—*Arabidopsis thaliana* (Columbia ecotype) was grown at 23 °C under continuous light in a chamber equipped with white fluorescent lamps at a light intensity of 80 μE m^{-2} s^{-1}. For the dark-induced leaf senescence experiments, 4-week-old *Arabidopsis* plants were kept in darkness at 23 °C for 2, 4, 6, and 8 days. The T-DNA insertion mutants, lacking either AT4G13250 (SALK_091664) (*NYC1*) or AT5G04900 (AL759262) (*NOL*) were obtained from the Arabidopsis Biological Resource Center (Ohio State University) and GABI-Kat (Cologne, Germany), respectively. Both mutants were crossed, and the double mutant was identified by PCR-based genotyping.

Expression and Purification of Recombinant NOL—The coding region of NOL lacking its transit peptide was amplified by PCR using the primers 5′-AATTCGAAGGGAAAAGAGAAACCTATGACGC-3′ (the underlined section is an engineered NspV site) and 5′-TTAAGCTTCTACTCTTCAGTAACATACCTG-3′ (the underlined section is an engineered HindIII site), and cloned into pET-30a(+) at NspV and HindIII sites.

The expression plasmid was introduced into *Escherichia coli* Rosetta DE3 (Novagen) cells. Two milliliters of an overnight culture of the transformed *E. coli* was diluted with 250 ml of Luria-Bertani medium containing kanamycin (50 μg/ml) and chloramphenicol (10 μg/ml). The culture was grown at 37 °C until the optical density at 600 nm reached 0.6. The expression of the *NOL* gene was induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 2 h. After incubation, the culture was harvested by centrifugation at 8000 × *g* for 10 min at 4 °C. The collected cells were resuspended in 100 mM phosphate buffer (pH 7.8) containing 300 mM NaCl and disrupted by sonication. Triton X-100 was added at a final concentration of 1%, and the mixture was incubated for 1 h at room temperature. It was then centrifuged at 8000 × *g* for 20 min at 4 °C to remove the cell debris. The soluble fraction containing recombinant NOL was loaded onto a nickel column (Novagen) pre-equilibrated with the buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole, and 0.8% Triton X-100). The unbound proteins were washed out with the buffer used for equilibration of the column. Subsequently, the recombinant proteins were eluted with 20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 300 mM imidazole, and 0.8% Triton X-100. The concentration of the purified NOL was 0.6 μg/ml. NOL was concentrated using an ultrafiltration column to a protein concentration of 6.0 μg/ml when LHCII was used as the substrate of NOL. The purified protein was stored at 4 °C and used within 24 h of purification.

Preparation of Chlorophyll Derivatives—Chlorophyllide *b* was prepared from chlorophyll *b* by hydrolysis with recombinant chlorophyllase (37). After hydrolysis in 1 ml of the mixture, the reaction was stopped by adding 1 ml of acetone, 2 ml of hexane, and 0.1 ml of 2 M Tris-HCl (pH 9.0). The reaction mixture was vigorously shaken and centrifuged at 8000 × *g* for 10 min. The aqueous layer was diluted with 4 ml of NaCl-saturated water, and chlorophyllide was transferred into 1 ml of diethyl ether. The ether was evaporated using nitrogen gas.

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Pheophytin *b* and pheophorbide *b* were prepared by the elimination of Mg^{2+} from chlorophyll *b* and chlorophyllide *b*, respectively, with HCl. Chlorophyll *b* and chlorophyllide *b* were dissolved in 2 ml of acetone containing 25 mM HCl and incubated for 5 min at room temperature. After incubation, the reaction mixture was diluted with 5 ml of NaCl-saturated water, and the pheophytin *b* or pheophorbide *b* was transferred into 1 ml of diethyl ether. The ether was evaporated by flushing with nitrogen gas.

Isolation and Purification of LHCII Trimer—Mature leaves of *Arabidopsis* were homogenized with a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 0.35 M sucrose. The homogenates were filtered and centrifuged at 6000 × *g* for 10 min. The precipitate was resuspended in 5 mM EDTA and then repelleted by centrifugation at 6000 × *g* for 10 min. The washed pellet was solubilized in 0.8% Triton X-100 to obtain a chlorophyll concentration of 0.8 mg/ml. The mixture was incubated with stirring for 1 min on ice, and centrifuged at 6000 × *g* for 15 min at 4 °C. The supernatant was loaded on 0.1–0.7 M linear sucrose gradients containing 0.08% Triton X-100. The gradients were centrifuged at 100,000 × *g* for 12 h at 4 °C. The LHCII fraction was removed from the gradients with a syringe. $MgCl_2$ and KCl were added to the final concentrations of 10 mM and 100 mM, respectively, and the sample was loaded onto a 0.5 M sucrose cushion in a centrifuge tube and centrifuged at 6000 × *g* for 10 min. The pellet was resuspended in distilled water to a chlorophyll concentration of 2.0 mg/ml.

Enzyme Assay—Chlorophyll derivatives dissolved in a small amount of acetone and NADPH (final concentration was 1 mM) were added to 50 μl of purified NOL solution. The reaction mixture was incubated for 10 min at 25 °C, and the reaction was stopped by adding 200 μl of acetone. After centrifugation at 10,000 × *g* for 10 min, the supernatant containing chlorophyll derivatives was subjected to high performance liquid chromatography (HPLC) as previously described (38). Elution profiles were monitored by absorbance at 441 nm. Chlorophyll derivatives were identified by their retention times on HPLC and their absorbance spectra.

Isolated LHCII was used as a substrate with various concentrations of LHCII and NOL as required (see legends for figures). Trimeric LHCII was incubated with NOL in the presence or absence of 1 mM NADPH at 25 °C. After incubation, the reaction mixtures were subjected to HPLC as described above.

Immunodetection of Chlorophyll-binding Proteins—*Arabidopsis* leaves were homogenized with solubilizing buffer containing 0.1 M Tris-HCl, pH 6.8, 2% SDS, 0.6% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol. The homogenate was heated at to 70 °C for 3 min and centrifuged at 10,000 × *g* for 5 min. The proteins in the supernatant were separated by the SDS-PAGE (10% acrylamide). For the two-dimensional gel electrophoresis (Fig. 6), LHCII was separated under the non-denaturing condition as described below, except that slave gels were used instead of disc gels. After electrophoresis, the gels were immersed in solubilizing buffer and heated at 90 °C for 1 min. The gels were loaded onto the top of a second gel and electrophoresed. Immunodetection was performed using the ECL-plus immunoblotting detection reagents according to the method supplied by the manufacturer (Amersham Biosciences). Specific primary antibodies for CP1 and CP43 were raised against purified proteins, and LHCs antibodies were obtained from AgriSera.

Green Gel Analysis—Chlorophyll-protein complexes of green tissues were analyzed as previously described (13). Detached leaves were homogenized in 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and centrifuged at 10,000 × *g* for 10 min. The green pellet was suspended in 5 mM EDTA and centrifuged at 10,000 × *g* for 10 min. The pellet was then suspended in water and mixed with the same volume of solubilizing buffer (0.6 M Tris-HCl, pH 8.8, 1% SDS, and 20% (v/v) glycerol) and centrifuged at 10,000 × *g* for 1 min. The green supernatant was applied on the polyacrylamide disc gel (5 mm in diameter). Electrophoresis was performed at a constant current of 0.5 mA per disc gel for 1.5 h at 4 °C. The reaction mixture containing LHCII was directly mixed with solubilizing buffer when purified LHCII was analyzed (Figs. 4 and 6). The chlorophyll contents of the green bands were analyzed as previously described (13).

Spectral Measurements of LHCII—After incubation of LHCII with NOL and NADPH, absorbance spectra of LHCII were recorded using an Hitachi 3310 spectrophotometer.

RESULTS

Degradation of Chlorophyll-binding Proteins during Dark Incubation—The first step of the conversion of chlorophyll *b* to chlorophyll *a* is catalyzed by chlorophyll *b* reductase, which reduces chlorophyll *b* to 7-hydroxymethyl chlorophyll *a*. Genetic and biochemical studies recently identified two chlorophyll *b* reductase genes, *NOL* and *NYC1*, in the rice genome (35). Homologous genes of *NOL* and *NYC1* also exist in the *Arabidopsis* genome. We constructed an *Arabidopsis nol/nyc1* double mutant by crossing *nol* and *nyc1* mutants to examine whether inactivation of these chlorophyll *b* reductases results in the stabilization of LHCII in *Arabidopsis*, as observed in rice. Western blotting analysis with antibodies against NYC1 and NOL revealed that these two proteins were completely missing in the mutant (data not shown). The wild type and the mutant were grown under a light-dark cycle for 3 weeks, and then transferred to darkness to trigger senescence. Pigment analysis clearly showed that almost all chlorophyll molecules disappeared after 8-day-dark incubation in the wild type. In the *nol* mutant, the levels of chlorophyll *b* were slightly higher than those of wild type, and the decrease in chlorophyll *b* level was substantially suppressed in the *nyc1* mutant. This observation was in contrast to that of the rice *nol* mutant in which chlorophyll *b* was retained during dark incubation (36). This discrepancy might be due to the differences in the *NOL* expression profiles between rice and *Arabidopsis* as discussed in our previous report (36). Furthermore, in the *nol*/*nyc1* double mutant of *Arabidopsis*, nearly complete suppression of chlorophyll *b* breakdown was observed (Fig. 1*A*). This indicates that chlorophyll *b* reduction by NOL and NYC1 is a unique or, at least, a major route for chlorophyll *b* degradation.

Subsequently, the levels of chlorophyll-binding proteins were examined by Western blotting (Fig. 1*B*). In wild-type plants, all chlorophyll-binding apoproteins examined, including core and peripheral antenna complexes, decreased during senescence, and these proteins finally disappeared after 8-day-

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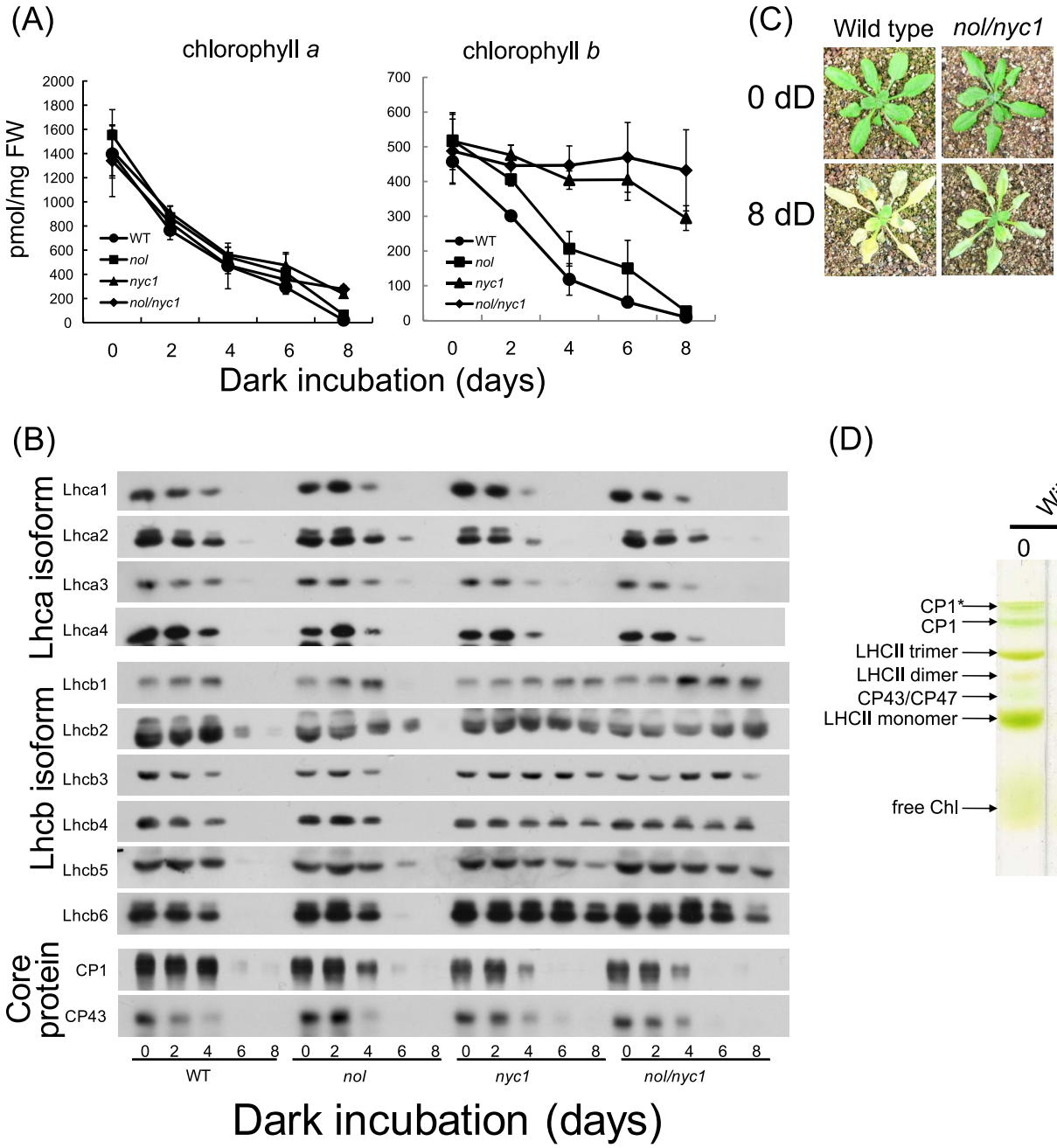

FIGURE 1. **Phenotypic differences between the wild type and the *nol*, *nyc1*, and double mutants during senescence.** *A*, changes in the chlorophyll content during senescence in the dark. Chlorophyll was extracted from leaves and analyzed by HPLC. Data are the average of three replicates, and *bars* denote means ± S.D. *B*, Western blot analysis of chlorophyll-binding proteins. The whole proteins were extracted from the leaves during 8-day dark incubation of the wild type and the mutants, and the apoproteins of LHC, CP43, and CP1 were analyzed by Western blotting. *C*, visible phenotype of the wild type and *nol/nyc1* mutant plants grown for 4 weeks under continuous light (*0 dD*) and transferred to the dark for 8 days (*8 dD*). *D*, separation of chlorophyll-protein complexes by native green gel. Thylakoid membranes were isolated from the leaves before and after 8-day dark incubation of the wild type, and the *nol/nyc1* mutants and chlorophyll-protein complexes were resolved by native green gel electrophoresis. Applied samples were normalized by the fresh weight of leaves. *CP1** is a supercomplex of PSI consisting of CP1 and Lhca.

dark incubation. The *nol* mutant exhibited a degradation profile similar to the wild-type plants. Degradation of core antenna complexes and Lhca isoforms was observed in both of the *nyc1* and *nol/nyc1* mutants as in the wild type; however, Lhcb isoforms remained at a constant level during dark incubation. Consistent to the results of chlorophyll measurements during dark incubation (Fig. 1*A*), the *nol*/*nyc1* double mutant was green after 8 days of dark incubation (Fig. 1*C*). Native green gel experiments also showed that LHCII was selectively stabilized in the mutant (Fig. 1*D*). These results indicate that chlorophyll *b* reductase plays a central role in LHCII degradation in *Arabidopsis*.

Substrate of Chlorophyll b Reductase—Experiments with *nol/nyc1* double mutant revealed that these two chlorophyll *b* reductases participate in chlorophyll *b* degradation in *Arabidopsis*. However, many chlorophyll *b* derivatives such as chlorophyllide *b*, pheophorbide *b*, and pheophytin *b* are expected to occur in the chloroplasts, because some of the degradation

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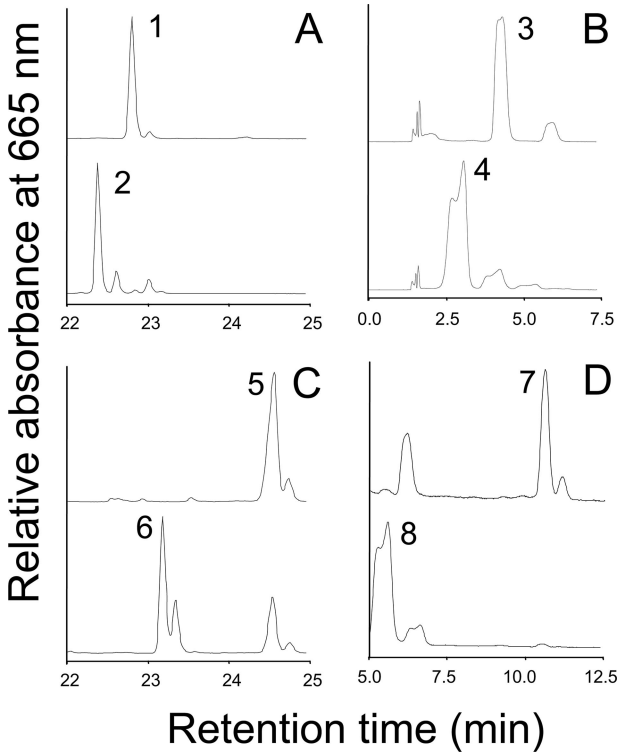

FIGURE 2. **Substrate specificity of chlorophyll *b* reductase.** Chlorophyll derivatives (1–4 μM) (*A*, chlorophyll *b*; *B*, chlorophyllide *b*; *C*, pheophytin *b*; and *D*, pheophorbide *b*) were incubated with recombinant NOL (0.3 mg/ml) and 1 mM NADPH for 10 min. After incubation, pigment compositions were analyzed by HPLC. *1*, chlorophyll *b*; *2*, 7-hydroxymethyl chlorophyll *a*; *3*, chlorophyllide *b*; *4*, 7-hydroxymethyl chlorophyllide *a*; *5*, pheophytin *b*; *6*, 7-hydroxymethyl pheophytin *a*; *7*, pheophorbide *b*; and *8*, 7-hydroxymethyl pheophorbide *a*.

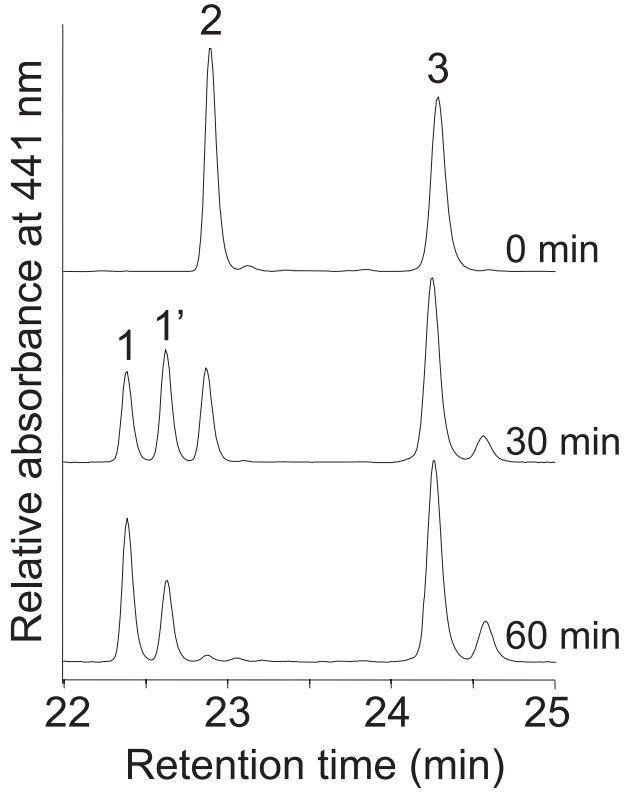

FIGURE 3. **Changes in chlorophyll composition after incubation of trimeric LHCII with recombinant NOL.** Purified LHCII trimer (10 μg of chlorophyll/ml) was incubated with the recombinant NOL (0.3 mg/ml) and 1 mM NADPH. After incubation, chlorophylls were extracted from the reaction mixture and analyzed by HPLC. Elution profiles were monitored by absorbance at 441 nm. *1*, 7-hydroxymethyl chlorophyll *a*; *1′*, 7-hydroxymethyl chlorophyll *a* epimer; *2*, chlorophyll *b*; and *3*, chlorophyll *a*.

enzymes such as chlorophyllase (39) and Mg-dechelatase (40) have wide substrate specificity. We examined whether a formyl group at the C-7 position of these pigments was converted to a hydroxymethyl group by chlorophyll *b* reductase. NOL was expressed in *E. coli*, and chlorophyll *b* derivatives were incubated with the purified recombinant NOL proteins. When chlorophyll *b* was used as a substrate, almost all chlorophyll *b* was converted to 7-hydroxymethyl chlorophyll *a* within 10 min (Fig. 2*A*). Chlorophyllide *b* was also efficiently converted to 7-hydroxymethyl chlorophyllide *a* (Fig. 2*B*). Interestingly, both pheophorbide *b* and pheophytin *b* were converted to their respective hydroxymethyl molecules (Fig. 2, *C* and *D*). These results indicate that chlorophyll *b* reductase has wide substrate specificity and reduces the formyl groups of all the chlorophyll *b* derivatives that potentially occur in chloroplasts.

All the chlorophyll molecules are believed to form chlorophyll-protein complexes in fully greened tissues. If chlorophyll *b* reductase cannot catalyze chlorophyll *b* in the complexes, chlorophyll *b* extraction by some unknown mechanism or degradation of the protein moiety by proteases must precede the reduction of chlorophyll *b*. In contrast, if chlorophyll *b* reduction is the first step of LHCII degradation, chlorophyll *b* reductase must catalyze chlorophyll *b* embedded in LHCII as a substrate. To answer this question, we carried out *in vitro* experiments with chlorophyll *b* reductase (NOL) and LHCII trimer. LHCII trimer, which was isolated by sucrose density gradient centrifugation, was incubated with chlorophyll *b* reductase, and the products were analyzed by HPLC (Fig. 3). Two peaks corresponding to chlorophyll *a* and chlorophyll *b* were observed before incubation. After 30-min incubation, new peaks corresponding to 7-hydroxymethyl chlorophyll *a* and its epimer appeared. As the incubation period prolonged, 7-hydroxymethyl chlorophyll *a* increased with a concomitant decrease in chlorophyll *b*. Finally, all the chlorophyll *b* in LHCII was converted to 7-hydroxymethyl chlorophyll *a*. In contrast, chlorophyll *a* remained at a constant level during the incubation periods. These results clearly indicate that chlorophyll *b* reductase is able to act on the substrate within the complex.

Fig. 4 shows the native green gel analysis of LHCII after incubation with chlorophyll *b* reductase. We employed two different experimental conditions to investigate the degradation processes of the LHCII trimer. In the first experiment, LHCII was incubated with a low NOL/LHCII ratio to investigate the initial process of LHCII degradation (Fig. 4*A*). A control was performed without NADPH, because chlorophyll *b* reductase requires NADPH as a reductant. When LHCII was incubated with chlorophyll *b* reductase in the absence of NADPH, most of the LHCII migrated as a trimer and levels of free chlorophyll and monomeric LHCII were low. The monomeric LHCII might have been formed during SDS-PAGE. When NADPH was added to the reaction mixture, monomeric LHCII slightly increased with a concomitant decrease in trimeric LHCII. The free pigment band significantly increased during incubation.

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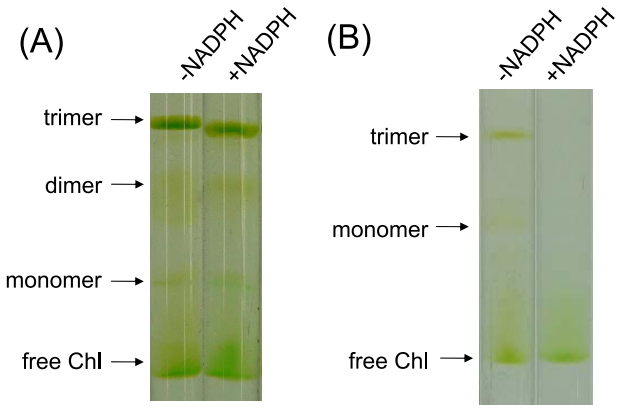

FIGURE 4. **Native green gel electrophoresis of LHCII.** *A*, isolated trimeric LHCII (0.4 mg of chlorophyll/ml) was incubated for 2 h with the recombinant NOL (0.24 mg/ml) and 1 mM NADPH. After incubation, LHCII was separated by native green gel electrophoresis. *B*, isolated trimeric LHCII (0.1 mg of chlorophyll/ml) was incubated with concentrated NOL (3.0 mg/ml) for 2 h with (+NADPH) or without (−NADPH) NADPH, and LHCII was separated by native green gel electrophoresis.

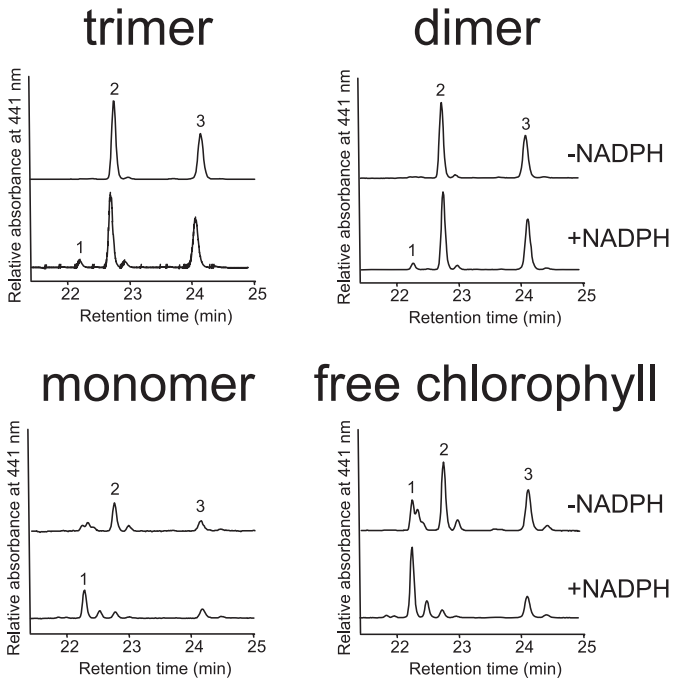

FIGURE 5. **Chlorophyll composition of LHCII after incubation with NOL.** Chlorophyll was extracted from the green bands (shown in Fig. 4*A*), and analyzed by HPLC. Elution profiles were monitored by absorbance at 441 nm. *1*, 7-hydroxymethyl chlorophyll *a*; *2*, chlorophyll *b*; and *3*, chlorophyll *a*.

Next, chlorophylls were extracted from the green bands on the gel and analyzed by HPLC (Fig. 5). When NADPH was absent in the reaction mixture, chlorophyll *a* and *b* were found in the green bands, whereas other chlorophyll derivatives were not detected. After incubation with NADPH for 2 h, both dimeric and trimeric LHCII contained a trace of 7-hydroxymethyl chlorophyll *a* in addition to chlorophyll *a* and chlorophyll *b*. In contrast, a large amount of 7-hydroxymethyl chlorophyll *a* appeared in free pigment bands after incubation. These results indicate that chlorophyll *b* reductase extracted chlorophyll *b* from trimeric LHCII and converted it to 7-hydroxymethyl chlorophyll *a* followed by a release of the pigment from the

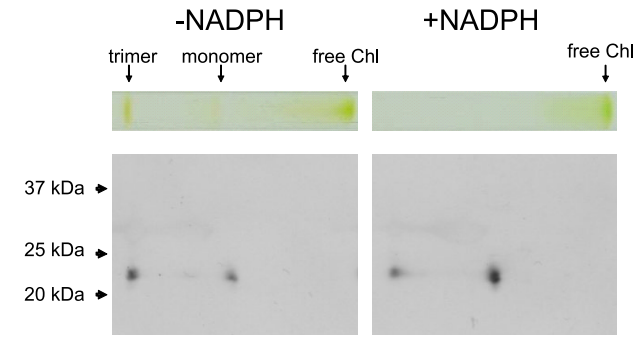

FIGURE 6. **Two-dimensional gel electrophoresis of LHCII after incubation with NOL.** After separation of LHCII by native green gel electrophoresis (Fig. 4*B*), the gel was immersed in solubilizing buffer and heated at 90 °C for 1 min. The gel was loaded on top of a slab gel and electrophoresed. Proteins were transferred to a polyvinylidene difluoride membrane, and LHCII was detected using an anti-LHCII antibody.

enzymes and LHCII. A small amount of 7-hydroxymethyl chlorophyll *a* was found in monomeric LHCII.

When LHCII was incubated with a high NOL/LHCII ratio, green bands corresponding to monomeric and trimeric LHCII disappeared, and all chlorophyll and carotenoid molecules migrated as free pigments on the gel (Fig. 4*B*). Next, we examined the distribution of LHCII apoproteins on a gel by Western blotting. Our antibodies only weakly reacted with trimeric LHCII; therefore, trimeric LHCII on the gels was dissociated into monomer by heat treatment in an SDS-containing buffer before conducting the second electrophoresis for Western blotting. Western blotting analysis clearly showed that LHCII apoproteins existed on the gel in the region corresponding to the LHCII trimer as well as the monomer, although chlorophyll was not found in this region (Fig. 6). These results indicate that LHCII apoproteins exist in the trimeric form, even when they lose all of their chlorophyll molecules. An increase in monomeric LHCII was observed, thus indicating that the trimeric form was unstable when it lost chlorophyll compared with the intact LHCII.

Spectral Changes of LHCII during Incubation with Chlorophyll b Reductase—Final folding conformation of LHCII requires both chlorophyll *a* and chlorophyll *b*. It is, therefore, reasonable to assume that the LHCII conformation is altered when chlorophyll *b* is released from LHCII during incubation with chlorophyll *b* reductase. These structural changes would also have an effect on the spectral properties of other chlorophylls in LHCII. We measured LHCII absorbance spectra during incubation with NOL to monitor the spectral changes of LHCII. Fig. 7 shows the difference absorbance spectra obtained before and after incubation. After 5-min incubation, a small decrease in the absorbance at around 650 nm was detected, thus indicating that a small amount of chlorophyll *b* had been converted to 7-hydroxymethyl chlorophyll *a*. In contrast, an extensive decrease in absorbance at 680 nm of chlorophyll *a* was observed with a corresponding increase in 664 nm during the first 5 min. When the incubation time was prolonged, the decrease in absorbance at 650 nm continued until 60 min, which was consistent with the conversion of chlorophyll *b* to 7-hydroxymehtyl chlorophyll *a* (Fig. 3). The decrease in 680 nm absorbance was almost complete after 30 min. It has been

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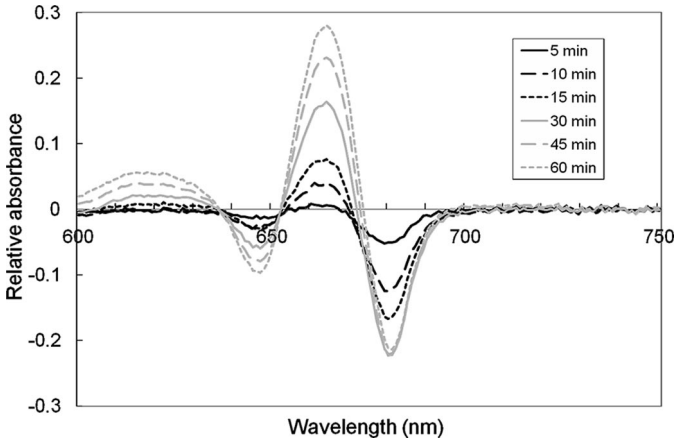

FIGURE 7. **Time difference spectra of LHCII during incubation with NOL.** LHCII (0.03 mg of chlorophyll/ml) was incubated with NOL (0.3 mg/ml) and 1 mM NADPH for various periods, and the absorbance spectra from 600 nm to 750 nm were recorded. Time difference spectra obtained before and after incubation were calculated from the absorbance spectra.

reported that strong interactions between chlorophylls or between chlorophyll and proteins is required for long wavelength absorption (41). A decrease in long wavelength absorption could result from the loss of these strong interactions due to conformational changes of LHCII induced by the loss of chlorophyll *b*.

DISCUSSION

The involvement of multiple chloroplast proteases in LHCII degradation has been suggested by *in vitro* and *in vivo* experiments. For example, thylakoid membranes prepared from high light-acclimated leaves degrade LHCII, whereas the mutant lacking FtsH6 does not degrade LHCII (26). However, the molecular mechanism of LHCII degradation is still unknown. It has been reported that LHCII with chlorophyll is more stable in thylakoid membranes compared with chlorophyll-depleted apoproteins (28, 42), probably because chlorophyll-binding promotes proper conformation, which contributes to the stability of the complex. Structural changes or denaturation processes of substrate LHCII might occur before degradation by proteases. FtsH, which was identified as a LHCII protease, belongs to the AAA-protein family; however, FtsH lacks a robust unfoldase activity (43), and ATP hydrolysis by FtsH is mainly used to sequentially translocate the unfolded substrates from the recognition signal to the active site. For example, FtsH cannot degrade stable proteins such as GFP, but GFP can be degraded by clp protease (43). This indicates that the unfolding of LHCII by unfoldase or by other mechanisms must precede LHCII degradation by FtsH. Chlorophyll *b* reductase solves this problem. When a small number of chlorophyll *b* molecules in trimeric LHCII was converted to 7-hydroxymethyl chlorophyll *a*, the absorbance spectra of chlorophyll *a* were drastically changed, thus suggesting the loss of the structural integrity of LHCII. All the chlorophyll molecules were released from the apoproteins during native green PAGE (Fig. 4*B*) when incubation periods were prolonged. LHCII apoproteins might not be able to retain chlorophyll *a* when they lose chlorophyll *b* by the action of chlorophyll *b* reductase, probably due to the loss of structural integrity. The same phenomena could occur in thylakoid membranes during disassembly of LHCII by chlorophyll *b* reductase. However, it cannot be excluded that chlorophyll molecules still loosely associate to LHCII apoproteins in thylakoid membranes. This free and/or loosely bound chlorophyll *b* in chloroplasts could be immediately converted to 7-hydroxymethyl chlorophyll *a*, because our biochemical studies clearly showed that chlorophyll *b* reductase could convert both free and bound chlorophyll *b*.

Two conflicting hypotheses concerning the substrate of LHCII proteases have been proposed. Most of the LHCII exists in a trimeric form in the thylakoid membranes. Proteolytic activity against LHCII has been co-purified with the trimeric LHCII. The protease degraded both monomeric and trimeric LHCII at a more or less equal rate (44). However, the experiments with reconstituted LHCII clearly showed that the monomeric LHCII was targeted by the protease, whereas trimeric LHCII was not (28). The latter hypothesis requires the dissociation of trimeric LHCII into monomers before degradation. Thermal stability experiments suggested the possibility of the dissociation of trimeric LHCII into monomers, if the monomeric state is trapped by some biological mechanism such as proteolytic digestion (45). However, we found that trimeric LHCII is targeted by chlorophyll *b* reductase, and that all the chlorophyll molecules were ultimately released from trimeric LHCII. Interestingly, we found trimeric LHCII apoproteins on the gel, which had no chlorophyll molecules, thus indicating that the trimeric LHCII need not dissociate into monomers during disassembly of pigments from the apoproteins. Chlorophyll-depleted trimeric apoproteins would be immediately degraded by proteases.

Based on *in vivo* and *in vitro* experiments, we propose that the LHCII degradation mechanism is as follows. First, chlorophyll *b* reductase acts on LHCII to extract chlorophyll *b* from the complexes and converts it to 7-hydroxymethyl chlorophyll *a*. Furthermore, 7-hydroxymethyl chlorophyll *a* is converted to chlorophyll *a* by 7-hydroxymethyl chlorophyll *a* reductase. Finally, all the chlorophyll *b* in LHCII is released from the apoproteins. Accompanied by the release of chlorophyll *b*, chlorophyll *a* also dissociates from the LHC protein. LHCII apoproteins are denatured by the loss of chlorophyll. Nonstructural LHCII apoproteins are immediately digested by proteases such as FtsH and SppA. Besides major LHCII proteins, some other minor LHCII proteins are degraded by the same process, because degradation of these complexes is retarded in the *nyc1* mutant (35). This scheme cannot be applied to the core antenna complexes of CP1, CP43/47, and D1/D2 complexes, because they have no chlorophyll *b* and are degraded during dark incubation in the *ncy1* mutant as in the wild type. Degradation of these complexes must therefore be regulated by a different mechanism. Different degradation mechanisms provide a reasonable hypothesis, because the degradation profile of LHCII and other core complexes are quite different. For example, LHCII is rapidly decreased, whereas other chlorophyll *a*-protein complexes increased during the dark incubation of greening tissues (22). It is also reasonable to assume that the chlorophyll degradation process precedes the degradation of the protein moiety. If protease triggers the initial step of LHCII degradation, free chlorophyll molecules would appear, which

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would potentially generate reactive oxygen species, and induce growth retardation and/or cell death. According to our model, chlorophyll directly enters the degradation pathway (46) or is immediately reused in the formation of other chlorophyll-protein complexes (47), reducing the level of free chlorophyll.

LHCII were not degraded in the mutant lacking chlorophyll *b* reductase, although other chlorophyll protein complexes such as CP1 and CP43/47 degraded as in the wild type (35). These observations indicate that the conversion of chlorophyll *b* to 7-hydroxymethyl chlorophyll *a* plays an essential role in LHCII degradation. It has been recently reported that LHCII degradation is also retarded in mutants such as *sgr* (48). SGR is a chloroplast protein and was thought to be directly involved in the destabilizing mechanism of LHC complexes by forming SGR-LHC complexes. However, we have shown that the purified LHCII trimer was entered into the degradation pathway by NOL without the addition of SGR, thus suggesting that chlorophyll *b* reductase activity does not directly require SGR protein. Further studies are required to clarify the relationship between chlorophyll *b* reductase and other stay-green genes.

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