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Defects in a Proteolytic Step of Light-Harvesting Complex II in an *Arabidopsis* Stay-Green Mutant, *ore10*, during Dark-Induced Leaf Senescence

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During dark-induced leaf senescence (DIS), the non-functional stay-green mutant *ore10* showed delayed chlorophyll (Chl) degradation and increased stability in its light-harvesting complex II (LHCII). These phenomena were closely related to the formation of aggregates that mainly consisted of terminal-truncated LHCII (Oh et al., 2003). The *ore10* mutant apparently lacks the protease needed to degrade the truncated LHCII. In wild-type (WT) plants, protease was found in the thylakoid fraction, but not the soluble fraction. A similar experiment using dansylated LHCII revealed that the protease degraded both WT and *ore10* LHCII, indicating that its stability in *ore10* perhaps did not result from a defect in the LHCII polypeptides themselves. Although protease activity was not present in non-senesced WT leaves, it was induced during DIS. It also was possible to diminish the high level of protease present in the thylakoids through high-salt washing, suggesting that this enzyme is extrinsically bound to the outer surface of the stroma-exposed thylakoid regions.

Keywords: Arabidopsis thaliana, leaf senescence, light-harvesting complex II, proteolysis, stay-green mutant

The development cycle of a plant leaf ends with its senescence, during which deteriorative events lead to cell death (Nooden, 1988). The most characteristic, visible change is leaf yellowing, a result of the preferential breakdown of chlorophyll (Chl) and the synthesis of other pigments with concomitant chloroplast degradation (Thimann, 1980; Thomas and Stoddart, 1980; Biswal and Biswal, 1988; Nooden, 1988; Matile, 1992; Smart, 1994). Inside the cells of senescing leaves, highly ordered disassembly and degradation processes of cellular components occur (Nooden et al., 1997).

Several mutations affect leaf senescence (Thomas and Smart, 1993; Bachmann et al., 1994; Nooden and Guiamet, 1996; Nam, 1997; Oh et al., 1997). For example, some *Arabidopsis* mutants show delayed onset (Oh et al., 1997, 2000; Woo et al., 2001). We have also investigated the disassembly process of Chl-protein complexes during dark-incubation of detached leaves of *Arabidopsis* (Oh and Lee, 1996; Oh et al., 2000). The photosystem II (PSII) reaction center complexes are degraded earlier than the light-harvesting complex II (LHCII). In addition, the photosystem I (PSI) antenna complexes are degraded before the breakdown of PSI reaction center complexes, indicating that the degradation of the PSI antenna is regulated by a different mechanism from that controlling the destruction of PSII (Oh and Lee, 1996; Yamazaki et al., 2000; Guiamet et al., 2002). However, Chl-protein complexes in a non-functional stay-green mutant, e.g., *ore*10, are degraded through abnormal processes; the stability of its Chls is probably related to the increased durability of the LHCII through aggregate formation (Oh et al., 2000, 2003). Similarly, the maternally inherited mutation *cytG* in soybean (Bernard and Weiss, 1973) also inhibits the degradation of LHCII and its associated pigments (Guiamet et al., 1991). Finally, the stay-green phenotype of a Chl catabolism mutant, Bf993, from the grass *Festuca pratensis*, has a lesion at the ring-opening step in the Chl degradation pathway and shows stable LHCII and D1 proteins (Vicentini et al., 1995; Matile et al., 1996). Although the mutation genes *cytG* and *Bf993* block Chl degradation, such a breakdown can also be prevented through the inhibition of protein degradation because Chls are surrounded by proteins in the LHCII.

About half the Chls in higher plants and green algae are bound to LHCII that plays an important role in the stacking of thylakoid membranes (Mullet and Arntzen, 1980) and in the regulation of energy distribution between PSI and PSII (Anderson, 1986), in addition to its main function, i.e., the collection of light energy.

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The susceptibility of Chl to light exposure is more pronounced in the thylakoid membrane, the site of light perception. Thylakoid membrane proteins, such as D1 and LHCII, manifest increased susceptibility to proteolysis when chloroplasts are exposed to light (Christopher and Mullet, 1994; Andersson and Aro, 1997). Regulatory proteolytic activity is also involved in the degradation of LHCII when spinach leaves are acclimating from low- to high-intensity light (Lindahl et al., 1995). This adaptation by the light-harvesting apparatus is generally thought to optimize photosynthetic efficiency and minimize light stress and PSII photoinhibition (Andersson and Barber, 1996; Melis, 1996; Park et al., 1997).

Previously we reported that the delayed Chl degradation and increased LHCII stability in mutant *ore10* is somewhat related to aggregate formation during dark-induced senescence (DIS), and that most of those aggregates consist primarily of trimmed LHCII (Oh et al., 2003). The objective of this current research was to investigate the process of LHCII proteolysis and elucidate the reason for this high stability in LHCII.

MATERIALS AND METHODS

Plant Material

Seeds of *Arabidopsis ore10* mutant and a wild type (WT) were sown in a 1:1:1 mixture of vermiculite:peat moss:perlite, and placed in a growth chamber with a 16-h photoperiod and a temperature cycle of 22°C/18°C (D/N). For the dark treatment, leaves were excised from approximately 21-d-old seedlings, just prior to bolting, and were floated on 3 mM MES buffer (pH 5.8) in the dark.

Measurement of Chl and Photochemical Efficiency of PSII

Leaves were ground in 2 ml of 80% acetone with a glass homogenizer. Chl content was calculated on a tissue fresh-weight basis according to the method of Arnon (1949). Leaf fluorescence was determined with a PAM-2000 fluorometer (Walz, Germany) after dark-adaptation for 10 min at room temperature. The photochemical efficiency of PSII was monitored by measuring Fv/Fm (Kitajima and Butler, 1975). Minimal fluorescence (Fo) -- with all open PSII reaction centers -- was obtained by measuring the modulated light sufficiently low enough (<0.1 μmol $m^{-2}s^{-1}$) not to induce any significant variable fluorescence. Maximal fluorescence (Fm) -- with all closed PSII reaction centers -- was determined after applying a 0.8-s saturating pulse at 3,200 μmol $m^{-2}s^{-1}$ to dark-adapted leaves. Variable fluorescence (Fv) was then calculated as Fm minus Fo.

Preparation of Thylakoid Membranes and Soluble Fractions

Thylakoid membranes were prepared as described by Aro et al. (1993), with some modifications. Detached leaves were ground with a glass homogenizer in ice-cold grinding buffer [50 mM Hepes (pH 7.6), 0.3 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$]. The homogenates were filtered through two layers of Miracloth (Calbiochem, USA) and spun for 7 min at 20,000*g* in a microcentrifuge (Micro 17R; Hanil, Korea). The pellet was then washed twice and re-suspended in a buffer containing 50 mM Hepes (pH 7.6), 0.1 M sorbitol, 10 mM NaCl, and 5 mM $MgCl_2$. This suspension was kept on ice in the dark until after the Chl concentration was measured, as described by Arnon (1949). The supernatant obtained via centrifugation was dialyzed overnight at 4°C and used as a soluble fraction for assaying proteolytic activity. Protein concentration in this soluble fraction was determined according to the method of Lowry et al. (1951).

Native Green Gel Electrophoresis and Fully Denaturing SDS-PAGE

Native Chl-protein complexes were separated as described by Allen and Staehelin (1991). The isolated thylakoid membrane suspension was centrifuged, and the pellet was washed twice in 2 mM Tris-maleate (pH 7.0). Afterward, it was re-suspended in a solubilization buffer of 0.45% octyl glucoside, 0.45% decyl maltoside, 0.1% lithium dodecyl sulfate, 10% glycerol, and 2 mM Tris-maleate (pH 7.0) to yield a 20:1 (w/w) ratio of total nonionic detergents to Chl. The samples were kept on ice for 30 min. An aliquot of the solubilized thylakoid membranes containing 13.5 μg of Chl per lane was loaded on a 1.5-mm-thick gel. The samples were then electrophoresed at 4°C and a constant 6 mA.

For the two-dimensional SDS-PAGE analysis of the Chl-protein complexes, 1.5-mm-thick strips were excised from the green gel lanes and incubated for 30 min at 37°C in a denaturing buffer that combined 1X stacking gel buffer [25 mM Tris-HCl (pH 6.3) and 50 mM glycine], as described by Allen and Staehelin (1991), plus 2% sodium dodecyl sulfate, 2% mercap-

toethanol, and 10% glycerol. The fully denatured gel strips were laid directly on a 1.5-mm-thick stacking gel, using the buffer system of Laemmli (1970). Electrophoresis was conducted at 30 mA constant current on a vertical electrophoresis unit (LKB 2001; LKB, Sweden). Afterward, the gels were stained with either silver nitrate or Coomassie Brilliant Blue R250 (Sambrook et al., 1989).

Proteolysis of LHCII

The putative protease for the proteolytic cleavage of *ore10* LHCII may reside in either the soluble or the thylakoid fraction. To test whether leaves from *ore10* plants lacked this protease, we isolated a thylakoid membrane fraction from the mutant samples and incubated it at 25°C in the dark for various intervals with either a soluble or a thylakoid fraction isolated from either WT or *ore10* leaves. When the two thylakoid fractions were mixed, an aliquot from each thylakoid fraction contained 13.5 μg Chl; when a thylakoid fraction was mixed with a soluble fraction, an aliquot of *ore10* thylakoids containing 27 μg Chl was mixed with an aliquot of a soluble fraction containing 500 μg soluble protein. After the incubation, the degradation of LHCII polypeptides was evaluated based on changes in the band intensities of LHCII following SDS-PAGE analysis of the reaction mixtures.

Dansylation of Senesced *ore10* Thylakoids and LHCII Proteolysis using Dansylated Thylakoids

Dansyl chloride [DNS; 5-(Dimethylamino)naphthalene-1-sulfonyl chloride] is frequently used as a reagent for the fluorescence-labeling of proteins (Kinoshita et al., 1974). Here, we dansylated the proteins in thylakoid membranes containing 1 mg Chl from 6-d-senesced *ore10* leaves by incubating them with 2 mM DNS for 2 h at 20°C, according to the technique of Jacquot et al. (1984). The reaction mixture was then washed with the thylakoid re-suspending buffer and pelleted by centrifuging it twice to remove unreacted DNS. For the proteolysis assay, the dansylated thylakoid proteins were mixed with thylakoid fractions isolated from 0-, 2-, 4-, or 6-d-senesced WT leaves, and then incubated at 25°C for 18 h. After the LHCII polypeptides were separated by SDS-PAGE, the gel was illuminated with ultraviolet light and the dansylated LHCII polypeptides were visualized using a camera and an attached 510-nm narrow-bandpass filter. The gel was then stained with Coomassie Brilliant Blue R250.

High-Salt Treatment of Thylakoid Membranes

Isolated WT thylakoids were washed twice with the thylakoid re-suspending buffer, which was supplemented with 0.5 M NaCl to release most of the loosely bound extrinsic polypeptides from the membranes. The supernatants obtained after centrifugation were desalted by overnight dialysis, and used for the proteolysis experiments.

RESULTS

Delayed Chl Degradation and LHCII Stability in *ore10*

After 6 d of dark-induced senescence (DIS), leaves of the *ore10* mutant still retained 57% as much Chl as the non-senescing controls, while nearly 90% of the Chl in the WT control leaves had been degraded at that point (Table 1). As we reported previously (Oh et al., 2003), the decrease in photochemical efficiency of PSII during DIS in the non-functional stay-green mutant, *ore10*, is not delayed at all, compared with that process in the WT, and efficiency becomes undetectable after 6 d of DIS. Our native green gel analysis showed that most of the retained Chls in the mutant leaves were located in a LHCII trimer band (Fig. 1A). The coupled two-dimensional SDS-PAGE indicated that almost all the Chls remaining after those 6 d may have been bound mainly to the LHCII polypeptides

Table 1. Changes in Chl contents of WT and *ore10* leaves during dark-induced senescence. Content was determined before (0 d) and 6 d after dark-incubation, and expressed on basis of fresh weight of leaves measured before onset of DIS. Digits in parentheses represent percent values of the controls. FW, fresh weight.

	WT		*ore10*	
Dark-incubation time (days)	0d	6d	0d	6d
μg Chl / g FW (%)	1247 ± 57 (100)	140 ± 34 (11)	1250 ± 40 (100)	712 ± 12 (57)

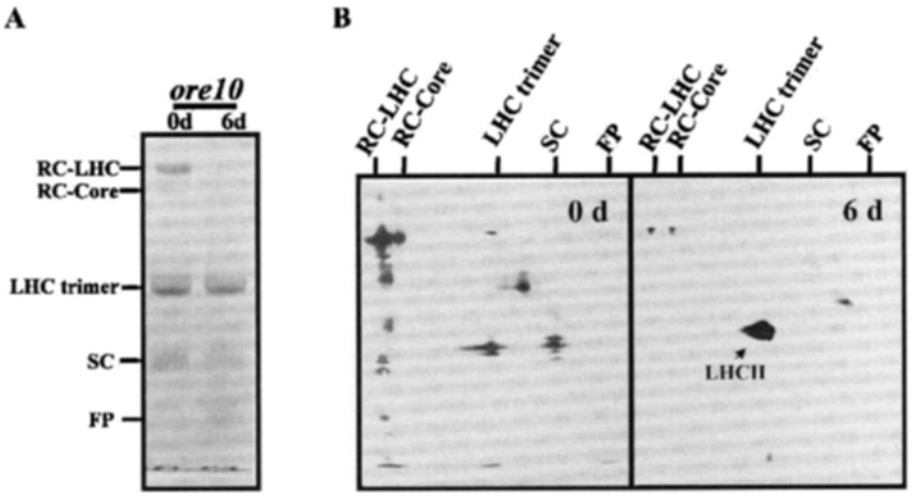

Figure 1. A, Native green gel band patterns of Chl-protein complexes in *ore10* leaves before and after dark-induced senescence for 6 d, and **B**, two-dimensional SDS-PAGE analysis of polypeptide composition of those complexes. Each lane was excised anc applied to top of SDS-PAGE gel after being rotated counterclockwise at 90° from direction of green-gel electrophoresis. Separated polypeptides were stained with Coomassie Brilliant Blue R250, and bands were labeled according to Allen and Staehelir (1991). Region labeled as RC-LHC contains a number of large PSI and PSII complexes attached with antennae. RC-Core regior comprises several partial PSI and PSII complexes largely stripped of antennae. LHCII trimer, trimeric form of PSII antenna LHCII; SC, small complexes; FP, free pigment.

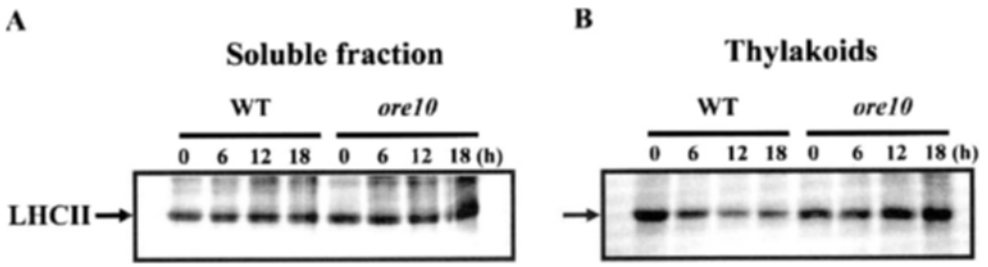

Figure 2. LHCII proteolysis of *ore10* thylakoids mixed with either soluble (**A**) or thylakoid (**B**) fractions isolated from either WT or *ore10* leaves during dark-induced senescence. Reaction mixtures were incubated at 25°C in dark for 0 to 18 h; band for LHCII polypeptides, indicated by arrow, was separated by SDS-PAGE and stained with Coomassie Brilliant Blue R250.

(Fig. 1B).

Proteolysis of LHCII by Incubation of *ore10* Thylakoids with either Soluble or Thylakoid Fractions Isolated from Either WT or *ore10* Leaves

In *ore10*, the delay in Chl degradation is probably due to its stable LHCIIs, an attribute that may result from a defect in the proteolytic process in that mutant. To test this possibility, we isolated thylakoids from 6-d-senesced *ore10* leaves and incubated them with either soluble or thylakoid fractions extracted from either 5-d-senesced WT leaves or 6-d-senesced mutant leaves (Fig. 2). LHCII degradation was pronounced only when the *ore10* thylakoids were mixed with the WT thylakoid fraction. This suggests that only the latter fraction contained an active protease for LHCII degradation, which was not present in the *ore10* thylakoid fraction or other soluble fractions.

Proteolysis of Dansylated *ore10* LHCII by Its Incubation with Thylakoid Fraction Isolated from WT Leaves

The decrease in the intensity of the LHCII band shown in Figure 2B can be due to the degradation of WT LHCII, *ore10* LHCII or both. To confirm that *ore10* LHCII really was degraded, we repeated the LHCII degradation experiment, using 6-d-senesced *ore10* thylakoids labeled by a reaction with 2 mM dansyl chloride (Fig. 3). A decrease in fluorescence emitted from the labeled LHCII polypeptides was clearly

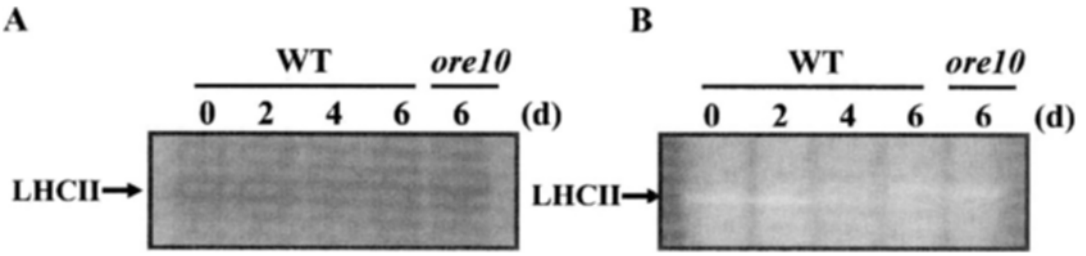

Figure 3. LHCII proteolysis of dansylated *ore10* thylakoids mixed with WT or *ore10* thylakoids during dark-induced senescence. Reaction mixtures were incubated at 25°C in dark for 18 h; band for LHCII polypeptides, indicated by arrow, was separated by SDS-PAGE and stained with Coomassie Brilliant Blue (**A**), after fluorescence from dansylated LHCII was observed with illumination by ultraviolet light passed through 510-nm narrow-bandpass filter (**B**).

observed in 4-d-senesced leaves. Such degradation was less obvious when the polypeptides were stained with Coomassie Brilliant Blue, but it did become apparent in the 6-d-senesced leaves. These results demonstrate that the LHCII polypeptides in *ore10* leaves were actually degraded by a protease contained in the thylakoid fractions of 4-d- or 6-d-senesced WT leaves, and that such a protease seemed to be missing or non-functional in our 6-d-senesced *ore10* leaves. In addition, protease activity probably was not occurring in the non-senesced leaves but was apparently induced during DIS. Furthermore, the protease in senescing WT thylakoids was able to recognize *ore10* LHCII polypeptides as well as WT LHCII polypeptides for degradation, and the increased stability of *ore10* LHCII was not due to possible structural changes that resulted from an alteration in some key amino acid sequences of the LHCII polypeptides that served as enzyme substrates.

High-Salt Washing of WT Thylakoids

The association of protease with WT thylakoids may be intrinsic or extrinsic. If the latter, washing the thylakoids with high-salt or alkaline media should release proteolytic activity from the thylakoid fraction, because these treatments are known to weaken the attractive forces between extrinsic membrane polypeptides and their anchoring protein (Lindahl et al., 1995). As expected, WT thylakoids washed with an incubation medium containing 0.5 M NaCl could not degrade *ore10* LHCII (Fig. 4). In contrast, when the high-salt solution that remained after washing the thylakoids, i.e., the 'wash supernatant', was re-added after the salt was removed by dialyzing the reaction mixture of *ore10* thylakoids and high-salt washed WT thylakoids, we noted that the LHCII polypeptides were degraded (Fig. 4). This washing experiment supported the theory that the proteolytic system was extrinsically associated with the WT thylakoids, which could be released from the thylakoids into solution as an active form.

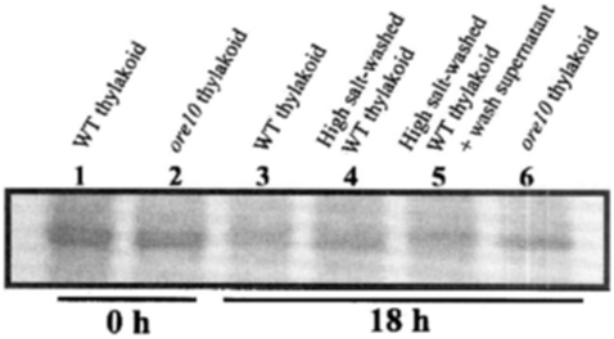

Figure 4. LHCII proteolysis of *ore10* thylakoids mixed with WT or *ore10* thylakoids during dark-induced senescence, as well as effect of high-salt washing. Reaction mixtures were incubated at 25°C in dark for 18 h; band for LHCII polypeptides, indicated by arrow, was separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Lanes 1 and 2 show LHCII bands before incubation; Lanes 3 and 6 include bands after 18 h of incubation for mixtures with WT and *ore10* thylakoids, respectively. Lanes 4 and 5 show bands after 18 h of incubation for mixtures with high salt-washed WT thylakoids and washed thylakoids plus 'wash supernatant', respectively.

DISCUSSION

Our study revealed that the *ore10* mutant has a defect in the degradation process of its LHCII, making that complex very stable during dark-induced senescence. Chls bound in the LHCII remain in the thylakoids during DIS, even after most of the unbound Chls are degraded. Therefore, we assume that the stay-green phenotype of the *ore10* mutant is due to

some imperfections in a proteolytic system or a protease for LHCII polypeptide. However, instead of the protease itself, its substrate might also be mutated to confer stability on those polypeptides, because some amino acid sequences in the *ore10* mutant can be modified during its ethylmethanesulfonate mutagenization (Oh et al., 1997). Nevertheless, this possibility is rare, because protease in the WT thylakoids is also able to recognize and degrade the DNS-labeled *ore10* LHCII (Fig. 3B).

Proteolysis of LHCII

As determined by our high salt-washing experiments, the protease responsible for LHCII degradation appears to be extrinsically located at the outer thylakoid surfaces. Moreover, the proteolytic activity for LHCII is not evident in non-senesced leaves from either the WT or *ore10*, although activity is induced in a late-senescence stage (Fig. 3). Therefore, the product of the mutated gene may be a protease for LHCII polypeptides or a regulatory protein that can induce or activate the protease for LHCII polypeptides during DIS.

In contrast to the steps involved with DIS, the adaptation of a plant to different light conditions is often accompanied by changes in the composition of chloroplast proteins. One notable adjustment is to the size of the photosynthetic antennae. Upon transition from low- to high-light intensity, the size of the LHCII is reduced as a result of specific degradation in a subpopulation of Chl a/b binding proteins (Lindahl et al., 1995). The protease involved in this process is of a serine or cysteine type, reported to be extrinsically bound to stroma-exposed thylakoids. A very prominent feature of this proteolytic process is its strict requirement for ATP. The high light-induced acclimative proteolysis of LHCII is somewhat similar to the DIS-activated proteolysis observed in this study, although it is induced by different stimuli. In contrast, detergent-induced proteolysis has been reported in isolated bean thylakoids (Anastassiou and Argyroudi-Akoyunoglou, 1995). That related enzyme is of a serine type and probably contains sulfhydryl groups essential for catalytic activity. The detergent-induced proteolytic activity of LHCII is independent of ATP, which contrasts with the acclimative degradation of LHCII. In fact, this detergent-induced proteolysis occurred equally in both our WT and *ore10* leaves (data not shown), indicating that the defective protease in *ore10* is not a detergent-inducible enzyme.

Many proteolytic processes in the chloroplast are well recognized, and several chloroplast proteases have been identified and cloned (Adam, 2000). Proteases in the chloroplast are distributed in all major cellular compartments where proteolysis is expected to take place: i.e., in the stroma (Clp protease), on both sides of the thylakoid membrane (FtsH protease), and in the thylakoid lumen (DegP, Tsp protease). Therefore, based on our results, we believe that the thylakoid-bound, stroma-exposed FtsH protease can be a candidate protease defective in *ore10*. In the case of light-induced degradation of the PSII D1 protein, the protein is first damaged via photooxidative stress, then cleaved by an unknown mechanism requiring GTP. The resulting 23-kD fragment is completely degraded by the FtsH protease (Lindahl et al., 2000). In fact, we have previously reported that LHCII polypeptides with truncated terminals are accumulated during DIS in the *ore10* mutant (Oh et al., 2003). Based on this observation, we assume that LHCII degradation proceeds in two steps, and that the N-terminal truncated polypeptides generated in the first step can be degraded fully in the second. The *ore10* mutant may have defects in that second step, causing N-terminal truncated polypeptides to be accumulated. Therefore, this FtsH for a 23-kD D1 fragment appears to be a reasonable candidate for the protease that participates in the dark-induced degradation of LHCII as well. Besides the FtsH, SppA supposedly is involved in the light-induced degradation of PSII and/or LHCII antenna proteins, although the substrates for that chloroplast enzyme are still unknown (Lindahl et al., 1995; Yang et al., 1998). These two candidate enzymes for LHC degradation -- FtsH and SppA -- have been described as membrane-bound proteases (Anastassiou and Argyroudi-Akoyunoglou, 1995; Tziveleka and Argyroudi-Akoyunoglou, 1998) and also as extrinsic components that can be reversibly removed from the thylakoids (Lindahl et al., 1995). Thus, both FtsH and SppA should be considered as potential candidates for the LHC degradation process.

LHCII Degradation and Aggregate Formation

Removal of the LHCII is a complicated process involving not only a specific proteolytic cleavage but also changes in the oligomeric state of the complex, as well as protein phosphorylation and lateral migration of the LHCII from appressed to non-appressed thylakoids. Our previous studies have shown that LHCII aggregates are formed during DIS of *ore10*

leaves (Oh et al., 2000, 2003). These aggregates differ, however, from those found in WT leaves with respect to the timing of their formation, their protein composition, and their spectroscopic properties (Oh et al., 2003). Moreover, aggregates are easily formed because of the increase in LHCII binding ability after its N-terminal region is trimmed (Yang et al., 2000; Oh et al., 2003). Therefore, aggregate formation may confer additional protection from the access of proteases, even though that may not be the primary reason for a stable LHCII.

In conclusion, *ore10* mutant plants seem to have defects in a proteolytic system for LHCII, which is inducible during DIS. The inactive protease is possibly involved in the accumulation of trimmed LHCII for the formation of stable aggregates. Identifying the gene responsible for this defect will increase our understanding of the proteolytic process for LHCII.

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