The Stromal Chloroplast Deg7 Protease Participates in the Repair of Photosystem II after Photoinhibition in Arabidopsis[1][W][OA]

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Light is the ultimate source of energy for photosynthesis; however, excessive light leads to photooxidative damage and hence reduced photosynthetic efficiency, especially when combined with other abiotic stresses. Although the photosystem II (PSII) reaction center D1 protein is the primary target of photooxidative damage, other PSII core proteins are also damaged and degraded. However, it is still largely unknown whether degradation of D1 and other PSII proteins involves previously uncharacterized proteases. Here, we show that Deg7 is peripherally associated with the stromal side of the thylakoid membranes and that Deg7 interacts directly with PSII. Our results show that Deg7 is involved in the primary cleavage of photodamaged D1, D2, CP47, and CP43 and that this activity is essential for its function in PSII repair. The double mutants *deg5 deg7* and *deg8 deg7* showed no obvious phenotypic differences under normal growth conditions, but additive effects were observed under high light. These results suggest that Deg proteases on both the stromal and luminal sides of the thylakoid membranes are important for the efficient PSII repair in Arabidopsis (*Arabidopsis thaliana*).

Chloroplasts of higher plants carry out one of the most important biochemical reactions: the capture of light energy and its conversion into chemical energy. Although light is the ultimate source of energy for photosynthesis, it can also be harmful to plants. Light-induced loss of photosynthetic efficiency, which is generally termed as photoinhibition, limits plant growth and lowers productivity, especially when combined with other abiotic stresses.

The main target of photoinhibition is PSII, which catalyzes the light-dependent water oxidation concomitantly with oxygen production (for review, see Prasil et al., 1992; Aro et al., 1993; Adir et al., 2003). In higher plants, PSII consists of more than 20 subunits, including the reaction center D1 and D2 proteins, cytochrome (Cyt) b_{559}, the light-harvesting chlorophyll *a*-binding proteins CP47 and CP43, the oxygen-evolving 33-kD protein (PsbO), and several low molecular mass proteins (Nelson and Yocum, 2006). The PSII reaction center D1 protein has been identified among PSII proteins as the primary target of light-induced damage (Kyle et al., 1984; Mattoo et al., 1984; Ohad et al., 1984; Adir et al., 1990), but several studies have shown that the D2, CP47, and CP43 proteins are degraded under photoinhibitory conditions (Schuster et al., 1988; Yamamoto and Akasaka, 1995; Jansen et al., 1999; Adir et al., 2003). Moreover, several small PSII subunits, such as PsbH, PsbW, and Cyt b_{559}, were also found to be frequently replaced within PSII (Hagman et al., 1997; Ortega et al., 1999; Bergantino et al., 2003). Evidence for the involvement of two families of proteases, FtsH and Deg, in the degradation of the D1 protein in thylakoids of higher plants has been recently described (Lindahl et al., 1996, 2000; Bailey et al., 2002; Sakamoto et al., 2003; Silva et al., 2003; Kapri-Pardes et al., 2007; Sun et al., 2007a, 2007b). However, it is still largely unknown whether degradation of D1 and other PSII proteins involves previously uncharacterized proteases.

DegP (or HtrA) proteases were initially identified based on the fact that they are required for the survival of *Escherichia coli* at high temperatures and for the degradation of abnormal periplasmic proteins (Lipinska et al., 1988; Strauch and Beckwith, 1988). DegP is an ATP-independent Ser endopeptidase, and it contains a trypsin-like protease domain at the N terminus, followed by two PDZ domains (Gottesman, 1996; Pallen and Wren, 1997; Clausen et al., 2002). PDZ domains appear to be important for complex assembly and substrate binding through three or four residues in the C terminus of their target proteins (Doyle et al.,

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1996; Harris and Lim, 2001). DegP switches between chaperone and protease functions in a temperature-dependent manner. The chaperone function dominates at low temperatures, and DegP becomes proteolytically active at elevated temperatures (Spiess et al., 1999). Crystal structures of different members of the DegP protein family (Krojer et al., 2002; Li et al., 2002; Kim et al., 2003; Wilken et al., 2004) have revealed the structure-function relationship of these PDZ-containing proteases. Trimeric DegP is the functional unit, and the hexameric DegP is formed via the staggered association of trimers (Clausen et al., 2002; Kim and Kim, 2005). At normal growth temperatures, the active site of the protease is located within the chamber of hexameric DegP, which is not accessible to the substrates. However, at high temperatures, conformational changes induce the activation of the protease function (Krojer et al., 2002). Recent studies have shed light on the substrate binding-induced formation of larger oligomeric complexes of DegP (Jiang et al., 2008; Krojer et al., 2008).

In Arabidopsis (*Arabidopsis thaliana*), 16 genes coding for DegP-like proteases have been identified, and at least seven gene products are predicted to be located in chloroplasts (Kieselbach and Funk, 2003; Huesgen et al., 2005; Adam et al., 2006; Sakamoto, 2006; Kato and Sakamoto, 2009). Based on proteomic data, four Deg proteases have been shown to be localized to the chloroplast (Peltier et al., 2002; Schubert et al., 2002) and functionally characterized. Deg1, Deg5, and Deg8 are located in thylakoid lumen, and Deg2 is peripherally associated with the stromal side of thylakoid membranes (Itzhaki et al., 1998; Haußühl et al., 2001; Sun et al., 2007a). Recombinant DegP1, now renamed Deg1, has been shown to be proteolytically active toward thylakoid lumen proteins such as plastocyanin and PsbO of PSII in vitro (Chassin et al., 2002). A 5.2-kD C-terminal fragment of the D1 protein was detected in vitro after incubation of recombinant Deg1 with inside-out thylakoid membranes. In transgenic plants with reduced levels of Deg1, fewer of its 16- and 5.2-kD degradation products were observed (Kapri-Pardes et al., 2007). Deg5 and Deg8 form a dodecameric complex in the thylakoid lumen, and recombinant Deg8 is able to degrade the photodamaged D1 protein of PSII in an in vitro assay (Sun et al., 2007a). The 16-kD N-terminal degradation fragment of the D1 protein was detected in wild-type plants but not in a *deg5 deg8* double mutant after high-light treatment. The *deg5 deg8* double mutant showed increased sensitivity to high light and high temperature in terms of growth and PSII activity compared with the single mutants *deg5* and *deg8*, suggesting that Deg5 and Deg8 have overlapping functions in the primary cleavage of the CD loop of the D1 protein (Sun et al., 2007a, 2007b). In vitro analysis has demonstrated that recombinant stroma-localized Deg2 was also shown to be involved in the primary cleavage of the DE loop of the D1 protein (Haußühl et al., 2001). However, analysis of a mutant lacking Deg2 suggested that Deg2 may not be involved in D1 degradation in vivo (Huesgen et al., 2006).

Here, we have expressed and purified a recombinant DegP protease, His-Deg7. In vitro experiments showed that His-Deg7 is proteolytically active toward the PSII proteins D1, D2, CP43, and CP47. In vivo analyses of a *deg7* mutant revealed that the mutant is more sensitive to high light stress than the wild-type plants. We demonstrated that Deg7 is a chloroplast stroma protein associated with the thylakoid membranes and that it interacts with PSII, which suggests that it can cleave the stroma-exposed region of substrate proteins. Our results also provide evidence that Deg7 is important for maintaining PSII function.

RESULTS

Deg7 Is a Chloroplast Stromal Protein

Deg7 was predicted to be located in chloroplasts by LOCtree. To determine the subcellular localization, the N-terminal 243 amino acids of Deg7 were fused to the N terminus of the synthetic GFP under the control of the 35S promoter with a S65T mutation. The Deg7-GFP fusion proteins were transiently expressed in protoplasts, and GFP fluorescence of Deg7-GFP fusion proteins was colocalized with the chloroplastic chlorophyll, which is consistent with the signals observed when the GFP was fused to the transit peptide of FtsH11 (Sakamoto et al., 2003). When the targeting signals of the fibrillarin and FRO1 proteins from Arabidopsis were fused to GFP (Cai et al., 2009), GFP signals were located in the nucleus and mitochondria, respectively (Supplemental Fig. S1). Thus, these results indicate that Deg7 is targeted to the chloroplast.

Next, intact chloroplasts were isolated, separated into chloroplast stroma and thylakoid membrane fractions, and then subjected to immunoblot analysis with a specific antibody against Deg7. The purity of chloroplasts was confirmed by immunoblot analysis using the antibodies against the nuclear histone H1 protein, the mitochondrial NDUFS1 protein, the cytosolic DHAR2 protein, and the thylakoid membrane protein LHCII (Supplemental Fig. S2). The identities of the different fractions were further verified by immunoblot analyses with the antibodies against the thylakoid membrane protein LHCII, the peripheral protein Deg2, and the stromal protein RbcL (Fig. 1A). To determine the localization of Deg7, polyclonal antiserum was raised and immunoblot analysis of chloroplast protein showed the specificity of the Deg7 antibody (Supplemental Fig. S3). We found that Deg7 is located in chloroplasts and that the majority of Deg7 is present in chloroplast stromal fractions (Fig. 1A). To further investigate whether Deg7 is a peripheral or an intrinsic membrane protein, the thylakoid membranes were incubated with trypsin. Protease digestion assays showed that Deg7 could be degraded

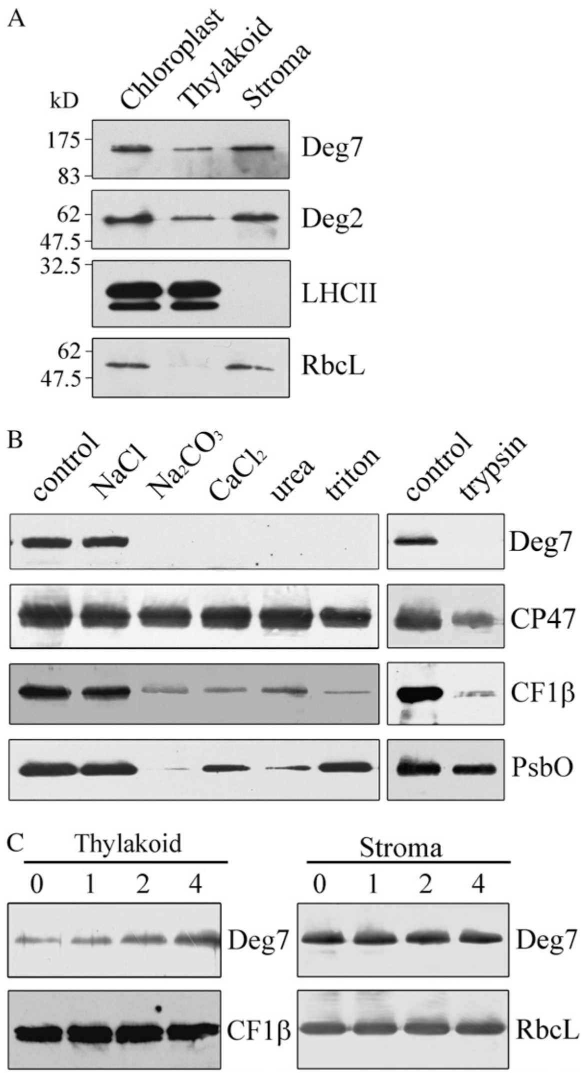

Figure 1. Immunolocalization and expression of Deg7. A, Intact chloroplasts were separated into stroma and thylakoid membranes. Proteins in each fraction were resolved by SDS-PAGE, and the location of Deg7 was determined by immunoblot analysis. B, The thylakoid membranes from mature Arabidopsis leaves were treated with trypsin for 30 min at 25°C or incubated with 250 mM NaCl, 200 mM Na_2CO_3, 1 M $CaCl_2$, 6 M urea, and 0.05% Triton X-100 for 30 min at 4°C. After these treatments, the thylakoid proteins were separated by SDS-PAGE and immunodetected with anti-Deg7, anti-CF1β, anti-PsbO, and anti-CP47 antibodies. CF1β, PsbO, and CP47 were used as markers. Membrane preparations that had not been subjected to any of these treatments were used as controls. The experiments were repeated five times independently, and similar results were obtained. Results of a representative experiment are shown. C, Expression of Deg7 associated with the thylakoid membranes. The detached leaves, floating adaxial side up on water, were illuminated with a photon flux density of 1,800 μmol m^{-2} s^{-1} for 1, 2, and 4 h. Then, the thylakoid and stroma proteins were prepared, and the proteins were subjected to SDS-PAGE and immunoblot analyses.

by trypsin, while the lumen protein PsbO was protected from trypsin treatment (Fig. 1B). These results indicate that the Deg7 protein is a chloroplast stroma protein and is associated with the membranes. Next, to examine the strength of Deg7's membrane association, we treated the thylakoid membranes with salts and chaotropic agents. Washing the membranes with 0.25 M NaCl did not release the Deg7 from the membranes, but Deg7 was barely detectable after washing the membranes with 0.2 M Na_2CO_3, 1 M $CaCl_2$, or 6 M urea. As a control, we found that the integral membrane protein CP47 was not released from the membranes by such treatments. High-light treatment increased the amount of Deg7 associated with the thylakoid membranes, and the levels of Deg7 in the stromal fractions remained almost unchanged after exposure to high-light treatment (Fig. 1C). This may reflect an increased expression of Deg7 after high-light treatment.

Proteolytic Activity and Physiological Targets of Deg7

The increased association of Deg7 with the membranes suggested that it might function in the degradation of photosynthetic proteins. To examine the proteolytic activity of the recombinant His-tagged Deg7, we incubated it with a mixture of α-, β-, and κ-forms of casein. β-Casein is the preferred substrate for assaying bacterial DegP activity under in vitro conditions (Lipinska et al., 1990). β-Casein was efficiently degraded by the fractions containing overexpressed Deg7, and the α- and κ-forms remained unchanged under our experimental conditions (Supplemental Fig. S4). More than 50% of the β-casein was degraded within the first 30 min. These results indicated that the recombinant Deg7 was proteolytically active.

Next, we examined whether Deg7 is proteolytically active toward photosynthetic proteins. Since the DegP protease has been shown to degrade damaged or misfolded proteins (Gottesman, 1996; Pallen and Wren, 1997; Clausen et al., 2002), the thylakoid membranes used in this experiment were first subjected to high-light treatment (1,800 μmol m^{-2} s^{-1}) for 90 min at 0°C. Treatment with high light causes irreversible oxidative protein damage, which subsequently induces conformational changes in proteins, making them vulnerable to proteolytic degradation (Prasil et al., 1992; Aro et al., 1993; Andersson and Aro, 2001). The thylakoid membranes isolated from leaves exposed to high light were treated with 50 mmol EDTA to inhibit the endogenous FtsH proteases. They were then incubated with or without recombinant Deg7 in the dark at 37°C. In the absence of recombinant Deg7, the D1, D2, CP43, and CP47 protein levels remained unchanged and no degradation products were detected (Fig. 2A). However, when recombinant Deg7 was added to isolated thylakoid membranes, the amounts of full-length D1, D2, CP43, and CP47 proteins decreased with time, and polypeptides of 20 kD for D1, 29 kD for D2, 19 kD for CP43, and 19 kD for CP47 were immunodetected using antibodies raised against the DE loop of the D1 protein, D2 protein, CP43 protein, and CP47 protein, respectively (Fig. 2A).

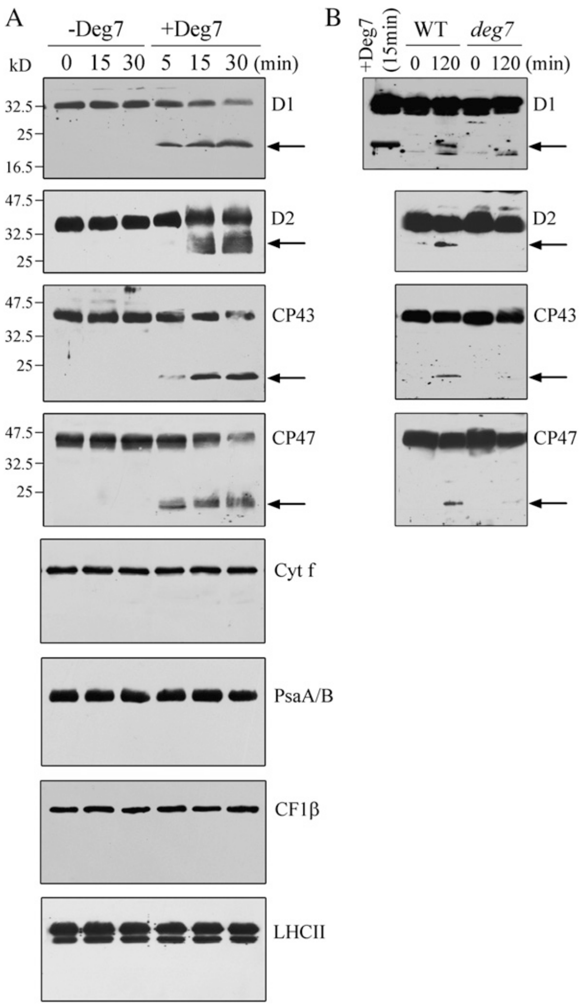

Figure 2. Degradation of photodamaged PSII proteins by recombinant Deg7. A, High-light-treated thylakoid membranes (10 μg of chlorophyll) were incubated with (+Deg7) or without (−Deg7) purified recombinant Deg7 (0.5 μg), and the degradation of thylakoid proteins was detected by SDS-PAGE and immunoblot analyses with anti-D1, anti-D2, anti-CP43, anti-CP47, anti-Cyt *f*, anti-CF1β, anti-PsaA/B, and anti-LHCII antibodies. Molecular mass markers (kD) are to the left. B, Thylakoid membranes isolated from wild-type (WT) and *deg7* leaves were exposed to high-light illumination for 2 h. The thylakoid proteins were separated by SDS-PAGE and immunodetected with antibodies raised against the D1, D2, CP43, and CP47 proteins. The degradation fragments are indicated with arrows. Similar results were obtained in two additional independent experiments. Results from a representative experiment are shown.

Immunoblot analysis with anti-Cyt *f*, anti-LHCII, anti-CF1β, and anti-PsaA/B antibodies showed that the levels of these proteins remained almost constant in the presence of recombinant Deg7 (Fig. 2A). When the thylakoid membranes were isolated from the plants that were not exposed to high-light treatment, the photosynthetic proteins were not degraded by recombinant Deg7 (Supplemental Fig. S5). The relative mole ratio of Deg7 and PSII was estimated to be about 1:10 using isolated PSII and anti-CP47 for calibration (data not shown), which showed a large excess of PSII over protease.

Functions of PDZ Domains with Respect to the Activities of Deg7

To determine the function of the PDZ domains in regulating the proteolytic activities of Deg7, we expressed a series of Deg7 proteases that contain zero to two PDZ domains in vitro (Supplemental Figs. S6–S8). Regardless of the presence of either one or two PDZ domains in the truncated Deg7 protein, the levels of PSII core proteins D1, D2, CP43, and CP47 decreased and the specific degradation fragments of their corresponding proteins were not detected. When the truncated Deg7 without any PDZ domains was incubated with the thylakoid membranes, the amounts of not only PSII core proteins D1, D2, CP43, and CP47 but also LHCII, PsaA/B, Cyt *f*, and CF1β were reduced.

Association of Deg7 with PSII

The proteolytic activity of Deg7 toward PSII core proteins suggests that Deg7 is associated with PSII. To test this possibility, the thylakoid protein complexes separated on a blue native gel were further subjected to denaturing SDS-PAGE and immunoblot analysis using specific antibodies. Our results showed that the Deg7 protein and PSII complex comigrated on the gel (Supplemental Fig. S9). Next, pull-down experiments were performed with recombinant Deg7 fused to an N-terminal His tag. The purified His-Deg7 fusion protein was incubated with *n*-dodecyl β-D-maltoside (DM)-solubilized thylakoid membranes. After washing nickel-nitrilotriacetic acid agarose (Ni-NTA) resin with buffer, the bound proteins were separated by SDS-PAGE and examined by immunoblot analysis (Fig. 3). The PSII proteins D1, D2, and CP47 were detected when the His-Deg7 fusion protein was used

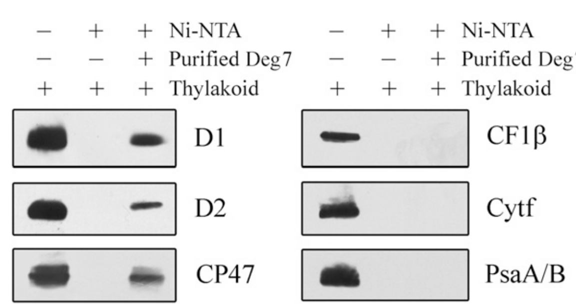

Figure 3. Interaction of Deg7 with PSII. Ten micrograms of the Deg7-His tag fusion protein coupled to Ni-NTA resin was incubated with 100 μg of DM-solubilized thylakoid membranes. Bound proteins were eluted, separated by SDS-PAGE, and subjected to immunoblot analysis with anti-PsaA/B, anti-D1, anti-D2, anti-CP47, anti-Cyt *f*, and anti-CF1β antibodies. Similar results were obtained in two additional independent experiments.

in the assay. In contrast, the PSI protein PsaA/B, the Cyt b_6f protein Cyt *f*, and the ATP synthase protein CF1β were not detected when the solubilized thylakoid membrane and the resin were incubated in the presence or absence of His-Deg7 (Fig. 3).

T-DNA Insertion Mutants of *deg7*

To study the in vivo function of Deg7, we obtained Arabidopsis lines from the SALK collection. The *deg7* mutant line (SALK_075584) contains a T-DNA insertion within the 1,417-bp sequence downstream of the ATG codon, and this was confirmed by PCR and subsequent sequencing of the amplified products (Supplemental Fig. S10, A and B). Reverse transcription (RT)-PCR analysis showed that expression of the *Deg7* gene was not detectable (Supplemental Fig. S10C). Further immunoblot analyses revealed that Deg7 protein was not detectable in the *deg7* mutant and that the levels of Deg7 in the complemented plants were comparable to those in the wild-type plants (Supplemental Fig. S10D). When grown under 120 μmol m^{-2} s^{-1} light, the growth rates of the *deg7* mutants were comparable to those of the wild type (Fig. 4A). Chlorophyll fluorescence analysis showed that the maximal photochemical efficiency of PSII (F_v/F_m) was similar between the *deg7* plants (0.82 ± 0.02) and wild-type plants (0.83 ± 0.01).

To examine the steady-state levels of thylakoid proteins, immunoblot analyses were performed with antibodies raised against specific subunits of the photosynthetic protein complexes (Supplemental Fig. S11). Our results showed that levels of the thylakoid proteins, including D1, D2, LHCII, PsbO, and CP43 of PSII, PsaA/B of PSI, Cyt *f* of the Cyt b_6f complex, and CF1β of ATP synthase, were not altered in the *deg7* mutant. The levels of Deg1, Deg5, Deg8, and FtsH were also not changed in the mutant.

Increased Sensitivity of the *deg7* Mutants to High Light

To determine whether Deg7 is involved in photoinhibition and repair processes, the F_v/F_m was measured in the wild-type and *deg7* plants under high-light illumination (1,800 μmol m^{-2} s^{-1}). In the absence of lincomycin, within 2 h of illumination at a light intensity of 1,800 μmol m^{-2} s^{-1}, F_v/F_m declined in the wild-type and mutant leaves to about 53% and 39%, respectively, of the dark-adapted values (Fig. 5A). These results clearly demonstrate the increased photosensitivity of the mutants. In the presence of lincomycin, the decline of F_v/F_m in the wild-type leaves was more rapid and continued until F_v/F_m values approached about 10% of the dark-adapted values (Fig. 5A). Interestingly, in the presence of lincomycin, the decline in F_v/F_m in the *deg7* mutants was similar to that observed in the wild-type leaves during the same photoinhibitory light treatment (Fig. 5A). Since lincomycin blocks the repair of PSII by inhibiting de novo protein synthesis in the chloroplast, these results suggest that the wild-type and mutant leaves display similar rates of PSII photoinhibition.

To establish whether the high susceptibility of the *deg7* mutants to photoinhibition is related to PSII protein turnover, we analyzed the PSII protein contents of the thylakoid membranes in the wild-type and mutant plants under high-light illumination in the presence of lincomycin. Under high-light illumination and in the presence of this inhibitor, the levels of the PSII proteins D1, D2, CP43, and CP47 gradually declined in the *deg7* and wild-type plants, but the rate of reduction was slower in the mutant (Fig. 5B). The levels of PsbO, another PSII protein, and the PSI protein PsaA/B were found to be relatively stable in both the wild-type and mutant plants.

Next, we performed an in vivo labeling experiment to follow the turnover of newly synthesized chloroplast proteins (Fig. 5C). Our results showed that the

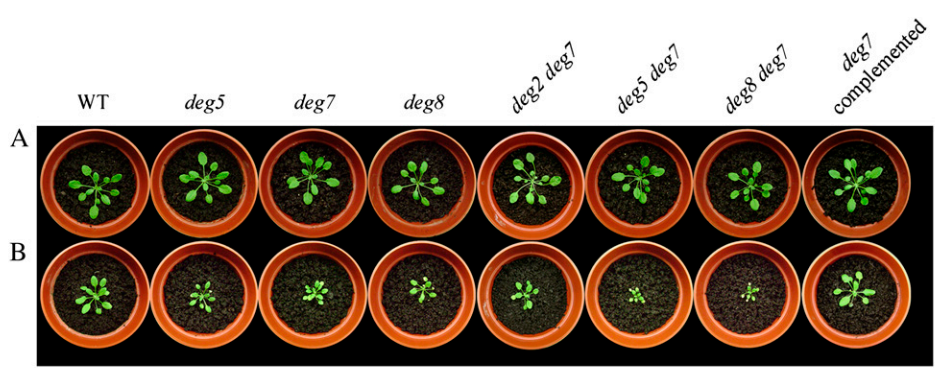

Figure 4. Phenotypes of *deg5*, *deg7*, *deg8*, *deg2 deg7*, *deg5 deg7*, and *deg8 deg7* mutants and wild-type (WT) plants. A, Four-week-old *deg5*, *deg7*, *deg8*, *deg2 deg7*, *deg5 deg7*, *deg8 deg7*, and *deg7* complemented mutants and wild-type plants grown under a photon flux density of 120 μmol m^{-2} s^{-1}. B, Two-week-old *deg5*, *deg7*, *deg8*, *deg2 deg7*, *deg5 deg7*, *deg8 deg7*, and *deg7* complemented mutants and wild-type plants grown in a growth chamber under a photon flux density of 120 μmol m^{-2} s^{-1} and then transferred to a greenhouse for another 2 weeks with a maximum photon flux density at noon of about 1,000 μmol m^{-2} s^{-1}.

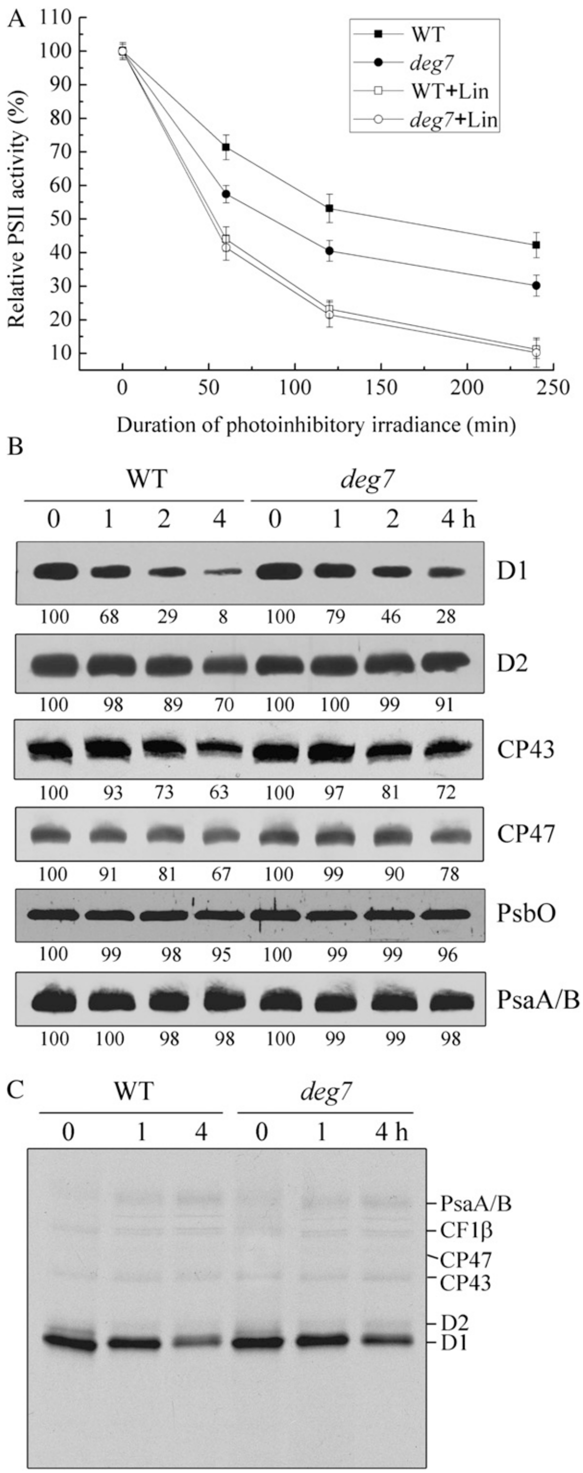

Figure 5. Changes in PSII activity and protein levels. A, The F_v/F_m was measured for detached leaves from wild-type (WT) plants (squares) and *deg7* mutant plants (circles) following high-light illumination (1,800 μmol m^{-2} s^{-1}) in the absence (white symbols) or presence (black symbols) of lincomycin (Lin). B, Immunoblot analysis of thylakoid proteins following high-light illumination. Thylakoid membranes were isolated from the wild-type and *deg7* mutant leaves during exposure to high-light illumination in the presence of lincomycin for 1, 2, or 4 h. The thylakoid proteins were separated by SDS-PAGE and immunodetected with specific antibodies directed against D1, D2, CP43, CP47, PsbO, and PsaA/B proteins. X-ray films were scanned and analyzed using an AlphaImager 2200 documentation and analysis system. The percentage protein levels shown below the lanes were estimated by comparison with levels found in corresponding samples taken at time 0. Similar results were obtained in two additional independent experiments. C, Pulse-chase analysis of chloroplast proteins. Two-week-old leaves of the wild-type and *deg7* mutant plants were labeled for 20 min followed by a chase after 1 or 4 h at 120 μmol m^{-2} s^{-1}. Thylakoid membranes with equal amounts of chlorophyll were isolated and separated by SDS-PAGE. The resolved proteins were visualized by autoradiography.

synthesis rates of the PSII core proteins D1, D2, CP47, and CP43, the PSI reaction center proteins PsaA/B, and the ATP synthase CF1α/β were similar between the wild-type and mutant plants. The PSII core proteins D1, D2, CP47, and CP43 showed increased stability in the mutant compared with the wild-type plants. The protein synthesis rates in pulse labeling for 20 min reflect the differences between the synthesis and degradation rates of photosynthetic proteins. Thus, the net synthesis rate of PSII reaction center protein D1 in the mutant was lower than that of the wild type, since the rate of D1 degradation was slowed in *deg7*.

To further analyze the physiological function of Deg7, photoinhibition was carried out with isolated thylakoid membranes to which 50 mmol EDTA had been added in order to inactivate the endogenous FtsH proteases. We found that the 20-kD polypeptide of D1, the 29-kD polypeptide of D2, the 19-kD polypeptide of CP43, and the 19-kD polypeptide of CP47 were detected in the samples from wild-type plants following photoinhibition but not in samples from the *deg7* mutant. An 18-kD polypeptide from D1 was detected both in the wild-type and mutant plants (Fig. 2B), which may be due to the function of lumen-localized Deg5 and Deg8 (Sun et al., 2007a).

Since Deg7 is involved in protection against photoinhibition, we analyzed the phenotypes of the mutants during growth under high irradiance. For this purpose, we transferred the wild-type and *deg7* mutant plants that were grown initially at 120 μmol m^{-2} s^{-1} to a greenhouse with a maximum intensity of 1,000 μmol m^{-2} s^{-1} at noon. Our results showed that when exposed to high light, the growth of the *deg7* mutant was inhibited compared with the wild-type plants (Fig. 4B).

Generation of *deg2 deg7*, *deg5 deg7*, and *deg8 deg7* Double Mutants and Their Growth under High Light

Three double mutants were constructed, *deg2 deg7*, *deg5 deg7*, and *deg8 deg7*, to test the physiological importance of stromal and luminal Deg proteases in photosynthesis. The growth rates and PSII activities of *deg2 deg7*, *deg5 deg7*, and *deg8 deg7* were comparable to those of wild-type plants when grown at 120 μmol

m^{-2} s^{-1} (Fig. 4A). However, when exposed to high light, the growth of the mutants was slower than that of the wild-type plants, and the extent of the phenotype in the *deg5 deg7* and *deg8 deg7* double mutants was more pronounced than in the single mutants, while that of *deg2 deg7* was similar to that of the *deg7* mutant (Fig. 4B). Moreover, some of the mutant leaves exhibited symptoms of yellowing in the *deg5 deg7* and *deg8 deg7* double mutants (Fig. 4B).

DISCUSSION

The adverse effect of high light on plant growth in the *deg7* mutant indicates the physiological importance of Deg7 for photoprotection under high-light illumination (Figs. 4 and 5). Such a role for Deg7 is also in agreement with our observation of a dramatic increase in the levels of proteins associated with the thylakoid membranes under high light based on immunoblot analysis (Fig. 1). There was no apparent difference in growth between wild-type and mutant plants under normal growth conditions at 120 μmol m^{-2} s^{-1}, which also reflected the similar F_v/F_m ratios. When the wild-type plants were subjected to high-light treatment, the PSII activity was reduced for the duration of treatment in the absence of lincomycin, and the reduction of PSII activity became more pronounced in the presence of lincomycin (Fig. 5). The PSII activity in the *deg7* mutant showed enhanced sensitivity to high-light treatment in the absence of lincomycin compared with the wild-type plants, while the rate of PSII photoinhibition was similar in the mutant and wild-type plants in the presence of the protein synthesis inhibitor lincomycin (Fig. 5). These results indicate that under high light, the repair of PSII was perturbed in the mutant. It is likely that the increased sensitivity of PSII to high light in the mutant could be ascribed to the impairment of the PSII repair cycle.

The repair of PSII involves the degradation and removal of damaged PSII proteins and their subsequent replacement with newly synthesized copies (Prasil et al., 1992; Aro et al., 1993; Andersson and Aro, 2001). The degradation of photodamaged PSII proteins is inherent to the repair cycle of PSII after photoinhibition. Removal of photooxidatively damaged PSII proteins and subsequent replacement with newly synthesized copies represents an efficient repair cycle. However, the detailed mechanisms of the repair of PSII are far from being understood. Impairment of the PSII repair cycle could result from the perturbation of either protein synthesis or degradation. Our pulse-labeling experiments showed that the synthesis rates of the PSII core proteins D1, D2, CP47, and CP43 were not affected in the mutant (Fig. 5C). Instead, examination of the degradation of PSII core proteins showed that the decrease in the levels of PSII core proteins was slower in the mutant than in the wild-type plants (Fig. 5B). Pulse-chase labeling experiments also revealed that the turnover rates of newly synthesized PSII core proteins were slowed in the *deg7* mutant (Fig. 5C). These results suggest that Deg7 is involved in the degradation of PSII core proteins.

Deciphering the physiological roles of specific proteases is intimately related to the identification of their substrates. The D1 protein of the PSII reaction center has been identified as the substrate of characterized chloroplast Deg proteases in vivo. This protein is susceptible to proteolytic cleavage at its lumen-exposed domains (the AB and CD loops and the C terminus) by Deg1 (Kapri-Pardes et al., 2007) and the Deg5-Deg8 complex (Sun et al., 2007a). The detection of a C-terminal D1 fragment of about 20 kD in in vitro experiments suggests that Deg7 catalyzes the cleavage of the photodamaged D1 protein at the stromal loop connecting the B and C transmembrane helices (Fig. 2). Another stroma-localized Deg2 protein was shown to be involved in the initial cleavage of the DE loop of the D1 protein in vitro (Haußühl et al., 2001); however, this possibility is not supported in vivo, since the *deg2* mutant showed a similar extent of PSII inactivation and a similar D1 protein turnover rate to that in the wild-type plants (Huesgen et al., 2006). Since Deg2 and Deg7 are located in chloroplast stroma, we analyzed a *deg2 deg7* double mutant, and the *deg2 deg7* and *deg7* mutants showed similar degrees of sensitivity to high light in terms of growth (Fig. 4) and PSII activity (Supplemental Fig. S12). Thus, Deg2, unlike Deg7, seems to have little effect on PSII repair in vivo.

Although the PSII reaction center protein D1 is best characterized as the main target of photodamage, the other PSII proteins D2, CP47, and CP43 are occasionally damaged and degraded, especially in response to increasing light intensity (Schuster et al., 1988; Yamamoto and Akasaka, 1995; Jansen et al., 1999; Adir et al., 2003). The detection of the 29-kD fragment of D2 indicates that the cleavage site of photodamaged D2 is located at its N terminus. The observation of the 19-kD fragments of CP47 and CP43 after treatment of thylakoid membranes with recombinant Deg7 suggests that cleavage may take place at the stromal loop connecting transmembrane domains D and E.

The PDZ domains in *E. coli* DegP proteases have been implicated in the formation of oligomers and also in the regulation of proteolytic activity (Doyle et al., 1996; Harris and Lim, 2001). It is interesting that Deg7 harbors three PDZ domains. The presence of three PDZ domains raises questions about their functions. Truncated Deg7 containing either one or two PDZ domains was also able to degrade PSII core proteins, but it did not generate specific fragments. The truncated Deg7 that lacked any PDZ domain lost its specificity for the substrate and was proteolytically active not only toward PSII core proteins but also toward other photosynthetic proteins (Supplemental Figs. S6–S8). These results highlight the importance of PDZ domains as regulators of proteolytic activity that recognize and discriminate between substrates.

The function of Deg7 in the degradation of photodamaged PSII core proteins suggests its possible interaction with PSII. The results presented in Figure 3 and Supplemental Figure S9 strongly suggest that Deg7 associates with PSII complexes but not with other photosynthetic complexes such as PSI, Cyt b_6f, or ATP synthase. This association is consistent with the physiological role of Deg7 in the repair of damage to PSII caused by photoinhibition. The finding of an association between a protease and a complex containing its substrates is intriguing. Our results suggest that, at least in the case of PSII (which is known to undergo continuous cycles of assembly and repair), protein complexes might be equipped with components that are essential for their repair.

The distribution of Deg proteases on both the stromal and luminal sides of the thylakoid membranes might have implications for the efficient degradation of damaged D1 protein. It is thus reasonable to speculate that Deg7 functions cooperatively with lumen-localized Deg1, Deg5, and Deg8, generating more cleavage sites on both sides of the membranes. This may increase the efficiency of D1 degradation. In mitochondria, the degradation of membrane proteins was greatly assisted by the cooperation of proteases functioning on both sides of the membranes (Leonhard et al., 2000). The single mutants *deg5*, *deg7*, and *deg8* all showed decreased growth compared with the wild-type plants under high light, although these mutants did not show any apparent differences in terms of phenotype under normal growth conditions at 120 μmol m^{-2} s^{-1} (Fig. 4; Sun et al., 2007a), which indicates that the Deg proteases are important for efficient PSII repair. The additive functions of Deg proteases were also demonstrated by the phenotypes of the *deg5 deg7* and *deg8 deg7* double mutants grown under high light.

Since Deg proteases are endopeptidases that can cleave intermembrane peptide regions exposed to the stromal or the luminal side, it is unlikely that PSII proteins are completely degraded by Deg proteases. The creation of D1 degradation intermediates may provide additional sites for the initiation of proteolysis. The function of FtsH in degrading *E. coli* integral membrane proteins involves relocation of regions of substrates from one side to the other (Ito and Akiyama, 2005). It may be that Deg proteases from both sides of the membranes enhance the efficiency of D1 degradation by increasing the number of D1 degradation intermediates that are accessible by FtsH. The Arabidopsis *var2* mutant lacking FtsH2 exhibited more increased sensitivity to high-light treatment than the *deg7* mutant (Supplemental Fig. S12), which suggests that FtsH2 is predominantly involved in the PSII repair cycle. The light-dependent degradation of D1 and D2 was slowed in the Arabidopsis *var2* mutant (Bailey et al., 2002). In *Synechocystis* species PCC6803, the FtsH protease slr0228, which shows the highest similarity to Arabidopsis FtsH2, has been shown to play a more general role in the removal of PSII proteins other than the D1 protein (Komenda et al., 2006). These studies suggest a possible function of FtsH in the degradation of other PSII core proteins. Considering the functional mode of FtsH, it is likely that other PSII core proteins may also be cleaved by peptidases in order to generate limited numbers of distinct fragments, followed by their complete digestion by the processive ATP-dependent FtsH protease.

MATERIALS AND METHODS

Plant Materials

Wild-type and mutant Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) plants were grown in soil under short-day conditions (10-h-light/14-h-dark cycles) with a photon flux density of 120 μmol m^{-2} s^{-1} at a constant temperature of 22°C. The T-DNA insertion line SALK_075584 (*Deg7*; *At3g03380*) was obtained from the T-DNA-transformed Arabidopsis collection from the Arabidopsis Biological Resource Center (Ohio State University). Homozygous *deg7* mutant plants were identified by PCR analyses using the following gene-specific and T-DNA-specific primers: LP (5′-GGTACGTT-CAGTGACACACCC-3′), RP (5′-CAAAGCTTTCTAGGGGTGCTC-3′), and T-DNA LB (5′-GCGTGGACCGCTTGCTGCAACT-3′). The precise location of the T-DNA insertion was determined by sequencing the PCR products. Confirmation of null mutants was carried out by RT-PCR using the same primer set described above for *Deg7*. Equal cDNA loading in each sample was monitored by RT-PCR analysis of the expression level of actin (5′-AACTGG-GATGATATGGAGAA-3′ and 5′-CCTCCAATCCAGACACTGTA-3′). The double mutants *deg2 deg7*, *deg5 deg7*, and *deg7 deg8* were obtained by PCR screening of an F2 population from crossed single mutant lines.

Complementation of the *deg7* mutant

The cDNA containing the coding region of *Deg7* was amplified with the following primers, which include *Xho*I and *Kpn*I restriction sites at their 5′ ends to facilitate cloning: sense primer (5′-GACCCGGGGGAGAT-CAAATGGGAGATCC-3′) and antisense primer (5′-GGGGTACCTTACTG-CAAGGCTTTCAATATA-3′). The resulting fragment was cloned into the *Sam*I and *Kpn*I sites of pSN1301 under the control of the cauliflower mosaic virus 35S promoter. The plasmid pSN1301-Deg7 was transformed into *Agrobacterium tumefaciens* strain C58 via electroporation and introduced into the homozygous *deg7* plants (Clough and Bent, 1998). Transformant plants were selected on medium containing half-strength Murashige and Skoog salt mix, 50 μg mL^{-1} hygromycin, and 0.8% agar. The resistant plants were transferred to soil to grow to maturity, and their transgenic status was further confirmed by PCR and immunoblot analyses.

Photoinhibition Treatments

Detached mature leaves were floated adaxial side up on water and exposed to a photon flux density of 1,800 μmol m^{-2} s^{-1}, and chlorophyll fluorescence was measured using a PAM-2000 fluorometer (Walz). The temperature of the water was kept at 22°C during photoinhibition treatments. Synthesis of chloroplast-encoded proteins was blocked by incubating detached leaves with their petioles submersed in 1 mM lincomycin solution at an irradiance of 20 μmol m^{-2} s^{-1} for 3 h prior to photoinhibitory light treatment.

To investigate the effects of high irradiance on plant growth, we transferred 2-week-old Arabidopsis plants grown in a growth chamber under a photon flux density of 120 μmol m^{-2} s^{-1} to a greenhouse under sunlight illumination for another 2 weeks. In the greenhouse, the maximum photon flux density at noon was about 1,000 μmol m^{-2} s^{-1} and average day/night temperatures were 25°C/22°C.

Thylakoid Membrane Preparation

Thylakoid membranes were isolated essentially as described by Zhang et al. (1999). Briefly, Arabidopsis leaves were homogenized by a mortar and pestle in an ice-cold isolation buffer (400 mM Suc, 50 mM HEPES-KOH, pH 7.8, 10 mM NaCl, and 2 mM $MgCl_2$), filtered through two layers of cheesecloth, and centrifuged at 5,000*g* for 10 min. The thylakoid pellets were resuspended in the isolation buffer and then centrifuged at 5,000*g* for 10 min. The thylakoids were finally suspended in isolation buffer, and their chlorophyll levels were determined.

SDS-PAGE and Immunoblot Analysis

The protein sample was mixed with the same volume of 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% [w/v] glycerol, 4% SDS, 5% β-mercaptoethanol, and 0.1% bromphenol blue) for 60 min and layered onto 15% SDS polyacrylamide gels containing 6 M urea (Laemmli, 1970). After electrophoresis, gels were stained with Coomassie Brilliant Blue. For immunoblot analysis, the proteins resolved by SDS-PAGE were blotted onto nitrocellulose membranes and reacted with specific antibodies, and the signals were visualized with the enhanced chemiluminescence method.

Primary antibodies used in this study were as follows: (1) a D1-specific antibody against the oligopeptide NYGYKFGQE containing the amino acids 234 to 242 in the DE loop of the D1 protein of *Synechocystis* species PCC 6803; (2) a D2-specific antibody against the oligopeptide NTFRAFNPTQAEETYS containing the amino acids 231 to 246 in the DE loop of the D2 protein of Arabidopsis; (3) an anti-peptide antibody specific for *E. coli* FtsH, which is potentially cross-reactive with all *Synechocystis* species PCC 6803 FtsH homologs; (4) a Cyt *f* antibody raised against the amino acids 146 to 257 in the Cyt *f* protein of Arabidopsis; (5) a CF1β antibody raised against the amino acids 206 to 387 in the CF1β protein of Arabidopsis; (6) a PsaA/B antiserum raised against the amino acids 3 to 77 in the N terminus of the PsaA protein of Arabidopsis; (7) a CP43 antibody raised against the amino acids 330 to 449 in the C terminus of the CP43 protein of Arabidopsis; (8) a CP47 antibody raised against the amino acids 330 to 475 in the C terminus of the CP47 protein of Arabidopsis; (9) a LHCII antibody raised against the amino acids 25 to 154 in the LHCB3 protein of Arabidopsis; (10) a Deg1 antibody raised against the amino acids 315 to 436 in the C terminus of the Deg1 protein of Arabidopsis; (11) a Deg5 antibody raised against the amino acids 91 to 211 in the N terminus of the Deg5 protein of Arabidopsis; and (12) a Deg8 antibody raised against the amino acids 297 to 423 in the C terminus of the Deg8 protein of Arabidopsis.

Antiserum Production and Immunolocalization Studies

The His-Deg7 fusion protein was purified on a Ni-NTA resin matrix, and polyclonal antibodies were raised in rabbit with the purified antigens. The intracellular localization of Deg7 was determined essentially according to Lennartz et al. (2001). The Arabidopsis membranes were suspended to a final concentration of 50 mg chlorophyll mL^{-1} in 10 mM HEPES-KOH, pH 8.0, 10 mM $MgCl_2$, 330 mM sorbitol, and 1 mM phenylmethylsulfonyl fluoride supplemented with 250 mM NaCl, 200 mM Na_2CO_3, 1 M $CaCl_2$, 6 M urea, and 0.05% Triton X-100 at 0°C for 30 min or 0.05 mg mL^{-1} trypsin at 25°C for 30 min. Membrane fractions without supplements were used as a control. After treatment, the membranes were pelleted at 100,000*g* for 2 h at 4°C, quickly washed with isolation buffer, and used for SDS-PAGE and immunoblot analysis.

Recombinant Expression of Deg7 and Pull-Down Assays

Full-length Deg7 cDNA was cloned into the pET28a plasmid and transformed into BL21 cells. The expression of the His-Deg7 fusion protein was induced by isopropylthio-β-D-galactoside (0.4 mM) for 2 h, and the overexpressed protein was purified using a Ni-NTA resin matrix. The purified protein was renatured through a Sephadex G-75 column by eluting with 20 mM NaH_2PO_4, pH 7.8, buffer, and the identity of the protein was confirmed by immunoblot analyses with specific antibodies.

For protein pull-down assays, thylakoid membranes (100 μg of chlorophyll) were solubilized with 1% (w/v) DM in 20% (w/v) glycerol, 25 mM BisTris-HCl, pH 7.0, and 1 mM phenylmethylsulfonyl fluoride for 15 min at 4°C, then centrifuged at 10,000*g* for 10 min. The supernatant obtained after centrifugation was incubated with His-Deg7 coupled to Ni-NTA resin. After incubation overnight with constant rotation at 4°C, the beads were washed five times with 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA buffer, and the bound proteins were eluted with SDS-PAGE sample buffer. The eluted proteins were resolved by SDS-PAGE followed by immunoblot analyses.

Proteolytic Degradation Assays

The proteolytic activity was assayed by incubating Deg7 with 15-μg mixtures of casein (α-, β-, and κ-casein; Sigma-Aldrich) in standard reaction mixtures including 0.5 μg of purified Deg7 in a 30-μL solution containing 50 mM Tris-HCl, pH 7.6. The mixtures were incubated for 0, 15, 30, 45, and 60 min at 37°C, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue.

For the in vitro degradation assay, wild-type Arabidopsis thylakoid membranes were illuminated at 1,800 $\mu mol\ m^{-2}\ s^{-1}$ for 90 min at 4°C and centrifuged at 15,000*g* for 15 min. The collected thylakoids were washed with 1.0 M $CaCl_2$ to reduce endogenous Deg7 activity before the addition of recombinant Deg7 or its derivatives and resuspended in 300 mM sorbitol and 10 mM HEPES-KOH, pH 8.0. After incubation of purified recombinant Deg7 protein with the thylakoid membranes at 37°C in the dark for various amounts of time, the samples were subjected to SDS-PAGE and immunoblot analyses.

In Vivo Labeling of Chloroplast Proteins

In vivo chloroplast protein labeling was carried out essentially according to Meurer et al. (1998). Leaves of 2-week-old Arabidopsis plants were preincubated for 30 min in the presence of 20 $\mu g\ mL^{-1}$ cycloheximide, which blocks the synthesis of nucleus-encoded proteins. Then, the leaves were radiolabeled with 1 $\mu Ci\ \mu L^{-1}$ [^{35}S]Met (specific activity > 1,000 Ci $mmol^{-1}$; Amersham Pharmacia Biotech) at 120 $\mu mol\ m^{-2}\ s^{-1}$ in the presence of 20 $\mu g\ mL^{-1}$ cycloheximide for 20 min at 22°C, followed by a chase of 1 or 4 h in buffer containing 10 mM cold Met. Afterward, the leaves were collected, the thylakoid membranes were isolated, and the proteins were separated by SDS-PAGE. For autoradiography, gels were stained, dried, and exposed to x-ray film.

GFP Fusion Constructs for Transient Expression in Protoplasts

A fragment encoding the N-terminal amino acids 1 to 243 of Deg7 was amplified by PCR using the following primers: 5′-GCGTCGACATGGGAGATCCGTTGGAGAG-3′ and 5′-GGCCATGGTTAACGCCCTAACAACTCG-3′. The PCR product was cloned into the *Sal*I and *Nco*I sites of expression vector pUC18-35S-sGFP to generate a fusion protein with the GFP as a reporter in the C terminus. The transit peptide (amino acids 1–245) of the FtsH11, the N-terminal part (amino acids 1–282) of AtFbr1, and the entire coding region of FRO1 were used as chloroplast, nuclear, and mitochondrial controls, respectively (Sakamoto et al., 2003; Cai et al., 2009). Isolation of the Arabidopsis protoplast and polyethylene glycol-mediated transfection were performed as described by Sakamoto et al. (2003). The cells with GFP signals were examined using a confocal laser scanning microscope (LSM510; Carl Zeiss).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number *At3g03380* (*DEG7*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Subcellular localization of the Deg7 protein.

Supplemental Figure S2. Immunoblot analysis of the purity of isolated chloroplasts.

Supplemental Figure S3. Immunoblot analysis of chloroplast proteins with the specific Deg7 antibody.

Supplemental Figure S4. Engineering of the Deg7 deletion construct and proteolytic activity of Deg7 with β-casein.

Supplemental Figure S5. Degradation of photosynthetic proteins by a recombinant Deg7 protein.

Supplemental Figure S6. Degradation of photodamaged PSII proteins by a recombinant Deg7 protein containing two PDZ domains.

Supplemental Figure S7. Degradation of photodamaged PSII proteins by a recombinant Deg7 protein containing one PDZ domain.

Supplemental Figure S8. Degradation of photodamaged PSII proteins by a recombinant Deg7 protein lacking PDZ domains.

Supplemental Figure S9. Immunoblot analysis of the association of Deg7 with photosynthetic protein complexes after blue native/SDS-PAGE.

Supplemental Figure S10. Identification of the *deg7* mutant.

Supplemental Figure S11. Analysis of thylakoid proteins from *deg7* mutant and wild-type plants.

Supplemental Figure S12. Changes in F_v/F_m of wild-type, *deg7*, *deg2 deg7*, and *ftsh2* plants following high-light illumination.

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