

Quality Control of Photosystem II: Direct Imaging of the Changes in the Thylakoid Structure and Distribution of FtsH Proteases in Spinach Chloroplasts under Light Stress

(Light stress response of thylakoids)

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Abbreviations: D1 and D2, the reaction center-binding proteins of photosystem II; PS, photosystem; FtsH, filamentation temperature sensitive H; LHCII, light-harvesting Chl-protein complex of PSII; TEM, transmission electron microscopy; CN-PAGE, clear native polyacrylamide gel electrophoresis; DM, *n*-dodecyl β -D-maltoside; 3D, three-dimensional; CP43, the antenna Chl binding protein of PSII core; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; Tricine, *N*-[Tris(hydroxymethyl)methyl]glycine; PO, propylene oxide

Abstract

Under light stress, the reaction center-binding protein D1 of Photosystem II is photo-oxidatively damaged and removed from Photosystem II complexes by proteases located in the chloroplast. A protease considered to be responsible for degradation of the damaged D1 protein is the metalloprotease FtsH. We showed previously that the active hexameric FtsH protease is abundant at the grana margin and the grana end membranes, and this homo-complex removes the photo-damaged D1 protein in the grana (Yoshioka et al. 2010). Here, we showed a change in the distribution of FtsH in spinach thylakoids during excessive illumination by transmission electron microscopy and immuno-gold labeling of FtsH. The change in distribution of the protease was accompanied by structural changes to the thylakoids, which we detected with spinach leaves by transmission electron microscopy after chemical fixation of the samples. Quantitative analyses showed several characteristic changes in the structure of the thylakoids, including shrinkage of the grana, outward bending of the marginal portions of the thylakoids, and an increase in the height of the grana stacks under excessive illumination. The increase in the height of the grana stacks may include swelling of the thylakoids and increase in the partition gaps between the thylakoids. These data strongly suggest that excessive illumination induces partial unstacking of the thylakoids, which enables FtsH to access easily the photo-damaged D1 protein. Finally three-dimensional tomography of the grana was recorded to observe the effect of light stress on the overall structure of the thylakoids.

Keywords: FtsH protease • Light stress • Photosystem II • Spinach chloroplast • TEM • Thylakoid

Introduction

When higher plants are exposed to excessive light, the reaction center-binding protein D1 of Photosystem (PS) II is damaged by reactive oxygen species produced near PSII, which is followed by a decrease in PSII activity. This phenomenon is known as photoinhibition of PSII (Barber and Andersson, 1992, Aro et al., 1993, Yamamoto, 2001, Yamamoto et al., 2008). The photo-oxidatively damaged D1 protein is removed from the PSII complex through successive proteolytic processes. Several proteases have been suggested to participate in these steps, and one of the proteins is a Zn-metalloprotease termed FtsH (filamentation temperature sensitive H), which has a prokaryote origin and is located in chloroplast thylakoids as an intrinsic protein (Bailey et al., 2002).

The FtsH protease from the chloroplasts of *Arabidopsis thaliana* (hereafter *Arabidopsis*) adopts a hexameric ring structure composed of six subunits; two of them are type A subunits (FtsH1 and 5), and the other four are type B subunits (FtsH2 and 8). Each subunit has two trans-membrane α -helices at the N-terminal side and a large hydrophilic part containing ATP binding and catalytic sites at the C-terminus (Zaltsman et al., 2005, Adam et al., 2006). Mutants of *Arabidopsis* lacking FtsH show increase sensitivity to high light (Bailey et al., 2002). The mutants, Δ FtsH2 (VAR2) and Δ FtsH5 (VAR1), showed yellow variegated leaves and impaired development of chloroplasts (Sakamoto et al., 2003, Janska et al., 2012, Kato and Sakamoto, 2010). Among the four subunits of FtsH, FtsH2 is the most abundant (Sinvany-Villalobo et al., 2004). By mass spectroscopic measurements, FtsH5 was shown to be four to five times more abundant than FtsH1 in type A subunits, whereas FtsH2 is two to three times

higher in amount than FtsH8 in type B subunits (Moldavski et al., 2012). The two FtsH proteases within the type A or B group are exchangeable with each other and functionally overlapping (Yu et al., 2004, Yu et al., 2005). Crystal structures of the FtsH protease were obtained from hyperthermophilic bacteria such as *Aquifex aeolicus* (Suno et al., 2006) and *Thermotoga maritima* (Bieniossek et al., 2006, Bieniossek et al., 2009). These bacterial FtsH proteases consist of a 6-fold symmetric protease domain and a 2-fold symmetric ATPase-associated activity ring. Recently, an open and closed conformational model was proposed for the molecular mechanism of proteolysis (Suno et al., 2006). Currently, crystal structure of FtsH has not been available in higher plants.

Information describing the change in the distribution of the proteases on the thylakoids during light stress is limited. To obtain the missing information, we have examined possible structural changes of the thylakoids upon excessive illumination where the FtsH proteases are located. In higher plant chloroplasts, the thylakoid membranes form the grana interconnected by unstacked stroma thylakoids, which may contribute to the stabilization of the thylakoid membranes and the membrane-embedded supercomplexes of the PSII/light-harvesting Chl protein (LHCII). Unstacking of the grana thylakoids under light stress and moderate heat stress was studied previously (Khatoon et al., 2009, Kirchhoff et al., 2011, Herbstova et al., 2012). In one of these studies, we suggested that the thylakoid unstacking increases the area of the grana margins where FtsH proteases are assembled into active hexamers and react with the damaged D1 protein (Khatoon et al., 2009). The thylakoid unstacking may also mobilize the PSII/LHCII supercomplexes in the grana and protect PSII from high light (Johnson et al.,

2011b). It remains unsolved, however, if the movement of PSII/LHCII facilitates repair of PSII (Herbstova et al., 2012, Goral et al., 2012).

In the present study we have visualized excessive light-induced changes in the distribution of FtsH in spinach thylakoids by transmission electron microscopy in combination with immuno-gold labeling of FtsH. To characterize the light-induced unstacking of the thylakoids quantitatively, we have examined the structural changes of the thylakoids at various light intensities. Finally, we have obtained three-dimensional (3D) tomography data of the grana to observe overall structural changes of the grana in spinach chloroplasts.

Results

Composition and molecular arrangement of FtsH proteases in the chloroplast thylakoids

Both type A (FtsH1/5) and type B (FtsH2/8) subunits are necessary for stable accumulation and function of FtsH proteases in the thylakoid membranes. It was reported previously with the thylakoids of *Arabidopsis* that in hexameric FtsH proteases type A and type B subunits assemble at a molecular ratio of 1:2 (Zaltsman et al., 2005). In dark-adapted spinach thylakoids, we detected monomeric, dimeric and hexameric forms of FtsH using an antibody against *Arabidopsis* FtsH2 that cross-reacts with all the spinach FtsH subunits including type A and B subunits (Yoshioka et al., 2010). Since the molecular arrangement of type A and type B subunits in active hexameric FtsH proteases is unknown, we have prepared two types of antibodies against type A and type B FtsH subunits, and determined how the

subunits are arranged in the hexamer using clear native polyacrylamide gel electrophoresis (CN-PAGE) and Western blot analysis of the proteins in spinach thylakoids. For this experiment, the thylakoids were solubilized with 1.5% *n*-dodecyl β -D-maltoside (DM) and CN-PAGE was carried out as described previously (Yoshioka et al., 2010). With the Western blot analysis, the antibody against type B subunits (FtsH2/8) detected the bands of FtsH monomer and hexamer, whereas the antibody against type A subunits (FtsH1/5) detected the bands of FtsH dimer and hexamer (Fig. 1A). Given the 1:2 stoichiometry of type A and B subunits in the hexamer (Zaltsman et al., 2005), we suggest that the hexameric FtsH is composed of two type A subunits and four type B subunits. The most likely arrangement is AA+B+B+B+B, namely a pair of type A subunits and four monomeric type B subunits. Two monomeric type A subunits exist adjoining each other because type A subunit was detected as a dimer. The monomeric type B subunit is unable to exist between two type A monomers (Fig. 1B below). All six subunits should be arranged in line symmetry (Fig. 1B above).

Light-induced accumulation of FtsH proteases in the grana

We next examined the light-induced accumulation of FtsH proteases in the grana using Western blot analysis (Fig. 2). In these experiments, spinach thylakoid membranes were illuminated with high light (light intensity: 0–2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) first and then fractionated into the grana and stroma thylakoids by treatment of the thylakoids with 1.2% Triton X-100 and subsequent centrifugation at 35,000 *g* for 15 min. At the light intensities used, unstacking of the thylakoids occurred and the amount of FtsH in the stroma thylakoids decreased, whereas the FtsH levels in the grana increased significantly.

Importantly, degradation of the D1 protein was detected in the grana under the same illumination conditions (Fig. 2). These results strongly suggest that movement of FtsH and degradation of the D1 protein take place simultaneously in the grana under light stress. The grana fraction used here probably contains both the grana cores and grana margins.

Light-induced change in distribution of FtsH in the thylakoid membranes was then assayed by TEM combined with immuno-gold labeling. Using the antibody against FtsH2/8 and the secondary antibody against FtsH with conjugated gold particles, we visualized the localization of FtsH proteases in the thylakoids from either dark-adapted spinach leaves or leaves illuminated with strong light (light intensity: $1,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$). In the dark-adapted leaves, FtsH proteases were detected in both the stroma thylakoids and grana end membranes (Fig. 3A). In contrast, in the thylakoids exposed to high light for 30 min, FtsH proteases were detected in the grana margins and grana stacks (Fig. 3B). Migration of the FtsH protease to the grana may have already started after illumination for 30 min. These results suggest that the FtsH proteases migrate from the stroma thylakoids to the grana where the photo-damaged D1 proteins are degraded.

Thylakoid unstacking under excessive illumination

We next examined structure of the grana membranes under light stress with TEM. In the dark-adapted spinach leaves, chloroplast thylakoids were tightly stacked in the grana from end to end (Fig. 4A). In contrast, the grana of the chloroplasts from the leaves exposed to high light (light intensity: $2,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 60 min) showed an increase in the height of the thylakoid layers, in

particular at the both ends of the grana where the thylakoids appeared to bend outward (Fig. 4B). We collected more data on the effects of light intensity and light treatment time on the structure of the thylakoids (Supplemental Fig. S1). The spinach leaves were illuminated (light intensity: 500, 1,000, 2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) for 0–60 min. The amount of thylakoid membrane unstacking increased as the illumination time increased. To show the light-induced structural changes of the grana quantitatively, the height of 10 thylakoid layers in 5 separate areas in the grana were measured using the software of Gatan Digital Micrograph (Gatan Inc., USA). Our data showed partial unstacking of the grana under light stress, which takes place prominently at the grana margins (Fig. 4C).

3D modeling of thylakoid membranes

Three-dimensional (3D) analysis of the grana should provide new insights into how the structure of the grana changes under light stress. We constructed a 3D model of the grana from spinach chloroplasts using images obtained by TEM. In the model of the dark-adapted chloroplasts (Fig. 5A), stacked thylakoids in the grana are shown in green, whereas the stroma thylakoids interconnecting the grana are depicted in orange. The thylakoids were stacked tightly in the dark, as described above (see the Supplemental Movies). A large number of small particles in the lumen were observed in the dark-adapted thylakoids, and these particles have not been identified. In the chloroplasts from the leaves illuminated with strong light, the height of the thylakoid membranes increased at the ends of the grana (Fig. 5B). The number of the unknown particles in the lumen decreased in the illuminated thylakoids.

Discussion

The damage and repair cycle of the reaction-center binding protein D1 of PSII is central in the quality control of PSII under light stress (Barber and Andersson, 1992, Aro et al., 1993, Yamamoto, 2001, Yamamoto et al., 2008). As shown through extensive studies carried out, PSII complexes containing photo-damaged D1 proteins migrate from the grana core, where PSII/LHCII super complexes are abundant, to the grana margins and stroma thylakoids where degradation of the D1 protein occurs. The D1 protein, and proteins including another reaction-center binding protein D2 and the core antenna Chl binding protein CP43, are phosphorylated by the action of redox-regulated specific kinases during light stress (Rintamaki et al., 1995, Vener, 2007, Vainonen et al., 2005, Bonardi et al., 2005). This phosphorylation event facilitates the repair of D1 protein. Removal of CP43 from the PSII complex is also needed not only for the degradation of the damaged D1 protein but for the synthesis of new D1 protein (Rintamaki et al., 1995, Rintamaki et al., 1996, Tikkanen et al., 2008). The release of CP43 may be easier when it is phosphorylated due to strong repulsive forces between the negative charges of the phosphate groups on D1 and CP43. Before degradation of the photo-damaged D1 protein takes place, the phosphate group of the D1 protein is removed by phosphatase named PBCP located in the chloroplast stroma (Samol et al., 2012). Proteases such as FtsH then recognize and react with the stroma-exposed portion of the photo-damaged D1 protein. However, FtsH access to the damaged D1 protein must be difficult if the grana thylakoids are tightly stacked and no suitable

space is available for the proteolytic reaction. In the study of relationship between phosphorylation and thylakoid membrane structure, it is suggested that phosphate groups unfold the appressed thylakoid grana through electrostatic repulsion and facilitate the lateral protein diffusion (Fristedt et al., 2009, Fristedt et al., 2010). FtsH proteases and the phosphatase PBCP in the stroma which dephosphorylates the PSII core proteins may be able to enter the region of grana core under excessive illumination. Thus it is reasonable to assume that excessive illumination induces partial unstacking of thylakoids by phosphate groups of grana proteins, which enables efficient dephosphorylation and subsequent degradation of the photo-damaged D1 protein.

In *Arabidopsis*, an active FtsH hexamer consists of two type A (FtsH1/5) and four type B (FtsH2/8) subunits (Zaltsman et al., 2005, Adam et al., 2006). The arrangement of FtsH subunits in a hexameric ring structure might be involved in the efficient activity for proteolysis as well as stability. What we first studied in this paper was molecular assembly of active hexameric FtsH proteases from constituent subunits in spinach thylakoids. Although we have not revealed the relationship between arrangement of FtsH subunits and its activity yet, it was found that one dimeric type A subunit and the remaining four monomeric type B subunits form the FtsH hexamer (Fig. 1). In contrast, another study *in silico* dealing with the heteromeric FtsH hexamer structure composed of *Arabidopsis* FtsH2 and FtsH5 suggested that two type A subunits (FtsH5) exist as monomers (Moldavski et al., 2012). This conclusion was based on the calculation of buried surface interfaces formed by the monomers and it was assumed that the larger the buried surface area, the larger the thermodynamic stability of the hexameric complex.

It is likely, however, that transmembrane domains of the subunits should also contribute to assembly of the hexamer. Furthermore, catalytic activity of the protease needs a certain conformational change in the pore formed by oligomerization (Geiger et al., 2011), suggesting that the flexibility of the hexameric structure is required for the function. Nonetheless, the determination of the molecular structure of chloroplast FtsH awaits X-ray crystallographic analysis, and such analysis should provide exact stoichiometry and assembly of the subunits as well as structural detail of the protease.

Oligomerization of FtsH probably leads to activation of the enzyme. It was demonstrated with bacterial FtsH derived from a hyperthermophile, *T. maritima*, that FtsH oligomerization led to high activity (Bieniossek et al., 2006). FtsH proteases in spinach thylakoids exist mainly as monomers, dimers and hexamers (Yoshioka et al., 2010), and the active proteases are hexamers. Therefore, there should be a mechanism to regulate oligomerization, in which FtsH proteases adopt proteolytic activity under light stress. As to the mechanism of FtsH hexamer formation, we have previously shown in a preliminary study using an uncoupler NH_4Cl that acidification of the thylakoid lumen under illumination is an important trigger for activation (Yoshioka and Yamamoto, 2011). The loop of FtsH protease connecting two N-terminal trans-membrane α -helices is exposed to the thylakoid lumen and is rich in acidic and basic amino acids. Acidification of the thylakoid lumen by illumination should induce an increase in the net positive charge in this loop region and may induce monomers to undergo conformational changes that facilitate oligomerization of FtsH. A study on the Deg1 protease, which is also claimed to participate in the proteolysis of photo-damaged D1 protein, showed that inactive Deg1

monomers are rapidly transformed into active hexamers under acidic pH conditions in the thylakoid lumen during illumination (Kley et al., 2011). Thus, excessive light damages the D1 protein, and at the same time it may activate the proteases responsible for repair of the damaged proteins.

Change in the distribution of FtsH in the thylakoids under excessive illumination is quite important, because it determines the effective diffusion distance of the protease on the thylakoids and therefore the rate of degradation and removal of the photo-damaged D1 protein in PSII complexes. Recently, we showed in spinach thylakoids that hexameric FtsH proteases are present in a relatively large amount in the PSII-enriched membranes obtained after treatment of dark-adapted thylakoids with Triton X-100 (Yoshioka et al., 2010). In this study the PSII membranes prepared contained the grana cores, and probably the grana margins and grana end membranes as well. Judging from bacterial FtsH complexes, the size of the hexameric enzyme is about 6.5 nm in diameter in its hydrophilic portion (Suno et al., 2006). Because the partition gap between the two adjacent grana thylakoids is a width of 2–4 nm (Nir and Pease, 1973, Dekker and Boekema, 2005, Kirchhoff et al., 2011), the hexameric FtsH protease in higher plant chloroplasts is most likely to be located in the grana margins and grana end membranes. In that study, we also showed the degradation of the photo-damaged D1 protein in the PSII membranes, which was stimulated by the addition of Zn^{2+} and inhibited by EDTA (Yoshioka et al., 2010). These results suggest participation of FtsH in protein degradation. In this study, an increase in the amount of FtsH in the PSII membranes was found following excessive illumination of the thylakoids when compared with the PSII membranes from dark-adapted thylakoids (Fig. 2). An increase in the

degradation of the D1 protein was also detected in the grana fractions obtained by Triton-treatment of the illuminated thylakoids. These results suggest that FtsH proteases are located both in the stroma thylakoids and grana in the dark, with higher amounts of hexameric FtsH in the grana margins as well as grana end membranes, whereas under excessive light the FtsH protease forms active hexamers and is more abundant in the grana margins. The TEM data with immuno-gold labeling of the thylakoids support the movement of FtsH to the grana under excessive illumination (Fig. 3).

The grana margin is an area of importance for active turnover of the D1 protein (Yoshioka et al., 2010). However, this area is actually a narrow space limited by tightly stacked grana membranes in dark adapted chloroplasts (Albertsson, 2001). If no expansion of the grana margins occurs during strong illumination, repair of photo-damaged D1 protein, and generation and insertion of a new copy of the protein to the PSII complex may be difficult. This is because the area is crowded with proteases, monomeric PSII complexes that require repair, phosphatases that dephosphorylate D1 in preparation for degradation, and many subunits of PSII and LHCII released after disorganization of PSII and then used for its reconstruction (Danielsson et al., 2006). The only possible way to increase the area of the grana margin is the unstacking of the grana thylakoids. We have observed outward bending of the thylakoids at both ends of the grana with TEM (Fig. 4). Actually the grana constitute a systematic membrane network whose conformation may be under tight regulation through cooperative forces working between the adjacent thylakoids (Mustardy and Garab, 2003). Former biochemical studies examining the effects of salts or pH on the thylakoid architecture in vitro showed that electrostatic

interactions between the thylakoids is most effective in determining the status of thylakoid stacking and unstacking (Barber, 1982). Thylakoid swelling and shrinkage were also observed through changes in the ionic conditions or pH of the thylakoid suspensions and by illumination (Murakami and Packer, 1970a, Murakami and Packer, 1970b, Johnson et al., 2011a). We must take care, however, as these type of in vitro experiments represent model cases, which are sometimes exaggerated and not representative of native conditions. In our TEM images obtained with spinach leaves, we observed marked structural changes in the grana margin regions under strong illumination. Chemical fixation was used for this TEM analysis, but the reproducibility of the data with different fixation conditions led us to conclude that this outward bending of the grana margin is occurring under natural high light stress conditions.

To further assess the excessive light-induced architectural changes in the grana in spinach chloroplasts, we obtained 3D tomography data of the grana from dark adapted and high light-treated spinach leaves (Fig. 5, also see the Supplemental Movies). From these data, clearly stacked layers of thylakoids in the grana were visible. These characteristics are compatible with the previous models obtained by TEM and 3D tomography showing organization of the grana (Mustardy and Garab, 2003, Shimoni et al., 2005). Following strong illumination of spinach leaves, we could detect a slight change at the grana margin areas, in which outward bending of the thylakoids was observed. This structural change was not as significant as that observed in Fig. 4, probably because the lower resolution of the images in the 3D tomography. In the dark control samples, numerous small particles between the

thylakoid membranes were observed, and the number of particles was reduced following strong illumination. The identity of the particles has not yet been determined.

In conclusion, we demonstrate here that excessive illumination of spinach leaves induces damage and degradation of the D1 protein, assembly of FtsH subunits to an active hexameric enzyme, migration of FtsH from stroma thylakoids to the grana margins, and partial unstacking of both ends of the grana, which is effective for increasing the area of the grana margins. These dynamic features that the thylakoid membranes show upon stress-induced illumination should be crucial for understanding the molecular mechanism of the quality control system of PSII.

Materials and Methods

Isolation of various thylakoid membrane fractions

Fresh spinach leaves were purchased from a local market at Okayama, Japan, and intact chloroplasts were isolated as reported previously (Yamamoto et al., 2004). Thylakoid membranes were obtained by osmolysis of the intact chloroplasts with a hypotonic solution containing 5 mM MgCl₂ and 10 mM HEPES-KOH (pH 7.5). The mixture was subsequently centrifuged at 15,000 g for 10 min, after which the pellets were suspended in a solution containing 0.1 M sorbitol, 15 mM NaCl, 5 mM MgCl₂, and 50 mM Tricine-KOH (pH 7.6) (TC buffer). After washing twice by centrifugation at 15,000 g for 5 min with TC buffer, the thylakoids were suspended in the same buffer solution. The grana and stroma thylakoids were separated by treating the thylakoids with 1.2% Triton X-100 and subsequent

centrifugation at 35,000 *g* for 15 min. We carried out all the preparation steps at 4 °C in a dark room under a green safe light. Chl levels were determined with 80% acetone extracts using a Hitachi U-2000 spectrophotometer (Japan). The Chl concentration was determined using the absorption coefficient of Mackinney (Mackinney, 1941).

SDS/urea-PAGE, clear native PAGE, and Western blot analysis

Sodium dodecyl sulfate (SDS)/urea-PAGE and Western blot analysis were carried out as described previously (Yamamoto et al., 2004). Thylakoid membranes were suspended in TC buffer, and the Chl concentration was adjusted to 0.25 mg Chl·ml⁻¹. CN-PAGE was used to determine the subunit structure of FtsH complexes in thylakoid membranes. The thylakoids samples were solubilized with 1.5% DM at 4 °C for 10 min, followed by centrifugation at 18,000 *g* for 20 min to remove insoluble materials. The supernatant was loaded onto a gel with a gradient of 5–13% acrylamide and electrophoresis was performed for 2 h at 4 °C where the voltage was gradually increased from 70 to 300 V. The composition of the electrode buffers for CN-PAGE was 50 mM Tricine, 7.5 mM imidazole (pH 7.0), 0.05% Triton X-100, and 0.05% deoxycholic acid sodium salt (Merck, USA) for the cathode buffer and 25 mM imidazole HCl (pH 7.0) for the anode buffer. The antibodies against the DE loop of the D1 protein, PsaA, *cyt f*, FtsH1/5 and FtsH2/8 were used for Western blot analysis. Horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad) was used as the secondary antibody. Immunodecorated bands were detected by fluorography with enhanced chemiluminescence (GE Healthcare, Japan).

Immuno electron microscopy

To visualize the distribution of the FtsH protease in the thylakoids, the thylakoids from the leaves of spinach were observed by electron microscopy with immuno-gold labeling. Fixation was performed with 4% paraformaldehyde, 0.1% glutaraldehyde and 1% tannic acid in 0.05 M cacodylate buffer (pH 7.4) at 4 °C for 2 h. The samples were dehydrated through a series of graded ethanol steps (50%, 70%) at 4 °C for 30 min. After embedding to the LR White resins, the samples were ultra-thin-sectioned at 80 nm with a diamond knife and the sections were placed on nickel grids. We used a primary antibody against FtsH2/8, and the anti-rabbit secondary antibody conjugated with 10 nm gold particles (BBI Solutions, UK). The grids were stained with 2% uranyl acetate. The specimens were observed by TEM (JEM-1200EX, JEOL Ltd., Japan) at an acceleration voltage of 80 kV.

TEM observation and tomographic reconstruction

The slices of spinach leaves were cut out and fixed in 2.5% glutaraldehyde in phosphate-buffered saline. After washing in phosphate buffer, samples were post fixed with 1% OsO₄ and washed with distilled water. Block dyeing of the sample was carried out by saturated uranyl acetate. The samples were washed in phosphate buffer and then dehydrated with 50 to 100% of ethanol. Substitution of samples was carried out in three operations: (1) only propylene oxide (PO); (2) mixed-solution of PO and Epoxy resin; and (3) pure Epoxy resin. The samples were embedded in Epoxy resin composed of a mixed-solution of Epon812, dodecyl succinic anhydride, methyl nadic anhydride and 2,4,6-Tris(dimethylaminomethyl)phenol, and polymerized for 3 days at 45 °C to 60 °C.

Ultra-thin sections (200 nm) were made by using an ULTRACUTUCT (Leica, Japan), and stained with uranyl acetate and lead citrate. TEM experiments were carried out on a JEM-2100 (JEOL Ltd., Japan) with a LaB6 electron gun. The accelerating voltage was 200 kV. The transmitted images were obtained by a charge-coupled device camera (Gatan Inc., USA) in the TEM mode. A series of TEM micrographs with 2,048 × 2,048 pixel elements were acquired at tilt angles ranging from -70° to $+70^{\circ}$ with 1° increments. One photograph was taken at 20,000-fold magnification with the TEM. Three-dimensional reconstructions of spinach thylakoids were performed by the software Tomography (JEOL Ltd., Japan) and Amira (FEI Visualization Sciences Group).

Funding

This work was supported by a Grant-in-Aid for Scientific Research 24570053 from the Ministry of Education, Culture, Sports, Science and Technology of Japan to Y.Y., and grants for the Okayama University Woman-Tenure-Track (WTT) staff from Okayama University, from the Japan Science Society (The Sasakawa Scientific Research Grant), from the Ryobi Foundation and from the Nippon Life Insurance Foundation to M.Y.-N.

Acknowledgments

We thank Drs. Yuichiro Takahashi and Jian-Ren Shen of Okayama University for valuable discussion.

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Figure legends

Fig. 1 Arrangement of the FtsH subunits in the thylakoids. *A*, Western blot analysis after CN-PAGE of spinach thylakoid membranes solubilized with 1.5% DM, showing the subunit composition of FtsH proteases. Two types of FtsH antibodies, namely those against FtsH1/5 (Type A) and FtsH2/8 (Type B), were used in the Western blotting. Each lane contains thylakoid membranes equivalent to 1 µg of Chl. The FtsH monomer and oligomers are indicated on the right-hand side of the gel. *B*, A model showing the molecular arrangement of the FtsH protease. The hexameric FtsH protease is composed of a pair of type A subunits and four type B subunits. A predicted arrangement of the FtsH subunits is AA+B+B+B+B, where type A subunits co-exist as a dimer and the remaining four subunits are type B monomers. The six subunits are arranged in line symmetry.

Fig. 2 Accumulation of FtsH and degradation products of the D1 protein in the grana. Quantification of

the amounts of FtsH2/8, the D1 protein, PsaA and cyt *f* in spinach stroma thylakoids (S) and grana (G) were performed (A). In the left halves of the gels, the amounts of the samples were equivalent to 2.5 μg of Chl in each lane. In the right halves of the gels, the gradient of each antigen that was used for quantification of the bands in the left half of each gel is shown. Western blot analysis of the thylakoid membranes fractionated into the stroma thylakoids and the grana after illumination at 0–2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 60 min. FtsH proteases in the stroma thylakoids (B) and the grana (C) were detected with the antibody against FtsH2/8. Degradation of the D1 protein was detected with the antibody against the DE-loop of the D1 protein. The sizes of the fragments of the D1 protein are shown on the right-hand side of the gel. At the bottom, the relative amount of FtsH protease in the stroma thylakoids or grana was shown. The data are derived from the Western blot analysis.

Fig. 3 Immunogold labeling of FtsH proteases in the thylakoid membranes. TEM images of the thylakoids illuminated with strong illumination (1,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$) for 30 (B) min. A is a dark control. The primary antibody against FtsH2/8 and the secondary antibody attached with 10 nm gold particles were used to visualize the location of the FtsH proteases. Scale bar = 200 nm.

Fig. 4 Structural changes of the thylakoid membranes under light stress. TEM images of thylakoid membranes in the dark-adapted (A) or the light-exposed (B) spinach leaves. The scale bar = 200 nm. The spinach leaves were kept at 4 °C in the dark and the light treatment was performed at a light

intensity of 2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 60 min. The height of 10 thylakoid membrane layers was measured in each area shown with red squares (No. 1–5) in the TEM images. The areas No. 1 and 5 correspond to the grana margins. The area No. 3 represents the grana core. The results are shown at the bottom of the figure (C). Blue and red lines indicate the dark-adapted and high light-treated samples, respectively. The data are the means \pm S.D. ($n = 10$).

Fig. 5 3D modeling of the thylakoid membranes. 3D movies were constructed from TEM images of thylakoid membrane structure. A series of TEM images with tilt angles ranging from -70° to $+70^\circ$ with 1° increments were used. The dark-adapted and the high light-treated (2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 60 min) spinach leaves were examined by TEM. The grana and the stroma thylakoids are colored with green and orange, respectively.

Supplementary data

Fig. S1 Thylakoid membrane structure under illumination. A-C, Structure of thylakoid membranes under various light conditions. The spinach leaves were incubated at 4 $^\circ\text{C}$ in the dark before light treatment. The leaves were illuminated at light intensities of 500, 1,000, 2,000 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$ for 0–60 min. The scale bar represents 200 nm.

Movie S1 A 3D-tomographic model of the thylakoids. This 3D movie was constructed using TEM

images of thylakoids in the dark-adapted spinach leaves. The stacked grana and the stroma thylakoids are colored with green and orange, respectively. The particles are shown with blue color. The scale bar represents 100 nm.

Movie S2 A 3D-tomographic model of thylakoids under high light. This 3D movie was constructed using TEM images of thylakoids in the light-treated spinach leaves. The spinach leaves were subjected to high light ($2,000 \mu\text{mol photons m}^{-2}\text{s}^{-1}$) for 60 min. The stacked grana and the stroma thylakoids are colored with green and orange, respectively. The particles are shown with blue color. The scale bar represents 100 nm.