Close relations between the PSII repair cycle and thylakoid membrane dynamics

Running head: PSII and thylakoid membranes under light stress

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Abbreviations: D1 and D2, the reaction center-binding proteins of PSII; Deg, degradation of periplasmic proteins; DGDG, digalactosyldiacylglycerol; EPR, electron paramagnetic resonance; FtsH, filamentation temperature sensitive H; LHCII, light-harvesting Chl-protein complex of PSII; MGDG, monogalactosyldiacylglycerol; PBCP, PSII core phosphatase; PG, phosphatidylglycerol; ROS, reactive oxygen species; SQDG, sulfoquinovosyl diacylglycerol; stn7, state transition 7; stn8, state transition 8; TEM, transmission electron microscopy.

Abstract

In chloroplasts, a three-dimensional network of thylakoid membranes is formed by stacked grana and interconnecting stroma thylakoids. The grana are crowded with photosynthetic proteins, where PSII/LHCII supercomplexes often show semi-crystalline arrays for efficient energy trapping, transfer, and use. Although light is essential for photosynthesis, PSII is damaged by reactive oxygen species that are generated from primary photochemical reactions when plants are exposed to excess light. Because PSII complexes are embedded in the lipid bilayers of thylakoid membranes, their functions are affected by the conditions of the lipids. EPR spin trapping measurements showed that singlet oxygen was formed through peroxidation of thylakoid lipids, suggesting that lipid peroxidation can damage proteins, including the D1 protein. After photodamage, PSII is restored by a specific repair system in thylakoid membranes. In the PSII repair cycle, phosphorylation and dephosphorylation of the PSII proteins control the timing of PSII disassembly and subsequent degradation of the D1 protein. Under light stress, stacked grana turn into unstacked thylakoids with bent grana margins. These structural changes may be closely linked to the mechanisms of the PSII repair cycle because PSII can move more easily from the grana core to the stroma thylakoids through an expanded stromal gap between each thylakoid. Thus, plants modulate the structure of thylakoid membranes under high light to carry out efficient PSII repair. This review

focuses on the behavior of the PSII complex and active role of structural changes to thylakoid membranes under light stress. (240 words)

Keywords: FtsH protease • Light stress • Photoinhibition • PSII • PSII repair cycle • Thylakoid membrane

Introduction

Acclimation is an efficient response by higher plants for survival in ever-changing environmental conditions. Light is necessary for photochemical reactions of photosynthesis; however, light is one of the most fluctuating environmental factors in nature. Thus, it is important for plants to optimize photosynthesis under various light conditions. PSII/LHCII supercomplexes are embedded in the thylakoid membrane and each complex consists of more than 30 subunits (Barber 1998; Hankamer et al. 1997). The PSII/LHCII complexes are mostly localized in the stacked grana, whereas the Photosystem I (PSI) and ATP synthase exist in the stroma thylakoids including grana margins and grana end membranes (Andersson and Anderson 1980; Miller and Staehelin 1976). Both the PSII and LHCII complexes have flat surfaces exposed to the stromal side (Nield and Barber 2006), and therefore these complexes can exist in the stacked grana. By contrast, the PSI and ATP synthase protrude into the stromal side (Amunts and Nelson 2008; Junge et al. 2009), which prevents them from localizing in the narrow membrane gaps of the stacked grana. Cytochrome b_6/f complexes are equally found in both stacked and unstacked thylakoid membranes (Allred and Staehelin 1985; Anderson 1982; Cox and Andersson 1981; Mansfield and Bendall 1984). Although PSII plays a key role in water splitting, it is vulnerable to photo-oxidative stress under high light and so-called photoinhibition occurs (Aro et al. 1993; Barber and Andersson 1992; Kyle et al. 1984; Ohad et al. 1984). To overcome these problems plants have developed various sophisticated

mechanisms.

Under high light conditions, the acceptor side of PSII is over-reduced and reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) are produced near PSII (Khatoon et al. 2009; Macpherson et al. 1993; Pospisil 2009; Telfer et al. 1994; Tiwari et al. 2013). $^1O_2$ is also produced through high light-induced lipid peroxidation in the thylakoids, and it was suggested that $^1O_2$ and the by-products of lipid peroxidation (malondialdehyde) damage the proteins near PSII, such as D1 and LHCII subunit proteins (Chan et al. 2012). Specific enzymes, antioxidants, and pigments for scavenging ROS work to protect the photosynthetic apparatus from oxidative damage (Dall'Osto et al. 2007; Havaux et al. 2005; Pospisil 2011, 2012). Structural changes to the thylakoid membrane are also a key factor, which functions to avoid further damage of the PSII by ROS. When the thylakoid membranes are subjected to high light, more hydroxyl radicals are detected in the stacked grana thylakoids than in the unstacked thylakoids (Khatoon et al. 2009). It is suggested that unstacking of the thylakoid membranes may prevent energy transfer which takes place intensively in the stacked grana crowded with PSII/LHCII complexes. Light-induced production of ROS associated with PSII over-reduction should be reduced by unstacking of the thylakoids (Yamamoto et al. 2014). In contrast to the stacked grana which enhance the ability of light harvesting system, thylakoid unstacking is a mechanism that plants protect the PSII from photo-oxidative damage under high light. Moreover, diffusion of small electron carriers is affected by the structural changes

of thylakoid membranes, which contributes to the efficiency of electron shuttling under light condition. In the stacked grana, diffusion of plastoquinone in the membranes and plastocyanin in the lumen which is slowed by the densely packed PSII/LHCII complexes is a rate-limiting factor in the electron transport (Kirchhoff et al. 2011; Kirchhoff et al. 2000; Kirchhoff et al. 2004; Lavergne et al. 1992; Yamamoto et al. 1981). It has been reported that light-induced expansion of the lumen increases the space for protein diffusion which facilitates electron transport mediated by plastocyanin, along with the reduced restriction of plastoquinone in the light (Kirchhoff 2014; Kirchhoff et al. 2011). Additionally, the thylakoid unstacking and the increased membrane fluidity under light condition (Yamamoto et al. 2013) may allow damaged PSII to move more easily to the stroma thylakoids for the repair of D1 protein. Thus, both the molecular strategy (ROS scavenging) and structural strategy (swelling and unstacking of the thylakoid membranes) protect plants from oxidative stress under high light.

Characterization of thylakoid membranes

In chloroplasts, the thylakoid membranes consist of two main regions, the stacked grana thylakoids and unstacked stroma thylakoids. In the grana, many thylakoids are layered and the partition gap width between two adjacent thylakoid membranes is 3.5 nm (Daum et al. 2010; Kirchhoff et al. 2011). The grana discs are 130–160 nm in height and about 500 nm in diameter and they are interconnected by the stroma thylakoids, forming a continuous

three-dimensional network (Austin and Staehelin 2011; Daum and Kuhlbrandt 2011). The lumenal space of the thylakoid membrane from dark-adapted *Arabidopsis thaliana* is 4.5 nm in width (Fig. 1), and the space expands in the light, which facilitates plastocyanin-mediated electron transport (Daum et al. 2010; Kirchhoff 2013a; Kirchhoff et al. 2011). The expansion of the thylakoid lumen is called thylakoid swelling and is postulated to be related to the osmotic potential formed through electron transport in the light (Anderson et al. 2012). The chloride ion influx through the voltage-gated channels in the thylakoid membranes caused by the light-induced proton motive force (pmf) is likely to be involved in thylakoid swelling (De Angeli et al. 2009; Hechenberger et al. 1996; Kirchhoff 2013a; Kirchhoff et al. 2011; Schonknecht et al. 1988; Spetea and Schoefs 2010). The exact explanation for the process of thylakoid swelling requires further investigation.

The number of stacked thylakoids increases in shade plants to collect more light energy (Anderson 1986; Lichtenthaler et al. 1981). The size of grana may be directly related to the capacity of acclimation to low light. Grana formation is controlled by multiple genes and regulation of grana formation is thought to be linked to phosphorylation of thylakoid proteins. Grana membranes were shown to become longer in length in the *stn8* mutant and *stn7stn8* double mutant of *Arabidopsis thaliana* lacking thylakoid protein phosphorylation (Fristedt et al. 2009). PSII core phosphatase (PBCP) working against STN8 kinase was also shown to be involved in grana formation. A decrease in the number of grana layers was found in a *pbcp*

mutant where the phosphatase is inactive (Samol et al. 2012). Moreover, the curvature thylakoid 1 (CURT1) proteins abundant in the grana margins, which directly induce membrane curvature, were suggested to regulate the formation of the grana stacks (Armbruster et al. 2013).

Organization of thylakoid membrane proteins

Thylakoid membranes are crowded with many photosynthetic proteins (Kirchhoff 2008; Kirchhoff et al. 2011). Electron microscopic studies show that supercomplexes of PSII and LHCII are embedded in the thylakoid membranes forming semi-crystalline arrays (Dekker and Boekema 2005). Formation of the PSII/LHCII supercomplexes enables efficient excitation energy transfer from LHCII complexes to PSII (Haferkamp et al. 2010). According to recent computer simulation analyses, it was demonstrated that ordered and disordered protein arrays coexist in the grana, suggesting that the thylakoid membranes are functionally flexible (Schneider and Geissler 2013). The PSII/LHCII arrays are abundant in the stacked grana, whereas they are absent in the unstacked thylakoid membranes. The electrostatic attraction between PSII/LHCII complexes in the layered thylakoid membranes is likely to be involved in grana stacking (Daum et al. 2010). The role of cations in thylakoid stacking has been proposed in studies on the crystal arrays of LHCII (Hind et al. 2014) and protein phosphorylation mutants (Fristedt et al. 2010).

Most recently, the impact of the highly ordered semi-crystalline arrays to the PSII repair cycle was examined using a fatty acid desaturase 5 (*fad5*) mutant of *Arabidopsis thaliana* (Tietz et al. 2015). The *fad5* mutant had much higher abundance of PSII/LHCII protein crystals than wild-type in the grana, and showed less degradation of the D1 proteins. This suggests that the crowded semi-crystalline arrays impede the migration of PSII from the stacked grana to the unstacked thylakoids where the PSII repair cycle takes place. However, the formation of ordered PSII/LHCII supercomplexes is favorable for the mobility of small hydrophobic molecules such as plastoquinone or xanthophylls (Tietz et al. 2015). The increased mobility of plastoquinone and xanthophylls assists electron transport between PSII and cytochrome b_6/f, and non-photochemical quenching (Jahns et al. 2009; Kirchhoff 2014; Macko et al. 2002). The effects of the ordered protein arrays on protein mobility in the thylakoid membranes have been proposed to be different and dependent on the size of the protein molecules.

Lipids of thylakoid membrane

Membrane fluidity and mobility of proteins are crucial points for understanding the effects of light stress on PSII at the molecular level (Kirchhoff 2008; Mullineaux 2008). Thylakoid membranes consist of two major galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which constitute 50–60 mol% and 20–30

mol% of the total lipids, respectively. The remaining lipids are phospholipids and sulfolipids-phosphatidylglycerol (PG) and sulfoquinovosyl diacylglycerol (SQDG) comprising 5–10 mol% of the total thylakoid lipids (Block et al. 1983; Dorne et al. 1990; Somerville et al. 2000). Lipid composition of thylakoid membranes is conserved from cyanobacteria to higher plants. The ratio of MGDG/DGDG is higher in the grana regions than in the stroma thylakoids (Gounaris et al. 1983), and MGDG is likely to form the curved thylakoids, whereas DGDG may stabilize the thylakoid membranes network (Rast et al. 2015). Recently, possible effects of the MGDG/DGDG ratio on membrane phase transition and membrane stacking have been studied (Deme et al. 2014a; Deme et al. 2014b). Membrane lipids are divided into two types: bound (boundary) lipids associated with membrane proteins; and free (bulk) lipids that diffuse freely within the membranes (Dahlquist et al. 1977; Jost et al. 1973a; Jost et al. 1973b; Owicki et al. 1978; Tieleman et al. 1997). Interactions among various proteins in the thylakoid membranes are supported by membrane fluidity. Membrane fluidity depends on the degree of saturation of the fatty acid in the free lipids, and a higher degree of unsaturation increases membrane fluidity. Membrane fluidity of thylakoids increases under moderate high light where LHCII complexes form reversible aggregates to dissipate excess light energy as heat, while the fluidity decreases under extremely high light which causes irreversible aggregation of PSII core subunits (Yamamoto et al. 2013). The reversible aggregation of proteins generates free space for PSII complexes to move across the

grana to the stroma thylakoids for repair of the D1 protein. However, under extremely high light, singlet oxygen is generated through photochemical reaction or lipid peroxidation, and may damage the thylakoid proteins and form irreversible aggregates (Chan et al. 2012; Yamashita et al. 2008). When the irreversible aggregates accumulate, they lead to a decrease in the membrane fluidity. Decreased mobility of thylakoid membranes should prevent rapid diffusion of protein molecules, and migration of the damaged PSII from the stacked grana to stroma thylakoids for repair will be delayed. Lipid peroxidation propagates in thylakoid membranes and may ultimately inhibit the process of quality control of PSII. To avoid the chain reaction of lipid peroxidation, thylakoid unstacking possibly facilitates the escape of ROS from the thylakoid membranes.

Proteases involved in the degradation of the D1 protein

The D1 protein of the PSII complex is photo-oxidatively damaged and degraded by proteases located in the chloroplast. Two proteases are the most likely candidates for the degradation of the D1 protein, FtsH (Komenda et al. 2006; Silva et al. 2003) and Deg (Itzhaki et al. 1998; Kapri-Pardes et al. 2007; Kato et al. 2012; Sun et al. 2007) proteases (Fig. 1). FtsH proteases bind to the thylakoid membranes with two trans-membrane helices and form the hexameric ring-shaped FtsH complex, composed of type A (FtsH1 and 5) and type B (FtsH2 and 8) subunits at the ratio of 1:2 (Adam et al. 2006; Zaltsman et al. 2005). FtsH5 is

present four to five times more than FtsH1, and FtsH2 is two to three times more abundant than FtsH8 (Moldavski et al. 2012; Sinvany-Villalobo et al. 2004). In the case of Deg proteases, they are found to be peripherally attached to thylakoid membranes. Deg1, 5, and 8 are located in the lumen, and Deg2 and 7 are located on the stromal side of thylakoid membranes (Huesgen et al. 2009; Schuhmann and Adamska 2012). Deg1 exists as both monomer and hexamer showing proteolytic activity at low pH, but the Deg2 hexamer is pH independent (Chassin et al. 2002; Kley et al. 2011; Sun et al. 2012). Deg5 forms a hetero-hexamer together with Deg8 (Sun et al. 2007), and Deg7 forms trimeric complexes (Schuhmann et al. 2011). These five Deg proteases participate in the degradation of the damaged D1 protein under light stress. The FtsH protease is dependent on the zinc ion and ATP initiates processive proteolysis, whereas the Deg protease is a serine-type endopeptidase that is ATP independent. Deg1, 5, and 8 proteases degrade the CD loop of the D1 protein (Kapri-Pardes et al. 2007; Sun et al. 2007), whereas Deg2, 7, and FtsH proteases are involved in degradation of the DE loop of the D1 protein (Haussuhl et al. 2001; Sun et al. 2010; Yoshioka et al. 2006). In subsequent studies, however, a *deg2* mutant of *Arabidopsis thaliana* showed D1 degradation under high light, which suggests the presence of redundant D1 degradation pathways (Huesgen et al. 2006).

Machinery of the PSII repair cycle

Thylakoid membranes are separated into three subdomains, namely the grana core, the grana margins, and the stroma thylakoids. A model of the PSII repair cycle focusing on the individual roles of the three thylakoid subdomains was recently proposed (Puthiyaveetil et al. 2014a). The thylakoid membranes are stacked in the dark as well as under low light conditions, suggesting that the stacked grana core harbor the PSII complexes to prevent degradation of D1 and D2 proteins (Anderson and Aro 1994). The stacked grana were shown to unstack under high light (Herbstova et al. 2012; Khatoon et al. 2009; Yoshioka-Nishimura et al. 2014), and it was demonstrated that thylakoid unstacking is accompanied by bending of the thylakoids outwards and an increase in the area of grana margins producing the new unstacked regions of the thylakoids (Puthiyaveetil et al. 2014a; Yoshioka-Nishimura et al. 2014). Unstacking of the thylakoid membranes should stimulate migration of membrane proteins, and promote the PSII repair cycle as detailed below (Fig. 2). Stacking and unstacking of thylakoid membranes are reversible events (Anderson 1999; Anderson and Aro 1994; Horton 1999); however, harsh light conditions can induce irreversible unstacking of the thylakoids (Khatoon et al. 2009).

When the PSII complex is damaged under high light, PSII phosphorylation catalyzed mainly by the STN8 kinase increases and triggers the disassembly of the PSII supercomplex in the grana core (Bonardi et al. 2005; Tikkanen et al. 2008; Vainonen et al. 2005; Wunder et al. 2013). In parallel with high light-induced unstacking of the thylakoids, phosphorylated and

disassembled PSII migrates from the stacked grana to the grana margins and to the stroma thylakoids. Conversely, FtsH proteases move from the stroma thylakoids to the grana margins and possibly to the grana core as well because the width of the stromal gap between thylakoid membranes increases upon unstacking of the grana (Yoshioka-Nishimura et al. 2014). Shrinkage of the grana diameter and increased mobility of the membrane proteins have been confirmed by confocal laser scanning microscopy analysis and fluorescence recovery after photobleaching measurements (Herbstova et al. 2012). Although many FtsH proteases were detected near the PSII complex in the grana (Yoshioka et al. 2010), it seems to be difficult for the protease to exist in the stacked grana core because of the large extrusion of its hydrophilic portion to the stromal side. Therefore, grana margins are considered to be the major site where the FtsH proteases reside. TEM observations and biochemical data revealed that the FtsH proteases are localized in the grana margins when thylakoid swelling and unstacking occurs (Puthiyaveetil et al. 2014a; Yoshioka-Nishimura et al. 2014). Thylakoid swelling allows the Deg proteases with a height of 7 nm to move and access the PSII within the lumen because the width of the lumen expands from 4.7 to 9.2 nm under moderate illumination (500 μmol photons m^{-2} s^{-1}) (Kirchhoff 2013b; Kirchhoff et al. 2011; Kley et al. 2011) (Fig. 1).

After migration, the PSII complexes are dephosphorylated by the PSII core phosphatase (PBCP) identified by reverse genetic screening (Samol et al. 2012). PBCP has no membrane-spanning region and the exact localization of PBCP is unclear, although it has been

identified as a chloroplast protein (Puthiyaveetil et al. 2014a). In *pbcp* mutants of *Arabidopsis thaliana*, reduced levels of degradation of the D1 protein were observed in the presence of lincomycin under high light, suggesting that dephosphorylation of PSII is necessary for degradation of the D1 protein (Puthiyaveetil et al. 2014b). The dephosphorylated PSII is recognized by FtsH protease, and the damaged D1 protein is degraded immediately in the grana margins and the stroma thylakoids. Rapid degradation of the D1 protein may occur especially in the increased areas of the grana margins under high light because PBCP and FtsH proteases are localized together. Briefly, the grana margins that increase under high light are the areas necessary for swift and efficient D1 degradation (Yoshioka-Nishimura et al. 2014; Yoshioka et al. 2010). Like FtsH proteases, Deg proteases that reach the PSII complexes by structural changes to the thylakoid degrade the damaged D1 protein. At the last stage of the D1 repair cycle, newly synthesized D1 is inserted into the PSII and reassembly of the PSII supercomplex is accomplished. It is assumed that these final steps occur in the unstacked thylakoids because the grana are crowded with PSII/LHCII supercomplexes (Aro et al. 2005; Danielsson and Albertsson 2009).

Concluding remarks

Thylakoid membranes show dynamic changes in structure under high light to control the quality of PSII. Through recent electron microscopic studies, grana margins have been shown

to be an important site for PSII repair. The swift degradation of the damaged D1 protein occurs in the grana margins without long migration to the stroma thylakoids, which avoids irrecoverable damage to the PSII caused by aggregation of the damaged proteins. Compartmentalization of the PSII repair cycle in each thylakoid subdomain regulates the timing of repair events, and light-induced conversion of a part of the grana core to the grana margins enables photosynthetic proteins to diffuse easily within the thylakoids. Thus, structural changes of the thylakoid membranes support the PSII repair cycle. For a better understanding of the structural changes of the thylakoid membranes, non-invasive techniques, such as small angle neutron scattering (Unnep et al. 2014a; Unnep et al. 2014b), live cell imaging (Iwai et al. 2014), and other new approaches will be essential.

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Disclosures

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Figure legends

Fig. 1 Structural changes of the thylakoid membranes under high light. Stacked thylakoid membranes (upper) are unstacked and bent, which is accompanied by thylakoid swelling (lower). The distance between two adjacent thylakoid membranes and the width of the lumen (Daum et al. 2010; Kirchhoff et al. 2011) are shown on the left side of the figure. Deg1, 5, and 8 are located on the lumenal side, whereas Deg2 and 7 are peripherally attached to the stromal side of thylakoid membranes. FtsH proteases bind to thylakoid membranes with two transmembrane helices. Thylakoid swelling allows Deg1, 5, and 8 to move freely within the thylakoid lumen. Under high light, Deg2, 7, and FtsH access the D1 protein in the grana region because membrane-stacking constraints dissipate.

Fig. 2 A model of the PSII repair cycle. After the PSII complex is phosphorylated by STN8 kinase under high light, monomerization of the PSII complex and detachment of LHCII from the PSII core complex occurs. At the same time, thylakoid membranes swell and unstack within minutes, which supports lateral migration of PSII from the grana to the unstacked thylakoids such as grana margins and stroma thylakoids. In the unstacked thylakoid regions, the PSII core complex is dephosphorylated by PBCP phosphatase before degradation of the D1 protein by Deg and FtsH proteases. A newly synthesized D1 protein is inserted into the

PSII core complex probably in the stroma thylakoids. The reassembled PSII complex returns to the stacked grana. The PSII repair cycle works cooperatively with the structural changes of the thylakoid membranes, which play a significant role in the diffusion of the photosynthetic proteins, kinases, phosphatases, and proteases.