The variegated mutants lacking chloroplastic FtsHs are defective in D1 degradation and accumulate reactive oxygen species

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In the photosynthetic apparatus, a major target of photodamage is the D1 reaction center protein of Photosystem II (PSII). Photosynthetic organisms have developed a PSII repair cycle in which photodamaged D1 is selectively degraded. A thylakoid membrane-bound metalloprotease FtsH was shown to play a critical role in this process.

Here, the effect of FtsHs in D1 degradation was investigated in Arabidopsis (Arabidopsis thaliana) mutants lacking FtsH2 (var2) or FtsH5 (var1). Because these mutants are characterized by variegated leaves that sometimes complicate biochemical studies, we employed another mutation fug1 that suppresses leaf variegation in var1 and var2 to examine D1 degradation. Two-dimensional blue-native PAGE showed that var2 has less PSII supercomplex and more PSII intermediate lacking CP43 termed RC47 than wild type under normal growth light. Moreover, our histochemical and quantitative analyses revealed that chloroplasts in var2 accumulate significant levels of reactive oxygen species, such as superoxide radical and hydrogen peroxide. These results implicate that the lack of FtsH2 leads to impaired D1 degradation at the step of RC47 formation in the PSII repair, and to photooxidative stress even under non-photoinhibitory conditions.

Our in vivo D1 degradation assays, carried out by non-variegated var2 fug1 and var1 fug1 leaves, demonstrated that D1 degradation was impaired in different light conditions. Taken together, our results suggest the important role of chloroplastic FtsHs, which was not precisely examined in vivo. Attenuated D1 degradation in the non-variegated mutants also suggests that leaf variegation seems to be independent of the PSII repair.
Excessive light often limits the growth of photosynthetic organisms by irreversibly inactivating the photosynthetic apparatus, a process called photoinhibition (for review, see Barber and Andersson, 1992; Aro et al., 1993). A major target of photodamage is Photosystem II (PSII) (Barber and Andersson, 1992; Aro et al., 1993; Murata et al., 2007), a large pigment-protein complex in the thylakoid membrane. In particular, the reaction center D1 protein, which binds cooperatively to D2 and carries cofactors required for electron flow from the manganese cluster of the water-oxidizing complex to the plastoquinone pool (Zouni et al., 2001; Loll et al., 2005), is the primary target of light-induced irreversible oxidative damage (Mattoo et al., 1981; Ohad et al., 1990). Because D1 can be damaged by even low light intensities, photosynthetic organisms cannot avoid photodamage (Barber and Andersson, 1992; Tyystjärvi and Aro, 1996). To overcome this, photosynthetic organisms have evolved an efficient PSII repair cycle, which involves disassembling of PSII, degrading photodamaged D1, and replacing newly synthesized D1 (for review, see Baena-Gonzalez and Aro, 2002). The rate of photodamage is proportional to light energy (Barber and Andersson, 1992; Tyystjärvi and Aro, 1996). When the light intensity exceeds the repair capacity, damaged D1 accumulates, resulting in photoinhibition.

In the PSII repair, recent studies in Synechocystis sp. PCC 6803 and Arabidopsis (Arabidopsis thaliana) suggest important roles of prokaryotic proteases (Lindahl et al., 1996, 2000; Bailey et al. 2002; Sakamoto et al., 2003; Silva et al., 2003; Komenda et al., 2006; Sun et al., 2007; Kapri-Pardes et al., 2007). Among them, FtsH appears to be a major protease. It is a membrane-anchored ATP-dependent zinc metalloprotease that belongs to the ATPases associated with a variety of cellular activities (AAA)+ protein family (for review, see Patel and Latterich, 1998; Ogura and Wilkinson, 2001). The ATPase and protease domain of FtsH was shown to form a hexameric-ring structure (Suno et al., 2006). In Synechocystis sp. PCC 6803, deletion of one of the thylakoidal FtsHs shr0228 results in light-sensitive growth, impairment of the PSII repair cycle, and slower D1 degradation under high-light conditions (Silva et al., 2003). In Arabidopsis, twelve FtsH homologues were identified, nine of which are targeted to chloroplasts (Sakamoto et al., 2003). FtsH2 and FtsH5 are the most abundant among all chloroplastic FtsHs and are located in thylakoid membranes (Sakamoto et al., 2003; Yu et al., 2004, 2005). Chloroplastic FtsHs predominantly exist as a hetero-complex consisting of at
least two types of isomers A and B, represented by FtsH1/5 and FtsH2/8, respectively. These two types are functionally distinguishable with each other (Sakamoto et al., 2003; Yu et al., 2004, 2005; Zaltsman et al., 2005b).

We have extensively studied Arabidopsis mutants lacking chloroplast FtsHs. A mutant lacking FtsH5 (called yellow variegated 1, var1) or lacking FtsH2 (var2) is highly vulnerable to PSII photodamage under high light (Chen et al., 2000; Takechi et al., 2000; Lindahl et al., 2000; Sakamoto et al., 2002, 2004). One notable feature in these mutants, in addition to the defective PSII repair, is the leaf-variegated phenotype that displays two sectors in the same leaf (green sectors containing normal chloroplasts and white sectors containing abnormal plastids lacking thylakoid membranes) (Supplemental Fig. 1). White sectors are made by living cells and appear to be comparable with green sectors, except for lacking photosynthetic proteins (Kato et al., 2007). These results demonstrated that white sectors in var2 are arrested in chloroplast development. It is thus proposed that FtsH is not only involved in the PSII repair but also in the formation of thylakoid membranes. Moreover, a series of genetic studies enabled us to identify trans-acting mutations that suppressed leaf variegation in var2 (Park and Rodermel, 2004; Miura et al., 2007; Yu et al., 2008). Many suppressors appeared to be associated with chloroplast translation, suggesting that the formation of variegated sectors is not simply governed by a specific factor but rather by factors related to chloroplast development (Miura et al., 2007; Yu et al., 2008). Since the variegated phenotype complicates our biochemical study in Arabidopsis, unlike cyanobacteria, a combined usage of var and its suppressors is necessary to further investigate the role of FtsH in the PSII repair cycle.

In this study, we show that chloroplasts in green sectors accumulate less amount of PSII supercomplex and more PSII partial complexes than wild-type chloroplasts, likely due to the compromised PSII repair. Interestingly, we found chloroplasts in green sectors accumulated significantly high levels of reactive oxygen species (ROS), suggesting that var2 indeed suffers from photo-oxidative stress. Given the essential role of FtsH, we evaluated impaired D1 degradation by the lack of FtsH2 and FtsH5 under different light conditions. Although a defect in degrading PSII reaction center proteins in var2 has been reported to occur under a photoinhibitory light condition (Bailey et al., 2002), D1 degradation examined in this study was very limited.
This is because in vivo degradation of D1 is very difficult to measure in variegated leaf tissues (e.g. the presence of green and white leaf sectors interferes with protein normalization). To hurdle the difficulty handling variegated sectors, non-variegated suppressor lines were subjected to these experiments. Our D1 degradation assays demonstrated that the lack of FtsH2 or FtsH5 significantly impairs D1 degradation. Collectively, our data corroborate important roles of FtsH2 and FtsH5 in avoiding photooxidative stress in chloroplasts.

RESULTS

PSII Protein Complexes in Thylakoid Membranes of the Wild Type and var2

The functional PSII core complex of higher plants is a dimer and is associated with a light-harvesting antenna chlorophyll complex. In the repair cycle of the PSII complex, assembly and disassembly of PSII are stepwise processes that involve the formation of several intermediates, such as a PSII core complex lacking CP43 (RC47) and a monomeric core complex with a reaction center core (PSII monomer) (Baena-Gonzalez and Aro, 2002). Thus, monitoring intermediates of PSII assembly/disassembly and analyzing them in the wild type and var2 should allow us to assess the repair state of PSII and the involvement of FtsH. To examine the repair intermediates, we purified chloroplasts using a Percoll gradient, extracted thylakoid membranes, solubilized the fraction with 0.4 % n-dodecyl β-D-maltoside, and finally separated PSII and other complexes using two-dimensional blue native BN/SDS-PAGE. We simultaneously performed an immunoblot analysis to also detect D1 in these preparations. For BN/SDS-PAGE, proteins from the wild type and var2-1 were equally loaded based on total chlorophyll content. To monitor PSII assembly under a minimized photooxidative condition, we first isolated thylakoid protein complexes from leaves adapted at 20 μmol m⁻² s⁻¹ (referred to low light condition) for 1 day prior to the extraction experiment, and BN/SDS-PAGE was performed as shown in Fig. 1. Silver staining of the gels revealed that the PSII-LHCII supercomplexes were detectable in var2-1 as well as in the wild type under low light, indicating that the assembly of the supercomplex proceeds normally in var2-1.
We subsequently isolated thylakoid proteins from leaves illuminated constantly at 150 µmol m\(^{-2}\)s\(^{-1}\) (referred to normal light condition). Under this normal light condition, the PSII-LHCII supercomplexes were hardly detectable in \(\text{var2-1}\). Whereas these complexes were still detectable in Col, indicating that increased light intensity affected assembly of PSII supercomplexes in \(\text{var2-1}\). Further immunoblot analysis of the PSII complex using D1 antibodies confirmed the decreased level of a PSII supercomplex and the PSII dimer in \(\text{var2-1}\) constant illumination under normal light. More importantly, the level of RC47 was higher in \(\text{var2-1}\). RC47 is a known intermediate that is formed during a disassembly step of PSII. Studies in cyanobacteria have demonstrated that detachment of CP43 from damaged PSII complexes is a prerequisite for FtsH to access and degrade photodamaged D1 (Komenda et al., 2006). Thus, the preferential accumulation of RC47 in \(\text{var2-1}\) after light irradiation strongly suggests that the selective D1 turnover was impaired at the step of the formation of RC47 in \(\text{var2}\). Normal accumulation of RC47 in \(\text{var2}\) adapted under low light also suggests a possibility that the residual level of FtsHs is sufficient or other proteases considerably participate in the PSII repair at low light intensity.

**High ROS Accumulated in \(\text{var2}\)**

As a consequence of defects in the PSII repair, \(\text{var2}\) leaves are very likely to receive photooxidative stress. Given the partial PSII complexes accumulated in \(\text{var2}\), we assumed that \(\text{var2}\) leaves are defective in transmitting excitation energy into water oxidation reaction even under normal light, and accumulate substantial ROS as a result of the oxidative stress. To test this possibility, we histochemically detected superoxide radical (\(\text{O}_2^-\)) and hydrogen peroxide (\(\text{H}_2\text{O}_2\)) by nitroblue tetrazolium (NBT) staining or by 3, 3′-deaminobenzidine (DAB) staining, respectively. NBT forms blue formazan in the presence of \(\text{O}_2^-\), and polymerization of DAB by peroxidase is visible as a brown precipitate in the presence of \(\text{H}_2\text{O}_2\).

Our NBT staining showed that \(\text{O}_2^-\) preferentially accumulated in \(\text{var2}\) (Fig. 2A). Detailed \textit{in situ} observation in Col and \(\text{var2}\) leaves revealed that Col had limited amounts of NBT stains, whereas \(\text{var2}\) leaves contained the blue precipitates that appeared to overlap green sectors (Fig. 2B). Observation of NBT stains under light microscopy demonstrated that the NBT stains in green sectors were detected within
organelles whose morphologies are typical of chloroplasts (Fig. 2C). Likewise, we performed DAB staining with different light intensities, and the results showed that H$_2$O$_2$ was detectable in green sectors of var2 leaves (Fig. 2D). Although DAB did not give us difference between Col and var2 leaves as clearly as NBT precipitates, we observed that generation of H$_2$O$_2$ appeared to be light-dependent: dark-grown leaves had little DAB stains, whereas a photoinhibitory light condition (800 μmol·m$^{-2}$·s$^{-1}$) deepened DAB stains both in Col and var2. Our results thus supported our assumption that the defective PSII repair capacity in var2 leads to high ROS production in chloroplasts.

To further characterize ROS accumulation in var2 quantitatively, we measured levels of three ROS from Col and var2 leaves as described in Materials and Methods. Because ROS were preferentially observed in chloroplasts, ROS amounts were normalized based on total chlorophyll content (Fig. 3). First, we assessed the levels of thiobarbituric acid-reactive substances (TBARS) including malondialdehyde as an index for lipid peroxidation. TBARS was 3.5-fold higher in var2 than in Col (Fig. 3A). Second, H$_2$O$_2$ and O$_2^-$ were quantitatively measured to confirm our histochemical experiments. To detect both ROS, we performed this measurement using not only 3 weeks-old seedlings grown on MS medium but also 10 weeks-old mature leaves (Fig. 3B, C). This was because our initial experiments showed that ROS levels varied in different developmental stages (data not shown). H$_2$O$_2$ levels increased dramatically in both Col and var2-1 mature leaves, but its levels were constantly higher in var2 than in Col (4.4-fold in young seedlings and 5.1-fold in mature leaves, Fig. 3B). In addition, O$_2^-$ levels were also shown to be constantly higher in var2 than in Col (10.4 fold in young seedlings and 5.5 fold in mature leaves, Fig. 3C). No significant difference in O$_2^-$ was detected between dark-treated var2 and Col seedlings (covered with aluminum foil for 2 d), which was consistent with our DAB stains and suggests that O$_2^-$ generation is light-dependent. We also calibrated each ROS level based on fresh weight and higher O$_2^-$ and H$_2$O$_2$ levels in var2 mature leaves were evident (Supplemental Fig. 2). Taken together, these results demonstrated that var2 leaves indeed suffer from photooxidative stress and generate substantial ROS in chloroplasts in a light dependent manner.

In Vivo Assessment of Impaired D1 Degradation in var2 Using a Variegation
Suppressor

Because photooxidative stress accessed by ROS production appeared to take place in var2 leaves under normal light, we attempted to design an experiment by which defective PSII repair cycle, as represented by D1 turnover, can be monitored in different light conditions. To perform this experiment, we encountered difficulty to utilize variegated var2 leaves, because D1 levels that can be detected by immunoblots were shown to fluctuate among leaf discs (Supplemental Fig. 3). Moreover, variegated sectors were very difficult to normalize protein contents (Kato et al., 2007). In vivo measurement of D1 turnover in var2 thus gave limited results as reported previously by Bailey et al. (2002). To minimize such an experimental problem, a non-variegated var2 mutant (var2 fug1) was used in this study. The FUG1 locus encodes a chloroplastic translation initiation factor 2 (cpIF2). A complete lack of cpIF2 in fug1-2 results in an embryo-lethal phenotype, whereas two leaky mutations in fug1-1 and fug1-3 are viable and recover leaf variegation when combined with var2 (Supplemental Fig. 1, 3). In this study, D1 turnover in the absence of FtsHs was assessed in var2 fug1, and we used fug1 as a control.

Prior to D1 measurement by immunoblots, leaf discs from Col and other mutant lines were infiltrated with lincomycin to inhibit chloroplast protein synthesis. In the presence of lincomycin, wild-type leaves showed a rapid decrease in Fv/Fm under high-light conditions 1200 µmol m⁻²s⁻¹ (~20% after 210 min, Fig. 4A). The rapid decrease of Fv/Fm in var2-1, fug1-3 and var2-1 fug1-3 was comparable to that in the wild type. We also measured Fv/Fm values of var2-1, fug1-3 and var2-1 fug1-3 under growth- and low-light conditions (100 and 20 µmol m⁻²s⁻¹ for 8 h, respectively, Fig. 4B, C). In the absence of lincomycin, a decrease in PSII activity was not detected in any of the genotypes, suggesting that PSII photodamage is within the capacity of the PSII repair cycle under these conditions. On the other hand, addition of lincomycin decreased Fv/Fm in all genotypes, and as expected, photoinhibitory effects appeared to be light-dependent. These results suggest that infiltration of lincomycin into leaf discs efficiently inhibits D1 synthesis and that PSII photodamage was similar in the wild type and the mutants.

In Vivo D1 Degradation Assay in var2 fug1
Following lincomycin infiltration, leaf discs were first incubated for 0, 60, 120, and 210 min under high light (1200 µmol m⁻² s⁻¹). To detect D1 levels during these periods, thylakoid membranes were isolated and subjected to immunoblot analysis (Fig. 5A). Rates of D1 degradation in *fug1-3* and *var2-1 fug1-3* were estimated based on the ratio of immunoreacted D1 to CBB-stained LHCII as shown in Fig. 5. Neither obvious decrease nor significant change of LHC levels were detected between wild-type and mutants in the conditions we employed (Supplemental Fig. 4). After 210 min incubation, D1 levels in *fug1-3* rapidly decreased to about 20% of the initial value. In contrast, D1 levels in *var2-1 fug1-3* remained at 40%. We also performed a D1 degradation assay in the wild type, which had a D1 degradation rate that was similar to *fug1-3* (Supplemental Fig. 5). These results are consistent with the previous study, which showed that the D1 degradation in *var2* was significantly slower than that in the wild type when plants are exposed to high light (Bailey et al., 2002).

Next, we examined D1 degradation in *fug1-3* and *var2-1 fug1-3* during non-photoinhibitory growth- and low-light irradiance (100 and 20 µmol m⁻² s⁻¹, respectively, Fig. 5B, C). Under growth light, D1 levels in *fug1-3* decreased to 40% of the initial amount, whereas *var2-1 fug1-3* remained at 70% of the initial D1 amount after 8-h of irradiation. Likewise, under low light, D1 levels in *fug1-3* decreased to 70% of the initial amount, whereas no or little D1 degradation was detected in *var2-1 fug1-3*. These results suggest that the rate of D1 degradation was significantly delayed and slowed in *var2-1 fug1-3* when compared to the control. We also performed a D1 degradation assay in the dark and found that D1 protein levels remained unchanged even after 480-min incubation (Supplemental Fig. 6). These data suggest that *var2-1 fug1-3* leaves are compromised in light-dependent D1 degradation under non-photoinhibitory light conditions.

To further characterize D1 turnover in vivo and exclude a possible secondary effect of lincomycin, we estimated D1 degradation by pulse labeling of chloroplast proteins (Fig. 6). De novo synthesized protein in leaf discs were labeled with ³⁵S-methionine for 60 min, and subsequently chased in unlabelled medium for 60, 120, and 180 min as previously reported (Nonardi et al., 2005). Our previous data showed that after 60-min labeling, D1 proteins detectable in *fug1-3* were comparable with those in Col (Miura et al., 2007). The bands corresponding to D1 (confirmed by immunoblot,
not shown) were shown to turnover very rapidly in fug1-3 during chase period, whereas we consistently observed that D1 turnover was impaired in var2-1 fug1-3. Collectively, our in vivo D1 degradation assays reinforced the important role of FtsHs in the PSII repair cycle.

**In Vivo D1 Degradation Assay in var1 fug1**

Although FtsH2 was shown to play a role in D1 degradation, involvement of other FtsH isomers such as FtsH5 is also possible. FtsH2 and FtsH5 are major FtsHs that were suggested to constitute a heterocomplex (Sakamoto et al. 2003; Yu et al., 2003; Zaltsman et al., 2005b). To test whether FtsH5 is indeed involved in D1 protein degradation, var1-1 fug1-1 was subjected to the aforementioned D1 degradation assay. (photosynthetic phenotypes of fug1-1 and fug1-3 were essentially identical). Immunoblot analysis of the D1 protein levels was performed at three different light conditions (Fig. 7). Similarly to var2, D1 levels in var1-1 fug1-1 remained high during high-light irradiation, whereas the control plant fug1-1 showed a rapid decrease of D1 (Fig. 7A). Relative amounts of D1 estimated by immunoblot signals showed that the rate of D1 degradation in var1-1 fug1-1 was slower than that in fug1-1 (Fig. 7). The defect in D1 protein degradation in var1-1 fug1-1 was also observed when plants were grown at growth- and low-light intensities (Fig. 7B, C). We conclude that FtsH5 as well as FtsH2 are functioning in the PSII repair cycles.

**PSII Activities of fug1-3 and var2-1 fug1-3 upon High-light Exposure**

To assess the photoinhibitory status of var2 along with fug1, we measured the maximum quantum yield of PSII (Fv/Fm) in leaf discs prepared from fully expanded rosette leaves (8 weeks old, grown in soil). The result shown in Fig. 4 indicated that Fv/Fm values were similar between the wild type, fug1-3 and var2-1 fug1-3, suggesting that the coexistence of fug1 and var2 not only recovers leaf variegation but also mitigates PSII photoinhibition. Because the effect of fug1 under long-term light irradiation complicates the evaluation of photoinhibitory status of PSII activity, we calibrated another photosynthetic parameter PSII electron transport rate (ETR) in this study. Light-dependent changes of ETRs were measured in leaves from the wild type, var2-1, fug1-3 and var2-1 fug1-3 (Fig. 8). No significant difference in ETRs was
observed between the wild type and the mutants when light intensity was below 100 µmol m$^{-2}$s$^{-1}$. A slower increase of ETRs in var2-1 and var2-1 fug1-3 was detectable at relatively low-light intensity (over 200 µmol m$^{-2}$s$^{-1}$), whereas ETRs in the wild type and fug1-3 elevated well until 400 µmol m$^{-2}$s$^{-1}$. At 1200 µmol m$^{-2}$s$^{-1}$, ETRs in var2-1 and var2-1 fug1-3 declined and reached only 70% of ETR in the wild type. We also found that fug1-3 itself does not affect ETR.

In addition to ETR, we also measured capability of recovery from photoinhibition in the wild type and the mutants. To analyze this, leaf discs from Col, var2-1, fug1-3, and var2-1 fug1-3 were illuminated until the initial Fv/Fm was reduced to approximately 50%, and subsequent recoveries of Fv/Fm were monitored under a low light condition (20 µmol m$^{-2}$s$^{-1}$). As shown in Supplemental Fig. 7, recovery of Fv/Fm was always faster in Col than in other mutant lines and reached above 85% of the initial value after 3 h. Similarly to Col, fug1-3 exhibited a fast recovery rate. Whereas, recoveries in var2-1 and var2-1 fug1-3 were apparently slower than in Col and reached only 70% after 3 h. Taken together, these results appeared to reflect the fact that the lack of FtsH2 has a significant effect on the PSII repair cycle.

**DISCUSSION**

An essential role of FtsHs in chloroplasts has been well documented previously by us and other groups (Chen et al., 2000; Takechi et al., 2000; Sakamoto et al., 2002; Sakamoto et al., 2003). Involvement of FtsHs in protecting photosystems has also been implicated in cyanobacteria (Mann et al., 2000; Silva et al., 2003). In particular, characterization of Arabidopsis var2 mutants demonstrated that the lack of FtsH2 leads to an impaired capacity in the PSII repair cycle and a defective D1 turnover (Bailey et al., 2002; Sakamoto et al., 2004). However, measurement of PSII activity was mostly conducted under photoinhibitory light conditions, where we cannot rule out secondary effects of excess light energy on damaging photosystems. Here we show that the impairment of the PSII repair cycle occurs under non-photoinhibitory light conditions. First, comparison of PSII supercomplex and other PSII complexes in var2 and Col leaves revealed that var2 results in accumulating less functional PSII supercomplex and
more RC47 intermediates than Col (Fig. 1). Second, we found that var2 leaves accumulate more ROS in chloroplasts than Col, suggesting that photooxidative stress is induced due to the impaired PSII cycle under growth condition (Fig. 2, 3). Finally, D1 degradation monitored in non-variegated var2 fug1 mutants was compromised under both growth and weak light conditions (Fig. 5). Collectively, our results demonstrated the critical role of FtsHs in the PSII repair cycle not only under photoinhibitory but also non-photoinhibitory light conditions, which was not precisely investigated in the previous studies (Bailey et al., 2002).

Our histochemical and quantitative analyses clearly indicated that chloroplasts in green sectors produced high levels of O$_2^-$, which has short lifetime and are converted rapidly to H$_2$O$_2$ by superoxide dismutase (SOD). Because O$_2^-$ cannot permeate across membranes, it is probably converted to H$_2$O$_2$ by chloroplastic SOD (for review, see Asada, 2006). H$_2$O$_2$ can then be exported to cytosol or to other organelles to ultimately convert it to water. As a consequence, high ROS in chloroplasts may cause damage in photosynthetic proteins. In contrast, ROS in var2 leaves may affect response to environmental stresses, since H$_2$O$_2$ not only causes cytotoxicity but also acts as a signaling molecule for various cellular activities (for review, see Apel and Hirt, 2004). Further studies may allow us to investigate a physiological role of chloroplast ROS. In photosynthetic electron transport, it is known that ROS can be mainly produced by Mehler reaction around PSI (Asada, 2006). However, considering that FtsH is involved in the PSII repair cycle, O$_2^-$ and H$_2$O$_2$ accumulated in var2 was likely generated around PSII. Other ROS such as singlet oxygen and hydroxy radicals may also be generated around PSII. To date, in vitro investigation of electron transport processes of PSII suggested ROS generation on the electron accepter and donor sides of PSII under the reducing and oxidizing conditions, respectively. However, these observations are yet to be confirmed in vivo (for review, see Pospíšil, 2009). Interestingly to note, high ROS generation in var2 is correlated with our finding that the less functional partial PSII complexes accumulate. It is thus possible that partial PSII complexes contribute, if not all, to ROS production. An effective PSII repair can be required not only to provide functional PSII but also to avoid cytotoxicity caused by these partial PSII complexes.

Proteases involved in the PSII repair cycle have been extensively studied in Synechocystis sp. PCC6803, in which FtsH (slr0228) was shown to play major roles in
degrading photodamaged D1 (Silva et al., 2003; Komenda et al., 2006). Phylogenetic analysis implicated that slr0228 is most closely related to Arabidopsis FtsH2 (Chen et al., 2000; Sakamoto et al., 2003). Participation of other cyanobacterial FtsH isomers in thylakoidal proteolysis is currently unknown. Loss of slr0228 was first suggested to affect PSI (Mann et al., 2000). However, later it was demonstrated that loss of slr0228 function results in high-light sensitive growth, which was suggested to be due to defects in the PSII repair cycle (Silva et al., 2003; Komenda et al., 2006). Similar to the results obtained with var2 in this study, a ΔFtsH (slr0228) strain attenuated D1 degradation and accumulated RC47 (Silva et al., 2003; Komenda et al., 2006). These observations further supported important roles of FtsH in light-dependent D1 degradation in photosynthetic organisms. In addition, deletion of 20 amino acids from the N-terminal end of D1 in Synechocystis was shown to significantly affect D1 degradation, suggesting that D1 turnover is processively carried out by FtsH from its N-terminal end (Komenda et al., 2007). Damaged D1 probably causes a conformational change of the PSII dimer, leading to partial disassembly of the PSII monomer, thus allowing access of FtsH to RC47 and degrade of D1 from N-terminal end.

Our D1 degradation assays showed that D1 turnover still occurred even in the absence of FtsH2 or FtsH5 (Fig.5, 7), suggesting the presence of a yet unidentified D1 degradation pathway in addition to the FtsH2/FtsH5 protease pathway in the repair cycle. One possibility is the contribution of other FtsH subunits, such as FtsH8 and FtsH1, because loss of FtsH2 or FtsH5 in var2 or var1 mutants is partially compensated by elevated levels of FtsH8 and FtsH1, respectively (Yu et al., 2004, 2005). Transcriptional analysis of several FtsH genes showed a remarkable increase of FtsH8 and FtsH1 mRNA levels during high-light exposure (Sinvany-Villalobo et al., 2004). An alternative possibility is the contribution of other proteases such as Deg, which encodes an ATP-independent Ser-type endopeptidase and which is peripherally attached to thylakoid membranes (Itzhaki et al., 1998). Deg1, Deg5 and Deg8, located in the thylakoid lumen, were suggested to act on the PSII repair under high light conditions (Sun et al., 2007; Kapri-Pardes et al., 2007). Deg was proposed to act as an endopeptidase to aid in processive degradation that is preceded by other proteases. The putative cooperation between FtsH and Deg in D1 degradation has been therefore feasible. Under our experimental conditions, however, we could not detect remarkable
accumulation of D1 degradation fragments, which were previously reported in wild-type but not in the deg mutants (data not shown). In contrast to the reports in Arabidopsis, physiological experiments using a triple deg mutant in Synechocystis sp. PCC 6803 showed that Deg is not essential for in vivo D1 degradation (Barker et al., 2006). Different roles of Deg in Arabidopsis and Synechocystis may indirectly suggest that Deg acts supplementary to FtsH-dependent D1 degradation pathway. Finally, we cannot rule out the possibility that unknown proteases contribute to D1 degradation under certain conditions.

Previous studies in var mutants have demonstrated that leaf variegation is not a consequence of the defect in the PSII repair cycle. This hypothesis is supported by the facts that i) the degree of variegation is independent of light intensities and is rather dependent on plant development (Zaltsman et al., 2005a), and ii) white leaf sectors in var2 are composed of living cells with undifferentiated plastids (Kato et al., 2007). In addition to these observations, our D1 degradation assays showed that PSII repair is attenuated in the non-variegated var2 fug1 and var1 fug1 mutants, further supporting the fact that variegation is independent of PSII repair. Thus, it is likely that FtsH plays an additive and distinct role in thylakoid development at an early stage of chloroplast differentiation, which is unrelated to PSII photodamage but is related to the variegation phenotype. This is supported by the fact that fug1 shows a phenotypic effect on chloroplast development in early developmental stages, but the mutant does not exhibit an altered phenotype at later developmental stages (Miura et al., 2007). Whether the protease activity of FtsH is required for this additive function remains unclear and requires further investigations.

It is interesting to note that photosynthetic organisms apparently possess multiplicated genes for proteases in thylakoid membranes, as exemplified by FtsH (nine in Arabidopsis and four in Synechocystis) and Deg (four in Arabidopsis and three in Synechocystis) (Sakamoto et al., 2003; Sokolenko et al., 2002). Although this raises the question of functional divergence between isomers, no experimental data on differential roles such as tissue-specificity have been reported so far. One exception is FtsH6, which was suggested to play a specific role in the degradation of LHCII during senescence, but the presence of an FtsH6 homocomplex has not yet been confirmed (Zelisko et al., 2005). Rather, our study demonstrates a redundant role of FtsH2 and FtsH5 in the PSII
repair cycle. Given the fact that two types of FtsH isomers, FtsH5 in Type A and FtsH2 in Type B, were shown to be required for a heteromeric complex formation, it is reasonable to assume that FtsH2 and FtsH5 equally contribute to PSII repair. This is supported by the fact that the degree of photoinhibition as assessed by a decline in Fv/Fm was similar in var1 and var2 (Sakamoto et al., 2004). Therefore, our data suggest that in Arabidopsis, the Type A/B FtsH heterocomplex plays a major role in degrading D1 and possibly other thylakoid proteins. Other isomers such as FtsH1 and FtsH8, the loss of which does not show any detectable phenotypes, may act supplementary to the major FtsH2/5 isomers. Thus, the presence of multipliclated FtsH isomers may be required to ensure proper quality control of thylakoid proteins that are highly vulnerable to photooxidative damage.

MATERIALS AND METHODS

Plant Materials, Growth Condition

Arabidopsis (Arabidopsis thaliana) Columbia ecotype was used as a wild type. The mutant lines used in this study, fug1-1, fug1-3, var1-1 fug1-1, var2-1 and var2-1 fug1-3, were described previously. All mutants are in the Columbia background (Miura et al., 2007). Plants were germinated and grown on 0.7% (w/v) agar plates containing Murashige and Skoog medium supplemented with Gamborg’s vitamins (Sigma-Aldrich, St. Louis, MO), 2 mM MES, pH5.8, and 1.5% (w/v) sucrose. Plants were maintained under 12-h light (~60 µmol m⁻² s⁻¹) at a constant temperature of 22°C. When plants were four weeks old, they were transferred onto soil and maintained under 12-h light (100 µmol m⁻² s⁻¹) at a constant temperature of 22°C.

Detection and Measurements of ROS

In situ detection of O₂⁻ was performed by treating leaves with nitroblue tetrazolium (NBT) as described by Kawai-Yamada et al. Leaves were detached from seedlings, vacuum-infiltrated with 10 mM NaN₃ in 10 mM potassium phosphate buffer (pH 7.8) for 1 min, using a syringe and incubated in 0.1% (w/v) NBT in 10 mM potassium phosphate buffer (pH 7.8) for 120 min at room temperature under light (100
μmol·m$^{-2}$·s$^{-1}$) or dark (covered with aluminum foil). Stained leaves were cleared by boiling in acetic acid: glycerol: ethanol (1: 1: 3, v/v/v) solution before photographs were taken. To quantify formazan generation, washed leaves were boiled in DMSO until formazan precipitates were eluted completely (Yaeno et al., 2004). The amount of formazan was determined spectrophotometrically by measuring absorbance at 560 nm. *In situ* detection of H$_2$O$_2$ was performed by treating leaves with 5mM DAB at pH3.8, as previously described (Orozco-Cardenas and Ryan, 1999). Stained leaves were completely cleared by boiling in ethanol. H$_2$O$_2$ levels were determined according to Velikova *et al*. Leaves (> 200 mg) were homogenized in liquid nitrogen with 500 μl 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 15,000g for 15 min at 4°C and 200 μl of the supernatant was added to 20 μl 100mM potassium phosphate buffer (pH 7.0) and 160 μl 5 M potassium iodide. Absorbance of the supernatant was read at 390 nm. The content of H$_2$O$_2$ was determined using a standard curve. Malondialdehyde (MDA) was measured according to the TBARS-method (Yaronskaya *et al*., 2003). MDA equivalents were calculated as described (Yaronskaya *et al*., 2003).

**Lincomycin Treatment, Protein Extraction and SDS-PAGE**

Three milligrams of leaf discs were harvested from approximately eight-week-old plants using a 5-mm diameter biopsy punch (Kai Medical, Japan). The leaf discs were placed in a glass vial containing 5 mM lincomycin in 0.2% (v/v) Tween 20 and the vial was sealed with a rubber cap. A syringe needle was pierced through the rubber cap and lincomycin was infiltrated into the leaf discs by repeated pressuring and de-pressuring the syringe for 60 s. Leaf discs treated with lincomycin were immediately placed on wet filter papers and irradiated with three different light intensities (20, 100 and 1200 μmol m$^{-2}$s$^{-1}$). To isolate thylakoid proteins, three leaf discs were collected at each time point. SDS-PAGE sample preparation of thylakoid membrane proteins and SDS-PAGE was carried out as described previously (Kato *et al*., 2007). Sample loadings were normalized based on fresh weight.

**In Vivo Labeling of Chloroplast Proteins**

The in vivo labeling was carried out as described previously (Bonardi *et al*., 2005) with slight modifications. To label the chloroplast proteins, leaf discs of
approximately eight-week-old plants were vacuum-infiltrated by syringe and incubated with 0.1 mCi/ml $^{35}$S-methionine in 0.2% Tween 20 at a light intensity of 100 µmol m$^{-2}$s$^{-1}$ for 60 min. After labeling, leaf discs treated with 0.1 mCi/ml $^{35}$S-methionine were immediately washed twice with 10 mM unlabelled L-methionine, 0.2 % Tween20 and further incubated in the presence of unlabelled L-methionine at a light intensity of 600 µmol m$^{-2}$s$^{-1}$ for 60, 120, 180 and 240 min after labeling. Two leaf discs were collected to isolate thylakoid proteins at each time point. SDS-PAGE sample preparation and SDS-PAGE was carried out as described previously (Kato et al., 2007). To detect labeled proteins, gels were stained by CBB, dried, and exposed to imaging plate (Fuji Photo Film, Japan). Radiolabels were detected by the BAS 1000 image analyzer (Fuji Photo Film, Japan).

Immunoblot Analysis

For immunoblot analysis, proteins were electroblotted onto PVDF membrane (ATTO, Japan) after SDS-PAGE. Prior to immunoreaction, the membrane was blocked with 1% (w/v) Bovine serum albumin (BSA) in 50 mM sodium phosphate buffer, pH 7.5, containing 155 mM NaCl and 0.05% (v/v) Tween 20 (PBST buffer) for 1h. After two washes with PBST buffer, the membrane was incubated with anti-D1 (dilution 1:5000). After two washes with PBST buffer, the membrane was incubated with secondary antibodies. Immunodetection was performed as previously described using the ECL system (GE Healthcare, Waukesha, WI). Relative amounts of signals from immunoblots were quantified by Image-J (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/) and normalized to the amount of LHCII detected by Coomassie blue (CBB).

Preparation of Thylakoid Membranes and Blue Native/SDS PAGE

For blue-native PAGE (BN-PAGE), samples were prepared according to the protocol described previously with the following modifications (Suorsa et al., 2004). The material plants were low-light (20 µmol m$^{-2}$s$^{-1}$, 10-h light/14-h dark) adapted for 1 day prior to the start of light treatment. Thylakoids membranes were isolated from low-light adapted leaves, and from leaves illuminated for 2h at 150 µmol m$^{-2}$s$^{-1}$. To isolate chloroplasts, 2 g of wild-type and var2-1 leaves from approximately
eight-week-old plants were harvested. Chloroplasts were isolated according to the protocol described previously (Miura et al., 2007). The homogenate containing intact and broken chloroplasts was diluted ten times with homogenation buffer and centrifuged at 60 x g for 2 min. The pellet was washed with 50 mM HEPES, pH 7.5, containing 5 mM mannitol and centrifuged at 2500 x g for 4 min. The pellet was then resuspended in 50 mM HEPES, pH 7.5, containing 100 mM mannitol and 10 mM MgCl₂. After centrifugation at 2500 x g for 4 min, the pellet was resuspended in the same buffer, and total chlorophyll concentration was measured. Thylakoid membrane suspensions containing 100 µg chlorophyll were centrifuged at 2500 x g for 4 min, and the pellet was washed with wash buffer (50 mM bis-Tris, 330 mM mannitol, pH 7.5). After centrifugation, thylakoid membranes were resuspended in buffer (25 mM bis-Tris, 20 % (w/v) Glycerol, pH 7.5) to a final concentration of 0.5 mg/ml chlorophyll. To solubilize thylakoid membranes, n-dodecyl ß-D-maltoside was added to a final concentration of 0.4 % (w/v). After centrifugation at 14,000 x g for 20 min, the supernatant was supplemented with one-tenth volume of loading buffer (50 mM bis-Tris, 500 mM ε -amino-n-capronic acid, 5 % (w/v) CBB-G-250, pH 7.5), and loaded onto a 4-12 % gradient native gel. Electrophoresis was performed at 4°C overnight at 50 V. After electrophoresis, each lane was excised from the gel and incubated in equilibration buffer (50 mM Tris-Cl, 6 M urea, 30 % (w/v) glycerol, 2 % (w/v) SDS, 0.05 % (w/v) BPB, 10 mM DTT, pH 8.8) for 30 min at room temperature. Then, proteins were separated by SDS-PAGE in a 10% polyacrylamide gel. After electrophoresis, the resolved gels were used for immunoblot analysis or stained using the PlusOne Silver Staining Kit (GE Healthcare, Waukesha, WI), according to the manufacturer’s instructions. Chlorophyll content was determined using 80 % (v/v) acetone-extracts of thylakoid membranes. The chlorophyll concentration was determined as previously described (Porra et al., 1989).

**Fluorescence Measurements**

Photosynthetic electron transport rate (ETR) was calculated as $\Phi_{II} * \text{PAR} * 0.5 * 0.84$, where $\Phi_{II}$ represents the overall photochemical quantum yield ($\Delta F_v/F_m'$), PAR is actinic irradiance in µmol m⁻² s⁻¹, 0.5 is the assumed proportion of photons absorbed by pigments associated with PS II, and the 0.84 standard factor is the
incident quanta absorbed by the leaf (for review see Baker, 2008). Chlorophyll fluorescence for ETR using fully expanded leaves was determined using the chlorophyll fluorometer PAM-2000 (Walz Effertrich, Germany). Changes in the maximum quantum yield of PSII (Fv/Fm) were measured by FluorCam700MF (Photon Systems Instruments, Czech Republic). Prior to the measurement, leaf discs were kept in the dark for 10 min to fully oxidize the plastoquinone pool. The results shown are the average of three biological repeat experiments.

**Recovery Treatment**

The recovery treatment was carried out as described previously (Ishihara et al. 2007). Saturating light (1200 µmol m⁻² s⁻¹) was illuminated on leaf discs until Fv/Fm reached about 50% of the initial Fv/Fm value in Col, fug1-3, var2-1 and var2-1 fug1-3. The discs were transferred under low light condition (20 µmol m⁻² s⁻¹), and the change of Fv/Fm values was measured at 30, 60, 90, 120, 180 and 360 min. Prior to the measurement, leaf discs were kept in the dark for 10 min. The chlorophyll fluorescence was measured by FluorCam700MF (Photon Systems Instruments, Czech Republic).

**Supplemental Data**

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

Supplemental Figure S1. Plant materials used in this study.

Supplemental Figure S2. Quantification of ROS in var2 compared to wild-type (Col).

Supplemental Figure S3. Accumulation of D1 protein in leaf discs prepared from var2 and var2 fug1 leaves.

Supplemental Figure S4. LHCII levels in the wild type and mutants after light irradiation.

Supplemental Figure S5. Immunoblot analysis of D1 protein in wild-type Col.

Supplemental Figure S6. D1 degradation in darkness.

Supplemental Figure S7. Recovery curves of the photochemical efficiency after photoinhibition.

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FIGURE LEGENDS

Figure 1. BN/SDS-PAGE analysis of thylakoid protein complexes from mature leaves of wild-type Col and var2-l. A, Thylakoid membranes (0.5 mg/ml chlorophyll) were purified from intact and broken chloroplasts of Col and var2-l leaves (approximately eight-week-old plants). Thylakoid protein complexes were solubilized with 0.4% n-dodecyl β-D-maltoside and separated on 4-12% BN-PAGE gels (10 µg chlorophyll per lane). Thylakoid membrane proteins were further separated by 14% SDS-PAGE and stained with silver. Thylakoids membranes were isolated from low-light adapted leaves (pretreated for 1 day at 20 µmol m^{-2} s^{-1}, left two panels), and from leaves illuminated constantly at 150 µmol m^{-2} s^{-1} followed by low-light adaptation (right two panels). B, Two-dimensional gels were also electroblotted onto PVDF membranes and were subjected to immunodetection of D1 protein. The positions corresponding to PSII supercomplexes, PSII dimmer, PSII monomer, and RC47 are indicated at the bottom. To confirm D1, CP47, CP43 in each complex, spots in the gel were subjected to MALDI-TOF mass spectrometric analysis (data not shown).

Figure 2. ROS accumulation in var2 green sectors. (A) In situ detection of O₂⁻ by staining with NBT (blue, bottom panels) in four-week-old wild-type (Col) and var2 was shown. (B) In situ detection of O₂⁻ by staining with NBT (blue, bottom panels) in four-week-old wild-type (Col) and var2 leaves was shown. (C) Higher magnification of var2 leaves of (A) was shown. (D) In situ detection of H₂O₂ by DAB staining (dark brown, bottom panels) in three-week-old wild-type (Col) and var2 grown on MS plates under different light conditions (growth light; 100 µmol m^{-2} s^{-1} and high light; 800 µmol m^{-2} s^{-1}) was shown. Plants that were dark-adapted for two days are also shown (dark).

Figure 3. Quantification of ROS in var2 compared to wild-type (Col). (A) The level of lipid peroxidation in 35-day-old plants without roots (young seedling) was measured by TBARS assay. (B) H₂O₂ accumulation in 30-day-old plants without roots (young seedling) and nine-week-old leaves (mature plant) was quantified using KI methods. (C) The level of blue formazan as an O₂⁻ in four-week-old leaves (young seedling),
nine-week-old leaves (mature plant), or four-week-old leaves after dark adaptation for two days (dark). NBT formazan precipitates were completely eluted and the amount was determined at 560 nm. Values of each graph were normalized to chlorophyll content. (mean ± SD, n = 3). Asterisks indicate significant difference from wild-type using the Student's t-test (** P < 0.01).

Figure 4. Maximal photochemical efficiency of PSII in the presence or absence of lincomycin. Fv/Fm as maximal photochemical efficiency was measured in leaf discs from Col (circle), var2-1 (diamond), fug1-3 (triangle) and var2-1 fug1-3 (square). Leaf discs were incubated for 0, 60, 120, and 210 min under high light condition (1200 µmol of photons m⁻²s⁻¹), or for 0, 120, 240, 360, 480 min under growth and low light conditions (100, 20 µmol of photons m⁻²s⁻¹) in the presence (closed symbols) or absence (open symbols) of lincomycin. At each time point, leaf discs were dark adapted for 10 min prior to measurement. Values were means ± SD (n = 3).

Figure 5. Immunoblot analysis of D1 protein in fug1-3 and var2-1 fug1-3 mutants under three different light conditions. Leaf discs harvested from fully expanded mature leaves of fug1-3 and var2-1 fug1-3 (approximately eight-week-old plants grown under normal conditions) were infiltrated with 5 mM lincomycin. Leaf discs were incubated for 0, 60, 120, and 210 min under high light condition (1200 µmol m⁻²s⁻¹), or for 0, 120, 240, 360, 480 min under growth and low light conditions (100 and 20 µmol m⁻²s⁻¹, respectively). Thylakoid membrane proteins were separated by 14% SDS-PAGE and stained with CBB. A representative immunoblot using anti-D1 antibodies and the band corresponding to CBB-stained LHCII are shown. Signals of immunoblots from three biological repeats were quantified using the ImageJ program and normalized to the amount of CBB-stained LHCII (SD with bars, n=3). Closed and open circles indicate fug1-3 and var2-1 fug1-3, respectively. To compare D1 levels, ratios at 0 min were adjusted to 1.

Figure 6. Pulse-chase analysis of thylakoid membrane proteins in fug1-3 and var2-1 fug1-3 mutants. Leaf discs were labeled for 60 min at 100 µmol m⁻²s⁻¹, and subsequently chased 60, 120, 180 and 240 min at a light intensity of 600 µmol m⁻²s⁻¹.
Thylakoid membrane proteins were separated by 14% SDS-PAGE, and labeled proteins were detected. A representative labeled D1 protein signals is indicated by arrowhead. The bands corresponding to LHCII on the CBB-stained gel were also shown at the bottom. Similar results were obtained in four additional independent experiments.

**Figure 7.** Immunoblot analysis of D1 protein in *fug1-1* and *var1-1 fug1-1* mutants under three different light conditions. Leaf discs harvested from fully expanded, mature leaves of *fug1-1* and *var1-1 fug1-1* (approximately eight-week-old plants grown under normal conditions) were infiltrated with 5 mM lincomycin. The leaf discs were incubated for 0, 60, 120, and 210 min under high light condition (1200 µmol m⁻² s⁻¹), or for 0, 120, 240, 360, 480 min under growth and low light conditions (100 and 20 µmol m⁻² s⁻¹, respectively). Thylakoid membrane proteins were separated by 14% SDS-PAGE and stained with CBB. A representative immunoblot using anti-D1 antibodies and the band corresponding to CBB-stained LHCII are depicted. Signals of immunoblots from three biological repeats were quantified as indicated in Fig. 5.

**Figure 8.** PSII capacities during high-light exposure. ETR was measured in leaf discs from the wild-type Col (opened circle), *var2-1* (closed circle), *fug1-3* (triangle) and *var2-1 fug1-3* (square) in response to increasing light intensities. PAR: photosynthetically active radiation. Means ± SD (n = 3) are shown.
Figure 4

A 1200 µmol of photons m\(^{-2}\)s\(^{-1}\)

B 100 µmol of photons m\(^{-2}\)s\(^{-1}\)

C 20 µmol of photons m\(^{-2}\)s\(^{-1}\)
Figure 8

The graph shows the electrical transfer rate (ETR) in different conditions of PAR (μmol photons m$^{-2}$s$^{-1}$) and plant genotypes. The y-axis represents ETR (μmol photons m$^{-2}$s$^{-1}$), and the x-axis represents PAR (μmol photons m$^{-2}$s$^{-1}$).

- **Col**: Circles
- **var2-1**: Black circles
- **fug1-3**: Triangles
- **var2-1 fug1-3**: Squares

Each genotype has a distinct line indicating their ETR response to varying PAR levels.