Thylakoid-Bound FtsH Proteins Facilitate Proper Biosynthesis of Photosystem I

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Thylakoid membrane-bound FtsH proteases have a well-characterized role in degradation of the photosystem II (PSII) reaction center protein D1 upon repair of photodamaged PSII. Here, we show that the Arabidopsis (Arabidopsis thaliana) var1 and var2 mutants, devoid of the FtsH5 and FtsH2 proteins, respectively, are capable of normal D1 protein turnover under moderate growth light intensity. Instead, they both demonstrate a significant scarcity of PSI complexes. It is further shown that the reduced level of PSI does not result from accelerated photodamage of the PSI centers in var1 or var2 under moderate growth light intensity. On the contrary, radiolabeling experiments revealed impaired synthesis of the PsaA/B reaction center proteins of PSI, which was accompanied by the accumulation of PSI-specific assembly factors. psaA/B transcript accumulation and translation initiation, however, occurred in var1 and var2 mutants as in wild-type Arabidopsis, suggesting problems in later stages of PsaA/B protein expression in the var mutants. Presumably, the thylakoid membrane-bound FtsH5 and FtsH2 have dual functions in the maintenance of photosynthetic complexes. In addition to their function as a protease in the degradation of the photodamaged D1 protein, they also are required, either directly or indirectly, for early assembly of the PSI complexes.

Photosynthetic light reactions are driven by the thylakoid membrane-embedded multisubunit pigment-protein complexes PSII and PSI (associated with their light-harvesting antennas, LHCI and LHCII, respectively) and the cytochrome (Cyt) b\textsubscript{6}f complex in cooperation with the mobile electron carriers plastoquinone and plastocyanin. PSII performs the highly oxidizing chemistry of water splitting, thereby inducing photooxidative damage to the D1 subunit of PSI (Tyystjärvi and Aro, 1996). In order to maintain the photosynthetic apparatus as functional, photodamaged PSI complexes are subjected to a multistep repair cycle (Aro et al., 1993; Baena-González and Aro, 2002; Nickelsen and Rengstl, 2013). PSI repair takes place primarily in the stroma-exposed thylakoid membranes and involves (1) partial disassembly of the PSII holocomplex, (2) degradation of the damaged D1 protein, (3) cotranslational insertion of the new D1 copy to PSII, (4) C-terminal processing of the newly synthesized D1 protein, and (5) reassembly and activation of the functional PSII complex. These processes are assisted by an array of auxiliary proteins such as kinases, phosphatases, proteases, and chaperones, which are located in the chloroplast stroma, thylakoid membrane, and thylakoid lumen (Mulo et al., 2008; Chi et al., 2012; Suorsa et al., 2014).

PSI generally has been regarded as a robust photosystem, and in plants, it is protected in vivo by a highly efficient antioxidant network, which scavenges reactive oxygen species that otherwise would have detrimental damaging effects on the complex (Asada, 1999). Likewise, PROTON GRADIENT REGULATION5 (PGR5) protein-dependent processes and photoinhibition of PSI protect plant PSI against photodamage (Munekage et al., 2002; Shikanai, 2014; Tikkanen et al., 2014). PSI photoinhibition has been reported to take place under specific conditions, such as a combination of chilling temperature and low light intensity, or under fluctuating light conditions, particularly in the absence of the PGR5 protein (Terashima et al., 1994; Sonoike et al., 1995; Tjus et al., 1998; Zhang and Scheller, 2004; Suorsa et al., 2012). The efficient repair mechanism has been considered not to exist for PSI; thus, its photoinhibition is likely to lead to a degradation of the entire PSI complex (Kudoh and Sonoike, 2002; Zhang and...
assembly steps remain elusive, several auxiliary proteins followed by de novo biogenesis of the complex. The PSI (Scheller, 2004; Jensen et al., 2007; Sonoike, 2011), followed by de novo biogenesis of the complex. The PSI assembly steps remain elusive, several auxiliary proteins have been identified that transiently assist the biogenesis and assembly of PSI (for review, see Schöttler et al., 2011), and novel PSI assembly factors are discovered constantly (Liu et al., 2012; Fristedt et al., 2014; Roose et al., 2014).

FtsH proteins constitute a family of ATP-dependent membrane-bound metalloproteases found in eubacteria, animals, and plants and are known to have a crucial role in the proteolysis of membrane-embedded proteins (Ito and Akiyama, 2005; Wagner et al., 2012). The Arabidopsis (Arabidopsis thaliana) genome encodes a total of 12 FtsH proteins. Of these, FtsH1, FtsH2, FtsH5, and FtsH8 have been shown to reside in the thylakoid membrane and to have a well-characterized role in the degradation of the damaged D1 protein (Lindahl et al., 2000; Bailey et al., 2002). FtsH-mediated degradation of the D1 protein occurs in cooperation with the lumenal DegP-type Ser proteases Deg1, Deg5, and Deg8 (Kapri-Pardes et al., 2007; Sun et al., 2007; Kato et al., 2012b). The homologous FtsH1 and FtsH5 proteins are referred to as type A and FtsH2 and FtsH8 as type B subunits of the FtsH complex. In its active form, the thylakoid membrane FtsH forms a ring-shaped heterohexameric complex, which has been reported to be composed of type A subunits and type B subunits (Yu et al., 2004, 2005; Zaltsman et al., 2005b; Moldavski et al., 2012). FtsH2 and FtsH5 are the major thylakoid membrane FtsH subunits, while FtsH1 and FtsH8 are less abundant (Sinvany-Villalobo et al., 2004). The amount of FtsH5 in wild-type Arabidopsis plants is approximately 60% of the amount of the dominant FtsH2 protein (Sinvany-Villalobo et al., 2004). Mutants deficient in FtsH5 (var1) or FtsH2 (var2) show a variegated phenotype, indicating the involvement of these proteins in the biogenesis (Zaltsman et al., 2005a; Kato et al., 2007) and maintenance (Kato et al., 2012a) of the thylakoid membrane. Moreover, var1 and var2 mutant plants show attenuated D1 degradation under high light intensities (Lindahl et al., 2000; Sakamoto et al., 2002; Kato et al., 2009). Nevertheless, attenuated degradation of the damaged D1 protein was shown not to contribute to the leaf variegation phenotype, at least in var2 (Kato et al., 2009).

Here, we took a more holistic approach, using the Arabidopsis mutant lines var1 and var2 to elucidate the consequences of the abnormally low amount of the FtsH protease in the thylakoid membrane on the components and composition of the entire photosynthetic electron transfer chain. It is demonstrated that, under moderate growth light (ML) intensities, var1 and var2 mutant plants do not suffer from inefficient PSII turnover. Instead, the loss of either FtsH2 or FtsH5 leads to significantly impaired biosynthesis of PSI.

RESULTS

Low Amount of PSI Subunits in var1 and var2 Thylakoids under ML Intensities

The role of the FtsH2 and FtsH5 proteins was addressed under ML intensities (120 μmol photons m⁻² s⁻¹ in an 8-h light regime at 23°C), instead of subjecting the var1 and var2 plants to high light intensities that induce extensive damage of the PSII centers followed by impaired D1 degradation (Lindahl et al., 2000; Sakamoto et al., 2002; Kato et al., 2009). The var2 plants, lacking the most abundant FtsH2 subunit of the FtsH holocomplex, showed drastic growth defects as compared with wild-type and var1 plants. The gels were loaded on a chlorophyll (Chl) basis to overcome normalization problems caused by the variegation phenotype.

The total amount of FtsH proteins was reduced in var1, and particularly in var2, as compared with the wild type, evidenced by immunoblotting with the antibody against recombinant FtsH1 that recognizes all four thylakoid-bound FtsHs (Fig. 1A). This is in line with a previous report (Sinvany-Villalobo et al., 2004). The amount of the PSII core protein D1 did not differ between wild-type, var1, and var2 plants. Somewhat higher amounts of Cyt b and ATP synthase, represented by Cyt f and Atpβ, respectively, were observed in var1 as compared with the wild type. Of these, Atpβ was present in higher amounts also in var2, when expressed on a Chl basis, as compared with the wild type. Instead, the amount of the PSI core protein PsbA was lower in both var1 and var2 mutant plants as compared with the wild type. Similar to PsbA, the LHClI docking proteins PsaL and Psah (Lunde et al., 2000) showed decreased accumulation in var1 and var2 plants as compared with the wild type (Fig. 1, B and C). The amounts of PsAD, PsaK, and the LHCl subunit Lhca2 were at rather similar levels to the wild type (Fig. 1). Between plants of different ages, only negligible differences were observed in the accumulation of representative subunits of the thylakoid membrane protein complexes (Fig. 1A). Bearing this in mind, the subsequent experiments were performed with 6-week-old wild type and var1 plants and 8-week-old var2 plants to gain more leaf material of var2.

The uneven accumulation of PSI subunits observed in var1 and var2 as compared with the wild type (Fig. 1B) was studied further using two-dimensional (2D) blue native (BN)-PAGE in combination with SYPRO Ruby protein gel stain, which allows linear quantitation of distinct subunits within the protein complex. To that end, thylakoid membrane was solubilized by n-dodecyl β-D-maltoside, individual subunits of the protein complexes...
were separated by 2D BN-PAGE, and protein spots were stained with SYPRO Ruby protein gel stain. Interestingly, the abnormal stoichiometry of the integral components of PSI-LHCI was evident in both var1 and var2 thylakoids (Supplemental Fig. S2). Indeed, the densitometry of the SYPRO Ruby-stained PSI-LHCI complex revealed that the ratio of PsaH to PsaD (well-separated subunits were selected for quantification in order to avoid overlapping of the protein spots) in the wild-type PSI-LHCI complex was $33\% \pm 3\%$, whereas both var1 and var2 demonstrated only half ($16\% \pm 5\%–6\%$) of the wild-type ratio. These results strongly imply that the subunit abundance of the PSI-LHCI complex differs between wild-type and var1 and var2 plants.

**Accumulation of PSI (Sub)complexes in var1 and var2 Thylakoids**

Next, we addressed the amount of low-molecular-mass PSI complexes, which might represent either degradation products or biogenesis intermediates, in the var1 and var2 plants. The thylakoid membranes of wild-type, var1, and var2 plants were solubilized using digitonin, and the samples were submitted to separation of the protein complexes by large-pore blue native (lpBN)-PAGE. Digitonin was used as a detergent, as it preferably solubilizes PSI (Anderson and Boardman, 1966). To avoid accumulation of the PSI-LHCI-LHCII complex, dark-adapted plants were used for the experiment (Pesaresi et al., 2009). Immunoblotting of the native gels with a PsbA-specific antibody revealed low accumulation of both PSI-LHCI and the PSI core subcomplex in the mutant thylakoids as compared with the wild type (Fig. 2). Similar coregulation of the PSI-LHCI complex and the PSI core subcomplex was reported earlier for an Arabidopsis mutant that lacks the PSI assembly factor PSA2 (Fristedt et al., 2014).

**Photosynthetic Electron Transfer in var1 and var2 Plants under ML Intensities**

Next, the functional status of the photosynthetic apparatus was investigated in detail in the var1 and var2 mutants grown under ML conditions. The maximum quantum yield of PSII in dark-adapted ($F_{v}/F_{m}$) and in light-adapted ($F'_{v}/F'_{m}$) leaves did not show any statistically significant difference between var1, var2, and the wild type (Table I). More detailed analyses of PSII acceptor side properties, measured as flash-induced increase and subsequent relaxation of Chl fluorescence yield from wild-type, var1, and var2 plants, likewise gave very similar results (Fig. 3A). Collectively, these results strongly suggest the presence of unaffected PSII in var1 and var2 upon growth of plants under ML intensities. This differs drastically from high-light conditions that induce severe PSII photoinhibition in the var1 and var2 plants, evidenced as a decrease in the $F_{v}/F_{m}$ parameter (Bailey et al., 2002; Sakamoto et al., 2003). However, the effective quantum yield of PSII ($\phi_{v}$) measured under moderate actinic light intensities was significantly lower in var1 and var2 mutant plants.
(0.30 ± 0.03 and 0.37 ± 0.04, respectively) compared with the wild type (0.50 ± 0.08). The decrease in Φ\textsubscript{II}, representing the fraction of absorbed energy utilized by photochemistry, could originate from an increase in the yield of regulated protective nonphotochemical energy dissipation (Φ\textsubscript{NQ}) and/or in the yield of nonregulated nonphotochemical energy loss (Φ\textsubscript{NO}), reflecting the fraction of energy that is dissipated passively in the form of heat and fluorescence, mainly due to closed PSI reaction centers (Hendrickson et al., 2004; Kramer et al., 2004). There was no statistically significant difference in Φ\textsubscript{NO} values between wild-type and mutant plants (Table I). However, a decrease in the Φ\textsubscript{II} was accompanied by a considerable increase of the Φ\textsubscript{NQ} in var1 and var2 (0.44 ± 0.03 and 0.39 ± 0.04, respectively) as compared with the wild type (0.22 ± 0.1; Table I). The induction kinetics of significantly higher NPQ level in the var1 and var2 lines compared with the wild type also is depicted in Figure 3B. The excitation pressure of PSI, which reflects the redox state of the plastocyanine pool, was slightly higher in var1 as compared with the wild type, whereas no statistically significant difference was recorded between var2 and the wild type.

Contrary to the similar \( F_c/F_m \) values between var1, var2, and wild type, lack of either FtsH2 or FtsH5 had a drastic effect on \( P_M \), the PSI parameter representing the maximum amount of photooxidizable \( P_700 \). The \( P_M \) values of the light-acclimated leaves of both var1 and var2 (0.6 ± 0.07 and 0.48 ± 0.17, respectively) were significantly lower as compared with the wild type (0.89 ± 0.14), indicating a low amount of photooxidizable \( P_700 \) in both mutant lines (Table I). Moreover, the performance of PSI under actinic light, measured as effective quantum yield of PSI (Φ\textsubscript{I}), was significantly lower in var1 and var2 (0.47 ± 0.06 and 0.62 ± 0.05, respectively) as compared with the wild type (0.77 ± 0.04). The drop in the Φ\textsubscript{I} value can be attributed to the donor and/or the acceptor side limitation of PSI. In both mutant lines, a significant donor side limitation of PSI was recorded, while no limitation on the acceptor side of PSI was evident between mutant lines and the wild type (Table I).

Moreover, rereduction of \( P_700 \) in darkness, after termination of far red light, was faster in both var1 and var2 mutants than in the wild type (Fig. 3C). This result implied a high capacity of the var1 and var2 plants for PSI cyclic electron transfer (CET) followed by protonation of the lumen, which is a prerequisite for triggering NPQ and a photosynthetic control of the Cyt b\textsubscript{6f} complex.

### Biosynthesis of PSI Is Impaired in var1 and var2 Plants under ML Intensities

Scarcity of PSI in the var1 and var2 lines (Fig. 1; Table I) could originate either from accelerated damage to PSI or serious defects in the biosynthesis of PSI. Thus, we next focused on both the biosynthesis and degradation of PSI complexes in the thylakoid membrane of wild-type, var1, and var2 plants. Based on RNA blots with specific probes, it was demonstrated that the mRNAs (psaA-psaB-rps14) encoding the PsaA/PsbA proteins accumulated in higher amounts in var1 and in similar amounts in var2 as in the wild-type plants (Fig. 4A) when loading was done based on the total RNA. Next, the efficiency of translation initiation of the psaA/B mRNAs was assessed by a polysome-loading experiment. Following the migration of the mRNA-ribosome complexes in Suc gradients upon ultracentrifugation revealed only a slight shift between mutant and wild-type plants in the polysome-loading efficiency of the psaA/B mRNAs (Fig. 4B). In line with this, polysome profiles of psbA and psbD mRNAs were similar in wild-type and var mutant plants (Supplemental Fig. S3). It should be noted that normalization in the polysome-loading experiment was performed on the basis of fresh weight of samples, and the presence of white sectors affected the amount of psaA/B mRNA in variegated plants as compared with the RNA-blot experiment. Collectively, the mRNA and polysome-loading

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<th>Table 1. Functional characteristics of the thylakoid membrane of wild-type, var1, and var2 plants</th>
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<td>Plants were grown under ML intensities (120 ( \mu \text{mol photons m}^{-2} \text{s}^{-1} )). Values shown are means ± SD (n = 7–10). Statistically significant differences comparing mutant plants with the corresponding wild type are marked with asterisks. See text for details. Col-0, Columbia-0 wild type.</td>
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<td><strong>Photosynthetic Parameter</strong></td>
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<td>Yield of nonregulated nonphotochemical energy loss, Φ\textsubscript{NO}</td>
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<td>Excitation pressure of PSI, 1 − qP</td>
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results indicate that the failure to accumulate the PsaB protein in var1 and var2 is likely not linked to the psaA/B transcription efficiency, mRNA stability, or translation initiation of the PsaB protein.

The remaining options for reduced PsaB accumulation in var1 and var2 as compared with wild-type plants included differences in the translation efficiency and stability of the PsaB protein. In order to distinguish these two possibilities, we applied in vivo pulse-chase labeling experiments using [35S]Met. The newly synthesized proteins were identified by 2D BN-PAGE separation of the wild-type pulse samples solubilized with n-dodecyl β-D-maltoside. PsaA/B proteins were found to migrate slightly above the ATP synthase subunits in the BN-PAGE autoradiogram films (Supplemental Fig. S4; Aro et al., 2005). Synthesis and degradation of the PsaA/B proteins were followed by separation of the [35S]Met-labeled proteins by one-dimensional SDS-PAGE (Fig. 4C).

Figure 3. Flash-induced increase and subsequent relaxation of the Chl fluorescence yield (FF-relaxation), nonphotochemical quenching (NPQ), and rereduction of P700$^+$ in darkness in the wild type (black line), var1 (gray line), and var2 (pale gray line). A, FF-relaxation in darkness. Curves, representing an average of 32 independent measurements, are normalized to the same amplitude for direct comparison of the kinetics. B, Induction and relaxation of NPQ monitored during the dark-to-light transition (120 μmol photons m$^{-2}$ s$^{-1}$). Bars at bottom indicate the light period (white bar) and the dark period (black bar). Curves represent averages of six independent measurements. C, Rereduction of P700$^+$ in darkness. P700 was oxidized by illumination of the leaf with far-red light (FR) for 30 s, and after the termination of far-red light illumination, P700$^+$ rereduction was monitored in darkness. Curves, representing an average of six independent measurements, are normalized to the same amplitude for direct comparison of the kinetics. Col-0, Columbia-0 wild type.

Figure 4. Biogenesis and degradation of PsaA/B in var1, var2, and wild-type plants. A, RNA-blot analysis of mRNAs encoding PSI core proteins. B, Association of mRNAs encoding PSI core proteins with polysomes. Polysomes were fractionated using ultracentrifugation, mRNAs were separated by agarose gel electrophoresis, and specific probes were used to identify mRNA. C, In vivo pulse labeling. Leaves were labeled under ML for 5 and 20 min (left) or for 2 h followed by a 2-h chase in the presence of unlabeled Met (right). [35S]Met-labeled proteins were separated by SDS-PAGE. The synthesis and degradation of the PsaA/B proteins were followed using autoradiogram films. A representative example from three independent biological replications is shown. Col-0, Columbia-0 wild type; M.B., methylene blue.
For optimal separation of the α- and β-subunits of ATP synthase from the PsA/B proteins, the running time on the SDS-PAGE gels was extended from that used for BN-PAGE and is presented in Supplemental Figure S4.

As demonstrated in Figure 4C, var1 and var2 plants accumulated clearly less radioactivity in the PSI reaction center proteins PsA/B than the wild-type plants, evidenced by denaturing SDS-PAGE of thylakoid membrane proteins after \( ^{35}S \)Met labeling of the leaves for 5 and 20 min. Such short labeling times allowed discrimination between possible defects in the biosynthesis (faster process) and degradation (slower process) of the PSI core proteins. In order to determine the relative synthesis rate of the PsA/B proteins (calculated as the amount of radioactivity incorporated into the PsA/B proteins relative to that incorporated into the ATP6 and D1 proteins), a longer duration of \( ^{35}S \)Met labeling of the leaves (2 h) was applied in order to accumulate more newly synthesized radiolabeled PsA/B proteins. These samples also were further chased for 2 h in the presence of nonradioactive Met to estimate the degradation rates of the PsA/B proteins. The calculated relative synthesis rates of the PsA/B proteins were only 37% ± 8% in var1 and 31% ± 11% in var2 of that recorded for the wild-type plants (n = 3). Instead, no drastic differences between wild-type and mutant plants were observed in the degradation of newly synthesized PsA/B proteins during the 2-h chase period (Fig. 4C). However, an exact evaluation of the degradation rate of PSI core proteins in mutants was not possible due to a low incorporation of radioactivity into the newly synthesized PsA/B proteins (i.e. a low synthesis rate) in the var1 and var2 plants.

To fully exclude the possibility that the scarcity of PSI in var1 and var2 originated from abnormally fast degradation of the complex under ML conditions, we applied lincomycin, a chloroplast translation inhibitor, to compare the degradation of the D1 protein and the PsA/B protein between var1, var2, and wild-type plants. In line with the pulse-chase labeling experiments, no statistically significant difference in the amount of PsA/B (and D1) was recorded in var1, var2, and wild-type leaves for up to 8 h of exposure to ML in the presence of lincomycin (Fig. 5, A and B). On the contrary, under high-intensity light (800 μmol photons m\(^{-2}\) s\(^{-1}\)), the lincomycin-treated var1 and var2 plants, as expected (Lindahl et al., 2000; Bailey et al., 2002), could not properly degrade the D1 protein, as occurred in the wild type (Fig. 5C). Although under high light intensity and in the presence of lincomycin, the PsA/B protein degraded slightly faster in the mutant plants as compared with wild-type plants, the results in Figure 5 do not support a superior degradation of PsA/B in var mutants under ML conditions (Fig. 1). We thus conclude that the scarcity of PSI in var1 and var2 did not originate from the instability of PsA/B but rather arose from the low translation efficiency of the PSI core proteins.

Figure 5. Degradation of D1 and PsA/B proteins in lincomycin-treated var1, var2, and wild-type leaves. Lincomycin (lin) at 1 ms was incorporated into detached leaves trough petioles overnight in darkness. Prior to thylakoid isolation, leaves floating on water were shifted from darkness to ML (120 μmol photons m\(^{-2}\) s\(^{-1}\); A and B) or high light (HL; 800 μmol photons m\(^{-2}\) s\(^{-1}\); C). A and C, Immunoblot analysis of the degradation of D1 and PsA/B proteins. B, Relative amounts of the D1 and PsA/B proteins in the wild type, var1, and var2 after the light treatment of leaves (as indicated) and expressed as a proportion of the respective dark controls. Error bars indicate SD. Gels were loaded on an equal Chl basis. Col-0, Columbia-0 wild type.

PSI Assembly Factors in Wild-Type, var1, and var2 Plants under ML Intensities

Defects in the biosynthesis of thylakoid protein complexes have been shown to influence the accumulation of auxiliary proteins assisting the assembly of the same complex (Cai et al., 2010). To address this, we first analyzed an accumulation of two well-known PSI assembly factors, Y3IP1 and Ycf4, in the thylakoid membrane of var1 and var2. Y3IP1 (Albus et al., 2010) accumulated in var1 in high amounts but was less abundant in var2 as compared with the wild type (Fig. 6). Instead, Ycf4 (Krech et al., 2012) was present in high amounts in both var1 and var2. Unlike the PSI assembly factors, no distinct differences occurred in the accumulation of the PSI auxiliary proteins LPA1, LPA2, and TLP18.3 (Peng et al., 2006; Ma et al., 2007; Sirpiö et al., 2007) between the wild type, var1, and var2. Instead, the amount of CYP38 (Fu et al., 2007) was higher in both var mutant plants as compared with the wild type. It should be noted, however, that even if CYP38 is referred to as an auxiliary protein of PSI, cyp38 plants

Järvi et al.
also contain a low amount of PSI (Sirpiö et al., 2008), similar to the var1 and var2 mutants.

### DISCUSSION

Type A and type B FtsH isomers have been identified in cyanobacteria, green and red algae, mosses, and flowering plants (Kato et al., 2012a). The var1 mutant is deficient of the type A FtsH isomer FtsH5 and var2 of the type B FtsH isomer FtsH2. Importantly, the type A isomer FtsH1 and the type B isomer FtsH8 can substitute for FtsH5 and FtsH2, respectively; therefore, var1 and var2 are, in practice, FtsH knockdown lines (Fig. 1A; Zaltsman et al., 2005b). The FtsH proteins play a crucial role in the proteolysis of membrane-embedded proteins, including the photodamaged PSII reaction center protein D1 (Lindahl et al., 2000; Bailey et al., 2002; Sakamoto et al., 2003). Likewise, thylakoid-bound FtsH proteases have been shown to participate in the quality control of the Cyt b6f complex (Ostersetzer and Adam, 1997; Malnoë et al., 2014) and possibly in the regulation of the PSI CET (Terashima et al., 2012; Szyszka-Mroz et al., 2014) and possibly in the regulation of the PSI CET (Ostersetzer and Adam, 1997; Malnoë et al., 2014) and possibly in the regulation of the PSI CET (Terashima et al., 2012; Szyszka-Mroz et al., 2014). Here, we demonstrate that FtsH2 and FtsH5 also play an important role in the biosynthesis of the PSI complex.

Under ML conditions, lower amounts of the FtsH proteases in the var1 and var2 mutant plants do not impair the function or turnover of the PSI complex (Figs. 1A and 3A; Table I). This corroborates earlier reports (Sakamoto et al., 2003; Zhang et al., 2010) and indicates that the photodamaged PSI is efficiently restored by a rapid PSI repair cycle in the wild type as well as in var1 and var2 plants under ML intensities. The low values of ΦII in var1 and var2 are not related to PSI photoinhibition but rather stem from a highly elevated ΦNPO (Table I; Fig. 3B).

Contrary to the PSI complex, whose function is fully secured in the absence of FtsH2 or FtsH5 under ML intensity, the PSI complex turned out to be affected significantly under the same conditions. Interestingly, the amount of PSI, evidenced by both biochemical (Fig. 1A) and biophysical (Pm value; Table I) measurements, was directly proportional to the amount of the thylakoid-bound FtsH proteins in var1 and var2. The low amounts of PSI observed in the mutant lines were somewhat surprising, considering earlier reports showing a similar amount of the Psaf subunit in var2 as compared with that in the wild type (Yu et al., 2008, 2011). Nonetheless, our data suggest that, in var1 and var2, several low-molecular-mass nucleus-encoded PSI subunits (PsaD, PsaK, and Lhca2) are present in high amounts within the PSI-LHCl complex (Fig. 1; Supplemental Fig. S2). Such nonstoichiometric amounts of different PSI subunits also have been observed in various PSI mutants. The tobacco (Nicotiana tabacum) Y31P1-1 suppression line accumulates more Psaf as compared with the PSI core (Albus et al., 2010), and a Chlamydomonas reinhardtii F15 mutant deficient in TAB1 (deficient in the translation of the psaB mRNA) is capable of accumulating Psaf and PsaD to some extent, despite the total absence of the PSI core (Boudreau et al., 1997). Similarly, high accumulation of LHCl as compared with the PSI core is a well-known phenomenon within the PSI auxiliary protein mutants (Amann et al., 2004; Lezhneva and Meurer, 2004; Lezhneva et al., 2004). The reason behind the nonstoichiometric accumulation of low-molecular-mass subunits of PSI as compared with the PSI core in the PSI auxiliary protein mutants is currently not known, and more efforts are needed to unravel the underlying mechanisms behind the phenomenon.

The scarcity of PSI in var1 and var2 under ML conditions (Figs. 1 and 2; Table I) could, in theory, originate either from serious damage to PSI or defects in the biosynthesis of PSI. In general, PSI can suffer from irreversible photoinhibition under conditions where the PSI acceptor side is overreduced, leading to the formation of reactive oxygen species, which, in turn, damage the Fe-S centers of PSI (Inoue et al., 1986; Sonoike et al., 1995). Similarly, high accumulation of LHCl as compared with the PSI core is a well-known phenomenon within the PSI auxiliary protein mutants (Amann et al., 2004; Lezhneva and Meurer, 2004; Lezhneva et al., 2004). The reason behind the nonstoichiometric accumulation of low-molecular-mass subunits of PSI as compared with the PSI core in the PSI auxiliary protein mutants is currently not known, and more efforts are needed to unravel the underlying mechanisms behind the phenomenon.
condition, where the leaves are illuminated by continuous white light, but rather reflects a maximum CET capacity of the leaves during the induction of photosynthesis. In line with these results, the PSI core does not show accelerated photodamage and degradation in var1 or var2 plants under ML conditions (Figs. 4C and 5).

Our results provide evidence that the deficiency of the FtsH2 and FtsH5 proteases impairs the translation efficiency of the PsA/B proteins (Fig. 4). Several reports have provided information indicating that the role of FtsH during the biogenesis of PSI might be direct. Indeed, FtsH2 has been observed to be attached to the thylakoid-associated polyosomes, which are responsible for the translation of chloroplast-encoded membrane proteins like PsA/B (Sirpiö et al., 2007). Furthermore, var2 suppressor screens have shown that the decreased translation rate caused by the secondary mutation in the var2 background leads to proper chloroplast development (Liu et al., 2013; Putarjunan et al., 2013), which indicates that the functional role of FtsH2 is linked to the chloroplast translation machinery. Moreover, the chloroplast-encoded PSI assembly factor Ycf4 accumulated in high amounts in var1 and var2 plants (Fig. 6), similar to a PSI mutant characterized by reduced translation efficiency of the psaA mRNA (Krech et al., 2012). On the other hand, modified electron transfer (Table I; Fig. 3) and/or the accumulation of reactive oxygen species (Kato et al., 2009) in var1 and var2 plants also might hamper the translation of PsA/B mRNA. Furthermore, a low amount of Y3IP1 has been shown to be connected with a deficiency in PSI (Albus et al., 2010), being in line with a lower amount of both Y3IP1 and PSI in var2 than in var1 (Figs. 1 and 6). Whether the thylakoid-bound FtsHs have a direct role during the translation elongation of the PSI core protein still remains to be verified.

If the role of FtsH in the biogenesis of PSI is direct, it would be interesting to know whether the thylakoid-bound FtsH functions as a structural scaffold or as a protease during the synthesis of the PSI core. A previous report has shown that the protease activity of the type B isoform of FtsH is unnecessary for chloroplast biogenesis (Zhang et al., 2010), which indicates either a redundancy of protease sites or the function of FtsH as a chaperone. Translation of the PsA/B proteins is down-regulated in var mutant plants (Fig. 4C), which suggests a role for FtsH as a structural scaffold. Also, the accumulation of the PSI assembly factors in var1 and var2 as a compensatory mechanism (Fig. 6) provides indirect evidence supporting its role as a chaperone. However, we cannot exclude the possibility that thylakoid-bound FtsHs play a role in the degradation of low-molecular-mass PSI subunits, like PsAD or PsAK (Fig. 1). To that end, the role of FtsH as a protease ensuring quality control during the biogenesis of PSI also is possible. In Escherichia coli, FtsH has long been known to be responsible for both the proteolysis of unassembled DrrB (for bacterial doxorubicin resistance protein B) and the refolding of misassembled DrrAB (Akiyama et al., 1994; Li et al., 2013). In addition, Arabidopsis Deg1 protease was shown earlier to be involved both in the degradation of damaged D1 protein and in the assembly of the PSII complex (Sun et al., 2010).

Further support for thylakoid-bound FtsH proteins as important auxiliary proteins for the proper biosynthesis of PSI comes from cyanobacteria research. Disruption of slr0228, encoding one of the four putative homologs of FtsH in Synechocystis sp. PCC 6803, was found to cause a major reduction in the abundance of PSI without affecting the cellular content of PSII or phycobilisomes (Mann et al., 2000). The slr0228 gene encodes an FtsH isomer that belongs to type B FtsHs together with Arabidopsis FtsH2 (Kato et al., 2012a). Moreover, in the C. reinhardtii ftsH1-1 mutant, the amount of PsaA was shown to be drastically down-regulated when cells were grown mixotrophically under 50 or 150 μmol photons m⁻² s⁻¹, while the amount of PsaD and PsaL remained unaltered as compared with wild-type cells (Malnoë, 2011). The similar function of FtsH in Synechocystis sp. PCC 6803, C. reinhardtii, and Arabidopsis provides evidence that the function of FtsH in the biosynthesis of PSI is evolutionarily conserved in oxygenic photosynthetic organisms.

Finally, the difference in the accumulation of FtsH in var2 and var1 is likely to explain the specific modifications of the photosynthetic electron transfer and the varying accumulation of PSI auxiliary proteins in the two var mutants (Table I; Fig. 6). Moreover, it is plausible that not only the quantity of FtsH matters but the FtsH isomers type A and type B possibly play partially different roles in the thylakoid membrane, also independently of the heterohexameric FtsH protease complex. Indeed, it was reported previously that specifically var1 mutant plants show a distinct temperature-sensitive phenotype (Sakamoto et al., 2002). To that end, the future goal is to reveal in detail the multitude of functions carried out by the thylakoid-bound FtsH proteins during the entire life cycle of the chloroplast.

CONCLUSION

We show here that the FtsH proteins are of utmost importance for the proper biosynthesis of PSI under normal growth conditions, while in high-light conditions, the function of FtsH proteins in the degradation of damaged D1 reaction center proteins of PSII becomes dominant. In line with these two most important roles of FtsH, the high-light treatment has been shown to lead to a migration of FtsH from stroma lamellae, where PSI is mainly located, to grana margins, where D1 degradation is likely to take place (Putthyaveetil et al., 2014). So far, the origin of the variegation phenotype of the FtsH2- and FtsH5-deficient plants has remained unknown. To that end, our results here raise the question of whether the variegation phenotype of the var2 and var1 mutants might partially
result from problems in the translation of the PSI core proteins. It is clear that defective PSI biosynthesis per se does not lead to a variegation phenotype, but it might be one of the factors that determine the threshold of FtsH activities necessary for proper chloroplast development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Six- or 8-week-old Arabidopsis (Arabidopsis thaliana), ecotype Columbia-0, wild-type, var1-1 (Nottingham Arabidopsis Stock Centre seed line N271), and var2-2 (Nottingham Arabidopsis Stock Centre seed line N272) plants were used for most of the experiments. Plants were grown under a photon flux density of 120 μmol photons m⁻² s⁻¹ in an 8-h light regime at 23°C. For specific experiments, plants were shifted to high-intensity light (800 μmol photons m⁻² s⁻¹) up to 3 h. Osmotic H2O-BT 400 W/D Metal Halide lamps were used as the light source. Oldest and youngest rosette leaves were excluded from the experiments. In experiments with the inhibition of translation, 1 μM lincomycin was incorporated into leaves through petioles overnight.

Isolation of the Thylakoid Membrane

Leaves were ground in ice-cold grinding buffer (50 mM HEPES-KOH pH 7.5, 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 5 mM ascorbate, 0.05% [w/v] bovine serum albumin, and 10 mM NaF), and suspension was filtered through two layers of Miracloth and centrifuged at 5,000 x g for 4 min. The supernatant was suspended in shock buffer (50 mM HEPES-KOH, pH 7.5, 5 mM sorbitol, 5 mM MgCl₂, and 10 mM NaF) and centrifuged at 5,000 x g for 4 min. The supernatant was suspended into storage buffer (50 mM HEPES-KOH, pH 7.5, 100 mM sorbitol, 10 mM MgCl₂, and 10 mM NaF) and centrifuged at 5,000 x g for 4 min. Finally, the pellet was resuspended into a small aliquot of storage buffer. The Chl content was determined according to Porra et al. (1989).

In Vivo Pulse Labeling of Thylakoid Proteins

Radioactive Met was incorporated into detached leaves through petioles (20 μCi) [³⁵S]Met and 0.4% [³⁵S]cysteine). The experiment was started by the addition of a single-turnover burst of measuring light reaching the photodetector at 120 μmol photons m⁻² s⁻¹ for 3 h. The samples were then harvested for the autoradiographic analysis, and the separation of the labeled proteins was performed by SDS-PAGE analysis. The numerical data were subjected to statistical analysis by Student’s t test with statistical significance at P < 0.05.
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