LPA19, a Psb27 Homolog in Arabidopsis thaliana, Facilitates D1 Protein Precursor Processing during PSII Biogenesis

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The biogenensis and assembly of photosystem II (PSII) are mainly regulated by the nuclear-encoded factors. To further identify the novel components involved in PSII biogenesis, we isolated and characterized a high chlorophyll fluorescence low psii accumulation lpa19 (lpa19) mutant, which is defective in PSII biogenesis. LPA19 encodes a Psb27 homolog (At1g05385). Interestingly, another Psb27 homolog (At1g03600) in Arabidopsis was revealed to be required for the efficient repair of photodamaged PSII. These results suggest that the Psb27 homologs play distinct functions in PSII biogenesis and repair in Arabidopsis. Chloroplast protein labeling assays showed that the C-terminal processing of D1 in the lpa19 mutant was impaired. Protein overlay assays provided evidence that LPA19 interacts with D1, and coimmunoprecipitation analysis demonstrated that LPA19 interacts with mature D1 (mD1) and precursor D1 (pD1). Moreover, LPA19 protein was shown to specifically interact with the soluble C terminus present in the precursor and mature D1 through yeast two-hybrid analyses. Thus, these studies suggest that LPA19 is involved in facilitating the D1 precursor protein processing in Arabidopsis.

Photosystem II (PSII),2 which catalyzes light-driven water oxidation and plastoquinone reduction, is a large pigment-protein complex found in the thylakoid membranes of cyanobacteria and chloroplasts. In higher plants, PSII contains more than 20 subunits including both integral and peripheral proteins (1, 2). The PSII reaction center proteins D1 and D2 bind most of the redox components essential for primary charge separation. Light energy transfer to the PSII reaction center is mediated by the intrinsic chlorophyll a-binding proteins, CP47 and CP43. The oxygen evolving complex, located on the luminal side of PSII, consists of a manganese cluster and several extrinsic proteins (3–6).

Structural studies have shed light on the function of PSII. However, the molecular mechanism concerning the biogenesis and assembly of PSII remains far from understood because of its structural complexity. The initial step in the biogenesis includes the formation of the PSII reaction center complexes (7–10). Subsequent assembly steps involve the association of the intrinsic antenna proteins CP47/CP43, integration of the small subunits, and assembly of the water-splitting system (11, 12). Recently, in several model organisms, a number of PSII assembly factors have been identified, such as PratA, Slr2013, Slr0286, Hcf136, Lpa1, and Lpa2 (13–19). A unique property of PSII is that the damage directed to it occurs at all light intensities (20). Among the PSII proteins, the PSI1 reaction center D1 protein is the main target of PSII damage and the damaged D1 protein must be replaced with a newly synthesized copy (21). The assembly of D1 into PSII includes several well-regulated processes, including the synthesis, targeting and membrane insertion of a newly synthesized D1 precursor (pD1) with a C-terminal extension, cleavage of the C-terminal extension by the C-terminal peptidase (CtpA) to form mature D1 (mD1) (22), assembly of the catalytic manganese cluster, and the binding of extrinsic proteins on the luminal side of the complex.

The Psb27 protein is widely present in cyanobacteria as well as higher plants and is localized to the thylakoid lumen (23, 24). Cyanobacterial Psb27 is tightly associated with the thylakoid membrane via its N-terminal lipoprotein motif, which is absent in higher plants (25). Several biochemical studies have shown that Psb27 co-purified with less active PSII, but it is not a component of functional PSII (26–29). A significantly higher amount of Psb27 was found to be associated with PSII in the cyanobacterial mutant lacking CtpA, in which the D1 precursor was not processed and subsequent association of the extrinsic proteins was blocked (27). Moreover, three-dimensional solution structure studies revealed that the core of the Psb27 protein is a right-handed four-helix bundle with an up-down-up-down topology, which is implicated in the transient binding to PSII during the biogenesis and repair of thylakoids (30, 31). These studies suggest that Psb27 functions in the early stage of PSII assembly. Recent studies have revealed the function of Psb27 in facilitating manganese cluster assembly in PSII and preventing the premature binding of the other PSII extrinsic subunits during the repair cycle (29–32). Our previous study has shown that a Psb27 homolog (At1g03600) is required for the efficient repair of photodamaged PSII in Arabidopsis (33). However, whether it is involved in PSII biogenesis is still unknown. Moreover, although the association of Psb27 with

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3 The abbreviations used are: PSII, photosystem II; pD1, precursor D1; mD1, mature D1; CtpA, the C-terminal peptidase; LHClII, light harvesting complex II; WT, wild type.

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PSII was observed, the nature of such an interaction remains unclear.

Here we report on the characterization of an Arabidopsis lpa19 mutant with high chlorophyll fluorescence. LPA19 encodes another Psb27 homolog (At1g05385) in Arabidopsis (33, 34). We present evidence that LPA19 is involved in facilitating the C-terminal processing of pD1. In addition, we show that LPA19 interacts with the soluble C-terminal regions of mD1 and pD1 in the lumen of thylakoids.

EXPERIMENTAL PROCEDURES

Mutant Isolation and Plant Growth Conditions—The lpa19-1 mutant was isolated from a collection of pSKI015 T-DNA mutagenized Arabidopsis thaliana (ecotype Columbia) lines from the ABRC (35) using a chlorophyll fluorescence imaging system (CF imager; Technologica) (19). The lpa19-2 mutant (T-DNA insertion line, SALK_093605) was obtained from the ABRC, and the homozygous mutant was confirmed by PCR using primers LP (5′-TGGTTTCTCTGTAGCCGT-CAT-3′) and RP (5′-AAGCGTTCTTCTATTTGCTTCA-3′). The T-DNA insertion was verified by PCR and sequenced using primers LB (5′-TCACACAGGATTCTCGGCTGT-3′) and RP. Wild-type and homozygous mutant plants were grown in soil under short-day conditions (10-h-light/14-h-dark) with a photon flux density of 120 μmol m⁻² s⁻¹ in a growth chamber at 22 °C.

Chlorophyll Fluorescence Analysis—Chlorophyll fluorescence was measured with a PAM-2000 portable chlorophyll fluorometer (Walz) attached to leaves, which were dark-adapted using a leaf-clip holder for 15 min before measurements were taken (model 2030-B; Walz). The Fo, Fm, Fv, and the Fv/Fm ratio were measured and calculated as previously described (36).

Map-based Cloning and Complementation—The lpa19-1 mutation was mapped with a series of SSLP and In/Del markers based on the polymorphisms between two Arabidopsis ecotypes, Columbia (Col) and Landsberg erecta (Ler) (37). The mutant plants (Col) were crossed with wild-type Ler to produce F1 plants. The heterozygotes were planted and allowed to self-fertilize to generate a segregating population. Homozygous F2 mutant plants (lpa19-1/lpa19-1 and wild-type lpa19-1/lpa19-1 mutant was isolated from a collection of pSKI015 T-DNA mutagenized Arabidopsis thaliana (ecotype Columbia) lines from the ABRC, and the homozygous mutant was confirmed by PCR using primers LP (5′-TGGTTTCTCTGTAGCCGT-CAT-3′) and RP (5′-AAGCGTTCTTCTATTTGCTTCA-3′). The T-DNA insertion was verified by PCR and sequenced using primers LB (5′-TCACACAGGATTCTCGGCTGT-3′) and RP. Wild-type and homozygous mutant plants were grown in soil under short-day conditions (10-h-light/14-h-dark) with a photon flux density of 120 μmol m⁻² s⁻¹ in a growth chamber at 22 °C.

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Thylakoid Membrane and Total Protein Preparations—Thylakoid membranes were prepared as previously described (10). Briefly, the leaves were homogenized on ice in isolation buffer (400 mM sucrose, 50 mM HEPES-KOH, pH 7.8, 10 mM NaCl, and 2 mM MgCl₂) and filtered through three layers of filter paper. The resulting filtrates were centrifuged at 5,000 × g for 10 min at 4 °C, and the thylakoid pellets were washed once with the isolation buffer. The chlorophyll contents were determined as previously described (39). Total protein samples were prepared as previously described (40).

SDS-PAGE and Western Blot Analysis—SDS-PAGE was performed mainly as previously described (41). For Western blot assays, the proteins were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat milk, incubated with specific primary antibodies and subsequently with the corresponding secondary antibodies. The signals were detected using enhanced chemiluminescence methods.

In Vivo Labeling of Chloroplast Proteins and Coimmunoprecipitation—In vivo chloroplast protein labeling was performed essentially as previously described (42). For pulse labeling, the primary leaves from 12-d-old seedlings were incubated in 1 μCi/μl [35S]Met (specific activity > 1000 Ci/mmol; PerkinElmer Biotech) in the presence of 20 μg/ml cycloheximide for 20 min at 25 °C after preincubation with cycloheximide for 30 min. After pulse labeling, the samples were chased in the presence of 10 mM cold Met for 10, 30, and 60 min. After labeling, thylakoid membranes were isolated as previously described (18), and the proteins were resolved by SDS-PAGE. After electrophoresis, the gels were stained with Coomassie Brilliant Blue and dried, and the labeled proteins were visualized by autoradiogram. For coimmunoprecipitation analysis, the thylakoid membranes isolated from 12-d-old wild-type plants that had been labeled for 10 min were solubilized with 0.5% DM. After centrifugation at 14,000 × g for 15 min, the thylakoid membranes were solubilized, incubated with anti-LPA19 for 1 h at 4 °C and then incubated with protein A-Sepharose (Sigma-Aldrich) for another 1 h. Coimmunoprecipitates were isolated by centrifugation, washed at least three times, and separated by SDS-PAGE. The resolved proteins were visualized by autoradiogram.

Antiserum Production—The nucleotide sequences encoding the LPA19 total mature protein (amino acids 65–199) were amplified by PCR using the primers 5′-ATCCTAGGGGCGCT-CAGGCCGTCAGAGG-3′ and 5′-GGCGCTTGAGCAAAACCTCTTCAGC-3′. The resulting DNA fragment was cleaved with Ncol and Xhol and ligated into the vector pET28a in-frame with the C-terminal His affinity tag. The BL21 cells were harvested after the addition of 0.8 mM isopropyl β-D-1-thio-
For the protease protection assay, the proteins were studied essentially as previously described (43). Resin (Promega), and the purified antigen was used for anti-fusion protein was purified using a HisLink Protein Purification Resin (Promega), and the purified antigen was used for antisera production in rabbit.

**Immunolocalization Studies**—The localization of the LPA19 proteins were studied essentially as previously described (43). For the protease protection assay, the Arabidopsis thylakoid membrane samples (0.1 mg chlorophyll/ml) were each sonicated three times for 15 s on ice. After sonication, trypsin was then added to a final concentration of 50 mg/ml and incubated at 25 °C for 15 min. After the treatments, the thylakoid membranes were solubilized in SDS sample buffer, and the proteins were separated by SDS-PAGE and immunodetected with specific antibodies. For the salt washing assay, the thylakoid membranes were suspended in a final concentration of 0.1 mg chlorophyll/ml in 10 mM HEPES-KOH, pH 8.0, 10 mM MgCl₂, 330 mM sorbitol, supplemented with 1 M NaCl, 1 M CaCl₂, or 6 M urea, respectively. These suspensions were sonicated three times for 15 s each while kept on ice. After the treatments, the membranes were pelleted at 100,000 × g for 2 h at 4 °C, solubilized and then subjected to SDS-PAGE and immunoblot analysis. The membrane fractions without supplements were used as a control.

**Overlay Assays**—BN/SDS-PAGE two-dimensional gels were performed as previously described (18, 44, 45). After electrophoresis, the resolved proteins were transferred to nitrocellulose membranes, blocked with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat milk for 6 h and then reacted with the recombinant His-LPA19 protein at a concentration of 0.05 mg/ml in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20, and 1% nonfat milk. Membranes were washed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.05% Tween 20, probed with His tag antibody, and the signals were visualized by the enhanced chemiluminescence method.

**Yeast Two-hybrid Assays**—The yeast two-hybrid assay was performed using the Matchmaker Gold Yeast Two-Hybrid System from Clontech according to the manual. The mature full-length LPA19 gene was obtained by PCR amplification and cloned into the bait vector pGBKT7 (LPA19-BD). The soluble pD1 C terminus (amino acids 296–353), mD1 C terminus (amino acids 296–344), D1 AB loop (amino acids 56–110), and CD loop (amino acids 165–194) were obtained by PCR amplification and cloned into the prey vectors pGADT7 AD (renamed AD-pD1C, AD-mD1C, AD-D1 AB loop, and AD-D1 CD loop, respectively). Interaction was determined by growth of diploid yeast colonies on S.D.-His-Leu-Trp-Ade plates supplemented with 40 μg/ml X-α-Gal and 70 ng/ml Aureobasidin A.

**RESULTS**

**Molecular Cloning of the lpa19-1 Gene**—The lpa19-1 mutant was isolated by screening for mutants from the Scheible and Somerville T-DNA Arabidopsis lines with a high chlorophyll fluorescence phenotype (36, 37). The leaves of the mutant appeared pale-green under normal growth conditions. The chlorophyll contents in lpa19-1 decreased to about 35% of the wild-type level and chlorophyll a/b decreased to about 2.30 compared with 3.31 in the wild-type (Table 1). The mutant plants showed reduced growth under medium light (Fig. 1A). When wild-type and lpa19-1 mutant plants, which were initially grown at 120 μmol m⁻² s⁻¹, were transferred to low light and high light growth conditions, the growth of the lpa19-1 mutant was greatly inhibited under high light. The mutants appeared greener under low light (Fig. 1, B and C). The ratio of variable fluorescence to maximum fluorescence (Fv/Fm), which reflects the maximum photochemical efficiency of PSII (39), was significantly lower in the mutant (0.48 ± 0.02) than in the wild-type plants (0.82 ± 0.02) (Fig. 1D). The decreased Fv/Fm ratio indicated that PSII function was impaired in the mutant.

### Table 1

|                      | Wild type | lpa19-1 |
|----------------------|-----------|---------|
| Chlorophyll (μg/cm²) | 18.4 ± 1.3| 6.5 ± 0.7|
| Chlorophyll a/b      | 3.31 ± 0.02| 2.30 ± 0.04|

**FIGURE 1.** Phenoypes and chlorophyll fluorescence analysis. A, 5-week-old plants grown in the growth chamber under medium light (ML) conditions of 120 μmol m⁻² s⁻¹, B, 2-week-old plants grown in a growth chamber under a photon flux density of 120 μmol m⁻² s⁻¹ and then transferred to low light (LL) conditions of 10 μmol m⁻² s⁻¹ for another 3 weeks. C, 2-week-old plants grown in a growth chamber under a photon flux density of 120 μmol m⁻² s⁻¹ and then transferred to a greenhouse with high light (HL) conditions of maximum photon flux density at noon of about 1,000 μmol m⁻² s⁻¹. D, chlorophyll fluorescence induction. Fm, maximum fluorescence yield when all PSII centers are closed, Fo, minimum fluorescence yield when all PSII centers are open. The ratios of variable to maximum fluorescence were calculated by Fv/Fm = (Fm-Fo)/Fm. SL, saturating light.
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FIGURE 2. Identification of the lpa19 mutation. A, a schematic diagram of the LPA19 gene. Exons (black bars) and introns (black lines) are indicated. The positions of the mutation corresponding to lpa19-1 and lpa19-2 are shown. ATG indicates the start codon, and TGA indicates the stop codon. LB, left border; RB, right border. The diagram is not drawn to scale. The numbers indicate the positions of the nucleic acid relative to 5′-untranslated region. B, RT-PCR analysis of lpa19. The analysis was performed with specific primers for At1g05385 and actin. C, immunoblot analysis of LPA19. The thylakoids (20 μg of total proteins), isolated from mutant and wild-type plants, were separated by SDS-urea-PAGE and immunodetected with antibodies raised against LPA19.

 Genetic analysis showed that the lpa19-1 mutation was recessive and that the lpa19-1 mutant phenotype did not cosegregate with the phosphinothricin resistance marker (data not shown). Thus, a map-based cloning approach, based on SSLP and In/Del molecular markers, was adopted to isolate the mutated gene responsible for the lpa19-1 mutant phenotype (supplemental Fig. S1). Between BACs YUP8H12 and F3F20, all genes with a predicted chloroplast transit peptide were sequenced, and all of these genes were identical to the wild-type except for At1g05385. In the second exon of the At1g05385 gene, a 14-bp deletion from nucleotides 708–721 resulted in a premature stop codon due to a frameshift mutation (Fig. 2A). An independent line from ABRC harboring a T-DNA insertion at nucleotide position of 706 in the second exon of the At1g05385 gene was designated lpa19-2. RT-PCR analysis showed that the abundance of At1g05385 transcripts in the lpa19-1 mutant was slightly decreased compared with the wild-type plants. The 14-bp deletion from 416 to 429 relative to the ATG codon in the coding sequence was confirmed by direct sequencing. No expression of At1g05385 in the lpa19-2 mutant was detected by RT-PCR assays (Fig. 2B). The lpa19-2 mutant exhibited retarded growth and pale green leaves, which are phenotypes similar to that of lpa19-1 (Fig. 1A). Of the two alleles (lpa19-1 and lpa19-2), lpa19-1 was used for further analysis.

To confirm that the phenotype of the lpa19-1 mutant was caused by the mutation in At1g05385, a complementation experiment was performed with the wild-type At1g05385 full-length coding sequence. Nine successfully complemented transgenic plants had phenotypes (Fig. 1A) and Fv/Fm ratios similar to those of the wild-type (Fig. 1D). Therefore, the inactivation of At1g05385 gene was responsible for the phenotype of the lpa19 mutant.

LPA19 Is a Psb27 Homolog—The open reading frame of LPA19 encodes a polypeptide that lacks membrane-spanning helices and has a calculated molecular mass of about 22 kDa. Arabidopsis luminal proteins identified by experimental mass spectrometry analyses combined with NCBI conserved domain searches and protein sequence alignments revealed that LPA19 is a homolog of cyanobacterial Psb27 and belongs to Psb27 superfamily (27, 34). In addition, there was another Psb27 homolog At1g03600 in Arabidopsis. To avoid confusion, we designated At1g03600 and At1g05385 as Psb27-H1 and Psb27-H2, respectively.

To confirm the localization of LPA19, polyclonal antiserum was raised against recombinant His-tagged LPA19 mature protein (amino acids 65–199). The ~15-kDa protein signals were detected in thylakoid membranes, and total protein extracted from the wild-type plants, but no signals were observed in the thylakoid membranes or total protein preparations from either the lpa19-1 or lpa19-2 plants (Fig. 2C). The level of LPA19 in the total protein preparations of complemented plants was higher than that of the wild-type plants. These results showed that the 14-bp deletion and the T-DNA insertion at the second exon of the AT1g05385 gene lead to the loss of LPA19 protein accumulation.

The subcellular localization and membrane association analyses of LPA19 were further investigated by immunoblot analysis. LPA19 was protected from trypsin treatment in intact thylakoid membranes, similar to the luminal PsbO protein of PSII (Fig. 3A). However, after sonication treatment of the thylakoid membranes, LPA19, and PsbO were sensitive to trypsin digestion (Fig. 3B). These results indicate that LPA19 is located in the thylakoid lumen. After incubation of sonicated thylakoid membranes with 1 M NaCl, considerable amounts of LPA19 were detected in the membrane, and LPA19 was effectively removed by treatment with 1 M CaCl2 or 6 M urea. These results suggested that LPA19 is a lumen protein peripherally associated with the membranes (Fig. 3C).

Reduced PSII Protein Contents in lpa19-1—The defect in PSII electron transfer of lpa19-1 may be associated with altered protein levels of PSII. To examine the PSII protein levels, immunoblot analysis was performed with equal amounts of total protein extracted from the leaves of 5-week-old mutant and wild-type plants (Fig. 4). The results showed that the levels of chloroplast-encoded PSII subunits D1, D2, CP47, and CP43 were reduced to ~25% of wild-type levels. The level of light harvesting complex II (LHCII) increased slightly in the mutant. The amounts of the PSI reaction center proteins Psaa/B decreased slightly and the β-subunit of the ATP synthase was almost unaffected. In contrast, the amount of cytochrome f of the cytochrome b6f complex increased by about 20% in the mutant.

The Processing of the D1 Precursor Is Perturbed in lpa19-1—The decreased PSII levels in lpa19-1 may be due to the altered synthesis and/or stability of PSII subunits. To test this, the synthesis of thylakoid membrane proteins was investigated by in
In vivo pulse-chase labeling experiments. For this assay, the 12-day-old leaf proteins were labeled with $[^{35}\text{S}]$Met in the presence of cycloheximide, which blocks the synthesis of nuclear-encoded proteins. As shown in Fig. 5A, the synthesis rates of the PSII subunits D2, CP47, and CP43, PSI reaction center PsaA/B proteins, and the $\alpha$- and $\beta$-subunits of the chloroplast ATP synthase (CF1-$\alpha/\beta$) were almost unchanged in the mutant. However, the incorporation of $[^{35}\text{S}]$Met into the D1 protein in $lpa19-1$ was dramatically reduced to about 10% of wild-type levels, and the pD1/mD1 ratio in $lpa19-1$ was higher compared with that of the wild-type after pulse labeling for 20 min. The pD1 protein was still not processed into mD1 even after a chase of 60 min in the mutant, whereas pD1 could be processed into mD1 within 10 min in the wild-type plants (Fig. 5B). These results suggest that the C-terminal processing of pD1 is inefficient in $lpa19-1$ relative to the wild-type.

$LPA19$ Interacts with the PSII Reaction Center Protein D1—Because the pD1 protein has been shown to be processed by CtpA and $LPA19$ belongs to Psb27 superfamily without the C-terminal peptidase domain, it is likely that $LPA19$ is involved in facilitating the processing of pD1 via interactions with CtpA or D1. Our efforts to detect the interaction between Psb27-H2 and CtpA was unsuccessful (data not shown) because CtpA perturbed this two-hybrid system, which was also observed in (13). The possibility that $LPA19$ interacts with D1 was examined by protein overlay assays. The thylakoid membranes were solubilized with dodecyl-$\beta$-D-maltopyranoside (DM), fractionated by blue-native (BN)/SDS-PAGE two-dimensional gel electrophoresis (Fig. 6A), and the resolved proteins were transferred onto nylon membranes. The membranes were first

![FIGURE 5. In vivo chloroplast protein labeling assays.](image-url)
incubated with purified recombinant His-LPA19 protein with a polyhistidine tag at its N terminus, and the proteins binding to LPA19 were identified by subsequent immunoblot analysis with the antibody specific to the polyhistidine tag (Fig. 6B). There was one detected spot that co-migrated with the distinct signal of the D1 protein in PSII monomer identified by immunoblotting analysis of a duplicate membrane with antibody against the D2 protein (Fig. 6B). The signal of B, nitrocellulose membrane was incubated with recombinant His-tagged LPA19 protein that subsequently reacted with His antibodies. C, nitrocellulose membrane was incubated with D2 and D1 antibodies, as indicated to the left. D, superposition of B and C. The signal of B was shown by thick line loops and denoted by arrowhead; the signal of C, which was immunodetection with anti-D2 and anti-D1 antibodies was shown by gray black line loops and labeled by D1 and D2. E, membrane was incubated with His antibody.

Because LPA19 is a thylakoid luminal protein, it is reasonable to speculate that it interacts with the luminal exposed segments of the PSII reaction center protein D1. To confirm the possibility, yeast two-hybrid assays were performed utilizing the MATCHMAKER Gold Yeast Two-Hybrid System (Clontech). The coding sequence of LPA19 was cloned into the yeast bait vector pGBK7T7 and DNA fragments encoding the D1 AB loop, CD loop, the soluble C-terminal segments of pD1 and mD1 were cloned into the prey vector pGADT7 AD. The resulting transformants were analyzed for growth on plates lacking Ade, His, Leu, and Trp (SD-Ade-His-Leu-Trp), supplemented with 70 ng/ml Aureobasidin A, a highly sensitive yeast antibiotic marker, and their α-galactosidase activities were assayed. As shown in Fig. 7, an interaction between LPA19 and the soluble C-terminal segments of D1 was detected in the coimmunoprecipitants. Conversely, preimmunization of the antibodies against the polyhistidine tag in the absence of LPA19 did not produce any signals (Fig. 6E). These results strongly support the hypothesis that LPA19 interacts directly with the PSII reaction center protein D1.

In addition, we performed coimmunoprecipitation analysis with anti-LPA19 antibody using the radiolabeled thylakoid membranes. As shown in Fig. 8, both pD1 and mD1 were detected in the coimmunoprecipitants. Conversely, preimmunization did not produce any signals. Therefore, we conclude that LPA19 interacts with pD1 and mD1 in vivo.

DISCUSSION

In this study, we report on the isolation and characterization of the lpa19 high chlorophyll fluorescence mutant of Arabidopsis. LPA19 encodes a thylakoid lumen Psb27 homolog protein, designated as Psb27-H2. In the mutant, the maximum photochemical efficiency of PSII was decreased (Fig. 1D), which is consistent with the greatly reduced PSII content (Fig. 4). This suggests that LPA19 is involved in PSII biogenesis and stabilization. There are two Psb27 homologs in Arabidopsis. Analysis of another Psb27 homolog in Arabidopsis, Psb27-H1, revealed that it is not essential for PSII biogenesis. However, recovery of PSII activity after photoinhibition was slowed in the Psb27-H1 mutant compared with the wild-type plants, which points to its important role in the efficient repair of photodamaged PSII (33). Similarly, inactivation of Psb27 in Synechocystis has no effect on photosynthetic performance, yet it is required for efficient PSII repair. Inspection of the detailed function of Psb27 in
The re-assembly process revealed that the Psb27 protein is involved in facilitating the manganese cluster assembly in PSII, at least in part by preventing the premature assembly of the extrinsic proteins during the photoactivation process (29). Thus, these two Psb27 homologs in Arabidopsis, Psb27-H1 and Psb27-H2, have distinct functions in the biogenesis and repair of PSII.

Inactivation of LPA19 resulted in a strong reduction in PSII content (Fig. 4) and the impairment of C-terminal processing of the D1 precursor (Fig. 5A). The ipa1 mutant displays a pale-green coloration and a reduced growth rate (Fig. 1A). This resembles the observations on the periplasmic protein PratA deletion mutant in Synechocystis (13, 14). In this mutant, the levels of PSII proteins were reduced to about 25%, and the D1 precursor processing was hampered. These two mutants are able to grow photoautotrophically. However, the Synechocystis mutants lacking the C-terminal processing D1 protease are not viable (22). This suggests that LPA19 is required, but not essential, for D1 precursor processing.

The Psb27 protein has been found to copurify with PSII protein complexes, but it is not present in the functional PSII (26–29). It is likely that Psb27 transiently interacts with the PSII subunits. Protein overlay analysis strongly suggests that Psb27-H2 directly interacts with the PSII D1 protein (Fig. 6). Coimmunoprecipitation analysis provided direct evidence that Psb27-H2 interacts with mD1 and pD1 in vivo (Fig. 8). We further demonstrate that the pD1 C-terminal extension is not involved in the interaction between Psb27-H2 and D1 using the yeast two-hybrid system (Fig. 7). Instead, the soluble C-terminal segment of the mature D1 protein is required for such interaction. Also, it is interesting to note that the Psb27-associated PSII lacks the extrinsic proteins PsbO, PsbU, PsbV, and PsbQ that are normally associated with the active PSII (26–29). In the recent PSII crystal structure, the extrinsic proteins PsbO, PsbU, and PsbV have some direct interactions with the luminal C-terminal portion of the mature D1 protein (3–6). The interaction of Psb27 with the luminal C-terminal portion of the mature D1 protein suggests that it functions in the PSII biogenesis steps facilitating the D1 precursor processing and the interaction between LPA19 and D1 would exclude the early association of extrinsic proteins, as observed in the cyanobacterial mutant lacking CtpA (27).

The involvement of additional components facilitating the D1 precursor processing has recently been suggested because the recombinant CtpA exhibits a relatively weak affinity toward its substrates in vitro (46). The PratA protein has been implicated in facilitating D1 processing probably through direct interaction with the C-terminal regions. The binding region was mapped to amino acids 314–328 of the D1 protein present in the precursor and mature D1 proteins. Blast searches did not reveal the presence of PratA homologue proteins in Arabidopsis. It is possible that Psb27-H2 has evolved to fulfill a similar function to PratA in assisting the processing of the D1 precursor. Two Psb27 homologs were also found in the genomes of higher plants that have been sequenced (supplementary Figs. S2 and S3). It is likely that the functions of Psb27 in PSII biogenesis and repair are highly conserved in higher plants. It is interesting to note that PratA and D1 were localized in a specific membrane fraction that may be a transfer and/or connecting region between the plasma and thylakoid membranes (14). However, the LPA19 protein was exclusively localized to the lumen. Therefore, it is likely that PSII biogenesis in higher plants and cyanobacteria may involve distinct mechanisms.

REFERENCES
1. Iwata, S., and Barber, J. (2004) Curr. Opin. Plant. Biol. 14, 447–453
2. Nelson, N., and Yocum, C. F. (2006) Annu. Rev. Plant Biol. 57, 521–565
3. Kamiya, N., and Shen, J. R. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 98–103
4. Ferreira, K. N., Iverson, T. M., Maghlaoui, K., Barber, J., and Iwata, S. (2004) Science 303, 1831–1838
5. Loll, B., Kern, J., Saenger, W., Zouni, A., and Biesiadka, J. (2005) Nature 438, 1040–1044
6. Guskov, A., Kern, J., Gabdulkhakov, A., Broser, M., Zouni, A., and Saenger, W. (2009) Nat. Struct. Mol. Biol. 16, 334–342
7. Adir, N., Shochat, S., and Ohad, I. (1990) J. Biol. Chem. 265, 12563–12568
8. van Wijk, K. J., Roobol-Boza, M., Kettunen, R., Andersson, B., and Aro, E. M. (1997) Biochemistry 36, 6178–6186
9. Müller, B., and Eichacker, L. A. (1999) Plant Cell 11, 2365–2377
10. Zhang, L., Paakkinen, V., van Wijk, K. J., and Aro, E. M. (1999) J. Biol. Chem. 274, 16062–16067
11. Suorsa, M., Regel, R. E., Paakkinen, V., Battchikova, N., Herrmann, R. G., and Aro, E. M. (2004) Eur. J. Biochem. 271, 96–107
12. Rokka, A., Suorsa, M., Saleem, A., Battchikova, N., and Aro, E. M. (2005) Biochem. J. 388, 159–168
13. Klinckert, B., Ossenbühl, F., Sikorski, M., Berry, S., Eichacker, L., and Nickelsen, J. (2004) J. Biol. Chem. 279, 44639–44644
14. Schottkowski, M., Gkalympoudis, S., Tzekova, N., Stelljes, C., Schümemann, D., Ankele, E., and Nickelsen, J. (2009) J. Biol. Chem. 284, 1813–1819
15. Kufryk, G. I., and Vermaas, W. F. (2001) Biochemistry 40, 9247–9255
16. Kufryk, G. I., and Vermaas, W. F. (2003) J. Bacteriol. 185, 6615–6623
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