LPA2 Is Required for Efficient Assembly of Photosystem II in Arabidopsis thaliana

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To elucidate the molecular mechanism of photosystem II (PSII) assembly, we characterized the low psii accumulation2 (lpa2) mutant of Arabidopsis thaliana, which is defective in the accumulation of PSII supercomplexes. The levels and processing patterns of the RNAs encoding the PSII subunits are unaltered in the mutant. In vivo protein-labeling experiments showed that the synthesis of CP43 (for chlorophyll a binding protein) was greatly reduced, but CP47, D1, and D2 were synthesized at normal rates in the lpa2-1 mutant. The newly synthesized CP43 was rapidly degraded in lpa2-1, and the turnover rates of D1 and D2 were higher in lpa2-1 than in wild-type plants. The newly synthesized PSII proteins were assembled into PSII complexes, but the assembly of PSII was less efficient in the mutant than in wild-type plants. LPA2 encodes an intrinsic thylakoid membrane protein, which is not an integral subunit of PSII. Yeast two-hybrid assays indicated that LPA2 interacts with the PSII core protein CP43 but not with the PSII reaction center proteins D1 and D2. Moreover, direct interactions of LPA2 with Albino3 (Alb3), which is involved in thylakoid membrane biogenesis and cell division, were also detected. Thus, the results suggest that LPA2, which appears to form a complex with Alb3, is involved in assisting CP43 assembly within PSII.

INTRODUCTION

Photosystem II (PSII) is a pigment-protein complex in the thylakoid membrane of cyanobacteria and chloroplasts that drives the electron transfer from water to plastoquinone with concomitant oxygen evolution. In higher plants, PSII consists of >20 subunits, including both membrane integral and extrinsically associated proteins (Wollman et al., 1999; Iwata and Barber, 2004; Nelson and Yocum, 2006). The PSII reaction center complex is composed of the D1 and D2 proteins, the α and β subunits of cytochrome b559, and the PsbI protein, which is capable of primary charge separation and subsequent electron transfer (Nanba and Satoh, 1987). The PSII core complex also contains the light-harvesting chlorophyll a binding proteins CP47 and CP43, the oxygen-evolving 33-kD protein, and several low molecular mass proteins (Bricker and Ghanotakis, 1996). CP47 and CP43 are closely associated with the PSII reaction center complex and are located on opposite sides of the complex (Hankamer et al., 1999; Zouni et al., 2001; Ferreira et al., 2004; Loll et al., 2005). The functional form of PSII in the thylakoid membrane consists of the PSII core and the associated light-harvesting complex (LHC). In PSII-LHCII supercomplexes, LHCII trimers consisting of Lhcb1 and Lhbc2 anchor to the PSII core dimers (Boekema et al., 1995; Hankamer et al., 1997). The minor antenna complex proteins CP29, CP26, and CP24 probably serve as linker proteins between LHCII and PSII (Nelson and Yocum, 2006).

The molecular mechanism underlying the biogenesis and assembly of PSII is poorly understood, although our knowledge of the structure and function of PSII has greatly advanced. Because of the structural complexity of PSII, the assembly process is likely to consist of multiple steps. The initial step in PSII biogenesis is the formation of the PSII reaction center complex (Adir et al., 1990; van Wijk et al., 1997; Müller and Eichacker, 1999; Zhang et al., 1999). During this process, the D1 protein is incorporated into a precomplex, probably consisting of the D2, cytochrome b559, and PsbI proteins, which functions as a receptor. CP47 is directly associated with the PSII reaction center complex. D1, D2, and CP47 appear to accumulate in a coordinated manner (Jensen et al., 1986; de Vitry et al., 1989; Yu and Vermaas, 1990). CP43 is synthesized independently and is a dynamic component of PSII, with dissociation and reassociation constantly recurring (de Vitry et al., 1989; Zhang et al., 2000). Integration of the low molecular mass proteins into PSII has been found to occur at different stages of the PSII assembly process (Hager et al., 2002; Suorsa et al., 2004; Rokka et al., 2005). The final establishment of a functional PSII involves the dimerization of PSII monomers and the association of LHCII trimers.

Genetic and biochemical studies have provided evidence for the nuclear control of the biogenesis and assembly of PSII (Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Rochaix, 2001; Zhang and Aro, 2002; Leister, 2003). A number of nucleus-encoded factors have been shown to be involved in the assembly of PSII. Notably, HCF136 is
essential for the stable assembly of PSII in Arabidopsis thaliana (Meurer et al., 1998). Protein-labeling analyses have shown that plastid-encoded proteins are synthesized in the hcf136 Arabidopsis mutant, which lacks functional HCF136 protein, but the assembly of PSII reaction centers is blocked and no stable PSII complexes appear to accumulate in it (Meurer et al., 1998; Plückem et al., 2002). In the low psii accumulation1 (lpa1) mutant of Arabidopsis, the newly synthesized PSII proteins are assembled into functional protein complexes, but the assembly is less efficient than in wild-type plants (Peng et al., 2006). Protein-protein interaction studies have revealed that LPA1 specifically interacts with the PSII reaction center protein D1. LPA1 appears to be an integral membrane chaperone that assists efficient PSII assembly, probably through direct interaction with D1 (Peng et al., 2006). In addition, Albino3.1 (Alb3.1), a homolog of Arabidopsis Alb3, has been found to be required for the efficient assembly of functional PSII in Chlamydomonas reinhardtii, although integration of D1 into the thylakoid membrane does not appear to be affected in the Chlamydomonas Alb3.1 deletion mutant ac29 (Ossenbühl et al., 2004). Alb3.1 probably acts as a membrane-integral chaperone in an early step of PSII assembly in Chlamydomonas (Ossenbühl et al., 2004). Sr1471p, a homolog of Alb3, has been found to be essential for the processing of the D1 precursor (Ossenbühl et al., 2006). In the mutant sr1471p, integration of the D1 precursor into the thylakoid membrane is perturbed and the D1 precursor is accumulated in the membrane. FKBP-2, an immunophilin of the chloroplast lumen, has been shown to function in the accumulation of the PSII supercomplex (Lima et al., 2006). In that study, inactivation of FKBP-2 resulted in elevated levels of PSII monomers and dimers and reduced accumulation of PSII supercomplexes. Thus, a number of proteins appear to participate in the assembly of PSII. Moreover, each stage of PSII assembly appears to be assisted by one or more nucleus-encoded proteins.

In the study presented here, we aimed to genetically dissect the molecular mechanism underlying the assembly of PSII and to characterize the low psii accumulation2 (lpa2) mutant with defective accumulation of PSII supercomplexes. We report the isolation of LPA2, a nucleus-encoded factor, which is required for the accumulation of PSII. The results of our functional analyses indicate that LPA2 is involved in assisting CP43 assembly within PSII.

RESULTS

PSII Activity Is Reduced in lpa2-1

The lpa2-1 mutant was obtained by screening the Scheible and Somerville T-DNA Arabidopsis lines (Weigel et al., 2000) for high chlorophyll fluorescence phenotypes (Meurer et al., 1996; Peng et al., 2006). Chlorophyll fluorescence induction experiments revealed that the ratio of variable fluorescence to maximum fluorescence (Fv/Fm), which reflects the maximum potential of photochemical reactions of PSII, was significantly lower in the lpa2-1 mutant (0.54 ± 0.03) than in wild-type plants (0.83 ± 0.02) (Figure 1A). The decreased Fv/Fm ratios suggest that the mutants have defects in electron transfer within PSII or a partial loss of PSII capacity. The redox kinetics of P700 showed that P700 can be oxidized in the lpa2-1 mutant, indicating that PSI is functional in it (Figure 1B). The amplitude of changes in A_{280} induced by far-red light was lower in the mutant than in wild-type plants. These spectroscopic analyses showed that the photosynthetic deviations of lpa2-1 are similar to those of lpa1 and primarily reflect reductions in PSII activity (Peng et al., 2006).

The lpa2-1 mutant displayed a slightly pale green phenotype, and its growth was greatly reduced (Figure 2A). The leaf areas of mutant plants were 70% smaller than those of wild-type plants at 26 days after germination (Figure 2B).

Molecular Cloning of the LPA2 Gene

The genetic defect in lpa2-1 segregated as a single recessive mutation. Cosegregation of the mutant phenotype and the phosphinotricin resistance conferred by the T-DNA confirmed that the mutation was induced by the T-DNA insertion (data not shown).

Figure 1. Spectroscopic Analysis of Wild-Type and lpa2-1 Plants.

(A) Chlorophyll fluorescence induction. The minimal level of fluorescence (Fo) of dark-adapted whole plants with all PSII reaction centers open was determined using a pulsed measuring beam of red light. The Fm level with all PSII reaction centers closed was determined using a saturating pulse and dark-adapted leaves. SL, saturating light; ML, measuring light.

(B) Redox kinetics of P700. The oxidation of P700 was investigated by measuring absorbance changes of P700 at 820 nm induced by far-red light (FR; 720 nm). ΔA_{max}, maximum oxidation induced by far-red light. AL, actinic light.
shown). To determine the genetic basis of the lpa2-1 phenotype, thermal asymmetric interlaced (TAIL) PCR was performed and the genomic region flanking the left border sequences of the T-DNA was isolated. Sequence analysis revealed that the T-DNA was inserted in the 5' untranslated region of At5g51545, at position −12 relative to the ATG codon (Figure 3A), and the gene was expressed at levels that were barely detectable by RT-PCR in the lpa2-1 mutant (Figure 3B). However, two annotated genes close to the insertion site (At5g51540 and At5g51550) were expressed at similar levels to those in wild-type plants, as shown by RT-PCR analysis (Figure 3B).

To confirm that the mutated gene is responsible for the observed phenotype, we analyzed an independent T-DNA insertion line, SAIL_293_G05, in the sequence-indexed Arabidopsis T-DNA insertion mutant stock. This homologous line, lpa2-2, carries a T-DNA insertion at nucleotide position 452 in the second exon of the At5g51545 gene (Figure 3A). RT-PCR analysis showed that the expression of At5g51545 was also suppressed in this mutant (Figure 3B), and its chlorophyll fluorescence induction kinetics were similar to those of lpa2-1 (data not shown). The lpa2-2 mutant also had a pale-green phenotype (Figure 2A) and reduced growth rate (data not shown).

To obtain direct evidence that disruption of the LPA2 gene was responsible for the lpa2 mutant phenotype, a complementation experiment was performed with the isolated At5g51545 full-length cDNA. The resulting clone containing the cDNA sequence under the control of the cauliflower mosaic virus 35S promoter was introduced into a homozygous lpa2-1 mutant using the floral dip method (Clough and Bent, 1998). Sixteen successfully complemented transgenic plants had similar growth rates and chlorophyll fluorescence induction kinetics to wild-type plants (Figures 1 and 2). Thus, it can be concluded that the lpa2 phenotype is due to the inactivation of At5g51545.

**PSII Proteins Are Severely Reduced in lpa2-1**

We hypothesized that the defect in the electron transfer of PSII could be associated with altered levels of proteins in the PSII complex. To examine steady state levels of thylakoid proteins in wild-type and lpa2-1 plants, thylakoid membranes were isolated from 5-week-old wild-type and lpa2-1 leaves and immunoblot analysis was performed using antibodies raised against specific

![Figure 2. Phenotypes of lpa2-1, lpa2-2, Wild-Type, and lpa2-1 Transformant Plants Complemented with the Open Reading Frame of the At5g51545 Gene. (A) Five-week-old plants grown in the growth chamber. (B) Growth kinetics of the lpa2-1 mutant compared with wild-type plants. Values shown are averages ± SE of six replicate experiments.](image2)

![Figure 3. Identification of the lpa2 Mutation. (A) Schematic diagram of the LPA2 gene. Exons (white boxes) and introns (lines) are indicated. The positions of the T-DNA insertions corresponding to lpa2-1 and lpa2-2 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. (B) RT-PCR analysis of mutant plants. RT-PCR was performed with specific primers for At5g51545, At5g51540, and At5g51550 and ubiquitin-specific primers.](image3)
subunits of the photosynthetic thylakoid membrane protein complexes. The results showed that the levels of plastid-encoded PSII subunits D1, D2, CP47, and CP43 were all decreased to ~30% of wild-type levels (Figure 4A). By contrast, levels of the nucleus-encoded 33-kD protein of the oxygen-evolving complex and LHCII were only slightly reduced in the mutant (Figure 4A).

To explore putative structural alterations of the thylakoid membrane protein complexes in the mutant, thylakoid membranes from mutant and wild-type plants were solubilized with dodecyl-β-D-maltopyranoside (DM) and the chlorophyll protein complexes (with equal amounts of chlorophyll) were separated by blue-native (BN) PAGE (Figure 5A). Six major bands labeled I to VI were resolved after the first-dimensional separation (Figure 5A), apparently representing PSII supercomplexes (band I), monomeric PSI and dimeric PSII (band II), monomeric PSII (band III), CP43-free PSII (band IV), trimeric LHCII/PSII reaction center (band V), and monomeric LHCII (band VI) (Guo et al., 2005). As clearly shown in Figure 5A, the largest chlorophyll-containing protein complexes (labeled I) were absent, and there was a nearly complete loss of band IV, in the mutant. Since the separated proteins were detected by Coomassie blue staining (Figure 5B), it did not show PSII reaction center composition but only the presence of CP43 together with some LHC and (probably) some minor antenna complexes (for additional information, see Figure 7E below). Analyses of the two-dimensional SDS-urea-PAGE gels after Coomassie blue staining showed that the relative levels of PSII core proteins D1, D2, CP47, and CP43 were reduced in the mutant, especially the PSII supercomplexes, which were barely detectable (Figure 5B). It is interesting that the CP43 doublet becomes a single band in the mutant (Figure 5B). In addition, the amount of PSI was decreased slightly and the contents of cytochrome b6f and ATP synthase were increased slightly per unit of chlorophyll in the mutant (Figure 5B).

CP43 is known to undergo two cotranslational or early post-translational changes: chlorophyll binding and phosphorylation. It has been observed previously that changes or defects in CP43 phosphorylation accompany changes in antenna-PSII core formation (de Vitry et al., 1989), with a spectacular loss of PSII-LHCII supercomplexes (Swiatek et al., 2001). To examine the phosphorylation of CP43, we performed immunoblot analysis with antiphosphothreonine antibodies. There were no apparent changes in the phosphorylation level of CP43 between the wild-type and mutant plants when normalized to the protein level of CP43 (Figure 5C). To examine the chlorophyll binding to CP43, we detected the presence of the chlorophyll binding form of CP43 by monitoring the abnormal migration of holo-CP43 versus apo-CP43 on two-dimensional gels (de Vitry et al., 1989). In the wild-type plants, CP47 and CP43 were detected as spots off the
diagonal by two-dimensional gel electrophoresis (Figure 5D, arrows). In the \textit{lpa2-1} mutant, the content of CP43 was reduced and CP43 was detected as spots off the diagonal in two-dimensional gel electrophoresis (Figure 5D, arrows). CP47 and CP43 were not detectable after heat treatment before the first-dimensional electrophoresis in the wild-type and mutant plants (data not shown). Thus, these two events may not account for the presence of the CP43 doublet observed in the wild type and the single CP43 band in the \textit{lpa2-1} mutant.

**Figure 5.** Accumulation of Chloroplast Proteins, Phosphorylation of CP43, and Binding of Chlorophyll to CP43 in Wild-Type and \textit{lpa2-1} Plants.

(A) BN gel analysis of thylakoid membrane protein complexes. Thylakoid membranes (10 \(\mu\)g of chlorophyll) from 5-week-old wild-type and \textit{lpa2-1} leaves were solubilized with 1% DM and separated by BN gel electrophoresis. The positions of protein complexes representing PSII supercomplexes (band I), monomeric PSI and dimeric PSII (band II), monomeric PSII (band III), CP43 minus PSII (band IV), trimeric LHCII/PSII reaction center (band V), and monomeric LHCII (band VI) were identified by immunodetection and matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Guo et al., 2005; Peng et al., 2006).

(B) Two-dimensional separation of protein complexes in the thylakoid membrane. BN-PAGE-separated thylakoid proteins in a single lane from a BN gel were separated in a second dimension by 15% SDS-urea-PAGE and stained with Coomassie blue. Names of the proteins resolved by the second-dimensional SDS-PAGE, previously identified, are indicated by arrowheads (Peng et al., 2006).

(C) Immunodetection of the phosphorylation of CP43. Thylakoid membranes were isolated from 5-week-old \textit{Arabidopsis} \textit{lpa2-1} (2.5 \(\mu\)g of chlorophyll) and wild-type (1 \(\mu\)g of chlorophyll) plants grown under normal growth conditions, separated by SDS-PAGE, and immunodetected with anti-phosphothreonine (top panel; P-CP43) and anti-CP43 (bottom panel; CP43) antibodies.

(D) Analysis of chlorophyll binding to CP43. Thylakoid membranes isolated from 5-week-old wild-type and \textit{lpa2-1} plants were solubilized at 4°C. After solubilization, the thylakoid membranes were separated in the first dimension on a 7.5 to 15% polyacrylamide gradient gel at 4°C followed by a second-dimensional 12 to 18% polyacrylamide gel in the presence of 6 M urea at room temperature. CP47 and CP43, on the diagonal, are indicated by arrows. The proteins identified by MALDI-TOF mass spectrometry are indicated at left.

**Steady State mRNA Levels and Polysome Association Are Unaffected in \textit{lpa2-1}**

The reduced PSII contents in the \textit{lpa2-1} mutant could be due to impaired accumulation of transcripts that encode one or more PSII proteins. To assess this possibility, we performed RNA gel blot hybridization to examine levels of the plastid-encoded PSII transcripts. Our results indicate that identical amounts of \textit{psbA} and \textit{psbC} (encoding the D1 protein and CP43 of PSII, respectively) accumulated in 5-week-old \textit{lpa2-1} and wild-type plants (Figure 6A). The abundance and pattern of transcripts of other PSII operons (such as the \textit{psbD/C}, \textit{psbEFLJ}, and \textit{psbKI} operons) and transcripts of \textit{psaA} and \textit{petA} (which encode the PSI reaction center protein PsaA and the cytochrome b_{6}f subunit cytochrome f, respectively) were also unaltered in the mutant (Figure 6A).

The effects of the \textit{lpa2-1} mutation on the protein synthesis capacity of chloroplasts were investigated by analyzing changes in the polysome association of \textit{psbA}, \textit{psbB}, \textit{psbC}, and \textit{psbD} transcripts following sucrose gradient fractionation. The results showed that the association of these transcripts with polysomes was largely unaffected in 5-week-old mutant plants (Figure 6B).

**Synthesis of Thylakoid Membrane Proteins in \textit{lpa2-1}**

The decreased accumulation of PSII may be due to either reduced protein synthesis or increased degradation of its protein components. Therefore, we studied the synthesis of plastid-encoded thylakoid membrane proteins by pulse-chase labeling of mutant leaves in the presence of cycloheximide, which inhibits the translation of nucleus-encoded proteins. As shown in Figures 7A and 7B, after pulse labeling for 20 min, the rates of synthesis of the PSII proteins CP47, D1, and D2, the PSI reaction center proteins PsaA/B, and the \(\alpha\) and \(\beta\) subunits of ATP synthase (CF1\(\alpha/\beta\)) in 12-d-old young mutant seedlings and 5-week-old plants were comparable to those in their wild-type counterparts. However, labeling of the PSII subunit CP43 in the \textit{lpa2-1} mutant was dramatically reduced, to <10% of wild-type levels in both young seedlings and 5-week-old plants (Figures 7A and 7B). When the time of pulse labeling was shortened to 10 min, the amount of radioactivity incorporated into CP43 in the \textit{lpa2} mutant was >25% of wild-type levels in young seedlings (Figure 7C).
Pulse labeling for 20 min was followed by a chase with unlabeled Met to monitor the turnover rates of plastid-encoded proteins in young seedlings. The results showed that the turnover rates of CP47 and the weakly synthesized CP43 are relatively unaffected in the mutant, and the turnover rate of D1 is much more strongly affected than that of D2 (Figure 7D).

The Assembly of PSII Is Impaired in lpa2-1

To study the assembly of photosynthetic protein complexes, thylakoid membrane proteins were separated by BN gel electrophoresis and the different PSII assembly intermediates of free PSII protein, PSII reaction center, CP43-free PSII monomers, intact PSII monomers, PSII dimers, and PSII supercomplexes were visualized autoradiographically. After a 20-min pulse, most of the radiolabeling was found in protein complexes of ~100 kD (PSII reaction centers), 220 kD (CP43-free PSII monomers), and 250 kD (PSII monomers) (Figure 7E). Protein complexes of 220, 250, and 500 kD (PSII dimers) were the most strongly labeled complexes after a 15-min chase, and larger protein complexes (>500 kD) became clearly visible in the autoradiogram obtained after a 30-min chase in the wild-type plants. During the chase period, the incorporation of radioactivity into PSII monomers, dimers, and supercomplexes gradually increased, with concomitant losses of label in PSII reaction centers and CP43-free PSII complexes. There was no evidence of the further assembly of PSII after 60 min of chasing (Figure 7E). However, in the lpa2-1 mutant, most of the radioactivity in the thylakoid membrane preparations was found to be associated with PSII reaction centers and CP43-free PSII complexes. The radioactivity in PSII monomers increased after a 15-min chase, and the PSII dimer was clearly labeled after a 30-min chase. The accumulation of radioactivity in PSII supercomplexes was barely detectable even after a 60-min chase (Figure 7E).

The pulse–chase samples were also subjected to BN-SDS-PAGE analysis to follow the assembly of the major PSII core proteins, CP47, CP43, D1, and D2, into PSII complexes (Figure 7F). The PSII reaction center protein D1 was the most heavily labeled protein, even though very young leaves were used for the pulse–chase experiments. After a 20-min pulse labeling, ~20% of the labeled D1 protein was found in PSII reaction centers, ~35% was found in CP43-free PSII monomers, and ~25% was found in PSII monomers (Figure 7F). As the chase time increased, the radiolabeled D1 protein appeared in larger PSII complexes,
such as PSII dimers and PSII supercomplexes. After a 30-min chase, most of the radiolabeled D1 protein was incorporated into PSII monomers (30%), dimers (25%), and supercomplexes (25%), and only 20% was found in CP43-free PSII monomers (Figure 7F). However, the assembly from CP43-free PSII monomers to PSII monomers and the formation of PSII supercomplexes were distinctively slower in the lpa2-1 mutant than in wild-type plants. In the mutant, 70% of the radiolabeled D1 protein was incorporated into CP43-free PSII monomers after a 20-min pulse, which is consistent with the results of the one-dimensional BN gel analysis described above (Figures 7E and 7F). Even after a 60-min chase, 40% of the labeled D1 protein was still in CP43-free PSII monomers, indicating that CP43 assembly was severely hampered in the mutant. Only a small amount of radiolabeled D1 protein (<5%) had accumulated in PSII supercomplexes after a 60-min chase (Figure 7F).

LPA2 Is an Intrinsic Thylakoid Membrane Protein

The open reading frame of LPA2 encodes a polypeptide of 185 amino acids with a calculated molecular mass of 20 kD. Database searches did not reveal the presence of any recognizable motif or domain in LPA2 (Figure 8A). However, the N-terminal sequence of LPA2 is rich in positive and hydroxylated amino acid residues, which is characteristic of chloroplast transit peptides. Analysis by the TMHMM program showed that LPA2 has two
transmembrane domains, in the regions 112 to 135 and 150 to 173, suggesting that LPA2 is probably a membrane protein (Figure 8A). BLAST searches of the complete Arabidopsis sequence indicated that the LPA2 gene is present in a single copy in the nuclear genome. Database searches and protein sequence alignments revealed that LPA2 shares significant sequence identity with an unknown rice (Oryza sativa) protein, XP_463951 (68% identity, 80% similarity) (Figure 8A). No homologous protein to LPA2 was found in cyanobacteria or Chlamydomonas. The homologous protein in rice also had a chloroplast transit peptide, suggesting that they may have similar functions.

To determine the localization of LPA2, polyclonal antiserum was raised against recombinant LPA2 protein (amino acids 7 to 114). An ~20-kD protein was detected in thylakoid membrane proteins and total proteins prepared from the wild-type plants, but no signal was detected in total protein preparations from either the lpa2-1 or lpa2-2 plants (Figure 8B). The level of LPA2 in total protein preparations of complemented plants was comparable to that in wild-type plants (Figure 8B). These findings indicate that the T-DNA insertion leads to the loss of LPA2 protein accumulation.

To further investigate whether LPA2 is a transmembrane protein, thylakoid membrane fractions of wild-type plants were sonicated and treated with different salts and the proteins were then subjected to immunoblot analysis. LPA2 was still retained in the membranes, even when membrane preparations were sonicated in the presence of 250 mM NaCl, 1 M CaCl₂, or 6 M urea (Figure 8C). During these treatments, PsbO, the 33-kD luminal protein, and CP47, the PSII core protein, were used as controls.

LPA2 Interacts with the PSII Core Protein CP43

Since LPA2 appears to be required for the efficient assembly of PSII, we tested the possibility that LPA2 is an integral subunit of PSII or part of a multiprotein complex. For this purpose, isolated thylakoid membranes of wild-type plants were solubilized by DM, separated by sucrose gradient sedimentation, and subjected to immunoblot analysis (Zhang et al., 1999; Peng et al., 2006). After centrifugation, 20 fractions of equal volume were collected from the top to the bottom of the gradients and the proteins in each fraction were immunodetected using specific antibodies. The immunoblots indicated that LPA2 was present in fractions at ~100 kD, based on the migration of molecular standards, and that it did not comigrate with PSII proteins. Thus, it appears that LPA2 is not a component of PSII (Figure 9).

However, if LPA2 is involved in the assembly of PSII, it may interact with one or more subunits of the PSII complex. To test this possibility, we examined the interaction of LPA2 with the subunits of PSII in vivo using a modified split-ubiquitin system.

Figure 8. Amino Acid Sequence Alignment and Immunolocalization of LPA2.

(A) The amino acid sequence of the At5g51545 protein was compared with a homologous sequence from O. sativa. Identical amino acids are marked with asterisks, and conserved exchanges are marked with colons. Two possible transmembrane domains are shown by blue bars. The sequences were aligned with ClustalW (Thompson et al., 1994).

(B) Immunoblot analysis of LPA2. Samples from wild-type, lpa2-1, lpa2-2, and complemented plants, consisting of total leaf proteins and thylakoids (equivalent to 3 mg of chlorophyll), were separated by SDS-PAGE and immunodetected with antibodies raised against LPA2.

(C) Salt washing of the thylakoid membranes. The thylakoid membranes were sonicated in the presence of 250 mM NaCl, 1 M CaCl₂, and 6 M urea for 30 min at 0°C, then incubated for another 30 min at 0°C. PsbO, the 33-kD luminal protein of PSII, and the PSII core protein CP47 were used as markers. Membranes that had not been subjected to any salt treatment (CK) were used as controls.

Figure 9. Sucrose Gradient Fractionation Analysis of Thylakoid Proteins.

Thylakoid membranes (0.5 mg chlorophyll/mL) were solubilized with 1% DM and separated by centrifugation on a linear 0.1 to 1 M sucrose gradient at 180,000g for 22 h. Twenty fractions were collected from the top to the bottom of the gradients, and the proteins from each fraction were separated by SDS-PAGE and immunodetected with anti-LPA2, anti-D2, anti-CP47, anti-CP43, anti-LHCII, anti-PsaA/B, and anti-Alb3 antibodies. The positions of 669-kD (thyroglobulin), 440-kD (ferritin), 232-kD (catalase), 140-kD (lactate dehydrogenase), and 66-kD (BSA) molecular mass markers are indicated.
designed to assess interactions of membrane proteins (Pasch et al., 2005; Peng et al., 2006). A prey construct plasmid, in which the NubG moiety was fused to the N terminus of LPA2, was prepared and subsequently transformed into the NWY32 strain in which bait proteins were expressed. The bait plasmids were constructed to generate the fusion proteins X-Cub-LexA-VP16 (where X represents D1, D2, or CP43). The resulting transformants were analyzed for growth on plates lacking His, Leu, and Trp (SD-His-Leu-Trp), and their β-galactosidase activities were assayed. As shown in Figure 10, coexpression of CP43-Cub-LexA-VP16 with NubG-LPA2 resulted in positive β-galactosidase activity and growth on SD-His-Leu-Trp plates (Figure 10). However, coexpression of D1-Cub-LexA-VP16 or D2-Cub-LexA-VP16 with NubG-LPA2 produced transformants that showed no β-galactosidase activity or growth on SD-His-Leu-Trp plates (Figure 10). These results suggest that LPA2 interacts with CP43 but not with D1 or D2. CP43-LPA2 complexes may not be sufficiently stable to accumulate in the membranes, based on immunoblot analysis of the distribution of proteins over the sucrose gradient fractions (Figure 9).

**LPA2 Interacts with Alb3**

Recent studies have revealed that Arabidopsis Alb3 interacts with the PSII proteins D1, D2, and CP43 (Pasch et al., 2005). To examine whether LPA2 interacts with Alb3, the fractions obtained from sucrose gradient fractionation were immunoblotted with anti-Alb3 antibodies. Alb3 was found to be present in two distinct complexes: one of ~60 to 140 kD, and one >600 kD (Figure 9). The interaction between LPA2 and Alb3 was further investigated by yeast two-hybrid analysis. Coexpression of Alb3-Cub-LexA-VP16 with NubG-LPA2 resulted in positive β-galactosidase activity and growth on SD-His-Leu-Trp plates (Figure 10).

In previous studies, we have shown that LPA1 is required for efficient PSII assembly (Peng et al., 2006). The interaction between LPA1 and LPA2 was also investigated. Coexpression of LPA1-Cub-LexA-VP16 with NubG-LPA2 produced transformants that showed no β-galactosidase activity or growth on SD-His-Leu-Trp plates (Figure 10). Thus, the above results suggest the interaction of LPA2 with Alb3, but not with LPA1.

**DISCUSSION**

The biogenesis and assembly of both the chloroplast- and nucleus-encoded proteins into PSII require the stoichiometric synthesis and sequential assembly of PSII proteins and the ligation of various cofactors. Thus, the assembly processes are likely to require additional auxiliary and regulatory factors to assist this complex, multistep process in chloroplasts. Due to the limited coding capacity of the chloroplast genome, the biogenesis and assembly of PSII is mainly regulated by nucleus-encoded proteins (Goldschmidt-Clermont, 1998; Barkan and Goldschmidt-Clermont, 2000; Rochaix, 2001; Leister, 2003). The identification of these factors and the clarification of their function will undoubtedly improve our understanding of the ways in which photosynthetically active protein complexes are assembled and functionally maintained. Here, we report the isolation and characterization of the lpa2 high chlorophyll fluorescence mutant of Arabidopsis, in which the efficient PSII assembly is blocked. Evidence regarding the function of the mutated gene LPA2 and its role in assisting CP43 assembly within PSII complexes is also presented and discussed.

In the mutant lpa2-1, the accumulation of component subunits of PSII was reduced to ~30% of wild-type levels (Figure 4), in accordance with spectroscopic data revealing a defect in its PSII activities (Figure 1). When thylakoid membranes from wild-type and mutant plants with equal amounts of chlorophyll were analyzed, in conjunction with the reductions in levels of PSII, the contents of other thylakoid membrane protein complexes, including cytochrome b6f and ATP synthase, were found to be slightly higher in the mutant, as expected (Figures 5A and 5B). There were also reductions in its amounts of functional PSI complexes (Figure 4), again in accordance with spectroscopic analyses (Figure 1B). These results show that like the lpa1 mutation (Peng et al., 2006), the mutation in lpa2-1 primarily affects the accumulation of the PSII complex.

There are several possible explanations that could account for the reduced accumulation of PSII. Examination of the abundance and patterns of PSII, PSI, and cytochrome b6f gene transcripts in
the lpa2-1 mutant (Figure 6A) indicated that the reduced PSII contents are not due to the absence of transcripts encoding any of the subunits of PSII. Furthermore, analysis of the association of psbA, psbB, psbC, and psbD transcripts with polysomes showed that polysome association of these genes was not perturbed in the mutant (Figure 6B). In vivo labeling of chloroplast proteins directly demonstrated that the rates of synthesis of the CP43 protein in Arabidopsis lpa2-1 mutants were >90% lower than in wild-type counterparts after pulse labeling for 20 min and were 25% of wild-type levels after 10 min of pulse labeling (Figures 7A to 7C). Thus, rapid degradation of CP43 in the absence of LPA2 could affect CP43 synthesis with no changes in polysome loading. Our results suggest that ~30% of PSII proteins that accumulated in the mutant (Figure 4) would escape degradation and be processed normally. The increased degradation of CP43 in the mutant may be regulated by the state of protein assembly. Indeed, the assembly of PSII is retarded in the mutant (Figures 7E and 7F), and the presence of unassembled proteins in the thylakoid membrane could account for this. Such assembly-dependent regulatory processes have been reported in the biogenesis of photosynthetic proteins. Deletion of the psbC gene leads to an increased turnover of the other PSII core proteins (D1, D2, and CP47) and to the reduced accumulation of PSII core proteins, although these PSII core proteins are synthesized at or near wild-type levels (Jensen et al., 1986; Rochaix et al., 1989; Yu and Vermaas, 1990, 1993). Therefore, it can be speculated that the decreased accumulation of the assembly partner may increase the turnover rates of proteins in the mutant.

In vivo chloroplast protein-labeling experiments revealed that functional assembly of PSII occurs in both wild-type and lpa2-1 plants (Figures 7E and 7F), and the photautotrophic growth of the mutant provides further support that it produces functional photosynthetic systems (Figure 2). The formation of PSII complexes was not delayed in the lpa2-1 mutant, but assembly within PSII complexes was less efficient and the level of formation of PSII supercomplexes was blocked (Figures 7E and 7F). These results suggest that the absence of LPA2 causes a cotranslational or early posttranslational degradation of ~70% of CP43. LPA2 may be required for the efficient translation of CP43, and that delayed synthesis could underpin the decreased assembly efficiency within PSII in the mutant (Figures 7E and 7F).

The presence of a chloroplast transit peptide in the N terminus of LPA2 indicates that the protein is targeted to chloroplasts. Chloroplast fractionation and immunoblot analysis confirmed that LPA2 is an intrinsic thylakoid membrane protein (Figure 8), in accordance with previous proteomic analysis (Peltier et al., 2004). Database searches suggest that the occurrence of LPA2 is restricted to higher plants. There are no gene sequences that are homologous with LPA2 in the sequenced genomes of cyanobacteria or Chlamydomonas. No function has been reported for LPA2. Thus, LPA2 is a novel protein that is essential for CP43 assembly, and it is very likely that the factor evolved after the divergence of the vascular plant lineage or has been lost in related lineages.

The unique occurrence of LPA2 in higher plants suggests that the function of this nuclear factor is associated with the biogenesis of the PSII complex rather than with a fundamental photosynthetic process. This hypothesis is also supported by the finding that the incorporation of CP43 into PSII is retarded in the mutant (Figures 7E and 7F). Sucrose gradient fractionation and immunoblot analysis showed that LPA2 is not a component of the PSII complex (Figure 9). Our yeast two-hybrid analysis provides direct evidence that LPA2 specifically interacts with CP43 but not with D1 or D2 (Figure 10). Thus, it is possible that LPA2 is involved in assisting CP43 assembly within PSII complexes.

Alb3 belongs to the Oxa1p/YidC protein family, members of which have been found in all organisms examined to date. Members of this protein family are mainly involved in the insertion and/or assembly of membrane proteins (Kuhn et al., 2003), and in vitro studies have shown that Alb3 is required for insertion of the LHC into the thylakoid membrane (Moore et al., 2000). The alb3 mutant in Arabidopsis shows an albino phenotype with strongly reduced thylakoid membranes and arrested chloroplast development (Sundberg et al., 1997). Chlamydomonas ac29 mutants, which also lack Alb3.1, are less strongly affected and are still able to grow photoautotropically (Bellafiore et al., 2002). In these mutants, the LHC pool is specifically depleted and PSII accumulation is reduced, but the extent of the depletion is dependent on the growth conditions. Alb3.1 has also been found to interact with the PSII reaction center protein D1 and to assist its assembly into PSII (Ossenbühl et al., 2004). Another member of the family, Alb3.2, has been found to be essential for the assembly of the photosystems and for cell survival in Chlamydomonas (Göhre et al., 2006). Only a single Alb3 gene has been found in all cyanobacterial genomes sequenced to date (Srl1471 in Synechocystis). Analysis of Srl1471 deletion mutants has shown that its product plays important roles in both cell division and membrane biogenesis (Spence et al., 2004). These findings indicate that Alb3 is essential for thylakoid membrane biogenesis and that it may have a broad spectrum of substrates. Indeed, protein–protein interaction studies using the yeast split-ubiquitin system have shown that Alb3 interacts with diverse subunits of thylakoid membrane proteins, including the PSII core proteins D1, D2, and CP43, the PSI reaction center protein PsaA, and the ATP synthase subunit CFoII (Pasch et al., 2005).

The function of Alb3 in thylakoid protein biogenesis raises the question of whether Alb3 operates on its own or requires one or more other interacting protein(s) to perform its function. Alb3 has been demonstrated to be required for the posttranslational integration of light-harvesting chlorophyll binding protein into the thylakoid membrane (Moore et al., 2000). Moreover, the association of Alb3 with cpSecY, the chloroplast homolog of bacterial SecY, has been detected (Klostermann et al., 2002; Pasch et al., 2005), suggesting that cpSecY and Alb3 may function together as a transloca. However, the integration of light-harvesting chlorophyll binding protein into the thylakoid membrane is independent of cpSecY. Similarly, the Escherichia coli protein YidC can function either in concert with, or independently of, the SecY transloca (Valent et al., 1998; Houben et al., 2000). Our sucrose gradient fractionation analysis of solubilized thylakoid membrane preparations showed that LPA2 cofractionates with Alb3 in a 100-kD protein complex (Figure 9), and the ability of LPA2 and Alb3 to interact was confirmed by yeast two-hybrid split-ubiquitin analysis (Figure 10). The presence of Alb3 in different protein complexes suggests that its functions may vary, depending on the substrates with which it associates. Thus,
LPA2 and Alb3 may form a complex that is involved specifically in the efficient assembly of CP43 in PSII.

METHODS

Mutant Isolation and Plant Growth Conditions

Arabidopsis thaliana was grown in a growth chamber with a 10-h photo-period, at a photon flux density of 120 μmol·m⁻²·s⁻¹ and constant temperature of 22°C, after incubating seeds in darkness for 48 h at 4°C to ensure synchronized germination. The lpa2-1 mutant was isolated as a recessive high chlorophyll fluorescence plant from a collection of pSKI015 T-DNA–mutagenized Arabidopsis (ecotype Columbia) lines from the ABRC. The T-DNA insertion line, named lpa2-2, was also obtained from the ABRC. The T-DNA insertion site is located in exon 2 of At5g51540, as confirmed by genomic PCR and sequencing analysis using the following primers: LB (5′-TAGCATTGAATTTCAACATTGATAC-3′), LP (5′-CACAGGA-ACATTGTGAGAGAGAAG-3′), and RP (5′-TCACCTTTGACCCCTTCTAC-3′). Segregation analysis confirmed that the high chlorophyll fluorescence phenotype is caused by a T-DNA insertion.

Chlorophyll Fluorescence Phenotype

Chlorophyll fluorescence was measured, using a PAM-2000 portable chlorophyll fluorometer (Walz), in plants that had been dark-adapted for 30 min. The minimum fluorescence yield (Fo) was measured under measuring light (650 nm) of very low intensity (0.8 μmol·m⁻²·s⁻¹). A saturating pulse of white light (3000 μmol·m⁻²·s⁻¹) was applied to determine the maximum fluorescence yield (Fm) and the ratio of variable (Fv) to maximum (Fm) fluorescence (Fv/Fm = (Fm – Fo)/Fm). All of these measurements were performed in a dark room with stable ambient conditions. In order to measure changes in light-induced P700 absorbance at 820 nm, the PAM chlorophyll fluorometer was equipped with an ED 800T emitter–detector unit (Walz) and the measurements were performed according to Meurer et al. (1996). Absorbance changes induced by saturating far-red light reflect the relative amounts of photo-oxidizable P700.

Thylakoid Membrane Preparation and Sucrose Gradient Analysis

To isolate thylakoid membranes, leaves were homogenized in an ice-cold isolation buffer containing 400 mM sucrose, 50 mM HEPES-KOH, pH 7.8, 10 mM NaCl, and 2 mM MgCl₂. After filtration, the thylakoids were centrifuged at 5000g for 10 min, washed twice with isolation buffer, and then resuspended in isolation buffer. The resulting thylakoids were either freshly or frozen in liquid N₂ and stored at −70°C before use. The chlorophyll content was determined spectrophotometrically according to Porra et al. (1989).

Sucrose gradient fractionation of the thylakoid membrane preparations was performed according to Peng et al. (2006). The thylakoid membranes were solubilized with 1% (w/v) DM and then loaded onto a linear 0.1 to 1 M sucrose gradient. After centrifugation at 180,000g for 22 h, the sucrose gradient was divided into 20 fractions of equal volume from the top to the bottom.

In Vivo Labeling of Chloroplast Proteins

In vivo protein labeling was performed essentially according to Meurer et al. (1998). Primary leaves of 12-d-old and 5-week-old Arabidopsis were preincubated in 20 μg/mL cycloheximide for 30 min and radiolabeled with 1 μCi/μL [³⁵S]Met (specific activity > 1000 Ci/mmole; Amersham Pharmacia Biotech) in the presence of 20 μg/mL cycloheximide at 25°C. Pulse labeling of the leaves was followed by a chase in the presence of 10 mM unlabeled Met. After labeling, the thylakoid membranes were isolated by the method described by Peng et al. (2006).

BN-SDS-PAGE and Protein Analysis

BN-PAGE was performed as described by Schägger et al. (1994) with minor modifications (Cline and Mori, 2001). Thylakoid membranes were suspended in resuspension buffer (20% [w/v] glycerol and 25 mM Bis-Tris-HCl, pH 7.0) at 1.0 mg chlorophyll/mL, and an equal volume of resuspension buffer containing 2% DM was added. The membranes were centrifuged at 4°C for 5 min and resuspended at 15,000g for 10 min. The supernatant was combined with one-tenth volume of 5% Serva Blue G in 100 mM Bis-Tris-HCl, pH 7.0, 0.5 M 6-amino-n-caproic acid, and 30% (w/v) glycerol and loaded onto a gel with a 6 to 12% acrylamide gradient on the separation gel using a Hoefer Mighty Small vertical electrophoresis unit connected to a cooling circulator. For two-dimensional analysis, BN-PAGE lanes were excised, incubated in SDS sample buffer containing 5% β-mercaptoethanol for 30 min, and layered onto 15% SDS polyacrylamide gels containing 6 M urea (Laemmli, 1970).

Immunoblot analysis was performed according to standard techniques using specific antibodies with the enhanced chemiluminescence method. For immunodetection of LPA2, the proteins were resolved on Tricine/SDS-PAGE gels (Schägger and von Jagow, 1987). X-ray films were scanned and analyzed using an Alphalager 2200 documentation and analysis system (Alpha Innotech). For autoradiography, gels were stained, dried, and exposed to x-ray films. The relative amounts of [³⁵S]Met in the D1 protein after SDS-PAGE were quantified by scanning the x-ray films and analyzing the acquired data, again using the Alphalager 2200 system.

Two-Dimensional SDS-PAGE and Protein Identification

Chlorophyll binding to CP43 was examined by two-dimensional gel analysis. Thylakoid membranes were solubilized at 4 or 25°C in 50 mM Na₂CO₃, 50 mM DTT, 12% sucrose, and 2% LiDolSO₄ to a final chlorophyll concentration of 1 mg/mL. The first-dimensional separation was performed on a 7.5 to 15% polyacrylamide gradient gel at 4°C, and the second-dimensional separation was performed on a 12 to 18% polyacrylamide gel in the presence of 6 M urea at room temperature. After gel electrophoresis, the proteins were stained with Coomassie Brilliant Blue R 250.

To identify the proteins separated on the two-dimensional gel, MALDI-TOF spectrometry was used, after in-gel digestion and sample preparation according to the method of Jensen et al. (1999). MALDI-TOF analysis was performed in reflector mode with a Kratos AXIMA-CFRPlus mass spectrometer (Kratos Analytical). Proteins were identified as the highest ranking results by Mascot Wizard freeware (Matrix Science). For positive identification, the score of the results [-10 x Log(P)] had to be over the P < 0.05 significance threshold level.

Nucleic Acid and Polysome Analysis

Total plant RNA was extracted from fresh tissues using a Trizol isolation reagent. To determine expression levels of the three genes located around the T-DNA insertion site, RT-PCR was performed using the following specific primers based on genomic sequence information: 5′-TACCTACC-GATCCACCTCAGGGGCTCA-3′ and 5′-GACGGGCAAATCTCTTCAGG- AGGCCGA-3′ for At5g51540; 5′-ATGGGCTAACATCCCTTCCTG-3′ and 5′-GGCAGGCTATAAGCTTCCATT-3′ for At5g51545; and 5′-CCTTACACATGGTCCAGTGC-3′ and 5′-CCACCTGGTCTCCATAAATAC-3′ for At5g51550. The amount of RNA loaded in each sample was monitored by RT-PCR. Polysome profiles were analyzed by RT-PCR using the level of ubiquitin cDNA using the following primers: 5′-GATCCATTGCCGCAAAACATTGAGGATG-3′ and 5′-CATGCTGAGGAAGAGAAGTCC-3′.

Polysomes were isolated from leaf tissues under conditions that maintain polysome integrity according to Barkan (1988). RNA was isolated, fractionated, and transferred onto nylon membranes. The filters were
hybridized with \(^{32}P\)-labeled cDNA probes. All of the blots were exposed to x-ray film for 1 to 3 d. The hybridization probes were labeled by random priming and prepared from the PCR fragments of the chloroplast genome (GenBank accession number AP000423). The sequences of the PCR primers used for the amplification of chloroplast genes were obtained from Peng et al. (2006).

**Antiserum Production**

For the production of polyclonal antibodies against LPA2, a cDNA fragment encoding the soluble part of LPA2 (amino acids 7 to 114, corresponding to nucleotide positions 19 to 342 of the LPA2 gene) was amplified by PCR using the primers 5’-CAATAAGCTTTCTCGGTGTCACCTCTCAGGAGACC-3’ and 5’-CAAGCTCGAGTTTTCTGTCTGCTGCTCTG-3’. The resulting DNA fragment was cleaved with HindIII and XhoI and fused in-frame with the N-terminal His affinity tag of PET28a. The BL21 cells were harvested after the addition of 0.6 mM isopropylthio-

**Immunolocalization Analysis**

The intracellular localization of LPA2 was determined essentially according to Lennartz et al. (2001). Arabidopsis thylakoid membranes were suspended to a final concentration of 40 g chlorophyll/mL and incubated for 30 min in 10 mM HEPES-KOH, pH 7.8, and 5 mM MgCl\(_2\) in the presence of a protease inhibitor cocktail and sonicated three times for 15 s on ice before salt treatment with 250 mM NaCl, 1 M CaCl\(_2\), or 6 M urea. After salt treatment, the membranes were centrifuged at 100,000 g for 2 h at 4°C, washed once with suspension buffer, and then used for immunoblot analysis. Thylakoids without supplements were used as controls.

**Cloning of LPA2 and Complementation of the lpa2-1 Mutant**

To clone the T-DNA–tagged gene in the lpa2-1 mutant, T-DNA left border sequences were isolated using a TAIL PCR strategy essentially according to Liu et al. (1995). The amplified TAIL PCR products were sequenced with SK3 primers.

For complementation of the lpa2-1 mutant, the cDNA containing the LPA2 coding region was amplified by PCR with the primers 5’-CTCTAGAATGGCGCTACAAATCCACTCTCCGT-3’ and 5’-CCGGTGTCAGTACTCTGACCCCTTCATTTCCTC-3’. The PCR product was cleaved with XbaI and XhoI and subcloned into the plant expression vector pBI121 under the control of the cauliflower mosaic virus 35S promoter. The construct was transformed into Agrobacterium tumefaciens C58 strain and introduced into Arabidopsis mutant plants by the floral dip method (Clough and Bent, 1998). Transgenic plants were plated on Murashige and Skoog medium containing 40 \(\mu\)g/mL kanamycin monosulfate. The resistant plants were transferred to soil and grown in a greenhouse to produce seeds. The success of the complementation was confirmed by chlorophyll fluorescence analysis.

**Yeast Two-Hybrid Assays**

Yeast two-hybrid assays were performed using the yeast strain NMY32 supplied by Dualsystems Biotech (Stagljar et al., 1998). To construct the bait plasmids, the vectors pCCW-SET and pCCW-SUC encoding the Cub-LexA-VP16 fragment were used. The prey plasmids were constructed from the vector pDSLNx, which encodes the NubG fragment (Dualsystems Biotech). The mature full-length LPA2 gene and the complete gene sequences of CP43, D1, D2, and Alb3 were obtained by PCR amplification. The LPA2 gene was cloned into the NubG prey vector, and the CP43, D1, D2, and Alb3 genes were cloned into bait vectors. The LPA1 bait plasmid was described by Peng et al. (2006). The authenticity of all constructs was confirmed by sequence analysis. Interactions were determined by growing diploid yeast colonies on SD-His-Leu-Trp plates and by β-Gal activity using the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside filter assay described by Stagljar et al. (1998).

**Accession Numbers**

Sequence data used for the alignment presented in this study can be found in the GenBank/EMBL data libraries under the following accession numbers: NP_1032057 for Arabidopsis thaliana At5g51545, and XP_463951 for Oryza sativa (japonica cultivar group).

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**REFERENCES**


