Running title: Role of LPA3 and LPA2 in PSII assembly

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Cooperation of LPA3 and LPA2 Is Essential for Photosystem II Assembly in Arabidopsis

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Abstract

Photosystem II (PSII) is a multisubunit membrane protein complex that is assembled in a sequence of steps. However, the molecular mechanisms responsible for the assembly of the individual subunits into functional PSII complexes are still largely unknown. Here we report the identification of a chloroplast protein, LPA3, which is required for the assembly of CP43 subunit in PSII complexes. LPA3 interacts with LPA2, a previously identified PSII CP43 assembly factor, and a double mutation of \textit{LPA2} and \textit{LPA3} is more deleterious for assembly than either single mutation, resulting in a seedling-lethal phenotype. Our results indicate that LPA3 and LPA2 have overlapping functions in assisting CP43 assembly, and that cooperation between LPA2 and LPA3 is essential for PSII assembly. In addition, we provide evidence that LPA2 and LPA3 interact with Alb3, which is essential for thylakoid protein biogenesis. Thus, the function of Alb3 in some PSII assembly processes is probably mediated through interactions with LPA2 and LPA3.
Introduction

Oxygenic photosynthesis, in which oxygen and organic carbon are produced from water and carbon dioxide using sunlight, provides energy for nearly all living organisms on Earth. Four major multi-protein complexes, located in thylakoid membranes, are responsible for the capture of light and its conversion to chemical energy in eukaryotic photosynthetic organisms: photosystem I (PSI), photosystem II (PSII), cytochrome b6/f, and ATP synthase (Wollman et al., 1999; Nelson and Yocum, 2006). PSII catalyzes one of the most important of all biochemical reactions, the light-induced transfer of electrons from water to plastoquinone, which generates most of the oxygen in the earth’s atmosphere. PSII consists of more than 20 subunits in higher plants (Wollman et al., 1999; Nelson and Yocum, 2006). The PSII reaction center consists of the D1 and D2 proteins, the α and β subunits of cytochrome b559, and the PsbI protein, and the D1 and D2 heterodimers bind all the redox components essential for the primary charge separation (Nanba and Satoh, 1987). The PSII core complex additionally contains CP47, CP43, the oxygen-evolving complex, and several low molecular mass proteins (Wollman et al., 1999; Nelson and Yocum, 2006). CP47 and CP43, two inner chlorophyll a-binding proteins, are closely associated with, and located on opposite sides of, the PSII reaction center (Hankamer et al., 1999). The functional form of PSII cores in thylakoid membranes is dimeric and is associated with light-harvesting complex (LHC). In PSII-LHCII supercomplexes, PSII core dimers are surrounded by LHCII trimers, which consist of Lhcb1 and Lhcb2 proteins (Wollman et al., 1999; Iwata and Barber, 2004; Nelson and Yocum, 2006).

Our knowledge of the molecular mechanisms involved in the biogenesis and assembly of PSII in the thylakoid membranes is still limited, although the structure and function of PSII have been extensively studied. Genetic and biochemical studies have elucidated several distinct steps that occur in PSII assembly. D2 and cytochrome b559 form an initial complex, which serves as a receptor for the co-translational assembly of D1 (Adir et al., 1990; van Wijk et al., 1997; Müller and Eichacker, 1999; Zhang et al., 1999). The next step involves the association of CP47 with the PSII reaction center (Zhang et al., 1999; Rokka et al., 2005), while CP43 is synthesized independently then continuously associates and dissociates with PSII (de Vitry et al., 1989; Zhang et al., 2000). The biogenesis of PSII involves “a control by epistasy of synthesis” process (Minai et al., 2006). D2 is required for D1 synthesis, which itself is needed for CP47
synthesis. However, many aspects of the processes involved in the oligomerization and coordination of the various PSII subunits are still unclear (Rochaix, 2001). Due to the structural complexity of PSII, its assembly consists of multiple assembly steps, which is likely to require the participation of a number of assembly factors.

Several assembly factors involved in the biosynthesis and assembly of the PSII complex have been identified recently. For instance, the thylakoid lumen protein HCF136 is known to be required for the formation of PSII since the hcf136 mutant is capable of synthesizing plastid-encoded proteins, but it does not appear to accumulate any stable PSII complexes due to blockage of the assembly of the PSII reaction center (Meurer et al. 1998; Plücken et al. 2002). Alb3.1, a homolog of Arabidopsis Alb3, is essential for the efficient assembly of PSII in Chlamydomonas reinhardii, probably through interactions with D1 following its insertion (Ossenbühl et al., 2004), and another Alb3 homolog, Alb3.2, appears to be required for photosystem assembly in Chlamydomonas (Göhre et al. 2006). Co-immunoprecipitation analysis has shown that Alb3.1 and Alb3.2 interact directly, while Alb3.2 reportedly interacts with the PSI and PSII reaction centers’ proteins (Göhre et al. 2006). The lumenal immunophilins, AtCYP38 and FKBP20-2, have also been shown to be involved in PSII assembly (Lima et al., 2006; Fu et al., 2007; Sirpiö et al., 2008). In addition, we recently identified two PSII assembly factors, LPA1 and LPA2, involved in PSII assembly. The LPA1 (Low PSII Accumulation1) protein appears to be an integral membrane chaperone required for efficient assembly of the PSII core complex, probably through direct interaction with D1 (Peng et al. 2006). LPA2, which interacts with Alb3, forms a protein complex that assists CP43 assembly within PSII (Ma et al., 2007). These findings suggest that each stage of the PSII assembly process is assisted by one or more specific assembly factors, most of which have not yet been identified.

Here we report the identification of a low photosystem ii accumulation3 (lpa3) mutant with reduced levels of PSII. Functional characterization points to the possible role of LPA3 in assisting CP43 assembly within PSII. In addition, biochemical and genetic analyses indicate that an assembly complex of LPA3 and LPA2 is essential for PSII assembly.

Results

Reduced Levels of PSII Protein Contents in lpa3
The high-chlorophyll-fluorescence lpa3 mutant was isolated by screening T-DNA Arabidopsis lines (Weigel et al., 2000). Homozygous mutants had pale green coloration and lower growth rates than their wild-type counterparts (Fig. S1). Analysis of chlorophyll fluorescence induction kinetics showed that the ratios of variable to maximum fluorescence ($F_v/F_m$), reflecting the maximum potential of PSII photochemical reactions, were significantly lower in the mutant (0.42±0.02) than in the wild-type plants (0.82±0.02) (Fig. S2), indicating that electron transfer within PSII was impaired, or some PSII capacity had been lost in the mutants. In addition, P700 redox kinetics analysis revealed that PSI was functional, although reductions in PSI contents were detected in the mutants (Fig. S2).

To investigate if the impaired PSII function was associated with changes in PSII protein levels, we performed immunoblot analyses of thylakoid membrane preparations with equal amount of chlorophyll from wild-type and mutant plants with specific antibodies. The results showed that levels of the PSII core proteins D1, D2, CP47 and CP43 were reduced to ~30% of wild-type levels, contents of the oxygen-evolving complex protein PsbO, LHCII and the PSI reaction center proteins PsaA/B were ~80%, 90% and 75% of wild-type levels, respectively, while contents of Cyt f of the cytochrome b6/f complex and the β-subunit of ATP synthase were slightly increased in both 12-d-old (data not shown) and 5-wk-old lpa3 plants (Fig. 1). Reduced PSI levels have also been observed in several other PSII mutants (Meurer et al., 1998; Plücken et al. 2002; Peng et al., 2006; Ma et al., 2007; Schult et al., 2007). One possibility is that levels of PSI may be light sensitive in the PSII mutants (Plücken et al. 2002; Schult et al., 2007). Alternatively, the perturbed photosynthetic activities of PSII in the mutant may down-regulate the levels of PSI (Bailey et al., 2001; Hager et al., 2002). To evaluate possible alterations of thylakoid protein complexes in the mutants, we also performed blue native gel electrophoresis analysis of thylakoid membranes solubilized with 1 % n-dodecyl-β-D-maltoside. The results showed that levels of the PSII supercomplex, PSII dimer and PSII monomer were reduced in the lpa3 mutant compared to the wild-type (Fig. S3).

**Steady State mRNA Levels and Polysome Association Are Unaltered in lpa3**

The observed reductions in levels of PSII proteins in lpa3 could have been caused not only by impaired transcription but also by post-transcriptional defects. Hence, northern
blot analysis was used to examine transcript levels of chloroplast-encoded PSII subunits in the mutant (Fig. 1B). The results showed there were similar amounts of psaA/B (encoding the PsaA/B proteins of PSI) and psbA (encoding the D1 subunit of PSII) transcripts, and almost identical amounts of psbD/C (encoding the D2 and CP43 subunits of PSII, respectively) transcripts in lpa3 and wild-type plants.

To examine if the mutant’s capacity for chloroplast protein synthesis is altered, we also analyzed the association of chloroplast mRNA with ribosomes following sucrose gradient fractionation (Fig. 1C). This analysis detected no significant differences in the polysome association of psbA and psbC transcripts between lpa3 and wild-type plants. The association of psaA/B transcripts with polysome was also largely unaffected in the mutant compared with the wild type.

Synthesis of Thylakoid Membrane Proteins in lpa3

The reduced levels of PSII proteins in the lpa3 mutant may have been caused either by their impaired translation or enhanced degradation. Therefore, we studied the synthesis rates of plastid-encoded proteins in lpa3 and wild-type plants via pulse-chase labeling in the presence of cycloheximide, which inhibits the translation of nuclear-encoded proteins. The synthesis rates of PSII proteins D1, D2 and CP47 in 12-d-old and 5-wk-old lpa3 mutants were comparable to those in wild-type counterparts, while labeling of the PSII protein CP43 was found to be dramatically (>90%) lower in both the young seedlings and 5-wk-old lpa3 mutants compared to wild-type counterparts after pulse labeling for 20 min (Figs. 2A and B). After shortening the pulse labeling duration to 10 min, the amount of radioactivity incorporated into CP43 in young lpa3 seedlings was somewhat higher, ca. 25% of wild-type levels per unit chlorophyll (Fig. 2C). In addition, pulse labeling for 20 min followed by a chase with unlabeled Met showed that the turnover rates of the PSII proteins D1 and D2 were much higher in the lpa3 mutant than in the wild-type (Fig. 3D). Our results showed that the amounts of labeled PsaA/B PSI proteins after 20-min labeling pulses were lower, and those of the α and β subunits of ATP synthase (CF1α/β) were higher, per unit chlorophyll, in the mutants than in wild-type plants (Figs. 2A and B). Increased incorporation of label into CF1α/β, reflecting enhanced synthesis of these proteins, has been previously observed in plants carrying other mutations affecting the biogenesis of PSI and PSII (Aman et al., 2004; Lezhneva and Meurer, 2004; Ma et al., 2007).
Assembly of PSII Is Impaired in lpa3

To monitor the assembly of newly synthesized proteins into photosynthetic protein complexes, thylakoid membranes from mutant and wild-type plants were solubilized and protein complexes from them were separated in BN gels following a pulse-chase experiment (Fig. 2E). In wild-type preparations, most of the radioactivity was found in various PSII complexes, subcomplexes, and constituents after 10 min of pulse labeling, indicating that PSII complexes were being efficiently assembled. The incorporation of radioactivity into PSII dimers and supercomplexes increased during the following 30 min of chasing, but there was no further evidence of PSII assembly thereafter. However, after 10 min of pulse labeling of the lpa3 samples, most of the radioactivity was associated with the PSII reaction center and the CP43-free PSII monomers. After a 30-min chase, PSII dimers and supercomplexes were clearly labeled, but there was still a considerable amount of radioactivity in the CP43-free PSII monomers.

Cloning of the LPA3 gene

The lpa3 mutant phenotype did not cosegregate with the phosphinotricin resistance marker, indicating that the T-DNA did not tag the mutated LPA3 gene (data not shown). Thus, the affected gene in lpa3 was identified by map-based cloning, which located the lpa3 mutation in a 300-kilobase region of chromosome 1. Comparison of the wild-type and lpa3 genomic sequences of the genes containing putative chloroplast targeting signals revealed a G to C mutation at nucleotide position 1081 bp in the sixth intron of the gene At1g73060 relative to the ATG codon in the mutant (Fig. 3A). RT-PCR analysis of the mutant showed the presence of two types of transcripts, one unspliced and the other incorrectly spliced, according to subsequent sequence analysis (Fig. 3B). Both transcripts encode truncated polypeptides that terminate immediately after the mutation site.

To confirm that the mutated gene was responsible for the observed lpa3 mutant phenotypic traits, complementation experiments were performed with isolated wild-type cDNA of the gene At1g73060. This complementation successfully restored normal wild-type growth and chlorophyll fluorescence kinetics (Figs. S1 and S2). Immunoblot analysis also showed that the protein levels of LPA3 were comparable between the complemented and wild-type plants (Fig. 3C), while no LPA3 protein was detected in
the total protein or thylakoid samples from the lpa3 mutant. These findings indicate that the gene mutation leads to the loss of LPA3, and that the lpa3 mutant phenotype is likely due to the loss of the function of the At1g73060 gene.

LPA3 Is a Chloroplast Protein

Lpa3 encodes a putative protein consisting of 340 amino acids, which does not contain any recognizable domain or motif according to database searches. In addition, analysis of the protein sequences by the TMHMM program indicated that LPA3 does not have any transmembrane domain. Highly conserved LPA3 sequences were found in Oryza sativa, Chlamydomonas reinhardtii, Ostreococcus lucimarinus, Ostreococcus tauri, and Physcomitrella patens. The program ChloroP 1.1 predicted that the first 58 amino acids of LPA3 comprise the plastid target signals (Fig. S4).

To confirm the prediction that LPA3 is a chloroplast protein, we isolated intact chloroplasts and performed immunoblot analysis with a specific anti-LPA3 antibody (Fig. 3D). As predicted, LPA3 was found to be located in the chloroplasts. To further resolve the localization of LPA3 within the chloroplasts, chloroplasts were fractionated into chloroplast stroma and thylakoid membrane factions. Immunoblot analysis revealed that some LPA3 is present in the stroma, but some is also associated with the thylakoid membrane fractions (Fig. 3D). The chloroplast stromal ribulose bisphosphate carboxylase large subunit (RbcL) and the integral thylakoid membrane protein D2 of PSII were used as controls for this experiment. To examine the strength of the membrane association of LPA3, we treated thylakoid membranes with salt and chaotropic agents, which are known to remove extrinsic proteins (Boudreau et al., 1997). LPA3 was completely removed from the membranes after they were incubated with 0.1 M Na2CO3 or 6 M urea. A considerable amount of LPA3 was detected in the thylakoid membranes after treatment with 1 M NaCl, like the peripheral protein CF1β of ATPase, while a small amount remained associated with the membranes after treatment with 1 M CaCl2 (Fig. 3E).

To assess if the association of LPA3 with the membranes is modulated by the light conditions, we isolated thylakoid membrane and stroma fractions for immunoblot analysis from plants that were illuminated at 20 µmol m−2 s−1 (low light intensity) and 1000 µmol m−2 s−1 (high light intensity) for 2 h after growth at 120 µmol m−2 s−1 for five weeks (Fig. 3F). Our results showed that the LPA3 protein contents associated with the
thylakoid membranes increased, but those in the stroma decreased under high-intensity light. Conversely, under low-intensity light, levels of the LPA3 protein associated with the thylakoid membrane decreased while its levels in the stroma increased.

**LPA3 Interacts with PSII Core Protein CP43**

Several assembly factors have been shown to be required for the assembly of photosystems through direct interaction with certain subunits of the protein complexes (Naver et al., 2001; Ossenbühl et al., 2004; Peng et al., 2006; Ma et al., 2007). Since the above findings showed that LPA3 is required for efficient PSII assembly (Fig. 2), we postulated that it may interact with specific subunits of PSII. To test this possibility, we first used a modified split-ubiquitin system to examine potential interactions between LPA3 and PSII proteins. The results showed that LPA3 interacts with CP43, but not D1, D2 or CP47 (Fig. 4A). However, in further co-immunoprecipitation analyses of these interactions CP43 was not detected in immunoprecipitates by either anti-LPA2 or anti-LPA3 antibodies under non-denaturing conditions (Fig. 4B).

**Interactions between LPA1, LPA2 and LPA3**

We have previously demonstrated the involvement of LPA1 and LPA2 in PSII assembly, through their respective interactions with the PSII proteins D1 and CP43 (Peng et al., 2006; Ma et al., 2007). To examine protein interactions between these two factors and LPA3, we performed split-ubiquitin analysis. The results revealed that LPA2 and LPA3 interact with each other, but there was no indication that either protein interacts with LPA1 in the yeast two-hybrid system (Fig. 4C). In order to further examine whether the interaction between these assembly factors occurs *in vivo*, we used bimolecular fluorescence complementation (BiFC) analysis (Fig. 4D). The results confirmed that LPA2 and LPA3 interact *in planta*, and that there was no interaction between either of these proteins and LPA1. Further co-immunoprecipitation analysis corroborated the observations regarding these interactions (Fig. 4B).

**LPA2 and LPA3 Interact with Alb3**

The Alb3/Oxl/YidC protein family plays essential roles in the assembly of membrane protein complexes (Kuhn et al., 2003; van der Laan et al., 2005; Kiefer and Kuhn, 2007), and we have previously shown that LPA2 interacts with Alb3 (Ma et al., 2007). Thus,
given the interaction between LPA3 and LPA2, we examined the possibility that LPA3 also interacts with Alb3. The results showed that LPA3 and Alb3 interact in the yeast two-hybrid system (Fig. 4A). BiFC and co-immunoprecipitation analyses further demonstrated that Alb3 directly interacts with LPA2 and LPA3, but not LPA1 (Figs. 4B and D).

**Analysis of lpa2lpa3 Double Mutant**

The above results strongly indicated that LPA2 and LPA3 interact with each other, but not with LPA1 (Fig. 5). *lpa2* and *lpa3* mutants all had reduced growth rates, pale green coloration and *Fv/Fm* values of ca. 0.4-0.5. To genetically assess the interactions between LPA2 and LPA3, we crossed the single mutants and identified double mutant by PCR analysis. *lpa2lpa3* double mutation was seedling-lethal for plants sown in soil (Fig. 5A), and even though they could be maintained on sucrose-supplemented medium they did not develop any flowers. Analysis of chlorophyll fluorescence showed that *Fv/Fm* ratio was close to zero in the *lpa2lpa3* double mutant (Fig. 5A).

Next we examined the effects of *lpa2* and *lpa3* double mutation on the steady state levels of photosynthetic proteins by immunoblot analysis. For immunoblot analysis, we grew the mutant and wild type plants under 20 μmol m⁻² s⁻¹ illumination to avoid photobleaching. Immunoblot analysis showed that PSII proteins D1, D2, CP47 and CP43 were hardly detectable in *lpa2lpa3* (Fig. 5B). The amounts of LHCII, Cytf and CF1 were not reduced, but the contents of PsaA/B were about 70% of wild type levels. Since the assembly of newly synthesized CP43 into PSII was slowed in either *lpa2* or *lpa3* mutants, we also performed *in vivo* protein labeling analysis to check the functional relationship of LPA2 and LPA3 in this assembly process. Pulse labeling for 20 min resulted in most of the radioactivity being incorporated into CP43-free PSII, and there were no improvements in the assembly process after a 30-min chase in the *lpa2lpa3* double mutant (Fig. 5C). These findings show that there is a block in the assembly of PSII complexes from CP43-free PSII complexes in the *lpa2lpa3* double mutant.

In subsequent analyses we investigated the effects of mutations affecting one of these proteins on the stability of the other factor. The amount of LPA3 associated with the thylakoid membrane was greatly reduced in the *lpa2* mutant, whereas the amount of LPA2 was unaffected in the *lpa3* mutant. In contrast, the total cellular LPA3 protein
content of the *lpa2* mutant was similar to wild-type levels (Fig. 5D).

**Discussion**

PSII is a crucial, complex component of the photosynthetic machinery. Its biogenesis and assembly proceed via a series of steps that appear to be regulated by an intricate array of interacting factors. Identification of these factors and clarification of their functions will undoubtedly enhance our understanding of the processes involved in the assembly of photosynthetically active multicomponent membrane protein complexes. In the present study we described the isolation and characterization of the *lpa3* high-chlorophyll-fluorescence mutant of *Arabidopsis*, in which the efficient assembly of PSII is hampered. Evidence regarding the role of LPA3 in assisting CP43 assembly and the cooperative function of LPA3 with LPA2 in PSII assembly is also presented.

**LPA3 Is Required for PSII Assembly**

Several key regulatory processes, including transcription, transcriptional stabilization, translation, and assembly, may be involved in the regulation of PSII levels (Wollman et al., 1999; Barkan and Goldschmidt-Clermont, 2000; Rochaix, 2001). Northern blot analysis demonstrated that the *LPA3* mutation does not affect levels of PSII transcripts (Fig. 1). These results suggest that the reductions in PSII may be regulated at a post-transcriptional level.

One of the possible roles of the LPA3 protein is that it may be required for the synthesis of one or more PSII proteins, since it has been shown that loss of any genes coding for PSII core proteins will profoundly affect levels of other components (Jensen et al., 1986; de Vitry et al., 1989; Yu and Vermaas, 1990; 1993). Accordingly, our *in vivo* labeling experiments showed that the synthesis rates of the PSII protein CP43 were dramatically decreased in the *lpa3* mutant, although those of the PSII core proteins D1, D2 and CP47 were not affected (Figs. 2A and B). In addition, polysome association analysis indicated that LPA3 is not required for the initiation of translation or early stages of translation elongation. The incorporation of radioactivity into CP43 increased by 2-3 folds after shortening the pulses from 20 min to 10 min, which suggests rapid degradation of CP43 in the mutant. The increased degradation of newly synthesized CP43 observed in the *lpa3* mutant may be regulated by the state of PSII protein complex assembly, as it has been well demonstrated that rapid degradation of core
protein occurs when it cannot be efficiently assembled into the PSII protein complex (Jensen et al., 1986; de Vitry et al., 1989; Yu and Vermaas, 1990). Indeed, the assembly of PSII complexes was less efficient in lpa3 than in wild-type plants (Fig. 2E), although functional PSII complexes were assembled in the lpa3 mutants, as shown by in vivo chloroplast protein labeling experiments (Fig. 2E) and their ability to grow photoautotrophically (Fig. S1). It is possible that rapid cotranslational, or early post-translational, degradation of CP43 in the absence of LPA3 may affect CP43 synthesis without altering translation.

Interestingly, the PSII protein CP43 was found at ca. 25% of wild-type levels in lpa3 (Fig. 1), although in the synthesis analyses the amount of labeled CP43 was well below 10% of wild-type levels (Fig. 3). This suggests that newly synthesized CP43 polypeptide is very rapidly degraded, by a co- or early post-translation degradation process. The greatly reduced CP43 synthesis rate may also suggest that translation elongation was slowed down in the mutant because of similar pattern of psbC transcript loading on polysome between the wild-type and mutant plants. The assembly-dependent regulation of translation elongation has been observed in a chloroplast translation system, where the PSII D1 protein translation elongation was greatly inhibited when the proper interactions of D1 and D2 were prevented (Zhang et al., 2000). Alternatively, considering the similar protein labeling pattern in very young seedlings and mature leaves (Fig. 3), it is possible that PSII repair rather than biogenesis is affected in the mutant, and the immunoblots revealed rates of biogenesis while the labeling following pulses primarily detected subunits that were destined to replace previously formed subunits.

Cooperation between LPA2 and LPA3 Is Essential for PSII Assembly

The analyses described above demonstrated that LPA3 is required for the efficient assembly of PSII, but the mechanisms involved remained to be elucidated. One possibility we explored is that LPA3 may be an integral constituent of PSII, since the assembly of PSII has been shown to be perturbed in several PSII mutants that lack specific subunits (de Vitry et al., 1989; Wollman et al., 1999; Suora et al., 2004; Dobáková et al., 2007). However, our sucrose gradient fractionation analysis showed that LPA3 is not an intrinsic component of the PSII complex (data not shown). Another possibility is that LPA3 transiently interacts with some PSII subunits, in a similar
manner to several assembly factors that have been shown to interact with specific subunits of the photosynthetic protein complexes and facilitate their assembly, but are not stably associated with the final functional complexes (Naver et al., 2001; Peng et al., 2006; Ma et al., 2007). Indeed, yeast two-hybrid analysis of protein interactions provided direct evidence that LPA3 specifically interacts with CP43 (Fig. 4A). However, such interactions between these proteins were not detected in co-immunoprecipitation analysis (Fig. 4B). The possibility for the absence of co-immunoprecipitation is that the interactions might be less stable or shorter in duration during the co-immunoprecipitation assays. Regardless of the reasons for the lack of stable interactions, the apparent transience of the interaction between LPA3 and CP43 suggests that LPA3 probably assists, through membrane association, the correct folding and assembly of CP43 into PSII complexes containing D1, D2 and CP47 proteins.

LPA3, together with the previously identified LPA2 (Ma et al., 2007), has been shown to assist CP43 assembly, raising questions about the functional relationship between LPA2 and LPA3 in the PSII assembly process. Our results showed that LPA2 directly interacts with LPA3 (Fig. 4), indicating that LPA2 and LPA3 may form a complex that regulates the insertion of CP43 into PSII. In addition, the seedling-lethal phenotype of the \textit{lpa2lpa3} double mutant (Fig. 5) suggests that LPA2 and LPA3 function redundantly in CP43 assembly. Further analysis of the steady state PSII protein levels and assembly of newly synthesized protein into PSII provided evidence that LPA2 and LPA3 play cooperative roles in CP43 assembly. Overall, these results suggest that LPA2 and LPA3 are both essential for PSII assembly.

LPA2 is an intrinsic membrane protein, while LPA3 is an extrinsic protein associated with the thylakoid membranes (Fig. 3). Tight association between LPA3 and membranes may make it available for interactions with another PSII assembly factor, LPA2, during the PSII assembly process. However, the association between LPA3 and the thylakoid membrane was decreased in \textit{lpa2} mutants (Fig. 5), but LPA3 could still assist PSII assembly by interacting with CP43, albeit at reduced efficiency, as manifested by reductions in levels of PSII supercomplexes in the mutants (Ma et al., 2007). The tight association of several RNA-binding proteins with the membranes has been implicated in efficient translation of chloroplast proteins (Trebitsh et al., 2001; Ossenbühl et al., 2002; Schult et al., 2007). In the present study we detected LPA3 in both stroma and membrane thylakoid fractions, suggesting that dynamic partitioning...
between the stroma and membrane may be an important modulator of the efficiency of PSII assembly. Notably, increased association of LPA3 with the membranes under high-light conditions may be important in promoting efficient PSII assembly, since degradation of PSII is enhanced by increases in light intensity, so increased rates of protein synthesis and assembly are required for the maintenance of photosynthetic activities (Aro et al., 1993). Since the dynamic regulation of synthesis and assembly of PSII core proteins in responses to changes in light conditions is crucial to ensure efficient photosynthesis (Anderson, 1986), it is possible that LPA3 may be involved in these regulatory responses via its light-dependent association with the membrane.

Role of Alb3 in PSII Assembly
Alb3/Oxa1/YidC proteins have important functions in the insertion, folding and assembly of membrane proteins in diverse biological systems (Kuhn et al., 2003; van der Laan et al., 2005; Kiefer and Kuhn, 2007). In Arabidopsis, alb3 mutants have been seen to display an albino phenotype with reduced amounts of thylakoid membranes, suggesting that Alb3 plays an essential role in thylakoid biogenesis (Sundberg et al. 1997). The Alb3 protein has been shown to be required for the insertion of light-harvesting proteins into thylakoid membranes (Moore et al., 2000). In addition, Alb3 has been demonstrated to interact directly with the D1, D2 and CP43 PSII core proteins, the PSI protein PsaA, and the ATPase subunit CFoIII (Pasch et al., 2005). Alb3 has also been shown to be involved in the assembly of D1 into PSII (Bellafiore et al., 2002). The role of Alb3 in the assembly of PSII raises questions about how it interacts with its substrate proteins and whether additional factors are required to assist this process. We found that Alb3 directly interacts with LPA2 and LPA3 (Fig. 4), both of which assist CP43 assembly within PSII. The interaction of LPA2 with CP43 was similar to that of LPA3, as seen in the yeast two-hybrid analysis, but the stable interaction between them was not detected in the co-immunoprecipitation analysis (Fig. 4). It may be speculated that LPA2/LPA3 transiently interacts with CP43 and subsequently pass it to Alb3. Thus, the function of Alb3 in some of the PSII assembly processes may be mediated through interactions with LPA2 and LPA3, ensuring that correct interactions occur during the assembly of multimeric PSII complexes in thylakoid membranes.
Materials and methods

Plant Materials and Growth Conditions

*Arabidopsis thaliana* (ecotype *Columbia*) plants were grown in a growth chamber under a constant temperature of 22 °C and a 10-h photoperiod (photon flux density, 120 µmol m⁻² s⁻¹). The lpa3 mutant was isolated from a collection of pSKI015 T-DNA-mutagenized *Arabidopsis* lines as recessive, high-chlorophyll-fluorescence plants. The double mutant lpa2lpa3 was obtained by PCR screening of an F2 population from crossed single mutant lines. In the immunoblot and pulse-chase labeling experiments involving the lpa2lpa3 mutant, the wild-type and mutant plants were grown at ~20 µmol m⁻² s⁻¹ to avoid photobleaching.

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was determined by dark-adapting plants for 30 min prior to measurements, which were performed with a PAM-2000 portable chlorophyll fluorometer (Walz, Effeltrich, Germany). The minimum fluorescence yield (*Fo*) was measured under light (650 nm) of very low intensity (0.8 µmol m⁻² s⁻¹). The maximum fluorescence yield (*Fm*) was determined by applying a saturating pulse of white light (6000 µmol m⁻² s⁻¹ for 0.8 s), and the ratio of variable (*Fv*) to maximum (*Fm*) fluorescence [*Fv/Fm= (Fm-Fo)/Fm*] was then calculated. The light-induced P700 absorbance at 820 nm was measured using a PAM chlorophyll fluorometer equipped with an ED 800T emitter-detector unit (Walz, Effeltrich, Germany), according to Meurer et al. (1998). The contribution of plastocyanin was corrected for by taking the difference between the transmission signals of plastocyanin and P700, through simultaneous measurement of the signals acquired using a Dual PAM system (Dual-PAM-100, Heinz Walz, Effeltrich, Germany). Measurements were performed on pre-illuminated *Arabidopsis* leaves with a fully activated Calvin cycle to exclude any acceptor-side limitation of P700 oxidation. PSI was selectively excited by photo-oxidizing P700 and plastocyanin with far-red light for 10 s. A saturating red light pulse (6000 µmol m⁻² s⁻¹, 100 ms duration) was then applied to completely photooxidize plastocyanin and P700, and allows their subsequent reduction after the end of the light pulse. Hence, the maximal transmission changes between fully oxidized and fully reduced P700 were obtained. The PSI content was obtained after normalizing of
transmission changes according to chlorophyll contents.

Chlorophyll a fluorescence images were captured in planta at room temperature, using a chlorophyll fluorescence imaging system (CF imager; Technologica). Image data obtained in each experiment were normalized to a false color scale, resulting in the highest and lowest $F_v/F_m$ values being represented by the red and blue extremes of the color scale, respectively.

**BN/SDS-PAGE and Protein Analysis**

BN-PAGE was performed as described by Schägger et al. (1994). Thylakoid membrane preparations, equivalent to 10 μg chlorophyll, were solubilized by mixing with 1% DM in 20% (w/v) glycerol and 25 mM BisTris-HCl (pH 7.0), to a final concentration of 0.5 mg chlorophyll/mL. Samples were incubated at 4°C for 5 min, centrifuged at 15,000 g for 10 min, and finally loaded onto a separation gel with a 6 to 12% acrylamide gradient.

The proteins were separated in 15% SDS polyacrylamide gels containing 6 M urea, prior to transference to a nitrocellular membrane. Immunodetection using specific antibodies was performed with the enhanced chemiluminescence method, according to standard techniques. X-ray films were scanned and analyzed using the AlphaImager™ 2200 system (Alpha Innotech, San Leandro, CA).

Polyclonal antibodies were raised against the soluble parts of LPA3 (amino acids 71 to 190). The corresponding cDNA fragments were cloned into pET28a, transformed into *Escherichia coli* strain BL21 cells, and the overexpressed proteins were induced by 0.6 mM isopropylthio-β-D-galactoside for 5 h. The proteins were purified on a Ni-NTA resin matrix using a nickel affinity column, and polyclonal antibodies were raised in rabbit using purified antigen.

**In Vivo Labeling of Chloroplast Proteins**

In vivo protein labeling was essentially performed according to Meurer et al. (1998). Primary leaves from 12-d-old and 5-wk-old *Arabidopsis* seedlings were preincubated in 20 μg/mL cycloheximide for 30 min prior to radiolabeling with 1 μCi/μL $^{35}$S]Met (specific activity >1000 Ci/mmol; 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 methionine. After labeling, thylakoid membrane...
proteins (2 μg chlorophyll) were separated by SDS-PAGE as described in Peng et al. (2006). For autoradiography, gels were stained, dried, and exposed to X-ray films.

**Cloning of LPA3 and Mutant Complementation**

The lpa3 mutation was mapped using molecular markers based on a simple sequence length polymorphism (Lukowitz et al., 2000). Genomic DNA was isolated from F2 plants derived from a cross between lpa3 (genetic background, Columbia) and wild-type plants (Landsberg erecta). Homozygous F2 plants (lpa3lpa3) exhibiting high-chlorophyll-fluorescence phenotypes were selected as previously described (Peng et al., 2006). Analysis of 643 F2 plants indicated that the lpa3 gene was located on chromosome 1 in a 300-kilobase region between the two markers nga111 (~27.3Mb) and ATPASE (~28.5Mb). Genomic sequences of genes containing predicted plastid-targeting signals were then compared between lpa3 and wild-type plants.

For complementation of the lpa3 mutation, cDNAs containing the LPA3 coding regions were amplified by PCR using the primers: 5′-CGTCTCTAGAATGGCGCTACAAATCCACTCTCCGT-3′ and 5′-CCGGTCTCGAGTCACTCTTGACCCTTCATTTTCTC-3′. The resulting PCR product was cleaved with XbaI and XhoI, and then subcloned into pBI121 under the control of the cauliflower mosaic virus 35S promoter. The construct containing LPA3 was then transformed into the Agrobacterium tumefaciens C58 strain and introduced into Arabidopsis lpa3 mutant plants (Clough and Bent, 1998). The transgenic plants were initially grown on Murashige and Skoog medium containing 40 μg/mL kanamycin monosulfate, then the resistant plants were transferred to soil and grown in a growth chamber to produce seeds. The success of the complementation was confirmed by chlorophyll fluorescence analysis.

**Immunolocalization Analysis**

Intact chloroplasts were isolated according to Munekage et al. (2002). Briefly, Arabidopsis leaves were homogenized in 330 mM sorbitol, 20 mM Tricine-NaOH, pH 7.6, 5 mM EGTA, 10 mM Na2CO3, 0.1% (w/v) BSA, and 330 mg/l ascorbate prior to filtration. The samples were then centrifuged for 5 min at 2000 g and each resultant pellet was resuspended in 330 mM sorbitol, 20 mM HEPES-KOH, pH 7.6, 5 mM MgCl2, 10 mM Na2CO3, 0.1% (w/v) BSA, and 2.5 mM EDTA. Intact chloroplasts were
then isolated through a 40%/70% step percoll gradient. Total leaf protein, intact chloroplasts, chloroplast stroma, and thylakoid membrane proteins were separated by SDS-PAGE, and the resolved proteins immunodetected with specific antibodies.

The association of LPA3 with the thylakoid membranes was essentially investigated according to Sun et al. (2007). The thylakoid membranes were suspended to a final concentration of 0.1 mg chlorophyll/mL in 10 mM Hepes-KOH, pH 8.0, 10 mM MgCl₂, 330 mM sorbitol, and 1 mM PMSF supplemented with 1 M NaCl, 1 M CaCl₂, 0.1 M Na₂CO₃, or 6 M urea. Membrane preparations without supplements were used as controls.

To examine the association of LPA3 with the thylakoid membranes under different light intensities, the chloroplast stroma and thylakoid membrane proteins were isolated from Arabidopsis plants that had been subjected to illumination at 20, 120, 1000 µmol m⁻² s⁻¹ for 2 h after growth for 5 weeks under standard growth conditions (see above).

Nucleic Acid and Polysome Analysis

Total plant RNA was extracted from fresh leaf tissues using the Trizol isolation reagent, then RT-PCR was performed to determine LPA3 expression levels, using the following specific primers, based on genomic sequence information: 5’-CAATTCTCTAGCTTTCGCTCTCCAG-3’ in the first exon and 5’-AAATTGAGAAAGAAACCGGTAGTGC-3’ in the seventh exon, spanning a fragment with a predicted size of 565bp for wild-type samples. After separation in an agarose gel, the DNA products (one band from wild type and two bands from lpa3 mutant samples) were recovered from the gel and directly sequenced. The amount of RNA loaded in each sample was monitored by RT-PCR analysis of the level of actin cDNA, using the following primers: 5’-AACTGGGATGATATGGAGAA-3’ and 5’-CCTCCAATCCAGACACTGTA-3’.

Polysomes were isolated from leaf tissues under conditions that maintained polysome integrity, according to Barkan (1988). Briefly, RNA was isolated, fractionated, and transferred onto nylon membranes. The filters were hybridized with ³²P-labeled cDNA probes, then exposed to an 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 PCR primer sequences used in
chloroplast gene amplifications were obtained from Peng et al. (2006).

**Yeast Two-hybrid Assays**
Yeast two-hybrid assays were performed using the yeast strain NMY32 supplied by Dualsystems Biotech (Stagljar et al., 1998). The mature full-length \(LPA1, LPA2, LPA3, Alb3,\) CP47, CP43, D1, D2, PsaA and PsaB genes were cloned into the Cub bait vector, and the \(LPA1, LPA2,\) and \(LPA3\) genes were cloned into the NubG prey vector. Interactions between them were then determined by growing diploid yeast colonies on SD-His-Leu-Trp plates prior to measuring their \(\beta\)-Gal activity using the X-Gal filter assay described by Stagljar et al. (1998).

**Protoplast Transformation and BiFC Assay**
Protoplasts were isolated from 5-wk-old Arabidopsis rosette leaves grown as previously described (Asai et al., 2002). Full-length cDNAs were cloned into pSAT4A-nEYFP-N1 and pSAT4A-cEYFP-N1, and plasmids were co-transformed into protoplasts (Citovsky et al., 2006). YFP fluorescence was observed under a confocal laser scanning microscope (LSM 510 Meta, Zeiss).

**Co-immunoprecipitation Analysis**
For co-immunoprecipitation, thylakoid membranes were solubilized with 1% (w/v) DM in 20% glycerol (w/v), 25mM BisTris-HCl, pH 7.0, and 1mM PMSF for 15min at 4°C, centrifuged at 15,000g for 10 min, and the supernatant was then incubated with antibodies for 3h at 4°C. Subsequently, 10μl of Protein A-Sepharose beads (Fast Flow; Amersham Pharmacia) were added and incubation continued overnight. The sepharose beads were washed three times with 0.03% DM in 50 mMTris-HCl, pH 7.5, 100mM NaCl, and 1mM EDTA buffer, and the precipitated proteins were dissociated in SDS sample buffer by heating at 95°C for 5min. Following SDS-PAGE, the precipitated proteins were subjected to immunoblot analyses.

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

**Figure S1.** Phenotypes of Wild-type, \(lpa3\), and the Complemented Plants

**Figure S2.** Spectroscopic Analysis of Wild-type, \(lpa3\) Mutant and the
Complemented Plants.

Figure S3. BN Gel Analysis of Thylakoid Membrane Protein Complexes.

Figure S4. Amino Acid Sequence Alignment of LPA3.

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Figure legends

Figure 1. Accumulation of chloroplast proteins, chloroplast transcripts and polysome association.

(A) Immunodetection of thylakoid proteins (10 μg chlorophyll) from 5-wk-old wild-type and *lpa3* mutant leaves grown under normal conditions, using specific antibodies for the indicated thylakoid protein complexes.

(B) RNA gel blot analysis of chloroplast mRNA from leaves of wild-type and *lpa3* plants. Chloroplast mRNAs were detected with the probes indicated to the right of the figure.

(C) Association of *psbA*, *psbC*, *psaA/B* with polysomes. Intact polysomes fractionated by ultracentrifugation and their associated mRNAs were analyzed by RNA blotting using the probes indicated to the left of the figure. rRNAs were detected by ethidium bromide staining.

Figure 2. *In vivo* synthesis and assembly of chloroplast proteins.

(A) Labeling of chloroplast proteins in 5-week-old *Arabidopsis* leaves following a 20-min *in vivo* pulse of [35S]Met in the presence of cycloheximide.

(B) and (C) *In vivo* pulse labeling of chloroplast proteins in 12-d-old *Arabidopsis* seedlings. 12-d-old *Arabidopsis* seedlings were pulse-labeled for 20 min (B) and 10 min (C) in the presence of cycloheximide.

(D) Pulse and chase labeling of chloroplast proteins in 12-d-old seedlings. A 20-min pulse of labeling in young *Arabidopsis* seedlings in the presence of cycloheximide was followed by 0.5, 1, 2, or 4 h chases with cold Met.

(E) Incorporation of 35S-methionine into PSII complexes in wild-type and *lpa3* mutant plants. A 10-min pulse in *Arabidopsis* 12-d-old seedlings in the presence of cycloheximide was followed by a chase of cold Met for 15, 30 or 60 min. Bands, indicated to the right, correspond to PSII supercomplexes in various assembly states: PSII supercomplexes; monomeric PSI superimposed on the PSII dimer; monomeric PSII; CP43-free PSII monomer; RC, reaction center; and free proteins. After pulse labeling (A-C) or pulse labeling with chases (D-E), the wild-type and *lpa3* thylakoid membrane protein samples with equal amounts of chlorophyll were separated by SDS-urea-PAGE (A-D) or BN-PAGE (E) and then visualized autoradiographically.
**Figure 3.** Identification of the *lpa3* mutation and localization of LPA3.

(A) Schematic diagram of the *LPA3* gene. Exons (boxes) and introns (lines) are indicated. ATG indicates the start codon, and TGA the stop codon. Direct sequencing of the genomic sequence revealed a G to C mutation at nucleotide position 1081 bp of the *At1g73060* gene relative to the ATG codon. The diagram is not drawn to scale.

(B) RT-PCR analysis of mutant plants, using specific primers for *At1g73060* and actin.

(C) Immunoblot analysis of LPA3, using anti-LPA3 antibodies, in thylakoid or total protein samples from wild-type, *lpa3* and complemented mutant plants.

(D) Immunolocalization analysis of LPA3. Intact chloroplasts (Chl) were isolated from wild-type leaves and then separated into stroma (Str) and thylakoid (Thy) membrane fractions. Total protein samples (Total) were also used. Polyclonal antisera were used against the abundant stroma protein ribulose biphosphate carboxylase large subunit (RbcL), the integral membrane protein D2, the mitochondrial NDUFS1 protein and LPA3. Sample loading was standardized by protein content (50 μg).

(E) Association of LPA3 with the thylakoid membranes. Thylakoid membrane preparations were treated with 1M NaCl, 1M CaCl2, 0.1 M Na2CO3 or 6 M urea, and the proteins were then separated by SDS-PAGE and immunodetected with anti-LPA3. The peripheral protein CF1β was used as a marker. CK, thylakoid membrane samples without treatment.

(F) Immunoblot analysis of LPA3 under different light intensities. After growth at 120 μmol m⁻² s⁻¹ (GL) for 5 weeks, *Arabidopsis* plants were exposed for 2 h to 20 μmol m⁻² s⁻¹ (LL), and 1000 μmol m⁻² s⁻¹ (HL). Thylakoids (Thy) and chloroplast stroma (Str) were isolated and proteins were subjected to immunoblot analysis following SDS-PAGE. X-ray films were scanned and analyzed using the AlphaImager 2000 documentation and analysis system. Percentages of protein levels shown below the lanes in the stroma and thylakoid fractions under HL and LL were estimated by comparison with levels found in corresponding samples obtained from plants grown under GL. Similar results were obtained in four additional independent experiments. The values that are followed by a different letter for LPA3 in stroma (a, b, c) and in membranes (d, e, f) are significantly different, respectively, at P < 0.05 using Student’s test.

**Figure 4.** Characterization of protein interactions.

(A) Growth and β-galactosidase (β-gal) activity of cells expressing X-Cub-LexA-VP16
(where X represents CP47, CP43, D1, D2, or Alb3) with NubG-LPA3 fusion proteins. Yeast cells containing the indicated combinations of bait and prey proteins were spotted on SD-His-Leu-Trp plates and incubated at 30 °C for 3 d. The activity of β-gal was visualized by transferring cells to Whatman filters, permeabilizing, and incubating them in the presence of X-Gal.

(B) Co-immunoprecipitation assays. Thylakoid membranes (50µg chlorophyll) solubilized by 1% DM were incubated with preimmune serum and an excess of antibodies against LPA1, LPA2, or LPA3. After immunoprecipitation, the proteins were separated by SDS-PAGE and then analyzed by immunoblotting with the respective anti-LPA1, LPA2, LPA3, CP43 and Alb3 antibodies. A sample of thylakoid membranes, equivalent to 2.5µg of chlorophyll, was loaded simultaneously. Similar results were obtained in two additional independent experiments.

(C) Interactions between LPA1, LPA2, and LPA3 in yeast. As a positive control, NMY32 was transformed with the NubI-Alg5 expression plasmid, and NMY32 was transformed with the plasmid expressing NubG-Alg5 as a negative control.

(D) BiFC analysis of the interactions between Alb3, LPA1, LPA2, and LPA3 in planta. Plasmids encoding fusion constructs with the N- or C-terminal part of YFP (YFP-N or -C) were transiently expressed in Arabidopsis protoplasts. YFP fluorescence indicates a direct interaction in planta localized to the chloroplasts.

Figure 5. Analysis of lpa2lpa3 and wild-type plants.

(A) Phenotypes and chlorophyll fluorescence imaging of 5-wk-old mutant and wild-type plants. (a) shows photograph of a representative set of seedlings, with genetic backgrounds indicated to the top left of each panel. Corresponding chlorophyll fluorescence image is shown in (b) directly beneath each photograph. The red and blue extremes of the color scale presented in the bottom right indicate Fv/Fm values ranging from 0 (blue) to 0.9 (red).

(B) Immunodetection of thylakoid proteins (10 µg proteins) from 5-wk-old wild-type and lpa2lpa3 mutant leaves grown under low-intensity light (~20 µmol m⁻² s⁻¹), using specific antibodies for the indicated thylakoid proteins.

(C) Incorporation of ³⁵S-methionine into PSII complexes in wild-type and lpa2lpa3 mutant plants. The 20-min pulse labeling in 12-d-old Arabidopsis seedlings grown at 20 µmol m⁻² s⁻¹ was followed by a chase of cold Met for 30 min. Thylakoid membranes
from the wild-type and \textit{lpa2lpa3} mutant leaves were separated by BN-PAGE and visualized by autoradiography. Bands corresponding to complexes in various assembly states, including PSII supercomplexes, monomeric PSI superimposed on the PSII dimer, monomeric PSII, CP43-free PSII monomer, reaction center (RC), and free proteins, are indicated to the right.

(D) Immunoblot analysis of thylakoid samples and total cellular proteins from wild-type, \textit{lpa1}, \textit{lpa2}, and \textit{lpa3} plants using specific anti-LPA1, anti-LPA2, and anti-LPA3 antibodies.
Figure 1. Accumulation of Chloroplast Proteins, Chloroplast Transcripts and Polysome Association. (A) Immunodetection of thylakoid proteins (10 μg chlorophyll) from 5-wk-old wild-type and lpa3 mutant leaves grown under normal conditions, using specific antibodies for the indicated thylakoid protein complexes. (B) RNA gel blot analysis of chloroplast mRNA from leaves of wild-type and lpa3 plants. Chloroplast mRNAs were detected with the probes indicated to the right of the figure. (C) Association of psbA, psbC, psaA/B with polysomes. Intact polysomes fractionated by ultracentrifugation and then associated mRNAs were analyzed by RNA blotting using the probes indicated to the left of the figure. rRNAs were detected by ethidium bromide staining.
Figure 2. *In Vivo* Synthesis and Assembly of Chloroplast Proteins. (A) Labeling of chloroplast proteins in 5-week-old Arabidopsis leaves following a 20-min in vivo pulse of [35S]Met in the presence of cycloheximide. (B) and (C) *In vivo* pulse labeling of chloroplast proteins in 12-d-old Arabidopsis seedlings. 12-d-old Arabidopsis seedlings were pulse-labeled for 20 min (B) and 10 min (C) in the presence of cycloheximide. (D) Pulse and chase labeling of chloroplast proteins in 12-d-old seedlings. A 20-min pulse of labeling in young Arabidopsis seedlings in the presence of cycloheximide was followed by 0.5, 1, 2, or 4 h chases with cold Met. (E) Incorporation of 35S-methionine into PSII complexes in wild-type and *lpa3* mutant plants. A 10-min pulse in Arabidopsis 12-d-old seedlings in the presence of cycloheximide was followed by a chase of cold Met for 15, 30 or 60 min. Bands, indicated to the right, correspond to PSII supercomplexes in various assembly states: PSII supercomplexes; monomeric PSI superimposed on the PSII dimer; monomeric PSII; CP43-free PSII monomer; RC, reaction center; and free proteins. After pulse labeling (A-C) or pulse labeling with chases (D-E), the wild-type and *lpa3* thylakoid membrane protein samples with equal amounts of chlorophyll were separated by SDS- or PAGE (A-D) or BN-PAGE (E) and then visualized autoradiographically.
Figure 3. Identification of the *lpa3* Mutation and Localization of LPA3. (A) Schematic diagram of the *LPA3* gene. Exons (boxes) and introns (lines) are indicated. ATG indicates the start codon, and TGA the stop codon. Direct sequencing of the genomic sequence revealed a G to C mutation at nucleotide position 1081 bp of the *Atlg73060* gene relative to the ATG codon. The diagram is not drawn to scale. (B) RT-PCR analysis of mutant plants, using specific primers for *Atlg73060* and actin. (C) Immunoblot analysis of LPA3, using anti-LPA3 antibodies, in thylakoid or total protein samples from wild-type, *lpa3*, and complemented mutant plants. (D) Immunolocalization analysis of LPA3. Intact chloroplasts (Chl) were isolated from wild-type leaves and then separated into stroma (Str) and thylakoid (Thy) membrane fractions. Total protein samples (Total) were also used. Polyclonal antisera were used against the abundant stroma protein ribulose biphosphate carboxylase large subunit (RubL), the integral membrane protein D2, the mitochondrial NDUF51 protein and LPA3. Sample loading was standardized by protein content (50 μg). (E) Association of LPA3 with the thylakoid membranes. Thylakoid membrane preparations were treated with 1 M NaCl, 1 M CaCl2, 0.1 M Na2CO3, or 6 M urea, and the proteins were then separated by SDS-PAGE and immunodetected with anti-LPA3. The peripheral protein CF1β was used as a marker. CK, thylakoid membrane samples without treatment. (F) Immunoblot analysis of LPA3 under different light intensities. After growth at 120 μmol m−2 s−1 (GL) for 5 weeks, Arabidopsis plants were exposed for 2 h to 20 μmol m−2 s−1 (LL), and 1000 μmol m−2 s−1 (HL). Thylakoids (Thy) and chloroplast stroma (Str) were isolated and proteins were subjected to immunoblot analysis following SDS-PAGE. X-ray films were scanned and analyzed using the Alphalanger 2000 documentation and analysis system. Percentages of protein levels shown below the lanes in the stroma and thylakoid fractions under HL and LL were estimated by comparison with levels found in corresponding samples obtained from plants grown under GL. Similar results were obtained in four additional independent experiments. The values that are followed by a different letter for LPA3, atlg73060, and in membranes (d, e, f) are significantly different, respectively, at P < 0.05 using Student’s test.
Figure 4. Characterization of Protein Interactions. (A) Growth and β-galactosidase (β-gal) activity of cells expressing X-Cub-LexA-VP16 (where X represents CP47, CP43, D1, D2, or Alb3) with NubG-LPA3 fusion proteins. Yeast cells containing the indicated combinations of bait and prey proteins were spotted on SD-His-Leu-Trp plates and incubated at 30 °C for 3 days. The activity of β-gal was visualized by transferring cells to Whatman filters, permeabilizing, and incubating them in the presence of X-Gal. (B) Co-immunoprecipitation assays. Thylakoid membranes (50 μg chlorophyll) solubilized by 1% DM were incubated with preimmune serum and an excess of antibodies against LPA1, LPA2, or LPA3. After immunoprecipitation, the proteins were separated by SDS-PAGE and then analyzed by immunoblotting with the respective anti-LPA1, LPA2, LPA3, CP43, and Alb3 antibodies. A sample of thylakoid membranes, equivalent to 2.5 μg of chlorophyll, was loaded simultaneously. Similar results were obtained in two additional independent experiments. (C) Interactions between LPA1, LPA2, and LPA3 in yeast. As a positive control, NMY32 was transformed with the NubI-Alg5 expression plasmid, and NMY32 was transformed with the plasmid expressing NubG-Alg5 as a negative control. (D) BiFC analysis of the interactions between Alb3, LPA1, LPA2, and LPA3 in planta. Plasmids encoding fusion constructs with the N- or C-terminal part of YFP (YFP-N or -C) were transiently expressed in Arabidopsis protoplasts. YFP fluorescence indicates a direct interaction of proteins localized to the chloroplasts.
Figure 5. Analysis of lpa2/lpa3 and Wild-type Plants. (A) Phenotypes and chlorophyll fluorescence imaging of 5-wk-old mutant and wild-type plants. (a) shows photograph of a representative set of seedlings, with genetic backgrounds indicated to the top left of each panel. Corresponding chlorophyll fluorescence image is shown in (b) directly beneath each photograph. The red and blue extremes of the color scale presented in the bottom right indicate Fv/Fm values ranging from 0 (blue) to 0.9 (red). (B) Immunodetection of thylakoid proteins (10 μg proteins) from 5-wk-old wild-type and lpa2/lpa3 mutant leaves grown under low-intensity light (~20 μmol m⁻² s⁻¹), using specific antibodies for the indicated thylakoid proteins. (C) Incorporation of ³⁵S-methionine into PSII complexes in wild-type and lpa2/lpa3 mutant plants. The 20-min pulse labeling in 12-d-old Arabidopsis seedlings grown at 20 μmol m⁻² s⁻¹ was followed by a chase of cold Met for 30 min. Thylakoid membranes from the wild-type and lpa2/lpa3 mutant leaves were separated by BN-PAGE and visualized by autoradiography. Bands corresponding to complexes in various assembly states, including PSII supercomplexes, monomeric PSI superimposed on the PSII dimer, monomeric PSI, CP43-free PSII monomer, reaction center (RC), and free proteins, are indicated to the right. (D) Immunoblot analysis of thylakoid samples and total cellular proteins from wild-type, lpa1, lpa2, and lpa3 plants using specific anti-LPA1, anti-LPA2, and anti-LPA3 antibodies.