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Running Title: Lack of LIL8/PSB33 affects energy transfer

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Deficiency of the Stroma-lamellar Protein LIL8/PSB33 Affects Energy Transfer around Photosystem I in Arabidopsis

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Abbreviations:

BN-PAGE, blue-native polyacrylamide gel electrophoresis

DM, dodecyl maltoside

DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

ELIP, Early light-induced protein

FDAS, fluorescence decay-associated spectra

LHC, light-harvesting chlorophyll-binding proteins

LIL, light-harvesting-like proteins

WT, wild type

Abstract

Light-harvesting-like (LIL) proteins are a group of proteins that share a consensus amino acid sequence with light-harvesting chlorophyll-binding (LHC) proteins. We hypothesized that they might be involved in photosynthesis-related processes. In order to gain a better understanding of a potential role within photosynthesis-related processes, we examined the most recently identified LIL protein, LIL8/PSB33. Recently, Fristedt and coworkers suggested that this protein is an auxiliary photosystem (PS) II core protein which is involved in the PSII supercomplex organization. However, we found that the majority of LIL8/PSB33 was localized in stroma lamellae, where PSI is predominant. Moreover, the PSI antenna sizes measured under visible light were slightly increased in the *lil8* mutants which lack LIL8/PSB33 protein. Analysis of fluorescence decay kinetics and fluorescence decay-associated spectra indicated that energy transfer to quenching sites within PSI was partially hampered in these mutants. On the other hand, analysis of the steady-state fluorescence spectra in these mutants indicate that a population of LHCII is energetically disconnected from PSII. Taken together, we suggest that LIL8/PSB33 is involved in the fine tuning of light harvesting and/or energy transfer around both PSs.

Keywords: Arabidopsis, thylakoids, light-harvesting

Introduction

Photosynthesis is a complex process that involves numerous proteins. In addition to the authentic subunits of photosystems that harvest and transfer light energy and transfer electrons, a variety of proteins are required to maintain photosynthetic activity. For example, regulators of electron flow (Munekage et al., 2004), protease (Järvi et al., 2016; Sakamoto et al., 2003), assembly factors (Chidgey et al., 2014), and enzymes for cofactor biosynthesis (Nagata et al., 2005) are all necessary for photosynthesis. It is anticipated that a number of chloroplast proteins involved in photosynthesis remain to be identified. Functional characterization of these potentially photosynthesis-related proteins is a promising approach to gain a better understanding of novel aspects pertaining to the regulation of photosynthesis.

A group of proteins termed light-harvesting-like (LIL) proteins were recently determined to play essential roles in multiple steps to sustain photosynthesis. The name LIL, which was designated by Jansson (1999), refers to a group of proteins that share a consensus amino acid motif with light-harvesting chlorophyll-binding (LHC) proteins (Engelken et al., 2010; Jansson, 1999; Neilson and Durnford, 2010). The consensus motif typically consists of 25–30 amino acid residues, most of which are composed of hydrophobic residues, and several charged residues are also conserved. Within LHC proteins, these charged residues bind chlorophyll or carotenoids. In contrast, the specific function(s) of the LHC motif in LIL proteins is not well understood.

A variety of LIL proteins have been identified in the genomes of oxygenic photosynthetic organisms. The functions of LIL proteins are best characterized in cyanobacteria, which contain several small LIL proteins (termed Scp or Hlip). It has been recently demonstrated recently that two cyanobacterial proteins (HliC and HliD) contribute to the assembly of photosystem (PS) II (Chidgey et al., 2014; Knoppová et al., 2014). The cyanobacterial

ferrochelatase which catalyzes the last step of heme biosynthesis is also categorized as a LIL protein with a single LHC motif (Sobotka et al., 2008).

Plants contain several LIL proteins. Early light-induced protein (ELIP) was the first LIL protein to be identified (Meyer and Kloppstech, 1984). ELIP is suggested to be involved in photoprotection (Hutin et al., 2003) or to function as a regulator of chlorophyll (Tzvetkova-Chevolleau et al., 2007). An isoform of ferrochelatase possesses an LHC motif (Smith et al., 1994) and catalyzes an insertion of a ferrous ion into protoporphyrin IX. Jansson (1999) identified several novel LIL-encoding genes by analyzing expressed sequenced tags. Some of them have assigned functions; the photosystem (PS) II protein PsbS is involved in pH-dependent regulation of excitation energy (Li et al., 2000). LIL3 functions to stabilize geranylgeranyl reductase, which is involved in phytol biosynthesis (Takahashi et al., 2014; Tanaka et al., 2010). LIL2 (OHP1) and LIL6 (OHP2) are involved in the assembly of PSII (Beck et al., 2017). At the present time, the functions of LIL4 (SEP1) and LIL5 (SEP5) are not well understood. More recently, two additional LIL proteins were identified in plants (Engelken et al., 2010; Neilson and Durnford, 2010). In this study, we focused on one of these LIL proteins that is encoded by the At1g71500 locus in the Arabidopsis genome. This protein was the eighth LIL protein identified in the green lineage of photosynthetic eukaryotes (Engelken et al., 2010). Accordingly, we named this protein LIL8. This protein was recently characterized in detail by Fristedt et al. (2015). Since they suggested that this protein is an auxiliary core protein of PSII, they named this protein PSB33. However, in this study, we show that this protein is mainly localized in stroma lamellae of thylakoid membranes where PSII is less abundant. The results indicate that the function of LIL8 (PSB33) may not be restricted to PSII. Therefore, we refer to this protein as LIL8 hereafter within this manuscript. Fristedt et al (2015) reported that Arabidopsis mutants lacking LIL8 show pleiotropic phenotypes. Specifically, the mutants grow more slowly than the wild

type, and have unstable photosystem (PS) II–LHCII supercomplexes, and also exhibit aberrant patterns of state-transitions. In addition, they showed that LIL8 co-migrates with the dimeric and monomeric forms of PSII in blue-native polyacrylamide gel electrophoresis (BN-PAGE). Accordingly, a role for LIL8 in the regulation and optimization of photosynthesis in response to environmental changes was suggested (Fristedt et al., 2015).

More recently, Cruz et al. (2016) analyzed the phenotypes of a *lil8-2* mutant (alternatively named *psb33-1*) under systematically programmed changing environments. The *lil8-2* (*psb33-1*) mutant did not show obvious phenotypes under the standard growth conditions, but showed altered photosynthetic efficiency when grown under prolonged light fluctuations. The results indicate that LIL8 is required for the response to dynamic light environments.

Our research group has investigated the function of LIL8 independently to explore a novel aspect of photosynthesis regulation through the analysis of LIL proteins. Under several different growth conditions of our laboratory, Arabidopsis mutants lacking LIL8 did not show observable growth retardation in relative comparison to wild type (WT) plants. In addition, PSII–LHCII super-complexes are relatively stable in the mutants in our laboratory conditions. In other words, the mutant phenotypes appeared to be less pleiotropic under our experimental conditions. We hypothesized that the phenotypes under such conditions would be more directly linked to the function of LIL8. Moreover, we observed that LIL8 is mostly localized in the stroma lamellae of thylakoid membranes where PSI operates. Furthermore, we show evidence that energy transfer around PSI is compromised in the LIL8-deficient mutants. Accordingly, we suggest that the function of LIL8 is not only linked to PSII but also to PSI. The function of LIL8 is more likely to be associated with the connectivity of LHCII to both core complexes.

Results

Basic Characterization of the *lil8* Mutants

To improve understanding of LIL8 function, we analyzed two *Arabidopsis* mutants (*lil8-1* and *lil8-2*) in which the *LIL8/PSB33* gene (At1g71500) was disrupted by a T-DNA insertion. The *lil8-1* mutant contained a T-DNA insertion in the 52 bp upstream of the initiation codon of the *LIL8/PSB33* gene (Figure 1A). The *lil8-2* mutant is identical to the *psb33-1* mutant, which contained a T-DNA insertion in the 38 bp upstream of the initiation codon of the *LIL8/PSB33* gene (Figure 1A). The *LIL8* gene is expressed in both young and mature leaves in our standard growth conditions of growth chambers conditions (22°C under a 14 h/10 h light/dark photoperiod with a fluorescent light source [$70 \mu\text{mol m}^{-2} \text{s}^{-1}$]) (Figure 1B); indicating that LIL8 may function under moderate light conditions. In both mutants, *LIL8* mRNA levels were approximately 1/100 of WT (Figure 1B). LIL8 protein levels within the *lil8-1* and *lil8-2* mutants were below the detectable level (Figure 1C).

Both mutants were indistinguishable from the WT under our standard experimental conditions and the composition of photosynthetic pigments was similar between the WT and *lil8* mutant plants (Figure 1D). We noted that the calculated chlorophyll *a/b* ratios for the *lil8-1* (2.97 ± 0.02) and *lil8-2* (3.04 ± 0.13) mutants were slightly lower than that of the WT (3.18 ± 0.06); indicating that the ratios of LHC proteins to the core-complex subunits were slightly higher in the *lil8* mutants.

We also grew the *psb33-3* mutant that was previously analyzed by Fristedt et al. (2015) to enable cross-comparisons under a variety of growth conditions including standard (Figure 2A), fluctuating light (a repeated cycle of $450 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 1 min and $50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 min: Figure 2B), high light ($200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ for two weeks: Figure 2C), and blue light ($130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$: Figure 2D). In addition, the plants were

also grown under short-day conditions (10-h light/14-h dark; data not shown). Under these aforementioned light conditions, the *lil8-1*, *lil8-2* and *psb33-3* plants were indistinguishable from WT. Thus, we concluded that LIL8 is not essential for the growth of the Arabidopsis plants under a wide range of growth conditions.

Localization of LIL8 Protein in Stroma Lamellae

Fristedt et al. (2015) demonstrated that LIL8 is localized in chloroplasts. We further examined the localization of LIL8 in the three domains of thylakoid membranes, namely stroma lamellae, grana core, and grana margins. It is known that PSI is enriched in stroma lamellae, whereas PSII is enriched in the grana core. Both PSI and PSII reside in the grana margins. Accordingly, we detected CP1 (the core subunit of PSI) in both grana margins and stroma lamellae, whereas LHCII was localized in both grana margins and the grana core (Figure 3). In our preparation of thylakoid membranes, LIL8 was enriched in stroma lamellae, though it was detected in the grana core and grana margins. At the present time, it is presently unclear whether the detection of LIL8 in the grana core and grana margins was due to contamination of stroma lamellae in the grana core or grana margin preparations. The possibility that a small population of LIL8 localizes in the grana lamellae cannot be excluded. It should be noted that the migration of LIL8 on a SDS-PAGE gel appeared slightly faster with the preparation of total thylakoids, the grana core and grana margins than with the preparation of stroma lamellae. This was most likely due to the migration of a large amount of LHCII just above LIL8 in these preparations.

Analysis of Native Forms of LIL8

Enrichment of LIL8 in the stroma lamellae preparation was further confirmed by resolving thylakoid proteins in two-dimensional BN-PAGE/SDS-PAGE (Figure 4A). We loaded protein extracts from the grana core, grana margins and stroma lamellae with an equal amount of chlorophyll per lane. To compare the amounts of LIL8 protein in different preparations, we blotted six gels that resolved grana core, grana margins and stroma lamellae proteins from WT and *lil8-1* leaves onto a single polyvinylidene difluoride (PVDF) membrane; which was subsequently immuno-reacted with anti-LIL8 antiserum. In this manner, the transfer efficiency of proteins from gels to PVDF membranes, and the efficiency of the immuno-reaction were equalized to all samples; enabling comparison of signals between the extracts. In addition, this analysis also showed that LIL8 was more abundant in the stroma lamellae fraction than in the other fractions (Figure 4A).

In all three preparations of thylakoid membranes from the WT, LIL8 yielded three spots on immunoblot membranes. The largest spot (labeled 3 in Figure 4A) co-migrated with the PSII monomer, the second largest spot (labeled 2) was located at a smaller (ca. 100 kD) position, and the smallest spot was located at the bottom of the first-dimension BN-PAGE (labeled 1). In addition, we observed that LIL8 appeared to form multiple oligomeric forms trailing over the entire range of the BN-PAGE. Since self-oligomerizing proteins often show such trailing signals on BN-PAGE (Liu et al., 2005), it is possible that LIL8 tends to oligomerize. All of these signals were absent in the preparations from *lil8-1*, showing that these signals represent the LIL8 protein. The position of the largest spot (spot 3 in Figure 4A) is close to that of the PSII monomer or the LHCII assembly complexes which comprises LHCII, Lhcb4 and Lhcb6 (Aro et al., 2005; Järvi et al., 2011).

We further examined the co-migration of LIL8 with the PSII monomer or the LHCII assembly by analyzing the migration profiles of LIL8 and the PSII subunits on the Protein Co-migration Database (PCoM-DB) (Takabayashi et al., 2013) (Figure 4B). This database

present migration profiles of chloroplast proteins on BN-PAGE. In particular, we analyzed data of chloroplast proteins were solubilized by β -dodecyl maltoside (β -DM) and resolved by BN-PAGE. In Figure 4B, the X axis indicates the position of each protein in a strip of a BN-PAGE-gel. The relative amounts of each protein in a single lane were estimated by the exponentially modified protein abundance index (Ishihama et al., 2005). This analysis showed that the peptides of LIL8 and D1 were concentrated in the same BN-PAGE gel slice, confirming that LIL8 and PSII co-migrated in the presence of β -DM. These results indicate a possible interaction of LIL8 with the PSII monomer. On the contrary, LIL8 did not co-migrate with the component of the LHC assembly including Lhcb1, Lhcb4 and Lhcb6 (Figure 4B). Due to the lack of an observed co-migration, these data suggest that LIL8 does not forms a complex with the LHCII assembly in the presence of β -DM.

Subsequently, we further analyzed the co-migration of LIL8 with known photosynthetic complexes by using digitonin which enables even milder solubilization of protein complexes from WT thylakoid membranes than β -DM (Järvi et al., 2011). After resolving digitonin-solubilized protein complexes with 2-dimensional BN-PAGE/SDS-PAGE, we analyzed the co-migration of LIL8 with CP47, Lhcb4, Lhcb6 and CP1 by immunoblotting using respective antisera (Figure 5). For better comparison of the migration profiles of each protein, we cut a single blot into three pieces and stained each separately with different antisera. One blot was stained with anti-CP47, anti-LIL8 and anti-Lhcb6 antisera. Subsequently, the membrane which was stained with an anti-LIL8 antiserum was once washed with a guanidine-HCl-based stripping solution (Yee-Guide Yeung, 2009) and reprobed with anti-Lhcb4 antiserum (Figure 5A). LIL8 formed a spot in at the top of the BN-PAGE (>1,236 kDa) in a position where the PSI-PSII megacomplex migrates (Järvi et al., 2011; Yokono et al., 2015b). Additionally, it formed another spot around 480 kDa, and a putative free form near the front of the BN-PAGE gel. The position of LIL8 around 480 kDa

was close to those of Lhcb4 and Lhcb6, while it was distinct from that of CP47 (Figure 5A). Another blot was also cut into three pieces and stained with anti-CP1, anti-CP47 and anti-LIL8 antisera (Figure 5B). The results show that the migrated position of LIL8 was different from those of CP47 and CP1.

There are two potential explanations for why LIL8 appears to form different complexes in the presence of β -DM or digitonin. A simple explanation is that LIL8 itself forms homo-oligomers and thus, co-migration of LIL8 with either PSII monomer complexes or the LHCII assembly at an approximate 480 kDa size was a coincidence. Another explanation is that LIL8 interacts with the PSI-PSII megacomplexes, and treatment with β -DM or digitonin partly and differentially dissociate the megacomplexes. If this is the case, LIL8 appears to maintain an interaction with the PSII monomer in the presence of β -DM, while it is staying with the LHCII assembly in the presence of digitonin.

Composition of Photosynthetic Complexes

We analyzed the composition of photosynthetic complexes in the WT and the *lil8* mutants by BN-PAGE (Figure 6). For comparison between plants of the three thylakoid fractions, we loaded the same amount of chlorophyll per lane. The amount of PSII–LHCII complexes in the grana core of the *lil8-1* and *lil8-2* mutants was slightly less than that of the WT, which is consistent with the observations of Fristedt et al. (2015). The composition of the photosynthetic complexes was similar between the WT and the *lil8* mutants in the grana margins and stroma lamellae. It should be noted that in the particular image presented in Figure 6, all bands of the grana margin of *lil8-2* appear slightly thicker than the other lanes. This is most likely due to a slightly larger loading of chlorophyll in this lane, which is within usual loading deviations. These results showed that LIL8 is not essential in the assembly of

either PSII or PSI super-complexes, although the absence of LIL8 showed a marginal impact on the PSII–LHC assembly.

Since LIL8 co-migrated with the putative PSI-PSII megacomplexes (Fig. 5), we explored the possibility that LIL8 contributes to the formation of the PSI-PSII megacomplexes by analyzing the amounts of the putative PSI-PSII megacomplexes in the *lil8* mutants with BN-PAGE. The results of three independent experiments in which digitonin-solubilized thylakoid membranes were analyzed with BN-PAGE show that the putative PSI-PSII megacomplexes (the topmost bands) were present in both WT and *lil8* mutant plants (Supplementary Figure 1). However, the intensities of these bands were variable between mutants and between experiments. In some cases, they were sometimes decreased in the *lil8-1* and *psb33-3* mutants, while no changes were detected in the *lil8-2* mutant in all three experiments. We hypothesize that the PSI-PSII megacomplexes are present but they become unstable in the *lil8-1* and *psb33-3* mutants. This assertion is supported by the observations that the stability of these topmost bands during electrophoresis was particularly sensitive to small changes in experimental conditions, such as processing time and temperatures of thylakoid preparation. Due to such technical limitations, we hypothesized that the topmost bands did not precisely reflect the levels of the PSI-PSII megacomplexes in the mutants. Therefore, we proceeded to examine the functionality of the photosynthetic complexes by analyzing chlorophyll fluorescence profiles in the following experiments.

Effects of LIL8 Deficiency on Chlorophyll Fluorescence Parameters

To further understand the effects of LIL8 deficiency, we analyzed several chlorophyll fluorescence parameters. Fristedt et al. (2015) previously reported that *psb33* mutants show a

lower quantum yield of PSII (F_v/F_m) under the growth conditions of their study. Likewise, in the present experimental conditions, the *lil8* mutants showed lower F_v/F_m values with increased F_o levels in comparison to WT (Table 1). These data indicated an increase in an antenna population that is energetically uncoupled from the core complexes and/or an increase in the population of closed PSII reaction centers. The qL and qP values were higher in the *lil8* mutants than the WT under illumination of $475 \mu\text{mol m}^{-2} \text{s}^{-1}$, indicating that the plastoquinone pools were more oxidized in the *lil8* mutants as compared to the WT. A possible explanation for this observation is a relative decrease of the PSII antenna size and/or an increase in PSI antenna size in the *lil8* mutants compared with those of the WT.

Fluorescence Measurement Indicates an Increased Population of Energetically Uncoupled Antennae

We estimated the proportion of energetically uncoupled antennae (so-called free antennae) using the method of Schmitz et al. (2012). In this method, as with the measurements shown in Table 1, the fluorescence yields of open PSII centers before and after qE quenching was induced were compared ($F_o - F_o'$) and represent an indicator of “free” antennae (Figure 7). The results indicated that the proportion of “free” antennae was slightly increased in the *lil8* mutants.

To further investigate the source of increased F_o levels, we compared the chlorophyll fluorescence spectra of WT and mutant leaves that were excited at 408 nm under 77K. As shown in Figure 8, the spectra were decomposed by three spectral components corresponding to LHCII, PSII and PSI (Ruban et al., 1995). Relative fluorescence amplitudes for LHCII were 1.4- and 1.2-times larger in the *lil8-1* and *lil8-2* mutants than in WT. Chlorophyll fluorescence at approximately 682.5 nm (equivalent to 14652 cm^{-1} wavenumber) was slightly

increased in the *lil8* mutants (See insets in Figure 8) as compared to WT. These results were consistent with a slight increase in the energetically uncoupled antennae (see Figure 7).

Altered State Transition Profiles of the *lil8* Mutants Resemble Those of the Lhcb4 Knockout Plant

We measured state transitions using a pulse-amplitude-modulated (PAM) fluorometer by illumination with blue (470 nm) and far-red (720 nm) light that preferentially excites PSII and PSI, respectively (Figure 9). We compared the changes in chlorophyll fluorescence during state transitions between the WT, *lil8-1* and *lil8-2*. In addition, we also included the Arabidopsis Lhcb4-less knockout plant (*lhcb4-ko*) for comparison. In this mutant, all three isoforms of Lhcb4 (CP29) are absent and, thus, the formation of the PSII super-complex is partially impaired (De Bianchi et al., 2011; 2008). Upon illumination with blue light, chlorophyll fluorescence was gradually decreased in the WT, which is explained by an induction of *qE* quenching and state 1 to state 2 transitions. The decrease in chlorophyll fluorescence was moderate in the *lil8-1*, *lil8-2* and *lhcb4-ko* plants, indicating that the plastoquinone (PQ) pool was oxidized to a greater degree in these plants. Upon illumination with far-red light, chlorophyll fluorescence sharply declined in the WT; whereas no decrease was observed in the *lil8-1* and *lhcb4-ko* plants. The decrease was less marked in the *lil8-2* mutant. These results indicated that blue light equally excited PSII and PSI in the *lil8-1*, *lil8-2* and *lhcb4-ko* plants, and thus the addition of far-red light did not change the excitation balance between PSII and PSI. This is partially explained by assuming that part of LHCII is energetically uncoupled from PSII. In addition, it is possible that far-red light excites PSI less efficiently in the *lil8* mutants. After far-red light was switched off, a sharp elevation followed by a more rapid decrease in chlorophyll fluorescence was observed in the *lil8-1*, *lil8-2* and

lhcb4-ko plants. These data indicated that a mechanism which balances PSII and PSI excitation is altered in the three plants. The state transition parameters (qT) reflecting the PSII cross section changes decreased in *lil8-1* and *lhcb4-ko* compared with those of WT (Table 2). Although the fluorescence kinetics were similar in *lil8-2* and the other two mutants, the qT parameters were similar in WT and *lil8-2*. We speculate that *lil8-2* possibly has an unidentified second site mutation which compromised its phenotype. It is important to note that state transitions of the *lil8* mutants were described by Fristedt et al (2015) as well. Their observations with the *psb33-1* (*lil8-2*) and *psb33-3* are apparently in accordance to our observations with *lil8-1* and *lhcb4-ko* in this study.

Energy Transfer Around PSI

The PSI antenna sizes were compared between the WT and the *lil8* mutants by measuring the changes in P700 absorption upon irradiation with far-red light (740 nm; Figure 10A). The PSI antennae that absorb far-red light were not altered in the *lil8* mutants. Far-red-absorbing chlorophylls are mostly present in LHCI (Qin et al., 2015), which indicates that LHCI connected to PSI was not affected by a lack of LIL8.

Subsequently, PSI antenna sizes were compared between the WT and the *lil8* mutants by illumination with red actinic light in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), which preferentially inhibits PSII activity (Figure 10B). In comparison to WT, the PSI antenna sizes under red light were slightly larger in the *lil8* mutants.

We measured the fluorescence decay kinetics by monitoring fluorescence either at 686 nm or 734 nm for PSII and PSI, respectively. The decay kinetics of PSII and PSI were described by the sum of three exponential lifetime components (Table 3). At 686 nm, the WT and the *lil8-1* mutant showed similar values at each lifetime component, indicating that the kinetics

of fluorescence decay was not altered by deficiency in LIL8 protein. In contrast, the fluorescence decay kinetics at 734 nm, which is specific for PSI, differed between WT and the *lil8-1* mutant. Most importantly, the faster decay component (200–270 ps) was decreased in the *lil8-1* mutant, whereas the slower decay component (2.3–2.4 ns) was increased in the *lil8-1* mutant as compared with WT. The amplitude of the faster and slower decay components indicates the numbers of chlorophyll molecules that transfer and do not transfer energy to a quenching site, respectively. Thus, the results indicated that the number of chlorophyll molecules that transfer energy to a presumed quenching site was decreased in the *lil8-1* mutant, whereas the chlorophyll molecules that do not transfer energy to the quenching site were increased in the mutant.

The fluorescence decay-associated spectra (FDAS) were measured in leaves of the WT and the *lil8-1* mutant (Figure 11). The six components shown in Figure 11 were required to describe the fluorescence kinetics. The first component (50–60 ps) showed energy transfer from the “bulk” chlorophyll to red chlorophyll. In both the WT and the *lil8-1* mutant, the spectral patterns were similar. The second component (200–220 ps) indicated energy transfer to a presumed quenching site. It was notable that the amplitude at 730 nm in the second lifetime component (200–220 ps) was smaller in the *lil8* mutant and the WT, which indicated that fewer chlorophyll molecules transferred energy to a quenching site in the *lil8* mutant. In contrast, the amplitudes in the slower lifetime components (2.1 and 3.7 ns) in a long wavelength region (>730 nm) were increased in the *lil8-1* mutant compared with those of the WT; indicating that part of the remaining energy was emitted at around 2.1 and 3.7 ns from red chlorophyll. In this region (>730 nm), the slower lifetime components represents the re-absorption and emission of the delayed fluorescence of PSII by PSI (Yokono et al., 2015b). Existence of these lifetime components indicates the presence of the PSI-PSII megacomplexes in both WT and the *lil8* mutants.

To further analyze the alterations in energy transfer around PSI, we calculated the average lifetime of chlorophyll fluorescence with frozen leaf samples. Since P700+ functions as an effective quencher (Schlödter et al., 2011), we compared the lifetime with dark-adapted leaves and those illuminated with a diode laser at an intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 15 min to generate P700+. The estimated average lifetimes of chlorophyll fluorescence at 735 nm were almost the same in the dark-adapted WT, *lil8-1* and *psb33-3* leaves (Table 4). In contrast, the average lifetimes were faster in illuminated WT leaves than in *lil8-1* or *psb33-3* leaves.

The analysis of fluorescence decay kinetics and FDAS indicated that energy transfer to quenching sites was altered in PSI of the *lil8-1* mutant. Considering that the composition of photosynthetic complexes was indistinguishable in the stroma lamellae of the WT and the *lil8* mutants, we hypothesized that a pool of LHCII that is loosely associated with PSI was affected by deficiency in LIL8 protein.

Discussion

The responses of photosynthesis to a range of environments are more dynamic and complicated than we had originally envisioned. Recently, novel mechanisms for photosynthetic responses continue to be implicated or discovered (Cruz et al., 2016; e.g. Yokono et al., 2015b). Furthermore, functional analyses of thylakoid proteins will also likely contribute to increasing our understanding of the mechanisms underlying photosynthetic responses.

LIL8 is an intriguing protein with a conserved LHC motif. Similar to other LIL proteins with known functions, this protein is expected to be involved in photosynthesis-related reactions.

Fristedt et al. (2015) showed that deficiency in LIL8 retards plant growth and impairs the assembly of PSII–LHCII super-complexes. However, we have never observed obvious growth retardation of the *lil8* mutants under the various growth conditions tested in our laboratory (see Figure 2) nor the drastic reduction of PSII–LHCII super-complexes (see Figure 6). Recently, Cruz et al. (2016) also showed that growth of the *lil8-2* mutant was indistinguishable under the typical light/dark growth conditions that are often used in laboratories. Therefore, we speculate that this protein is involved in the fine regulation of photosynthesis rather than functioning as an essential subunit of PSII–LHCII. Although the possibility that LIL8 specifically functions under extreme light conditions cannot be ruled out, it is reasonable to assume that LIL8 also functions under low-light conditions because LIL8 is abundant in low-light conditions (see Figures 1 and 3). A large-scale analysis of Arabidopsis gene expression under various light conditions showed that the *LIL8* gene is expressed in a constitutive manner regardless of light conditions (Michael et al., 2008). Therefore, it is plausible that LIL8 may function under a wide range of light conditions.

We found that LIL8 is mainly localized in stroma lamellae (Figure 4). In addition, deficiency in LIL8 affected energy transfer around PSI (Figure 11 and Table 4). Both chlorophyll fluorescence decay (Table 3) and FDAS (Figure 11) indicated that the frequency of energy transfer from “bulk” chlorophyll to a quenching site was decreased in the *lil8* mutants compared with WT. These results indicate that LIL8 may be directly or indirectly involved in the regulation of energy transfer around PSI. At the same time, we also confirmed the observation of Fristedt et al. (2015) by an analysis of 77K chlorophyll fluorescence which indicated that the higher F_0 level was due to a fraction of peripheral LHCII antennae that are energetically uncoupled to PSII (Figure 8). Considering that LHCII is not only connected with PSII, but also with PSI, it is possible that LIL8 functions in the control of LHCII dynamics. In this aspect, Benson et al. (2015) reported existence of an extra LHC pool that is

loosely attached to PSs. It is intriguing to hypothesize that LIL8 is involved in the regulation of such an extra LHC pool.

On the other hand, our BN-PAGE analysis with digitonin-solubilized thylakoid membranes (Figure 5) raises a possibility that LIL8 interacts with the PSI-PSII megacomplexes comprising PSI-LHCI and PSII cores with minor and major LHCs (Yokono et al., 2015b). This model would be in line with the report of Fristedt et al. (2015), who showed that the LIL8 protein can be co-immunoprecipitated with PSII subunits. ~~A hypothesis that LIL8 is involved in the regulation of energy transfer or dynamics of PSI-PSII megacomplexes is consistent with our observations that LIL8 deficiency affects energy transfer around PSI (Figure 11 and Table 4) and increases a pool of energetically uncoupled LHCII (Figures 7 and 8).~~

The potential binding of LIL8 to the PSI-PSII megacomplexes allowed us to hypothesize that LIL8 might be involved in the formation of the PSI-PSII megacomplexes. However, two lines of evidence indicate the presence of the PSI-PSII megacomplexes in the *lil8* mutants: First, in the BN-PAGE analysis with digitonin-solubilized thylakoid membranes, we observed the bands near the top of the gel which most likely represent the PSI-PSII megacomplexes in the LIL8-deficient mutants (see Supplementary Figure 1). Secondly, we detected the slow lifetime components in the long wavelength region (>730 nm; see Fig. 11) which likely indicate the energy transfer (spillover) from PSII to PSI (Yokono et al., 2015b). Therefore, we think it is unlikely that LIL8 is involved in the formation of the PSI-PSII megacomplexes. On the other hand, it would be more reasonable to hypothesize that LIL8 is involved in the regulation of LHC dynamics, as the representative phenotypes of the *lil8* mutants (e.g. high F_0 and modified state-transition profiles) could be explained by an impairment in LHC dynamics. In addition, modified PSI energy transfer in the *lil8* mutants could be also explained by assuming that LIL8 deficiency might affect a specific form of the

PSI-PSII megacomplexes. Grieco et al. (2015) hypothesized that two forms exist within PSI-PSII interactions in the PSI-PSII megacomplexes, in which PSI and PSII could directly interact with each other, or their interactions could be mediated by LHC proteins. Based on their model, we also hypothesized that the latter form of the PSI-PSII megacomplexes increases relative to the former form of the PSI-PSII megacomplexes in the *lil8* mutants. Our observation that the PSI-PSII megacomplexes of the *lil8* mutants apparently become more unstable during our CN-PAGE analysis directly support this hypothesis (Supplementary Fig. 1).

Considering that a large amount of LHC (so-called LHCII lake) participates in the energy transfer to both PSI and PSII (Grieco et al., 2015), the control of LHC connectivity has a primary importance to balance the excitation energy distribution between PSI and PSII. Thus, it would be reasonable to assume that plants contain multiple mechanisms to control the LHC connectivity other than the well-known mechanism involving phosphorylation. We hypothesize that LIL8 is involved in one of the regulatory mechanisms of LHC dynamics. Nevertheless, our understanding on the identity of the PSI-PSII megacomplexes and the dynamics of LHC are still limited at this time (Yokono et al., 2015b). Further elucidation of LIL8 function will contribute to an improved understanding of the dynamics of light harvesting that respond to changing environments.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The *lil8-1* mutant (SAIL681_F11) and *lil8-2* (SALK_098173) mutants were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA). The *psb33-3* mutant (GABI_573D02) was obtained from the European Arabidopsis Stock Centre (Nottingham,

UK). For most of the experiments, plants were grown on a mixture of vermiculite (Taiheiyo Coal Services and Transportation Co. Ltd., Kushiro, Japan) and a fertilized soil (Kumiai-Engei-Baidok, Hokusan Co. Ltd., Kita-Hiroshima, Japan) at 23°C under 14-h/10-h (light/dark) photoperiod with a photosynthetic photon flux density of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ supplied by standard fluorescent lamps (Mitsubishi Electric Co. Ltd, Tokyo, Japan). For the isolation of thylakoid membranes, plants were grown under short-day conditions (10-h light/14-h dark) and all other conditions (besides photoperiod) were identical to those used within the long-day conditions as described above. We confirmed that typical chlorophyll fluorescent characteristics were similar under both long-day and short-day conditions (data not shown). Fully expanded leaves from 4- to 5-week-old plants were used for the experiments in which we measured chlorophyll fluorescence or extracted RNA. Senescing leaves were excluded from the analyses. For the isolation of thylakoids, all leaves were used from the plants.

In addition, plants were also grown under a few different light conditions as follows. For fluctuating light conditions, white fluorescent lamps were kept on for 8 hours per day while white LED lamps (LDA9N, Toshiba, Tokyo, Japan) were repeatedly turned on for 1 min in every 5 min during daytime. For high-light conditions, plants were grown for two 2 weeks under standard growth conditions as described above, and then they were then transferred to another growth chamber equipped with high-pressure sodium lamps ($200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; NH450, Toshiba) and kept for 2 weeks. For blue-light conditions, plants were grown under a 12 h blue light source ($130 \mu\text{mol photons m}^{-2} \text{s}^{-1}$; EP30, Edison, Taipei, Taiwan) and a 12 h dark cycle for 4 weeks.

Analysis of Photosynthetic Pigments

Leaves were harvested from 5-week-old plants grown under long-day conditions as described above, and frozen in liquid nitrogen. Pigments were extracted with chilled acetone (-30°C) by mechanical disruption of the leaf tissue. Pigments were separated by high-performance liquid chromatography in accordance with the methods of Zapata et al. (2000) and quantified by spectrometry.

Fractionation of Thylakoid Membranes

Thylakoid membranes were isolated from 7-week-old plants grown under short-day conditions following the method of Järvi et al. (2011). In brief, thylakoid membranes were solubilized at the concentration of $0.8\text{ mg chlorophyll mL}^{-1}$ with 0.8% digitonin (Calbiochem 300410, Merck Millipore, Darmstadt, Germany). Isolated membranes were further fractionated into the grana core, grana margins and stroma lamellae in accordance with the method of Lu et al. (2011) with slight modification. In particular, thylakoid pellets were mixed by gentle stirring with 0.8% digitonin for 10 min at 25°C . Subsequently, the pellet was dissolved in 0.1% digitonin to isolate stroma lamellae.

BN-PAGE Analysis

Thylakoid membranes were isolated as described by Takabayashi et al. (2011). The membranes were suspended in suspension buffer (50 mM imidazole-HCl [pH 7.0], 20% glycerol, 5 mM 6-aminocaproic acid, 1 mM EDTA) to a final concentration of $0.8\text{ mg chlorophyll mL}^{-1}$ (w/v). Membranes were solubilized by adding an equal volume of 2% α -dodecyl maltoside solution for 5 min on ice, and were centrifuged at $18,800 \times g$ at 4°C for 10 min to remove insoluble materials. Next, 5% (v/v) CBB solution, containing 5% Serva

Blue G, 500 mM 6-aminocaproic acid and 50 mM imidazole-HCl, was added to the supernatant; which was then loaded onto a 4–16% polyacrylamide gradient gel. Electrophoresis was performed in accordance with the method of Wittig et al. (2006).

For the co-migration analysis described in Figure 4, thylakoid membranes were solubilized by adding an equal volume of 2% β -DM solution (Takabayashi et al., 2013). For co-migration analysis that is described in Figure 5, thylakoid membranes were solubilized by incubation with an equal volume of 2% digitonin solution for 5 min at 20°C.

The grana core, grana margins and stroma lamellae fractions were solubilized with thylakoid buffer containing 15 mM tricine (pH 7.5), 0.1 M sorbitol, 10 mM NaCl, and 5 mM MgCl_2 to a final concentration of 0.8 mg chlorophyll mL^{-1} . An equal volume of DMG buffer containing 2% α -DM and 20% glycerol was added, and incubated for 5 min on ice. After centrifugation at $18,800 \times g$ at 4°C for 20 min, the supernatant was directly loaded onto a 5–13% polyacrylamide gradient gel.

After electrophoresis, the BN-PAGE gel was cut into strips and incubated in solution containing 10% (w/v) SDS and 0.5% (w/v) 2-mercaptoethanol at 28°C for 15 min. A strip from a gel was horizontally placed on a 14% polyacrylamide gel and subjected to conventional SDS-PAGE analysis.

Measurement of Chlorophyll Fluorescence with PAM Fluorometer

Chlorophyll fluorescence parameters were measured with a PAM2000 fluorometer (Heinz-Walz GmbH, Effeltrich, Germany). Plants were kept in darkness for at least 15 min prior to measurement. With a weak measuring light, F_o was determined, which was followed by the illumination of the saturating pulse to estimate F_m and F_v ($F_m - F_o$). One minute after the start of the analysis, red actinic light ($435 \mu\text{mol m}^{-2} \text{s}^{-1}$) was switched on, and after 2 min

and 2.5 min, saturating pulses were emitted to estimate qP , qL , qE and NPQ. The fluorescence amplitude just before the third saturating pulse was defined as F_s , and the maximum fluorescence during the third saturating pulse was defined as F_m' . After 30 s of the third saturating pulse, the actinic light was switched off and far-red light ($160 \mu\text{mol m}^{-2} \text{s}^{-1}$) was switched on. The minimal fluorescence when the far-red light was on was defined as F_o' . Ten minutes after switching on the far-red light, the fourth saturating pulse was emitted, and F_m'' was measured. Chlorophyll fluorescence parameters were calculated as follows: $qP = (F_m' - F_s)/(F_m' - F_o')$, $qL = qP \cdot F_o'/F_s$, $\text{NPQ} = (F_m - F_m')/F_m'$, $qE = F_m/F_m' - F_m/F_m''$. See Kramer et al. (2004) for more detailed description of chlorophyll fluorescence parameters.

State transitions were measured in accordance with Lunde et al. (2000) with substitution of a blue light source with blue LED (470 nm, $90 \mu\text{mol m}^{-2} \text{s}^{-1}$, EP30-A0E0-00, EDISON, Taipei, Taiwan). Far-red light was supplied by PAM-2000 fluorometer (735 nm, $165 \mu\text{mol m}^{-2} \text{s}^{-1}$). Briefly, the blue LED was on after the measurement of F_m , and was then maintained for the remainder of the experiment which finished at 46 min. A far-red light source was turned on 15 min after the onset of the blue LED treatment, and was maintained for a total of 15 min (see Figure 9). qT was calculated as $(F_{m1} - F_{m2})/F_{m1}$, where F_{m1} and F_{m2} indicate F_m' values in State 1 and State 2, respectively.

The relative yield of “free antennae” was measured in accordance with the method of Schmitz et al. (2012) using a Dual-PAM-100 fluorometer (Walz) with slight modification as follows: Briefly, 1.5 min after measuring F_o and F_m , far-red light ($6 \mu\text{mol m}^{-2} \text{s}^{-1}$) was switched on and emitted for 2 min. Then, after 2.5 min, actinic light ($2400 \mu\text{mol m}^{-2} \text{s}^{-1}$) was emitted for 5 min. The minimal fluorescence value immediately after switching off the actinic light was defined as F_o' , and the yield of free antennae was defined as $(F_o - F_o')/F_m$.

Immunoblotting analysis

Immunoblotting was performed as previously described (Takabayashi et al., 2011). Anti-Lhcb4, Lhcb6, CP47 and PSB33 (LIL8) antisera purchased from AgriSera (Vännäs, Sweden). Anti-CP1 antiserum was raised using purified spinach CP1 as an antigen{Tanaka:1991p114}. For detection of LIL8, anti-PSB33 antiserum from AgriSera was used for nearly all experiments in this study, with the exception for what is shown in Figure 1. For the results shown in Figure 1, we used antiserum which was raised against recombinant Arabidopsis LIL8 from our laboratory. It is important to note that the anti-PSB33 (LIL8) antiserum from AgriSera, and the one raised in our laboratory, always showed similar reactivity against LIL8 protein.

Measurement of P700+

The PSI antenna size was measured with far-red light in accordance with the method of Kim et al. (2009) using a Dual-PAM-100 fluorometer. For measurement of relative PSI antenna sizes under visible light, fresh leaves were incubated in 200 μ M DCMU for 1 h in darkness. Actinic light ($2,800 \mu\text{mol m}^{-2} \text{s}^{-1}$) was emitted and the absorbance of P700+ was measured (Benson et al., 2015).

Time-resolved Fluorescence Analysis

Time-resolved fluorescence decay curves at 77K were measured as described previously (Yokono et al., 2015b) using a diode laser (PIL040X controlled by EIG2000DX-40, 408 nm; Advanced Laser Diode Systems GmbH, Berlin, Germany) as an excitation source. The curves were deconvoluted using free-running exponential components with an instrument function.

The amplitudes are summarized in Table 1. Fluorescence decay-associated spectra were constructed as previously described (Yokono et al., 2015b; 2015a).

Chlorophyll fluorescence lifetime of dark-adapted leaves and those illuminated with a diode laser at an intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 15 min was measured at 735 nm. For this measurement, leaves were excited with the same diode laser as describe above with the minimum light intensity available ($<10 \mu\text{mol m}^{-2} \text{s}^{-1}$).

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Tables

	WT	<i>lil8-1</i>	<i>lil8-2</i>
<i>F_o</i>	0.25 ± 0.01	0.33 ± 0.02	0.28 ± 0.01
<i>F_v/F_m</i>	0.80 ± 0.01	0.73 ± 0.01	0.77 ± 0.01
<i>qE</i>	1.24 ± 0.06	1.19 ± 0.17	1.23 ± 0.10
<i>qL</i>	0.15 ± 0.03	0.33 ± 0.11	0.26 ± 0.03
<i>qP</i>	0.30 ± 0.05	0.49 ± 0.11	0.43 ± 0.05
NPQ	1.37 ± 0.06	1.34 ± 0.17	1.36 ± 0.11

Table 1. Chlorophyll Fluorescence Parameters

Chlorophyll fluorescence parameters of the wild type (WT) and the *lil8* mutants measured with a pulse amplitude modulation (PAM) chlorophyll fluorometer. The parameters *qE*, *qL*, *qP* and NPQ parameters were measured under light intensity of 435 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Values represent the means of five biological replicates $\pm$ SD

	WT	<i>lil8-1</i>	<i>lil8-2</i>	<i>lhcb4-KO</i>
qT	0.088 ± 0.024	0.049 ± 0.029	0.086 ± 0.009	0.055 ± 0.026

Table 2. State Transition Parameters

State transition parameters of the wild type (WT), the *lil8* mutants and *lhcb4-KO* measured with a pulse amplitude modulation (PAM) chlorophyll fluorometer. qT was calculated from the data presented in Figure 9 as $(Fm_1 - Fm_2)/Fm_1$. Values represent the means of three biological replicates $\pm$ SD

PSII (686 nm)			PSI (734 nm)		
	WT	<i>lil8-1</i>		WT	<i>lil8-1</i>
180-190 ps	0.795	0.799	50-60 ps	-0.766	-0.585
725-730 ps	0.194	0.187	200-270 ps	0.248	0.135
5 ns	0.011	0.014	2.3-2.4 ns	0.752	0.865

Table 3. Fluorescence Decay Kinetics Measured by Monitoring Fluorescence Either at 686 nm (PSII) or 734 nm (PSI) with Frozen Leaves.

	WT	<i>lil8-1</i>	<i>psb33-3</i>
Dark Adapted Leaves	2758 ± 116	2747 ± 110	2797± 16
Illuminated Leaves	2552 ± 40	2650 ± 105	2708± 29

Table 4. Chlorophyll Fluorescence Lifetime at 735 nm. Average chlorophyll fluorescence lifetime (ns) was measured at 735 nm with dark-adapted leaves or 15-min illuminated leaves as described in Materials and Methods. Values represent the means of three biological replicates ± SD

Figure Legends

Figure 1. Phenotypes of the *lil8* Mutants Analyzed in this Study.

(A) Positions of the tags inserted in the *LIL8/PSB33* gene of the *lil8* mutants. UTR, untranslated region. (B) Relative mRNA levels for the *LIL8* gene in the WT, *lil8-1* and *lil8-2* estimated by semi-quantitative real-time PCR. The expression levels were normalized with the relative expression levels of the *UBC21* gene (At5g25760). (C) LIL8 protein levels in leaves of 5-week-old WT and *lil8* plants. The amount of protein loaded on a lane was equivalent to extracts from 0.5 mg leaf material. The LIL8 protein was detected with anti-LIL8 antiserum. (D) Pigment compositions in leaves of 5-week-old WT and *lil8* plants.

Figure 2. Growth of the *lil8* mutants under various light conditions.

(A) Phenotype of 5-week-old plants of the *lil8* mutants and the wild type (WT) plants grown under our standard growth conditions, (14-h/10-h (light/dark) photoperiod with light intensity of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$). (B) Four-week-old plants grown under fluctuating light conditions (a repeated cycle of $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 min and $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 5 min). (C) Three-week-old plants grown under the standard growth conditions described above and then transferred to high-light conditions ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). (D) Four-week-old plants grown under blue LED ($130 \mu\text{mol m}^{-2} \text{s}^{-1}$).

Figure 3. Localization of LIL8 Protein within Thylakoid Domains.

LIL8/PSB33 was detected with anti-LIL8/PSB33 serum. Signals for CP1 (the PsaA/B subunit of PSI) and LHCII (the major light-harvesting complexes of PSII) are shown to indicate the purity of each membrane fraction. Chlorophyll *a/b* ratios of each fraction are shown above the immunoblotting images. Thy, total thylakoid membranes; SM, stroma lamellae; GC, grana core regions of thylakoid membranes; GM, grana margins.

Figure 4. Analysis of the Native Forms of LIL8.

(A) Thylakoid membrane fractions (grana core, grana margins and stroma lamellae) were isolated from the wild type (WT) and *lil8-1* plants as described in the Materials and Methods section. Equal amounts of chlorophyll were loaded per lane and proteins were resolved by two-dimensional BN-PAGE/SDS-PAGE. The gel was blotted onto a PVDF membrane and LIL8/PSB33 was detected with anti-LIL8/PSB33 antiserum (images in the left panel).

Subsequently, the PVDF membrane was stained with CBB (images in the middle panel). The immunoblotting images and CBB stained images were merged and are shown in the right panel.

(B) Protein migration profiles of LIL8, D1, Lhcb4 and Lhcb6. Proteins were solubilized with 1% β -DM on a BN-PAGE gel that was analyzed by mass spectroscopy and deposited on the PCoM database are selectively shown with that of LIL8. In this analysis, protein complexes with a large molecular size were observed in fewer gel slices, whereas those complexes with a small molecular size were observed in a larger number of gel slices.

Figure 5. Co-migration analysis of LIL8 and representative PS subunits. Thylakoid membranes were solubilized with 1% digitonin and were analyzed by two-dimensional BN-PAGE/SDS-PAGE followed by immunoblotting as described in Materials and Methods section. Assignment of each complex was performed according to Aro et al. (2005) and Järvi et al. (2011). (A) For a better comparison of protein migration, we cut a single immunoblot into three pieces and stained with three different antibodies including anti-CP47, anti-LIL8 and anti-Lhcb6 antisera. Furthermore, the piece of blot that was reacted with an anti-LIL8 antiserum was washed with a stripping solution containing 6M Guanidine-HCl, 0.2% Nonidet P-40, 0.1M β -mercaptoethanol, and 20mM Tris-HCl, pH7.5 (Yee-Guide Yeung, 2009) and re-stained with an anti-Lhcb4 antiserum. (B) Another single blot of WT Arabidopsis thylakoids was cut into three pieces and each piece was stained with anti-CP1, anti-CP47 and anti-LIL8 antisera, respectively.

Figure 6. BN-PAGE Analysis of Thylakoid Proteins.

(A) Thylakoid membranes were isolated from the wild type (WT), *lil8-1* and *lil8-2* plants, and were solubilized with 1% α -dodesyl maltoside. Solubilized proteins were resolved on a 4–16% gradient blue-native polyacrylamide gel. Equal amounts of chlorophyll (8 mg) were loaded per lane.

(B) Thylakoid membranes were further fractionated into three domains (grana core, grana margins and stroma lamellae) and resolved on a 5–13% blue-native polyacrylamide gel. Equal amounts of chlorophyll (8 mg) were loaded per lane.

Figure 7. Relative Fluorescence Yield of the Wild Type and the *lil8* Mutants.

The analysis was based on the same time-course experiments as shown in Table 1. Following the method of Schmitz et al. (2012), $F_0 - F_0'$ is an indicator of “free” antennae that are

energetically uncoupled from PSII. Values represent the means of three biological replicates. Error bars indicate the SD. F_v , variable fluorescence from dark-adapted leaves; F_o , and F_o' , minimal fluorescence from dark adapted and actinic-light adapted leaves, respectively.

Figure 8. Chlorophyll Fluorescence of Leaves at 77K.

Steady-state fluorescence spectra of intact leaves harvested in darkness. The excitation wavelength was 408 nm and the measurement temperature was 77K. The spectra were decomposed by three spectral components attributed to LHCII (red), PSII (blue) and PSI (gray). In the *lil8-1* and *lil8-2* mutants, the relative amplitude of LHCII/PSII was 1.4- and 1.2-times larger than in WT. Insets are fluorescence difference spectrum (mutant - WT) in the LHCII wavenumber region.

Figure 9. State Transitions in the *lil8* Mutants Compared with the Wild Type and the Lhcb4 Knockout Plant.

Plants were illuminated with blue light (470 nm, $90 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 45 min after the measurement of F_m . Far-red light (735 nm, $165 \mu\text{mol m}^{-2} \text{s}^{-1}$) was turned on after 15 min of the onset of the blue light treatment, and was maintained for 15 min. Saturating pulses were applied prior to switching off the far-red light and before the end of the measurement period.

Figure 10. Measurement of PSI Antenna Sizes under Far-red or Red Light.

(A) PSI antenna sizes were measured by illumination of fresh leaves of the wild type (WT), *lil8-1* and *lil8-2* with far-red light (740 nm). Fluorescent profiles obtained from three biological replicates are overlapped and shown in the images. On the lower-right image, all fluorescent profiles from WT, *lil8-1* and *lil8-2* were overlapped.

(B) PSI antenna sizes were measured by illuminating red light in the presence of DCMU. From the kinetics of P700+ changes, relative antenna sizes were estimated and shown with the mean values for the WT set to 1. Data represent the means of three biological replicates. Error bars indicate the SD.

Figure 11. Fluorescence Decay Associated Spectra of Intact Leaves.

Spectra for the leaves of the wild type (black lines) and the *lil8-1* mutant (blue lines) plants are shown. Spectra were normalized at 686 nm, as the decay kinetics were similar at this wavelength between the two samples (see Table 1).

Supplementary Figure 1. BN-PAGE analysis of digitonin-solubilized thylakoid membranes.

Thylakoid membranes were isolated from WT and *lil8* mutants that were grown under different growth conditions. (1) WT, *lil8-1* and *lil8-2* were grown under short-day conditions (10-h light/14-h dark) at the light intensity of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ and leaves were harvested under darkness, and were ground under dim light for thylakoid preparation. A stroma lamellae preparation that contains 8 mg chlorophyll was solubilized with 1% digitonin, and it was subjected to BN-PAGE analysis. Note that since the CBB was not destained so that the gel looked bluer than the gels in Experiments 2 and 3. (2) WT, *lil8-1*, *lil8-2* and *psb33-3* plants were grown under long-day conditions (14-h light/10-h dark) at the light intensity of $70 \mu\text{mol m}^{-2} \text{s}^{-1}$ and leaves were harvested under darkness, and were ground under dim light for thylakoid preparation. Thylakoids containing a total of 8 mg chlorophyll were solubilized with 1% digitonin and subsequently loaded on the gel. (3) Plants were grown at the same conditions as Experiment 2, and they were illuminated with blue-light (470 nm) for 10 min just before harvest. After harvest, thylakoids were prepared and analyzed just like Experiment 2.

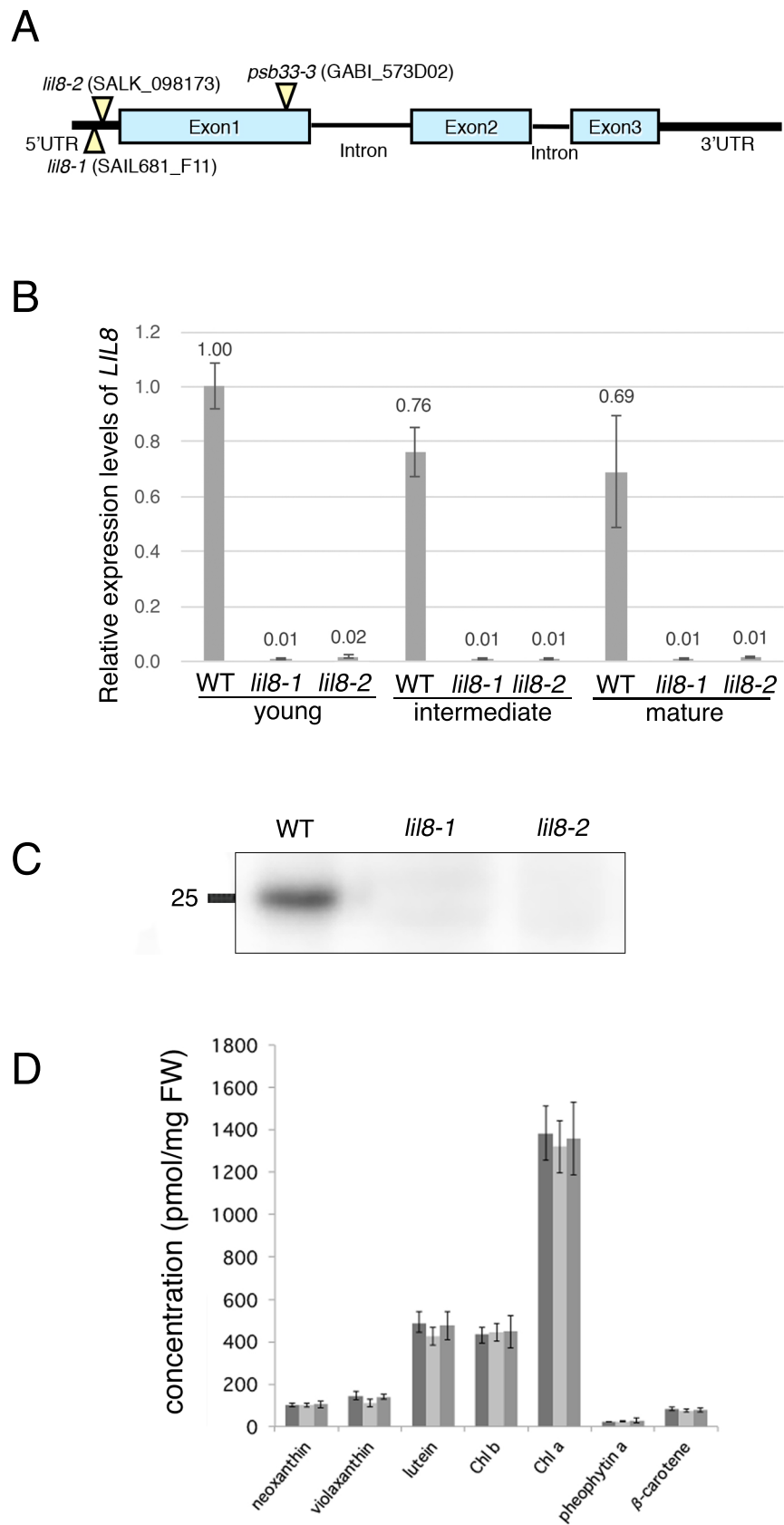
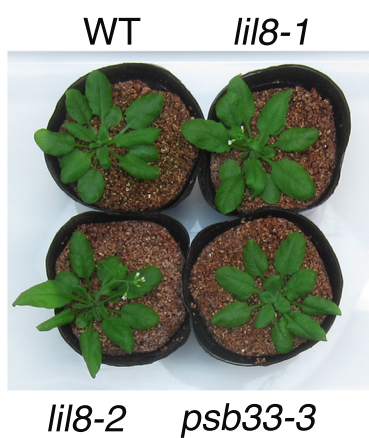
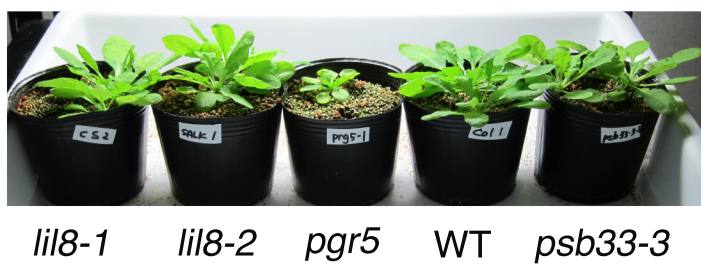


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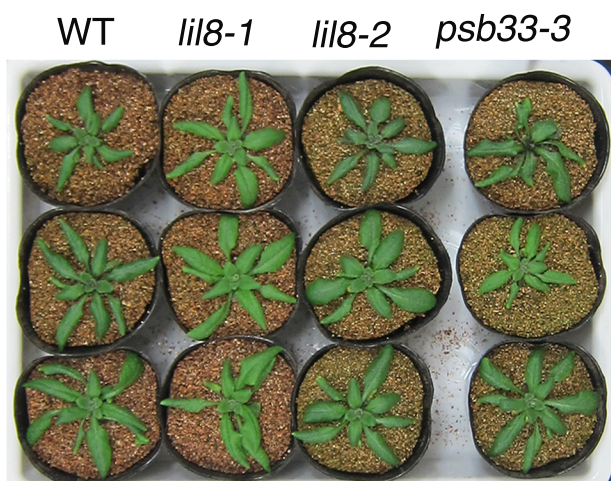
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B



C



D

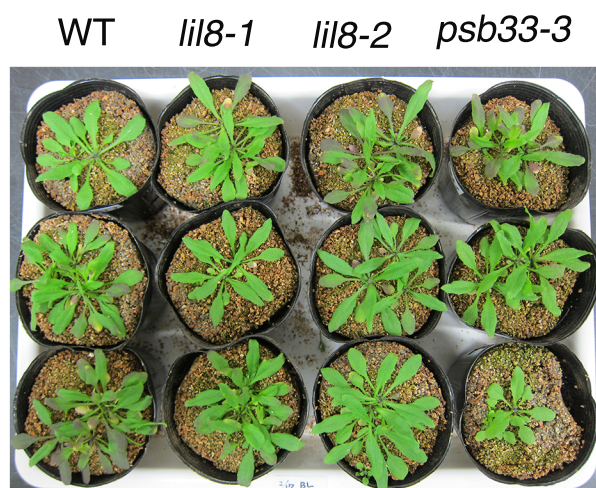


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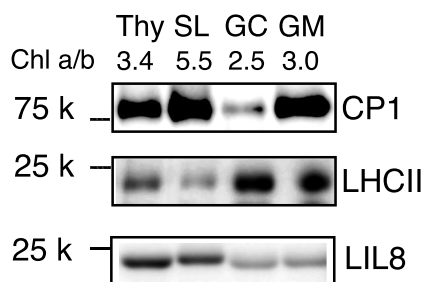


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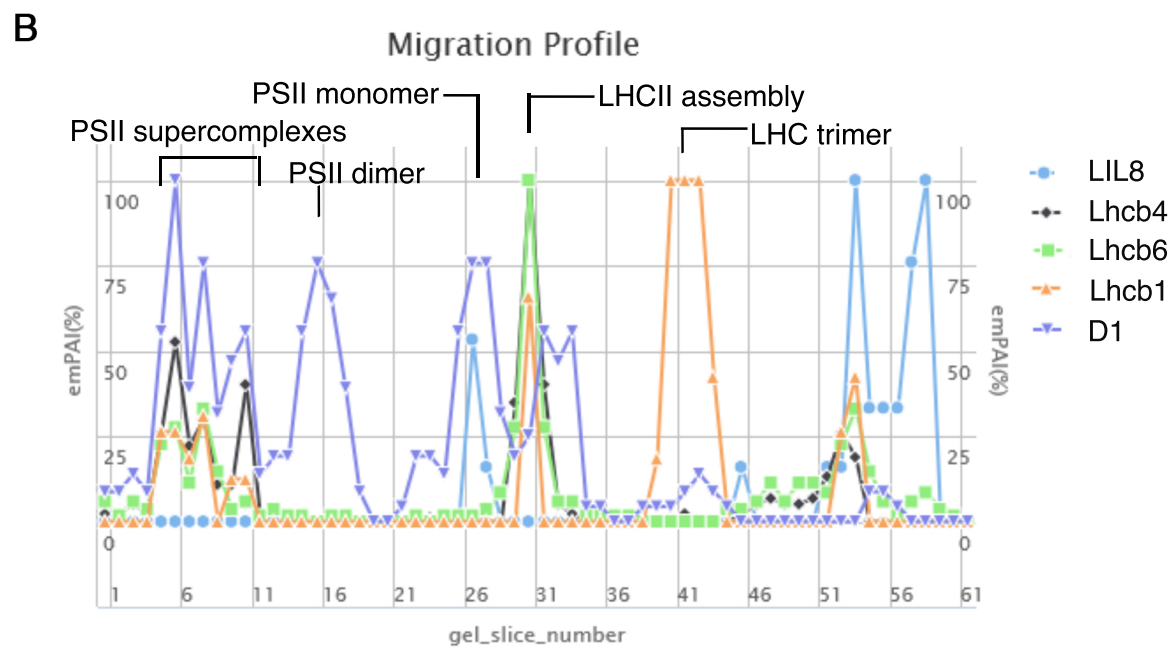
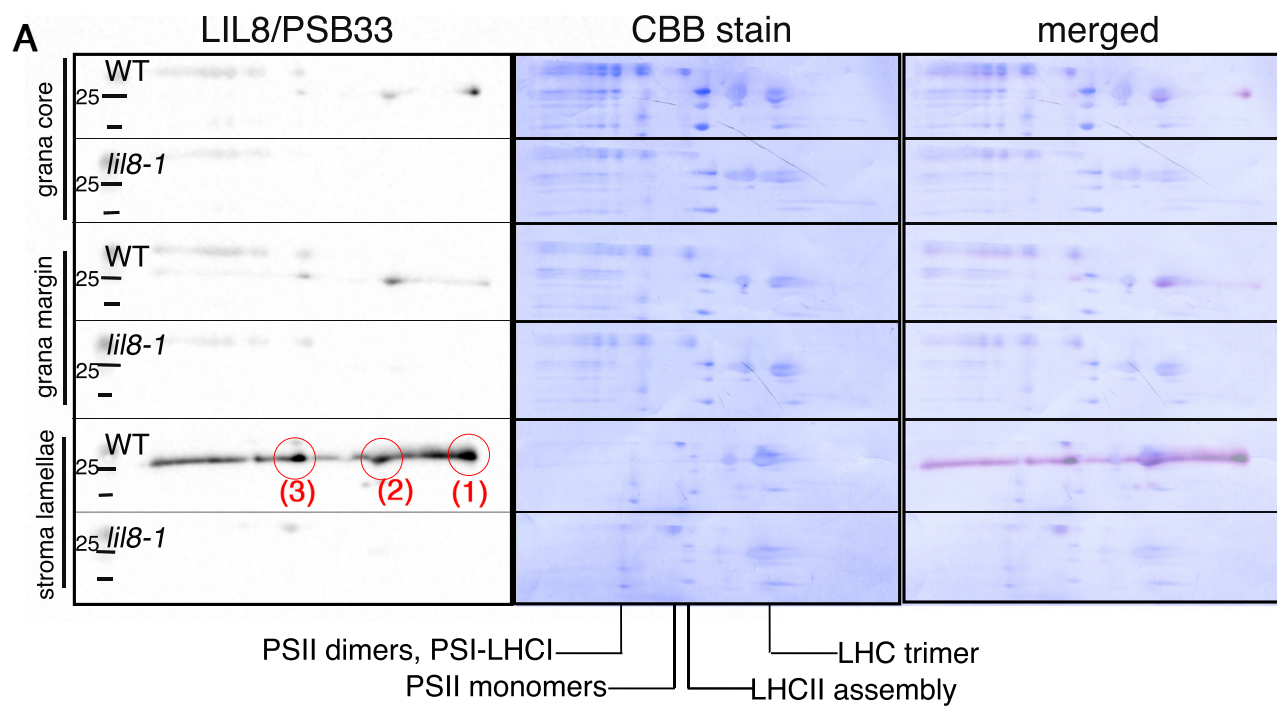


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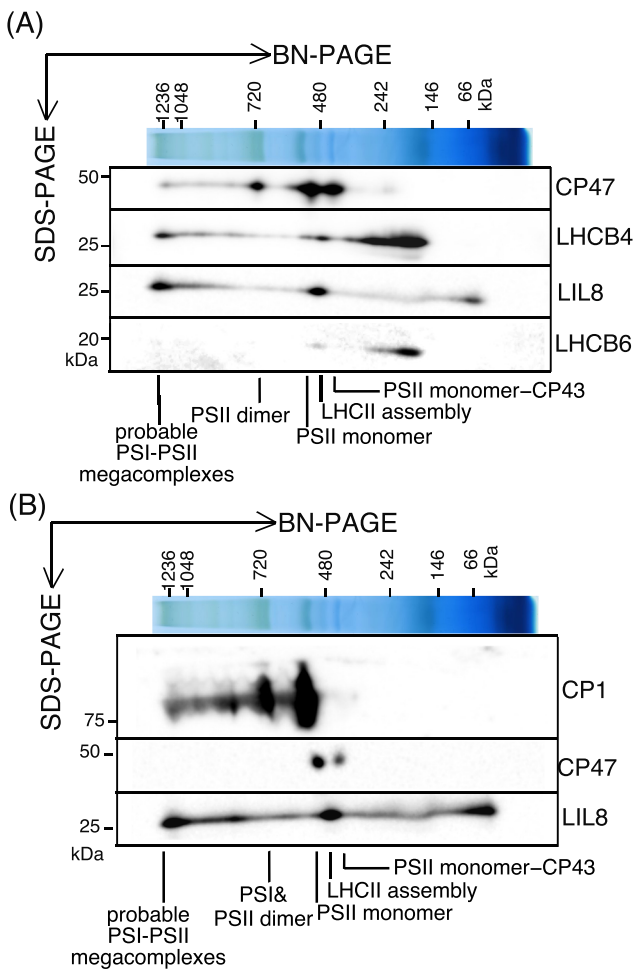


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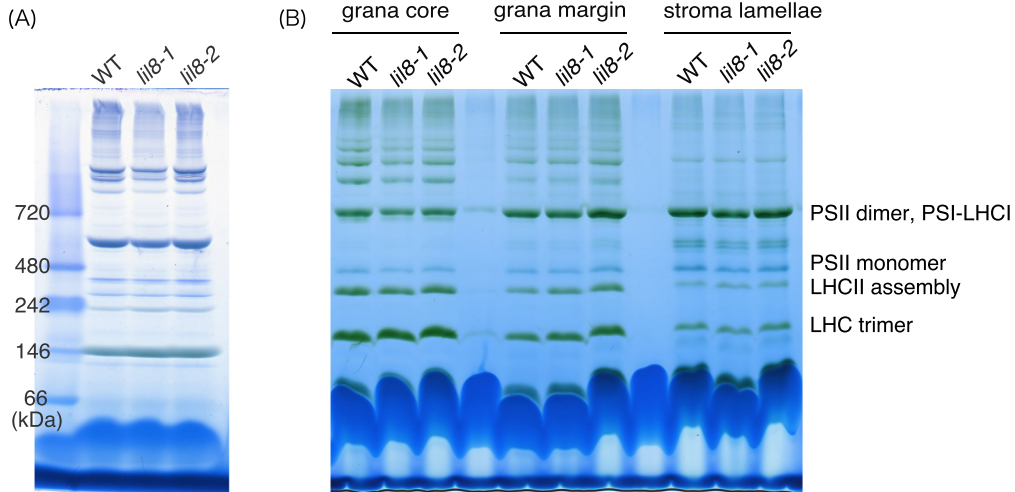


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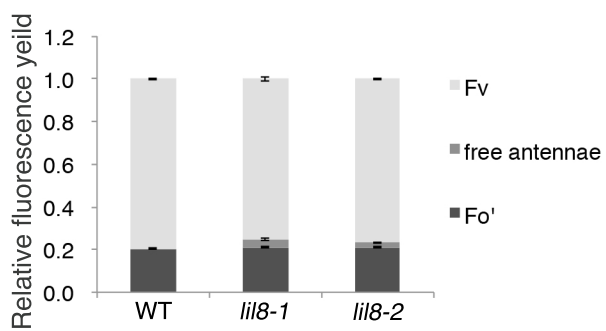


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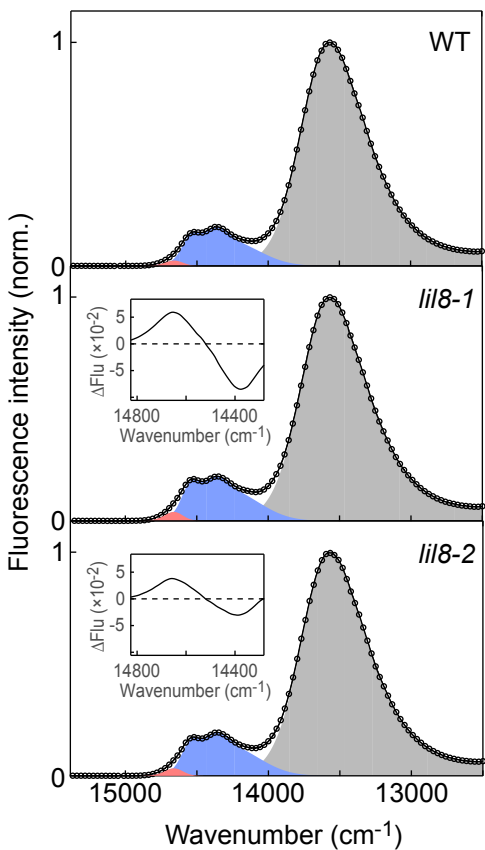


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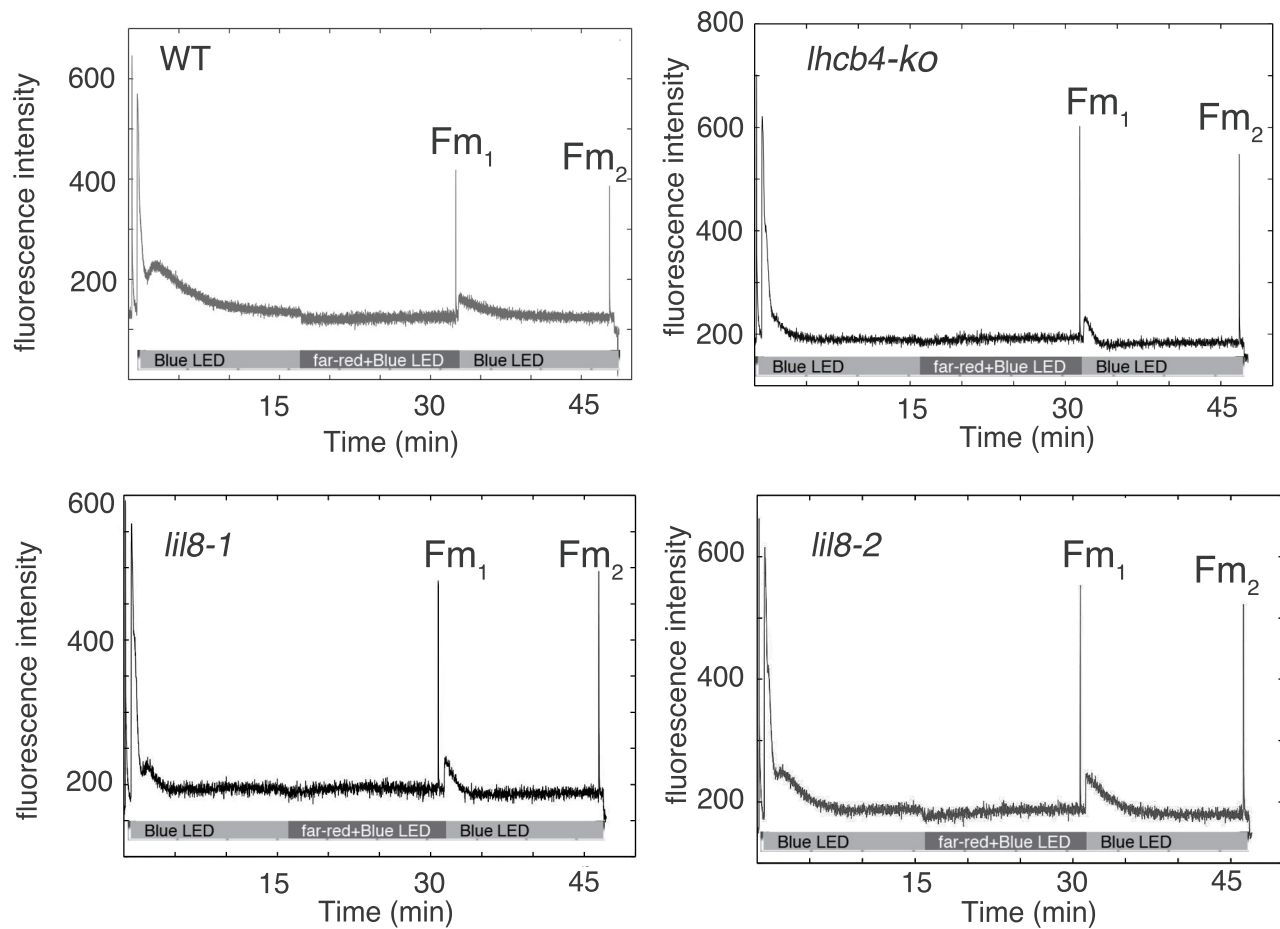


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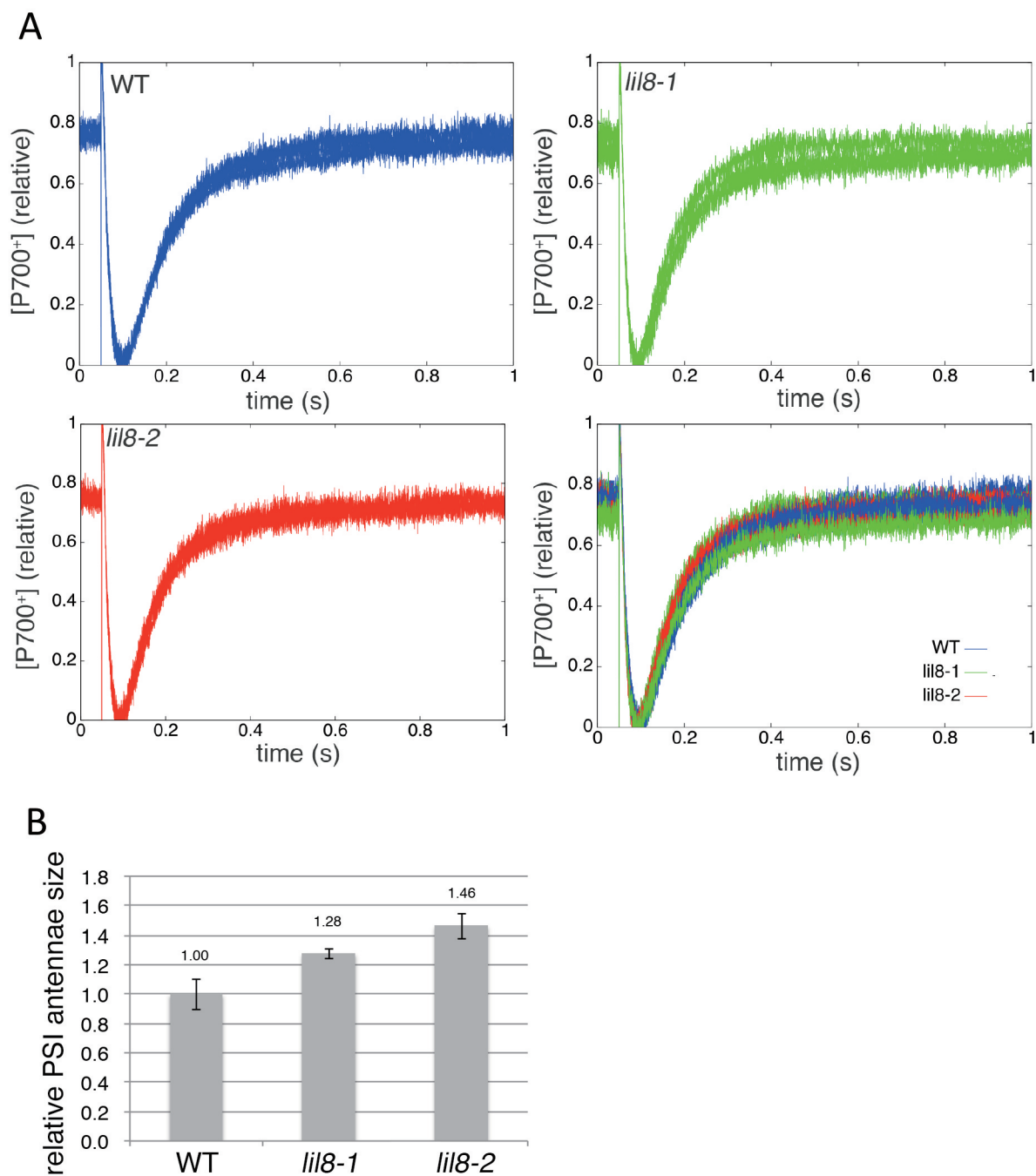


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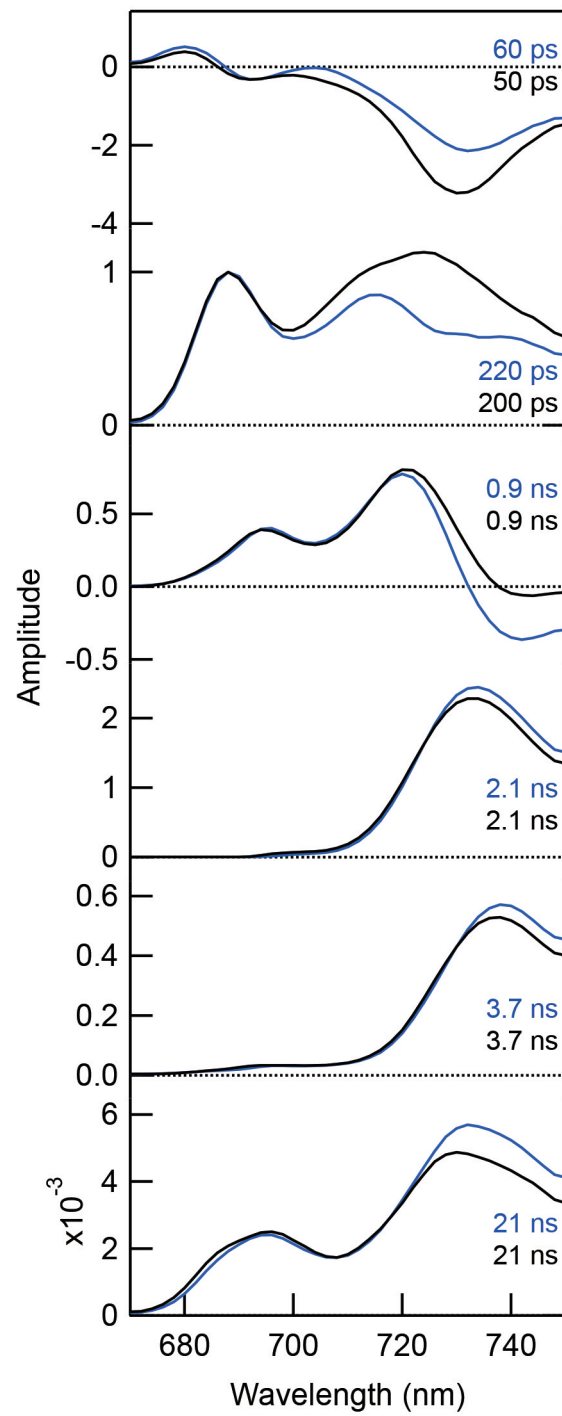
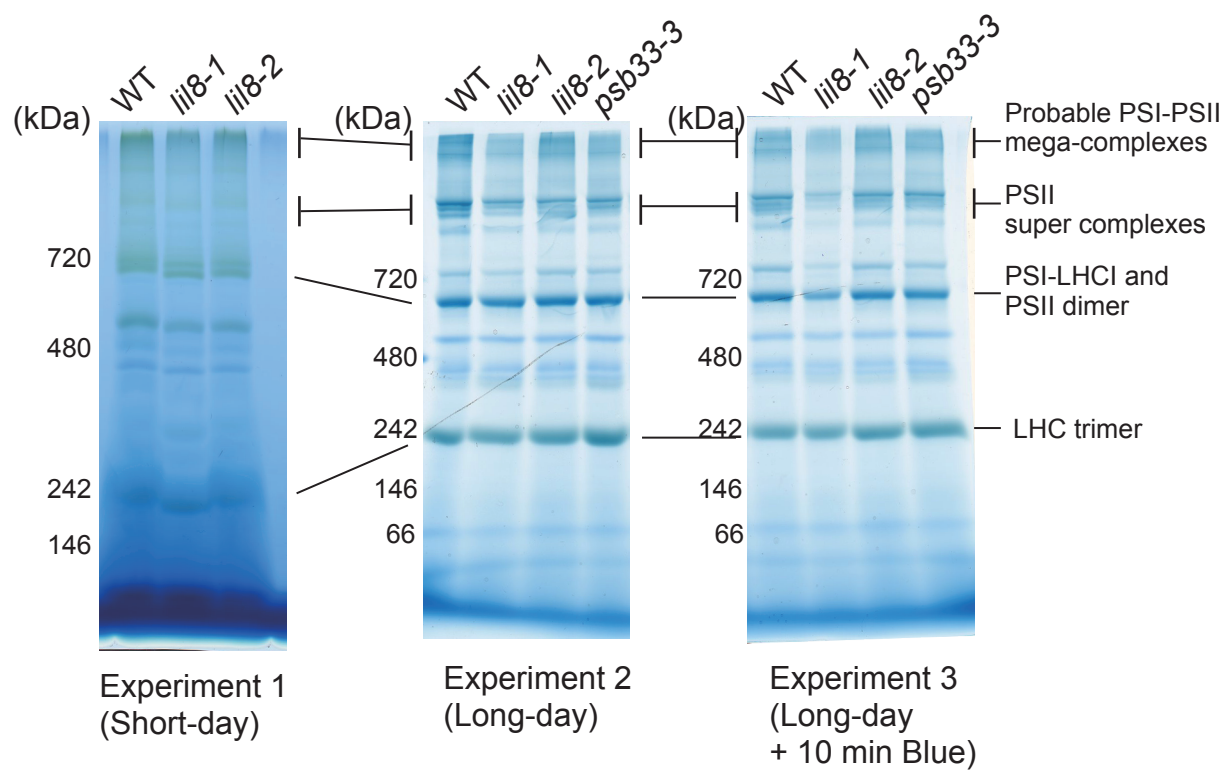


Figure 11



Supplementary Figure 1