HYPERSENSITIVE TO HIGH LIGHT1 Interacts with LOW QUANTUM YIELD OF PHOTOSYSTEM II1 and Functions in Protection of Photosystem II from Photodamage in Arabidopsis

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Under high-irradiance conditions, plants must efficiently protect photosystem II (PSII) from damage. In this study, we demonstrate that the chloroplast protein HYPERSENSITIVE TO HIGH LIGHT1 (HHL1) is expressed in response to high light and functions in protecting PSII against photodamage. Arabidopsis thaliana hhl1 mutants show hypersensitivity to high light, drastically decreased PSII photosynthetic activity, higher nonphotochemical quenching activity, a faster xanthophyll cycle, and increased accumulation of reactive oxygen species following high-light exposure. Moreover, HHL1 deficiency accelerated the degradation of PSII core subunits under high light, decreasing the accumulation of PSII core subunits and PSII–light-harvesting complex II supercomplex. HHL1 primarily localizes in the stroma-exposed thylakoid membranes and associates with the PSII core monomer complex through direct interaction with PSII core proteins CP43 and CP47. Interestingly, HHL1 also directly interacts, in vivo and in vitro, with LOW QUANTUM YIELD OF PHOTOSYSTEM II1 (LQY1), which functions in the repair and reassembly of PSII. Furthermore, the hhl1 lqy1 double mutants show increased photosensitivity compared with single mutants. Taken together, these results suggest that HHL1 forms a complex with LQY1 and participates in photodamage repair of PSII under high light.

INTRODUCTION

The photosynthetic apparatus of cyanobacteria, eukaryotic algae, and vascular plants is located in the specialized thylakoid membrane system and is mediated by the integral membrane protein complexes photosystem II (PSII), cytochrome b6/f, photosystem I (PSI), and ATP synthase, which harvest light and transduce solar energy into chemical energy (Eberhard et al., 2008). The multisubunit pigment-protein complex of PSII catalyzes the light-driven water oxidation and reduction of plastoquinone (Chi et al., 2012a). Although green plants require PSII to absorb energy from sunlight via the photosystem, harnessing this tremendous light energy during photosynthesis carries great risks, including inevitable damage to the photosynthetic apparatus, especially PSII (Takahashi and Badger, 2011). Plants have therefore evolved diverse photoprotection mechanisms, including light avoidance associated with the movement of leaves and chloroplasts, screening of photoradiation, reactive oxygen species (ROS) scavenging systems, dissipation of absorbed light energy as thermal energy (energy-dependent quenching [qE]), cyclic electron flow around PSI, and the photorespiratory pathway (Takahashi and Badger, 2011).

When these protective measures fail to prevent damage to PSII, plant cells deploy a complex PSII repair system that involves disassembly and reassembly of a thylakoid membrane protein complex comprising dozens of proteins (Baena-González and Aro, 2002; Mulo et al., 2008). Therefore, most PSII assembly factors also play a role in PSII repair. Although our current knowledge of PSII structure and function is extensive, relatively little is known about how disassembly and reassembly of PSII occur. However, two major findings have recently emerged: PSII repair is a highly ordered process, and large numbers of accessory factors are involved in forming this multi-protein complex and incorporating the various cofactors (Nickelsen and Rengstl, 2013). The PSII repair processes can be generalized as follows: (1) Photodamage induces a reversible phosphorylation and disassembly of the PSII-light-harvesting complex II (LHCII) supercomplexes; (2) damaged PSII core monomers migrate from grana stacks to stroma-exposed membranes; (3) PSII core monomers partially disassemble and release CP43; (4) photodamaged PSII subunits are degraded by proteases; (5) new PSII subunits are synthesized and reassembled into new PSII core monomers; (6) PSII core monomers migrate to grana stacks and reform functional PSII-LHCII supercomplexes (Aro et al., 2005; Mulo et al., 2008).

Genetic and biochemical studies have provided evidence for the protection and repair of PSII. The repair cycle of PSII requires the participation of hundreds of auxiliary proteins (Mulo et al., 2008; Nickelsen and Rengstl, 2013), and those in higher plants are different from those of more primitive photosynthetic organisms (Nixon et al., 2010). However, some PSII assembly
and repair factors are conserved in cyanobacteria and higher plants, including HIGH CHLOROPHYLL FLUORESCENCE136 (Komenda et al., 2008), ALBINOS (Luirink et al., 2001; Göhre et al., 2006), Psb27 (Nowaczyk et al., 2006; Komenda et al., 2012), Psb28 (Dobáková et al., 2009; Sakata et al., 2013), Psb29 (Keren et al., 2005), and YCF39 (Ermakova-Gerdes and Vermaas, 1999), as well as immunophilins, such as CYCLOPHILIN38 (CYP38)/THYLAKOID LUMEN PEPTIDYLPROLYL ISOMERASE OF 40 kD (TLP40) (Siriò et al., 2008). Presumably, therefore, the roles of these factors in PSII biogenesis or maintenance have been retained during evolution. In contrast, other factors are specific to cyanobacteria, such as PROCESSING ASSOCIATED TPR PROTEIN (Schottkowski et al., 2009), Sir0286 (Kufryk and Vermaas, 2001), and Sir2013 (Kufryk and Vermaas, 2003), or specific to higher plants, such as LOW PSII ACCUMULATION1 (LPA1)/PSII REPAIR27 (Park et al., 2007; Dewez et al., 2009) and LPA2 (Ma et al., 2007), which may represent evolutionary adaptations to specific environments. Notably, LOW QUANTUM YIELD OF PHOTOSYSTEM II (LQY1), a small thylakoid zinc-finger protein exclusive to land plants, was also recently found to be involved in the repair cycle and reassembly of PSII (Lu et al., 2011).

The ceaseless identification of new auxiliary proteins associated with PSII confirms that much remains to be learned about the regulation of structure and function of PSII. However, compared with the structural subunits of PSII complex, the auxiliary proteins of PSII for research are challenging for a variety of reasons, including the low protein abundance, labile interactions with core and antenna complexes, and functional redundancy (Mulo et al., 2008). Analysis of in vivo chlorophyll fluorescence is a powerful, noninvasive technique used to identify mutations affecting photosynthesis (Maxwell and Johnson, 2000; Müller et al., 2001; Kramer et al., 2004; Baker, 2008). Alterations in chlorophyll fluorescence indicate defects in the photosynthetic electron transport chain due to changes in the structure and function of the thylakoid membrane (Baker, 2008). Therefore, screening mutants with altered chlorophyll fluorescence provides a more specific way to obtain and characterize photosynthetic mutants.

To gain further insight into the maintenance and repair of PSII, we used the chlorophyll fluorescence system to screen a pool of Arabidopsis thaliana mutants and identified hypersensitive to high light (hhl) mutants, which show lower photosynthesis efficiency under high-light conditions and exhibit high-light-induced defects in PSII photochemistry and accumulation of PSII supercomplexes, both of which are surprisingly similar to defects observed in lqy1 mutants. We found that the HHL1 protein associates with PSII directly, specifically with PSII core proteins CP43, CP47, and LOY1 both in vivo and in vitro. The results of this study suggest that HHL1 and LOY1 may form a complex that is involved in the repair and reassembly cycle of the PSII-LHCII supercomplex under high-light conditions in land plants.

RESULTS

Isolation and Identification of hhl1 Mutants

To identify regulatory components involved in the photoprotection and repair of PSII, we screened more than 50,000 Arabidopsis mutants obtained from RIKEN (ichikawa et al., 2006) using chlorophyll fluorescence imaging. The ratio of variable fluorescence/maximal fluorescence from dark-adapted leaves (Fv/Fm) can be used to estimate the maximum photochemical efficiency of PSII (Baker, 2008). Arabidopsis mutants that exhibited aberrant Fv/Fm after exposure to high light were identified using a chlorophyll fluorescence video imaging system, and a new series of Arabidopsis hhl mutants were identified. Interestingly, one of the hhl mutants, hhl1-0, was found to have a lower maximum photochemical efficiency of PSII (Fv/Fm) after high-light treatment (−1200 μmol photons m⁻² s⁻¹) than the wild type (Columbia-0 [Col-0]; Supplemental Figure 1A), although the growth phenotype and Fv/Fm of the mutants were identical to the wild type grown under growth light conditions (−100 μmol photons m⁻² s⁻¹). However, Fv/Fm returned to normal levels following a 2-d recovery under growth light conditions (Supplemental Figure 1B). The results were confirmed with twice-backcrossed mutants and imply that the hhl1-0 mutant has defects in energy transfer within PSII or a partial loss of PSII capacity after high-light stress.

To determine the genetic basis of the hhl1-0 phenotype, we performed thermal asymmetric interlaced PCR to determine the genomic region flanking the left border sequences of the T-DNA (Supplemental Figure 1C). Sequence analysis revealed that the T-DNA was inserted in the translated region of the nuclear-encoded gene At1g67700 (Supplemental Figure 1D). To confirm that the mutated gene is responsible for the observed phenotype, we analyzed two other independent homozygous T-DNA insertion mutants of At1g67700 (SALK_129146C and SALK_010366C) from the sequence-indexed Arabidopsis T-DNA insertion mutant stock. The T-DNA insertion positions in these mutants were determined by PCR and subsequent sequencing of the amplified products (Figure 1B). The mutants carry T-DNA insertions in the first exon and last intron of At1g67700, respectively (Figure 1A). Quantitative real-time RT-PCR analysis showed that the transcription of At1g67700 was suppressed in both mutants (Figure 1C). We subsequently named SALK_129146C and SALK_010366C as hhl1-1 and hhl1-2, respectively, and used these names throughout the article. The hhl1-0 mutant described above exhibited an identical phenotype to that of both the hhl1-1 and hhl1-2 mutants: The Fv/Fm value decreased from 0.82 to 0.70 in the wild type after high-light treatment, while in the mutants, this value decreased from 0.82 to 0.51 (Figure 1D; Supplemental Table 1). Thus, it can be concluded that the hhl1 phenotype is due to the inactivation of At1g67700.

Carotenoid Composition but Not Chlorophyll Content Is Slightly Altered in the hhl1 Mutants after High-Light Treatment

Under growth light conditions, hhl1 plants showed no phenotypic differences from the wild type (Figure 1D). In addition, spectrofluorometric analyses indicated that there were also no differences in the chlorophyll or total carotenoid contents (Supplemental Table 1).

Carotenoids are involved in the regulation of photosynthetic light harvesting and photoprotection (Holt et al., 2005). To more precisely investigate whether the carotenoids compositions were affected in the hhl1 mutants, we performed HPLC...
HHL1 Functions in Photoprotection of PSII

PSII Activity Is Markedly Reduced in the hhl1 Mutants after High-Light Treatment

Initial Fv/Fm measurements preliminarily suggested that PSII activity is disturbed in the hhl1 mutants after high-light stress. To further investigate the primary target of the hhl1 mutation, the minimum (F0) and maximum (Fm) fluorescence of dark-adapted leaves, Fv/Fm, and other chlorophyll parameters were quantitatively determined under a fixed light intensity. Under growth light conditions, the hhl1 mutants possessed much higher non-photochemical quenching (NPQ) than wild-type plants (Figure 2B), which indicates that the mutants dissipate more excess excitation energy via nonphotochemical pathways in growth light, although this did not affect the normal growth of mutants under growth light conditions. After a 3-h high-light treatment, the hhl1 mutants had much lower Fm and Fv/Fm than wild-type plants. Interestingly, the F0 was slightly higher in the hhl1 mutants than in the wild type, instead of being impaired after high-light treatment. Thus, we speculate that the zeaxanthin level may also be slightly higher in the hhl1 mutants after high light, although this was not detected successfully in this assay due to the lower level of this compound. However, the level of chlorophyll and other carotenoid components, including neoxanthin, lutein, and β-carotene, showed no difference in hhl1 compared with wild-type plants (Supplemental Figure 2). These results suggest that the photosensitivity of hhl1 mutants is not due to perturbation of carotenoids.

green, blue, and violet to purple (1) as indicated at the bottom. GL, growth light (−100 μmol photons m−2 s−1); HL, high light (−1200 μmol photons m−2 s−1). Six biological replicates were performed in all experiments, and similar results were obtained.
To further characterize the photosynthetic apparatus, we analyzed the light intensity dependence of two chlorophyll fluorescence parameters, the light-response curves of PSII quantum yield \( F_{PSII} \) and the electron transport rate (ETR) before and after a 3-h high-light treatment. After a short-term high-light treatment (3 h), \( F_{PSII} \) and ETR were much lower in hhl1 mutants than in the wild type (Figure 2A), as was also observed for \( F_v/F_m \) (Figure 1; Supplemental Table 1). Interestingly, after an extended high-light treatment, the difference between wild-type and hhl1 plants was the same as that observed after the 3-h treatment (Figure 2A). However, \( F_{PSII} \) and ETR in the hhl1 mutants almost reached wild-type levels after the plants were returned to growth light conditions for 2 d (Figure 2A).

In addition, to assess the capacity of the mutants to recover from photoinhibition, we monitored changes in \( F_v/F_m \) after high-light treatment, which resulted in an \(-50\%\) reductions for both wild-type and mutant leaves. As shown in Figure 2C, hhl1 mutants recovered from photoinhibition more slowly than wild-type leaves. After an 8-h recovery period, \( F_v/F_m \) of wild-type and hhl1 mutants leaves reached 87 and 79% of their initial values, respectively. Even after a 24-h recovery period, \( F_v/F_m \) was still \(-89\%\) of the initial \( F_v/F_m \) values in the hhl1 mutants and 97% in the wild type. Taken together, detailed chlorophyll fluorescence analyses showed that PSII photochemical activity decreased to a greater extent and showed delayed recovery of PSII activity from photoinhibition in hhl1 mutants after high-light treatment.

**ROS Level in the hhl1 Mutants Is Higher after High-Light Treatment**

Under high-light conditions, the production of ROS is accelerated in chloroplasts (Takahashi and Badger, 2011), and \( H_2O_2 \), superoxide \( (O_2^-) \), and \( 1O_2 \) are primary ROS (Li et al., 2009a). To test whether the accumulation of ROS in the hhl1 mutants differed from that in the wild type after high-light treatment, we examined the accumulation of \( H_2O_2 \), \( O_2^- \), and \( 1O_2 \). We first measured \( H_2O_2 \) and \( O_2^- \) levels using diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining. Following a 2-d high-light treatment, \( H_2O_2 \) and \( O_2^- \) were present at considerably higher levels in the hhl1 mutants than in the wild type (Figures 3A and
3B). Specifically, \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) levels were higher in younger \( hhl1 \) leaves, but older leaves showed no differences from the wild type (Figures 3A and 3B). Detection of \( ^1\text{O}_2 \) was performed on detached leaves using the singlet oxygen sensor green (SOSG) fluorescence indicator. A stronger fluorescence signal was detected in the high-light-treated mutants (Figure 3C). To further determine total intracellular ROS concentrations, we compared with the level of ROS accumulation using the fluorescent dye 2',7'-dichlorofluorescein diacetate (Pei et al., 2000). Again, a stronger fluorescence signal was detected in high-light-treated \( hhl1 \) mutants (Figure 3D), further indicating that ROS accumulated at higher levels in mutant plants. Together, the results from \( \text{O}_2^- \), \( \text{H}_2\text{O}_2 \), \( ^1\text{O}_2 \), and total ROS detection indicated that the \( hhl1 \) mutants accumulate more ROS than the wild type after high-light treatment. Recent studies have also demonstrated that ROS accelerates photoinhibition by inhibiting the repair of photodamaged PSII (Nishiyama et al., 2006). Thus, high-light intensity may cause more extensive oxidative damage in \( hhl1 \) mutants by inhibiting the repair of photodamaged PSII.

**HHL1 Is a Chloroplast Thylakoid Transmembrane Protein**

To understand the mechanism of photoprotection by HHL1, we first determined the subcellular localization of this protein. **HHL1** encodes a predicted 231-amino acid protein of unknown function based on The Arabidopsis Information Resource annotation (www.arabidopsis.org). HHL1 protein also contains a predicted N-terminal chloroplast transit peptide (1 to 39) based on TargetP prediction (Emanuelsson et al., 2000), as well as a transmembrane domain (84 to 109) based on SPLT prediction (Juretić et al., 2002) and a Von Willebrand factor type A (VWA) domain (110 to 158 amino acids) based on SMART prediction (Letunic et al., 2012). VWA domains often appear to involve protein–protein interactions and assembly of multiprotein complexes (Hynes and Zhao, 2000; Whittaker and Hynes, 2002; Tornko and Hochstrasser, 2013). In addition, HHL1 shares a close evolutionary relationship to the Thimet Oligopeptidase family (Mi et al., 2013). Thimet Oligopeptidase specifically degrades peptides of 9 to 17 residues into aminopeptides (Polge et al., 2009). To confirm whether HHL1 is located in the chloroplast, a 35S::HHL1-GFP (green fluorescent protein) construct was introduced into Arabidopsis protoplasts by transient transformation. Analysis of the subcellular localization of the HHL1-GFP fusion proteins by confocal laser scanning microscopy revealed that HHL1 is localized in the chloroplast as expected (Figure 4A). To further determine the location of HHL1 within the chloroplast, we separated the thylakoid membrane and stroma fractions from wild-type Arabidopsis chloroplasts and performed precise sublocation analysis. Immunoblot analysis of the soluble and membrane fractions from Percoll-purified chloroplasts demonstrated that HHL1 is primarily associated with the thylakoid membranes.

![Figure 3. Analysis of ROS in the Wild Type and hhl1 Mutants.](image-url)
of isolated chloroplasts and is not found in the stoma (Figure 4B), in agreement with previous proteomic studies (Ferro et al., 2010).

To localize the HHL1 protein more precisely, wild-type thylakoids isolated from growth light conditions were fractionated into the grana core-, grana margin-, and stroma lamellae-enriched thylakoid membranes by digitonin. The purity of the fractions was evaluated by both determining chlorophyll a/b ratio (Supplemental Figure 3) and by using PSII core protein D1 and PSI core protein PsA as controls. As expected, the PSI core proteins were detected exclusively in the stroma-exposed thylakoids and the PSII proteins in the grana thylakoids, except for a small amount of PSII proteins in the stroma-exposed membrane representing PSII complexes undergoing repair (Figure 4D). The HHL1 protein was found to be distributed among the grana core-, grana margin-, and stroma lamellae-enriched thylakoid membranes thylakoids (Figure 4D). More interestingly, HHL1 protein was more abundant in the grana margin than in the grana core, and it was even more abundant in the stroma lamellae (Figure 4D), indicating that the protein may function in all of these locations.

To confirm whether HHL1 is a transmembrane protein, wild-type thylakoid membrane fractions were isolated and the proteins from these fractions were subjected to immunoblot analysis. After the membrane preparations were sonicated in the presence of various salts, HHL1 protein remained in the membranes (Figure 4C). During these treatments, PsbO (the 33-kD luminal protein of PSII) and CP47 (the PSII core protein) were used as controls (Figure 4C). HHL1 behaved like the integral protein CP47 but not like the peripheral protein PsbO, strongly indicating that HHL1 is an intrinsic membrane protein.

**The HHL1 Protein Associates with PSII Complex**

The reduction in PSI activity and the increased accumulation of ROS in high-light-treated hhl1 mutants suggest a possible role for HHL1 in protection of PSII from photodamage. To test whether HHL1 is directly involved in the protection and repair of PSII, we first asked whether HHL1 protein is directly associated with PSII complexes. HHL1 comigrates with PSII core monomer, as determined by Blue Native (BN) gel immunoblotting (Figure 5A). To further confirm the colocalization of HHL1 and PSII, we separated the proteins of the complexes by SDS-PAGE in the second dimension and immunoblotted the proteins with antibodies or antisera against HHL1 and PSII core proteins. The results further confirmed that HHL1 comigrates with PSII core monomer (Figure 5B). Interestingly, a small amount of HHL1 was also detected in the free protein fraction (Figure 5B), suggesting that the majority of HHL1 may associates with PSII monomer complex, but a small fraction exists as free protein.

Overlay assays were performed to further confirm the direct association of HHL1 with PSII complexes. Chlorophyll-protein complexes were separated by BN-PAGE and blotted onto polyvinylidene fluoride (PVDF) membranes, which were then incubated with purified His-HHL1 fusion protein. Possible HHL1 binding proteins were subsequently detected by immunoblotting with a His-tag-specific antibody. Interestingly, in vitro, as well as primarily comigrating with PSII core monomers, HHL1 associated with other PSII complexes including the PSII-LHCII supercomplexes, PSII dimer complexes, and even free CP43. A negative control without His-HHL1 was included (Figure 5C). Taken together, these results suggest that HHL1 interacts with the PSII monomer complex.

Comigration of HHL1 with free CP43 in an overlay assay indicates a possible direct interaction between HHL1 and CP43. To investigate this possibility, we first examined the interaction of HHL1 with the subunits of PSII in vivo using coimmunoprecipitation (Co-IP). Interestingly, besides PSII core protein CP43, CP47 was also detected with 2% dodecyl β-o-maltoside (DM)–solubilized thylakoid membranes from the wild type, but not from hhl1 (Figure 5D). However, PSII core proteins D1 and D2 were not detected in repeated attempts of this assay (Figure 5D). These indirect or weak interactions may be unstable during the washing steps or long time scales of the experiments. To further confirm the interaction of HHL1 with PSII subunits CP43 and CP47, pull-down experiments were performed with recombinant N-terminal His-tagged HHL1 protein. Consistent with the results of Co-IP in vivo, PSII core proteins CP43 and CP47 were detected in this assay, but no signal was detected with just solubilized thylakoid membranes or the resin (Figure 5E). The PSII core proteins D1 and D2 were not detected in this assay either (Figure 5E). Taken together, these results suggest that HHL1 probably associates with PSII core complex by directly interacting with PSII core subunits CP43 and CP47 specifically, rather than with D1 and D2.

**HHL1 Interacts with LQY1 in Vivo and in Vitro**

Recent studies have revealed that LQY1 also associates with the PSII, probably by directly interacting with CP43 and CP47, during PSII repair and reassembly following photodamage (Lu et al., 2011). The phenotype of high-light hypersensitivity observed in hhl1 mutants in this study is also surprisingly similar to that of lqy1 mutants (Lu et al., 2011). To determine whether HHL1 interacts with LQY1, we first performed bimolecular fluorescence complementation (BiFC) analysis using an Arabidopsis protoplast transient expression system. Coexpression of both nYFP-tagged HHL1 and cYFP-tagged LQY1 resulted in significant fluorescence in the chloroplasts of Arabidopsis protoplast cells (Figure 6A), suggesting that the HHL1 protein interacts with the LQY1 protein. No fluorescence was detected in Arabidopsis protoplast cells cotransformed with nYFP-HHL1 and cYFP, or nYFP and cYFP-LQY1 (Figure 6A). These results strongly indicate that HHL1 interacts with LQY1 in vivo.

To further confirm the interaction between HHL1 and LQY1, a pull-down experiment was performed with recombinant N-terminal His-tagged LQY1 protein. His-HHL1 and His-LQY1 fusion proteins were first expressed in an Escherichia coli expression system (Supplemental Figure 4). Purified His-LQY1 was incubated with 2% DM-solubilized thylakoid membranes from wild-type Arabidopsis or recombinant HHL1 protein expressed in E. coli. After washing with the buffer, proteins bound to the cyanogen bromide (CNBr)–activated resin were separated by SDS-PAGE and examined by immunoblot analysis. As shown in Figures 6B and 6C, HHL1 protein was successfully pulled down by His-LQY1 in the presence of membranes or recombinant HHL1, and no signal was detected when only the solubilized thylakoid membrane, recombinant HHL1 protein, or resin was used. LQY1 could...
therefore pull down HHL1 from solubilized thylakoid membranes and recombinant HHL1 protein, further indicating that there is a direct interaction between HHL1 and LQY1 proteins in vitro. Collectively, these results suggest that HHL1 directly interacts with LQY1 in vivo and in vitro.

**PSII Subunits Levels Are Perturbed in the hhl1 Mutants after High-Light Treatment**

The above analysis showed that the hhl1 mutants have reduced photosynthetic efficiency after high-light treatment, suggesting HHL1 is involved in photoprotection against high-light stress. BiFC and pull-down analysis showed that HHL1 interacts directly with LQY1. Therefore, we speculate that HHL1, which may play a similar role in photoprotection to that of LQY1, is involved in maintaining PSII activity under high-light conditions by regulating the repair and reassembly of PSII complexes. To confirm this hypothesis, we first investigated the influence of high light on the transcription of photosynthesis-related genes in the absence of HHL1. Most photosynthesis-related genes were slightly downregulated after high-light treatment, including PsaA, PsbE, PsbA, PsbD, and PsbS, although stress-related genes ZAT10 and ZAT12 were significantly upregulated (Supplemental Table 2), further indicating that hhl1 mutants suffered more extensive photodamage than wild-type plants.

To explore the abundance of HHL1 in thylakoid membranes, titration of HHL1 protein abundance in thylakoid membranes was performed, referring to the well-characterized stoichiometry of LhcSR/PSII (0.17 ± 0.11) in high-light acclimated thylakoids (Bonente et al., 2011). The results showed that the chlorophyll/HHL1 ratio is 3616.5 ± 330.2 (Supplemental Figure 6). Based on a chlorophyll/PSII ratio of 440 (Pätsikkä et al., 2002), we calculated a HHL1/PSII ratio of 0.122 ± 0.01. The abundance of HHL1 protein in wild-type leaves increased by 31.76% after a 2-d high-light treatment (Figure 7; Supplemental Figure 7).
Figure 5. Association of HHL1 Protein with PSII Protein Complexes.

(A) Immunoblot analysis of HHL1 after separation by the first dimension of BN-PAGE.
(B) Immunoblot analysis of thylakoid proteins separated in the first dimension in a BN-PAGE gel followed by SDS-PAGE in the second dimension. The resolved proteins were immunodetected with anti-D1, anti-D2, anti-CP47, anti-CP43, and anti-HHL1 antibodies.
(C) Overlay assays of the interactions of HHL1 protein with the PSII complex. (a) Thylakoid membrane proteins were separated by BN-PAGE. (b) After blotting, the PVDF membrane without incubation with His-HHL1 protein was subjected to immunodetection with anti-His tag antibody. (c) After blotting, the PVDF membrane was incubated with His-HHL1 protein and subsequently immunodetected with anti-His tag antibody. (d) After blotting, the PVDF membrane was directly subjected to immunodetection with anti-CP43 tag antibody.
(D) Co-IP assay of the interaction between HHL1 and PSII core proteins D1, D2, CP43, and CP47 interaction. Solubilized thylakoid membranes from wild-type (Col-0) and hhl1 mutant leaves were incubated with Protein A/G-coupled anti-HHL1 antiserum. The immunoprecipitates were probed with specific antibodies, as indicated on the left.
(E) Pull-down assay of the interaction between HHL1 and PSII core proteins D1, D2, CP43, and CP47 interaction. Recombinant His-HHL1 bound to CNBr resin was incubated with solubilized thylakoid membranes, and the bound proteins eluted were analyzed immunobiologically employing the antibodies as in (D). All experiments were repeated three times with similar results.

[See online article for color version of this figure.]
LHC B1, ATP synthase subunit B, and cytochrome b6 were relatively stable in both wild-type and mutant plants after high-light treatment (Figure 7). Taken together, these results suggest that deficiency of HHL1 perturbs the relative levels of PSII core subunits after high-light treatment.

Absence of HHL1 Affects the Accumulation of PSII Complexes after High-Light Treatment

Under high irradiance, proteins in the PSII reaction center are damaged and undergo rapid repair and reassembly to enable photosynthetic electron transport to continue (Nickelsen and Rengstl, 2013). The defects in photosynthesis displayed by the hhl1 mutants were possibly caused by a reduced level or malfunction of protein complexes in the electron transport chain. To further investigate the effects of a lack of functional HHL1 on PSII structure and function, we analyzed the accumulation of various PSII complexes in wild-type and hhl1 plants. Thylakoid membranes were solubilized in 2% DM, membrane protein complexes were separated by BN-PAGE (Figure 8A), and the complexes were analyzed by immunoblotting with antibodies specific for PSII core proteins. Results with anti-CP43 antisera showed that hhl1 mutants contain less PSII-LHCII supercomplex than wild-type thylakoid membranes (Figure 8B). The reduction of the PSII-LHCII complex in the hhl1 mutants was further confirmed by immunoblot analysis with anti-D1 antisera (Figure 8C), suggesting that absence of HHL1 may affect the formation and stability of the PSII-LHCII supercomplex after high-light treatment.

To study the stability of the assembled PSII complex, we treated leaves with chloramphenicol to block chloroplast-encoded protein synthesis and measured the PSII protein content by immunoblot analysis. In the absence of chloramphenicol, levels of PSII proteins D1, D2, CP43, and CP47 declined gradually in both wild-type and hhl1 mutant plants and showed no apparent difference after a short (0, 2, or 4 h) high-light treatment (Figure 9A). However, in the presence of chloramphenicol, the degradation rates of PSII core subunits D1, D2, CP43, and CP47 were more rapid in the hhl1 mutants, and degradation of D1 and D2 was more rapid than that of CP43 and CP47 (Figure 9B). This may be because D1 and D2 are more rapidly photodamaged and degraded by chloroplast proteases than CP43 and CP47 (Chi et al., 2012b).

More interestingly, the HHL1 abundance was rapidly increased by short-term high-light treatment in the absence of chloramphenicol but degraded following short-term high-light stress in the presence of chloramphenicol (Figure 9B), indicating that HHL1 protein is highly dynamic under high-light treatment. However, HHL1 transcription is not affected by high light (Supplemental Figure 8), which indicates that HHL1 is regulated at the posttranscriptional level. Taken together, these results suggest that the absence of HHL1 protein affects the reassembly and stability of PSII core proteins under high-light treatment.

The Homozygous hhl1 lqy1 Double Mutant Is More Sensitive to High Light

The above analysis showed that HHL1 has surprising similarities to HHL1, which is involved in PSII repair and reassembly, suggesting that HHL1 and LQY1 appear to share a functional redundancy or synergy in PSII repair and reassembly. To confirm this hypothesis, we next investigated the genetic relationship between HHL1 and LQY1. We first obtained the homozygous...
lqy1 mutant and generated hhl1 lqy1 double mutants by crossing (Figures 10A to 10C). In growth light conditions, the hhl1, lqy1, and hhl1 lqy1 double mutants showed no obvious differences from the wild type (Figure 10D). To compare PSII activity, we measured the maximum photochemical efficiency of PSII (Fv/Fm) before and after high-light treatment. The hhl1, lqy1, and hhl1 lqy1 double mutants showed a markedly lower Fv/Fm after a 3-h high-light treatment than the wild type, even though the growth phenotype and Fv/Fm were identical under growth light conditions. Interestingly, the Fv/Fm of the hhl1 lqy1 double mutant declined to a lower level (47%) than the single mutants (55 and 56%) (Figure 10D). However, Fv/Fm declined considerably (72%) in the wild type.

The further analysis of the light-response curves of F700 and the ETR before and after high-light treatment were also performed. After a 3-h high-light treatment, both F700 and ETR in the hhl1 lqy1 double mutant were much lower significantly than in the single mutants. Taken together, detailed chlorophyll fluorescence analyses showed that the homozygous hhl1 lqy1 double mutant is more sensitive to high light than the single mutants, suggesting that HHL1 and LQY1 possess functional redundancy or synergy in PSII repair.

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Figure 7. Analysis of HHL1 and Thylakoid Membrane Protein Accumulation in the Wild Type and hhl1 Mutants.

Thylakoid membrane proteins from the wild type (Col-0) and hhl1 mutants were separated by 15% SDS-urea-PAGE, electroblotted to PVDF membranes, and probed with affinity-purified anti-HHL1 or antisera against known thylakoid membrane proteins obtained from Agrisera. Samples were loaded on an equal chlorophyll basis. Cytb6/f, cytochrome b6/f complex; ATPase, ATP synthase complex; ATPB, the β-subunit of ATP synthase complex; GL, growth light; HL, after 2-d high-light treatment. Similar results were obtained in four independent biological replicates.

Figure 8. Immunological Analysis of PSII Complexes Separated by BN-PAGE.

(A) Representative unstained BN-PAGE gel. Thylakoid membranes from the wild type (Col-0) and hhl1 mutants were solubilized with 2% DM and separated by a native PAGE. A sample with an equal amount of chlorophyll (10 μg) was loaded in each lane. GL, growth light; HL, after 2-d high-light treatment. (B) A representative immunoblot with anti-CP43 antiserum used to probe a BN-PAGE gel. An equal amount of chlorophyll (1.5 μg) was loaded in each lane. (C) A representative immunoblot with anti-D1 antisera used to probe a BN-PAGE gel. An equal amount of chlorophyll (1.5 μg) was loaded in each lane. All experiments were performed in three additional independent biological replicates, and similar results were obtained. [See online article for color version of this figure.]
max), Medicago truncatula, Fragaria ananassa, Prunus trichocarpa, and tomato (Solanum lycopersicum), and the monocots rice (Oryza sativa), maize (Zea mays), barley (Hordeum vulgare), and Aegilops tauschii. However, HHL1 homologs were not found in more primitive photosynthetic organisms, such as cyanobacteria and algae (Supplemental Figures 9 and 10). The evolutionary distribution of HHL1 is therefore also similar to that of LQY1, suggesting that the role of HHL1 may be conserved among other higher land plants. This mode of evolution may reflect the adaptation of plants to excess light stress during the transition to land by evolution of a set of specific auxiliary factors to protect the photosynthetic apparatus.

**DISCUSSION**

PSII repair requires the sequential disassembly and reassembly of PSII proteins and the ligation of various cofactors, and many additional auxiliary and regulatory factors are needed to facilitate this complex, multistep process in chloroplasts (Mulo et al., 2008; Nixon et al., 2010; Nickelsen and Rengstl, 2013). In this study, we established that the nuclear-encoded thylakoid transmembrane protein HHL1 plays an important role in maintaining PSII activity, especially under high-light stress.

**HHL1 Is a Thylakoid Protein Involved in PSII Repair**

Chlorophyll fluorescence measurements showed that mutation of HHL1 significantly decreased the activity of PSII after high-light treatment (Figures 1D and 2A). Recovery rate following photoinhibition was slower in hhl1 than the wild type, suggesting HHL1 may be involved in repairing photodamage to PSII (Figure 2C). Furthermore, we found that 1-qp and 1-qL were also lower in the hhl1 mutants following high-light treatment (Supplemental Table 1), suggesting that there is a more highly oxidized plastoquinone pool in the hhl1 mutants, a response more likely due to a PSII deficiency rather than to downstream defects (Fu et al., 2007). This hypothesis was confirmed by analysis of thylakoid protein accumulation. Deficiency of HHL1 led to a reduction in the accumulation of PSII core subunits after high-light treatment, but accumulation of PSI, Cytb6/f, LHC, or ATPase complexes was not affected (Figure 7), further supporting this hypothesis. Deficiency of HHL1 specifically affected the accumulation of the PSII-LHCII complex (Figure 8), indicating that HHL1 is involved in PSII repair and reassembly.

There are several lines of evidence suggesting that deficiency of HHL1 results in more extensive photooxidative damage and photoinhibition in the chloroplast. First, the mutants had higher qL and accumulation of ROS following high-light treatment (Figure 3). Expression of zinc-finger transcription factors ZAT10 and ZAT12 are activated by high-light-induced oxidative stress (Iida et al., 2000; Gadjev et al., 2006), and these stress-related genes were upregulated more in hhl1 mutants in this study (Supplemental Table 2), indicating greater photooxidative damage and photoinhibition in the mutants. Additionally, in growth light, the hhl1 mutants possessed normal carotenoid levels but higher NPQ than the wild type, indicating that a deficiency in HHL1 dose not directly affect the carotenoid components, and the higher NPQ observed in the mutants under this condition was not due to an alteration of carotenoid components. However after high-light treatment, photooxidative damage resulted in a faster xanthophyll cycle in mutant plants, as shown by increased antheraxanthin and decreased violaxanthin (Supplemental Figure 2), as well as higher qE (Figure 2B), compensating for the defect of PSII repair pathway by dissipating more excess excitation energy as a feedback regulation mechanism. The higher NPQ may be due, at least in part, to the faster xanthophyll cycle in the hhl1 mutants. Finally, extensive photooxidative damage in the mutants also downregulated many photosynthesis-associated genes (Supplemental Table 2) and caused more rapid degradation of PSII core subunits (Figure 9).

**The Role of HHL1 in PSII Repair**

The repair of photodamaged PSII complexes is a complicated process involving multiple steps, including monomerization, partial disassembly of the dimeric complexes in grana stacks, migration of
the PSII monomers to the stroma-exposed thylakoids, partial disassembly of PSII core monomers, release of CP43 into the CP43-less monomer, and proteolysis of the damaged D1 protein. After cotranslational insertion of the newly synthesized D1 subunit into the membrane, D1 is posttranslationally processed, and PSII monomers are reassembled and trafficked to the grana thylakoids, where dimerization takes place (Nixon et al., 2010).

To explore the possible role of HHL1 in PSII repair, we first determined that HHL1 is a thylakoid transmembrane protein (Figures 4B and 4C), as expected given the chloroplast signal sequence and transmembrane domain in this protein (Supplemental Figure 10). PSII repair involves migration of PSII complex from grana to stroma thylakoids (Mulo et al., 2008), and the distribution of HHL1 in both grana and stroma...
HHL1 Functions in Photoprotection of PSII

Despite evidence supporting the role of HHL1 in efficient PSII repair and reassembly of photodamaged PSII proteins, HHL1 appears not to be absolutely essential for PSII core repair, suggesting functional redundancy. To identify other potential PSII repair factors, we screened potential proteins that interact with the PSI monomer core proteins after disassembly of photodamaged PSII-LHCII supercomplexes and PSII core dimers into PSII core monomers. Given that HHL1 simultaneously dissociates from PSII core monomer with CP43, we predict that HHL1 may function in guiding the release of CP43 from PSII core monomers.

This hypothesis was supported by Co-IP and pull-down studies that showed a direct interaction between HHL1 and CP43 in vivo and in vitro. Interestingly, in addition to interacting with CP43, HHL1 also specifically interacts with PSII core subunits CP47, but not with D1 or D2 (Figures 5D and 5E). The reaction center of PSII contains the D1/D2/cytochrome b559 complex, which houses the cofactors necessary for electron transport, which together with the nearest light-harvesting chlorophyll binding proteins CP43 and CP47, form the core complex (Takamiya et al., 2004). Therefore, HHL1 may interact with CP43 and CP47 specifically to help form the binding site(s) for these extrinsic proteins to PSII (Bricker and Frankel, 2002). Database searches revealed that the C-terminal region of the HHL1 protein includes one incomplete VWA domain, which is known to mediate protein–protein interactions in integrins and extracellular matrix proteins (Hynes and Zhao, 2000). The roles of VWA domains in plants is unclear but are often involved in forming multiprotein complexes (Fu et al., 2001; Lin et al., 2011), and the VWA domain of HHL1 may mediate protein–protein interactions involved in the assembly or functioning of PSII complexes.

HHL1 and LQY1 May Form a Complex Involved in PSII Repair Specifically

Despite evidence supporting the role of HHL1 in efficient PSII repair and reassembly of photodamaged PSII proteins, HHL1 appears not to be absolutely essential for PSII core repair, suggesting functional redundancy. To identify other potential PSII repair factors, we screened potential proteins that interact with HHL1-interacting proteins by comparing the precise locations of proteins in the chloroplast and the phenotypes of the mutants. Inactivation of certain factors affected the repair cycle after PSII photodamage in Arabidopsis, including thylakoid lumen protein of 18.3 kD (TLP18.3) (Sirió et al., 2007), the zinc-finger protein LQY1 (Lu et al., 2011), and the PsbP-Like Protein PPL1 (Ishihara et al., 2007). However, it has also been reported that TLP18.3 and PPL1 are not associated with PSII complexes (Ishihara et al., 2007; Sirió et al., 2007). Similarly, we found no interaction of HHL1 with TLP18.3 or PPL1 using BiFC (Supplemental Figure 5).

We noticed a striking functional similarity between HHL1 and LQY1, a small zinc-finger thylakoid protein reportedly involved in maintaining PSI activity under high light by regulating repair and reassembly of PSII complexes (Lu et al., 2011). Both lqy1 and hhl1 mutant plants showed enhanced sensitivity to high light stress, and both suffered extensive photooxidative damage and photoinhibition. Moreover, the accumulation of PSII core subunits and PSII-LHCII complexes significantly decreased in these mutants. LQY1 or HHL1 deficiency also resulted in faster turnover or degradation of PSII core subunits (Figures 2. 3, 8, and 9) (Lu et al., 2011). However, deficiency of either HHL1 or LQY1 resulted in a relatively mild and reversible phenotype. In stark contrast, the hhl1 lqy1 double mutant showed slightly higher photosensitivity compared with hhl1 and lqy1 single mutants (Figure 10), which is reminiscent of the photosensitive phenotype of the deg5 deg8 double mutant (Sun et al., 2007). This suggests functional redundancy or synergy for HHL1 and LQY1 in PSII repair and reassembly.

To explore the molecular relationship between HHL1 and LQY1, we first analyzed whether HHL1 and PSII were in the same complex. Immunoblotting of BN-SDS-PAGE and overlay analysis showed that HHL1 mainly associates with PSII monomeric complexes (Figures 5A to 5C) and specifically interacts with PSII core proteins CP43 and CP47 (Figures 5D and 5E). LQY1 also comigrates with PSII monomers, and Co-IP results suggest that LQY1 also directly interacts with CP43 and CP47 (Lu et al., 2011). These observations indicate that HHL1 and LQY1 may form a complex that functions in a similar process of PSII repair. As suspected, BiFC and pull-down experiments showed that HHL1 directly interacts with LQY1 in vivo (Figure 6). Phylogenetic analysis of HHL1 protein showed that HHL1 is specific to land plants (Supplemental Figures 9 and 10), which is also consistent with the mode of evolution described previously for LQY1 (Lu, 2011). Taken together, our results suggest that HHL1 and LQY1 may form a complex specifically involved in PSII repair and reassembly that has evolved to protect the sensitive photosynthetic machinery of land plants.

METHODS

Plant Materials and Growth Conditions

Mutant pools used for screening were obtained from RIKEN (Ichikawa et al., 2006), T-DNA and transgenic Arabidopsis thaliana lines used in this study were in the Col-0 background. The hhl1-1, hhl1-2, and lqy1-1 mutants were obtained from the ABRC (stock numbers SALK_129146C, SALK_010366C, and SALK_021824C). Arabidopsis was grown in soil in a growth chamber (100 μmol photons m⁻² s⁻¹, 12-h/12-h light/dark photoperiod, 21°C, and 60% relative humidity). Plants for the chlorophyll fluorescence assays and protein analysis were 3 and 4 weeks old, respectively. To study the effects of high light, plants were placed in a high-light growth chamber (1200 to 1500 μmol photons m⁻² s⁻¹) while maintaining the 12-h-light/12-h-dark cycle.

Pigment Analysis

Chlorophyll from 3-week-old plants was extracted with 80% acetone in 2.5 mM HEPES-KOH, pH 7.5, and the amount of chlorophyll was determined as previously described (Wellburn, 1994). Carotenoids were extracted and analyzed as previously described using spectrofluorometry (Yang et al., 2012) and HPLC (Pogson et al., 1996; Li et al., 2009b). Pigments were identified by comparing retention times to references.

Chlorophyll Fluorescence

Chlorophyll fluorescence parameters were measured with the MAXIM version of the Imaging-PAM M-Series chlorophyll fluorescence system.
In situ detection of H$_2$O$_2$, O$_2$, and $\text{^1}O_2$ was performed using DAB, NBT, and SOSG reagent fluorometric probes (FluorChem Q Imaging System; excitation 504 nm and emission 525 nm) as previously described by Lu et al. (2011). Total ROS production was detected by 2′,7′-dichlorofluorescin diacetate fluorometric probes (FluorChem Q Imaging System; excitation 488 nm and emission 525 nm) as previously described (Pei et al., 2000).

Isolation of Thylakoid Membranes

Thylakoid membranes were prepared as previously described (Robinson and Yocum, 1980). Isolated thylakoid membranes were quantified based on total chlorophyll as described (Porra et al., 1989). Total proteins extracted from leaf thylakoid membrane preparations were prepared as described (Liu et al., 2012). Protein concentrations were determined using the Bio-Rad detergent-compatible colorimetric protein assay according to the manufacturer’s protocol (Bio-Rad).

Production of Anti-HHL1 Polyclonal Antibodies

Affinity-purified anti-HHL1 polyclonal antibodies were made by GenScript. A 15-amino acid peptide (corresponding to amino acids 212 to 225 of HHL1) with an additional N-terminal Cys residue, C5LNPOGSDEKTEETS, was synthesized, conjugated with keyhole limpet hemocyanin, and used to raise antibodies against HHL1.

RT-PCR and Quantitative Real-Time RT-PCR

Total RNA was extracted from Arabidopsis rosette leaves using an RNeasy plant mini kit (Qiagen). The RNA samples were reverse transcribed into first-strand cDNA using a PrimeScript RT reagent kit (Takara). For RT-PCR, UBQ10 was used as a control gene. Quantitative real-time RT-PCR was performed using gene-specific primers and SYBR Premix ExTaq reagent (Takara) with a real-time RT-PCR system (Roche-LC480) following the manufacturer’s instructions. Reactions were performed in triplicate for each sample, and expression levels were normalized against ACTIN and UBQ4 gene (for primers used for RT-PCR and quantitative real-time RT-PCR, see Supplemental Table 3).

BN-SDS-PAGE and Immunoblot Analyses

BN-PAGE was performed as described (Schägger et al., 1994) with the modifications described (Peng et al., 2006). Two-dimensional analysis and immunodetection of proteins on a PVDF membrane were performed as described by Lu et al. (2011). For quantification of thylakoid proteins, gels were loaded on an equivalent chlorophyll basis, in amounts ensuring immunodetection was in the linear range. Except for anti-His (which was raised in mouse), all other primary antibodies and antisera were raised in rabbits. Antisera against photosynthetic proteins were purchased from Agrisera.

Protein Overlay Assays

Protein overlay assays were performed as described (Liu et al., 2012).

Subcellular Localization of GFP Fusions and BiFC

Subcellular localization of GFP fusion proteins and BiFC was performed as previously described (Zhang et al., 2011) (for primers used for the fusion constructs, see Supplemental Table 3).

Chloroplast Fractionation and Immunolocalization Studies

Separation of thylakoid and stroma phases and salt washing of thylakoids were performed as described (Armbruster et al., 2010). Subfractionation of grana core-, grana margin-, and stroma lamellae-enriched thylakoids was performed as described by Lu et al. (2011).

Analysis of Protein Degradation

Analysis of protein degradation was performed as described by Ishihara et al. (2007) with minor modifications. Detached leaves, placed adaxial side up on filter papers soaked in sodium phosphate buffer (NaOH/PH 7.0), were illuminated at 1200 μmol photons m$^{-2}$ s$^{-1}$. Detached leaves were incubated with the same buffer containing 200 μg mL$^{-1}$ chloramphenicol under reduced pressure for 30 min prior to photoinhibitory light treatment. To analyze the accumulation of PSII proteins under high-intensity light, proteins were extracted from leaves ground in liquid nitrogen and used for SDS-PAGE and subsequent immunoblotting against PSII protein antibodies.

Immunoprecipitation

Immunoprecipitation of PSII core subunits by anti-HHL1 antibody was performed as described (Klöstermann et al., 2002) with minor modifications. Thylakoid membrane proteins were solubilized with 2% (w/v) DM in 20% (w/v) glycerol, 25 mM BisTris-HCl, pH 7.0, and 1 mM PMSF for 20 min at 4°C. After centrifugation, the supernatant was diluted in an equal volume of the same buffer without DM and preincubated with affinity-purified anti-HHL1 antibody at 4°C for 2 h. Preincubated thylakoid membrane proteins (200 mL, 0.5 mg chlorophyll/mL) were incubated at 4°C overnight with Protein A/G Plus agarose in Pierce spin columns. After incubation overnight with constant rotation at 4°C, the resin was washed five times with ice-cold PBS buffer, pH 7.8, and bound proteins were eluted with SDS-PAGE sample buffer, resolved by SDS-PAGE, and subjected to immunoblot analysis.

Pulldown Assays

One microliter of each recombinant fusion protein was incubated with 1 mL of a 50% suspension (v/v) of CNBr resin in equilibration buffer for 50 min at 4°C. After the thylakoid membranes (100 mg chlorophyll) were solubilized in 2% (w/v) DM in 20% glycerol (w/v), 25 mM BisTris-HCl pH 7.0, and 1 mM PMSF for 15 min at 4°C, they were centrifuged at 10,000 g for 10 min, and supernatant or purified protein was incubated with His-HHL1 or His-LQY1 overnight with constant rotation at 4°C. After washing five times with ice-cold 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA buffer, bound proteins were eluted with SDS-PAGE sample buffer resolved by SDS-PAGE and subjected to immunoblot analyses.

Accession Numbers

Amino acid sequence data for HHL1 and its homologs in other species can be found in the GenBank/EMBL databases under the following accession numbers: Arabidopsis, NP_564903; Physcomitrella patens, XP_001765741; Selaginella moellendorffii, XP_002983221; Picea stichensis, ABK23133; Populus trichocarpa, XP_002314565; grape (Vitis vinifera), XP_002722721; cucumber (Cucumis sativus), XP_004142961; castor bean (Ricinus communis), XP_002515011; soybean (Glycine max), NP_001237795; Medicago
truncatula, XP_003601843; Fragaria ananassa, XP_004310022; Prunus trichocarpa, EM25099; tomato (Solanum lycopersicum), XP_004239174; rice (Oryza sativa), EEC73537; maize (Zea mays), NP_001141155; barley (Hordeum vulgare), BAJ85479; and Aegilops tauschii, EMT22924. The photosynthesis and stress-related gene accession numbers are as follows: LQY1 (At1g75690), PsbA (At1g00020), PsbB (At5g00270), PsbO (At3g50820), PsbS (At1g44575), PsbA (At1g00030), PsaE (At2g20260), LHCBl (At1g29810), ATPB (At1g00480), YCF2 (At5g00860), ELIP2 (At4g14690), ZAT7 (At1g27730), and ZAT12 (At5g9820).

Supplemental Data

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

**Supplemental Figure 1.** Isolation of hhl1-0 Mutant.

**Supplemental Figure 2.** HPLC Analysis of Pigments in the Wild-Type (Col-0), hhl1-1, and hhl1-2 Plants.

**Supplemental Figure 3.** The Chlorophyll a/b Ratios of Grana Core-, Grana Margin-, Stroma Lamellae-Enriched, and Total Thylakoids.

**Supplemental Figure 4.** Expression and Purification of HHL1 and LQY1.

**Supplemental Figure 5.** BiFC Analyses Show HHL1 Interacts with LQY1, but Not with PPL1 or TLP18.3.

**Supplemental Figure 6.** Example of Titration Used for Stoichiometry Determination.

**Supplemental Figure 7.** Relative Abundance of HHL1 and PSII Proteins.

**Supplemental Figure 8.** Transcript Analysis of HHL1 Gene under High-Light Treatment.

**Supplemental Figure 9.** Phylogenetic Analysis of HHL1 Protein and Its Homologs in Other Land Plants.

**Supplemental Table 1.** Alignment Analysis of HHL1 Protein in Other Land Plants.

**Supplemental Table 2.** Chlorophyll Contents and Chlorophyll Fluorescence Parameters in Wild-Type (Col-0) and hhl1 Mutants.

**Supplemental Table 3.** A List of Primers Used in This Study.

**Supplemental Data Set 1.** Text File of the Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 9.

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**AUTHOR CONTRIBUTIONS**


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