The conversion of light energy to chemical energy by photosynthesis requires the concerted action of large protein complexes in the thylakoid membrane. Recent work has provided fundamental insights into the three-dimensional structure of these complexes, but how they are assembled from hundreds of parts remains poorly understood. Particularly little is known about the biogenesis of the cytochrome \( b_6f \) complex (Cyt\( b_6f \)), the redox-coupling complex that interconnects the two photosystems. Here we report the identification of a factor that guides the assembly of Cyt\( b_6f \) in thylakoids of chloroplasts. The protein, DE-ETIOLATION-INDUCED PROTEIN 1 (DEIP1), resides in the thylakoid membrane and is essential for photoautotrophic growth. Knock-out mutants show a specific loss of Cyt\( b_6f \), and are defective in complex assembly. We demonstrate that DEIP1 interacts with the two cytochrome subunits of the complex, PetA and PetB, and mediates the assembly of intermediates in Cyt\( b_6f \) biogenesis. The identification of DEIP1 provides an entry point into the study of the assembly pathway of a crucial complex in photosynthetic electron transfer.
The thylakoidal protein complexes involved in photosynthetic electron transfer represent large macromolecular assemblies comprised of plastid genome-encoded and nuclear genome-encoded protein subunits. In addition, they bind a large number of pigments (chlorophylls, carotenoids), lipids, and redox-active co-factors such as hemes and iron-sulfur clusters. Recent work has provided high-resolution three-dimensional structures of the complexes. The biogenesis of the complexes and their assembly from hundreds of individual parts represents a challenging task involving (i) the co-ordinated expression of nucleus-encoded and plastid-encoded protein subunits, (ii) the post-translational import of the nucleus-encoded subunits into the chloroplast and their faithful targeting to the thylakoid membrane, (iii) the sequential integration of the protein subunits into the membrane in a spatially and temporally highly controlled manner, and (iv) the synthesis and co-ordinated insertion of a large number and variety of co-factors into the nascent complexes.

Little is known about the molecular mechanisms involved, but it has become clear that complex biogenesis is dependent on numerous accessory proteinaceous factors that collectively are referred to as assembly factors. They act as chaperones that support protein folding, promote protein-protein interactions and/or subunit insertion into membranes, help with co-factor attachment to protein subunits, or protect assembly intermediates from degradation or photo-oxidative damage.

Several assembly factors have been identified for photosystem II (PSII; e.g., SCO2, HCF247, HCF136, and PAM68), photosystem I (PSI; e.g., Ycf3, Y31P1, PYG7, and Ycf4), and the chloroplast ATP synthase (e.g., BFA3 and CGL16019,20). Based on mutant analyses, protein interaction studies and identification of assembly intermediates, assembly pathways for these complexes have been proposed. By contrast, little is known about the assembly of the cytochrome b6f (Cytb6f) complex. Cytb6f is a dimer composed of two identical monomeric complexes. Each monomer consists of a core formed by the chloroplast-encoded proteins PetA (cytochrome f), PetB (cytochrome b6) and PetD (subunit IV), and the nucleus-encoded subunit PETC (Rieske iron-sulfur protein). Additional small polypeptide subunits are localized at the periphery of the complex and are believed to contribute to complex stabilization and/or regulate complex activity: the plastid-encoded small subunits PetG, PetL, and PetN, and the nucleus-encoded PETM.

In *Chlamydomonas*, several proteins involved in biogenesis of co-factors of Cytb6f have been described, including the CCB pathway (Cofactor assembly, complex C (b6f), subunit B) composed of the CCB1-4 proteins that deliver heme to the cytochrome b6 apoprotein. Additional proteins such as CPLD38 and CPLD49 have been implicated in Cytb6f accumulation by a yet undefined mechanism.

Homologs of the CCB pathway genes encoding plastid-localized proteins have been identified also in the vascular plant *Arabidopsis thaliana*, and the corresponding knock-out mutants display severe growth phenotypes. Besides the CCB homologs, only a handful of genes have been described to influence the content of the Cytb6f complex in plants. Loss-of-function mutants for the locus *DETECTIVE ACCUMULATION OF Cytb6f* (DAC) display decreased accumulation of the Cytb6f complex (10–20% of wild-type levels) and exhibit pale phenotypes. DAC appears to interact with PetD, but its mode of action and its possible contribution to Cytb6f assembly or stability has remained unknown. The DnaJ-like protein *HIGH CHLOROPHYLL FLUORESCENCE* 222 (HCF222) and the thioredoxin-like protein *HIGH CHLOROPHYLL FLUORESCENCE* 164 (HCF164) were also shown to influence Cytb6f accumulation, likely by their chaperone and disulfide reductase activity, respectively. However, most of the factors implicated in Cytb6f accumulation act in co-factor integration, and very little is known about the pathway governing the assembly of the protein subunits of the Cytb6f complex.

In the present work, we have identified and characterized an essential assembly factor for Cytb6f complex biogenesis in *Arabidopsis*. The protein, encoded by locus AT2G27290, facilitates the assembly of the core protein subunits of the complex. The corresponding gene was found in a time-resolved analysis of gene expression during the greening of etiolated tobacco (*Nicotiana tabacum*) plants. The gene, referred to as *DE-etiolation-induced* protein 1 (DEIP1), responded very early to the light signal by showing pronounced induction of mRNA accumulation. We show that knock-out mutants of DEIP1 are incapable of photoautotrophic growth and almost completely lack fully assembled Cytb6f complexes. Blue-native polyacrylamide (PAA) gel electrophoresis (BN-PAGE), co-immunoprecipitation and bimolecular fluorescence complementation (BiFC) assays revealed that DEIP1 interacts with the Cytb6f subunits PetA and PetB. The deip1 mutants exhibit altered accumulation of Cytb6f complex intermediates, suggesting a role in the initial steps of Cytb6f complex assembly. The identification of DEIP1 provides an entry point into disentangling the assembly process of the Cytb6f complex that previously has not been amenable to experimental investigation.

### Results

**DEIP1** is a thylakoid-anchored protein essential for photosynthesis and autotrophic growth. To identify new regulators of light-induced chloroplast biogenesis, we screened our previous highly time-resolved RNAseq datasets of greening tobacco leaves for genes whose transcripts responded early to the de-etiolation signal. One of the earliest light-induced genes of unknown function was locus 104247735, identified in the nuclear genome of the diploid progenitor species *Nicotiana sylvestris* of the allotetraploid tobacco, *Nicotiana tabacum*. Expression of the locus showed pronounced induction already after 60 min of illumination and peaked as early as after 120 min (Supplementary Fig. 1a). We preliminarily named the corresponding putative gene product de-etiolation induced protein 1 (DEIP1). Its putative ortholog in *Arabidopsis* is encoded by locus AT2G27290 and shows high expression in all green tissues, including cotyledons and true leaves (Supplementary Fig. 1b). The predicted protein comprises 201 amino acids, and contains a predicted chloroplast transit peptide (according to the SUBA4 database) at the N-terminus, an annotated domain of the uncharacterized function, DUF1279, at the C-terminus, and a single predicted transmembrane segment (Supplementary Fig. 1c).

The DUF1279 of *DEIP1* is conserved in all embryophytes, and a putative ortholog is also found in the green alga *Chlamydomonas reinhardtii* (Supplementary Figs. 2, 3; Supplementary Table 1). However, no homologs of DEIP1 were found in cyanobacteria.

De-etiolation experiments conducted with *Arabidopsis* seedlings germinated in the dark revealed that, similar to tobacco, *DEIP1* transcripts are strongly upregulated during light-induced greening, displaying a 12-fold induction 48 h after the onset of illumination (Supplementary Fig. 4a). The light-induced expression of *DEIP1* followed a similar kinetics as the *LHCBC2* transcript (encoding a component of the PSII light-harvesting antenna), while mRNA accumulation of *LPOR* (encoding the light-dependent photochlorophyllide oxidoreductase) showed a rapid decrease within the first 6 h of light (Supplementary Fig. 4a), as previously reported.

To address the physiological function of the DEIP1 gene product, collections of T-DNA insertion lines were screened for mutants potentially affecting locus AT2G27290. Two potential
mutant alleles were identified and the predicted insertions were experimentally verified. One of the mutants harbors the T-DNA insertion in the first intron (deip1-1; SALK_037263), whereas the second mutant carries it in the 5' untranslated regions (5' UTR; deip1-2; SALK_067267C; Supplementary Fig. 4b, c). Since T-DNA insertions in introns and UTRs often produce leaky mutants that do not represent true null alleles, we used CRISPR/Cas9-based genome editing to additionally produce genuine knock-out mutants. A genome-edited line harboring a large deletion of 1084 bp in the coding region of AT2G27290 was selected for further analysis (deip1-3; Supplementary Fig. 4b–d).

Homozygous deip1-1 and deip1-3 mutants displayed striking pigment-deficient phenotypes and were incapable of photoautotrophic growth under moderate light (95 μmol m⁻² s⁻¹) conditions, thus necessitating their maintenance as heterozygous plants (Fig. 1a, Supplementary Fig. 5a). By contrast, the T-DNA insertion line deip1-2 resembled the wild type (Fig. 1a). To correlate the observed phenotypes with DEIP1 gene expression, the transcript accumulation was measured by qRT-PCR analysis. The data revealed that, while DEIP1 mRNA accumulation was reduced to undetectably low levels in mutant lines deip1-1 and deip1-3, the deip1-2 mutant accumulated the mRNA to ~30% of the wild-type level (Fig. 1b). These results suggest that both the T-DNA insertion into intron 1 (deip1-1) and the CRISPR/Cas9 deletion of the coding region (deip1-3) represent true knock-out alleles, while the deip1-2 mutant represents a mild knock-down allele (Supplementary Fig. 4b; Fig. 1b) that may not appreciably affect DEIP1 function. This interpretation gained further support from chlorophyll content measurements that revealed strongly reduced chlorophyll contents in the deip1-1 and deip1-3 mutants, but not in the deip1-2 mutant (Supplementary Fig. 5b).

To ultimately confirm that DEIP1 loss of function was causally responsible for the observed mutant phenotypes and, at the same time, be able to determine the subcellular and suborganellar localization of the protein, a transformation constructs for genetic complementation was generated by fusing the coding sequence of
DEIP1 to a C-terminal GFP tag. The construct was introduced into heterozygous deip1-1 mutant lines by Agrobacterium-mediated transformation, followed by isolation of transgenic lines that were homozygous for both the deip1-1 T-DNA insertion and the P\textsubscript{UBQ10}:DEIP1-GFP construct by genetic segregation (Supplementary Fig. 6a). Four independent doubly homozygous complemented lines were isolated (subsequently referred to as deip1-1-C1 to deip1-1-C4). Transcript accumulation from the DEIP1-GFP fusion gene in the complemented lines was 20 to 80 times higher than that from the native DEIP1 locus in wild-type plants (Supplementary Fig. 6b), and accumulation of the DEIP1-GFP fusion protein was readily detectable by immunoblot analysis (Supplementary Fig. 6c). Full complementation of the deip1-1 mutant phenotype was observed in all four lines (Fig. 1a), including restoration of the chlorophyll content to wild-type levels (Supplementary Fig. 6d). Analysis of GFP fluorescence by confocal laser-scanning microscopy revealed chloroplasts localization of DEIP1-GFP (Fig. 1c), consistent with the predicted presence of an N-terminal transit peptide for import into plastids. Immunoblot analysis of soluble and thylakoid fractions showed that DEIP1 is associated with thylakoids (Fig. 1d). To resolve the localization within thylakoids in greater detail, thylakoid preparations were fractionated into grana cores, grana margins and stroma lamellae (Fig. 1e). Immunoblot analyses using an anti-GFP antibody (for detection of the DEIP1-GFP fusion) and a set of marker proteins as controls revealed that DEIP1 predominantly localizes to the grana margins. Its distribution in the various thylakoid fractions was strikingly similar to that of PetA, the cytochrome f subunit of the Cytb\textsubscript{f} complex (Fig. 1e). Washes of isolated thylakoid membranes with 1 M NaCl showed that DEIP1 is strongly anchored to the membrane (Fig. 1f) and suggested that it is a membrane-spanning protein. To test this assumption and determine the topology of the protein in the thylakoid membranes, trypsin digestion assays were performed. Protease susceptibility of the GFP tail indicated that the C-terminus of DEIP1 is exposed to the stromal side, while the N-terminus is likely exposed to the luminal side of the thylakoid membrane (Fig. 1g).

Next, we explored the role of DEIP1 in photosynthesis by pulse amplitude modulation (PAM) measurements in the wild-type and the deip1-1, deip1-2 and deip1-3 mutants (Fig. 2). The maximum quantum yield of photosystem II (F\textsubbox{V}/F\textsubbox{m}) and the effective quantum yield (Φ\textsubbox{PSII}) were found to be strongly reduced under both low-light (LL; 56 PAR) and high-light (HL; 461 PAR) conditions in the deip1-1 and deip1-3 mutants (Fig. 2a). While none of the mutants showed pronounced defects in non-photochemical quenching (NPQ) under low light, NPQ was much reduced in the deip1-1 and deip1-3 mutants under high light (Fig. 2b). Finally, the chlorophyll fluorescence parameter qL (reflecting the fraction of PSII reaction centers that are ‘open’, with the primary quinone-type acceptor QA being in the oxidized state) and the electron transport rate (ETR) were severely reduced in deip1-1 and deip1-3 under both low light and high light, and mildly reduced in deip1-2 under high light (Fig. 2c, d). Next, we measured chlorophyll a fluorescence induction kinetics in the wild-type and the deip1-2 mutant (Fig. 2e). While the fluorescence curves were similar in the wild-type and the deip1-2 mutant, the curves for deip1-1 and deip1-3 showed the typical pattern occurring when electron transfer from PSII to downstream components of the photosynthetic electron transport chain is impaired (Fig. 2e). As expected, all measured photosynthetic parameters were restored to wild-type-like levels in the DEIP1-GFP complemented lines (Supplementary Fig. 7).

The deip1-2 mutant displayed no visible phenotype upon growth under standard conditions (Fig. 1a), but showed mild defects in qL and ETR under high light (Fig. 2c, d). This observation prompted us to examine the photosynthetic performance of this mutant during acclimation from moderate light (95 μmol m\textsuperscript{-2} s\textsuperscript{-1}) to high-light conditions (600 μmol m\textsuperscript{-2} s\textsuperscript{-1}; Supplementary Fig. 8). We observed a pronounced decrease in F\textsubbox{v}/F\textsubbox{m} in cotyledons of deip1-2 upon exposure to high light (Supplementary Fig. 8a), but not under standard light conditions. However, chlorophyll contents did not differ significantly between the wild type and the deip1-2 mutant in any of the tested conditions (Supplementary Fig. 8b).

Finally, we examined morphology and ultrastructure of deip1-1, deip1-2, and deip1-3 mutant chloroplasts (Fig. 3). Confocal laser-scanning microscopy revealed that mesophyll cells of the two knock-out mutants, deip1-1, and deip1-3, harbored smaller chloroplasts than cells of the wild-type and the mild knock-down...
mutant *deip1-2* (Fig. 3a). However, the typical dotted fluorescence pattern originating mainly from LHCII complexes residing in grana stacks was still detectable in the knock-out mutants. Further analysis of thylakoid architecture by transmission electron microscopy (TEM; Fig. 3b) confirmed the smaller chloroplast size in the *deip1-1* and *deip1-3* mutants. Although grana stacking was observed in all three mutants, aberrant membrane splits were seen near the grana stacks, and also some discontinuous membranes in the non-appressed thylakoid regions (Fig. 3b).

Taken together, our characterization of the mutant phenotypes demonstrates that DEIP1 is essential for photoautotrophic growth, localizes to the grana margins, resides within the membrane, and is required for photosynthesis and proper chloroplast development.

**DEIP1 is required for the accumulation of the Cytb6f complex, but does not contribute to synthesis or degradation of its subunits.** To test whether the pale phenotypes observed in the knock-out mutants *deip1-1* and *deip1-3* are the results of photo-
reached wild-type levels (Supplementary Fig. 9a). This finding raised the possibility that the light sensitivity of the deip1 mutants is due to an imbalance in the photosynthetic electron transfer chain, which causes photo-oxidative stress and triggers rapid destruction of the protein complexes in the thylakoid membrane. We, therefore, investigated the accumulation of the major thylakoidal protein complexes in total protein extracts from wild-type and mutant seedlings by immunoblot analyses (Fig. 4b). When seedlings were grown in moderate light, we observed complete absence of the Cytb6f subunits PetA and PetB, and reduced accumulation of subunits of PSII, PSI, and cpATP synthase (to ~25%) in the deip1-1 and deip1-3 knock-out mutants, while the deip1-2 mutant displayed a reduction in Cytb6f subunit accumulation to ~50–75% of the wild-type level and no reduction in the other protein complexes (Fig. 4b). Based on these patterns, we suspected that the primary defect in the deip1 mutants is in Cytb6f, and the disruption of the photosynthetic electron transfer chain by the Cytb6f deficiency causes severe photo-oxidative damage that secondarily results in lower accumulation of all other thylakoidal protein complexes. To test this assumption, protein accumulation was investigated in plants grown for 5 weeks under extreme low-light conditions (5 µmol m$^{-2}$ s$^{-1}$), where no phenotypic differences were observed between the wild type and the mutants. These immunoblot analyses revealed that the Cytb6f subunits are virtually undetectable in the deip1-1 and deip1-3 mutants (Fig. 4b; Supplementary Fig. 9b), whereas the subunits of the other protein complexes accumulated to wild-type-like levels (Fig. 4b). To verify that the accumulation patterns observed in total protein samples faithfully reflect the composition of the thylakoid membranes, we also examined the abundance of Cytb6f subunits in thylakoids purified from 7-day-old seedlings grown under moderate light and 5-week-old plants grown under low light by immunoblot analyses. These experiments confirmed the absence of all major Cytb6f subunits (PetA, PetB, PETC, and PetD) from the deip1 knock-out mutants in moderate light, and their extremely low accumulation in low light (Fig. 4c). When the complemented lines were analyzed, we observed wild-type levels of PetA and PETC in all deip1-C lines, suggesting that the overaccumulation of DEIP1 in these plants does not result in excessive accumulation of the Cytb6f complex (Supplementary Fig. 9c).

Next, we analyzed the main photosynthetic complexes by blue-native polyacrylamide (PAA) gel electrophoresis (BN-PAGE) of thylakoid samples from wild-type seedlings and the deip1-1, deip1-2, and deip1-3 mutants grown under moderate light (Fig. 5a, b). These experiments confirmed the absence of the Cytb6f complex in the knock-out mutants and revealed decreased abundance of PSII supercomplexes, the PSI/PSII dimer band, and the LHClII assembly in deip1-1 and deip1-3 grown under moderate light (Fig. 4a). Subsequent 2D-SDS-PAGE further confirmed the absence of the Cytb6f complex from both deip1-1 and deip1-3, and decreased complex abundance in the deip1-2 mutant (Supplementary Fig. 10). In agreement with our immunoblot analyses (Fig. 4c), the PSI and PSI complexes accumulated to wild-type-like levels in deip1 knock-out mutants grown under low light (Fig. 5b), while no detectable accumulation of the Cytb6f complex occurred.

The function of DEIP1 in the chloroplast and its requirement for Cytb6f accumulation would be compatible with a role in the expression of plastid-encoded Cytb6f subunits, or alternatively, a function in complex assembly and/or stability. To test for a possible function in transcription, mRNA turnover or translation, we conducted array-based RNA and ribosome profiling in wild-type plants and the deip1-1 knock-out mutant grown under extremely low light, where the effects of photo-oxidative damage are minimal (Fig. 5c, d; Supplementary Fig. 11; Supplementary Data 1). The biological replicates showed high reproducibility in oxidative damage, plants were grown for 5 weeks under extreme low-light conditions (5 µmol m$^{-2}$ s$^{-1}$) in heterotrophic culture. Indeed, the mutants were visibly indistinguishable from wild-type plants (Fig. 4a) under these conditions, and chlorophyll contents Fig. 3 Confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM) images of chloroplasts in the wild type (WT) and the deip1-1, deip1-2, and deip1-3 mutants. a CLSM images of chloroplasts. The red fluorescence corresponds to the chlorophyll fluorescence in chloroplasts of mesophyll cells, the dotted pattern reflects the arrangement of grana stacks. Representative images were selected based on observation of 50 chloroplasts per genotype. n = 2 independent biological replicates. b TEM micrographs obtained from chloroplasts of mesophyll cells. The arrowheads mark fork-like structures close to appressed membranes within thylakoids of deip1-1 and deip1-3. Representative images were selected based on observation of 15 chloroplasts. n = 2 independent biological replicates.
Fig. 4 Light-dependent accumulation of thylakoid proteins in deip1 mutants.  

(a) Phenotypes of 5-week-old plants grown in vitro under extreme low-light conditions (5 µmol m\(^{-2}\) s\(^{-1}\)).

(b) Accumulation of diagnostic protein subunits of the major thylakoidal protein complexes in the wild type (WT), and the deip1-1, deip1-2, and deip1-3 mutants grown under moderate light (ML; 95 µmol m\(^{-2}\) s\(^{-1}\)) or extreme low light (LL; 5 µmol m\(^{-2}\) s\(^{-1}\)). Samples of 20 µg total protein were loaded for deip1-1, deip1-2, and deip1-3 (1×), and blots were probed with antibodies against proteins of the PSII complex (PsbA, PsbD, and LHCB2), the PSI complex (PsaB, PSAD and LCHA2), the Cyt\(b_6f\) complex (PetA, PetB) and the chloroplast ATP synthase (AtpB and AtpE). As a control for equal loading, the Coomassie-stained PAA gel (CBB) is shown below each series of immunoblots.  

(c) Protein accumulation in thylakoids purified from the wild type (WT) and the deip1 mutants grown under moderate light (ML) or extreme low light (LL). Thylakoid protein samples equivalent to 1 µg of chlorophyll were resolved in 12.5% PAA gels and blotted. Immune detection was conducted with antibodies against the Cyt\(b_6f\) subunits PetA, PetB, PETC, and PetD. The light-harvesting complex protein LHCB2 served as a marker for equal chlorophyll-based loading, and the Coomassie-stained PAA gel (CBB) is shown below each series of immunoblots. Note that, due to their low chlorophyll content, the amount of loaded protein is substantially higher in the deip1-1 and deip1-3 mutants grown under ML. n = 3 independent biological replicates.
ribosome footprints and RNA accumulation data across the plastid genome (with average $r$-values > 0.99 for both transcriptomic and ribosome profiling data; Supplementary Data 1). No pronounced changes in transcript accumulation were observed between the wild-type and the deip1-1 mutant (Fig. 5c). However, we observed a significantly (>2-fold) lower ribosome footprint abundance for the psbA mRNA (2.3-fold) in deip1-1 compared to the wild type (Fig. 5d; Supplementary Fig. 11), which appears to be caused by a reduced translation efficiency of unaltered psbA transcript levels. Translation of psbA is known to be the step in plastid gene expression that is most sensitive to environmental changes and genetic perturbations (e.g., changes in light intensity\textsuperscript{48}), suggesting that its down-regulation in the deip1 knock-out plants represents a response to the Cytb$_{6f}$ deficiency (Fig. 5d). Importantly, no differences in the translation output or transcript accumulation for chloroplast-
encoded Cytb6f genes (or any other chloroplast gene besides psbA) were seen in the mutant, indicating that DEIP1 does not act at the level of Cytb6f subunit expression in the chloroplast.

The potential involvement of DEIP1 in Cytb6f complex stability was assessed by blocking de novo synthesis with the chloroplast translation inhibitor lincomycin and following complex accumulation over time (Fig. 5e). Due to the long lifetime of the Cytb6f complex, and to avoid extreme photodamage due to the lack of D1 turnover, the seedlings were infiltrated with lincomycin (or water as control) and incubated in the dark at 4 °C for 16 h, and then transferred to liquid media for 4 days under low light intensities. Immunoblot analysis showed that the decline in the contents of the Cytb6f subunit PetB and the PSII subunit PsbD (analyzed as a control) was similar in lincomycin-infiltrated wild-type leaves and deip1-2 mutant leaves, suggesting that DEIP1 does not act in the degradation or stabilization of Cytb6f subunits (Fig. 5e).

DEIP1 interacts with PetA and PetB and acts as an assembly factor for the Cytb6f complex. To further explore the role of DEIP1 in the accumulation of Cytb6f complex, we employed BN-PAGE and 2D-SDS-PAGE to analyze thylakoid samples purified from 7-day-old wild-type seedlings grown under moderate light and 5-week-old deip1-1 plants grown under low light (Fig. 6a, b). In the wild type, several putative assembly intermediates of Cytb6f could be detected. Based on co-migration of Cytb6f subunits, these intermediate complexes include: (i) three complexes containing PetA and PFTC (intermediates IV, V, and VI), (ii) three complexes containing PetB (intermediates II, III, and V), and (iii) two complexes containing PetD (intermediates I and V). Interestingly, the deip1-1 mutant accumulated monomeric forms of both PetA and PetC, and intermediates IV and V were not detected (Fig. 6b). In addition, the deip1-1 mutant exhibited lower accumulation of the complex intermediate III (containing PetB), and accumulated an additional putative assembly intermediate containing PetD, which did not co-migrate with PetA, PetB, or PETC (Fig. 6b). Next, we assessed the assembly intermediates in thylakoid samples extracted from deip1-C4 seedlings (Fig. 6c). DEIP1-GFP co-migrated with PetB (in intermediate III), with the intermediate complex containing all four core subunits (intermediate V) and with the complex containing PetA and PETC (intermediate III). DEIP1 does not co-migrate with the fully assembled Cytb6f complex, providing further evidence against a role in complex stabilization.

To verify the association of DEIP1 with other thylakoid proteins in protein complexes, bis(sulfosuccinimidyl)suberate (BS5)–mediated crosslinking of thylakoid fractions from the deip1-C4 complemented line and, as a control, stromal fractions of a transgenic line expressing a soluble chloroplast-targeted GFP (ctGFP line) was performed (Fig. 7a). DEIP1 was found to be present in the thylakoids within high molecular weight complexes (hmDEIP1-GFP; Fig. 7a).

To provide direct evidence for the protein-protein interactions suggested by the co-migration patterns of Cytb6f subunits and DEIP1 (Fig. 7b), co-immunoprecipitation (co-IP) experiments were conducted (Fig. 7b; Supplementary Fig. 12a, b). The crosslinker dithiobis(succinimidyl propionate) (DSP) was applied to stabilize protein interaction in thylakoid membranes isolated from deip1-C4 and the wild type prior the solubilisation and co-IP. These experiments revealed strong co-IP enrichment of DEIP1–GFP with PetA and PetB and much weaker with PetD. No interaction was detected with PETC and all control proteins from

![Fig. 6 DEIP1 co-migrates with core subunits of the Cytb6f complex. 2D-SDS-PAGE analyses of thylakoids purified from a wild-type plants grown under moderate (ML) light for 7 days, b deip1-1 mutants grown under low light (LL) for 5 weeks, and c the deip1-C4 complemented line grown under moderate light (ML) for 7 days. Thylakoid samples equivalent to 20 μg chlorophyll were solubilized in β-DDM, and resolved in an 8-13.8% gradient gel. SDS-PAGE was performed as second dimension, and immunodetection was conducted with anti-PetA, anti-PetB, anti-PETC, and anti-PetD antibodies. For the deip1-C4 line, the antibody against GFP was included, and the band that corresponds to DEIP1–GFP is indicated by the black arrowhead. Putative assembly intermediates are indicated as I, II, III, IV, V, and VI. See text for details. n = 3 independent biological replicates.](image-url)
other thylakoidal protein complexes: the PSII subunit PsbA (as a protein with a high turnover rate), the ATP synthase subunit AtpB (as a protein enriched in grana margins), and the PSI subunit PsAC (Fig. 7b). The presence of weak signals for PetA, PetB, PetD, and AtpB in the co-IPs with wild-type samples is likely attributable to non-specific immunoprecipitation due to the crowding of proteins in the (highly protein-rich) thylakoid membranes.

To confirm the interactions of DEIP1 with Cytb6f subunits by an independent method, bimolecular fluorescence complementation (BiFC) assays were performed (Fig. 8). Interaction of DEIP1 with itself was observed (YFP fluorescence detected in 54% of the transfected protoplasts showing RFP fluorescence), suggesting that DEIP1 can undergo dimerization. Strong interactions were also seen with PetA (in 30% of the protoplasts) and with PetB (in 28% of the protoplasts; Fig. 8). No interaction was observed between DEIP1 and PETC, while the interaction with PetD was extremely weak and only observed in 17% of the protoplasts (Fig. 8). Overall, the results of the BiFC assays confirm the protein-protein interactions detected in our co-IP experiments and suggest that DEIP1 mediates essential steps in Cytb6f assembly by interacting with the subunits PetA and PetB.

**Discussion**

The Cytb6f complex plays a central role in photosynthesis by functionally connecting PSII with PSI in the thylakoid membrane. In contrast to the two photosystems, very little is known about the biogenesis of Cytb6f and the auxiliary factors involved in the Cytb6f assembly process. In this work, we have identified and characterized a protein factor, DEIP1, that plays a crucial role in Cytb6f assembly. Our data show that DEIP1 is a thylakoid-anchored protein that is specifically required for the biogenesis of Cytb6f. As photo-oxidative damage can secondarily lead to reduced levels of other thylakoidal protein complexes, we analyzed plant material grown under extreme low-light conditions. This approach minimizes photo-oxidative destruction of pigment-protein complexes in the thylakoid membrane of mutants with severe defects in the photosynthetic electron transport chain. The results demonstrated that Cytb6f is the only complex that is primarily affected in deip1 loss-of-function mutants (Fig. 4). Consistent with this finding, the deip1-2 knock-down mutant exhibits lower QL and ETR exclusively under high light (Fig. 2c, d), a finding that can be attributed to the decreased activity of the Cytb6f complex. As the Cytb6f complex catalyzes the rate-limiting step in photosynthetic electron transfer, decreased complex activity in the mutants directly impacts luminal acidification under high light and, in this way, photosynthetic control. Similar observations were made in the pgr1 mutant (harboring a point mutation in PETC), thus lending additional support to the conclusion that reduced Cytb6f complex accumulation represents the primary defect in the deip1 mutants.

The absence of Cytb6f complex from deip1-1 and deip1-3 knockout mutants resulted in smaller chloroplasts, and the thylakoid ultrastructure displayed aberrant fork-like splits, as commonly observed in immature thylakoids. This observation confirms that Cytb6f contributes to thylakoid maturation and shows that impaired thylakoid development negatively affects chloroplast development and size (Fig. 3). All deip1 mutants formed grana stacks, reinforcing the notion that grana stacking is mainly, though not exclusively, mediated by the accumulation of LHCSR.

The DEIP1 gene was identified based on its very rapid induction upon reillumination of etiolated leaves (Supplementary Fig. 1). However, the Cytb6f complex accumulates already in the prolamellar bodies (PLBs) of etioplasts, suggesting that the low-level basal expression of DEIP1 is sufficient to facilitate the assembly of the Cytb6f amounts required in etioplast seedlings. Clearly, the function of DEIP1 in the assembly of Cytb6f in the dark warrants further study.

Our genome-wide analyses of mRNA accumulation, ribosome footprint abundance, and translation efficiency demonstrated that the expression of all chloroplast-encoded subunits of the Cytb6f complex is unchanged in the deip1 mutants, excluding a function of DEIP1 in the expression of complex subunits (Fig. 5c, d). However, we noted a decreased translation output of psbA, the gene encoding the D1 subunit of PSII. Similar changes in psbA expression were previously reported for other mutants with reduced Cytb6f content, and therefore, likely represent secondary effects that are due to psbA being the most highly regulated chloroplast gene that is most sensitive to genetic perturbations and environmental changes.

The symmetric composition of the Cytb6f dimer argues for formation of a monomeric Cytb6f (containing all subunits) prior to formation of the functional Cytb6f dimer. Complex assembly has

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**Fig. 7 DEIP1 is associated in high molecular weight complexes and interacts primarily with PetA and PetB.**

*a* BS3-mediated crosslink of stroma fractions and thylakoid samples obtained from transgenic plants expressing a soluble chloroplast-targeted GFP (ctpGFP) and the deip1-C4 line. The treated samples were separated in a 10% SDS-PAA gel. An anti-GFP antibody was used to detect the soluble GFP, the monomeric DEIP1 (mDEIP1-GFP) and the high molecular weight complexes containing DEIP1 (hmwDEIP1-GFP). *n* = 3 independent biological replicates. *b* Co-IP from deip1-C4 and the wild type (WT). Thylakoid samples were treated with 1.5 mM DSP crosslinker prior to co-IP. The eluate was resolved by SDS-PAGE, and the immunodetection was done with anti-GFP, anti-PetA, anti-PetB, anti-PETC, anti-PetD, anti-PsbA, anti-AtpB and anti-PsaC antibodies. *n* = 3 independent biological replicates.
been proposed to initiate with the membrane insertion of the four core subunits PetA, PetB, PETC and PetD (Fig. 9). Of the three chloroplast genome-encoded subunits, PetB and PetA are co-translationally inserted into the thylakoid membrane, while this may not be the case for PetD. Previous in vitro experiments suggested that PetB and PetD are inserted into the thylakoid membrane by the signal recognition particle (SRP) pathway and the general insertase ALBINO3 (ALB3). In our two-dimensional gel electrophoresis experiments conducted to resolve putative assembly intermediates, we did not observe co-migration of PetB and PetD in low molecular weight complexes (assembly intermediate I containing PetD; assembly intermediates II and III containing PetB; Fig. 6a). Our data are consistent with a model, in which Cyt_{b6 f} assembly initiates with PetB and PetA insertion into the membrane prior to addition of PetD to PetB, and PETC to PetA (Fig. 9). In the wild type, none of the four core subunits accumulate as a free protein, suggesting that the initial steps of complex assembly in the membrane occur very rapidly. This may include the association of PetB with auxiliary factors that remain to be identified (Fig. 6a; assembly intermediates II and III). Our BN-PAGE and two-dimensional gel electrophoresis suggested that DEIP1 accumulates as a dimer in the thylakoid membrane, and co-migrates with the PetB assembly intermediate III (Fig. 6c). In the absence of DEIP1, increased amounts of free and incompletely assembled PetD subunits accumulate (Fig. 6b), presumably because the PetB-DEIP1 interaction is required for formation of the PetB-PetD heterodimer (or other bottleneck effects that restrict assembly of the monomeric Cyt_{b6 f}; Fig. 9).

Based on the intermediate complexes and the protein-protein interactions identified (Figs. 6–8), we propose that DEIP1 mediates the assembly of the monomeric Cyt_{b6 f} by interacting with

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**Fig. 8 BiFC analyses to verify the interaction of DEIP1 with Cyt_{b6 f} complex subunits in chloroplasts.** Bimolecular fluorescence complementation (BiFC) assays with the DEIP1-cYFP fusion protein in tobacco protoplasts are shown. Interactions were assessed with the nYFP fused to the C-terminus of DEIP1, PetA, PetB, PETC and PetD. The endogenous transit peptide was employed for DEIP1, and the pea RBCS chloroplast transit peptide was employed for all Cyt_{b6 f} subunits. The bright field (BF), YFP (green), chlorophyll auto fluorescence (red) and the merged images are depicted. Representative images were selected based on the observation of 80 protoplasts in two independent experiments for each interaction. Scale bars: 20 μm.
both the PetB-PetD and the PetA-PETC subcomplexes (assembly
intermediate V; Figs. 6, 9). The abnormal accumulation of low
molecular weight signals for PetA, PETC and PetD, and the
decreased abundance of the PetB-containing assembly inter-
mediate III in the deip1-1 mutant (Fig. 6b) support the proposed
critical role of DEIP1 in the assembly of the monomeric Cyt_{b6f}
complex (Fig. 9). Although it appears likely that the stabilizing
small subunits PetG, PetL, PETM, and PetN are added only after
the four core subunits have been assembled (Fig. 9)26,28, the exact
temporal order of their incorporation into the complex remains
to be defined. Finally, it needs to be borne in mind that the Cyt_{b6f}
complex is tightly associated to lipids that influence complex
assembly and stability62,64. It, therefore, cannot be excluded that
some of the intermediate complexes and unassembled subunits
detected in the knock-out mutant (Fig. 6b) are the result of
detergent treatments together with the complex-destabilizing
effects of the absence of DEIP1.

Although the assembly of the Cyt_{b6f} is largely unclear, our co-
immunoprecipitation analyses and BiFC assays indicate that
DEIP1 interacts primarily with PetA and PetB, but not with the
PETC, and only weakly with PetD (Figs. 7, 8). These data suggest
that DEIP1 acts in Cyt_{b6f} complex assembly by mediating the
interaction between PetB and the PetA-containing subcomplexes
to form an assembly intermediate that contains all four core
subunits (Fig. 9). DEIP1 could act as a scaffolding protein that
brings PetA and PetB together, possibly aided by DEIP1 self-
dimerization. The exact protein composition of the DEIP1-
containing assembly intermediates II and III (containing PetA
and PETC; Fig. 6a) as well as the composition of the assembly
intermediates II and III (containing PetB) remains to be deter-
mined in future analyses.

In summary, our work reported here has identified an essential
assembly factor for the biogenesis of the Cyt_{b6f} complex in thyl-
akoid membranes. By interacting with the two cytochromes of
the complex, cytochrome f (PetA) and cytochrome b_{6} (PetB), the
DEIP1 protein appears to promote the association of these two
key subunits of the core complex and facilitate the assembly of at
least two transiently accumulating intermediate complexes in
Cyt_{b6f} biogenesis. The identification of DEIP1 will facilitate
future investigations into the challenging problem of resolving the
assembly pathway of the Cyt_{b6f} complex.

Methods
Plant material and growth conditions. Arabidopsis thaliana plants were grown in
0.5x MS medium supplemented with 1% sucrose under long-day conditions (16 h
light, 8 h dark) at 22 °C, and with a light intensity of 5 μmol m^{-2} s^{-1} (low light;
LL), 95 μmol m^{-2} s^{-1} (moderate light; ML) or 600 μmol m^{-2} s^{-1} (high light; HL).
The ecotype Col-0 (CS60000) was used as wild type. The T-DNA insertion mutants
deip1-1 (SALK_037263) and deip1-2 (SALK_0672267) were obtained from NASC.
The transgenic line expressing a chloroplast-targeted GFP under the control of the
UBIQUITIN10 promoter was described previously65. All other transgenic lines
were generated in this study.

Phylogenetic tree construction and sequence alignment. Putative orthologs of
AT2G27290 were identified in PhyloGenes66, and protein sequences were retrieved
from UniProt67. Phylogenetic tree construction and sequence alignment were
conducted using phylogeny.fr68. Detailed information on the proteins included in
the analyses is provided in Supplementary Table 1.

Etiolation and de-etiolation kinetics. Arabidopsis seeds were surface sterilized
and stratified at 4 °C in the dark for 2–3 days54. The plates were placed in the light
for 1 h, followed by 7 days in dark. After 7 days, the plants were placed under
continuous moderate light (95 μmol m^{-2} s^{-1}), and samples were taken after 0, 3, 6,
12, 24, and 48 h of illumination.

RNA extraction, cDNA synthesis, and transcript analysis by qRT-PCR. Total
RNA was extracted using the NucleoSpin® RNA plus Plant kit (Macherey-Nagel).
For cDNA synthesis, 0.5 μg of total RNA was employed and the reverse tran-
scription reaction was conducted using the SuperScriptIII reverse transcriptase and
equal molar amounts of both oligo-dT and random hexamer primers. The primers
used in this study for DNA amplification are listed in Supplementary Table 1.
Transcript accumulation in qRT-PCR was calculated by the ΔΔC_{T} method74,75, using
the accumulation of UBIQ10 as internal control.

Genome editing with CRISPR-Cas9. To generate deip1 deletion mutants, we
employed the CRIPR-Cas9 method as previously described75. Briefly, the single-
guide RNAs (sgRNAs) were incorporated in the sequences of primers
cc_AT2G27290_sgRNA1_Fo and cc_AT2G27290_sgRNA1_Re (Sup-
plementary Table 1), and used for PCR amplification using as a template the
previously described plasmid pHHEE-TRI74. The resulting PCR product was
digested with BsaI, and ligated into the BsaI digested binary vector pJF103171. Col-
0 plants were transformed by Agrobacterium-mediated transformation76, and
selected in the presence of 20 μg mL^{-1} hygromycin. The deletion of the targeted
sequence was confirmed by genomic DNA extraction, PCR amplification with
primers cL_AT2G27290_Fo and cL_AT2G27290_Rev (Supplementary Table 2) and
DNA sequencing.
Generation of PβR005·DEI1-GFP·dei1-1 complemented lines. The GreenGate cloning method was employed to clone the complemented lines. To this end, the coding region of DEI1 was inserted into pPUBQ10 as Gateway® cassette [138 bp: 5′-ATGCTGAGCCGTTCTGCTG-3′ (for) and 5′-ATTGATCATCTGATTCTTTATG-3′ (Rev)]. The resulting plasmid pPUBQ10 was transformed into Agrobacterium tumefaciens by electroporation. The resulting plasmid pGGC-DEI1 was mixed with the vectors pGGA006, pGGC000 were mixed in 5:1 ratio, digested with BsaI and ligated with T4 DNA ligase. The resulting plasmid pGGZ-PUBQ10:DEI1-GFP by Agrobacterium-mediated transformation and transgenic lines were selected in the presence of 20 μg mL−1 hygromycin. Isolation of PβR005·DEI1-GFP·dei1-1 lines (dei1-1 C lines) was carried out using the resistant cassette of pPUBQ10. pGGZ-PUBQ10:DEI1-GFP, and PCR-based genotyping with the primers cl_AT2G27290_For and gt_AT2G27290-Introm_Rev for the wild-type allele, and cl_AT2G27290_For and LBL3 for the T-DNA mutant allele (dei1-1) (Supplementary Table 2).

Confocal laser scanning microscopy (CLSM). Confocal microscopy was conducted with a Leica TCS SP8 microscope. The excitation/emission settings used were 488/53(55)-540 (for GFP), 512/520-540 nm (for YFP), 566/50-600 nm for RFP, and 488/680-720 nm for chlorophyll fluorescence. All images were acquired using sequential scanning settings. For analysis of chloroplast ultrastructure, chlorophyll autofluorescence was employed, and 5–10 z-stacks of 0.2–0.5 μm were acquired at low resolution, to avoid quenching of the fluorescence. The images were combined using the ImageJ software, considering the maximum intensity of each stack.

Protein extraction and immunoblotting. Plant material from 7-day-old seedlings or 5-week-old plants was ground in liquid nitrogen, and total protein extracts were obtained by grinding tissue with 2% (w/v) n-dodecyl-β-D-maltoside (β-DM) for 2 min at 4°C in the dark. Trypsin digestion for protein topology determination was performed as described above. For trypsin digestion, the samples were fragmented by incubation in fragmentation buffer [40 mM Tris-OAc, pH 8.3, 150 mM MgCl2, 5 mM 2-mercaptoethanol, 100 µg mL−1 dithiothreitol (DTT), 2× protease inhibitor cocktail (Roche)]. For immunoblot analyses, total protein extracts or purified thylakoid proteins were resolved in 12.5% SDS-PAGE gels and blotted onto PVDF membranes. All antibodies used in this study are listed in Supplementary Table 3.

Thylakoid isolation and fractionation. Plant material (leaves or cotyledons) was harvested and ground at 4°C under dim light in homogenization buffer [50 mM HEPES pH 7.5, 330 mM sorbitol, 1 mM MgCl2, 2 mM EDTA, 5 mM sodium ascorbate anhydrous, 0.05% (w/v) formaldehyde and 0.0005% (w/v) sodium thiosulfate] for 15 min at 4°C, and the soluble fraction was transferred to a new tube and centrifuged at 150,000 × g for 90 min at 4°C in a Ti 70 rotor (Beckman Coulter). The supernatant was measured.

Salt washes to assess protein anchoring in membranes. Thylakoid samples equivalent to 1 mg of chlorophyll were resuspended in storage buffer75. For thylakoid subfractionation, thylakoid samples equal to 1 mg of chlorophyll were resuspended in storage buffer to a final concentration of 0.5 mg mL−1. The thylakoid samples were mixed with equal volume of 2% (w/v) dithionitrobenzoic acid (DTT) and incubated on ice with stirring for 5 min. The suspension was then homogenized in a potter and centrifuged at 1000 × g for 2 min at 4°C to remove insoluble material. Subsequently, the soluble fraction was centrifuged at 40,000 × g for 30 min at 4°C, the pellet was resuspended in storage buffer (grana core fraction), and the soluble fraction was transferred to a new tube and centrifuged at 150,000 × g for 90 min at 4°C in a fixed-angle rotor. The loose pellet (grana margins), and the strongly anchored pellet (stroma lamellae) were diluted in storage buffer75.

Salt washes to assess protein anchoring in membranes. Thylakoid samples equivalent to 0.5 mg mL−1 of chlorophyll were incubated with 1 M NaCl for 1 h at 4°C. The thylakoid samples were then centrifuged at 12,000 × g for 5 min, and proteins present in the pellet and soluble fractions were resolved by electrophoresis in a 12.5% SDS-PAA gel76.

Tryptsin digestion for protein topology determination. For trypsin digestion, thylakoid samples equivalent to 0.5 μg chlorophyll mL−1 were incubated with or without 10 μg mL−1 trypsin at 37°C for 15 min. Sample buffer for electrophoresis was added and the samples were immediately boiled at 98°C for 5 min. Finally, the samples were centrifuged for 5 min at 16,000 × g to remove insoluble material, and proteins were resolved in a 12.5% SDS-PAA gel76.

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relative abundances of ribosome footprints, total RNA, and translation efficiency were calculated for each reading frame from three biological replicates.

Significances of changes were calculated using the Empirical Bayes method in the LIMMA package and the P values were adjusted for multiple testing according to FDR approach to correct for multiple comparisons. Genes with twofold or more than twofold change and an adjusted P value < 0.05 were considered significantly differentially expressed.

**BS2 crossing linking.** For BS2 crossing experiments, 7-day-old seedlings grown under standard conditions were used as starting material. Membrane fractions equivalent to 0.5 μg chlorophyll μL−1 obtained from complemented line dep1-C4, or soluble fractions equivalent to 5 μg of protein μL−1 obtained from ctpGFP, were incubated with 1.5 mM BS2 for 1 h on ice. The samples were then resuspended in reducing sample buffer for PAA gel electrophoresis, boiled at 98 °C for 5 min, and separated in a 10% PAA gel.

**DPS crossing linking and co-immunoprecipitation (co-IP).** Co-immunoprecipitation experiments were performed using the GFP-Trap Kit (Chromotek). Thylakoids extracted from the wild type and the destination vector for BiFC and strain GV3101 harboring the p19 suppressor of supplementary information files. Genetic material and all raw data are available upon request from the corresponding author. Source data are provided with this paper.

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Author contributions
O.S-I. and R.B. designed research, O.S-I., D.R., R.G., A.P.H., T.A-M., A.S., and R.Z. performed research. All authors contributed to data analysis. O.S-I., D.R., R.G., A.P.H., T.A-M., R.Z., and R.B. wrote the manuscript.

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