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Supplemental Information

A Thylakoid Membrane Protein Functions Synergistically with GUN5 in Chlorophyll Biosynthesis

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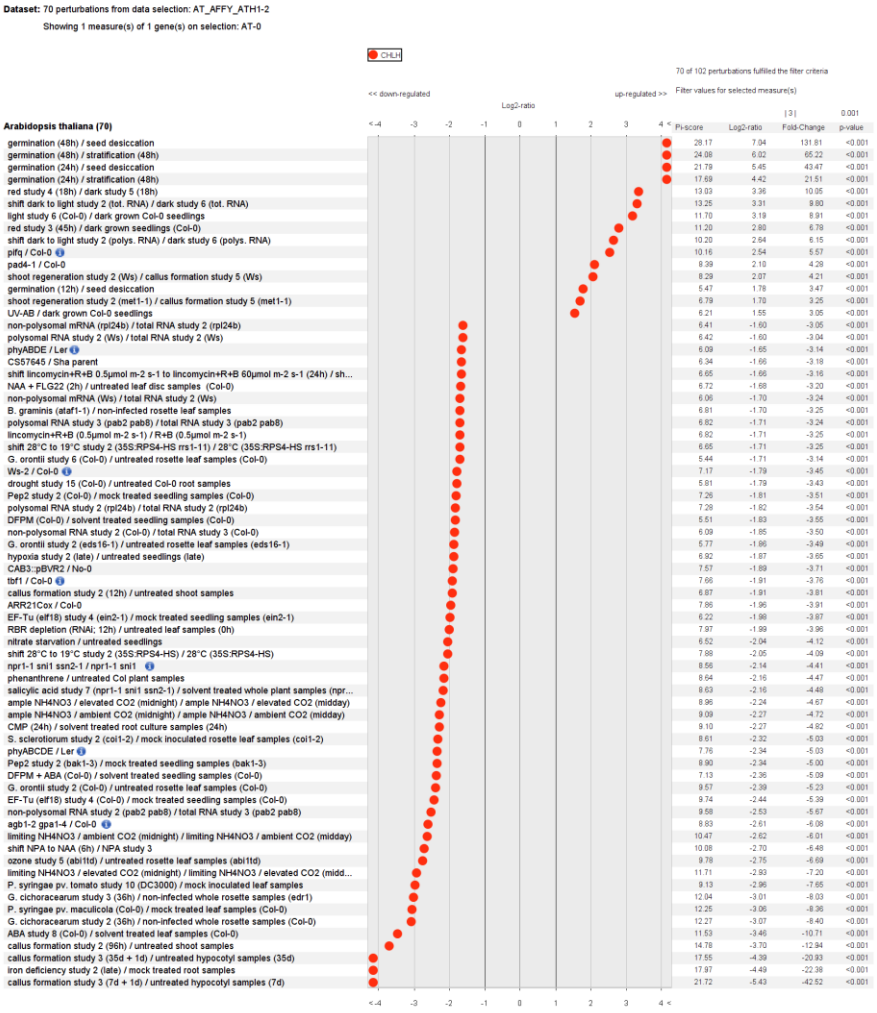
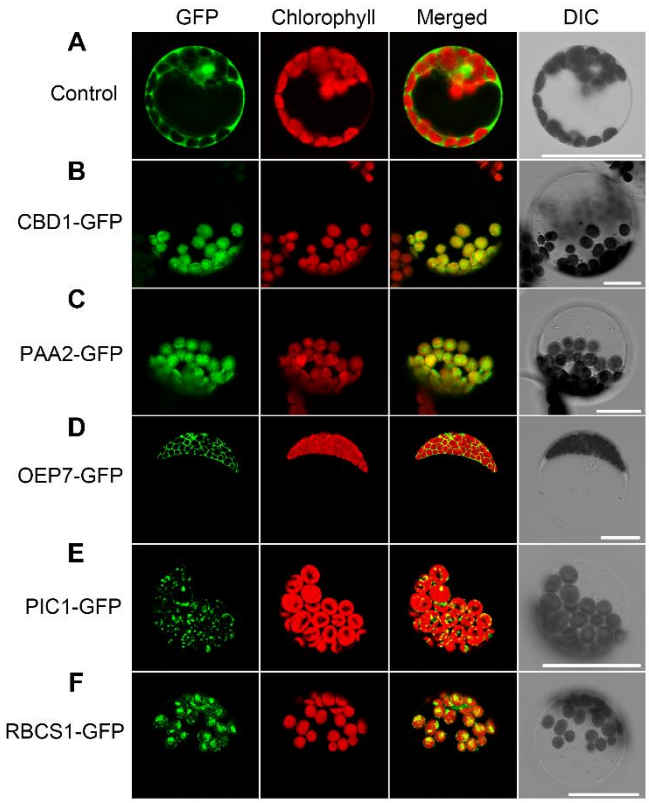
Supplemental Materials



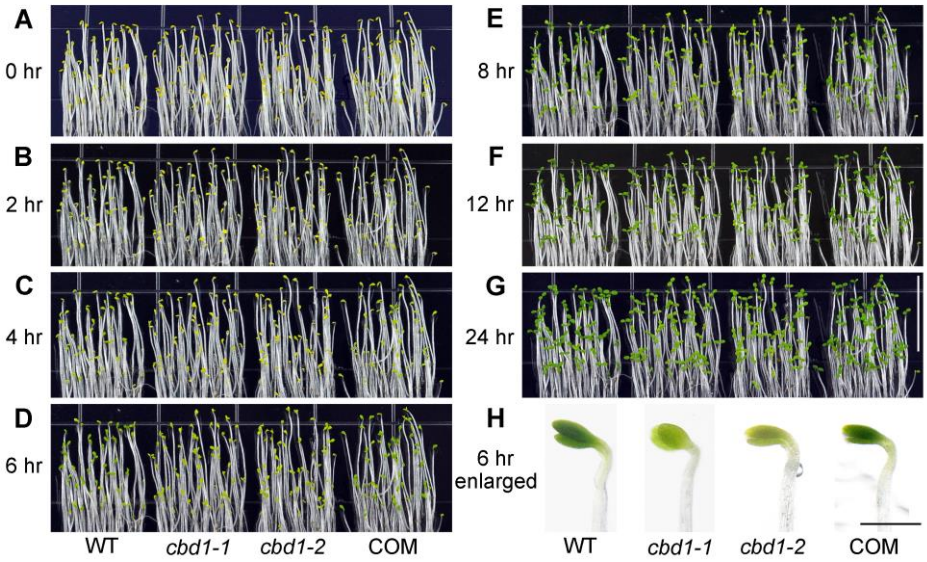
Supplemental Figure 1. Expression Profiles of *CHLH/GUN5* under Perturbation Conditions.

The dataset were 70 perturbations which the expression profiles of *CHLH/GUN5* were most regulated. Data were collected from the Genevestigator database.



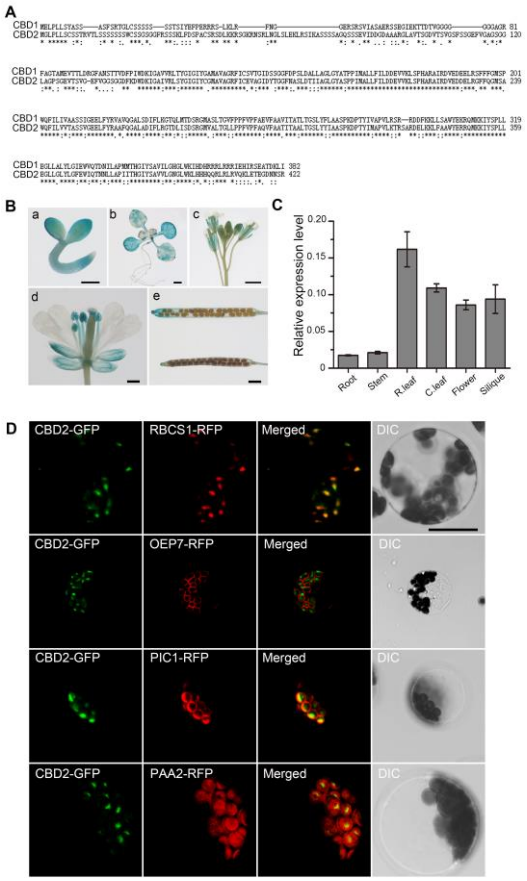
Supplemental Figure 2. Subcellular Localization of CBD1 and Marker Proteins in Chloroplast.

Arabidopsis leaf protoplasts were transformed with plasmids that express the indicated gene constructs under control of the constitutive 35S Cauliflower mosaic virus promoter. **(A)** The unfused GFP vector served as a control. **(B)** The GFP coding sequence was fused to the C terminus of the CBD1 coding region in the pEZSNL vector. **(C-F)** Fluorescence signals of marker proteins localized in different compartments of chloroplast. PAA2-GFP, PIC1-GFP, OEP7-GFP, and RBCS1-GFP were used as markers of thylakoid membrane, inner envelope, outer envelope, and stroma respectively. After 16 h of expression, protoplasts were observed using a confocal laser scanning microscope. Green fluorescence signals, chlorophyll red auto-fluorescence, merged signals from GFP and chlorophyll channels, and bright-field images that showed intactness of the protoplasts are shown separately from left to right in each lane. Scale bars, 20 μm.



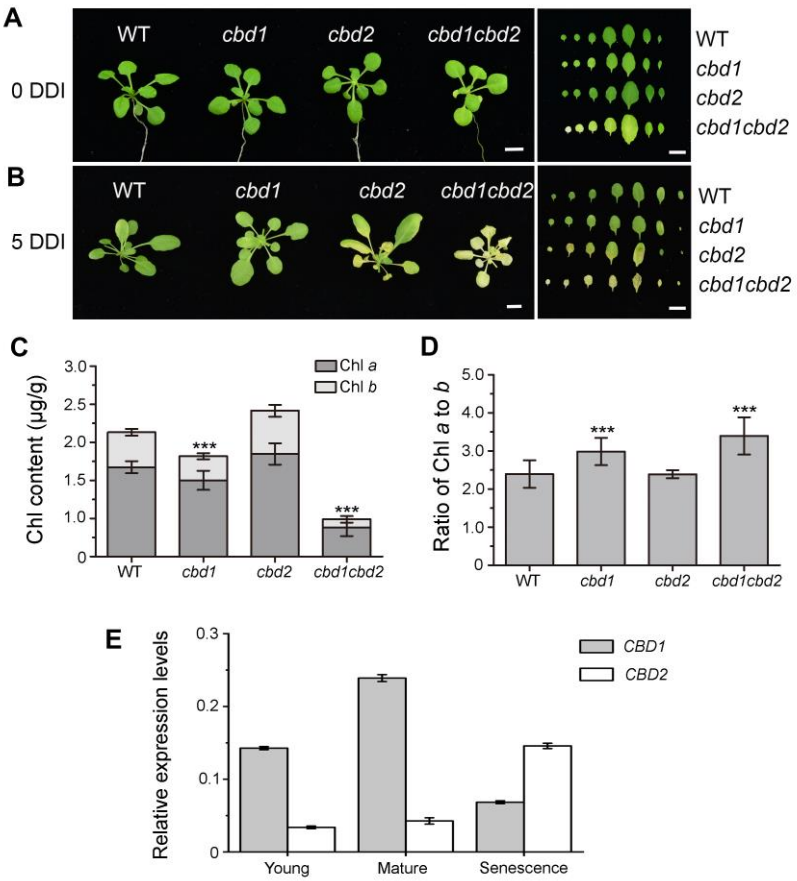
Supplemental Figure 3. *cbd1* Exhibited a Slower Rate in Chl Biosynthesis.

(A) to **(G)** 3-day-old dark-grown etiolated WT, *cbd1-1*, *cbd1-2*, and COM seedlings exposed to continuous light for indicated time periods. Scale bar, 1 cm. **(H)** Enlarged representative WT, *cbd1-1*, *cbd1-2*, and COM seedling after light exposure for 6 h. Scale bar: 1 mm.



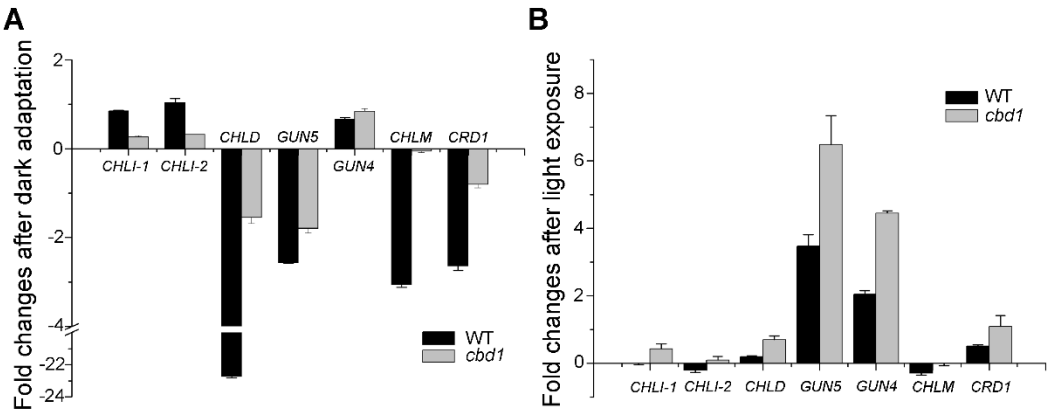
Supplemental Figure 4. Expression Profiles of *CBD2* and Subcellular Localization of CBD2.

(A) Sequence alignment of CBD1 and CBD2 using Clustal X. **(B)** GUS staining of **(a-c)** 1-3 days old seedlings after germination, **(d)** Inflorescence and silique from 4-week-old plants, and **(e)** Rosette leaf from 3-week-old plants in soil. Scale bars, 200 μm in **(a)** and **(b)**, 1 mm in **(c)** and **(f)**, 2 mm in **(d)** and **(e)**. Data are mean ± SD. n = 4. **(C)** Relative expression levels of *CBD2* in various tissues are revealed by qRT-PCR analysis. **(D)** The construct of *35S::CBD2-GFP* was co-transformed with the marker RBCS1-RFP (stroma), OEP7-RFP (outer envelope), PIC1-RFP (inner envelope), or PAA2-RFP (thylakoid membrane) into *Arabidopsis* leaf protoplasts for fluorescence observation using confocal laser scanning microscope after 16 h of expression. Green fluorescence signals, chlorophyll red auto-fluorescence, merged signals from GFP and chlorophyll channels, and bright-field images that showed intactness of the protoplasts are shown separately from left to right in each lane. Scale bar, 20 μm.



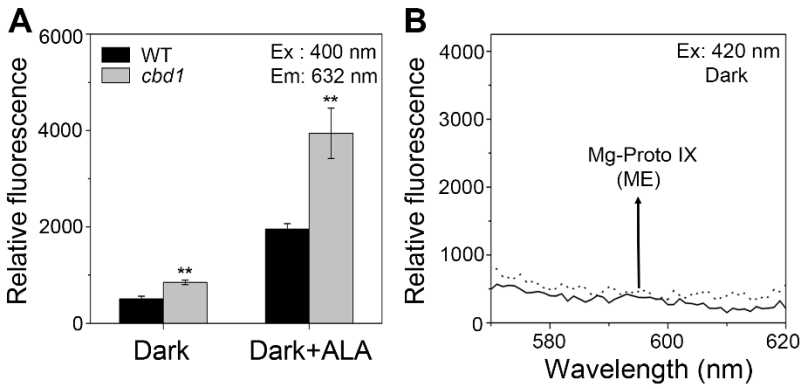
Supplemental Figure 5. Phenotypic Analysis of WT, *cbd1*, *cbd2*, and *cbd1cbd2* Mutants.

(A) 3-week-old WT, *cbd1*, *cbd2*, and *cbd1cbd2* mutant grown in normal conditions. Detached leaves were shown in the right. **(B)** 3-week-old WT, *cbd1*, *cbd2*, and *cbd1cbd2* mutant were dark incubated for 5 days. Detached leaves were shown in the right. Scale bars, 1 cm. **(C)** Chlorophyll content and **(D)** ratio of Chl *a* to *b* of seedlings in **(A)** (Student's *t* test, *** $P<0.001$). **(E)** Relative expression levels of *CBD1* and *CBD2* in leaves of different ages were detected by qRT-PCR analysis. Young leaves: the two leaves that newly emerged; mature leaves: the middle leaves; senescence leaves: the two true leaves close to the base of plants.



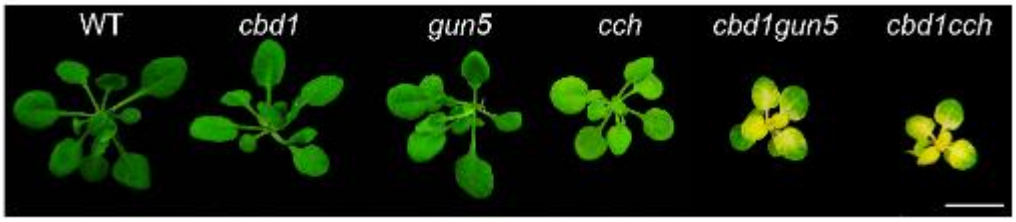
Supplemental Figure 6. Altered Expression Levels of Genes Encoding Key Enzymes in Chl Biosynthetic Pathway.

(A) 3-week-old WT and *cbd1* were dark-adapted for 1 hour before harvest. **(B)** Dark-grown WT and *cbd1* were harvested after 1 hour light exposure. Data are mean ± SD, n=4. *Actin2* was used as an internal reference.



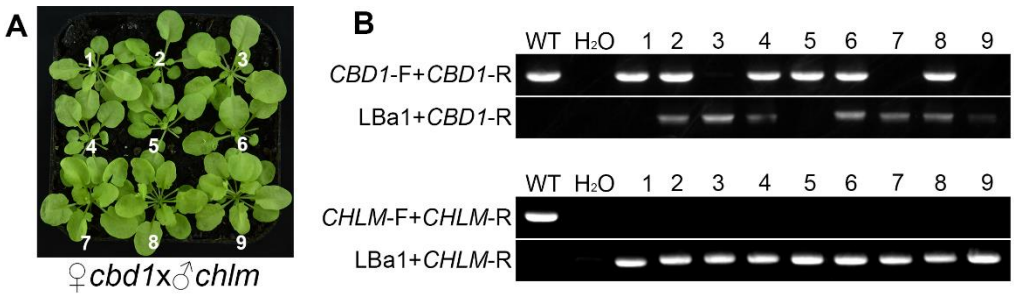
Supplemental Figure 7. Spectrofluorometric analysis of Chl precursors.

(A) Levels of Proto IX in dark-adapted WT and *cbd1* seedlings are determined by spectrofluorometry. 3-week-old seedlings were dark-adapted for 3 days before harvested for measurement. For ALA treatment, 10 mM ALA was added. Excitation at 400 nm produces an emission peak at 632 nm corresponding to the Proto IX content. **(B)** In dark-adapted seedlings, excitation at 420 nm did not produce an emission peak at 595 nm, indicating that the no Mg-Proto IX (ME) was detected by spectrofluorometry in this condition. Data are mean ± SD. n=4. Asterisks indicate statistically significant differences compared with the wild type (Student's *t*-test, $**P<0.01$).



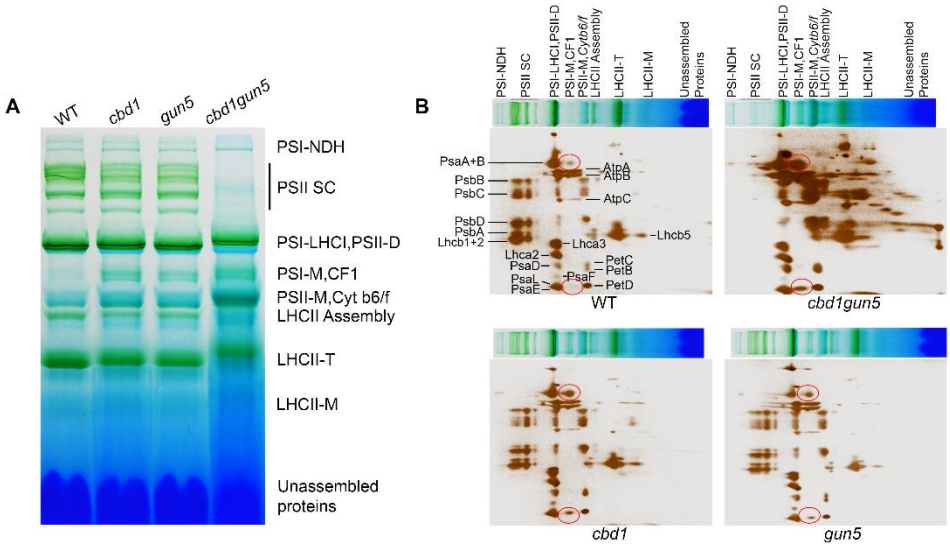
Supplemental Figure 8. Double Mutant *cbd1gun5* and *cbd1cch* Exhibits the Same Phenotype.

3-week-old WT and mutant lines grown in half-strength MS medium. Scale bar, 1 cm.



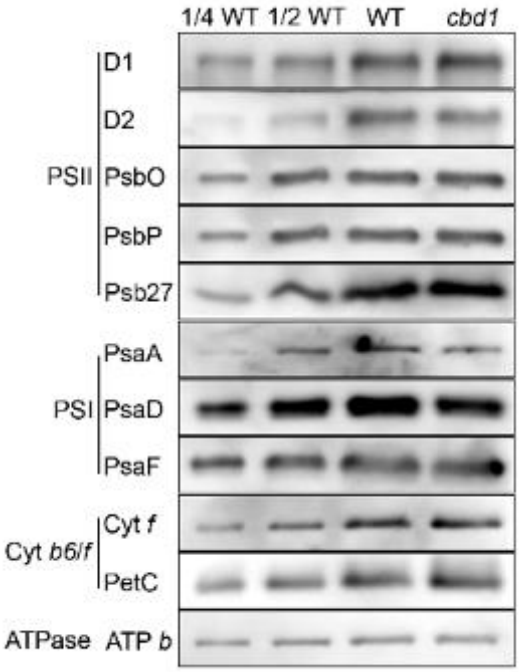
Supplemental Figure 9. Identification and phenotype of *cbd1chlm* double mutant.

(A) Nine *cbd1chlm* double mutant lines grown in soil in greenhouse. **(B)** PCR amplification of *CBD1* and *CHLM* genomic fragment from WT and the nine mutant lines. Primer pair CBD1-F and CBD1-R was used to amplify genomic fragment of *CBD1*. Primer pair CBD1-R and LBa1 (the T-DNA left border flanking sequence) was used to identify T-DNA insertion in *CBD1*. Primer pair CHLM-F and CHLM-R was used to amplify genomic fragment of *CHLM*. Primer pair LBa1 and CHLM-R was used to identify T-DNA insertion in *CHLM*. Wild type (WT) was used as a positive control and water was used as a negative control (H_2O).



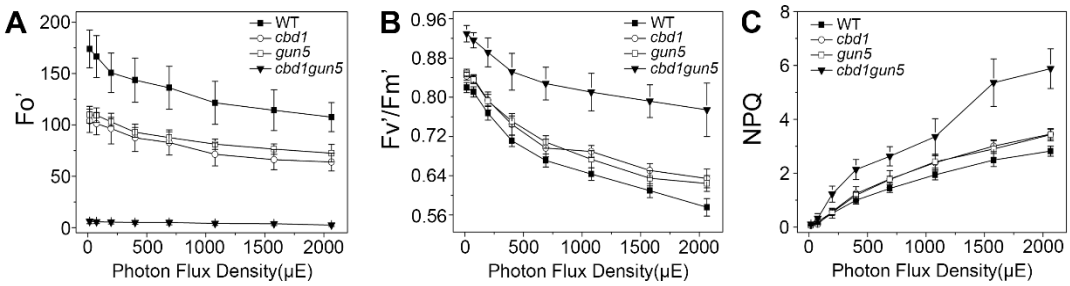
Supplemental Figure 10. Disturbed Organization of Thylakoid Membrane Protein Complexes in Mutants.

(A) BN-PAGE analysis of equal thylakoid proteins (10 μg chlorophyll) of 3-week-old WT, *cbd1*, *gun5*, and *cbd1gun5* grown in half-strength MS. Thylakoid membranes were solubilized with 1% DM by 5%-13.5% gradient gel. Designation of thylakoid membrane protein complexes are labeled to the right. Six biological replicates were performed and a representative one is shown. **(B)** 2D-SDS-PAGE fractionation of thylakoid membrane protein complexes. After separation in the non-denaturing gradient gel in the first dimension, the gels were sliced and laid on the top of the denaturing 12.5% 2D SDS-PAGE gel for the further separation in the second dimension. The followed silver stain was applied for the visualization of specific proteins. The red oval circle indicates the location of PsaA+B and Psa E proteins.



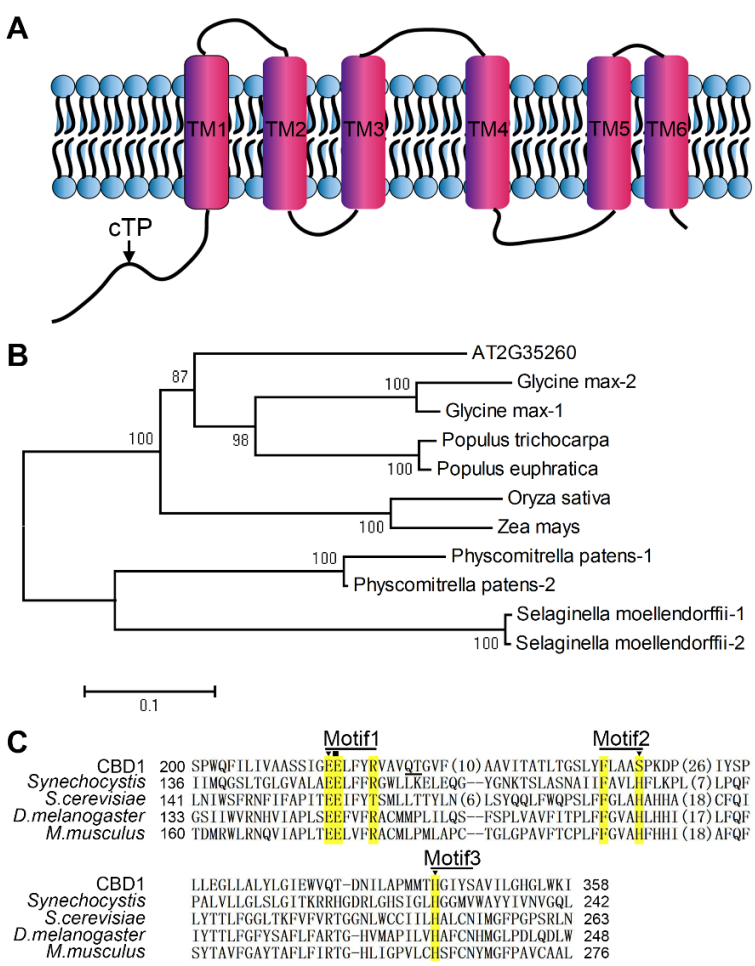
Supplemental Figure 11. Immunoblotting Analysis of Subunits in Photosynthetic Supercomplexes.

SDS-PAGE immunoblotting analysis of thylakoid proteins (1 μg chlorophyll) from WT and *cbd1*. Antibodies were applied as indicated.



Supplemental Figure 12. Photosynthetic Activity of PSII in WT, *cbd1*, *gun5*, and *cbd1gun5*.

Chlorophyll *a* fluorescence parameters of 3-week-old WT, *cbd1*, *gun5*, and *cbd1gun5*. Plants were dark-adapted for 40 min before measurement. **(A)** Fo': the minimal fluorescence; **(B)** Fv'/Fm': the PSII maximum efficiency; **(C)** NPQ: the non-photochemical quenching. Data are mean ± SD. n = 5.



Supplemental Figure 13. Phylogenetic Analysis of CBD1.

(A) Predicted topology structure for CBD1 based on the TMHMM Server v.2.0. (http://www.cbs.dtu.dk/services/TMHMM/). **(B)** The phylogenetic tree was constructed using the Neighbor-Joining method based on the Poisson model of MEGA 6. Bootstrap values (1000 replicates) were shown for corresponding nodes. The analysis involved 11 amino acid sequences (*Arabidopsis thaliana*, AT2G35260; *Populus euphratica*, XP_011018585.1; *Populus trichocarpa*, XP_006368412.1; *Oryza sativa*, XP_015633215.1; *Glycine max*, NP_001239886.1; *Glycine max*, KRH77201.1; *Physcomitrella patens*, XP_001769984.1; *Physcomitrella patens*, XP_001764584.1; *Selaginella moellendorffii*, XP_002967687.1; *Selaginella moellendorffii*, XP_002964348.1; *Zea mays*, NP_001137056.1). **(C)** Multiple sequence alignment of CBD1 with its Type II CAAX homologues (Pei and Grishin, 2001).

Supplemental Table 1. Metal Ion Content Analysis

Genotype	Fraction	Fe	Mn	Cu	Ca
WT	Leaf	0.13 ±0.02	0.11 ±0.02	0.02 ±0.002	28.23 ±1.19
cbd1	Leaf	0.13 ±0.01	0.11 ±0.01	0.02 ±0.001	29.24 ±2.25
WT	Thylakoid	7.16 ±1.81	1.58 ±0.06	0.54 ±0.02	15.10 ±5.14
cbd1	Thylakoid	6.99 ±1.77	1.22 ±0.02	0.49 ±0.03	13.16 ±2.30

Metal ion contents were measured for leaves of wild-type and *cbd1* plants grown on soil as well as for intact thylakoids isolated from these plants. Values indicate amounts of metal ions (µg/mg dry weight for leaf fractions, µg/10^9 thylakoids for intact thylakoids). All values are averages of four replicates ± SD.

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344 **Supplemental Table 2. Primers Used in This Study**

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Primer Name	Prime Sequence (5'-3')	Application
CBD1-F	CGGGATCCATGGAGCTTCCGTTACTCTCGTATG	Mutant identification
CBD1-R	GGAATTCTTAAATCAACTTATCCGTGGCCTCCG	
Lba1	TGGTTCACGTAGTGGGCCATCG	
gun5-point-F	GGTGGTCATGGACAACGAAC	
gun5-point-R	CCAAAGAACCTGCCCAAGAG	
chlm-F	GCTTTCAGACTGTGTTCCAATTG	
chlm-R	CCATAGCAGCAGAAATATCGG	
CBD1-genomic-F	CGGGATCCTGCTAAGTTATTTTTAGTTTACCTAG	Genomic complementation
CBD1-genomic-R	CGCTGCAGGCAGGGCACAAAGCAC	
CBD1-GFP-F	GGAATTCGATGGAGCTTCCGTTACTCTCGTATG	Subcellular localization
CBD1-GFP-R	CGGGATCCGCAATCAACTTATCCGTGGCCTCCG	
CBD2-GFP-F	GGAATTCGATGGGTCTTCCTTTATTGTCTTGTAGTTCC	
CBD2-GFP-R	CGGGATCCGCTCTTGAGTTGTTGTCACCTTCAGTTTCAAG	
PAA2-GFP-F	CGGGTACCATGGCGAGCAATCTTCTCC	
PAA2-GFP-R	CGCCCGGGTAGATGCTTGAAGCTTTGCTCTTT	
PIC1-GFP-F	CGCTCGAGATGCAATCACTACTCTTGCCG	
PIC1-GFP-R	CGGGATCCAGAGCAACCTTAGGAACTACGAC	
OEP7-GFP-F	CGCTCGAGATGGGAAAAACTTCGGGAGC	
OEP7-GFP-R	CGGGATCCAGCAAACCCTCTTTGGATGTGG	
RBCS1-GFP-F	CGCTCGAGATGGCTTCCTCTATGCTCTC	
RBCS1-GFP-R	CGGGATCCACACCGGTGAAGCTTGGTGG	
CBD1pro-F	CCCAAGCTTTGCTAAGTTATTTTTAGTTTACCTAG	Promoter cloning
CBD1pro-R	CGGGATCCTGAAGGTTATTCGAAGTAACCG	
CBD2pro-F	CCCAAGCTTCTTTGTAACTAAAGCAATTTAATGATG	
CBD2pro-R	CGGGATCCTTATAACATGCGACAACTCTCAG	

CBD1-M-F	GGAATTCATGGAGCTTCCGTTACTCTCG	MM281 complementation
CBD1-M-R	CGGGATCCTTAAATCAACTTATCCGTGGCCTCC	
CBD1-Q-F	ACGGAGCAGTGAAGGGATTG	qRT-PCR
CBD1-Q-R	TCAACGGTGGTGGAGTTAGC	
CBD2-Q-F	TTGACTGGACTCTTGCCACC	
CBD2-Q-R	GAGAGAACCAGTGAGAGCGG	
GUN4-Q-F	CACTTACCGCTCACAAACGC	
GUN4-Q-R	GCTCCTACTCCTGCCTGTTC	
CHLI-1-Q-F	ACCCGGCGAGGTTTATCTTG	
CHLI-1-Q-R	GCTTGTCCTGCTCGGTTTTG	
CHLI-2-Q-F	GCTGCATCTGGTTGGAACAC	
CHLI-2-Q-R	TTAACTCTCAGCTCGGCGTC	
CHLD-Q-F	TCCCTCCCCAAACGAAACAG	
CHLD-Q-R	AGGGAAAAACTGTCGGCCAT	
CHLH-Q-F	GCTTACCTCGCTTCTTGGGT	
CHLH-Q-R	CCACCAACTTCAGGCACTCT	
CHLM-Q-F	CGTTTGCTCCTTCCTTGTTGTC	
CHLM-Q-R	CCGAGTACGGCGATTGTTGT	
CRD1-Q-F	CCCAAAGCTCTCAAACCCGA	
CRD1-Q-R	TCGTTCCCTTCTTGGACTTCG	
Actin2-F	ACTCTCCCGCTATGTATGTCGCC	
Actin2-R	ATTTCCCGCTCTGCTGTTGTGGT	
$CBD2_{CRISPR}$-F	CGTGTAGACATGCAAAGTGGT	CRISPR
$CBD2_{CRISPR}$-R	CCTTCTTACAGACATGTGCC	
CBD2gRT1+	CCTGAATCCATTCAAAGCCGGTTTTAGAGCTAGAAAT	
CBD2gRT2+	GATCGACGACGGAGATGCGGGTTTTAGAGCTAGAAAT	
CBD2gRT3+	GGAGACTTTGGACCGTTGGAGTTTTAGAGCTAGAAAT	
CBD2-AtU6-29-T1	CGGCTTTGAATGGATTCAGGCAATCTCTTAGTCGACT	
CBD2-AtU3b-T2	CCGCATCTCCGTCGTCGATCTGACCAATGTTGCTCC	
CBD2-AtU3d-T3	TCCAACGGTCCAAAGTCTCCTGACCAATGGTGCTTTG	

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