Plant Communications, Volume 1

Supplemental Information

A Thylakoid Membrane Protein Functions Synergistically with GUN5 in

Chlorophyll Biosynthesis

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1 Supplemental Materials

•	Dataset: 70 perturbations from data selection: AT AFFY ATH1-2											
2	Showing 1 measure(s) of 1 gene(s) on selection: AT-0											
		_										
		😑 CHLH										
3									70 ef 102 p	erturbations fulfille	the filter criteria	
0		<< down-re	egulated			Lon2-ratio		up-regulate	id >> Fitter values	for selected mea	sure(s)	
	Analida a la Aleita a 1761	< .4	-9	-2	-1	0	1 2	9	4 <		3	0.001
1	Arabicopsis trainana (70)								Pi-score	Log2-ratio	Fold-Change	p-value
4	germination (46h) / steet desiccation germination (48h) / stratification (48h)								24.08	6.02	65.22	<0.001
	germination (24h) / seed desiccation								21.79	5.45	43.47	< 0.001
	germination (24h) / stratification (48h) red study 4 (18h) / dark study 5 (18h)								17.69	4.42	21.51	<0.001
5	shift dark to light study 2 (tot. RNA) / dark study 6 (tot. RNA)							ĕ	13.25	3.31	9.90	<0.001
0	light study 6 (Col-0) / dark grown Col-0 seedlings							•	11.70	3.19	8.91	< 0.001
	shift dark to light study 2 (polys, RNA) / dark study 6 (polys, RNA)							•	10.20	2.60	6.15	<0.001
•	pifq / Col-0 🚯							•	10.16	2.54	5.57	<0.001
6	pad4-1 / Col-0 shoot regeneration study 2 (Ms) / callus formation study 5 (Ms)								8.39 8.29	2.10	4.28	<0.001
-	germination (12h) / seed desiccation						•		5.47	1.78	3.47	< 0.001
	shoot regeneration study 2 (met1-1) / callus formation study 5 (met1-1)								6.79	1.70	3.25	<0.001
7	non-polysomal mRNA (rpl24b) / total RNA study 2 (rpl24b)						•		6.41	-1.60	-3.05	< 0.001
1	polysomal RNA study 2 (Ws) / total RNA study 2 (Ws)			•					6.42	-1.60	-3.04	<0.001
	phyABDE / Ler ()								6.09	-1.65	-3.14	<0.001
	shift lincomycin+R+B 0.5µmol m-2 s-1 to lincomycin+R+B 60µmol m-2 s-1 (24h) / sh								6.65	-1.66	-3.16	<0.001
8	NAA + FLG22 (2h) / untreated leaf disc samples (Col-0)								6.72	-1.68	-3.20	< 0.001
0	non-polysomal mRNA (Ws) / total RNA study 2 (Ws) B. graminis (ataf1-1) / non-infected rosatte leaf samples								6.05	-1.70	-3.24	<0.001
	polysomal RNA study 3 (pab2 pab8) / total RNA study 3 (pab2 pab8)			ĕ					6.82	-1.71	-3.24	<0.001
_	lincomycin+R+B (0.5µmol m-2 s-1) / R+B (0.5µmol m-2 s-1)								6.82	-1.71	-3.25	< 0.001
9	G. orontii study 6 (Col-0) / untreated rosette leaf samples (Col-0)								5.44	-1.71	-3.14	<0.001
0	Ws-2 / Col-0 🕕			•					7.17	-1.79	-3.45	<0.001
	drought study 15 (Col-0) / untreated Col-0 root samples Ren2 study 2 (Col-0) / mock treated seadling samples (Col-0)								5.81	-1.79	-3.43	<0.001
10	polysomal RNA study 2 (rpl24b) / total RNA study 2 (rpl24b)			ĕ					7.28	-1.82	-3.54	< 0.001
10	DFPM (Col-0) / solvent treated seedling samples (Col-0)			•					5.51	-1.83	-3.55	<0.001
	G. orontii study 2 (cds16-1) / untreated rosette leaf samples (cds16-1)								6.03	-1.05	-3.50	<0.001
	hypoxia study 2 (late) / untreated seedlings (late)			•					6.92	-1.87	-3.65	<0.001
11	CAB3::pBVR2 / No-0 thf1 / CoL0 0								7.57	-1.89	-3.71	<0.001
11	callus formation study 2 (12h) / untreated shoot samples			÷.					6.87	-1.91	-3.81	<0.001
	ARR21Cox / Col-0			•					7.86	-1.96	-3.91	< 0.001
	RBR depletion (RNAi; 12h) / untreated leaf samples (0h)								7.97	-1.99	-3.96	<0.001
12	nitrate starvation / untreated seedlings								6.52	-2.04	-4.12	<0.001
.=	shift 28°C to 19°C study 2 (35S:RPS4-HS) / 28°C (35S:RPS4-HS)								7.88	-2.05	-4.09	<0.001
	phenanthrene / untreated Col plant samples			ĕ					8.64	-2.16	-4.47	< 0.001
10	salicylic acid study 7 (npr1-1 sni1 ssn2-1) / solvent treated whole plant samples (npr			•					8.63	-2.16	-4.48	<0.001
13	ample NH4NO3 / amblent CO2 (midnight) / ample NH4NO3 / amblent CO2 (midday) ample NH4NO3 / amblent CO2 (midnight) / ample NH4NO3 / amblent CO2 (midday)								9.09	-2.27	-4.72	<0.001
	CMP (24h) / solvent treated root culture samples (24h)								9.10	-2.27	-4.82	<0.001
	S. scierotiorum study 2 (coi1-2) / mock inoculated rosette leaf samples (coi1-2) hyABCDE / Let								8.61	-2.32	-5.03	< 0.001
11	Pep2 study 2 (bak1-3) / mock treated seedling samples (bak1-3)								8.90	-2.34	-5.00	<0.001
14	DFPM + ABA (CoI-0) / solvent treated seedling samples (CoI-0)								7.13	-2.36	-5.09	<0.001
	EF-Tu (elf18) study 4 (Col-0) / mock treated seedling samples (Col-0)								9.74	-2.44	-5.39	<0.001
	non-polysomal RNA study 2 (pab2 pab8) / total RNA study 3 (pab2 pab8)		•						9.58	-2.53	-5.67	<0.001
15	agb1-2 gpa1-4 / CoF0 € limiting NH4NO3 / ambient CO2 (midnight) / limiting NH4NO3 / ambient CO2 (midday)								8.83	-2.61	-6.08	<0.001
	shift NPA to NAA (6h) / NPA study 3		ĕ						10.08	-2.70	-6.48	<0.001
	ozone study 5 (abi1td) / untreated rosette leaf samples (abi1td)								9.78	-2.75	-6.69	<0.001
10	P. syringae pv. tomato study 10 (DC3000) / mock inoculated leaf samples								9.13	-2.96	-7.65	<0.001
10	G. cichoracearum study 3 (36h) / non-infected whole rosette samples (edr1)		•						12.04	-3.01	-8.03	< 0.001
	P. syringae pv. maculicola (Col-0) / mock treated leaf samples (Col-0) G. cichoracearum study 2 (36h) / non-infected whole rosette samples (Col-0)								12.25	-3.06	-8.36	<0.001
	ABA study 8 (CoI-0) / solvent treated leaf samples (CoI-0)	•							11.53	-3.46	-10.71	< 0.001
17	callus formation study 2 (96h) / untreated shoot samples	•							14.78	-3.70	-12.94	<0.001
17	callus formation study 3 (35d + 1d) / untreated hypocotyl samples (35d) iron deficiency study 2 (late) / mock treated root samples								17.55	-4.49	-20.93	<0.001
	callus formation study 3 (7d + 1d) / untreated hypocotyl samples (7d)	•							21.72	-5.43	-42.52	<0.001
		<-4	-3	-2	-1	0	1 2	з	4 <			
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19 Supplemental Figure 1. Expression Profiles of CHLH/GUN5 under Perturbation

20 **Conditions.**

- 21 The dataset were 70 perturbations which the expression profiles of CHLH/GUN5
- 22 were most regulated. Data were collected from the Genevestigator database.
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Supplemental Figure 2. Subcellular Localization of CBD1 and Marker Proteins in Chloroplast.

41 Arabidopsis leaf protoplasts were transformed with plasmids that express the 42 indicated gene constructs under control of the constitutive 35S Cauliflower mosaic 43 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 44 45 vector. (C-F) Fluorescence signals of marker proteins localized in different compartments of chloroplast. PAA2-GFP, PIC1-GFP, OEP7-GFP, and RBCS1-GFP 46 47 were used as markers of thylakoid membrane, inner envelope, outer envelope, and 48 stroma respectively. After 16 h of expression, protoplasts were observed using a 49 confocal laser scanning microscope. Green fluorescence signals, chlorophyll red 50 auto-fluorescence, merged signals from GFP and chlorophyll channels, and 51 bright-field images that showed intactness of the protoplasts are shown separately 52 from left to right in each lane. Scale bars, 20 µm.

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

58 (A) to (G) 3-day-old dark-grown etiolated WT, *cbd1-1*, *cbd1-2*, and COM seedlings

59 exposed to continuous light for indicated time periods. Scale bar, 1 cm. (H) Enlarged

60 representative WT, *cbd1-1*, *cbd1-2*, and COM seedling after light exposure for 6 h.

- 61 Scale bar: 1 mm.

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90 Supplemental Figure 4. Expression Profiles of *CBD2* and Subcellular 91 Localization of CBD2.

(A) Sequence alignment of CBD1 and CBD2 using Clustal X. (B) GUS staining of 92 93 (a-c) 1-3 days old seedlings after germination, (d) Inflorescence and silique from 94 4-week-old plants, and (e) Rosette leaf from 3-week-old plants in soil. Scale bars, 200 95 μ m in (a) and (b), 1 mm in (c) and (f), 2 mm in (d) and (e). Data are mean \pm SD. n = 4. (C) Relative expression levels of *CBD2* in various tissues are revealed by qRT-PCR 96 97 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 98 99 PAA2-RFP (thylakoid membrane) into Arabidopsis leaf protoplasts for fluorescence 100 observation using confocal laser scanning microscope after 16 h of expression. Green 101 fluorescence signals, chlorophyll red auto-fluorescence, merged signals from GFP and chlorophyll channels, and bright-field images that showed intactness of the 102 103 protoplasts are shown separately from left to right in each lane. Scale bar, 20 µm.



115 Supplemental Figure 5. Phenotypic Analysis of WT, *cbd1*, *cbd2*, and *cbd1cbd2*116 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.



131 Supplemental Figure 6. Altered Expression Levels of Genes Encoding Key 132 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.

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152 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 \pm SD. n=4. Asterisks indicate statistically significant differences compared with the wild type (Student's t-test, ***P*<0.01).

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174	Supplemental Figure 8. Double Mutant <i>cbd1gun5</i> and <i>cbd1cch</i> Exhibits the Same
175	Phenotype.
176	3-week-old WT and mutant lines grown in half-strength MS medium. Scale bar, 1 cm.
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193 Supplemental Figure 9. Identification and phenotype of *cbd1chlm* double 194 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) .



218 Supplemental Figure 10. Disturbed Organization of Thylakoid Membrane 219 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 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 \pm SD. n = 5.



305 Supplemental Figure 13. Phylogenetic Analysis of CBD1.

306 (A) Predicted topology structure for CBD1 based on the TMHMM Server v.2.0. (http://www.cbs.dtu.dk/services/TMHMM/). **(B)** The phylogenetic 307 tree was 308 constructed using the Neighbor-Joining method based on the Poisson model of MEGA 6. Bootstrap values (1000 replicates) were shown for corresponding nodes. 309 310 The analysis involved 11 amino acid sequences (Arabidopsis thaliana, AT2G35260; Populus euphratica, XP_011018585.1; Populus trichocarpa, XP_006368412.1; 311 Oryza sativa, XP_015633215.1; Glycine max, NP_001239886.1; Glycine max, 312 KRH77201.1; Physcomitrella patens, XP_001769984.1; Physcomitrella patens, 313 314 XP_001764584.1; Selaginella moellendorffii, XP_002967687.1; Selaginella moellendorffii, XP_002964348.1; Zea mays, NP_001137056.1). (C) Multiple 315 sequence alignment of CBD1 with its Type II CAAX homologues (Pei and Grishin, 316 317 2001).

319 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	

322 Metal ion contents were measured for leaves of wild-type and *cbd1* plants grown on

323 soil as well as for intact thylakoids isolated from these plants. Values indicate amounts

324 of metal ions (μ g/mg dry weight for leaf fractions, μ g/10⁹ thylakoids for intact

thylakoids). All values are averages of four replicates \pm SD.

344 Supplemental Table 2. Primers Used in This Study

		1	
Primer Name	Prime Sequence (5'-3')	Application	
CBD1-F	CGGGATCCATGGAGCTTCCGTTACTCTCGTA		
	TG		
CBD1-R	GGAATTCTTAAATCAACTTATCCGTGGCCTC		
	CG	Mutont	
Lba1	TGGTTCACGTAGTGGGCCATCG	identification	
gun5-point-F	GGTGGTCATGGACAACGAAC	Identification	
gun5-point-R	CCAAAGAACCTGCCCAAGAG		
chlm-F	GCTTTCAGACTGTGTTCCAATTG		
chlm-R	CCATAGCAGCAGAAATATCGG		
CBD1-genomi	CGGGATCCTGCTAAGTTATTTTAGTTTACC		
c-F	TAG	Genomic	
CBD1-genomi	CGCTGCAGGCAGGGCACAAAGCAC	complementa	
c-R		tion	
CBD1-GFP-F	GGAATTCGATGGAGCTTCCGTTACTCTCGTA		
	TG		
CBD1-GFP-R	CGGGATCCGCAATCAACTTATCCGTGGCCTC		
	CG		
CBD2-GFP-F	GGAATTCGATGGGTCTTCCTTTATTGTCTTG		
	TAGTTCC		
CBD2-GFP-R	CGGGATCCGCTCTTGAGTTGTTGTCACCTTC		
	AGTTTCAAG		
PAA2-GFP-F	CGGGTACCATGGCGAGCAATCTTCTCC	Cuballular	
PAA2-GFP-R	CGCCCGGGTAGATGCTTGAAGCTTTGCTCTT		
	Т	localization	
PIC1-GFP-F	CGCTCGAGATGCAATCACTACTCTTGCCG		
PIC1-GFP-R	CGGGATCCAGAGCAACCTTAGGAACTACGA		
	С		
OEP7-GFP-F	CGCTCGAGATGGGAAAAACTTCGGGAGC		
OEP7-GFP-R	CGGGATCCAGCAAACCCTCTTTGGATGTGG		
RBCS1-GFP-F	CGCTCGAGATGGCTTCCTCTATGCTCTC		
RBCS1-GFP-	CGGGATCCACACCGGTGAAGCTTGGTGG		
R			
CBD1pro-F	CCCAAGCTTTGCTAAGTTATTTTTAGTTTAC		
	CTAG		
CBD1pro-R	CGGGATCCTGAAGGTTATTCGAAGTAACCG	Promoter	
CBD2pro-F	CCCAAGCTTCTTTGTAACTAAAGCAATTTAA	cloning	
	TGATG		
CBD2pro-R	CGGGATCCTTATAACATGCGACAACTCTCAG		

CBD1-M-F	GGAATTCATGGAGCTTCCGTTACTCTCG	MM281
CBD1-M-R	CGGGATCCTTAAATCAACTTATCCGTGGCCT	complementa
	CC	tion
CBD1-Q-F	ACGGAGCAGTGAAGGGATTG	
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	qRI-PCK
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	
CBD2 _{CRISPR} -R	CCTTCTTACAGACATGTGCC	
CBD2gRT1+	CCTGAATCCATTCAAAGCCGGTTTTAGAGCT	
	AGAAAT	
CBD2gRT2+	GATCGACGACGGAGATGCGGGTTTTAGAGC	
	TAGAAAT	
CBD2gRT3+	GGAGACTTTGGACCGTTGGAGTTTTAGAGCT	CRISPR
	AGAAAT	
CBD2-AtU6-2	CGGCTTTGAATGGATTCAGGCAATCTCTTAG	
9-T1	TCGACT	
CBD2-AtU3b-	CCGCATCTCCGTCGTCGATCTGACCAATGTT	
T2	GCTCC	
CBD2-AtU3d-	TCCAACGGTCCAAAGTCTCCTGACCAATGGT	
T3	GCTTTG	