A Thylakoid Membrane Protein Functions Synergistically with GUN5 in Chlorophyll Biosynthesis

Chi Zhang1,2,5, Bin Zhang1,2,5, Baicong Mu2,3, Xiaojiang Zheng1,4, Fugeng Zhao2, Wenzhi Lan2,*, Aigen Fu1,.* and Sheng Luan4,*

1The Key Laboratory of Western Resources Biology and Biological Technology, College of Life Sciences, Northwest University, Xi’an 710069, China
2Nanjing University-Nanjing Forestry University Joint Institute for Plant Molecular Biology, College of Life Sciences, Nanjing University, Nanjing 210093, China
3Temasek Life Sciences Laboratory, Singapore 117604, Republic of Singapore
4Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
5These authors contributed equally

*Correspondence: Wenzhi Lan (lanw@nju.edu.cn), Aigen Fu (aigenfu@nwu.edu.cn), Sheng Luan (sluan@berkeley.edu)

https://doi.org/10.1016/j.xplc.2020.100094

ABSTRACT

Chlorophyll (Chl) is essential for photosynthetic reactions and chloroplast development. While the enzymatic pathway for Chl biosynthesis is well established, the regulatory mechanism underlying the homeostasis of Chl levels remains largely unknown. In this study, we identified CBD1 (Chlorophyll Biosynthetic Defect1), which functions in the regulation of chlorophyll biosynthesis. The CBD1 gene was expressed specifically in green tissues and its protein product was embedded in the thylakoid membrane. Furthermore, CBD1 was precisely co-expressed and functionally correlated with GUN5 (Genome Uncoupled 5). Analysis of chlorophyll metabolic intermediates indicated that cbd1 and cbd1gun5 mutants over-accumulated magnesium protoporphyrin IX (Mg-Proto IX). In addition, the cbd1 mutant thylakoid contained less Mg than the wild type not only as a result of lower Chl content, but also implicating CBD1 in Mg transport. This was supported by the finding that CBD1 complemented a Mg2+ uptake-deficient Salmonella strain under low Mg conditions. Taken together, these results indicate that CBD1 functions synergistically with CHLH/GUN5 in Mg-Proto IX processing, and may serve as a Mg-transport protein to maintain Mg homeostasis in the chloroplast.

Keywords: chlorophyll biosynthesis, magnesium, thylakoid membrane, GUN5, magnesium protoporphyrin

Zhang C., Zhang B., Mu B., Zheng X., Zhao F., Lan W., Fu A., and Luan S. (2020). A Thylakoid Membrane Protein Functions Synergistically with GUN5 in Chlorophyll Biosynthesis. Plant Comm. 1, 100094.

INTRODUCTION

Chlorophyll (Chl) is a tetrapyrole macrocycle critical for the photosynthetic process that underpins life on earth. As the most abundant pigment, Chl plays vital roles in harvesting light energy in the antenna system and driving electron transfer in the reaction center for the initiation of photosynthesis in all green lineages. Chl in algae and plants is synthesized from a branched tetrapyrole biosynthetic pathway localized entirely within plastids (Larkin, 2016). This pathway begins with the synthesis of 5-aminolevulinic acid (ALA), a universal precursor of all tetrapyrroles (Tanaka et al., 2011; Brzezowski et al., 2015). In the subsequent enzymatic steps, eight molecules of ALA are converted into protoporphyrin IX (Proto IX), which is then allocated into two distinct branches, namely, the heme-synthesizing Fe branch and the Chl-synthesizing Mg branch.

In the Fe branch, Fe^{2+} is inserted into Proto IX by ferrochelatase (FeCh) to form heme, a cofactor for cytochromes and other redox proteins (Chow et al., 1998; Tanaka et al., 2011; Scharfenberg et al., 2015). The Mg branch results in Chl biosynthesis and begins with ATP-dependent Mg^{2+} insertion into Proto IX, which is catalyzed by magnesium chelatase (MgCh) to produce Mg-Proto IX. Unlike FeCh, MgCh is a complex composed of three subunits, CHLI, CHLD, and CHLH (Gibson et al., 1995; Jensen et al., 1998, 1999). Catalysis by MgCh involves a two-step reaction, namely, an enzyme activation step, followed by an Mg^{2+} insertion step (Masuda, 2008; Tanaka et al., 2011). In the first step, the
CHLD hexamer and CHLI hexamer interact to form a CHL–CHLD–Mg-ATP complex without ATP hydrolysis. This complex then binds to the substrate-binding CHLH subunit to form a transient holoenzyme complex. Mg chelation occurs with ATP hydrolysis to generate Mg-Proto IX, followed by the disassembly and turnover of the MgCh complex (Walker and Willows, 1997; Jensen et al., 1998; Masuda, 2008; Tanaka et al., 2011). Subsequently, the propionate side chain of Mg-Proto IX is methylated by Mg-Proto IX methyltransferase (MgMT) to generate Mg-Proto IX monomethyl ester (Mg-Proto IX ME). The enzyme MgMT, which is encoded by the CHLM gene, has been reported to form a complex with MgCh (Alawady et al., 2005; Shepherd et al., 2005). In the next step, a Mg-Proto IX ME cyclase encoded by the CHL27/CRD1 gene in Arabidopsis catalyzes the conversion of Mg-Proto IX ME into protochlorophyllide, the key precursor of mature Chl (Bang et al., 2008; Tanaka et al., 2011).

As the catalytic subunit of MgCh, CHLH has also been proposed to interact with and enhance MgMT catalysis by accelerating the formation and breakdown of the catalytic intermediate. Interactions between MgMT and MgCh facilitate the methylation step by channeling Mg-Proto IX, the product of MgCh and substrate of MgMT (Alawady et al., 2005; Shepherd et al., 2005; Pontier et al., 2007). CHLH (also known as GENOMES UNCOUPLED 5 [GUN5]) also functions in other processes, including plastid-to-nucleus retrograde signaling and the ABA response (Mochizuki et al., 2001; Shang et al., 2010). Absence of CHLH/ GUN5 resulted in a lethal phenotype in Chlamydomonas reinhardtii (Chekounova et al., 2001); however, several chlh/ gun5 mutants, including the T-DNA insertional gun5 mutant, are viable in Arabidopsis (Chen et al., 2017), implying that additional components might function collaboratively with CHLH/GUN5 in Arabidopsis. In this study, we identified a previously unknown protein that functionally interacts with CHLH/GUN5 in Chl biosynthesis.

RESULTS

CBD1 Is Co-expressed with CHLH/GUN5 and Localized in the Thylakoid Membrane

Genes involved in related biological pathways are often co-expressed in space and time to establish their common functions perturbation conditions (Supplemental Figure 1) were isolated (Figure 1A). The 20 most correlated genes included known genes of the Chl biosynthetic pathway, such as CHL27/CRD1, CHLH (encoding the geranyl/geranyl pyrophosphate reductase), and HEMA1 (encoding the glutamyl-tRNA reductase), as well as genes encoding the subunits of photosynthetic complexes, such as LHCB3, LHCB6, PSBY, PSAH2, PSAD1, and PSAF. Many other co-expressed genes in the list were also reported to function in the chloroplast (Figure 1B). However, the co-expressed gene AT2G35260 encodes a novel protein of unknown function. We hypothesized that this gene may play a role in Chl biosynthesis, and designated it as CBD1 (Chlorophyll Biosynthetic Defect1) based on our research described in this report.

Genes in the Chl biosynthetic pathway were grouped into four categories from cluster 1 (c1) to cluster 4 (c4) (Matsumoto et al., 2004). The genes in the c1 cluster (HEMA1, CHLH/GUN5, CHL27/CRD1, CAO, GUN4, and CHLP), which are coordinately regulated by light and the circadian rhythm, form a tight co-expression network with each other and are closely correlated (Kobayashi and Masuda, 2016; Masuda and Fujita, 2008), and CBD1 was co-expressed with most of the genes in this cluster. The expression level of CBD1, similar to that of CHLH/GUN5, rapidly increased after etiolated seedlings were exposed to light (Figure 2A). This light-inducible expression pattern is typical for genes involved in Chl biosynthesis (Matsumoto et al., 2004). To determine the rhythmic expression profile of CBD1, samples were harvested from 3-week-old Arabidopsis seedlings grown under 12-h light/12-h dark conditions. After data processing, we found that the phases and amplitudes of CBD1 and CHLH/ GUN5 were synchronized (Figure 2B). This again supported the finding from our initial co-expression analysis of CBD1, pointing to a tight correlation between CBD1 and the Chl biosynthetic genes (Masuda and Fujita, 2008; Kobayashi and Masuda, 2016). To confirm the expression pattern of CBD1 in Arabidopsis, we conducted quantitative real-time PCR (qRT–PCR) analysis and found that the CBD1 mRNA was specifically expressed in green tissues, but not detected in roots (Figure 2C). Consistent with the qRT–PCR results, the GUS staining of transgenic plants expressing the proCBD1::GUS construct revealed that CBD1 promoter activity was restricted to green tissues, including leaves, stems, flowers, and immature siliques, and it was
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

The subcellular localization of a protein is a critical indicator of its function. Among the top 20 candidates co-expressed with CHLH/GUN5, all known proteins are located and function in the chloroplast. We speculated that CBD1 may also be localized in the chloroplast. We tested this possibility by transiently expressing the CBD1::GFP fusion protein in Arabidopsis mesophyll protoplasts and examining the subcellular localization of the fusion protein by confocal microscopy. The GFP signal from the CBD1::GFP fusion protein was specifically associated with chloroplast clusters, whereas the signal from the control GFP vector was detected in the cytosolic portions of the cell, except for areas covered with chloroplasts and vacuoles (Supplemental Figure 2A and 2B).

The chloroplast is surrounded by a dual-membrane envelope composed of the outer and inner envelope, and contains an internal membrane system known as the thylakoid (Sandellius and Aronsson, 2009). Because CBD1 is a multi-transmembrane domain protein, we expected it to be embedded into one of these membrane systems in the chloroplast. To determine the exact location of CBD1 within the chloroplast, we used marker proteins fused to GFP with known sub-chloroplast locations to serve as references (Supplemental Figure 2C-2F). For example, PAA2 resides in the thylakoid membrane (Abdel-Ghany et al., 2005), whereas PIC1 and OEP7 are located in the inner and outer envelopes, respectively (Duy et al., 2007). RBCS1, a small subunit of the Rubisco enzyme, is present in the stroma (Lee et al., 2006). We found that CBD1 showed the same localization pattern as PAA2, a thylakoid membrane protein. To further confirm this localization, we co-transformed CBD1::GFP with PAA2::RFP, PIC1::RFP, or OEP7::RFP into Arabidopsis protoplasts, respectively (Figure 2E). The results demonstrated that CBD1::GFP was co-localized with PAA2::RFP, but not with other markers. Thus, we concluded that CBD1 is localized in the thylakoid membrane of chloroplasts.

Cbd1 Mutant Plants Display Lower Chl Content

The putative function of CBD1 was explored using two T-DNA insertion mutant alleles of the gene. The first allele, referred to as cbd1-1 (SALK_031802), contained a T-DNA insertion in the first exon, whereas the second allele, cbd1-2 (SALK_058830), contained a T-DNA insertion in the eighth exon of the coding region (Figure 3A). The full-length CBD1 transcript was not detected by RT-PCR in either mutant (Figure 3B). The two different cbd1 mutant lines both exhibited a consistent pale-green phenotype and a 40% reduction in total Chl content compared with the wild type (WT), which indicated possible defects in Chl synthesis (Figure 3C and 3D). There was a stronger reduction in Chl b content than Chl a, leading to a higher Chl a/b ratio in the cbd1 mutant than in the WT (Figure 3E). To further confirm that the phenotype of the cbd1 mutant resulted from the loss-function of CBD1, a 3.4-kb genomic fragment of CBD1 (including the putative promoter, untranslated region, and coding sequence) was cloned into pCAMBIA1300, and the construct was used to transform the cbd1-1 mutant line by Agrobacterium-mediated gene transformation. Both the Chl...
Cbd1-1 Works Synergistically with GUn5 in Chl Biosynthesis

Because ChlH/Gun5 is an essential component in the Chl biosynthetic pathway and Cbd1-1 was co-expressed with ChlH/Gun5, we hypothesized that Cbd1-1 might participate in the same pathway as ChlH/Gun5. Moreover, the decreased Chl content and the slower rate of “greening” of cbd1-1 seedlings also supported a function of Cbd1-1 in Chl biosynthesis. To explore the mechanism of action of Cbd1-1, we surveyed the expression profiles of genes involved in the Chl biosynthesis pathway in mutant and WT plants during the dark-to-light switch. When...
plants were transferred from light to dark, the expression levels of these genes were downregulated but the down-turn was slower in the *cbd1* mutant (Supplemental Figure 7A). While most of the genes in this pathway were upregulated upon the dark-to-light switch, they were induced to a higher level in the *cbd1* mutant than in the WT (Supplemental Figure 7B). The perturbed expression levels of Chl biosynthetic genes also supported the idea that CBD1 was functionally involved in this pathway.

We next assessed the levels of Chl intermediates in 3-week-old WT and *cbd1* seedlings by spectrofluorometry. In dark-adapted seedlings, the content of Proto IX was increased in the *cbd1* mutant compared with the WT (Supplemental Figure 8A), implying that loss-of-function of CBD1 may hinder fluxes through the Chl biosynthesis pathway (Figure 3G). In spectrofluorometry, excitation at 420 nm produces an emission peak at 595 nm, corresponding to Mg-Proto IX and Mg-Proto IX ME content and a higher Chl to C ratio compared with the WT (Supplemental Figure 7A). While most of the *cbd1* mutants exhibited a reduced level in the PSI–LHCI complex (PSII SC). In addition, the *cbd1gun5* double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L).

To assess chloroplast development in the mutants, we examined the ultrastructure of chloroplasts and thylakoids in 4-week-old plants by transmission electron microscopy. Chloroplasts and thylakoids in *cbd1* and *gun5* single mutants showed a more chlorotic phenotype compared with the two single mutants (Figure 4A and 4B). After being transferred to soil, *cbd1gun5* plants showed stunted growth; however, they were able to produce seeds (Figure 4C and 4D). After assessing the Chl content, we found that the *cbd1gun5* double mutant showed an additive effect of the two single mutants and retained a dramatically lower Chl content and a higher Chl a/b ratio compared with its parental double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.

We next assessed the levels of Chl intermediates in 3-week-old WT and *cbd1* seedlings by spectrofluorometry. In dark-adapted seedlings, the content of Proto IX was increased in the *cbd1* mutant compared with the WT (Supplemental Figure 8A), implying that loss-of-function of CBD1 may hinder fluxes through the Chl biosynthesis pathway (Figure 3G). In spectrofluorometry, excitation at 420 nm produces an emission peak at 595 nm, corresponding to Mg-Proto IX and Mg-Proto IX ME content and a higher Chl to C ratio compared with the WT (Supplemental Figure 7A). While most of the *cbd1* mutants exhibited a reduced level in the PSI–LHCI complex (PSII SC). In addition, the *cbd1gun5* double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.

CBD1 Functionally Interact with GUN5 in Chl Biosynthesis and Chloroplast Development

Based on the aforementioned results, CBD1 and CHLH/GUN5 appear to work together at the step of Mg-Proto IX conversion in the Chl biosynthesis pathway. To further investigate the functional connection between CBD1 and CHLH/GUN5, we generated double mutants of *cbd1* and *chlm/gun5*. The *gun5* mutant (hereafter called *gun5*) with a point mutation (Ala990→Val) in CHLH/GUN5 (Mochizuki et al., 2001) was used in this experiment. In the F2 generation from a *cbd1* x *gun5*–1 cross, we found several pale-yellow seedlings that were homozygous *cbd1gun5* double mutants, as verified by genomic PCR and sequencing. The homozygous *cbd1gun5* F2 seedlings showed a more chlorotic phenotype compared with the two single mutants (Figure 4A and 4B). After being transferred to soil, *cbd1gun5* plants showed stunted growth; however, they were able to produce seeds (Figure 4C and 4D). After assessing the Chl content, we found that the *cbd1gun5* double mutant showed an additive effect of the two single mutants and retained a dramatically lower Chl content and a higher Chl a/b ratio compared with its parental double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.

We next assessed the levels of Chl intermediates in 3-week-old WT and *cbd1* seedlings by spectrofluorometry. In dark-adapted seedlings, the content of Proto IX was increased in the *cbd1* mutant compared with the WT (Supplemental Figure 8A), implying that loss-of-function of CBD1 may hinder fluxes through the Chl biosynthesis pathway (Figure 3G). In spectrofluorometry, excitation at 420 nm produces an emission peak at 595 nm, corresponding to Mg-Proto IX and Mg-Proto IX ME content and a higher Chl to C ratio compared with the WT (Supplemental Figure 7A). While most of the *cbd1* mutants exhibited a reduced level in the PSI–LHCI complex (PSII SC). In addition, the *cbd1gun5* double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.

We next assessed the levels of Chl intermediates in 3-week-old WT and *cbd1* seedlings by spectrofluorometry. In dark-adapted seedlings, the content of Proto IX was increased in the *cbd1* mutant compared with the WT (Supplemental Figure 8A), implying that loss-of-function of CBD1 may hinder fluxes through the Chl biosynthesis pathway (Figure 3G). In spectrofluorometry, excitation at 420 nm produces an emission peak at 595 nm, corresponding to Mg-Proto IX and Mg-Proto IX ME content and a higher Chl to C ratio compared with the WT (Supplemental Figure 7A). While most of the *cbd1* mutants exhibited a reduced level in the PSI–LHCI complex (PSII SC). In addition, the *cbd1gun5* double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.

We next assessed the levels of Chl intermediates in 3-week-old WT and *cbd1* seedlings by spectrofluorometry. In dark-adapted seedlings, the content of Proto IX was increased in the *cbd1* mutant compared with the WT (Supplemental Figure 8A), implying that loss-of-function of CBD1 may hinder fluxes through the Chl biosynthesis pathway (Figure 3G). In spectrofluorometry, excitation at 420 nm produces an emission peak at 595 nm, corresponding to Mg-Proto IX and Mg-Proto IX ME content and a higher Chl to C ratio compared with the WT (Supplemental Figure 7A). While most of the *cbd1* mutants exhibited a reduced level in the PSI–LHCI complex (PSII SC). In addition, the *cbd1gun5* double mutant, which may have resulted from the degradation of the thylakoid membrane (Figure 5L). Furthermore, the *cbd1gun5* double mutant showed the same phenotype as the *chlm* single mutant (Supplemental Figure 10), supporting the notion that CBD1 functions upstream of CHLM. Therefore, we suggested that CBD1 and CHLH/GUN5 work collaboratively in trafficking Mg-Proto IX to the next step in the pathway.
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

moss, ferns, and flowering plants (Supplemental Figure 14B), although none of these proteins has been functionally characterized. A sequence alignment based on Position-Specific Iterated-BLAST identified a C-terminal region of 100 amino acids in CBD1 containing three conserved motifs characteristic for type II CAAX prenyl proteases that were conserved in diverse prokaryotic and eukaryotic species (Pei and Grishin, 2001) (Supplemental Figure 14C). In these motifs, the conserved amino acids required for chelating or binding metal ions were also present in CBD1, suggesting a possible involvement of CBD1 in metal homeostasis. To test this possibility, we conducted an elemental analysis using inductively coupled plasma-mass spectrometry (ICP–MS). Although no significant differences in the contents of metals were found in the leaves of the WT and cbdl mutant (Supplemental Table 1), the Mg content in intact leaves of cbdl showed a 40% decrease compared with the WT (Figure 6A), whereas the contents of Fe, Mn, Cu, and Ca were not significantly different (Supplemental Table 1). As the total Chl content was reduced by 19% in the cbdl mutant compared with the WT, the 40% reduction in thylakoid Mg content was not simply attributed to the decrease in Chl levels.

Considering the presence of six putative transmembrane domains in the CBD1 protein, we speculated that CBD1 may have Mg transport activity and tested this idea using the Salmonella mutant strain MM281, which lacks Mg2+ transport systems. We cloned the full-length cDNA of CBD1 into the pTrc99A vector and then transformed it into MM281. While the MM281 strain transformed with the empty pTrc99A vector barely grew in medium containing less than 2 mM Mg2+, MM281 expressing AtMGT10, a high-affinity Mg2+ transporter, grew well in medium containing only 10 μM Mg2+. MM281 transformed with the CBD1-containing construct grew in medium with 100 μM Mg2+ (Figure 6B), suggesting that CBD1 may mediate Mg2+ transport in the high μM range. This affinity of CBD1 for Mg binding is in the physiological range relevant to chloroplast Mg levels. Taken collectively, these results indicated that CBD1 may bind/translocate Mg2+ and regulate its incorporation into Chl biosynthesis processes in synergy with CHLH/GUNS and other enzymes.

**DISCUSSION**

Despite the indispensable role of Chl in photosynthetic light harvesting, photo-oxidative damage to cells can result from Chl and its highly photoactive intermediates, that is, if they are not rapidly integrated into the protein complexes. Therefore, the biosynthesis and homeostasis of Chl are precisely controlled to fine-tune the metabolic flow and protect plants from photodamage (Triantaphylides and Havaux, 2009). For this purpose, enzymes involved in Chl biosynthesis often form protein complexes to enable the efficient channeling of intermediates (Tanaka and Tanaka, 2007). In the Mg-chelating reaction, protoporphyrinogen IX oxidase (PPOX), the enzyme that produces Proto IX in chloroplasts, is in close contact with CHLH/GUN5 to form a super-complex that enables efficient reactions to occur (Chen et al., 2015). Thereafter, the association of CHLH to the chloroplast membrane is prompted by the GUN4-porphyrin complexes (Adhikari et al., 2009, 2011). The PPOX-CHLH/GUN5-GUN4 complex ensures the transformation of Proto IX to Mg-Proto IX. In the following step, a tight complex formed between
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

While preparing this study for publication, a report (Wang et al., 2020) described the analysis of BCM1 (Balance of Chlorophyll Metabolism1) that is identical to CBD1. BCM1 was also identified as a thylakoid membrane protein playing a role in Chl biosynthesis. It is important to compare several points between the two studies, which may help to better understand the mechanism underlying BCM1/CBD1 function in Chl biosynthesis. Both studies addressed the step in Chl biosynthesis at which BCM1/CBD1 may play a role. In the bcm1 mutant (Wang et al., 2020), the authors observed a decrease in the ALA synthesis rate and reduced accumulation of Proto IX, Mg-Proto IX, and Mg-Proto IX ME. It is difficult to pinpoint which step of Chl biosynthesis is affected based on this profile of metabolites. The fact that BCM1 physically and genetically interacts with GUN4 prompted the conclusion that BCM1 facilitates the biosynthesis of Mg-Proto IX. We took a different approach to examine the later steps in Chl biosynthesis by feeding adequate ALA to the cbd1 mutant and WT plants and investigated the intermediate accumulation. We observed Mg-Proto IX/ME over-accumulation in cbd1, and this accumulation was more dramatic in the cbd1gun5 double mutant (Figures 3H and 4G). Therefore, we suggest that CBD1 likely functions in synergy with CHLH/GUN5 in Mg-Proto IX trafficking to the methylation step. This is consistent with the idea that CHLH/GUN5 not only serves as the catalytic subunit in the Mg chelation step, but also as the “activator” of CHLM in the methylation step. With regard to the mechanism of action, BCM1 is shown to interact with GUN4 and by doing so serves as an important “scaffold” to promote the organization and targeting of a MgCh–MgMT complex at the thylakoid membrane (Wang et al., 2020). We also speculated that the thylakoid membrane-localized CBD1 may serve as an anchor protein of MgCh for its subsequent interaction with MgMT. Consistent with this hypothesis, double mutation of CBD1 and CHLH/GUN5 resulted in severely disturbed MgMT function as reflected by the over-accumulation of Mg-Proto IX/ME upon ALA feeding. In addition to the functional interaction between CBD1 and GUN5, we also provided evidence that CBD1 may bind/translocate Mg2+ at the thylakoid membrane and affect Mg2+ homeostasis (Figure 6), thus leading to Mg2+ accumulation in the stroma. Although adequate free Mg2+ is required for the Mg-chelating reaction in Chl biosynthesis, excessive Mg2+ in the stroma is known to inhibit MgMT activity due to the aggregation of the enzyme at a high free-Mg2+ concentration (Savicki and Willows, 2007, 2010). The exact mechanism of how CBD1 affects the Mg balance between the stroma and thylakoid warrants further study.

METHODS

Plant Materials and Growth Conditions

The Arabidopsis thaliana WT (ecotype Columbia-0) and T-DNA insertion line cbd1-1 (SALK_031802) and cbd1-2 (SALK_058830) were ordered from the Arabidopsis Biological Resource Center. The homozygous mutants were obtained from heterozygous plants and screened by genomic PCR using the primers listed in Supplemental Table 2. Homozygous individuals were further identified using RT–PCR analysis. Seeds were surface sterilized in 75% ethanol three times before being sown on half-strength Murashige and Skoog (MS) medium. To ensure synchronized germination, seeds were incubated in darkness for 2 days at 4 °C before transferring to a growth chamber. For culture on agar plates in the growth chamber, WT and mutant plants used in this study were grown in

**Figure 5. Transmission Electron Microscopy Analysis of Chloroplasts in WT, *cbd1*, *gun5*, and *cbd1gun5.*

(A–C) Chloroplasts containing starch grains and thylakoids from the leaves of 4-week-old WT, *cbd1*, and *gun5*. (D–F) Chloroplasts from the leaves of 4-week-old *cbd1gun5*. (G–I) Thylakoids containing grana and stroma thylakoids from the leaves of 4-week-old WT, *cbd1*, and *gun5*. (J–L) Thylakoids from the leaves of 4-week-old *cbd1gun5*. The white arrow in (K) indicates the rudimentary thylakoids and the white circle in (L) indicates the aggregated osmiophilic granules.

Scale bars correspond to 2 μm in (A–C), 1 μm in (D–F), and 500 nm in (G–L).

MgCh and MgMT enables the direct channeling of Mg-Proto IX from CHLH/GUN5 to CHLM for the methylation (Gorchtein, 1972). In fact, the direct interaction of CHLH/GUN5 with the CHLM protein enhances the overall activity of MgMT (Jensen et al., 1999; Alawady et al., 2005; Alawady and Grimm, 2005; Shepherd et al., 2005; Chen et al., 2014).

In this study, we uncovered a thylakoid membrane localized protein, CBD1, as a critical component in Chl biosynthesis. Detailed analysis indicated that CBD1 may work synergistically with CHLH/GUN5 in promoting the formation and activity of the MgCh–MgMT complex. CBD1 was identified by co-expression analysis using CHLH/GUN5 as a query gene (Figure 1). The *cbd1* mutant in *Arabidopsis* showed a pale-green phenotype, reduced total Chl content, and an increased Chl a/b ratio, reminiscent of the *gun5* mutant (Figure 3C–3E). Furthermore, the *cbd1gun5* double mutant had more severe chlorotic leaves and more stunted growth (Figure 4A–4F), suggesting that CBD1 and CHLH/GUN5 might participate in similar biological processes.
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

Chl Content Determination
To analyze Chl content, plants (~20 mg fresh weight) were harvested. Leaf samples were ground in 1 ml 80% acetone and placed in the dark at 4°C for 30 min before centrifugation. The supernatants were used for spectrophotometric measurements at 645 and 663 nm, respectively, using a spectrometer (Biomate 3S, Thermo Scientific).

Subcellular Localization of CBD1
Arabidopsis mesophyll protoplasts were prepared from 4-week-old rosette leaves by soaking leaf slices with an enzymatic mixture containing 1% (w/v) cellulase R10 and 0.4% (w/v) macerozyme R10 (Yakult Pharmaceutical) for 2–3 h. The digested protoplasts were resuspended in the W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES [pH 5.8]), and transfected in combination with 20 µg recombinant plasmids (CBD1-PEZSNL, PAA2-PEZSNL, PIC1-PEZSNL, OEP7-PEZSNL, and RBCS1-PEZSNL) using a PEG-mediated transformation protocol. The transformed protoplasts were incubated in the dark at 22°C overnight before imaging using a laser scanning confocal microscope (Lei TCS SP8-MaTai MP and Leica TCS-SL).

Histochemical GUS Analysis
Approximately 70 transformants harboring the CBD1pro::GUS fusion protein were selected and a representative line was used in this assay. For histochemical analysis, plant materials were fixed in 90% ice-cold acetone for 30 min and then washed three times with distilled water (10 ml X-Glc free staining buffer (0.5 mM K₄Fe(CN)₆, 0.5 mM K₃Fe(CN)₆, 0.1% Triton X-100, 10 mM EDTA, and 100 mM sodium phosphate). Samples were then immersed in the staining buffer supplemented with 1 ml X-Glu. After a short vacuuming period, samples were incubated at 37°C for 24 h. To clear the background, the stained materials were then rinsed in 75% ethanol overnight to remove Chl. Representative seedlings were photographed under a microscope (Olympus, SZX16).

Generation of a cdb2 Knockout Mutant by CRISPR/Cas9
For the identification of CRISPR/Cas9 target sites in CBD2, the online CRISPR-P software (http://cbi.hzau.edu.cn/crispr/) was used. Secondary structure analysis of target single-guide (sgRNA) sequences was carried out with the program RNA Folding Form (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3). After screening, three sgRNAs were selected. For each target sequence, primer pairs were designed to form a dimer with two Bsal overhangs. The three sgRNA expression cassettes were generated by adaptor-ligation and PCR amplification, and subsequently ligated into the pyLCRISPR/Cas9 vector by Golden Gate cloning. The recombinant constructs were then transformed into the WT or cdb1 mutant using the flk2 dip method with the Agrobacterium tumefaciens strain EHA105. To check for mutations in CBD2, genomic DNA was extracted, and PCR amplifications were carried out using primer pairs flanking the target sites. These PCR products were sequenced to detect frame-shift or deletion mutations in CBD2. All primers used are listed in Supplemental Table 2.

Functional Complementation of Mg²⁺ Transport by Salmonella typhimurium Mutant MM281
The competent MM281 cells were transformed with the empty pTrc99A vector, recombinant MGT10-pTrc99A, and CBD1-pTrc99A by electroporation (Gene Pulser Xcell, Bio-Rad). Transformed cells were plated onto LB medium containing 10 mM MgCl₂ and indicated antibiotics (17 µg/ml chloramphenicol and 100 µg/ml ampicillin). After overnight incubation at 37°C, transformants were selected and confirmed by PCR. Positive ones were selected and further grown in liquid LB medium containing 10 mM MgCl₂ and antibiotics as indicated above; 50 µg/ml IPTG was applied to induce protein expression. The liquid cultures were adjusted to an optical density at 600 nm of 1.0, 5 µl of a 10-fold serial dilution was spotted onto the N-minimal medium plates as indicated above, further diluted in a 10-fold series, and 4 µl spotted onto N-minimal medium (5 mM KCl, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 7.5 mM (NH₄)₂SO₄, 0.1 mM Tris, 0.02%...
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

[g/w] glucose, and 0.1% [w/v] casein hydrolysate supplemented with indicated MgSO₄ and antibiotics. The plates were incubated at 37°C for 48 h before being photographed.

Isolation of Total RNA, RT–PCR, and qRT–PCR Analysis

Total RNA was extracted using the TRIzol reagent (Invitrogen) following the manufacturer’s instructions. The first-strand cDNA was synthesized by M-MLV Reverse Transcriptase (Promega). Semi-quantitative RT–PCR analysis of gene expression using cDNA of WT, two mutant lines (cbd1-1 and cbd1-2), and the COM line, followed by 26 cycles of PCR, was carried out. qRT–PCR analysis was performed using the FastStart Universal SYBR Green Master mix (Roche) on a CFX Connect Real-time system (Bio-Rad). Target quantifications were performed with specific primer pairs designed using the Primer designing tool in NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Actin2 (AT3G18780) was used as an internal reference in both RT–PCR and qRT–PCR analysis. All primers used are listed in Supplemental Table 2.

Analysis of Intermediates in the Chl Biosynthetic Pathway

Wild-type, cbd1, gun5, cbd1gun5, and chlm were grown horizontally in a growth chamber for 3 weeks, and plates were dark adapted for another 3 days, after which ALA feeding solution (10 mM ALA, 5 mM MgCl₂, and 10 mM HEPES in 10 mM NaPO₄ [pH 7.0]) was added to each plate. The seedlings were incubated overnight and harvested under dim green light. Approximately 20 mg of leaf material was homogenized in extraction solution (acetone:NH₄OH = 9:1 [v/v]). Samples were centrifuged for 10 min at 12,000 g. The supernatants were then transferred to fresh microcentrifuge tubes and extracted three times with hexane to remove carotenoids and Chls. Fluorescence emission spectra were recorded from 560 to 700 nm using an F7000 luminescence spectrophotometer (HITACHI) at room temperature. The samples were excited at 400 and 420 nm for the detection of ProtoX and Mg-ProtoX (ME), respectively.

BN Gel, 2D SDS–PAGE Electrophoresis and Immunoblot Analysis

BN gel electrophoresis was performed as described previously (Zhang et al., 2018). Thylakoid membrane samples containing equal amounts of Chl (10 μg) were loaded onto a BN gradient gel. Electrophoresis was performed at 100 V at 4°C with a 1–8 mA current (PowerSac Universal, Bio-Rad). The cathode buffer initially contained 0.01% (w/v) Serva Blue G dye and was replaced with cathode buffer free of dye halfway through the run. For 2D SDS–PAGE analysis, the excised BN gel lanes were soaked for 10 min in SDS sample buffer (12.5 mM Tris, 4% [w/v] SDS, 20% [v/v] glycerol, and 0.02% [w/v] bromophenol blue) supplied with 5% (v/v) β-mercaptoethanol. Each lane with denatured proteins was placed on top of 12% SDS–PAGE and electrophorated at 100 V at 4°C for 4 h. After electrophoresis, gels were stained using a protein silver stain kit (CWBIO) and photographed (Gel Doc XR+, Bio-Rad). Protein samples corresponding to equal amounts of Chl (1 μg) were separated on 15% SDS–PAGE gel and transferred to polyvinylidene fluoride membrane. After blocking non-specific binding with 5% (w/v) milk, the blot was subsequently incubated with antibodies generated against the indicated proteins and detected using a SuperSignal West Pico Trial kit (Thermo Scientific).

Chl Fluorescence Measurement

Intact leaves of 3-week-old WT and mutant plants grown on half-strength MS agar plates were used in this assay. Plants were dark adapted for 40 min before each measurement. To assess the photosynthetic performance of PSIi, basic parameters F₀, Fₚ, F_em, and Fm'; and Fv/Fm' were measured with an FMS2 fluorometer (Hansatech, Norfolk, UK). F_v/F_m' and NPQ were calculated according to a previous study (Fu et al., 2007).

Transmission Electron Microscopy

For transmission electron microscopy processing, rosette leaves were collected from 3-week-old WT and mutant plants grown in half-strength MS medium in a growth chamber. The leaves were cut into slices and fixed in 2.5% (v/v) glutaraldehyde under mild vacuuming. The fixed samples were used to make ultrathin sections using a diamond knife on an ultramicrotome (PowerTome-XL, RMC). Micrographs were taken under a transmission electron microscope (H7650, HITACHI).

Isolation of Intact Chloroplasts and Thylakoids

Leaves of 3-week-old WT and mutant plants grown on half-strength MS agar plates were harvested in the daytime. To isolate intact chloroplasts, leaf samples (at least 10 g fresh weight) were homogenized in ice-cold grind buffer (0.33 M sorbitol, 10 mM EDTA, 50 mM HEPES, and 0.5 g/L BSA [pH was adjusted to 8.0 with KOH]). The homogenate was filtered through two layers of Miracloth (Calbiochem) and concentrated by centrifugation for 5 min at 700 g at 4°C using a swing-out rotor (Centrifuge 5810R, Eppendorf). The pellets were gently resuspended in grinding buffer and then layered onto a discontinuous 40% (w/v)/80% (w/v) Percoll (GE Healthcare) gradient. After centrifugation for 15 min at 2000 g at 4°C, intact chloroplasts were isolated from the interface between the two layers. The interface was gently sucked into a new tube and washed in SH (0.33 M sorbitol and 50 mM HEPES [pH adjusted to 8.0 with KOH]) to remove the remaining Percoll. For the further isolation of thylakoids, intact chloroplasts were lysed in lysis buffer (10 mM HEPES [pH adjusted to 8.0 with KOH]) for 10 min on ice. After centrifugation at 700 g at 4°C for 10 min, the transparent supernatant containing stroma fractions was discarded, and the pellets were intact thylakoids.

Metal Ion Content Determination

Leaves, intact chloroplasts, and thylakoids were collected and evaporated for further analysis. Dried samples were digested with concentrated HNO₃ (69%) at 80°C in a digester (DigiBlock ED16, LabTech). The ion concentration was measured by ICP–MS (NexION 300, PerkinElmer).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at Plant Communications Online.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant no. 31900220 to C.Z., and 31770267 to W.L.) and the National Science Foundation (MCB-1714795 to S.L.).

AUTHOR CONTRIBUTIONS

C.Z., B.Z., A.F., W.L., and S.L. designed the research. C.Z., B.Z., and B.M. carried out the experiments. C.Z., B.Z., B.M., A.F., W.L., and S.L. analyzed the data. X.Z. and F.Z. provided technical assistance. C.Z., B.Z., and S.L. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Nobuyoshi Mochizuki for the gun5-1, cch, and chlm mutant seeds used in this work, Dr Yaoguang Liu for the CRISPR/Cas9 vectors, and Dr. Fangjie Zhao for advice on ICP–MS analysis. No conflict of interest declared.

Received: April 30, 2020
Revised: June 24, 2020
Accepted: July 2, 2020
Published: July 3, 2020

REFERENCES

Abdel-Ghany, S.E., Muller-Moule, P., Niyogi, K.K., Pilon, M., and Shikanai, T. (2005). Two P-type ATPases are required for copper delivery in Arabidopsis thaliana chloroplasts. Plant Cell 17:1233–1251.

Adhikari, N.D., Froehlich, J.E., Strand, D.D., Buck, S.M., Kramer, D.M., and Larkin, R.M. (2011). GUN4-porphyrin complexes bind the ChlH/GUN5 subunit of Mg-chelatase and promote chlorophyll biosynthesis in Arabidopsis. Plant Cell 23:1449–1467.
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

insertion, and the MgATP-dependent interaction of the Chl and ChlD subunits. Biochem. J. 339 (Pt 1):127–134.

Kobayashi, K., and Masuda, T. (2016). Transcriptional regulation of tetrapyrrole biosynthesis in Arabidopsis thaliana. Front. Plant Sci. 7:1811.

Larkin, R.M. (2016). Tetrapyrrole signaling in plants. Front. Plant Sci. 7:1588.

Lee, D.W., Lee, S., Lee, G.J., Lee, K.H., Kim, S., Cheong, G.W., and Hwang, I. (2006). Functional characterization of sequence motifs in the transit peptide of Arabidopsis small subunit of rubisco. Plant Physiol. 140:466–483.

Masuda, T. (2008). Recent overview of the Mg branch of the tetrapyrrole biosynthesis leading to chlorophylls. Photosynth. Res. 96:121–143.

Masuda, T., and Fujita, Y. (2008). Regulation and evolution of chlorophyll metabolism. Photochem. Photobiol. Sci. 7:1131–1149.

Matsumoto, F., Obayashi, T., Sasakisekimoto, Y., Ohta, H., Takamika, K., and Masuda, T. (2004). Gene expression profiling of the tetrapyrrole metabolic pathway in Arabidopsis with a mini-array system. Plant Physiol. 135:2379.

Mochizuki, N., Brusslan, J.A., Larkin, R., Nagatani, a., and Chory, J. (2001). Arabidopsis genomes uncoupled 5 (GUN5) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc. Natl. Acad. Sci. U S A. 98:2053–2058.

Mochizuki, N., Tanaka, R., Tanaka, A., Masuda, T., and Nagatani, A. (2008). The steady-state level of Mg-protoporphyrin IX is not a determinant of plastid-to-nucleus signaling in Arabidopsis. Proc. Natl. Acad. Sci. U S A. 105:15184–15189.

Obayashi, T., Nishida, K., Kasahara, K., and Kinoshita, K. (2011). ATTED-II updates: condition-specific gene coexpression to extend coexpression analyses and applications to a broad range of flowering plants. Plant Cell Physiol 52:213–219.

Pei, J., and Grishin, N.V. (2001). Type II CAAX prenyl endopeptidases belong to a novel superfAMILY of putative membrane-bound metalloproteases. Trends Biochem. Sci. 26:275–277.

Pontier, D., Albrieux, C., Joyard, J., Lagrange, T., and Block, M.A. (2007). Knock-out of the magnesium protoporphyrin IX methyltransferase gene in Arabidopsis. Effects on chloroplast development and on chloroplast-to-nucleus signaling. J. Biol. Chem. 282:2297–2304.

Sandelin, A.S., and Aronsson, H. (2009). The Chloroplast: Interactions with the Environment (Berlin Heidelberg: Springer-Verlag).

Sawicki, A., and Willows, Robert D. (2007). S-Adenosyl-L-methionine:magnesium-protoporphyrin IX O-methyltransferase from Rhodobacter capsulatus: mechanistic insights and stimulation with phospholipids. Biochem. J. 406:469–478.

Sawicki, A., and Willows, R.D. (2010). BchN and BchM interact in a 1:1 ratio with the magnesium chelatase BchH subunit of Rhodobacter capsulatus. FEBS J. 277:4709–4721.

Scharfenberg, M., Mittermayr, L., E, V.O.N.R.-L., Schlicke, H., Grimm, B., Leister, D., and Kleine, T. (2015). Functional characterization of the two ferrochelatases in Arabidopsis thaliana. Plant Cell Environ 38:280–298.

Shang, Y., Yan, L., Liu, Z.-Q., Cao, Z., Mei, C., Xin, Q., Wu, F.-Q., Wang, X.-F., Du, S.-Y., Jiang, T., et al. (2010). The Mg-chelatase H subunit of Arabidopsis antagonizes a group of WRKY transcription repressors to relieve ABA-responsive genes of inhibition. Plant Cell 22:1909–1935.

Shepherd, M., McLean, S., and Hunter, C.N. (2005). Kinetic basis for linking the first two enzymes of chlorophyll biosynthesis. FEBS J. 272:4532–4539.

Tanaka, R., Kobayashi, K., and Masuda, T. (2011). Tetrapyrrole metabolism in Arabidopsis thaliana. The Arabidopsis book 9:e0145.
CBD1 Works Synergistically with GUN5 in Chl Biosynthesis

Tanaka, R., and Tanaka, A. (2007). Tetrapyrrole biosynthesis in higher plants. Annu. Rev. Plant Bio. 58:321–346.

Triantaphylides, C., and Havaux, M. (2009). Singlet oxygen in plants: production, detoxification and signaling. Trends Plant Sci. 14:219–228.

Walker, C.J., and Willows, R.D. (1997). Mechanism and regulation of Mg-chelatase. Biochem. J. 327 (Pt 2):321–333.

Wang, P., Richter, A.S., Kleeberg, J.R.W., Geimer, S., and Grimm, B. (2020). Post-translational coordination of chlorophyll biosynthesis and breakdown by BCMs maintains chlorophyll homeostasis during leaf development. Nat. Commun. 11:1254.

Zhang, B., Zhang, C., Liu, C., Jing, Y., Wang, Y., Jin, L., Yang, L., Fu, A., Shi, J., Zhao, F., et al. (2018). Inner envelope CHLOROPLAST MANGANESE TRANSPORTER 1 supports manganese homeostasis and phototrophic growth in Arabidopsis. Mol. Plant 11:943–954.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol. 136:2621–2632.