The *GDC1* Gene Encodes a Novel Ankyrin Domain-Containing Protein That Is Essential for Grana Formation in Arabidopsis[1][OA]

Yong-Lan Cui, Qi-Shi Jia, Qian-Qian Yin, Guan-Nan Lin, Meng-Meng Kong, and Zhong-Nan Yang*

Biology Department, Life and Environmental College, Shanghai Normal University, Shanghai 200234, China (Y.-L.C., Q.-S.J., Q.-Q.Y., G.-N.L., Z.-N.Y.); and National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200032, China (M.-M.K.)

In land-plant chloroplasts, the grana play multiple roles in photosynthesis, including the potential increase of photosynthetic capacity in light and enhancement of photochemical efficiency in shade. However, the molecular mechanisms of grana formation remain elusive. Here, we report a novel gene, *Grana-Deficient Chloroplast1* (*GDC1*), required for chloroplast grana formation in Arabidopsis (*Arabidopsis thaliana*). In the chloroplast of knockout mutant *gdc1-3*, only stromal thylakoids were observed, and they could not stack together to form appressed grana. The mutant exhibited seedling lethality with pale green cotyledons and true leaves. Further blue native-polyacrylamide gel electrophoresis analysis indicated that the trimeric forms of Light-Harvesting Complex II (LHCII) were scarcely detected in *gdc1-3*, confirming previous reports that the LHCII trimer is essential for grana formation. The Lhcb1 protein, the major component of the LHCIIb trimer, was substantially reduced, and another LHCIIb trimer component, Lhcb2, was slightly reduced in the *gdc1-3* mutant, although their transcription levels were not altered in the mutant. This suggests that defective LHCII trimer formation in *gdc1-3* is due to low amounts of Lhcb1 and Lhcb2. *GDC1* encodes a chloroplast protein with an ankyrin domain within the carboxyl terminus. It was highly expressed in Arabidopsis green tissues, and its expression was induced by photosignaling pathways. Immunoblot analysis of the GDC1-green fluorescent protein (GFP) fusion protein in 35S::GDC1-GFP transgenic plants with GFP antibody indicates that GDC1 is associated with an approximately 440-kD thylakoid protein complex instead of the LHCII trimer. This shows that GDC1 may play an indirect role in LHCII trimerization during grana formation.

Photosynthesis is the primary process through which solar energy is converted into chemical energy of organic substances, which serves as the substrate and energy source for various biological events, not only in plants but also in animals. The process is carried out in the thylakoid membranes of plants, algal chloroplasts, and photosynthetic bacteria. The thylakoid membranes of land-plant chloroplasts have a remarkably complex structure and organization. One of the most striking features is the presence of grana (Mustárdy and Garab, 2003). Grana consist of regular stacks of appressed thylakoids, typically 300 to 600 nm in diameter, containing approximately 10 to 20 layers of thylakoid membranes, which are interconnected by unstacked membrane regions known as the stromal lamellae (Mullineaux, 2005). This structural differentiation of thylakoids into appressed granal and nonappressed stromal lamellae is accompanied by functional differentiation (Kim et al., 2005). PSII and its associated integral membrane chlorophyll *a*/*b*-binding light-harvesting complex (LHCII) reside mainly in the grana membranes (Andersson and Anderson, 1980; Anderson and Andersson, 1982; Chow et al., 1991). PSI and ATP synthase are predominant in the stroma lamellae, and the cytochrome b_6/f complex is evenly distributed between the two types of membranes (Miller and Staehelin, 1976; Allred and Staehelin, 1984).

Grana of vascular plant chloroplasts are relatively recent and successful products of evolution (Mullineaux, 2005). Their ubiquitous presence in vascular plants suggests that grana play critical roles in the optimization of photosynthetic functions (Albertsson, 2001; Goss et al., 2007). The tightly appressed arrangement of granum thylakoid membranes enhances light capture through a vastly increased area-to-volume ratio and connectivity of several PSIIs with large functional antenna size (Dekker and Boekema, 2005). Grana control lateral separation of PSII from PSI and therefore limit spillover of excitation from PSII to PSI (Anderson, 1981; Trissl and Wilhelm, 1993). Grana have been implicated in the reversible fine-tuning of energy distribution between

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* Corresponding author; e-mail znyang@shnu.edu.cn.

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the photosystems by state 1-state 2 transition (Bennett et al., 1980; Chow et al., 1981). Anderson and Aro (1994) hypothesized that grana stacking plays an important role in protecting PSII under sustained high-light irradiance conditions. Furthermore, grana have been proposed to be involved in adaptation to terrestrial environments. Under these conditions, plants were subjected to direct sunlight, and consequently land plants required higher photosynthetic capacities. Therefore, organized thylakoid stacking was necessary to acquire more light energy and generate more volume for macromolecular diffusion in the stroma of land-plant chloroplasts. The evolution of grana was favored by selection, as it enabled higher plants to survive and thrive in ever-fluctuating light environments, from limiting to saturating light and even under prolonged intense light exposure (Chow et al., 2005).

The stacking of stroma lamellae to form grana is the last step in the structural changes of photosynthetic membranes (Paolillo, 1970; Wellburn and Wellburn, 1971; Argyroudi-Akoyunoglou et al., 1976). The extent of stacking is greatly dependent on light conditions (Anderson et al., 1973), the protein complex within the thylakoid membranes (McDonnel and Staehelin, 1980), and the ionic environment of the chloroplasts (Akoyunoglou and Argyroudi-Akoyunoglou, 1974). Izawa and Good (1966) observed marked effects of cations on the chloroplast membrane structure. Incubation of chloroplasts in a low-salt medium resulted in the unstacking of grana that could be reversed upon the addition of high concentrations of monovalent cations or low concentrations of divalents. These effects have since been confirmed by others (Goodenough and Staehelin, 1971; Jennings et al., 1978, 1981). LHCIIb has been thought to play an important role in the formation of grana (Allen and Forsberg, 2001) and is the major LHCII antenna complex, occurring in a trimeric association state (Butler and Kühlbrandt, 1988). It consists of various combinations of three very similar proteins, encoded by the *Lhcb1*, *Lhcb2*, and *Lhcb3* genes, that usually occur in a ratio of approximately 8:3:1 (Jansson, 1994). In addition, there are three "minor" antenna complexes, which are designated Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24), and usually exist in monomeric aggregation states. However, Andersson et al. (2003) questioned the requirement of LHCIIb for grana stacking, as there is a case where grana can form normally in Lhcb1 and Lhcb2 protein-deficient plants. At present, there remains uncertainty surrounding the roles of LHCII in grana formation.

Several studies have clarified grana stack formation and the spatial distribution of protein complexes within the membranes; however, the nature of adhesion factors involved in the stacking process is unclear. In this study, we report a novel gene, *Grana-Deficient Chloroplast1* (*GDC1*), essential for grana formation in Arabidopsis (*Arabidopsis thaliana*). Our results suggest that the trimeric form of LHCII is important for thylakoid stacking and grana formation.

RESULTS

Isolation of the *gdc1-1* Mutant

We screened young seedlings to isolate novel genes required for chloroplast biogenesis and identified a pale green mutant, named *gdc1-1*, from a collection of T-DNA insertion lines (Qin et al., 2003). The *gdc1-1* mutants can survive for approximately 5 to 6 weeks with a pale green phenotype when germinated on soil (Fig. 1). The pale coloration was observed throughout the life of the mutant and uniformly affected all the aerial tissues. The *gdc1-1* plants grew much slower than the wild type, and growth ceased at the vegetative growth stage before bolting.

To establish whether *gdc1-1* is a nuclear recessive mutant, 353 progeny obtained from self-pollinated heterozygotes (*GDC1/gdc1*) were segregated at a green plants-to-pale green plants phenotypic ratio of 268:85 [χ^2 (3:1) = 0.16; $P > 0.50$]. This ratio was expected for a character inherited as a single recessive Mendelian trait. Cosegregation of resistance to Basta (conferred by T-DNA) and the pale green phenotype indicated that the pale coloration of the *gdc1-1* mutant cosegregated with the T-DNA insertion (data not shown).

Cloning of the *GDC1* Gene

To identify the corresponding *GDC1* gene, thermal asymmetric interlaced (TAIL)-PCR was used to obtain a genomic DNA fragment that flanked the left border of T-DNA (Liu et al., 1995). Sequencing of the TAIL-PCR products suggested that the T-DNA is inserted in the only intron of a predicted open reading frame (AT1G50900; Fig. 2A). PCR analysis using T-DNA and genome-specific primers indicated that all mutant plants analyzed were homozygous for the insertion (data not shown), which indicated that AT1G50900 was responsible for the *gdc1-1* pale green phenotype. Genetic complementation was subsequently performed to confirm the results. A 3,538-bp DNA fragment, which included the genomic sequences of AT1G50900 and 1,886-bp sequences upstream from the initiation codon, was cloned from the wild type and introduced into the heterozygous (*GDC1/gdc1*) plants. A total of 17 transgenic plants were generated, and each exhibited normal morphology (Fig. 1A). PCR analysis confirmed that two of 17 transgenic plants were homozygous for the *gdc1* mutation. Transmission electron microscopy analysis showed that the ultrastructure of the complemented *gdc1-1* chloroplast resembled that of the wild type (Fig. 3G). These results verified that AT1G50900 was *GDC1* and that the 3,538-bp genomic region was sufficient for *GDC1* function.

In addition, we obtained another two *gdc1-1* alleles from the SIGnAL collection at the Arabidopsis Biological Resource Center (ABRC): *gdc1-2* (SALK_151530) and *gdc1-3* (SALK_126967). PCR analysis confirmed that the T-DNAs were inserted into the only intron and the last exon of the AT1G50900 locus in the *gdc1-2* and *gdc1-3* alleles, respectively. Both *gdc1-2* and *gdc1-3*

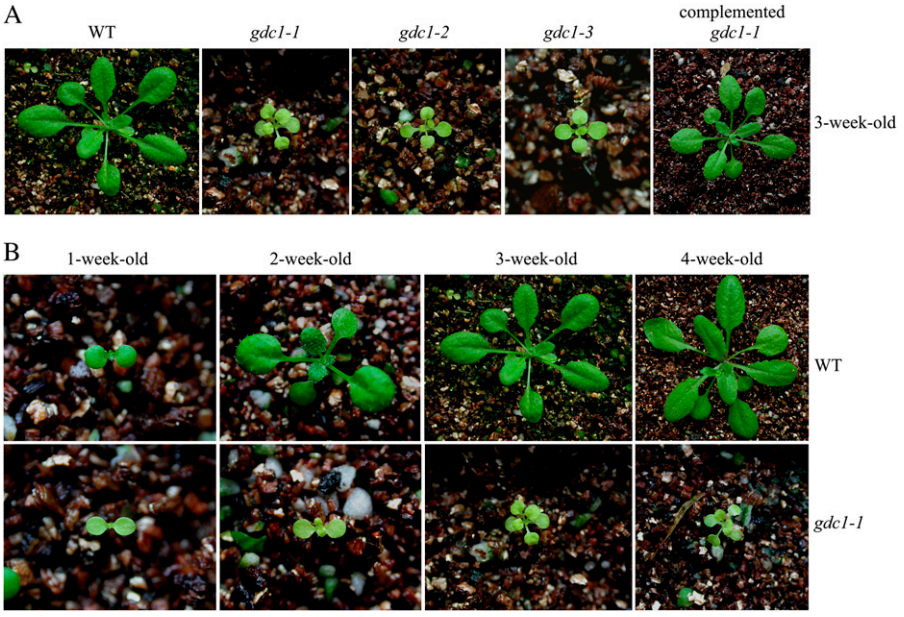

Figure 1. Characterization of the *gdc1-1* mutant. A, Phenotypes of the wild-type (WT) Columbia-0 ecotype, *gdc1-1*, *gdc1-2*, and *gdc1-3* mutants, and complemented *gdc1-1*. B, Growth time course of *gdc1-1* and wild-type plants.

showed similar phenotypes to that of *gdc1-1* (Fig. 1A). AT1G50900 expression was examined in the allelic mutant plants using reverse transcription (RT)-PCR and real-time RT-PCR analysis with gene-specific primers. Gene expression levels in *gdc1-1* and *gdc1-2* were 5.07% and 3.58% of the wild type, respectively. However, *GDC1* expression was not detected in *gdc1-3* (Fig. 2, B and C). Therefore, *gdc1-3* was chosen for further analysis.

Grana Are Deficient in *gdc1-3*

Previous reports demonstrated that leaf coloration mutants are related to chloroplast biogenesis (Reiter et al., 1994; Sundberg et al., 1997). Therefore, to determine if the *GDC1* mutation resulted in the chloroplast biogenesis defect, the *gdc1-3* mutant leaf chloroplast ultrastructure was analyzed by transmission electron microscopy. In mature chloroplasts of the wild-type plants, the internal membranes were present as either stroma thylakoids or stacked grana thylakoids (Fig. 3, A and B). In *gdc1-3* chloroplasts, only stromal thylakoids were observed; they could not stack together to form appressed grana (Fig. 3, C and D). Typical grana were not detected in the *gdc1-1* and *gdc1-2* mutants, in which the unstacked thylakoid lamella resembled *gdc1-3* (Fig. 3, E and F). These results indicated that the GDC1 protein is essential for the formation of stacked grana.

Decreased Chlorophyll Content in the *gdc1-3* Mutant

The pale green phenotype suggested that major pigment levels in the mutant were reduced. Therefore, we measured the concentration of chlorophylls in the leaves of mutant and wild-type plants. The total chlorophyll content of the 3-week-old wild-type leaves was 1,302.57 $\pm$ 60.56 μg g^{-1} fresh weight, while the total chlorophyll content in *gdc1-3* was reduced by approximately 82%, with only 245.32 $\pm$ 1.49 μg g^{-1} fresh weight. However, the chlorophyll *a*/*b* ratio was increased from 3.44 in the wild type to 13.81 in the *gdc1-3* mutant (Table I). This indicated that the high chlorophyll *a*/*b* ratio primarily resulted from a more substantial reduction of chlorophyll *b* in the mutant.

PSII and LHCII are mainly located in the appressed granal domain. PSII activity in the grana-deficient *gdc1-3* mutant was analyzed by measuring the ratio of variable fluorescence to maximum fluorescence as follows: $F_v/F_m = (F_m - F_o)/F_m$, where F_o and F_m are minimum and maximum chlorophyll *a* fluorescence of dark-adapted leaves, respectively. F_v/F_m reflects the maximum potential capacity of the PSII photochemical reactions (Krause and Weis, 1991). The F_v/F_m value of 3-week-old leaves was 0.81 $\pm$ 0.01 in wild-type plants, and the *gdc1-3* value was 0.79 $\pm$ 0.01. Despite the fact that the F_v/F_m value was very close for the wild type and the mutant, the F_o and F_m in the mutant were dramatically lower than in the wild type. This demonstrates that the photochemical efficiency of PSII was not affected by the mutation in *gdc1-3*.

gdc1-3 Accumulated Very Low Amounts of LHCII Trimer

The effects of grana deficiency on protein complexes embedded in thylakoid membranes were examined. The chlorophyll-protein complexes were solubilized from thylakoid membranes using dodecyl-β-D-maltopyranoside and separated by blue native (BN)-PAGE (Schägger et al., 1994). After the first-dimensional separation in the presence of Coomassie Brilliant Blue G-250, the major bands representing PSII supercomplexes, monomeric PSI and dimeric PSII, monomeric PSII, dimeric cytochrome b_6f, trimeric LHCII, and monomeric LHCII were resolved in the wild type (Fig. 4A). Most of the bands were also detected in the *gdc1-3*

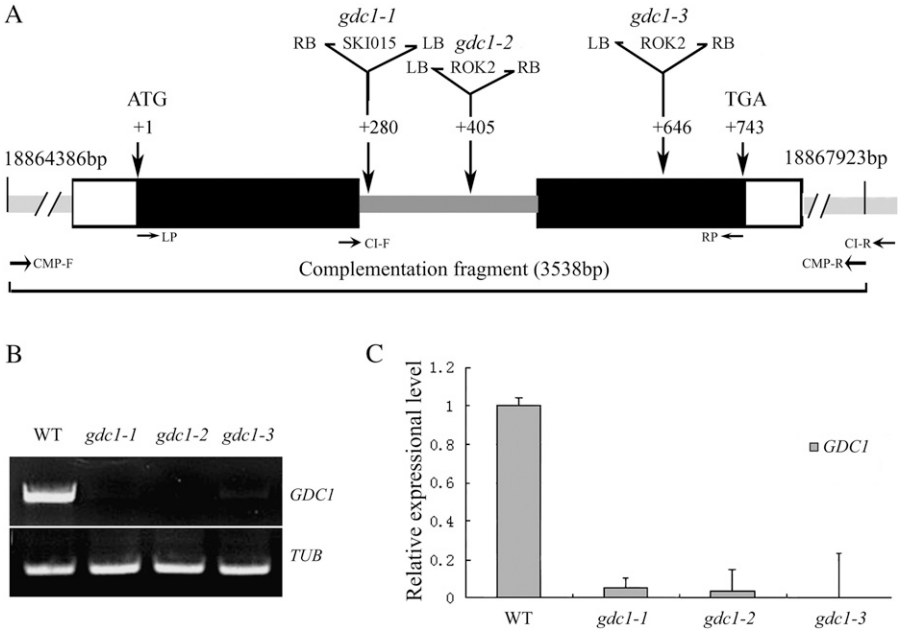

Figure 2. Characterization of the *GDC1* gene. A, Gene structure of the *GDC1* and T-DNA insertion. Black boxes, Exons; white boxes, 5′- and 3′-untranslated regions; gray lines, introns; light gray lines, upstream region of transcription initiation sites and downstream region of 3′-untranslated regions; LP/RP, primers used for T-DNA insertion validation in *gdc1-1*, *gdc1-2*, and *gdc1-3*; CMP-F/CMP-R, primers used for PCR amplification of *GDC1* complement fragments; CI-F/CI-R, primers used to validate the homozygous background. The *gdc1-1* mutant was screened in this study; *gdc1-2* and *gdc1-3* are allelic mutant lines, which were obtained from ABRC. Their insertion sites and the orientations of the T-DNA are indicated. The complementary fragment is shown, and the numbers represent the chromosome locus. B, Effects of *gdc1-1*, *gdc1-2*, and *gdc1-3* mutation on *GDC1* transcript accumulation. Transcripts were detected by RT-PCR. PCR products obtained with *GDC1* gene-specific primers and control primers for the *TUBULIN* (*TUB*) gene were analyzed on a 1% (w/v) agarose gel. WT, Wild type. C, Real-time RT-PCR analysis of *GDC1* expressional levels in *gdc1-1*, *gdc1-2*, and *gdc1-3* mutants.

mutant. However, the trimeric LHCII was negligible, and band I of the wild type disappeared in the *gdc1-3* mutant.

In the grana of higher plants, the LHCII trimer is composed of Lhcb1, Lhcb2, and Lhcb3 in a ratio of approximately 8:3:1 (Jansson, 1994). The steady-state levels of LHCII proteins were examined by immunoblot analyses performed with antibodies raised against specific subunits of LHCIIb and one other minor monomeric CP26 protein. The results showed that Lhcb1 levels were substantially reduced, and Lhcb2 was slightly reduced in *gdc1-3*. Lhcb3 and CP26 accumulated at the same level between the mutant and the wild type (Fig. 4B). We further investigated LHCIIb and CP26 gene expression at the transcriptional level by RT-PCR. The results demonstrated no differences on transcription level between the *gdc1-3* mutant and the wild type (Fig. 4C). Therefore, the accumulation of Lhcb1 and Lhcb2 was affected by the GDC1 mutation. We also characterized the subunits of PSII, PSI, cytochrome b_6f, and ATP synthase complexes in the mutant. In the *gdc1-3* mutant, the D1 and D2 core subunits of PSII accumulated to low levels, while the PsaA subunit of PSI, the cytochrome *f* subunit of cytochrome b_6f, and the β-subunit of ATP synthase accumulated to similar levels as the wild type (Fig. 4B). These results suggested that PSII was severely impaired by the GDC1 mutation and grana deficiency.

The *GDC1* Gene Encodes a Novel Ankyrin Protein That Localizes to the Chloroplast

To verify the *GDC1* gene primary structure, its full genomic DNA and cDNA were cloned and sequenced. The *GDC1* gene genomic structure was 1,008 bp in length and was composed of two exons and one intron, which is consistent with The Arabidopsis Information Resource gene predictions. The cDNA we cloned from seedlings is identical to the cDNA in The Arabidopsis Information Resource database.

The *GDC1* mRNA encodes an unknown protein of 175 amino acids with a putative molecular mass of 19 kD. Domain analysis indicated that the GDC1 protein is an ankyrin domain-containing protein (amino acids 117–149). This domain (Fig. 5A) exhibits conservation of the consensus motif in Arabidopsis with 55% identity (defined by Becerra et al., 2004). This is congruent with 54% to 100% of those observed among hundreds of ankyrin repeats (Bork, 1993). BLASTP search showed that the homologs of the GDC1 protein were also present in various plant species, including castor (*Ricinus communis*), poplar (*Populus trichocarpa*),

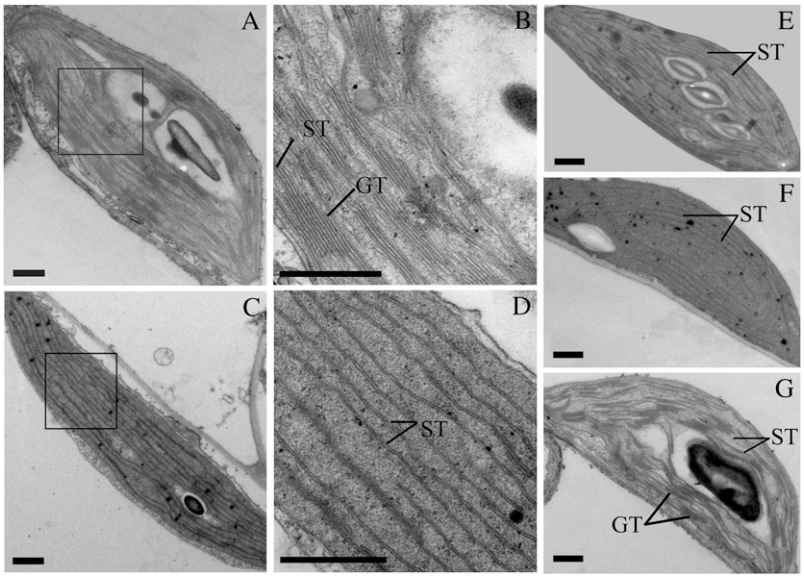

Figure 3. Electron micrographs of *gdc1-3* and its allelic mutant chloroplasts. A, Chloroplast of the wild-type Columbia-0. B, Closeup view of the wild-type Columbia-0 chloroplast. GT, Grana thylakoid stacks; ST, stromal thylakoids. C, Abnormal chloroplast of the pale green mutant *gdc1-3*. D, Closeup view of the abnormal chloroplast of the pale green mutant *gdc1-3*. E, Chloroplast of *gdc1-1*. F, Chloroplast of *gdc1-2*. G, Chloroplast of the complemented *gdc1-1* restored the ultrastructure to that of the wild-type. Bars = 500 nm.

grape (*Vitis vinifera*), soybean (*Glycine max*), rice (*Oryza sativa*), sorghum (*Sorghum bicolor*), maize (*Zea mays*), moss, and green algae. For example, the homolog from poplar, ABK94006, shared the highest identity (76%) and similarity (85%) with GDC1; and the homolog from grape, CAN65357, shared an identity of 70% and similarity of 82% with GDC1. ClustalX2 was used to perform an alignment with these protein sequences (Fig. 5B). Phylogenetic analysis indicated that GDC1 formed a basal clade within the dicotyledons (Fig. 5C). These results indicated that GDC1 was conserved in the evolutionary process, and its putative homologs were present in various chloroplast-containing organisms, including unicellular green algae and mosses.

TargetP program analysis showed that GDC1 is a chloroplast-targeted protein (http://www.cbs.dtu.dk/services/TargetP/; Emanuelsson et al., 2000). The subcellular localization of the protein was confirmed by fusing the full-length coding sequence of GDC1 with the GFP gene and introduced into wild-type plants under the control of the cauliflower mosaic virus 35S promoter. Stable transgenic plants were obtained, and GFP fluorescence of transgenic plants was observed with confocal laser microscopy. GFP fluorescence was colocalized with chlorophyll autofluorescence. This confirmed that GDC1 is a chloroplast-localized protein (Fig. 6A).

To further determine the localization of GDC1 within the chloroplast, we investigated the GDC1-GFP fusion protein in 35S::GDC1-GFP transgenic plants with GFP antibody. A positive signal was detected in the total proteins and thylakoid membrane proteins from 35S::GDC1-GFP transgenic plants (Fig. 6B). To understand which complex GDC1-GFP might be associated with, the thylakoid membrane protein complexes of 35S::GDC1-GFP transgenic plants were separated by BN-PAGE, denatured on the gel, and directly used for two-dimensional SDS-PAGE. Immunoblot analysis indicated that the GDC1-GFP fusion protein was associated with a complex of approximately 440-kD thylakoid membrane (Fig. 6C). There are two bands of about 440 kD in the wild type; however, only one band is present in the mutant (Fig. 4A). This indicates that the GDC1 might be associated with the approximately 440-kD protein complex that is absent in the mutant.

GDC1 Gene Expression Pattern

Expression data from Genevestigator showed that GDC1 was widely expressed in Arabidopsis green tissues (Zimmermann et al., 2004; http://www.genevestigator.com). Therefore, northern-blot analysis was performed to confirm these data. The results showed that *GDC1* was highly expressed in leaves and seedlings; however, it was weakly expressed in roots (Fig. 7A). This result was consistent with the microarray data.

GDC1 expression patterns in Arabidopsis were also investigated using a GUS reporter gene fused to its promoter (Fig. 7, B–G). The observed patterns suggested that *GDC1* was not expressed in germinating seeds (Fig. 7B). In 15-d-old seedlings, GUS activity was detected in the basal rosette leaves but not in the roots (Fig. 7C). GUS staining was also observed in the stems

Table I. *Chlorophyll content of leaves*

Total chlorophylls were obtained from 100 mg of fresh tissue from 3-week-old *Arabidopsis* basal rosette leaves using 100% acetone. Spectrophotometric quantification was carried out, and then the ratio of chlorophyll *a* to chlorophyll *b* was calculated. Values shown are $\mu g\ g^{-1}$ fresh weight.

Chlorophyll	Wild Type	*gdc1-3*
Total chlorophyll	1,302.57 ± 60.56	245.32 ± 1.49
Chlorophyll *a*	1,008.95 ± 44.90	228.75 ± 1.48
Chlorophyll *b*	293.62 ± 15.66	16.57 ± 0.01
Chlorophyll *a*/*b*	3.44 ± 0.04	13.81 ± 0.09

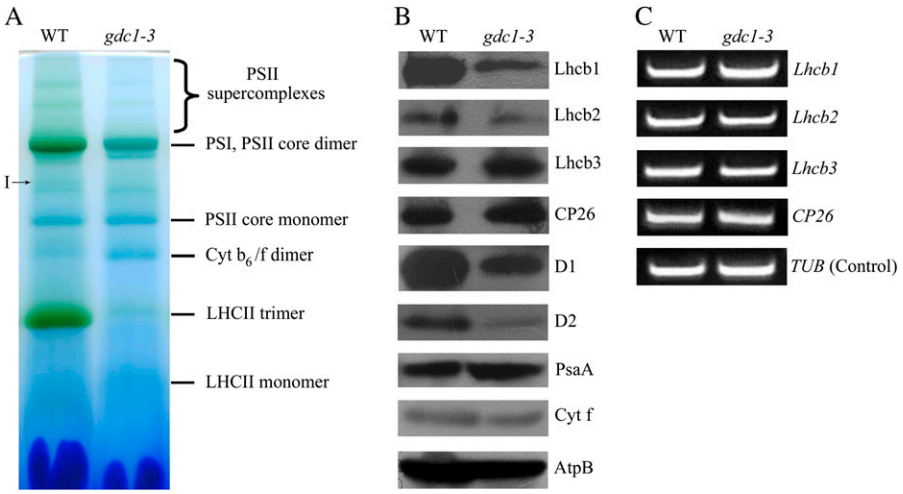

Figure 4. Analysis of thylakoid membrane proteins from 3-week-old *gdc1-3* and the wild-type. A, BN gel analysis of thylakoid membrane protein complexes. Thylakoid membranes (10 μg of chlorophyll) from wild-type (WT) and *gdc1-3* mutant leaves were solubilized with 2% dodecyl-β-D-maltopyranoside and separated by BN gel electrophoresis. B, Immunodetection of thylakoid proteins. The total proteins (40 μg) were separated by SDS-urea-PAGE, and blots were probed with specific antibodies for Lhcb1, Lhcb2, Lhcb3, CP26, D1, D2, PsaA, cytochrome *f*, and AtpB. C, RT-PCR analysis of *Lhcb1*, *Lhcb2*, *Lhcb3*, and *CP26* gene expression at the transcriptional level.

and siliques but not in mature seeds (Fig. 7, E and G). In the flower, GUS activity was detected in the sepals, stamens, and styles but not in the petals (Fig. 7E). GUS staining for transgenic lines showed that *GDC1* was only expressed in green tissues containing chloroplasts, which was consistent with its putative roles in chloroplast grana formation.

In addition, we examined the effect of light on GDC1 expression (Fig. 7H). Real-time RT-PCR analysis was carried out using total RNA isolated from leaves harvested at 0, 1, 3, 6, 12, or 24 h after the transfer of 7-d-old dark-grown wild-type plants to light conditions. *GDC1* was weakly expressed in the dark (0 h), but after transfer of etiolated plants to light conditions, *GDC1* mRNA accumulated in a time-dependent manner. This result suggests that the expression of GDC1 is induced by photosignaling pathways.

DISCUSSION

In this study, we isolated a pale green Arabidopsis mutant and cloned the *GDC1* gene (AT1G50900) that encodes a protein with an ankyrin domain. Transmission electron microscopy analysis indicated that GDC1 is essential for grana formation, and gene expression pattern and protein subcellular localization analysis suggested that GDC1 is related to chloroplast biogenesis. The trimeric form of LHCII in the *gdc1-3* mutant was scarcely detected, indicating that the LHCII trimer is essential for grana formation. The substantially reduced Lhcb1 protein in the *gdc1-3* mutant suggested that GDC1 might be involved in importing LHCP into the thylakoid membrane in the chloroplast.

GDC1 Is Essential for Grana Formation

Many mutants with abnormal grana structure have been isolated from different plant species. Chlorophyll deficiency is responsible for the majority of mutants with abnormal grana, and these plants exhibit pale green or yellow phenotypes. In barley (*Hordeum vulgare*), chlorophyll-deficient mutants can be divided into two groups based on their chlorophyll content and chlorophyll *a*/*b* ratio. The group with chlorophyll *a*/*b* ratios lower than the wild type is referred to as grana-rich mutants, in which most the lamellae are stacked into grana. The other group exhibits high ratios of chlorophyll *a*/*b* and low chlorophyll content. The chloroplasts have very few grana and are referred to as grana-deficient mutants (Nielsen et al., 1979). In this study, stacked grana were not observed in the *gdc1-3* mutant and it had a high chlorophyll *a*/*b* ratio. This suggested that *gdc1-3* is allied with the grana-deficient mutant group.

All genes for enzymes responsible for chlorophyll biosynthesis in higher plants have been identified, which include 15 enzymes and 27 genes in Arabidopsis (Nagata et al., 2005). In the allele mutants with mutations occurring in the CHLI1, which is a subunit of magnesium chelatase, chlorophyll can accumulate at lower levels than in the wild type, with an elevated chlorophyll *a*/*b* ratio ranging from 4.0 to 10.4 relative to the wild-type ratio of 3.0, and grana stacking disappears (Rissler et al., 2002; Apchelimov et al., 2007). In the CHLI1 null *ch42-3* mutant, Lhcb1 levels were not altered and Lhcb2 and Lhcb3 proteins were absent. DVR encodes 3,8-divinyl protochlorophyllide, an 8-vinyl reductase that is another key enzyme of the chlorophyll biosynthesis pathway. The DVR mutation could result in a pale green phenotype with increased chlorophyll *a*/*b* ratios (Nagata et al., 2005) and a disorderly arrangement of thylakoid membranes without distinct grana stacks (Nakanishi et al., 2005). In this study, the phenotype of *gdc1-3* was similar to the chlorophyll-deficient mutants, with an increased chlorophyll *a*/*b* ratio and no grana formation; however, *GDC1* is not a gene in the chlorophyll biosynthesis pathway.

Chlorophyll *b* is synthesized from chlorophyll *a* by chlorophyll *a* oxygenase in Arabidopsis (AtCAO). In the AtCAO null *ch1-3* allelic mutant, six major LHCII proteins do not accumulate (Espineda et al., 1999). In the *gdc1-3* mutant, both chlorophyll *b* and Lhcb1 were markedly decreased. However, Lhcb3 and Lhcb5

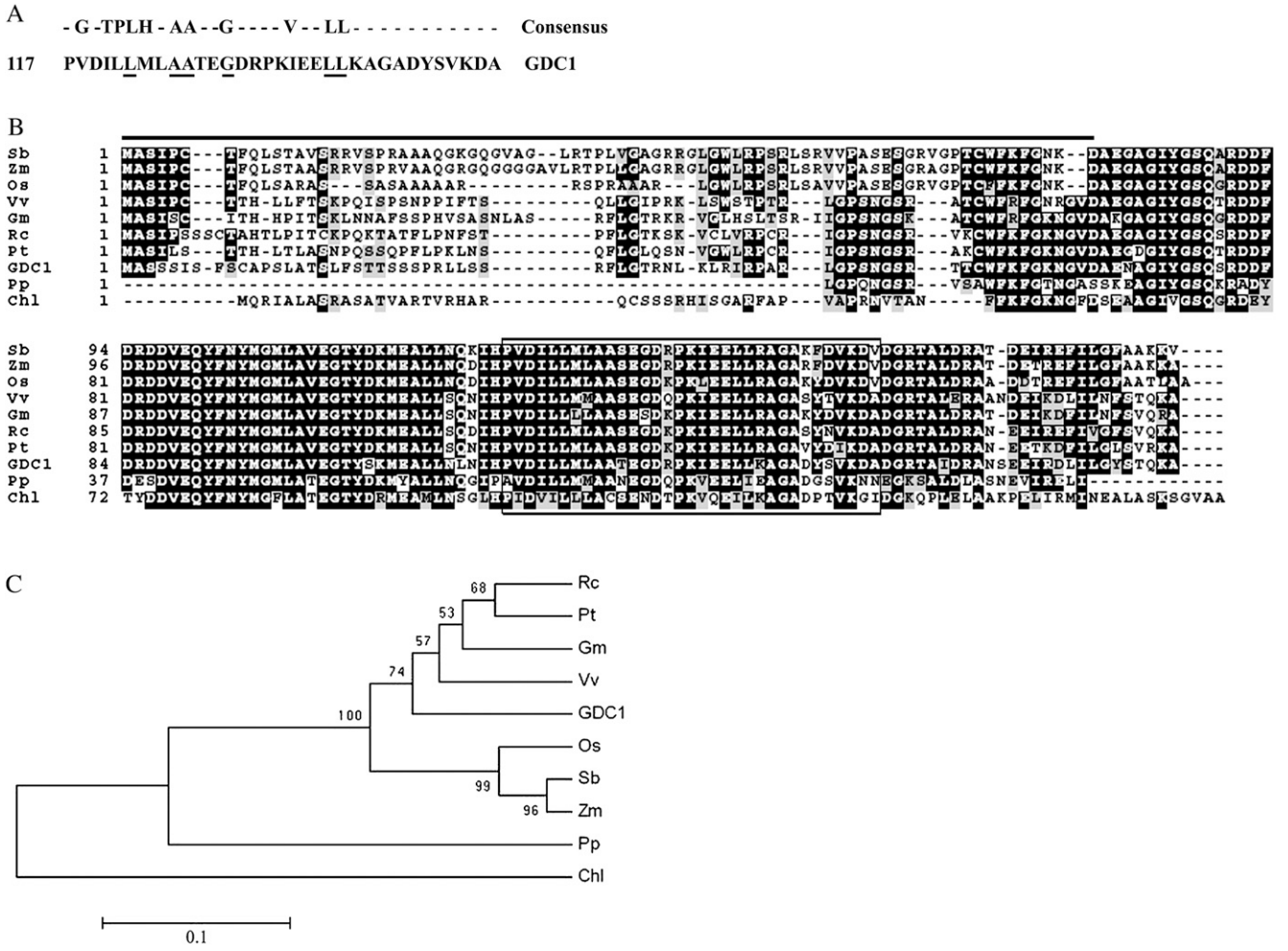

Figure 5. Phylogenetic analysis of GDC1 homologous proteins. A, Similarity to ankyrin consensus motifs (defined in Becerra et al., 2004) in Arabidopsis. B, Multiple alignments of GDC1 and homologous proteins. Black bars indicate putative transit peptide sequences, and boxes indicate the ankyrin domain. Protein sequence files are as follows: Rc, *Ricinus communis*, 29666.m001480; Pt, *Populus trichocarpa*, ABK94006; Vv, *Vitis vinifera*, CAN65357; Gm, *Glycine max*, ACU14277; Os, *Oryza sativa*, Os07g0520800; Sb, *Sorghum bicolor*, SORBIDRAFT_02g034910; Zm, *Zea mays*, LOC100277526; Pp, *Pyscomitrella patens*, EDQ65558; Chl, *Chlamydomonas reinhardtii*, EDP03071. C, Unrooted phylogenetic tree of GDC1 and homologous proteins. Amino acid sequences of GDC1 homologous proteins were analyzed using the neighbor-joining method with genetic distance calculated by MEGA3.1. The numbers at the nodes represent percentage bootstrap values based on 1,000 replications. The length of the branches is proportional to the expected numbers of amino acid substitutions per site, with a scale provided at the bottom of the tree.

proteins accumulated to the same levels as that of wild-type plants. Concurrently, AtCAO mRNA levels remained unchanged in the mutant. LHCP family members are the only proteins that bind chlorophyll *b* (Klimyuk et al., 1999). Therefore, chlorophyll *b* deficiency in *gdc1-3* was probably due to a reduced amount of Lhcb1 instead of the failure of chlorophyll *b* synthesis.

The Trimeric Form of LHCII Is Important for Grana Formation in Arabidopsis

LHCIIb, which consists of Lhcb1, Lhcb2, and Lhcb3, is the major LHCII of PSII. It constitutes approximately one-third of the total thylakoid proteins and binds half of the chlorophylls (Yamamoto and Bassi, 1996). Early evidence has shown that LHCIIb is involved in the stacking process (Allen and Forsberg, 2001). In vitro studies with isolated purified LHCII reconstituted into proteoliposomes demonstrated that LHCII is capable of mediating membrane adhesion (McDonnel and Staehelin, 1980). Reconstitution of isolated purified LHCII into native membranes lacking the complex serves to restore the ability to stack under physiological conditions (Day et al., 1984). In this work, the trimer was barely detected (Fig. 4) and grana formation was defective (Fig. 3) in *gdc1-3*. This is congruent with previous reports that the trimeric form is essential for grana formation.

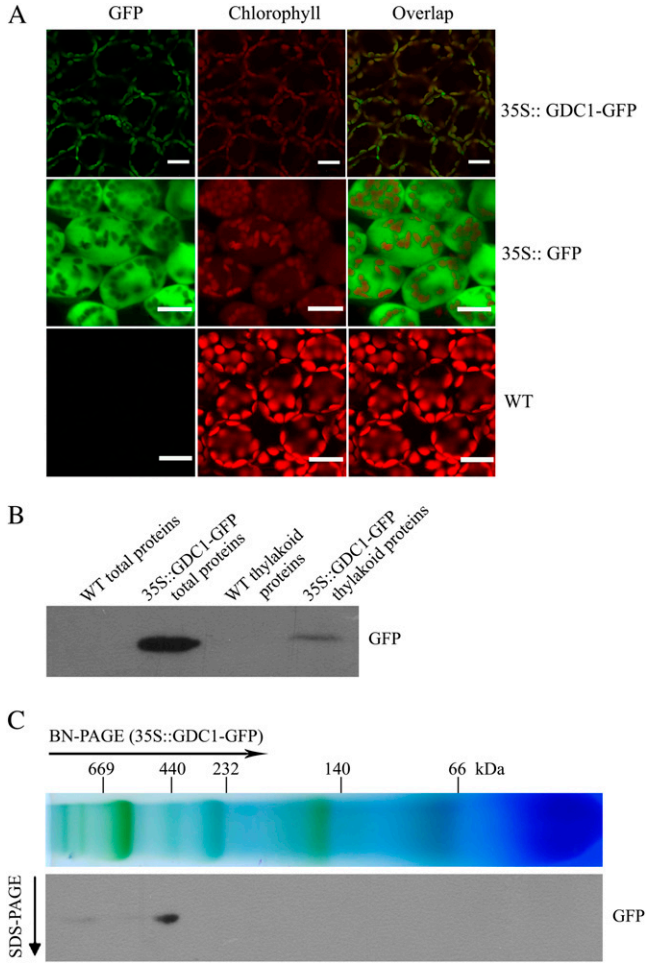

Figure 6. Localization of the GDC1 protein. A, Subcellular localization of the GDC1 protein by GFP assays. Fluorescence signals were visualized using confocal laser scanning microscopy. Green fluorescence indicates GFP, red fluorescence shows chlorophyll autofluorescence, and yellow-green fluorescence shows overlay images of the two types of fluorescence. Bars = 10 μm. WT, Wild type. B, Immunoblot analysis of GDC1. Samples from wild-type and 35S::GDC1-GFP transgenic plants consisting of total proteins (10 μg) and the thylakoid proteins (equivalent to 10 μg of chlorophyll) were separated by SDS-PAGE and immunodetected with the GFP antibody. C, Analysis of thylakoid protein complexes isolated from the 35S::GDC1-GFP transgenic plants. Complexes were separated by BN-PAGE and further subjected to two-dimensional SDS-PAGE. The proteins were immunodetected with GFP antibody.

The LHCII trimer constitutes Lhcb1, Lhcb2, and Lhcb3 proteins with a ratio of 8:3:1 (Jansson, 1994). Immunolocalization of GDC1-GFP indicated that the fusion protein was not associated with the LHCII trimer (Fig. 6C). This suggests that GDC1 is unlikely to be directly involved in the LHCII trimerization process. It was reported that LHCP proteins, including Lhcb1 and Lhcb2, are encoded in the nucleus and synthesized in the cytosol, imported into the chloroplast, and posttranslationally targeted to the thylakoid membrane by chloroplast signal recognition particle (cpSRP; Hutin et al., 2002; Schünemann, 2004; Stengel et al., 2008). However, the mechanism of LHCP insertion into the thylakoid membrane remains unclear. In this work, GDC1-GFP was associated with a thylakoid membrane protein complex of approximately 440 kD (Fig. 6C). This suggests that GDC1 might be involved in Lhcb1 and Lhcb2 integrating into the thylakoid membrane. As the other two LHCPs including Lhcb3 and CP26 were unchanged between *gdc1-3* and the

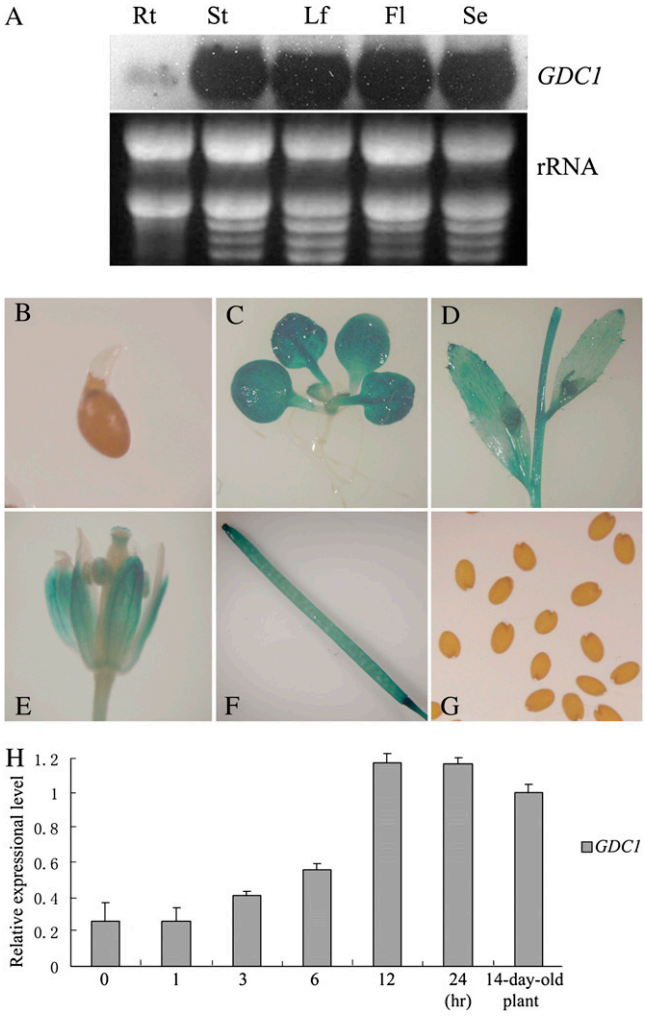

Figure 7. Expression analysis of the *GDC1* gene. A, RNA gel-blot analysis of *GDC1* gene expression in various organs. Transcript of *GDC1* was detected by probing the filter with *GDC1*-specific probe. Rt, Root; St, stem; Lf, leaf; Fl, inflorescence; Se, seedling. B, GUS activity was not observed in germinating seeds. C, GUS activity was observed in basal rosette leaves but not in roots. D, GUS activity was observed in stems and cauline leaves. E, GUS activity was observed in sepals, stamens, and styles but not in petals. F and G, GUS activity was observed in green siliques (F) but not in mature seeds (G). H, Expression of the *GDC1* gene after exposure to light by real-time RT-PCR analysis. Total RNA was isolated from 7-d-old dark-grown plants exposed to light for 0, 1, 3, 6, 12, or 24 h. Total RNA was also isolated from 14-d-old Columbia ecotype plants grown under a cycle of 16 h of light/8 h of dark at 22°C.

wild type (Fig. 4B), it is not clear if GDC1 is involved in all LHCPs integrating into the thylakoid membrane. The further identification of the 440 kD protein complex should provide clues that GDC1 functions in LHCII trimer formation.

Lhcb1 is the major component of the LHCII trimer. Lhcb1 is largely reduced in *gdc1-3*, and Lhcb2 is slightly reduced in *gdc1-3* (Fig. 4). Therefore, the low level of trimer formed in the mutant is due to the reduced Lhcb1 and Lhcb2 proteins. However, the trimeric form can also be assembled in the asLhcb2 plant with the absence of Lhcb1 and Lhcb2, as CP26 can take the place of Lhcb1 and Lhcb2 (Ruban et al., 2003, 2006). In *gdc1-3*, the amount of CP26 was similar to that in the wild type. This also suggests that GDC1 is associated with CP26 integrating into the thylakoid membrane, and the excessive accumulation of CP26 is important for its replacement of Lhcb1 and Lhcb2 to form the trimeric form.

The *GDC1* Gene Encodes an Ankyrin Domain-Containing Protein

GDC1 encodes a putative 19-kD polypeptide precursor. It possesses a transit peptide that functions in chloroplast targeting in the N terminus and an ankyrin domain in the C terminus. The ankyrin repeats are one of the most common protein sequence motifs, which are present in prokaryotes, eukaryotes, and some viruses (Sedgwick and Smerdon, 1999). The primary structure of the ankyrin repeats consists of 33 residues repeated in tandem that build a specific secondary and tertiary structure (Bork, 1993). Some ankyrin proteins contain more than two ankyrin repeats; however, GDC1 contains only a single ankyrin domain. Ankyrin repeats are thought to mediate protein-protein interactions among diverse groups of proteins, and their diverse functions are well illustrated in all groups of organisms (Breeden and Nasmyth, 1987; Artavanis-Tsakonas et al., 1991; Bennett, 1992). In Arabidopsis, a total of 105 genes encode ankyrin repeat-containing proteins. The number of ankyrin repeats in the same array rank between two and 10, with an average of 4.5 (Becerra et al., 2004). Twenty-six of 105 gene products have been localized in the chloroplast by TargetP analysis. One of the chloroplast-localized ankyrin proteins is cpSRP43. It contains four tandem ankyrin repeats and is a component of the cpSRP pathway, which functions in targeting the PSII light-harvesting proteins into thylakoid membranes (Klimyuk et al., 1999). However, currently, the function of proteins with a single ankyrin has not been reported. As GDC1 is essential for thylakoid stacking, this might also suggest that a single ankyrin domain protein is also involved in protein-protein interaction.

In this study, *GDC1* was widely expressed in green tissues, such as leaves and stems, and expressed at very low levels in the nongreen organs, including roots, petals, and mature and germinating seeds. GDC1 expression patterns suggested that it is involved in chloroplast biogenesis. The presence of grana is the most striking feature of land-plant chloroplasts, and grana stacking is greatly dependent on light conditions. Expression of *GDC1* was induced by photosignaling pathways (Fig. 4H), and grana were deficient in *GDC1* knockout mutants. These results suggested that *GDC1* is essential for grana formation. Phylogeny reconstruction indicated that the GDC1 protein is highly conserved in higher plants and green algae. Although unicellular green algae invariably have thylakoid membranes with both LHCII and GDC1 homologs, their thylakoid ultrastructure is often significantly different from that of land plants. GDC1 may be necessary for grana formation, but clearly it is not sufficient for the formation of characteristic land-plant grana. In nongranal photosynthetic organisms such as cyanobacteria, GDC1 homologous proteins were not detected. This indicates that GDC1 is conserved in LHCII-containing photosynthetic organisms, which suggests that the GDC1 protein was required in the evolution of grana.

MATERIALS AND METHODS

Plant Growth and Mutant Isolation

Arabidopsis (*Arabidopsis thaliana*) in the Columbia-0 background was used in the study. Seeds were planted on vermiculite and plant nutrition solution medium agar plates after imbibition for 3 d at 4°C. Plants were grown under long-day conditions (16 h of light/8 h of dark) in an approximately 22°C growth room. A *gdc1-1* mutant was characterized from pSKI15 activation tagging T-DNA mutant pools (Qin et al., 2003).

Phenotype Characterization and Microscopic Observations

Plants were photographed with a Nikon digital camera (Coolpix 4500). Transmission electron micrographs were obtained exactly as described by Motohashi et al. (2001). Small leaf segments from 3-week-old plants grown on soil were obtained. The specimens were examined with a Hitachi H7650 transmission electron microscope (http://www.hitachi.com).

TAIL-PCR and Molecular Cloning of the *GDC1* Gene

The presence of the T-DNA insertion in the mutant was validated using primers that specifically amplified the T-DNA BAR gene (Bar-F, 5′-GCACCATCGTCAACCACTAC-3′; Bar-R, 5′-TGCCAGAAACCCACGTCAT-3′). For TAIL-PCR, T-DNA left-border primers (AtLB1, 5′-ATACGACGGATCGTAATTTGTC-3′; AtLB2, 5′-TAATAACGCTGCGGACATCTAC-3′; AtLB3, 5′-TTGACCATCATACTCATTGCTG-3′) and genomic DNA isolated from mutant plants were used. The TAIL-PCR procedure and arbitrary degenerate primers were as described by Liu et al. (1995). Cosegregation of the T-DNA insertion site and the mutant phenotype were analyzed with AtLB3 and plant-specific primers (LP, 5′-ATGGCTTCTTCTTCAATC-3′; RP, 5′-AGCCTTTTGAGTCGAGTA-3′). For the mutant plants, only PCR using AtLB3 and RP primers could successfully amplify a DNA fragment of approximately 500 bp. For wild-type plants, only PCR using LP and RP primers could amplify a DNA fragment of 740 bp. PCR with both primer pairs showed positive results for heterozygous mutant plants.

Complementation was achieved as follows: a DNA fragment of 3,538 bp, including an 1,886-bp upstream sequence and a 909-bp downstream sequence, was amplified using LA-Taq polymerase (Takara; CMP-F, 5′-AGTCGACACCTTTGGCTCTGTTTAGTTGA-3′; CMP-R, 5′-GGAATTCATTGGTAAGGGCATAGCGTTGA-3′). Following sequence verification, the fragment was cloned into the pCAMBIA1300 binary vector (CAMBIA; www.cambia.org.au) and introduced into heterozygous plants using the infiltration method by *Agrobacterium tumefaciens* strain LBA4404. The transformants were selected on

plant nutrition solution culture medium with 80 mg L^{-1} hygromycin and screened for green phenotype plants with a homozygous background. The homozygous background could be verified because the LP/RP amplified sequences are included in the complementation fragment, and primer sets were used as follows: AtLB3/RP primers were used to validate the existence of a T-DNA insertion in *GDC1*; LP/RP primers were used to detect either the GDC1 genomic sequence or a transgenic complementation fragment; and genome-specific primers CI-F/CI-R (CI-F, 5′-ACAGAGACGACGTCGAACAGGT-3′; CI-R, 5′-GCACCGATCCACTAAGTAGACAGAC-3′) were used to validate the homozygous background. The CI-R primer was designed 59 bp downstream of the CMP-R primer; consequently, PCR with the CI-F/CI-R primer set was not able to amplify a 1,486-bp fragment in homozygous plants even if the complementation fragment was integrated into the genome.

SALK mutant identification verified the T-DNA insertion sites as described above by pROK2 vector-specific primer pROK2-LB3 (5′-GACCGCTTGCTGCAACTCT-3′) and genome-specific primers as follows: SALK_126967-LP (5′-CGTTTGTTTCTCCTCTTGGAG-3′), SALK_126967-RP (5′-CGACCAAGAAGTCAGAAGACG-3′), SALK_151530-LP (5′-ATGCAGACGAAAACGGATATG-3′), and SALK_151530-RP (5′-CTCATTCTCCTGTGCACCTTC-3′).

Pigment Content and Chlorophyll Fluorescence Analysis

Total chlorophyll was determined according to the method described by Lichtenthaler and Wellburn (1983). Extracts were obtained from 100 mg of fresh tissue from 3-week-old Arabidopsis basal rosette leaves and homogenized in 10 mL of 100% acetone. Spectrophotometric quantification was carried out in a Shimadzu UV-VIS-2450. Chlorophyll fluorescence measurements were performed using a pulse amplitude-modulated fluorometer (PAM 101; Walz; http://www.walz.com) equipped with a data-acquisition system to record fast changes (Meurer et al., 1996).

BN-PAGE, SDS-PAGE, and Immunoblot Analysis

The leaves from 3-week-old wild-type plants, *gdc1-3* mutants, and 35S::GDC1-GFP transgenic plants were homogenized in an ice-cold isolation buffer containing 400 mM Suc, 50 mM Tris-HCl, pH 7.6, and 10 mM NaCl with a chilled mortar and pestle and filtrated through two layers of cheesecloth. The filtrate was centrifuged at 5,000*g* for 10 min. The thylakoid pellets were washed with isolation buffer, recentrifuged, and suspended in isolation buffer. The resulting thylakoid membrane pellets were either used fresh or frozen in liquid N_2 and stored at −70°C before use. The chlorophyll content was determined spectrophotometrically according to Porra et al. (1989). Thylakoid membrane complexes were separated by BN-PAGE (Schägger et al., 1994; Cline and Mori, 2001; Zhou et al., 2009). Albumin bovine monomer (66 kD), lactate dehydrogenase (140 kD), catalase (232 kD), ferritin (440 kD), and porcine thyroid (669 kD) from Amersham Life Sciences were loaded alongside as molecular marker proteins. Thylakoid membrane complexes of 35S::GDC1-GFP transgenic plants were directly denatured for SDS-PAGE and immunolocalization analysis.

For two-dimensional analysis, excised BN-PAGE lanes were soaked in SDS sample buffer and 5% β-mercaptoethanol for 15 min and layered onto 15% SDS-polyacrylamide gels. Total proteins for immunological detection were extracted from 3-week-old wild-type plants and *gdc1-3* mutants as described previously (Motohashi et al., 2001). Proteins were separated by 15% SDS-PAGE. After electrophoresis, the total proteins and the thylakoid membrane proteins were transferred electrophoretically to BioTrace polyvinylidene difluoride transfer membranes (Pall Corp.; http://www.pall.com) and immunoblotted with various thylakoid membrane protein antibodies (Agrisera; http://www.agrisera.com) and GFP antibody (Beijing CoWin; www.cwbiotech.com). Antibodies were detected using an enhanced chemiluminescence detection system (ECL; Amersham Life Sciences; http://gehealthcare.com/lifesciences) following the manufacturer's instructions.

Subcellular Localization of GDC1-Fused GFP Protein

For GFP fusion, the full-length coding sequence without the TAG stop codon was cloned from the seedling cDNA of the wild type with the following primers: GDC1-Subcellular-F (5′-GGAGATCTATGGCTTCTTCTTCAATCTC-3′) and GDC1-Subcellular-R (5′-AAGGTACCGGAGCCTTTTGAGTCGAGTA-3′). The coding sequence was fused with eGFP by subcloning and cloned into the pMON530 binary vector. Transformation was performed as described above, and transformants were selected using 50 mg L^{-1} kanamycin. The GFP fluorescence of transgenic plants was observed using a Zeiss confocal laser scanning microscope (LSM 5 PASCAL; http://www.zeiss.com).

Phylogenetic Analysis

The multiple sequence alignment of full-length protein sequences was performed using the ClustalX2 tool and displayed using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed and tested by MEGA3.1 (http://www.megasoftware.net) based on the neighbor-joining method.

RNA Isolation, cDNA Synthesis, and RT-PCR Analysis

Total RNA from both wild-type and *gdc1* plants, which were grown at a constant temperature of 22°C under a 16-h-light/8-h-dark cycle, was isolated using the TRIzol Reagent (Invitrogen; http://www.invitrogen.com) and DNase I treated by an RNeasy kit (Qiagen; http://www.qiagen.com) following the manufacturer's instructions. The first-strand cDNA was synthesized with the Revert-Aid first-strand cDNA synthesis kit (Toyobo; http://www.toyobo.co.jp) following the manufacturer's instructions. RT-PCR was applied to assess the *GDC1* expression levels using the following primer set: GDC1-F (5′-ATGGCTTCTTCTTCAATC-3′) and GDC1-R (5′-TCAAGCCTTTTGAGTCGA-3′). The transcriptional expression of Lhcb1, Lhcb2, Lhcb3, CP26, and AtCAO genes in the wild type and the *gdc1-3* mutant was performed by RT-PCR with the following primer sets: Lhcb1F (5′-TAGAAGTTATCCACAGCA-3′) and Lhcb1R (5′-CGAAGAATCCAAACATAG-3′); Lhcb2F (5′-CGGACCAGACCGTCCCAA-3′) and Lhcb2R (5′-ATGCTTTGCGCGTGGATC-3′); Lhcb3F (5′-ATGGCATCAACATTCACG-3′) and Lhcb3R (5′-TATGCAACCAAAAGCTCC-3′); CP26F (5′-ATGGCGTCTTTGGGTGTG-3′) and CP26R (5′-ACCAAATGGGTCATAACC-3′); and AtCAOF (5′-CGTGAAAGGAGAATTTAG-3′) and AtCAOR (5′-CATGCTTGAGATCTGCAG-3′).

Quantitative Real-Time PCR Analysis

For expression analysis of *GDC1* in mutants and wild-type plants, total RNA was obtained from 3-week-old wild-type and *gdc1* allelic mutant plants. For light induction analysis, total RNA was isolated from 7-d-old dark-grown plants exposed to light for 0, 1, 3, 6, 12, or 24 h and from 14-d-old Columbia ecotype plants grown under a cycle of 16 h of light/8 h of dark at 22°C. Quantitative real-time PCR amplifications were carried out in an ABI 7300 Real-Time PCR System (Applied Biosystems; http://www.appliedbiosystems.com) using the following primer set, GDC1realF (5′-CACCCAGTTGATATCTTG-3′) and GDC1realR (5′-AGCCTTTTGAGTCGAGTA-3′), and the relative quantification of gene expression data was analyzed as described by Hricová et al. (2006). The data set was normalized using β-tubulin as a control.

RNA Gel-Blot Hybridization

Northern-blot analysis of GDC1 expression was performed for specific organ types; therefore, RNA was extracted from roots, basal rosette leaves, seedlings, and inflorescences using TRIzol (Invitrogen) following the manufacturer's protocol. Twenty micrograms of total RNA from wild-type and *gdc1-3* mutant plants was size fractionated by 1.0% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes, and probed with digoxigenin-labeled cDNA probe. Probe was generated by RT-PCR amplification from the specific GDC1-F/R primer set. Chemiluminescence detection was carried out as described in the Roche digoxigenin manual.

Assay of GUS Activity

The GDC1 promoter:GUS gene was constructed by PCR amplification of a fragment 1,886 bp upstream from the translation start point of GDC1 using the primers GDC1PF (5′-GGAGATCTACCTTTGGCTCTGTTTAG-3′) and GDC1PR (5′-AAGGATCCGGCTGTTCTCTTAATGCT-3′). The fragment was cloned into the modified pBI121 vector, in which the region containing the 35S promoter between *Hin*dIII and *Xba*I was replaced with the fragment containing *Hin*dIII, *Kpn*I, *Bgl*II, *Xba*I. Transformation was performed as described above, and transformants were selected using 50 mg L^{-1} kanamycin. GUS activity was assayed as described by Caissard et al. (1994). Tissues were examined using an Olympus SZ-CTV dissecting microscope interfaced with an Olympus DP70 digital camera (http://www.olympus.com.cn) and ACT-1 image-capture software.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At1g50900.

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