Running title:

The *GDC1* is required for grana formation in Arabidopsis

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The GDC1 Gene Encodes a Novel Ankyrin Domain Containing Protein That is Essential for Grana Formation in Arabidopsis thaliana **

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ABSTRACT

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 Chloroplast 1* (*GDC1*), required for chloroplast grana formation in *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 BN-PAGE 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 (ANK) domain within the C terminal. It was highly expressed in *A. thaliana* green tissues, and its expression was induced by photo signaling pathways. Immunoblotting analysis of GDC1-GFP fusion protein in 35S::GDC1-GFP transgenic plant with GFP antibody indicates that GDC1 is associated with ~440 kDa thylakoid protein complex instead of the LHCII trimer. This shows that GDC1 may play an indirect role in LHCII trimerization during grana formation.
INTRODUCTION

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 and 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 300-600 nm in diameter, containing ~10-20 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 non-appressed stromal lamellae are accompanied by functional differentiation (Kim et al., 2005). PSII and its associated integral membrane chlorophyll a/b binding light-harvesting complex (LHCII) resides 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/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 grana play critical roles in the optimization of photosynthetic functions (Albertsson, 2001; Goss et al., 2007). The tightly appressed arrangement of granal thylakoid membranes enhances light capture through a vastly increased area-to-volume ratio and connectivity of several PSIIIs 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 the photosystems by state1- state2 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 form grana is the last step in the structural changes of photosynthetic membranes (Argyroudi-Akoyunoglou et al., 1976; Paolillo, 1970; Wellburn, 1971). 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 (1961) 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 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 \textit{Lhcb1}, \textit{Lhcb2} and \textit{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 \textit{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. Presently, there remains uncertainty surrounding the roles of LHCII in grana formation.
Several studies have clarified grana stack formation and 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-Deficicent Chloroplast 1 (GDC1)*, essential for grana formation in *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 *grana-deficient chloroplast 1-1* (*gdc1-1*) from a collection of T-DNA insertion lines (Qin et al., 2003). The *gdc1-1* mutants can survive for approximately 5~6 weeks with a pale green phenotype when germinated on soil (Fig. 1, A and B). 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 the *gdc1-1* is a nuclear recessive mutant, 353 progenies obtained from self-pollinated heterozygotes (*GDC1/gdc1*) were segregated at a green plants to pale-green plants phenotypic ratio of 268 : 85 ($\chi^2 (3 : 1) =0.16; P>0.50$). This ratio was expected for a character inherited as a single recessive Mendelian trait. Co-segregation of resistance to Basta (conferred by T-DNA) and the pale green phenotype indicated that the pale coloration of the *gdc1-1* mutant co-segregated with the T-DNA insertion (data not shown).

Cloning of the *GDC1* Gene

To identify the corresponding *GDC1* gene, thermal asymmetric interlaced PCR (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 (ORF) (AT1G50900) (Fig. 2A). PCR analysis using T-DNA and genomic-specific primers indicated that all mutant plants analyzed were homozygous for the insertion (data not shown), which indicated AT1G50900 was responsible for the *gdc1-1* pale green phenotype. Genetic complementation was subsequently performed to confirm the results. A 3538bp DNA fragment, which included the genomic sequences of AT1G50900 and 1886bp 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 (TEM) analysis showed the ultrastructure of the complemented gdc1-l chloroplast resembled that of wild type (Fig. 3G). These results verified that AT1G50900 was GDC1 and the 3538bp genomic region was sufficient for GDC1 function.

In addition, we obtained another two gdc1-l alleles from the SIGnAL collection in ABRC, i.e. gdc1-2 (SALK_151530) and gdc1-3 (SALK_126967). PCR analysis confirmed the T-DNAs were inserted into the only intron and last exon of the AT1G50900 locus in the gdc1-2 and gdc1-3 alleles, respectively. Both gdc1-2 and gdc1-3 showed similar phenotypes with that of gdc1-1 (Fig. 1A). AT1G50900 expression was examined in the allelic mutant plants using RT-PCR and real time RT-PCR analysis with gene specific primers. Gene expression levels in gdc1-l 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 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 (TEM). 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-l 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 the mutant and wild type. The total chlorophyll content of the 3-week-old wild-type was 1302.57±60.56 μg g⁻¹ fresh weight, while the total chlorophyll content in \textit{gdc1-3} was reduced by approximately 82% with only 245.32±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 \textit{gdc1-3} mutant (Table 1). 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 \textit{gdc1-3} mutant was analyzed by measuring the ratio of variable fluorescence to maximum fluorescence as follows: \( Fv/Fm = (Fm - Fo)/Fm \) value (\( Fo \) and \( Fm \) are minimum and maximum chlorophyll \( a \) fluorescence of dark-adapted leaves, respectively). \( Fv/Fm \) reflects the maximum potential capacity of the PSII photochemical reactions (Krause and Weis, 1991). The \( Fv/Fm \) value of 3-week-old leaves was 0.81±0.01 in wild-type plants and the \textit{gdc1-3} value was 0.79±0.01. Despite the fact that the \( Fv/Fm \) value was very close for the wild-type and the mutant, the \( Fo \) and \( Fm \) in mutant were dramatically lower than in wild-type. This demonstrates that the photochemical efficiency of PSII was not affected by the mutation in \textit{gdc1-3}.

\textit{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-\( \beta \)-D-maltopyranoside (DM) and separated by blue native PAGE (BN-PAGE) (Schägger et al., 1994). After the first-dimensional separation in the presence of Coomassie blue G-250, the major bands representing PSII supercomplexes, monomeric PSI and dimeric PSII, monomeric PSII, dimeric cytb\(_6\)/f, trimeric LHCII and monomeric LHCII were resolved in the wild-type (Fig. 4A). Most of the bands were also detected in the \textit{gdc1-3} mutant. However, the trimeric LHCII was negligible, and the band I of wild type disappeared in the
In the grana of higher plants, the LHCII trimer is comprised 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 \textit{gdc1-3}. Lhcb3 and CP26 accumulated at the same level between the mutant and the wild type (Fig. 4B). We further investigated the LHCIIb and CP26 gene expressions on transcriptional level by RT-PCR. The results demonstrated no differences on transcription level between the \textit{gdc1-3} mutant and the wild type (Fig. 4C). Therefore, the accumulation of Lhcb1 and Lhcb2 were affected by the GDC1 mutation. We also characterized the subunits of PSII, PSI, cytb6f and ATP synthase complexes in the mutant. In \textit{gdc1-3} mutant, the D1 and D2 core subunits of PSII accumulated to low levels, while PsaA subunit of PSI, Cyt f subunit of cytb6f and beta subunit of ATP synthase accumulated to the similar levels as that of wild type (Fig. 4B). The results suggested PSII was severely impaired by the GDC1 mutation and grana deficiency.

\textbf{The GDC1 Gene Encodes a Novel Ankyrin Protein That Localizes to the Chloroplast}

To verify the \textit{GDC1} gene primary structure, its full genomic DNA and cDNA were cloned and sequenced. The \textit{GDC1} gene genomic structure was 1008bp in length and was comprised of two exons and one intron, which is consistent with the TAIR gene predictions. The cDNA we cloned from seedlings is identical to the TAIR database.

The \textit{GDC1} mRNA encodes an unknown protein of 175 amino acids with a putative molecular weight of 19 kD. Domain analysis indicated that the GDC1 protein is an ankyrin domain containing protein (aa117-149). This domain (Fig. 5A) exhibits conservation of the consensus motif in \textit{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 homologues of the GDC1 protein were also present in various plant species, including castor, poplar, grape, soybean, rice, sorghum, maize, moss
and green algae. For example, the homologue from poplar, ABK94006, shared the highest identity (76%) and similarity (85%) with GDC1; and the homologue 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). The results indicated that GDC1 was conserved in the evolutionary process, and its putative homologues 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 CDS of GDC1 with the green fluorescence protein (GFP) gene and introduced into wild type plants under control of the CaMV 35S promoter. Stable transgenic plants were obtained and GFP fluorescence of transgenic plants was observed with confocal laser microscopy. GFP fluorescence was co-localized 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 GDC1-GFP fusion protein in 35S::GDC1-GFP transgenic plant with GFP antibody. A positive signal was detected in the total proteins and thylakoid membrane proteins from 35S::GDC1-GFP transgenic plant (Fig. 6B). To understand which complex GDC1-GFP might be associated with, the thylakoid membrane protein complexes of 35S::GDC1-GFP transgenic plant were separated by BN-PAGE, denatured in the gel, and directly used for two-dimensional SDS-PAGE. Immunoblotting analysis indicated that the GDC1-GFP fusion protein was associated with a complex of ~440 kDa of thylakoid membrane (Fig. 6C). There are two bands with about 440 kDa 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 ~440 kDa 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 β-glucuronidase (GUS) reporter gene fused to its promoter (Fig. 7, B-G). The observed patterns suggested GDC1 was not expressed in germinating seeds (Fig. 7B). In 15-day-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 and siliques, but not in mature seeds (Fig. 7, E and G). In the flower, GUS activity was detected in the sepals, the 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-day-old dark-grown wild-type plants to light conditions. GDC1 was weakly expressed in the dark (0h), 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 photo signaling pathways.
DISCUSSION

In the present study, we isolated a pale green mutant *Arabidopsis*, and cloned the *GDC1* gene (At1g50900) that encodes a protein with an ankyrin domain. TEM 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.

**GDC 1 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, 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 the present 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 *A. thaliana* (Nagata et al., 2005). In the allele mutants with mutations occurring in the CHLI1, which is a subunit of magnesium (Mg) chelatase, chlorophyll can accumulate at lower levels than 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.
encodes 3,8-Divinyl protochlorophyllide, an 8-Vinyl reductase 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 to 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 proteins accumulated at the same levels as that of wild-type plant. 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.

The LHCII trimer constitutes Lhcb1, Lhcb2 and Lhcb3 proteins with a ratio of 8:3:1 (Jansson.
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 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 cpSRP (Hutin et al., 2002; Schünemann, 2004; Stengel et al., 2008). However, the mechanism of LHCP insertion into thylakoid membrane remains unclear. In this work, GDC1-GFP was associated with a thylakoid membrane protein complex of ~ 440 kDa (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 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 kDa 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 was slightly reduced in gdc1-3 (Fig. 4). Therefore the low levels 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 (ANK 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 ANK repeats consists of 33 residues repeated in tandem that build a specific secondary and tertiary structure (Bork, 1993). Some ANK proteins
contain more than two ankyrin repeats, however GDC1 contains only a single ANK domain. ANK 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-Tsalandis et al., 1991; Bennett, 1992). In *Arabidopsis*, a total of 105 genes encode ANK repeat containing proteins. The number of ANK 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 ANK proteins is cpSRP43. It contains four tandem ankyrin repeats and is a component of the chloroplast signal recognition particle (cpSRP) pathway, which functions in targeting the PSII light-harvesting proteins LHCP into thylakoid membranes (Klimyuk et al., 1999). However, currently the function of proteins with a single ANK has not been reported. As GDC1 is essential for thylakoid stacking, this might also suggest that a single ANK domain protein is also involved in protein-protein interaction.

In the present study, *GDC1* was widely expressed in green tissues, such as leaves and stems, and expressed at very low levels in the non-green organs, including roots, petals and mature and germinating seeds. GDC1 expression patterns suggested 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 photo signaling pathways (Fig. 4H), and grana were deficient in *GDC1* knocked-out 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 membrane with both LHCII and GDC1 homologue, 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 non-granal photosynthetic organisms such as cyanobacteria, GDC1 homologous proteins were not detected. This indicates that GDC1 is conserved in LHCII-containing photosynthetic organisms, which suggests the GDC1 protein was required in the evolution of grana.
MATERIALS AND METHODS

Plant Growth and Mutant Isolation

*Arabidopsis thaliana* in the Columbia-0 background was used in the study. Seeds were planted on vermiculite and PNS medium agar plates after imbibition for 3-days at 4°C. Plants were grown under long-day conditions (16 hours light/8 hours dark) in a ~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 (Nikon, Japan). Transmission electron micrographs (TEMs) 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 (Hitachi, 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’- GCACCATCGTCAACCACACTAC-3’; Bar-R: 5’-TGCCAGAAACCCACGTCAT-3’). For Tail-PCR, T-DNA LB primers (AtLB1: 5’-ATACGACGGATCGTAATTTGTC-3’; AtLB2: 5’-TAATAACGCTGCGGACATCTAC-3’; AtLB3: 5’-TTGACCATCATCATACTCATTGCTG-3’) and genomic DNA isolated from mutant plants was used. The Tail-PCR procedure and AD 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’-ATGGGCTTCTTCTTCAATC-3’, RP: 5’-AGCCTTTTGAGTCGAGTA-3’). For the mutant plants, only PCR using AtLB3 and RP primers could successfully amplify a DNA fragment of approximately 500bp. For wild type plants, only PCR using LP and RP primers could amplify a DNA fragment of 740bp. PCR with both primer pairs showed positive results for heterozygous mutant plants.
Complementation was achieved as follows: a DNA fragment of 3538bp, including an 1886bp upstream and 909bp downstream sequence was amplified using LA-Taq polymerase (TAKARA, Japan) (CMP-F: 5’-AGTCGACACCTTTGGCTCTGTTAGTTGA-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 strain LBA4404. The transformants were selected on PNS culture medium with 80 mg/L 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 genomic 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 59bp downstream of the CMP-R primer, consequently PCR with the CI-F/CI-R primer set was not able to amplify a 1486bp 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 genomic specific primers as follows: SALK_126967-LP: 5’-CGTTTGTTTCTCCTCTTGAG-3’; SALK_126967-RP: 5’-CGACCAAGAAGTCGACAGACG-3’; SALK_151530-LP: 5’-ATGCAGACGAAACCGGATATG-3’; 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 100mg 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 (Japan). 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 400mM sucrose, 50 mM Tris-HCl, pH 7.6, and 10mM NaCl with a chilled mortar and pestle and filtrated through two layers of cheesecloth. The filtrate was centrifuged at 5000 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 N2 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 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa), and porcine thyroid (669 kDa) from Amersham Lifesciences 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™ PVDF transfer membrane (Pall corporation, 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 Lifesciences, http://gehealthcare.com/lifesciences) following the manufacturer’s instructions.

**Subcellular Localization of GDC1 fused GFP protein**

For GFP fusion, the full-length CDS 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’; GDC1-Subcellular-R: 5’-AAGGTACCGGAGCCTTTTGAGTCGAGTA-3’. The CDS 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 under a ZEISS Confocal Laser Scanning Microscope (LSM 5 PASCAL; ZEISS, 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 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 RNase 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 sets: 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 gdc1-3 mutant were performed by RT-PCR with the following primer sets: Lhcb1F: 5’-TAGAAGTTATCCACAGCA-3’, Lhcb1R: 5’-CGAAGAATCCAAAACATAG-3’; Lhcb2F: 5’-CGGACCAGACCGTCCCAA-3’, Lhcb2R: 5’-ATGCTTTGCCTGCTGGATC-3’; Lhcb3F: 5’-ATGGCATCAACATCAG-3’, Lhcb3R: 5’-TATGCAACAAAGCTCC-3’; CP26F: 5’-ATGGCGTCTTTGGGTGTA-3’, CP26R: 5’-ACCAAATGGGTCAATAAAC-3’; AtCAOF: 5’-CGTGAAGGAGAATTTAG-3’, AtCAOR: 5’-CATGCTTGAATCTGCAG-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 mutants. For light induction analysis, total RNA was isolated from 7-day-old dark-grown plants exposed to light for 0, 1, 3, 6, 12, or 24h, and from 14-day-old Columbia ecotype plants grown under a cycle of 16-h light/8-h 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 following primer sets: GDC1realF (5’-CACCCAGTTGGATCTCGG-3’) and GDC1realR (5’-AGCCTTTTGAGTCGAAGTA-3’), and the relative quantification of gene expression data was analyzed as described in 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 were size-fractionated by 1.0% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes, and probed with digoxigenin-labelled cDNA probe. Probe was generated by RT-PCR amplification from with the specific GDC1-F/R primer set. Chemiluminescent detection was carried out as described in the Roche DIG manual.
Assay of GUS Activity

The GDC1 promoter:GUS gene was constructed by PCR amplification of a fragment 1886bp upstream from the translation start point of GDC1 using the primers GDC1PF(5’-GGAGATCTACCTTTGGCTCTGTTTAG-3’) and GDC1PR (5’-AAGGATCCGGCTTCTCTAATGCT-3’). The fragment was cloned into the modified pBI121 vector, in which the region containing 35S promotor between the HindIII and XhaI was replaced with the fragment containing HindIII, KpnI, BglII, XbaI. 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.
ACKNOWLEDGEMENTS

We would like to thank ABRC Bioresources, who kindly offered the transgenic Arabidopsis lines (SALK_126967 and SALK_151530). We are grateful to anonymous reviewers for their suggestions to improve this manuscript, and Mrs Hui-Qi Zhang from SHNU for her skillful technical assistance in transmission electron microscopy.
LITERATURE CITED


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Figure Legends

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

Figure 2. Characterization of the GDC1 gene. A, Gene structure of the GDC1 and T-DNA insertion. Black boxes, exons; white boxes, 5’-UTR and 3’-UTR; gray lines, intron; light gray lines, upstream region of transcription initiation site and downstream region of 3’-UTR. 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 gene were analyzed on a 1% (w/v) agarose gel. C, Real-time RT-PCR analysis of GDC1 expressional levels in gdc1-1, gdc1-2, gdc1-3 mutants.

Figure 3. Electron micrographs of gdc1-3 and its allelic mutant chloroplasts. A, Chloroplast of the wild-type Columbia-0. B, Close-up 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, Close-up 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. Scale bars: 500nm.

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 chlorophyll) from the wild-type and gdc1-3 mutant leaves were solubilized
with 2% dodecyl-β-D-maltopyranoside (DM) and separated by BN gel electrophoresis. B, Immunodetection of thylakoid proteins. The total proteins (40 μg) were separated by SDS-urea-PAGE, and blots probed with specific antibodies for Lhcb1, Lhcb2, Lhcb3, CP26, D1, D2, PsaA, Cyt f and AtpB. C, RT-PCR analysis of Lhcb1, Lhcb2, Lhcb3 and CP26 gene expressions at the transcriptional level.

**Figure 5.** Phylogenetic analysis of GDC1 homologous proteins. A, Similarity to ankyrin consensus motifs (defined by Becerra et al., 2004) in *Arabidopsis*. B, Multiple alignments of GDC1 and homologous proteins. Black bars, putative transit peptide sequences. Boxes, ankyrin domain. Protein sequence files: 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 Mega 3.1. The numbers at the nodes represent percent bootstrap values based on 1000 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.

**Figure 6.** Localization of 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. Scale bars: 10μm. B, Immunoblot analysis of GDC1. Samples from wild-type and 35S::GDC1-GFP transgenic plants consisting of total proteins (10 μg) , the thylakoid proteins (equivalent to 10 μg 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 2D SDS-PAGE. The
proteins were immunodetected with GFP antibody.

**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; Inf, inflorescence; Sd, seedling. B, β-Glucuronidase (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, GUS activity was observed in green siliques, 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-day-old dark-grown plants exposed to light for 0, 1, 3, 6, 12, or 24h. Total RNA was also isolated from 14-day-old Columbia ecotype plants grown under a cycle of 16-h light/8-h dark at 22°C.
### Table 1. Chlorophyll content of leaves (μg g⁻¹ fresh weight)

<table>
<thead>
<tr>
<th></th>
<th>Wild type</th>
<th>gdc1-3</th>
</tr>
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<tbody>
<tr>
<td>Total chlorophyll</td>
<td>1302.57±60.56</td>
<td>245.32±1.49</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>1008.95±44.90</td>
<td>228.75±1.48</td>
</tr>
<tr>
<td>Chlorophyll b</td>
<td>293.62±15.66</td>
<td>16.57±0.01</td>
</tr>
<tr>
<td>Chlorophyll a/b</td>
<td>3.44±0.04</td>
<td>13.81±0.09</td>
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**Table Legend**

Table 1. Total chlorophylls were obtained from 100mg of fresh tissue from 3-week-old *Arabidopsis* basal rosette leaves using 100% acetone. Spectrophotometric quantification was carried out, then calculated the ratio of chlorophyll a to b.
A

Complementation fragment (3538bp)

WT  gdc1-1  gdc1-2  gdc1-3

GDC1

TUB

B

C

Relative expression level

WT  gdc1-1  gdc1-2  gdc1-3

GDC1

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