

# Galactolipids Are Essential for Internal Membrane Transformation during Etioplast-to-Chloroplast Differentiation

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Etioplasts developed in angiosperm cotyledon cells in darkness rapidly differentiate into chloroplasts with illumination. This process involves dynamic transformation of internal membrane structures from the prolamellar bodies (PLBs) and prothylakoids (PTs) in etioplasts to thylakoid membranes in chloroplasts. Although two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are predominant lipid constituents of membranes in both etioplasts and chloroplasts, their roles in the structural and functional transformation of internal membranes during etioplast-to-chloroplast differentiation are unknown. We previously reported that a 36% loss of MGDG by an artificial microRNA targeting major MGDG synthase (*amiR-MGD1*) only slightly affected PLB structures but strongly impaired PT formation and protochlorophyllide biosynthesis. Meanwhile, strong DGDG deficiency in a DGDG synthase mutant (*dgd1*) disordered the PLB lattice structure in addition to impaired PT development and protochlorophyllide biosynthesis. In this study, thylakoid biogenesis after PLB disassembly with illumination was strongly perturbed by *amiR-MGD1*. The *amiR-MGD1* expression impaired the accumulation of Chl and the major light-harvesting complex II protein (LHCB1), which may inhibit rapid transformation from disassembled PLBs to the thylakoid membrane. As did *amiR-MGD1* expression, *dgd1* mutation impaired the accumulation of Chl and LHCB1 during etioplast-to-chloroplast differentiation. Furthermore, unlike in *amiR-MGD1* seedlings, in *dgd1* seedlings, disassembly of PLBs after illumination was retarded. Because DGDG but not MGDG prefers to form the bilayer lipid phase in membranes, the MGDG-to-DGDG ratio may strongly affect the transformation of PLBs to the thylakoid membrane during etioplast-to-chloroplast differentiation.

**Keywords:** Chloroplast • Digalactosyldiacylglycerol • Etioplast • Monogalactosyldiacylglycerol • Prolamellar body • Thylakoid membrane.

## Introduction

Biogenesis of the thylakoid membrane, the site of photochemical reactions, in chloroplasts requires coordinated assembly of proteins, cofactors and lipids. Glycerolipids function as building blocks of the thylakoid membrane and provide the lipid bilayer matrix for protein–pigment complexes and hydrophobic cofactors. In the thylakoid membrane, two uncharged galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), account for approximately 50% and 30%, respectively, of the total membrane lipids (Dorne et al. 1990). Despite only one galactose difference in their polar head groups, MGDG and DGDG show different phase behaviors in aqueous media. DGDG can self-assemble into lamellar bilayers in mixtures with water, whereas MGDG is a nonbilayer-forming lipid and forms an inverse hexagonal phase in aqueous media (Shipley et al. 1973). The ratio of bilayer-forming DGDG and nonbilayer-forming MGDG largely affects the phase behavior of lipid bilayers and may be involved in the organization of thylakoid membrane structures (Demé et al. 2014).

In plant cells, MGDG is synthesized from diacylglycerol (DAG) and UDP-galactose by MGDG synthase (Benning and Ohta 2005). A portion of MGDG is further galactosylated with UDP-galactose by DGDG synthase to yield DGDG. The *Arabidopsis thaliana* genome has three MGDG synthase genes (*MGD1*, *MGD2* and *MGD3*) and two DGDG synthase genes (*DGD1* and *DGD2*). *MGD1* in the inner envelope and *DGD1* in the outer envelope of plastids synthesize the bulk of MGDG and DGDG, respectively, in photosynthetic tissues. The alternative pathway by *MGD2*–*MGD3* and *DGD2*, which are all localized to the outer envelope of plastids, particularly contributes to DGDG accumulation under phosphate starvation (Kobayashi et al. 2009b). In addition, SENSITIVE TO FREEZING 2, which transfers a galactose residue from MGDG to a second galactolipid to yield oligogalactolipids, is involved in

galactolipid biosynthesis specifically under freezing conditions (Moellering et al. 2010).

DAGs used for galactolipid biosynthesis are derived from two different pathways (Browse and Somerville 1991, Ohlrogge and Browse 1995). The plastid pathway produces DAGs entirely within plastids, whereas the endoplasmic reticulum (ER) pathway first produces phospholipids in ER, then DAGs metabolized from the phospholipids are used in plastids. In both pathways, fatty acids (FAs) are synthesized in conjugation with acyl carrier protein (ACP) within plastids. In the plastid pathway, 16:0-ACP and 18:1-ACP, the latter made from 18:0-ACP by stearoyl-ACP desaturase (SAD) in plastids, are directly used for glycerolipid biosynthesis. Meanwhile, a portion of these acyl-ACPs is hydrolyzed by plastid thioesterases to free FAs, exported from plastids, then used for the ER pathway after conjugation with CoA (Li-Beisson et al. 2013). DAGs from the plastid pathway contain C-18 FAs at the *sn*-1 position and C-16 FAs at the *sn*-2 position of the glycerol backbone. In contrast, DAGs from the ER pathway contain C-18 or C-16 FAs at the *sn*-1 position and C-18 FAs at the *sn*-2 position. C-18 FAs of galactolipids are mostly unsaturated to linolenic acids (18:3) in the plastid or ER pathway. Specifically in MGDG from the plastid pathway, C-16 FAs at the *sn*-2 position are unsaturated to 16:3. Molecular lipid species analyses indicate that in *Arabidopsis*, half of MGDG in chloroplasts is synthesized via the plastid pathway, whereas much of DGDG is derived from the ER pathway (Browse and Somerville 1991, Ohlrogge and Browse 1995).

The thylakoid membrane is characterized by its high protein density: 70–80% of the membrane area is occupied by proteins (Kirchhoff et al. 2002). Thus, integration of protein complexes into the lipid bilayer matrix is a crucial process for thylakoid membrane biogenesis. The light-harvesting complex proteins (LHCPs), in particular the major light-harvesting complex II (LHCII), are highly abundant in the thylakoid membrane and concentrated in the grana region (Pribil et al. 2014). In vitro analysis revealed that despite the nonbilayer-forming nature of MGDG, this lipid can form ordered lamellar structures in the presence of LHCII, which indicates the importance of the MGDG–LHCII interaction for lipid phase behavior in the thylakoid membrane (Simidjiev et al. 2000). Mutant analysis of the ALBINO3 protein, which mediates insertion of LHCPs into the thylakoid membrane, in *Arabidopsis* suggests that integration of LHCPs into the membrane is essential for the development of the thylakoid membrane and grana formation (Sundberg et al. 1997). PSI and PSII are also major protein–cofactor complexes in the thylakoid membrane. Disruption of PSI or PSII strongly affects the structure of the thylakoid membrane in plant chloroplasts. In particular, PSI is required for the formation of stroma lamellae, whereas PSII is more important for the organization of grana stacks (Pribil et al. 2014).

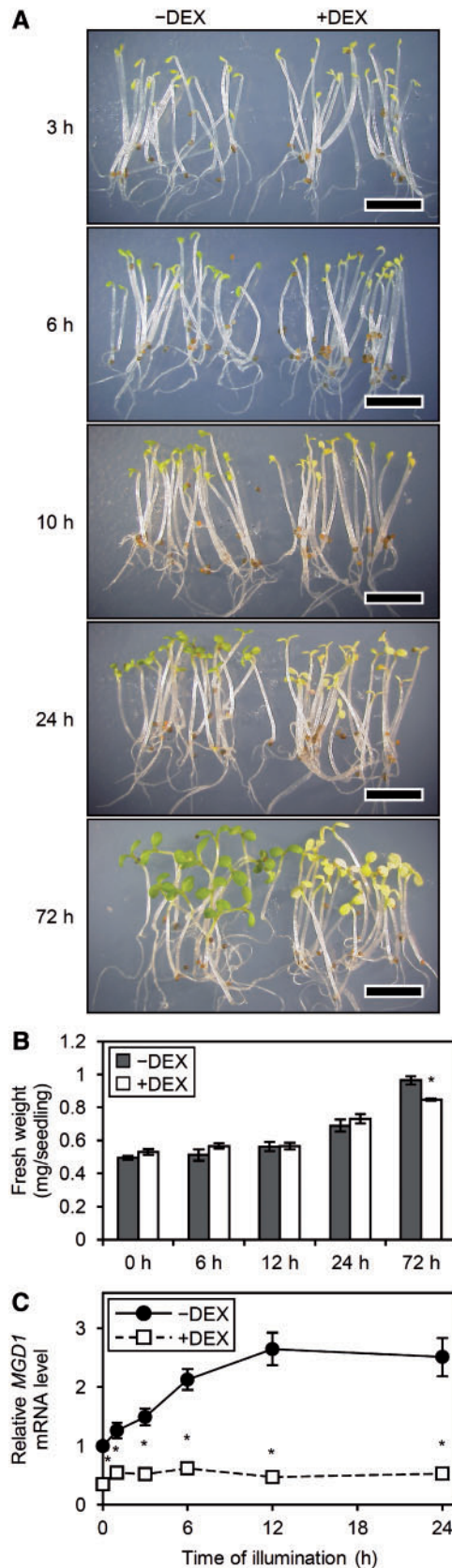
In chloroplasts, the major portion of total Chl is bound to LHCPs and is required for the assembly of the complexes. Many mutants deficient in Chl biosynthesis showed severely impaired thylakoid biogenesis and grana stacking, with decreased or no accumulation of LHCPs (Frick et al. 2003, Pontier et al. 2007, Bang et al. 2008, Apitz et al. 2014). Moreover, a Chl *b*-deficient *Arabidopsis* mutant showed decreased LHCII accumulation and loss of LHCII trimers, which resulted in decreased grana stacking

of the thylakoid membrane (Kim et al. 2009). These data indicate that Chl is required for the accumulation of LHCPs and is involved in thylakoid membrane biogenesis. Chl biosynthesis is also essential for the accumulation of D1, the core protein of PSII, and the assembly of PSII (Müller and Eichacker 1999).

During germination of angiosperms under light, undifferentiated plastids in cotyledon mesophyll cells differentiate into chloroplasts with the thylakoid membrane. However, in the absence of light, proplastids differentiate into etioplasts with unique lattice membrane structures called prolamellar bodies (PLBs) and lamellar prothylakoid (PTs) membranes (Solymosi and Schoefs 2010). PLBs accumulate a Chl precursor, protochlorophyllide (Pchl) *a*, and light-dependent NADPH:Pchl oxidoreductase (LPOR) in the lipid bilayer matrix. Although some photosynthesis-associated proteins, such as subunits of ATP synthase and the *Cytb<sub>f</sub>* complex, already exist in etioplasts (Blomqvist et al. 2008), LHCPs are almost absent and, if present, are not yet inserted in the membrane (Kuttkat et al. 1997, Müller and Eichacker 1999). PSI and PSII proteins also show minor abundance in etioplasts and are not assembled into mature complexes (Müller and Eichacker 1999).

The lipid composition of PLBs is similar to that of the thylakoid membrane: in PLBs of wheat etioplasts, MGDG and DGDG account for approximately 50% and 30%, respectively, of total glycerolipids (Selstam and Sandelius 1984). Expression of a dexamethasone (DEX)-inducible artificial microRNA targeting *MGD1* (*amiR-MGD1*) in dark-grown *Arabidopsis* seedlings reduced *MGD1* mRNA level by 70% as compared with the control, which resulted in 36% loss of MGDG without affecting DGDG content (Fujii et al. 2017). Although the loss of MGDG in etioplasts only slightly changed the lattice structure of PLBs, it strongly impaired the elongation of PTs and the formation and oligomerization of Pchl–LPOR complexes in membranes. Moreover, in the tetrapyrrole biosynthesis pathway, three sequential membrane-associated processes from the Mg-protoporphyrin IX formation to Pchl synthesis, particularly the monomethyl esterification of Mg-protoporphyrin IX, were severely inhibited by loss of MGDG in etioplasts. We previously investigated the role of DGDG in etioplasts (Fujii et al. 2018) by analyzing a knockout mutant of *DGD1* (*dgd1*; Dörmann et al. 1995). In the dark-grown *dgd1* mutant, DGDG content was reduced by 80% as compared with the wild type and, as in *amiR-MGD1* seedlings, membrane processes of Pchl biosynthesis were impaired. Furthermore, the lattice structure of PLBs was strongly disordered and PTs were severely underdeveloped in mutant etioplasts. These data demonstrate the essential roles of galactolipids in the formation of internal membranes and membrane-associated processes in etioplasts.

Once the etiolated seedlings are exposed to light, most Pchl molecules in PLBs are immediately converted to chlorophyllide by LPOR and then to Chl via some enzymatic processes (Schoefs and Bertrand 2000, Schoefs 2001). At the same time, light triggers de novo Chl biosynthesis and the expression of photosynthetic proteins to establish photosynthetic systems. In morphology, PLBs and PTs are directly transformed to the thylakoid membrane and etioplasts differentiate into chloroplasts (Kowalewska et al. 2016). We previously reported that MGDG biosynthesis is essential for the initiation of chloroplast biogenesis in light-grown seedlings



**Fig. 1** Greening of dark-grown *amiR-MGD1* seedlings after illumination. Plants were grown for 4 d in the dark and then illuminated for indicated hours in the absence (–) or presence (+) of DEX. (A) Phenotype and (B) fresh weight of *amiR-MGD1* seedlings. Bars in

(Kobayashi et al. 2007, Kobayashi et al. 2013, Fujii et al. 2014). However, the roles of these galactolipids during etioplast-to-chloroplast differentiation remain unclear. To reveal how the deficiency of galactolipid biosynthesis affects thylakoid biogenesis from PLBs during etioplast-to-chloroplast differentiation, we examined dark-grown *amiR-MGD1* and *dgd1* seedlings after illumination.

## Results

### *MGD1* suppression impairs greening after dark growth

To assess how *MGD1* suppression affects conversion from etioplasts to chloroplasts on illumination, we grew a strong line (L4w) of *amiR-MGD1* transgenic plants (Fujii et al. 2017) in complete darkness for 4 d in the absence (–) or presence (+) of DEX and then illuminated them with continuous white light. In +DEX seedlings, the greening of cotyledons was strongly impaired compared with the –DEX control (Fig. 1A), although fresh weight was similar between +DEX and –DEX seedlings before and at 24 h of illumination (Fig. 1B). After 72-h illumination, +DEX seedlings had lower fresh weight than the –DEX control, with cotyledon greening remained strongly impaired.

To ascertain whether *MGD1* expression during illumination was suppressed by DEX treatment in the *amiR-MGD1* line, we determined *MGD1* mRNA levels by quantitative reverse transcription-PCR (qRT-PCR) analysis (Fig. 1C). As we previously reported (Fujii et al. 2017), *MGD1* mRNA level was approximately 30% of –DEX control levels before illumination. In –DEX seedlings, *MGD1* level increased gradually with illumination and reached 2.5-fold of the initial level after 12 h of illumination. However, in +DEX seedlings, *MGD1* mRNA level did not notably increase in response to illumination. Thus, *MGD1* mRNA level in +DEX seedlings was only approximately 20% of the –DEX levels after 12 h of illumination. We also examined the mRNA levels of *MGD2*, *MGD3*, *DGD1* and *DGD2* during the greening process of *amiR-MGD1* plants (see Supplementary Fig. S1). In +DEX seedlings, *MGD2* mRNA level decreased compared with the –DEX control after 12-h illumination, whereas those of *MGD3*, *DGD1* and *DGD2* showed no remarkable difference between +DEX and –DEX seedlings.

### *MGD1* suppression strongly impairs thylakoid membrane biogenesis with illumination

We next analyzed the ultrastructure of plastids in cotyledons after illumination for 0, 3, 6 and 20 h (Fig. 2 and Supplementary Fig S2). In etioplasts of –DEX *amiR-MGD1* seedlings grown in the dark

#### Fig. 1 Continued

(A) = 5.0 mm. Data in (B) are means  $\pm$  SE from three (0 h) or four (6, 12, 24, 72 h) independent experiments. (C) *MGD1* mRNA levels presented as fold difference from the –DEX control at 0 h of illumination after normalization to the control gene *ACTIN8*. Data are means  $\pm$  SE from 13 (0) or three (1, 3, 6, 12, 24 h) independent experiments. Asterisks indicate significant difference from the –DEX control ( $P < 0.05$ , Student's *t*-test).

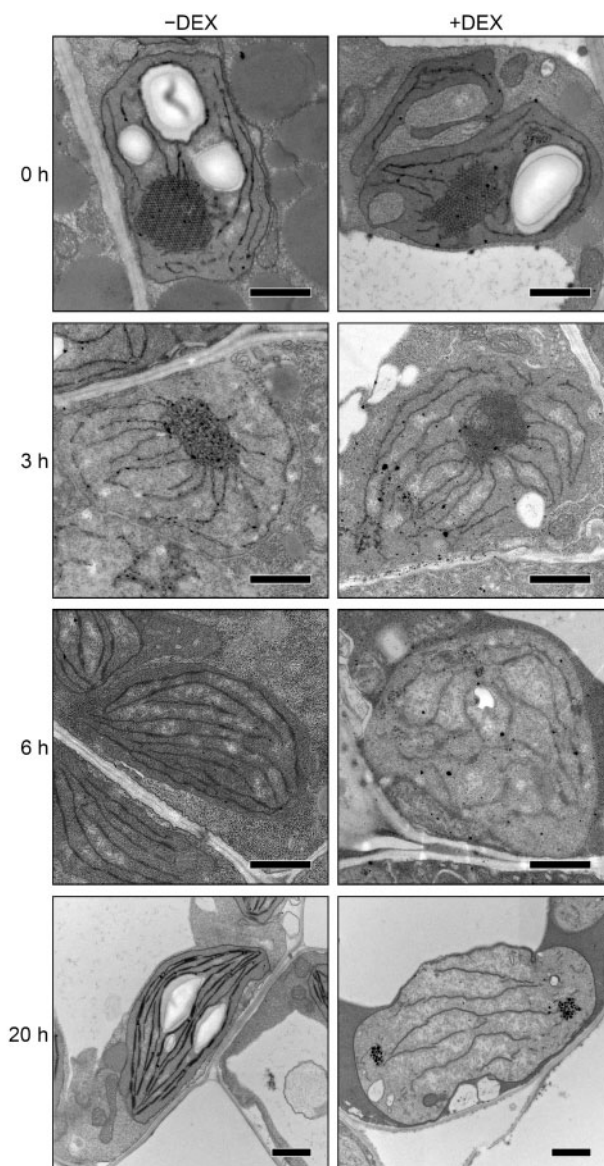
(0 h), regular lattice PLB structures were observed with PTs connected to the PLBs. After 3-h illumination, the crystalline structure of PLBs was disrupted and transformed into aggregates of short membranes. Most plastids had round shapes. After 6-h illumination, PLB remnants were no longer detected in all plastids observed in –DEX seedlings. Instead, lamellar thylakoid membranes had developed and frequently formed short stacks of two or three membranes. The plastids had a spindle or lens-like shape, with thylakoid membranes oriented toward each apex. After 20-h illumination, the thylakoid membrane networks were extended and grana stacks were markedly developed in cotyledon plastids of –DEX seedlings. Starch granules were formed between thylakoid membranes in these plastids.

We previously reported that +DEX *amiR-MGD1* seedlings grown in the dark showed reduced PT development and slightly disordered PLB structures in etioplasts (Fujii et al. 2017; Fig. 2). After 3-h illumination of +DEX *amiR-MGD1* seedlings, the PLB lattice membranes were transformed into irregular membrane aggregates as in the –DEX control. After 6-h illumination, PLB remnants were scarcely observed in plastids of +DEX cotyledons as in the –DEX control. However, unlike in –DEX plastids, thylakoid membranes were underdeveloped and did not align horizontally in many +DEX plastids, although some plastids showed thylakoid-like membrane structures with immature grana stacks (see Supplementary Fig. S2A). After 20-h illumination, the development of internal membranes was strongly perturbed and only a few fragmented stromal lamellae were observed. In addition, high electron-dense particles similar to plastoglobuli were often observed in the stromal region (Fig. 2 and Supplementary Fig. S2B). Plastids in +DEX cotyledons had a distorted circular outline and did not contain starch granules inside.

### Galactolipid biosynthesis during etioplast-to-chloroplast differentiation is impaired by disruption of *MGD1*

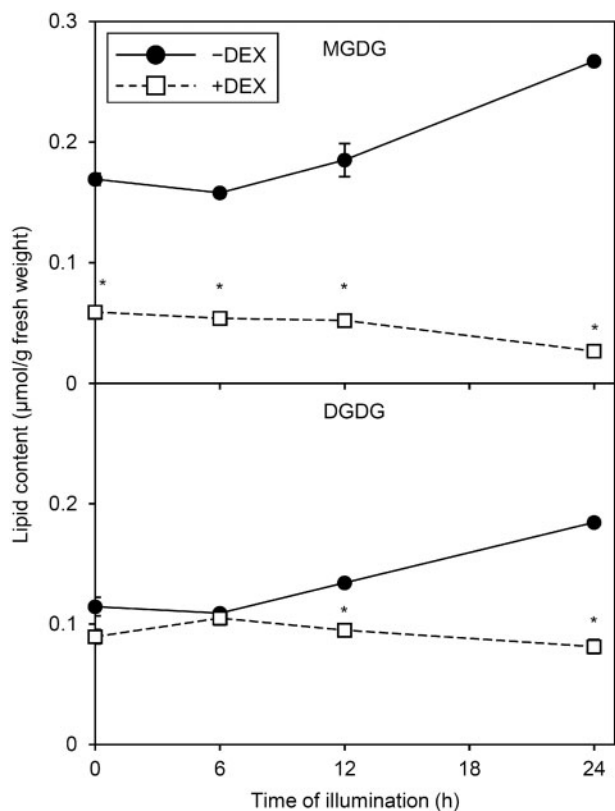
To examine whether the morphological changes in internal membranes of plastids during the greening process were linked to changes in galactolipid content, we determined the amount of MGDG and DGDG in –DEX and +DEX *amiR-MGD1* seedlings before and after illumination. In –DEX *amiR-MGD1* seedlings, although the proportion of MGDG and DGDG in total membrane lipids slightly increased after 6-h illumination (Supplementary Fig. S3), absolute MGDG and DGDG content per fresh weight was not significantly increased (Fig. 3). Similar trends continued until 12-h illumination. At 24 h after illumination, –DEX seedlings showed a significant increase in MGDG and DGDG content. In +DEX seedlings grown in the dark (0 h), MGDG but not DGDG content substantially decreased as compared with the –DEX control, which agreed with a previous report (Fujii et al. 2017). Moreover, under the +DEX condition, both MGDG and DGDG content decreased with illumination: after 24-h illumination, MGDG and DGDG content in +DEX seedlings was only 10% and 44%, respectively, of the –DEX control levels.

We then analyzed the FA composition of galactolipids before and after illumination. As we reported previously



**Fig. 2** Changes in ultrastructure of cotyledon plastids during greening of dark-grown *amiR-MGD1* seedlings. Plants were grown for 4 d in the dark and then illuminated for indicated hours in the absence (–) or presence (+) of DEX. Bars = 1.0  $\mu$ m.

(Fujii et al. 2017), before illumination, the FA composition of MGDG and DGDG did not notably differ between +DEX and –DEX seedlings (Fig. 4). After illumination, –DEX seedlings showed only small changes in FA composition of MGDG and DGDG: the proportion of 16:3 in MGDG was slightly decreased with illumination for 12 h but recovered after 24 h, and that of 18:3 in DGDG slightly increased, with decreased proportions of 18:2 and 16:0. In contrast, in +DEX seedlings, the proportions of 16:3 and 18:3 in MGDG remarkably decreased and those of 16:1 and 18:0 increased during 6 to 12 h of illumination (Fig. 4A). In addition, the proportion of 16:0 in MGDG gradually increased during illumination. Also, the absolute amounts of 16:0, 16:1 and 18:0 in MGDG increased from 6 to 12 h of illumination but decreased later with a decrease in total MGDG content

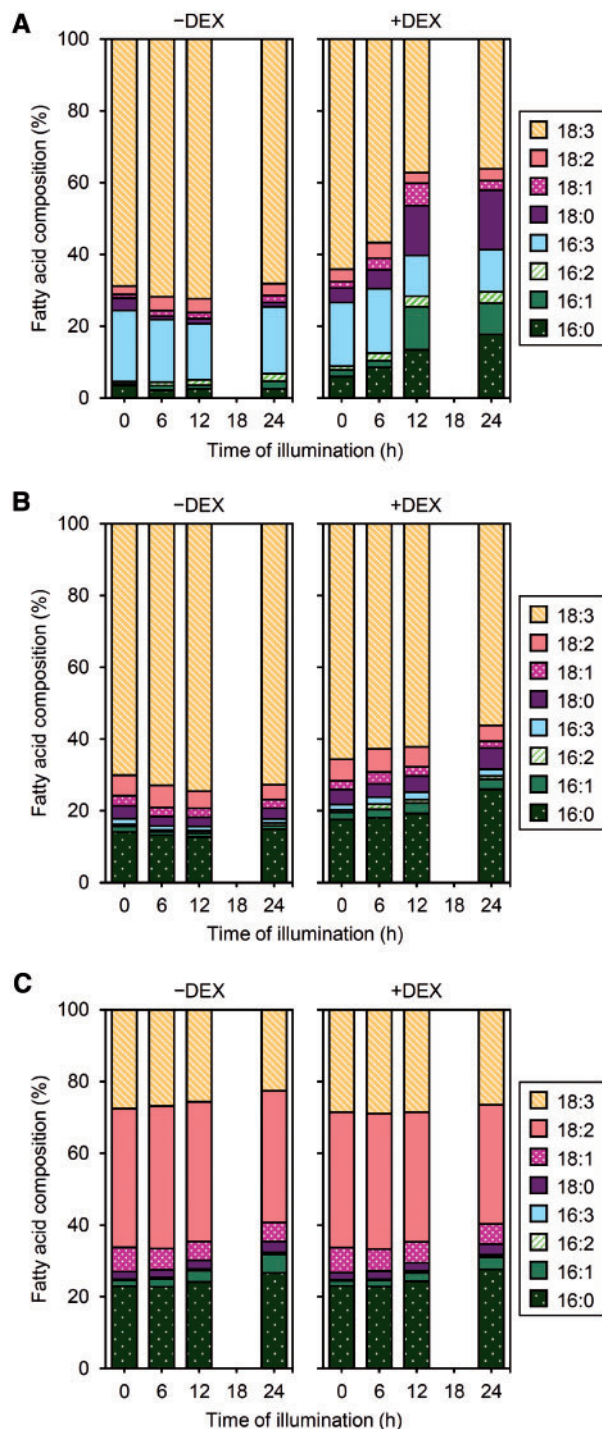


**Fig. 3** Changes in MGDG and DGDG content during greening of dark-grown *amiR-MGD1* seedlings. Seedlings were illuminated for indicated hours after 4-d growth in the dark in the absence (–) or presence (+) of DEX. Data are means  $\pm$  SE from three independent experiments. Asterisks indicate significant difference from the –DEX control ( $P < 0.05$ , Student’s *t*-test).

(see Supplementary Fig. S4A). In DGDG, the proportion of 18:3 decreased and that of 16:0 increased in +DEX seedlings during illumination (Fig. 4B). Similar patterns were observed for absolute amounts (see Supplementary Fig. S4B). Meanwhile, the composition of total FAs from other membrane lipids was not notably changed by DEX treatment before and after illumination (Fig. 4C). These results suggest that *MGD1* suppression specifically affected FA metabolism of galactolipids during etioplast-to-chloroplast differentiation.

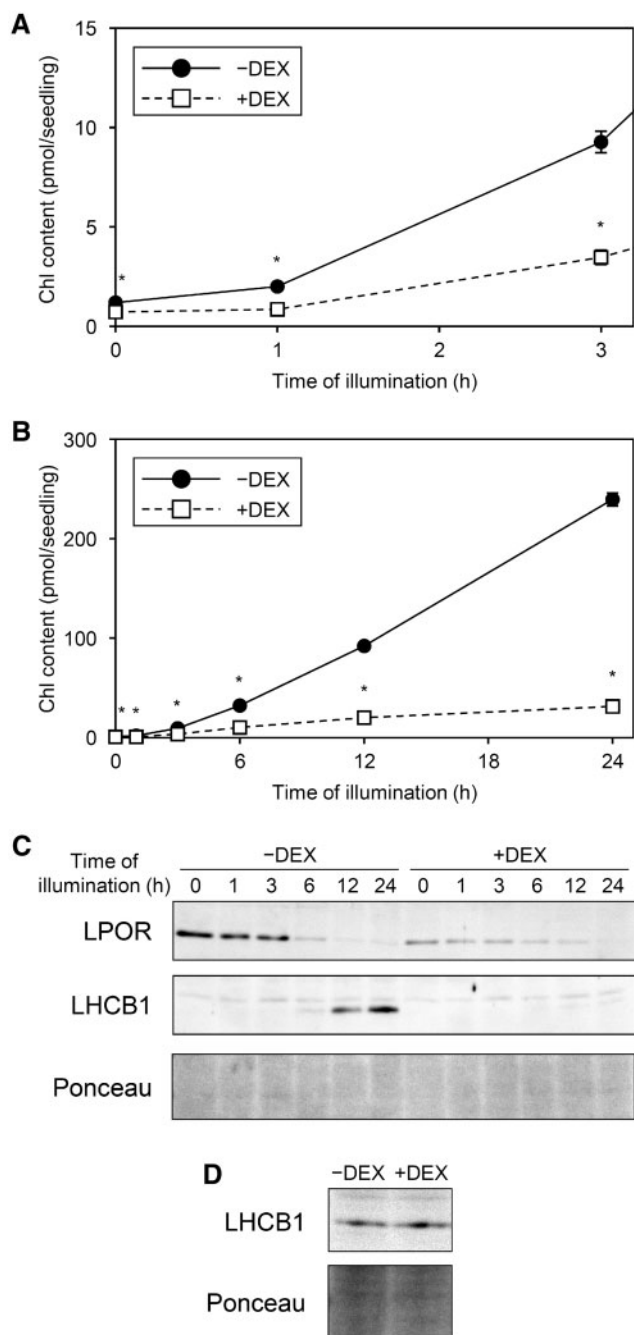
### MGD1 suppression affects Chl and membrane protein levels during etioplast-to-chloroplast differentiation

*MGD1* suppression by *amiR-MGD1* impaired Pchl<sub>ide</sub> accumulation in dark-grown seedlings (Fujii et al. 2017). Reflecting the decreased Pchl<sub>ide</sub> content by DEX treatment, the amount of Chl at 1 h after illumination, which would mainly originate from Pchl<sub>ide</sub> accumulated in the dark, was lower in +DEX *amiR-MGD1* than –DEX control seedlings (Fig. 5A). After 3-h illumination, Chl content rapidly increased in –DEX control plants but not much in +DEX seedlings. The accumulation of Chl was continuously impaired in +DEX seedlings during illumination; indeed, Chl level in +DEX plants was only 13% of the –DEX

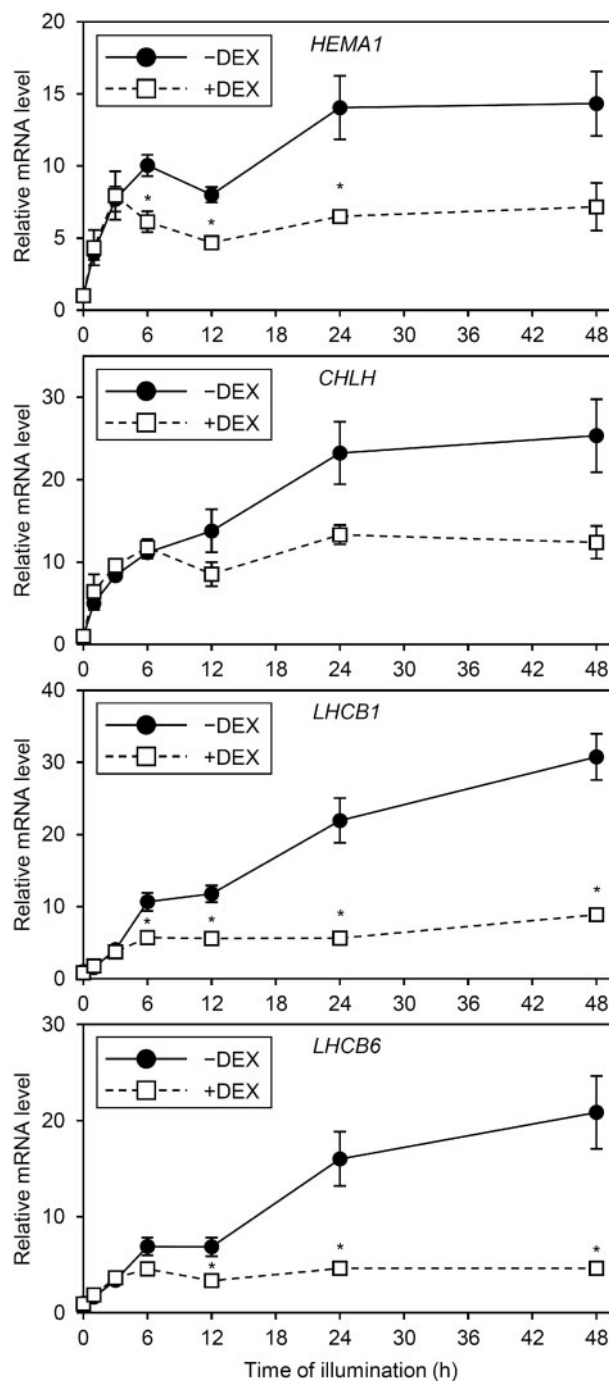


**Fig. 4** Changes in FA composition of galactolipids during greening of dark-grown *amiR-MGD1* seedlings. FA composition of (A) MGDG, (B) DGDG and (C) other membrane lipids. Seedlings were illuminated for indicated hours after 4-d growth in the dark in the absence (–) or presence (+) of DEX. Data are means from three independent experiments.

control at 24 h after illumination (Fig. 5B), consistent with the strong impairment of cotyledon greening in +DEX seedlings (Fig. 1). To confirm the relationship between the *MGD1* suppression level and the impaired Chl accumulation, we also



**Fig. 5** Changes in Chl and protein levels during greening of dark-grown *amiR-MGD1* seedlings. Seedlings were illuminated for indicated hours after 4-d growth in the dark in the absence (–) or presence (+) of DEX. (A, B) Chl content per seedling. The amount of protochlorophyllide (Fujii et al. 2017, www.plantphysiol.org. Copyright American Society of Plant Biologists) is shown for 0 h instead of Chl amount. (A) is an enlarged view of (B) within 3 h of illumination. Data are mean  $\pm$  SE from 12 (0, 1, 3, 6, 12 h) or 6 (24 h) independent experiments. Asterisks indicate significant difference from –DEX control ( $P < 0.05$ , Student's *t*-test). (C) Time course immunoblot analysis of LPOR and LHCBI proteins and (D) detection of LHCBI proteins in seedlings illuminated for 3 h. In (D), 50  $\mu$ g of total protein was used to detect faint LHCBI signals from 3-h illuminated samples, whereas 10  $\mu$ g of total protein was used for the time course analysis in (C). Unspecific bands were constantly observed above the LHCBI bands. Data are representative of three independent experiments. Ponceau-stained proteins were shown as a loading control.



**Fig. 6** mRNA levels of photosynthesis-related genes during greening of dark-grown *amiR-MGD1* seedlings. Seedlings were illuminated for 1, 3, 6, 12, 24 and 48 h after 4-d growth in the dark (0 h) in the absence (–) or presence (+) of DEX. Fold difference from the –DEX control at 0 h after normalization to the control gene *ACTIN8* is shown for each gene. Data are mean  $\pm$  SE from 10 (0 h) or 3 (1, 3, 6, 12, 24, 48 h) independent experiments. Asterisks indicate significant difference from the –DEX control ( $P < 0.05$ , Student's *t*-test). Data for *HEMA1*, *CHLH* and *LHCBI6* before illumination (0 h) were shown based on Fujii et al. (2017) www.plantphysiol.org. Copyright American Society of Plant Biologists.

analyzed Chl content in a weak *amiR-MGD1* line (L4g), where *MGD1* mRNA level decreased to approximately 50% of the control levels (see Supplementary Fig. S5A). The accumulation rate of Chl in +DEX L4g seedlings was similar to that in the

–DEX control until 12-h illumination, but diminished at 24 h (see Supplementary Fig. S5B). We also confirmed that DEX treatment itself had no effect on Chl accumulation in wild-type *Arabidopsis* seedlings (*Landsberg erecta*) illuminated for 24 h (see Supplementary Fig. S5C). Thus, the diminished Chl accumulation in +DEX L4g seedlings would result from the partial *MGD1* suppression.

We previously reported that *MGD1* suppression by *amiR-MGD1* did not decrease the amount of LPOR protein, which determines the size of PLBs, in the dark (Fujii et al. 2017). However, in this study, we continually found lower LPOR level in +DEX than –DEX control seedlings in the dark (Fig. 5C). Because the signal intensities of proteins in immunoblot analysis often include a large deviation, the small difference in LPOR levels between dark-grown +DEX and –DEX plants might have been masked in the previous study. In the –DEX control, LPOR level gradually decreased with illumination but remained detectable at 24 h after illumination. However, in +DEX seedlings, LPOR level decreased to almost undetectable after 24-h illumination.

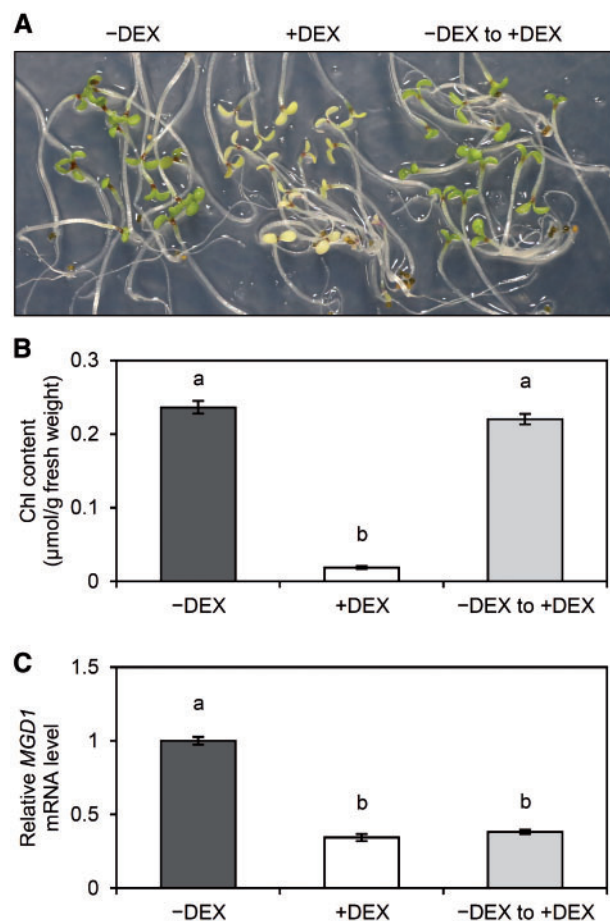
LHCII plays a crucial role in thylakoid membrane biogenesis and maintenance (Pribil et al. 2014). Consistent with a previous report (Müller and Eichacker 1999), LHCBI protein, the main subunit of LHCII, was undetectable before illumination in both +DEX and –DEX seedlings (Fig. 5C). At 3 h after illumination, LHCBI became detectable with both treatments (Fig. 5D), although the protein signals, which were similar between +DEX and –DEX plants, were very faint (Fig. 5C). In –DEX seedlings, LHCBI level further increased during illumination and showed an intense signal at 24 h after illumination. In contrast, in +DEX seedlings, LHCBI protein accumulation was strongly impaired at 6 h after illumination and the signal remained minor even after 24 h.

### **MGD1 suppression does not affect acute induction of genes for Chl biosynthesis and light-harvesting after illumination**

To examine whether changes in gene expression are involved in impaired accumulation of Chl and LHCBI protein in +DEX *amiR-MGD1* seedlings, we measured mRNA levels of genes for Chl biosynthesis and light-harvesting before and after illumination (Fig. 6). The mRNA levels of *HEMA1* encoding glutamyl-tRNA reductase and *CHLH* encoding the H subunit of Mg-chelatase, key genes for Chl biosynthesis (Tanaka et al. 2011), were not changed by *MGD1* suppression in dark-grown seedlings (Fujii et al. 2017). In addition, the mRNA levels of *LHCBI* for the major LHCII protein and *LHCB6* for the minor PSII antenna protein (CP24) were similar between –DEX and +DEX plants in the dark. After illumination, the mRNA levels of all genes examined increased, with no significant differences between –DEX and +DEX plants before and at 3 h. The mRNA levels of these genes further increased in –DEX but not +DEX seedlings with illumination for 6 h or more. As a result, +DEX seedlings showed much lower expression of these genes than the –DEX control after 24 to 48 h of illumination.

### **MGD1 expression during dark growth is essential for greening after illumination**

To examine whether the *MGD1* expression during etioplast development is important for etioplast-to-chloroplast differentiation after illumination, we grew *amiR-MGD1* seedlings on DEX-free media in the dark for 3 d, then treated them with DEX for 24 h to fully downregulate the *MGD1* expression before illumination. After illumination for 24 h in the presence of DEX, seedling phenotypes were compared with those continuously grown under –DEX or +DEX. Consistent with data in Figs. 1, 5, Chl accumulation was strongly impaired in +DEX seedlings (Fig. 7A, B). Meanwhile, seedlings treated with DEX before illumination showed a phenotype similar to the –DEX control and accumulated Chl to the control level, even though the short DEX treatment suppressed *MGD1* mRNA accumulation



**Fig. 7** Greening of dark-grown *amiR-MGD1* seedlings treated with DEX after the development in darkness. (A) Phenotype, (B) Chl content and (C) mRNA abundance of *MGD1* were analyzed in *amiR-MGD1* seedlings illuminated for 24 h after grown in the dark for 4 d. Seedlings indicated as –DEX and +DEX were grown continuously under DEX-free or DEX-fed conditions, respectively, whereas those indicated as ‘–DEX to +DEX’ were treated with DEX 24 h before illumination. Different letters indicate significant differences ( $P < 0.05$ , Tukey–Kramer multiple comparison test).

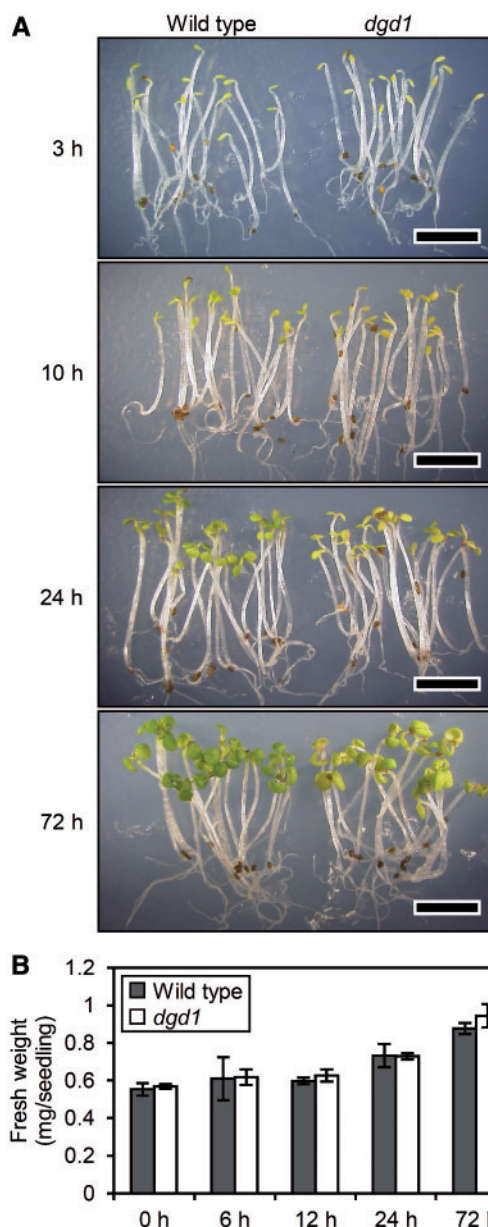
to the minimal level (Fig. 7C). The data demonstrate that *MGD1* expression during etioplast development in the dark is sufficient for chloroplast development after illumination.

### Loss of DGDG retards conversion of the PLB to the thylakoid membrane during etioplast-to-chloroplast differentiation

To assess the effect of DGDG deficiency on the etioplast-to-chloroplast differentiation after illumination, we analyzed the greening process of dark-grown *dgd1* seedlings, in which etioplast development is strongly affected (Fujii et al. 2018). The cotyledon greening after illumination was delayed in *dgd1* as compared with the wild-type *Arabidopsis* Columbia (Col) plants (Fig. 8A), although fresh weight of the *dgd1* seedlings increased similar to the wild type (Fig. 8B). After 72-h illumination, *dgd1* recovered from impaired greening to some extent, which was in contrast to the continuous impairment of greening in +DEX *amiR-MGD1* seedlings (Fig. 1A).

We then investigated plastid ultrastructure in cotyledon cells of dark-grown *dgd1* seedlings illuminated for 0, 3, 10, 24 and 72 h with continuous white light (Fig. 9 and Supplementary Fig. S6). As in –DEX *amiR-MGD1* seedlings, in wild-type *Arabidopsis* Col plants, the crystalline structure of PLBs observed in etioplasts was disrupted and replaced by irregular membrane aggregates after 3-h illumination. In addition, PTs were further elongated from the PLB remnants, and short stacks of two or three membranes were observed. Most plastids had distorted circular shapes. After 10-h illumination, PLB remnants were no longer detected in all wild-type plastids, and instead, lamellar thylakoid membranes were extensively developed. In addition, long-stacked regions of two or three membranes were frequently observed, occasionally forming thick stacks of four to six membranes. The plastids had elliptical or spindle shapes with thylakoid membranes oriented toward each apex. After 24-h illumination, plastids in wild-type cotyledons contained large starch granules between thylakoid membranes, which were further enlarged after 72 h, and appeared as typical chloroplasts with a lens-like shape.

As we previously reported (Fujii et al. 2018), in dark-grown *dgd1* etioplasts, alignment of PLB lattices was strongly disturbed and the development of PTs was severely impaired. After 3-h illumination, the PLB lattice membranes were converted to aggregates of irregular membrane tubules as in the wild type, but the growth of PTs was still impaired and no stacks of lamellar membranes were observed. After 10-h illumination, large circular aggregates of membrane tubules, which would be remnants of PLBs, were frequently observed in *dgd1* plastids. Development of thylakoid membranes was strongly impaired, and stacked membrane regions were rarely observed in the mutant. After 24-h illumination, the thylakoid membrane development was still strongly impaired and no grana stacks were formed. Some plastids still contained circular aggregates of membrane tubules, presumably PLB remnants (see Supplementary Fig. S6A). Of note, high electron-dense particles similar to plastoglobuli were often observed around the tubule aggregates. After 72-h illumination, circular aggregates of membrane tubules were not observed in *dgd1* cotyledons. Some mutant plastids developed wild-type-like thylakoid membrane networks and accumulated starch granules, although



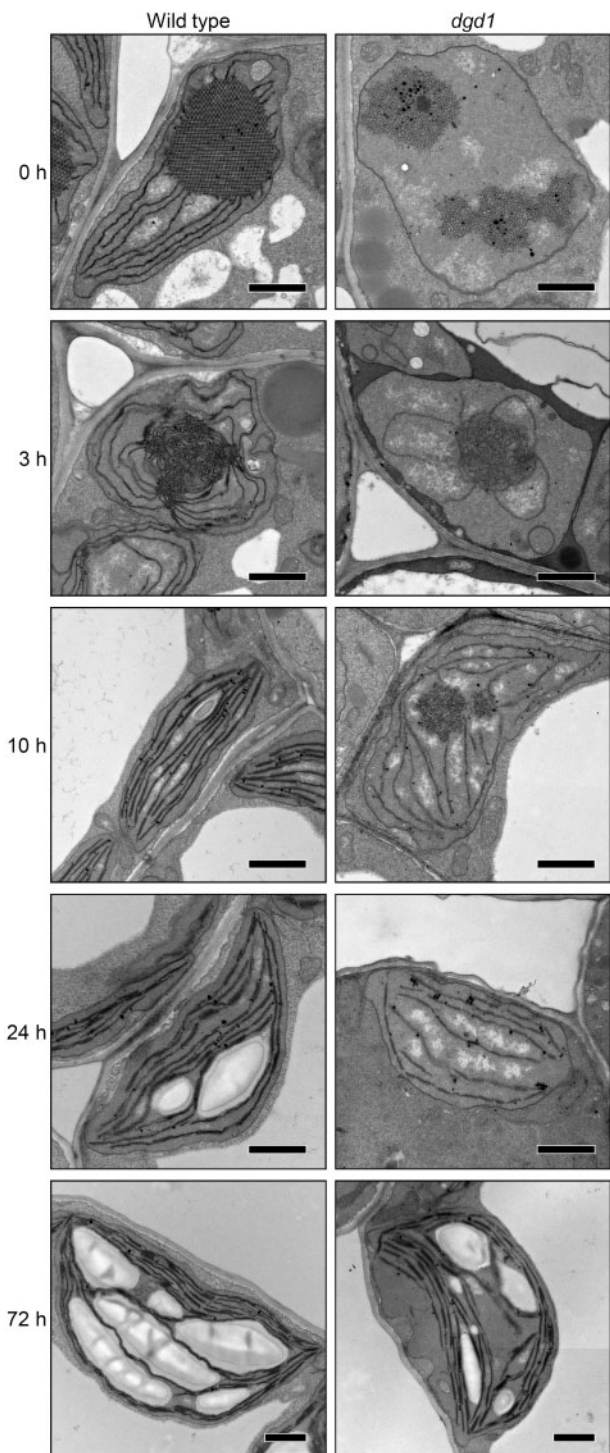
**Fig. 8** (A) Phenotype and (B) fresh weight of dark-grown wild-type and *dgd1* seedlings after illumination. Plants were grown for 4 d in the dark and then illuminated for indicated hours. Bars in (A) = 5.0 mm. Data in (B) are mean  $\pm$  SE from three (0 h) or four (6, 12, 24, 72) independent experiments. No significant difference in fresh weight was observed between wild type and *dgd1* ( $P > 0.05$ , Student's *t*-test).

thylakoid-free areas were often observed, as reported previously (Dörmann et al. 1995). Plastids with underdeveloped thylakoid membranes were still observed in cotyledon cells of some *dgd1* seedlings at 72 h after illumination (see Supplementary Fig. S6B).

### Galactolipid biosynthesis during etioplast-to-chloroplast differentiation is impaired by disruption of DGD1

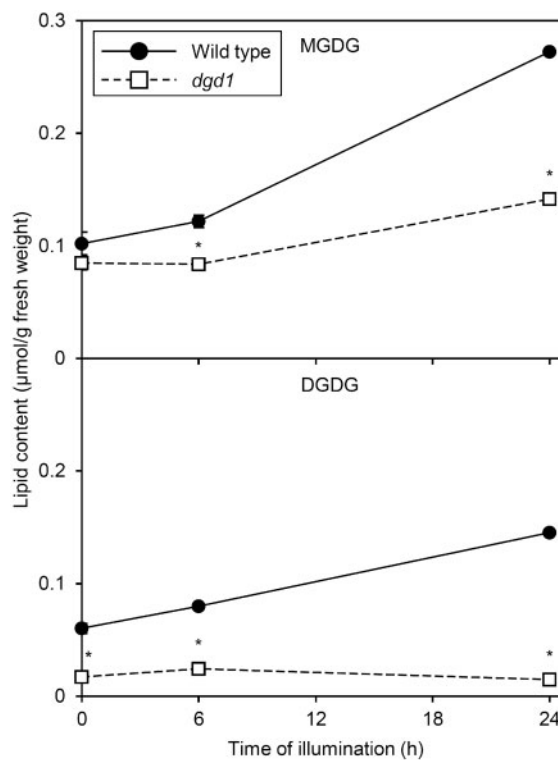
In contrast to *amiR-MGD1* lines, in dark-grown *dgd1* seedlings, DGDG but not MGDG content was remarkably decreased





**Fig. 9** Changes in ultrastructure of cotyledon plastids during greening of dark-grown seedlings of the *Arabidopsis* Columbia wild type and the *dgd1* mutant. Plants were grown for 4 d in the dark and then illuminated for indicated hours. Bars = 1.0  $\mu\text{m}$ .

(**Fig. 10**; Fujii et al. 2018). In the wild type, but not *dgd1*, both MGDG and DGDG content slightly increased after 6 h of illumination. After 24-h illumination, MGDG content increased in *dgd1* seedlings but to a lesser extent than in the wild type, so MGDG content in *dgd1* was about half of the wild-type level.

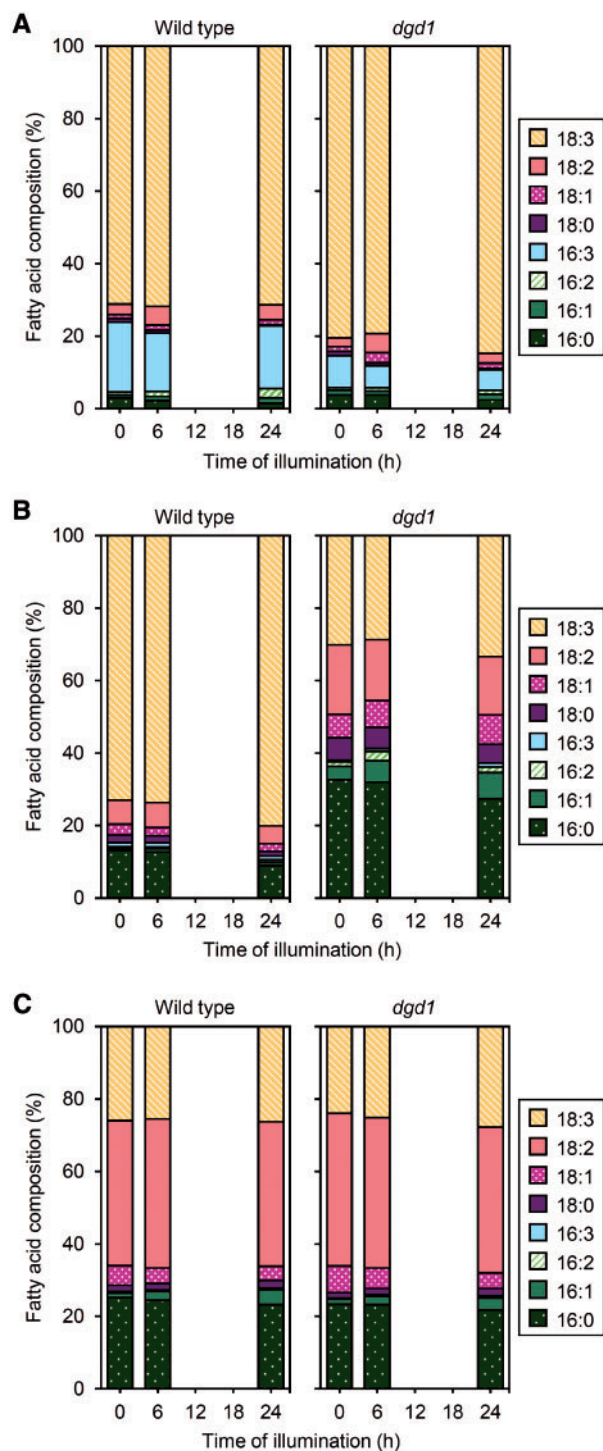


**Fig. 10** Galactolipid content during greening of dark-grown seedlings of the *Arabidopsis* Columbia wild type and the *dgd1* mutant. Plants were grown for 4 d in the dark and then illuminated for indicated hours. Data are mean  $\pm$  SE from three independent experiments. Asterisks indicate significant difference from the wild type ( $P < 0.05$ , Student's *t*-test).

Moreover, unlike in the wild type, in *dgd1*, DGDG content did not increase in response to illumination. Thus, DGDG content in *dgd1* seedlings was only 10% of the wild-type level after 24-h illumination. Similar results were observed in proportion of galactolipids in total membrane lipids (see Supplementary Fig. S7).

### Disruption of DGD1 changes FA composition of galactolipids during etioplast-to-chloroplast differentiation

Similar to  $-\text{DEX } \textit{amiR-MGD1}$  plants with a *Landsberg erecta* background, wild-type Col seedlings showed only slight changes in FA composition of MGDG with illumination (**Fig. 11A**). In DGDG, the proportion of 18:3 was increased and those of 18:2 and 16:0 were slightly decreased in the illuminated wild type (**Fig. 11B**). We previously reported that FA composition of MGDG and DGDG in dark-grown *dgd1* seedlings greatly differed from that in the wild type (Fujii et al. 2018). The proportions of 16:3 and 18:3 in MGDG were lower and higher, respectively, in dark-grown *dgd1* than the wild type (**Fig. 11A**). The proportion of 18:3 in DGDG was lower and those of other FAs were higher in the mutant than the wild type (**Fig. 11B**). Altered FA composition was also observed in *dgd1* after illumination. In MGDG of the mutant, the proportions of 16:3 and 18:3 were further decreased and increased,



**Fig. 11** Changes in FA composition of galactolipids during greening of dark-grown seedlings of wild type and the *dgd1* mutant. FA composition of (A) MGDG, (B) DGDG and (C) other membrane lipids in seedlings illuminated for indicated hours after 4-d growth in the dark. Data are means from three independent experiments.

respectively, after 24-h illumination. Moreover, the absolute amount of 18:3 was specifically increased in the mutant MGDG after 24-h illumination (see Supplementary Fig. S8A). In the mutant DGDG, the proportions of 18:3, 18:1 and 16:1

were increased and those of 16:0 and 18:2 were decreased with 24-h illumination (Fig. 11B). However, on a fresh weight basis, the amounts of 18:3, 18:1 and 16:1 in DGDG did not increase during this period (see Supplementary Fig. S8B), which agrees with the decreased total DGDG content after 24-h illumination (Fig. 10). Meanwhile, *dgd1* mutation did not affect the composition of total FAs from other membrane lipids before and after illumination (Fig. 11C).

### Disruption of *DGD1* strongly impairs accumulation of Chl and LHCB1 during etioplast-to-chloroplast differentiation

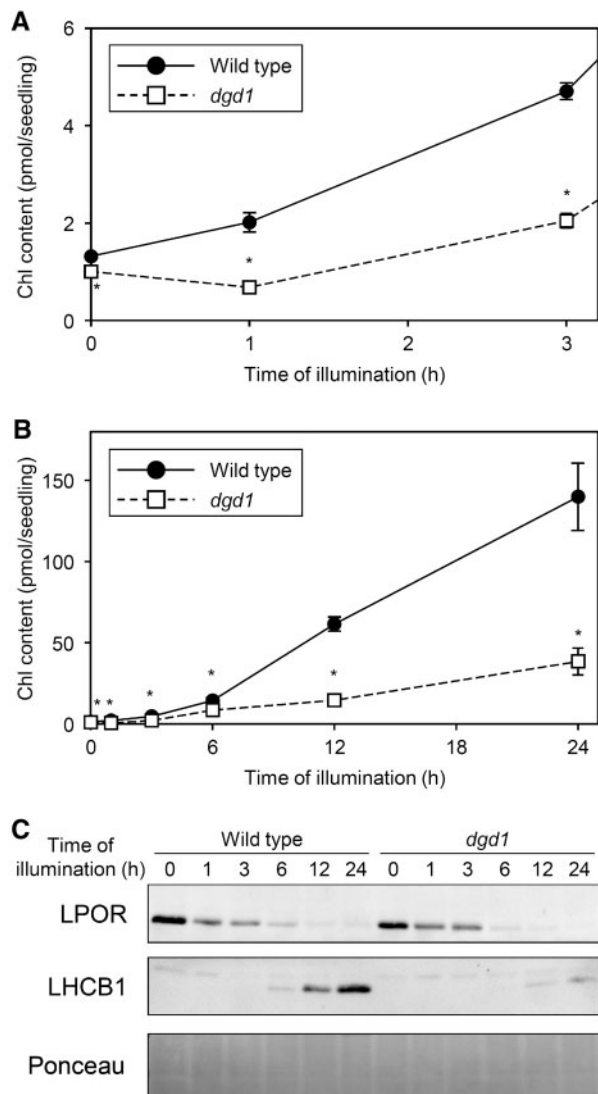
Consistent with our previous study showing that *dgd1* mutation impaired Pchlide accumulation in dark-grown seedlings (Fujii et al. 2018), the amount of Chl after 1-h illumination was lower in *dgd1* than wild-type seedlings (Fig. 12A). In *dgd1*, Chl content at 1 h after illumination was 0.68 pmol/seedling, which was lower than Pchlide content before illumination (1.00 pmol/seedling; Fujii et al. 2018). Although Chl content increased in *dgd1* afterward, the accumulation rate was remarkably slower than in wild-type seedlings (Fig. 10B), resulting in the delayed greening of the mutant cotyledons (Fig. 8).

We next examined whether the *dgd1* mutation affects protein accumulation during the greening process. LPOR protein level, which was slightly lower in dark-grown *dgd1* than wild-type seedlings as observed previously (Fujii et al. 2018), rapidly decreased both in *dgd1* and the wild type with illumination (Fig. 12C). In contrast to LPOR protein level, LHCB1 level increased with illumination in wild-type seedlings. However, in *dgd1*, the accumulation of LHCB1 was very slow and the signals were faint even after 24-h illumination.

## Discussion

### Galactolipids deficiency impairs accumulation of Chl and Chl-binding proteins during etioplast-to-chloroplast differentiation

We previously reported that MGDG deficiency in *amiR-MGD1* etioplasts impaired membrane-associated processes of the tetrapyrrole biosynthesis pathway and thus decreased Pchlide accumulation in the dark (Fujii et al. 2017). In +DEX seedlings, Chl content after 1-h illumination was comparable to the Pchlide level before illumination, which indicates strong inhibition of de novo Chl biosynthesis by *MGD1* suppression (Fig. 5A). In contrast, the mRNA levels of *HEMA1* and *CHLH*, which show strong coexpression with other Chl biosynthesis genes (Kobayashi and Masuda 2016), rapidly increased in +DEX seedlings as in the –DEX control until 3 or 6 h of illumination (Fig. 6). The data suggest that in +DEX *amiR-MGD1* seedlings, inhibited accumulation of Chl within the first several hours after illumination is not caused by transcriptional downregulation. As observed in dark-grown seedlings, MGDG deficiency likely impairs the membrane processes of the tetrapyrrole biosynthesis pathway after illumination. Because *dgd1* mutation also impaired membrane processes of the tetrapyrrole biosynthesis in etioplasts



**Fig. 12** Changes in Chl and protein contents during greening of dark-grown seedlings of the *Arabidopsis* Columbia wild type and *dgd1* mutant. Seedlings were illuminated for indicated hours after 4-d growth in the dark. (A, B) Chl content per seedling. Amount of protochlorophyllide (Fujii et al. 2018, www.plantphysiol.org. Copyright American Society of Plant Biologists) is shown for 0 h instead of Chl amount. Data are mean  $\pm$  SE from 18 (0 h) or 3 (1, 3, 6, 12, 24 h) independent experiments. Asterisks indicate significant difference from the wild-type control ( $P < 0.05$ , Student's *t*-test). (A) is an enlarged view of (B) within 3 h of illumination. (C) Immunoblot analysis of LPOR and LHCBI proteins. Data are representative of three independent experiments. Unspecific bands were constantly observed above the LHCBI bands. Ponceau-stained proteins are shown as a loading control.

(Fujii et al. 2018), the inhibited Chl accumulation in *dgd1* seedlings shortly after illumination may be explained by the same mechanism. Of note, in *dgd1*, Chl content at 1 h after illumination was even lower than Pchlde content before illumination (Fig. 12A; Fujii et al. 2018). We previously reported that *dgd1* mutation retarded the regeneration of photoactive Pchlde-LPOR complexes from nonphotoactive forms, which is required

for subsequent steps of Chl biosynthesis (Fujii et al. 2018). In the mutant, the decreased Chl formation at the beginning of greening may be explained in part by the retarded regeneration of photoactive Pchlde-LPOR complexes.

Accumulation of the LHCBI protein was also impaired in +DEX *amiR-MGD1* (Fig. 5C) and *dgd1* seedlings (Fig. 12C). Because Chl is important for the accumulation of LHCPs in membranes, impaired Chl accumulation might result in decreased LHCBI levels in these plants. However, we found no noticeable differences in LHCBI levels between +DEX and -DEX *amiR-MGD1* seedlings at 3 h after illumination (Fig. 5D), although the Chl content was remarkably lower in +DEX than -DEX control seedlings (Fig. 5A). Because LHCBI level was very low at this period, reduced Chl level in +DEX seedlings may be sufficient for the initial LHCBI accumulation.

Although +DEX seedlings showed an acute induction of *LHCBI* gene expression similar to the -DEX control during 3-h illumination, unlike the -DEX control, +DEX seedlings showed no enhancement of *LHCBI* expression thereafter (Fig. 6). In addition to the impaired Chl biosynthesis, inhibited induction of the *LHCBI* gene expression might decrease the accumulation of LHCBI protein at later greening stages in +DEX seedlings. Similar transcriptional trends were observed in *HEMA1*, *CHLH* and *LHCBI6* genes, which is consistent with our previous report that decreased MGDG biosynthesis downregulated the expression of photosynthesis-associated genes in light-grown seedlings (Kobayashi et al. 2013, Fujii et al. 2014). In +DEX seedlings, some impairments in the process of chloroplast development, caused by *MGD1* dysfunction within 6 h after illumination, might activate a regulatory system to suppress the expression of photosynthesis-associated genes. In addition, we do not exclude that galactolipid deficiency directly destabilizes LHCII proteins and impairs their accumulation in membranes. In fact, single-molecule force spectroscopy analysis of LHCII embedded in lipid bilayers revealed that MGDG with a conical shape increased the mechanical stability of LHCII (Seiwert et al. 2017), which suggests a requirement of MGDG for stable accumulation of LHCII in the thylakoid membrane.

### Mechanism of impaired PLB-to-thylakoid transformation by galactolipid deficiency during illumination

In -DEX *amiR-MGD1* seedlings, PLB remnants observed at 3 h after illumination were mostly transformed into thylakoid membranes after 6 h (Fig. 2). Previous studies showed that the lipid composition of PLBs is similar to that of the thylakoid membrane (Selstam and Sandelius 1984, Dorne et al. 1990). Moreover, both MGDG and DGDG content did not largely increase during the early greening stages (Figs. 3, 10), which agrees with no increase in galactolipid content in dark-grown barley seedlings with illumination for several hours (Selldén and Selstam 1976). These data suggest that most galactolipids in PLBs are used for constructing the thylakoid membrane. This assumption is consistent with the observation that tubular structures of PLBs are directly transformed into flat slats of the thylakoid membrane during the etioplast-to-chloroplast

differentiation (Kowalewska et al. 2016). In addition, we observed that *MGD1* suppression from 24 h before illumination did not inhibit greening of seedlings after illumination (Fig. 7). Thus, although *MGD1* is light-inducible, the *MGD1* protein or galactolipids already synthesized during the dark growth may be sufficient for the initial processes of the etioplast-to-chloroplast differentiation. *DGD1* is also weakly light-inducible (Kobayashi et al. 2014). The light-induced expression of these genes may contribute to increased galactolipid content at the later stages of chloroplast development. In fact, +DEX seedlings of a weak *amiR-MGD1* line (L4g) showed decreased Chl levels only after 24-h illumination (see Supplementary Fig. S5), which suggests that full expression of *MGD1* is required for chloroplast development at the later greening stage when galactolipid content increases.

As in the –DEX control, in +DEX *amiR-MGD1* seedlings, PLB remnants were mostly disassembled after 6-h illumination (Fig. 2). However, in these seedlings, many plastids failed to develop stacked thylakoid membrane networks. Seiwert et al. (2018) proposed that nonbilayer-forming MGDG promotes membrane stacking by easing the membrane curvature stress in the margin domains, which suggests a structural importance of MGDG for thylakoid membrane development. In addition to a direct effect of MGDG deficiency on membrane biogenesis, in +DEX seedlings, lack of LHCII and possibly other LHCPs may negatively affect thylakoid membrane formation after disassembly of PLBs. This assumption is consistent with the observation that grana stacking, with LHCII playing a crucial role (Pribil et al. 2014), was severely underdeveloped in +DEX chloroplasts. Although some plastids in +DEX seedlings could form a thylakoid membrane at 6 h after illumination, all plastids observed after 20-h illumination showed poorly developed internal membrane networks. This observation is consistent with the decreased content of both galactolipids from 6- to 24-h illumination in +DEX seedlings (Fig. 3), which would further impair thylakoid biogenesis in chloroplasts. In +DEX seedlings, down-regulation of photosynthesis-associated genes in response to decreased MGDG biosynthesis may secondarily suppress chloroplast biogenesis and result in stronger impairment of the thylakoid membrane development at later stages of greening.

In contrast to +DEX *amiR-MGD1*, the *dgd1* mutant showed PLB remnants in plastids at 10 h after illumination and even after 24-h illumination in some cases (Fig. 9 and Supplementary Fig. S6). Because LPORs, which determine the size of PLBs as essential constituents (Sperling et al. 1998, Franck et al. 2000, Masuda et al. 2003), were rapidly degraded in *dgd1* as in the wild-type control (Fig. 12C), LPOR protein may not be involved in the retarded disassembly of PLBs in illuminated *dgd1* seedlings. Moreover, inhibited accumulation of Chl and LHCBI would not be a cause of retarded PLB disassembly in *dgd1* because *amiR-MGD1* having similar inhibition in these processes showed rapid PLB disassembly. Considering that the *dgd1* mutant had a remarkably high MGDG-to-DGDG ratio (Fig. 10) and MGDG cannot form bilayer membranes by itself in vitro (Shiple et al. 1973), the high proportion of MGDG in PLBs may inhibit disassembly of PLBs and subsequent formation of lamellar lipid bilayer membranes even after

degradation of LPORs. After 24-h illumination, development of the thylakoid membrane with grana stacks was still strongly arrested in *dgd1* chloroplasts (Fig. 9). Because LHCII is required for thylakoid membrane stacking, lack of LHCII protein may perturb the development of multilamellar thylakoid membrane networks in this mutant. In addition to LHCII and MGDG, DGDG may be involved in stacking the thylakoid membranes, as Kanduč et al. (2017) suggested with molecular dynamics simulations showing that saccharide headgroups of DGDG play an important role in tight cohesion of photosynthetic membranes with stable multilamellar structures. Of note, the *dgd1* mutant showed cotyledon greening (Fig. 8A) and could form a thylakoid membrane (Fig. 9) after longer exposure to light. Because MGDG can form lamellar membranes with LHCII in vitro (Simidjiev et al. 2000), the gradual increase in LHCII content in *dgd1* during illumination may induce thylakoid membrane formation with a lipid pool rich in MGDG. This assumption is supported by the *dgd1* mutant grown under light from the beginning of germination showing accumulated LHCII protein to the wild-type level and forming the highly developed thylakoid membrane as did wild-type chloroplasts (Dörmann et al. 1995, Härtel et al. 1997).

In both +DEX *amiR-MGD1* and *dgd1* seedlings, clusters of high electron-dense particles similar to plastoglobuli were frequently observed along with underdeveloped thylakoid membranes in immature chloroplasts. Etioplasts contain a number of plastoglobuli in PLBs. However, during the etioplast-to-chloroplast differentiation, the abundance of plastoglobuli decreases concomitant with the transformation of PLBs to the thylakoid membrane (Rottet et al. 2015). This observation suggests that plastoglobuli, which include neutral lipids such as prenylquinones, carotenoids, phytylesters and triacylglycerols, contribute to thylakoid development presumably by supplying these hydrophobic compounds. However, in +DEX *amiR-MGD1* and *dgd1* seedlings, the thylakoid development was strongly impaired, so neutral lipids that cannot be distributed to the thylakoid membrane might accumulate in plastoglobule-like lipid droplets.

### Galactolipid metabolism during etioplast-to-chloroplast differentiation

In addition to impaired MGDG accumulation, the light-induced increase in DGDG content was impaired in +DEX *amiR-MGD1* seedlings. Because DGDG is synthesized from MGDG, the impaired DGDG accumulation in +DEX seedlings can be explained by decreased MGDG biosynthesis. After illumination, MGDG content as well as DGDG content was lower in the *dgd1* mutant than the wild type, although MGDG is the substrate for DGDG biosynthesis. Because the expression of both *MGD1* and *DGD1* is downregulated in response to chloroplast dysfunction (Kobayashi et al. 2014), the decrease in one of the galactolipids and consequent impaired chloroplast development may down-regulate the expression of galactolipid biosynthesis genes and decrease the biosynthesis of the other galactolipid. Moreover, *MGD1* needs to be reduced by thioredoxin for activation (Yamaryo et al. 2006), so photosynthetic perturbation may also decrease MGDG biosynthesis activity in these plants.

In  $-DEX$  *amiR-MGD1* and wild-type Col seedlings, FA composition in galactolipids did not largely change before and after illumination (Figs. 4, 11). The data suggest that the galactolipid metabolic pathway is not largely altered during etioplast-to-chloroplast differentiation. In contrast to the  $-DEX$  control,  $+DEX$  *amiR-MGD1* seedlings showed remarkable changes in FA composition of MGDG with illumination, particularly during 6 to 12 h (Fig. 4A). Because galactolipid content did not largely change during this period (Fig. 3), MGDG may be subject to active turnover. Considering that the 16:3-containing MGDG, which is exclusively produced via the plastid pathway, is scarcely used for DGDG biosynthesis as a substrate (Browse and Somerville 1991, Ohlrogge and Browse 1995), the decrease in 16:3 and 18:3 proportions in MGDG of  $+DEX$  seedlings implies active degradation of 18:3/16:3-MGDG molecular species. In contrast, the proportions of 16:1 and 18:0 were increased in  $+DEX$  seedlings. In *Arabidopsis*, 16:1 does not originate from the ER pathway because of lack of particular desaturases (Li-Beisson et al. 2013), so the increase in 16:1 proportion in MGDG may reflect decreased activity of FA desaturase (FAD) 6, which desaturates 16:1 and 18:1 to 16:2 and 18:2 in plastids. This suggestion also implies that FAD5, which specifically desaturates 16:0 at the *sn-2* of MGDG to 16:1 in plastids (Heilmann et al. 2004), is functional in  $+DEX$  seedlings. In addition, the accumulation of 18:0 in MGDG under the  $+DEX$  condition may reflect decreased desaturation of 18:0-ACP to 18:1-ACP by SAD. Because the major function of MGD2 and MGD3 is to supply MGDG to DGDG biosynthesis at the outer envelope (Kobayashi et al. 2009a), presumably via the ER pathway, most of the MGDG in  $+DEX$  seedlings may be derived from MGD1 activity leaked from the *amiR-MGD1* suppression.

In  $+DEX$  seedlings, the FA composition of DGDG changed after illumination, with 16:0 and 18:3 proportions showing an increase and decrease, respectively (Fig. 4B). Considering that the alternative pathway by MGD2, MGD3 and DGD2 preferentially synthesizes 16:0-containing DGDG species (Kelly et al. 2003, Kobayashi et al. 2009a), the contribution of this pathway may be enhanced in  $+DEX$  seedlings vs. the  $-DEX$  control. In this context, the gradual increase in 16:0 proportion in MGDG of  $+DEX$  seedlings may also reflect increased contribution of MGD2 and MGD3 to MGDG biosynthesis. However, no compensatory increases in the mRNA levels of *MGD2*, *MGD3*, *DGD1* and *DGD2* in response to *MGD1* suppression were observed in  $+DEX$  seedlings (see Supplementary Fig. S1). Thus, the increased contribution of the alternative pathway in  $+DEX$  seedlings may simply result from altered metabolic flow from the main MGD1 pathway to the MGD2-MGD3 pathway due to the MGD1 deficiency. Of note, unlike in MGDG, in DGDG of  $+DEX$  seedlings, the 18:0 proportion did not strongly increase after illumination. As discussed earlier, in  $+DEX$  seedlings, 18:0-ACP may be accumulated in addition to 18:1-ACP because of decreased SAD activity. One possibility is that 18:1-ACP is immediately hydrolyzed to free FAs after desaturation by SAD and used for the ER pathway, whereas 18:0-ACP, which is not a good substrate for plastid thioesterases (Ohlrogge and Browse 1995, Li-Beisson et al. 2013), is retained within plastids and used for the plastid pathway. Therefore, in  $+DEX$  seedlings, MGDG produced via the plastid pathway may contain high levels of 18:0, whereas DGDG produced via the ER pathway may be devoid of 18:0. The reason why some desaturase

activities appear to be specifically downregulated in  $+DEX$  seedlings after illumination remains elusive.

FA compositions of MGDG and DGDG greatly differed in the dark-grown *dgd1* mutant from those in the wild type, and the difference continued even after 24 h of illumination (Fig. 11). In the *Arabidopsis* wild type, MGD1 synthesizes C18/C16-MGDG via the plastid pathway and C18/C18-MGDG via the ER pathway (Browse and Somerville 1991, Ohlrogge and Browse 1995). Among these MGDG species, DGD1 mainly consumes the C18/C18-MGDG to form 18:3/18:3-DGDG, and therefore, 18:3/16:3-MGDG remains at a high proportion. In the *dgd1* mutant, many C18/C18-MGDG molecules remain unconverted to DGDG because of lack of DGD1, leading to an accumulation of 18:3/18:3-MGDG, so the proportion of 18:3 in MGDG might increase and that of 16:3 relatively decrease. In the mutant DGDG, the proportion of 18:3 substantially decreased and those of other FAs, particularly 16:0, increased. These changes would be due to the enzymatic property of DGD2 as reported previously (Kelly et al. 2003).

### Requirements of galactolipids for internal membrane transformation during etioplast-to-chloroplast differentiation

In summary, our data show that MGDG deficiency by *MGD1* suppression during the etioplast-to-chloroplast differentiation did not perturb degradation of LPORs and disassembly of PLBs but inhibited the development of thylakoid membrane networks after PLB disassembly. Chl accumulation shortly after illumination was impaired by the MGDG deficiency, presumably because of inhibited membrane-associated processes of the Chl biosynthesis pathway. The *MGD1* suppression did not impair an initial increase in mRNA and protein levels of *LHCB1* at the beginning of greening but abolished their accumulation as well as the light-induced increase in DGDG content at later stages. Downregulation of *LHCB* gene expression and destabilization of LHClI by Chl deficiency both decrease LHClI levels, which, in addition to the direct effect of galactolipid deficiency on membrane formation, may inhibit thylakoid membrane biogenesis after PLB disassembly.

DGDG deficiency by loss of *DGD1* also impaired the accumulation of Chl and *LHCB1* protein after illumination. Unlike MGDG deficiency, DGDG deficiency retarded disassembly of PLB remnants after illumination despite rapid degradation of LPORs. Because loss of *DGD1* results in a high ratio of nonbilayer-forming MGDG to bilayer-forming DGDG, the high proportion of MGDG may impair transformation from PLB remnants to lamellar membranes. However, after long exposure to light, *dgd1* seedlings developed thylakoid membrane networks with grana stacks, presumably with gradually accumulating LHClI, which can induce MGDG to form bilayer membranes.

## Materials and Methods

### Plant materials and growth conditions

The *amiR-MGD1* transgenic lines (Fujii et al. 2014, Fujii et al. 2017) and *dgd1* mutant (Dörmann et al. 1995) were Landsberg *erecta* and Col ecotypes, respectively, of *A. thaliana*. Surface-sterilized seeds were incubated at 4°C for 3 or 4 d in the dark. Seeds were sown on 0.8% (w/v) agar-solidified Murashige and

Skoog medium containing 1% (w/v) sucrose and illuminated for 3 h under room lights to synchronize germination. Then seeds were incubated at 23°C for 4 d in complete darkness. Etiolated seedlings were illuminated with continuous white light (50  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at 23°C. For DEX treatment, DEX (Wako) was added to a final concentration of 10  $\mu\text{M}$  in the medium from a 50 mM stock in dimethyl sulfoxide.

### Fresh weight measurement

Twenty dark-grown seedlings were collected from agar-solidified media before or after illumination and weighed immediately after quick removal of water attached to the sample surface.

### Transmission electron microscopy

Ultrathin sections of fixed cotyledon samples were stained with uranyl acetate and lead citrate and observed by transmission electron microscope as described (Fujii et al. 2017).

### Lipid analysis

Total lipids were extracted from seedlings and separated by thin-layer chromatography as described (Kobayashi et al. 2006, Fujii et al. 2017). The content of MGDG, DGDG and a mixture of other glycerolipids was quantified by gas chromatography (GC-17A; Shimadzu) as described (Fujii et al. 2017).

### Determination of Chl content

To extract Chl, intact seedlings were incubated in 80% (v/v) acetone at 4°C in darkness for 2 or 3 d. Chl content in extracts was determined by measuring the absorbance at 663 and 645 nm with a V-370 BIO (JASCO) spectrophotometer as described (Melis et al. 1987) or measuring fluorescence emission at 666 nm under 440-nm excitation with a RT-5300PC spectrofluorometer (Shimadzu) by using the Chl standard sample of known concentration as described previously (Fujii et al. 2014).

### Immunoblot analysis

For the data in **Figs. 5C, 12C**, 10  $\mu\text{g}$  total protein extracted from seedlings was separated by SDS-PAGE as described (Fujii et al. 2017). For **Fig. 5D**, 50  $\mu\text{g}$  total protein was used to detect faint LHCB1 signals from 3-h illuminated samples. Polyclonal antibodies that react with total LPOR protein (Masuda et al. 2003) and LHCB1 (AgriSera) were used as primary antibodies to detect total LPOR (PORA, PORB and PORC) and LHCB1 protein, respectively. Protein signals were measured as described (Fujii et al. 2017). Ponceau-stained proteins between 35 and 50 kDa blotted on nitrocellulose membranes (Amersham Protran Premium 0.2 NC; GE Healthcare) were loading controls.

### qRT-PCR analysis

Transcript abundance was quantified as described (Fujii et al. 2017). Gene-specific primers shown in Supplementary Table S1 were used to amplify cDNA. To quantify LHCB1 mRNA levels, primers were designed to amplify LHCB1.2 (At1g29910), but might also amplify LHCB1.1 (At1g29920) and LHCB1.3 (At1g29930) with the nucleotide length (381 bp) being the same as that from LHCB1.2, because the forward primer perfectly binds to the LHCB1.1 and LHCB1.3 sequences and the reverse primer has only a single mismatch to the binding sites of LHCB1.1 and LHCB1.3.

### Supplementary Data

Supplementary data are available at PCP online.

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### Disclosures

The authors have no conflicts of interest to declare.

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