Role of MGDG in etioplasts

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Article title
Monogalactosyldiacylglycerol facilitates synthesis of photoactive protochlorophyllide in etioplasts

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One sentence summary
Monogalactosyldiacylglycerol is required for membrane-associated processes of the protochlorophyllide synthesis pathway and formation of protochlorophyllide-enzyme complexes in Arabidopsis etioplasts.

**List of author contributions**

S.F. designed and performed most experiments, analyzed the data and wrote the article; K.K. conceived the project, designed the study and wrote the article; N.N. performed electron microscopic analysis; T.M. and H.W. supervised and complemented the writing.

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Abstract

Cotyledon cells of dark-germinated angiosperms develop etioplasts that are plastids containing unique internal membranes called prolamellar bodies (PLBs). Protoporphyrilide (Pchlide), a precursor of chlorophyll, accumulates in PLBs and forms a ternary complex with NADPH and light-dependent NADPH:Pchlide oxidoreductase (LPOR), which allows for rapid formation of chlorophyll after illumination while avoiding photodamage. PLBs are 3D lattice structures formed by lipid bilayer rich in monogalactosyldiacylglycerol (MGDG). Although MGDG was found to be required for the formation and function of the thylakoid membrane in chloroplasts in various plants, the roles of MGDG in PLB formation and etioplast development are largely unknown. To analyze roles of MGDG in etioplast development, we suppressed MGD1 encoding the major isoform of MGDG synthase by using a dexamethasone-inducible artificial microRNA in etiolated Arabidopsis seedlings. Strong MGD1 suppression caused 36% loss of MGDG in etiolated seedlings, together with a 41% decrease in total Pchlide content. The loss of MGDG perturbed etioplast membrane structures and impaired formation of the photoactive Pchlide-LPOR-NADPH complex and its oligomerization, without affecting LPOR accumulation. The MGD1 suppression also impaired the formation of Pchlide from protoporphyrin IX via multiple enzymatic reactions in etioplast membranes, which suggests that MGDG is required for the membrane-associated processes in the Pchlide biosynthesis pathway. Suppressing MGD1 at several germination stages revealed that MGDG biosynthesis at an early germination stage is particularly important for Pchlide accumulation. MGDG biosynthesis may provide a lipid matrix for Pchlide biosynthesis and formation of Pchlide–LPOR complexes as an initial step of etioplast development.

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Introduction

Angiosperms germinating in darkness develop etioplasts instead of chloroplasts, in cotyledon cells (Solymosi and Schoefs, 2010). Etioplasts have unique internal membrane structures called prolamellar bodies (PLBs), 3D lattice structures of membrane tubules, in addition to lamellar prothylakoid (PT) membranes (Gunning, 1965; Kowalewska et al., 2016). Etioplasts do not contain chlorophyll (Chl), but rather accumulate small amounts of a Chl precursor, protochlorophyllide (Pchlide) (Masuda, 2008). Accumulation of Pchlide in the dark may be an advantage for forming Chl rapidly after light exposure. However, as for Chl and other Chl intermediates, Pchlide is a photosensitizer, and excess accumulation of Pchlide causes photodamage and cell death with light irradiation (Triantaphylidès and Havaux, 2009). Thus, the amount of Pchlide in the dark is strictly controlled by multiple regulatory mechanisms of the biosynthetic pathway (Tanaka et al., 2011). In addition, plants develop a photoprotective system in etioplasts to avoid oxidative damage from photoreactive Pchlide (Solymosi and Schoefs, 2010).

In PLBs, a major portion of Pchlide forms the ternary complex with light-dependent NADPH:Pchlide oxidoreductase (LPOR) and NADPH, which further aggregates into large oligomers (Schoefs and Franck, 2003). LPOR is the membrane-associated enzyme that reduces Pchlide to chlorophyllide (Chlide), the immediate precursor of Chl, by using NADPH and light energy (Heyes and Hunter, 2005). The photoactive form of Pchlide bound by LPOR at the active site is converted instantaneously to Chlide in the light without generating singlet oxygen, whereas nonphotoactive Pchlide, which is not bound to the LPOR active site, easily generates singlet oxygen and causes photobleaching with light (op den Camp et al., 2003). After
photoconversion of Pchlide, LPOR also functions in preventing photodamage from Chlide by forming Chlide-LPOR-NADPH ternary complexes (Solymosi and Schoefs, 2010). Thus, the formation of pigment–LPOR complexes in PLBs is essential for the rapid and safe conversion of etioplasts to chloroplasts during the dark-to-light transition.

The tetrapyrrole biosynthetic pathway in plants has been well characterized as described in comprehensive reviews (Beale, 1999; Moulin and Smith, 2005; Masuda and Fujita, 2008; Tanaka et al., 2011; Brzezowski et al., 2015) and briefly summarized as follows. The biosynthesis of all tetrapyrroles including Pchlide starts from the formation of 5-aminolevulinic acid (ALA) in plastids. ALA biosynthesis is the rate-limiting process of the tetrapyrrole biosynthesis pathway, with glutamyl-tRNA reductase (GluTR) subjected to strict transcriptional and post-translational regulation as a key enzyme of this step. Subsequently, a cascade of enzymatic reactions takes place to form protoporphyrin IX (Proto IX), the last common precursor shared by Chl and heme biosynthesis pathways. Insertion of Mg\(^{2+}\) into Proto IX by Mg-chelatase (MgCh) yields Mg-Proto IX for Chl biosynthesis, whereas insertion of Fe\(^{2+}\) by ferrochelatase results in heme \(b\) in one step. In the Chl biosynthesis pathway, S-adenosyl-L-methionine:Mg-Proto IX methyltransferase (MgMT) esterifies Mg-Proto IX into Mg-Proto IX monomethylester (Mg-Proto IX ME), which is further metabolized to Pchlide by Mg-Proto IX ME cyclase (MgCY). Then Pchlide is converted to Chlide by Pchlide reductase. Angiosperms possess only LPOR, which absolutely requires light for catalysis, whereas cyanobacteria, algae and most other plants have the dark-operative (light-independent) type of Pchlide reductase in addition to LPOR. Therefore, in angiosperms, the Chl biosynthesis pathway is paused at Pchlide in the dark; only after light exposure does LPOR reduce Pchlide into Chlide. In addition, 3,
8-divinyl Pchlide a 8-vinyl reductase, which reduces the 8-vinyl group on the B pyrrole ring, is involved in Chlide formation. Chlide is subsequently esterified with a phytol chain by Chl synthase to result in Chl.

PLB is a lipid bilayer membrane structure rich in glycerolipids relative to proteins and pigments (Selstam and Sandelius, 1984; Williams et al., 1998). The lipid composition of the PLB membrane is similar to that of the thylakoid membrane in chloroplasts, with two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), accounting for ~50% and ~30% of total lipids, respectively, in both membranes (Selstam and Sandelius, 1984; Dorne et al., 1990). These galactolipids are also predominant lipid constituents of the PTs and the envelopes of etioplasts. In plants, MGDG is synthesized in plastid envelopes in a one-step reaction by MGDG synthase, which transfers the Gal moiety from UDP-Gal to diacylglycerol (Benning and Ohta, 2005). DGDG is synthesized from MGDG, so MGDG biosynthesis is also essential for DGDG biosynthesis. Three isoforms of MGDG synthase, namely MGD1, MGD2 and MGD3, have been identified in *Arabidopsis (Arabidopsis thaliana)*; inner-envelope–localized MGD1 is responsible for most of the MGDG biosynthesis for thylakoid biogenesis, whereas outer-envelope–localized MGD2 and MGD3 mainly provide MGDG for DGDG biosynthesis specifically under phosphate-starved conditions (Kobayashi et al., 2009b).

The requirement of MGDG biosynthesis for chloroplast development has been demonstrated by analyses of *MGD1* mutants. Partial deficiency of MGDG by knockdown mutations in *MGD1* decreased the amount of the thylakoid membrane, Chl content, and photosynthetic activity in *Arabidopsis* (Jarvis et al., 2000; Fujii et al., 2014) and tobacco (Wu et al., 2013). A knockout mutation of *MGD1 (mgd1-2)* in
Arabidopsis, which resulted in severe loss of both galactolipids, strongly impaired thylakoid membrane development and completely abolished photosynthetic activity (Kobayashi et al., 2007; Kobayashi et al., 2013). MGDG biosynthesis is also required for coordinated expression of nuclear- and plastid-encoded photosynthesis-associated genes responsible for chloroplast development (Kobayashi et al., 2013; Fujii et al., 2014). Thus, MGDG biosynthesis is one of the determinant steps of chloroplast biogenesis in plants.

Considering the abundance of galactolipids in PLBs, MGDG biosynthesis may also play a crucial role in etioplast development in the dark. In fact, MGDG is involved in making PLB-like cubic structures in vitro (Brentel et al., 1985), and the interaction between LPOR and MGDG has been hypothesized to contribute to PLB formation (Klement et al., 1999; Engdahl et al., 2001; Selstam et al., 2002). Very recently, Gabruk et al. (2017) demonstrated that MGDG promotes oligomerization of the Pchlide-LPOR complexes in vitro. However, mutant analyses so far have not provided conclusive information on the role of MGDG in PLB formation and etioplast development. Double knockout mutations of MGD2 and MGD3 did not affect galactolipid content in etiolated seedlings, which suggests that the remaining MGD1 is the main isoform responsible for galactolipid biosynthesis in etioplasts as in chloroplasts (Kobayashi et al., 2009a). However, Jarvis et al. (2000) reported that the MGD1 knockdown mutant (mgd1-1), with 42% reduced MGDG content in light-grown leaves, showed no noticeable defects in etioplast development. Meanwhile, the knockout mgd1-2 mutant cannot be used for analysis of etioplast development because it does not develop cotyledons owing to severe inhibition of embryogenesis (Kobayashi et al., 2007).

Recently, we generated Arabidopsis transgenic lines carrying a dexamethasone
(DEX)-inducible artificial microRNA targeting \textit{MGD1} (ami\textit{R-MGD1}) (Fujii et al., 2014). In the previous study, these lines showed up to 75\% reduction in \textit{MGD1} expression in a DEX-dependent manner, which resulted in up to 90\% reduction in MGDG content in light-grown seedlings as compared with the DEX-free (−DEX) control. In ami\textit{R-MGD1} lines, we could eliminate the effect of the \textit{MGD1} deficiency on embryo development by harvesting seeds under −DEX conditions. Taking advantage of the inducible knockdown system of ami\textit{R-MGD1} lines, we investigated the roles of MGD1 in galactolipid biosynthesis, Pchlide biosynthesis, formation of photoactive Pchlide-LPOR-NADPH complexes and development of PLBs during etiolated seedling growth.
Results

Screening of phenotypically homogeneous lines of amiR-MGD1

We previously reported that the T3 generation of amiR-MGD1 lines grown under DEX-treated (+DEX) conditions showed various color phenotypes from green to white in cotyledons even within a single homozygous line, presumably due to fluctuations of suppression levels of MGD1 expression (Fujii et al., 2014). Because the heterogeneity in a single amiR-MGD1 line prevented detailed analyses of etiolated seedlings, we attempted to isolate phenotypically homogeneous amiR-MGD1 lines. Screening of T4 generations of the amiR-MGD1 line 4 (L4) (Fujii et al., 2014) under +DEX conditions identified several lines showing homogeneous cotyledon phenotypes (Supplemental Fig. S1A). In L4-01, L4-03, L4-04, L4-07, L4-09, and L4-11 lines, all seedlings had albino cotyledons under +DEX conditions, with Chl content decreased to 10% of the −DEX control (Supplemental Fig. S1B). By contrast, in L4-02, L4-05, L4-06, L4-08 and L4-10, all seedlings had green cotyledons regardless of DEX treatment. In these green lines, Chl content was only slightly decreased with DEX treatment (Supplemental Fig. S1B). Phenotypically homogeneous lines were also obtained from the amiR-MGD1 L2 line (Supplemental Fig. S1C), so these phenomena are not specific to the L4 line.

To examine the relationship between cotyledon phenotypes and suppression levels of the MGD1 expression, we determined MGD1 mRNA levels in 5-d-old seedlings of homogeneous L4 lines (Supplemental Fig. S1D). In DEX-induced albino lines (L4-01, L4-03 and L4-04), MGD1 mRNA levels were decreased to 20% or less, whereas those in green lines (L4-02, L4-05 and L4-06) ranged from ~35% to ~50% as compared with the −DEX control. These data are consistent with a previous report showing that the reduced MGD1 expression to ~25% but not to ~40% of wild-type
levels caused an albino cotyledon phenotype (Fujii et al., 2014). We conclude that L4-01, L4-03, L4-04, L4-07, L4-09, and L4-11 were homogeneous lines that strongly suppressed the \textit{MGD1} expression in a DEX-dependent manner. Because these 6 lines, originally derived from a single T2 plant, showed almost the same phenotype in \textit{MGD1} expression, Chl content, and seedling growth under +DEX conditions, we used a mixture of these lines, called L4w, for further analyses. We also used a mixture of L4-02, L4-05, L4-06, L4-08 and L4-10, called L4g, showing the homogeneous green cotyledon phenotype under +DEX conditions. Likewise, we used L2-01, called L2w, and a mixture of L2-03 and L2-04, called L2g, for homogeneous L2 lines with white and green cotyledons, respectively, under +DEX conditions.

\textbf{MGD1 suppression decreases MGDG content in etiolated seedlings}

To reveal the roles of MGD1 during etioplast development, we investigated etiolated \textit{amiR-MGD1} seedlings under −DEX and +DEX conditions. In 4-d-old L4w and L4g seedlings grown under +DEX conditions in the dark, \textit{MGD1} mRNA levels were decreased to 35% and 53%, respectively, of the −DEX control (Fig. 1A). The result confirms stronger \textit{MGD1} suppression in L4w than L4g even in etiolated seedlings. A similar result was observed in L2 seedlings (Supplemental Fig. S2A). To assess whether the \textit{MGD1} suppression affected galactolipid biosynthesis in etiolated seedlings, we analyzed galactolipid content in etiolated L4w seedlings (Fig. 1B). In L4w seedlings, the proportion of MGDG in total membrane lipids was decreased from 6.0 mol% under −DEX conditions to 3.8 mol% under +DEX conditions. By contrast, the proportion of DGDG did not noticeably differ between +DEX and −DEX seedlings, so the MGDG to DGDG ratio decreased from 1.37 in the −DEX control to 0.96 in +DEX seedlings. For
both MGDG (Fig. 1C) and DGDG (Fig. 1D), fatty acid compositions were not greatly altered by DEX treatment. The DEX-dependent decrease in relative MGDG content without altered DGDG content in etiolated L4w seedlings allowed us to investigate the specific effects of the MGDG deficiency in etioplast development.

Figure 1. Effect of MGD1 suppression on galactolipid biosynthesis in etiolated seedlings. A, Quantitative reverse transcription-PCR analysis of MGD1 mRNA level in 4-d-old etiolated seedlings of amiR-MGD1 under +DEX and −DEX conditions. Data are presented as fold difference from the −DEX control after normalizing to the control gene ACTIN8. Data are mean ± SE from 13 (L4w) or 3 (L4g) independent experiments. B, Accumulation of MGDG and DGDG in 4-d-old etiolated seedlings of amiR-MGD1 L4w. C and D, Fatty-acid composition of MGDG (C) and DGDG (D) in 4-d-old etiolated seedlings of amiR-MGD1 L4w. In B, C and D, data are mean ± SE from three independent experiments. In A to D, asterisks indicate significant differences from the −DEX control (* P < 0.05, ** P < 0.01, *** P < 0.001, Student’s t-test).
MGD1 suppression impairs Pchlide accumulation in etiolated seedlings

To reveal the contribution of MGDG to Pchlide accumulation, we quantified the total amount of Pchlide in amiR-MGD1 seedlings (Fig. 2A). DEX treatment from the beginning of germination decreased total Pchlide content in etiolated L4w seedlings to 59% of the −DEX control without affecting the size of cotyledons (Supplemental Fig. S3). By contrast, total Pchlide content in L4g seedlings was not changed by DEX treatment (Fig. 2A). Similar data were obtained from L2 lines (Supplemental Fig. S2, B and C). Thus, similar to Chl content, Pchlide content was affected only when MGD1 expression was strongly suppressed. The data also show that DEX treatment itself had no effect on Pchlide accumulation. To examine when MGDG biosynthesis was required for Pchlide accumulation, we delayed the start time of DEX treatment after seeding and determined Pchlide content in etiolated 4-d-old L4w seedlings (Fig. 2B). DEX treatment from 1 d after seeding decreased Pchlide accumulation in L4w seedlings but to a slightly smaller extent than with DEX treatment from the beginning. By contrast, DEX treatment from 2 d after seeding did not decrease Pchlide content. The data suggest that the MGD1 expression at the very early stage of germination is particularly important for Pchlide accumulation.

Pchlide in etiolated seedlings is distinguished as photoactive and nonphotoactive forms; only photoactive Pchlide can be immediately converted into Chlide by short light treatment (Schoefs, 2001). To assess which type of Pchlide was decreased by MGDG deficiency in etiolated L4w seedlings, we determined Pchlide content after a 0.7-ms light flash, which represented the amount of nonphotoactive Pchlide (Fig. 2A). The amount of nonphotoactive Pchlide was not changed by DEX treatment and so the
proportion of photoactive to total Pchlide was decreased from 78% in −DEX seedlings to 63% in +DEX seedlings. A similar result in L2w (Supplemental Fig. S2B) supports
that $MGD1$ suppression mainly decreases photoactive Pchlide content in the dark. Meanwhile, etiolated L4g seedlings showed a slight increase in nonphotoactive Pchlide content (Fig. 2A).

We also performed a time-course analysis of Pchlide accumulation during etioplast development in L4w seedlings (Fig. 2C). In 2-d-old etiolated seedlings, Pchlide content was very low under both +DEX and −DEX conditions. However, in −DEX seedlings, Pchlide content was sharply increased until 5 d after seeding. Although Pchlide content was also gradually increased in +DEX seedlings, the rate was much lower than in the −DEX control. Because photoactive Pchlide is associated with LPOR proteins, we examined total LPOR content in 4-d-old etiolated L4w seedlings by using polyclonal anti-LPOR antibodies that recognize all *Arabidopsis* LPOR isoforms (Rowe and Griffiths, 1995; Masuda et al., 2003). Immunoblot analysis showed that the $MGD1$ suppression by DEX treatment did not noticeably change LPOR levels in etiolated seedlings (Fig. 2D).

According to Moro et al. (2004), inhibition of carotenoid biosynthesis in etiolated seedlings impairs the accumulation of photoactive Pchlide and formation of PLBs without altering LPOR protein levels. To investigate whether MGDG deficiency affects carotenoid biosynthesis in etiolated seedlings, we measured total carotenoid content in 4-d-old etiolated L4w seedlings (Fig. 2E). Carotenoid content was not affected by DEX treatment in etiolated seedlings. In addition, absorption spectra between ~420 nm and ~490 nm, mostly derived from a composite of carotenoids in etiolated seedlings (Böddi et al., 1989), were similar in +DEX and −DEX seedlings (Supplemental Fig. S4), so the carotenoid composition may also be unchanged by the $MGD1$ suppression.

Although our data from the amiR-$MGD1$ lines suggest that MGD1 is required for
accumulation of photoactive Pchlide, Aronsson et al. (2008) previously reported that the ratio of photoactive to nonphotoactive Pchlide was increased in the etiolated mgd1-1 mutant. Thus, we investigated the amount of total and nonphotoactive Pchlide in etiolated seedlings of mgd1-1 and corresponding wild-type (Columbia) in the same experimental condition as amiR-MGD1 (Supplemental Fig. S2D). In our experiments, the mgd1-1 mutation decreased total Pchlide content in etiolated seedlings without affecting the ratio of photoactive to nonphotoactive Pchlide. The discrepancy between our results and those by Aronsson et al. (2008) might be due to some differences in experimental conditions; for example, Aronsson et al. grew seedlings on soil for 5 d, whereas we grew them on agar-solidified media containing 1% Suc for 4 d.

MGDI suppression impairs the formation of the photoactive Pchlide-LPOR-NADPH complex and its oligomerization

To evaluate the role of MGDG in the formation of the Pchlide-LPOR-NADPH ternary complex in PLBs, we measured fluorescence spectra of Pchlide at 77K in etiolated amiR-MGD1 L4w seedlings (Fig. 3A). Photoactive and nonphotoactive forms of Pchlide can be optically distinguished with their fluorescence peaks at about 655 and 633 nm, respectively, under 77K (Schoefs, 2001; Solymosi et al., 2007). Furthermore, photoactive Pchlide includes 2 forms, one emitting fluorescence at ~645 nm at 77K in the dimeric ternary complex, which is minor in typical etioplasts in several plants, and the other, the major form in PLBs, emitting fluorescence at ~657 nm in oligomeric aggregates of the complex (Böddi et al., 1989; Schoefs, 2001). In our experimental conditions, we observed two fluorescence bands peaking at about 630 nm (P_{630}) and 653 nm (P_{653}) in the −DEX control. In +DEX seedlings, the peak position of P_{653} but not
P_630 was slightly blue-shifted as compared to that in the −DEX control (Fig. 3A and Table 1). A difference spectrum between +DEX and −DEX samples revealed increased fluorescence at ~645 nm with decreased fluorescence at ~655 nm in +DEX seedlings relative to the −DEX control (Fig. 3B). The data suggest an increase in dimeric ternary
complex and a decrease in large aggregates with DEX treatment. The blueshift of P$_{653}$ was not found in etiolated seedlings of +DEX L4g (Table 1 and Supplemental Fig. S5A) and mgd1-1 (Supplemental Fig. S5, B and C), confirming that the spectral change observed in L4w was caused by strong MGD1 suppression.

To examine whether the MGD1 suppression affected the rapid photoconversion of Pchlide, we treated etiolated L4w seedlings with a 0.7-ms light flash before 77K fluorescence measurement (Fig. 3C). In both $-$DEX and +DEX seedlings, P$_{653}$ was no longer observed after flash irradiation, whereas P$_{630}$ remained, which substantiates that these two bands were attributed to photoactive and nonphotoactive Pchlide, respectively. In addition, a prominent band peaking at 689 nm (C$_{689}$), which originates from the Chlide-LPOR-NADP$^+$ complex (Schoefs, 2001; Solymosi et al., 2007), emerged after flash irradiation of both $-$DEX and +DEX seedlings. Thus, the Pchlide-LPOR-NADPH complex was efficiently converted to the Chlide-LPOR-NADP$^+$ complex with flash irradiation even when MGDG biosynthesis was suppressed.

After illumination, the fluorescence maximum around 690 nm gradually shifted to $\sim$680 nm in the dark, presumably as a result of disaggregation or rearrangement of large oligomers of Chlide-LPOR complexes (Shibata, 1957; Smeller et al., 2003; Solymosi et al., 2007). During this process, called “Shibata shift”, major spectral changes are completed within 20 min after irradiation, followed by small shifts continuing until 2 or 3 h. To investigate the contribution of MGDG to subsequent processes after Pchlide photoconversion, we incubated etiolated L4w seedlings in the dark for 20 min (Fig. 3D) or 2 h (Fig. 3E) after flash irradiation and measured fluorescence spectra at 77K. In both $-$DEX and +DEX seedlings, C$_{689}$ was completely shifted to a peak at 678 nm (C$_{678}$) during the first 20 min after flash irradiation. After dark incubation for 2 h, the peak
wavelength of C$_{678}$ was slightly shifted to 677 nm, regardless of DEX treatment. These data suggest that the *MGDI* suppression did not remarkably affect the Shibata shift in etiolated seedlings. Meanwhile, DEX treatment largely affected regeneration of photoactive Pchlide during dark incubation after illumination. In −DEX seedlings, the P$_{653}$ emission from photoactive Pchlide emerged at 20 min after dark incubation and became more prominent after 2 h (Fig. 3, D and E). The P$_{653}$ emission was also increased in +DEX seedlings during dark incubation but to a lesser extent than in the −DEX control. Moreover, the peak wavelength of P$_{653}$ after 2 h of dark incubation was slightly blue-shifted in +DEX seedlings, as was observed before flash irradiation (Table 1). Consistent with this result, a band peaking at around 645 nm, which would originate from the dimer of the Pchlide-LPOR-NADPH ternary complex, was again observed in a difference spectrum between +DEX and −DEX samples (Fig. 3B). To evaluate the regeneration activity of photoactive Pchlide after flash irradiation, we determined Pchlide content in L4w seedlings incubated in the dark for 20 min after flash irradiation (Fig. 3F). In +DEX seedlings, total Pchlide content was 55% lower than the −DEX control, mainly due to a reduction in photoactive Pchlide content. These data suggest that MGDG deficiency impairs regeneration of the photoactive Pchlide-LPOR-NADPH complex after flash irradiation.

**MGDI suppression affects membrane organization in etioplasts**

To assess the role of MGDG in PLB formation, we observed the ultrastructure of etioplasts in 4-d-old L4w seedlings by transmission electron microscopy (Fig. 4 and Supplemental Fig. S6). In the −DEX control, etioplasts had the regular lattice membrane
structure connecting to short lamellae (Fig. 4, A and B, Supplemental Fig. S6, A to H).

Although DEX treatment did not abolish the development of etioplasts (Fig. 4, C and D, Supplemental Fig. S6, I to S), some etioplasts in +DEX seedlings had irregular-shaped PLBs. Quantitative analysis revealed that the circularity but not the total size of PLBs

Figure 4 Ultrastructure of etioplasts in cotyledon cells of 4-d-old etiolated umiR-MGD1 14w seedlings. A and C, Images of whole etioplasts in cotyledons grown under -DEX (A) and +DEX (C). Bars = 1.0 μm. B and D, Magnified images of PLB lattices in A (B) and C (D). Bars = 200 nm. For more images, see Supplemental Fig. S6. E-K, Quantitative data of circularity index (E) and area (F) of PLBs, area of a single PLB unit (G), relative SD value (SD/average) of the unit area in a PLB (H), length of PTs (I), and circularity index (J) and area (K) of etioplasts. The horizontal line in each box represents the median value of the distribution. The top and bottom of each box represent the upper and lower quartiles, respectively. The whiskers represent the range. Data were obtained from 46 different etioplasts. In H, the relative SD value was calculated from 20 units of the PLB in each etioplast. Distribution of the PLB unit area in each etioplast is shown in Supplemental Fig. S7. Asterisks indicate significant differences from the -DEX control (* P < 0.05, ** P < 0.01, *** P < 0.001, Welch's t-test).
was decreased in +DEX seedlings compared with the −DEX control (Fig 4, E and F). In addition, the unit size of the PLB lattice was increased by DEX treatment (Fig. 4G), with relative SD values of the unit area in a PLB, used as an index of irregularity of the lattice crystalline structure, also increased in +DEX seedlings (Fig. 4H and Supplemental Fig. S7). Besides the morphological changes in PLBs, the length of PTs was shorter in +DEX seedlings than the −DEX control (Fig. 4I). Moreover, the shape of etioplasts was disordered by MGD1 suppression, as represented by decreased circularity of etioplasts in +DEX seedlings (Fig. 4J). Intrusion of cytosolic regions into etioplasts was more frequently observed in +DEX seedlings (13 of 46 etioplasts; Supplemental Fig. S6, K, P, Q, R and S; red arrowheads) than the control (1 of 46 etioplasts), although the size of etioplast was not largely different in both conditions (Fig. 4K).

**MGD1 suppression impairs membrane-associated processes of the Pchlide biosynthesis pathway**

The decreased content of total Pchlide in +DEX L4w seedlings suggested impaired Pchlide biosynthesis by MGDG deficiency. To evaluate the effect of MGDG deficiency on the Pchlide biosynthesis pathway, we measured porphyrin intermediates in *amiR-MGD1* etiolated seedlings. Porphyrin pigments in the Chl biosynthesis pathway, such as Proto IX, Mg-Proto IX, Mg-Proto IX ME and Pchlide, can be distinguished by their own unique fluorescence characteristics, whereas ALA and other non-porphyrin intermediates do not emit fluorescence. Porphyrin pigments extracted from 4-d-old etiolated seedlings were separated and detected by high-performance liquid chromatography (HPLC) with a fluorescence detector (Supplemental Fig. S8A). In both −DEX and +DEX seedlings, no porphyrin pigments besides Pchlide were detected.
Because porphyrin metabolism in the dark is strictly regulated particularly at the ALA biosynthesis step (Brzezowski et al., 2015), it is not surprising that toxic porphyrin intermediates were not detected in *amiR-MGD1* even under +DEX conditions.

To bypass the rate-limiting step of ALA biosynthesis, we fed 10 mM ALA to dark-grown *amiR-MGD1* L4w seedlings for 1.5 h (Fig. 5A and Supplemental Fig. S8A) and 24 h (Fig. 5B). This method is commonly used for measuring the activity of porphyrin metabolism *in planta* (Terry and Kendrick, 1999; Tottey et al., 2007). In −DEX seedlings, Pchlide content was increased 3- and 30-fold with ALA feeding for 1.5 h and 24 h, respectively (Fig. 5, A and B; cf. Fig. 2A). Small amounts of Proto IX and Mg-Proto IX ME were also accumulated with ALA feeding, but Mg-Proto IX was undetectable even after ALA feeding for 24 h. In +DEX seedlings, Pchlide accumulated more slowly than in the −DEX control; the content after 24-h ALA feeding was only 30% of the −DEX control level (Fig. 5B). Instead, +DEX seedlings accumulated a substantial amount of Mg-Proto IX, which was not detected in the −DEX control, in addition to larger amounts of Proto IX and Mg-Proto IX ME than in −DEX seedlings.

Excess accumulation of porphyrin intermediates was already observed after 1.5 h of ALA feeding (Fig. 5A) and enhanced after 24 h (Fig. 5B). We also measured porphyrin levels in *amiR-MGD1* L4g seedlings treated with ALA for 24 h (Fig. 5C). DEX treatment to L4g etiolated seedlings neither decreased Pchlide content nor enhanced the accumulation of other intermediates, so changes in the porphyrin profile in L4w were not due to side effects of DEX treatment but resulted from strong *MGD1* suppression and consequent MGDG deficiency. In addition, there were no notable differences in porphyrin accumulation between etiolated *mgd1-1* and wild-type seedlings with ALA feeding for 24 h (Supplemental Fig. S8C).
To address which processes of porphyrin biosynthesis are particularly affected by MGDG deficiency in etioplasts, we compared porphyrin profiles in +DEX L4w seedlings with those in Chl biosynthesis mutants after ALA feeding (Fig. 5, D and E, Supplemental Fig. S8A). *chlm* (Mochizuki et al., 2008) and *chl27/crd1* (Ankele et al.,...
The chlm mutant displayed higher accumulation of Mg-Proto IX and Proto IX with ALA feeding, particularly with 24-h feeding. Mg-Proto IX ME, the product of the MgMT reaction, was undetectable in chlm with 1.5-h ALA feeding and remained at low levels with 24-h feeding. In contrast, the high accumulation of Mg-Proto IX and Proto IX, Pchlide formation was strongly inhibited in this mutant. In the chl27 mutant, which is partially deficient in MgCY activity converting Mg-ProtoIX ME to Pchlide, Mg-Proto IX ME was strongly accumulated along with Proto IX and Mg-Proto IX with ALA feeding. However, the chl27 mutation did not notably affect the Pchlide accumulation with ALA feeding. These data reveal that +DEX amiR-MGD1 L4w seedlings showed a porphyrin profile similar to that of chlm, but unlike chlm, L4w also highly accumulated Proto IX and Mg-Proto IX ME even after short ALA feeding (Fig. 5A).

To reveal the mechanism how MGD1 suppression affects the Pchlide biosynthesis pathway, we investigated mRNA levels of genes involved in Pchlide biosynthesis in amiR-MGD1 etiolated seedlings (Fig. 5F). HEMAI encodes the major isoform of GluTR. CHLH, CHLD and CHLII encode H, D and the major isoform of I subunits of MgCh, respectively, whereas GUN4 encodes the GUN4 protein, which is required for MgCh activity. CHLM is a single gene for MgMT. CHL27 and LOW CHLOROPHYLL ACCUMULATION A (LCAA) encode two membrane-bound subunits constituting MgCY (Tanaka et al., 2011; Albus et al., 2012). In etiolated L4w seedlings, steady-state mRNA levels of these Pchlide synthesis genes were unchanged by DEX treatment.
However, the mRNA level of \textit{CHL27} was slightly decreased in +DEX L4w, but a similar decrease was also observed in +DEX L4g seedlings. Because +DEX L4g seedlings showed normal porphyrin metabolism (Fig. 5C), the decreased \textit{CHL27} expression does not likely affect the MgCY activity.

\textit{MGD1} suppression is unlikely to affect expression of photosynthesis-associated genes in etiolated seedlings

We previously revealed in the \textit{mgd1-2} mutant, galactolipid biosynthesis and subsequent thylakoid development is crucial for the expression of photosynthesis-associated genes encoded in the nucleus and plastids in light-grown \textit{Arabidopsis} seedlings (Kobayashi et al., 2013). Indeed, \textit{MGD1} suppression in \textit{amiR-MGD1} during an early stage of chloroplast development downregulated photosynthesis-associated genes (Fujii et al., 2014). We investigated whether the decreased MGDG content in etioplasts also affects the mRNA expression of plastid-encoded photosynthesis-associated genes in etiolated \textit{amiR-MGD1} L4w seedlings (Fig. 6). The genes \textit{psaA} and \textit{psbA} encode PsaA and D1 proteins in reaction centers of PSI and PSII, respectively, and \textit{petB} encodes the cytochrome \textit{b}_6 subunit. The genes \textit{rbcL} and \textit{rps14} are for the large subunit of Rubisco and the 14S subunit of plastidic 30S ribosome, respectively. The genes \textit{accD} and \textit{rpoB} encode the βCT subunit of acetyl-CoA carboxylase and the β subunit of plastid-encoded RNA polymerase, respectively. The mRNA levels of these genes were unchanged with DEX treatment in etiolated L4w seedlings, which suggests that partial MGDG deficiency does not affect plastid-encoded gene expression in etioplasts. We also examined the mRNA levels of photosynthesis-associated nuclear genes, \textit{LHCA4} and \textit{LHCB6}, encoding the
light-harvesting complex (LHC) I subunit 4 and the LHCII subunit 6, respectively, and found no altered expression of these genes with *MGD1* suppression in etiolated seedlings (Fig. 6B).

Impaired porphyrin metabolism often causes production of reactive oxygen species.
and oxidative damage under light (Triantaphylidès and Havaux, 2009). Moreover, a
chlorophyll metabolite, pheophorbide $a$, is reported to induce cell death with increased
hydrogen peroxide even in the dark (Hirashima et al., 2009). $AAA$-$ATPase$ and $BON$
$ASSOCIATED PROTEIN1$ ($BAP1$) are singlet oxygen-induced genes (Šimková et al.,
2012), whereas $ASCORBATE PEROXIDASE2$ ($APX2$) and $ZAT ZINK FINGER$
$PROTEIN10$ ($ZAT10$) are induced by superoxide and altered plastid redox state (Pogson
et al., 2008). mRNA levels of these 4 nuclear-encoded genes were not changed by
$MGD1$ suppression (Fig. 6B), so the partial MGDG deficiency in the dark does not
globally affect gene expression in plastids and nucleus.
Discussion

Identification of homogeneous albino lines of amiR-MGD1 transgenic Arabidopsis

In this study, we identified homogeneous T4 amiR-MGD1 lines that develop albino cotyledons in the presence of DEX under light (Supplemental Fig. S1). MGD1 mRNA levels in these lines were reduced to less than 20% of the −DEX control with DEX treatment. We also obtained homogeneous green amiR-MGD1 lines, showing MGD1 mRNA levels reduced by DEX treatment to 35~50% of the −DEX control but Chl content only slightly decreased. These results are consistent with a previous observation in T3 amiR-MGD1 lines that strong suppression of MGD1 expression less than 30% of wild-type levels caused a white cotyledon phenotype, whereas milder MGD1 suppression (~50% of wild-type levels) resulted in a wild-type–like green cotyledon phenotype (Fujii et al., 2014). Similar results in two independent amiR-MGD1 lines (L2 and L4) (Supplemental Fig. S1) suggest that these phenomena are not due to the positional effect of the transgene. Under our growth conditions, mgd1-1 seedlings grown in the light showed a phenotype similar to that of +DEX green seedlings of amiR-MGD1 lines: Chl content was only slightly decreased in mgd1-1, whereas the MGD1 mRNA level was reduced to 38% of the wild-type level (Supplemental Fig. S9).

These data suggest the existence of a threshold level of the MGD1 expression at ~35% of the wild-type level to maintain regular chloroplast development in cotyledons (Supplemental Fig. S10). Galactolipid biosynthesis would be one of the determinant processes in chloroplast development (Kobayashi, 2016), and decreased MGD1 expression below the threshold may cause severe deficiency of MGDG and subsequent discontinuation of chloroplast development at early stages in cotyledons. We note that the homogeneity of the T4 amiR-MGD1 lines is not genetically fixed across generations.
L4-01 plants, one of the DEX-dependent albino T4 lines, generated T5 lines with various cotyledon color phenotypes under +DEX conditions (Supplemental Fig. S11). The suppression levels of \( MGD1 \) expression in \( amiR-MGD1 \) lines may be prone to fluctuation in response to growth conditions of parent plants, although the underlying mechanism remains to be elucidated.

**MGD1 is responsible for MGDG biosynthesis in etioplasts**

Our analyses with homogeneous \( amiR-MGD1 \) L4w plants revealed that DEX treatment suppressed the \( MGD1 \) expression in etiolated seedlings to 35% of the −DEX control, which resulted in decreased MGDG content to 64% of the control level (Fig. 1). By contrast, loss of function of both MGD2 and MGD3 did not affect galactolipid content in etiolated seedlings (Kobayashi et al., 2009a). Therefore, MGD1 plays a central role in MGDG biosynthesis during etioplast development as it does during chloroplast development. Although MGDG is used as a substrate for DGDG biosynthesis, \( MGD1 \) suppression did not change DGDG content and so the MGDG/DGDG ratio was reduced in +DEX etiolated L4w seedlings (Fig. 1B). This result is consistent with previous reports of light-grown seedlings showing that partial decreases in MGD1 activity primarily result in a loss of MGDG without affecting DGDG biosynthesis (Jarvis et al., 2000; Wu et al., 2013; Fujii et al., 2014). Although we cannot exclude that MGD2 and MGD3, localized to the outer envelope membrane of plastids and using diacylglycerol pools different from the MGD1 pathway localized to the inner envelope (Kobayashi et al., 2009b), specifically function to produce DGDG in etioplasts, unchanged fatty acid compositions in both galactolipids by DEX treatment (Fig. 1, C and D) imply a negligible contribution of the MGD2/MGD3-mediated
galactolipid biosynthesis pathway.

**MGDG is required for the Pchlide biosynthesis pathway in etioplasts**

Strong suppression of the *MGD1* expression decreased the total amount of Pchlide in etiolated L2w and L4w seedlings (Fig. 2A, Supplemental Fig. S2B). Moreover, the rate of Pchlide accumulation was remarkably lower in +DEX L4w seedlings than the −DEX control (Fig. 2C), which suggests that Pchlide biosynthesis is strongly retarded by MGDG deficiency. ALA feeding experiments revealed that Mg-Proto IX metabolism in the Pchlide biosynthesis pathway was particularly impaired by strong *MGD1* suppression (Fig. 5, B and C). The porphyrin profile in +DEX L4w seedlings after ALA feeding (Fig. 5, A and B) was similar to that in the ALA-fed *chlm* mutant (Fig. 5, D and E), so conversion of Mg-Proto IX to Mg-Proto IX ME by *CHLM*-encoded MgMT may be strongly impaired by MGDG deficiency. However, unlike the *chlm* mutant, +DEX L4w seedlings also rapidly accumulated Proto IX and Mg-Proto IX ME after ALA feeding. Thus, MGDG deficiency may also impair the metabolisms of Proto IX by MgCh and Mg-Proto IX ME by MgCY. Despite strong impairments of the Pchlide biosynthesis pathway, particularly Mg-Proto IX metabolism, no porphyrin intermediates were accumulated in +DEX etiolated L4w seedlings in the absence of ALA (Supplemental Fig. S8A). Deficiency of MgCh, MgMT and MgCY activities by genetic manipulation is reported to suppress ALA biosynthesis (Papenblock et al., 2000; Alawady and Grimm, 2005; Peter et al., 2010; Schlicke et al., 2014), possibly via increased metabolic flow from Proto IX to heme and consequent feedback inhibition of GluTR activity by heme (Vothknecht et al., 1996; Terry and Kendrick, 1999; Goslings et al., 2004), which prevents the accumulation of porphyrin intermediates in mutant plants.
Therefore, impaired porphyrin metabolism by MGDG deficiency likely downregulates ALA biosynthesis in a feedback manner, which may further reduce Pchlide accumulation in +DEX L4w seedlings in the dark. Meanwhile, the biosynthetic pathway from ALA to Proto IX may not be affected by MGDG deficiency, because the sum of

Figure 7. Roles of MGDG in Pchlide biosynthesis and formation of photoactive Pchlide-LPOR-NADPH complexes during etiolated development. Arrowheads indicate enzymatic steps in the Pchlide biosynthesis pathway from 5-aminolevulinic acid (ALA). Most of the Pchlide synthesized in etioplasts forms the photoactive ternary complex with LPOR and NADPH, and the photoactive complex exists as the dimer or further aggregates into oligomeric complexes. MGDG is required for the Pchlide biosynthesis pathway from protoporphyrin IX (Proto IX) to Pchlide (arrow 1), the formation of the photoactive Pchlide-LPOR-NADPH ternary complex (arrow 2), and oligomerization of the ternary complex (arrow 3).
Proto IX, Mg-Proto IX, Mg-Proto IX ME and Pchlide content after ALA feeding was not obviously reduced in +DEX seedlings, or seemed even higher, as compared with that in the −DEX control (Fig. 5, A and B). Therefore, MGDG is required for efficient porphyrin metabolism from Proto IX to Pchlide (Fig. 7, arrow 1) and somehow affects ALA biosynthesis but is less important for the pathway from ALA to Proto IX.

Quantitative reverse transcription-PCR analysis suggested that impaired Pchlide biosynthesis in +DEX L4w seedlings was not attributed to transcriptional modification of genes involved in this pathway (Fig. 5F). Considering that the Chl biosynthesis pathway downstream from Proto IX formation takes place in plastid membranes rich in MGDG, several possibilities for impaired porphyrin metabolisms by the MGD1 suppression can be considered. A 36% loss of MGDG in +DEX L4w seedlings slightly but significantly changed membrane structures in etioplasts, although it did not severely perturb etioplast development (Fig. 4 and Supplemental Fig. S6). Thus, a disordered local lipid environment by MGDG deficiency rather than inhibited etioplast biogenesis may affect the Pchlide biosynthesis pathway. Because MgCh, MgMT and MgCY are bound to plastid membranes (Masuda and Fujita, 2008), altered lipid compositions with reduced MGDG content, which would change fluidity and/or local structures of the membrane (Demé et al., 2014), may affect the functional status of these enzymes. Moreover, enzymes in the Chl biosynthesis pathway including MgCh, MgMT and MgCY may form heterocomplexes to channel Chl intermediates efficiently (Tanaka and Tanaka, 2007; Wang and Grimm, 2015). Thus, MGDG deficiency may perturb the localization of MgCh, MgMT or MgCY to the proper sites of membranes in etioplasts or may impair the formation of multiple complexes and channeling of Chl intermediates, which results in the accumulation of the intermediates. Recently, Kopečná et al. (2015)
showed that deficiency of phosphatidylglycerol (PG), a major phospholipid in the thylakoid membrane of chloroplasts and cyanobacteria, in *Synechocystis* PCC 6803 inhibits the Chl biosynthesis pathway particularly at the conversion of Mg-Proto IX ME to Pchlide. The authors hypothesized that PG is an essential component in a membrane microdomain where Chl biosynthesis may take place along with the synthesis of PSI proteins. Although the exact site of the last several steps of Pchlide biosynthesis in plant etioplasts remains unclear, MGDG may also function to provide a specific lipid environment for the Pchlide biosynthesis pathway. Another possibility is that MGDG acts as an essential cofactor or activator of enzymes in the Pchlide biosynthesis pathway. However, *Synechocystis* MgCh subunits expressed in *Escherichia coli* (Jensen et al., 1996) and tobacco subunits expressed in yeast (Papenbrock et al., 1997) could reconstitute the chelatase activity *in vitro*. Recombinant *Arabidopsis* MgMT expressed in *E. coli* also exerted its catalytic activity *in vitro* (Block et al., 2002). *E. coli* and yeast do not contain galactolipids, so MGDG is not essential for MgCh and MgMT activity *in vitro*, although we do not exclude that MGDG functions to enhance the activity of these enzymes.

**MGDG facilitates the formation of the photoactive Pchlide-LPOR-NADPH complex and its oligomerization**

Photoconversion assay of Pchlide revealed that the strong *MGD1* suppression particularly impaired the accumulation of photoactive Pchlide (Fig. 2A and Supplemental Fig. S2B). Consistent with this finding, regeneration of photoactive Pchlide after flash irradiation was retarded in *MGD1*-suppressed seedlings (Fig. 3F). By contrast, the total amount of LPOR proteins was not changed with *MGD1* suppression.
(Fig. 2D). Thus, decreased photoactive Pchlide in +DEX L4w seedlings is not due to deficiency of LPOR proteins. These results suggest that MGDG deficiency perturbs the formation of the photoactive Pchlide-LPOR-NADPH ternary complex. The impaired complex formation together with reduced Pchlide biosynthesis resulted in a preferential decrease in photoactive Pchlide content in +DEX L4w and L2w seedlings, with nonphotoactive Pchlide levels virtually unchanged owing to the increased ratio of the nonphotoactive to photoactive form (Fig. 2A and Supplemental Fig. S2B). The amount of photoactive Pchlide was also slightly decreased in +DEX L4g seedlings, while the total Pchlide level unchanged (Fig. 2A). Thus, the weak MGD1 suppression in L4g seedlings may partially impair formation of the photoactive Pchlide complex without affecting total Pchlide biosynthesis activity. Meanwhile, in mgd1-1 seedlings, which showed no retarded porphyrin metabolism after ALA feeding (Supplemental Fig S8C), amounts of both Pchlide forms were similarly decreased, resulting in a significant decrease in the total Pchlide content (Supplemental Fig. S2D). Unlike in amiR-MGD1 lines, MGD1 expression in mgd1-1 is continuously suppressed by a T-DNA insertion in the MGD1 promoter region. The different profiles of MGD1 suppression between mgd1-1 and amiMGD1 lines may partially change the profiles of Pchlide accumulation during etioplast development.

In addition to the impaired formation of the photoactive Pchlide complex, strong MGD1 suppression caused a blue-shift of the fluorescence band emitted from photoactive Pchlide, with increased fluorescence around 645 nm (Fig. 3, A, B and E, and Table 1). Fluorescence bands peaking at ~645 and ~655 nm may be derived from the dimer of the Pchlide-LPOR-NADPH ternary complex and its large aggregates, respectively (Schoefs, 2001). Increased fluorescence at 645 nm with decreased
fluorescence at 655 nm in +DEX seedlings relative to the −DEX control (Fig. 3B)
suggests that the MGDG deficiency impairs the formation of large aggregates of the
Pchlide-LPOR-NADPH ternary complex from the dimer. Consistently, in vitro
experiment showed that MGDG strongly enhances oligomerization of the
Pchlide-LPOR complexes presumably by interacting with LPOR (Gabruk et al., 2017).
Therefore, an MGDG-rich lipid environment in etioplasts may contribute to forming the
photoactive ternary complex efficiently and developing it into large aggregates (Fig. 7,
arrows 2 and 3). Meanwhile, all photoactive Pchlide in large aggregates or the dimeric
complex was instantaneously photoconverted to Chlide by flash irradiation even in
MGDI-suppressed seedlings, which indicates that the high ratio of MGDG in
membranes is not essential for the catalytic activity of the ternary complex. The Shibata
shift after flash irradiation was also unaffected by MGDG deficiency, so MGDG may
not play a crucial role in disaggregation of large aggregates of Chlide-LPOR complexes.

**MGDG contributes to formation of the membrane structure in etioplasts**

A 36% reduction in the relative MGDG content in etiolated L4w seedlings
disordered the entire shape and the lattice structure of PLBs in etioplasts (Fig. 4, E, G
and H). Because MGDG in PLBs has been suggested to facilitate the formation of the
cubic phase structure (Brentel et al., 1985), the high presence of MGDG may be
required for the structural organization of the PLB membrane in addition to or through
formation of the photoactive Pchlide-LPOR-NADPH complex and its oligomerization.
Carotenoids, the major lipophilic pigments in etioplasts, are also deeply involved in the
formation and maintenance of PLB structures, as demonstrated by disrupted PLB
structure in etiolated seedlings with disordered carotenoid biosynthesis (Park et al.,
However, \textit{MGDI} suppression altered neither total carotenoid content nor carotenoid composition in etiolated L4w seedlings (Fig. 2E, Supplemental Fig. S4), so carotenoid functions would not be associated with the distorted PLB structures by MGDG deficiency.

Comprehensive studies with LPOR-overexpressing and LPOR-deficient etiolated seedlings indicated that the total amount of LPOR proteins is well associated with the size of PLBs (Sperling et al., 1998; Franck et al., 2000; Masuda et al., 2003). In fact, in etiolated +DEX L4w seedlings, which showed no significant reduction in LPOR levels (Fig. 2D), the size of PLBs was similar to that of the −DEX control despite decreased MGDG content (Fig. 4F). By contrast, the size of PTs was reduced by the \textit{MGDI} suppression (Fig. 4I). The maintained PLB size in MGDG-deficient L4w seedlings by LPORs may result in decreased formation of PTs due to deficiency of membrane lipids, although we cannot exclude the possibility that MGDG plays a specific role in PT development. In addition, irregular-shaped etioplasts and intrusion of cytosolic regions into etioplasts were frequently observed in +DEX L4w cotyledons (Fig. 4J, Supplemental Fig. S6). Because MGDG accounts for 47% of the total membrane lipids in the etioplast envelope (Selstam and Sandelius, 1984), MGDG may also be important for maintaining the structure of the etioplast envelope.

\textbf{MGDG biosynthesis at an early germination stage is a prerequisite for Pchlide accumulation}

Although the DEX treatment from the beginning of or 1 day after seeding decreased Pchlide content in etiolated L4w seedlings, that from 2 days after seeding no longer inhibited Pchlide accumulation (Fig. 2B). Considering that Pchlide begins to
accumulate from 2 d after seeding (Fig. 2C), the *MGD1* suppression after this time would be less effective, presumably because the MGD1 protein or galactolipids is already synthesized to some extent at that stage. In fact, public transcriptome data show that *MGD1* is expressed in dry seeds and during the early germination stage (Winter et al., 2007; Bassel et al., 2008). Moreover, lipid analysis in cucumber (*Cucumis sativus*) indicates that galactolipid biosynthesis starts early after germination in the dark (Ohta et al., 1995). Thus, the *MGD1* expression and subsequent galactolipid biosynthesis likely occur before Pchlide biosynthesis, which may be essential for effective Pchlide biosynthesis and formation of the Pchlide-LPOR-NADPH ternary complex. A similar result was obtained in +DEX *amiR-MGD1* seedlings grown under continuous light (Fujii et al., 2014); although *MGD1* suppression initiated within 3 d after seeding severely impaired Chl accumulation, suppression after 3 d did not inhibit greening of cotyledons. MGDG biosynthesis may be a prerequisite as one of the initial processes of etioplast and chloroplast biogenesis, because galactolipid-rich lipid bilayers provide a matrix for various processes on the membrane, such as Pchlide biosynthesis and the formation of Pchlide–LPOR complexes during etioplast development and Chl accumulation and formation of photosynthetic Chl–protein complexes during chloroplast development.

**Materials and methods**

**Plant materials, growth conditions and light treatment**

The *amiR-MGD1* transgenic lines were the Landsberg *erecta* ecotype (Fujii et al., 2014) and the *mgd1-1* (Jarvis et al., 2000), *chlm* (SALK_110265) (Mochizuki et al., 2008) and *chl27/crd1* (SALK_009052) (Ankele et al., 2007; Mochizuki et al., 2008) mutants were
the Columbia ecotype of *Arabidopsis* (*Arabidopsis thaliana*). Seeds were surface-sterilized, then cold-treated in water at 4°C for 4 d in the dark before seeding.

Plants were grown on Murashige and Skoog (MS) medium (adjusted to pH 5.7 with KOH) containing 1% (w/v) Suc solidified with 0.8% (w/v) agar except for the experiments in Figure 2B, in which plants were grown in liquid media with gentle rotation. All plants were grown at 23°C in a growth chamber. For light-grown seedlings, plants were illuminated with continuous white light (~30 μmol photos m⁻² s⁻¹). For etiolated seedlings, cold-treated seeds were illuminated with room light for ~3 h at room temperature to synchronize germination and then germinated in darkness. Unless stated otherwise, light-grown and etiolated seedlings were grown for 5 and 4 d, respectively.

For DEX treatment, DEX (Wako; http://www.wako-chem.co.jp/english/) was added to a final concentration of 10 μM in the medium from a 50-mM stock in dimethyl sulfoxide.

Etiolated seedlings were sampled under dim green light unless otherwise stated. To obtain seeds, parental plants were grown on watered soil in the absence of DEX at ~23°C under continuous white light (~30 μmol photos m⁻² s⁻¹).

Photoconversion of photoactive Pchlide into Chlide involved a single flash of white light for 0.7 ms (Power ratio 1/2) from a PZ42X electronic flash equipment (Sunpak; http://www.sunpak.jp/english/).

**Quantitative reverse transcription-PCR analysis**

Total RNA extraction, genomic DNA digestion, reverse transcription, complementary DNA amplification and normalization of transcript abundance were performed as described (Fujii et al., 2014). Gene-specific primers used in complementary DNA amplification are in Supplemental Table S1.
**Immunoblot analysis**

Total proteins were extracted and solubilized from seedlings crushed into powder in liquid nitrogen by adding sample buffer and incubating at 95°C for 5 min. Protein content was determined by using RC DC Protein Assay (Bio-Rad; http://www.bio-rad.com/) with bovine serum albumin as a standard. 10 and 20 μg of total proteins were subjected to SDS-PAGE on a gel containing 12.5% (w/v) polyacrylamide for separation, then electrotransferred to nitrocellulose membranes (Amersham Protran Premium 0.2 NC; GE Healthcare; http://www3.gehealthcare.com/). Protein bands reacting with a primary antibody against total LPOR protein (Rowe and Griffiths, 1995; Masuda et al., 2003) were secondarily labeled with goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Thermo Scientific; http://www.thermoscientific.com/). The secondary antibody was detected using a chemiluminescence reagent (Pierce Western Blotting Substrate Plus; Thermo Scientific) and an imager (ImageQuant LAS 4000 mini; GE Healthcare). For the loading control, proteins blotted on the membranes were stained with 0.1% (w/v) Ponceau S in 5% (v/v) acetic acid solution.

**Lipid analysis**

Extraction of total lipids, separation by thin-layer chromatography and visualization of lipids were performed as described (Kobayashi et al., 2006; Fujii et al., 2014). MGDG, DGDG, and a mixture of other glycerolipids were isolated from silica gel plates. Fatty acids in each lipid fraction were methyl-esterified by incubation in 1 M HCl in methanol at 85°C for 1.5 h and quantified by gas chromatography (GC-17A; Shimadzu;...
Determination of Chl, Pchlide and carotenoids

Pigments were extracted by incubating intact seedlings in 1 mL of 80% (v/v) acetone at 4°C in the dark for 3 d (for Chl) or overnight (for Pchlide and carotenoids). Chl content was determined spectrophotometrically by measuring the absorbance of the extract at 663 and 645 nm with an Ultrospec 2100 pro (GE healthcare; http://www3.gehealthcare.com/) or a V-730 BIO (JASCO; http://www.jascoinc.com/) spectrophotometer as described (Melis et al., 1987). In 20 etiolated Arabidopsis seedlings used for carotenoid determination, Chl and Pchlide were spectrophotometrically undetectable, and thus carotenoid content was determined by simply measuring the absorbance of the extract at 470 nm with a following formula; 1000 × A_{470}/198 (mg carotenoids mL⁻¹) (Lichtenthaler, 1987).

Pchlide content was determined by measuring fluorescence emission at 634 nm under 433-nm excitation with an RT-5300PC spectrofluorometer (Shimadzu) by using a Pchlide standard of known concentration. The concentration of the Pchlide standard, which was extracted from etiolated cucumber cotyledons, was determined by measuring the absorbance of the extract at 663, 645 and 626 nm (Anderson and Boradman, 1964). Many etiolated angiosperms including Arabidopsis accumulate both monovinyl-Pchlide and divinyl-Pchlide (Tanaka et al., 2011). Because molecular weights of monovinyl- and divinyl-Pchlide, 613 and 611, respectively, were very close, we used the molecular weight of monovinyl-Pchlide, the major form in mature etiolated Arabidopsis seedlings (Nagata et al., 2007), for calculation. For determining nonphotoactive Pchlide content, intact seedlings were irradiated with a single flash of light before extraction.
ALA feeding and HPLC analysis of porphyrin pigments

For porphyrin determination, intact seedlings were incubated in the dark in a solution containing 10 mM ALA, 10 mM MES-KOH (pH 5.7) and 5 mM MgCl₂, with or without 10 μM DEX, with gentle rotation at 23°C in a growth chamber. Pigments were extracted by incubating intact seedlings in 100 μL N, N-dimethylformamide at 4°C in the dark overnight. HPLC analysis was performed basically as described (Zapata et al., 2000) with some modifications. Pigments in 10 μL extract were separated by using a HPLC system consisting of an L-2130 pump (Hitachi; http://www.hitachi.com/), a Rheodyne 7725i injector with a 20-μL sample loop (IDEX Health and Science; https://www.idex-hs.com/), a COSMOSIL 5C₁₈-MS-II guard column (Nakalai Tesque, http://www.nacalai.co.jp/global/) and a reverse-phase C₈ column (Symmetry C₈ Column, 100Å, 3.5 μm, 4.6 X 150 mm, 1/pkg; Waters; http://www.waters.com/), and detected by using an RF-550 spectrofluorometric detector (Shimadzu). The mobile phase consisted of two solvents: A [50% (v/v) methanol, 25% (v/v) acetonitrile, 25% (v/v) of 0.25 M pyridine in ultrapure water (adjusted to pH 5.0 with acetic acid)] and B [20% (v/v) methanol, 60% (v/v) acetonitrile, 20% (v/v) acetone]. Pigments were eluted with a linear gradient from 100% A to 70% A plus 30% B over 8 min and to 2% A plus 98% B over 0.5 min for Proto IX detection; 100% A to 76% A plus 24% B over 6.4 min and to 2% A plus 98% B over 0.6 min for Mg-Proto IX (ME) detection; 100% A to 79% A plus 21% B over 5.6 min and to 2% A plus 98% B over 0.4 min for Pchlide detection, followed by isocratic elution with 2% A and 98% B for 5 min for all cases (see Supplemental Fig. S8B). Flow rate was 1.2 mL min⁻¹. Pigments were detected by measuring fluorescence emission at 634 nm under 400-nm excitation (Proto IX), at 595
nm under 420-nm excitation (Mg-Proto IX (ME)), and at 634 nm under 440-nm excitation (Pchlide) (Supplemental Fig. S8A). Pigments were identified and quantified by comparing retention times and absorption spectra of standard pigments of Proto IX, Mg-Proto IX (Frontier Science) and Pchlide (from cucumber as described above). The concentration of standard Proto IX and Mg-Proto IX was determined by absorption at 404 and 417 nm by using the V-730 BIO spectrophotometer (JASCO) and calculated with extinction coefficients of 1.08244 and 1.659 × 10⁵ M⁻¹ cm⁻¹, respectively (Kopetz et al., 2004). For Mg-Proto IX ME quantification, standard curves of Mg-Proto IX were used because they have the same spectral property.

**In situ fluorescence spectroscopy**

Fluorescence emission spectra were obtained directly from excised cotyledons placed between two thin acrylic resin plates by using an RF-5300PC spectrofluorometer (Shimadzu) under 440-nm excitation at 77K in liquid nitrogen. Slit widths for excitation and emission were 3 and 5 nm, respectively. Fluorescence data were obtained every 1 nm (for Fig. 3 and Supplemental Fig. S5, A and B) and 0.2 nm wavelength (for Table 1 and Supplemental Fig. S5C). Obtained spectra were normalized at the maxima between 620 and 640 nm as 1 and the fluorescence at 750 nm as 0. For measurement before and after photoconversion, cotyledons placed between plates were frozen before or immediately after flash treatment. For measurement of Shibata shift and regeneration of photoactive Pchlide, intact seedlings on the agar-solidified medium were flash-irradiated and incubated at 23°C in darkness for 20 min or 2 h before spectrum measurements.
Cotyledon size measurement

Etiolated cotyledons were observed by using an MZ16 FA stereomicroscope (Leica; http://www.leica-microsystems.com) with a VB-7010 CCD camera (KEYENCE; http://www.keyence.com/). Cotyledons were excised from the seedlings and stuck on adhesive tape to observe the front view. The area of cotyledons was determined by using the ImageJ software (https://imagej.nih.gov/ij/).

Transmission electron microscopy analysis

Samples were fixed with 4% glutaraldehyde and 4% paraformaldehyde in a 50-mM sodium cacodylate buffer, pH 7.0, at 4°C for 2 h and washed with the same buffer at 4°C overnight. Then they were post-fixed with 2% OsO₄ in a 50-mM sodium cacodylate buffer at 4°C for 2 h. The fixed samples were run through an alcohol series and embedded in Spurr resin. Ultra-thin sections (80 nm thick) were cut with a diamond knife on an ULTRACUT E ultra-microtome (Leica), and transferred to Formvar-coated grids. They were double-stained with 1% (v/v) uranyl acetate for 20 min and with lead citrate solution for 10 min. After washing with distilled water, the samples were observed under a JEM-1400 transmission electron microscope (JEOL; https://www.jeol.co.jp/en/).

Quantitative analysis of etioplast ultrastructures was performed with the ImageJ software. Etioplasts with no clear PLBs or two or more PLBs were eliminated from the analysis. The circularity index of PLBs and etioplasts was calculated as follows: $4 \times \pi \times \text{area}(\text{perimeter}^2)$. The unit of PLBs was defined as a low electron density region surrounded by a high density membrane area in the lattice of PLBs.
Accession numbers

Sequence data of the genes investigated in this article can be found in The Arabidopsis Information Resource under the following accession numbers: ACT8 (AT1G49240), MGD1 (AT4G31780), HEMAI (AT1G58290), CHLH (AT5G13630), CHLD (AT1G08520), CHLII (AT4G18480), GUN4 (AT3G59400), CHLM (AT4G25080), CHL27 (AT3G56940), LCAA (AT5G58250), psaA (ATCG00350), psbA (ATCG00020), petB (ATCG00720), rbcL (ATCG00490), rps14 (ATCG00330), accD (ATCG00500), rpoB (ATCG00190), LHCA4 (AT3G47470), LHC6B (AT1G15820), AAA-ATPase (AT3G28580), BAPI (AT3G61190), APX2 (AT3G09640) and ZAT10 (AT1G27730).

Acknowledgements

We thank Paul Jarvis (Department of Plant Sciences, University of Oxford) for supplying the mgd1-1 mutant, Nobuyoshi Mochizuki (Department of Botany, Graduate School of Science, Kyoto University) for the chlm and chl27 mutants, and Megumi Kobayashi (Department of Chemical and Biological Sciences, Faculty of Science, Japan Women’s University) for technical assistance in transmission electron microscopy analysis.

Supplemental Data

Supplemental Figure S1. T4 generation of amiR-MGD1 transgenic lines.

Supplemental Figure S2. MGD1 expression and Pchlide accumulation in etiolated seedlings of amiR-MGD1 L2 lines and mgd1-1.

Supplemental Figure S3. The size of cotyledons in 4-d-old etiolated seedlings of amiR-MGD1 L4w.
Supplemental Figure S4. Absorbance spectra of pigments extracted from 4-d-old etiolated seedlings of amiR-MGD1 L4w grown under −DEX and +DEX conditions.

Supplemental Figure S5. In situ 77K Pchlide fluorescence spectra in etiolated cotyledons of amiR-MGD1 L4w and mgd1-1.

Supplemental Figure S6. Ultrastructure of etioplasts in cotyledon cells of 4-d-old etiolated seedlings of amiR-MGD1 L4w.

Supplemental Figure S7. Distribution of the PLB unit area in each etioplast of amiR-MGD1 L4w seedlings.

Supplemental Figure S8. HPLC analysis of 4-d-old etiolated seedlings.

Supplemental Figure S9. MGD1 expression and Chl accumulation in mgd1-1 seedlings grown under the light.

Supplemental Figure S10. Correlation between MGD1 mRNA level and Chl content.

Supplemental Figure S11. T5 generation of amiR-MGD1 transgenic lines.

Supplemental Table S1. Oligonucleotide primers used for quantitative reverse transcription-PCR analysis.
Table 1. Peak position of Pchlde fluorescence bands in 4-d-old etiolated cotyledons of amiR-\textit{MGD1} L4w and L4g under 77K. Fluorescence data were obtained every 0.2 nm.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Conditions</th>
<th>Fluorescence bands</th>
<th>DEX treatment</th>
<th>Peak wavelength (nm)</th>
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<tbody>
<tr>
<td>L4w</td>
<td>Dark</td>
<td>( P_{630} )</td>
<td>−DEX</td>
<td>630.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>(before flash)</td>
<td>( P_{653} )</td>
<td>+DEX</td>
<td>630.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−DEX</td>
<td>653.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+DEX</td>
<td>652.6 ± 0.1**</td>
</tr>
<tr>
<td>L4g</td>
<td>Dark</td>
<td>( P_{630} )</td>
<td>−DEX</td>
<td>629.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>(before flash)</td>
<td>( P_{653} )</td>
<td>+DEX</td>
<td>630.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>−DEX</td>
<td>653.1 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+DEX</td>
<td>653.1 ± 0.1</td>
</tr>
<tr>
<td>L4w</td>
<td>Flash + 2 h dark</td>
<td>( P_{653} )</td>
<td>−DEX</td>
<td>653.3 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+DEX</td>
<td>652.1 ± 0.2*</td>
</tr>
</tbody>
</table>

Data are mean ± SE from 3 to 6 independent experiments. Asterisks indicate significant difference from the −DEX control (* P < 0.05, ** P < 0.01, Student’s t-test).
Figure Legends

Figure 1. Effect of MGD1 suppression on galactolipid biosynthesis in etiolated seedlings. A, Quantitative reverse transcription-PCR analysis of MGD1 mRNA level in 4-d-old etiolated seedlings of amiR-MGD1 under +DEX and −DEX conditions. Data are presented as fold difference from the −DEX control after normalizing to the control gene ACTIN8. Data are mean ± SE from 13 (L4w) or 3 (L4g) independent experiments. B, Accumulation of MGDG and DGDG in 4-d-old etiolated seedlings of amiR-MGD1 L4w. C and D, Fatty-acid composition of MGDG (C) and DGDG (D) in 4-d-old etiolated seedlings of amiR-MGD1 L4w. In B, C and D, data are mean ± SE from three independent experiments. In A to D, asterisks indicate significant differences from the −DEX control (* P < 0.05, ** P < 0.01, *** P < 0.001, Student’s t-test).

Figure 2. Contribution of MGDG to accumulation of Pchlide and carotenoids in etiolated seedlings. A, Pchlide content in 4-d-old etiolated seedlings of amiR-MGD1 grown under −DEX and +DEX conditions. Total and nonphotoactive Pchlides were extracted before and after flash treatment, respectively. Data are mean ± SE from 12 (L4w) or 15 (L4g) independent experiments. The amount of photoactive Pchlide was estimated by subtracting the amount of nonphotoactive Pchlide from total Pchlide. B, Pchlide content in 4-d-old etiolated L4w seedlings treated with DEX at different times after seeding. L4w plants were treated with DEX from the beginning of seeding (+DEX), 1 d after seeding (1-d-DEX), or 2 d after seeding (2-d DEX). Seedlings grown in the absence of DEX were analyzed as the untreated control (−DEX). Data are mean ± SE from 7 to 12 independent experiments. Different letters indicate significant
differences (P < 0.05, Tukey-Kramer multiple comparison test). C, Pchlide accumulation in etiolated amiR-MGD1 L4w seedlings grown for 2-5 d under −DEX and +DEX conditions. Data are mean ± SE from 6 to 12 independent experiments. D, Immunoblot analysis of total LPOR proteins (~37 kDa) in 4-d-old etiolated seedlings of amiR-MGD1 L4w. As a loading control, ponceau-stained proteins between ~25 and ~50 kDa blotted onto a membrane are shown. Representative data from 3 biologically independent experiments are shown. E, Total carotenoid content in 4-d-old etiolated seedlings of amiR-MGD1 L4w. Data are mean ± SE from 8 independent experiments. In A, C, and E, asterisks indicate significant differences from the −DEX control (* P < 0.05, ** P < 0.01, *** P < 0.001, Student’s t-test).

**Figure 3.** Role of MGDG in the formation of the Pchlide-LPOR-NADPH complex and processes after illumination. A, C, D and E, in situ 77K fluorescence spectra under 440-nm excitation in etiolated cotyledons of amiR-MGD1 L4w grown for 4 d under +DEX and −DEX conditions. Samples were frozen in liquid nitrogen without flash treatment (A), immediately after 0.7-ms flash (C), and after additional dark-incubation for 20 min (D) or 2 h (E) following flash treatment. Representative data from 3 or more biologically independent experiments are shown. Vertical dotted lines in A and E represent peak wavelength of fluorescence from photoactive Pchlide (P653) in −DEX seedlings. B, +DEX minus −DEX difference spectra in continuous dark (Dark) or 2-h dark after flash irradiation. Data are means of 8 (Dark) or 3 (Flash + 2 h dark) independent experiments. An arrow indicates fluorescence peak from the dimeric Pchlide-LPOR-NADPH complex at ~645 nm. F, Pchlide content in 4-d-old etiolated amiR-MGD1 L4w seedlings dark incubated for 20 min after flash treatment. Data are
mean ± SE from 5 to 7 independent experiments. The amount of photoactive Pchlide was estimated by subtracting the amount of nonphotoactive Pchlide from total Pchlide. Asterisks indicate significant differences from each form of Pchlide of the −DEX control (* P < 0.05, *** P < 0.001, Student’s t-test).

Figure 4. Ultrastructure of etioplasts in cotyledon cells of 4-d-old etiolated amiR-MGD1 L4w seedlings. A and C, Images of whole etioplasts in cotyledons grown under −DEX (A) and +DEX (C). Bars = 1.0 μm. B and D, Magnified images of PLB lattices in A (B) and C (D). Bars = 200 nm. For more images, see Supplemental Fig. S6. E-K, Quantitative data of circularity index (E) and area (F) of PLBs, area of a single PLB unit (G), relative SD value (SD/average) of the unit area in a PLB (H), length of PTs (I), and circularity index (J) and area (K) of etioplasts. The horizontal line in each box represents the median value of the distribution. The top and bottom of each box represent the upper and lower quartiles, respectively. The whiskers represent the range. Data were obtained from 46 different etioplasts. In H, the relative SD value was calculated from 20 units of the PLB in each etioplast. Distribution of the PLB unit area in each etioplast is shown in Supplemental Fig. S7. Asterisks indicate significant differences from the −DEX control (* P < 0.05, ** P < 0.01, *** P < 0.001, Welch’s t-test).

Figure 5. Effect of MGD1 suppression on Pchlide biosynthesis in the dark. A-C, Accumulation of porphyrin pigments in etiolated amiR-MGD1 L4w seedlings fed ALA for 1.5 h (A) and 24 h (B), and L4g seedlings fed ALA for 24 h (C). Seedlings were grown in the dark under +DEX or −DEX conditions for 4 d before ALA feeding. D and
E, Accumulation of porphyrin pigments in 4-d-old etiolated wild-type (WT), chlm and chl27 seedlings fed ALA for 1.5 h (D) and 24 h (E). In A to E, data are mean ± SE from 3 to 6 independent experiments. ND, not detected. Trace, trace amount. F, Quantitative reverse transcription-PCR analysis of mRNA expression of genes involved in Pchlide biosynthesis in L4w and L4g seedlings grown in the dark for 4 d. mRNA levels in +DEX seedlings are presented as fold difference from −DEX controls (a broken line) after normalizing to the control gene ACTIN8. Data are mean ± SE from 10 (L4w) or 3 (L4g) independent experiments. In A, B, C and F, asterisks indicate significant differences from the −DEX control (* P < 0.05, *** P < 0.001, Student’s t-test). In D and E, different letters indicate significant differences (P < 0.05, Tukey-Kramer multiple comparison test).

Figure 6. Quantitative reverse transcription-PCR analysis of mRNA levels of photosynthesis-associated and reactive oxygen species-responsive genes in amir-MGD1 L4w etiolated seedlings grown for 4 d under −DEX and +DEX conditions. A, Genes encoded in plastid genome. B, Photosynthesis-associated and reactive oxygen species-responsive genes encoded in nucleus. In A and B, mRNA levels are presented as fold difference from the −DEX control after normalizing to the control gene ACTIN8. Data are mean ± SE from 10 independent experiments. None of the genes showed significant differences between +DEX and −DEX seedlings (P > 0.05, Student’s t-test).

Figure 7. Roles of MGDG in Pchlide biosynthesis and formation of photoactive Pchlide-LPOR-NADPH complexes during etioplast development. Arrowheads indicate enzymatic steps in the Pchlide biosynthesis pathway from 5-aminolevulinic acid (ALA).
Most of the Pchlide synthesized in etioplasts forms the photoactive ternary complex with LPOR and NADPH, and the photoactive complex exists as the dimer or further aggregates into oligomeric complexes. MGDG is required for the Pchlide biosynthesis pathway from protoporphyrin IX (Proto IX) to Pchlide (arrow 1), the formation of the photoactive Pchlide-LPOR-NADPH ternary complex (arrow 2), and oligomerization of the ternary complex (arrow 3).
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