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Role of galactolipids in organelle differentiation

Research Area:
Biochemistry and Metabolism

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Inducible knockdown of *MONOGALACTOSYLDIACYLGLYCEROL SYNTHASE 1* reveals roles of galactolipids in organelle differentiation in Arabidopsis cotyledons

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**One-sentence summary:**

Biosynthesis of the major thylakoid membrane lipids mediates coordinated differentiation of chloroplasts with peroxisomes and mitochondria during greening of Arabidopsis cotyledons.
Footnotes:

1This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (No. 24770055) and JST CREST from the Ministry of Education, Sports, Science and Culture in Japan. YN was supported by JST, PRESTO and core budget from Institute of Plant and Microbial Biology, Academia Sinica.

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ABSTRACT

Monogalactosyldiacylglycerol (MGDG) is the major lipid constituent of thylakoid membranes and is essential for chloroplast biogenesis in plants. In Arabidopsis, MGDG is predominantly synthesized by inner envelope-localized MGDG synthase1 (MGD1); its knockout causes albino seedlings. Because of the lethal phenotype of the null *MGD1* mutant, functional details of MGDG synthesis at seedling development have remained elusive. In this study, we used an inducible gene-suppression system to investigate the impact of MGDG synthesis on cotyledon development. We created transgenic Arabidopsis lines that express an artificial microRNA targeting *MGD1* (*amiR-MGD1*) under a dexamethasone (DEX)-inducible promoter. The induction of *amiR-MGD1* resulted in up to 75% suppression of *MGD1* expression, although the resulting phenotypes related to chloroplast development were diverse, even within a line. The strong *MGD1* suppression by continuous DEX treatment caused substantial decreases in galactolipid content in cotyledons, leading to severe defects in formation of thylakoid membranes and impaired photosynthetic electron transport. Time-course analyses of the *MGD1* suppression during seedling germination revealed that MGDG synthesis at the very early germination stage is particularly important for chloroplast biogenesis. The *MGD1* suppression downregulated genes associated with the photorespiratory pathway in peroxisomes and mitochondria as well as those responsible for photosynthesis in chloroplasts and caused high expression of genes for the glyoxylate cycle. MGD1 function may link galactolipid synthesis with coordinated transcriptional regulation of chloroplasts and other organelles during cotyledon greening.
INTRODUCTION

In dicotyledonous plants, cotyledons, which are formed during embryogenesis, initially serve as storage organs during seed germination but mainly function in photosynthesis after seedling establishment. During the developmental switch from heterotrophic to autotrophic growth in germinated seedlings, metabolic activities change greatly in cotyledon cells. Before the development of photosynthetic capacity in cotyledons, the seedlings of oilseed plants such as Arabidopsis grow heterotrophically depending on triacylglycerol (TAG) stored within oil bodies in cotyledon cells. In this stage, peroxisomes function as the glyoxysome, which converts fatty acids bound to TAG to succinate via β-oxidation and the glyoxylate cycle, to provide carbon sources and energy for growth. After chloroplast development, plants rely on photosynthesis, which converts solar energy into chemical energy and fixes carbon dioxide into carbohydrates. Concomitant with photosynthesis, photorespiration, performed by cooperation among chloroplasts, peroxisomes and mitochondria, is activated to recycle 2-phosphoglycolate, the product of oxygenation reaction instead of carboxylation by Rubisco (Peterhansel et al., 2010).

Chloroplast biogenesis involves the remarkable development of thylakoid membranes consisting of photosynthetic protein–pigment complexes and the membrane lipid bilayer. In chloroplasts, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) account for approximately 50 and 25 mol% of thylakoid membrane lipids, respectively (Block et al., 1983). In addition to providing a lipid matrix for thylakoid membranes, these galactolipids may be required for the structure and function of photosynthetic complexes (Mizusawa and Wada, 2012).

The last step of MGDG synthesis is catalyzed by MGDG synthase (MGD), which
transfers a galactose moiety of UDP-Gal to diacylglycerol in plastid envelopes (Kobayashi et al., 2009b). Because DGDG is synthesized by galactosylation of MGDG (Dörmann and Benning, 2002), MGD is the key enzyme for the biosynthesis of both of these galactolipids and therefore biogenesis of thylakoid membranes. Three MGD isoforms identified in Arabidopsis are MGD1, MGD2 and MGD3 (Kobayashi et al., 2009b). The contribution of MGD2 and MGD3 to galactolipid synthesis is limited; the isoforms are localized at the outer envelope membrane of chloroplasts (Awai et al., 2001) and affect galactolipid synthesis only under phosphate (Pi)-limited conditions (Kobayashi et al., 2009a). By contrast, MGD1 is targeted to the chloroplast inner-envelope membrane and is expressed actively in photosynthetic tissues (Awai et al., 2001). The significance of MGD1 in galactolipid biosynthesis and thylakoid membrane biogenesis was demonstrated by study of two Arabidopsis mgd1 mutants: the knockdown mutant mgd1-1 (Jarvis et al., 2000) and the knockout mutant mgd1-2 (Kobayashi et al., 2007). The mgd1-1 mutant reduces MGDG to 58% of that in wild-type plants and has chloroplasts with fewer thylakoid membranes (Jarvis et al., 2000). These defects result in impaired thylakoid membrane energization and decreased photoprotective capacity (Aronsson et al., 2008). Similar results were recently reported in an MGD1 knockdown mutant (M18) in tobacco, which further revealed an involvement of MGD1-mediated MGDG biosynthesis in photosynthetic electron transport between PSII and PSI (Wu et al., 2013). However, the mgd1-2 knockout mutant accumulated only negligible amounts of both MGDG and DGDG, thus resulting in the absence of thylakoid membranes in leaf plastids and the complete dysfunction of photosynthetic activities (Kobayashi et al., 2007).

Recently, we revealed that the loss of galactolipids in mgd1-2 caused strong
downregulation of both plastid- and nuclear-encoded photosynthesis-associated genes, whereas partial complementation of the mutation by the alternative MGD2/MGD3 pathway under Pi starvation attenuated the downregulation of such genes (Kobayashi et al., 2013). Our findings suggest that galactolipid biosynthesis plays a crucial role in coordinating the formation of photosynthetic protein–pigment complexes with the development of thylakoid membranes during chloroplast biogenesis. However, how the genes and processes associated with photosynthesis are intertwined with the development of thylakoid membrane bilayers remains largely unknown because of the strong pleiotropic effects of the constitutive loss of galactolipids on plant growth and development. Furthermore, no detailed analysis has been reported on the effect of galactolipid biosynthesis and consequent thylakoid membrane biogenesis on the differentiation of other organelles such as peroxisomes and mitochondria during leaf development, although a tight metabolic coordination occurs between chloroplasts and these organelles during the transition from heterotrophic to photoautotrophic growth in leaf cells (Peterhansel et al., 2010; Nunes-Nesi et al., 2011).

To gain insight into the role of galactolipid biosynthesis through MGD1 in the coordinated formation of photosynthetic complexes and thylakoid membranes, we constructed an artificial microRNA gene (amiR-MGD1) targeting Arabidopsis MGD1 under the control of the dexamethasone (DEX)-inducible promoter and introduced it into Arabidopsis Landsberg erecta (Ler) ecotype. The resulting plants showed a wide phenotypic variation in cotyledon development with strongly reduced MGD1 expression with DEX treatment, which allowed us to analyze the function of the MGD1-mediated galactolipid biosynthesis during the initial stage of chloroplast biogenesis. Moreover, we investigated a linkage between MGD1 function and gene expression associated with
peroxisomal and mitochondrial functions during early seedling development.

RESULTS AND DISCUSSION

Specific suppression of *MGD1* by the *amiR-MGD1* transgene is DEX-dependent

To control the timing of *MGD1* expression during chloroplast development, we used a microRNA-mediated gene suppression technique (Schwab et al., 2006) combined with a glucocorticoid-inducible system (Craft et al., 2005) (Fig. 1A). We obtained 7 DEX-responsive lines from 48 individual transgenic Arabidopsis lines that harbor an artificial microRNA targeting *MGD1* (*amiR-MGD1*). We chose Lines 2, 4, 5, and 7 (L2, L4, L5, L7) for subsequent analyses. First we examined the effect of continuous DEX treatment of early seedling growth of *amiR-MGD1* transgenic lines. The phenotypes of all *amiR-MGD1* lines were indistinguishable from the wild type in the absence of DEX (Fig. 1B). In the presence of 10 µM DEX, cotyledon greening was impaired in L2, L4, and L5, although a wide variety of color phenotypes, from albino-like to wild-type–like, were observed in these lines. Meanwhile, L7 showed no remarkable difference from the wild type in visible phenotype even in the presence of 10 µM DEX. Chlorophyll measurements in 5-d-old seedlings supported these results (Fig. 1C). Although the chlorophyll content considerably deviated in each line, the median values were substantially lower in DEX-treated L2, L4 and L5 seedlings than in the DEX-untreated control of each line. However, chlorophyll content was almost the same in DEX-treated L7 seedlings as the DEX-untreated control.

To address the relationship between the *amiR-MGD1* phenotypes and suppression of *MGD1* expression, we examined the expression of *MGD1* in 5-d-old *amiR-MGD1* seedlings by quantitative RT-PCR (qRT-PCR) (Fig. 1D). In the absence of DEX, no
lines showed decreased $MGD1$ expression, which confirms that the $amiR-MGD1$ transgene does not suppress $MGD1$ without DEX treatment. By contrast, in the presence of 10 µM DEX, $MGD1$ expression decreased by 70% to 80% in L2, L4, and L5 seedlings as compared with the wild type. Even in L7 seedlings, which did not show reduced chlorophyll content with DEX treatment, $MGD1$ expression was decreased by half of the wild-type level with DEX treatment (Fig. 1D).

Because Arabidopsis possesses two paralogs of $MGD1$, namely $MGD2$ and $MGD3$ (Kobayashi et al., 2009b), we also examined whether the $amiR-MGD1$ transgene targets the expression of $MGD2$ and $MGD3$ in L4 seedlings. Under Pi-sufficient normal growth conditions, the expression of $MGD2$ and $MGD3$ was too low to quantify in both the wild type and $amiR-MGD1$ lines. Then we grew seedlings under the Pi-deficient condition to upregulate $MGD2$ and $MGD3$ as described previously (Awai et al., 2001; Kobayashi et al., 2004). In Pi-deficient L4 seedlings, DEX treatment suppressed the expression of $MGD1$ but not $MGD2$ or $MGD3$ (Fig. 1E). Therefore, the $amiR-MGD1$ construct specifically targeted the $MGD1$ transcript for suppression and had no effect on the expression of $MGD2$ and $MGD3$. We confirmed DEX responsiveness and target specificity of the $amiR-MGD1$ system.

**Tight link between chlorophyll accumulation and galactolipid biosynthesis in $amiR-MGD1$ seedlings**

To further analyze phenotypes of L2 and L4, we classified DEX-treated seedlings of these lines into three groups, namely “white”, “pale-green”, and “green”, using a chlorophyll-deficient $hemA1$ mutant (Kobayashi et al., 2013) as a color reference. The “white” seedlings had paler cotyledons than those of $hemA1$ mutants, whereas the
cotyledon color of “green” seedlings was similar to that of DEX-untreated seedlings (Fig. 2A). The remaining seedlings with intermediate phenotypes between white and green seedlings were defined as “pale-green”, which were the majority in both L2 and L4 (Fig. 1B). This classification was supported by quantifying chlorophyll content in each class of DEX-treated seedlings (Fig. 2B).

We compared the expression of $MGD1$ in white and green seedlings grown for 5 d in the presence of DEX and in DEX-untreated controls (Fig. 2C). In both L2 and L4 lines, $MGD1$ expression was lower in white seedlings than in green seedlings. Together with the results in Figure 1D, our data show a link between the $MGD1$ suppression and impaired cotyledon greening and support our previous conclusion that galactolipids are necessary for chloroplast development (Kobayashi et al., 2007). Why wide variation in cotyledon phenotypes is found in L2, L4, and L5 remains elusive; the $amiR-MGD1$ activity may differ individually even within a single line and the small differences in the $amiR-MGD1$ activity may lead to the considerable disparity in chlorophyll accumulation and chloroplast development in cotyledons.

Next we analyzed galactolipid content in each color class of 5-d-old DEX-treated L4 seedlings and in the untreated control (Fig. 2D). The proportion of MGDG in total glycerolipids decreased by 25%, 77% and 85% in green, pale-green and white seedlings, respectively, as compared with the DEX-untreated control. The MGDG level in these seedlings was associated with their chlorophyll content (Fig. 2B), which suggests that MGDG biosynthesis strongly affects chlorophyll accumulation and thus the formation of photosynthetic machinery, presumably by varying the size and/or the quality of thylakoid membrane bilayers. In green seedlings, the proportion of DGDG did not change and thus the MGDG/DGDG ratio decreased. These findings agree with
the previous report of the Arabidopsis mgd1-1 mutant and tobacco M18 line showing that the decrease in MGD1 activity primarily results in loss of MGDG (Jarvis et al., 2000; Wu et al., 2013). A possible explanation for the decrease in MGDG/DGDG ratio by partial defects of the MGD1 function is that the decreased MGD1 activity results in an overbalance of DGDG biosynthesis relative to MGDG biosynthesis and consequent consumption of MGDG in the formation of DGDG, thus leading to reduced distribution of MGDG to membrane bilayers. In the pale-green and white seedlings, the proportion of DGDG decreased together with MGDG, which reduced the proportion of total galactolipids by 80% in white seedlings. The ratio of non-bilayer-forming MGDG to bilayer-forming DGDG may affect properties of chloroplast membranes and so is tightly regulated through a yet unknown mechanism (Dörmann and Benning, 2002). The inhibition of MGD1 expression may induce coordinated downregulation of DGDG biosynthesis in the paler amiR-MGD1 seedlings so that the MGDG/DGDG ratio is maintained in photosynthetic membranes.

In addition to examining membrane lipids, we analyzed levels of TAG in these seedlings. The proportion of TAG was 1.5-fold higher in white L4 seedlings than the DEX-untreated control, with the proportion in the green or pale-green seedlings unchanged (Fig. 2D). As in wild type, TAG in white seedlings contained eicosenoic acid (20:1) as major constituents (Supplemental Fig. S1), which may reflect high retention of seed TAG in white seedlings after germination because eicosenoic acid is specifically accumulated in TAG during seed maturation (Li et al., 2006). The strong MGD1 suppression in white seedlings may decrease the demand of diacylglycerol for galactolipid biosynthesis, thereby influencing TAG metabolism. However, unlike white seedlings, pale-green seedlings showed no change in TAG content, despite the
substantial decrease in galactolipid content. Therefore, changes in other metabolic processes caused by severe defects in chloroplast biogenesis may affect TAG metabolism in white seedlings.

**Impaired chloroplast biogenesis and cotyledon cell organization with MGD1 suppression**

Partial deficiency of MGDG by MGD1 knockdown leads to decreased amount of thylakoid membranes with altered architecture (Jarvis et al., 2000; Wu et al., 2013), whereas crucial lack of both galactolipids by MGD1 knockout results in no or severely underdeveloped internal membrane structures in leaf plastids (Kobayashi et al., 2007). We examined the effect of MGD1 suppression on chloroplast development in green or white cotyledons from DEX-treated L4 seedlings by transmission electron microscopy. Consistent with previous reports (Jarvis et al., 2000; Wu et al., 2013), chloroplasts in green cotyledons, with slightly reduced MGDG content (Fig. 2D), appeared a little less mature than those from DEX-untreated controls (Fig. 3, A and B, and Supplementary Fig. S2, A to D). However, white cotyledons had severely undeveloped plastids with few internal membrane structures (Fig. 3C and Supplementary Fig. S2, E and F). In addition, immature plastids in white cotyledons had oval or flattened shapes and lacked the lens-like structures typically observed in mature chloroplasts in green and DEX-untreated seedlings. The defective phenotype of chloroplasts in white L4 cotyledons, which is milder than in the mgd1-2 knockout mutant (Kobayashi et al., 2007) and more severe than in the mgd1-1 knockdown mutant (Jarvis et al., 2000), agrees with their phenotype in galactolipid content, which indicates a direct impact of the MGD1-derived MGDG synthesis on the development of thylakoid membrane
networks. In addition to the abnormal chloroplasts, oil bodies were frequently observed in close contact with glyoxysomes in the cotyledon cells of the white seedlings (Supplemental Fig. S2, G and H), which is consistent with the high TAG level in the seedlings.

The **MGD1** suppression also influenced the structure and organization of cotyledon cells (Fig. 3, D to F). Both epidermal and mesophyll cells in the L4 white cotyledons had irregular shapes, often with indented cell outlines, whereas those in the green cotyledons appeared similar to the DEX-untreated control. In the white cotyledons, the alignment of mesophyll cells was disordered, with irregular organization of palisade cells and spongy cells. Moreover, the size of mesophyll cells in the white cotyledons was smaller than those in the green cotyledons of DEX-treated and DEX-untreated seedlings. Because seeds of all **amiR-MGD1** lines were collected from parents grown in the absence of DEX and therefore the embryonic development progressed normally in all these lines, the morphological disorder of the white cotyledons could be attributed to impaired postgerminative development. Considering that cell proliferation for cotyledon formation is nearly completed during embryogenesis and the cell expansion mainly contributes to the postgerminative cotyledon growth in the light (Tsukaya et al., 1994; Stoynova-Bakalova et al., 2004), the morphological disorder and the dwarf phenotype in the white L4 cotyledons may be due to distorted mesophyll cell expansion during postgerminative growth. The distorted organization of mesophyll cells has been observed in several mutants with defective chloroplast development (Wang et al., 2000; Wycliffe et al., 2005; Garcion et al., 2006; Sulmon et al., 2006) including the **mgd1-2** mutant (Kobayashi et al., 2007), thus showing a tight developmental link between chloroplasts and host mesophyll cells.
Furthermore, application of norflurazon, which inhibits carotenoid biosynthesis and impairs chloroplast development, to Arabidopsis seedlings was recently found to affect the transition of leaf cells from the proliferation to expansion stage, thus resulting in decreased cell size in young true leaves (Andriankaja et al., 2012). Our data reveal that inhibition of cell expansion also occurred in cotyledons with impaired MGDG biosynthesis during postgerminative growth, which suggests that the thylakoid membrane biogenesis and consequent chloroplast development play a role in regulating cell development even in cotyledons with basic structure established during embryogenesis.

Requirement of galactolipids for photosynthetic electron transport

A partial loss of MGDG (~50% of wild-type levels) results in decreased intersystem electron transport between PSII and PSI (Wu et al., 2013) and increased conductivity of thylakoid membranes (Aronsson et al., 2008), whereas the absence of MGDG (~5% of wild-type levels) causes severe disorder in photosystem complexes and complete deficiency of photosynthetic electron transport (Kobayashi et al., 2013). Because the amiR-MGD1 L4 seedlings showed wide variation in MGDG content, from 15% to 75% of wild-type levels with DEX treatment (Fig. 2D), we used this line and the similar L2 line to investigate the relationship between MGDG biosynthesis and the functionality of the photosynthetic electron transport chain. The maximum quantum efficiency of PSII in the dark (Fv/Fm), which represents the intrinsic photosynthetic efficiency of PSII, and the actual PSII efficiency (ΦII) under light (36 µmol photons m⁻² s⁻¹) were determined in individual cotyledons by using a chlorophyll fluorescence imaging system based on pulse-amplitude-modulation (PAM) techniques. Maximal chlorophyll fluorescence after
dark adaptation (Fm) was used as a measure of chlorophyll accumulation in cotyledons to indicate severity of *amiR-MGD1*-mediated suppression of galactolipid biosynthesis. Unlike in wild type, both Fv/Fm and ΦII were decreased in L2 and L4, being correlated with a decrease in Fm values (Fig. 4, A and B). The result suggests that the galactolipid biosynthesis through MGD1 is closely associated with the efficiency of light utilization in PSII. Very similar scatter diagrams between L2 and L4 indicate that these lines are mostly equivalent.

To elucidate the influence of MGD1 deficiency on photosynthetic electron transport in cotyledons in more detail, several photosynthetic parameters were characterized in three color classes of 5-d-old DEX-treated L2 seedlings. Fv/Fm was most decreased in white seedlings, followed by pale-green and green seedlings (Fig. 4C). A *dgd1* mutant showed a slight decrease in Fv/Fm (~0.74) as compared with the wild type (~0.81), although the mutant contains only ~10% DGDG of the wild-type level (Hölzl et al., 2009), which indicates that the loss of DGDG has only a minor impact on the maximal PSII efficiency. Thus, in the white *amiR-MGD1* cotyledons, the deficiency in MGDG may be primarily responsible for the substantial decrease in maximal PSII efficiency.

Next we analyzed quantum yields of PSII under low photosynthetically active radiation (45 µmol photons m⁻² s⁻¹). Whereas ΦII in wild-type and green seedlings increased within 2 min after actinic illumination started, that in white and pale-green seedlings remained very low during the measurement (Fig. 4D). The similar pattern was observed in the coefficient of photochemical quenching (qP) (Fig. 4E). Because qP represents QA redox status and thus the openness of PSII, the low qP in white and pale-green seedlings suggests acceptor-side limitation in PSII resulting from defective electron transfer downstream of PSII. MGDG deficiency decreased levels of the
cytochrome \( b_{6f} \) complex and blocked the intersystem electron transport in the tobacco \( MGD1 \) mutant (Wu et al., 2013). Therefore, the PSII acceptor-side limitation in the \( amiR-MGD1 \) cotyledons may be attributed at least in part to inhibition of intersystem electron transport around the cytochrome \( b_{6f} \) complex. In addition, the maximum quantum efficiency of open PSII (\( F_{v'}/F_{m'} \)) was constantly lower for white and pale-green seedlings than the wild-type and green seedlings (Fig. 4F). The low \( F_{v'}/F_{m'} \) indicates dysfunctional photochemical reactions in open PSII and is in line with the low \( F_{v}/F_{m} \) in these seedlings (Fig. 4C). In the white and pale-green seedlings, defective electron transfer both within and downstream of PSII could cause decreased light utilization for photosynthesis represented in the low \( \Phi_{II} \).

We also evaluated the quantum yield of light-induced non-photochemical quenching (\( \Phi_{NPQ} \)) and quantum yield of non-light induced non-photochemical quenching (\( \Phi_{NO} \)), which represents the proportion of regulated and non-regulated dissipation of light energy in PSII, respectively (Kramer et al., 2004). In both white and pale-green seedlings, the low \( \Phi_{II} \) was inversely related to the high \( \Phi_{NO} \) (Fig. 4G) whereas \( \Phi_{NPQ} \) stayed low levels (Fig. 4H), which indicates that severe galactolipid deficiency causes non-regulated dissipation of excess light energy from PSII even under low light. The high value of \( \Phi_{NO} \) shows the limited photo-protective capacity in DEX-treated \( amiR-MGD1 \) cotyledons, consistent with the observation of accelerated photodamage in the tobacco MGDG-deficient mutant under high light conditions (Wu et al., 2013). Although pale-green seedlings retained a certain amount of DGDG compared with the strong reduction in MGDG, the seedlings showed severe photosynthetic defects similar to white seedlings. Thus, deficiency of MGDG may be the major cause of photosynthetic dysfunction in these seedlings.
To dissect the functionality of PSII in the white cotyledons of amiR-MGD1 lines, we analyzed the transient kinetics of chlorophyll fluorescence in a logarithmic timing series (Fig. 4I). Wild-type cotyledons showed a typical polyphasic fluorescence increase called origin-inflection-intermediary peak-peak (OJIP) transient (Govindjee, 1995). The O-J phase and the J-I phase reflect the photochemical process in PSII and the reduction in the plastoquinone pool, respectively, whereas the I-P phase is related to the process in PSI. White cotyledons of L2 and L4 showed a very fast increase in chlorophyll fluorescence during the photochemical phase (the O-J phase), which indicates impaired electron transfer from excited chlorophylls to the downstream within the PSII. In the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a rapid reduction of total QA, the primary electron acceptor of PSII, occurs by illumination due to the inhibition of electron transfer from QA to QB, thus resulting in a fast increase in chlorophyll fluorescence without inflection, as observed in wild-type cotyledons (Fig. 4J). The fluorescence increase in the presence of DCMU was much faster for L2 and L4 than the wild type, which suggests decreased electron-accepting capacity of QA in these transgenic lines. The limitation in electron-accepting capacity within PSII decreases the utilization of light energy for photosynthesis and thus increases excess energy, which would be dissipated in non-regulated forms of heat and fluorescence in DEX-treated amiR-MGD1 cotyledons as indicated by the high ΦNO value (Fig. 4G). Crystallographic studies in cyanobacteria have revealed that many galactolipid molecules reside in the PSII complex and some are present in the vicinity of the reaction center (Guskov et al., 2009; Umema et al., 2011). Therefore, deficient galactolipids in the PSII reaction center may be involved in the dysfunction of the PSII photochemical reaction in DEX-treated amiR-MGD1 cotyledons, as was proposed in the mgd1-2 mutant (Kobayashi et al.,
Role of MGD1 in formation of Photosystems

Previously, we revealed by chlorophyll fluorescence analysis at 77K that the lack of MGDG in the Arabidopsis *mgd1-2* knockout mutant caused severely disordered formation of both PSII and PSI complexes (Kobayashi et al., 2013). Meanwhile, knockdown of MGD1 in Arabidopsis and tobacco had only a slight impact on chlorophyll fluorescence spectra at 77 K (Aronsson et al., 2008; Wu et al., 2013). To evaluate the importance of galactolipids in formation of photosystem complexes, we determined chlorophyll fluorescence spectra at 77 K in white cotyledons of L2 and L4, whose phenotypes are intermediate between the *mgd1-2* knockout mutant (Kobayashi et al., 2007) and the knockdown mutants (Aronsson et al., 2008; Wu et al., 2013). In wild type, three major emission peaks were detected at 682, 690, and 729 nm (Fig. 5A). The peak at 729 nm is attributed to the PSI–light-harvesting complex (LHC) I complex whereas peaks at 682 and 690 nm primarily originate from CP43 and CP47 in PSII, respectively (Govindjee, 1995). In white cotyledons of L2 and L4, peak wavelengths did not differ from wild type, except for a very slight shift of the peak at 732 nm, which suggests that the global formation of photosystem complexes is not largely perturbed in the L2 and L4 white cotyledons. When fluorescence intensities were normalized to the emission maximum at 682 nm among these samples, emission peaks at 729 nm were lower for both L2 and L4 than the wild type. However, the fluorescence intensity from PSII can vary by chlorophyll concentration in samples because of self-absorbance of the PSII emission by the PSI complex. Therefore, next we compared 77 K chlorophyll fluorescence spectra in membrane fractions from each color class of DEX-treated L2...
seedlings at the same chlorophyll concentration (0.8 µg/ml) (Fig. 5B). Fluorescence from PSI largely decreased in white and pale-green seedlings compared with wild type (Fig. 5B), which suggests a decrease in PSI proteins or reduced energy transfer to the PSI reaction center from antenna complexes. Even in green seedlings, which had reduced MGDG but wild-type level of DGDG, the emission from PSI decreased, which suggest a specific requirement of MGDG for formation of the PSI-LHC complexes.

Moreover, emission peaks from PSII and PSI were both blue-shifted in L2 seedlings in a correlation with the extent of the $MGD1$ suppression. The result suggests that LHCs in membrane fractions from the L2 seedlings are largely dissociated from core complexes in both photosystems. Considering that emission peaks were not blue-shifted in intact white seedlings (Fig. 5A), LHCs may be weakly interacted with both photosystems in vivo, but dissociated from core complexes during membrane fractionation. Indeed, an in vitro analysis revealed that MGDG intensifies the physical interactions between LHCII and PSII core complexes and increases their energy coupling (Zhou et al., 2009). Thus, galactolipids, and particularly MGDG, may be essential for strong interactions between photosystem core complexes and LHCs.

To address whether the $MGD1$ suppression affect the abundance of membrane photosynthetic proteins, we performed immunoblot analysis in DEX-treated L2 pale-green seedlings. Total membrane proteins (20 µg) from pale-green seedlings were analyzed together with a dilution series (1, 5, and 20 µg) of membrane proteins from wild type. Although both PSI (PsaA/B, LHCA1 and LHCA3) and PSII proteins (D1, D2, LHCB1 and LHCB3) largely decreased in pale-green seedlings compared with wild type, the balance of protein abundance between PSI and PSII were maintained. Thus, the reduced PSI fluorescence at 77 K (Fig. 5B) is not due to a decrease in PSI protein
levels but presumably due to reduced energy transfer to PSI reaction center from LHCl or from the PSII antenna system, both of which may be dissociated from reaction centers in the membrane fraction from pale-green seedlings (Fig. 5B).

The immunoblot analysis also revealed that core proteins (PsaA/B, D1 and D2) were more decreased than antenna proteins (LHCA1, LHCA3, LHCB1 and LHCB3) in pale-green seedlings. Similar results were observed in the Pi-deficient mgd1-2 mutant (Kobayashi et al., 2013). Thus, MGDG may be required for accumulation or maintenance of photosystem core complexes.

**Involvement of galactolipid biosynthesis in coordinated regulation among genes associated with photosynthesis, photorespiration and glyoxylate cycle during cotyledon development**

We recently reported in the mgd1-2 mutant that galactolipid biosynthesis and subsequent membrane biogenesis inside the plastid strongly influence the expression of both plastid- and nuclear-encoded photosynthetic genes independent of photosynthesis (Kobayashi et al., 2013). To investigate the effect of the MGD1 suppression on photosynthetic gene expression during cotyledon development, we examined the expression of \( psaA \) and \( psbA \), which are plastid-encoded genes for PsaA and D1 of PSI and PSII core complexes, respectively. Consistent with the substantial decrease in PsaA and D1 proteins (Fig. 5C), both \( psaA \) and \( psbA \) were downregulated in white cotyledons and also green cotyledons of DEX-treated L4 seedlings (Fig. 6A). In developing plastids, a large DNA–protein complex named nucleoids exists in close contact with envelope and thylakoid membranes (Sato, 2001). Considering that plastid transcription is regulated by the activity of the RNA polymerase complexes and by the structural
organization of plastid DNA (Sekine et al., 2002), the galactolipid biosynthesis in the plastid envelope and subsequent thylakoid membrane biogenesis may affect transcriptional activities in the nucleoid. In fact, we previously showed that galactolipid biosynthesis modifies the morphology of nucleoids in leaf plastids, which infers an association between galactolipid biosynthesis and nucleoid activity (Kobayashi et al., 2013).

We then examined expression of \textit{LHCB6} and \textit{CHLH}, which are nuclear genes encoding the LHCII subunit 6 and the H subunit of magnesium chelatase involved in chlorophyll biosynthesis, respectively. Coordinately with plastid-encoded genes (\textit{psaA} and \textit{psbA}), nuclear-encoded photosynthetic genes (\textit{LHCB6} and \textit{CHLH}) were downregulated in both white and green cotyledons of DEX-treated L4 seedlings (Fig. 6A). Consistent with their galactolipid phenotypes, the suppression of both plastid- and nuclear-encoded genes was stronger in white than green cotyledons, which suggests that MGDG biosynthesis is tightly linked to photosynthetic gene expression during chloroplast biogenesis. Inhibition of chloroplast protein translation results in downregulated nuclear photosynthetic gene expression during early seedling growth, which suggests an involvement of plastid translation in plastid signaling that downregulates nuclear photosynthetic gene expression in response to chloroplast dysfunction (Nott et al., 2006). Furthermore, plastid gene expression mediated by nuclear-encoded sigma factors plays a role in the plastid signaling (Woodson et al., 2013). Therefore, one possibility is that the decreased expression of plastid-encoded genes in DEX-treated \textit{amiR-MGD1} seedlings triggers the downregulation of photosynthetic genes in the nucleus through plastid signaling pathways.

During cotyledon greening, peroxisomes and mitochondria transform their
functions in concert with chloroplast development (Hayashi and Nishimura, 2006). To examine whether the MGD1 suppression affects organelle differentiation early during seedling development, we analyzed the expression of genes associated with peroxisomal and mitochondrial functions in 5-d-old L4 seedlings (Fig. 6B). GOX1 and HPR1, which encode glycolate oxidase and hydroxypyruvate reductase of the photorespiratory pathway, respectively, were used as leaf peroxisome markers. In addition, GLDP1 and SHM1, which encode the P-protein of the glycine decarboxylase complex and serine hydroxymethyltransferase, respectively, were used as markers for the functionality of photorespiration in mitochondria. All of these photorespiratory genes are reported to be specifically expressed in photosynthetic tissues (Kamada et al., 2003; Voll et al., 2006; Engel et al., 2007; Timm et al., 2008). Meanwhile, ICL and MLS, which encode isocitrate lyase and malate synthase of the glyoxylate cycle, respectively, were used as markers for glyoxysomes. ICL and MLS are transiently expressed during the very earliest post-germinative growth stages, when peroxisomes actively operate the glyoxylate cycle as glyoxysomes (Eastmond et al., 2000; Cornah et al., 2004). Our qRT-PCR analysis revealed that the photorespiratory genes GOX1, HPR1, GLDP1 and SHM1 were downregulated concomitantly with the photosynthetic genes by DEX treatment, particularly in white seedlings (Fig 6B). The photorespiratory metabolism is associated with photosynthetic functionalities (Peterhansel et al., 2010) and therefore co-downregulation of the photorespiratory genes with photosynthesis-associated genes is reasonable. Because many photorespiratory genes are downregulated together with photosynthesis-associated nuclear genes in norflurazon-treated seedlings through plastid signaling pathways (Strand et al., 2003), the MGD1 suppression may downregulate these genes coordinately with nuclear photosynthetic genes by activating plastid
signaling. By contrast, the expression of glyoxysomal genes *ICL* and *MLS* remained at higher levels in both DEX-treated green and white seedlings than in the untreated control (Fig 6C). The low expression of photorespiratory genes and the high expression of glyoxylate cycle-associated genes suggest that differentiation of peroxisomes and mitochondria to leaf-specific types during cotyledon development is inhibited by *amiR-MGD1*-mediated suppression of galactolipid biosynthesis. Considering that many glyoxysomes were observed close to oil bodies in 5-d-old white L4 cotyledon cells (Supplemental Fig. S2, G and H), which is typically observed during the early seed germination stage in the case of wild type (Hayashi et al., 2001), the white plants may keep the β-oxidation and the glyoxylate cycle activities high to maintain heterotrophic growth in response to disrupted chloroplast biogenesis. Of note, we observed high expression of glyoxysomal *ICL* and *MLS* genes in green DEX-treated seedlings (Fig. 6C), although they did not show extra TAG possession in cotyledons (Fig. 2D), which implies that the glyoxysomal gene expression is not simply associated with TAG levels but rather appears to be linked to lipid metabolism in plastids. Furthermore, the high expression of *ICL* and *MLS* without strong downregulation of *GOX1* and *HPR1* in green seedlings indicates that the expression of glyoxylate cycle genes can be regulated independent of photorespiratory genes during peroxisome differentiation in cotyledons.

**Requirement of galactolipids for the initiation of chloroplast biogenesis**

To elucidate the role of *MGD1* expression and galactolipid biosynthesis in the initial stage of chloroplast development during seed germination, we suppressed *MGD1* expression early in chloroplast biogenesis by adding 10 µM DEX after a lapse of a certain period after seeding. Application of DEX within 36 h after seeding strongly
suppressed chlorophyll accumulation in 5-d-old seedlings, whereas DEX application 48 h after seeding decreased the inhibition in chlorophyll accumulation. In 5-d-old seedlings with DEX added 72 h after seeding, chlorophyll content was almost equal to that in DEX-untreated controls (Fig. 7A), although MGD1 expression was suppressed to nearly minimal levels of that of amiR-MGD1 constructs (Fig 7B). Even when the seedlings were treated with DEX for 5 d from 72 h after seeding, chlorophyll content did not substantially differ from that in untreated control (Supplemental Fig. S3A). Consistent with the chlorophyll levels, the expression of both nuclear- and plastid-encoded genes were not impaired in 5-d-old and 8-d-old L4 seedlings treated with DEX 72 h after seeding (Fig. 7C and Supplemental Fig. S3B), which suggests that galactolipid biosynthesis and presumably thylakoid membrane biogenesis at the initiation of chloroplast biogenesis are sufficient to ensure the induction of photosynthesis-associated genes. The expression of genes associated with peroxisomal and mitochondrial functions were also unchanged by the DEX treatment after initial seedling growth (Fig. 7C). Therefore, once MGDG is properly synthesized by MGD1 at the beginning of chloroplast biogenesis, development of chloroplasts and other organelles in cotyledons proceeds without severe retardation even if MGD1 expression is inhibited afterward. MGD1 expression may be crucial particularly at the initiation of chloroplast biogenesis in cotyledons but less required as seedlings grow.

**Effect of MGD1 suppression on development of true leaves**

The continuous induction of amiR-MGD1 also affected the development of true leaves in L2 and L4 seedlings, although the effect was milder than in cotyledons. In the L2 and L4 seedlings with white cotyledons, first and second true leaves appeared smaller and
paler than those in the wild type in the presence of DEX (Fig. 8A). Consistent with color, chlorophyll content in L4 true leaves were decreased with DEX treatment (Fig. 8B). When the PSII activity was monitored by measuring $F_v/F_m$ in the presence of DEX, we consistently detected lower values in the true leaves of both L2 and L4 white seedlings than in the wild type (Fig. 8C). However, in these lines, the PSII impairment in the true leaves was considerably weaker than in the cotyledons, which shows a diminished impact of $amiR-MGD1$ in true leaves.

To evaluate the suppression effect of the $amiR-MGD1$ transgene on the $MGD1$ expression in true leaves, we grew L4 seedlings with white cotyledons on DEX-containing media for 10 d and compared $MGD1$ expression in the true leaves with that in the DEX-untreated control. DEX treatment suppressed the $MGD1$ expression in the true leaves (Fig. 8D) to the same level as in the white cotyledons of 5-d-old seedlings (Fig. 2C), so the weak effect of the DEX treatment in the true leaves was not due to release of $MGD1$ suppression. To investigate whether the reduced $MGD1$ expression was sufficient for galactolipid biosynthesis in true leaves, we analyzed galactolipid content in true leaves in the white L4 class seedlings grown for 14 d in the presence of DEX (Fig. 8E). The proportion of MGDG in total-membrane glycerolipids was decreased by 25% in DEX-treated true leaves as compared with the DEX-untreated control, whereas the proportion of DGDG was unchanged. Consistent with the lipid data, MGDG synthesis rate estimated by $^{14}$C-acetate incorporation was partially decreased in true leaves by DEX treatment, whereas that in cotyledons was very low compared with the DEX-untreated control (Fig. 8F). These data indicate that MGDG biosynthesis was also decreased in true leaves by $amiR-MGD1$ although the suppression effect was weaker than that in the white cotyledons. The reason why the MGDG synthesis activity
largely differs from the \textit{MGD1} transcriptional level in the true leaves remains unclear. \textit{MGD2} and \textit{MGD3} expression appeared even decreased in true leaves of \textit{amiR-MGD1} and their contribution to leaf MGDG synthesis could be negligible (Supplemental Fig. S4) (Kobayashi et al., 2009a; Kobayashi et al., 2013). The MGD1 enzyme is reduced and activated in vitro by chloroplast thioredoxins, which could be coupled with photosynthetic activity in vivo (Yamaryo et al., 2006). Thus, the difference in photosynthetic activity (Fig. 8C) may explain the discrepancy in MGDG synthesis rate between true leaves and cotyledons in DEX-treated \textit{amiR-MGD1}. In addition, the MGDG synthesizing activity is regulated by anionic membrane lipids (Dubots et al., 2010) and also could be influenced by substrate supply. These factors may also affect the MGDG synthetic activity differently between true leaves and cotyledons. Processes of chloroplast differentiation differ considerably between cotyledons and true leaves (Pogson and Albrecht, 2011); in cotyledons, plastids partially developed during embryogenesis differentiate simultaneously into chloroplasts on light-induced germination (Mansfield and Briarty, 1996), whereas in true leaves, proplastids in the shoot apical meristem gradually differentiate into mature chloroplasts during leaf biogenesis (Charuvi et al., 2012). Thus, the requirement of \textit{MGD1} gene expression for sufficient galactolipid accumulation may vary between these two organs.

To address whether the reduced MGDG biosynthesis modifies gene expression in true leaves as observed in cotyledons (Fig. 6), we examined the expression of genes associated with organelle functions in true leaves of the 10-d-old white L4 seedlings (Fig. 8G). The expression of genes associated with photosynthesis and photorespiration was not decreased with DEX treatment, and \textit{psbA} expression even appeared to be increased in true leaves. In addition, \textit{ICL} expression was undetectable in true leaves
regardless of DEX treatment, which implies no prominent expression of glyoxysomal genes in response to MGD1 suppression in true leaves. Our data indicate that MGD1 suppression differently affects gene expression between cotyledons and true leaves. Impaired MGDG biosynthesis in the mgd1-2 mutant strongly downregulates both nuclear- and plastid-encoded photosynthetic genes in true leaves, which indicates a requirement of galactolipid biosynthesis in photosynthetic gene expression in true leaves (Kobayashi et al., 2013). Therefore, the 75% reduction in the MGD1 expression would be sufficient to downregulate photosynthetic genes in cotyledons but not true leaves. Distinct regulation of chloroplast differentiation between cotyledons and true leaves is evident from identification of several mutants with cotyledon-specific defects in chloroplast biogenesis (Pogson and Albrecht, 2011). Most of the cotyledon-specific mutants show impaired transcription and translation of plastid-encoded genes (Shirano et al., 2000; Yamamoto et al., 2000; Ishizaki et al., 2005; Albrecht et al., 2006; Ruppel and Hangarter, 2007; Chi et al., 2010; Woodson et al., 2013) and homeostasis of plastid proteins (Shimada et al., 2007; Albrecht et al., 2008; Tanz et al., 2012), which suggests that cotyledons possess a particular set of components essential for photosynthetic gene expression during early seedling growth, in which galactolipid biosynthesis by MGD1 may be involved.

MATERIALS AND METHODS

Plant materials and growth conditions

All plants used in this study were Ler ecotype of Arabidopsis thaliana. Seeds were surface-sterilized and then cold-treated 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 1.0% (w/v) sucrose solidified with 0.8% (w/v) agar except for experiments in Figures 1C and 7, A and B in which the liquid form of the medium was used. All plants were grown at 23°C under continuous white light (30 µmol photons m⁻² s⁻¹) in a growth chamber. For DEX treatment, DEX (Wako, http://www.wako-chem.co.jp/english/) was added to a final concentration of 10 µM in the media from a 50 mM stock in dimethyl sulfoxide. Unless otherwise stated, DEX was added to plants from the start of the cold treatment at the seed stage.

For the analyses in Figures 1C and 7, A and B, seeds cold-treated in deionized water in the dark were seeded in liquid MS medium and grown for 3 d in the chamber with gentle rotation (90 rpm). Only germinated seedlings were grown on solidified MS medium for another 2 d or 5 d to synchronize the germination time in the experiments. For the experiment in Figure 7, A and B, DEX was added to the medium after a lapse of each time from the start of the light growth.

For the Pi-deficient condition in Figure 1E, the concentration of KH₂PO₄ was reduced to 10 µM in the MS medium. Seedlings for the DEX-untreated control were grown on Pi-deficient medium without DEX for 5 d from germination. For DEX treatment, seedlings were first grown on Pi-deficient medium without DEX for 3 d and then on Pi-deficient medium containing 10 µM DEX for another 2 d.

**Construction of transgenic line**

The artificial microRNA targeting MGD1 was designed (miR-sense: 5’-TAGATTATTAGGCAGTGCAAC-3’) according to WMD2 software and instructions at the website (http://wmd2.weigelworld.org/cgi-bin/mirnatools.pl). The assembled amiRNA precursor fragment was cloned into pENTR entry vector (Invitrogen,
which as pOp6 was inserted at the *Not*I site. We cloned 35S::GRLhG4 fragment into the *Asc*I site of the same pENTR vector and selected the orientation of two promoters outgoing with each other (Fig. 1A). Then, 35S::GRLhG4/pOp6:amiR-MGD1 fragment in pENTR was transferred to the pBGW destination vector by LR recombination. Wild-type plants (*Ler* ecotype) were transformed with the resulting plasmid via transfection with *Agrobacterium tumefaciens* strain GV3101.

Forty-eight transgenic plant lines were isolated with 0.1% (w/v) Basta spray at the T1 generation. At the T2 generation, Basta-resistant seedlings germinated on soil were sprayed with 1 μM DEX solution (pH 7.0) once daily until true leaves appeared. Seven lines showing color change of true leaves with DEX treatment were isolated as candidates of functional amiR-MGD1 transgenic lines. Seeds harvested individually from these seven lines showed approximately 75% of Basta resistance, indicating a single copy of transgene insertion. We further harvested 8 independent T3 seeds for each line (56 lines in all) and selected those showing 100% Basta resistance to be homozygous in the transgene. In this study, lines 2, 4, 5, and 7 were used for detailed analyses.

**qRT-PCR analysis**

Total RNA was extracted by using the RNeasy Plant Mini kit (Qiagen, http://www.qiagen.com/). Genomic DNA digestion and reverse transcription involved the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa Bio, http://www.takara-bio.com/). cDNA amplification involved the Thunderbird PreMix kit (Toyobo, http://www.toyobo-global.com/) and 200 nM gene-specific primers.
(Supplemental Table S1). Thermal cycling consisted of an initial denaturation step at 95°C for 10 sec, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. Signal detection and quantification were performed in duplicate by use of MiniOpticon (Bio-Rad, http://www.bio-rad.com/). The relative abundance of all transcripts amplified was normalized to the constitutive expression level of ACTIN8 (Pfaffl, 2001). Three independent biological experiments were performed for each sample.

**Lipid analysis**

Total lipids were extracted from seedlings crushed into powder in liquid nitrogen and were separated by thin-layer chromatography with a solvent system of aceton/toluene/water (136:45:12, v/v/v) as described (Kobayashi et al., 2006). Lipids were visualized with 0.01% (w/v) primuline in 80% (v/v) acetone under UV light. MGDG, DGDG, TAG, and a mixture of other glycerolipids consisting of phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylglycerol, and sulfoquinovosyldiacylglycerol 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 2 h and quantified by gas chromatography (GC-17A, Shimadzu, http://www.shimadzu.com/) with myristic acid as an internal standard.

For [14C]acetate labeling assay, cotyledons from 5-d-old seedlings or true leaves from 10-d-old seedlings were incubated for 30 min with sodium [1-14C]acetate (1.9 MBq/ml). Lipids were extracted and separated by TLC as described above. Radioactivity detected by Imaging Plate (Fujifilm, http://www.fujifilm.com/) and an imaging analyzer (BAS-2500, Fujifilm) was quantified by ImageQuant (GE Healthcare, http://www3.gehealthcare.com/).
**Chlorophyll determination**

For chlorophyll determination in Figures 2B and 8B, seedlings crushed in liquid nitrogen were homogenized in 80% (v/v) acetone, and debris was removed by centrifugation at 15,000 rpm for 5 min. The chlorophyll content of samples was determined spectrophotometrically by measuring the absorbance of the supernatant at 720, 663, 647, and 645 nm with an Ultrospec 2100 pro spectrophotometer (GE Healthcare) according to the previous report (Melis et al., 1987). For the analyses in Figures 1C and 7A, chlorophyll was extracted from single intact seedlings by incubating each seedling in 1 mL 80% (v/v) acetone at 4°C for 3 d. Chlorophyll content in single seedlings was determined by measuring fluorescence emission at 666 nm under 440-nm excitation with an FP-6200 spectrofluorometer (JASCO, http://www.jascoinc.com/) with a chlorophyll sample of known concentration used as a standard.

**Photosynthetic chlorophyll fluorescence analysis**

Photosynthetic activity was determined with a chlorophyll fluorescence imaging system (IMAGING-PAM MAXI version, Walz, http://www.walz.com/) (Fig. 4, A and B, and Fig. 8C) or a chlorophyll fluorometer (JUNIOR-PAM, Walz) (Fig. 4, C-H). Seedlings on MS agar plates (Fig. 4, A and B, and Fig. 8C) or detached cotyledons (Fig. 4, C-H) were dark-incubated for 15 min before measurement. After measuring minimum chlorophyll fluorescence (F_o) in the dark, maximal chlorophyll fluorescence (F_m) was determined with a saturating pulse. After treatment with actinic light (36 and 45 μmol photons m^{-2} s^{-1} for IMAGING-PAM and JUNIOR-PAM, respectively) for 10 min, stationary fluorescence (F) and maximum fluorescence under the actinic light (F_m')...
were determined. Minimal fluorescence of illuminated samples \( (F_o') \) was computed with the approximation of Oxborough and Baker (1997): \( F_o' = F_o/(F_v/F_m + F_o/F_m') \).

From these fluorescence yields, photosynthetic parameters were calculated as follows (van Kooten and Snel, 1990; Maxwell and Johnson, 2000): \( F_v/F_m = (F_m-F_o)/F_m, F_v'/F_m' = (F_m'-F_o')/F_m' \), \( \Phi_{II} = (F_m'-F)/F_m' \), \( qP = (F_m'-F)/(F_m'-F_o') \). The actual PSII efficiency \( \Phi_{II} \) can be transformed into a product of the PSII openness \( (qP) \) and the quantum efficiency of open PSII \( (F_v'/F_m') \): \( \Phi_{II} = (F_m'-F)/F_m' = qP \cdot F_v'/F_m' \). \( \Phi_{NPQ} \) and \( \Phi_{NO} \) were determined according to the method of Kramer et al. (2004).

Measurement parameters for IMAGING-PAM were as follows: measuring light intensity = 2, measuring light frequency = 4, damping = 4, gain = 1, saturation pulse intensity = 10. For quantification, averaged fluorescence values in a circular area were collected from a cotyledon or true leaf for each seedling by using the software ImagingWin (Walz). For the analysis with JUNIOR-PAM, the automated induction program provided by the WinControl-3 software (Walz) was used with default settings for measuring light and saturation pulse.

For chlorophyll fluorescence induction experiments (Fig. 4, I and J), 5-d-old seedlings were dark-incubated for 5 min before experiments. Eight seedlings were used for an experiment in a batch. When required, the seedlings were infiltrated with 40 µM DCMU and 150 mM sorbitol by depression before dark incubation. Chlorophyll fluorescence transients were measured in a logarithmic time series between 30 µs and 10 s after the onset of strong actinic light (1650 µmol photons m\(^{-2}\) s\(^{-1}\)) with a LED pump-probe spectrometer (JTS-10, BioLogic, http://www.bio-logic.info/).

Fluorescence emission spectra of chlorophyll proteins at 77 K were obtained directly from plant tissues (Fig. 5A) or from membrane fractions (Fig. 5B) in liquid nitrogen by using an RF-5300PC spectrofluorometer (Shimadzu) under 435-nm
excitation. To prepare membrane fractions, seedlings were pulverized in liquid nitrogen and homogenized in a cold buffer (0.33 M sorbitol, 5 mM MgCl₂, 5 mM EDTA, 50 mM HEPES-KOH, pH 7.6). The homogenate was filtered through a single layer of Miracloth (Merck Millipore, http://www.merckmillipore.com/) with gentle hand pressure. After centrifugation at 5,000 g for 10 min at 4°C, the supernatant was discarded and the pellet was resuspended in a cold buffer to obtain 0.8 μg/ml chlorophyll-containing membrane fractions.

Microscopy analysis

Arabidopsis seedlings were fixed in 2.5% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.0 at room temperature for 4 h. After rinses with phosphate buffer (3 times for 20 min each), samples were postfixed in 1% (w/v) OsO₄ in the same buffer for 4 h at room temperature and rinsed with phosphate buffer (3 times for 20 min each). Samples were dehydrated in an ethanol series and propyene oxide, embedded in Spurr’s resin, and sectioned with use of an ultramicrotome (Reichert Ultracut S or EM UC6, Leica, http://www.leica-microsystems.com/). For light microscopy, sections (0.8 μm) were stained with 1% (w/v) Toluidine blue with sodium Borex and analyzed under a light microscope (BX60, Olympus, http://www.olympus-global.com/en/). For electron microscopy observation, ultrathin sections (70-90 nm) were stained with 5% (w/v) uranyl acetate in 50% (v/v) methanol and 0.4% (w/v) lead citrate in 0.1 N NaOH. Sections were observed by transmission electron microscopy (CM 100, Philips, http://www.fei.com/) at 80KV and images were obtained by use of a Gatan Orius CCD camera (http://www.gatan.com/).
**Immunoblot analysis**

The membrane protein fraction was prepared as described previously (Kobayashi et al., 2013) from 5-d-old wild-type and L2 pale-green seedlings treated with 10 µM DEX. Twenty micrograms of total membrane protein from L2 sample was electrophoresed together with a dilution series (0.2-5 µg) of total membrane protein from wild-type and electrotransferred onto nitrocellulose membranes (Amersham Protran Premium 0.2 NC, GE Healthcare) as described (Kobayashi et al., 2007). Protein bands that reacted with primary antibodies were secondarily labeled with goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (Thermo Scientific, http://www.thermoscientific.com/) and detected using a chemiluminescence reagent (Pierce Western Blotting Substrate Plus, Thermo Scientific) and an imager (ImageQuant LAS 4000 mini, GE Healthcare). Antibodies against PsaA/PsaB was kindly provided by R. Tanaka, Hokkaido University, Sapporo, Japan and those against D1, and D2 were kindly provided by M. Ikeuchi, The University of Tokyo, Tokyo, Japan. Antibodies against LHCA1, LHCA3, LHCB1, and LHCB3 were from AgriSera (http://www.agrisera.com/).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Fatty acid composition of TAG.

**Supplemental Figure S2.** Organelle ultrastructure of 5-d-old amiR-MGD1 L4 cotyledons.

**Supplemental Figure S3.** Effect of 5-d DEX treatment from 72 h after seeding in the amiR-MGD1 L4 line.

**Supplemental Figure S4.** Expression of MGD2 and MGD3 in true leaves of 10-d-old
amiR-MGD1 L4 seedlings grown on DEX-free or DEX-containing media.

Supplemental Table S1. Oligonucleoide primers used for quantitative reverse transcription-PCR analysis.

ACKNOWLEDGEMENTS

We thank the technical support in electron microscopy by Dr. Wann-Neng Jane, Plant Cell Biology Care Laboratory, Institute of Plant and Microbial Biology, Academia Sinica. We also thank Dr. Krishna K. Niyogi, Department of Plant and Microbial Biology, University of California, Berkeley, for providing access to equipment for photosynthetic measurements and valuable comments.
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FIGURE LEGENDS

Figure 1. Characterization of the *amiR-MGD1* seedlings. A, Schematic representation of inducible *amiR-MGD1* construction. The amiRNA precursor fragment designed to target *MGD1* was cloned under 6-times repeat of the OP promoter. The rat glucocorticoid receptor (GR) fused to the transcription activator LhG4, which consists of a high-affinity DNA-binding mutant of lac repressor (lacI<sup>His17</sup>) fused to transcription-activation domain II of GAL4 from *Saccharomyces cerevisiae* (Gal4-II), was cloned under the CaMV 35S promoter. *amiR-MGD1* was designed to target 20 nt across the stop codon of *MGD1* mRNA. B, Photograph of wild-type (WT) and *amiR-MGD1* seedlings (L2, L4, L5, and L7) grown on dexamethasone (DEX)-free (−DEX) or 10 μM DEX-containing (+DEX) media. C, Chlorophyll content per seedling in each *amiR-MGD1* line grown on −DEX or +DEX media. The horizontal line in each box represents the median value of the distribution. The top and the bottom of each box represent the upper and lower quartile, respectively. The whiskers represent the range (n = 24). D, Expression of *MGD1* in WT and each *amiR-MGD1* line under +DEX or −DEX conditions. E, Expression of three *MGD* genes in phosphate-deficient L4 seedlings grown under +DEX or −DEX conditions. Data are fold difference from the +DEX WT in D and from the −DEX control in E (means ± SE, n = 3). Plants used in B to E are all 5-d-old seedlings.

Figure 2. Diverse phenotypes of *amiR-MGD1*. A, Classification of DEX-treated L4 seedlings into three color groups (green, pale-green and white). B, Chlorophyll content in each color class of DEX-treated L2 and L4 seedlings and DEX-untreated (−DEX) control. C, *MGD1* expression in green and white seedlings of DEX-treated L2 and L4.
Data are fold difference from −DEX control of each line. D, Glycerolipid composition in L4 seedlings grown under +DEX or −DEX conditions. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; TAG, triacylglycerol. In B to D, data are mean ± SE from three independent experiments. Plants used in A to D are all 5-d-old seedlings.

**Figure 3.** Plastid ultrastructure and cotyledon morphology of *amiR-MGD1*. Electron micrographs of plastids (A to C) and light micrographs of cross-sections (D to F) from 5-d-old L4 seedlings. A and D, DEX-untreated (−DEX) control. Green (B, E) and white (C, F) cotyledons of DEX-treated seedlings. Scale bars = 1.0 μm in A to C and 50 μm in D to F.

**Figure 4.** Photosynthesis characteristics in 5-d-old *amiR-MGD1*. A and B, Scatter diagrams between maximal chlorophyll fluorescence (Fm) (x-axis) and (A) maximum quantum yield of PSII (Fv/Fm) or (B) effective quantum yield of PSII (ΦII) (y-axis) in wild type (WT), L2 and L4 under DEX-treated conditions. Each point represents the fluorescence data in one cotyledon (n = 75-100). C, Fv/Fm in WT and each color class of DEX-treated L2 seedlings. D to H, Induction curves of (D) ΦII, (E) coefficient of photochemical quenching (qP), (F) maximum quantum yield of PSII under light conditions (Fv’/Fm’), (G) quantum yield of non-regulated energy dissipation (ΦNO), and (H) quantum yield of regulated energy dissipation (ΦNPQ). I and J, Transient fluorescence induction kinetics of chlorophyll in WT and white seedlings of L2 and L4 (I) in the absence or (J) presence of 40 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Two inflections, labeled J and I, were observed between the levels O (origin)
and P (peak) only in the −DCMU samples.

**Figure 5.** Role of MGD1 in formation of photosystem complexes. A and B, 77-K chlorophyll fluorescence spectra (A) in wild-type (WT) and white seedlings of L2 and L4 in the presence of DEX and (B) in the membrane fraction (0.8 µg/ml chlorophyll) from WT and each color class of DEX-treated L2 seedlings. C, Immunoblot analysis of membrane photosynthetic proteins from WT and L2 pale-green seedlings in the presence of DEX. A 20 µg aliquot of total membrane protein from the L2 seedlings was compared with a dilution series (20, 5 and 1 µg) of membrane proteins from WT.

**Figure 6.** Expression of genes associated with organelle functions in 5-d-old cotyledons. Quantitative RT-PCR analysis of expression of genes associated with photosynthesis (A), photorespiration (B) and glyoxylate cycle (C) in DEX-untreated (−DEX) or DEX-treated green or white L4 seedlings. Data are fold difference from −DEX control (means ± SE, n = 3).

**Figure 7.** Involvement of galactolipids in initial organelle development. A, Inhibition of chlorophyll accumulation in 5-d-old L2 and L4 seedlings by DEX treatment at different times from 0 to 72 h after seeding. Chlorophyll content in 5-d-old seedlings continuously grown in the absence of DEX shown as untreated control (−DEX). The horizontal line in each box represents the median value of the distribution. The top and the bottom of each box represent the upper and lower quartile, respectively. The whiskers represent the range (n = 24). B, *MGD1* expression in 5-d-old L2 and L4 seedlings with DEX added 72 h after seeding (72 h) or continuously grown in the
absence of DEX (−DEX). C, Expression of genes associated with photosynthesis, photorespiration and glyoxylate cycle in 5-d-old L4 seedlings with DEX added 72 h after seeding (72 h) or continuously grown in the absence of DEX (−DEX). In B and C, data are fold difference from −DEX control (means ± SE, n = 3).

Figure 8. Characteristics of amiR-MGD1 true leaves. A, 8-d-old seedlings of wild type (WT), L2 and L4 continuously grown on DEX-containing media. B, Chlorophyll content in true leaves of 14-d-old L4 seedlings grown on DEX-free (−DEX) or DEX-containing (+DEX) media. C, Changes in maximum PSII quantum yield (Fv/Fm) in true leaves (left panel) or cotyledons (right panel) of DEX-treated WT, L2 and L4 seedlings from day 7 to day 10 after seeding. Data are mean ± SE (n = 10). D, MGD1 expression in true leaves of 10-d-old L4 seedlings grown on −DEX or +DEX media. E, Proportion of galactolipids in total glycerolipids in true leaves of 14-d-old L4 seedlings grown on −DEX or +DEX media. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol. F, Relative radioactivity in MGDG per total radioactivity in lipid fractions extracted from [14C]acetate-labeled leaves of L4 seedlings grown on −DEX or +DEX media. True leaves from 10-d-old seedlings or cotyledons from 5-d-old seedlings were used. G, Expression of genes associated with photosynthesis, photorespiration and glyoxylate cycle in true leaves of 10-d-old L4 seedlings grown on −DEX or +DEX media. * not detected. In D and G, data are fold difference from DEX-untreated control. In B and D to G, data are mean ± SE from three or four independent experiments. All +DEX amiR-MGD1 seedlings used in A to G had white cotyledons.
Figure A: Chlorophyll content (nmol/seedling) over time with different DEX addition times after seeding. The data is represented in box plots for L2 and L4 samples.

Figure B: Relative expression of MGD1 gene under -DEX and 72 h conditions for L2 and L4 samples.

Figure C: Relative expression of various genes (psaA, psbA, LHCb6, CHLH, GOX, GLDP1, ICL) under -DEX and 72 h conditions.
