Eukaryotic lipid metabolic pathway is essential for functional chloroplasts and CO_2 and light responses in *Arabidopsis* guard cells

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Edited by Krishna K. Niyogi, Howard Hughes Medical Institute and University of California, Berkeley, CA, and approved July 27, 2018 (received for review June 22, 2018)

Stomatal guard cells develop unique chloroplasts in land plant species. However, the developmental mechanisms and function of chloroplasts in guard cells remain unclear. In seed plants, chloroplast membrane lipids are synthesized via two pathways: the prokaryotic and eukaryotic pathways. Here we report the central contribution of endoplasmic reticulum (ER)-derived chloroplast lipids, which are synthesized through the eukaryotic lipid metabolic pathway, in the development of functional guard cell chloroplasts. We gained insight into this pathway by isolating and examining an *Arabidopsis* mutant, *gles1* (*green less stomata 1*), which had achlorophyllous stomatal guard cells and impaired stomatal responses to CO_2 and light. The *GLES1* gene encodes a small glycine-rich protein, which is a putative regulatory component of the trigalactosyldiacylglycerol (TGD) protein complex that mediates ER-to-chloroplast lipid transport via the eukaryotic pathway. Lipidomic analysis revealed that in the wild type, the prokaryotic pathway is dysfunctional, specifically in guard cells, whereas in *gles1* guard cells, the eukaryotic pathway is also abrogated. CO_2-induced stomatal closing and activation of guard cell S-type anion channels that drive stomatal closure were disrupted in *gles1* guard cells. In conclusion, the eukaryotic lipid pathway plays an essential role in the development of a sensing/signaling machinery for CO_2 and light in guard cell chloroplasts.

stomata | chloroplast | lipid metabolism | CO_2 | *Arabidopsis*

Stomatal pores allow an influx of CO_2 in exchange for transpirational water loss. The stomatal aperture is regulated by environmental and physiological factors, especially CO_2, the plant hormone abscisic acid (ABA), humidity, light, and ozone (1–4). Chloroplasts in the guard cells of stomata have been proposed to play an important role in osmoregulatory mechanisms mediating stomatal movements (5, 6), although their functions have been a subject of debate. To date, studies on guard cell chloroplasts have largely focused on their photosynthetic activities (7–9), whereas the relevance of lipid synthesis remains poorly investigated.

Chloroplast development accompanies the biogenesis of thylakoid membranes, which requires the coordinated synthesis of membrane proteins and glycerolipids. The thylakoid membranes consist of the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol and the phospholipid phosphatidylglycerol (PG). Fatty acids are exclusively synthesized de novo within plastids, but the assembly of fatty acids into the glycerolipids of thylakoid membranes occurs via two distinct pathways: the prokaryotic pathway and the eukaryotic pathway (10–12). In the prokaryotic pathway, all reaction steps take place within the chloroplast (hence called the plastidial pathway), whereas in the eukaryotic pathway or the cooperative pathway, fatty acids are exported from the chloroplast to the cytosol to be assembled into glycerolipids in the endoplasmic reticulum (ER). Some of the ER-localized glycerolipids return to the chloroplast to serve as a substrate for glycolipid synthesis (10–12) (*SI Appendix*, Fig. S1). The prokaryotic pathway contributes to chloroplast PG synthesis in all plants, and to glycolipid synthesis only in the so-called 16:3 plants, which contain 16:3 (acyl carbons: double bonds) in the *sn*-2 position of MGDG. In contrast, the eukaryotic pathway contributes to the glycolipid synthesis in all plants. MGDG synthesized via the eukaryotic pathway contains C18 fatty acids, most abundantly 18:3, in the *sn*-2 position, and those plants, including pea (*Pisum sativum*) and rice (*Oryza sativa*), that exclusively use the eukaryotic pathway in plastid glycolipid synthesis have been referred to as 18:3 plants. In 16:3 plants, the contribution of the prokaryotic and the eukaryotic pathways varies among different plant species (13). The contribution of the lipid flux through the prokaryotic pathway is the highest in nonseed plants, such as mosses and ferns, whereas it has been estimated as 38% in *Arabidopsis* (14). Moreover, even in the same16:3 plant species, the prokaryotic and the eukaryotic pathways do not necessarily work at a fixed proportion in all tissues. For example, in *Arabidopsis*, the prokaryotic pathway appears to be strongly diminished during embryo development (15–18). Although each of the prokaryotic and the eukaryotic pathways produces lipid

Significance

Guard cells have photosynthetically active chloroplasts in most plant species. However, the significance of their existence in guard cells or their developmental mechanisms is unknown. Here, through a forward-genetic approach, we have identified a key feature and a function of guard cell chloroplasts. We observed that a mutation that impaired chloroplast biogenesis in guard cells also disrupted the regulation of stomatal movements by CO_2 and light. We demonstrated that guard cell chloroplasts, compared with those in mesophyll cells, display a unique lipid metabolism, in which the prokaryotic pathway is diminished and the eukaryotic pathway gains control. Our findings highlight the importance of the eukaryotic pathway for developing functional chloroplasts in guard cells.

Author contributions: J.N. and K.I. designed research; J.N., S.M., B.S., R.T., M.F., T.A.-S., C.B.E., and K.K. performed research; J.N. contributed new reagents/analytic tools; J.N., I.N., J.I.S., and K.I. analyzed data; and J.N., I.N., J.I.S., and K.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1810458115/-/DCSupplemental.

Published online August 20, 2018.

molecular species with distinct fatty acid distribution, the physiological significance of such metabolic divergence remains unclear.

The eukaryotic pathway requires lipid transfer from the ER to chloroplasts. The trigalactosyldiacylglycerol (TGD) protein complex has been identified as playing a major role in the lipid transfer from the ER to chloroplasts (19). The TGD protein complex itself is an ABC-type transporter consisting of three subunits: TGD1, TGD2, and TGD3. TGD1 is an ABC transporter permease located in the inner envelope membranes of chloroplast, TGD3 is an ATPase or a nucleotide-binding protein facing to stroma, and TGD2 is a substrate-binding protein, which is suggested to bind a lipid substrate in outer envelope membranes to deliver it to TGD1. An additional subunit, TGD4, is located in outer envelope membranes to play a tethering role between the ER and the chloroplast envelope (20, 21). Furthermore, TGD5, a small glycine-rich protein, has been hypothesized to play a regulatory role in the ER-to-chloroplast lipid transfer (22).

To date, the studies of lipid metabolism in guard cells have been confined to an 18:3 plant species, *Vicia faba* (23, 24). Using [^{14}C] acetate labeling, guard cell protoplasts from *V. faba* have been shown to produce eukaryotic lipid molecular species (23). Guard cells are known to contain a large amount of the triacylglycerols produced by the eukaryotic lipid metabolic pathway (24). Recently, it has been reported that triacylglycerols stored in guard cells are used to produce ATP required for light-induced stomatal opening (25). However, the distinct roles of prokaryotic and eukaryotic lipid metabolic pathways in guard cells have not been understood.

In this study, we have found, through a forward-genetic approach, that lipid synthesis in guard cells is distinct from that in mesophyll cells, and that the prokaryotic pathway is extensively retarded in *Arabidopsis* guard cells. As a consequence, lipid transfer from ER to chloroplast through the eukaryotic pathway gains more significance and seems essential for guard cell chloroplast development and for stomatal CO_2 and light responses in *Arabidopsis* guard cells.

Results and Discussion

Isolation of *Arabidopsis gles1* Mutant That Develops Abnormal Chloroplasts in Guard Cells. Previously, we isolated a CO_2-insensitive mutant line (*cdi6*) from an M2 population of ~30,000 ethyl methanesulfonate-mutagenized *Arabidopsis* plants, using leaf infrared imaging thermography (3). This technology enabled us to isolate a number of mutants that showed abnormal leaf temperature resulting from malfunction in stomatal movement (3). The *cdi6* mutant line showed two phenotypes [irregularly shaped stomata (26) and achlorophyllous stomata], but these phenotypes were segregated by backcrossing with WT. In this study, we separated a

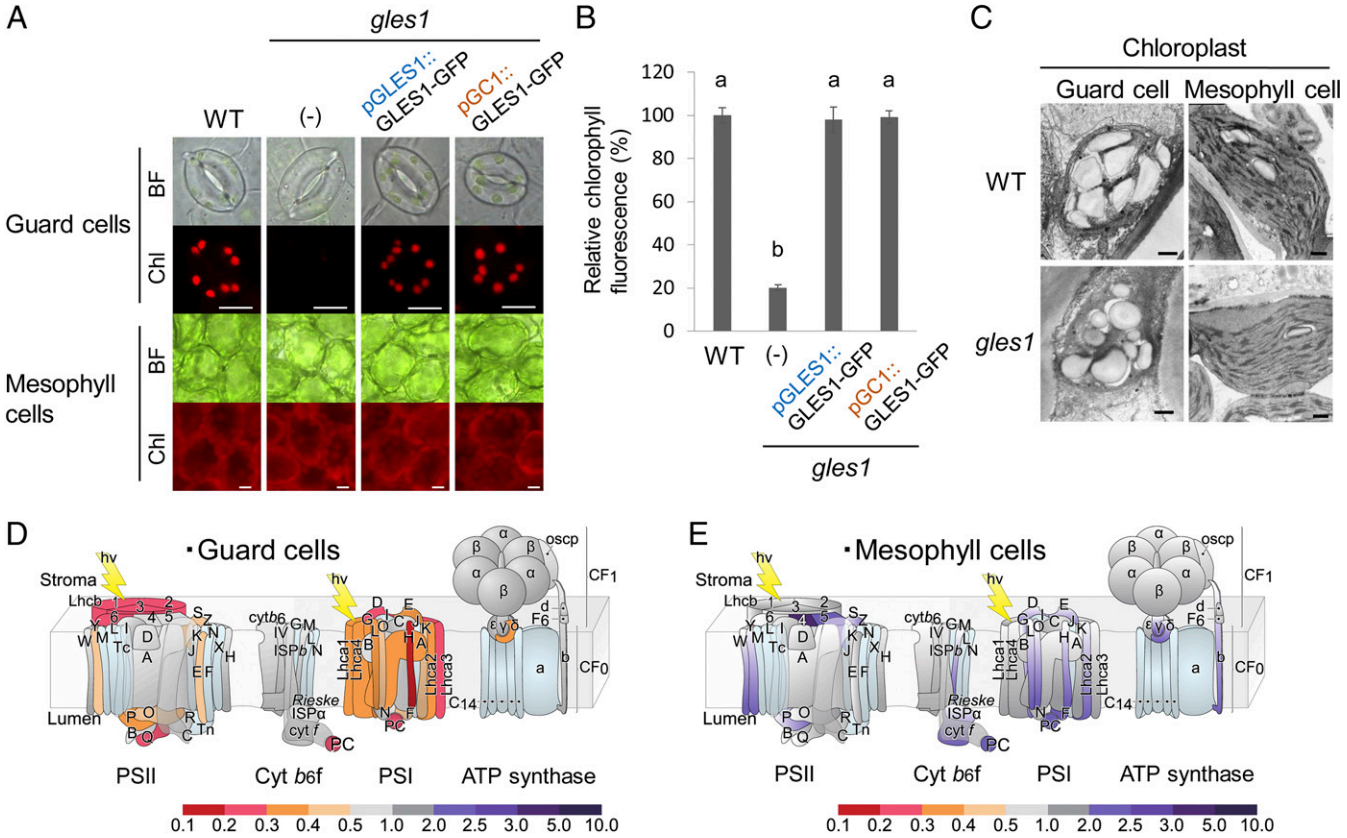

Fig. 1. Mutation in *GLES1* impairs chloroplast development in guard cells. (*A*) Guard cells in WT contain well-developed chloroplasts, but the imaged *gles1* stomata have a negligible amount of chloroplasts and chlorophyll autofluorescence in their guard cells. In contrast, the *gles1* mutant has normal chlorophyllous mesophyll cells. (Scale bars, 10 μm.) These *gles1* phenotypes were restored in transformants expressing GLES1-GFP driven by GLES1's own promoter or guard cell-specific GC1 promoter. BF, Bright Field; Chl, Chlorophyll fluorescence. Percentage relative chlorophyll fluorescence values ± SE ($n > 100$) are shown in *B*. The statistical significance was determined by a one-way ANOVA with Tukey–Kramer multiple comparison tests. Same letters (a and b) indicate no significant difference ($P > 0.05$). (*C*) Ultrastructure of plastids in WT and *gles1* mutant plants. Mutant chloroplasts are as developed as WT chloroplasts in mesophyll cells. However, they fail to develop thylakoid membranes in guard cell. (Scale bars, 10 μm.) (*D* and *E*) Schematic view of gene expression of thylakoid-associated photosynthetic components integrated with microarray data. Fold-changes were calculated as *gles1*/WT control and are shown as a heat map. In achlorophyllous *gles1* guard cell protoplasts that were sorted by FACS (*SI Appendix*, Fig. S2*B*: type I), the expression of photosynthesis genes is significantly down-regulated (*D*). In contrast, the expression of these genes is not down-regulated in the *gles1* mesophyll cell protoplasts (*E*). Note that only transcripts for nuclear-encoded proteins could be detected on the microarrays used. Further information is available in *SI Appendix*, Table S1.

recessive mutation responsible for achlorophyllous stomata from the *cdi6* line and designated it as *gles1*. Although WT plants displayed normal chlorophyll fluorescence in guard cells and mesophyll cells (Fig. 1*A*), *gles1* exhibited reduced chlorophyll fluorescence specifically in some guard cells (Fig. 1*A*): *gles1* mutants developed different types of stomata with differentially reduced chlorophyll fluorescence, which were categorized as achlorophyllous (*SI Appendix*, Fig. S2*A*, type I; 12.2 ± 1.2%), faintly chlorophyllus (*SI Appendix*, Fig. S2*A*, type II; 70.0 ± 1.5%), and chlorophyllous stomata (*SI Appendix*, Fig. S2*A*, type III; 17.8 ± 1.5%). The fluorescence values differed substantially from WT controls (*SI Appendix*, Fig. S2*A*). Similar trends were also observed in guard cell protoplasts (GCPs) isolated from WT and *gles1* using flow cytometry. Chlorophyll fluorescence decreased in more than 70% *gles1* GCPs (*SI Appendix*, Fig. S2*B*).

We then observed the ultrastructure of WT and mutant chloroplasts from guard cells and mesophyll cells by electron microscopy. In mesophyll cells, WT and *gles1* chloroplasts showed comparable thylakoid membrane structure and development with similar numbers of granal stacks and some starch granules (Fig. 1*C*). In guard cells, however, *gles1* chloroplasts showed fewer thylakoid membranes with smaller granal stacks compared with WT (Fig. 1*C*). These results indicate that the thylakoid-less phenotype of *gles1* is specific to guard cells.

Using GCPs and mesophyll cell protoplasts (MCPs) isolated from WT and *gles1*, we also measured transcription levels of thylakoid membrane system components in guard cells and mesophyll cells by microarray experiments. Compared with WT control cells, the expression of thylakoid-associated photosynthetic components was significantly reduced in the type I population of *gles1* guard cells isolated by cell sorting (Fig. 1*D* and *SI Appendix*, Table S1). In contrast, the expression was not reduced but was slightly up-regulated in *gles1* mesophyll cells (Fig. 1*E* and *SI Appendix*, Table S1). The *gles1* mutant showed no differences in the maximum efficiency of photosystem II (F_v/F_m; *SI Appendix*, Fig. S3*A*) or CO_2 assimilation rates at 360 ppm compared with WT in whole-plant measurements (*SI Appendix*, Fig. S3*B*). These results suggested that *gles1* mutation did not affect photosynthetic activity in whole leaves.

GLES1 Was Identical to TGD5, a Putative Regulatory Component of the TGD Protein Complex. By map-based cloning, we originally identified that *gles1* mutation had a single ^{160}G-to-A substitution in *At1g27695*, which caused a ^{54}Gly-to-Arg exchange in a small glycine-rich protein (Fig. 2*A* and *SI Appendix*, Fig. S4). The ^{54}Gly residue was conserved among four GLES1 orthologs from a variety of higher plant species (Fig. 2*A*). Introduction of a genomic *GLES1* sequence fused in-frame with green fluorescent protein (GFP) marker gene (*GLES1-GFP*) into *gles1* plants fully restored the WT phenotype, verifying that *At1g27695* is *GLES1* (Fig. 1 *A* and *B*). Furthermore, the distribution of GFP fluorescence in the guard cells of GLES1-GFP plants was similar to that of AtOUTER ENVELOPE MEMBRANE PROTEIN 7 (AtOEP7)-GFP (27), which is a marker protein localized to the envelope of plastids (Fig. 2*B*). These results demonstrated that GLES1 is a chloroplast envelope-localized protein in guard cells. To investigate the expression patterns of GLES1, we examined the expression of the β-glucuronidase reporter driven by the *GLES1* promoter in transgenic plants. GLES1 expression was detected in the whole plant, including guard cells (*SI Appendix*, Fig. S5). However, the molecular function of GLES1 had remained unidentified until Fan et al. (22) reported that *At1g27695* encodes TGD5, a putative regulatory component for the TGD protein complex. We therefore investigated whether mutation in components of TGD protein complex and its related protein TDG4 could exhibit *gles1*-like phenotypes. We found that the null mutant *tgd4-3* (20) exhibited similar *gles1* phenotypes in guard cells; that is, reduced chlorophyll fluorescence (Fig. 2 *C* and *D*). A knockdown mutant *tgd3-1* (28) also exhibited reduced chlorophyll fluorescence in guard cells (Fig. 2 *C* and *D*), but less severely than *tgd4-3*. These results suggested that the TGD proteins are important for the development of mature chloroplasts in guard cells.

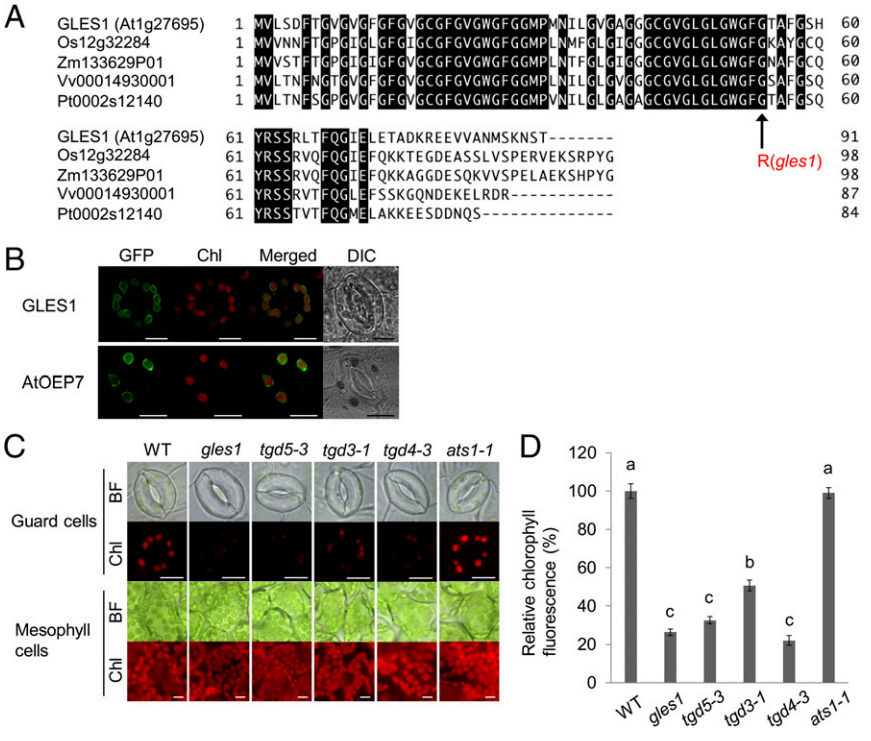

Fig. 2. TGD proteins involved in lipid transport from ER to chloroplasts are essential for guard cell chloroplast development. (*A*) Alignment of amino acid sequence of the GLES1(At1g27695) protein and its orthologs assessed using Clustal W. GLES1-like proteins are found in various plants, including *Oryza sativa* (Os12g32284), *Zea mays* (Zm133629P01), *Vitis vinifera* (Vv00014930001) and *Populus trichocarpa* (Pt0002s12140). Amino acid identity is indicated by black boxes. The *gles1* mutation site Glycine 53 is conserved among the orthologs, as indicated by the arrowhead. (*B*) Chloroplast envelope localization of GLES1-GFP. Expression of GLES1–GFP fusion protein resulted in functional complementation of the *gles1* mutant phenotypes (Fig. 1 *A* and *B*). GFP fluorescence, autofluorescence of chlorophyll, the overlay of all fluorescence signals, and the differential interference contrast images (DIC) are shown for a representative example. AtOEP7-GFP was used as the chloroplast envelope marker. Chl, Chlorophyll fluorescence. (Scale bars, 10 μm.) (*C* and *D*) GLES1 is identical to TGD5, a subunit of TGD protein involved in the ER-to-plastid transport. The T-DNA insertion allele *tgd5-3* and loss of function mutants of other TGD complex subunits exhibited achlorophyllous phenotypes in guard cells. (Scale bars, 10 μm.) Total chlorophyll autofluorescence of individual guard cells was analyzed (*D*). The average chlorophyll autofluorescence measured for WT plants was designated as 100%, and relative fluorescence has been plotted (%). Values shown are means ± SE ($n > 100$) of five independent experiments. The statistical significance was determined by a one-way ANOVA with Tukey-Kramer multiple comparison tests. Same letters (a–c) indicate no significant difference ($P > 0.05$).

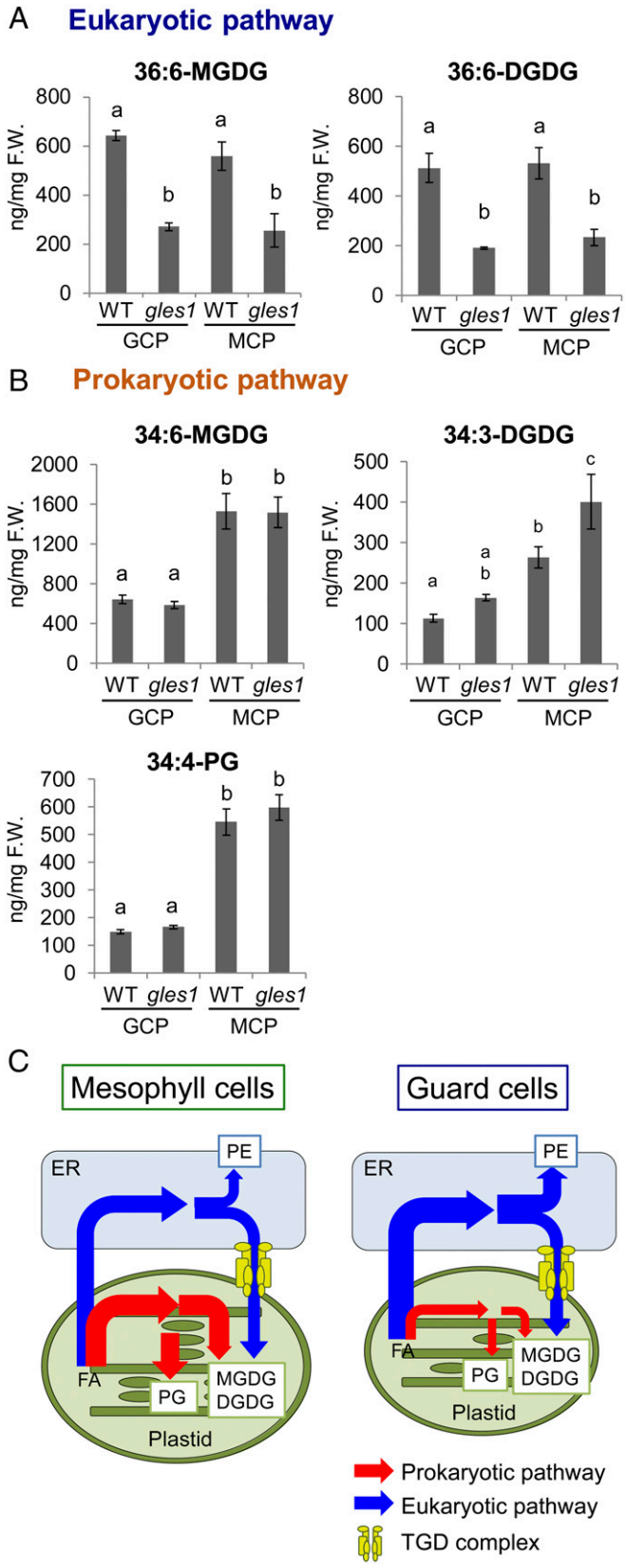

Fig. 3. The prokaryotic pathway of lipid synthesis is suppressed in guard cells. (*A* and *B*) Chloroplast polar lipid species were determined in guard cell protoplasts GCPs and MCPs, which were isolated from WT and *gles1* mutants. As an overview, we have depicted representatives of the most abundant species derived from the eukaryotic pathway (*A*) or prokaryotic pathway (*B*). MGDG, DGDG, and PG content are shown. Values shown are means ± SE ($n = 4$). The statistical significance was determined by a one-way ANOVA with Tukey-Kramer multiple comparison tests. Same letters (a–c) indicate no significant difference ($P > 0.05$). A complete dataset with details on analysis is given in *SI Appendix*, Fig. S6. (*C*) Schematic diagram of deduced lipid flux model in *Arabidopsis* mesophyll cells and guard cells. We propose that the contribution of the prokaryotic and eukaryotic lipid pathways is different between mesophyll cells and guard cells, and that the guard cells rely on the eukaryotic pathway exclusively. Therefore, the ER-to-plastid lipid trafficking mediated by the TGD complex is essential for the formation of guard cell chloroplasts. FA, fatty acid; PE, phosphatidylethanolamine.

The Prokaryotic Lipid Metabolic Pathway Is Severely Down-Regulated in Guard Cells. In *Arabidopsis*, *ATS1* encoding plastid-targeted glycerol-3-phosphate acyltransferase catalyzes the biosynthesis of lysophosphatidic acid, the first step of the prokaryotic pathway or the glycerolipid biosynthesis within plastids (*SI Appendix*, Fig. S1). We found that the *ats1-1* mutant that is blocked in the prokaryotic pathway (29) had no effect on chlorophyll fluorescence in guard cells (Fig. 2 *C* and *D*). Because the TGD proteins have been shown to be involved in lipid transfer from the ER to plastids in the eukaryotic pathway (30), and the loss-of-function mutants of TGD complex subunits had a reduced chlorophyll fluorescence in guard cells (Fig. 2 *C* and *D*), we hypothesized that ER-derived lipid precursors are essential for the synthesis of chloroplast glycolipids, and hence, the development of chloroplasts in guard cells. To address this hypothesis, we determined the content of chloroplast lipids in GCPs and MCPs isolated from leaves of WT and *gles1* plants. In both GCPs and MCPs, the *gles1* mutation reduced the contents of 36:6-MGDG and 36:6-DGDG, both of which are synthesized from diacylglycerol derived from the eukaryotic pathway (Fig. 3*A* and *SI Appendix*, Fig. S6). In WT plants, it is noteworthy that the contents of the prokaryotic glycerolipid molecular species such as 34:6-MGDG, 34:3-DGDG, and 34:4-PG were reduced in guard cells compared with those in mesophyll cells (Fig. 3*B* and *SI Appendix*, Fig. S6). In contrast, phosphatidylethanolamine, which is an ER-produced phospholipid, increased in guard cells compared with that in mesophyll cells (*SI Appendix*, Fig. S6). The *Arabidopsis* Δ7-desaturase FAD5, which is specific to palmitate esterified at the *sn*-2 position of MGDG, is responsible for the synthesis of 34:6-MGDG in the prokaryotic pathway, and this enzyme is not present in 18:3 plants (31). To exclude the possibility that the down-regulation of *FAD5* decreased the content of 34:6-MGDG in guard cells, we conducted microarray analysis between the transcripts of guard cells and mesophyll cells. The results showed that the expression level of *FAD5* in guard cells was comparable to that in mesophyll cells (GCP/MCP ratio = 1.35 ± 0.23; $P = 0.4$). Thus, the lower content of 34:6-MGDG in guard cells compared with mesophyll cells should be ascribed to a decreased flux of the prokaryotic pathway, and not to the changes in *FAD5* expression levels. These results suggest that guard cells have a limited contribution from the prokaryotic pathway and rely on the eukaryotic pathway for chloroplast development (Fig. 3*C*). Therefore, when the *gles1* mutation disrupted the eukaryotic pathway, it must have caused drastic defects in the development of guard cell chloroplasts.

Guard Cell Chloroplasts Are Essential for Light-Induced Stomatal Opening and CO_2-Induced Stomatal Closure. To evaluate the roles of guard cell chloroplasts in stomatal movement in WT and *gles1* leaves, we measured stomatal responses to CO_2, light, and ABA. The type I (achlorophyllous) stomata of *gles1* mutants showed significantly reduced responses to CO_2 (Fig. 4*A* and *SI Appendix*, Fig. S7*A*) and light (Fig. 4*B* and *SI Appendix*, Fig. S7*B*). Compared with the WT, *gles1* stomata opened more slowly and less extensively in response to a CO_2 shift from 360 to 0 ppm (Fig.

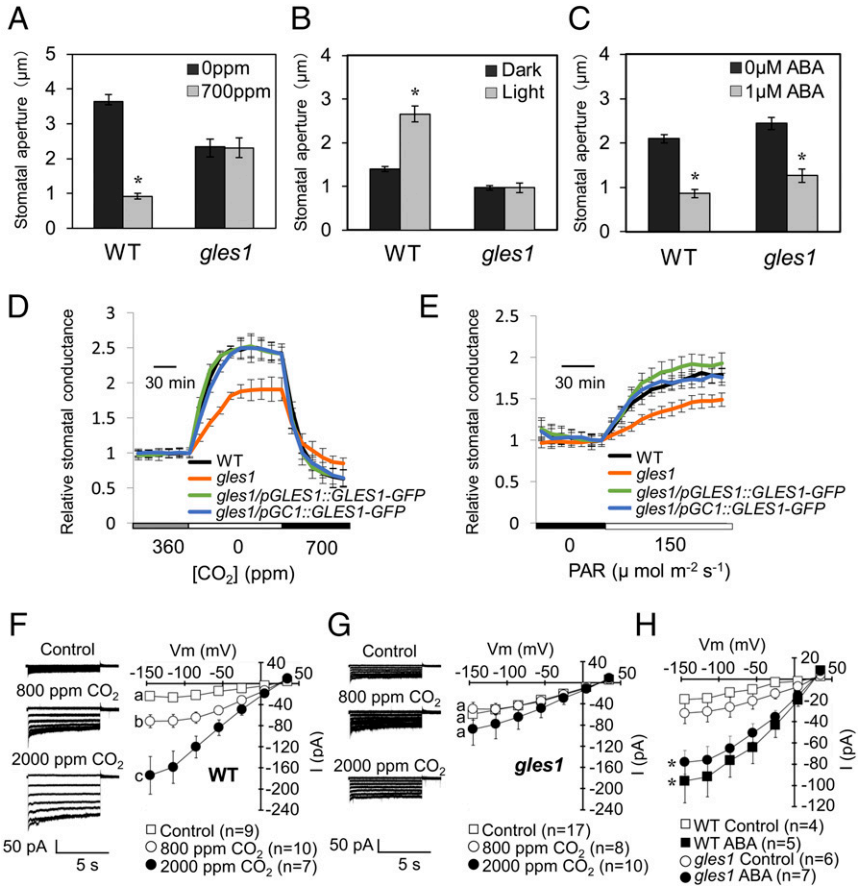

Fig. 4. Chloroplasts in guard cells have a central role in the regulation of CO_2- induced stomatal closure via S-type anion channel activation. (*A*–*C*) Stomatal aperture in *gles1* mutant and WT. The achlorophyllous stomata (*SI Appendix*, Fig. S2*A*: type I) in *gles1* mutant fail to respond to high [CO_2] (*A*) and light (*B*), but show a normal response to ABA (*C*). Values shown are means ± SE (n = 4 independent experiments with >50 stomata per experiment). Asterisks indicate significant differences ($P < 0.05$, Student's t test). (*D* and *E*) CO_2 and light responses are impaired in the *gles1* mutant. Time course of stomatal conductance in *gles1* mutant, *gles1*/pGLES1:GLES1-GFP, *gles1*/pGC1:GLES1-GFP, and WT in response to changes in CO_2 concentrations (*D*) or in light intensity (*E*). Stomatal conductance was normalized to the average conductance at the last 360 ppm CO_2 data point (*D*) and the last 0 μmol $m^{-2}{\cdot}s^{-1}$ PAR data point (*E*). Values shown are means ± SE ($n = 5$). (*F* and *G*) CO_2 activation of S-type anion channels is impaired in *gles1* GCPs. Representative current traces (*Left*) and steady state current–voltage relationships (*Right*) are shown. CO_2 activates S-type anion channel currents in WT GCPs (*F*), but not in *gles1* GCPs (*G*). Values shown are means ± SE. Different lowercase letters indicate significant differences at −145 mV ($P < 0.05$, Tukey-Kramer test). (*H*) ABA activation of S-type anion channels remains intact in *gles1* guard cell protoplasts. Steady state current-voltage relationships of the whole-cell currents recorded in the WT (squares) and *gles1* mutant (circles) with (black) or without (white) ABA are shown. Error bars indicate ± SE. Asterisks indicate significant differences (without ABA vs with ABA at −145 mV; $P < 0.02$, Student's t test).

4*D*), and closed more slowly in response to a CO_2 shift from 0 to 700 ppm (Fig. 4*D*). Similarly, compared with the WT, *gles1* stomata opened more slowly and less extensively in response to illumination at 150 μmol $m^{-2}{\cdot}s^{-1}$ (Fig. 4*E*). In contrast, the stomatal aperture showed a similar decrease in size in both WT and achlorophyllous *gles1* stomata in response to applied ABA, indicating that the *gles1* mutation did not affect stomatal responses to applied ABA (Fig. 4*C* and *SI Appendix*, Fig. S7*C*). These results indicate that the function of guard cell chloroplasts is important not only for the light-induced stomatal opening but also for the CO_2-induced stomatal closing. The guard cell S-type anion channel, SLOW ANION CHANNEL 1 (SLAC1), has a crucial role for CO_2- and ABA-induced stomatal closure (32, 33). We therefore tested whether the S-type channel activity is impaired in *gles1* mutants, using whole-cell patch clamp techniques. Our results showed that elevated CO_2 concentrations activated S-type anion channel currents in WT guard cells (Fig. 4*F*), but the CO_2 response was much diminished in *gles1* guard cells (Fig. 4*G*). Interestingly, ABA activation of the S-type anion channel current in the *gles1* guard cells was not impaired (Fig. 4*H*), indicating that the *gles1* mutation did not cause structural defects in the S-type anion channel. These results suggest that the *gles1* mutation affected the signaling pathway from CO_2 sensing to the S-type anion channel activation, and that guard cell chloroplasts could have an important role in CO_2-dependent activation of S-type anion channels.

We found that *tgd4-3* (20) stomata also showed reduced CO_2 sensitivity in intact *Arabidopsis* leaves (*SI Appendix*, Fig. S8). Interestingly, the knockdown mutant *tgd3-1* (28) also exhibited reduced chlorophyll fluorescence in guard cells, but its stomatal CO_2 responsiveness was not affected (*SI Appendix*, Fig. S8), suggesting that the residual activity of lipid transfer from the ER to chloroplasts in *tgd3-1* mutants was not sufficient to maintain the WT levels of photosynthetic activity, but was sufficient to maintain the perception and signaling events involved in CO_2-induced stomata closure. In contrast, the prokaryotic pathway mutant *ats1-1* (29) had no effect on the stomatal CO_2 response (*SI Appendix*, Fig. S8). These results suggested that ER-derived lipids have an important role within the chloroplast, possibly in an osmoregulatory mechanism mediating stomatal movements. Alternatively, down-regulation of the lipid flux from the ER to chloroplasts may have a secondary effect on the lipid metabolism in the extrachloroplastic compartments, which could eventually affect the perception and signaling events involved in CO_2-induced stomatal closure. However, our study did not exclude the possibility that, in addition to the lipid transfer, GLES1/TGD5 could play an unknown regulatory role in CO_2-induced signaling processes.

It has been proposed that stomatal conductance is affected by photosynthetic activity in the mesophyll (34, 35). However, *gles1* mutants showed normal leaf photosynthetic activity in our experimental conditions (*SI Appendix*, Fig. S3). Therefore, it seems unlikely that photosynthetic activity of the mesophyll cells could have affected stomatal CO_2 and light responses in *gles1* mutants, despite the fact that *GLES1* expression was recognized in whole-plant tissues (*SI Appendix*, Fig. S5). However, to exclude this possibility, we created a transgenic *gles1* plant that expressed *GLES1* under the control of the guard-cell-specific promoter pGC1 (*SI Appendix*, Fig. S9). The resultant transformant developed normal chloroplasts in guard cells (Fig. 1 *A* and *B*) and showed normal stomatal responses (Fig. 4 *D* and *E*). These results demonstrated that *GLES1* plays a crucial role in the regulation of stomatal movements by CO_2 and light when expressed

in guard cells, and that *gles1* mutation in mesophyll cells had little influence on the stomatal responses in *gles1* guard cells.

Conclusions

We conclude that the lipid supply pathway from the ER to chloroplasts has a significant contribution in the development of guard cell chloroplasts and the regulation of stomatal movements in response to CO_2 and light. Chloroplasts of nonseed plants are essentially autonomous in membrane lipid synthesis, but the relative contributions of prokaryotic pathways to plastid glycolipid synthesis have diminished during the course of evolution, and have even become extinct in 18:3 plants (13). To date, the physiological relevance of the prokaryotic pathways in the extant 16:3-plants has not been well described, except that it is dispensable for the development both of male and female gametophytes and of embryos before the heart stage (15–18). Thus, our present finding that the prokaryotic pathway to chloroplast glycolipids is significantly down-regulated in guard cells compared with mesophyll cells in *Arabidopsis thaliana*, a 16:3 plant (Fig. 3), provides additional evidence for the advantage of the eukaryotic pathway in plant lipid metabolism and would help unravel the physiological significance of the evolution of lipid metabolic pathways in plants. The present study also points to key functions of the eukaryotic lipid pathway in the physiological regulation of stomatal movements.

Materials and Methods

The details and procedures of plant materials and growth conditions, fluorescence microscopy, electron microscopy, isolation of guard cell protoplasts and mesophyll cell protoplasts, cell sorting of guard cell protoplasts, *Arabidopsis* gene expression microarray, construction of binary vectors for plant transformation, transgene expression analysis, measurement of lipid content, whole-plant stomatal conductance and photosynthesis measurements, microscopic analysis of stomatal responses, and patch clamp analyses are provided in *SI Appendix, Supplementary Materials and Methods*.

ACKNOWLEDGMENTS. We thank Dr. F. Beisson for critical reading of the manuscript. We also thank N. Kawahara and Y. Johno for the technical assistance. We appreciate the technical assistance from The Research Support Center, Research Center for Human Disease Modeling, Kyushu University Graduate School of Medical Sciences. This work was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (JP25891020 and JP15K18556 to J.N., and JP26221103 to K.I.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Core Research for Evolution Science and Technology, Japan Science and Technology Agency (JPMJCR1505 to K.I.) and grants from the National Science Foundation (MCB-1616236 to J.I.S.), and in part the National Institutes of Health (GM060396 to J.I.S.).

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