Supporting Information

Supplementary Materials and Methods

Plant Materials and Growth Conditions. All lines of *Arabidopsis* used were derived from the Columbia ecotype. EMS-mutagenized M2 seeds were purchased from Lehle Seeds (Round Rock, TX). The mutant lines: *tgd3-1* (SALK_040335), *tgd4-3* (SAIL_133_H06), *tgd5-3* (SAIL_254_A11) and *ats1-1* (CS200) were obtained from the Arabidopsis Biological Resource Center. Transgenic plants were generated by *Agrobacterium*-mediated transformation as previously described (1). Plants were grown on solid Murashige-Skoog (MS) medium for 19 days in a growth chamber (constant white light of 35 μmol m^{-2} s^{-1} at 22°C, 70% RH), and then transplanted into vermiculite pots supplemented with mineral nutrients.

Fluorescence Microscopy. Chlorophyll autofluorescence images were obtained using a fluorescence microscope (BZ-9000; Keyence). The filter set used was Cy5 (exciter, 590-650 nm; emitter, 665-735 nm). For guard cell imaging, epidermal tissues were peeled. For mesophyll cell imaging, whole leaves were cut into small squares (5 mm^2) with a razor blade. Quantification of chlorophyll autofluorescence signals in each guard cell was obtained using an image analysis software (Metamorph; Molecular Devices).

Electron Microscopy. For transmission electron microscopy (TEM), epidermal peels (~2 to 4 mm^2) were collected from rosette leaves of 4-weeks-old wild-type (WT) and *gles1* plants, and were fixed immediately with 2.0% glutaraldehyde in 100 mM sodium phosphate buffer, pH 7.3, and remained for 3 h on ice. These tissue samples were then washed twice with 100 mM sodium phosphate buffer for 30 min, and post-fixed with 2% osmic acid in 100 mM sodium phosphate buffer for 2 h. The fixed tissues were further dehydrated serially with 30, 50, 65, 75, 85, 95 and 100% ethanol on ice. The samples were retained for 10 min in each ethanol solution except the 100% solution, in which they remained for 2 h. The tissue samples were subjected to two 20 min dehydration steps in propylene oxide and then placed in 1:1 and 3:1 (v/v) mixtures of the Spurr's resin (TAAB, UK) and propylene oxide for 1 h and 3 h, respectively.

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Finally, the samples were incubated for 24 h in undiluted Spurr's resin and sandwiched between an Aclar film (Honeywell International, USA) and a slide glass. The samples were polymerized at 70°C for 24 h. Sectioning and microphotography were carried out at the Hanaichi Electron Microscopic Laboratory, Inc. (Okazaki, Aichi, Japan).

Isolation of Guard Cell Protoplasts and Mesophyll Cell Protoplasts Guard cell protoplasts and mesophyll cell protoplasts were isolated enzymatically from 4- to 5-week-old *Arabidopsis* plants as described previously (2, 3).

Cell Sorting of Guard Cell Protoplasts. Guard cell protoplasts (3×10^6 cells) isolated from WT and *gles1* mutant plants were loaded in the cell sorter (EPICS ALTRA; Beckman Coulter) and were selected based on fluorescence emission intensity in the red (665-685 nm) channel. 0.4 M mannitol containing 1 mM $CaCl_2$ was used as sorting buffer. For transcriptome analysis, protoplasts were collected in tubes until the total cell count reached 100,000.

***Arabidopsis* Gene Expression Microarray.** Total RNA was isolated from guard cell protoplasts and mesophyll cell protoplasts using an RNeasy Plant Mini Kit (Qiagen). Total RNA was used to prepare Cyanine-3 (Cy3) labeled probes using a low RNA input linear amplification/labeling kit (Agilent). Labeled cRNA probes were fragmented using fragmentation buffer (Agilent) and hybridized to the whole *Arabidopsis* Gene Expression Microarray (V4, design ID 21169; Agilent) for 17 h at 65°C in a hybridization oven. The arrays were air-dried and scanned using the high-resolution array scanner (Agilent) with the appropriate settings for one-color gene expression arrays. Raw data files were imported into GeneSpring software (Agilent) and normalized using the intra-array percentile shift normalization (threshold of 75 and above). P values for the comparison of genotypes were calculated using GeneSpring software.

Construction of Binary Vectors for Plant Transformation. To construct a binary vector for functional complementation of the *gles1* mutant, a 2.79-kb fragment of the

BAC clone T22C5 (49,190 to 51,982) was inserted between SalI and SacI sites of a binary vector pBI101, together with *GFP* gene from the plasmid CaMV35S-sGFP(S65T) (4). To generate pGC1::GLES1–GFP, the SalI/NcoI fragment obtained by digestion of the PCR-amplified *GLES1* ORF was inserted into the SalI/NcoI sites of the plasmid CaMV35S-sGFP(S65T). The CaMV35S promoter was replaced by 1,716bp of the GC1 promorter regions (5). The gene cassette of pGC1::GLES1–GFP was inserted into the BamHI/SacI sites of the binary vector pBI101. To generate 35S::AtOEP7–GFP, the SalI/NcoI fragment obtained by digestion of the PCR-amplified *AtOEP7* ORF was inserted into the SalI/NcoI sites of the plasmid CaMV35S-sGFP(S65T). The gene cassette of 35S::AtOEP7–GFP was inserted into the HindIII/SacI sites of the binary vector pBI101. A binary vector, pGLES1::GUS, a 2.0-kb SalI/NcoI fragment of the BAC clone T22C5 (49,190 to 51,196) was inserted between SalI and SacI of pBI101, together with a NcoI/SacI fragment of pSLAC1::GUS（6）that harbors *GUS* gene for examination of GUS expression under the control of the *GLES1* promoter.

Transgene Expression Analysis. Histochemical staining of GUS activity in the pGLES1::GUS transformants was performed as described previously (2). Two-week-old pGLES1::GUS transformants grown on MS medium were incubated for 6 h in staining buffer that contained 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 0.1% Triton X-100, and 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid. On the other hand, leaf epidermal peels prepared from three-week-old plants that expressed the GLES1-GFP fusion protein were analyzed using a confocal microscope (LSM510; Carl Zeiss) (3) or a fluorescence microscope (BZ-9000; Keyence) (2)

Measurement of Lipid Contents. Guard cell protoplasts and mesophyll cell protoplasts (20-50 mg FW) were kept in liquid nitrogen and a 4-fold volume of extraction solvent [methanol (MeOH) : methyl tert-butyl ether (MTBE) = 1:4, v/v] was added followed by vigorous stirring on a vortex mixer and sonication. After a subsequent addition of a 3.25-fold volume of mixture of MeOH and water (1:3, v/v), the homogenate was centrifuged at 20,000 g for 10 min. The supernatant samples were

diluted with extraction solvent. LC-MS was performed with an Agilent 1200 system (Agilent Technologies) coupled to a LTQ Orbitrap XL (ThermoFisher Scientific), which was equipped with an electrospray source operating in the positive-ionization mode, and with a lock-spray interface for accurate mass measurement. An aliquot of the extracted sample (5 µL) was injected into a HALO C18 column (2.7 µm, 3 x 30 mm; Advanced Materials Technologies) with mobile phases containing solvent A (MeOH : water : acetate : 25% ammonium hydroxide solution = 500:500:1:1) and solvent B (isopropanol : acetate : 25% ammonium hydroxide solution = 1000:1:1). The gradient program was as follows: 0-100% solvent B for the first 15 min, 100% solvent B for the next 5min, and 0% solvent B from 20 to 25 min, with a flow rate of 0.8 mL/min. The column oven temperature was set at 50°C. LC-MS analysis was performed in the positive-ionization mode of ESI. All data obtained from the LC-MS analysis were acquired with the Xcalibur™ software (ThermoFisher Scientific). The lipids in each class were quantified in comparison to standards, such as MGDG (Avanti, 840523P), DGDG (Avanti, 840524P), 18:0–18:1-PG (Avanti, 840503P) and di18:0-PE (Avanti, 850715P).

Whole-Plant Stomatal Conductance and Photosynthesis Measurements The whole plant stomatal conductance to water vapor (g_s), the chlorophyll fluorescence (F) and the CO_2 assimilation rate were measured with a portable gas exchange fluorescence system (GFS-3000, Heinz Walz) equipped with a 3010-A *Arabidopsis* chamber and an LED array unit 3055-FL as actinic light source. The response of g_s to CO_2 was measured at constant light intensity (150 µmol m^{-2} s^{-1}) and 60% RH. The g_s response to light was measured under constant [CO_2] (350 ppm) and 60% RH. The flow rate in the system was kept constant (750 µmol s^{-1}) throughout the gas exchange experiments. Measurements were recorded every minute. For measurement of the chlorophyll fluorescence (F), the leaves were adapted to dark conditions for 3 hours before measurement. The maximum fluorescence (Fm) and minimum fluorescence (F_0) were determined by applying a saturating light pulse as described manufacture protocol. The maximum PS II quantum yield (Fv/Fm) was calculated as Fv/Fm = (Fm- F_0)/Fm.

Microscopic Analysis of Stomatal Responses. Abaxial epidermal peels of three-week-old plants were used to measure stomatal apertures. In the CO_2 treatment, plants were

incubated at the indicated [CO_2] in growth cabinets. After 90 min of adaptation to low [CO_2] (0 ppm) or high [CO_2] (700 ppm), epidermal tissues were peeled. Plants used for measurements of light response were pre-incubated overnight under dark condition. After 3 hours of illumination with white light (60 μmol m^{-2} s^{-1}), epidermal tissues were peeled. In the ABA treatment, epidermal peels were floated on a test medium containing 30 mM KCl, 10 mM MES-KOH (pH 6.15) and 0.1 mM $CaCl_2$ and were incubated in the growth chamber. ABA was added to the solution after 1 h of illumination and stomatal apertures were measured 2 h later. The stomatal apertures in epidermal peels were observed with a fluorescence microscope (IX71, Olympus).

Patch Clamp Analyses. Patch clamp analyses of S-type anion channel currents were performed as previously reported (7, 8). To isolate *Arabidopsis* GCPs, one or two rosette leaves of 4- to 5-week-old plants were blended in a blender with cold deionized water. Epidermal tissues were collected using a 100-μm nylon mesh and rinsed well with cold deionized water. Then the epidermal tissues were incubated in 10 ml of an enzyme solution containing 1% (wt/vol) cellulase R-10 (Yakult, Japan), 0.5% (wt/vol) macerozyme R-10 (Yakult, Japan), 0.5% (wt/vol) bovine serum albumin, 0.1% (wt/vol) kanamycin sulfate, 10 mM ascorbic acid, 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-mannitol for 16 hr at 25°C on a circular shaker at 40 rpm. After the incubation, GCPs were collected through a 20-μm nylon mesh, and then washed twice with a washing solution containing 0.1 mM KCl, 0.1 mM $CaCl_2$, and 500 mM D-sorbitol (pH 5.6 with KOH). The isolated GCP suspension was kept on ice before use. The pipette solution contained 150 mM CsCl, 2 mM $MgCl_2$, 5.86 mM $CaCl_2$, 6.7 mM EGTA, 5 mM Mg-ATP, and 10 mM Hepes-Tris (pH 7.1). Free $[Ca^{2+}]_{cyt}$ (2 μM) was calculated with the WEBMAXC standard program (http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm). The bath solution contained 30 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM MES-Tris (pH 5.6). The osmolality of the pipette solution and bath solution was adjusted to 485 mmol/L and 500 mmol/L using D-sorbitol, respectively. To investigate CO_2-activation of S-type anion channels, the bath solution was bubbled with 2000 ppm CO_2, 800 ppm CO_2, or CO_2-free air (through soda lime, as control experiments) for 1 hr before the experiments. GCPs were pre-incubated with the CO_2-equilibrated bath solution for 10 min. The bath solution was constantly perfused using a peristatic pump and bubbled

during the pre-incubation and during experiments. To investigate ABA-activation of S-type anion channels, GCPs were pre-incubated with 10 μM ABA for 30 min before experiments. Voltage was stepped from +35 mV to −145 mV with 30 mV-decrements and the holding potential was adjusted to +30 mV.

Supplementary Figures

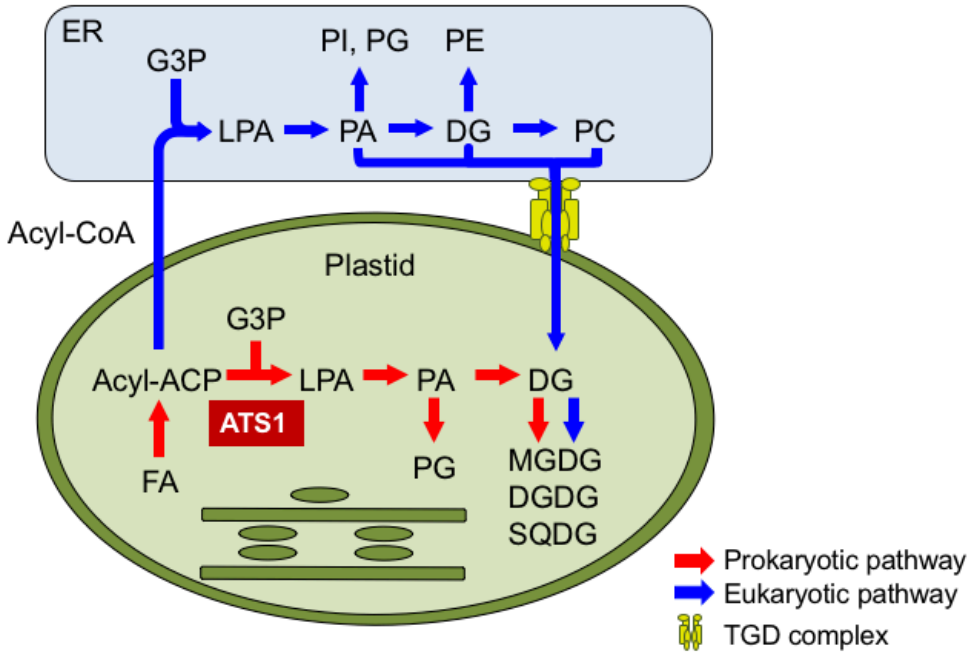

Fig. S1. A schematic diagram of the metabolism of lipids to chloroplast glycolipids, MDGD, DGDG, and SQDG. In seed plants, two distinct pathways have been described, i.e., the prokaryotic and the eukaryotic pathways. The prokaryotic pathway produces C18/C16 DG within the chloroplast whereas the eukaryotic pathway produces C18/C18 DG, using lipid precursors imported from the ER. The TGD complex contributes to the ER-to-chloroplast lipid transfer. The *ATS1* gene encodes a chloroplast-localized glycerol-3-phosphate acyltransferase that initiates the prokaryotic pathway. FA, fatty acid; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PG, phosphatidylglycerol; DG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; ER, endoplasmic reticulum.

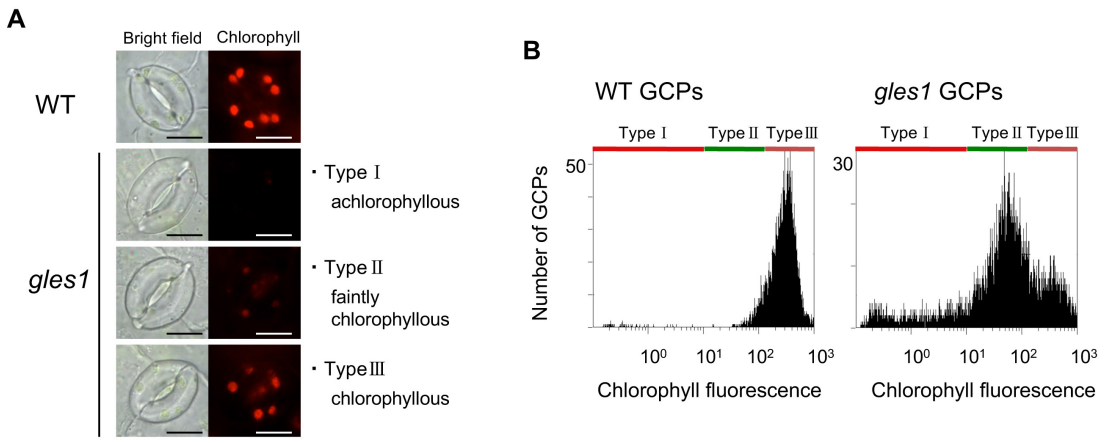

Fig. S2. In the *gles1* mutant, most guard cells have negligible chlorophyll autofluorescence. (*A*) The *gles1* stomata were classified into three groups based on the intensity of chlorophyll fluorescence. Representative images of Type I (achlorophyllous), Type II (faintly chlorophyllous), Type III (chlorophyllous) are shown. (*B*) Chlorophyll fluorescence histogram of wild-type (WT) and *gles1* guard cell protoplasts (GCPs). GCPs isolated from WT and *gles1* were subjected to flow cytometry. These GCPs were categorized into three types according to relative intensity of chlorophyll fluorescence; Type I ($<10^1$), Type II (10^1 to 10^2) and Type III ($>10^2$). In WT GCPs, Type I is 1.0%, Type II is 4.6% and Type III is 93.7%. In *gles1* GCPs, Type I is 14.6%, Type II is 52.3% and Type III is 31.8%.

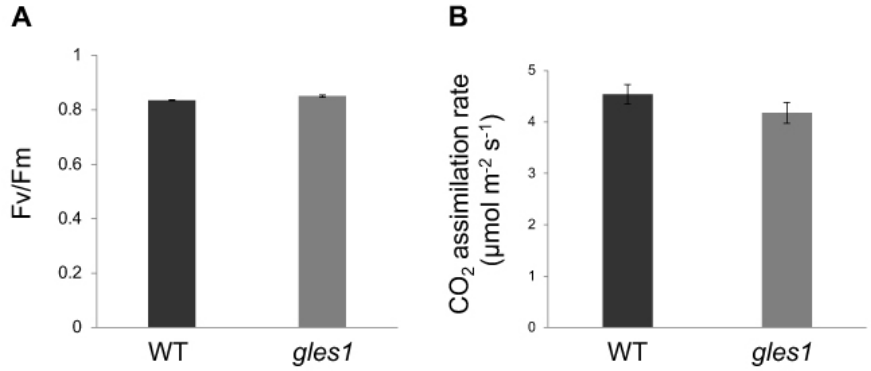

Fig. S3. Photosynthetic activity parameters in whole leaves (*A*) F_v/F_m in dark-adapted leaves was unaffected in *gles1* mutant plants. Values shown are means ± S.E. (*n* = 4). (*B*) CO_2 assimilation rate at 380 ppm [CO_2] was not impaired in *gles1* plants. Plants were kept under constant white light of 150 µmol m^{-2} s^{-1} at 22°C in 60% relative humidity. Values shown are means ± S.E. (*n* = 9).

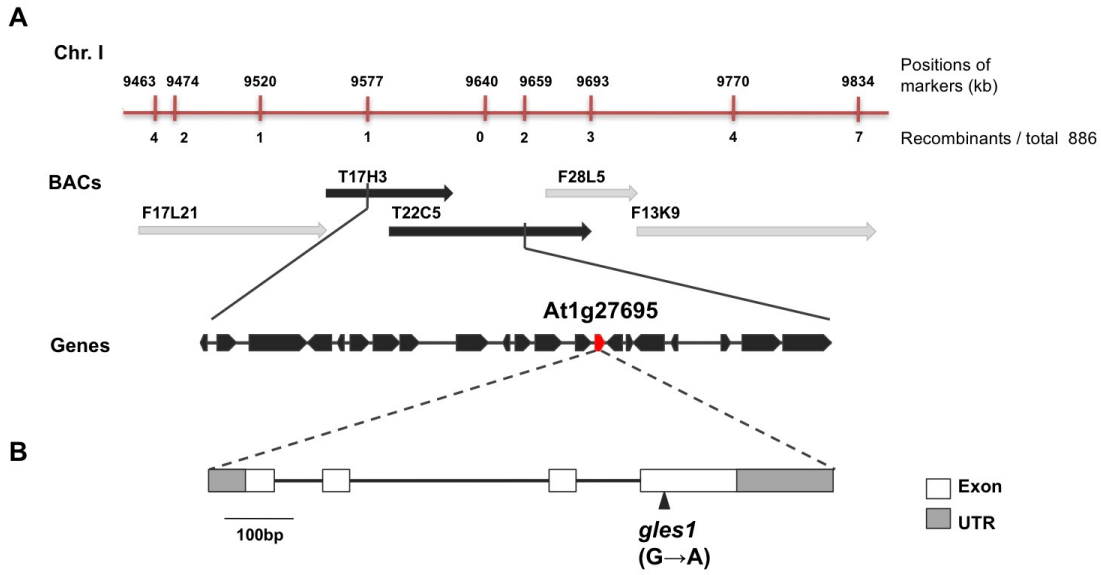

Fig. S4. Map-based cloning of the *GLES1* gene. (*A*) Genetic map of the *GLES1* locus. The location of molecular markers, the number of recombinants in 886 chromosomes, and the corresponding BAC clone are indicated. Open reading frames within the 120 kb interval are shown at the bottom. GLES1 encodes At1g27695. (*B*) Exon-intron structure of *GLES1* gene. Position of mutation in *gles1* is indicated by arrowheads. The substitution of a single nucleotide, from G to A, resulting in the substitution an amino acid from 54G to R was found in *gles1*.

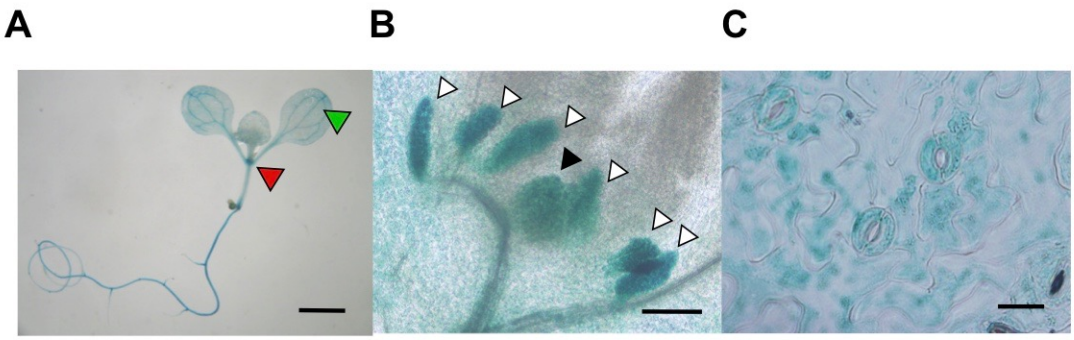

Fig. S5. Expression pattern of *GLES1*. (*A* to *C*) GLES1 promoter activity was histochemically analyzed using a GLES1 promoter-GUS reporter gene that was introduced into wild-type plants. The GLES1 gene was widely expressed throughout the plant and showed predominant expression in vascular tissues and meristem. Young seedling at 7 days after germination (*A*). A magnification of the region indicated by red arrows in (*A*) is shown in (*B*). Young leaf primordia (indicated by white arrows) and shoot apical meristem (indicated by black arrows) were stained in particular. A magnification of the region indicated by green arrows in (*A*) is shown in (*C*). Guard cells and epidermal cells were stained. Scale bars; 25mm (*A*), 100μm (*B*), 20μm (*C*).

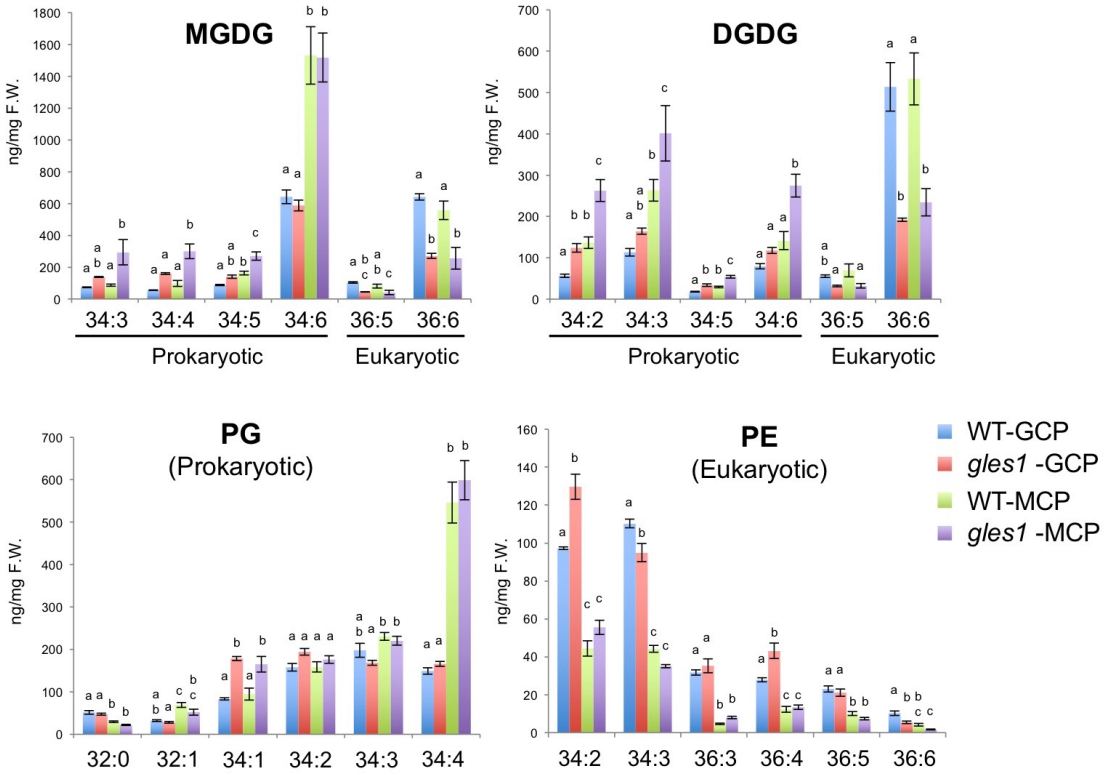

Fig. S6. Profiling of molecular species of glycerolipid classes in guard cell protoplasts and mesophyll cell protoplasts. Monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG) and Phosphatidylethanolamine (PE) content values are shown as means ± S.E. (n = 4). The statistical significance was determined by a one-way ANOVA with Tukey-Kramer multiple comparison tests. Same letters indicate no significant difference ($P > 0.05$).

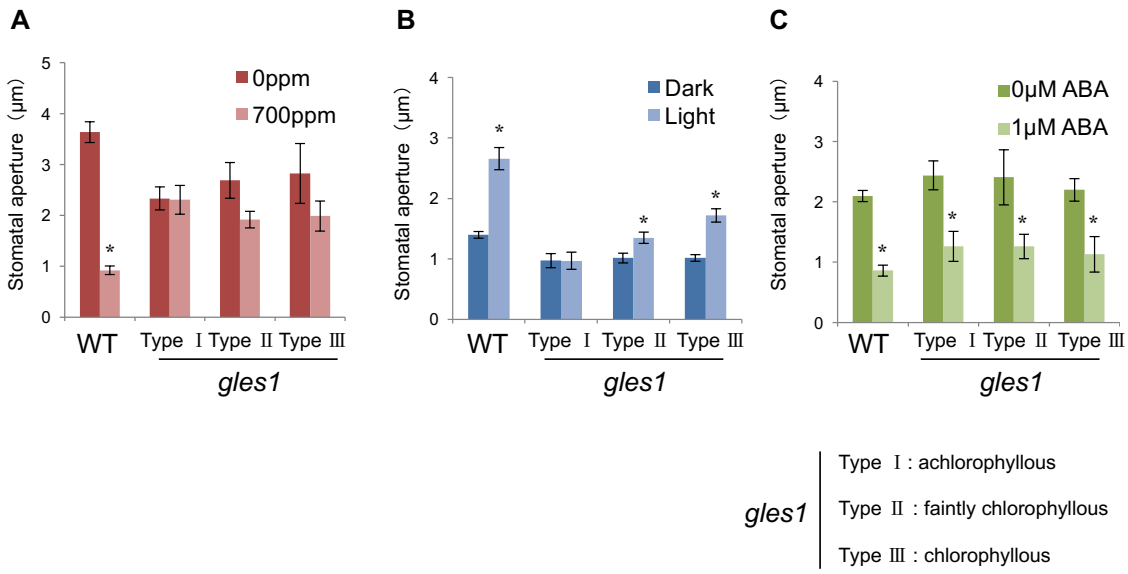

Fig. S7. Stomatal aperture changes in response to CO_2, light and ABA. (*A* to *C*) Stomatal aperture of achlorophyllous, faintly chlorophyllous and chlorophyllous *gles1* stomata are measured in response to changes in CO_2 concentrations (*A*) or in light intensity (*B*) or in ABA concentrations (*C*). Values shown are means ± S.E. ($n = 4$ independent experiments with >50 stomata per experiment). Asterisks indicate significant differences ($P < 0.05$, Student's t-test).

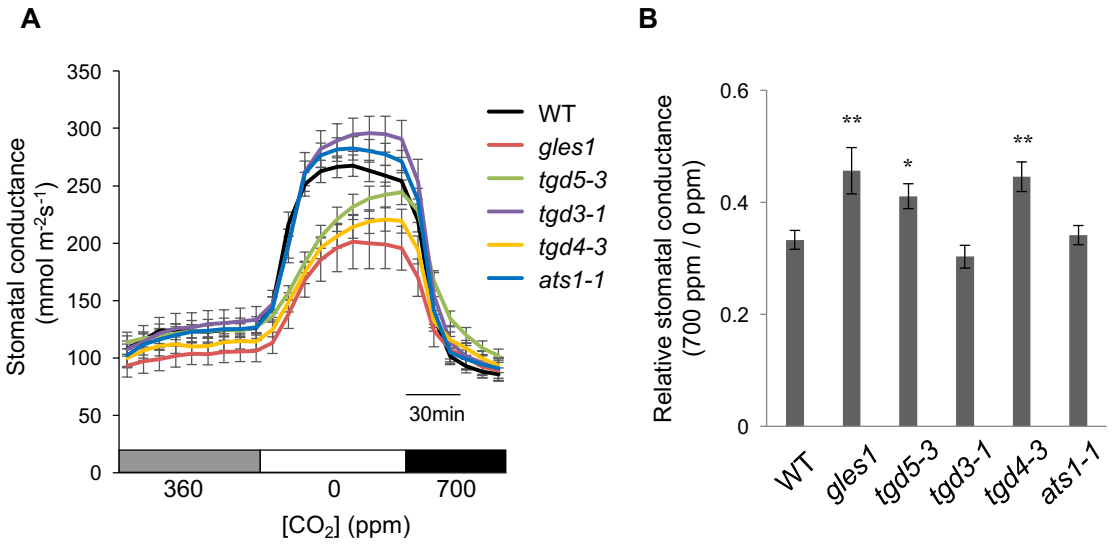

Fig. S8. Stomatal conductance in the *tgd* mutants in response to [CO_2] changes. Time-resolved relative stomatal conductance responses to [CO_2] in the *tgd* mutants and *ats1-1* mutant (*A*). Plants were kept under constant white light of 150 µmol m^{-2} s^{-1} at 22°C in 60% relative humidity. Values shown are means ± S.E. (n > 10). Relative stomatal conductance (*B*) were calculated as (gH_2O at 700 ppm CO_2)/(gH_2O at 0 ppm CO_2); large values represent small responses. Values shown are means ± S.E. (n > 10). Asterisks denote comparison with WT. **P < 0.05 and *P < 0.1 by one-way ANOVA with Dunnett's post hoc test.

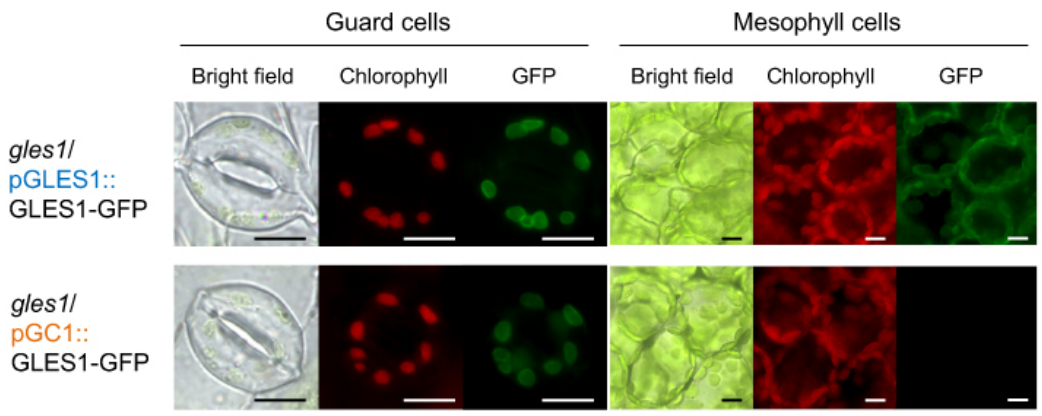

Fig. S9.

Bright field images, autofluorescence of chlorophyll and GFP fluorescence in guard cells or mesophyll cells are shown for a representative example. The *gles1*/pGC1::GLES1-GFP transformant expressed GLES1-GFP in guard cell-specific manner. Bars = 10μm.

Supplementary Table

Table S1. Expression profile of thylakoid-associated photosynthetic components in guard cell protoplasts and mesophyll cell protoplasts.

			GCP		MCP	
	GeneName	ProbeName	*gles1*/WT	p value p<0.05	*gles1*/WT	p value p<0.05
<PS II>	PsbA	A_84_P769753	0.62	0.244	1.06	0.828
	PsbB	A_84_P769873	0.50	0.331	1.18	0.550
	PsbC	A_84_P770018	0.68	0.361	1.21	0.585
	PsbD	A_84_P769933	0.60	0.235	1.14	0.713
	PsbE	A_84_P769945	0.46	0.129	1.13	0.605
	PsbF	none				
	PsbH	A_84_P769837	0.54	0.315	1.43	0.209
	PsbI	none				
	PsbJ	none				
	PsbK	A_84_P769861	0.69	0.336	1.19	0.543
	PsbL	none				
	PsbM	none				
	PsbN	none				
	PsbO	A_84_P808897	0.48	0.176	1.94	0.021 *
	PsbP	A_84_P807362	0.35	0.032 *	2.27	0.026 *
	PsbQ	A_84_P17567	0.25	0.068	1.80	0.004 *
	PsbR	A_84_P855736	0.50	0.130	1.80	0.002 *
	PsbS	A_84_P307130	0.34	0.101	2.40	0.003 *
	PsbT	none				
	PsbW	A_84_P23842	0.53	0.065	2.06	0.008 *
	PsbX	none				
	PsbY	A_84_P18912	0.50	0.015 *	2.11	0.033 *
	PsbZ	none				
	Lhcb1.1	A_84_P210788	0.24	0.044 *	1.42	0.455
	Lhcb2.1	A_84_P240805	0.26	0.004 *	1.21	0.698
	Lhcb3	A_84_P15956	0.21	0.007 *	1.32	0.544
	Lhcb4.3	A_84_P20179	0.58	0.102	4.21	0.006 *
	Lhcb5	A_84_P19470	0.54	0.341	2.09	0.042 *
	Lhcb6	A_84_P14174	0.24	0.018 *	1.67	0.202
<Cyt b6f>	PetB	A_84_P769694	0.82	0.438	1.25	0.095
	PetD	A_84_P769903	0.77	0.477	1.33	0.224
	PetA	A_84_P769965	1.13	0.249	1.20	0.439
	PetC	A_84_P14724	0.73	0.205	2.20	0.011 *
	PetL	none				
	PetM	A_84_P13483	0.78	0.461	2.07	0.007 *
	PetN/YCF6	none				
	PetG	none				
<PS I >	PsaA	A_84_P769750	0.36	0.232	1.21	0.473
	PsaB	A_84_P769817	0.59	0.283	1.12	0.694
	PsaC	A_84_P769744	0.85	0.415	1.24	0.647
	PsaD	A_84_P18505	0.29	0.008 *	1.95	0.055
	PsaE	A_84_P11967	0.36	0.045 *	2.06	0.007 *
	PsaF	A_84_P22465	0.41	0.094	1.99	0.027 *
	PsaG	A_84_P14291	0.32	0.019 *	2.07	0.006 *
	PsaH	A_84_P19062	0.20	0.054	3.03	0.003 *
	PsaI	none				
	PsaJ	none				
	PsaK	A_84_P19035	0.42	0.157	1.87	0.046 *
	PsaL	A_84_P21372	0.38	0.138	2.58	0.003 *
	PsaM	none				
	PsaN	A_84_P10324	0.53	0.236	2.41	0.008 *
	PsaO	A_84_P15184	0.41	0.103	1.99	0.031 *
	PsaX	none				
	Lhca1	A_84_P861734	0.39	0.135	1.48	0.187
	Lhca2	A_84_P808589	0.35	0.173	2.34	0.037 *
	Lhca3	A_84_P802372	0.25	0.055	1.25	0.385
	Lhca4	A_84_P17477	0.32	0.080	1.47	0.369
	PetE1(minor)	A_84_P14364	0.24	0.037 *	2.08	0.037 *
<ATP synthase>	beta	A_84_P769899	0.97	0.869	1.18	0.282
	alpha	A_84_P769781	1.06	0.862	1.44	0.057
	gamma	A_84_P297434	0.53	0.068	2.51	0.002 *
	delta	A_84_P12848	0.39	0.030 *	2.49	0.032 *
	epsilon	A_84_P769998	0.94	0.716	1.11	0.713
	c	none				
	a	none				
	b	A_84_P18601	1.00	0.996	2.03	0.001 *

References

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