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Activation of the Chloroplast Monogalactosyldiacylglycerol Synthase MGD1 by Phosphatidic Acid and Phosphatidylglycerol[§]

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One of the major characteristics of chloroplast membranes is their enrichment in galactoglycerolipids, monogalactosyldiacylglycerol (MGDG), and digalactosyldiacylglycerol (DGDG), whereas phospholipids are poorly represented, mainly as phosphatidylglycerol (PG). All these lipids are synthesized in the chloroplast envelope, but galactolipid synthesis is also partially dependent on phospholipid synthesis localized in non-plastidial membranes. MGDG synthesis was previously shown essential for chloroplast development. In this report, we analyze the regulation of MGDG synthesis by phosphatidic acid (PA), which is a general precursor in the synthesis of all glycerolipids and is also a signaling molecule in plants. We demonstrate that under physiological conditions, MGDG synthesis is not active when the MGDG synthase enzyme is supplied with its substrates only, *i.e.* diacylglycerol and UDP-gal. In contrast, PA activates the enzyme when supplied. This is shown in leaf homogenates, in the chloroplast envelope, as well as on the recombinant MGDG synthase, MGD1. PG can also activate the enzyme, but comparison of PA and PG effects on MGD1 activity indicates that PA and PG proceed through different mechanisms, which are further differentiated by enzymatic analysis of point-mutated recombinant MGD1s. Activation of MGD1 by PA and PG is proposed as an important mechanism coupling phospholipid and galactolipid syntheses in plants.

Chloroplast membrane lipids are mostly composed of non-phosphorous galactoglycerolipids *i.e.* monogalactosyldiacylglycerol (MGDG)² and digalactosyldiacylglycerol (DGDG) that represent more than 50 and 30% of thylakoid lipids, respectively. Phosphatidylglycerol is the main phospholipid in plastids, representing about 10% of thylakoid lipids. Each of those

glycerolipid classes is represented by a range of molecular species differing in the acyl composition at *sn*-1 and *sn*-2 positions of the glycerol backbone. Based on the model of cyanobacterial lipids, the prokaryotic type of glycerolipids contains a 16-carbon fatty acid at the *sn*-2 position of glycerol. The eukaryotic type contains an 18-carbon fatty acid at the *sn*-2 position. Some plants, such as *Arabidopsis* or spinach, have both prokaryotic- and eukaryotic-type MGDG, whereas other plants, such as pea or cucumber, have only eukaryotic-type MGDG. DGDG is mostly of eukaryotic type in all plants. Chloroplast PG contains exclusively a prokaryotic-type DAG moiety in contrast to non-plastidial PG. These different chloroplast lipids are assembled in the chloroplast envelope (1). Most enzymes have now been identified, but their functioning and regulation remain largely unknown.

MGDG is synthesized by MGDG synthase (MGD), which transfers galactose from UDP-gal to DAG. In *Arabidopsis*, there are three MGDG synthases, and among them, MGD1, is necessary for synthesis of galactolipids and for development of photosynthetic membranes (2, 3). MGD1 utilizes DAG derived from two main sources, either from a DAG *de novo* synthesized in plastid envelope by double acylation of glycerol-3 phosphate and PA dephosphorylation (prokaryotic DAG) or from a DAG derived from endoplasmic reticulum phosphatidylcholine (PC) possibly through a phospholipase D enzymatic step (eukaryotic DAG) (1). MGD1 can produce prokaryotic- and eukaryotic-type MGDG with the same efficiency (4). MGD1 localizes at the inner envelope membrane (IEM). The protein anchors to the membrane as a monotopic protein through protein-lipid interaction to the external monolayer of the IEM (5, 6). Native and recombinant MGD1 are known to be active as homodimers (5). Each MGD1 monomer is likely to be organized in two Rossman folds (N- and C-domains) (7). Visualization of surface hydrophobic regions suggested that MGD1 interacts with the membrane surface by its N-domain, whereas the C-domain protrudes above the membrane. Although MGD1 is a membrane protein and synthesizes MGDG using a lipid substrate (DAG), its mechanisms of interaction with hosting membrane, lipid substrate, and product remain unclear. Moreover, the enzyme activity might potentially be regulated by anionic lipids: PG, SQDG, or PA (8–10). Because (i) PA is a general precursor in the glycerolipid synthesis pathway and a signaling molecule

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² The abbreviations used are: MGDG, monogalactosyldiacylglycerol; MGD, MGDG synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; PA, phosphatidic acid; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; DAG, diacylglycerol; AEBFSF, 4-2-aminoethyl benzenesulfonyl fluoride hydrochloride; PLD, phospholipase D; DTT, dithiothreitol; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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in plants, and (ii) MGD1 is essential for chloroplast development, regulation by PA potentially represents a way of controlling chloroplast biogenesis during plant development. Using both native and recombinant MGD1 protein, we therefore analyzed in this report the enzymatic characteristics of regulation of MGD1 by PA. We also compared the regulation effects of PA and PG on the enzyme.

EXPERIMENTAL PROCEDURES

Chemicals

Lipids were purchased as follows, DAG (Sigma D0138), PA (Sigma P9511), 18:1/18:1-PA (Sigma P2767), 16:0/18:1-PA (Avanti Polar lipids 840857c), 16:0/16:0-PA (Sigma P4013), PG (Sigma P0514), and PC (Sigma P3017). 18:1/16:0-PA was purified by two-dimensional TLC after hydrolysis of 18:1/16:0-PC (Sigma P4142) by PLD activity (Sigma L4384) according to the manufacturer's protocol. Identification of the fatty acid in the *sn*-2 position was determined by gas chromatography after hydrolysis with the *Rhizopus arrhizus* lipase (Roche, 50 units· μl^{-1} in 50 mM Tris-HCl, pH 7.2, 0.5% Triton X-100) for 1 h at room temperature. AEBSE, 4-2-aminoethyl benzenesulfonyl fluoride hydrochloride, was purchased from Fluka 76307.

Plant Materials and Growth Conditions

Arabidopsis plants were grown on Murashige and Skoog (MS, Duchefa) agar containing 0.5% sucrose for 15 days between 22 to 26 °C under a daily 16-h light cycle at 100 μmol photons $\text{m}^{-2} \text{s}^{-1}$. The *pld2* mutant (SALK_094369 T-DNA line of At3g05630 (11)), ecotype Columbia-0, corresponds to the *pld2* line analyzed by (12). The homozygous line was initially selected from the SALK seed stock and further from a back cross of this line with the parental wild type Columbia line by kanamycin selection and PCR genotyping using P1 (5'-CGC-ACCACGAGAAAATAGTG-3') and P2 (5'-CACTCTGCT-TCCCAATCTGC-3') for the wild-type gene, and P1 and LB (5'-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3') for the tagged gene. We verified by RT-PCR with primers 5'-CGACGACGGTTTGGGGAG-3' and 5'-CTTCCCAATCTGCCACCA-CCAGTC-3' that the mutant line had no expression of the *PLD2* gene. Spinach leaves were purchased on the local market.

Lipid Analysis

Lipids were analyzed by TLC generally in CHCl_3 /acetone/MeOH/acetic acid/ H_2O 50:20:10:10:5 (v/v) and quantified by gas chromatography as described by Ref. 13.

Chloroplast Envelope Isolation from Spinach Leaves

Intact chloroplasts were obtained from spinach (*Spinacia oleracea* L.) leaves and purified by isopycnic centrifugation using Percoll gradients (14). Purified envelope membranes from thermolysin-treated chloroplasts were prepared as described by Ref. 15. Briefly, Percoll-purified intact chloroplasts (final concentration, 1 mg of chlorophyll/ml) were incubated in the following medium: 0.3 M sucrose, 20 mM MOPS-NaOH, pH 7.8, 1 mM CaCl_2 , for 1 h at 4 °C with 600 $\mu\text{g}\cdot\text{ml}^{-1}$ of thermolysin from *Bacillus thermoproteolyticus* (Sigma). The digestion was terminated by the addition of 10 mM EGTA. Intact chloroplasts

were then recovered on a second Percoll gradient before lysis in a hypotonic medium and isolation of the envelope fraction on a sucrose gradient.

Preparation of MGD1 Recombinant Proteins

Arabidopsis atMGD1 sequence beginning at A138 and fused with a His₆ tag at the C terminus was expressed in *Escherichia coli* using the pET expression system (Novagen Inc.) as described by Ref. 7. Expression was induced by 1 mM isopropyl-1-thio- β -D-galactopyranoside for 5 h at 18 °C. The protein was recovered in buffer containing 50 mM Tris-HCl, pH 8.0, 12% (w/v) glycerol, 1 M NaCl, 1 mM β -mercaptoethanol, subjected to chromatography on a Ni²⁺-charged resin (Ni-NTA-agarose, Qiagen GmbH, Germany), from which it was eluted with 50–100 mM imidazole. Imidazole was removed from the protein fraction by gel filtration on BiogelP6DG (Bio-Rad). In this buffer, the recombinant protein was soluble, and its purity was estimated to be more than 95% after SDS-PAGE and Coomassie Blue staining. Recombinant atMGD1 proteins were obtained by site-directed mutagenesis of the protein described above. They were expressed and purified as the wild-type protein. Cucumber csMGD1 beginning at Val-105 and fused to a His₆ tag at the C terminus was produced as in Ref. 16. Spinach soMGD1 beginning at Leu-99 was expressed and purified as in Ref. 7.

Measurement of MGDG Synthase Activity

Measurement on Leaf Homogenates—Rosette leaves of 2-week-old plants were grounded in liquid nitrogen. The powder was resuspended in 50 mM MOPS-NaOH, pH 7.8, 1 mM DTT, 6 mM CHAPS, 25 mM KCl, 25 mM KH_2PO_4 , 2% glycerol, 150 mM NaCl, and immediately used for measurement of MGDG synthase activity. The measurement was monitored at 23 °C with 7 mol% DAG, 350 μM [¹⁴C]UDP-gal (22.9 GBq·mol⁻¹) and 1.5 to 4 $\mu\text{g}\cdot\mu\text{l}^{-1}$ protein. As lipid concentration, the surface concentration of the relevant lipid relative to all mixed micelle components ($[\text{lipid}]/([\text{detergent}] + \sum[\text{lipids}])$ expressed in mol%) was used as a rough representation of the lipid concentration in the vicinity of the enzyme. Labeled galactolipids were analyzed, and radioactivity was measured by liquid scintillation.

Measurement on Chloroplast Envelope—An envelope fraction purified from thermolysin-treated spinach chloroplasts was incubated in 10 mM Tricine-NaOH, pH 9, 5 mM EDTA, 1 mM DTT for 15 min at 23 °C. One volume was then added to 1 volume of 100 mM MOPS-NaOH, pH 7.8, 1 mM DTT, 12 mM CHAPS, 50 mM KCl, 50 mM KH_2PO_4 with 7 mol% DAG, and 750 μM UDP-[¹⁴C]gal (22.9 GBq·mol⁻¹) for a protein concentration of 0.9–1.3 $\mu\text{g}\cdot\mu\text{l}^{-1}$.

Measurement on Recombinant MGD1—Wild-type or mutant MGD1 proteins were usually incubated in 50 mM MOPS-NaOH, pH 7.8, 6 mM CHAPS, 150 mM NaCl, 2% glycerol, 1 mM DTT, 25 mM KCl, 25 mM KH_2PO_4 with 7 mol% DAG and 750 μM UDP-[¹⁴C]gal (22.9 GBq·mol⁻¹) for a protein concentration of 11 $\text{ng}\cdot\mu\text{l}^{-1}$ or as indicated. Kinetic data were fitted with the Hill equation (Equation 1),

$$V = V_{\text{max}} \times [S]^n / (K_{0.5}^n + [S]^n) \quad (\text{Eq. 1})$$

where *V* is the enzyme velocity, *S* the PA or PG or UDP-gal

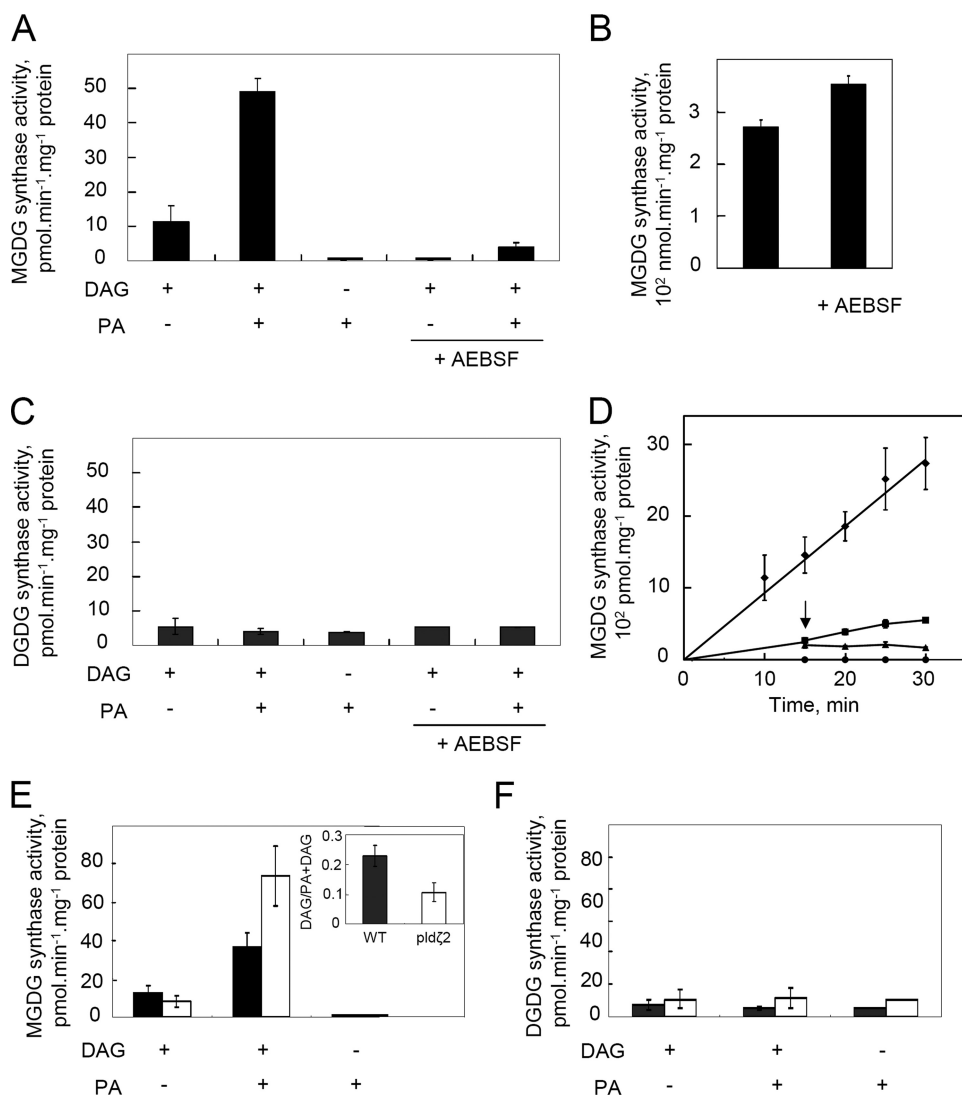


FIGURE 1. Activation of MGDG synthase by PA in *Arabidopsis* leaf homogenates. Activity was measured as described under "Experimental Procedures." *A*, effect of PA (1.5 mol%), DAG (7 mol%), and AEBSF (6 mM) on MGDG synthase activity. *B*, effect of AEBSF (6 mM) on purified at Δ 1–137MGD1. 175 ng of purified protein was incubated with 1.5 mol% PA under the same condition as described for leaf homogenates. *C*, effect of PA (1.5 mol%), DAG (7 mol%), and AEBSF (6 mM) on DGDG synthase activity. *D*, kinetic analysis of MGDG synthesis: ■, control; ●, in the presence of AEBSF (6 mM), ▲, with addition of AEBSF after 15 min of incubation (arrow), ◆, with 1.5 mol% of PA. *E*, comparison of MGDG synthase activity in wild-type and in *pld2*. PA (1.5 mol%), DAG (7 mol%) were added as indicated. The inset shows the ratio of the activities measured with addition of DAG in the absence of PA reported to in the presence of PA. *F*, comparison of DGDG synthase activity in wild-type and in *pld2*. PA (1.5 mol%), DAG (7 mol%) were added as indicated. Results are average values \pm S.D. for three independent replicates.

concentration, $K_{0.5}$ the concentration of S where $V = \frac{1}{2}V_{\max}$, and n the Hill coefficient using the nonlinear least squares method (17) implemented in the package from the R software (18).

Protein-Lipid Binding Assays

Lipid-Protein Overlay Assay—1 μ l of lipid solution containing 0.1–10 nmol of PA, DAG, or PG dissolved in chloroform was spotted on to nitrocellulose membranes (Schleicher & Schuell) and allowed to dry at room temperature for 1 h. The membrane was blocked in 5% (w/v) fatty acid-free bovine serum albumin in TTBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl with 0.1%, v/v, Tween 20) for 1 h. The membrane was then incubated overnight at 4 °C with gentle stirring in the same solution containing about 0.5–1.5 μ g \cdot μ l⁻¹ of MGD1 protein. The membrane was washed

four times for 5 min in TTBS and then incubated for 1 h with anti-His monoclonal antibody 1/5000 (Sigma) or anti-spinach MGD1 (1/500) in TTBS with 3% skimmed milk. The membrane was washed as before, incubated with anti-mouse-horse-radish peroxidase (HRP) or anti-rabbit-HRP conjugate 1/5000 (Jackson ImmunoResearch) in TTBS with 3% skimmed milk, washed four times in TTBS, followed by once in TBS before ECL detection.

Liposome Binding Assay—Dried lipids were resuspended at 2 μ g \cdot μ l⁻¹ in 50 mM Tris-HCl, pH 7, 100 mM NaCl, 1 mM DTT for 1 h at 37 °C. Liposomes were then vigorously vortexed for 5 min before centrifugation at 20,000 \times g for 10 min. Purified recombinant MGD1 was diluted in the same medium at 0.1 μ g \cdot μ l⁻¹ and collected as a supernatant after centrifugation at 20,000 \times g for 10 min. Protein and liposomes were incubated together for 30 min at 30 °C. Liposomes were finally harvested as a pellet by centrifugation at 20,000 \times g for 10 min, and protein was analyzed by SDS-PAGE with silver nitrate staining.

RESULTS

Activation of the MGDG Synthase Activity of Leaf Homogenates by PA

In a first evaluation of galactolipid synthesis tuning by specific lipids, MGDG synthase activity was measured in leaf homogenates of 2-week-old *Arabidopsis* plants. The level of activity was quantified following transfer of radioactive galactose from UDP-gal onto DAG

added to the homogenate. We observed a moderate MGDG synthase activity that was stimulated 5 times by addition of 1.5 mol% of PA (Fig. 1A). Stimulation by PA was not due to conversion of PA into DAG, because in the absence of exogenous DAG, no activity was detected. PLDs are highly active in ground leaves and could generate enough PA to maintain a basal level of MGDG synthase activity in leaf homogenates. We therefore considered inhibiting the presumed formation of PA by phospholipases D using AEBSF. AEBSF has been previously reported as an inhibitor of PLD activity in leaf extracts (19, 20), a property we confirmed in our system (supplemental Fig. S1). We verified that pure recombinant atMGD1, expected as the most active MGDG synthase in leaf homogenates (see below), was not inhibited by AEBSF (Fig. 1B). We observed that the

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MGDG synthase activity of leaf homogenates was lower in the presence of AEBSF and that, upon addition of PA, some MGDG synthase activity was restored (Fig. 1A). DGDG synthase activity in leaf homogenates was low and independent of DAG and PA (Fig. 1C). Our conclusion was therefore that PA was an activator of the MGDG synthase of leaf homogenates.

PA in leaf homogenates is likely to be very transient dependent on both anabolism and catabolism. This may dynamically regulate MGDG synthase activity. Kinetics analysis of the MGDG synthase activity of leaf homogenates showed that the incorporation of galactose into MGDG was linear with time and stimulated by addition of PA (Fig. 1D). When AEBSF was added at the beginning of the kinetics, no activity was detected in the absence of additional PA. Interestingly, the addition of AEBSF after 15 min of incorporation very rapidly stopped MGDG synthase activity (Fig. 1D). These results were consistent with the following: (i) the MGDG synthase activity of leaf homogenates was dependent on the constant presence of PA, (ii) decrease of PA production led to a decrease of the MGDG synthase activity, and (iii) PLDs were able to produce PA necessary for MGDG synthase activation.

However, the AEBSF effect was only partially restored by PA addition (Fig. 1A). To confirm that PA issued from PLDs had an impact on the level of MGDG synthase activity in leaf homogenates, we assayed the MGDG synthase activity of leaf homogenates in a mutant deleted of PLD. Twelve PLDs have already been reported in *Arabidopsis* (21). Most of them are expected to be active only in the presence of Ca^{2+} except the 2 PLD ζ proteins. Because no Ca^{2+} was added in our assay and PLD ζ 2 had been previously shown to be involved in the phosphate deprivation-induced synthesis of galactolipids in roots (12, 22), we chose to assay a mutant deleted of PLD ζ 2. We observed that the mutant exhibited a little less MGDG synthase activity compared with the wild type (Fig. 1E). When PA was added, MGDG synthase activity was more highly increased in the mutant than in the wild type. No activity was detected in the mutant as in the wild type with the addition of PA alone (no addition of DAG). As a result, the ratio of MGDG synthase activity obtained in the absence to presence of exogenous PA was 2-fold lower in *pld ζ 2* than in the wild type. The DGDG synthase activity was a little higher in the mutant than in the wild type but still insensitive to addition of DAG or PA (Fig. 1F). These data therefore (i) confirmed that PA was important for activation of the MGDG synthase, and (ii) indicated that PLD ζ 2 was a potent source of PA for activation of the MGDG synthase in leaf homogenates.

Effect of PA on the MGDG Synthase Activity of Chloroplast Envelope

In leaves, the MGDG synthase enzyme is located in the chloroplast envelope (6). To date, the MGDG synthase activity has been classically measured in chloroplast envelope fractions isolated from leaves. A sufficient amount of DAG is present in the purified envelope to sustain the activity except when the envelope is isolated from thermolysin-treated chloroplasts (15). Indeed thermolysin hydrolyzes the galactolipid-galactolipid galactosyltransferase, which is present on the surface of chloroplasts and responsible for DAG formation during isolation of the chloroplast envelope. The supply of exogenous DAG is

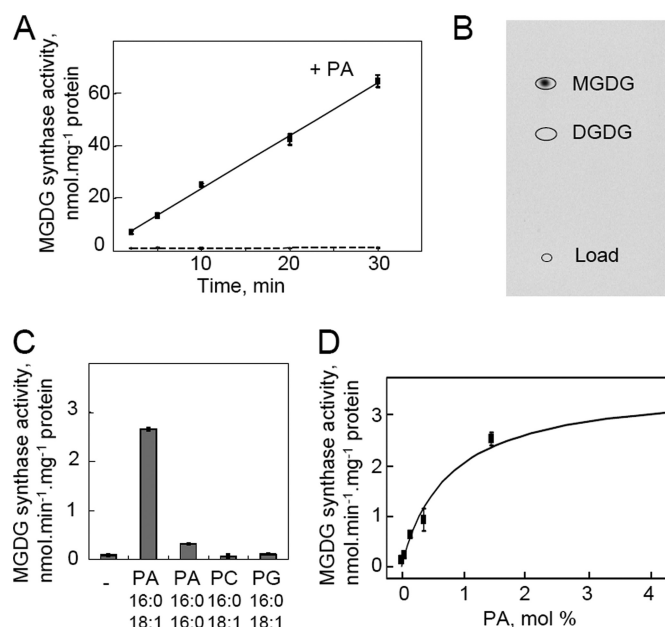


FIGURE 2. Effect of PA on MGDG synthase measured in isolated spinach chloroplast envelope. Before the experiment, the envelope fraction was prepared from thermolysin-treated chloroplasts and incubated in conditions favorable for PA phosphatase activity as described under "Experimental Procedures." MGDG synthase was then measured as described under "Experimental Procedures." *A*, kinetic analysis of MGDG synthase activity: control, broken line; with PA 1.5 mol%, solid line. Results are average values \pm S.D. for three independent measurements. *B*, analysis of [¹⁴C]galactose-labeled lipids on TLC. *C*, effect of different lipids on MGDG synthase activity. Activity was measured as described under "Experimental Procedures." The presence of DAG and additional lipid is indicated below the graph, with for additional lipid fatty acid in *sn-1* position on the first line and fatty acid in *sn-2* position on the second line. *D*, effect of 16:0/18:1-PA level on the MGDG synthase activity. Activity was measured as described under "Experimental Procedures." Results are average values \pm S.D. for three independent measurements. The curve equation is: $V = 3 \times [\text{PA}] / (0.5 + [\text{PA}])$.

therefore required for measurement of MGDG synthase activity in the envelope isolated from thermolysin-treated chloroplasts. In contrast, PA addition was never reported to be required to detect MGDG synthase activity in the envelope from either thermolysin-treated or non-treated chloroplasts. The PA level in the isolated chloroplast envelope is low and undetectable by conventional techniques. However, our data suggested that a low concentration of PA could be sufficient to activate MGDG synthase. We questioned whether PA was necessary for activation of the chloroplast envelope MGDG synthase and was present in sufficient amounts in the isolated envelope to allow MGDG synthase activity.

To decrease endogenous PA, a chloroplast envelope fraction was prepared from thermolysin-treated spinach chloroplasts and preincubated to favor envelope-associated PA phosphatase activity. High pH and absence of DAG during pretreatment were expected to allow PA phosphatase activity (23). Then, the envelope fraction was supplemented with DAG and incubated in MGDG synthase activity buffer. We thus observed a barely detectable MGDG synthase activity (Fig. 2A). When exogenous PA was added, the MGDG synthase activity was high in contrast. Only radiolabeled MGDG was formed indicating the absence of DGDG synthase and galactolipid-galactolipid galactosyltransferase activities (Fig. 2B). Several molecular species of PA, either of eukaryotic- or prokaryotic-type, were able to stim-

ulate the MGDG synthase activity, although the 16:0/16:0 molecular species was less active than the 16:0/18:1 (Fig. 2C). PG had only a slight effect, whereas PC was not active. The MGDG synthase activity was dependent on the PA concentration and a range of about 0.5 mol% PA (16:0/18:1-PA) was sufficient to reach half-maximal activity of the enzyme (Fig. 2D). Our conclusion was that PA had a regulatory effect on the native MGDG synthase present in chloroplast envelope.

Effect of PA on a Recombinant Form of atMGD1

MGDG Synthase Activity—In *Arabidopsis*, atMGD1 is the main MGDG synthase expressed in leaves and is essential for the development of photosynthetic tissues (2, 3, 6). The full-length mature protein associates with membranes (5). In a previous work (7), it has been shown that a $\Delta 1-137$ truncated form of atMGD1 is expressed in *E. coli* as a soluble and active form. Using this form of the enzyme, the effect of PA was analyzed. In the absence of PA, the enzyme alone had no activity (Fig. 3A). In contrast, several acyl species of PA were able to activate the enzyme although less efficiently for the 16:0/16:0 molecular species. PG was partially effective but not PC. A conversion of PA into DAG was excluded because almost no activity was found with PA when exogenous DAG was absent. These results suggested that the MGDG synthase was allosterically activated by PA and that PG could play a similar role as PA.

The MGDG synthase enzymatic velocity *versus* PA concentration showed a similar sigmoid curve with two molecular species of PA: a eukaryotic-type form, 16:0/18:1, and a prokaryotic-type form, 18:1/16:0 (Fig. 3B). In each case, half-maximal activation was obtained for ~ 0.2 mol% PA. The cooperative effect of PA on the enzyme could illustrate a relationship with dimerization of the enzyme, because it has been previously shown that the enzyme is active as a dimer (5).

PA binding can change the enzyme behavior regarding substrate handling. The enzyme velocity *versus* UDP-gal concentration was compared in the presence of 0.15 and 1.5 mol% of PA (Fig. 3C). With 0.15 mol% of PA, the enzyme velocity *versus* substrate curve was sigmoid, whereas with 1.5 mol% of PA, the curve was hyperbolic. There was therefore a clear allosteric change of the enzyme for UDP-gal handling depending on PA concentration. PA predominantly affected the apparent V_{\max} of the enzyme because $V_{\text{App}} V_{\max}$ was increased five times when PA concentration was changed from 0.15 and 1.5 mol%, whereas the $S_{0.5}$ for UDP-gal was kept in the same range (80 μM) in both conditions. The allosteric activation of the enzyme by PA finally resulted in an increase of the $V_{\text{App}} V_{\max}$ of the enzyme. These data therefore indicated that the allosteric activation of the enzyme by PA resulted either from an increase of the apparent affinity of the enzyme for the DAG co-substrate or from an increase of the true V_{\max} of the enzyme.

Lipid Binding Assay of MGD1—To confirm the direct interaction of MGDG synthase with PA, binding of the protein on PA was tested. By lipid-protein overlay assay, the protein was detected on PA spots (Fig. 4, A and B). The binding started to be visible on 0.5 nmol of PA. Only a weak signal was visible on PG and DAG. A similar binding was observed with other MGD1 protein forms issued from cucumber and spinach (Fig. 4A). *Arabidopsis* and cucumber proteins contained a His₆ tag that

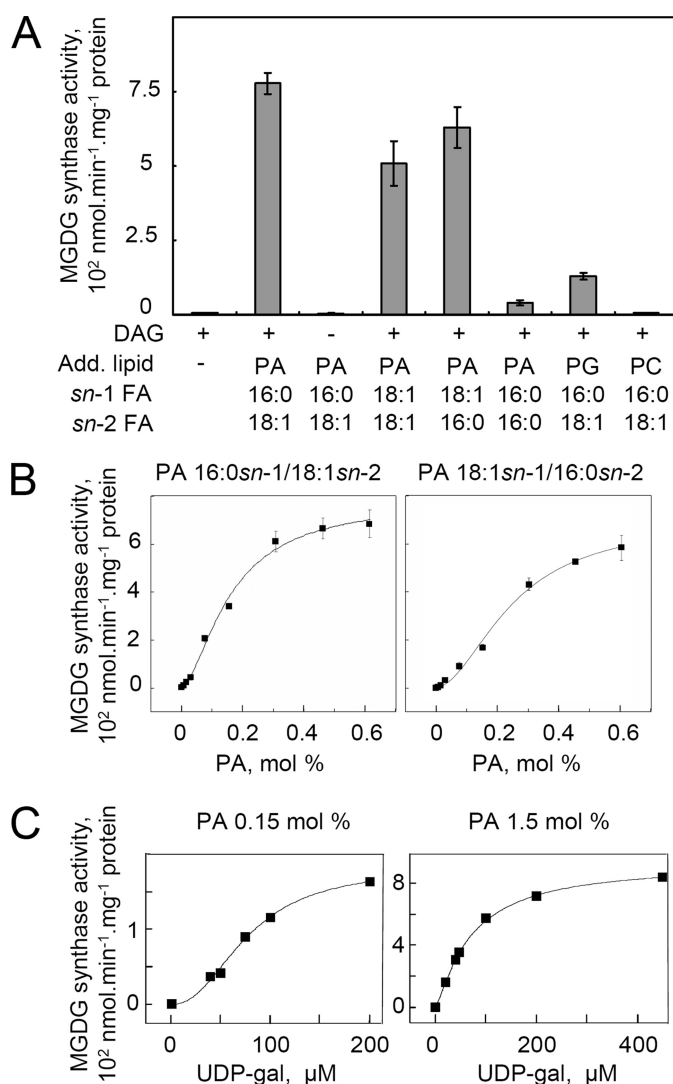


FIGURE 3. Effect of PA on the MGDG synthase activity of at $\Delta 1-137$ MGD1. MGDG synthase activity was measured as described under "Experimental Procedures." A, effect of different lipids on the activity. The presence of DAG and additional lipid is indicated below the graph, with for an additional lipid fatty acid in *sn-1* position on the first line and fatty acid in *sn-2* position on the second line. B, 16:0/18:1-PA and 18:1/16:0-PA concentration dependence of the activity. The measures were done with 5 $\text{ng}\cdot\mu\text{l}^{-1}$ of protein. The curve equation is $V = 7.8 \times [\text{PA}]^{1.6} / (0.2^{1.6} + [\text{PA}]^{1.6})$ for 16:0/18:1 - PA and $V = 7.1 \times [\text{PA}]^{1.9} / (0.2^{1.9} + [\text{PA}]^{1.9})$ for 18:1/16:0 - PA. C, UDP-gal concentration dependence of the activity for two different concentrations of PA, 0.15 mol% and 1.5 mol%. The curve equation is $V = 1.8 \times [\text{UDP-gal}]^{2.7} / (78^{2.7} + [\text{UDP-gal}]^{2.7})$ for 0.15 mol% of PA and $V = 9.2 \times [\text{UDP-gal}]^{1.2} / (71^{1.2} + [\text{UDP-gal}]^{1.2})$ for 1.5 mol% of PA. Results are average values \pm S.D. for three independent measurements on a single batch of purified protein whereas each part of the figure was done with a separated batch of purified protein.

could interact with PA, but, because the spinach MGD1 protein did not contain a tag, the binding was apparently not related to the tag. Liposome binding assays showed that atMGD1 bound to PC liposomes and that the binding was increased when 5 mol% PA was included in the liposomes (Fig. 4C).

Comparative Characterization of MGD1 Activation by PA and PG

To analyze the specific role of PA and PG on the functioning of the enzyme, we compared the enzymatic characteristics of atMGD1 with both activators. In contrast to what was observed

Activation of MGD1 by PA and PG

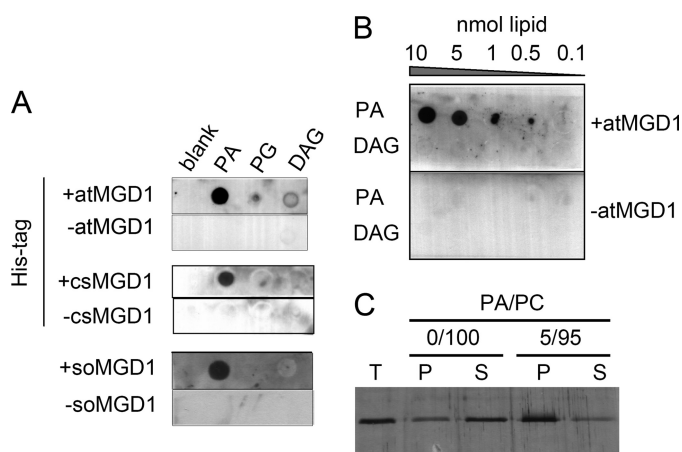


FIGURE 4. Lipid binding assay of MGD1. *A*, lipid-protein overlay assay of three different MGD1 from *Arabidopsis*, cucumber, and spinach. at Δ 1–137MGD1 and cs Δ 1–104MGD1 harbor a His₆ tag at their C terminus, whereas so Δ 1–98MGD1 has no His tag. Different lipids were spotted onto nitrocellulose membranes as indicated. The experiment was conducted as described under “Experimental Procedures.” *B*, different amounts of PA or DAG were spotted onto nitrocellulose membranes. *C*, liposome binding assay with at Δ 1–137MGD1. Liposomes were formed of 100% PC or 5% PA and 95% PC. *T*, total amount of protein; *P*, liposome pellet; *S* supernatant.

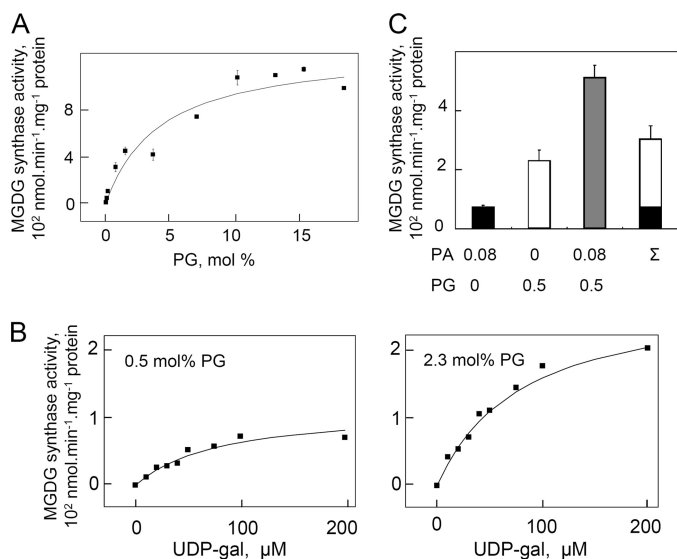


FIGURE 5. Effect of PG on the MGDG synthase activity of at Δ 1–137MGD1. Experiments were conducted as described in the legend to Fig. 3. *A*, PG concentration dependence of the activity. The curve equation is $V = 13 \times [\text{PG}] / (4 + [\text{PG}])$. *B*, synergistic effect of PG and PA on the activity. The activity was measured with a combination of different mol% concentration of PA and PG as indicated under the graph. Σ indicates the sum of the activities with only PA or PG. *C*, UDP-gal concentration dependence of the activity for two different concentrations of PG, 0.5 mol% and 2.3 mol%. The curve equation is $V = 1.8 \times [\text{UDP-gal}] / (80 + [\text{UDP-gal}])$ for 0.5 mol% of PG and $V = 4.5 \times [\text{UDP-gal}] / (80 + [\text{UDP-gal}])$ for 2.3 mol% of PG. Results are average values \pm S.D. for three independent measurements on a single batch of purified protein, whereas each part of the figure was done with a separated batch of purified protein.

with PA, the enzyme velocity *versus* PG concentration curve was not sigmoid but hyperbolic (Fig. 5A) indicating a simpler way of interaction of the enzyme with PG than with PA. Half-maximal activation was obtained with \sim 4 mol% PG, suggesting a lower affinity of the enzyme for PG than for PA. However the maximal activity attained with PG was more elevated than with PA. The apparent affinity of the enzyme for UDP-gal was not modified by different levels of PG but in contrast to what was

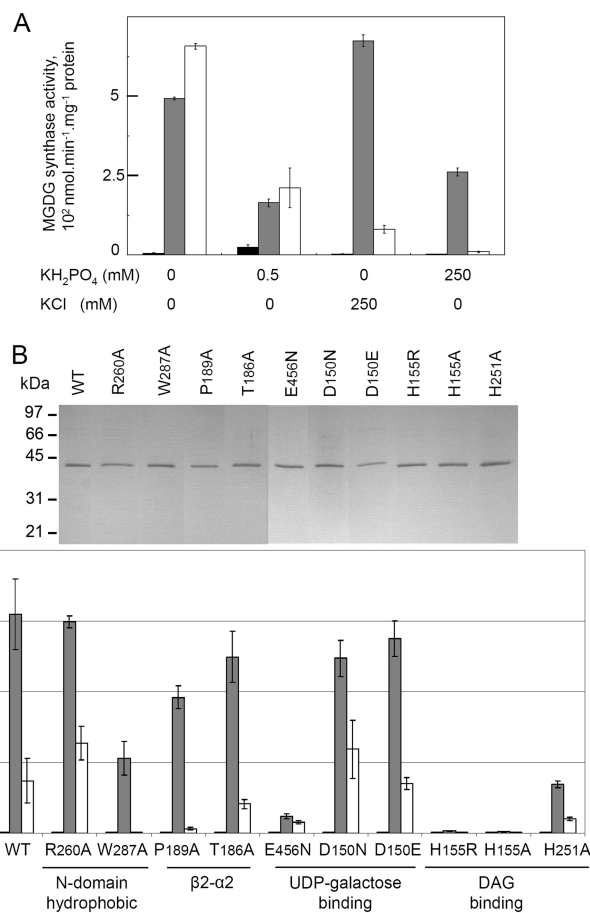


FIGURE 6. Molecular discrimination of PA and PG binding on at Δ 1–137MGD1. The activity was measured as described under “Experimental Procedures” without PA or PG (black bars), with 1.5 mol% of PA (gray bars) or PG (white bars). Results are average values \pm S.D. for three independent measurements. *A*, effect of salts on MGDG synthase activity. Concentrations of KH_2PO_4 and KCl in the incubation medium are indicated below the graph. *B*, effect of point mutations of the protein on activation by PA and PG. SDS-PAGE analysis of the purified point-mutated at Δ 1–137MGD1 proteins used for activity measurement is shown above the graph.

observed with PA, at a low concentration of PG, no cooperative effect between UDP-gal concentration and enzymatic activity was observed. This suggested that there was no interference between PG binding and UDP-gal handling by the enzyme (Fig. 5B).

Because the effects of PA and PG look different, we addressed then the question of whether they were synergic. We measured the enzyme activity at concentrations of PA (0.08 mol%) and PG (0.5 mol%) far below those giving the maximal activation of the enzyme. The activity in the presence of both PA and PG was much higher than the sum of the activities obtained separately in the presence of either PA or PG (Fig. 5C). This was clearly in support of distinct binding sites and different roles of PA and PG on the enzyme.

Molecular Discrimination of PA and PG Binding Sites

In a preliminary investigation to discriminate between PA and PG binding sites, we analyzed whether the activations by PA or PG were differentially sensitive to salt and phosphate. Data show that with 250 mM KCl, activation by PG was decreased whereas activation by PA was increased (Fig. 6A).

This suggested that the activation by PG was specifically dependent on electrostatic interaction. Because hydrophobic interactions are strengthened by salt, this suggested, on the other hand, that the activation by PA was dependent on hydrophobic interaction. Incubation with 250 mM KH_2PO_4 confirmed that activation by PG was inhibited by high salt concentrations. The inhibition was even stronger than with 250 mM KCl. Activation by PA was, in contrast, reversed using KH_2PO_4 instead of KCl. KH_2PO_4 had an inhibitory effect on activation by PA, the opposite to KCl. Because a low concentration of KH_2PO_4 (0.5 mM) was sufficient to slightly activate the enzyme, we concluded that the phosphate anion directly interacted with the enzyme and that this interaction was competing with PA and PG. It is therefore likely that phosphate is involved in the binding of both phospholipids to the enzyme. Altogether, main components of PG binding were electrostatic linkage and interaction through the phosphate group, whereas for PA binding they were of hydrophobic linkage and interaction through the phosphate group.

As another way to discriminate between PA and PG binding sites, we analyzed the differential activation by PA and PG of proteins exhibiting point mutations. To our knowledge, no information is available in the literature concerning PG binding sites. Concerning PA binding, there is no consensus sequence, but basic, hydrophobic, and aromatic residues were often reported to be important (24). Some uncharged polar residues such as serine and glutamine might also play a role (25, 26). A first model of plant MGDG synthase has been previously established using *E. coli* MURG as a template (7). The model for an MGD monomer comprises 2 Rossmann domains (C- and N-domains). The binding site for UDP-gal was predicted in the cleft separating the two Rossmann folds involving residues of a conserved UDP-sugar binding pocket in the C-domain. The N-domain is enriched in basic and hydrophobic residues and was proposed to be involved in lipid binding, either diacylglycerol binding or membrane anchorage. In this domain, a $\langle\beta_2\text{-}\alpha_2\rangle$ loop, which has no counterpart in the MURG template, could play a specific role in lipid binding necessary for activity. Based on this information, we decided to test several residues possibly important for substrate binding or lipid binding. We targeted in the N-domain Arg-260, Trp-287, Pro-189, Thr-186, Asp-150, His-155, His-251 and in the C-domain Glu-456. The Arg-260, Trp-287, Pro-189, and Thr-186 residues were selected for a possible interaction with PA. Glu-456 and Asp-150 were selected for a possible interaction with UDP-galactose and His-155 and His-251 with diacylglycerol. The residue replacement was decided on a case by case basis to either change the charge or more generally test the structure of the lateral chain. None of the proteins is active without the addition of either PA or PG (Fig. 6B). Change of the lateral chain of Arg-260 (R260A) did not alter the activity except for a slight improvement of the activation by PG. In contrast, W287A was modified in activation by both PA and PG and, furthermore, activation by PG was abolished in this mutant. Mutants in the $\langle\beta_2\text{-}\alpha_2\rangle$ loop, P189A and T186A, were also modified for both activations. P189A had a notably severe reduction of activation by PG. Mutant E456N was altered for activations by PA and PG. D150N and D150E were almost not affected except for possibly a slight enhance-

ment of activation by PG in D150N. His-155 looked essential because H155R and H155A had no activity at all. H251A was activated by both PA and PG but less efficiently than the wild-type protein. Altogether most of the mutants were affected in both types of activation. Only two mutants, W287A and P189A, showed a specific severe reduction of activation by PG. Because activation by PA was only slightly affected in these mutants, our conclusion was that the catalytic capability of the W287A and P189A proteins was conserved whereas their PG binding capability was modified. R260A and D150N had possibly a light enhancement of activation by PG, whereas their activation by PA was not modified. In conclusion, our data confirm that the N-domain is important for lipid binding and that Trp-287 and Pro-189 play a critical role in the interaction of MGD1 with PG. Finally, drastic reduction of activation by PA was not detected without drastic reduction of activation by PG.

DISCUSSION

In this report, we have shown that MGD1 is allosterically activated by PA and that this activation is mechanistically different from the activation by PG. The native enzyme present in leaf homogenates or in the chloroplast envelope as well as the recombinant *Arabidopsis* $\Delta 1\text{-}137$ MGD1 were activated by PA. The level of PA required for activation was low ($k_{0.5}$ of about 0.2 mol%) and not sufficient to feed MGDG synthesis by transformation of PA into DAG, demonstrating an allosteric regulation of MGD1 by PA.

Modification of PA metabolism by inhibiting its production in leaf homogenates or enhancing its degradation in the chloroplast envelope hindered the MGDG synthase activity. The PA steady state concentration in the chloroplast envelope where MGD1 is located is assumed to be very low. It is undetectable in the isolated envelope fraction by conventional techniques. The PA level in the envelope is possibly close to PA levels in thylakoids, which was reported to be 0.08 mol% mainly prokaryotic PA (27). MGD1 was activated by several molecular species of PA from either a prokaryotic or eukaryotic nature. All PA molecular species were however not similarly efficient, because only partial restoration of PLD inhibition was obtained with exogenous 16:0/18:1-PA in leaf homogenates and also one prokaryotic molecular species of PA, 16:0/16:0-PA, was less efficient than others on MGD1. There are several possible sources of PA in the chloroplast envelope. Prokaryotic forms of PA are produced in the envelope by acylation of glycerol-3-phosphate by GAT (*ATS1* gene) and LPAAT (*ATS2* gene) (28–30). Formed PA, mostly 18:1/16:0-PA and a low level of 16:0/16:0-PA, are then precursors in synthetic pathways mainly synthesis of PG and prokaryotic MGDG and also partly of SQDG. Eukaryotic forms of PA are imported from extra-plastidial membranes. They likely result from activity of phospholipase(s) D and transport to the chloroplast by a system involving the TGD proteins (31). Phospholipase D activity has never been detected in the isolated chloroplast envelope, although proteomics analysis of the envelope reported the presence of PLD $\alpha 1$ (32). Our results on *pld $\zeta 2$* indicated that without the addition of exogenous PA, the MGDG synthase activity of the mutant leaves was a little reduced compared with the wild type, whereas, after addition of exogenous PA, the activity was much

higher. These results are consistent with (i) PLD ζ 2 being a potent source of PA for activation of MGD1 with (ii) a counterbalance effect of the PLD ζ 2 mutation such as an overexpression of MGD1. However, considering that PLD ζ 2 has been located in the tonoplast (33), it is not yet clear how PA generated by PLD ζ 2 could reach MGD1 present in the inner envelope membrane. Besides PLD ζ 2, some other PLDs could contribute to PA supply. One other candidate is PLD ζ 1, which was shown to be involved like PLD ζ 2 in galactolipid synthesis in roots under phosphate deprivation. It is possible that overexpression of PLD ζ 2/1 in the initial stage of phosphate deprivation could enhance MGD1 activity independently of DAG supply increase. Several other enzymes determine the fate of PA in the envelope. Plastid PAP hydrolyzes PA into diacylglycerol, which is the substrate for MGDG or SQDG synthesis (23, 34). Alternatively, a CDP-diacylglycerol synthetase transfers cytidyl from CTP onto PA and sets off the first step for PG synthesis (35). Finally some reports suggest that lipid kinases and acylhydrolases are active in the chloroplast, which could therefore modify the envelope PA level (36–38).

Ultimately, the question is the role of MGD1 activation by PA. Because PA is a key signal for many processes in the cell (24, 39), MGD1 activation by PA could relate to a signaling process necessary to couple chloroplast and plant development. In this sense, with regards to the role of PLD ζ 2 under the initial stage of phosphate deprivation and PLD ζ 2 tonoplast localization (12, 22, 33, 40, 41), PA produced by PLD ζ 2 could be a switch related to phosphate sensing. Formation of prokaryotic PA could also be important for MGD1 activity. In support of this, *ats2*, a mutant devoid of LPAAT, has no formation of prokaryotic PA and is impaired in embryo development (29) although neither formation of prokaryotic galactolipids is essential at this stage (*ats1* mutant analysis; Ref. 28, 30), nor envelope-specific PG synthesis (*pgp1* mutant analysis; Ref. 35). Another possibility is that MGD1 activation by PA plays a role in the tuning of the different lipid syntheses that occur in the envelope: galactolipid, PG, and SQDG syntheses. For instance, activation of MGD1 by PA could enhance the enzyme functioning when the envelope is initially fed with DAG, while PA is preferentially sustaining PG synthesis. Galactolipid synthesis could thus start instantly.

A second point of our results is that activation by PA and PG proceed through different mechanisms. First, we have shown that there is a synergic effect of PA and PG on MGD1 activity. Second, kinetic analysis of enzyme velocity according to activator concentration indicated (i) a more complex and cooperative way of activation by PA than by PG, (ii) a higher $\text{App } V_{\text{max}}$ of the enzyme with PG although the $k_{0.5}$ was much higher than with PA. Third, the activation by PA and PG was affected differently by salts indicating that the binding of MGD1 with each activator was driven by different types of chemical bond, electrostatic bond for PG activation and hydrophobic bond for PA. Altogether, this suggests that PG plays a specific role in the regulation of MGD1. PG level in the inner membrane is close to 8 mol%, which is not very far from the $k_{0.5}$ calculated for PG on the recombinant *Arabidopsis* Δ 1–137 MGD1 (5 mol%). Moreover almost no activity was found in the chloroplast envelope after treatment to deplete PA but activity was enhanced by

addition of PG, indicating that the envelope PG in our assay was not present in sufficient concentration to sustain MGD1 activity. Two residues, Pro-189 and Trp-287, which play a specific role in the activation by PG, are present in the N domain of MGD1. Because the N domain of MGD1 was previously proposed to interact with the membrane (7), it is possible that PG is important to anchor MGD1 into the membrane. However, we observed that the recombinant MGD1 could easily associate with PC liposomes. This suggests that PG is not essential for anchoring. MGD1 activation by PG could finally reflect, as suggested above for activation by PA, a reinforced coupling of MGDG synthesis with PG synthesis, considering that MGDG and PG are two essential components of photosynthetic membranes.

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Activation of the Chloroplast Monogalactosyldiacylglycerol Synthase MGD1 by Phosphatidic Acid and Phosphatidylglycerol

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