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ORIGINAL PAPER

Specific role of phosphatidylglycerol and functional overlaps with other thylakoid lipids in Arabidopsis chloroplast biogenesis

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Abstract

Key message **With phosphate deficiency, the role of phosphatidylglycerol is compensated by increased glycolipid content in thylakoid membrane biogenesis but not photosynthetic electron transport in Arabidopsis chloroplasts.**

Abstract In plants and cyanobacteria, anionic phosphatidylglycerol (PG) is the only major phospholipid in thylakoid membranes, where neutral galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) are predominant. In addition to provide a lipid bilayer matrix, PG plays a specific role in photosynthetic electron transport. Non-phosphorous sulfoquinovosyldiacylglycerol (SQDG) is another anionic lipid in thylakoids; it substitutes for PG under phosphate (Pi) deficiency to maintain proper balance of anionic charge in thylakoid membranes. Although the crucial role of PG in photosynthesis has been deeply analyzed in cyanobacteria, its physiological function in seed plants other than photosynthesis remains unclear. To reveal specific roles of PG and functional overlaps with other thylakoid lipids, we characterized a PG-deficient Arabidopsis mutant (*pgp1-2*) under Pi-controlled conditions. Under Pi-sufficient conditions, the proportion of PG and other thylakoid lipids was decreased in *pgp1-2*, which led to severe disruption of thylakoid membrane biogenesis. Under Pi-deficient conditions, the proportion of all glycolipids in the mutant was greatly increased, with that of PG further decreased. In Pi-deficient *pgp1-2*, thylakoid membranes remarkably developed, which was accompanied by a change in nucleoid morphology and restored expression of nuclear- and plastid-encoded photosynthesis genes. Increase in glycolipid content with Pi deficiency may compensate for the loss of PG in terms of thylakoid membrane biogenesis. Although Pi deficiency increased chlorophyll and photosynthesis protein content in *pgp1-2*, it critically decreased photochemical activity in PSII. Further deprivation of PG in photosynthesis complexes may abolish the PSII activity in Pi-deficient *pgp1-2*, which suggests that glycolipids cannot replace PG in photosynthesis.

Keywords Chloroplast · Galactolipid · Phosphatidylglycerol · Photosynthesis · Sulfoquinovosyldiacylglycerol · Thylakoid membrane

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Introduction

The thylakoid membrane is the site of the photochemical and electron transport reactions of oxygenic photosynthesis in cyanobacteria and plant chloroplasts. The lipid bilayer of the thylakoid membrane provides a matrix embedded with

photosynthesis protein–pigment complexes forming the electron transport chain. The major groups of lipids in the thylakoid membrane are the neutral galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which together account for about 80 % of total thylakoid lipids in plant chloroplasts (Kobayashi et al. 2009b). The rest is composed of the anionic lipids sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) (Wada and Murata 2007).

PG is the only major phospholipid in the thylakoid membrane and, besides providing a lipid bilayer matrix, plays a specific role in oxygenic photosynthesis (Mizusawa and Wada 2012). X-ray crystallography studies of cyanobacterial photosystems (PS) demonstrated PG molecules present near reaction centers in both PSI and PSII (Jordan et al. 2001; Guskov et al. 2009; Umena et al. 2011), which suggests an involvement of PG in photosynthetic reactions. In-depth analyses of PG-deficient mutants in cyanobacteria revealed that PG depletion greatly decreased PSII activity by inhibiting electron transport from plastoquinone Q_A–Q_B and dissociating extrinsic proteins required for stabilization of the oxygen-evolving complex (Sato et al. 2000; Hagio et al. 2000; Gombos et al. 2002; Sakurai et al. 2003, 2007). PG is also required for PSI activity and trimerization of PSI complexes in cyanobacteria (Domonkos et al. 2004; Sato et al. 2004).

In higher plants, the involvement of PG in photosynthesis was first revealed in phospholipase-treated thylakoid membranes. Phospholipase treatment eliminated 50–70 % of the original PG from pea thylakoid membranes, which eliminated electron transport in PSII without any notable effect on PSI activity (Jordan et al. 1983; Droppa et al. 1995). In addition, genetic approaches in Arabidopsis showed the need for PG for chloroplast biogenesis. Mutants deficient in chloroplast PG biosynthesis had pale yellow–green leaves and failed to develop thylakoid membrane networks inside leaf chloroplasts (Hagio et al. 2002; Babiychuk et al. 2003; Haselier et al. 2010). These data indicate that PG is needed for plant photosynthesis.

For synthesis of PG, phosphatidic acid is first converted to CDP-diacylglycerol (DAG) by CDP-DAG synthase. Then PG phosphate (PGP) is formed from CDP-DAG and glycerol 3-phosphate by PGP synthase. The last step of this pathway is dephosphorylation of PGP by PGP phosphatase, which results in PG (Wada and Murata 2007).

In plant cells, PG is synthesized in plastids, mitochondria and endoplasmic reticulum (ER) membranes (Wada and Murata 2007). Two genes encoding PGP synthase, *PGP1* and *PGP2*, have been identified and characterized in Arabidopsis. PGP1 is dual localized in plastids and mitochondria (Babiychuk et al. 2003), whereas PGP2 is targeted to the ER (Tanoue et al. 2014). Analyses of mutants revealed that knockout of *PGP1* greatly reduced total PG content in leaves, with a severe defect in thylakoid membrane development (Hagio et al. 2002; Babiychuk et al. 2003). The remaining PG in *PGP1*-knockout mutants could be derived from PGP2 activity, because a *pgp1pgp2* double mutant could not synthesize PG and showed a lethal phenotype during embryogenesis (Tanoue et al. 2014). Despite a strong impairment of chloroplast development in *PGP1*-knockout mutants, loss of PGP1 did not affect mitochondria features (Hagio et al. 2002; Babiychuk et al. 2003). The data suggest that ER-localized PGP2 can complement the PGP1 function in mitochondria.

Another thylakoid anionic lipid, SQDG, is not indispensable for chloroplast biogenesis during normal growth conditions but is important to maintain the amount of total anionic lipids in chloroplasts under phosphate (Pi)-deficient conditions (Yu et al. 2002; Yu and Benning 2003; Okazaki et al. 2013). SQDG content increases during Pi deficiency in parallel with decreased PG content, which may maintain proper balance of anionic charge in the thylakoid membrane. When SQDG biosynthesis was disrupted along with a mild defect in PG biosynthesis, plants showed impaired photosynthesis and strong growth retardation (Yu and Benning 2003). These data suggest the importance of SQDG in plant chloroplasts in association with the role of PG. However, which part of the PG function SQDG can substitute under Pi-deficient conditions remains elusive.

Although functions of anionic lipids in photosynthesis have been extensively investigated in cyanobacteria, their role in chloroplast biogenesis and plant growth is still largely unclear. To elucidate the specific roles of PG and functional overlaps with other thylakoid lipids in chloroplast biogenesis, we characterized the *pgp1-2* mutant under Pi-deficient conditions. Pi-deficient *pgp1-2* accumulated SQDG and galactolipids in parallel with reduced PG content, which led to the development of thylakoid membrane networks inside chloroplasts. The partial complementation of chloroplast development in the mutant allowed us to elucidate functional relationships between PG and thylakoid glycolipids in plants.

Materials and methods

Plant materials and growth conditions

The wild-type and *pgp1-2* mutant (the KG10062 line) (Hagio et al. 2002) were the Columbia ecotype of *Arabidopsis thaliana*. Seeds were surface sterilized, then cold treated at 4 °C for 4 days in the dark before seeding. For all conditions, plants were grown with 1.0 % (w/v) sucrose at 23 °C under continuous white light (40 μmol photons m^{-2} s^{-1}) in a growth chamber (CLE-303, Tomy Seiko, Tokyo, Japan). Wild type and *pgp1-2* were grown on

Murashige and Skoog medium (adjusted to pH 5.7 with KOH) solidified with 0.8 % (w/v) agar for 5 and 10 days, respectively, to equalize their developmental stages. Then plants were transferred to liquid Pi-sufficient (1.0 mM) or -deficient (0 mM) medium (Kobayashi et al. 2013) and grown with gentle rotation for 7 days.

Lipid analysis

Total lipids were extracted from seedlings that had been pulverized into powder in liquid nitrogen and separated by 2D thin-layer chromatography (TLC) as described (Kobayashi et al. 2007). Lipids visualized with 0.01 % (w/v) primuline in 80 % (v/v) acetone under UV light were collected with silica gel and incubated with 5 % (v/v) HCl in methanol at 85 °C for 90 min to hydrolyze and methylesterify acyl moieties. Then fatty acid methyl esters extracted with *n*-hexane were quantified with gas liquid chromatography (GC-17, Shimadzu, Kyoto, Japan) with myristic acid used as an internal standard (Fig. 1a, b and Supplementary Fig. S1).

Gene expression analysis

Gene expression was examined by real-time quantitative RT-PCR analysis (Pfaffl 2001) (Figs. 1c, 5). Total RNA extracted with use of the RNeasy Plant Mini kit and the RNase-Free DNase Set (Qiagen K.K., Tokyo, Japan) was reverse transcribed by the use of RNA PCR kit 3.0 (Takara Bio, Otsu, Japan). cDNA amplification involved use of the Thunderbird PreMix kit (Toyobo, Osaka, Japan) and 200 nM gene-specific primers (Supplementary Table S1). Thermal cycling consisted of an initial denaturation step at 95 °C for 10 s, followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. Melting curve runs were performed at the end of each PCR to verify the specific amplification of each gene. Signal detection and quantification were performed in duplicate by the use of MiniOpticon (Bio-Rad, Hercules, CA, USA). The relative abundance of all amplified transcripts was normalized to the constitutive expression of *ACTIN8* (Pfaffl 2001). Three independent biological experiments were performed for each sample.

Chlorophyll determination

Whole seedlings were pulverized in liquid nitrogen and homogenized in 80 % (v/v) acetone and debris was removed by centrifugation at 10,000×*g* for 5 min. The absorbance at 720, 663, and 645 nm was measured by the use of an Ultrospec 2100 *pro* spectrophotometer (GE Healthcare Bioscience, Amersham, UK). The chlorophyll (*a* and *b*) concentration of samples was determined as described (Melis et al. 1987) (Fig. 2).

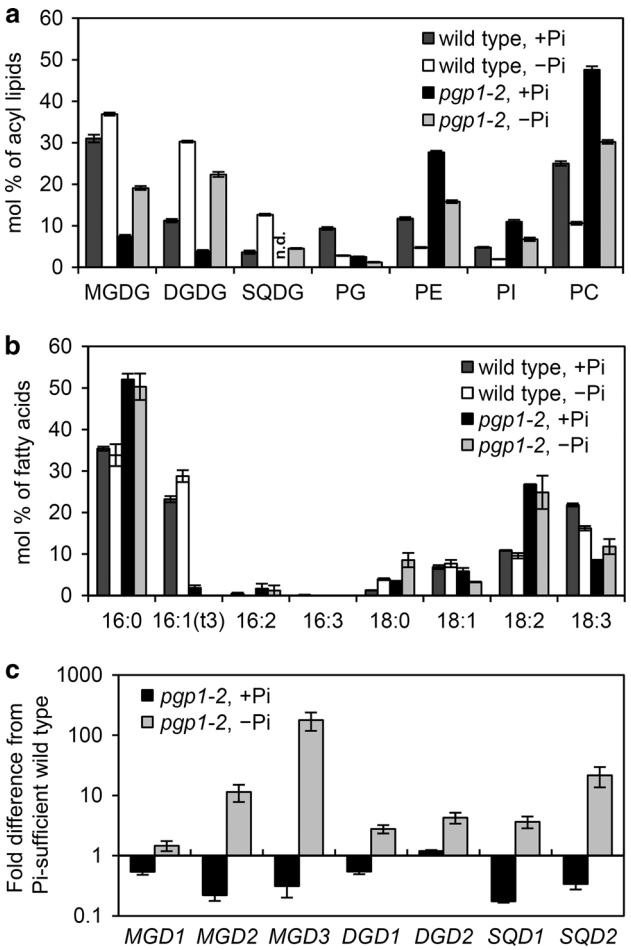

Fig. 1 Lipid remodeling in *pgp1-2* mutant with phosphate (Pi) deficiency. Composition of **a** polar glycerolipids and **b** fatty acids of phosphatidylglycerol (PG) in wild type and *pgp1-2* under Pi-sufficient (+Pi) or -deficient (−Pi) conditions. *MGDG* monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *PE* phosphatidylethanolamine, *PI* phosphatidylinositol, *PC* phosphatidylcholine. **c** Expression of glycolipid biosynthesis genes in *pgp1-2* under +Pi or −Pi conditions. Data are fold difference from the Pi-sufficient wild type after normalization to the control gene *ACTIN8*. **a**–**c** Data are mean ± SE ($n = 3$), *n.d.* not detected

Microscopy analyses

Bright-field images and chlorophyll autofluorescence (Fig. 2a) were observed under a stereomicroscope (MZ 16FA, Leica) with a color CCD camera (VB-7010, Keyence). Settings of the microscope and the CCD camera were consistently fixed to compare chlorophyll fluorescence intensities in different samples.

For microscopy analysis of leaf sections (Fig. 3 and Supplementary Fig. S2), segments from wild-type and *pgp1-2* leaves were fixed and embedded in a resin as described previously (Toyooka et al. 2000) with modification. Tissues were fixed with 4 % (w/v) paraformaldehyde and 2 % (w/v) glutaraldehyde in 100 mM sodium

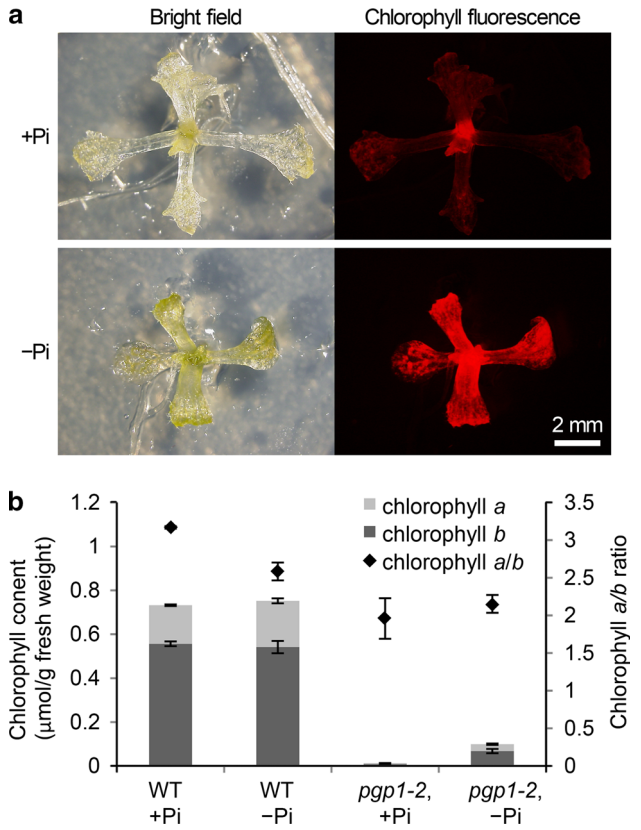

Fig. 2 Chlorophyll accumulation in *pgp1-2* with Pi deficiency. **a** Color phenotype (*left panels*) and chlorophyll autofluorescence (*right panels*) of *pgp1-2* under Pi-sufficient (+Pi) or -deficient (−Pi) conditions. **b** Content and ratio of chlorophyll *a* and *b* in wild type and *pgp1-2* under +Pi or −Pi. Data are mean ± SE ($n = 4$)

cacodylate buffer, pH 7.4, overnight at 4 °C, then post-fixed with 1 % (w/v) osmium tetroxide in 50 mM sodium cacodylate buffer, pH 7.4, for 3 h at room temperature. After dehydration in a graded methanol series, the samples were embedded in Epon812 resin. Samples sectioned to 1 μm were stained with 0.1 % (w/v) toluidine blue and analyzed under a light microscope (BX-51, OLYMPUS, Tokyo, Japan) (Fig. 3a and Supplementary Fig. S2a). Ultrathin sections (80 nm) were stained with 4 % (w/v) uranyl acetate and lead citrate and observed under a JEM-1400 electron microscope (JEOL, Akishima, Japan) and merged by the use of Adobe Photoshop CS5 (Fig. 3b and Supplementary Fig. S2b).

To detect plastid nucleoids (Fig. 4a), protoplasts were prepared from leaves and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described (Kobayashi et al. 2013). The diameter of DAPI fluorescence signals was measured by the use of ImageJ (US National Institutes of Health) to determine nucleoid size (Fig. 4b).

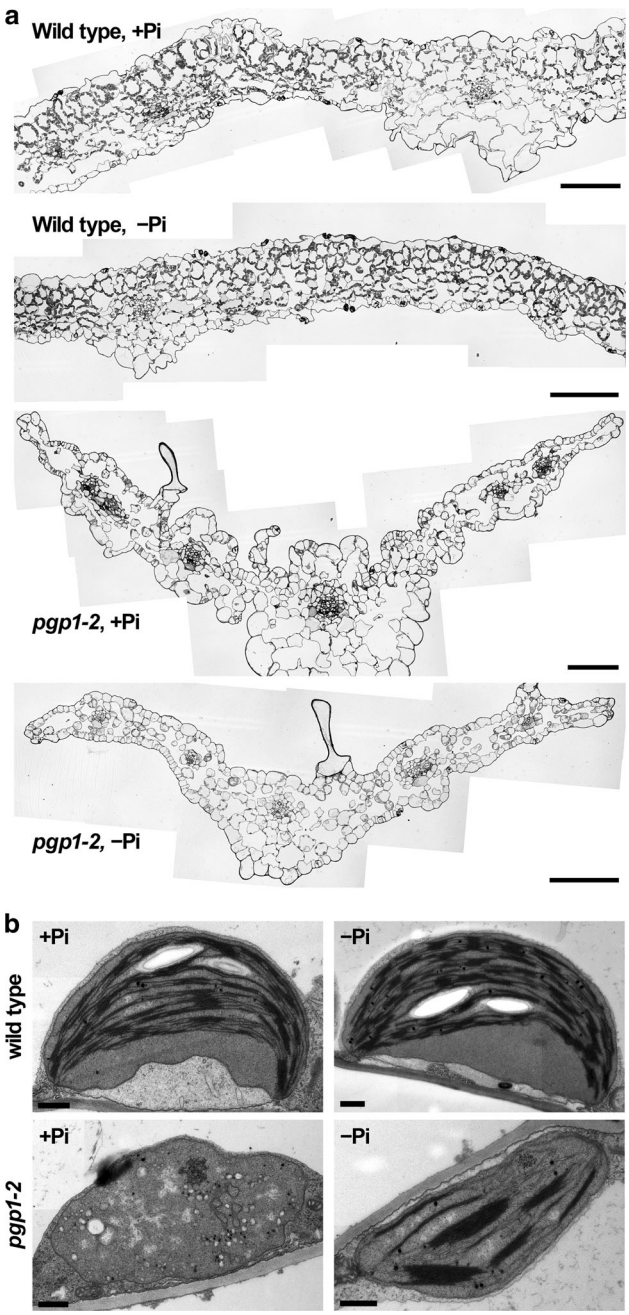

Fig. 3 **a** Leaf morphology and **b** ultrastructure of leaf plastids in wild type and *pgp1-2* under Pi-sufficient (+Pi) or -deficient (−Pi) conditions. *Bars* 0.5 mm in (**a**) and 0.5 μm in (**b**)

Protein analysis

The total membrane protein fraction was prepared as described (Kobayashi et al. 2013). For total protein analysis (Fig. 6a), the protein sample (40 μg) underwent 12.5 % (w/v) SDS-PAGE followed by staining with 0.25 % (w/v) Coomassie brilliant blue. For immunoblot analysis (Fig. 6b), total membrane proteins from *pgp1-2*

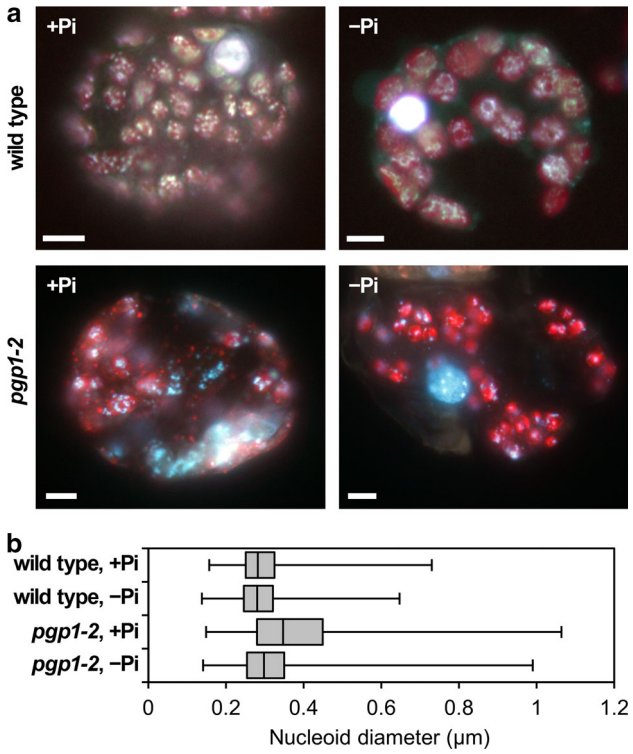

Fig. 4 **a** Morphology and **b** diameter of plastid nucleoids in protoplasts from wild-type and *pgp1-2* leaves under Pi-sufficient (+Pi) or –deficient (−Pi) conditions. DNA was stained with 4′,6-diamidino-2-phenylindole. In **a**, small fluorescent particles corresponding to plastid nucleoids were observed in addition to large fluorescence from nuclei. *Bars* 5.0 μm. In **b**, the *vertical line* in each *box* represents the median value of the distribution. The *left* and the *right end* of each *box* represent the upper and lower quartile, respectively. The whiskers represent the range

(40 μg) and wild-type samples (4 μg) were electrophoresed and electro transferred onto nitrocellulose membranes (Amersham Protran Premium 0.2 NC, GE Healthcare). Target proteins labeled with primary antibodies were then labeled with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Thermo Fisher Scientific K.K., Yokohama, Japan). Chemiluminescence signals were detected with the use of reagent (Pierce Western Blotting Substrate Plus, Thermo Fisher Scientific K.K.) and an imager (ImageQuant LAS 4000 mini, GE Healthcare Japan, Tokyo). Antibodies against PsaA/PsaB and light-harvesting chlorophyll protein complexes (LHC) were kindly provided by R. Tanaka (Hokkaido University, Sapporo, Japan) and against D1, and D2 by M. Ikeuchi (The University of Tokyo).

Photosynthetic chlorophyll fluorescence analysis

For chlorophyll fluorescence analysis at 77 K (Fig. 6c), membrane fractions (2 μg chlorophyll/ml) were prepared as described (Fujii et al. 2014). Chlorophyll fluorescence from membrane fractions was measured in liquid nitrogen with use of an RF-5300PC spectrofluorometer (Shimadzu, Kyoto, Japan) under 435 nm excitation.

The maximum photosynthetic efficiency of PSII (*Fv*/*Fm*) (Fig. 6d) was determined with the use of a chlorophyll fluorometer (JUNIOR-PAM, Heinz Walz, Effeltrich, Germany) as described (Fujii et al. 2014).

Results

Lipid remodeling in *pgp1-2* with Pi deficiency

In higher plants, Pi deficiency induces the replacement of phospholipids with non-phosphorous glycolipids to economize phosphorus in membranes (Essigmann et al. 1998; Kelly et al. 2003; Yu and Benning 2003; Kobayashi et al. 2009b). To assess whether glycolipids accumulated in response to Pi deficiency can complement PG deficiency, we characterized the *pgp1-2* mutant grown under Pi-controlled conditions. With Pi sufficiency (1 mM Pi), in addition to PG, the proportion of MGDG and DGDG was lower in *pgp1-2* than the wild type (Fig. 1a). Moreover, SQDG was undetectable in Pi-sufficient *pgp1-2* under our experimental conditions. With Pi deficiency (0 mM Pi), the proportion of galactolipids and SQDG in the wild type increased in parallel with a decrease in phospholipids, as was reported previously (Essigmann et al. 1998). The increase in galactolipids was greater in Pi-deficient *pgp1-2* seedlings; MGDG and DGDG increased 2.6- and 5.8-fold, respectively, with Pi deficiency. Moreover, SQDG became detectable in Pi-deficient *pgp1-2* and accounted for 4.5 mol% of total membrane lipids. By contrast, with Pi deficiency, the proportion of phospholipids including PG was decreased in the mutant, as was observed in the wild type.

Fatty acid (FA) composition differed between the wild-type and the *pgp1-2* seedlings. Consistent with previous reports (Gao et al. 2009), PG in the wild type contained a large amount of 16:1(3t) FAs in both conditions (Fig. 1b). However, PG in *pgp1-2* contained a low proportion of 16:1(3t) FAs; instead the proportion of 16:0 FAs was increased. In addition, the 18:3 FA content was decreased with increased 18:2 FA content. MGDG, DGDG and other phospholipids showed decreased 18:3 FA content with the *pgp1-2* mutation (Supplementary Fig. S1). The FA composition of MGDG and PG in the *pgp1-2* seedlings was not largely changed by Pi deficiency, but the composition in DGDG greatly differed. The 16:0 FA content in DGDG was increased by Pi deficiency in the wild type, as previously reported (Kelly et al. 2003), and was very high in *pgp1-2* with Pi sufficiency but decreased with Pi deficiency (Supplementary Fig. S1).

Next, we examined the expression of glycolipid synthesis genes in *pgp1-2* under Pi-controlled conditions. *MGD1*, *MGD2* and *MGD3* encode isoforms of MGDG synthase (Awai et al. 2001) and *DGD1* and *DGD2* encode isoforms of DGDG synthase (Dörmann et al. 1995; Kelly and Dörmann 2002). *SQD1* and *SQD2* encode UDP-sulfoquinovose synthase and SQDG synthase, respectively, both essential for SQDG biosynthesis in Arabidopsis (Yu et al. 2002; Okazaki et al. 2009). *MGD1* is unresponsive to Pi deficiency, whereas all other genes examined are strongly upregulated by Pi deficiency (Essigmann et al. 1998; Awai et al. 2001; Yu et al. 2002; Kelly et al. 2003; Kobayashi et al. 2004). Under Pi sufficiency, the expression of most glycolipid biosynthesis genes was lower in *pgp1-2* than the wild type (Fig. 1c). However, under Pi deficiency, all genes were upregulated in the mutant, which is consistent with the substantial increase in glycolipids in Pi-deficient *pgp1-2*.

Chlorophyll accumulation in *pgp1-2* with Pi deficiency

As reported previously (Hagio et al. 2002), under Pi sufficiency, greening was strongly inhibited, with severe growth retardation in *pgp1-2* (Fig. 2a). However, under Pi deficiency, the *pgp1-2* seedlings became pale green and showed stronger chlorophyll fluorescence than under Pi sufficiency, although the growth retardation was not recovered. Consistent with the visible phenotype, the chlorophyll content in the mutant was increased eightfold with Pi deficiency (Fig. 2b). In the wild type, the chlorophyll *a*/*b* ratio was decreased with Pi deficiency, with no change in the total chlorophyll content per gram fresh weight. In *pgp1-2* seedlings, the chlorophyll *a*/*b* ratio was lower than in the wild type and was unchanged by Pi deficiency.

Leaf development and thylakoid membrane biogenesis in *pgp1-2* with Pi deficiency

The *pgp1-2* mutation impaired not only chlorophyll accumulation but also leaf development (Fig. 2a). Consistent with a previous report (Hagio et al. 2002), the leaf architecture in *pgp1-2* was strongly disordered; Pi-sufficient *pgp1-2* leaves showed extensive large intercellular spaces (Fig. 3a and Supplementary Fig. S2a). The mutant leaves contained mesophyll cells only around vascular structures and their number was substantially decreased as compared with wild-type leaves. The epidermal cell layers in the Pi-sufficient mutant leaves were distorted and abnormally crooked. However, in Pi-deficient *pgp1-2* leaves, intercellular spaces were decreased as compared with Pi-sufficient mutant leaves (Fig. 3a and Supplementary Fig. S2a) and the disordered in epidermal cell layers was moderated.

To address the effect of Pi deficiency-induced glycolipid accumulation on thylakoid biogenesis in *pgp1-2*, we observed the ultrastructure of leaf chloroplasts under Pi-controlled conditions (Fig. 3b and Supplementary Fig. S2b). In wild-type leaves, chloroplast structures were not notably affected by Pi deficiency. Pi-sufficient *pgp1-2* leaves showed many vesicles or enlarged vacuolated structures, barely detected in wild-type chloroplasts, instead of thylakoid membrane networks. A few chloroplasts in Pi-sufficient leaves formed internal membrane structures, although they were underdeveloped, as was observed previously (Hagio et al. 2002). The *pgp1-2* chloroplasts under Pi-deficiency showed vesicular structures (Supplementary Fig. S2b). However, these chloroplasts also showed highly stacked thylakoid membranes in addition to such vesicles.

Change in nucleoid morphology in the *pgp1-2* plastid with Pi deficiency

The morphology and distribution of nucleoids in plastids are associated with thylakoid membrane biogenesis (Powikrowska et al. 2014), so we examined DAPI-stained plastid nucleoids in leaf protoplasts under Pi-controlled conditions (Fig. 4). In wild-type chloroplasts, the distribution and size of nucleoids did not differ greatly between seedlings grown under Pi-sufficient and -deficient conditions. In the *pgp1-2* chloroplasts, some nucleoids appeared aggregated and thus the nucleoid diameter was increased under Pi sufficiency. However, the nucleoid size in the *pgp1-2* chloroplasts was decreased to close to wild-type levels with Pi deficiency.

Upregulation of photosynthesis-associated genes in *pgp1-2* with Pi deficiency

To assess the effect of Pi deficiency on photosynthesis gene expression in *pgp1-2*, we examined the expression of nuclear-encoded photosynthesis-associated genes (*LHCA4*, *LHCB6*, *CHL27* and *GLK1*) and plastid-encoded genes (*psaA*, *psbA* and *rpoB*). *LHCA4* and *LHCB6* encode subunits of LHCI and LHCII, respectively, whereas *CHL27* encodes the membrane subunit of Mg-protoporphyrin IX monomethyl ester cyclase involved in chlorophyll biosynthesis. The transcription factor GLK1 induces the expression of photosynthesis-associated nuclear genes such as *LHCA4*, *LHCB6* and *CHL27* (Waters et al. 2009). The *psaA* and *psbA* genes encode core proteins of PSI (PsaA) and PSII (D1), respectively. Plastid-encoded *psaA* and *psbA* are mainly transcribed by plastid-encoded RNA polymerase (PEP), and PEP genes, including *rpoB*,

encoding the β-chain of PEP, are transcribed by nuclear-encoded plastid RNA polymerase (NEP) (De Santis-MacIossek et al. 1999).

Under Pi sufficiency, all photosynthesis-associated genes investigated were strongly downregulated in *pgp1-2* as compared with the wild type (Fig. 5a). The *rpoB* expression was also slightly decreased in *pgp1-2*, although the reduction was milder than for *psaA* and *psbA*. The expression of photosynthesis-associated genes is decreased in the wild type in response to Pi deficiency (Kobayashi et al. 2013). However, in *pgp1-2*, except for *rpoB*, the expression was increased with Pi deficiency.

We next examined the expression of genes for sigma factors (SIGs), which are subunits of the PEP complex functioning redundantly in transcription initiation of PEP-dependent plastid genes (Schweer et al. 2010). The expression patterns of *SIG* genes varied, although the pattern of *SIG1* and *SIG4* was similar to that of the photosynthesis-associated genes (Fig. 5b), *SIG3* expression was unchanged between the wild type and *pgp1-2* and *SIG5* expression was even increased in *pgp1-2*. The expression of *SIG2* and *SIG6* was reduced in Pi-sufficient *pgp1-2* and was not recovered by Pi deficiency.

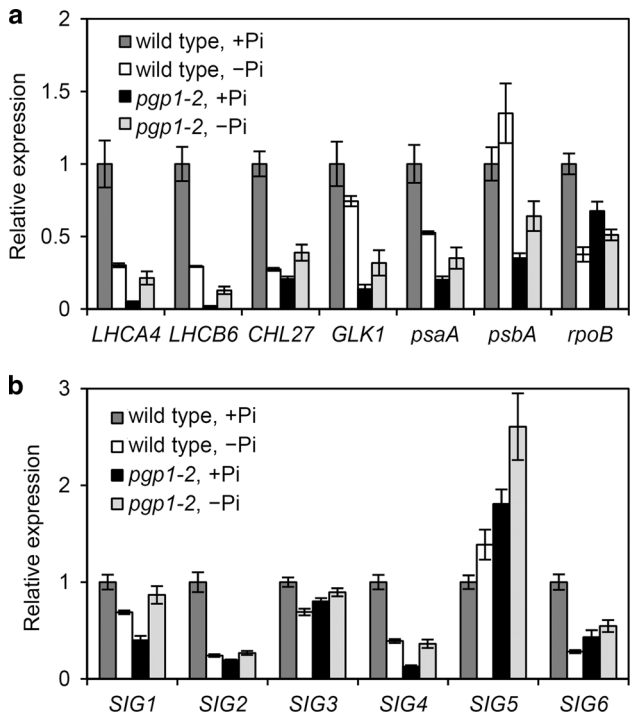

Fig. 5 Quantitative RT-PCR of expression of **a** genes associated with chloroplast biogenesis and photosynthesis and **b** genes encoding sigma factors in wild-type and *pgp1-2* under Pi-sufficient (+Pi) or -deficient (−Pi) conditions. Data (mean ± SE, $n = 3$) are fold difference from the wild type (+Pi) after normalization to the control gene *ACTIN8*

Complete dysfunction of PSII in *pgp1-2* with Pi deficiency

To investigate the effect of Pi status on photosynthesis proteins in *pgp1-2* seedlings, we analyzed proteins in membrane fractions from the wild type and the *pgp1-2*. Coomassie brilliant blue staining for total membrane proteins was similar between *pgp1-2* and the wild type under both Pi conditions, with some exceptions, including a strong reduction in bands at approximately 25 kDa, which presumably correspond to LHC proteins (Fig. 6a). We next compared the level of membrane photosynthesis proteins among these samples by immunoblot analysis. Total membrane proteins (40 μg) from *pgp1-2* seedlings were compared with one-tenth the amount (4 μg) of wild-type proteins (Fig. 6b). As compared with the wild type, Pi-sufficient *pgp1-2* seedlings contained only a low amount of membrane photosynthesis proteins. Photosynthesis protein content in *pgp1-2* was higher with Pi deficiency than sufficiency, although the content was still lower than in the wild type. In *pgp1-2*, levels of PS core proteins (PsaA/B, D1 and D2) were decreased more than LHC levels under both conditions as compared with the wild type, which is consistent with the lower chlorophyll *a*/*b* ratio in the mutant than the wild type.

To assess the effect of Pi deficiency on the formation of photosynthesis complexes in Pi-deficient *pgp1-2* seedlings, we analyzed chlorophyll fluorescence at 77 K in membrane fractions from wild-type and *pgp1-2* seedlings (Fig. 6c). Emission peaks around 728 and 683 nm, originating from PSI and PSII, respectively (Govindjee 1995), in the wild type were both blue shifted to 718 and 679 nm, respectively, in *pgp1-2*. Moreover, chlorophyll fluorescence from PSI was largely decreased in *pgp1-2*. Under Pi deficiency, the emission around 718 nm was increased in *pgp1-2*, with an emission shoulder around 700 nm, but the emission spectra were not largely changed in the wild type with Pi deficiency.

To assess the effect of Pi deficiency on photosynthesis activity in the *pgp1-2*, we analyzed maximum quantum efficiency of PSII (*Fv*/*Fm*) under Pi-controlled conditions (Fig. 6d). In wild-type seedlings, *Fv*/*Fm* was slightly decreased by Pi deficiency. *Fv*/*Fm* was much lower in *pgp1-2* than wild-type leaves. Moreover, the value was further deceased by Pi deficiency in the mutant.

Discussion

Pi deficiency increases glycolipids in *pgp1-2*

In *pgp1-2* seedlings with Pi sufficiency, the amount of PG and also that of thylakoid glycolipids was substantially

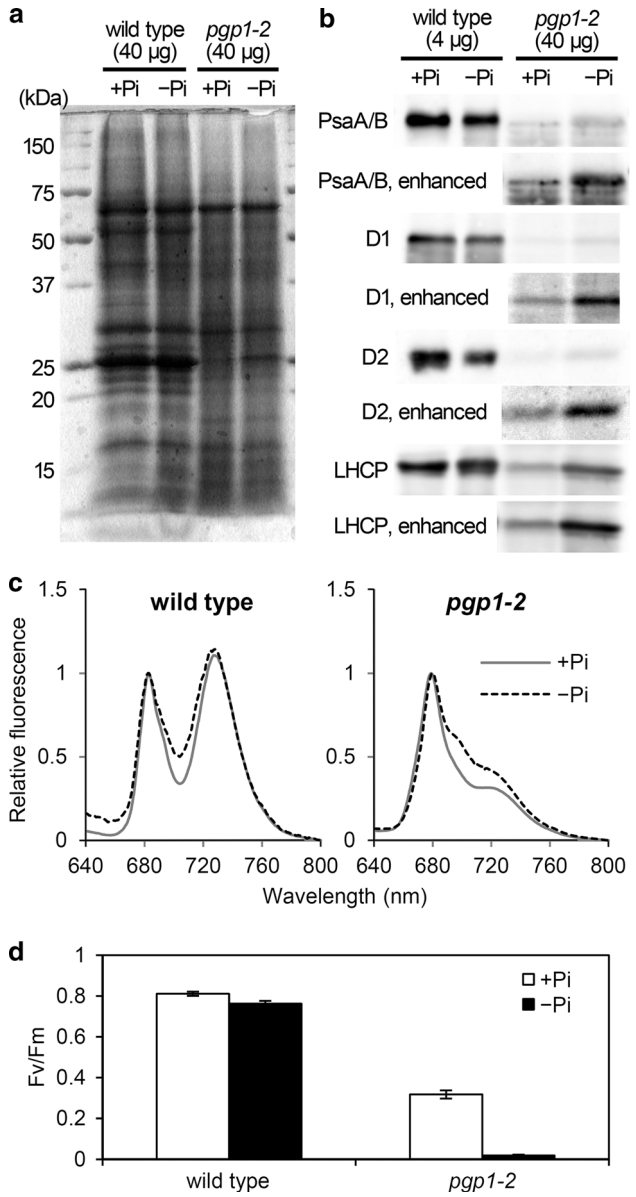

Fig. 6 Photosynthetic characteristics in *pgp1-2*. **a** Coomassie brilliant blue staining of SDS-PAGE-separated total membrane proteins (40 µg) from wild type and *pgp1-2* grown under Pi-sufficient (+Pi) or -deficient (−Pi) conditions. Molecular weight is indicated on the *left*. **b** Immunoblot analysis of membrane photosynthesis proteins from wild type (4 µg) and *pgp1-2* (40 µg) under +Pi or −Pi conditions. Contrast-enhanced images are also shown for *pgp1-2* samples. **c** 77 K chlorophyll fluorescence emission spectra and **d** maximum PSII quantum yield (*Fv*/*F*m) in wild type and *pgp1-2* under +Pi or −Pi conditions

decreased as compared with the wild type (Fig. 1a). Most glycolipid biosynthesis genes were downregulated in the Pi-sufficient mutant (Fig. 1c). Because *MGD1* and *DGD1* are responsible for the bulk of galactolipid synthesis in photosynthetic tissues under Pi sufficiency (Dörmann et al. 1995; Jarvis et al. 2000; Kobayashi et al. 2007), the reduced *MGD1* and *DGD1* expression may be involved in the decrease in MGDG and DGDG in the Pi-sufficient mutant. We recently reported that *MGD1* and *DGD1* are negatively regulated by plastid signaling via GENOMES-UNCOUPLED1 (GUN1) in response to defects in chloroplast biogenesis (Kobayashi et al. 2014). Because *pgp1-2* showed strong chloroplast dysfunction under Pi sufficiency (Fig. 3b), *MGD1* and *DGD1* may be downregulated via plastid signaling in *pgp1-2*. *SQD1* and *SQD2* were also strongly downregulated in Pi-sufficient *pgp1-2* seedlings (Fig. 1c), which may reduce the SQDG content to undetectable levels in the mutant. Although the downregulation mechanism for *MGD2*, *MGD3*, *SQD1* and *SQD2* in the Pi-sufficient mutant remains elusive, some of these genes might be co-regulated with *MGD1* and *DGD1* during chloroplast development.

Under Pi deficiency, all of the Pi-inducible glycolipid biosynthesis genes were strongly upregulated in *pgp1-2* (Fig. 1c), which would trigger accumulation of glycolipids in the mutant (Fig. 1a). Moreover, *MGD1*, which is unresponsive to Pi deficiency (Awai et al. 2001; Kobayashi et al. 2004), was increased in level with Pi deficiency in the mutant (Fig. 1c). Because *MGD1* expression is regulated in response to the chloroplast functionality (Kobayashi et al. 2014), chloroplast biogenesis in the Pi-deficient *pgp1-2* may positively affect *MGD1* expression, as was observed with other photosynthesis-associated genes (Fig. 5). Of note, *pgp1-2* showed no PSII activity under Pi deficiency (Fig. 6d) and thus photosynthetic electron transport activity would not be required for the upregulation of *MGD1* or other photosynthesis-associated genes. In addition to increased DGDG and SQDG levels, both reported to be increased by Pi deficiency (Essigmann et al. 1998), MGDG level was largely increased in the Pi-deficient mutant (Fig. 1c). Because MGD2 and MGD3 mainly have a role in DGDG accumulation during Pi deficiency (Kobayashi et al. 2009a), the restored expression of *MGD1* in Pi-deficient *pgp1-2* may mainly contribute to the MGDG accumulation. In this context, *MGD1* expression and galactolipid accumulation may affect reciprocally during chloroplast biogenesis.

Plastidic lipids are synthesized via two distinct pathways, namely the prokaryotic and eukaryotic pathways. The prokaryotic pathway is restricted to the plastid, whereas the eukaryotic pathway includes reactions at the ER in addition to plastids (Li-Beisson et al. 2013). Consistent with a previous report (Babiychuk et al. 2003), content of 16:3 FA of MGDG, which is restricted to the molecules derived from the prokaryotic pathway, was only moderately decreased in *pgp1-2* as compared with the wild type under both Pi conditions (Supplementary Fig. S1). Therefore, the prokaryotic pathway is still active in *pgp1-2*. However, 16:1(3t) FA of PG, another prokaryotic pathway-specific FA species, was absent in *pgp1-2* (Fig. 1b). Thus,

desaturation of 16:0 to 16:1(3t) FA in PG or the prokaryotic PG biosynthesis itself would be inhibited in *pgp1-2*. Considering that the remaining PG in *pgp1-2* could be derived from ER-localized PGP2 (Tanoue et al. 2014), PGP1 is likely indispensable for the prokaryotic PG biosynthesis and thus the *pgp1-2* null mutation abolishes it. Of note, the FA composition of PG in *pgp1-2* was similar to that of phosphatidylinositol (PI) (Fig. 1b and Supplementary Fig. S1). Because the biosynthesis of PI and PG at the ER uses the same CDP-DAG pool for substrates (Zhou et al. 2013), the similar FA composition between PG and PI in *pgp1-2* may reflect the same origin of DAG backbone from the ER pathway.

In addition to 16:1(3t) FA, 18:3 FA content decreased in parallel with increased 18:2 FA content in *pgp1-2* PG (Fig. 1b). As observed in another Arabidopsis *pgp1* mutant allele (*jovtenky*) (Babiychuk et al. 2003), the decreased 18:3 FA content was also observed in other lipids such as MGDG, DGDG, and PC in *pgp1-2* (Supplementary Fig. S1). Furthermore, the decreased 18:3 FA content in *pgp1-2* glycerolipids was not recovered by Pi deficiency, so glycolipid biosynthesis and subsequent thylakoid biogenesis and leaf greening cannot compensate for loss of PG with respect to FA metabolism. As proposed previously (Babiychuk et al. 2003), the PG level in plastid membranes may be important for the homeostasis of 18:3 FA groups. Alternatively, the impaired photosynthetic electron transport in PSII (Fig. 6d) may influence 18:3 formation in *pgp1-2*, because FA desaturases require electron donors for their activity.

Pi deficiency induces chloroplast development in *pgp1-2*

In *pgp1-2* seedlings, thylakoid membranes developed with glycolipid accumulation under Pi deficiency (Fig. 3b). A similar phenomenon was observed in a galactolipid-deficient mutant, *mgd1-2* (Kobayashi et al. 2013). The *mgd1-2* mutant has a knockout mutation in the *MGD1* gene for the major galactolipid biosynthesis pathway, which results in lack of galactolipids under Pi sufficiency (Kobayashi et al. 2007). However, under Pi deficiency, MGD2 and MGD3, involved in an alternative MGDG biosynthesis pathway, were activated and partially complemented the MGD1 knockout in the mutant (Kobayashi et al. 2013). Because MGD2 and MGD3 mainly contribute to DGDG accumulation with DGD2 (Kobayashi et al. 2009a), Pi-deficient *mgd1-2* seedlings mostly accumulated DGDG (Kobayashi et al. 2013), which may serve as a structural component of thylakoid membranes as well as extraplastidic membranes. For Pi-deficient *pgp1-2*, DGDG but also MGDG and SQDG were substantially accumulated (Fig. 1a), which may lead to the pronounced development of thylakoid membranes in the mutant chloroplasts. This result also suggests that PG is not necessarily required for thylakoid membrane biogenesis when glycolipids are sufficiently synthesized. Meanwhile, the vesicular or vacuolated membrane structures detected in the Pi-sufficient *pgp1-2* (Fig. 3b; Hagio et al. 2002) were also often observed under Pi-deficient conditions (Supplementary Fig. S2b). PG may be essential for some sort of internal membrane organization, which cannot be compensated by glycolipids.

In *pgp1-2* chloroplasts, nucleoid morphologic features were changed with thylakoid membrane development during Pi deficiency (Fig. 4). Membrane biogenesis within plastids is closely associated with the morphology and distribution of plastid nucleoids, involving several nucleoid-associated proteins (Powikrowska et al. 2014). Therefore, the impaired thylakoid biogenesis in the Pi-sufficient *pgp1-2* chloroplasts may cause nucleoid aggregation, which is relaxed by thylakoid membrane development in Pi-deficient chloroplasts, as observed in the *mgd1-2* mutant (Kobayashi et al. 2013).

In addition to chloroplast development, leaf morphologic features distorted in Pi-sufficient *pgp1-2* seedlings were improved by Pi deficiency (Fig. 3a). Considering that thylakoid lipid biosynthesis and chloroplast development is associated with the organization of mesophyll cells (Hagio et al. 2002; Yu and Benning 2003; Kobayashi et al. 2007), the glycolipid accumulation and consequent thylakoid biogenesis by Pi deficiency likely moderates the defective mesophyll cell organization in *pgp1-2*, which may also affect epidermal cell alignment (Fig. 3a).

Pi deficiency upregulates photosynthesis-associated genes in *pgp1-2*

In *pgp1-2* seedlings, plastid-encoded photosynthesis genes were strongly downregulated under Pi sufficiency (Fig. 5a). A similar downregulation was observed with the defect in thylakoid biogenesis in *mgd1-2* (Kobayashi et al. 2013). Because mutants deficient in chlorophyll biosynthesis did not show strong downregulation of these genes (Kobayashi et al. 2013); thylakoid lipid biosynthesis may specifically affect the expression of plastid-encoded photosynthesis genes. Moreover, the downregulation of *psaA* and *psbA* was partially attenuated in *pgp1-2* with Pi deficiency (Fig. 5a), as was reported in *mgd1-2* (Kobayashi et al. 2013). As we discussed previously (Kobayashi et al. 2013), thylakoid biogenesis and subsequent changes in nucleoid morphology during Pi deficiency may alter the transcriptional activity of plastid genes. In addition, in *pgp1-2*, *SIG1* and *SIG4* were coexpressed with *psaA* and *psbA* in response to Pi deficiency (Fig. 5b). Although *SIG* genes showed various expression patterns in *pgp1-2*, *SIG1* and *SIG4* may contribute to upregulation of these plastid

genes. The *SIG5* expression in the *pgp1-2* mutant was also slightly increased with Pi deficiency. However, this gene was even upregulated in the Pi-sufficient mutant, indicating that the *SIG5* expression is not tightly linked with the expression of *psaA* and *psbA* in this condition. Unlike in *mgd1-2* (Kobayashi et al. 2013), in *pgp1-2*, *rpoB* expression was not increased by Pi deficiency (Fig. 5a). Therefore, PG biosynthesis and galactolipid biosynthesis may differentially affect NEP activity in plastids.

As observed in *mgd1-2* (Kobayashi et al. 2013), in the Pi-sufficient *pgp1-2*, nuclear-encoded photosynthesis-associated genes were strongly downregulated (Fig. 5a). However, the expression of these genes was derepressed in part under Pi deficiency. Because the transcription factor GLK1, which directly upregulates photosynthesis-associated nuclear genes including *LHCA4*, *LHCB6* and *CHL27* (Waters et al. 2009), was coexpressed with these target genes, GLK1 may play a role in the transcriptional regulation of photosynthesis-associated nuclear genes in *pgp1-2* in response to the developmental state of thylakoid lipid bilayers. Considering that GLK1 operates downstream of GUN1-mediated plastid signaling to synchronize the expression of nuclear photosynthesis genes with chloroplast development (Kakizaki et al. 2009), the plastid signaling may link thylakoid lipid biosynthesis and GLK1-mediated regulation of photosynthesis-associated nuclear genes.

Pi deficiency abolishes electron transport in PSII in *pgp1-2*

In *pgp1-2*, the amount of membrane photosynthesis proteins was low under Pi sufficiency but increased under Pi deficiency (Fig. 6b). Meanwhile, the formation of PS complexes was largely perturbed in *pgp1-2* under both conditions (Fig. 6c). The blue shift of chlorophyll fluorescence emitted from PSI and PSII suggests dissociation of LHCs from each reaction center. Specifically, PSI in *pgp1-2* may lose most LHCs and thus decrease fluorescence emission, because PSI lacking LHC antennas emits fluorescence at 720 nm (Kuang et al. 1984). In addition, under Pi deficiency, the *pgp1-2* membrane fraction showed fluorescence emission around 700 nm, which may have originated from aggregated LHCII (Kirchhoff et al. 2003). PG has an important role in trimerization of LHCII (Hobe et al. 1994, 1995), and thus loss of PG in Pi-deficient *pgp1-2* may perturb proper formation of LHCII complexes. In *Chlamydomonas reinhardtii*, deficiency of 16:1(3t) FA-binding PG decreased a trimeric LHCII complex and grana stacking of thylakoid membranes (El Maanni et al. 1998). However, in Pi-deficient *pgp1-2*, grana stacking appeared enhanced in some chloroplasts (Fig. 3b and Supplementary Fig. S2b). Requirement of PG for grana stacking may differ between seed plants and green algae as seen in the different requirement of 16:1(3t) FA-containing PG for the assembly of the LHCII trimer between Chlamydomonas (Dubertret et al. 1994) and Arabidopsis (McCourt et al. 1985). As well, SQDG and/or galactolipids increased by Pi deficiency may induce stacking in Pi-deficient *pgp1-2* chloroplasts.

Despite thylakoid membrane biogenesis and accumulation of membrane photosynthesis proteins with chlorophylls, the PSII photochemical reaction was completely abolished in Pi-deficient *pgp1-2* leaves (Fig. 6d). PG content was further decreased by Pi deficiency in *pgp1-2* (Fig. 1a), so PG molecules essential for the PSII activity may be lost under such conditions. Many PG molecules were found in the crystal structure of cyanobacterial PSII, and some were located near the photochemical reaction center (Guskov et al. 2009; Umena et al. 2011). Thus, Pi deficiency in *pgp1-2* may have depleted the PG molecule(s) from the PSII reaction center, for complete impairment of the PSII photochemical reaction. Therefore, glycolipids accumulated during Pi deficiency cannot compensate for the loss of PG in terms of the PSII activity. Although SQDG has an anionic property similar to PG and can compensate for the decrease in PG (Yu and Benning 2003), its compensatory role is limited to some parts related to chloroplast development other than the PSII photochemical reaction.

Author contribution statement KK conceived of the project, designed the study, perform experiments, analyzed data and wrote the paper. SF, MS and KT performed experiments and analyzed data. HW supervised and complemented the writing.

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Conflict of interest The authors declare that they have no conflict of interest.

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