Chloroplast-localized PITP7 is essential for plant growth and photosynthetic function in Arabidopsis

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Abstract
Recent studies of chloroplast-localized Sec14-like protein (CPSFL1, also known as phosphatidylinositol transfer protein 7, PITP7) showed that CPSFL1 is necessary for photoautotrophic growth and chloroplast vesicle formation in Arabidopsis (Arabidopsis thaliana). Here, we investigated the functional roles of CPSFL1/PITP7 using two A. thaliana mutants carrying a putative null allele (pitp7-1) and a weak allele (pitp7-2), respectively. PITP7 transcripts were undetectable in pitp7-1 and less abundant in pitp7-2 than in the wild-type (WT). The severity of mutant phenotypes, such as plant developmental abnormalities, levels of plastoquinone-9 (PQ-9) and chlorophylls, photosynthetic protein complexes, and photosynthetic performance, were well related to PITP7 transcript levels. The pitp7-1 mutation was seedling lethal and was associated with significantly lower levels of PQ-9 and major photosynthetic proteins. pitp7-2 plants showed greater susceptibility to high-intensity light stress than the WT, attributable to defects in nonphotochemical quenching and photosynthetic electron transport. PITP7 is specifically bound to phosphatidylinositol phosphates (PIPs) in lipid-binding assays in vitro, and the point mutations R82, H125, E162, or K233 reduced the binding affinity of PITP7 to PIPs. Further, constitutive expression of PITP7H125Q or PITP7E162K in pitp7-1 homozygous plants restored autotrophic growth in soil but without fully complementing the mutant phenotypes. Consistent with a previous study, our results demonstrate that PITP7 is essential for plant development, particularly the accumulation of PQ-9 and photosynthetic complexes. We propose a possible role for PITP7 in membrane trafficking of hydrophobic ligands such as PQ-9 and carotenoids through chloroplast vesicle formation or direct binding involving PIPs.

1 | INTRODUCTION

Phosphatidylinositol (PtdIns) and its phosphorylated derivatives, phosphoinositides (PIPs), though comprising only a small percentage of...
eukaryotic cell membranes, play crucial roles in diverse intracellular processes such as signal transduction and regulation of membrane trafficking (Bankaitis et al., 2010; Bella, 2013; Heilman, 2016). Although the physiological functions of PIPs remain incompletely understood, previous studies have shown that PtdIns transfer proteins (PITPs) play important roles as signal integrators in PIPs metabolism (Ghosh & Bankaitis, 2011; Grabon et al., 2015; Wiedemann & Cockcroft, 1998). PITPs are highly conserved and present in all eukaryotic cells, and they fall into two distinct groups, namely Sec14-like PITPs and START (steroidogenic acute response related transfer domain)-like PITPs. The Sec14 domain (CRAL_TRIO, cellular retinaldehyde binding-triple response) is an ancient and versatile protein structural fold. Fungal and plant PITPs are typically Sec14-like and they are also found in metazoan. Mammalian Sec14-like PITPs have conserved inositol-lipid binding capacities and bind hydrophobic ligands such as phosphatidylcholine (PtdCho), sterols, or α-tocopherols in a hydrophobic cavity, indicating their diverse cellular functions (Kono et al., 2013; Saito et al., 2007). The roles and mechanism of Sec14-like PITPs have been studied mostly in budding yeast (Saccharomyces cerevisiae); Sec14p (Sec14 protein) was identified as the prototypical PITP and is a peripheral membrane protein of the Golgi apparatus that functions in trafficking within the trans-Golgi network (TGN) (Bankaitis et al., 1989). PITPs are around 35 kD and facilitate the transfer of either PtdIns or PtdCho between membrane bilayers in the cell (Cleves et al., 1991; Wirtz, 1991). Sec14p serves as a molecular sensor to regulate levels of PtdIns, PtdCho, and diacylglycerol in the Golgi apparatus (Kearns et al., 1998; Skinner et al., 1995).

Plant genomes encode three types of Sec14-like PITPs, classified according to domain arrangement (Huang et al., 2016; Mousey et al., 2007). The Arabidopsis (Arabidopsis thaliana) genome encodes 32 Sec14-like proteins, of which 14 belong to the Sec14-nodulin group with two functional domains (Sec14 Homolog 1 [SFH1] to SFH14), six are Sec14-Gold proteins (PATTLIN 1 [PATT1] to PATT6), and 12 are single-domain Sec14 proteins (PITP1 to PITP12) (Huang et al., 2016). Similarly, the rice (Oryza sativa) genome harbors 27 SEC14-like genes (Huang et al., 2013). The great number and diversity in domain organization of plant Sec14-like proteins suggest that they have diverse functions in growth or development; however, only a few have been functionally characterized. Members of the Sec14-nodulin protein family generally play critical roles in regulating extreme modes of polarized membrane growth in land plants (Vincent et al., 2005). The PITPs can of WORMS1 (COW1, also named SFH1) and Sec14-nodulin domain-containing protein (OxsNPD1) contribute to root hair growth and elongation in Arabidopsis (Böhme et al., 2004) and rice (Huang et al., 2013), respectively. Sec14-GOLD patellins are expressed in many tissues during plant development and are regulated by plant hormones and responsive to abiotic stresses (Montag et al., 2020; Zhou et al., 2019). In dividing cells, AtPATLs are localized to the cell plate, suggesting a role during cytokinesis, and are redundantly required for auxin-mediated root development (Tejos et al., 2018).

The single-domain Sec14-like proteins AtPITP11 and soybean (Glycine max) Sec14p homolog protein 1 (Ssh1p) and Ssh2p were identified from their ability to rescue the yeast sec14 mutant (Kearns et al., 1998). Ssh1p exhibits a binding preference for PtdIns(3,5)P₂, whose biosynthesis is strongly enhanced under hyperosmotic stress in both yeast and plants (Dove et al., 1997). Functions of single-domain Sec14-like proteins have been explored in barley (Hordeum vulgare), maize (Zea mays), Arabidopsis, and other species. barley Sec14 (HvSec14p) is highly expressed at specific developmental stages and in drought-tolerant barley genotypes, suggesting that HvSec14p has important roles in membrane biogenesis in expanding cells (Kießbickowicz-Matuk et al., 2016). Similarly, maize ZmSEC14p was isolated from cold-tolerant cultivars and encodes a nuclear-localized protein. Heterologous expression of ZmSEC14p in Arabidopsis increases germination rate, root length, and plant survival rate and reduces the production of reactive oxygen species (ROS) such as superoxide and hydrogen peroxide when plants are exposed to cold treatment (Wang et al., 2016). Recently, Hertle et al. (2020) reported that the Arabidopsis chloroplast-localized Sec14-like protein CPSF1 (which is the same protein as PITP7) is required for autotrophic growth and vesicle formation at the chloroplast inner membrane. Similarly, the cpsf1 mutant of Chlamydomonas (Chlamydomonas reinhardtii) displays reduced carotenoid content, abnormal chloroplast development, susceptibility to high light, and failure to maintain a stable organization of photosystem II (PSII) complexes when grown in the dark (Garcia-Cerdan et al., 2020).

Thylakoid membrane biogenesis is a determinant process of chloroplast biogenesis; however, its exact mechanisms remain incompletely understood, even though these membranes' structure and function are well known (Wietrzynski & Engel, 2021). Thylakoid membrane formation and function require coordinated biosynthesis of lipids, proteins, pigments, and cofactors (Kelly & Dormann, 2004; Vothknecht & Westhoff, 2001). Two main routes have been postulated for the deposition of these components from the envelope to the thylakoids: (1) lipids are produced at the envelope membrane, transported in vesicles, and then transferred into the thylakoid membrane (Andersson et al., 2001; Garcia et al., 2010); or (2) a continuum exists between the inner envelope and the developing internal membrane in the undifferentiated plastid (Charuvi et al., 2012; Morre et al., 1991). Direct contact between the envelope membrane and stroma lamellae of the thylakoid membrane has been observed only in young, undifferentiated proplastids of lettuce (Lactuca sativa) plants (Shimoni et al., 2005). However, rare observation of mature chloroplasts of pea (Pisum sativum) found invagination of the inner envelope toward the thylakoid membrane (Morre et al., 1991). Several proteins putatively linked to vesicle transport and/or thylakoid formation, such as thylakoid formation 1 (THF1; At2g20890), snowy cotyledon 2 (SCO2; At3g19920), and vesicle-inducing protein in plastids 1 (VIPP1; At1G20890), have been reported (Lindquist & Andersson, 2018). Light harvesting chlorophyll a/b-binding (LHCB) proteins are proposed as a possible cargo in vesicles (Khan et al., 2013; Tanz et al., 2012) and reported to interact with THF1 and SCO2, both involved in vesicle formation. VIPP1 is suggested to mediate lipid transport between the envelope and thylakoids (Kroll et al., 2001; Vothknecht et al., 2012). In Chlamydomonas, VIPP1 binds strongly to liposomes containing PtdIns(4)P, and VIPP rods engulf
membranes containing PtdIns(4)P (Theis et al., 2019). The pitp1 Arabidopsis mutant displays impaired thylakoid biogenesis, fewer vesicles, and a disturbed photosynthetic electron transport chain compared with the wild-type (WT).

In this study, we investigated the function of the chloroplast-localized Sec14-like PITP7 protein encoded by At5g63060 in Arabidopsis using three T-DNA insertion alleles at the PITP7 locus. Hertle et al. (2020) previously reported this protein as CPSFL1, but we refer to it as PITP7 according to Huang et al. (2016). Our data demonstrate that PITP7 is necessary for autotrophic growth, chloroplast development, and thylakoid ultrastructure, as well as for the biosynthesis of photosynthetic membrane proteins, consistent with previous observations (Hertle et al., 2020). We showed that a PITP7-deficient mutant displays defects in electron transport in PSII and formation of PSII supercomplexes, reduced plastoquinone-9 (PQ-9) content, and altered lipid composition. We also identified the amino acid residues involved in the specific in vitro interaction between PITP7 and phosphatidylinositol phosphates (PIPs).

2 | MATERIALS AND METHODS

2.1 | Plant materials, growth conditions, and transformation

The T-DNA insertion mutants pitp7-1 (SALK_116723), pitp7-2 (SALK_023278C), and pitp7-3 (SALK_023278C) were obtained from the Arabidopsis Biological Resource Center (ABRC). Arabidopsis accession Columbia-0 (Col-0) was grown in soil or on agar plates containing half-strength Murashige and Skoog (MS) medium supplemented with 1% (w/v) sucrose, when indicated, under a 16-h-light/8-h-dark photoperiod with 100 μmol m−2 s−1 light intensity provided by fluorescent light bulbs at 22°C. Transgenic plants were generated using Agrobacterium (Agrobacterium tumefaciens)-mediated transformation. T-DNA insertion sites in the mutants were confirmed by sequencing the T-DNA junction using primers specific to PITP7 and to the left border of the T-DNA. Genomic DNA was extracted from Arabidopsis leaves and used as a template for PCR genotyping. Primers used in this study are listed in Table S1.

2.2 | Measurement of transcript levels

Transcript levels were determined by reverse-transcription quantitative PCR (RT-qPCR). Total RNA was extracted from various tissues using an RNAeasy Plant total RNA isolation Kit (QiaGen) and pretreated with RNase-free DNase I (Takara) to remove traces of contaminating genomic DNA. First-strand cDNA synthesis was conducted using RNA to cDNA EcoDry™ premix (Clontech) following the manufacturer’s instructions. RT-qPCR reactions were performed using a StepOnePlus Real-time PCR system (Applied Biosystems) and TB Green Premix Ex Taq II (Tli RNaseH Plus, Takara). The three-step thermal cycling profile was performed following the manufacturer’s instructions. Reactions were performed in biological triplicates using different RNA samples extracted from three independent plants. Relative transcript levels were evaluated against those of ACTIN2 as a reference gene. The comparative ΔΔCt method was used to evaluate the relative levels of individual amplified products in the samples according to Livak and Schmittgen (2001). Primers used in this study are listed in Table S1.

2.3 | Complementation of the Arabidopsis pitp7-1 mutant

The full-length PITP7 coding sequence, flanked by EcoRV and BamHI restriction sites, was amplified from cDNA prepared from Arabidopsis leaves using KOD+ Hot Start DNA Polymerase (Novagen). The amplified PITP7 sequence was cloned in-frame with either the superfolder green fluorescent protein (sGFP) gene in vector p326sGFP (PITP7-GFP) or the sequence of the HA tag in vector p326HA (PITP7-HA) under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The entire expression cassette was excised by restriction digestion with EcoRI and cloned into the plant transformation vector pCAMBIA3300. Site-directed mutagenesis was performed according to the protocol from the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) using the PITP7-sGFP construct as template and specific primers (Table S1); mutations (PITP7H125Q and PITP7E162K) were verified by sequencing. The entire cassette was excised by restriction digestion with EcoRI and cloned into the plant transformation vector pCAMBIA3300. The resulting constructs (35S::PITP7-sGFP, 35S::PITP7-HA, 35S::PITP7H125Q-sGFP, and 35S::PITP7E162K-sGFP) were introduced into Agrobacterium strain GV3101 using the freeze–thaw method. Heterozygous pitp7-1/PITP7 plants were transformed with Agrobacterium as described in Section 2.1 using the floral dip transformation method (Clough & Bent, 1998) and selected for resistance to 0.3% (v/v) BASTA herbicide (Bayer). Genomic DNA was extracted from the leaves of all BASTA-resistant plants and used to identify homozygous pitp7-1 plants.

2.4 | GUS staining

A promoter fragment of approximately 2.1 kb upstream of the transcription start site of the PITP7 gene was amplified by PCR using genomic DNA as template and the appropriate primer pair (Table S1). The resulting PCR product was cloned into pENTR/D-TOPO (Invitrogen) and then transferred into the pMDC163 destination vector using the LR reaction, resulting in PITP7:: B-GLUCURONIDASE (GUS) (Curtis & Grossniklaus, 2003). This construct was transformed into Col-0 plants via Agrobacterium-mediated transformation. GUS staining of harvested fresh tissues was performed using a GUS Reporter gene staining kit (Sigma–Aldrich), with 0.1% (w/v) Triton X-100 added to the staining solution. Plant samples were incubated at 37°C for 18–24 h and then incubated in a graded ethanol series from 35% (v/v) to 70% (v/v) to remove chlorophylls.
2.5 | Subcellular localization

For transient transfection assays, approximately 1.5 × 10⁶ protoplasts (prepared from Arabidopsis mesophyll cells) were transfected with 20 μg p326sPITP7-GFP construct. The transfected protoplasts were then incubated at 22°C in the dark (Jin et al., 2001). Fluorescence images of the protoplasts were acquired with a fluorescence microscope (Axioplan 2; Carl Zeiss) equipped with a 40×/0.75 objective (Plan-Neofluar) and a cooled charge-coupled device camera (Senicam; PCO Imaging). XF116 filter sets for sgFP were used (exciter, 474AF20; dichroic, 500DRLP; emitter, 510AF23; Omega Inc.).

2.6 | Measurement of photosynthetic parameters

A portable FluorPen FP 100 chlorophyll fluorometer (Photon System Instrument) was used to measure photosynthetic parameters. Arabidopsis leaves were dark-adapted for 10 min before measurement. The maximal photochemical activity of PSII (Fm/Fo) was calculated as (Fm − Fo)/Fo, where Fo is the initial chlorophyll fluorescence level and Fm is the maximal fluorescence level, determined using an intense pulse of white light (3000 μmol m⁻² s⁻¹). Stress-dependent reduction in Fv/Fm values was interpreted as PSII photoinhibition. Measurements of OJIP were used to denote the flow of energy through PSII (Stirbet & Govindjee, 2011). Minimum fluorescence was measured at 50 μs when all PSII reaction centers were open and was defined as the O step, followed by the J step (at 2 ms) and the I step (at 60 ms); maximum fluorescence (Fm) was measured when all PSII reaction centers were closed, known as the P step. Leaves were infiltrated with 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) or water, and excess solution was removed by air drying at room temperature before use.

2.7 | Analysis of prenyl lipids

To analyze plastoquinone (PQ-9), plastochromanol-8 (PC-8), tocopherols, carotenoids, and chlorophylls, total lipids were extracted and analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu LC-20AD chromatograph, as previously described (Kim et al., 2015). Chromatography was conducted at 30°C on a C18 reverse-phase column (5 μm Supelco Discovery C18 column, 250 × 4.6 mm). Frozen tissues were ground in liquid nitrogen, and their total lipids were extracted using cold ethyl acetate. After centrifugation for 10 min at 13,000 g and 4°C, the supernatant was transferred to a new tube and evaporated under nitrogen flow. The extract was dissolved in 95% (v/v) ethanol. PQ-9, PC-8, and tocopherols were analyzed using an isocratic solvent system comprising methanol: hexane (9:1, v/v) at a flow rate of 1.0 ml min⁻¹. PQ-9 was detected by absorption at 255 nm, while PC-8 and tocopherols were detected fluorometrically (290 nm excitation and 330 nm emission). The compounds were quantified against their respective external calibration standards, and data were corrected by comparison with the recovery rate of rac-Tocol (Matreya) as an internal standard. Chromatographic conditions for analysis of carotenoids and chlorophylls were as above, using solvent A (acetonitrile: water = 9:1, v/v, with 0.1% trimethylamine, v/v) and solvent B (ethyl acetate), with the following gradient: 0–5 min, 0%–33.3% B; 5–33 min, 33.3%–66.7% B; 33–33.5 min, 66.7% to 100% B; 33.5–38 min, 100% B; 38–38.5 min, 0% B; 38.5–43 min, 0% B. HPLC peak areas at 440 nm were integrated to determine the abundance of each compound.

2.8 | Analysis of chloroplast ultrastructure by transmission electron microscopy (TEM)

Leaf fragments from 3-week-old Col-0 and pitp7-1 plants grown on MS medium supplemented with 1% (w/v) sucrose were fixed in 4% (v/v) formaldehyde and 2.5% (v/v) glutaraldehyde in Kamovsky’s fixative overnight at 4°C, washed three times for 10 min each in cacodylate buffer, pH 7.2, and fixed in 1% (v/v) OsO₄ for 2 h at 4°C. After washing twice for 10 min each in water, samples were incubated in 0.5% aqueous uranyl acetate overnight at 4°C. Samples were dehydrated in a graded ethanol series, washed twice in propylene oxide, and then infiltrated in a 1:1 mixture of propylene oxide and Spurr’s resin. Samples were then embedded in Spurr’s resin overnight, placed in fresh resin, and incubated overnight at 70°C. Ultrathin sections (100 nm) were examined using a LEO 912AB transmission electron microscope (Carl Zeiss) at 100 kV.

2.9 | Isolation of intact chloroplasts and differential isolation of stroma and thylakoid membranes

Arabidopsis chloroplasts were isolated by the direct homogenization method as described previously (Aronsson & Jarvis, 2002; Bruce et al., 1994) with the following modifications. Approximately 20 g of aerial parts was homogenized in 400 ml blending buffer (50 mM HEPES-KOH, pH 7.8, 330 mM sorbitol, 2 mM EDTA, 1 mM MnCl₂, 1 mM MgCl₂, and 50 mM sodium ascorbate [freshly added as powder]) with or without 5 mg ml⁻¹ protease inhibitor cocktail for plant extracts (Sigma-Aldrich) in five 2-s pulses in a kitchen blender equipped with disposable razor blades. The homogenate was filtered through four layers of Miracloth (Calbiochem) and then centrifuged at 4000g for 3 min at 4°C. The crude chloroplast pellet was resuspended in HS buffer (50 mM HEPES-KOH, pH 7.8, 330 mM sorbitol) and overlaid onto a 30% (v/v) Percoll solution in HS buffer. After centrifugation at 1350g for 15 min at 4°C in a swing-bucket rotor, the pellet was washed twice with HS buffer. Purified intact chloroplasts were kept on ice in the dark and used within 3 h. The chlorophyll concentration in intact chloroplasts was calculated from chlorophyll extracts in 80% acetone according to Porra et al. (1989). Stroma and thylakoid membranes of intact chloroplasts were isolated using sucrose gradient ultracentrifugation. Pellets of intact chloroplasts containing 1 mg of chlorophyll were resuspended in 1 ml chloroplast lysis (CL) buffer containing 50 mM HEPES (pH 7.5) and 2 mM MgCl₂ and incubated on ice.
for 1 min. A two-step sucrose gradient (0.9 M and 0.6 M) was prepared in Cl buffer, and the lysate was gently transferred to the top of the sucrose gradient and then centrifuged at 100,000g for 1 h at 4°C (Beckman SW32-Ti rotor). After centrifugation, stroma proteins at the top of the 0.6 M sucrose layer and thylakoid membranes as a pellet were collected for experiments.

2.10 | Isolation of thylakoid membranes and blue native (BN)-PAGE

Isolation of thylakoid membranes and blue native (BN)-PAGE were performed according to Järvi et al. (2011) with minor modifications for native gels. Chlorophyll concentration in isolated thylakoids was calculated from chlorophyll extracts in 80% (v/v) acetone according to Porra et al. (1989). Two different methods were applied for isolating different thylakoid membrane protein complexes. First, to separate thylakoid membrane protein complexes from grana stacks, an aliquot of thylakoid membranes equivalent to 15 μg chlorophyll was solubilized in 1% (w/v) n-dodecyl-β-D-maltoside (DM) for 5 min at 4°C with gentle agitation. Samples were then centrifuged at 18,000 g for 20 min at 4°C, and solubilized (supernatant) fractions were loaded onto NativePAGE™ 4–16% Bis-Tris Gel of 1.0 mm thickness (Invitrogen). Similarly, to separate thylakoid membrane protein complexes from stroma lamellae, thylakoid membranes were solubilized in 1% digitonin for 5 min at 20°C before the solubilized fraction was loaded onto a manually prepared 5%–13.5% gradient BN gel.

2.11 | SDS-PAGE and immunoblot analysis

For immunological localization of PITP7-HA in chloroplast subfractions from 355:PITP7-HA, pellets containing chloroplasts, thylakoids, and stroma regions, were resuspended in lysis buffer (10 mM Tris–HCl, pH 7.5, 1% (w/v) SDS and 1 mM EDTA) for 30 min at 4°C. After centrifugation at 11,400 g for 10 min at 4°C, the supernatant was removed and its protein concentration was determined using a Qubit Protein Assay Kit.

2.12 | Thin-layer chromatography and fatty acid composition analysis

Lipid extraction and thin-layer chromatography (TLC) were performed according to the method described by Wang and Benning (2011). Total lipids were extracted in chloroform:methanol (2:1, v/v) from 30 mg of Arabidopsis leaf tissue ground in liquid nitrogen. Total lipids from leaf tissue were spotted onto silica gel G60 plates (Merck Millipore), submerged in 0.15 M ammonium sulfate, dried for 2 days, baked at 120°C for 2.5 h, and separated using acetonetoctane:water (91:30:7.5, v/v/v) in a TLC developing tank in a fume hood for 50 min at room temperature. The silica gel plate was sprayed with 0.1% (w/v) primuline (Sigma) in 80% (v/v) acetone, and lipid spots were visualized under an ultraviolet transilluminator. Spots corresponding to monogalactosyldiacylglycerol (MGD), phosphatidyglycerol (PtdGro), digalactosyldiacylglycerol (DGDG), PtdCho, and phosphatidylethanolamine (PtdEtn) were scraped off the TLC plate using a razor blade and subjected to fatty acid methyl ester (FAME) analysis. Scraped silica gel samples containing lipid spots were transmethylated at 85°C for 60 min in 1 ml of 5% (v/v) H2SO4 in methanol containing pentadecanoic acid (100 μg ml−1) as an internal standard. After transmethylation, 1.0 ml of 0.9% (w/v) NaCl solution was added to the sample and the FAMEs were extracted three times with 1.0 ml n-hexane. Concentrated FAMEs were analyzed using a GC-2010 plus (Shimadzu) gas chromatograph with flame ionization detector and a 30 m × 0.25 mm (inner diameter) HP-FFAP column (Agilent) while increasing the oven temperature from 190°C to 230°C at 3°C min−1. Ultrapure nitrogen gas was used as the carrier gas with 150 kPa pressure and a split ratio of 5:1.

2.13 | Recombinant PITP7 protein production and purification

The sequence encoding an N-terminally truncated PITP7 lacking the chloroplast-targeting peptide was amplified by PCR using primers pitp7-2-F and pitp7-2-R. The resulting PCR amplicon was cloned into the pCR-Blunt vector (Invitrogen) using T4 DNA ligase and sequenced. Point mutants were generated according to the protocol from the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies) using PITP7 cDNA in pCR-Blunt vector as template. Primers and mutation sites are listed in Table S1. After digestion with the restriction enzymes NdeI and BamHI, the insert was ligated into the pET27b+ vector (Novagen), placing the PITP7 coding sequence in-frame with the sequence of the His tag. Production of recombinant protein in Escherichia coli strain BL21(DE3) cells was induced by the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside when cultures reached an OD600 of 0.6 at 28°C, followed by overnight incubation. Induced cell pellets were stored at −70°C until use. The His-tagged proteins produced were purified under native conditions using Proteo Ni-TED 1000 Packed Columns (Macherey-Nagel) according to the manufacturer’s protocol. Samples were dialyzed into TBS buffer using Pierce protein concentrator PIS (Thermo Scientific). Protein concentration was determined using a Qubit Protein Assay Kit.
(Life Technologies). Fusion proteins were analyzed for purity using SDS-PAGE followed by Coomassie Brilliant Blue staining and stored at −20°C in 50% (v/v) glycerol.

2.14 Lipid–protein interaction assays

To determine the specific lipid binding of recombinant purified PITP7-His proteins lacking the chloroplast transit peptide, in vitro lipid–protein interaction assays were performed. PIP strips (P-6001) and membrane lipid strips (P-6002) were purchased from Echelon Biosciences. The strips were first blocked with 3% (w/v) fatty acid-free BSA in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% [v/v] Tween 20) for 3 h and then incubated overnight at 4°C in blocking buffer containing 0.5 μg ml⁻¹ recombinant PITP7-His protein. Blots were washed three times with 1× TBST, followed by incubation with an anti-His mouse monoclonal antibody (Abcam) for PITP7-His in 1× TBST at a 1:2000 dilution for 1 h at room temperature. After washing with 1× TBST, membranes were incubated with a peroxidase-conjugated anti-mouse antibody (Thermo Fisher Scientific) at a 1:20,000 dilution in 1× TBST for 1 h at room temperature. After washing with 1× TBST three times, chemiluminescence was detected using a camera-based gel documentation system (Vilber fusion SL).

2.15 Phylogenetic analysis of SEC14-like proteins

Deduced protein sequences of the Arabidopsis Sec14-like protein family and other previously reported plant Sec14-like proteins were aligned using the ClustalW utility included in MEGA6.0 (Tamura et al., 2013). The corresponding phylogenetic tree was generated using the maximum-likelihood method with 1000 bootstraps using MEGA6.0.

3 RESULTS

3.1 PITP7 is a member of the Sec14-like protein family

Arabidopsis PITP7 encodes an N-terminal chloroplast transit peptide (amino acids 1–31), a CRAL/TRIO N-terminal domain (amino acids 46–112), and a conserved Sec14-like domain (amino acids 114–261, CRAL-TRIO domain). We reconstructed a phylogenetic tree using the sequences of all 17 Arabidopsis Sec14-like proteins as well as Sec14-like proteins from other species (Figure S1). As previously described (Huang et al., 2016), we determined that Arabidopsis Sec14-like proteins are distributed into five groups. Sec14-nodulins (Group I) and patellin Sec14 domain proteins (Group II) are each forming their own clade. The stand-alone Sec14 proteins forming Groups III–V are more diverse. Therefore, the topology of the phylogenetic tree indicates the functional and evolutionary divergence of PITP proteins in Arabidopsis. PITP7 belongs to group III and is closely related to PITP5 and PITP6. PITP7 is the only member of the Arabidopsis PITP family predicted to be targeted to the chloroplast.

The conserved residues associated with PtdIns binding (the PtdIns barcode) are conserved among several Arabidopsis PITP proteins (PITP1, PITP3, PITP4, PITP11, and PITP12), whereas the PtdCho-binding barcode is not found among PITP proteins (Huang et al., 2016). Alignment of the PITP7 protein sequence with the sequences of other PITP proteins revealed that residues R65, T236, and K239 from the PtdIns barcode are not conserved in PITP7 (Figure S2); residue 65 is a W instead of an R, and residues 236 and 239 are replaced by A and V, respectively. However, the residues from the core G-module (TDKGDR), which is central to controlling Sec14 conformational transitions (Huang et al., 2016), are conserved in PITP7 as the motif LDVKGR. Therefore, this analysis suggests that PITP7 might have a ligand other than PtdCho or PtdIns.

3.2 Identification of three PITP7 T-DNA insertion alleles

To determine the molecular function of PITP7, we identified three T-DNA insertion mutants in the Col-0 background: pitp7-1 (SALK_116723), pitp7-2 (SALK_023278C), and pitp7-3 (SALK_023278C) (Figure 1A). We confirmed the T-DNA insertion sites by sequencing the T-DNA junctions using primers specific to PITP7 and to the left border of the T-DNA. The T-DNA was inserted into the first exon in pitp7-1, the sixth exon in pitp7-2, and the promoter region in pitp7-3 (Figure 1A,B). The pitp7-1 allele was seedling lethal to plants grown in soil, but the plants survived in MS medium supplemented with 1% (w/v) sucrose. We isolated homozygous lines for each mutant grown in MS medium supplemented with 1% (w/v) sucrose (Figure 1C). RT-qPCR analysis of the WT and homozygous pitp7 mutants showed that PITP7 transcripts were undetectable in pitp7-1 and less abundant in pitp7-2 than in the WT; however, we observed no difference in pitp7-3 relative to the WT (Figure 1D). pitp7-1 seedlings displayed severe growth and developmental defects, eventually dying even on MS medium supplemented with sucrose (Figure 1B). pitp7-2 mutant plants were substantially smaller than WT plants but able to grow on MS medium lacking sucrose and in soil, producing seeds. The pitp7-3 T-DNA insertion line did not show any differences in growth or development compared with the WT, indicating that a normal PITP7 transcript level is required for plant growth and development. The quantum yield of PSII (Fv/Fm) was also lower in pitp7-1 and pitp7-2 plants than in WT plants grown on MS medium supplemented with 1% (w/v) sucrose under normal growth conditions (Figure 1E). These results demonstrated that PITP7 is essential for autotrophic plant growth and development.

To confirm that the pitp7-1 null mutation is responsible for the observed seedling-lethal phenotype, we introduced a construct consisting of the PITP7 cDNA cloned in-frame with the coding sequence of either the superfolder green fluorescence protein (sGFP) or the
three results indicate that the pitp7-1 mutation causes the seedling-lethal phenotype and that PITP7 is required for autotrophic growth.

### 3.3  | PITP7 is expressed in various tissues

To investigate the expression pattern of PITP7 in various tissues, we analyzed PITP7 transcript levels using RT-qPCR. PITP7 was expressed in various tissues in WT adult plants, including leaves, stems, flowers, roots, and siliques (Figure 2A). We also generated transgenic lines in the WT background expressing the GUS reporter gene under the control of a PITP7 promoter fragment. High PITP7 transcriptional activity was visualized in leaves, pistils, filaments, flower buds, roots, and siliques but not in petals, anthers, or developing seeds (Figure 2B).

### 3.4  | PITP7 localizes to the chloroplast

To determine the localization of PITP7, we transiently transfected Arabidopsis mesophyll protoplasts with a 35S::PITP7-sGFP construct. Results showed an overlap between the green fluorescence signal of the sGFP-tagged PITP7 protein and chlorophyll autofluorescence, indicating that PITP7-sGFP is localized in the chloroplast (Figure 2C). However, GFP and chlorophyll fluorescence did not entirely overlap, indicating that PITP7 localizes to thylakoids as well as stroma. We then used subcellular fractionation to refine the location of PITP7 within chloroplasts, using chloroplasts prepared from pitp7-1 + 35S::PITP7-HA transgenic leaves and subjected to fractionation into thylakoids and stroma. Immunoblotting of fractionated chloroplasts with anti-HA antibody showed that PITP7 accumulates in intact chloroplasts, thylakoids, and stroma (Figure 2D). In a previous study, CPSFL1-FLAG was localized in the plastid stroma exclusively, with only traces within the envelope and thylakoid fractions (Hertle et al., 2020). We performed SDS-PAGE using ~10 μg proteins extracted from chloroplasts, thylakoids, and stroma. Since Rubisco is the most abundant protein in the stroma, it is likely that PITP7 is present in smaller amounts in the stroma fraction than in the thylakoid fraction. Since pitp7-1 + 35S::PITP7-HA lines are strong overexpressors (40-fold greater expression than the WT), the consequent excess of PITP7, which has neither a hydrophobic patch nor a transmembrane domain, is expected to accumulate mainly in the stroma, in line with previous results (Hertle et al., 2020). To investigate the nature of the association between PITP7 and the thylakoid, we washed thylakoids with various buffers. PITP7 was largely removed from thylakoid membranes by 0.1 M NaOH and 1% (v/v) Triton X-100 and was partially washed out with 0.1 M Na2CO3 (Figure 2E). However, treating thylakoids with 1 M NaCl or resuspension buffer alone did not remove PITP7 from thylakoid membranes. This result indicated that PITP7 is peripherally associated with thylakoids.

### 3.5  | The pitp7 mutant shows reduced prenyl lipid accumulation

Since the pitp7 mutants exhibited a lower quantum yield of PSII, we measured the levels of PQ-9, tocopherols, carotenoids, and...
Accumulation and localization of PITP7 in Arabidopsis.

The amount of PQ-9 was reduced to 13.6%, 36.2%, and 87% of WT levels in the pitp7-1, pitp7-2, and pitp7-3 mutants, respectively. Chlorophyll in the leaves of 8-day-old WT and pitp7 mutant seedlings. The amount of PQ-9 was reduced to 13.6%, 36.2%, and 87% of WT levels in the pitp7-1, pitp7-2, and pitp7-3 mutants, respectively.

FIGURE 2 Accumulation and localization of PITP7 in Arabidopsis. (A) Relative PITP7 transcript levels in various tissues of 5-week-old Col-0 plants, as determined by RT-qPCR. (B) GUS staining patterns in PITP7pro::GUS transgenic seedlings and different tissues of plants. Scale bars = 2 mm. (C) Subcellular localization of PITP7-GFP in Arabidopsis mesophyll protoplasts transiently transfected with the 35S::PITP7-GFP construct. Chlorophyll autofluorescence is shown in red. Scale bar = 20 mm. (D) Immunoblot analysis of PITP7 localization in various chloroplast fractions. M, molecular marker; IC, intact chloroplasts; TM, thylakoids; ST, stroma. (E) PITP7 is peripherally associated with thylakoids. Thylakoids were washed in the indicated solutions. PITP7-HA was detected by immunoblotting with an anti-HA antibody. Similar results were obtained in two independent experiments. M, molecular marker; 1, 0.1 M Na2CO3; 2, 1 M NaCl; 3, 0.1 M NaOH; 4, 1% (v/v) Triton X-100; 5, resuspension buffer; P, pellet; and S, soluble fraction.

FIGURE 3 Quantification of plastoquinone-9, tocopherols, carotenoids, and chlorophylls and expression of their biosynthetic genes in the WT and pitp7-1 mutants. (A–D) Reverse-phase high-performance liquid chromatography (HPLC) was used to analyze prenyl lipids and pigments in leaves of 8-day-old WT and pitp7-1 seedlings grown on half-strength MS medium supplemented with 1% (w/v) sucrose. (A) PQ-9 and PC-8 levels. (B) Individual (γ-TC and α-TC) and total tocopherol (total) levels. (C) Carotenoid levels. Neo, neoxanthin; Vio, violaxanthin; Ant, antheraxanthin; Lut, lutein; β-car, β-carotene; total, total carotenoids. (D) Chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total chlorophyll (total) contents. Data are means ± SD (n = 5 biological replicates, each 50 mg of fresh weight leaf tissues were pooled from multiple WT plants and 20 pitp7-1 mutants). (E, F) RT-qPCR analysis of relative transcript levels of prenyl lipid and pigment biosynthetic genes. SPS1-2, solanesyl diphasate synthase 1 and 2; HST, homogentisic acid solanesyl diphosphate synthase; FBNS, fibrillin 5; VTE1, tocopherol cyclase 1; VTE2, HGA phytol transferase; VTE3, 1-tocopherol methyl transferase; VTE4, 2-methyl-6-phytlybenzoquinone methyl transferase; HPPDAE, P-hydroxyphenylpyruvate dioxygenase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, beta-carotene desaturase; CRTISO, carotene isomerase; LBC, lycopene beta-cyclohexanoid; LEB, lycopene epsilon-cyclohexanoid; BHY1, beta carotene hydroxylase 1; LUT1, cytochrome p450-type beta-hydroxylase; CAO, chlorophyll a oxygenase; CHLG, chlorophyll synthase. Asterisks represent a significant difference relative to the WT by Student’s t test: *p < 0.05, **p < 0.01, and ***p < 0.001. Data are means ± SD (n = 3–4 technical replicates, 300 mg of fresh leaf tissues were pooled from multiple WT plants and pitp7-1 mutants). N.D., not detected.
3.6 The pitp7-2 mutant is hypersensitive to high light exposure

pitp7-2 plants grown in soil showed a reduced $F_{v}/F_{m}$ ratio of between 0.25 and 0.78, depending on leaf age, in contrast to the stable ratio of $0.81 \pm 0.02$ observed in the WT. Lower $F_{v}/F_{m}$ values were recorded in older leaves, indicating that the loss of quantum efficiency with aging was faster in pitp7-2 plants than in the WT. In agreement, the average $F_{v}/F_{m}$ value in rapidly expanding young leaves detached from the upper part of pitp7-2 plants was high, with a range of 0.66–0.78. To determine whether loss of PITP7 function resulted in enhanced sensitivity to a sudden increase in light intensity, we exposed 4-week-old WT and pitp7-2 plants to high light (1000 μmol photons m$^{-2}$ s$^{-1}$) for 3 h, 6 h, 1 day, and 2 days and measured $F_{v}/F_{m}$ at each time point. pitp7-2 plants experienced a marked drop in $F_{v}/F_{m}$ after 3 h in high light, which further decreased before stabilizing after 24 h; meanwhile, the WT showed only subtle variation in $F_{v}/F_{m}$ (Figure 4A).

To evaluate whether pitp7-2 plants lack the capability to protect the photosynthetic machinery from photoinhibition, we measured non-photochemical quenching (NPQ) at each actinic light intensity level (Figure 4B). NPQ of the WT increased rapidly with increasing actinic light intensities (2.0 at 500 μmol m$^{-2}$ s$^{-1}$), whereas NPQ in pitp7-2 increased slowly with increasing actinic light intensities (0.5 at 500 μmol m$^{-2}$ s$^{-1}$). OJIP transients reflect the reduction of the photosynthetic electron transport chain. WT plants showed a typical OJIP curve, and $F_{m}$ reached its peak at around ~2 ms in the presence of DCMU (Figure 4C). In the presence of DCMU, $F_{o}$ was higher and $F_{m}$ was lower compared with those in untreated pitp7-2 leaves, suggesting that electron transport of pitp7-2 plants is inhibited or slowed down at the acceptor side of PSII (Q$_{A}$ re-oxidation) (Tóth et al., 2007). pitp7-1 plants were too small and delicate, even when grown on MS medium supplemented with sucrose, for us to measure these parameters in the strong mutant background. These results indicated that the higher photosensitivity of pitp7-2 compared with the WT can be ascribed to lower NPQ, possibly due to loss of the capacity for ΔpH-triggered conformational changes in thylakoids caused by slower electron transport on the acceptor side of PSII.

![Figure 4](https://physiologia-plantarum.com/) Photosynthetic properties of WT and pitp7-2 plants. (A) Maximum photosystem II quantum efficiency ($F_{v}/F_{m}$) measured in plants exposed to high light (1000 μmol photons m$^{-2}$ s$^{-1}$). (B) Nonphotochemical quenching (NPQ) and (C) OJIP fluorescence transients in the WT and pitp7-2 in the presence (DCMU) or absence (control) of 10 μM DCMU. Data are means ± SD ($n = 5$ biological replicates, each from individual WT plants and pitp7-2 mutants).
3.7 | pitp7 mutants show defective chloroplast structure and deficiency in photosynthetic complexes

To investigate whether loss of PITP7 causes any defects in chloroplast development, we determined chloroplast ultrastructure using transmission electron microscopy (TEM) in the WT and pitp7-1. Chloroplasts were irregular in shape in the pitp7-1 mutant compared with the WT. Several notable features in the structure of thylakoid membranes were observed in pitp7-1 chloroplasts, including a lower extent of grana stacking, poor interconnection of grana stacks by stroma lamellae, swollen thylakoids, and reduced relative volume of plastoglobules compared with the WT (Figure S4). These data indicate that PITP7 plays a critical role in the development of chloroplasts and thylakoid membranes, as revealed by previous studies (García-Cerdán et al., 2020; Hertle et al., 2020).

Given the structural changes in thylakoids caused by loss of PITP7, we analyzed the assembly of protein complexes in grana and stroma lamellae of thylakoids upon solubilization using DM or digitonin. The abundances of PSI-NADPH dehydrogenase megacomplexes, PSII-light-harvesting complex II (LHCII) supercomplexes, and PSI/PSII dimers were slightly reduced in pitp7-2 compared with those in the WT (Figure 5A). However, these complexes were drastically lacking in pitp7-1, along with PSII monomers, Cytb6f, and LHCII assembly (Figure 5A,B). The levels of LHCII trimers and monomers were comparable in the WT and pitp7-2, but were slightly decreased in pitp7-1 (Figure 5A,B). Since LHCII is critical for grana stacking, in which PSII-LHCII complexes are also involved (Wan et al., 2014), LHCII trimers and monomers in pitp7-1 may result in grana stacking to some extent.

3.8 | pitp7 mutants exhibit lower abundance of thylakoid proteins

In light of the lower abundance of photosynthetic protein complexes and the lower photochemical efficiency of PSII (Fv/Fm) in pitp7 mutants, we determined the levels of representative subunits of photosynthetic proteins by immunoblotting. Levels of D1 (PSII core protein), CP47 (PSII core antennae protein), PsAC (PSI core protein), and Cyt f (Cytb5f subunit) were significantly reduced in pitp7-1 compared with the WT (Figure 5C). However, pitp7-2 possessed substantial levels of these proteins in comparison with the WT. Interestingly, levels of Lhcb1 and PsbS in both pitp7-1 and pitp7-2 were similar to those in the WT. The unchanged Lhcb1 level in pitp7 mutants was consistent with the similar accumulation of LHCII trimers and monomers compared with the WT (Figure 5A,B). These data indicated that the significant reduction in thylakoid protein complexes in pitp7-1 resulted from the loss of major photosynthetic proteins. Thus, loss of PITP7 strongly suppresses the accumulation of major thylakoid proteins, resulting in fewer protein complexes and eventually leading to loss of the proper organization of thylakoid membranes and chloroplast ultrastructure, which is essential for proper photosynthetic activity (Poudyal et al., 2020).

3.9 | pitp7 mutants exhibit repression of chlorophyll biosynthetic genes

Photosynthetic pigments—chlorophylls and carotenoids, including xanthophylls—play an important role in plant growth and development. The pitp7-1 mutant showed a pale green phenotype and contained lower levels of total chlorophylls (Figures 1B and 3D). To test whether PITP7 affects the expression of genes involved in chlorophyll biosynthesis, transcript levels of these genes were measured using RT-qPCR. As shown in Figure 5D, CAO, HCAR (7-hydroxymethyl chlorophyll a reductase), HEMA1 (glutamyl-TRNA-reductase 1), HEMA2, NYC1 (non-yellow coloring 1), NOL (NYC1-like), PorA (protochlorophyllide oxidoreductase A), and PorB were expressed at lower levels in both pitp7-1 and pitp7-2 mutants compared with the WT. However, DVR (3,8-divinyl protochlorophyllide A 8-vinyl reductase) transcript levels increased twofold in pitp7-2 relative to the WT, but not in pitp7-1 (Figure 5D). These data indicated that pitp7 mutants (especially the pitp7-1 allele) substantially affect the expression levels of chlorophyll biosynthetic genes, resulting in lower chlorophyll a and b levels (Figure 3D).

3.10 | pitp7-2 accumulates more PtdGro containing 16:1Δ3trans fatty acid

To determine whether the fatty acid composition of leaf lipids changed in pitp7 mutants, we analyzed the lipid contents of WT and pitp7-2 leaves; the small size of pitp7-1 leaves precluded to obtain sufficient lipid leaf extracts for analysis. Accordingly, we first separated leaf lipids extracted from 30 mg of WT and pitp7-2 leaves using thin-layer chromatography (TLC), and then performed gas chromatographic determination of the fatty acid composition of each phospholipid spot (Figure 6). The major chloroplastic lipids consist of galactolipids, such as MGDG and DGDG, and PtdGro, the only chloroplast phospholipid. Specifically, PtdGro contains a 16:1Δ3trans (16:1Δ3t) fatty acid at the sn-2 position (Dubacq & Tremolieres, 1983). Trienoic acids such as 16:3 and 18:3 MGDG accounted for 86.2% of the total fatty acids in the WT. pitp7-2 showed a similar fatty acid profile for MGDG as the WT (Figure 6A), with a slight increase in the trienoic acid content of MGDG, reaching 89.7%, accompanied by a corresponding decrease in monoenoic acid and dienoic acid content. DGDG of the WT had a higher 16:0 content and a lower 16:3 content than MDGD from the WT. DGDG in pitp7-2 showed a slight decrease in 18:1 and 18:2 content compared with DGDG from the WT, but the 18:3 content of the mutant increased by 3.5% compared with that of DGDG from the WT (Figure 6B).

PtdGro was the lipid exhibiting the most dramatic differences in fatty acid composition between pitp7-2 and the WT. In the WT, 16:0 and 16:1Δ3t-PtdGro species contributed equally to total PtdGro levels, accounting for 24.3% and 22.5%, respectively; the pitp7-2 mutant accumulated less 16:0 (17.5% of total) and more 16:1Δ3t (30.8% of total). In addition, as observed with DGDG, the contents of
18:1 and 18:2 PtdGro decreased slightly in the mutant relative to the WT, while 18:3 contents increased slightly, likely to compensate for the decrease in 18:1 and 18:2 species (Figure 6C). The relative content of chloroplast lipids was also different in *pitp7-2* compared with the WT, as the 18:3 content was increased slightly and the content of 16:1Δ3t-PtdGro was substantially increased. By contrast, the relative content of saturated fatty acids in the extraplastidial lipids PtdEtn and PtdCho increased while the 18:1 and 18:2 contents decreased slightly compared with those of the WT (Figure 6D,E). However, we observed no significant differences in the contents of leaf lipids between the WT and *pitp7-2* (Figure 6F).

### 3.11 | Recombinant PITP7 specifically binds to PIPs

We next investigated the lipid-binding properties of recombinant PITP7 using an in vitro protein-lipid overlay assay. We produced PITP7 (lacking the chloroplast targeting sequence) fused with a C-terminal His tag in *E. coli*. We then tested binding the purified recombinant protein to various lipids using commercial lipid strips. PITP7-His strongly binds to PtdIns(3)P, PtdIns(4)P, PtdIns(5)P, cardiolipin, and sulfatide (3-sulfogalactosylceramide) (Figure 7A,C). PITP7-His did not bind to PtdEtn or PtdCho, as expected from our analysis of the
Fatty acid composition of total leaf lipids. Total lipids were extracted from leaf tissues of the WT and pitp7-2, and the fatty acid composition of (A) MGDG, (B) DGDG, (C) PtdGro, (D) PtdEtn, (E) PtdCho, and (F) leaf lipids was analyzed. Fatty acids representing less than 1% are not shown. Data are means ± SD (n = 4 biological replicates, each 200 mg of fresh leaf tissues were pooled from multiple WT plants and pitp7-2 mutants, and then freeze-dried. 30 mg of dry weight samples were used for experiments). Asterisks indicate statistical significance by Student’s t test (*p < 0.05, **p < 0.001).

3.12 Identification of PITP7 residues required for PIP binding

To further identify critical residues involved in the binding of PIPs by PITP7, we generated five versions of PITP7 with single-point mutations. Human α-tocopherol (α-Toc) transfer protein (ATTP) contains a Sec14-like domain that specifically binds to α-Toc and regulates the secretion of α-Toc from the liver (Traber & Arai, 1999). It is known that missense mutations in ATTP associated with the neurodegenerative disorder disease “ataxia with vitamin E deficiency” (AVED) prevent ATTP from interacting with PIPs, which regulate the structure of ATTP and promote the release of α-Toc (Kono et al., 2013; Lamprakis et al., 2015). The three-dimensional structures of PITP7...
and ATTP predicted using the Protein Model Portal ([https://proteinnmodelportal.org](https://proteinnmodelportal.org)) were similar (Figure S5). To find residues responsible for the interaction with the phosphate groups from PIPs, we aligned the protein sequences of ATTP and PITP7. There were conserved residues between PITP7 and ATTP, and the two proteins showed 24.2% identity and 53.9% similarity using LALIGN software (Figure S5). We generated point mutations of five PITP7 residues that were identical or similar to the following point mutations in ATTP related to AVED: R59W, H101Q, E141K, L183P, and R221W (Kono et al., 2013; Meier et al., 2003; Min et al., 2003). The corresponding residues in PITP7 were R82W, H125Q, E162K, L213P, and K233W. As assessed by lipid-binding assays (Figure 7B), recombinant PITP7 proteins harboring the R82W, H125Q, and K233W point mutations individually showed a reduced ability to bind PtdIns(4)P, with R82W producing the greatest decrease in binding ability. The E162K protein exhibited stronger binding to PtdOH, whereas the L213P protein bound to PtdIns(4)P, PtdIns(4,5)P2, PtdIns(3,4,5)P3, and PtdOH more strongly than intact recombinant PITP7. This result indicates that the R82, H125, E162, and K233 residues of PITP7 are involved in PIP binding.

To examine the functional importance of the interaction between PITP7 and PIPs, we introduced the constructs 35S::PITP7*ΔH125Q-sGFP and 35S::PITP7*ΔE162K-sGFP into heterozygous pitp7-1/PITP7 plants and selected homozygous pitp7-1 transgenic lines. Expression of the transgene rescued the seedling lethality of the pitp7-1 mutant but only partially rescued the growth phenotype of pitp7-1; transgenic plants remained smaller than the WT plants. In contrast, transgenic plants expressing the 35S::PITP7-sGFP and 35S::PITP7*HA constructs rescued fully pitp7-1. Hence, we suggest that the H125 and E162 residues are possibly significant for the PITP7 function (Figure S6).

4 | DISCUSSION

PITP7 is the only single-domain Sec14-like protein in Arabidopsis predicted to be targeted to the chloroplast. Recently, Hertle et al. (2020) demonstrated that this protein (which they called CPSFL1) is required for autotrophic growth and vesicle formation at the chloroplast inner membrane. CPSFL1 binds PIPs and PtdOH and acts as a PITP in vitro. CPSFL1 can transfer PIP into PtdOH-rich membrane bilayers in vitro, suggesting that CPSFL1 may facilitate vesicle formation by trafficking PtdOH and/or PIP, two known regulators of membrane trafficking between organelar subcompartments.

In this study, we characterized three Arabidopsis T-DNA insertion lines with varying degrees of visible phenotypes associated with the position of the T-DNA insertion and the reduction in PITP7 transcript levels. Our data showed that deficiency of pitp7 gene expression leads to critical changes in structural and functional properties of plants. The severity of mutant phenotypes such as plant developmental abnormalities, levels of PQ-9 and chlorophylls, and photosynthetic performance was related to PITP7 transcript levels (Figure 1). The pitp7-1 mutant exhibited pale green leaves and eventually died even when grown on MS medium supplemented with sucrose, whereas the pitp7-2 mutant was viable and produced seeds in the soil. In pitp7-1, PSII-LHCII/PSII protein complexes were greatly reduced along with the major photosynthetic proteins D1, CP47, Cytf, and PsaC (Figure 5A,C), suggesting that PITP7 is essential for the biosynthesis/organization of photosynthetic complexes in the thylakoid membrane. Consistent with this, a previous study reported that cpsfl1 is associated with the loss of major photosynthetic protein subunits in Arabidopsis (Hertle et al., 2020). In line with the decrease in PSII-LHCII/PSII protein complexes, chloroplasts of the pitp7-1 mutant showed a deficiency in grana stacking (Figure S4). However, the pitp7-2 mutant showed no significant changes in photosynthetic proteins, indicating that the PITP7 content of pitp7-2 might be enough to function in the biosynthesis/organization of photosynthetic complexes in the thylakoid membrane. Furthermore, the leaf lipid content of pitp7-2 was comparable to that of the WT, with slight changes in the acyl composition of MGDG, DGDG, PtdGro, PtdCho, and PtdEtn. Higher content of 16:1Δ3t-PtdGro in the pitp7-2 mutant relative to the WT likely represents a photoprotective strategy against the photoinhibition observed even under normal light intensity. 16:1Δ3t participates in the replacement of the D1 protein damaged by excessive light energy, thereby alleviating PSII photoinhibition and promoting faster recovery from photoinhibition (Gray et al., 2005; Siegenthaler & Trémolières, 1998). In addition, 16:1Δ3t-PtdGro contents of winter rye (Secale cereale) grown in intense light increase by 1.8-fold compared with those of winter rye grown in a weak light environment, favoring a greater predominance of LHCII oligomers over their monomeric form (Gray et al., 2005).

In our study, 8-day-old pitp7-1 and pitp7-2 mutants exhibited markedly lower PQ-9 levels than the WT, while the overall accumulation of carotenoids and tocopherols was not significantly different between them. PQ-9 deficient Arabidopsis, maize, and rice mutants show similar phenotypes, such as severe growth or developmental defects, that are almost identical to that of pitp7-1 (Block et al., 2013; Cook & Miles, 1992; Kim et al., 2017; Otsubo et al., 2018). The fbn5 Arabidopsis mutant displays abnormalities in chloroplast and thylakoid morphology, including swollen thylakoid vesicles in grana and disorganized grana stacks (Otsubo et al., 2018), indicating that PQ-9 is important for normal chloroplast development. In addition, PQ-9-deficient Arabidopsis mutants suppress NPQ development at high actinic light intensities and have elevated susceptibility to high-intensity light stress (Block et al., 2013; Otsubo et al., 2018). Interestingly, fbn5 Arabidopsis and rice mutants and the sps1sp2 Arabidopsis mutant (all having an interruption in PQ-9 biosynthesis) exhibit comparable amounts of tocopherols as the WT, consistent with observations in pitp7-1 (Block et al., 2013; Kim et al., 2015, 2017). PQ-9 is also involved in photoprotection as a potent antioxidant and in chloroplast metabolism as a cofactor for metabolite biosynthesis in chloroplasts, in addition to its essential role as an electron carrier in the electron transport chains of thylakoids (Havaux, 2020). PQ-9 oxidation by plastid terminal oxidase (PTOX) is required for carotenoid biosynthesis via phytoene desaturase (Carol et al., 1999), indicating that lower carotenoid levels might result from lower PQ-9 contents in the pitp7-1 mutant compared to the WT. Recent studies have identified a
functional connection between the redox status of the PQ-9 pool in thylakoids and the biosynthesis of chlorophylls (Brezowski et al., 2019; Steccanella et al., 2015). Thus, lower transcriptional levels of most chlorophyll biosynthetic genes and hence lower chlorophyll contents in pitp7 mutants compared with the WT might be attributable to feedback regulation between PQ-9 redox and chlorophylls.

Decreased PSII photosynthetic capacity \( (F_v/F_m) \) in pitp7-2 (~25% lower than WT) in the presence of apparently normal PSII-LHCII/PSII protein complexes might be attributed to a defective electron transport component; PQ-9 was reduced to 36.2% of the WT level (Figures 1–3). In addition, lower NPQ induction as actinic light intensity increased and abnormal OJIP fluorescence transients in pitp7-2 can be explained mostly by lower PQ-9 content in thylakoids, resulting in impaired electron transport at the acceptor side of PSII (Figure 4B,C). Furthermore, PQ-9 displays singlet oxygen \( (^1O_2) \) scavenger activity during photooxidative stress (Hundal et al., 1995; Ksas et al., 2018; Szymańska et al., 2014). High total PQ-9 level is associated with tolerance to photooxidative stress related to reduced lipid peroxidation and \(^1O_2 \) production in vet1SP5106 Arabidopsis (devoid of tocopherol and PC-8, and overexpressing the SPS1 gene) (Ksas et al., 2018). Thus, low PQ-9 levels in pitp7-2 plants might diminish the role of PQ-9 as a \(^1O_2 \) scavenger in photoprotection as well as its function as an electron carrier in photosynthetic electron transport, resulting in lower photosynthetic efficiency and higher photosensitivity.

In our study, PITP7 lacking the chloroplast targeting peptide (cTP) sequence specifically bound to PIPs. (Figure 7A,C). Our point mutants in vitro suggest that R82, H125, E162, and K233 are important residues for PtdIns(4)P binding (Figure 7B). Further, \( \text{pitp7-1+3SS::PITP7}^{H125Q} \)-sGFP and \( \text{pitp7-1+3SS::PITP7}^{E162K} \)-sGFP transgenic plants displayed restored autotrophic growth in soil but were not fully complemented, unlike \( \text{pitp7-1+3SS::PITP7} \)-sGFP plants (Figure 5A). This result indicates that H125 and E162 residues are important for PITP7 function in plants. However, the preferential binding of PITP7 to PIPs on lipid strips is consistent with previous studies. Full-length CPSFL1 preferentially binds to PtdOHi and PIPs, whereas a version of CPSFL1 lacking the CRAL_TRIO_N domain (which overlaps with cTP) well as PIPs, indicating that a slight structural change in the PITP7 protein could affect its lipid selection.

Thylakoid biogenesis from proplastids into mature chloroplasts requires a supply of lipids and proteins from the chloroplast envelope to thylakoids. Prenyl lipids such as carotenoids, tocopherols, and PQ-9 play crucial roles in plant development, photosynthesis, and protection against environmental stresses. Proteomic studies suggest that most biosynthetic enzymes for these prenyl lipids are found in envelope membranes (Joyard et al., 2009), but they are widely distributed in the chloroplast envelope, thylakoids, and plastoglobules (Ksas et al., 2018; Nisar et al., 2015; Vidi et al., 2006). It remains to be determined how these compounds are transported from envelope membranes to other compartments in response to chloroplast development and environmental changes. Chloroplast vesicle transport could be involved in the trafficking of these prenyl lipids (Rast et al., 2015). Recently, it was suggested that CPSFL1 in Chlamydomonas is involved in the regulation of phytoene biosynthesis and carotenoid transport (García-Cerdán et al., 2020). CPSFL1 proteins expressed in carotenoid-producing \( E. coli \) strains bind phytoene and \( \beta \)-carotene. Meanwhile, the \( \text{cpsf1} \) Chlamydomonas mutant shows decreased accumulation of carotenoids, resulting from impaired biosynthesis at a step prior to phytoene. CPSFL1 is hypothesized to be involved either in the delivery of isoprenoid intermediate precursors to the chloroplast envelope or in the direct, or indirect (vesicle transport pathway), transfer of carotenoids from the envelopes to other destinations such as thylakoids and plastoglobules. PtdOH binding might be the mechanism triggering the release of bound isoprenoid intermediate precursors or carotenoids from CPSFL1 to a target membrane. Similarly, CPSFL1 of Arabidopsis is proposed to play a role in vesicle formation by PtdOH and/or PIP, which is critical for thylakoid biogenesis and/or maintenance (Hertle et al., 2020). Low carotenoid levels in \( \text{cpsf1} \) Arabidopsis and Chlamydomonas mutants might result from a defect in the vesicle-mediated transfer of carotenoids from the envelope (Hertle et al., 2020). In our study, contents of neoxanthin and \( \beta \)-carotene in 8-day-old \( \text{pitp7-1} \) plants were decreased to 61.3% and 36% of WT levels, respectively, whereas the PQ-9 level was decreased to 11% of that of the WT. Based on the previously reported functions of CPSFL1, we speculate that PITP7 plays an important role in the transport of hydrophobic molecules, including carotenoids and particularly PQ-9, from envelope membranes to thylakoids and plastoglobules either by direct transfer or through involvement in vesicle formation. Dynamic transfer of PQ-9 from envelope membranes, its place of biosynthesis, to its functional sites in thylakoids and plastoglobules is necessary to maintain a constant pool in thylakoids. Lower PQ-9 levels observed in PITP7-deficient mutants might be explained through transcriptional feedback inhibition of PQ-9 biosynthetic genes by a defect in the vesicle-mediated transfer of PQ-9 (Figure 3A,E).

5 | CONCLUSIONS

Our work has shown that a chloroplast-localized PITP7 in Arabidopsis is required for plant development and maintenance of optimized photosynthetic performance. A significantly lower abundance of
photosynthetic protein complexes, PQ-9, carotenoids, and chlorophylls was observed in 8-day-old pitp7-1 mutants than in WT plants, indicating that PITP7 plays essential roles in the formation of the photosynthetic machinery in thylakoids. Lower PQ-9 level in the pitp7-2 mutant might have resulted in inefficient \( \text{O}_2 \) quenching and electron transport rates in thylakoids, explaining the decreased maximum photosynthetic capacity compared with the WT and the susceptibility to high light. Moreover, PITP7 bound to PIPs, with higher affinity for mono PIPs, in protein-lipid overlay assays. Various point mutations in recombinant PITP7 revealed that the residues R82, H125, E162, and L213 are important for PtdIns(4)P binding in vitro. Furthermore, we suggest that H125 and E162 residues are involved in PITP7 function in plants, mediating PIP binding. Given the functional role of CPSFL1 in chloroplast vesicle formation, we conclude that PITP7 plays an important role in membrane trafficking by transferring hydrophobic molecules, including PQ-9, which is important for the formation and/or maintenance of functional thylakoids.

**AUTHOR CONTRIBUTIONS**

Eun-Ha Kim performed gene cloning, vector construction, prenyl lipid analysis, GUS assays, and TEM; Roshan Sharma Poudyal carried out immunoblot analysis and BN-PAGE; Hami Yu purified recombinant PITP7 protein in *E. coli* and performed lipid–protein interaction assays and complementation tests using site-directed mutated PITP7::sGFP; Eunji Gi conducted RT-qPCR and complementation tests using PITP7::HA; Hyun Uk Kim conceived the research plans; Kyeong-Ryeol Lee performed TLC and fatty acid analysis; Eun-Ha Kim, Roshan Sharma Poudyal, Hyun Uk Kim, and Kyeong-Ryeol Lee analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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**DATA AVAILABILITY STATEMENT**

The data supporting the findings of this study are available from the corresponding authors Kyeong-Ryeol Lee and Hyun Uk Kim upon request.

**ACCESSION NUMBERS**

Sequence data from this study can be found in the GenBank/EMBL libraries under the following accession numbers: ACT2 (At3g18780), BHY1 (At4g25700), CAO (At1g44446), CHLG (At3g51820), CRTISO (At1g06820), FBNS (At5g09820), HPPDase (At1g06570), HST (At3g11945), LBC (At3g10230), LEB (At5g57030), LUT1 (At3g3130), PDS (At1g06570), PITP7 (At5g63060), PSY (At5g17230), SPS1 (At1g78510), SPS2 (At1g17050), VTE1 (At4g32770), VTE2 (At2g18950), VTE3 (At3g34110), VTE4 (At1g64970), ZDS (At3g04870), DVR (At5g18660), HCAR (At1g04620), HEMA1 (At1g58290), HEMA2 (At1g09940), NOL (At5g04900), NYC1 (At4g13250), PorA (At5g54190), and PorB (At4g27440).

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**SUPPORTING INFORMATION**

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