**Arabidopsis** PFA-DSP-Type Phosphohydrolases Target Specific Inositol Pyrophosphate Messengers


**ABSTRACT:** Inositol pyrophosphates are signaling molecules containing at least one phosphoanhydride bond that regulate a wide range of cellular processes in eukaryotes. With a cyclic array of phosphate esters and diphosphate groups around myo-inositol, these molecular messengers possess the highest charge density found in nature. Recent work deciphering inositol pyrophosphate biosynthesis in Arabidopsis revealed important functions of these messengers in nutrient sensing, hormone signaling, and plant immunity. However, despite the rapid hydrolysis of these molecules in plant extracts, very little is known about the molecular identity of the phosphohydrolases that convert these messengers back to their inositol polyphosphate precursors. Here, we investigate whether Arabidopsis Plant and Fungi Atypical Dual Specificity Phosphatases (PFA-DSP1-5) catalyze inositol pyrophosphate phosphohydrolase activity. We find that recombinant proteins of all five Arabidopsis PFA-DSP homologues display phosphohydrolase activity with a high specificity for the 5-β-phosphate of inositol pyrophosphates and only minor activity against the β-phosphates of 4-InsP$_7$, and 6-InsP$_7$. We further show that heterologous expression of Arabidopsis PFA-DSP1 rescues wortmannin sensitivity and deranged inositol pyrophosphate homeostasis caused by the deficiency of the PFA-DSP-type inositol pyrophosphate phosphohydrolase Siw14 in yeast. Heterologous expression in Nicotiana benthamiana leaves provided evidence that Arabidopsis PFA-DSP1 also displays 5-β-phosphate-specific inositol pyrophosphate phosphophosphohydrolase activity in planta. Our findings lay the biochemical basis and provide the genetic tools to uncover the roles of inositol pyrophosphates in plant physiology and plant development.

**INTRODUCTION**

Inositol pyrophosphates (PP-InsPs), such as InsP$_7$ and InsP$_9$, are molecules derived from myo-inositol (Ins) esterified with unique patterns of monophosphates (P) and diphosphates (PP) and have been described as versatile messengers in yeast, amoeba, and animal cells. With recent discoveries that PP-InsPs regulate nutrient sensing and immunity in plants, these molecules are a novel focus of research in plant physiology. The synthesis of PP-InsPs is partially conserved in eukaryotes, with some important distinctions in plants. In baker’s yeast and mammals, 5-InsP$_7$ is synthesized by Kcs1/IP6K-type proteins, whereas Vip1/PPISP5K-type kinases phosphorylate the C1 position of both InsP$_6$ (also termed phytic acid) and 5-InsP$_7$, generating 1-InsP$_7$ and 1,5-InsP$_8$, respectively. In plants, detection, quantification, and characterization of PP-InsPs have been challenging due to the low abundance of these molecules and their susceptibility to hydrolytic activities during extraction. Employing $^3$H myo-inositol labeling and subsequent analysis of plant extracts by strong-anion exchange high-performance liquid chromatography (SAX-HPLC) allowed the detection of PP-InsPs in different plant species. The recent development of capillary electrophoresis (CE) coupled to electrospray ionization mass spectrometry (ESI-MS), has enabled the detection and quantification of many InsP and PP-InsP isomers in various cell extracts including all InsP$_7$ isomers, except enantiomers (labeled, e.g., as 1/3 or 4/6-InsP$_7$). Similar to yeast and mammals, the Arabidopsis PPISP5K isoforms VHI and VIH2 catalyze the synthesis of InsP$_5$ and are likely involved in the synthesis of 1/3-InsP$_7$. However, Kcs1/IP6K-type proteins are absent in...
land plants. The question of how plants synthesize 5-InsP7 has been partially solved by work on Arabidopsis inositol (1,3,4) triphosphate 5/6 kinases ITPK1 and ITPK2. Notably, ITPK1 and ITPK2 were reported to catalyze the synthesis of 5-InsP7 from InsP6 in vitro45−48 and consequently iptk1 mutant plants display reduced 5-InsP7 levels.49

In Arabidopsis, disturbances in the synthesis of InsP5 and/or InsP8 result in defective signaling of the plant hormones jasmonate43 and auxin,5 as well as defects in salicylic acid-dependent plant immunity45 and impaired phosphate (P) homeostasis.29,41,52 In the case of auxin and jasmonate perception, 5-InsP7 and InsP8, respectively, are proposed to function as co-ligands of the respective receptor complexes.29,52 The role of PP-InsPs in P signaling is related to their ability to bind to SPX proteins, which act as receptors for these messenger molecules.53 InsP8 has been found as the preferred ligand for stand-alone SPX proteins in vivo.5,10,28

In vitro, the phosphatase domain of Arabidopsis PIPPSK VIH2 hydrolyzes PP-InsPs to InsP6,10 similar to the respective C-terminal domains of fission yeast and mammalian PIPPSKs.35,36 Although Arabidopsis ITPK1 harbors no phosphatase domain, under conditions of low adenylate charge, it can shift its activity in vitro from kinase to ADP phosphotransferase activity using 5-InsP7 but no other InsP, isomer.11,26 Apart from relying on the reversible activities of ITPK1 and Vip1/PIIPPSKs, the degradation of PP-InsPs may also be controlled by specialized phosphohydrolases.

In mammalian cells, diphosphoinositol polyphosphate phosphohydrolases (DIPPs), members of the nudix hydrolase family, have been shown to catalyze the hydrolysis of the diphosphate groups of InsP7 and InsP8 at the C1 and C5 position.5,37,38 The baker’s yeast genome encodes a single homologue of mammalian DIPPI, named diadenosine and diphosphoinositol polyphosphate phosphohydrolase (DDP1), which hydrolyzes various substrates including diadenosine polyphosphates, 5-InsP7, and InsP8, but has a preference for inorganic polyphosphates (poly-P) and for the β-phosphate of 1-InsPβ.39−41 In addition, baker’s yeast has an unrelated PP-InsP phosphohydrolase, Sis14 (also named Oca3) with a high specificity for the β-phosphate at position C5 of 5-InsPβ.42,43 This enzyme is a member of the Plant and Fungi ATPypical Dual Specificity Phosphatases (PFA-DSPs) that belong to a large family of protein tyrosine phosphatases (PTPs).45,46

Blast search analyses revealed that the Arabidopsis thaliana genome encodes five PFA-DSPs, with AtPFA-DSP1 sharing 61% identity and 76% similarity with yeast Sis14.44,45 X-ray crystallography revealed that the protein adopts an α/β-fold typical for cysteine phosphatases, with the predicted catalytic cysteine (Cys150) residing at the bottom of a positively charged pocket.46 Of a number of putative phosphate substrates tested, recombinant AtPFA-DSP1 displayed the highest activity against inorganic polyphosphate, as well as against deoxyribob- and ribonucleoside triphosphates, and less activity against phosphotyroline-containing peptides and phosphoinositides.46 Here, we investigated whether Arabidopsis PFA-DSPs might function as PP-InsP phosphohydrolyases.

### METHODS

#### Plant Materials and Growth Conditions

Seeds of A. thaliana T-DNA insertion lines pfa-dsp1-3 (WiscD Slew_473_B10, Col-0), pfa-dsp1-4 (CSHL_GT1415, Ler-0), pfa-dsp1-6 (SAIL_116_C12, Col-0) and mrp5 (GK-068B10) were obtained from The European Arabidopsis Stock Centre (http://arabidopsis.info/). To identify homozygous lines, F2 and F3 plants were genotyped by PCR using the primers indicated in Table S2.

For sterile cultures, Arabidopsis seeds were surface sterilized in 1.2% (v/v) NaHClO2 and 0.05% (v/v) Triton X-100 for 3 min, in 70% (v/v) ethanol and 0.05% (v/v) Triton X-100 for 3 min and in 100% (v/v) ethanol before transferring onto sterile filter paper. Sterilized seeds were sown onto half-strength Murashige and Skoog (MS) medium59 containing 1% sucrose, pH 5.7 and solidified with 0.7% (w/v) Phytgel (Sigma-Aldrich). After 2 days of stratification at 4 °C, the plates were transferred to a growth incubator and the seedlings were grown under short-day conditions with the following regime: 8/16 h light/dark; light intensity 120 μmol m−2 s−1; temperature 22 °C/20 °C.

#### Constructs

The following full-length ORFs were amplified by PCR from an Arabidopsis whole seedling cDNA preparation: PFA-DSP1 (At1g050000), PFA-DSP2 (At2g32960), PFA-DSP3 (At3g02800), PFA-DSP4 (At4g03960), and PFA-DSP5 (At5g16480). Likewise, the SIW14 ORF sequence was amplified from yeast genomic DNA. Primers used for amplification are listed in Table S2. The reverse primers contained a V5 sequence (underlined) allowing a translational fusion of the resulting gene products with a C-terminal V5 epitope tag. Amplification products were cloned into pDONR221 (Invitrogen) via BP clonase II (Invitrogen) reaction following the manufacturer’s instructions. The ORFs were then swapped into the episomal yeast expression vector pDRF1−GW40 by the LR clonase II (Invitrogen) reaction following the manufacturer’s instructions. For expression of SIW14 under control of the endogenous promoter from a CEN-based plasmid, the SIW14 gDNA was amplified from purified yeast gDNA using the primers listed in Table S2. The SIW14 gDNA was inserted into pCIPac33 (ATCC #87586) using the restriction enzymes PsfI and EcoRI.

For protein expression, PFA-DSP1−5 were amplified as described before but with a reverse primer containing a stop codon. Amplified products were cloned into pDONR221 (Invitrogen), then swapped by LR clonase II (Invitrogen) into the bacterial expression vector pDEST56 (Addgene plasmid #11517), which contains a sequence encoding an N-terminal His6−maltose-binding protein (MBP) epitope tag. Free His-tagged MBP protein was expressed from a modified pet28 vector carrying an N-terminal sequence encoding a His6−maltose-binding protein (MBP) epitope tag.

For transient expression in Nicotiana benthamiana, the ORF of PFA-DSP1 (wild-type sequence and with a mutated sequence encoding the C150S substitution) was swapped by LR clonase II (Invitrogen) from pDONR221 into the plant
expression vector pGWB641,61 which harbors a viral CaMV 3SS promoter to allow gene expression and a sequence encoding a C-terminal EYFP tag. Site-directed mutagenesis was performed on the respective plasmids with the primers listed in Table S2.

N. benthamiana Infiltration. A single colony of transformed Agrobacteria was inoculated in 2 mL of LB media containing the appropriate antibiotics and cultivated overnight at 26 °C in a spinning wheel. On the next morning, 1 mL of overnight culture was added to 5 mL of fresh LB with antibiotics and grown for another 4 h at 26 °C. Afterward, the cultures were harvested by centrifugation at 4 °C with 3000g for 20 min. The pellet was then resuspended in 3 mL of infiltration solution containing 10 mM MgCl₂, 10 mM MES-KOH (pH 5.6), and 150 μM acetoxyringone. OD₆₀₀ was determined using a 1:10 dilution and adjusted to 0.8 in infiltration solution. Then, the working solution was prepared by pooling equal amounts of cultures (e.g., P19 + PFA-DSP1), which were then co-infiltrated in the abaxial surface of the leaf using a 1 mL syringe without a needle. Afterward, the plants were placed in a dark incubator at 26 °C for ~1 day before keeping them for another 4 days on the workbench. The leaves were then harvested and frozen in liquid nitrogen before continuing with the extraction of inositol phosphates.

Yeast Strains. Different strains of the budding yeast Saccharomyces cerevisiae were used. The BY4741 wild-type (MATa his3Δ leu2Δ met15Δ ura3Δ), siw14Δ (YNL032c::kanMX4), kcs1Δ (YDR017c::kanMX4), and ipk2Δ (YDR173c::kanMX4) were obtained from Euroscarf. vip1Δ (siw14Δ), kcs1Δ siw14Δ, ipk2Δ siw14Δ were generated using loxP/Cre gene disruption and the ble resistance marker, which confers phleomycin/Zeocin (In-vitrogen) resistance67 using the primers listed in Table S2. In addition, the following mutants in the DDY1810 background (MATa; leu2Δ ura3-52 trp1Δ; prb1-1122 pep4-3 pre1-451)65 were used: kcs1Δ and kcs1Δ ddp1Δ, kcs1Δ siw14Δ, kcs1Δ ddp1Δ siw14Δ, and siw14Δ were generated in this background as described before. For all assays, the yeast cells were transformed by the Li-acetate method64 and cultured in either YPD or YPD + CSM medium or selective synthetic de medium and grown overnight at 28 °C while shaking (200 rpm). Then, OD₆₀₀ was measured, adjusted to 1.0, and an 8-fold dilution series was prepared in a 96-well plate. Subsequently, 10 μL of each dilution were spotted on selective solid media as described earlier65 and incubated at 26 °C for 2–4 days. To prepare selective solid media supplemented with wortmannin, autoclaved media was cooled down to 60 °C, wortmannin was added from a 10 mM stock in DMSO (Sigma-Aldrich) to a final concentration of 1–3 μM. Since the activity of wortmannin changed by age and by the number of freezing/thawing cycles, aliquots were kept at ~20 °C and were not thawed more than five times. In addition, several concentrations were employed for the spotting assays to be able to identify the activity at which growth differences between siw14Δ, kcs1Δ, and their isogenic wild-type transformants became most obvious. Pictures were taken with a Bio-Rad ChemiDoc MP imager using white backlight.

Protein Preparation. His₆-MBP-PFA-DSP protein fusions or free His₆-MBP were expressed in Escherichia coli BL21 CodonPlus (DE3)-RIIL cells (Stratagene). Overnight bacterial cultures were inoculated 1:1000 into fresh 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with 100 mg/L ampicillin (pDEST566) or 50 mg/L kanamycin (pET28) and 25 mg/L chloramphenicol. Cells were grown at 37 °C while shaking (200 rpm) for 4 h (~0.6 OD₆₀₀), and protein expression was induced at 16 °C overnight with 0.1 mM isopropyl-1-thiogalactopyranoside. The cells were lysed as described66 using the following lysis buffer: 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 25 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Tween 20, 5 mM β-mercaptoethanol, and EDTA-free complete ULTRA protease inhibitor cocktail (Roche). Proteins were batch-purified using Ni-NTA agarose resin (Macherey-Nagel) and eluted using the above-mentioned lysis buffer with increased imidazole concentration (250 mM). Three elutions were combined and dialyzed using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific) following the manufacturer’s instructions and a buffer containing 50 mM Tris-Cl, pH 7.5 and 100 mM NaCl. The concentrated protein preparations were then stored at ~20 °C. Purified proteins were analyzed using SDS-PAGE followed by Coomassie blue staining. Proteins were compared with PageRuler plus prestained protein ladder (Thermo Fisher) and with designated amounts of a BSA standard to estimate target protein concentrations.

In Vitro PP-InsP Phosphohydrolase Assay. The phosphohydrolase assay was carried out in a 15 μL reaction mixture containing 0.35–2 μM recombinant PFA-DSP or Siw14 protein, 50 mM HEPES (pH 7.0), 10 mM NaCl, 5% (v/v) glycerol, 0.1% (v/v) β-mercaptoethanol, and 0.33 mM of various InsP₃ and InsP₄ isomers as indicated, and was incubated for 1, 2, or 24 h at 22 °C. The PP-InsP₃ isomers were synthesized as described previously.67,68 Reactions were separated by 33% PAGE and visualized by toluidine blue or DAPI staining.

Titanium Dioxide Bead Extraction and PAGE/CE-ESI-MS. Purification of inositol polyphosphates using TiO₂ beads and analysis via PAGE was performed as described previously.11 CE-ESI-MS analyses of in vitro, yeast and plant samples were performed as described previously.11,23

Inositol Polyphosphate Extraction from Yeast Cells and Seedlings and HPLC Analyses. For inositol polyphosphate analyses from yeast, transformants were inoculated into a selective synthetic deficiency (SD) medium and grown overnight at 28 °C while shaking (200 rpm). They were then diluted 1:200 in 2 mL of fresh medium supplemented with 6 μCi mL⁻¹ [³H]-myo-inositol (30–80 Ci mmol⁻¹; Biotrend; ART-0261-5) and grown overnight at 28 °C in a spinning wheel. After centrifugation and washing of the cell pellet, inositol polyphosphates were extracted and analyzed as described before.5,25,70

Extraction of [³H]-myo-inositol polyphosphates from Arabidopsis seedlings and subsequent SAX-HPLC analyses were performed as described previously.70

RNA Isolation and Quantitative Real-Time PCR. Fifteen-day-old seedlings were transferred from solid half-strength MS plates to liquid half-strength MS media (supplemented with 1% sucrose) for 5 days before harvest and immediately frozen in liquid N₂. Total RNA was extracted with NucleoSpin RNA Plant and Fungi kit (Macherey-Nagel). cDNA was synthesized using RevertAid RT reverse transcription kit (Thermo Fisher). Quantitative PCR reactions were conducted with the CFX384 real-time system (Bio-Rad) and the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad).
Figure 1. *In vitro*, *Arabidopsis* PFA-DSPs display Mg$^{2+}$-dependent PP-InsP phosphohydrolase activity with high specificity for 5-InsP$_7$. Recombinant His-MBP-PFA-DSPs and His-MBP-Siw14 (indicated with the plus symbol in (C) and (E)) were incubated with 0.33 mM InsP$_7$ at 22 °C. His-MBP served as a negative control (as indicated with the minus symbol in (C) and (E)). (A) 0.4 μM His-MBP-PFA-DSP1 was incubated for 1 h with 1-InsP$_7$, 5-InsP$_7$, and 1 mM EDTA, MnCl$_2$, MgCl$_2$, CaCl$_2$, or ZnCl$_2$ as indicated. The reaction products were then separated by 33% PAGE and visualized by toluidine blue. (B-D) The InsP$_7$ phosphohydrolase activity of ∼0.4 μM His-MBP-PFA-DSPs and His-MBP-Siw14 was analyzed in the presence of 1 mM MgCl$_2$. After 1 h, the reaction products were then (B, D) spiked with isotopic standards mixture ([13C$_6$] 1,5-InsP$_8$, [13C$_6$]5-InsP$_7$, [13C$_6$]1-InsP$_7$, [13C$_6$]InsP$_6$, [13C$_6$]2-OH InsP$_5$) and subjected to CE-ESI-MS analyses or (C) separated by 33% PAGE and visualized by toluidine blue/DAPI staining. (D) Data represent mean ± SEM (n = 3). Representative extracted-ion electropherograms are shown in Figure S2. Asterisks indicate values that are significantly different from the MBP control reactions (according to Student’s t test, P < 0.05 (*); P < 0.01 (**)).

(E) Recombinant His-MBP-PFA-DSP5 (2 μM) was incubated with 0.33 mM InsP$_7$ isomers for 2 h. The reaction product was separated by 33% PAGE and visualized with toluidine blue. (A, C, E) Identity of bands was determined by migration compared to TiO$_2$-purified mrp5 seed extract.
Rad) using the primers listed in Table S2. TIP41-like and PP2A A3 were used as reference genes to normalize relative expression levels of all tested genes. Relative expression was calculated using the CFX Maestro software (Bio-Rad).

Yeast Protein Extraction and Immunodetection. Multiple transformants were inoculated into 4 mL of YPD (with 3% glucose) or selective SD-media and grown for up to 24 h at 28 °C. On the following day, the yeast was reinoculated into 4 mL of fresh media and grown for another day. Afterward, the cells were harvested and resuspended in 500 μL of extraction buffer (300 mM sorbitol, 150 mM NaCl, 50 mM Na₂HPO₄, 1 mM EDTA, pH 7.5), supplemented with 100 mM β-mercaptoethanol and a 1:50 dilution of protease inhibitor cocktail for fungal extracts (Sigma-Aldrich). The cells were lysed with bead beating using 150−200 μL of glass beads (ø 0.5 mm). The lysate was spun down and the supernatant boiled for 10 min after the addition of sample buffer. The protein extracts were then resolved by SDS-PAGE. Target proteins were detected by immunoblot. As the primary antibody, a mouse anti-V5 (Invitrogen, R960-25, 1:2000 dilution) or a goat anti-mouse HRP antibody (Bio-Rad, 1:10 000 dilution). As a loading control, Gal4 was detected using a rabbit polyclonal anti-Gal4 antibody (Santa Cruz, 1:1000 dilution), followed by a goat anti-rabbit StarBright Blue 700 antibody (Bio-Rad, 1:2500 dilution). The signal was detected using the multi-plex function of the ChemiDoc MP imager (Bio-Rad). Alternatively, for blots where a secondary antibody coupled to HRP was used, the chemiluminescence signal of the ECL reagent was detected, followed by Ponceau staining as loading control.

■ RESULTS AND DISCUSSION

Arabidopsis PFA-DSP Proteins Display In Vitro PP-InsP Phosphohydrolase Activity with High Specificity for 5-InsP₇. To explore the potential role of Arabidopsis PFA-DSP proteins in PP-InsP hydrolysis, we first generated translational fusions of PFA-DSPs with an N-terminal hexahistidine tag followed by a maltose-binding protein (MBP) and expressed recombinant proteins in bacteria. Corresponding His-MBP-Siw14 and free His-MBP constructs were generated as controls. All constructs allowed the purification of soluble recombinant proteins (Figure S1). We
then tested potential PP-InsP phosphohydrolase activities of PFA-DSP1 with 1-InsP₇ or 5-InsP₇ in the presence of various divalent cations. Notably, PFA-DSP1 failed to catalyze the hydrolysis of 1-InsP₇ or 5-InsP₇ in the presence of Mn²⁺, Ca²⁺,
or Zn[2]. However, in the presence of the cytoplasmic prevalent cation Mg[2], PFA-DSP1 displayed a robust hydrolytic activity against 5-InsP[7], likely resulting in the generation of InsP[6] as deduced from the mobility of the reaction product compared to TiO[2]-purified mrp5 (multidrug resistance-associated protein S) seed extract separated by polyacrylamide gel electrophoresis (PAGE) and visualized by toluidine blue staining (Figure 1A). Seeds of Arabidopsis mrp5 mutants that have a defective ABC-transporter involved in vacuolar loading of InsP[6] display reduced InsP[6] levels and simultaneously increased InsP[7] and InsP[8] levels.[5,24] Therefore, TiO[2]-purified mrp5 seed extract serves as a marker to visualize InsP[6], InsP[7], and InsP[8] on PAGE. CE-ESI-MS analysis of the reaction product spiked with a [53C] InsP[6] standard confirmed that the resulting product indeed had the migration behavior and the mass of phytic acid (Figure 1B). In contrast, 1-InsP[7] was largely resistant to PFA-DSP1 also in the presence of Mg[2] (Figure 1A). In the absence of divalent cations (i.e., in buffer not supplemented with divalent cations but instead supplemented with EDTA, a condition unlikely to represent any cellular condition), both InsP[7] isomers were hydrolyzed to InsP[6] as deduced from the mobility of the reaction product by PAGE (Figure 1A).

We then tested the hydrolytic activities of the Arabidopsis PFA-DSP homologues with all six “simple,” synthetic InsP[7] isomers and with the two enantiomeric InsP[7] isomers 1,5-InsP[7] and 3,5-InsP[7] in the presence of Mg[2]. Of note, myo-inositol is a meso compound with a mirror plane dissecting the C2 and C3 positions. Derivatives differentially (pyro)phosphorylated at the C1 and C3 positions, as well as at the C4 and C6 positions are enantiomeric forms that can only be distinguished in the presence of appropriate chiral selectors.[48,49] Yeast Siw14 and all Arabidopsis PFA-DSPs with the exception of PFA-DSP5 displayed robust activity with a high specificity toward 5-InsP[7] (Figure 1C), confirming earlier reports that 5-InsP[7] is a preferred substrate for yeast Siw14 compared to 1-InsP[7].[22] A5 PFA-DSP1−4 and Siw14 also displayed partial hydrolytic activities against the enantiomers 4-InsP[7] and 6-InsP[7], as well as very weak hydrolytic activities against enantiomeric 1-InsP[7] and 3-InsP[7] (Figure 1C). The latter activities were more pronounced in PFA-DSP1 and PFA-DSP3 compared to Siw14 and PFA-DSP2. As for 5-InsP[7], the reaction products with the other InsP[7] isomers had the mass and the migration behavior of the InsP[7] isomer phytic acid, as deduced from CE-ESI-MS analyses (Figures 1D and S2).

Notably, PFA-DSP5 only showed very weak activities at the 0.4 μM concentration tested in our assay. However, when the reaction time was extended from 1 h to 2 h and the enzyme concentration was increased to 2 μM, PFA-DSP5 displayed robust activity with a substrate specificity similar to PFA-DSP1-4 and yeast Siw14, with a high selectivity for 5-InsP[7] and only weak hydrolytic activities against 4-InsP[7] and 6-InsP[7] (Figure 1E).

Notably, the meso InsP[7] isomer 2-InsP[7] was completely resistant to Siw14 or any of the Arabidopsis PFA-DSP proteins under the assay conditions. This was also the case in the absence of divalent cations (i.e., in buffer not supplemented with divalent cations but instead supplemented with EDTA), a condition unlikely to represent any cellular condition, both InsP[7] isomers were hydrolyzed to InsP[6] as deduced from the mobility of the reaction product by PAGE (Figure 1A).

Figure 4. Heterologous expression of Arabidopsis PFA-DSPs complements siw14Δ-associated wortmannin sensitivity in yeast. (A) Growth complementation assays of an siw14Δ yeast strain. Wild-type yeast (BY4741) and an isogenic siw14Δ yeast mutant were transformed with either the empty episomal pDRf1-GW plasmid or different pDRf1-GW plasmids carrying the respective PFA-DSP gene or Siw14. Yeast transformants were then spotted in 8-fold serial dilutions (starting from OD[600] 1.0) onto selective media supplemented with either wortmannin or DMSO as control. Plates were incubated at 26 °C for 2 days before photographing. The yeast growth assay was repeated twice (n = 3) with similar results. (B) Relative amounts of InsP[7] of wild-type yeast, siw14Δ and siw14Δ transformed with pDRf1-GW carrying the PFA-DSP genes are shown as InsP[7]/InsP[6] ratios. InsP[6] and InsP[7] levels were determined by analysis of SAX-HPLC profiles using OriginPro 8. Data represent mean ± SEM (n = 3). Asterisks indicate values that are significantly different from siw14Δ (according to Student’s t test, P < 0.05 (*); P < 0.01 (**)).
InsP₆ to an InsP₇ isomer based on the mobility of the reaction product in PAGE analyses (Figure 3A). Also the enantiomeric 3,5-InsP₇ was efficiently hydrolyzed by Siw14 and PFA-DSP1–4 (Figure 3A), and CE-ESI-MS analysis of the reaction products showed the migration behavior and the mass of 1/3-4 InsP₈ (Figure 3A), and CE-ESI-MS analysis of the reaction products showed the migration behavior and the mass of 1/3-4 InsP₈ (Figure 3A). Reduced growth of siw14Δ transformants expressing PFA-DSP4 on media supplemented with wortmannin is therefore likely not caused by inefficient expression of this homologue in yeast but might rather be a consequence of excess protein activity in this heterologous expression system. To investigate the contribution of PFA-DSPs in InsP metabolism, we monitored InsP profiles using SAX-HPLC analyses of various [³H]-myo-inositol labeled yeast transformants. Of note, conventional SAX-HPLC analyses as employed here do not allow the discrimination of different InsP₇ or InsP₈ isomers. Heterologous expression of PFA-DSPs in siw14Δ restored InsP₇/InsP₈ ratios to wild-type levels, indicating that Arabidopsis PFA-DSP proteins are functionally similar to Siw14 (Figure 4B).

Notably, the InsP₇ signal was the only one consistently affected by the loss of Siw14 and heterologous expression of any PFA-DSP gene (Figure S9). We generated variants of Siw14 or PFA-DSP1, in which the catalytic cysteine was replaced by a serine resulting in a C214S and a C150S substitution in Siw14 and PFA-DSP1, respectively, and selected the fungal toxin wortmannin that caused a severe siw14Δ-associated growth defect. Previous observations that kcs1Δ yeast cells are resistant to wortmannin suggest that wortmannin sensitivity of siw14Δ yeast might be related to Kcs1-dependent PP-InsPs. The siw14Δ-associated growth defect was fully complemented by heterologous expression of either of the five Arabidopsis PFA-DSP homologues or of yeast Siw14 from episomal plasmids under control of a PMA1 promoter fragment (Figure 4A). Immunoblot analyses taking advantage of a C-terminal V5-tag revealed that all PFA-DSP homologues were expressed in yeast with PFA-DSP1 and PFA-DSP4 showing the highest protein abundance (Figure S8). Reduced growth of siw14Δ transformants expressing PFA-DSP4 on media supplemented with wortmannin is therefore likely not caused by inefficient expression of this homologue in yeast but might rather be a consequence of excess protein activity in this heterologous expression system. To investigate the contribution of PFA-DSPs in InsP metabolism, we monitored InsP profiles using SAX-HPLC analyses of various [³H]-myo-inositol labeled yeast transformants. Of note, conventional SAX-HPLC analyses as employed here do not allow the discrimination of different InsP₇ or InsP₈ isomers. Heterologous expression of PFA-DSPs in siw14Δ restored InsP₇/InsP₈ ratios to wild-type levels, indicating that Arabidopsis PFA-DSP proteins are functionally similar to Siw14 (Figure 4B).

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wortmannin tolerance and InsP₇ homeostasis and that complementation of the siw₁₄Δ-associated defects depends on the catalytic activity of these proteins.

**Growth Defects of siw₁₄Δ Yeast on Wortmannin Require Kcs1-Dependent 5-InsP₇ Synthesis.** For a deeper understanding of the wortmannin phenotype of siw₁₄Δ yeast, we investigated genetic interactions between Siw14 and different InsP kinases. We generated different double mutants with defects in Siw14 and the PP-InsP synthases Kcs1 and Vip1, and tested their performance on wortmannin-containing media (Figure 5A). Again, siw₁₄Δ cells did not survive on media supplemented with 3 μM wortmannin, a defect that was fully complemented by the expression of SIW₁₄ under control of the endogenous promoter from a CEN-based single-copy plasmid (Figure 5A). The growth of vip₁Δ cells was comparable to wild-type yeast. In contrast, the vip₁Δ siw₁₄Δ double mutant showed a severe growth defect on media supplemented with wortmannin similar to single siw₁₄Δ cells (Figure 5A). Like the vip₁Δ yeast strain, a kcs₁Δ strain did not show growth defects on media supplemented with wortmannin compared to control media. In contrast, at increased concentrations, we observed kcs₁Δ-associated wortmannin resistance (Figure 5B), as reported earlier. Importantly, deletion of KCS1 in siw₁₄Δ cells rescued siw₁₄Δ-associated wortmannin sensitivity since the resulting kcs₁Δ siw₁₄Δ double-mutant yeast strain, despite growing overall weaker than the kcs₁Δ single-mutant strain, showed no increased sensitivity to wortmannin (Figure 5A). These findings indicate that the presence of Kcs1 is critical for the growth defects displayed by siw₁₄Δ single-mutant cells on wortmannin. We then investigated whether the presence of Kcs1 itself or of Kcs1-dependent PP-InsPs such as 5-InsP₇ or PP-InsP₄ synthesis. Neither of the strains showed growth defects on media supplemented with wortmannin compared to the isogenic wild-type yeast strain, suggesting that also the loss of IPK2 rescues siw₁₄Δ-associated wortmannin sensitivity (Figure 5A). We further tested wortmannin sensitivity of kcs₁Δ and kcs₁Δ siw₁₄Δ yeast transformants in a different genetic background and observed similar results (Figure 5B,C). Taken together, these results provide a causal link...
between Kcs1 (but not Vip1)-dependent PP-InsPs and Siw14Δ-associated wortmannin sensitivity with Kcs1 and Siw14/PFA-DSPs playing antagonistic roles in regulating this sensitivity.

**Increased PFA-DSP1 Expression Coincides with Decreased InsP7 Levels In Planta.** To gain insight into PFA-DSP functions in planta, we searched for Arabidopsis T-DNA insertion lines of PFA-DSP1 and were able to identify three lines, pfa-dsp1-3 and pfa-dsp1-6 in the Col-0 background and pfa-dsp1-4 in the Ler-0 background, for which homozygous progeny could be obtained. None of these lines displayed an obvious growth phenotype under our standard growth conditions. SAX-HPLC profiles of extracts of 20-day-old [3H]-myo-inositol labeled pfa-dsp1-3 and pfa-dsp1-4 seedlings did not reveal a significant difference compared to the respective wild-types (Figure S11A,B). However, SAX-HPLC analyses of the pfa-dsp1-6 line revealed a significant average reduction (around 36%) of the InsP7/InsP6 ratio compared to Col-0 (Figure 6B). The levels of other InsP species remained largely unaffected (Figure 6A,B). The available sequencing data for this line, as well as our analysis, indicated that the insertion of the T-DNA is 18 bp upstream of the start codon, suggesting that the full-length transcript and PFA-DSP1 protein might be expressed in this line. We therefore conducted qPCR analyses of pfa-dsp1-6 seedlings that were grown under identical conditions as the seedlings for SAX-HPLC analyses and detected ca. 6-fold increased expression of PFA-DSP1 in pfa-dsp1-6 in comparison to Col-0 seedlings (Figure 6C).

Since the analyses of the pfa-dsp1-6 line indicated that the T-DNA insertion causes an overexpression of PFA-DSP1, resulting in decreased InsP7 levels, we investigated whether PP-InsP phosphohydrolase activity is also observed in a heterologous plant expression system. To this end, we transiently expressed a translational fusion of PFA-DSP1 with a C-terminal EYFP under control of the strong viral CaMV 35S promoter in N. benthamiana using agrobacterium-mediated transfection. The respective catalytically inactive PFA-DSP1C150S-EYFP fusion protein was also expressed and InsPs were then extracted from N. benthamiana leaves and purified 5 days after infiltration. PAGE analyses showed that transient expression of PFA-DSP1 or expression of its catalytic inactive version did not alter InsP6 levels (Figure 7A). In contrast, InsP7 levels were reduced by the transient expression of PFA-DSP1 but not by the expression of its catalytic inactive version (Figure 7A). These findings were strengthened by subsequent CE-ESI-MS analyses that revealed no changes in the ratios of 1/3-InsP7/InsP6 or 4/6-InsP7/InsP6 compared to control leaves infiltrated with agrobacteria carrying the silencing inhibitor P19 alone (Figure 7B). In contrast, the 5-InsP7/InsP6 ratio was significantly reduced in plants expressing PFA-DSP1 compared to plants expressing the
inactive version of PFA-DSP1 or P19 alone (Figure 7C). The InSp$_6$/InSp$_9$ ratio, in turn, was strongly reduced by the expression of PFA-DSP1 (Figure 7D) in agreement with a partial hydrolytic activity of PFA-DSP proteins against InSp$_8$ isomers (Figures 3 and S7) and in agreement with the finding that 5-InSp$_9$, a substrate hydrolyzed by PFA-DSP1, represents the major precursor for InSp$_9$ synthesis. In summary, these results demonstrate that ectopic expression of Arabidopsis PFA-DSP1 results in a specific decrease of 5-InSp$_2$ and InSp$_9$ in planta.

**CONCLUSIONS**

Recent studies elucidating the identity and substrate specificity of InSp$_9$/PP-InSp kinases have allowed us to establish important functions of PP-InSpS in nutrient sensing, hormone signaling, and plant immunity. In contrast, information on enzymatic activities removing PP-InSpS to switch off their signaling functions in plants is sparse. Intriguingly, the first robust detection of PP-InSpS messengers in mammalian cells was made possible by blocking mammalian PP-InSp phosphohydrolases with fluoride. While substantial progress in elucidating the role of various PP-InSp phosphohydrolases in regulating these messengers in yeast and mammalian cells has been made, we are unaware of any study about PP-InSp degrading enzymes in plants at the onset of this study. Here, we provide evidence that the Arabidopsis PFA-DSP proteins are functional homologues of yeast Siw14 with high phosphohydrolase activity against 5-InSp$_7$, 1,5-InSp$_8$, and 3,5-InSp$_8$, respectively (Figure S2); in the absence of divalent cations, all InSp$_i$, isomers with the exception of 2-InSp$_7$, become substrates for selected Arabidopsis PFA-DSPs in vitro (Figure S3); in the presence of Mg$^{2+}$, PFA-DSP1 and PFA-DSP3 display robust in vitro InSp$_7$ phosphohydrolase activity with high specificity for the 5-$\beta$-phosphate (Figure S4); under prolonged incubation time, Arabidopsis PFA-DSP1 efficiently hydrolyzes 5-InSp$_7$, 4-InSp$_7$, and 6-InSp$_7$ but only displays partial activities against 1-InSp$_7$ and 3-InSp$_7$, and a very weak activity against 2-InSp$_7$ (Figure S5); Arabidopsis PFA-DSP1 maintains 5-InSp$_7$, phosphohydrolase activity during prolonged incubation times in vitro (Figure S6); in vitro, Arabidopsis PFA-DSPs display robust 1/3,5-InSp$_7$ phosphohydrolase activity (Figure S7); all five PFA-DSP homologues are stably expressed in the swi14$\Delta$ yeast strain (Figure S8); heterologous expression of Arabidopsis PFA-DSPs complements swi14$\Delta$-associated defects in InSp$_7$/InSp$_9$ ratios in yeast (Figure S9); complementation of swi14$\Delta$-associated growth defects depends on catalytic activity (Figure S10); single-mutant Arabidopsis pfa-dsp1 loss-of-function lines do not display InSp$_7$/PP-InSp$_9$ defects (Figure S11); Arabidopsis PFA-DSP1, 2, and 4 are strongly induced by P$_i$ deficiency (Figure S12); overview of Arabidopsis PFA-DSP substrate specificities in the presence of Mg$^{2+}$ showing a robust PP-InSp phosphohydrolase activity against 5-InSp$_7$, 1,5-InSp$_9$, and 3,5-InSp$_9$, in vitro (Table S1); and oligonucleotide sequences (Table S2) (PDF).

**Accession Codes**

DNA and Protein Sequences can be obtained from the Saccharomyces Genome database (https://www.yeastgenome.org/), TAIR (https://www.arabidopsis.org/), and UniProt (https://www.uniprot.org/) under the following accession numbers: SIW14 (YNL032W, NC_001146.8), Arabidopsis PFA-DSP1 (At1g05000, NM_100379.3), Arabidopsis PFA-DSP2 (At2g32960, NM_128856.5), Arabidopsis PFA-DSP3 (At3g02800, NM_111148.3), Arabidopsis PFA-DSP4 (At4g03980, NM_116634.4), Arabidopsis PFA-DSP5 (At5g16480, NM_121653.4), Arabidopsis PFA-DSP6 (At6g75700, NM_111457.3), Arabidopsis PFA-DSP7 (At6g75700, NM_111457.3), and Arabidopsis TIP41-like (At5g54000, NM_115260). PDB ID: 1XRI.

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**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.2c00145.

Purification of PFA-DSP proteins (Figure S1); in vitro, Arabidopsis PFA-DSP1 displays robust PP-InSp phosphohydrolase activity against 5-Insp$_7$, and partial phosphohydrolase activity against 4-Insp$_7$, and 6-Insp$_7$, respectively (Figure S2); in the absence of divalent cations, all InSp$_i$, isomers with the exception of 2-Insp$_7$, become substrates for selected Arabidopsis PFA-DSPs in vitro (Figure S3); in the presence of Mg$^{2+}$, PFA-DSP1 and PFA-DSP3 display robust in vitro Insp$_7$ phosphohydrolase activity with high specificity for the 5-$\beta$-phosphate (Figure S4); under prolonged incubation time, Arabidopsis PFA-DSP1 efficiently hydrolyzes 5-Insp$_7$, 4-Insp$_7$, and 6-Insp$_7$ but only displays partial activities against 1-Insp$_7$ and 3-Insp$_7$, and a very weak activity against 2-Insp$_7$ (Figure S5); Arabidopsis PFA-DSP1 maintains 5-Insp$_7$, phosphohydrolase activity during prolonged incubation times in vitro (Figure S6); in vitro, Arabidopsis PFA-DSPs display robust 1/3,5-Insp$_7$ phosphohydrolase activity (Figure S7); all five PFA-DSP homologues are stably expressed in the swi14$\Delta$ yeast strain (Figure S8); heterologous expression of Arabidopsis PFA-DSPs complements swi14$\Delta$-associated defects in Insp$_7$/Insp$_9$ ratios in yeast (Figure S9); complementation of swi14$\Delta$-associated growth defects depends on catalytic activity (Figure S10); single-mutant Arabidopsis pfa-dsp1 loss-of-function lines do not display Insp$_7$/PP-Insp$_9$ defects (Figure S11); Arabidopsis PFA-DSP1, 2, and 4 are strongly induced by P$_i$ deficiency (Figure S12); overview of Arabidopsis PFA-DSP substrate specificities in the presence of Mg$^{2+}$ showing a robust PP-Insp phosphohydrolase activity against 5-Insp$_7$, 1,5-Insp$_9$, and 3,5-Insp$_9$, in vitro (Table S1); and oligonucleotide sequences (Table S2) (PDF).
Complete contact information is available at: https://pubs.acs.org/10.1021/acs.biochem.2c00145

Author Contributions

- P.G. and R.S. contributed equally to this manuscript.
- D.L., G.S., and P.G. conceived the study.
- P.G. generated yeast mutants and performed all yeast experiments, generated constructs, isolated T-DNA insertion lines, performed HPLC analyses of plants, performed qPCR analyses, performed plant infiltration and TiO₂ pull-downs, and analyzed most of the experiments. R.S. purified recombinant proteins and carried out and analyzed in vitro kinase assays. G.L. and D.Q. performed CE-ESI-MS/MS analysis and isomer identification. J.W. and J.S. generated constructs and established the expression and purification of recombinant proteins. N.J., M.H., and K.R. synthesized InsP₇ and InsP₁₀ isomers. N.F.-R. isolated T-DNA insertion lines, performed HPLC analyses of plants, generated constructs, and performed qPCR analyses. R.F.H.G. generated plant samples for CE-ESI-MS analysis and did transcriptome analysis. M.N.T. synthesized ¹³C-InsP standards. V.G. analyzed and quantified HPLC analyses. P.G., G.S., D.L., H.J.J., and D.F. supervised the experimental work. P.G., G.S., R.S., D.L., and R.Y. wrote the manuscript with input from all authors.

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Notes

The authors declare no competing financial interest.

During the revision of this manuscript, a study by Wang and colleagues¹ reported high-resolution crystal structures of Arabidopsis PFA-DSPI in complex with 5-InsP₇, 6-InsP₇, and 5-InsP₁₀ analogues and provided evidence for efficient in vitro phosphatase activity of this enzyme against 5-InsP₁₀ as well as weaker in vitro activities against 4-InsP₇ and 6-InsP₇ in agreement with our findings.

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