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**Alternative metabolic fates of phosphatidylinositol produced by PI-synthase isoforms in *Arabidopsis thaliana***

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**Abbreviations:** CDP-DAG, cytidinediphospho-diacylglycerol; CFP, cyan fluorescent protein; DAG, diacylglycerol; ER, endoplasmic reticulum; EYFP, enhanced yellow fluorescent protein; GC, gas chromatography; HFT, NFT, types of beam splitters; ; MBS, main beam splitter; PIS, phosphatidylinositol synthase; PLD, phospholipase D; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol-4-phosphate; PtdIns(4,5)P<sub>2</sub>, phosphatidylinositol-4,5-bisphosphate PtdOH, phosphatidic acid; TLC, thin layer chromatography

## Synopsis

Phosphatidylinositol (PtdIns) is an important precursor for inositol-containing lipids, including polyphosphoinositides, which have multiple essential functions in eukaryotic cells. It was previously proposed that different regulatory functions of inositol-containing lipids may be performed by independent lipid-pools, however, it remains unclear how such subcellular pools are established and maintained. Here, a previously-uncharacterised *Arabidopsis* gene-product with similarity to the known *Arabidopsis* PI-synthase, PIS1, is shown to be an active enzyme, PIS2, capable of producing PtdIns *in vitro*. PIS1 and PIS2 diverged slightly in substrate preferences for cytidinediphospho-diacylglycerol species differing in fatty acid composition, PIS2 preferring unsaturated substrates *in vitro*. Transient expression of fluorescence-tagged PIS1 or PIS2 in onion epidermal cells indicates localisation of both enzymes in the endoplasmic reticulum and, possibly, Golgi, as was reported previously for fungal and mammalian homologues. Constitutive ectopic overexpression of PIS1 or PIS2 in *Arabidopsis* plants resulted in elevated levels of PtdIns in leaves. PIS2-overexpressors additionally exhibited significantly-elevated levels of PtdIns-4-phosphate and PtdIns-4,5-bisphosphate, whereas polyphosphoinositides were not elevated in plants overexpressing PIS1. In contrast, PIS1-overexpressors contained significantly-elevated levels of diacylglycerol and phosphatidylethanolamine, an effect not observed in plants overexpressing PIS2. Biochemical analysis of transgenic plants regarding fatty acids associated with relevant lipids indicates that lipids increasing with PIS1-overexpression were enriched in saturated or monounsaturated fatty acids, whereas lipids increasing with PIS2-overexpression, including polyphosphoinositides, contained more unsaturated fatty acids. The data indicate that PtdIns-populations originating from different PI-synthase isoforms may enter alternative routes of metabolic conversion, possibly based on specificity and immediate metabolic context of the biosynthetic enzymes.

## Introduction

The complex interplay of physiological processes in eukaryotic organisms, including plants, relies in part upon the regulation by inositol-containing lipids, such as polyphosphoinositides [1-4]. As regulatory functions of polyphosphoinositides require tight spatio-temporal coordination [5, 6], it has been proposed for a range of eukaryotic model systems that alternative regulatory functions of inositol-containing lipids are performed by discrete lipid pools of different metabolic origin [5-11]. For instance, we have previously demonstrated that stress-induced and constitutive polyphosphoinositides of *Arabidopsis* plants differ in the composition of their respective associated fatty acids, indicating an origin of these lipid pools from precursors differing in fatty composition [11]. So far, it is unresolved how independent pools of inositol-containing lipids can be established and maintained.

The key biosynthetic precursor of inositol-containing lipids is phosphatidylinositol (PtdIns), formed by phosphatidylinositol synthase (PI-synthase, EC 2.7.8.11) catalyzing the transfer of *myo*-inositol onto an activated cytidinediphospho-diacylglycerol (CDP-DAG) [12]. PI-synthases from different biological sources have previously been characterised, including examples from mammals [13, 14], fungi [15] and plants [16-18]. A characteristic property of reported PI-synthase sequences is a CDP-alcohol phosphatidyltransferase-domain and the possession of four hydrophobic membrane-spanning domains identifying PI-synthases as membrane-integral proteins. A blast-search (blastp) with the amino acid sequence of the *Arabidopsis* PI-synthase, PIS1, against publicly-accessible genomic databases indicates the presence of more than one PI-synthase isoform in most eukaryotic organisms, including plants, fungi and mammals, suggesting that distinct PI-synthase isoforms may contribute to the establishment of different physiological pools of inositol-lipids. Besides the known *PIS1* gene locus (At1g68000), the *Arabidopsis* genome contains at least one more gene (At4g38570, *PIS2*) with similarity to PI-synthase-coding sequences, which has not been functionally characterised. Because mammalian and fungal PI-synthases have been reported to reside at the interface of the endoplasmic reticulum (ER) and Golgi-stacks [19, 20], and plant PI-synthase activity has been found associated with microsomal fractions [18], PtdIns-production is thought to take place in the ER and/or the Golgi, although precise localisation studies have not been performed on plant enzymes. From multiple reports of polyphosphoinositide-formation in cellular compartments other than ER or Golgi [4, 9, 21-24] the question arises how PtdIns is channeled to those other cellular locations in order to provide PtdIns necessary for polyphosphoinositide production. Several mechanisms have been proposed by which PtdIns may be distributed throughout the cell, and possible

explanations include the action of PtdIns-specific lipid transfer proteins [25, 26], a distribution by targeted vesicle delivery, or a combination of both.

PtdIns can be converted into other lipid-intermediates by several alternative pathways. The inositol-headgroup can be successively phosphorylated at the D3, D4, or D5-positions by phosphatidylinositol-kinases (PI-kinases) and phosphatidylinositolphosphate-kinases (PIP-kinases), giving rise to a variety of polyphosphoinositides, many of which with reported regulatory effects on physiology [1, 3, 5]. Alternatively, PtdIns can be degraded by phospholipase D (PLD) to phosphatidic acid (PtdOH), which is a central biosynthetic intermediate of phospholipid metabolism and can be transformed into any other glycerophospholipid [27-29]. In addition to the pathways mentioned, a number of other lipid-modifying enzymes, including phospholipases C, A1 and A2, and inositolphosphoceramide synthase may convert PtdIns to a variety of other products [30]. In this context we aimed to address the question whether PtdIns produced by different *Arabidopsis* PI-synthase isoforms may enter discernible alternative routes of further metabolic conversion.

Here we show that recombinant PIS2 protein is catalytically active as a PI-synthase *in vitro* and that *Arabidopsis*, thus, contains at least two PI-synthase isoforms. In the course of experiments for *in vitro* characterisation, subtle preferences of PIS1 and PIS2 for saturated and unsaturated CDP-DAG substrates, respectively, were observed. PIS1 and PIS2 are both localised in the ER with only very slight differences in localisation between the isoforms. Increased PtdIns-levels with overexpression of PIS1 or of PIS2 in transgenic *Arabidopsis* plants were correlated to either increased DAG- and PtdEtn-levels (PIS1) or to increased levels of polyphosphoinositides (PIS2). The data suggest a role for PI-synthase isoforms in the channeling of PtdIns towards different pools of PtdIns-derived lipids.

## Experimental

### cDNA-constructs

The cDNA sequences encoding *Arabidopsis* PIS1 and PIS2 were amplified from cDNA prepared from flowers and siliques using the primer combinations 5'-GATCGAGTCGACATGGCTAAAAAGGAGAGAC-3' (primer A)/5'-GATCGAGCGCCGCTCAAGGCTTCTGCTGCTTCTCTATA-3' and 5'-GATCGAGTCGACATGGCTAAACAGAGACCGGCG-3' (primer B)/5'-GATCGAGCGCCGCTCAAGGCTTCTTATGCTGTTTCTC-3', respectively. For constructs to be c-terminally tagged by enhanced yellow-fluorescent protein (EYFP), *PIS1*

and *PIS2* cDNA fragments were amplified using the primer combinations primer A/5'-GATCGAGCGGCCGCTAGGCTTCTGCTGCTTCTCTATA-3' and primer B/5'-GATCGAGCGGCCGCTAGGCTTCTTATGCTGTTTCTC-3', respectively, leaving out the stop-codons and introducing one additional nucleotide base for in-frame-fusion with the tag. The cDNAs encoding the enhanced yellow fluorescent protein (EYFP)- and cyan fluorescent protein (CFP)-tags were amplified from plasmids carrying the authentic clones provided by Dr. Martin Fulda (Göttingen), in both cases using the primer combination 5'-GATCGCGGCCGCCCATGGTGAGCAAGGGCGAG-3'/5'-GATCGATATCTTACTTGTACAGCTCGTCCATG-3'. All amplifications were carried out using Phusion High-Fidelity DNA polymerase (Finnzymes) and a PCR-protocol of 2 min denaturing at 95°C followed by 36 cycles of 30 s denaturing at 95°C, 30 s annealing at 60°C and 1 min elongation at 72°C, with a terminal elongation step for 5 min at 72°C. Amplicons were cloned into the pGEM-T-Easy vector (Promega) according to manufacturer's instructions and sequenced. *PIS1*- and *PIS2*-cDNA clones were moved as *SalI/NotI*-fragments into the bacterial expression vector pET28b (Novagen). For plant expression, the plasmid pUC18 (Fermentas) was modified by the addition of multiple cloning site and gateway cassette of the pENTR2b-vector (Invitrogen), yielding the plasmid pUC18-ENTR. The cDNAs encoding EYFP or CFP were introduced into pUC18-ENTR as *NotI/EcoRV*-fragments, yielding the plasmids pUC18-ENTR-EYFP and pUC18-ENTR-CFP, respectively. *PIS1*- and *PIS2*-cDNA clones were moved as *SalI/NotI*-fragments first into pUC18-ENTR; *PIS1*- and *PIS2*-cDNA-fragments lacking the stop-codon were moved as *SalI/NotI*-fragments into pUC18-ENTR-EYFP or into pUC18-ENTR-CFP. Subsequently, cDNAs were moved from the pUC18-derived vectors into a pCAMBIA plasmid (pCAMBIA3300-OGC) modified to contain a Gateway cassette (Invitrogen), using Gateway technology (Invitrogen) according to manufacturer's instructions.

### Heterologous expression in *E. coli*

*PIS1*- and *PIS2*-pET28b constructs were heterologously expressed in *E. coli* C43(DE3) cells optimised for the accumulation of integral membrane proteins [31]. Cells were transformed by heat-shock-treatment [32]. Expression cultures (400 mL) were grown in Luria-Bertani-medium [33] in 1-L Erlenmeyer flasks at 37°C shaking at 200 rpm. Protein expression was induced by addition of 1 mM isopropyl- $\beta$ -D-thiogalactoside at an optical density (600 nm) of 0.8. Cultures were harvested 4 h after induction, and cells were disrupted on ice by three 30 s-pulses of ultrasound using a Sonifier Cell disruptor B15 (Branson) at 50

% performance. The homogenate was centrifuged for 10 min at 840 x g at 4°C, and the cleared supernatants were subjected to ultracentrifugation at 100,000 x g for 60 min at 4°C. Membrane pellets were resuspended in 1 mL of 20 mM Tris-HCl, 1 mM EDTA, 8 mM β-mercaptoethanol, pH 8.0, containing 20 % (v/v) glycerol. Protein contents were determined by the Bradford method (BioRad) with BSA as a standard [34].

### Synthesis of CDP-DAG substrates

CDP-DAG substrates not available commercially were synthesized from PtdOH by chemical modification via a lipid-morpholidate as previously described [35]. PtdOH-species used for chemical conversion were obtained from Avanti (1,2-dipalmitoyl-glycerin-3-phosphate, 1,2-dioleoyl-glycerin-3-phosphate and 1,2-dilinoleoyl-glycerin-3-phosphate) or Sigma (1,2-diheptadecanoyl-glycerin-3-phosphate). In addition to synthetic lipids, PtdOH was extracted from 50 g of green leaf tissue [36], purified by thin-layer-chromatography (TLC) and used for conversion to CDP-DAG. CDP-DAG species obtained comigrated with authentic CDP-DAG standards (Avanti) and were purified by TLC. Samples were dissolved in CHCl<sub>3</sub>, dried under streaming N<sub>2</sub> and diluted in CHCl<sub>3</sub> to a lipid content of approximately 0.3 mg mL<sup>-1</sup>.

### PI-synthase activity tests

The activity of recombinant PIS1 and PIS2 proteins was detected in bacterial membrane pellets by monitoring the production of PtdIns from CDP-DAG (Avanti) and non-labelled *D-myo*-inositol (Sigma). Before addition of soluble components, CDP-DAG was dissolved in ethanol and dried into glass reaction tubes under streaming nitrogen. The assay mixture contained 50 mM Tris-HCl, pH 8.0, 20 mM MgCl<sub>2</sub>, 1.5 mM *D-myo*-inositol, 0.9 mM CDP-DAG, and 1 % (v/v) Triton-X100 in a reaction volume of 200 μL, as described [37]. In experiments to determine the fatty acid compositions of CDP-DAG substrates and PtdIns formed, CDP-DAG substrates were used at a concentration of 0.2 mM. Reactions were started by the addition of 20 μg of bacterial membranes and incubated for 30 min at room temperature. Lipid reaction products were extracted [36], and the organic phase was washed with 1 % (w/v) NaCl solution and dried under streaming nitrogen. Samples were subjected to TLC using a developing solvent containing methylacetate, 2-propanol, CHCl<sub>3</sub>, CH<sub>3</sub>OH and 0.25 % (w/v) aqueous KCl-solution at volume ratios of 25:25:25:10:9 [38]. Lipids were visualised by submerging dried chromatography plates in an aqueous solution of 10 % (w/v) CuSO<sub>4</sub> (Sigma) containing 8 % (v/v) H<sub>3</sub>PO<sub>4</sub> (Sigma) and subsequent heating to 180°C. Lipid

products were identified according to comigration with authentic standards (Avanti). Alternatively to destructive visualization, lipids were reisolated from TLC-plates and analyzed for their fatty acid composition as described below.

### Lipid extraction and biochemical analysis

Plant material was ground under liquid nitrogen to a fine powder. Phospholipids were extracted from powdered plant material by using an acidic extraction protocol [39]. Lipids were separated by TLC on silica gel plates (Merck) using developing solvents for optimal resolution: for phosphoinositidies and PtdOH,  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{NH}_4\text{OH}:\text{H}_2\text{O}$  (57:50:4:11(v/v/v/v)) [40]; for PtdEtn, acetone:toluol: $\text{H}_2\text{O}$  (91:30:7 (v/v/v)) [41]; for isolating phosphatidylinositol, the same developing solvent was used as described as part of the PI-synthase assay. Lanes with lipid standards (5  $\mu\text{g}$ ) run in parallel to biological samples or to lipids formed in *in vitro*-assays were cut and lipids visualised as described above. Unstained lipids were located on the remaining parts of the TLC plates according to standard migration, were scraped, redissolved in their respective developing solvents and dried under  $\text{N}_2$  flow. Lipids were transmethylated [42], fatty acid methyl esters dissolved in acetonitril and analysed using a GC6890 gas chromatograph with flame ionization detection (Agilent) fitted with a 30 m x 250  $\mu\text{m}$  DB-23 capillary column (Agilent). Helium flowed as a carrier gas at 1  $\text{ml min}^{-1}$ . Samples were injected at 220°C. After one min at 150°C, the oven temperature was raised to 200°C at a rate of 8°C  $\text{min}^{-1}$ , then to 250°C at 25°C  $\text{min}^{-1}$ , and then kept at 250°C for 6 min. Fatty acids were identified according to authentic standards and by their characteristic mass spectrometric fragmentation patterns (data not shown), and quantified according to internal tri-pentadecanoic acid standards of known concentration. Due to limiting material in samples representing isolated minor lipids, fatty acids of low abundance may be absent from fatty acid patterns.

### Transient gene expression by particle bombardment

One-mm epidermal sections of quartered onion (*Allium cepa*) bulbs were placed on moist whatman paper and were transformed by bombardment with plasmid-coated 1  $\mu\text{m}$  gold-particles using a helium-driven particle accelerator (PDS-1000/He; BIO-RAD) with 1350 psi rupture discs at a vacuum of 28 inches of mercury. Gold-particles (1.25 mg) were coated with 3-7  $\mu\text{g}$  of plasmid DNA of the pCAMBIA-vectors also used for plant transformation. After bombardment onion sections were incubated in covered petri dishes for 24 h at room temperature in a closed moist styrofoam box.

### Fluorescence microscopy.

Images of onion epidermal peels were recorded using a Zeiss LSM 510 meta confocal microscope. EYFP was excited at 514 nm and imaged using an HFT 405/514/633 nm major beam splitter (MBS) and a 530-600 nm band pass filter; CFP was excited at 405 nm and imaged using an HFT 405/514/633 nm MBS and a 470-500 nm band pass filter. In coexpression experiments, CFP and EYFP were synchronously excited at 405 nm and 514 nm, respectively, and imaged using an HFT 405/514/633 nm and NFT 515 nm MBSs and 470-500 nm and 530-600 nm band pass filters, respectively.

### Plant culture and transformation

*Arabidopsis thaliana* (L.), ecotype Columbia-0 (col-0) plants were grown on soil in growth chambers (York) at 22-25 °C under a regime of 8 h exposure to 130 -150  $\mu\text{mol photons m}^{-1}\text{s}^{-1}$  and 16 h darkness and at approximately 60 % humidity. Recombinant DNA-constructs were introduced into *Arabidopsis* plants by *Agrobacterium tumefaciens*-mediated transformation, using *A. tumefaciens* strain EHA105 [43] and the floral-dip method [44]. Independent transformants were selected according to resistance against aerosolic glufosinate-ammonium (Bayer). All analyses were carried out on six-week-old plants. Plant growth was estimated according to leaf area of plants of the same age. Leaf areas were determined using Blattfläche 1.0.4.6 software (DatInf GmbH) according to manufacturer's instructions.

## Results

### ***Arabidopsis* contains two distinct PI-synthase isoforms capable of generating phosphatidylinositol**

A search of publicly-accessible databases [30, 45] suggested the gene locus At4g38570 to encode a previously-uncharacterised PI-synthase isoform in *Arabidopsis* in addition to the known PIS1 encoded by the locus At1g68000. The amino acid sequence of the deduced PIS2 protein indicates strong similarity to other PI-synthases from plants, fungi or mammals (Figure 1). According to published transcript-array data accessible through the Genevestigator portal [45] the *PIS1*- and *PIS2*-genes are ubiquitously expressed at modest levels throughout the plant, indicating no tissue-specific functions for either isoenzyme. Sequencing of independently-cloned full-length *PIS2*-cDNAs consistently indicated a

mismatch against the published *Arabidopsis* genomic sequence at position 444 (G instead of C), resulting in the amino-acid exchange C148W, as indicated (Figure 1). As C148 is conserved in PI-synthases from plants and fungi, but not in mammalian enzymes (Figure 1), at present the relevance of the polymorphism is unclear. In order to test the functionality of the PIS2 gene product, the cDNA was recombinantly expressed in *E. coli*. Production of the correct recombinant proteins was verified by immunodetection of the poly-His-tagged proteins, and PIS1- and PIS2-proteins were present only in pellet-fractions representing insoluble membrane-proteins (data not shown). Protein levels of PIS1 and PIS2 preparations tested were adjusted according to immunodetection. Recombinant PIS2 (C144W) protein exhibited substantial activity, producing PtdIns from CDP-DAG and *D-myo*-inositol substrates (Figure 2). In side-by-side expression experiments, PIS2 activity was comparable to that of the previously reported PIS1 (Figure 2). Affinity-purification of poly-His-tagged recombinant enzymes was not successful, likely because of the inherent difficulties with reconstituting membrane-integral enzymes in an active form, and, therefore, no specific activity was calculated. Even so, the data presented establish PIS2 as an active PI-synthase isoform in *Arabidopsis*.

### **PIS1 and PIS2 proteins have preferences for different CDP-DAG fatty acyl species**

In order to test the substrate preferences of PIS1 and PIS2, catalytic activity was assayed in the presence of CDP-DAG species differing in fatty acid composition (Figure 3). CDP-DAG substrates tested included 1,2-dipalmitoyl-CDP-DAG; 1,2-diheptadecanoyl-CDP-DAG; 1,2-dioleoyl-CDP-DAG and 1,2-dilinoleoyl-CDP-DAG. In a first experiment PI-synthase activity was assayed under identical conditions against substrates presented individually (Figure 3 A). Catalytic activity was consistently higher with substrates containing C18-fatty acids than with those containing medium-chain fatty acids. For PIS1, the highest activity was determined using 1,2-dioleoyl-CDP-DAG, whereas PIS2 was more active with 1,2-dilinoleoyl-CDP-DAG. In order to assess substrate preferences in a competition assay, activity tests were performed against a mixture of all four available synthetic CDP-DAG substrates (Figure 3 B). The preference of PIS1 and PIS2 for substrates containing C18-fatty acids was confirmed. In the competitive assay PIS1 preferentially converted 1,2-dioleoyl-CDP-DAG, whereas PIS2 preferred 1,2-dilinoleoyl-CDP-DAG. All CDP-DAG species tested so far were of synthetic origin and symmetrical with regard to the associated fatty acids. As biological lipids usually are not symmetrical and contain different fatty acids, CDP-DAG synthesized from PtdOH isolated from *Arabidopsis* leaves was also tested as a substrate for

PIS1 and PIS2 (Figure 3 C). The pattern of fatty acids associated with the "biological" CDP-DAG input-mixture in these experiments resembled that of structural phospholipids of *Arabidopsis* leaves [11, 46, 47]. After *in vitro*-conversion of the CDP-DAG by PIS1 or PIS2, the fatty acid composition of CDP-DAG not converted (CDP-DAG residue) and that of newly-formed PtdIns (PtdIns product) was determined. The biological CDP-DAG mixture was converted to PtdIns with high efficiency by PIS2, whereas conversion by PIS1 was only moderate (Figure 3 C). PtdIns formed by PIS1 contained mainly palmitic acid and, to a lesser degree, linoleic acid. The CDP-DAG residue after PIS1 conversion contained high proportions of linoleic acid and linolenic acids, indicating that CDP-DAG species containing these fatty acids were not effectively converted by PIS1. In contrast, PtdIns formed by PIS2 from biological CDP-DAG contained predominantly palmitic acid, linoleic acid and linolenic acid, concomitant with a severe depletion of these fatty acids in the corresponding CDP-DAG residue. Together the *in vitro* data suggest a preference of recombinant PIS2 for polyunsaturated CDP-DAG species.

### **PIS1 and PIS2 proteins localise in the ER of onion epidermal cells**

In a previous study, plant PI-synthase activity was reported to associate with microsomal fractions [18], and because mammalian and fungal PI-synthases have been reported to reside in both ER and Golgi-membranes [19, 20], experiments were initiated to test the subcellular localisation of *Arabidopsis* PIS1 and PIS2 proteins. In-frame fusions of PIS1 or PIS2 with c-terminally-attached EYFP- CFP-tags were transiently expressed in onion epidermal cells, and the fluorescence distribution of the PIS1-EYFP and PIS2-CFP proteins synchronously monitored by laser-scanning confocal microscopy (Figure 4). PIS1-EYFP and PIS2-CFP both exhibited clear ER-localisation (Figure 4 A). Differences in the localisation of PIS1-EYFP and PIS2-CFP were restricted to small punctate structures of limited occurrence (Figure 4 A, arrowheads) where PIS1 was observed in the centre and PIS2 at the periphery (Figure 4 B). The distribution of PIS1 and PIS2 was evaluated in relation to that of the Golgi-associated signal peptide of the rat sialyl-S-transferase (Sial) [48]. When PIS1-EYFP or PIS2-EYFP were coexpressed with CFP-Sial, a limited degree of overlap was observed, as shown for PIS1-EYFP in Figure 4 C. The combined localisation data indicate that PIS1 and PIS2 are present in the ER, including tubular domains in close proximity to Golgi-particles, and exhibit slight but reproducible differences in localisation patterns.

### **Constitutive ectopic overexpression of PIS1 or PIS2 in *Arabidopsis* affects plant growth**

In order to test whether the metabolism of polyphosphoinositides or other phospholipids could be disturbed by overproduction of PtdIns in plants, PIS1 and PIS2 were individually overexpressed in transgenic *Arabidopsis* plants under the constitutive cauliflower mosaic virus 35S-promoter. Successful transformation was confirmed by PCR-based genotyping of glufosinate-ammonium-resistant transformants and detection of the 35S-PIS1 or 35S-PIS2 transgene (data not shown). The T2-progeny of resistant plants exhibited severely diminished growth (Figure 4 A), indicating a substantial influence of transgene expression on physiology. Evaluation of plant size by automated leaf-area detection indicates a significant difference ( $p < 0.01$ ) in plant size compared to wild type plants grown in parallel (Figure 5 B).

### **Increased formation of PtdIns and PtdOH with ectopic overexpression of PIS1 or PIS2 in *Arabidopsis* plants**

Of the transgenic *Arabidopsis* lines expressing either PIS1 or PIS2, three lines each were chosen for detailed biochemical analysis of leaf lipids. The first question asked was whether PI-synthase overexpression was correlated with increased accumulation of PtdIns in the transgenic plants. GC-based quantification of fatty acids associated with PtdIns [11] indicates that PtdIns levels were significantly ( $p < 0.01$ ) increased in all PI-synthase overexpressing lines tested (Figure 6 A). This result contrasts somewhat with previous data indicating that the levels of PtdIns are tightly controlled and kept at a constant level with either metabolic disturbance or with applied stress-treatments [49]. When the levels of PtdOH as a potential breakdown-product of PtdIns were tested in the PI-synthase overexpressing lines, PtdOH-levels were found to also be significantly increased ( $p < 0.01$ ) over those detected in wild type plants (Figure 6 B), suggesting action of regulatory mechanisms to limit PtdIns-accumulation.

### **Alternative metabolic fates of PtdIns produced by PIS1 or PIS2**

When other lipids were included in the analysis of PIS1- or PIS2-overexpressors, it was found that PIS1 and PIS2 exerted different effects on downstream metabolites (Figure 7). Whereas overexpression of PIS1 resulted in a significant increase in the levels of DAG and phosphatidylethanolamine (PtdEtn) in all cases, no such increases were observed with expression of PIS2 (Figure 7 A and B). In contrast, the levels of the polyphosphoinositides, PtdIns<sub>4</sub>P and PtdIns(4,5)P<sub>2</sub>, were significantly ( $p < 0.01$ ) increased in plants overexpressing PIS2, but not in those overexpressing PIS1 (Figure 7 C and D). The lipid analyses performed

indicate that despite of equivalent accumulation of PtdIns with overexpression of PIS1 or PIS2 (Figure 6 A) different lipid-metabolites were affected by PtdIns-overproduction through each PI-synthase isoform.

### **Lipid-backbone fatty acid composition indicates metabolic origin of downstream metabolites**

In order to provide evidence for the metabolic origin of phospholipids exhibiting altered levels correlated with PIS1 or PIS2 overexpression (Figure 8), the flow of intermediates into alternative pathways must be traced. As [<sup>3</sup>H]-*myo*-inositol headgroup-labeling is not feasible at a scale required for the detection of polyphosphoinositides in intact *Arabidopsis* tissue, and because the label would be lost from the part of the PtdIns-molecule that enters pathways for conversion to other phospholipids including PtdEtn, metabolites were traced according to the fatty acid composition of their glycerolipid-backbones, as previously described [11]. The fatty acid composition of relevant phospholipids isolated from wild type plants (Figure 8 A) indicates a high proportion (50-60 mol%) of polyunsaturated fatty acids associated with PtdIns, PtdOH and PtdEtn, whereas the polyphosphoinositides, PtdIns4P and PtdIns(4,5)P<sub>2</sub>, contained more saturated and monounsaturated fatty acids, as was reported previously [11]. With overexpression of PIS1 the fatty acid composition of PtdIns, PtdOH, DAG and PtdEtn shifted to contain less (30-45 mol%) polyunsaturated fatty acids and a greater proportion (55-65 mol%) of saturated and monounsaturated fatty acids (Figure 8 B), whereas the fatty acid composition of polyphosphoinositides was not obviously altered. In contrast, overexpression of PIS2 resulted in an increased proportion of 55-75 mol% polyunsaturated fatty acids associated with all lipids tested, most remarkably in PtdIns4P and PtdIns(4,5)P<sub>2</sub> (Figure 8 C). The proportion of polyunsaturated fatty acids associated with PtdIns4P and PtdIns(4,5)P<sub>2</sub> changed notably from only 25 and 15 mol% of the associated fatty acids in wild type plants, respectively, to 50 and 45 mol%, respectively, in plants overexpressing PIS2 (compare Figure 8 A with C). The data indicate that the degree of unsaturation of fatty acids associating with phospholipids affected by PI-synthase overexpression changes differentially for PIS1 or PIS2. Changes in the degree of unsaturation of fatty acids associated with phospholipids resulting from overexpression of PIS1 or PIS2 are summarized in supplemental Table 1.

## Discussion

The present study was initiated to explore effects of increased PtdIns-levels on plant lipid metabolism as a result of PI-synthase overexpression. In addition to a known *Arabidopsis* PI-synthase, PIS1, the previously uncharacterized PIS2-protein was included in this study and was first demonstrated to have PI-synthase activity *in vitro* (Figure 2). Activity tests were performed using crude bacterial extracts, because *E. coli* does not contain endogenous PI-synthase activity [16]. The lack of chaperones or posttranslational modifications required for correct functionality of many eukaryotic proteins expressed recombinantly in *E. coli* did not obviously affect the catalytic activity of PIS1 or PIS2 (Figure 2), indicating that chaperones or modifications are not essential for PI-synthase function. The presence of an unusual number of amino acid positions (>20 out of ~220 residues) conserved in PI-synthases either as T or S (Figure 1), some across organismic kingdoms, may indicate the presence of phosphorylation sites possibly regulating enzyme activity or localisation *in vivo*. A detailed investigation of catalytic activity of PIS1 and PIS2 *in vitro* against CDP-DAG species differing in associated fatty acyl moieties indicated that PIS1 and PIS2 differ in their substrate preferences (Figure 3). Whereas PIS1 preferred CDP-DAG substrates containing a combination of palmitic acid and linoleic acid, PIS2 was most active with CDP-DAG substrates containing the polyunsaturated linoleic acid and linolenic acid. The observation that PIS1 exhibited substantially less activity than PIS2 against the biological CDP-DAG suggests that the endogenous PIS1 substrate may not have been represented in the mixture of CDP-DAG-species tested.

By monitoring the subcellular distribution of fluorescence-tagged PIS1 and PIS2 proteins, ER-localisation is suggested for both plant enzymes (Figure 4). Both PIS1 and PIS2 associated with tubular ER-structures in close proximity to Golgi-particles (Figure 4 C), consistent with reports of fungal and mammalian PI-synthases which localise to the interface of ER and Golgi [19, 20]. While PIS1 and PIS2 generally colocalised in the ER, punctate ER-patterns were regularly observed to which both PIS1 and PIS2 associated, however with subtle differences in localisation (Figure 4 B). In this context it should be noted that algorithms for the prediction of subcellular targeting such as Predotar V1.03 [50] or iPSORT [51] did not yield consistent targeting predictions for PIS1 and PIS2, despite of substantial similarity of primary sequences (Figure 1). The interpretation of the visual information obtained by confocal microscopy was aided by the use of the Golgi-marker constructs, CFP-Sial [52], which is an established organellar marker for use in plants and has been used in previous studies [53]. The results support a view of PtdIns biosynthesis at the interface of the

lipid biosynthetic pathways in the ER and the secretory machinery of the Golgi. It is our working hypothesis that PI-synthases act in subtly-different metabolic contexts, and that the action of PIS1 and PIS2 may supply different pools of inositol-containing lipids with PtdIns.

Ectopic overexpression of PIS1 or the previously uncharacterised PIS2 resulted in significantly delayed growth of transgenic plants, suggesting that transgene expression was placing strain on metabolism or energy balance. It has previously been reported that the levels of PtdIns are not readily perturbed by stress-treatments affecting the levels of other phospholipids, including PtdIns-derived lipids, suggesting PtdIns-levels are tightly regulated [49]. Increases in PtdIns-levels in leaves of plants overexpressing PIS1 or PIS2 coincided with raised levels of PtdOH, the immediate PLD-breakdown product of PtdIns (Figure 6). The inhibitory effect of PI-synthase overexpression on plant growth (Figure 5) may, thus, be due to the establishment of a futile cycle of increased PtdIns-generation and breakdown and consequentially increased energy consumption. An alternative explanation are possible effects of altered polyphosphoinositide formation on signalling processes affecting plant growth or development [2, 3]. In order to monitor downstream metabolic effects of increased PtdIns-turnover in the transgenic plants, a range of candidate sinks for PtdIns-derived lipid backbones was analysed (Figure 7). Overexpression of PIS1 resulted in elevated DAG and PtdEtn levels in leaves of all transgenic lines tested (Figure 7 A and B), whereas that of PIS2 consistently supported increased polyphosphoinositide production (Figure 7 C and D). The hypothesis that PtdIns produced by PIS1 or PIS2 was channeled towards different routes of metabolic conversion was tested by tracing the glycerolipid backbones of phospholipid intermediates according to their fatty acid composition (Figure 8). The observation that PtdIns and other phospholipids increasing with PIS1-overexpression contained fatty acids with a lower degree of unsaturation, whereas PtdIns and phospholipids increasing with PIS2-overexpression contained fatty acids with a higher degree of unsaturation, suggests that the particular phospholipids may have their metabolic origin in distinct PtdIns-populations formed by PIS1 or PIS2, respectively. The data are consistent with the results of the *in vitro* characterisations, indicating differences in substrate preferences of PIS1 and PIS2 for CDP-DAG-species with particular fatty acid compositions. Influences of lipid-substrate fatty acid compositions on enzyme functionality have been previously demonstrated for other enzymes involved in the biosynthesis of inositol-lipids, including invertebrate PIP-kinases and phospholipase C [54], and mammalian phosphoinositide phosphatases [55].

In contrast to the situation with PIS1, overexpression of PIS2 resulted in several-fold increased levels of PtdIns4P and PtdIns(4,5)P<sub>2</sub> (Figure 7 B and C), most likely as a

consequence of increased substrate availability for PI-kinases and PIP-kinases, respectively. As the phosphorylation sequence leading to the formation of PtdIns(4,5)P<sub>2</sub> is likely not ER-associated in plants, PtdIns originating from PIS2-activity and entering this sequence must be delivered for further metabolic conversion by a different route than PtdIns produced by PIS1.

In summary, the data suggest that PIS1 and PIS2 may act in different metabolic contexts, resulting in PtdIns channeling towards different physiological pools. While it is clear that independent pools of inositol-lipids are present in plant cells [8-11], the establishment of such pools may also be controlled by the distribution of lipids other than PtdIns. Future experiments will be aimed at elucidating the contribution of other phospholipid-biosynthetic enzymes in the formation of distinct lipid pools.

### Acknowledgments

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## Figure Legends

**Figure 1. Alignment of deduced amino acid-sequences of PI-synthases from different biological origins.** Sequences were aligned using ClustalW and the alignment was optimised manually. Residues identical in five or more sequences are indicated in black, similar amino acids in grey. The dotted line indicates the conserved CDP-alcohol phosphatidyltransferase-domain. Black lines indicate hydrophobic domains suggested by analysis using TMpred [56]. The arrow indicates amino acid-position 148 of the deduced *Arabidopsis* PIS2-sequence affected (C148W) by the single-nucleotide-polymorphism (G444C) of the *PIS2*-allele isolated in this study. Origin of sequences: At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Zea mays*; Nc, *Neurospora crassa*; Mm, *Mus musculus*; Hs, *Homo sapiens*.

**Figure 2. PI-synthase activity of recombinant *Arabidopsis* PIS1 and PIS2 proteins *in vitro*.** PIS1 and PIS2 proteins were heterologously expressed in *E. coli* C43(DE3) cells. Protein contents were adjusted according to immunodetection. Catalytic activity of recombinant PIS1 and PIS2 proteins was tested in total solubilised membrane extracts by monitoring the conversion of CDP-DAG and D-*myo*-inositol substrates to PtdIns. Lipids were visualised by charring and identified according to authentic standards, as indicated. Data shown are from two representative experiments. The experiment was performed three times with similar results.

**Figure 3. Substrate preferences of recombinant PIS1 and PIS2 for different CDP-DAG species.** Recombinant PIS1 and PIS2 enzymes were tested *in vitro* against various species of CDP-DAG differing in the associated fatty acids. CDP-DAG substrate prior to conversion (white) and PtdIns formed by PIS1 (grey) or PIS2 (black) after conversion were reisolated from TLC-plates and quantified by GC-analysis of associated fatty acids. A, PtdIns formation from individual synthetic substrates. B, PtdIns formation from a mixture of synthetic substrates. C, A mixture of "biological" CDP-DAG species was synthesized from PtdOH isolated from *Arabidopsis* rosette leaves, and PtdIns formation from this substrate mixture was tested. First, the fatty acid composition of the CDP-DAG substrate was analysed (CDP-DAG input). After *in vitro* conversion by PIS1 or PIS2, the fatty acid compositions of non-converted CDP-DAG (CDP-DAG residue) and of PtdIns produced (PtdIns product) was analysed, as indicated. Fatty acids are indicated at the bottom and are also distinguished by the

bars: white, 16:0; light grey, 16:1<sup>9</sup>; diamonds, 18:0; dark grey, 18:1<sup>9</sup>; stripes, 18:2<sup>9,12</sup>; black, 18:3<sup>9,12,15</sup>.

**Figure 4. Subcellular distribution of *Arabidopsis* PIS1 and PIS2 transiently expressed in onion epidermal cells.** PIS1 and PIS2 were transiently expressed in onion epidermal cells as fusion proteins to c-terminally-attached EYFP- or CFP-reporters. The fluorescence distribution was synchronously monitored for EYFP (left panels) and CFP (middle panels). The right panels show the merged image. Yellow colour indicates colocalisation. A, Coexpression of PIS1-EYFP and PIS2-CFP. Arrowheads, punctate structures with distinct patterns of EYFP and CFP-fluorescence. Inset, Magnified view of punctate structure. B, Gathering of punctate structures sometimes observed with coexpression of PIS1-EYFP and PIS2-CFP. C, Coexpression of PIS1-EYFP with the Golgi-marker, CFP-Sial. Bars, 10  $\mu$ m (A, C); 5  $\mu$ m (B)

**Figure 5. Influence of constitutive ectopic PI-synthase overexpression on *Arabidopsis* growth.** PIS1 and PIS2 were ectopically expressed in *Arabidopsis* plants under the cauliflower-mosaic-virus 35S-promoter. A, Habitus of five-week-old T2-offspring of glufosinate-ammonium-resistant transformants expressing PIS1 or PIS2 in comparison to wild type plants, as indicated. B, Mean leaf area of wild type plants (white), and five-week-old T2-offspring of glufosinate-ammonium-resistant transformants expressing PIS1 (grey) or PIS2 (black). Data are the means of 7-10 plants each for wild type or the independent transgenic lines  $\pm$  SD. Differences in the growth of transgenic lines are significant according to a student's t-test ( $p < 0.01$ ).

**Figure 6. Increased levels of PtdIns and PtdOH in *Arabidopsis* plants constitutively overexpressing PIS1 or PIS2.** The levels of isolated phospholipid classes were determined by GC-quantification of associated fatty acids relative to data obtained with total lipid extracts. A, PtdIns; B, PtdOH. Data are the means of 7-10 plants each for wild type (WT, white) and independent PIS1 (grey) or PIS2 (black) overexpressor lines, as indicated  $\pm$  SD. Lipid levels in the transgenic lines are significantly increased over those of wild type plants according to a student's t-test ( $p < 0.01$ ).

**Figure 7. Differential effects of PIS1 or PIS2 overexpression on lipid metabolism.** The levels of isolated phospholipid classes or DAG were determined by GC-quantification of

associated fatty acids relative to data obtained with total lipid extracts. A, DAG; B, PtdEtn; C, PtdIns4P; D, PtdIns(4,5)P<sub>2</sub>. Data are the means of 7-10 plants each for wild type (WT, white) and independent PIS1 (grey) or PIS2 (black) overexpressor lines, as indicated  $\pm$  SD. Asterisks indicate significant increases in lipid levels over those of wild type plants according to a student's t-test ( $p < 0.01$ ).

**Figure 8. Differential effects of PIS1 or PIS2 overexpression on phospholipid fatty acid composition.** The fatty acid compositions of PtdIns, PtdOH, DAG, PtdEtn, PtdIns4P and PtdIns(4,5)P<sub>2</sub> were analysed by GC for wild type plants (left panel) and for plants overexpressing PIS1 (middle panel) or PIS2 (right panel). The proportion of each fatty acid is given as mol% of the associated fatty acids of each lipid. Fatty acids correspond to the bar segments, as indicated at the right. Data represent the means of 7-10 plants for wild type and 7-10 plants of the representative overexpressor lines, PIS1-1 and PIS2-1. The experimental error is approximately 15-20% and is not marked in the graphs for better clarity. Fatty acids are indicated by the bars: white, 16:0; light grey, 16:1<sup>9</sup>; diamonds, 18:0; dark grey, 18:1<sup>9</sup>; stripes, 18:2<sup>9,12</sup>; black, 18:3<sup>9,12,15</sup>. For an estimate of fatty acid unsaturation in individual lipids in wild type and more transgenic lines, please refer to supplemental Table 1.

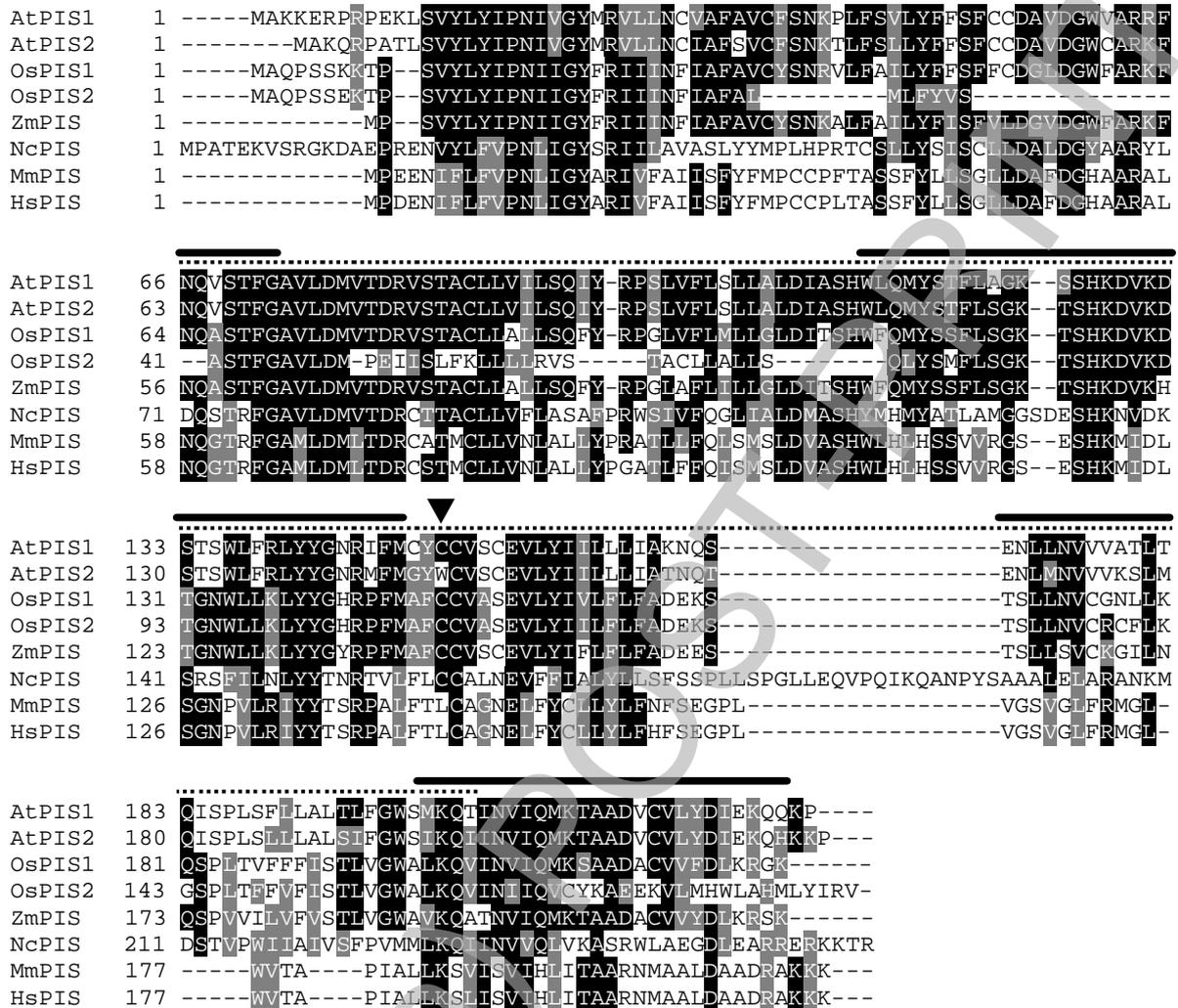
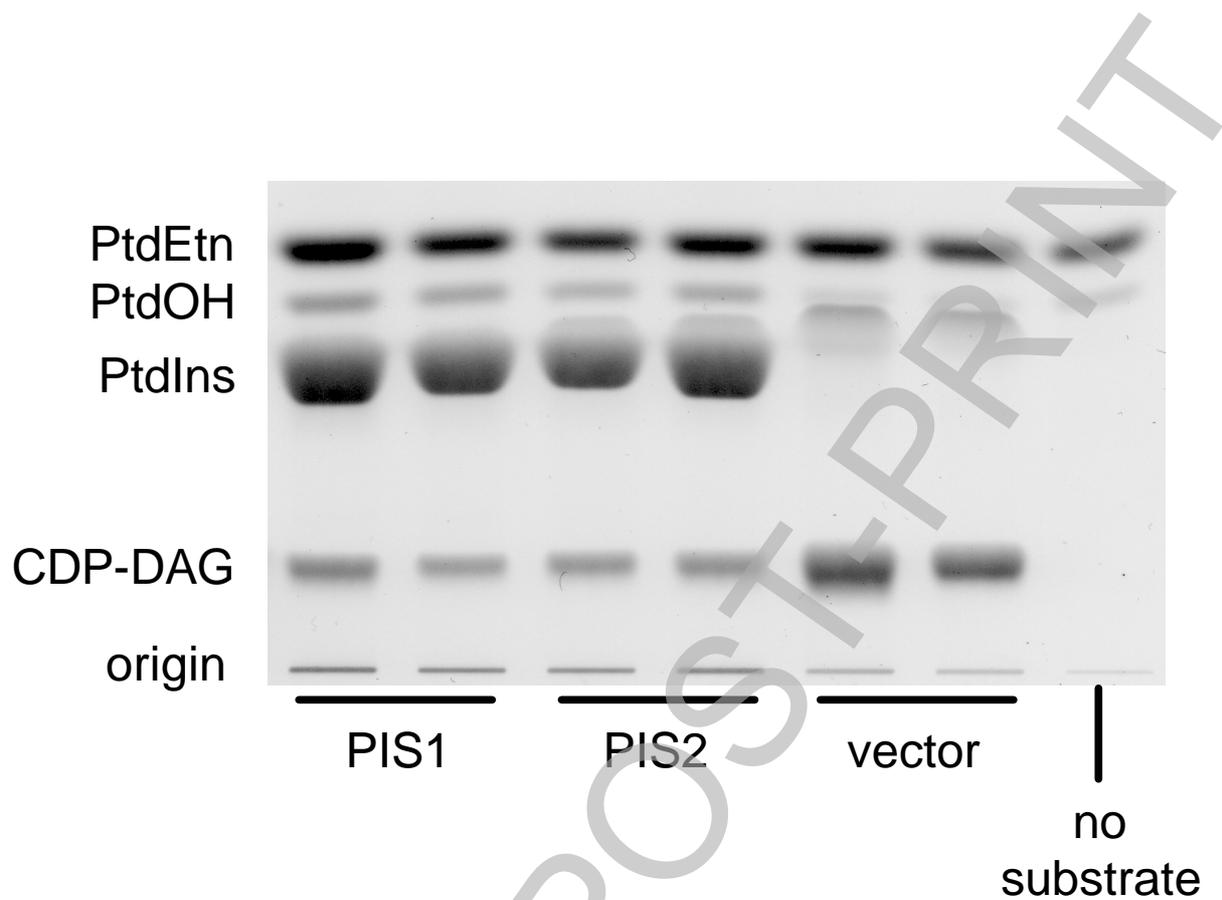
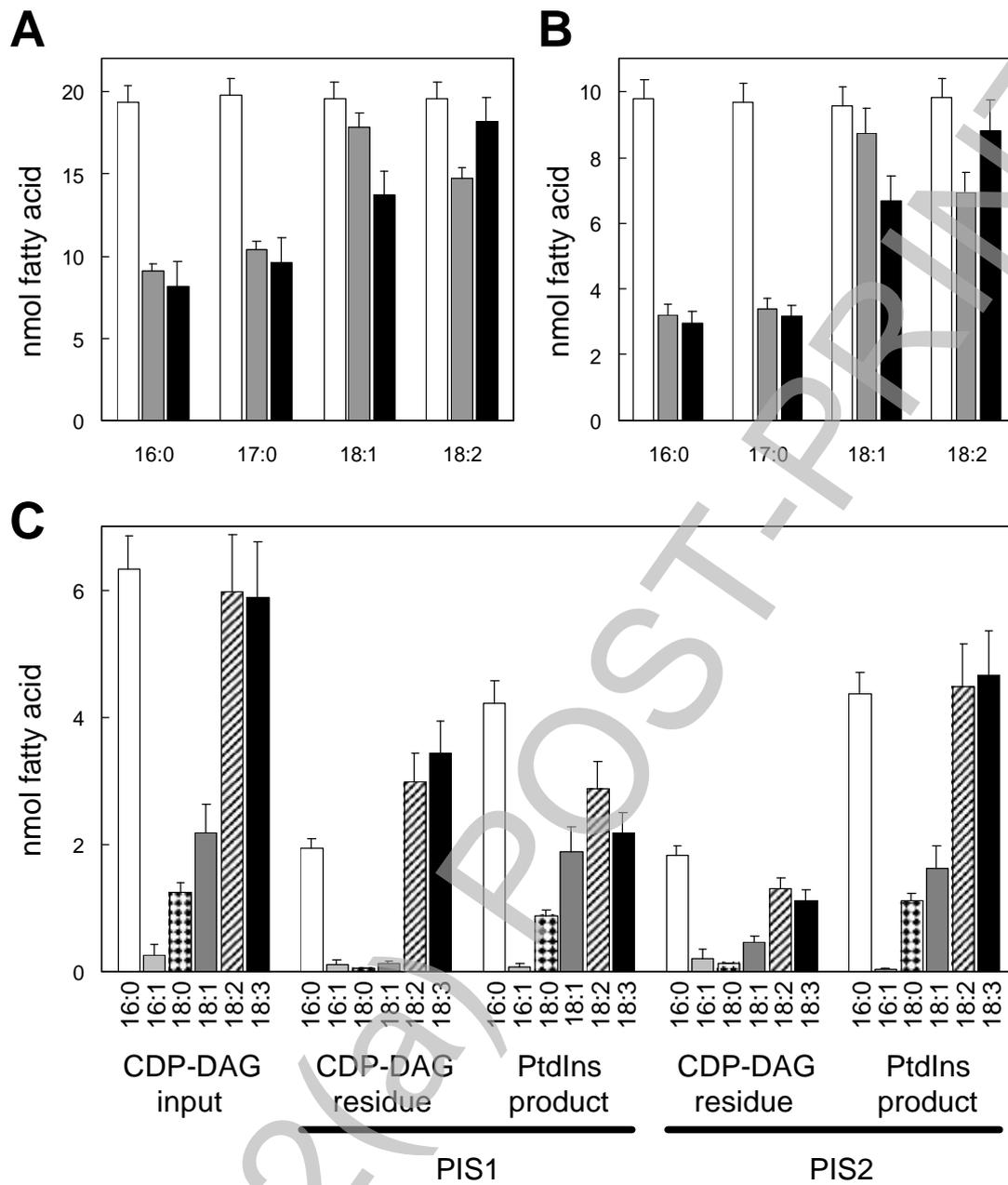


Figure 1



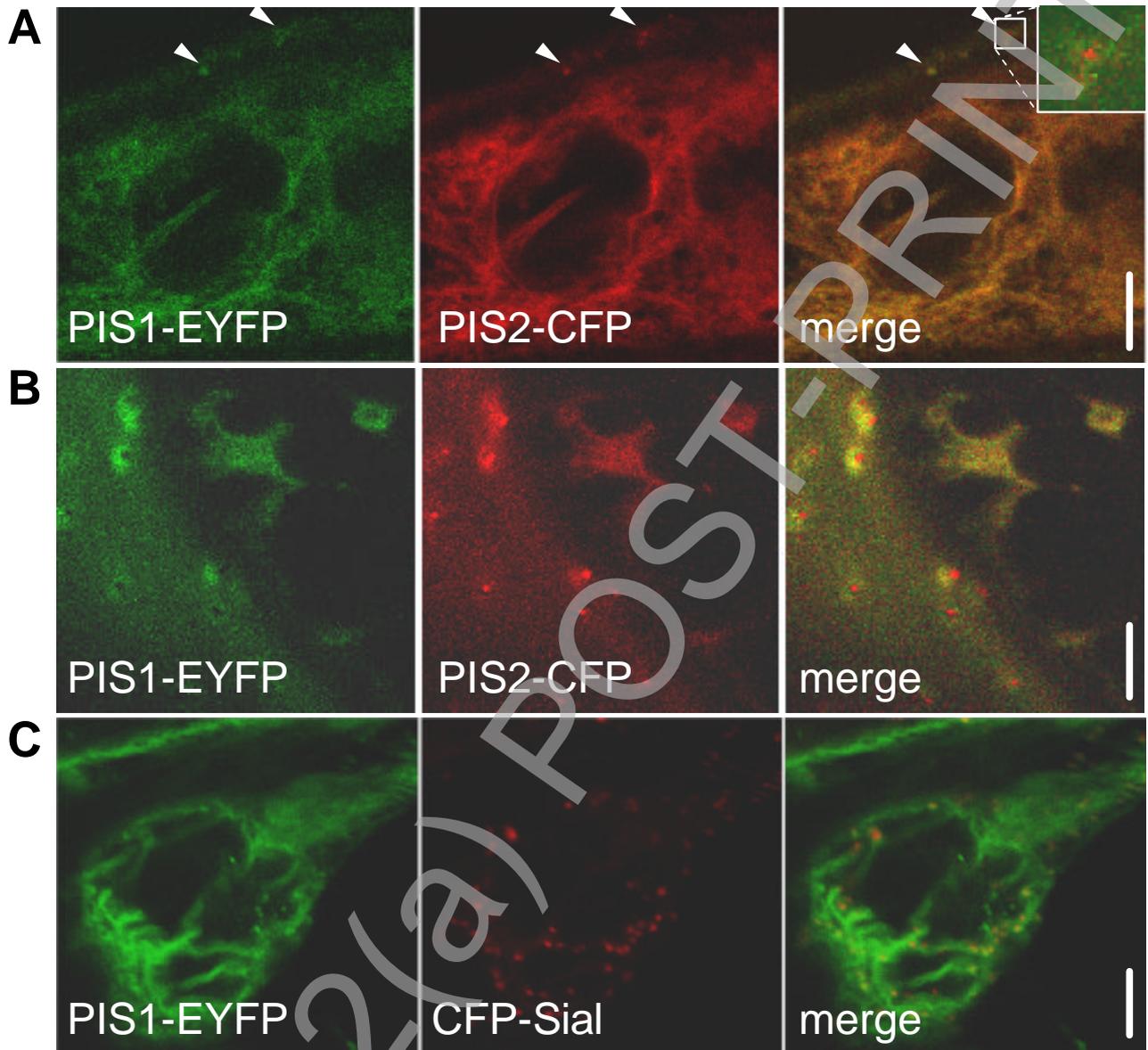
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Figure 2



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Figure 3



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Figure 4

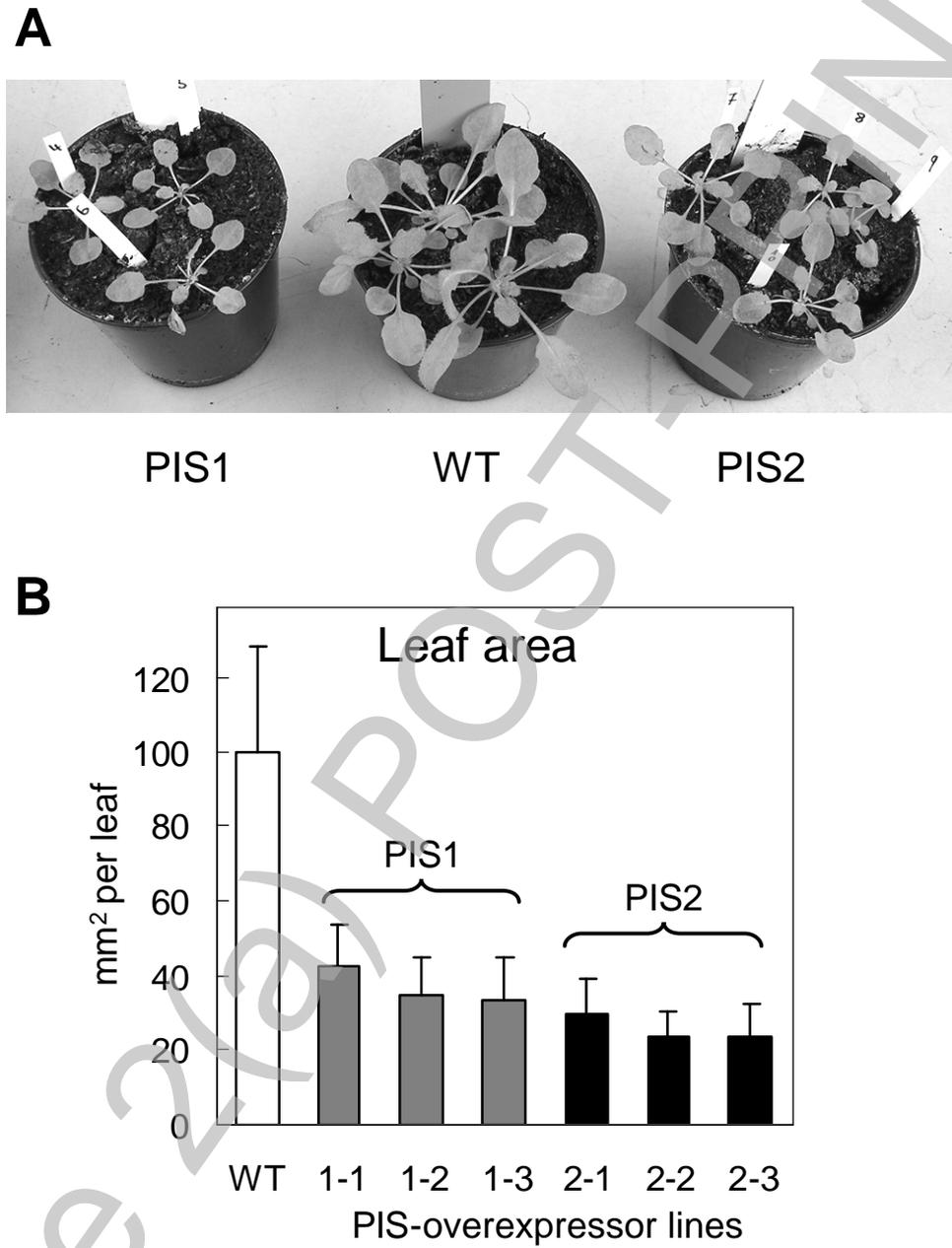
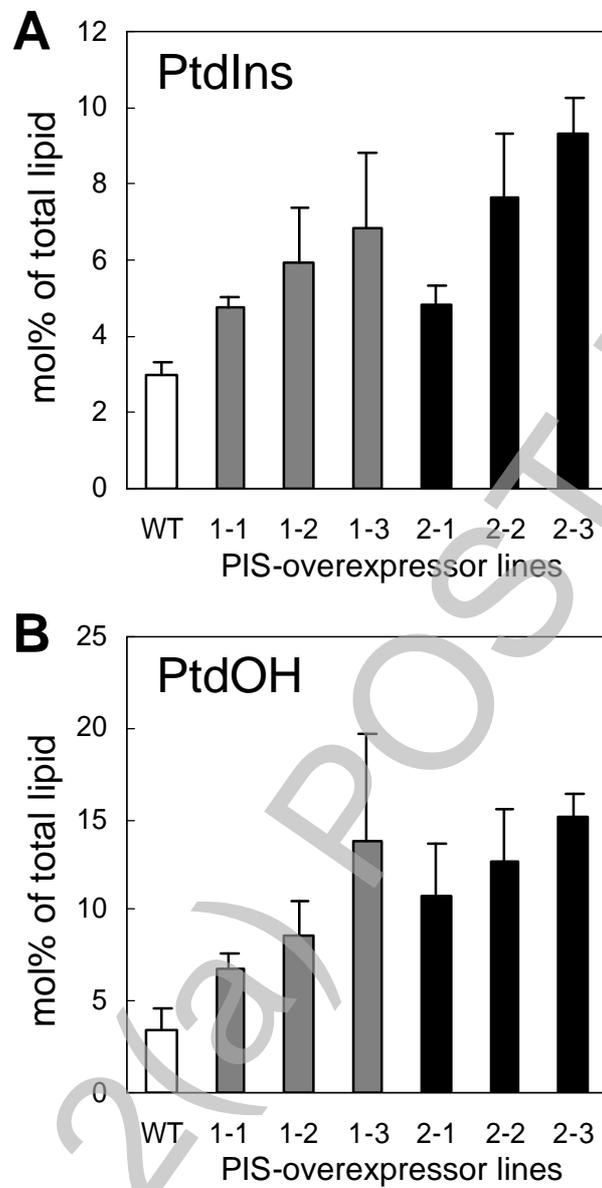


Figure 5



THIS IS NOT THE FINAL VERSION - see doi:10.1042/BJ20071371

Figure 6

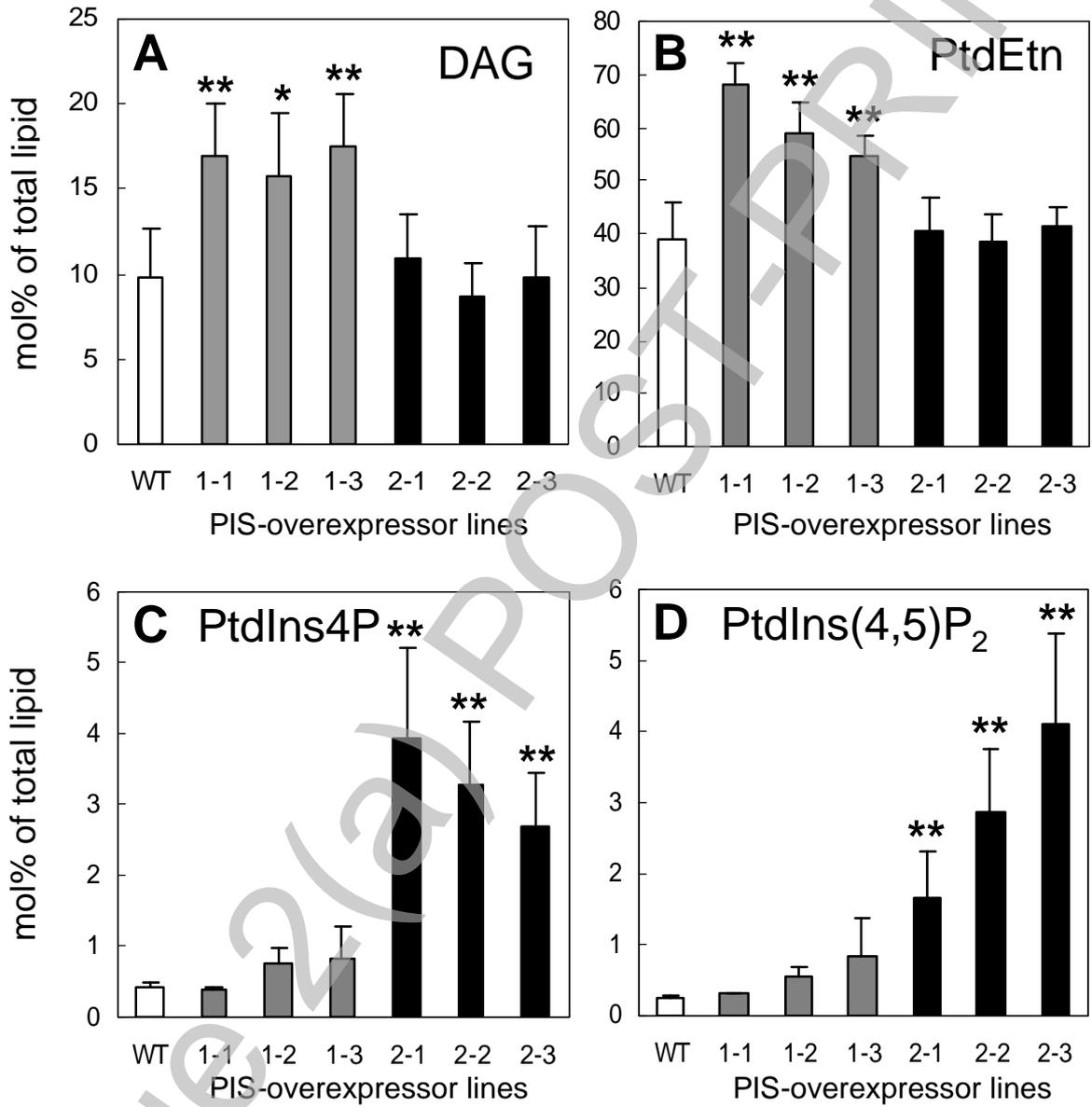
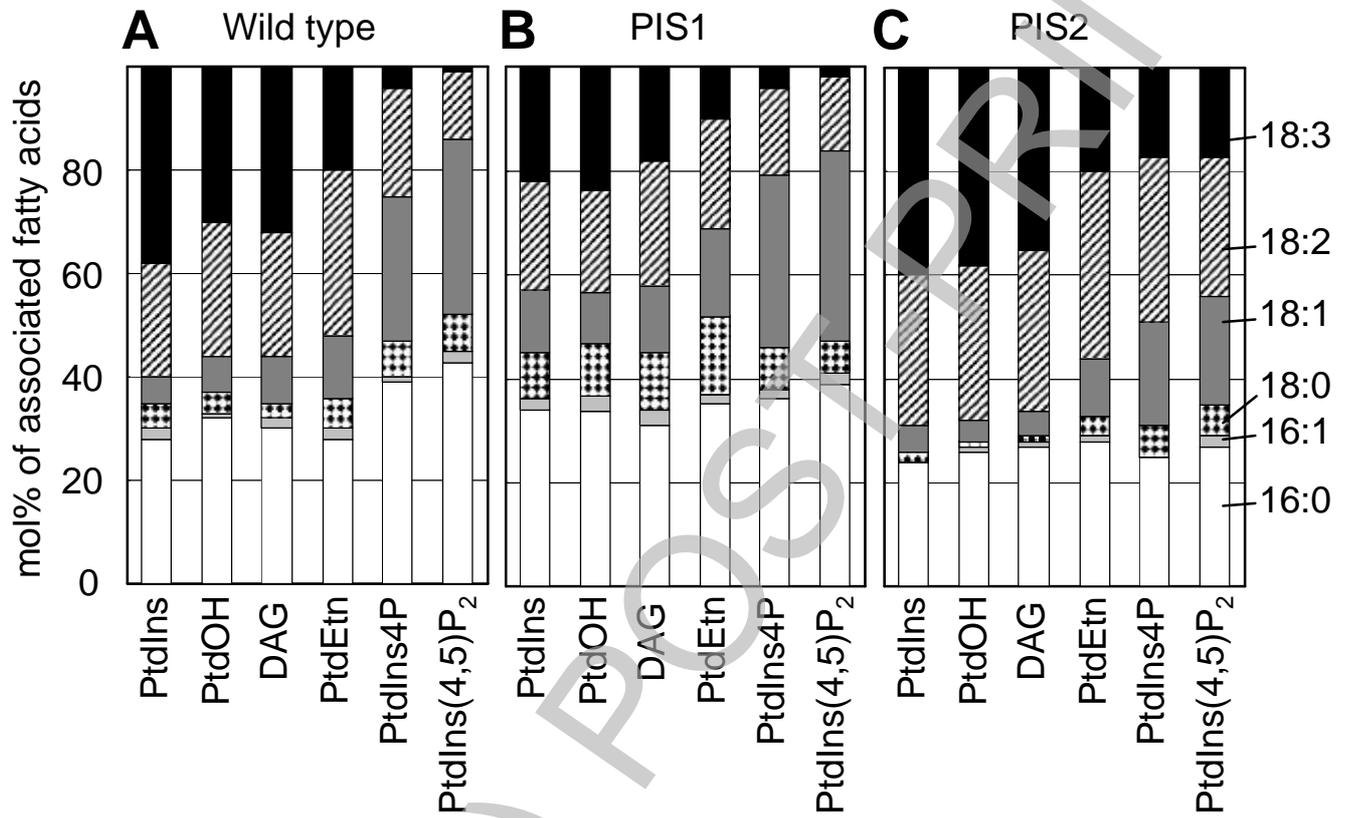


Figure 7



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Figure 8