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Identification and characterization of the phosphatidic acid-binding *A. thaliana* phosphoprotein PLDrp1 that is regulated by PLDα1 in a stress-dependent manner

Guido Ufer, Anke Gertzmann, Francisco Gasulla[†], Horst Röhrig and Dorothea Bartels*
Institute of Molecular Physiology and Biotechnology of Planta (IMBIO), University of Bonn, Kirschallee 1, D-53115 Bonn, Germany

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*For correspondence (e-mail dbartels@uni-bonn.de).
[†] Present address: Department of Life Sciences, University of Alcalá, Spain.

SUMMARY

Phospholipase D (PLD) and its cleavage product phosphatidic acid (PA) are crucial in plant stress-signalling. Although some targets of PLD and PA have been identified, the signalling pathway is still enigmatic. This study demonstrates that the phosphoprotein At5g39570, now called PLD-regulated protein1 (PLDrp1), from *Arabidopsis thaliana* is directly regulated by PLDα1. The protein PLDrp1 can be divided into two regions with distinct properties. The conserved N-terminal region specifically binds PA, while the repeat-rich C-terminal domain suggests interactions with RNAs. The expression of PLDrp1 depends on PLDα1 and the plant water status. Water stress triggers a *pldα1*-like phenotype in *PLDrp1* mutants and induces the expression of PLDrp1 in *pldα1* mutants. The regulation of PLDrp1 by PLDα1 and environmental stressors contributes to the understanding of the complex PLD regulatory network and presents a new member of the PA-signalling chain in plants.

Keywords: *Arabidopsis thaliana, At5g39570*, dehydration stress, phosphatidic acid-binding, phospholipase Dα1, phosphoprotein.

INTRODUCTION

In plants, members of the phospholipase D (PLD) family are involved in many cellular processes. PLDs play important roles in salt and dehydration stress (Frank *et al.*, 2000; Munnik, 2001; Hou *et al.*, 2016). PLDs catalyse the hydrolysis of structural phospholipids to produce phosphatidic acid (PA), which acts as a second messenger in signal transduction pathways (Munnik, 2001).

The PLD family in *Arabidopsis thaliana* consists of 12 isoforms, which are divided into six classes (PLDα, PLDβ, PLDγ, PLDδ, PLDε and PLDζ), based on sequence homologies and enzymatic activities (Bargmann and Munnik, 2006). PLDα1 is the most abundantly expressed member of this family, and an important enzyme in stress responses to dehydration by regulating stomata closure (Sang *et al.*, 2001; Zhang *et al.*, 2004). Stress-signalling pathways lead to rapid physiological changes in the cell (e.g. pH-shift and altered Ca^{2+} levels) and trigger the expression of stress genes (Yu *et al.*, 2010; Hou *et al.*, 2016). Activities of phospholipases C (PLC) and D are closely connected to Ca^{2+} levels and intracellular pH changes (Kopka *et al.*, 1998; Pappan *et al.*, 1998; Pappan and Wang, 1999). PLC and PLD isoforms are involved in the maintenance of the intracellular PA pool (Figure 1). A large portion of PA produced in the cell serves as a component for the biosynthesis of structural phospholipids (Munnik, 2001). Signalling PA is rapidly and transiently generated in response to environmental stress.

While PA produced via the PLC pathway is mainly composed of PA 16:0/18:2 and PA 16:0/18:3 species, PLD generates additional PA species like PA 18:3/18:2 and PA 18:2/18:2 (Vergnolle *et al.*, 2005; Hou *et al.*, 2016). Guo *et al.* (2011) suggested that different molecular species of PA exhibit different affinities to proteins. It was shown that the PA species 16:0/18:2 bind and activate the mitogen-activated protein kinase MPK6, and thus regulate downstream pathways (Yu *et al.*, 2010). PA regulates the abundance and activity of transcription factors, as reported by Klimecka *et al.* (2011) and Yao *et al.* (2013).

Phosphatidic acid functions by recruiting protein targets and subsequently modulating their activities (Hou *et al.*, 2016). However, there is still very little knowledge of direct PA targets and how PA exerts its function in plants. In

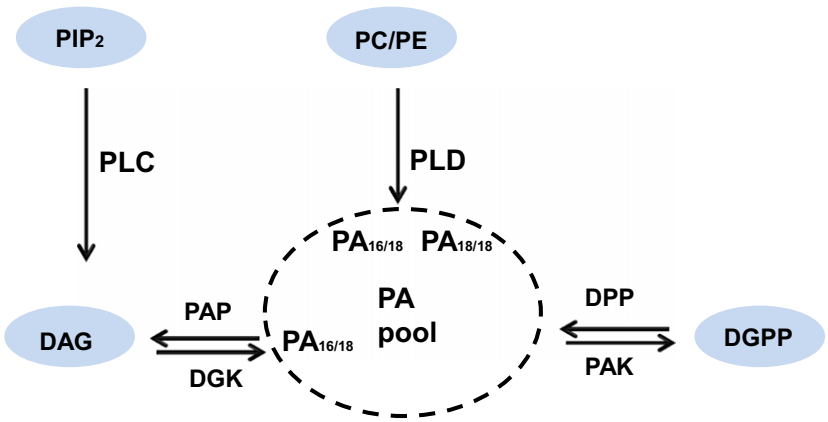


Figure 1. Schematic illustration of the formation of phosphatidic acid (PA) species.
Phospholipase C (PLC) hydrolyses phosphatidylinositol-(4,5)-bisphosphate (PIP_2) to generate the intermediate product diacylglycerol (DAG), which is phosphorylated by the diacylglycerol kinase (DGK) to mainly produce PA 16:0/18:2 and PA 16:0/18:3 species. Phospholipase D (PLD) produces additional PA species like PA 18:3/18:2 and PA 18:2/18:2 by the hydrolysis of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The dephosphorylation of diacylglycerol pyrophosphate (DGPP) to PA is catalysed by the DGPP phosphatase (DPP). The dephosphorylation of PA by PA phosphatases (PAP) and the phosphorylation of PA by PA kinases (PAK) contribute to the homeostasis of the PA pool. [Colour figure can be viewed at wileyonlinelibrary.com].

some cases, PA acts synergistically with other signalling lipids, such as diacylglycerol or phosphatidylinositol (Karathanassis *et al.*, 2002; Lopez-Andreo *et al.*, 2003; Lindsay and McCaffrey, 2004). The interplay of different PA-molecular species, their abundance and interaction with target proteins in combination with other lipid-signalling molecules and plant second messengers indicates a complex regulation. Although potential targets of PA have been identified by affinity chromatography (Testerink *et al.*, 2004), the signal transduction pathway still needs to be uncovered. Here, a combination of genetic and proteomic approaches was used to identify targets of PLDα1. This resulted in the identification of the PLD-regulated protein1 (PLDrp1), which selectively binds PA and is a target of PLD.

RESULTS

PLDα1 controls the expression of PLDrp1

The aim of this study was to identify proteins that are regulated downstream of PLD. Phosphoproteins are often involved in signal transduction pathways and thus they are candidates for the PLD-regulated pathway. Therefore, a phosphoproteomic screen was performed with *A. thaliana* wild-type plants in combination with *pldα1* and *pldδ3* homozygous mutants. Phosphoproteins from leaf extracts of these lines were enriched by metal oxide affinity chromatography (MOAC; Röhrig *et al.*, 2008) and analysed by 2D gel electrophoresis. A protein was identified that is present in wild-type and *pldδ3*, but not in extracts of the *pldα1* mutant and the double-mutant line *pldα1/pldδ3* (Figure 2). Thus, the presence of this protein is correlated with the functional *PLDα1* gene. The protein spot was excised from a Coomassie-stained gel and subjected to MALDI-MS and MS/MS analyses after a tryptic digestion. The MS spectra identified this protein as At5g39570, termed PLDrp1, an *A. thaliana* protein with unknown function. The protein is listed as a phosphoprotein in the plant protein phosphorylation database (http://www.p3db.org). Serine 339, serine 346 and serine 357 had been identified as phosphorylated amino acids by Laugesen *et al.* (2006) and De la Fuente van Bentem *et al.* (2008).

Characteristics of the PLDrp1 protein

The transcribed *PLDrp1* gene encompasses a length of 1559 bp coding for two exons separated by an intron close to the C-terminal end (Figure 3a). The encoded protein is acidic with an isoelectric point of pH 4.7 and consists of 381 amino acids. The PLDrp1 protein can be divided into two parts. The N-terminal region (first 181 N-terminal amino acids) is characterised by a high content of glycine (17.7%) and hydrophobic patches. The C-terminus (last 200 C-terminal amino acids) is dominated by charged amino acids (20.5% glutamic acid and 12.5% arginine), and is made up of 10 imperfect tandem repeats of 15 amino acids (Figure 3b and c). Protein blast with the amino acid sequence of PLDrp1 identified high sequence similarities with proteins in angiosperms within the N-terminal half of the protein and the last 50 C-terminal amino acids (Figure S2). The remaining C-terminal part shows only homologies to close relatives within the Brassicaceae (e.g. *Capsella rubella* and *Arabidopsis lyrata*).

Bioinformatic analysis predicts a disordered structure for the PLDrp1 protein, especially for the C-terminal half (Figure S3). Functional domain search against the conserved domain database, using DELTA-BLAST (E-value cut-off 0.05), resulted in the identification of two weakly conserved domains (PRK12678: transcription termination factor Rho;

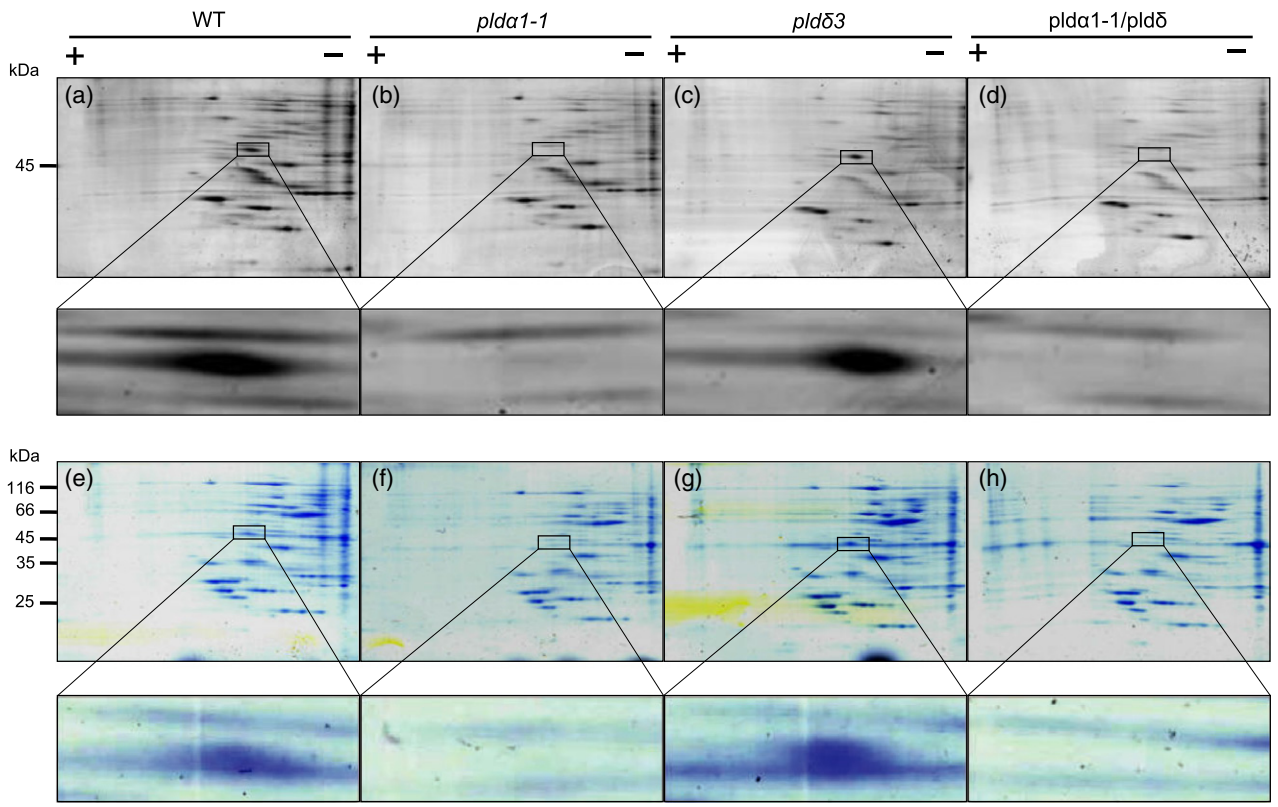


Figure 2. Enriched phosphoprotein extracts from leaves of *Arabidopsis thaliana* wild-type plants and the mutant lines *pldα1*, *pldδ3* and *pldα1/pldδ3* were analysed by 2D isoelectric focusing (IEF)/sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using immobilised non-linear pH gradients of pH 3–6.
The gels were stained with Pro-Q Diamond for detection of phosphoproteins (a–d) or with Coomassie Brilliant Blue for total proteins (e–h). Enlarged gel sections below the respective gels show the region of the phospholipase D-regulated protein1 (PLDrp1).

e-value: $5.16*e^{-5}$ and pfam02724: a member of the CDC45 superfamily involved in the initiation of DNA replication in *Saccharomyces cerevisiae*; e-value:0.02) in this protein region (Figure 3a).

Phospholipase D-regulated protein1 is present as a single copy in the *A. thaliana* genome. Database searches identified the glycine-rich protein At3 g29075 as the closest homologue (Figure S4). At3 g29075 occurs as a single copy, and it has 49% sequence identity in the first 181 N-terminal amino acids with PLDrp1. While the repeats in At3 g29075 are dominated by aspartic acid and lysine ('KKKKKHYNDDDDE'), PLDrp1 harbours glutamic acid-rich repeats ('YGRSEEQEEGGYRKPS') in its C-terminal end (Figure S5).

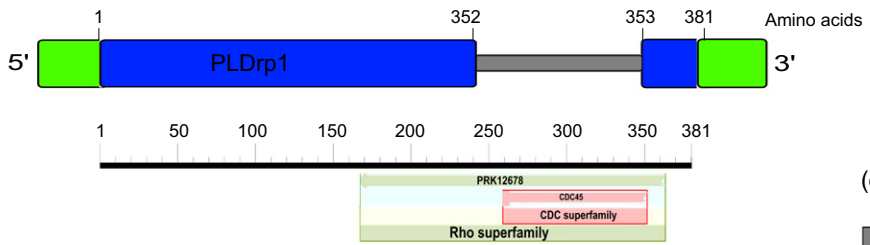
Tissue-specific expression of the *PLDrp1* gene

To analyse the spatial-temporal expression pattern of the *PLDrp1* gene promoter-GUS fusions were made and analysed in wild-type and *pldα1*-mutant *A. thaliana* plants. GUS expression was driven by a 733-bp *PLDrp1* promoter fragment and the expression was monitored in various organs throughout plant development in T2 transgenic lines (Figure 4). In wild-type plants, GUS expression was prominent in seedlings, cotyledons, primary leaves, pollen and radicles, but not in seeds or siliques (Figure 4a–h). In radicles, GUS was mainly expressed in root hairs (Figure 4c). The expression pattern in young leaves suggests that GUS is produced in the accessory cells of trichomes and it diffuses to the surrounding cells, which causes staining of larger leaf areas (Figure 4b, e and f). The GUS expression in *pldα1* plants was close to zero in Arabidopsis leaves. A slight GUS-expression was only found in roots and anthers.

PLDα1 controls the expression of the protein PLDrp1

Antibodies were raised against the recombinant proteins PLDrp1 and PLDα1 to monitor protein expression in wild-type, *pldα1* and PLDrp1 mutants. Immunoblot analyses of total proteins identified PLDrp1 and PLDα1 in wild-type plants, and verified their absence in the corresponding mutant lines *pldrp1* and *pldα1*, respectively (Figure 5a). The expression of PLDrp1 was strongly reduced in the *pldα1* mutant. To identify the protein PLDrp1 in the phosphoproteome of *A. thaliana*, enriched phosphoproteins from wild-type, *pldrp1* and *pldα1* lines were separated by 2D polyacrylamide gel electrophoresis (PAGE; Figure 5b).

(a)



(b)

1 MPYYTRDDNDVDDFDEFDPTPYSGGYDITVIYGRPIPPSDETCYPLSSGV

51 DDDFEYERPEFTQIHEPSAYGDEALNTEYSSYSRPKPRPAFRPDSGGGGH

101 VQGERPNPGYGSESGYGRKPESEYGSGYGGQTEVEYGRRPEQSYGSGYGG

151 RTETESEYGSGGGGRTEVEYGRRPESGLGSG YGGRSESEYERKPSYGRSE

201 EQEEGYRKPS YGRSEEQEEGYRKPS YGRSEEQEEGYRKPS YGRSEEEQEE

251 GYRKPS YGRSEEQEEGSYRKPS YGRSDDQVESYIKPS YGRSEEQEEGSYR

301 KPSYGRSEEQEEGSYRKQPS YGRGNDDDDDEQRRNRSGSGDDEEGSYGRK

351 KYGGNDSDEDEEKKKHRHKHHHQKRRDEDDE

(c)

Repeat type 1			
ID	Sequence	Start	Size
1	YGSESGYGRKPESEY	110	15
2	YGGRSESEYERKPS	182	14
3	YGRSEEQEEGYRKPS	196	15
4	YGRSEEQEEGYRKPS	210	15
5	YGRSEEQEEGYRKPS	225	15
6	YGRSEEEQEEGYRKPS	241	16
7	YGRSEEQEEGSYRKPS	257	16
8	YGRSDDQVESYIKPS	273	15
9	YGRSEEQEEGSYRKPS	288	16
10	YGRSEEQEEGSYRKQPS	304	17
11	YGRGNDDDDDEQRRNR	321	16

Figure 3. Structure and amino acid sequence of phospholipase D-regulated protein1 (PLDrp1).
(a) The gene model of PLDrp1 consists of two exons (blue) separated by an intron (grey). Untranslated regions are marked in green. Sequence comparison by DELTA-BLAST (Domain Enhanced Lookup Time Accelerated BLAST) identified a putative domain for PLDrp1 in the C-terminal half of the protein. The CDC45 superfamily (e-value 0.02) comprises the amino acids 261–351 and represents a domain involved in the initiation of DNA replication in *Saccharomyces cerevisiae*. The PRK12678 domain (transcription termination factor Rho-like; e-value $5.16e^{-05}$) includes the amino acids 172–362 and denotes a potential RNA-binding domain in PLDrp1.
(b) The translated protein sequence can be separated into two halves. Phosphorylated serine residues are marked by an asterisk. The C-terminal half of the protein contains 10 tandem repeats (indicated in red) that are dominated by charged amino acids. The glycine-rich N-terminal region contains only one repeat unit.
(c) Alignment of the tandem repeats.

The proteins were blotted on a nitrocellulose membrane, which was sequentially probed with antibodies against PLDrp1 (Figure 5b–I), PLDα1 (Figure 5b-I I) and Rubisco as a control (Figure 5b-II I). The protein PLDrp1 was detected at a pI of 4.7 and a molecular weight of 56 kDa in protein extracts of wild-type leaves by immunoblotting (Figure 5b). The PLDrp1 protein (indicated by I) is strongly reduced in the *pldα1* mutant, which demonstrates that the absence of PLDα1 suppresses PLDrp1 protein accumulation. The PLDα1 protein (indicated by II) is present in three isoforms in wild-type and *pldrp1* lines.

PLDrp1 accumulates in response to water stress

Transcript and protein expression were analysed in fully turgescent *A. thaliana* plants and plants dehydrated to a relative water content (RWC) of approximately 50%. The analysis of *PLDrp1* by reverse transcriptase-polymerase chain reaction (RT-PCR) showed that the transcript is present in wild-type plants, but it is hardly detectable in leaves of hydrated *pldα1* mutants (Figure 6a). Dehydration did not change the expression of *PLDrp1* in wild-type plants, but the *PLDrp1* transcript accumulated in *pldα1* mutants (Figure 6a). This finding suggests that *PLDrp1* transcript accumulation depends on the water status of the *pldα1* mutant. Likewise, the PLDrp1 protein expression was very low in fully turgescent *pldα1* mutants, but it increased to a similar level as in wild-type plants upon dehydration (Figure 6b). This indicates a dehydration-dependent regulation of PLDrp1 expression in the *pldα1* mutant.

Proteins of wild-type plants and *pldα1* mutants were extracted in the presence of 0.2% v/v proteasome inhibitor cocktail (P9599 Sigma, http://www.sigmaaldrich.com), to prevent a putative degradation of PLDrp1 in the *pldα1* mutant. Immunological detection of the PLDrp1 protein did not show any difference in samples extracted with or without the inhibitor (Figure 6c). These findings indicate that the PLDrp1 protein is not degraded and the abundance is mainly regulated on the transcriptional level.

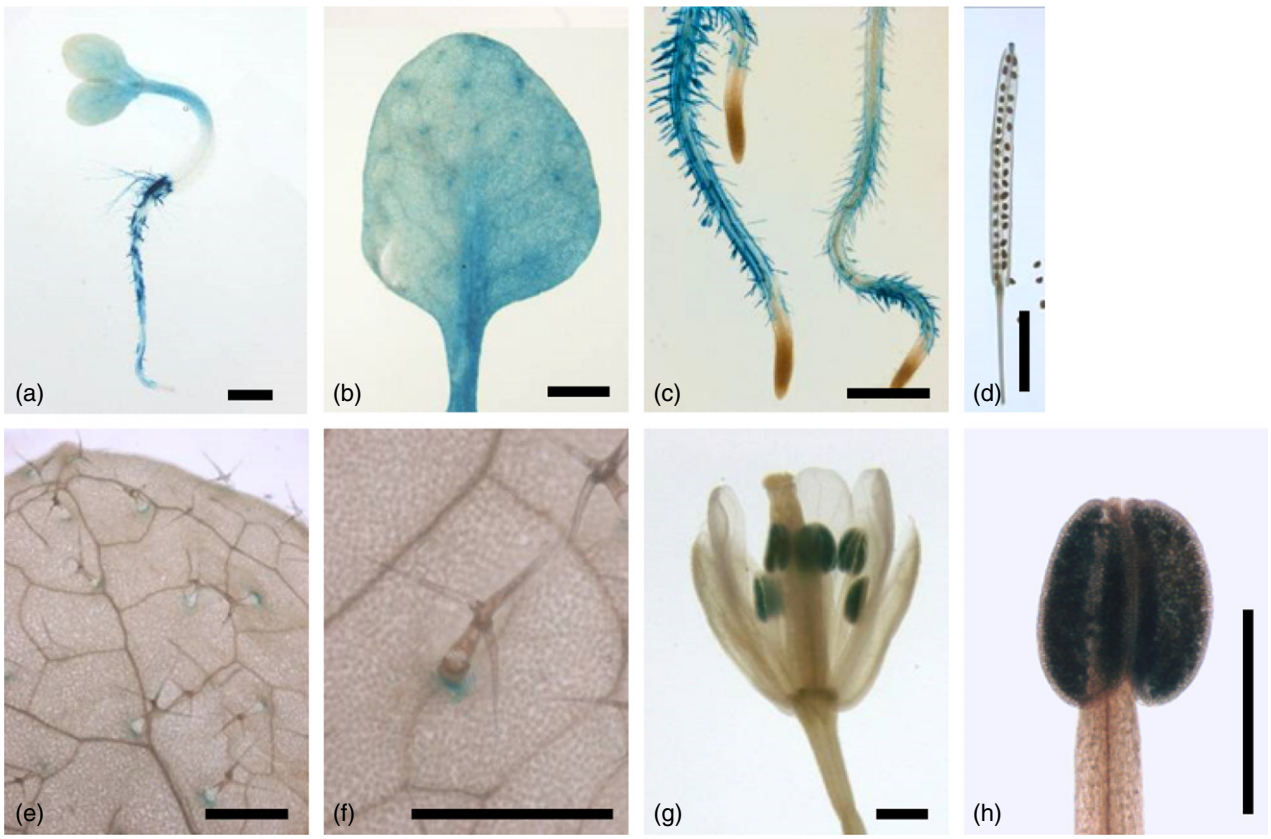


Figure 4. Histochemical localisation of GUS activity in representative transgenic *Arabidopsis thaliana* plants carrying phospholipase D-regulated protein1 (PLDrp1)_promotor::GUS constructs.
(a) One-day old seedling, (b) leaf, (c) root detail, (d) silique, (e, f) trichomes and accessory cells, (g) flower, (h) anther detail. Scale bars: 500 μm.

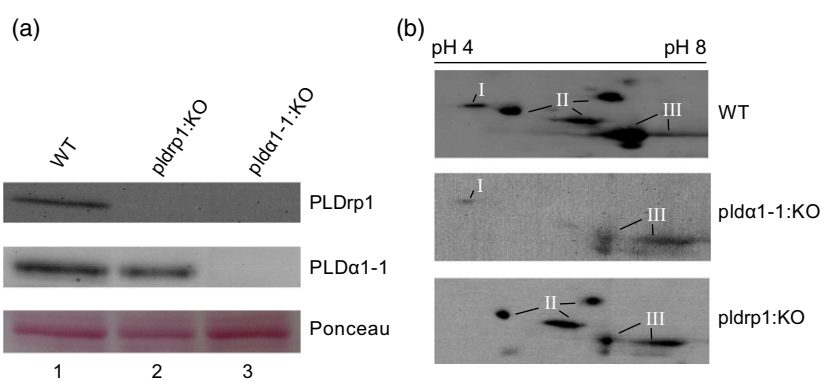


Figure 5. (a) Total proteins isolated from wild-type, *pldrp1* and *pldα1* mutant plants were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Antisera against phospholipase D (PLD)α1 and phospholipase D-regulated protein1 (PLDrp1) proteins were used to detect the corresponding proteins.
(b) Protein blot analysis of PLDα1 and PLDrp1 proteins in wild-type and *pldα1*- and *pldrp1* mutants. Enriched phosphoproteins were separated by 2D-PAGE using 7-cm strips pH 3–10 in the first dimension followed by separation on SDS–PAGE. Antibodies raised against PLDrp1 (I), PLDα1 (II) and Rubisco (III) were sequentially used for protein detection. Enlarged parts of the blots spanning the region of the respective proteins are shown. [Colour figure can be viewed at wileyonlinelibrary.com].

Phosphoprotein analyses revealed that, in contrast to the abundance of PLDrp1, phosphorylation of this protein slightly increases upon dehydration (RWC < 75%) in wild-type plants (Figure 6d).

Sodium chloride treatments (100–400 mM NaCl) of wild-type plants slightly induced the expression of the PLDrp1 protein (twofold induction; Figure 7a). However, a strong upregulation of the PLDrp1 protein was detected in *pldα1*

mutants upon increasing sodium chloride stress (Figure 7b). Thus, sodium chloride treatment of wild-type and *pldα1* mutant lines had a similar effect as dehydration on the expression of the PLDrp1 protein.

PLDrp1 mutants display a pldα1-like phenotype

Pldrp1 mutant plants did not show any phenotypic changes in morphology or life cycle in control conditions. However, it seems that long-term salt-treatment (3 weeks) triggers a similar phenotype as in *pldα1* mutants (Figure 8). This needs to be further verified by physiological measurements. While low concentrations of NaCl (≤ 200 mM) influenced all plants in a similar manner, high concentrations (400 mM) affected the mutants more than the wild-type plants in terms of necrosis and wilting of leaves.

Role of other PLD isoforms in the regulation of PLDrp1

To test whether besides PLDα1 other PLD isoforms influence the expression of PLDrp1, protein accumulation was investigated in *pldα3-*, *pldδ3-* and in the *pldε* mutants. The analysis shows that PLDα1 is the main regulator of PLDrp1 expression. The other PLD isoforms have only minor effects, if any, on PLDrp1 accumulation (Figure 9).

Interaction of PLDα1 with PLDrp1

Expression of PLDrp1 is linked to the presence of PLDα1, but the absence of PLDrp1 has no effect on the expression of PLDα1, which suggests that *PDLα1* acts upstream of *PLDrp1*. Pull-down and co-immunoprecipitation assays did not show a direct interaction of PLDα1 and PLDrp1. To test whether PLDα1 and PLDrp1 interact via PA, binding of PLDrp1 to lipids was analysed. Lipid-binding to histidine-tagged PLDrp1 was examined using the full-size recombinant protein and N- or C-terminal protein fragments. The histidine-tagged nusA protein from *Escherichia coli* was used as a negative binding control. All recombinant proteins used in the protein-lipid binding assays are listed in Table 1.

Binding of the PLDrp1 protein to selected lipids was analysed in lipid binding assays (Figure 10). The full-length protein PLDrp1 showed strong binding to PA but not to any other tested lipid (Figure 10a). The binding affinity of PLDrp1 to PA is predominantly enabled via its N-terminal domain. The C-terminal region of PLDrp1 has only a weak binding affinity to PA. These results were supported by liposome-binding assays (Figure 10b), which reflect the intracellular and molecular arrangement of lipid–protein interactions. Incubation of PLDrp1 with phosphatidylcholine (PC), the main substrate of PLDα1, retained the proteins in the unbound supernatant fractions, whereas PA-bound proteins can be detected in the pellet fraction (Figure S6). Strong binding of the PLDrp1 full-size protein and its N-terminal fragment to PA were observed, while the C-terminal fragment only showed faint binding to PA. However, the C-terminal protein showed a weak signal in the pellet fraction, indicating insufficient solubilisation. A protein-aggregation assay revealed that PLDrp1 forms aggregates *in vitro*, which can only be partly disrupted upon severe, denaturing conditions (Figure S7). The C-terminal fragment of PLDrp1 seems to be involved in this self-aggregation process. Therefore, liposome-turbidity assays, which monitor lipid binding to proteins in real-time, were used to analyse PA-binding to the PLDrp1 full-size and N-terminal protein. This assay demonstrates an interaction between PA and PLDrp1 after the first 5 min of incubation (Figure 10c).

DISCUSSION

The objective of this study was to use a proteomics approach to identify genes that are regulated by phospholipases. As a result, the phosphoprotein PLDrp1 was identified as a target, downstream of PLDα1. PLDrp1 has biochemical properties that suggest molecular interactions with lipids and RNAs.

The bipartite structure of PLDrp1 separates the protein into two regions

The N-terminus of the protein is dominated by glycine-rich motifs (GGX, GXGX and GGGX), which classify PLDrp1 as a glycine-rich protein according to Sachetto-Martins *et al.* (2000). This part of the protein is highly conserved across the plant kingdom (Figure S2), pointing to an important function encoded in the N-terminus. The overall hydrophobic composition of the N-terminal protein region might facilitate binding of PA to basic amino acids of PLDrp1, as suggested by the 'electrostatic/hydrogen-bond switch model', which explains binding of PA to basic residues of target proteins (Kooijman *et al.*, 2007).

The hydrophilic C-terminal region is mainly characterised by 10 tandem repeats, which are unusually rich in the polar amino acids lysine and aspartic acid. No function can so far be assigned to these repeats, but aggregation experiments have shown that PLDrp1 forms aggregates *in vitro* due to the amino acid composition of the C-terminus. Jorda *et al.* (2010) reported that such a repeat perfection often points to recent evolutionary events. The exclusive occurrence of these repeats in members of the Brassicaceae family supports this theory. The C-terminal region harbours two weakly conserved domains. The PRK12678 motif shares similarities with the transcriptional termination factor Rho and is linked to RNA-binding (Figure 3a; Dolan *et al.*, 1990; Boratyn *et al.*, 2012). Two short (18 bp and 24 bp), highly conserved nucleotide fragments, which are found in several RNA-associated genes, exist within close vicinity to this PRK domain (Table S1). The conserved 24-nucleotide fragment A was identified at position 969–993 bp coding for the seven amino acids $AA_{325–331}$ (GNDDDDD). The second conserved fragment B was found

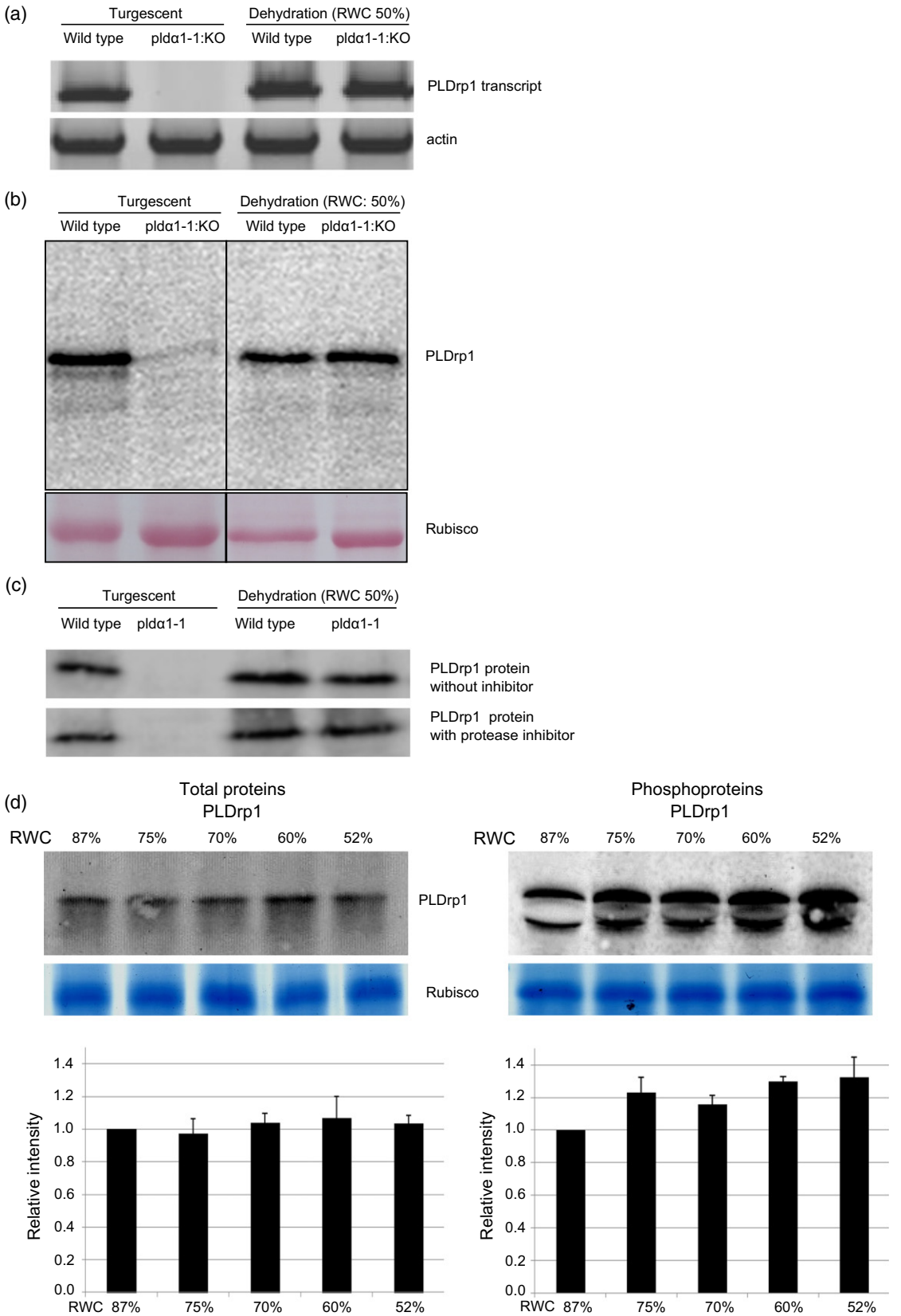

(a)
Turgescent
Dehydration (RWC 50%)
Wild type
pldα1-1:KO
Wild type
pldα1-1:KO
PLDrp1 transcript
actin
(b)
Turgescent
Dehydration (RWC: 50%)
Wild type
pldα1-1:KO
Wild type
pldα1-1:KO
PLDrp1
Rubisco
(c)
Turgescent
Dehydration (RWC 50%)
Wild type
pldα1-1
Wild type
pldα1-1
PLDrp1 protein
without inhibitor
PLDrp1 protein
with protease inhibitor
(d)
Total proteins
PLDrp1
Phosphoproteins
PLDrp1
RWC 87% 75% 70% 60% 52%
RWC 87% 75% 70% 60% 52%
PLDrp1
Rubisco
Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RWC 87% 75% 70% 60% 52%
Relative intensity
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
RWC 87% 75% 70% 60% 52%

Figure 6. Phospholipase D-regulated protein1 (*PLDrp1*) expression in response to dehydration in wild-type and *pldα1* mutants.
(a) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of the *PLDrp1* transcript in 2 μg total RNA extracted from wild-type and *pldα1* mutant. Actin transcripts served as loading controls. PCR products were separated on an agarose gel and visualised by ethidium bromide.
(b) Immunological detection of PLDrp1 protein in total protein extracts from leaves of fully turgescent and dehydrated wild-type plants and *pldα1* mutants. Ponceau-S staining of the nitrocellulose membrane is shown as loading control.
(c) Immunological detection of PLDrp1 protein in total protein extracts prepared in the presence or absence of the protease inhibitor cocktail.
(d) Comparison of PLDrp1 protein abundance in total and phosphoprotein extracts prepared from leaves of wild-type *Arabidopsis thaliana*. Leaves of fully hydrated and dehydrated plants were used; the relative water contents (RWCs) are indicated on the top of the corresponding lanes. Top panels: PLDrp1 protein detection and below Coomassie-stained Rubisco. Bottom panels: mean values and standard deviation of protein levels of three independent repetitions. Expression levels in comparison to a fully turgescent plant (RWC 87%) are indicated in the ordinates (relative intensity). [Colour figure can be viewed at wileyonlinelibrary.com].

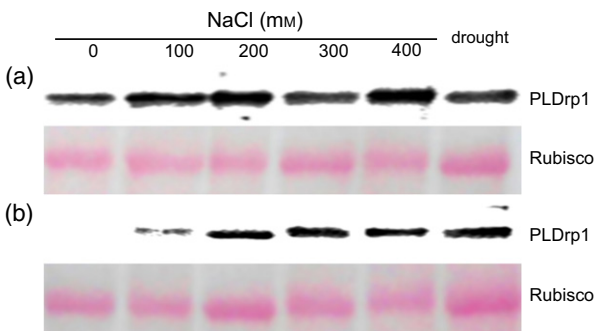


Figure 7. Phospholipase D-regulated protein1 (PLDrp1) protein expression in response to sodium chloride treatments.
Anti-PLDrp1 antiserum was used to detect the PLDrp1 protein in sodium chloride-treated leaves.
(a) Expression of the PLDrp1 protein in wild-type in response to increasing concentrations of NaCl.
(b) Abundance of the PLDrp1 protein in *pldα1* mutants in response to increasing concentrations of NaCl. Ponceau-S staining of the nitrocellulose membrane is shown as loading control (bottom). [Colour figure can be viewed at wileyonlinelibrary.com].

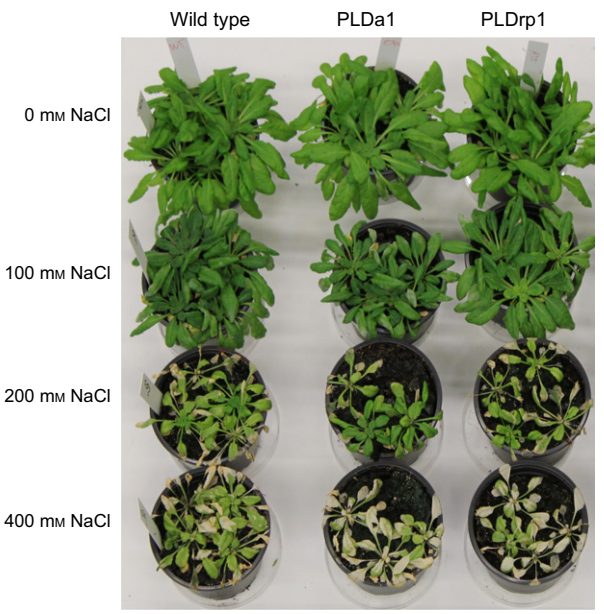


Figure 8. *Arabidopsis thaliana* wild-type and mutant plants after 3 weeks of salt treatment.
Wild-type, *pldα*1- and *pldrp1* mutants were grown under short-day conditions and watered with 50 ml water every week. After 6 weeks, the water was replaced by salt solutions containing up to 400 mM NaCl and the plants were stressed for 3 weeks. The experiment was conducted with four sets of plants. Here, a representative photo of one of these sets is shown.

at position 1083–1101 bp coding for the six amino acids $AA_{361–367}$ (EKKKHR) at the C-terminal end of the identified PRK-domain. Involvement of tandem repeat-rich proteins in RNA-binding has been well studied in the pentatricopeptide repeat protein family that is involved in RNA-editing (Lurin *et al.*, 2004). Members of this family match with the

Figure 9. Expression of phospholipase D-regulated protein1 (PLDrp1) varies in different pld-mutants.
(a) Total proteins from wild-type and *pld* mutant lines (*pldα*1, *pldα*3, *pldδ*3 and *pldε*) were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Antisera against PLDrp1 proteins were used to detect the proteins. Mean values and standard deviation of protein levels of three independent repetitions. The mean value for the wild-type is set as reference.

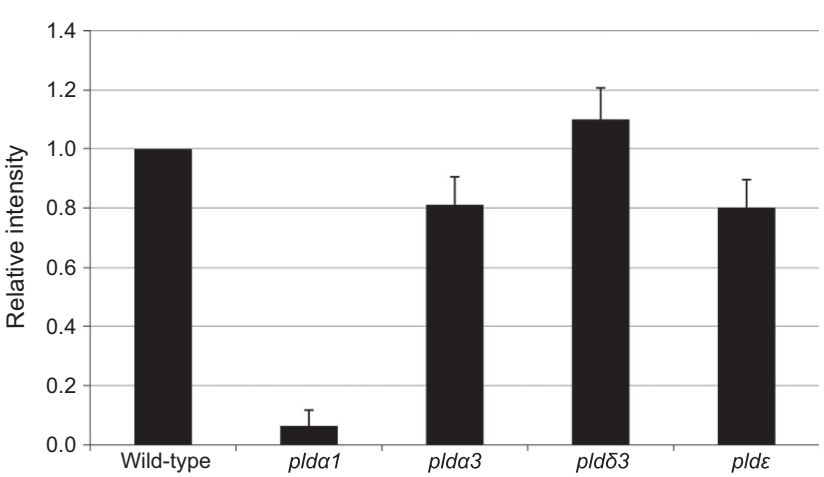


Table 1 Recombinant proteins used in lipid-binding assays and capacity to bind PA

Construct	Hybrid gene[a]	Position of the HIS-tag	Binding affinity to PA
PLDrp1-HIS	Full-length PLDrp1 CDS (1–1146 bp) fused to a HIS tag	C- terminal	+++
5′ PLDrp1-HIS	1–585 bp 5′ of PLDrp1 fused to a HIS tag	C- terminal	++
3′ PLDrp1-HIS	480–1143 bp 5′ of PLDrp1 fused to a HIS tag	N- terminal	+
nusA-HIS	Full-length nusA CDS (1–1485 bp) fused to a HIS tag	C-terminal	0

[a]Nucleotides are counted from the first ATG of the PLDrp1 gene coding sequence.
PA, phosphatidic acid; PLDrp1, phospholipase D-regulated protein1.

putative RNA-binding fragments of PLDrp1 (Figure S8). Thus, RNA binding properties are proposed to be anchored in the repeat-rich structure in the C-terminal half of PLDrp1.

Phospholipase D-regulated protein1 occurs as a single copy gene in the *A. thaliana* genome, but At3g29075, a protein with high sequence similarities (> 50%) in the N-terminal part, exists. The amino acid sequence in the middle part of both proteins is made up of tandem repeats, although the repeats differ in each protein. These repeats might have been originated through gene duplication. Gene duplications arise from unequal crossing-over events

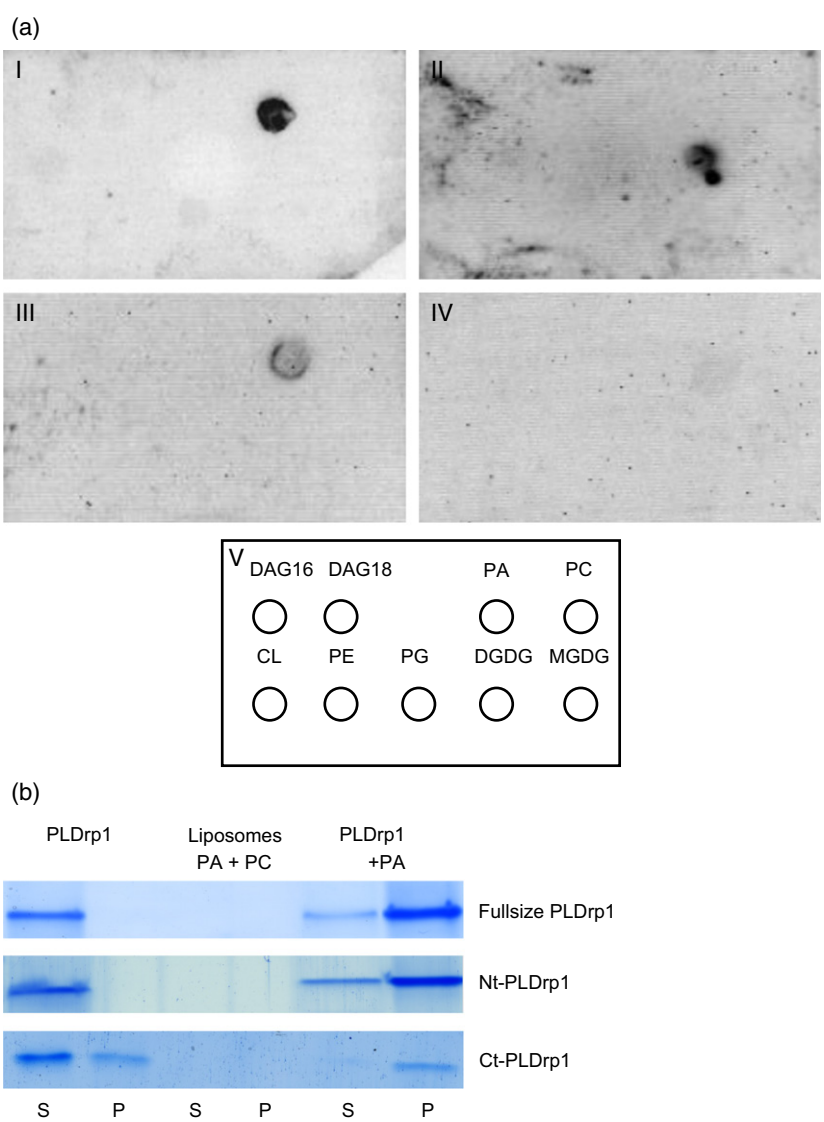


Figure 10. Protein-lipid binding assays.
(a) Protein-lipid overlay assays using the recombinant phospholipase D-regulated protein1 (PLDrp1)-His protein at pH 7.0. Anti-His antibody was used to detect the full-size protein PLDrp1-His (I), the N-terminal fragment of PLDrp1 (II), the C-terminal fragment of PLDrp1 (III) and the control protein NusA with a His-tag (IV). The loading scheme (V) displays the positions of the immobilised lipids on the membrane: diacylglycerol 16:0 (DAG16), diacylglycerol 18:3 (DAG18), phosphatidic acid (PA), phosphatidylcholine (PC), cardiolipin (CL), phosphatidylethanolamin (PE), phosphatidylglycerin (PG), digalactosyldiacylglyceride (DGDG) and monogalactosyldiacylglyceride (MGDG). In case of the C-terminal fragment, only PA and PC were applied on the membrane.
(b) Liposome-binding assay with PLDrp1. Binding of PA to the full-size PLDrp1 protein and the N-terminal fragment (Nt-PLDrp1) and the C-terminal fragment (Ct-PLDrp1) was tested. Purified recombinant proteins and liposomes were used as binding controls. S, supernatant; P, pellet.
(c) Liposome turbidity assay of PLDrp1. Unilamellar liposomes (75% PC/25% PA) were incubated with PLDrp1 proteins. The measurements were taken after the addition of the protein. The increase of light scattering in response to increasing vesicle diameters was monitored. Red line: PLDrp1 full-size; green line: Nt-PLDrp1; blue line: NusA protein as control. Binding of PA is monitored in a spectrophotometer at OD_{350} for 5 min. [Colour figure can be viewed at wileyonlinelibrary.com].

that possibly result in the formation of repetitive genetic elements (Zhang, 2003). High sequence similarities of genes that are located on separate chromosomes (At5 g39570 and At3 g29075) promote gene duplication processes at duplication breakpoints (Zhang, 2003) and could explain the origin of the unique tandem repeats in both proteins. Like PLDrp1, At3 g29075 displays an acidic pI and high amounts of charged amino acids (aspartic acid 19.3% and lysine 21.1%). The exceptionally high numbers of lysine residues in At3 g29075 render the protein lysine-rich. Lysine, as one of the essential amino acids, is the limiting amino acid in most cereal grains (Young and Pellett, 1994), and therefore lysine-rich proteins are of special interest for nutrition improvements.

Expression of PLDrp1 correlates with PA production

Analysis of promotor GUS constructs revealed strong gene expression in seedlings, cotyledons, primary leaves, pollen and radicles. In agreement with this, the activities of PLD isoforms are tissue specific, and contribute to different PA concentrations and compositions (Bargmann and Munnik, 2006). The highest relative concentration of PA is found in roots (approximately 10%) of *A. thaliana* wild-type plants (Devaiah *et al.*, 2006), which correlates with the strong expression levels of *PLDrp1* in radicles. It seems that PA concentration and PA composition influence the expression of *PLDrp1*. A recent study found that the *PLDrp1* mRNA transcript is post-transcriptionally transported from shoot to root tissue (Thieme *et al.*, 2015). The authors suggest that mobile RNAs are translated at their destination site, and thus postulated that tissue-specific gene expression profiles might not be predictive for the actual location in which the transcript exerts its function. Therefore, we mainly focused on the protein expression.

PLDrp1 is under the control of PLDα1

The PLDrp1 protein data showed a strong correlation of the expression of both PLDrp1 and PLDα1, which is supported by *in silico* analysis (Warde-Farley *et al.*, 2010). However, it is a one-sided dependence of PLDrp1 on PLDα1: while the PLDα1 expression is not affected by the absence of PLDrp1, protein and transcript levels of PLDrp1 depend on the presence of PLDα1. Nevertheless, no direct physical interaction was found. Possibly, PLDrp1 is regulated by PA, the enzymatic product of PLDα1. The absence of PLDα1 in leaves of *pld* mutants in *A. thaliana* does not result in reduced PA levels, but affects the lipid composition in the cells (Devaiah *et al.*, 2006). While the expression of a few PA species, such as PA 18:3/18:3 or PA 16:0/18:3, is slightly reduced in roots or seeds in *pldα1* mutants, most PA levels are unchanged compared with the wild-type (Devaiah *et al.*, 2006). Especially the leaf extracts, which contain relatively low amounts of PA in Arabidopsis, did not show any significant differences in PA content between the wild-type and the mutant.

This suggests that PLDα1 activity is substituted by other PLD isoforms or the generation of PA via another pathway, which generates different molecular forms of PA (Figure 1). A reduction of specific PA species (e.g. 16:0/18:2 PA and 16:0/18:3 PA) could explain the downregulation of PLDrp1 in *pldα1* mutants. Activation, inhibition and degradation of target proteins by binding to specific PA species have been reported (Ruelland *et al.*, 2002; Arisz *et al.*, 2009; Li *et al.*, 2009; Guo *et al.*, 2011; Kim *et al.*, 2013; Hou *et al.*, 2016). PLD activity is strongly regulated during different developmental stages and in response to stress (Qin *et al.*, 2006; Wang *et al.*, 2014), which impedes the identification of a correlation between distinct PA species and downstream effects.

The PLDα1 isoform is specific for the regulation of PLDrp1

All members of the PLD family produce PA via the hydrolysis of phospholipids. PLDα3, PLDδ3 and PLDε share similar requirements as PLDα1 for their enzymatic activities, such as Ca^{2+} dependence and membrane localisation (Pappan *et al.*, 1998), but out of the corresponding genes affected the expression of PLDrp1 only to a minor extent, suggesting that the expression of PLDrp1 is mainly regulated by PLDα1 under non-stress conditions. Knockout of a specific *pld* gene (e.g. *pldα1*) might lead to the absence of associated PA species that are (in high levels) only specifically synthesised by these PLD isoforms and may contribute to the PLDrp1 pathway. The PLD isoforms PLDα3, PLDδ3 and PLDε seem to play a minor role in the regulation of PLDrp1 under non-stress conditions. Possibly only a specific PA species, produced mainly by PLDα1 in hydrated conditions, can activate the expression of PLDrp1. The PLD isoforms PLDα3 and PLDε might only partly generate these PA species. The isoform-specific PA species would thereby explain the slightly reduced expression levels of *PLDrp1* in *pldα3* and *pldε* mutants, and the strong reduction in *pldα1* mutants.

Expression of PLDrp1 is transcriptionally regulated under water stress

Phospholipase D-derived PA species may synergistically promote expression of PLDrp1. The co-operation of different PLD isoforms was previously reported for PLDα1 and PLDδ3 in response to reactive oxygen species (Bargmann and Munnik, 2006). Abrogation of one or more PLDs would therefore result in reduced amounts and altered compositions of PA species that are involved in the regulation of PLDrp1.

As PLDα1 and its cleavage product PA are known to be important players in plant stress responses (Testerink and Munnik, 2005), the effect of abiotic stress on the expression of the protein PLDrp1 was investigated. While drought

and salinity had no effect on the expression of the PLDrp1 protein level in wild-type plants, the PLDrp1 transcripts and proteins were strongly induced in the *pldα1* mutant under dehydration and salt stress. These findings suggest that water-limiting conditions might trigger the activation of additional PA-producing pathways (Figure 1), resulting in the production of more and specific PA-species, which eventually lead to a compensation of the PLDrp1 protein expression in *pldα1* mutants.

Salinity and drought trigger the expression of PLD and PLC genes (Hirayama *et al.*, 1995; Kocourková *et al.*, 2011; Wang *et al.*, 2014; Hou *et al.*, 2016), resulting in increased production of PA species. The Arabidopsis PLD isoforms have distinguishable catalytic and regulatory properties, such as Ca^{2+} requirements, substrate preferences and phosphorylation status (Wang *et al.*, 2014), which determine their activity in osmotic stress conditions. How plant PLC activity is regulated is still unknown, but elevated Ca^{2+} levels, interaction with proteins or increased phosphorylation levels as a response to dehydration might trigger PLC activity and raise local PA concentrations independent from PLDs (Munnik, 2014). Members of the PLD family were reported to act cooperatively in response to water-limiting conditions (Uraji *et al.*, 2012), and the PLC gene family is suggested to have functional redundancy (Munnik and Nielsen, 2011). Elevated PA levels originating from other PLD isoforms or PLC-derived PA could therefore compensate the absence of PA produced by a specific PLD isoform like PLDα1 during dehydration.

The potential of PA to activate, reduce or inhibit the expression of target genes and proteins has widely been reported (Hou *et al.*, 2016). A putative inhibition of *PLDrp1* gene-expression by a repressor could explain the expression patterns. In the absence of PLD(α1)-specific PA-species, repression of the gene takes place. Upon drought-induced PA production repressor binding and deactivation leads to transcriptional activation. PLDrp1 could be a candidate for this repressor, as it was shown to bind to PA. Binding of PA to proteins was shown previously to induce conformational changes in unstructured proteins (Hou *et al.*, 2016). The 3D structure of the mainly unstructured protein PLDrp1 might be dependent on its phosphorylation state and the availability of specific PA species, as PLDrp1 is phosphorylated in response to dehydration. Phosphorylation of proteins resulting in conformational changes or disorder-to-order transitions has been reported (Metskas and Rhoades, 2015). This self-regulatory process under the control of two distinct pathways could explain our phenotypic findings that drought stress has no influence on the protein level in wild-type plants, where the repressor is already in its unbound form. Taken together, water-limiting conditions seem to promote the expression of *PLDrp1* in a PLD(α1)-independent manner. A possible model for the regulation of these processes is depicted in Figure 11.

The function of PLDrp1 remains unknown, but its abundant expression and distribution in plants suggests an important role in the PLD pathway. The *pldα1*-like phenotype in dehydrated *PLDrp1* mutant plants emphasizes the importance of PLDrp1 in the stress response.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and stress treatment

Arabidopsis thaliana wild-type (Columbia-0 ecotype) and mutants were sown on soil and cultivated after a vernalisation period with an 8 h photoperiod (about 120–150 μE m^{-2} sec^{-1}) and a day/night temperature regime of 22/20°C. Flowering was induced by a 16 h photoperiod.

T-DNA insertion mutants *pldα1* (SALK_067533), *pldα3* (SALK_130690) and *pldδ3* (SALK_023247) were obtained from the SALK collection (Bargmann *et al.*, 2009). Dr Koncz (MPI Cologne) provided the T-DNA mutant *pldε2* (Ríos *et al.*, 2002). The double-mutant *pldα1/pldδ3* was obtained by crossing the single mutants. The *pldrp1* line (GK–167C05) derived from the GABI-KAT collection (Rosso *et al.*, 2003). PCR genotyping identified homozygous T-DNA plants with the gene-specific primer PLDrp1-fwd and the T-DNA-specific primer GK-LB. The T-DNA insertion sites are depicted in a gene model (Figure S1). Homozygous T2 plants were used.

Six-week old plants were watered with 50 ml dH_2O every week. For dehydration treatments, watering was stopped for 14 days to reach a RWC of about 50%. The RWC was determined according to Bartels *et al.* (1990). For salt stress treatments, the water was replaced by saline solutions (100 mM, 200 mM, 300 mM or 400 mM NaCl) and used to treat plants for 14 days.

Protein analyses

Extraction of proteins and enrichment of phosphoproteins by MOAC were performed as described (Röhrig *et al.*, 2008; Colby *et al.*, 2011). Denatured proteins were purified from leaves with a phenol-based extraction and used for affinity binding to Al $(OH)_3$ in incubation buffer (30 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.1, 0.2 M sodium glutamate, 0.2 M potassium aspartate, 0.25% w/v CHAPS, 8 M urea, 20 mM imidazole). After several washing steps with incubation buffer, the Al-bound phosphoproteins were eluted with 300 mM potassium pyrophosphate pH 9.0 containing 8 M urea. Phosphoproteins were concentrated in Amicon Ultracel-10K centrifugal concentrators (MILLIPORE, http://www.millipore.com) before they were precipitated with 10% (w/v) trichloroacetic acid using 0.02% (w/v) sodium deoxycholate as co-precipitant. Proteins were quantified using the protein assay kit (BIO-RAD, http://www.bio-rad.com) with bovine serum albumin (BSA) as standard.

Proteins for 1D gel-electrophoresis were dissolved in 1 × sodium dodecyl sulphate (SDS) gel-loading buffer (Laemmli, 1970) at a concentration of 1 mg/ml and heat-treated at 95°C for 5 min. For the protease inhibitor assay 0.5% (v/v) protease inhibitor cocktail (P9599 SIGMA, http://www.sigmaaldrich.com) was added to the extraction buffer to avoid proteolytic degradation. Proteins (20 μg) were separated by 12% (w/v) SDS–PAGE. Isoelectric focusing (IEF) for 2D gel-electrophoresis was performed using 7 cm immobilized pH gradient strips with a pH range of 3–10 (GE HEALTHCARE, http://www.gehealthcare.com) to separate 40 μg of proteins (Röhrig *et al.*, 2006). In the second dimension the proteins were separated on 12% (w/v) SDS–PAGE gels. Phosphoproteins were stained with Pro-Q Diamond fluorescent gel stain

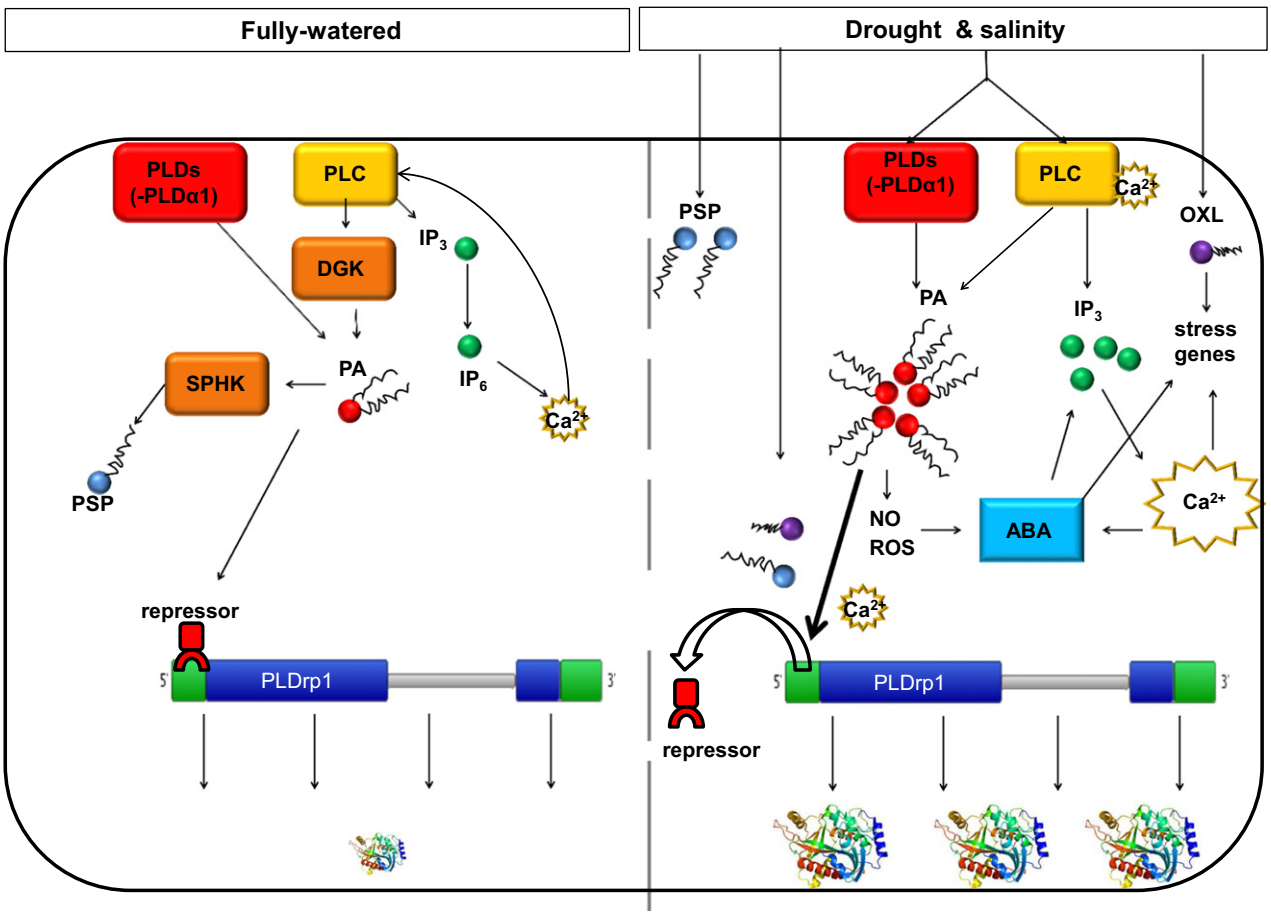


Figure 11. Model for the regulation of expression of phospholipase D-regulated protein1 (PLDrp1) in response to water-limiting conditions in the absence of PLDα1.

Under hydrated conditions, a repressor (e.g. PLDrp1) prevents transcription of *PLDrp1*. In the absence of PLDα1, the production of phosphatidic acid (PA) species is impaired and PA-generated via the phospholipase C (PLC) pathway is insufficient to separate the repressor from the gene. Water-limiting conditions promote PLC and phospholipase D (PLD) activity, resulting in the increased production of PA species. Additionally, production of other signalling molecules, such as OXL, PSP, NO, ROS and IP_3 is promoted by oxidative stress, and has a positive feedback on the generation of PA and stress genes. Elevated levels of distinct PA species in combination with other signalling molecules separate the repressor from *PLDrp1*, resulting in increased protein biosynthesis. Arrows indicate positive regulatory events. ABA, abscisic acid; DGK, diacylglycerol kinase; IP3, inositol trisphosphate; NO, nitric oxide, OXL, oxylipins; PA, phosphatidic acid; PLC, phospholipase C; PLD, phospholipase D; PSP, phytosphingosine phosphate; ROS, reactive oxygen species; SPHK, sphingolipid kinase.

(INVITROGEN, http://www.invitrogen.com). For staining with Coomassie, gels were fixed for 1 h in fixation solution (10% v/v acetic acid, 40% v/v methanol) and washed three times with water. Staining was done overnight using staining solution (10% w/v ammonium sulphate, 1% v/v phosphoric acid, 0.1% w/v Coomassie G250 and 20% v/v methanol), subsequently gels were destained in H_2O.

For immunoblot analyses, proteins were transferred from the SDS gel onto a nitrocellulose membrane (Towbin *et al.*, 1979). The membranes were stained with Ponceau Red (0.2% w/v Ponceau S and 3% w/v trichloroacetic acid) to monitor protein loading. Unspecific binding of antiserum was blocked by incubating the membrane with 4% (w/v) non-fat milk powder in TBST (20 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl and 0.1% v/v Tween 20). PLDrp1 and PLDα1 antisera were used for the immunoblot detection at a 1:5000 dilution.

Polyclonal antisera were raised against the recombinant protein PLDrp1 in *E. coli* using an N-terminal PCR-fragment containing the first 710 bp of the *PLDrp1* cDNA. The fragment was amplified using the primers PLDrp1–5Fwd and PLDrp1–5Rev to introduce *Nco*I and *Xho*I restriction sites (Table S2) to clone the fragment into the pET28a expression vector (NOVAGEN, http://www.novagen.com/). Antibodies were produced by immunising rabbits (SEQLAB, http://www.seqlab.de/) with 1 mg affinity-purified recombinant protein.

To produce anti-PLDα1 antibodies, a PLDα1 restriction fragment was cloned into the pGEX4-T expression vector (PHARMACIA DIAGNOSTICS, http://www.phadia.com/) as a glutathione-S-transferase fusion to yield the plasmid pGEX-PLDα1, which was transformed into *E. coli* BL21 cells (Studier and Moffatt, 1986). The expression of the recombinant protein was induced by adding isopropyl-β-D-thiogalactopyranoside to a concentration of 1 mM. Bacterial cells were lysed by sonification and the fusion protein was purified on a glutathione agarose column (Harper and Speicher, 2001). Antiserum was raised in rabbits (BIOGENES, http://www.biogenes.de/).

Detection of the proteins was performed with a peroxidase-coupled anti-rabbit IgG secondary antibody and the ECL Western Blotting detection kit (GE HELATHCARE, http://www.gehealthca

re.com). Visualisation of signals was done in the Intelligent Dark Box II (FUJIFILM CORP., http://www.fujifilm.com) and quantification was done by ImageJ (https://imagej.net/).

Identification of the PLDrp1 protein by MS

Proteins were identified by in-gel digest with trypsin and MALDI-MS and MS/MS analyses on an Ultraflex III system (BRUKER, http://www.bdal.com/; Röhrig *et al.*, 2008). The resulting peptide data were used to search the NCBInr database with the search engine MASCOT (MATRIX SCIENCE, http://www.matrixscience.com). The PLDrp1 protein was identified with a Mascot score of 133 for peptide mass fingerprint (significance threshold of 79) and a sequence coverage of 46.7%. A sub-threshold score of 44.3 for peptide fragment fingerprint (threshold 47) was obtained for the peptide 'SEEQEEGYRKPSYGR' of the same protein.

Bioinformatics

Tandem repeat units were identified in the protein sequence of PLDrp1 by TRUST ('Tracking Repeats Using Significance and Transitivity'; http://www.ibi.vu.nl/programs/trustwww/) and RADAR (http://www.ebi.ac.uk/Tools/pfa/radar/).

PLDrp1 promoter GUS constructs and GUS analyses

The promoter fragment was amplified by nested-PCR. The forward primer PLDrp1-promoter-F1 in the 5′-UTR of the gene *PLDrp1* and the reverse primer PLDrp1-promoter-R1 in *PLDrp1* were used for the first amplification. The second pair of primers was designed to bind inside the first amplified fragment. The primer pair PLDrp1-promoter-F2 and PLDrp1-promoter-R2 were used to generate a 733-bp fragment, which was cloned into the pBT10-GUS vector (Sprenger-Haussels and Weisshaar, 2000). The resulting PLDrp1_promoter::GUS fragment was inserted into the binary vector pBIN19 (Bevan, 1984). The construct was introduced into *Agrobacterium tumefaciens* cells by electroporation (Tung and Chow, 1995) and transformed into *A. thaliana* wild-type and *pldα1* mutant plants using floral dip (Clough and Bent, 1998).

The GUS detection was performed according to Jefferson *et al.* (1987). Seedlings were immersed in X-Gluc solution [0.5 mM X-Gluc, 50 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.1% v/v Triton X–100] and incubated for 16–24 h at 37°C. The chlorophyll was removed by incubation in 80% v/v ethanol at 80°C. The samples were observed under a dissecting microscope (NIKON SMZ-800, http://www.nikon.com) and a compound microscope (NIKON Eclipse-80i, http://www.nikon.com) equipped with a DS-2Mv digital camera.

Gene expression analysis

Total RNA was extracted according to Missihoun *et al.* (2011). For RT-PCR analysis, total RNA was treated with RNase-free DNase I prior to the cDNA-synthesis using the 'RevertAid H Minus First Strand cDNA Synthesis kit' (FERMENTAS, http://www.fermentas.de) according to the manufacturer. The gene-specific primers are listed in Table S3. The reactions were performed using the following programme: 5 min 94°C, 30 cycles (30 sec 94°C, 30 sec 65°C and 30 sec 72°C), 10 min 72°C and pause at 4°C.

Protein-lipid overlay assay

To assess the PA-binding properties of PLDrp1, a protein-lipid overlay assay was performed (Deak *et al.*, 1999). Nitrocellulose membranes with immobilised lipids (5 μg each) were blocked overnight and incubated with the recombinant protein in 5% (w/v) BSA in TBST for 1 h at 4°C. The membranes were washed four times with TBST (5 min) and incubated with the primary antibody (1 h, RT). Washing steps were repeated and the membrane was incubated for 45 min with the secondary antibody.

Liposome-binding assay

Phosphatidic acid and PC were dissolved in chloroform/methanol (2:1) solution (final concentration 4 μg/μl) and stored at −20°C. For each assay, 250 μg lipids (150 μg PC and 100 μg PA) were transferred to fresh glass tubes. Solvents were evaporated and resuspended in liposome-binding buffer [0.5 μl/μg (20 mM MES, 30 mM Tris–HCl (pH 7), 0.5 mM NaCl, 2 M urea, 0.5% w/v CHAPS, 1 mM dithiothreitol)]. The liposome-binding assay was performed according to Zhang *et al.* (2004). The mixture was incubated at 37°C on a shaker for 1 h, vortexed and centrifuged (10 min, 20 000 ***g***, 4°C). The pelleted liposomes were resuspended in liposome-binding buffer containing 0.1 μg μl^{-1} of the protein of interest. The mixture was incubated at 30°C for 30 min and centrifuged (10 min, 10 000 ***g***, 4°C). Supernatants and pellets were analysed on polyacrylamide gels.

Liposome-turbidity assay

The liposome-turbidity assay was prepared as described by Roston *et al.* (2011). The liposome solution was filtered through a Mini-Extruder (AVANTI, https://avantilipids.com) and extruded until the solution was clear. Unilamellar liposomes were transferred to UV-cuvettes and the OD_{350} was determined. The protein PLDrp1 (5 μg) was added to the solution, mixed and the OD_{350} was measured every 2 sec for 8 min.

ACCESSION NUMBERS

The Arabidopsis Information Resource accession numbers used here are At3G15730.1 (PLDα1), At5G25370 (PLDα3), At4G35790.1 (PLDδ3), At1 g55180 (PLDε), At5 g39570 (PLDrp1) and At3 g29075.

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest to declare.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. T-DNA insertion sites for mutant lines.

Figure S2. Amino acid sequence alignment of 25 selected angiosperms and phospholipase D-regulated protein1 (PLDrp1; *Arabidopsis thaliana*).

Figure S3. Intrinsically disordered structure of phospholipase D-regulated protein1 (PLDrp1).

Figure S4. Protein sequence alignment of phospholipase D-regulated protein1 (PLDrp1) and At3 g29075.

Figure S5. Comparison of phospholipase D-regulated protein1 (PLDrp1) and At3 g29075.

Figure S6. Pre-experiment to determine optimal pH-conditions for liposome-binding assays.

Figure S7. Protein-aggregation assay for phospholipase D-regulated protein1 (PLDrp1).

Table S1. Arabidopsis-specific nucleotide-blast of PLDrp1-fragments identified nucleotide-binding proteins and members of the pentatricopeptide-repeat protein family with antisense complementarity.

Table S2. Primers for the amplification of *PLDrp1* fragments used to construct GFP fusions.

Table S3. Primers used for genotyping T-DNA insertion mutants and RT-PCR.

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