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Chapter 6

Hypersalinity-induced phosphatidic acid signalling in *Arabidopsis* seedlings

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Abstract

As the devastating impact of high soil salinity on worldwide crop production is becoming clear, much research is focussed on the question how various plant species respond to low environmental water potential and salinity. The regulation of these responses depends on signal transduction pathways, and several have now been implicated. A recent addition is the hypersalinity-induced activation of phospholipase D (PLD), producing the lipid second messenger, phosphatidic acid (PtdOH). This lipid can also be formed through the combined activities of phospholipase C (PLC), hydrolyzing phosphoinositides, and diacylglycerol kinase (DGK), phosphorylating the resulting diacylglycerol (DAG). In this study, we establish the rapid accumulation of PtdOH in salt-treated *Arabidopsis* seedlings, and investigate its metabolic sources at different stress levels. Evidence is presented for the involvement of two distinct pathways. One, active at medium-high NaCl concentrations (≥ 100 mM), involving the activity of diacylglycerol kinase (DGK), and another, under the experimental conditions, active only at very high concentrations (> 500 mM), involving PLD. Using T-DNA insertion mutants the latter activity was found to depend solely on the PLD δ isozyme. However, a successful identification of the activated DGK and PLC isozymes, of which the *Arabidopsis* genome encodes 7 and 9 genes, respectively, awaits the generation of effective knock-out mutants. A study using the *sos1*, *sos2*, *sos3*, *fry1*, *los1*, and *hos1* mutants, provided evidence that the PtdOH response should be positioned upstream of each of these components. Finally, in an assay of seedling root growth at 25-75 mM NaCl, a *pld α* knock-out mutant, but not the *pld δ* knock-out, displayed enhanced sensitivity compared to wild-type.

Introduction

Excessive soil salinity imposes major constraints on plant growth and limits the arable land. In particular, agricultural potential is compromised by accumulated salts from irrigation water, and from rising underground water tables that bring natural salts in the soil to the surface. Understanding the mechanisms by which plants respond to salt stress is a major goal in plant physiology. Such responses range from the acute phase of stress signal transduction, down to acclimatory responses by means of physiological, developmental and biochemical changes. High soil salt concentrations decrease the extracellular water potential, and challenge turgor maintenance and water uptake. The water potential gradient can be restored by synthesis of cytosolic compatible osmolytes and vacuolar ion storage. However, intracellular accumulation of ions, particularly Na^+ , can reach toxic levels that interfere with enzymatic activities and membrane structure, and disturb cellular ionic balances. To prevent this, Na^+ has to be actively removed from the cytosol to the apoplast or to the vacuole, which is driven by the electrochemical potential gradients across the plasma membrane and the tonoplast, and, in *Arabidopsis*, involves the Na^+ - H^+ antiporters, *AtSOS1* and *AtNHX1*, respectively [1, 2].

A number of components in the salt stress response have been found in screens of mutants affected in salt tolerance and stress-related gene activation [3-5]. Thus, not only *AtSOS1*, but also *AtSOS2*, a serine-threonine kinase, and *AtSOS3*, a Ca^{2+} -regulated protein phosphatase, were found. The latter two form a complex to regulate *AtSOS1* activity, and are thus essential in re-establishing ionic homeostasis. Genes activated in abiotic stresses have conserved promoter elements, including ABRE and DRE/C-repeat, which are the basis for ABA and CBF/DREB dependent regulation of transcription. Using a luciferase reporter under the control of the stress-responsive *RD29A* promoter, several mutants were isolated in *Arabidopsis*, that exhibited altered induction of stress-response genes under cold, drought, salt, and ABA treatments. Thus, the induction was increased in *fry1* and *hos1*, and decreased in *los1*. The physiological functions of the wildtype proteins are subject of current research.

The signalling network underlying responses to hypersalinity is largely unknown, but a high degree of complexity is suggested by the multiplicity of components and pathways involved. They include calcium [6], reactive oxygen species, nitric oxide, cGMP [7], and a variety of protein kinases, including mitogen-activated protein kinases (MAPKs, [8]) calcium-dependent protein kinase (CDPK), SNF-related protein kinases (SnRKs), and S6 kinase. Moreover, the generation of lipid second messengers has been established as one of the fastest responses to hypersalinity [9-11].

Recently, interest has focussed on the minor phospholipid phosphatidic acid (PtdOH) and the enzymes that form it during various stress responses. In a variety of plant systems, it accumulates rapidly in response to osmotic and salt stress [11], drought [9, 12, 13], and low [14] (see Chapter 7), as well as high temperatures [15]. Two different pathways have been

implicated in its formation under these stresses [16-18]. Phospholipase D generates PtdOH by hydrolysis of structural phospholipids such as phosphatidyl ethanolamine (PtdEtn) or phosphatidyl choline (PtdCho), and diacylglycerol kinase (DGK) produces PtdOH by phosphorylation of diacylglycerol (DAG). In the latter pathway, DAG can be derived from PLC-catalyzed hydrolysis of polyphosphoinositides, possibly phosphatidylinositol 4-phosphate (PtdIns4P) or phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂). This activity would liberate the soluble headgroups, inositol bisphosphate (InsP₂) or inositol trisphosphate (InsP₃), respectively. The latter is believed to function in the generation of a cytosolic calcium signal, perhaps because multiple phosphorylation generates inositol hexaphosphate (InsP₆), a potent activator of calcium release in stomatal guard cells [19] (Munnik PCE). Increased levels of InsP₃ have been found in salt-stressed *Arabidopsis* cells [20] and plants [21-23] within minutes after onset of the treatment, suggesting the activation of PLC.

Numerous studies have pointed to a role for different PLD isozymes in water stresses [11, 13, 24-26] (add Wang, Welti). *AtPLDδ* transcripts accumulated within 1 h of salt stress in *Arabidopsis* plants, and antisense suppression decreased its induced activity upon dehydration [13]. In leaf discs, both *AtPLDα1* and *AtPLDδ* contributed to salt-and dehydration-induced PtdOH increases and, using knock-out mutants, the same PLDs were suggested to be essential for root growth under osmotic stress conditions [9]. While the activation of PLC under similar conditions suggested another pathway of PtdOH formation, namely via the phosphorylation of PLC-generated DAG by DGK, this possibility remained largely unexplored.

This study uses whole *Arabidopsis* seedlings as model, and focusses on the question which enzymes are responsible for the rapid PtdOH response that we found. We used ³²P-radiolabelling techniques to dissect the pathways of PtdOH formation, and tried to identify the isozymes involved using T-DNA insertion mutants. Using loss-of-function mutants of *SOS1*, *SOS2*, *SOS3*, *LOS1*, *HOS2*, *FRY1*, and *SNOW1*, we assessed whether the PtdOH response was downstream of any of these elements in the response to high salinity.

Results

In vivo ³²P-radiolabelling assays are well-suited for the study of rapid phospholipid responses to environmental stresses, which in general are very fast (minutes), and they allow quantitatively minor phospholipid changes to be analyzed, while they simultaneously provide evidence for precursor-product relations. *Arabidopsis* seedlings rapidly take up ³²P-P_i added to the liquid medium and incorporate it into organic compounds such as phospholipids. Seedlings are useful in the study of responses to abiotic stress because they are particularly sensitive to environmental cues and capable of stress signalling and

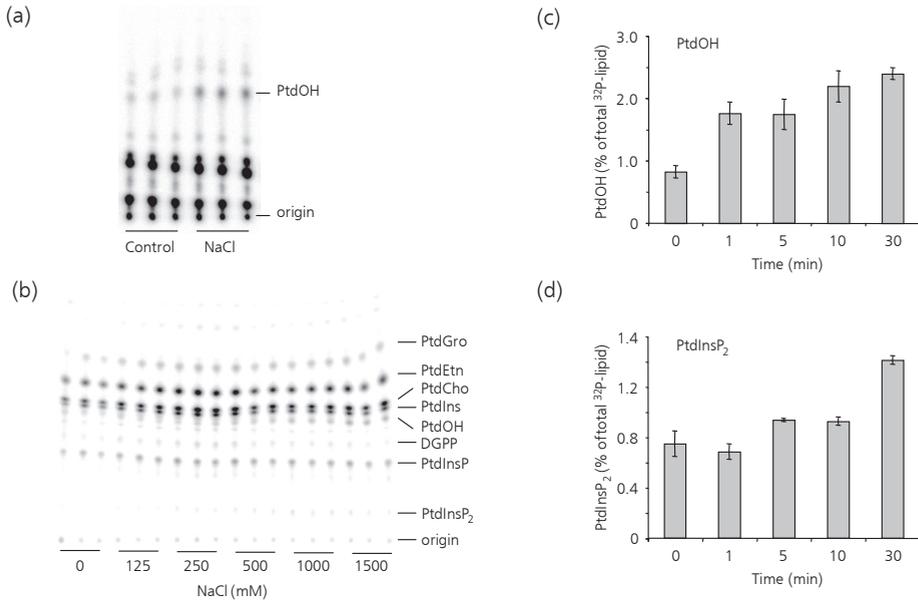


Figure 1. Hypersalinity conditions rapidly induce the formation of PtdOH in *Arabidopsis*. 5-day-old seedlings were O/N labelled with $^{32}\text{P}_i$ and then treated with NaCl. Extracted phospholipids were separated by TLC and analyzed by phosphorimaging. (a) The treatment of seedlings with 300 mM NaCl (5 min) was characterized by massive PtdOH formation. (b) Applying a range of NaCl concentrations (5 min) revealed a dose-dependent PtdOH response. In addition, there was an increase in PtdInsP₂. The time course of the accumulation of PtdOH (c) and PtdInsP₂ (d) in response to 300 mM NaCl shows a very rapid and sustained increment in PtdOH and a slower PtdInsP₂ response. The levels are expressed as radioactivity percentage of the total radiolabelled phospholipids. Values represent the means of triplicates of a representative experiment; error bars indicate standard deviations.

acclimatory responses. We set out to investigate the early phospholipid response to salt stress, focussing on the accumulation of ^{32}P -PtdOH in seedlings, and the enzyme activities underlying this.

Seedlings were prelabelled for 16 hr and then exposed to 300 mM NaCl for 5 min. When their phospholipids were extracted, separated by TLC and visualized by autoradiography, a consistent and substantial increase in PtdOH was detected (Fig. 1a). Moreover, a dose-response experiment revealed two other changes in the phospholipid pattern (Fig. 1b). An increase in DGPP was witnessed, signifying the phosphorylation of PtdOH. In addition, an increase in the minor phosphoinositide PtdInsP₂ was detected at higher osmolarity (> 500 mM NaCl). Time-course experiments using 300 mM NaCl doses showed a rapid PtdOH accumulation, with a marked increase already after 1 min. (Fig. 1c) and a slower PtdInsP₂ accumulation (Fig. 1d).

Since PtdOH is a metabolite in several phospholipid signalling pathways, we set out to determine its metabolic origin under conditions of salt stress. For this purpose a differential

labelling strategy was followed. Using this protocol, the formation of ^{32}P -PtdOH through the activities of DGK and PLD can be distinguished, as the first depends on the ^{32}P -labelling of ATP and the second depends on the ^{32}P -labelling of the phosphodiester of the PLD substrate, e.g. PtdEtn or PtdCho. Hence, after short prelabelling, when the ATP pool has a high degree of radiolabelling, but the PLD substrates are hardly labelled, the formation of ^{32}P -PtdOH reflects DGK rather than PLD activity. However, long labelling times warrant sufficient $^{32}\text{P}_i$ -incorporation into the PLD substrates, and hence, the ^{32}P -PtdOH it produces. To specifically assay PLD, its ability to catalyze the transphosphatidylation of a primary alcohol is used; i.e. in the presence of 0.5% n-ButOH, PLD transfers the

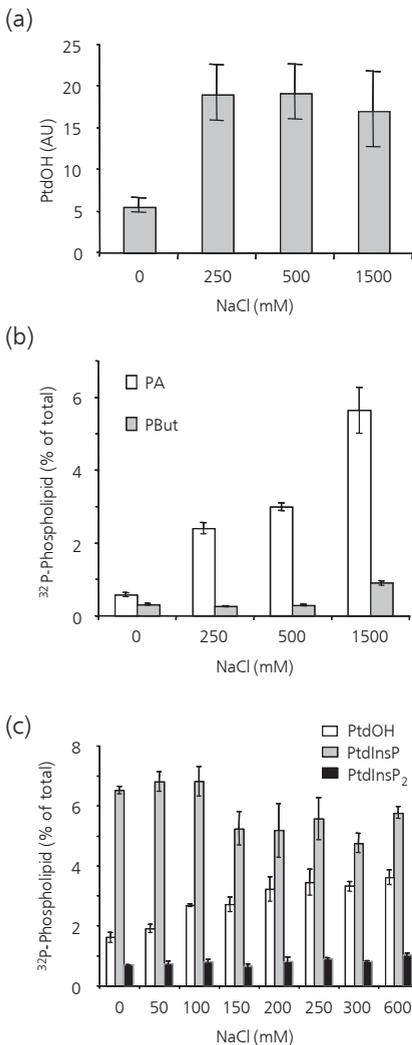


Figure 2. Distinguishing between DGK- and PLD-mediated PtdOH formed in response to 5 min. of hypersalinity. (a) To discriminate DGK- and PLD-mediated PtdOH a differential labelling strategy was applied [46]. Probing DGK activity, seedlings were metabolically labelled with $^{32}\text{P}_i$ for 15 min. followed by 5 min NaCl treatment. Lipids were extracted, separated by TLC, and quantified by phosphoimaging. Data are in arbitrary units (AU) because after a short labelling time the amount of PtdOH cannot be expressed relative to the weakly labelled structural lipids. (b) Alternatively, plants were labelled O/N and treated with salt in the presence of 0.5% ButOH. The accumulation of PtdOH (white bars) was monitored and the formation of PBut (grey bars) was taken as a measure of PLD activity and expressed as percentage of the total labelled lipid. (c) Detailed dose-response study, using O/N $^{32}\text{P}_i$ labelling, showing PA and concurrent changes in the PPIs.

phosphatidyl moiety of the phospholipid substrate to the alcohol to produce the artificial lipid PtdBut. The accumulation of PtdBut is used as a measure of the activity of PLD [27]. Fig. 2a shows the ^{32}P -PtdOH response of seedlings, prelabelled for 15 min and subsequently challenged with different concentrations of NaCl. There was a clear increase of ^{32}P -PtdOH at 250 mM NaCl, but higher concentrations did not elicit greater increments. This was in contrast with the pattern after long prelabelling (Fig. 2b) which featured an increase at 250 mM, but an additional, larger rise between 500 and 1500 mM NaCl. After 15 min prelabelling the structural phospholipids were hardly labelled (not shown), so the ^{32}P -PtdOH accumulation could not be the result of PLD activity. Rather, an activation

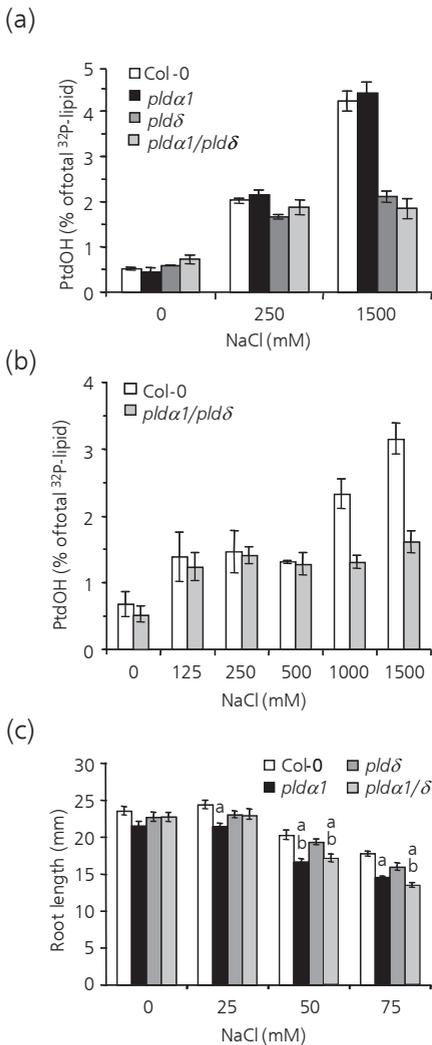


Figure 3. Salt-induced PtdOH formation and root growth inhibition in PLD-knockout lines. (a) The PtdOH response to severe hypersalinity is abrogated in the *pldδ* and the *pldα1/pldδ* double mutants. Seedlings were prelabelled O/N with ^{32}P -P_i and treated 5 min with the NaCl concentrations indicated. Incubations were terminated by the addition of PCA to a final conc of 5%, and lipids were extracted, analyzed by TLC and quantified by phosphoimaging (values \pm SD). (b) Similarly, a more detailed study of the *pldα1/pldδ* double mutant showed saturation of the PtdOH response at 250 mM NaCl. (c) Primary root length (mean \pm SE) of Col-0, and the mutants *pldα1*, *pldδ* and *pldα1/pldδ*, after 7 d of seedling growth. Data were analyzed for significance ($p < 0.01$) by one-way ANOVA; a = different from Col-0 at the same salt conc; b = different from *pldδ* at the same salt conc.

Table 1. Hypersalinity-induced PtdOH formation in DGK T-DNA insertion lines 5-day old *Arabidopsis* seedlings were labelled O/N with $^{32}\text{P}_i$ and treated with 300 mM NaCl for 5 min. Lipids were extracted, separated by TLC and quantified by phosphoimaging. PtdOH levels are expressed as a percentage of the total ^{32}P -labelled lipids and represent the averages of multiple samples containing 2 seedlings each, followed by stdev's. The total numbers of replications and experiments per genotype are listed in the last column. Wt = Col-0

gene	AGI ID	Line	Name	Control	Salt
wt		-		0.75 ± 0.18	1.73 ± 0.29
<i>AtDGK1</i>	At5g07920	SALK 053412	<i>dgk1-1</i>	0.67 ± 0.09	1.83 ± 0.10
<i>AtDGK2</i>	At5g63770	SAIL 718_G03	<i>dgk2-1</i>	0.77 ± 0.11	1.95 ± 0.31
		SAIL 71_B03	<i>dgk2-2</i>	0.63 ± 0.10	1.74 ± 0.33
<i>AtDGK3</i>	At2g18730	SALK 082600	<i>dgk3-1</i>	0.72 ± 0.03	1.94 ± 0.12
<i>AtDGK4</i>	At5g57690	SAIL 339_C01	<i>dgk4-1</i>	0.73 ± 0.09	1.77 ± 0.19
		SALK 069158	<i>dgk4-2</i>	0.70 ± 0.08	1.53 ± 0.14
<i>AtDGK5</i>	At2g20900	SAIL 1212_E10	<i>dgk5-1</i>	0.57 ± 0.07	1.63 ± 0.12
		SAIL 253_E12	<i>dgk5-2</i>	0.62 ± 0.06	1.82 ± 0.21
<i>AtDGK6</i>	At4g28130	SALK 016285	<i>dgk6-1</i>	0.79 ± 0.05	1.85 ± 0.28
<i>AtDGK7</i>	At4g30340	SAIL 51_E04	<i>dgk7-1</i>	0.70 ± 0.07	1.90 ± 0.27
		SALK 059060	<i>dgk7-2</i>	0.87 ± 0.26	2.04 ± 0.16
		SALK 007896	<i>dgk7-3</i>	0.79 ± 0.11	2.04 ± 0.20

Table 2. Hypersalinity-induced PtdOH formation in PLC T-DNA insertion lines. Experiment as in Table 1. PtdOH levels are expressed as a percentage of the total ^{32}P -labelled lipids with stdev's in brackets.

Wild type	Gene	AGI ID	Line	Name	Control	300 mM NaCl
Columbia gl 1	-	-	-	-	0.57 ± 0.03	1.88 ± 0.18
	<i>PLC1</i>	At5g58670	ET Jack	<i>plc1-1</i>	0.49 ± 0.04	1.83 ± 0.11
Columbia-0	-	-	-	-	0.63 ± 0.09	1.76 ± 0.07
	<i>PLC2</i>	At3g08510	SAIL 224_F02	<i>plc2-1</i>	0.55 ± 0.05	1.84 ± 0.20
	<i>PLC3</i>	At4g38530	SAIL 1219_G08	<i>plc3-1</i>	0.63 ± 0.09	1.80 ± 0.19
	<i>PLC4</i>	At5g58700	SAIL 791_05	<i>plc4-1</i>	0.50 ± 0.07	2.02 ± 0.14
	<i>PLC5</i>	At5g58690	SALK 144469	<i>plc5-1</i>	0.62 ± 0.10	1.84 ± 0.20
	<i>PLC6</i>	At2g40116	SALK 090508	<i>plc5-1</i>	0.54 ± 0.09	1.88 ± 0.31
			SALK 041365	<i>plc6-2</i>	0.61 ± 0.10	1.62 ± 0.36
	<i>PLC7</i>	At3g55940	SAIL 303_H07	<i>plc7-1</i>	0.63 ± 0.08	1.78 ± 0.15
			SAIL 636_F05	<i>plc7-2</i>	0.62 ± 0.05	1.73 ± 0.06
			SALK 030333	<i>plc7-3</i>	0.74 ± 0.10	2.01 ± 0.17
			SALK 148821	<i>plc7-4</i>	0.56 ± 0.03	1.58 ± 0.24
	<i>PLC9</i>	At3g47220	SALK 025949	<i>plc9-1</i>	0.71 ± 0.07	1.94 ± 0.24
			SALK 021982	<i>plc9-2</i>	0.71 ± 0.08	1.99 ± 0.18

Stim.Fold	Replicates (exp)
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2.3	10 (3)
2.7	6 (2)
2.5	6 (2)
2.8	6 (2)
2.8	3 (1)
2.4	6 (2)
2.2	6 (2)
2.9	3 (1)
2.9	3 (1)
2.3	3 (1)
2.7	6 (2)
2.4	6 (2)
2.6	6 (2)

Stim. Fold	Replicates (exp)
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3.30	6 (2)
3.73	6 (2)
2.80	6 (2)
3.37	6 (2)
2.87	6 (2)
4.06	6 (2)
2.94	6 (2)
3.50	6 (2)
2.64	6 (2)
2.82	3 (1)
2.79	3 (1)
2.72	3 (1)
2.84	6 (2)
2.75	6 (2)
2.79	6 (2)

of DGK could account for this, since the ATP pool is rapidly labelled. However, the additional increase at 1500 mM, visualized only after long ^{32}P -labelling, is most likely the consequence of PLD activation. To test this possibility, the transphosphatidylation assay was applied. When 0.5% ButOH was added to the medium, ^{32}P -PtdBut formation was witnessed (Fig. 2b). Its level remained constant at salt concentrations up to 500 mM, but showed a marked increase at 1500 mM NaCl, correlating with the second large rise in ^{32}P -PtdOH. This suggested the activation of PLD at high NaCl concentrations, and the involvement of DGK at concentrations < 250 mM. A detailed dose-response experiment (Fig. 2c) showed a distinct increase in ^{32}P -PtdOH at 100 mM NaCl, while higher concentrations up to 600 mM resulted in gradual increments. Also, ^{32}P -PtdInsP₂ increased slightly under these conditions. Interestingly, ^{32}P -PtdInsP levels declined at concentrations of 150 mM NaCl or higher. This is suggestive of its hydrolysis by PLC, which could be the basis for the increase in ^{32}P -PtdOH at low NaCl levels as it would provide the DAG substrate for DGK. The *Arabidopsis* genome encodes 7 DGKs and 9 PLCs, some of which have been implicated in responses to abiotic stresses. E.g. *DGK2* is transcriptionally induced by cold and *PLC1* by drought, salt and cold [28, 29]. In an attempt to identify isozymes responsible for the early PtdOH response in *Arabidopsis* seedlings, we made use of T-DNA insertion lines. Several homozygous lines containing an insert in or close to the respective coding regions were obtained from SAIL and SALK collections (Suppl. Tables 1 and 2) and tested for their PtdOH response (Table 1, Table 2, Suppl. Fig. 1). In the DGK set of mutants, the control levels of PtdOH were similar, and stimulations varied between 2.2 and 2.9 times control levels. In the PLC lines, stimulations ranged from 2.8 to 4.06 times control levels. Variations in values obtained from different experiments sometimes made it difficult

to compare them, and assess reproducible significant alterations. None of the T-DNA insertion lines displayed a decreased PtdOH response.

By a similar genetic approach, the two predominant PLD isozymes in *Arabidopsis*, previously implicated in the response to high salt concentrations, viz. PLD α 1 and PLD δ , were tested for their contribution to the PtdOH increase in salt-stressed seedlings, using the previously described *plda1* and *pldd* knock-out lines, and the *plda1/pldd* double mutant [9]. Since our biochemical data implied a role for PLD at high concentrations NaCl (> 500 mM), mutant and wild-type seedlings were challenged with a range of concentrations up to 1500 mM (Fig. 3a and b). Interestingly, the PA increase of *plda1* seedlings was unaffected, but clearly suppressed in *pldd* and the double mutant (Fig. 3a). This suppression was most clearly visible at 1500 mM NaCl, effectively wiping out any increases in excess of the level attained at 250 mM. A more detailed analysis of the double mutant confirmed that the mutations affected responses at salt concentrations higher than 500 mM (Fig. 3b). These results are in good agreement with our biochemical ^{32}P -labeling data suggesting PLD involvement under severe salt stress (Fig. 2), and indicate a principal role for AtPLD δ in seedlings.

Salt and osmotic stress affects growth and development of the root system [30]. In particular, high salt concentrations have been shown to inhibit primary root growth and the formation of lateral roots. The inhibition of primary root growth of seedlings grown on agar supplemented with 150 mM NaCl has previously been found to be more severe in *plda1*, *pldd* and double knock-out lines [9]. Similarly, we tested the same mutants at concentrations of 0, 25, 50 and 75 mM NaCl, quantifying root length after 7 d of seedling growth (Fig. 3c). Seeds were sown directly on agar containing the appropriate salt concentrations, their germination being hardly affected by the supplement. At concentrations of 50 mM and 75 mM NaCl, primary root lengths in wt plants were decreased by 14% and 25%, respectively. In *plda1* seedlings, control root lengths were unaffected, but the inhibition at 50 and 75 mM NaCl was stronger, showing reductions of 23% and 36% the control values, respectively. Although *pldd* root length tended to be more inhibited, it was not statistically significant. The *plda1/pldd* double mutant displayed a reduced root growth phenotype similar to *plda1*.

The accumulation of PtdOH is a fast response to hypersalinity, and as such one would predict it to be upstream of other components in salt stress signalling. The SOS proteins function in restoring ionic balance in the presence of high $[\text{Na}^+]$ in the environment. *SOS3* encodes a Ca^{2+} -binding protein which binds to *SOS2*, a serine/threonine protein kinase. One of the functions of the *SOS2-SOS3* complex is to activate the Na^+/H^+ exchange activity of *SOS1*. KO mutants of these genes are impaired in salt stress adaptation. To investigate whether the PtdOH response occurs up- or downstream of these protein functions, we analyzed PA accumulation in the *sos1*, *sos2* and *sos3* mutants.

Since growth of *sos1* seedlings was generally retarded, they were grown for seven days instead of five, to allow them to reach the same developmental state as the other strains.

Basal levels of ^{32}P -PtdOH (Fig. 4a) and other ^{32}P -lipids (not shown) were similar. Treatment with 300 mM NaCl for 5 min. resulted in stimulations in PtdOH, varying between 2.0 and 2.4 times the control levels.

Finally, several mutants were tested which are altered in their responses to water-stress: *fry1*, *hos1*, *los1* (Fig. 4b). The results showed no reproducible changes in the levels of ^{32}P -PtdOH. Taken together, these data suggest a position of PtdOH signalling in the NaCl response, upstream of these previously characterized components. In contrast, *myb15* a KO mutation in *AtMYB15*, an R2R3 transcription factor, which is thought to be a negative regulator of CBF/DREB1 in cold acclimation [31], and may function in ABA signalling under drought stress [32], was associated with an increased PtdOH response of, on average, 3.7 times control values, compared to wt stimulations of 2.8 times control values (Fig. 4c). In contrast, the response to cold shock treatment was unaffected in *myb15*.

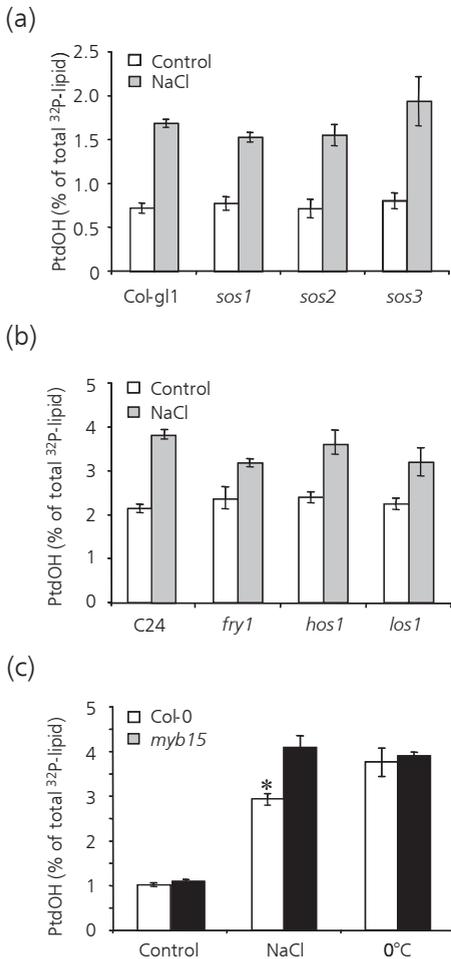


Figure 4. Salt-induced PtdOH response in several mutants. (a) 5-day old *sos1*, *sos2*, *sos3* and Col-gl seedlings were labelled O/N with $^{32}\text{P}_i$ and then challenged with 300 mM NaCl for 5 min. Lipids were subsequently extracted, separated by TLC and quantified by phosphoimaging. The results shown are representative of 3 independent experiments and the values are means of triplicates of 2 seedlings each. Similarly, *fry1*, *hos1*, *los1* and their genetic background control C24RD29A-luc were investigated (B), while *myb15* seedlings (C) were assayed with 300 mM NaCl or at 0°C for 5 min. Asterisk marks a significant difference between wt and mutant values ($p=0.003$).

Discussion

³²P-PtdOH accumulates under salt stress in Arabidopsis seedlings

This study shows that high environmental NaCl concentrations induce the rapid accumulation of PtdOH in *Arabidopsis* seedlings. Within 1 min of 300 mM salt treatment, an average 2.3-fold increase in ³²P-PtdOH was found. A sigmoidal dose-response relation (Fig. 2b) suggested the involvement of different metabolic pathways at moderate (250 mM NaCl) and severe (1500 mM NaCl) salt stress levels. PtdOH, traditionally just regarded as common precursor in glycerolipid synthesis and product of basal turn-over, is now emerging as a potential phospholipid signal in environmental stress responses. PtdOH metabolism is complex, because of the diversity of enzymes that are involved in its synthesis and breakdown (Chapter 1).

Previous studies have found osmotic stress- and salt-induced accumulation of PtdOH in a variety of other plant models, ranging from leaves of *Arabidopsis* [9, 23] and rice [10], to suspension-cultured cells of tomato and alfalfa [9, 11] and *Chlamydomonas* [11, 33]. Frequently, it was explained in terms of the activation of PLD activity, the evidence being based on both biochemical and genetic approaches. Nevertheless, PtdOH is also the product of the phosphorylation of DAG by DGK activity, which has been implicated in several environmental stress responses of biotic as well as abiotic origin. Moreover, several *AtPLC* genes are induced under conditions of environmental stress, including salt stress [34, 35], and the accumulation of Ins(1,4,5)P₃ in *Arabidopsis* cells [20] and plants [21, 23] suggested its activation. These data suggested that salt-induced PtdOH may involve not only PLD activity but also DGK, acting on PLC-generated DAG. Hence, we decided to investigate the source(s) for PtdOH in salt-stressed seedlings.

DGK generates PtdOH at moderate and high levels of salt stress

To dissect the pathways a differential phospholipid radiolabelling protocol was followed [16, 36]. In experiments using short prelabelling times to probe DGK activity, we found a distinct increase in PtdOH at moderate stress levels (Fig. 2a). Studies using *Chlamydomonas* cells similarly implicated DGK activity in salt-induced PtdOH accumulation [11]. Interestingly, in another study, analysis of the fatty acid profiles of PtdInsP₂, DAG and PtdOH of salt-stressed *Arabidopsis* plants, provided evidence not only for DAG phosphorylation, but for DAG production through PLC-mediated hydrolysis of a subpool of polyphosphoinositides [23]. Accordingly, the ³²P-PtdInsP decrease, which accompanied PtdOH formation (Fig. 2c), may reflect its hydrolysis by PLC. It could also be due to its phosphorylation to PtdInsP₂, but this seems less likely as the increase in the latter does not complement the PtdInsP decrease (Fig. 2c), and is much slower (Fig. 1d).

PLD generates PtdOH at levels of severe salt stress in seedlings: AtPLD δ is responsible

As the first part of the PtdOH response (< 500 mM NaCl) was tentatively ascribed to DGK activity, the source for the second part (> 500 mM NaCl) reflected PLD activity. This hypothesis was substantiated by the enhanced formation of the artificial phospholipid PtdBut through PLD-catalyzed transphosphatidylation as a measure of PLD activity (Fig. 2b). The increase in ^{32}P -PtdBut at 1500 mM NaCl suggested that at this concentration PLD activity was stimulated, responsible for the second part of the PtdOH response curve (Fig. 2b). The first part was not accompanied by ^{32}P -PtdBut formation, in agreement with our conclusion that this reflected DGK activity rather than PLD.

Several previous studies have argued for PLD functioning in responses to dehydration and salinity stress, and in *Arabidopsis*, four PLD isozymes have been implicated [25], PLD α 1, PLD δ , PLD α 3 and PLD ϵ . The latter two have been linked to the regulation of root architecture under hyperosmotic and salinity stress. The most abundant PLDs in *Arabidopsis* seedlings, PLD α 1 and PLD δ , are thought to be involved in the ABA-dependent regulation of stomatal aperture (PLD α 1) and defence against oxidative stress (PLD δ), and their silencing or knock-out leads to altered water stress responses.

In the present study, knock-out mutants of *PLD α 1* and *PLD δ* , and a double mutant, were tested for their salt-induced PtdOH responses, and, surprisingly, the activity of PLD δ was found to account for the entire increment in PtdOH at concentrations in excess of 1 M NaCl (Fig. 2b). Previously, using leaf discs of the same knock-out lines, not only PLD δ but also PLD α 1 has been suggested to contribute to the PtdOH response under dehydration and hypersalinity stress [9]. Also, in *PLD δ* antisense-suppressed *Arabidopsis* leaf discs a partial reduction in dehydration-induced PtdOH accumulation has been found [13]. The apparent difference between results from seedlings and leaves could merely reflect the distribution of the PLD isozymes. Interestingly, whereas *PLD α 1* is constitutively expressed, *PLD δ* is upregulated by dehydration within one hour, most clearly in the vascular tissues of cotyledons, which could indicate a localized early activation of the enzyme [25].

PLD α 1, but not PLD δ , is required for normal seedling primary root growth on media supplemented with NaCl (25-75 mM)

High environmental salt concentrations inhibit root growth, as a result of high osmotic strength [9, 25, 30], and we used this response in a physiological test, to investigate whether the sensitivities of *pld α 1*, *pld δ* and double mutant seedlings were altered. Compared to Col-0, primary root growth of *pld α 1* and *pld α 1-pld δ* seedlings was reduced at NaCl concentrations \geq 25 mM, and \geq 50 mM, respectively. PLD δ -deficient seedlings were not affected, which indicated a discongruency with the reduced early PtdOH formation at higher stress levels in this mutant. Another study found that abrogation of *PLD δ* does not compromise drought tolerance [37], although *pld δ* plants generated less

PtdOH in response to drought [13]. This does not preclude a physiological role of this enzyme in the response, since different PLD enzymes might substitute for one another. For example, *PLD α 1*-silenced plants display an enhanced induction of *PLD δ* under drought stress, which was speculated to mitigate the adverse effects of the loss of *PLD α 1* function [37]. Of course, *PLD δ* may have a function which is not associated with root growth. Nevertheless, earlier studies of *pld α 1* and *pld δ* seedlings showed reduced root growth in both single and the double mutants at 150 mM salt, suggesting that *PLD δ* could play a role at more severe stress levels. In addition, a recent study [38] showed that also *AtPLD ϵ* contributed to the PtdOH response and was required for root growth under salt stress, since corresponding knock-out lines displayed excessive growth reductions at 50 mM NaCl.

PtdOH formation is 'upstream' of SOS1/2/3, FRY1, HOS1 and LOS1, and is enhanced in Atmyb15 seedlings

The Ca²⁺-dependent SOS pathway functions under salt stress to remove cytoplasmic Na⁺, and, as a consequence, the *sos1*, *sos2* and *sos3* KO mutants displayed retarded growth on salt-supplemented agar plates. The interesting question is now raised, whether the SOS proteins function upstream of PtdOH signalling. To this end, the salt-induced PtdOH responses in the SOS mutants were analyzed. Neither the accumulation in PtdOH (Fig. 4a), nor that of PtdInsP₂ (not shown), was significantly altered in any of the mutants. Also the lipid responses in the *fr1*, *los1* and *hos1* mutant seedlings were not affected (Fig. 4b). These results suggest that the accumulation of PtdOH does not occur downstream of these components, as one might expect based on its extremely rapid kinetics. This adds PtdOH to the list of potential second messengers with a function in salt stress signalling, together with PtdInsP₂ and Ca²⁺. Interestingly, abrogation of *MYB15*, a transcription factor which has been implicated in different abiotic stress responses, increases salt-induced PtdOH, which may reflect altered transcript levels of PLC or DGK genes. Remarkably, cold stress-induced PtdOH accumulation was unaltered (Fig. 4c).

In summary, we characterized the early salt-induced accumulation of PtdOH in *Arabidopsis*, and found two distinct parts of the response, one occurring at medium-high salt stress levels featuring DGK activity, and the other at high salt concentrations involving PLD. The separation of the two pathways offers the unique possibility to identify signalling components associated with either one of the activities. E.g. the formation of the enigmatic plant stress lipid DGPP, witnessed at 250 mM, is most likely the phosphorylation product of DGK-derived PtdOH, and not of PLD-derived PtdOH, since the latter is only stimulated at much higher NaCl concentrations.

Our attempt to identify the responsible DGK isozyme, and the PLC providing its substrate DAG, was not successful, likely due to the missing out of effective T-DNA insertion mutants for each of the genes, and redundancy problems. In contrast, surprisingly, the PLD-dependent increment in PtdOH at very high salinity levels was demonstrated to be entirely the result of PLD δ activity. For seedling growth on salt media several PLDs are required and it will be interesting to learn how they work. Some answers to questions of functionality might result from the use of multiple mutants, which will more effectively abrogate the enzyme's activity. Furthermore, determining the distribution and subcellular localization of the PtdOH-generating enzymes using fluorescently-tagged proteins may give important clues which will help to fit them into the current models of environmental stress.

Materials and Methods

Plant material and culture

Unless indicated otherwise, Arabidopsis thaliana ecotype Col-0 was used.

RT-QPCR of DGK T-DNA insertion lines was performed as described elsewhere (see Chapter 7). Inserts in *dgk2-1* (SAIL718_G03), *dgk2-2* (SAIL71_B03), *dgk7-2* (SALK059060) and *dgk7-3* (SALK007896) caused suppressed expression, and *dgk5-1* (SAIL1212_E10) showed gene knock-out (see Supplementary table 1). Six of the PLC insertion mutant lines (see Supplementary table 2), and *pld α 1*, *pld δ* , and *pld α 1/pld δ* double mutants [9] displayed abrogation of gene expression. Seeds of the *sos1* [39], *sos2* [40], *sos3* [41], *fry1* [42], *hos1* [43], *los1* [44] and *myb15* [45] mutant lines were kindly provided by Dr. J.K. Zhu.

Surface-sterilized seeds were sown on 1% agar plates containing 2.15 g/l MS salts and 0.5 g/l MES buffer. For uniform germination and growth of the mutants, media were supplemented with 1 % sucrose. After a 2-day period at 4°C in the dark, plates were transferred to a climate room, under a 12 h light/ 12 h dark regime at 21°C and 70% humidity.

Treatments and lipidology

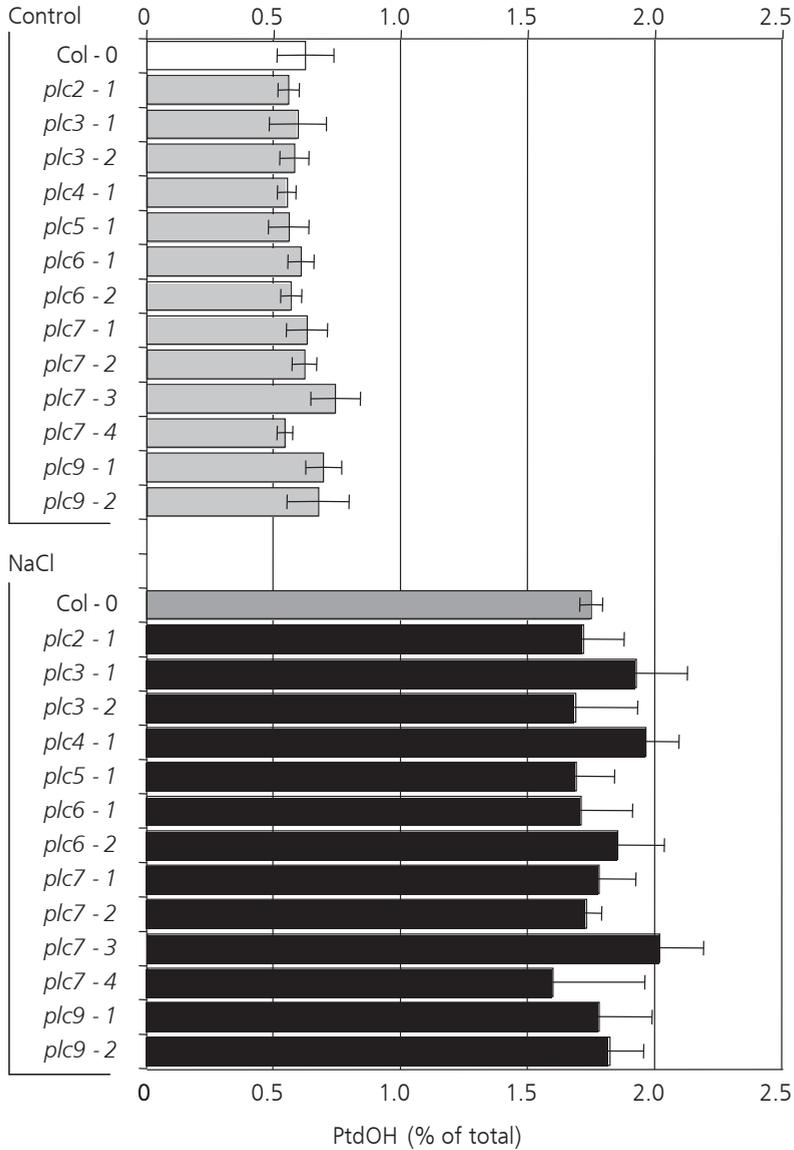
5-day-old seedlings were transferred to 2.0 ml Eppendorf tubes, containing MES(2-[N-Morpholino]ethane sulfonic acid)-based buffer of 2.56 mM MES (pH 5.7) and 10 mM KCl. To label phospholipids, 5 μ Ci carrier-free 32 P-PO $_4^{3-}$ (32 P-P $_i$) per tube was added for different periods of time. Treatments were started by adding the MES buffer supplemented with the appropriate NaCl concentrations, and stopped by addition of perchloric acid to a final concentration of 5% (w/v) and 10 min of subsequent shaking. The total solvent was removed and 375 μ l CHCl $_3$ /MeOH/HCl (50:100:1, [v:v:v]) was added to extract the lipids. After 10 min of vigorous shaking, two phases were induced by adding 375 μ l

CHCl₃ and 200 µl 0.9% (w/v) NaCl. The organic lower phase was then transferred to a tube containing 375 µl CHCl₃/MeOH/1M HCl (3:48:47, [v:v:v]). Shaking, spinning and removing the upper phase yielded a purified organic phase, which was dried down in a vacuum centrifuge at 50°C. The residue was resuspended in 50 µl CHCl₃, and sampled for lipid analysis.

Phospholipids were analyzed by thin-layer chromatography (TLC) on silica gel 60 plates (20x20 cm) using one of the following solvent systems (ratios by vol.): A ethylacetate/*iso*-octane/formic acid/H₂O (13:2:3:10), B CHCl₃/MeOH/NH₄OH(25%) /H₂O (90:70:4:16). Only the organic phase of A was used for TLC. Radiolabelled phospholipids were visualized by autoradiography and quantified by phosphoimaging.

Root growth assay

Seeds were sown directly on agar plates supplemented with the appropriate NaCl concentrations. Genotypes were represented by 3 sets of 12 seedlings each. After at least 2 days dark-incubation at 4°C, plates were transferred to the growth chamber. Primary root lengths of 7-day-old seedlings were microscopically measured using Object Image Software.

Supplementary Figure 1. Hypersalinity-induced PtdOH formation in PLC T-DNA insertion lines. Experiment of Table 2.

Supplementary Table 1. DGK T-DNA insertion lines used in this study.

Name	AGI ID	Line	Position insert relative to ATG	Status
<i>dgk1-1</i>	At5g07920	SALK 053412	>+ 1398	KD
<i>dgk2-1</i>	At5g63770	SAIL 718_G03	>+ 1367	
<i>dgk2-2</i>	At4g38530	SAIL 71_B03	<+ 698	
<i>dgk3-1</i>	At2g18730	SALK 082600	<+ 2819	
<i>dgk4-1</i>	At2g20900	SAIL 339_C01	>+ 2407	
<i>dgk4-2</i>		SALK 069158	>+ 2277	
<i>dgk5-1</i>	At2g20900	SAIL 1212_E10	>+ 2795	KO
<i>dgk5-2</i>		SAIL 253_E12	> - 511	
<i>dgk6-1</i>	At4g28130	SALK 016285	<+ 1625	
<i>dgk7-1</i>	At4g30340	SAIL 51_E04	>+ 1480	
<i>dgk7-2</i>		SALK 059060	> - 300	KD
<i>dgk7-3</i>		SALK 007896	>+ 1743	KD

Supplementary Table 2. PLC T-DNA insertion lines used in this study.

Name	AGI ID	Line	Position insert relative to ATG	Status
<i>plc1-1</i>	At5g58670	ET Jack	< - 400	
<i>plc2-1</i>	At3g08510	SAIL 224_F02	> - 600	
<i>plc3-1</i>	At4g38530	SAIL 1219_G08	> - 41	
<i>plc3-2</i>		SALK 037453	>+ 698	KO
<i>plc4-1</i>	At5g58700	SAIL 791_05	>+ 255	
<i>plc5-1</i>	At5g58690	SALK 144469	>+ 2242	KO
<i>plc6-1</i>	At2g40116	SALK 090508	<+ 480	KO
<i>plc6-2</i>		SALK 041365	<+ 2234	
<i>plc7-1</i>	At3g55940	SAIL 303_H07	> - 133	
<i>plc7-2</i>		SAIL 636_F05	> - 279	
<i>plc7-3</i>		SALK 030333	>+ 1768	KO
<i>plc7-4</i>		SALK 148821	<+ 13	
<i>plc9-1</i>	At3g47220	SALK 025949	<+ 711	KO
<i>plc9-2</i>		SALK 021982	<+ 934	KO

References

- [1] J.K. Zhu, Salt and drought stress signal transduction in plants, *Annu Rev Plant Biol* 53 (2002) 247-273.
- [2] D. Bartels, R. Sunkar, Drought and salt tolerance in plants, *Crit Rev Plant Sci* 24 (2005) 23-58.
- [3] V. Chinnusamy, K. Schumaker, J.K. Zhu, Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants, *Journal of experimental botany* 55 (2004) 225-236.
- [4] K. Yamaguchi-Shinozaki, K. Shinozaki, Organization of cis-acting regulatory elements in osmotic- and cold-stress-responsive promoters, *Trends in plant science* 10 (2005) 88-94.
- [5] T. Urao, T. Katagiri, T. Mizoguchi, K. Yamaguchi-Shinozaki, N. Hayashida, K. Shinozaki, An *Arabidopsis thaliana* cDNA encoding Ca(2+)-dependent protein kinase, *Plant physiology* 105 (1994) 1461-1462.
- [6] H. Knight, A.J. Trethewey, M.R. Knight, Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity, *Plant J* 12 (1997) 1067-1078.
- [7] L. Donaldson, N. Ludidi, M.R. Knight, C. Gehring, K. Denby, Salt and osmotic stress cause rapid increases in *Arabidopsis thaliana* cGMP levels, *FEBS Lett* 569 (2004) 317-320.
- [8] M. Teige, E. Scheikl, T. Eulgem, R. Doczi, K. Ichimura, K. Shinozaki, J.L. Dangl, H. Hirt, The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*, *Mol Cell* 15 (2004) 141-152.
- [9] B.O. Bargmann, A.M. Laxalt, B. ter Riet, B. van Schooten, E. Merquiol, C. Testerink, M.A. Haring, D. Bartels, T. Munnik, Multiple PLDs required for high salinity and water deficit tolerance in plants, *Plant & cell physiology* 50 (2009) 78-89.
- [10] E. Darwish, C. Testerink, M. Khalil, O. El-Shihy, T. Munnik, Phospholipid signaling responses in salt-stressed rice leaves, *Plant & cell physiology* 50 (2009) 986-997.
- [11] T. Munnik, H.J. Meijer, B. Ter Riet, H. Hirt, W. Frank, D. Bartels, A. Musgrave, Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate, *Plant J* 22 (2000) 147-154.
- [12] W. Frank, T. Munnik, K. Kerkmann, F. Salamini, D. Bartels, Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*, *The Plant cell* 12 (2000) 111-124.
- [13] T. Katagiri, S. Takahashi, K. Shinozaki, Involvement of a novel *Arabidopsis* phospholipase D, AtPLDdelta, in dehydration-inducible accumulation of phosphatidic acid in stress signalling, *Plant J* 26 (2001) 595-605.
- [14] E. Ruelland, C. Cantrel, M. Gawer, J.C. Kader, A. Zachowski, Activation of phospholipases C and D is an early response to a cold exposure in *Arabidopsis* suspension cells, *Plant physiology* 130 (2002) 999-1007.
- [15] M. Mishkind, J.E. Vermeer, E. Darwish, T. Munnik, Heat stress activates phospholipase D and triggers PIP accumulation at the plasma membrane and nucleus, *Plant J* 60 (2009) 10-21.
- [16] S.A. Arisz, C. Testerink, T. Munnik, Plant PA signaling via diacylglycerol kinase, *Biochimica et biophysica acta* 1791 (2009) 869-875.
- [17] T. Munnik, C. Testerink, Plant phospholipid signaling: "in a nutshell", *Journal of lipid research* 50 Suppl (2009) S260-265.
- [18] C. Testerink, T. Munnik, Phosphatidic acid: a multifunctional stress signaling lipid in plants, *Trends in plant science* 10 (2005) 368-375.
- [19] F. Lemtiri-Chlieh, E.A. MacRobbie, A.A. Webb, N.F. Manison, C. Brownlee, J.N. Skepper, J. Chen, G.D. Prestwich, C.A. Brearley, Inositol hexakisphosphate mobilizes an endomembrane store of calcium in guard cells, *Proceedings of the National Academy of Sciences of the United States of America* 100 (2003) 10091-10095.

- [20] S. Takahashi, T. Katagiri, T. Hirayama, K. Yamaguchi-Shinozaki, K. Shinozaki, Hyperosmotic stress induces a rapid and transient increase in inositol 1,4,5-trisphosphate independent of abscisic acid in *Arabidopsis* cell culture, *Plant & cell physiology* 42 (2001) 214-222.
- [21] D.B. DeWald, J. Torabinejad, C.A. Jones, J.C. Shope, A.R. Cangelosi, J.E. Thompson, G.D. Prestwich, H. Hama, Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-trisphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*, *Plant physiology* 126 (2001) 759-769.
- [22] B.K. Drobak, P.A. Watkins, Inositol(1,4,5)trisphosphate production in plant cells: an early response to salinity and hyperosmotic stress, *FEBS Lett* 481 (2000) 240-244.
- [23] S. Konig, A. Mosblech, I. Heilmann, Stress-inducible and constitutive phosphoinositide pools have distinctive fatty acid patterns in *Arabidopsis thaliana*, *Faseb J* 21 (2007) 1958-1967.
- [24] B.O. Bargmann, T. Munnik, The role of phospholipase D in plant stress responses, *Curr Opin Plant Biol* 9 (2006) 515-522.
- [25] Y. Hong, W. Zhang, X. Wang, Phospholipase D and phosphatidic acid signalling in plant response to drought and salinity, *Plant, cell & environment* (2009).
- [26] L. Thiery, A.S. Leprince, D. Lefebvre, M.A. Ghars, E. Debarbieux, A. Savoure, Phospholipase D is a negative regulator of proline biosynthesis in *Arabidopsis thaliana*, *The Journal of biological chemistry* 279 (2004) 14812-14818.
- [27] T. Munnik, S.A. Arisz, T. De Vrije, A. Musgrave, G Protein Activation Stimulates Phospholipase D Signaling in Plants, *The Plant cell* 7 (1995) 2197-2210.
- [28] F.C. Gomez-Merino, C.A. Brearley, M. Ornatowska, M.E. Abdel-Haliem, M.I. Zanon, B. Mueller-Roeber, AtDGK2, a novel diacylglycerol kinase from *Arabidopsis thaliana*, phosphorylates 1-stearoyl-2-arachidonoyl-sn-glycerol and 1,2-dioleoyl-sn-glycerol and exhibits cold-inducible gene expression, *The Journal of biological chemistry* 279 (2004) 8230-8241.
- [29] T. Hirayama, C. Ohto, T. Mizoguchi, K. Shinozaki, A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana*, *Proceedings of the National Academy of Sciences of the United States of America* 92 (1995) 3903-3907.
- [30] K.I. Deak, J. Malamy, Osmotic regulation of root system architecture, *Plant J* 43 (2005) 17-28.
- [31] K. Miura, J.B. Jin, J. Lee, C.Y. Yoo, V. Stirn, T. Miura, E.N. Ashworth, R.A. Bressan, D.J. Yun, P.M. Hasegawa, SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in *Arabidopsis*, *The Plant cell* 19 (2007) 1403-1414.
- [32] Z. Ding, S. Li, X. An, X. Liu, H. Qin, D. Wang, Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in *Arabidopsis thaliana*, *J Genet Genomics* 36 (2009) 17-29.
- [33] S.A. Arisz, F. Valianpour, A.H. van Gennip, T. Munnik, Substrate preference of stress-activated phospholipase D in *Chlamydomonas* and its contribution to PA formation, *Plant J* 34 (2003) 595-604.
- [34] L. Hunt, J.E. Gray, ABA signalling: a messenger's FIERY fate, *Curr Biol* 11 (2001) R968-970.
- [35] I.M. Tasma, V. Brendel, S.A. Whitham, M.K. Bhattacharyya, Expression and evolution of the phosphoinositide-specific phospholipase C gene family in *Arabidopsis thaliana*, *Plant Physiol Biochem* 46 (2008) 627-637.
- [36] T. Munnik, Phosphatidic acid: an emerging plant lipid second messenger, *Trends in plant science* 6 (2001) 227-233.
- [37] S.P. Mane, C. Vasquez-Robinet, A.A. Sioson, L.S. Heath, R. Grene, Early PLD α -mediated events in response to progressive drought stress in *Arabidopsis*: a transcriptome analysis, *Journal of experimental botany* 58 (2007) 241-252.

- [38] Y. Hong, S.P. Devaiah, S.C. Bahn, B.N. Thamasandra, M. Li, R. Welti, X. Wang, Phospholipase D epsilon and phosphatidic acid enhance Arabidopsis nitrogen signaling and growth, *Plant J* 58 (2009) 376-387.
- [39] S.J. Wu, L. Ding, J.K. Zhu, SOS1, a Genetic Locus Essential for Salt Tolerance and Potassium Acquisition, *The Plant cell* 8 (1996) 617-627.
- [40] J. Liu, M. Ishitani, U. Halfter, C.S. Kim, J.K. Zhu, The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance, *Proceedings of the National Academy of Sciences of the United States of America* 97 (2000) 3730-3734.
- [41] M. Ishitani, J. Liu, U. Halfter, C.S. Kim, W. Shi, J.K. Zhu, SOS3 function in plant salt tolerance requires N-myristoylation and calcium binding, *The Plant cell* 12 (2000) 1667-1678.
- [42] L. Xiong, B. Lee, M. Ishitani, H. Lee, C. Zhang, J.K. Zhu, FIERY1 encoding an inositol polyphosphate 1-phosphatase is a negative regulator of abscisic acid and stress signaling in Arabidopsis, *Genes Dev* 15 (2001) 1971-1984.
- [43] M. Ishitani, L. Xiong, H. Lee, B. Stevenson, J.K. Zhu, HOS1, a genetic locus involved in cold-responsive gene expression in arabidopsis, *The Plant cell* 10 (1998) 1151-1161.
- [44] Y. Guo, L. Xiong, M. Ishitani, J.K. Zhu, An Arabidopsis mutation in translation elongation factor 2 causes superinduction of CBF/DREB1 transcription factor genes but blocks the induction of their downstream targets under low temperatures, *Proceedings of the National Academy of Sciences of the United States of America* 99 (2002) 7786-7791.
- [45] M. Agarwal, Y. Hao, A. Kapoor, C.H. Dong, H. Fujii, X. Zheng, J.K. Zhu, A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance, *The Journal of biological chemistry* 281 (2006) 37636-37645.
- [46] T. Munnik, J.A.J. Van Himbergen, B. Ter Riet, F.-J. Braun, R.F. Irvine, H. Van den Ende, A. Musgrave, Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipases C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan, *Planta* 207 (1998) 133-145.

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