The Plant Journal (2000) **22**(2), 147–154

Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate

Teun Munnik[1,*], Harold J.G. Meijer[1], Bas ter Riet[1], Heribert Hirt[2], Wolfgang Frank[3], Dorothea Bartels[4] and Alan Musgrave[1]

[1]*Institute for Molecular Cell Biology, BioCentrum Amsterdam, University of Amsterdam, Kruislaan 318, NL-1098 SM Amsterdam, The Netherlands,*

[2]*Vienna Biocenter, Department of Microbiology and Genetics, Vienna, Austria,*

[3]*Fraunhofer Institut für Umweltchemie und Ökotoxikologie, Schmallenberg, Germany, and*

[4]*Max Planck-Institute für Züchtungsforchung, Cologne, Germany*

Received 15 November 1999; revised 10 February 2000; accepted 21 February 2000.
*For correspondence (fax +31 20 5257934; e-mail munnik@bio.uva.nl).

Summary

In mammalian cells, phospholipase D (PLD) and its product phosphatidic acid (PA) are involved in a number of signalling cascades, including cell proliferation, membrane trafficking and defence responses. In plant cells a signalling role for PLD and PA is also emerging. Plants have the extra ability to phosphorylate PA to produce diacylglycerol pyrophosphate (DGPP), a newly discovered phospholipid whose formation attenuates PA levels, but which could itself be a second messenger. Here we report that increases in PA and its conversion to DGPP are common stress responses to water deficit. Increases occur within minutes of treatment and are dependent on the level of stress. Part of the PA produced is due to PLD activity as measured by the *in vivo* transphosphatidylation of 1-butanol, and part is due to diacylglycerol kinase activity as monitored via ^{32}P-PA formation in a differential labelling protocol. Increases in PA and DGPP are found not only in the green alga *Chlamydomonas moewusii* and cell-suspension cultures of tomato and alfalfa when subjected to hyperosmotic stress, but also in dehydrated leaves of the resurrection plant *Craterostigma plantagineum*. These results provide further evidence that PLD and PA play a role in plant signalling, and provide the first demonstration that DGPP is formed during physiological conditions that evoke PA synthesis.

Introduction

Plant cells experience osmotic stress when the solute concentration in their apoplasts changes, and quickly respond with compensatory adaptations to re-establish the osmotic equilibrium. In order to understand osmotic adaptation, components of the stress-induced signal transduction pathways must be identified.

Osmotic stress varies from hypo- to hyperosmotic, and probably activates different receptors depending on the nature and level of stress. This is the case in yeast where the stress receptor Sho1p responds to NaCl concentrations between 200 and 300 mM, while a second receptor, Sln1p, responds to concentrations between 100 and 600 mM (Maeda *et al.*, 1995). Another, as yet unidentified, yeast receptor responds only to NaCl concentrations above 900 mM (Dove *et al.*, 1997). As a consequence of activating different receptors, different signalling pathways will be activated. The osmo-stress pathways identified so far in plants include abscisic acid (ABA, Bonetta and McCourt, 1998), MAP kinases (Hirt, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Munnik *et al.*, 1999), Ca^{2+} (Bressan *et al.*, 1998), phospholipases (Chapman, 1998; Munnik *et al.*, 1998a), and lipid kinases (Dove *et al.*, 1997; Meijer *et al.*, 1999). The first plant osmosensor has just been identified in *Arabidopsis* and resembles the Sln1 sensor in yeast (Urao *et al.*, 1999).

Early research on osmo-stress-induced lipid signalling implicated phospholipase C (PLC) activation. The potential signals generated – inositol 1,4,5-trisphosphate ($InsP_3$), diacyl glycerol (DAG) and Ca^{2+} – are thought to cause cell shrinkage (reviewed by Coté, 1995; Munnik *et al.*, 1998a).

© 2000 Blackwell Science Ltd

Whether DAG is directly involved in signalling is dubious, because a protein kinase C (PKC) has not been isolated from plants (Munnik *et al.*, 1998a). However, a metabolic product of DAG could be an active signal.

In mammalian cells, DAG is rapidly phosphorylated to phosphatidic acid (PA) by DAG kinase. Originally this was thought to down-regulate DAG levels and to attenuate PKC activation, but currently PA is seen as a novel lipid second messenger (reviewed by English, 1996; McPhail *et al.*, 1999; Munnik *et al.*, 1998a; Topham and Prescott, 1999). Phosphatidic acid targets identified so far include NADPH oxidase (Erickson *et al.*, 1999; Waite *et al.*, 1997), PtdInsP 5-OH-kinase (type I enzymes, Jenkins *et al.*, 1994; Moritz *et al.*, 1992), and a variety of protein kinases such as protein kinase C ζ (Limatola *et al.*, 1994), Raf-1 kinase (Ghosh and Bell, 1997; Ghosh *et al.*, 1996; Rizzo *et al.*, 1999) and novel kinases (reviewed in McPhail *et al.*, 1999). In addition, PA action has been tightly coupled to membrane trafficking, regulation of the cytoskeleton and the oxidative defense reactions in neutrophils (English, 1996; McPhail *et al.*, 1999; Topham and Prescott, 1999).

PA is not only produced indirectly via PLC and DAG kinase, but can be directly generated by phospholipase D (PLD). PLD hydrolyzes structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine and, like PLC, it is activated in response to a variety of signals and is implicated in multiple signalling pathways (Munnik *et al.*, 1998a).

A picture of PLD and PA signalling in plant cells is slowly emerging (reviewed by Munnik *et al.*, 1998a; Wang, 1999). In general, PLD activity is associated with plant stress responses. More specifically, it is thought to play a role in the response to pathogens (Chapman, 1998; Young *et al.*, 1996), wounding (Lee *et al.*, 1997; Ryu and Wang, 1998), water stress (Frank *et al.* 2000; Munnik *et al.*, unpublished data), and the plant stress hormones ethylene (Fan *et al.*, 1997; Lee *et al.*, 1998) and abscisic acid (Fan *et al.*, 1997; Jacob *et al.*, 1999; Ritchie and Gilroy, 1998). Some of these effects may be mediated by G-protein-coupled stress receptors, because G-protein activators such as mastoparans, alcohols and cholera toxin activate PLD in the absence of stress (De Vrije and Munnik, 1997; Munnik *et al.*, 1995; Munnik *et al.*, 1998b; Van Himbergen *et al.*, 1999). As yet few PA-induced responses have been reported, but treatment of the green alga *Chlamydomonas moewusii* immediately resulted in deflagellation (Munnik *et al.*, 1995), while similar treatment of barley aleurone cells inhibited the secretion of α-amylase (Ritchie and Gilroy, 1998), and recently PA has been shown to induce stomatal closure (Jacob *et al.*, 1999). In both the latter responses PA mimicked the effects of abscisic acid.

Another potential lipid signal is produced when PA is phosphorylated further to the novel phospholipid diacylglycerol pyrophosphate (DGPP). While this lipid was originally identified as an *in vitro* product of PA kinase activity (Wissing and Behrbohm, 1993a), we discovered that it was rapidly produced *in vivo* during G-protein activated signalling (Munnik *et al.*, 1996; Munnik *et al.*, 1998b). As high DGPP levels are rapidly attenuated (Munnik *et al.*, 1998b), it is attractive to think of this lipid as another intracellular signal (Munnik *et al.*, 1996; Munnik *et al.*, 1998a; Wu *et al.*, 1996). This idea was recently supported by demonstrating that DGPP activates a signalling cascade in human macrophages that involves PLA_2, MAP kinases and subclasses of protein kinase C (Balboa *et al.*, 1999). Despite DGPP's general presence in plants (Munnik *et al.*, 1996; Munnik *et al.*, 1998a; Wissing and Behrbohm, 1993b), yeast (Toke *et al.*, 1998a; Toke *et al.*, 1998b; Wu *et al.*, 1996) and trypanosomes (Marchesini *et al.*, 1998), changes in the level of DGPP under physiological conditions have yet to be established.

In this study we investigated the phospholipid signalling events induced by hyperosmotic stress. We report here that salts, sugars and drought induce immediate activation of PLD and the accumulation of PA and DGPP, suggesting that these phospholipids are involved in signalling water stress in plant cells.

Results and Discussion

NaCl stimulates the formation of PA and DGPP in a time- and dose-dependent manner

In order to test whether lipid metabolism was affected by hyperosmotic stress, we first prelabelled *Chlamydomonas moewusii* cells in HMCK medium with ^{32}Pi and then added NaCl to produce final concentrations ranging from 50–500 mM. After 5 min lipids were extracted, separated by TLC and the radioactivity in each spot quantified by phosphoimaging. Salt concentrations above 150 mM stimulated the accumulation of PA and DGPP, whereby the amounts of these two phospholipids were proportional to the concentrations of NaCl added, as shown in Figure 1(a,b). The effect of severe treatment was dramatic: within 5 min cells in 500 mM NaCl had increased their PA level sevenfold and their DGPP level 14-fold. The increase in PA appeared to be less because of the higher background levels in non-stimulated cells, as PA is an important intermediate in the biosynthesis of phospholipids, and because DGPP contains two phosphates whereas PA contains only one. However, under the conditions applied the radioactivity in the structural phospholipids did not increase (data not shown). Therefore these results suggest that the accumulation of PA and DGPP is not a general metabolic reaction to salt stress, but may primarily function in signal transduction.

A general property of intracellular signals is that any increase in concentration is transient so that the signal

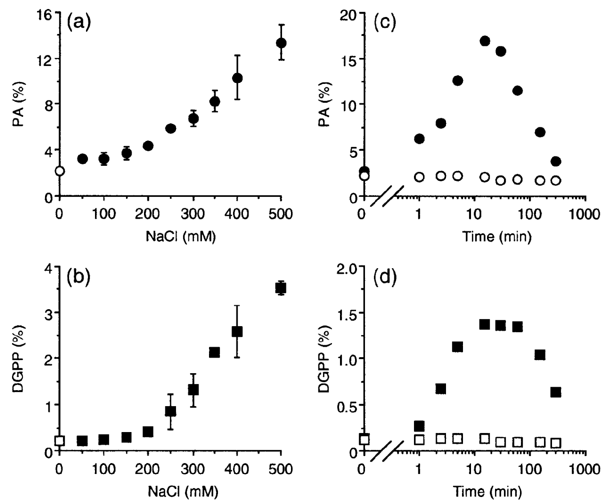

Figure 1. NaCl stimulates the formation of PA and DGPP in the green alga *Chlamydomonas moewusii* in a time- and dose-dependent manner.
$^{32}P_i$-prelabelled cells were treated with different concentrations of NaCl for 5 min (a,b) or with 300 mM for the times indicated (c,d). Phospholipids were extracted, separated by TLC, and the radioactivity in PA (circles; a,c) and DGPP (squares; b,d) quantified by phosphoimaging. Data are presented as percentages of the total radioactivity in phospholipids. The dose–response curve (a,b) is represented by the means (±SE) of three independent experiments, while for the time-course (c,d) a typical experiment is shown (n=3). Closed symbols, NaCl; open symbols, control.

level can increase again in response to another stimulus. To study the nature of the increase in PA and DGPP, radiolabelled cells were treated with 300 mM NaCl and samples were taken at subsequent intervals to follow changes in phospholipid levels. A typical result is shown in Figure 1(c,d), illustrating the temporary nature of the increase in both lipids, with maxima at about 15 min. As DGPP is synthesized from PA during signalling by the activity of PA-kinase (Munnik *et al.*, 1996), changes in PA levels slightly preceded those in DGPP, while the latter response lasted longer (note log scale).

PA and DGPP elevations are general responses to hyperosmotic stress

Although increasing the external NaCl concentration can represent an osmotic stress, it could simply represent ion toxicity, especially as this alga is cultivated in the absence of NaCl. Therefore other osmotica were applied for 5 min in concentrations equivalent to 300 mM NaCl. As shown in Table 1, they all activated PA and DGPP synthesis. In separate time-course experiments the kinetics of synthesis were found to be very similar to those observed for 300 mM NaCl (results not shown; Figure 1). Together these data illustrate that accumulation of PA and DGPP is rapidly induced by all osmotica tested, and is therefore a general response to hyperosmotic stress in *C. moewusii*.

Table 1. Hyperosmotic stress triggers PA and DGPP formation

	Fold increase	
Osmolyte	PA	DGPP
Control	1	1
NaCl	2.7 ± 0.2	6.8 ± 4.2
KCl	3.1 ± 0.2	7.4 ± 4.7
Glycerol	2.6 ± 0.4	8.6 ± 3.0
Sucrose	1.9 ± 0.1	6.5 ± 2.9
Mannitol	2.8 ± 1.0	9.2 ± 1.7

^{32}P-prelabelled *Chlamydomonas* cells were stimulated for 5 min with buffer alone or with 0.55 os kg^{-1} (equivalent to 300 mM NaCl) of one of the compounds described above. Results are presented as the means of three independent experiments (± SD).

Hyperosmotic stress activates PLD

The synthesis of PA can result from different lipid signalling pathways. PA can be produced either through an increase in DAG kinase activity, for example due to high levels of DAG being formed via the PLC pathway, or through increased PLD activity. As PLD has not yet been invoked in signalling hyperosmotic stress, we measured the effect of 300 mM NaCl on *in vivo* PLD activity by repeating the time-course experiments in the presence of 0.25% 1-butanol. This was done because PLD can transfer the phosphatidyl group of its substrate not only to its natural substrate, water, but also to butanol, producing phosphatidylbutanol (PBut) which can be easily determined as a measure of the *in vivo* PLD activity (De Vrije and Munnik, 1997; Munnik *et al.*, 1995; Munnik *et al.*, 1998b). It should be noted that this assay cannot determine absolute PLD activities, because most phosphatidyl groups are still transferred to water, forming PA. Figure 2(b) illustrates a typical time-course experiment, with the synthesis of PBut increasing within the first minute of treatment and reaching a maximum at 10 min. The increase in the synthesis of PBut was always coupled to a larger increase in PA (Figure 2a), establishing that PLD is rapidly activated by high concentrations of NaCl as well as by other osmotica (data not shown).

Part of the PA response is due to phosphorylation of DAG

The formation of PBut provides a relative measure of PLD activity, yet the increase after a 10 min treatment with 300 mM NaCl was about 2.5-fold (Figure 2b), whereas the increase in PA was about eightfold (Figure 2a), suggesting that PLD activity was not the only contributor to PA formation. To investigate the possible contribution of DAG

kinase-generated PA, a differential labelling technique (Munnik *et al.*, 1998b) was used. In short, *Chlamydomonas* cells were prelabelled with $^{32}P_i$ for 5 min before treating them with a range of NaCl concentrations. Under such short labelling conditions, the structural lipids that are hydrolysed by PLD are hardly labelled, and consequently the PA generated by PLD is hardly labelled. In contrast, lipids synthesized by the incorporation of ^{32}P from ATP, for example PA synthesized by DAG kinase, are strongly labelled. Therefore any increase in [^{32}P]PA synthesis under these conditions cannot be due to PLD but is due to DAG kinase (for a detailed validation of this technique see Munnik *et al.*, 1998b).

To test whether [^{32}P]PA was formed under these circumstances, cells were treated for 5 min with NaCl concentrations ranging from 100 to 400 mM. As shown in Figure 3, treatment again stimulated [^{32}P]PA synthesis in a dose-dependent manner, indicating that DAG kinase also makes a strong contribution to PA synthesis during osmotic stress. The simplest explanation for this increase is that DAG production was increased by PLC activity; we have previously shown that when PLC hydrolyses phosphatidylinositol bisphosphate the DAG generated is rapidly phosphorylated to PA (Munnik *et al.*, 1998b). This is the most likely explanation: a number of reports have shown that hyperosmotic stress activates polyphosphoinositide metabolism (Cho *et al.*, 1993; Heilmann *et al.*, 1999), an increase in $InsP_3$ (Coté, 1995; Heilmann *et al.*, 1999; Smolenska-Sym and Kacperska, 1996), and an increase in intracellular Ca^{2+} (Bressan *et al.*, 1998; Knight *et al.*, 1997). Because vacuolar Ca^{2+} stores become more sensitive to $InsP_3$ after osmo-stress (Allen and Sanders, 1994), and the expression of genes involved in PLC signalling is also increased after water stress (Hirayama *et al.*, 1995; Katagiri *et al.*, 1996; Mikami *et al.*, 1998), it is likely that a PLC pathway is involved in osmo-signalling in plants and *Chlamydomonas*. Nonetheless, we cannot exclude the possibility that part of the response is due to direct activation of DAG kinase.

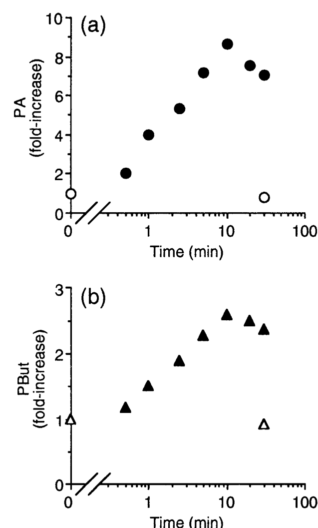

Figure 2. NaCl rapidly stimulates PLD activity.
^{32}P-prelabelled *Chlamydomonas moewusii* cells in HMCK buffer containing 0.2% 1-butanol were treated with (closed symbols) or without (control; open symbols) 300 mM NaCl for the times indicated. Phospholipids were subsequently extracted, separated by TLC and quantified by phosphoimaging. Radioactivity is expressed as the *x*-fold increase over control samples.
(a) PA formation; (b) PBut formation. Closed symbols, 300 mM NaCl; open symbols, control. A typical result is shown from four independent experiments.

Activation of PLD and elevation of PA-and DGPP levels are common plant responses to water stress

As osmotic stress consistently stimulated PLD activity and the formation of PA and DGPP in *Chlamydomonas*, the question remained as to whether this was a common plant response to hyperosmotic conditions. Therefore tomato and alfalfa cell-suspension cultures were tested. Cell suspensions were chosen rather than intact tissues because they take up $^{32}P_i$ and react to treatment more synchronously. Accordingly, cells were prelabelled for 3 h with $^{32}P_i$ before treating them for 5 min with different NaCl concentrations in the presence of 0.25% 1-butanol. After extracting and separating the lipids, the radioactivity in the appropriate spots was quantified. As shown in Figure 4, alfalfa and tomato cells also respond to NaCl treatment by increasing the synthesis of PA and DGPP in a dose-dependent manner, and part of the response is again due to PLD activity, as witnessed by the formation of PBut. We therefore conclude that the response is typical of many plant cells.

Although it is convenient to add solutions of osmotica to cell cultures, in nature plants frequently experience osmotic stress under drought conditions. To test whether dehydration activates similar changes in lipid metabolism, we used leaf discs of the dessication-tolerant resurrection plant *Craterostigma plantagineum*. They were prelabelled by floating them for 16 h on a $^{32}P_i$-containing solution and exposed to dehydration by transferring to dry filter paper in a fume cupboard for 30 min. As shown in Figure 5, drought stress dramatically increased the levels of PA and DGPP. Part of the PA increase was due to the activation of PLD, because a similar increase in PBut formation was found. A more detailed analysis of PLD activation during drought in *C. plantagineum* is presented by Frank *et al.* (2000), but the data shown here illustrate that the activation of PLD and the production of PA and DGPP are typical not only for cell cultures under artificial conditions, but also for plant tissues under more natural forms of osmotic stress.

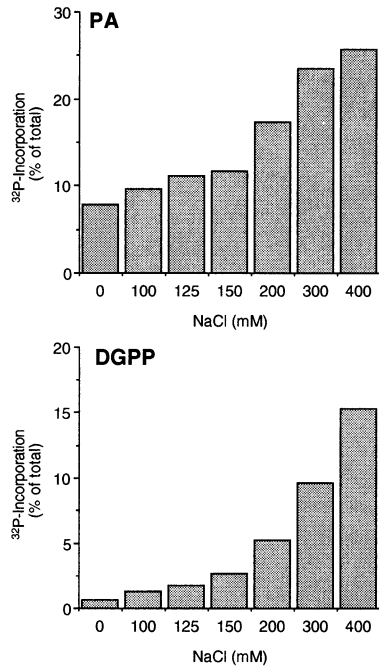

Figure 3. Part of the NaCl-induced PA formation is generated through phosphorylation of DAG.
Chlamydomonas moewusii cells were prelabelled with $^{32}P_i$ for just 5 min. Nearly all the radioactivity incorporated into the phospholipids was then in PA and the polyphosphoinositides. The radioactivity in the structural phospholipids hydrolysed by PLD was <10% of the total and therefore PLD could hardly contribute to the production of [^{32}P]PA. Under these conditions, cells were treated for 5 min with the NaCl concentrations indicated. The lipids were then extracted and separated. The radioactivity in PA (top) and DGPP (bottom) was quantified by phosphoimaging. Data are presented as percentages of the total radioactivity in the phospholipids. The fact that NaCl treatment stimulated [^{32}P]PA formation in short-labelled cells provides evidence that it was generated by DAG kinase. Three independent experiments produced similar results, one of which is shown here.

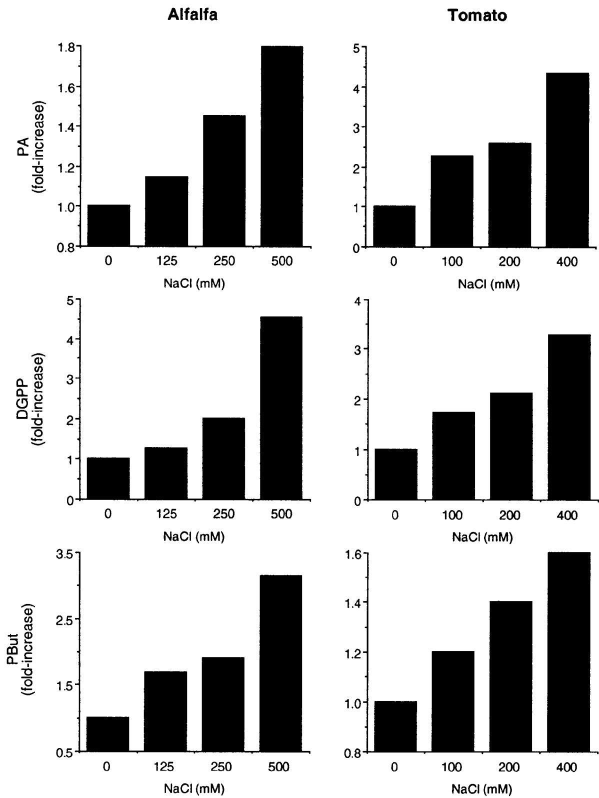

Figure 4. Osmotic stress stimulates PLD activity and PA and DGPP formation in higher plant cells.
Suspension-cultured alfalfa (left) and tomato cells (right) were prelabelled for 3 h and stimulated for 5 min with the concentrations of NaCl indicated in the presence of 0.25% 1-butanol, to monitor the activation of PLD. Lipids were extracted, separated by TLC and quantified by phosphoimaging. The data for PA (top), DGPP (middle) and PBut (below, as a measure of PLD activity) are represented as *x*-fold increases compared with the non-stressed control cells. Results of a representative experiment (alfalfa, $n=4$; tomato, $n=2$) are shown. ^{32}P percentages for the alfalfa and tomato controls, respectively, were 1.44 and 1.0% PA, 0.31 and 0.53% DGPP, 0.042 and 0.11% PtdBut.

In conclusion, PLD is one of the signalling enzymes that is rapidly activated by hyperosmotic stress. In support of its potential importance to osmo-signalling, a *PLD* gene has recently been shown to be one of the first expressed in resurrection plants subjected to drought (Frank *et al.*, 2000). This response is similar to the osmo-stress-induced expression of genes involved in PLC and MAP kinase signalling in *Arabidopsis* and alfalfa (Hirt, 1997; Munnik *et al.*, 1999; Shinozaki and Yamaguchi-Shinozaki, 1997), two other pathways thought to be involved in osmo-signalling. Thus PLD signalling could be subject to positive feedback regulation in order to prime cells for further osmo-stress. In this way plants could adapt to stress, making them more tolerant (Knight *et al.*, 1998). Within the lipid signalling pathways activated by osmotic stress, PA and its phosphorylated derivative DGPP could be key players. Future studies will reveal whether these phospholipids regulate the expression of osmo-stress-inducible genes, or whether they serve other functions.

Experimental procedures

Materials

Silica 60 TLC plates and reagents for lipid extraction and analyses were from Merck (Darmstadt, Germany). [^{32}P]-Orthophosphate (carrier-free) was from Amersham International (Hertogenbosch, The Netherlands). Radioactive DGPP was prepared and identified as described by Munnik *et al.* (1996).

Plant material, metabolic radiolabeling and osmo-stress

Chlamydomonas cells (*C. moeuwusii* strain UTEX 10) were cultivated as described earlier (Munnik *et al.*, 1994) and suspended in HMCK buffer (10 mM HEPES pH 7.4 [KOH], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM KCl) at a final concentration of 1–2 × 10^7 cells per ml.

Phospholipids were metabolically labelled by incubating cells (1–2 × 10^7 cells per ml) with 100 μCi carrier-free PO_4^{3-} per ml in HMCK, usually for 2–4 h but, in a short-term labelling protocol, for

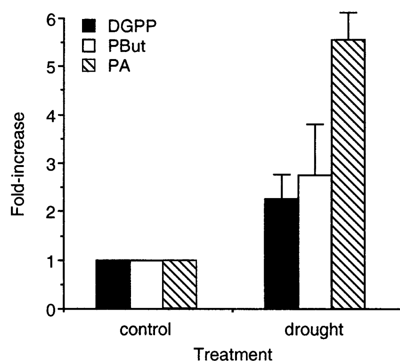

Figure 5. Drought activates PLD activity and PA and DGPP formation in the resurrection plant.
Leaf discs of *Craterostigma plantagineum* were prelabelled for 16 h by floating them on $^{32}P_i$ solution. Some were transferred to filter paper in a fume cupboard to simulate drought. After 30 min their lipids were extracted, separated and quantified. The radioactivity in PA, DGPP and PBut is represented as *x*-fold increases compared with non-stressed control cells. ^{32}P percentages for the control were 2.75% PA, 0.49% DGPP and 0.37% PtdBut.

only 5 min (Figure 3; Munnik *et al.*, 1998b). Routinely, 100 µl cell suspension was treated in a total volume of 200 µl for the time and at the stress levels indicated. Incubations were stopped by adding 3.75 vol $CHCl_3$/MeOH/HCl (50 : 100 : 1, by vol), and lipids were extracted, separated and quantified as described earlier (Munnik *et al.*, 1996).

Suspension-cultured alfalfa cells (*Medicago sativa* ssp. *varia* cv Rambler, line A2) were cultivated at 24°C in MS medium supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid and 0.1 mg l^{-1} kinetin (Munnik *et al.*, 1999). Tomato suspension cells (*Lycopersicon esculentum* cv. Moneymaker) were grown in the same medium supplemented with B_5 vitamins (Munnik *et al.*, 1996).

Higher plant cell suspensions were grown on a rotary shaker (125 r.p.m.) at 24°C in the dark. Cells were subcultured at intervals of 1 week and used 3–5 days after transfer. They were labelled at room temperature in their own medium using 100 µCi $^{32}P_i$ ml^{-1} for the times indicated. Cells were osmotically stressed by adding 90 µl of a 2× concentrated solution to an equal volume of cells. Incubations were stopped by adding 20 µl 50% (w/v) perchloric acid and freezing them into liquid nitrogen. Lipids were extracted by adding 3.75 vol $CHCl_3$/MeOH/HCl (50 : 100 : 1 v/v) and further processed as described (Munnik *et al.*, 1996).

Leaf discs of the resurrection plant *Craterostigma plantagineum* were $^{32}P_i$-prelabelled and exposed to drought stress as described in detail by Frank *et al.* (2000).

PA and DGPP analysis

Lipids were separated by TLC using an alkaline solvent system ($CHCl_3$/MeOH/25% NH_4OH/H_2O; 90 : 70 : 4 : 16 by vol; Munnik *et al.*, 1994). Radiolabelled phospholipids were detected by autoradiography and quantified by phosphoimaging (Storm, Molecular Dynamics, Sunnyvale, CA). Identities of DGPP and PA were confirmed by de-acylation and head-group analysis as described by Munnik *et al.* (1996). Non-radioactive phospholipid standards (approximately 10 µg) were visualized by exposure to iodine vapour.

PLD measurements

PLD activity was measured as the production of PBut in living cells essentially as described by Munnik *et al.* (1995). Briefly, ^{32}P-prelabelled cells were treated in the presence of 0.25% *n*-butanol. At subsequent times incubations were stopped and the lipids extracted as described above. The ^{32}P-labelled PBut was separated from the rest of the phospholipids by a modified ethyl acetate TLC system (organic upper phase of a mixture of ethyl acetate/*iso*-octane/HCOOH/water 12 : 2 : 3 : 10 by vol; Munnik *et al.*, 1998b). Radioactivity was visualized by autoradiography and quantified by phosphoimaging (Molecular Dynamics, Sunnyvale, CA, USA).

Acknowledgements

TM is funded by The Netherlands Organisation for Scientific Research (NWO-PULS; 805-48-005). This work was co-supported by an EMBO fellowship awarded to T.M. We are grateful to our colleagues in the laboratory for helpful discussions, and to Arnold van der Luit, Aveline van Doorn and Claudia Jonak in particular for the tomato and alfalfa suspension cultures.

References

Allen, G.J. and Sanders, D. (1994) Osmotic stress enhances the competence of *Beta vulgaris* vacuoles to respond to inositol 1,4,5-trisphosphate. *Plant J.* **6**, 687–695.

Balboa, M.A., Balsinde, J., Dillon, D.A., Carman, G.M. and Dennis, E.A. (1999) Proinflammatory macrophage-activating properties of the novel phospholipid diacylglycerol pyrophosphate. *J. Biol. Chem.* **274**, 522–526.

Bonetta, D. and McCourt, P. (1998) Genetic analysis of ABA signal transduction pathways. *Trends Plant Sci.* **3**, 231–235.

Bressan, R.A., Hasagawa, P.M. and Pardo, J.M. (1998) Plants use calcium to resolve salt stress. *Trends Plant Sci.* **3**, 411–412.

Chapman, K.D. (1998) Phospholipase activity during plant growth and development and in response to environmental stress. *Trends Plant Sci.* **3**, 419–426.

Cho, M.H., Shears, S.B. and Boss, W.F. (1993) Changes in phosphatidylinositol metabolism in response to hyperosmotic stress in *Daucus carota* L. cells grown in suspension culture. *Plant Physiol.* **103**, 637–647.

Coté, G.G. (1995) Signal transduction in leaf movement. *Plant Physiol.* **109**, 729–734.

De Vrije, T. and Munnik, T. (1997) Activation of phospholipase D by calmodulin antagonists and mastoparan in carnation petal tissue. *J. Exp. Bot.* **48**, 1631–1637.

Dove, S.K., Cooke, F.T., Douglas, M.R., Sayers, L.G., Parker, P.J. and Michell, R.H. (1997) Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. *Nature*, **390**, 187–192.

English, D. (1996) Phosphatidic acid: a lipid messenger involved in intracellular and extracellular signalling. *Cell. Signal.* **8**, 341–347.

Erickson, R.W., Langel-Peveri, P., Traynor-Kaplan, A.E., Heyworth, P.G. and Curnutte, J.T. (1999) Activation of human neutrophil NADPH oxidase by phosphatidic acid or diacylglycerol in a cell-free system. Activity of diacylglycerol is dependent on its conversion to phosphatidic acid. *J. Biol. Chem.* **274**, 22243–22250.

Fan, L., Zheng, S. and Wang, X. (1997) Antisense suppression of phospholipase D alpha retards abscisic acid- and ethylene-

promoted senescence of postharvest *Arabidopsis* leaves. *Plant Cell*, **9**, 2183–2196.

Frank, W., Munnik, T., Kerkmann, K., Salamini, S. and Bartels, D. (2000) Water-deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum. Plant Cell*, **12**, 111–123.

Ghosh, S. and Bell, R.M. (1997) Regulation of Raf-1 kinase by interaction with the lipid second messenger, phosphatidic acid. *Biochem. Soc. Trans*, **25**, 561–565.

Ghosh, S., Strum, J.C., Sciorra, V.A., Daniel, L. and Bell, R.M. (1996) Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. *J. Biol. Chem*, **271**, 8472–8480.

Heilmann, I., Perera, I.Y., Gross, W. and Bos, W.F. (1999) Changes in phosphoinositide metabolism with days in culture affect signal transduction pathways in *Galdieria sulphuraria. Plant Physiol.* **119**, 1331–1340.

Hirayama, T., Ohto, C., Mizoguchi, T. and Shinozaki, K. (1995) A gene encoding a phosphatidylinositol-specific phospholipase C is induced by dehydration and salt stress in *Arabidopsis thaliana. Proc. Natl Acad. Sci. USA*, **92**, 3903–3907.

Hirt, H. (1997) Multiple roles of MAP kinases in plant signal transduction. *Trends Plant Sci.* **2**, 11–15.

Jacob, T., Ritchie, S., Assmann, S.M. and Gilroy, S. (1999) Abscisic acid signal transduction in guard cells is mediated by phospholipase D activity. *Proc. Natl Acad. Sci. USA*, **96**, 12192–12197.

Jenkins, G.H., Fisette, P.L. and Anderson, R.A. (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J. Biol. Chem.* **269**, 11547–11554.

Katagiri, T., Mizoguchi, T. and Shinozaki, K. (1996) Molecular cloning of a cDNA encoding diacylglycerol kinase (DGK) in *Arabidopsis thaliana. Plant Mol. Biol.* **30**, 647–653.

Knight, H., Trewavas, A.J. and Knight, M.R. (1997) Calcium signalling in *Arabidopsis thaliana* responding to drought and salinity. *Plant J.* **12**, 1067–1078.

Knight, H., Brandt, S. and Knight, M.R. (1998) A history of stress alters drought calcium signalling pathways in *Arabidopsis*. *Plant J.* **16**, 681–687.

Lee, S., Suh, S., Kim, S., Crain, R.C., Kwak, J.M., Nam, H.-G. and Lee, Y. (1997) Systemic elevation of phosphatidic acid and lysophospholipid levels in wounded plants. *Plant J.* **12**, 547–556.

Lee, S.H., Chae, H.S., Lee, T.K., Kim, S.H., Shin, S.H., Cho, B.H., Cho, S.H., Kang, B.G. and Lee, W.S. (1998) Ethylene-mediated phospholipid catabolic pathway in glucose-starved carrot suspension cells. *Plant Physiol.* **116**, 223–229.

Limatola, C., Schaap, D., Moolenaar, W.H. and Blitterswijk, W.J. (1994) Phosphatidic acid activation of protein kinase C-overexpressed in COS cells: comparison with other protein kinase C isotypes and other acidic lipids. *Biochem. J.* **304**, 1001–1008.

Maeda, T., Takehara, M. and Saito, H. (1995) Activation of yeast PBS2 MAPKK by MAPKKKs or binding of an SH3-containing osmosensor. *Science*, **269**, 554–558.

Marchesini, N., Santander, V. and Machado-Domenech, E. (1998) Diacylglycerol pyrophosphate: a novel metabolite in the *Trypanosoma cruzi* phosphatidic acid metabolism. *FEBS Lett.* **436**, 377–381.

McPhail, L.C., Waite, K.A., Regier, D.S., Nixon, J.B., Qualliotine-Mann, D., Zhang, W.X., Wallin, R. and Sergeant, S. (1999) A novel protein kinase target for the lipid second messenger phosphatidic acid. *Biochim. Biophys. Acta*, **1439**, 277–290.

Meijer, H.J.G., Divecha, N., van den Ende, H., Musgrave, A. and Munnik, T. (1999) Hyperosmotic stress induces rapid synthesis of phosphatidylinositol 3,5-bisphosphate (PtdIns (3,5) P_2) in plant cells. *Planta*, **208**, 294–298.

Mikami, K., Katagiri, T., Iuchi, S., Yamaguchi-Shinozaki, K. and Shinozaki, K. (1998) A gene encoding phosphatidylinositol-4-phosphate 5-kinase is induced by water stress and abscisic acid in *Arabidopsis thaliana*. *Plant J.* **15**, 563–568.

Moritz, A., de Graan, P.N.E., Gispen, W.H. and Wirtz, K.W.A. (1992) Phosphatidic acid is a specific activator of phosphatidylinositol-4-phosphate kinase. *J. Biol. Chem.* **267**, 7207–7210.

Munnik, T., Irvine, R.F. and Musgrave, A. (1994) Rapid turnover of phosphatidylinositol 3-phosphate in the green alga *Chlamydomonas eugametos*: signs of a PI 3-kinase signalling pathway in lower plants? *Biochem. J.* **298**, 269–273.

Munnik, T., Arisz, S.A., de Vrije, T. and Musgrave, A. (1995) G-protein activation stimulates phospholipase D signalling in plants. *Plant Cell*, **7**, 2197–2210.

Munnik, T., de Vrije, T., Irvine, R.F. and Musgrave, A. (1996) Identification of diacylglycerol pyrophosphate as a novel metabolic product of phosphatidic acid during G-protein activation in plants. *J. Biol. Chem.* **271**, 15708–15715.

Munnik, T., Irvine, R.F. and Musgrave, A. (1998a) Phospholipid signalling in plants. *Biochem. Biophys. Acta*, **1389**, 222–272.

Munnik, T., van Himbergen, J.A.J., ter Riet, B., Braun, F.-J., Irvine, R.F., van den Ende, H. and Musgrave, A. (1998b) Detailed analysis of the turnover of polyphosphoinositides and phosphatidic acid upon activation of phospholipase C and D in *Chlamydomonas* cells treated with non-permeabilizing concentrations of mastoparan. *Planta*, **207**, 133–145.

Munnik, T., Ligterink, W., Meskiene, I., Calderini, O., Beyerly, J., Musgrave, A. and Hirt, H. (1999) Distinct osmo-sensing protein kinase pathways are involved in signalling moderate and severe hyper-osmotic stress. *Plant J.* **20**, 381–388.

Ritchie, S. and Gilroy, S. (1998) Abscisic acid signal transduction in the barley aleurone is mediated by phospholipase D activity. *Proc. Natl. Acad. Sci. USA*, **95**, 2697–2702.

Rizzo, M.A., Shome, K., Vasudevan, C., Stolz, D.B., Sung, T.-C., Frohman, M.A., Watkins, S.C. and Romero, G. (1999) Phospholipase D and its product, phosphatidic acid mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. *J. Biol. Chem*, **274**, 1131–1139.

Ryu, S.B. and Wang, X. (1998) Increase in free linolenic and linoleic acids associated with phospholipase D-mediated hydrolysis of phospholipids in wounded castor bean leaves. *Biochim. Biophys. Acta*, **1393**, 193–202.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997) Gene expression and signal transduction in water-stress response. *Plant Physiol.* **115**, 327–334.

Smolenska-Sym, G. and Kacperska, A. (1996) Inositol 1,4,5-trisphosphate formation in leaves of winter oilseed rape plants in response to freezing, tissue water potential and abscisic acid. *Physiol. Plant.* **96**, 692–698.

Toke, D.A., Bennett, W.L., Dillon, D.A. *et al.* (1998a) Isolation and characterization of the *Saccharomyces cerevisiae* DPP1 gene encoding diacylglycerol pyrophosphate phosphatase. *J. Biol. Chem.* **273**, 3278–3284.

Toke, D.A., Bennett, W.L., Oshiro, J., Wu, W.-I., Voelker, D.R. and Carman, G.M. (1998b) Isolation and characterization of the *Saccharomyces cerevisiae* LPP1 gene encoding a Mg^{2+}-independent phosphatidate phosphatase. *J. Biol. Chem.* **273**, 14331–14338.

Topham, M.K. and Prescott, S.M. (1999) Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. *J. Biol. Chem.* **274**, 11447–11450.

Urao, T., Bakhtiyor, B., Satoh, R., Yamaguchi-Shinozaki, K., Seki, M., Hirayama, T. and Shinozaki, K. (1999) A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. *Plant Cell*, **11**, 1743–1754.

Van Himbergen, J.A.J., ter Riet, B., Meijer, H.J.G., van den Ende, H., Musgrave, A. and Munnik, T. (1999) Mastoparan analogues activate phospholipase C and phospholipase D activity in *Chlamydomonas*: a comparative study. *J. Exp. Bot.* **50**, 1735–1742.

Waite, K.A., Wallin, R., Qualliotine-Mann, D. and McPhail, L.C. (1997) Phosphatidic acid-mediated phosphorylation of the NADPH oxidase component p47-phox. Evidence that phosphatidic acid may activate a novel protein kinase. *J. Biol. Chem.* **272**, 15569–15578.

Wang, X. (1999) The role of phospholipase D in signaling cascades. *Plant Physiol.* **120**, 645–651.

Weigert, R., Silletta, M.G., Spanò, S. *et al.* (1999) CtBP/BARS induces fission of golgi membranes by acylating lysophosphatidic acid. *Nature*, **402**, 429–433.

Wissing, J.B. and Behrbohm, H. (1993a) Diacylglycerol pyrophosphate, a novel phospholipid compound. *FEBS Lett.* **315**, 95–99.

Wissing, J.B. and Behrbohm, H. (1993b) Phosphatidate kinase, a novel enzyme in phospholipid metabolism. Purification, subcellular localization, and occurence in the plant kingdom. *Plant Physiol.* **102**, 1243–1249.

Wu, W.-I., Liu, Y., Riedel, B., Wissing, J.B., Fisch, A.S. and Carman, G.M. (1996) Purification and characterization of diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **271**, 1868–1876.

Young, S.A., Wang, X. and Leach, J.E. (1996) Changes in the plasma membrane distribution of rice phospholipase D during resistant interactions with *Xanthomonas oryzae* pv *oryzae*. *Plant Cell*, **8**, 1079–1090.

Note added in proof

While this paper was in review, a paper by Weigert *et al.* (1999) was published describing another interesting route of PA synthesis that could be important for signalling, namely by acylation of lysophosphatidic acid. However, we have found no evidence that would support such a pathway in response to osmotic stress.