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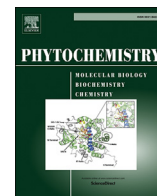
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Acclimation to salt modifies the activation of several osmotic stress-activated lipid signalling pathways in *Chlamydomonas*

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ABSTRACT

Osmotic stress rapidly activates several phospholipid signalling pathways in the unicellular alga *Chlamydomonas*. In this report, we have studied the effects of salt-acclimation on growth and phospholipid signalling. Growing cells on media containing 100 mM NaCl increased their salt-tolerance but did not affect the overall phospholipid content, except that levels of phosphatidylinositol phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] were reduced by one-third. When these NaCl-acclimated cells were treated with increasing concentrations of salt, the same lipid signalling pathways as in non-acclimated cells were activated. This was witnessed as increases in phosphatidic acid (PA), lyso-phosphatidic acid (L-PA), diacylglycerol pyrophosphate (DGPP), PI(4,5)P₂ and its isomer PI(3,5)P₂. However, all dose-dependent responses were shifted to higher osmotic-stress levels, and the responses were lower than in non-acclimated cells. When NaCl-acclimated cells were treated with other osmotica, such as KCl and sucrose, the same effects were found, illustrating that they were due to hyperosmotic rather than hyperionic acclimation. The results indicate that acclimation to moderate salt stress modifies stress perception and the activation of several downstream pathways.

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1. Introduction

Plants are often exposed to drought, salinity and freezing that result in osmotic stress. Since they cannot avoid such conditions, they must withstand them. Therefore plants perceive hyperosmotic stress and acclimate by modifying their development, structure, physiology and metabolism. Many studies have focused on identifying compounds that accumulate during osmo-stress e.g. ions, proteins, amino acids and sugars, because they play a role in osmotic adjustment and osmo-protection (Flowers et al., 2015; Gechev et al., 2012; Julkowska and Testerink, 2015; Kumari et al., 2015; Munns and Tester, 2008; Slama et al., 2015).

Stress signalling involves perception and transduction to signalling cascades that activate the appropriate responses. Mechanisms to detect osmotic stress exist in plants and putative osmosensors have been identified (Haswell and Versluys, 2015;

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Kurusu et al., 2015; Kushwaha et al., 2014; Yuan et al., 2014). Different phospholipid signalling pathways are also rapidly activated (Delage et al., 2013; Hong et al., 2010; Hou et al., 2015; Li et al., 2009; Munnik and Meijer, 2001; Munnik and Vermeer, 2010; Xue et al., 2009). One such route has been suggested to result from phospholipase C (PLC) activation, producing inositol 1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG; DeWald et al., 2001; Drøbak and Watkins, 2000; König et al., 2008; Takahashi et al., 2001; Williams et al., 2005). Increased cytosolic concentrations of Ca²⁺ have been shown to be released from internal stores during salinity and drought (Knight et al., 1997, 1998) but it is not clear whether this is generated via InsP₃ or through its conversion into InsP₆ (Lemtiri-Chlieh et al., 2003; Munnik, 2014). A gene encoding for an InsP₃ receptor has been identified for *Chlamydomonas* but not for any of the higher plant genomes sequenced so far (Kuin et al., 2000; Munnik, 2014; Wheeler and Brownlee, 2008). The main target for DAG in mammalian systems, i.e. protein kinase C (PKC), is also lacking, in both algae and higher plant cells. Instead, DAG is rapidly phosphorylated by DAG-kinase (DGK) to phosphatidic acid (PA) whose role in plant signalling has clearly emerged (Arisz et al., 2009, 2013; Li et al., 2009; Munnik, 2001, 2014; Munnik et al., 1998b, 2000; Munnik and Testerink, 2009; Testerink and Munnik, 2011). Phospholipase D (PLD) also contributes to this PA

rise through hydrolysis of structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine (Arisz et al., 2000, 2003; Beligni et al., 2015; Bargmann et al., 2009; Frank et al., 2000; Hou et al., 2015; Katagiri et al., 2001; Munnik et al., 2000; Wang et al., 2006). Thus two different routes produce PA in response to osmotic stress (reviewed in Hong et al., 2010; Hou et al., 2015; Li et al., 2009; Munnik and Vermeer, 2010; Wang et al., 2006 osmotic; Testerink and Munnik, 2011).

Hyperosmotic stress also promotes the formation of lyso-phosphatidic acid (L-PA) by activating a phospholipase A₂ (PLA₂; Einspahr et al., 1988a; Meijer et al., 2001a; Arisz et al., 2011). Apart from producing new potential signals (L-PA and a free fatty acid), it also metabolizes and therefore attenuates the PA signal. The PA signal is also attenuated by the formation of diacylglycerol pyrophosphate (DGPP) when PA-kinase is activated (Munnik et al., 1996, 2000; Pical et al., 1999; Zalejski et al., 2005, 2006). Both L-PA and DGPP have signalling properties in animal cells and their role in plant cells is emerging (Arisz et al., 2013; Hou et al., 2015; Meijer et al., 2001a; Van Schooten et al., 2006; Zalejski et al., 2005, 2006).

Osmotic stress results in the formation of the lipid phosphatidylinositol 3,5-bisphosphate [PI(3,5)P₂]; Meijer et al., 1999; Zonia and Munnik, 2004). Its formation was originally reported for yeast where it is involved in regulating the homeostasis of the vacuolar membrane (Dove et al., 1997, 2009). Other poly-phosphoinositides (PPIs) such as phosphatidylinositol phosphate (PIP) and PI(4,5)P₂ are reported to change in osmotically stressed plant cells (Cho et al., 1993; Darwish et al., 2009; DeWald et al., 2001; Einspahr et al., 1988a,b; König et al., 2008; Meijer et al., 2001b; Munnik et al., 2000; Pical et al., 1999; Takahashi et al., 2001; Zonia and Munnik, 2004).

The unicellular, biflagellated green alga *Chlamydomonas* is an excellent system for studying phospholipid metabolism because it rapidly takes up and incorporates ³²P_i into all phospholipids (Arisz et al., 2000; Munnik et al., 1998b) and, because treatment synchronously affects all cells. Metabolism can then be followed by monitoring the changes in lipid radioactivity patterns. What is more, all osmotic stress-induced signalling mechanisms described above have already been documented for this alga and, more intriguingly, each individual pathway seems to be activated in a characteristic dose-dependent manner (Arisz and Munnik, 2011; Meijer et al., 1999, 2001a,b; Munnik et al., 2000; Munnik and Meijer, 2001).

When plant cells are subjected to low stress levels they need to acclimate and modify their cellular processes to restore growth and development. Acclimation has been correlated with changes in (phospho)lipid and fatty acid compositions that are generally thought to affect the biophysical properties of the membranes rather than change their signalling properties (Aziz and Larher, 1998; Surjes and Durand, 1996; Wu et al., 1998). However, since osmotic stress is known to activate phospholipid signalling, these changes may help to regulate signalling in acclimated plants. In this study, *Chlamydomonas* cells were acclimated to 100 mM NaCl before assessing changes in osmotic stress-induced phospholipid signalling pathways in order to establish to what extent signalling was affected.

2. Results

2.1. Effect of NaCl on cell growth and phospholipid composition of *Chlamydomonas*

To determine whether salt-pretreatment results in salt tolerance, *Chlamydomonas moewusii* cells were pre-cultured in M1-medium with or without 100 NaCl. When cells were transferred to fresh liquid media containing different NaCl concentrations, the

growth of acclimated and non-acclimated cells in low concentrations (0, 100, 200 mM) of NaCl was similar (Fig. 1A; not shown). However, non-acclimated cells did not grow at 400 mM NaCl and their growth was delayed in 300 mM NaCl, whereas pre-treated cells grew at both concentrations without a lag-phase (Fig. 1A). When salt acclimated cells were grown on salt-containing agar media, acclimated cells grew better on 200 mM NaCl plates, and grew well on 300 mM NaCl whereas control cells did not grow at all (Fig. 1B). These results illustrate that salt-pretreatment leads to increased salt-tolerance.

In order to study whether acclimation affects phospholipid-based signalling, cells were directly cultured on M1-agar medium supplemented with 0, 50, 100 or 200 mM NaCl. However, when 200 mM plate cultures were flooded with buffer containing the

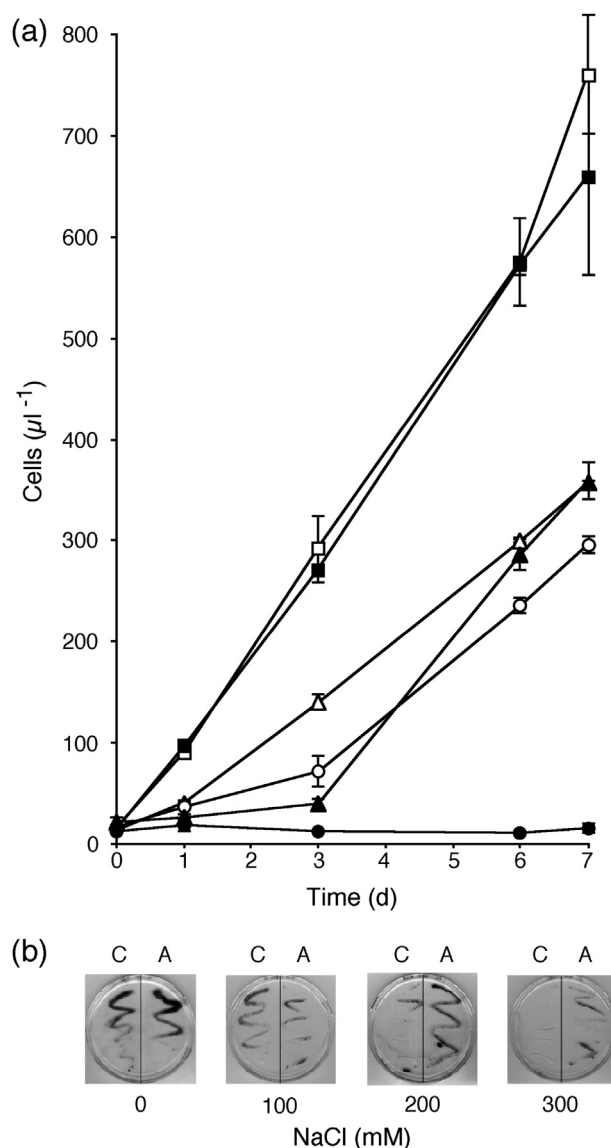


Fig. 1. Salt-acclimation affects the growth of *Chlamydomonas* cells. Cells were pre-grown for two weeks with or without 100 mM salt and then (A) transferred to fresh liquid media containing a range of NaCl concentrations. Cell numbers were registered with time. Values represent the means of two independent experiments \pm standard deviation. Data are shown for control cells (closed symbols) and acclimated cells (open symbols) growing in 0 mM NaCl (■, □), 300 mM NaCl (▲, △) or 400 mM NaCl (●, ○). (B) Other cells were plated on M1-agar plates, supplemented with different NaCl concentrations. The growth after two weeks is shown (C = control cells; A = acclimated cells).

same concentration of NaCl, no swimming cells were generated, whereas cultures grown and flooded with 50 or 100 mM NaCl did generate masses of swimming cells. Since flagella are required for P_i take-up, swimming cells grown on 0, 50 or 100 mM NaCl were incubated with $^{32}P_i$ to label the phospholipids. When lipids were extracted and separated by TLC, the phospholipid patterns seen by autoradiography were similar to those observed for control cells (see Fig. 2). We therefore focused on cells grown up to 100 mM NaCl, the highest concentration to which they could acclimate without losing vitality. The quantified results from TLC analysis are represented in Table 1. No significant changes in the structural phospholipids were found but radiolabelled PI(4,5) P_2 and PIP were reduced by about one-third in salt-acclimated cells (Table 1).

2.2. NaCl-acclimated cells are less sensitive to salt-shock

To determine how cells grown in 100 mM NaCl responded to higher NaCl concentrations, pre-labelled control and acclimated cells were treated with a range of NaCl concentrations for 5 min. Treatments consisted of mixing 50 μ l cell suspension with 50 μ l cell-free medium containing the extra salt. Cell-free medium from control cells contained less than 1 mM NaCl while that from acclimated cells contained approximately 100 mM NaCl, since this was previously added to the HMCK-buffer before flooding the cells. Lipids were extracted, separated by TLC and visualized by autoradiography of which a typical result is shown in Fig. 2. It should be noted that the concentrations listed in the figure refer to the extra NaCl salt in the cell-free medium.

Whereas the structural phospholipids (PI, PE and PG) were relatively unaffected by salt treatment of non-acclimated cells, the metabolism of other lipids was profoundly affected (Fig. 2A). For example, the synthesis of PI(3,5) P_2 was stimulated by as little as 50 mM, whereas PI(4,5) P_2 levels started to increase at slightly higher concentrations of NaCl. PA levels responded above 150 mM, while L-PA and DGPP increased at 150 mM and 250 mM, respectively. These changes reflect increases in the activation of PI- and PIP-kinases, PLD, PLC, DGK, PLA₂ and PA-kinase, confirming previous reports (Arisz and Munnik, 2011, 2013; Meijer et al., 1999, 2001a,b; Meijer et al., 2002; Munnik et al., 2000). At the two highest concentrations tested (500 and 600 mM), radioactivity in most signalling lipids decreased. Since cells were prelabelled, this

Table 1

Effect of salt acclimation on the phospholipid composition of *Chlamydomonas*. Cells were grown for 2–3 weeks on agar-containing medium with 100 mM NaCl (acclimated) or without (control). Plates were then flooded for 12–16 h with buffer and the cells labelled with $^{32}P_i$ for 2.5–3 h. Phospholipids were extracted, separated by alkaline TLC and quantified by phosphoimaging. Data represents the percentage of total ^{32}P -labelled lipids and are the means \pm SD from six independent experiments. Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositolphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

Phospholipid	Control	Salt-acclimated
PI(4,5) P_2	5.2 \pm 0.7	2.9 \pm 0.2
PIP	6.6 \pm 0.7	4.6 \pm 0.3
PA	2.4 \pm 0.3	2.5 \pm 0.4
PI	38.8 \pm 3.6	41.9 \pm 2.7
PE	8.7 \pm 1.9	10.5 \pm 1.6
PG	23.3 \pm 3.4	22.5 \pm 1.9

means that catabolism exceeded synthesis, perhaps because cell vitality was lost. This is not apparent from the structural lipids, because their turnover rate is much lower (Munnik et al., 1998a; 1998b).

In salt-acclimated cells, extra salt-stress activated the same lipid signalling pathways, but to a lesser extent (Fig. 2B). This is best illustrated in Fig. 3 (NaCl, left panel), where radioactivity levels in each signalling phospholipid are represented with those in non-acclimated cells. For all phospholipids, a clear shift can be observed in the dose-response curves towards higher NaCl concentrations and the signal levels are reduced. For example, whereas DGPP formation was clearly stimulated in non-acclimated cells by 250 mM (0.46 Os kg⁻¹), a comparable stimulation in salt-acclimated cells was only reached at 500 mM NaCl (0.93 Os kg⁻¹). Furthermore, the maximum increase in DGPP in acclimated cells was 13-fold, in contrast to a 23-fold increase in non-acclimated cells (Fig. 3). Whereas this phenomenon was typical of most lipid signalling responses, a clear exception were PIP and PI(4,5) P_2 , that exhibited increases similar to those in non-acclimated cells, although at higher osmolarities.

2.3. Acclimation does not affect phospholipid signalling kinetics

Osmotic stress results in the activation of several signalling

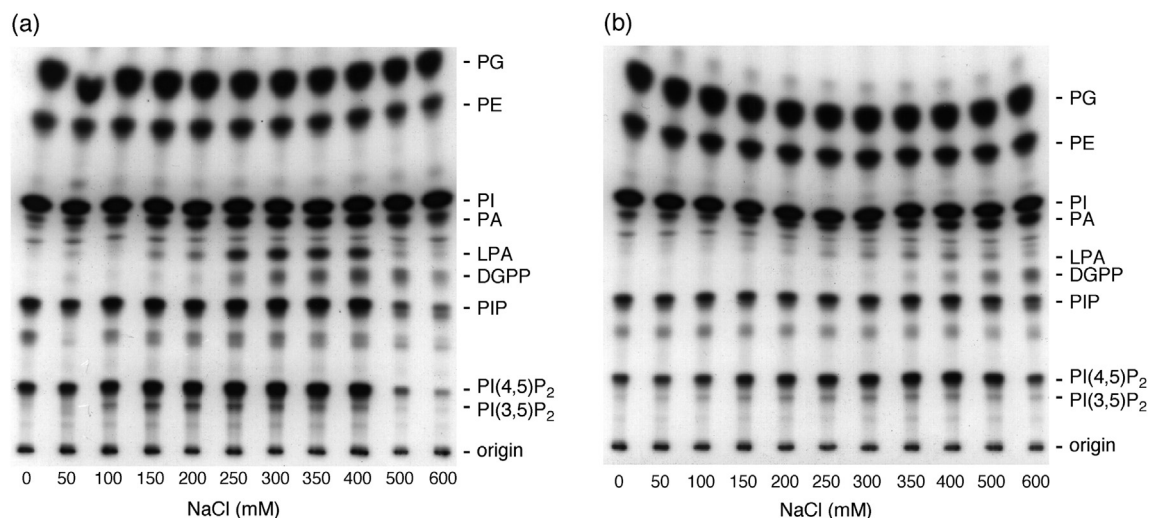


Fig. 2. Salt-induced phospholipid signalling in control and acclimated cells. Control (A) and acclimated cells (B) were prelabelled with $^{32}P_i$ for 2.5 h and then treated for 5 min with the concentrations of NaCl indicated. Note that these are the concentrations added to cell-free medium. The lipids were then extracted and separated by alkaline TLC and visualized by autoradiography. Abbreviations: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositolphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate.

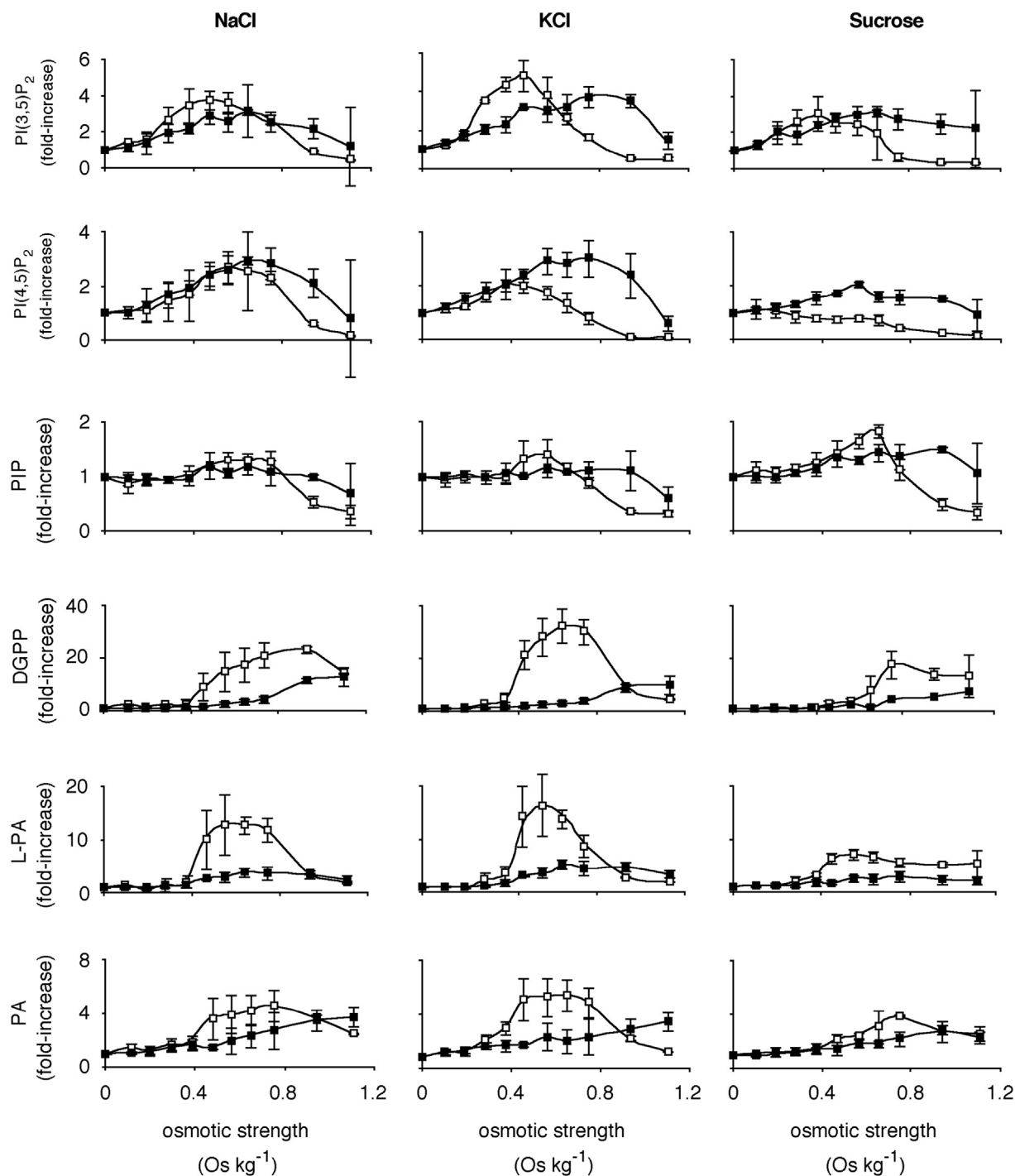


Fig. 3. Effects of NaCl, KCl and sucrose on the metabolism of signalling phospholipids in *Chlamydomonas*. Prelabelled *Chlamydomonas* cells were treated in a dose-dependent manner with NaCl, KCl or sucrose for 5 min at concentrations that produced the osmotic values indicated. They are equivalent to 0, 50, 100, 150, 200, 250, 300, 350, 400, 500 and 600 mM NaCl. Lipids were extracted, separated by TLC and quantified by phosphoimaging. The radioactivity levels in the responsive phospholipids are presented for control (open symbols) and acclimated cells (closed symbols). Each point is the average of two independent experiments with the standard errors indicated. *Abbreviations:* see legend Fig. 2.

pathways, each activated over a specific concentration range with typical kinetics (Arisz et al., 2011; Meijer et al., 2001a,b; 2002; Munnik and Meijer, 2001). To see whether acclimated cells exhibited an accelerated or retarded response that was undetected by measurements after 5 min, radiolabelled control and acclimated cells were treated with 300 mM NaCl and harvested at different time-points. Samples were taken and the fold-increase in signalling

lipids was determined. The results from a representative experiment ($n = 2$) are shown in Fig. 4. NaCl treatment increased the levels of signalling phospholipids with similar kinetics, reaching maximum levels at the same time for both control and acclimated cells. Therefore the apparent insensitivity of acclimated cells was not due to modifications of signalling kinetics but rather to lower signal levels being generated.

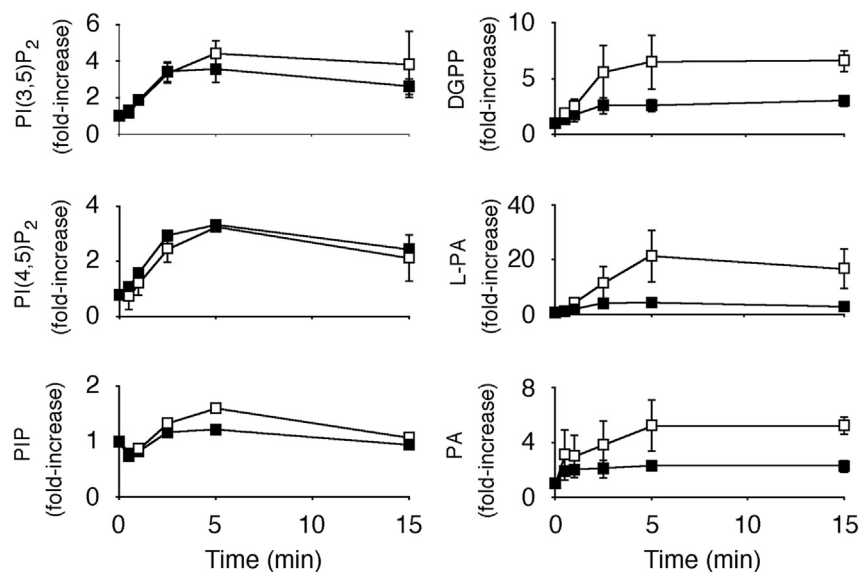


Fig. 4. NaCl induced phospholipid signalling in control and acclimated cells generates similar kinetics. *Chlamydomonas* cells were labelled for 2.5 h and then treated with 300 mM NaCl for the times indicated. Lipids were isolated, separated by TLC and quantified by phosphoimaging. The results are shown for the phospholipids that were responsive to osmotic stress in control (open squares) and acclimated cells (closed squares). Data from two independent experiments are included (\pm SE). Abbreviations: see legend Fig. 2.

2.4. NaCl-grown cells are acclimated to osmotic rather than ionic stress

Prolonged growth on 100 mM NaCl-containing medium can result in two types of acclimation, ionic and osmotic. To establish whether *Chlamydomonas* cells became acclimated to salinity or to hyperosmotic stress, we tested their response to a range of KCl- and sucrose concentrations. Control and acclimated cells were treated for 5 min with the osmolytes added to the cell-free medium, as described above. The changes in phospholipid labelling were quantitated and summarized in Fig. 3 (middle and right panel).

Before considering the effects of acclimation, it is worth emphasising that the changes in lipid metabolism described for non-acclimated cells mixed with NaCl, recurred when mixed with equivalent concentrations of KCl or sucrose (compare open squares, Fig. 3). Thus the dominant factor inducing these signalling responses is hyperosmotic stress. Nonetheless, it is also clear that NaCl and KCl induced stronger responses than sucrose. Therefore hyperionicity is more than hyperosmolarity alone, although the cell translates both treatments into common phospholipid signalling responses.

Treatment of NaCl-acclimated cells with KCl or sucrose (Fig. 3, filled squares) underlines the common effects of hyperosmotic adjustment, for in both cases the responses in lipid metabolism were similar to those observed for NaCl-treatment of salt-acclimated cells. Again the signalling maxima in the NaCl-acclimated cells were shifted to higher concentrations and generally reduced in magnitude. Thus acclimation to 100 mM NaCl bestowed resistance to high concentrations of other osmolytes, whether ionic or not (Fig. 3).

2.5. PLD activity is affected during acclimation

PA is a reporter lipid for both DGK and PLD activities. In a previous report we have shown that both signalling pathways are activated in *Chlamydomonas* during hyperosmotic stress (Munnik et al., 2000) and that PLD is more strongly activated at relatively low salt concentrations (Meijer et al., 2002). In order to determine whether PLD activation was affected by acclimation, both control and NaCl-acclimated cells were treated with a range of NaCl

concentrations in the presence of 0.25% *n*-butanol to report the formation of PLD-catalyzed phosphatidylbutanol (PBut; Munnik et al., 1995). As is evident from Fig. 5, non-acclimated cells responded to NaCl treatment in a dose-dependent manner, resembling the response to KCl (Meijer et al., 2002). When salt-acclimated cells were used, the PBut increase was greatly reduced, and the response was much broader and shifted towards higher concentrations. Similar results were found when KCl was used (data not shown). This illustrates that acclimation affects the activation of PLD in a manner similar to the other lipid signalling pathways described above.

3. Discussion

We have studied the effect of salt-acclimation on *Chlamydomonas* cells. When grown under low salt-stress conditions (100 mM NaCl), cells tolerated still higher salt concentrations that were inhibitory for control cells, suggesting they acclimate to salt stress. Since osmotic stress has been reported to activate different phospholipid signalling pathways (Meijer et al., 1999, 2001a, 2001b, 2002; Munnik et al., 2000; Munnik and Meijer, 2001), we studied the effects of salt-acclimation (100 mM) on osmo-stress-induced phospholipid metabolism. Whether acclimated or not, cells exhibited dramatic changes in their minor lipids within 5 min of increasing the osmolyte level. Since these lipids have already been assigned signalling functions (Bak et al., 2013; Boss and Im, 2012; Heilman, 2009, 2016; Hirano et al., 2015, 2016; Hong et al., 2010; Hou et al., 2015; Munnik and Nielsen, 2011; Munnik and Vermeer, 2010; Testerink and Munnik, 2011; Xue et al., 2009; Wang et al., 2006), we propose that these changes represent dose-dependent signalling responses to hyperosmotic stress.

Although specific changes in lipid signalling have been reported previously for osmotic-stressed cells (Arisz et al., 2000, 2013; Cho et al., 1993; Darwish et al., 2009; DeWald et al., 2001; König et al., 2009; Meijer et al., 1999, 2001a, 2001b, 2002; Munnik et al., 2000; Pical et al., 1999; Zonia and Munnik, 2004), what is new is that all changes are activated at the same time and monitored in the same cells. It epitomises the complexity of signalling abiotic stress, especially since phospholipid pathways represents only one facet of intracellular signalling. Five independent routes can be quickly

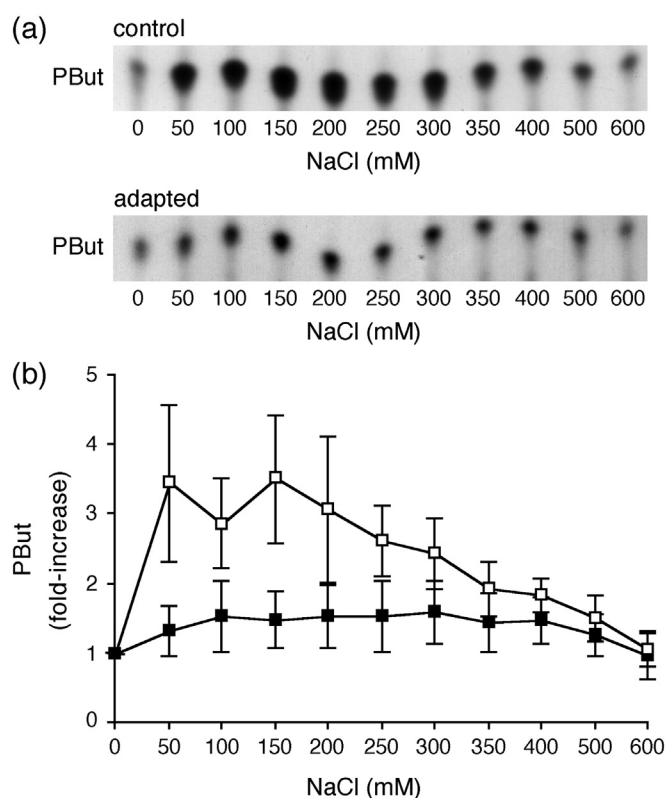


Fig. 5. Osmotic stress-induced PBut formation in control and acclimated cells. ^{32}P -prelabelled *Chlamydomonas* cells were treated with a range of NaCl concentrations in the presence of *n*-butanol for 5 min. Phospholipids were then extracted and subjected to ethyl acetate TLC analysis and (A) the radioactivity in PBut was visualized by autoradiography, control (upper panel) and NaCl-acclimated (lower panel). The results of a representative experiment are shown. (B) ^{32}P -levels of PBut were determined by phosphoimaging and shown for control (open squares) and acclimated cells (closed squares) ($n = 3$). Abbreviations: PBut, phosphatidylbutanol. Rest, see legend Fig. 2.

distinguished, those represented by increases in PI(3,5)P₂, PI(4,5)P₂, PA, DGPP and L-PA. This is justified because of known independence, e.g. PI(3,5)P₂ and PI(4,5)P₂, or because lipid signals formation over different stress ranges. For example, the formation of L-PA by a PLA₂ (Meijer et al., 2001a; Arisz et al., 2013) depends on PA synthesis but peaks at lower osmotic stresses than does PA formation (Fig. 3). Similarly, DGPP formation only occurs at high salt concentrations, whereas the PA that is generated via PLD at relatively low salt concentrations is not phosphorylated to DGPP (see also Meijer et al., 2002). In addition, there are treatments that trigger dramatic increases in PA, e.g. mastoparan, that result in the concomitant formation of DGPP (Munnik et al., 1998b; Van Himbergen et al., 1999) but do not result in the formation of L-PA (Meijer et al., 2001a; Arisz et al., 2013).

Hyperosmotic stress activates the formation of PA via both PLD and DGK (Arisz and Munnik, 2011; Arisz et al., 2000, 2003, 2009; Munnik et al., 2000). However, while PLD was strongly activated by lower concentrations of salts, the increase in ^{32}P -PA was much more apparent at higher concentrations (Meijer et al., 2002). This is because i) ^{32}P -PBut is made at the cost of PLD-generated ^{32}P -PA, ii) most of PLD's substrate is not labelled within the three hours labelling period and therefore PA_{PLD} is not well ^{32}P -labelled, and iii) in contrast to the structural lipids, ATP is rapidly labelled as soon as $^{32}\text{P}_i$ is taken up by the cells, so when DAG_{PLC} is converted to ^{32}P -PA_{DGK}, it becomes well labelled, making ^{32}P -PA_{DGK} stand out more strongly than ^{32}P -PA_{PLD} (Munnik, 2001; Arisz et al., 2009, 2013). Interestingly, only at high osmotic concentrations, ^{32}P -PA is

converted to ^{32}P -DGPP. This means that either the two PA pools, generated at low or high salt conditions, resides at different locations in the cell while PA-kinase is only present at one of them, or that PA-kinase is only activated at hyperosmotic conditions. This emphasizes that PA signals can be of different nature, and that we can speak of a PA signature that incorporates parameters such as concentration increase, cellular localisation and biochemical context (Hong et al., 2010; McLoughlin and Testerink, 2013; Munnik and Testerink, 2011; Wang et al., 2006).

The rapid activation of signals with different stress-reaction profiles probably reflects the presence of different osmosensors. The concept is strongly supported by work on yeast, where two osmotically regulated receptors (Sho1p and Sln1p) have been characterized, operating at different stress profiles between 100 and 600 mM NaCl (Maeda et al., 1995; Munnik and Meijer, 2001). A third receptor can be postulated because at very high salt concentrations (>900 mM), yeast also synthesizes PI(3,5)P₂ (Dove et al., 1997). In higher plants, only putative osmosensors have been cloned so far (Haswell and Verslues, 2015; Kurusu et al., 2015; Kushwaha et al., 2014; Urao et al., 1999; Yuan et al., 2014). However, protein kinase activities responding to discrete osmotic levels have been documented (Munnik and Meijer, 2001; Munnik et al., 1999; Fujii and Zhu, 2012). These data suggest that environmental stimuli of graded intensity are detected by different osmosensors, each responding to a limited intensity scale, rather than one osmosensor accommodating the whole scale. Although the nature of these sensors is not known, the shift in response towards higher concentrations during salt-acclimation suggests that they respond to the difference between external and internal water potentials, rather than to a distinct range of external water potentials. For example, they could be stretch-activated, responding to shrinkage of the plasma membrane as water is lost from the cell (Haswell and Verslues, 2015). Their function is to translate a stress continuum into 'signal blends' that are specific for small stress ranges. For example, non-acclimated cells respond in the low osmotic ranges with an increase in PI(3,5)P₂, PI(4,5)P₂ and PA_{PLD} (PBut), while at intermediate concentrations all signalling routes are activated to a lesser or higher extent. Each signal blend then activates responses necessary to compensate that level of osmotic stress e.g. K⁺ channel regulation, H⁺-ATPase activity or osmoticum synthesis (Bak et al., 2013; Delage et al., 2013; Heilmann, 2009; Leprince et al., 2015; Li et al., 2009; Meijer and Munnik, 2003; Munnik and Meijer, 2001; Munnik and Nielsen, 2011; Munnik and Vermeer, 2010; Xue et al., 2009; Wang et al., 2006).

When plant cells acclimate to 100 mM NaCl, they not only compensate the cytoplasmic water potential, but also desensitize detection and signalling. This was seen as the lower signal levels generated by osmo-stress in acclimated compared with non-acclimated cells. This could be because acclimated cells experience a less severe stress when shifted from 100 to 200 mM NaCl, compared with non-acclimated cells (1–100 mM NaCl). However, we think that at least part of the effect is due to the down-regulation of sensors or their effector enzymes like PLC, PLD, PLA₂, DGK and PA-kinase. We think this because acclimated cells responded less strongly, not only to osmo-stress but also to mastoparan, producing only half of the normal amounts of PA and DGPP (data not shown; see Munnik et al., 1998b; Van Himbergen et al., 1999). This effect could be explained by a reduction in osmosensors or signalling enzymes or by a modification that reduces their sensitivity. This acclimation response, measured in weeks, should not be confused with the up-regulated expression of genes involved in phospholipid signalling that can be detected within the first hours of osmotic stress (Boss and Im, 2012; Hirayama et al., 1995; Katagiri et al., 1996, 2001; Laxalt et al., 2001; Lin et al., 2004; Mikami et al., 1998; Tasma et al., 2008).

Since PLC together with DGK play a major role in the production of PA during osmo-stress, as well as during mastoparan treatment (Arisz et al., 2000, 2003; Arisz and Munnik, 2011, 2013; Van Himbergen et al., 1999; Munnik et al., 1998b, 2000), lowering their substrate levels would automatically down-regulate the signalling response. In practice, acclimated cells contained less PI(4,5)P₂ than control cells and therefore lipid substrate availability rather than enzyme levels could also account for the down-regulation mechanism. Paradoxically, when acclimated cells were subjected to osmo-stress, they rapidly synthesised PI(4,5)P₂, as if re-sensitizing the PLC/DGK pathway. This was not translated into a subsequent increase in PA, either because these enzymes were themselves down-regulated or because the PI(4,5)P₂ was not available for hydrolysis. PI(4,5)P₂ is known to have a strong regulatory function independent of PLC signalling, for example, it is also involved in cytoskeletal rearrangements, vesicle trafficking, and K⁺-channel activity, regulating stress responses as well as growth development (Balla, 2013; Heilman, 2016; Ischebeck et al., 2010, 2011, 2013; Kost et al., 1999; Kusano et al., 2008; Lee et al., 2007; Ma et al., 2009, 2015; Mei et al., 2012; Rodriguez-Villalon et al., 2015; Simon et al., 2014; Sousa et al., 2008; Stenzel et al., 2008; Tejos et al., 2014; Wigoda et al., 2010; Zhao et al., 2010).

In conclusion, our results provide a first illustration of how plant cells acclimate to stress by modifying the activity of those phospholipid signalling pathways that triggered the initial response.

4. Experimental

4.1. Cell cultures, growth and acclimation

The unicellular, biflagellate green alga *Chlamydomonas moewusii* (strain UTEX 10; mating type minus, Culture Collection of Algae, University of Texas, Austin, TX, USA) was cultivated on agar-containing M1 medium in Petri dishes as described earlier (Meijer et al., 1992). Cultures were maintained at 20 °C in a 12 h light/12 h dark regime with an average photon flux of 30 μE m⁻² sec⁻¹ which was provided by Philips (Eindhoven, The Netherlands) TL 65W/33 fluorescent tubes. Osmotically acclimated cells were produced by cultivating them on M1-plates, supplemented with 100 mM NaCl.

Swimming cell suspensions containing 1–2 × 10⁷ cells/ml were obtained by flooding 2- to 3-weeks-old plates for 14–18 h with 20 ml HMCK (10 mM HEPES-KOH, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 1 mM KCl). Acclimated cell plates were flooded with HMCK supplemented with 100 mM NaCl.

To analyse the growth rate in liquid cultures, Erlenmeyer flasks containing 100 ml liquid M1-medium were supplemented with various concentrations of NaCl (0, 300, or 400 mM), inoculated with either acclimated or non-acclimated cells, and their growth monitored for seven days while shaking 125 rpm at 20 °C in a 12 h light/12 h dark regime with an average photon flux of 30 μE m⁻² sec⁻¹. Samples were daily harvested and the cells fixed in 1.25% glutaraldehyde. Cell numbers were monitored using a Coulter counter (Coulter Multisizer II; Coulter Electronics Ltd. Luton, Bedfordshire, UK). For the growth analyses on solid media, cells were plated on M1-agar, supplemented with a range of NaCl concentrations and grown for two weeks in the same growth chamber (20 °C; 12 h light/12 h dark).

4.2. Metabolic radiolabelling and phospholipid analyses

Phospholipids were metabolically labelled by incubating 100 μl cell suspension (1–2 × 10⁷ cells per ml) for 2.5–3 h with 100 μCi of carrier-free ³²PO₄³⁻ ml⁻¹. Cells were then incubated for the times indicated in cell-free medium containing the appropriate

concentration of osmoticum. Cell-free medium refers to the fluid collected after centrifuging cell-suspension cultures. Incubations were stopped by adding 3.75 vol. CHCl₃/MeOH/HCl (50:100:1, by vol.). Lipids were extracted, separated by TLC plates using an alkaline TLC solvent, detected by autoradiography and quantified by phosphoimaging as described by Munnik and Zarza (2013).

For *in vivo* PLD activity measurements, *n*-butanol was included at a concentration of 0.25% (v/v) during treatments. After lipid extraction, samples were separated by TLC using an ethyl acetate solvent system (Munnik and Laxalt, 2013). Radiolabelled phospholipids were visualized by autoradiography and quantified by phosphoimaging.

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