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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)P_2] 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)P_2 and its isomer PI(3,5)P_2. 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.

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; 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 ($InsP_3$) 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 Ca^{2+} 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 $InsP_3$ or through its conversion into $InsP_6$ (Lemtiri-Chlieh et al., 2003; Munnik, 2014). A gene encoding for an $InsP_3$ 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

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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 A_2 (PLA_2; 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)P_2]; 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 polyphosphoinositides (PPIs) such as phosphatidylinositol phosphate (PIP) and PI(4,5)P_2 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 $^{32}P_i$ 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

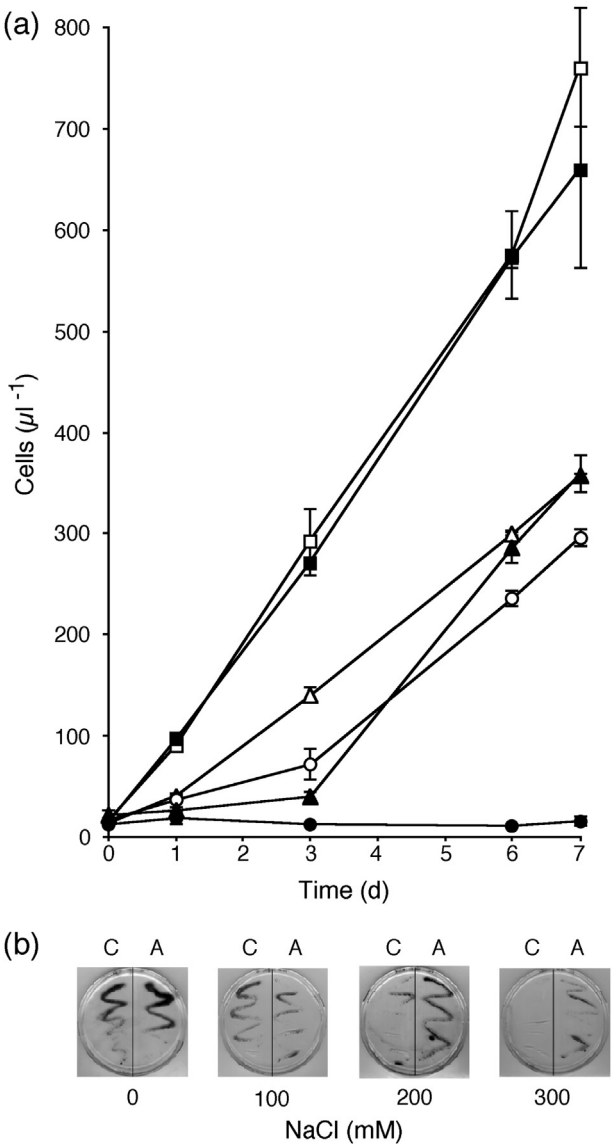

Fig. 1. Salt-acclimation affects the growth of *Chlamydomonas* cells. Cells were pre-grown for two weeks with or without 100 mM salt and than (A) transferred to fresh liquid media containing a range of NaCl concentrations. Cell numbers were registered with time. Values represent the means of two independent experiments ± standard deviation. Data are shown for control cells (closed symbols) and acclimated cells (open symbols) growing in 0 mM NaCl (■,□), 300 mM NaCl (▲,Δ) 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).

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