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Saline and osmotic stresses stimulate PLD/diacylglycerol kinase activities and increase the level of phosphatidic acid and proline in barley roots

Maria V. Meringer[a], Ana L. Villasuso[a,*], Micaela Peppino Margutti[a], Javier Usorach[a], Susana J. Pasquaré[b], Norma M. Giusto[b], Estela E. Machado[a], Graciela E. Racagni[a]

[a] Química Biológica, FCEFQN, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina
[b] INIBIBB (CONICET), Universidad Nacional del Sur, Bahía Blanca, Argentina

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ABSTRACT

Soil salinity is one of the major abiotic stresses that affect the crop productivity. Understanding the mechanisms by which plants transmit the signals to cellular machinery to trigger adaptive responses is essential to develop more stress tolerant crops. Barley (*Hordeum vulgare* L.) is an important cereal and its production is affected by increasing dryland salinity. This severely limits growth and reduces yields. In barley seedling, NaCl and mannitol stresses modulated the level of phosphatidic acid (PA), the proline accumulation and the reduced root length. PA is a well-known lipid signal and an intermediary in the lipid synthesis. However, little is known about their role during the saline and osmotic stresses in barley roots. PA increased by phospholipase D (PLD, E.C. 3.1.4.4) and by diacylglycerol kinase activities (DAG-k, EC 2.7.1.107) and its conversion to DGPP suggested that they are part of stress responses to salinity in barley. In contrast, saline stress decreased the activity of the Mg^{2+}-independent, NEM-insensitive form of phosphatidate phosphohydrolase (PAP2, E.C. 3.1.3.4), keeping the PA levels. The application of 1-butanol also stimulated proline accumulation while the DGK-inhibitor treatment decreased proline levels. Endogenous phytohormone levels measured by liquid chromatography-tandem mass spectrometry revealed that, under stress, the barley roots decreased the GA_3 and ABA levels and increased the SA and JA endogenous amounts. The results presented here suggest that PA may modulate the cellular signal of barley roots by differentially affecting components of the abiotic stress – response cascade.

1. Introduction

Salinity is one of the most severe environmental stresses that cause crop yield loss. Plants have developed different mechanisms to adapt to saline stress involving complex physiological and biochemical changes (Hasegawa et al., 2000; Widodo et al., 2009). Salinity causes ionic stress, osmotic stress, and secondary stresses including nutritional imbalance and oxidative stress (Zhu, 2002). High concentrations of Na disturb the osmotic balance causing "physiological drought", which prevents plant water uptake. To survive to the harmful effects of salt stress, plants have developed a series of biochemical and molecular mechanisms, mainly those including selective build up or exclusion of salt ions, control of ion uptake by roots and transport into leaves, ion compartmentalization, synthesis of compatible osmolytes, and induction of antioxidative enzymes (Shabala and Lew, 2002; Chen et al., 2007a; Cuin and Shabala, 2008; Rodriguez-Rosales et al., 2008; Munns and Gilliham, 2015). The amino acid proline is an osmolyte that accumulates in a wide range of plant species in response to stress (Szabados and Savoure, 2010). The exact role of proline

Abbreviations: ABA, abscisic acid; CL, cardiolipin; DAG, diacylglycerol; DAG-k, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DGPPase, diacylglycerol pyrophosphate phosphatase; IAA, indole-3-acetic acid; IP_3, inositol 1,4,5-trisphosphate; GA_3, gibberellic acid; GPL, glycerophospholipids; JA, jasmonic acid; LPPs, lipid phosphate phosphatases; PA, phosphatidic acid; PA-k, phosphatidate kinase; PAP2, phosphatidate phosphohydrolase type 2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI4-k, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI-k, phosphatidylinositol kinases; PLC, phospholipase C; PLD, phospholipase D; SA, salicylic acid; TLC, thin layer chromatography.

* Corresponding author at: Dpto. Biología Molecular, FCEFQN, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina.
E-mail address: lvillasuso@exa.unrc.edu.ar (A.L. Villasuso).

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accumulation in stress tolerance is still unknown, although, several studies have suggested that it may play other roles to limit damage stress (Shabala and Lew, 2002; Chen et al., 2007a; Shabala, 2013; Munns and Gilliham, 2015). Consequently, understanding the plant's mechanisms to salt tolerance will provide effective means to breed or genetically engineer salt tolerant crops. Barley (*Hordeum vulgare* L.) is a valuable cereal, grown primarily for animal feed and beer production. Barley production is affected by increasing dryland salinity, which severely limits growth and reduces yields (Rengasamy et al., 2003). Although some of the late responses to saline and osmotic stresses in barley are relatively well studied (Chen et al., 2007a; Widodo et al., 2009), the role of phospholipids in the signalling pathways is still unknown. During osmotic stress, several phospholipid-based signalling pathways in plants are rapidly activated. They include phospholipase D (PLD) and phospholipase C (PLC) coupled with diacylglycerol kinase (DAG-k) pathways that result in the increase of phosphatidic acid (PA) (Munnik et al., 1998, 2000; Arisz et al., 2009; Li et al., 2009; Kolesnikov et al., 2012; Pokotylo et al., 2014). PA is converted into diacylglycerol pyrophosphate (DGPP) by a phosphatidate kinase (PA-k) (Wissing and Behrbohm, 1993; Van Schooten et al., 2006; Racagni et al., 2008). Thus, the enzymes that metabolize PA/DGPP play important roles in switching the PA/DGPP signal on/off (Villasuso et al., 2013).

PA is the glycerophospholipid with the simplest chemical structure in biological membranes. Its behavior is crucial for cell survival since it is a phospholipid involved in the synthesis of phospholipids and triacylglycerols, thus playing a central role in cell signalling (Athenstaedt and Daum, 1999). PA signalling acts by binding effector proteins and recruiting them to a membrane, which regulates the proteins' activity in cellular pathways (Testerink and Munnik, 2011). Binding is mainly dependent on the concentration of the lipid in the bilayer and it depends on nonspecific electrostatic interactions between clusters of positively charged amino acids in the protein and the negatively charged phosphomonoester headgroup of PA (Shin and Loewen, 2011).

The formation of PA is an integral part of the adaptation of plants to saline environments (Hou et al., 2015; Julkowska and Testerink, 2015) and its levels increase when plants are exposed to salinity (Munnik et al., 2000; Yu et al., 2010). Recently, it has been shown that PA mediates important adaptive mechanisms including the maintenance of root architecture and cytoskeletal organization. PA and DGPP were shown to bind to glyceraldehyde-3-phosphate dehydrogenase and modulate its activity, a key glycolysis enzyme, in response to salt stress in roots (Kim et al., 2013; Mcloughlin et al., 2013; Astorquiza et al., 2016). PA is implicated in controlling the growth of the primary root (Kim et al., 2013). Besides, members of the sucrose non-fermenting 1-related protein kinase 2 (SnRK2s) family with PA-binding affinities were modulated by saline stress (Testerink et al., 2004; Mcloughlin et al., 2012). Mcloughlin et al. (2012) suggests an involvement of PA in membrane trafficking and cellular re-organization during salt stress. The PLD-induced PA under saline conditions also affects the organization of the cytoskeleton (Lee et al., 2003; Zhang et al., 2012). The activity of the microtubule-associated protein MAP65-1, that bundles and stabilises adjacent microtubules, was increased after PA binding in response to salinity, while mutants with low PA concentrations (*PLDα1, PLDα3, PLDδ* and *PLDε*) were more sensitive to salt stress (Zhang et al., 2012). Furthermore, the plants have developed other survival strategies, including also the synthesis of stress-related hormones like abscisic acid (ABA) and salicylic acid (SA) to protect themselves from the detrimental surroundings. Although hormones are likely to play important roles in root growth regulation under water-stressed conditions, the involvement of most of these compounds has not been still elucidated in barley roots. The aim of this work was to study the lipid-signalling pathway to better understand the physiological and biochemical responses induced by the saline and osmotic stresses in barley roots.

2. Materials and methods

2.1. Plant materials, growth conditions, and separation roots

Barley seeds (*H. vulgare*, cv. Carla INTA) were surface sterilized and soaked in sterilized water for 4 days in the dark at 25 °C. Seedlings were obtained from whole grains, surface sterilized, grown in a growth chamber on disks of filter paper moistened with sterilized water for control seedling and with 100 mM NaCl or 200 mM mannitol solutions for seedling under stress, in Petri dishes (10-cm diameter), for 4 days in the dark at 25 °C, and then harvested. Roots were separated and kept frozen in liquid nitrogen at −80 °C until use. When seedlings were treated with 1-butanol, R59949 (DAG-k inhibitor type I) or R59022 (DAG-k inhibitor type I), these inhibitors were added to the other treatment.

2.2. Preparation of membranes

Control and stressed roots prepared as above were thawed and homogenized in 10 vols of 50 mM HEPES (pH 7.4) containing 0.25 M sucrose, 5 mM KCl, 1 mM EDTA, and protease inhibitors (1 mg mL^{-1} leupeptin, 1 mM phenyl methane sulfonyl fluoride (PMSF), 1 mg mL^{-1} aprotinin). The homogenate was centrifuged at 1000*g* for 15 min at 4 °C to remove unbroken cells and cell debris, and the resulting supernatant was further centrifuged at 105,000*g* for 60 min at 4 °C. The supernatant was eliminated, and the pellet was resuspended in 50 mM HEPES (pH 7.4) and used as crude membrane fraction. Protein concentration of samples was measured using Bradford reagent with BSA as standard (Bradford, 1976).

2.3. Lipid kinase activity and phospholipid extraction and separation

The membrane fraction isolated as above (60 μg protein) was added to thermally equilibrated (30 °C) 50 mM HEPES buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM DTE, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 mM Mg^{2+}-ATP, and [γ-^{32}P]ATP (370 MBq). Lipid kinase activities were assayed simultaneously using endogenous lipids as substrates. Lipid phosphorylation was allowed to proceed for 2 min at 30 °C in a final volume of 100 μL, and reaction was stopped by addition of 1.5 mL chloroform/methanol (1:2, v/v). Lipids were extracted from membranes and phospholipids were separated by TLC as described by Racagni et al. (2008). Plates were developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v/v/v/v) in a plate of 20 cm. Positions of radiolabeled lipids were determined by autoradiography on Kodak film.

2.4. [^{32}P]Pi phospholipid labelling, extraction and separation

Roots (6 tips root) were incubated in 1 mL label medium (20 mM $CaCl_2$ and 20 mM sodium-succinate, pH 6.5) containing 50 mCi carrier-free [^{32}P]orthophosphate, abbreviated as [^{32}P]Pi. Treatments were stopped at specified times by adding 250 μL of 25% v/v perchloric acid vortexing for 5 min, and maintaining sample-containing tubes for 30 min at room temperature. After discarding perchloric acid, root lipids were extracted by adding 400 μL chloroform/methanol/hydrochloric acid (50:100:1, v/v/v) and freezing and thawing the mixture by means of liquid nitrogen. After 5 min of vigorous mixing, lipid extracts were transferred to clean tubes and 400 μL chloroform and 214 μL 0.9% (v/v) NaCl were added to produce a two-phase system. After vortexing

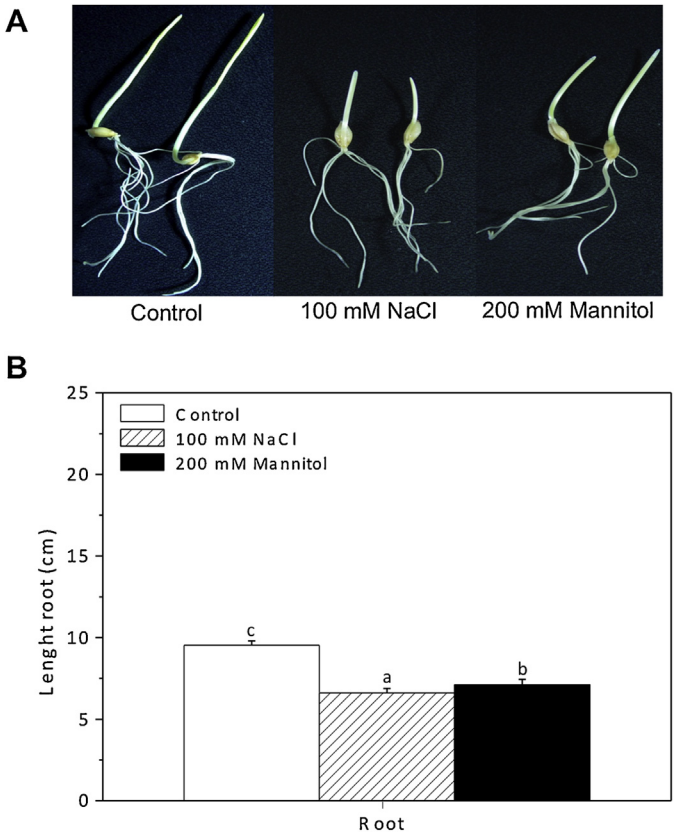

Fig. 1. (A) Effect of saline and osmotic stresses on root length of barley. Results are expressed in centimetres (cm) and shown as mean ± S.D., n = 30, different lowercase letters indicate significance at $P < 0.05$. (B) Image of 4 d plant treated with 100 mM NaCl or 200 mM mannitol.

(1 min) and centrifugation (1000*g*, 5 min), the upper phase was removed and the lower phase washed with 500 μL chloroform/methanol/1 M hydrochloric acid (3:48:47, v/v/v). Lipid extracts were dried by vacuum centrifugation, dissolved in a suitable volume of chloroform, and immediately subjected to TLC analysis. An alkaline solvent system, chloroform/methanol/25% ammonium hydroxide/water (45:35:2:8, v/v/v/v), was used to separate labelled GPL as described by Villasuso et al. (2013).

2.5. Lipid phosphate phosphatase activity assays

For determination of phosphatidate phosphohydrolase type 2 (PAP2) activity, the assay mixture consisted of 50 mM Tris-maleate buffer, pH 6.5, 1 mM DTT, 1 mM EDTA plus 1 mM EGTA, 4.2 mM NEM, and 100 μg of membrane protein in a volume of 0.1 mL. The reaction was started by addition of 0.6 mM [2-^{3}H]phosphatidate, continued for 30 min at 37 °C, and stopped by addition of chloroform/methanol (2:1, v/v). PAP activity product 1,2-diacyl [^{3}H]glycerol was isolated and measured. Radiolabeled PA was obtained from [2-^{3}H]phosphatidylcholine, which was synthesized as described by Pasquare De Garcia and Giusto (1986). PAP activity was expressed as the sum of nmol ([^{3}H]diacylglycerol and [^{3}H] monoacylglycerol) × (h × mg protein)$^{-1}$.

DGPP phosphatase activity was assayed as described by Meringer et al. (2012), based on release of water-soluble [^{32}P]Pi from chloroform-soluble [β-^{32}P]DGPP (2000 cpm/pmol). The reaction mixture contained 50 mM citrate buffer (pH 5.0), 0.1 mM DGPP, 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein, in a total volume of 0.1 mL. DGPP was synthesized from PA using enriched-membrane fraction *H. vulgare* PA kinase.

2.6. Protein gel blot analysis

Barley roots homogenates were prepared as described above for lipid kinase assay. Extract samples were separated by 12.5% SDS-PAGE, and proteins were electrotransferred to nitrocellulose membranes. Protein blots were blocked with 5% skim milk powder in phosphate-buffered saline (4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl). Antibodies directed to C-terminal DGPP phosphatase (a generous gift from Dr. George Carman) were added at 1:1000 dilution and incubated for 2 h. Secondary antibody coupled to horseradish peroxidase was used to visualize target proteins, in combination with chromogenic substrates.

2.7. Phospholipase D activity

PLD activity was measured by production of phosphatidyl butanol (PBut), mainly as described by Villasuso et al. (2013). After labelling lipids with [^{32}P]Pi, controls and stresses roots were incubated in the presence of 0.75% v/v *n*-butanol for 30 min at 24 °C. Reactions were stopped and lipids were extracted for separation of PA and PBut of the other phospholipids, which was achieved by using the organic upper phase of the biphasic system, ethyl acetate/isooctane/formic acid/water (13:2:3:10, v/v/v/v) as the TLC solvent. Radiolabelled lipids were located by autoradiography on Kodak film. Spots were scraped off the plates, and fractions were counted in a liquid scintillation counter.

2.8. Phytohormone analysis

Levels of GA_3, ABA, IAA, SA, and JA were analyzed simultaneously by electrospray ionization/tandem mass spectrometry (LC-ESI–MS-MS), essentially as described by Durgbanshi et al. (2005). Briefly, tissues samples (200 mg) were ground in liquid nitrogen, extracted with acetone/water/acetic acid (80:19:1, v/v/v), added with 5 μL of a mixture of internal standards (50 ng/sample of $^{2}H_5$-IAA, $^{2}H_6$-ABA, $^{2}H_6$-JA, and $^{2}H_4$-SA, and 100 ng $^{2}H_2$-GA_3,), and centrifuged at 500*g* for 15 min. The resulting supernatant was collected and evaporated, and the solid residue was dissolved in 500 μL of methanol and evaporated. The resulting residue was dissolved in methanol/1% acetic acid (99:1, v/v), and then passed through a DEAE Sephadex A-25 column. Aliquots of the resulting solution were injected directly into the LC-ESI–MS–MS system. MS/MS experiments were performed on a Micromass Quatro Ultima™ Pt double quadrupole mass spectrometer (Micromass, Manchester City, UK). The conditions used for a MS/MS were the same as those in Meringer et al. (2012).

2.9. Proline accumulation

Proline was determined from roots as described by Bates et al. (1973). Briefly, 500 mg of roots were homogenized in 10 mL of 3% aqueous sulfosalicylic acid and the homogenate filtered through filter paper. 2 mL of homogenate filtrated reacted with 2 mL acid ninhydrin and 2 mL glacial acid acetic in the tube at 100 °C, and the reaction was terminated in ice bath. The reaction mixture was extracted with 4 mL toluene, and mixed vigorously with a tube stirrer for 15–20 s. The chromophore containing toluene was aspirated from aqueous phase, warmed to room temperature and the absorbance read at 520 nm using toluene for a blank. The proline concentration was determined from a standard curve and calculated on a fresh weight.

2.10. Root Na^+ and K^+ contents

Barley roots (1 g) were homogenized with liquid nitrogen and then they were centrifuged to 40,000*g*, during 60 min. The Na^+ and

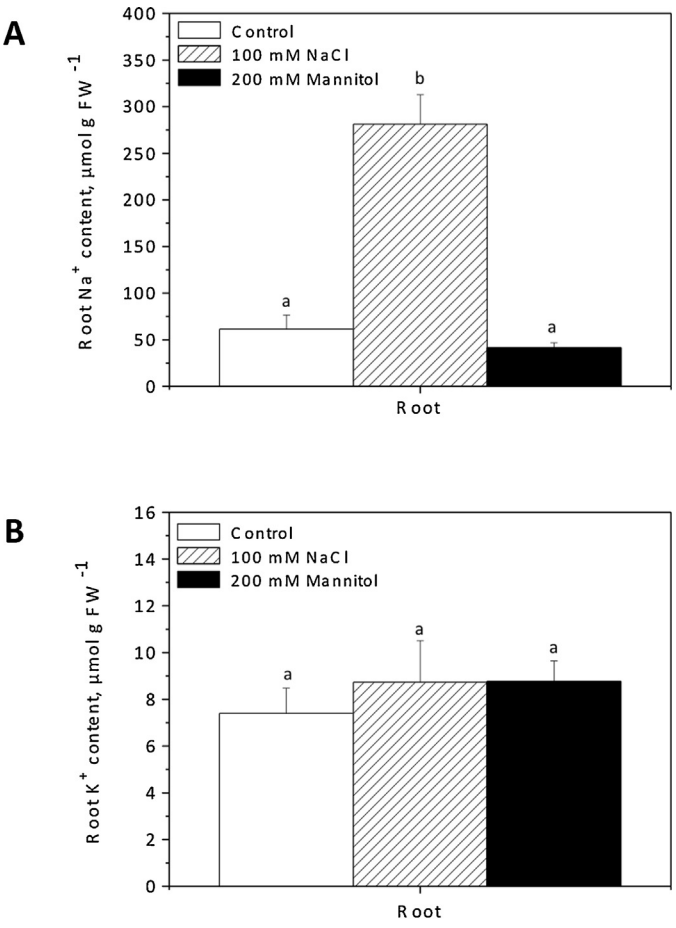

Fig. 2. Determination of root Na^+ (A) and K^+ (B) contents. Barley roots (1 g) were homogenized with liquid nitrogen and then, they were centrifuged to 40,000*g*, during 60 min. The Na^+ and K^+ contents were determined in the supernatant using a flame photometer. Results are expressed in µmol g FW^{-1} and represent the mean ± S.D., (n = 3). Different lowercase letters indicate significance at $P < 0.05$.

K^+ contents were determined in the supernatant using a flame photometer.

2.11. Statistical analysis

To determine the statistical difference between at least one pair of means, analysis of variance test (ANOVA) was used. When the assumptions of homogeneity of variance (Levene test) and normality (Shapiroe Wilk test) were not checked, corresponding transformations were performed using the appropriate functions. To determine significant differences between treatments, Tukey's Test was applied, with a significance level of 0.05 ($P < 0.05$). The statistical program used was InfoStat (2015e version, Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Argentina)

3. Results

Saline stress triggers a wide range of plant responses, from altered gene expression and cellular metabolism to change in growth rates and crop yields. To evaluate the effect of saline stress on barley seedling, a growth analysis for the characterization of plant's response was carried out. Fig. 1A shows that the root length (cm) of stressed seedlings decreased after 96 h of imbibition, compared to that of control seedling. The effect was more significant in the saline than in the osmotic stress (Fig. 1B). Reduction on the length of the roots of 40.5 ± 10.06% with 100 mM

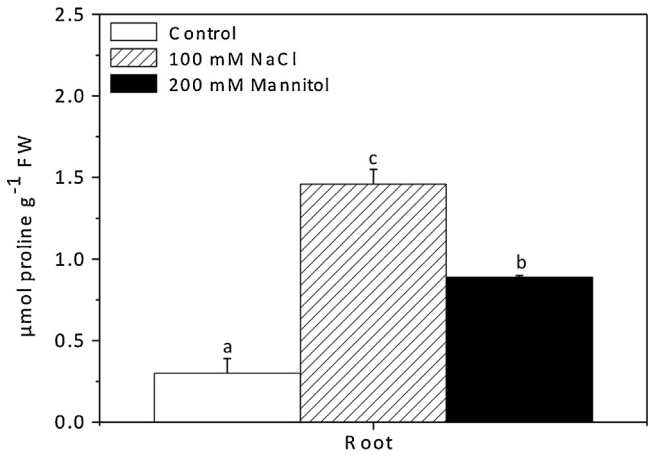

Fig. 3. NaCl and mannitol treatment induce proline accumulation in barley roots. The determination of proline concentration was performed according to Bates (1973). Results are expressed in µmol g FW-1 and represent the mean ± S.D., n = 3, different lowercase letters indicate significance at $P < 0.05$.

NaCl and 18.98 ± 4.77% with 200 mM mannitol was observed. Likewise, both 100 mM NaCl and 200 mM mannitol reduced the percentage of germination, although the effect induced by mannitol was lower than that evoked by NaCl (data not shown). This difference in growth was also reflected on root Na^+ and K^+ content (Fig. 2). In absence of salt, the Na^+ content was around 50 µmol/g FW. Treatment with 100 mM NaCl resulted in a significant 6-fold increase in the Na^+ content in seedling roots (Fig. 2A). In contrast, the K^+ content did not change significantly as result of NaCl or mannitol stress (Fig. 2B).

It is well known that accumulation of compatible solutes, as the amino acid proline, is a shared stress signal by plants. To investigate this response, the proline level was measured in roots obtained from control and stress seedlings. Fig. 3 shows that proline content in roots increased significantly 3-fold by 100 mM NaCl. Similarly, the root treated with 200 mM mannitol increased 2-fold the proline level.

Lipid signalling associated to accumulation of PA is a rapid response to saline stress in plants. To investigate the possible role of PA signalling, the lipid kinase activities in NaCl and mannitol treated barley roots were assayed. A sequential measurement system was used, with both endogenous substrates and DAG-k and PA-k present in the same membrane fraction, prepared by centrifugation at 105,000*g*. Phospholipids present in this fraction were identified by autoradiography as PA and DGPP (Fig. 4A). Saline and osmotic stresses induced changes on phosphorylated products of barley lipid kinase activities (Fig. 4B). An increase in the PA (150 ± 5%; n = 5) formation was observed after 100 mM NaCl and 200 mM mannitol treatment, with respect to unstimulated control (100%). In contrast, NaCl treated root displayed lower PA-k activities, and higher activities in response to mannitol. This reduced activity of PA-k may be correlated with increased activities of the enzymes that regulate PA and DGPP levels. PAP2 activity (measured with [^{3}H]PA as substrate) and DGPPase activity (measured with [β-^{32}P]DGPP as substrate) of root tissues, expressed as lipid phosphate phosphatase activities relative to values in unstimulated barley root, are shown in Fig. 5A and B.

DGPPase activity was 20% higher, whereas PAP2 activity was 25% lower in stimulated root tissues relative to control roots. In vitro phosphatase activities decreased in stressed roots compared to control. For this reason, a protein blotting was performed to determine whether protein levels were different. Protein fraction

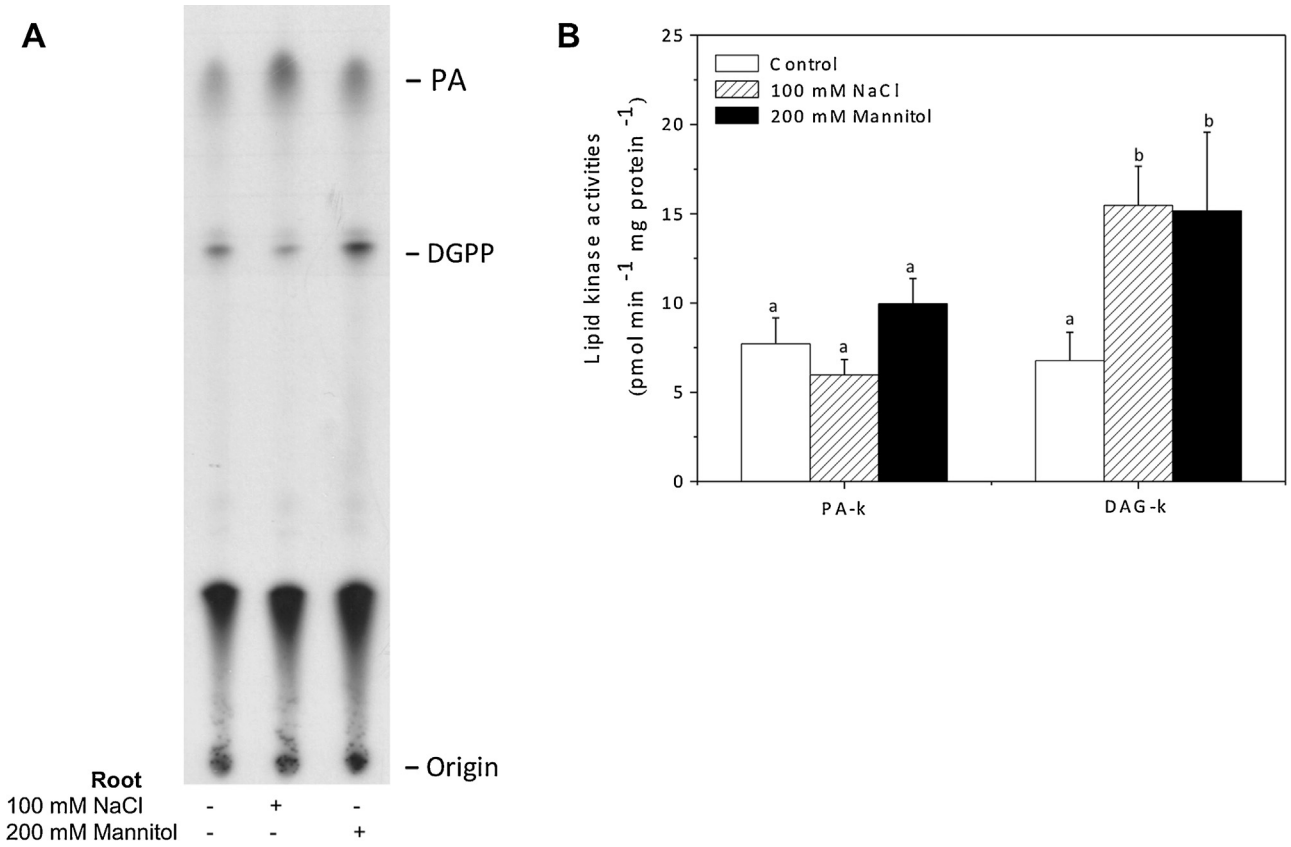

Fig. 4. Effect of saline and osmotic stresses on DAG-k and PA-k activities in barley roots. (A) Representative autoradiography of lipid products from barley roots treated with 100 mM NaCl or 200 mM mannitol. Phosphorylation assay of endogenous phospholipids was carried out with the 105,000g membrane fraction in the presence of 370 MBq [γ-^{32}P]ATP for 2 min at 30 °C. Labelled lipids were extracted and separated by 1-dimensional TLC with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). (B) Results are expressed as specific activity (pmol min–1 mg protein-1) and shown as mean ± S.D., n = 3, different lowercase letters indicate significance at $P < 0.05$.

isolated from barley tissues was separated by SDS-PAGE and immunoblotted. Antibody directed to C-terminus of *S. cerevisiae* DGPPase, used for detection of DGPPase protein levels, showed band with 30 kDa. Signal levels were significantly different from stressed roots compared to control (Fig. 5C). DGPPase signal levels were 0.41 ± 0.1 and 0.62 ± 0.1 (n = 4, $P < 0.05$) in NaCl and mannitol treated root compared to control.

PA levels can also be increased through the hydrolysis of PC/PE catalysed by PLD. In order to know the PLD's contribution to PA signal, *in vivo* transphosphatidylation activity of PLD was measured. Barley roots were labelled O/N and then treated with salt or mannitol in the presence of 0.5% (v/v) 1-butanol. After 30 min, reactions were stopped, and lipids were extracted and separated by ethyl acetate TLC to monitor the PLD-catalyzed phosphatidylbutanol (PBut) formation by liquid scintillation. As shown in Fig. 6, increased PBut levels were found after NaCl and manitol treatment. PLD activity was 0.5-fold higher in presence of salt (Fig. 6A) and 0.25-fold higher in presence of manitol (Fig. 6B) in relation to that of the control values. Under these conditions, 1-butanol did lead to the formation of PBut instead of PA. Accordingly, in Fig. 6C we show that the stress-induced PA levels in the presence of 1-butanol are below the level obtained in the absence of 1-butanol. These results indicate that the increase of PA, in response to saline and osmotic stresses, is also as consequence of PLD activation.

Saline and osmotic stresses could trigger changes in the phospholipid patterns. In order to analyse whether stress treatment can produce a change in the PLs turnover (either structural or minor PLs), studies on the lipid profiles were carried out (Fig. 7). First, barley seedlings were grown-up for 4 d with NaCl or mannitol and then labelled with [^{32}P]Pi for 18 h (isotopic equilibrium). In control roots, it was determined that structural PLs had a high percentage of [^{32}P]Pi-incorporation, being PE ones of the most abundant phospholipids and representing 60% of total PLs, followed by CL, PG and PC, with values of 18, 9 and 4%, respectively. The rest of the phospholipids had a percentage of [^{32}P]Pi-incorporation lower than structural lipid, being all similar around less than 5%. As shown in Fig. 7B, differences on the lipid profiles in presence of NaCl or mannitol were observed. In NaCl treatment, several PLs *i.e.*, PI, DGPP and PIP, showed higher percentages of [^{32}P]Pi-incorporation than the control treated with water. Regarding structural lipids, the lipid turnover was similar to that of the control for PC, PE and LPE, with the exception of CL, which showed a significant decrease in the lipid turnover, while PA showed a lower [^{32}P]Pi-incorporation compared to the control.

We found that the formation of PA during saline and osmotic stresses is a consequence of both, PLD action and DAG-k activity. This fact could be related to proline accumulation. Consequently, the possibility that DGK and PLD inhibition (by using 1-butanol and DAG-k inhibitors) may modulate the proline levels during stress responses was tested. Fig. 8 shows that in response to 4-d of treatments with either 100 mM NaCl or 200 mM mannitol, proline was accumulated to 3- and 2-fold, respectively, compared to control seedlings. In presence of 1-butanol, proline increased to 4- and 3 fold during treatments with either 100 mM NaCl or 200 mM mannitol, respectively. On the contrary, in presence of DAG-k inhibitors the proline accumulation was abolished.

Changes in phytohormone concentrations in plants mediate a wide range of developmental processes, many of which involve interactions with phospholipid metabolism. Endogenous

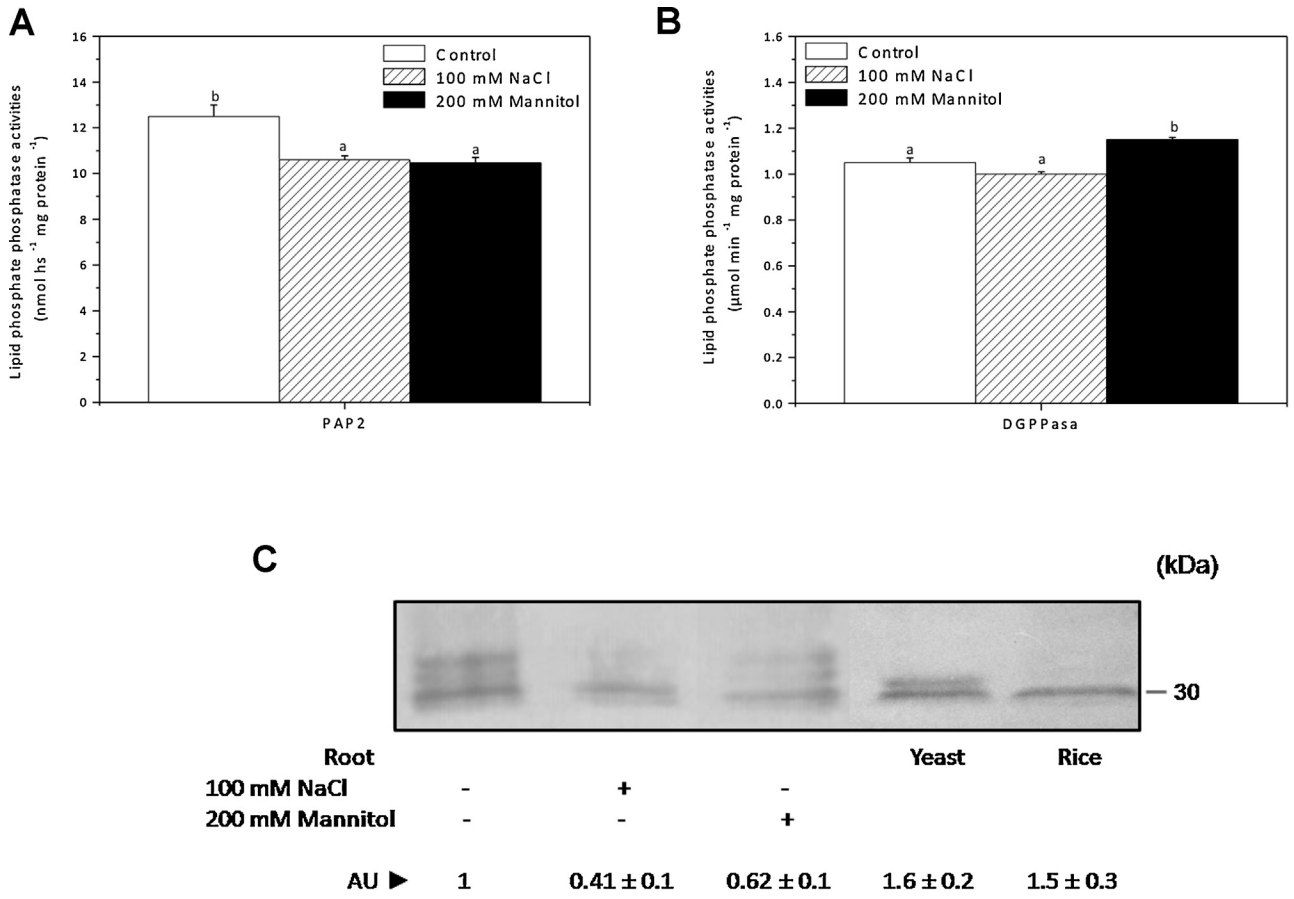

Fig. 5. Effect of saline and osmotic stresses on phosphatidate phosphohydrolase and diacylglycerol pyrophosphate phosphatase. Enzymatic activities were assayed in the 105,000*g* membrane fraction obtained from barley roots. PAP2 activity was determined using [^{3}H]-PA (0.6 mM) as substrate. DGPP phosphatase activity was measured by the release of water-soluble [^{32}P]Pi using chloroform-soluble [β-^{32}P]DGPP. Results are expressed as specific activity and shown as mean ± S.D., n = 3, different lowercase letters indicate significance at $P < 0.05$.

phytohormone concentrations in barley roots, expressed as ng per g dry weight, are summarized in Table 1. GA_3 level was 25% lower in stressed roots than in control tissue. ABA level was 50% lower in stressed than in control roots. IAA content was similar among the three tissues. SA level was 0.8-fold higher in mannitol treated roots. Similarly, JA level was ∼4-fold higher in plants treated with mannitol.

4. Discussion

In our present work, the effects of NaCl and mannitol on barley roots were investigated in relation to lipid signalling. In response to NaCl, the barley root accumulated Na+, although K+ content did not change significantly. This result could be explained through the barley root's ability to retain K+. It was also observed that NaCl and mannitol treatment evoked a reduction on the length of the primary roots. Adaptation to salinity is related to cell's ability to maintain the optimal cytosolic K/Na ratio and this fact involves orchestrated regulation of a large number of Na^+ and K^+ transporters (Shabala and Lew, 2002). In presence of salt, the cytosolic K^+/Na^+ ratio decreases due to excessive Na^+ accumulation in the cytosol and enhanced K^+ leakage. This salinity-induced K^+ loss from cells (Cuin and Shabala, 2005; Shabala et al., 2006; Chen et al., 2007b) is a result of NaCl-induced membrane depolarization, leading to the activation of depolarization-activated outward-rectifying K^+ channels (Shabala et al., 2006, 2016).

Studies on barley have suggested that the magnitude of this NaCl-induced efflux from the roots of young seedlings has a negative correlation with salt tolerance (Chen et al., 2007a,b). Therefore, the root's ability to retain K^+ is significant to salt tolerance (Cuin et al., 2008). We have also observed that NaCl and mannitol treatment in barley seedlings evoked a reduction on the length of the primary roots and an increased development of new roots (data not shown). This event could be due to a process of salt-induced programmed cell death (PCD) of primary roots as an adaptive response and they could also be related to ionic homeostasis (Affenzeller et al., 2009; Shabala, 2009). The elimination of a primary root that makes the plant susceptible to a saline medium, and its replacement with roots that can facilitate nutrient and water acquisition, may modulate a plant's capacity to regulate nutrients and water uptake. Likewise, accumulation of compatible solutes is often regarded as a basic strategy for the protection and survival of plants under abiotic stress (Cuin and Shabala, 2005; Shabala, 2013; Munns and Gilliham, 2015). In barley seedling roots, we have observed an increased proline accumulation in response to salt and mannitol. Compatible solutes are suggested to act as low- molecular-weight chaperones, stabilizing the photosystem II complex, protecting the structure of enzymes and proteins, maintaining membrane integrity and scavenging ROS (Szabados and Savoure, 2010). Recently, it was also shown that some of these compatible solutes are very efficient in reducing the extent of K^+ loss in response to both salinity and oxidative stress in barley and Arabidopsis roots (Cuin and Shabala, 2005; 2008). However, signalling cascades modulating proline accumulation are still poorly characterized in barley. We also found that PA is rapidly and transiently formed

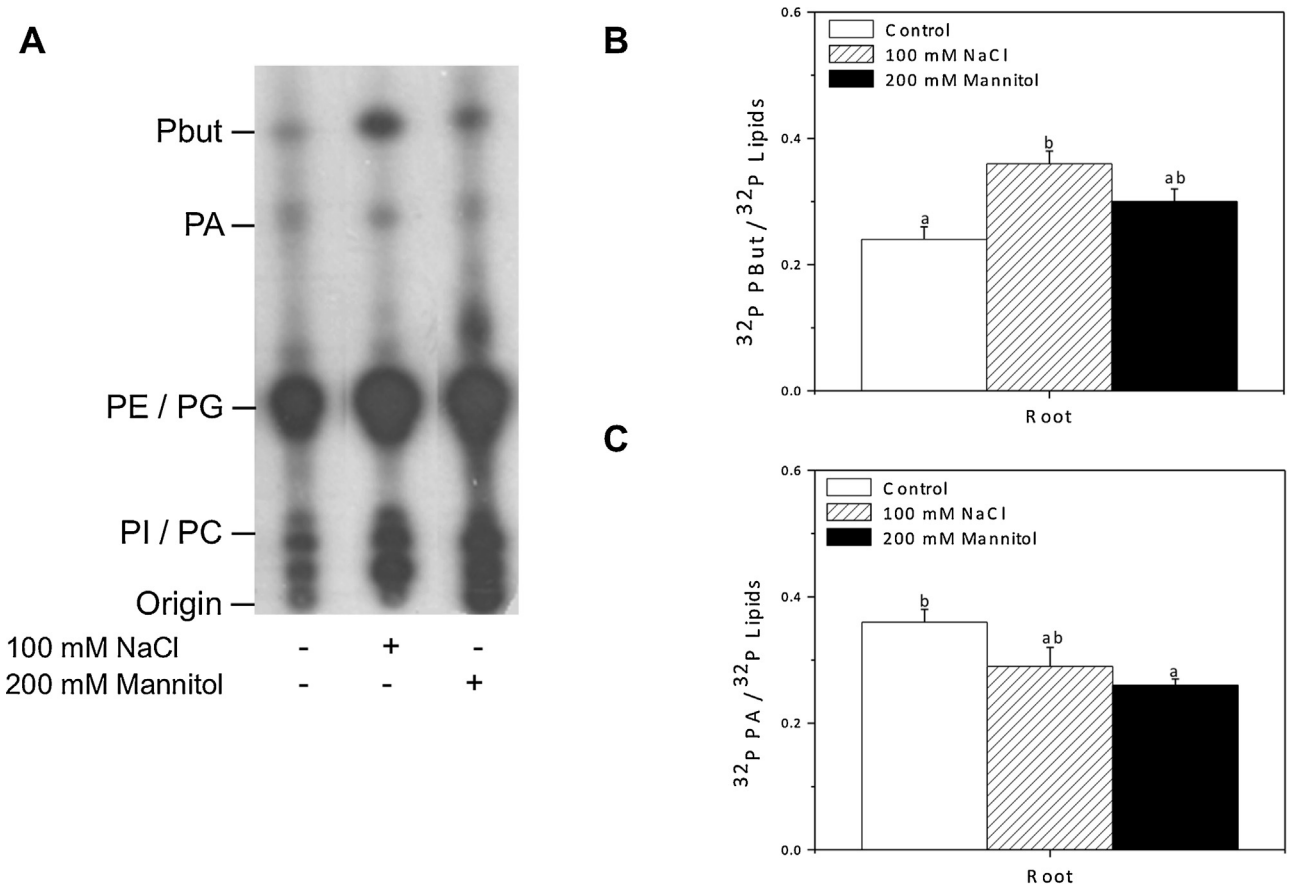

Fig. 6. Effect of saline and osmotic stresses on PLD activity. (A) Representative TLC, with lipids separated with the solvent containing ethyl acetate. Barley roots were incubated in labelling buffer in presence of [^{32}P]Pi overnight, and then treated with 0.5% (v/v) 1-butanol. Lipids were extracted, separated by TLC and detected by autoradiography. Results are expressed as ^{32}P-PtdBut (B) or ^{32}P PA (C) formation percentage over the total ^{32}P-(PC-PtdBut-PA). Results are shown as mean values ± S.D., n = 3, different lowercase letters indicate significance at $P < 0.05$.

during saline and osmotic stresses. PA may be produced by multiple enzymes: (a) PLD, acting hydrolytically on membrane phospho- lipids; (b) DGK, phosphorylating DAG; (c) acyl transferase, adding a fatty acid to lysoPA; and (d) the enzymes of the de novo pathway from glyceraldehyde 3-phosphate. Available data suggest that PLD and DGK are the two principal routes that produce signalling PA. These enzymes are subject to complex and tight regulation. In barley, the two main pathways involved in PA levels during the response to abiotic stress are PLD and DAG-k. Therefore, we evaluated whether inhibiting these enzymes could affect the proline accumulation. Application of 1-butanol stimulated proline increased, indicating a negative correlation between proline and PLD/PA (Thiery et al., 2004). Nevertheless, PA pool triggered by DAG-k activity could be related to the proline synthesis, since the DGK-inhibitors abolished proline accumulation. In barley, cellular PA level is highly dynamic and its production and removal are mediated by several complex families of enzymes (Meringer et al., 2012; Villasuso et al., 2013). PA formed in response to salt stress has been suggested to function as a signalling molecule directing the plant's acclimation responses to salt stress. In Arabidopsis, PA can bind and affect the activity of various signalling proteins, including protein kinases and phosphatases. The protein kinase SnRK2 was found in a proteomic screen for PA targets (Testerink et al., 2004) and it has been shown to be activating in response to salt stress (Boudsocq et al., 2004). In barley, PA binds and modulates the glyceraldehyde 3-phosphate dehydrogenase activity (Astorquiza et al., 2016). However, the specificity in which molecular species of PA transduce saline stress signals remains largely unknown. Here, we suggest that the different PA pools could modulate the ability to sense and response stress condition.

Cardiolipin (CL), a minor structural phospholipid that is predominantly present in mitochondria, was found to decrease in response to salt stress. This fact seems to be associated with the response to saline stress in crops like in rice (Darwish et al., 2009). CL is a key component of both prokaryotic and eukaryotic membranes, with unique structure and functions (Lewis and Mcelhaney, 2009). It is an anionic phospholipid with a dimeric structure and contains a triple glycerol backbone and four acyl groups, most of which are highly unsaturated. In barley and rice, it is still unclear what the decrease in CL in response to salt stress means (Darwish et al., 2009); although we speculate that CL could be a substrate of PLD and produce PA and PG. However, under our condition PG increase was not observed. Considering this, more experiments are necessary to solve this point.

The adaptive response of salt-stressed plants is controlled by chemical signals that compensate adjustment of growth and development in response to such unfavourable conditions. Some of these signals play a dual role; they can act as signals triggering adaptation or they accompany stress-related damage (Hazman et al., 2015). In barley, endogenous ABA concentrations were found to decrease in roots upon 4-d treatment with salt. In contrast, in maize roots, ABA increased in response to saline stress. We consider that in barley root samples, ABA could be released into NaCl solutions (Jia et al., 2002). Conversely, JA only accumulated in response to mannitol stress. Presumably, a decrease in turgor could have generated membrane damage and hence, triggered the release of the lipid precursors for jasmonate synthesis in roots

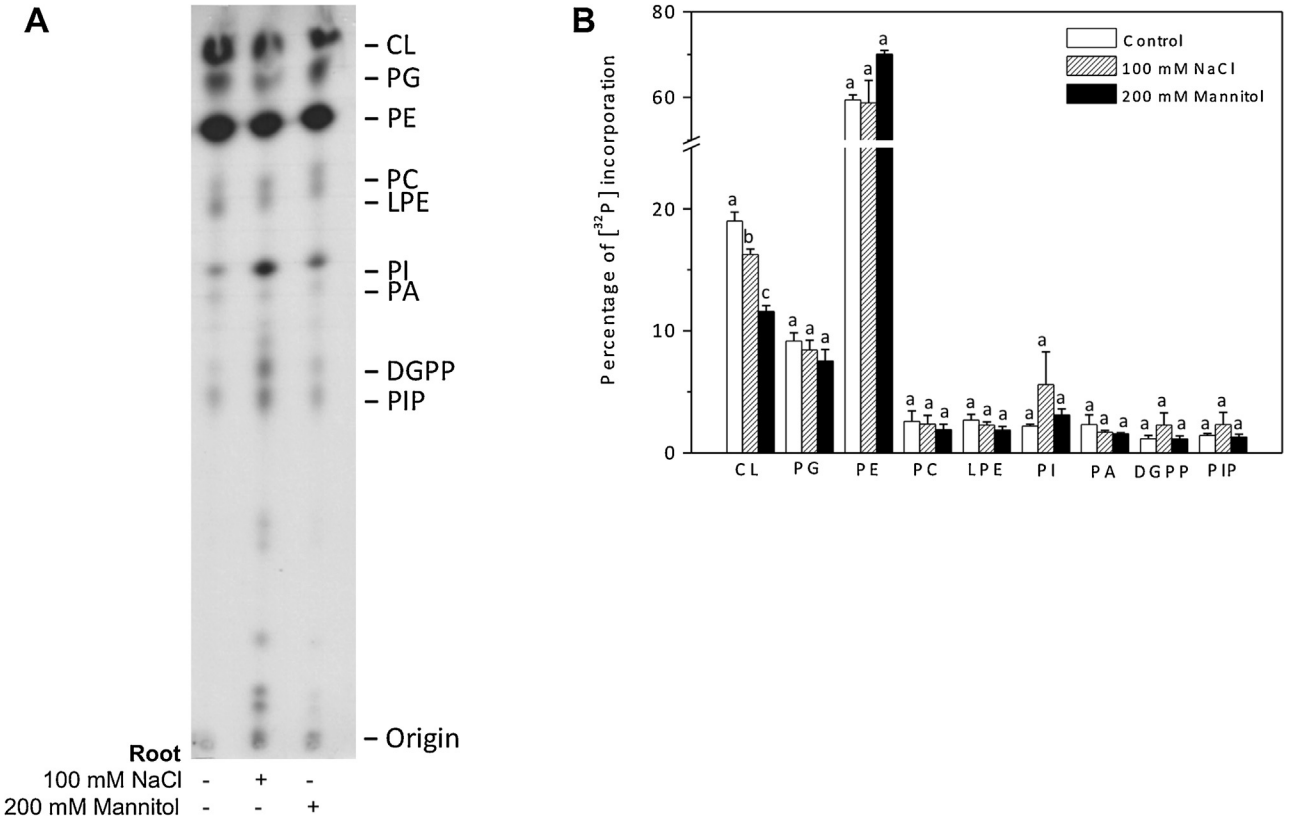

Fig. 7. Effect of saline and osmotic stresses on phospholipid turnover in barley roots. (A) Autoradiography of products from lipids radiolabelled with [^{32}P]Pi. Barley roots were incubated in presence of [^{32}P]Pi, extracted and separated by TLC. (B) Results are expressed as percentage distribution of each lipid class and shown as means ± S.D., n = 3, different lowercase letters indicate significance at $P < 0.05$.

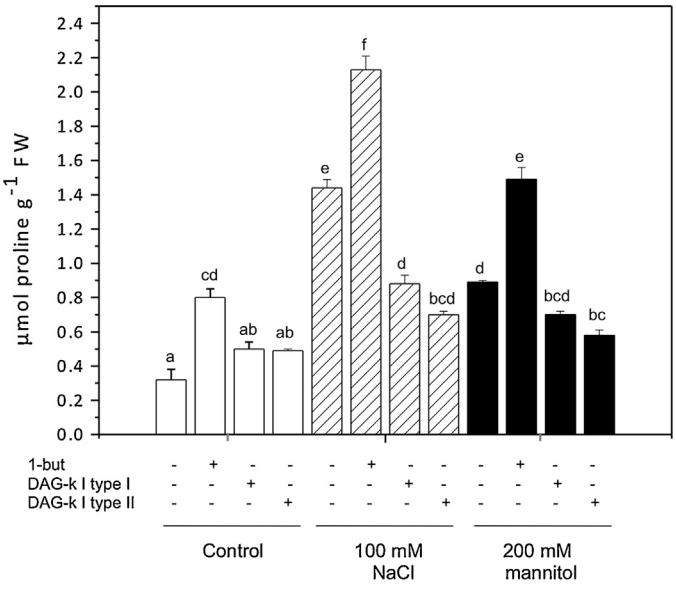

Fig. 8. Effect of 1-butanol and DGK inhibitors (R59022 and R59949) on proline accumulation in barley roots. Seedling were incubated with 0.5% v/v 1-butanol, 100 µM R59022 or 150 µM R59949. The determination of proline concentration was performed as described in materials and methods. Results are expressed in µmol g FW-1 and represent the mean ± S.D., n = 4, different lowercase letters indicate significance at $P < 0.05$.

exposed to salt stress. JA has been reported to accumulate in response to saline stress in tomato (Pedranzani et al., 2003) and rice (Moons et al., 1997). Whether this accumulation is a signal triggering adaptation is not very clear. However, the fact that a salt-tolerant cultivar of rice shows higher endogenous JA contents as compared to a salt-sensitive cultivar indicates a function for JAs in salt adaptation (Kang et al., 2005). Signalling triggered by JA is complex, because this signal has been found to interact with the signalling triggered by other plant hormones known to be involved in the adaptation to salt stress such as ABA. These interactions, often referred to as 'hormonal cross-talk', need more investigations, especially in crops of economic importance (Hazman et al., 2015). Most of our knowledge about stress signalling has been learnt through the study of *Arabidopsis thaliana*, although our understanding about cereals as barley is quite incomplete.

Finally, we hope that the future studies will reveal the role of PA and its relation to others partners in the lipid cascade signalling as part of a mechanism by which barley responds and adapts to salt stress.

Table 1
Endogenous amounts of phytohormones for different roots condition.

Roots	GA_3	ABA	IAA	SA	JA
	Amount(ng/dry weight)				
Control	46	286	18	404	86
100 mM NaCl	35	126	17	396	71
200 mM mannitol	34	111	19	497	382

Levels of GA_3, ABA, IAA, SA, and JA were analyzed simultaneously by LC-ESI–MS–MS essentially as described by Durgbanshi et al. (2005). Results are expressed as ng/dry weight of barley roots; they correspond to a representative experiment performed by triplicate.

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