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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

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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.

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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