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Research article

Lipid signalling mediated by PLD/PA modulates proline and H_2O_2 levels in barley seedlings exposed to short- and long-term chilling stress

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ABSTRACT

Phospholipase D (PLD) hydrolyses phospholipids to yield phosphatidic acid (PA) and a head group, and is involved in responses to a variety of environmental stresses, including chilling and freezing stress. Barley responses to chilling stress (induced by incubating seedlings at 4 °C) are dynamic and the duration of stress, either short (0–180 min) or long-term (24–36 h) had a significant impact on the response. We investigated the roles of PLD/PA in responses of barley (*Hordeum vulgare*) seedlings to short and long-term chilling stress, based on regulation of proline and reactive oxygen species (ROS) levels. Short-term chilling stress caused rapid and transient increases in PLD activity, proline level, and ROS levels in young leaves. PLD has the ability to catalyse the transphosphatidylation reaction leading to formation of phosphatidylalcohol (preferentially, to PA). Pre-treatment of seedlings with 1-butanol significantly increased proline synthesis but decreased ROS (H_2O_2) formation. These observations suggest that PLD is a negative regulator of proline synthesis, whereas PA/PLD promote ROS signals. Exogenous PA pre-treatment reduced the proline synthesis but enhanced H_2O_2 formation. Effects of long-term chilling stress on barley seedlings differed from those of short-term chilling stress. *E.g.*, PLD activity was significantly reduced in young leaves and roots, whereas proline synthesis and ROS signals were increased in roots. Exogenous ROS application enhanced proline level while exogenous proline application reduced ROS level and modulated some effects of long-term chilling stress. Our findings suggest that PLD contributes to signalling pathways in responses to short-term chilling stress in barley seedling, through regulation of the balance between proline and ROS levels. In contrast, reduced PLD activity in the response to long-term chilling stress did not affect proline level. Increased ROS levels may reflect an antioxidant system that is affected by chilling stress and positively compensated by changes in proline level. Implications of our findings are discussed in regard to adaptation strategies of barley seedlings to low temperatures.

1. Introduction

Low temperatures greatly limit plant growth and significantly restrict productivity and spatial distribution of crop plants. In regard to cold temperatures, it is important to distinguish between positive cold temperatures (chilling) and negative cold temperatures (freezing). The response of plants to low-temperature stress is a highly complex process involving multiple levels of regulation (Penfield, 2008; Ruelland et al., 2009). The molecular mechanisms underlying plant responses to chilling stress, including changes in gene expression and in cellular signal transduction, have been analysed extensively, and a variety of transcription factors and signalling molecules have been shown to play important roles in cellular homeostasis under chilling and freezing stress conditions (Ruelland and Zachowski, 2010). Perception of chilling by plants may occur through several mechanisms (Knight and Knight, 2012). Alterations in membrane fluidity may play a role in perception of a temperature drop outside a plant cell,

Abbreviations: ABA, abscisic acid; DAG, diacylglycerol; DGK, diacylglycerol kinase; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D; ROS, reactive oxygen species; TLC, thin layer chromatography.

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leading to elevated intracellular calcium concentration, which is decoded by calcium responsive proteins, with consequent signalling leading to altered expression of cold responsive genes (Ruelland and Zachowski, 2010). Exposure to chilling in certain plant species promotes resistance to freezing stress, a process termed "cold acclimation" (Ruelland et al., 2009). Cold acclimation is a complex phenomenon involving multiple genetic regulatory networks. Transcriptome profiling and screening of mutant strains of *Arabidopsis thaliana* have resulted in characterization of multiple genes involved in initiation of chilling acclimation and freezing tolerance (Vergnolle et al., 2005; Ruelland and Zachowski, 2010; Knight and Knight, 2012). The multigenic CBF/DREB1 family is the most extensively studied family of transcription factors (TFs) responsible for cold hardening and frost tolerance. These TFs are members of the AP2/ERF (APETALA2/ethylene-responsive factor) superfamily, and have the ability to bind to CRT/DREs (C-repeat/dehydration-responsive elements) in the promoters of several cold-induced genes (Skinner et al., 2005). In response to chilling stress, transcription levels of CBF/DREB1 genes increase rapidly and transiently, followed by accumulation of cold-responsive (COR) gene transcripts (Thomashow, 1999). Expression of *CBF* genes is also a crucial factor in strong induction of *COR* genes (*e.g.*, COR14b, DHN5) in barley (*Hordeum vulgare*) (Choi et al., 2002; Dal Bosco et al., 2003). Little is known regarding the lipid-signalling pathway that occurs earlier during expression of CBF genes in barley. Studies using pharmacological approaches have shown that blocking of certain phospholipases reduces expression of chilling-induced CBF genes, suggesting that lipid signalling modulates upstream cascade signalling that leads to gene induction (Vergnolle et al., 2005; Ruelland et al., 2009; Marozsan-Toth et al., 2015).

Phospholipases are enzymes that hydrolyse phospholipids into fatty acids or lipophilic substances. They influence chilling and freezing tolerance through alterations in plasma membrane lipid composition (Ruelland et al., 2009). Several phospholipid-based signalling pathways in plants are rapidly activated during cold stress. These pathways include the phospholipase D (PLD) and phospholipase C coupled with diacylglycerol kinase (PLC/DGK) pathways that result directly or indirectly in production of phosphatidic acid (PA) (Ruelland et al., 2002; Li et al., 2004; Arisz et al., 2009; Delage et al., 2012; Liu et al., 2013). PA comprises a minor class of membrane lipids in which phosphorylglycerol is esterified with two fatty acids chains. PA is a key intermediate in biosynthesis of phospholipids, galactolipids, and triacylglycerols (Athenstaedt and Daum, 1999). A temperature reduction may be perceived as a change in membrane rigidity (Vigh et al., 2007). The content and molecular forms of PA play significant roles in chilling and freezing tolerance. During cold acclimation, significant increases of unsaturated fatty acids are observed in lipid profiles (Welti et al., 2002; Zheng et al., 2016).

Chilling is also associated with accumulation of reactive oxygen species (ROS). Activities of scavenging enzymes are reduced at low temperatures, resulting in the inability of scavenging systems to offset the constant ROS formation associated with mitochondrial and chloroplastic electron transfer reactions (Mittler et al., 2004). Development of cold tolerance and freezing tolerance is correlated with changes in levels of certain metabolites, *e.g.*, accumulation of proline, sugars, and other cryoprotectant molecules (Kaplan et al., 2007). Proline plays multifunctional roles; it can act as a potent nonenzymatic antioxidant (Szabados and Savoure (2010), as a singlet oxygen quencher (Alia et al., 1991) and as a scavenger of hydroxyl radicals (Smirnoff and Cumbes, 1989). Proline accumulated in plant tissues may help prevent ROS-induced oxidative damage (Ben Rejeb et al., 2014; Kishor and Sreenivasulu, 2014). Proline metabolism is involved in regulation of intracellular redox potential, and in storage and transfer of energy and reducing power (Szabados and Savoure, 2010; Sharma et al., 2011; Giberti et al., 2014). The harmful effects and the signalling functions of ROS are well documented; however, the relationship between ROS and proline metabolism is poorly understood.

We found that lipid signalling triggered by PLD plays a key role in short- and long-term chilling stress in barley. A PLD/PA signal appears to be involved in the relationship between ROS signalling and proline metabolism. Our findings suggest that lipid signalling triggered by chilling stress is a part of plant adaptation to low-temperature environments.

2. Materials and methods

2.1. Plant materials, growth conditions, and stress treatment

Barley (*Hordeum vulgare*, cv. Carla INTA) seeds were germinated at 25 °C for 4 days (control) (Meringer et al., 2012). Short-term chilling stress was induced by incubating seedlings at 4 °C for 30, 60, or 180 min. Long-term chilling stress was induced by incubating seedlings at 4 °C for 24 or 36 h. For 1-butanol experiments, seedlings were pre-incubated with 1-butanol (0.5%, v/v) for 1 h at 25 °C and then subjected to short- or long-term chilling stress as above. For proline and H_2O_2 experiments, seeds were germinated in the presence of 20 mM proline or 40 mM H_2O_2 in Petri dishes (10 cm diameter) for 4 days in the dark at 25 °C, and seedlings were subjected to short- or long-term chilling stress as above. Roots and leaves were separated for corresponding experiments. For PA experiments, seedlings were pre-incubated with 50 mM dioleoyl-PA for 20 min (Racagni et al., 2008).

2.2. Plant growth analysis

Seedlings were separated into root and young leaves, and length, fresh weight (FW), and dry weight (DW) of these parts were determined.

2.3. Protein extraction, and determination of in vitro PLD activity

2.3.1. Protein extraction

Total proteins from roots and leaves were extracted as described previously (Astorquiza et al., 2016). Protein content was determined by Bradford method. PLD activity was determined directly from supernatant.

2.3.2. In vitro PLD activity assay

PLD activity was determined by TLC as synthesis of phosphatidylbutanol (NBD-PtdBut) in relation to NBD-PA and NBD-PC levels. NBD-PC (Avanti Polar Lipids) was stored at −80 °C in chloroform (1 mg mL^{-1}), dried prior to use under N_2 stream, resuspended in Hepes (50 mM, pH 7.4), and added to PLD assay mixture as liposome (Ibañez et al., 2016). Fluorescence from lipids (excitation wavelength 460 nm, emission wavelength 534 nm) was measured using a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream, Molecular Imaging) and quantified by the ImageJ software program. PLD activity was determined by formation of NBD-PtdBut. NBD-PtdBut was expressed as percentage of NBD-PtdBut fluorescence, normalised to NBD-PC, and expressed as fold increase relative to time 0 value.

2.4. Proline content analysis

Proline content of roots and leaves was determined as described by Bates et al. (1973).