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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).

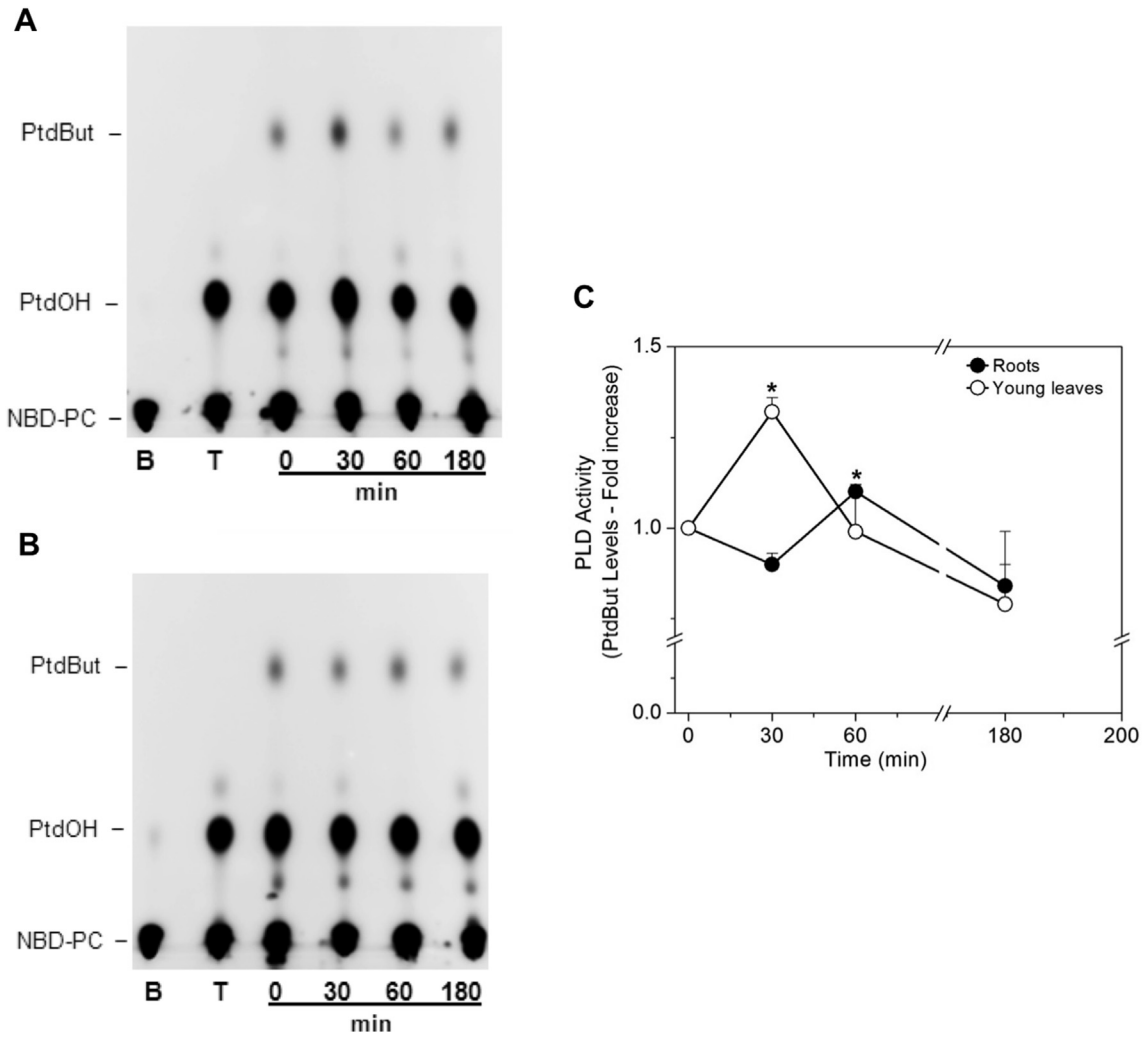

Fig. 1. Effects of short-term chilling stress (4 °C) on PLD activity of barley roots and young leaves. Representative TLC blots are shown for: **(A)** short-term chilling stress (min) in young leaves; **(B)** roots with lipids separated by solvent containing ethyl acetate. B: blank without proteins, T: blank without 1-butanol, 0: control tissue. **(C)** Relative values of PtdBut obtained using image analysis program (ImageJ). Values shown are mean ± S.D. of three or more independent experiments (n = 5). Leaves and roots grown at 25 °C were defined as having 100% activity. Asterisks indicate significance at $p < 0.05$.

2.5. Staining methods and microscopy

ROS levels were determined by staining with 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Invitrogen). Leaf and root tissue samples were placed in DCFH-DA solution (10 μM, pH 7) in the dark for 5 min (Bustos et al., 2008), washed with distilled water to remove excess dye, and examined with an epifluorescence microscope (Axio Lab; Zeiss) with excitation filter 492–495 and emission filter 517–527. Images were taken with a Canon G10 camera.

Cell death was evaluated by the method of Duan et al. (2010), with some modifications. Root and leaf tissue samples were immersed in a 0.25% solution of Trypan Blue in distilled water (w/v), incubated in the dark for 5 min, washed several times with PBS, and visualized by epifluorescence microscopy as above.

2.6. H_2O_2 content

H_2O_2 content was determined as described by Sergiev et al. (1997). In brief, 500 mg tissue was ground by mortar with 2 mL of 100 mM potassium phosphate buffer at 4 °C, homogenate was centrifuged at 12,000×*g* for 15 min at 4 °C, and supernatant was collected. The reaction mixture consisted of 0.5 mL of 0.1% trichloroacetic acid (TCA), 0.5 mL supernatant, 0.5 mL of 100 mM potassium phosphate, and 2 mL reagent (1 M KI [w/v] in distilled water). The blank consisted of 0.1% TCA without tissue extract. The reaction was developed for 1 h in the dark, and absorbance of the solution at 390 nm was measured. H_2O_2 concentrations were calculated using a standard curve. Concentrations of diluted solutions were standardized based on measurement of UV absorption at 240 nm prior to dilution, using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}.

3. Results

The effects of short-term (0–180 min) vs. long-term (24–36 h) chilling stress were analysed. Short-term chilling stress was induced by incubating seedlings at 4 °C for 30, 60, or 180 min. To evaluate lipid signalling triggered by short-term chilling stress in barley seedlings, we focused on the roles of PLD and its product PA, which we previously identified as key components of lipid signalling in response to abiotic stress in this species (Meringer et al.,

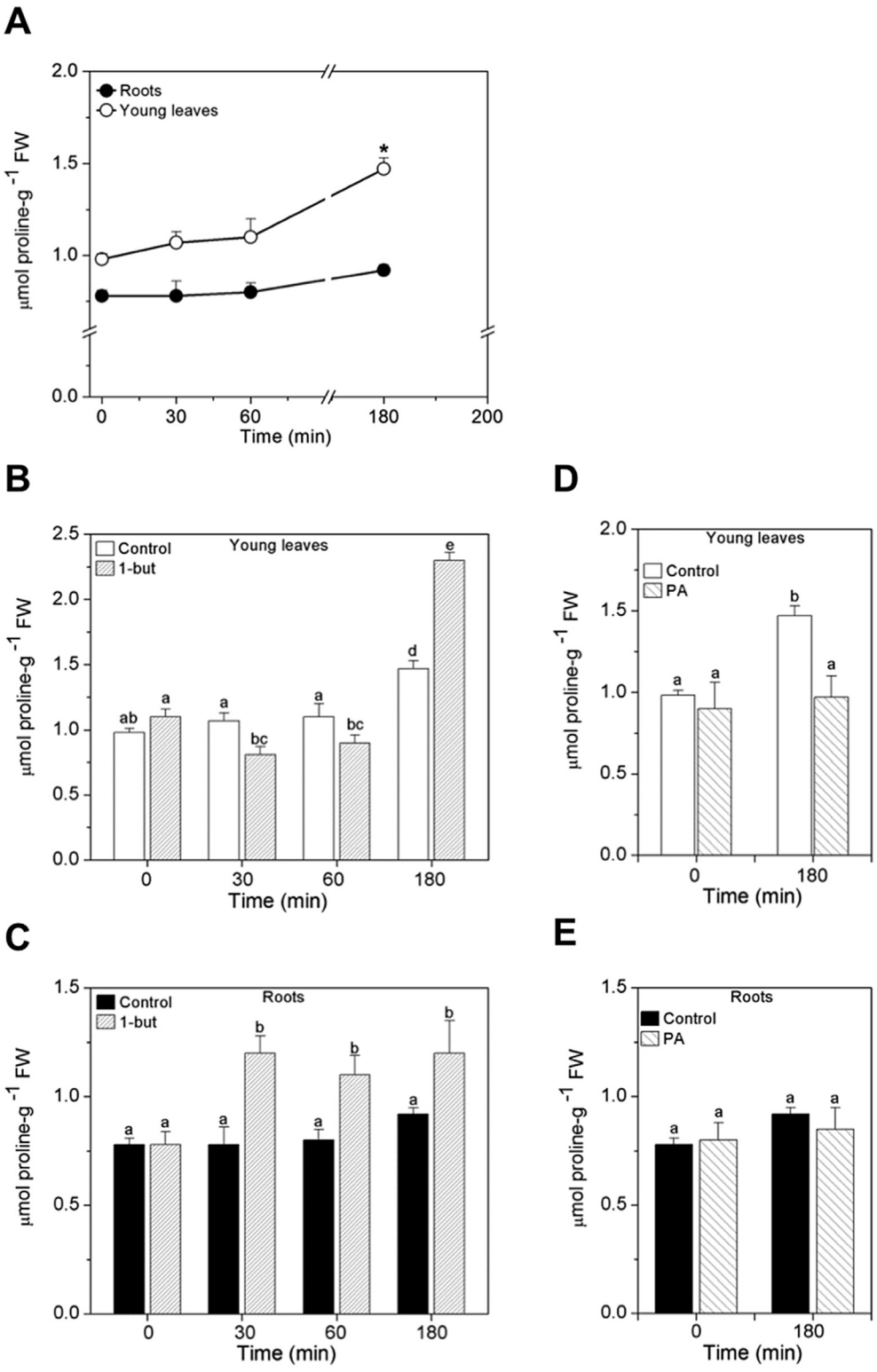

Fig. 2. Proline accumulation as a function of time during root and leaf tissue responses to chilling stress. **(A)** Free proline content in fresh plant material was measured as described by Bates et al. (1973) with some modification. Values shown are mean ± S.D., n = 5. (**B, C**) Effects of 1-butanol on proline accumulation during responses of young leaves (B) and roots (C) to chilling stress. Seedlings were pre-incubated with 1-butanol (0.5%, v/v) for 1 h at 25 °C, and then subjected to short-term chilling stress. Proline concentration was determined as described in M&M. Results are expressed as µmol g FW^{-1}; values shown are mean ± S.D., n = 3. Differing lowercase letters indicate significance at $p < 0.05$. (**D, E**) Effects of PA on proline accumulation during responses of young leaves (D) and roots (E) to chilling stress. Seedlings were pre-incubated with 50 µM C18:1 PA for 3 h at 25 °C, and then subjected to short-term (180 min) chilling stress. Results are expressed as µmol proline g FW^{-1}; values shown are mean ± S.D., n = 3.

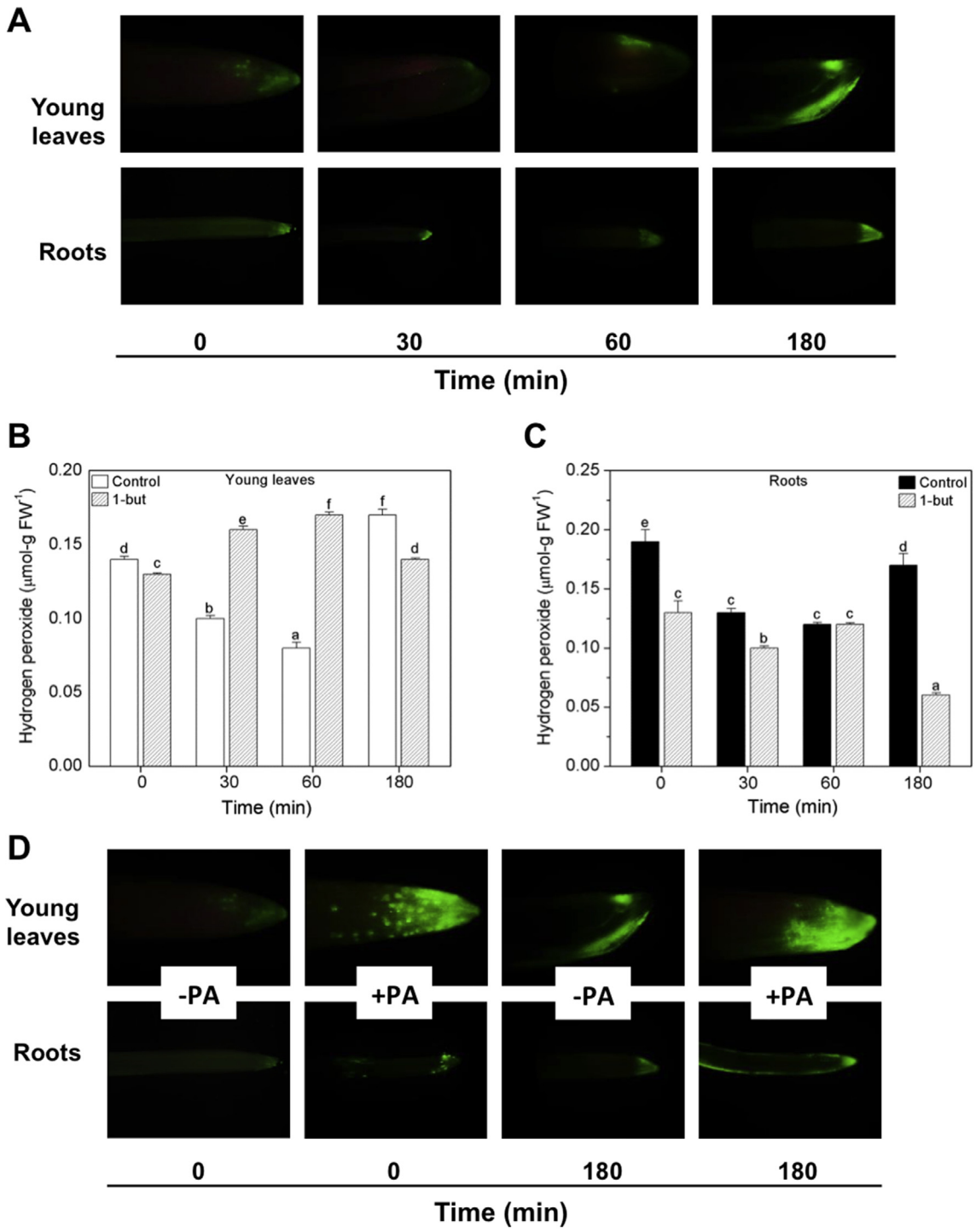

Fig. 3. (A) Qualitative distribution of ROS in leaf and root tissues under short-term chilling stress. Tissues were stained with DCFH-DA for 5 min. Images from epifluorescence microscopy were obtained as described in M&M. (**B, C**) Effects of 1-butanol on H_2O_2 accumulation in (B) young leaves and (C) roots. Seedlings were pre-incubated with 1-butanol (0.5%, v/v) for 1 h at 25 °C, and then subjected to short-term chilling stress. Values shown are mean ± S.D., n = 3. Differing lowercase letters indicate significance at $p < 0.05$. **(D)** Effects of PA on ROS accumulation in young leaves and roots subjected to short-term (180 min) chilling stress. Seedlings were pre-incubated with 50 µM C18:1 PA for 3 h at 25 °C. Tissues were stained with DCFH-DA for 5 min, and images from epifluorescence microscopy were obtained.

2016). Changes of PLD activity in response to chilling stress (4 °C) were assessed by incubating protein extract with fluorescent substrate NBD-PC in the presence of 1-butanol. Phospholipids were extracted, separated by ethyl acetate TLC, and analysed based on fluorescence intensity. The lipid pattern reveals an increase of NBD-PtdBut in response to short-term chilling stress (Fig. 1A and B).

1-butanol was used because PLD can transfer the phosphatidyl moiety from PA to a primary alcohol, and this activity is specific to PLD, thus providing a specific indicator of PLD activity. PLD activity increased 25% during 30 min incubation at 4 °C in leaves (Fig. 1A, C), but was unaffected by the same treatment in roots (Fig. 1B and C).

Free proline is accumulated in many plants in response to a wide

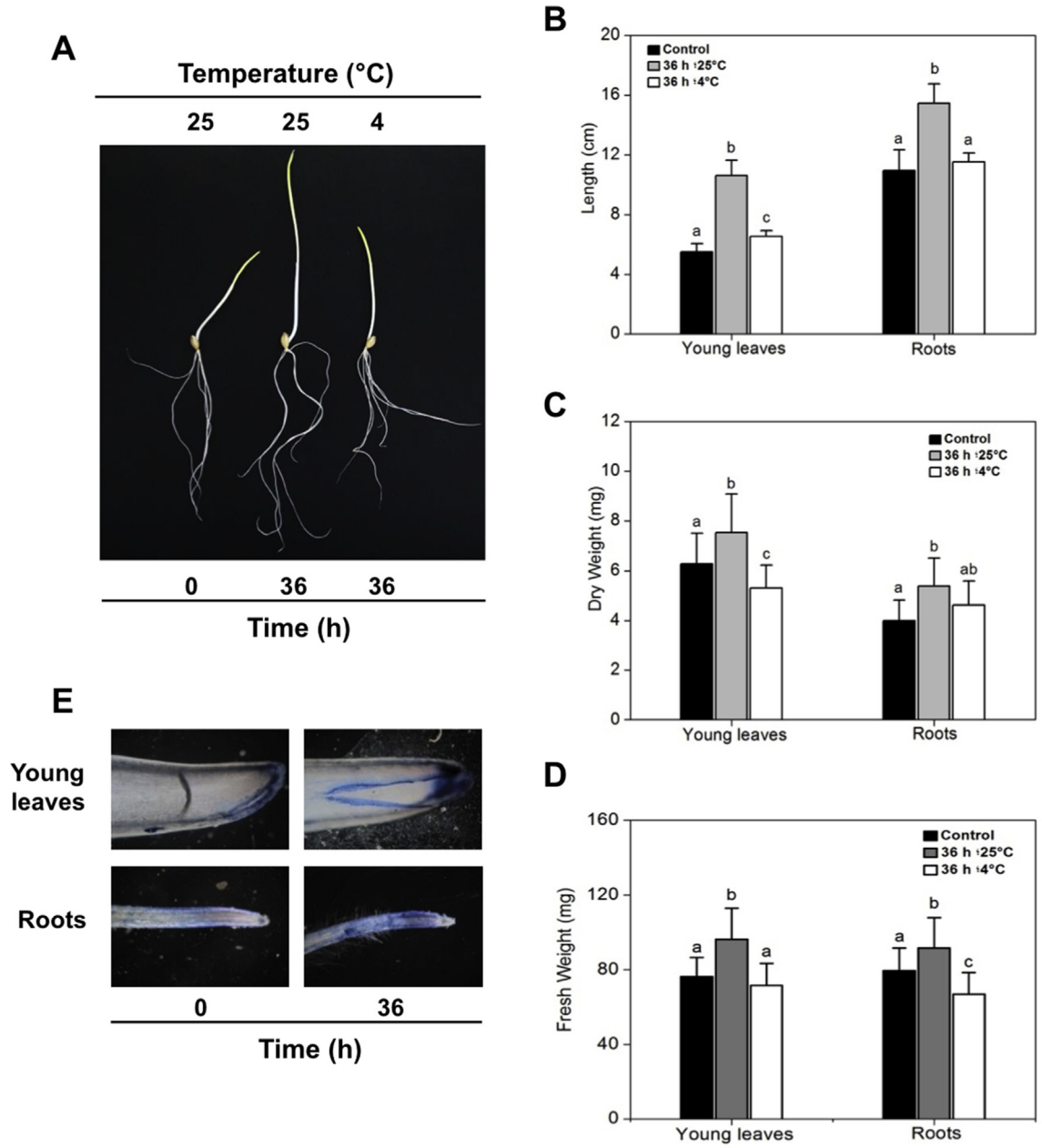

Fig. 4. Effects of long-term chilling stress on barley seedling. **(A)** Photographs of stressed seedlings. (**B, C, D**) Growth responses at 36 h for length (B), DW (C), and FW (D). (E) Images after Trypan Blue staining of young leaves and roots at 0 and 36 h. Values shown are mean ± S.D., n = 20. Differing lowercase letters indicate significance at $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

variety of biotic and abiotic stresses. We measured proline levels in 4-day-old barley seedlings in response to various periods of exposure to low temperature. Untreated control young leaves contained ~1 μmol proline g-1 FW (Fig. 2A). Exposure to 4 °C triggered proline accumulation in young leaves in a time-dependent manner, with maximal level (1.5 μmol proline g-1 FW) at 180 min. Similar results were obtained for roots.

To investigate the role of PLD in proline metabolism, PA produced by PLD activity was reduced by 1-butanol pre-treatment and free proline content was measured (Fig. 2B and C). Following 1-butanol pre-treatment, proline levels of leaves increased in response to short-term chilling stress, with maximal increase (225%) observed at 180 min (Fig. 2B). 1-butanol pre-treatment also increased proline content of roots (Fig. 2C). We also conducted assays with tert-butanol; application of tert-butanol did not notably alter proline level (Table S1). We examined the possibility that PA can modulate this effect of 1-butanol in barley seedlings. Pre-treatment of dioleoyl-PA reduced proline level in leaf tissue (Fig. 2D), suggesting that PA blocks the 1-butanol effect and that PLD negatively regulates proline metabolism. Dioleoyl-PA pre-treatment had no such effect on root tissue (Fig. 2E).

Cold exposure has been reported to increase ROS concentration (Ruelland et al., 2009). To determine whether short-term chilling stress induces ROS increase in barley seedlings, we studied ROS localization using a membrane-permeable fluorescent probe. DCFH-DA staining provides a qualitative estimate of ROS product

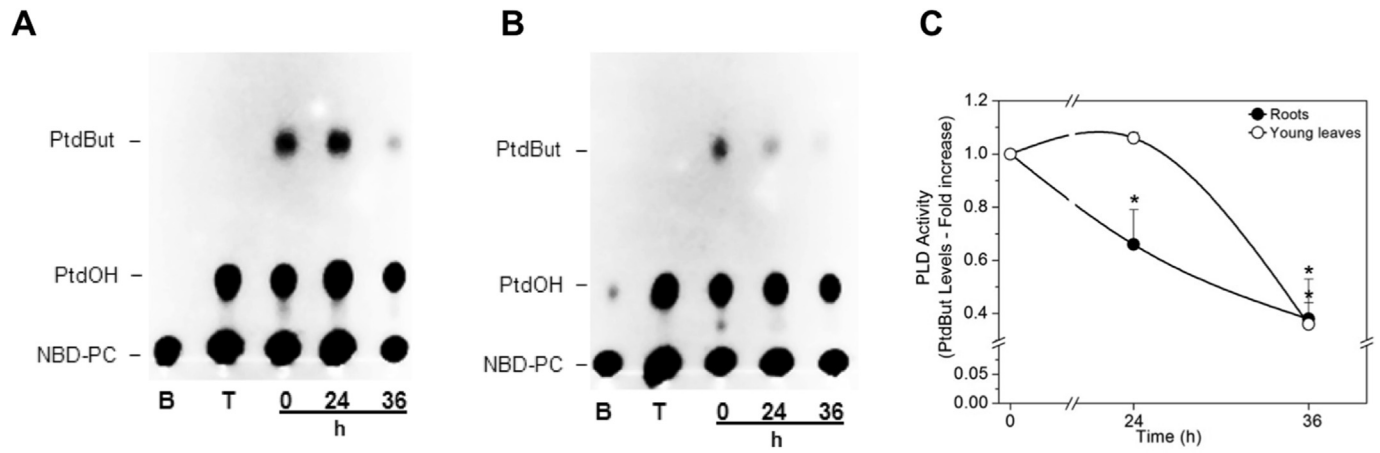

Fig. 5. Effects of long-term chilling stress on *in vitro* PLD activity of roots and young leaves. (**A, B**) Representative TLC blots showing PLD activity under long-term chilling stress in young leaves (A) and roots (B). B: blank without proteins, T: blank without 1-butanol, 0: control tissue. **(C)** Relative values of PtdBut obtained using ImageJ. Values shown are mean ± S.D. of three or more independent experiments (n = 5). Leaves and roots grown at 25 °C were defined as having 100% activity. Asterisks indicate significance at $p < 0.05$.

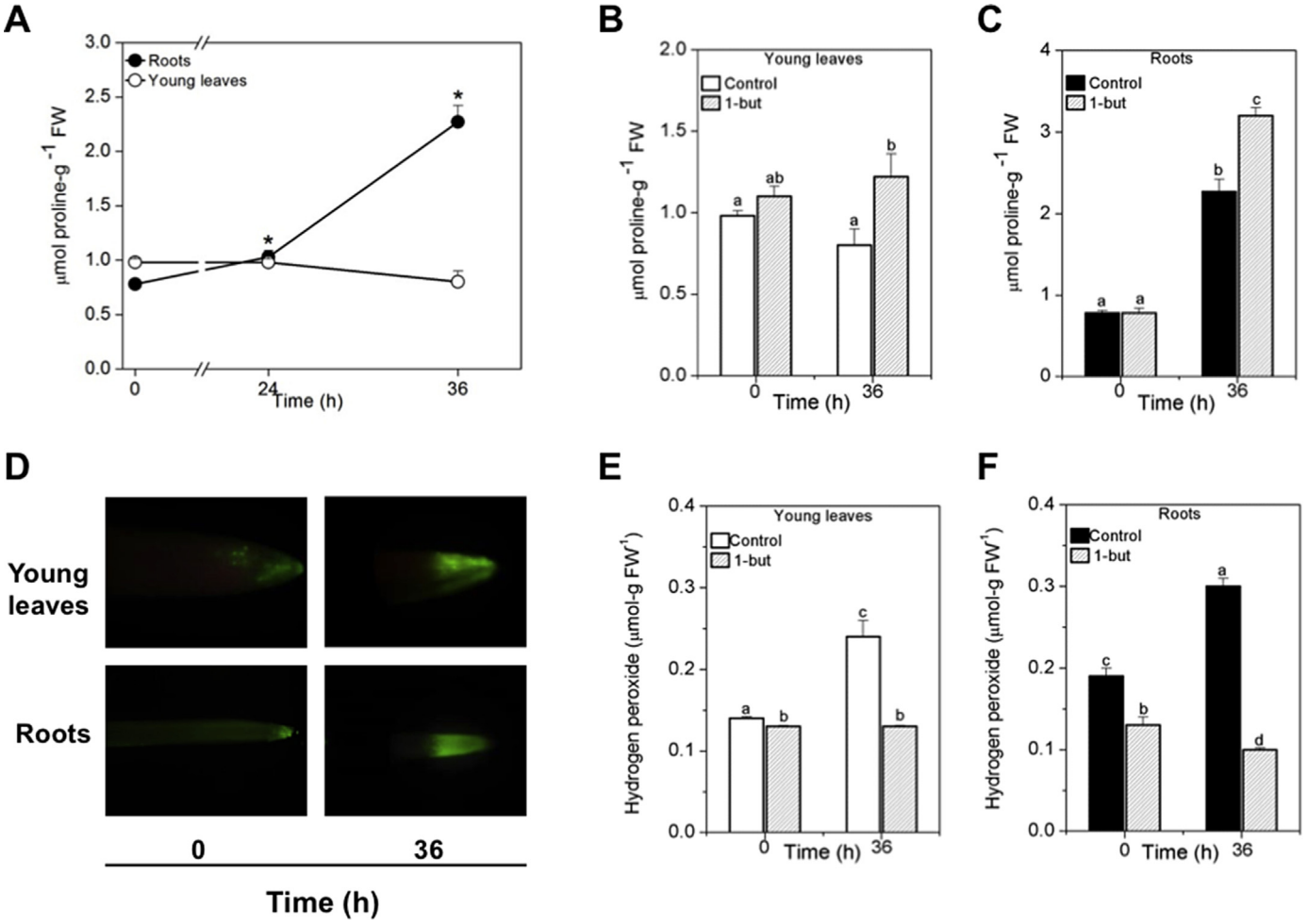

Fig. 6. **(A)** Proline accumulation in young leaves and roots under long-term chilling stress (24–36 h). Free proline content in fresh plant material was determined as described in M&M. Values shown are mean ± S.E., n = 5, *$p < 0.05$. **(B,C)** Effects of 1-butanol on proline accumulation in leaves and roots. Seedlings were pre-incubated with 1-butanol (0.5%, v/v) for 1 h at 25 °C, and then subjected to 36 h at 4 °C. Results are expressed as µmol proline g FW^{-1}; values shown are mean ± S.D., n = 3. Differing lowercase letters indicate significance at $p < 0.05$. **(D)** Qualitative distribution of ROS in young leaves and roots. Images from epifluorescence microscopy were obtained. **(E,F)** Effect of 1-butanol on H_2O_2 accumulation. Values shown are mean ± S.D., n = 3. Differing lowercase letters indicate significance at $p < 0.05$.

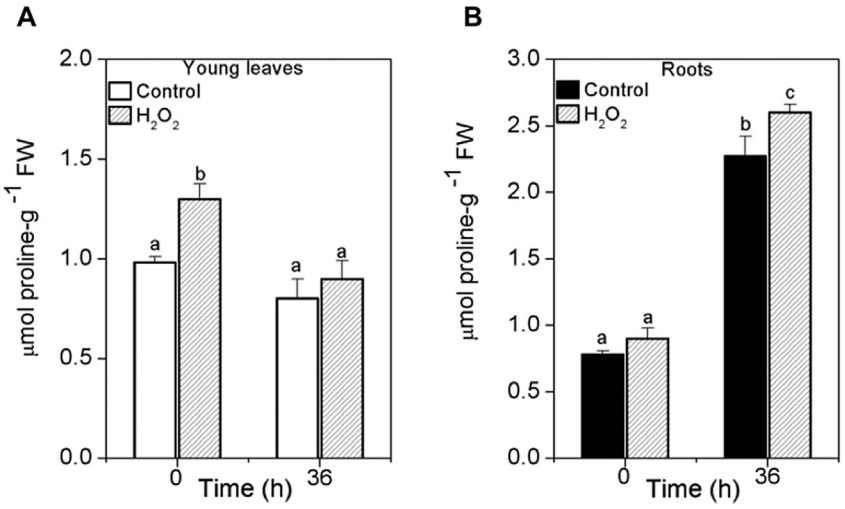

Fig. 7. (A,B) Effect of H_2O_2 on proline accumulation. Seedlings were grown with water (control) or with 40 mM H_2O_2 at 25 °C, and then subjected to 36 h at 4 °C. Results are expressed as µmol proline g FW^{-1}; values shown are mean ± S.D., n = 3.

formation and information on localization. Non-fluorescent DCFH-DA is converted to fluorescent molecules in the presence of ROS. In control roots, ROS were detected in both cell division and elongation areas (Fig. 3A). Short-term chilling stress led to increased ROS levels in both these areas.

H_2O_2 level was quantified by spectrophotometry. In response to short-term chilling stress, H_2O_2 level in leaves was reduced from 30 to 90 min, but slightly increased at 180 min (Fig. 3B). Similar results were obtained in roots (Fig. 3C). 1-butanol pre-treatment caused considerable reduction of H_2O_2 levels in control roots and leaves, but enhanced H_2O_2 levels at 30–60 min of chilling stress. The inhibitory effect of 1-butanol on H_2O_2 levels suggests that PLD/PA contribute to ROS formation. To test this hypothesis, we examined the ability of PA (the product of PLD) to modulate the 1-butanol effect. Dioleoyl-PA pre-treatment enhanced H_2O_2 production in seedlings exposed to short-term (180 min) chilling stress (Fig. 3D).

The duration, severity and rate at which a stress is imposed all influence how a plant responds. The effects of short-term vs. long-term chilling stress were compared. Long-term chilling stress was induced by incubating seedlings at 4 °C for 24 or 36 h. Morphology of seedlings under these two conditions is illustrated in Fig. 4. Exposure to low temperature for 24–36 h inhibited root elongation (Fig. 4A). After 36 h at 4 °C, root length and leaf length were similar to control values (Fig. 4B). Long-term chilling stress had significant effects on plant growth and biomass production, *e.g.*, it caused a ~50% reduction of length, FW, and DW of roots and leaves (Fig. 4C and D). Cell viability was evaluated by Trypan Blue staining. Long-term chilling stress reduced cell viability in cell division areas (Fig. 4E). Long-term chilling stress had a strong effect on PLD activity (Fig. 5A and B), *e.g.*, a 50% reduction at 36 h (Fig. 5C). These observations are indicative of reduced metabolic activity.

Proline levels in response to long-term chilling stress were increased by 150% in roots but unchanged in leaves (Fig. 6A). Proline content of both roots and leaves was significantly increased by pre-treatment with 1-butanol (Fig. 6B and C). Long-term chilling stress also enhanced ROS formation (Fig. 6D), including a 100% increase of H_2O_2 levels (Fig. 6E and F). Such increased ROS levels in roots may play key roles in intracellular communication that promotes acclimation ability and survival under adverse environmental conditions. To evaluate the ability of ROS to mediate proline accumulation during long-term chilling stress, seeds were germinated in the presence of 40 mM H_2O_2 for 4 days at 25 °C, and seedlings were subjected to long-term chilling stress (36 h at 4 °C) and proline level was determined. Proline formation was enhanced by H_2O_2 treatment (Fig. 7A and B). We have performed assays using lower concentrations of H_2O_2 (0.4 and 4 mM). Similar results regarding proline levels were obtained, indicating that responses observed under our conditions were independent of H_2O_2 concentration (Table S2). In view of the complex relationship between proline metabolism and ROS, we also examined the ability of proline to modulate ROS level. Seed germinated with 20 mM proline for 4 days at 25 °C, and seedlings were subjected to long-term chilling stress (36 h at 4 °C) improved growth of both control (36 h at 25 °C) and cold-stressed seedlings (Fig. 8A), and greatly reduced H_2O_2 level (Fig. 8B and C).

4. Discussion

We conducted biochemical analyses of barley seedlings subjected to short-term (0–180 min) and long-term (24–36 h) chilling stress. Previous studies based on transcriptome profiling and screening of mutant strains have helped characterize the signalling pathways involved in responses to chilling and freezing (Knight and Knight, 2012; Zheng et al., 2016). The "model higher plant" *A. thaliana* has been extensively studied (Wang et al., 2006). Far less is known regarding cold temperature response processes in winter cereals such as barley. In *A. thaliana* seedlings, PA formation clearly plays a role in regulation of the acclimation process in response to low temperatures (Ruelland et al., 2002; Gomez-Merino et al., 2004; Li et al., 2004; Vergnolle et al., 2005; Rajashekar et al., 2006). Our findings indicate that short-term chilling stress in barley triggers a rapid and transient accumulation of PA. Cellular PA levels are dynamic, and production and metabolism of PA are catalysed by diverse, complex families of enzymes. Our previous studies indicate that PA signalling involves the hydrolytic action of PLD on membrane phospholipids and DGK, during phosphorylation of DAG (Racagni et al., 2008; Villasuso et al., 2013). Transphosphatidylation assays based on formation of phosphatidylbutanol also suggest the involvement of PLD activity. In contrast, our assays indicate that short-term chilling stress does not stimulate DGK activity (Peppino Margutti, unpubl. data). Thus, PLD appears to be the principal agent for triggering of PA signalling in response to chilling stress. Our

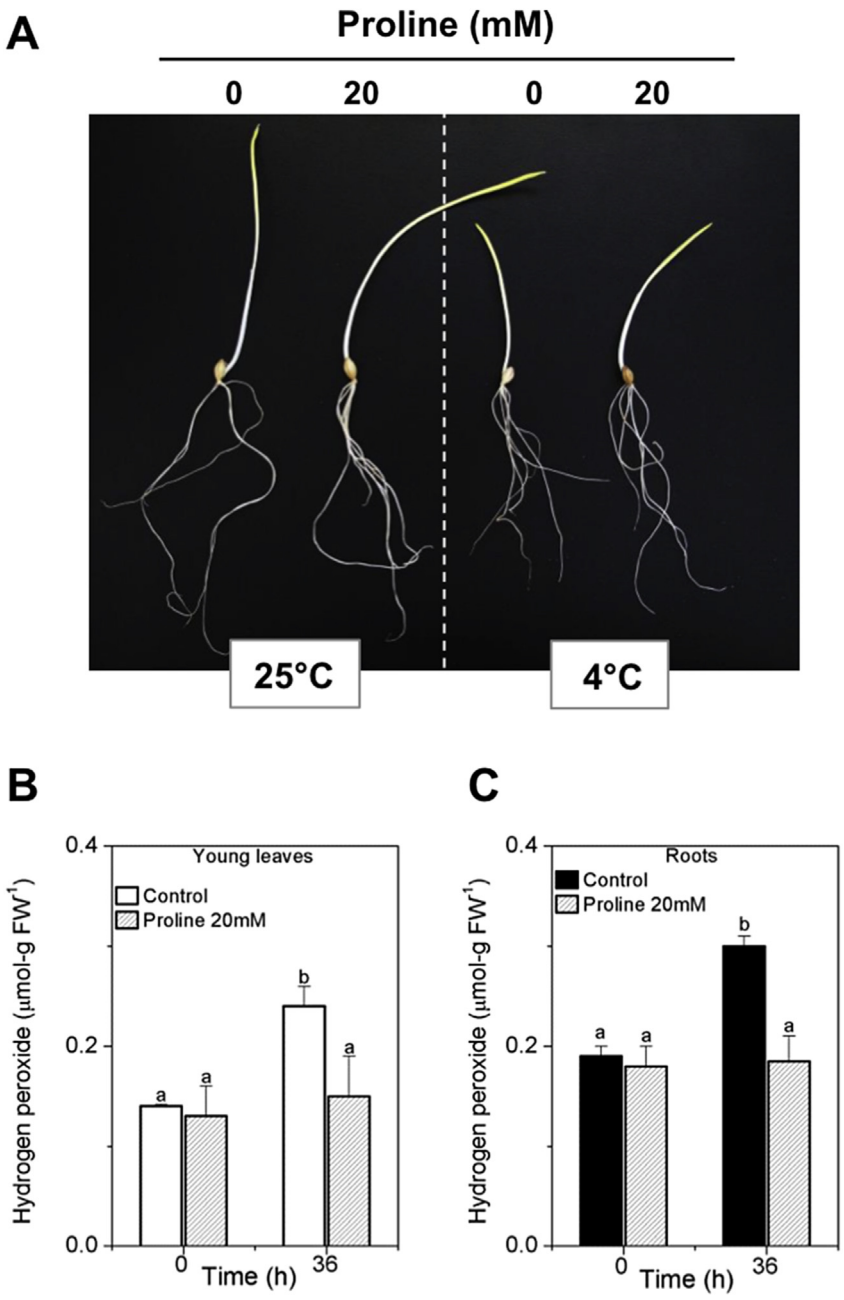

Fig. 8. Effects of exogenous proline on seedlings grown at 25 °C and 4 °C for 36 h. **(A)** Images of seedlings grown with water (control) or with 20 mM proline. **(B,C)** Accumulation of H_2O_2 in young leaves and roots. Values shown are mean ± S.D., n = 3, *p < 0.05.

findings support a role of PLD in the chilling transduction pathways that function upstream of gene expression and involve PA as signalling molecule. PLD-generated PA has been proposed to act by binding effector proteins and recruiting them to a membrane, thereby regulating activity of proteins in cellular pathways (Testerink and Munnik, 2011; Astorquiza et al., 2016).

Low temperature stress induces various responses that lead to cold tolerance and/or freezing tolerance. These responses include proline accumulation (Szabados and Savoure, 2010). In the present study, proline level oscillated during cold exposure. In young leaves, maximal proline level (50% higher than baseline) was observed at 180 min. Proline accumulation in response to stress results from both activation of biosynthesis and inhibition of degradation. During osmotic stress, proline is synthesized primarily from glutamate; the bifunctional enzyme pyrroline-5-carboxylate synthetase (P5CS) reduces glutamate to glutamyl-5-semialdehyde (GSA), which is spontaneously converted to pyrroline-5-carboxylate (P5C). P5C is then reduced to proline by P5C reductase (P5CR). Degradation of proline occurs in mitochondria through sequential action of proline dehydrogenase (ProDH) and P5C dehydrogenase.

Under normal growth conditions, PLD functions as a negative regulator of proline biosynthesis in *Arabidopsis* (Thiery et al., 2004). When this regulator is eliminated, plants display increased proline sensitivity to hyperosmotic stress. Proline synthesis in barley increased in response to water stress, abscisic acid (ABA) level, and

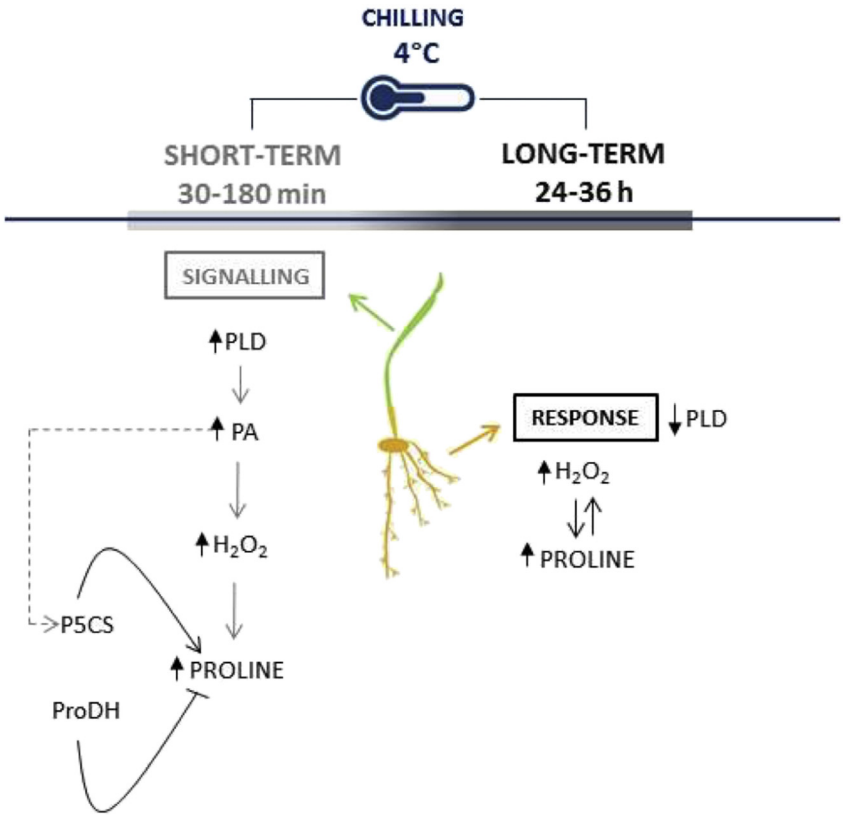

Fig. 9. Proposed role (schematic) of PLD/PA in regulation of proline and ROS levels during the chilling signal transduction process in barley seedlings. Solid-headed arrows: events during responses of seedlings to short- and long-term chilling stress.

salinity (Ueda et al., 2007). Barley also uses variations in ABA and compatible solutes as protective mechanisms associated with cold resistance (Murelli et al., 1995; Bravo et al., 1998). When we uncoupled PA signalling from PLD activity by 1-butanol pre-treatment, proline level was increased, and was then reduced by pre-treatment with exogenous PA. As mentioned above, proline accumulation results from simultaneous increase of biosynthesis and decrease of degradation. More specifically, such accumulation may result from increased P5CS transcription level and/or increased P5CS activity and/or inhibition of proline degradation by ProDH. The first possibility is supported by our preliminary findings (unpubl.) that P5CS activity increases in response to chilling. Regulation of proline metabolism in barley is poorly understood, and the metabolic role of P5CS remains to be elucidated.

Effects of long-term chilling stress in barley differed from those of short-term chilling stress. Under long-term chilling stress, *in vitro* PLD activity was significantly reduced in seedlings (we can not discard changes *in vivo* PLD activity), whereas proline and ROS synthesis were enhanced in roots. Plants continuously synthesize ROS as a by-product of various metabolic pathways (Ben Rejeb et al., 2014). Excessive levels of ROS result in oxidative damage. Cold stress triggers an oxidative burst, characterized by increased production of superoxide ion, H_2O_2, and free radicals (Hussain et al., 2016). Notwithstanding their adverse effects on cell metabolism, ROS play key roles in intracellular communication that promotes acclimation ability in plants (Gilroy et al., 2014). In maize seedling, H_2O_2 treatment stimulated proline synthesis during long-term chilling stress Yang et al., (2009). In rice seedling leaves, H_2O_2 treatment increased P5CS expression (Uchida et al. (2002). In co-leoptiles and roots of maize seedlings, H_2O_2 treatment led to significant proline accumulation through increased P5CS activity and reduced ProDH activity (Yang et al., 2009). Proline metabolism has a direct connection to redox balance, suggesting that proline functions as a redox shuttle (Sharma et al., 2011; Giberti et al., 2014). Enhancement of proline synthesis under stress conditions may be a mechanism whereby redox potential is maintained at values suitable for normal metabolism (Szabados and Savoure, 2010). The ability of proline to counteract damage by ROS could explain its role as a component of an antioxidative network involved in mitigating the effects of stress-induced oxidative damage. We observed that exogenous proline addition reduced ROS level and reversed some effects of long-term chilling stress. Proline may act as a source of nitrogen and carbon, thereby enhancing growth and regeneration of barley seedlings exposed to chilling stress (Szabados and Savoure, 2010).

In view of the present observations, we propose a role of PLD/PA in regulation of proline and ROS levels during the chilling signal transduction process in barley seedlings as illustrated schematically in Fig. 9. This scheme provides a conceptual basis for further functional studies of barley seedling responses to chilling stress. Cellular regulation of proline metabolism, and the molecular targets of PA, remain to be identified. This knowledge will help clarify the roles of proline and PA signalling in responses of barley to low temperatures.

Author contributions

Conceived and designed the experiments: MPM ALV. Performed the experiments: MPM MR. Analysed the data: MPM MR ALV. Result discussion ALV MVM GER. Wrote the paper: ALV.

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.plaphy.2017.02.008.

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