Plant Physiology and Biochemistry 132 (2018) 174–182

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Differential phosphatidic acid metabolism in barley leaves and roots induced by chilling temperature

Micaela Peppino Margutti[a,1], Virginia L. Gaveglio[b,c,1], Mercedes Reyna[a], Susana J. Pasquaré[b,c], Graciela E. Racagni[a], Ana Laura Villasuso[a,*]

[a] Dpto. de Biología Molecular, FCEFQN, Universidad Nacional de Río Cuarto, X5804BYA, Río Cuarto, Córdoba, Argentina
[b] Instituto de Investigaciones Bioquímicas de Bahía Blanca INIBIBB, UNS-CONICET, Edificio E1, Camino La Carrindanga Km 7, 8000, Bahía Blanca, Argentina
[c] Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional del Sur, San Juan 670, 8000, Bahía Blanca, Argentina

ARTICLE INFO

Keywords:
Barley
Chilling temperature
Lipid phosphate phosphatases
Monoacylglycerol lipase
Phosphatidic acid

ABSTRACT

Phosphatidic acid (PA) is an important bioactive lipid that mediates chilling responses in barley. Modifications in the lipid composition of cellular membranes during chilling are essential to maintain their integrity and fluidity. First, we investigated the molecular species of PA present in leaves and roots by ESI-MS/MS, to evaluate the modifications that occur in response to chilling. We demonstrated that PA pools in leaves differ from PA fatty acid composition in roots. Compared with plants grown at 25 °C, the short-term and long-term chilling for 3 h and 36 h at 4 °C not produced significant changes in PA molecular species. The endogenous DAG and PA phosphorylation by *in vitro* DAG and PA kinase activities showed higher activity in leaves compared with that in root, and they showed contrasting responses to chilling. Similarly, PA removal by phosphatidate phosphohydrolase was tested, showing that this activity was specifically increased in response to chilling in roots. The findings presented here may be helpful to understand how the PA signal is modulated between tissues, and may serve to highlight the importance of knowing the basal PA pools in different plant organs.

1. Introduction

Plants are continuously confronted with a great diversity of chemical and physical signals during their life cycle. To cope with these variable environmental conditions, they have developed sensing and signalling mechanisms (Ruelland et al., 2015; Shabala, 2017). Chilling is a physical change that takes place when the plant is exposed to low, non-freezing temperatures. According to their relative chilling sensitivity, plants are classified as resistant, sensitive and extremely sensitive (Kratsch and Wise, 2000). Barley (*Hordeum vulgare*) is a chilling-resistant crop species with considerable economic importance in central-eastern Argentina (García et al., 2018). It has spring and winter crop growth habits, with the latter requiring a period of cold-chilling to induce flowering. This fact reflects the tolerance of barley to different environmental conditions (Gürel et al., 2016). Chilling-resistant plants have also developed diverse strategies such as transcriptome reprogramming and biochemical membrane remodelling to survive chilling and freezing stress (Ruelland and Zachowski, 2010; Shabala, 2017). Modifications in the lipid composition of cellular membranes during chilling are essential to maintain their integrity and fluidity (Li et al., 2004; Ruelland and Zachowski, 2010; Zheng et al., 2016). In general, and under drought conditions, the remodelling of membrane lipid composition comprises two phenomena. The first one involves changes in the proportions of the polar lipid head group (Li et al., 2014), while the second includes changes in the saturation level and in the chain length of fatty acids forming glycerolipid non-polar tails (Yu and Li, 2014).

Phosphatidic acid (PA) is a minor phospholipid but an important signal lipid (Liu et al., 2013; Hong et al., 2016). It is also known as a key precursor for the synthesis of major glycerophospholipids and galactolipids (Dubots et al., 2012). PA is structurally the simplest type of glycerophospholipids, although it comprises a number of molecular

Abbreviations: DAG, diacylglycerol; DAGL, diacylglycerol lipase; DAGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; FAs, fatty acids; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; NEM, N-ethylmaleimide; PA, phosphatidic acid; PAK, phosphatidate kinase; PAP1, NEM-sensitive Mg^{2+}-dependent phosphatidate phosphohydrolase; PAP2, NEM-insensitive Mg^{2+}-independent phosphatidate phosphohydrolase; PC, phosphatidylcholine; PLC, phospholipase C; PLD, phospholipase D; TLC, thin layer chromatography; WSP, water soluble products

* Corresponding author.
E-mail address: lvillasuso@exa.unrc.edu.ar (A.L. Villasuso).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.plaphy.2018.08.038
Received 12 July 2018; Received in revised form 28 August 2018; Accepted 28 August 2018
Available online 01 September 2018

species, which differ in acyl chain length, number of acyl double bonds, and acyl linkage at the sn-1 and sn-2 positions (Testerink and Munnik, 2011). PA levels increase fast and transiently within minutes in response to chilling (Peppino Margutti et al., 2017). Families of multiple enzymes that regulate the timing, location, amount, and molecular species of PA mediate the metabolism of signalling PA (Arisz et al., 2009). Available data suggest that phospholipase D (PLD), which acts hydrolytically on membrane phospholipids, and diacylglycerol kinase (DAGK), which phosphorylates diacylglycerol (DAG), are the two main routes that produce signalling PA (Testerink and Munnik, 2011). On the other hand, enzymes that metabolize PA play an important role in switching off the PA signal. It has been shown that it can be carried out by several enzymes: (1) phosphatidate phosphohydrolase type 2 (PAP2) that dephosphorylates PA to produce DAG, (2) PA kinase that phosphorylates PA to form DGPP, and (3) PA-selective A type phospholipases (PLA) that deacylate PA to produce LPA and free fatty acids (FFAs). In addition to attenuating PA function, the activity of these enzymes can also generate new lipid signals, such as DAG, DGPP, FFAs and LPA (Testerink and Munnik, 2011; Liu et al., 2013). PA mediates cellular processes through several modes of action. These include binding to its targeted proteins to modulate their activity and modifying the biophysical properties of the membrane (Villasuso et al., 2014; Astorquiza et al., 2016; Pokotylo et al., 2018). PA acts as a signalling lipid in the plant's response to the cold. However, the accumulation of PA can affect membrane integrity (Ruelland and Zachowski, 2010). Several studies show that in model plants such as *Arabidopsis*, PLD and PA play important and complex roles in the chilling and freezing response. The cellular functions of two PLDs, PLDα and PLDδ, have been implicated in the freezing induced hydrolysis of phospholipids. PLDα1-deficient *Arabidopsis* plants displayed less phospholipid degradation and greater freezing tolerance than wild-type plants, suggesting a hydrolytic role for PLDα1 under severe stress conditions (Welti et al., 2002). By contrast, a knockout of PLDδ renders *Arabidopsis* plants more sensitive to freezing. This opposite behaviour is likely evoked by PLDα-triggered ROS production and PLDδ-mediated ROS reduction (Zhang et al., 2009). Some biophysical evidence suggests that polar lipids such as PA, which lack a head group, can form a destabilized hexagonal II-type phase and thus have a negative effect on membrane stability during freezing (Kooijman and Burger, 2009; Testerink and Munnik, 2011). By contrast, the metabolism of PA toward DAG or DGPP formation may prevent or avoid membrane destabilization (Arisz et al., 2018; Pokotylo et al., 2018). Although some mechanisms by which PA mediates specific processes in the response of barley to chilling are well known, our understanding of PA metabolism and remodelling is still fragmentary. Thus, the aim of this study was to investigate molecular species of PA present in leaves and roots and to evaluate the modifications that occur in response to chilling. We demonstrated that PA pools in leaves differ from PA fatty acid composition in roots. We also showed that short- and long-term chilling modulated enzymatic activities in barley tissues.

2. Methods

2.1. Materials

[2-^{3}H]glycerol (0.8 Ci/mmol) was obtained from Perkin Elmer (Boston, MA, USA) and pre-blended dry fluor 2a70 (98% PPO and 2% bis-MSB) was obtain from Research Products International Corp. (Mount Prospect, IL, USA). The [^{32}P]γ-ATP (370 Mbq ml^{1}) was obtained from Amersham Pharmacia Biotech (UK). Solvents and TLC plates were purchased from Merck (Darmstadt, Germany). The fluorescence-labelled phospholipid was obtained from Avanti Polar Lipids. Other chemicals were from Sigma.

2.2. Plant materials, growth conditions, and stress treatment

Barley (*Hordeum vulgare*, cv. scarlett) seeds were surface sterilized, and soaked in sterilized water for 4 days in the dark at 25 °C (control). Seedlings were obtained from whole grains, surface sterilized, grown in a growth chamber on disks of filter paper moistened with sterilized water, in Petri dishes (10-cm diameter), for 4 days in the dark at 25 °C, and then harvested. Chilling stress was induced by incubating seedlings at 4 °C for 3 and 36 h (Peppino Margutti et al., 2017). Roots and leaves were separated and placed in liquid nitrogen for corresponding experiments.

2.3. Lipid extraction and ESI-MS/MS analysis

After stress treatment, samples were immediately immersed in liquid nitrogen and used for ESI-MS/MS lipid profiling. Lipid extraction was performed as described by Welti et al. (2002). For each sample, 3 roots or leaves (approximately 200 mg) were immersed in 3 ml of isopropanol (0.01% w/v 2,6-Di-tert-butyl-4-methylphenol) at 75 °C for 15 min. These conditions led to the inactivation of lipid-modifying or -degrading enzymes (Munnik and Heilmann, 2013). Then, 1.5 ml of chloroform and 0.6 ml of distilled water were added. The tubes were shaken for 1 h at room temperature, and then the extract was removed. The tissues were re-extracted with chloroform/methanol (2:1) with 0.01% 2,6-Di-tert-butyl-4-methylphenol five times, with 30 min of agitation each time, until all of the remaining plant tissue appeared white. The combined extracts were washed once with 1 ml of 1 M KCl and once with 2 ml of water. The organic phase was recovered, and the solvent was evaporated under a nitrogen stream. The lipid extracts were sealed in a glass tube with a Teflon tape screw cap and stored at −20 °C until the analysis. The remaining plant tissues were heated overnight at 105 °C and weighed. These weights are described as plant “dry weight”. Lipid samples were analysed on a triple quadrupole MS/MS equipped for ESI. The lipid analyses described here were performed at the Kansas Lipidomics Research Center Analytical laboratory. Data processing was performed as previously described by Li et al. (2008). The lipids in each class were quantified in comparison to two internal class standards. Three replicates of each treatment for each tissue were analysed. The quantification, expressed as nmol mg^{-1} dry wt, from all three replicates was averaged and used to calculate the standard deviations. The Q-test was performed on the total amount of lipid in each head group class, and data from discordant samples were removed (Li et al., 2008).

2.4. Enzymatic assays

2.4.1. Preparation of membranes

The prepared controls and stressed tissues were thawed and homogenized in 10 vol 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; Meringer et al., 2012).

2.4.2. DAG and PA kinase assays

DAG and PA kinase lipid kinases were simultaneously assayed using endogenous lipids as substrates, unless otherwise stated. This method involve monitoring the appearance of labelled lipids as ^{32}P-PA or ^{32}P-DGPP after phosphotransfer from [γ-^{32}P]ATP to DAG or PA, respectively. The membrane fraction isolated (60 μg protein) as above was added to a thermally equilibrated (30 °C) 50 mM HEPES buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM 1,4-Dithioerythritol, 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 (Racagni et al., 2008). 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).

2.4.3. PA and DGPP extraction and separation

Lipids were extracted from membranes following Racagni et al. (2008). Phospholipids were separated by TLC. Samples were spotted on silica gel plates presoaked with 1% of potassium oxalate solution and heated at 110 °C for 60 min just before use. Plates were developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v) for the first dimension, and chloroform/pyridine/formic acid (35:30:7, v/v) for the second dimension. The position of radiolabeled lipids was determined by autoradiography on NEM™ (Life Science Products) film. Spots were scraped off the plates, and fractions were counted in a scintillation counter.

2.4.4. Preparation of radiolabeled PA and phosphatidate phosphohydrolase type 2 activity assays

Radioactive PA was obtained from [2-^{3}H]glycerol-PC ([^{3}H]PC), which was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described (Gaveglio et al., 2011). [^{3}H]PA specific activity was calculated by measuring the phosphorous and radioactivity of an aliquot of the substrate (Gaveglio et al., 2011). PAP activities are differentiated according to NEM-sensitivity. This thiol-reactive compound inhibits PAP1 activity but does not affect PAP2 activity. To determine NEM-insensitive 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 50 µg of membrane protein in a volume of 0.1 mL. The reaction was started by addition of 0.1 mM [2-^{3}H]phosphatidate, continued for 20 min at 37 °C, and stopped by addition of chloroform/methanol (2:1, v/v). DAG (the product of PAP_2 activity) was separated by gradient-thickness thin-layer chromatography on silica gel G and developed with hexane:diethyl ether:acetic acid (35:65:1, v/v). In this system, solvent DAG migrates to the three fourths of the plate and PA and MAG stay in the origin. To separate MAG from PA, the chromatogram was rechromatographed up to the middle of the plate by using hexane:diethyl ether:acetic acid (20:80:2.3, v/v) as developing solvent. Lipids were measured by scintillation after the addition of 0.5 ml water and 10 ml of 5% pre-blended dry fluor 2a70 toluene/detergent (4:1, v/v) (Gaveglio et al., 2016). PAP2 activity was expressed as the sum of DPM([^{3}H]diacylglycerol and [^{3}H] monoacylglycerol).(mg protein.20min)$^{-1}$.

2.4.5. Preparation of radiolabeled MAG and MAGL assays

Radioactive MAG were obtained from [2-^{3}H]glycerol-TAG ([^{3}H]TAG), which was synthesized from bovine retinas incubated with [2-^{3}H]glycerol (200 mCi/mmol) as previously described Pascual et al. (2013). [^{3}H]MAG specific activity was calculated by measuring the fatty acid content and the radioactivity of an aliquot the substrate (Pascual et al., 2013). Fatty acid composition of MAG was determined by gas chromatography: C16:0 45.1, C16:1 0.8, C17:0 0.9, C16:0 45.1, C18:0 26.5, C18:2n-6 1.8, C18:3n-6 1.2, C20:3n-6 1.2, C20:4n-6 11.5 (% composition). For enzymatic tests, protein (100 µg) was incubated with [^{3}H]MAG (10 µM) previously sonicated with fatty acid-free BSA (0.125% w/v), in a total volume of 200 µL Tris-HCl buffer (10 mM, pH 7.2) containing 1 mM EDTA, for 15 min at 37 °C. MAGL enzymatic reaction was stopped by adding 400 µL chloroform:methanol (1:1,v/v), followed by vortex mixing the tubes twice and placing them on ice. Phases were separated by centrifugation for 5 min at 2500 rpm, and the aqueous phase from Folch extraction containing radiolabeled water-soluble products (glycerol and/or glycerol 3- phosphate) was concentrated to dryness and counted by liquid scintillation. Radiolabeled samples were counted as was indicated in 2.4.4.

2.4.6. PLA assay

For enzymatic tests, protein (80 µg) was incubated with 3 µL of liposomes in a total volume of 100 µL of MES-KOH, pH 6.8, and 1 mM $CaCl_2$ at 33 °C for 30 min. Reactions were stopped with 2 vol of the stopping solution (methanol:chloroform 2:1, v/v). 1 vol 0.1 M KCl was subsequently added, followed by incubation at −20 °C for 20 min. After centrifugation for 2 min at 3300 × g, the organic phase was evaporated under a stream of nitrogen and redissolved in 20 µL chloroform for thin-layer chromatography on silica gel 60 (Merck, Darmstadt, Germany) in a solvent made up of chloroform:methanol:water (65:25:4, v/v). Plates were dried and scanned. Fluorescence (excitation 460 nm, emission 534 nm) from lipids was measured in a fluorescence spectrophotometer (Image Station 4000 MM PRO-Carestream Molecular Imaging). Fluorescently labelled lipids were visualized with a UV light box (FBTIV-88, Fisher Scientific). The spots marked were scraped from the plates and placed in 600 µL chloroform:methanol:water (5:5:1, v/v/v), vortexed, and centrifuged for 5 min at 15,000 × *g*. Fluorescence (excitation 460 nm, emission 534 nm) from the eluted lipids was measured with a fluorescence spectrofluorometer (Horiba-Fluoromax-3).

3. 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, Duncan'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). Software R version 3.1.2 was used for ESI-MS/MS analysis from Excel-grouped data. Factor Analysis was performed as exploratory multivariate analysis tools that allow the reduction of the dimensionality of large datasets generated by ESI-MS/MS. The factor analysis was performed using the principal factor method, as the extraction method was applied iteratively. The correlations were calculated as co-variance analysis.

For the enzymatic assay the results are shown as the means ± SEM for at least three independent experiments. Statistical analyses were evaluated by Duncan's test and by Student's test (Fig. S1), and performed using Origin Pro 8 Copyright 1991–2008. Statistical significance was set at $P < 0.05$.

4. Results

4.1. ESI-MS/MS analysis of PA molecular species

We have demonstrated the action of PLD on membrane phospholipids in response to short- and long-term chilling stress in barley seedlings. Besides, PA was shown to be a key metabolic intermediate and a second messenger that differentially modulates chilling response in barley tissues (Peppino Margutti et al., 2017). We wondered whether short- and long-term chilling stress was also able to modulate PA total content. To answer this, seedlings were incubated at 4 °C for 3 and 36 h and then PA levels were evaluated in leaves and roots using ESI-MS/MS. Fig. 1 shows that neither short- nor long-term chilling treatment modified PA levels in barley tissues. This may indicate that chilling exposure was a moderate stress condition. As mentioned earlier, PA is a group of molecules that differ in their fatty acids moieties. To gain further insight into the metabolism of PA, we studied the PA fatty acid composition of barley tissues incubated for 3 and 36 h at 4 °C (Fig. 2). The analysis allowed the identification and relative quantification of 10 different molecular species of PA, each species being characterized by the couple of acyl chains that composes it. PA molecular species

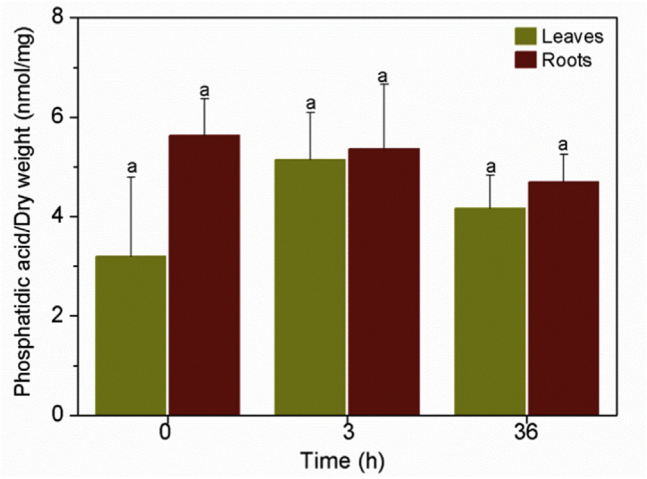

Fig. 1. ESI-MS/MS quantification of phosphatidic acid in barley tissues. After chilling treatment, induced by incubating seedlings at 4 °C for 3 and 36 h, leaves (green bars) and roots (red bars) were separated and immersed in 3 ml of isopropanol (0.01% w/v 2,6-Di-tert-butyl-4-methylphenol) at 75 °C for 15 min. Then, phosphatidic acid was extracted and analysed as described in Section 2.3. The values are the means ± SEM (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified contained C16 and/or C18 acyl chains (Welti et al., 2002). The major molecular species of PA are C34:2 (18:2–16:0) and C36:4 (di18:2 or 18:1–18:2), total acyl chain: doble bonds) (Welti et al., 2002). Compared with plants grown at 25 °C (T0), short-term and long-term chilling for 3 h (T3) and 36 h (T36) at 4 °C not produced significant changes in PA molecular species (Fig. 2A and B). Although a slight increasing trend can be observed in molecular species containing C34:2 and C36:4 of leaves (Fig. 2A).

Fig. 3 shows the comparison between tissues of relative molecular species compositions of PA pools in the 3 conditions (T0, T3 and T36). Fig. 3A shows that fatty acids moieties composition in leaves and roots from plants grown at 25 °C were similar. However, some difference tissue specific can be observed. The proportion of C34:2 and C36:4 chains in PA molecular species was higher in roots than in leaves. In contrast, the proportion of C34:1, C34:3, C36:3, C36:5 and C36:6 was higher in leaves than in roots.

When plants were submitted to short- (Fig. 3B) or long-term (Fig. 3C) chilling, relative molecular species compositions of PA pools was the same for leaves and roots in the both chilling condition.

As PA composition clearly differs between roots and leaves, a Factorial data analyse was performed. This factor analysis of data from leaf and root tissues during short- and long-term chilling stress shows that one axis (F1) is sufficient to discriminate between data for each one, with leaf data being associated with positive values on the F1 axis and root data being associated with negative values on the F2 axis (Fig. 4A). Interestingly, factor F1 is highly correlated with the PA species containing C18:3 (C34:3, C36:3, C36:4, C36:5, C36:6) (Fig. 4B).

4.2. Enzyme activities involved in PA metabolism

The PA signal is closely related to the production of another lipid signal. PA may be phosphorylated to DGPP, dephosphorylated to DAG or deacylated to LPA. Besides, it has been shown that DAG may act as a precursor to PA via DAGK. As mentioned before, PA molecules differ in their fatty acid moieties. As a consequence, the production of specific PA pools may affect enzyme activities. In the next experiment, we assayed the enzymatic activities involved in PA signal attenuation. Lipid kinase activities (DAGK and PAK) were assayed simultaneously using endogenous lipids as substrates. A phosphorylation assay of endogenous DAG and PA was carried out with the 105.000 × *g* membrane fraction in the presence of radiolabeled ATP. Fig. 5A shows that basal DAGK was more active in leaves than in roots (23 ± 2.3 (pmol (mg prot min.)$^{-1}$) *vs* 13.6 ± 2 (pmol (mg prot min.)$^{-1}$, $P < 0.05$). The opposite behaviour was observed in leaves with respect to roots when plants were exposed to chilling. In leaves, DAGK activity (3 ± 0.7 (pmol (mg prot min.)$^{-1}$); n = 3) decreased after short-term chilling treatment, with respect to the control (23 ± 2 (pmol (mg prot min.)$^{-1}$); n = 3). This effect was also observed after long-term chilling. By contrast, DAGK activity (25 ± 4 (pmol (mg prot min.)$^{-1}$); n = 3) increased in roots after chilling treatment for 3 h, with respect to the control (14 ± 2 (pmol (mg prot min.)$^{-1}$); n = 3). This indicates that short-term chilling modified DAGK activity in roots in a positive manner, although this effect was not observed after long-term chilling. A decrease in DAG phosphorylation (5 ± 1 (pmol (mg prot min.)$^{-1}$); n = 3) occurred after chilling treatment for 36 h, with respect to the control (14 ± 2 (pmol (mg prot min.)$^{-1}$); n = 3). Similar trends were observed in PAK activity. In leaves, PAK activity was detected in 105,000 × *g* membrane fractions with a specific activity of 12 (pmol (mg prot min.)$^{-1}$). PAK activity decreased with short-term chilling (Fig. 5B). The opposite effect was observed in roots, where PAK activity increased ~50% with short-term chilling stress (Fig. 5B).

In the next experiment, PA desphorylation was studied. The activity of PAP2 is coupled mainly to PLD activity because the main product of hydrolytic PLD activity, PA, is the substrate used by PAP2 to yield DAG. DAGL activity that produces MAG was assayed in a coupled reaction

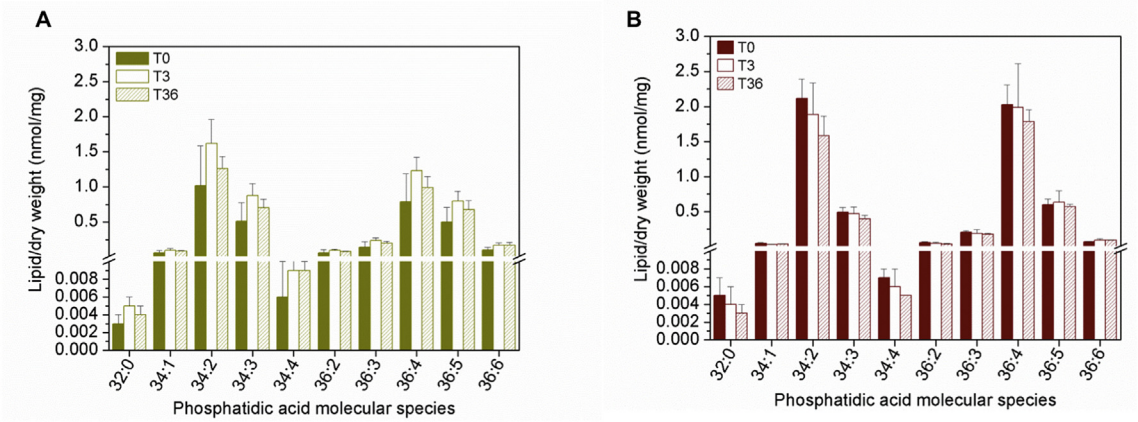

Fig. 2. PA molecular species of barley tissues in response to chilling. Barley leaves (A) and roots (B) were incubated at 25 °C (T0) or at 4 °C for 3 h (T3) and 36 h (T36). The analysis was performed by ESI-MS/MS on three independent samples. The results are expressed as Lipid/Dry weight (nmol/mg). The values are the means ± SEM (n = 3).

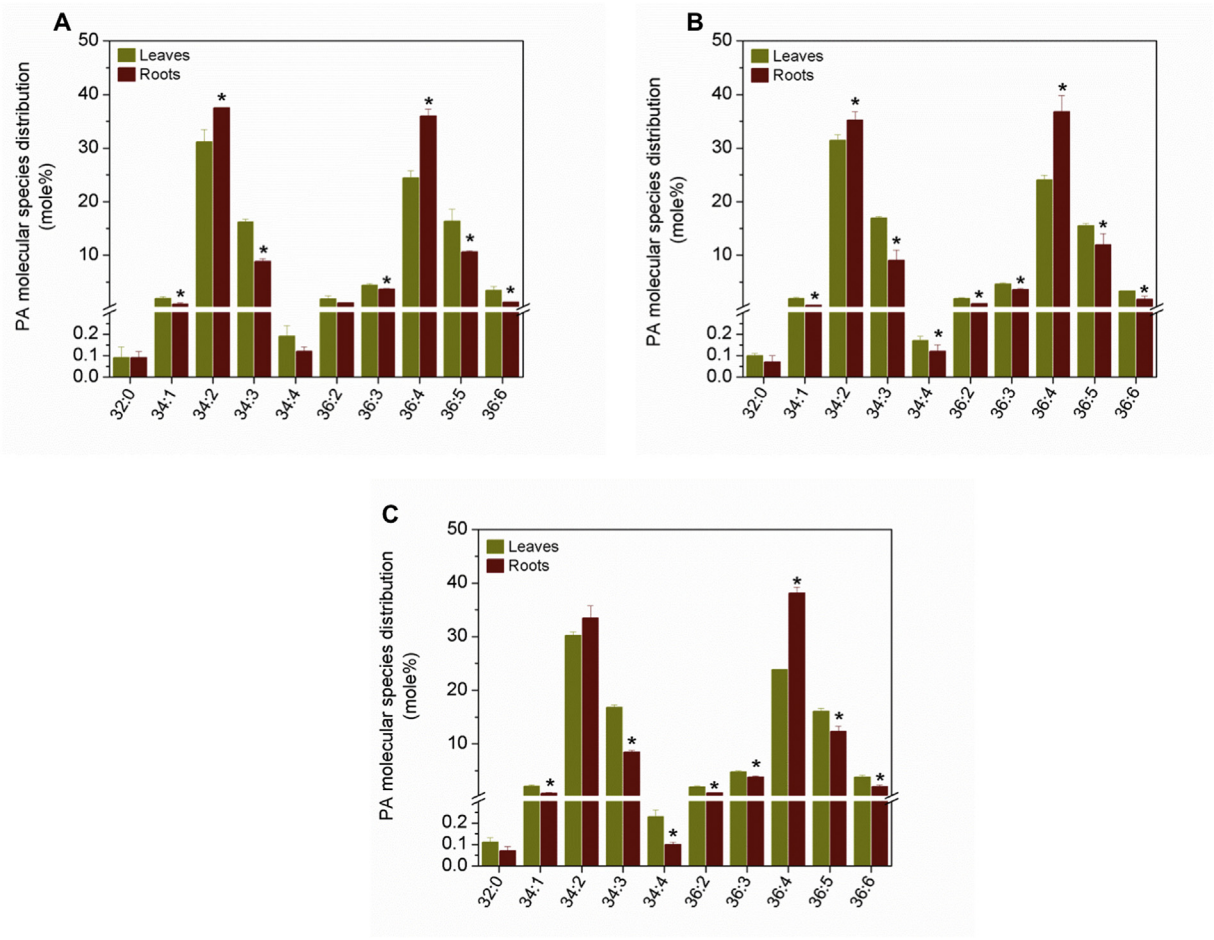

Fig. 3. Comparative analysis of the compositions of PA molecular species in leaves and roots in response to chilling. Barley seedlings were incubated at 25 °C (A) or at 4 °C for 3 h (B) and 36 h (C). The analysis was performed by ESI-MS/MS on three independent samples. The results are expressed as PA molecular species distribution (mole%). The values are the means ± SEM (n = 3). Asterisks indicate significant differences between leaves and roots (Duncan's Test, $P < 0.05$).

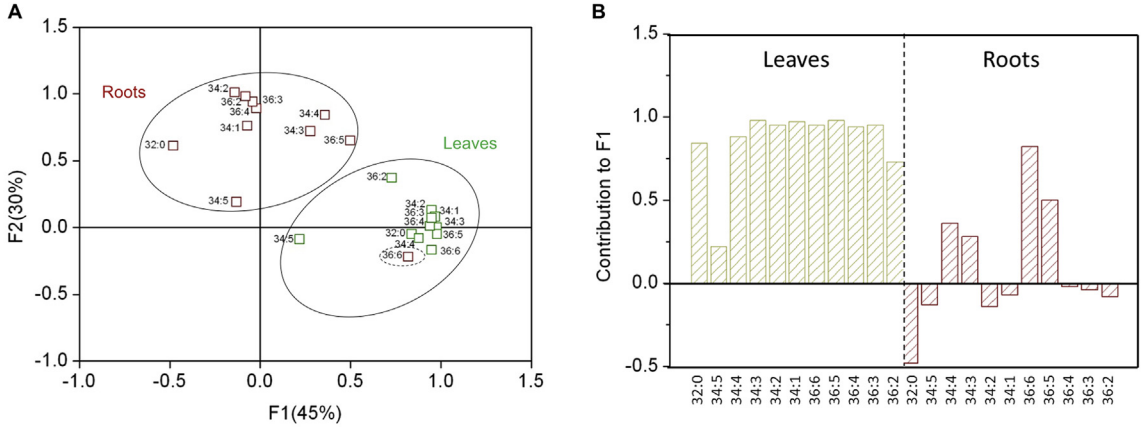

Fig. 4. Factor analysis of PA molecular species from roots and leaves during chilling. (A) Positions of the observations on the two principal factors. Each observation corresponds to the PA fatty acid composition after chilling in roots (red square) and leaves (green square) tissues. Contribution of variables to F1 factor (B). In the analysis, the molecular species were used as variables. Factor analysis was performed with R, using the principal factor method as the extraction method applied iteratively. Correlations were calculated as co-variance analysis. The 11 variables that contributed the most to factor F1 were selected and their loading on the F1 axis represented in (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

using DAG, which is produced by PAP2 activity, as a substrate.

DAG, MAG and WSP (water soluble products, glycerol and/or glycerol 3-P) were identified when microsomal membranes from leaves were incubated with [^{3}H]PA (Fig. 6A, B, C). With 100 µM of [^{3}H]PA, the formation of DAG and MAG (3 DPM(mg prot.20 min)$^{-1}$) was similar, while the values for WSP were 4 times higher than those for DAG and MAG. This is indicative of the presence of active MAGL in this tissue (Fig. 6E). In leaves, short- and long-term chilling stress did not significantly alter PAP2 and MAGL activities (Fig. 6D and E).

DAG and MAG formation in roots was 6 times lower than in leaves, 0.4 ± 0.1 DPM (mg prot.20 min)$^{-1}$ *vs* 3.2 ± 0.42 DPM (mg prot.20 min)$^{-1}$; n = 3, $P < 0.05$, while WSP formation was similar to

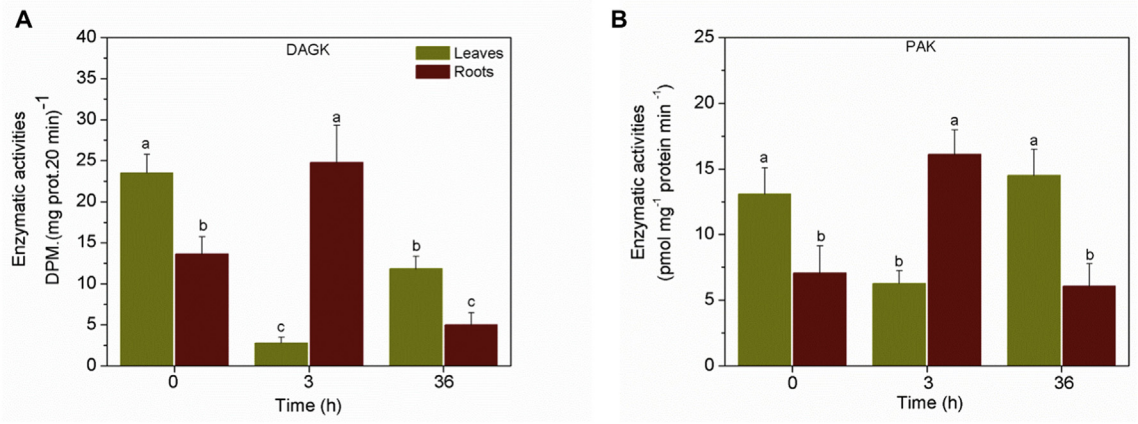

Fig. 5. Effect of short- and long-term chilling treatment on DAGK and PAK activities. Phosphorylation assay of endogenous DAG (A) or PA (B) was carried out with the 105,000 × *g* membrane fraction in the presence of 370 MBq [γ-^{32}P]ATP for 2 min at 30 °C. Labelled lipids were extracted and separated by 1-dimensional TLC with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v). Results are expressed as specific activity (pmol (mg prot min.)$^{-1}$) and shown as mean S.E.M., n = 3. Different letters indicate significant differences (Duncan's Test, $P < 0.05$).

that observed in leaves (Fig. 6A and B). In roots, long-term chilling increased PAP2 activity significantly (Fig. 6D). Besides, as shown in Fig. 6C, it was observed that WSP increased in response to long-term chilling stress. Under this assay condition, WSP are produced from PA by PAP2 action. However, WSP (glycerol and/or glycerol 3-P) can be formed by the activity of MAGL or PA-PLA, respectively. Thus, we considered studying the contribution of MAGL to WSP formation. When microsomal membranes were incubated with [^{3}H]MAG, MAGL activity was similar in leaves and roots (Fig. 6E). In leaves, no changes were observed in response to chilling, on MAGL enzymatic activity. On the other hand, short- and long-term chilling treatment in roots significantly decreased and increased MAGL and PAP2 activities, respectively (Fig. 6D and E). A similar trend was observed for PA-PLA activity, which was only affected by long-term chilling in roots (Fig. 6F).

5. Discussion

The results presented here show that short- and long-term chilling modify PA metabolism in barley. ESI-MS/MS assays demonstrated that PA species molecules are not uniform and differ in their fatty acid moieties (Wang et al., 2006; Liu et al., 2013; Ruelland et al., 2015; Pokotylo et al., 2018). We found that basal PA pools in leaves are different that PA pools in roots. Several factors modulate the PA signal, while PA accumulation can trigger another undesired response (Li et al., 2008). Membranes are the initial sites of temperature sensing and the remodelling of membrane lipids is one of the most important mechanisms used by plants to respond to low temperatures (Perlikowski et al., 2016; Tshabuse et al., 2018). Some studies have used a lipidomic approach to investigate the influence of freezing on the glycerolipid profile in *Arabidopsis*. In these studies, PA formation from PC hydrolysis by PLDα activity is believed to be a key feature in freezing-induced lipid hydrolysis. In line with this, *Arabidopsis* plants that are deficient in PLDα have improved tolerance to freezing (Welti et al., 2002; Li et al., 2008). Therefore, the levels of accumulated PA as a product of PLD action during stress may compromise membrane integrity (Li et al., 2004).

In plants, PA is well established as a signal lipid since rapid PA accumulation is a common reaction to a variety of stresses (Ruelland et al., 2015; Pokotylo et al., 2018). Our data demonstrate that the short- and long-term chilling does not alter PA content in barley tissues. However, a transient PA accumulation was reported in plants that were exposed to moderate cold (Ruelland et al., 2002; Arisz et al., 2013). The fact that PA levels remain unchanged may be the consequence of a moderate stress application or a rapid PA metabolism. In agreement with this, short-term chilling stress caused rapid but transient increases in PLD activity in young barley leaves (Peppino Margutti et al., 2017). On the other hand, the accumulation of ROS and proline in barley roots was PA-mediated, suggesting that the PA signal differs depending on the plant organs where it is evaluated (Pokotylo et al., 2018). Here, we found that PA molecules of barley tissues have a different fatty composition. The PA in barley leaves contains less unsaturated fatty acid than in roots. Although, the chilling exposition not evoked significant changes in leaves, a slight increasing trend can be observed in molecular species containing C34:2, C36:4 and C36:6 (Fig. 2A). An increase in highly unsaturated PA molecular species might be related to the organization of PA-enriched micro domains, which may contribute to the physical state and the dynamics of other membrane components such as protein binding. For example, one effect of lipid-protein interaction is the modulation of protein catalytic activity, which can be inhibitory or stimulatory, depending on the effector protein (Liu et al., 2013). ABA signalling in *Arabidopsis* requires the functioning of many proteins and PA has been found to interact with several of them, including negative effector ABI1 and positive effector NADPH oxidase. In lipid-protein binding assays, it has been shown that PA composition may affect the protein binding to the target. RbohD and RbohF NADPH-oxidases have a preference for binding to PA that contains at least one unsaturated or poly-unsaturated FA, while no binding was observed for di16:0- or di18:0- PA (Zhang et al., 2009). In other experiments, it has been reported that di18:1-PA also appears to bind ABI1 more efficiently than di18:2-PA (Zhang et al., 2004). Otherwise, PA species enriched with 34:2 and 36:4 acyl chains could also modulate the initial PA signal during chilling response through a still unrevealed mechanism. Alternatively, it may also reflect accumulation of PA as precursor in eukaryotic galactolipids synthesis. Low temperature may cause inhibition of import and consequent accumulation of 34:2 and 36:4 PA species, being main substrates in this biosynthetic route, associated with chloroplast-rich plant tissues (Boudiere et al., 2014; Kelly et al., 2016).

Another report indicates that the PA signalling mechanism relies on the fact that PA can be metabolised by multiple enzymes in multiple pathways (Pokotylo et al., 2018).

PA signals can be enhanced or attenuated by conversion to other lipids. This may also be connected to the fatty acid composition of the lipids used as a substrate. In leaves, the lipid kinases DAGK and PAK were more active than the enzymes in roots, although chilling considerably affected their activity, whereas short-term chilling stimulated both kinases in roots. Thus, it seems likely that the PA fatty acid composition observed in roots may have some relation with lipid kinase activity. On the contrary, the increase in unsaturated moieties observed in leaves was not reflected in an increase in kinase activity. Still, these events may not be related, and the fact that we had to measure the

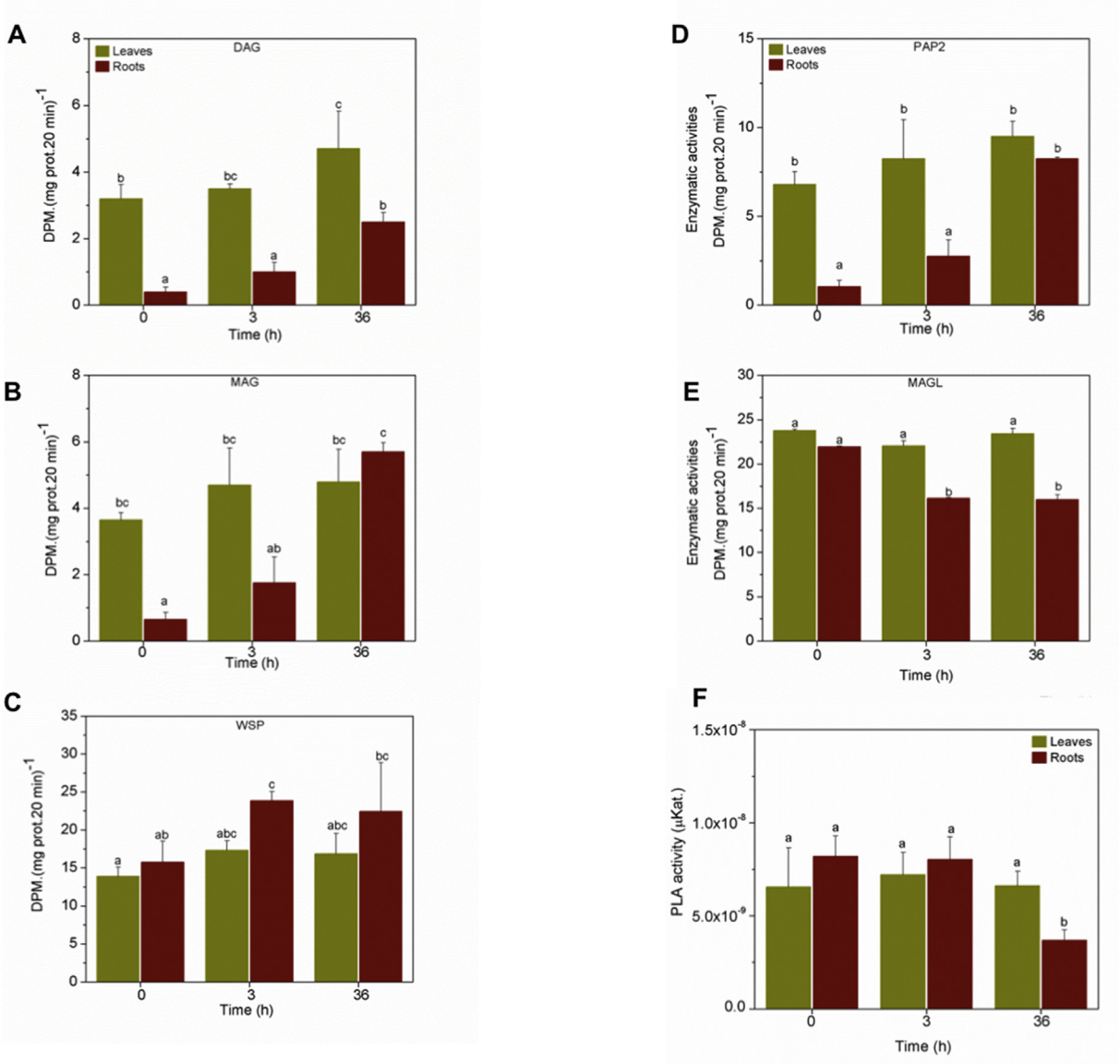

Fig. 6. Chilling-related changes in the enzymatic pathways involved in PA/DAG/MAG metabolism in leaves (green bars) and roots (red bars). (A) DAG, (B) MAG and (C) WSP production as a function of time chilling exposure. (D) PAP2 activity was determined using 0.1 mM [^{3}H]PA and 50 µg of 105,000 × *g* membrane fraction in a final volume of 100 µL, as described in Section 2.4.4. (E) MAGL activity was determined by monitoring the formation rate of [2-^{3}H]glycerol-WSP using monoacyl [2-^{3}H]glycerol. The subsequent procedure is specified in Section 2.4.5. (F) PLA activity was determined using NBD-PA and 80 µg of 105.000 × g membrane fraction in a final volume of 100 µL, as described in Section 2.4.6. Lipids were extracted and separated by 1-dimensional TLC with chloroform:methanol:water (65:25:4, v/v). Results represents the mean ± S.E.M., n = 3. Different letters indicate significant differences (Duncan's Test, $P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

kinases with an endogenous substrate would support this idea.

On the other hand, the fact that DGPP formation occurs only in roots after short-term chilling would indicate that it might act as a molecular switch that may turn the PA signal on and off. Physicochemical studies have shown that DGPP stabilizes the bilayer phase at neutral pH, unlike PA, which induces negative curvature stress on the membrane. In addition, DGPP will carry more negative charge in its head group than PA. Therefore, the conversion of PA to DGPP will affect curvature and local membrane electrostatics. (Kooijman et al., 2007; Kooijman and Burger, 2009; Strawn et al., 2012). Furthermore, adding complexity to the PA signal, PA can also be dephosphorylated to DAG by PAP2 activity in barley roots (Racagni et al., 2008). Basal PAP2 activity in control roots is indeed much less active than in leaves. However, it was stimulated several times in response to long-term chilling. This stimulatory effect seems to occur only in roots responding to chilling. PAP2 activity is sensitive to abiotic stress, but it was inhibited in response to ABA in aleurone and during saline stress in barley roots (Racagni et al., 2008; Meringer et al., 2016). Thus, the results suggest that barley roots might modulate the DAG-PA-DGPP balance during exposure to the cold. Both the change in MAG formation by DAGL activity and MAGL inhibition are more pronounced with long-term chilling. We propose that this effect, linked to unmodified PA levels, might facilitate tolerance during a long term-chilling situation.

In view of the present observations, the PA signal is differentially modulated by changes in the enzymatic activities during the chilling signal transduction process in barley seedlings, as illustrated schematically in Fig. 7.

The findings presented here may be helpful in understanding how the PA signal is modulated between tissues during moderate chilling stress, and highlight the importance of knowing the basal PA pools in different plant organs.

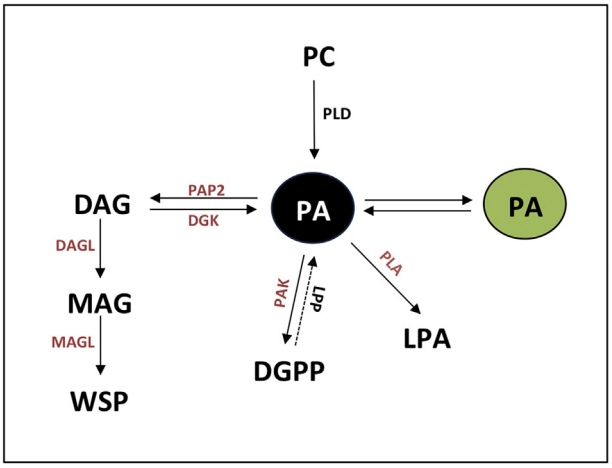

Fig. 7. Scheme of the contribution of the present work with PA metabolism in barley. PA pools in leaves (green circle) differ from PA fatty acid composition in roots (black circle). Chilling treatment modified different enzymatic activities (red letters) involved in the attenuation of the PA signal in roots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Author contributions

Conceived and designed the experiments: MPM, ALV. Performed the experiments: MPM, VG. Analyzed the data: MPM, VG, MR, ALV. Contributed reagents/materials/analysis tools: ALV, SJP. Result discussion MPM, VG, SJP, ALV. Wrote the paper: ALV. Edited the manuscript, wrote figure legends, and helped with the outline of the paper: MPM, ALV.

Acknowledgements

This work was supported by Universidad Nacional de Río Cuarto (SECyT-UNRC, grant number 18/C426); PPI-CONICET (grant number 11220150100206CO), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, grant number PICT 1108/15 Préstamo BID, Argentina. INTA-Bordenave generously provided seeds. ALV and VLG are Career Investigators of CONICET. SJP is UNS Investigator. MPM and MR are CONICET fellowship holders. Instrument acquisition and method development at the Kansas Lipidomics Research Center were supported by grants from the National Science Foundation (EPS 0236913, MCB 1413036, MCB-0920663, DBI-1228622, Kansas Techonology Enterprise Corporation K-IDeA Networks of Biomedical Research Excellence (INBRE) of National Institutes of Health (P206M103418), and Kansas State University. The authors are grateful to Florencia Sgarlatta for language/editing assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.plaphy.2018.08.038.

References

Arisz, S.A., Testerink, C., Munnik, T., 2009. Plant PA signaling via diacylglycerol kinase. Biochim. Biophys. Acta 1791, 869–875. https://doi.org/10.1016/j.bbalip.2009.04.006.

Arisz, S.A., Van Wijk, R., Roels, W., Zhu, J.K., Haring, M.A., Munnik, T., 2013. Rapid phosphatidic acid accumulation in response to low temperature stress in Arabidopsis is generated through diacylglycerol kinase. Front. Plant Sci. 4, 1. https://doi.org/10.3389/fpls.2013.00001.

Arisz, S.A., Heo, J.Y., Koevoets, I.T., Zhao, T., Van Egmond, P., Meyer, J., Zeng, W., Niu, X., Wang, B., Mitchell-Olds, T., Schranz, M.E., Testerink, C., 2018. Diacylglycerol acyltransferase 1 contributes to freezing tolerance. Plant Physiol. (Wash. D C). https://doi.org/10.1104/pp.18.00503.

Astorquiza, P.L., Usorach, J., Racagni, G., Villasuso, A.L., 2016. Diacylglycerol pyrophosphate binds and inhibits the glyceraldehyde-3-phosphate dehydrogenase in barley aleurone. Plant Physiol. Biochem. 101, 88–95. https://doi.org/10.1016/j.plaphy.2016.01.012.

Boudiere, L., Michaud, M., Petroutsos, D., Rebeille, F., Falconet, D., Bastien, O., Roy, S., Finazzi, G., Rolland, N., Jouhet, J., Block, M.A., Marechal, E., 2014. Glycerolipids in photosynthesis: composition, synthesis and trafficking. Biochim. Biophys. Acta 1837, 470–480. https://doi.org/10.1016/j.bbabio.2013.09.007.

Bradford, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Dubots, E., Botté, C., Boudière, L., Yamaryo-Botté, Y., Jouhet, J., Maréchal, E., Block, M.A., 2012. Role of phosphatidic acid in plant galactolipid synthesis. Biochimie 94, 86–93. https://doi.org/10.1016/j.biochi.2011.03.012.

García, G.A., Mirallesa, D.J., Serrago, R.A., Alzueta, I., Huthd, N., Dreccer, F., 2018. Warm nights in the Argentine Pampas: modelling its impact on wheat and barley shows yield reductions. Agric. Syst. 162, 259–268. https://doi.org/10.1016/j.agsy.2017.12.009.

Gaveglio, V.L., Pasquare, S.J., Giusto, N.M., 2011. Metabolic pathways for the degradation of phosphatidic acid in isolated nuclei from cerebellar cells. Arch. Biochem. Biophys. 507, 271–280. https://doi.org/10.1016/j.abb.2011.01.001.

Gaveglio, V.L., Pascual, A.C., Giusto, N.M., Pasquare, S.J., 2016. Age-related changes in retinoic, docosahexaenoic and arachidonic acid modulation in nuclear lipid metabolism. Arch. Biochem. Biophys. 604, 121–127. https://doi.org/10.1016/j.abb.2016.06.017.

Gürel, F., Öztürk, Z.N., Uçarlı, C., Rosellini, D., 2016. Barley genes as tools to confer abiotic stress tolerance in crops. Front. Plant Sci. 7, 1137. https://doi.org/10.3389/fpls.2016.01137.

Hong, Y., Zhao, J., Guo, L., Kim, S.-C., Deng, X., Wang, G., Zhang, G., Li, M., Wang, X., 2016. Plant phospholipases D and C and their diverse functions in stress responses. Prog. Lipid Res. 62, 55–74. https://doi.org/10.1016/j.plipres.2016.01.002.

Kelly, A.A., Kalisch, B., Holzl, G., Schulze, S., Thiele, J., Melzer, M., Roston, R.L., Benning, C., Dormann, P., 2016. Synthesis and transfer of galactolipids in the chloroplast envelope membranes of Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A. 113, 10714–10719. https://doi.org/10.1073/pnas.1609184113.

Kooijman, E.E., Burger, K.N., 2009. Biophysics and function of phosphatidic acid: a molecular perspective. Biochim. Biophys. Acta 1791, 881–888. https://doi.org/10.1016/j.bbalip.2009.04.001.

Kooijman, E.E., Tieleman, D.P., Testerink, C., Munnik, T., Rijkers, D.T., Burger, K.N., De Kruijff, B., 2007. An electrostatic/hydrogen bond switch as the basis for the specific interaction of phosphatidic acid with proteins. J. Biol. Chem. 282, 11356–11364. https://doi.org/10.1074/jbc.M609737200.

Kratsch, H.A., Wise, R.R., 2000. The ultrastructure of chilling stress. Plant Cell Environ. 23, 337–350.

Li, W., Li, M., Zhang, W., Welti, R., Wang, X., 2004. The plasma membrane-bound phospholipase Ddelta enhances freezing toler- ance in Arabidopsis thaliana. Nat. Biotechnol. 22, 427–433.

Li, W., Wang, R., Li, M., Li, L., Wang, C., Welti, R., Wang, X., 2008. Differential degradation of extraplastidic and plastidic lipids during freezing and post-freezing recovery in Arabidopsis thaliana. J. Biol. Chem. 283, 461–468. https://doi.org/10.1074/jbc.M706692200.

Li, A., Wang, D., Yu, B., Yu, X., Li, W., 2014. Maintenance or collapse: responses of extraplastidic membrane lipid composition to desiccation in the resurrection plant Paraisometrum mileense. PLoS One 9, e103430. https://doi.org/10.1371/journal.pone.0103430.

Liu, Y., Su, Y., Wang, X., 2013. Phosphatidic acid-mediated signaling. Adv. Exp. Med. Biol. 991, 159–176. https://doi.org/10.1007/978-94-007-6331-9_9.

Meringer, M.V., Villasuso, A.L., Pasquare, S.J., Giusto, N.M., Machado, E.E., Racagni, G.E., 2012. Comparative phytohormone profiles, lipid kinase and lipid phosphatase activities in barley aleurone, coleoptile, and root tissues. Plant Physiol. Biochem. 58, 83–88. https://doi.org/10.1016/j.plaphy.2012.06.013.

Meringer, M., Villasuso, A., Peppino Margutti, M., Usorach, J., Pasquaré, S., Giusto, N., Machado, E., Racagni, G., 2016. Saline and osmotic stresses stimulate PLD/diacylglycerol kinase activities and increase the level of phosphatidic acid and proline in barley roots. Environ. Exp. Bot. 69–78.

Munnik, T., Heilmann, I., 2013. Plant Lipid Signaling Protocols 1009 Springer Protocols Methods in Molecular Biology.

Pascual, A.C., Gaveglio, V.L., Giusto, N.M., Pasquare, S.J., 2013. Aging modifies the enzymatic activities involved in 2-arachidonoylglycerol metabolism. Biofactors 39, 209–220. https://doi.org/10.1002/biof.1055.

Peppino Margutti, M., Reyna, M., Meringer, M.V., Racagni, G.E., Villasuso, A.L., 2017. Lipid signalling mediated by PLD/PA modulates proline and H2O2 levels in barley seedlings exposed to short- and long-term chilling stress. Plant Physiol. Biochem. 113, 149–160. https://doi.org/10.1016/j.plaphy.2017.02.008.

Perlikowski, D., Kierszniowska, S., Sawikowska, A., Krajewski, P., Rapacz, M., Eckhardt, A., Kosmala, A., 2016. Remodeling of leaf cellular glycerolipid composition under drought and Re-hydration conditions in grasses from the lolium-festuca complex. Front. Plant Sci. 7, 1027. https://doi.org/10.3389/fpls.2016.01027.

Pokotylo, I., Kravets, V., Martinec, J., Ruelland, E., 2018. The phosphatidic acid paradox: too many actions for one molecule class? Lessons from plants. Prog. Lipid Res. 71, 43–53. https://doi.org/10.1016/j.plipres.2018.05.003.

Racagni, G., Villasuso, A.L., Pasquare, S.J., Giusto, N.M., Machado, E., 2008. Diacylglycerol pyrophosphate inhibits the alpha-amylase secretion stimulated by gibberellic acid in barley aleurone. Physiol. Plantarum 134, 381–393. https://doi.org/10.1111/j.1399-3054.2008.01148.x.

Ruelland, E., Zachowski, A., 2010. How plants sense temperature. Enviromental and

Experimental Botany 69, 225–232.
Ruelland, E., Cantrel, C., Gawer, M., Kader, J.C., Zachowski, A., 2002. Activation of phospholi- pases C and D is an early response to a cold exposure in Arabidopsis suspension cells. Plant Physiol. (Wash. D C) 130, 999–1007.
Ruelland, E., Kravets, V., Derevyanchukb, M., Martinec, J., Zachowski, A., Pokotylo, I., 2015. Role of phospholipid signalling in plant environmental responses. Environ. Exp. Bot. 114, 129–144.
Shabala, S., 2017. Plant Stress Physiology, second ed. CABI, pp. 1–353.
Strawn, L., Babb, A., Testerink, C., Kooijman, E.E., 2012. The physical chemistry of the enigmatic phospholipid diacylglycerol pyrophosphate. Front. Plant Sci. 3, 40. https://doi.org/10.3389/fpls.2012.00040.
Testerink, C., Munnik, T., 2011. Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. J. Exp. Bot. 62, 2349–2361. https://doi.org/10.1093/jxb/err079.
Tshabuse, F., Farrant, J.M., Humbert, L., Moura, D., Rainteau, D., Espinasse, C., Idrissi, A., Merlier, F., Acket, S., Rafudeen, M.S., Thomasset, B., Ruelland, E., 2018. Glycerolipid analysis during desiccation and recovery of the resurrection plant Xerophyta humilis (Bak) Dur and Schinz. Plant Cell Environ. 41, 533–547. https://doi.org/10.1111/pce.13063.
Villasuso, A.L., Wilke, N., Maggio, B., Machado, E., 2014. Zn(2+)-dependent surface behavior of diacylglycerol pyrophosphate and its mixtures with phosphatidic acid at different pHs. Front. Plant Sci. 5, 371. https://doi.org/10.3389/fpls.2014.00371.
Wang, X., Devaiah, S.P., Zhang, W., Welti, R., 2006. Signaling functions of phosphatidic acid. Prog. Lipid Res. 45, 250–278. https://doi.org/10.1016/j.plipres.2006.01.005.
Welti, R., Li, W., Li, M., Sang, Y., Biesiada, H., Zhou, H.E., Rajashekar, C.B., Williams, T.D., Wang, X., 2002. Profiling membrane lipids in plant stress responses. Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. J. Biol. Chem. 277, 31994–32002. https://doi.org/10.1074/jbc.M205375200.
Yu, B., Li, W., 2014. Comparative profiling of membrane lipids during water stress in Thellungiella salsuginea and its relative Arabidopsis thaliana. Phytochemistry 108, 77–86. https://doi.org/10.1016/j.phytochem.2014.09.012.
Zhang, W., Qin, C., Zhao, J., Wang, X., 2004. Phospholipase D alpha 1-derived phosphatidic acid interacts with ABI1 phosphatase 2C and regulates abscisic acid signaling. Proc. Natl. Acad. Sci. U. S. A. 101, 9508–9513. https://doi.org/10.1073/pnas.0402112101.
Zhang, Y., Zhu, H., Zhang, Q., Li, M., Yan, M., Wang, R., Wang, L., Welti, R., Zhang, W., Wang, X., 2009. Phospholipase dalpha1 and phosphatidic acid regulate NADPH oxidase activity and production of reactive oxygen species in ABA-mediated stomatal closure in Arabidopsis. Plant Cell 21, 2357–2377. https://doi.org/10.1105/tpc.108.062992.
Zheng, G., Li, L., Li, W., 2016. Glycerolipidome responses to freezing- and chilling-induced injuries: examples in Arabidopsis and rice. BMC Plant Biol. 16, 70. https://doi.org/10.1186/s12870-016-0758-8.