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Research article

Cold stress affects H$^+$-ATPase and phospholipase D activity in Arabidopsis

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ABSTRACT

Low temperature is an environmental stress that greatly influences plant performance and distribution. Plants exposed to cold stress exhibit modifications of plasma membrane physical properties that can affect their functionality. Here it is reported the effect of low temperature exposure of Arabidopsis plants on the activity of phospholipase D and H$^+$-ATPase, the master enzyme located at the plasma membrane. The H$^+$-ATPase activity was differently affected, depending on the length of cold stress imposed. In particular, an exposure to 4 °C for 6 h determined the strong inhibition of the H$^+$-ATPase activity, that correlates with a reduced association with the regulatory 14-3-3 proteins. A longer exposure first caused the full recovery of the enzymatic activity followed by a significant activation, in accordance with both the increased association with 14-3-3 proteins and induction of H$^+$-ATPase gene transcription. Different time lengths of cold stress treatment were also shown to strongly stimulate the phospholipase D activity and affect the phosphatidic acid levels of the plasma membranes.

Our results suggest a functional correlation between the activity of phospholipase D and H$^+$-ATPase mediated by phosphatidic acid release during the cold stress response.

1. Introduction

Plants are constantly exposed to different environmental stresses such as extreme temperatures, high salinity, excess or lack of water, light and nutrients that strongly limit plant growth and significantly reduce crop yields (Levitt, 1980). In order to cope with unfavourable environmental conditions, plants have evolved specific molecular mechanisms allowing them to adapt to different stresses.

Low temperature is one of the most relevant stress that, besides adversely affecting the crop productivity, determines the geographical distribution of agricultural crops. Response of plants to low temperatures depends on duration and intensity of stress. In many plants originating from temperate regions, as Arabidopsis and spinach, a gradual exposure to moderately low temperatures induces an adaptation response known as cold acclimation that allows plants to increase freezing tolerance. This response is due to reprogramming gene expression which results in a metabolic adjustment (Thomashow, 1999). In Arabidopsis the C-repeat binding factors (CBF), transcriptional activators rapidly induced in response to low temperatures (Gilmour et al., 1998), have been demonstrated to regulate the expression of cold responsive genes (*COR*) playing a central role in freezing tolerance.

How plants can perceive cold signal is still under investigation, since no plant cold sensor has been so far identified. Several data suggest that plants may sense low temperature through modification in the membrane physical properties, as membrane fluidity decreases during cold stress and membrane rigidification induces at 25 °C the same responses induced by low temperature (Orvar et al., 2000). Accordingly enzymes, as phospholipase D (PLD), involved in the regulation of membrane composition have been reported to play a role during cold signaling (Bargmann and Munnik, 2006). Modification on membrane fluidity and composition may strongly affect the activity of enzymes located at the plasma membrane. Among them, the H$^+$-ATPase, that extrudes protons from the plant cell thus generating an electrochemical gradient across the plasma membrane, provides the driving force for the secondary transport of ions and nutrients into and out of cells (Palmgren, 2001). The H$^+$-ATPase is often referred to as the

Abbreviations: AHA1, *Arabidopsis thaliana* H$^+$-ATPase isoform 1; AHA2, *Arabidopsis thaliana* H$^+$-ATPase isoform 2; CBF, C-repeat binding factor; DAG, diacylglycerol; FC, fusicoccin; GST, glutathione S-transferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; PLD, phospholipase D; RT-qPCR, quantitative real time PCR.

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"master enzyme" of plant cells playing a pivotal role in different aspects of plant physiology and biochemistry, such as cell expansion, solute uptake, phloem loading, and stomatal aperture (Palmgren, 1998).

Because of its importance, H^+-ATPase activity has to be modulated to deal with environmental stress conditions and metabolic changes. Recent evidence has shown that the H^+-ATPase is very sensitive to abiotic stresses, such as salinity, drought and temperature (Gong et al., 2010; Wakeel et al., 2010; Janicka-Russak et al., 2012, 2013; Kim et al., 2013), even though its role in the plant response has not yet been fully clarified.

The H^+-ATPase activity is regulated by association of 14-3-3 proteins with the C-terminal autoinhibitory domain (Fullone et al., 1998; Visconti et al., 2003). The H^+-ATPase binding site for 14-3-3s is generated upon phosphorylation of a conserved threonine residue within the sequence YTV, located at the very end of the C terminus (Fuglsang et al., 1999). 14-3-3 proteins are a family of evolutionary conserved dimeric proteins that accomplish a wide range of regulatory roles in eukaryotes (Fu et al., 2000). In plants, in addition to the plasma membrane transport through the regulation of the H^+-ATPase activity, 14-3-3s are involved in the control of gene expression, in the cellular trafficking, in the control of the activities of diverse enzymes of metabolism, in the hormone signaling, and in general in the coordination of different signal transduction pathways (Aducci et al., 2002; Sottocornola et al., 2006; Camoni et al., 2011; Schoonheim et al., 2009; Denison et al., 2011). Lots of evidence suggest also a role of 14-3-3s in the plant response to stress conditions (Gökirmak et al., 2010): environmental and biotic stresses can affect the expression levels of 14-3-3 genes (Chevalier et al., 2009) and also a large number of stress–related proteins have been identified as 14-3-3 clients (Chang et al., 2009). Recently the involvement of 14-3-3 proteins in the plant response to salinity has been clear demonstrated for two 14-3-3 Arabidopsis isoforms that have been reported to regulate the Salt Overly Sensitive pathway, known to control sodium homeostasis during salt stress (Zhou et al., 2014). Moreover, the Arabidopsis 14-3-3 psi isoform has been proposed to modulate constitutive freezing tolerance and cold acclimation connecting the low-temperature response with ethylene biosynthesis (Catalá et al., 2014).

In the present study, we have investigated the role of the H^+-ATPase and regulatory 14-3-3 proteins in the response to low-temperature conditions. Given the central role of PLD in plant adaptation to cold stress, we have also explored the relationships between H^+-ATPase and the PLD signaling pathway.

2. Materials and methods

2.1. Chemicals

[γ^{32}P]ATP (specific activity 111 TBq/mmol) was from Perkin Elmer (Boston, MA). Fusicoccin (FC) was prepared according to Ballio et al. (1968). The catalytic subunit of protein kinase A, thrombin, diacylglycerol (DAG), phosphatidylcholine (PC) were from Sigma (St. Louis, MO). Chemicals for gel electrophoresis were from Bio-Rad (Hercules, CA).

2.2. Plant growth and low temperature treatment

Arabidopsis thaliana, ecotype Columbia (Col-0), seedlings were grown in a growth chamber at 22 °C, 80% humidity, under a 16 h light/8 h dark cycle. Three-week old plants were subjected to cold stress treatment by incubating them in the dark, at 4 °C, for 1, 6 12 and 18 h. Control plants were incubated at 21 °C in the same conditions, in the dark. Leaves from stressed and control seedlings were then sampled and immediately subjected to further assays, as described below.

2.3. Electrolyte leakage and lipid peroxidation tests

Electrolyte leakage test was performed as described by Kim et al. (2013). Arabidopsis leaves (0.2 g) were cut into 5 mm slices and shaken at 25 °C in 30 ml of deionized water for 2 h. The electrical conductivity of solution was measured by using an electrical conductivity meter. Boiled samples were used to determine the maximum percentage of electrolyte leakage.

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) production during 4 °C exposure, following the protocol described by Taulavuori et al. (2001).

2.4. Purification of plasma membrane from Arabidopsis leaves

Two-phase partitioned plasma membranes were obtained from 200 g of Arabidopsis leaves as previously described (Pallucca et al., 2014).

2.5. SDS-PAGE and immunoblotting

SDS–PAGE was performed as described by Laëmmli (1970), in a Mini Protean apparatus from Bio-Rad (Hercules, CA). For immunoblotting analysis, proteins were separated by SDS–PAGE, then electroblotted onto a PVDF membrane with 39 mM glycine, 48 mM Tris, 0.1% SDS and 10% methanol. After blocking for 1 h in TTBS (20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 0.05% Tween-20) with 5% no-fat dried milk at room temperature, the membrane was incubated with anti-14-3-3 antibodies (1:1000) or anti-H^+-ATPase antibodies (1:1000) (Marra et al., 2000). Following three washes with TTBS, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:5000) from Bio-Rad (Hercules, CA).

2.6. Overlay assay

The overlay assay was carried out according to Camoni et al. (2001) with minor modifications; 20 μg of Arabidopsis plasma membrane fractions, purified from leaves of Arabidopsis seedlings subjected to low temperature for different period of time, were separated on SDS-PAGE and blotted on nitrocellulose membrane by semidry electroblotting from Bio-Rad (Hercules, CA). The membrane was blocked with 5% fatty acid-free milk in 25 mM HEPES-OH, 75 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA, 0.05% Tween-20, pH 7.5 (buffer HT) and then cut into identical strips which were incubated overnight at 4 °C in the same buffer containing 3% fatty acid-free milk, 10 μM FC and 0.1 μM ^{32}P-labeled GF14ω isoform (corresponding to 9 kBq ml^{-1}) used as the probe. The nitrocellulose membrane was then extensively washed three times with buffer HT and radioactivity detected by autoradiography. Densitometric analysis (Abramoff et al., 2004) was performed using the ImageJ image processing program. Densitometric data are expressed as a percentage of the maximum Integrated Densitometric Value (the product of area and mean gray value).

GF14ω 14-3-3 isoform was expressed in *Escherichia coli* as fusion protein with the glutathione S-transferase (GST) and [^{32}P]ATP labeled on the phosphorylation site present at junction between GST and 14-3-3 protein using the catalytic subunit of protein kinase A as already described (Visconti et al., 2008).

2.7. PLD activity

PLD activity was determined by measuring the amount of

choline released from PC following the colorimetric-enzymatic method developed by Imamura and Horiuti (1978). The reaction mixture (0.5 ml) was composed of 10 mM $CaCl_2$, 2 mM PC emulsion in 0.2 M acetate buffer (pH 5.5) in the presence or in the absence of 0.6 mM oleic acid. To verify the specific effect of oleic acid on PLD activity, the reaction was performed in the presence of a reduced $CaCl_2$ concentration (10 μM) The reaction was started by adding the plasma membrane from Arabidopsis leaves (60 μg) and continued for 25 min at 25 °C. It was stopped by adding 0.1 ml of 50 mM EDTA in Tris-HCl buffer (pH 8). The resulting mixture was mixed with 0.3 ml of a solution of 3 units of choline oxidase from Sigma (St. Louis, MO), 0.2 units of peroxidise from Sigma (St. Louis, MO), 1.5 μmol of 4-aminoantipyrine and 2.1 μmol of phenol in 10 mM Tris-HCl buffer (pH 8). The reaction was stopped by adding 2.0 ml of 1% (v/v) Triton X-100 solution. The amount of choline liberated was calculated from absorbance change at 500 nm, using a standard curve for absorbance change versus amount of choline made with a standard solution of choline chloride.

2.8. Phosphohydrolytic activity

The phosphohydrolytic activity of the plasma membrane preparation from Arabidopsis leaves was assayed according to Camoni et al. (2006) using 50 μg of protein.

2.9. Protein quantification

Protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard (Bradford, 1976).

2.10. Extraction of total lipids

2.5 g of plantlets were ground in a mortar with a pestle for 3 min at room temperature. The homogenate was heated in boiled water for 3 min for inactivation of lipolytic enzymes. Lipids were extracted from the homogenates by the Bligh and Dyer method (Bligh and Dyer, 1959). The lower layer (organic phase) was dried under N_2 and dissolved in 0.2 ml of 1/1 chloroform/methanol (v/v).

2.11. Phosphatidic acid and DAG quantification

Phosphatidic acid (PA) was quantified according to manufacturer's instruction of Total Phosphatidic Acid Fluorometric Assay kit from Cayman Chemical (Ann Arbor, MI). Briefly: lipase is used to hydrolize PA to glycerol-3-phosphate, which in turn is oxidized by glycerol-3-phosphate oxidase generating hydrogen peroxide. In the presence of a peroxidise, hydrogen peroxide reacts with 10-acetyl-3,7 dihydroxyphenoxazine producing the fluorescent compound resorufin. Resorufin fluorescence can be analysed using the excitation wavelength of 530 nm and the emission wavelength of 585 nm. DAG was quantified by TLC on silica gel with petroleum ether/diethyl ether (75/25, v/v) as solvent system. After spraying the plate with a solution of cupric acetate 3% and H_2SO_4 8% (v/v), and dried for 30 min at 120 °C, an image of the spots on the plate was captured. DAG amount was calculated using a DAG standard curve obtained with standard solutions of DAG emulsion.

2.12. PA phosphohydrolase activity

The PA phosphohydrolytic activity of plasma membrane preparations from Arabidopsis leaves was measured by a colorimetric assay quantifying the phosphate inorganic released from PA, accordingly with the method described by Ullah et al. (2012). Briefly, 50 μg of plasma membrane fractions were incubated in 100 μl of a reaction mixture containing 50 mM Tris-HCl pH7.5, 1 mM $MgCl_2$ and 0.2 mM phosphatidic acid for 20 min at 30 °C. Reaction was blocked by adding 1 ml of solution containing 0.5% ammonium molybdate, 0.5% SDS, 2% sulphuric acid and 0.5% ascorbic acid. The absorbance of blue colour developed was read at 740 nm. Specific activity of PAP was determined subtracting the value of absorbance obtained carrying out the reaction in the absence of PA. The amount of phosphate released was determined by interpolating a standard curve obtained with Na_2HPO_4 at different concentrations.

2.13. RT-qPCR analysis

Total RNA was extracted from 100 mg of Arabidopsis leaves using the RNeasy Plant Mini Kit RNA from Qiagen (Hilden, GE). Two micrograms of RNA were used for retro-transcription with M-MLV from Invitrogen (Carlsbad, CA). qPCR was performed in triplicates by using validated qPCR primers from Sigma (St. Louis, MO) specific for *AHA1* and *AHA2* transcripts, KAPA SYBR FAST qPCR Master mix from KapaBiosystems (Boston, MA) and the Real-Time PCR Light-Cycler II from Roche Diagnostics (Indianapolis, IN). mRNA levels were normalized to beta 8-tubulin mRNA (TUB8), and the relative mRNA levels were determined by using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001).

2.14. Statistics

Statistical analysis was performed by means one-way (Figs. 3 and 5) or two-way (Figs. 1, 2, 4 and 6) analysis of variance using GraphPad Prism software.

3. Results

3.1. Low temperature exposure for diverse time lengths differently affects H^+-ATPase and PLD activity

Cold stress affects plant cells at different levels. Plasma membrane damage is a known effect detectable when plants are exposed to cold conditions (Yu et al., 2006; Kim et al., 2013) and it can be measured as increased ion leakage and lipid peroxidation. When Arabidopsis plantlets were treated at 4 °C for different periods of time, both the effects were observed after 6 h as shown in Fig. 1, accordingly with data already reported (Ahn et al., 2000; Kim et al., 2013; Liu et al., 2013).

The effect of cold stress was studied on the activity of the H^+-ATPase and PLD, two fundamental plasma membrane enzymes involved in the plant response to different stresses (Bargmann and Munnik, 2006; Duby and Boutry, 2009).

The activities of both enzymes were determined in plasma membrane fractions purified from Arabidopsis leaves exposed to 4 °C for different time lengths. As reported on Fig. 2, after a short time (6 h) an increase of activity was detectable for PLD (Fig. 2A) and it became even more significant at longer treatments. At difference, the H^+-ATPase after 6 h cold exposure showed a strong decrease of the activity compared to the control (Fig. 2B). The ATP hydrolysis activity was recovered after 12 h treatment, turning out to be almost as the same as that of control plantlets.

Interestingly, a longer exposure to 4 °C (18 h) caused the significant induction of the H^+-ATPase activity of about fourfold, similarly to PLD.

It is known that PLD enzyme exists as various isoforms whose activities may be differentiated by requirement as calcium and oleic acid (Hong et al., 2016). To determine which isoforms were induced by 4 °C treatment, PLD activity was measured at millimolar and micromolar Ca^{2+} concentration and in the presence or in the

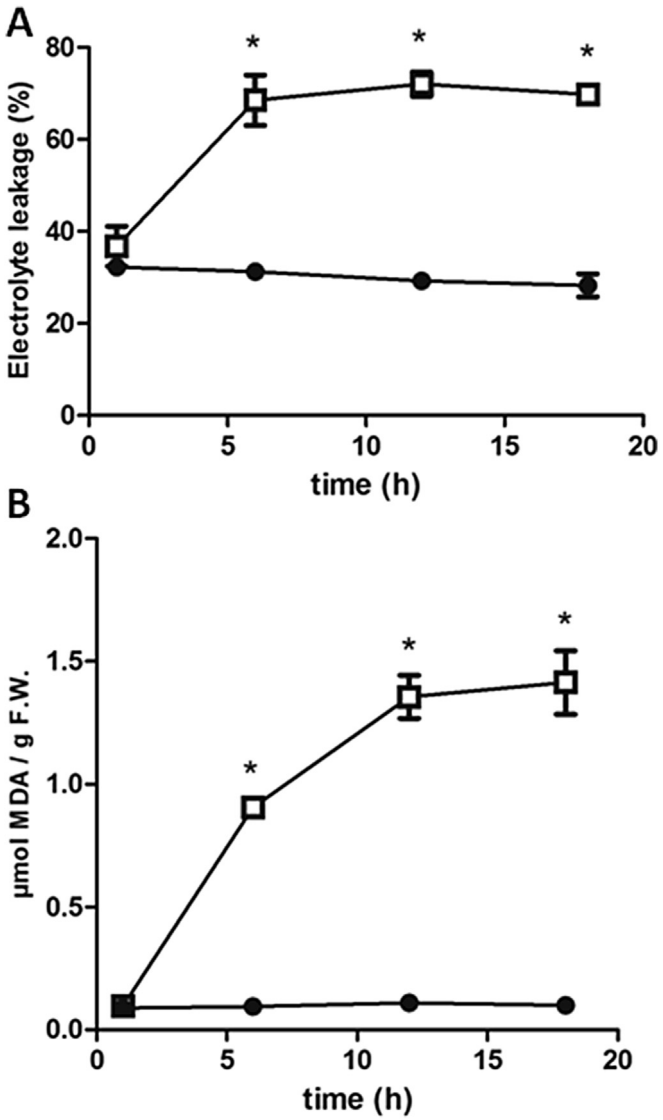

Fig. 1. Plasma membrane damage induced by 4°C exposure. Plasma membrane damage induced by 4 °C treatment for different time lengths was evaluated as ion leakage release (A) and lipid peroxidation, measuring MDA production (B). ●, control plants; □, plants exposed to 4 °C. The illustrated data are the means ± standard error for three independent experiments. *, $p < 0.05$.

absence of oleic acid. As shown in the Fig. 2C, PLD activity was stimulated by oleic acid also at lower Ca^{2+} concentration, indicating that, besides the Ca^{2+}-dependent PLD isoforms, also the oleate-dependent PLDδ isoform (Wang and Wang, 2001) was activated by low temperature.

These results indicate that, when Arabidopsis plantlets were exposed to 4 °C, a rapid response was given at the plasma membrane level by H^+-ATPase and PLD.

3.2. H^+-ATPase and 14-3-3 levels in plasma membrane fractions from Arabidopsis leaves

It is well known that H^+-ATPase activation is dependent on the interaction with 14-3-3 proteins, occurring after phosphorylation of threonine 948 in the C-terminal domain of the enzyme (Jahn et al., 1997; Fuglsang et al., 1999). Since the major portion of 14-3-3 proteins present at the plasma membrane level is associated to the H^+-ATPase, the effect of cold stress treatment of Arabidopsis

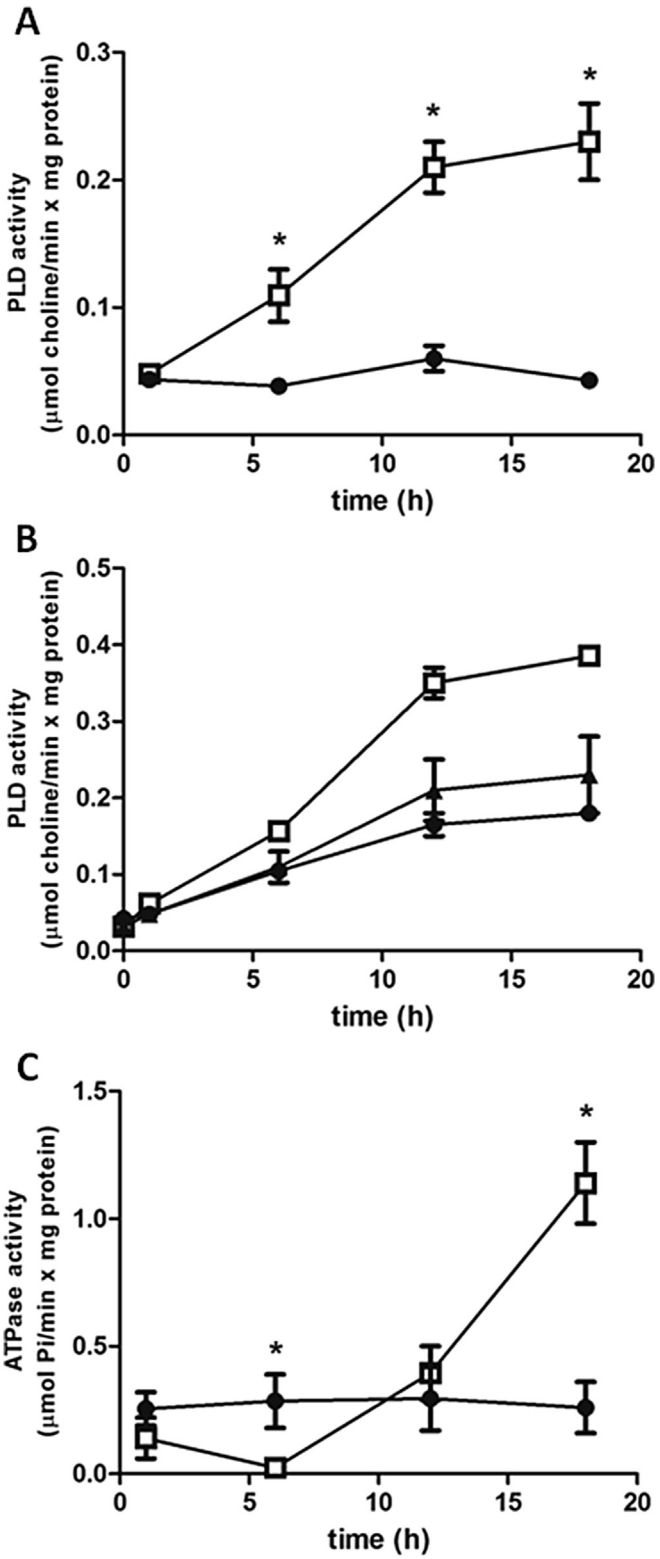

Fig. 2. Effect of 4°C exposure on the H^+-ATPase and PLD activity. A, PLD activity of Arabidopsis plasma membranes purified from control (●) and plants exposed to 4 °C (□).B, Ca^{2+} and oleate dependence of PLD activity from stressed plants. PLD activity in the presence of: □, 10 mM $CaCl_2$ and 0.6 mM oleic acid; ▲, 10 µM $CaCl_2$ and 0.6 mM oleic acid; ●, 10 mM $CaCl_2$. C, H^+-ATPase phosphohydrolytic activity of Arabidopsis plasma membranes purified from control (●) and plants exposed to 4 °C (□). The illustrated data represent activity means ± standard error for three independent experiments. *, $p < 0.05$.

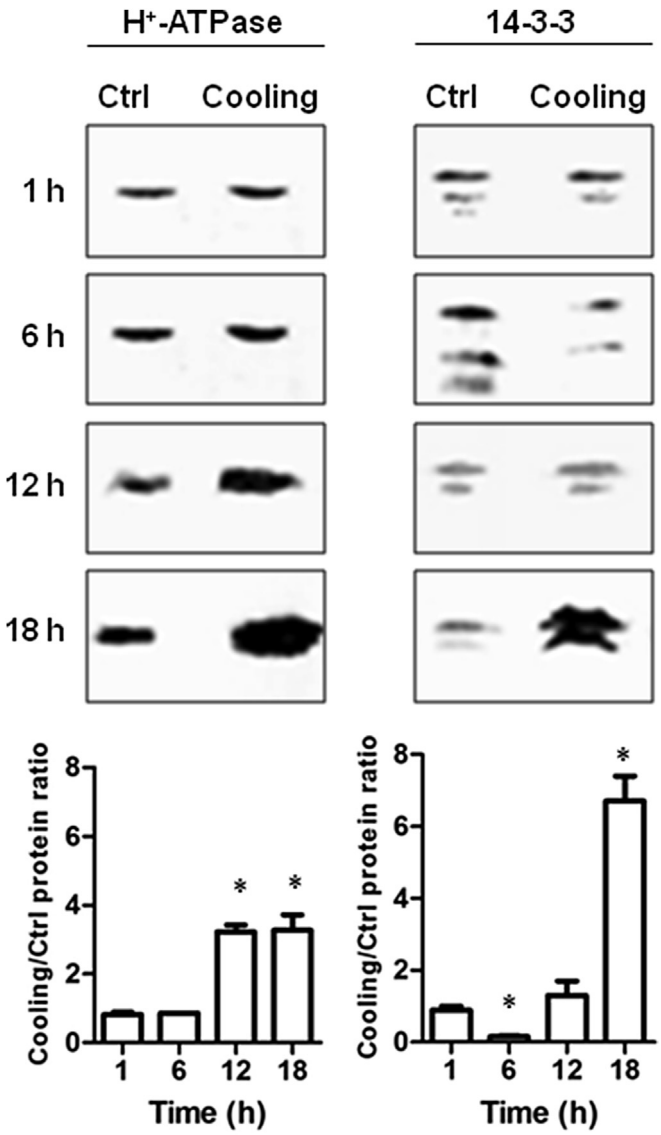

Fig. 3. H^+-ATPase and 14-3-3 levels in plasma membrane fractions purified from Arabidopsis plants exposed to 4°C. Upper panels, left: immunoblotting with anti-H^+-ATPase on plasma membrane purified from control (Crtl) and 4°C-exposed plants (Cooling) for the indicated times. Right, immunoblotting with anti-14-3-3 antibodies. Lower panels, densitometric analysis of immunodecorated bands from three independent experiments, performed using the ImageJ software. *, $p < 0.05$.

plantlets on 14-3-3 and H^+-ATPase levels in leaf plasma membranes was measured. Plasma membrane fractions from Arabidopsis leaves were subjected to SDS-PAGE, transferred on nitrocellulose membrane and incubated with anti H^+-ATPase or anti 14-3-3 antibodies. As reported in Fig. 3 (right panels), western blot analysis of 14-3-3 proteins showed a reduced level of 14-3-3 associated to plasma membrane fraction after 6 h of 4 °C treatment.

Western blots for plasma membrane H^+-ATPase showed (Fig. 3, left panels) that the amount of enzyme was similar to controls after 1 and 6 h of 4 °C exposure, while after 12 h and 18 h, a four-fold increase of H^+-ATPase levels was observed, as indicated by the densitometric analysis of bands reported in Fig. 3, lower panels.

The observed higher concentration of plasma membrane H^+-ATPase after 4 °C treatment could be due to activation of gene transcription. To investigate this point, a real-time PCR analysis of Arabidopsis AHA1 and AHA2 encoding genes was performed.

As shown in Fig. 4, after 12 h cold stress both the isoforms showed an appreciable increase in gene transcription which became even more significant after 18 h. At this length time of exposure to 4 °C both the *AHA1* (Fig. 4, left panel) and *AHA2* (Fig. 4, right panel) genes were subjected to 100 fold higher transcription levels compared to controls.

3.3. Interaction between H^+-ATPase and 14-3-3 proteins during cold stress

Since it is known that the activation status of H^+-ATPase depends on 14-3-3 binding occurring upon H^+-ATPase phosphorylation, the effect of low temperature on the ability of H^+-ATPase to interact with 14-3-3 proteins was investigated by an overlay assay. To this purpose plasma membrane fractions purified from 4 °C treated plantlets were subjected to SDS-PAGE, transferred on nitrocellulose membrane and incubated with ^{32}P-labeled GF14ω isoform. The experiment was also performed in the presence of fusicoccin (FC), a fungal toxin known to activate the H^+-ATPase by strongly stabilizing the interaction with 14-3-3 proteins (Fullone et al., 1998). The results are reported on Fig. 5. Densitometric analysis of radioactive bands detected in overlay experiments showed that the H^+-ATPase present in plasma membrane purified from Arabidopsis plantlets subjected to 4 °C treatment for 6 h interacted with GF14ω at a very low extent compared to control. At longer 4 °C exposure (12 h) the interaction of H^+-ATPase with GF14ω became higher than control and was significantly increased at 18 h, in agreement with data on the phosphohydrolitic activity reported in Fig. 2B.

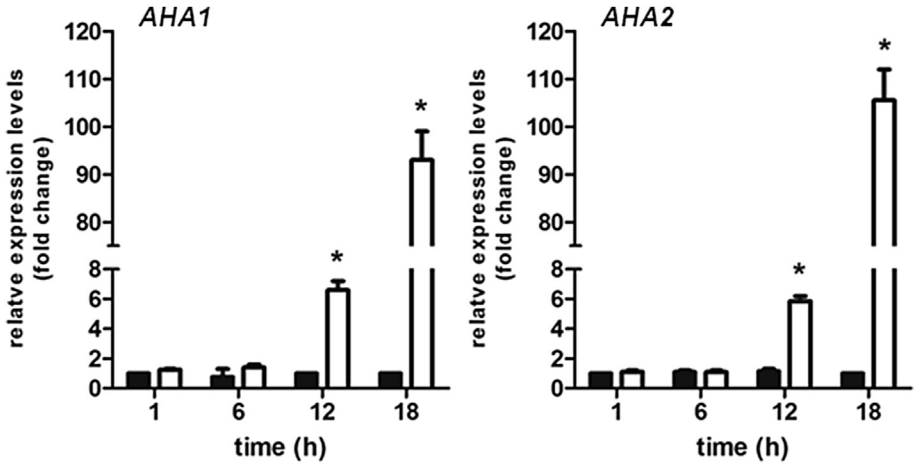

Fig. 4. Relative expression of H^+-ATPase genes in Arabidopsis leaves exposed to 4°C. To determine the expression of *AHA1* and *AHA2* H^+-ATPase genes, total RNA was extracted from Arabidopsis seedlings exposed to 4 °C for different time lengths, retro-transcribed and subjected to a real-time PCR analysis. mRNA levels were normalized to beta 8-tubulin mRNA (TUB8). Values are means of 3 replications ±standard error. *, $p < 0.05$.

3.4. Effect of low temperature exposure on PA and diacylglycerol production

The activities of the two enzymes H^+-ATPase and PLD under cold stress could be interrelated. It has been shown that the product of PLD activity PA acts as a second messenger in response to several biotic and abiotic stresses (Testerink and Munnik, 2011). It has been also demonstrated that PA binds to 14-3-3 proteins and consequently hampers their ability to interact with H^+-ATPase or other target proteins (Camoni et al., 2012). As shown in Fig. 2A during cold treatment PLD activity was markedly and continuously increased, thus producing a consequent enhancement of PA concentration that could explain the observed H^+-ATPase inhibition detected after 6 h cold treatment (Fig. 2B).

The PA release during the plantlets exposure to 4 °C was measured at the same different periods of time and reported on Fig. 6A, left panel. At 6 h a sharp increase in PA concentration was observed followed by a decline at longer time lengths. As PA is rapidly converted *in vivo* to DAG by phosphatidic acid phosphatase (PAP), the DAG concentration was measured in the same extracts. As shown in Fig. 6A, right panel, a rapid increase in DAG concentration was observed after 12 h treatment. The hydrolysis of PA was evaluated by measuring the activity of PAP in plasma membrane fractions. In Fig. 6B can be observed that PAP activity was stimulated at 12 h and 18 h of 4 °C treatment, while no activation was detected at shorter exposures.

Since it has been already demonstrated that PA, but not DAG, in vitro bind to 14-3-3 proteins (Camoni et al., 2012) in order to verify whether PA binding to 14-3-3 proteins may disrupt the 14-3-3/H^+-ATPase complex, the interaction studies between H^+-ATPase and 14-3-3 proteins were carried out in the presence or in the absence of PA and DAG. As shown in the overlay assay reported on Fig. 6C, binding of recombinant 14-3-3 proteins to the H^+-ATPase was markedly hampered by PA. The inhibitory effect was maximum at 150 μM PA, but evident also at 50 μM, a concentration range estimated to occur *in vivo* (Welti et al., 2002). As expected, no inhibition of 14-3-3-H^+-ATPase association was detected in the presence of DAG.

The change of both PA and DAG concentrations can be consistent with the H^+-ATPase inhibition at 6 h and its following recovery at 12 h.

4. Discussion

Plasma membranes from Arabidopsis plantlets treated under cold stress conditions (4 °C) were damaged as results from the increase of ion leakage and lipid peroxidation. This effect is known when plants are exposed to cold conditions. The activity of two important plasma membrane enzymes, the H^+-ATPase and the phosphoplipase D, has been investigated in the present work.

Data here reported show that the activity of the H^+-ATPase was strongly affected by low temperature treatment and that the effect was depending on the length of exposure. In particular, a relatively short time (6 h) induced a significant inhibition of the enzymatic activity (Fig. 2B) that correlates with a reduced amount of 14-3-3 proteins associated with its C-terminal regulatory domain. It is known that H^+-ATPase is fully activated by binding with 14-3-3 proteins occurring at the extreme C-terminal end of the enzyme as the consequence of the phosphorylation of a specific threonine residue (Fuglsang et al., 1999; Camoni et al., 2000) in the mode III 14-3-3 binding motif. Overlay assay demonstrated that upon 6 h 4 °C treatment, the H^+-ATPase lost the ability to bind 14-3-3 proteins in vitro (Fig. 5), suggesting that exposure to low temperature induces the dephosphorylation of the H^+-ATPase 14-3-3 binding site. Accordingly, the amount of 14-3-3 proteins associated to plasma membrane preparations resulted significantly reduced while no variation of the H^+-ATPase levels was observed (Fig. 3). Interestingly, a longer period of 4 °C exposure determined a recovery of the H^+-ATPase activity: upon 12 h of cold stress, the H^+-ATPase activity resulted comparable to that of control plants. An increase of the enzyme level was determined, due to the induction of *AHA1* and *AHA2* gene transcription, as shown by RT-qPCR analysis (Fig. 4). However this was not associated to a higher enzymatic activity, probably because the H^+-ATPase, even though increased, was not fully phosphorylated at the C-terminal 14-3-3 binding site. This hypothesis was corroborated by overlay data, where a similar 14-3-3 association with H^+-ATPase purified from control and

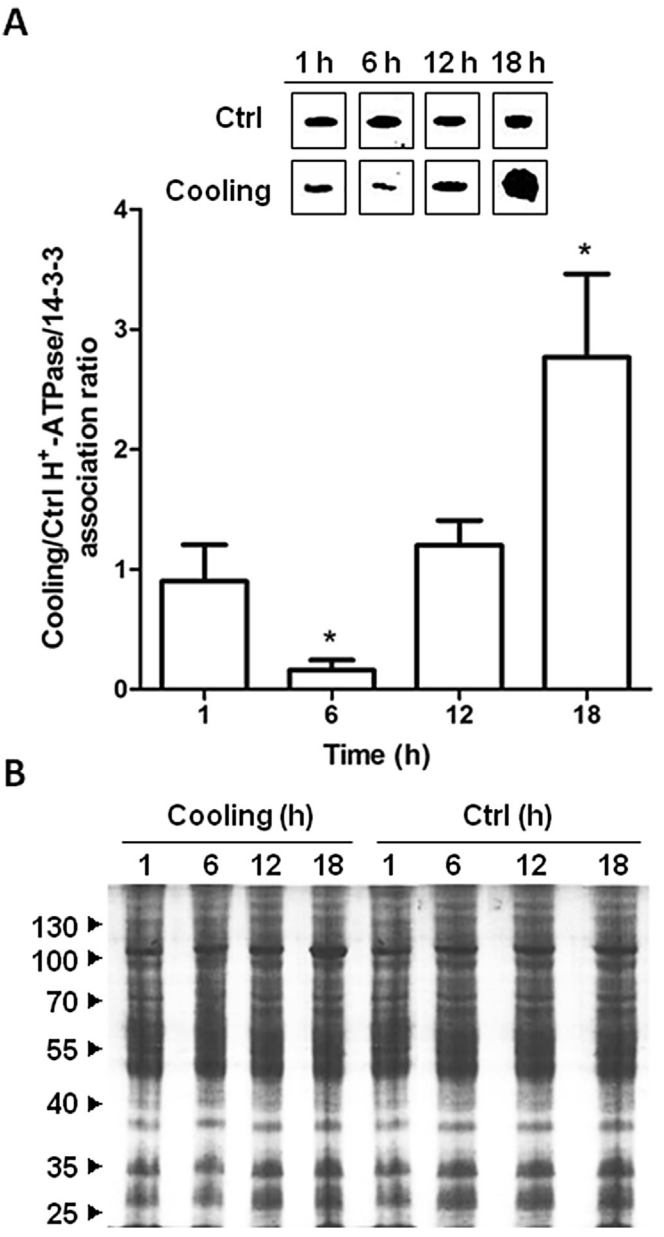

Fig. 5. Effect of 4°C exposure on the interaction between the H^+-ATPase and 14-3-3 proteins. A, upper panel: overlay assay of plasma membranes purified from control (Ctrl) and cold-exposed Arabidopsis plants (Cooling). Plasma membrane fractions (20 μg of proteins) were subjected to SDS-PAGE, blotted onto nitrocellulose membrane and incubated in buffer H with ^{32}P-labeled GF14ω as described in the Material and Methods. A, lower panel, densitometric analysis of bands from three independent experiments, performed using the ImageJ software. *, $p < 0.05$. B, Coomassie staining of plasma membrane fractions utilized in overlay assay.

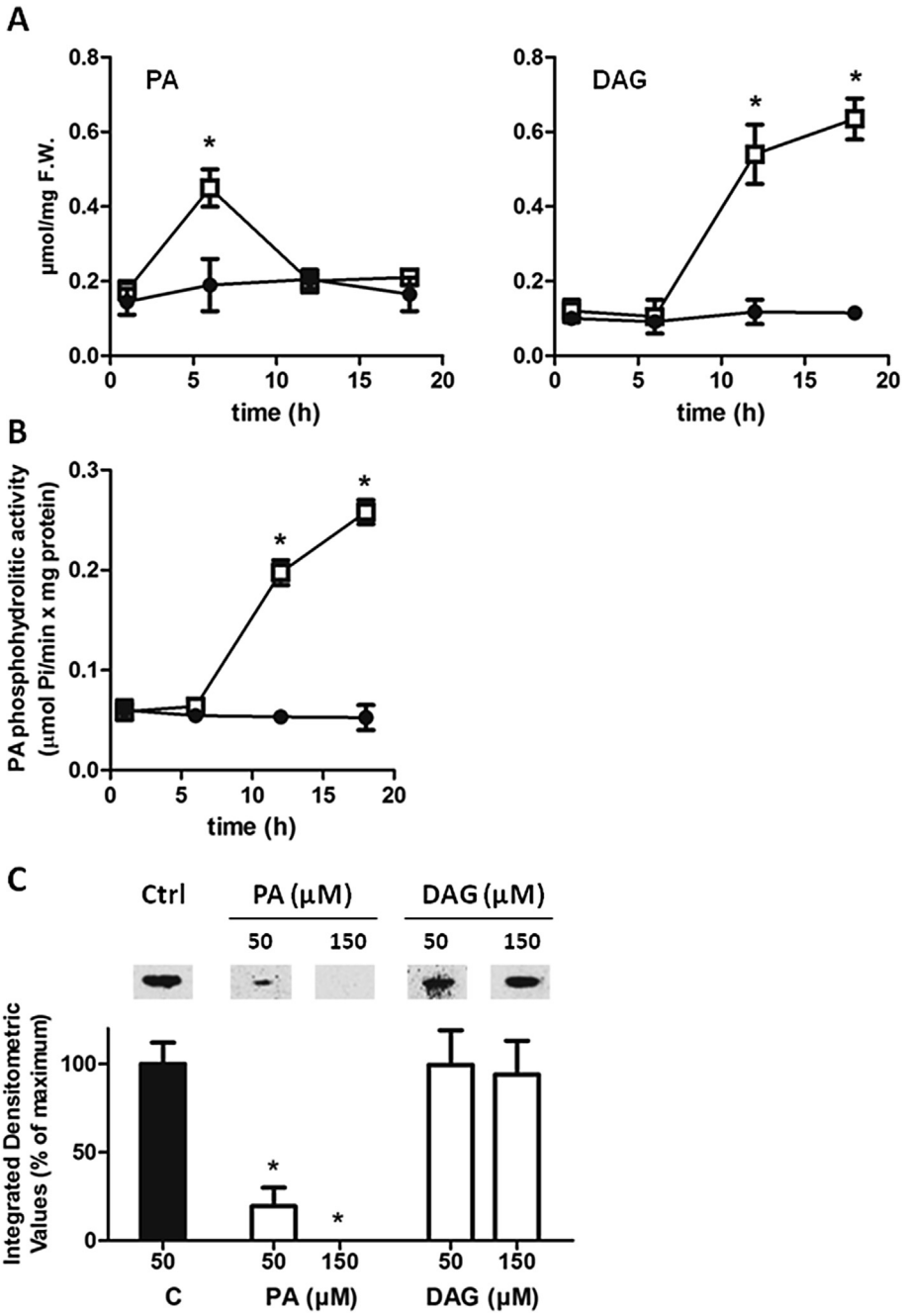

Fig. 6. 4°C-induced changes of PA and DAG levels. A, amount of PA (left panel) and DAG (right panel) in control (●) and in 4°C-exposed Arabidopsis plants (□). The illustrated data are mean ± standard error of three independent experiments. *, $p < 0.05$. B, PA phosphohydrolitic activity measured in plasma membrane fractions purified from control (●) and 4°C-exposed Arabidopsis plants (□). The illustrated data represent activity means ± standard error for three independent experiments. *, $p < 0.05$. C, overlay assay. PA and DAG effect on the interaction between H^+-ATPase and 14-3-3 proteins. Densitometric analysis of bands was performed on three independent overlay assays. *, $p < 0.05$.

stressed plants was observed in vitro (Fig. 5), since it is known that the in overlay experiments the in vitro interaction of H^+-ATPase with a recombinant 14-3-3 protein is completely dependent on the phosphorylation status of the enzyme (Fuglsang et al., 1999; Camoni et al., 2000). Accordingly, the levels of 14-3-3s present in the plasma membrane of control and stressed plants were almost identical after 12 h 4 °C treatment (Fig. 3). It is remarkable to note that the H^+-ATPase is dephosphorylated by a protein phosphatase 2A (PP2A) (Camoni et al., 2000), which has been reported to be involved in cold stress response (Pais et al., 2009; Zhao, 2015).

When the 4 °C exposure was extended to 18 h, the H^+-ATPase activity strongly rose. The effect may be due both to the higher phosphorylation level with the consequent 14-3-3 binding and to the remarkable activation of the H^+-ATPase gene transcription which resulted more than 100-fold increased compared to the control. The activation of H^+-ATPase observed for 4 °C exposure longer than 12 h was in accordance with that already reported for a cold-tolerant species as camelina (Kim et al., 2013). Interestingly, this H^+-ATPase behaviour, which resulted first inhibited by short periods of 4°C-induced stress and then activated after prolonged exposures, was similar to that reported by Janicka-Russak et al. (2012) on the H^+-ATPase activity in cucumber roots exposed at

10 °C, even though for a longer time span.

Low temperature induced the strong activation of PLD activity, evident at 6 h and reaching the maximum after 12 h of exposure (Fig. 2), in agreement with literature data demonstrating that PLD activation represents an early response to cold stress (Ruelland et al., 2002) and plays a crucial role in cold-induced freezing tolerance (Li et al., 2004). Accordingly with PLD activation, PA levels rose in plantlets exposed to cold stress (Fig. 6). PA is involved in many plant physiological responses and considered a lipid second messenger during plant stress, metabolism, and development (Li et al., 2009; Testerink and Munnik, 2011). Although the molecular mechanism by which PA exerts its effects is not fully clarified, several data indicate that PA functions as a membrane-localized signal, that affects downstream responses by interacting with specific protein targets. Interestingly, PA has been reported to bind 14-3-3 proteins, thus hampering their interaction with H^+-ATPase and consequently its enzymatic activation (Camoni et al., 2012). Therefore, PA produced by PLD during cold stress may bind 14-3-3 proteins thus inhibiting their association with the H^+-ATPase and consequently bringing about the inactivation of the enzyme observed after 6 h of low temperature exposure. Higher PA levels may account for the residual presence of 14-3-3 proteins at the plasma membrane as they were not bound to the H^+-ATPase which resulted completely inactive. Accordingly, the recovery of the H^+-ATPase activity observed at longer period of low temperature stress could be ascribable to the conversion of PA into DAG by PAP (Fig. 6).

It is also relevant to consider that recent studies have shown that PA is also able to bind PP2A subunit A1, thus recruiting it at the plasma membrane and activating PP2A activity (Gao et al., 2013). Therefore, it is possible that the high PA levels and the H^+-ATPase inhibition observed at 6 h of cold stress were related.

The H^+-ATPase inhibition can in fact be due both to its dephosphorylation and additionally to inhibition of its interaction with 14-3-3 proteins. Both the effects are affected by PA which can regulate PP2A activity and 14-3-3 binding.

Authors' contribution

SV and PA conceived the project. CM, LC and SV designed the experiments. CM, LC, and SV performed the experiments and data analysis. SV and PA wrote the manuscript. All authors participate to the critical revision of the manuscript.

Disclosures

The authors declare that there is no conflict of interest.

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