Plant Physiology and Biochemistry 101 (2016) 88–95

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

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

Research article

Diacylglycerol pyrophosphate binds and inhibits the glyceraldehyde-3-phosphate dehydrogenase in barley aleurone

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ARTICLE INFO

Article history:
Received 15 October 2015
Received in revised form
14 January 2016
Accepted 18 January 2016
Available online 27 January 2016

Keywords:
Aleurone
Abscisic acid
Diacylglycerol pyrosphoshate
Phosphatidic acid
Glyceraldehyde-3-Phosphate dehydrogenase

ABSTRACT

The aleurona cell is a model that allows the study of the antagonistic effect of gibberellic acid (GA) and abscisic acid (ABA). Previous results of our laboratory demonstrated the involvement of phospholipids during the response to ABA and GA. ABA modulates the levels of diacylglycerol, phosphatidic acid and diacylglycerol pyrophosphate (DAG, PA, DGPP) through the activities of phosphatidate phosphatases, phospholipase D, diacylglycerol kinase and phosphatidate kinase (PAP, PLD, DGK and PAK). PA and DGPP are key phospholipids in the response to ABA, since both are capable of modifying the hydrolitic activity of the aleurona. Nevertheless, little is known about the mechanism of action of these phospholipids during the ABA signal. DGPP is an anionic phospholipid with a pyrophosphate group attached to diacylglycerol. The ionization of the pyrophosphate group may be important to allow electrostatic interactions between DGPP and proteins. To understand how DGPP mediates cell functions in barley aleurone, we used a DGPP affinity membrane assay to isolate DGPP-binding proteins from *Hordeum vulgare*, followed by mass spectrometric sequencing. A cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) was identified for being bound to DGPP. To validate our method, the relatively abundant GAPDH was characterized with respect to its lipid-binding properties, by fat western blot. GAPDH antibody interacts with proteins that only bind to DGPP and PA. We also observed that ABA treatment increased GAPDH abundance and enzyme activity. The presence of phospholipids during GAPDH reaction modulated the GAPDH activity in ABA treated aleurone. These data suggest that DGPP binds to GAPDH and this DGPP and GAPDH interaction provides new evidences in the study of DGPP-mediated ABA responses in barley aleurone.

1. Introduction

Biological membranes are composed of different types of lipids with distinct properties and functions. Besides the structural functions of phospholipids, some of them provide spatial information for cell signalling in plants. Signal lipids are synthesized rapidly and transiently in response to external stimuli to activate downstream signalling pathway. In plants, the minor phosphatidic acid (PA) plays a key role as a precursor in the biosynthesis of glycerophopsholipids (GPL) and triacylglycerols (TAG), and as an important signal-transducing molecule (Testerink and Munnik, 2011).

In addition to *de novo* synthesis in the endoplasmic reticulum, PA may be generated from plasma and chloroplast membrane glycrophospholipids (GPL) like phosphatidylcholine (PC), phosphatidylethanolamine (PE) or phosphoinositides by the hydrolytic actions of phospholipases. Such actions are phospholipase D (PLD), or phospholipase C (PLC) followed by diacylglycerol kinase (DGK) (Pokotylo et al., 2014). PLD- or PLC/DGK-generated PA, has been proposed to have intracellular messenger functions, since it activates a number of physiological events in plants through the activation of specific protein targets (Liu et al., 2013).

PA has indeed been shown to interact with abscisic acid-insensitive 1 (ABI1), a protein phosphatase, constitutive triple response (CTR1), a protein kinase, and phosphoethanolamine N-methyltransferase (PEAMT). It also inhibits their phosphatase or kinase activities (Zhang et al., 2004; Testerink et al., 2007; Jost et al.,

Abbreviations: ABA, abscisic acid; DAG, diacylglycerol; DAGK, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DGPPase, diacylglycerol pyrophosphate phosphatase; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GA, gibberellic acid; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; TAG, triacylglycerols; TLC, thin layer chromatography.

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http://dx.doi.org/10.1016/j.plaphy.2016.01.012
0981-9428/

2009). PA stimulates the catalytic activity of a phosphoinositide-dependent protein kinase (PDK1), sphingosine kinases (SPHK1/2), NADPH oxidases (RbohD/F), a mitogen-activated protein kinase (MPK6), and a SNF1-related kinase (SnRK2) by direct interaction (Anthony et al., 2004; Zhang et al., 2009; Yu et al., 2010; Guo et al., 2011).

In addition to enzymes involved in PA generation, those responsible for PA removal, shortly after its increase, may play a role in terminating PA signalling events. Reduction of PA levels after PLD activation may be effected by an active PA kinase (PAK) that phosphorylates PA to yield diacylglycerol pyrophosphate (DGPP) or by lipid phosphate phosphatases (LPPs), which dephosphorylate PA to produce DAG.

Diacylglycerol pyrophosphate (DGPP) is a minor phospholipid found in biological membranes, with a relatively simple chemical structure within the glycerophospholipid family (Wissing and Behrbohm, 1993a). DGPP is synthesized from phosphatidic acid (PA) and ATP via the reaction catalysed by phosphatidate kinase (PAK) and dephosphorylated to PA by the enzyme DGPP phosphatase (Wissing and Behrbohm, 1993b). The average concentration of DGPP in cell membranes is usually very low but evidence suggests that DGPP may act as a novel second messenger with important roles in diverse cellular processes in plants that are related to drought and osmotic stress or salinity (van Schooten et al., 2006). DGPP formation is transient and it is always associated with variations of the amount of PA. Therefore, its synthesis may also be involved in attenuating PA levels (Munnik et al., 1996; van Schooten et al., 2006; Racagni et al., 2008; Paradis et al., 2011).

DGPP is an anionic phospholipid with a pyrophosphate group attached to diacylglycerol. It was shown that, depending on the pH, the pyrophosphate moiety of DGPP could display 2 or 3 negative charges, making it a highly polar molecule (Villasuso et al., 2010; Strawn et al., 2012). Consequently, the ionization of the pyrophosphate group may be important for allowing electrostatic interactions between DGPP and proteins as well as with bivalent cations such as Zn^{2+} and Ca^{2+} (Han et al., 2001; Zalejski et al., 2006; Strawn et al., 2012). Physicochemical studies have shown that DGPP stabilizes the bilayer phase at neutral pH, unlike PA that 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). Recent studies of the surface properties of DGPP in Langmuir monolayers showed that DGPP and PA exhibit expanded and condensed phase states depending on pH, on the proportion of each lipid in the film and on the presence of Zn^{2+} (Villasuso et al., 2014). Although its levels are very low under control conditions, DGPP levels are induced upon several biotic and abiotic stimulus (van Schooten et al., 2006). Moreover, treatment of cells, aleurones or seeds with abscisic acid (ABA) was also shown to induce an increase in DGPP (Katagiri et al., 2005; Zalejski et al., 2006; Paradis et al., 2011; Villasuso et al., 2013). The kinetics of synthesis of PA and DGPP, as well as labelling assay has shown that DGPP is increased by phosphorylation of PA. However, the gene encoding the enzyme PAK still has not been identified. It is evident that our understanding of DGPP signalling is still incomplete.

In barley aleurone layers, synthesis of hydrolytic enzymes (mainly α-amylase) is induced by gibberellin (GA), thus providing resources for seed germination and early seedling growth, whereas ABA inhibits this response (Lovegrove and Hooley, 2000). PA and DGPP levels regulate ABA response in barley aleurone. However, whether both molecules are engaged in the lipid–protein interactions remains to be elucidated.

To understand how DGPP functions as a lipid mediator, a DGPP affinity membrane approach was used to identify DGPP-interacting proteins. It was found that cytosolic GAPDH as a potential DGPP interacting protein. GAPDHs are enzymes conserved in all living organism, where they catalyse the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate in glycolysis. In this work, we also described how the GAPDH activity is regulated by ABA and phospholipids.

2. Results

2.1. Identification of DGPP-binding protein

To isolate DGPP-binding proteins, we used a DGPP-on-nitrocellulose membrane to incubate with total proteins extracted from *Hordeum vulgare* in an initial attempt to study the DGPP signalling in aleurone lipid metabolism. Proteins bound to the membrane were eluted and subjected to SDS-PAGE. Fig. 1A shows barley aleurone protein profile obtained from control crude extract. Fig. 1B shows the proteins that bound the DGPP-nitrocellulose in the absence of soluble DGPP, but not in its presence. The most clear examples are marked with arrows in Fig. 1B. Attention was focussed on proteins whose binding could be competed by DGPP. Protein bands whose presence in the DGPP-binding fractions were reduced in the presence of excess free DGPP, were excised from the gel, treated with trypsin, and the resulting tryptic peptides were analysed using mass spectrometry (Fig. 2). One of the proteins, a 36 kDa band (Fig. 2, marked between asterisks) was identified as GAPDH. Unfortunately, most of the proteins could not be identified, either because the amounts were too limited or because the MS fingerprint did not give a hit in the barley databases.

2.2. Confirmation of the DGPP – GAPDH binding

To validate our method, the relatively abundant GAPDH was further characterized with respect to its lipid-binding properties, using an antibody. Fig. 3A shows protein profile obtained from aleurone control and ABA-stimulated by 30 min Fig. 3B shows that the antibody against GAPDH cross-reacted with barley GAPDH. In this way, the identity of the 36 kDa protein as GAPDH was

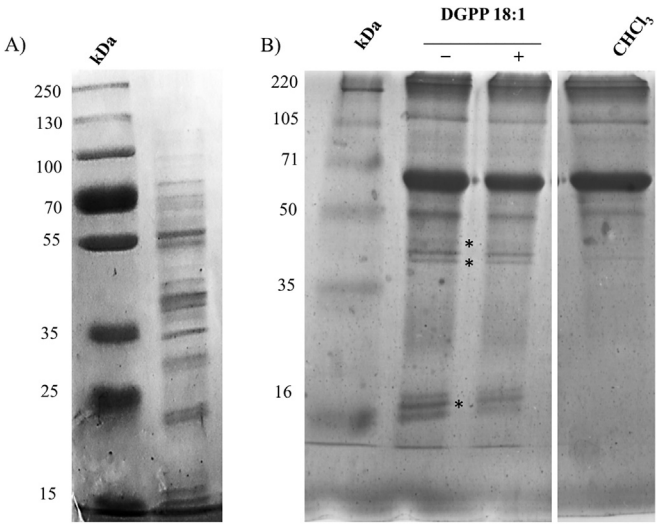

Fig. 1. Identification of DGPP-binding proteins. SDS-PAGE image of the candidate DGPP-binding proteins. (A)Total proteins profile from *H. vulgare* were incubated with DGPP-spotted nitrocellulose membrane, and proteins bound to the membrane were resolved by SDS-PAGE. (B) Asterisks indicate the proteins present in the DGPP-spotted membrane but not in the presence of excess free DGPP or in the solvent-only spotted membrane.

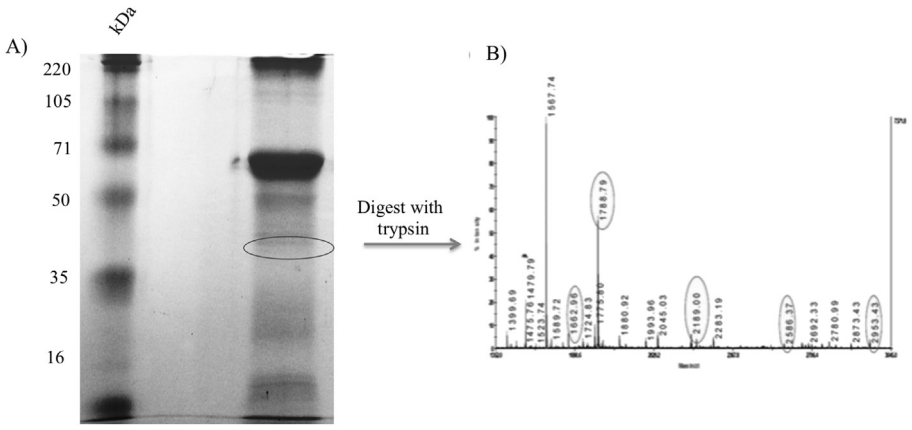

Fig. 2. Identification of a DGPP-binding protein as glyceraldehyde-3-phosphate dehydrogenase. A 36 kDa band was cut out from the gel (A), digested with trypsin and the tryptic peptides were analysed using MALDI-TOF-TOF (B).

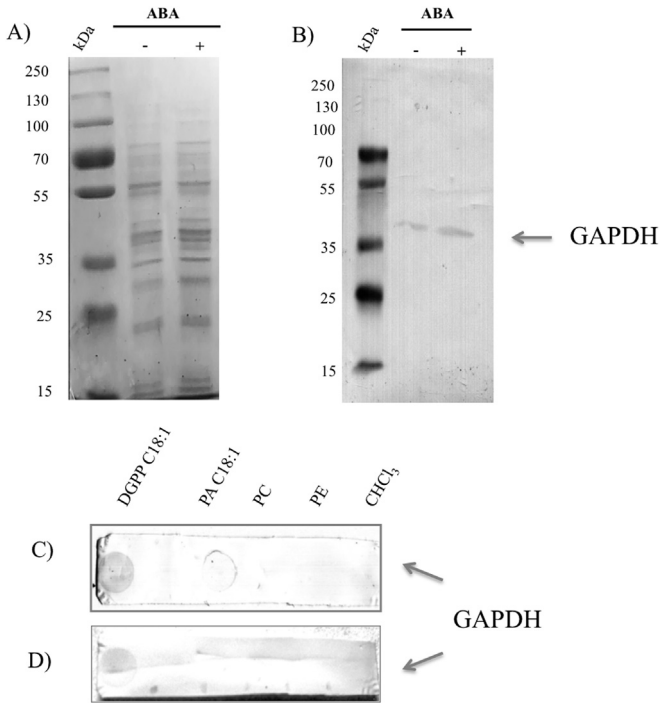

Fig. 3. Increased abundance of some proteins in the DGPP-binding fraction in response to ABA. (A) Barley aleurone cells were treated for 30 min with $CaCl_2$ or ABA 5 μM. Soluble proteins were extracted, eluted and separated on SDS-PAGE and detected by coomasie. (B) Aliquots of the soluble fractions were subjected to SDS-PAGE, proteins were transferred to nitrocellulose sheets and GAPDH content was determined by Western blotting using an anti-GAPDH as described in Materials and Methods. (C) Approximately 10 μg or 1 μg (D) of DGPP C18:1, PA C18:1, PC, PE were spotted on nitrocellulose membrane, incubated with soluble protein, and GAPDH was detected by using an anti-GAPDH and detected by the 4-chloro-1-naphthol method.

confirmed. When comparing the control and ABA-treated aleurone, an important difference was observed in the GAPDH level (Fig. 3B). As GAPDH was shown that bind to PA in *Arabidopsis thaliana* (Kim et al., 2013), a dot blots with others phospholipids were carried out. For this, different concentration of the phospholipids (10 μg and 1 μg; Fig. 3C and D, respectively) were spotted onto a nitrocellulose membrane; then incubated with crude extract of protein and the blot was subsequently incubated with an anti-GAPDH antibody. Fig. 3C and D shows that the antibody reacts only with proteins that bind to PA and DGPP. These results confirm that DGPP interact with GAPDH.

2.3. Effect of ABA and phospholipids on GAPDH activity

Later GAPDH activity was assayed in the crude extract from control and ABA-stimulated aleurone. Fig. 4A shows that GAPDH activity was 0.33 ± 0.06 U mg of protein^{-1} in aleurone control, while that ABA treatment increased approximately 1.5 folds its activity (0.512 ± 0.08 U mg of protein^{-1}, $n = 10$, $P < 0.05$, *t*-test). To test the possible role of DGPP as a signalling molecule in the ABA signal, GAPDH activity was assayed in presence of DGPP. Using ABA-treated aleurone, addition of 50 μM dioleoyl-DGPP caused ~50% inhibition in GAPDH activity relative to maximal activity obtained with ABA. The DGPP effect was also observed with addition of 5 μM dioleoyl-DGPP (Fig. 4B) and it was also depending on the pre-incubation time (Fig. 4C). Other phospholipids were also tested to determine the specificity of the DGPP effect on GAPDH activity (Fig. 4D). GAPDH activity was measured in the crude extract from ABA-stimulated aleurone. The pre-treatment with 5 μM dioleoyl-DGPP reduced ~30% the GAPDH activity. The pre-incubation with 5 μM dioleoyl-PA or dipalmitoyl-PA, reduced ~40% the GAPDH activity. The water soluble short-chain C8:0 variant of PA decreased ~20% the GAPDH activity. The pre-incubation with phosphatidylcholine, phosphatidylethanolamine or phosphatidylinositol, led to a ~15% decrease of the GAPDH activity (Fig. 4D). The effects of the pre-incubation with a 10-fold higher concentration of phospholipids were tested. Fig. 4E shows, that the addition of 50 μM DGPP or PA decreased GAPDH activity to approximately 50% of the ABA treated aleurone activity. Similarly, a lower degree of inhibition of GAPDH activity was observed with PC, PE or PI.

2.4. Identification of barley GAPDH gene

To better understand barley GAPDH, we identified the predicted *GAPDH* gene in the barley database. The GenBank at NCBC website (www.ncbi.nlm.nih.gov) was searched for homologues of *GAPDH* genes. This search identified one predicted gene, which was noted at GenBank as X6034 and assigned as glyceraldehyde-3-phosphate dehydrogenase. BLAST searches showed that the sequences of barley have high similarity or identity to that of *Arabidopsis* GAPDH. It was therefore called *HvGAPDH* (*Hordeum vulgare* glyceraldehyde-3-phosphate dehydrogenase), and its product called HvGapdg2 (P08477). There was 72% or more amino acid sequence similarity (55% identity) between *Arabidopsis* and *Hordeum vulgare* GAPDHs. The size of *HvGAPDH* is 1365 bp, and they code for putative protein of

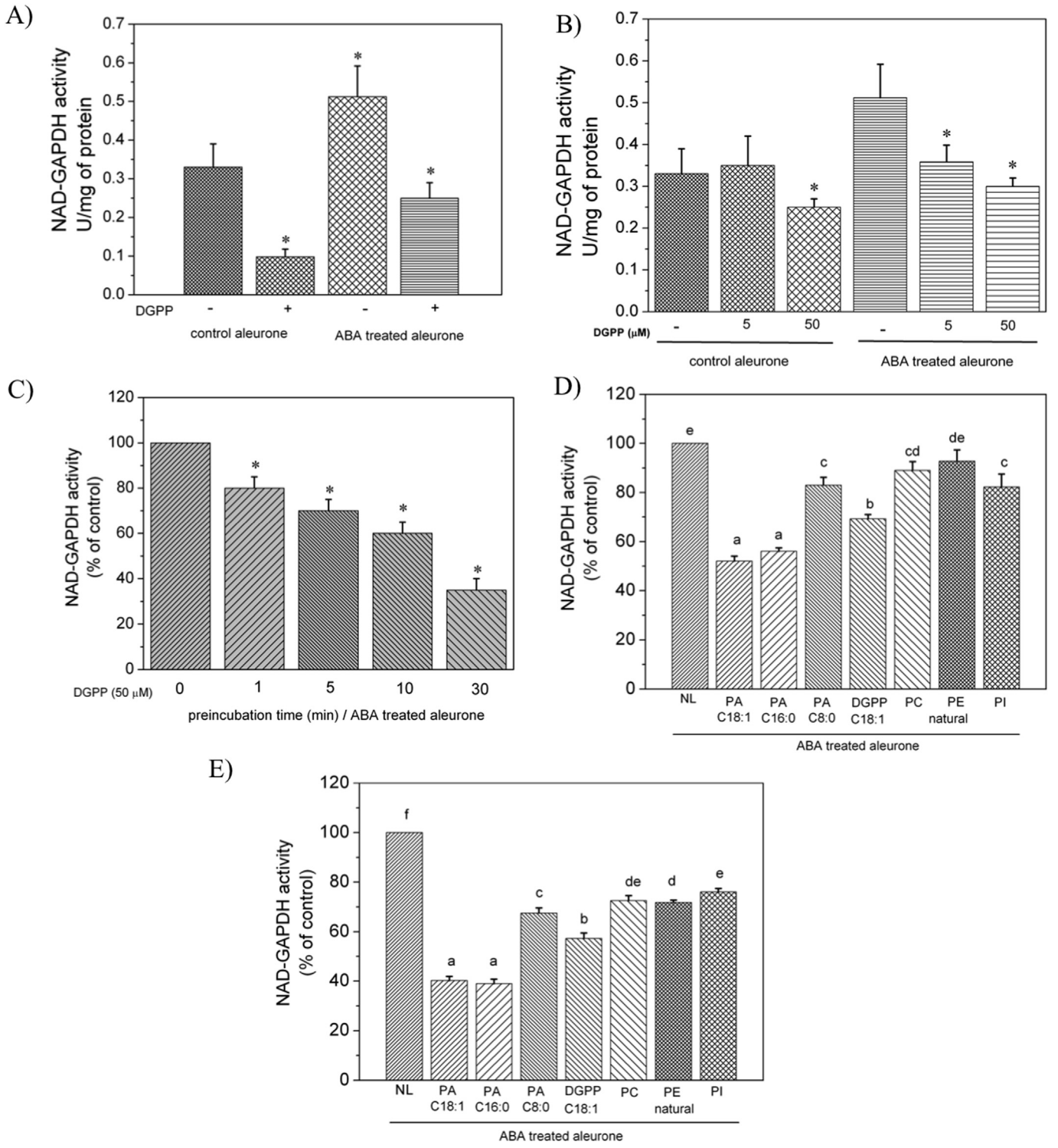

Fig. 4. NAD-GAPDH activity was determined in barley aleurone control and ABA treated by 30 min. GAPDH was assayed as described in materials and methods. (A) Effect of DGPP on aleurone barley NAD-GAPDH control and ABA-stimulated. (B) Effect of DGPP concentration on aleurone barley NAD-GAPDH activity. Data are means SEM, n = 5, and were compared using Student's t test. Asterisks indicate that the mean value is significantly different from that of the control (*$P < 0.05$). (C) Effect of DGPP time of pre-incubation on NAD-GAPDH activity. (D) Effect of phospholipids on NAD-GAPDH activity, 5 μM or 50 μM (E) of indicated phospholipids were added to enzyme assay and pre-incubated during 10 min and the GAPDH assayed as described in material and methods. Results are expressed as percent of GAPDH activity obtained in ABA treated aleurone (defined as 100%) SEM, n = 5. Data were compared using Tukey's test. Different letters indicate significant differences (Tukey's Test, $p < 0.05$). NL: no lipid added.

305 amino acids. The open reading frame of 1365 nucleotides on the cDNA codes for a putative 36-kDa protein. The barley protein showed the presence of conserved amino acid residues which are important for GAPDH activity as a cysteine residue at 22 position (Kim et al., 2013). Similar results were obtained in analysis of AtGapdh. A phylogenetic tree was constructed for members of the glyceraldehyde-3-phosphate dehydrogenase family, including HvGapdh. The *H. vulgare* protein was grouped with that of *A. thaliana* (Fig. 5B).

3. Discussion

DGPP is a minor lipid that accumulated transiently during ABA response in aleurone. However, several aspects of DGPP are completely unknown. Here, we have showed that affinity membrane assay followed by mass spectrometric allows to identify a DGPP-binding protein from *Hordeum vulgare*. Indeed, this could lead on to the detection and isolation of GAPDH as target of DGPP. Binding of GAPDH to DGPP was validated using an antibody and GAPDH activity was stimulated by ABA, and modulated by DGPP.

In barley, Glyceraldehyde-3-phosphate dehydrogenase is a 305 amino acid long polypeptide, which is highly conserved across the phylogenetic scale. It contains two major domains, the NAD + binding domain and the catalytic or glyceraldehyde-3-phosphate domain, which has an active site cysteine.

Initially, GAPDH was well characterized for its role in glycolysis, and recent studies in mammalian indicate those GAPDH amino acid sequences that are utilized for its new functions. In particular, its NAD + binding site is the loci for its role in determining mRNA stability and translation (Sirover, 2014). In *A. thaliana*, GAPDH is a target of PLDδ and PA (Guo et al., 2012; Kim et al., 2013). Moreover, GAPDH protein is a candidate PA targets in salt stress (Mcloughlin et al., 2013).

Several approaches have been applied to identify the molecular targets of PA (Testerink et al., 2004; McLoughlin et al., 2013). In addition, it has also been shown that the specificity of PA-protein interaction is likely related to the ionization properties of the phosphomonoester head group, and a model for such mechanism has been proposed on the basis of the ionization properties (Kooijman and Burger, 2009).

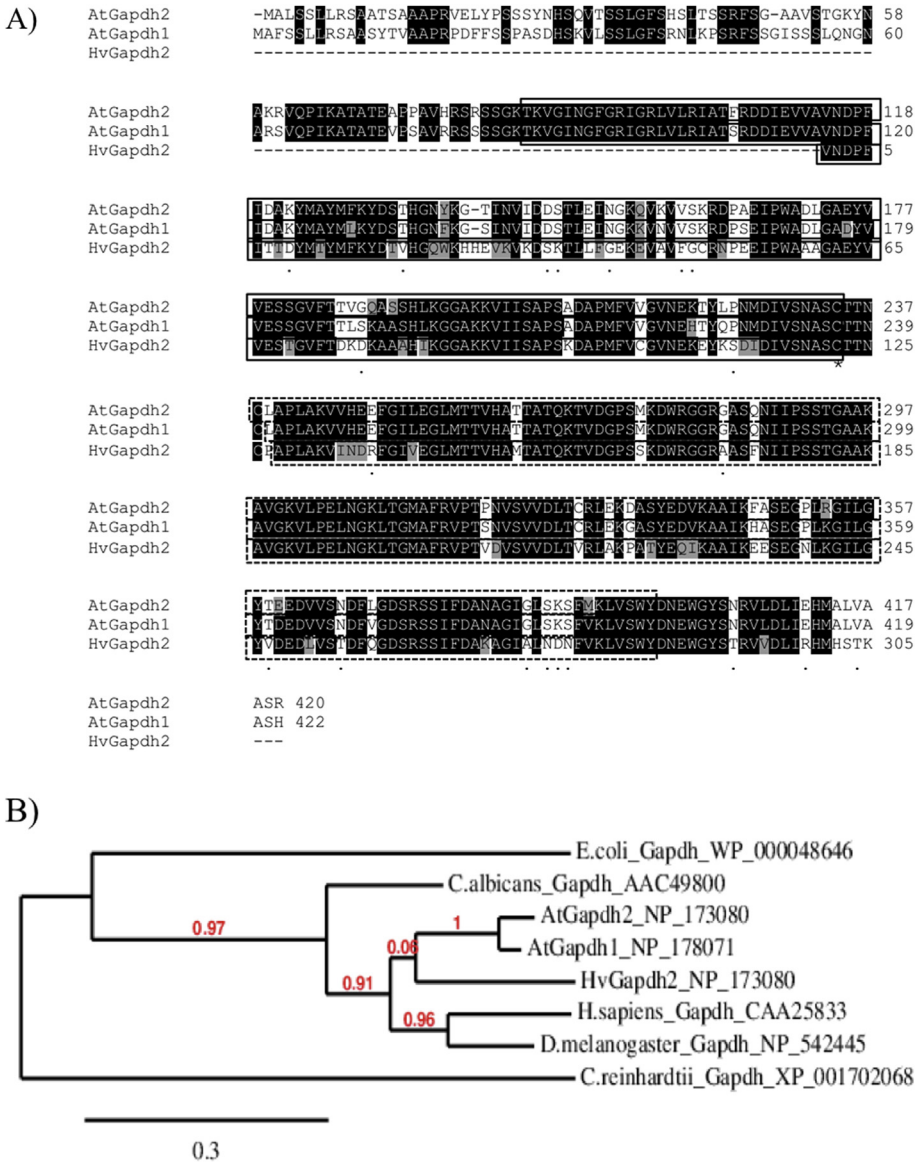

Fig. 5. Amino acid sequence comparison between the deduced HvGAPDH and *Arabidopsis thaliana* glyceraldehyde-3-phosphate dehydrogenase. (A) In the aligned amino acid sequences, invariant amino acids are boxed in grey and conserved amino acids are boxed in black. The black box indicates catalytic site, straight line indicates the NAD binding site and dashed line indicates *c*-terminal site. Asterisks indicate conserved cysteine residue of all GAPDH proteins. (B) Phylogenetic analysis of the glyceraldehyde-3-phosphate dehydrogenase family. The *Hordeum vulgare* GAPDH protein, the *Arabidopsis* protein, the *C. elegans* proteins, mammalian proteins, the *Drosophila* protein, as well as the *D. melanogaster*, *Chlamydomonas*, and *E. coli* genomes were used for comparison. The tree was built applying the Neighbor Joining Method to PAM distances computed on complete reliably aligned sites. SwissProt accession numbers (in *brackets*) designate all protein sequences. The length of horizontal branches is such, that the evolutionary distance between two proteins is proportional to the total length of the horizontal branches that connect them. Bootstrap values are shown at the nodes.

However, our understanding of DGPP formation and degradation in aleurone is still fragmentary and its molecular function is still unknown. On the other hand, recent physicochemical studies of DGPP suggest that the effective lipid molecular shape and the ionization properties of the phosphomonoester head group of DGPP are similar to PA. Therefore, the ionization properties of the phosphomonoester of DGPP mimic those of PA following the electrostatic–hydrogen bond switch model. In spite of this, DGPP is not a cone-shaped lipid, i.e., it is not capable of imparting negative curvature stress to the membrane that could facilitate the insertion of hydrophobic protein domains in the membrane (Strawn et al., 2012). This fact suggested that PA binding proteins might no longer be able to bind to DGPP due to its lack of negative curvature, higher negative charge, and different position of the phosphomonoester in the headgroup interface of the lipid bilayer. Until now, no specific DGPP binding protein has been identified to date aside from the observation that Opi1 has some affinity for DGPP (Young et al., 2010). Our data shows that DGPP could have the ability to interact with protein in a both similar/different fashion as PA. We hypothesized that this fact could modulate the initial PA signal during ABA response. The exact mechanism of DGPP action in this process is not yet clear, although some possibilities are to occur. When PA is phosphorylated to DGPP by the enzyme PAK, local membrane curvature stress is decreases and local electrostatic negative charge increases. This change in lipid packing may attenuate the PA signal. Alternatively, DGPP could strengthen the docking of the protein to the membrane or act as a signal itself. Additional experiments are necessary to dilapidate the DGPP role in the membrane packing during protein interaction.

On the other hand, one effect of lipid–protein interaction is the modulation of the protein catalytic activity, which can be inhibitory or stimulatory, depending on the effector protein. For example, ABA signalling in plant requires the function of many proteins and PA has been found to interact with several of them, including negative effector ABI1 and positive effectors NADPH oxidase and phytosphingosine kinase (Zhang et al., 2009; Guo et al., 2011). Similar results were observed on phosphoenolpyruvate carboxylase (PEPC), a key enzyme in photosynthesis of C4 plant. The anionic phospholipids as PA (C18:1) inhibit C_4 PEPC activity and it localizes it to membrane (Monreal et al., 2010).

In barley aleurone, we studied the ABA transduction pathway that inhibits amylase secretion. We demonstrated that a part of the signalling cascade is located at the plasma membrane. ABA stimulated PLD activity whose product, PA, could be the substrate of PAP1 and/or PAK activities and that DGPP is likely to be an important signalling partner. Application of ABA stimulates a transient increase in PA followed by an increase in DGPP. Interestingly, application of dioleoylDGPP and dioleoylPA, inhibit amylase secretion mimicking ABA. Moreover, we showed in vitro PA kinase - that allows the production of 18:2-DGPP from 18:2-PA - is stimulated by ABA (Racagni et al., 2008; Villasuso et al., 2013).

Now, we provide evidence that ABA increases the GAPDH activity and we identify the anionic phospholipid as DGPP and PA as novel regulators that inhibit the GAPDH activity through an unknown mechanism. Experiments in progress attend to dilucidate the physicochemical properties of DGPP-GAPDH interaction and its relation with PA levels.

4. Materials and methods

4.1. Plant material

Barley grains (*Hordeum vulgare* L, cv. Himalaya) were deembryonated, surface sterilized, and allowed to imbibe in sterile water in the dark for 4 days at room temperature. Aleurone layers were isolated by gently scraping away the starchy endosperm with metal spatulas. Layers were incubated for 20 min at 25 °C in the dark, with gentle shaking, in a medium containing 20 mM $CaCl_2$ in the absence (controls) or in the presence of ABA (5 μM) 30 min (Racagni et al., 2008).

4.2. Nitrocellulose membrane binding assay and protein identification by mass spectrometry

Approximately 1 g barley aleurone (*Hordeum vulgare*) was ground with liquid nitrogen. Proteins were extracted by adding 4 ml of protein extraction buffer (50 mM Tris–HCl, pH 7.3, 50 mM NaCl, 5% (v/v) glycerol, 1 mM DTT, 0.5 mM PMSF) and cleared by centrifugation at 1500 × g for 10 min at 4 °C. Protein concentration in the supernatant was determined by the Bradford assay, and 500 μg of total proteins was used for nitrocellulose membrane binding assay. The assay was carried out as described previously (Stevenson et al., 1998) with some modifications. Approximately 10 μg of lipid dissolved in chloroform was spotted on a piece of nitrocellulose membrane (0.45-μm pore; Whatman) and air-dried for at least 30 min. The membrane was incubated with TBST buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 0.1% (v/v) Tween 20) containing 0.5% (w/v) fatty acid-free BSA for 1 h to block the membrane, it was washed three times with TBST buffer, and incubated with proteins overnight at 4 °C. The membrane was washed three times with TBST buffer to remove unbound proteins, and bound proteins were either eluted or probed by immunoblotting (dot blot) as described below. Proteins bound to the DGPP-nitrocellulose membrane were eluted by incubation of the membrane with 9 M urea for 1 h and recovered as described previously (Wessel and Flugge, 1984) with modification. An aliquot (0.4 ml) of methanol was added to 0.1 ml of protein sample and the samples were vortexed and centrifuged (10 s at 9000 × g) for total collection of the sample. Then, chloroform (0.1 ml) was added and the samples were vortexed and centrifuged again (10 s at 9000 × g). For phase separation, 0.3 ml of water was added, and the samples were vortexed vigorously and centrifuged for 1 min at 9000 × g. The upper phase was carefully removed and discarded. A further 0.3 ml methanol was added to the rest of the lower chloroform phase and the interphase with the precipitated protein. The samples were mixed and centrifuged again for 2 min at 9000 × g to pellet the protein. The supernatant was removed and the protein pellet was dried under a stream of air. The dried pellet can be stored now until use. The resulting protein pellet was dissolved in 50 μl of SDS-PAGE sample buffer, boiled for 5 min, and subjected to SDS-PAGE separation. The protein bands were carefully excised from the gel, and proteins were in-gel digested with 20 μl of trypsin (20 ng ml^{-1}, Sigma–Aldrich). The digested peptides were run on mass spectrometer 4800 MALDI TOF TOF *plus* de ABI. The database search was done with peptide mass fingerprint data using MASCOT PMF, MASCOT MSMS and database of nr/swissprot" of MASCOT (www.matriscience).

4.3. SDS-PAGE and immunoblotting

Protein samples were dissolved in SDS-PAGE sample buffer, boiled for 5 min, and loaded on 12% (v/v) polyacrylamide gel. The gel was run at 100 V for 1 h and stained with Coomassie Brilliant Blue for 1 h, followed by washing with methanol:water:acetic acid (3:6:1, v/v/v) to remove background stain. For immunoblotting, proteins were electrophoretically transferred from the gel onto a nitrocellulose sheet using the Semidry Trans-Blot apparatus (Bio-Rad). The membrane was blocked in TBST buffer containing 5% (w/v) non fat milk for 1 h, followed by washing it three times with TBST buffer. The membrane was incubated with primary antibodies (anti-GAPDH from Ambion Cat #AM4300) dilution 1:1000 for 1 h.

After washing three times with TBST buffer, the membrane was incubated with peroxidase-conjugated anti-IgG secondary antibody (Sigma–Aldrich) for 1 h, followed by colourimetric detection of the proteins stained by the 4-chloro-1-naphthol method. For Dot Blot assay: the lipids were spotted on the nitrocellulose membrane, the membrane was incubated with TBST buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 0.1% (v/v) Tween 20) containing 0.5% (w/v) fatty acid-free BSA for 1 h to block the membrane. It was washed three times with TBST buffer, and incubated with proteins overnight at 4 °C. The membrane was washed three times with TBST buffer to remove unbound proteins, and bound proteins were probed by immunoblotting with anti-GAPDH and detected by the 4-chloro-1-naphthol method as indicated before.

4.4. GAPDH enzymatic activity

Barley aleurones (200 mg) were washed, frozen under liquid nitrogen, and ground to a powder. The powdered material was homogenized with 600 mL of buffer containing 50 mM Tris–HCl, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 40 mM 2-mercaptoethanol. The homogenate was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was collected. GAPDH was assayed spectrophotometrically at 340 nm at 30 °C as described previously (Rius et al., 2008). GAPDH activity was measured by following the reduction of NAD+. The medium contained 50 mM triethanol-amine-HCl, pH 8.5, 4 mM NAD+, 10 mM Na3AsO4, 1.2 mmol D-Ga3P (Glyceraldehyde 3-phosphate), and 3 mM dithiothreitol. Reactions were initiated by the addition of Ga3P, and the rate of increase in absorbance was linear for at least 3–5 min. Activity incremented linearly with increasing enzyme concentration. One unit (U) is defined as the amount of enzyme that catalyses the formation or consumption of 1 mmol min NADH or NAD under each specified assay condition. Phospholipids of different fatty acyl chain composition (synthetic C16:0, 18:1 or 8:0 as indicated, and natural PI, PC and PE species) were all from Avanti Polar Lipids, USA. Lipids in chloroform stocks were dried, rehydrated in 50 mM triethanol-amine-HCl, pH 8.5 to a concentration of 1 mM and sonicated prior to adding them to the GAPDH activity assay mixture. Water-soluble C8:0 DGPP was added directly from an aqueous stock; the concentration used was below the predicted critical micellae concentration (CMC) of this lipid (King and Marsh, 1987).

4.5. Sequence alignment and phylogenetic analysis

Sequence analysis was performed using tools provided by the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov/), ExPASy Molecular Biology Server (http://us.expasy.org), and Arabidopsis Information Resource (www.arabidopsis.org). Amino acid sequences of open reading frames were initially aligned using ClustalW (Thompson et al., 1994) with BioEdit Sequence Alignment Editor 4.8.8, and the alignment was then visually refined. Phylogenetic analysis was performed using the neighbor-joining method with PAM distances computed on total aligned sites.

4.6. 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, Tukey'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).

Author contributions

Conceived and designed the experiments: PLA ALV. Performed the experiments: PLA JIU. Analysed the data: ALV PLA GER. Result discussion ALV PLA GER. Wrote the paper: ALV.

Acknowledgements

This work was supported by ANPCyT, SECyT-UNRC (grant number: 18/C426), Río Cuarto, and MINCyT Córdoba, PPI-CONICET (PPI 2012-2015) (grant numbers: 4541/12 and 3646/14). Argentina. ALV is Career Investigator of CONICET. We thank to Dr M Bollo and A Liffourrena for GAPDH assays assistance and MV Di Palma for language assistance.

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