Plant Physiology and Biochemistry 58 (2012) 83–88

Contents lists available at SciVerse ScienceDirect

Plant Physiology and Biochemistry

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

Short communication

Comparative phytohormone profiles, lipid kinase and lipid phosphatase activities in barley aleurone, coleoptile, and root tissues

Maria V. Meringer [a], Ana L. Villasuso [a], Susana J. Pasquaré [b], Norma M. Giusto [b], Estela E. Machado [a], Graciela E. Racagni [a,*]

[a] Dpto. Química Biológica, FCEFQN, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina
[b] INIBIBB (CONICET), Universidad Nacional del Sur, Bahía Blanca, Argentina

ARTICLE INFO

Article history:
Received 28 May 2012
Accepted 14 June 2012
Available online 23 June 2012

Keywords:
Barley
Lipid kinases
Lipid phosphate phoshatases
Phospholipids
Phytohormones

ABSTRACT

We analyzed lipid kinase and lipid phosphatase activities and determined endogenous phytohormone levels by liquid chromatography–tandem mass spectrometry in root and coleoptile tissues following germination of barley (*Hordeum vulgare*) seeds. The enzymes showing highest activity in aleurone cells were diacylglycerol kinase (DAG-k, EC 2.7.1.107) and phosphatidate kinase (PA-k). The ratio of gibberellins (GAs) to abscisic acid (ABA) was 2-fold higher in aleurone than in coleoptile or root tissues. In coleoptiles, phosphatidylinositol 4-kinase (PI4-k, EC 2.7.1.67) showed the highest enzyme activity, and jasmonic acid (JA) level was higher than in aleurone. In roots, activities of PI4-k, DAG-k, and PA-k were similar, and salicylic acid (SA) showed the highest concentration. In the assays to evaluate the hydrolysis of DGPP (diacylglycerol pyrophosphate) and PA (phosphatidic acid) we observed that PA hydrolysis by LPPs (lipid phosphate phosphatases) was not modified; however, the diacylglycerol pyrophosphate phosphatase (DGPPase) was strikingly higher in coleoptile and root tissues than to aleurone. Relevance of these findings in terms of signaling responses and seedling growth is discussed.

1. Introduction

Lipid-mediated signaling in plant cells is an increasingly well-documented phenomenon. A crucial early event in the regulation of a variety of plant cell functions is hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), by a specific phospholipase C (PLC, EC 3.1.4.11). This reaction gives rise to two intracellular messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [1]. DAG is rapidly phosphorylated to phosphatidic acid (PA) by diacylglycerol kinase (DAG-k, EC 2.7.1.107) primarily in order to down-regulate DAG levels; however, PA also functions as a lipid second messenger [2]. PA can also be produced rapidly in stimulated cells as a direct result of phospholipase D (PLD, EC 3.1.4.4) activation [3]. PA is converted into diacylglycerol pyrophosphate (DGPP) by a phosphatidate kinase (PA-k) [4]. Enzymes that metabolize PA/DGPP play important roles in switching the PA/DGPP signal on/off [5]. Barley (*Hordeum vulgare*) displays an increase in the PA/DGPP ratio in response to abscisic acid (ABA), and contains multiple PA phosphohydrolase genes [6]. Studies on ABA response have focused on metabolic functions of these enzymes; however, it remains unclear whether any of these proteins have regulatory functions following germination.

Phosphatidylinositol 3-kinase (PI3-k, EC 2.7.1.137) and phosphatidylinositol 4-kinase (PI4-k, EC 2.7.1.67), which synthesize phosphatidylinositol 3-phosphate (PI3P) and phosphatidylinositol 4-phosphate (PI4P), plays an important role in the barley aleurone. PI4-k activity was shown to be increased by treatment of aleurone layers with gibberellic acid (GA_3), suggesting that PI4-k helps regulate secretory processes [7].

Several studies have demonstrated the involvement of phospholipid signaling in aleurone responses to GA_3 and ABA, but little is known regarding the role of phospholipids following seed germination. Two tissues become very important after germination: the coleoptile and the root. The main functions of the coleoptile are to protect the shoot apex during initial seedling

Abbreviations: ABA, abscisic acid; IAA, indole-3-acetic acid; DAG, diacylglycerol; DAG-k, diacylglycerol kinase; DGPP, diacylglycerol pyrophosphate; DGPPase, diacylglycerol pyrophosphate phosphatase; IP_3, inositol 1,4,5-trisphosphate; GA_3, gibberellic acid; JA, jasmonic acid; LPA, lysophosphatidic acid; LPPs, lipid phosphate phosphatases; PA, phosphatidic acid; PA-k, phosphatidate kinase; PAP2, phosphatidate phosphohydrolase type 2; PI, phosphatidylinositol; PI3-k, phosphatidylinositol 3-kinase; PI3P, phosphatidylinositol 3-phosphate; PI4-k, phosphatidylinositol 4-kinase; PI4P, phosphatidylinositol 4-phosphate; PI-k, phosphatidylinositol kinases; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; SA, salicylic acid.

* Corresponding author. Fax: +54 358 4676232.
E-mail address: gracagni@exa.unrc.edu.ar (G.E. Racagni).

0981-9428/$ – see front matter © 2012 Published by Elsevier Masson SAS.
http://dx.doi.org/10.1016/j.plaphy.2012.06.013

establishment [8]. The main functions of roots are absorption of water and inorganic nutrients from the external environment, and anchoring of the plant body to the ground. Phospholipid signaling is well known to be involved in plant responses to treatment with exogenous phytoregulators, but few studies have addressed functions of lipid kinases/phosphatases in relation to endogenous levels of phytohormones in barley tissues.

Metabolism of various phytohormones is clearly interdependent and interrelated, but it has been difficult to quantify them simultaneously in a given type of plant tissue because of their diverse chemical nature. Recent advances in hormone analytical techniques have helped overcome this barrier [9]. The aim of the present study was to evaluate lipid kinase and lipid phosphatase activities and the hormone profile in barley tissues. This is useful information for future studies of cellular responses in barley tissues.

2. Materials and methods

2.1. Plant materials, growth conditions, and separation of aleurones, coleoptiles, and roots

Barley seeds (*H. vulgare*, cv. Carla INTA) were surface sterilized, and soaked in sterilized water for 4 days in the dark at 25 °C. Aleurone layers were separated by gently scraping away the starchy endosperm from barley seedlings with a metal spatula, and kept frozen in liquid nitrogen at −80 °C until use. 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. Coleoptiles (the new leaves were also included) and roots were separated, and kept frozen in liquid nitrogen at −80 °C until use.

2.2. Preparation of membranes

Aleurones, coleoptiles, and roots prepared as above were thawed and homogenized in 10 volumes 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 105000× 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 [10].

2.3. Lipid kinase activity and phospholipid extraction and separation

The membrane fraction isolated as above (60 μg protein) was added to thermally equilibrated (30 °C) 50 mM HEPES buffer (pH 7.4), 0.1 mM EDTA, 0.5 mM DTE, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate (inhibitor of lipid phosphate phosphatases), 1 mM Mg^{2+}-ATP, and [γ-^{32}P]ATP (370 MBq). Lipid kinase activities were assayed simultaneously using endogenous lipids as substrates. 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). Lipids were extracted from membranes, and phospholipids were separated by TLC as described by Racagni-Di Palma et al. [11]. Plates were developed with chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v/v/v/v) for the first dimension, and chloroform/pyridine/formic acid (35:30:7, v/v/v) for the second dimension. Positions of radiolabeled lipids were determined by autoradiography on Kodak film. PI3-k assay was performed using pellet and supernatant fractions from a homogenate of *Saccharomyces cerevisiae* (∼4 μg protein) as control, and sonicated PI as substrate (0.2 mg mL^{-1}). Phosphorylated lipids were extracted as described by Stack et al. [12], and separated by TLC/boric acid system [13].

2.4. Lipid phosphate phosphatase activity assays

For determination of 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 100 μg of membrane protein in a volume of 0.1 mL. The reaction was started by addition of 0.6 mM [2-^{3}H]phosphatidate, continued for 30 min at 37 °C, and stopped by addition of chloroform/methanol (2:1, v/v). PAP activity product 1,2-diacyl[^{3}H]glycerol was isolated and measured. Radiolabeled PA was obtained from [2-^{3}H]phosphatidylcholine, which was synthesized as described by Pasquaré and Giusto [14]. PAP activity was expressed as the sum of nmol ([^{3}H]diacylglycerol and [^{3}H]monoacylglycerol) × (h × mg protein)$^{-1}$.

DGPP phosphatase activity was assayed as described by Han and Carman [15], based on release of water-soluble ^{32}P from chloroform-soluble [β-^{32}P]DGPP (2000 cpm/pmol). The reaction mixture contained 50 mM citrate buffer (pH 5.0), 0.1 mM DGPP, 2 mM Triton X-100, 10 mM 2-mercaptoethanol, and enzyme protein, in a total volume of 0.1 mL. DGPP was synthesized from PA using enriched-membrane fraction *H. vulgare* PA kinase. Enzyme assays were conducted at 30 °C in duplicate.

2.5. Protein gel blot analysis

Barley tissue homogenates were prepared as described above for lipid kinase assay. Extract samples were separated by 12.5% SDS-PAGE, and proteins were electrotransferred to nitrocellulose membranes. Protein blots were blocked with 5% skim milk powder in phosphate-buffered saline (4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl). Antibodies directed to C-terminal DGPP phosphatase (a generous gift from Dr. George Carman) were added at 1:1000 dilution and incubated for 2 h. Secondary antibody coupled to horseradish peroxidase was used to visualize target proteins, in combination with chromogenic substrates.

2.6. Phytohormone analysis

Levels of GA_1, GA_3, ABA, IAA, SA, and JA were analyzed simultaneously by electrospray ionization/tandem mass spectrometry (LC-ESI-MS-MS), essentially as described by Durgbanshi et al. (2005). In brief, tissues samples (200 mg) were ground in liquid nitrogen, extracted with acetone/water/acetic acid (80: 19: 1, v/v/v), added with 5 μL of a mixture of internal standards (50 ng/sample of $^{2}H_5$-IAA, $^{2}H_6$-ABA, $^{2}H_6$-JA, and $^{2}H_4$-SA, and 100 ng $^{2}H_2$-GA_3, $^{2}H_2$-GA_1), and centrifuged at 500× g for 15 min. The resulting supernatant was collected and evaporated, and the solid residue was dissolved in 500 μL of methanol and evaporated. The resulting residue was dissolved in methanol/1% acetic acid (99:1, v/v), and then passed through a DEAE Sephadex A-25 column. Aliquots of the resulting solution were injected directly into the LC-ESI-MS-MS system. MS/MS experiments were performed on a Micromass Quatro Ultima™ Pt double quadrupole mass spectrometer (Micromass, Manchester City, UK). Precursor and product ions specific for each hormone were identified, using authentic compounds and appropriate precursor-to-product ion transitions representing

Table 1
Optimized MS/ MS conditions for the analysis of barley tissues hormones.

Analytes	Scan mode	Retention Time (min)	Transition		Collision Energy (eV)	Internal standard	Transition	
GA_1	Negative	3.7	348	242	13	$^2H_2$-GA_1	350	244
GA_3	Negative	6.2	345	221	13	$^2H_2$-GA_3	347	223
IAA	Negative	5.2	175	130	10	$^2H_5$-IAA	180	135
ABA	Negative	10.2	263	163	5	$^2H_6$-ABA	269	169
JA	Negative	14.3	209	59	5	$^2H_6$-JA	215	59
SA	Negative	4.5	137	93	5	$^2H_4$-SA	141	97

a major fragmentation path and unique for each phytohormone were chosen MS/MS conditions were optimized to produce maximal signal (Table 1).

3. Results

In order to determine lipid kinase activities in aleurones, coleoptiles, and roots, we used a sequential measurement system, with both endogenous substrates and kinases in the same membrane fraction, prepared by centrifugation at 105000× g. Compounds present in this fraction were identified by autoradiography as PA, lysophosphatidic acid (LPA), DGPP, PIP, and PIP_2 (Fig. 1A). The three tissues displayed important differences in lipid kinase activities. In coleoptiles, PIP was the predominant lipid species, comprising 45.25 ± 4.19% of total phosphorylated products. In aleurones, the major lipid component was DGPP (41 ± 3.61%), followed by PA (28.67 ± 3.79%), and PIP was much lower (13.5 ± 2.65%). The same three components were present in roots, but with a more homogeneous distribution: PA 20.5 ± 4.43%; DGPP 24.75 ± 3.86%; PIP 28.67 ± 8.62%. Percentages of LPA and PIP_2 were much lower in all three tissues. Coleoptiles displayed lower DAG-k and PA-k activities, and higher activity of PI-k, with its phosphorylated product PIP as the major lipid. Enzyme activities for each of the three tissues, expressed as pmol min^{-1} mg $protein^{-1}$, are summarized in Fig. 1B.

These reduced activities of DAG-k and PA-k may be correlated with increased activities of the enzymes that regulate PA and DGPP levels. PAP2 activity (measured with [^{3}H]PA as substrate) and DGPPase activity (measured with [β-^{32}P]DGPP as substrate) in coleoptile and root tissues, expressed as fold increase relative to values in aleurone, are shown in Fig. 2. DGPPase activities were ~1 and 0.5-fold higher, whereas PAP2 activities were essentially unchanged, in coleoptile/root tissues relative to aleurones. *In vitro* phosphatase activities were different in coleoptile/root tissues compared to aleurones, so protein blotting was performed to determine whether protein levels were different. Protein fraction isolated from barley tissues was separated by SDS-PAGE and immunoblotted. Antibody directed to C-terminus of *S. cerevisiae* DGPPase, used for detection of DGPPase protein levels, showed bands with 30 and 35 kDa. Signal levels were significantly different for coleoptile/root tissues compared to aleurones (Fig. 2, inset). DGPPase signal levels were ~2.04 ± 0.6 and 0.85 ± 0.31(n = 4, $P < 0.05$, t-test) in coleoptile/root tissues relative to aleurones.

PIP isomers were identified using pellet and supernatant fractions from a homogenate of *S. cerevisiae*. Autoradiography showed the presence of PI3P and PI4P, with Rf values 0.44 and 0.48, respectively (Fig. 3). In all three tissues, PI4P levels were higher than PI3P levels. Analysis of radioactive lipids revealed PI4-k activity (4.6 pmol min^{-1} mg $protein^{-1}$) 2.5-fold higher than PI3-k activity

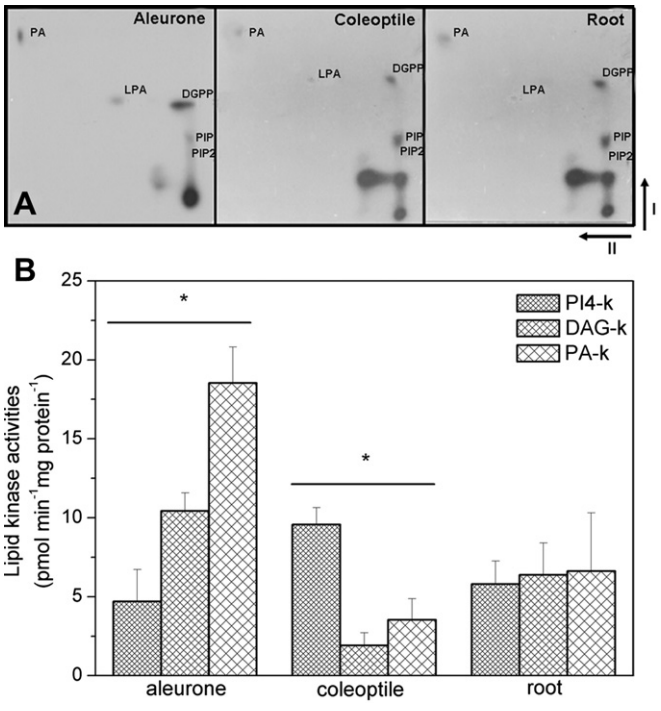

Fig. 1. (A) Autoradiography of lipid kinase products from barley aleurone, coleoptile, and root. Membrane lipids were phosphorylated with [γ-^{32}P]ATP, extracted, and separated by 2-dimensional TLC. Solvent I: chloroform/methanol/acetone/acetic acid/water (40:14:15:12:7, v/v/v/v/v). Solvent II: chloroform/pyridine/formic acid (35:30:7, v/v/v). (B) Results are expressed as specific activity (pmol min^{-1} mg $protein^{-1}$) and shown as mean ± S.D., n = 3, $^*P < 0.05$, t-test.

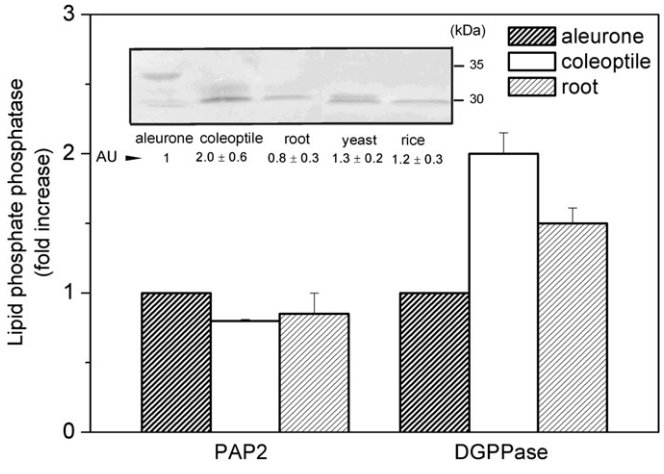

Fig. 2. PAP2 activity was determined using [^{3}H]PA (0.6 mM) as substrate. DGPPase activity was determined using [β-^{32}P]DGPP as substrate. Phosphatases activities in coleoptile and root are expressed as fold increase relative to values in aleurone. Inset: immunoblotting of homogenates from barley tissues. Barley, rice, and yeast proteins were separated by SDS-PAGE, and analyzed by protein blotting using yeast anti-DGPPase antibody. Size of molecular weight marker is indicated at right. DGPPase protein levels (band with 30 kDa) were quantified (optical density in arbitrary units) using the Scion Image program, with optical density of aleurone band defined as 1 AU (arbitrary units).

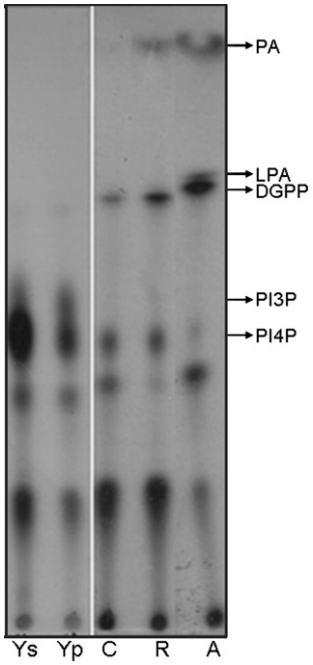

Fig. 3. Autoradiography of PI4P and PI3P isomers in aleurone, coleoptile, and root. PI4-k and PI3-k activities were identified in membrane fraction (60 μg protein) by phosphorylation of corresponding endogenous substrates with [γ-^{32}P]ATP, and then extracted and separated by TLC/boric acid system. Proteins prepared from yeast (yeast soluble, Ys) and membrane homogenate (Yp) were assayed for kinase activity to generate reference markers for PI3P and PI4P. Products of phosphorylation of lipid kinases in aleurone (A), coleoptile (C) and root (R) are shown.

(2 pmol min^{-1} mg protein^{-1}) in aleurones, and PI4-k activity (9.5 pmol min^{-1} mg protein^{-1}) 4-fold higher than PI3-k activity (2 pmol min^{-1} mg protein^{-1}) in coleoptiles, showing that coleoptiles have an equal level of PI3P and a higher PI4P level relative to aleurones. Lipid kinase activities in roots (PI3-k = 2.1 pmol min^{-1} mg protein^{-1}; PI4-k = 5.8 pmol min^{-1} mg protein^{-1}) were higher than in aleurones.

Changes in phytohormone concentrations in plants mediate a wide range of developmental processes, many of which involve interactions with phospholipid metabolism. Endogenous phytohormone concentrations in barley aleurones, coleoptiles, and roots, expressed as ng per g dry weight, are summarized in Table 2. GA_1 + GA_3 levels were ~4-fold higher in aleurone than in coleoptiles and root tissues. IAA level was ~4-fold higher in aleurones than in coleoptile and root tissues. ABA content was similar among the three tissues. SA level was 2.7-fold higher in roots than in coleoptile and 12-fold higher in roots than in aleurone tissues. JA level was ~3-fold higher in coleoptiles than in aleurone and roots tissues. For better understanding of these results, we calculated ratios between various pairs of hormones, and compared these ratios among the three tissues (Table 3). The GAs/ABA ratio was 2-fold higher, in aleurones than in coleoptile or root tissues. The SA/GAs ratio was highest in roots, and the SA/IAA ratio was 22-fold higher in roots than in aleurone or coleoptile tissues, illustrating the importance of SA for roots function. JA/GA_3 ratio was higher in coleoptiles than in roots, and 13-fold higher in coleoptiles than in aleurones.

Table 2
Endogenous amounts of phytohormones for different barley tissues.

Barley tissues	Amount (ng/dry weight)					
	GA_1	GA_3	ABA	IAA	SA	JA
Aleurones	325	220	248	71	33	52
Coleoptiles	24	108	274	21	147	153
Roots	60	46	286	18	404	86

Levels of GA_1, GA_3, ABA, IAA, SA, and JA were analyzed simultaneously by LC-ESI-MS-MS essentially as described by Durgbanshi et al. (2005). Results are expressed as ng/dry weight of barley tissues; they correspond to a representative experiment performed by triplicate.

Table 3
Pairs of hormones: ratios among the three tissues.

Ratio	Aleurones	Coleoptiles	Roots
GAs/ ABA	2.0	0.5	0.3
SA/ GAs	0.6	1.1	3.8
SA/ ABA	0.13	0.5	1.4
SA/ IAA	0.46	7.0	22.0
IAA/ GAs	0.13	0.15	0.16
IAA/ ABA	0.28	0.08	0.06
JA/ GAs	0.09	1.16	0.80
JA/ ABA	0.21	0.5	0.3
JA/ SA	1.60	0.9	0.21

GAs: Sum of GA_1 and GA_3.

4. Discussion

Our previous studies demonstrated that treatment of barley aleurones with GA_3 and ABA modulates activity of lipid kinases/phosphatases, and these effects are correlated with stimulation or inhibition of α-amylase secretion [6,7].

Results of the present study show important differences in lipid kinase and lipid phosphatase activities, and phytohormone profiles, in aleurones (isolated from the seedling) in comparison to coleoptile and root tissues during barley seed germination. Phospholipid metabolism, and related lipid kinases/phosphatases, are important regulators of normal plant growth and development [16]. PA and DGPP comprise >50% of total phosphorylated lipids in barley aleurones. Similarly, in a study of *Arabidopsis thaliana*, Katagiri et al. [17] reported that PA and DGPP reached maximal levels within the first 12 h after germination, followed by a decrease after 96 h. Thus, these two phospholipids appear to play important roles in germination.

The balance among various phytohormones, particularly GAs and ABA, has been shown to determine the onset or progress of germination or dormancy in several plant species [18]. We found that the concentration in aleurones of GAs is 2-fold higher than that of ABA, consistent with findings by Durgbanshi et al. [9]. Our previous study showed that PA and DGPP play important metabolic roles during *in vitro* stimulation of aleurone secretory activity [6]. In the present study, DAG-k activity correlated positively with an increase of PA level, with simultaneous DGPP formation through activation of PA-k. The exact mechanism of DGPP's role in aleurones activity remains unclear, although our recent study based on film packing suggests that DGPP may act as a structural signal transducer in membrane-mediated cellular processes [19].

Our analysis of hormone ratios showed important difference in SA/GAs and SA/ABA ratios in the three tissues studied. Signaling cross-talk occurs between SA and GA_3 in barley aleurones, and SA is capable of blocking barley seed germination by suppressing induction of amylase by GA_3 [20]. The role of SA in seed germination is ambiguous; it appears to be capable of either inhibiting germination or enhancing seed vigor [21]. Few studies have addressed the interaction of SA with phospholipids [22,23]. We hypothesize that the lower levels of SA observed in aleurones may affect the PA/DGPP levels. SA treatment of *Capsicum chinense* cells in suspension was recently shown to modulate PA levels via DAG-k activity [24]. The observed decrease of DGPP and PA levels in seedlings suggests the involvement of enzymes that utilize DGPP

and PA as substrates. The present study showed the presence of PAP2 and DGPPase activities in barley seedling. Differences in behavior of these two enzymes suggest the presence of distinct LPP isoforms [5]. Immunoblot analysis of phosphatases in the present study showed that they are expressed in all three tissues examined, as well as in rice, but with differences in abundance of phosphatase protein. These findings are consistent with those of Eastmond et al. [25]. Plants contain multiple PA phosphohydrolase isoforms, but it has not been established whether any of these proteins play a regulatory role in plant lipid metabolism, either directly or indirectly. The study by Eastmond et al. [25], suggested that PAH1/2 may govern phospholipid synthesis through changes in the level of PA or DAG. In the present study, DGPPase activity was much higher in barley coleoptile and root tissues than in aleurones, whereas PAP2 activity was essentially the same in the three tissues. We therefore suggest that DGPPase activity is the primary phosphatase activity involved in regulation of PA/DGPP levels in coleoptile and root cells. We found that the GAs level was 4-fold higher in aleurone than in coleoptiles or root tissues, and JA level was respectively 3-fold higher in coleoptiles and 2-fold higher in roots compared to aleurones. It has been shown that the analysis of the GAs is particularly complex as there are a very large number of these molecules in most plant tissues. In this work we have determinated the biologically active GA_1 and its 1,2-double bond analog, GA_3. GA_1 is believed to be the predominant bioactive GA responsible for α-amylase production in germinated mature barley grains [26] and that GA_3 applied to de-embrionated half-grains stimulates amylolytic activity in endosperm. However, a study by Green et al. [27] of barley embryos showed the highest levels of GAs inactive and lowest amount of GA_1 and GA_3 was not determinated. Now, we show that aleurone tissue possess also GA_3. The difference between Green's studies and this work could be due to the methods used in the phytohormones measure as well as the grains cultivar. Although, there are several earlier reports that show the presence of GA_3 in germinating grain and leaf sheaths [28,29] therefore the presence of GA_3 should be also considered.

GAs is well known to stimulate growth and elongation of rice seedlings, but the role of JA is less known. A few reports indicate a relationship between endogenous JA and GAs levels and PI4-k activity. Regente et al. [30] observed that treatment of sunflower seeds with JA altered the profiles of extracellular phospholipids, and led to accumulation of PI4P. We found previously that GA_3 treatment causes major fluctuations in PI4P levels in aleurone cells, suggesting that PI4P is part of the GA pathway [7]. Here, we show that level of PI4P is an important feature of coleoptiles. However, the regulation of PI4P levels in barley tissues is poorly understood. In both, aleurone [7] and coleoptiles, most PI4P is formed by phosphorylation of PI by PI4-k. Recent studies have shown PI4P has multifaceted roles in plants, and regulates diverse plant processes [31], including polarized membrane trafficking during tip-growth. This highly polarized form of cell expansion occurs in growing root hairs and pollen tubes, and requires focused delivery of secretory vesicles to an apical plasma membrane domain. A role for PI4P was revealed when PI4Kb1 was found to be recruited to tip-localized secretory vesicles by the regulatory GTPases, RabA4b in root hair, and RabA4d in pollen tubes [32,33]. As in this study the coleoptiles included the young leaves we propose the PI4P could be involved in the elongating and dividing of barley tissues since PI4P also regulates the establishment of polarity during cell differentiation in other plant developmental contexts. During cell division, it accumulates at the cell plate as well as it has also been implicated in cell polarity establishment during stem cell maintenance [34]. In this way, both PI4-k and DGPPase activities in coleoptile would be also related with lipid turnover as well as phospholipid synthesis through changes in the level of PA or DAG.

Of the three tissues studied, roots showed the fewest differences among lipid kinase activities; however, SA level was 2.5-fold higher in root than in coleoptile or aleurone tissues. SA was reported to display growth-stimulatory effects in soybean and wheat [21]. The SA/IAA ratio was ~3-fold higher in roots than in aleurone or coleoptile tissues. Some studies have shown evidence of cross-talk between SA and IAA signaling during plant vegetative growth [21], but the precise mechanism is unknown. Thus, we propose that the different hormonal regulation observed in the barley tissues would be reflected in the lipid metabolism through lipid kinase activities. Consequently, the role of phospholipids in phytohormone function and plant seedling growth is an important topic for future studies.

Acknowledgments

This work was supported by ANPCyT (PICT 2007-02212, BID 1728/OC-AR), SECyT-UNRC, Río Cuarto, and MINCyT Córdoba, Argentina. Seeds were generously provided by Ing. JC Tomaso of INTA-Bordenave. Careful reviews by anonymous reviewers greatly improved the manuscript. MVM is a fellow of MINCyT-CONICET. ALV is a Career Investigator of CONICET.

References

[1] T. Munnik, T. Testerink, Plant phospholipid signaling: "in a nutshell", J. Lipid Res. 50 (2009) 260–265.
[2] T. Munnik, Phosphatidic acid: an emerging plant lipid second messenger, Trends Plant Sci. 6 (2001) 227–233.
[3] M. Li, Y. Hong, W. Wang, Phospholipase D- and phosphatidic acid-mediated signaling in plants, Biochim. Biophys. Acta 1791 (2009) 927–935.
[4] J.B. Wissing, B. Kornak, A. Funke, B. Riedel, Phosphatidate kinase a novel enzyme in phospholipid metabolism, Plant Physiol. 105 (1994) 903–909.
[5] O. Pierrugues, C. Brutesco, J. Oshiro, M. Gouyi, Y. Deveaux, G. Carman, P. Thuriaux, M.J. Kazmaier, Lipid phosphate phosphatases in Arabidopsis, J. Biol. Chem. 276 (2001) 20300–20308.
[6] G. Racagni, A.L. Villasuso, S. Pasquaré, N. Giusto, E. Machado, Diacylglycerol pyrophosphate inhibits the alpha-amylase secretion stimulated by gibberellic acid in barley aleurone, Physiol. Plant 134 (2008) 381–393.
[7] A.L. Villasuso, G. Racagni, E. Machado, Phosphatidylinositol kinases as regulators of GA-stimulated alpha-amylase secretion in barley (*Hordeum vulgare*), Physiol. Plant 133 (2008) 157–166.
[8] E.M. Wiedenroth, G. Wernicke, P. Hoffman, Morphological and anatomical characterization of the coleoptile of *Triticum aestivum* with regard to the evolution of forms with different ploidy levels, Ann. Bot. 66 (1990) 531–540.
[9] A. Durgbanshi, V. Arbona, O. Pozo, O. Miersch, J. Sancho, A.J. Goämez-Cadenas, Simultaneous determination of multiple phytohormones from plant extracts by liquid chromatography-electrospray tandem mass spectrometry, J. Agric. Food Chem. 53 (2005) 8437–8442.
[10] M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[11] G. Racagni-Di Palma, L. Brito-Argáez, S.M.T. Hernández-Sotomayor, Phosphorylation of signaling phospholipids in *Coffea arabica* cells, Plant Physiol. Biochem. 40 (2002) 899–906.
[12] J. Stack, P. Herman, P. Schu, S. Emr, A membrane-associated complex containing the Vpsl 5 protein kinase and the Vps34 Pi 3-kinase is essential for protein sorting to the yeast lysosome-like vacuole, EMBO J. 12 (1993) 2195–2204.
[13] J. Walsh, K. Caldwell, P. Majerus, Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9184–9187.
[14] S.J. Pasquaré de García, N.M. Giusto, Phosphatidate phosphatase activity in isolated rod outer segment from bovine retina, Biochim. Biophys. Acta 875 (1986) 195–202.
[15] G. Han, G. Carman, Assaying lipid phosphate phosphatase activities, Methods Mol. Biol. 284 (2004) 209–216.
[16] H.W. Xue, X. Chen, Y. Mei, Function and regulation of phospholipid signalling in plants, Biochem. J. 421 (2009) 145–156.
[17] T. Katagiri, K. Ishiyama, T. Kato, S. Tabata, M. Kobayashi, K. Shinozaki, An important role of phosphatididc acid in ABA signalling during germination in *Arabidopsis thaliana*, Plant J. 43 (2005) 107–117.
[18] P.C. Bethke, I.G. Libourel, N. Aoyama, Y.Y. Chung, D.W. Still, R.L. Jones, The Arabidopsis aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy, Plant Physiol. 143 (2007) 1173–1188.

[19] A.L. Villasuso, N. Wilke, B. Maggio, E. Machado, The surface organization of diacylglycerol pyrophosphate and its interaction with phosphatidic acid at the air-water interface, Chem. Phys. Lipids 163 (2010) 771–777.
[20] Z. Xie, Z.L. Zhang, S. Hanzlik, E. Cook, Q.J. Shen, Salicylic acid inhibits gibberellin-induced alpha-amylase expression and seed germination via a pathway involving an abscisic-acid-inducible WRKY gene, Plant Mol. Biol. 64 (2007) 293–303.
[21] M. Rivas-San Vicente, J. Plasencia, Salicylic acid beyond defence: its role in plant growth and development, J. Exp. Bot. 62 (2011) 3321–3338.
[22] O. Krinke, M. Flemr, C. Vergnolle, S. Collin, J.P. Renou, L. Taconnat, A. Yu, L. Burketová, O. Valentová, A. Zachowski, E. Ruelland, Phospholipase D activation is an early component of the salicylic acid signaling pathway in Arabidopsis cell suspensions, Plant Physiol. 150 (2009) 424–436.
[23] O. Krinke, E. Ruelland, O. Valentová, C. Vergnolle, J.P. Renou, L. Taconnat, M. Flemr, L. Burketová, A. Zachowski, Phosphatidylinositol 4-kinase activation is an early response to salicylic acid in Arabidopsis suspension cells, Plant Physiol. 144 (2007) 1347–1359.
[24] A.R. Altúzar-Molina, J.A. Muñoz-Sánchez, F. Vázquez-Flota, M. Monforte-González, G. Racagni-Di Palma, S.M.T. Hernández-Sotomayor, Phospholipidic signaling and vainillin production in response to salicylic acid and methyl jasmonate in *Capsicum chinense* J. cell, Plant Physiol. Biochem. 49 (2011) 151–158.
[25] P.J. Eastmond, A.L. Quettier, J.T. Kroon, C. Craddock, N. Adams, A.R. Slabas, A phosphatidate phosphatase double mutant provides a new insight into plant membrane lipid homeostasis, Plant Cell. 22 (2010) 2796–2811.
[26] J.R. Lenton, N.E.J. Appleford, S.J. Croker, Gibberellins and α-amylase gene expression in germinating wheat grains, Plant Growth Regul. 15 (1994) 261–270.
[27] L.S. Green, E.M. Faergestad, A. Poole, P.M. Chandler, Grain development mutants of barley ([alpha]-amylase production during grain maturation and its relation to endogenous gibberellic acid content), Plant Physiol. 114 (1997) 203–212.
[28] K. Yamada, Determination of endogenous gibberellins in germinating barley by combined gas chromatography-mass spectrometry, J. Am. Soc. Brew. Chem. 40 (1982) 18–25.
[29] S.J. Croker, P. Hedden, J.R. Lenton, J.L. Stoddart, Comparison of gibberellins in normal and slender barley seedlings, Plant Physiol. 94 (1990) 194–200.
[30] M. Regente, G. Corti Monzón, L.V. de la Canal, Phospholipids are present in extracellular fluids of imbibing sunflower seeds and are modulated by hormonal treatments, J. Exp. Bot. 59 (2008) 553–562.
[31] E. Delage, E. Ruelland, I. Guillas, A. Zachowski, J. Puyaubert, Arabidopsis type-III phosphatidylinositol 4-kinases β1 and β2 are upstream of the phospholipase C pathway triggered by cold exposure, Plant Cell Physiol. 53 (2012) 565–576.
[32] M.L. Preuss, A.J. Schmitz, J.M. Thole, H.K. Bonner, M.S. Otegui, E. Nielsen, A role for the RabA4b effector protein PI-4Kbeta1 in polarized expansion of root hair cells in *Arabidopsis thaliana*, J. Cell Biol. 172 (2006) 991–998.
[33] A. Szumlanski, E. Nielsen, The *Arabidopsis thaliana* Rab GTPase, RabA4d, is required for the regulation of pollen tube tip growth, Plant Cell. 21 (2009) 526–544.
[34] J.M. Gagne, S.E. Clark, The Arabidopsis stem cell factor POLTERGEIST is membrane localized and phospholipid stimulated, Plant Cell. 22 (2010) 729–743.