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RESEARCH PAPER

Phospholipids are present in extracellular fluids of imbibing sunflower seeds and are modulated by hormonal treatments

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Abstract

Phospholipids are well known messengers involved in developmental and stress responses mediating intracellular signalling. It has been hypothesized that phospholipids exist which could participate in intercellular communication events through the apoplast of sunflower (*Helianthus annuus*) seeds. Here it is shown that extracellular washing fluids (EWFs) obtained from seeds imbibed for 2 h contain diverse phospholipids. Lipid profiling by electrospray ionization tandem mass spectrometry revealed that the EWFs have a particular composition, with phosphatidic acid (PA) and phosphatidylinositol (PI) being the major phospholipids. These profiles are clearly distinct from those of seed extract (SE), and comparative SDS–PAGE of EWF and SE, followed by intracellular and plasma membrane marker analyses, allowed a significant contamination of the EWF to be discarded. Treatment of the seeds with 100 μM jasmonic acid (JA) induces changes in the profile of EWF phospholipids, leading to a decrease in PI content, while the accumulation of phosphatidylinositol 4-phosphate (PI4P) and specific PA species is observed. On the other hand, the EWF from seeds subjected to 50 μM abscisic acid (ABA) treatment exhibit an increase in PA and phosphatidylglycerol levels. To our knowledge, this is the first report on the existence of phospholipids as extracellular components of seeds. Moreover, the modulation of PA, PI, and PI4P levels by hormonal treatments further suggests their contribution to intercellular communication *in planta*.

Key words: Apoplast, phospholipids, seed, sunflower.

Introduction

The apoplast is the aqueous phase external to the plasma membrane which basically includes cell walls, intercellular spaces, and conducting cells of the xylem. It is generally described as an interconnected medium surrounding the cells and involved in water and solute movement within tissues and organs. However, the apoplast is a dynamic compartment participating in many processes, including maintenance of tissue shape, development, nutrition, signalling, detoxification, and defence. Signalling molecules for the transmission of short or medium distance signals could be expected in this compartment, but those molecules and the mechanisms involved are largely unknown. Although controversial, plant neurobiology is an emerging field of research which focuses on the combined molecular, chemical, and electrical components of intercellular plant signalling (Brenner *et al*., 2006). A challenge of this discipline is the discovery of signal molecules participating in the integrated control of plant behaviour. Hence, besides phytohormones, recently discovered intercellular signals involved in development and defence pathways include RNA molecules, peptide hormones, and transcriptional activators (Brenner *et al*., 2006).

Increasing evidence has shown that lipids participate in crucial plant processes such as signal transduction, membrane trafficking, and cytoskeletal rearrangements, apart from their classical function for energy storage and as the structural basis of membranes. Plant signalling lipids include diverse molecules such as phospholipids, oxylipins, fatty acids, sterols, and sphingolipids (Wang, 2004; Shah, 2005). However, most of the accumulated information on lipid-based signalling concerns intracellular

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events, except for the described role of fatty acids and oxylipins in wounding and defence responses triggered by pathogens (Farmer *et al*., 2003). In addition, two other lipid types have been implicated in plant extracellular signalling events: *N*-acylethanolamines (NAEs) and sphingosine-1-phosphate (S1P). NAEs are amides of fatty acids that accumulate in the extracellular milieu of tobacco cells and leaves upon treatment with an elicitor of defence responses (Chapman, 2004). Moreover, treatment of cells or leaves with NAEs activates the expression of defence genes and reduces the alkalinization of the extracellular medium caused by elicitors, suggesting their participation in signal transduction events (Tripathy *et al*., 1999). A role for NAEs in seed germination has also been proposed (Chapman, 2004). On the other hand, Coursol *et al*. (2003) identified S1P as a molecular signal involved in the regulation of guard cell turgor mediated by abscisic acid (ABA), and implicating heterotrimeric G proteins. Although its extracellular location in plants has not been demonstrated yet, S1P is an extracellular lipid mediator involved in the regulation of numerous processes in animals (Hait *et al*., 2006). Attention has been focused on phospholipids, taking into account the vast knowledge accumulated in the last few years demonstrating their crucial roles in intracellular plant signalling (Meijer and Munnik, 2003; Wang, 2004). Among phospholipids, phosphatidic acid (PA) has been implicated in signalling events related to environmental stimuli as well as normal plant growth and development (Testerink and Munnik, 2005; Wang *et al*., 2006). Other signalling phospholipids described in plants are the phosphoinositides. They are produced by phosphorylation of phosphatidylinositol (PI) to give mono- or diphosphorylated derivatives (PIP or PIP_2). Three hydroxyl groups of PI can be phosphorylated, generating six different derivatives in plant cells whose functions are barely known but are clearly implicated in plant growth (Stevenson *et al*., 2000) and stress responses (Meijer and Munnik, 2003). Phospholipids as well as diacylglycerol, triacylglycerol, steryl and wax esters, and unesterified fatty acids have been described in the phloem sap from canola, and their detection has prompted the authors to suggest the existence of a putative long-distance transport of lipids in plants (Madey *et al*., 2002).

The aim of this work was to introduce the concept of phospholipids in the extracellular space of plants, determining their presence and composition in apoplastic fluids of sunflower (*Helianthus annuus* L) seeds under basal conditions and upon stimulation with hormonal treatments.

Materials and methods

Plant material and collection of extracellular washing fluid

Sunflower seeds (*H. annuus* L, line 10347) were provided by Advanta Semillas SAIC, Centro Biotecnológico Balcarce, Argentina. The seeds were subjected to imbibition for 2 h in water (control), 50 μM ABA, or 100 μM jasmonic acid (JA) and then carefully peeled prior to the extraction of the extracellular washing fluid (EWF). The EWF was collected by a standard technique allowing the recovery of the components present in the intercellular spaces based on a vacuum infiltration and centrifugation procedure (Pinedo *et al*., 1993; Olivieri *et al*., 1998). Briefly, seeds were immersed in cold infiltration buffer (50 mM TRIS-HCl pH 7.5, 0.6% NaCl, 0.1% 2-mercaptoethanol) and subjected to three vacuum pulses of 10 s, separated by 30 s intervals. A pressure of 45 kPa was applied, generating a soft vacuum which is half of the standard condition (Lohaus *et al*., 2001). The infiltrated seeds were recovered, dried on filter paper, placed in fritted glass filters inside centrifuge tubes, and centrifuged for 20 min at 400 *g* at 4 °C. The EWF was recovered in the filtrate.

A seed extract (SE) was obtained by homogenization of 0.4 g of decoated seeds subjected to imbibition in water for 2 h in 5 vols of infiltration buffer. The resultant homogenate was clarified by centrifugation at 10 000 *g* for 15 min at 4 °C and the supernatant obtained was called the SE.

Protein analysis

Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin (BSA) as standard. Electrophoretic separation was performed using standard SDS–PAGE 12% gels, which were stained with Coomassie blue.

Western blot assays were performed by standard procedures (Regente and de la Canal, 2000). Briefly, after electrophoretic migration, the proteins were transferred to nitrocellulose membranes using a semi-dry apparatus. Blots were incubated with different antibodies, and alkaline phosphatase was used for signal visualization. The polyclonal anti-oleosin antiserum, provided by Dr Beaudoin, was raised against sunflower purified oil bodies obtained by the urea-washed method (Millichip *et al*., 1996). The antibody raised against an *Aradidopsis thaliana* H^+-ATPase proved to react with plasma membrane H^+-ATPase from sunflower (Parets-Soler *et al*., 1990). A commercial monoclonal anti-actin antibody was purchased from Sigma (A0480) and a polyclonal antibody raised against the sunflower extracellular lipid transfer protein Ha-AP10 was previously obtained in the laboratory (Regente and de la Canal, 2000).

Glucose-6-phosphate (Glu-6-P) dehydrogenase activity was assayed at 25 °C in 700 μl of solution containing 65 mM $MgCl_2$, 1.3 mM $NADP^+$, 85 mM triethanolamine buffer pH 7.6, and the protein sample of EWF or SE (positive control). The reaction was initiated by adding 3 mM Glu-6-P and the activity was determined by following the appearance of NADPH spectrophotometrically at 340 nm (Pinedo *et al*., 1993).

Lipid extraction

Lipid extraction was performed according to Bligh and Dyer (1959) with modifications. The EWF obtained from 20 g of seeds and SE samples were first treated with 0.1 vol. of 50% (v/v) perchloric acid and then submitted to lipid extraction with 4.5 vols of chloroform: methanol:HCl (200:100:1, v/v/v) and 1 vol. of 0.9 % (w/v) NaCl (Munnik *et al*., 1996). After vigorous agitation the organic phase was obtained by centrifugation and washed with 4.5 vols of theoretical upper phase (chloroform:methanol:1 M HCl, 3:45:47, v/v/v). The organic phase was recovered and the solvent was evaporated under a nitrogen stream.

Phospholipid measurement

The lipid pellets were analysed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) at the Facility of the Kansas

Lipidomics Research Center (Kansas State University, USA). Automated ESI-MS/MS, data acquisition and analysis, and acyl group identification were carried out as described elsewhere (Welti *et al*., 2002; Devaiah *et al*., 2006) with minor modifications. Briefly, unfractionated lipid extracts were introduced by continuous infusion into the ESI source on a triple quadrupole MS/MS (API 4000, Applied Biosystems). Sequential precursor and neutral loss scans of the extracts produce a series of spectra, with each spectrum revealing a set of lipid species containing a common head group fragment. The scans were those previously described by Welti *et al*. (2002) and Brügger *et al*. (1997), except for the addition of neutral loss of 87 in the negative mode for phosphatidylserine (PS). The spectral background was subtracted, the data were smoothed, and peak areas integrated using a custom script and Applied Biosystems Analyst software. Isotopic overlap corrections were applied, and the lipids in each class were quantified in comparison with the two internal standards of that class, and using correction curves determined for the API 4000 mass spectrometer. Internal standards, obtained and quantified as previously described (Welti *et al*., 2002), were 0.630 nmol di14:0-PC (phosphatidylcholine), 0.540 nmol di24:1-PC, 0.660 nmol 13:0-lysoPC, 0.660 nmol 19:0-lysoPC, 0.381 nmol di14:0-PE (phosphatidylethanolamine), 0.309 nmol di24:1-PE, 0.378 nmol 14:0-lysoPE, 0.344 nmol 18:0-lysoPE, 0.313 nmol di14:0-PG (phosphatidylglycerol), 0.233 nmol di24:1-PG, 0.352 nmol 14:0-lysoPG, 0.347 nmol 18:0-lysoPG, 0.302 nmol di14:0-PA, 0.317 nmol di20:0(phytanoyl)-PA, 0.228 nmol di14:0-PS, 0.230 nmol di20:0(phytanoyl)-PS, 0.146 nmol 16:0-18:0-PI (phosphatidylinositol), and 0.068 nmol di18:0-PI. The source temperature (heated nebulizer) was 100 °C, the interface heater was on, +5.5 kV or –4.5 kV were applied to the electrospray capillary, the curtain gas was set at 20 (arbitrary units), and the two ion source gases were at 45 (arbitrary units).

Samples were introduced using an autosampler (LC Mini PAL, CTC Analytics AG, Zwingen, Switzerland) fitted with the required injection loop for the acquisition time and presented to the ESI needle at 30 ml min^{-1}. The collision gas pressure was set at 2 (arbitrary units). Lipid species were detected, using the scans previously described (Brügger *et al*., 1997; Kim *et al*., 1999; Welti *et al*., 2002). The collision energies, with nitrogen in the collision cell, were 28 V for PE, 40 V for PC, –58 V for PI, –57 V for PA and PG, and –34 V for PS. Declustering potentials were 100 V for PE and PC, and –100 V for PA, PG, PI, and PS. Entrance potentials were 15 V for PE, 14 V for PC, and –10 V for PI, PA, PG, and PS. Exit potentials were 11 V for PE, 14 V for PC, –15 V for PI, –14 V for PA and PG, and –13 V for PS. The mass analysers were adjusted to a resolution of 0.7 D full width at half height. For each spectrum, 9–150 continuum scans were averaged in multiple channel analyser (MCA) mode.

Afer every 11th set of mass spectra, the spectrum of the internal standards alone was acquired. Peaks corresponding to the target lipids were identified and molar amounts were calculated for these peaks in the same way as from peaks in spectra obtained from the sample infusions (i.e. in comparison with the internal standards). The molar amount of each lipid profile component detected in the spectra obtained for internal standards only was subtracted from the molar amount of each component calculated in each set of sample spectra. The data from an 'internal standards alone' set of spectra was used to correct the spectra from the following 10 samples.

In the first ESI-MS/MS analyses, the samples were run in triplicate to evaluate the reproducibility of the method. As they showed high reproducibility, further biological replicates were analysed once. From each ESI-MS/MS run, quantitative data were obtained for the phospholipid species indicated in Supplememtary Table S1 available at *JXB* online, distinguishing chain length and unsaturation degree of the whole molecule. The means obtained for three repetitions of the SE, seven independent experiments for control EWF, and five independent experiments for EWF prepared from ABA- and JA-treated seeds are presented in Supplememtary Table S1 at *JXB* online. As ESI-MS/MS identifies individual species for each phospholipid, in Figs 2 and 4 the total content of each phospholipid class, i.e. PC, was calculated by adding together all the species of PC and is expressed as a percentage of total phospholipids present in each independent experiment. The same criterion was used for the analysis of lipid species within a phospholipid class (Figs 3, 5). Data were statistically analysed using the Mann–Whitney test (SigmaStat Software).

Protein–lipid overlay assay

The protein–lipid overlay assay was based on the procedure described by Dowler *et al*. (2002). A 3 µl aliquot of serial dilutions of phosphatidylinositol 4-phosphate (PI4P) (Avanti Polar Lipids, Inc.) and of EWF lipid samples was spotted onto a Hybond-C extra membrane using chloroform:methanol:water (20:9:1) as dissolvent. After air drying and blocking in 2% fatty acid-free BSA in 50 mM TRIS-HCl pH 7.5, 150 mM NaCl, and 0.1% (v/v) Tween-20, the membrane was incubated overnight in the same solution containing 1 µg ml^{-1} of purified anti-PI4P IgM (Echelon Biosciences Inc.), which does not cross-react with PI3P and PI5P (supplier certificate analysis and own assays). A horseradish peroxidase-conjugated anti-mouse antibody (dilution 1:3000) and ECL substrate (Amersham ECL Western Blotting Systems) were employed for chemiluminescence detection.

Results

Presence of phospholipids in the apoplast of imbibed sunflower seeds

The first aim of this work was to analyse the presence of phospholipids in apoplastic fluids of sunflower seeds under imbibition. A standard infiltration procedure was used to yield ~100 µl of EWF per g of seed, with an estimated protein concentration of 1 mg ml^{-1}. The presence of a previously characterized apoplastic lipid transfer protein (Ha-AP10) was verified in this extracellular fluid (Fig. 1). The EWF obtained was evaluated with different approaches to assess the occurrence of intracellular and plasmalemma contamination. First, clearly different protein patterns were observed on SDS–PAGE when comparing equal protein amounts from EWF and SE (Fig. 1A). A second piece of evidence was contributed by the analysis of marker proteins. Hence, western blot analysis of EWF fractions failed to detect plasma membrane H^+-ATPase, actin, and oleosin (Fig. 1). The latter is a protein located in intracellular oil bodies and enriched in seed extracts (Millichip *et al*., 1996). In addition, no enzymatic activity of Glu-6-P dehydrogenase could be detected in the EWF even when testing 600-fold more fresh weight than the SE control (not shown). These pieces of evidence indicate that the apoplastic fraction obtained was devoid of any significant contamination and allowed the characterization of its phospholipid components.

The lipid profiling approach based on ESI-MS/MS (Brügger *et al*., 1997) has proven to be sensitive, quantitative, and efficient for the analysis of plant extracts

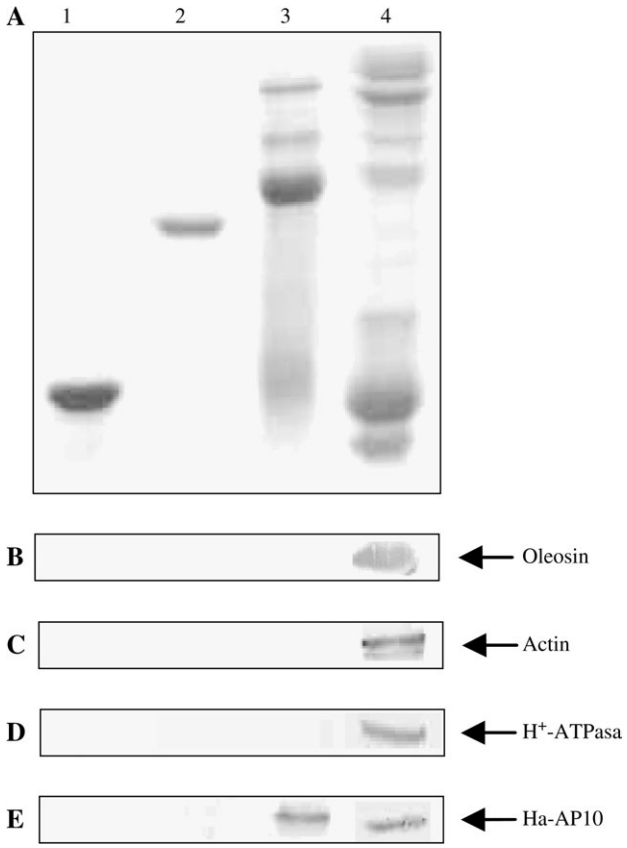

Fig. 1. Comparative protein electrophoretic profiles and immunodetection analysis of extracellular washing fluid (EWF) and seed extract (SE) fractions from sunflower seeds. EWF and SE (50 µg of protein) were analysed by SDS–PAGE and subjected to Coomassie blue staining (A). Fractionated proteins were transferred to nitrocellulose and immunodetected with 1:5000 anti-oleosin antiserum (B), 1:2500 anti-actin antibodies (C), 1:5000 anti-H^+-ATPase antiserum (D), and 1:6000 anti-Ha-AP10 antiserum (E). A 50 µg aliquot of protein was loaded in B and C, 200 µg in D, and 100 µg in E. Lane 1, 14 kDa molecular weight marker; lane 2, 29 kDa molecular weight marker; lane 3, EWF; lane 4, SE.

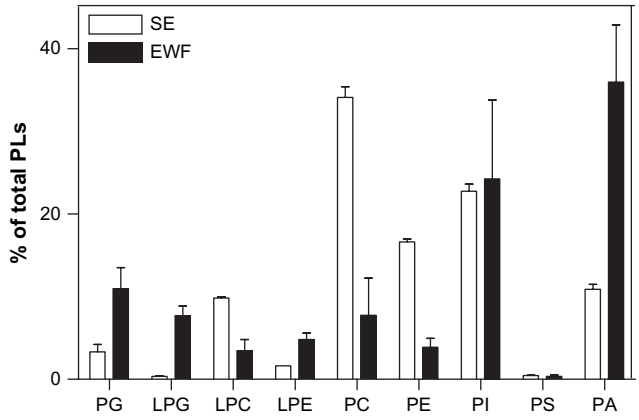

Fig. 2. Phospholipid composition of sunflower extracellular washing fluid (EWF) and seed extract (SE). The lipid fraction from EWF and SE was analysed by ESI-MS/MS for determination of phospholipids. The content of each phospholipid class was calculated by adding all the species detected (Supplementary Table S1 at *JXB* online), and is expressed as a percentage of total phospholipids. Values are means of three repetitions for SE and seven independent experiments for EWF. Bars indicate the SD.

(Welti and Wang, 2004). Hence, the lipid fractions from both the EWF and SE were prepared by organic extraction and subjected to ESI-MS/MS for the analysis of phospholipid s. Although detectable, levels of phospholipids in the EWF were substantially lower than those in the SE ($\sim 2\times10^5$-fold). A concentration of 5 µmol of phospholipids was detected in the SE, and only 21 pmol were obtained in EWF preparations, both from 1 g of seeds. Hence, phospholipid measurement in fluids was routinely performed with samples prepared from 20 g of seeds, which proved to generate reproducible profiles in independent experiments. The content of each phospholipid species determined by ESI-MS/MS in the SE and EWF is presented in Supplememtary Table S1 at *JXB* online. These data were first used for the calculation of the total content of each phospholipid class in both samples. Figure 2 shows that the phospholipid composition detected in the SE and EWF was clearly different. Major phospholipids present in the EWF were PA and PI, which proved to be enriched in all the repetitions performed. PG, PC, lysoPG, lysoPE, PE, and lysoPC were also detected as minor components of the EWF. In addition, barely detectable levels of PS were seen.

On the other hand, and in accordance with previous reports (Salas *et al.*, 2006), PC is the most abundant phospholipid in the SE, followed by PI and PE. The molar ratio of each phospholipid class in both samples is presented in Table 1. Their relative levels comparing SEs and EWFs vary depending on the lipid type, indicating again that it is unlikely that the lipids detected in the EWF originated from cellular leakage. The results presented in Fig. 2 and Table 1 account for the total bulk of each phospholipid class, calculated as the sum of all the species determined. However, each class is composed of several molecular species whose acyl chains vary in the degree of saturation and carbon number (Supplememtary Table S1 available at *JXB* online), and a detailed inspection gives additional information which is presented in Fig. 3 for the major phospholipid classes detected. The most abundant species found in PA from the EWF are not the same as those that are present in the SE. For example, 34:6 PA was not detected in the SE, while it represents the major species in the EWF. Similarly, 34:1 PA is also enriched in the EWF compared with the SE. On the other hand, 36:4 PA is the most abundant form in SEs but is not a well-represented species in the EWF. To discard the possibility of PA production by phospholipases during sample handling, the phospholipase D (PLD) activity was measured in the EWF using [^{32}P]PC as substrate. Positive controls (SE) displayed a clear production of PA at the expense of PC, while it was not possible to detect PA

Table 1. *Molar ratio of SE phospholipids in relation to EWF phospholipids*

The lipid fraction from the EWF and SE was analysed by ESI-MS/MS for determination of phospholipids. The content of each phospholipid class was calculated by adding all the species containing fatty acids with a different degree of saturation and chain length. The molar ratio was calculated as the mean nanomoles of each phospholipid class present in the SE from 1 g of seeds in relation to the total mean nanomoles of the same phospholipid present in the EWF obtained from 1 g of seeds.

Phospholipid class	Molar ratio SE/EWF ($\times 10^5$)
Phosphatidylglycerol	0.61
Lysophosphatidylglycerol	0.11
Lysophosphatidylcholine	5.38
Lysophosphatidylethanolamine	0.76
Phosphatidylcholine	10.43
Phosphatidylethanolamine	8.96
Phosphatidylinositol	1.97
Phosphatidylserine	2.45
Phosphatidic acid	0.70
Total PLs	2.23

production in the EWF (not shown). Along the same lines of evidence, the major species of PC in the EWF are 34:2 and 36:4. If they were partially hydrolysed to render PA, the same acyl species would be expected in PA. However, the major species of PA in the EWF are 34:1 and 34:6. These observations strongly suggest that the PA detected in the EWF does not originate from PLD activity during sample preparation.

Concerning PI species, the 34:2 form is highly abundant in SEs while the EWF has several species in minor abundance. These data indicate that a particular composition of fatty acids is associated with extracellular PA and PI, the major phospholipids detected in the apoplast of imbibed sunflower seeds.

Effect of ABA and JA treatments on extracellular phospholipids

ABA and JA play regulatory roles in several physiological processes in plants, including seed germination (Wasternack and Hause, 2002; Nambara and Marion-Poll, 2003). Accumulated experimental evidences also suggests that phospholipids are involved in the signal transduction pathways triggered by both hormones (Ercetin and Gillaspy, 2004; Lin *et al*., 2004; Katagiri *et al*., 2005). Hence, ABA and JA were chosen to evaluate whether their application causes changes in extracellular phospholipids during sunflower seed imbibition. Seeds were incubated for 2 h with water, 50 μM ABA, or 100 μM JA, then the lipids from the EWF were extracted and the phospholipids analysed by ESI-MS/MS to allow their measurement (Supplememtary Table S1 available at *JXB* online). Although ESI-MS/MS is very sensitive and showed the same qualitative profiles among repetitions,

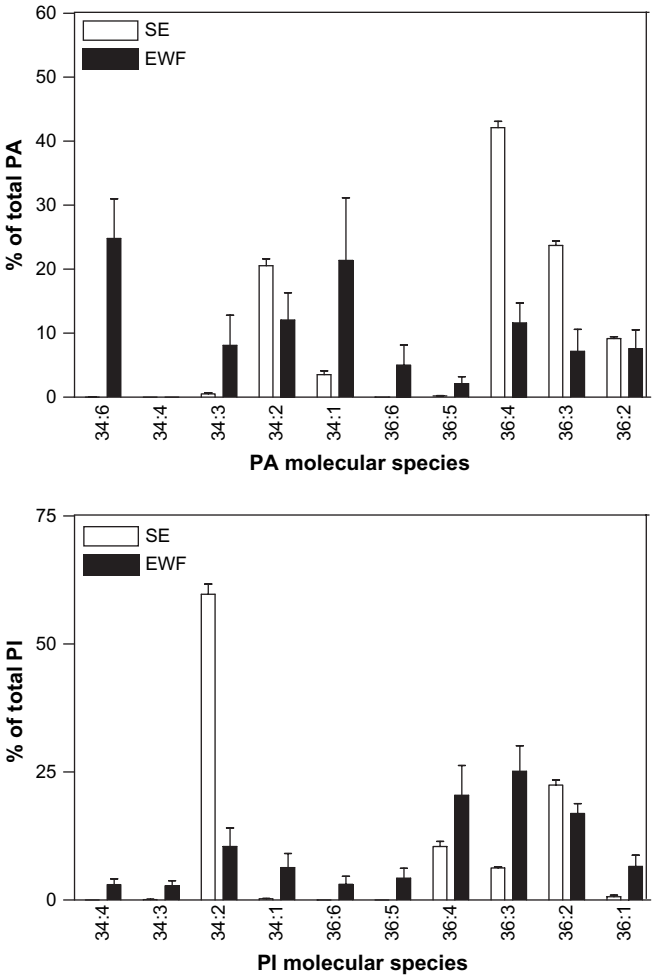

Fig. 3. Molecular species of the major EWF phospholipids compared with the same phospholipid species present in the SE. The lipid fraction from the EWF and SE was analysed by ESI-MS/MS for determination of phospholipids. Results in each column indicate individual species for each phospholipid class, distinguishing chain length and degree of unsaturation. Two numbers separated by a colon denote the total number of carbon atoms and the total number of double bonds present in the acyl chains. Values are means of three repetitions for the SE and seven independent experiments for the EWF. Bars indicate the SD.

the low quantity of each molecular species present in the samples produced great variability in quantitative data. Efforts were thus made to increase the volume of EWF analysed, but this was not practicable for several repetitions if treating >20 g of seeds. Under these conditions, statistics carry the risk that physiologically relevant changes can remain undetected due to the high SD of the data. To solve this point, the stringency of the significance test was reduced, accepting as significant *P*-values $\leqslant 0.1$.

When analysing the sum of the lipid species of each phospholipid class, only JA treatment resulted in significant changes in the phospholipid profile, showing the accumulation of PG ($P=0.003$) and a decrease in PI ($P=0.052$) (Fig. 4). Considering that the sum of each phospholipid species present in the EWF may be masking

a change in certain molecular species, a detailed inspection was performed and is presented in Fig. 5 for PA, PI, and PG species. The levels of different PA species showed little change upon hormonal treatment, except for 34:1 PA which increased in JA-treated seeds (P=0.1) and 36:3 PA which accumulates with ABA treatment (P=0.073). Also, 36:2 PI diminished significantly when JA was applied to seeds (P=0.016). Concerning PG species, the only significant modification was observed in 34:3 PG which accumulates in the EWF from ABA-treated seeds (P=0.018).

The decrease in PI levels observed upon JA treatment (Figs 4, 5) could probably originate from its phosphorylation to yield PIP derivatives and/or PIP_2 isomers. In plants, six different phosphoinositides can be generated by the action of specific PI kinases, but ESI-MS/MS is not optimized for their detection. Hence, a different experimental approach was used to evaluate whether phosphoinositides accumulate while PI decreases. Taking into account that PI4P is the most important monophoshorylated form of PI in both quantitative and biological terms, a specific anti-PI4P antibody was employed in protein–lipid overlay assays to assess the presence of PI4P in EWF samples. Commercial PI4P was detected in the 50–200 pmol range, and EWF samples clearly display a positive PI4P signal. Figure 6A shows a representative assay demonstrating the presence of PI4P in the lipid fraction of the EWF from non-treated imbibed seeds and the accumulation of this phospholipid in extracellular fluids from JA-treated seeds tested at different dilutions. Chemiluminescence detection of PI4P from protein–lipid overlay assays shows a constant JA/control ratio at 1:4

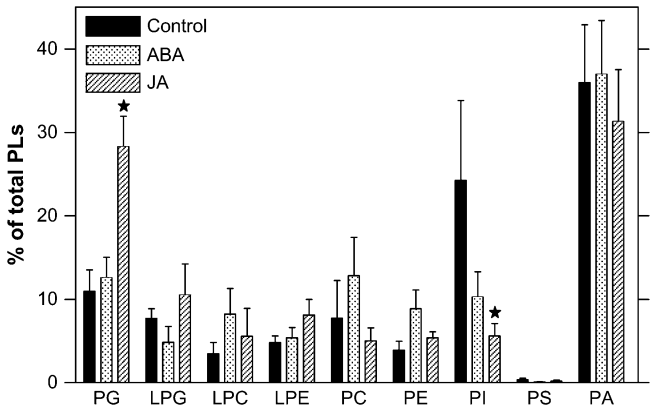

Fig. 4. EWF phospholipid profile from seeds treated with abscisic acid (ABA) or jasmonic acid (JA). The lipid fraction of the EWF from seeds imbibed in water (control) or seeds subjected to 50 μM ABA or 100 μM JA treatment were analysed by ESI-MS/MS for determination of phospholipids. The content of each phospholipid class was calculated by adding all the species detected (Supplementary Table S1 at *JXB* online), and is expressed as a percentage of total phospholipids. Values are means of seven independent experiments for control EWF and five independent experiments for EWF prepared from ABA- and JA-treated seeds. Bars indicate the SD.

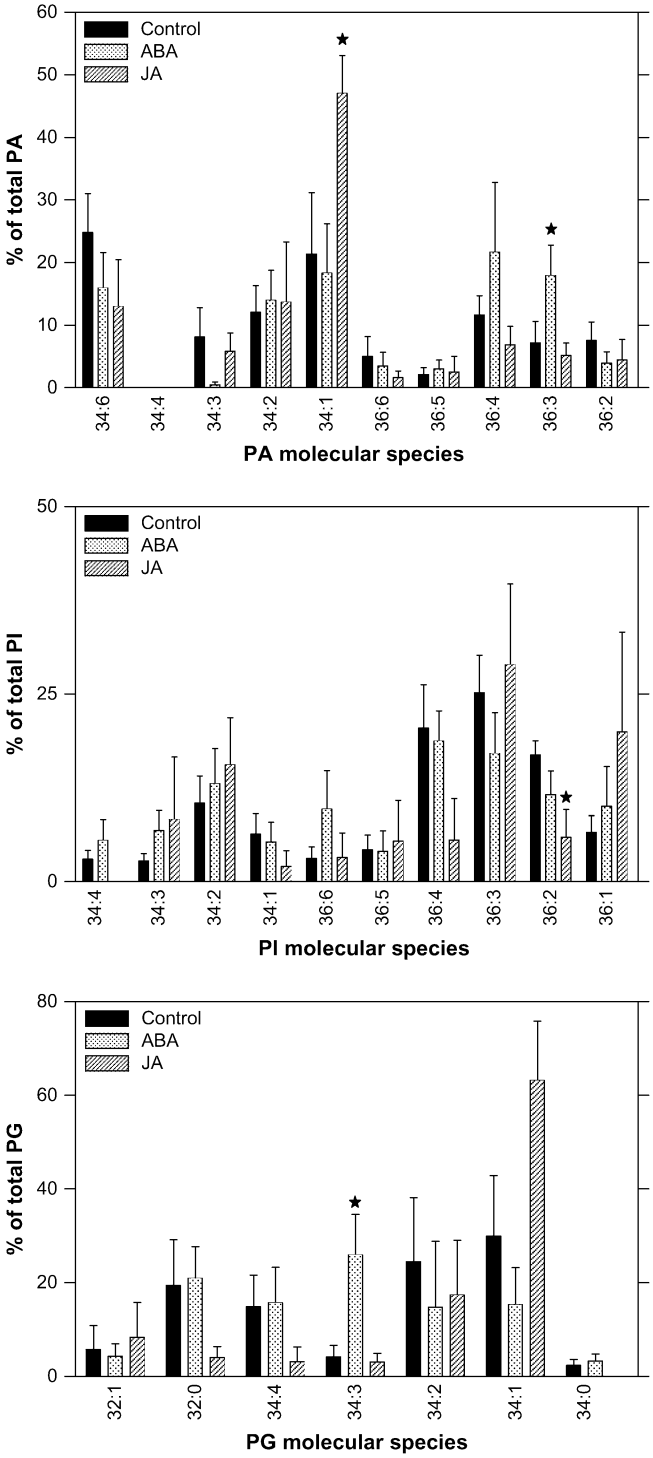

Fig. 5. Molecular species of PA, PI, and PG in the EWF from seeds treated with ABA or JA. The lipid fraction of the EWF from seeds imbibed in water (control) or seeds subjected to ABA or JA treatment was analysed by ESI-MS/MS for determination of phospholipids. Results in each column indicate individual species for each phospholipid class, distinguishing chain length and unsaturation degree. Two numbers separated by a colon denote the total number of carbon atoms and the total number of double bonds present in the acyl chains. Values are means of seven independent experiments for control EWF and five independent experiments for EWF prepared from ABA- and JA-treated seeds. Bars indicate the SD.

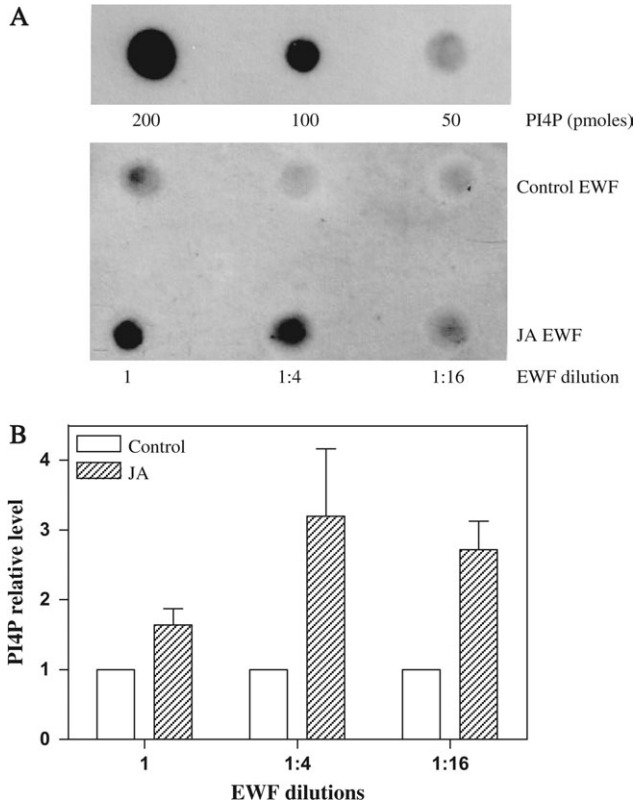

Fig. 6. Presence of phosphatidylinositol 4-phosphate (PI4P) in the EWF from sunflower seeds. (A) Serial dilutions of PI4P (upper panel) or EWF lipid fractions (lower panel) from control and JA-treated seeds were immobilized on a Hybond-C extra membrane. The membrane was submitted to protein–lipid overlay assay using an anti-PI4P antibody and detected by chemiluminescence. For EWF samples, dilutions 1, 1:4, and 1:16 correspond to the lipid fraction of fluid obtained from 1, 0.25, and 0.06 g of seeds, respectively. (B) PI4P signals from JA-treated EWF samples detected in protein–lipid overlay assays were quantified relative to control EWF. Data were obtained from three independent experiments and bars indicate the SD.

and 1:16 EWF dilutions, allowing it to be estimated that PI4P increases ~2.5 fold in seeds treated with 100 μM JA for 2 h (Fig. 6B).

Discussion

The results presented here show the presence of phospholipids in EWFs from sunflower seeds. Several pieces of evidence indicate that the detection of these phospholipids cannot be attributed to contamination of the EWF with intracellular contents. In fact, the suitability of the infiltration–centrifugation method for the collection of apoplastic fluids with negligible contamination has already been demonstrated (Lohaus *et al.*, 2001), and the controls performed here have shown the absence of detectable levels of intracellular and plasma membrane components. Last, but not least, clearly distinct phospholipid patterns are detected in the EWF compared with preparations of seed homogenates. Note that the levels of phospholipids in the SE are substantially higher (~2×10^5-fold) than those from the EWF; therefore, a minimum contamination of cellular components originating from cellular leakage would be expected to generate a phospholipid pattern in the EWF similar to that seen in SEs. Moreover, *in vitro* studies have shown the existence of specific domains present in the plasmalemma of petals and leaves which, in turn, have been suggested to participate in microvesiculation events (Madey *et al.*, 2001). However, those domains contain H^+-ATPase and this protein has even been used as a target for their isolation (Madey *et al.*, 2001). The fact that it was not possible to detect plasma membrane H^+-ATPase in the EWF isolated strongly suggests that this putative microvesiculation is not at the origin of the phospholipids detected extracellularly. Hence, although the possibility of contamination with as yet unknown cellular compartments cannot be completely excluded, the exhaustive controls performed indicate the absence of cytosolic and plasma membrane components in the extracellular fluids.

The phospholipid profile observed in seeds, with PC being the most abundant one, is similar to those previously described in other species (Moreau *et al.*, 1998) and to that reported for sunflower developing kernels (Salas *et al.*, 2006). Concerning the content of phospholipids in the EWF, it has been possible to obtain reproducible profiles by ESI-MS/MS. The amounts of certain species are extremely low, making it difficult to obtain an accurate value for the concentration, but other species, particularly PA and PI, are clearly enriched.

The composition determined for the EWF does not correlate with that of previously reported plant cell compartments. In fact, although the relative levels of each phospholipid class vary among the endoplasmic reticulum, Golgi, tonoplast, and plasma membranes, PC is always the most abundant phospholipid (Moreau *et al.*, 1998) and no reports could be found describing PA and PI as major components of any endomembrane structure. Indeed, the composition of this EWF is clearly distinguishable from that of plant plasma membranes and also different from plant lipid particles such as oil bodies. PC is also the major polar lipid present in oil bodies, which are enriched in sunflower seeds (Millichip *et al.*, 1996).

An unexpected result reported here is the detection of high levels of PA in the EWF. PA is normally present in small amounts in membranes and constitutes 1–9% of total phospholipids (Moreau *et al.*, 1998; Wang *et al.*, 2006). Levels of PA are known to depend on PLD activity during sample preparation. That is why the extractions of EWFs were always performed under cold conditions and perchloric acid was added to the EWF prior to lipid extraction. In fact, no PLD activity could be detected in these EWFs, supporting the conclusion that PA is naturally enriched in the apoplast of sunflower seeds. Two principal pathways have been identified in plants for

the generation of intracellular signalling PA: (i) production of diacylglycerol from membrane phospholipids by PLC, followed by diacylglycerol kinase activity which produces PA; and (ii) PLD activity which acts hydrolytically on membrane phospholipids yielding PA (Wang *et al*., 2006). However, the existence of extracellular PA has now been described and, to our knowledge, no extracellular PLD nor PLC activity has been described in plants.

The enrichment in apoplastic fluids of phospholipids such as PI and PA, which are known to participate in intracellular signalling events directly or as precursors of signal molecules, prompted the analysis of whether their extracellular levels were modified upon hormonal treatments. Besides ABA, which is a well-known hormone involved in germination, jasmonates are also capable of modulating both germination and seedling development (Staswick *et al*., 1992; Andrade *et al*., 2005). Exogenous treatments with JA or methyl-JA have been shown to exert inhibitory effects on the germination of *Lactuca* (Yamane *et al*., 1981), sunflower (Corbineau *et al*., 1988), rapeseed, and flax (Wilen *et al*., 1991). It has been verified that the JA concentration used in this study, 100 μM, partially reduces sunflower seed germination and root growth (Pinedo *et al*., 2006). Moreover, levels of jasmonates in sunflower seeds have been analysed, demonstrating their accumulation upon imbibition (Vigliocco *et al*., 2007) and reinforcing a physiological role for these regulators in sunflower germination. It is shown here that both ABA and JA induced changes in the pattern of phospholipids from the EWF. Even though they are modest responses, ABA induced the accumulation of 34:3 PG and 36:3 PA, and JA affected the levels of 34:1 PA and 36:2 PI.

Concerning PI, its metabolic pathway is considered critical in plant responses to many environmental factors and development, generating signal molecules and facilitating membrane traffic, and both exo- and endocytosis (Kim *et al*., 2001; Meijer and Munnik, 2003). PI is phosphorylated by different kinases forming signal molecules that participate in multiple cellular processes. Besides the reduction in PI levels determined by ESI-MS/MS in the EWF from JA-treated seeds, protein–lipid overlay assays performed in this work enabled the detection of increased levels of extracellular PI4P. In fact, this phosphoinositide was already detected in the lipid fraction of the EWF from control imbibed seeds, but higher levels of PI4P were observed upon JA treatment. Although other isoforms were not analysed in this work, it is demonstrate that at least PI4P levels are modulated in apoplastic fluids. Limited information is available on the role of this PIP isoform in plants, but it is clearly implicated in stomatal movements (Jung *et al*., 2002). PI4P is considered a precursor of $PI(4,5)P_2$ which, in turn, is the precursor of the second messengers inositol(1,4,5)P_3, diacylglycerol, and PA (Mueller-Roeber and Pical, 2002; Meijer and Munnik, 2003). Taking into account the documented role of phosphoinositides in plant signalling, the modulation of PI and PI4P levels by JA reported in this work further supports the participation of apoplastic phospholipids in intercellular communication events. The role of these extracellular phosphoinositides in a model system is currently under examination (G Gonorazky *et al*., unpublished).

Here it is shown that certain species of extracellular PA are modulated by ABA and JA during seed imbibition. Jacob *et al*. (1999) first suggested that intracellular PA plays a signalling role in seed physiology and germination. Later, the occurrence of high levels of PA during early germination of *Arabidopsis* and its regulation by ABA were also reported (Katagiri *et al*., 2005). Although the origin of extracellular PA is a matter of future study, the fact that this phospholipid has been shown to be implicated in early germination supports a biological significance of its presence in the apoplast. On the other hand, a rather surprising observation was the accumulation of PG upon hormonal treatment, the metabolic significance of which cannot be fully interpreted yet. The fact that PG levels are not reduced upon treatment suggests that this putative substrate of phospholipases may not be the origin of the high levels of PA detected.

Numerous pieces of evidence presented in the last few years have shown that exogenously applied phospholipids exert strong effects on plant growth and development. That is the case, for example, of PA, lysoPE, and lysoPC (Cowan, 2006). Although the underlying mechanism of this phenomenon is not known, it reflects that phospholipids added extracellularly are incorporated by the cells or eventually exert their effect in contact with the membrane. The occurrence of phospholipids in the apoplast of sunflower seeds reported here suggests that such responses to extracellular phospholipids could effectively be operating *in planta*. Interestingly, the characterization of DIR1 (defective in induced resistance), which encodes an apoplastic lipid transfer protein (LTP) from *A. thaliana*, has suggested that lipids are implicated in systemic signalling in plants (Maldonado *et al*., 2002). Although JA has been proposed as a putative ligand of LTPs, phospholipids also bind to these proteins (Oliveira Carvalho and Moreira Gomes, 2007) and the identity of the transmitted molecule responsible for systemic signalling still remains elusive (Grant and Lamb, 2006). Moreover, a dihydroxyacetone phosphate reductase, which forms the glycerol-3-phosphate backbone of glycerolipids, has been implicated in the generation, translocation, and/or perception of the mobile signal of systemic acquired resistance (Nandi *et al*., 2004).

Much of the effort currently in progress in plant sciences is directed towards discovering new systemic signals translocated extracellularly through the apoplast.

The present report now describes the existence of PI, PA, and PI4P in the apoplast of plants and their modulation by hormonal treatments. The extracellular occurrence of phospholipids recognized as key components in signalling events suggests that they could play a role in communicating messages between cells and/or organs, opening up novel opportunities to understand plant intercellular signalling.

Supplementary data

Table S1. PL molecular species of sunflower seed extract (SE) and extracellular washing fluid (EWF) from seeds imbibed in water (control), 50 μM ABA or 100 μM JA.

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