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RESEARCH PAPER

Phosphatidylinositol 4-phosphate is associated to extracellular lipoproteic fractions and is detected in tomato apoplastic fluids

G. Gonorazky[1], A. M. Laxalt[1], H. L. Dekker[2], M. Rep[3], T. Munnik[4], C. Testerink[4] & L. de la Canal[1]

1 Instituto de Investigaciones Biológicas, Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de Mar del Plata, Mar del Plata, Argentina

2 Mass Spectrometry of Biomacromolecules, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

3 Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

4 Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, the Netherlands

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Correspondence
Laura de la Canal, Instituto de Investigaciones Biológicas – CONICET, Universidad Nacional de Mar del Plata, CC 1245, CP 7600, Mar del Plata, Argentina.
E-mail: ldelacan@mdp.edu.ar

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ABSTRACT

We have recently detected phosphatidylinositol-4-phosphate (PI4P) in the extracellular medium of tomato cell suspensions. Extracellular PI4P was shown to trigger the activation of defence responses induced by the fungal elicitor xylanase. In this study, by applying a differential centrifugation technique, we found that extracellular PI4P is associated with fractions composed of diverse phospholipids and proteins, which were pelleted from the extracellular medium of tomato cell suspensions grown under basal conditions. Using mass spectrometry, we identified the proteins present in these pelleted fractions. Most of these proteins have previously been characterised as having a role in defence responses. Next, we evaluated whether PI4P could also be detected in an entire plant system. For this, apoplastic fluids of tomato plants grown under basal conditions were analysed using a lipid overlay assay. Interestingly, PI4P could be detected in intercellular fluids obtained from tomato leaflets and xylem sap of tomato plants. By employing electrospray ionisation tandem mass spectrometry (ESI-MS/MS), other phospholipids were also found in intercellular fluids of tomato plants. These had a markedly different profile from the phospholipid pattern identified in entire leaflets. Based on these results, the potential role of extracellular phospholipids in plant intercellular communication is discussed.

INTRODUCTION

Phospholipids are amphipathic molecules that constitute the major lipids present in eukaryotic membranes. They can be classified according to their main function into structural and signalling molecules. Phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) are structural phosphopholipids that have distinct and specific distributions in the cellular membranes, contributing to their identity (van Meer *et al.* 2008). Distinctively, phosphatidic acid (PA), phosphatidylinositolmonophosphate (PIP), phosphatidylinositolbisphosphate (PIP_2) and lysophospholipids such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and lysophosphatidic acid (LPA) can be produced from structural phospholipids by specific enzymatic pathways and are present in minor amounts in the cell membranes (Meijer & Munnik 2003). They can play a role as mediators in diverse cellular functions, such us regulation of cell growth, proliferation, membrane trafficking and signalling pathways involved in responses to hormones, biotic and abiotic stress (Moolenaar *et al.* 2004; Ryu 2004; Wang *et al.* 2006; Balla *et al.* 2009; Munnik & Vermeer 2010). Particularly in plants, phospholipids have been mainly characterised as intracellular molecules exclusively involved in processes that occur inside the cell. However, extracellular phospholipids have been recently detected in intercellular fluids of sunflower seeds by employing electrospray ionisation tandem mass spectrometry (ESI-MS/MS) (Regente *et al.* 2008). It has been demonstrated that these phospholipids are associated to vesicular fractions present in the apoplast of sunflower seeds (Regente *et al.* 2009). In addition, we have reported the detection of an extracellular phospholipid profile in tomato cell suspensions, which is clearly different from the intracellular phospholipid pattern (Gonorazky *et al.* 2008). The most abundant extracellular phospholipid detected by radiolabelling in cell suspensions grown under basal conditions was identified as phosphatidylinositol-4-phosphate (PI4P), previously characterised as an intracellular signal molecule in animal and plant systems (Balla *et al.* 2009; Munnik & Vermeer 2010). Extracellular PI4P was shown to be involved in the activation of defence responses induced by the fungal elicitor xylanase. Based on these results, we postulated a novel role for PI4P as an intercellular signalling molecule involved in the induction of plant defence responses (Gonorazky *et al.* 2008).

The first aim of this work was to study whether, due to its amphipathic nature, PI4P would be present in the form of particles in an aqueous environment, such as the extracellular medium of tomato cell suspensions. In this system, we have found that extracellular PI4P is associated with lipoproteic fractions, *i.e.* pellets composed of phospholipids and proteins, which were obtained upon high-speed centrifugation of the extracellular medium. We also demonstrate the presence of PI4P in apoplastic fluids of tomato plants by employing a lipid overlay assay. In addition, using ESI-MS/MS we detected the presence of other phospholipids distinct from PI4P in intercellular fluids of tomato leaflets.

MATERIAL AND METHODS

Cell suspensions

Tomato cell suspensions (*Solanum lycopersicum* cv. Money-Maker, line Msk8) were grown at 25 °C in the dark at 125 rpm in MS medium (Gonorazky *et al.* 2008). Total extracellular medium was isolated from the cell suspensions as previously described (Gonorazky *et al.* 2008). Briefly, 2 ml of cell suspensions were carefully transferred to 2-ml reaction vials and centrifuged at 100 *g* for 2 min. One microliter of the supernatant, which constitutes the extracellular medium, was subsequently recovered by filtering through a 5-μm and then through a 0.2-μm membrane filter in order to avoid cell contamination. Total lipid or proteic extraction was then performed, as indicated.

Plant material and collection of intercellular fluids and xylem sap

Tomato plants (*Solanum lycopersicum* cv GCR 161) were grown in soil under a 16-h light (200 $\mu mol \cdot m^{-2} \cdot s^{-1}$)/8-h dark regime, at 25 °C. After 6–8 weeks, leaflets were cut to obtain intercellular fluids. This was collected using a standard technique that allowed recovery of apoplastic components based on a vacuum infiltration and centrifugation procedure (Joosten & De Wit 1989; Regente *et al.* 2008). Briefly, leaflets were immersed in an infiltration solution [150 mM NaCl, 0.2% (v:v) Tween 20, 0.1% (v:v) 2-mercaptoethanol] for 45 min and subjected to 10 vacuum pulses of 20 s, at 5-s intervals. A pressure of 45 kPa was applied. Infiltrated leaflets were carefully blotted dry on filter paper to remove excess of infiltration solution and then were placed on perforated-base acrylic vessels, which, in turn, were located over collector vessels made of the same material. These were centrifuged for 60 min at 650 *g* at 4 °C. Intercellular fluid samples were recovered in the collector acrylic vessels. One gram of fresh weight of leaflets yielded 80 μl of intercellular fluid. Entire leaflets were also harvested in order to extract total proteins or lipids.

Xylem sap was collected from the root side of cut stems, as previously described (Kehr & Rep 2007). In short, stems of 6- to 8-week-old plants were cut off below the second true leaf. The plant was placed horizontally and sap emerging from the cut surface through root pressure was collected in tubes placed on ice. The first 300 μl of exudate was discarded in order to avoid contamination with cell debris.

[$^{32}P_i$]-Phospholipid labelling and analysis

For ^{32}PIP production *in vivo*, 85 μl of cell suspension aliquots were labelled for 3 h with 5 μCi carrier-free $^{32}PO_4^{-3}$. For differential centrifugation assays, 2 ml of cell suspension aliquots were labelled for 3 h with 40 μCi carrier-free $^{32}PO_4^{-3}$ and the extracellular medium was subsequently isolated. Total lipids were extracted from 85 μl of cell aliquots, total extracellular medium or pellets and the supernatant obtained upon differential centrifugation of the extracellular medium, as described earlier (Gonorazky *et al.* 2008). Lipids were resolved on silica-60 thin layer chromatography (TLC) plates employing an alkaline solvent system as a mobile phase (Gonorazky *et al.* 2008). Radiolabelled phospholipids were visualised using autoradiography or phosphoimaging (Storm; Amersham Biosciences, Piscataway, NJ, USA).

Protein analysis of extracellular medium and tomato cells

Total proteins were extracted from filtered tomato cells frozen in liquid nitrogen. Cells were ground in liquid nitrogen and protein isolation buffer was added [50 mM Tris, pH 7.5, 80 mM NaCl, 2 mM EGTA, 1 mM EDTA, 2 mM DTT, 300 mM sucrose, 1% (v:v) PVPP, PMSF 1 mM] (Testerink *et al.* 2004). The extract was centrifuged for 10 min at 2000 *g* at 4 °C. The supernatant was spun again employing the same conditions. The resulting supernatant was used for SDS-PAGE analysis and GAPDH detection. Proteins from 500 μl aliquots of extracellular medium were precipitated overnight at −20 °C with three volumes of precipitation buffer [0.1 M sodium acetate, 95% (v:v) ethanol]. Precipitated proteins were spun down by centrifugation for 20 min at 15,000 *g* at 4 °C. The pellet was resuspended in 10 mM Tris-HCl pH 7. Protein concentration was determined using the Bradford method with serum albumin as standard. Electrophoretic separation was performed using 12% SDS-PAGE gels. Proteins obtained from cells and extracellular medium were visualised with colloidal Coomasie blue or transferred to nitrocellulose membranes for GAPDH detection using an anti-GAPDH polyclonal antibody provided by Dr M.C. Shih (Wang *et al.* 1997). Binding of anti-GAPDH antibody to GAPDH was detected using anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Biosciences) and visualised using enhanced chemoluminescence and autoradiography.

Differential centrifugation assay

Extracellular medium isolated from two aliquots of 2 ml-[$^{32}P_i$] pre-labelled tomato cell suspensions (for phospholipid profile analysis) or from four aliquots of 2 ml non-labelled cell suspensions (for protein profile analysis) were subjected to fractionation using sequential centrifugation steps at 1000 *g* for 10 min, 10,000 *g* for 10 min and 50,000 *g* for 60 min.

Identification of proteins by mass spectrometry

Proteins from total extracellular medium were precipitated overnight at −20 °C with three volumes of precipitation buffer, as described earlier. Pellets obtained from total protein precipitation and 50,000 *g* centrifugation of the extracellular

medium were resuspended in 1× Laemmli loading buffer, resolved with 12% SDS-PAGE and visualised by colloidal Coomassie staining. Bands corresponding to the 50,000 *g* pellet were cut out from the gel and subsequently processed, digested with trypsin and analysed using mass spectrometry, as previously described (Testerink *et al.* 2004). The identification of the tryptic peptides was performed using a Mascot MSDB database, release 20063108 (http://www.matrixscience.com) or an updated version of a predicted tryptic fragment database built up from the Sol Genomics Network (SGN) unigene sequences database (http://www.solgenomics.net) (Houterman *et al.* 2007). For this, the tomato unigene set 'Tomato_200607_build_1.seq.gz' was downloaded from SGN and then the largest open reading frame contained within each sequence was translated into protein using a homemade PERL script. A Fasta file with these protein sequences was then queried with Masslynx protein probe software (Waters Corp., Milford, MA, USA).

Lipid extraction and phospholipid profile analysis by ESI-MS/MS

Total lipids were extracted from entire leaflets as previously described (Welti *et al.* 2002). Lipid extraction from intercellular fluids and xylem sap was performed by adding 0.5% (v:v) perchloric acid and 3.75 vol. $CHCl_3$/MeOH/HCl (50:100:1, v:v:v) and subsequently processed as detailed before (Gonorazky *et al.* 2008). Lipid extracts were dried under a stream of nitrogen and stored at −80 °C. Phospholipid profiles and acyl composition analysis of intercellular fluids and entire leaflets lipid extracts were carried out using an automated ESI-MS/MS facility available at the Kansas Lipidomics Center.

Lipid overlay assay

Lipid extracts obtained from intercellular fluids, xylem sap and entire leaflets were resuspended in 1 ml $CHCl_3$/CH_3OH/NH_4COOH (5:10:2, v:v:v) and incubated with 20 µl of neomycin beads (Echelon Biosciences, Salt Lake City, UT, USA) in order to purify total phosphoinositides, *i.e.* PI, PIP and PIP_2, which further improves PI4P detection. Phosphoinositide extracts were dried under a stream of nitrogen. Purified phosphoinositides were resuspended in $CHCl_3$/MeOH/H_2O (20:9:1, v:v:v) and analysed by lipid overlay assay for PI4P detection, as previously described (Gonorazky *et al.* 2008).

Incubation of tomato leaves with *in vivo*-produced ^{32}PIP

The ^{32}PIP produced *in vivo* by tomato cell suspensions was scrapped from the TLC plate, eluted from the silica gel and resuspended in water using sonication. Tomato leaves were cut from a 6-week-old plant and their petioles were subsequently submerged in the ^{32}PIP suspension for 5 h. Total lipids were extracted from the petiole portion that had not been in contact with the ^{32}PIP suspension and from the leaflets, as described above. Lipids were resolved using TLC, employing an alkaline solvent system as a mobile phase, and radiolabelled phospholipids were visualised with phosphoimaging.

RESULTS

In animal systems, extracellular phospholipids are generally detected in microparticles or vesicles (Meziani *et al.* 2008; Schorey & Bhatnagar 2008). Therefore, the identification of extracellular PI4P in tomato cell suspensions (Gonorazky *et al.* 2008) prompted us to study whether, due to its amphipathic nature, this phospholipid would be associated with lipidic or lipoproteic particles in an aqueous environment such as the extracellular medium. To this aim, extracellular medium isolated from [$^{32}P_i$]-pre-labelled tomato cell suspensions grown under basal conditions was sequentially centrifuged at 1000 *g* 10 min, 10,000 *g* 10 min and 50,000 *g* 60 min. Phospholipid profiles of total extracellular medium, 1000, 10,000, 50,000 *g* pellets and 50,000 *g* supernatant were analysed with TLC. Figure 1 shows that the phospholipid pattern of the extracellular medium was different from the intracellular phospholipid profile, as previously described (Gonorazky *et al.* 2008). This indicates that the extracellular medium was not contaminated with cellular debris, in which case similar extracellular and intracellular phospholipid patterns would be expected, as demonstrated in snap-freezing experiments (Figure S1). As we reported earlier (Gonorazky *et al.* 2008), Fig. 1 shows that the most abundant extracellular phospholipid was ^{32}PIP, which, in addition, proved to be stable in the extracellular medium (Figure S2). Phospholipids

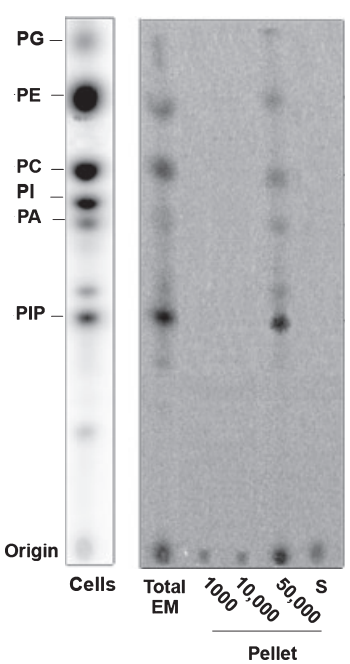

Fig. 1. Detection of PI4P in fractions pelleted from the extracellular medium of tomato cell suspensions. Extracellular medium was isolated from [$^{32}P_i$]-prelabelled tomato cell suspensions and sequentially centrifuged at 1000 *g* 10 min, 10,000 *g* 10 min and 50,000 *g* 60 min. Total lipids were extracted from extracellular medium (Total EM), from the 1000 *g*, 10,000 g and 50,000 *g* pellets (Pellet) and from the 50,000 *g* supernatant (S) obtained upon centrifugation. Phospholipids were resolved by TLC and visualised by phosphoimaging. Phospholipids extracted from tomato cells (cells) were employed as markers. Results of a typical experiment are shown (n = 5).

could not be detected in the 1000 and 10,000 *g* pellets (Fig. 1). In contrast, ^{32}PIP was detected in the 50,000 *g* pellet as the most abundant phospholipid. Upon longer TLC exposure, radioactive PA, PC and PE could also be observed in the 50,000 *g* pellet fraction. Interestingly, none of these phospholipids were detected in the 50,000 *g* supernatant, suggesting that they would be entirely associated with the fractions pelleted from the extracellular medium of tomato cell suspensions. To determine the nature of these 50,000 *g* pelleted fractions, we analysed their proteic profile using SDS-PAGE. Figure 2a shows that proteic bands (indicated with the numbers 1–7) were observed in the 50,000 *g* pellet. These proteins did not originate from cell damage, since the patterns observed in the total extracellular medium and 50,000 *g* pellet were different from the intracellular protein pattern (Fig. 2a). Moreover, the cytosolic enzyme glyceraldehyde phosphate dehydrogenase (GAPDH) was not detected in total extracellular medium or in the 50,000 *g* pellet fraction (Fig. 2b). Mass spectrometry analysis of bands 1–7 observed in the 50,000 *g* pellet (Fig. 2a) allowed the identification of six proteins in bands number 2, 3, 4, 5 and 7, which presented significant scores, having similar theoretical and experimental molecular weights (Table 1). All have been previously identified in tomato as extracellular proteins and most of them are potentially involved in defence responses. These sequences include an inhibitor protein of *Aspergillus acueleatus* xyloglucan-specific endoglucanase, previously detected in the extracellular medium of tomato cell suspensions (Qin *et al.* 2003; Bargmann *et al.* 2006) and in xylem sap of tomato plants, where it accumulates upon infection with *Fusarium oxysporum* (Houterman *et al.* 2007), an aspartyl protease (Bindschedler *et al.* 2008), an inhibitor protein of *Botrytis cinerea* polygalacturonase (Stotz *et al.* 1994), a pectin acetylesterase that plays a role in plant cell wall extension (Bordenave *et al.* 1995; Breton *et al.* 1996), a peroxidase that accumulates during the compatible interaction between tomato and *Pseudomonas syringae* (Coego *et al.* 2005) and an osmotin-like protein that belongs to the PR-5 family of pathogenesis-related proteins (Chen *et al.* 1996). No significant hit was obtained for band 1, whereas a pectin acetylesterase was identified in band 6 but its score was low. On the other hand, no intracellular proteins were detected in bands 1–7 of the 50,000 g pellet of the extracellular medium. This confirms that the results obtained could not be due to contamination with cellular debris. Other bands different from those indicated in Fig. 2a could not be identified because the amount of protein was too limited for mass spectrometry analysis.

As mentioned earlier, we identified PI4P as the most abundant extracellular phospholipid present in the medium of tomato cell suspensions using a radiolabelling technique (Gonorazky *et al.* 2008). To further determine the significance of this observation, the presence of extracellular PI4P *in planta* was evaluated. For this, intercellular fluids and xylem sap samples were analysed, since these represent the apoplastic system that transports distinct molecules along the plant (Kehr & Rep 2007; Seifert & Blaukopf 2010). Intercellular fluids were extracted from tomato leaflets while xylem sap was obtained from cut stems of tomato plants grown under basal conditions. As revealed by GAPDH analysis, no contamination with intracellular components could be detected in intercellular fluids or in xylem sap (Figure S3). Since it

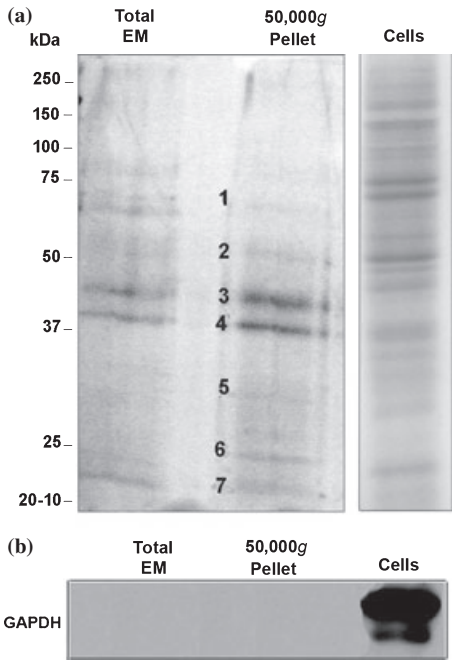

Fig. 2. SDS-PAGE analysis of the proteins associated with phospholipid fractions pelleted from the extracellular medium of tomato cell suspensions. Extracellular medium of tomato cell suspensions was sequentially centrifuged at 1000 *g* 10 min, 10,000 *g* 10 min and 50,000 *g* 60 min. a: Twenty micrograms of proteins isolated from total extracellular medium (Total EM), 50,000 *g* fractions pelleted from the extracellular medium (50,000 *g* pellet) and total proteins obtained from tomato cells (Cells) were resolved with 12% SDS-PAGE and visualised using colloidal Coomasie staining. The protein bands in the 50,000 *g* pellet (1–7) were cut from the gel, digested with trypsin and further analysed using mass spectrometry. b: Immunodetection of glyceraldehyde phosphate dehydrogenase (GAPDH, 40 kDa) with 1:50,000 anti-GAPDH antibody.

was not possible to use a radiolabelling method in an entire plant system to detect extracellular PI4P, we employed a lipid overlay assay using a PI4P-specific antibody (Gonorazky *et al.* 2008). Total lipids were extracted from intercellular fluids, xylem sap and also from entire leaflets as a positive control. Figure 3 shows that positive PI4P signals were observed in intercellular fluids when employing four hundred times more of these samples compared to entire leaflets. Similar results were obtained when xylem sap was analysed (Fig. 3). This indicates that PI4P is present in apoplastic fluids of tomato plants, although at substantially lower levels compared to entire leaflets.

In plants, PI4P can play a role as signal molecule *per se* or it could act as a substrate for the production of $PI(4,5)P_2$ (Munnik & Vermeer 2010). Hence, the detection of PI4P in intercellular fluids and xylem sap prompted us to analyse whether extracellular PI4P would be metabolised to $PI(4,5)P_2$ *in planta*. To this aim, we employed radiolabelled ^{32}PIP produced *in vivo* by [$^{32}P_i$]-labelled tomato cell suspensions, since PI4P constitutes the most abundant monophosphorylated isoform of the PIP pool (Munnik *et al.* 1994; Gonorazky *et al.* 2010). This was resuspended in water through sonication and subsequently supplied to a tomato leaf by submerging the basal portion of the petiole in the ^{32}PIP suspension

Table 1. Identification of proteins associated with phospholipid fractions pelleted from the extracellular medium of tomato cell suspensions.

band number[a]	protein identity	SGN unigen[b]	theoretical MW (kDa)[c]	experimental MW (kDa)[d]	matching peptides[e]	score	references
2	Xyloglucan-specific fungal endoglucanase inhibitor protein (*)	U314071	51	53	4	298	Qin *et al.* (2003), Bargmann *et al.* (2006), Houterman *et al.* (2007)
3	Aspartyl protease (+)	U322187	45	47	11	685	Bindschedler *et al.* (2008)
	Polygalacturonase inhibitor protein (*)	U315727	35–41	47	6	336	Stotz *et al.* (1994)
4	Pectinacetyl estearase (+)	U318294	43	43	15	1045	Bordenave *et al.* (1995),
	Polygalacturonase inhibitor protein (*)	U315727	35–41	43	2	274	Breton *et al.* (1996) Stotz *et al.* (1994)
5	Peroxidase (*)	U313714	33	32	4	389	Coego *et al.* (2005)
7	Osmotin-like protein (*)	U318558	24	17	3	178	Chen *et al.* (1996)

[a]Numbers correspond to bands in Fig. 2a.
[b]As defined by Sol Genomics Network (SGN) unigen sequences database (http://www.solgenomics.net).
[c]Molecular weight predicted from the coding sequence after removal of the predicted signal peptide.
[d]Molecular weight estimated from the position in the SDS-PAGE gel.
[e]Number of tryptic peptides, obtained by mass spectrometry, matching the protein sequence
(*) Tryptic peptides identified with Mascot MSDB database, release 20063108 (http://www.matrixscience.com).
(+) Tryptic peptides identified using a predicted tryptic fragment database built up from SGN.

(Fig. 4a). After 5 h of incubation, the incorporation of ^{32}PIP into the leaf was evaluated by analysing the phospholipid profiles obtained from the petiole portion that had not been in contact with the ^{32}PIP suspension and from leaflets 1, 2, 3 and 4, as indicated in Fig. 4a. Total lipids were extracted from the leaflets and the petiole and resolved using TLC. Figure 4b shows that ^{32}PIP was taken up by the tomato leaf, being detected in the petiole and in leaflets 1 and 2. The ^{32}PIP signal diminished from the bottom part of the leaf, where the ^{32}PIP uptake began, and moved to the upper part. The phospholipid profile of the petiole was similar to the applied ^{32}PIP, in which ^{32}lysoPIP, probably as an artefact, was also detected (Fig. 4b). Similarly, only ^{32}PIP was detected in the leaflets (Fig. 4b). These results suggest that the incorporated ^{32}PIP was not metabolised to ^{32}PI(4,5)P_2 during the incubation. Similar results were obtained when the ^{32}PIP suspension was supplied to the tomato leaf by infiltrating one of its leaflets with a needless syringe (data not shown).

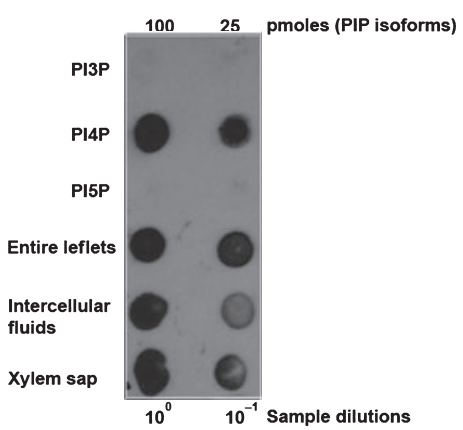

Fig. 3. Detection of PI4P in intercellular fluids and xylem sap of tomato plants. Total lipids were extracted from 0.24 g of entire leaflets, 5.5 ml of intercellular fluids obtained from 100 g of leaflets, and 5.5 ml of xylem sap of tomato plants. Phosphoinositides were isolated from each lipid extract using neomycin beads. Phosphoinositide extracts and synthetic PI3P, PI4P and PI5P were spotted on a nitrocellulose membrane. PI4P was immunodetected with 1 $\mu g \cdot ml^{-1}$ purified mouse monoclonal anti-PI4P antibody. Representative results are shown (n = 2).

The detection of extracellular PI4P in tomato plants prompted us to analyse the presence of other phospholipids in the apoplast using ESI-MS/MS. This technique allows the identification and quantification of various phospholipid molecules, with the exception of PIP and PIP_2 (Welti & Wang 2004). The phospholipids detected are resolved into their molecular species, which differ in the number of carbons and degree of saturation of their fatty acid chains (Welti & Wang 2004). Hence, total lipids were extracted from 3 ml of intercellular fluids and 0.35 g of entire leaflets of tomato plants grown under basal conditions. Lipid extracts were subjected to ESI-MS/MS in order to perform a comparative analysis. The sum of the molecular species identified (Table S1) was used to calculate the content of each phospholipid class and total phospholipid amounts. In this way, we determined that total phospholipid content in intercellular fluids was in the order of 37 $pmol \cdot g^{-1}$, whereas in entire leaflets it was 14 $\mu mol \cdot g^{-1}$, *i.e.* approximately 4×10^5-fold lower in intercellular fluids than in entire leaflets. For this reason, the phospholipid composition of these samples was expressed as a percentage of each phospholipid class in relation to the total content. Figure 5 shows the results obtained from five intercellular fluid and three entire leaflet independent samples. It can be seen that PC was the most abundant phospholipid in both intercellular fluids and entire leaflets. Nevertheless, while PG, PE and PI were present in major proportions in entire leaflets, as previously described in *Arabidopsis* leaves (Welti *et al.* 2002), in intercellular fluids these phospholipids were significantly lower (Fig. 5). Moreover, in intercellular fluids PA was the second most abundant phospholipid detected, making up approximately 15% of the total

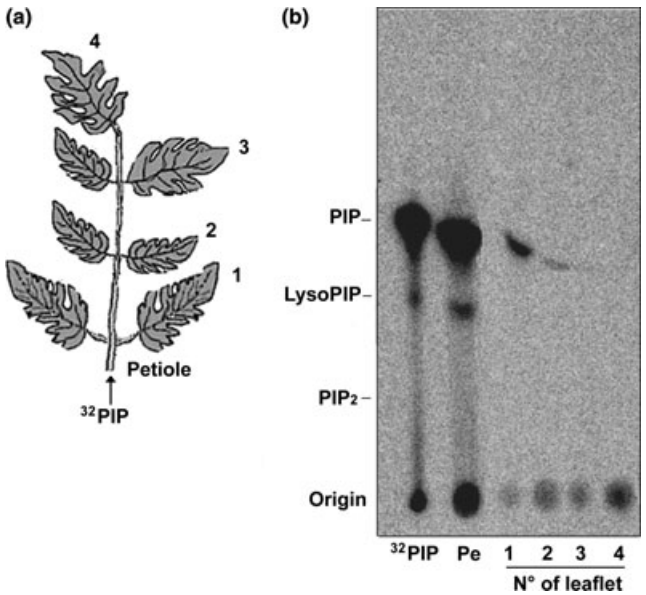

Fig. 4. Analysis of supplied ^{32}PIP incorporation and metabolism in tomato leaves. ^{32}PIP was produced *in vivo* by [$^{32}P_i$]-labelled tomato cell suspensions and resolved using TLC as described in Material and Methods. The ^{32}PIP spot was isolated from the TLC plate and resuspended in water. A tomato leaf was cut from a 6-week-old plant and its petiole was submerged in the ^{32}PIP suspension. After 5 h of incubation, total lipids were extracted from the leaflets and the upper part of the petiole that had not been in contact with the ^{32}PIP suspension. The extracted lipids were resolved using TLC and the radiolabelled phospholipids were visualised with phosphoimaging. a: Schematic representation of the tomato leaf that was incubated in ^{32}PIP suspension. Numbers (1–4) indicate the leaflets from which total lipids were subsequently extracted. b: Phospholipid profiles obtained from the upper part of the petiole (Pe) and leaflets 1, 2, 3 and 4. ^{32}PIP isolated from a TLC plate was used as a control. The expected position of PIP_2 is indicated. R_f values: PIP, 0.35; lysoPIP, 0.24; PIP_2, 0.13.

(Fig. 5). In addition, the intercellular fluid phospholipid profile revealed significantly higher percentages of PS, LPC and LPE than those observed in entire leaflets, where they were present at very low percentages (Fig. 5). These results indicate that phospholipids are present in intercellular fluids constituting a particular profile, in which the proportion of potential phospholipid signal molecules, such as PA, LPC and LPE, is higher than in entire leaflets.

The molecular species profile of each phospholipid class was further analysed by performing a comparative study of their molecular species. This was expressed as the sum of total carbons and double bonds, since the ESI-MS/MS method cannot identify specific acyl groups (Welti & Wang 2004). Figure 6 shows that for most molecular species patterns, differences could be detected between intercellular fluids and entire leaflets. For PC molecular species profiles, only slight differences between intercellular fluids and entire leaflet samples were detected. These include PC 36:2 and PC 36:5, whose proportions respectively were twice higher and threefold lower in intercellular fluids than entire leaflets (Fig. 6a). More extensive dissimilarities were detected between intercellular fluids and entire leaflets in the patterns of PG, PI, PE, PS, PA and LPC (Fig. 6). Regarding PG, the most abundant molecular species in intercellular fluids were 32:0 and 32:1, which were present in only minor amounts in entire leaflets (Fig. 6a). For PI, 32:2 was detected at higher percentages in intercellular fluids compared to entire leaflets, together with 32:0, 32:1, 34:4 and 36:3 (Fig. 6a). PE 40:2, 42:2 and 42:3 species were also present in higher amounts in intercellular fluids than in entire leaflets (Fig. 6a). Regarding PS, 36:2 and 36:6 were twice and twenty times higher in intercellular fluids than in entire leaflets, respectively (Fig. 6a). Concerning the molecular species of PA, the ratio of 32:0 was around fifty times higher in intercellular fluids than entire leaflets (Fig. 6 a). The comparison between molecular species profiles of PA and the structural phospholipids detected in intercellular fluids may be indicative of potential sources for the production of extracellular PA (Welti & Wang 2004). For instance, PA 32:0 could be produced from PG 32:0, which is a substrate of phospholipase D (PLD), and/or PI 32:0 *via* phospholipase C (PLC), while the source of PA 34:2 could be PC 34:2 or PE 34:2, which could be hydrolysed by PLD, and/or PI 34:2 *via* PLC. Regarding LPC, 16:0 was the major species detected in intercellular fluids, while 18:0 was 25-fold higher in intercellular fluids than in entire leaflets (Fig. 6b). The proportions of LPE molecular species in both samples were found to be similar (Fig. 6b).

DISCUSSION

In this study, we show that extracellular PI4P can be completely pelleted together with PA, PC, PE and various proteins when the extracellular medium of tomato cell suspensions is subjected to high-speed centrifugation. Interestingly, the proteins associated with this pelleted fraction have been previously identified as extracellular proteins, and most of them are potentially involved in defence responses. These include a xyloglucan-specific endoglucanase inhibitor protein (Qin *et al.* 2003; Bargmann *et al.* 2006, Houterman *et al.* 2007), an aspartyl protease (Bindschedler *et al.* 2008), a polygalacturonase inhibitor protein (Stotz *et al.* 1994), a pectinacetylesterase (Bordenave *et al.* 1995; Breton *et al.* 1996), a peroxidase (Coego *et al.* 2005) and an osmotin-like protein that belongs to the PR-5 family of pathogenesis-related proteins (Chen *et al.* 1996). These results suggest the existence of putative lipoproteic particles in the extracellular medium. Interestingly, extracellular vesicular bodies of 50–200 nm have been recently detected in 40,000 and 100,000 *g* pellets of centrifuged intercellular fluids of sunflower seeds (Regente *et al.* 2009). On the other hand, the association of signalling phospholipids, such as PI4P and PA, and defence proteins to extracellular particles could constitute a mechanism for delivery of these molecules in a concentrated manner. This could enable more efficient induction of responses in a target cell. This hypothesis has been previously postulated in animal systems, in which extracellular vesicles composed of diverse phospholipids and proteins have been described (Meziani *et al.* 2008; Schorey & Bhatnagar 2008). Interestingly, extracellular vesicles have also recently been described in plants (An *et al.* 2007; Regente *et al.* 2009; Wang *et al.* 2010).

Our discovery of extracellular PI4P associated with a phospholipid profile different from the intracellular pattern in tomato cell suspensions (Gonorazky *et al.* 2008) prompted us to analyse whether this reflects an entire plant system. Here,

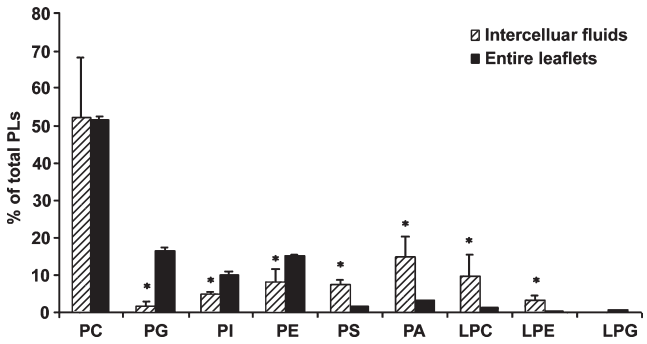

Fig. 5. Intercellular fluids of leaflets and entire leaflets of tomato plants are different in their phospholipid composition. Total lipids were extracted from 3 ml intercellular fluids obtained from 45 g of leaflets, and 0.35 g of entire leaflets of tomato plants. Lipid extracts of intercellular fluids and entire leaflets were analysed using ESI-MS/MS to determine their phospholipid composition. The quantity of each phospholipid class was calculated by adding the molecular species detected (Table S1) and is expressed as a percentage of total phospholipids. Error bars indicate standard deviation of mean values of five intercellular fluid and three entire leaflet independent samples. Asterisks denote that means are significantly different ($P < 0.05$, *t*-test). PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine, PS, phosphatidylserine; PA, phosphatidic acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol.

we demonstrate, for the first time, the presence of PI4P in intercellular fluids and xylem sap of tomato plants. From the results obtained in lipid overlay assays, it was estimated that the content of apoplastic PI4P would be 6 pmol·g^{-1} of leaflets. This would represent 14% of total phospholipids detected in intercellular fluids using ESI-MS/MS. This estimation suggests that PI4P would be among the major extracellular phospholipids identified in tomato plants. Extracellular PI4P could act as a signal molecule *per se* or as a substrate for the production of $PI(4,5)P_2$, which, in turn, can be used by the PLC–diacylglycerol kinase (DGK) enzymatic pathway to yield PA (Munnik & Vermeer 2010). We could not, however, detect the metabolism of exogenously incorporated PIP into PIP_2 *in planta*. Interestingly, similar results were obtained in tomato cell suspensions (Gonorazky *et al.* 2010). Hence, it could be that extracellular PI4P acts as a direct substrate of the PLC–DGK pathway for the production of PA, as previously postulated (Munnik & Testerink 2009; Gonorazky *et al.* 2010). In this sense, the potential metabolism of apoplastic PI4P to PA should be further analysed. Recently, Kale *et al.* (2010) reported that phosphatidylinositol-3-phosphate (PI3P) is present in the outer surface of plant cell plasma membranes, where it enables entry of pathogens into the cells through its binding to host protein effectors (Kale *et al.* 2010). PI4P also binds to oomycete and fungal protein effectors *in vitro*, although it has not been detected in the outer surface of plasma membranes. The detection of both, PI3P and PI4P, has been analysed by peptide-binding studies (Kale *et al.* 2010). Nevertheless, Kale *et al.* (2010) have not chemically measured the presence of PI4P or PI3P, as we have, to demonstrate the existence of PI4P in the extracellular medium of tomato cell suspensions (Gonorazky *et al.* 2008).

In this study, we also show that phospholipids distinct from PI4P are present in intercellular fluids of tomato leaflets. Total phospholipid content was approximately 4×10^5-fold lower in intercellular fluids than in extracts of entire leaflets. Similar results were obtained earlier from intercellular fluids of sunflower seeds (Regente *et al.* 2008). Moreover, the phospholipid profile identified in intercellular fluids of tomato plants was clearly different from that observed in extracts of entire leaflets, showing that the detection of extracellular phospholipids is not due to contamination with intracellular material. Most importantly, the levels of each phospholipid detected in entire leaflets were at the nanomolar scale, while the levels of the distinct apoplastic phospholipids were at the picomolar scale (Table S1). Therefore, any contamination with cell debris, even at a picomolar level, should be reflected in the extracellular profile. In this sense, PA, LPC and LPE, which were detected in minor amounts in entire leaflets, were significantly enriched in intercellular fluids. In contrast, extracts of entire leaflets were mainly composed of structural phospholipids, as previously reported for other plant species (Welti *et al.* 2002; Regente *et al.* 2008). In addition, the molecular species composition of most phospholipids of intercellular fluids revealed differences compared to those of extracts of entire leaflets. A distinct phospholipid profile was identified as well in intercellular fluids of sunflower seeds (Regente *et al.* 2008). There, PA and LPE were also enriched in the apoplast, with PA representing the highest proportion (Regente *et al.* 2008). In spite of these similarities between intercellular fluids of tomato plants and sunflower seeds, differences were observed in their extracellular molecular species composition. For instance, in the case of extracellular PA, PA 34:6 and 34:1 were the most abundant in intercellular fluids of sunflower seeds (Regente *et al.* 2008), whereas in tomato plants PA 32:0 and 34:2 were the major molecular species. In plants, PA has been shown to be involved in diverse biological processes, such as germination, stomatal movement, growth regulation, wounding, biotic and abiotic defence responses (Wang *et al.* 2006). LPC and LPE have been less characterised. Nevertheless, different reports indicate that they play a role in the regulation of plant growth and defence responses (Ryu 2004). Therefore, the presence of these phospholipids in intercellular fluids of tomato plants may suggest their potential role as intercellular signalling molecules. In this sense, PA and LPC have been previously described as extracellular phospholipids in mammalian systems (Moolenaar *et al.* 2004). We have also observed an enrichment of PS in intercellular fluids of tomato plants. Interestingly, extracellular PS has been characterised as an intercellular signal molecule in animal systems (Freyssinet 2003; Meziani *et al.* 2008).

The nature of the cell-to-cell signal(s) in plants has been the subject of decades of research and, although several candidates have emerged (Vlot *et al.* 2008), this major question still remains largely unresolved. In this report, the association of extracellular PI4P to lipoproteic fractions pelleted from the extracellular medium is described, as well as the detection of a distinct apoplastic phospholipid profile in tomato plants, which includes PI4P together with an enrichment of PA, LPC, LPE and PS. These phospholipids may act in still unidentified intercellular signalling pathways, contributing to communication between plant cells.

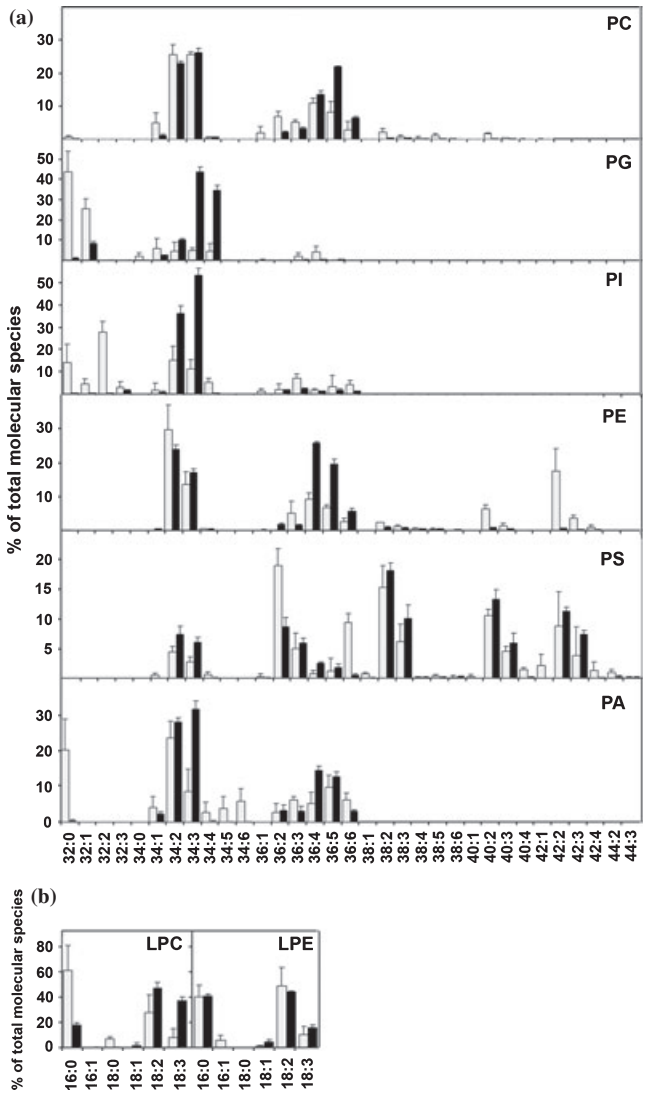

Fig. 6. Comparative analysis of the molecular species composition of each phospholipid detected in intercellular fluids of leaflets and entire leaflets of tomato plants. Total lipids were extracted from intercellular fluid of leaflets (grey bars) and entire leaflets (black bars) of tomato plants and analysed using ESI-MS/MS, as described in Fig. 5. The molecular species detected in intercellular fluids and entire leaflets are expressed as a percentage of the total for each phospholipid class and are identified by chain length (total number of carbon atoms) and degree of unsaturation (total number of double bonds in the acyl chains), separated by a colon. Error bars indicate standard deviation of mean values of five intercellular fluid and three entire leaflet independent samples.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Phospholipid profile of the extracellular medium of snap-frozen tomato cells.

Figure S2. PIP is not hydrolysed upon overnight incubation in extracellular medium.

Figure S3. Analysis of GAPDH detection in apoplastic fluids obtained from non-damaged and damaged tomato plant tissues.

Table S1. Molecular species composition of each phospholipid detected in intercellular fluids of leaflets and entire leaflets of tomato plants.

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REFERENCES

An Q., van Bel A.J.E., Hückelhoven R. (2007) Do plant cells secrete exosomes derived from multivesicular bodies? *Plant Signaling & Behavior*, **2**, 4–7.

Balla T., Szentpetery Z., Kim Y.J. (2009) Phosphoinositide signaling: new tools and insights. *Physiology*, **24**, 231–244.

Bargmann B.O., Laxalt A.M., ter Riet B., Schouten E., van Leeuwen W., Dekker H.L., de Koster C.G., Haring M.A., Munnik T. (2006) LePLDbeta1 activation and relocalization in suspension-cultured tomato cells treated with xylanase. *Plant Journal*, **45**, 358–368.

Bargmann B.O., Laxalt A.M., ter Riet B., Testerink C., Merquiol E., Mosblech A., Leon-Reyes A., Pieterse C.M.J., Haring M.A., Heilmann I., Bartels D., Munnik T. (2009) Reassessing the role of phospholipase D in the *Arabidopsis* wounding response. *Plant, Cell & Environment*, **32**, 837–850.

Bindschedler L.V., Palmblad M., Cramer R. (2008) Hydroponic isotope labelling of entire plants (HILEP) for quantitative plant proteomics; an oxidative stress case study. *Phytochemistry*, **69**, 1962–1972.

Bordenave M., Goldberg R., Huet J.C., Pernollet J.C. (1995) A novel protein from mung bean hypocotyl cell walls with acetyl esterase activity. *Phytochemistry*, **38**, 315–319.

Breton C., Bordenave M., Richard L., Pernollet J.C., Huet J.C., Perez S., Goldberg R. (1996) PCR cloning and expression analysis of a cDNA encoding a pectinacetylesterase from *Vigna radiata* L. *FEBS Letters*, **388**, 139–142.

Chen R., Wang F., Smith A.G. (1996) A flower-specific gene encoding an osmotin-like protein from *Lycopersicon esculentum*. *Gene*, **179**, 301–302.

Coego A., Ramirez V., Ellul P., Mayda E., Vera P. (2005) The H_2O_2-regulated Ep5C gene encodes a peroxidase required for bacterial speck susceptibility in tomato. *Plant Journal*, **42**, 283–293.

Freyssinet J.M. (2003) Cellular microparticles: what are they bad or good for? *Journal of Thrombosis and Haemostasis*, **1**, 1655–1662.

Gonorazky G., Laxalt A.M., Testerink C., Munnik T., de la Canal L. (2008) Phosphatidylinositol 4-phosphate accumulates extracellularly upon xylanase treatment in tomato cell suspensions. *Plant, Cell and Environment*, **31**, 1051–1062.

Gonorazky G., Laxalt A.M., de la Canal L. (2010) Involvement of phospholipase C in the responses triggered by extracellular phosphatidylinositol 4-phosphate. *Journal of Plant Physiology*, **167**, 411–415.

Houterman P., Speijer D., Dekker H., De Koster C., Cornelissen B., Rep M. (2007) The mixed xylem sap proteome of *Fusarium oxysporum*-infected tomato plants. *Molecular Plant Pathology*, **8**, 215–221.

Joosten M.H., De Wit P.J. (1989) Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn Fulvia fulva) as 1,3-beta-Glucanases and Chitinases. *Plant Physiology*, **89**, 945–951.

Kale S.D., Gu B., Capelluto D.G.S., Dou D., Feldman E., Rumore A., Arredondo F.D., Hanlon R., Fudal I., Rouxel T., Lawrence C.B., Shan W., Tyler B.M. (2010) External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. *Cell*, **142**, 284–295.

Kehr J., Rep M. (2007) Protein extraction from xylem and phloem sap. *Methods in Molecular Biology*, **355**, 27–35.

van Meer G., Voelker D.R., Feigenson G.W. (2008) Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology*, **9**, 112–124.

Meijer H.J., Munnik T. (2003) Phospholipid-based signaling in plants. *Annual Review of Plant Biology*, **54**, 265–306.

Meziani F., Tesse A., Andriantsitohaina R. (2008) Microparticles are vectors of paradoxical information in vascular cells including the endothelium: role in health and diseases. *Pharmacological Reports*, **60**, 75–84.

Moolenaar W.H., van Meeteren L.A., Giepmans B.N. (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays*, **26**, 870–881.

Munnik T., Testerink C. (2009) Plant phospholipid signaling: ''in a nutshell''. *Journal of Lipid Reseaerch*, **50**, S260–S265.

Munnik T., Vermeer J. (2010) Osmotic stress-induced phosphoinositide and inositol phosphate signalling in plants. *Plant, Cell and Environment*, **33**, 655–669.

Munnik T., Musgrave A., de Vrije T. (1994) Rapid turnover of polyphosphoinositides in carnation flower petals. *Planta*, **193**, 89–98.

Qin Q., Bergmann C.W., Rose J.K., Saladie M., Kolli V.S., Albersheim P., Darvill A.G., York W.S. (2003) Characterization of a tomato protein that inhibits a xyloglucan-specific endoglucanase. *Plant Journal*, **34**, 327–338.

Regente M., Corti Monzón G., de la Canal L. (2008) Phospholipids are present in extracellular fluids of imbibing sunflower seeds and are modulated by hormonal treatments. *Journal of Experimental Botany*, **59**, 553–562.

Regente M., Corti-Monzon G., Maldonado A.M., Pinedo M., Jorrin J., de la Canal L. (2009) Vesicular fractions of sunflower apoplastic fluids are associated with potential exosome marker proteins. *FEBS Letters*, **583**, 3363–3366.

Ryu S.B. (2004) Phospholipid-derived signaling mediated by phospholipase A in plants. *Trends in Plant Sciences*, **9**, 229–235.

Schorey J.S., Bhatnagar S. (2008) Exosome function: from tumor immunology to pathogen biology. *Traffic*, **9**, 871–881.

Seifert G.J., Blaukopf C. (2010) Irritable walls: the plant extracellular matrix and signaling. *Plant Physiology*, **153**, 467–478.

Stotz H.U., Contos J.J., Powell A.L., Bennett A.B., Labavitch J.M. (1994) Structure and expression of an inhibitor of fungal polygalacturonases from tomato. *Plant Molecular Biology*, **25**, 607–617.

Testerink C., Dekker H.L., Lim Z.Y., Johns M.K., Holmes A.B., Koster C.G., Ktistakis N.T., Munnik T. (2004) Isolation and identification of phosphatidic acid targets from plants. *Plant Journal*, **39**, 527–536.

Vlot A.C., Klessig D.F., Park S.W. (2008) Systemic acquired resistance: the elusive signal(s). *Current Opinion in Plant Biology*, **11**, 436–442.

Wang Q., Kuo L., Sjölund R., Shih M.C. (1997) Immunolocalization of glyceraldehyde-3-phosphate dehydrogenase in *Arabidopsis thaliana*. *Protoplasma*, **198**, 155–162.

Wang X., Devaiah S.P., Zhang W., Welti R. (2006) Signaling functions of phosphatidic acid. *Progress in Lipid Research*, **45**, 250–278.

Wang J., Ding Y., Wang J., Hillmer S., Miao Y., Lo S.W., Wang X., Robinson D.G., Jiang L. (2010) EXPO, an exocyst-positive organelle distinct from multivesicular endosomes and autophagosomes, mediates cytosol to cell wall exocytosis in Arabidopsis and tobacco cells. *Plant Cell*, **22**, 4009–4030.

Welti R., Wang X. (2004) Lipid species profiling: a high-throughput approach to identify lipid compositional changes and determine the function of genes involved in lipid metabolism and signaling. *Current Opinion in Plant Biology*, **7**, 337–344.

Welti R., Li W., Li M., Sang Y., Biesiada H., Zhou H.E., Rajashekar C.B., Williams T.D., Wang X. (2002) Profiling membrane lipids in plant stress responses Role of phospholipase D alpha in freezing-induced lipid changes in Arabidopsis. *Journal of Biological Chemistry*, **277**, 31994–32002.

Supplementary Data Fig. S1

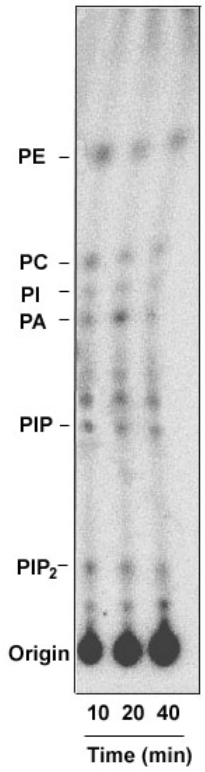

Fig S1. Phospholipid profile of the extracellular medium of snapfrozen tomato cells. Tomato cell suspensions were prelabelled with [^{32}Pi] for 180 min and subsequently snapfrozen in liquide nitrogen and thawed for the indicated times in order to cause mechanical damage of the cells, as previously described (Bargmann et al. 2009). Phospholipids were extracted from the extracellular medium of snapfrozen cells, resolved by alkaline TLC and visualized by phosphoimaging.

Reference

Bargmann BO, Laxalt AM, ter Riet B, Testerink C, Merquiol E, Mosblech A, Leon-Reyes A, Pieterse CM, Haring MA, Heilmann I, Bartels D, Munnik T (2009) Reassessing the role of phospholipase D in the Arabidopsis wounding response. *Plant Cell and Environment* 32**:** 837-850

Supplementary Data Fig. S2

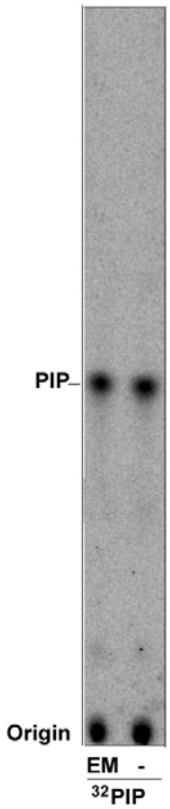

Fig. S2. PIP is not hydrolyzed upon over night incubation in extracellular medium. Radioactive extracellular PIP originated from [^{32}Pi]-prelabelled tomato cells was isolated from a TLC silica plate, resuspended in 10 mM HCl-Tris pH 7.5, sonicated and incubated with or without extracellular medium (EM) for 15 hours at 25ºC. Lipids were then extracted, separated by alkaline TLC and visualized by phosphoimaging.

Supplementary Data Fig. S3

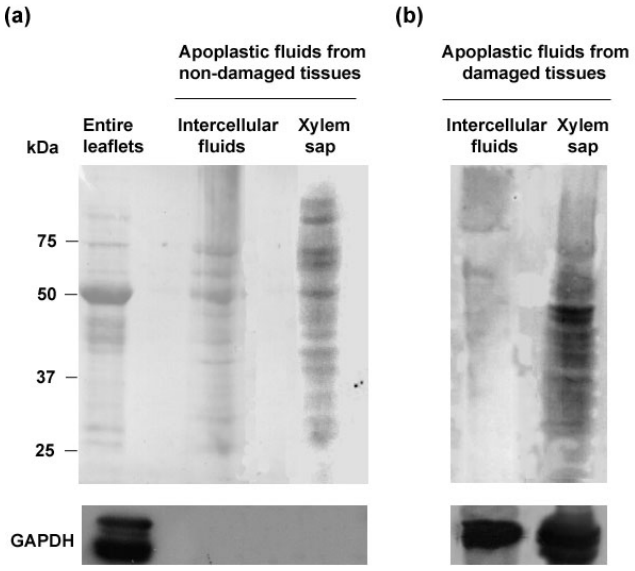

Fig. S3. Analysis of GAPDH detection in apoplastic fluids obtained from non-damaged and damaged tomato plant tissues. (a) Total proteins extracted from 4 mg of entire leaflets; 500 μL of intercellular fluids obtained from 6.25 g of leaflets through soft extraction conditions as described in materials and methods (ten 45 kPa vacuum pulses of 20 s followed by 60 min centrifugation at 650*g*); and from 500 μL of xylem sap collected from the root side of cut stems, upon discarding the first 300 μL of exudates. **(b)** Total proteins extracted from 500 μL of intercellular fluids obtained from 6.25 g of leaflets by performing more stringent extraction conditions (two 45 kPa vacuum pulses of 6 min each followed by 60 min centrifugation at 1,500*g*); and from the first 300 μL of xylem sap exudated from cut stems of tomato plants. Twenty micrograms of proteins from each sample were resolved by SDS-PAGE, transferred to a nitrocellulose membrane and visualized by red ponceau staining (upper panels). Glyceraldehyde phosphate dehydrogenase (GAPDH, 40 kDa) was immunodetected with 1:50,000 anti-GAPDH antibody (lower panels).

Supplementary Data Table S1

[1] Molecular species	[2] Intercellular fluids (pmoles.g^{-1})			[3] Entire leaflets (nmoles.g^{-1})		
PC 32:0	0.12	±	0.05	1.68	±	0.00
PC 34:1	1.03	±	0.71	7.61	±	0.00
PC 34:2	4.87	±	1.00	169.64	±	0.03
PC 34:3	4.94	±	1.44	194.00	±	0.02
PC 34:4	0.09	±	0.03	4.01	±	0.00
PC 36:1	0.36	±	0.35	0.00	±	0.00
PC 36:2	1.31	±	0.30	15.98	±	0.00
PC 36:3	1.03	±	0.42	24.35	±	0.00
PC 36:4	2.14	±	0.80	100.00	±	0.02
PC 36:5	1.64	±	0.98	161.54	±	0.02
PC 36:6	0.52	±	0.46	47.54	±	0.00
PC 38:2	0.39	±	0.13	3.20	±	0.00
PC 38:3	0.17	±	0.18	2.80	±	0.00
PC 38:4	0.05	±	0.09	1.72	±	0.00
PC 38:5	0.21	±	0.11	1.01	±	0.00
PC 38:6	0.04	±	0.02	0.70	±	0.00
PC 40:2	0.33	±	0.10	1.28	±	0.00
PC 40:3	0.07	±	0.06	0.83	±	0.00
PC 40:4	0.02	±	0.04	0.15	±	0.00
PC 40:5	0.02	±	0.03	0.17	±	0.00
Total PC	19.35	±	5.88	738.19	±	0.10
PG 32:0	0.37	±	0.05	2.11	±	0.00
PG 32:1	0.23	±	0.08	18.51	±	0.00
PG 34:0	0.01	±	0.01	0.11	±	0.00
PG 34:1	0.06	±	0.06	5.74	±	0.00
PG 34:2	0.04	±	0.05	23.43	±	0.00
PG 34:3	0.04	±	0.03	103.70	±	0.02
PG 34:4	0.04	±	0.05	81.68	±	0.02
PG 36:1	ND			0.00	±	0.00
PG 36:2	ND			0.21	±	0.00
PG 36:3	ND			0.38	±	0.00
PG 36:4	ND			1.01	±	0.00
PG 36:5	0.02	±	0.01	0.65	±	0.00
PG 36:6	0.04	±	0.01	0.17	±	0.00
Total PG	0.86	±	0.28	238.15	±	0.04
PI 32:0	0.19	±	0.12	0.42	±	0.00
PI 32:1	0.06	±	0.04	0.44	±	0.00
PI 32:2	0.42	±	0.08	0.46	±	0.00
PI 32:3	0.05	±	0.05	2.31	±	0.00
PI 34:1	0.02	±	0.05	1.05	±	0.00
PI 34:2	0.39	±	0.12	52.92	±	0.01
PI 34:3	0.30	±	0.07	77.06	±	0.01
PI 34:4	0.07	±	0.03	0.52	±	0.00
PI 36:1	0.02	±	0.02	0.13	±	0.00
PI 36:2	0.03	±	0.05	2.56	±	0.00
PI 36:3	0.12	±	0.03	3.32	±	0.00
PI 36:4	0.02	±	0.01	1.70	±	0.00
PI 36:5	0.05	±	0.09	2.49	±	0.00
PI 36:6	0.06	±	0.04	1.93	±	0.00
Total PI	1.72	±	0.24	147.31	±	0.03

PE 34:1	0.00	±	0.00	0.71	±	0.00
PE 34:2	0.92	±	0.52	51.65	±	0.01
PE 34:3	0.42	±	0.28	36.87	±	0.01
PE 34:4	0.01	±	0.00	0.66	±	0.00
PE 36:1	ND			0.11	±	0.00
PE 36:2	ND			3.75	±	0.00
PE 36:3	0.18	±	0.14	2.96	±	0.00
PE 36:4	0.25	±	0.09	55.37	±	0.01
PE 36:5	0.20	±	0.11	41.94	±	0.00
PE 36:6	0.08	±	0.05	12.02	±	0.00
PE 38:2	0.07	±	0.03	1.92	±	0.00
PE 38:3	0.03	±	0.01	1.39	±	0.00
PE 38:4	0.01	±	0.01	0.80	±	0.00
PE 38:5	0.01	±	0.01	0.94	±	0.00
PE 38:6	ND	±		0.27	±	0.00
PE 40:2	0.17	±	0.06	1.60	±	0.00
PE 40:3	0.04	±	0.03	0.68	±	0.00
PE 42:2	0.41	±	0.11	1.19	±	0.00
PE 42:3	0.10	±	0.04	0.48	±	0.00
PE 42:4	0.02	±	0.03	0.22	±	0.00
Total PE	2.93	±	1.33	215.54	±	0.03
PS 34:1	0.01	±	0.01	0.00	±	0.00
PS 34:2	0.11	±	0.01	1.91	±	0.00
PS 34:3	0.07	±	0.03	1.55	±	0.00
PS 34:4	0.02	±	0.01	0.01	±	0.00
PS 36:1	0.01	±	0.01	0.02	±	0.00
PS 36:2	0.50	±	0.04	2.23	±	0.00
PS 36:3	0.14	±	0.08	1.49	±	0.00
PS 36:4	0.02	±	0.02	0.62	±	0.00
PS 36:5	0.02	±	0.04	0.46	±	0.00
PS 36:6	0.25	±	0.04	0.11	±	0.00
PS 38:1	0.02	±	0.01	0.01	±	0.00
PS 38:2	0.40	±	0.09	4.65	±	0.00
PS 38:3	0.16	±	0.07	2.55	±	0.00
PS 38:4	0.002	±	0.003	0.04	±	0.00
PS 38:5	0.01	±	0.01	0.02	±	0.00
PS 38:6	0.01	±	0.01	0.05	±	0.00
PS 40:1	0.01	±	0.01	0.00	±	0.00
PS 40:2	0.28	±	0.06	3.43	±	0.00
PS 40:3	0.12	±	0.03	1.52	±	0.00
PS 40:4	0.04	±	0.01	0.04	±	0.00
PS 42:1	0.06	±	0.06	0.00	±	0.00
PS 42:2	0.25	±	0.18	0.06	±	0.00
PS 42:3	0.11	±	0.15	0.03	±	0.00
PS 42:4	0.04	±	0.05	2.88	±	0.00
PS 44:2	0.03	±	0.04	1.86	±	0.00
PS 44:3	0.004	±	0.005	0.02	±	0.00
Total PS	2.68	±	0.46	25.56	±	0.00
PA 32:0	0.94	±	0.48	0.19	±	0.00
PA 34:1	0.24	±	0.25	1.04	±	0.00
PA 34:2	1.12	±	0.50	12.42	±	0.00
PA 34:3	0.52	±	0.56	13.82	±	0.00
PA 34:4	0.12	±	0.10	0.11	±	0.00
PA 34:5	0.16	±	0.11	0.02	±	0.00
PA 34:6	0.25	±	0.16	0.07	±	0.00
PA 36:2	0.10	±	0.09	1.56	±	0.00

PA 36:3	0.29	±	0.09	1.33	±	0.00
PA 36:4	0.28	±	0.20	6.36	±	0.00
PA 36:5	0.49	±	0.32	5.58	±	0.00
PA 36:6	0.32	±	0.19	1.36	±	0.00
Total PA	4.84	±	2.07	43.86	±	0.01
LysoPC 16:0	2.10	±	1.06	3.17	±	0.00
LysoPC 16:1	ND			0.02	±	0.00
LysoPC 18:0	0.24	±	0.15	0.05	±	0.00
LysoPC 18:1	0.00	±	0.01	0.29	±	0.00
LysoPC 18:2	1.09	±	0.94	8.55	±	0.00
LysoPC 18:3	0.24	±	0.19	6.80	±	0.00
Total LysoPC	3.67	±	2.06	18.88	±	0.01
LysoPE 16:0	0.38	±	0.26	1.68	±	0.00
LysoPE 16:1	0.04	±	0.03	0.002	±	0.00
LysoPE 18:1	0.004	±	0.01	0.18	±	0.00
LysoPE 18:2	0.41	±	0.21	1.82	±	0.00
LysoPE 18:3	0.10	±	0.11	0.64	±	0.00
Total LysoPE	0.93	±	0.54	4.31	±	0.00
LysoPG 16:0	ND			1.33	±	0.00
LysoPG 16:1	ND			2.77	±	0.00
LysoPG 18:1	ND			0.00	±	0.00
LysoPG 18:2	ND			2.71	±	0.00
LysoPG 18:3	ND			1.28	±	0.00
Total LysoPG	ND			8.09	±	0.01

Table S1. Molecular species composition of each phospholipid detected in intercellular fluids of leaflets and entire leaflets of tomato plants.

[1] Each molecular species is identified by its chain length (total number of carbon atoms) and degree of unsaturation (total number of double bonds in the acyl chains), separated by a colon denote.

[2] Total lipids were extracted from 3 ml of intercellular fluids obtained from 45 g of tomato leaflets. The results shown are mean values ± SD of five independent samples analyzed by ESI-MS/MS.

[3] Total lipids were extracted from 0.35 g of entire tomato leaflets. The results shown are mean values ± SD of three independent samples analyzed by ESI-MS/MS