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Phosphatidylinositol 4-phosphate accumulates extracellularly upon xylanase treatment in tomato cell suspensions

GABRIELA GONORAZKY[1], ANA M. LAXALT[1], CHRISTA TESTERINK[2], TEUN MUNNIK[2] & LAURA DE LA CANAL[1]

[1]*Instituto de Investigaciones Biológicas, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata CC (1245), CP (7600) Mar del Plata, Argentina and* [2]*Section of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Kruislaan 318, NL-1098 SM, Amsterdam, the Netherlands*

ABSTRACT

Various phosphoinositides have been implicated in plant defence signalling. Until now, such molecules have been exclusively related to intracellular signalling. Here, evidence is provided for the detection of extracellular phosphatidylinositol 4-phosphate (PI4P) in tomato cell suspensions. We have analysed and compared the intracellular and extracellular phospholipid profiles of [$^{32}P_i$]-prelabelled tomato cells, challenged with the fungal elicitor xylanase. These phospholipid patterns were found to be different, being phosphatidylinositol phosphate (PIP) the most abundant phospholipid in the extracellular medium. Moreover, while cells responded with a typical increase in phosphatidic acid and a decrease in intracellular PIP upon xylanase treatment, extracellular PIP level increased in a time- and dose-dependent manner. Using two experimental approaches, the extracellular PIP isoform was identified as PI4P. Addition of PI4P to tomato cell suspensions triggered the same defence responses as those induced by xylanase treatment. These include production of reactive oxygen species, accumulation of defence-related gene transcripts and induction of cell death. We demonstrate that extracellular PI4P is accumulated in xylanase-elicited cells and that exogenous application of PI4P mimics xylanase effects, suggesting its putative role as an intercellular signalling molecule.

Key-words: cell death; extracellular lipids; lipid signalling; phosphoinositides; plant defence; oxidative burst.

INTRODUCTION

Lipids are major components of biological membranes and provide energy for metabolism. Evidence is also emerging that certain lipids can function as signalling molecules during cell growth and differentiation and in response to diverse stimuli. Phosphatidylinositol (PI) is one of the most abundant phospholipids in eukaryotic cells. Three of the five free hydroxyl groups of the inositol ring of PI can be phosphorylated on the D3-, D4- and D5-position, yielding different phosphorylated derivatives which are collectively termed phosphoinositides (PPIs). Unlike PI, PPIs are only present in small amounts and their levels rapidly change in response to stimulation. In eukaryotes, PPIs are considered key regulators of different cellular processes such as vesicle trafficking, cytoskeletal organization, regulation of cellular metabolism and production of second messengers (Mueller-Roeber & Pical 2002; Boss *et al*. 2006; Zonia & Munnik 2006; Krauss & Haucke 2007; Sasaki *et al*. 2007; Strahl & Thorner 2007). Particularly in plants, the responses elicited by both biotic and abiotic stress are among the best-characterized PPI-signalling networks (Zonia & Munnik 2006). The monophosphorylated forms of PPIs described in eukaryotes are PI 4-phosphate (PI4P), PI3P and PI5P (Boss *et al*. 2006; Sasaki *et al*. 2007). PI4P plays a role as precursor of PI 4,5-bisphosphate [PI(4,5)P_2], which is implicated in the phospholipase C (PLC)-signalling pathway that leads to the production of inositol 1,4,5-triphosphate (IP_3), diacylglycerol and phosphatidic acid (PA), which has been characterized as a novel second messenger in plants (Boss *et al*. 2006; Zonia & Munnik 2006; Sasaki *et al*. 2007; Strahl & Thorner 2007). PI4P has been implicated as well in stomatal movement (Jung *et al*. 2002) and vesicular trafficking in plant cells (Vermeer *et al*. 2006a). PI3P and PI5P have been discovered later in plants (Munnik, Irvine & Musgrave 1994; Meijer *et al*. 2001). PI3P has bee involved in vesicular transport (Kim *et al*. 2001; Vermeer *et al*. 2006b), abscisic acid-induced formation of reactive oxygen species (ROS) in guard cells (Park *et al*. 2003), stomatal movement (Jung *et al*. 2002), and regulation of endocytosis and ROS production induced by salt stress (Leshem, Seri & Levine 2007). PI5P has been postulated to play a role during osmotic stress (Meijer *et al*. 2001).

Until today, phospholipid molecules implicated in plant signal transduction have been exclusively related to intracellular signalling. Nevertheless, different lines of evidence have been presented over the last few years suggesting that

Correspondence: L. de la Canal. Fax: +54 223 4753150; e-mail: ldelacan@mdp.edu.ar

phospholipids could also play a role in intercellular signalling. Nandi, Welti & Shah (2004) characterized *Arabidopsis sfd1* gene that encodes a dihydroxyacetone phosphate reductase, which forms the glycerol-3-phosphate backbone of glycerophospholipids. This gene has been implicated in systemic acquired resistance (SAR), a mechanism of plant defence response triggered by an unknown signal that is transported from infected to non-infected tissues, providing enhanced systemic immunity (Grant & Lamb 2006). It was suggested that an SFD1 lipid product would be a signal required for SAR establishment (Nandi *et al*. 2004). This idea is supported by studies of Maldonado *et al*. (2002), who reported an extracellular lipid transfer protein, DIR1, that is detected in the petiole exudates of *Arabidopsis* and plays a role in SAR induction. Interestingly, overexpression of DIR1 in a *dir1* mutant background was not sufficient to trigger SAR, suggesting that DIR1 functions in cooperation with another signal that also has to be induced. Taking into account the ability of lipid transfer proteins to bind acylated compounds through their hydrophobic pocket (Kader 1997), the extracellular mobile signal could be a lipid molecule that is translocated and/or transmitted systemically by DIR1 (Maldonado *et al*. 2002). Despite all evidence implicating lipids in extracellular signalling, the identity of the transmitted lipid molecule still remains elusive (Grant & Lamb 2006).

In this context, the aim of this work was to evaluate the existence of extracellular phospholipid-signalling molecules in an elicited plant system. A well-studied plant-defence elicitor is the fungal molecule xylanase (Bailey, Dean & Anderson 1990; Felix, Regenass & Boller 1993; Avni *et al*. 1994; Yano *et al*. 1998; Tripathy, Venables & Chapman 1999; van der Luit *et al*. 2000; Laxalt *et al*. 2001, 2007; Elbaz, Avni & Weil 2002; Ron & Avni 2004; Bargmann *et al*. 2006). Nevertheless, the signals involved in the xylanase-induced responses have not been fully defined. In tomato cell suspensions, xylanase treatment activates intracellular phospholipid signalling that involves PLC and phospholipase D (PLD) pathways, which also leads to the production of the secondary messenger PA (van der Luit *et al*. 2000; Laxalt *et al*. 2001, 2007; Bargmann *et al*. 2006). Here we show that xylanase triggers PI4P accumulation in the extracellular medium (EM) of tomato cell suspensions. Moreover, addition of this phospholipid to tomato cells mimics xylanase effects by triggering typical defence responses.

MATERIALS AND METHODS

Cell suspensions

Tomato cell suspensions (*Lycopersicon esculentum* cv. Money Maker; line Msk8) (Felix *et al*. 1991) were grown at 25 °C in the dark at 125 r.p.m. in MS medium (Duchefa, Haarlem, the Netherlands) supplemented with 3% (w/v) sucrose, 5.4 μM 1-naphtylacetic acid, 1 μM 6-benzyladenine and vitamins (Duchefa) as described earlier (Felix *et al*. 1991).

Reagents

Xylanase (*Trichoderma viride*) was purchased from Fluka (Buchs, Switzerland). Reagents for lipid extractions and subsequent analysis, as well as silica-60 thin layer chromatography (TLC) plates (20×20 cm) were purchased from Merck (Darmstadt, Germany). The fluorescent probes 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA), 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (pyranine) and SYTOX green nucleic acid stain were purchased from Molecular Probes (Eugene, OR, USA). The natural long fatty acid chain phosphatidylcholine (PC) (Kachroo *et al*. 2005) (Product Number: 840051), phosphatidylglycerol (PG) (Product Number: 841138C), PI4P (Product Number: 840045) and diC18:1 phosphatidylserine (PS) (Product Number: 840035) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Purified mouse monoclonal anti-PI4P antibody was purchased from Echelon Biosciences (Salt Lake City, UT, USA). Hybond-C extra membrane and anti-mouse IgG antibody conjugated to horseradish peroxidase were purchased from Amersham Pharmacia Biosciences (Piscataway, NJ, USA).

Treatments

Tomato cell suspensions of 4 to 5 d old were treated for the indicated time periods with xylanase or different concentrations of PI4P, PC, PS or PG. For the addition of PI4P, PC, PS or PG to tomato cell suspensions, lipids were prepared as follows. PI4P [dissolved in $CHCl_3$/MeOH/H_2O (20:9:1 by vol.)], PC, PS and PG (dissolved in $CHCl_3$) were dried by vacuum centrifugation. The required volume of 10 mM HCl-Tris pH 7.5 buffer was then added to the lipid film, allowed to hydrate for at least 30 min and sonicated three times for 5 s immediately before treatment. Assays were done in Petri dishes (for lipid assays and RNA isolation), in 96-well microtiter plates (for ROS production assays), or in 2 mL reaction vials (for cell death assays) at 25 °C without shaking.

[^{32}Pi] Phospholipid labelling and analyses

Two millilitres of Msk8 cells was labelled for 180 min with 40 μCi carrier-free $^{32}PO_4^{-3}$ (Amersham, Buckinghamshire, UK) prior to incubation with xylanase or cell-free medium, as a control. When the treatments ended, 85 μL of cell suspension was withdrawn and the incubation was stopped with 0.5% (v/v) perchloric acid. The rest of the cell suspension was carefully transferred to 2 mL reaction vials and centrifuged at 100 *g* for 2 min. One millilitre of the supernatant, which constitutes the EM, was subsequently recovered by filtering through a 5 μM and then through a 0.2 μM membrane filter in order to avoid cell contamination (Chapman *et al*. 1998; Oh *et al*. 2005). Total lipid extraction from the cell aliquots and the filtered EM was performed by adding 3.75 vol. of $CHCl_3$/MeOH/HCl (50:100:1 by vol.) and processed as described before (van der Luit *et al*. 2000). Lipids were separated on silica-60 TLC plates employing an

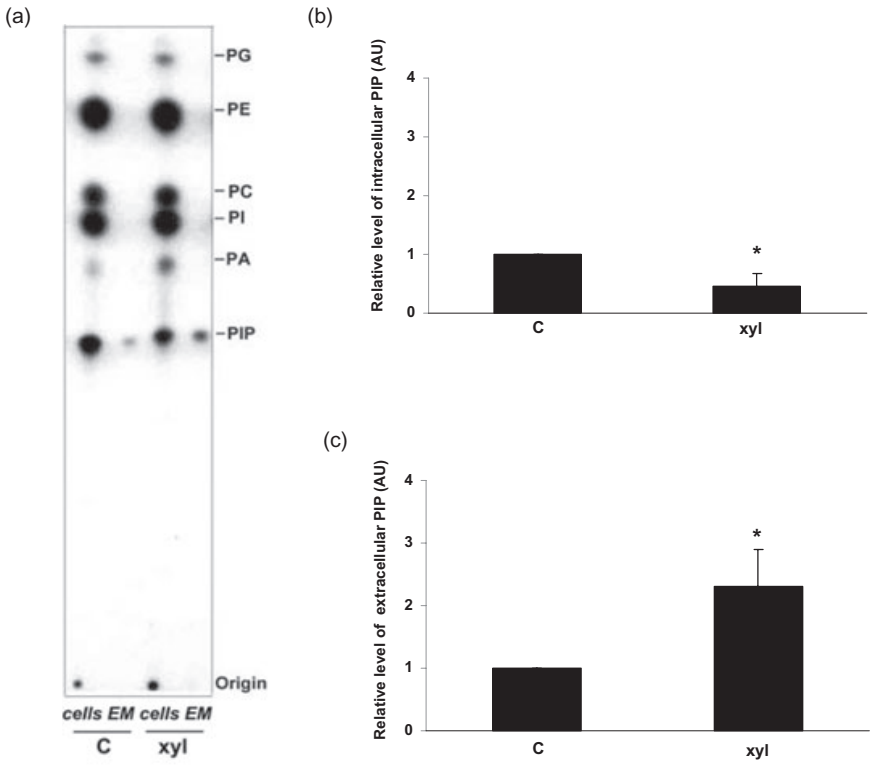


Figure 1. Phosphatidylinositol phosphate (PIP) detection in the extracellular medium (EM) of tomato cell suspensions. Tomato cell suspensions were prelabelled with [^{32}Pi] for 180 min and subsequently treated with 100 μg mL^{-1} xylanase (xyl) or without xylanase (control, C) for 40 min. Phospholipids extracted from cells and EM were resolved by thin layer chromatography and visualized by autoradiography. (a) Representative autoradiograph showing total phospholipid profiles from cells and EM. (b,c) Quantification of PIP levels from six independent experiments. Intracellular (b) and extracellular (c) PIP levels of xylanase treated cells are, respectively, expressed as fold decrease and fold increase relative to control samples. Error bars indicate standard error of means. Asterisks denote that means are significantly different ($P < 0.001$) from control treatments, according to *t*-test. PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid; AU, arbitrary units.

alkaline solvent system as a mobile phase (van der Luit *et al*. 2000). Radiolabelled phospholipids were visualized by autoradiography or by phosphoimaging (Storm; Amersham). Quantification of PI phosphate (PIP) level in Fig. 1 was performed by plot analysis of autoradiography using ImageJ (version 1.37v) (Wayne Rasband, NIH, Bethesda, MD, USA).

Kinase assays

Phosphatidylinostol phosphate 4- and PIP 5-kinases were kindly provided by Dr Nullin Divecha (Division of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, the Netherlands). Myc-tagged PIP 4-kinase was immunoprecipitated from transgenic *Escherichia coli* cells, and myc-tagged PIP 5-kinase was immunoprecipitated from transiently transfected COS cells (Meijer *et al*. 2001). Kinase assays were performed as described previously (Meijer *et al*. 2001). Briefly, phospholipids were labelled *in vivo* and cells were treated with 100 μg mL^{-1} xylanase, as described earlier. Extracellular lipids were extracted and separated by TLC. Subsequently, the radiolabelled PIP spot was localized and eluted from the silica gel. After resuspending it in 10 mM HCl-Tris pH 7.5 by sonication, 1 vol. of double-strength PIP kinase buffer (100 mM HCl-Tris pH 7.5, 20 mM $MgCl_2$, 2 mM EGTA, 140 mM KCl) was added together with 200 μM ATP and PIP 4- or PIP 5-kinase. The PIP kinase assay was carried out at 30 °C for 15 h. The reaction was stopped with 1 mL of cold chloroform:methanol (1:2 vol.) and 200 μL of 2.4 M HCl. Lipids were isolated and separated by an alkaline solvent system (described earlier).

Lipid overlay assay

Determination of the extracellular PIP isoform was performed by a lipid overlay assay, essentially as described by Dowler, Kular & Alessi (2002), except that it was modified to employ an anti-PI4P antibody instead of a PI4P-binding protein for the detection of PI4P. Phospholipids were [^{32}P$_i$]-labelled *in vivo* and cells were treated with 100 μg mL^{-1} xylanase, as described earlier. Extracellular lipids were extracted, separated by TLC and visualized by phosphoimaging. Five-month-decayed non-radioactive extracellular PIP spot was eluted from the silica gel. The eluted extracellular PIP and the three PIP isomers were resuspended in $CHCl_3$/MeOH/H_2O (20:9:1 vol.), spotted onto a Hybond-C extra membrane and subsequently incubated overnight with 1 μg mL^{-1} purified mouse monoclonal anti-PI4P antibody at 4 °C. Specific binding of anti-PI4P antibody to PI4P was detected by using goat anti-mouse immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase and visualized by chemoluminescence.

Visualization of ROS production

Reactive oxygen species production was detected by incubating 80 μL of tomato cell suspension with 0.025 μM

H_2DC-FDA (Ubezio & Civoli 1994), and 10 mM HCl-Tris pH 7.5 (control), 100 μg mL^{-1} xylanase or the indicated concentrations of PI4P, PC, PS or PG in a final volume of 100 μL for 30 min at 25 °C in the dark. The cells were then mounted on microscope slides and visualized with fluorescence microscopy with an excitation filter of 495 nM and a barrier filter of 515 nM, and bright-field microscopy. The production of green fluorescence under these conditions is due to ROS production.

Quantification of H_2O_2 production by fluorometry

The quantification of H_2O_2 production by fluorometry was performed as described previously by de Jong *et al*. (2004), except that it was modified to employ 96-well microtiter plates. Five-day-old tomato cells were pelleted at 450 *g* for 5 min, resuspended in 50 mL assay buffer (5 mM Mes/NaOH pH 5.7, 175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$) and allowed to equilibrate at 25 °C in the dark for 20 min on a rotary shaker (125 r.p.m.). This procedure was repeated twice after which the cells were allowed to equilibrate overnight as described previously. To measure oxidative burst, aliquots of 75 μL of cells equilibrated in assay buffer were carefully pipetted into a 96-well microtiter plate. Then, 25 μL of a mix composed by 13 μL of assay buffer, 2 μL of 200 μg mL^{-1} pyranine (Apostol, Heinstein & Low 1989) and 10 μL of 10 mM HCl-Tris pH 7.5 (control), 100 μg mL^{-1} xylanase or the indicated concentrations of PI4P, PC, PS or PG, was added to the cells. The quenching of pyranine fluorescence because of H_2O_2 production was recorded at 1 or 2 min intervals using an excitation wavelength of 405 nM and an emission wavelength of 525 nM in a Fluoroskan Acsent microwell fluorometer (Thermo Electron Company, Vantaa, Finland). During the measurements, temperature was maintained at 25 °C. Each treatment was performed in triplicate.

RNA isolation and northern blot analysis

Total RNA from tomato cells was isolated using Trizol as described by the manufacturer (Invitrogen, Grand Island, NY, USA). Total RNA at 15 μg was denatured using glyoxal and subsequently separated on a 1.5% agarose gel electrophoresis and transferred onto Hybond-XL (Amersham). Membranes were hybridized in modified Church solution [0.5 M phosphate buffer pH 7.2, 7% sodium dodecyl sulphate (SDS) and 10 mM ethylenediaminetetraacetic acid] overnight at 65 °C. Probes consisted of fragments of *HSR*203J (Pontier *et al*. 1994), *PAL* (Joos & Hahlbrock 1992), *PLDβ1* (Laxalt *et al*. 2001), *ACC oxidase 3* (Hamilton, Bouzayen & Grierson 1991) or *PR1* (van Kan *et al*. 1992). Fragments were radiolabelled with the Megaprime DNA Labelling System according to the manufacturer's recommendation (Amersham) using Redivue α^{32}P-dCTP (Amersham). The membranes were washed twice with 2× SSC, 0.1% SDS for 15 min, 1 × SSC, 0.1% SDS for 30 min and 0.2 × SSC (300 mM NaCl, 30 mM trisodium citrate dihydrate), 0.1% SDS for 15 min at 65 °C. Hybridization patterns were visualized by autoradiography. Before re-hybridization, blots were stripped by washing the membrane with boiling 1% SDS.

Detection of cell death

Ninety microlitre batches of tomato suspension cells were treated with 10 mM HCl-Tris pH 7.5 (control), 100 μg mL^{-1} xylanase or the indicated concentrations of PI4P, PC, PS or PG for 20 h at 25 °C in the dark, and then incubated with 0.2 μM SYTOX green (Thevissen, Terras & Broekaert 1999) for 10 min. Afterwards the cells were washed once with fresh culture medium and carefully pipetted into 96-well microtiter plates. The microwell plate was transferred to a Fluoroskan Acsent microwell fluorometer for fluorometric measurements using filters D480-40 and D525-30 for excitation and emission, respectively. Then, the cells were mounted on microscope slides and visualized as described previously for detection of ROS production. The production of green fluorescence under these conditions is due to the binding of the probe SYTOX green to the nuclear DNA of permeabilized cells. Each treatment was performed in triplicate.

RESULTS

Phosphatidylinostol phosphate is detected in the EM of tomato cells and accumulates upon xylanase treatment

To analyse whether tomato cells produce extracellular phospholipids upon elicitor treatment, we used an experimental approach based on [$^{32}P_i$]-labelling. Cell suspensions were incubated with [$^{32}P_i$] to label all phospholipids, and then treated with or without xylanase. Total lipids were extracted from the cells and from the EM, which was filtered to reduce a putative contamination with cellular debris. Figure 1a shows that a typical phospholipid profile was detected in the cells. This includes structural phospholipids [PG, phosphatidylethanolamine (PE), PC, PI] and the minor signalling phospholipids PA and PIP. This pattern was clearly different from that observed in the EM, where a major spot of PIP was detected under basal conditions (Fig. 1a). Figure 1a & b shows that treatment of tomato cell suspensions with xylanase led to an increase in PA and a decrease in PIP in the cells, confirming the results of van der Luit *et al*. (2000). Interestingly, the opposite effect was observed in the EM (Fig. 1a), revealing a statistically significant 2- to 2.5-fold increase in extracellular PIP level upon xylanase treatment (Fig. 1c). The effect of different doses of xylanase on both intracellular and extracellular phospholipid profiles was further evaluated. Figure 2a shows the intracellular phospholipid profile, in which PA level increases while PIP level decreases upon treatment with increasing concentrations of xylanase. The extracellular PIP, on the other hand, increased in a dose-dependent

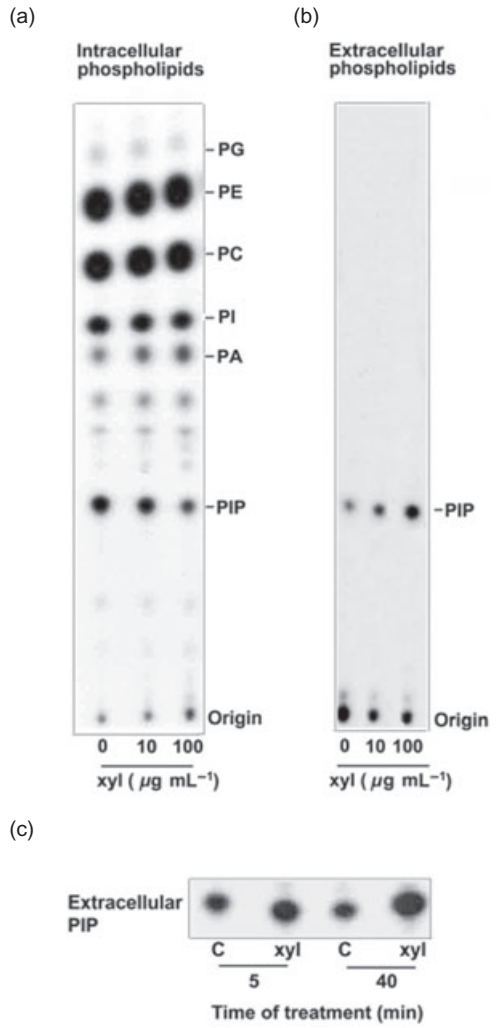


Figure 2. Extracellular phosphatidylinositol phosphate (PIP) level increases upon xylanase treatment in a dose- and time-dependent manner. (a,b) [^{32}Pi]-prelabelled tomato cells were treated with the indicated concentrations of xylanase for 40 min. (c) [^{32}Pi]-prelabelled tomato cells were treated with 100 μg mL^{-1} xylanase (xyl) or without xylanase (control, C) for the indicated times. Phospholipids extracted from cells (a) and extracellular medium (b,c) were resolved by thin layer chromatography and visualized by autoradiography. In panel (c), from the extracellular phospholipid profile only the relevant (PIP) part is shown. Results of a typical experiment are shown ($n = 3$). PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid.

manner (Fig. 2b). Figure 2c shows that extracellular PIP also accumulates in a time-dependent manner. It is already augmented within 5 min of treatment with xylanase and its level kept on increasing upon longer incubations (40 min).

The differences observed between the EM and total cellular phospholipid profiles, as well as the opposite effect of xylanase on intracellular and extracellular PIP levels, indicate that extracellular PIP could not be originated from cellular debris. Moreover, as shown in Figs 1a & 2b, the major phospholipids of plant plasma membranes and endomembranes -PC and PE- (Moreau *et al*. 1998) are not detected in the EM, allowing to discard a significant contamination of this fraction with cellular components.

Phosphatidylinositol 4-phosphate is the PIP isoform that accumulates in the EM of xylanase-treated cells

There are three PIP isomers in plants: PI3P, PI4P and PI5P (Boss *et al*. 2006). To identify which isoform is accumulated in the EM, specific PIP kinases were used that phosphorylate either the D4- or D5-position of the inositol ring (Meijer *et al*. 2001). Accordingly, extracellular ^{32}P-PIP was isolated from [^{32}P$_i$]-prelabelled xylanase-treated tomato cell suspensions and incubated with either PIP 4-kinase or PIP 5-kinase, in the presence of non-radioactive ATP. Reactions were stopped and lipids were extracted and resolved by TLC. If the extracellular PIP would be PI4P, it should be converted to PI(4,5)P$_2$ via PIP 5-kinase. If the extracellular PIP would be PI5P, it should be converted to PI(4,5)P$_2$ via PIP 4-kinase. Finally, if extracellular PIP would be PI3P, it should be converted into two PIP$_2$ products: PI(3,5)P$_2$ via PIP 5-kinase, and PI(3,4)P$_2$, via PIP 4-kinase. As shown in Fig. 3a, only PIP 5-kinase was able to phosphorylate the extracellular ^{32}P-PIP into ^{32}P-PIP$_2$. This indicates that PI4P is the extracellular PIP isoform.

In order to support this conclusion, lipid overlay assays were performed (Dowler *et al*. 2002). For this, the extracellular PIP and synthetic PIP isomers were spotted onto a nitrocellulose membrane and the blot was subsequently incubated with a monoclonal anti-PI4P antibody. Figure 3b shows that only PI4P and the extracellular PIP were recognized by the antibody, which did not bind PI3P or PI5P. These results confirm that PI4P is the PIP isoform that accumulates in the EM of xylanase-treated tomato cell suspensions.

Phosphatidylinositol 4-phosphate treatment induces defence responses in tomato cell suspensions

To investigate whether the xylanase-induced extracellular PI4P response has any significance for defence signalling, we tested whether PI4P treatment would trigger defence responses in tomato cell suspensions.

Reactive oxygen species production is one of the earliest responses triggered after pathogen recognition and has been shown to occur upon xylanase elicitation (Yano *et al*. 1998; Bargmann *et al*. 2006; Laxalt *et al*. 2007). Hence, cell suspensions were treated with xylanase, 10 μM or 100 μM PI4P, or buffer only, in the presence of the probe H$_2$DCFDA, which reacts with intracellular ROS to generate the fluorescent 2′,7′-dichlorofluorescein (Ubezio & Civoli 1994).

Figure 4a shows that exogenous application of PI4P triggers ROS production. The effect was barely detectable with 10 μM PI4P but clearly visible when 100 μM PI4P was

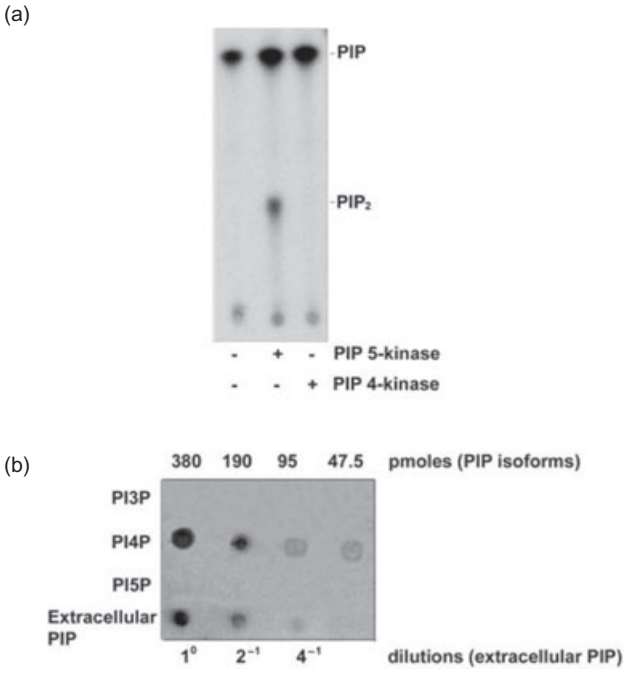


Figure 3. Phosphatidylinositol 4-phosphate (PI4P) is the extracellular phosphatidylinositol phosphate (PIP) isoform. (a) Radioactive extracellular PIP spot from [^{32}Pi]-prelabelled xylanase-treated tomato cells was isolated from a TLC plate and incubated for 15 h without kinase, with PIP 5-kinase or PIP 4-kinase at 30 °C in the presence of 200 μM ATP. Lipids were then extracted, separated by thin layer chromatography (TLC) and visualized by phosphoimaging. (b) Different concentrations of non-radioactive extracellular PIP isolated from xylanase-treated tomato cells and synthetic PI3P, PI4P and PI5P were spotted onto a Hybond-C extra membrane. The blot was incubated overnight with 1 μg mL^{-1} purified mouse monoclonal anti-PI4P antibody and subsequent binding was detected by an anti-mouse IgG antibody and enhanced chemoluminescence, which was visualized by autoradiography. Results of typical experiments are shown ($n = 3$).

applied. Cells were also treated with the zwitterionic phospholipid PC and the anionic phospholipids PS and PG as control treatments. Incubation of the cells with 100 μM PC or 100 μM PS had no effect on ROS production, while 100 μM PG had a minor effect compared with 100 μM PI4P (Fig. 4a).

The oxidative burst was also analysed by using the fluorescent probe pyranine (Apostol *et al*. 1989), which is readily degraded by the H_2O_2 that is produced by challenged cells. Figure 4b shows that exogenous application of 100 μM PI4P induced a rapid and significant decrease in pyranine fluorescence, while no change was detected upon PC treatment. Addition of 10 μM PI4P induced H_2O_2 production only after 35 min of treatment (data not shown), while 50 μM PI4P caused a faster decrease in pyranine fluorescence. These results show that treatment of tomato cells with PI4P induces ROS (Fig. 4a), and particularly H_2O_2 production (Fig. 4b). Figure 4c shows that treatment of the cells with 100 μM PS had no effect on H_2O_2 production, while application of 100 μM PG had less effect than 100 μM PI4P. These results indicate that the effect of PI4P is not exclusively due to its negative charge.

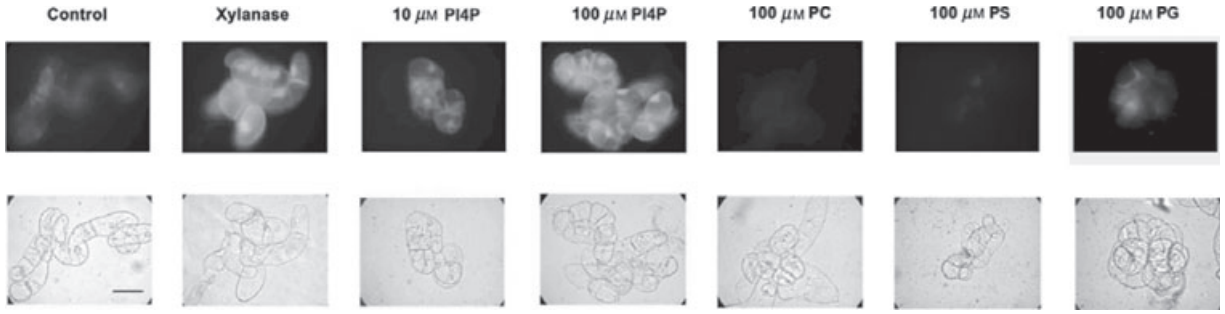
Xylanase has been reported to induce the expression of several defence-related genes such as *phenylalanine ammonia-lyase* (*PAL*) (Tripathy *et al*. 1999), involved in the phenylpropanoid metabolism, *PLDβ1*, which is involved in xylanase-induced PLD signalling (Laxalt *et al*. 2001; Bargmann *et al*. 2006), and *PR1* (Laxalt *et al*. 2001), a pathogenesis-related protein of tomato cells and leaves (van Kan *et al*. 1992). Hence, the effect of PI4P treatment on the transcript levels of these genes was investigated. Because xylanase also induces hypersensitive response (Ron & Avni 2004) and ethylene production (Bailey *et al*. 1990), we also analysed the expression of the hypersensitive response marker *HRS203*J (Pontier *et al*. 1994) and *1-aminocyclopropane* (*ACC*) *oxidase*, involved in ethylene biosynthesis (Hamilton *et al*. 1991), which transcript level is increased as well upon xylanase treatment (Avni *et al*. 1994). Accordingly, tomato cell suspensions were treated with xylanase, 10 or 100 μM PI4P or PC, or buffer only. Samples were harvested after 1 or 8 h of treatment and transcript levels of the individual genes were determined by RNA gel blot analysis (Fig. 5). Gene expression levels in response to applied PC were similar to control treatment, whereas xylanase induced an early increase of *HSR203*J, *PAL* and *PLDβ1* transcript levels, and a late increase of *ACC oxidase* and *PR1*. Applied 100 μM PI4P was able to mimic these responses. *HSR203*J, *PAL* and *PLDβ1* transcript levels were induced within 1 h of 100 μM PI4P treatment, while *ACC oxidase* and *PR1* expression increased after 8 h of treatment. Figure 5 also shows that PI4P triggers the induction of defence response genes in a dose-dependent manner.

As oxidative burst and the induction of defence gene expression often lead to cell death, we also analysed whether applied PI4P was able to trigger this response. For this, an assay was performed based on the uptake of the fluorogenic dye SYTOX Green (Thevissen *et al*. 1999). This reagent only penetrates cells that have lost their membrane integrity and subsequently fluoresces upon DNA binding. Tomato cell suspensions were treated with xylanase, PI4P, PC, PS, PG or buffer for 20 h, after which SYTOX Green was added to evaluate cell death. Figure 6a shows that no fluorescent cells were observed upon control, PC, PS and PG treatments. In contrast, the dye was incorporated into cells treated with xylanase or PI4P. Figure 6b shows that PI4P induced cell death in a dose-dependent manner. Moreover, 100 μM PI4P triggers the same levels of cell death as xylanase treatment, which is demonstrated by a 2.6- to 3.2-fold increase in fluorescence with respect to control cells.

DISCUSSION

In the current report, we show that PIP is present in the EM of tomato cell suspensions and that it accumulates upon xylanase treatment in a dose- and time-dependent manner. Moreover, extracellular PIP, identified as PI4P, was already increased within 5 min of xylanase treatment, indicating that it is a rapid response triggered by the elicitor. As PI4P

(a)



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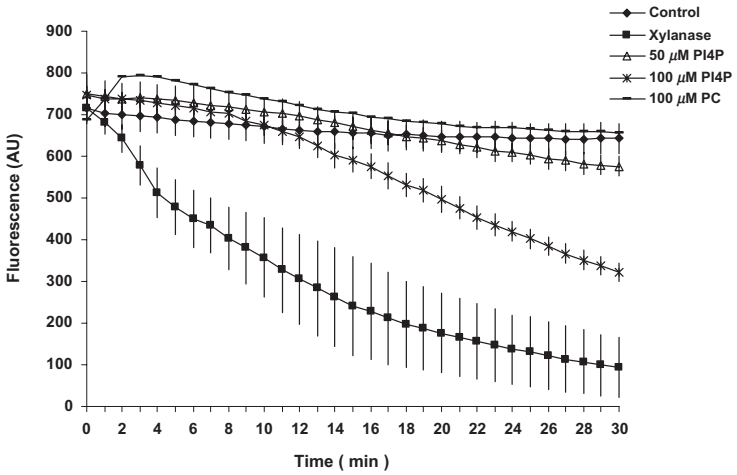

(c)

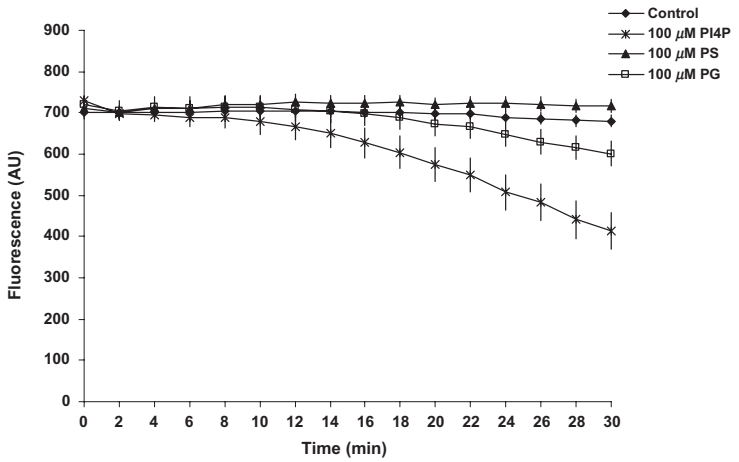

Figure 4. Phosphatidylinositol 4-phosphate (PI4P) treatment induces oxidative burst. Tomato cell suspensions were treated with 10 mM HCl-Tris pH 7.5 (control), 100 μg mL^{-1} xylanase (positive control), the indicated concentrations of PI4P or 100 μM PC, 100 μM PS or 100 μM PG for 30 min. (a) Reactive oxygen species production was detected by incubating the cells with the probe H_2DC-FDA during the treatments. Tomato cells were observed with a fluorescence microscope. Fluorescence (upper panels) and bright-light images (lower panels) are shown. Bar = 5 μM. Results of a typical experiment are shown ($n = 3$). (b,c) Pyranine fluorescence quenching was recorded as a measure of H_2O_2 production directly after adding the indicated treatments. Each treatment was performed in triplicate. Error bars indicate standard error of means. Results of a typical experiment are shown ($n = 5$). PI4P, phosphatidylinositol 4-phosphate; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol; AU, arbitrary units.

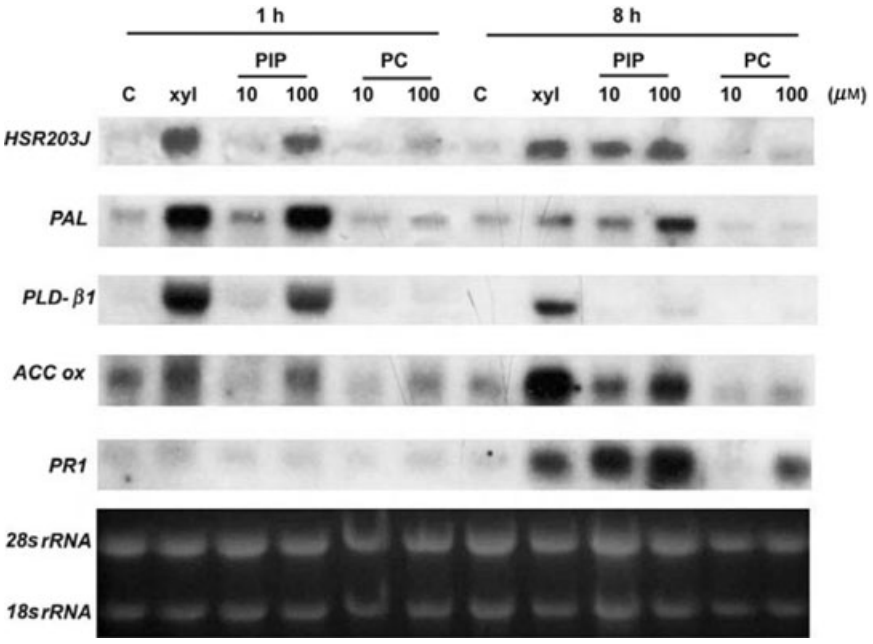

Figure 5. Phosphatidylinositol 4-phosphate (PI4P) treatment induces the expression of *HRS203*J, *PAL*, *PLDβ1*, *ACC oxidase* and *PR1* in tomato cells. Tomato cell suspensions were treated with 10 mM HCl-Tris pH 7.5 (C, control), 100 μg mL^{-1} xylanase (positive control) or the indicated concentrations of PI4P or PC for 1 or 8 h. Total RNA was isolated, subjected to electrophoresis in glyoxal gels, transferred to nylon membranes and hybridized with *HRS203*J, *PAL*, *PLDβ1*, *ACC oxidase* or *PR1* probes. 28S and 18S rRNA are shown as loading controls. Results of a typical experiment are shown ($n = 3$). PIP, phosphatidylinositol phosphate; PI4P, phosphatidylinositol 4-phosphate; PC, phosphatidylcholine.

is classically involved in signalling (Boss *et al*. 2006), these results support a putative role of PI4P as an intercellular phospholipid-signalling molecule which could be involved in the induction of plant-defence responses. Until now, the detection of extracellular phospholipid signals that participate in the regulation of defence responses has only been described in animals. These include the platelet-activating factor (PAF; Prescott *et al.* 2000; Zimmerman *et al*. 2002), oxidized phospholipids (McIntyre, Zimmerman & Prescott 1999; Leitinger 2003), lysoPA (Sano *et al*. 2002; Jin *et al*. 2003) and sphingosine-1-phosphate (Rosen & Liao 2003; Spiegel & Milstien 2003).

We demonstrate that PI4P treatment can induce defence responses that are also triggered by xylanase itself. This effect is not exclusively due to its negative charge, as shown in experiments performed with control anionic phospholipids. We show that ROS production, which is one of the earliest cellular responses following pathogen or elicitor recognition (Torres, Jones & Dangl 2006), was induced within 15 min of PI4P treatment, and that addition of PI4P also triggers the accumulation of *PAL*, *PLDβ1*, *ACC oxidase* and *PR1* transcripts. These responses are related with the onset of hypersensitive response, which is defined as a rapid cell death occurring in plants in response to pathogen attack (Jones & Dangl 2006). Interestingly, we found that PI4P induced the expression of the hypersensitive response gene *HRS203*J (Pontier *et al*. 1994). This result correlates with the fact that application of PI4P to tomato cell suspensions triggered cell death. Different applied phospholipids have been previously shown to boost plant defence (Cowan 2006). For instance, PA induces oxidative burst in tobacco cells (de Jong *et al*. 2004). In *Arabidopsis* leaves and rice cell suspensions, PA treatment induces defence responses such as ROS production (Park *et al*. 2004; Yamaguchi *et al*. 2005), expression of defence genes (Yamaguchi *et al*. 2005; Andersson *et al*. 2006), enhanced phytoalexin biosynthesis (Yamaguchi *et al*. 2005) and cell death (Park *et al*. 2004; Yamaguchi *et al*. 2005; Andersson *et al*. 2006). Recently, Alvarez-Venegas *et al*. (2006) showed that treatment of *Arabidopsis* seedlings with PI4P or PI5P differentially affected the expression of diverse gene families involved in plant-defence responses, apoptosis and plant growth. Among other processes, exogenously administered PI(4,5)P_2 has been reported to induce stomatal opening while PI3P, PI4P and PI5P do not affect this response (Lee *et al*. 2007). In addition, application of PI3P and, in a less extent, PI4P addition, rescued salt stress responses (endocytosis and ROS production) in PI 3-kinase *Arabidopsis* mutants and in seedlings treated with wortmannin, which inhibits PI 3- and PI 4-kinase (Leshem *et al*. 2007). These results, together with the defence responses induced in tomato cells by application of PI4P, indicate that addition of different PPIs have distinct and specific effects on cellular processes. In spite of this evidence, the mechanism of action of applied phospholipids is still unknown (Cowan 2006). Moreover, application of phospholipids has rather been used as an experimental approach to mimic intracellular responses. However, the fact that we are now demonstrating the existence of extracellular PI4P highlights a putative and still unexplored role of PPIs in intercellular signalling.

The detection of extracellular PI4P leads to several novel questions such as: how is extracellular PI4P so rapidly produced upon xylanase treatment? Is it produced extracellularly or is it generated intracellularly and transported out of the cell? If PI4P would be produced extracellularly, the presence of an extracellular PI kinase or an extracellular PIP_2 phosphatase would be required. Until today, these kinds of extracellular enzymes have not been described in plants or in animals and we were not able to detect such activities in preliminary assays. Nevertheless, Kuin

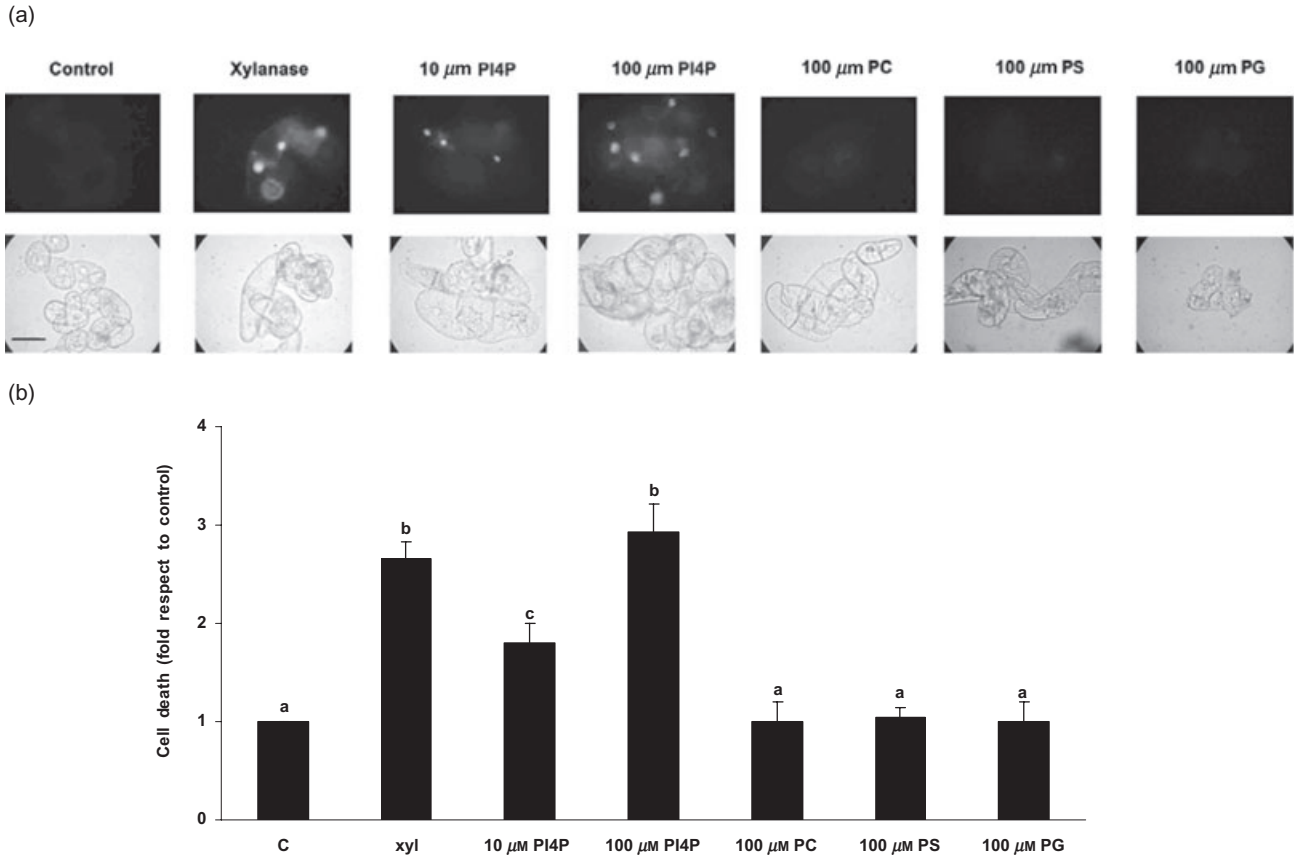

Figure 6. Phosphatidylinositol 4-phosphate (PI4P) treatment induces cell death in a dose-dependent manner. Tomato cell suspensions were treated with 10 mM HCl-Tris pH 7.5 (C, control), 100 μg mL^{-1} xylanase (positive control), the indicated concentrations of PI4P, 100 μM PC, 100 μM PS or 100 μM PG for 20 h at 25 °C. At the end of the treatments, cells were incubated with the probe SYTOX green for 10 min. Cell death was examined using a fluorescence microscope and quantified by fluoroscanning. (a) Fluorescence (upper panels) and bright-light images (lower panels) are shown. Bar = 5 μM. Results of a typical experiment are shown. (b) Quantification of cell death by fluoroscanning. Fluorescence is expressed as fold relative to control cells. Error bars indicate standard error of means ($n = 4$). Means denoted with the same letter do not significantly differ ($P < 0.05$) according to one-way analysis of variance. PI4P, phosphatidylinositol 4-phosphate; PC, phosphatidylcholine; PS, phosphatidylserine; PG, phosphatidylglycerol.

and Munnik (personal communication) found that ^{32}P-PIP could be produced by the unicellular green alga *Chlamydomonas* upon labelling the cells with [γ-^{32}P] ATP. As ATP would not be able to cross the plasma membrane, this result suggests the existence of an extracellular PI-kinase that would use extracellular ATP to phosphorylate PI and produce PIP. Chivasa *et al*. (2005) reported the detection of extracellular ATP in *Arabidopsis*. Nonetheless, even if extracellular PI kinases are present in plants, a new question arises on the existence of PI in an aqueous extracellular compartment. Interestingly, extracellular PI has been detected in apoplastic fluid of sunflower seeds (Regente, Corti Monzón & de la Canal 2008). Alternatively, PI4P could be produced within cells and secreted to the EM. According to animal systems, one possible mechanism would be the production of ectosomes, which are particles generated by vesiculation of the plasma membrane of apoptotic animal cells (Heijnen *et al*. 1999; Morel *et al*. 2004). Exosomes constitute another class of extracellular vesicles released by exocytosis from endocytic multivesicular bodies produced by different cell types (Heijnen *et al*. 1999; Denzer *et al*. 2000). Both exosomes and ectosomes are considered vectors in the intercellular exchange of biological information which are involved in the induction of immune responses, among other processes (Denzer *et al*. 2000; Stoorvogel *et al*. 2002; Freyssinet 2003; Morel *et al*. 2004; Pilzer *et al*. 2005). Until now, this kind of lipid vectors has not been described in plant systems. However, several lines of evidence support the occurrence of exosome-like vesicles in plants and their involvement in different processes such as defence responses (An, van Bel & Hückelhoven 2007).

Phosphoinositides are frequently recognized by specific proteins to trigger signalling cascades (Boss *et al*. 2006; Krauss & Haucke 2007; Sasaki *et al*. 2007). As the detection of extracellular PI4P suggests its putative role as an intercellular signalling molecule, another question is if extracellular PI4P could interact with an extracellular signalling protein. Extracellular PPI-binding proteins have not been reported in plants, but have been detected in mammalian

cells. One of them was identified as glycosyl-PI (GPI)-anchored CD14. This is a cell surface receptor that binds to PI and PI4P with high affinity, and this binding may modulate cellular responses *in vivo* (Wang, Kitchens & Munford 1998; Wang & Munford 1999). The other two extracellular PPI-binding proteins characterized in mammalian cells are the soluble isoform of CD14, which lacks the GPI anchor, and a lipopolysaccharide (LPS)-binding protein. Both of these proteins can transfer PI to plasma lipoproteins and lipid membranes (Yu, Hailman & Wright 1997; Sugiyama & Wright 2001). Furthermore, it was shown that LPS-binding protein facilitates the binding of PI to GPI-anchored CD14 (Wang *et al*. 1998). Finally, Mueller *et al*. (2005) reported that PI binding to LPS-binding protein regulates defence responses. Performing an *in silico* analysis with BlastP program (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) to find putative homologs of CD14 or LPS-binding protein in plant genomes, we were unable to find plant sequences similar to CD14. However, we did find an *Arabidopsis* protein sequence with a good degree of identity with a human LPS-binding protein. Interestingly, this protein would have an extracellular localization and it is identified as a lipid-binding glycoprotein (accession number: NP_563724, NCBI database), making it a good candidate for future research.

In conclusion, our results demonstrate the existence of an extracellular PPI in tomato cell suspensions that would be involved in still non-identified xylanase-induced pathways, suggesting a novel mechanism of intercellular signalling in plant-defence responses.

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