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SHORT COMMUNICATION

# Involvement of phospholipase C in the responses triggered by extracellular phosphatidylinositol 4-phosphate

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ABSTRACT

Phosphatidylinositol 4-phosphate (PI4P) is a minor phospholipid signal molecule involved in diverse biological processes. It is also the main precursor of phosphatidylinositol 4,5-bisphosphate [PI(4,5)$P_2$], which is well described as the substrate of the phospholipase C (PLC)–diacylglycerol kinase (DGK) signalling pathway. Recently, we have demonstrated that PI4P plays a role as an extracellular signalling molecule involved in the activation of xylanase-induced defence responses in tomato cell suspensions. Here we make an approach to the possible mode of action of extracellular PI4P by analysing the involvement of PLC/DGK signalling pathway. We show that extracellular PI4P can be incorporated into tomato cells and further metabolized to PI, although its conversion to PI(4,5)$P_2$ could not be detected. In addition, treatment of tomato cells with the PLC inhibitor U73122 inhibited the oxidative burst triggered by PI4P treatment, suggesting the involvement of PLC in the induction of this response. Nevertheless, we demonstrate that PI4P treatment does not induce DGK activity. These results indicate that PLC but not DKG activation would be triggered by extracellular PI4P. In this sense, a possible mode of action of extracellular PI4P as a direct substrate of PLC to induce this signalling pathway is discussed.


## Introduction

Phosphoinositides (PPIs) are phosphorylated inositol phospholipids, which represent approximately 1% of total phospholipids present in eukaryotic membranes (Lemmon, 2008). They differ in the number and/or position of the phosphate groups present on the *myo*-inositol ring of phosphatidylinositol. In plants, three phosphatidylinositolmonophosphate (PIP) isomers have been identified, i.e. PI3P, PI4P and PI5P, and three phosphatidylinositolbiphosphate ($PIP_2$) isoforms, i.e. PI(4,5)$P_2$, PI(3,4)$P_2$ and PI(3,5)$P_2$, all of which are formed by specific kinases and phophatases (Mueller-Roeber and Pical, 2002). These PPIs can play different roles as signalling molecules in diverse biological processes, such as vesicle trafficking, or as substrates for the production of other second messengers (Meijer and Munnik, 2003). In plant and animal systems, PI4P has been found to be the most abundant PPI, making up approximately 80–90% of the monophosphorylated isoforms (Munnik et al., 1994; Rameh et al., 1997; Meijer et al., 2001). PI4P has been mainly characterized as the precursor of PI(4,5)$P_2$, implicated in the phospholipase C (PLC)-mediated signalling. In this pathway, PI(4,5)$P_2$ is hydrolyzed by PLC producing inositol 1,4,5-triphosphate [I(1,4,5)$P_3$] and diacylglycerol (DAG), which can be phosphorylated by a diacylglycerol kinase (DGK) to produce phosphatidic acid (PA) (Meijer and Munnik, 2003). In plants, both I(1,4,5)$P_3$ and PA, have been described as molecules involved in the regulation of cellular responses triggered by diverse stimuli (Boss et al., 2008). In addition to be important for PLC signalling as a precursor of PI(4,5)$P_2$, PI4P also plays a role as a signalling molecule involved in membrane trafficking and cytoskeletal organization (Boss et al., 2006). Particularly in plants, PI4P has been implicated in the regulation of vesicular trafficking (Kim et al., 2001), stomatal movement (Jung et al., 2002), development of root hairs (Preuss et al., 2006; Thole et al., 2008; Vermeer et al., 2009), regulation of the expression of genes involved in plant defence responses, apoptosis and plant growth (Alvarez-Venegas et al., 2006), endosome formation in response to salt stress (Konig et al., 2008) and plate cell formation during cell division (Vermeer et al., 2009). Moreover, we have recently described a novel role for PI4P as an extracellular signalling molecule involved in the induction of defence responses triggered by the fungal elicitor xylanase (Gonorazky et al., 2008). We have shown that treatment of tomato cell suspensions with xylanase triggers PI4P accumulation in the extracellular medium. Furthermore, addition of PI4P to tomato cells mimics xylanase effects by inducing oxidative burst, expression of defence genes and cell death.

*Abbreviations:* DAG, diacylglycerol; DGK, diacylglycerol kinase; EM, extracellular medium; I(1,4,5)$P_3$, inositol 1,4,5-triphosphate; PA, phosphatidic acid; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)$P_2$, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PPIs, phosphoinositides; TLC, thin layer chromatography

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The aim of this work was to make an approach to the possible mode of action of extracellular PI4P. We have hypothesized that extracellular PI4P is incorporated into tomato cells inducing the production of PI(4,5)$P_2$, and activating, in this way, the PLC/DGK signalling pathway. Here we show that extracellular PI4P can be incorporated into tomato cells and that PLC would be involved in the induction of oxidative burst triggered by this phospholipid. A possible mode of action of extracellular PI4P as a direct substrate of PLC to induce this signalling pathway is discussed.

## Materials and methods

### *Cell suspensions*

Tomato cell suspensions (*Solanum lycopersicum* cv. Money Maker; line Msk8) were grown at 25 °C in dark in MS medium as previously described (Gonorazky et al., 2008).

### *Handling of commercial PI4P*

The natural long fatty acid chain PI4P (Product Number: 840045) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Prior to use, PI4P [dissolved in $CHCl_3$/MeOH/$H_2O$ (20:9:1 by vol.)] was dried under a stream of nitrogen. The required volume of cell-free medium or 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.

### *[$^{32}$Pi] Phospholipid labelling and analyses*

For $^{32}$PIP production *in vivo*, 85 μL of cell suspension aliquots were transferred to 2-mL Eppendorf tubes and labelled for 3 h with 5 μCi carrier-free $^{32}PO_4^{-3}$. For short labelling experiments, 65 μL of cell suspension aliquots were equilibrated during 3 h in 2-mL Eppendorf tubes. Two minutes before addition of the treatments, 20 μL of cell-free medium containing 5 μCi carrier-free $^{32}PO_4^{-3}$ was added to the cells. Two hundred micromolar PI4P dissolved in 85 μL of cell-free medium was subsequently added to the cells. Control treatments were performed by adding cell-free medium. Incubations were stopped with 0.5% (v/v) perchloric acid. Total lipid extraction from the cell aliquots was performed by adding 3.75 vol. of $CHCl_3$/MeOH/HCl (50:100:1 by vol.) and processed as described before (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 visualized by phosphoimaging and quantified using ImageQuant version 5.2 (Molecular Dynamics, Sunnyvale CA, USA).

### *Incubation of tomato cell suspensions with $^{32}$PIP*

*In vivo* produced $^{32}$PIP spot was localized on the TLC plate, eluted from the silica gel and resuspended in water by sonication. One hundred microlitres of tomato cell suspensions were incubated during 60 min with 100 μL of the $^{32}$PIP suspension. Then the extracellular medium was transferred to a new Eppendorf tube and the cells were washed three times with cell-free medium. Incubations were stopped by adding 0.5% (v/v) perchloric acid to the washed cells and the extracellular medium. Total lipids were extracted from both fractions and resolved by TLC, as described earlier.

### *Quantification of $H_2O_2$ production by fluorometry*

The quantification of $H_2O_2$ production by fluorometry was performed as described previously by Gonorazky et al. (2008). Briefly, aliquots of 75 μL of cells equilibrated in assay buffer (5 mM Mes/NaOH pH 5.7, 175 mM mannitol, 0.5 mM $K_2SO_4$, 0.5 mM $CaCl_2$) were carefully pipetted into a 96-well microtitre plate. Thirty minutes prior to the treatments, 1% DMSO, as a negative control of the inhibitor, or 20 μM U73122 dissolved in 1% DMSO was added to the cells. Then 20 μL of a mix composed by 8 μL of assay buffer, 2 μL of 200 μg mL$^{-1}$ pyranin and 10 μL of 10 mM HCl–Tris pH 7.5 (control), or 1 mM PI4P dissolved in HCl–Tris buffer was added to the cells, in a final volume of 100 μl. The quenching of pyranin fluorescence due to $H_2O_2$ production was recorded at 1 min intervals using an excitation wavelength of 405 nm and an emission wavelength of 525 nm in a Fluoroskan Acsent microwell fluorometer. Each treatment was performed in triplicates.

## Results

To analyse whether extracellular PI4P is incorporated into tomato cells and metabolized to PI(4,5)$P_2$, we used an experimental approach based on incubating tomato cell suspensions with radiolabelled $^{32}$PIP produced *in vivo*. As mentioned previously, the PIP pool is mainly composed by PI4P. This was corroborated for the $^{32}$PIP pool obtained from tomato cells by analysing tomato cells phospholipid profile with a borate TLC system (data not shown; Munnik et al., 1994; Krinke et al., 2007). Hence, after 60 min of incubation with $^{32}$PIP, the extracellular medium was isolated, the cells were washed and the total lipids were extracted from both fractions. Fig. 1 shows that the phoshopolipid profile of the extracellular medium was similar to

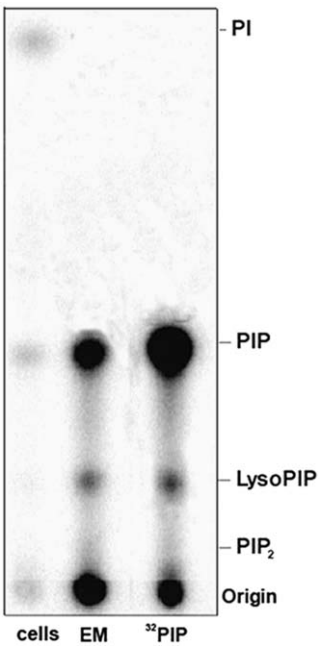


**Fig. 1.** Incorporation and metabolization of extracellular PIP by tomato cells. Radioactive $^{32}$PIP produced by [$^{32}P_i$]-prelabelled tomato cells was isolated from a TLC plate and incubated for 60 min with tomato cell suspensions. Lipids were extracted from cells and extracellular medium (EM) and resolved by TLC. $^{32}$PIP isolated from a TLC plate was used as a control. Results of a typical experiment are shown ($n$=3). The expected position of $PIP_2$ is also indicated.