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Visualization of phosphatidylinositol 4,5-bisphosphate in the plasma membrane of suspension-cultured tobacco BY-2 cells and whole Arabidopsis seedlings

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Summary

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is an important signalling lipid in mammalian cells, where it functions as a second-messenger precursor in response to agonist-dependent activation of phospholipase C (PLC) but also operates as a signalling molecule on its own. Much of the recent knowledge about it has come from a new technique to visualize PtdIns(4,5)P_2 *in vivo*, by expressing a green or yellow fluorescent protein (GFP or YFP) fused to the pleckstrin homology (PH) domain of human PLCδ1 that specifically binds PtdIns(4,5)P_2. In this way, YFP-$PH_{PLC\delta1}$ has been shown to predominantly label the plasma membrane and to transiently translocate into the cytoplasm upon PLC activation in a variety of mammalian cell systems. In plants, biochemical studies have shown that PtdIns(4,5)P_2 is present in very small quantities, but knowledge of its localization and function is still very limited. In this study, we have used YFP-$PH_{PLC\delta1}$ to try monitoring PtdIns(4,5)P_2/PLC signalling in stably-transformed tobacco Bright Yellow-2 (BY-2) cells and Arabidopsis seedlings. In both plant systems, no detrimental effects were observed, indicating that overexpression of the biosensor did not interfere with the function of PtdIns(4,5)P_2. Confocal imaging revealed that most of the YFP-$PH_{PLC\delta1}$ fluorescence was present in the cytoplasm, and not in the plasma membrane as in mammalian cells. Nonetheless, four conditions were found in which YFP-$PH_{PLC\delta1}$ was concentrated at the plasma membrane: (i) upon treatment with the PLC inhibitor U73122; (ii) in response to salt stress; (iii) as a gradient at the tip of growing root hairs; (iv) during the final stage of a BY-2 cell division. We conclude that PtdIns(4,5)P_2, as in animals, is present in the plasma membrane of plants, but that its concentration in most cells is too low to be detected by YFP-$PH_{PLC\delta1}$. Hence, the reporter remains unbound in the cytosol, making it unsuitable to monitor PLC signalling. Nonetheless, YFP-$PH_{PLC\delta1}$ is a valuable plant PtdIns(4,5)P_2 reporter, for it highlights specific cells and conditions where this lipid becomes abnormally concentrated in membranes, raising the question of what it is doing there. New roles for PtdIns(4,5)P_2 in plant cell signalling are discussed.

Keywords: polyphosphoinositide, lipid binding proteins, pleckstrin homology, salt stress, stress signalling.

Introduction

Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] is a minor lipid that is thought to be in the plasma membranes of most, if not all, eukaryotic cells, where it plays various roles in cell signalling. It was first discovered as a substrate for phospholipase C (PLC) and phosphatidylinositide 3-kinase (PI3K) to generate second messengers such as inositol

1,4,5-trisphosphate (InsP_3)/diacylglycerol (DAG) and PtdIns(3,4,5)P_3, respectively. Later, it was found to be a signal in its own right, binding proteins involved in signalling and regulating their activity. PtdIns(4,5)P_2 binds proteins with defined domains, such as PH-, ENTH- and FERM-domains, but also binds other proteins of which the three-dimensional structures complement the head group of this lipid (Carlton and Cullen, 2005; Heo *et al.*, 2006; Yu and Lemmon, 2003). Upon binding PtdIns(4,5)P_2 protein targets can become activated, for example small G-proteins, PLDs and certain ion channels (Suh and Hille, 2005; Toker, 2002). Alternatively, PtdIns(4,5)P_2-rich membrane locations can 'assemble' complexes of signalling proteins that, via their juxtaposition, activate signalling or cellular reorganization. In this way PtdIns(4,5)P_2 has been postulated to regulate membrane trafficking and actin polymerization (Balla, 2007; Carlton and Cullen, 2005; De Matteis *et al.*, 2005; Downes *et al.*, 2005; Halstead *et al.*, 2005; Haucke, 2005; Hilpela *et al.*, 2004; Varnai *et al.*, 2006; Zoncu *et al.*, 2007).

Most of our understanding of PtdIns(4,5)P_2 as a signal and signal precursor was established for animal cells, but similar roles may exist in plants. The first plant proteins that bind and/or are activated by this lipid are now being characterized (Allwood *et al.*, 2002; Monks *et al.*, 2001; Pappan *et al.*, 1997; Peterman *et al.*, 2004; Qin *et al.*, 2002), and more are predicted, based on PH-, ENTH- and FERM-domain sequences, in plant genomes (van Leeuwen *et al.*, 2004; Meijer and Munnik, 2003). Similarly, several plant stress treatments have been claimed to induce PtdIns(4,5)P_2 hydrolysis (Cho *et al.*, 1993, 1995; DeWald *et al.*, 2001; van der Luit *et al.*, 2000; Perera *et al.*, 1999, 2006), whereas the different lipid kinases, phosphatases and PLCs that are required for its metabolism seem abundantly present (Hunt *et al.*, 2004; Mueller-Roeber and Pical, 2002; Torabinejad and Gillaspy, 2006). PtdIns(4,5)P_2 has also been reported to rapidly accumulate: i.e. during salt stress in Arabidopsis seedlings and suspension cultures, where levels increased from 8- to 20-fold within 15–30 min (DeWald *et al.*, 2001; Pical *et al.*, 1999).

Not all PtdIns(4,5)P_2 signalling roles seem duplicated. For example, plant genomes lack type-I and type-II PI3-kinases and the mammalian lipid–second messenger, PtdIns(3,4,5)P_3, has indeed never been found in plant lipid extracts (Meijer and Munnik, 2003).

To study PtdIns(4,5)P_2 in plants it has been convenient to radio-label plant material with ^{32}P orthophosphate and to subsequently monitor treatment-induced changes in its radioactivity after extraction and thin layer chromatography. A limitation of that technique is that it only presents an average response. We do not know which cells respond or in which membrane(s) the response is located. In animal cells, a powerful new technique has been developed that provides such information. By transforming cells with a gene coding for a pleckstrin homology (PH) domain that specifically binds PtdIns(4,5)P_2, and is fused to the GFP gene, the expressed GFP-PH chimera was shown to bind PtdIns(4,5)P_2 in the plasma membrane *in vivo*. Moreover, upon PLC activation, the PtdIns(4,5)P_2 was shown to be rapidly metabolized, with the fluorescence dissipating into the cytosol until the lipid was resynthesised (Stauffer *et al.*, 1998; Varnai and Balla, 1998). The PH domain of human PLCδ1 was used because it is specific for PtdIns(4,5)P_2 compared with other phosphoinositides.

In the plant field, there are some preliminary studies that have used the PH$_{PLC\delta1}$-GFP approach, i.e. in pollen tubes of Arabidopsis and tobacco (Dowd *et al.*, 2006; Kost *et al.*, 1999) and in Arabidopsis root hairs (Vincent *et al.*, 2005). However, expression in pollen was only transient, and its use in plants in general has never been validated: i.e. it was never demonstrated to monitor intracellular PtdIns(4,5)P_2 and does not cause detrimental effects on lipid signalling. In this paper, we have stably expressed a YFP-PH$_{PLC\delta1}$ chimera in tobacco Bright Yellow-2 (BY-2) cells and Arabidopsis plants, and have shown that it monitors increases in PtdIns(4,5)P_2 in the plasma membrane but is unsuitable to report the activation PLC.

Results

Verification of the phospholipid-binding specificity of PH$_{PLC\delta1}$

Prior to using the PH domain of human PLCδ1 (PH$_{PLC\delta1}$) as a PtdIns(4,5)P_2 reporter, we first wanted to confirm its lipid-binding specificity. To this end a glutathione-*S*-transferase (GST) fusion was made and the chimera was overexpressed in *Escherichia coli*. Proteins were isolated and the purified GST-PH$_{PLC\delta1}$ was tested in a protein–lipid overlay assay (Dowler *et al.*, 2000, 2002). Of all seven naturally occurring polyphosphoinositides, only PtdIns(4,5)P_2 bound PH$_{PLC\delta1}$ in a dose-dependent manner, with 0.8 pmol still being detected (Figure 1). GST itself did not bind any of these lipids (data not shown). These results confirm that PH$_{PLC\delta1}$ binds PtdIns(4,5)P_2 with relatively high specificity and affinity, as previously documented (Dowler *et al.*, 2000, 2002).

Validation of the use of the PH$_{PLC\delta1}$ domain in tobacco BY-2 cells

To test the ability of the domain to function as a PtdIns(4,5)P_2 biosensor in living plant cells, we fused the PH$_{PLC\delta1}$ domain to an enhanced version of the yellow fluorescent protein (eYFP) and stably expressed it in tobacco BY-2 cells via *Agrobacterium*-mediated transformation. As a control, a cell line was created expressing a construct without the PH$_{PLC\delta1}$ domain (i.e. only YFP).

After 5–6 weeks, stably growing cell suspensions were obtained that were subcultured every week. No growth

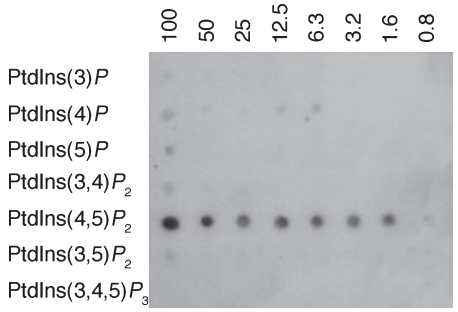

Figure 1. Lipid-binding specificity of $PH_{PLC\delta1}$. A protein–lipid overlay assay was conducted by spotting all naturally occuring polyphosphoinositide (PPI) isomers (indicated on the left) on a Hybond-C extra membrane, ranging from 100 to 0.8 pmol (indicated on the top). The blot was incubated overnight with purified GST-$PH_{PLC\delta1}$ protein (0.5 μg ml^{-1}), and subsequent binding was detected by an anti-GST antibody and enhanced chemiluminescence.

differences between yfp, yfp-ph or wild-type (wt) BY-2 cells were ever observed over a period of ~2 years (data not shown), indicating that there was no deleterious effect from overexpressing YFP or YFP-$PH_{PLC\delta1}$.

Imaging both transgenic cell lines under the confocal microscope revealed, to our surprise, very similar patterns (Figure 2a–f): the fluorescence was predominantly localized in the cytosol and there was no apparent membrane localization of YFP-$PH_{PLC\delta1}$. At first glance, fluorescence appears to be in the plasma membrane, but that is because the cytosol is squeezed against the plasma membrane by the huge vacuole. A true plasma membrane localization gives a homogenous, tight, fluorescent line, whereas a squeezed vacuole against the plasma membrane is much more heterogenous, i.e. diffuse. This can be checked by co-staining the cells with a membrane dye such as FM4-64, and this indeed confirmed our idea that YFP-$PH_{PLC\delta1}$, just like YFP alone, is predominantly localized in the cytosol (Figures S1 and S2), in contrast to mammalian cells.

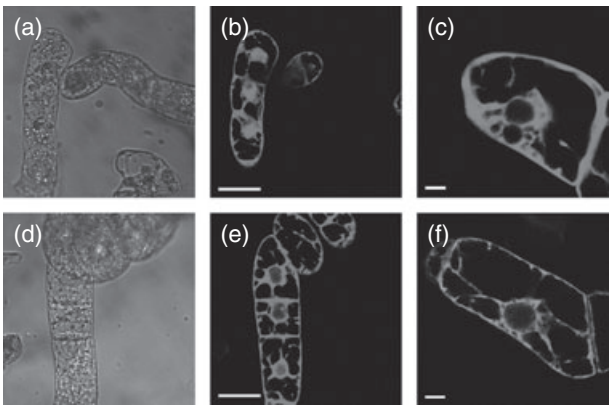

Figure 2. Fluorescence patterns of tobacco BY-2 cells stably expressing YFP or YFP-$PH_{PLC\delta1}$. (a–c) Control 'yfp' cells, expressing YFP; (d–f) 'yfp-ph' cells expressing YFP-$PH_{PLC\delta1}$. Panels (a) and (d) show the differential interference contrast (DIC) image of panels (b) and (e), respectively. The scale bar in panels (b) and (e) is 50 μm; the scale bar in panels (c) and (f) is 10 μm.

As plant cells are notorious for their low PtdIns(4,5)P_2 levels (Meijer and Munnik, 2003; Zonia and Munnik, 2006), we reasoned that the level of PtdIns(4,5)P_2 in the membrane might be too low for detection.

Effect of the PLC inhibitor U73122 on the YFP-$PH_{PLC\delta1}$ localization in tobacco BY-2 cells

To test this idea, the effect of the PLC inhibitor U73122 was studied. The inactive analogue, U73343, was used a positive control. As shown in Figure 3, U73122 induced two striking effects. The first was an accumulation of YFP-$PH_{PLC\delta1}$ at the plasma membrane over a 2-h incubation period (Figure 3a–f). The second was that the fluorescence in the cytosol became more evenly dispersed: fewer cytoplasmic strands were visible and the size of the vacuoles was reduced, appearing as smooth, circular black holes in the fluorescent cytosol (Figure 3c,f). None of these effects were found when the inactive U73343 was used (Figure 3b,e). In yfp cells U73122 did not induce YFP translocation to the membrane, but it did reveal the second phenomenon (Figures 3c and S3).

To investigate whether the increased fluorescence was occurring at, and not just near, the plasma membrane, two additional types of experiments were performed. In the first, the red-fluorescent lipophylic membrane marker FM4-64 was used. As shown in Figure 3(g–i), co-incubation of the yfp-ph cells with 10 μM U73122 and 2 μM FM4-64 revealed that YFP-$PH_{PLC\delta1}$ co-localized with FM4-64 fluorescence at the plasma membrane, whereas there was no co-localization observed in control cells (data not shown). In the second type of experiments, a FRAP (fluorescence recovery after photobleaching) approach was used. With FRAP analyses membrane-bound YFP-$PH_{PLC\delta1}$ molecules can be distinguished from free cytosolic YFP-$PH_{PLC\delta1}$ molecules, because the latter have a much higher diffusion rate. In short, a small region of membrane and cytosol, not too close to cytoplasmic strands, was bleached for 0.6 sec after which fluorescence recovery was measured with 100-msec intervals. Accordingly, FRAP analyses were performed 2 h after cells were treated either with or without 10 μM U73122. The results from a typical experiment are represented in Figure 3(m–p), showing the average fluorescence recovery (solid black line) measured in five different cells, with the surrounding grey area indicating the standard deviation. For the yfp cells, U73122 treatment had no significant effect on the fluorescence recovery (Figure 3m,n). In contrast, for yfp-ph cells the effect was dramatic ($P < 0.0001$; Figure 3o,p), revealing a strongly decreased diffusion rate, indicating that many of the YFP-$PH_{PLC\delta1}$ molecules were bound to the plasma membrane.

Together, these results suggest that YFP-$PH_{PLC\delta1}$, stably expressed in tobacco BY-2 cells, is a biosensor for PtdIns(4,5)P_2, but that the concentration of the lipid in the membrane is normally too low for detection. Hence, most of

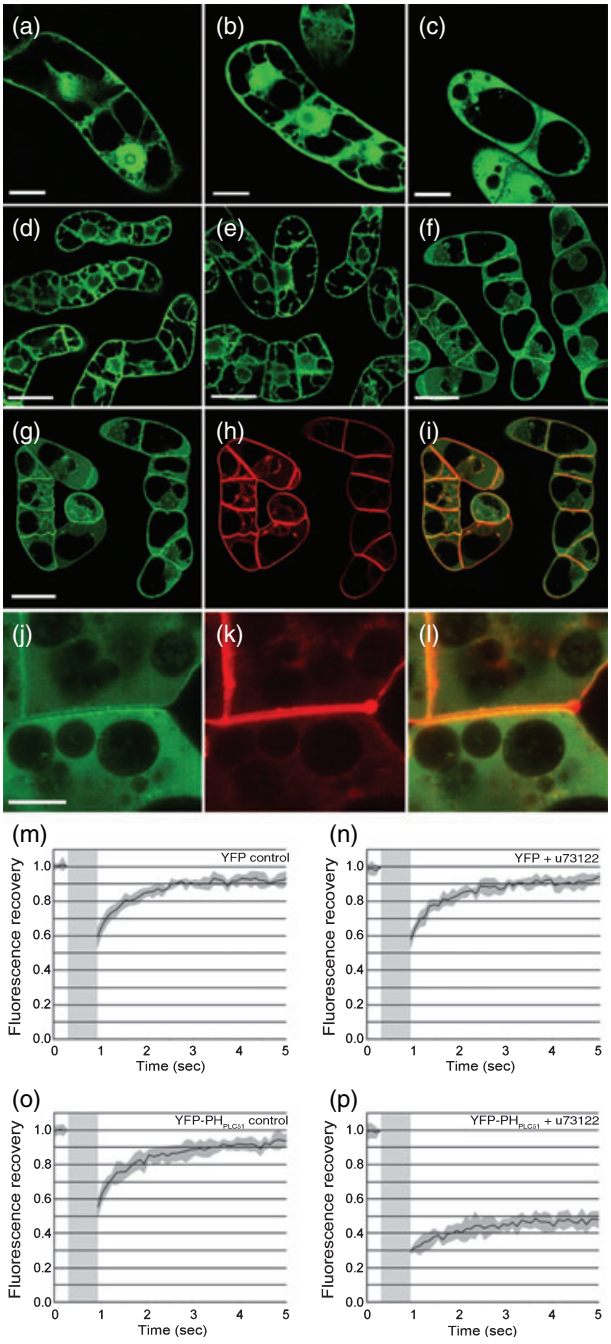

Figure 3. Effect of the phospholipase C (PLC) inhibitor U73122 on the localization of YFP-PH$_{PLC\delta1}$. Confocal scanning laser microscope (CLSM) imaging of yfp (a–c) or yfp-ph cells (d–i) that were treated for 2 h with buffer (control, a, d), 10 μM of the PLC inhibitor U73122 (c, f–l) or its inactive analogue U73343 (10 μM; b, e). Panels (g–l) show the *in vivo* co-localization of YFP-PH$_{PLC\delta1}$ fluorescence with the membrane marker FM4-64 in U73122-treated yfp-ph cells. Whole cells are shown in panels (g–i), whereas part of a cell is shown in panels (j–l). Green shows YFP-PH$_{PLC\delta1}$ fluorescence (g, j) and red shows FM4-64 fluorescence (h, k), whereas yellow shows their co-localization (i, l). FRAP analyses on yfp (m, n) and yfp-ph cells (o, p) were performed. Fluorescence in a 6.6-μm-wide area (containing plasma membrane and cytosol) was bleached and subsequently analysed for fluorescence recovery in cells treated for 2 h either with (n, p) or without 10 μM U73122 (m, o). The average fluorescence is shown for five independently measured cells, relative to the level of fluorescence prior to bleaching, measured for 5 s. The grey area surrounding the curve indicates the standard deviation. The grey rectangle between 0.3 and 0.9 s indicates the time that the area was bleached. Scale bars: (a–c), 20 μm; (d–i) 50 μm; (j–l) 10 μm.

the YFP-PH$_{PLC\delta1}$ is freely moving in the cytosol without bound PtdIns(4,5)P_2. The U73122 data suggests a role for PLC in maintaining these low PtdIns(4,5)P_2 levels, but also implies a role for PLC/PtdIns(4,5)P_2 in the maintenance of cytoplasmic strands.

The effect of salt stress on the formation and localization of PtdIns(4,5)P$_2$ in tobacco BY-2 cells

Salt stress has been shown to rapidly increase PtdIns(4,5)P_2 levels in Arabidopsis seedlings and cell suspensions (DeWald *et al.*, 2001; Pical *et al.*, 1999). Where this PtdIns(4,5)P_2 accumulates is unknown. We reasoned that salt stress could be a good system to validate whether PtdIns(4,5)P_2 responses can be monitored with YFP-PH$_{PLC\delta1}$ and if so, where in the cell this would be. First, we checked whether our tobacco cells indeed responded to salt stress with an increase in PtdIns(4,5)P_2. Therefore, cells were pre-labelled with $^{32}P_i$ for 3 h and were then treated with 125 mM NaCl for different times (0–20 min). Lipids were extracted, separated by thin layer chromatography, and their radioactivity was visualized and quantified by phosphoimaging. A typical response is shown in Figure 4(a–d). PtdIns(4,5)P_2 levels quickly rose by between six- and eightfold within 20 min of adding salt. In general, the ^{32}P responses of the yfp and yfp-ph cells were always very similar (Figure 4a,b; not shown) and were indistinguishable from that of wild-type BY-2 cells (not shown).

To image the cells with a confocal microscope an eight-well Nunc chamber was used, allowing the addition of salt in a controlled manner, i.e. by adding an equal volume of cell-free medium either with or without salt. Although the final concentration of salt (125 mM) was lower than that frequently used by others, extensive plasmolysis was already visible within a minute, and continued for up to 10 min (Figure 4e–l). Because of the movement after the salt addition, it was difficult to image cells within the first minute and so images were routinely captured ~1 min after salt addition. In yfp cells, the cytosolic localization of fluorescence never changed upon salt treatment (data not shown). In yfp-ph cells, however, plasma membrane fluorescence increased between 2 and 5 min (Figure 4e–i, arrows; Video Clip S1), revealing a local increase in the concentration of PtdIns(4,5)P_2. This increased fluorescence coincided with the process of plasmolysis. After maximum shrinkage and during slow recovery (building up turgor, after 5–10 min), the fluorescence of the plasma membrane became indistinguishable from the fluorescence in the cytosol again (Figure 4l).

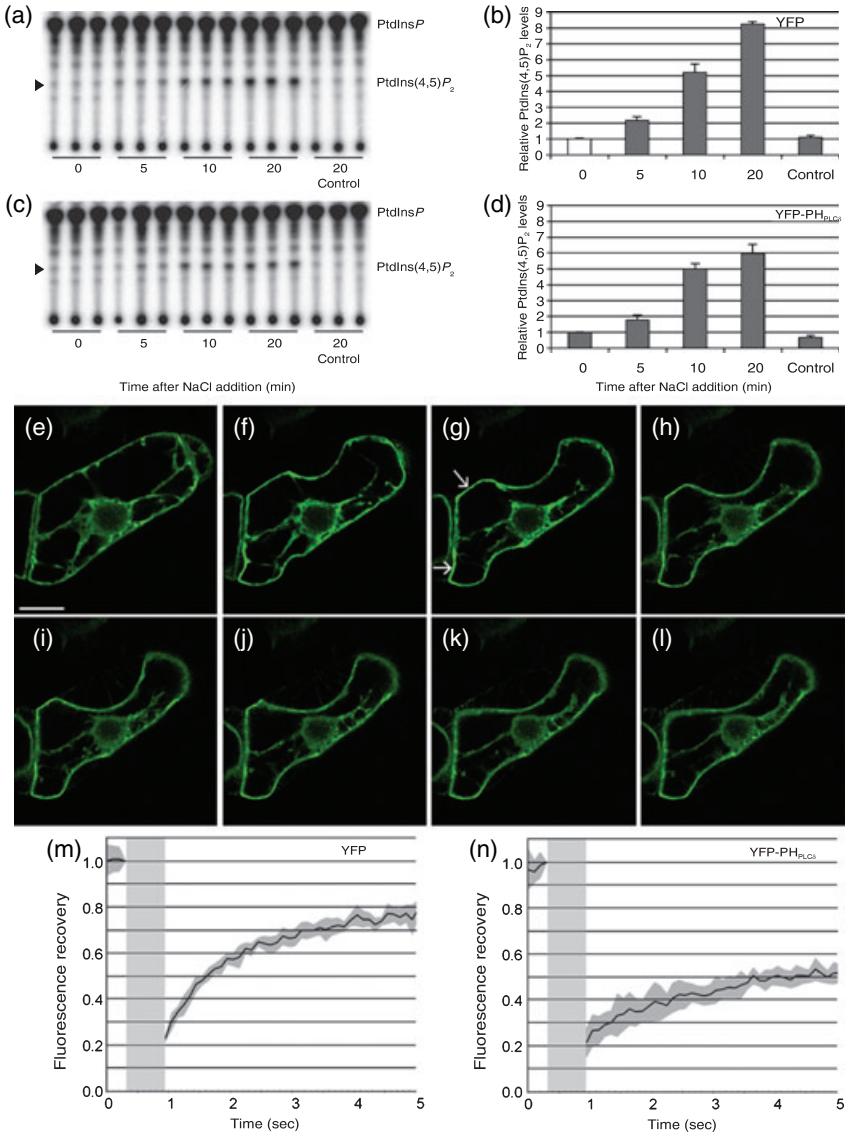

Figure 4. Effect of salt stress on the formation and localization of PtdIns(4,5)P_2 in tobacco BY-2 cells. The change in ^{32}P-labelled PtdIns(4,5)P_2 on an alkaline TLC is shown in response to 125 mM NaCl after 0, 5, 10 and 20 min, and for 20 min of vehicle without salt (control). The response of yfp (a) and yfp-ph cells (c) is shown. Arrowheads indicate PtdIns(4,5)P_2. Panels (b) and (d) show the quantification of PtdIns(4,5)P_2 in the yfp and yfp-ph cells, respectively, plotted relative to the control PtdIns(4,5)P_2 levels at $t = 0$ and $t = 20$ min (control). Panels (e–l) show the *in vivo* pattern of YFP-PH$_{PLC\delta1}$ fluorescence in BY-2 cells imaged with a confocal scanning laser microscope (CLSM) after 1, 2, 3, 4, 5, 6, 7 and 8 min of treatment, respectively, with 125 mM NaCl. The arrows in panel (g) indicate membranes of which the fluorescence levels are increased (see also Video Clip S1). The scale bar in panel (e) is 20 μm. Panels (m–n) show a FRAP analysis of yfp (m) or yfp-ph (n) cells, respectively, 5 min after treatment with 125 mM NaCl. Fluorescence in a 6.6-μm-wide area (containing plasma membrane and cytosol) was bleached and subsequently analysed for fluorescence recovery. The average fluorescence is shown for 5 s, measured in five independent cells, plotted relative to the level of fluorescence prior to bleaching. The grey area surrounding the curve indicates the average level ± the standard deviation. The grey rectangle between 0.3 and 0.9 s indicates the time that the area was bleached. Bar = 10 μm.

Not every yfp-ph cell that we imaged revealed a clear increase in plasma membrane fluorescence. We therefore also analysed cells using the FRAP technique. A typical result is presented in Figure 4(m,n), showing FRAP analyses for five independent cells 5 min after adding 125 mM NaCl to yfp and yfp-ph cells, respectively. The average fluorescence recovery is again depicted as a black line with the grey area indicating the standard deviation. FRAP analysis clearly revealed a significantly slower recovery of fluorescence than in yfp cells ($P < 0.0001$).

Together, these results suggest that YFP-PH$_{PLC\delta1}$ can monitor rapid changes in PtdIns(4,5)P_2 concentrations in plant membranes, and provides evidence that the salt-induced PtdIns(4,5)P_2 response actually occurs at the plasma membrane.

Expression of YFP-PH$_{PLC\delta1}$ in Arabidopsis thaliana

To determine whether the PtdIns(4,5)P_2 biosensor could also be used in whole plants, transgenic Arabidopsis plants were generated, stably expressing YFP-PH$_{PLC\delta1}$ behind a 35S promoter. Homozygous T3 lines grew normally and were indistinguishable from YFP-transformed or untransformed plants (data not shown).

As shown in Figure 5, YFP-PH$_{PLC\delta1}$ was expressed throughout the plant, as can be expected from 35S promoter-driven gene expression. Analyzing root and leaf tissue in more detail revealed that YFP-PH$_{PLC\delta1}$ was predominantly localized in the cytosol again (Figure 5a–m), and was clearly excluded from the nucleus (e.g. Figure 5b, indicated by *). Overall, fluorescence images resembled those observed in the BY-2 cells expressing YFP-PH$_{PLC\delta1}$ (the yfp-ph cells).

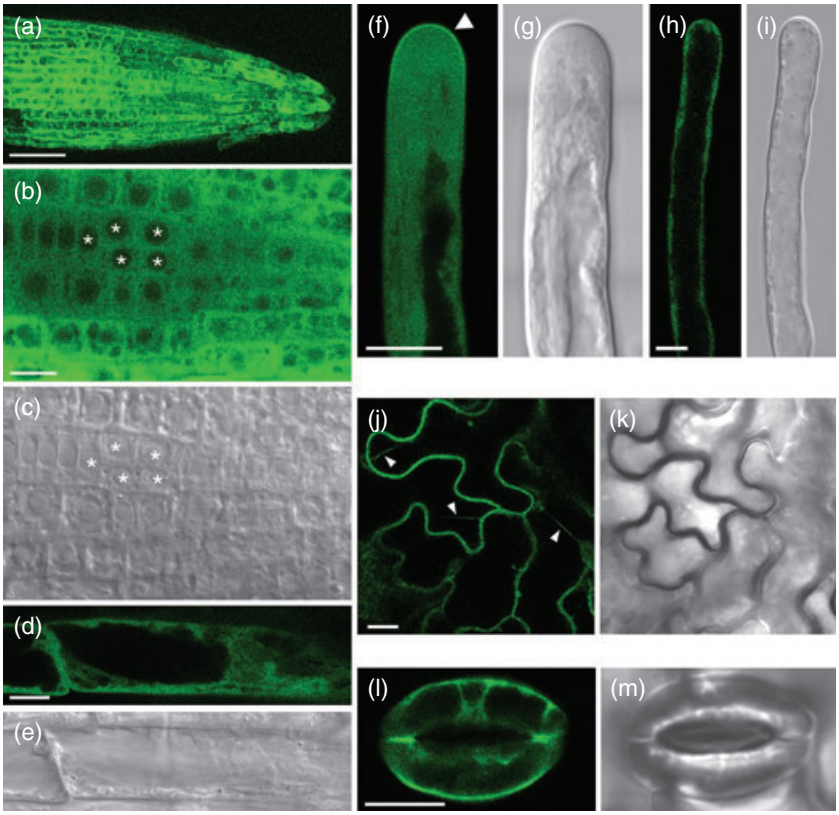

Figure 5. YFP-$PH_{PLC\delta1}$ localization in Arabidopsis plants. Confocal fluorescence images taken from different cell types from Arabidopsis plants stably expressing YFP-$PH_{PLC\delta1}$. (a) Maximal projection of an image stack taken from a root tip, (b, c) root cortex cells, (d, e) root epidermal cells, (f, g) growing root hair, (h, i) older, not growing root hair, (j, k) leaf epidermal cells and (l, m) a guard cell. Bars: (a) 50 μm; (b–m) 10 μm. Images (a, b, d, f, h, j and l) show YFP fluorescence, whereas (c, e, g, i, k and l) show differential interference contrast (DIC) images (see also Video Clips S2–S4).

Interestingly, when analysing growing root hairs, YFP-$PH_{PLC\delta1}$ clearly labelled the plasma membrane at the tip (Figure 5f,g; Video Clip S2). In contrast, root hairs that had stopped growing failed to display such a tip-labelling gradient (Figure 5h,i). In leaf epidermal and guard cells YFP-$PH_{PLC\delta1}$ appears to be in the plasma membrane, but it is the cytosol again that is squeezed into a thin layer against the plasma membrane by the vacuole. In Video Clips S3 and S4, a 3D stack of leaf epidermal and guard cells is shown, clearly revealing that the fluorescence is located in the cytosol (note the presence of cytoplasmic strands).

As salt stress induced the translocation of YFP-$PH_{PLC\delta1}$ to the plasma membrane of tobacco BY-2 cells (Figure 4e–l), its effect on Arabidopsis seedlings was analysed. $^{32}P_i$ labelling of 6-day-old seedlings gave a clear PtdIns(4,5)P_2 increase, essentially as described by DeWald *et al.* (2001; data not shown). Under the confocal microscope, a clear translocation of YFP-$PH_{PLC\delta1}$ to the plasma membrane was observed in both root epidermal and cortex cells within 20 min of salt application (Figure 6).

Epidermal cells first showed a strong plasmolysis, but after ~15 min YFP-$PH_{PLC\delta1}$ fluorescence was visible at the plasmolysed membrane (Figure 6k–o, arrowhead; Video Clip S5). Root cortex cells, on the other hand, did not exhibit plasmolysis, but still displayed an accumulation of YFP-$PH_{PLC\delta1}$ fluorescence at the plasma membrane (Figure 6c,d). These results provide further evidence that the PtdIns(4,5)P_2 response to salt stress occurs at the plasma membrane.

YFP-$PH_{PLC\delta1}$ dynamics during tobacco BY-2 cell division

While analysing numerous cells in the yfp and yfp-ph BY-2 cell suspensions, a striking phenomenon was observed when a cell was caught during the process of cell division. On examining more dividing cells a picture started to emerge, of which a typical example is represented in Figure 7, which shows a time-lapse series of a dividing yfp-ph cell over a period of 50 min. To follow the formation of the new cell membrane (the cell plate), we co-incubated cells with the red fluorescent lipophylic dye FM4-64 (lower panels, in red; Bolte *et al.*, 2004; Dhonukshe *et al.*, 2006). From time t = 1–31 min (Figure 7) the YFP-$PH_{PLC\delta1}$ fluorescence pattern was more or less identical to the YFP images in the dividing yfp cells (not shown), reflecting the distribution of non-bound YFP-$PH_{PLC\delta1}$ throughout the cytosol. However, at the final stage of cytokinesis, when the new growing membrane approached the original plasma membrane (Figure 7, t = 41–51 min), YFP-$PH_{PLC\delta1}$ started to accumulate on the edge of the cell plate; this was never observed for YFP in yfp cells (data not shown).

These results suggest that the cell plate becomes rich in PtdIns(4,5)P_2 just prior to fusing with the 'old' plasma membrane, indicating a role for this lipid in membrane

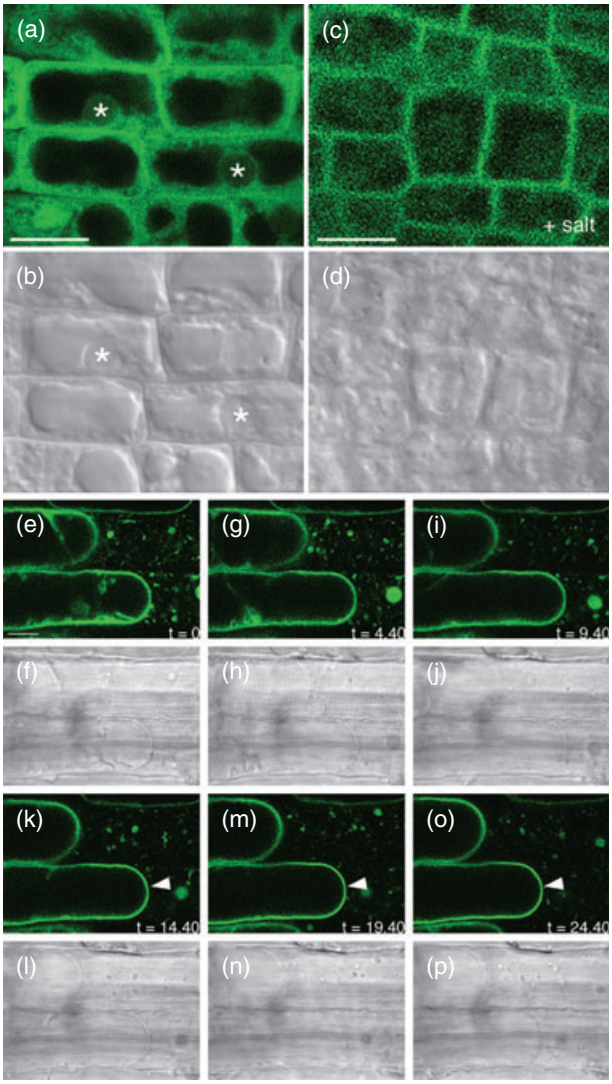

Figure 6. YFP-$PH_{PLC\delta1}$ translocation in Arabidopsis roots in response to salt stress. Confocal images of YFP-$PH_{PLC\delta1}$ distribution in root cortex cells of 7-day-old seedlings after 15 min of incubation either without (a–d) or with 250 mM NaCl. Panels (e–p) show a confocal time-lapse following root epidermal cells after 250 mM NaCl with the time (min) indicated at the bottom right (see also Video Clip S5). Bar = 10 μm; * nuclei.

fusion and cell division. Such transient, local, high concentrations of a specific lipid represent new information for plant cells, and provide a striking example of how useful this lipid biosensor can be. For a movie of this cell division, see Video Clip S6.

Discussion

YFP-$PH_{PLC\delta1}$, a non-invasive PtdIns(4,5)P_2 reporter

In this paper a YFP fusion with the PH domain of human PLCδ1 has been used to visualize PtdIns(4,5)P_2 in living plant cells. Overexpression of the construct did not cause any deleterious effects in either stably-transformed BY-2 cells or whole Arabidopsis plants: both grew and developed normally. This is important, as it is unlikely that overexpression produces abnormal images of the distribution of PtdIns(4,5)P_2.

Plant cells contain large vacuoles, which squeeze the cytoplasm into a thin layer against the plasma membrane, making it difficult to discriminate between cytosolic- and plasma membrane-fluorescence. However, in this case it was clearly localized in the cytosol and not in the plasma membrane, as in animal cells (Stauffer *et al.*, 1998; Varnai and Balla, 1998). This does not mean that plant cells contain their PtdIns(4,5)P_2 in the cytosol. On the contrary, it simply reflects the fact that there is not enough PtdIns(4,5)P_2 in the plant plasma membrane for YFP-$PH_{PLC\delta1}$ to bind to. Compared with animal cells and green algae, higher plant cells contain very low levels of PtdIns(4,5)P_2 (Meijer and Munnik, 2003; Munnik *et al.*, 1994b, 1998b; Vermeer, 2006; Zonia and Munnik, 2006). Overexpressing YFP-$PH_{PLC\delta1}$ may have saturated the PtdIns(4,5)P_2 in the membrane, with the excess suffusing the cytoplasm with fluorescence, thereby masking the fluorescence in the membrane. Using FRAP analysis and co-labelling with FM4-64 we were able to shown that small quantities of PtdIns(4,5)P_2 were indeed present in the plasma membrane. Using the PLC inhibitor U73122, a strong, although slow (1–2 h), accumulation of YFP-$PH_{PLC\delta1}$ at the plasma membrane was found, suggesting that these low PtdIns(4,5)P_2 levels, at least in part, may be caused by a constitutive active PLC system that constantly hydrolyses PtdIns(4,5)P_2. This would not only result in lower PtdIns(4,5)P_2 levels but could also generate high levels of Ins(1,4,5)P_3 in the cytosol. In theory, cytosolic Ins(1,4,5)P_3 could also bind the PH domain because Ins(1,4,5)P_3 is nothing more than the head group of the lipid. However, in plants, Ins(1,4,5)P_3 levels are also extremely low (DeWald *et al.*, 2001; Stevenson-Paulik *et al.*, 2005; Williams *et al.*, 2005), and we know that the affinity of $PH_{PLC\delta1}$ for the lipid head group is much higher than for the water-soluble Ins(1,4,5)P_3 (van der Wal *et al.*, 2001). Independently we confirmed this by expressing a tandem construct of $PH_{PLC\delta1}$ (i.e. YFP-2x$PH_{PLC\delta1}$) in cowpea protoplasts, and showed that this increased the localization of the sensor at the plasma membrane, rather than enhancing its 'stay' in the cytosol (Vermeer, 2006).

This study shows that PtdIns(4,5)P_2 is in the plasma membrane, but that the levels are very low. Extra proof of plasma membrane-localized PtdIns(4,5)P_2 came from the salt stress experiments. Treatment of BY-2 cells with 125 mM NaCl increased PtdIns(4,5)P_2 levels up to ~sevenfold within 20 min, and visually increased the concentration of YFP-$PH_{PLC\delta1}$ at the plasma membrane, which was confirmed with FRAP experiments (Figure 4). Similarly, the level of PtdIns(4,5)P_2 was increased in Arabidopsis seedlings (not shown; DeWald *et al.*, 2001), and was shown to translocate

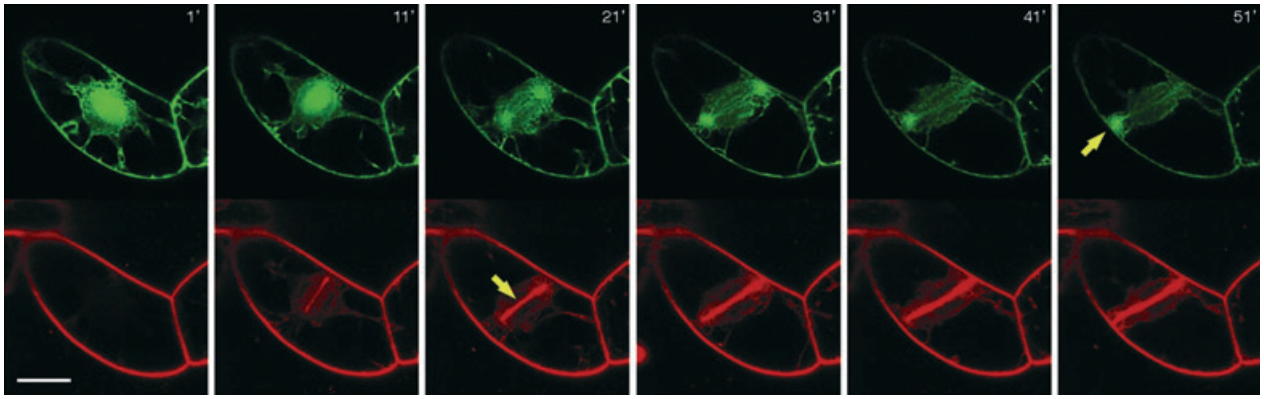

Figure 7. YFP-$PH_{PLC\delta1}$ dynamics during cell division of BY-2 cells. Upper panel shows YFP-$PH_{PLC\delta1}$ fluorescence and lower panel shows FM4-64 fluorescence (red). Time is indicated in min (also see Video Clip S6). Arrow at t = 21 min indicates cell plate-labelling with FM4-64, whereas the arrow at t = 51 min points to local elevated PtdIns(4,5)P_2 levels at the final stage of cytokinesis. Bar = 20 μm.

YFP-$PH_{PLC\delta1}$ to the plasma membrane in root epidermal and cortex cells. Increased plasma membrane localization was also found in growing root hairs (but not in non-growing root hairs) and during cell division, when old and new plasma membranes were about to fuse.

The importance of validation studies such as these and by Vermeer *et al.* (2006) is illustrated by a recent research paper in which GFP-$PH_{PLC\delta1}$ was wrongly used as an Ins(1,4,5)P_3 reporter in plant cells (Tang *et al.*, 2007). It has been clearly demonstrated by van der Wal *et al.* (2001), and by our own studies (Vermeer, 2006), that GFP-$PH_{PLC\delta1}$ detects PtdIns(4,5)P_2 and not Ins(1,4,5)P_3. Moreover, Tang *et al.*, (2007) claim that GFP-$PH_{PLC\delta1}$ was localized to the plasma membrane, which is clearly not the case if one studies their figure carefully and takes into consideration the results presented here (Figure 5). They misinterpreted cytosolic fluorescence, squeezed against the plasma membrane by the large vacuole, as a plasma membrane signal, and overlooked the cytoplasmic strands disappearing from the cell periphery into the cell.

Is PtdIns(4,5)P_2 the prime PLC substrate in plants?

In mammalian cells, YFP-$PH_{PLC\delta1}$ primarily labels the plasma membrane. Upon PLC activation, the biosensor can then be seen to translocate transiently into the cytosol, after which it returns back to the membrane. In this way, PLC signalling was visualized for the first time in a living cell (Stauffer *et al.*, 1998; Varnai and Balla, 1998). In plants, very little PtdIns(4,5)P_2 is present in the membrane; hence, most of the YFP-$PH_{PLC\delta1}$ is already in the cytosol. Although this demonstrates that the biosensor cannot be used as a PLC signalling reporter in plant cells, it also emphasizes that there is a big difference between the function of PtdIns(4,5)P_2 in plants and animals (van Leeuwen *et al.*, 2004; Testerink and Munnik, 2005; Zonia and Munnik, 2006).

The low concentration of PtdIns(4,5)P_2 is also not supportive of a major role in PLC signalling in plants. In the literature, the rapid turnover of PtdIns(4,5)P_2 has always been claimed to be able to compensate for this. However, if one looks in more detail at the $^{32}P_i$ incorporation studies into the different phospholipid pools of various plant systems, i.e. suspension cultured tomato, Medicago and Arabidopsis cells, tobacco pollen, and Medicago and Arabidopsis seedlings (DeWald *et al.*, 2001; den Hartog *et al.*, 2001, 2003; van der Luit *et al.*, 2000; Pical *et al.*, 1999; Ruelland *et al.*, 2002; Zonia and Munnik, 2004), then the PtdIns(4,5)P_2 levels are always extremely low, and its turnover characteristics are by far not as striking as in mammalian cells or green algae (Munnik *et al.*, 1998b). In animal cells and green algae, PtdIns(4,5)P_2 and PtdIns$P$ levels are more or less the same (~1:1), whereas in higher plants the PtdIns$P$:PtdIns(4,5)P_2 ratio ranges from 10:1 to 100:1 (Meijer and Munnik, 2003; Munnik *et al.*, 1994a,b, 1998a,b). PtdIns$P$ quantities seem normal in higher plants, although ^{32}P-labelling studies tend to overestimate their levels because of rapid $^{32}P_i$ uptake, the high turnover of ATP and PtdIns$P$, and because each lipid contains two radioactive phosphates (Munnik *et al.*, 1994a; Vermeer, 2006).

In vitro, plant PLCs hydrolyse PtdIns4P as efficiently as PtdIns(4,5)P_2, whereas PtdIns is only hydrolysed when millimolar Ca^{2+} concentrations are present (reviewed in Munnik *et al.*, 1998a). As plants have 'normal' PtdIns$P$ levels, perhaps this lipid is a much more realistic precursor for the production of InsP_3, InsP_6 and PtdOH. Recently, Dowd *et al.* (2006) provided evidence that in specialized cells, i.e. tip-growing pollen tubes, a correlation between PLC and the maintenance of a tip-focussed PtdIns(4,5)P_2 gradient can be found. Using GFP-$PH_{PLC\delta1}$ and GFP-PetPLC1 they showed that GFP-$PH_{PLC\delta1}$ only accumulated at the plasma membrane where GFP-PetPLC1 was lacking, namely at the plasma membrane of the growing tip.

Although the authors concluded that this must be PLC hydrolysing PtdIns(4,5)P_2 (Dowd *et al.*, 2006), the data may also reflect the hydrolysis of PtdIns4*P* limiting its phosphorylation to PtdIns(4,5)P_2, which has functions on itself (see below). Using a PtdIns4*P*- specific biosensor (YFP-PH_{FAPP1}) we observed a similar gradient as for PtdIns(4,5)P_2 (J. E. M. Vermeer, T. W. J. Gadella, T. Munnik, unpublished data). *In vivo* evidence for a PtdIns*P*-specific PLC was recently provided in Arabidopsis leafs upon exposure to a bacterial effector protein (Andersson *et al.*, 2006).

In summary, it seems unlikely that PtdIns(4,5)P_2 simply fulfils the classic substrate role in plant PLC signalling. Interestingly, the unicellular green algae *Chlamydomonas* and *Dunaliella* do contain PtdIns*P* and PtdIns(4,5)P_2 levels and turnover characteristics that resemble those of mammalian systems. Moreover, PLC signalling can be readily activated (Brederoo *et al.*, 1991; Einspahr *et al.*, 1988; Irvine *et al.*, 1992; Munnik *et al.*, 1998b; Musgrave *et al.*, 1992, 1993; Quarmby *et al.*, 1992), and in a more recent proteomic screen of *Chlamydomonas* flagellar proteins, a putative Ins(1,4,5)P_3 receptor has been identified (Pazour *et al.*, 2005). Putative Ins(1,4,5)P_3 receptor homologues are still missing from higher plant genomes. This may represent an interesting divergence in PLC signalling between algae and higher plants (see also Zonia and Munnik, 2006).

PtdIns(4,5)P_2 function in plants

What other roles could PtdIns(4,5)P_2 possibly fulfil in the membrane of a higher plant cell? In animal cells it is clear that PtdIns(4,5)P_2 is not only a substrate for PLC (or PI3K), but is also emerging as a signalling molecule itself, regulating the activity and/or localization of a number of target proteins, affecting various cell biological processes. A few examples, with special emphasis to the observations made in this study, have been summarized below, to increase our understanding of what might be going on.

(i) Ion channel regulation. PtdIns(4,5)P_2 is increasingly being recognized as a key regulator of ion channel activity: especially K^+ channels (Suh and Hille, 2005). The first plant report suggesting a role for PtdIns(4,5)P_2 in K^+-channel regulation has recently appeared (Liu *et al.*, 2005). In principal, the salt stress-induced PtdIns(4,5)P_2 accumulation in plant plasma membranes could reflect such a function.

(ii) Membrane fusion and fission. During mammalian cell division, PtdIns(4,5)P_2 is predominantly localized in the furrow membrane and is required for the proper completion of cytokinesis (Brill *et al.*, 2000; Emoto *et al.*, 2005; Saul *et al.*, 2004; Stock *et al.*, 1999; Wong *et al.*, 2005; Zhang *et al.*, 1999). It is thought to provide an active zone in the cleavage furrow for efficient membrane fusion, as well as fission. The highly localized PtdIns(4,5)P_2 formation during plant cell division might reflect a similar role. Interestingly, Patellin1, a novel Sec14-like protein, was recently shown to bind PtdIns(4,5)P_2 and to localize to the cell plate of dividing Arabidopsis cells (Peterman *et al.*, 2004).

(iii) Organization of the cytoskeleton. An additional role of the assembly of PtdIns(4,5)P_2 at the cleavage furrow is to regulate cytoskeletal dynamics. PtdIns(4,5)P_2 binds directly to a variety of actin-regulatory proteins, and can modulate their function (Carlton and Cullen, 2005; Emoto *et al.*, 2005; Hilpela *et al.*, 2004). Alternatively, its local increase may anchor proteins to the plasma membrane via specific PtdIns(4,5)P_2-binding modules. Besides the PH domain, these include ENTH, FERM, PDZ, Tubby and WASP (Balla, 2005; Carlton and Cullen, 2005). Plants are predicted to contain several of these domains, including proteins involved in the organization of the cytoskeleton (van Leeuwen *et al.*, 2004; Meijer and Munnik, 2003). The low PtdIns(4,5)P_2 levels in plants predict that these binding domains must display a higher affinity for this lipid than their mammalian counterparts, or that additional protein domains displaying affinity for other plasma membrane components are required for efficient plasma membrane tethering. The responses observed during cell division and salt stress, but also the U71322-induced changes on the cytoplasmic strands and vacuole morphology, may be signs of such functions. In growing pollen tubes, PtdIns(4,5)P_2 has also been suggested to play a role in the regulation of the actin cytoskeleton (Dowd *et al.*, 2006).

(iv) Membrane trafficking. In mammalian cells, many polyphosphoinositides (PPIs) are involved in endo- and/or exocytosis (Carlton and Cullen, 2005; De Matteis *et al.*, 2005; Haucke, 2005). Although we found no evidence for PtdIns(4,5)P_2 accumulation in small vesicular structures, as we did for PtdIns3*P* and PtdIns4*P* (Vermeer, 2006; Vermeer *et al.*, 2006), the polar gradient of PtdIns(4,5)P_2 in growing root hairs may reflect a membrane trafficking event, as has been proposed earlier (Dowd *et al.*, 2006; Vincent *et al.*, 2005). Of course, ion channel regulation and organization of the cytoskeleton are other interesting possibilities that may reflect this gradient.

Summarizing, we conclude that the PtdIns(4,5)P_2 biosensor is a useful tool. Although higher plants clearly exhibit a different PLC signalling mechanism than that found in animals (i), minor levels of PtdIns(4,5)P_2 are present in the plasma membrane of plants (ii), which was discovered to accumulate in the newly formed membrane of dividing cells (iii) and to accumulate in the plasma membrane upon salt stress (iv).

Experimental procedures

Plant material

Tobacco (*Nicotiana tabacum*) BY-2 cells were grown in Murashige and Skoog (MS) medium, supplemented with Gamborg B5 vitamins, 3% (w/v) sucrose and 1 mg l^{-1} 2,4-dichlorophenoxy-acetic

acid (2,4-D; Duchefa, http://www.duchefa.com). Cells were cultured in the dark at 24°C, and were shaken in 300-ml Erlenmeyer flasks containing 50 ml MS medium at 125 rpm.

Seedlings of *A. thaliana* cv. Columbia were cultivated on 0.5x MS plates in a growth chamber at 21°C with a 16-h light period. Mature plants were grown on soil in the greenhouse following the same regime.

Constructs and generation of transgenic BY-2 cells and Arabidopsis plants

The human PLCδ1-PH domain (amino acids 1–175) was fused to the C-terminus of eYFP (Clontech, http://www.clontech.com), which had an additive Q70K mutation to enhance its stability. The resulting eYFP-$PH_{PLC\delta1}$ sequence was *Xba*I-*Bam*HI cloned into the pGreen-1K vector, containing a double C*aMV* 35S promoter (from –343 to –90 and from –343 to +8) and the *NPTII* gene as a kanamycin resistance marker. An eYFP-Q70K was cloned for control experiments.

Vectors were transformed into *Agrobacterium tumefaciens* strain EHA105 and subsequently into BY-2 cells. In short, 200 μl of an overnight (O/N) bacterial culture was mixed with 8 ml of (5-days old) BY-2 cells, supplemented with 0.2 mM acetosyringone. Cells were kept for 3 days in a sterile Petri dish, at 24°C in the dark, and were subsequently transferred to fresh MS plates (with 2,4-D, 3% sucrose and 0.8% agar), containing carbenicillin (250 mg l^{-1}) and increasing concentrations of kanamycin, every 3 days: two times 40 mg l^{-1}, two times 100 mg l^{-1} and two times 200 mg l^{-1} kanamycin, respectively. Calli were maintained on 200 mg l^{-1} kanamycin plates and were transferred to fresh plates every 3 weeks. Transgenic calli were checked for YFP fluorescence using a fluorescence stereomicroscope, and were selected for inoculation into liquid medium. Afer 3–6 weeks a stably growing cell suspension was obtained, which was subcultured every week and used after 4–6 days for both microscopic and biochemical analysis.

Arabidopsis thaliana cv. Columbia plants were transformed using either the eYFP or the eYFP-$PH_{PLC\delta1}$ constructs by standard floral-dip transformation (Clough and Bent, 1998). Kanamycin-resistant T2 plants were selected for high fluorescence levels using a fluorescence stereomicroscope. Homozygous T3 plants were used for further analysis. Seeds were germinated on 0.5x MS plates with 0.8% agar after 48 h of stratification, and were grown at 21°C as described above.

Overexpression and purification of GST fusion proteins

Both GST and GST-$PH_{PLC\delta1}$ constructs were cloned into the pGEX-KG vector using PCR and pfu polymerase, and were used to transform into the *Escherichia coli* strain BL21 (DE3). Expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) for 9 h at 20°C, after which total protein was extracted using lysozyme, freeze/thaw and sonication. GST fusion proteins were further purified using 1-ml GSTrap FF columns (Amersham, http://www.amersham.com), after which the pure protein was quantified and stored at –20°C until use in the protein lipid overlay assay.

Protein lipid overlay assay

Serial dilutions of all seven known PPI isomers (CellSignals, Inc., Lexington, KY, USA) were spotted onto a Hybond-C extra membrane (Amersham), ranging from 0.8 to 100 pmol. The membrane was dried, washed in binding buffer and incubated with the purified GST-$PH_{PLC\delta1}$ fusion protein, essentially as described by Dowler *et al.* (2000, 2002). Binding was detected using an anti-GST antibody (Santa Cruz Biotechnology, Inc., http://www.scbt.com) followed by ECL.

Confocal imaging

Routinely, 4–6-days-old weekly subcultured BY-2 cells were imaged in eight-well Nunc chambers (Nunc, Inc., http://www.nuncbrand.com). Cells (150 μl), at a standard concentration of 20 mg fresh weight ml^{-1} medium, were treated by adding an equal volume of cell-free medium either with or without 20 μM U73122 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)-hexyl]-1H-pyrrole-2,5-dione; 20 μM U73343 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)-hexyl]-2,5-pyrrolidinedione) (Calbiochem, http://www.merckbiosciences.co.uk/html/CBC/home.html) or with 250 mM NaCl. Confocal images were acquired using a confocal scanning laser microscope (CLSM; Zeiss LSM510; Zeiss, http://www.zeiss.com) with a Zeiss c-apochromat 40× (NA 1.2) water-immersion objective and a pinhole corresponding to 1–2 Airy units. YFP was excited using a 514-nm argon ion laser line, and fluorescence was detected using a 540/20-nm bandpass filter. FM4-64 (N-(3-triethylammonium-propyl)-4-(6-(4-(diethylamino)phenyl) hexatrienyl) pyridinium dibromide; Invitrogen, http://www.invitrogen.com) was added to a final concentration of 2 μM, and its fluorescence was detected using the same laser line and a 650-nm long-pass filter. Each scan was the result of averaging eight time-frames to produce a high signal-to-noise ratio.

$^{32}P_i$ phospholipid labelling, extraction and analysis

BY-2 cells were labelled in their own growth medium using 100 μCi ml^{-1} $^{32}PO_4^{3-}$ (carrier-free; Amersham). Labelling took place in 2-ml Eppendorf 'safelock' tubes, containing 85-μl aliquots. After 3 h, cells were treated with an equal volume of cell-free medium either with or without 20 μM U73122, 20 μM U73343 or 250 mM NaCl (to give half the final concentration) for the times indicated. Incubations were stopped by adding 20 μl 50% perchloric acid. Lipids were extracted by adding 3.75 vol. $CHCl_3$/MeOH/HCl (50:100:1 by vol.). A two-phase system was induced by adding 3.75 vol. $CHCl_3$ and 1 vol. NaCl (0.9% w/v), and was processed as described previously (Munnik *et al.*, 1996).

Lipids were separated on Silica-60 TLC plates (Merck, http://www.merck.com) employing an alkaline TLC solvent composed of $CHCl_3$/MeOH/25% NH_4OH/H_2O (90:70:4:16, by vol.; Munnik *et al.*, 1994a). Radioactivity was visualized by autoradiography and quantified by phospho-imaging (Storm; Molecular Dynamics, http://www.gelifesciences.com). The radioactivity in PtdIns(4,5)P_2 was calculated by dividing it by the radioactivity incorporated in the total lipid fraction. The fold increase was calculated by dividing the radioactivity in the stimulated cells by the radioactivity in the corresponding control. Autoradiographs shown represent general phenomena, representative for 3–6 independent experiments.

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Supplementary Material

The following supplementary material is available for this article online:

Figure S1. BY-2 cells expressing YFP co-labelled with 2 μM FM4-64 for 5 min showing no plasma membrane labelling.

Figure S2. BY-2 cells expressing YFP-$PH_{PLC\delta1}$ co-labelled with 2 μM FM4-64 for 5 min showing no plasma membrane labelling.

Figure S3. BY-2 cells expressing YFP co-labelled with 2 μM FM4-64 after 2-h treatment with 10 μM U73122 showing no accumulation of YFP at the plasma membrane.

Video Clip S1. Dynamics of YFP-$PH_{PLC\delta1}$ in BY-2 cells during salt stress.

Video Clip S2. Localization of YFP-$PH_{PLC\delta1}$ in growing Arabidopsis root hair.

Video Clip S3. Localization of YFP-$PH_{PLC\delta1}$ in an Arabidopsis guard cell.

Video Clip S4. Localization of YFP-$PH_{PLC\delta1}$ in Arabidopsis epidermal leaf cells.

Video Clip S5. Effect of 250 mM salt on the localization of YFP-$PH_{PLC\delta1}$ in Arabidopsis root epidermal cells.

Video Clip S6. Dynamics of YFP-$PH_{PLC\delta1}$ during BY-2 cell division.

This material is available as part of the online article from http://www.blackwell-synergy.com

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