Imaging phosphatidylinositol 4-phosphate dynamics in living plant cells

Joop E.M. Vermeer1,2,†, Julie M. Thole3, Joachim Goedhart1, Erik Nielsen4, Teun Munnik2,‡ and Theodorus W.J. Gadella Jr1

1Department of Molecular Cytology, Centre for Advanced Microscopy, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands, 2Department of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, Amsterdam, The Netherlands, 3Department of Biology, Washington University, One Brookings Drive, St Louis, MO 63130, USA, and 4Department of Molecular, Cellular & Developmental Biology, University of Michigan, Ann Arbor, MI 48109, USA

Received 3 April 2008; revised 4 August 2008; accepted 21 August 2008; published online 27 October 2008.

*For correspondence (fax +31 205257924; e-mail T.munnik@uva.nl).
†Present address: Section of Plant Physiology, Swammerdam Institute for Life Sciences, University of Amsterdam, The Netherlands.

Summary

Polyphosphoinositides represent a minor group of phospholipids, accounting for less than 1% of the total. Despite their low abundance, these molecules have been implicated in various signalling and membrane trafficking events. Phosphatidylinositol 4-phosphate (PtdIns4P) is the most abundant polyphosphoinositide. 32Pi-labelling studies have shown that the turnover of PtdIns4P is rapid, but little is known about where in the cell or plant this occurs. Here, we describe the use of a lipid biosensor that monitors PtdIns4P dynamics in living plant cells. The biosensor consists of a fusion between a fluorescent protein and a lipid-binding domain that specifically binds PtdIns4P, i.e. the pleckstrin homology domain of the human protein phosphatidylinositol-4-phosphate adaptor protein-1 (FAPP1). YFP–PHFAPP1 was expressed in four plant systems: transiently in cowpea protoplasts, and stably in tobacco BY-2 cells, Medicago truncatula roots and Arabidopsis thaliana seedlings. All systems allowed YFP–PHFAPP1 expression without detrimental effects. Two distinct fluorescence patterns were observed: labelling of motile punctate structures and the plasma membrane. Co-expression studies with organelle markers revealed strong co-labelling with the Golgi marker STtmd–CFP, but not with the endocytic/pre-vacuolar marker GFP–AtRABF2b. Co-expression with the PtdIns3P biosensor YFP–2FYVE revealed totally different localization patterns. During cell division, YFP–PHFAPP1 showed strong labelling of the cell plate, but PtdIns3P was completely absent from the newly formed cell membrane. In root hairs of M. truncatula and A. thaliana, a clear PtdIns4P gradient was apparent in the plasma membrane, with the highest concentration in the tip. This only occurred in growing root hairs, indicating a role for PtdIns4P in tip growth.

Keywords: phosphoinositides, GFP, membrane trafficking, microscopy, lipid binding domain.

Introduction

Phosphatidylinositolmonophosphate (PtdInsP) is a minor lipid present in all eukaryotic membranes, accounting for less than 1% of total phospholipids (Meijer and Munnik, 2003; Munnik et al., 1998). In nature, three isomers occur, which differ in the position of the phosphate group on the D-myoinositol ring, i.e. PtdIns3P, PtdIns4P and PtdIns5P, which are formed by specific kinases and phosphatases (Mueller-Roeber and Pical, 2002; Meijer and Munnik, 2003; Shisheva, 2008). PtdIns5P, the last isomer that was discovered, accounts for approximately 15% of the 32P-labelled PtdInsP pool in plants, and has been implicated in the response to osmotic stress (Meijer et al., 2001). Similar amounts are present in mammalian cells, where it has been implicated in host-
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Pathogen interactions and apoptosis (Gozani et al., 2003; Pendaries et al., 2006). PtdIns3P accounts for approximately 10% of a plant’s 32P-labelled PtdIns3P pool (Meijer et al., 2000; Munnik et al., 1994a,b). In yeast and mammalian cells, PtdIns3P is an important regulator of endocytosis and vesicular trafficking (Corvera et al., 1999; Simonsen et al., 2001; Stenmark and Gillooly, 2001), and this is probably also the case for plants (Kim et al., 2001; Vermeer et al., 2006). PtdIns4P is the most abundant isomer, making up approximately 80% of the plant PtdIns4P pool (Meijer and Munnik, 2003; Meijer et al., 2001; Munnik et al., 1994ab). In addition to being important for phospholipase C (PLC) signalling, as a substrate itself, or as a precursor for PtdIns(4,5)P2, PtdIns4P has also been recognized as an important signalling molecule involved in membrane trafficking and cytoskeletal organization as known from mammalian and yeast studies (Audhya and Emr, 2002; Audhya et al., 2000; Balla et al., 2002, 2005).

In plants, functional data on PtdIns4P are still very limited. In the genome of Arabidopsis, 12 putative phosphoinositide (PI) 4-kinases have been predicted, classified as PI4K\(a\)-2, \(\beta\)-1-2 and \(\gamma\)-1-8 (Mueller-Roever and Pical, 2002). So far, kinase activity has only been established for PI4K\(a\)-1 and \(\beta\). Moreover, PI4K\(a\)-1-8 are likely to represent a novel protein kinase family (Galvão et al., 2008). Over-expression of a truncated version of AtPI4K\(a\)-1 in Arabidopsis protoplasts was shown to affect vesicle trafficking (Kim et al., 2001). Knocking out both AtPI4K\(a\) and \(\beta\) genes in Arabidopsis resulted in root hairs with disrupted polarity (Preuss et al., 2006). Moreover, a mutant with a defect in root hair development was recently discovered to carry a mutation in a PtdIns4P phosphatase (Thole et al., 2008). Together, these data indicate an important role for PtdIns4P in root hair growth.

In vitro, PI 4-kinase activity has been detected in several cellular fractions, including cytosol, plasma membrane, microsomal membranes and actin-enriched fractions (Okpodu et al., 1995; Stevenson et al., 1998; Stevenson-Paulik et al., 2003; Yang et al., 1993). GFP fusions in insect cells revealed that GFP–AtPI4K\(a\)-1 was localized to the peri-nuclear region, whereas GFP–AtPI4K\(a\)-1 was localized in undefined punctate structures (Stevenson-Paulik et al., 2003). More recently, AtPI4K\(a\)-1 was shown to localize to the tip of growing root hairs (Preuss et al., 2006). Where PtdIns4P resides is largely unknown.

In this study, we have used a fusion between a fluorescent protein and the PtdIns4P-specific pleckstrin homology domain of human phosphatidylinositol-4-phosphate adapter protein-1 (HsFAPP1) (Dowler et al., 2000; Levine and Munro, 2002) to monitor PtdIns4P dynamics in living plant cells. Four commonly used model systems were validated: transient expression in cowpea protoplasts, and stable expression in tobacco BY-2 cells, Medicago truncatula roots and Arabidopsis thaliana seedlings. All four systems allowed PtdIns4P visualization without noticeable effects on cell growth and development. Two PtdIns4P pools could be distinguished: one at the plasma membrane and one at the Golgi. During cell division in BY-2 cell, and polarized tip growth in Medicago and Arabidopsis root hairs, interesting observations were made that suggest PtdIns4P-specific functions that are independent of the PtdIns(4,5)P2 and PLC signalling paradigm.

**Results**

**YFP–PH\(a\)-FAPP1 expression in cowpea protoplasts**

In yeast and mammalian cells, GFP–PH\(a\)-FAPP1 has been shown to function as a robust in vivo marker for PtdIns4P (Balla et al., 2005; Dowler et al., 2000; Levine and Munro, 2002). To investigate the use of this biosensor in living plant cells, we re-cloned it into a plant vector and expressed it transiently in cowpea protoplasts under the control of a 35 S promoter. As shown in Figure 1(a–d), YFP–PH\(a\)-FAPP1 typically labelled the plasma membrane and certain motile punctate structures. These structures were approximately 0.73 ± 0.1 \(\mu\)m in size (\(n\) = 50), and their motility was inhibited by latrunculin A but not by oryzalin, indicating that movement was dependent on the actin cytoskeleton and not microtubules (data not shown). To determine the identity of the motile structures, protoplasts were co-transfected with the endosomal/pre-vacuolar marker GFP–AtRABF2b (Lee et al., 2004; Ueda et al., 2004) or the trans-Golgi marker STtmd–CFP (Boevink et al., 1998). YFP–PH\(a\)-FAPP1 fluorescence showed a strong overlap with STtmd–CFP (Figure 1e–h) but not with GFP–AtRABF2b (Figure 1i–l), indicating that the YFP–PH\(a\)-FAPP1-labelled structures were Golgi stacks.

**Mutational analysis of YFP–PH\(a\)-FAPP1**

To investigate whether the localization of YFP–PH\(a\)-FAPP1 was dependent on PtdIns4P, two point mutations were introduced (i.e. K7E and R18L) that are known to abolish PtdIns4P binding (Godi et al., 2004; Levine and Munro, 2002; Park et al., 2003). When expressed in cowpea protoplasts, YFP–PH\(a\)-FAPP1-K7E and YFP–PH\(a\)-FAPP1-R18L no longer labelled the plasma membrane (Figure 1m,q) YFP–PH\(a\)-FAPP1-R18L was still faintly localized to the Golgi stacks but YFP–PH\(a\)-FAPP1-K7E was mainly cytosolic and partly nuclear (compare Figure 1m,n and Figure 1q,r). The large increase in cytosolic localization indicates a loss of binding of the sensor to PtdIns4P-containing membranes. Labelling of the nucleus is probably due to diffusion of the sensor into the nucleus. When these constructs were expressed in mammalian HeLa cells, similar residual Golgi localization of YFP–PH\(a\)-FAPP1-R18L was observed, but YFP–PH\(a\)-FAPP1-K7E was again completely cytosolic (Figure S1a–c).
Figure 1. Expression of YFP–PHFAPP1 in cowpea protoplasts and identification of YFP–PHFAPP1-labelled punctate structures. Confocal micrographs of cowpea protoplasts expressing YFP–PHFAPP1 (a–d), YFP–PHFAPP1 plus STmtd–CFP (e–h), YFP–PHFAPP1 plus GFP–AtRABF2b (i–l), YFP–PHFAPP1-K7E plus STmtd–CFP (m–p), and YFP–PHFAPP1-R18L plus STmtd–CFP (q–t). (a, b, e–t) Median confocal sections of a protoplast expressing YFP–PHFAPP1. (c, d) Maximum projection of an image stack of the same protoplast. Arrowheads in (a) indicate punctate structures labelled by YFP–PHFAPP1, arrowheads in (q) show residual Golgi labelling by YFP–PHFAPP1. YFP fluorescence is shown in yellow (a–d) or red (e, i, m, q), GFP and CFP fluorescence are shown in green (f, j, n, r), and chlorophyll is shown in red (b, d) or blue (g, k, o, s). The images are representative of at least five independent transfections. Overlay of fluorescence is shown in (b, d, h, j, l, p, t). Scale bars = 10 μm.
Localization of PtdIns4P in stably transformed BY-2 cells

While the advantage of a transient protoplast system is that GFP constructs can be quickly assessed, a limitation is that protoplasts are often physiologically stressed by the procedure of making them and/or by the sudden over-expression of a recombinant protein (Vermeer, 2006). To further investigate the expression pattern of YFP–PHFAPP1, stably transformed tobacco BY-2 cells were generated.

Cells stably expressing YFP–PHFAPP1 or mRFP–PHFAPP1 grew and appeared normal, indistinguishable from wild-type cells (data not shown). Fluorescence was mostly associated with the cell periphery and motile punctate structures (Figure 2a–d, Figure S2 and Movie S1). A faint cytoplasmic and nucleoplasmic background was also observed, probably reflecting unbound YFP–PHFAPP1 (Figure 2a–d, Figure S2 and Movie S1). Mannitol-induced plasmolysis and co-labelling with the endocytic tracer FM4-64 confirmed that the PtdIns4P biosensor did indeed label the plasma membrane (Figure 2a–h). Following endocytic uptake of FM4-64 over 20 min using time-lapse confocal microscopy revealed hardly any co-labelling between FM4-64 and YFP–PHFAPP1 (Figure 3 and Movie S2). After 20 min, compartments started to appear closer to each other and sometimes even overlapped (arrowheads in Figure 3i–k). This could be the Golgi, as Bolte et al. (2004) reported that Golgi membranes can be labelled with FM4-64 within 30 min. Nonetheless, these results clearly indicate that PtdIns4P is absent from early endocytic compartments. To confirm that YFP–PHFAPP1 also detected a PtdIns4P pool in the Golgi, co-expression studies with STtmd–CFP were performed. As shown in Figure 4 and Movie S3, strong co-labelling between mRFP–PHFAPP1 and STtmd–CFP was found.

PtdIns4P and PtdIns3P in BY-2 cells

The difference between PtdIns3P and PtdIns4P is the phosphate group on the 3- or 4-position of the D-myo-inositol ring. PtdIns3P has previously been imaged using the biosensor YFP–2·FYVE (Vermeer et al., 2006; Voigt et al., 2005). To investigate whether there is any overlap between PtdIns3P and PtdIns4P pools, stably transformed BY-2 cells expressing both biosensors were generated.

mRFP–PHFAPP1 was present on the plasma membrane and Golgi structures, but YFP–2·FYVE clearly labelled a different population of membrane structures (Figure 5 and Movie S4) that were previously identified as late endosomal compartments (Vermeer et al., 2006; Voigt et al., 2005). Frequently, however, we observed these pools in close proximity to each other (Figure 5h).

Are phenylarsine oxide and wortmannin PI 4-kinase inhibitors in plant cells?

In mammalian cells, phenylarsine oxide (PAO) has been shown to decrease PtdIns4P levels, presumably through inhibition of a PI 4-kinase activity (Wiedemann et al., 1996). Wortmannin is widely used as PI 3-kinase inhibitor (Arcaro and Wymann, 1993; Stephens et al., 1994), but has also been reported to affect PtdIns4P levels in both mammalian and plant cells (Balla et al., 2005; Matsuoka et al., 1995). Recently, higher concentrations of wortmannin were found to inhibit isoform-specific (type III) PI 4-kinases, which have kinase domains that closely resemble those of PI 3-kinases (Krinke et al., 2007). Having the BY-2 cells express both biosensors allowed us investigate the effect of these inhibitors on PtdIns3P and PtdIns4P pools simultaneously.

Within 20 min after adding 20 μM PAO, most of the membrane-localized mRFP–PHFAPP1 was lost and reappeared in the cytoplasm and nucleus, indicating unbound mRFP–PHFAPP1. In contrast, the fluorescence pattern of YFP–2·FYVE hardly changed (Figure 6a–d and Movie S5). The cyto-architecture of the cells did change: cytoplasmic...
strands vanished and large vacuoles appeared (Figure 6a–
d). This was even more evident after 45 min (Figure 6e–h).
Confocal time-lapse imaging revealed that cytoplasmic
streaming and the movement of YFP–2·FYVE-labelled
vesicles was greatly impaired (Movie S6). As treatment with
20 μM PAO had such a strong effect on the mRFP–PHFAPP1
localization, the effect of PAO on PtdIns4P levels was
investigated further. BY-2 cells were pre-labelled for 5 min
with 32Pi, and than treated with either 20 μM PAO for 15, 30
and 45 min or 0.05% v/v DMSO as a control. Lipids were
subsequently extracted, separated by TLC, and quantified by
phosphoimaging. In contrast to the strong effect of PAO on

Figure 3. YFP–PHFAPP1-labelled structures are not early endocytic structures.
Cells expressing YFP–PHFAPP1 were imaged immediately after addition of 2 μM FM4-64 or
after 20 min. Partial co-labelling with FM4-64-positive compartments is only visible after
20 min (i, k).
(i–l) Close-ups of the boxed area in (e) showing compartments with only FM4-64 (yellow arrows)
and partial overlap between YFP–PHFAPP1- and FM4-64-labelled compartments (arrowheads).
YFP fluorescence is shown in red (a, e, i), FM4-64 fluorescence is shown in red (b, f, j), and
DIC is shown in grey (d, h, l).
(c, g, k) Merged images. Results are representa-
tive of three independent experiments. Scale
bar = 10 μm (a–h) or 5 μm (i–l).

Figure 4. mRFP–PHFAPP1-labelled punctate structures are Golgi membranes.
Tobacco BY-2 cells expressing mRFP–PHFAPP1 and the Golgi marker STtmd–
CFP show clear co-localization at Golgi stacks.
(a) mRFP–PHFAPP1 fluorescence (red);
(b) STtmd–CFP fluorescence (green);
(c) overlay;
(d) DIC image. Arrowheads indicate several mRFP–PHFAPP1-labelled Golgi
stacks. Scale bar = 10 μm.

Figure 5. Simultaneous imaging of PtdIns3P and PtdIns4P.
(a–d) Tobacco BY-2 cells stably expressing YFP–2·FYVE and mRFP–PHFAPP1.
(e–h) Close-ups of the same cell showing no overlap between fluorescent
signals. (a, e) mRFP fluorescence; (b, f) YFP fluorescence; (c, g) DIC image;
(d, h) overlay. Scale bar = 10 μm.
mRFP–PHFAPP1 localization, no effect was found on 32P-PtdIns₄P levels (Figure S3). We do not understand why this is, and this is discussed further below.

Treatment of the cells with 10 μM wortmannin resulted in a complete loss of YFP–2·FYVE labelling of the punctate structures within 20 min and a subsequent increase of labelling in the cytosol, but the localization of mRFP–PHFAPP1 remained unaffected (Figure 6i–l and Movie S7). After 45 min, the fluorescence pattern of mRFP–PHFAPP1 was still not affected, while YFP–2 × FYVE started to appear on larger membrane structures (Figure 6m–p and Movie S8) (Vermeer et al., 2006). In contrast to PAO, wortmannin hardly affected the cyto-architecture of the cells (compare Figure 6h and Figure 6p). When a threefold higher wortmannin concentration (i.e. 30 μM) was used, no alterations in the above-described patterns were observed (Figure S4).

PtdIns₄P dynamics during cytokinesis in BY-2 cells

In dividing BY-2 cells, we previously found that YFP–2 × FYVE was predominantly present on vesicular structures in the vicinity of the expanding cell plate, but was never actually found on the newly formed cell membrane (Vermeer et al., 2006). Interestingly, monitoring both sensors simultaneously revealed that mRFP–PHFAPP1 labelled the growing cell plate right from the start (Figure 7a,b and Movie S9). When YFP–PHFAPP1 cells were co-labelled with the endosomal tracer FM4-64 (2 μM), FM4-64 was found to label the cell plate just before YFP–PHFAPP1 fluorescence was detected (Figure 7c,d and Movie S10). This implies that PtdIns₄P appears just after the cell plate has been initiated, which might reflect its role in cell-plate expansion. The results demonstrate beautifully the differential roles that the PtdIns₄P isomers can play.

Localization of YFP–PHFAPP1 and YFP–2 × FYVE in Medicago truncatula root hairs

Next, we tested the expression of both biosensors in the stably transformed root system of the model legume Medicago truncatula (Boisson-Dernier et al., 2001; Mirabella et al., 2004). As shown in Figure 8(a–m), YFP–PHFAPP1 clearly labelled the plasma membrane and punctate structures, the latter probably again being Golgi stacks. Interestingly, in emerging and growing root hairs, a clear tip-localized gradient of YFP–PHFAPP1 at the plasma membrane was observed, which was absent in root hairs that had stopped growing (compare Figure 8e,g,i–k with Figure 8m). Control roots transformed with the mutated mRFP–PHFAPP1-K7E only showed cytosolic fluorescence (Figure 8o–t). In comparison, YFP–2 × FYVE labelled numerous vesicular structures and vacuolar membranes, but was not enriched in the tip area (Figure 8u–x). These results indicate a specific role for PtdIns₄P in root hair growth.

Localization of YFP–PHFAPP1 in Arabidopsis seedlings

To image PtdIns₄P dynamics in whole plants, stably transformed Arabidopsis thaliana lines were generated.

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Homozygous T3 lines, expressing YFP–PHFAPP1 under the control of the 35S promoter, grew normally and were indistinguishable from wild-type (Col-0) or mRFP–PHFAPP1-K7E-transformed plants (not shown). Seedlings from at least three independent transformed lines expressing YFP–PHFAPP1 all revealed similar fluorescence patterns to those described above for the other model systems, i.e. strong labelling of the plasma membrane, with occasionally some punctate structures moving through the cytosol (Figure 9a,d). Plasma membrane labelling was seen in all cell

Figure 7. PtdIns3P and PtdIns4P dynamics during cell division in BY-2 cells. 
(a, b) Confocal time-lapse images of a dividing BY-2 cell co-expressing mRFP–PHFAPP1 (red) and YFP–2 × FYVE (green). mRFP–PHFAPP1 labels the cell plate (double arrow heads), but YFP–2 × FYVE fluorescence appears to be completely absent. 
(c, d) Confocal time-lapse images of a dividing BY-2 cell expressing YFP–PHFAPP1 (green) co-labelled with 2 μM FM4-64 (red). The time series shows that FM4-64 accumulates at the cell plate prior to YFP–PHFAPP1 fluorescence. Time is indicated in minutes corresponding to Movies S9 and S10. For both experiments, at least 3–5 cell divisions were followed. Scale bar = 10 μm.
types, including leaf epidermal and guard cells (Figure 9s,u). Seedlings expressing mRFP–PHFAPP1-K7E only showed fluorescence in the cytosol (Figure S5). In growing root hairs, a tip-localized PtdIns4P gradient at the plasma membrane was again observed, which was always lacking in non-growing root hairs (Figure 10g,k and Figures S6 and S7).
This tip-localized plasma membrane gradient appeared to resemble that of PtdIns(4,5)P_2, as shown previously using YFP–PHPLC d_1 (van Leeuwen et al., 2007; Vincent et al., 2005). However, as shown in Figure 9(j,q), YFP–PHPLC d_1 only labels the extreme tip, whereas YFP–PHFAPP1 labelled the whole plasma membrane, increasing in intensity towards the tip. In contrast, as indicated by YFP–2·FYVE labelling, PtdIns3P was only present in punctate structures, probably late endosomes (Vermeer et al., 2006) that were evenly distributed throughout the growing root hair (Figure 9m,n). To confirm whether the YFP–PHFAPP1-labelled punctate structures were Golgi membranes, seedlings were treated with brefeldin A (BFA), a fungal toxin that inhibits Golgi trafficking, which results in the appearance of larger so-called BFA compartments (Geldner et al., 2003; Ritzenthaler et al., 2002). Incubation with 50 μM BFA for 30 min resulted in the appearance of large YFP–PHFAPP1-labelled compartments (Figure S8a,b). Washout of the BFA completely reversed this effect (Figure S8c,d). These results suggest that, in Arabidopsis, PtdIns4P is present on both plasma membrane and the Golgi.

Recently, it was shown that the root hair defective 4 mutant is mutated in the AtSAC7 gene, which was shown to encode a PtdIns4P phosphatase (Thole et al., 2008). rhd4 mutants exhibited approximately 50% more PtdIns4P (Thole et al., 2008). Expression of YFP–PHFAPP1 in rhd4-1 revealed that most of the fluorescence was localized on internal membranes, whereas, in the wild-type background (Col-0), it was mostly the plasma membrane that was labelled (compare Figure 10a–c with Figure 10d–i), essentially confirming the results of Thole et al. (2008). This differential localization...
of YFP–PHFAPP1 was also observed in root cells close to the tip (Figure 10j,k). In addition to strong plasma membrane labelling, accumulation of YFP–PHFAPP1 on enlarged Golgi structures, some of which displayed a ‘donut-like’ shape, was also observed (arrows in Figure 10j,k).

Discussion

Using YFP–PHFAPP1 as a PtdIns4P biosensor

The YFP–PHFAPP1 biosensor has been used to monitor PtdIns4P dynamics in animal cells (Balla et al., 2005, 2008; Godi et al., 2004) and yeast (Levine and Munro, 1998, 2002; Roy and Levine, 2004). Here, we describe its use to study the localization and dynamics of PtdIns4P in living plant cells. Four plant systems were tested: transient expression in cowpea prooplasts, and stable expression in tobacco BY-2 cells, Medicago truncatula roots and Arabidopsis seedlings. All systems allowed expression of YFP–PHFAPP1, without any adverse effects caused by the 35 S-driven expression of the biosensor. Point mutations in PHFAPP1, known to be essential for PtdIns4P binding, revealed that the observed localization was PtdIns4P-dependent. In addition, changes in cellular PtdIns4P levels were detected (Figure 10) (Thole et al., 2008). We conclude that, as in yeast mammalian cells (Balla et al., 2005; Dowler et al., 2000; Strahl et al., 2005), YFP–PHFAPP1 or mRFP–PHFAPP1 can be used as a genuine PtdIns4P sensor in living plant cells.

Identification of multiple PtdIns4P pools in plants

Expression of YFP–PHFAPP1 in protoplasts labelled punctate structures moving through the cytosol and the plasma membrane (Figure 1). Similar labelling patterns were
observed in stably transformed BY-2 cells, *M. truncatula* roots and Arabidopsis seedlings (Figures 2, 8 and 9). This suggests that plants contain two spatially separated PtdIns4P pools. An additional lower level of fluorescence in the cytoplasm and nucleoplasm probably reflects the dynamic equilibrium between binding and non-binding that is typical for these biosensors (Balla, 2007; van Leeuwen et al., 2007; Vermeer, 2006).

Using co-expression studies with various markers, YFP–PHFAPP1 clearly co-localized with the Golgi marker STtmd–CFP but not with the endocytic/pre-vacuolar marker GFP–ArABF2b. BY-2 cells co-expressing mRFP–PHFAPP1 and YFP–2×FYVE, which binds PtdIns3P and labels a late endocytic compartment (Vermeer et al., 2006), also showed no overlap in fluorescence, emphasizing that the PtdIns3P- and PtdIns4P pools are spatially separated. When observed in more detail, the punctate mRFP–PHFAPP1 structures were frequently found in close proximity to the YFP–2×FYVE-labelled compartments, resembling the observations in BY-2 cells stably expressing YFP–2×FYVE and STtmd–CFP (Vermeer et al., 2006). Together these data suggest that plant cells contain at least two distinct PtdIns4P pools: plasma membrane and the Golgi.

PtdIns4P localization may be functionally conserved because yeast and mammalian cells also revealed these two pools (Audhya and Emr, 2002; Balla et al., 2005; Roy and Levine, 2004; Weixel et al., 2005). However, while in yeast and mammalian cells, YFP–PHFAPP1 is mainly localized to the Golgi (Balla et al., 2005; Levine and Munro, 2001, 2002), it is clearly the plasma membrane that is predominantly labelled in plant cells. In mammalian cells, the plasma membrane is only clearly labelled after receptor stimulation (Balla et al., 2005; Levine and Munro, 2001, 2002).

PtdIns4P synthesis is catalysed by PI 4-kinases. These enzymes are classified as type II or III forms (type I was found to be a PI 3-kinase) based on their enzymatic properties and size (Balla, 1998; Balla and Balla, 2006). Type III kinases are relatively large, usually soluble and sensitive to wortmannin, whereas the smaller type II enzymes are particulate and insensitive to this inhibitor.

The yeast *Saccharomyces cerevisiae* contains three PI 4-kinases, encoded by Pik1p, Stt4p (both type III) and Lsb6p (type II). Knockout strains of *Lsb6p* exhibit no significant differences in PtdIns4P levels and are viable, whereas the *Pik1p* and *Stt4p* genes are essential and responsible for at least 95% of the PtdIns4P pool (Audhya and Emr, 2002; Han et al., 2002; Shelton et al., 2003). Pik1p genetically interacts with Sec14p and is important for exocytic trafficking from the Golgi (Hama et al., 1999; Walch-Solimena and Novick, 1999). The PtdIns4P generated by Pik1p facilitates the binding of Kes1p to the Golgi, a factor that controls the function of the small G-protein ARF (ADP-ribosylation factor) and budding (Li et al., 2002). A temperature-sensitive mutant of Pik1p exhibits defects in endocytosis and vacuolar dynamics (Audhya et al., 2000). Pik1p has also been suggested to play an essential role in the nucleus but this could be independent of its kinase activity, as no nuclear labelling by GFP–PHFAPP1 was observed (Strahl et al., 2005). In contrast, Stt4p binds to the plasma membrane via the protein Sfk1p, where it promotes cell-wall assembly and cytoskeletal rearrangements (Audhya and Emr, 2002; Audhya et al., 2000). Lsb6p plays a role in actin-dependent endosome motility; however, this is independent of its PI 4-kinase activity (Chang et al., 2005).

In mammalian cells, four PI 4-kinases have been identified, i.e. α and β isoforms for both type II and III enzymes (Balla et al., 2002, 2005, 2008; Godi et al., 1999; Weixel et al., 2005). Type II enzymes are present in various cellular membranes but enriched in the plasma membrane and have been suggested to play a role in post-trans-Golgi network trafficking. Nonetheless, the plasma membrane localization of PtdIns4P was recently shown to be dependent on the activity of wortmannin-sensitive, type III PI4 kinases, particularly PI4KIIα (Balla et al., 2008).

In Arabidopsis, 12 PI 4-kinase genes have been predicted, which have been classified into three subfamilies: α1–2 and β1–2 (all type III) and γ1–8 (type II). So far, lipid kinase activity has only been shown for AtPI4Kα1 and AtPI4Kβ1 (Stevenson-Paulik et al., 2003). Moreover, it is possible that the γ subfamily represents protein kinases rather than lipid kinases (Galvão et al., 2008). GFP localization experiments for AtPI4Kα1 and β1 in insect cells revealed that the proteins were confined to distinct punctate structures and absent from the plasma membrane (Stevenson-Paulik et al., 2003). Immunolocalization studies on AtPI4Kβ1 in growing root hairs of Arabidopsis revealed co-localization with a Rab GTPase on Golgi membranes, concentrated at the tip (Preuss et al., 2006). It will be interesting to determine which kinase(s) is/are responsible for the relatively large plasma membrane pool of PtdIns4P.

**Do PAO and wortmannin inhibit PI 4-kinase activity in plant cells?**

Wortmannin is a typical PI 3-kinase inhibitor, but at higher concentrations it is also able to inhibit mammalian type III PI 4-kinase activity, presumably because the kinase domains closely resemble each other. PAO also inhibits type III PI 4-kinase activity and is relatively ineffective against type II enzymes. The mechanism by which this sulphydryl-reactive agent works is unknown (Balla et al., 2008). In plant extracts, a relatively high concentration of wortmannin (30 μM) has been reported to inhibit PI 4-kinase activity *in vitro* (Matsuoka et al., 1995). *In vivo* analysis, using 32P-labelled BY-2 cells and HPLC headgroup analysis of PPIs revealed that wortmannin reduced the PtdIns3P levels by more than one-third within 15 min of treatment and that the 32P-labeling of PtdIns4P was also slightly affected, being reduced by
approximately 5–10% (Vermeer et al., 2006). Wortmannin had no effect on the localization of mRFP–PHFAPP1, even with the higher 30 μM concentration, while that of YFP–2 × FYVE was dramatically affected (Figure 6i–p and Figure S4). In contrast, treatment of cells with 20 μM PAO resulted in a rapid loss of membrane labelling by mRFP–PHFAPP1 in both plasma membrane and punctate structures (Fig 6a–d), while YFP–2 × FYVE labelling was hardly affected. We also observed a strong effect on cytoplasmic streaming and vacuolar morphology of the cells. However,32P radiolabeling experiments did not reveal any changes in32P-PtdIns4P levels (Figure S3). However, using longer labelling times, we did observe that PAO treatment resulted in a decrease of32P-labelled structural lipids (data not shown). This could mean that PAO is toxic to the cells, rather than acting as a PI 4-kinase-specific inhibitor. However, this does not explain why the mRFP–PHFAPP1 localization was affected whereas localization of YFP–2 × FYVE was not. It should be noted that in another system, Arabidopsis suspension cells, Krinke et al. (2007) reported that PAO could inhibit PI 4-kinase activation.

Together, these results indicate that, in tobacco BY-2 cells, wortmannin primarily acts an inhibitor for PI 3-kinase and not PI 4-kinase activity. As PtdIns3P is involved in various membrane trafficking events, it is not unlikely that wortmannin has a small indirect effect on PtdIns4P synthesis. Our data also show that the results obtained with PAO should be interpreted with caution.

**Role for PtdIns4P during plant cytokinesis**

Recently, Dhonukshe et al. (2006) showed that cell-plate initiation can still occur in the absence of Golgi stacks and/or protein synthesis. Moreover, they provided evidence that endocytic material was required to form this cell plate (Dhonukshe et al., 2006). In dividing BY-2 cells co-expressing mRFP–PHFAPP1 and YFP–2 × FYVE, we show that PtdIns3P is completely excluded from the cell plate, whereas PtdIns4P is strongly enriched. Previously, we found that PtdIns3P-containing compartments completely surround the expanding cell plate (Vermeer et al., 2006) and that wortmannin was able to delay cell-plate formation (Dhonukshe et al., 2006). These data imply that, although PtdIns3P does not localize to the cell plate, it could have a role in the transport of membrane vesicles to or from the growing cell plate. The fact that we found PtdIns4P on Golgi stacks and on the forming cell plate could mean that there is a flow of vesicles from the Golgi to the cell plate. However, no Golgi stacks were found in close proximity to the cell plate during its initiation, and when BFA was used, Golgi compartments were dissolved, but this did not abolish cell-plate initiation (Dhonukshe et al., 2006). This suggests that the PtdIns4P in the cell plate is not derived from vesicle flow from the Golgi. Using co-labelling with the endocytic tracer FM4-64, we found that FM4-64 labelled the cell plate slightly earlier than did YFP–PHFAPP1 (Figure 7c,d and Movie S10). This implies that PtdIns4P is synthesized locally by a cell plate-associated PI 4-kinase, although we cannot exclude the possibility that some PtdIns4P also enters the cell plate via active transport (Golgi or endocytosis). Knockout mutants of PI 4-kinases and GFP-tagged versions of the enzymes may shed further light on this in the near future. The fact that Arabidopsis pi4k1/1/2 double mutants only exhibit a slight cytokinetic phenotype (E.N., unpublished results) indicates a role for PtdIns4P in cell-plate expansion rather than initiation.

**Does PtdIns4P regulate tip growth?**

Previously, a PtdIns(4,5)P2 gradient in the plasma membrane of growing root hairs and pollen tubes has been implicated in regulating tip growth (Dowd et al., 2006; Helling et al., 2006; Kost et al., 1999; van Leeuwen et al., 2007; Vincent et al., 2005). This gradient was proposed to be created by local stimulation of PtdIns4P 5-kinase activity via a Rop GTPase (Kost et al., 1999). Our observation here, that PtdIns4P is already present in a tip-localized gradient, implies that it is not necessarily the regulation of the PtdIns4P 5-kinase that creates the PtdIns(4,5)P2 gradient, as a uniformly spatial distributed kinase would also create this. Recently, an Arabidopsis PI 4-kinase that plays a crucial role in the polarized expansion of root hairs has been identified (Preuss et al., 2006). This AtPI4K1J1 localizes to the tip of growing root hairs through interaction with a Rab GTPase and a Ca2+ sensor. Together, this was proposed to result in tip-localized PI 4-kinase activity, with subsequent enrichment of PtdIns4P (Preuss et al., 2006). Our data confirm this hypothesis. More recently, a root hair mutant exhibiting huge defects in polarity (bulging, swelling) was discovered to carry a mutation in a PtdIns4P phosphatase (Thole et al., 2008). Together, these data indicate an essential role for PtdIns4P in polar tip growth. Recent data on a PtdIns4P 5-kinase mutant (AtPI4P63K) exhibiting a root hair phenotype indicate that PtdIns(4,5)P2 is more important for growth rather than polarity (Kusano et al., 2008; Stenzel et al., 2008). The observation of enlarged Golgi structures in rhd4-1 suggests that proper regulation of PtdIns4P levels at the Golgi is required for maintaining its structure.

It is still not known what the precise function of PtdIns4P is. In general, PtdIns4P is usually regarded as the precursor of PtdIns(4,5)P2, which has emerged as a very important signalling molecule in three ways: (i) as a substrate for PI 3-kinase signalling to produce the lipid second messenger PtdIns3(3,4,5)P3; (ii) as substrate for PLC signalling, generating second messengers Inositol 3,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which activates protein kinase C (PKC); and (iii) as a second messenger itself, targeting proteins to membranes via specific lipid-binding domains, regulating ion-channel

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activity, organizing the actin cytoskeleton and modulating vesicular trafficking (for review, see Gamper and Shapiro, 2007; Huang, 2007; McLaughlin et al., 2002). While these signalling systems have been clearly established in mammalian cells over the last decade (van Leeuwen et al., 2007), a completely different picture is emerging for plants. There is no PI 3-kinase that phosphorylates PtdIns(4,5)P_2 into PtdIns(3,4,5)P_3, there is no Ca^{2+}-gated InsP_3 receptor, and no PKC homologues have been identified in plants (Zonia and Munnik, 2006). In the past, InsP_3 has been shown to release Ca^{2+} from intracellular stores when micro-injected or released via photo-activation of a caged variant, but work from Brearley’s lab shows this is probably caused by InsP_6 (Lemtrí-Chlieh et al., 2003; Zonia and Munnik, 2006). The latter can release Ca^{2+} at a 10-fold lower concentration than InsP_3, and when InsP_6 is micro-injected, it is rapidly converted into InsP_3. The hormonal stimulation via ABA to which it was linked was also shown to generate an InsP_6 response rather than InsP_3 (Lemtrí-Chlieh et al., 2003). In yeast, InsP_6 signalling is not related to Ca^{2+} but directly regulates gene transcription and mRNA export from the nucleus. The pathway involves PLC and two inositol polyphosphate multi-kinases that can stepwise phosphorylate InsP_3 to InsP_6 (Ives et al., 2000; Odom et al., 2000; Perera et al., 2004; York et al., 1999, 2001).

Another difference is that the PtdIns(4,5)P_2 concentrations in higher plants are extremely low compared to those of mammalian cells or green algae. The latter systems usually have a 1:1 ratio of PtdIns4P and PtdIns(4,5)P_2, whereas higher plant cells exhibit 30–100-fold lower PtdIns(4,5)P_2 ratios (van Leeuwen et al., 2007; Munnik et al., 1994a). The huge difference in PtdIns4P and PtdIns(4,5)P_2 levels shows that PtdIns4P cannot be simply regarded as a precursor of PtdIns(4,5)P_2. Their differential localization during, for example, cell-plate formation (this study; van Leeuwen et al., 2007) also suggests this, as do the strong mutant phenotypes with respect to root hair growth of a PI 4-kinase (Preuss et al., 2006) and PtdIns4P 4-phosphatase (Thole et al., 2008), as opposed to the weak phenotype of a phosphatidylinositol 4-phosphate 5-kinase mutant (Kusano et al., 2008; Stenzel et al., 2008).

We anticipate that PtdIns4P fulfils additional signalling roles. Arabidopsis is predicted to contain more than 50 proteins with a pleckstrin homology domain (van Leeuwen et al., 2004). For most of them, it is still unknown which phosphoinositide they bind or whether they bind at all, but, as for the human FAPP1 protein, PtdIns4P might be a targeting determinant itself. Alternatively, PtdIns4P might be responsible for many of the effects shown for mammalian PtdIns(4,5)P_2, e.g. regulating the actin cytoskeleton or ion-channel activity (Hilgemann et al., 2001; Hilpela et al., 2004). PtdIns4P could also be the substrate for PLC, generating Ins(1,4)P_2 that is phosphorylated to InsP_6 (Zonia and Munnik, 2006). Alternatively, PLC could simply function as a PtdIns4P attenuator. In vitro, plant phosphoinositide specific phospholipase C (PI-PLCs) do not distinguish between PtdIns4P or PtdIns(4,5)P_2 (reviewed by Munnik et al., 1998). So far, we have assumed that PtdIns(4,5)P_2 is the in vivo substrate only because of the mammalian paradigm.

Whatever the function of PtdIns4P turns out to be, the use of lipid biosensors together with mutant analyses will be of great help in further uncovering its role in cell signalling and membrane trafficking.

**Experimental procedures**

**Constructs**

All constructs were produced using standard molecular biological methods. To create pEYFP-PHFAPP1 and pmRFP-PHFAPP1, the pleckstrin homology domain of HsFAPP1 were amplified from plasmid pEGFP-PHFAPP1, kindly provided by Dr T. Levine (Institute of Ophthalmology, London, UK) using primers FAPP1Bgl2fw (5′-GAAGATCTATGAGGGGGTTGTGACATCGGACACATCTACAGCC-3′) and FAPP1Terrev (5′-GCGACTTCTTATGATCAGTGACATCGGACACATCTACAGCC-3′) [restriction sites are underlined]. Subsequently, the pleckstrin homology domain was cloned behind EYFP or mRFP using Bgl2 and EcoRI. The EYFP/mRFP-PHFAPP1 fusions were excised using Nhel and EcoRI, and transferred to pMONid35 S using its XbaI and EcoRI sites. The plasmid containing mRFP1 was kindly provided by Dr R.Y. Tsien (University of California, La Jolla, CA, USA). To generate the point mutations in the pleckstrin homology domains of HsFAPP1, the primers FAPP1K7Efw (5′-CTATGAGGGGGTTGTGACATCGGACACATCTACAGCC-3′) and FAPP1K7Erev (5′-GCGACTTCTTATGATCAGTGACATCGGACACATCTACAGCC-3′) were used. To generate pMONid35S::YFP-PHFAPP1_R18L, the PHFAPP1 domain was amplified from plasmid pEGFP-dIfAPP1R18L (kindly provided by Dr M.A. de Matreis, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy) using the same primers and strategy as described for pMONid35S::YFP-PHFAPP1. To generate stably transformed A. thaliana plants, both YFP-PHFAPP1 and mRFP-PHFAPP1_R18L were transferred to pCAMBIA-35 S using Ncol/EcoRI and Nhel/EcoRI, respectively. The plasmid bearing GFP·ARA7 was kindly provided by Dr A. Nakano (RIKEN, Tokyo, Japan). For BY-2 cell and Medicago truncatula root transformation, all fusions were cloned into the pCAMBIA35S vector.

**Transient expression in cowpea protoplasts**

Cowpea (Vigna unguiculata L.) protoplasts were prepared from 10-day-old plants and transfected with 10 μg of plasmid DNA using the polyethylene glycol method as described previously (Vermeer et al., 2006).

**32P, phospholipid labelling, extraction and analysis**

BY-2 cells (5–6 days old, sub-cultured weekly) were pre-labelled with 32PO4 (carrier-free, PerkinElmer, http://www.perkinelmer.nl) for 5 min and subsequently treated with an equal volume of cell-free medium containing 40 μM PAO or 0.1% DMSO as a control for the solvent. Lipids were extracted, separated by TLC and quantified by phosphoinositol as described previously (Vermeer et al., 2006).
Stable transformations

Tobacco BY-2 cells were transformed and sub-cultured as described previously (Vermeer et al., 2006). For stable expression of GFP fusions in *Medicago truncatula* (Jemalong A17), *Agrobacterium rhizogenes*-mediated root transformation was performed as described by Limpens et al. (2004). Transgenic roots were selected for fluorescence using a Leica MZFLIII stereo fluorescence microscope (http://www.leica-microsystems.com). Fluorescent roots were mounted between two cover glasses in liquid root medium (Gamborg B5 medium including vitamins [Duchefa, http://www.duchefa.nl] supplemented with 3% sucrose, pH 5.8). *Arabidopsis thaliana* cv. Columbia plants were transformed using floral-dip transformation (Clough and Bent, 1998). Homozygous T3 plants were used for further analysis. Seeds were germinated on 0.5× MS containing 1% sucrose and 0.9% agar.

Confocal microscopy

BY-2 cells (4–5 days old) or protoplasts (17 h after transfection) were mounted in eight-chambered cover slides (Nalge Nunc International, http://www.nuncbrand.com). Arabidopsis seedlings were imaged as described previously (Vermeer et al., 2004). Transgenic roots were selected for fluorescence using a Zeiss LSM 510 confocal laser scanning microscope (http://www.zeiss.com/), implemented on an inverted microscope (Zeiss Axiovert 100). Excitation was provided by the 458, 488 and 514 nm Ar laser, 543 nm HeNe and 568 nm Kr lines, controlled by an acousto-optical tuneable filter. For YFP/chlorophyll fluorescence, we used excitation/emission combinations of 514 nm/band pass 530–600 for YFP and long pass 650 for chlorophyll in combination with the HFT458/514 primary, NFT635 secondary and NFT515 tertiary dichroic splitters. For YFP/FM-64 fluorescence, we used excitation/emission combinations of 488 nm/band pass 505–550 for YFP and 543 nm/long pass P650 for FM-64 in combination with HFT 488/543 primary and NFT545 secondary dichroic splitters. For CFP/YFP/chlorophyll detection, we used the excitation/emission combinations of 488 nm/band pass 470–500 for CFP and 514 nm/band pass 530–600 for YFP in combination with the HFT458/514 primary, NFT635 secondary and NFT490 tertiary dichroic splitters. For GFP/YFP/chlorophyll detection, settings were used as described for CFP/YFP/chlorophyll detection.

For YFP/mRFP dual scanning, we used the excitation/emission combinations of 488 nm/band pass 505–550 for YFP and 568 nm/long pass 585 for mRFP in combination with the HFT488/568 primary, NFT570 secondary and NFT515 tertiary dichroic splitters. Cross-talk-free images were acquired by operating the microscope in the multi-tracking mode. A Zeiss water-immersion C-Apochromat 40 x objective lens (numerical aperture 1.2), corrected for cover glass thickness, was used for scanning. Images were captured and analyzed using Zeiss LSM510 software (version 3.2 SP3).

Acknowledgements

We thank Tim Levine (Institute of Ophthalmology, London, UK), Maria Antonietta De Matteis (Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy), Akihiki Nakano (IRKEN, Tokyo, Japan), and Roger Tsien (University of California, La Jolla, CA, USA) for kindly providing us with various constructs. This work was supported by the Netherlands Organization for Scientific Research (NWO), Earth and Life Sciences (ALW) project numbers 810.66.012 and 864.05.001. T.W.J.G.’s lab was additionally supported by the EU integrated project on Molecular Imaging (LSHG-CT-2003-503259) and T.M.’s lab by NWO grant numbers 810.66.011, 813.06.003, 864.05.001 and 700.56.007 and the Royal Netherlands Academy of Arts and Sciences (KNAW).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Localization of various YFP–PHFAPP1 constructs in mammalian cells.

**Figure S2.** Stable expression of mRFP–PHFAPP1 in tobacco BY-2 cells.

**Figure S3.** Effect of 20 μM PAO on 32P-PtdIns4P levels in tobacco BY-2 cells.

**Figure S4.** Effect of 30 μM wortmannin on the localization of mRFP–PHFAPP1 and YFP–2 × FYVE.

**Figure S5.** Localization of PHFAPP1 fusion is PtdIns4P-dependent.

**Figure S6.** YFP–PHFAPP1 is present as a tip-localized plasma membrane gradient in growing Arabidopsis root hairs.

**Figure S7.** YFP–PHFAPP1 is not present as a tip-localized plasma membrane gradient in a root hair that has stopped growing.

**Figure S8.** Effect of 50 μM BFA on the localization of YFP–PHFAPP1 in *A. thaliana*.

**Movie S1.** mRFP–PHFAPP1 is present on the plasma membrane and motile punctate structures.

**Movie S2.** Absence of co-labelling of YFP–PHFAPP1 and FM-64.

**Movie S3.** mRFP–PHFAPP1 labels PtdIns4P at the Golgi membranes.

**Movie S4.** Co-expression of mRFP–PHFAPP1 and YFP–2 × FYVE in stably transformed BY-2 cells.

**Movie S5.** Effect of the PI 4-kinase inhibitor phenylarsine oxide (PAO) on mRFP–PHFAPP1 and YFP–2 × FYVE labelling over 20 min.

**Movie S6.** Effect of the PI 4-kinase inhibitor phenylarsine oxide (PAO) on mRFP–PHFAPP1 and YFP–2 × FYVE labelling over 25 min.

**Movie S7.** Effect of the PI 3-kinase inhibitor wortmannin (WM) on mRFP–PHFAPP1 and YFP–2 × FYVE labelling over 25 min.

**Movie S8.** Effect of the PI 3-kinase inhibitor wortmannin (WM) on mRFP–PHFAPP1 and YFP–2 × FYVE labelling over 45 min.

**Movie S9.** Dynamics of PtdIns3P and PtdIns4P during cell division in BY-2 cells.

**Movie S10.** Dynamics of PtdIns4P and FM-64-labelled membranes during cell division in BY-2 cells.

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