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Lipid Metabolism, Compartmentalization and Signalling in the Regulation of Pollen Tube Growth

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Abstract To understand the biological context of lipid metabolism and signalling in pollen, we have to consider male gametophytes as organisms optimised for their role in sexual reproduction, but also for survival in dry conditions. While our knowledge of molecular mechanisms governing pollen development and pollen tube growth is based on the studies of a few model species (mostly Arabidopsis, tobacco, petunia and lily), important aspects of pollen development may vary substantially among species. Moreover, current understanding of pollen lipid biochemistry is rather fragmentary, since biochemically tractable amounts of pollen material are difficult to obtain, and knowledge of sporophytic lipid metabolism and signalling cannot be simply transferred to the study of male gametophytes.

1 Introduction

Lipid signalling is crucial for the organization of membrane domains and membrane trafficking. Conserved lipid-binding domains, such as PH, FYVE, PX, PHD, ENTH, and C2 target relevant proteins towards specific lipid-enriched membrane domains (van Leeuwen et al. 2004). Phosphorylated derivatives of the phospholipid phosphatidylinositol (phosphoinositides) translate the complex "language" of lipid signalling into membrane traffic (Czech 2003; Downes et al. 2005; Roth 2004), assembly of specific membrane domains (Carlton and Cullen 2005), and signal-triggered reorganization of the actin cytoskeleton (Huang et al. 2003; Yin and Janmey 2003; Downes et al. 2005). These interactions modulate activities of actin-binding proteins (Huang et al. 2003), which have an immediate impact on the actin cytoskeleton (Downes et al. 2005; Yin and Janmey 2003). Pollen tubes provide an excellent model object for the study of these aspects, since their extremely polarized tip growth requires a complex molecular machinery integrating

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both the secretory pathway and dynamic actin structures regulated by lipid signalling.

2
Pollen-specific Features of Lipid Metabolism

2.1
Pollen Tubes Use Aerobic Ethanolic Fermentation to Support Lipid Biosynthesis

Pollen tubes grown aerobically in vitro in sucrose media with extremely high respiratory rate produce copious amounts of ethanol (Bucher et al. 1995). It has been suggested that this energy metabolism pathway may provide a competitive advantage in areas of low oxygen pressure within the style (Gass et al. 2005).

More than half of the carbohydrate flows through the fermentative pathway; the flux towards ethanol is regulated by sugar availability rather than by oxygen (Tadege et al. 1999). Products of aerobic fermentation – acetaldehyde and ethanol – are further metabolised in a pathway that involves pyruvate decarboxylase (PDC), alcoholdehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and acetyl-CoA synthetase (ACS), but bypasses mitochondrial pyruvate dehydrogenase, similar to situation in yeast (Tadege et al. 1999; Mellema et al. 2002). PDC and ADH are among the major pollen proteins. Interestingly, maize mutants lacking ADH produce viable pollen, since ADH+/adh– heterozygotes segregate in a Mendelian fashion (Freeling and Bennett 1985), while a petunia mutant impaired in the pollen-specific PDC2 locus produces pollen unable to outcompete wild-type pollen in mixed pollination/fertilization experiments. The genetically proven non-essentiality of ADH suggests that this enzyme may act mainly to prevent accidental accumulation of reactive acetaldehyde during pollen tube growth, while the main flux leads via PDC and ALDH towards high molecular weight compounds, especially lipids (Gass et al. 2005).

2.2
Triacylglycerols, Surface/Exine Lipids and Oleosins in Pollen-pistil Recognition

The variability of Angiosperm species with respect to the contents of storage compounds (starch or lipids) in mature, post-anthesis pollen grains have been known since the 1950s (Baker and Baker 1979). Prominent lipid accumulation in mature pollen correlates with insect pollination, while starch-accumulating species are often pollinated by wind. Major interspecific diversity with respect to metabolic flux directions and timing has to be therefore expected. However, it is reasonable to assume that this diversity will affect mostly triacylglycerol storage and mobilization, rather than membrane lipid metabolism, transport and signalling.

Intracellular triacylglycerols are stored in oil bodies, in complex with phospholipids and amphipatic structural proteins – oleosins. Oleosins form large gene families, with 16 different proteins in *Arabidopsis*. Apart from oleosins expressed in pollen grains (Kim et al. 2002), there are paralogues expressed in tapetal cells and involved in oil bodies development during tapetum maturation and disintegration. During anther dehiscence, these proteins accumulate inside cavities of the exine; at pollination, they are released to the stigma surface. In *Arabidopsis*, oleosins and lipases comprise more than 90% of pollen coat proteins, suggesting their crucial function in pollen-stigma recognition interaction (Mayfield et al. 2001). Rapid evolution of pollen oleosins in *Arabidopsis* and closely related species further supports this hypothesis (Schein et al. 2004).

The functional importance of surface pollen lipids was first documented by conditional pollen sterility of cuticle-deficient Arabidopsis *cer6* mutant that may be rescued by high humidity (Preuss et al. 1993). Mutant pollen lacks very-long-chain fatty acids (VLCFA); cloning of the *CER6/CUT1* locus showed that the mutated protein resembles fatty acid-condensing enzymes (Fiebig et al. 2000). Disruption of the locus CER10 encoding enoyl-CoA reductase also compromises VLCFA synthesis, resulting in a general cell expansion and endocytosis defect (Zheng et al. 2005). VLCFA are also essential components of the stigmatic surface needed for the pollen tube penetration (Wolters-Arts et al. 1998).

3
Membrane Lipids and Phospholipids in Pollen Tubes

3.1
Membrane Lipid Metabolism

There are very few biochemical studies addressing specifically membrane lipid metabolism in pollen tubes. In the first detailed analysis of the dynamics of phospholipid metabolism in elongating lily pollen tubes, Helsper et al. (1986b) observed considerable incorporation of [^{32}P] orthophosphate into phospholipids, mainly phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylinositol monophosphate (PIP), phosphatidylglycerol (PG) and phosphatidic acid (PA). As the total amount of lipid-bound phosphorus did not change during germination, continuous incorporation of labelled phosphorus indicated high phospholipid turnover. Radioactively marked *myo*-inositol also exhibited high turnover; it was incorporated not only into phospholipids, but also in pectic polysaccharides. The dynamics of phospholipids was shown to be linked to the activity of lipases. Activities of phospholipase C (PLC) and phospholipase D (PLD) in pollen tubes and the presence of their products diacylglycerol (DAG), Ins(1,4,5)P_3

and PA have been documented (Helsper et al. 1986b, 1987). Dorne et al. (1988) found that in *Nicotiana sylvestris* the total amount of lipids does not increase during pollen germination and tube growth, despite membrane surface expansion and continuous incorporation of radioactively labelled acetate into all lipid classes (dominated by neutral lipids – triacyl glycerols and sterols). In fact, the total phospholipid content decreased after an initial increase preceding germination. All these approaches suggest a rapid turnover of membrane components in growing pollen tubes.

3.2 Phospholipid Signalling and Metabolism – Phospholipase C and Inositol Trisphosphate

Polyphosphate inositol in the form of phytic acid was recognized as an important phosphate-rich storage compound accumulated in the mature pollen (Jackson and Lisnkens 1982). Molecular analysis of *Arabidopsis* inositol polyphosphate kinases (IPK) uncovered two genes known as AtIPK2α and β lacking, in contrast to some animal orthologues, the CAM binding domain implying Ca^{2+}-independent regulation (Xu et al. 2005). Antisense transgenic plants with reduced amounts of AtIPK2α transcripts showed increased pollen germination and pollen tube growth as compared to the wild type, especially at suboptimal Ca^{2+} concentrations; other phenotypes of these plants suggest an important role of inositol polyphosphate metabolism in Ca^{2+} signalling, possibly via modification of inositol trisphosphate activity (Xu et al. 2005). Alkaline phytase, catalysing hydrolysis of phytic acid and activated by Ca^{2+}, was recently characterized from pollen (Jog et al. 2005).

Based on the known central role of Ca^{2+} tip-focused gradient in pollen tube growth, as well as knowledge of Ca^{2+}-related PI signalling from animal systems, early work in membrane-related pollen signalling was centered on the expected role of PLC activity in phosphatidylinositol 4,5 bisphosphate (PIP_2) cleavage and DAG and Ins(1,4,5)P_3 production. Franklin-Tong et al. (1996) showed that the Ca^{2+} dynamics is correlated with Ins(1,4,5)P_3 in poppy pollen tubes. However, in this particular study extraordinary high concentrations of mastoparan (25 μM) were used to stimulate Ins(1,4,5)P_3 production. Our unpublished data (Zarsky and Obermeyer) show that at this concentration mastoparan causes membrane permeabilization, making the interpretation of data very difficult. Further experiments with caged Ins(1,4,5)P_3 and other drugs (Franklin-Tong et al. 1996; Malhó 1998) showed clearly that Ins(1,4,5)P_3 is involved in $[Ca^{2+}]_c$ dynamics and appears to participate in the reorientation of tube growth.

Small GTPases are well-known regulators of cell polarity; members of the Rop family are localized to the growing pollen tube tip and participate in the regulation of the Ca^{2+} gradient (Li et al. 1999; Yang and Cheung, this volume). Furthermore, they were shown to interact at the pollen tube tip

with the phosphatidylinositol monophosphate kinase (PI-PK) whose product, PIP_2, accumulates in the Rop-containing tip-focused membrane domain (Kost et al. 1999). Overexpression of the PIP_2-binding domain of PLC inhibited, as predicted, pollen tube elongation. Rop GTPases may control local activity of PI-PK in the tip of growing pollen tubes, and PIP_2 might serve as a substrate for the PLC producing Ins(1,4,5)P_3. The abundant monomeric G-actin-binding protein profilin may also be involved in the PIP_2-dependent regulation of actin cytoskeleton and signalling activities, including those mediated by the Ca^{2+} gradient (Clarke et al. 1998). There are two functionally distinct classes of profilin isoforms in plants, differing in their affinity for PIP_2 (Yokota and Shimmen, this volume). Von Witsch et al. (1998) observed membrane localization of profilin in microspores and pollen. The role of PIP_2 was further re-examined by Monteiro et al. (2005) who showed that photorelease of both caged PIP_2 and Ins(1,4,5)P_3 modified growth and caused reorientation of the growth axis. However, measurements of cytosolic free calcium ($[Ca^{2+}]_c$) and apical secretion revealed significant differences between effects of PIP_2 or Ins(1,4,5)P_3. Release of PIP_2 led to transient growth arrest, increase of cytosolic Ca^{2+}, and inhibition of apical secretion. By contrast, a concentration of Ins(1,4,5)P_3 which caused a $[Ca^{2+}]_c$ transient of similar magnitude, stimulated apical secretion and caused severe growth perturbation. Therefore, there may be different targets of these two signalling intermediates.

The *Arabidopsis* genome encodes nine PI-PLC isoforms, with three of the proteins being most probably inactive. Plant PI-PLCs do not have PH domain, similar to animal PI-PLCζ (Hunt et al. 2004). Partially purified PI-PLCs from different plant species are Ca^{2+}-dependent in their activities; e.g. AtPLC2 preferentially hydrolyses PIP_2 and maximal activity in vitro is achieved at 1 μM $[Ca^{2+}]_c$ (Otterhag et al. 2001). Systematic analysis of expression and Ca^{2+} sensitivity of Arabidopsis PI-PLC isoforms revealed overlapping but specific expression and Ca^{2+} optimum ranging from 1 μM for AtPLC2,4 and 5 to 3 μM for AtPLC1 and 3. AtPLC4 is highly expressed in mature and developing Arabidopsis pollen (Hunt et al. 2004). This particular PLC isoform was the only one that retained in vitro activity (20%) even in the presence of 2 mM EGTA (Hunt et al. 2004). High levels of AtPLC4 accumulation in mature dehydrated pollen may assist early Ins(1,4,5)P_3-induced Ca^{2+} release during pollen germination. Tip-localization of AtPLC4-GUS fusion in growing pollen tubes implies function in tip growth and fertilization (Hunt et al. 2004). Since animal spermatic PLC triggers $[Ca^{2+}]_c$ oscillations within mouse egg, leading to egg activation (Saunders et al. 2002); it is tempting to speculate that similar function might be linked to pollen PI-PLC during final stages of embryosac double-fertilization (Hunt et al. 2004). Pan et al. (2005) described PI-PLC activity and cloned two PLC isoforms, LdPLC1 and LdPLC2, from *Lillium daviddi* pollen. PI-PLC activity in pollen protoplasts was enhanced by exogenous calmodulin and G protein agonist cholera toxin, and

decreased by G protein antagonist, pertussis toxin. Application of the PI-PLC inhibitor U-73122 abolished the cholera toxin-mediated stimulation of PLC activity and led to the decrease of $[Ca^{2+}]_c$ in pollen grains, further indicating the presence of PIP_2-PLC- Ins(1,4,5)P_3-Ca^{2+} cascade in pollen.

3.3 Phospholipid Signalling – Phospholipase D and Phosphatidic Acid

Alongside with phosphoinositides, other (phospho)lipids such as PA have been shown to control cell polarity and vesicular trafficking in yeast and animal cells (for plants see Testerink and Munnik 2005). PLD activity and accumulation of its product PA was observed already in the early pollen phospholipids analyses (Dorne et al. 1988; Helsper et al. 1986b). Although PLDs activities in vegetative plant cells in respect to stress signalling and adaptation have been intensively studied (see e.g., Testerink and Munnik 2005), less attention has been paid to their role in housekeeping cellular functions. The analysis of PLD functions in plants is complicated by the overlapping activities of different PLD isoforms in the same cell (for plant PLD family phylogenetic analysis see Elias et al. 2002). PLDζ1 was localized to the clear zone vesicles of root hairs; its inducible overexpression caused branching of root hairs while PLD inhibition resulted in loss of root hairs (Ohashi et al. 2003). This reveals that PLD and PA are essential signalling molecules driving tip-growth of root hairs, suggesting their role in other tip-growing cells as well.

PA can be produced in plant cells either by cleavage of PIP_2 by PLC followed by phosphorylation of DAG by DAG kinase (connecting thus phosphoinositide and PA signalling), or directly by PLD-mediated hydrolysis of structural phospholipids such as PC and PE. During hydrolysis, PLD covalently binds to the phosphatidyl group and then transfers it to a nucleophile. The nucleophile is usually water, but in the presence of 1-butanol, stable and biologically inactive phosphatidylbutanol is formed preferentially. Thus, incubation of plant cells in 0.2% to 0.5% butanol inhibits PA production. Using this approach, Potocky et al. (2003) showed that PLD-mediated production of PA is crucial for the tip growth of tobacco pollen tubes. PLD inhibition in vivo by 0.25% 1-butanol led to rapid arrest of pollen tube growth, whereas application of PA-containing liposomes increased the growth rates. The dynamics of BODIPY-labelled PA internalization and pollen tube endomembrane localization was followed (Fig. 1). Distinct PLD activities with specific PIP_2 and Ca^{2+} requirements and with different dynamics were detected during pollen germination and tube elongation suggesting multiple roles of PA in signalling and membrane domain formation (Potocky et al. 2003). Pollen tube growth inhibition caused by 1-butanol was partly relieved by taxol, supporting the hypothesis that some plant PLDs may function as a MAP in the regulation of MT dynamics (Gardiner et al. 2001; Potocky et al. 2003). PLD inhibition rapidly disturbs the dynamics of pollen tube endomembrane system,

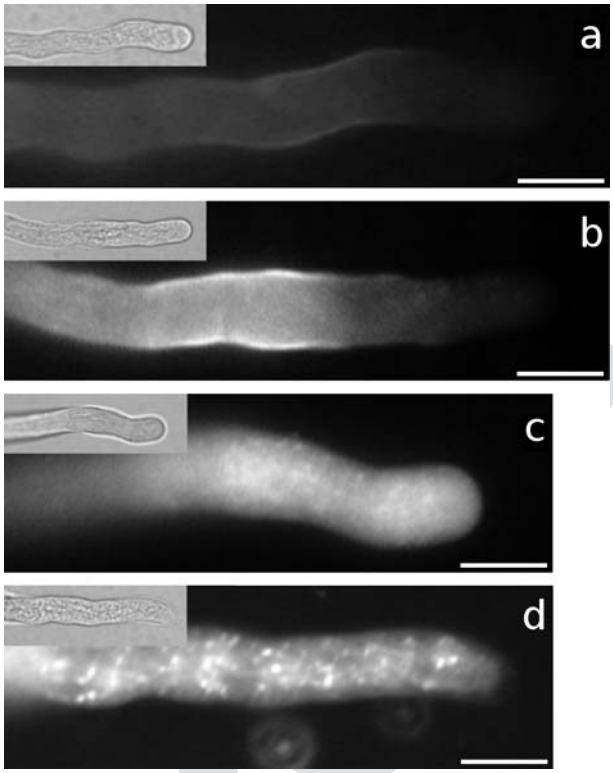

Fig. 1 Cellular distribution of the BODIPY-PA in growing pollen tubes of tobacco. Incorporation of 0.5 [TS d] M BODIPY-PA after 5 min (**a**), 30 min (**b**), 60 min (**c**), and 90 min (**d**) is shown. Bar = 10 mm. Adapted from Potocky et al. 2003

as visualised by FM1-43 dye. Recovery of pollen tube elongation by external addition of PA liposomes is correlated also with the recovery of tip membrane dynamics (Potocky et al. 2004). A similar disturbance of tip localized secretory machinery by 1-ButOH treatment was observed also in *Agapanthus umbellatus* pollen tubes, resulting in pollen tube growth arrest, disappearance of tip-focused calcium gradient, profound rearrangement (bundling) of F-actin microfilaments, apex expansion and disappearance of secretory vesicles (Monteiro et al. 2005). Although we have repeatedly observed fragmentation of long F-actin filaments under comparable conditions (Potocky et al., in preparation), data of both groups point to the profound effect of PLD activity on the pollen tube cytoskeleton.

Our understanding of the regulatory function of PA in the plant cell F-actin dynamics was recently augmented by the finding that PA added to *Arabidopsis* cell suspension cultures as well as to poppy pollen leads to a significant increase in F-actin levels, a process mediated by the heterodimeric capping protein from *Arabidopsis* (AtCP; Chris Staiger, pers. comm). AtCP binds not

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only to PA, but also to PIP_2 with a similar affinity. However, the intracellular concentration of PA is orders of magnitude higher, making it the major binding partner of AtCP. Interaction of PA with AtCP inhibits the actin-binding activity of AtCP, rendering it unable to block the barbed end of actin filaments allowing rapid filament assembly from an actin monomer pool that is buffered with profilin (Chris Staiger, pers. comm).

Stress-induced activation of multiple phospholipid pathways was also documented in growing pollen tubes (Zonia and Munnik 2004). Although PLD activity is constitutive during tube growth, hypo-osmotic treatment rapidly increases PLD-derived PA content and hyper-osmotic stress is characterised by elevated levels of phosphatidylinositol bisphosphates. Specific patterns of membrane modifications in response to osmotic stress were recognized – hypoosmotic treatment stimulated pollen tube expansion assisted by the stimulation of PLD activity and 7-fold accumulation of PA. In contrast, hyperosmotic treatment (50 to 400 mM NaCl) caused pollen tube shrinking, inhibited PLD activity, reduced PA levels and induced an increase in PIP_2 isomers (Zonia and Munnik 2004). This work also indicates that the majority of PA in pollen tubes is produced via PLD pathway and not PLC/DAG kinase pathway. Interpretation of the data with hyperosmotic treatment in this study is unfortunately compromised by the fact that NaCl at the concentration used exhibits general toxic effects (like KCl and/or LiCl). Inhibition of pollen tube growth imposed by 100 mM NaCl can be completely restored by the addition of 40 mM $CaCl_2$ – i.e., even further increase in osmotic potential of the in vitro growth medium (Brink 1924). Further analysis of this topic is thus required.

Despite the developmental dehydration before anthesis, the possible role of ABA signalling in pollen development is unclear (e.g., Rose et al. 1996). *Arabidopsis* transcriptome analysis suggests two ABA related genes relatively overexpressed in pollen (Pina et al. 2005) and the ABI1 PP2C protein phosphatase, a negative regulator of ABA response, was recently discovered as a binding partner of PA. Three closely related PP2C isoforms are expressed in tricellular and mature *Arabidopsis* pollen, including ABI1 mRNA itself (At3g11410; At1g72770; At4g26080). It is therefore highly probable that PA in developing pollen might intervene also in putative signalling pathways regulated by PP2C phosphatases activities (Zhang et al. 2005).

4
Lipid Signalling in the Cellular and Whole-plant Context

4.1
Expression Analysis of the Phosphoinositide Pathway in Pollen

Recent studies reported unique characteristics of the pollen transcriptome emphasizing a functional skew of pollen transcriptome toward vesicle traf-

ficking, cytoskeleton and signalling (Twell, Oh and Honys, this volume). Using publicly available expression data, we attempted a more detailed analysis of the expression of genes putatively involved in generation and transduction of PPI signalling. We selected 176 genes, from which 158 were found on Affymetrix ATH1 chip. These included phospholipases, PI and PIP kinases, phosphoinositide phosphatases, inositol polyphosphate kinases and phosphatases together with proteins containing known phosphoinositide binding modules such as PH, PX, FYVE and ENTH domains (Fig. 2, Table 1, see also http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). Interestingly, 89 genes (58.4%) were called "present in the pollen", which is nearly twice as much compared to whole pollen transcriptome, where only 29% of genes are called present in pollen (Pina et al. 2005). Strong presence of PPI signalling related genes in pollen is also evident from the number of pollen-exclusive and pollen-enriched genes (7.0% and 26.8% respectively). In comparison, there are no PPI related genes selective for leaves and only 1–3.4% are enriched in this tissue (depending on the dataset). Same conclusion can be drawn from roots (data not shown). Overall analysis also indicates that PPI signalling is equally important in all stages of pollen development, as from 116 PPI genes called present during pollen development, 45 are expressed preferentially during unicellular and bicellular stage and 54 genes show maximum expression in tricellular stage and in mature pollen.

If we look closely at specific gene families, the most striking feature is the absence of Vps34 transcript, coding for type III PI3-kinase (Fig. 3). Vps34 is the only PI3-kinase identified so far in *Arabidopsis*. Although Vps34 is absent in pollen, other components of PI3P signalling such as FYVE domain and PX domain containing proteins and Fab1 (PI3P-5 kinase) orthologues are present in pollen (see below) and PI(3,5)P_2 can be detected during growth of tobacco pollen tubes (Zonia and Munnik 2004). It is difficult to accept absence or substantial down-regulation of PI3P in developing pollen and pollen tubes, as it seems to be an important specific component of plant endosomes (Samaj et al. 2005). The genes putatively involved in PIP_2 pathway have generally the strongest signal in pollen (Fig. 2) and often contain novel sequence features, further pointing to the central role of this phosphoinositide in pollen.

Both type II and type III PI4 kinases are expressed in pollen with type II being the dominant subfamily, including some of the most expressed pollen loci (PI4Kγ3, PI4Kγ4, PI4Kγ8, Fig. 2). Type II PI4 kinases, albeit yet not functionally characterised in plants, are supposed to be insensitive to wortmannin, well-known inihibitor of PI3- and type III PI4-kinases. The expression profile of PI-kinase isotypes yielded conclusions similar to that of PI-4 kinases; both PIP kinases synthetising PIP_2, PI(3,4)P_2 (type I/II), and PI(3,5)P_2 (type III) are expressed in pollen. Again, the pollen-specific and most expressed isoform PIPK10 belongs to plant specific subfamily A, and

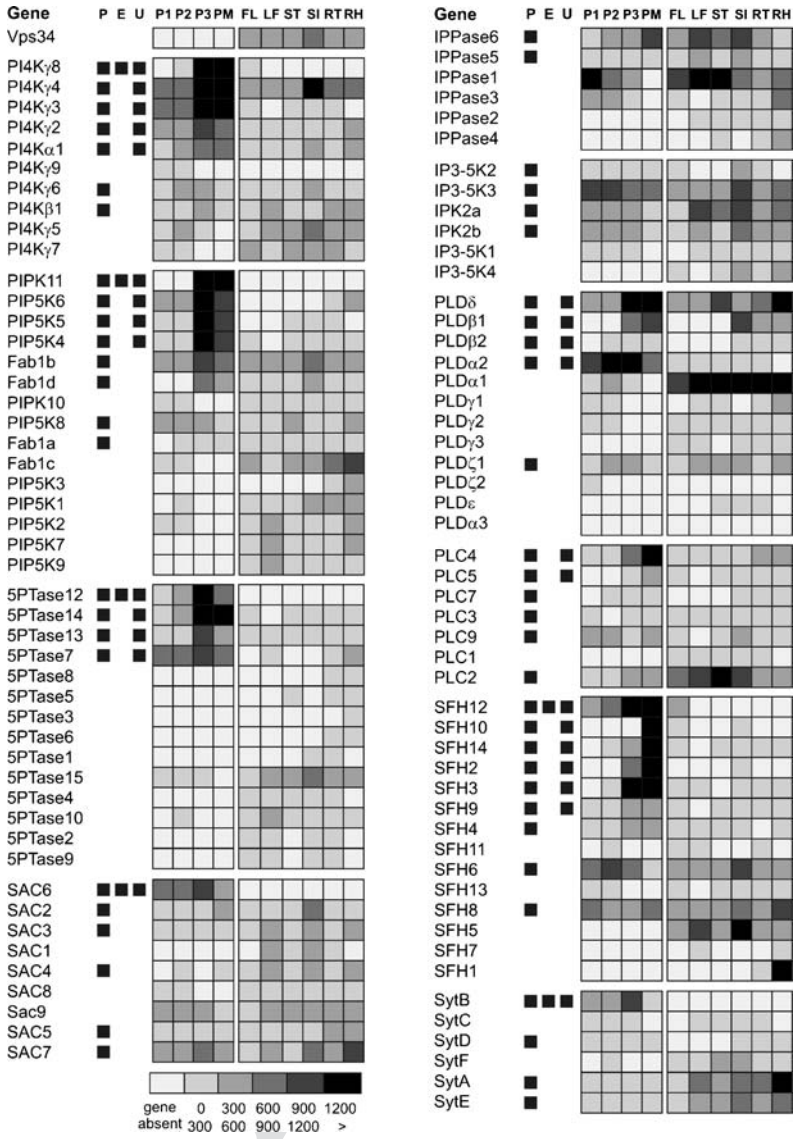

Fig. 2 Expression analysis of selected phosphoinositide pathway genes from Arabidopsis. Expression data for haploid male gametophyte development was extracted from the works of Honys and Twell (2005) together with selected data from sporophytic tissues that have been subject to normalization and statistical analysis. Also selected data from Pina et al. (2005) and AtGenExpress database (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) were renormalized and included into analysis. For additional information, see (http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). P, gene present in pollen; E, gene exclusive in pollen; U, gene upregulated in pollen; P1, uninucleate microspores; P2, bicellular pollen; P3, immature tricellular pollen; PM, mature pollen grains; FL, flowers; LF, leaves; ST, stems; SI, siliques; RT, roots; RH, root hairs

Table 1 Expression analysis of *Arabidopsis* genes with phosphoinositide binding domains. Expression data for haploid male gametophyte development was extracted from the works of Honys and Twell (2004) together with selected data from sporophytic tissues which has been subject to normalization and statistical analysis. Also selected data from Pina et al. (2005) and the AtGenExpress database (http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) were renormalized and included into analysis. For additional information, see (http://home.ueb.cas.cz/potocky/pollen_lipid_signalling.htm). P, gene present in pollen; E, gene exclusive in pollen; U, gene upregulated in pollen; S, expression signal in pollen; FC, fold change

Gene	AGI code	P	E	U	S	FC	Gene	AGI code	P	E	U	S	FC
PH domain containing proteins													
ArfGap1	At1g1087	–	–	–	–	–	PLDzeta2	At3g0563	–	–	–	–	–
ArfGap2	At1g6086	*	–	–	147	–	PH8	At1g7320	*	–	–	219	–
ArfGap3	At5g6198	*	–	*	729	14.1	Praf1	At1g6592	–	–	–	–	–
ArfGap4	At5g1330	–	–	–	–	–	Praf2	At3g4766	*	*	*	346	6.9
ADL6	At1g1029	*	–	–	241	–	Praf4	At1g7695	–	–	–	–	–
ADL3	At1g5961	*	–	–	267	–	Praf5	At5g4214	–	–	–	–	–
PH2	At2g2970	*	–	–	154	–	Praf6	At3g2327	*		*	339	16.0
PH3	At5g0571	*	–	–	298	–	Praf7	At4g1437	*	*	*	141	9.6
PH4	At1g7773	*	*	*	637	19.7	Praf8	At5g1235	–	–	–	–	–
OBP1	At2g3103	–	–	–	–	–	Praf9	At5g1942	–	–	–	–	–
OBP2	At4g0818	*	–	*	598	4.2	PH9	At4g3278	–	–	–	–	–
OBP3	At1g1317	–	–	–	–	–	PH10	At3g6330	–	–	–	–	–
OBP4	At4g1246	–	–	–	–	–	PH11	At5g4387	*	–	–	131	
OBP5	At4g2254	*	–	–	169	–	PH12	At3g2281	–	–	–	–	–
START1	At3g5480	*	–	*	10354	107	PH14	At4g1667	–	–	–	–	–
START2	At4g1904	*	–	*	934	2.8	PH15	At4g1735	*	–	–	181	–
START3	At5g4556	–	–	–	–	–	PH16	At5g4744	–	–	–	–	–
START4	At5g3518	*	–	–	609	–	PDK1–1	At3g1054	*	–	*	811	5.8
START5	At2g2832	*	–	*	866	3.0	PDK1–2	At5g0451	–	–	–	–	–
RhoGAP1	At4g2458	*	–	*	826	13.9	Vps34	At1g6049	–	–	–	–	–
RhoGAP2	At5g1215	*	–	–	124	–	PI4Ka1	At1g4934	*		*	621	2.8
PH5	At1g4809	*	–	–	355	–	PI4Kb1	At5g6407	*	–	–	238	–
PLDzeta1	At3g1678	*	–	–	240	–	–	–	–	–	–	–	–
FYVE domain containing proteins													
Praf1	At1g6592	–	–	–	–	–	Praf9	At5g1942	–	–	–	–	–
Praf2	At3g4766	*	*	*	346	6.9	Fab1a	At4g3324	*	–	–	233	–
Praf4	At1g7695	–	–	–	–	–	Fab1b	At3g1427	*	–	–	868	–
Praf5	At5g4214	–	–	–	–	–	Fyve1	At1g2011	*	–	–	558	–
Praf6	At3g2327	*		*	339	16.0	Fyve2	At3g4323	*	–	–	343	–
Praf7	At4g1437	*	*	*	141	9.6	Fyve4	At1g6169	*	–	–	172	–
Praf8	At5g1235	–	–	–	–	–	RingFyve	At1g6162	*	–	–	547	–
PX domain containing proteins													
PX-PXA1	At2g1590	–	–	–	–	–	PX6	At5g5844	*	–	–	92	–
PX3	At2g2535	–	–	–	–	–	PLDzeta1	At3g1678	*	–	–	240	–
PX-SPEC	At4g3216	*	–	–	350	–	PLDzeta2	At3g0563	–	–	–	–	–
PX4	At5g0614	–	–	–	–	–	–	–	–	–	–	–	–

Table 1 (continued)

Gene	AGI code	P	E	U	S	FC		Gene	AGI code	P	E	U	S	FC	
ENTH domain containing proteins															
ENTH1	At2g4316	*	–	–	234	–	–	ENTH4	At1g0867	–	–	–	–	–	
ENTH2	At3g5929	*	*	*	1937	15.5	–	ENTH5	At3g4654	–	–	–	–	–	
ENTH3	At5g1171	*	–	–	400	–	–	ENTH6	At3g2335	*	*	*	159	5.0	

bears unique sequence motifs. Also other pollen-enriched isoforms, PIPK4-6, form a specific cluster in the phylogeny of type B PIP kinases (Mueller-Roeber and Pical 2002), further indicating pollen-specific regulation of PIP_2 synthesis. As noted previously, despite the absence of PI3-kinase, 3 of 4 Fab1 genes, coding for putative PI3P-5 kinase, are expressed in pollen. PITPs (Sec14p nodulin domain phosphatidylinositol transfer proteins) are characterised by their ability to transfer PI or PC monomers between membrane bilayers in vitro. PITP dependence has been observed in reconstitutions of constitutive exocytosis, regulated exocytosis, intra-Golgi membrane trafficking, and plasma membrane signalling, suggesting that PITPs play important roles in regulating PPI production in vivo (Routt and Bankaitis 2004). Vincent et al. (2005) recently showed that AtSfh1, member of plant PITP family, controls PIP_2 synthesis and regulates cytoskeleton in root hairs, demonstrating the necessity of PITPs for tip growth.

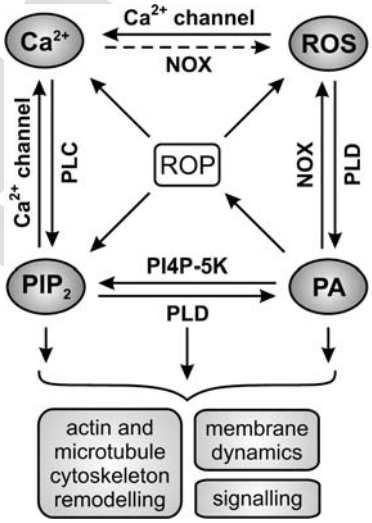

Fig. 3 Hypothetical scheme depicting suggesting central role of PIP_2/PA tandem in regulation of tip growth in pollen tubes

PPI degradation genes exhibit trends similar to the PIP_2 synthesis pathway. From 15 genes coding for inositol polyphosphate 5-phosphatases (5PTases), 4 loci are expressed and upregulated in pollen. Three of these genes, 5PTase12, 5PTase13 and 5PTase14, belong to type II subfamily of 5PTases, which in yeast and animals have been known to regulate vesicle trafficking and actin organization (Suchy and Nussbaum 2002). 5PTase14, the most abundant pollen isoform, has highest substrate specificity to PIP_2, while 5PTase12 and 5PTase13 hydrolyse only Ins(1,4,5)P_3 (Zhong and Ye 2004). The remaining member of plant type II 5PTase family, 5PTase15, has been demonstrated recently to be the FRA3 gene, which controls actin organisation and secondary cell wall synthesis in fiber cells (Zhong et al. 2004), hence analogous functions can be hypothesised for pollen isoforms. Actin disorganisation was also shown in *fra7* mutant, coding for SAC domain containing PPI phosphatase that preferentially cleaves membrane PPIs (Zhong et al. 2005).

Expression data indicate that PIP_2-regulated PLDs, particularly PLDδ and PLDβ1 are involved in pollen PPI signalling (Fig. 2). PLDβ1 interacts with the actin cytoskeleton (Kusner et al. 2003) and PLDδ is connected to ROS-mediated stress signalling in vegetative cells (Zhang et al. 2003) and our data suggest that these isoforms fulfil similar roles in pollen tubes (Potocky et al., unpublished data).

Over the past ten years, several PPI-binding modules have been recognised: pleckstrin homology (PH) domains, which may bind broad spectrum of PPIs such as PI(4,5)P_2, PI(3,5)P_2, PI(3,4)P_2, PI3P, PI4P, PI5P and other acidic phospholipids such as PA with various specificity and affinity (Yu et al. 2004), FYVE domains, which are specific towards PI3P, PX domains, which are mostly specific to PI3P, and ENTH domains, which are mostly specific for PI(4,5)P_2 (Lemmon 2003). From 53 *Arabidopsis* PH domain-containing proteins, 26 are present in pollen transcriptome, covering all 11 subfamilies (van Leeuwen et al. 2004). 12 genes from 7 subfamilies are upregulated in pollen, including regulators of small GTPases (ArfGAP3, RhoGap1, putative RabGEFs Praf2, Praf6, Praf7, also containing FYVE domain), putative oxysterol-binding proteins (OBP2) and proteins bearing the PH domain and other putative lipid-binding module, START domain (START1, START2 and START5). Interestingly, protein kinase PDK1-1, a PH domain containing protein, which is specifically activated upon PA binding (Anthony et al. 2004) is also upregulated in pollen, whereas both its known target AGC2-1 and its interaction partner AGC1-1are absent from pollen. Similarly, 4 out of 6 ENTH domain are called "present in pollen" with 2 pollen selective genes (Table 1). On the other hand, none of the PX domain and FYVE domain proteins is selective or enriched in pollen (with the exception of Praf proteins, which bear also PH domain, see above), providing another indirect clue to PIP_2 as central phosphoinositide in pollen.

4.2
PTEN-like Domains as Possible Targets of Lipid Signalling

Proteins of the PTEN superfamily are emerging as candidates for a link between signalling lipids, in particular PI(3,4,5)P_3(PIP_3), and downstream effectors. The PTEN molecule exhibits structural similarity to dual specificity protein phosphatases, contains the protein phosphatase active site signature, HCXXGXXR, and possesses a phosphatase activity towards both lipids and proteins. However, its protein tyrosine phosphatase activity is rather weak, compared to the strong affinity towards PIP_3 (Li and Sun 1997).

Gupta et al. (2002) identified three PTEN homologues in the *Arabidopsis* genome and demonstrated that one of them, AtPTEN1 (At5g39400), exhibits a lipid- and protein-phosphatase activity similar to the mammalian prototype. The failure to isolate T-DNA insertion mutants in AtPTEN1 suggested that the gene might be essential, which led the authors to construct "knock-down" *Arabidopsis* lines using the RNAi technology. They found that AtPTEN1 depletion results in loss of pollen viability, which is consistent with the observed pollen-specific activity of the AtPTEN1 promoter. The affected pollen grains revealed cell surface defects, namely local separation of plasma membrane from the intine, as well as both intine and exine lesions, again supporting possible role of PTEN in development of surface structures of the pollen grain. Interestingly, the AtPTEN proteins (and their relatives from other species) are not the only representatives of the PTEN superfamily in the plant kingdom. A divergent PTEN-related domain is present in the variable N-terminal extension of many plant formins (Cvrčkova et al. 2004b), i.e., members of the formin (FH2 protein) family.

Higher plants have a prolific family of FH proteins (encoded by 21 loci in *Arabidopsis*). Plant formins can be divided into two distinct subfamilies (classes) based on the sequence of the conserved FH2 domain (Cvrčkova et al. 2004a; Deeks et al. 2002). Presence of PTEN-like domains is characteristic for Class II plant formins, while most Class I proteins carry a N-terminal membrane-insertion signal (Cvrčkova 2000), suggesting a possible plant-specific mechanism of cytoskeleton-membrane connection. No functional studies on Class II formins have been reported so far. It is not even known yet whether Class I and Class II FH2 proteins can form mixed heterodimers, which would greatly increase the diversity of formin complexes and their regulation. In *Arabidopsis* pollen, only three Class II formins (AtFH13, AtFH14 and AtFH17) are expressed to a significant extent (data from Honys and Twell 2004 and Pina et al. 2005); two of them, AtFH13 and AtFH14, possess the PTEN-related domain.

The only available knowledge on the PTEN-like domain present in many Class II formins results from bioinformatic analyses of a collection of the four domains of *Arabidopsis* formins AtFH13, AtFH14, AtFH18 and AtFH20, as well as related sequences from rice and *Medicago truncatula* (Cvrčkova

et al. 2004a). Surprisingly, all these formin-associated PTEN domains contain mutations that make catalytic activity as either lipid or protein phosphatase extremely unlikely. A crucial arginine residue in the phosphatase active site is replaced by hydrophobic or small polar residues in the plant proteins, and a conserved and functionally important asparagine is substituted by glycine. We therefore believe that the function of plant PTEN domains is rather structural than catalytic – perhaps analogous to the role of the transmembrane segments in Class I formins. A variant PTEN domain that lacks catalytic activity but retains its intracellular phospholipids-dependent localization ability might contribute to intracellular positioning of the actin-organizing FH2 domains. Such a structural role could perhaps be attributed to the C-terminal portion of the conserved PTEN core, which is related to a class of domains collectively referred to as C2; curiously, this portion appears to be lost in the AtPTEN proteins, while it has been retained in the formin-associated PTEN domains, albeit in a highly diverged form (Cvrčkova et al. 2004a; Gupta et al. 2002). The C2 domains found in PTEN-like part of plant formins resemble the C2 domain of human PTEN, including the residues that make PTEN Ca^{2+} independent.

5 Perspectives

There are two major aspects of phosphoinositides function in eukaryotic cells – as signalling molecules and as localization cues, enabling the recruitment of phosphoinositide-binding proteins to specific phosphoinositide-containing membranes or membrane domains (Carlton and Cullen 2005). In pollen tubes these two aspects are likely to relate. A dynamic gradient in plasmalemma lipid composition from the growing tip to less active sub-apical, non-growing regions can be expected. The paradigm for secretory pathway and tip growth in plants claims that Golgi-produced secretory vesicles are vectorially transported, along F-actin cables, towards the growing cell walls or tips where they accomplish local exocytosis. Fusing vesicles deliver both new cell wall material and membranes for extending plasma membrane. Endocytosis is implicated in this classical model to remove the excess of the inserted membranes. However, recent data revealed new aspects that urge a revision of this model. In vivo observations of the vesicle-rich apical zone in both root hairs and pollen tubes revealed large amounts of vesicles that move chaotically throughout this clear zone and which only seldom accomplish complete fusion with the apical plasma membrane (Ovecka et al. 2005). Surprisingly, in both pollen tubes and root hairs exposed to endocytic tracers FM1-43 and FM4-64 all secretory vesicles of the clear zone rapidly become labelled; the internalization – i.e., endocytosis – of tracers is very rapid pointing to the overlap between pollen tube exocytosis and endocytosis (Ovecka et al. 2005;

Samaj et al. 2005). Similar data have been obtained with the recycling endosome marker GFP-RabA/Rab11 which accumulated within the clear zone of tip-growing pollen tubes (de Graaf et al. 2005). Endocytosis and endosomes are known to be tightly linked to the actin cytoskeleton and lipid domains enriched with structural sterols play an active role in this process (Golub and Caroni 2005; Yin and Janmey 2003). Ectopic expression of activated AtRAC10 disrupted the actin cytoskeleton, compromised endocytosis, and resulted in aberrant root hair formation (Bloch et al. 2005). Interestingly, AtRAC10 localizes to an insoluble fraction of the plasma membrane suggesting that it is enriched within lipid rafts of plant cells. Our unpublished data revealed accumulation of filipin-positive sterols within outgrowing bulges and at tips of growing but not non-growing root hairs (Ovecka et al., in preparation); in pollen tube, however, filipin did not result in differential staining of the apex. Recent analyses of pollen GPI proteins showed that lesions in some of them results in reduced pollen germination and tube growth (Lalanne et al. 2004). As GPI anchored proteins are well known "inhabitants" of cholesterol enriched lipid rafts their prominent representation in pollen transcriptome suggests a role of lipid rafts in pollen tube growth regulation.

PIP_2 is highly enriched at the tips of root hairs (Braun et al. 1999) and AtSfh1p is essential for the enrichment of PIP_2 at tips of root hairs as well as for the maintenance of clear zone vesicles and dense meshworks of F-actin (Bohme et al. 2004; Vincent et al. 2005). AtSfh1p might stimulate activity of PLD that would result in PIP_2 synthesis on the clear zone vesicles via activation of PIPK by PA. This suggests a presence of reciprocal positive feedback regulation of PA and PIP_2 enabling creation of distinct PA and PIP_2 enriched membrane domain at the expanding pollen tube tip or root hair (Fig. 3). Kusner et al. (2002) has shown that F-actin not only binds but also activates plant PLD, while G-actin inhibits its activity. Activation of PLD thus results in PA generation, which might feed into another lipid-signalling pathway – PA-induced and phosphoinositide-dependent kinase-1 (PDK1) stimulation (Anthony et al. 2004). PDK1 is expressed in pollen but further data are required to assign a role to this signalling pathway.

Another signalling pathway that might crosstalk with phosphoinositides is the Reactive Oxygen Species (ROS) pathway. We have recently described polarized and calcium stimulated NADPH-oxidase (gp91 NOX subunit homologue)-dependent ROS production at the tips of growing pollen tubes (Jones et al. submitted), similar to the ROS production machinery of root hair tips (Foreman et al. 2003). In the case of pollen tubes we hypothesize that, just like $[Ca^{2+}]_c$, ROS production oscillates with the growth rate oscillation (Jones et al. submitted). From this point of view we also reinterpret the negative chemotaxis of pollen tubes towards nitric oxide (NO; Prado et al. 2004) as an outcome of inhibitory or interference effects of NO on NOX activity or ROS metabolism at the tip (Fujii et al. 1997). We predict that significant proportion of O_2 consumption accompanying pollen tube germination and

growth is brought about by NOX catalyzed ROS production and NO production. NADPH oxidase producing ROS molecules may also be localized to endosomes. Rho/Rac GTP-binding proteins are well known activators of NOX dependent ROS production also in plants (Kawasaki et al. 1999; Moeder et al. 2005; Carol et al. 2005) and recently described oscillations in Rop activity at the pollen tube tip (Hwang et al. 2005) might account, along with Ca^{2+}, for putative NOX dependent ROS oscillations in a feed-back regulatory circuit comprising activation effect of Ca^{2+} and regulatory effects of activated Rop on NOX activity. PA in plant cells can induce ROS production (Zhang et al. 2005), acting probably via Rops (Park et al. 2004). Recently discovered family of plant specific Rop GEFs (Berken et al. 2005) and their interaction with pollen receptor kinases (Kothien et al. 2005) allows us to hypothesize, that PA might activate Rops by changing lipid environment of receptor kinases. We suggest the possibility that Rop, Ca^{2+}, putative NOX/ROS oscillations and PA/PIP_2 reciprocal regulation are components of a positive feed-back process producing a distinct tip-localized membrane domain facilitating highly polarized cell expansion of pollen tubes (Fig. 3).

Considering the accumulation pattern of recycling proteins and pectins, tips of pollen tubes/root hairs and cytokinetic cell plates resemble endocytic BFA-induced compartments (Jaillais et al. 2004), suggesting that similar endocytic secretory pathways drive tip growth and cell plate formation. The majority of vesicle trafficking at the pollen tube tip is most probably related to the activity of pollen specific recycling endosome, an assumption supported by our recent discovery of known Rho and Rab effector exocyst complex and their importance for pollen tube growth (Elias et al. 2003, Zarsky et al. 2004, Cole et al. 2005).

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