Distinct roles of PI4P 5-kinase isoforms in polar tip growth of pollen tubes

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Abbreviations

ABP	Actin binding protein
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
CDP	Cytidine diphosphate
CDP-DAG	Cytidine diphosphate-diacylglycerol
CFP	Cyan fluorescent protein
CMP	Cytidine monophosphate
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DNA	Desoxyribonucleic acid
dNTPs	Desoxynucleotidetriphosphates
ER	Endoplasmatic reticulum
EtOH	Ethanol
EYFP	Enhanced yellow fluorescent protein
FS	Freezer stock
G proteins	Guanine nucleotide-binding proteins
GFP	Green fluorescent protein
GTPases	Guaninetriphosphatases
IP_3	Inositol 1,4,5-trisphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MBP	Maltose binding protein
MES	2-(N-morpholino)ethanesulfonic acid
MORN	Membrane occupation and recognition nexus
Nag	N-acetylglucosaminyl transferase I
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PH domain	Pleckstrin homology domain
P_i	Inorganic phosphate
PP_i	Inorganic pyrophosphate
PI	Phosphatidylinositol
PI 4-kinase	Phosphatidylinositol 4-kinases
PI3P	Phosphatidylinositol-3-phosphate
PI4P	Phosphatidylinositol-4-phosphate
PI5P	Phosphatidylinositol-5-phosphate
$PI(3,4)P_2$	Phosphatidylinositol-3,4-bisphosphate
$PI(3,5)P_2$	Phosphatidylinositol-3,5-bisphosphate
$PI(4,5)P_2$	Phosphatidylinositol-4,5-bisphosphate
$PI(3,4,5)P_3$	Phosphatidylinositol-3,4,5-trisphosphate
PI4P 5-kinase	Phosphatidylinositol-4-phosphate 5-kinase

PIK	Phosphatidylinositol-4-phosphate 5-kinase
PIP	Phosphatidylinositol-monophosphates
PIP-kinase	Phosphatidylmonophosphate kinase
PIP5K	Phosphatidylinositol-4-phosphate 5-kinase
PLC	Phospholipase C
PME	Pectin methylesterase
PTEN	Phosphatase and tensin homolog
RFP	Red fluorescent protein
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
WT	Wild type
X-gluc	5-bromo-4-chloro-3-indolyl-glucuronide

1. Introduction

1.1 The cell and the endomembrane system

All living beings are organized in cells. While some organisms like many bacteria and protista are unicellular, there are other life forms, like mammals, containing numerous types of highly specialized cells (Alberts, 1994).

All cells are surrounded by a membrane composed of a lipid bilayer, consisting mostly of phospholipids and proteins, called the plasma membrane (Figure 1.1). This bilayer allows lateral diffusion as described by the fluid mosaic model, diffusion across the membrane, however, is abridged. This restricted permeability allows cells to selectively take up or extrude molecules from their cytoplasm (Alberts, 1994; Horton et al., 2006).

Eukaryotes, which include protists, animals, fungi and plants, possess not only the plasma membrane that separates them from their environment but also other intracellular membrane-surrounded compartments, called organelles (Horton et al., 2006). Examples for these organelles are mitochondria, where oxidative phosphorylation takes place, and, in plants and some protists, chloroplasts, as sites of photosynthesis. Both these organelles reproduce autonomously (Alberts, 1994). Other organelles that are part of the endomembrane system are the nuclear envelope, which surrounds the nucleus, where most of the genetic material is situated, and where RNA synthesis takes place, the endoplasmatic reticulum (ER), connected to the nucleus, where many proteins, lipids and other important molecules are synthesized, and the Golgi apparatus, where compounds originating from the ER are further processed and sorted (Alberts, 1994). Also part of the endomembrane system are lysosomes, organelles specialized in the degradation of cellular structures, including whole organelles, and vacuoles, large storage organelles that also play a role in turgor regulation, especially in plants. The membranes that surround these organelles all originate from the ER. Vesicles, spheroid structures of 0.5 - 2 µm in diameter that are surrounded by a lipid bilayer containing lipids, proteins and other

metabolites, constantly bud and move between organelles, leading to a continuous flux of cellular membranes (Horton et al., 2006).

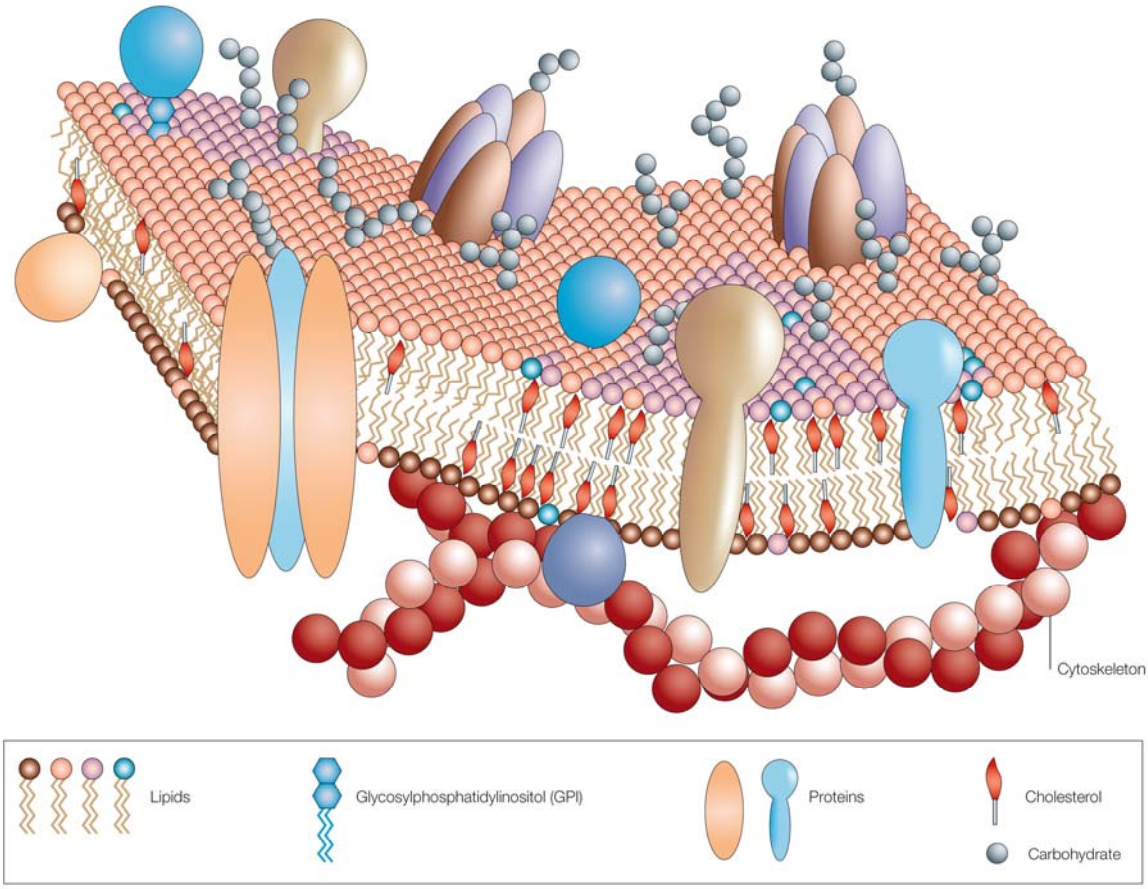

Figure 1.1. The eukaryotic plasma membrane.

The plasma membrane is composed of lipids, mostly phospholipids that form the lipid bilayer, and proteins. While the bilayer is impassable to hydrophilic substances, ions and metabolites can be selectively imported into the cell via channel or transport proteins. The cytoskeleton is attached to the plasma membrane. (Pietzsch, 2004)

An example for such membrane flux is the exocytotic pathway by which proteins, small molecules and lipids are transported from the ER via the Golgi apparatus to the plasma membrane in so called exocytotic vesicles (Battey et al., 1999). These vesicles fuse with the plasma membrane, and lipids and lipid-bound proteins are incorporated into the plasma membrane. Vesicular contents are, thus, secreted by the cell, a process called exocytosis. Key structures of a plant cell are illustrated in Figure 1.2.

Plants and fungi generate an extracellular cell wall that surrounds the plasma membrane, protecting the cell against mechanical damage but also from bursting due to turgor pressure (Cosgrove, 2005; Latge, 2007). The cell wall particularly of plants defines the cell shape. Cell walls consist of carbohydrate polymers, such as cellulose, callose and pectin, and of embedded proteins. Some of these cell wall components, such as pectin, are produced and secreted by the cell via the secretory pathway, while others such as cellulose and callose are synthesized directly through the plasma membrane by specialized protein complexes (Cosgrove, 2005). Another structure that helps to stabilize

the cell is the cytoskeleton (Wasteneys and Galway, 2003). Cytoskeletal structures consist of protein fibres such as microtubules (an α- and β-tubulin polymer), microfilaments (an actin polymer) and the heterogeneous group of intermediate filaments only known from animal cells. As will be described in more detail further down, cytoskeletal structures also define routes for vesicle and organelle trafficking and can thus influence the shape of a cell. Cellular functions like exocytosis and the dynamcis of the cytoskeleton have to be tightly regulated. Some of the factors controlling these regulatory processes will be discussed in the following chapters.

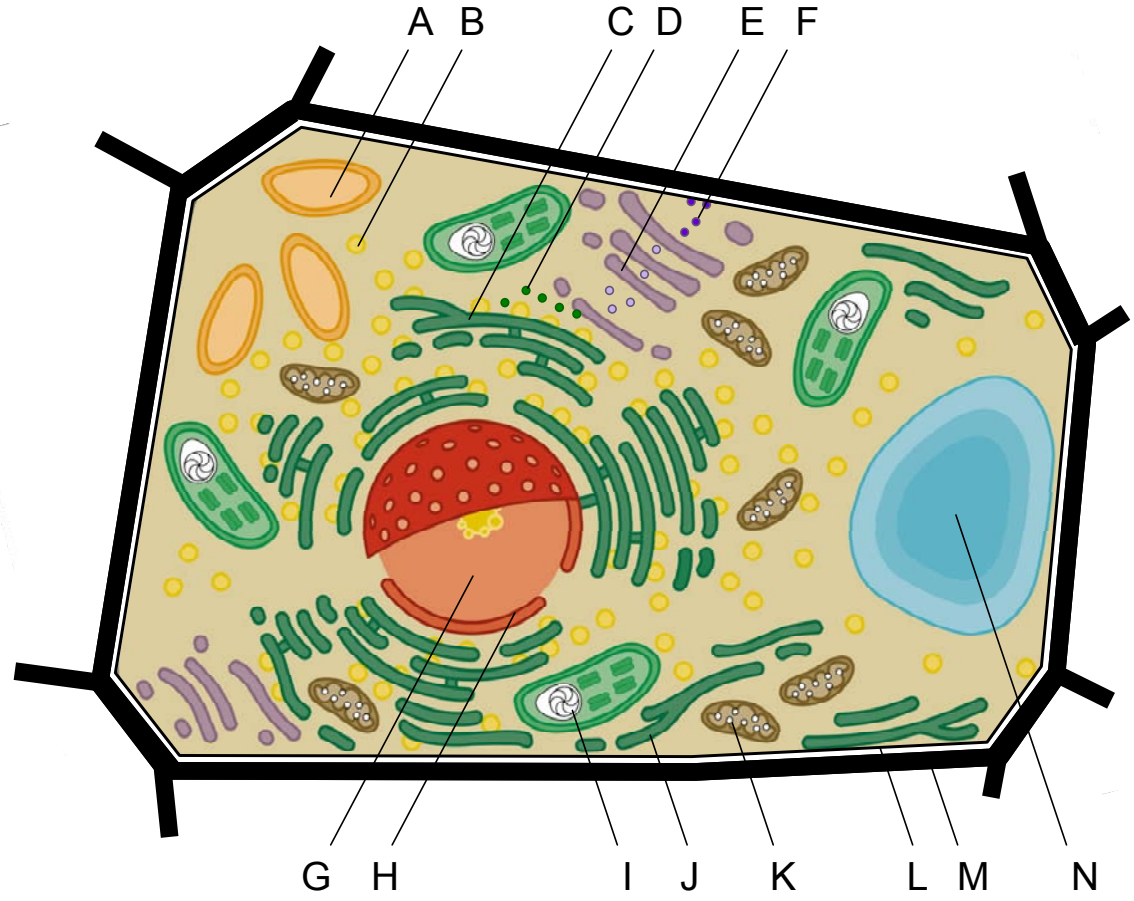

Figure 1.2. Subcellular structures of a plant cell.

A, leucoplast; B, free ribosome; C, rough ER; D, transport vesicle; E, Golgi apparatus; F, secretory vesicle; G, nucleus; H, nuclear envelope; I, chloroplast; J, smooth ER; K, mitochondrion; L, plasma membrane; M, cell wall; N, vacuole. Modified from http://idw-online.de/pages/de/image17537.

1.2 Signal transduction

During their lifespan, cells not only have to adapt to changes in their surroundings, but, even under stable environmental conditions, they have to maintain homeostasis of cellular functions to remain viable. To ensure functionality, cells have to transduce and integrate numerous extracellular and intracellular signals which can lead to changes in cell shape, cell motility, proliferation, metabolism and export or import of molecules. Cells contain different mechanisms for perception and transduction of signals coming from outside the cell. Such stimuli are called hormones if they are released by other cells of the same

organism. Signaling molecules can be detected either by receptors at the plasma membrane, as for instance the phytohormone abscisic acid (Liu et al., 2007b), or at intracellular receptors, as for example the phytohormone jasmonic acid (Chini et al., 2007; Thines et al., 2007). Signaling molecules can be effective at very low concentrations, because the perceiving cells are able to amplify weak local signals by signaling cascades to obtain strong cell-wide responses. A major part in these signaling pathways is played by signaling proteins that can change the conformation of - and, thus, activate or inactivate - target proteins by simply binding them, by transferring phosphate groups to the amino acid side chains of serine, threonine or tyrosine of the target protein, or by facilitating the exchange of bound GDP to GTP in the target protein, to name but a few examples. Proteins can also regulate other ptoteins indirectly by the production of small diffusible molecules, called secondary messengers (Krauss, 2003). Some of these messengers are soluble, such as cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP), Ca^{2+} ions and inositol-1,4,5-trisphosphate (IP_3), while others like diacylglycerol (DAG) or phosphoinositides are incorporated in membranes due to their hydrophobicity. Many proteins have been demonstrated to change their activity or their localization by specifically binding to secondary messengers (Krauss, 2003). Changes in localization can also be achieved by direct protein-protein interaction, for example by “recruiting” a soluble protein from the cytosol to a protein anchored to the plasma membrane. One of the most profound possibilities is also to influence the abundance of a protein by regulating its synthesis via transcription and translation or by altering its rate of degradation.

Target proteins of signaling factors often trigger other proteins, which leads to signaling pathways involving numerous different proteins. To minimize diffusion distances, proteins of the same signaling pathway are often located in close proximity, for example in distinct membrane areas or bound to so called scaffold proteins. Since effector proteins such as guanine nucleotide-binding proteins (G proteins) often target more than one other protein, there is often cross talk between signaling pathways, which means that pathways interact and influence each other. In summary, cells contain large and complex signaling networks rather than distinct signaling pathways, which enable the cells to adapt, proliferate or move, and to perform their distinct roles in a larger organism (Krauss, 2003).

1.3 Phosphoinositides - synthesis and degradation

Phospholipids account for the majority of lipids constituting most prokaryotic and eukaryotic membranes, with the exceprion of thylakoid membranes. While some phospholipids, such as phosphatidylethanolamine (PE) or phosphatidylcholine (PC) have a high abundance and mostly structural roles, others like phosphoinositides occur to a much smaller extent (Figure 1.3). Despite their low abundance, especially the phosphorylated representatives of phosphoinositides play a very important role in eukaryotic signaling cascades (Stevenson et al., 2000; Meijer and Munnik, 2003). Since this work is mostly concerned with phosphoinositides, their synthesis, interconversion and effects on cellular functions will be highlighted in the following chapters.

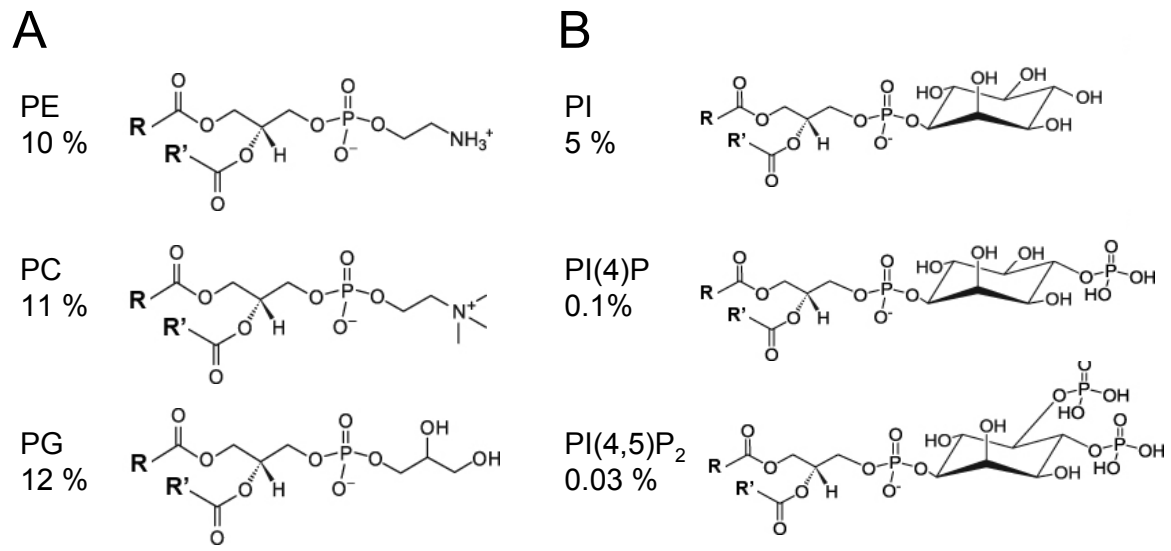

Figure 1.3. Structure and relative abundance of structural glycerophospholipids and of phosphoinositides relevant for this study.

A, structural glycerophospholipids: PE, phosphatidylethanolamin; PC, phosphatidylcholine; PG, phosphatidylglycerol. B, phosphoinositides: PI, phosphatidylinositols; PI(4)P, Phosphatidylinositol-4-phosphate; PI(4,5)P_2, phosphatidylinositols-4,5-bisphosphate. Abundance is given as percentage of total leaf glycerolipids. The main glycerolipids found in leaves are the plastidial galactolipids, monogalactosyldiacylglycerol and digalactosyldiacylglycerol, which are not a concern of this study. Data for PE, PC, PG and PI from (Bonaventure et al., 2003). Amounts of PI(4)P and PI(4,5)P_2 estimated from (König et al., 2007).

1.3.1. Formation of the phospholipid phosphatidylinositol

Phospholipid synthesis starts with the formation of fatty acids in the chloroplasts of plants or, in the case of all other organisms, in the cytosol. After transport to the ER, two of these fatty acids are esterified with glycerol-3-phosphate to obtain phosphatidic acid. While the synthesis of some phospholipids, such as PE or PC, requires the dephosphorylation of phosphatidic acid (PA) to form DAG, the synthesis of others begins with the activation of phosphatidic acid with CTP, generating cytidine diphosphate-diacylglycerol (CDP-DAG), a reaction catalyzed by the CDP-DAG synthase (Figure 1.4). Phosphatidylinositol (PI), the precursor of all phosphoinositides, is generated by PI synthases (PIS) that catalyze the condensing reaction of CDP-DAG and inositol. In contrast, other phospholipids such as PE and PC are formed by condensation of their activated head group precursors with DAG (Somerville et al., 2000).

PI synthesis has been detected in a number of organisms, including mammals (Takenawa and Egawa, 1977; Parries and Hokin-Neaverson, 1984), yeast (Fischl and Carman, 1983), plants (Justin et al., 1995), but also in some prokaryotes like actinomycetes (Jackson et al., 2000). The first gene encoding a PIS was identified in yeast (Nikawa et al., 1987), and later the *Arabidopsis* homolog, PIS1, was described (Collin et al., 1999; Justin et al., 2002). *Arabidopsis* also contains a second isoform, PIS2, with high similarity to PIS1 (Löfke et al., 2008). Both PISs from *Arabidopsis* are membrane-bound due to several transmembrane helices and are localized in the ER (Löfke et al., 2008).

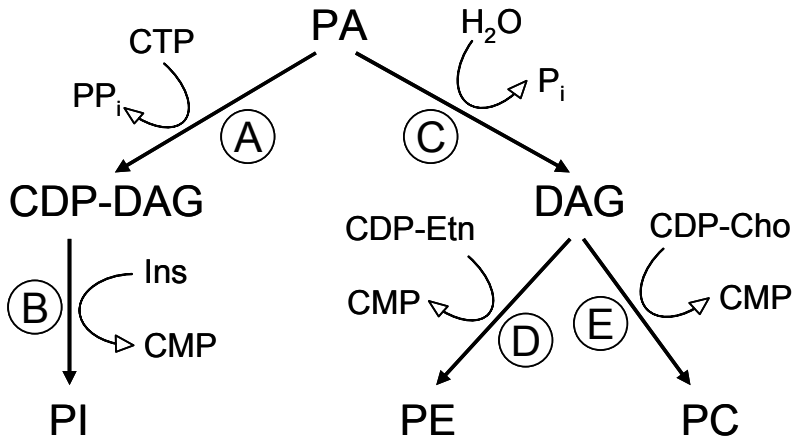

Figure 1.4. Synthesis of the phospholipids PI, PE and PC starting from PA

A, The penultimate step in the biosynthesis of PI catalyzed by the CDP-DAG synthase is the activation of PA with CTP to form CDP-DAG. B; PI is synthesized from inositol (Ins) and CDP-DAG, releasing CMP. This reaction is catalyzed by PIS. C, Alternative to the reaction described in A, PA can be dephosphorylated to DAG catalyzed by the PA-phosphatase. D, E, The structural phospholipids PE and PC are generated by condensation of their activated head group precursors, CDP-ethanolamine (CDP-Etn) and CDP-choline (CDP-Cho), respectively, with DAG, catalyzed by the CDP-ethanolamine:1,2-diacylglycerol ethanolamine-phosphotransferase and the CDP-choline:1,2-diacylglycerol cholinephosphotransferase, respectively.

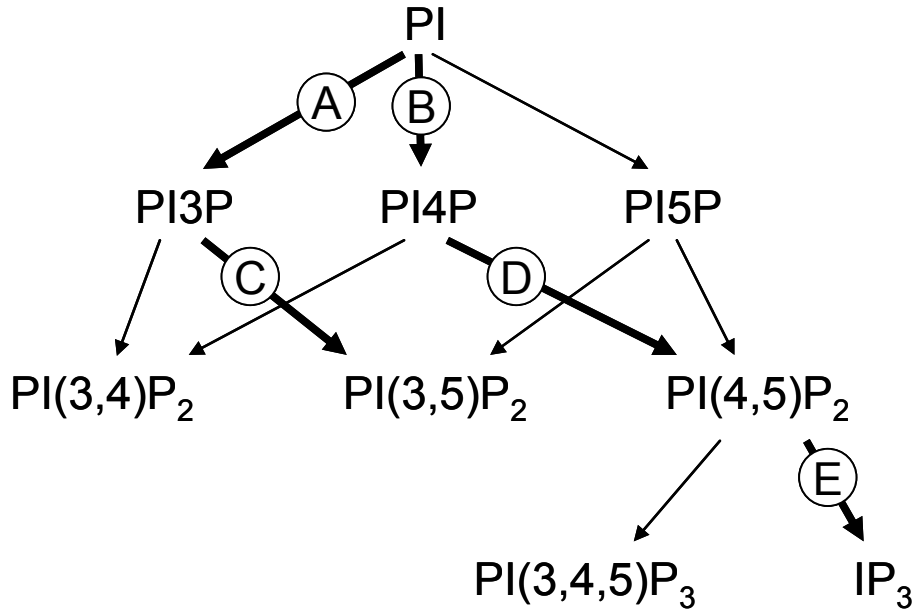

Figure 1.5. The synthesis of phosphoinositides

Phosphoinositides found in eukaryotes and their routes of synthesis. Bold arrows indicate major reactions found in plants as for example *Arabidopsis*. Thin arrows indicate reactions found in other eukaryotes. A-E Enzymes catalyzing phosphoinositide interconversions in plants. A, Type III phosphoinositide 3-kinase; B, phosphatidylinositol 4-kinase; C, Type III phosphatidylinositol-monophosphate-kinase; D, Type I phosphatidylinositol-monophosphate-kinase, also referred to as phosphatidylinositol-4-phosphate 5-kinase. E, Phosphoinositide-specific Phospholipase C. PI3P, phosphatidylinositol-3-phosphate; PI4P, phosphatidylinositol-4-phosphate; PI5P, phosphatidylinositol-5-phosphate; $PI(3,4)P_2$, phosphatidylinositol-3,4-bisphosphate; $PI(3,5)P_2$, phosphatidylinositol-3,5-bisphosphate; $PI(4,5)P_2$, phosphatidylinositol-4,5-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol-3,4,5-trisphosphate.

The inositol ring of PI can be further phosphorylated at the D-3, D-4 and D-5 position by specific kinases that occur only in eukaryotes (Anderson et al., 1999; Drobak et al., 1999). The various phosphorylations can be carried out in consecutive steps, giving rise to a total of seven structurally-related phosphoinositides (Figure 1.5) as for example phosphatidylinositol 4-phosphate (PI4P) and phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) most important for this work. The individual reactions will be described in more detail in the next paragraphs.

1.3.2. Phosphoinositide 3-kinases

Phosphatidylinositol 3-kinases can be grouped in three different classes according to their substrate specificity. Class I enzymes can phosphorylate all phosphoinositide species, such as PI, PI4P and $PI(4,5)P_2$ (Vanhaesebroeck et al., 2001). Even though class I enzymes play an important role in animals, there is no evidence for their occurence in plants or fungi. Class II phosphoinositide 3-kinases can only phosphorylate PI and PI4P, while enzymes of class III accept only PI as a substrate. Enzymes of the latter class bear similarities to the protein Vps34p (vacuolar protein sorting 34) from yeast (Schu et al., 1993) and are the only phosphoinositide 3-kinases identified in plants and fungi. In *Arabidopsis* there is only one gene, *AtVPS34*, present that encodes a phosphoinositide 3-kinase (Welters et al., 1994).

1.3.3. Phosphoinositide 4-kinases

Phosphoinositide 4-kinases that use exclusively PI as a substrate and generate PI4P are called phosphatidylinositol 4-kinases (PI 4-kinases). Activities of this enzyme have been found in animals (Endemann et al., 1987; Pike, 1992), but also in plants, such as carrot (*Daucus carota*) (Okpodu et al., 1995) and spinach (*Spinacia oleracea*; (Westergren et al., 1999)). The first gene encoding a PI 4-kinase isolated from yeast was *PIK1* (phosphatidylinositol kinase 1) (Flanagan et al., 1993). The first genes from *Arabidopsis* were identified some years later (Stevenson et al., 1998; Xue et al., 1999). From genomic (Mueller-Roeber and Pical, 2002) and expression data (Schmid et al., 2005) it is now known that there are four ubiquitously expressed genes present in *Arabidopsis*, which can be divided in two subfamilies α and β that consist of two members each (Figure 1.6). A third subfamily γ containing eight members with similaritiy to mammalian PI-kinases (Mueller-Roeber and Pical, 2002) and to the yeast PI 4-kinase, Lsb6p, (Han et al., 2002) have not been shown to harbour PI-kinase activity but rather act as protein kinases (Galvao et al., 2008). The two kinases from the α subfamily differ in size (α1, 200 kD; α2, 60 kD), with the larger of the two isoforms containing a pleckstrin homology (PH) domain with a preference for binding PI4P (Figure 1.6 Stevenson et al., 1998). According to microarray PI4-kinases of the α subfamily are both ubiquitously expressed (Schmid et al., 2005). Stt4p (staurosporine and temperature sensitive 4), a yeast homolog of *Arabidopsis* type α PI 4-kinases (Yoshida et al., 1994), has been shown to localize at the plasma membrane by binding Sfk1p (suppressor of four kinase), a transmembrane spanning protein (Audhya and Emr, 2002).

The two type β PI 4-kinases from *Arabidopsis* with a size of ~120 kD (Xue et al., 1999) are soluble cytosolic proteins. Nonetheless, type β PI 4-kinases are localized at trans-Golgi vesicles in *Arabidopsis* root hairs (Preuss et al., 2006), tobacco pollen tubes (Jin and Heilmann, personal communication) and insect cells (Stevenson-Paulik et al., 2003). Similar localization has been seen for homologs in yeast (Walch-Solimena and Novick, 1999) and mammals (Olsen et al., 2003). In *Arabidopsis* it has been proposed that type β PI 4-kinases are recruited to these trans-Golgi vesicles by the action of the small G protein, RabA4b (Ras associated binding protein A4b) and of AtCBL1 (calcineurin B-like protein 1), a calcium sensing protein (Preuss et al., 2006). A similar interaction partner as AtCBL1, frequenin, has previously been described in yeast and animals (Hendricks et al., 1999; Strahl et al., 2003).

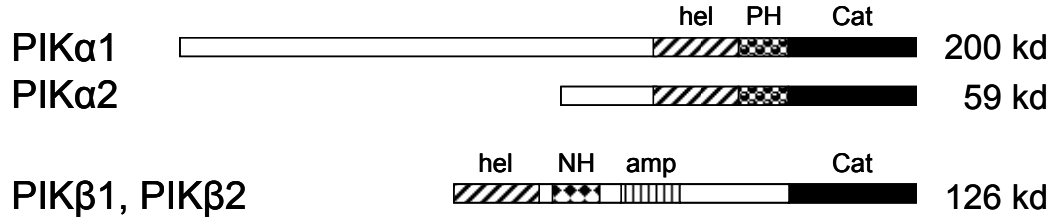

Figure 1.6. Domain structure of the PI 4-kinases of *Arabidopsis*

Arabidopsis contains two subfamilies of PI 4-kinases with different domain structures. Note that the catalytic domain (Cat) and the helical domain (hel) are conserved in all four kinases. Type α PI 4-kinases harbor a PH domain (PH), whereas type β PI 4-kinases contain a NH (neurohypophysial hormones) domain and a amphipatic repeat domain (amp).

Phosphoinositide 4-kinases using substrates other than PI, such as PI3P or PI5P, and, thus, generating $PI(3,4)P_2$ and $PI(4,5)P_2$, respectively, have not been reported in plants. The formation of $PI(3,4)P_2$ in plants is so far only considered as a side reaction of type I PIP-kinases (Westergren et al., 2001; Stenzel et al., 2008), as will be described in the next paragraph. In animals, an enzyme specifically catalyzing the formation of $PI(3,4)P_2$ from PI3P has been identified (Banfic et al., 1998). Phosphoinositide 4-kinases phosphorylating PI5P are also called type II phosphatidylinositol monophosphate kinases (PIP-kinases). These enzymes are similar in sequence to the PI4P 5-kinases described in the next paragraph, but differ distinctly in key amino acids of the active center (Kunz et al., 2000; Kunz et al., 2002). Such enzymes have been described to date neither in plants nor in fungi and are also not found in the *Arabidopsis* genome (Mueller-Roeber and Pical, 2002).

1.3.4. Phosphoinositide 5-kinases

Phosphorylation of phosphatidylinositol-monophosphates (PIPs) can be performed by two types of kinases, classified by their substrate specificity. Type I and type III PIP-kinases use PI4P and PI3P as substrates, synthesizing $PI(4,5)P_2$ and $PI(3,5)P_2$, respectively (Mueller-Roeber and Pical, 2002). It has been postulated for many years that type I PIP-kinases are present in plant plasma membranes (Sommarin and Sandelius, 1988; Mueller-Roeber and Pical, 2002), the first gene, however, was cloned and characterized

many years later (Elge et al., 2001). The *Arabidopsis* genome contains eleven genes coding for type I PIP-kinase isoforms (Mueller-Roeber and Pical, 2002), and four of them have been shown to perform mainly as phosphatidylinositol-4-phosphate 5-kinases (PI4P-5-kinases) with minor PI3P 4-kinase activity *in vitro* (Elge et al., 2001; Perera et al., 2005; Lee et al., 2007; Kusano et al., 2008; Stenzel et al., 2008).

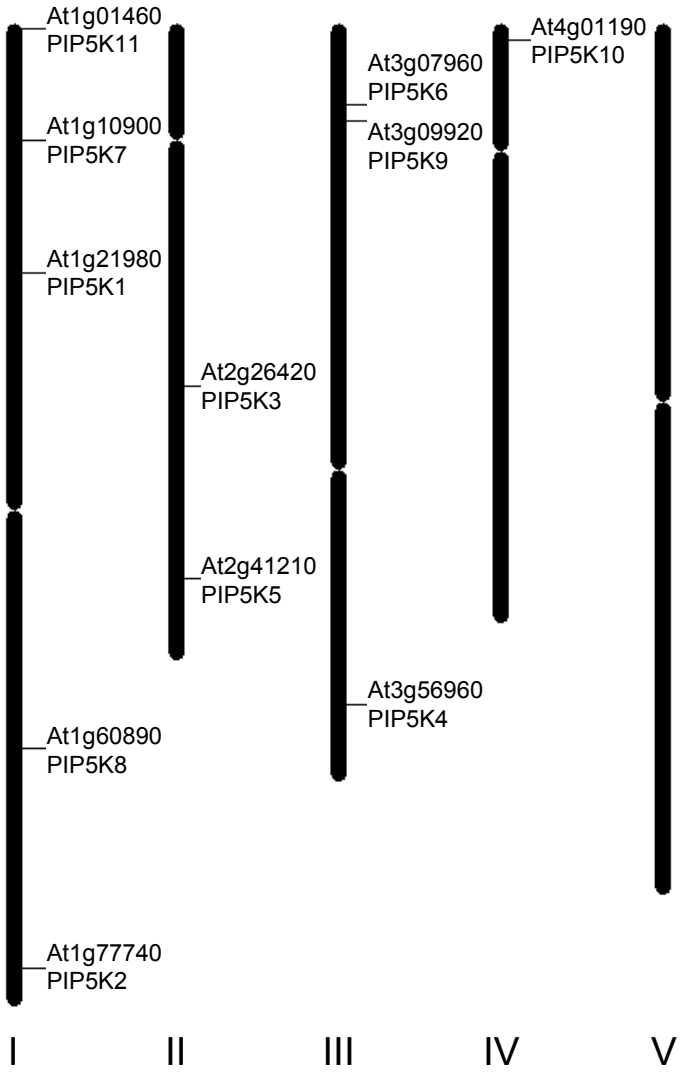

Figure 1.7. Map of the *Arabidopsis* genome displaying gene loci encoding PI4P 5-kinases

The map was created by the Chromosome Map Tool from The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). Roman numbers indicate the chromosomes. PI4P 5-kinase genes are identified by their AGI numbers and the annotation of isoforms according to Mueller-Roeber and Pical, 2002.

Based on their domain structure, *Arabidopsis* PI4P 5-kinases have been grouped in two subfamilies, A and B, consisting of two and nine members, respectively (Mueller-Roeber and Pical, 2002). As indicated in Figure 1.8 the members of subfamily B are larger than those of subfamily A (81-92 kD compared to 46-48 kD, respectively), partially due to the presence of several N-terminal membrane occupation and recognition nexus (MORN) domains (Mueller-Roeber and Pical, 2002) with a suspected function in plasma membrane binding (Takeshima et al., 2000; Im et al., 2007a). According to the Genevestigator portal

(Zimmermann et al., 2004), six isoforms are ubiquitously expressed, whereas four other kinases are exclusively expressed in pollen (Schmid et al., 2005). One isoform has been shown to be expressed solely in root epidermal cells that form root hairs (Kusano et al., 2008; Stenzel et al., 2008).

Figure 1.8. Type A and type B PI4P 5-kinases differ in their domain structure

Type A PI4P 5-kinases have a domain structure resembling that of mammalian PIP-kinaases. The larger type B PI4P 5-kinases contain an N-terminal extension with several characterisitic domains as indicated. NT, N-terminus; MORN, region containing several membrane occupation and recognition nexus domains; Lin, linker region connecting the MORN domain region with the dimerisation domain and the catalytic domain; Dim, dimerisation domain; Cat, catalytic domain.

Even though PI4P 5-kinases are soluble proteins targeted to the cytoplasm, according to TargetP prediction (Emanuelsson et al., 2007), the enzymes have been found to be localized at the plasma membrane of animal cells, yeast, and recently also of *Arabidopsis* cells (Lee et al., 2007; Kusano et al., 2008; Stenzel et al., 2008). Enzyme activities have been found in different eukaryotic systems not only at the plasma membrane (Perera et al., 1999; Heilmann et al., 2001; Kobayashi et al., 2005; Santarius et al., 2006), but also in other subcellular fractions such as the nucleus of mammalian and yeast cells (Ciruela et al., 2000; Audhya and Emr, 2003; Santarius et al., 2006), the actin cytoskeleton of yeast and plants (Desrivieres et al., 1998; Doughman et al., 2003; Davis et al., 2007) and endomembranes of animal and plant cells (Whatmore et al., 1996; Heilmann et al., 1999; Im et al., 2007a). The recruiting mechanism for plasma membrane localization of PI4P 5-kinases in plants is unknown; however sphingolipids have been shown to be involved in plasma membrane localization of Mss4p (multicopy suppressor of stt4), a PI4P 5-kinase from yeast (Kobayashi et al., 2005).

About the type III PIP-kinases that phosphorylate PI3P in the D-5 position of the inositol ring comparably less is known in plants. The *Arabidopsis* genome contains four genes encoding these enzymes, but none of them has been cloned or characterized. All isoforms with deduced sizes of approximately 200 kD show similar ubiquitous expression patterns and contain a FYVE domain, named after the four proteins Fab1p YOTB, Vac1p and EEA1 (Gaullier et al., 1998). This domain is capable of binding the catalytic substrate, PI3P (Gaullier et al., 1998).

1.3.5. Phospholipase C and phosphoinositide phosphatases

Since phosphoinositides are potent messenger molecules, organisms have evolved mechanisms to turn off these signals by either dephosphorylating the inositol polyphosphate headgroup or by cleaving it from the diacylglycerol backbone. Especially PI4P and PI(4,5)P_2 have been shown to have high turnover rates in plant cells (Meijer et al., 1999; Perera et al., 2002; Im et al., 2007b; Krinke et al., 2007).

The *Arabidopsis* genome contains multiple genes coding for putative phosphoinositide phosphatases. The SAC (suppressor of actin mutations) family contains nine proteins with similarity to the yeast phosphoinositide phosphatases Sac1p (Hughes et al., 2000; Foti et al., 2001) and Fig4p (Rudge et al., 2004) that dephosphorylate PI4P and $PI(3,5)P_2$, respectively. SAC1-5 have the highest similarity to Fig4p and at least SAC1 catalyzes the same reaction as Fig4p (Zhong et al., 2005). *Arabidopsis* SAC6-8 are more closely related to yeast Sac1p, and Sac7 has been recently shown to mainly dephosphorylate PI4P with additional substantial activity towards all other phosphoinositides (Thole et al., 2008). SAC9 is unique in its domain structure (Zhong et al., 2004), and even though its enzyme activity has not been reliably determined in *in vitro* tests, *Arabidopsis* mutants deficient in this protein have constitutively elevated $PI(4,5)P_2$ levels (Williams et al., 2005). Three other phosphatases show sequence similarity to the family of PTEN (phosphatase and tensin homolog) phosphatases known from the mammalian field, as for example PTEN1 (Gupta et al., 2002). Another group of phosphatases dephosphorylate inositol polyphosphates at the D-5 position (Zhong et al., 2004; Zhong and Ye, 2004). The phosphoinositide phosphatases from the SAC family dephosphorylate both phosphoinositides and soluble inositol polyphosphates and are therefore referred to as type II inositol polyphosphate 5-phosphatases. Other enzymes capable of dephosphorylating only soluble inositol polyphosphates as for example IP_3 are called type I inositol polyphosphate 5-phosphatases (Berdy et al., 2001).

Phospholipases C (PLCs) are enzymes that cleave phospholipids between the *sn-1* phosphate group and the diacylglycerol backbone. While some PLCs are active towards structural lipids (non-specific PLCs), there are others that only cleave phosphoinositides (PI-PLCs), releasing the soluble messenger molecule IP_3 if $PI(4,5)P_2$ is the substrate. While in mammals multiple gene families encoding PI-PLCs are present, the *Arabidopsis* genome contains only one seven member family with similarity to mammalian type ζ PLCs, that depend in their activity on the presence of Ca^{2+} (Mueller-Roeber and Pical, 2002). In plant cells, PLCs have been found to be localized at the plasma membrane (Melin et al., 1992; Dowd et al., 2006; Helling et al., 2006). Some of them are stress-induced at the transcriptional level (Hirayama et al., 1995).

1.4 Physiological functions of PI4P and $PI(4,5)P_2$ and IP_3

Since phosphoinositides and other derived molecules, such as DAG and IP_3, control a large number of cellular processes, it would not be possible to exhaustively cover all aspects of this topic in this introduction. Therefore, there will be a focus on processes most relevant for this work, which are the control of membrane trafficking, of the cytoskeleton and of ion channels. The enzymes forming the relevant secondary messengers, such as PI 4-kinases, PI4P 5-kinases and PI-PLCs, will also be discussed.

Phosphoinositides are potent secondary messengers because of the high density of negative charges of their head groups that are extending further from the hydrophobic bilayer into the cytoplasm than those of other membrane lipids (Sansom et al., 2005). These particular properties of phosphoinositide headgroups enable proteins to bind phosphoinositides electrostatically via positively charged amino acid side chains with high

affinity and high specificity (Lemmon, 2003). There are several families of protein domains known that can bind the different phosphoinositides. Two examples for these families are the FYVE domains (Misra et al., 2001; Stenmark et al., 2002) and the diverse group of PH domains (Lemmon and Ferguson, 2000). FYVE domains specifically bind PI3P and with high affinity (Bravo et al., 2001; Stenmark et al., 2002). PH domains show a broad variety of PI binding specificities (Lemmon and Ferguson, 2000), most of the PH domains not discriminating between different phosphoinositides at all (Lemmon and Ferguson, 2000). An exception is the PH domain of the human PLCδ1 that specifically binds $PI(4,5)P_2$ and IP_3 (Lemmon, 2003). Another excemption is the PH domain from the human FAPP protein (family A phosphoinositol 4-phosphate adaptor protein 1) that exclusively binds PI4P (Blumental-Perry et al., 2006). Because the PH domains from PLCδ1 and FAPP are independent in their binding capabilities from the rest of the protein they can be used as molecular probes for $PI(4,5)P_2$ and PI4P, respectively (Varnai and Balla, 1998; Weixel et al., 2005; Blumental-Perry et al., 2006).

1.4.1. Control of membrane trafficking

It has been shown in yeast and animal cells that phosphoinositides play an important role in the secretory pathway from the Golgi to the plasma membrane. In yeast it is known that the PI 4-kinase Pik1p is essential for membrane traffic from the Golgi to the plasma membrane. When Pik1p is eliminated, secretion is diminished (Uno et al., 1988; Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000) and abnormal membranous structures called “Berkeley bodies” accumulate in the cytoplasm (Walch-Solimena and Novick, 1999; Audhya et al., 2000; Strahl et al., 2005). On the other hand, when PI4P levels are elevated in cells overexpressing Pik1p or in cells deficient in the phosphoinositide phosphatase Sac1p, exocytosis of the chitin synthase Chs3p and most likely also of other proteins is enhanced, leading to cell wall defects (Schorr et al., 2001). The only PI4P 5-kinase in yeast, Mss4p is also suspected to play a role in exocytosis. It has been hypothesized that $PI(4,5)P_2$ generated by Mss4p is involved in the fusion of secretory vesicles with the plasma membrane (Strahl and Thorner, 2007), because overexpression of Mss4p can rescue several temperature sensitive mutants defective in proteins of the secretory machinery. Examples are Sec8p (protein with a role in secretion), Sec10p and Sec15p (TerBush et al., 1996; Guo et al., 1999; Hsu et al., 1999), which are all members of the multiprotein complex, exocyst, required for the tethering of exocytotic vesicles to the plasma membrane. Other examples include the syntaxin-binding protein Sec1p (Aalto et al., 1991; Halachmi and Lev, 1996; Carr et al., 1999) and the plasma membrane t-SNARE (target soluble N-ethylmaleimide sensitive factor attachment receptor) Sec9p (Sollner et al., 1993; Brennwald et al., 1994; Hay and Scheller, 1997).
In animal cells phosphoinositides are involved in exocytosis in a similar way. In neuronal cells the phosphoinositides PI4P and $PI(4,5)P_2$ stimulate exocytosis by priming dense core vesicles for fusion and by recruiting the protein CAPS (calcium-activated protein for secretion) to the plasma membrane, which is essential for vesicle fusion (Hay and Martin, 1993; Hay et al., 1995; Berwin et al., 1998). A similar mechanism has been shown for the exocytosis of insulin-containing vesicles in pancreatic β-cells (Olsen et al., 2003). Similar

as in yeast, $PI(4,5)P_2$ targets the exocyst complex to the plasma membrane also of mammalian cells (He et al., 2007; Liu et al., 2007a).
$PI(4,5)P_2$ is not only involved in exocytosis but also plays a crucial role in endocytosis at the plasma membrane. The temperature sensitive yeast mutant *mss4^{ts}*, deficient in its only PI4P 5-kinase, displays defects in endocytic uptake (Desrivieres et al., 2002) when shifted to restrictive temperature. Similar results were obtained with mammalian neuronal cells, where endocytosis is increased, when $PI(4,5)P_2$ levels are enhanced (Cremona et al., 1999), and decreased, when $PI(4,5)P_2$ levels are reduced (Di Paolo et al., 2004). $PI(4,5)P_2$ could be important in endocytosis due to the fact that many proteins of the endocytotic machinery are targeted to the plasma membrane by $PI(4,5)P_2$ in yeast and in mammalian cells (Jost et al., 1998; Gaidarov and Keen, 1999; Takei et al., 1999; Vallis et al., 1999; D'Hondt et al., 2000; Ford et al., 2001; Itoh et al., 2001; Friesen et al., 2006). Even though association of $PI(4,5)P_2$ with clathrin-coated vesicles during stress-induced endocytosis has been shown for plant cells recently (König et al., 2008b), much less is known about membrane trafficking and the roles of phosphoinositides in related processes in plants.

1.4.2. Control of the actin cytoskeleton

Eukaryotic cells contain a dynamic network of protein filaments called the cytoskeleton, which not only mediates stability of the cells, but also is involved in protein and vesicle movement, in cell shaping and cell division, and in sensing and signaling (Wasteneys and Galway, 2003). As was mentioned above, three different types of protein filaments can be distinguished in eukaryotic cells. With a diameter of 25 nm microtubuli are the thickest filaments. Microtubuli are composed from α- and β-tubulin subunits. Intermediate filaments are about 8 to 11 nm thick and consist of a diverse group of proteins. This type of filaments is not known in plants. The most dynamic and complex cytoskeletal network is formed by 7 nm thick microfilaments composed of actin subunits bound to ATP or ADP. In its polymeric filamentous form actin is also called F-actin. The filamentous actin polymer is a polar structure containing a barbed (+) and pointed (-) end. Actin can also occur as a soluble globular monomer called G-actin. Even though actin can polymerize spontaneously in the presence of ATP, through monomers being added to both the barbed and the pointed end, *in vivo* such polymerization process is regulated by actin binding proteins (ABPs) that can promote or inhibit polymerization, induce depolymerization, or can lead to branching or breaking of actin strands.
A number of proteins with roles in the regulation of the cytoskeleton were found and studied in non-plant organisms. After the completion of the *Arabidopsis* genome sequence (Arabidopsis-Genome-Initiative, 2000) it became apparent that a number of ABPs known from non-plant species have homologous counterparts in plants (Wasteneys and Yang, 2004), whereas other ABPs - for example talin, vitronectin and vinculin - appear to be missing in plants (Wasteneys and Galway, 2003). Therefore, it can be assumed that many aspects of the plant cytoskeleton are regulated in a similar fashion as in animals or yeast, while others may be profoundly different. One aspect that might be similar in plant- and non-plant cells is the regulation of the actin cytoskeleton by phosphoinositides. Even

though actin does not bind phosphoinositides, itself, some ABPs that regulate actin dynamics have, however, been shown to be regulated by $PI(4,5)P_2$.

The actin binding protein complex ARP2/3 (actin related protein 2/3) binds to existing actin filaments and serves as a nucleation site for new daughter filaments that grow out from the mother filament at a 70° angle (Blanchoin et al., 2000). ARP2/3 is not regulated by $PI(4,5)P_2$ directly, but is activated by N-WASP (neural Wiskott-Aldrich syndrome protein) family proteins that are activated by $PI(4,5)P_2$ (Prehoda et al., 2000). Even though the proteins of the Arp2/3 complex are present in plants, genes coding for N-WASP proteins are missing in the *Arabidopsis* genome (Hussey et al., 2002). However, it can not be precluded that the ARP2/3 complex might be indirectly regulated by $PI(4,5)P_2$ through plant specific effectors.

ABPs of the ADF (actin depolymerising factor)/cofilin family destabilize actin filaments by promoting depolymerization at the pointed end and by severing them (Bailly et al., 2001). ADF/cofilin proteins are strongly inhibited by phosphoinositides, particularly by $PI(4,5)P_2$ (Ojala et al., 2001), and it can be hypothesized that regions of the plasma membrane enriched in $PI(4,5)P_2$ may, thus, stabilize actin filaments (Yin and Janmey, 2003). In plants a similar regulation mechanism of ADF/cofilin has been demonstrated (Gungabissoon et al., 1998), and it has been shown that these ABPs have important functions in cellular morphogenesis (Dong et al., 2001) and in polar tip growth of root hairs (Dong et al., 2001) and pollen tubes (Chen et al., 2002).

Gelsolin is another ABP regulated by $PI(4,5)P_2$ and Ca^{2+}, originally identified in alveolar macrophages (Yin and Stossel, 1979) that can sever actin strands and cap several of them at the barbed end (Janmey et al., 1998; Sun et al., 1999). Whereas Ca^{2+} stimulates severing and capping, $PI(4,5)P_2$ is essential for removing gelsolin from actin (Janmey and Stossel, 1987). While a protein with similarity to gelsolin was identified in *Papaver rhoeas* pollen (Huang et al., 2004), no gene with similarities to sequences coding for gelsolin has been reported from *Arabidopsis* (Wasteneys and Yang, 2004).

Profilins are small ABPs that can bind monomeric G-actin, exerting positive or negative effects on microfilament elongation *in vitro*, depending on the conditions. If the concentration of G-actin is high, profilin can promote elongation at the barbed end. However it can also sequester G-actin from the barbed end of F-actin, leading to depolymerization (Theriot and Mitchison, 1993). Besides actin, profilin can also bind $PI(4,5)P_2$ (Sohn et al., 1995; Kovar et al., 2000). Since binding of $PI(4,5)P_2$ or G-actin are mutually exclusive (Drobak et al., 1994), profilin releases free G-actin when recruited to the plasma membrane by $PI(4,5)P_2$ (Witke, 2004). On the other hand, when $PI(4,5)P_2$ is cleaved by the action of PLCs, free profilin can be liberated and participate in elongation or degradation of actin. Plant profilins have important functions in cell elongation and cell shape maintenance in the sporophyte (Ramachandran et al., 2000) and also occur at very high concentrations in pollen and pollen tubes (Vidali and Hepler, 1997). Profilins have also been found at the plasma membrane of the tip of growing root hairs in close proximity to $PI(4,5)P_2$ (Braun et al., 1999).

AtCP (*Arabidopsis* thaliana capping protein) is a heterotrimeric protein found in *Arabidopsis* that is homologous to the vertebrate muscle protein, CapZ (actin capping

protein Z), that can cap the barbed end of actin fibers, protecting it from loss and addition of subunits at this end. AtCP can also act as a nucleation site of filament assembly. AtCP can be dislodged from the barbed end of actin by $PI(4,5)P_2$ (Huang et al., 2003) in a similar way as homologs in animals and fungi (Haus et al., 1991; Heiss and Cooper, 1991; Schafer et al., 1996), and as was described above for profilins.

Since $PI(4,5)P_2$ can influence ABPs in so many ways, it is not surprising that manipulation of $PI(4,5)P_2$ levels in living cells can have severe effects on the cytoskeleton. When phosphoinositide levels are diminished, for example in budding yeast deficient in the PI 4-kinase Stt4p or the PI4P 5-kinase Mss4p, the actin cytoskeleton behaves abnormal and cell polarity is lost (Strahl and Thorner, 2007). It has also been described for mammalian fibroblasts that lowering the amount of free $PI(4,5)P_2$ decreases the adhesion of the cytoskeleton to the plasma membrane, demonstrating that $PI(4,5)P_2$ acts in the attachment of actin to the plasma membrane (Raucher et al., 2000). *Arabidopsis* lines mutated in PIP5K3 (Kusano et al., 2008; Stenzel et al., 2008), PIP5K4 (Lee et al., 2007) and PIP5K9 (Lou et al., 2007) have been isolated, however, the cytoskeletal structures in these plants have so far not been investigated.

Increases in $PI(4,5)P_2$ levels have also been shown to have effects on the actin cytoskeleton as for example in mammalian cell lines (Shibasaki et al., 1997; Rozelle et al., 2000; Yamamoto et al., 2001). In petunia pollen tubes an increase in $PI(4,5)P_2$ levels by a reduction of PLC activity led to a disorganization of the actin cytoskeleton and to a loss of polar growth (Dowd et al., 2006).

1.4.3. Control of ion channels

Another important function of phosphoinositides in eukaryotic cells is the control of ion channels. For all living organisms it is important to keep the internal environment of their cells at a dynamic equilibrium with their surroundings. In addition, transient and localized changes in ion concentrations, especially of Ca^{2+}, are essential for various signal transduction pathways. Since the hydrophobic core of the plasma membrane is impassable for charged ions, cells contain protein pores called ion channels that allow the diffusion of ions into or out of the cell. Other transport proteins use energy to pump ions against their concentration gradients. All these channels and transporters are selective for certain ions, and their activity is regulated by a number of factors, including voltage across the membrane, phosphorylation, ligand binding or a combination of these. One important ligand regulating ion channels is $PI(4,5)P_2$.

Unlike other proteins that are soluble and are recruited from the cytoplasm to the plasma membrane by their affinity to $PI(4,5)P_2$, ion channels are regulated differently, because they are membrane-bound due to their membrane spanning domains (Lee, 2004). It is presumed that ion channels bind $PI(4,5)P_2$ located in close proximity at the inner leaflet of the plasma membrane via N- and/or C-terminal binding domains (Suh and Hille, 2008). The examination of ion channel regulation by $PI(4,5)P_2$ in living cells is difficult due to the complex cellular responses to $PI(4,5)P_2$ and because the methods and reagents used are not optimal (Suh and Hille, 2005). Methods used so far include the increase of $PI(4,5)P_2$ levels by addition of soluble $PI(4,5)P_2$ analogs, such as dioctanoyl-$PI(4,5)P_2$, or the

overexpression of PIP-kinases. $PI(4,5)P_2$ levels can also be decreased by overexpression of lipases or phosphatases specific for $PI(4,5)P_2$. The amount of $PI(4,5)P_2$ available for ion channel gating can also be reduced by overexpression of $PI(4,5)P_2$-binding proteins such as PH-PLCδ1 or the application of $PI(4,5)P_2$ antibodies. Another possibility to block $PI(4,5)P_2$ synthesis is the unspecific inhibition of PI-kinases by wortmannin (Suh and Hille, 2008).

The inward-rectifier K^+-channels were the first channels found to be regulated by $PI(4,5)P_2$ and they are activated by binding this lipid (Hilgemann and Ball, 1996). For inward-rectifier K^+-channels there are also the best partial structures available (Nishida and MacKinnon, 2002; Pegan et al., 2005), even though no complete structure has been reported to date. From structural data and additional mutagenesis studies it has been concluded, that these channel proteins bind $PI(4,5)P_2$ with each of their four subunits via six basic residues situated at the C-terminal cytosolic domain (Haider et al., 2007). Voltage-gated K^+-channels are activated similarly (Delmas and Brown, 2005; Li et al., 2005), and their $PI(4,5)P_2$ binding sites are also suspected to reside in the C-terminal cytosolic domain (Zhang et al., 2003). Transient receptor potential (TRP) channels are another diverse group of cation channels that are affected in their activity by $PI(4,5)P_2$ binding. This class of ion-channels is particularly interesting because some representatives such as TRPV5 (Rohacs et al., 2005), are activated while others like TRPL (Estacion et al., 2001) are inhibited by their binding to a $PI(4,5)P_2$ ligand. The regulating mechanism seems to be similar as in the ion channels described above (Rohacs, 2007). All the channels described so far were of animal origin and much less is known about ion channel regulation by $PI(4,5)P_2$ in plants. It was reported that some shaker-type K^+-channels are activated *in vitro* by $PI(4,5)P_2$ (Liu et al., 2005). Recently it was proposed that $PI(4,5)P_2$ plays a role in the inactivation of slow anion channels in guard cells during stomatal opening (Lee et al., 2007). All channels that have been reported to be regulated by $PI(4,5)P_2$ are localized at the plasma membrane. Therefore, these channels remain inactive during their trafficking from the ER via the Golgi to the plasma membrane or also during recycling after endocytosis (Hilgemann et al., 2001) since $PI(4,5)P_2$ is not localized in the ER or Golgi vesicles. Another advantage of ion channel regulation by $PI(4,5)P_2$ is that $PI(4,5)P_2$ levels can rapidly be changed by the cell, allowing for short-term regulation and, possibly, links to other signal transduction pathways.

1.4.4. Distinct Phosphoinositide pools

Phosphoinositides can control different physiological functions in eukaryotic cells, as has been described in the previous paragraphs. Changes in $PI(4,5)P_2$ abundance have been considered as one distinct cellular signal. However, it becomes more and more clear from experiments in a number of eukaryotic model systems that discrete phosphoinositide pools with different metabolic origin and/or different spatio-temporal localization (Doughman et al., 2003; Santarius et al., 2006) can control alternative cellular processes (King et al., 1987; Heilmann et al., 1999; Kost et al., 1999; Heilmann et al., 2001; Doughman et al., 2003; Santarius et al., 2006; König et al., 2007). How these different phosphoinositide pools are generated and maintained is still unclear. Since all the

enzymes participating in the synthesis of phosphoinositides in *Arabidopsis* are present in multiple isoforms, analysis of these different isoforms should be considered. Differences in substrate preferences for CDP-DAG containing saturated or unsaturated acyl chains have been recently reported for the PIS isoforms, PIS1 and PIS2 (Löfke et al., 2008). In addition, while PIS1 and PIS2 are both localized predominantly in the ER, their suborganellar localization is not completely identical (Löfke et al., 2008). Overexpression of PIS1 or of PIS2 in *Arabidopsis* plants indicates that the enhanced amounts of PI produced by each isoform were channelled into different metabolic routes (Löfke et al., 2008), suggesting that different pools of phosphoinositides may exist already at the level of PI. It is known from the animal field, that enzymes such as phosphoinositide kinases and phosphatases, can have preferences for PI-substrates with certain fatty acid compositions (Carricaburu and Fournier, 2001; Schmid et al., 2004). It has also been shown that phosphoinositides increasing after osmotic stress in *Arabidopsis* differ in their acyl-chain composition from PI4P and PI(4,5)P_2 present under non-stressed conditions (König et al., 2007). In consequence, differences in fatty acid composition may be one factor underlying changed lateral mobility of phosphoinositides (Mukherjee et al., 1999; Cho et al., 2006), possibly leading to interactions with alternative partner proteins, as previously reviewed (Heilmann, 2008). The concept of discrete localization of phosphoinositide pools is especially important in polar growing cells, because phosphoinositides are considered to play crucial roles in establishing and maintaining polar cell growth.

1.5 Polar tip growth

Eukaryotic organisms show polarity at all levels of their organization. As it is obvious that whole organisms and their organs are polar, polarity can also be found at the level of the cell and even in subcellular structures, such as the Golgi apparatus. Polar growth, occuring in some cell types, is strictly regulated and phosphoinositides are known to play important roles in this process (Fischer et al., 2004). Polar tip growth is known from many organisms, including fungal hyphae and mammalian cells such as neurones. In plants two well known cell types exhibiting polar growth are root hairs and pollen tubes.

Root hairs are tube-shaped protrusions from single root epidermal cells that can reach a length of ~ 800 µm with a diameter of ~ 11 µm in *Arabidopsis* (Galway et al., 1997). Root hairs greatly increase the surface of plant roots and are crucial for the uptake of nutrients.

Pollen tubes are outgrowths from pollen grains and represent the male gametophyte of higher land plants, which is reduced to the pollen tube cell containing a vegetative nucleus (Figure 1.9 C) and two sperm cells (Figure 1.9 E). After landing on the stigma of the pistil, pollen grains hydrate and extrude unicellular pollen tubes that invade the pistil tissue, target the ovules, and deliver the sperm cells for double fertilization (Lord and Russell, 2002). Pollen tubes can elongate at a tremendous speed of up to 1 mm per hour in lily pollen tubes growing *in vitro* (Cheung et al., 1995). Since pollen tubes have to grow longitudinally through the entire pistil, these cells can reach tremendous lengths: While only ~2.5 mm long in *Arabidopsis*, pollen tubes can reach a length of ~ 30 cm in maize plants (Cheung et al., 1995). Some known cellular structures and signaling pathways that

allow root hairs and pollen tubes to grow in a polar fashion will be discussed in the following paragraphs.

1.5.1. Polar tip growth - growth mechanisms

In order for a cell to grow, several conditions must be met. First of all, an expanding cell needs a constant addition of membrane lipids to the plasma membrane due to the increasing of cellular surface. Another problem rapidly growing cells have to cope with is the increase in cellular volume. In order to prevent dilution of cytosolic compounds, such as proteins, these compounds have to be either synthesized at a high rate, or the increase in cytosol has to be abridged by a volume increase of cellular compartments, in plant cells for instance the vacuole (Figure 1.9 A). Cells that contain a cell wall, also require continuous exocytosis of cell wall material to prevent a thinning of the extracellular cell wall. This process has to be tightly regulated, however, since a cell wall that is too thick and rigid would prevent cell expansion. Another factor required is a force to promote cellular growth. In plant cells cell expansion is driven mainly by turgor pressure. Since turgor is an undirected force that acts in all directions of the cell, this force has to be counteracted if a certain cell shape has to be obtained, as for example during polar growth of pollen tubes and root hairs.

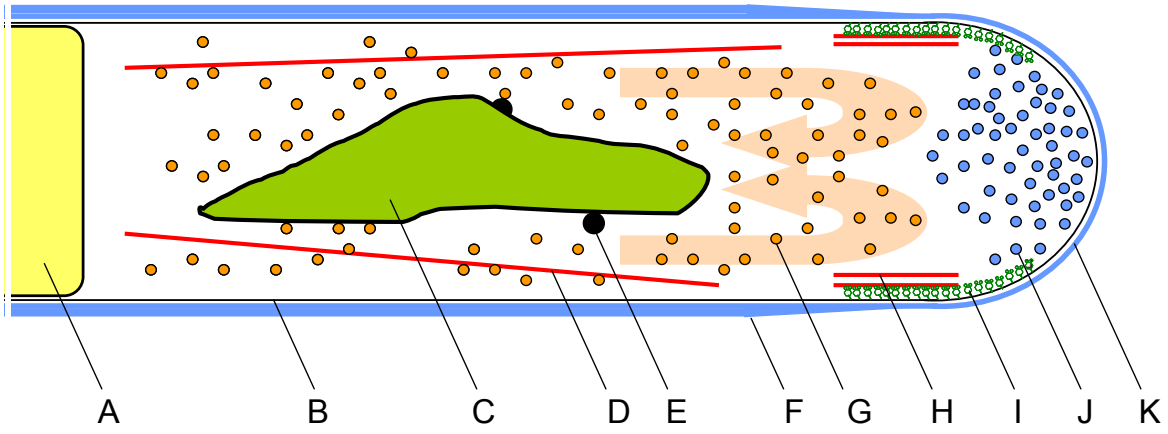

Figure 1.9. Pollen tube ultrastructure

Subcellular structures with particular relevance for this work are indicated. A, vacuole; B, plasma membrane; C, vegetative nucleus; D, actin bundles; E, sperm cells; F, rigid cell wall containing cellulose, callose and deesterified pectin complexed with Ca^{2+}; G, Golgi vesicles moving in the reverse fountain pattern; H, fine actin filaments arranged in a structure called actin fringe; I, $PI(4,5)P_2$; J, secretory vesicles containing cell wall precursors localized in the clear zone; K, flexible cell wall composed of pectin methylesters. Arrows indicate the pattern of reverse fountain streaming.

Pollen tubes and root hairs meet these premises in similar ways. Both pollen tubes and root hairs have a large pool of exocytotic vesicles containing cell wall precursors at the apex of the cell (Figure 1.9 J; Sherrier and VandenBosch, 1994), where exocytosis takes place at a high rate. This vesicle-rich region of pollen tubes and root hairs contains no larger organelles or other cellular structures and is also referred to as the “clear zone” (Figure 1.9 J) due to the absence of structures visible under the light microscope. The

cytosol, including other cellular organelles, flows in pollen tubes and growing root hairs in a pattern referred to as “reverse fountain” streaming: cellular contents move peripherally in apical direction up to the clear zone, where the flow turns to move backwards in the central region of the cell (Figure 1.9 G; Hepler et al., 2001). Cytoplasmic streaming allows the continuous recycling of membrane material and transport vesicles. In pollen tubes, the amount of cell wall material required for growth exceeds that of membrane material, and it is estimated that as much as 80 % of the secreted membrane is recycled by endocytosis (Picton and Steer, 1983).

Cell elongation in pollen tubes and root hairs is driven, at least predominantly, by an internal turgor pressure that reaches values around 0.2 MPa in pollen tubes of *Lilium longiforum* (Benkert et al., 1997). To limit cell growth to the very apex, the cell wall of pollen tubes and root hairs at the flanks is inflexible, to prevent lateral cell expansion. The lateral cell wall of pollen tubes consists of an inner callose and an outer cellulose layer and is rigid (Figure 1.9 B; Taylor and Hepler, 1997; Cheung and Wu, 2008). In contrast, the cell wall at the tip is thin and flexible (Figure 1.9 K; Bosch and Hepler, 2005) and consists almost exclusively of pectin (Ferguson et al., 1998), which is secreted in its esterified form at the apex of the growing pollen tube. Demethylesterification of pectin compounds, such as homogalacturonan by pectin methylesterases (PME), followed by complexation with Ca^{2+} leads to stiffening of the cell wall in subapical regions (Parre and Geitmann, 2005). The importance of the action of PMEs has been shown by the addition of exogenous PMEs to pollen tubes growing *in vitro*, which leads to an increase in cell wall stiffness and growth arrest (Bosch et al., 2005; Parre and Geitmann, 2005). Analysis of an *Arabidopsis* mutant lacking the pollen-specific PME, VGD1 (vanguard 1), revealed that pollen tubes of this mutant burst when growing *in vitro* and are also greatly reduced in fertility, even though the overall PME activity was only reduced by less than 20 % (Jiang et al., 2005).

In order to target vesicles containing cell wall precursors to the apical tip of the growing cell, both pollen tubes and root hairs depend on actin filaments that act as tracks for vesicle trafficking. It is long known that Golgi dynamics depend on actin (Boevink et al., 1998) and that polar growth, cytoplasmic streaming and accumulation of exocytotic vesicles in the apex are inhibited in pollen tubes (Vidali et al., 2001) and root hairs (Bibikova et al., 1999; Miller et al., 1999; Baluska et al., 2000), when the actin cytoskeleton is disrupted. In the pollen tube shank actin filaments are organized in longitudinal cables parallel to the direction of growth (Figure 1.9 D). The actin cables reach up to the base of the clear zone, where actin is organized in a dynamic ring-like network of short filaments called the “actin fringe” (Figure 1.9 H; Lovy-Wheeler et al., 2005). In root hairs the organization of actin cytoskeletal structures in regions distal of the tip is similar to the organization found in pollen tubes. In the tip, however, the fine actin mesh resembles a cap rather than a ring-like structure (Baluska et al., 2000).

The growth of pollen tubes (Holdaway-Clarke et al., 2003; Iwano et al., 2004) and root hairs (Wymer et al., 1997) is not linear but oscillates in phases of faster and slower growth. For instance, the growth rate of lily pollen tubes oscillates between 100 and 400 nm s^{-1} with a periodicity of 15 to 50 s (Holdaway-Clarke et al., 1997; Messerli and

Robinson, 1997); the oscillation of root hair growth is less substantial, ranging from 20 to 25 nm s^{-1} with a periodicity of 700 s (Wymer et al., 1997).
The described structures required for polar tip growth are regulated by many factors, including proteins and secondary messengers, such as $PI(4,5)P_2$ (Figure 1.9 I). Manipulating just one of these factors can have dramatic effects on polar tip growth.

1.5.2. Polar tip growth - signaling molecules

One important signaling molecule in pollen tubes is Ca^{2+}. Ca^{2+} can influence the activities of many proteins directly, or indirectly by Ca^{2+}-binding proteins such as calmodulins. Pollen tubes (Taylor and Hepler, 1997) and root hairs (Gilroy and Jones, 2000) contain a Ca^{2+} gradient with the highest concentration in the tip close to the plasma membrane, reaching a concentration of 10 µM in pollen tubes (Messerli et al., 2000) and 1.5 µM in root hairs (Wymer et al., 1997), respectively. If this tip-focused Ca^{2+} gradient is disrupted, cellular expansion is stopped (Felle and Hepler, 1997; Taylor and Hepler, 1997; Wymer et al., 1997), underlining its importance for polar tip growth. It is assumed that Ca^{2+} involved in the maintenance of the tip-focussed Ca^{2+} gradient comes from outside the cell, entering at the apex via plasma membrane-located channel proteins (Vincent et al., 2005). Evidence supporting this hypothesis comes from the observation that Ca^{2+} concentration is highest in pollen tubes right beneath the plasma membrane (Messerli et al., 2000). It has also been reported that drugs blocking the uptake of extracellular Ca^{2+} lead to growth arrest of pollen tubes and root hairs (Schiefelbein et al., 1992; Malho et al., 2000). In root hairs an inward-rectifying Ca^{2+}-conductance was measured at the tip of the plasma membrane (Véry and Davies, 2000). It is likely that Ca^{2+} levels influence exocytosis and actin dynamics, and many calcium binding signaling proteins have been found to be expressed in pollen (Honys and Twell, 2004; Pina et al., 2005; Becker and Feijo, 2007). Despite of all data indicating an involvement of Ca^{2+} in the control of polar tip growth, it remains unclear how Ca^{2+} exactly regulates cellular expansion (Cheung and Wu, 2008). It is actually debated whether Ca^{2+} changes are a cause for or rather a consequence of pollen tube elongation (Hepler et al., 2001). In pollen tubes it was found that calcium levels oscillate with the same phase as the growth rate (Messerli et al., 2000). The Ca^{2+} peak, however, lags the growth peak by 4 s (Messerli et al., 2000). These observations show that cell elongation is not controlled by Ca^{2+} but rather the other way around. Turgor-driven cell elongation stretches the plasma membrane, and it has been speculated that Ca^{2+} channels are stretch-activated (Pierson et al., 1996; Holdaway-Clarke et al., 1997; Messerli et al., 2000). The ensuing increase in Ca^{2+} levels could then increase exocytosis, leading to a relief of plasma membrane tension and starting a new turgor-driven oscillatory wave of growth.
While Ca^{2+} is clearly an important factor of - so far - unresolved function, other signaling factors with importance in plant polar cell growth have also been reported. The roles of one group of proteins, Rho guanine triphosphatases (GTPases) of the Rac-Rop subfamily, in polar tip growth have been the focus of a number of studies and have recently been reviewed (Kost, 2008). Like other small GTPases, Rac-Rop GTPases are monomers that are active when bound to GTP and inactive in their GDP-bound form. Rac-Rop GTPases

localize at the apex of growing pollen tubes and root hairs, and it has been hypothesized that they play a role in membrane trafficking and the organization of the actin cytoskeleton (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002). Overexpression of these GTPases leads to a loss of polar growth as well as tip swelling in pollen tubes and root hairs (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Jones et al., 2002), whereas overexpression of inactive variants leads to growth arrest (Kost et al., 1999; Li et al., 1999; Jones et al., 2002). Rac-Rop GTPases are negatively regulated by RhoGAPs (Rho GTPase activating proteins) that increase the intrinsic GTPase activity of the GTPases, which, in turn, leads to their inactivation (Klahre and Kost, 2006). The tobacco protein RhoGDI2 (Rho GDP-dissociation inhibitor 2), is exclusively expressed in pollen tubes (Klahre et al., 2006), and inactivates Rac-Rop GTPases by binding and displacing them from the plasma membrane to the cytosol (DerMardirossian and Bokoch, 2005). Rho-GEFs, on the other hand, which were just recently discovered in plants (Berken et al., 2005) activate inactive GDP-bound Rac-Rop GTPases by replacing GDP with GTP. Since RhoGAPs and RhoGDIs inactivate Rac-Rop GTPases whereas Rho-GEFs activate them, it is not surprising that overexpression of these proteins in pollen tubes leads to growth arrest (Klahre et al., 2006; Klahre and Kost, 2006) or tip swelling (Gu et al., 2006; Zhang and McCormick, 2007), respectively.

One enigmatic factor in polar tip growth are phosphoinositides, the topic of this thesis. It has been previously shown that root hairs and pollen tubes have a $PI(4,5)P_2$ (Braun et al., 1999; Kost et al., 1999; Kusano et al., 2008; Stenzel et al., 2008) and a PI4P domain (Jin and Heilmann, personal communication, Thole et al., 2008) at the apical part of their plasma membrane (Figure 1.9 I). Phosphoinositides play an important role in membrane trafficking, organization of the cytoskeleton, and in ion channel regulation, as has been described above. Therefore, it comes as no surprise that polar tip growth depending on these processes can be disturbed by manipulation of phosphoinositide levels. For instance, disruption of the PLC-mediated $PI(4,5)P_2$ degradation leads to a loss of polar growth and tip swelling in pollen tubes (Dowd et al., 2006; Helling et al., 2006), accompanied by distortion of the cytoskeleton (Dowd et al., 2006) and an increase of intracellular calcium levels (Dowd et al., 2006). *Arabidopsis* plants mutated in the *PIP5K3* gene which is specifically expressed in root epidermal cells have very short root hairs (Kusano et al., 2008; Stenzel et al., 2008), and overexpression of PIP5K3 also leads to aberrant root hair morphology (Kusano et al., 2008; Stenzel et al., 2008). Regulation of PI4P levels is also important for normal root hair growth, since plants mutated in type β PI 4-kinases or the PI4P-degrading phosphatase, RHD4, display aberrant root hair morphology (Preuss et al., 2006; Thole et al., 2008).

Phosphoinositides are very important for the regulation of exocytosis, as has been outlined in previous paragraphs. One aspect of this regulation is the recruitment of the exocyst complex to the plasma membrane via $PI(4,5)P_2$. Members of the plant exocyst complex, Sec3/Rth1 (roothairless1) in *Zea* mays (Wen et al., 2005) and Sec8 in *Arabidopsis* (Cole et al., 2005), have been found to be involved in the formation of root hairs and pollen tubes, respectively, highlighting their importance in polar tip growth. Even though a putative interaction of these proteins with $PI(4,5)P_2$ was not investigated,

$PI(4,5)P_2$ might be involved in the targeting of the exocyst complex to the plasma membrane in plants in analogy to other eukaryotic models, suggesting $PI(4,5)P_2$ as an important regulator of exocytosis in tip growing cells.

1.6 Microscopy

Because the main data presented in this thesis was obtained using fluorescence microscopy, key aspects of fluorescent reporters and their detection by different microscopic methods will be introduced in the following paragraphs.

1.6.1. Epifluorescence and confocal microscopy

Epifluorescence microscopy has been used for the investigation of biological questions for more than 30 years. In epifluorescence microscopy, light from a source such as a mercury lamp is limited to a certain band of wavelengths by a filter. This light is focused on the object of interest and can excite fluorophores that emit, then, light of a higher wavelength. The emitted light is filtered again, to block reflected and scattered light, so that only the emitted light is viewed or recorded. One of the problems inherent to epifluorescence microscopy is that especially thicker regions of cells or tissue sections cannot be observed, because fluorescence from above and below the focal plane blurs the image. Confocal microscopy was already invented over 50 years ago (Minsky, 1961), but was only seriously developed for biological imaging around 30 years later. Since then, confocal microscopy has helped to obtain fluorescence images with higher resolution and made it possible to overcome the described problems of epifluorescence microscopy (White et al., 1987; Amos and White, 2003). The principle of modern laser scanning confocal microscopy is similar to that of epifluorescence microscopy, however, with some crucial differences. First of all, a laser is used rather than a mercury lamp. Secondly, the object is only illuminated in one point, and there is a pinhole in front of the detector situated in an optically conjugated plane to eliminate emitted light from planes that are out of focus. Since only one point is illuminated at a time, the microscope has to scan a raster of lines to obtain an image of a defined optical plane. The thickness of this plane can be altered by adjusting the aperture of the pinhole. When many adjacent planes are scanned, the information can be projected to obtain a three-dimensional representation of the object. Since most of the biological substances exhibit no specific fluorescence, structures or proteins have to be stained or labeled to obtain information about their localization. How this can be achieved will be described in the next paragraph.

1.6.2. Fluorescent dyes and chimeric fluorescent proteins

In the early days of fluorescence, microscopy proteins were localized by specific antibodies that were conjugated to fluorescent labels. Other fluorescent molecules that would bind to certain subcellular structures were also developed. One example is phalloidin (Small et al., 1999), coupled to a fluorescent dye that specifically binds F-actin. The problem of these approaches is, that antibodies or other molecules specifically binding certain target proteins are not always available, and that antibodies often exhibit cross-reactivity with other proteins. Another problem is that it is not always easy to perfuse

fluorescent probes into the cells to access the protein of interest due to restrictions by the cell wall and the plasma membrane. Therefore, cells often have to be fixed before staining, which makes it impossible to monitor them *in vivo*. Another possibility to determine the subcellular localization of proteins is to express them as fusion constructs with fluorescent proteins. The first fluorescent protein found was the green fluorescent protein (GFP) of the jelly fish *Aequorea victoria* (Shimomura et al., 1962). This protein has a β-barrel structure containing the fluorophore in the centre (Ormo et al., 1996). This fluorophore forms autocatalytically from the three amino acids S65-Y66-G67 without help of accessory proteins. Because the original GFP found was not optimal for biological applications, amino acid changes were introduced to make this protein more stable and brighter (Janke et al., 2004). It was also possible to change the absorption and emission spectra by amino acid exchanges in the vicinity of the fluorophore (Patterson et al., 1997; Tsien, 1998), giving rise to a multitude of different fluorescent proteins (Janke et al., 2004). Two of them, cyan fluorescent protein (CFP) and enhanced yellow fluorescent protein (EYFP), were used in this work. Other fluorescent proteins, Redstar (Janke et al., 2004) and mCherry (Shu et al., 2006), also used in this work, derive from a different fluorescent protein, DsRed, from the sea anemone *Discosoma striata*. Its fluorophore is similar to that of GFP but contains one more double bond, leading to the red shift of the absorption and emission spectra (Gross et al., 2000; Yarbrough et al., 2001). Since the fluorophores of the described proteins form autocatalytically, it is feasible to express these fluorescent proteins as fusions with any other protein in any cell type that can be stably or transiently transformed. Cells can be observed *in vivo* and dynamic protein localization can be studied in real time.In this thesis, often two different fluorescent reporters were coexpressed as fusions to different proteins, allowing for the simultaneous observation of these proteins *in vivo*.

1.7 Goals

To this date the enzymes responsible for $PI(4,5)P_2$ production in pollen tubes have not been characterized. In this thesis putative PI4P 5-kinases with suspected roles in $PI(4,5)P_2$ production in pollen tubes were the main focus of investigation.
The detailed goals of this thesis are outlined as follows:

- The first goal of this work was to identify PI4P 5-kinases that could play a role in pollen tube growth according to their expression pattern and to characterize the recombinant enzymes biochemically.
- The next goal was to identify mutants disrupted in the genes encoding the PI4P 5-kinases identified and to analyze their pollen and pollen tube morphology.
- Another goal was to monitor the localization of the selected PI4P 5-kinases in pollen tubes, especially in relation to the described $PI(4,5)P_2$ domain in the apical plasma membrane.
- To increase $PI(4,5)P_2$ levels in pollen tubes, PI4P 5-kinases should be overexpressed, and effects on pollen tube morphology were to be studied.
- Further experiments were to be carried out to identify cellular functions responsible for the observed phenotypical changes.

The study of these enzymes could lead to a better understanding of the role of $PI(4,5)P_2$ in the polar growth of pollen tubes. Pollen tubes are model systems not only for polar growing cells but all cell types because they are especially suited to study actin dynamics, exocytosis, cell wall deposition and ion channel activity. Therefore results obtained in this work could give insights into cellular processes occurring not only in pollen tubes but also in other cell types of plants and other organisms.

2. Material and Methods

Methods used in this thesis are mostly dealing with the identification of T-DNA insertion mutants, the molecular cloning of cDNA constructs encoding chimaeric fusions of PI4P 5-kinases of various origins, their stable or transient expression in plant cells, and the subsequent examination of fluorescence distribution by epifluorescence microscopy or laser scanning confocal microscopy. Various fluorescent dyes were used to specifically stain subcellular structures. Proteins of interest were recombinantly expressed in *E. coli* and biochemically characterized.

2.1 Material

2.1.1. Chemicals

γ-[^{32}P]ATP	Hartmann Analytics, Brunswick, Germany
Agar	Invitrogen, Karlsruhe, Germany
Agarose	Duchefa Biochemie, Haarlem, The Netherlands
Glufosinate-Ammonium (BASTA)	Bayer Crop Science, Germany
Brome phenol blue sodium salt	Serva, Heidelberg, Germany
Carbenicillin	Duchefa Biochemie, Haarlem, The Netherlands
Desoxynukleotid triphosphate (dNTPs)	Roche Molecular Biochemicals, Mannheim, Germany
Yeast extract	Sigma, Deisenhofen, Germany
Isopropyl-β-D-thiogalactosylpyranosid (IPTG)	AppliChem, Darmstadt, Germany
Kanamycin	Duchefa Biochemie, Haarlem, The Netherlands

2-(N-morpholino)ethanesulfonic acid (MES)	Serva, Heidelberg, Germany
Murashige & Skoog medium	Duchefa Biochememie, Haarlem, The Netherlands
Pepton	Invitrogen, Karlsruhe, Germany
Phosphoinositide standards	Avanti Polar Lipids, Inc, Alabaster, AL, USA
Phytagel	Sigma, Deisenhofen, Germany
Rifampicin	Duchefa Biochemie, Haarlem, The Netherlands
Szintillation fluid	Zinsser Analytics, Frankfurt, Germany
Silwet-Copolymer	OSi Specialties Inc., South Charleston, WI USA

All other chemicals were obtained from the companies Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany), Fluka (Steinheim, Germany) or Sigma (Deisenhofen, Germany).

2.1.2. Enzymes and size markers

Go*Taq*-DNA-Polymerase	Promega, Mannheim, Germany
Phusion High Fidelity DNA-Polymerase	Finnzymes, Espoo, Finnland
MasterAmp *Tfl*-DNA-Polymerase	EPICENTRE Biotechnologies, Madison, WI USA
Takara *Ex Taq*™ DNA Polymerase	Takara Bio Inc, Madison, WI, USA
Gateway® LR Clonase™ Enzyme Mix	Invitrogen, Karlsruhe, Germany
RevertAid H Minus M-MuLV Reverse Transcriptase	MBI Fermantas, St. Leon Rot, Germany
Restriktionsendonukleasen	
T4-DNA-Ligase	
Klenow-Fragment	
GeneRuler™ 1kb DNA-Ladder	
RNase-free DNAseI	

2.1.3. Kits

Big Dye Terminator v1.1 Cycle Sequenzier-Kit	Applied Biosystems, Darmstadt, Germany
pGEM-Teasy Ligation Kit	Promega, Heidelberg, Germany
Nucleospin Plasmid Kit	Machery Nagel, Düren, Germany
Nucleospin Extract II Kit	
Plasmid Midi Core Kit	Qiagen, Hilden, Germany

2.1.4. Staining reagents used in microscopy

Alexa 543-phalloidin	Invitrogen, Karlsruhe, Germany
FM 4-64	Molecular Imaging Products, Bend, OR, USA
Toluidine blue O	Sigma, Deisenhofen, Germany

5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc)	Glycosynth, Warrington, UK
Aniline blue coloring fluid	Euromex, Arnheim, The Netherlands

2.1.5. Equipment

UV imager raytest IDA	Herolab, Wiesloch, Germany
Shaking mill MM200	Retsch, Haan, Germany
Helium driven particle accelerator PDS-1000	Bio Rad, Munich, Germany
Stereo microscope SZX12	Olympus, Hamburg, Germany
Epifluorescence and bright field microscope BX51	
F41-028 HQ Filterset for Yellow GFP	
UMWU 2 UV filterset	
P31-044 Filterset for CFP	
F41-007 HQ-filterset for Cy3	
Color View II Camera	
Confocal microscope LSM510	Carl Zeiss Inc., Jena, Germany

2.1.6. Single-use materials

Glass beads 2,85-3,3 mm	Roth, Karsruhe, Germany
Cellulose Acetate Filter 0.2 µm	Sartorius, Göttingen, Germany
Filter paper	Whatman, Maidstone, UK
Silica thin layer chromatography plates Si-60, 20 x 20 cm	Merck, Darmstadt, Germany
Sterile filters 0.45 µm	Sarstedt, Nümbrecht, Germany
1.0 Micron Gold Microcarriers, Stopping Screens, Rupture Disks	Bio Rad
Autoradiographie film X-Omat	Kodak Stuttgart, Germany

2.1.7. Software

analySIS Docu 3.2	Soft Imaging Systems GmbH, Münster, Germany
LSM 510 software v 4.0	Carl Zeiss Inc., Jena, Germany
Photoshop	Adobe Systems, Munich, Germany
Chromas Lite v 2.0	Technelysium, Tewantin, Australia

2.1.8. Plasmids

Vector	Selection marker	Obtained from	Plasmid structure
pGEM®-T Easy	Amp^R	Promega, Mannheim, Germany	
pUC18ENTRY	Amp^R	Ellen Hornung, Göttingen, Germany	pUC18 backbone with the MCS from pENTR2b flanked by the attL1 and attL2 sequences required for the Gateway®-System
pENTR2b	Km^R	Invitrogen, Karlsruhe, Germany	Plasmid contains a MCS flanked by the attL1 and attL2 sequences required for the Gateway®-System
pLatGW	Amp^R	Wolfgang Dröge-Laser, Göttingen, Germany	Plasmid contains a Lat52 promotor used for pollen-specific expression in front of a Gateway cassette containing attR1 and attR2 sequences required for the Gateway®-System
pCAMBIA3300-0GC	Amp^R	Ellen Hornung, Göttingen, Germany	Plasmid contains a BASTA resistence under a 35S promoter and a Gateway cassette containing attR1 and attR2 sequences required for the Gateway®-System
pET-M41	Amp^R	Achim Dickmanns, Göttingen, Germany	

2.1.9. Oligonucleotides

All oligonucleotides were obtained from Invitrogen, Karlsruhe, Germany. A complete list and the respective sequences are comprised in the Appendix.

2.1.10. Bacterial strains

Organism	Strain	Genotypy	Reference	Obtained from
E. coli	XL1Blue	recA1endA1gyrA96 thi-1hsdR17 supE44 relA1lac[F`proAB lac9zM15 Tn19(Tetr)]	(Bullock et al., 1987)	Stratagene, Heidelberg, Germany
E. coli	BL21-AI	F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm araB::T7RNAP-tetA		Invitrogen, Karlsruhe, Germany
A. tumefaciens	pEHA105	pTiBo542ΔT-DNA RifR	(Hood et al., 1993)	-

2.1.11. Plant lines

Name	Species	Transgenes	Resistance	obtained from
Tobacco WT	*Nicotiana tabacum* Ecotype Samsun NN	-	-	
Arabidopsis WT	*Arabidopsis thaliana* Ecotype Col 0	-	-	
Arabidopsis PIP5K1 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K1::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K2 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K2::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K3 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K3::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K4 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K4::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K5 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K5::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K6 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K6::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K7 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K7::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K8 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K8::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K9 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K9::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K10 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K10::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis PIP5K11 promotor GUS line	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K11::uidA; 35S::Npt1*	Kanamycin	Irene Stenzel, Göttingen, Germany
Arabidopsis Lat52::PIP5K:EYFP	*Arabidopsis thaliana* Ecotype Col 0	*Lat52::PIP5K5:EYFP 35S::Bar gene*	BASTA	Generated during this work
Arabidopsis Lat52::PIP11K:EYFP	*Arabidopsis thaliana* Ecotype Col 0	*Lat52::PIP5K11:EYFP 35S::Bar gene*	BASTA	Generated during this work
Arabidopsis PIP5K11::PIP5K11:EYFP	*Arabidopsis thaliana* Ecotype Col 0	*PIP5K11::PIP5K11 :EYFP 35S::Bar gene*	BASTA	Generated during this work
Arabidopsis pipk4 mutant	*Arabidopsis thaliana* Ecotype Col 0	SALK cassette SALK_001138	Kanamycin	SALK institute, La Jolla, CA, USA
Arabidopsis pipk5 mutant	*Arabidopsis thaliana* Ecotype Col 0	SALK cassette SALK_147475	Kanamycin	SALK institute, La Jolla, CA, USA

continued on next page

2.1.11 Plant lines (continued)

Arabidopsis pipk4 pipk5 double mutant	*Arabidopsis thaliana* Ecotype Col 0	SALK cassette SALK_001138; SALK cassette SALK_147475	Kanamycin	Generated by crossing during this work
Arabidopsis pipk4 pipk5 double mutant PIPK4::PIP4K:EYFP	*Arabidopsis thaliana* Ecotype Col 0	SALK cassette SALK_001138; SALK cassette SALK_147475; *PIPK4::PIP4K:EYFP*	Kanamycin, BASTA	Generated during this work
Arabidopsis pipk4 pipk5 double mutant PIPK5::PIP5K:EYFP	*Arabidopsis thaliana* Ecotype Col 0	SALK cassette SALK_001138; SALK cassette SALK_147475; *PIPK5::PIP5K:EYFP*	Kanamycin, BASTA	Generated during this work
Arabidopsis pipk10 mutant	*Arabidopsis* thaliana Ecotype Col 0	SALK cassette SALK_119243	Kanamycin	SALK institute, La Jolla, CA, USA
Arabidopsis pipk11 mutant	*Arabidopsis* thaliana Ecotype Col 0	GABIKat cassette GABI _284F05	Sulfadiazin	IGS, Bielefeld, Germany

2.2 Methods

2.2.1. Preparation of chemically competent *E. coli* cells

In preparation of transformation experiments *E.coli* XL1-Blue were made chemically competent (Inoue et al., 1990). Cells were picked from plate and precultured in 2 ml SOB medium (2 % peptone, 0.5 % (w/v) yeast extract, 10 mM NaCl 2.5 mM KCl) overnight. Cultures were diluted 1: 100, 1 ml was transferred to 250 ml of SOB medium and cells were grown at 37 °C until an OD_{600} of 0,45 - 0,75 was reached. Cells were collected by centrifugating at 1000 g and 4 °C for 10 min and resuspended in 80 ml of TFP buffer (10 mM Pipes, pH 6.7, 15 mM $CaCl_2$, 250 nM KCl, 55 mM $MnCl_2$) and incubated on ice for 10 min. Afterwards centrifugation was repeated and cells were resuspended in 21.5 ml TFB buffer containing 7 % (v/v) DMSO and incubated again for 10 min on ice prior to aliquoting, shock freezing in liquid nitrogen and storage at -80 °C.

2.2.2. Transformation of chemically competent *E. coli* cells

For transformation according to Inoue (Inoue et al., 1990) 100 µl of *E. coli* cells were thawed and added to the plasmid DNA in a 2 ml reaction tube. After incubation on ice for 20 min the cells were heat shocked at 42 °C for 45 s, immediately incubated on ice for 5 min. After addition of 300 µl SOC medium (1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl, 20 mM sucrose, 20 mM $MgCl_2$) cells were incubated at 37° C for 1 h under constant shaking and plated on solid LB medium (1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1 % (w/v) NaCl solidified with 1.5 % (w/v) agar) containing the adequate selection antibiotic (carbenicillin 100 µg/ml kanamycin 50µg/ml). For blue/white selection when using the pGEM-T Easy plasmid plates contained additionally 0.004 % (w/v) X-Gal and 0.2 mM IPTG. In liquid culture *E. coli* cells were grown in LB medium (as above but without agarose; antibiotics were used in the same concentrations as in plates).

2.2.3. Preparation of chemically competent *Agrobacterium tumefaciens* cells

The *Agrobacterium tumefaciens* strain EH 105 (Hood et al., 1993) was made competent as follows. Cells were taken from a plate containing 50 µg/ml rifampicin and grown in a 2 ml YEB (0.5 % (w/v) beef extract, 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.5 % (w/v) saccharose, 5 mM $MgSO_4$) culture at 28 °C over night. This culture was transferred to a flask containing 50 ml YEB and grown at 28 °C for approximately 4 h until an OD_{600} of 0.5 was reached. Cells were collected by centrifugation at 5000 g and 4 °C for 5 min and resuspended in 10 ml of ice cold 0.15 M NaCl solution. After another centrifugation step under the same conditions cells were resuspended in 1 ml of ice cold 75 mM $CaCl_2$ solution, aliquoted, shock frozen in liquid nitrogen and stored at -80 °C.

2.2.4. Transformation of chemically competent *Agrobacterium tumefaciens* cells

For transformation according to (Hofgen and Willmitzer, 1988) 100 µl of competent *Agrobacterium tumefaciens* cells were thawed, placed on ice and 3 µg of plasmid DNA were added. After incubation for 5 min the cells were shock frozen in liquid nitrogen for 5 min and immediately heat shocked for 5 min in a 37°C water bath. After an addition of

900 µl of YEB medium cells were incubated at 28 °C for 4 h under constant shaking and plated on solid YEB (solidified with 1.5 % (w/v) agarose) medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin.

2.2.5. Isolation of plasmid DNA from bacterial cultures

Plasmids were isolated from small scale (4 ml) liquid *E. coli* cultures using the NucleoSpin Plasmid Kit (Macherey-Nagel, Düren, Germany) as recommended by the manufacturer. To generate larger amounts of DNA, plasmids where isolated from medium scale (40 ml) *E. coli* cultures using the CompactPrep Plasmid Midi Core Kit (Qiagen, Hilden, Germany) following manufacturer's recommendations. For purification of DNA from solutions or agarose gel pieces the NucleoSpin Extract II Kit (Macherey-Nagel, Düren, Germany) was used following manufacturer's recommendations.

2.2.6. Reverse transcription

Reverse transcription was performed with RevertAid H Minus M-MuLV *Reverse Transcriptase* (MBI Fermentas, St-Leon Rot, Germany) in the presence of oligo(dT) primers using 5 µg of floral RNA (kindly provided by Irene Stenzel, Göttingen, Germany) as a template according to manufacturer's recommendations. 0.5 µl of the obtained cDNA solution were used as template for the amplification of specific gene sequences in subsequent polymerase chain reactions (PCR).

2.2.7. Amplification of specific sequences by PCR

For DNA amplification from cDNA or genomic DNA for application in cloning procedures *Phusion High-Fidelity PCR Master Mix* (Finnzymes, Espoo, Finland) was used following manufacturer's recommendations. For the reaction the following temperature program was applied: 98 °C 30 s, [98 °C 15 s, 60 °C 30 s, 72 °C 1 min/ 1000 bp gene length] x 35, 72°C 5 min.
30 s at 98 °C; 28 cycles of 15 s at 98 °C, 30 s at 60 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72°C.
For colony PCR MasterAmp *Tfl* DNA *Polymerase*, (EPICENTRE Biotechnologies, Madison, WI, USA) was used following manufacturer's recommendations and the following temperature program was used: 2 min at 95 °C; 28 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72°C.

2.2.8. Electrophoretic DNA separation

DNA was separated after addition of 1/5 volume of sample buffer (250 mM EDTA, 0.58 M sucrose, 50 % (w/v) glycerol and 0.4 % (w/v) Orange G) on TAE agarose gels (40 mM TRIS/HCL, pH 7.0, 20 mM acetic acid, 1 mM EDTA solidified with 1 % (w/v) agarose) using GeneRuler 1 kb DNA Ladder as a size marker and subsequently stained for 10 min with ethidium bromide in a TAE staining solution containing 2 µg/ml ethidium bromide. DNA bands were then visualized using a UV-imager (raytest IDA; Herolab, Wiesloch, Germany)

2.2.9. Assembly of DNA constructs

For conventional DNA construct assembly, plasmids were restricted and ligated according to the recommendations of the enzyme manufacturer (MBI Fermentas, St.Leon Rot, Germany).

For cloning of PCR products into the pGEM-T Easy vector an adenosine nucleotide was added to the 3'-end of the purified PCR products by using 0.5 µl of MasterAmp *Tfl* DNA *Polymerase*, (EPICENTRE Biotechnologies, Madison, WI, USA) and dATP at a final concentration of 100 pM in a total volume of 50 µl buffered solution. Ligation was carried out using the pGEM-T Easy vector system Kit (Promega, Heidelberg, Germany) according to manufacturer's recommendations.

When DNA constructs were generated using the GATEWAY system the reaction was performed using 75 ng of donor and target plasmid and 0.25-0.5 µl of Gateway LR Clonase enzyme mix in a total volume of 5 µl containing 1 µl of 5x buffer. After transformation as described cells from individual colonies were resuspended in 100 µl of H_2O and selected on plates for kanamycin, ampicillin and chloramphenicol resistance. Only colonies with a single resistance were further investigated because all other colonies contained unwanted vectors generated by side reaction of the Clonase enzyme mixture.

2.2.10. Site-directed mutagenesis

Base pair exchanges were introduced into genes using QuickChange technology (Stratagene, La Jolla, CA, USA). In this method the template plasmid is completely replicated by a DNA polymerase starting from the primers that contain the desired base pair exchanges. In this reaction 50 ng of plasmid DNA were used in a total volume of 25 µl containing 10 pmol of each primer, 0.75 µl DMSO and 12.5 µl of 2x *Phusion* High-Fidelity PCR Master *Mix* (Finnzymes, Espoo, Finland). For the reaction the following temperature cycle was used: 30 s at 95 °C; 21 cycles of 30 s at 95 °C, 1 min at 55 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72°C. The mixture of product and template was subsequently restricted over night with 10 U of DpnI that recognizes and cleaves the methylated restriction site Gm6ATC (MBI Fermentas, St. Leon Rot, Germany). This led to the elimination of most of the methylated template leaving the unmethylated product intact which was transformed into *E. coli* as described.

2.2.11. DNA sequencing

DNA was sequenced by the dideoxy termination method using 50 ng of plasmid in a total volume of 10 µl containing 1.5 µl sequencing buffer, 1.5 µl reaction mix and 10 pmol of primer. For the reaction the following temperature cycle was used: 2 min at 95 °C; 25 cycles of 30 s at 95 °C, 15 s at 55 °C, 4 min at 60 °C. The reaction product was separated by Andreas Nolte (University Göttingen) using capillary electrophoresis.

2.2.12. Cloning strategies

DNA fragments amplified by PCR were precloned into the pGEM-T Easy vector (Promega, Heidelberg, Germany) and sequenced to exclude falsely amplified DNA fragments.

For transient expression in pollen tubes DNA fragments were transferred from the pGEM-T Easy vector in frame into the pENTR2B (Invitrogen, Karlsruhe, Germany) vector that had been previously modified to contain a gene encoding either an N-terminal or C-terminal fluorescent protein such as EYFP, CFP or Redstar (Janke et al., 2004). This vector harbored sites for directional cloning using the GATEWAY system, and the DNA fragment of interest could be transferred with the fluorescence tag into the vector pLatGW, which was also suitable for using the GATEWAY system and contained the strong pollen-specific promoter Lat52 (Twell et al., 1990). The pLatGW plasmid was kindly provided by Wolfgang Dröge-Laser (Göttingen, Germany)

The strategy for stable expression of genes in *Arabidopsis* was similar. Here the specially modified vector pUC18ENTRY suitable to be used as a donor vector in the GATEWAY system was used. First the gene coding a fluorescent protein was transferred into this vector followed by the promoter and finally the gene of interest. These three DNA fragments were transferred together to the pCambia3300.0GC *Arabidopsis* expression vector suitable for using the *Agrobacterium*-mediated stable *Arabidopsis* transformation by the floral dip method (Clough and Bent, 1998). Both the pUC18ENTRY and pCambia3300.0GC plasmids were kindly provided by Ellen Hornung (Göttingen, Germany).

2.2.13. Heterologous expression in *E. coli*

Recombinant enzymes were expressed in *E. coli* strain BL21-AI (Invitrogen, Karlsruhe, Germany) at 25 °C for 18 h after induction with 1 mM IPTG and 0.2 % (w/v) L-arabinose. Cell lysates were obtained by sonification in a lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, and 10 % (v/v) glycerol at pH 8.0.

2.2.14. Lipid kinase assays

Lipid kinase activity was assayed by monitoring the incorporation of radiolabel from γ[^{32}P]ATP (Hartmann Analytics, Bonn, Germany) into defined lipid substrates (Avanti Polar Lipids, Alabaster, AL, USA) as described (Cho and Boss, 1995), using 30 µl of total extracts of BL-21-AI expression cultures in a total volume of 50 µl containing 15 mM $MgCl_2$, 1 mM Na Molybdate, 0.9 mM ATP, 9 µCi γ-[^{32}P]ATP, 25 ng lipid substrate 0.2 % (v/v) Triton X-100 and 1 mM Tris, pH 7.2. Recombinant expression levels were adjusted between individual cultures according to immunodetection of expressed MBP-tagged proteins. Radiolabeled lipid reaction products were extracted by addition of 1.5 ml $CHCl_3$:CH_3OH (1:2), 250 µl 0.5 M EDTA, 500 µl 2.4 M HCl, 500 µl $CHCl_3$ mixing the sample after addition of each compound. The organic phase was taken out and the aqueous phase reextracted twice with 500 µl $CHCl_3$. The combined organic phases were washed once with 1.5 ml 0.5 M HCl in CH_3OH:H_2O (1:1), dried and resolubilized in 20 µl $CHCl_3$. The samples were then separated by thin-layer-chromatography using silica S60 plates (Merck, Darmstadt, Germany) and $CHCl_3$:CH_3OH:NH_4OH:H_2O (57:50:4:11 v/v/v/v) as a developing solvent. Radiolabeled lipids were visualized by autoradiography using Kodak X-Omat autoradiography film (Eastman Kodak, Stuttgart, Germany). Reaction products were identified by comigration with authentic standards (Avanti Polar Lipids,

Alabaster, AL, USA). Lanes with lipid standards (5 µg) that were run next to the radiolabeled samples were visualized by the application of aqueous 10 % (w/w) $CuSO_4$ containing 8 % (w/v) H_3PO_4 followed by heating to 180 °C (König et al., 2007). Phosphatidylinositol-bisphosphate bands were scraped according to autoradiography, and incorporated radiolabel was quantified using liquid scintillation counting (Analyzer Tricarb 1900 TR, Canberra Packard, Schwadorf, Austria).

2.2.15. Plant growth conditions

Tobacco plants were grown on soil (Frühstorfer Erde type: T25 Str. 1 (Fein), Industrie Erdwerk Archut, Lauterbach-Wallenrod, Germany) in the green house under 16 h of light (~130- 150 µmol photons m^{-2} s^{-1}).

Arabidopsis lines were precultured in Petri dishes containing MS growth medium (0.22 % (w/v) Murashige & Skoog medium (Duchefa Biochemie, Haarlem, The Netherlands), 1 % (w/v) sucrose, 0.8 % (w/v) agar) for two weeks under constant light (~130- 150 µmol photons m^{-2} s^{-1}) before transfer on soil and growth under conditions as described above.

2.2.16. Extraction of DNA from *Arabidopsis* leaves

DNA was extracted from *Arabidopsis* material as follows: One medium-sized leaf per sample was frozen in liquid nitrogen and pulverized in a 2 ml reaction tube containing three 3 mm glass beads (Roth, Karlsruhe, Germany) using a type MM200 shaking mill (Retsch, Haan, Germany). Material was kept frozen during the whole procedure until 500 µl of CTAB extraction solution (2 % (w/v) CTAB (Cetyltrimethylammoniumbromid), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl) were added, followed by immediate agitation. The samples were heated to 65 °C for 15 min and cooled before an equal volume of $CHCl_3$:isoamyl alcohol (24:1, v/v) was added. Samples were mixed followed by a centrifugation step at 20000 g for 2 min. 400 µl of the upper (aqueous) phase were transferred to a new reaction tube containing 40 µl of an aqueous solution of 10 % (w/v) CTAB and 0.7 % (w/v) and mixed. After incubating the samples for two minutes 500 µl of isopropanol were added and the samples were mixed. To precipitate DNA, samples were centrifuged at 20000 g for 15 min and the supernatant carefully removed. After a washing step with 70 % EtOH, the pellets were dried briefly and dissolved over night at 4 °C in 100 µl of H_2O.

2.2.17. Identification of T-DNA mutants

Arabidopsis T-DNA insertion mutants were genotyped by PCR using total DNA (isolated as described above) as a template. PCR was performed using *TaKaRa Ex Taq*™ (TAKARA Bio Inc. Madison, WI, USA) polymerase according to manufacturer's recommendations. For the reaction the following temperature program was used: 2 min at 95 °C; 28 cycles of 30 s at 95 °C, 30 s at 62 °C, 1 min per 1000 bp length at 72 °C; 5 min at 72°C.

To identify mutant and WT alleles present in the plants the following primer combinations were used: *pip5k4* (21 + 22 for WT allele and 51 + 22 for mutant allele) *pip5k5* (23 + 24 for

WT allele and 51 + 24 for mutant allele) *pip5k10* (31 + 32 for WT allele and 51 + 32 for mutant allele) *pip5k11* (136 + 137 for WT allele and 136 + 138 for mutant allele).

2.2.18. RT-PCR analysis of specific transcript abundance

Semiquantitative RT-PCR analysis was performed to study the expression of PIP5K4, 5, 10 and 11 in various tissues of wild-type *Arabidopsis* plants. Total RNA was extracted from roots, stems, leaves and flowers using Plant RNA Purification Reagent (Invitrogen, Karlsruhe, Germany). Total RNA was incubated with RNase-free DNAseI (Fermentas, St. Leon Rot, Germany) for 30 min at 37 °C to remove genomic DNA contamination, and RNA was precipitated with 75 µM Na-acetate pH 5,2 in 80 % ethanol. Five µg of total RNA were used as a template for reverse transcription with RevertAid H Minus M-MuLV reverse transcriptase (MBI Fermentas, St. Leon Rot, Germany) in the presence of oligo(dT) primers as described. Equal amounts of first-strand cDNAs were used as templates for PCR amplification using the following primer combinations: PIP5K4 S1+S2; PIP5K5 S3 + S4; PIP5K6 S5+ S6 PIP5K10 S7 + S8 PIP5K11 S9 + S10. The *Arabidopsis* actin gene, *ACT8*, was amplified using primer combination S11 and S12 and served as an internal positive control, as previously described (Bustin, 2000).
For the reaction the following temperature cycle was used: 2 min at 95 °C; 31 or 36 cycles of 30 s at 95 °C, 30 s at 60 °C, 1 min/ 1000 bp length at 72 °C; 10 min at 72 °C.

2.2.19. *Arabidopsis* transformation

Recombinant constructs were introduced into *Arabidopsis* plants through *Agrobacterium*-mediated transformation using the floral dip method (Clough and Bent, 1998). Agrobacteria were precultured over night (30 °C and shaking at 200 rpm) from plates or freezer stocks in 5 ml YEB (see above) medium with 50 µg/ml kanamycin and 50 µg/ml rifampicin. Precultures were used to inoculate 400 ml of the same medium and grown over night at identical conditions. Cells were collected by centrifugation at 10000 g for 20 min and resuspended in a total volume of 600 ml 5 % (w/v) sucrose 0.025 % (v/v) Silwet-Copolymer (OSi Specialties Inc., South Charleston, WI, USA). *Arabidopsis* flowers were dipped in the bacterial suspension and plants hooded over night. This procedure was repeated one week later. Offspring of these plants was grown on soil and sprayed after 7 and 14 days with a solution containing 0.5 % Basta solution (Bayer crop science, Monheim, Germany). Resistant seedlings were individualized after 2–3 weeks; T2 plants were used for further analysis.

2.2.20. Tobacco pollen tube growth and transient gene expression

Mature pollen was collected from 4-6 tobacco (*Nicotiana tabacum*) flowers of 8-week-old plants. Pollen was resuspended in growth medium (5 % (w/v) sucrose, 12.5 % (w/v) PEG-6000, 0.03 % (w/v) casein hydrolysate, 15 mM MES-KOH pH 5.9, 1 mM $CaCl_2$, 1 mM KCl, 0.8 mM $MgSO_4$, 1.6 mM H_3BO_3, 30µM $CuSO_4$, 10µg/ml rifampicin; (Read et al., 1993)), filtered onto cellulose acetate filters (Sartorius, Göttingen, Germany) which were transferred to filter paper (Whatman, Maidstone, UK) moistened with growth medium. Within 5-10 min of harvesting, pollen was transformed by bombardment with plasmid-

coated 1 µm gold-particles using a helium-driven particle accelerator (PDS-1000/He; BIO-RAD, Munich, Germany) using 1350 psi rupture discs and a vacuum of 28 inches of mercury. Gold-particles (1.25 mg) were coated with 5-8 µg of plasmid DNA, by precipitating the DNA with 1 M CaCl and 16 µM Spermidine, and washing the gold particles three times with 95% EtOH. After bombardment pollen was resuspended in growth medium and grown for 5-14 h in small droplets of media directly on microscope slides.

2.2.21. *Arabidopsis* pollen tube growth

Newly opened *Arabidopsis* flowers were collected and prehydrated for 1 h. Pollen of ten flowers was transferred to solid growth medium (0.01 % (w/v) boric acid/KOH, pH 7.5, 5 mM $CaCl_2$, 5 mM KCl, 1 mM $MgSO_4$, 10 % (w/v) sucrose, 0.5 % (w/v) phytagel according to (Boavida and McCormick, 2007), covering an area of 1 cm^2, and incubated for 30 min at 30 °C before further incubation at 22 °C.

2.2.22. Histochemical staining for GUS activity

Histochemical staining of plant tissue for GUS activity was performed as previously described (Jefferson et al., 1987). Tissue samples were vacuum-infiltrated for 5 min in a GUS substrate solution of 100 mM sodium phosphate, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1 % (v/v) Triton-X100, and then incubated at 37 °C for 3 h. Subsequently, the samples were transferred to 70 % (v/v) ethanol to remove chlorophyll pigmentation. Pollen tubes were stained without vacuum-infiltration for 14h and were not destained with ethanol. GUS-positive samples were examined with a bright field microscope (Olympus BX51, Olympus, Hamburg, Germany) or a stereo microscope (Olympus SZX12, Olympus, Hamburg, Germany) at low magnification (4x to 10x) and digital images were recorded. All GUS-stained samples shown represent typical results of at least three independent transgenic lines for each construct.

2.2.23. Staining of cell wall, plasma membrane and F-actin structures

The steryl dye FM 4-64 (Molecular Imaging Products, Bend, OR, USA) was added to pollen tubes at a final concentration of 10 µM (Parton et al., 2001) and visualized after 5-10 min of incubation. During this time range the dye stains only the plasma membrane and no endomembranes.

For cell wall staining according to O'Brien (O'Brien et al., 1964) toluidine blue O (Sigma, Deisenhofen, Germany) was added to pollen tubes at a final concentration of 0.125 % (w/v) 5 min before imaging.

Callose was stained with aniline blue (Fukumoto et al., 2005) by addition of 1/3 volume of aniline blue staining solution to pollen tubes 5 min before imaging under the epifluorescence microscope. The aniline blue staining solution was prepared from equal amounts of aniline blue colouring fluid (Euromex, Arnhem, the Netherlands) and double concentrated tobacco pollen medium (see above).

For staining of actin strands in fixed pollen tubes by phalloidin, transformed pollen grains were spread onto a 24 mm x 32 mm wide and 1 mm-thick growth medium solidified by addition of 0.25 % (w/v) phytagel and grown for 10 h at room temperature. The pollen tubes were fixed at RT for 2 h by the addition of 30 µl of fixation buffer (100 mM PIPES adjusted with TRIS to pH 9, 5 mM $MgSO_4$, 0.05 mM $CaCl_2$, 0.05 % (v/v) Triton-X-100 and freshly added 1.5% (w/v) formaldehyde, 0.05 % (w/v) glutaraldehyde and 5 mM ethylene glycol bis[sulfosuccinimidylsuccinate] (sulfo-EGS)) and washed twice with washing buffer (100 mM PIPES TRIS pH 7, 5 mM $MgSO_4$, 0.05 mM $CaCl_2$, 10 mM EGTA, 0.05 % (v/v) Triton-X-100). The actin cytoskeleton was stained for at least 1 h by the addition of 6.6 µM (10 µl/ml) Alexa 543-phalloidin (Invitrogen, Karlsruhe, Germany) in washing buffer (Lovy-Wheeler et al., 2005).

2.2.24. Microscopy and imaging

Images were recorded using either an Olympus BX51 (Olympus, Hamburg, Germany) epifluorescence microscope or a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Jena, Germany). BX51: Samples were imaged using the following filter sets: F41-028 HQ for EYFP, UMWU 2 UV for aniline blue, P31-044 for CFP and F41-007 HQ for mCherry and RFP (all filters were obtained from Olympus, Hamburg, Germany), an Olympus ColorView II camera (Olympus, Hamburg, Germany) and analySIS Docu 3.2 software (Soft-Imaging-Systems GmbH, Münster, Germany). LSM 510: EYFP was excited at 514 nm and imaged using an HFT 405/514/633 nm major beam splitter (MBS) and a 530-600 nm band pass filter; CFP was excited at 405 nm and imaged using an HFT 405/514/633 nm MBS and a 470-500 nm band pass filter; RedStar and mCherry were excited at 561 nm and imaged using an HFT 405/488/561 nm MBS and a 583-604 nm band pass filter; Phalloidin was excited at 561 nm and imaged using an HFT 458/561 nm MBS and a 575-615 nm band pass filter. In coexpression experiments, CFP and EYFP were synchronously excited at 405 nm and 514 nm, respectively, and imaged using an HFT 405/514/633 nm and NFT 515 nm MBSs and 470-500 nm and 530-600 nm band pass filters, respectively; EYFP and RedStar were synchronously excited at 488 nm and 561 nm, respectively, and imaged using an HFT 405/488/561 nm MBS and a 518-550 nm band pass filter and a 583-636 nm band pass filer, respectively; EYFP and FM 4-64 were synchronously excited at 488 nm and 561 nm, respectively, and imaged using an HFT 405/488/561 nm MBS and a 518-550 nm band pass-filter and a 657-754 nm band pass filter, respectively. If not specified otherwise, images were obtained by confocal microscopy at 630 x magnification using the Zeiss LSM510 image acquisition system and software (v 4.0, Carl Zeiss Inc., Jena, Germany). Fluorescence and transmitted light images were contrast-enhanced by adjusting brightness and γ-settings using image-processing software (Photoshop; Adobe Systems, Munich, Germany). Video sequences were assembled using ImageReady software (Adobe Systems, Munich, Germany). Pollen tube widths, length and fluorescence intensity were analyzed from epifluorescence images using analySIS Docu 3.2 software (Soft Imaging Systems GmbH, Münster, Germany).

2.2.25. Accession numbers

Sequences used in this study can be identified by their accession numbers as follows: PIP5K1, At1g21980; PIP5K2, At1g77740; PIP5K3, At1g77740; PIP5K4, At3g56960; PIP5K5, At2g41210; PIP5K6, At3g07960; PIP5K7, At1g10900; PIP5K8, At3g09920; PIP5K9, At3g09920 PIP5K10, At4g01190; PIP5K11, At1g01460; ACT8, At1g49240; Nag, AJ243198; NtPLC3, EF043044; PIKα1, At1g49340; PIKα2, At1g51040;PIKβ1, At5g64070; EXP4, At2g39700, NCPIPK, NCU022295; HsPIPKIα, P70182.

3. Results

3.1 *Arabidopsis* PI4P 5-kinase isoforms 4, 5, 6, 10, and 11 are expressed in pollen tubes

The *Arabidopsis* genome contains eleven genes with similarities to sequences encoding PI4P 5-kinase isoforms (Mueller-Roeber and Pical, 2002). In order to investigate, which of these isoforms play a role in pollen tube growth and to verify the transcript array information accessible through the Genevestigator portal (Zimmermann et al., 2004), the relevant expression patterns were studied by promoter-GUS experiments and by RT-PCR. In the promoter-GUS experiments transgenic *Arabidopsis* lines, each containing one of the eleven 1500 bp- PI4P 5-kinase promoter fragments driving expression of a GUS-reporter, were histochemically stained in a way that the colourless dye X-gluc was transferred to a blue dye in those tissues that were expressing the GUS gene. From these experiments it could be seen that expression levels in pollen were highest in lines expressing the GUS-gene under the promoter of PI4P 5-kinase genes 4, 5, 6, and 11 (Figure 3.1). Some GUS activity could also be observed in PI4P 5-kinase 1, 2, 8, 9 promoter lines (Figure 3.1), whereas no or very weak GUS-staining was observed in the promoter lines of PI4P 5-kinase 3, 7 and 10 (Figure 3.1).

When pollen grains were grown *in vitro* prior to histochemical staining, the results reflected those obtained with non germinated pollen (Figure 3.2), with the difference that the PI4PK10 promoter line showed greatly increased expression levels after pollen tube germination (Figure 3.2). GUS expression of PI4P 5-kinase promoter lines 10 and 11 was so strong in pollen tubes that staining could be observed in pollen tubes that invaded the pistil (Figure 3.3).

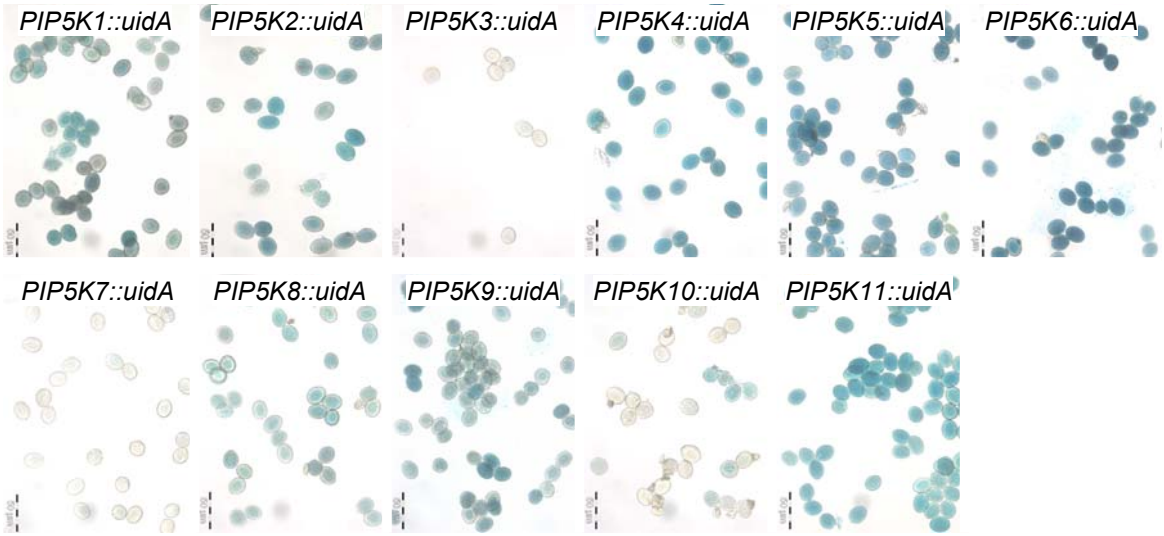

Figure 3.1. PI4P 5-kinase gene expression in pollen.

Histochemical staining for GUS-activity in pollen of transgenic plants expressing the GUS reporter gene *uidA* under putative (1500 bp 5'-UTR) promoter fragments of the genes indicated. Bars, 50 µm. Samples were stained for 24 h at room temperature. Images shown are representative for images obtained from three independent lines. Images were obtained by Irene Stenzel.

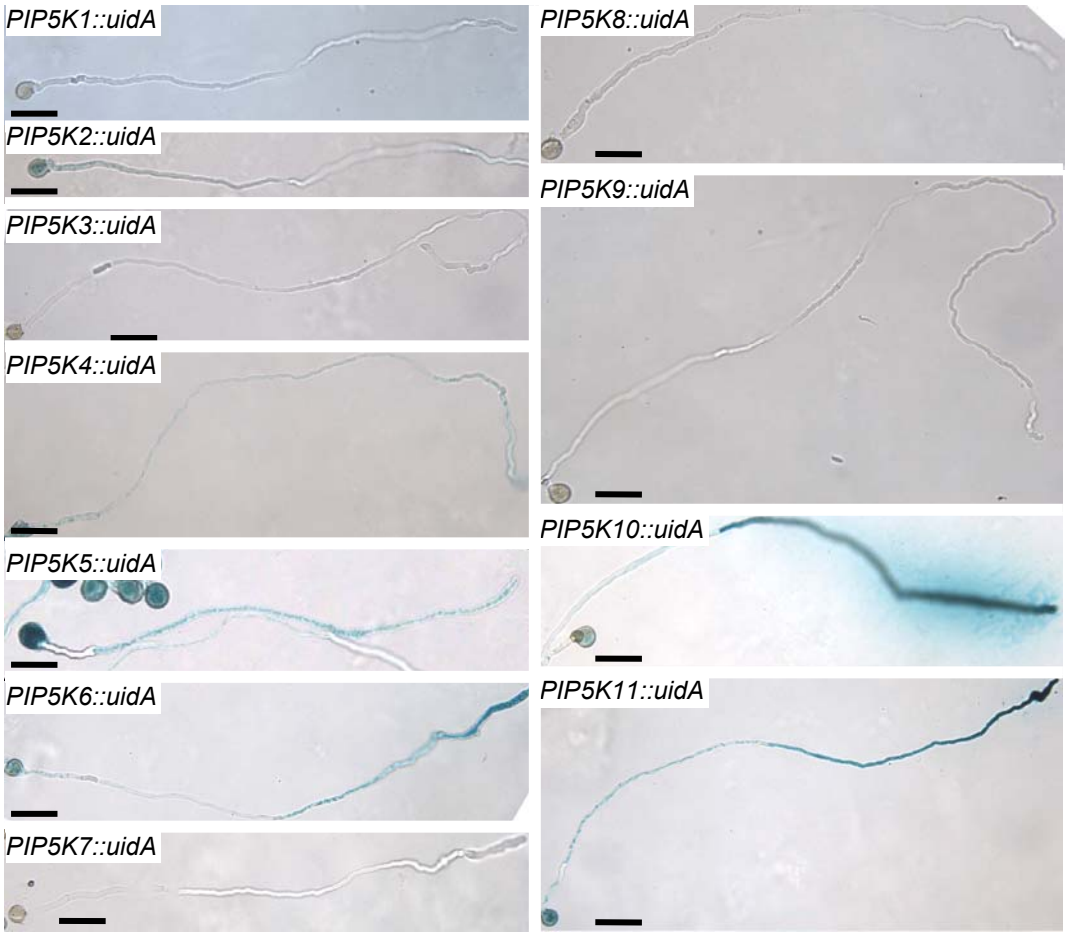

Figure 3.2. PI4P 5-kinase gene expression in pollen tubes

Histochemical staining for GUS-activity in pollen tubes of transgenic plants expressing the GUS reporter gene under putative (1500 bp 5'-UTR) promoter fragments of the genes indicated. Pollen were germinated *in vitro*. Bars, 50 µm. Samples were stained for 24 h at room temperature. Images shown are representative for images obtained from three independent lines.

Figure 3.3. PI4P 5-kinase gene expression in pollen tubes

Histochemical staining for GUS-activity in pollen tubes of transgenic plants expressing the GUS reporter gene under putative (1500 bp 5'-UTR) promoter fragments of the genes indicated. Pollen tubes were stained and imaged after pistil invasion. Bars, 50 µm. Samples were stained for 24 h at room temperature. Images shown are representative for images obtained from three independent lines. Images by Irene Stenzel.

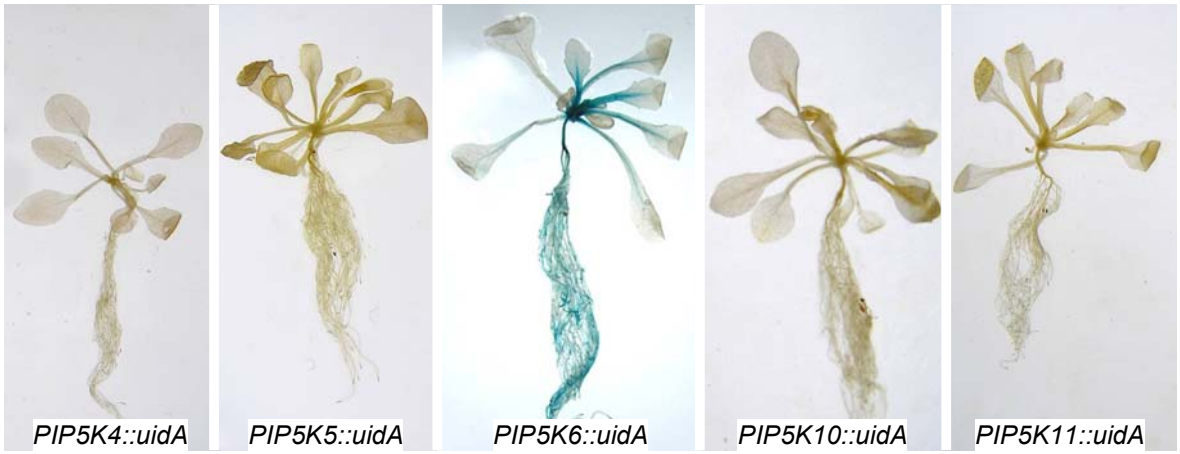

Figure 3.4. PI4P 5-kinase gene expression in whole 21 d old seedlings

Histochemical staining for GUS-activity of 21 d old transgenic plants expressing the GUS reporter gene under putative (1500 bp 5'-UTR) promoter fragments of the genes indicated. Samples were stained for 24 h at room temperature. Images shown are representative for images obtained from three independent lines. Images by Irene Stenzel.

To investigate whether the pollen-expressed PI4P 5-kinase isoforms 4, 5, 6, 10, 11 were also present in other organs, whole seedlings of the *Arabidopsis* promoter-GUS lines were stained. These experiments indicated no or very low expression of PI4P 5-kinase isoforms

4, 5, 10, and 11 in organs other than pollen (Figure 3.4), whereas PIP5K6 was also expressed in the vascular tissues of leaves and roots (Figure 3.4).
RT-PCR analysis of expression in different *Arabidopsis* organs (Figure 3.5) indicates an overall low expression for all PI4P 5-kinase isoforms tested. Expression of PI4P 5-kinases 5, 10 and 11 appears to be restricted to flowers, whereas some PIP5K4 and PIP5K6-transcript was detected by RT-PCR also in leaf- and root-samples.

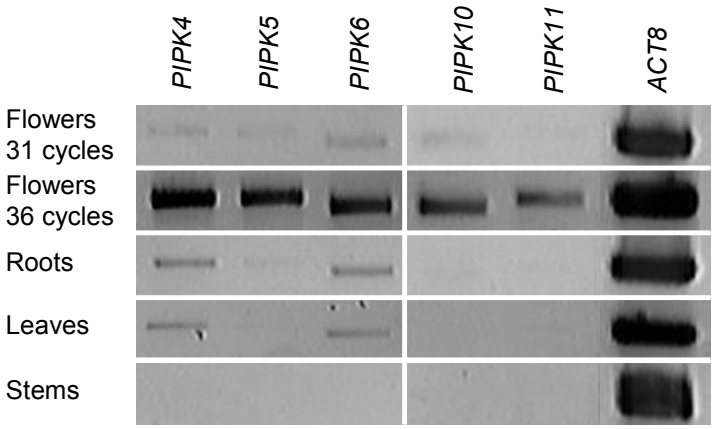

Figure 3.5. Transcript-abundance in different *Arabidopsis* organs according to RT-PCR analysis

The transcript abundance of *PIP5K4, 5, 6, 10* and *11* was determined by RT-PCR as indicated. PCR was run for 31 cycles if not differently annotated. Abundance of *ACT8* transcript was used as a control. Images are from a representative experiment that was performed twice with similar results. Analysis by Irene Stenzel.

3.2 *Arabidopsis* PI4P 5-kinase isoforms 4, 5, 6, 10, and 11 catalyze the conversion of PI4P to PI(4,5)P_2

With the exception of PIP5K10 (Perera et al., 2005) and in the meantime also of PIP5K4 (Lee et al., 2007) the putative PI4P 5-kinase genes used in this study have not been functionally characterized. Therefore, the corresponding cDNAs were cloned, heterologously expressed in *E. coli* as fusions to maltose-binding-protein (MBP) tags, and the recombinant proteins tested for activity *in vitro*. Catalytic activities of the recombinant enzymes with the preferred substrate, PI4P, are given in Figure 3.6. When different potential lipid substrates were tested, minor phosphorylation activity against PI3P was also observed, whereas no activity was detected with PI5P as a substrate (Table 5.1). The *in vitro* activity assays indicate that in addition to isoforms 4 and 10 the sequences annotated as PI4P 5-kinase isoforms 5, 6 and 11 also represent active PI4P 5-kinases. It is also noteworthy that the type B PI4P 5-kinases showed substantially higher enzyme activities *in vitro* than representatives of the type A family (Figure 3.6). Variants of PI4P 5-kinases 5, 10 and 11 mutated in a specific conserved lysine residue of their ATP-binding sites (Ishihara et al., 1998) were also tested, and catalytic activity of the mutant proteins PIP5K5 (K497A) and PIP5K10 (K86A) against PI4P was reduced to background levels (Figure 3.6).

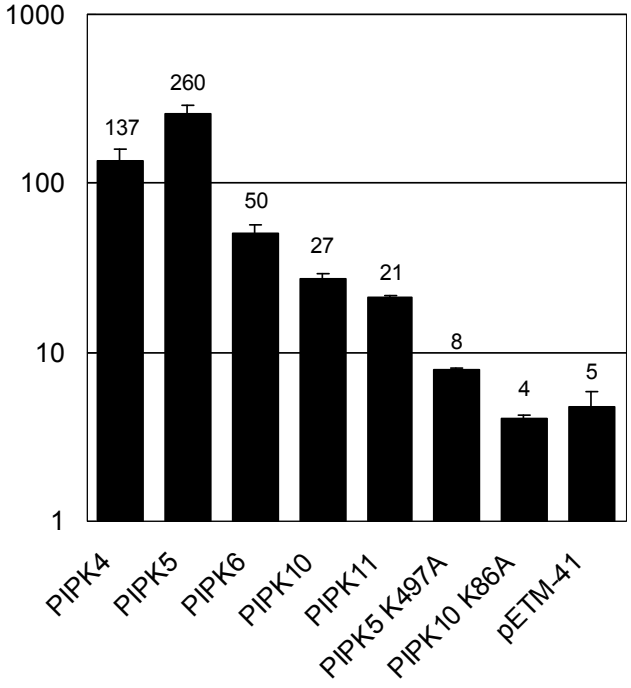

Figure 3.6. *Arabidopsis* PI4P 5-kinase isoforms 4, 5, 6, 10, and 11 catalyze the conversion of PI4P to PI(4,5)P_2

The gene products PIP5K4, 5, 6, 10, and 11 were heterologously expressed in *E. coli* as fusion proteins to N-terminal MBP-tags and the recombinant extracts tested *in vitro* for enzymatic activity against PI4P. In addition, two variants with dysfunctional ATP-binding sites, PIP5K5 K497A and PIP5K10 K86A, were also tested. pETM-41, maltose binding protein control. Concentrations of recombinant proteins expressed in *E. coli* were adjusted according to western blot analysis. Data are the mean from three independent experiments ± standard deviation. Note the logarithmic scale; numbers indicate the mean PI(4,5)P_2-formation in fmol min^{-1}.

Table 3.1. Relative activities of *Arabidopsis* PI4P 5-kinase isoforms.

Catalytic activities were tested *in vitro* against different phosphatidylinositol-monophosphate substrates and indicate the rate of product formation in fmol min^{-1}. Recombinant protein concentrations were balanced according to western blot analysis. Data are the means of two independent experiments ± standard deviation. MBP, maltose binding protein

Substrate	PI3P	PI4P	PI5P	PI4P/PI3P activity
Protein				ratio
MBP	2 ± 0.3	4 ± 0	1 ± 0.1	-
PIP5K1	5 ± 0.8	205 ± 17	2 ± 0.2	41
PIP5K4	12.3 ± 2.0	137 ± 24	1 ± 0.3	16
PIP5K5	19.7 ± 27	260 ± 28	1 ± 0.2	16
PIP5K6	9 ± 3	120 ± 17	1 ± 0.5	13
PIP5K10	4.4 ± 0.3	27 ± 2.4	2 ± 0.3	4.9
PIP5K11	7.5 ± 1.0	21 ± 0.4	2 ± 0.3	2.8

3.3 T-DNA mutant analysis

Since several PI4P 5-kinases were found to be expressed in pollen, the question arose whether the pollen-expressed PI4P 5-kinases performed redundant functions. Therefore, T-DNA mutant lines were acquired with exon insertions in the genes for *PIP5K4* (SALK_001138), *PIP5K5* (SALK_147475), *PIP5K10* (SALK_119243), *PIP5K11* (GABI _284F05) (Figure 3.7 A). No T-DNA insertion line was available for *PIP5K6*. Homozygous

mutant lines were identified by successful amplification of the T-DNA-tagged alleles and concomitant failure to amplify a WT allele (Figure 3.7 B). The sites of the T-DNA insertions were determined by sequencing (Figure 3.7 A). RNA was extracted and analyzed by semi-quantitative RT-PCR showing that the respective transcripts were lacking or at least greatly reduced in their abundance in the mutants (Figure 3.7 C).

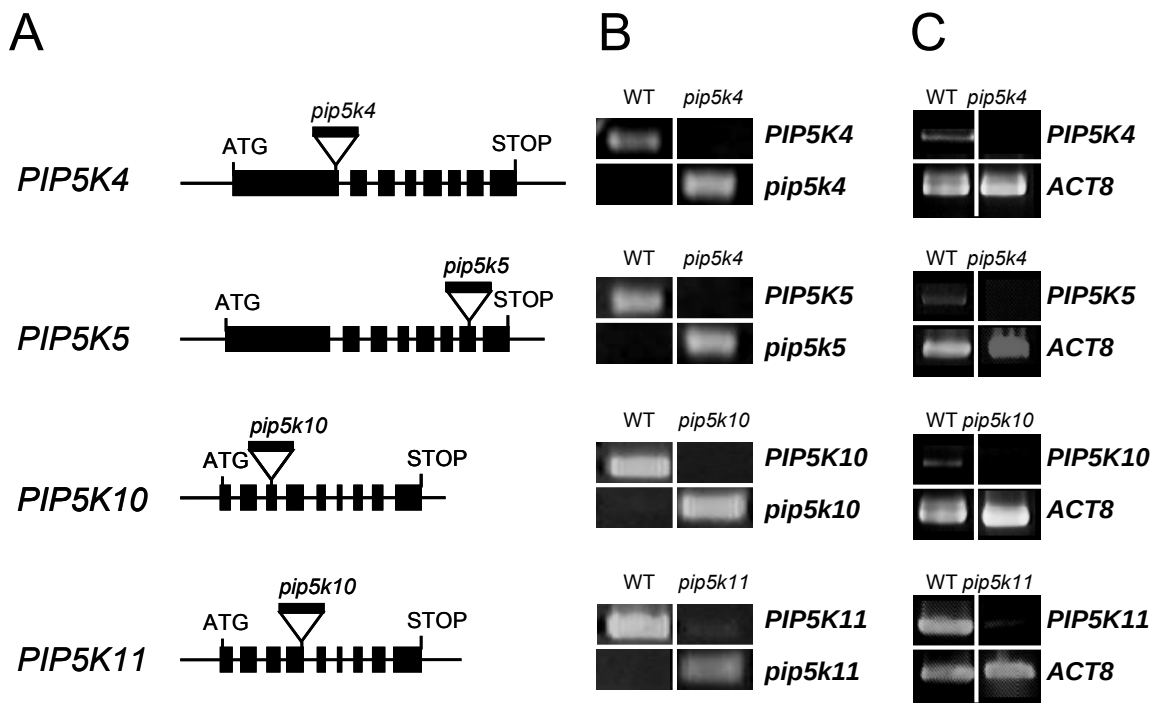

Figure 3.7. Position of T-DNA insertions in genomic loci encoding PI4P 5-kinases

The T-DNA mutant lines indicated were selected and their insertion sites confirmed by sequencing. A, Structure of the gene loci of *PIP5K4*, *PIP5K5*, *PIP5K10* and *PIP5K11* as indicated, and the position of the corresponding T-DNA insertions. B, PCR-based genotyping of isolated homozygeous lines. Homzygosity was inferred from the positive amplification of the T-DNA-tagged mutant alleles (lower case) with concomitant failure to amplify wild-type alleles (upper case). C, Abundance of PI4P 5-kinases transcripts as quantified by 31 cycles of RT PCR in WT controls and the respective mutants. Upper panels, PI4P 5-kinase transcripts; lower panel, *ACT8* control. Images are from a representative experiment that was performed twice with similar results.

Single homozygous mutants showed no obvious sporophytic or gametophytic phenotypes, suggesting some functional redundancy between pollen-expressed PI4P 5-kinases. Therefore, mutants deficient in pollen expressed type B PI4P 5-kinases, *pip5k4* and *pip5k5*, and mutants deficient in pollen expressed type A PI4P 5-kinases, *pip5k10* and *pip5k11* were crossed, respectively. The offspring was further propagated in order to find homozygous double mutants.

3.4 The *pip5k4 pip5k5* double mutant is impaired in pollen germination and pollen tube growth

The crossing of the *pip5k4* and *pip5k5* plants yielded the double mutant. The *pipk4 pipk5* plants proved to be fertile, demonstraing that the disrupted genes were not essential for pollen development, pollen tube growth and targeting, or for fertilization of the female gametophyte. However, when pollen of the double mutant was germinated *in vitro* (Figure 3.8 A-D), the rate of germination was substantially reduced (Figure 3.9 A), and emerging pollen tubes showed significantly shorter tube growth (Figure 3.9 B). These data indicate that PIP5K4 and PIP5K5 contribute to the functionality of the pollen tube.

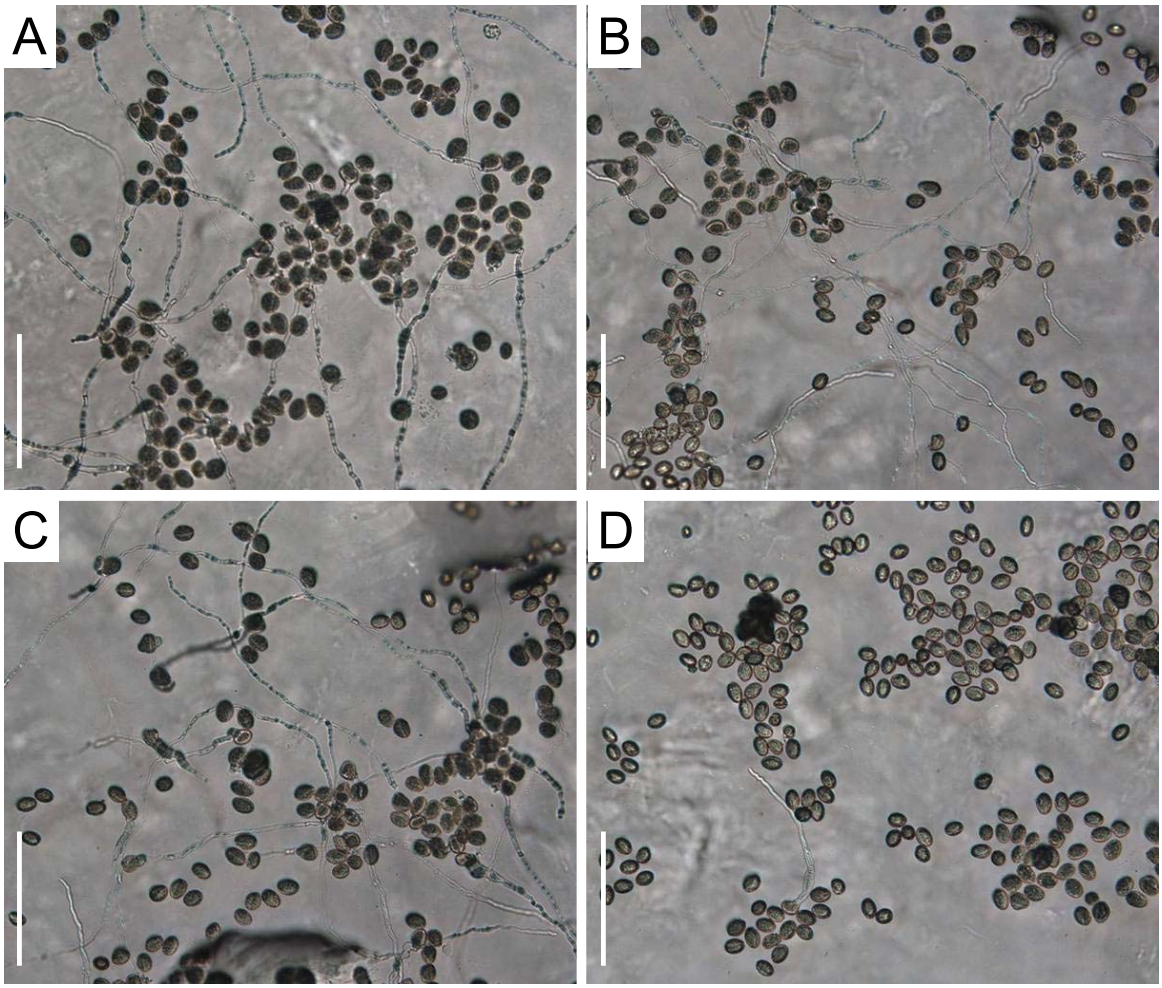

Figure 3.8. The *pipk4 pipk5* double mutant is inhibited in pollen germination and growth

Pollen grains from *Arabidopsis* were germinated, grown *in vitro* for 12 h and visually examined by light microscopy. A, WT; B, *pip5k4*; C, *pip5k5*; D, *pip5k4 pip5k5*. Bars, 200 µm. Images were obtained in a representative experiment that was repeated three times with similar results.

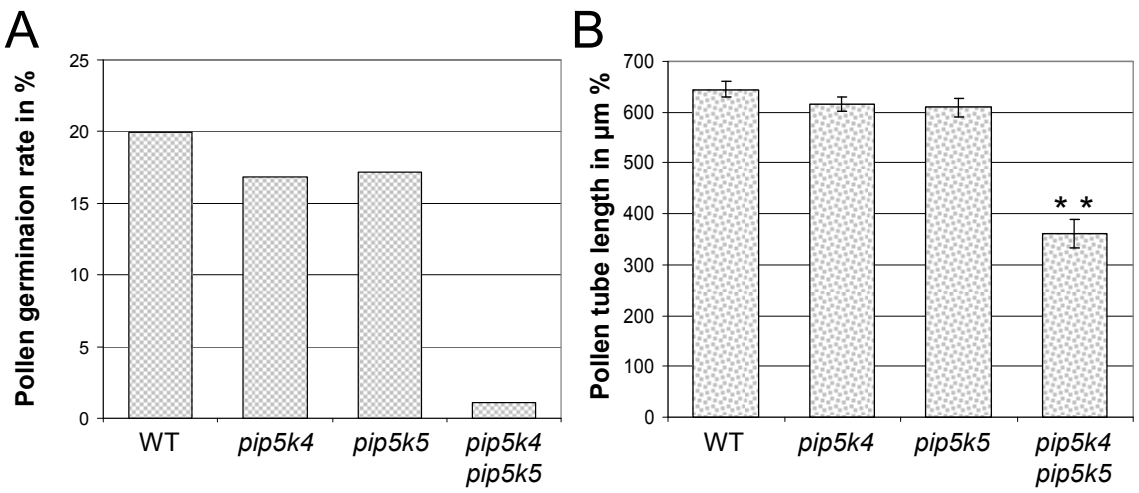

Figure 3.9. Germination rate and pollen tube length in *pipk4 pipk5* double mutants

Pollen germination rate and pollen tube length was quantified from pollen tubes cultured *in vitro* for 12 h. A, the pollen germination rate was slightly reduced in *pip5k4* and *pip5k5* and greatly reduced in *pip5k4 pip5k5* in comparison to WT (n>1200 for each line). B, pollen tube length was slightly reduced in *pip5k4* and *pip5k5* and significantly ($p<0.0001$ according to a Student's t-test) reduced in *pip5k4 pip5k5* mutants in comparison to WT (WT, n=178; *pip5k4*, n=138; *pip5k5*, n=148; *pip5k4 pip5k5*, n=59; ± standard error). The data shown is from a representative experiment that was performed three times with similar results. Pollen used in this experiment was harvested from 30 flowers of 10 different plants of each line.

3.5 The *pip5k4 pip5k5* double mutant can be complemented with *PIP5K4*:EYFP or *PIP5K4*:EYFP

In order to verify that the pollen germination and growth phenotype of the *Arabidopsis* mutant *pip5k4 pip5k5* was due to the disruption of the *PIP5K4* and *PIP5K5* genes, *pip5k4 pip5k5* double mutant plants were stably transformed with *PIP5K4*:*EYFP* or *PIP5K5*:*EYFP* each under the control of their intrinsic *PIP5K4* or *PIP5K5* 1500bp promoter-fragments, respectively. Pollen from five plants expressing PIP5K4-EYFP or PIP5K5-EYFP as indicated by pollen fluorescence were pooled and germinated *in vitro*. Pollen germination and growth length were monitored under the light microscope. It was apparent that both constructs *PIP5K4::PIP5K4*:*EYFP* and *PIP5K5::PIP5K5*:*EYFP* rescued the germination and the growth phenotype *pip5k4 pip5k5* double mutant pollen in T1 plants (Figure 3.10 A-F), and germination and pollen tube growth even exceeded those of WT pollen (Figure 3.10 E). Fluorescing pollen grains containing either *PIP5K4*:*EYFP* or *PIP5K5*:*EYFP* had a higher germination frequency than pollen from the same flower lacking these genes (Figure 3.10 G). In one example 47 % (n=200) of the pollen grains from one flower showed EYFP fluorescence, indicating only one *PIP5K5*:*EYFP* insertion site (Figure 3.10 G). From 150 pollen tubes germinated from these pollen grains 144 showed strong EYFP fluorescence and only 6 were free of obvious EYFP fluorescence. This result indicates successful complementation of the *pip5k4 pi5k5* double mutant by a single insertion of the ectopic construct encoding *PIP5K5*:*EYFP*.

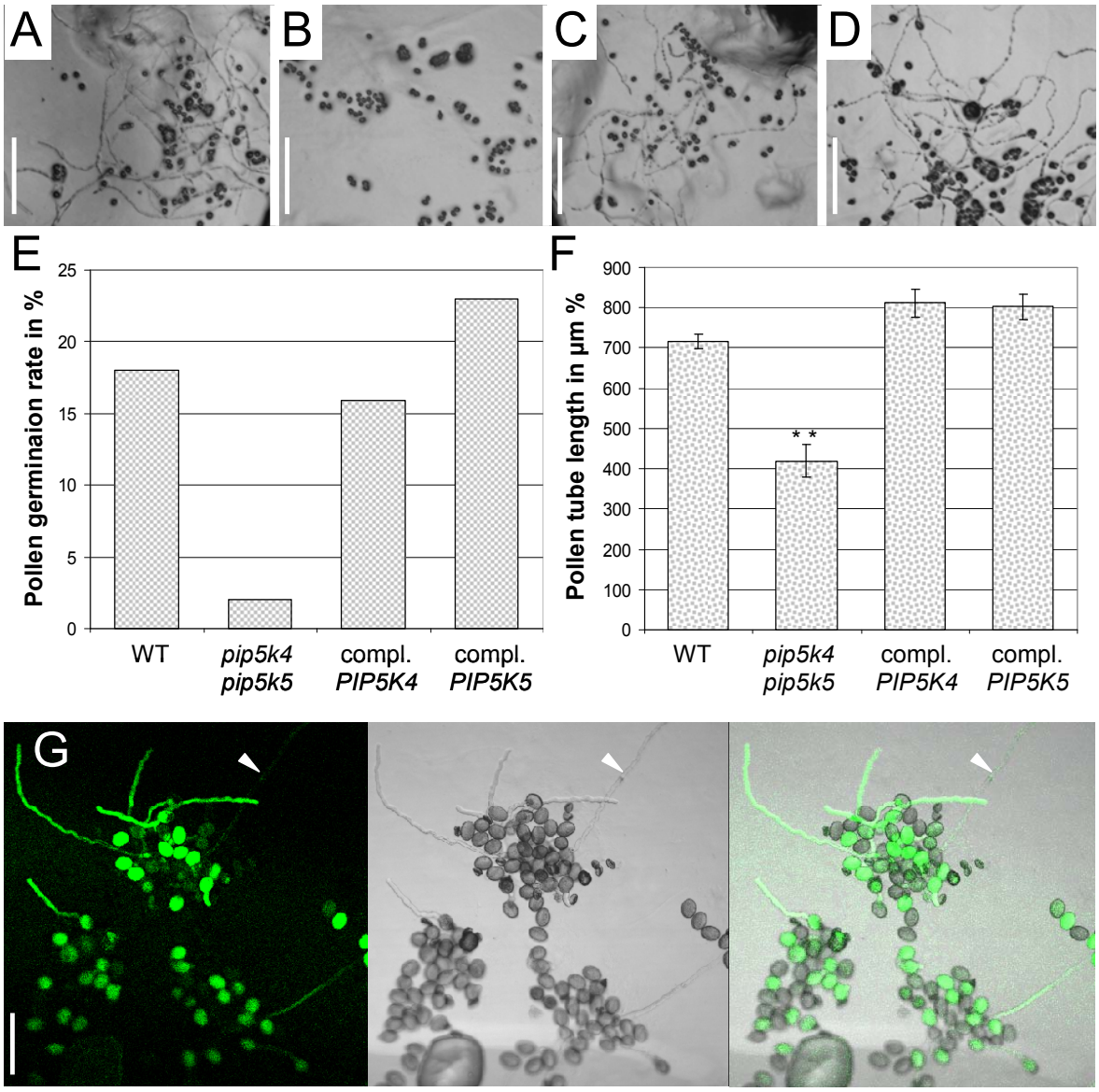

Figure 3.10. The *pip5k4 pip5k5* double mutant can be complemented by *PIP5K4*:*EYFP* and *PIP5K5*:*EYFP*

A-D, Pollen from *Arabidopsis* WT (A), *pip5k4 pip5k5* (B), *pip5k4 pip5k5 PIP5K4::PIP5K4:EYFP* (compl. *PIP5K4*) (C) and *pip5k4 pip5k5 PIP5K5::PIP5K5:EYFP* (compl. *PIP5K5*) (D) where germinated and grown *in vitro* for 12 h. Pollen from 5 independent complementation lines was pooled. Germination rate and pollen tube length were monitored by light microscopy. Bars, 500 µm. E, the pollen germination rate was greatly reduced in *pip5k4 pip5k5* in comparison to WT. In the compl. *PIP5K4* and compl. *PIP5K5* lines germination rate was restored to WT levels (n>1300 for each line). F, pollen tube length was significantly ($p<0.0001$ according to a Student's t-test) shorter in *pip5k4 pip5k5* mutants in comparison to WT and to compl. *PIP5K4* and compl. *PIP5K5* lines. In the compl. *PIP5K4* and compl. *PIP5K5* lines the growth rate was restored to levels slightly higher as in WT. Pollen tubes of the complemented lines are significantly longer ($p<0.0001$ according to a Student's t-test) than pollen of the *pip5k4 pip5k5* double mutant (WT, n=129;; *pip5k4 pip5k5*, n=30 compl. *PIP5K4*, n=74; compl. *PIP5K5*, n=107 ± standard error). G, Pollen from one flower of a compl. *PIP5K5* plant germinated and grown *in vitro* for 4 h. Approximately 50 % of the pollen grains showed fluorescence indicating a single copy insertion of the transgene. All observed pollen tubes were fluorescent. None of the non-transgenic pollen grains germinated. The pollen tube that seems to contain no fluorescence, as indicated by the arrow, showed fluorescence in the more apical part of the tube. Left, EYFP; middle, bright field; right, merge. Bar, 100 µm The data and pictures shown are from a representative experiment that was performed twice with similar results. Pollen used in this experiment was harvested from 30 flowers of 5 different plants of each line. Pollen from transgenic plants was pooled from five independent lines.

Some of the lines containing multiple copies of the *PIP5K4*:*EYFP* or the *PIP5K5*:*EYFP* transgene, as apparent by high fluorescence levels, showed aberrant pollen tube morphology such as tube branching (Figure 3.11 A-D) or a zigzag growth pattern (Figure 3.11 B). Even though tube branching also occurs in *Arabidopsis* WT pollen tubes the frequency was up to eight times higher in the transgenic type B PI4P 5-kinase overexpressors (Figure 3.11 E).

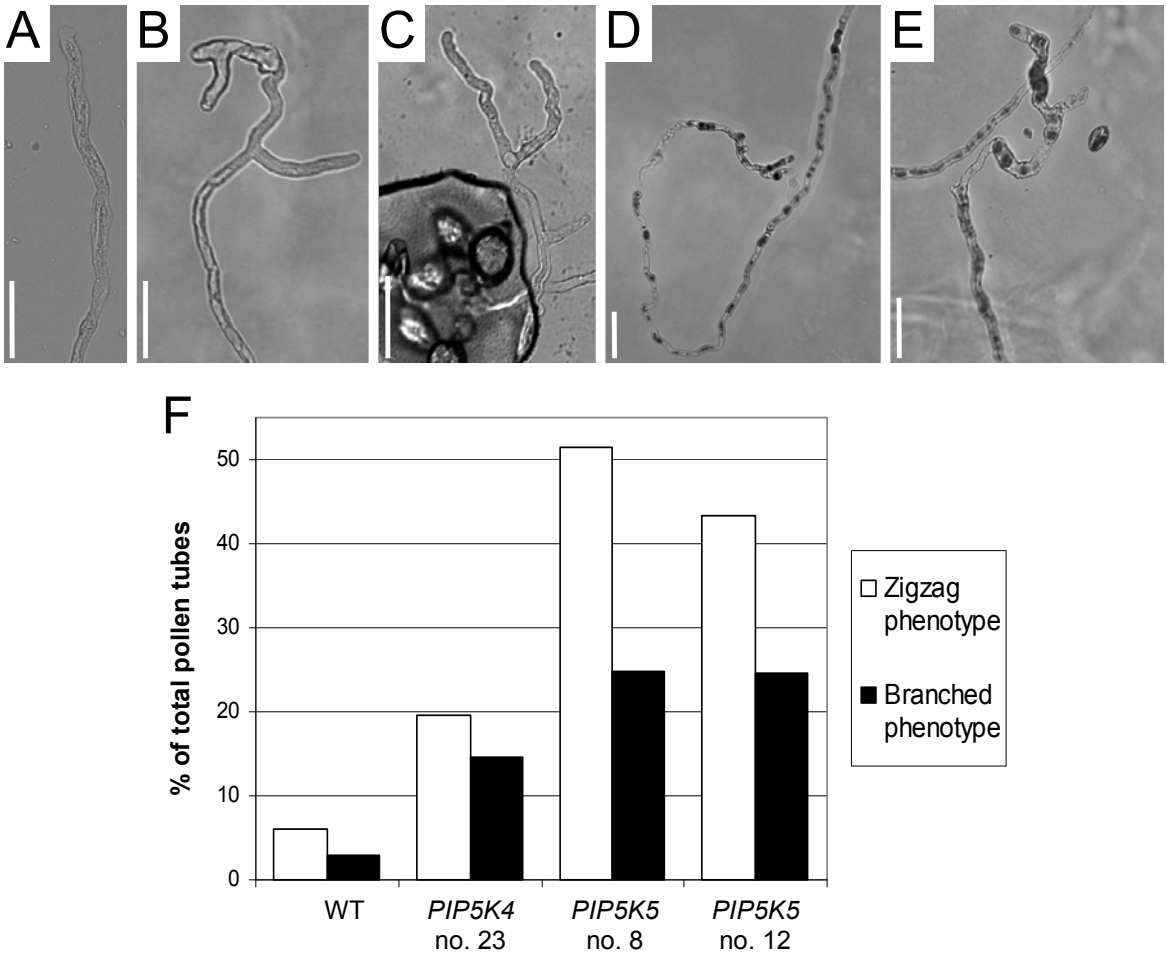

Figure 3.11. Phenotypes in *Arabidopsis* pollen tubes overexpressing type B PI4P 5-kinase

Pollen grains from *Arabidopsis* were germinated, grown *in vitro* for 12 h and visually examined by light microscopy. A, Morphology of a WT *Arabidopsis* pollen tube. B-E, Pollen tubes with high expression levels of PIP5K4:EYFP (B) or PIP5K5:EYFP (C-E) as visible from high fluorescence intensity displayed abnormal growth phenotypes, such as tube branching (B-E) or a zigzag pattern of growth (D-E). Bars, 50 µm. F, Pollen tube branching and the zigzag pattern of growth occurred at much higher frequencies in three independent lines *pip5k4 pip5k5 PIP5K4::PIP5K4:EYFP* no. 23 (*PIP5K4* no. 23) and *pip5k4 pip5k5 PIP5K5::PIP5K5:EYFP* no. 8 and no. 12 (*PIP5K5* no. 8 and no. 12) than in a WT control. WT, n=200; compl *PIP5K4* Nr.23, n=117; compl *PIP5K4* Nr.8, n= 169; compl *PIP5K5* Nr.12, n= 159. The data and images shown are from a representative experiment that was performed twice with similar results. Pollen was harvested from 10 flowers for each line.

3.6 Pollen and ovules deficient in PIP5K10 and PIP5K11 are sterile

A different set of phenotypes was observed in pollen of double mutant *Arabidopsis* plants deficient in the type A PI4P 5-kinases PIP5K10 and PIP5K11. When the F2 generation of the crossed mutants *pip5k10* and *pip5k11* was genotyped, none of 50 plants analyzed

showed homozygosity for one of the mutant alleles *pip5k10* or *pip5k11* and was at the same time homo- or heterozygous for the other allele. Instead, all the plants found homozygous for either *pip5k10* or *pip5k11* possessed two WT alleles for the other gene. This observation indicates that the expression of PIP5K10 and PIP5K11 is not only essential for the development of the male gametophyte but also of the female gametophyte; lethality restricted to either the female or male gametophytes should still result in offspring homozygous for one of the mutant alleles and heterozygous for the other (Figure 3.12). Embryo-lethality resulting from homozygeous double-mutations can also be excluded as the reason for the genotype distribution found (Figure 3.12). It should be noted that the genes *PIP5K10* and *PIP5K11* are situated on different chromosomes (Figure 1.7), and are, therefore, inherited independently.

♀ \ ♂	*PIP5K10 PIP5K11*	*pip5k10 PIP5K11*	*PIP5K10 pip5k11*	*pip5k10 pip5k11*
PIP5K10 PIP5K11	*PIP5K10 PIP5K11* / *PIP5K10 PIP5K11*	*pip5k10 PIP5K11* / *PIP5K10 PIP5K11*	*PIP5K10 pip5k11* / *PIP5K10 PIP5K11*	*pip5k10 pip5k11* / *PIP5K10 PIP5K11*
pip5k10 PIP5K11	*pip5k10 PIP5K11* / *PIP5K10 PIP5K11*	*pip5k10 PIP5K11* / *pip5k10 PIP5K11*	*pip5k10 pip5k11* / *PIP5K10 PIP5K11*	*pip5k10 pip5k11* / *pip5k10 PIP5K11*
PIP5K10 pip5k11	*PIP5K10 pip5k11* / *PIP5K10 PIP5K11*	*pip5k10 pip5k11* / *PIP5K10 PIP5K11*	*PIP5K10 pip5k11* / *PIP5K10 pip5k11*	*pip5k10 pip5k11* / *PIP5K10 pip5k11*
pip5k10 pip5k11	*pip5k10 pip5k11* / *PIP5K10 PIP5K11*	*pip5k10 pip5k11* / *pip5k10 PIP5K11*	*pip5k10 pip5k11* / *PIP5K10 pip5k11*	*pip5k10 pip5k11* / *pip5k10 pip5k11*

Figure 3.12. Genetic distribution of the F2 progeny of crossed pip5k10 and pip5k11 mutants

The alleles derived from the male gametophyte are marked in blue while the alleles from the female gametophyte are marked in purple. If double mutants are pollen or ovule lethal, alleles marked with * or # respectively can not be passed on to the F2 generation. Therefore, F2 plants with the genotypes marked in orange and green would be missing in the F2 generation in case of pollen or ovule lethality, respectively. F2 plants with the genotype marked in orange and green stripes should be missing in both cases. The genotype marked in orange and green stripes would also be the only one missing in the case of embryo lethality. The F2 offspring of crossed *pip5k10* and *pip5k11* showed only genotypes marked in yellow. The data, thus, indicate pollen and ovule lethality.

Based on the results and the crossing scheme pollen lethality was concluded. Therefore, pollen from *pip5k10/PIP5K11 pip5k11/PIP5K11* plants was visually examined. This investigation showed that 46 % (n = 396) of the pollen grains were deformed and did not germinate *in vitro* (Figure 3.13 A). The data suggest that PIP5K10 and PIP5K11 are already essential during pollen maturation and not only at the stage of pollen tube growth. Since only 25 % of the pollen grains should carry the *pip5k10 pip5k11* genotype, it must be concluded that some of the pollen grains mutated only in *PIP5K10* or *PIP5K11* are also crippled. A possible explanation is that the maturation of the pollen grains may depend on the gene dose of type A PI4P 5-kinase. As a possible explanation, some of the pollen that already received only half of the gene dose from their heterozygous mother plant at the microspore stage before meiosis and that now carry only one WT allele for *PIP5K10* or *PIP5K11* during later maturation could fail to develop normally. In this context, it should

also be considered that expression of *PIP5K10* has been observed only late in pollen development (Figure 3.1, Figure 3.2).

To confirm the hypothesized ovule lethality, siliques from plants heterozygous for both genes, *pip5k10/PIP5K11 pip5k11/PIP5K11*, were examined. From this investigation, it was obvious that not all of the ovules had developed to form seeds. Instead, a substantial proportion remained small and white (Figure 3.13 B). These observations are consistent with the proposed ovule lethality.

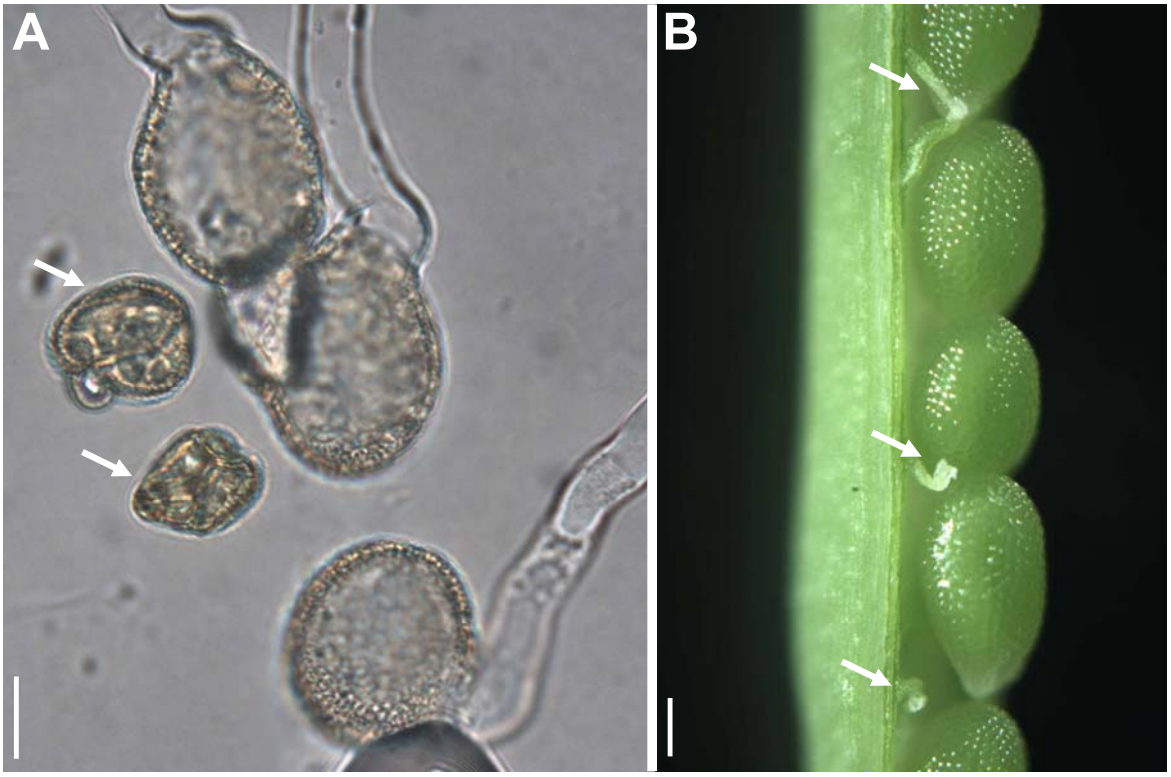

Figure 3.13. The *pip5k10 pip5k11* mutants are pollen and ovule lethal

A, Part of the pollen grains obtained from plants heterozygous for the *pip5k10* and *pip5k11* mutant alleles were reduced in size and did not germinate, as indicated by arrows while others appeared normal. B, Siliques obtained from the same plants also showed developmentally retarded seeds possibly due to defects in ovule development. Bars, 10 µm (A) and 200 µm (B). The phenotypes shown were observed in 14 independent parent plants heterozygous for the *pip5k10* and *pip5k11* mutant alleles. The phenotypes were not observed in WT or *pip5k10* or *pip5k11* single mutants.

3.7 Type A and B PI4P 5-kinases localize to different plasma membrane microdomains of the pollen tube tip

Even though the mutant analysis revealed that both type A and type B PI4P 5-kinase isoenzymes are important in pollen and likely not redundant in their function, their exact functions in polar growth remained unclear. Since it is known that pollen tubes posses a $PI(4,5)P_2$ domain in the apical plasma membrane of the cell (Kost et al., 1999; Dowd et al., 2006), experiments were carried out to test if type A and type B PI4P 5-kinases also localized in this region, indicating possible roles in the generation of the tip-localized $PI(4,5)P_2$ domain. Constructs coding for PIP5K5 and PIP5K11 each fused to the fluorescence tag EYFP were stably transformed into *Arabidopsis* under the control of the

pollen-specific *Lat52* promoter (Twell et al., 1989). Pollen of the transgenic offspring was germinated *in vitro* and monitored under the microscope. These experiments showed a ring-like localization of PIP5K5 and PIP5K11 close to the apex of the pollen tubes (Figure 3.14 C, D) moving forward as the pollen tubes grew. However, localization of PIP5K5 and PIP5K11 differed greatly in the covered area. While PIP5K5 only localized close to the apex of the growing pollen tubes, PIP5K11 covered an area of the plasma membrane extending further away from the apex (Figure 3.14 C, D). Whereas PIP5K11 was almost exclusively found at the plasma membrane, PIP5K5 was found mostly in the cytosol, and plasma membrane localization was not always apparent.

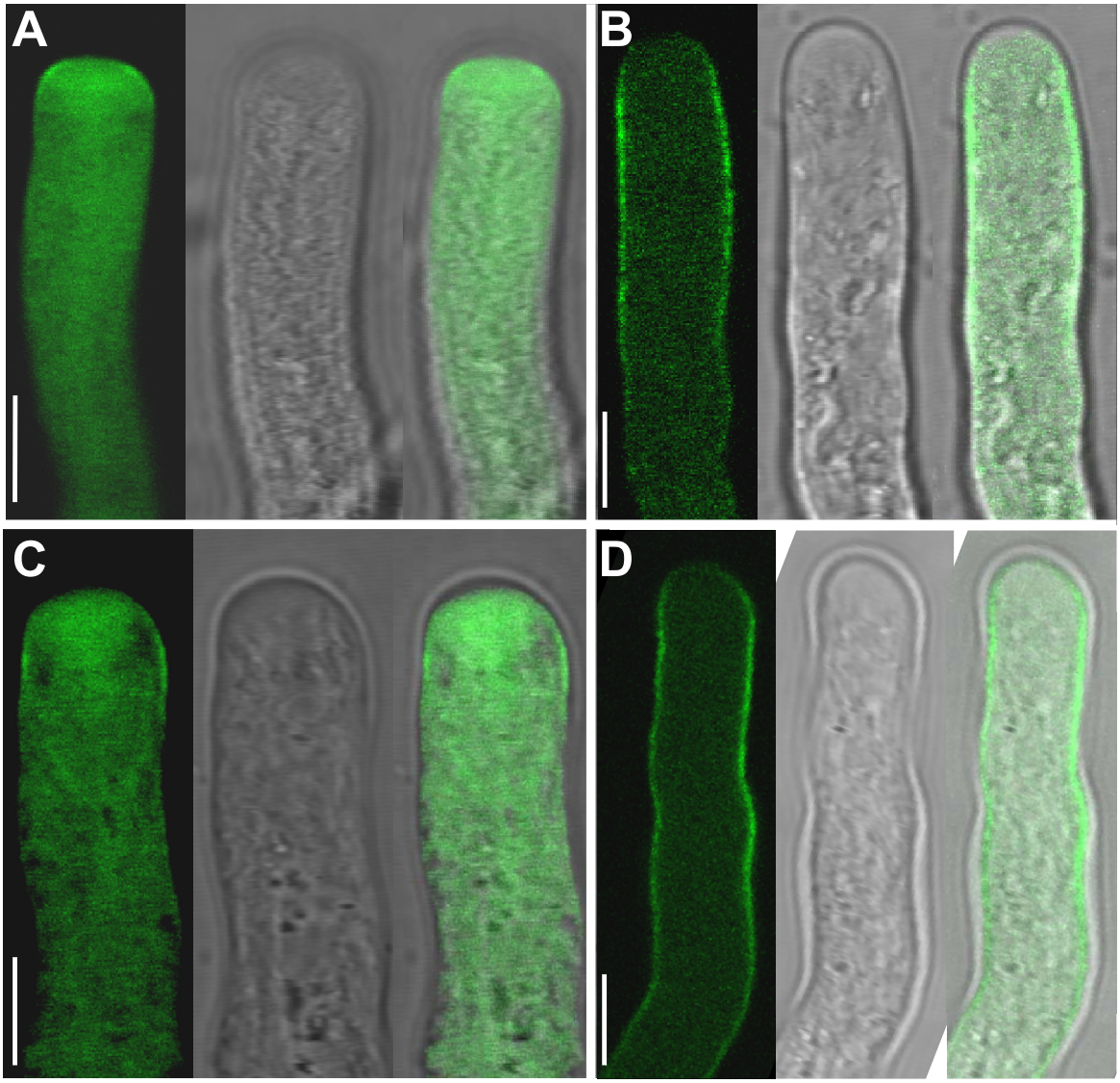

Figure 3.14. Type A and B PI4P 5-kinases localize to distinct plasma membrane microdomains of the *Arabidopsis* pollen tube tip

Subcellular distribution of stably expressed EYFP-tagged PIP5K5 (type B) and PIP5K11 (type A) enzymes was monitored during growth by fluorescence microscopy. Expression was performed either under the *Lat52* promoter or under intrinsic 1500 bp promoter fragments. A, *Lat52*::*PIP5K5*:*EYFP*; B, *Lat52*::*PIP5K11*:*EYFP*; C, *PIP5K5*::*PIP5K5*:*EYFP*; D, *PIP5K11*:: *PIPK5K11*:*EYFP*. Left panels, YFP fluorescence; middle panels bright field; right panels merge. Bars, 5 µm. Images are from a representative experiment that was performed twice with at least three independent lines with similar results.

From the localization of PIP5K5 and PIP5K11 it can be concluded that both type A and type B PI4P 5-kinases could play roles in the generation of the tip-localized $PI(4,5)P_2$ domain. It should be noted, however, that the localization of this domain is only known from pollen of other species than *Arabidopsis* (Kost et al., 1999; Dowd et al., 2006). When expression of the PI4P 5-kinases was performed under their intrinsic promoters, expression levels similar to the expression under the *Lat52* promoter were found. The localizations were also similar (Figure 3.14 C, D), and expression could be observed during the whole time-range of pollen tube growth.

3.8 Type A and B PI4P 5-kinases localize to overlapping plasma membrane microdomains of the pollen tube tip

Even though it is preferable to express genes in a homologous system there can be substantial drawbacks if an organism is not well suited for the required experiments. *Arabidopsis* pollen tubes, for instance, are not as reliable in growth as pollen tubes from other species (Boavida and McCormick, 2007). Another drawback of the *Arabidopsis* system for the analyses performed in this thesis is that successful transformation of *Arabidopsis* pollen by particle bombardment has not yet been reported and was also not possible in our hands. The generation of stably transformed *Arabidopsis* plants makes protein localization experiments much more time-consuming, especially when coexpression of different protein combinations is desired. These problems can be circumvented by the choice of a suitable alternative model system, in which gene expression in pollen is performed. The tobacco pollen tube system has long been established as being very reliable in its growth and germination characteristics and makes easy and efficient transient expression possible (Kost et al., 1998). In our work transient expression levels of proteins in tobacco were also much higher than those obtained using the same promoter for stable expression in *Arabidopsis*, allowing for the observation of additional phenotypic effects described further down. Because of these advantages further expression experiments were performed in tobacco pollen tubes, still using PI4P 5-kinases isoforms originating from *Arabidopsis*. A possible artifact of heterologous expression is that proteins are prone to mislocalization. Therefore, it was first tested whether PI4P 5-kinase isoforms showed a similar localization pattern in tobacco pollen tubes as had been observed in *Arabidopsis* pollen tubes (Figure 3.14). The different pollen-expressed *Arabidopsis* PI4P 5-kinases were transiently expressed under the pollen-specific *Lat52* promoter in tobacco pollen grains as in frame-fusions with EYFP, the pollen were germinated, and pollen tube growth was monitored (Figure 3.15). Tobacco pollen tubes exhibiting only weak expression levels of the transgenes, i. e., with levels comparable to those achieved with stable expression in *Arabidopsis*, were selected for this analysis to allow for the documentation of meaningful distribution patterns. In growing pollen tubes PIP5K4, PIP5K5 and PIP5K6 localized laterally in a ring-like plasma membrane domain close to the growing tip (Figure 3.15 A-C). PIP5K10 and PIP5K11 localized in a similar ring-like pattern, which was positioned in the plasma membrane slightly further back from the tip (Figure 3.15 D, E).

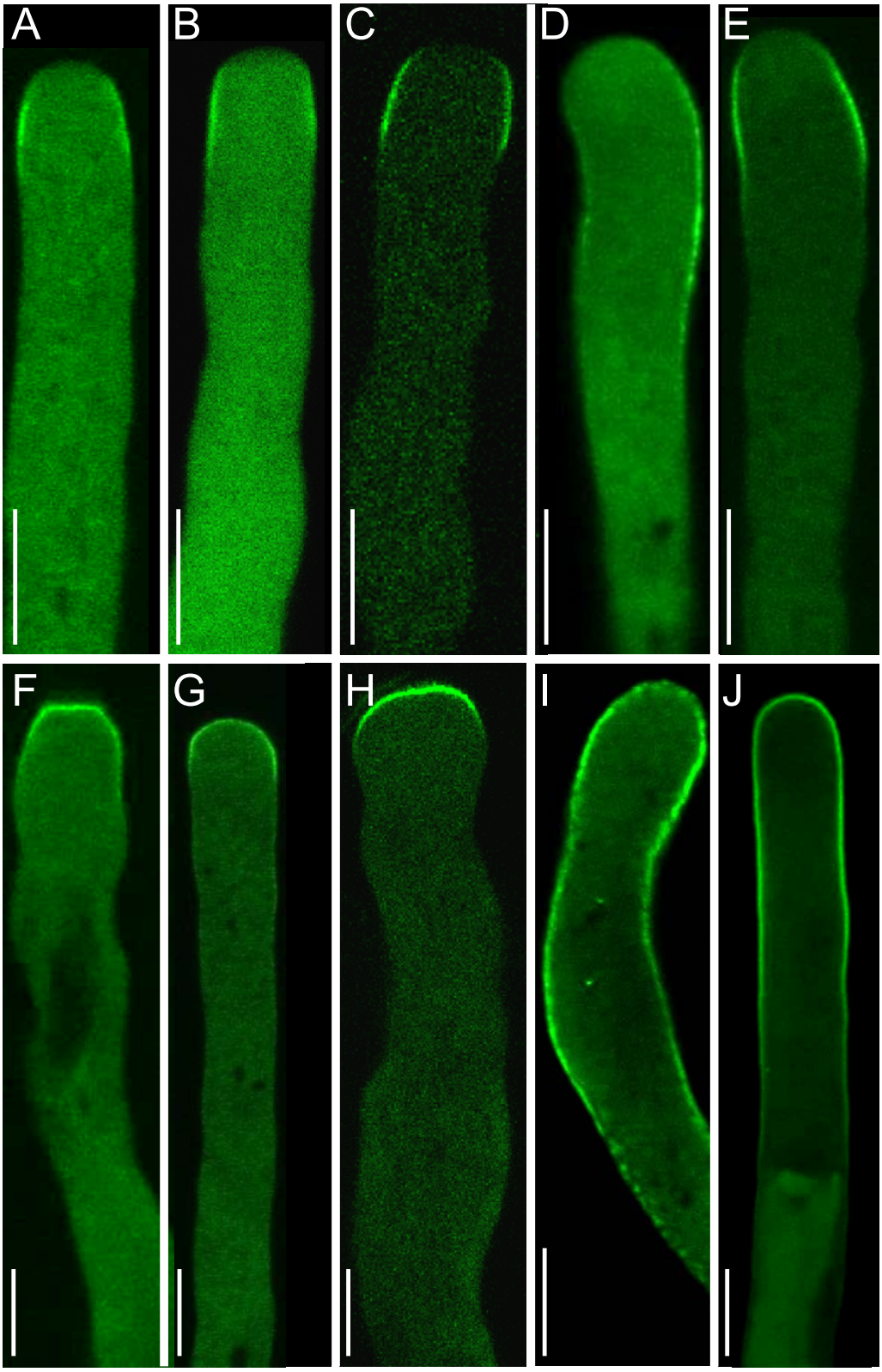

Figure 3.15. Type A and B PI4P 5-kinases localize to distinct plasma membrane microdomains of the tobacco pollen tube tip

Fluorescence distribution of EYFP-tagged type B and type A PI4P 5-kinases was monitored during growth (A-E) and after cessation of growth (F-J) in pollen tubes weakly expressing the respective enzymes. A, F, PIP5K4; B, G, PIP5K5; C, F, PIP5K6; D, I, PIP5K10; E, J, PIP5K11. Bars, 10 µm. Images are representative for at least 50 independently transformed pollen tubes.

Localization of PIP5K5 and PIP5K11 during progression of growth can also be assessed from an image series in the appendix (Figure 6.2, Figure 6.1). Whereas differences in plasma membrane localization of type A and type B enzymes were not as easily apparent in growing tubes as they were in *Arabidopsis*, distinct localization patterns were observed in non-growing tubes. With cessation of growth, all five PI4P 5-kinase isoforms localized to plasma membrane areas that included the pollen tube apex (Figure 3.15 F-H). In contrast to type B enzymes, type A PI4P 5-kinases now also localized to an extended area further back from the tip (Figure 3.15 I-J), resembling the pattern observed in *Arabidopsis* pollen tubes (Figure 3.14).

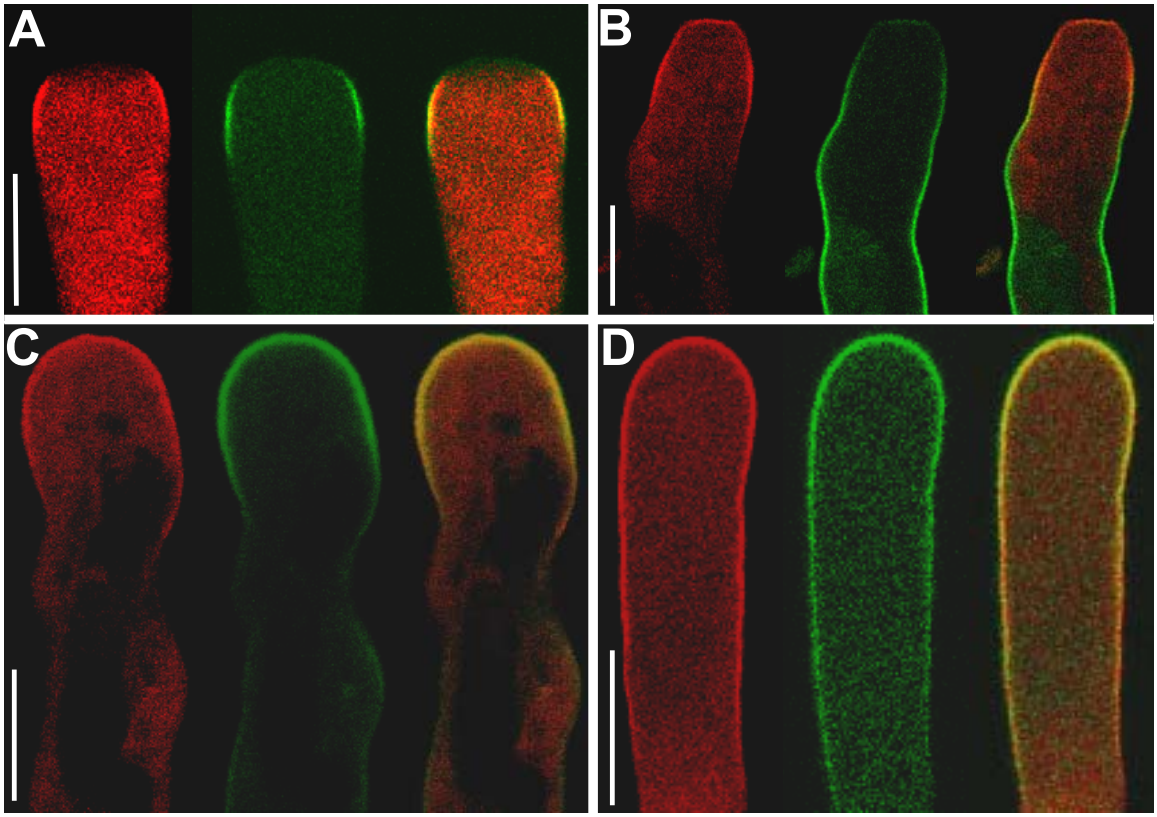

Figure 3.16. Type A and B PI4P 5-kinases localize to overlapping plasma membrane microdomains of the tobacco pollen tube tip

Fluorescence distribution of fluorescence-tagged PIP5K4/5 (type B) and PIP5K10/11 (type A) enzymes was monitored when coexpressed in different combinations in pollen tubes weakly expressing the respective enzymes. A, B, coexpression of CFP-tagged PIP5K5 with EYFP-tagged PIP5K11; C, coexpression of CFP-tagged PIP5K5 with EYFP-tagged PIP5K4; D, coexpression of CFP-tagged PIP5K11 with EYFP-tagged PIP5K10. A, Growing pollen tube; B, C, D, non-growing tubes. Red, CFP; green, EYFP; yellow, overlap. Bars, 10 µm. Images are representative for at least 50 independently transformed pollen tubes.

In order to document distinct localization patterns, the subcellular distribution of CFP-tagged PIP5K5 (type B) was directly compared to that of EYFP-tagged PIP5K11 (type A) in a coexpression experiment (Figure 3.16). In growing tubes, the localization of PIP5K5 and PIP5K11 was similar (Figure 3.16 A), or differences could not be resolved by the methods used. In contrast, in older tubes the resulting pattern (Figure 3.16 B) indicates

distinct localization of PIP5K5 and PIP5K11 to a domain close to the tip and one extending further back from the tip, respectively. Coexpression of EYFP-PIP5K4/CFP-PIP5K5 or CFP-PIP5K10/EYFP-PIP5K11 indicated colocalization of the respective enzymes pairs in growing and non-growing tubes (Figure 3.16 C, D).
Plasma membrane-localization of PI4P 5-kinase isoforms was tested by colocalization of EYFP-tagged enzymes with the steryl-dye, FM 4-64, and is shown for PIP5K5 (Figure 3.17 A) and PIP5K11 (Figure 3.17 B). While PIP5K11 shows exact colocalization with FM 4-64 (Figure 3.17, B), the type B PIP5K5 decorated a less-well defined diffuse pattern at and immediately inside the plasma membrane (Figure 3.17, A).

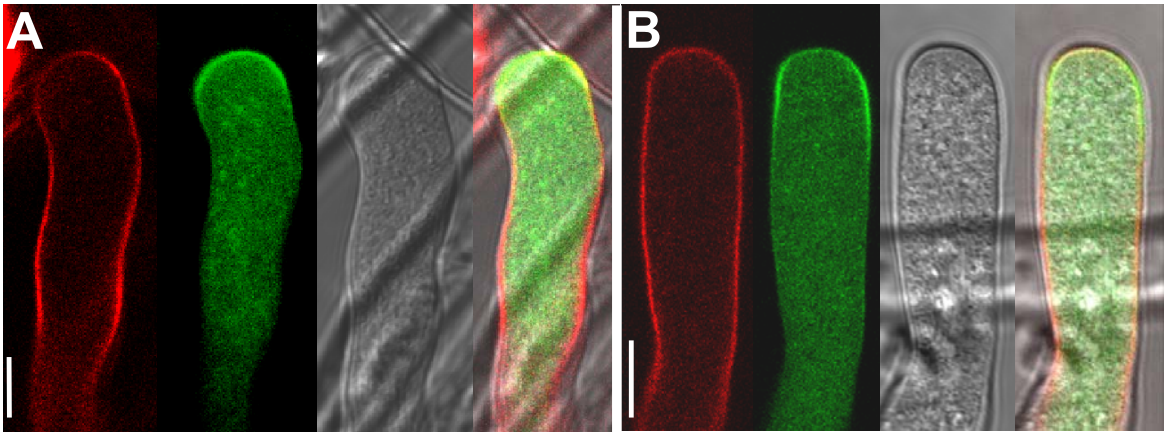

Figure 3.17. PIP5K5 and PIP5K11 colocalizes with the plasma membrane-steryl dye FM 4-64

Plasma membrane association of EYFP-tagged PI4P 5-kinases was analyzed in reference to the steryl-dye FM 4-64 in tobacco pollen tubes. A, PIP5K5; B, PIP5K11. Panels left to right, FM 4-64, EYFP, bright field, merge. Overlap of FM 4-64 and EYFP in yellow indicates plasma membrane localization of PIP5K5 and PIP5K11. Bars, 10 µm. Images are representative for at least 50 independently transformed pollen tubes.

Even though the localization of PI4P 5-kinases observed in tobacco pollen tubes (Figure 3.15) was slightly different than in *Arabidopsis* pollen tubes (Figure 3.14), no mislocalization was observed. Tobacco pollen tubes can, thus, be considered as an acceptable model system to study proteins originating from *Arabidopsis*.

3.9 Type A and type B PI4P 5-kinases associate with different plasma membrane PI(4,5)P_2-domains

To test the relative localization of the type A and type B PI4P 5-kinases to the PI(4,5)P_2 domain previously reported in tobacco pollen tubes (Kost et al., 1999; Dowd et al., 2006), EYFP-tagged PIP5K5 and PIP5K11 were coexpressed with the PIP(4,5)P_2-specific PH-domain of human PLCδ1 (Varnai and Balla, 1998), fused to the red-fluorescent protein, RedStar (Janke et al., 2004). Since it has been demonstrated that the expression of the human PLCδ1-PH-domain influences phosphoinositide levels of the host cells (Balla et al., 2000; Balla and Varnai, 2002), and because we observed decreased pollen tube-growth with high expression of the RedStar-PLCδ1-PH-reporter, expression levels of the PI(4,5)P_2-reporter were kept as low as possible in the experiments shown to ensure that

effects on PI(4,5)P_2-functionality remained small. The RedStar-PLCδ1-PH-reporter alone decorated a plasma membrane microdomain at the tips of growing (Figure 3.18 A) or a larger plasma membrane area of non-growing pollen tubes (Figure 3.18 B), as was previously reported for PI(4,5)P_2 in this system (Kost et al., 1999; Dowd et al., 2006). When coexpressed with EYFP-tagged PIP5K5 (Figure 3.18 C, D) or PIP5K11 (Figure 3.18, E, F), localization of either enzyme in growing tubes overlapped largely with that of PI(4,5)P_2 (Figure 3.18 C, E). In non-growing tubes the localization of type A and type B PI4P 5-kinases differed with respect to PI(4,5)P_2 (Figure 3.18 D, F).

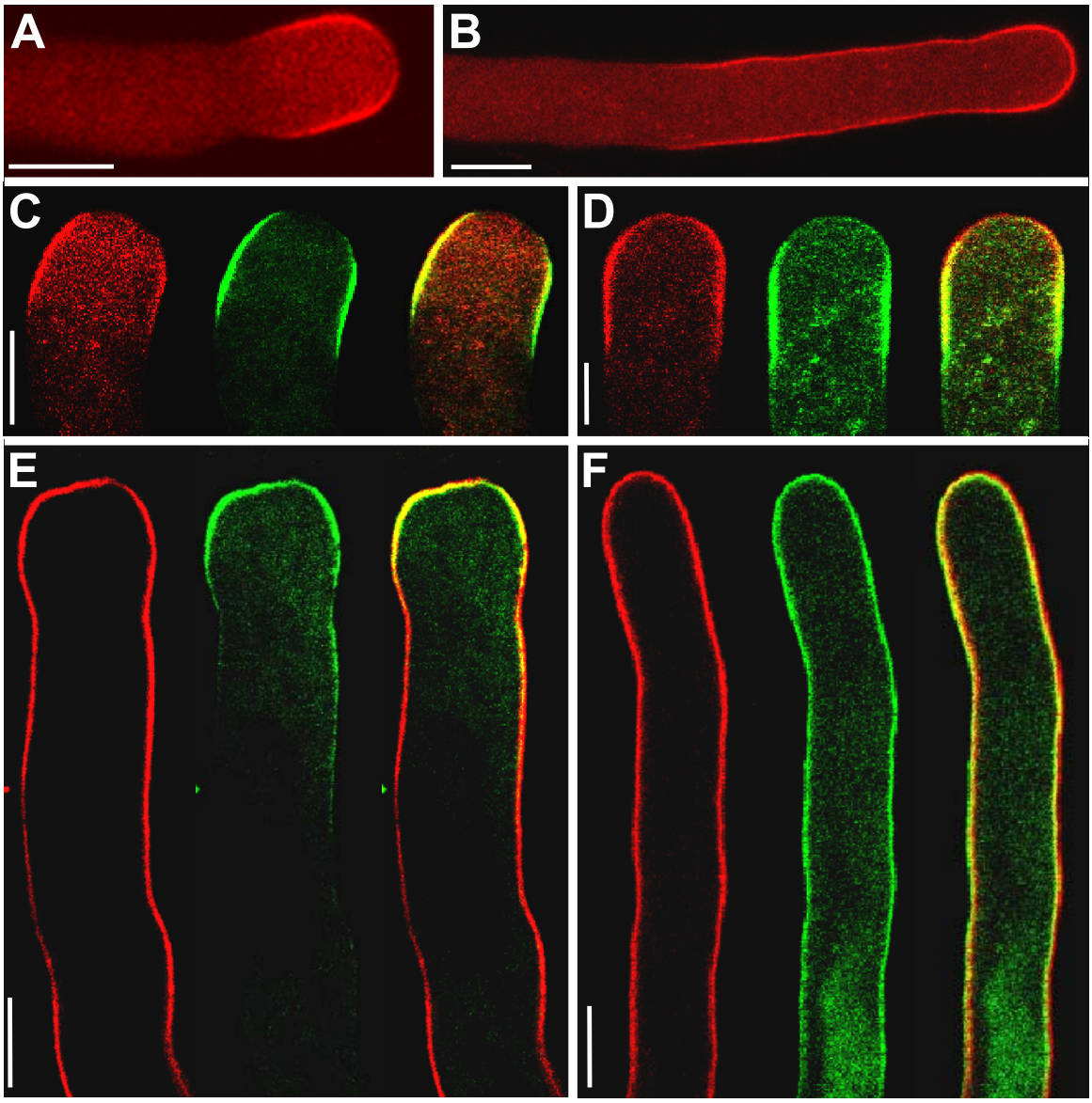

Figure 3.18. Distribution of PI(4,5)P_2 relative to type A and B PI4P 5-kinases

Tobacco pollen tubes were transiently transformed with the PI(4,5)P_2 reporter RedStar-PLCδ1-PH and PIP5K5-EYFP or PIP5K11-EYFP. A, B, Distribution of PI(4,5)P_2 in a growing (A) and a non-growing tobacco pollen tube (B). C-F, PI(4,5)P_2 (red) was visualized in combination with PIP5K5 or PIP5K11 (green) in growing (C, E) and non-growing pollen tubes (D, F). C, D, PIP5K5; E, F, PIP5K11. Yellow indicates colocalization. Bars, 10 µm. Images are representative for at least 50 independently transformed pollen tubes.

Localization of RedStar-PLCδ1-PH exceeded that of EYFP-tagged PIP5K5 with overlap only at the apex, indicating a PI(4,5)P_2-rich plasma membrane region further back from the tip that was not associated with PIP5K5 (Figure 3.18 D). In contrast, coexpression of RedStar-PLCδ1-PH with EYFP-tagged PIP5K11 (Figure 3.18 F) resulted in exact colocalization over a larger plasma membrane-area of non-growing tubes, including the tip and the region further back. Since both PIP5K5 and PIP5K11 are catalytically active PI4P 5-kinases (Figure 3.6) and overlapped at least partially with the PI(4,5)P_2 domain visualized, it can be concluded that both type A and B PI4P 5-kinases may play a role in the establishment of the PI(4,5)P_2 domain.

3.10 Overexpression of type A or type B PI4P 5-kinases exerts different effects on pollen tube morphology

The images illustrating the localization of *Arabidopsis* PI4P 5-kinases in tobacco pollen tubes were all obtained from pollen tubes expressing the kinases at the lowest possible levels. PI4P 5-kinase overexpressors, however, were also monitored, in which PI(4,5)P_2 levels were likely increased. More so than the mutant analysis described above, the observation of pollen tubes with enhanced PI(4,5)P_2 levels could lead to insights into the possible physiological roles of PI(4,5)P_2 generated by type A and B PI4P 5-kinases on pollen tube growth.

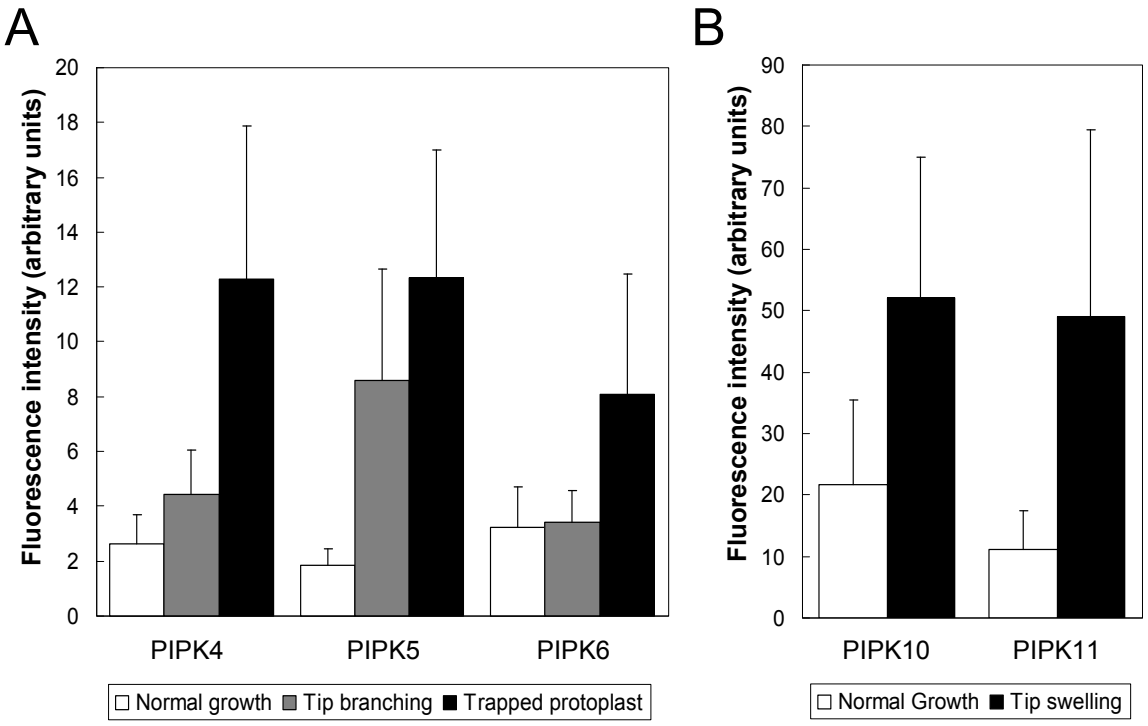

Figure 3.19. Morphological changes are observed in pollen tubes strongly expressing PI4P 5 kinase isoforms

Pollen tubes displaying aberrant phenotypes due to type B (A) and type A (B) PI4P 5-kinase activity showed higher fluorescence levels. Expression levels found in pollen tubes displaying the "trapped protoplast" phenotype were higher than the levels found in branched tubes. Data are means of ten individually transformed pollen tubes. Error bars indicate standard deviation; n = 10. The data shown is from a representative experiment that was performed twice with similar results.

Fluorescing pollen tubes overexpressing either type of PI4P 5-kinase regularly exhibited severe morphological alterations, namely loss of polar growth and tip swelling in type A overexpressors, or pollen tube branching or the “trapped protoplast” phenotype in type B overexpressor,s as will be described in detail in the next paragraphs. Morphological effects were restricted to tubes strongly expressing the transgenes (Figure 3.19). While it could be expected that polar tip-growth of pollen tubes is altered with significant PI4P 5-kinase overexpression, it was surprising that two different categories of morphological changes were observed, although type A and B PI4P 5-kinases catalyze the same reaction and localize similarly during growth.

3.11 Pollen tube branching with overexpression of type B PI4P 5-kinases

Overexpression of either of the type B enzymes, PIP5K4, PIP5K5 or PIP5K6 in tobacco pollen tubes, resulted in the induction of a branching pattern of tube growth at time points beyond 5 h after transformation (Figure 3.20), similar to pollen tube branching observed in *Arabidopsis* (Figure 3.11). While some pollen tubes branched once (Figure 3.20 A, C, E), branching could also occur at multiple sites (Figure 3.20 A, C), giving rise to pollen tubes with multiple tips. Separate branches were not affected in their individual growth dynamics, which can be observed in a series of Images recorded from a branched growing pollen tube in the appendix (Figure 6.3). The branching phenotypes shown were recorded after 14 h of pollen tube growth and were obvious in 42 out of 247 (17 %), 65 out of 253 (26 %) and 26 out of 265 (10 %) scored pollen tubes expressing PIP5K4, PIP5K5 or PIP5K6, respectively.

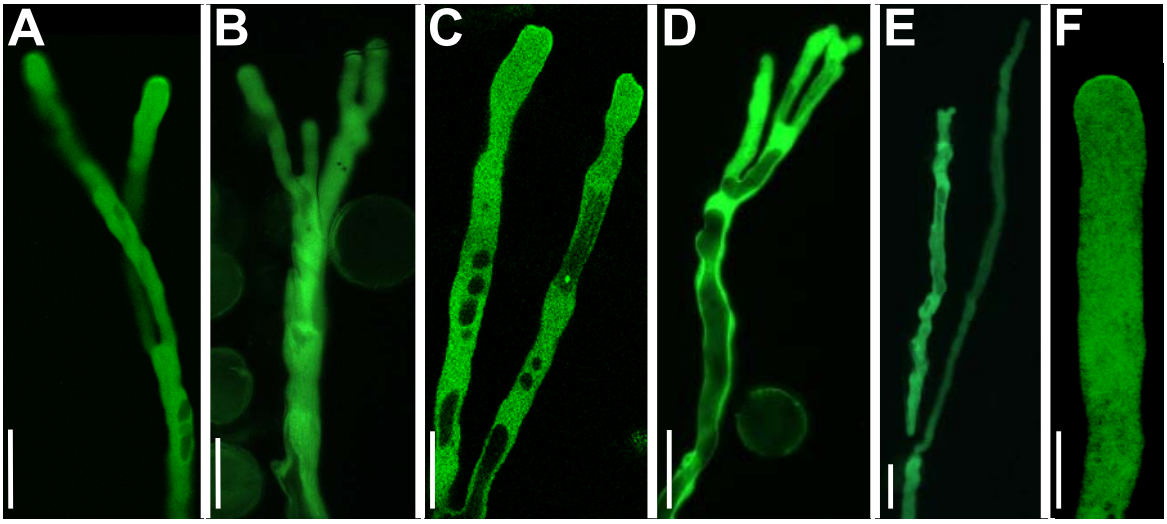

Figure 3.20. Branching of tobacco pollen tubes strongly expressing type B PI4P 5-kinases

Phenotypes of pollen tubes expressing EYFP-tagged enzymes were monitored by epifluorescence (A, B, D, E) or confocal microscopy (C, F). A, B, Dichotomous (A) and multiple (B) branching with expression of PIP5K4. C, D, Dichotomous (C) and multiple (D) branching with expression of PIP5K5. E, Dichotomous branching with expression of PIP5K6. F, Unaffected pollen tube growth with expression of the inactive PIP5K5 K497A protein. Bars, 25 µm (A-E) or 10 µm (F). Images represent phenotypes that were observed in more than 100 independently transformed pollen tubes in at least two independent experiments.

Expression of the inactive PIP5K5 K497A did not result in altered pollen tube-morphology, and zero tubes expressing the inactive PIP5K5 K497A exhibited the branching phenotype (n = 75). Importantly, however, the localization pattern PIP5K5 K497A remained unchanged (Figure 3.20 F) compared to that of the active PIP5K5. The growth dynamics of pollen tubes expressing the inactive PIP5K5 K497A were not altered, indicating the absence of dominant-negative effects.

3.12 Massive cell wall-deposition and plasma membrane invagination in pollen tubes overexpressing type B PI4P 5-kinases

While a substantial proportion of pollen tubes overexpressing type B enzymes showed the branching phenotype (Figure 3.20), a large subset of strong overexpressors of type B PI4P 5-kinases was arrested in growth shortly after germination and morphologically altered in the apical region of the cells (Figure 3.21). After 14 h of pollen tube growth this phenotype was obvious in 93 out of 247 (38 %), 103 out of 253 (41 %) and 158 out of 265 (60 %) scored pollen tubes expressing PIP5K4, PIP5K5 or PIP5K6, respectively.

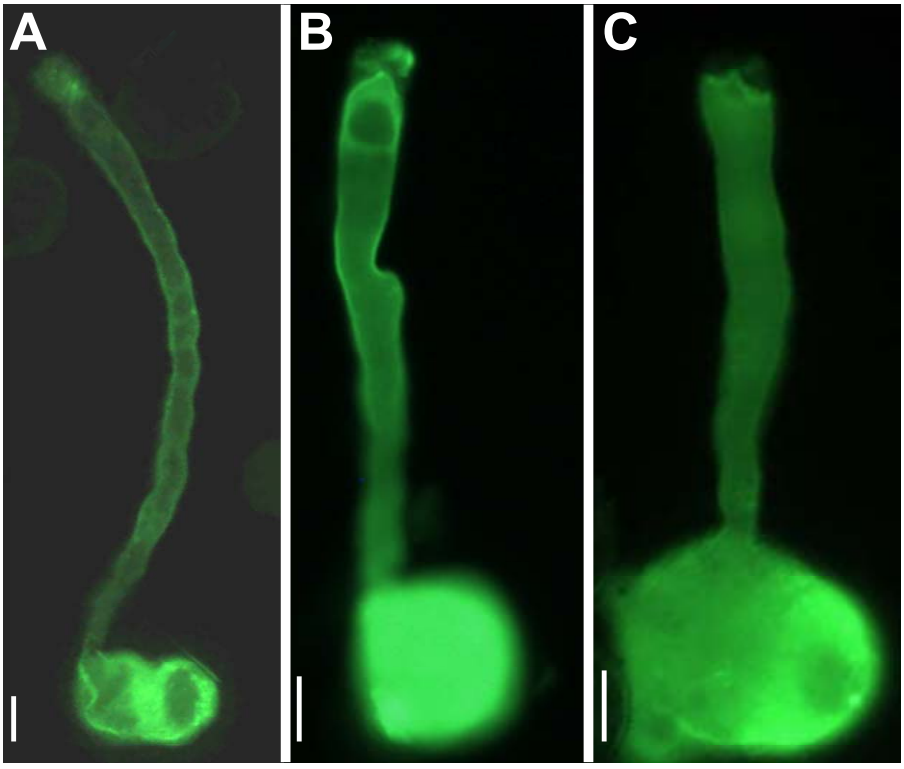

Figure 3.21. Tobacco pollen tubes overexpressing type B PI4P 5-kinases are arrested in growth and morphologically altered

Epifluorescence images of pollen tubes strongly overexpressing PIP5K4, A; PIP5K5, B; PIP5K6, C. Bars, 25 µm. Images represent phenotypes that were observed in more than 100 independently transformed pollen tubes in at least two independent experiments.

In morphologically altered pollen tubes overexpressing type B PI4P 5-kinases increased deposition of cell wall-material was observed (Figure 3.22 A-D). The cell wall at the tip of the pollen tube is composed of a flexible pectin network, whereas the cell wall at the shank is additionally strengthened by incorporated cellulose and callose. Side-by-side

toluidine blue O staining (O'Brien et al., 1964) of non-transformed pollen tubes and of tubes overexpressing type B PI4P 5-kinases indicates that the increased apical secretion of cell wall material was substantial (Figure 3.22 A-D). Toluidine blue O stains pectin in pink or purple, depending on the pH, but does not stain callose. The toluidine blue O staining pattern, thus, indicates an increased deposition of pectin in the apical cell wall.

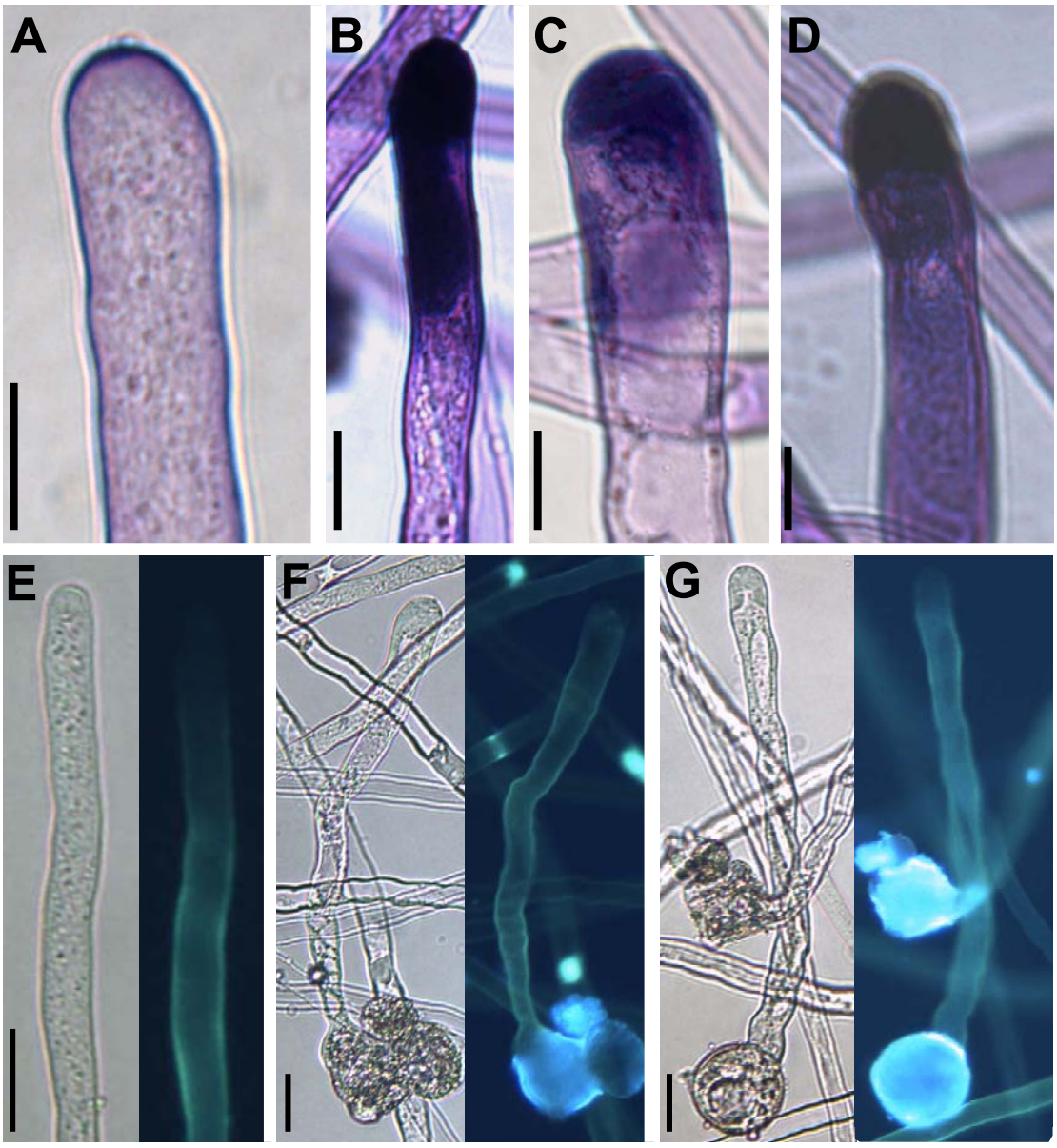

Figure 3.22. Apical accumulation of cell wall material in tobacco pollen tubes overexpressing type B PI4P 5-kinases

A substantial proportion of pollen tubes strongly expressing either PIP5K4 or PIP5K5 were altered in pollen tube tip fine structure. A, Toluidine blue O stain of non-transformed control B-D, Increased deposition of primary cell walls as stained by toluidine blue O with overexpression of PIP5K4 (B) PIP5K5 (C) PIP5K6 (D) and imaged by light microscopy. E-G, Callose staining by aniline blue in a non-transformed control (E) or in pollen tubes overexpressing PIP5K5 (F, G) reveals no differences in callose deposition. Bars, A-D, 10 µm; E-G 20 µm. Images represent phenotypes that were observed in more than 20 independently transformed pollen tubes in at least two independent experiments.

To find out if misplacement of callose also played a role in the observed growth arrest (Figure 3.21), PIP5K5 overexpressing tobacco pollen tubes were stained with aniline blue (Figure 3.22 F-G), a dye that specifically stains callose (Fukumoto et al., 2005). Non-transformed pollen tubes were used as a control. In this control experiment aniline blue fluorescence could be observed in the cell wall of the shank but not of in that of the tip of the pollen tube (Figure 3.22 E). Results from PIP5K5 overexpressors were comparable (Figure 3.22 F-G) to those of the non-transformed controls, indicating no excess incorporation of callose into the cell wall of PIP5K5 overexpressors.

An effect often accompanying increased cell wall-deposition was the formation of plasma membrane invaginations in the apical regions of the pollen tubes (Figure 3.23). Plasma membrane invaginations were only observed in pollen tubes that also exhibited excessive cell wall-deposition and had ceased to grow. It was concluded that since the pollen tube is arrested in growth due to a rigidification of the cell wall, excess plasma membrane that can no longer expand folds inward, a phenomenon that was named “trapped protoplast”.

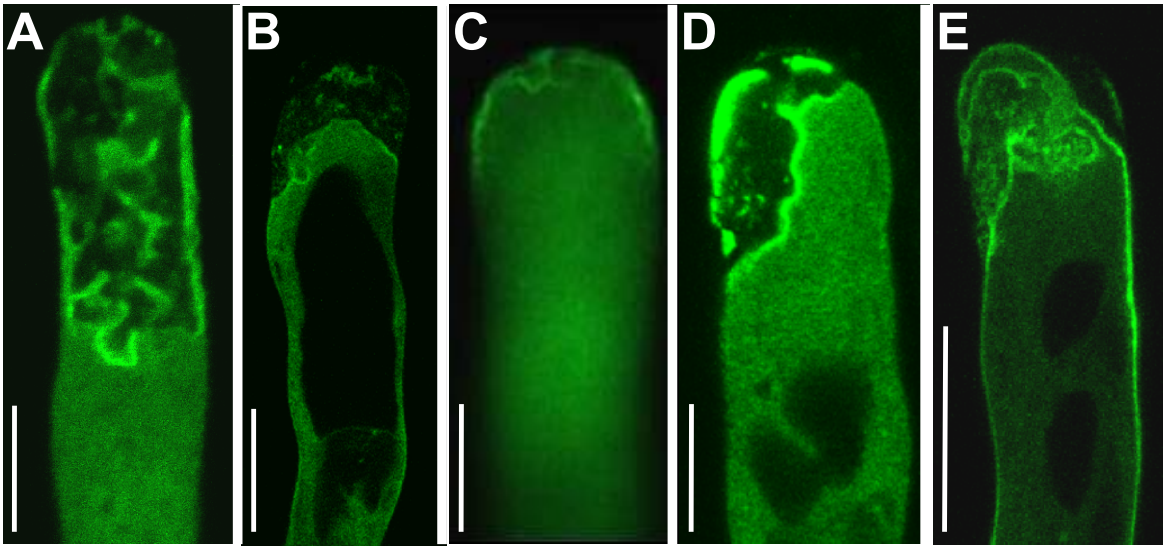

Figure 3.23. Apical Plasma membrane invaginations in tobacco pollen tubes overexpressing type B PI4P 5-kinases

Plasma membrane invaginations with expression of EYFP-tagged PIP5K4 (A, B), PIP5K5 (C, D) or PIP5K6 (E) All tubes were imaged by epifluorescence (C) or confocal microscopy (A, B, D, E) after cessation of growth, but showed vigorous cytoplasmic streaming. Bars, 20 µm. Images represent phenotypes that were observed in more than independently transformed 100 pollen tubes in at least two independent experiments.

3.13 Tip swelling with overexpression of type A PI4P 5-kinases

In the 3.10 it was described that overexpression of type A and type B PI4P 5-kinases resulted in surprisingly different phenotypes. In contrast to the branching pattern observed with type B enzymes, overexpression of type A enzymes, PIP5K10 or PIP5K11, resulted in severe swelling of the pollen tube tip at time points beyond 7 h after transformation (Figure 3.24). Branching or increased deposition of cell wall-material was never observed with expression of type A enzymes. For both PIP5K10 (Figure 3.24 A, B, C) and PIP5K11 (Figure 3.24 D, E), swollen tube-tips could exceed the size of the pollen grain. Tip swelling

dynamics can be followed in the appendix from a series of images recorded from a swelling pollen tube (Figure 6.4). From this image series it is obvious that only the very apex of the pollen tube swells up and not a larger area further back from the apex, as might have been expected. The extent of tip swelling was evaluated by scoring categories of different tip diameters (Figure 3.24 G).

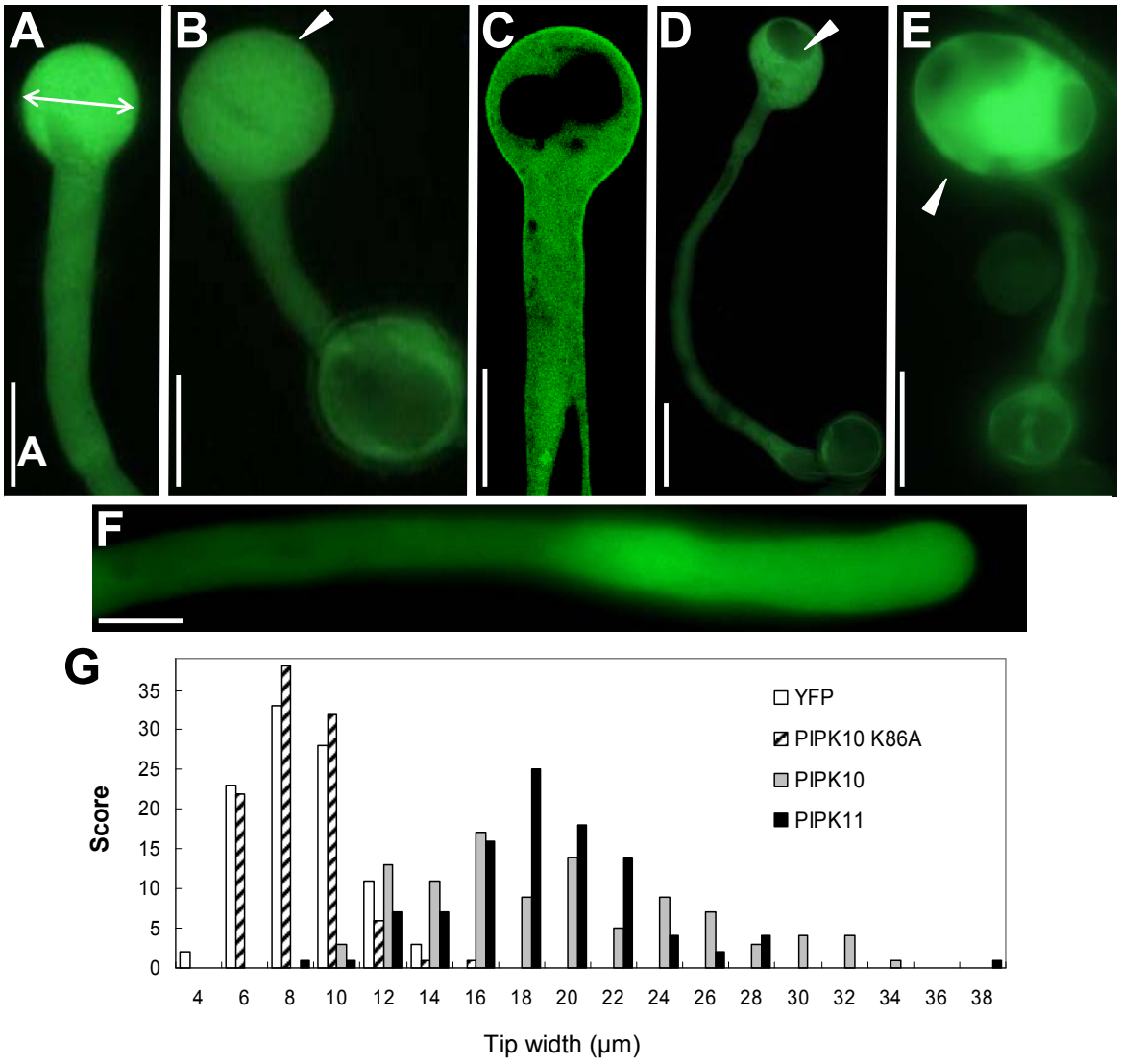

Figure 3.24. Swelling of tobacco pollen tubes strongly expressing type A PI4P 5-kinases

Phenotypes of tobacco pollen tubes expressing EYFP-tagged enzymes were monitored by epifluorescence (A, B; D-F) or confocal microscopy (C). A, B, Tip swelling with expression of PIP5K10. Arrow indicates maximal tip-width, as determined for statistical evaluation (see panel G). C, Tip swelling and plasma membrane localization of EYFP-tagged PIP5K11. D, E, Tip swelling with expression of PIP5K11. Arrowheads indicate the tip. Note dark vacuoles in the tips of tubes shown in C-E. F, Unaltered pollen tube growth with expression of the inactive PIP5K10 K86A protein. Bars, 20 µm (A-E) or 10 µm (F). G, Range of tip widths observed in pollen tubes expressing EYFP alone (white bars), EYFP-tagged PIP5K10 K86A (striped), EYFP-tagged PIP5K10 (grey), or EYFP-tagged PIP5K11 (black). Pollen tubes were scored according to digital image analysis. Images represent phenotypes that were observed in more than 100 independently transformed pollen tubes in at least four independent experiments.

Mean tip diameters of tubes expressing PIP5K10 or PIP5K11 were 20 ± 6 and 20 ± 4 µm, respectively, and both significantly ($p<0.001$ according to a Student's t-test; n = 100) larger than those of tubes expressing EYFP (10 ± 2 µm) or the inactive PIP5K10 K86A (10 ± 2 µm), according to a Student's t-test (Figure 3.24 G). Tip diameters of ≥ 16 µm after 14 h of pollen tube growth were observed in 73 out of 100 (73 %) and 84 out of 107 (79 %) of scored pollen tubes transformed with PIP5K10 or PIP5K11, respectively. Expression of the inactive PIP5K10 K86A at similar levels did not result in altered pollen tube-morphology (Figure 3.24 F), and only 1 out of 100 tubes (1 %) expressing PIP5K10 K86A exhibited modest tip swelling. The growth dynamics of pollen tubes expressing the inactive PIP5K10 K86A were not altered, indicating the absence of dominant-negative effects.

3.14 Callose distribution in pollen tubes overexpressing PIP5K11

The cell wall at the tip of pollen tubes is composed mostly of pectin (Bosch and Hepler, 2005) and is very flexible. As was already mentioned above, the cell wall further down the shank becomes thicker and inflexible due to the incorporation of cellulose and callose. It was one working hypothesis that only the tips of pollen tubes that are surrounded by a flexible cell wall could swell up, as for example due to PIP5K11 overexpression, since in lower regions the cell wall would not be flexible enough to allow expansion.

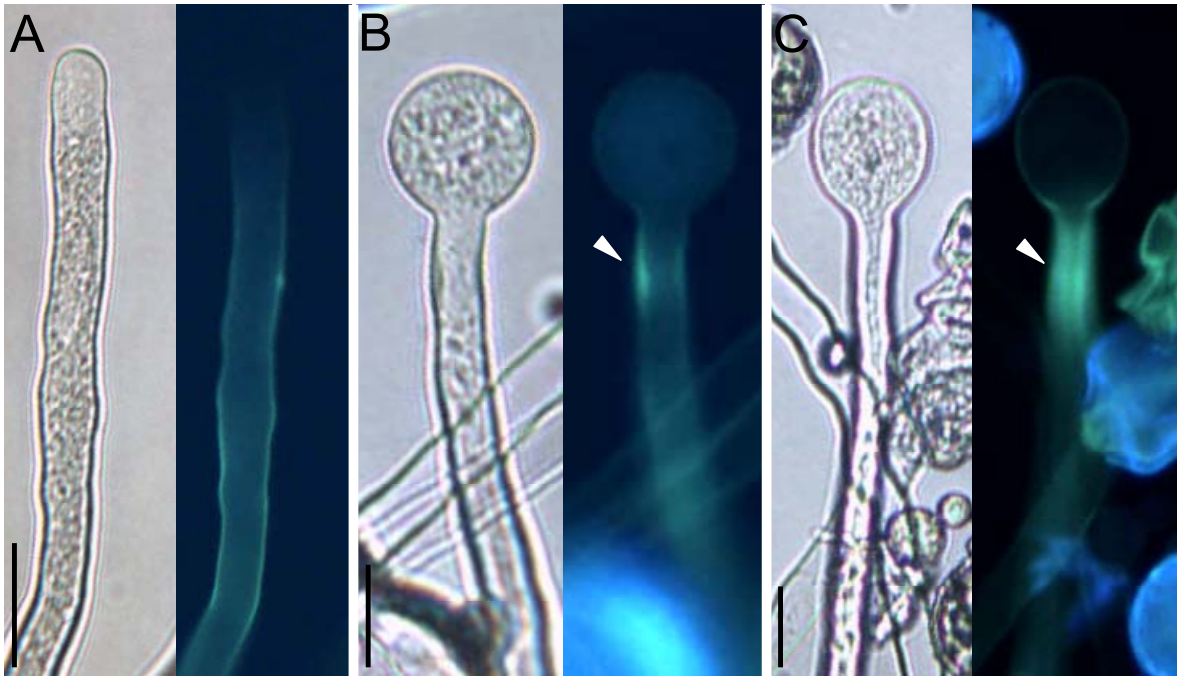

Figure 3.25. Callose distribution in pollen tubes overexpressing PIP5K11

Transiently transformed pollen tubes overexpressing PIP5K11-EYFP were stained with aniline blue after 14 h of growth. Non-transformed pollen tubes were used as a control. Aniline blue fluorescence indicating callose deposition was monitored by epifluorescence microscopy. A, Non-transformed control. The tip region was callose free. B, C, Pollen tubes overexpressing PIP5K11 showed very weak callose deposition in the bulge but increased callose deposition in a collar-like structure right below the bulge, as indicated by arrowheads. Left panels, bright field; right panel, aniline blue fluorescence. Bars, 20 µm. Images represent cell wall stainings that were observed in more than 10 independently transformed pollen tubes.

To verify this hypothesis, callose in the cell wall of tobacco pollen tubes that exhibited tip swelling due to PIP5K11 overexpression was stained with aniline blue (Figure 3.25). Non-transformed pollen tubes were used as controls. As expected the cell wall of the control pollen tubes was only stained in regions about 20-40 µm away from the apex (Figure 3.25 A). Pollen tubes overexpressing PIP5K11 and exhibiting tip swelling displayed weak to no callose deposition in the cell wall of the bulge, but a strong accumulation of callose in a collar-like structure immediately below the bulge (Figure 3.25 B, C).

3.15 Actin cytoskeletal structures in pollen tubes overexpressing PI4P 5-kinases

Because tip swelling of pollen tubes with overexpression of Rac-type GTPases or an inactive PLC-variant has been shown to be mediated in part by changes in the actin cytoskeleton (Kost et al., 1999; Gu et al., 2003; Dowd et al., 2006), it was tested whether morphological alterations observed in pollen tubes overexpressing PI4P 5-kinases would also manifest through regulatory effects on actin cytoskeletal dynamics. In order to document the status of the actin cytoskeleton in cells overexpressing type A or type B PI4P 5-kinases, the pollen tube actin cytoskeleton was visualized after embedding and staining (Lovy-Wheeler et al., 2005) with Alexa-Fluor 568-phalloidin (Figure 3.26). Non-transformed pollen tubes exhibited well-developed actin fringes (Figure 3.26 A). Pollen tubes expressing PIP5K5 and exhibiting tube branching were not obviously altered in actin cytoskeletal structures or, importantly, in the formation of the actin fringe (Figure 3.26 B). In contrast, actin fringes were absent from tubes exhibiting the swelling phenotype with expression of PIP5K11 (Figure 3.26 C, D, E). The absence of the fringe in the swelling tubes was accompanied by the formation of prominent actin cables or massive aggregates which extended into the clear zone of the pollen tube tip (Figure 3.26 C, D, E).

3.16 Altered Golgi-movement in pollen tubes overexpressing type A PI4P 5-kinases

As overexpression of PIP5K11 interfered with formation of the actin fringe (Figure 3.26 C, D, E) required for maintenance of directed vesicle trafficking in the pollen tube tip (Chen et al., 2007), experiments were initiated to visualize the movement of Golgi-particles required for secretion in growing pollen tubes (Figure 3.27). In pollen tubes expressing a CFP-tagged N-terminal protein fragment of N-acetylglucosaminyl transferase I (Nag; Dixit and Cyr, 2002) as a Golgi-marker, cell polarity was not perturbed. In these tubes Golgi-particles were excluded from the clear-zone defining the site of polar tip-growth (Figure 3.27 A), and reverse fountain movement of fluorescent particles was observed. With overexpression of PIP5K11, trafficking of Nag-CFP-decorated Golgi particles was severely disturbed, and CFP-fluorescence was not excluded from the clear zone anymore (Figure 3.27 B, C). Besides Golgi, large vacuoles frequently moved into the swelling tip and remained there despite of ongoing cytoplasmic streaming (Figure 3.27 C).

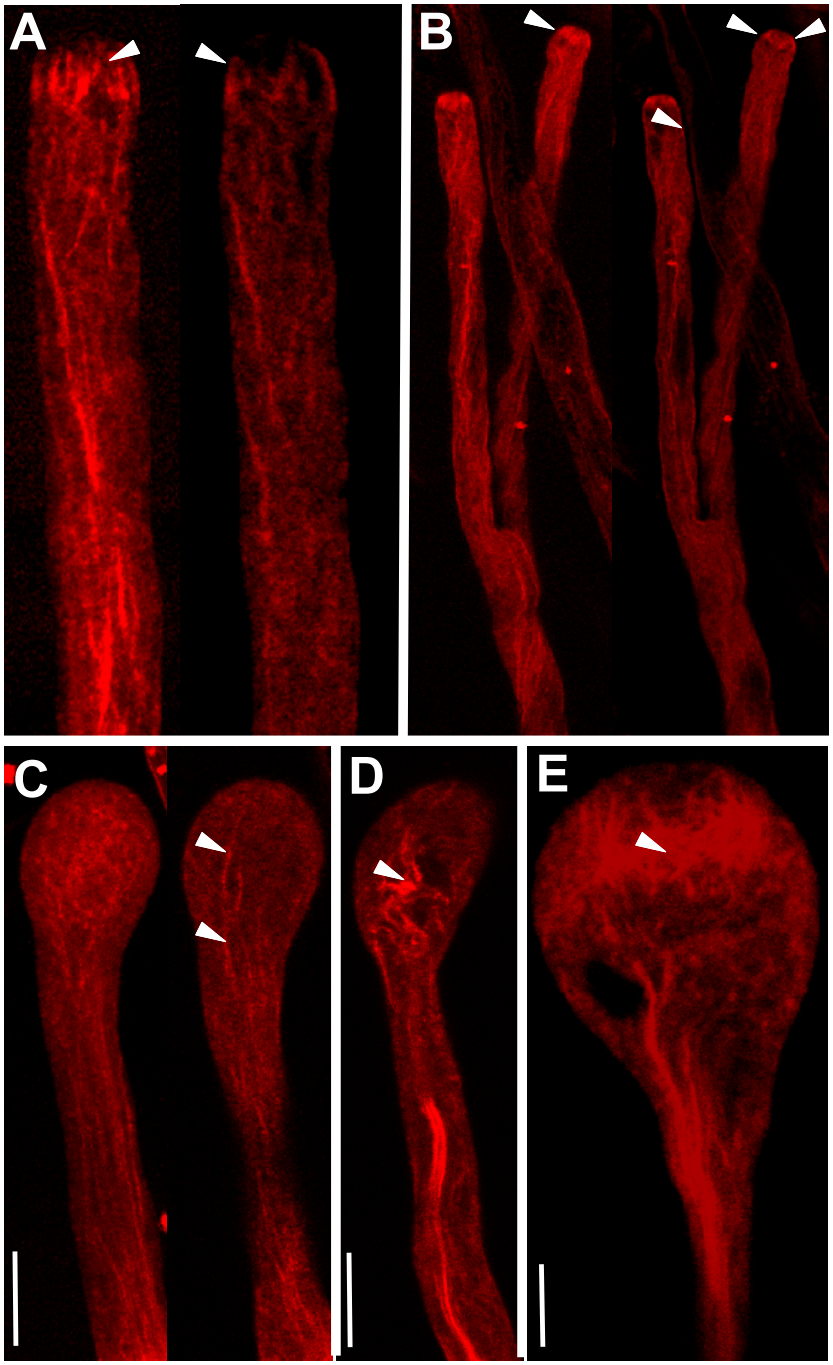

Figure 3.26. Visualization of F-actin in tobacco pollen tubes strongly expressing PI4P 5-kinases

Pollen tubes were embedded and F-actin structures stained with AlexaFluor568-phalloidin. Fluorescence was visualized by confocal microscopy and stacks of sequential confocal sections were recorded. A, Untransformed pollen tubes. Left, Projection of 18 confocal planes (total depth, 12 µm); arrowhead indicates the actin fringe. Right, Single midplane section (depth, 0.7 µm); arrowheads indicate peripheral actin localization. B, Pollen tubes expressing PIP5K5 and exhibiting the branching phenotype. Left, Projection of 15 planes (total depth, 19 µm); arrowhead indicates the actin fringe. Right, Single midplane section (depth, 1.3 µm); arrowheads indicate peripheral actin localization. C-E, Pollen tubes expressing PIP5K11 and exhibiting tip swelling. C, Projection of 27 planes (total depth, 19 µm), showing random actin aggregation. Note the absence of the actin fringe. Right, Midplane section (depth 2.1 µm), indicating absence of peripheral actin and formation of central actin cables (arrowheads). D, E, Single midplane sections (depth, 1 µm) of pollen tubes, illustrating central actin aggregation (D, arrowhead) and the formation of an extensive central actin network (E, arrowhead). Bars, 10 µm. Images show actin stainings representative for more than 50 independently transformed pollen tubes.

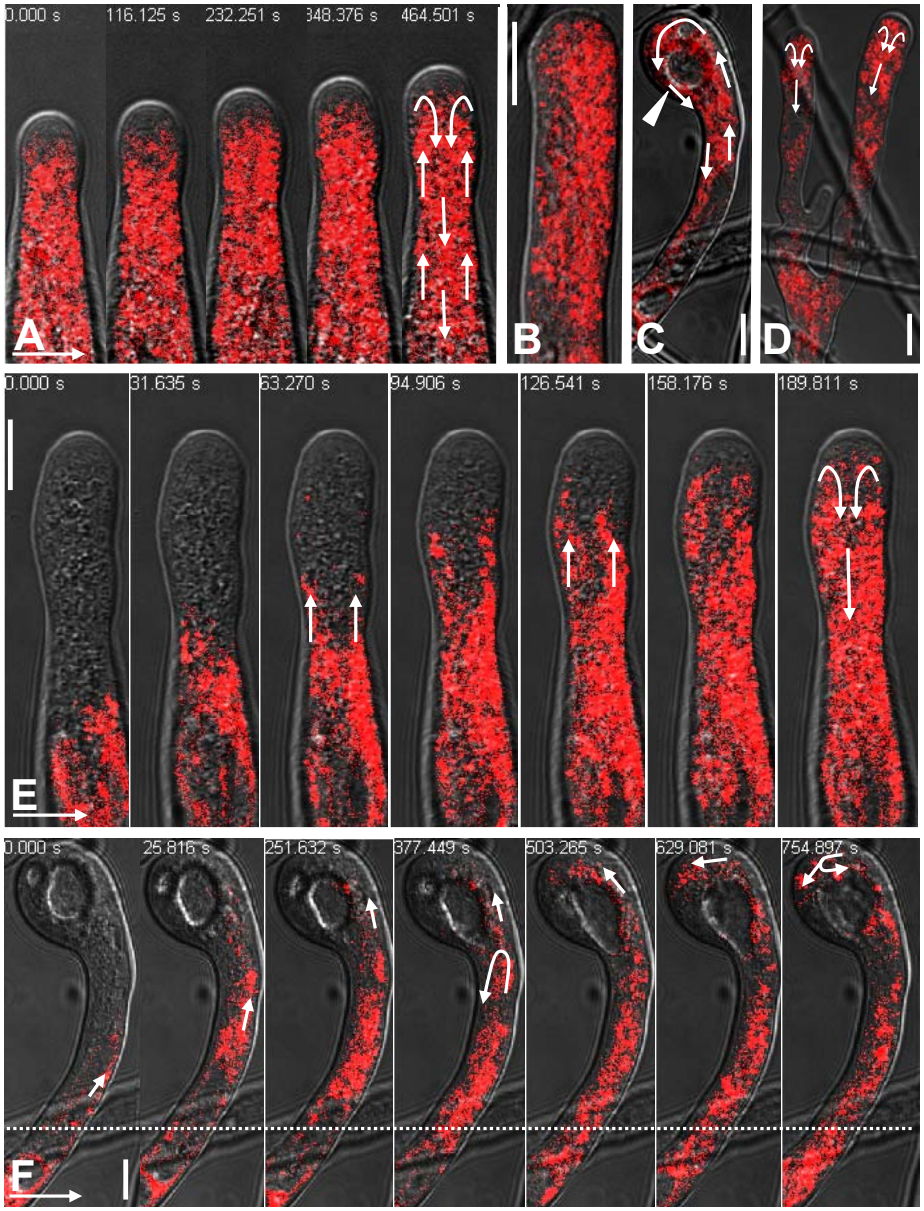

Figure 3.27. Altered Golgi-movement in tobacco pollen tubes strongly expressing PIP5K11

Golgi-particles decorated by the Nag-CFP-reporter and bright-field images were synchronously recorded and the images merged. A, Exclusion of Golgi-particles from the site of polar vesicle-accumulation ("clear-zone"), indicated by the bracket. The progression illustrates continuous growth of the pollen tube and movement of the clear-zone over a period of ~10 min. Arrows indicate reverse fountain streaming of Golgi vesicles. B, C, Loss of the clear-zone and extension of Golgi-movement into the tip in pollen tubes exhibiting weak (B) or strong (C) expression of PIP5K11. Note the appearance of large vacuoles in the swelling tip of the tube shown in panel C. D, normal, reverse fountain pattern of Golgi-dynamics observed in a branched pollen tube expressing PIP5K5. E, F, FRAP-analysis of Golgi-movement in pollen tubes exhibiting normal growth (E) or during tip swelling with strong expression of PIP5K11 (F). Dotted lines mark the lower limit of photo bleaching. E, The progression illustrates the directional movement of Nag-CFP-fluorescence into the growing tip (reverse fountain pattern), as indicated by the arrows over a period of ~3 min. Note the spiralling pattern of peripheral forward-movement and the central retrograde-movement. F, Loss of reverse fountain Golgi-movement and random Golgi-motility in the tip over a period of ~13 min. Note the decelerated and circular or random movement of Golgi-particles and vacuoles in the tip. Bars, 10 µm. Similar Golgi Movement was observed in more than 10 independently transformed pollen tubes.

Laser bleaching of a large area of the growing pollen tube tip and subsequent monitoring of fluorescence recovery (FRAP) indicates the reverse fountain movement in pollen tubes expressing Nag-CFP alone (Figure 3.27 E), whereas this pattern was lost with additional expression of PIP5K11. Instead, circular streaming occurred in areas set back from the tip, while streaming into the tip and former clear zone was slowed and followed a circular or random pattern (Figure 3.27 F).Cell polarity and the reverse fountain streaming pattern were retained in each limb of branched tubes expressing EYFP-tagged PIP5K5, indicating that PIP5K5 overexpression does not interfere with this cellular process (Figure 3.27 D).

3.17 The N-terminal MORN-domain of type B PI4P 5-kinases is not required for tip branching

Because of the identical biochemical activities *in vitro* (Figure 3.6) and the overlapping localization pattern (Figure 3.14, Figure 3.15), the different phenotypes observed with overexpression of type A and type B PI4P 5-kinases presented an enigma. From the phenotypic effects on exocytosis and the cytoskeleton it is clear that different physiological processes are controlled by $PI(4,5)P_2$ produced by type A and type B PI4P 5-kinases, prompting the question, what structural properties of the enzymes were underlying these differences in $PI(4,5)P_2$-dependent regulation. The most obvious difference between type A and type B PI4P 5-kinases is the different domain structure and the presence of the large N-terminal extension containing multiple MORN repeats in type B enzymes (Figure 1.8; Mueller-Roeber and Pical, 2002). Thus, experiments were initiated to test whether the observed phenotypic differences with overexpression of type A and type B enzymes could be attributed to the presence or absence of the N-terminal MORN-repeat domain. A truncated variant of the type B enzyme, PIP5K5, was generated that lacks the N-terminal 252 amino acids representing the extreme N-terminus and the MORN-repeat domain. This truncated version was still an active PI4P 5-kinase when tested *in vitro* (Stenzel and Heilmann, personal communication).

The phenotypes observed with expression of the truncated PIP5K5 protein indicate that the MORN repeat domain was not required for the manifestation of the branching pattern of pollen tube growth (Figure 3.28 A), as this phenotype could be seen in 5 % (13 of 262) of transformed pollen tubes. All other facets of the type B phenotype were also observed with expression of $PIP5K5^{253-772}$ in 54 % (141 of 262) of all transformed pollen tubes, including increased cell wall deposition and stunted growth (Figure 3.28 B-D), or plasma membrane invaginations due to “protoplast trapping” (Figure 3.28 E). The data also document that the N-terminal region of PIP5K5 was not required for correct localization, as the truncated enzyme could still be observed at the plasma membrane and the adjacent cytoplasm when expressed at low levels (Figure 3.29 A, B). In strong overexpressors, however, the truncated $PIP5K5^{253-772}$ localized not only at the plasma membrane, but also in punctate structures of unknown nature (Figure 3.29 C).

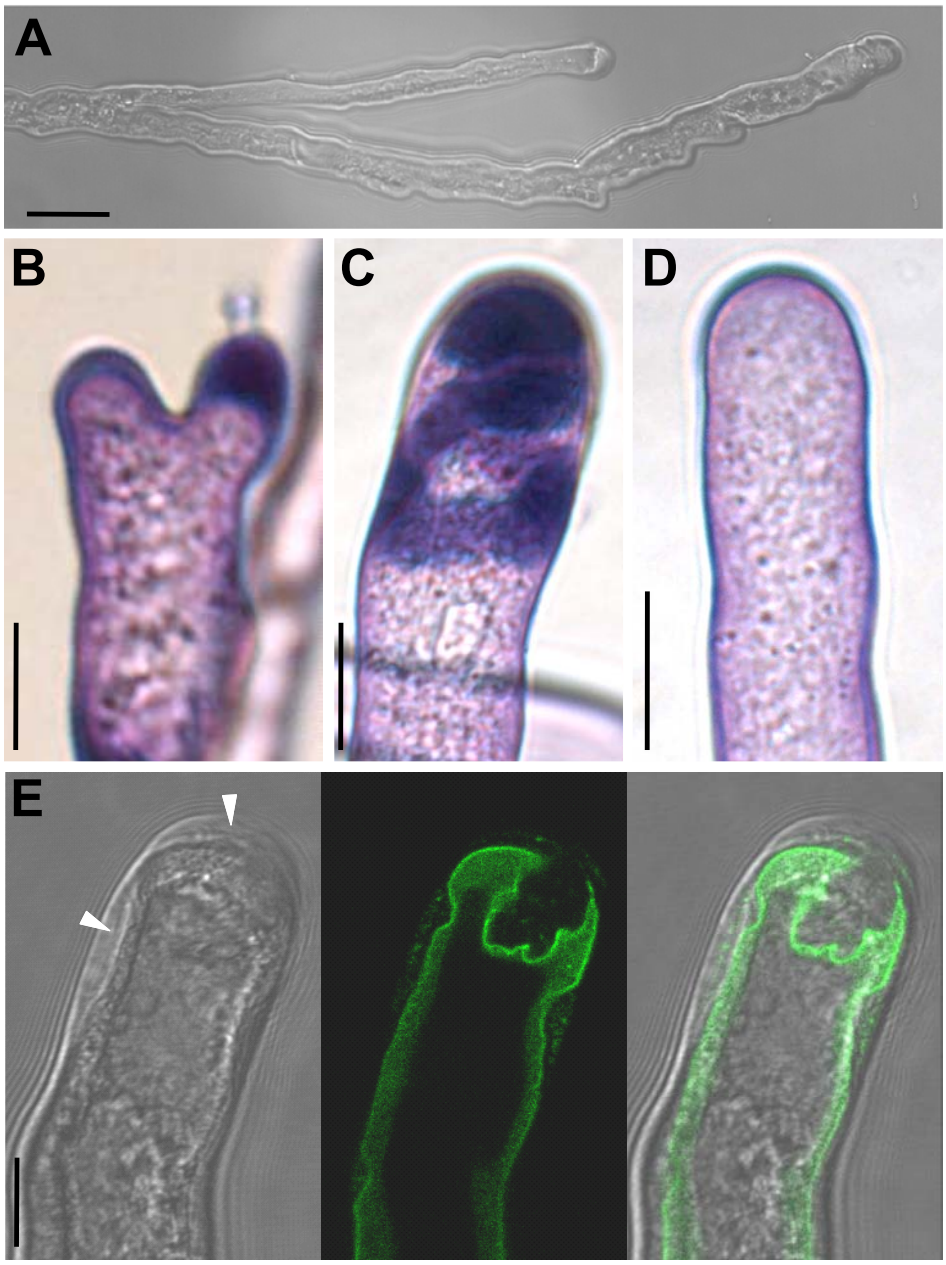

Figure 3.28. The N-terminus of type B PI4P 5-kinases is not required for induction of increased exocytosis

Tobacco pollen tube morphology was analyzed with overexpression of a truncated variant of PIP5K5 lacking the N-terminus and the MORN-repeat domain. A, Tip branching with expression of the truncated PIP5K5$^{253-772}$. B, C, Increased cell wall deposition with strong expression. D, Non-transformed control. E, Plasma membrane invaginations in pollen tubes with massive apical and lateral (arrowheads) cell wall deposition. Bars, 50 µm (A), 10 µm (B-E). Images represent phenotypes that were observed in more than 50 independently transformed pollen tubes.

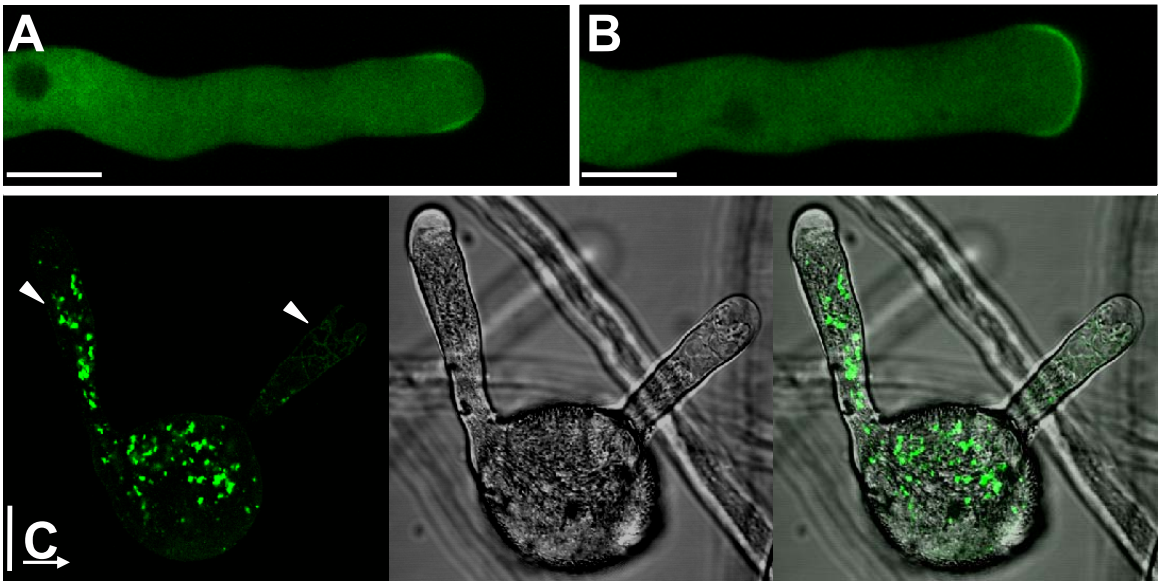

Figure 3.29. The N-terminus of type B PI4P 5-kinases is not required for plasma membrane localization

A truncated variant of PIP5K5, PIP5K5$^{253-772}$.lacking the N-terminus and the MORN-repeat domain was transiently expressed as an EYFP fusion protein in tobacco pollen tubes and the localization was monitored by confocal microscopy. A, B, Localization of PIP5K5$^{253-772}$ at the plasma membrane of a growing (a) and non-growing (B) pollen tube. C, Strong overexpressor of PIP5K5$^{253-772}$ showing localization of PIP5K5$^{253-772}$ not only at the plasma membrane (right arrow) but also in punctate structures of unknown nature (left arrow). C, left, EYFP fluorescence; middle, bright field; right, merge. Bars, 10 µm (A, B), 20 µm (C). Images represent phenotypes that were observed in more than 30 independently transformed pollen tubes.

3.18 Expression of non-plant PI4P 5-kinases in pollen tubes

The differences in phenotypes resulting from overexpression of *Arabidopsis* type A or type B PI4P 5-kinases suggested that the precise subcellular context of PI(4,5)P_2 production may be important for the ensuing physiological effects. In order to test whether a complex signaling microenvironment is required for the mediation of the branching or swelling phenotypes, effects of the only distantly related PI4P 5-kinases HsPIPK1α from *Homo sapiens* and the sole PI4P 5-kinase isoform, NcPIP5K, from the filamentous ascomycete, *Neurospora crassa*, on pollen tube growth were investigated.

HsPIPK1α had previously been demonstrated to harbour PI4P 5-kinase activity (Anderson et al., 1999), whereas NcPIP5K previously had not been characterized. Recombinant NcPIP5K displayed specific PI4P 5-kinase activity (Stenzel and Heilmann, personal communication).

HsPIPK1 and NcPIP5K were expressed in tobacco pollen tubes to determine whether the localization of the non-plant PI4P 5-kinases would be similar to that of the *Arabidopsis* PI4P 5-kinases, and whether the non-plant enzymes would be able to induce similar phenotypes. In contrast to the distribution of the *Arabidopsis* enzymes, both non-plant PI4P 5-kinases localized not only to the apical region of the pollen tube, but to the entire plasma membrane and the nucleus (Figure 3.30 A-E). In growing pollen tubes localization of NcPIP5K to the subapical part of the plasma membrane was enhanced (Figure 3.30 A). Strong overexpression of NcPIP5K led to an arrest of polar growth and tip swelling (Figure

3.30 B-C), a phenotype that was also induced by overexpression of type A PI4P 5-kinases (Figure 3.24). In contrast, overexpression of HsPIPK1α did not result in phenotypical changes (Figure 3.30 D-E) or in growth arrest.

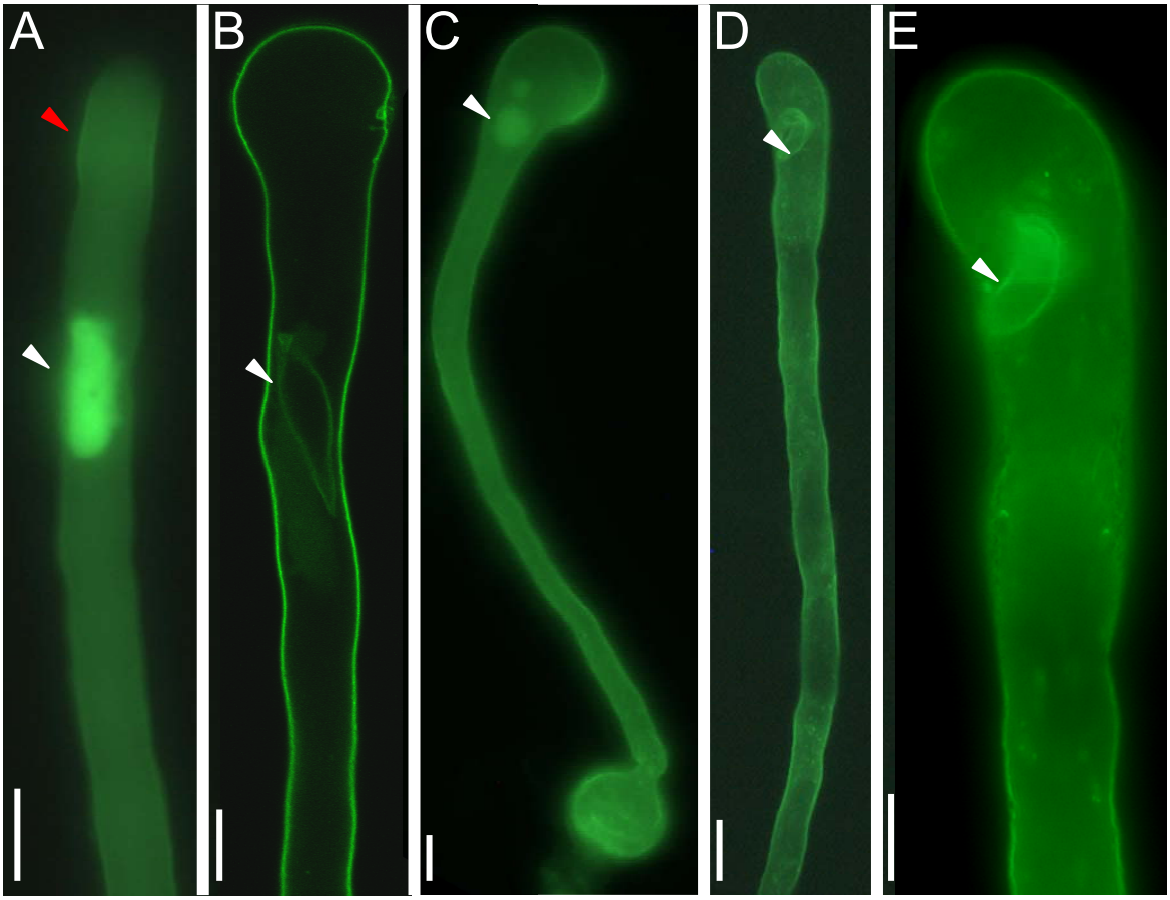

Figure 3.30. Expression of non-plant PI4P 5-kinases in pollen tubes

NcPIP5K and HsPIPK1α were transiently expressed as EYFP fusion proteins in tobacco pollen tubes and monitored by epifluorescence (A, C-E) or confocal (B) microscopy. A, NcPIP5K localization was stronger in the subapical part than in other parts of the plasma membrane as indicated by the red arrow. B, C, tip swelling induced by overexpression of NcPIP5K. D, E plasma membrane localization of HsPIPK1α. A-E, White arrow indicates the nucleus. Bars A, E, 10µm; B-D, 20 µm. Images represent phenotypes that were observed in more than 50 independently transformed pollen tubes from two independent experiments.

3.19 PI(4,5)P_2 microdomains are restricted by PLC

Previous work has suggested that the dimension of the plasma membrane PI(4,5)P_2-microdomain was restricted by PLC (Dowd et al., 2006; Helling et al., 2006). When red fluorescent protein (RFP)-tagged tobacco PLC3 (NtPLC3) (Helling et al., 2006) was coexpressed with EYFP-tagged PIP5K11, the resulting distribution indicated that NtPLC3 not only colocalized over the whole range with PIP5K11 but was also situated in a defined area behind PIP5K11 in the pollen tube plasma membrane (Figure 3.31). Localization of NtPLC3 in relation to that of PIP5K11 during pollen tube growth can also be viewed in a series of images in the appendix (Figure 6.5). The observed pattern is consistent with a role for NtPLC3 in restricting PI(4,5)P_2 formed by both type A and type B PI4P 5-kinases.

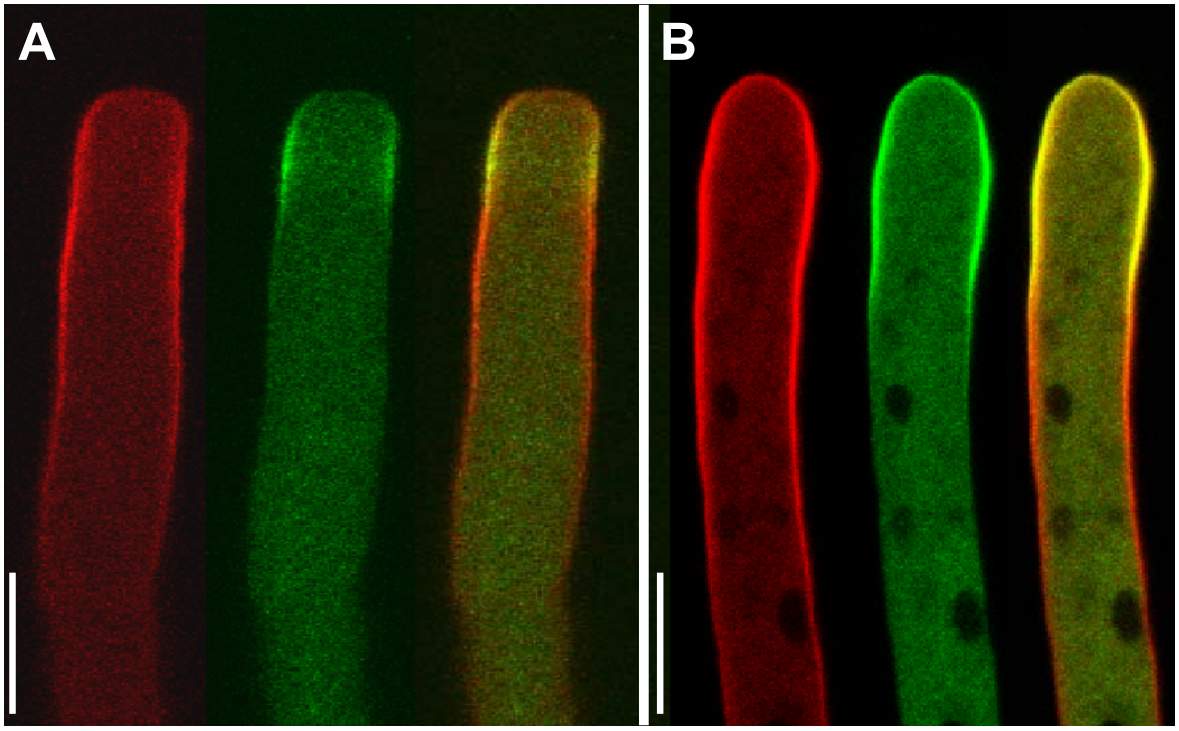

Figure 3.31. Localization of NtPLC3 in respect to PIP5K11

NtPLC3 was coexpressed as a RFP fusion protein with PIP5K11:YFP. NtPLC3 covers a larger area of the plasma membrane than PIP5K11 in growing (A) and non-growing (B) tobacco pollen tubes. Left, RFP fluorescence; middle YFP fluorescence; right, merge, yellow indicates overlap. Bars, 10 µm. Images represent phenotypes that were observed in more than 50 independently transformed pollen tubes.

3.20 PIP5K5 acts in a common pathway with PI 4-kinase β1

Since PI(4,5)P_2 production by PI4P 5-kinases depends on the availability of their substrate, PI4P, increased PI4P levels could also lead to higher PI(4,5)P_2 formation, and possibly cause similar phenotypes as the direct increase of PI(4,5)P_2 by overexpression of PI4P 5-kinases. To augment PI4P levels the *Arabidopsis* PI 4-kinase β1 (PIKβ1; Xue et al., 1999) was transiently expressed at high levels in tobacco pollen tubes, and the phenotypes were analyzed after 14h of tube growth. Surprisingly, only phenotypes such as pollen tube branching and the “trapped protoplast” (Figure 3.32 A, B) associated with overexpression of type B PI4P 5-kinases were observed, albeit, at a lower frequency (Jin and Heilmann, personal communication). Tip swelling as seen in pollen tubes overexpressing type A PI4P 5-kinases was not observed. The combined data gave rise to the hypothesis that PI4P generated by PIKβ1 was mostly a substrate for type B PI4P 5-kinases. To test this hypothesis, PIKβ1 was coexpressed with PIP5K5 and PIP5K11, respectively, in tobacco pollen tubes, and the phenotypes were monitored. The coexpression experiment of PIP5K5 with PIKβ1 showed a very strong increase in the occurrence of the “trapped protoplast” phenotype compared to the control experiment where PIP5K5 was expressed with EYFP (Figure 3.32 C). This observation suggests that PIKβ1 and type B PI4P 5-kinases act synergistically since the “trapped protoplast” is

associated with the strongest disturbance of $PI(4,5)P_2$ levels generated by type B PI4P 5-kinases.

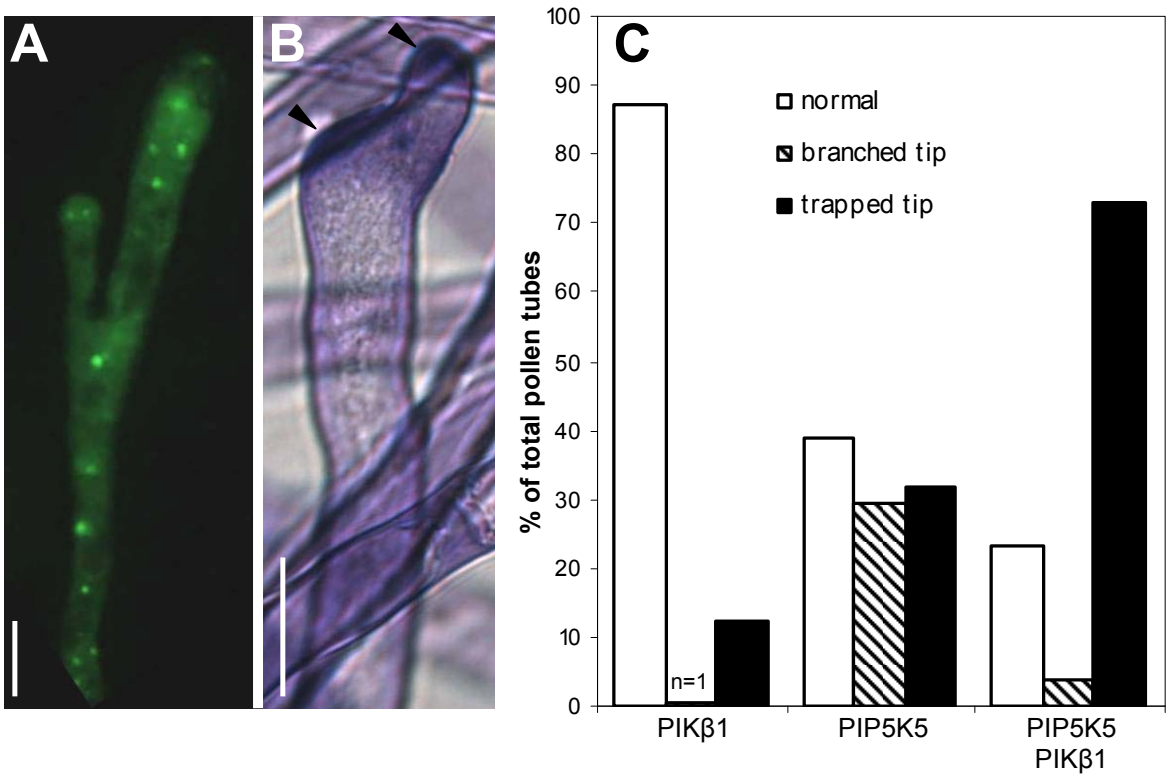

Figure 3.32. PIKβ1 acts in a common pathway with PIP5K5

A, B, Tobacco pollen tubes overexpressing PIKβ1 display similar phenotypes as overexpression of type B PI4P 5-kinases (Figure 3.20, Figure 3.21, Figure 3.22) such as pollen tube branching and excess incorporation of cell wall material. A, EYFP fluorescence; B bright field image after toluidine blue O staining. Arrows indicate excess cell wall incorporation. Bars, 20 µm. C Overexpression of PIP5K5 and PIKβ1 has synergistic effects. The number of pollen tubes that display the "trapped protoplast" is greatly increased when both proteins are overexpressed in comparison to overexpression of either PIKβ1 or PIP5K5 alone. Images are representative for at least five pollen tubes from two independent experiments. Data was scored from more than 110 independently transformed pollen tubes from each line.

In contrast, coexpression of PIKβ1 with PIP5K11 in tobacco pollen tubes led to no increase in the PIP5K11-induced tip swelling phenotype (Figure 3.33) that was described above (Figure 3.24). Quite contrary, with coexpression of PIP5K11 and PIKβ1 the average tip diameter was significantly smaller than in tubes coexpressing PIP5K11 and the EYFP control (Figure 3.33). This result could be due to an increase of cell wall strength caused by a PIKβ1 mediated increase of pectin exocytosis, which could lead to a decrease in tip swelling.

Considering the data of the experiments performed with PIKβ1 it can be concluded that this enzyme together with PIP5K5 regulates exocytosis of cell wall material but does not act in a common pathway with type A PI4P 5-kinases.

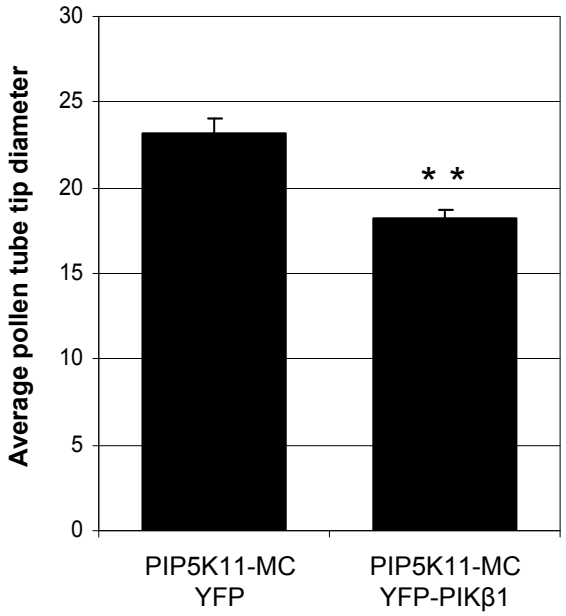

Figure 3.33. Coexpression of PIKβ1 decreases the PIP5K11 induced tip swelling phenotype

Tobacco pollen tubes were transiently expressing either PIP5K11:mCherry and EYFP as a control or PIP5K11:mCherry and EYFP:PIKβ1 were grown for 16 h. The tip diameter of 100 pollen tubes each was measured. The average tip diameter was significantly smaller ($p<0.0001$ according to a Student's t-test) in pollen tubes expressing PIP5K11:mCherry and EYFP:PIKβ1 as compared with the control. ± standard error. Data represents one experiment.

3.21 PIKβ1 localizes to secretory vesicles of pollen tubes

As we found that PIKβ1 acts in a common pathway with PIP5K5 which is localized to the plasma membrane, it was interesting to see where this kinase would localize in the pollen tube. Transient expression of *Arabidopsis* PIKβ1 with a fluorescence tag revealed localization to punctate structures spanning all the way to the tip of growing (Figure 3.34 A) and non-growing pollen tubes (Figure 3.34 B-D). It could also be observed that the expression level of PIKβ1 correlated with an increase of the size of the decorated punctate structures (Figure 3.34 A), possibly due to aggregation. The tip of growing pollen tubes - the so called clear zone - is free of larger organelles but also of Golgi particles and is presumed to contain secretory vesicles (Sherrier and VandenBosch, 1994). The punctate structures decorated with the PIKβ1 clearly penetrated into this clear zone. This observation was confirmed by coexpressing the kinase with a fluorescence-tagged marker for Golgi vesicles, Nag. This experiment demonstrates that the Golgi vesicles are unable to penetrate the clear zone, while the vesicles bound by PIKβ1 were present there (Figure 3.35 C). It was further observed that there was no overlap between these two pools of particles (Figure 3.35 C).

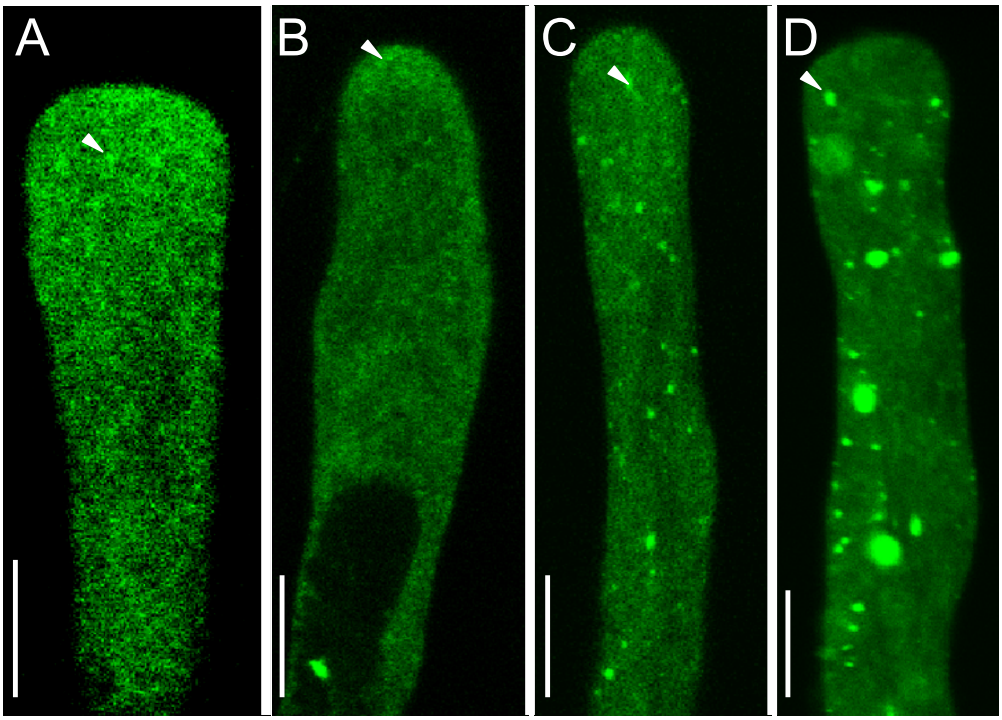

Figure 3.34. PIKβ1 localizes to punctate structures in tobacco pollen tubes and changes their morphology

PIKβ1 was transiently expressed as a EYFP fusion protein in tobacco pollen tubes and the localization was monitored by confocal microscopy in growing (A) and non growing (B-D) pollen tubes. A, B, Arrow indicates vesicle accumulation at the tip in the clear zone. C, D, increasingly stronger overexpression of PIKβ1 leads to an increase in vesicle size. Bars, 10 µm. Images obtained by Xu Jin. Images are representative for localization patterns observed in more than 50 independently transformed pollen tubes in four independent experiments.

It had previously been proposed that PI 4-kinases of the β subfamily are associated with trans-Golgi secretory vesicles in root hairs (Preuss et al., 2006). The data presented here confirm this notion for pollen tubes. In order to define possible cargo of secretory vesicles, it was tested if PIKβ1 associated with vesicles containing the secreted protein expansin 4 from *Arabidopsis*, which plays a role in cell wall loosening (Cosgrove, 2000) and is normally exclusively expressed in pollen according to the Genevestigator portal (Zimmermann et al., 2004). The fluorescence-tagged expansin protein was transiently expressed in tobacco pollen tubes and was found in vesicles but also in the cell wall outside the cell (Figure 3.35 A). Since the fluorescence of the expansin overlapped partially with that of the Nag-Golgi marker (Figure 3.35 B), it can be concluded that the expansin protein is secreted by the cell via the secretory pathway and that the vesicles only containing expansin 4 are secretory vesicles. Coexpression of mCherry-tagged expansin 4 and EYFP-tagged PIKβ1, however, revealed no overlap of fluorescence (Figure 3.35 D). This observation indicates that if PIKβ1 localizes to secretory vesicles that contain pectin, as hypthesized above, these vesicles must be independent from the vesicle pool that is responsible for the exocytosis of proteins, such as expansin 4.

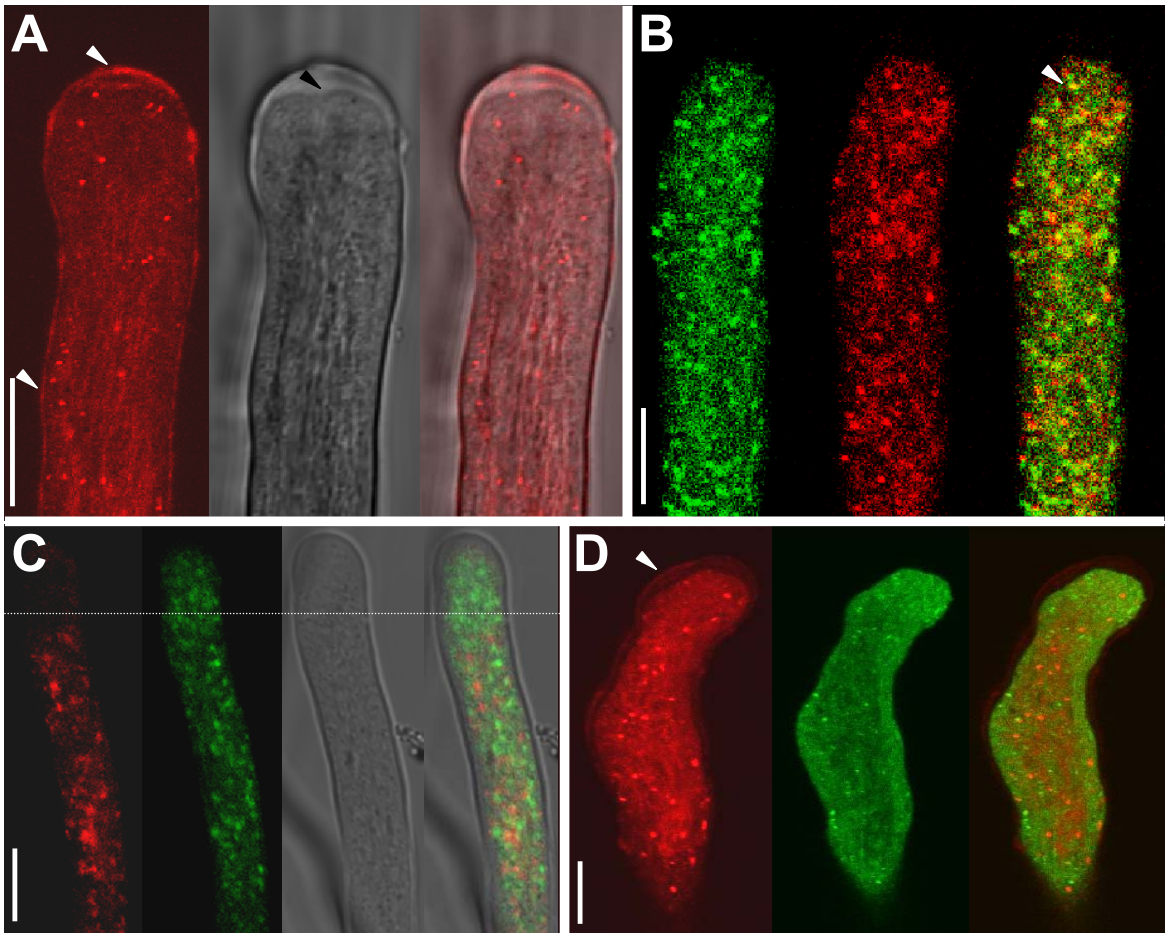

Figure 3.35. Localization pattern of EXP4, NAGT and PIKβ1

EXP4-mCherry, Nag-CFP and YFP-PIKβ1 were transiently expressed in different combinations in tobacco pollen tubes and localization was monitored by confocal microscopy. A, Expression of EXP4-mCherry alone revealed localization in vesicles and in the cell wall as indicated by white arrows. Plasma membrane localization can be ruled out, because the plasma membrane has drawn back from the very apex of the cell wall as can be seen in the bright field image, as indicated by the black arrow. Left, mCherry; middle, bright field; right, merge. B, Coexpression of EXP4-mCherry and the Golgi marker Nag-CFP reveals that some of the vesicles containing EXP4 are independent from the pool of Golgi vesicles while in others fluorescence overlaps as can be seen from yellow colour in the merged image as indicated by the arrow. Note that the absence of the clear zone is due to an arrest in growth. Left, CFP; middle, mCherry; right, merge. C, Vesicles associated with EYFP-PIKβ1 are independent from Golgi vesicles marked by Nag-CFP. While the clear zone at the pollen tube apex as indicated by the dotted line is free of Golgi vesicles, EYFP-PIKβ1-associated vesicles can penetrate this zone. Left to right, CFP; EYFP; bright field; merge. D, Coexpression of EYFP-PIKβ1 and EXP4-mCherry shows no fluorescence overlap indicating independence of the vesicle pools containing EXP4 and associated with EYFP-PIKβ1. Arrows indicates cell wall staining by EXP4-mCherry. Left, mCherry; middle, EYFP; right, merge. Bars, 10 µm. Images represent phenotypes that were observed in more than 20 independently transformed pollen tubes.

3.22 PIKβ1 overexpression leads to changes in the distribution of PI4P

Similar to the visualization of PI(4,5)P_2 with the PH domain of HsPLCδ1 (Varnai and Balla, 1998), PI4P pools can be detected using the PI4P-binding domain of HsFAPP (Weixel et al., 2005; Blumental-Perry et al., 2006). When a fluorescence-tagged FAPP domain was expressed in tobacco pollen tubes (Figure 3.36), it localized predominantly at the plasma

membrane of the tip, overlapping with the PH domain of HsPLCδ1, but not at vesicles in the cytosol. This observation suggests that the pool of PI4P generated by type β PI-kinases at secretory vesicles must be small compared to the amount of PI4P present at the plasma membrane, either because the rate of synthesis is low or because the PI4P is quickly degraded. Another reason could be that the PI4P in vesicles is not accessible to the HsFAPP domain, because it is tightly bound to other proteins. Overexpression of PIKβ1 in tobacco pollen tubes led to a redistribution of the HsFAPP domain, which now also localized not only to the plasma membrane but also to vesicles associated with PIKβ1 (Figure 3.36). From this redistribution of the marker it may be concluded that the amount of PI4P at the vesicles was now increased versus the amount of PI4P at the plasma membrane in comparison to untransformed controls. The data, thus, support the notion that vesicle-associated PI4P originates from a different source than plasma membrane-associated PI4P.

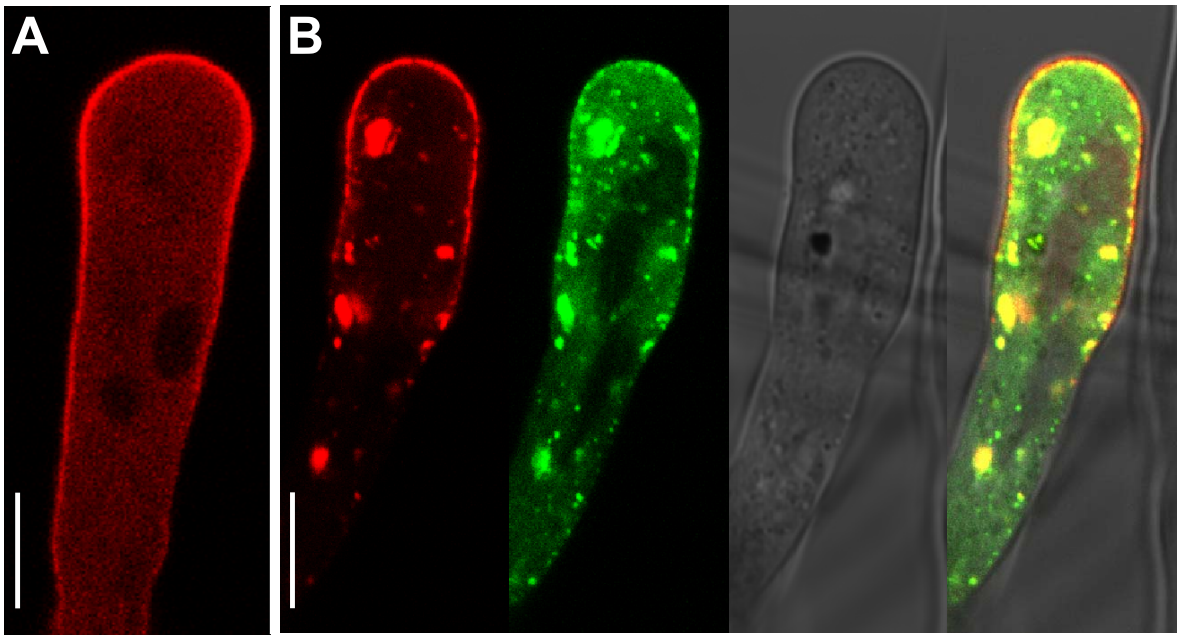

Figure 3.36. Overexpression of PIKβ1 leads to changes in PI4P distribution

The PI4P probe Redstar-HsFAPP was expressed with EYFP-PIKβ1 in tobacco pollen tubes, and the localization was monitored by confocal microscopy in non-growing tubes. A, HsFAPP localizes only to the plasma membrane of control pollen tubes not expressing PIKβ1. This indicates occurrence of PI4P predominantly at the plasma membrane. Image obtained by Xu Jin. B, When PIKβ1 was coexpressed with HsFAPP, the PI4P marker localized not only to the plasma membrane but also to vesicles labeled by PIKβ1, indicating an increase of PI4P at the vesicles due to PIKβ1 overexpression. Left to right, Redstar; EYFP, bright field, merge. Yellow indicates overlap. Images represent distribution pattern that were observed in more than 20 independently transformed pollen tubes.

4. Discussion

Phosphoinositides regulate many aspects of cellular physiology, such as membrane trafficking, the dynamics of the cytoskeleton and ion channel activity, as was introduced above. Various aspects of phosphoinositide signaling have been previously studied in animals and in yeast. In plants, however, substantially less is known. There are several possible approaches to study the function of phosphoinositides in plants. A broad approach to study the role of phosphoinositides *in vivo* is to manipulate phosphoinositide levels inside the cells. Such manipulations have been attempted before by addition of exogenous $PI(4,5)P_2$ to plant cells (Ozaki et al., 2000), as for example pollen tubes (Monteiro et al., 2005) and guard cells (Lee et al., 2007). The unclear efficiency of delivery and the unspecific "flooding" of the cells with often unphysiological amounts of the compounds applied have seriously limited interpretation in those cases mentioned. As a more specific alternative approach, in this work genetic tools were used to obtain cells with increased or decreased levels of $PI(4,5)P_2$ by overexpression or disruption of PI4P 5-kinase genes. The advantage of the approach chosen here is that $PI(4,5)P_2$ pools are generated or depleted by modulation of endogenous enzymes at their site of action and not in the whole cell as for example when the cell is flooded with exogenous $PI(4,5)P_2$. If independent pools of $PI(4,5)P_2$ exist in the cell, as has been proposed (King et al., 1987; Augert et al., 1989; Heilmann et al., 1999; Heilmann et al., 2001; König et al., 2007; Heilmann, 2008) and they are generated by different PI4P 5-kinase isoforms, it was the rationale of this study to test whether such pools could be individually manipulated by our approach.

4.1 A genetic approach to perturb PI(4,5)P_2 biosynthesis in plant cells

In this study the phosphoinositide network was studied in pollen tubes. The pollen tube system was chosen for several reasons, most importantly, because pollen tubes were the first plant cell type in which a defined PI(4,5)P_2 microdomain had been described (Kost et al., 1999). In addition, pollen grains are easily transformable, and the localization of proteins fused to fluorescent markers in emerging pollen tubes can be studied *in vivo* under the microscope. From several published examples it is known that the manipulation of signaling pathways in pollen tubes can have dramatic effects on polar tip growth behaviour (Clarke et al., 1998; Franklin-Tong, 1999; Gupta et al., 2002; Holdaway-Clarke et al., 2003; Palanivelu et al., 2003; Huang et al., 2004; Gu et al., 2005; Cole and Fowler, 2006; Klahre et al., 2006; Malho et al., 2006; Chen et al., 2007; Krichevsky et al., 2007; Cheung and Wu, 2008; Kost, 2008). Finally, a big advantage is that pollen tubes often exclusively express their own set of isoforms of different gene families, indicating that they have a distinct metabolic identity (Becker et al., 2003). This is especially interesting for genetic studies, since pollen tubes can be studied autonomously from other parts of the plant.

The *Arabidopsis* genome contains eleven genes coding for putative PI4P 5-kinases (Figure 1.7), and in the course of this work these eleven *Arabidopsis* genes were characterized. By expression analysis it was found that five of the PI4P 5-kinase isoforms were almost exclusively expressed in pollen tubes (Figures 3.1, 3.2, 3.3, 3.4 and 3.5). The expression patterns found were in accordance with expression data from microarray experiments that can be viewed via the Genevestigator portal (Zimmermann et al., 2004). One exception was the gene *PIP5K10* that exhibited overall low expression levels in the microarrays, including low expression in pollen. The experiments in this work as well as work published by others (Perera et al., 2005) showed, however, that low expression levels in pollen grains were followed by much stronger expression in germinating pollen tubes (Figures 3.1, 3.2 and 3.3). This result points out that genes with important roles in pollen tube growth can be missed if only expression data from microarray experiments in non-germinated pollen grains are considered.

As a prerequisite for all studies on putative PI4P 5-kinases performed in this thesis, the enzymes were first biochemically characterized. The characterization is particularly important, because functional annotation based solely on sequence comparison can be misleading. For instance, a protein family annotated as PI4-kinases of the γ subfamily according to sequence similarity to proteins from other organisms (Mueller-Roeber and Pical, 2002) was recently reported to represent protein kinases rather than PI 4-kinases (Galvao et al., 2008). The five pollen-expressed PI4P 5-kinases analyzed in this work were all tested not only with regard to their catalytic activity towards PI4P, but also towards other substrates. All five isoforms exhibited PI4P 5-kinase activity (Figure 3.6 and Table 3.1) and a minor amount of PI3P 4-kinase activity (Table 3.1), similar to the activity observed for the other six isoforms (Elge et al., 2001; Perera et al., 2005; Lee et al., 2007; Kusano et al., 2008; Stenzel et al., 2008; Stenzel and Heilmann, personal communication). A PI5P 4-kinase activity previously also taken under consideration

(Mueller-Roeber and Pical, 2002) was not detected (Table 3.1). The biochemical evidence is in accordance with the architecture found in the active centers of the enzymes tested that predicts PI4P 5-kinase activity rather than PI5P 4-kinase activity (Kunz et al., 2000; Kunz et al., 2002).

4.2 Individual functions and redundancy of PI4P 5-kinases

Since multiple PI4P 5-kinase isoforms are expressed in pollen tubes, experiments were initiated to test whether all these isoforms are redundant in their function or whether they play individual roles during pollen grain maturation, germination and growth.

Mutant analysis revealed that the single mutants *pip5k4*, *pi5k5*, *pip5k10* and *pip5k11* had no obvious gametophytic or sporophytic phenotypes. From this observation it can be concluded that some redundancy concerning PI4P 5-kinases must be present in pollen. Since the type A PI4P 5-kinases, PIP5K10 and PIP5K11, and the type B PI4P 5-kinases, PIP5K4 and PIP5K5, are most similar to each other (Mueller-Roeber and Pical, 2002), crosses of mutants lacking the respective proteins (*pip5k4* x *pip5k5* or *pip5k10* x *pip5k11*) were performed, and the offspring was analysed. These experiments revealed that pollen as well as ovules deficient in both type A PI4P 5-kinases showed severe developmental defects (Figures 3.12 and 3.13), despite of the presence of several pollen-expressed type B PI4P 5-kinases. The observed ovule lethality (Figure 3.13 B) is of particular interest, because it was not suspected from promoter GUS analyses and raises new possibilities for ongoing experiments to study the roles of phosphoinositides in ovule development. With regard to the male gametophyte, *pip5k10 pip5k11* double mutant pollen grains were misshapen, smaller in size and failed to germinate (Figure 3.13 A). In consequence, it can be concluded that type A PI4P 5-kinases have important functions already during pollen maturation. To get a better understanding of the role of PI4P 5-kinases in pollen development, future experiments need to be performed. It would be interesting to find out at what stage pollen grains lacking type PI4P 5-kinases fail to develop normally. Another experiment could be to observe the localization of type A PI4P 5-kinases during early developmental stages of the pollen grain. Evidence for roles of type A PI4P 5-kinases not only in pollen development but also in polar tip-growth comes from the localization studies performed. PIP5K11-EYFP was found at the plasma membrane of growing *Arabidopsis* pollen tubes when ectopically expressed under its intrinsic promoter, suggesting a function in polar tip growth of the emerging pollen tubes.

In contrast to the observations on mutants deficient in type A PI4P 5-kinases, the *pip5k4 pip5k5* double mutant did not exhibit male nor female gametophytic lethality, however, pollen grains from these plants were greatly reduced in their *in vitro* germination rate (Figure 3.9 A). In addition pollen tubes protruding from those grains that did germinate were significantly shorter than those of WT controls (Figure 3.9 B). Since the effect on the germination rate was most striking, it can be concluded that PIP5K4 and PIP5K5 are important during the germination phase and - possibly - less important during later phases of pollen tube growth. The germination and growth defects in *pip5k4 pip5k5* double mutants were reversed by the ectopic introduction of either *PIP5K4*:*EYFP* or *PIP5K5*:*EYFP* into the double mutant plants (Figure 3.10). This observation proves that

the pollen phenotype was due to the combined disruption of the *PIP5K4* and *PIP5K5* genes rather than to independent T-DNA insertions elsewhere in the genome. The results also demonstrate that the fluorescence-tag is not obstructive and that PI4P 5-kinase-EYFP fusion proteins are fully functional *in vivo*, as had been previously reported (Stenzel et al., 2008). Pollen germination and the length of pollen tubes were actually increased in *pip5k4 pip5k5* double mutants overexpressing *PIP5K4*:*EYFP* or *PIP5K5*:*EYFP* under intrinsic promoter fragments in comparison to non-transformed WT pollen (Figure 3.10 E). This observation is especially surprising since T1 plants are only heterozygous for the ectopically introduced genes, and only some of the pollen grains contain the newly introduced genes and some not. It follows that the germination rate would be even higher if only transgenic pollen were considered, indicating that overexpression of type B PI4P 5-kinases exerts a positive effect on the pollen germination rate. The heterogeneity of the pollen has, however, only little effect on the growth of pollen tubes, because the bulk of germinated pollen will in fact be transgenic due to the low germination rate of the untransformed *pip5k4 pip5k5* pollen. Since plants with increased and decreased levels of pollen-specific type B PI4P 5-kinases were generated in this work, future experiments could be undertaken to determine if these differences in the abundance of PI4P 5-kinases lead to changes in the PI4P 5-kinase activity or in the lipid levels of pollen grains harvested from these plants.

As outlined above, the *pip5k10 pip5k11* double mutant proved to be pollen lethal (Figure 3.13 A), whereas the *pip5k4 pip5k5* double mutant pollen was viable (Figure 3.8). A possible explanation for this observation is that PIP5K10 and PIP5K11 are the only type A PI4P 5-kinases present in *Arabidopsis* and that type A enzymes have different functions than type B PI4P 5-kinases, even though both type A and type B enzymes are expressed at the same time and in the same cell. Besides PIP5K4 and PIP5K5, several additional type B PI4P 5-kinases isoforms are expressed in pollen (Figure 3.2), and it may be necessary to eliminate other isoforms in addition to PIP5K4 and PIP5K5 to obtain mutants completely deficient in type B PI4P 5-kinase function in order to study the ensuing effects. Especially the isoform PIP5K6 was found to be expressed in pollen and pollen tubes at high levels (Figures 3.1, and 3.2), however, the fact that no T-DNA insertion lines disrupted in this gene are available complicates the generation of a *pip5k4 pip5k5 pip5k6* triple mutant. An RNAi approach was undertaken to reduce PIP5K6 levels in *pipk4 pipk5* mutant plants, but the T1 offspring has yet to be analysed.

In pollen tubes it may be possible that $PI(4,5)P_2$ produced by type B PIP4P 5-kinases supports certain physiological functions such as exocytosis, but that the type B kinases are not absolutely necessary, because these physiological functions can also be regulated by $PI(4,5)P_2$ generated by type A PI4P 5-kinases. In yeast, for example, a third PI 4-kinase was recently discovered that was not essential for cell proliferation despite a demonstrated unique functionality (Han et al., 2002). Therefore, the possibility that type B PI4P 5-kinases, even though being important, are not essential for pollen tube growth must also be taken under consideration.

The combined results of the mutant analyses (Figures 3.8, 3.9 and 3.13) and overexpression studies (Figures 3.20, 3.21, 3.22, 3.23 and 3.24) discussed in more detail

further down indicate that type A and type B PI4P 5-kinases have different roles in pollen and pollen tubes, but are redundant in their function within their respective subfamilies.

4.3 Role of type A PI4P 5-kinases in polar tip growth of pollen tubes

The overexpression of type A PI4P 5-kinases in tobacco pollen tubes resulted in a loss of polarity with respect to several cellular functions and led to tip swelling (Figures 3.24, 3.26 and 3.27). Since overexpression of an inactive type A PI4P 5-kinase variant failed to induce these phenotypical changes (Figure 3.24) it can be assumed that the production of $PI(4,5)P_2$ and not structural properties of the enzyme itself was responsible for the morphological changes. $PI(4,5)P_2$, however, is not only a secondary messenger, but also a substrate for PLC and, thus, for IP_3 production. Therefore, an increase in $PI(4,5)P_2$ levels could also increase IP_3 levels. IP_3 has been considered to influence pollen tube growth (Monteiro et al., 2005). When a human type I inositol polyphosphate 5-phosphatase, that has been shown to greatly reduce IP_3 levels in plant cells (Perera et al., 2002; Perera et al., 2006; König et al., 2007; Mosblech et al., 2008) was strongly expressed in pollen tubes, however, pollen tube morphology and growth was not altered (data not shown). This result indicates that IP_3 has either no important role in pollen tube growth or that it is as quickly degraded by intrinsic phosphatases even in non-transformed pollen tubes. Another indication that an increase in $PI(4,5)P_2$ and not IP_3 levels is responsible for the tip swelling phenotype is that the same phenotype was observed in pollen tubes disrupted in PI- PLC activity (Dowd et al., 2006; Helling et al., 2006), which were suspected to be increased in $PI(4,5)P_2$ levels but decreased in IP_3 levels.

Recently it has been proposed that the tip swelling of pollen tubes results from an expanded area of exocytosis (Kost, 2008). It must be noted, however, that a pollen tube does not necessarily grow in the direction of exocytosis, but rather in the direction where the cell wall yields most easily to the turgor pressure and to the force applied by actin microfilaments as will be discussed further down. One aspect observed with PIP5K11 overexpression in tobacco pollen tubes was that the actin cytoskeleton especially in the tip region was disorganized and that the fine subapical actin network - referred to as the actin fringe - was lost (Figure 3.26). Instead, an unorganized tuft of actin fibres was observed in the swollen tip region (Figure 3.26). The dynamics of the actin fringe depend not only on F-actin polymerization but also on its breakdown (Cole and Fowler, 2006). Since actin depolymerising ABPs, such as cofilin or gelsolin, are inhibited by $PI(4,5)P_2$ (Hepler et al., 2001; Ojala et al., 2001; Hussey et al., 2002), it can be concluded that an increase of cellular $PI(4,5)P_2$-levels with overexpression of type A PI4P 5-kinases would hyperstabilize the actin cytoskeleton, disrupting its dynamic structure. In this work the actin cytoskeleton was visualized by fixing and staining with phalloidin (Lovy-Wheeler et al., 2005). Another possibility to visualize actin filaments *in vivo* is to decorate them with fluorescence-tagged actin binding domains of proteins such as fimbrin (Wang et al., 2004) or talin (Kost et al., 1998), and such *in vivo* methods were also tested during this work (data not shown). These methods, however, have been found to influence the actin cytoskeleton by themselves (Lovy-Wheeler et al., 2005), and during this work it was hard to decide whether observed changes in the actin cytoskeleton had been due to

overexpression of the PI4P 5-kinases or due to expression of the actin reporters used. It must be noted that methods involving fixation also have certain drawbacks, because the fixation procedure can lead to artifacts. Therefore, here a fixation method was used that had previously been proven to yield similar results as cryofixation, a method considered to very accurately reflect the *in vivo* situation (Lovy-Wheeler et al., 2005).

The changes observed in the cytoskeleton with overexpression of PIP5K11 were also confirmed by the study of Golgi dynamics, which have been shown to be actin dependent (Boevink et al., 1998). Correlated to the alterations in F-actin structures, altered Golgi-motility (Figure 3.27) and the appearance of large vacuoles (Figures 3.24 C-E and 3.27 C) in the clear zone of pollen tubes indicates a loss of cellular polarity in the apical region of the cells.

The observation of altered cytoskeletal structures and cell polarity raised the question how these effects were mechanistically linked to the tip swelling phenotype that also occurred with overexpression of type A PI4P 5-kinases (Figure 3.24). From the animal field it is known that actin strands can influence cell shape directly in a mechanical manner (Pollard et al., 2000). Since plant cells, however, are surrounded by a cell wall, much greater forces are required to alter their cell shape. The turgor pressure in pollen tubes reaches around 0.2 MPa and it has been speculated that this force alone is responsible for cell expansion (Benkert et al., 1997; Bosch et al., 2005). However, it has been shown that the depolymerization of the actin cytoskeleton by the toxin, latrunculin B, results in a slower growth rate, a reduction in the capacity to invade a mechanical obstacle (Gossot and Geitmann, 2007) and, when latrunculin B is applied at higher concentrations, even growth arrest of pollen tubes (Vidali et al., 2001). In consequence it has been proposed that actin filaments themselves exert a mechanical force that drives pollen tube elongation as is illustrated in the model scheme (Figure 4.1 A; Gossot and Geitmann, 2007) and that turgor alone is not able to drive cell wall expansion. The tip swelling phenotype shown in this work has been previously observed in pollen tubes treated with the actin-stabilizing drugs jasplakinolide and chondramide B (Cardenas et al., 2008), indicating that strengthening of the cytoskeleton, as observed in pollen tubes overexpression type A PI4P 5-kinases, could be the cause of the tip swelling phenotype. If the cytoskeleton is considered as an important mechanical factor in pollen tube expansion (Figure 4.1 A-C), a possible explanation for the observed tip swelling phenotype is that the force applied by the actin cytoskeleton acts in all directions of the pollen tube tip after its distortion due to PIP5K11 overexpression (Figure 4.1 B), resulting in tip swelling (Figure 4.1 C). In untransformed pollen tubes, however, it would act only towards the pollen tube apex, which leads to polar elongation.

Nevertheless, the role of the cytoskeleton as a mechanical force driving polar expansion of plant cells is a matter of ongoing debate and it has been proposed that pollen tube elongation depends solely on turgor pressure counteracted by the mechanical properties of the cell wall (Zonia et al., 2006; Kroeger et al., 2008). If turgor pressure alone is responsible for cell expansion, an alternative model for tip swelling needs to be considered, that takes the apical cell wall structure of pollen tubes into account, which is presented in the second part of the model scheme (Figure 4.1 D-F).

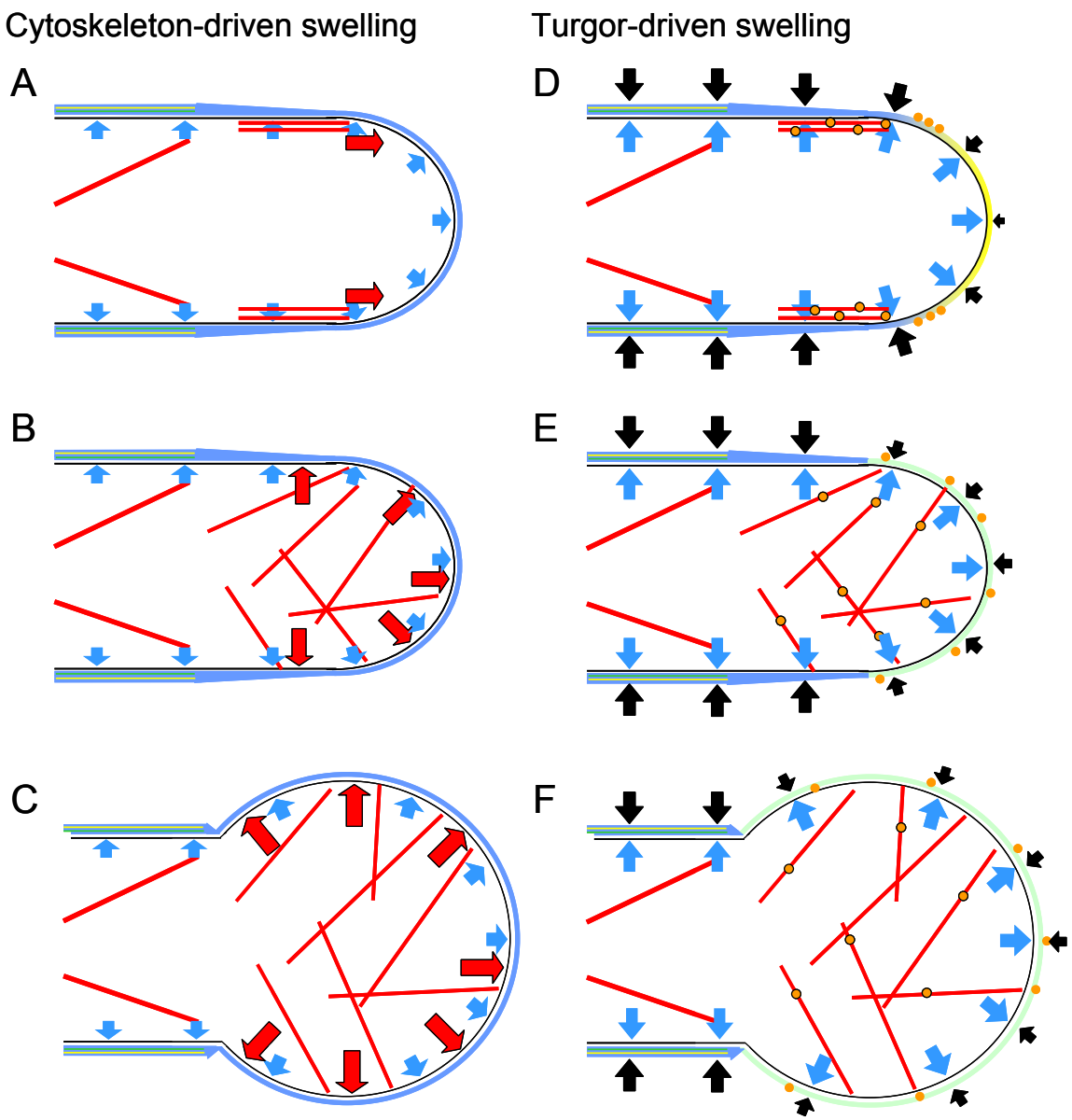

Figure 4.1. Alternative models to explain tip swelling

A-C, Model for pollen tube tip swelling considering the actin cytoskeleton as a major force in cell expansion. The cell wall is composed of pectin (blue) and is additionally strengthend by callose and cellulose in the pollen tube shank (green and yellow). Cellular expansion is driven by turgor pressure (blue arrows) and a mechanical force (red arrows) deriving from the cytoskeleton (red bars). A, Normal pollen tube growth. The actin cytoskeleton, especially the actin fringe (parallel subapical red lines), drives cell expansion only in the direction of growth. B, Disorganisation of the actin cytoskeleton due to overexpression of type A PI4P 5-kinases. The force exerted now by the cytoskeleton acts equally in all directions resulting in tip swelling (C). Tip swelling is restricted to the apex, because the cell wall is inflexible in the shank due to cellulose and callose. D-F, Model for pollen tube tip swelling considering only turgor pressure and cell wall plasticity, but no direct mechanical force by the actin cytoskeleton as factors of polar growth. Cellular expansion is driven by turgor pressure counteracted by the mechanical force of the cell wall (black arrows). D, Normal pollen tube growth. Pectin occurring in its methylated form at the very tip (indicated by yellow colour) is flexible and the resistance against the turgor is smaller (indicated by a small black arrow). PME is transported in exocytotic vesicles (orange dots encircled in black) along the actin fringe and secreted in the subapical region (orange dots), where it converts pectin to its rigid, deesterified, Ca^{2+} bound form (indicated by blue colour), resulting in a stronger counteracting force of the cell wall (longer black arrows). E, After disturbance of the cytoskeleton PME distribution is disorganized, resulting in a uniformly deesterified and flexible apical cell wall, indicated by smaller equally long arrows for the counteracting force of the cell wall. F, In consequence, the cell wall expands in all directions, resulting in tip swelling (F).

The cell wall of the region between the apex up to 10-20 µm away from the pollen tube tip consists mostly of pectin, and is not uniformly flexible due to the activity of PMEs (Figure 4.1 D; Bosch et al., 2005; Parre and Geitmann, 2005; Tian et al., 2006). How PMEs - or other proteins modulating cell wall plasticity - are targeted to their site of action in the pollen tube is unknown. Even though the role of the actin fringe is not well understood (Lovy-Wheeler et al., 2005), this fragile structure may be involved in the vectorial transport of secretory vesicles containing cell wall material and/or cell wall-modifying enzymes to the plasma membrane (Figure 4.1 D). If the actin fringe is perturbed, for example due to downstream effects of type A PI4P 5-kinase overexpression, the polarity in the apical cell wall could be lost, because enzymes such as PME are no longer targeted to the exactly required region but are more randomly distributed (Figure 4.1 E). If in consequence the apical cell wall becomes uniformly flexible, turgor pressure may expand the pollen tube in this region evenly in all directions, ultimately leading to the observed tip swelling (Figure 4.1 F). Again, changes of the cell wall structure due to manipulation of the cytoskeleton, however, cannot be the sole reason for tip swelling, because no tip swelling was observed in pollen tubes in which the cytoskeleton had been disrupted by the addition of toxins (Vidali et al., 2001). A possible explanation for the effects seen in this thesis is that PIP5K11 overexpression not only manipulates the cytoskeleton but also turgor pressure. Evidence for this hypothesis comes from studies that found that phospholipid and phosphoinositide signals can regulate the cell volume of pollen tubes (Zonia et al., 2002; Zonia et al., 2006). Studying turgor and ion fluxes of pollen tubes overexpressing type A PI4P 5-kinases could lead to a better understanding of the tip swelling phenotype. Future experiments will be devised to address these points.

To understand why only the apical part of the pollen tube swells up and not also regions further away from the tip, the cell wall architecture at the pollen tube shank must be taken under consideration. The region of the cell wall starting 10-20 µm away from the apex is inflexible due to incorporated cellulose and callose (Figure 1.9), making cell expansion impossible (Ferguson et al., 1998; Tian et al., 2006). Therefore, only the pollen tube apex can expand. The results of this work demonstrate clearly that tip swelling occurred only in the apical region where the cell wall contained no callose (Figure 3.25).

4.4 Role of type B PI4P 5-kinases in pollen tubes

From evidence contended in the previous paragraph it follows that exocytosis of pectin at the pollen tube tip has to be tightly regulated to preserve the plasticity of the apical cell wall, which is crucial for pollen tube elongation. It can be assumed that the exocytosis of pectin at the cell wall is coupled to growth, because pollen tubes inhibited in elongation due to toxification (Vidali et al., 2001), or due to expression of transgenes such as dominant negative GTPases (Kost et al., 1999) have not been reported to accumulate pectin as a consequence of continuing exocytosis after growth arrest. Tobacco pollen tubes overexpressing type B PI4P 5-kinases, however, did not only terminate growth (Figure 3.21), but also exhibited massive incorporation of pectin into the apical cell wall (Figure 3.22). Since pectin is secreted by exocytosis at the pollen tube tip (Bosch and Hepler, 2005; Tian et al., 2006), it can be concluded that exocytosis of pectin was over-

stimulated by overexpression of type B PI4P 5-kinases. Importantly, an inactive variant of a type B PI4P 5-kinase could not induce this phenotype (Figure 3.20). Therefore, it follows that not the enzyme itself - which was not changed in its localization (Figure 3.20 F) - but PI(4,5)P_2 was responsible for the observed phenotype. Other phenotypical changes, such as pollen tube-branching and membrane invaginations, can also be explained with an increased rate of exocytosis of pectin.

If the secretion of pectin is strongly increased due to strong overexpression of type B PI4P 5-kinases, the apical cell wall becomes thicker and less flexible, as illustrated in the model scheme (Figure 4.2 E). In consequence, the turgor pressure may no longer be strong enough to expand the cell wall, and pollen tube elongation will cease (Figure 4.2 E). Since exocytosis is uncoupled from tube growth due to overexpression of type B PI4P 5-kinases, exocytosis will continue even after cell expansion has ceased, leading to substantial pectin accumulation in the apical cell wall (Figures 3.22 and 4.2 F). Exocytosis leads not only to cell wall deposition, but also increases the incorporated area of apical plasma membrane. If the protoplast inside a rigidified cell wall cannot be expanded anymore by turgor pressure, the membrane area continuously incorporated at the apical plasma membrane may fold inward (Figure 4.2 G), resulting in the “trapped protoplast” phenotype reported in this work (Figure 3.23).

Branching of tobacco pollen tubes (Figure 3.20) was observed with type B PI4P 5-kinase overexpression levels that were weaker than in pollen tubes showing the “trapped protoplast” phenotype (Figure 3.19). Pollen tube branching was also observed in *Arabidopsis* pollen tubes overexpressing type B PI4P 5-kinases (Figure 3.11), indicating that the phenotype observed in tobacco pollen tubes upon overexpression of type B PI4P 5-kinase originating from *Arabidopsis* was not an artifact of the heterologous expression. A possible explanation for the branching phenotype is that increased pectin deposition may not be strong enough to terminate growth, but sufficiently altered to cause aberrant plasticity of the apical cell wall (Figure 4.2 B). Such changes in pectin deposition in the apical cell wall could occasionally lead to two zones of flexible cell wall divided by a more rigid region, which would ultimately lead to a splitting of the pollen tube tip at the very apex (Figure 4.2 C) and the observed branching pattern (Figure 4.2 D). Both arms of a branched pollen tube could continue growing independently for long distances (Figures 3.20 and 6.3), indicating that pollen tube elongation is a self-sustainable process not dependent on the close vicinity of the vegetative nucleus only present in one branch, as was previously hypothesized (Cole and Fowler, 2006).

The question why increased PI(4,5)P_2 levels lead to an increase of exocytosis of vesicles loaded with pectin cannot be easily answered, because information about the secretory machinery in plants with respect to its regulation by PI(4,5)P_2 is limited. From the animal and yeast fields it is known that PI(4,5)P_2 recruits subunits of the exocyst complex, required for secretion to the plasma membrane (He et al., 2007; Liu et al., 2007a). Even though the interactions of plant exocyst proteins with phosphoinositides have not been studied, it has been shown that these proteins are important for polar growth (Cole et al., 2005).

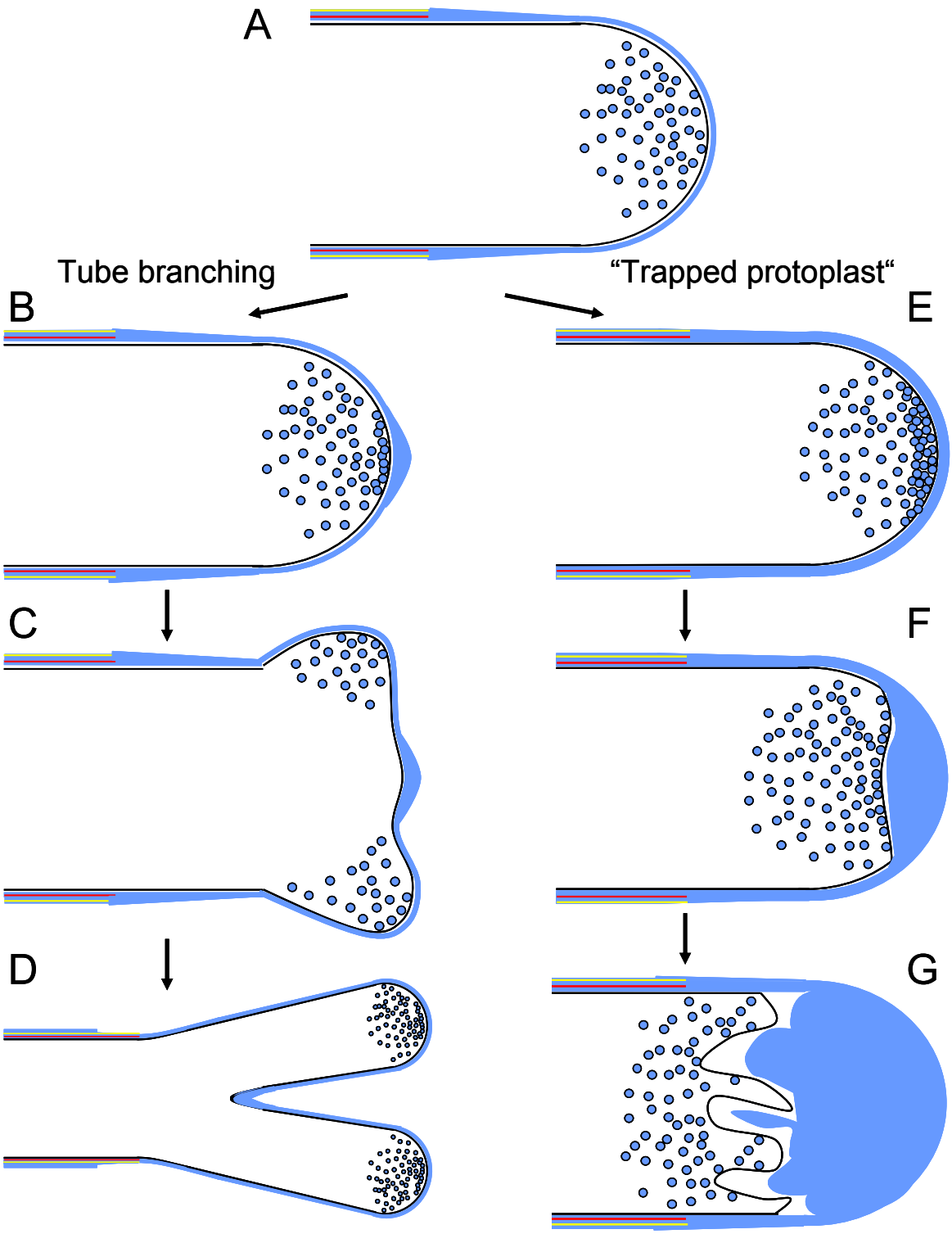

Figure 4.2. Model for the pollen tube branching and "trapped protoplast" phenotypes

A, Normal pollen tube growth. Secretion of pectin-containing exocytotic vesicles is tightly regulated to sustain cell wall plasticity. B, When secretion is increased due to type B PI4P 5-kinase overexpression, pectin occasionally blocks apical expansion. C, Pollen tube expansion in subapical regions with a cell wall more flexible than that at the very apex leads to splitting of the pollen tube tip. D, Growth continues simulatanously at both tips, resulting in the branched pollen tube phenotype. E, If pectin secretion is even higher due to very strong overexpression of type B PI4P 5-kinases cellular expansion is completely inhibited due to the thickening of the cell wall. F, Continuous exocytosis leads to additional cell wall accumulation at the pollen tube apex. G, The plasma membrane of the "trapped protoplast" folds inwards.

The *Arabidopsis* genome contains a total of 35 genes coding for putative exocyst subunits (Ehlert et al., 2006), six of them being exclusively expressed in pollen according to Genevestigator analysis (Zimmermann et al., 2004).
From the animal field it is also known that $PI(4,5)P_2$ stimulates soluble N-ethylmaleimide-sensitive-factor attachment receptor (SNARE)-complexes that are required for fusion of vesicles with their target membranes (Vicogne et al., 2006). This observation could be founded in the fact that two proteins, CAPS and synaptotagmin, that play roles in this SNARE-mediated fusion process are activated by phosphoinositides (Sugita, 2008). While the *Arabidopsis* genome contains no genes with obvious similarity to CAPS genes, three genes have been found that encode putative synaptotagmin isoforms (Craxton, 2004). One of these isoforms is exclusively expressed in pollen according to Genevestigator analysis (Zimmermann et al., 2004). Pollen tubes could be a suitable model system to study the role of proteins involved in exocytosis and their dependence on $PI(4,5)P_2$ to get a better understanding of exocytosis not only in pollen tubes but in all plant cells.

4.5 Localization pattern of PI4P 5-kinases in pollen tubes

As discussed in the previous paragraphs, type A and type B PI4P 5-kinases appear to perform distinct functions in pollen tube growth. One reason for this effect could be different subcellular localization. When expressed as EYFP-fusion proteins, however, localization overlapped near the apex of the plasma membrane of the pollen tube (Figures 3.14, 3.15, 3.16 and 3.17). This overlap was almost complete in growing tobacco pollen tubes (Figure 3.16), whereas the region of overlap was smaller in *Arabidopsis* pollen tubes (Figure 3.14), where PIP5K11 covered a much larger area than PIP5K5 (Figure 3.14). In non-growing tobacco pollen tubes localization between type A and type B PI4P 5-kinases also differed greatly in a way comparable to results from *Arabidopsis* (Figure 3.16). It should be noted that the affinity of type A PI4P 5-kinases to the plasma membrane appeared higher than that of type B PI4P 5-kinases, as evident from a sharper definition of plasma membrane localization in comparison to a more diffuse pattern for type B enzymes (Figures 3.14 and 3.16) which has also been reported for PIP5K3 in root hairs (Stenzel et al., 2008). Since both types of PI4P 5-kinases are soluble proteins, their recruitment to the plasma membrane must be facilitated by interaction with membrane-bound proteins and/or lipids. So far, the membrane-recruiting mechanisms of PI4P 5-kinases are unresolved. The distinct localization patterns observed for type A and type B PI4P 5-kinases indicate that possible interaction partners must be different for these two kinase subfamilies. Small G proteins of the Rac-Rop family have been proposed as potential interaction partners of PI4P 5-kinases (Kost et al., 1999; Davis et al., 2007). In coimmunoprecipitation experiments on whole *Arabidopsis* cell extracts both types of PI4P 5-kinases were found to be associated with such small GTPases (Davis et al., 2007), and a PI4P 5-kinase activity could be precipitated with a Rac-Rop GTPase from pollen tube extracts (Kost et al., 1999). Overexpression of Rac-Rop GTPases in pollen tubes also induces similar phenotypes as overexpression of type A PI4P 5-kinases, such as tip swelling and disorganization of the actin cytoskeleton (Li et al., 1999; Molendijk et al., 2001; Gu et al., 2003; Gu et al., 2005; Preuss et al., 2006; Kost, 2008). Despite of

extensive experimental effort, however, direct interactions of PI4P 5-kinases and small GTPases could not be verified by bimolecular fluorescence complementation (BiFC) or fluorescence resonance energy transfer (FRET) experiments (Jin and Heilmann, personal communication). The expression of a dominant negative Rac-Rop variant had also no effect on the localization of PIP5K11 or the phenotype induced by overexpression of this PI4P 5-kinase, casting doubt whether Rac-Rop proteins act as upstream factors of PIP5K11 signaling as has recently been proposed (Kost, 2008). An interaction of the type B PI4P 5-kinase PIP5K1 with PIKβ1 has been demonstrated *in vitro*. In pollen tubes, however, PIKβ1 localized to vesicles and it can, thus, be ruled out that type β PI 4-kinases play a role in the targeting of type B PI4P 5-kinases to the plasma membrane.

In order to explain the distinct phenotypes associated with type A or type B PI4P 5-kinases overexpression and, possibly, their different modes of membrane association, the structural differences of the proteins must be considered. One of the biggest differences in the domain architecture of type A and B PI4P 5-kinases is the N-terminal domain containing MORN repeats present in type B PI4P 5-kinases but missing in type A PI4P 5-kinases. These MORN repeats have previously been proposed to bind membrane lipids (Mueller-Roeber and Pical, 2002) and an affinity for all phosphorylated phosphoinositides could be detected *in vitro* (Rasche, König and Heilmann, personal communication). It was shown that these domains are crucial for the proper function of PIP5K3 in root hairs (Stenzel et al., 2008), however when a truncated version of PIP5K5 lacking the N-terminus including the MORN-repeat domain was expressed in tobacco pollen tubes, the localization was not changed (Figure 3.29) and the truncated version was still able to induce the abnormal growth phenotypes (Figure 3.28) equally well as the full length enzyme. This result indicates that the MORN-repeat domain was not essential for plasma membrane localization and proper function of at least of PIP5K5. Taken all the experiments into account it must be summarized that the mechanism that recruits PI4P 5-kinases to the plasma membrane of pollen tubes remains unclear. The only information gained is that the mechanism must be different for type A and B PI4P 5-kinases. Future work will be directed towards the identification of the recruiting mechanism of PI4P 5-kinases in pollen tubes by screening for interaction partners and testing the canididates *in vivo*.

Studying the localization of PI4P 5-kinases in relation to other signaling proteins is also interesting in order to understand the interplay of different proteins of the signaling machinery. In this work the relative localizations of PIP5K11 and a phosphoinositide-specific PLC were monitored, revealing that their localization partially overlapped (Figure 3.31). The finding that enzymes synthesizing and degrading $PI(4,5)P_2$ are found in close proximity within the cell is in accordance with the notion that $PI(4,5)P_2$ has a high turnover rate in plant cells (Perera et al., 2002; Im et al., 2007b; Krinke et al., 2007). The observation that the localization of PIP5K11 matches exactly the localization of $PI(4,5)P_2$ (Figure 3.18) also shows that newly formed $PI(4,5)P_2$ must be quickly degraded, because diffusion of this lipid out of the zone occupied by PIP5K11 was never observed. NtPLC3 covered a larger area of the membrane than PIP5K11 (Figure 3.31). This coordinated

situation may ensure that any $PI(4,5)P_2$ diffusing out of its zone of synthesis cannot accumulate further down the pollen tube and will be completely degraded.

4.6 Different phosphoinositide pools in pollen tubes

In growing tobacco pollen tubes PIP5K5 and PIP5K11 resided in plasma membrane domains that were almost completely overlapping with the $PI(4,5)P_2$ domain at the apex (Figure 3.18). In non-growing pollen tubes, however, PIP5K5 overlapped with $PI(4,5)P_2$ only at the apical plasma membrane but not with regions further away from the pollen tube tip (Figure 3.18). In contrast, PIP5K11 localization matched the $PI(4,5)P_2$-distribution well (Figure 3.18). The observed patterns indicate that type A and type B PI4P 5-kinases are associated with different $PI(4,5)P_2$ pools, since it is unlikely that $PI(4,5)P_2$ produced by type B PI4P 5-kinases can contribute to the $PI(4,5)P_2$ domain further away from the pollen tube tip; instead, $PI(4,5)P_2$ would be degraded by PLC before it could diffuse into this area.

During growth the $PI(4,5)P_2$ pools generated by type A and type B PI4P 5-kinases must overlap in the plasma membrane, because of the similar localization of type A and type B PI4P 5-kinases (Figure 3.14 and Figure 3.16), as was observed especially in tobacco pollen tubes (Figure 3.16). The most striking and unexpected result of this work was that the two overlapping $PI(4,5)P_2$ pools were functionally distinct, as obvious from the different observed phenotypes (Figures 3.19, 3.20, 3.21, 3.22, 3.23 and 3.24), and that this functional distinction was observed despite of the close proximity of the two $PI(4,5)P_2$ pools involved. Interestingly, overexpression of a human PI4P 5-kinase did not induce any growth phenotypes even though this kinase was also localized in the tip region. This finding indicates that $PI(4,5)P_2$ generated by PI4P 5-kinases in the apical plasma membrane does not necessarily affect pollen tube growth. Overexpression of a PI4P 5-kinase from *Neurospora crassa* with a crucial role in filamentous growth (Seiler and Plamann, 2003), however, led to tip swelling. This PI4P 5-kinase was found to be enriched in the subapical region of the plasma membrane. This could hint to conserved localization and regulating mechanisms in polar growth of plants and fungi.

The molecular identity of the postulated $PI(4,5)P_2$ pools is unclear but might be founded, at least partially, in differences at the molecular level. As described in the introduction, two pools of phosphoinositides with distinct differences in their fatty acid composition have been discovered in plant leaves (König et al., 2007). If differences in acyl-chains were the only difference of the two proposed $PI(4,5)P_2$ pools in pollen tubes, however, downstream target proteins that bind to $PI(4,5)P_2$ would have to discriminate between the different acyl moieties of $PI(4,5)P_2$ species present in the different pools. Since most phosphoinositide domains have been reported to bind only the headgroup of phosphoinositides, as they attach peripherally to the plasma membrane (Lemmon, 2003), a discrimination of phosphoinositides by means of their acyl moieties by phosphoinositide binding proteins seems unlikely, but cannot be ruled out. It must be noted that for enzymes of phosphoinositide metabolism preferences for substrates containing certain acyl moieties have been reported, including for invertebrate PLC and PI4P 5-kinases (Carricaburu and Fournier, 2001) and for mammalian phosphoinositide phosphatases (Schmid et al., 2004).

So far, however, the physiological relevance of such preferences observed *in vitro* is not clear.

Another explanation for the distinct functions of two $PI(4,5)P_2$ pools is the presence of $PI(4,5)P_2$ microdomains that are too small or too interwoven to be distinguished under the microscope. $PI(4,5)P_2$ has only a very short lifespan in plants before being degraded (Perera et al., 2002; Im et al., 2007b; Krinke et al., 2007). This lifespan could be even shorter for unbound $PI(4,5)P_2$ that can freely diffuse across the plasma membrane. Therefore, it can be concluded that $PI(4,5)P_2$ can act only in very close proximity of the PI4P 5-kinase by which it was generated, because it gets either quickly bound by a target protein or degraded by PLC before it can diffuse further away. This hypothesis is supported by the fact that $PI(4,5)P_2$ was never detected by the PLCδ1-PH reporter in regions of the pollen tubes not associated with a PI4P 5-kinase (Figure 3.18 E, F). Many proteins known to act in a common pathway have been found to be organized in complexes or on scaffolds (Yeaman et al., 1999; Moe, 2003). It could be hypothesized that $PI(4,5)P_2$ synthesized by PI4P 5-kinases that are parts of particular signaling complexes, would predominantly affect PI-regulated target proteins within these complexes. $PI(4,5)P_2$ would, thus, be channelled towards certain signaling functions by close spatial association, whereas other functions may be precluded.

Another possibility that has been previously discussed (Stenzel et al., 2008) is that type B PI4P 5-kinases could produce $PI(4,5)P_2$ not only at the plasma membrane but also on secretory vesicles. In consequence these kinases may bind at the plasma membrane, but have their active center oriented towards the cytoplasm, acting on secretory vesicles that come in close proximity of the plasma membrane. Evidence for this hypothesis comes from the observation that type B PI4P 5-kinases expressed in *Arabidopsis* pollen tubes did not only localize at the plasma membrane, but also in a “cloud” right below the apex (Figure 3.14). Another finding that strengthens this hypothesis is the observation that a truncated PIP5K5 variant missing the N-terminus with the MORN-repeat domain was sometimes associated with punctate structures (Figure 3.29). A similar localization pattern was reported from a comparably truncated variant of the PIP5K3 in root hairs (Kusano et al., 2008). If these structures were secretory vesicles, this finding could indicate that type B PI4P 5-kinases contain also a certain affinity towards secretory vesicles. The observation that type B PI4P 5-kinases can interact with PIKβ1 (Davis et al., 2007) supposedly localized at secretory vesicles (Preuss et al., 2006), and the fact that both enzyme classes act in a common pathway in pollen tubes (Figure 3.32) indicates that these enzymes may at least transiently associate in a larger complex required for exocytosis. Support for this notion comes from the mammalian field, and it is known that $PI(4,5)P_2$ in the membranes of secretory vesicles, and not at the plasma membrane, promotes vesicle fusion (Vicogne et al., 2006). Since two distinct pools of $PI(4,5)P_2$ were hypothesized, the question arose, whether also different pools of PI4P exist in pollen tubes. In yeast, three different types of independent PI4P pools have been described (Strahl and Thorner, 2007), two of them, attributed to the PI4-kinases, Pis1p and Stt4, with crucial functions in exocytosis and actin dynamics, respectively. Therefore, it was tempting that the respective homologs of *Arabidopsis*, PI 4-kinases from the β and α

subfamily were involved in similar processes, providing the precursors for the type B and type A PIP-kinases, respectively.

PIKβ1 and PIP5K5 induced similar phenotypes (Figures 3.20, 3.22 and 3.32), and showed synergistic effects (Figure 3.32) with coexpression. Therefore, it follows that these enzymes act in a common pathway and that PI4P synthesized by type β PI 4-kinases acts as a substrate only for type B PI4P 5-kinases, but not for type A PI4P 5-kinases. In support of this notion coexpression of PIKβ1 and a type A PI4P 5-kinase revealed antagonistic rather than synergistic effects (Figure 3.33). As outlined before, a possible explanation for this effect is that PIKβ1 strengthens the cell wall by enhancing pectin exocytosis, which would reduce tip swelling.

PIKβ1 localized in pollen tubes to punctate structures (Figure 3.34) similar to observations in root hairs (Preuss et al., 2006) and also led to pectin accumulation in the pollen tube apex (Figure 3.32). It may be concluded that PIKβ1 binds on the outside of secretory vesicles containing pectin and synthesizes PI4P in the membrane of these vesicles. To verify this hypothesis pollen tubes simultaneously stained for pectin and type β PI 4-kinases should be observed under the electron microscope in future studies.

Since the type β PI 4-kinases localized to vesicles (Figure 3.34) and not to the plasma membrane, it remains unclear why the bulk of PI4P was detected at the plasma membrane and what PI 4-kinase isoforms are responsible for the generation of the PI4P at the plasma membrane (Figure 3.36). Besides the two PI 4-kinases of the β subfamily, the *Arabidopsis* genome contains two more PI 4-kinases of the α subfamily (Mueller-Roeber and Pical, 2002). A yeast homolog of this subfamily, Stt4p, has been found at the plasma membrane, suggesting the type α PI 4-kinases as candidates possibly responsible for PI4P production at the plasma membrane of pollen tubes. When the respective type α PI 4-kinases were expressed as EYFP fusion constructs in tobacco pollen tubes, however, fluorescence was only found in the cytoplasm (data not shown). Strong expression of type α PI 4-kinases also did not alter pollen tube morphology (data not shown). If the type α PI 4-kinases are not mistargeted due to the expression as a fusion construct or due to heterologous expression, which should be checked by further studies, their role in pollen tube growth is unclear. If type α PI 4-kinases are not responsible for generation of PI4P at the plasma membrane, there must either be PI 4-kinases present in pollen tubes that are so far unknown, or PI4P is produced by proteins previously annotated as type γ PI 4-kinases but later classified as protein kinases and suggested to have no PI 4-kinase activity. Since the PI 4-kinase γ proteins were only investigated *in vitro*, however, it is still possible that they generate PI4P *in vivo*. If these last two possibilities are not the case, PI4P at the plasma membrane must originate from PI4P synthesis by type β PI 4-kinases at the secretory vesicles. This situation can well be imagined, because PI4P generated in the membrane of the secretory vesicles that had not been phosphorylated to $PI(4,5)P_2$ would be automatically incorporated in the plasma membrane after vesicle fusion. PI4P has normally a higher abundance in plant cells than $PI(4,5)P_2$ (König et al., 2007; König et al., 2008a), and it is likely that the conversion PI4P to $PI(4,5)P_2$ in secretory vesicles is not quantitative. If type β PI 4-kinases are the sole source of PI4P in pollen tubes, the question is, why an *Arabidopsis* double mutant

reported to be totally deficient in type β PI 4-kinases due to T-DNA insertions would be viable and fertile (Preuss et al., 2006). The only conclusive explanation is that type β PI 4-kinase expression was not completely diminished in this *pikβ1 pikβ2* double mutant, but only reduced to non-detectable levels. This explanations appears likely since both T-DNA insertions found in the respective PI 4-kinase β genes are situated in introns (Preuss et al., 2006). Future biochemical analyses may reveal changes in PI 4-kinase activity and/or phosphoinositides levels in pollen of WT and *pikβ1 pikβ2* double mutant plants.

4.7 Conclusions

The data presented in this thesis indicate that the multifunctional regulatory lipid, $PI(4,5)P_2$, can simultaneously control different aspects of polar growth of a pollen tube cell. Importantly, the different physiological functions of $PI(4,5)P_2$ are sorted based on the biosynthetic origin from type A or type B PI4P 5-kinases, even if the enzymes occurred in close proximity to one-another within the same plasma membrane area of the growing apex of the cell. The observations made may have far-reaching ramifications for the understanding of phosphoinositide functions in polar tip growth of pollen tubes, but possibly also in other cell types of plants or even cells of non-plant origin, such as fungal hyphae. Although many more experiments will have to be performed before a definite statement can be made, it is already clear today that the molecular components controlled by phosphoinositides, i.e., those mediating actin-dynamics, vesicle fusion and exocytosis, or the regulation of ion-channels, are conserved in evolution in organisms of all eukaryotic kingdoms. While the phosphoinositide system and its regulatory effects on physiology may, thus, be conserved at the molecular level and may be similar between plants, fungi and mammalian cells, clear differences exist when it comes to the organismic functions manifesting as consequences of actin-dynamics, exocytosis, or ion-channel activation. For instance, $PI(4,5)P_2$ controlling exocytosis of acetylcholine at the synaptic cleft may act similarly to $PI(4,5)P_2$ controlling exocytosis of pectin in tip-growing pollen tubes, however, consequences of the exocytosed material on the organismic function will differ by a lot. The differences in appearance, sessile vs. motile life-style, or in the organization of tissues and organs between representatives of the plant, fungal or animal kingdoms offer a large and fascinating area of research, which will ultimately reveal how phosphoinositides fit in to control plant function and development.

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6. Appendix

6.1 List of plasmids

No.	FS	Construct	Vector	Insert	Additional remarks
1	-	pGEM-T Easy			Vector obtained from Promega
2	-	pENTR2B			Vector obtained from Invitrogen
3	-	pENTR2B-ccdb	pENTR2B cut with EcoRI releasing the ccdb gene followed by religation		
4	57	pLatGW			Vector obtained from Wolfgang Dröge- Laser
5	-	PIPK4 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S1 + S2 from *Arabidopsis* floral cDNA	Vector obtained from Irene Stenzel
6	-	PIPK5 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S3 + S4 from *Arabidopsis* floral cDNA	Vector obtained from Irene Stenzel
7	-	PIPK10 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S7 + S8 from *Arabidopsis* floral cDNA	Vector obtained from Irene Stenzel
8	-	PIPK11 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S9 + S10 from *Arabidopsis* floral cDNA	Vector obtained from Irene Stenzel
9	30	EYFP pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 82 + 61 from plasmids carrying the authentic clones (Clonetech)	
10	29	+1EYFP pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 81 + 61 from plasmids carrying the authentic clones (Clonetech)	
11	28	+2EYFP pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 60 + 61 from plasmids carrying the authentic clones (Clonetech)	
12	159	CFP pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 82 + 61 from plasmids carrying the authentic clones (Clonetech)	
13	158	+2CFP pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 60 + 61 from plasmids carrying the authentic clones (Clonetech)	
14	-	N term. RedStar pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 259 + 260 from plasmids carrying the authentic clones (Janke et al., 2004))	

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6.1 List of plasmids (continued)

15	374	mCherry pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 302 + 303 from plasmids carrying the authentic clones (Clonetech)	
16	32	EYFP pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	EYFP NotI EcoRV from EYFP pGEM-T Easy (9)	
17	99	+1EYFP pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	+1EYFP NotI EcoRV from +1EYFP pGEM-T Easy (10)	
18	31	+2EYFP pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	+2EYFP NotI EcoRV from +2EYFP pGEM-T Easy (11)	
19	188	CFP pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	CFP NotI EcoRV from CFP pGEM-T Easy (12)	
20	187	+2CFP pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	+2CFP NotI EcoRV from +2CFP pGEM-T Easy (13)	
21	-	N term. RedStar pENTR2B	pENTR2B-ccdb (3) SalI NotI	N term. RedStar SalI NotI from N term. RedStar pGEM-T Easy (14)	
22	393	mCherry pENTR2B	pENTR2B-ccdb (3) NotI EcoRV	mCherry NotI EcoRV from mCherry pGEM-T Easy (15)	
23	56	EYFP pLatGW	pLatGW (4)	EYFP by Clonase reaction from EYFP pENTR2B (16)	
24	352	CFP pLatGW	pLatGW (4)	CFP by Clonase reaction from CFP pENTR2B (19)	
25	47	PIPK4 EYFP pENTR2B	+2EYFP pENTR2B (18) NotI	PIPK4 NotI from PIPK4 pGEM-T Easy (5)	
26	52	PIPK4 EYFP pLatGW	pLatGW (4)	PIPK4 EYFP by Clonase reaction from PIPK4 EYFP pENTR2B (25)	
27	48	PIPK5 EYFP pENTR2B	+2EYFP pENTR2B (18) NotI	PIPK5 NotI from PIPK5 pGEM-T Easy (6)	
28	53	PIPK5 EYFP pLatGW	pLatGW (4)	PIPK5 EYFP by Clonase reaction from PIPK5 EYFP pENTR2B (27)	
29	299	PIPK5 K497A EYFP pENTR2B	PIPK5 EYFP pENTR2B (27)		Plasmid generated by Quickchange reaction using primers 241 and 242

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6.1 List of plasmids (continued)

30	304	PIPK5 K497A EYFP pLatGW	pLatGW (4)	PIPK5 K497A EYFP by Clonase reaction from PIPK5 K497A EYFP pENTR2B (29)	
31	204	PIPK5 CFP pENTR2B	+2CFP pENTR2B (20) NotI	PIPK5 NotI from PIPK5 pGEM-T Easy (6)	
32	211	PIPK5 CFP pLatGW	pLatGW (4)	PIPK5 CFP by Clonase reaction from PIPK5 CFP pENTR2B (31)	
33	139	PIPK10 EYFP pENTR2B	EYFP pENTR2B (16) NotI	PIPK10 NotI from PIPK10 pGEM-T Easy (7)	
34	259	PIPK10 EYFP pLatGW	pLatGW (4)	PIPK10 EYFP by Clonase reaction from PIPK10 EYFP pENTR2B (33)	
35	300	PIPK10 K86A EYFP pENTR2B	PIPK10 EYFP pENTR2B (33)		Plasmid generated by Quickchange reaction using primers 243 and 244
36	305	PIPK10 K86A EYFP pLatGW	pLatGW (4)	PIPK10 K86A EYFP by Clonase reaction from PIPK10 K86A EYFP pENTR2B (35)	
37	50	PIPK11 EYFP pENTR2B	EYFP pENTR2B (16) NotI	PIPK11 NotI from PIPK11 pGEM-T Easy (8)	
38	55	PIPK11 EYFP pLatGW	pLatGW (4)	PIPK11 EYFP by Clonase reaction from PIPK11 EYFP pENTR2B (37)	
39	292	PIPK11 K94A EYFP pENTR2B	PIPK11 EYFP pENTR2B (37)		Plasmid generated by Quickchange reaction using primers 245 and 246
40	293	PIPK11 K94A EYFP pLatGW	pLatGW (4)	PIPK11 K94A EYFP by Clonase reaction from PIPK11 K94A EYFP pENTR2B (39)	
41	205	PIPK11 CFP pENTR2B	CFP pENTR2B (19) NotI	PIPK11 NotI from PIPK11 pGEM-T Easy (8)	
42	212	PIPK11 CFP pLatGW	pLatGW (4)	PIPK11 CFP by Clonase reaction from PIPK11 CFP pENTR2B (41)	

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6.1 List of plasmids (continued)

43	-	HsPLCδ1 PH pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 255 + 139 from plasmid-DNA provided by Dr. Tamas Balla (Varnai and Balla, 1998)	PCR product contains a N-terminal seven-amino acid linker (gly-gly-ala-gly-ala-ala-gly)
44	313	RedStar HsPLCδ1 PH pENTR2B	N term. RedStar pENTR2B (21) NotI EcoRV	HsPLCδ1 PH NotI EcoRV from HsPLCδ1 PH pGEM-T Easy (43)	
45	316	RedStar HsPLCδ1 PH pLatGW	pLatGW (4)	RedStar HsPLCδ1 PH by Clonase reaction from RedStar HsPLCδ1 PH pENTR2B (44)	
46	-	N-Acetyl-Glucosaminyl-transferase 1-79 (NAG) pGem-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 256 + 257 from *Arabidopsis* floral cDNA	
47	302	NAG CFP pENTR2B	CFP pENTR2B (19) SalI NotI	NAG SalI NotI from NAG pGem-T Easy (46)	
48	308	NAG CFP pLatGW	pLatGW (4)	NAG CFP by Clonase reaction from NAG CFP pENTR2B (48)	
49	-	Expansin4 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 235 + 236 from *Arabidopsis* floral cDNA	
50	456	Expansin4 mCherry pENTR2B	mCherry pENTR2B (22) SalI NotI	Expansin4 SalI NotI from Expansin4 pGEM-T Easy (49)	
51	464	Expansin4 mCherry pLatGW	pLatGW (4)	Expansin4 mCherry by Clonase reaction from Expansin4 mCherry pENTR2B (50)	
52	95	HsInsP-5-Ptase pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 127 + 128 from genomic DNA of transgenic HsInsP-5-Ptase *Arabidopsis* plants	Additional primer for sequencing 135
53	100	HsInsP-5-Ptase YFP pENTR2B	+1EYFP pENTR2B (17) SalI EcoRI	HsInsP-5-Ptase SalI EcoRI from HsInsP-5-Ptase pGEM-T Easy (52)	
54	110	HsInsP-5-Ptase YFP pLatGW	pLatGW (4)	HsInsP-5-Ptase YFP by Clonase reaction from HsInsP-5-Ptase YFP pENTR2B (53)	
55	-	HsPIPK1α pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 237 + 238 from plasmid containig HsPIPK1α (obtained from Wendy Boss)	

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6.1 List of plasmids (continued)

56	288	HsPIPK1α YFP pENTR2B	EYFP pENTR2B (16) SalI NotI	HsPIPK1α SalI NotI from HsPIPK1α pGEM-T Easy (55)	
57	289	HsPIPK1α YFP pLatGW	pLatGW (4)	HsPIPK1α YFP by Clonase reaction from HsPIPK1α YFP pENTR2B (56)	
58	240	NcPIP5K pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers 156 + 157 from Neurospora crassa cDNA	Additional primers for sequencing 168-172
59	242	NcPIP5K YFP pENTR2B	EYFP pENTR2B (16) EcoRI NotI	NcPIP5K EcoRI NotI from NcPIP5K pGEM-T Easy (58)	
60	250	NcPIP5K YFP pLatGW	pLatGW (4)	NcPIP5K YFP by Clonase reaction from NcPIP5K YFP pENTR2B (59)	
61	-	PIPK5 ΔMORN (253-772) YFP pLatGW			Vector obtained from Irene Stenzel
62	336	RedStar HsFAPP1 PH domain pLatGW			Vector obtained from Xu Jin
63	447	YFP PIKβ1 pLatGW			Vector obtained from Xu Jin
64	-	YFP PIPK6 pLatGW			Vector obtained from Irene Stenzel
65	58	pUC18ENTRY			Vector obtained from Ellen Hornung
66	136	pCambia 3300.0 GC			Vector obtained from Ellen Hornung
67	-	EYFP pUC18ENTRY	pUC18ENTRY (65) NotI EcoRV	EYFP NotI EcoRV from EYFP pGEM-T Easy (9)	
68	-	+2EYFP pUC18ENTRY	pUC18ENTRY (65) NotI EcoRV	+2EYFP NotI EcoRV from +2EYFP pGEM-T Easy (11)	
69	138	Promotor Lat52 pGem-T-Easy	pGEM-T Easy (1)	PCR product amplified with primers 120 + 119 from pLatGW	
70	154	Promotor Lat52 EYFP pUC18ENTRY	EYFP pUC18ENTRY (67) SalI NotI	Promotor Lat52 SalI NotI from Promotor Lat52 pGem-T-Easy (69)	
71	153	Promotor Lat52 +2EYFP pUC18ENTRY	+2EYFP pUC18ENTRY (68) SalI NotI	Promotor Lat52 SalI NotI from Promotor Lat52 pGem-T-Easy (69)	

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6.1 List of plasmids (continued)

72	165	Promotor Lat52 PIPK4 EYFP pUC18ENTRY	Promotor Lat52 +2EYFP pUC18ENTRY (71) NotI	PIPK4 NotI from PIPK4 pGEM-T Easy (5)	
73	199 228	Promotor Lat52 PIPK4 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor Lat52 PIPK4 EYFP by Clonase reaction from PIPK4 EYFP pUC18ENTRY (72)	
74	166	Promotor Lat52 PIPK5 EYFP pUC18ENTRY	Promotor Lat52 +2EYFP pUC18ENTRY (71) NotI	PIPK5 NotI from PIPK5 pGEM-T Easy (6)	
75	193 222	Promotor Lat52 PIPK5 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor Lat52 PIPK5 EYFP by Clonase reaction from PIPK5 EYFP pUC18ENTRY (74)	
76	168	Promotor Lat52 PIPK10 EYFP pUC18ENTRY	Promotor Lat52 EYFP pUC18ENTRY (70) NotI	PIPK10 NotI from PIPK10 pGEM-T Easy (7)	
77	195 224	Promotor Lat52 PIPK10 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor Lat52 PIPK10 EYFP by Clonase reaction from PIPK10 EYFP pUC18ENTRY (76)	
78	174	Promotor Lat52 PIPK11 EYFP pUC18ENTRY	Promotor Lat52 EYFP pUC18ENTRY(70) NotI	PIPK11 NotI from PIPK11 pGEM-T Easy (8)	
79	194 223	Promotor Lat52 PIPK11 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor Lat52 PIPK11 EYFP by Clonase reaction from PIPK11 EYFP pUC18ENTRY (78)	
80	-	Promotor PIPK4 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S13 + S14 from genomic *Arabidopsis* DNA	Vector obtained from Irene Stenzel
81	-	Promotor PIPK4 EYFP pUC18ENTRY	+2EYFP pUC18ENTRY (68) SalI NotI	Promotor PIPK4 SalI NotI from Promotor PIPK4 pGEM-T Easy (80)	
82	70	Promotor PIPK4 PIPK4 EYFP pUC18ENTRY	Promotor PIPK4 EYFP pUC18ENTRY (81) NotI	PIPK4 NotI from PIPK4 pGEM-T Easy (5)	
83	142	Promotor PIPK4 PIPK4 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor PIPK4 PIPK4 EYFP by Clonase reaction from Promotor PIPK4 PIPK4 EYFP pUC18ENTRY (82)	

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6.1 List of plasmids (continued)

84	-	Promotor PIPK5 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S15 + S16 from genomic *Arabidopsis* DNA	Vector obtained from Irene Stenzel
85	37	Promotor PIPK5 EYFP pUC18ENTRY	+2EYFP pUC18ENTRY (68) SalI NotI	Promotor PIPK5 SalI NotI from Promotor PIPK5 pGEM-T Easy (84)	
86	40	Promotor PIPK5 PIPK5 EYFP pUC18ENTRY	Promotor PIPK5 EYFP pUC18ENTRY (85) NotI	PIPK5 NotI from PIPK5 pGEM-T Easy (6)	
87	43 64	Promotor PIPK5 PIPK5 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor PIPK5 PIPK5 EYFP by Clonase reaction from Promotor PIPK5 PIPK5 EYFP pUC18ENTRY (86)	
88	-	Promotor PIPK11 pGEM-T Easy	pGEM-T Easy (1)	PCR product amplified with primers S17 + S18 from genomic *Arabidopsis* DNA	Vector obtained from Irene Stenzel
89	39	Promotor PIPK11 EYFP pUC18ENTRY	EYFP pUC18ENTRY (67) SalI NotI	Promotor PIPK11 SalI NotI from Promotor PIPK11 pGEM-T Easy (88)	
90	42	Promotor PIPK11 PIPK11 EYFP pUC18ENTRY	Promotor PIPK11 EYFP pUC18ENTRY (89) NotI	PIPK11 NotI from PIPK11 pGEM-T Easy (8)	
91	68 66	Promotor PIPK11 PIPK11 EYFP pCambia 3300.0 GC	pCambia 3300.0 GC (66)	Promotor PIPK11 PIPK11 EYFP by Clonase reaction from Promotor PIPK11 PIPK11 EYFP pUC18ENTRY (90)	
92	418	PIPK11 mCherry pENTR2B	mCherry pENTR2B (22) NotI	PIPK11 NotI from PIPK11 pGEM-T Easy (8)	
93	423	PIPK11 mCherry pLatGW	pLatGW (4)	PIPK11 mCherry by Clonase reaction from PIPK11 mCherry pENTR2B (92)	
		EYFP:PIKα2 pLatGW			Vector obtained from Irene Stenzel

6.2 List of oligonucleotides

No.	Name	Sequence
21	SALK_001138 PIPK4 forw.	TGTAATATGCTGCAAGCATCCTCA
22	SALK_001138 PIPK4 reverse	GACGTGTTTACTAATCTGAGTGAG
23	SALK_147475 PIPK5 forw.	CATTTCATTTACAAGAATTTCCAGAC
24	SALK_147475 PIPK5 reverse	CCATTATACTTTACTCTATCCATAGT
31	SALK_119243 PIPK10a forw.	AAGAATGAAGAATGTTGGTAAGGATT
32	SALK_119243 PIPK10a reverse	TTGGAAGGATTACTGTCCCGTTG
33	SALK_079111 PIPK10b forw.	CAGAGATATATATGTTCCAACTGAAA
34	SALK_079111 PIPK10b reverse	AATCCTTACCAACATTCTTCATTCTT
51	LBa1 SALK forw.	TGGTTCACGTAGTGGGCCATCG
60	YFP forward Not I A	GATCGCGGCCGCCCATGGTGAGCAAGGGCGAG
61	YFP reverse Eco RV	GATCGATATCTTACTTGTACAGCTCGTCCATG
77	Arf1 Forw. EcoRI	GCATGAATTCATGGGGTTGTCATTCGGAAAGTT
78	Arf1 rev. XhoI	GCATCTCGAGCTATGCCTTGCTTGCGATGTTG
81	YFP forward Not I B	GATCGCGGCCGCCATGGTGAGCAAGGGCGAG
82	YFP forward Not I C	GATCGCGGCCGCATGGTGAGCAAGGGCGAG
119	Lat52 rev. Not	GATCGCGGCCGCGTAATTGGAAATTTTTTTTTTGGTG
120	Lat52 forw. Sal	GATCGTCGACTCGACATACTCGACTCAGAAGG
127	HsIns-Ptase for Sal	GATCGTCGACATGGCGGGGAAGGCGGCCGC
128	HsIns-Ptase rev EcoRI	GCATGAATTCCTGCACGACACAACACTTGTG
135	HsInsPtase 400bp forw.	GATCTACTCGGATACCTTAGA
136	GABI 284F05 PIPK11 forw.	GTCCAAGAATTATGGAGCTCACT
137	GABI 284F05 PIPK11 rev.	CACTGAGTTCTCACTATCAACTG
138	GABI 284F05 PIPK11 TDNA rev.	ATATTGACCATCATACTCATTGC
139	HsPLCδ1 Rev. Stop EcoRV	GATCGATATCTTAGATCTTGTGCAGCCCCAGCA
141	YFP 150 bp reverse	GTGGTGCAGATGAACTTCAG
142	pENTR-5'	CTACAAACTCTTCCTGTTAGTT
143	SeqL-B (pENTR rev)	CATCAGAGATTTTGAGACAC
156	PIPk N. crassa forw. EcoRI	GATCGAATTCATGACCTCTTCGCCAAATGGAG
157	PIPk N. crassa rev. +1 Not	GATCGCGGCCGCTTGACACGCCTTGGGGCCGA
160	Lat 52 100 bp vom Ende forw.	AAGACACACACAAAGAGAAGGAGC
161	pLat Gw rev.	TGGAACATCGTATGGATAACC
168	PIPk N. crassa seq 400	CTTCTTCACCGCATCGTTAC
169	PIPk N. crassa seq 800	CGACGAGACACATGCCAAC
170	PIPk N. crassa seq 1200	GATTACTTCAAGCATGTTACCG
171	PIPk N. crassa seq 1600	ACAAGACCCTACGTGTGTTC
172	PIPk N. crassa seq 2000	GCGGTTCATCAATTTCATTGAG
235	EXPA4 At2g39700 forw. Sal	GATCGTCGACATGGCTATTAAACTAGCAATTCTATT
236	EXPA4 At2g39700 rev. Not	GATCGCGGCCGCTAACCCTGAAATTCTTCCCGACAA
237	HsPIPK1a forw. Sal	GATCGTCGACATGGCGTCGGCCTCCTCCGG
238	HsPIPK1a rev. Not	GATCGCGGCCGCTATGGGTGAACTCTGACTCTGCA
241	PIPK5 K497A QC for	CGATGATCGTTACATGATAGCAACAATGAAGAAGTCGG
242	PIPK5 K497A QC rev	CCGACTTCTTCATTGTTGCTATCATGTAACGATCATCG
243	PIPK10 K86A QC for	GATAACCGTTTTCTCATCGCGATTCTTCGAAAATCCG
244	PIPK10 K86A QC rev	CGGATTTTCGAAGAATCGCGATGAGAAAACGGTTATC
245	PIPK11 K94A QC for	GACCGTTTTCTCATTGCGATTCTACGCAAATCCGAAATC
246	PIPK11 K94A QC rev	GATTTCGGATTTGCGTAGAATCGCAATGAGAAAACGGTC
255	HsPLCδ1 Forw. NotI + GGAGAAG	GATCGCGGCCGCCGGTGGAGCTGGAGCTGCAGGAGATGAGGATCTACAGGCGCTG
256	N-Acetyl-Glycosamyl-Trans forw. SalI	GATCGTCGACATGGCGAGGATCTCGTGTGAC
257	N-Acetyl-Glycosamyl-Trans rev. Not +1	GATCGCGGCCGCCAAGTTCTTCGTCCTGGCGGTTC
259	Red Star forw. SalI	GATCGTCGACATGAGTGCTTCTTCTGAAGATGTC
260	Red Star rev. NotI	GATCGCGGCCGCCAAGAACAAGTGGTGTCTACCTT

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6.1 List of oligonucleotides (continued)

302	mcherry for C NotI	GATCGCGGCCGCATGGTGAGCAAGGGCGAGGAG
303	mcherry rev STOP EcoRV	GATCGATATCTTACTTGTACAGCTCGTCCATGCC
S1	PIPK4 forw.	GATCCATGGGCAAGGAACAAAGCTGTGTTCTAAAGG
S2	PIPK4 rev.	GATCCATGGCATTATCCTCAGTGAAGACCTTGA
S3	PIPK5 forw.	GATTCATGAGCAAGGACCAAAGCTATGTTCTAAAAG
S4	PIPK5 rev.	GATTCATGACATTGTCATCTGTGAAGACCTTGA
S5	PIPK6 forw.	GATACATGTCGGTAGCACACGCAGATGACGCCGAC
S6	PIPK6 rev.	GATACATGT CAGCGTCTTCAACGAAGACCC
S7	PIPK10 forw.	GATCCATGGAGCTGAGAGCCACGGTGGAGAATCGAA
S8	PIPK10 rev.	GATCCATGGCATATTTCGAAGAGAGATCATCGT
S9	PIPK11 forw.	GATCCATGGTTACGCGGGAGATAACAGCAAAGGA
S10	PIPK11 rev.	GATCCATGGCGTGAGAAGGATCTTCGTCTGG
S11	actin forw.	GCTGGATTCGCTGGAGATGATGCT
S12	actin rev.	TCTCTCAGCACCGATCGTGATC
S13	Promotor 1500 bp PIPK4 forw. SalI	GATCGTCGACGACTCGAGCCATATAGTCGGCACCATG
S14	Promotor 1500 bp PIPK4 rev. NotI	GATCGCGGCCGCTATGTTAAACTAAGGAAACGTCTATC TAAAAAC
S15	Promotor 1500 bp PIPK5 forw. SalI	GATCGTCGACGACTCGAGCCATATAGTCGGCACCATG
S16	Promotor 1500 bp PIPK5 rev. NotI	GATCGCGGCCGCTATGTTAAACTAAGGAAACGTCTATC TAAAAAC
S17	Promotor 1500 bp PIPK11 forw. SalI	GATCGTCGACCTTTCCGTTAGGGTTTCTAAAATC
S18	Promotor 1500 bp PIPK11 rev. NotI	GATCGCGGCCGCTTCCAAATTACCCTCTCTCTCTCAAC TTCTCC

6.3 Supplementary image series

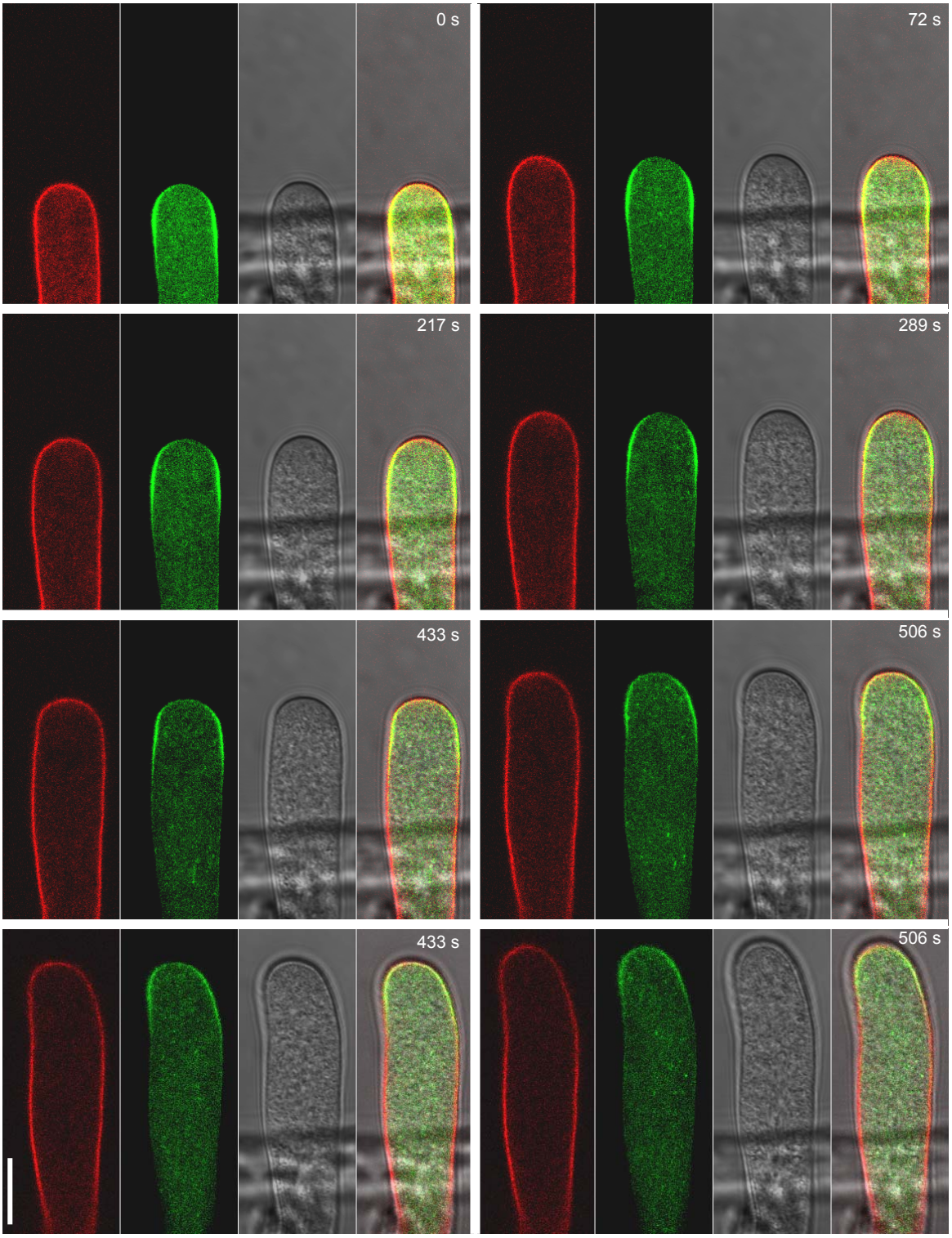

Figure 6.1 Progression of PIP5K11 localization in a growing tobacco pollen tube

Image series of a growing tobacco pollen tube expressing PIP5K11-EYFP as monitored by confocal microscopy. The pollen tube was additionally stained with the plasma membrane dye FM 4-64. Left to right, FM 4-64; EYFP; bright field; merge. Bar 10 µm.

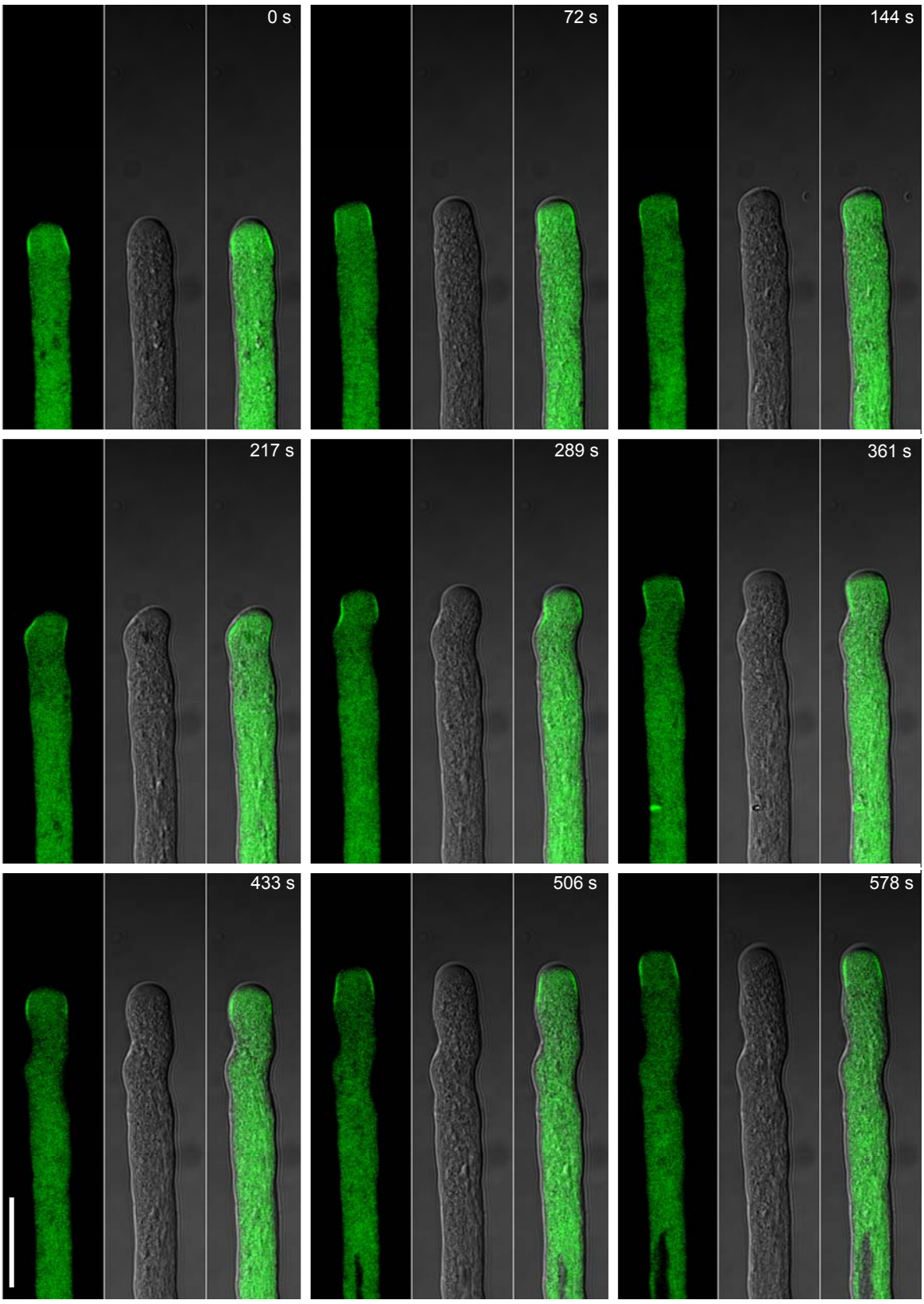

Figure 6.2 Progression of PIP5K5 localization in a growing tobacco pollen tube

Image series of a growing tobacco pollen tube expressing PIP5K11-EYFP as monitored by confocal microscopy. Left, EYFP; middle, bright field; right, merge. Bar, 20 µm.

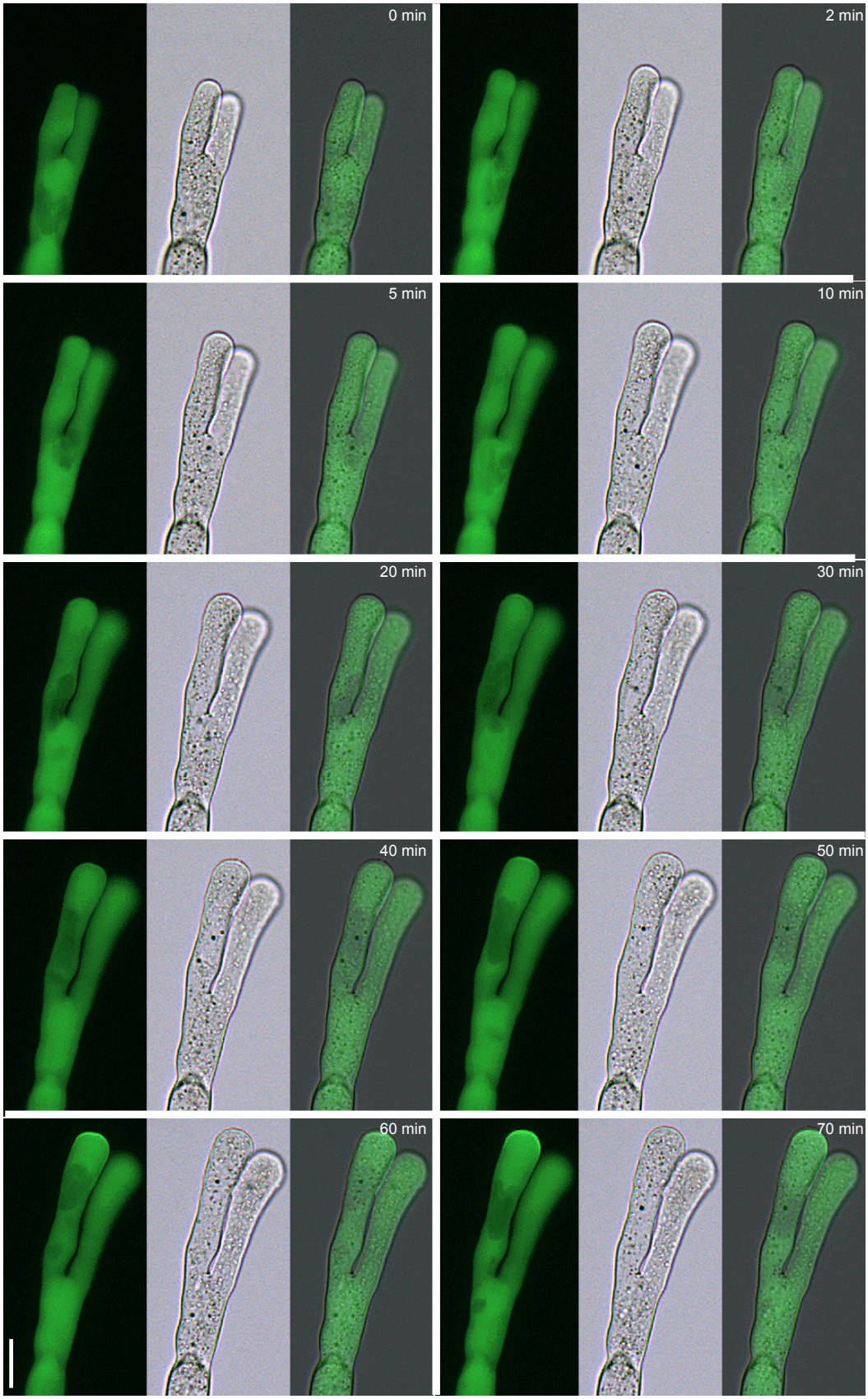

Figure 6.3 Growth progression of a branched pollen tube overexpressing PIP5K5

Image series of a growing tobacco pollen tube that has branched due to overexpression of PIP5K5-EYFP as monitored by epifluorescence microscopy. Left, EYFP; middle, bright field; right, merge. Bar, 20 µm.

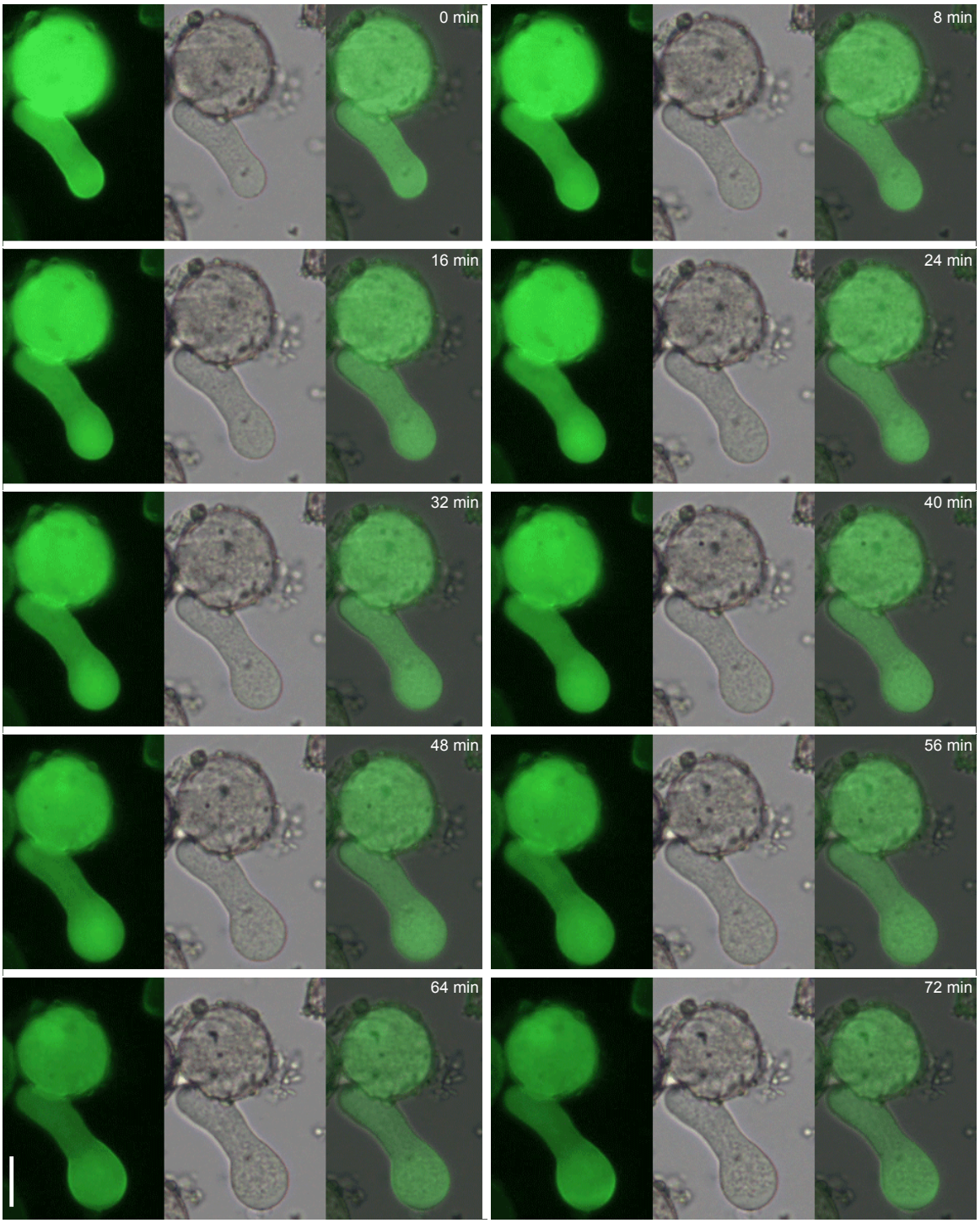

Figure 6.4 Progression of a tobacco pollen tube exhibiting tip swelling due to overexpression of PIP5K11

Image series of a pollen tube that exhibits tip swelling due to overexpression of PIP5K11-EYFP as monitored by epifluorescence microscopy. Left, EYFP; middle, bright field; right, merge. Bar, 25 µm

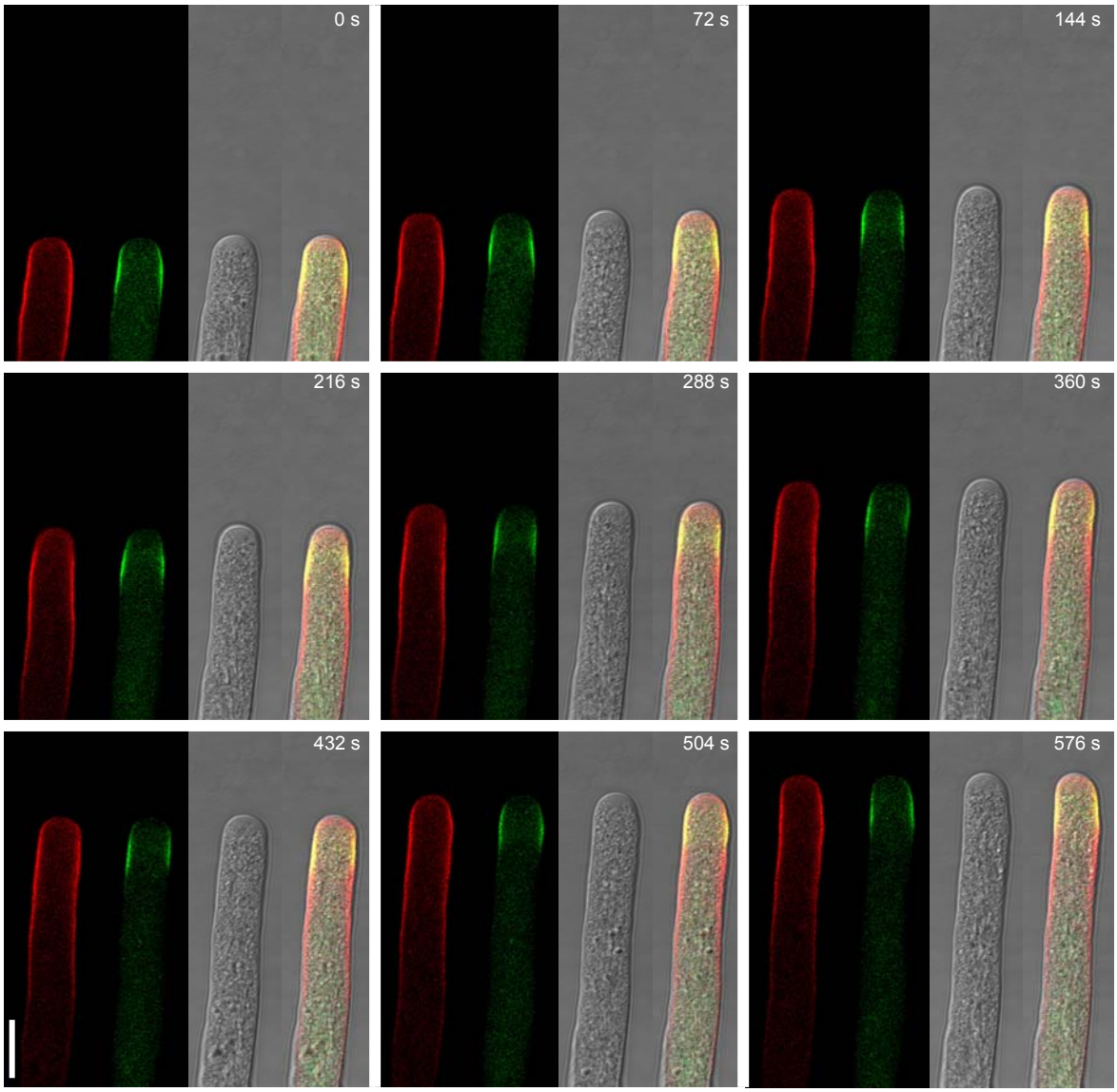

Figure 6.5 Progression of PIP5K11 and NtPLC3 localization in a growing tobacco pollen tube

Image series of a growing tobacco pollen tube expressing RFP-NtPLC3 and PIP5K11-EYFP as monitored by confocal microscopy. Left to right, RFP; EYFP; bright field; merge. Bar 10 µm.

6.4 Supplemental movies

Image series presented in the result section and in the appendix were taken from movie sequences. The respective movie sequences are provided as animated gif files and as mov files on the enclosed CD-ROM.

Supplemental Movie 1. Subcellular localization of PIPK5 in a growing pollen tube. EYFP-tagged PIPK5 (type B) was transiently expressed in tobacco pollen, and EYFP-fluorescence distribution was monitored 6 h after transformation over an 11.5 min period. Left, EYFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 2. Subcellular localization of PIPK11 in a growing pollen tube. EYFP-tagged PIPK11 (type A) was transiently expressed in tobacco pollen in the presence of the plasma membrane dye, FM 4-64, and fluorescence distribution was monitored 6 h after transformation over a 12.5 min period. Left to right, FM 4-64; EYFP-fluorescence; brightfield; merge.

Supplemental Movie 3. Pollen tube branching with strong expression of PIPK5. EYFP-tagged PIPK5 (type B) was transiently expressed in tobacco pollen. A typical morphological phenotype with strong expression was tube branching. EYFP-fluorescence was monitored 7 h after transformation over a 105 min period. Left, EYFP-epifluorescence; middle, brightfield; right, merge.

Supplemental Movie 4. Pollen tube swelling with strong expression of PIPK11. EYFP-tagged PIPK11 (type A) was transiently expressed in tobacco pollen. A typical morphological phenotype with strong expression was tube swelling. EYFP-fluorescence was monitored 7 h after transformation over a 90 min period. Left, EYFP-epifluorescence; middle, brightfield; right, merge.

Supplemental Movie 5. Golgi-movement in a "reversed-fountain"-pattern in pollen tubes without PI4P 5-kinase overexpression. The Nag-CFP Golgi-marker was transiently expressed in tobacco pollen. CFP-fluorescence was monitored 6 h after transformation over a 2 min period. Left, CFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 6. FRAP-analysis of Golgi-movement in pollen tubes without PI4P 5-kinase overexpression. The Nag-CFP Golgi-marker was transiently expressed in tobacco pollen. CFP-fluorescence was monitored ~6 h after transformation. A large portion of the pollen tube tip was bleached, and the recovery of CFP-fluorescence was monitored. The "reversed-fountain" pattern of Golgi-movement is obvious. Note that peripheral tip-bound streaming follows a spiralling pattern. CFP-fluorescence was monitored over a 5 min period. Left, CFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 7. Golgi-movement in a "reversed-fountain"-pattern in pollen tubes expressing PIPK5. The Nag-CFP Golgi-marker was transiently coexpressed with PIPK5 in tobacco pollen. CFP-fluorescence was monitored 6 h after transformation over a 3 min period. The branching pattern induced by PIPK5-expression does not interfere with Golgi-movement in the individual limbs. Left, CFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 8. Disturbed Golgi-movement in pollen tubes expressing PIPK11. The Nag-CFP Golgi-marker was transiently coexpressed with PIPK11 in tobacco pollen. CFP-fluorescence was monitored 6 h after transformation over a 6.5 min period. The swelling pattern induced by PIPK11-expression is accompanied by random/circular Golgi-movement. Note the large vacuoles in the swollen tip. Left, CFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 9. FRAP-analysis of Golgi-movement in pollen tubes expressing PIPK11. The Nag-CFP Golgi-marker was transiently coexpressed with PIPK11 in tobacco pollen. CFP-fluorescence was monitored ~6 h after transformation. A large portion of the pollen tube tip was bleached, and the recovery of CFP-fluorescence was monitored. The "reversed-fountain" pattern of Golgi-movement is lost. CFP-fluorescence was monitored over a 13 min period. Left, CFP-fluorescence; middle, brightfield; right, merge.

Supplemental Movie 10. Subcellular localization of NtPLC3 in relation to PIPK11 in a growing pollen tube. EYFP-tagged PIPK11 (type A) was transiently coexpressed with NtPLC3 in tobacco pollen tubes. Left to right, RFP-NtPLC3; EYFP-PIP5K11; brightfield; merge. Images were recorded 6 h after transformation over a 15 min period.

6.5 Index of tables

6.6 Index of figures

7. *Curriculum vitae*

7.1. Personal data

Name, Given name:	Ischebeck, Till
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Home address:	Karl-Grüneklee-Str. 1, 37077 Göttingen (0551) 305-7828

7.2. Scientific career

7.2.1	1999	Abitur at the Pascal-Gymnasium in Münster (Westf.)
	1999-2000	Zivildienst (Klinik und Polyklinik für Augenheilkunde, Universität Münster)
7.2.2	2000 - 2005	Studies of Biochemistry, Free University Berlin

7.2.3 Scientific Exams:

2005	Diploma Thesis with Prof. Dr. Peter Dörmann, Max-Planck-Institute for Molecular Plant Physiology, Potsdam/Golm

7.2.4 Scientific employment:

2003-2005	Student worker with Prof. Dr. Peter Dörmann, Max-Planck-Institute for Molecular Plant Physiology, Potsdam/Golm
since 2005	Ph.D. student with Dr. Ingo Heilmann Department of Plant Biochemistry, Georg-August-University Göttingen

7.2.5 Scientific efforts outside Germany:

1996 - 1997	Academic Year at Anna High School, Anna, TX, USA
2004	Three months of project work with Dr. John Shanklin, Department of Biology, Brookhaven National Laboratory, Upton, NY, USA

7.2.6 List of publications:

Ischebeck T, Zbierzak AM, Kanwischer M, Dormann P (2006) A salvage pathway for phytol metabolism in Arabidopsis. J Biol Chem 281: 2470-2477

König S, Ischebeck T, Lerche J, Stenzel I, Heilmann I (2008) Salt stress-induced association of phosphatidylinositol-4,5-bisphosphate with clathrin-coated vesicles in plants. Biochem J: in press

Löfke C, Ischebeck T, König S, Freitag S, Heilmann I (2008) Alternative metabolic fates of phosphatidylinositol produced by PI-synthase isoforms in Arabidopsis thaliana. Biochem J 413: 115-124

Pidkowich MS, Nguyen HT, Heilmann I, Ischebeck T, Shanklin J (2007) Modulating seed beta-ketoacyl-acyl carrier protein synthase II level converts the composition of a temperate seed oil to that of a palm-like tropical oil. Proc Natl Acad Sci U S A 104: 4742-4747

Stenzel I, Ischebeck T, König S, Holubowska A, Sporysz M, Hause B, Heilmann I (2008) The type B phosphatidylinositol-4-phosphate 5-kinase 3 is essential for root hair formation in Arabidopsis thaliana. Plant Cell 20: 124-141

8. Summary

Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) occurs in the tip-plasma membrane of growing pollen tubes. Because enzymes responsible for $PI(4,5)P_2$-production at that location are uncharacterized, $PI(4,5)P_2$-functions in polar tip-growth of pollen tubes are unresolved. Five candidate genes for pollen-expressed *Arabidopsis* phosphatidylinositol-4-phosphate 5-kinases (PI4P 5-kinases) were identified and the recombinant proteins characterized regarding catalytic activity and substrate preferences. All candidate enzymes are class I PI4P 5-kinases and represent the subfamilies A and B based on their respective domain structures. Mutant analysis revealed that type A PI4P 5-kinases were essential for pollen development, while type B PI4P 5-kinases had important roles especially in pollen germination. All five enzymes studied localized in a ring-like pattern in the apical plasma membrane of pollen tubes. Side-by-side comparison of PI4P 5-kinase localization in pollen tubes of *Arabidopsis* and tobacco shows that the tobacco model faithfully resembles the *Arabidopsis* system. Additional information on the roles of PI4P 5-kinases during pollen tube growth was obtained from transient overexpression of PI4P 5-kinases in tobacco pollen tubes that had strong effects on pollen tube morphology, characteristic for the type of enzyme tested. With overexpression of type A-enzymes pollen tubes were arrested in polar growth, exhibited tip-swelling and were altered in actin-cytoskeleton and Golgi-/vacuolar movement, whereas type B-enzymes induced tip branching or growth arrest due to increased apical pectin secretion. Morphological effects depended on $PI(4,5)P_2$-production, as evident from results with inactive enzyme-variants. The data indicate that PI4P 5-kinase isoforms generate distinct $PI(4,5)P_2$-pools that control either actin-dynamics or exocytosis of cell wall-material, respectively, and that these pools coexist within close proximity of the apical plasma membrane of pollen tubes. Overexpression of a type β PI 4-kinase induced similar effects on tobacco pollen tube morphology as was observed with type B PI4P 5-kinases and both enzymes showed synergistic effects in coexpression experiments, indicating that type β PI 4-kinases and type B PI4P 5-kinases act in a common pathway promoting pectin exocytosis.

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Ich möchte folgenden Personen danken:

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