INTRODUCTION

Pollen tubes are an excellent model system in which to investigate the molecular control of polar plant cell growth. These cells elongate rapidly in a strictly polarized manner based on massive actin-dependent tip-directed secretion (Hepler et al., 2001). Quantitative analysis of electron micrographs has suggested that cell wall biogenesis at the tip of tobacco (Nicotiana tabacum) pollen tubes requires the fusion of secretory vesicles with the plasma membrane at a rate ~8.5 times higher than necessary for membrane surface extension (DerkSEN et al., 1995). The analysis of intracellular distributions of clathrin or clathrin-coated pits (DerkSEN et al., 1995; Blackbourn and Jackson, 1996) and time lapse imaging of the endocytic uptake of the membrane dye FM4-64 (Parton et al., 2001) have indicated that excess plasma membrane material is retrieved by endocytosis at the flanks of the pollen tube tip and rapidly recycled to the secretory system.

Cytoplasmic Ca^{2+} concentrations are low in the pollen tube shank but increase gradually at the tip and reach highest levels just below the plasma membrane at the extreme apex (Pierson et al., 1996). This tip-focused Ca^{2+} gradient is essential for pollen tube elongation, presumably because it directly and/or indirectly promotes vesicle fusion with the plasma membrane (Hepler et al., 2001). Rac/Rop-type Rho family small GTPases accumulate at the plasma membrane specifically at the tip of elongating pollen tubes and are key regulators of polar cell expansion (ZhenG and Yang, 2000). Rac/Rop overexpression depolarizes pollen tube growth and results in massive ballooning at the tip, whereas Rac/Rop inactivation strongly inhibits this process (Kost et al., 1999; Li et al., 1999). Similar to homologous proteins in other systems, pollen tube Rac/Rop appears to coordinate F-actin organization, membrane traffic, Ca^{2+} signaling, and possibly other cellular processes essential for tip growth by controlling multiple signal transduction pathways.

In a previous study, we have shown that pollen tube Rac/Rop interacts with a lipid kinase activity that is responsible for the accumulation of the signaling lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5-P_2)) in the plasma membrane specifically at the apex (Kost et al., 1999). We have proposed that PI(4,5-P_2) may act as a key effector of pollen tube Rac/Rop by directly controlling actin organization and membrane traffic and by serving at the same time as a substrate for a phosphoinositide-specific phospholipase C (PI-PLC) (Kost et al., 1999). Because inositol 1,4,5-trisphosphate (IP_3) and diacyl glycerol (DAG) produced by PI-PLC-mediated PI(4,5-P_2) hydrolysis have important signaling functions in animal cells, which include IP_3-induced Ca^{2+} release from internal stores into the cytoplasm (Clapham, 1995; Yang and Kazanietz, 2003), we have postulated that PI-PLC activity may play an important role in the control of pollen tube tip growth (Kost et al., 1999).

Two types of PI-PLC activities have been biochemically identified in plant extracts (Munnik et al., 1998). One of these was...
present in cytosolic fractions, preferred phosphatidyl inositol as a substrate, and required millimolar Ca\(^{2+}\) concentrations for activity. The second activity was membrane-associated, preferred PI 4,5-P\(_2\), and was active at low micromolar Ca\(^{2+}\) concentrations. cDNAs encoding functional PI-PLCs have been cloned from different plant species (Mueller-Roeber and Pical, 2002). These PI-PLCs hydrolyzed PI 4,5-P\(_2\) at micromolar concentrations when assayed as recombiant proteins in vitro (Hirayama et al., 1995; Kopka et al., 1998; Venkataraman et al., 2003; Dowd et al., 2006), indicating that they are responsible for the membrane-associated activity detected in extracts. All cloned PI-PLCs share an identical domain structure and belong to the same plant-specific family, which is closely related to animal PI-PLC-\(\delta\) isoforms (Mueller-Roeber and Pical, 2002).

The structurally and biochemically well-characterized animal PI-PLC-\(\delta\) isoforms are composed of four functional domains (Essen et al., 1996; Katan and Williams, 1997; Rebecchi and Pentyala, 2000). (1) A PH domain responsible for membrane association. This domain has no enzymatic activity but is thought to tether animal PI-PLCs to PI 4,5-P\(_2\)-enriched membranes and thereby to allow processive catalysis. (2) An EF domain containing four EF hands. The first two EF hands bind Ca\(^{2+}\), display a high degree of structural disorder, and are thought to form a flexible linker between the PH domain and the rest of the protein. EF hands 3 and 4 are functionally poorly characterized. (3) A catalytic domain consisting of the highly conserved X and Y domains linked by a more variable region. (4) A C2 domain thought to bind to lipids in a Ca\(^{2+}\)-dependent manner (Rizo and Sudhof, 1998). This domain is not sufficient for the membrane association of animal PI-PLCs but appears to be responsible for productive positioning of the catalytic XY domain relative to the plasma membrane.

Interestingly, plant PI-PLCs, although they appear to be at least partially membrane-associated (Shi et al., 1995; Kim et al., 2004), are missing the PH domain and the first two EF hands required for membrane targeting of animal PI-PLC-\(\delta\)s. It has been suggested that the C2 domain of plant PI-PLCs alone may mediate the membrane association of these proteins (Mueller-Roeber and Pical, 2002). Consistent with this proposal, deletion of the C2 domain abolished the membrane association of mung bean (Vigna radiata) PLC3 (Kim et al., 2004), and the EF hands of Arabidopsis thaliana PLC2 (which correspond to EF hands 3 and 4 of animal PI-PLC-\(\delta\)s) were not required for lipid binding in vitro, although they were essential for catalytic activity (Otterhag et al., 2001).

Whereas little is known about the physiological functions of animal PI-PLC-\(\delta\)s, the expression of different plant PI-PLCs has been shown to be induced under stress conditions (Kopka et al., 1998; Kim et al., 2004; Vergnolle et al., 2005). Abscisic acid induction of seed dormancy (Sanchez and Chua, 2000) and stomatal closure (Stasin et al., 1999; Hunt et al., 2003) was blocked by reducing PI-PLC activity using genetic knockdown or the specific inhibitor U-73122, which establishes a function of PI-PLCs in abscisic acid signaling. Consistent with our observation that PI 4,5-P\(_2\) accumulates at the tip of pollen tubes (Kost et al., 1999; see above), a number of reports have hinted at possible roles of PI-PLCs, IP\(_3\), and/or PI 4,5-P\(_2\) in the control of the normal growth of these cells (Franklin-Tong et al., 1996; Malhó, 1998; Monteiro et al., 2005; Pan et al., 2005). The PI-PLC isofrom Pet PLC1 was recently shown to be associated with the plasma membrane throughout the tip of petunia (Petunia hybrida) pollen tubes during phases of slow growth (Dowd et al., 2006). In rapidly elongating pollen tubes, Pet PLC1 dissociated from the plasma membrane at the extreme apex and allowed PI 4,5-P\(_2\), which was evenly distributed in the plasma membrane of slowly growing cells, to accumulate to higher levels at this location. Pet PLC1 was completely removed from the pollen tube plasma membrane by the overexpression of an inactive mutant variant of this protein, or of its isolated C2 domain, which resulted in PI 4,5-P\(_2\) accumulation, arrest of cell elongation, and growth depolarization. Based on these observations, the Pet PLC1 C2 domain was proposed to be a key determinant of the intracellular localization of this protein (Dowd et al., 2006).

Here, we demonstrate that both the EF and C2 domains of Nt PLC3, a PI-PLC isofrom expressed exclusively at high levels in tobacco pollen and pollen tubes, are required to target this protein to the plasma membrane specifically at the flanks of the tips of these cells. In elongating pollen tubes, Nt PLC3 displayed a constant distribution pattern and never accumulated at the extreme apex. Our data strongly suggest that Nt PLC3 not only prevents lateral spreading of its substrate PI 4,5-P\(_2\), which is produced at the pollen tube apex in a Rac/Rop-dependent manner (see above), but also generates DAG at the flanks of the tip, which is transported to the apex by endocytic membrane recycling. Treating tobacco pollen tubes with the PI-PLC inhibitor U-73122, but not the overexpression of inactive Nt PLC3, effectively blocked pollen tube tip growth, caused PI 4,5-P\(_2\) spreading, and prevented DAG accumulation at the apex.

RESULTS

Cloning of Nt PLC3, a Tobacco PI-PLC Isoform Preferentially Expressed in Pollen and Pollen Tubes

A cDNA library representing genes expressed in tobacco pollen tubes after 3 h of culture (Klahre et al., 2006) was screened by colony hybridization with a PCR fragment amplified from the same library using primers flanking the highly conserved predicted catalytic core of the putative tobacco PI-PLC isoform Nt PLC1 (AF223351). This resulted in the identification of a 208-bp tobacco pollen tube cDNA (accession number EF043044) containing an open reading frame, which codes for a novel PI-PLC isofrom called Nt PLC3 hereafter. RNA gel blot analysis using probes corresponding to the catalytic core of Nt PLC3 (Figure 1A), or to gene-specific Nt PLC3 3’ untranslated region sequences (see Supplemental Figure 1 online), showed that hybridizing transcripts with the expected size of ~2 kb accumulated to high levels specifically in tobacco pollen grains and pollen tubes.

**Nt PLC3 Is Homologous with Characterized Plant and Animal PI-PLCs**

The Nt PLC3 cDNA encodes a 588–amino acid protein that shares 90.5% identical amino acids with petunia pollen tube Pet PLC1 (Dowd et al., 2006). Nt PLC3 is also highly similar to lily (Lilium daviddii) pollen PI-PLCs (Ld PLC1 and Ld PLC2, 53.6 and
53.7% identical amino acids, respectively; Pan et al., 2005), as well as to Arabidopsis At PLC4, At PLC5, and At PLC7 (51.1 to 66.0% identical amino acids; Mueller-Roeber and Pical, 2002), which are preferentially expressed in pollen based on microarray data (Zimmermann et al., 2004) and promoter–β-glucuronidase (GUS) fusion analysis (Hunt et al., 2004). Interestingly, phylogenetic analysis showed that pollen PI-PLCs cluster into two clearly distinct groups composed of Nt PLC3, Pet PLC1, and At PLC7 and of Ld PLC1, Ld PLC2, At PLC4, and At PLC5 (see Supplemental Figure 2 online).

An alignment of Nt PLC3 with At PLC2, a closely related Arabidopsis homolog that has been biochemically and functionally well characterized (67.9% identical amino acids; Hirayama et al., 1997; Otterhag et al., 2001), is shown in Figure 1B. Particularly highly conserved regions of Nt PLC3 include short N-terminal α-helical stretches (predicted by Garnier-Osguthorpe-Robson analysis) that may form EF hand–like structures (Otterhag et al., 2001), central X and Y domains predicted to form the catalytic core of the protein, and a C-terminal C2 domain (Figures 1B and 1C). Nt PLC3 contains all residues required for the activity of the rat homolog Rn PLC-d1 (Figure 1B) (Essen et al., 1996; Ellis et al., 1998), with which it shares 29.7% identical amino acids in the catalytic core (Nt PLC3, amino acids 111 to 437; Rn PLC-d1, amino acids 299 to 606) (Figure 1C). Like all other plant PI-PLC isoforms identified to date (Mueller-Roeber and Pical, 2002), Nt PLC3 is missing a PH domain and part of the EF domain (EF hands 1 and 2) at the N terminus, which are required for membrane association of closely related animal and yeast homologs (Figure 1C).

In Vitro Activity of Recombinant Nt PLC3 Shows Characteristic Ca2+ Dependence and Is Inhibited by U-73122 or Mutations in the Active Site

A recombinant glutathione S-transferase (GST):Nt PLC3 fusion protein purified from Escherichia coli displayed PI 4,5-P2–hydrolyzing activity (Figure 2A), which depended on the Ca2+ concentration in a similar manner as the in vitro activity of previously characterized plant PI-PLC isoforms (Hirayama et al., 1995; Kopka et al., 1998; Venkataraman et al., 2003; Dowd et al., 2006). The rate at which Nt PLC3 hydrolyzed PI 4,5-P2 (470 nmol/C min/C255 1/C1 mg/C255 1 at 10 mM Ca2+; average of seven experiments) was within the range of specific activities reported for other plant PI-PLCs (Hirayama et al., 1995; Kopka et al., 1998; Staxe´ n et al., 1999; Venkataraman et al., 2003). U-73122, an aminosteroid widely used to interfere with PI-PLC–dependent processes in animal, fungal, and plant cells (Stam et al., 1998; Staxe´ n et al., 1999; Silverman-Gavrila and Lew, 2002), inhibited the in vitro activity of Nt PLC3 in a dose-dependent manner and completely prevented PI 4,5-P2 hydrolysis at 80 mM (Figure 2B). A comparable sensitivity to U-73122 was displayed by recombinant Nicotiana rustica and Digitaria sanguinalis PI-PLC isoforms (Staxén et al., 1999; Coursol et al., 2002). To generate Nt PLC3 variants with reduced activity, His-124 and/or Asp-156 in the catalytic X domain (Figure 1B) were replaced by Ala and Arg, respectively. Corresponding amino acid exchanges greatly decreased the in vitro activity of Rn PLC-d1 (Ellis et al., 1998). Recombinant GST:Nt PLC3 fusion proteins carrying one of

Figure 1. Nt PLC3 Expression Pattern and Protein Structure.

(A) Nt PLC3 expression in different tissues and cell types as determined by RNA gel blotting. Five micrograms of total RNA was separated by gel electrophoresis (bottom panel), blotted, and hybridized with a 735-bp probe corresponding to the catalytic core of Nt PLC3. The asterisk indicates the 2-kb band. Fb, flower bud; Fm, mature flower; L, leaf; S, stem; R, root; P, mature pollen; PT, pollen tube.

(B) ClustalW (Higgins et al., 1992) alignment of the amino acid sequences of Nt PLC3 and a well-characterized Arabidopsis homolog (At PLC2). Gray shading, identical amino acids; dotted underlining, predicted α-helical regions possibly forming two EF hands; black underlining, X domain; gray underlining, Y domain; broken underlining, C2 domain; open arrowheads, conserved amino acids essential for catalytic activity.

(C) Comparison of the domain structures of Nt PLC3 and Rn PLC-d1 from rat (drawn to scale).
these two amino acid exchanges alone (GST:Nt PLC3H124A or GST:Nt PLC3D156R) or a combination of both mutations (GST:Nt PLC3H124A-D156R) hydrolyzed PI 4,5-P2 at significantly reduced rates (Figure 2C). As demonstrated by SDS-PAGE analysis (Figure 2C, inset), a significant proportion of each of the purified wild-type and mutant GST:Nt PLC3 fusion proteins used to generate the data shown in Figure 2 displayed the expected size (calculated molecular mass of 94.4 kD), although degradation products with lower molecular mass were also detected.

**Increased PI-PLC Activity Resulting from Transient Overexpression of Nt PLC3 Moderately Reduces the Growth Rate of Tobacco Pollen Tubes**

In overexpression experiments performed by Dowd et al. (2006), Pet PLC1 did not detectably affect the growth of petunia pollen tubes, whereas an inactive mutant of this protein (Pet PLC1H126A) arrested and depolarized this process. Similar experiments that we performed to analyze Nt PLC3 function in tobacco pollen tubes generated different results.

Cultured tobacco pollen tubes transiently overexpressing Nt PLC3 after particle bombardment of germinating pollen grains were ~20% shorter 6 h after gene transfer than control pollen tubes expressing the noninvasive marker protein GUS (Figures 3A, 3B, and 3D). In these experiments, yellow fluorescent protein (YFP) coexpression was used to identify transformed pollen tubes. The expression of all introduced transgenes was under the control of the Lat52 promoter (Twell et al., 1991). Although Nt PLC3–overexpressing pollen tubes displayed a reduced length,

**Figures 2. Sensitivity of Nt PLC3 in Vitro Activity to Ca²⁺ Levels, U-73122, and Mutations in the Active Site.**

(A) Nt PLC3 in vitro activity as determined by scintillation counting of water-soluble radioactivity after incubation of micellar [³H]PI 4,5-P2 with recombinant Nt PLC3 at different concentrations of free Ca²⁺. A representative example of seven independent data sets is shown.

(B) Dose-dependent reduction of the in vitro activity of Nt PLC3 at 10 μM Ca²⁺ by the PI-PLC inhibitor U-73122. A representative example of five independent data sets is shown.

(C) Reduced in vitro activity at 10 μM Ca²⁺ of Nt PLC3 carrying point mutations in the active site. A representative example of three independent data sets is shown. Inset, analysis of assayed wild-type and mutant Nt PLC3 by SDS-PAGE and Coomassie blue staining. The top band corresponds to GST:Nt PLC3 (calculated molecular mass, 94.4 kD), and the lower bands are degradation products.

**Figures 3. Effects of Transient Overexpression of Wild-Type and Mutant Nt PLC3 in Tobacco Pollen Tubes.**

(A) to (C) Low-magnification (5× lens) fluorescence images showing pollen tubes transiently coexpressing YFP along with GUS (A), Nt PLC3 (B), or Nt PLC3H124A D156R (C) 20 h after gene transfer. Only the cytoplasm-rich tip region (t) of most pollen tubes is visible. Two short pollen tubes are depicted from grain (g) to tip. Bar = 500 μm.

(D) Statistical analysis of pollen tube length 6 h after gene transfer. WT, Nt PLC3; H124A D156R, Nt PLC3H124A D156R. Error bars indicate 95% confidence intervals (n = 55 to 100).
they generally were morphologically normal (Figures 3A and 3B) and, similar to control pollen tubes, continued to elongate for at least 24 h (data not shown). It is interesting that other proteins with important functions in tobacco pollen tubes can cause much more drastic effects when transiently overexpressed in these cells under the same conditions (Kost et al., 1999; Klahre et al., 2006; Klahre and Kost, 2006). The moderate reduction of pollen tube length caused by Nt PLC3 overexpression depended on the enzymatic activity of this protein. Tobacco pollen tubes transiently overexpressing mutant Nt PLC3 with reduced PI 4,5-P2–hydrolyzing activity (Figure 2C) displayed the same length as control pollen tubes 6 h after gene transfer (Nt PLC3H124A-D156R, Figures 3C and 3D; Nt PLC3H124A and Nt PLC3D156R, unpublished results).

**Nt PLC3 Accumulates Laterally at the Plasma Membrane at the Tip of Growing Pollen Tubes in a Pattern Complementary to the Distribution of Its Substrate PI 4,5-P2**

Dowd et al. (2006) recently showed that green fluorescent protein (GFP) fused to the N terminus of Pet PLC1 (GFP:Pet PLC1) accumulates at the plasma throughout the tip of slowly elongating petunia pollen tubes. During phases of rapid growth, GFP:Pet PLC1 was found to dissociate from the membrane at the extreme apex, which resulted in increased PI 4,5-P2 levels at this location, as visualized using a GFP:PH fusion protein. Inactive GFP:Pet PLC1H126A, which disrupted pollen tube growth (see above), was associated with the plasma membrane throughout the tip.

Analysis by confocal microscopy showed that Nt PLC3 fused to YFP at the C terminus (Nt PLC3:YFP; Figure 4A) or the N terminus (YFP:Nt PLC3; Figure 4B) strongly associated with the tobacco pollen tube plasma membrane specifically at the flanks of the tip, but not at the apex, when transiently expressed under the control of the Lat52 promoter. By contrast, free YFP expressed under the control of the same promoter did not show any association with the pollen tube plasma membrane (Figure 4C). Amino acid exchanges reducing the in vitro PI 4,5-P2–hydrolyzing activity of Nt PLC3 (H124A, D156R, and H124A-D156R; Figure 2C) had no effect on the distribution pattern of transiently expressed YFP:Nt PLC3 (see Supplemental Figure 3 online), demonstrating that the intracellular localization of Nt PLC3 does not depend on its enzymatic activity. Time-lapse imaging established that the Nt PLC3:YFP distribution pattern remained constant over time in normally elongating pollen tubes (see Supplemental Movie 1 and Supplemental Figure 7 online). Stopping pollen tube elongation (e.g., by treatment with the actin-depolymerizing drug latrunculin B at 50 nM) caused Nt PLC3:YFP to redistribute to the apex and to display even labeling of the plasma membrane at the tip (see Supplemental Figure 3 online).

Nt PLC3 with YFP attached to its C terminus (Nt PLC3:CYP) accumulated at the plasma membrane particularly strongly and reduced the growth rate of transiently transformed pollen tubes (Figure 4D) to a similar extent as free Nt PLC3 (Figure 3D), indicating that this fusion protein was normally functioning. Weakly fluorescent pollen tubes that expressed this fusion protein at low levels displayed the same labeling pattern as shown in Figure 4A and were able to grow at the same rate as control pollen tubes expressing free YFP (see Supplemental Movie 1 and Supplemental Figure 7 online). Compared with Nt PLC3:CYP (Figure 4A), YFP:Nt PLC3 displayed reduced membrane association (Figure 4B; note the significantly brighter labeling of the cytoplasm) and caused a more pronounced inhibition of pollen tube growth (Figure 4D). This indicates that blocking the N terminus of Nt PLC3 with YFP affected the intracellular targeting and the activity of this protein.

In a previous study, we used GFP fused to the PH domain of human PLC-δ1 (GFP:PH), a specific marker for the PI-PLC substrate PI 4,5-P2 in living cells (Stauffer et al., 1997), to show that this lipid accumulates in the plasma membrane exclusively at the apex of normally elongating pollen tubes (Kost et al., 1999). Time-lapse imaging of a transiently expressed YFP:PH fusion protein stably coexpressing Nt PLC3 with YFP affected the intracellular targeting and the activity of this protein. **Figure 4.** Intracellular Localization of Nt PLC3 Fused to YFP and of the PI-PLC Substrate PI 4,5-P2.

| Single confocal sections show the distribution of Nt PLC3:YFP (A), YFP:NT PLC3 (B), YFP (C), Nt PLC3:mRFP1 (E), and the PI 4,5-P2 marker YFP:PH (F) in transiently transformed tobacco pollen tubes 6 to 8 h after gene transfer. (E) and (F) show an individual pollen tube coexpressing Nt PLC3:mRFP1 and YFP:PH. In all cases, central sections through pollen tubes lying flat on the cover slip surface are shown, representing at least 20 similar images collected in three independent experiments. Bars = 10 μm. (D) shows statistical analysis of the growth rate of pollen tubes displaying Nt PLC3:CYP, YFP:NT PLC3, and YFP distribution patterns as seen in (A) to (C). Pollen tubes showing YFP:PH/PI 4,5-P2 labeling similar to that in (F) can elongate normally (Kost et al., 1999). Error bars indicate 95% confidence intervals (n = 16 to 45).
protein demonstrated that the PI 4,5-P₂ labeling pattern in normally elongating tobacco pollen tubes remained constant over time, although labeling intensity displayed some fluctuation (see Supplemental Movie 2 and Supplemental Figure 8 online). Transient coexpression of Nt PLC3 fused to mRFP1 (for monomeric red fluorescent protein1) (Campbell et al., 2002) at the C terminus (Nt PLC3:mRFP1) did not detectably affect PI 4,5-P₂ labeling by YFP:PH (cf. Figure 4F and Figures 7A and 7D; see Supplemental Movie 2 and Supplemental Figure 8 online) and established that Nt PLC3 and PI 4,5-P₂ display complementary distribution patterns in tobacco pollen tubes (Figures 4E and 4F). This indicated that a key function of Nt PLC3 may be to prevent lateral spreading of its substrate PI 4,5-P₂ in the plasma membrane at the tip of these cells.

The EF and C2 Domains Together Are Required and Sufficient for the Correct Intracellular Targeting of Nt PLC3

Dowd et al. (2006) have reported that coexpression of inactive Pet PLC1H126A, or of the isolated C2 domain of this protein, in petunia pollen tubes dissociated GFP:Pet PLC1 from the plasma membrane. Based on these observations, the C2 domain was proposed to be a key determinant of the intracellular localization of this protein.

To identify the Nt PLC3 domains responsible for correct intracellular targeting, truncated forms of this protein missing one or two of the three major conserved regions (Figure 1C) were transiently expressed as YFP fusion proteins in tobacco pollen tubes under the control of the Lat52 promoter. Expression of these Nt PLC3 fragments, which carried YFP attached to their C termini unless specified otherwise, did not strongly affect pollen tube growth, although possible minor effects detectable by statistical growth rate analysis cannot be excluded. The intracellular distribution of all fusion proteins was imaged using confocal microscopy 6 to 8 h after gene transfer. Interestingly, we found that not only the deletion of a C-terminal fragment (amino acids 432 to 588) containing the C2 domain (EF-X:YFP; Figure 5A) but also the deletion of an N-terminal fragment (amino acids 1 to 110) containing the EF domain (XY-C2:YFP; Figure 5B) completely abolished the membrane association of Nt PLC3. Fragments containing just the EF domain (amino acids 1 to 106, EF:YFP; Figure 5C), the catalytic X and Y domains (amino acids 111 to 431, XY:YFP; Figure 5D), or the C2 domain (amino acids 437 to 588, C2:YFP; Figure 5E) also did not detectably accumulate at the plasma membrane. The fragment containing the C2 domain also failed to show membrane association when YFP was attached to its N terminus (YFP:C2; unpublished results). By contrast, YFP with the EF domain fragment (amino acids 1 to 106) fused to its N terminus and the C2 domain fragment (amino acids 437 to 588) attached to its C terminus showed an intracellular distribution (EF:YFP:C2; Figure 5F) indistinguishable from that of intact Nt PLC3 (Figures 4A and 4B). This finding demonstrates that the EF and C2 domains together mediate the normal intracellular localization of Nt PLC3 in tobacco pollen tubes.

U-73122 Strongly Inhibits Tobacco Pollen Tube Growth and Can Depolarize This Process

U-73122, a potent inhibitor of the in vitro activity of recombinant Nt PLC3 (Figure 2B), strongly inhibited the growth of cultured tobacco pollen tubes in a dose-dependent manner (Figure 6A). By contrast, this process was not affected by U-73343, an inactive analog of U-73122 (Bleasdale et al., 1990) (Figure 6A). Pollen tubes growing in the presence of U-73122 at low concentrations (0.1 to 2 μM) displayed reduced growth (Figure 6A) but were morphologically indistinguishable from pollen tubes growing in medium containing only solvent (Figure 6B; unpublished results). Interestingly, pollen tubes formed in the presence of U-73122 at concentrations at which pollen tube elongation...
was almost completely abolished (4 to 10 μM; Figure 6A) often displayed massively swollen tips resulting from depolarized cell expansion (Figure 6C). A comparable growth depolarization was not observed when pollen tube elongation was inhibited to the same extent with the actin-depolymerizing drug latrunculin B (10 nM; Figure 6D), although F-actin disruption sometimes causes a minor enlargement of pollen tube tips (Figure 7B) (Vidali et al., 2001).

Remarkably, the growth of cultured tobacco pollen tubes was significantly more sensitive to U-73122 than the in vitro PI 4,5-P₂ hydrolysis by recombinant Nt PLC3. Whereas pollen tube elongation was nearly abolished at 4 μM U-73122 (Figure 6A), completely blocking the in vitro activity of Nt PLC3 required a 20 times higher concentration (Figure 2B). Similar observations were made when the sensitivity to U-73122 of other plant or animal PI-PLC activities in vitro was compared with that of cellular processes depending on these activities (Staxén et al., 1999; Feisst et al., 2005). Because the molecular mechanism of the inhibition of PI-PLC activity by U-73122 is not understood, the reasons for this apparent discrepancy are not clear (Feisst et al., 2005). However, substantially different substrate presentation in vitro and in vivo (Drebak and Heras, 2002) may be at least partially responsible.

We have performed experiments (see below; Figures 7 and 8) showing that U-73122 causes an accumulation of the PI-PLC substrate PI 4,5-P₂, and a depletion of the PI-PLC product DAG, in cultured tobacco pollen tubes at the same concentration at which it strongly affects cell expansion. These data establish that endogenous PI-PLC activity in living tobacco pollen tubes displays the same sensitivity to U-73122 as the growth of these cells.

![Figure 6](image)

**Figure 6.** Effects of the PI-PLC Inhibitor U-73122 on Tobacco Pollen Tube Growth in Vitro.

(A) Statistical analysis of the length of tobacco pollen tubes after 6 h of culture in liquid medium containing U-73122 at different concentrations, the inactive analog U-73343 (10 μM), or solvent (1% DMSO). A representative example of three independent data sets is shown. Error bars indicate 95% confidence intervals (n = 70 to 80).

(B) to (D) Transmitted light micrographs showing the typical morphology of pollen tubes after 6 to 8 h in medium containing 1% DMSO (B), 6 μM U-73122 (C), or 10 nM latrunculin B (Lat B) (D). Pollen tubes shown in (C) and (D) had stopped elongating. Images were taken using either a 5× lens (top panels; bar = 200 μm) or a 40× lens (bottom panels [differential interference contrast]; bar = 50 μm).

U-73122 Induces Lateral Spreading of the Nt PLC3 Substrate PI 4,5-P₂ at the Pollen Tube Tip

Tobacco pollen tubes transiently expressing the PI 4,5-P₂ marker YFP-PH under the control of the Lat52 promoter 6 to 8 h after gene transfer were treated with U-73122 to determine the effects of the inhibition of PI-PLC activity on PI 4,5-P₂ distribution. The addition of solvent (1% DMSO) to the culture medium in control experiments did not interfere with pollen tube elongation (Figure 6A) and had no effect on PI 4,5-P₂ labeling (Figures 7A; 4F and Kost et al., 1999). By contrast, 5 μM U-73122 stopped pollen tube growth and consistently caused transiently expressed YFP-PH to display an enhanced association with a much larger area of the plasma membrane at the pollen tube tip (Figure 7C). A comparable

![Figure 7](image)

**Figure 7.** Effects of U-73122 on the Intracellular Distribution of the PI-PLC Substrate PI 4,5-P₂.

Single confocal sections show the distribution of the PI 4,5-P₂ marker YFP-PH in transiently transformed tobacco pollen tubes 6 to 8 h after gene transfer.

(A) A pollen tube elongating normally in medium containing DMSO.

(B) and (C) Pollen tubes 30 min after the addition of 50 nM latrunculin B (Lat B) or 5 μM U-73122, respectively, to the culture medium. These pollen tubes had stopped growing at the time of imaging.

(D) An individual pollen tube immediately (left; normally elongating) and 30 min (right; stopped growing) after treatment with an inhibitory dose of U-73122 (see Methods).

In all cases, central sections through pollen tubes lying flat on the cover slip surface are shown, representing at least 15 (A to C) or 5 (D) similar images collected in at least three independent experiments. Bars = 10 μm.
accumulation and spreading of PI 4,5-P2 in the plasma membrane at the tip was not observed (Figure 7B) when pollen tube growth was stopped using 50 nM latrunculin B, which demonstrates that this effect was specifically induced by U-73122 and was not a consequence of abolished growth.

Treatment of pollen tubes with U-73122 at a concentration of 0.1 μM, at which it barely affects cell growth (Figure 6A), had no discernible effect on PI 4,5-P2 labeling by YFP:PH (see Supplemental Figure 4A online). Together with the data described above, this establishes a tight correlation between the doses of U-73122 required to affect cell growth and intracellular PI 4,5-P2 distribution. PI 4,5-P2 accumulation and spreading could also be observed in individual pollen tubes that were treated with U-73122 directly on the microscope stage and imaged immediately before and 30 min after drug application (Figure 7D). The data summarized in this section, which are consistent with the complementary distribution patterns of Nt PLC3 and PI 4,5-P2 at the tip of normally growing tobacco pollen tubes (Figures 4E and 4F), strongly support the hypothesis that Nt PLC3 restricts lateral spreading of its substrate PI 4,5-P2 in the plasma membrane at the pollen tube tip.

**DAG Accumulates in the Plasma Membrane at the Pollen Tube Tip Depending on PI-PLC Activity**

Functionally significant local accumulations of DAG, the lipid product of PI-PLC activity, can be visualized in living cells using GFP fused to the C terminus of the first Cys-rich domain (Cys1) of protein kinase C (Cys1:GFP) (Oancea et al., 1998). Interestingly, we found that a Cys1:YFP fusion protein transiently expressed under the control of the Lat52 promoter strongly accumulated at the plasma membrane specifically at the tip of morphologically normal tobacco pollen tubes (Figures 8A and 8D) that were elongating at the same rate (5.1 ± 0.9 μm/min [95% confidence interval]; n = 11) as YFP-expressing pollen tubes (Figure 4D). Time-lapse imaging of such pollen tubes showed that the Cys1:YFP labeling remained constant over time (see Supplemental Movie 5 and Supplemental Figure 9 online). Coexpression of Nt PLC3:YFP did not detectably affect Cys1:mRFP1 labeling (cf. Figures 8A and 8D; see Supplemental Figure 5 online) and revealed essentially complementary distribution patterns of the two fusion proteins (see Supplemental Figure 5 online). However, Nt PLC3 fusion proteins consistently overlapped more significantly with the DAG marker Cys1:mRFP1 than with the PI 4,5-P2 marker YFP:PH at the flanks of the pollen tube tip (cf. Figures 4E and 4F; see Supplemental Figure 5 online).

The association of Cys1:YFP with the plasma membrane of transiently transformed pollen tubes was completely abolished when 5 μM U-73122 was added to the culture medium 6 to 8 h after gene transfer (Figure 8B), a treatment that stopped pollen tube growth and caused PI 4,5-P2 spreading at the tip (see above; Figures 7C and 7D). By contrast, the Cys1:YFP distribution pattern in transiently transformed pollen tubes was insensitive to solvent (1% DMSO) (Figure 8A), to the inhibition of cell expansion by 50 mM latrunculin B (Figure 8C), and to U-73122 at a concentration of 0.1 μM (see Supplemental Figure 4B online), at which this drug slightly reduced cell expansion without detectably changing PI 4,5-P2 distribution (see above). The disappearance of Cys1:YFP labeling from the plasma membrane could also be observed when individual pollen tubes were treated with U-73122 directly on the microscope stage and imaged immediately before and at 15-min intervals after drug application (Figure 8D). Together, these data strongly suggest that Nt PLC3 not only prevents lateral spreading of its substrate PI 4,5-P2 but is also responsible for the accumulation of its product DAG in the plasma membrane at the tip of normally elongating pollen tubes.

Nt PLC3 activity presumably generates DAG at the flanks of pollen tube tips, where the distribution pattern of this enzyme overlaps with that of its substrate PI 4,5-P2 (Figures 4E and 4F). The observed even distribution of DAG throughout the plasma membrane at the tip may be attributable to lateral DAG diffusion from the flanks to the apex. However, such diffusion would have to occur against a retrograde flow of plasma membrane material away from the tip that is expected to result from the constant fusion of secretory vesicles with the apical plasma membrane (Hepler et al., 2001). Therefore, we have suspected that endocytic plasma membrane recycling from the flanks to the apex, which was proposed to balance membrane traffic at the tip of growing tobacco pollen tubes (Derksen et al., 1995; see Introduction), may contribute to the DAG distribution pattern observed in these cells. Small, even-sized fluorescent dots were often visible on confocal images in the cytoplasm of Cys1:YFP-expressing cells after U-73122 treatment (Figures 8B and 8D).
These dots could corresponded to DAG-containing cell organelles that are only visible in nongrowing pollen tubes when cytoplasmic levels of Cys1:YFP are increased because DAG accumulation in the plasma membrane is prevented. As our attempts to demonstrate the colocalization of available markers for early endosomes (fluorescent Rab5 and FYVE domain fusion proteins) with the Cys1:YFP-labeled dots in U-73122–treated pollen tubes were not successful (unpublished results), the identity of these organelles remains to be determined.

DAG, but Not PI 4,5-P2, Undergoes Endocytic Recycling at the Tip of Tobacco Pollen Tubes

To establish a function of endocytic recycling in the transport of DAG to the pollen tube apex, we have blocked this process in tobacco pollen tubes by transiently overexpressing fluorescent FYVE domain fusion proteins at high levels. At low expression levels, these fusion proteins colocalize with Rab5 homologs to the surface of early endosomes in animal (Gillooly et al., 2000) and plant (Voigt et al., 2005; Vermeer et al., 2006) cells, including tobacco pollen tubes (unpublished results). When expressed at high levels in all of these cell types, fluorescent FYVE domain fusion proteins cause the formation of enlarged endocytic compartments, with which they are associated (Gillooly et al., 2000; Voigt et al., 2005) (Figures 9B to 9D, bottom panels). The styryl dye FM4-64 is an established tracer for endocytic membrane traffic in plant cells (Meckel et al., 2004). When applied to growing pollen tubes, the dye is incorporated into the plasma membrane, internalized by endocytosis, and recycled back to the plasma membrane by secretory vesicles, which accumulate in an apical region called the clear zone (Parton et al., 2001). In normally elongating tobacco pollen tubes, FM4-64 labels the plasma membrane and a cone-shaped clear zone in the apex after an incubation period of 20 min (Figure 9A).

When FM4-64 was applied to tobacco pollen tubes that displayed significantly reduced growth rates as a consequence of high-level transient expression of a YFP:FYVE domain fusion protein 6 to 8 h after gene transfer, the dye was internalized and accumulated in enlarged endocytic compartments showing bright YFP:FYVE labeling (Figure 9B). No FM4-64 labeling of secretory vesicles was visible, indicating that FM4-64 may have been trapped within these compartments. Interestingly, the DAG marker Cys1:mRFP1 also accumulated in YFP:FYVE-labeled enlarged endocytic compartments when transiently coexpressed with high levels of this fusion protein in tobacco pollen tubes (Figure 9C). No or only weak Cys1:mRFP1 accumulation at the plasma membrane was observed under these conditions (Figure 9C). These results demonstrate that DAG is endocytically internalized by living tobacco pollen tubes and strongly suggest that recycling from endocytic compartments is essential for the accumulation of this lipid in the plasma membrane at the pollen tube tip. Consistent with this conclusion, Cys1:YFP colocalized with FM4-64 to intracellular compartments formed in tobacco pollen tubes after treatment with brefeldin A (see Supplemental Figure 6 online). In plant cells (Nebenführ et al., 2002), including pollen tubes (Parton et al., 2003), brefeldin A induces the formation of aggregates of endocytic compartments and other cell organelles, in which plasma membrane markers that undergo endocytic recycling are retained.

Visualization of PI 4,5-P2 distribution by transiently expressed YFP:PH in tobacco pollen tubes coexpressing a mRFP1:FYVE domain fusion protein at high levels showed that this lipid was not trapped in enlarged endocytic compartments and accumulated in the plasma membrane at the tip, as it does in normally elongating pollen tubes (Figure 9D). In contrast with DAG, PI 4,5-P2 does not seem to be endocytically internalized by tobacco pollen tubes and shows an intracellular distribution that appears to be independent of membrane recycling. The effects of

Figure 9. Effects of High-Level Expression of FYVE Domain Fusion Proteins on the Intracellular Distribution of FM4-64, DAG, and PI 4,5-P2.

Single confocal sections show the distribution of FM4-64 and/or the indicated fluorescent fusion proteins in living tobacco pollen tubes. (A) FM4-64 labeling of a normally elongating pollen tube. (B) to (D) Analysis of colocalization of lipid markers (top panels) with YFP:FYVE or mRFP1:FYVE fusion proteins (bottom panels) expressed at high levels in transiently transformed pollen tubes. Expression of these fusion proteins at high levels caused the formation of brightly labeled enlarged endocytic compartments (open arrowheads). In all cases, central sections through pollen tubes lying flat on the cover slip surface are shown, representing at least 15 similar images collected in at least three independent experiments. Bars = 10 μm.
brefeldin A treatment on PI 4,5-P_2 distribution could not be analyzed, because this drug completely abolished PI 4,5-P_2 labeling in tobacco pollen tubes (unpublished results).

**DISCUSSION**

**PI-PLC–Dependent PI 4,5-P_2/DAG Accumulation at the Tip Is Essential for Pollen Tube Growth**

Our data firmly establish an essential function of PI-PLC activity during pollen tube elongation, for which only indirect evidence has previously been presented in the literature (see Introduction). The growth of cultured tobacco pollen tubes could be completely blocked by treatment with the PI-PLC inhibitor U-73122 and was reduced by Nt PLC3 overexpression. Remarkably, PI 4,5-P_2 and DAG, the substrate and a product of PI-PLC activity, both accumulate in an apical domain of the plasma membrane at the tip of normally elongating tobacco pollen tubes. These two membrane lipids are essential regulators of various cellular processes in different cell types and are likely to play important roles in the regulation of pollen tube tip growth (see below). Consistent with the hypothesis that the maintenance of an apical plasma membrane domain enriched in PI 4,5-P_2 and DAG is a key function of pollen tube PI-PLC activity, disruption of this domain and strong effects on pollen tube tip growth were caused by U-73122 with the same concentration dependence. Treatment with U-73122 at nanomolar concentrations, or Nt PLC3 overexpression (Figure 4; see Supplemental Figure 5 online), which moderately inhibited pollen tube growth, did not noticeably affect PI 4,5-P_2 or DAG accumulation in the apex. Conceivably, minor alterations in the levels of these lipids not detectable using transiently expressed in vivo markers reduced the rate of pollen tube elongation but did not abolish this process. By contrast, the massive inhibition and partial depolarization of pollen tube growth induced by U-73122 at micromolar concentrations was accompanied by PI 4,5-P_2 spreading as well as a complete loss of DAG accumulation in the plasma membrane at the tip.

**Nt PLC3 Is a Major PI-PLC Isoform in Tobacco Pollen Tubes**

Nt PLC3, which is highly similar to other pollen PI-PLC isoforms, is likely to contribute significantly to the endogenous PI-PLC activity in normally elongating tobacco pollen tubes. Transcripts coding for this protein specifically accumulate to high levels in tobacco pollen and pollen tubes, as demonstrated by RNA gel blotting. Recombinant Nt PLC3 displays PI 4,5-P_2–hydrolyzing activity characteristic of plant PI-PLC isoforms, which is sensitive to U-73122 as the endogenous PI-PLC activity in tobacco pollen tubes. The intracellular localization of Nt PLC3 fused to YFP is consistent with PI 4,5-P_2 and DAG distributions in normally elongating and in U-73122–treated pollen tubes.

However, tobacco pollen tubes presumably express more than one PI-PLC. Three PI-PLC isoforms are preferentially expressed in *Arabidopsis* pollen and pollen tubes based on microarray and promoter-GUS fusion analysis (see above) (Hunt et al., 2004; Zimmermann et al., 2004). The relative contributions of these three isoforms to the PI-PLC activity in *Arabidopsis* pollen tubes remain to be determined, as available data do not allow quantitative comparison of gene expression levels or protein activities. The observation that pollen PI-PLCs from *Arabidopsis* and other organisms cluster into distinct groups based on phylogenetic analysis may indicate the presence in pollen tubes of two different types of PI-PLC activities, potentially with distinct functions. Additional work is clearly required to understand the functional significance of the expression of multiple PI-PLC isoforms during pollen tube growth.

**Nt PLC3–Mediated PI 4,5-P_2 Hydrolysis and DAG Generation at the Flanks of the Tip, Together with Endocytic DAG Recycling, Maintain an Apical Plasma Membrane Domain Enriched in These Lipids**

YFP-tagged Nt PLC3 was associated with the plasma membrane of growing tobacco pollen tubes specifically at the flanks of the tip in a pattern complementary to the distribution of its substrate PI 4,5-P_2, which accumulated at the apex. The Nt PLC3 and PI 4,5-P_2 distribution patterns remained constant during pollen tube elongation. Inhibiting PI-PLC activity by U-73122, but not blocking pollen tube growth with latrunculin B, caused PI 4,5-P_2 accumulation and spreading in the plasma membrane at the tip. Together, these observations strongly suggest that during normal pollen tube growth Nt PLC3–mediated hydrolysis limits the lateral spreading of PI 4,5-P_2 (Figure 10), which is generated at the apex by a Rac/Rop-associated lipid kinase (Kost et al., 1999). Although DAG, a product of PI-PLC activity, also localizes to the pollen tube apex, this lipid appears to accumulate there based on a completely different mechanism. DAG is presumably generated at the flanks of the tip, where the distribution patterns of...
plasma membrane throughout the tip, whereas PI 4,5-P2, visu-
petunia pollen tubes, GFP-tagged Pet PLC1 accumulated at the
phases of slow growth (Dowd et al., 2006). In slowly elongating
cultured petunia pollen tubes appear to display rapid elongation,
which is spontaneously interrupted at irregular intervals by
phases of slow growth (Dowd et al., 2006). In slowly elongating
petunia pollen tubes, GFP-tagged Pet PLC1 accumulated at the
plasma membrane throughout the tip, whereas PI 4,5-P2, visual-
alized using a GFP:PH fusion protein, was present in all areas of
the plasma membrane at a constant low level. During rapid cell
elongation, Pet PLC1 was removed from the plasma membrane
at the apex, which allowed PI 4,5-P2 to accumulate to higher
levels at this location and established a PI 4,5-P2 gradient
stretching from tip to base all along the pollen tube length.
Despite the high sequence conservation between Pet PLC1 and
Nt PLC3, and the very similar distribution patterns of these two
proteins in rapidly growing pollen tubes, they do not appear to be
functionally identical. Pet PLC1 was reported to oscillate on and
off the apical plasma membrane during petunia pollen tube
growth, and thereby to regulate a PI 4,5-P2 gradient stretching all
along the plasma membrane, which controls the rate of tip
growth (Dowd et al., 2006). We propose that Nt PLC3 constantly
remains associated with the plasma membrane at the flanks of
the tip of tobacco pollen tubes, prevents lateral spreading of PI
4,5-P2 that is generated at the apex, produces DAG that is
transported to the apex via endocytic membrane recycling, and
thereby maintains an apical plasma membrane domain mas-
sively enriched in these two signaling lipids, which is essential for
tip growth.

Control of the Intracellular Localization of Nt PLC3

As discussed above, the specific association of Nt PLC3 with the
plasma membrane at the flanks of the pollen tube tip is crucial for
the functions of this enzyme. Previously available evidence has
suggested that the C-terminal C2 domain of plant PI-PLCs,
which are lacking a C-terminal PH domain, may be responsible
and sufficient for the accumulation of these proteins at the
plasma membrane (see Introduction). Our results establish that
not only the C2 domain but also the N-terminal EF hand domain is
required for the membrane association of Nt PLC3 in vivo and
that these two domains together are sufficient to confer the
normal intracellular localization of this protein.

Interestingly, expression in petunia pollen tubes of mutant Pet
PLC1 displaying reduced in vitro PI 4,5-P2 hydrolysis, or of the
isolated C2 domain of this protein, was reported to arrest cell
expansion and to cause growth depolarization by displacing PI-
PLCs from the plasma membrane. YFP fused to inactive mutant
Pet PLC1 accumulated at the plasma membrane throughout the
pollen tube tip (Dowd et al., 2006). We demonstrate that tobacco
pollen tubes expressing inactive mutants of Nt PLC3 were as
long as control pollen tubes at 6 h after gene transfer. YFP
tagging showed that inactive Nt PLC3 mutants accumulated at
the plasma membrane specifically at the flanks of the tip like
wild-type Nt PLC3, whereas the isolated Nt PLC3 C2 domain did
not show membrane association. The reasons for these discrep-
ancies are not clear. They may be attributable to variations in the
mode of function (see above), or in the regulation of the local-
ization, of PI-PLCs in tobacco and petunia pollen tubes. A
saturable interaction of the C2 domain with a regulatory factor
may be required for the association of Pet PLC1, but not of Nt
PLC3, with the pollen tube plasma membrane. However, Dowd
et al. (2006) did not exclude unspecific effects of the expression
of inactive Pet PLC1, or of the isolated Pet PLC1 C2 domain, on
the functions of other proteins in petunia pollen tubes. In fact, the
expression of mutant or truncated Pet PLC1 increased cyto-
plasmic Ca2+ levels (Dowd et al., 2006), an effect that may rather
be expected to result from a stimulation of PI-PLC-mediated IP3
production than from reduced PI-PLC activity.

The molecular mechanisms that prevent the accumulation of
PI-PLCs at the plasma membrane in the apex of rapidly growing
pollen tubes remain to be identified. Nt PLC3 partially colo-
ralized at the pollen tube plasma membrane with coexpressed
cativative active mutant Nt Rac5 (unpublished results) and with
markers for PI 4,5-P2 or DAG when cell expansion was inhibited
by latrunculin B (cf. Figures 7B and 8C; see Supplemental Figure
3D online). This finding indicates that PI 4,5-P2, DAG, and active
Rac/Rop, which accumulate at the apex in normally elongating
pollen tubes, are not directly responsible for the inability of
PI-PLCs to accumulate at this location. As proposed by Dowd
et al. (2006), the high cytoplasmic Ca2+ concentration in the apex
may interfere with the ability of the PI-PLC C2 domain to bind to
membrane lipids. Alternatively, interactions with other proteins
laterally associated with the pollen tube plasma membrane (e.g.,
inactive Rac/Rop [Klahre et al., 2006] and Nt RhoGAP1 [Klahre
and Kost, 2006]) may influence the intracellular distribution of
pollen tube PI-PLCs.

Regulation of Nt PLC3 Activity

Overexpression of Nt PLC3 reduced the growth rate of tobacco
pollen tubes only moderately. Dowd et al. (2006) did not detect
any effects of Pet PLC1 overexpression on petunia pollen tubes,
possibly because of functional differences between Pet PLC3
and Nt PLC3 (see above) and/or because a statistical growth rate
analysis was not performed in their study. In any case, the results
of the analysis of PI-PLC overexpression in pollen tubes are
consistent with the observation that At PLC1 overexpression
barely affected abscisic acid signaling, a process that was highly
sensitive to reduced levels of At PLC1 activity (Sanchez and
Chua, 2001). In all of these cases, overexpression of plant PI-PLC
isoforms displaying typical PI 4,5-P2-hydrolyzing activity in vitro
had little effect on cellular processes known to depend on the
activity of these isoforms. This strongly suggests that PI-PLC
activity is strictly regulated in plant cells.

Although the control of Nt PLC3 activity appears to depend
largely on molecular mechanisms governing substrate availability
by determining the intracellular distributions of this protein and of PI-PLC\(3\), other regulatory factors also seem to be important. When tobacco pollen tube growth was stopped using latrunculin B, Nt PLC3 and PI-PLC\(3\) partially colocalized at the tip (cf. Figure 7B and Supplemental Figure 3D online). At least in this situation, Nt PLC3 appeared to be inactive despite the availability of substrate. Cytoplasmic Ca\(^{2+}\) concentrations may affect Nt PLC3 activity not only by controlling membrane association (see above) but also by directly regulating catalytic activity. However, the in vitro activity of recombinant Nt PLC3 did not vary greatly in the physiological range of Ca\(^{2+}\) concentrations (Figure 2A). Interestingly, Rho family small GTPases (Hodson et al., 1998) and regulators of these proteins (Hosoda and Emori, 1995) were shown to interact directly with and/or to affect the activity of animal PI-PLC-\(\delta\) isoforms. Conceivably, effects of Rac/Rop signaling on the catalytic activity of Nt PLC3 at the pollen tube tip may create additional crosstalk in the complex regulatory network that controls polar cell growth. Consistent with this hypothesis, we have found that recombinant Nt PLC3 and Nt Rac5 interact in vitro (unpublished results).

Functions of the PI-PLC Substrate PI-4,5-P\(_2\) in Tobacco Pollen Tubes

We previously proposed that PI-4,5-P\(_2\) may function as a Rac/Rop effector, which promotes the fusion of secretory vesicles with the plasma membrane at the tip of tobacco pollen tubes (Kost et al., 1999). In animal systems, it has been demonstrated that PI-4,5-P\(_2\) can stimulate membrane association and the activation of Rac/Rop homologs by destabilizing the interactions of these proteins with guanine nucleotide dissociation inhibitors (Fauré et al., 1999). Together with our recent observation that guanine nucleotide dissociation inhibitor–mediated recycling is required for the accumulation of active Nt Rac5 at the apex of tobacco pollen tubes (Klahre et al., 2006), this suggests that PI-4,5-P\(_2\) may act not only as an effector, but also as a positive regulator, of Nt Rac5. Stimulation of PI-4,5-P\(_2\) generation by Nt Rac5 (Kost et al., 1999), together with the promotion of Nt Rac5 activation by PI-4,5-P\(_2\), may create a positive feedback loop that helps polarizing Rac/Rop signaling and cell growth at the pollen tube tip.

Independently of whether PI-4,5-P\(_2\) acts as a Rac/Rop effector, promotes Rac/Rop activation, or has both of these functions, Nt PLC3 appears to play an important role in the maintenance of polarized Rac/Rop signaling and cell expansion by preventing the lateral spreading of this lipid in the plasma membrane at the tip of tobacco pollen tubes. Consistent with this hypothesis, treatment with U-73122 at concentrations that caused PI-4,5-P\(_2\) spreading depolarized the growth of germinating tobacco pollen tubes, an effect that is also caused by Rac/Rop overexpression (Klahre et al., 2006). Interestingly, U-73122 treatment caused moderate tip swelling, whereas Rac/Rop overexpression resulted in the formation of much larger vacuolated balloons. U-73122–induced PI-4,5-P\(_2\) spreading apparently depolarized pollen tube growth only to a limited extent, possibly because products of PI-PLC–mediated PI-4,5-P\(_2\) hydrolysis, which are not formed in the presence of U-73122, also have important functions in promoting cell expansion (see below).

Functions of the PI-PLC Products DAG and IP\(_3\) in Tobacco Pollen Tubes

Although the accumulation of the PI-PLC product DAG at the pollen tube tip strongly suggests an important role of this lipid in the control of cell expansion, the exact nature of this role remains to be determined. The regulatory function of DAG in animal cells largely depends on the stimulation of protein kinase C homologs (Yang and Kazanietz, 2003). Because sequences encoding such homologs are not present in completely sequenced plant genomes (Meijer and Munnik, 2003), the identification of other proteins interacting with DAG will be important to understand how this lipid influences pollen tube growth.

Interestingly, stimulation of PI-PLC–dependent signaling did not result in DAG accumulation in different types of plant cells investigated by Meijer and Munnik (2003). Therefore, it was speculated that DAG may not have signaling functions in plant cells but may generally be rapidly phosphorylated by DAG kinases to phosphatidic acid, a membrane lipid generated by different metabolic pathways (e.g., by phospholipase D activity) that seems to regulate cellular processes in various cell types (Wang, 2005), including pollen tubes (Potocky et al., 2003; Monteiro et al., 2005). As discussed above, our data indicate that at least in pollen tubes, as in animal cells, DAG does accumulate and is likely to act as a signaling molecule. A possible additional role of DAG at the pollen tube tip as a phosphatidic acid precursor, which would establish a link between PI-PLC and phosphatidic acid signaling, remains to be demonstrated, as our attempts to visualize phosphatidic acid accumulation in living tobacco pollen tubes using an established in vivo marker (Hs Raf1AA390–426:YFP; Rizzo et al., 2000) were not successful.

IP\(_3\), the soluble second product of PI-PLC activity, opens channels to release Ca\(^{2+}\) from internal stores into the cytoplasm and plays a key role in Ca\(^{2+}\) signaling in animal cells (Clapham, 1995). Therefore, it is tempting to speculate (Kost et al., 1999) that IP\(_3\) generated by Nt PLC3–mediated PI-4,5-P\(_2\) hydrolysis at the flanks of the pollen tube tip may have a function in the establishment of the tip-focused cytoplasmic Ca\(^{2+}\) gradient essential for the growth of these cells. This idea is consistent with the observation that photoactivation of caged IP\(_3\) can increase the cytoplasmic Ca\(^{2+}\) concentration in pollen tubes (Franklin-Tong et al., 1996; Monteiro et al., 2005). However, substantial evidence suggests that IP\(_3\)-independent Ca\(^{2+}\) influx across the plasma membrane may maintain the Ca\(^{2+}\) gradient at the tip of these cells (Hepler et al., 2001), and IP\(_3\)-sensitive Ca\(^{2+}\) channels have not been identified in plants to date (Meijer and Munnik, 2003). To establish a direct link between PI-PLC activity and cytoplasmic Ca\(^{2+}\) signaling in pollen tubes, it will be necessary to analyze the effects of U-73122 treatment on cytoplasmic Ca\(^{2+}\) levels.

METHODS

cDNA Isolation by Colony Hybridization

A pollen tube cDNA library (Klahre et al., 2006) representing genes expressed in cultured tobacco (Nicotiana tabacum) pollen tubes after 3 h was screened for sequences encoding PI-PLC homologs by colony hybridization as described by Klahre et al. (2006). Colony lifts were probed with a PCR fragment amplified from the same pollen tube library using two
RNA gel blot analysis was performed as described by Klahre et al. (2006) using a digoxigenin-labeled 745-bp PCR fragment corresponding to the catalytic core of Nt PLC3 (nucleotides 386 to 1131) as a probe.

Recombinant DNA Construction

Plasmids, materials, and procedures described by Klahre et al. (2006) were used to generate mutations in the Nt PLC3 coding sequence and to introduce cDNAs encoding wild-type, mutant, or truncated forms of Nt PLC3 into the multiple cloning sites of expression vectors suitable (1) for transient expression of these proteins in tobacco pollen tubes under the control of the Lat52 promoter (Twell et al., 1991), either free or attached via a 5'-Gly-Ala linker to the N or C terminus of YFP, or (2) to express them as GST fusion proteins in *Escherichia coli* for purification. Plasmids used to transiently express GUS or YFP in pollen tubes were described previously (Klahre et al., 2006).

cDNA fragments encoding the first Cys-rich domain (Cys1; amino acids 26 to 89) of mammalian protein kinase Cγ (Oancea et al., 1998), the PH domain (amino acids 2 to 175) of human PLCδ1 (Stauffer et al., 1997), or two FYYE domains (amino acids 147 to 223) of mouse Hrs linked by QGQGS (Gillooly et al., 2000) were introduced into the pollen tube expression vectors described above either at the N terminus (Cys1) or the C terminus (PH, FYVE) of YFP to generate plasmids that allow the expression of Cys1:YFP, YFP:PH, and YFP:FYVE fusion proteins in pollen tubes.

The YFP sequences in the Nt PLC3:YFP, Cys1:YFP, and YFP:FYVE pollen tube expression vectors were replaced by POR-amplified mRFP1 cDNA fragments (Campbell et al., 2002) to generate constructs encoding corresponding RFP fusion proteins.

In Vitro Activity Assays

Standard procedures (Sambrook and Russell, 2001) were used to purify recombinant wild-type and mutant Nt PLC3 fused to the C terminus of GST from *E. coli* BL-21 and to assess protein quality/amount based on SDS-PAGE, Coomassie Brilliant Blue R 250 staining, and BSA reference solutions. PI-PLC activity of purified GST fusion proteins was determined as described by Melin et al. (1992) with some modifications. Reaction mixtures (50 μL) contained 100 ng of recombinant Nt PLC3 protein, free Ca<sup>2+</sup> (Owen, 1976) at the indicated concentration, 50 mM Tris-maleate at pH 6.0, 0.02% (w/v) sodium deoxycholate, 0.2 mM cold PI 4,5-P<sub>2</sub> (Fluka), and 0.74 kBq of [3H]PI 4,5-P<sub>2</sub> (240.5 GBq/mmol, head group labeled; Hartmann Analytic). To prepare micellar lipid substrate solutions, cold and labeled PI 4,5-P<sub>2</sub> were dried from solvent (chloroform) under a stream of nitrogen, followed by 10 min of sonication in a solution containing 0.1% (w/v) sodium deoxycholate and 50 mM Tris-Cl at pH 6.0. Reactions were started by the addition of micellar substrate solutions to reaction mixtures and stopped after 20 min at 25°C. After phase separation, 450 μL of the aqueous phase was mixed with 3 mL of scintillation cocktail (Optiphase HiSafe III; Perkin-Elmer) and analyzed in a liquid scintillation counter. Background radioactivity detected in control samples incubated in the absence of protein was subtracted from all data. To perform inhibitor experiments, required amounts of 2 mM U-73122 (Calbiochem) in chloroform were dried from solvent by evaporation and redissolved in substrate-free reaction mixtures. Reactions were started after a 15-min preincubation period by the addition of micellar substrate solutions.

Plant Material and Cell Culture

Mature pollen was collected immediately before use from tobacco (cv Petit Havana SR1) plants growing in a greenhouse under standard conditions and germinated in culture as described previously (Read et al., 1993; Kost et al., 1998).

Transient Gene Expression

Expression vectors were transferred into tobacco pollen grains germinating on solid culture medium using a gene gun (PDS-1000/He; Bio-Rad) as described previously (Kost et al., 1998). Particles were coated with 2 μg of each expression vector (circular, 4.3 to 6.5 kb) to be introduced into cells.

Microscopy and Image Analysis

Transiently transformed pollen tubes were transfected onto cover slips for microscopic analysis as described previously (Kost et al., 1998). Fluorescence images were acquired using a confocal laser scanning microscope (LSM510 Meta; Zeiss). Low-magnification fluorescence images (Figure 3) and high-resolution confocal sections were recorded through 5× (Plan-Neofluar 5×/0.15, dry; Zeiss) and 63× (C-Apochromat 63×/1.2, water immersion; Zeiss) lenses, respectively. A 514-nm laser line and a 530- to 600-nm band-pass emission filter, or a 543-nm laser line and a 560-nm long-pass emission filter, were used for YFP or mRFP1 imaging, respectively. YFP and mRFP1 coimaging was performed in the multitracking mode. To measure growth rates, the distance traveled by the apex of individual pollen tubes during at least 2 min was determined using sequentially recorded high-magnification (63×) images and the LSM510 software. An inverted microscope (DM IR; Leica) equipped with differential interference contrast optics was used to acquire transmission light micrographs of cultured pollen tubes. Pollen tubes mounted between two cover slips were imaged through 5× (N PLAN 5×/0.12, dry; Leica) or 40× (HCX PL FL L 40×/0.6, dry; Leica) lenses using a digital camera (DFC350FX R2; Leica). Pollen tube length after 6 h of culture was determined on digital low-magnification (5×) images using image-analysis freeware (ImageJ; http://rsb.info.nih.gov/ij/). Fluorescence and transmitted light images were contrast-enhanced by adjusting brightness and γ settings using image-processing software (Photoshop; Adobe Systems).

Drug and FM4-64 Application to Cultured Pollen Tubes

Stock solutions of U-73122 (1 mM in DMSO; Calbiochem), U-73343 (1 mM in DMSO; Calbiochem), latrunculin B (5 mM in DMSO; Calbiochem), and FM4-64 (1 mM in water; Molecular Probes) were stored in aliquots at −20°C and applied to pollen tubes diluted at least 100 times in culture medium. To analyze the effects of drug treatments on pollen tube growth (Figure 6), pollen collected from different flowers (two anthers per sample) was pooled and evenly distributed to 200 μL of liquid medium containing drugs at the indicated concentrations in individual wells of 24-well plates. Pollen cultures were incubated in the dark at room temperature (25°C) for the indicated time periods before analysis.

Drugs were applied to transiently transformed pollen tubes growing on the surface of solid medium (Figures 7A to 7C and 8A to 8C) by adding an equal volume of liquid medium containing drugs at twice the final concentration to culture dishes. Alternatively, a droplet (~20 μL) of culture medium containing drugs at 10 times the effective concentration was applied onto thin layers of solid culture medium (~1×1×0.2 cm) covering pollen tubes on the surface of cover slips directly on the microscope stage to observe drug effects on individual pollen tubes (Figures 7D and 8D). FM4-64 labeling of internal membranes was obtained by distributing 500 μL of culture medium containing the dye at a concentration of 7.5 μM on the surface of 3 mL of solid culture medium covered with growing pollen tubes (6 to 8 h after plating), followed by at least 20 min of incubation in the dark at room temperature.

Whereas latrunculin B completely inhibited the growth of germinating tobacco pollen tubes in liquid medium at a concentration of 10 nM, 50 nM
was required to stop pollen tube growth on solid medium several hours after germination.

**Accession Number**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EF043044.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Nt PLC3 Expression Pattern (RNA Gel Blot Analysis: 3’ Untranslated Region Probe).

**Supplemental Figure 2.** Phylogenetic Analysis of Pollen and Arabidopsis PI-PLCs.

**Supplemental Figure 3.** Intracellular Localization of Inactive Nt PLC3 Mutants, and of Wild-Type Nt PLC3, after Blocking Pollen Tube Growth with Latrunculin B.

**Supplemental Figure 4.** Normal PI 4,5-P2 and DAG Distributions in Pollen Tubes Treated with U-73122 at a Low Concentration, or with an Inactive Analog of This Drug (U-73343).

**Supplemental Figure 5.** Colocalization of Nt PLC3 and DAG in Growing Pollen Tubes.

**Supplemental Figure 6.** DAG Colocalizes with FM4-64 to Brefeldin A (BFA)–Induced Compartments.

**Supplemental Figure 7.** First, Middle, and Last Frames of Supplemental Movie 1.

**Supplemental Figure 8.** First, Middle, and Last Frames of Supplemental Movie 2.

**Supplemental Figure 9.** First, Middle, and Last Frames of Supplemental Movie 3.

**Supplemental Movie 1.** Dynamic Imaging of Nt PLC3 Localization.

**Supplemental Movie 2.** Dynamic Imaging of PI 4,5-P2 Localization.

**Supplemental Movie 3.** Dynamic Imaging of DAG Localization.

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Activity and Endocytic Membrane Recycling

Pollen Tube Tip Growth Depends on Plasma Membrane Polarization Mediated by Tobacco PLC3

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