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**DIACYLGlycerol Kinase 5 regulates polar tip growth of tobacco pollen tubes**

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**Summary**

- Pollen tubes require a tightly regulated pectin secretion machinery to sustain the cell wall plasticity required for polar tip growth. Involved in this regulation at the apical plasma membrane are proteins and signaling molecules, including phosphoinositides and phosphatidic acid (PA). However, the contribution of diacylglycerol kinases (DGKs) is not clear.
- We transiently expressed tobacco DGKs in pollen tubes to identify a plasma membrane (PM)-localized isomorph, and then to study its effect on pollen tube growth, pectin secretion and lipid signaling. In order to potentially downregulate DGK5 function, we overexpressed an inactive variant.
- Only one of eight DGKs displayed a confined localization at the apical PM. We could demonstrate its enzymatic activity and that a kinase-dead variant was inactive. Overexpression of either variant led to differential perturbations including misregulation of pectin secretion. One mode of regulation could be that DGK5-formed PA regulates phosphatidylinositol 4-phosphate 5-kinases, as overexpression of the inactive DGK5 variant not only led to a reduction of PA but also of phosphatidylinositol 4,5-bisphosphate levels and suppressed related growth phenotypes.
- We conclude that DGK5 is an additional player of polar tip growth that regulates pectin secretion probably in a common pathway with PI4P 5-kinases.

**Introduction**

In flowering plants, male sperm cells are nonmotile and depend on a vegetative cell to form a pollen tube for delivery to the female embryophyte (Johnson *et al*., 2019). Growth of pollen tubes occurs by unidirectional tip expansion, whose mechanical demands are reflected in the pollen tube’s cell wall composition (Fayant *et al*., 2010; Grebnev *et al*., 2017). The cell wall at the shank of the pollen tube provides a rigid scaffold for the pollen tube preventing lateral extension while the higher flexibility at the pollen tube tip enables turgor-driven expansion (Fayant *et al*., 2010; Chebli *et al*., 2012). This cell wall at the tip is composed mainly of pectin that is continuously secreted together with modifying enzymes during pollen tube growth (Bosch *et al*., 2005; Röckel *et al*., 2008; Chebli *et al*., 2012). Secretion of pectin has to be tightly controlled: if the cell wall is too rigid, the turgor will not be enough to drive further expansion and tube growth will be aborted (Bosch *et al*., 2005; Zerzour *et al*., 2009). On the other hand, lack of secreted pectin will lead to thinning of the tip cell wall and finally bursting of pollen tubes (Zerzour *et al*., 2009; Kroeger *et al*., 2011). Consequently, pectin deposition is subject to a plethora of signaling factors including phosphoinositides and derived lipids (Heilmann & Ischebeck, 2016; Scholz *et al*., 2020).

So far, the strongest connection between pectin secretion and signaling phospholipids has been described for phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2; Ischebeck *et al*., 2008), which is enriched at the apical plasma membrane (PM) of pollen tubes (Kost *et al*., 1999). PI(4,5)P2 is formed by phosphatidylinositol 4-phosphate 5-kinases (PIP5Ks) that catalyze the phosphorylation of phosphatidylinositol 4-phosphate. Interference with their normal enzyme function in Arabidopsis has been reported to impair tube growth (Ischebeck *et al*., 2008, 2011; Sousa *et al*., 2008; Zhao *et al*., 2010). Overexpression of *AtPIP5K4* or *AtPIP5K5* in tobacco (*Nicotiana tabacum*) pollen tubes leads to enhanced pectin accumulation at the tip (Ischebeck *et al*., 2008).

Decreased activity of phosphoinositide-specific phospholipase C (PLC) can also result in increased PI(4,5)P2 levels and defects...
in pollen tube growth (Dowd et al., 2006). Overexpression of an inactive variant of petunia PLC1 in petunia pollen tubes caused the PI(4,5)P2 localization to be more spread out rather than tip-localized. Furthermore, this overexpression led to pollen tube growth arrest (Dowd et al., 2006). This indicates that PLC activity is a major route to degrade PI(4,5)P2 and to switch off downstream signaling. However, the lipid product of PLC hydrolysis, diacylglycerol (DAG), might in turn serve as substrate for another signaling phospholipid, phosphatidic acid (PA; Munnik, 2001; Testerink & Munnik, 2005, 2011).

In animals, DAG is a signaling lipid in its own right, modulating members of the protein kinase C family (Nishizuka, 1988). By contrast, a signaling function of DAG in plant cells is still under debate, especially since homologs of protein kinase C and other typical DAG targets are missing from plants (Munnik & Testerink, 2009; Vermeer et al., 2017). Instead, PA has been implicated in many signaling pathways, especially in the reaction to abiotic and biotic stresses, where it is rapidly synthesized within seconds/minutes (Munnik, 2001; Testerink & Munnik, 2005, 2011; Yao & Xue, 2018; Kim & Wang, 2020; Noack & Jaillais, 2020). In tobacco pollen tubes, PA was localized to the subapical PM, partially overlapping with PI(4,5)P2 and DAG biosensors (Potocký et al., 2014).

The reaction to form PA from DAG is a critical step to generate signaling PA, and is catalyzed by diacylglycerol kinases (DGKs) (Wissing et al., 1989; Arisz et al., 2009). There is another pathway that generates signaling PA, which is by cleavage of structural phospholipids by phospholipase D (PLD) (Hanahan & Chaikoff, 1947). The functions of PLD and PLD-derived PA have been more studied than the physiological role of DGKs (Hong et al., 2016; Li & Wang, 2019). Nevertheless, different studies indicate a role for the PLC/DGK pathway in plant stress responses (Arisz et al., 2009, 2013). In suspension-cultured plant cells, the majority of initial PA synthesis in response to cold shock and cryptogein elicitation was traced back to PLC/DGK activities in Arabidopsis (Ruelland et al., 2002) and tobacco, respectively (Cacas et al., 2017). The same was true for cold-treated Arabidopsis seedlings (Arisz et al., 2013). Arabidopsis contains seven DGKs (Arisz et al., 2009) of which AtDGK2 was induced upon cold stress and winding (Gómez-Merino et al., 2004). Single Arabidopsis DGK-knockout (KO) mutants (i.e. dgk2, dgk3 and dgk5) were described to be more freezing-tolerant (Tan et al., 2018). Despite these studies on DGKs in stress signaling, their potential role in plant development has just recently come into focus.

The involvement of PA in pollen tube growth has been studied, albeit produced by PLD activity (Potocký et al., 2003, 2014), and connected to the organization of the actin cytoskeleton (Pleskot et al., 2010, 2012, 2013). There are limited reports on the impact of pollen tube-expressed AtDGK4 (Honys & Twell, 2004; Arisz et al., 2009) on pollen tube growth. Some studies reported impaired pollen fitness and tube growth for dgk4 (Vaz Dias et al., 2019; Wong et al., 2020), while another found reduced germination rates and effects on pollen development and pollen tube growth if DGK2 was also affected (Angkawijaya et al., 2020). Interestingly, the localization of AtDGK4 in pollen tubes was reported to be cytosolic (Vaz Dias et al., 2019), despite the fact that both substrate (DAG) and product (PA) are membrane-localized. However, in another study, localization was described to be at the endoplasmic reticulum (ER) in transiently transformed Nicotiana benthamiana leaves and Arabidopsis protoplasts (Angkawijaya et al., 2020). DGKs localized at the PM of pollen tubes remain to be identified.

Materials and Methods

Details of methods for phylogenetic and gene expression analysis, molecular dynamics simulation, molecular cloning, immunodetection, details of fluorescence recovery after photobleaching (FRAP) experiments and quantitative analysis of micrographs are provided in the Supporting Information (Methods S1–S7).

Heterologous protein expression and purification

pGEX-6P-1 constructs were transformed and proteins expressed as GST-tag fusions in Escherichia coli BL21 star (DE3) cells (Thermo Fisher Scientific, Waltham, MA, USA) at 16°C for 3 d after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside. The original pGEX-6P-1 vector was expressed and used as a control. Cell pellets of 500 ml cultures were resuspended in lysis buffer (1 × phosphate-buffered saline (PBS), pH 7.4, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and cells were disrupted by ultrasonication. Protein purification was performed by GST-affinity chromatography with GSTrapTM Fast Flow columns (Cytiva, Chalfont St Giles, UK), using 1 × PBS pH 7.4, 5 mM EDTA for washing and 50 mM Tris-HCl pH 8.0, 20 mM reduced glutathione for elution. Purified proteins were stored at −80°C in storage buffer (50 mM Tris-HCl pH 7.4, 100 mM NaCl, 10 mM MgCl2, 10% glycerol) until further use for enzyme activity assays and the protein-lipid overlay assays.

Analysis of DGK activity

Diacylglycerol kinase activity was assayed by measuring the incorporation of 32P-phosphate from [γ-32P]-ATP into DAG to produce PA. For enzyme assays, 50 ng of protein was mixed with 200 µl of assay buffer with or without 500 µM 1,2-dioleyl-sn-glycerol (DOG) as substrate, prepared in liposomes (Julkowska et al., 2013). Liposomes contained DOG, 1,2-dioleyl-phosphatidylcholine (DOPC) and 1,2-dioleyl-phosphatidylethanolamine (DOPE; all Sigma-Aldrich) in a molar ratio of 1:2:2 or DOPE and DOPC in a molar ratio of 3:2 without DAG. Liposome suspensions were pelleted by centrifugation at 100 000 g for 35 min at room temperature and the liposome pellet was subsequently resuspended in assay buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na2VO3, 500 µM ATP containing c. 2.5 µCi [γ-32P]-ATP). Reactions were started by the addition of protein, and incubated for 30 min at 25°C while gently shaking. Reactions were stopped by adding 750 µl chloroform : methanol (1 : 2, v/v) and the lipids extracted as described previously (Munnik & Laxalt, 2013; Munnik & Zarza, 2013). In brief, two phases were established by adding 750 µl chloroform and 200 µl 0.9% NaCl.
The organic lower phase was washed once with chloroform: methanol: 1 M HCl (3:48:47, v/v), dried under a stream of nitrogen, dissolved in chloroform and spotted on a thin layer chromatography (TLC) plate (Silica gel 60; Merck KGaA, Darmstadt, Germany). TLC plates were developed using a running system of chloroform: methanol: NH₄OH: water (45:45:4:11, v/v). A photostimulable phosphor plate (Fujiﬁlm, Tokyo, Japan) was exposed to the TLC plate for 3 d. The nonradioactive standard PA was detected by iodine staining. Phosphoimager screens were read using the FLA 3000 scanner (Fujiﬁlm) with BASREADER 3.14 software (Elysiay-raytest, Straubenhardt, Germany) and quantiﬁed with AIDA IMAGE data analyzer 3.24 (Elysiay-raytest).

Lipid-binding assays

GST-NtDGK5 and GST-NtDGK5G118A were used to establish protein–lipid interactions on membrane lipid strips (Echelon Biosciences, Salt Lake City, UT, USA) by a protein–lipid overlay assay. Briefly, lipid strips were incubated for 1 h with 2–2.5 µg mL⁻¹ of GST-tagged protein in a solution of 4% BSA in 1× PBS. Subsequently, successful protein–lipid interactions were analysed by immunodetection of the GST-tag.

Lipid overlay assays with in vitro translated protein, the TNT² SP6 High-Yield Wheat Germ Protein Expression System (Promega) was used according to the manufacturer’s instructions. Respective proteins of NtDGK5, NtDGK5G118A, NtDGK6 and NtDGK8 carried an N-terminal HA-tag for later detection. To remove membrane-bound proteins of the in vitro system, samples were centrifuged at 72 000 g and 4°C for 20 min and the supernatant was incubated with membrane lipid strips (Echelon Biosciences) preblocked for 30 min with 4% BSA in 1× PBS.

The vesicle cosedimentation assay was adapted from Julkowska et al. (2013). Control liposomes were prepared from 80% DOPC and 20% DOPE, and PA-containing liposomes consisted of 60% DOPC, 20% DOPE and 20% DOPA. Each sample contained 400 nmol of total lipids. Liposomes were prepared as described in Julkowska et al. (2013) and diluted in modiﬁed 1× binding buffer (400 mM KCl, 25 mM Tris-HCl pH 7.5, 1 mM DTT, 0.5 mM EDTA). Subsequently, liposomes were incubated with the supernatant of in vitro protein reactions, prepared as described above. Protein was incubated with liposomes at 45 min at room temperature. Liposomes and bound protein were then pelleted by centrifugation at 72 000 g for 30 min. Total nonbound protein in the supernatant fraction was precipitated by acetone precipitation; the pellet of liposomes and liposome-interacting proteins was washed once with 1× binding buffer. Finally, both protein fractions were dissolved in Laemmli sample buffer and used for subsequent Western blot analysis (Methods S6).

Plant lines, plant growth conditions and Arabidopsis in vivo pollen tube assay

Arabidopsis and tobacco plants were grown as previously described (Rotsch et al., 2017; Müller & Ischebeck, 2018). The Arabidopsis plant lines named here as dkg4, dkg6 and ms1 have already been described (Wilson et al., 2001; Angkawijaya et al., 2020). Plant line dkg5 was obtained as SAIL_1212-E10 from the SAIL collection (Sessions et al., 2002) and the double mutant dkg5 dkg6 was generated by crossing.

In vivo pollen tube assays of Arabidopsis dkg mutant lines were carried out after Mori et al. (2006) and Müller & Ischebeck (2018); however, instead of wild-type pistils, pistils of ms1 plants were pollinated with pollen of dkg strains.

Pollen transformation

Pollen grains were transformed by particle bombardment and pollen tubes were grown as described by Müller et al. (2017) or based on previous protocols (Read et al., 1993; Kost et al., 1998). FRAP analysis and spinning disk imaging was performed 6–8 h after transformation, and microscopic imaging for phenotype and cell wall analysis was carried out 14 h after transformation. For pectin staining of pollen tube cell walls, 10 µM propidium iodide was added to the pollen tubes just before imaging.

For cotransformation, plasmids were mixed during gold precipitation before particle bombardment. For cotransformation with Atp5P5K5 or markers for endomembrane compartments, plasmids were mixed at a ratio of 1:1; for cotransformation of lipid-binding domains, NtDGK5 and NtDGK5G118A were used at a 10:1 ratio and the ﬂuorescent tag control at a ratio of 3:1.

Epifluorescence and confocal microscopy

Epifluorescence microscopy of transformed tobacco pollen tubes was done with an Olympus BX51 by using an UPlanSapo ×10/0.40 objective (Olympus, Tokyo, Japan). For mVenus-tagged constructs, the ﬁlter cube U-MWIB was used. Images were taken with an ORCA flash 4.0 V2 Digital Camera using the software HOKAWO 2.10 (Hamamatsu Photonics, Hamamatsu, Japan).

Confocal imaging of pectin stained by propidium iodide was performed with a Zeiss LSM510 confocal microscope using a Plan-Neofluar ×40/1.30 oil immersion objective (Carl Zeiss, Oberkochen, Germany). Fluorescence of propidium iodide was observed after excitation with 561 nm and detected at 689–721 nm using the HFT 405/488/561 major beam splitter.

Fluorescence recovery after photobleaching analyses were carried out with a Zeiss LSM880 equipped with C-Apochromat ×40/1.2 water immersion objective (Carl Zeiss). For mVenus imaging, optimal singletrack acquisition parameters were used (mVenus ﬂuorescence was excited by a 514 nm laser, and the emission at 520–590 nm was recorded using a GaAsP detector).

Spinning disk confocal microscopy was carried out on the Nikon Ti-E platform with Yokogawa CSU-X1 spinning disk and sCMOS camera Andor Zyla, using a Plan Apo VC ×60/1.20 water immersion objective (Nikon, Tokyo, Japan). Fluorescence of mVenus/yellow ﬂuorescence protein (YFP) and mCherry/red ﬂuorescence protein (RFP)-tagged proteins were excited with the respective 488 and 561 nm laser lines (laser box Agilent MLC400, Agilent Technologies, Inc., Santa Clara, CA, USA) and detected with the ﬁlter cubes Semrock brightline Em 542/27 and Semrock brightline Em 607/36.

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Results

DGKs diverged into three conserved clades during early plant evolution

To describe the DGK family in tobacco, we searched the tobacco proteome and genome drafts available at NCBI and solgenomics.net, using BLAST searches with Arabidopsis DGK1, DGK4 and DGK5 as queries. Additionally, the draft genomes of *Nicotiana sylvestris* and *Nicotiana tomentosiformis* were analyzed. We found 16 genes coding for putative DGKs in the tobacco genome (Figs 1a, S1). These could be further divided into eight pairs of almost identical paralogs, reflecting that *N. tabacum* originated as a hybrid of *N. sylvestris* and *N. tomentosiformis*. To classify tobacco DGKs unambiguously and better understand the evolution of plant DGK genes, we significantly expanded our initial analysis (Pleskot *et al*., 2012) and performed an exhaustive genome-wide search and reconstruction of DGK phylogeny in Viridiplantae. We analyzed genomes of representative angiosperm, gymnosperm, lycophyte, bryophyte, charophyte and chlorophyte species. This yielded a dataset of 95 DGK isoforms from 20 species (Table S1; Notes S1). Phylogenetic analysis of plant DGKs performed with two independent algorithms showed a deep evolutionary split dividing DGK isoforms into three distinct clades (Fig. S1). All clade I genes harbor a transmembrane domain followed by two C1 domains, implicated in lipid binding (Coliongonzalez & Kazanietz, 2006), in addition to DGK catalytic and accessory domains, which constitute clades II and III (Fig. 1b; Table S2). In tobacco, six *NtDGKs* are grouped in clade I, four in clade II and six in clade III (Fig. 1a,b; Table S2). Clade I can be subdivided into two subclades (containing *NtDGK1-2* and *NtDGK3*, respectively) that probably split in early angiosperms. On the other hand, multiple DGK paralogs in clades II and III probably originated through recent gene or genome duplication events, and no apparent orthology could be assigned beyond Solanaceae (Fig. S1).

To estimate whether there is a trend in the expression pattern of DGK isoforms in the three clades, we first analyzed tobacco DGK expression in pollen by remapping tobacco pollen raw RNA sequencing (RNA-seq) data (Conze *et al*., 2017) on the reference genome sequence. Publicly available pollen, leaf and root RNA-seq data from selected angiosperm species were also collected and mapped onto the phylogenetic tree (see Methods S3 for details). Our data suggest that a similar expression pattern is retained within the two subclades of clade I, with one subclade exhibiting lower expression in the pollen compared to the sporophytic tissues, while the opposite pattern could be seen for the second subclade. Conversely, no trend is apparent for clade II genes (Fig. S1).

![Fig. 1](image-url) Tobacco diacylglycerol kinase isoforms show distinct localization patterns in pollen tubes. (a) Phylogeny of Arabidopsis and *Nicotiana tabacum* diacylglycerol kinase (DGK) isoforms and their distribution into three clades as extracted from a full phylogenetic tree (see Supporting Information Fig. S1). Syl-like and tom-like denote *NtDGK* sequences derived from ancestors of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*, respectively. An asterisk denotes isoforms cloned in this study. *At*, *Arabidopsis thaliana*; *Nt*, *Nicotiana tabacum*. (b) Schematic representation of the domain structure of tobacco isoforms. The green rectangle denotes the transmembrane domain. C1, diacylglycerol/phorbol esters binding domain; DAGKa, DGK accessory domain; DAGKc, DGK catalytic domain. (c) Localization of YFP-tagged *NtDGK*–8 in actively growing pollen tubes. Pollen was transiently transformed with 1 μg DNA and analyzed 6–8 h after transformation. Bar, 10 μm.
NtDGK5 specifically localized to the subapical PM of growing pollen tubes

Phosphatidic acid is an important second messenger and enriched in the subapical PM of tobacco pollen tubes (Potocký et al., 2014; Pejchar et al., 2020). To identify which of the DGK isoforms are localized at the PM and could generate PA near the pollen tube tip, we cloned eight DGK coding sequences (one of each pair, Fig. 1a; for the list of primers see Table S3) and fused them C- or N-terminally with YFP. As the isoforms DGK1–3 are predicted to have a transmembrane region close to the N-terminus with the very N-terminus facing the noncytosolic side, we chose a C-terminal tag for these isoforms.

When transiently expressed in growing tobacco pollen tubes, DGK isoforms 1–3 from clade I did not localize to the PM but rather to endomembrane compartments (Fig. 1c). To further analyze the localization, pollen tubes were cotransformed with NtDGK1-3:YFP and the ER marker AtbCH-SP:CFP:HDEL; NtDGK1:YFP was also cotransformed with the Golgi marker CFP:StGnT1.70 and CFP:ArVHA-a1 (Stephan et al., 2014) as a marker for the trans-Golgi network (TGN). Colocalization was observed only with the ER marker (Fig. S2). Clade II isoforms 4 and 7 displayed strikingly different localization, with NtDGK4 being purely cytosolic and NtDGK7 localizing homogeneously along the PM with no obvious enrichment. Among clade III isoforms, NtDGK6 was present in the cytoplasm and NtDGK8 was localized to an extended PM region. Finally, the localization of NtDGK5 was restricted to the PM region close to the pollen tube apex (Figs 1c, S3a,b).

Next, we tested whether observed localization differences within clade III isoforms were correlated with structural features and membrane-binding properties. Therefore, we constructed 3D homology models of NtDGK5, NtDGK6 and NtDGK8 and we mapped the electrostatic potential onto the solvent-excluded surface of the respective NtDGK protein (Fig. S3c–e; Methods S2).

As the plant PM is highly negatively charged, the presence of positively charged regions (caused by cationic amino acids: Lys, Arg and His) on the protein surface could be seen as a proxy for the electrostatic interaction and its strength. Indeed, we observed clear differences in the positive charge distribution among the different NtDGK isoforms of clade III correlating well with their localization in tobacco pollen tubes (Fig. S3). Furthermore, we tested the binding properties of clade III isoforms to membrane lipids by protein–lipid overlay assays. In line with previous results, NtDGK6 showed only limited binding to the plant PM lipids. By contrast, NtDGK5 and NtDGK8 displayed a similar tendency to bind to anionic phospholipids such as PA, PI4P and PI(4,5)P2 (Fig. S4).

Molecular dynamics simulations predict a direct interaction between NtDGK5 and PM

As the membrane-bound DGK isoforms of clade III have no prominent membrane-binding domain, we wondered how these members could interact with the PM. To analyze this, coarse-grained molecular dynamics (CG-MD) simulations were used. This computational approach is highly accurate in describing the membrane-bound state of peripheral membrane proteins (Yamamoto et al., 2020). The simulated system consisted of an NtDGK5 molecule, ions, water and a complex lipid bilayer with negatively charged phospholipids (Fig. 2a), similar to a PM of plants (Im et al., 2007; König et al., 2008; Furt et al., 2010). We performed 10 1 μs runs of CG-MD simulations. In all replicas, we observed that NtDGK5 quickly interacted with the lipid bilayer, and remained stably bound to the membrane through the remaining simulation time (Figs 2b, S5a). Detailed inspection of different CG-MD replicas revealed distinct membrane-binding modes of NtDGK5. To further analyze and categorize these binding modes, we monitored the distance between conserved glycine G118 located in the catalytic domain, which is

![Fig. 2](image-url) Molecular dynamics simulation indicates mechanistic details of the NtDGK5–membrane interaction. (a) Simulated system composed of NtDGK5, the complex lipid bilayer, ions and water molecules. (b) Snapshots from the coarse-grained molecular dynamics simulation. Left, initial configuration (time = 0 ns). Right, the membrane-bound protein (time = 1000 ns). (c) NtDGK5 together with negatively charged lipids 0.8 nm from the protein. Both catalytic and accessory domains contribute to binding. The protein is displayed in the ribbon representation with its transparent solvent-excluded surface. Negatively charged phospholipids are shown in the van der Waals representation, PA in yellow, PI4P in orange and PI(4,5)P2 in purple. DAGKa, DGK accessory domain; DAGKc, DGK catalytic domain; PA, phosphatidic acid; PI4P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate.

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NtDGK5 is a DAG kinase whose activity is disrupted by a single G118A amino acid exchange

While different Arabidopsis DAG kinases have been shown to harbor DAG-kinase activity (Gómez-Merino et al., 2004, 2005), NtDGK5 was previously not biochemically investigated. To confirm its putative DGK activity, we expressed and purified GST-tagged NtDGK5 in E. coli. Similarly, we generated a potentially kinase-dead mutant by replacing the conserved glycine 118 in the catalytic domain with an alanine (G118A). The methyl group introduced by the mutation probably interferes with ADP/ATP binding: the structural model NtDGK5 and NtDGK5G118A with a superimposed ADP molecule suggested a sterical clash between A118 in NtDGK5G118A and the γ-phosphate group of ADP (Fig. S6). We therefore hypothesized that NtDGK5G118A will be unable to complete the phosphorylation reaction. This variant might then act in a dominant negative manner in vivo, based on observations from a mammalian homolog carrying the corresponding substitution (Sanjuán et al., 2001).

Purified GST-NtDGK5, GST-NtDGK5G118A or GST-tag alone were subsequently used for enzyme activity assays, providing DAG and [γ-32P]-ATP as substrates (Fig. 3a). Following the reaction, lipids were extracted and separated by TLC, and radioactive PA was quantified by phosphoimaging (Figs 3b, S7). Enzyme reactions with NtDGK5 led to a 30-fold increase of PA levels compared to reactions with GST only, while the NtDGK5G118A lost ca. 95% of its activity, exhibiting only a twofold increase compared to GST. These results indicate that NtDGK5 functions as a DAG kinase, and that this activity is almost completely disrupted in the mutated variant.

PM localization of NtDGK5 and NtDGK5G118A differs

Next, we investigated if the PM association of NtDGK5 was affected by the amino acid exchange. Therefore, the localization of N-terminal fusions of mVenus to NtDGK5 and NtDGK5G118A was compared. Interestingly, when overexpressed in tobacco pollen tubes, the mutated variant showed a more pronounced PM localization compared to wild-type NtDGK5 based on quantification of the fluorescent signal length measured from the pollen tube tip (Fig. 4a–c). For further analysis, we calculated the PM index (PMI), the ratio between the fluorescence intensities at the PM and in the cytoplasm (Fig. 4d,e). This ratio decreased for both NtDGK5 and NtDGK5G118A in strongly overexpressing pollen tubes with high fluorescence values, indicating a limited binding capacity at the PM (Fig. 4d). In strong overexpressors, PMI values converge towards 1 for NtDGK5 and NtDGK5G118A, but NtDGK5G118A displayed a more pronounced PM localization than NtDGK5 in low-expressing pollen tubes (Fig. 4a,b,d).

To check for possible differences in protein mobility that could explain the observed localization pattern, we performed FRAP experiments. In pollen tubes overexpressing either mVenus:NtDGK5 or mVenus:NtDGK5G118A, a region in the subapical membrane was bleached, and replacement of the fluorescent signal for both mVenus:NtDGK5 and mVenus: NtDGK5G118A was followed for ca. 30 s. The resulting FRAP curves were fitted with a single exponential fit, which allowed

Fig. 3. NtDGK5 is a DAG-kinase that loses activity from the G118A mutation. NtDGK5 variants were expressed in Escherichia coli and purified protein was used for enzyme assays. Purified GST-tag alone served as a negative control. (a) For the assays, DAG and radioactively labelled [γ-32P]-ATP were supplied as substrates resulting in 32P-labeled phosphatidic acid (PA) as a potential product. Lipid products were extracted and separated via TLC and detected with a phosphorimag. (b) Identification of the radioactive product as PA was achieved by comigration of a commercial nonradioactive standard. (c) Intensity of the radioactive signal was quantified for three reactions per construct and normalized to the average signal for enzyme assays with NtDGK5. For each protein, three independent values were obtained and are displayed as gray dots. Error bars show the 95% confidence interval around the mean.
estimates of the mobile fraction of the enzyme and the halftime of its interaction with the PM (Fig. 4f,g; Methods S7). Membrane fluorescence was recovered with a halftime of 3.0 s for NtDGK5, while recovery of fluorescence for NtDGK5$^{G118A}$ took slightly longer, with a halftime of 3.8 s. The mobile fraction of the respective enzymes differed more strongly: for NtDGK5, membrane fluorescence after bleaching reached 91% of the original values. Fluorescence recovery of NtDGK5$^{G118A}$

Fig. 4 NtDGK5 and NtDGK5$^{G118A}$ differ in protein localization and mobility. NtDGK5 variants carrying an N-terminal mVenus-tag were transiently expressed in tobacco pollen tubes and transformed pollen tubes were imaged by spinning disk (a, b) or laser scanning (c–g) confocal microscopy after 6 h of growth. Both NtDGK5 (a) and NtDGK5$^{G118A}$ (b) localized to the plasma membrane. However, NtDGK5$^{G118A}$ localization was more pronounced with less cytosolic background. (c) The onset of plasma membrane localization relative to the tip and the total length of localization was determined. (d) Furthermore, the ratio between the fluorescence intensity at the plasma membrane and in the cytosol (plasma membrane index, PMI) was calculated. For low expression values, a more pronounced membrane localization of NtDGK5$^{G118A}$ was observed. With increasing fluorescence intensities, the PMI decreases and reaches comparable values for NtDGK5 and NtDGK5$^{G118A}$ at high fluorescence. Overall, PMI values of NtDGK5$^{G118A}$ differ significantly from PMI values of NtDGK5 (e). (f, g) Protein mobility was analyzed by fluorescence recovery after photobleaching. Relative intensities of single pollen tubes at the different time points are shown in gray, while average values ± SEM are displayed in red. An exponential fit was applied to the data and is shown as blue curves. Fluorescence recovery of both NtDGK5 (f) and NtDGK5$^{G118A}$ (g) have halftimes ($t_{1/2}$) below 4 s, but NtDGK5 recovers to higher fluorescence values than NtDGK5$^{G118A}$. Accordingly, the mobile fraction as estimated from the fitted curve amounted to 91% for NtDGK5 and 77% for NtDGK5$^{G118A}$. Boxplots in (c, e) display the first quartile, median and third quartile values and whiskers extend to the extreme data points inside of 1.5 x interquartile range. Single data points are shown as black circles. Statistical analysis was done by Kruskal-Wallis and Kruskal-Wallis post-hoc tests. Different letters indicate significant differences at $P < 0.05$. In the display of (d) a linear model was fitted to the single data points and is shown with the standard error shaded. $n = 8$ pollen tubes for NtDGK5 and $n = 18$ pollen tubes for NtDGK5$^{G118A}$ in (c); $n = 20$ pollen tubes for NtDGK5 and $n = 22$ pollen tubes for NtDGK5$^{G118A}$ in (d–g). Regression curves in (f, g) were calculated using the model $f(t) = Mf \times (1 - \exp(-t/\tau))$ with $Mf =$ mobile fraction, $\tau =$ time constant, $t =$ time and $f(t) =$ relative intensity. Halftime ($t_{1/2}$) was calculated as $t_{1/2} = \ln(2) \times \tau$. Bar, 10 µm.

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amounted to 77% of prebleach intensities. The mutation in NtDGK5<sup>G118A</sup> thus seems to induce an increased association to the PM, impeding dissociation of the enzymes to the cytoplasm. For further study of the changes induced by the G118A mutation, we evaluated the lipid binding behavior of NtDGK5 and NtDGK5<sup>G118A</sup> by a protein–lipid overlay assay (Fig. 5a). No qualitative changes were observed, as both protein variants were able to bind to the anionic phospholipids PA, PI<sub>4</sub>P and PI(4,5)P<sub>2</sub>. In addition, binding ability to cardiolipin, sulfatide and phosphatidylinositol 3,4,5-trisphosphate was observed, although these lipids have so far not been detected in the plant PM (Furt et al., 2011). To support these findings, we additionally tested lipid binding of NtDGK5 and NtDGK5<sup>G118A</sup> to large unilamellar vesicles (LUVs) by a vesicle cosedimentation assay. LUVs were constituted either from phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as control or PC, PE and PA, which we chose as proxy for anionic phospholipids. Both NtDGK5 and NtDGK5<sup>G118A</sup> showed increased propensity to bind to LUVs containing PA, but no increase of LUV binding was observed for NtDGK5<sup>G118A</sup> compared to NtDGK5 (Fig. 5b,c).

Overexpression of NtDGK5 or NtDGK5<sup>G118A</sup> induce distinct aberrant growth phenotypes in tobacco pollen tubes

As a DAG kinase, NtDGK5 could be potentially involved in the conversion of signaling phospholipids that regulate pollen tube growth (Scholz et al., 2020). We therefore checked for phenotypes in pollen tubes with altered NtDGK5 levels. To that end, we overexpressed either NtDGK5 or NtDGK5<sup>G118A</sup> carrying an N-terminal mVenus-tag in tobacco pollen tubes. Fourteen hours after transformation, growth phenotypes were analyzed to investigate potential effects of mVenus:NtDGK overexpression.

Overexpression of the two enzyme variants caused distinct phenotypes (Fig. 6; Videos S1–S3). Both mVenus:NtDGK5 and mVenus:NtDGK5<sup>G118A</sup> induced a wavy growth pattern (mV: NtDGK5<sup>G118A</sup> at a much higher frequency), which is rarely observed when expressing the mVenus-tag alone (Fig. 6a–d). Together with the wavy growth pattern, mVenus:NtDGK5<sup>G118A</sup> expressing pollen tubes often displayed swollen tips (Fig. 6c,d). mVenus:NtDGK5<sup>G118A</sup> expression also strongly increased the occurrence of short pollen tubes less than 500 µm long (Fig. 6d), an effect that was not observed for mVenus:NtDGK5.

Overall, overexpression of both mVenus:NtDGK5 variants disturbs pollen tube growth, though differences between active and suppressed enzymes are observed. A comparison with other NtDGKs and AtDGK5 shows that the described wavy tip growth is quite specific for NtDGK5, while also NtDGK4 and NtDGK7, and the Arabidopsis DGK5 induce stunted growth (Fig. S8a–c). On the other hand, PM localization of YFP:AtDGK5 close to the pollen tube apex resembles YFP:NtDGK5 in growing tobacco pollen tubes (Fig. S8d). We also assayed in vivo pollen tube growth of the Arabidopsis mutants dgk4, dgk5, dgk6 and dgk5 dgk6 in two independent experiments. However, despite some variations, the results did not show consistent alterations of pollen tube length (Fig. S9).

Overexpression of active NtDGK5 increases pectin deposition

In previous studies on signaling lipids and their converting enzymes in pollen tube growth, connections to the deposition of cell wall components have been described (Ischebeck et al., 2008). Altered cell wall deposition could also offer a possible explanation for the observed phenotypes. Consequently, we investigated the distribution of pectin, the main cell wall component of the pollen tube tip, in nontransformed cells and cells expressing mVenus-tagged NtDGK5 or NtDGK5<sup>G118A</sup>. Pectin was stained with propidium iodide and cross-sections of the cell wall were imaged by confocal microscopy (for methodology see also Fig. S10). Initial inspection of microscopic images indicated more intense pectin staining in pollen tubes overexpressing NtDGK5 (Fig. 7a–d). We therefore quantified propidium iodide staining of transformed pollen tubes normalized to respective neighboring nontransformed pollen tubes. Pectin accumulation was analyzed in the subapical region c. 20 µm behind the tip, as this pectin was probably deposited during growth and not after cessation of growth. NtDGK5 induced an increase of staining intensity in contrast to NtDGK5<sup>G118A</sup> and the mVenus control (Fig. 7e).

NtDGK5<sup>G118A</sup> expression influences PA and PI(4,5)P<sub>2</sub> signal intensity in the PM

Taking into account the localization of various lipid markers (Helling et al., 2006; Potocký et al., 2014; Pejchar et al., 2020)
and NtDGK5 in tobacco pollen tubes, we next tested whether there is an overlap in their distribution. To this end, we transiently cotransformed tobacco pollen tubes with mVenus: NtDGK5 and mRFP-tagged genetically encoded lipid marker for DAG (mRFP:2xCys1), PA (mRFP:2xSpo20p-PABD) or PI(4,5)P2 (mRFP:2xPHPLCδ1), and followed their localization. In growing pollen tubes with low levels of transgene expression, membrane mVenus:NtDGK5 was fully included within the range of mRFP:2xCys1 and mRFP:2xPHPLCδ1 signals. Similarly, the distribution of NtDGK5 overlaps with mRFP:2xSpo20p-PABD in the pollen tube subapex, although the area of the mVenus:NtDGK5 signal close to the tip is devoid of mRFP:2xSpo20p-PABD (Fig. S11).

Overexpression of PA-synthesizing enzymes can lead to an increase of PA in the PM of pollen tubes, detectable by an increased PMI of mRFP:2xSpo20p-PABD PA biosensor (Pejchar et al., 2020). In light of these hypotheses, we coexpressed mVenus:NtDGK5 or mVenus:NtDGK5G118A together with the PA biosensor and used a ratiometric approach for quantification of the mRFP:2xSpo20p-PABD signal (Fig. 8a–c). Confirming visual microscopic observations, calculated PMI did not increase upon overexpression of NtDGK5 compared to expression of free mVenus. However, overexpression of the inactive NtDGK5G118A led to a significant decrease of the median PMI to c. 70% that of the control (Fig. 8d).

Previously, we found that overexpression of PI4P 5-kinases also led to increased pectin deposition (Ischebeck et al., 2008), and pollen PI4P 5-kinases might be regulated by PA (Kost et al., 1999; Im et al., 2007). Hence, we also assayed PI(4,5)P2 using a similar ratiometric approach with the PI(4,5)P2 biosensor.
mRFP:2xPHPLC (Fig. 8e–h). Overexpression of NtDGK5 slightly reduced the median PMI of the biosensor to 72% of the control. Overexpression of NtDGK5G118A caused an even stronger decrease of the median PMI to 27% of control values, as the PMI in many cases came down close to 1.

NtDGK5 or NtDGK5G118A and AtPIP5K5 reciprocally influence pollen tube phenotypes

Based on the observed connection between PI(4,5)P2 levels and overexpression of NtDGK5 and NtDGK5G118A, we decided to analyze the effects of NtDGK5 variants on pollen tubes with disturbed PI(4,5)P2 metabolism. To increase PI(4,5)P2 levels, we overexpressed the enzyme AtPIP5K5 in tobacco pollen tubes (Ischebeck et al., 2008). On a phenotype level, mVenus:AtPIP5K5 expression causes the appearance of branched pollen tubes or pollen tubes with stunted growth (Fig. 9a,b), probably caused by pectin accumulation at the tip (Ischebeck et al., 2008). mVenus-tagged NtDGK5 and NtDGK5G118A were then coexpressed with mVenus:AtPIP5K5 or free mVenus as a control, and respective characteristic phenotypes were analyzed, including wavy growth and tip swelling for NtDGK5 variants, or pollen tube branching for AtPIP5K5. Pollen tube lengths were also evaluated (Fig. 9c,d).

In agreement with previous results, a wavy growth pattern was observed for NtDGK5 and NtDGK5G118A in combination with the mVenus control. Interestingly, AtPIP5K5 was able to suppress this growth pattern in combination with NtDGK5, but not in combination with NtDGK5G118A. By contrast, combining NtDGK5G118A with AtPIP5K5 led to a strong decrease in the frequency of swollen pollen tube tips in comparison with expression of NtDGK5G118A with the mVenus control. Vice versa, AtPIP5K5-induced branching was strongly reduced by coexpression of NtDGK5 or NtDGK5G118A.

Concerning pollen tube length, both NtDGK5G118A and AtPIP5K5 caused a decrease in average tube length of 39% and 36%, respectively (Fig. 9d). This was also represented by an increasing frequency of pollen tubes shorter than 500 or 1000 µm (Fig. 9c). Expression of NtDGK5 with the mVenus control did not alter pollen tube length. However, the comparison of AtPIP5K5 effects on pollen tube length with or without NtDGK5 displayed an increased abundance of short pollen tubes and an additional drop in average length induced by NtDGK5. Strikingly, the combination of AtPIP5K5 with NtDGK5G118A did not equal their negative effects on pollen tube length. Instead, the length distribution shifted towards an increased frequency of pollen tubes longer than 1000 µm. Compared to the control, pollen tube length decreased by 16%, which is a lower reduction than either AtPIP5K5 or NtDGK5G118A induced alone.
Fig. 8 NtDGK5<sup>G118A</sup> overexpression reduces the plasma membrane affinity of lipid sensors for phosphatidic acid (PA) and PI(4,5)P₂. NtDGK5, NtDGK5<sup>G118A</sup> tagged to fluorescent N-terminal mVenus (mV) and mVenus alone as a control were transiently coexpressed in tobacco pollen tubes together with the lipid sensors mRFP:2xSpo20p-PABD (a–c) or mRFP:2xPH<sub>PLCδ1</sub> (e–g) for PA and PI(4,5)P₂, respectively. For RFP-labeled lipid sensors the plasma membrane index (PMI) was calculated, dividing the fluorescence intensity at the plasma membrane by the fluorescence intensity in the cytosol. In the case of 2xSpo20p-PABD, plasma membrane localization decreased upon overexpression of NtDGK5<sup>G118A</sup>, but was not significantly altered by NtDGK5 (d). By contrast, both NtDGK5 and NtDGK5<sup>G118A</sup> caused a significant decrease of plasma membrane localization of mRFP:2xPH<sub>PLCδ1</sub>. Loss of mRFP:2xPH<sub>PLCδ1</sub> membrane localization was more severe for overexpression of NtDGK5<sup>G118A</sup> compared to NtDGK5 (h). In (d, h), boxplots display the first quartile, median and third quartile values and whiskers extend to the extreme data points inside of 1.5 × interquartile range. Single data points are shown as black circles. \( n = 52 \) (mVenus), 79 (mVenus:NtDGK5) and 71 (mVenus:NtDGK5<sup>G118A</sup>) pollen tubes for the PA sensor (d); \( n = 51 \) (mVenus), 63 (mVenus:NtDGK5) and 48 (mVenus:NtDGK5<sup>G118A</sup>) pollen tubes for the PI(4,5)P₂ sensor (h). Statistical analysis was done by one-way ANOVA with Tukey’s post-hoc test. Different letters indicate significant differences at \( P < 0.05 \). Bar, 10 μm.
NtDGK5 and NtDGK5\textsuperscript{G118A} interfere with AtPIP5K5-induced apical pectin accumulation

Length measurements and phenotypic analysis of pollen tubes coexpressing mVenus-tagged NtDGK5 variants with mVenus:AtPIP5K5 showed that NtDGK5\textsuperscript{G118A} partially counteracts pollen tube phenotypes resulting from the AtPIP5K5 overexpression-induced increase of PI(4,5)P\textsubscript{2} levels. As PI(4,5)P\textsubscript{2}-induced phenotypes have been traced to excessive apical pectin secretion, we then analyzed the impact of NtDGK5 variants on...
pollen tubes where pectin secretion was increased by the expression of AtPIP5K5. We again used the propidium iodide staining approach to analyze pectin accumulation at the pollen tube tip. In line with previous studies, AtPIP5K5 overexpression caused a massive increase of pectin accumulation at the pollen tube tip (Fig. 10a,b). Since expression levels of AtPIP5K5 and subsequent PI(4,5)P₂ concentrations vary considerably, the effects were highly variable. In some pollen tubes, pectin accumulation was scarcely changed, whereas in extreme cases the pectin staining intensity of transformed pollen tubes was more than 100-fold higher than in untransformed control pollen tubes. Overall, an increased pectin secretion at the tip was observed, as the ratiometric median calculated as log₂-change was altered from /C₀ to 4.28 (Fig. 10g). The pectin staining intensity was similarly broadly distributed in pollen tubes coexpressing AtPIP5K5 either with NtDGK5 or NtDGK5G118A (Fig. 10c–f). Nevertheless, in...
both populations of pollen tubes, median values of normalized staining intensity decreased compared to coexpression of AtPIP5K5 and mVenus, to a log$_2$-change of 4.04 or 3.39, respectively (Fig. 10g).

In conclusion, while the interplay of PA and phosphoinositides remains to be studied in depth, our data indicate that expression of NtDGK5$^{G118A}$ leads to reduced PI(4,5)P$_2$ levels and thereby counteracts AtPIP5K5-derived phenotypes, especially the reduction in pollen tube length.

Discussion

NtDGK5 has limited binding capacity to the subapical pollen tube PM

NtDGK5 sequence analysis did not indicate transmembrane helices or covalent attachment of lipid anchors. Furthermore, considerable cytoplasmic fluorescence and short FRAP times (Fig. 4a,f) suggest that NtDGK5 is a soluble protein transiently recruited by one or several binding partners for peripheral membrane association, similar to other lipid-converting enzymes (Ischebeck et al., 2008; Pejchar et al., 2020; Noack et al., 2021).

As saturation effects were observed for the membrane association of NtDGK5 (Fig. 4d), a limited number of such binding partners can be assumed. One possible mediator of membrane binding of peripheral membrane proteins are lipid-binding domains that recognize specific lipids at the PM (Noack & Jaillais, 2020; de Jong & Munnik, 2021). For example, PLCs from tobacco and petunia localized in the apical PM of pollen tubes depend for this targeting on their C2 domains (Dowd et al., 2006; Helling et al., 2006) described to bind anionic lipids in a Ca$^{2+}$-dependent manner (Corbalan-Garcia & Gómez-Fernández, 2014). For the apical PM targeting of AtPIP5K6, on the other hand, not the presumably lipid binding MORN (membrane occupation and recognition nexus) repeat domain is important, but a variable linker domain that might confer protein–protein interactions (Stenzel et al., 2012).

In comparison to some mammalian DGKs (Franks et al., 2017), but also AtDGK1 and 2 (Arisz et al., 2009) and NtDGK1–3 (Fig. 1b), NtDGK5 does not harbor any conserved domains implicated in lipid binding. It is still conceivable, however, that it is recruited to the PM by binding its substrate DAG enriched in the apical PM of pollen tubes (Helling et al., 2006). However, the membrane association of NtDGK5 was confined to a smaller region than has been observed with a biosensor for DAG (Fig. S10). Hence, the exact localization of NtDGK5 is probably mediated, at least in part, by factors other than substrate availability. Indeed, our MD simulations and lipid-binding assays show a direct interaction between NtDGK5 and anionic phospholipids that are enriched at the plant PM (Simon et al., 2016; Platre & Jaillais, 2017; Platre et al., 2018).

Still the question remains why the membrane-binding region in planta of DGK5 is so confined, especially given that other DGK isoforms and the G118A variant of DGK5 that harbors a similarly charged surface, bind to a much larger area of the PM (Figs 1, 4, S3).

Limited binding capacity for DGKs at the PM might contribute to a possible dominant negative effect of NtDGK5$^{G118A}$

Mutated protein variants that mediate dominant-negative effects have been proven to be valuable tools to investigate pollen tube signaling (Cheung et al., 2002; Dowd et al., 2006; Klahre et al., 2006; Chang et al., 2009). These dominant-negative effects can derive from mutated protein variants that outcompete the naturally occurring variants for crucial binding partners/regulators. Such an explanation is also possible here. Alteration of the glycine-rich loop in NtDGK5$^{G118A}$ did not interfere with the protein’s membrane association. By contrast, the association with the PM was even stronger and covered a longer stretch (Fig. 4c). Our FRAP experiments also suggested that a subpopulation of NtDGK5$^{G118A}$ remains more strongly attached to the PM (Fig. 4g). In this way, NtDGK5$^{G118A}$ might block the DAG substrate and possible physiological binding partners of the endogenous NtDGK5 and closely related paralogs, leading to a dominant-negative effect upon overexpression in pollen tubes and subsequent growth perturbations, as well as explain the reduced binding of the PA biosensor at the PM (Figs 6, 8). An alternative is that NtDGK5, and to a stronger degree NtDGK5$^{G118A}$ due to its enhanced PM recruitment, induces phenotypic changes by masking PA that we found to be bound by both variants. While the active variant could partially counter this effect by producing PA, the inactive variant cannot, thereby reducing the amount of free PA in the region. Previous inhibition of DGK activity in tobacco pollen tubes with the DGK inhibitor R59022 was partially shown to decrease PA levels at the PM, but did not induce the wavy growth effects on pollen tube growth as was observed for the overexpression of NtDGK5$^{G118A}$ (Pleskot et al., 2012; Potocký et al., 2014). This might be explained due to its less specific inhibition also affecting ER-localized DGKs. Disturbance of these DGKs’ role in phospholipid synthesis and ER lipid composition might hamper pollen tube growth differentially from NtDGK5. While PA produced at the PM might contribute directly to polar tip growth by regulating secretory processes, DGKs at the ER might be involved in the synthesis of phosphatidylinositol and phosphatidylglycerol (Angkawijaya et al., 2020). On the other hand, PA synthesized at the ER (e.g. by Arabidopsis DGK2 or DGK4; Angkawijaya et al., 2020) could also be transported to the PM via the secretory pathway thereby contributing to PA signaling in the tip region. Either way, R59022-related effects might be too strong, covering subtler effects induced by NtDGK5$^{G118A}$.

Cell wall plasticity shapes the pollen tube

Plant cells are shaped by turgor pressure and cell wall plasticity (Ivakov & Persson, 2013). While the apical cell wall of pollen tubes is somewhat more simple than most cell walls (e.g. lacking cellulose), its plasticity can still be regulated by targeted pectin secretion and pectin-modifying enzymes (Scholz et al., 2020). This allows not only balance between giving too much resistance against elongation and bursting, but also enables uniform tubular
growth. Such growth requires an uneven cell wall plasticity in the very tip region of the pollen tube, maintained by continuous targeted secretion of pectin and its modifiers orchestrated by a combination of feed-forward and feed-back regulations. Many studies have highlighted that this dynamic equilibrium can be disturbed by overexpression of various signaling proteins (Scheible & McCubbin, 2019; Scholz et al., 2020). Overexpression of AtPIP5K4 and 5, for example, leads to a premature growth arrest, accompanied by massive pectin deposition and membrane invaginations (Ischebeck et al., 2008). A similar phenotype was observed after overexpression of PLDs (Pejchar et al., 2020). Furthermore, studies of the Arabidopsis mutant dgk4 reported differences in cell wall properties of pollen tubes as determined by atomic force microscopy (Vaz Dias et al., 2019). Here we show that overexpression of NtDGK5 also leads to increased pectin deposition and shorter pollen tubes (Figs 6, 7). The increased deposition could first lead to thicker cell walls and finally terminate tube elongation when the cell wall resistance becomes greater than the turgor pressure.

In addition, the tip-swelling phenotype observed upon overexpression of the NtDGK5G118A variant might be explained by effects on cell wall deposition. Here, even though no effect on cell wall thickness was measured in our assay, subtle changes in pectin secretion could lead to wall thinning in certain regions, perturbing the plasticity in a way that leads to tip-swelling and ultimately growth arrest rather than regular, continuous tip growth.

More difficult to explain is the wavy growth pattern observed with its periodic nature. Pollen tube growth as an oscillating process has long been described and observed with regard to, inter alia, growth speed, Ca\(^{2+}\) concentrations and wall thickness (Feijö et al., 2001; Zonia et al., 2006). Experimental observations have mainly been described along the longitudinal axis of the growth direction; however, Haduch-Sendecka et al. (2014) also reported transverse oscillations in the width of tobacco pollen tubes that are correlated to longitudinal growth speed oscillations in tobacco pollen tubes. The observed wavy growth is therefore possibly the result of a slight asymmetry in the regulating networks that control transverse oscillations on which DGK variant was used. This might result in an asymmetric pectin deposition causing one side of the pollen tube to expand more strongly, resulting in a turning of growth direction. A correction by internal feed-back loops in combination with the transverse oscillations could possibly result in the wavy shape.

NtDGK5 might influence pectin secretion as part of a larger regulatory network

As we propose that the phenotypes observed are caused by alterations in the secretion of pectin and possibly also pectin-modifying enzymes, the question arises of how DGK-produced PA could regulate such secretory processes. Generally, PA could have a direct effect on pectin secretion or an indirect effect by regulating other signaling proteins. PA has, for example, been linked to the regulation of PIP5K activity: in vitro experiments demonstrated an activation of Arabidopsis AtPIP5K1 via its N-terminal MORN domain by PA, which is a conserved domain in Arabidopsis AtPIPK51–AtPIP5K5 (Im et al., 2007).

Phosphatidic acid and PI(4,5)P\(_2\) could then influence the rate of secretion in pollen tubes in several ways, including by the availability of secretory vesicles in the apical region, the rate of tethering of these vesicles to the PM as well as the actual rate of fusion events.

Important for the tethering of secretory vesicles to the PM is foremost the exocyst complex (TerBush et al., 1996; Elías et al., 2003). This complex consists of eight protein subunits and is recruited to the PM by anionic phospholipids including PA and PI(4,5)P\(_2\) (Bloch et al., 2016; Synek et al., 2021). In addition, Sekereš et al. (2017) analyzed the localization of different members of the tobacco EXO70 protein family in tobacco pollen tubes and described localization to distinct membrane domains for NtEXO70A1a and NtEXO70B. The apical localization of NtEXO70A1a appeared to be limited by the presence of PA in the membrane: the onset of NtEXO70A1a coincided with the onset of the PA sensor mRFP:2xSpo20p-PABD.

Vesicle fusion could also be regulated by PA directly, or through a potential connection to PI(4,5)P\(_2\) synthesis. In the animal field, PA was described to promote exocytosis through its cone shape, and for yeast interactions of PA with different SNARE proteins were described to influence membrane fusion events (Zhukovsky et al., 2019). Regarding a potential regulation of vesicle fusion by PI(4,5)P\(_2\), mechanistic knowledge in the plant system is very limited. However, PI(4,5)P\(_2\) is established as a key player in the neuron system by regulating the activity of ion channels that influence secretion (Hille et al., 2015). Furthermore, several proteins directly involved in fusion bind PI(4,5)P\(_2\), including SNARE proteins and the proteins CAPS and Munc13 that prime vesicles for exocytosis (Martin, 2012).

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Author contributions

PS, PP, RP, TM, MP and TI designed the research; PS, PP, MF, EŠ, RP, MP, KB and TI performed research; PS, PP, EŠ, RP, MP and TI analyzed the data; PS, PP, RP, TM, MP and TI wrote the paper with the help of all authors.
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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Phylogenetic and transcriptomic analysis of canonical plant diacylglycerol kinases from 20 diverse species.

Fig. S2 Coexpression of clade I NtDGKs with markers for endomembrane compartments.

Fig. S3 Tobacco diacylglycerol kinase isoforms of clade III differ in their charge distribution and plasma membrane localization patterns in pollen tubes.

Fig. S4 Lipid binding properties of clade III NtDGKs.

Fig. S5 Molecular dynamics simulations reveal several membrane binding modes.

Fig. S6 G118A mutation of NtDGK5 probably interferes with ADP/ATP binding.

Fig. S7 Enzyme assays show diacylglycerol kinase activity.

Fig. S8 AtDGK5 and NtDGK isoforms show differences in induced overexpression phenotypes of tobacco pollen tubes.

Fig. S9 In vivo assays of Arabidopsis pollen tube growth did not show drastic alterations of pollen tube length in different dgk mutants.

Fig. S10 Quantitative analysis of pectin staining by propidium iodide.

Fig. S11 NtDGK5 coexpression with lipid markers in growing pollen tubes.

Methods S1 Phylogenetic analysis of diacylglycerol kinase in Viridiplantae.

Methods S2 Prediction of diacylglycerol kinase domains and protein structure.

Methods S3 Diacylglycerol kinase gene expression analyses.

Methods S4 Molecular dynamics simulation.

Methods S5 Molecular cloning of diacylglycerol kinase constructs.

Methods S6 Immunodetection of lipid strips and Western blot analysis.

Methods S7 Quantitative analyses of micrographs.

Notes S1 Sequences of diacylglycerol kinases used for the phylogenetic analysis.

Table S1 Number of DGK isoforms in each clade based on DGK phylogeny in Viridiplantae.

Table S2 Tobacco DGK isoforms, their accession numbers and predicted domain structure.

Table S3 List of primers used in this study.

Video S1 Examples of pollen tube growth phenotypes in tobacco overexpressing NtDGK5.

Video S2 Examples of pollen tube swelling tip phenotypes in tobacco overexpressing NtDGK5G118A.

Video S3 Examples of pollen tube arrested growth phenotypes in tobacco overexpressing NtDGK5G118A.

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