A nanodomain anchored-scaffolding complex is required for PI4Kα function and localization in plants

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Abstract:
Phosphoinositides are low-abundant lipids that participate in the acquisition of membrane identity through their spatiotemporal enrichment in specific compartments. PI4P accumulates at the plant plasma membrane driving its high electrostatic potential, and thereby facilitating interactions with polybasic regions of proteins. PI4Kα1 has been suggested to produce PI4P at the plasma membrane, but how it is recruited to this compartment is unknown. Here, we pin-point the mechanism that tethers PI4Kα1 to the plasma membrane via a nanodomain-anchored scaffolding complex. We identified that PI4Kα1 is part of a heterotetrameric complex composed of proteins from the NO-POLLEN-GERMINATION, EFR3-OF-PLANTS, and HYCCIN families. Comprehensive knock-out and knock-down strategies revealed that subunits of the PI4Kα1 complex are essential for pollen, embryonic and post-embryonic development. We further found that the PI4Kα1 complex is immobilized in plasma membrane nanodomains. Using synthetic mis-targeting strategies, we demonstrate that a combination of lipid anchoring and scaffolding localizes PI4Kα1 to the plasma membrane, which is essential for its function. Together, this work opens new perspectives on the mechanisms and function of plasma membrane nanopatterning by lipid kinases.

Introduction

Eukaryotic cells are composed of several membrane-surrounded compartments. Each compartment has a unique physicochemical environment delimited by a membrane with a specific biochemical and biophysical identity (Bigay and Antonny, 2012). The membrane identity includes the nature of the lipids, the curvature, the electrostaticity and the density of lipids at the membrane. The identity of each membrane allows the proper localization of membrane-associated proteins.

Phosphoinositides are rare anionic lipids present in membranes. Five types of phosphoinositides exist in plants - PI3P, PI4P, PI5P, PI(4,5)P_2 and PI(3,5)P_2 - depending on the number and position of phosphates around the inositol ring. They accumulate differently at the plasma membrane and intracellular compartments and interact with proteins through stereo-specific or electrostatic interactions (Barbosa et al., 2016; Lemmon, 2008; Simon et al., 2016). Recent work uncovered that PI4P concentrates according to an inverted gradient by comparison to their yeast and animal counterpart (Hammond et al., 2009, 2014; Levine and Munro, 1998, 2002; Noack and Jaillais, 2017, 2020; Roy and Levine, 2004; Simon et al., 2016). Indeed, in yeast, the major PI4P pool is in the Golgi/trans-Golgi Network (TGN) compartments while a minor pool is present at the plasma membrane (Roy and Levine, 2004). The plasma membrane pool of PI4P is produced by the PI4-Kinases (PI4K) Stt4p, while Pik1p produces the PI4P pool at the TGN (Audhya and Emr, 2002; Audhya et al., 2000; Balla et al., 2005; Nakatsu et al., 2012; Roy and Levine, 2004). These two PI4P pools are essential for yeast survival and at least partially independent (Roy and Levine, 2004). In animal, three PI4K isoforms, PI4KIIIβ/PI4KIIα/PI4KIIβ, are responsible for synthetizing PI4P at the Golgi/TGN and in endosomes (Balla et al., 2002; Wang et al., 2003; Wei et al., 2002). Similar to the Δ*stt4* in yeast, PI4KIIIα loss-of-function mutant is lethal in mammals (Nakatsu et al., 2012). In PI4KIIIα conditional mutants, the pool of PI4P disappears from the plasma

membrane while the TGN structures seem to remain untouched suggesting that the two pools could be independent (Nakatsu et al., 2012).

In plants, PI4P massively accumulates at the plasma membrane and is less abundant at the TGN (Simon et al., 2014, 2016; Vermeer et al., 2009). This PI4P accumulation at the cell surface drives the plasma membrane electrostatic field, which in turn recruits a host of signalling proteins to this compartment (Barbosa et al., 2016; Platre et al., 2018; Simon et al., 2016). Moreover, the plant TGN is the site of vesicular secretion but also involved in endocytic sorting and recycling, which might imply regulatory mechanisms of lipid exchanges or maintenance of membrane identity between plasma membrane and TGN (Noack and Jaillais, 2017).

The *Arabidopsis* genome codes four PI4-kinases: PI4Kα1, PI4Kα2, PI4Kβ1 and PI4Kβ2 (Szumlanski and Nielsen, 2010). Due to the absence of Expressed Sequence Tags of *PI4Kα2,* it is considered as a pseudogene (Mueller-Roeber and Pical, 2002). *pi4kβ1pi4kβ2* double mutant displays mild growth defects including tip growth phenotype with bulged root hairs and cell plate defects, which suggest defective secretory pathway (Antignani et al., 2015; Delage et al., 2012; Kang et al., 2011; Lin et al., 2019; Preuss et al., 2006; Šašek et al., 2014). In addition, *pi4kβ1pi4kβ2* presents fewer and misshaped secretory vesicles at the TGN (Kang et al., 2011). PI4Kβ1 and PI4Kβ2 have first been described to be localized to the Trans-Golgi Network/Early Endosomes (TGN/EE) in root hairs (Preuss et al., 2006). This localization, as well as its accumulation at the cell plate, has later been validated by electron tomography and confocal microscopy in root meristematic cells (Kang et al., 2011; Lin et al., 2019). The targeting mechanism of PI4Kβ1 at the TGN involves RabA4b, a small GTPase (Preuss et al., 2006). In addition, PI4Kβ1 recognizes, and interacts with, the curved electronegative membrane of the TGN/EE via an amphipatic lipid packing sensor (ALPS) motif preceded by cationic amino acids (Platre et al., 2018).

In contrast, PI4Kα1 localizes at the plasma membrane (Okazaki et al., 2015) and its catalytic activity was confirmed *in vitro* (Stevenson-Paulik et al., 2003). Thus, it is a prime candidate for producing PI4P at the plasma membrane. However, PI4Kα1 is a soluble protein with no protein-lipid interaction domains or anchoring mechanism known. How PI4Kα1 is recruited and participates to the architecture of the plasma membrane are open questions.

Here, we uncovered that PI4Kα1 belongs to a 4-subunit complex composed of proteins from the NPG (NO POLLEN GERMINATION), HYC (HYCCIN) and EFOP (EFR3 OF PLANTS) protein families. Using fluorescent protein tagging, immunolocalization and subcellular fractionation, we confirmed the presence of the PI4Kα1 complex at the plasma membrane. Furthermore, we show that *pi4kα1* loss-of-function leads to full male sterility. Mutant pollen grains collapse and display abnormal cell walls. Knockout of any subunits of the PI4Kα1 complex mimics *pi4kα1* pollen lethality. Moreover, we established that the four subunits of the complex are essential for *PI4Kα1* function. By using mutant variants and chimeric constructs, we showed that the function of this complex is to target PI4Kα1 to the plasma membrane via lipid anchors. Finally, we observed that this heterotetrameric complex

is not homogenously present on the plasma membrane but enriched in nanodomains. Although all the subunits of the complex are peripheral proteins and lack a transmembrane domain, they show very little lateral mobility at the plasma membrane. These results suggest that PI4Kα1 is not localized homogeneously at the plasma membrane but rather accumulates in distinct hotspots at the inner leaflet of the plasma membrane. Consequently, the targeting of this lipid kinase by a multiprotein scaffold might allow its precise spatiotemporal recruitment in order to maintain the proper electrostatic landscape of plant cell membranes.

Results

PI4Kα1 is a soluble protein with a potential lipid-binding domain

To determine how PI4Kα1 is recruited to the plasma membrane, we first analysed its protein sequence *in silico*. Using TMHMM Server v. 2.0, no transmembrane helices could be predicted in PI4Kα1 protein sequence suggesting that PI4Kα1 is a soluble cytosolic protein. We then looked for lipid binding domains. Indeed, a Plecthrin Homology (PH) domain has been found in its C-terminal, upstream from the catalytic domain (Stevenson et al., 1998; Stevenson-Paulik et al., 2003; Xue et al., 1999). This PH domain was first thought to localize PI4Kα1 at the plasma membrane through interaction with anionic phospholipids but its role is now discussed (De Jong, F. and Munnik, T., 2021). Fat blot experiments showed affinity of the putative PH domain for PI4P and in a lesser extent for PI(4,5)P_2 (Stevenson et al., 1998; Stevenson-Paulik et al., 2003). However, no experiment *in planta* validates this result and using the Simple Modular Architecture Research Tool (SMART) software, we were not able to retrieve the PH domain. Because of the lack of predicted domain (except for the kinase domain), we decided to consider other targeting mechanisms involving possible protein partners. Indeed, protein targeting to a membrane can be multifactorial and may require coincidence binding of lipids and protein partners.

PI4Kα1 interacts with NO POLLEN GERMINATION proteins

To investigate this last hypothesis, we screened for PI4Kα1-protein partners. We performed a yeast-two-hybrid screen with the large N-terminal part of PI4Kα1 (1-1468 aa) **(Figure 1A)**. We recovered 267 in frame clones, which corresponded to 48 different proteins. Among them, the screen revealed interactions between PI4Kα1 and the three members of a protein family called NO POLLEN GERMINATION (NPG): NPG1 (*At2g43040*), NPG-Related 1 (NPGR1 – *At1g27460*) and NPGR2 (*At4g28600*) (Golovkin and Reddy, 2003). In the screen, we retrieved 39 clones (7 independent clones) for NPG1, 32 clones (6 independent clones) for NPGR1 and 2 clones (1 independent clone) for NPGR2. The clones from the NPG family corresponded to about 30% of the total clones obtained from the screen, suggesting that they were over-represented.

NPG proteins contain tetratricopeptide repeats (TPR) motifs that are protein-protein interaction motifs. In the yeast-two-hybrid screen, the selected interaction domain identified

for NPG1, NPGR1 and NPGR2 correspond to the C-terminal part of the proteins (aa 444-704 for NPG1; 501-694 for NPGR1 and 414-739 for NPGR2). This is also the part of the sequence that contains the highest density of predicted TPR motifs suggesting that the interaction between PI4Kα1 and NPGs could be mediated by the C-terminal TPR motifs.

Because all three members of the NPG family interacted with PI4Kα1 in yeast-two hybrids, and given their high degree of identity and similar architecture, we decided to focus on one member of the family to confirm the interaction *in planta*. We guided this choice based on the RNAseq expression data compiled on eFP browser (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We chose *NPGR2*, as it is the family member with the highest and more widespread expression. Indeed, *NPG1* was predicted to be specifically expressed in the pollen, while *NPGR1* expression matched that of *NPGR2* but was predicted to be much weaker.

To confirm the interaction between NPGR2 and PI4Kα1, we produced stable transgenic lines expressing *UBQ10::NPGR2-mCITRINE*. We raised antibodies against the native PI4Kα1 (residues 1 to 344 of PI4Kα1). In western blot the antibody recognized PI4Kα1 around the expected size (225kDa) and the tagged version of PI4Kα1 with mCITRINE and 2xmCHERRY slightly higher **(Supplemental Figure 1A; Table S1)**. We immunoprecipitated NPGR2-mCITRINE or the plasma membrane protein Lti6b-CITRINE as control using anti-GFP antibodies and probed whether they could co-immunoprecipitate PI4Kα1, using our native antibody. We efficiently immunoprecipitated NPGR2-mCITRINE or Lti6b-CITRINE, but PI4Kα1 was only co-immunoprecipitated with NPGR2-mCITRINE **(Figure 1B)**. Together, these experiments suggest that PI4Kα1 can interact in yeast with the C-terminus of all the three members of the NPG family and is at least found in complex with NPGR2 *in planta*.

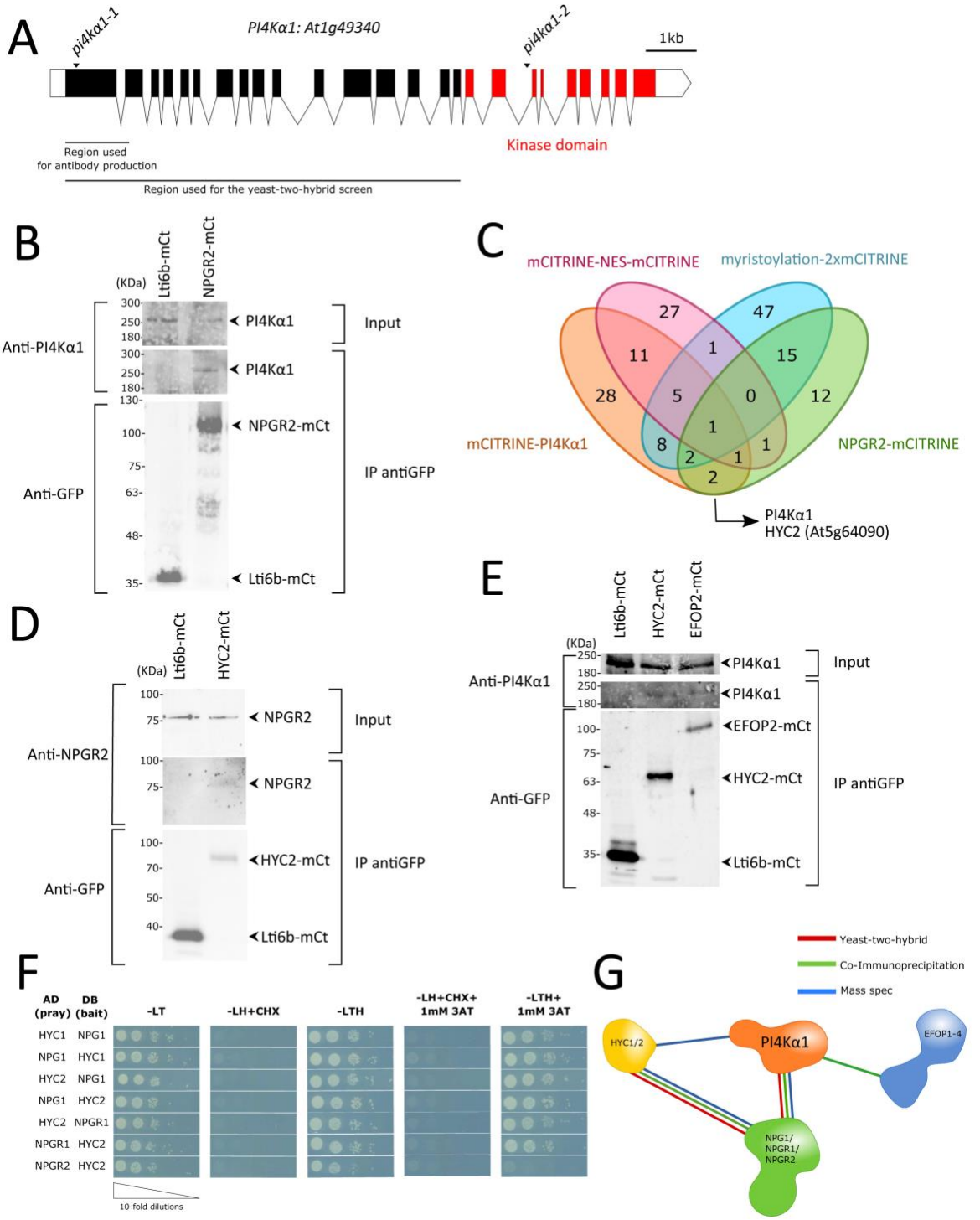

Figure 1. PI4Kα1 belongs to a 4-subunit complex in *Arabidopsis*, which includes proteins from the NPG, HYC and EFOP families. **(A)** Co-immunoprecipitation of PI4Kα1 with NPGR2. Arabidopsis transgenic plants overexpressing NPGR2-mCITRINE (NPGR2-mCt) or Lti6b-mCITRINE (Lti6b-mCt) were used for immunoprecipitation using anti-GFP beads. Immunoblots used anti-PI4Kα1 (upper panel) and anti-GFP (lower panel). **(B)** Venn diagram of proteins identified by mass spectrometry from immunoprecipitation of mCITRINE-PI4Kα1, NPGR2-mCITRINE, mCITRINE-NES-mCITRINE and myristoylation-2x-mCITRINE. **(C)** Co-immunoprecipitation of NPGR2 with HYC2. Arabidopsis transgenic plants overexpressing HYC2-mCITRINE (HYC2-mCt) or Lti6b-mCITRINE were used for immunoprecipitation using anti-GFP beads. The immunoblots used anti-GFP (lower panel) and anti-NPGR2 (upper panel). **(D)** Co-immunoprecipitation of PI4Kα1 with EFOP2 and HYC2. Arabidopsis transgenic plants overexpressing EFOP2-mCITRINE (EFOP2-mCt), HYC2-mCITRINE or Lti6b-mCITRINE were used for immunoprecipitation using anti-GFP beads. Immunoblots using anti-PI4Kα1 (upper panel) or anti-GFP (lower panel). **(E)** Yeast-two hybrid assay of HYC1 with NPG1, HYC2 with NPG1 or

NPGR1 and NPGR2. Indicated combinations of interactions between HYCCIN and NPG proteins were assessed by growth on plates with yeast growth media lacking Leu, Trp, and His (-LTH). Yeast growth on plates lacking Leu and Trp (-LT) shows the presence of the bait and prey vectors. Absence of growth when cycloheximide was added (+CHX) shows the absence of auto-activation of the DB vectors. Addition of 3-Amino-1,2,4-Triazol (+3AT) shows the strength of the interaction. **(F)** Summary of experiments showing interactions between PI4Kα1, NPG, HYC and EFOP2 proteins.

NPG proteins interact with HYCCIN proteins

Next, we asked whether the PI4Kα1 complex could include additional subunits. To this end, we used the lines expressing mCITRINE-PI4Kα1 and NPGR2-mCITRINE to perform immunoprecipitation (IP) followed by mass spectrometry analyses. Two lines expressing membrane-associated (myristoylation-2xmCITRINE) and nuclear-excluded (mCITRINE-NES-mCITRINE) proteins were also used as generic controls for plasma membrane and cytosolic proteins, respectively. In the NPGR2 IP, we found PI4Kα1, further confirming that these two proteins are present in the same complex in plants. Only one common protein was found in both NPGR2 and PI4Kα1 IPs but excluded from the two controls **(Figure 1C)**. This protein was coded by the *At5g64090* locus and contains a HYCCIN domain. *Arabidopsis* genome codes for only two proteins with a HYCCIN domain subsequently called HYCCIN1 (HYC1, *At5g21050*) and HYCCIN2 (HYC2, *At5g64090*).

HYC2 is broadly expressed, while *HYC1* expression is restricted to pollen according to the eFP browser data set (https://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Hence, we chose to confirm whether HYC2 was present in the PI4Kα1/NPGR2 sporophytic complex. To this end, we raised a *UBQ10::HYC2-mCITRINE* expressing lines and successfully isolated antibodies raised against NPGR2 (residues 1 to 273 of NPGR2). The expected size of NPGR2 is 82kDa. The antibody recognized a band at *ca.* 80kDa that is not present in *npgr2-1* or *npgr2-3* knock out mutants or *npgr1npgr2-1* double mutant **(Supplemental Figure 1B; Table S1)**. Moreover, the antibody recognized NPGR2-mCITRINE around 110kDa, but did not recognize NPGR1-mCITRINE indicating that the antibody specifically recognized NPGR2 **(Supplemental Figure 1B)**. We found that NPGR2 co-immunoprecipitated with HYC2-mCITRINE but not Lti6b-mCITRINE **(Figure 1D)**. Similarly, PI4Kα1 also co-immunoprecipitated with HYC2-mCITRINE but not with Lti6b-mCITRINE **(Figure 1E)**. Next, we used yeast-two hybrid to check whether the two HYCCIN family members may directly interact with PI4Kα1/NPGs **(Figure 1F)**. We found that the two isoforms that are pollen specific, HYC1 and NPG1 interacted in yeast. Likewise, HYC2 interacted with NPG1, NPGR1 and NPGR2 in yeast. Together, our results indicate that HYCCIN family members directly interact with NPG proteins. Furthermore, HYC2 is present in complex with PI4Kα1/NPGR2 in the *Arabidopsis* sporophyte, while HYC1/NPG1/PI4Kα1 could form a similar complex in the male gametophyte.

EFOPs proteins are part of the PI4Kα1-NPG-HYC complex

HYC domain containing proteins are also found in metazoan. In human cells, one of the members of the HYCCIN family, FAM126, has been shown to be part of a complex containing PI4KIIIα (Baskin et al., 2016; Dornan et al., 2018). FAM126 directly interacts with a protein called TETRATRICOPEPTIDE REPEAT PROTEIN 7 (TTC7), which bridges PI4KIIIα, FAM126 and a third protein called EFR3 (Baskin et al., 2016; Dornan et al., 2018;

Lees et al., 2017; Wu et al., 2014). While no HYCCIN containing protein are found in yeast, the plasma membrane PI4-Kinase, Stt4p is also anchored to the plasma membrane by a complex containing Ypp1p, which like NPG1 contains TPR motives, and Efr3p (Baird et al., 2008; Nakatsu et al., 2012; Wu et al., 2014).

Based on these information, we hypothesized that NPGs and HYCs in plants could be functional homologs of TTC7/Ypp1p and FAM126 in *Arabidopsis*, respectively. We thus wondered whether EFR3 homologs could exist in *Arabidopsis* using blast and sequence alignments. We found four potential candidates that we named EFR3 OF PLANT (EFOPs): EFOP1 (*At5g21080*), EFOP2 (*At2g41830*), EFOP3 (*At1g05960*) and EFOP4 (*At5g26850*).

Yeast Efr3p is a rod-shaped protein made of ARMADILLO-(ARM) and HEAT-like repeats. ARM and HEAT repeats are difficult to distinguished bioinformatically, but all four EFOP proteins belong to the ARM-repeat super family, which includes both ARM and HEAT repeat containing proteins (Wu et al., 2014). In *Marchantia polymorpha*, mutants for *MpPI4Kα1* (the homolog of *PI4Kα1)* and *MpSRI4*, the homolog of *EFOP2*, display short rhizoids, suggesting that they could act in the same pathway and/or protein complex (Honkanen et al., 2016). In addition, based on RNAseq data, *EFOP2* have a rather large pattern of expression. Thus, we decided to concentrate on EFOP2 to test whether it is indeed present in the sporophytic PI4Kα1/NPGR2/HYC2 complex. To this end, we raised *UBQ10prom::EFOP2-mCITRINE* transgenic lines and immunoprecipitated EFOP2-mCITRINE and Lti6b-mCITRINE using an anti-GFP antibody. We found that PI4Kα1 co-immunoprecipitated with EFOP2-mCITRINE while it did not with Lti6b-mCITRINE **(Figure 1E)**, suggesting that EFOP2 may belong to the PI4Kα1/NPGR2/HYC2 complex.
The summary of these interactions suggests that PI4Kα1 is part of an heterotetrameric complex in which NPG proteins may act as a scaffold that bridges EFOP, HYC and PI4Kα1 proteins together **(Figure 1G)**.

pi4kα1 mutants are pollen lethal

Next, we took a genetic approach to confirm whether NPG, HYC and EFOP family members indeed may function together with PI4Kα1 *in planta*. To this end, we isolated single mutants for all the genes encoding for a subunit of the PI4Kα1 complex **(Table S2).** We started our analysis with *PI4Kα1* because it is the catalytic subunit and it is present as a single-copy gene in the *Arabidopsis* genome for which we isolated two T-DNA insertion alleles. The first allele (*pi4kα1-1; GK*_502D11) had an insertion in the first exon, while the second insertion (*pi4kα1-2;* FLAG_275H12) was located in the 20th intron **(Figure 1A)**. T-DNAs of the first and second allele possess a sulfadiazine and glufosinate resistant gene, respectively. We failed to obtain homozygous mutant plants for both alleles. The segregations obtained after self-fertilization of heterozygous plants were 38% of sulfadiazine resistant seedlings for *pi4kα1-1* and 9% of glufosinate resistant seedlings for *pi4kα1-2* **(Table 1)**. Because there were less than 50% of resistant plants in the progeny of self-fertilized *pi4kα1* alleles, these segregations indicated likely gametophyte lethality, which might explain the absence of homozygous *pi4kα1* mutant. To address whether *pi4kα1* could be female and/or male gametophytic lethal, we performed reciprocal crosses, using *pi4kα1*+/- and wild-type as either male or female. For

both alleles, we recovered 0% resistant plants when *pi4kα1*+/- was used as the male indicating no transmission of the mutation via the pollen and thus complete male sterility **(Table 2)**. When *pi4kα1*+/- was used as female, we obtained 39% and 9% of resistant plants for each allele **(Table 2)**. This result shows that the *pi4kα1* mutation did not fully impair the transmission through the female gametophyte but led to a partial distortion of the segregation.

Seggregation						
Gene	Allele	Cross		% of resistant plants	% expected	n
		Female	Male			
PI4Kα1	*pi4kα1-1*	*pi4kα1-1 +/-*	*pi4kα1-1 +/-*	38,2	75	418
	pi4kα1-2	*pi4kα1-2 +/-*	*pi4kα1-2 +/-*	9,4	75	359
NPG1	*npg1-1*	*npg1-1 +/-*	*npg1-1 +/-*	50,3	75	296
	npg1-2	*npg1-2 +/-*	*npg1-2 +/-*	32	75	504
HYC1	*hyc1*	*hyc1 +/-*	*hyc1 +/-*	38,2	75	128
HYC2	*hyc2-2*	*hyc2-2 +/-*	*hyc2-2 +/-*	59,9	75	133
	hyc2-3	*NO RESISTANCE*				

Table 1: Segregation analyses of the indicated self-fertilized heterozygous mutants. n represent the number of seedling analysed.

Next, we observed *pi4kα1* pollen grains using scanning electron microscopy (SEM) to test whether they showed morphological defects **(Figure 2A)**. For both alleles, half of the pollen grains were shrivelled and likely not able to germinate explaining the pollen lethality **(Figure 2A and B)**. However, using Alexander staining, we observed that the *pi4kα1-1* pollens were still alive **(Supplemental Figure 2B).** DAPI staining also revealed the presence of the vegetative nucleus and the two sperm cell nuclei indicating that meiosis likely occurred normally **(Supplemental Figure 2C)**. Further analysis by transmission electron microscopy showed that the *pi4kα1-1* pollen grains displayed an abnormally thick cell wall **(Figure 2C)**.

The reintroduction of a copy of *PI4Kα1* under the control of its own promoter in *pi4kα1-1* background fully complemented the *pi4kα1-1* lethality as shown by the possibility to obtain homozygous mutant plants (three independent complemented lines, see **Supplemental Figure 2A)**. In addition, self-fertilized *pi4kα1-1-/-; PI4Kα1prom::PI4Kα1* plants showed a low number of shrivelled pollen grains, comparable to control plants, indicating that a wild-type copy of *PI4Kα1* is required for transmission through the male gametophyte and normal pollen morphology **(Figure 2A and B)**. Together, these results show that *PI4Kα1* is an essential gene for pollen development.

Reciprocal Crosses						
Gene	Allele	Cross		% of resistant plants	% expected	n
		Female	Male			
PI4Kα1	*pi4kα1-1*	*pi4kα1-1 +/-*	Col0	39	50	424
		Col0	*pi4kα1-1 +/-*	0	50	268
	pi4kα1-2	*pi4kα1-2 +/-*	Col0	9,3	50	106
		Col0	*pi4kα1-2 +/-*	0	50	293
NPG1	*npg1-1*	*npg1-1 +/-*	Col0	40,6	50	214
		Col0	*npg1-1 +/-*	0	50	163
	npg1-2	*npg1-2 +/-*	Col0	30,8	50	26
		Col0	*npg1-2 +/-*	0	50	111
HYC1	*hyc1*	*hyc1 +/-*	Col0	47	50	185
		Col0	*hyc1 +/-*	0	50	58
HYC2	*hyc2-2*	*hyc2-2 +/-*	Col0	29,6	50	54
		Col0	*hyc2-2 +/-*	29,8	50	47
	hyc2-3	*NO RESISTANCE*				
ELP3 ELP4	*elp3-1 elp4-2*	*elp3-1 +/- elp4-2-/-*	Col0	55,7	50	97
		Col0	*elp3-1 +/- elp4-2-/-*	2,2	50	45
	elp3-2 elp4-2	*elp3-2 +/- elp4-2-/-*	Col0	32,4	50	68
		Col0	*elp3-2 +/- elp4-2-/-*	0	50	31
	elp3-1 elp4-4	*elp3-1 +/- elp4-4-/-*	Col0	48,1	50	27
		Col0	*elp3-1 +/- elp4-4-/-*	ND	50	ND
	elp3-2 elp4-4	*elp3-2 +/- elp4-4-/-*	Col0	50	50	12
		Col0	*elp3-2 +/- elp4-4-/-*	0	50	6

Table 2: Segregation analyses of reciprocal crosses between wild-type and the indicated mutants. n represent the number of seedling analysed.

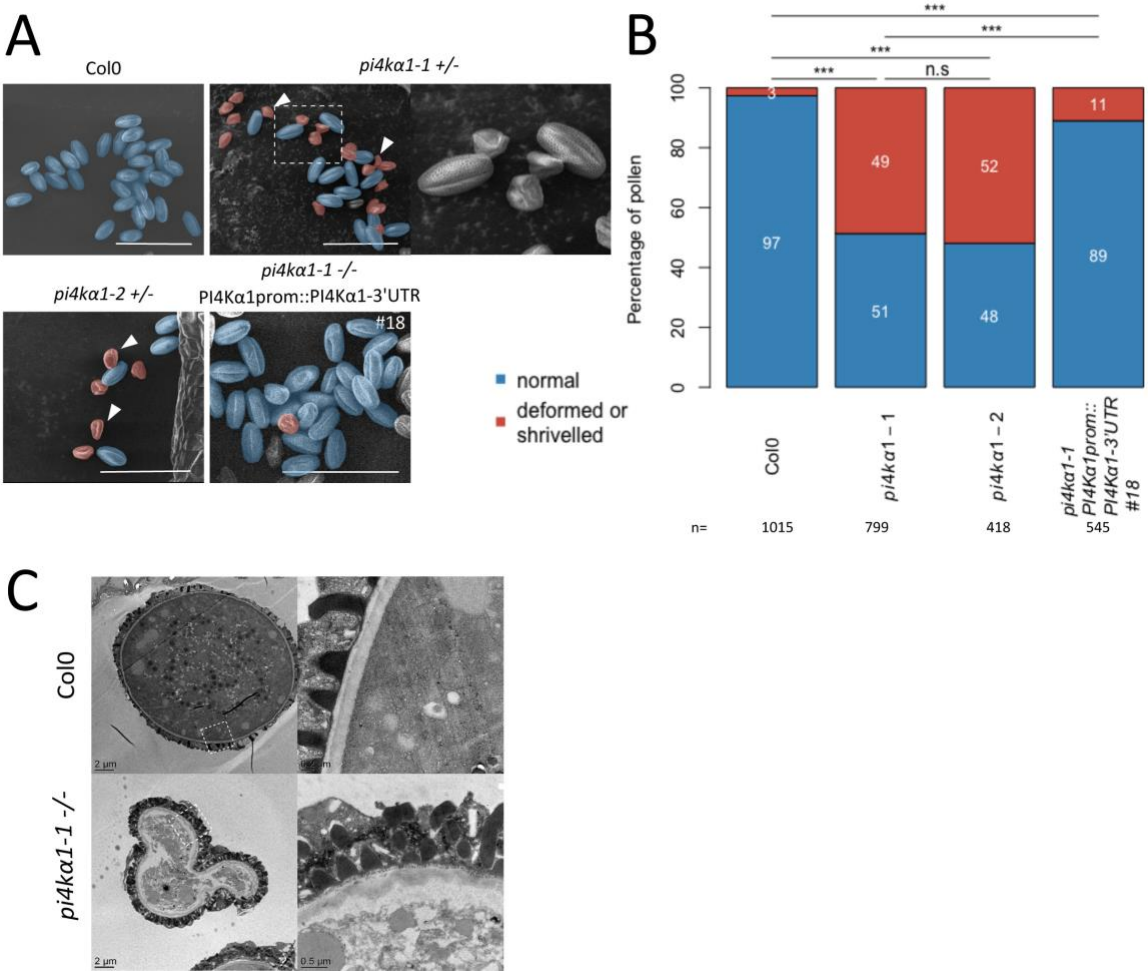

Figure 2. Analysis of the *pi4kα1* pollen phenotype. (A) Scanning electron microscope micrograph of pollen grains from Col-0, self-fertilized *pi4ka1-1* heterozygous plants, self-fertilized *pi4ka1-2* heterozygous plants, and self-fertilized *pi4ka1-1* homozygous plants expressing *PI4Ka1prom::PI4Ka1-3'UTR* (insertion n°18). Shrivelled pollen grains are coloured in red and normal pollen grains are coloured in blue. Close-up is shown for *pi4ka1-1* pollen on the right. Scale bar: 50 µm **(B)** Quantification of the pourcentage of normal (blue) versus deformed/shrivelled (red) pollen grains from Col-0, self-fertilized *pi4ka1-1* heterozygous plants, self-fertilized *pi4ka1-2* heterozygous plants, and self-fertilized *pi4ka1-1* homozygous plants expressing *PI4Ka1prom::PI4Ka1-3'UTR* (insertion n°18). n indicates the number of pollens counted for each genotype. Statistics used chi-square test. n.s, non-significant; ***, p<0.001. **(C)** Observation of Col-0 and *pi4ka1-1* shrivelled pollen grains by transmission electron microscopy. Right panels show close–up of the region indicated on the left panel.

Disturbing subunits of the PI4Kα1 complex mimics pi4kα1 pollen phenotypes

Next, we isolated single mutants for all the genes encoding for *NPG*, *HYC* and *EFOP* subunits in order to ask whether they would recapitulate *pi4kα1* loss-of-function phenotype **(Table S2)**.

The *npg1* mutant was previously published as not able to germinate giving its name NO POLLEN GERMINATION to the family (Golovkin and Reddy, 2003). We reproduced this result by characterizing two new T-DNA mutant alleles of *NPG1*. The self-progeny of *npg1-1+/-* and *npg1-2+/-* had segregation rate of 50,3% and 32% resistant seedlings, respectively, indicating gamete lethality **(Table 1)**. Reciprocal crosses confirmed their male sterility phenotype, with 0% of transmission of the mutation through the pollen, while the female

gametophyte might be affected only for the second allele with a weak distortion of the segregation rate **(Table 2)**. However, the observation of *npg1-1* and *npg1-2* pollen grains by SEM did not show any morphological defect, unlike *pi4kα1* pollens **(Figure 3A-B; Supplemental Figure 3A-B)**. The reintroduction of *NPG1* fused with mCITRINE under the control of its own promoter complemented the male sterility in *npg1-2* background leading to *npg1-2* homozygous plants **(Supplemental Figure 3C)**. Similarly, the expression of NPGR2 fused to the mCITRINE under the control of the *NPG1* promoter also complemented *npg1-2* male sterility. These experiments indicate that NPGR2 can substitute for NPG1 function in pollen and that both NPG1-mCITRINE and NPGR2-mCITRINE fusion are fully functional **(Supplemental Figure 3C)**.

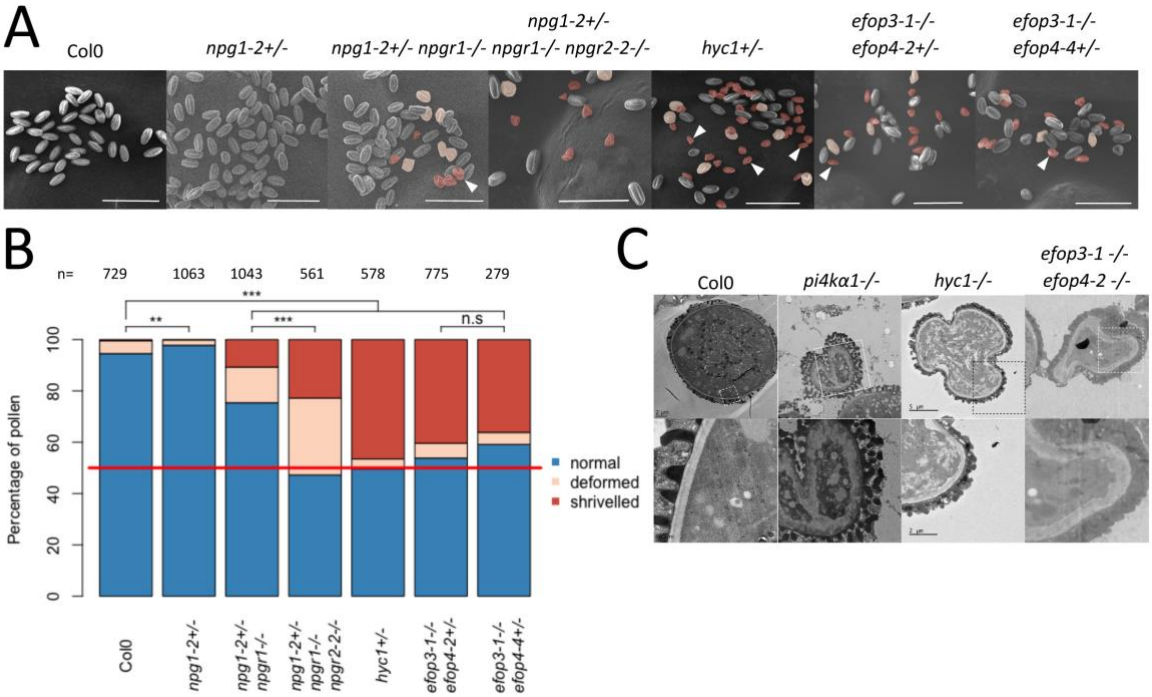

Figure 3. Analysis of the phenotype of indicated mutants from the *npg, hyc* and *efop* families. (A) Pollen grains observed by scanning electron microscopy of self-fertilized Col-0, *npg1-2 +/-, npg1-2+/- npgr1-/-, npg1-2+/- npgr1-/- npgr2-2-/-, hyc1+/-, efop3-1-/-efop4-2+/-* and *efop3-1-/-efop4-4+/-* plants. Deformed pollens and shrivelled pollens are colored in red and orange, respectively. Scale bar: 50 µm **(B)** Quantification of % of normal (blue), deformed (orange) and shrivelled (red) pollen grains from self-fertilized Col-0, *npg1-2 +/-, npg1-2+/- npgr1-/-, npg1-2+/- npgr1-/- npgr2-2-/-, hyc1+/-, efop3-1-/-efop4-2+/-* and *efop3-1-/-efop4-2+/-* plants. n indicates number of pollens counted for each genotype. Statistics used chi-square test. n.s, non-significant; ***, p<0.001. **(C)** Observation of pollen grains from self-fertilized Col0, *pi4kα1-1, hyc1* and *efop3-1-/-efop4-2-/-* by transmission electron microscopy. Lower panel shows close–up of region indicated on upper panel.

Because, NPGR2 can substitute for NPG1 in pollen, we speculated that a certain degree of functional redundancy between NPG1, NPGR1 and NPGR2 or compensatory effects during pollen development could lead to the weaker phenotype of *npg1* pollen compared to *pi4kα1* pollen and thus explain why *npg1* pollen did not present morphological defect by SEM. To test this hypothesis we generated higher order mutant combination within the *NPG* family **(Table S2)**. The *npg1-2+/- npgr1-/-* mutant combination presented about 10% of *pi4kα1*-like shrivelled pollen grains while *npgr1npgr2-1* and *npgr1npgr2-2* double homozygous mutants

displayed about 25% of deformed (but not shrivelled) pollen grains **(Figure 3A- B; Supplemental Figure 3A-B)**. Finally, the *npg1-2+/-npgr1-/-npgr2-1-/-* and *npg1-2+/-npgr1-/-npgr2-2-/-* displayed about 35 and 50% of deformed and shrivelled pollen grains, respectively. These data indicate that combinations of *npg* mutants partially mimic *pi4kα1* pollen phenotype.

Next, we addressed the loss-of-function phenotypes of *HYCCIN* family members. The self-progeny of a *hyc1+/-* single mutant presented a segregation rate around 50% indicating a gametophytic lethality **(Table 1)**. As *HYC1* expression is restricted to pollen, we were expecting that the segregation bias was caused by defects of the male gametophyte. As anticipated, reciprocal crosses showed male sterility while the T-DNA transmission through the female gametophyte was not affected **(Table 2)**. Observation of *hyc1+/-* pollen grains by SEM revealed that half of the pollen grains were shrivelled **(Figure 3A and B)**. In addition, transmission electron microscopy also showed a thickening of the cell wall of the *hyc1* mutant pollen grains, which was similar to the phenotype observed for *pi4kα1-1* **(Figure 3C)**. Finally, the male sterility, as well as the pollen morphological defects, were complemented by the reintroduction of *HYC1prom::HYC1-mCITRINE* **(Supplemental Figure 3A-B and D)**. All together, these data show that *hyc1* knockout phenotype fully mimic *pi4kα1* knockout regarding pollen development.

None of the *efop* single mutant presented any pollen morphological defects or distortion of segregation likely because of redundancy between the four members of this family **(Supplemental Figure 3B)**. We thus generated all the possible combinations of double mutants **(Supplemental Figure 3B; Table 3)**. We were able to obtain *efop2efop3* double homozygous mutants, suggesting no strong synthetic lethality. However, they presented from 19% to 25% of shrivelled pollen grains, resembling those of the *pi4kα1* and *hyc1* mutants, and from 43% to 65% of deformed pollens (resembling those of *npgr1npgr2* double mutants). Thus, depending on the alleles, these double mutant combinations presented from 70% to 90% of abnormal pollens **(Supplemental Figure 3A and B)**. In addition, it was not possible to generate *efop3efop4* double homozygous mutant no matter the alleles used. Indeed, reciprocal crosses indicated 0% of transmission of *efop3* mutant allele when *efop3+/-efop4-/-* plants were used as male, revealing that *efop3efop4* pollens were lethal **(Table 2)**. SEM showed that about 45% of the pollen grains present abnormal morphology **(Figure 3A and B; Supplemental Figure 3A and B)**. Finally, observation of *efop3-1efop4-2* shrivelled pollen grain by electron transmission microscopy revealed a thick cell wall similar to the phenotype observed for *pi4kα1-1* and *hyc1*. *efop2efop3* and *efop3efop4* double mutants mimic partially and fully *pi4kα1* and *hyc1* pollen phenotypes, respectively. Altogether, our genetic analyses indicate that all the protein classes in the putative PI4Kα1 complex are essential for the male gametophyte in *Arabidopsis* and that certain mutant combinations in the *NPG*, *HYC* or *EFOP* families either fully (*hyc1+/-*, *efop3+/-efop4-/-)* or partially (*npg1+/-*, *npg1+/-npgr1-/-*, *npgr1-/-npgr2-/-*, *npg1-2+/-npgr1-/-npgr2-2-/-*, *efop2efop3-/-*) mimic the *pi4kα1* phenotype. Thus, these proteins likely act as a single protein complex in plants.

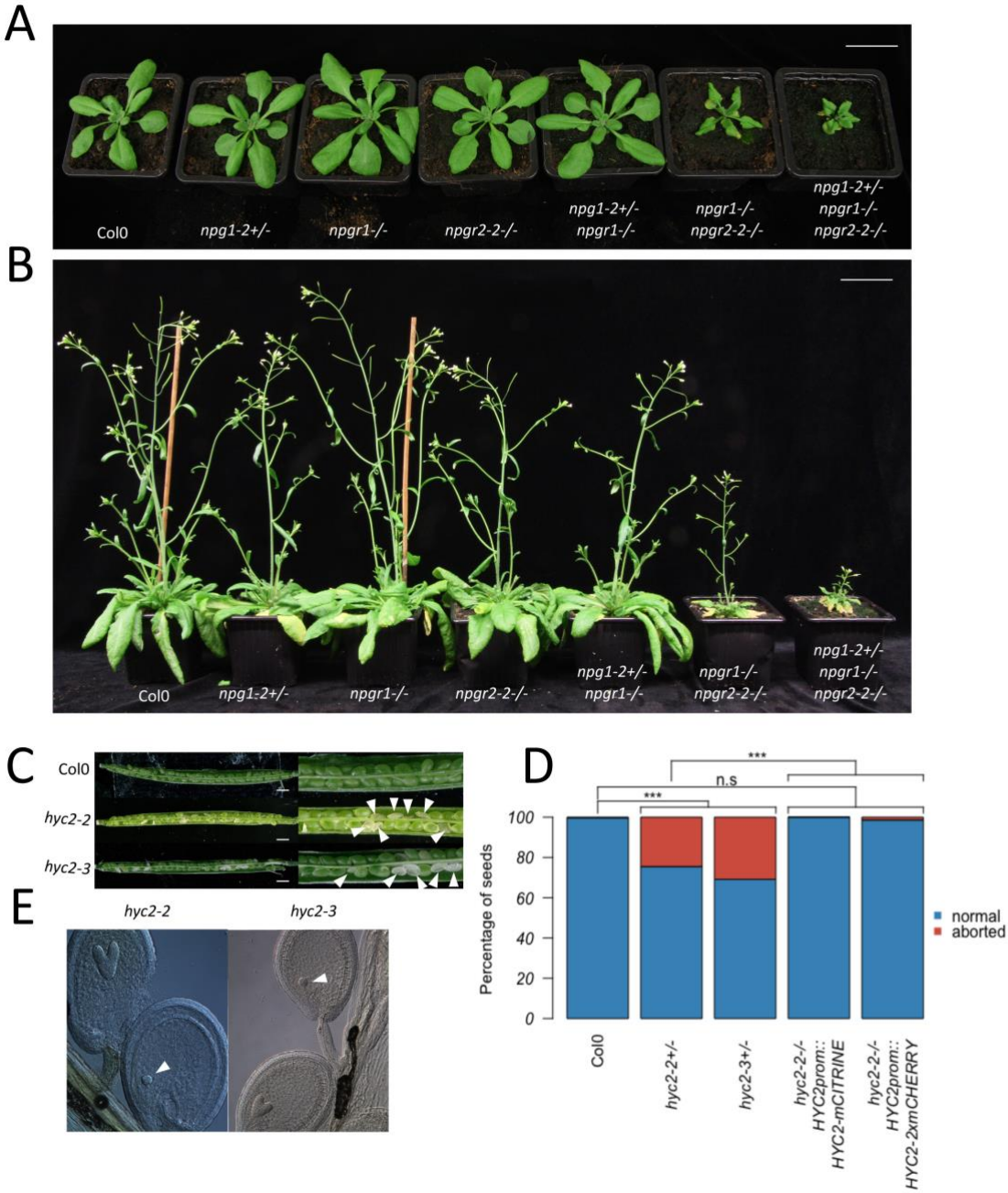

Figure 4. Sporophytic phenotypes induced by mutations of the PI4Kα1 complex (A) Twenty seven-days-old Col-0, *npg1-2+/-, npgr1-/-, npgr2-2-/-, npg1-2+/- npgr1-/-, npgr1-/- npgr2-2-/-* and *npg1-2+/- npgr1-/- npgr2-2-/-* plants. Scale bar: 2 cm **(B)** Forty-one-days-old Col-0, *npg1-2+/-, npgr1-/-, npgr2-2-/-, npg1-2+/- npgr1-/-, npgr1-/- npgr2-2-/-* and *npg1-2+/- npgr1-/- npgr2-2-/-* plants. Scale bar: 2cm **(C)** Opened siliques of self-fertilized Col-0, *hyc2-2* and *hyc2-3* heterozygous mutant plants. White arrowheads indicate aborted seeds. **(D)** % of aborted seeds in Col0, *hyc2-2+/-, hyc2-3+/-, hyc2-2-/- HYC2prom::HYC2-mCITRINE* (insertion n°10), and *hyc2-2-/- HYC2prom::HYC2-2xmCHERRY* (insertion n°11) siliques. The number of seed counted is superior at 250 for each genotype. Statistics used chi-square test. n.s, non-significant; ***, p<0.001. **(E)** Cleared seeds from *hyc2-2* and *hyc2-3* heterozygous mutant plants. White arrowheads indicate globular embryo that have stopped development.

Disturbing the PI4Kα1 complex results in various sporophytic phenotypes

The pollen lethality of *pi4kα1* knockout did not allow us to further study the role of PI4Kα1 in plant development. However, combinations of *npg* mutants presented growth defect phenotypes. Indeed, *npgr1npgr2-2* double mutant and *npg1+/-npgr1-/-npgr2-1-/-* present a mild growth phenotype while *npg1+/-npgr1-/-npgr2-2-/-* is dwarf **(Figure 4A-B; Supplemental Figure 4A-B)**. Reintroduction of *NPGR1prom::NPGR1-mCITRINE* was able to rescue the growth phenotype of *npgr1npgr2-2* double mutant **(Supplemental Figure 4C)**. This suggests that the PI4Kα1 complex is essential not only for pollen development but also for other developmental and growth processes.

While *HYC1* is specifically expressed in pollen and is male sterile, *HYC2* is predicted to be expressed in the sporophyte. We characterized two T-DNA alleles corresponding to two putative *hyc2* loss-of-function mutants. The segregation rate of *hyc2-2* heterozygous plants was of 60% **(Table 1)**. Moreover, it was not possible to retrieve homozygous plants in the self-progeny of both *hyc2-2* and *hyc2-3*. Reciprocal crosses indicated a transmission of the allele through the male and the female gametophytes even if a weak distortion could be observed in both cases **(Table 2)**. Siliques from *hyc2-2* and *hyc2-3* heterozygous plants presented around 25% to 30% of aborted seeds **(Figure 4C-D; Supplemental Figure 4D)**. Observations of the embryo after clearing showed that in those siliques, some embryo stopped their development at the globular stage before degenerating while the rest of the embryos pursued their development normally **(Figure 4E)**. This phenotype was lost and homozygous mutant plants were obtained when HYC2-mCITRINE or HYC2-2xmCHERRY were reintroduced under the control of the *HYC2* promoter **(Figure 4D; Supplemental Figure 4D-E)**. Thus, the loss of HYC2 leads to embryo lethality at the globular stage, suggesting that HYC1 is essential for the male gametophyte, while HYC2 is essential for sporophytic development. These results are consistent with the idea that the four-subunit PI4Kα1 complex is essential in plants.

The PI4Kα1 complex is associated with the plasma membrane

To confirm the localization of PI4Kα1 at the plasma membrane, we first raised stable transgenic lines expressing PI4Kα1 tagged with mCITRINE at either its N- or C-terminal ends and the red fluorescent protein 2xmCHERRY at the C-terminal end under the control of either its own promoter or the *UBQ10* promoter. Consistent with the hypothesis that PI4Kα1 acts at the plasma membrane, the three constructs mCITRINE-PI4Kα1, PI4Kα1-mCITRINE, and PI4Kα1-2xmCHERRY localized at the plasma membrane and in the cytosol **(Figure 5A)**. However, the introduction *PI4Kα1::PI4Kα1-mCITRINE*, *PI4Kα1::mCITRINE-PI4Kα1* or *PI4Kα1::PI4Kα1-2xmCHERRY* constructs in the *pi4kα1-1+/-* mutant background failed to complement the pollen lethality and we never recovered *pi4kα1-1-/-* plants **(data not shown)**. We used the same *PI4Kα1* promoter used for the rescue experiment with the untagged *PI4Kα1* **(Figure 2B-C; Supplemental Figure 2A)**, suggesting that PI4Kα1 fused with a fluorescent protein is non-functional. Expression of *PI4Kα1* fused to smaller tags (i.e. PI4Kα1-6xHA or Flag-PI4Kα1) also failed to complement *pi4kα1-1* **(data not shown)**.

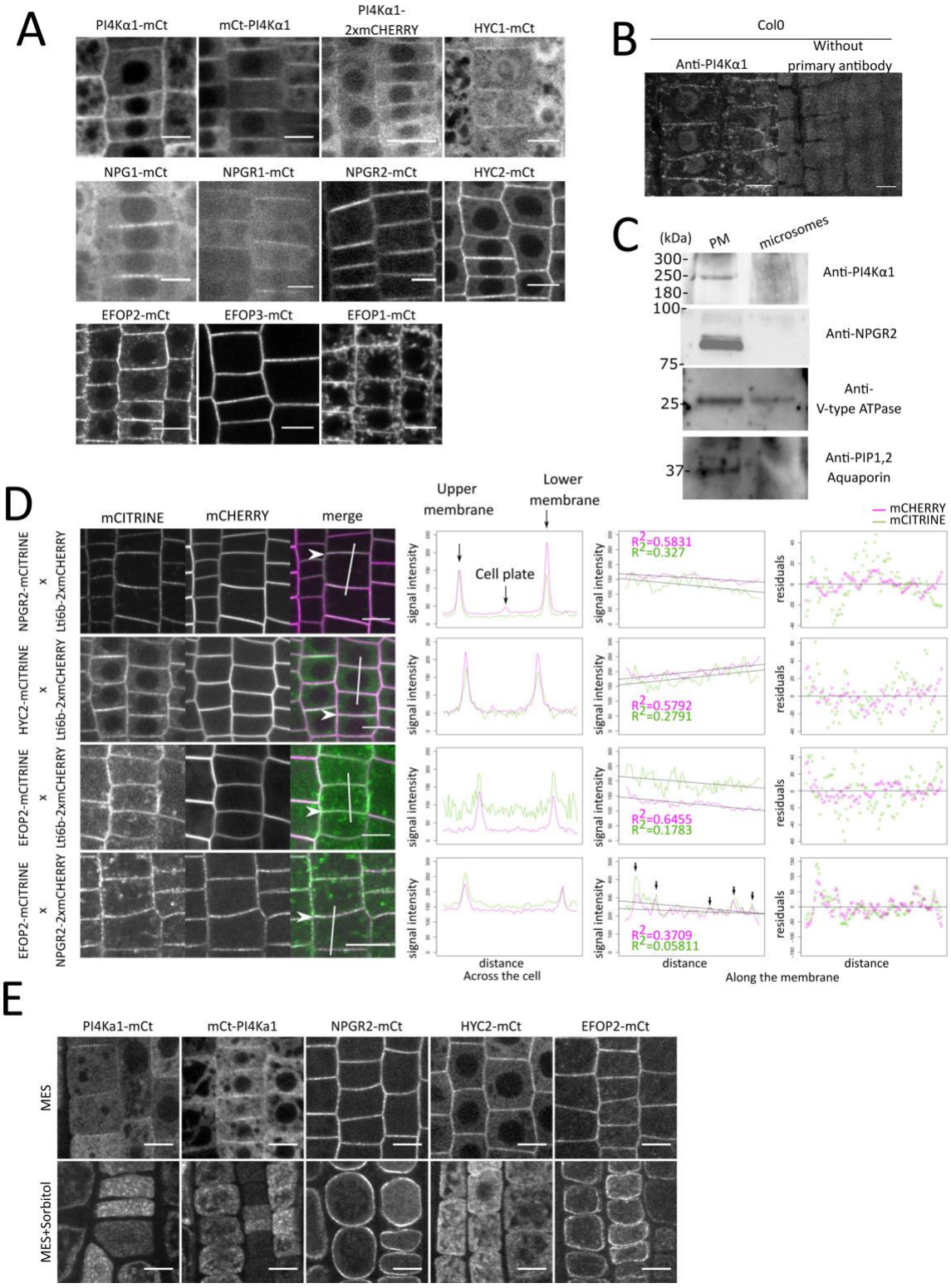

Figure 5. All the members of the PI4Kα1 complex localize at the plasma membrane. (**A)** Confocal images of PI4Kα1, NPG1, NPGR1, NPGR2, HYC1, HYC2, EFOP1, EFOP2 and EFOP3 fused to mCITRINE (mCt) or mCHERRY under control of the *UBQ10* promoter in root epidermal cells. Scale bars: 10μ m. **(B)** Confocal images of PI4Kα1 using an anti-PI4Kα1 antibody in epidermal root cells on WT seedlings. Control background without primary antibody is shown. Scale bar: 10 μm. **(C)** Western blot using anti-PI4Kα1, anti-NPGR2, anti Anti-V-type ATPase, and anti-PIP1,2 aquaporin antibodies on plasma membrane and microsomal fractions from WT seedlings. **(D)** Confocal images of seedlings co-expressing Lti6b-2xmCHERRY (under control of *2x35S* promoter), NPGR2-mCITRINE, HYC2-mCITRINE or EFOP2-mCITRINE (under control of *UBQ10* promoter). Graphics on the left represent intensity of each signal across the cell along the white line. Graphics in the middle represent intensity of

each signal along the membrane indicated by white arrow. pics with matching signal intensities are indicated by black arrows. Linear regression and adjusted R square for each signal are indicated. Graphics on the right represent residuals in y between signal along the membrane and linear regression. **(E)** Confocal images of PI4Kα1-mCt, mCt-PI4Kα1, NPGR2-mCt, HYC2-mCt, EFOP2-mCt under control of *UBQ10* promoter in root epidermal cells in control (MES) and during plasmolysis (MES+Sorbitol). Scale bars: 10μm.

To confirm the localization obtained with mCITRINE fusion, we used the antibodies against the native PI4Kα1 and performed whole mount immunolocalization in roots. Similar to the mCITRINE-PI4Kα1 and PI4Kα1-mCITRINE fusions, we observed again a signal at the plasma membrane **(Figure 5B)**. We also noticed signal in the cytosol and in nuclei **(Figure 5B)**. To further confirm the preferential association of PI4Kα1 with the plasma membrane, we used cellular fractionation and PEG/Dextran phase partition of whole seedling and compared the signal obtained on a purified plasma membrane or whole microsomal fractions. We confirmed the presence of proteins in the two fractions using an antibody against V-type ATPase. The purity of the plasma membrane fraction was evaluated with antibodies against the PIP1,2 aquaporin, a known plasma membrane resident protein **(Figure 5C)**. When loading the same amount of protein in each fraction, this experiment revealed the presence of a band around 225kDa, corresponding to PI4Kα1 in the plasma membrane fraction and only a very faint signal in the total microsomal fraction, showing that PI4Kα1 is enriched in the plasma membrane fraction **(Figure 5C)**. Together, fluorescent fusion, immunolocalization and cellular fractionation show that PI4Kα1 is associated with the plasma membrane.

We next addressed the subcellular localization of NPG, HYC and EFOP proteins at the plasma membrane. NPG1 was previously found to be an extracellular protein in pollen grains (Shin et al., 2014). In our hand, NPG1-mCITRINE, NPGR1-mCITRINE and NPGR2-mCITRINE all localized at the periphery of the cell in root meristem **(Figure 5A)**. To distinguish between the plasma membrane and cell wall, we co-expressed NPGR2-mCITRINE with Lti6b-mCHERRY. We observed that the two signals perfectly colocalized indicating that NPGR2 is present at the plasma membrane **(Figure 5D)**. Furthermore, we performed plasmolysis of the epidermal root cell by addition of sorbitol. In this context, the plasma membrane detaches from the cell wall. We observed that the signal of NPGR2-mCITRINE remains at the plasma membrane and is not present in the cell wall **(Figure 5E)**. Moreover, in western blot using an anti-NPGR2 antibody, NPGR2 was found enriched in the plasma membrane fraction compared to the microsomal fractions **(Figure 5C)**.

Similarly, HYC1-mCITRINE, HYC2-mCITRINE, EFOP1-mCITRINE, EFOP2-mCITRINE and EFOP3-mCITRINE expressed under the control of the *UBQ10* promoter were found at the plasma membrane **(Figure 5A and D)**. In addition to the plasma membrane localization, we noticed that EFOP1 and EFOP2 were associated with intracellular compartments, which were more prominently labelled for EFOP1 than EFOP2. Also, NPG1 and HYC1 were highly soluble in the cytosol. As HYC1 and NPG1 are normally mainly expressed in pollens, they might need each other to localize at the plasma membrane. This could explain why when we overexpressed them alone in root epidermal cells, we saw most of the proteins in the cytosol.

Upon plasmolysis, PI4Kα1-mCITRINE, mCITRINE-PI4Kα1, HYC2-mCITRINE and EFOP2-mCITRINE signals are found inside the cell and absent from the cell wall. EFOP2 remained associated with the plasma membrane while PI4Kα1 and HYC2 seem to delocalize to internal compartments **(Figure 5E)**. In any case, all the protein classes in the putative PI4Kα1 complex were associated to some extent with the plasma membrane.

The PI4Kα1 complex is present in plasma membrane nanodomains

Using confocal microscopy, we noticed that for several of the translational reporters of the PI4Kα1 complex, the signal at the plasma membrane was not continuous, raising the question of a possible subcompartimentalization of the proteins. This is notably the case for PI4Kα1-mCITRINE, NPG1-mCITRINE, NPGR2-mCITRINE, HYC2-mCITRINE, EFOP2-mCITRINE, and EFOP3-mCITRINE **(Figure 5A and D and FIGURE 6A)**. Using plant co-expressing Lti6b-2xmCHERRY and NPGR2-mCITRINE, HYC2-mCITRINE or EFOP2-mCITRINE, we observed that NPGR2-mCITRINE, HYC2-mCITRINE and EFOP2-mCITRINE signals along the plasma membrane is less homogeneous than Lti6b, and accumulated in patches of high intensity **(Figure 5D)**. We calculated the linear regression of each signal along the membrane and observed that the R square of NPGR2-mCITRINE, HYC2-mCITRINE and EFOP2-mCITRINE are always smaller than the one of Lti6b-2cmCHERRY indicating a higher dispersion of the intensity. In addition, plants co-expressing NPGR2-2xmCHERRY and EFOP2-mCITRINE show similar intensity pattern with signals partially localized along the plasma membrane **(Figure 5D).** Similarly, when we observed the plasma membrane in tangential sections, NPGR2-2xmCHERRY and EFOP2-mCITRINE subdomains were partially colocalized **(Figure 6A)**. As control, EFOP2-mCITRINE containing plasma membrane domains did not colocalize with the mostly uniformed localization of Lti6b-2xmCHERRY **(Figure 5D and Figure 6A)**. In order to get a better axial resolution, we used Total Internal Reflection Fluorescence Microscopy (TIRF) microscopy and confirmed that NPGR2-mCITRINE, HYC2-mCITRINE and EFOP2-mCITRINE were present in nanodomains of the plasma membrane **(Figure 6B)**.

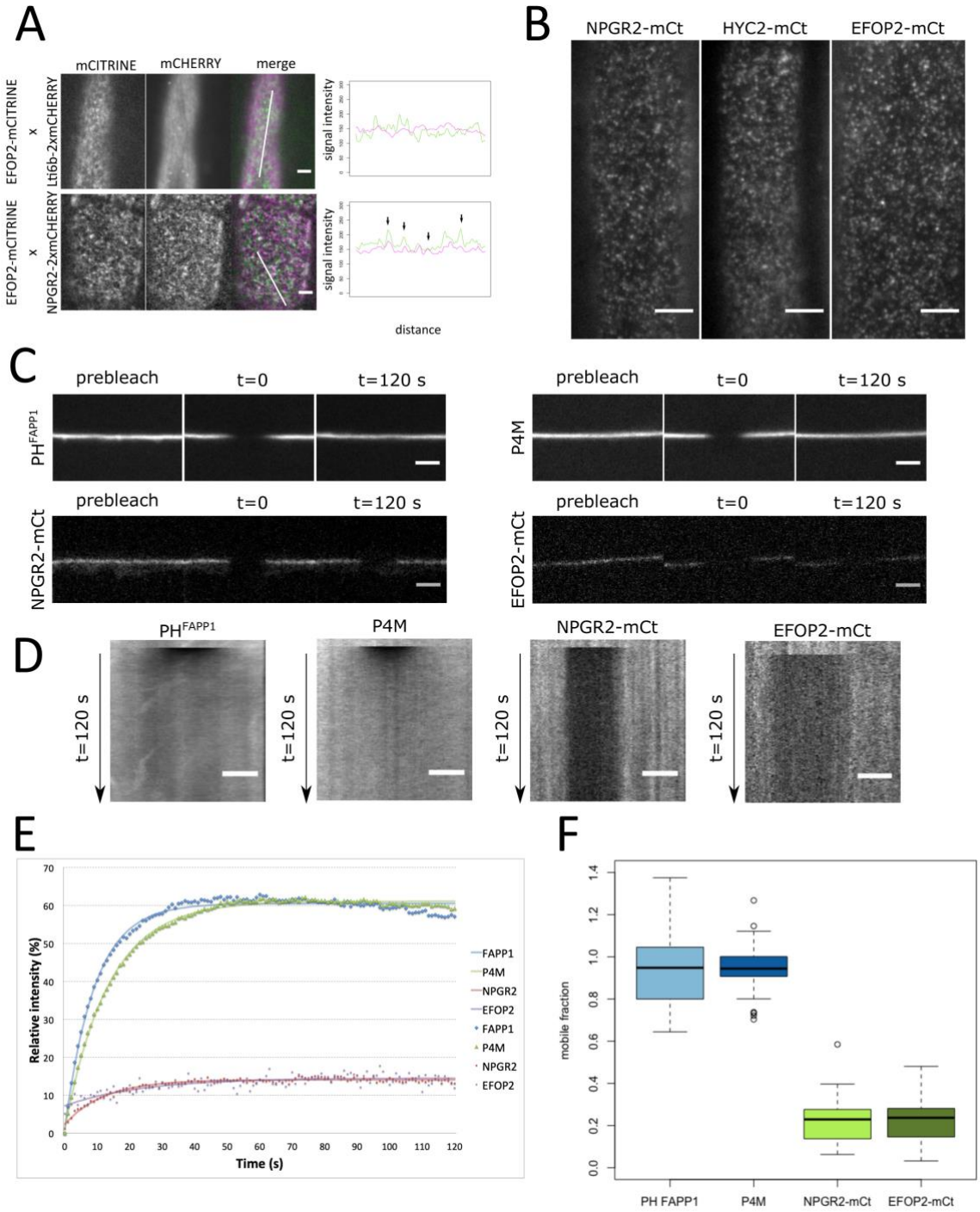

Figure 6. The PI4Kα1 complex localizes is highly static nanodomains at the plasma membrane. (A) Confocal images of seedlings co-expressing Lti6b-2xmCHERRY (under control of the *2x35S* promoter), NPGR2-mCITRINE or EFOP2-mCITRINE (under control of *UBQ10* promoter). Graphics represent intensity of each signal across the cell along the white line. Black arrows indicate matching signals. Scale bars: 5 µm. **(B)** Confocal images of TIRF microscopy of NPGR2-mCt, HYC2-mCt and EFOP2-mCt. Scale bars: 5 µm. **(C)** Confocal images of P4M, PH^{FAPP1}, NPGR2-mCt and EFOP2-mCt before photobleaching (prebleach) and 120s after photobleaching. Scale bars: 5 µm. **(D)** Kymographs along the membrane for the time laps in (C). Scale bars: 5 µm. **(E)** Graphic presenting the recovery of the signal intensity over time after bleaching. The number of zones measured is 37, 32, 30 and 13 for P4M, PH^{FAPP1}, NPGR2-mCt and EFOP2-mCt, respectively. The fitting curves are represented. **(F)** Graphic presenting the mobile fraction of P4M, PH^{FAPP1}, NPGR2-mCt and EFOP2-mCt after 2min postbleaching taking in account the inherent bleaching due to imaging.

PI4Kα1 complex-containing nanodomains are static at the plasma membrane

Next, we investigated the lateral dynamics of the PI4Kα1 complex at the plasma membrane. To do so, we used fluorescence recovery after photobleaching (FRAP). After bleaching, the signal of NPGR2-mCITRINE, HYC2-mCITRINE and EFOP2-mCITRINE did not recover after 2 min of acquisition **(Figure 6C-E; Supplemental Figure 5)**. In comparison, PI4P sensors (P4M and PH^{FAPP1}) recover in less than a minute after bleaching. Accordingly, the mobile fraction calculated of NPGR2-mCITRINE and EFOP2-mCITRINE was low (around 20%) while the mobile fraction of the sensor reached 100% **(Figure 6F)**. This indicates that if PI4P sensors are rapidly diffusing at the membrane, the PI4Kα1 complex is relatively static. Furthermore, the identical dynamics of NPGR2-mCITRINE, HYC2-mCITRINE and EFOP2-mCITRINE further reinforce the notion that these subunits are part of a single protein complex in vivo.

EFOPs localize at the plasma membrane via a lipid anchor

We next decided to investigate the mechanism by which the PI4Kα1 complex is targeted at the plasma membrane. The four subunits of the PI4Kα complex are soluble proteins, without known lipid binding domains. The *efop3-1efop4-4* and *efop3-2efop4-4* mutants showed the same pollen lethality phenotype as the *efop3-1efop4-2* and *efop3-2efop4-2* mutant **(Figure 3A-B, Supplemental Figure 3A-B)**. While *efop4-2* led to a very small-truncated protein (42 aa), the *efop4-4* allele led to near full-length protein with only a small in frame deletion of 19 residues close to EFOP4 N-terminus. This suggested that this N-terminal region is crucial for EFOP4 function **(Figure 7, A)**.

The residues corresponding to this deletion are well conserved among the four EFOPs and include both a cysteine-rich motif, which could be S-acylated, and a polybasic region, which could contact anionic lipids at the plasma membrane **(Figure 7A)**. We thus tested the potential role of those two elements in the regulation of EFOP localization and potentially the recruitment of the PI4Kα1 complex at the plasma membrane.

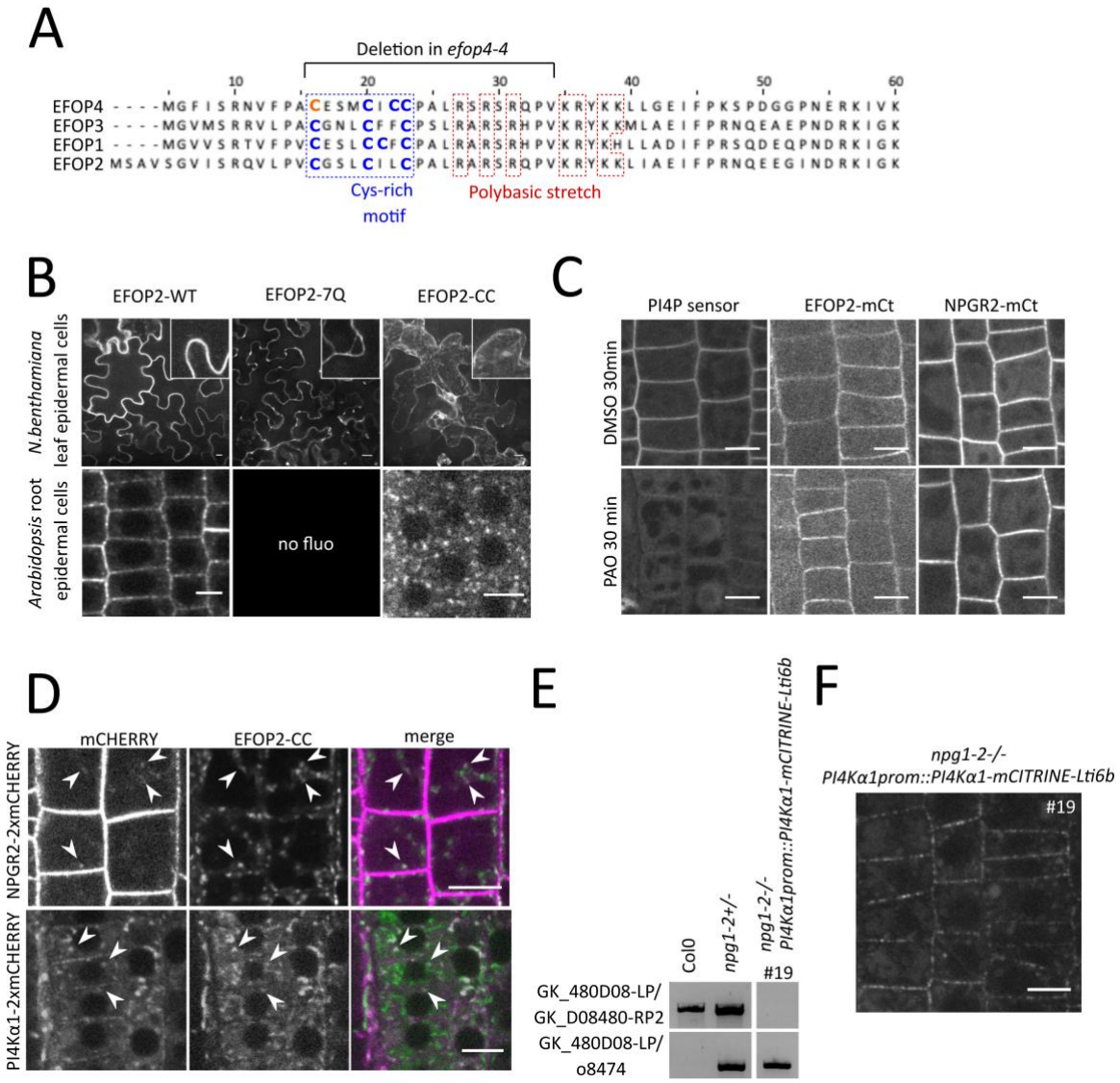

Figure 7. PI4Kα1 is targeted to the plasma membrane by a combination of lipid anchoring of the EFOP subunits and scaffolding activity of the NPG subunits. (A) N-terminal sequence alignment of EFOPs proteins. Conserved cys-rich motif (blue) and polybasic patch (red) are indicated, as well as, the deletion in *efop4-4* CrisPr allele. Bold cysteines are predicted as S-acetylated on the SwissPalm database with high (blue) and medium (orange) confidence level. **(B)** Confocal images of EFOP2-mCITRINE (wild type), EFOP2-7Q-mCITRINE and EFOP2-CC-mCITRINE in N. benthamiana leaf epidermal cells and Arabidopsis root epidermal cells. Scale bar: 10 µm. **(C)** Confocal images of PI4P sensor (PH domain of FAPP1 carrying the mutation E50A), EFOP2-mCITRINE (EFOP2-mCt) and NPGR2-mCITRINE (NPGR2-mCt) treated for 30min with 30µM PAO or the equivalent volume of DMSO. Scale bars: 10 µm. **(D)** Confocal images of Arabidopsis root epidermal cells co-expressing *EFOP2-CC-mCITRINE* and *NPGR2-2xmCHERRY* or *PI4Ka1-2xmCHERRY*. White arrows indicate internal structures where the two signals colocalize. Scale bar: 10 µm. **(E)** Genotyping of Col0, *npg1-2* heterozygous plants and *npg1-2* homozygous plants complemented with *PI4Ka1prom::PI4Ka1-mCITRINE-Lti6b* (insertion n°19). Upper panel shows amplification of the gene sequence. Lower panel shows amplification of T-DNA border. **(F)** Confocal images of *PI4Ka1::PI4Ka1-mCITRINE-Lti6b* in *npg1-2-/-* background (insertion n°19).

First, we evaluated the role of the polybasic patch in the N-terminus of EFOP proteins. Indeed, this region could be involved in targeting the entire PI4Kα1 complex to the plasma membrane through electrostatic interactions with anionic lipids, notably PI4P. In EFOP2, this region goes from the aa 27 to the aa 39 and contains 7 positively charged residues (**Figure**

7A). We mutated all lysines/arginines into neutral glutamines and generated a *UBQ10prom::EFOP2-7Q-mCITRINE* construct. We observed that EFOP2-7Q-mCITRINE was soluble when transiently expressed in *Nicotiana benthamiana* leaf cells while wild-type EFOP2-mCITRINE was localized to the plasma membrane indicating that polybasic patch in EFOP2 could be essential for plasma membrane targeting **(Figure 7B)**. We next introduced the *UBQ10prom::EFOP2-7Q-mCITRINE* construct in *Arabidopsis* epidermal root cells. However we did not retrieve any lines with a detectable fluorescent signal. It is likely that *EFOP2-7Q-mCITRINE* is unstable either because of miss folding or because EFOP2 needs to be associated with membrane to remain stable when expressed in *Arabidopsis*. Finally, we directly investigated the role of PI4P in the recruitment of the PI4Kα1 complex, by using PAO, a PI4K inhibitor. In this condition, the PI4P sensor is detached from the plasma membrane and relocalized in the cytosol **(Figure 7C)**. However, neither NPGR2-mCITRINE nor EFOP2-mCITRINE were mislocalized upon PAO treatment. Thus, PI4P might not be involved in the targeting of the PI4Kα1 complex at the plasma membrane or the depletion of PI4P is not sufficient to delocalize the PI4Kα1 complex. In any case, this indicates that the presence of the PI4Kα1 complex at the plasma membrane relies, at least in part, on another mechanism.

We then investigated the role of the Cys-rich motif, which was deleted in the *efop4-4* allele. Such motif could be a site of S-Acylation; a lipid posttranslational modification that can anchor protein to the plasma membrane (Zaballa and Goot, 2018). Indeed, according to the SwissPalm prediction software, this motif is predicted as S-acetylated with a high (in blue) or medium level of confidence (in orange) **(Figure 7A)**. Confirming this hypothesis, all four *Arabidopsis* EFOP proteins were found to be S-Acylated in a recent proteomic study (Kumar et al., 2020). Notably, all Cys-residues (underlined in Figure 7, A) within the Cys-rich region of EFOP3 and EFOP4 were found to be S-acylated with high confidence *in planta* (Kumar et al., 2020). To experimentally test the importance of such lipid modification in EFOP localization, we mutated the two conserved cysteines (C20 and C23) into serine and generated a *UBQ10prom::EFOP2-CC-mCITRINE* construct. Similar to EFOP2-7Q-mCITRINE, we observed that EFOP2-CC-mCITRINE was soluble when transiently expressed in *Nicotiana benthamiana* leaf cells **(Figure 7B)**. Next, we transformed the *UBQ10prom::EFOP2-CC-mCITRINE* construct into *Arabidopsis* and found that EFOP2-CC-mCITRINE was not localized at the plasma membrane of root meristematic cells and instead accumulated in intracellular structures **(Figure 7B)**. All together, this data suggest that EFOP2 is likely targeted to the plant plasma membrane using lipid acylation anchoring.

The EFOP/NPG/HYC complex targets PI4Kα1 to the plasma membrane

We then asked if EFOP proteins were sufficient to determine the localization of PI4Kα1 in the cell. Taking advantage of the EFOP2-CC construct localized in intracellular structures, we introgressed NPGR2-2xmCHERRY or PI4Kα1-2xmCHERRY in plants expressing EFOP2-CC-mCITRINE and analyzed if EFOP2-CC was able to recruit NPGR2 or PI4Kα1 in those intracellular structures. We observed a weak signal of NPGR2 labelling intracellular compartments that partially colocalize with EFOP2-CC-containing structures. Similarly, PI4Kα1 was not only at the plasma membrane and soluble in the cytosol but also associated

with the EFOP2-CC-containing structures **(Figure 7D)**. This showed that EFOP is able to recruit NPGR2 and PI4Kα1 in different compartments of the cell.

Because NPG proteins likely bridges PI4Kα1 to the membrane-binding EFOP subunits, we reasoned that they should contribute to the targeting of PI4Kα1 at the plasma membrane. To test this hypothesis, we generated a fusion between PI4Kα1-mCITRINE and the transmembrane protein Lti6b in order to artificially target PI4Kα1 at the plasma membrane and thus bypass the role of the NPG/HYC/EFOP complex. We transformed the *npg1-2* mutant with the *PI4Kα1prom::PI4Kα1-mC*ITRINE-*Lti6b* construct, and found a line able to complement the *npg1-2* mutant **(Figure 7E)**. This indicates that *npg1* pollen lethality is likely due to the absence of PI4Kα1 at the plasma membrane during pollen development. In these plants, PI4Kα1-mCITRINE-Lti6b is located in clusters at the plasma membrane while Lti6b is known as being rather homogenously localized at the plasma membrane **(Figure 5D and 7G)**. The chimeric PI4Kα1-mCITRINE-Lti6b proteins might be restricted in clusters by other factors, which indicates that the subcompartimentalization of PI4Kα1 complex might not be an intrinsic property of the complex but rather come from interactions between PI4Kα1 and other lipids or proteins. Furthermore, it might indicate that the subcompartimentalization of PI4Kα1 complex is an essential feature for the proper function of the complex.

Discussion and conclusions

The plant PI4Kα1 complex is essential for cell survival

In this study, we showed that the loss-of-function of the PI4Kα1 leads to lethality of the male gametophyte. Similarly, knockouts of HYC1 and EFOP proteins mimic this phenotype supporting the idea that these proteins act as a complex. Surprisingly, *npg1* single mutant is pollen lethal but do not present the same morphological defects that *pi4kα1* or *hyc1* mutants. The combination of loss of function of NPG1, NPGR1 and NPGR2 gives rise to deformed and shrivelled pollen grains indicating that during pollen development the three NPGs are expressed and partially redundant. NPG1 specifically could be needed for later steps of pollen development and germination explaining the pollen lethality of NPG1 despite the absence of morphological defect. Indeed, pollen apertures are established in distinct membrane domains enriched in PI4P and PI(4,5)P_2 (Lee et al., 2018). Like *npg1* mutant, loss-of-function of *SEC3A*, a gene coding for a subunit of the exocyst complex, is pollen lethal (Bloch et al., 2016; Li et al., 2017). *sec3a* mutant pollen grains do not present morphological defect but do not germinate. SEC3a is recruited at the pollen germination sites and its binding to the plasma membrane depends on positively charged amino acids. Thus, NPG1 could participate in the recruitment of PI4Kα1 at aperture sites, which could be necessary to landmark the germination site, and subsequent recruitment of SEC3a or other proteins. However, we showed that NPGR2 complements the *npg1* phenotype when expressed under the *NPG1* promoter, which suggest that the difference between NPG1 and NPGR2 function is mostly at the transcriptional level.

In addition to gametophytic phenotypes, we also observed that the loss of *HYC2* induces embryo lethality while *npg* triple sesquimutant display severe growth phenotypes. This is concordant with the idea that PI4Kα1 has a critical role for the cell function, not only during gametophytic but also sporophytic development. PI4P is crucial for the plasma membrane surface charge (Platre et al., 2018; Simon et al., 2016). Thus, it is likely that the loss of the PI4Kα1 affects the membrane surface charge and lead to the mislocalization of a whole set of proteins (Barbosa et al., 2016; Noack and Jaillais, 2020; Simon et al., 2016).

The genome of Arabidopsis codes for two other PI4Kinases, PI4Kβ1 and PI4Kβ2 localized at the TGN/EE and at the cell plate (Kang et al., 2011; Lin et al., 2019; Preuss et al., 2006). As the plasma membrane and the TGN/EE are intimately linked through vesicular trafficking, the interdependence of the TGN/EE and the plasma membrane pool of PI4P remained an open question. *pi4kβ1pi4kβ2* double mutant and *pi4kα1* mutant display developmental phenotypes, which indicates that these lipid kinases are not fully redundant. This is coherent with the different subcellular localization of PI4Kβ1/PI4Kβ2 and PI4Kα1 in plant cells. However, only PI4Kα1 is essential in plants, while the *pi4kβ1pi4kβ2* double mutant harbours mild phenotypes. This contrasts with yeast, in which the loss of either *pik1* or *sst4* is lethal (Audhya et al., 2000). It is thus possible that in plants, the lack of PI4Kβs at the TGN is compensated by PI4Kα1 activity, perhaps through the endocytosis of plasma membrane PI4P. In fact, a large portion of the *pi4kβpi4k1β2* double mutant phenotype can be attributed to its function in cytokinesis (Lin et al., 2019). Thus, PI4Kα1 activity is not able to compensate for PI4Kβs function during cell division.

Function of the NPG-HYC-EFOP complex

Our study also showed that PI4Kα1's plasma membrane localization is likely mediated by interactions with NPG and EFOP proteins rather than by a PH domain. At first, this PH domain was thought to localize PI4Kα1 at the plasma membrane through interaction with anionic phospholipids (Stevenson et al., 1998; Stevenson-Paulik et al., 2003; Xue et al., 1999). However, recent cryoEM studies characterized this region as a N-lobe and helical domain involved in the interaction with Ypp1/TTC7 in yeast and human (Dornan et al., 2018; Lees et al., 2017). We found that PI4Kα1's plasma membrane localization depends of the S-acylation of EFOP proteins. Several S-acylated peptides in addition to the one in the N-terminal region have been found in EFOP proteins (Kumar et al., 2020). As S-acylation is a reversible posttranslational modification. Differential S-acylation could be a mechanism explaining the different localization observed for EFOP1 and EFOP2 in the cell.

NPGs are bridging PI4Kα1 and HYC with EFOP proteins. In addition, NPG proteins are annotated as calmodulin (Cam)-binding protein. Indeed, NPG1 can interact with several Cam isoforms in presence of Ca^{2+} and has been suggested to play a role in Ca^{2+} dependent pollen tube germination (Golovkin and Reddy, 2003). Ca^{2+} is also intimately connected with phosphoinositides, membrane properties and endomembrane trafficking (Himschoot et al., 2017). Ca^{2+} can directly bind anionic phospholipids modulating locally membrane electrostatics, preventing or promoting the recruitment of lipid binding proteins, inducing the formation of $PI(4,5)P_2$ clusters and facilitating membrane fusion (Li et al., 2014; McLaughlin

and Murray, 2005). As phosphoinositides can bind Ca^{2+}, diffuse in the membrane and release Ca^{2+} somewhere else, they have been suggested to buffer and modulate Ca^{2+} signalling at the subcellular level. Ca^{2+} is also known to regulate many actors of endomembrane trafficking including regulators of the cytoskeleton, TPLATE complex, ANNEXINs and SYNAPTOTAGMINs (Bürstenbinder et al., 2013, 2017; Carroll et al., 1998; Hepler, 2016; Schapire et al., 2008; Van Damme et al., 2006). For instance, the ER-Plasma membrane tethers protein SYT1 contains C2 domains that bind Ca^{2+} and phosphoinositides (Giordano et al., 2013; Idevall-Hagren et al., 2015; Lee et al., 2019; Pérez-Sancho et al., 2015; Ruiz-Lopez et al., 2020; Yamazaki et al., 2008). As previously mentioned, the PI4Kα1 complex could be localized at ER- Plasma membrane contact sites and participate in the Ca^{2+} signalling at this junction through calmodulin binding.

If EFOPs are anchoring the complex at the membrane and NPGs are bridging PI4Kα1 and EFOPs, the role of HYCCIN in the complex is less clear. In mammals, FAM126A, which has a HYCCIN domain, is involved in the stability of the complex and is used as a scaffold by TTC7 to be shaped around (Dornan et al., 2018). In human, FAM126A mutations lead to severe case of hypomyelination and congenital cataract (Baskin et al., 2016; Miyamoto et al., 2014). However, even complete knockout of FAM126A is not lethal while loss-of-function of PI4KIIIα is (Baskin et al., 2016). This subunit is not present in yeast, suggesting that HYCCIN-domain containing proteins may have an accessory function in the PI4Kα1 complex, rather than an essential one. By contrast, we found that in Arabidopsis both *hyc1* and *hyc2* mutants are lethal as they are required for male gametophyte and embryo development, respectively. Our result thus suggests that HYC is an essential subunit for the function of the PI4Kα1 complex in plants. In human, there are additional HYCCIN-containing proteins, including FAM126B. Our results thus open the possibility that FAM126A is not the only HYCCIN-containing proteins that may contribute to PI4KIIIα function.

Formation and possible function of PI4Kα1-containing nanoclusters

The PI4Kα1 complex localizes at the plasma membrane in nanodomains. In yeast, Stt4 is found in large clusters called PIK patches (Baird et al., 2008). However, the clusters of Stt4 do not correlate with clusters of PI4P that probably diffuse laterally in the membrane. Similarly in *Arabidopsis*, PI4P biosensors are not clustering at the plasma membrane (Simon et al., 2014, 2016; Vermeer et al., 2009). This is in accordance with *in vitro* data showing that PI4P inhibits the catalytic activity of PI4Kα1 (Stevenson-Paulik et al., 2003). In addition, we showed that these nanodomains are immobile in plants, despite the fluidity of the plasma membrane (Jaillais and Ott, 2020). It is possible that unknown interactions with transmembrane protein, cytoskeleton or lipid rafts stabilize the PI4Kα1 complex.

Do these nanodomains correspond to a functional unit? Among many possibilities, they could correspond to ER-plasma membrane contact sites. Indeed, in yeast, Stt4 reside at these contacts (Omnus et al., 2018). Another hypothesis is that they could be a zone of attachment between the plasma membrane and the actin cytoskeleton. PI4Kα1 has been purified from F-actin-rich fraction from carrots and to associate with polymerized F-actin *in vitro* (Stevenson

et al., 1998). Additionally, in yeast, Stt4p is necessary for a proper actin cytoskeleton organization (Audhya et al., 2000; Foti et al., 2001). The two hypotheses are not mutually exclusive. Indeed, the actin disorganization phenotype of stt4 mutant is rescued in yeast by the knockout of Sac1p, a PI4P phosphatase that resides in the ER membrane (Foti et al., 2001). Stt4p and Sac1p together control the PI4P gradient at membrane contact sites (Noack and Jaillais, 2020).

A recent preprint shows that PI(4,5)P_2 is enriched in nanodomains at the plasma membrane in pollen tubes (Fratini et al., 2020). These nanodomains contain PIP5K2 and are involved in actin dynamics. We hypothesize that the PI4Kα1 complex is also targeted to these nanodomains in order to catalyse a phosphorylation cascade from PI to PI4P to PI(4,5)P_2. In this model, PI4Kα1 inhibition by its substrate could help to coordinate the following phosphorylation by PIP5K2. In any case, given the absolute requirement on the PI4Kα1 complex for plant cell survival, deciphering the mechanisms behind the precise spatiotemporal regulation of this complex and the associated function will be a fascinating challenge for the future.

Material and methods

Plant Material

Arabidopsis thaliana, ecotype Columbia (Col0) l was used in this study, except for *pi4kα1-2*, which is in Ws background. The plants expressing *2xp35S::myrimCITRINE-mCITRINE* were obtained from Jaillais et al., 2011. The PI4P sensors lines, *pUBQ10::mCITRINE-1xPH^{FAPP1}* and *pUBQ10::mCITRINE-P4M^{SidM}* were obtained from (Simon et al., 2014, 2016) **(Table S3)**.

In vitro culture conditions

Seeds were surface-sterilized by addition of 4ml of HCl 37% in 100mL of bleach for four hours before plating on Murashige and Skoog (MS, Duchefa Biochemie®) media supplemented with 0.8% plant agar and containing the appropriate antibiotic or herbicide. Glufosinate, Kanamycin, Hygromycin and Sulfadyacin have been used at 10mg.L^{-1}, 50mg.L^{-1}, 30mg.L^{-1} and 75mg.L^{-1} respectively. Plates were placed under continuous white light conditions for 7 to 10 days. Resistant and sensitive seedlings were counted for segregations.

Culture condition on soil

Seeds were directly sown in soil or 7-day-old seedlings were transferred from in vitro plates to soil. Plants were grown at 21°C under long day condition (16 hrs light, LED 150µmol/m^2/s).

Sequence Analysis

Sequence alignments have been performed using the Muscle WS software and edited with Jalview 2.0 software. Clustal colour code has been used. Domains have been identified using the SMART (Simple Modular Architecture Research Tool) software. Predicted lipid modification sites have been found using the GPS-Lipid software.

Cloning of reporter lines

Lti6b/pDONR207, UBQ10prom/pDONR P4-P1r, 2X35S/pDONR P4-P1r, mCITRINE/pDONR 221, mCITRINE/pDONR P2R-P3, 2xmCHERRY-4xmyc/pDONR P2R-P3 entry vectors were obtain from Elsayad et al., 2016; Jaillais et al., 2011; Marquès-Bueno et al., 2016; Simon et al., 2014, 2016 **(Table S4)**. For other entry vectors, coding gene or genomic gene sequences and 3'UTR sequences were cloned using Gateway® technology (Invitrogen®). Promoter sequences and Lti6b-FRB were cloned by Gibson Assembly method (Biolab®). The coding sequences were amplified by PCR using the high fidelity Phusion Hot Start II (Thermo Fisher Scientific®) taq polymerase and the indicated primers and template **(Table S5)**. The PCR products were purified using the NucleoSpin Gel and PCR Clean kit (Macherey-Nagel®) and recombined in the indicated vector using BP Clonase™ II Enzyme Mix (Invitrogen®) or Gibson Assembly mix (Biolab®) **(Table S5)**. Thermocompetent DH5α *E. coli* were transformed with the corresponding vectors and plate on LB (Difco™ LB Broth,

Lennox, #214010, 20 g/L, 15% agar, Difco™ Bacto Agar) containing the Kanamycin at 50mg.L^{-1}. Plasmids were purified using the Nucleospin Plasmid kit (Macherey-Nagel®) and inserts sequenced. Expression vectors containing the promoter-gene-fluorescent tag cassette or 3'UTR were obtained by using LR clonase-based three-fragment recombination system (Invitrogen®), the pB7m34GW/pH7m34GW/pK7m34GW/pS7m43GW/ pLOK180_pR7m34g (gift from L. Kalmbach) destination vectors, and the corresponding entry vectors **(Table S3-S4)**. Only the EFOP2-7Q was introduce in the destination vector pK7FWG2 using the LR clonase-based one-fragment recombination system (Invitrogen®) (Karimi et al., 2002). Thermocompetent DH5α cells were transformed and selected on LB plate (Difco™ LB Broth, Lennox, #214010, 20 g/L, 15% agar, Difco™ Bacto Agar) containing 100mg.L^{-1} of Spectinomycin. Plasmids were purified using the Nucleospin Plasmid kit (Macherey-Nagel®) and inserts sequenced.

Site directed mutagenesis

Plasmids were amplified by PCR using the high fidelity Phusion Hot Start II (Thermo Fisher Scientific®) taq polymerase and the indicated primers carrying mutations **(Table S6)**. PCR products were digested using and cutsmart buffer (Biolab®). Thermocompetent DH5α *E. coli* were transformed with the digested PCR product and plate on LB (Difco™ LB Broth, Lennox, #214010, 20 g/L, 15% agar, Difco™ Bacto Agar) containing the appropriate antibiotic. Plasmids were purified using the Nucleospin Plasmid kit (Macherey-Nagel®) and mutations were sequenced.

Agrobacterium transformation

Electrocompetent *A. tumefaciens* (C58pmp90) were transformed by electroporation. 1µL of DNA plasmid at a concentration of 0.25-1 µg/µl was added into 50 µL of electrocompetent agrobacterium on ice. The agrobacterium were transfered into cold 1mm wide electroporation chamber (Eurogentec, #CE00150). A pulse of 2 kV, 335 Ω, 15µF, for 5 ms was performed on the electroporation chamber using the MicropulserTM (Bio-Rad, #165-2100). 1 mL of liquid LB media was added and the bacteria were placed into a new tube and incubated at 29°C for 2-3h. The agrobacterium were selected on LB (Difco™ LB Broth, Lennox, #214010, 20 g/L, 15% agar, Difco™ Bacto Agar) or YEB (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 1.5% bactoagar, pH7.2) plates containing the appropriate antibiotics to select the agrobacterium strain (50mg.L-1 of Rifampicin and 20mg.L-1 of Gentamycin) and the target construct (250mg.L-1 of Spectinomycin). Plates were incubated at 29°C for 48h.

Plant transformation and selection

Agrobacterium were spun and resuspended in 5% sucrose and 0.02% Silwet L-77 detergent. *Arabidopsis* were transformed by floral dipping.

Transformed seedlings were selected on Murashige and Skoog (MS, Duchefa Biochemie®) media supplemented with 0.8% plant agar containing the appropriate antibiotic or herbicide. Basta, Kanamycin, Hygromycin and Sulfadiazin have been used at 10mg.L^{-1}, 50mg.L^{-1}, 30mg.L^{-1} and 75mg.L^{-1} respectively. Selection of the pLOK180_pR7m34g plasmid was done using the FastRed method (i.e. red fluorescent seeds).

Protein extraction, immunoprecipitation and western blot analysis

Leaf tissue from wild-type plants and/or transgenic lines were collected, frozen in liquid nitrogen and ground to powder. The powder was resuspended in -20°C MetOH; 1% Protease inhibitor (P9599, Sigma-Aldrich®) and incubated at -20°C for 5min. Proteins were pelleted, resuspended in -20°C acetone and incubated at -20°C for 5min. Proteins were pelleted. The pellet was dried and resuspended in Protein Extraction Reagent (C0856, Sigma-Aldrich®) supplemented with 1% Protease inhibitor (P9599, Sigma-Aldrich®). Protein extraction were then kept at -20°C.

Protein samples in 1xSDG (Tris-HCL 0.25 M, 10% glycerol, 2% DTT, 2.5% SDS) supplemented with bromophenol blue were denaturated at 95°C for 5min. Samples migrated on 7.5% (for proteins over 200kDa) or 10% SDS-PAGE polyacrylamide gel at 140V with 1xTris-Glycine SDS running buffer (Euromedex®). Proteins were transferred on nitrocellulose membrane 0.45 µm in 1xTris-Glycine (Euromedex®), 20%EtOH transfer buffer at 100 V for 1h. Membrane were blocked with 5% milk, 1X PBS-T (Phosphate Buffer Saline) (Dominique Dutscher®), 2% Tween® 20 for 1h. Primary antibodies **(Table S1)** were used at 1/2000^{e} in 5% milk, 1X TBS, 0.1% Tween 20 overnight. Secondary antibodies were used at 1/5000^{e} in 1X TBS, 0.1% Tween 20 for 1h. Protein revelation was done using the Clarity or Clarity Max ECL Western Blotting Substrates (Biorad®) and the Chemidoc MP imaging system (Biorad®).

For co-immunoprecipitation, leaf tissue from transgenic lines were collected, frozen in liquid nitrogen and ground to powder. Proteins fused to mCITRINE were immunoprecipitated using the pull down kit Miltenyi µMacs anti-GFP (Miltenyi Biotec®). Crude extracts were put in 1XSDG supplemented with bromophenol blue and denaturated at 95°C for 5 min while IP samples were directly ready for migration on polyacrylamide gels.

Mass spectrometry analysis

Mass spectrometry analysis had been performed by the Protein Science Facility of SFR (Structure Fédérative de Recherche) Bioscience Lyon. Trypsine-digested samples were analysed by LC-MS/MS (Orbitrap, ThermoFischer Scientific®). The peptides identified were compare to the UniProtKb database.

Yeast-two-hybrid

The initial screen was performed by hybrigenics services (https://www.hybrigenics-services.com/contents/our-services/interaction-discovery/ultimate-y2h-2), using the

ULTImate Y2H screen against their Universal Arabidopsis Normalized library obtained using oligo_dT. The residues 2 to 1479 of PI4Kα1 were used. The screen was performed on 0.5 mM 3AT, 58.6 million interactions were analyzed, and 313 positive clones were sequenced.
AD vectors (prey) and DB vectors (bait) were transformed in the Y8800 and Y8930 yeast strains, respectively. Transformed yeasts were plated on SD (0.67% yeast nitrogen base, 0.5% dextrose anhydrous, 0.01% adenine, 1.8% agar) with all amino acids except tryptophan (SD-T) for AD vectors and SD-L for DB vectors for selection.
Yeast colony transformed with AD- or DB- vectors were grown in SD-T and SD-L liquid media, 30°C, 200rpm for 48h. Mating was performed at 30°C, 200rpm, overnight using 10µl of AD and 10µl DB clones in 180µl YEPD (1% yeast extract, 2% peptone, 2% dextrose anhydrous, 0.01% adenine) liquid media. Diploid yeasts were selected by addition of 100µl of SD-LT liquid media. Yeast were grown for 48h at 30°C, 200rpm. Diploids yeasts were plates on the different selective media: SD-LT to verify the mating; SD-LTH to select the positive interactions; SD-LTH+3AT (3-Amino-1,2,4-Triazol at 1mM final) to select strong interactions only: SD-LH+CHX (cycloheximide at 10µg/mL final) to determine the autoactivated DB clones; SD-LH+CHX+3AT to determine which concentration of 3AT erase the autoactivation of DB clones.

Crispr lines

20bp target sequence upstream to a 5'-NGG -3' sequence in an exon of the gene of interest were found using the CrisPr RGEN tool (www.rgenome.net/). Primers were designed following the methods developed in (Wang et al., 2015; Xing et al., 2014) **(Table S7)**.

T-DNA and Crispr Mutant Genotyping

Leaf tissue from wild-type plants, T-DNA insertion lines and Crispr lines were collected, frozen in liquid nitrogen and ground to powder. The powder was resuspended in the extraction buffer (200mM of Tris pH 7.5, 250mM of NaCl, 25mM of EDTA, 0.5% of SDS). DNA was precipitated with isopropanol and the DNA pellet was washed with 75% ethanol before resuspension in water.
Plants were genotyped by PCR using the GoTaq® polymerase (Promega®) and the indicated primers **(Table S8)**. PCR products were migrated on 1% agarose gel or the percentage indicated **(Table S8)**. When sequencing was required, the bands were purified using the NucleoSpin Gel and PCR Clean kit (Macherey-Nagel®) and sequenced.

Pollen observation by SEM

Pollen grains from mutant or WT flowers were placed on tape and observed using the mini SEM Hirox® 3000 at -10°C, 10kV.

Pollen inclusion and observation by TEM

For transmission electron microscopy, anthers were placed in a fixative solution of 3.7% paraformaldehyde and 2.5% glutaraldehyde, Na_2HPO_4 0.1 M, NaH_2PO_4 0.1 M overnight and postfixed in 1% OsO_4, Na2HPO4 0.1 M, NaH_2PO_4 0.1 M. Anthers were dehydrated through a graded ethanol series from 30% to 100% and embedded in SPURR resin. Sections were made using an ultramicrotome Leica UC7 at 70-80nm and poststained with Acetate Uranyle 5% (in Ethanol), Citrate Plomb (in NaOH). Pollen were observed using a transmission electron microscope Philips CM120.

Pollen staining

To perform Alexander staining, flowers that were about to open were dissected and anthers were put between slide and coverslip in Alexander staining solution (25% glycerol, 10% EtOH, 4% acetic acid, 0.05% acid fuchsin, 0.01% Malachite green, 0.005% phenol, 0.005% chloral hydrate, 0.005% Orange G,) for 7h at 50°C before observation under a stereomicroscope.

For DAPI staining, flowers that were about to open were dissected and their anthers were put between slide and coverslip in DAPI solution (10% DMSO, 0.1% NP-40, 50mM PIPES, 5mM EGTA, 0.01% (4',6-diamidino-2-phénylindole) DAPI) for 5min at RT before observation at the confocal microscope. DAPI was excited with a 405nm laser (80mW) and fluorescence emission was filtered by a 447/60 nm BrightLine® single-band bandpass filter (Semrock, http://www.semrock.com/).

Seed clearing

Siliques were opened. The replums with the seeds attached were placed between slide and coverslip in clearing solution (87.5% chloral hydrate, 12.5% glycerol) for 1 hour before observation at the microscope with differential interference contrast.

Confocal Imaging setup

7 to 10 days-old seedlings were observed with an Zeiss microscope (AxioObserver Z1, Carl Zeiss Group, http://www.zeiss.com/) with a spinning disk module (CSU-W1-T3, Yokogawa, www.yokogawa.com) and a Prime 95B camera (Photometrics, https://www.photometrics.com/) using a 63x Plan-Apochromat objective (numerical aperture 1.4, oil immersion) and the appropriated laser and bandpath filter.

GFP was excited with a 488 nm laser (150mW) and fluorescence emission was filtered by a 525/50 nm BrightLine® single-band bandpass filter (Semrock, http://www.semrock.com/). mCITRINE was excited with a 515nm laser (60mW) and fluorescence emission was filtered by a 578/105 nm BrightLine® single-band bandpass filter (Semrock, http://www.semrock.com/). mCHERRY was excited with a 561nm laser (80mW) and fluorescence emission was filtered by a 609/54 nm BrightLine® single-band bandpass filter (Semrock, http://www.semrock.com/). In the case of seedling expressing mCHERRY and

mCITRINE markers, mCITRINE was excited and emission was filtered using the GFP settings.

TIRF microscopy used an objective based azimuthal ilas2 TIRF microscope (Roper Scientific) with 100x Apo NA 1.46 Oil objective. Exposure time used was 1s. HYC2-mCITRINE and NPGR2-mCITRINE were excited and emission was filtered using the GFP settings while EFOP2-mCITRINE was excited and emission was filtered using the YFP settings.

Immunolocalization

Five-days old seedlings were fixed in PFA 1%, MTBS (50mM PIPES, 5mM EGTA, 5mM MgS04, pH7) for 1h. Roots were Superfrost Plus® slides (Thermo Scientific, #10149870) and dried at RT for 1h and rehydrated using MTBS + 0.1% tritonx100. Permeabilization was done using 2% Driselase (Sigma, # D9515) in MTSB for 30 min. Roots were treated with 10% dimethylsulfoxide, 3% Igepal (Sigma, #CA-630) in MTSB for 1 h. Blocking was done using 5% normal goat serum (NGS Sigma #G9023) in MTSB. Roots were incubating overnight with the primary antibody diluted at 1/100 (anti-PI4Kα1) and with secondary antibody diluted at 1/500 for 3h (anti Rat IgG, alexa Fluor 488 conjugate, InVitrogen-Molecular Probes, #A-21210). Roots were placed between slide and coverslip in Vectashield® (Vectorlabs, #H-1000-10) and observed using a confocal microscope Zeiss 800.

Microsomes and plasma membrane purification

Microsomes were purified as described in (Simon-Plas et al., 2002) and resuspended in phosphate buffer (5 mM, pH 7,8) supplemented with sucrose (300 mM) and KCl (3 mM). Plasma membrane were obtained after cell fractionation by partitioning twice in a buffered polymer two-phase system with polyethylene glycol 3350/dextran T-500 (6.6% each).

For PI4Kα1, proteins were precipitated using 5 volumes of -20°C acetone for 1 volume of protein extraction and incubated for 10min at -20°C. Proteins were pelleted. This process was repeated 2 more times. Pellet was dried and resuspended in 1xSDG supplemented with bromophenol blue. All steps were performed at 4 °C.

Plasmolysis

7 to 10 days-old seedlings were placed between side and coverslip in MES 10 mM at pH 5.8 with or without 0.75M sorbitol and observed using a confocal microscope.

Fluorescence recovery after photobleaching (FRAP)

Fluorescence in a rectangle ROI (5x1,7µm) at the plasma membrane was bleached in the root by successive scans at full laser power (150 W) using the iLas2 FRAP module (Roper scientific, http://www.biovis.com/ilas.htm). Fluorescence recovery was measured in the ROIs and in controlled ROIs (rectangle with the same dimension in unbleached area). FRAP was recorded continuously during 120 s with a delay of 1s between frames. Fluorescence intensity data were normalized as previously described (Martinière et al., 2012). The mobile fraction was calculated at t=120s with the following formula: I(t)-Min(I)/Ictrl(t)/Min(I) where I(t) and Ictrl(t) are the intensity of the bleached and control region at a time t, repectively.

Nicotiana benthamiana leaf infiltration

Transformed agrobacterium were directly taken from plate with a tip and resuspended into 2 mL of infiltration media (10 mM MES pH 5.7, 10 mM $MgCl_2$, 0.15 mM acetosyringone (Sigma-Aldrich®, #D134406)) by pipetting. The OD_{600} was measured using a spectrophotometer (Biophotometer, Eppendorf) and adjusted to 1 by adding infiltration media.

The infiltration was performed on « heart shape » tobacco leaves from 2-3 weeks old plants. Using 1 mL syringe (Terumo®, #125162229), the infiltration solution with the agrobacterium was pressed onto the abaxial side of the chosen tobacco leaf. The plants were put back to the growth chamber for 2-3 days under long day conditions.

5 mm^2 regions of the leaf that surround the place where the infiltration has been made were cut. The pieces of leaf were mounted in water between slide and coverslip with the abaxial side of the leaf facing the coverslip. Using the appropriate wavelength, an epifluorescent microscope and the smallest objective (10X), the surface of the leaf was screened to find the transformed cells. Then, the subcellular localization of the fluorescent protein was observed using a spinning confocal microscope and 63X objective.

PAO treatment

Seedlings (7 to 10-days-old) were incubated in liquid MS with 30µM PAO (Sigma, www.sigmaaldrich.com, PAO stock solution at 60 mM in DMSO) for 30 min before observation using a confocal microscope.

Acknowledgments:

We thank the present and past members of the SiCE group as well as G. Vert and G. Ingram for discussions and comments, the NASC/ABRC collection for providing transgenic Arabidopsis lines, L. Kalmbach and M. Barberon for the gift of pLOK180_pFR7m34GW, A. Lacroix, J. Berger and P. Bolland for plant care. We acknowledge the contribution of SFR Biosciences (UMS3444/CNRS, US8/Inserm, ENS de Lyon, UCBL) facilities: C. Lionet, E. Chatre and J. Brocard at the LBI-PLATIM-MICROSCOPY for assistance with imaging and V. Gueguen-Chaignon, A. Page and F. Delolme at the Protein Science Facility (PSF) for assistance with mass spectrometry. YJ. was funded by ERC no. 3363360-APPL under FP/2007-2013 and ANR caLIPSO (ANR18-CE13-0025), M-C.C. by a start-up package “font de recherche – projet émergent” from ENS de Lyon, SM by project PlayMobil ANR-19-CE20-0016-02 and by project PhosphoREM-domain ANR-19-CE13-0021-01, L.C.N. by a PhD fellowship from the French Ministry of Higher Education.

Author contributions:

L.C.N. was responsible of all the experiments described in this manuscript with the following exception: V.B. performed transmission electron microscopy experiments and helped with microscopy image acquisition and analyses; F.R. performed and imaged whole mount immunolocalization; A.M-C. and S.M. purified plasma membranes; M-C.C. initiated the

yeast-two hybrid screen; L.A. helped with yeast-two hybrid experiments. L.C.N. and Y.J. wrote the manuscript and all authors commented on the manuscript.

Conflict of Interest:

The authors declare that they have no conflict of interest

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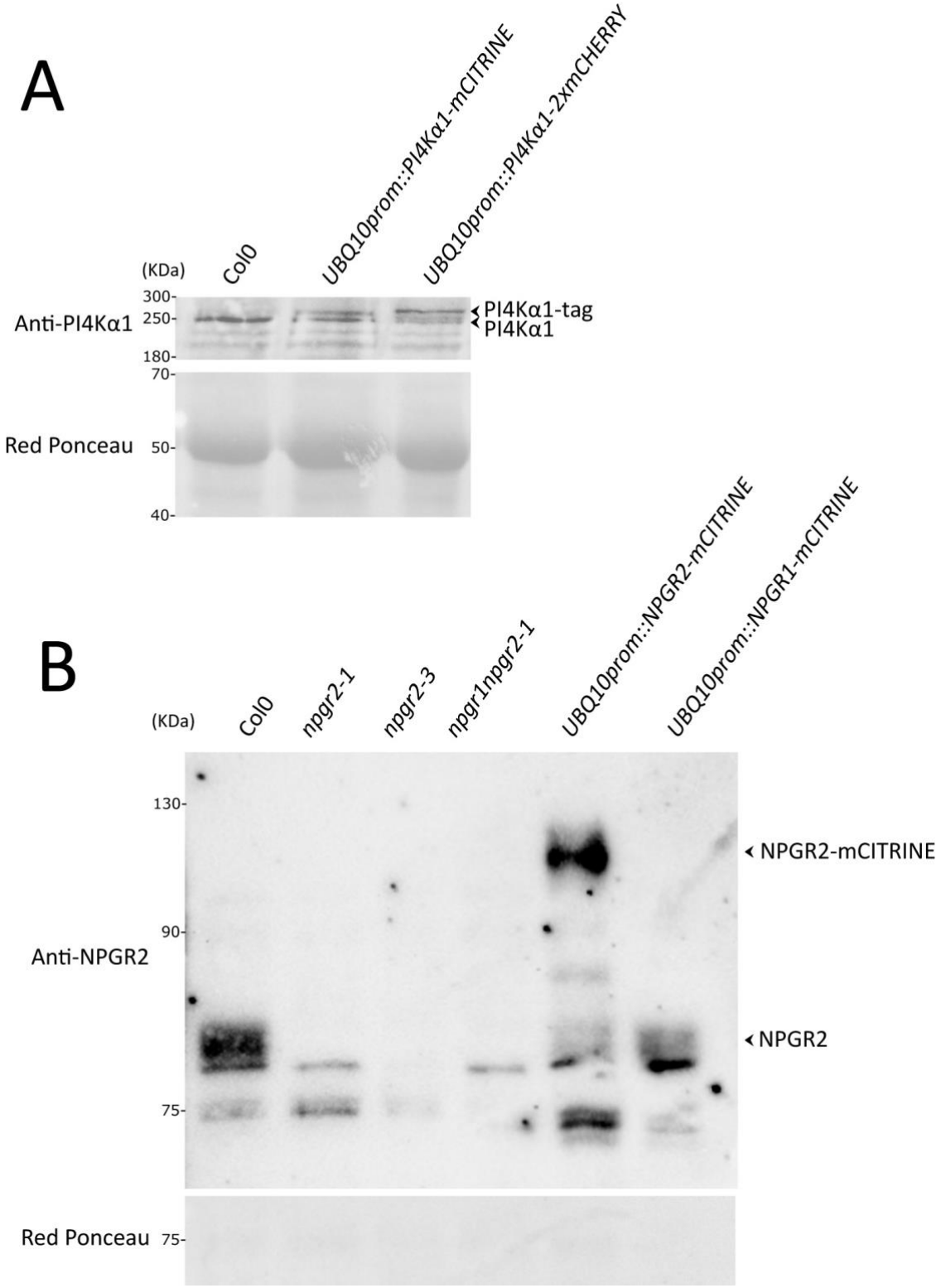
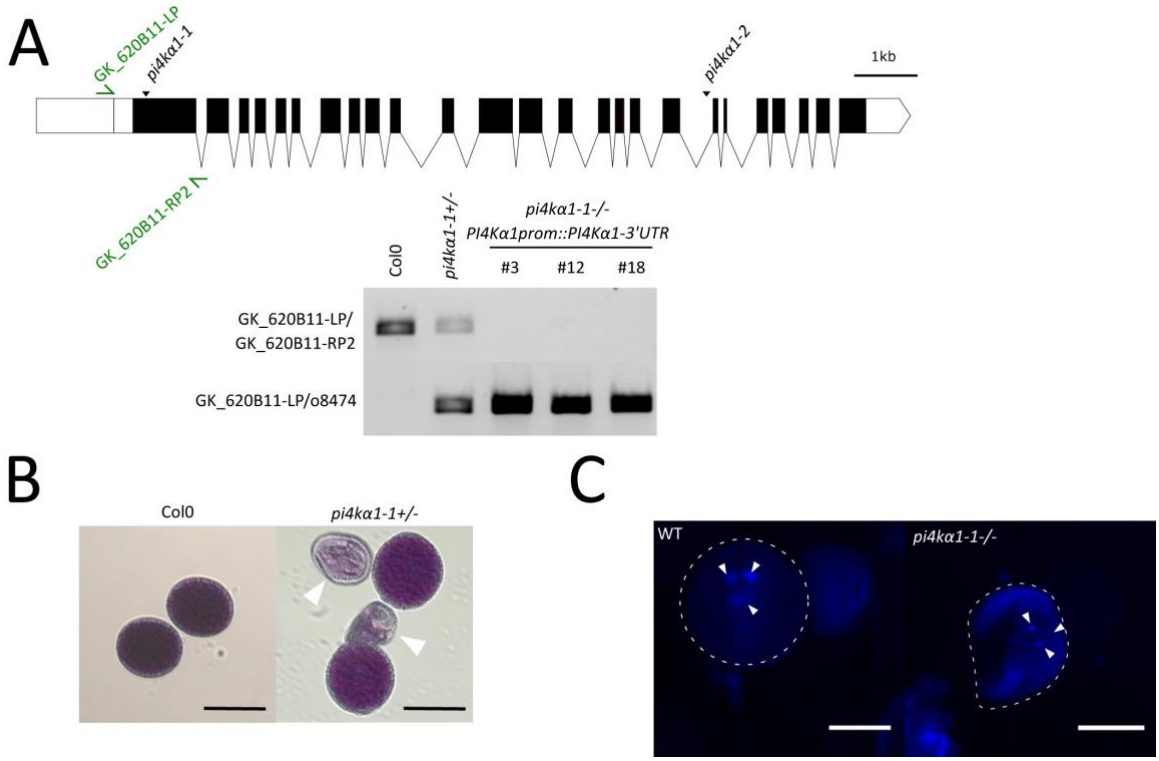
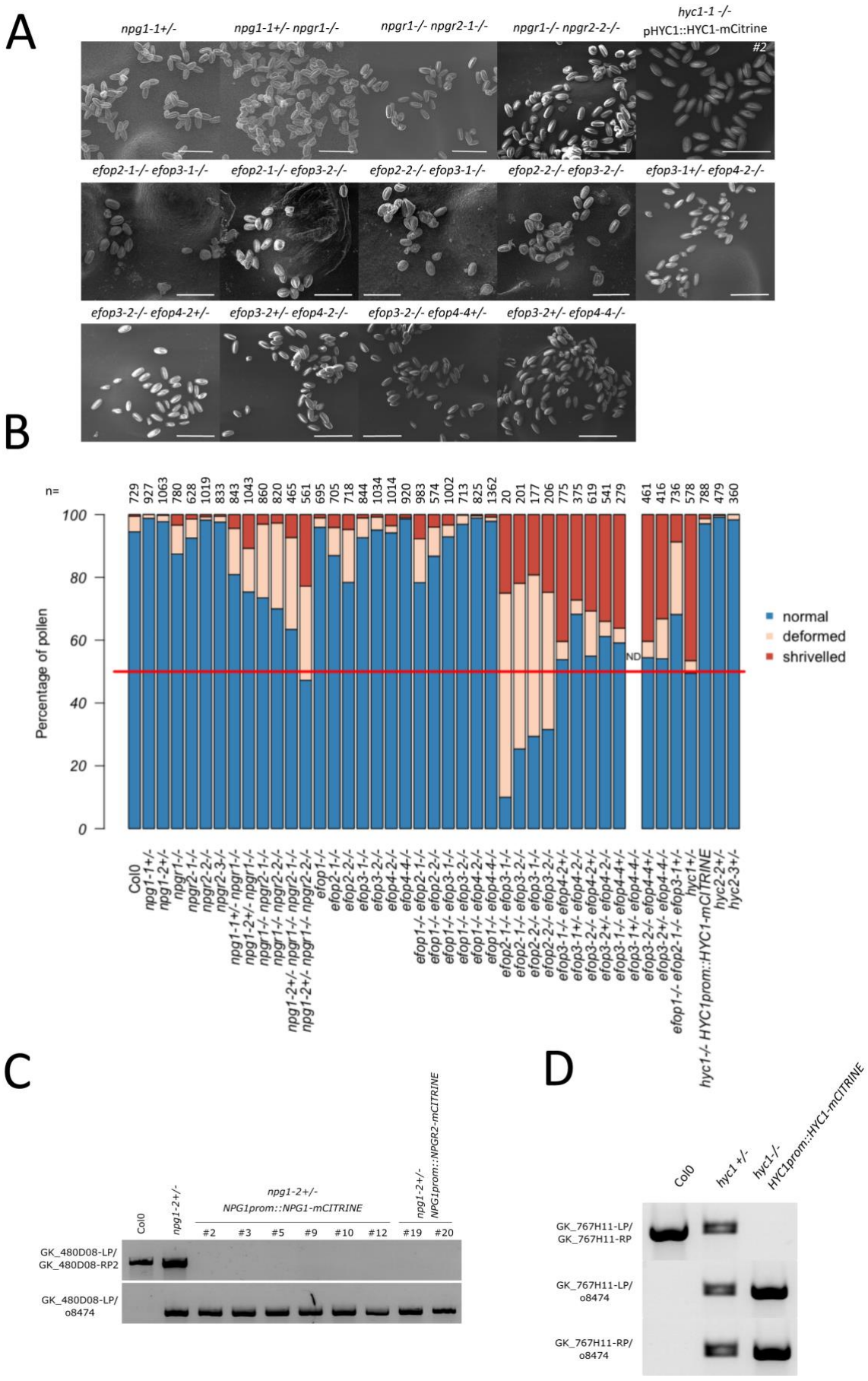


Supplemental Figure 1. Western-blot validation of the custom-made anti-PI4Kα1 and anti-NPGR2 antibodies. (A) Western blot anti-PI4Kα1 on total proteins extract of Col-0 seedlings, seedling expressing *UBQ10prom::PI4Ka1-mCITRINE* and seedling expressing *UBQ10prom::PI4Ka1-2xmCHERRY*. Red ponceau shows similar protein loading in every well. **(B)** Western blot anti-NPGR2 on total proteins extract of Col0*, npgr2-1, npgr2-3, npgr1npgr2-1* seedlings, seedling expressing *UBQ10prom::NPGR2-mCITRINE* and seedling expressing *UBQ10prom::NPGR1-mCITRINE*. Red ponceau shows similar protein loading in every well.



Supplemental Figure 2. Characterization of *pi4kα1* pollen phenotype (A) Genotyping of Col0, *pi4ka1-1* heterozygous plants, and *pi4ka1-1* homozygous plants expressing *PI4Ka1prom::PI4Ka1-3'UTR* (insertion n°3, 12 and 18). Upper panel shows the amplification of gene sequence. Lower panel shows amplification of T-DNA border. **(B)** Alexander staining of pollen grains from Col-0 and self-fertilized *pi4ka1-1* heterozygous plants. Shrivelled pollen grains are indicated by white arrowheads. Scale bars: 20 µm. **(C)** DAPI staining of pollen grains from self-fertilized *pi4ka1-1* heterozygous plants with normal (WT, left) and shrivelled (*pi4ka1-1 -/-,* right) shape. Nuclei are indicated with white arrowheads. Scale bars: 10 µm.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npg1-1+/- npgr1-/-
npgr1-/- npgr2-1-/-
npgr1-/- npgr2-2-/-
hyc1-1 -/- pHYC1::HYC1-mCitrine
#2
efop2-1-/- efop3-1-/-
efop2-1-/- efop3-2-/-
efop2-2-/- efop3-1-/-
efop2-2-/- efop3-2-/-
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efop3-2+/- efop4-2-/-
efop3-2-/- efop4-4+/-
efop3-2+/- efop4-4-/-
B
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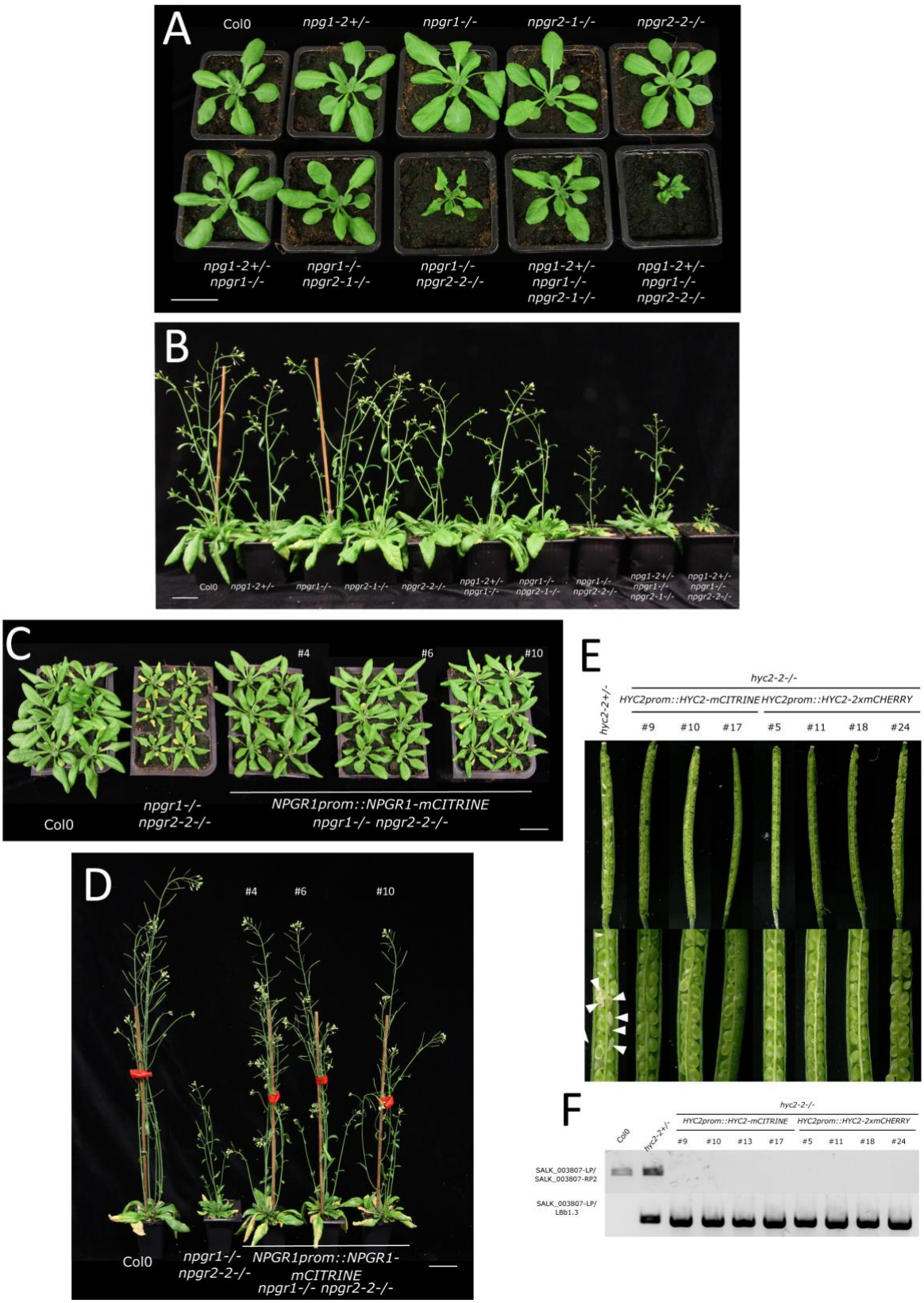
Percentage of pollen
normal
deformed
shrivelled
ND
C
Col0
npg1-2+/-
npg1-2+/- NPG1prom::NPG1-mCITRINE
#2 #3 #5 #9 #10 #12
npg1-2+/- NPG1prom::NPGR2-mCITRINE
#19 #20
GK_480D08-LP/ GK_480D08-RP2
GK_480D08-LP/ o8474
D
Col0
hyc1 +/-
hyc1-/- HYC1prom::HYC1-mCITRINE
GK_767H11-LP/ GK_767H11-RP
GK_767H11-LP/ o8474
GK_767H11-RP/ o8474

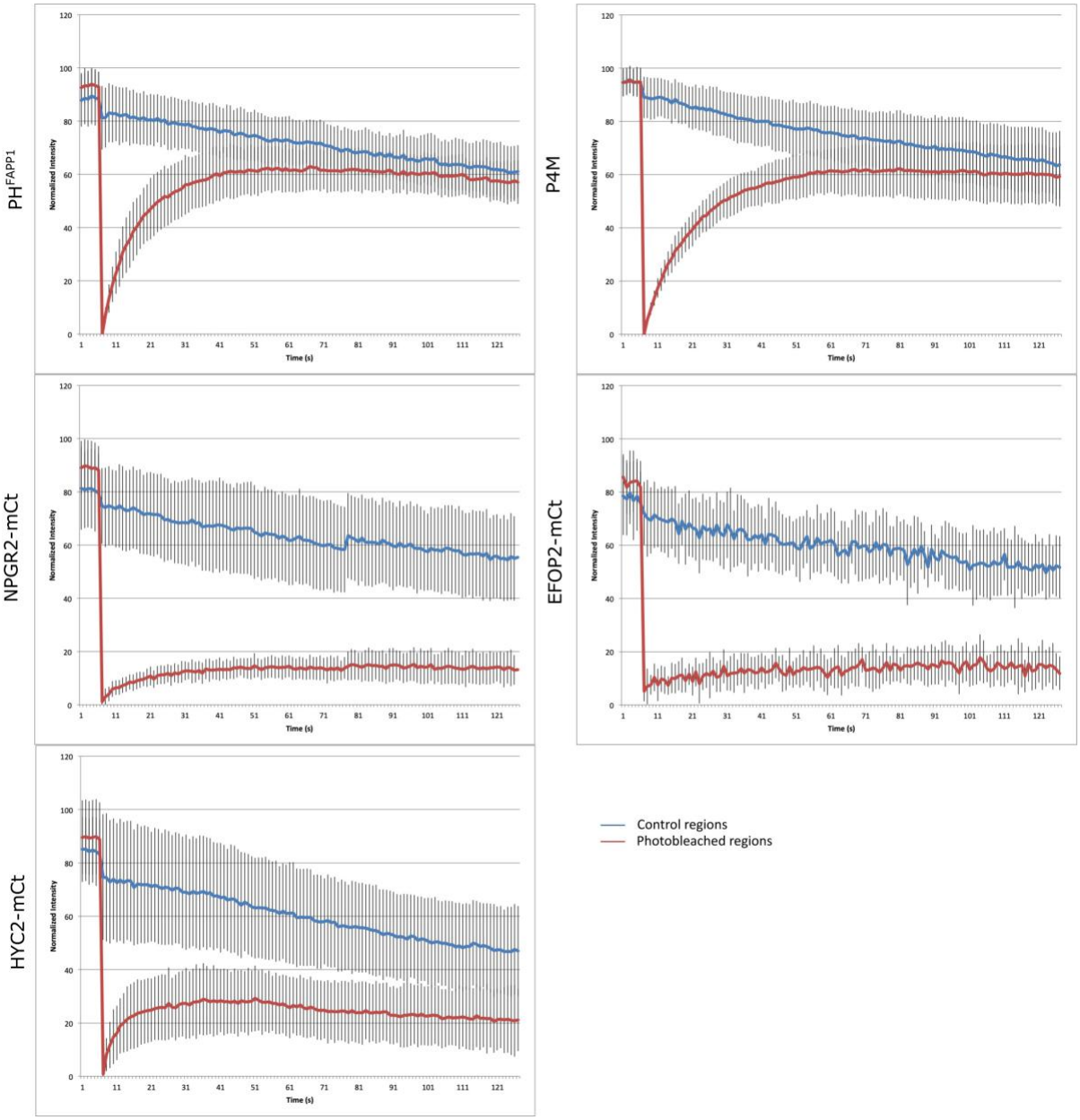
1363

Supplemental Figure 3. Characterization of the pollen phenotype of single and multiple *npg*, *hyc* and *efop* mutants. (A) Pollen grains observed at scanning electronic microscope of self-fertilized *npg1-1+/-, npg1-1+/- npgr1-/-, npgr1-1-/- npgr2-1-/-, npgr1-1-/- npgr2-2-/-, hyc1-/-* expressing *HYC1prom::HYC1-mCIT*RINE*, efop2-1-/- efop3-1-/-, efop2-1-/- efop3-2-/-, efop2-2-/- efop3-1-/-, efop2-2-/- efop3-2-/-, efop3-1+/- efop4-2-/-, efop3-2-/- efop4-2+/-, efop3-2+/- efop4-2-/-, efop3-2-/- efop4-4+/-,* and *efop3-2+/- efop4-4-/-* plants. Scale bars: 50 µm **(B)** Quantification of the % of normal (blue), deformed (orange) and shrivelled (red) pollen grains of all indicated genotypes. ND is indicated when no quantification of this genotype is available. n indicates the number of pollen grains counted. **(C)** Genotyping of Col-0, *npg1-2* heterozygous plants and *npg1-2* homozygous plants complemented with *NPG1prom::NPG1-mCITRINE* (insertion n° 2, 3, 5, 9, 10 and 12) and *NPG1prom::NPGR2-mCITRINE* (insertion n° 19 and 20). Upper panel shows amplification of the gene sequence. Lower panel shows amplification of T-DNA border. **(D)** Genotyping of Col-0, *hyc1* heterozygous plants and *hyc1* homozygous plants complemented with *HYC1prom::HYC1-mCITRINE* (insertion n° 2). Upper panel shows amplification of gene sequence. Lower panel shows amplification of T-DNA border.



Supplemental Figure 4. Sporophytic phenotypes of *npg1+/-npgr1-/-npgr2-/-* triple mutant *and hyc2-/-* single mutant and complementation (A) Twenty-seven-days-old Col-0*, npg1-2+/-, npgr1-/-, npgr2-2-/-, npg1-2+/- npgr1-/-, npgr1-/- npgr2-2-/-* and *npg1-2+/- npgr1-/- npgr2-2-/-* plants. Scale bar: 2 cm **(B)** Forty-one-days-old Col-0, *npg1-2+/-, npgr1-/-, npgr2-2-/-, npg1-2+/- npgr1-/-, npgr1-/- npgr2-2-/-* and *npg1-2+/- npgr1-/- npgr2-2-/-* plants. Scale bar: 2 cm **(C)** Twenty-seven days-old Col-0, *npgr1-/- npgr2-2-/-* and *npgr1-/- npgr2-2-/-* expressing NPGR1prom::NPGR1-mCITRINE. Several independent insertions are shown. Scale bar: 2 cm **(D)** Forty days-old

Col-0, *npgr1-/- npgr2-2-/-* and *npgr1-/- npgr2-2-/-* expressing NPGR1prom::NPGR1-mCITRINE. Several independent insertions are shown. Scale bar: 2 cm **(E)** Opened siliques of self-fertilized *hyc2-2* heterozygous mutant plants and self-fertilized hyc2-2 homozygous plants complemented by the expression of *HYC2prom::HYC2-mCITRINE* (insertion n°9, 10 and 17) and *HYC2prom::HYC2-2xmCHERRY* (insertion n°11, 18 and 24). White arrowheads indicate aborted seeds. **(F)** Genotyping of Col-0, *hyc2-2* heterozygous plants and *hyc2-2* homozygous plants complemented with *HYC2prom::HYC2-mCITRINE* (insertion n°9, 10, 13 and 17) and *HYC2prom::HYC2-2xmCHERRY* (insertion n° 11, 18 and 24). Upper panel shows amplification of gene sequence. Lower panel shows amplification of T-DNA border.



Supplemental Figure 5. FRAP analysis of NPGR2/EFOP2/HYC2 fused with mCITRINE in Arabidopsis root. Graphics presenting signal intensity over time for photobleached (red) and control regions (blue). Standard deviations are shown. The number of zones measured is 37, 32, 30, 13 and 29 for P4M, PH^{FAPP1}, NPGR2, EFOP2-mCt and HYC2-mCt, respectively.

	Antibody	References	Company	Host	Concentration used
Primary	anti-GFP	A-6455	ThermoFisher	Rabbit	1/2000
	anti-mCherry	ab167453	Abcam	Rabbit	1/1000
	anti-PI4Ka1	A03 rat #1	Proteogenix	Rat	1/1000
	anti-NPGR2	9094-A01 rabbit #2	Proteogenix	Rabbit	1/2000
Secondary	anti-Rat IgG, HRP conjugate	AP136P	Merck	Goat	1/5000
	anti-Rabbit IgG, HRP conjugate	W4011	Promega	Goat	1/5000

Table S1. Antibodies

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	Gene	Locus	Name	T-DNA or CrisPr line	Zygosity	Phenotype observed	Detail
Single Mutant	PI4Kα1	At1g49340	*pi4kα1-1*	GK502_D11	Heterozygous	Male sterility	Insertion in the 1st exon (208bp from ATG) of 26bp followed by two T-DNA in inverted tandem
			pi4kα1-2	FLAG_275H12	Heterozygous	Male sterility	Insertion in the 22nd intron (9087bp from ATG) of 28bp followed by one T-DNA
	NPG1	At2g43040	*npg1-1*	SAIL_262_A01	Heterozygous	Male sterility	Insertion in the 3rd exon (734bp from ATG) of 22bp followed by two T-DNA in inverted tandem
			npg1-2	GK_480D08	Heterozygous	Male sterility	Insertion in the 3rd exon (1585bp from ATG) of 2bp followed by two T-DNA in inverted tandem and 16bp
	NPGR1	At1g27460	*npgr1*	SALK_090514	Homozygous	None	Insertion in the 3rd exon (1152bp from ATG) of 13bp followed by two T-DNA in inverted tandem and 49bp
	NPGR2	At4g28600	*npgr2-1*	CrisPr	Homozygous	None	Insertion of 1 T in the first exon at the 236 pb after the ATG leading a early codon stop and a truncated protein of 82aa
			npgr2-2		Homozygous	None	Deletion of 17 bp leading a early codon stop and a truncated protein of 76aa
	HYC1	At5g21050	*hyc1*	GK_767H11	Heterozygous	Male sterility	Deletion of 10bp and insertion of two T-DNA ininverted tandem at 786bp from ATG
	HYC2	At5g64090	*hyc2-2*	SALK_003807	Heterozygous	Embryo lethality	Insertion of 9bp followed by one T-DNA at 611bp from ATG
			hyc2-3	SALK_040977	Heterozygous	Embryo lethality	Deletion of 30bp and insertion of two T-DNA ininverted tandem at 705bp from ATG
	EFOP1	At5g21080	*efop1*	GK_620B11	Homozygous	None	Insertion in the 9th exon (2118bp from ATG) of 11bp followed by two T-DNA in inverted tandem and 213bp
	EFOP2	At2g41830	*efop2-1*	SALK_128017	Homozygous	None	Insertion in the 6th intron (1976bp from ATG) of one T-DNA
			efop2-2	GK_387B07	Homozygous	None	Insertion in the 11th intron (3829bp from ATG) of 10bp followed by two T-DNA in inverted tandem and 78bp
	EFOP3	At1g05960	*efop3-1*	SALK_121262	Homozygous	None	Insertion in the 1st intron (136bp from ATG) of one T-DNA
			efop3-2	SALK_133976	Homozygous	None	Insertion in the 10th intron (2488bp from ATG) of 58bp followed by two T-DNA in inverted tandem and 28bp
	EFOP4	At5g26850	*efop4-2*	CrisPr	Homozygous	None	Insertion of 1A in position 34 leading a early codon stop and a truncated protein of 42aa
			efop4-4		Homozygous	None	Deletion of 57bp leading to deletion of 19 aa including palmytoylation site and positive charges

	Gene	Locus	Name	T-DNA or CrisPr line	Zygosity	Phenotype observed
Double Mutant	NPG1 NPGR1	At2g43040 At1g27460	*npg1-1 npgr1*	SAIL_262_A01 / SALK_090514	NPG1 Heterozygous / NPGR1 Homozygous	About 10% of shrivelled pollen
			npg1-2 npgr1	GK_480D08 / SALK_090514		
	NPGR1 NPGR2	At1g27460 At4g28600	*npgr1 npgr2-1*	SALK_090514 / CrisPr	Double Homozygous	About 10% of shrivelled pollen
			npgr1 npgr2-2	SALK_090514 / CrisPr		Weak pollen phenotype / Growth phenotype
	EFOP1 EFOP2	At5g21080 At2g41830	*efop1 efop2-1*	GK_620B11 / SALK_128017	Double Homozygous	None
			efop1 efop2-2	GK_620B11 / GK_387B07		
	EFOP1 EFOP3	At5g21080 At1g05960	*efop1 efop3-1*	GK_620B11 / SALK_121262	Double Homozygous	None
			efop1 efop3-2	GK_620B11 / SALK_133976		
	EFOP1 EFOP4	At5g21080 At5g26850	*efop1 efop4-2*	GK_620B11 / CrisPr	Double Homozygous	None
			efop1 efop4-4	GK_620B11 / CrisPr		
	EFOP2 EFOP3	At2g41830 At1g05960	*efop2-1 efop3-1*	SALK_128017 / SALK_121262	Double Homozygous	From 70 to 90% of deformed or shrivelled pollen grains
			efop2-1 efop3-2	SALK_128017 / SALK_133976		
			efop2-2 efop3-1	GK_387B07 / SALK_121262		
			efop2-2 efop3-2	GK_387B07 / SALK_133976		
	EFOP2 EFOP4	At2g41830 At5g26850	*efop2-1 efop4-1*	SALK_128017 / SAIL_642H01	Double Homozygous	None
			efop2-2 efop4-1	GK_387B07 / SAIL_642H01		
	EFOP3 EFOP4	At1g05960 At5g26850	*efop3-1 efop4-2*	SALK_121262 / CrisPr		About 50% of deformed or shrivelled pollen grains
			efop3-1 efop4-4	SALK_121262 / CrisPr		
			efop3-2 efop4-1	SALK_133976 / SAIL_642H01		About 50% of shrivelled pollens when efop3-2 is homozygous and efop4-1 is
			efop3-2 efop4-2	SALK_133976 / CrisPr		About 50% of deformed or shrivelled pollen grains
			efop3-2 efop4-4	SALK_133976 / CrisPr		
Triple Mutant	NPG1 NPGR1 NPGR2	At2g43040 At1g27460 At4g28600	*npg1-2 npgr1 npgr2-1*	SAIL_262_A01 / SALK_090514 / CrisPr	NPG1 Heterozygous / NPGR1 and NPGR2 Homozygous	Dwarfism
			npg1-2 npgr1 npgr2-2	SAIL_262_A01 / SALK_090514 / CrisPr		
	EFOP1 EFOP2 EFOP3	At5g21080 At2g41830 At1g05960	*efop1 efop2-1 efop3-1*	GK-620B11 / SALK_128017 / SALK_121262	Triple homozygous	None

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Table S3: Transgenic lines

Name	Full name	Background	Entry vectors			Destination vector	Use	Figure	Sources	
Lti6b-mCITRINE/Lti6b-mCt	2x35Sprom::Lti6b-FRB-CITRINE	Col0	2X35S/pDONR P4-P1r	Lti6b-FRB/pDONR 207	mCITRINE/pDONR P2R-P3	pB7m34GW	Coimmunoprecipitation	1	This study	
NPGR2-mCITRINE/NPGR2-mCt	UBQ10prom::NPGR2-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	NPGR2(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Coimmunoprecipitation, mass spectrometry, antibody test, Confocal microscopy, FRAP, PAO treatment	1, S1, 5, 6, S5, 7	This study	
mCITRINE-PI4Kα1/mCt-PI4Kα1	UBQ10prom::mCITRINE-PI4Kα1	Col0	UBQ10prom/pDONR P4-P1r	mCITRINE/pDONR 221	PI4Kα1(CDS)/pDONR P2R-P3	pB7m34GW	mass spectrometry, Confocal microscopy	1, 5	This study	
mCITRINE-NES-mCITRINE	2x35Sp::mCITRINE-NES-mCITRINE	Col0	2X35S/pDONR P4-P1r	mCITRINE/pDONR 221	NES-mCITRINE/pDONR P2R-P3	pB7m34GW	mass spectrometry	1	This study	
myristoylation-2XmCITRINE	2xp35S::myrimCITRINE-mCITRINE	Col0				pB7m34GW	mass spectrometry	1	Jaillais et al., 2011	
HYC2-mCITRINE/HYC2-mCt	UBQ10prom::HYC2-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	HYC2(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Coimmunoprecipitation, Confocal microscopy, FRAP	1, 5, 6, S5	This study	
EFOP2-mCITRINE/EFOP2-mCt	UBQ10prom::EFOP2-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	EFOP2(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Coimmunoprecipitation, Confocal microscopy, FRAP, PAO treatment	1, 5, 6, S5, 7	This study	
PI4Kα1-mCITRINE/PI4Kα1-mCt	UBQ10prom::PI4Kα1-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	PI4Kα1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Antibody test, Confocal microscopy	S1, 5	This study	
PI4Kα1-2XmCHERRY	UBQ10prom::PI4Kα1-2xmCHERRY-4xmyc	Col0	UBQ10prom/pDONR P4-P1r	PI4Kα1(CDS)/pDONR 221	2xmCHERRY-4xmyc/pDONR P2R-P3	pH7m34GW	Antibody test, Confocal microscopy	S1, 5	This study	
NPGR1-mCITRINE/NPGR1-mCt	UBQ10prom::NPGR1-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	NPGR1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pLOK180_pR7m34g	Antibody test, Confocal microscopy	S1, 5	This study	
PI4Kα1prom::PI4Kα1-3'UTR		*pi4kα1* GK502D11	PI4Kα1prom/pDONR P4-P1r	PI4Kα1(CDS)/pDONR 221	PI4Kα1-3'UTR/pDONR P2R-P3	pB7m34GW	Complementation	2	This study	
NPG1prom::NPG1-mCITRINE		*npg1-2* +/- GK_480D08	NPG1prom/pDONR P4-P1r	NPG1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Complementation	S3	This study	
NPG1prom::NPGR2-mCITRINE		*npg1-2* +/- GK_480D08	NPG1prom/pDONR P4-P1r	NPGR2(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Complementation	S3	This study	
NPGR1prom::NPGR1-mCITRINE		*npgr1 npgr2-2* SALK_090514/CrisPR	NPGR1prom/pDONR P4-P1r	NPGR1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Complementation	S4	This study	
HYC1prom::HYC1-mCITRINE		*hyc1* GK_767H11	HYC1prom/pDONR P4-P1r	HYC1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Complementation	S3	This study	
HYC2prom::HYC2-mCITRINE		*hyc2-2* SALK_003807	HYC2prom/pDONR P4-P1r	HYC2(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Complementation	4, S4	This study	
HYC2prom::HYC2-2xmCHERRY	HYC2prom::HYC2-2xmCHERRY-4xmyc	*hyc2-2* SALK_003807	HYC2prom/pDONR P4-P1r	HYC2(CDS)/pDONR 221	2xmCHERRY-4xmyc/pDONR P2R-P3	pH7m34GW	Complementation	4, S4	This study	
HYC1-mCITRINE/HYC1-mCt	UBQ10prom::HYC1-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	HYC1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Confocal microscopy	5	This study	
NPG1-mCITRINE/NPG1-mCt	UBQ10prom::NPG1-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	NPG1(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Confocal microscopy	5	This study	
EFOP1-mCITRINE/EFOP1-mCt	UBQ10prom::EFOP1-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	EFOP1genomic/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Confocal microscopy	5	This study	
EFOP3-mCITRINE/EFOP3-mCt	UBQ10prom::EFOP3-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	EFOP3(CDS)/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Confocal microscopy	5	This study	
Lti6b-2XmCHERRY	UBQ10prom::Lti6b-2xmCHERRY-4xmyc	Col0	UBQ10prom/pDONR P4-P1r	Lti6b/pDONR207	2xmCHERRY-4xmyc/pDONR P2R-P3	pH7m34GW	Confocal microscopy	5	Platre et al., 2018	
NPGR2-2XmCHERRY	UBQ10prom::NPGR2-2xmCHERRY-4xmyc	Col0	UBQ10prom/pDONR P4-P1r	NPGR2(CDS)/pDONR 221	2xmCHERRY-4xmyc/pDONR P2R-P3	pH7m34GW	Confocal microscopy	5	This study	
PHFAPP1	pUBQ10::mCITRINE-1xPHFAPP1 (P5Y)	Col0				pB7m34GW	FRAP, PAO treatment	6, S5, 7	Simon et al., 2014	NASC# N2105607
P4M	pUBQ10::mCITRINE-P4MSidM	Col0				pB7m34GW	FRAP	6, S5	Simon et al., 2016	NASC# N2107346
EFOP2-7Q	UBQ10prom::EFOP2-7Q-GFP	Col0	EFOP2-7Q/pDONR 221			pK7FWG2	Confocal microscopy	7	This study	
EFOP2-CC	UBQ10prom::EFOP2C20SC23S-mCITRINE	Col0	UBQ10prom/pDONR P4-P1r	EFOP2C20SC23S/pDONR 221	mCITRINE/pDONR P2R-P3	pB7m34GW	Confocal microscopy	7	This study	
PI4Kα1prom::PI4Kα1-mCITRINE-Lti6b	PI4Kα1prom:PI4Kα1-mCITRINE-3xSAG-Lti6b	*npg1-2* +/- GK_480D08	PI4Kα1prom/pDONR P4-P1r	PI4Kα1(CDS)/pDONR 221	mCITRINE-3xSAG-Lti6b/pDONR P2R-P3	pB7m34GW	Complementation	7	This study	

Name	Source	
Empty gateway entry vector: pDONR221	thermofisher	cat# 12536017
Empty gateway entry vector: pDONR P4P1R	thermofisher	cat# 12537023
Empty gateway entry vector: pDONR P2RP3	thermofisher	cat# 12537023
Empty gateway destnation vector: pB7m34GW	Karimi et al., 2007	N/A
Empty gateway destnation vector: pH7m34GW	Karimi et al., 2007	N/A
Empty gateway destnation vector: pLOK180_pR7m34g	L. Kalmbach and M. Barberon	N/A
Empty gateway destnation vector:pK7FWG2	Karimi et al., 2002	N/A
Entry vector: UBQ10prom/pDONR P4-P1r	Jaillais et al., 2011	NASC N2106315
Entry vector: 2X35S/pDONR P4-P1r	Mar Marquès-Bueno, M. et al. 2016	NASC N2106316
Entry vector: mCITRINE/pDONR 221	Simon et al., 2014	NASC N2106287
Entry vector: mCITRINE/pDONR P2R-P3	Jaillais et al., 2011	NASC N2106288
Entry vector: 2xmCHERRY-4xmyc/pDONR P2R-P3	Simon et al., 2014	NASC N2106292
Lti6b/pDONR207	Elsayad et al., 2016	N/A
pEGFP-FRB (Gift of Klaus Hahn)	Gift of Klaus Hahn	plasmid addgene #25919

Table S4: Published vectors used in this study

Table S5: Primers used for cloning into gateway entry vectors

			Cloning of reporter lines			
	Name	Sequence	Primer Sequence (5'-3'; forward then reverse)	Modifications	Template	Destination vector
Genes	PI4Kα1	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGAGGCACTGACGGAGCTTTGTG	B1-Kozak sequence	cDNA from seedlings	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTACTTCTCGATGCCTTGTTGCAAATACTG	B2-stop mutated		
			GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGAGGCACTGACGGAGCTTTGTG	B1-Kozak sequence		pDONR P2R-P3
			GGGGACCACTTTGTACAAGAAAGCTGGGTATTACTTCTCGATGCCTTGTTGCAAATACTG	B2-STOP		
			GGGGACAGCTTTCTTGTACAAAGTGGCTATGGAGGCACTGACGGAGCTTTGTGAC	B2R		
			GGGGACAACTTTGTATAATAAAGTTGCTTACTTCTCGATGCCTTGTTGCAAATACTG	B3		
	NPG1	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGCTCGGGAATCAATCCG	B1-Kozak sequence	cDNA from seedlings	pDONR221
			GGGGACCACTTTGTACAAGAAAGCTGGGTATTAAAGAATGGTTGAGAAGCTTTCAA	B2-stop mutated		
	NPGR1	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGTTGTGTGCTTGTTCAGGC	B1-Kozak sequence	cDNA from seedlings	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAATGAAACTCTGTACCGGAGC	B2-stop mutated		
	NPGR2	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGAAGAACAGCGAGATTAGGCC	B1-Kozak sequence	cDNA from seedlings	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTATCATCTGAATGGCTCCACTGG	B2-stop mutated		
	HYC1	Coding sequence/genomic sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGTCTGATTCTTCCTCAAGCCATG	B1-Kozak sequence	genomic DNA	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTAAACGGAGAGAACGGTGTCGTTTGATAGCTC	B2-stop mutated		
	HYC2	Coding sequence/genomic sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGACTTCTCCGTCAAACCTTCCGG	B1-Kozak sequence	genomic DNA	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTACTTTGATGCCAGAAGAATCTCAGG	B2-stop mutated		
	ELP1	Genomic sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGGGGTTGTTTCAAGAACC	B1-Kozak sequence	genomic DNA	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTACTAATCAAGAGGAACCAACGGC	B2-STOP		
	ELP2	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGAGCGCAGTCTCGGGTG	B1-Kozak sequence	cDNA from seedlings	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTAACAGCCAGCAGCTTTAAGGAAG	B2-stop mutated		
	ELP3	Coding sequence from ATG to Stop	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACCATGGGGGTTATGTCCAGACG	B1-Kozak sequence	cDNA from seedlings	pDONR 221
			GGGGACCACTTTGTACAAGAAAGCTGGGTATTAACACCCGGCTGCTTTC	B2-STOP		
	NES-mCitrine	Addition of NES sequence uptream of mCitrine on mCITRINE/pDONR P2RP3 entry vector	/5PHOS/GTTAGAGGAGCTGGAGCTAGACGAACAGCAAGGAGGAATGGTGAGCAAGGGCGAGGAGC	5'Phosphorylated	mCITRINE/pDONR P2RP3 entry vector from Jaillais et al., 2011	pDONR P2R-P3
			/5phos/TTCTTTTGAAGGTCAACCAAATTCGGAGCCACTTTGTACAAGAAAGTTGAACGAGAAACG	5'Phosphorylated		
	FRB-Lti6b	Lti6b with homology to FRB linker and pDONR207	TACCCAGCTTTCTTGTACAAAGTG		Lti6b/pDONR207 from Elsayad et al., 2016	pDONR 207
			CTTGGTGATGATATAAAGAGCG			
		FRB linker with homology to Lti6b and pDONR207	CTTTATATCATCACCAAGGCGGGAGCAGATAGTGCT		pEGFP-FRB (plasmid addgene #25919 Gift of	
			TACAAGAAAGCTGGGTAACTAGTCTTTGAGATTCGTCGG			
Promoters	PI4Kα1prom	1316bp upstream ATG with homology to pDONR P4-P1r	TTTGTATAGAAAAGTTGCTGTTAGGTTGATTCTCCTATCCTGGGTTTG	B4	genomic DNA	pDONR P4-P1r
			AACTTTTTTTGTACAAACTTGCCTCCGATTAAACAAAAAAAACAACGTTACG	B1		
		pDONR P4-P1r with homology to pPI4Kα1	TTTTGTTTAATCGGAGGCAAGTTTGTACAAAAAAGTTGAACGAG	B4	pDONR P4-P1r	
			AGGATAGGAGAATCAACCTAACAGCAACTTTTCTATACAAAGTTGGC	B1		
	NPG1prom	3983bp uspstream ATG with homology to pDONR P4-P1r	ACTTTGTATAGAAAAGTTGCTTATAGTTATGAAACTTAATGATTAGCGAGTTG	B4	genomic DNA	pDONR P4-P1r
			TTCAACTTTTTTTGTACAAACTTGCTGTTCTTCACCTTTTAGACTAAACCC	B1		
		pDONR P4-P1r with homology to pNPG1	AGTCTAAAAGGTGAAGAACAGCAAGTTTGTACAAAAAAGTTGAACGAGAAACG	B4	pDONR P4-P1r	
			TCATTAAGTTTCATAACTATAAGCAACTTTTCTATACAAAGTTGGCATTATAAAAAAGC	B1		
	NPGR1prom	1226bp upstream ATG with homology to pDONR P4-P1r	ACTTTGTATAGAAAAGTTGCTGGAAAGGAGAAACTAGGAGAGACGTCGCTATTTG	B4	genomic DNA	pDONR P4-P1r
			CGTTCAACTTTTTTGTACAAACTTGCCTTCTCCTCTTTCTCCTCACAAACGCGGAAATGC	B1		
		pDONR P4-P1r with homology to pNPGR1	GTGAGGAGAAAGAGGAGAAGGCAAGTTTGTACAAAAAAGTTGAACGAGAAACG	B4	pDONR P4-P1r	
			CTCTCCTAGTTTCTCCTTTCCAGCAACTTTTCTATACAAAGTTGGC	B1		
	HYC1prom	1212bp upstream ATG with homology to pDONR P4-P1r	GTATAGAAAAGTTGCTGAAAAGCCAGTCAGCGTCCGATGTAGCCCG	B4	genomic DNA	pDONR P4-P1r
			TTTGTACAAACTTGCTTTGGGGAAGATGATGATCGCTTCTGAG	B1		
		pDONR P4-P1r with homology to pHYC1	GCTGACTGGCTTTTCAGCAACTTTTCTATACAAAGTTGGC	B4	pDONR P4-P1r	
			CATCATCTTCCCCAAAGCAAGTTTGTACAAAAAAGTTGAACGAG	B1		
	HYC2prom	1430bp upstream ATG with homology to pDONR P4-P1r	GTATAGAAAAGTTGCTTGTGTGGGATTTTGATTCTTCTTCTC	B4	genomic DNA	pDONR P4-P1r
			TTTGTACAAACTTGCGGGAATTGAGTCTGACTTGAGAGAC	B1		
		pDONR P4-P1r with homology to pHYC2	AGTCAGACTCAATTCCCGCAAGTTTGTACAAAAAAGTTGAACGAG	B4	pDONR P4-P1r	
			TCAAAATCCCACACAAGCAACTTTTCTATACAAAGTTGGC	B1		
	ELP1prom	2594bp upstream ATG with homology to pDONR P4-P1r	TGTATAGAAAAGTTGCTAATCCAATGACTAGAAAAGTCTACAC	B4	genomic DNA	pDONR P4-P1r
			TACGTTTCTCGTTCAACTTTTTTGTACAAACTTGCCGATCTATCTCTCTTCC	B1		
		pDONR P4-P1r with homology to pELP1	CGAATATTCGCGGAAGAGAGATAGATCGGCAAGTTTGTACAAAAAAGTTG	B4	pDONR P4-P1r	
			GACTTTTCTAGTCATTGGATTAGCAACTTTTCTATACAAAGTTGGC	B1		
	ELP3prom	2411bp upstream ATG with homology to pDONR P4-P1r	TATAGAAAAGTTGCTTGCCCACACGCAGGATCGAACTACGGACCTTC	B4	genomic DNA	pDONR P4-P1r
			CAACTTTTTTGTACAAACTTGCTTTCTTCTCCGCCTAATATCC	B1		
		pDONR P4-P1r with homology to pELP3	TAGGCGGAGAAGAAAGCAAGTTTGTACAAAAAAGTTGAACGAGAAACG	B4	pDONR P4-P1r	
			CGATCCTGCGTGTGGGCAAGCAACTTTTCTATACAAAGTTGGC	B1		
3'UTR	PI4Kα1 3'UTR	395bp dowstream STOP codon	GGGGACAGCTTTCTTGTACAAAGTGGCTGAAGAAGAAGAGGGAACTTAATGATATCCG	B2R	genomic DNA	pDONR P2R-*P3*
			GGGGACAACTTTGTATAATAAAGTTGCGAATACAAATACCTCTACATGTTAATAG	B3		

Directed mutagenesis		
Name	**Mutation**	**Primer Sequence (5'-3'; forward then reverse)**
ELP1noSTOP/pDONR 221	Revertion of the STOP codon (TAG-->TTG leu) in ELP1gwSTOP/p221	cctcttgattTgtacccagctttcttgtacaaagtt
		agctgggtacAaatcaagaggaaccaacggcttggc
ELP3noSTOP/pDONR 221	Deletion of the stop (TAT)	gcagccgggTGTtacccagctttcttgtacaaagttgg
		gaaagctgggtaACAcccggctgctttcaagaatttatc
ELP2-CC/pDONR 221	Site directed mutagenesis C20 and C23 to S (TGT->AGT and TGC->AGC) to mutate the palmytoylation	tgtggtagccttAgtattcttAgccctgcgcttcgtgcaaggtctagacag
		acgaagcgcagggcTaagaatacTaaggctaccacaaacaggcaaaacttgcctc
ELP2-7Q/pDONR 221	site directed mutagenesis R29Q (AGG-->CAA) on ELP2	attctttgccctgcgctt**CGT**gca**CAG**ct**AGA**cagcctgtgaagaggtac
		cttgtacctcttcacaggctgTCTagaCTGtgcACGaagcgcagggcaaag
	site directed mutagenesis R27Q (CGT-->CAA)and R31Q (AGA-->CAA) on ELP2 to use on ELP2-R29Q	attctttgccctgcgcttCAAgcaCAGtctCAAcagcctgtgaagaggtac
		cttgtacctcttcacaggctg**TTG**aga**CTG**tgc**TTG**aagcgcagggcaaag
	site directed mutagenesis K35Q (AAG-->CAG)and K38Q (AAG-->CAG) on ELP2 to use on ELP2-3Q	CAGtctCAAcagcctgtgCAGAGGtacCAGAAGctcattgctgagatttttcc
		aaaaatctcagcaatgagCTTCTGgtaCCTCTGcacaggctgTTGagaCTGtgc
	site directed mutagenesis R36Q (AAG-->CAG) and K39Q (AAG-->CAG) on ELP2 to use on ELP2-5Q	CAGtctCAAcagcctgtgCAGCAGtacCAGCAGctcattgctgagatttttcc
		aaaaatctcagcaatgagCTGCTGgtaCTGCTGcacaggctgTTGagaCTGtgc

Table S6: Primers used for site directed mutagenesis

Gene mutated	Primer Name	Description	Primer Sequence (5'-3'; forward then reverse)
NPGR2	NPGR2-BsF	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Forward primer to clone a Single gRNA1 NPGR2 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers NPGR2-F0 and NPGR2-R0 and NPGR2-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence and the BSAI cutting site. Original primer name in the protocol: DT1-BsF.	ATATATGGTCTCGATTGTTACGTAAATGCAACTTCTCGTT
	NPGR2-F0	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Forward primer to clone a Single gRNA1 NPGR2 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers NPGR2-BsF and NPGR2-R0 and NPGR2-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence. Original primer name in the protocol: DT1-F0.	TGTTACGTAAATGCAACTTCTCGTTTTAGAGCTAGAAATAGC
	NPGR2-R0	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Reverse primer to clone a Single gRNA2 NPGR2 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers NPGR2-BsF and NPGR2-F0 and NPGR2-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence. Original primer name in the protocol: DT2-R0.	AACagttatctctgcgtgagacgCAATCTCTTAGTCGACTCTAC
	NPGR2-BsR	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Reverse primer to clone a Single gRNA2 NPGR2 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers NPGR2-BsF and NPGR2-F0 and NPGR2-R0. Amplicon size around 620 bp. This primer contains de gRNA sequence and the BSAI cutting site. Original primer name in the protocol: DT2-BsR.	ATTATTGGTCTCGAAACagttatctctgcgtgagacgCAA
EFOP4	EFOP4-BsFR	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Forward primer to clone a Single gRNA1 EFOP4 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers EFOP4-F0 and EFOP4-R0 and EFOP4-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence and the BSAI cutting site. Original primer name in the protocol: DT1-BsF.	ATATATGGTCTCGATTGATGCACATACTCTCGCATGCGTT
	EFOP4-F0	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Forward primer to clone a Single gRNA1 EFOP4 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers EFOP4-BsF and EFOP4-R0 and EFOP4-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence. Original primer name in the protocol: DT1-F0.	TGATGCACATACTCTCGCATGCGTTTTAGAGCTAGAAATAGC
	EFOP4-R0	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Reverse primer to clone a Single gRNA2 EFOP4 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers EFOP4-BsF and EFOP4-F0 and EFOP4-BsR. Amplicon size around 620 bp. This primer contains de gRNA sequence. Original primer name in the protocol: DT2-R0.	AACgtcaagcgttacaagaaattCAATCTCTTAGTCGACTCTAC
	EFOP4-BsR	To obtain new Crispr lines using the egg especific promoter or 35S promoter vectors from Wang et al. Genome biology 2015. Cloning using Golden Gate reaction. Reverse primer to clone a Single gRNA2 EFOP4 using pCBC-DT1T2 vector as a template. 4 primers PCR with primers EFOP4-BsF and EFOP4-F0 and EFOP4-R0. Amplicon size around 620 bp. This primer contains de gRNA sequence and the BSAI cutting site. Original primer name in the protocol: DT2-BsR.	ATTATTGGTCTCGAAACgtcaagcgttacaagaaattCAA

Table S7: Primers used for crispr constructs

Gene	Locus	Mutant Name	T-DNA or CrisPr line	Primer Name	Primer Sequence (5'-3'; forward then reverse)	Additional information for genotyping
PI4Kα1	At1g49340	*pi4kα1-1*	GK502_D11	GK502_D11-LP	ACCGGTTATACCGATTTGGTC	
				GK502_D11-RP2	TTGTTTCAGCAAGATATATCG	
		pi4kα1-2	FLAG_275H12	FLAG_275H12-RP	AGTCCTACCCTCCTGAACGAG	
				FLAG_275H12-LP	TCACGACATCTTTTGTCCAAAC	
NPG1	At2g43040	*npg1-1*	SAIL_262_A01	SAIL_262_A01-LP	AGTGAGAAGGGGGAAGATGAG	
				SAIL_262_A01-RP	AGTGTTCCAACGCTCAATACG	
		npg1-2	GK_480D08	GK_480D08-LP	AAGAAGTAATGCCTGGTGTGTTTA	
				GK_480D08-RP2	TGTGTTATTGTACAAGTTCTTC	
NPGR1	At1g27460	*npgr1*	SALK_090514	SALK_090514-LP	CCCTGGAATTGCTTCCTTTAC	
				SALK_090514-RP	TTTCTCTTGCGCTCGAATTAG	
NPGR2	At4g28600	*npgr2-1*	CrisPr	NPGR2-CPR-1	CTGTTCTTTCTCGAAATATG	Sequencing
				NPGR2-CPR-2	CCTTTAGACCGTCTTCTATG	
		npgr2-2		NPGR2-CPR-1	CTGTTCTTTCTCGAAATATG	Migration on 4% agarose gel
				NPGR2-CPR-3	CCTTTAGACCGTCTTCTATG	
HYC1	At5g21050	*hyc1*	GK_767H11	GABI_767H11-LP	CTTCCTCAAGCCATGACTCAC	
				GABI_767H11-RP	ACATCATCGACTTGCAGGATC	
HYC2	At5g64090	*hyc2-2*	SALK_003807	SALK_003807-LP	TGCCCCATTAGAAACAAACTG	
				SALK_003807-RP	ACTCAACTATACATCATAACC	
		hyc2-3	SALK_040977	SALK_040977-LP	TCAGACTCAATTCCCATGGAC	
				SALK_040977-RP	GAGAGGCTCTTGCGTACAATG	
EFOP1	At5g21080	*efop1*	GK_620B11	GABI_620B11_RP	GAGGATGCTAAAAACCCCAAG	
				GABI_620B11_LP	TTCGTCGTCAAAACTTTCACC	
EFOP2	At2g41830	*efop2-1*	SALK_128017	SALK_128017_RP	TGTGCCAATTAGGACTGGAAG	
				SALK_128017_LP	TTAAAACAGGGAATGCAATCG	
		efop2-2	GK_387B07	GABI387B07ch2_LP	TTCAAGCTATGGTCCATCCAG	
				GABI387B07ch2_RP	TTCAAACATAGCTCTCCCTCG	
EFOP3	At1g05960	*efop3-1*	SALK_121262	SALK_121262_RP	TTCTGGGTTTTCAGGGTTCTC	
				SALK_121262_LP	TCGACAAGAGTATTGCAACCC	
		efop3-2	SALK133976	SALK_133976_LP	AGGGCCAAGAAGATACCAAAG	
				SALK_133976_RP	CATGGGACTCATAACCACTGG	
EFOP4	At5g26850	*efop4-2*	CrisPr	EFOP4-CPR-1	GTATAGATGAGGTCTGGTTTC	Msl1 digestion and migration on 4% agarose gel
				EFOP4-CPR-2	GCAGGACAGCAAATGCACATACaCTCGCAT	
		efop4-4		EFOP4-CPR-1	GTATAGATGAGGTCTGGTTTC	Migration on 2% agarose gel
				EFOP4-CPR-3	TTAGGGATACGGATTGGGTTC	

Table S8: Primers used for genotyping