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Phosphatidylinositol 4-phosphate 5-kinases 1 and 2 are involved in the regulation of vacuole morphology during *Arabidopsis thaliana* pollen development

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ABSTRACT

The pollen grains arise after meiosis of pollen mother cells within the anthers. A series of complex structural changes follows, generating mature pollen grains capable of performing the double fertilization of the female megasporophyte. Several signaling molecules, including hormones and lipids, have been involved in the regulation and appropriate control of pollen development. Phosphatidylinositol 4-phophate 5-kinases (PIP5K), which catalyze the biosynthesis of the phosphoinositide PtdIns(4,5)P_2, are important for tip polar growth of root hairs and pollen tubes, embryo development, vegetative plant growth, and responses to the environment. Here, we report a role of PIP5Ks during microgametogenesis. *PIP5K1* and *PIP5K2* are expressed during early stages of pollen development and their transcriptional activity respond to auxin in pollen grains. Early male gametophytic lethality to certain grade was observed in both *pip5k1*$^{-/-}$ and *pip5k2*$^{-/-}$ single mutants. The number of *pip5k* mutant alleles is directly related to the frequency of aborted pollen grains suggesting the two genes are involved in the same function. Indeed *PIP5K1* and *PIP5K2* are functionally redundant since homozygous double mutants did not render viable pollen grains. The loss of function of *PIP5K1* and *PIP5K2*results in defects in vacuole morphology in pollen at the later stages and epidermal root cells. Our results show that *PIP5K1*, *PIP5K2* and phosphoinositide signaling are important cues for early developmental stages and vacuole formation during microgametogenesis.

1. Introduction

In flowering plants, male gametes develop inside male gametophytes structure, the pollen grains. The male gametophytes emerge after meiosis of diploid pollen mother cells within the anthers, generating a tetrad of haploid cells. The tetrad cells are then released as free microspores and undergo a series of complex processes that finally produce the mature pollen grain (MPGs). In *Arabidopsis thaliana*, an MPG consists of two generative cells encapsulated in the cytoplasm of a larger vegetative cell. While the last one contributes to the MPG survival and pollen tube formation, the two smaller germline cells participate in the ovule fertilization that will produce the zygote, the endosperm and seed tissues [1].

Microspore development is tightly associated with processes of cell wall deposition and vacuole biogenesis. After the first pollen mitosis, pre-existing small vacuoles fuse into a large vacuole before the generative cell formation. Later on, after the second mitosis, the mature tricellular pollen contains small dispersed vacuoles [1]. This progression in vacuole biogenesis must be tightly regulated during pollen development to appropriately control pollen growth and maturation.

In yeast, vacuole biogenesis dynamics requires a set of lipids that play regulatory roles in vesicle trafficking and membrane fusion, including phosphatidylinositol 3-phosphate (PtdIns3P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2), diacylglycerol (DAG) and phosphatidic acid (PA) [2–6]. The abundance and metabolism of these lipids play important roles in lipid signaling and membrane trafficking. Phosphatidylinositol metabolism

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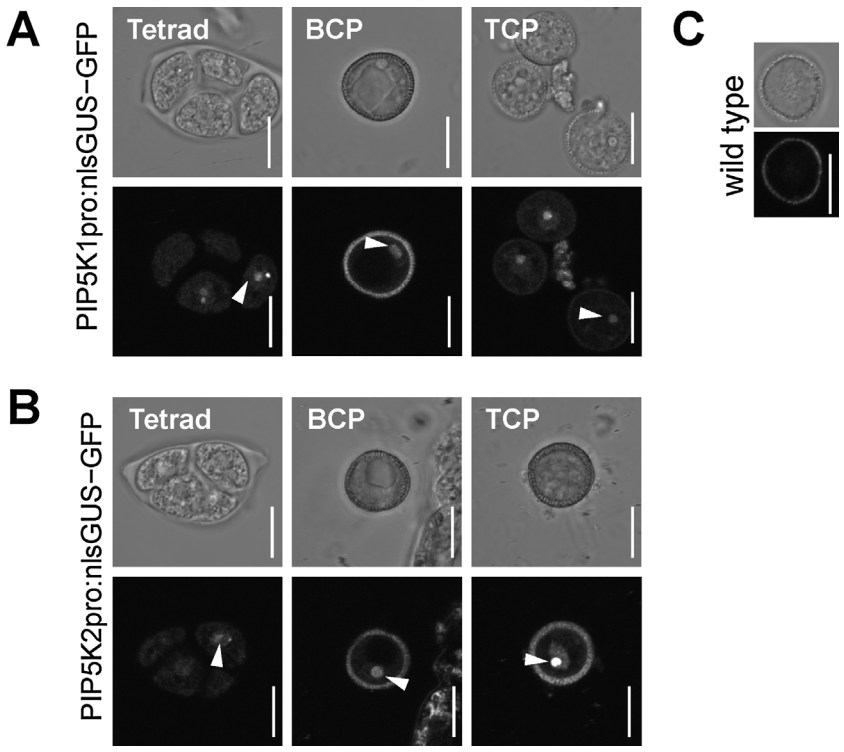

Fig. 1. *PIP5K1* and *PIPK2* are expressed in early stages of pollen development.
Flowers of the indicated reporter lines and wild type were dissected under a binocular stereomicroscope. Pollen grains were extracted by squeezing the anthers over a glass slide and immediately imaged by confocal microscopy. Bright field (upper panel) and GFP fluorescence images (lower panel)are shown for each stage of pollen development. BCP: bicellular pollen; TCP: tricellular pollen. Arrowheads indicate GFP-positive nuclei fluorescence. Wild type Col-0 was used as a control for pollen autofluorescence. Scale bar is 10 μm
(A) PIP5K1pro:nlsGUS-GFP
(B) PIP5K2pro:nlsGUS-GFP
(C) Wild type Col-0

determines the relative abundance of each lipid in intracellular compartments and defines the identity of vesicles and directionality of membrane trafficking. In *Arabidopsis*, the impairment of genes implicated in phosphatidylinositol metabolism has deleterious consequences on the function and morphology of vacuoles. The overexpression of the phosphatases *Suppressor of Actin (SAC)* genes, which are presumably involved in the metabolism of PtdIns(3,5)P_2 to PtdIns3P, leads to larger and fewer vacuoles in root tips, whereas decreased *SAC* expression has the opposite effect [2]. In *sac* loss and gain of function mutants, endocytic and vacuolar trafficking of the auxin efflux carrier PINFORMED2 (PIN2) is impaired, suggesting that the protein trafficking to the vacuole also depend on the levels of PtdIns(3,5)P_2 and PtdIns3 P [2]. Two out of the four *Arabidopsis* PtdIns3 P 5-kinase genes, *FAB1A* and *FAB1 B* are expressed in pollen. The double mutant *fab1a*$^{-/-}$ *fab1b*$^{-/-}$ exhibits pollen lethality as a consequence of a failure in vacuole rearrangement [3]. Thus, PtdIns3 P and PtdIns(3,5)P_2 synthesis is crucial for vacuole morphogenesis and essential for proper male gametophyte development. Nevertheless, little information exists on the role of other phosphoinositides in plant vacuole dynamics and during microgametogenesis.

Here, we present evidence indicating that the enzymes PIP5K1 and PIP5K2, which are involved in the synthesis of PtdIns(4,5)P_2, are essential for early pollen development. The single homozygous *pip5k1*$^{-/-}$ and *pip5k2*$^{-/-}$ as the double heterozygous mutant *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$ display defects during early microgametogenesis generating pollen grain abortion. Pollen grains from flowers of the *pip5k1*$^{+/-}$*pip5k2*$^{+/-}$mutants show defects in vacuoles and exine wall formation. These vacuole defects of pollen are consistent with the defects observed in vacuole morphology and protein trafficking in root cells of *pip5k1*$^{-/-}$*pip5k2*$^{-/-}$ mutants. Overall our data suggest that PIP5K1 and PIP5K2 are important for vacuole biogenesis and early pollen development.

2. Results

2.1. PIP5K1 and PIP5K2 are expressed during early pollen development and their transcript levels increased in response to auxin

In *Arabidopsis thaliana*, phosphatidylinositol 4-phosphate 5-kinases (PIP5 K) form of a family of 11 members (PIP5K1−PIP5K11) [4] which are functionally redundant [5–7]. *PIP5K1* and *PIP5K2* are part of the subgroup of ubiquitously expressed *PIP5Ks*. Both genes are expressed in several developmental contexts, including seedlings, embryos, root tips, leaves, and inflorescence stems [5,7]. Interestingly, *PIP5K1* and *PIP5K2* transcripts were also detected in closed and open flowers (Supplementary Fig. 1A), suggesting they may be expressed during early and late stages of gametogenesis. We checked published transcriptomic data from different microgametogenesis developmental stages and found *PIP5K1* and *PIP5K2* are expressed early in pollen development in unicellular microspores (UNMs) and bicellular pollen (BCP) (Supplementary Table 1, [8]). We confirmed the pollen expression for *PIP5K1* and *PIP5K2* using promoter transcriptional reporters lines [7]. These lines confirmed the activity of the *PIP5K1* and *PIP5K2* promoters at the tetrad, bicellular (BCP) and tricellular (TCP) pollen developmental stages (Fig. 1), indicating they may perform an important function during reproductive development.

During pollen development, auxin plays a fundamental regulatory role. Auxin maxima are detected from the UNM to TCP stages, which declines when pollen maturation and anther dehis-