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Mutation of Aquaporin Gene *PIP2;5* Postpones Pollen Hydration in Arabidopsis

Su Jian Tan[1], Juan Wang[1], Na Sui[2], Xian Sheng Zhang[1], Fang Wang[1*]

[1]State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Taian, Shandong 271018, China

[2]Shandong Provincial Key Laboratory of Plant Stress, College of Life Science, Shandong Normal University, Jinan 250014, China

[*]Corresponding author E-mail: wangf@sdau.edu.cn

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Abstract

In flowering plants with dry stigmas, pollen hydration involves water movement, which may be facilitated by aquaporins. To explore the possibility underlying this biological process, we identified and characterized a mutant with a T-DNA insertion in *PIP2;5*, which encodes an aquaporin with water channel activity in the PIP2 subfamily. We monitored the pollination process (pollen hydration, germination, and pollen tube growth) of wild type pollen on stigmas of the mutant and wild type. Pollen hydration was postponed on the stigmas of the mutant, compared with that on wild type stigmas. However, pollen tube germination and growth was unaffected in the mutant. The PIP2;5 protein was located in the cell plasma membrane and was preferentially expressed in the stigma. Based on our results, we concluded that PIP2;5 might play an important role in water movement during pollen hydration.

Keywords: Aquaporin, Pollen hydration, Stigma, PIP2;5, Arabidopsis

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1. Introduction

During sexual reproduction in flowering plants, water movement between cells and tissues is essential for anther dehiscence and pollen tube development. Pollination is initiated when desiccated pollen grains hydrate on the stigma [1]. The regulated passage of water from the stigma to pollen is important for successful pollen tube development in flowering plants with dry stigmas. The pollen grains that land on dry stigmas mobilize their lipid-rich pollen coat to form an interface between the two cell surfaces. This interface converts into a histochemically distinguishable structure that is thought to promote water flow [2, 3]. Water, nutrients, and other small molecules must be transported through two membranes from the stigma papillae to the pollen grain for successful pollen hydration and germination.

Aquaporins are membrane proteins that permit fast transport of water through cellular membranes. These proteins contain six membrane-spanning helices and five loops (loops A to E) with N- and C-termini residing in the cytosol [4, 5]. Plant aquaporins can be subdivided into four major groups: plasma membrane-intrinsic proteins (PIPs), tonoplast-intrinsic proteins (TIPs), nodulin26-like-intrinsic proteins (NIPs), and small and basic membrane-intrinsic proteins (SIPs) [6, 7]. The abundance of aquaporins in reproductive organs suggests that these proteins function in plant reproduction. In tobacco, PIP1 and PIP2 show distinct expression patterns during stigma and anther development, and may contribute to the development of these organs in different ways [8, 9]. In potato, PIP2a was shown to be highly expressed in the pistil and anther tissues [10]. PIP1 aquaporins are expressed in the anther and stigma epidermis in *Brassica* sp. [11-12] and *OsPIP1;1* and *OsPIP4;1* are highly expressed in rice anthers [13]. Six out of 35 aquaporin genes in the Arabidopsis genome are known to be expressed in pollen [11]. Among them, *TIP1;3* is expressed in vesicles and vacuoles of vegetative cells, and *TIP5;1* is expressed in vacuoles of sperm cells [11, 14-16]. TIP5;1 and TIP1;3 are involved in the nitrogen metabolic pathway during pollen tube growth [17, 18]. NIP4;1 and NIP4;2 are two pollen-specific aquaporins; NIP4;1 functions during pollen development and germination, while NIP4;2 functions exclusively during pollen tube growth [19]. As well as being expressed in pollen, *AtNIP7;1*, *AtSIP1;1*, and *AtSIP2;1* are expressed in other tissues. *AtNIP7;1* is expressed in microspores [20], while *AtSIP1;1* and *AtSIP2;1* are expressed in the vascular tissues of the flower petal, stigma, stamens, and pollen [21].

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As pollen hydration involves the movement of water, it is possible that aquaporins function in the stigma to facilitate this process. Previously, PIPs were found to be expressed in the stigma of Arabidopsis [12, 13]. To explore the role of PIP aquaporins expressed in the stigma in pollen hydration, we screened a number of *PIP* mutants obtained from Arabidopsis Biological Resource Center (ABRC). We found a mutant with a T-DNA insertion in *PIP2;5* that disrupted expression of the gene and resulted in slower pollen hydration on the stigma during pollination, comparing with that in wild type. Thus, we conclude that PIP2;5 played an important role in water movement during pollen hydration.

2. Results

2.1 Characterization of *pip2;5* mutant

To understand the role of aquaporins in pollen hydration, we tried to isolate Arabidopsis T-DNA insertion mutants showing delayed pollen hydration from the T-DNA insertion collection of ABRC. One mutant (SALK_072405) with delayed hydration was identified. In this mutant, the T-DNA was inserted in the second intron of the genomic sequence of *AT3G54820* (1,215 bp downstream from the initiation codon ATG) (Figure 1A). *AT3G54820* encodes the protein PIP2;5, which belongs to the PIP2 subfamily. The genotype of the heterozygous plants (*pip2;5*/+) among the progenies was identified by a PCR-based method using gene-specific primers and the T-DNA primer. The genotype ratio of the wild type, heterozygous, and homozygous plants among the offspring of heterozygous plants was about 1:2:1 (59:121:62), indicating the presence of a single T-DNA insertion. We investigated the effect of the T-DNA insertion on the transcript level of *PIP2;5* by RT-PCR. For *pip2;5* homozygous mutant plants, no product was obtained, indicating full knock-out of *PIP2;5* (Figure 1B). The *pip2;5* homozygous plants were used for further analysis.

2.2 Postponed hydration of pollen grains on *pip2;5* stigma

We examined the viability of *pip2;5* pollen grains by Alexander staining. No difference in viability was identified between the mature pollen grains of the *pip2;5* mutant and wild type (supplemental Figure 1A, B). Next, we examined the germination and tube growth of pollen *in vitro*. The pollen germination ratio and pollen tube morphology of the *pip2;5* mutant was

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comparable with those of wild type *in vitro* (Supplemental Figure 1C, D).

Pollen grains were considered to be fully hydrated when the width remained constant for 10 min. We determined the hydration completion time for pollen from wild type and the *pip2;5* mutant on stigmas of the *pip2;5* mutant and wild type. First, when wild type and *pip2;5* pollen grains were placed on a wild type stigma, the hydration completion time show no difference between the wild type and *pip2;5* pollen grains (data not showed). Next, the pollen grains were placed on wild type and *pip2;5* stigmas. Wild type pollen initiated hydration rapidly following it contact with on the wild type stigma (hydration completed by 9.5 ± 2.7 min after pollination. n = 61, Figure 2), however the pollen showed a severe delay in the completion of hydration on the *pip2;5* stigma (12.8 ± 5.2 min to complete hydration, n = 61, P<<0.01; Figure 2). By 12 min after pollination, 92% of wild type pollen grains had completed hydration on the wild type stigma, whereas only 36% had completed hydration on the stigma of the *pip2;5* mutant. The results suggest that pollen hydration is postponed on the *pip2;5* stigma.

After hydration, the pollen grain germinates and produces a pollen tube that will penetrate the stigma and grow through the style and along the transmitting tract to enter the ovule. To detect whether pollen tube growth was affected in the *pip2;5* mutant, we examined pollen tube growth in the pistil by histochemical analysis of emasculated wild type and *pip2;5* pistils hand-pollinated with pollen of P_{LAT52}::*GUS* expressing plants at 0.5, 1.5, 2.5, 4.5, 6, or 9 hours after pollination (HAP). The pollen tube growth in the style and transmitting tract was normal in *pip2;5* plants (Supplemental Figure 2). Seed set was also normal in *pip2;5* self-pollinated plants (data not shown). These results imply that the PIP2;5 mutation affected pollen hydration on the stigma.

2.3 Complementation of *pip2;5* mutant

To confirm that the *pip2;5* delayed-pollen-hydration phenotype was indeed due to a mutation in *PIP2;5*, we amplified the predicted promoter (3,800 bp) and coding sequence (CDS) of *PIP2;5*, subcloned them into PMDC107 vector (conferring hygromycin resistance to transgenic plants), and introduced them into the *pip2;5* homozygote via infiltration with *A. tumefaciens*. Seven lines harboring $P_{PIP2;5}$::*PIP2;5-GFP* were obtained by resistance screening and fluorescence detection. Five transformants can rescue the hydration defect of the mutants (average pollen hydration completion completion time, 9.4 ± 2.6 min; n = 56, combined data; P<<0.01, Figure 2, 3). Therefore, the *pip2;5* mutant phenotype could be converted to a normal phenotype by

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constitutively expressing the wild type PIP2;5 CDS.

The function of aquaporins is closely linked to their subcellular localization; therefore, we constructed P_{35S}::*GFP-PIP2;5* to investigate the subcellular localization of PIP2;5. The T-DNA insertion caused a truncated protein, designated as PIP2;5m, which contains the first and second exons of *PIP2;5*. The subcellular localization of PIP2;5 was in the plasma membrane (PM). In contrast, GFP-PIP2;5m showed a clear cytoplasmic and PM localization (Figure 3). This result indicates that the N terminus is important for determining the PM localization of the PIP2;5 protein.

2.4 Preferential expression of PIP2;5 in the stigma

The papilla cells of the stigma control whether a pollen grain hydrates or not, so, if PIP2;5 does play a role in regulating water transport at the stigma surface, it might be expressed in the papilla cells of the stigma. Previous Affymetrix analysis showed *PIP2;5* had a particularly strong transcript expression level in the stigma [22]. To determine whether this was the case, we detected the expression level of PIP2;5 protein in the developing flowers of transgenic Arabidopsis harboring $P_{PIP2;5}$::*PIP2;5-GUS*. The results showed that PIP2;5 was expressed in the style and the papilla cells of stages 9-14 flowers (Figure 4 A-H). In the pistil, GUS activity was detected in the papilla cells and the top part of the style close to the stigma. Strong GUS activity was detected in the transmitting tract. In stage 15 flowers, the weak GUS signals were detected in the papilla and style. In stage 16 flowers, GUS activity was observed at low levels in the anthers but not in pollen grains (data not shown).

2.5 Upregulation of *PIP2;5* by cold treatment

Abiotic stresses have adverse effects on plant growth and productivity. PIP2;5 is thought to be involved in water transport in plants and, as a consequence, it may play a role in water deficit tolerance. The overexpression *PIP2;5* in transgenic Arabidopsis plants showed no advantage under normal conditions but presented a rapid water loss under drought stress conditions [23]. In our study, pip2;5 mutants did not show any growth alteration in standard medium and on plates with 50 mM NaCl, 100 mM NaCl. pip2;5 mutants only showed reduced slightly roots length on plates with 200 mM sorbitol (Supplemental Figure 3). It indicated that pip2;5 mutants might be sensitive to and Osmotic stress, but not to salt stress.

Similar to water and salt stresses, cold stress is an important abiotic stress factor that

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significantly limits plant growth and development. To understand the effect of cold stress on *PIP2;5* expression, transgenic Arabidopsis seedlings (20 days after germination and flowers at stage 14) harboring $P_{PIP2;5}$::*PIP2;5-GUS* were subjected to a cold treatment at 4°C for 6 days. In response to the cold treatment, GUS signals were enhanced in the root, leaves, and papilla cells (Figure 4I-L). Real-time PCR analysis confirmed that the *PIP2;5* transcript levels were upregulated in the inflorescences and seedlings after the cold treatment (Figure 4M).

3. Discussion

The pollen hydration process is typically completed quickly. The water used for pollen hydration is derived from the stigma in flowering plants with dry stigmas. It is impossible that the free diffusion of water molecules provides sufficient water for timely pollen hydration. The plasma membrane is the first barrier limiting water exchange in plant cells, and the rate and capacity of its water transport is mainly determined by aquaporins. Therefore, there must be different aquaporins that are expressed and functioning in the stigma. The PIP aquaporins appear to play a major role in controlling membrane water permeability. This class of aquaporins can be grouped into two subfamilies: PIP1 and PIP2. We found that *PIP2;5* was expressed in papilla cells and the transmitting tract with increasing strength at stages 9-14 before pollination. The pollen grains on the homozygous *pip2;5* mutant stigma showed delayed hydration, longer average hydration time, and lower hydration frequency at every time point (Figure 2). However, subsequent pollen tube growth in the style and transmitting tract was normal in *pip2;5* plants (Supplemental Figure 2). Based on our results, we speculate that the *PIP2;5* mutation affects the transport activity or density of aquaporins in the stigma papillae, thereby affecting the pollen hydration rate. The molecular mechanisms of this process require further study.

Cold stress, including freezing and chilling, is one of the major stresses affecting the growth and development of plants, and it can result in huge crop losses. In plants, aquaporins are believed to play an important role in maintaining water homeostasis not only under normal growth conditions but also under various stress conditions. For example, the cotton aquaporin GhTIP1;1 was shown to play a role in the response to cold stress [24] and PIPs in rice have also been shown to play important roles in re-establishing the water balance after chilling [25]. Overexpression of *PIP2;5* was shown to alleviate inhibition of plant growth under low temperature and to facilitate

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seed germination of transgenic Arabidopsis under cold stress [23, 26]. The Columbia and Ler ecotypes have a defined a narrow temperature window for pollen germination and pollen tube growth. Another study reported that pollen germination rates dropped to <50% at temperatures above and below 22°C [27]. In our study, we found that PIP2;5 was preferentially expressed in the stigma and that the transcript level of *PIP2;5* increased after a cold treatment (Figure 4I-M). Based on these results, we speculate that at optimum temperature, the PIP2;5 protein may be involved in the pollen-stigma interaction, and under cold conditions, increased expression of *PIP2;5* in the stigma would create more favorable moisture conditions for pollen germination. Next, our research will focus on this this problem.

4. Materials and methods

4.1 Arabidopsis strains and growth conditions

Arabidopsis mutant *pip2;5* (SALK_072405) seeds were obtained from ABRC. The genotypes of *pip2;5* mutant plants were determined by a PCR-based method (primers LB1.3, LP1, and RP1). Seeds of the *pip2;5* and transgenic lines were plated onto medium supplemented with 50 mg/L kanamycin for *pip2;5* and with 30 mg/L hygromycin for transgenic lines harboring pMDC107 and pMDC163-based constructs. Wild type (Col-0) and mutant Arabidopsis plants were grown as reported previously by Xu et al. [28]. The primers used to detect the T-DNA insertion were as follows: LP1: GAAAGTGACGTTGGTGAGAGC, RP1: AAACTAGCCCTCAACACAAACTGATG, and LB1.3: ATTTTGCCGATTTCGGAAC.

4.2 Plasmid construction and plant transformation

For genetic complementation analyses, a genomic *PIP2;5* fragment was PCR-amplified with the following primers: F, ACCAACATAGAGAAGATGATTCGATTTCCATGCGTTGAA and R, TTAAACGTGAGGCTGGCTCCTG. The fragment was inserted into pMDC107 using Gateway Technology (Invitrogen, Carlsbad, CA, USA). The construct was sequenced, introduced into *Agrobacterium tumefaciens* (strain GV3101), and transformed into *pip2;5* plants. To analyze the expression pattern of PIP2;5, the 3,800 bp promoter region and PIP2;5 coding sequence (CDS) were inserted into the pMDC163 vector containing *GUS* (encoding β-glucuronidase) to generate $P_{PIP2;5}$::*PIP2;5-GUS*. To determine the subcellular localization of PIP2;5, PIP2;5 and PIP2;5m

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coding sequence were inserted into the pMDC107 vector containing *GFP* (encoding green fluorescent protein) to generate the P_{35S}::*GFP-PIP2;5* and P_{35S}::*GFP-PIP2;5m* constructs.

4.3 Pollen assays

Pollen hydration assays on the stigma were performed by hand-pollinating emasculated wild type and mutant stigmas and observing hydration of pollen grains under a Zeiss Axioscope microscope (Carl Zeiss MicroImaging Inc. Thornwood, NY, USA) until no further change in pollen diameter was observable. During this process, images were captured every 30 s. Pollen diameter was measured using ImageJ software and data were analyzed using SigmaPlot 11.0.

4.4 Confocal microscopy

The P_{35S}::*GFP-PIP2;5* and P_{35S}::*GFP-PIP2;5m* constructs were introduced into tobacco leaves via infiltration with *A. tumefaciens* for transient expression. The excitation and emission wave lengths for GFP fluorescence were 488 nm and 505-530 nm, respectively. Images were captured with a TCS SP5II confocal laser scanning microscope (Leica, Wetzlar, Germany).

4.5 GUS assays

Staining to detect GUS activity was performed according to Sieburth and Meyerowitz [29]. Flowers and seedlings of $P_{PIP2;5}$::*PIP2;5-GUS* plants were incubated in GUS staining solution [1 mg/mL 5-bromo-4-chloro-3-indolyl-b-glucuronic acid (Biosynth AG, Staad, Switzerland), 2 mM $K_4Fe(CN)_6$, 2 mM $K_3Fe(CN)_6$, and 0.1% (v/v) Triton X-100 in 50 mM sodium phosphate buffer, pH 7] for 1 to 3 d at 37°C. The stained samples were cleared in 70% (v/v) ethanol. Stained specimens were visualized and photographed under an Olympus BX51 microscope equipped with a CCD camera (Olympus, Tokyo, Japan).

4.6 Cold treatment

Transgenic plants harboring $P_{PIP2;5}$::*PIP2;5-GUS* were subjected a cold treatment 4°C for 6 days. The seedlings used in these experiments were 20 days old and had stage 14 flowers. The expression of the construct was determined by GUS staining, as described above, and by RT-PCR.

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Figure legends

Figure 1. (A) Schematic representation of *PIP2;5* genomic organization and T-DNA position in SALK-072405. Exons of *PIP2;5* are represented by black boxes and introns by lines. Triangle

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shows location of T-DNA insertion of SALK-072405. Primer LB1.3 is for left border of T-DNA, and RP1 and LP1 are gene-specific primers at upstream and downstream locations, respectively. (B) RT-PCR showing *PIP2;5* and *TUBE2* expression in wild type type and *pip2;5* flowers.

Figure 2. Hydration of wild type pollen on wild type and *pip2;5* homozygous stigma. (A-D) Wild type pollen status at 1 minute hydration of pollen (MHP), 9 MHP, 16 MHP, and 20 MHP on wild type stigma; (E-H) Wild type pollen status at 1 MHP, 9 MHP, 16 MHP, and 20 MHP on *pip2;5* homozygous stigma; (I-L) Wild type pollen status at 1 MHP, 9 MHP, 16 MHP, and 20 MHP on *pip2;5*+$P_{PIP2;5}$:: *PIP2;5* transgenic stigma. Bar =50 µm in (A)-(L). (M) Statistical analysis of pollen hydration time; (N) Box plot showing completion of hydration. In (M) and (N), Completion is defined as the time elapsed from the first change in diameter to the time at which final diameter is achieved. Box plot depicts median, 25th and 75th percentiles, and full range of values. Single spots represent outlying points, defined as being further from 75th percentile than spread between 25th and 75th percentiles.

Figure 3. Subcellular localization of PIP2;5 and PIP2;5m in tobacco leaf epidermal cells. Bar =20 µm.

Figure 4. Histochemical localization of $P_{PIP2;5}$:: *PIP2;5-GUS* activity in flowers and seedlings. (A-H), flowers at stage 9-16. (I) and (J), seedlings 20 day after germination before (I) and after (J) cold treatment. (K) and (L), pistils at stage 14 before (K) and after (L) cold treatment. (M), Real-time PCR analysis of *PIP2;5* in seedlings and inflorescences under cold treatment. Asterisks indicate significant difference (Student's test, $P<0.01$).

Supplemental Figure 1. Pollen viability and seed set were not affected in *pip2;5* homozygous plants. (A) and (B), Alexander staining of mature anthers from wild type and *pip2;5* homozygous plants. (C) and (D), Germination of pollen from wild type and homozygous *pip2;5* homozygous plants *in vitro*. Bars =100 µm.

Supplemental Figure 2. Normal pollen tube growth in transmitting tract. (A), Representative histochemical staining of Pro_{LAT52}:GUS pollen on emasculated wild type pistils at 0.5, 1.5, 2.5, 4.5, or 6 hours after pollination (HAP). (B), Representative histochemical staining of Pro_{LAT52} :GUS pollen on emasculated *pip2;5* pistils at 0.5, 1.5, 2.5, 4.5, or 6 HAP. Images shown are representative of three independent experiments. Seven or eight pistils were analyzed at each time

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point in each experiment. Bar =250 µm.

Supplemental Figure 3. Growth of mutants under various water stresses show differential sensitivity. Wild type (WT) and *pip2;5* mutants were germinated on GM gelrite plates containing various solutes (50 or 100 mM NaCl as well as 200 mM Sorbitol) to imposed water stress. Seedlings were grown for 15 days photographed.

Figure 1

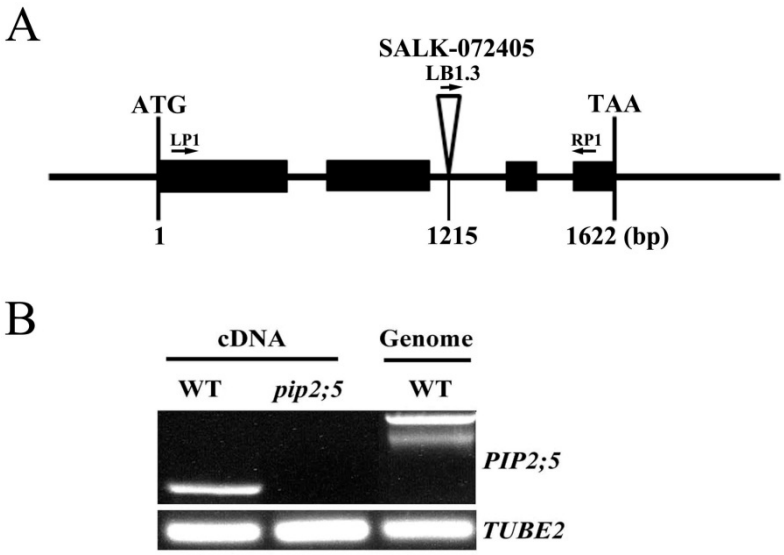

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Figure 2

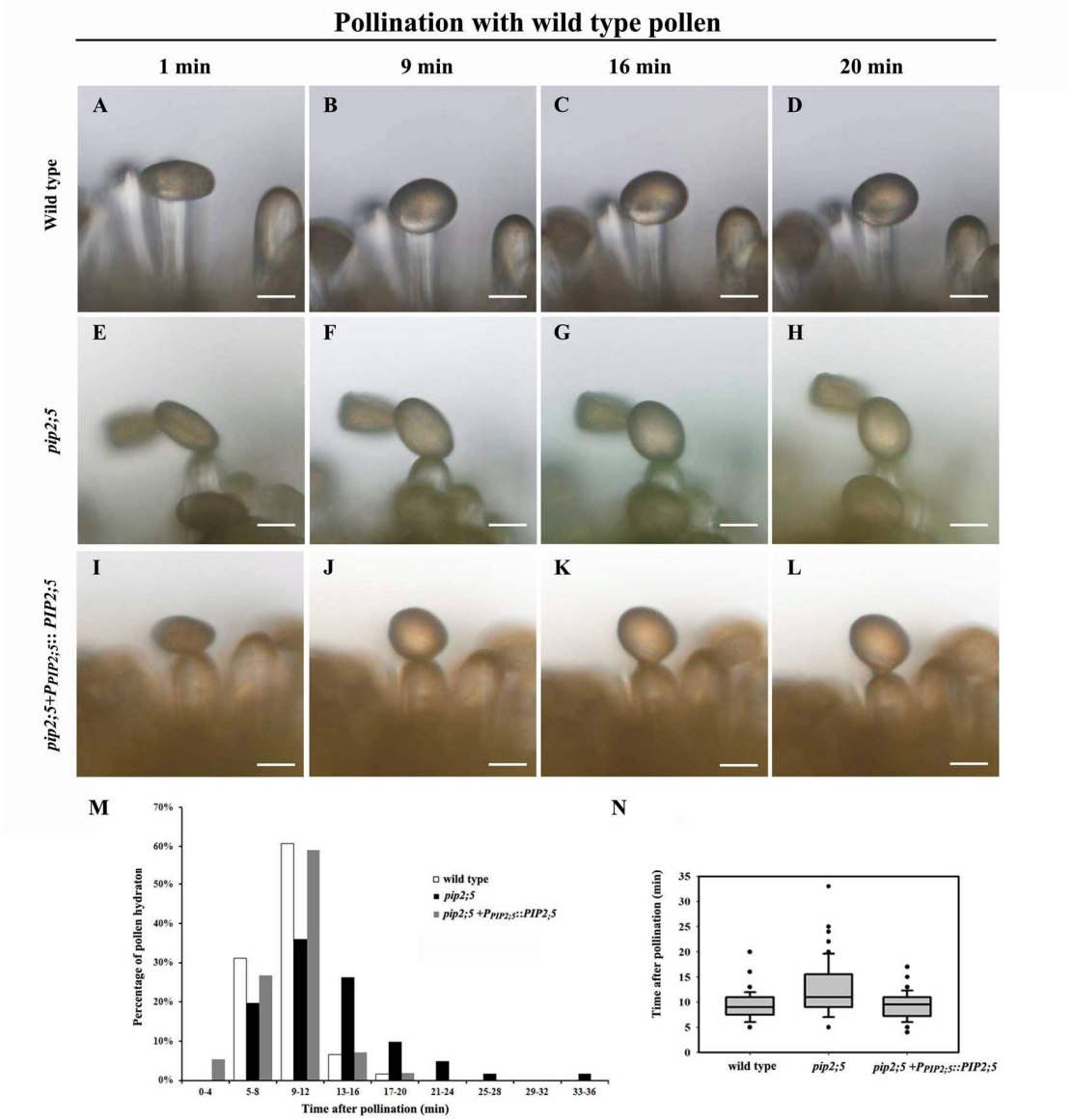

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Figure 3

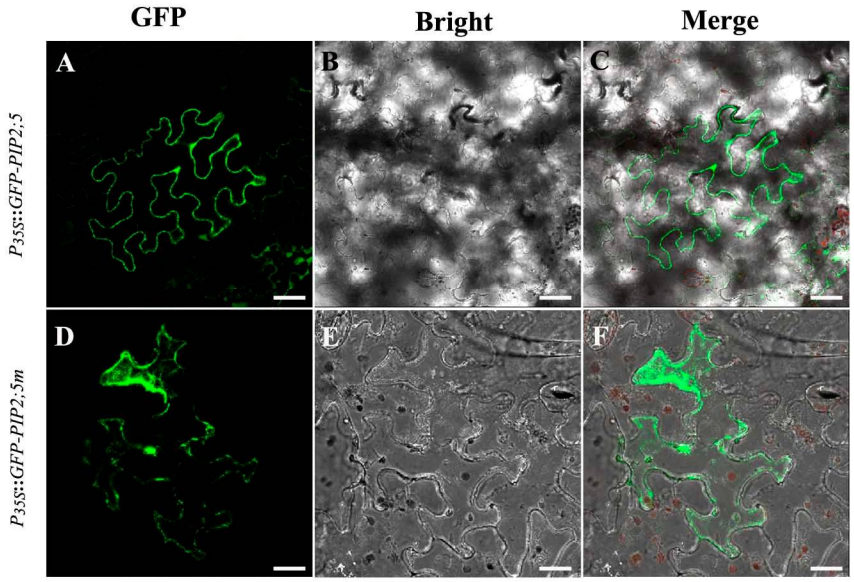
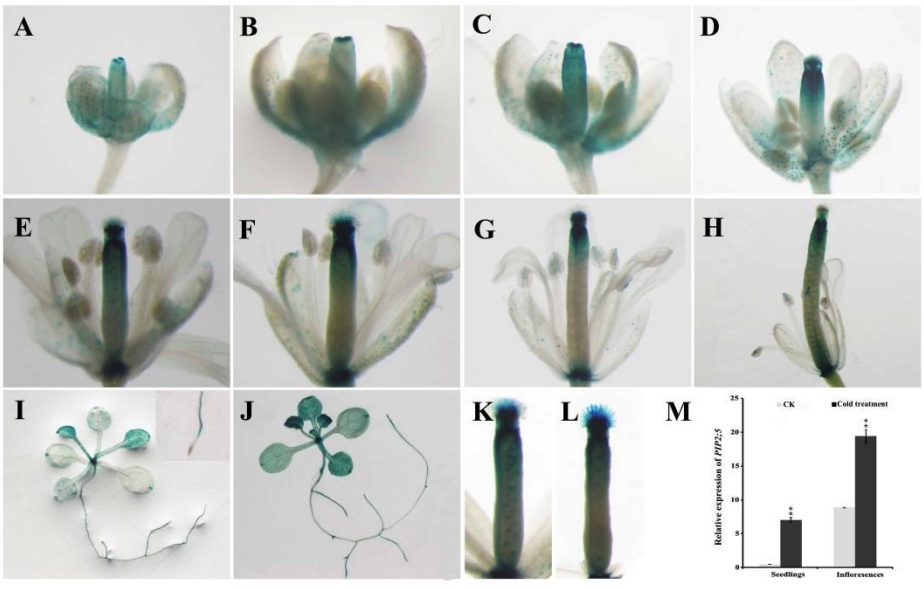


Figure 4



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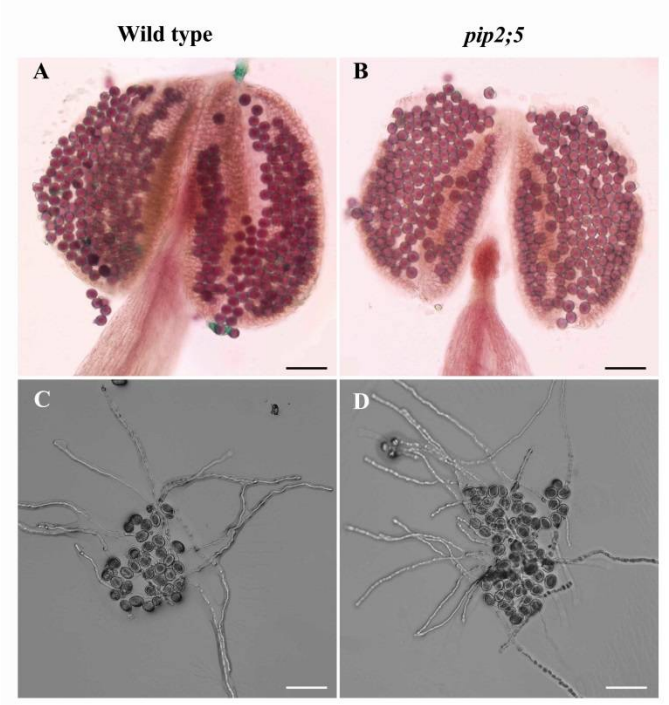
Supplemental Figure 1



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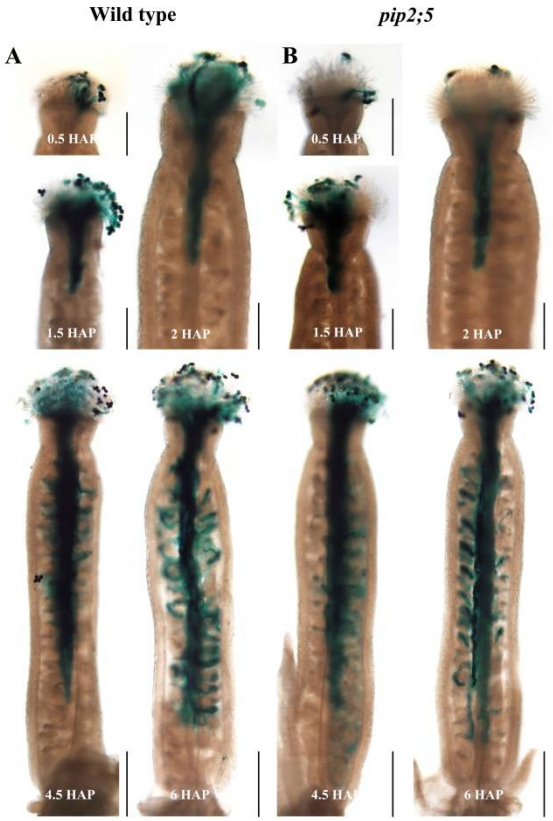
Supplemental Figure 2



Supplemental Figure 3