A Loss of Function Mutation in Protein phosphatase 1 Regulatory Subunit *INH3* Improves Poor *hpat1/3* Fertility and Pollen Tube Growth

by

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An honor thesis submitted to fulfill the requirement of the Honors program

Molecular, Cellular, and Developmental Biology

University of Michigan

Class of 2022

Acknowledgements

I really appreciate how much financial and emotional support my family have been providing to me. Without you all, I would not be able to fully focus on and enjoy my academic and personal development. I know I have missed some many treasurable times staying with you all, but I hope I can be a better granddaughter, daughter, and sister in the future.

I would like to thank Dr. Cora MacAlister for giving me an opportunity to do research in her lab and begin to learn about the world of research. I would also like to thank her for being a reader on my honors thesis in her lab.

I would like to say thank you to Steven Beuder for all the effort he spent on me. Besides teaching me how to do experiments, he always guided me through the principle behind each experiment and offered to help me in and outside the lab.

I would like to thank Cecilia Lara Mondragon for teaching me how to use R to make professional and beautiful figures for my paper.

I would like to thank Dr. Gary Huffnagle and Dr. John Schiefelbein for being my readers for this thesis and offering valuable suggestions and comments."

I would like to thank the University of Michigan for providing the best education. I am glad that I have become a person who knows how to learn, act, think, love, and appreciate.

Forever, go blue!!!

Abstract

The MacAlister lab previously found that *HYDROXYPROLINE O-ARABINOSYLTRANSFERASE 1* and *3* (*HPAT1* and *HPAT3*) were required for full pollen fertility in *Arabidopsis thaliana* by initiating post-translational protein modification on cell wall structural proteins (Beuder, et al., 2020). The *hpat1 hpat3* double knockout mutants *(hpat1/3)* showed low male fertility and disruption in pollen tube growth, which led to a large decrease in seed set (the average number of seeds per silique) compared to wild-type (WT) (MacAlister et al., 2016). They noticed that with the *hpat1/3* mutation, the pollen tube cell wall would frequently rupture, the pollen tube would sometimes initiate a second point of growth leading to pollen tube branching, and the growth rate of the pollen tubes would be slower. To learn more about how HPATs contributed to pollen tube growth, they looked for suppressor genes involved in the HPAT pathway that affects pollen tube growth and fertility and analyzed the role of each gene and looked for genes that were involved with pollen tube cell wall. Finally, they determined the suppressor was in the gene *INHIBITOR-3* (*INH3*), which codes for a regulatory subunit of protein phosphatase 1 (PP1), and has been reported to be required for the embryo development and negative phosphoprotein phosphatase regulation (Zhang, et al., 2008). The suppressor mutation in the gene *INH3* was caused by a G to A single-nucleotide substitution, which was predicted to cause an early truncation after amino acid W43. This allele was named *inh3-3* because *inh3-1* (SALK_0445593) and *inh3-2* (SAIL_806_C02) T-DNA insertion alleles had been previously characterized (Takemiya et al., 2009).

In order to better understand the mechanism behind *inh3-1* and *inh3-3* mutations and the *hpat1/3* pollen fertility pathway, the objectives of the research in my honor's thesis are to study the following: 1) how does *inh3-3* mutation suppress *hpat1/3* pollen tube fertility defects, 2) if *inh3-1* will also suppress the *hpat1/3* fertility defect as *inh3-3* does, and 3) if *inh3-3* and *inh3-1* can both lead to the decrease in fertility in the WT background. The experiments in this thesis show that knocking out *INH3* with either *inh3-1* or the *inh3-3* allele will suppress *hpat1/3* pollen fertility defects. Both *inh3-3* and *inh3-1* mutants in pollen could increase the transmission in *hpat1/3* pollen and the fertility of *hpat1/3* plants. However, the *inh3-3* pollen actually decreased the transmission through the pollen in the WT background without *hpat1/3* mutants as *inh3-1* did. However, the exact mechanism of how the loss-of-function *INH3* interacts with the hpat1/3 mutant protein in the pollen tube fertility pathway at the cellular level is still unknown and requires further investigation.

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Introduction

Arabidopsis thaliana is a common and wide-spread plant from the mustard family. *Arabidopsis thaliana* is a commonly used model plant in molecular biology experiments because of its 6-week short life cycle, high seed production, and small number of chromosomes. Beside these characters, the procedure for chemical and insertional mutagenesis, plant transformation through DNA insertion, plant crosses techniques, and a more complete chromosome maps of molecular markers and gene mutation are well developed in *Arabidopsis thaliana* (Meinke et al, 1998).

Sexual reproduction in *Arabidopsis thaliana* is required to produce progeny. The male gametes are called pollen, which are produced by the stamen; while the female gametes are generated in the ovule inside the flower ovary (Barrett, 2010). After the maturation of pollen, the pollen will be delivered to the ovary, combine with the female gamete, and fertilize it. If the fertilization is successful, then seeds are produced in siliques, slender fruits that enclose and protect seeds (Meinke et al, 1998). Later, when planted in the soil, the seed will germinate and grow into individual plants.

Since the female gametes are located within the ovary, flowering plants, including *Arabidopsis thaliana* developed a structure called a pollen tube, that can safely transport the pollen's cargo of sperm nuclei to the ovule (Schlupmann et al., 1994). Because of its function in pollen transport, pollen tubes cannot grow and expand in all directions. Instead, pollen tubes grow through tip growth, going anisotropically down the pistil towards the ovule (Krichevsky et al., 2007). Due to tip growth, pollen tubes expand exclusively at their extensible tip, while no active growth occurs in the shaft regions which refers to the rest of the pollen tube outer structure. (Schlupmann et al., 1994, Krichevsky et al., 2007). As a result, the pollen tube increases its length instead of expanding horizontally to increase its diameter.

The difference in expansion ability between the tip and the shaft region are due to the differences in the composition of the cell wall. The plant cell wall is important for providing mechanical strength and protection to cells and for allowing cells to change the direction of growth in response to extracellular and intracellular signals (Houston et al., 2016). The cell wall is primarily composed of various carbohydrates. The cell wall of the pollen tube contains two layers. The inner cell wall of the shaft is reinforced with callose, and the outer cell wall contains pectin with cellulose and hemicellulose, which are synthesized at both the plasma membrane and from the ER-Golgi secretory pathway (Taylor & Hepler, 1997). The pollen tube tip region cell wall is primarily composed of extensible pectin, which is converted to a rigid form of pectin in the shaft region; while cellulose is mainly concentrated in the shaft region with minimal cellulose in the pollen tube tip cell wall (Chebli et al., 2012).

In addition to carbohydrates, proteins are another essential component to build the cell wall at the tip of the pollen tube. To facilitate the active pollen tube tip growth, at the molecular level, the post-Golgi vesicles that contain cell wall material and cell wall modifying enzymes target the tip growth domain, fuse, and secrete their contents into the cell wall, which thickens the cell wall, increases pollen tube growth rate, and allows the pollen tube to elongate (McKenna et al., 2009). One of the most abundant secreted proteins are the glycosylated protein of the hydroxyproline-rich glycoprotein (HRGP) superfamily. Proline residues in the protein backbone are hydroxylated by prolyl hydroxylase. Some HRGPs, namely members of the Extensin family can form a 3D network with the polysaccharide to provide mechanical properties to the cell wall (Bacete & Hamann, 2020). Previous research found that hydroxyproline (Hyp) O-arabinosylation which is frequently found on Extensins, is required for pollen tube growth and fertilization (MacAlister *et al*., 2016). Hyp O-arabinosylation requires the enzymatic activity of Golgi-localized HYDROXYPROLINE *O*-ARABINOSYLTRANSFERASEs (HPATs), which add a single arabinose sugar to Hyp residues on target proteins including extensins and small signaling peptides (Ogawa-Ohnishih, et al., 2013).

The MacAlister lab previously found that *HYDROXYPROLINE O-ARABINOSYLTRANSFERASE 1* and *3* (*HPAT1* and *HPAT3*) were required for full pollen fertility in *Arabidopsis* (Beuder, et al., 2020). The *hpat1 hpat3* double knockout mutants *(hpat1/3)* show low male fertility and disrupted pollen tube growth, which leads to a large decrease in seed set (the average number of seeds per silique) compared to wild-type (WT) (MacAlister et al., 2016). They noticed that with the *hpat1/3* mutation, the pollen tube cell wall would frequently rupture, the pollen tube would sometimes initiate a second point of growth leading to pollen tube branching, and the growth rate of the pollen tubes would be slower due to disrupted cell wall organization (Beuder et al., 2020).

To learn more about how HPATs contributed to pollen tube growth, we looked for suppressor genes involved in the HPAT pathway that affects pollen tube growth and fertility. This includes second mutations that suppress the phenotypic effect caused by the first mutation (Hodgkin, 2005). The discovery of a suppressor was expected to provide more information about the genetic interaction that involves *hpat1/3*. To identify suppressors of the *hpat1/3* pollen tube fertility defect, the MacAlister lab performed a suppressor screen in which *hpat1/3* seeds were mutagenized, and then plants were screened for increased seed production. The experiment detail was referenced from Beuder & MacAlister (2020) and Beuder et al. (2020). They started by treating the *hpat1/3* seeds with 0.2% ethyl methanesulfonate that caused genetic mutation in *hpat1/3* genome and screened for the increased silique length and seed numbers in the progeny. They were able to identify several families that produced long siliques with more seeds than the *hpat1/3* background and confirmed that these families were homozygous for the original *hpat1* and *hpat3* insertion mutations. They also found that the suppressors increased seed set even when the plants were heterozygous for the suppressive mutation (Beuder et al., 2020). The possible reason behind this is that when a heterozygous suppressor, suppressor in general, plant goes through meiosis, 50% of the resulting pollen carries the suppressor mutation. If the pollen with the suppressor mutant has increased fertility, half of the total pollen is still fertile enough to fertilize most of the available ovules.

Specific candidate causative suppressor mutations were identified using genetic and bioinformatic approaches. First, the suppressor was backcrossed to *hpat1/3* plants for several generations to remove un-linked background mutations followed by a self-fertilization. In the resulting generation (BC5F2), test crosses were carried out to determine if suppressed plants were homozygous or heterozygous for the suppressor.For my thesis, I worked with one of the suppressors, which was named *fertility restored in hpat1/3* (frh) before genotyping this suppressor. Unexpectedly, it was found that all of the suppressed plants were heterozygous, suggesting that the homozygous mutants were non-viable. At the same time, it was noticed that a portion of the seeds produces by the heterozygous suppressors appeared abnormal and may represent the missing homozygous mutants. To clone the underlying mutation, these abnormal seeds were germinated on sterile plant plates and DNA was extracted for whole genome sequencing along with sibling homozygous WT *hpat1/3* (non-suppressed) plants.

After being able to collect the *hpat1/3; sup/sup* genotype plants, they wanted to find out the exact location of this suppressor mutant. They did the whole-genome sequencing on DNA of the homozygous suppressor plants (*sup/sup*), the homozygous non-suppressed plants (*SUP/SUP*), and the background (*hpat1/3*) plants. They identified 15 mutations that were unique to the presumed homozygous suppressor plants. By subtracting the *sup/sup* sequence to the *SUP/SUP* and *hpat1/3* background sequence, they got a list of 15 mutants that were possible suppressors using bioinformatics tools.

They then analyzed the role of each gene and looked for genes that were involved with pollen tube cell wall. The candidate mutation in the gene *INHIBITOR-3* (*INH3*). *INH3* gene was a G to A single-nucleotide substitution, which was predicted to cause an early truncation at amino acid W43. This allele was named *inh3-3* because *inh3-1* (SALK_0445593) and *inh3-2* (SAIL_806_C02) T-DNA insertion alleles

had been previously characterized (Takemiya et al., 2009). The *INH3* gene codes for a regulatory subunit of protein phosphatase 1 (PP1), and has been reported to be required for embryo development and negative phosphoprotein phosphatase regulation (Zhang, et al., 2008). *INH3* is a homolog of human *INH3* and yeast phosphatase inhibitor 1 (Ypi1) and occurs as a single gene in *Arabidopsis* instead of a gene family (Takemiya et al., 2009). The G to A single nucleotide polymorphism (SNP) found in *inh3-3* was located in the RVxF motif (KVSW in INH3), which served as a binding site for PP1c (protein phosphatase 1 catalytic subunit) as a ubiquitous serine/threonine phosphatase subunit (Takemiya et al., 2009). The PP1c was a catalytic and regulatory subunit of phosphorylase phosphatase that regulated cell wall formation (Takemiya et al., 2009).

However, *inh3-3* was only a candidate suppressor mutation. Further data would be required to prove that it is responsible for the suppression of the *hpat1/3* fertility phenotype. My thesis focused on establishing a causal link between *inh3-3* and *hpat1/3* suppression and understanding how this suppressor improves seed set in the *hpat1/3* plants. If *inh3-3*, which is an early truncation allele suppresses the *hpat1/3* fertility defect, other loss of function alleles of *inh3* should also suppress. The previously described *inh3-1* allele is caused by insertion of a T-DNA in the second exon of *INH3*, 146 bp downstream of the ATG initiation codon (Takemiya et al., 2009). This mutation was likely to decrease seed production in the WT background (Takemiya et al., 2009). Their research also proved that *inh3-1* was a full loss-of-function mutation in the *INH3* gene. We questioned if the *inh3-1* mutation could also suppress *hpat1/3* that functioned in a similar way as the *inh3-3* mutation in the *hpat1/3* plants. Finally, I also wanted to determine if *inh3-3* had a pollen phenotype in the absence of the *hpat1/3* mutations.

Objectives

In order to better understand the mechanism behind *inh3-1* and *inh3-3* mutations and the *hpat1/3* pollen fertility pathway, the objectives of the research in my honor's thesis are to study the following:

1) how does *inh3-3* mutation suppress *hpat1/3* pollen tube fertility defects,
2) if *inh3-1* will also suppress the *hpat1/3* fertility defect as *inh3-3* does, and
3) if *inh3-3* and *inh3-1* can both lead to the decrease in fertility in the WT background.

Methods

DNA extraction

Young *Arabidopsis thaliana* leaf tissues were collected at the early life stage and extracted DNA using organic extraction. Tissues are frozen with liquid nitrogen and lysed the cell. Then, 300µl of CTAB buffer (2% cetyl-trimethyl-ammonium bromide, 1.4 M NaCl, 100mM Tris HCl pH 8.0, 20 mM EDTA) was added to each tissue tube and incubated it at 65 °C for 20 minutes. Mixture was cooled down for 5 minutes and 300µl of chloroform was added. The mixture was vortexed thoroughly and spun in a microfuge for 2 minutes at 13300 RPM. There was a separation of two layers. The top layer was collected into a new tube and 500µl of 2-propanol was added. The mixture was vortexed thoroughly and spun in a microfuge for 10 minutes at 13300 RPM. The supernatant was discarded, and 500µl of 70% ethanol was added to clean up the unwanted substances on the pellet. The mixture was spun in a microfuge for 2 minutes at 13300 RPM. The ethanol liquid portion was discarded, and the pellet was dried upside down until there was no liquid associated with the pellet. Before use, 30µl TE buffer was added, and the mixture was vortexed thoroughly.

Polymerase Chain Reaction (PCR)

For each 2µl of DNA extracted using the DNA extraction method above, 2µl of 10 x PCR buffer (100mM Tris HCl pH 8.0, 500mM KCl, pH 8.3), 25mM $MgCl_2$, and 2mM dNTPs were added. Depending on the target strand for amplification, forward and reverse primers were added. 2µl each of the front primers and reverse primer (10 µM each) and 7.8µl of DI water were added into the mixture. Finally, 0.2µl of Taq polymerase was added. The mixture was slightly vortexed, transferred to a PCR tube, run in a thermal cycler. The exact time of denaturation, annealing, and extension temperature depended on the size of the target amplifying gene sequence. For the PCR cycle this thesis used, the denaturation temperature was 95 degrees Celsius, the annealing temperature was 60 degrees Celsius, and the extension temperature was 72 degrees Celsius.

Primers

For *inh3-3* genotyping PCR, INH3-W43*f and INH3-43*r primers that bind to the INH3 gene were designed. The primer sequences were tcttcagctATGAGCACAGC and ggttagataggagaagagacc, respectively. For the *inh3-1* transgene PCR, the SALK LB 1.3 and SALK_044593 RP primers bond to the INH3 gene, and primer sequences were ATTTTGCCGATTTCGGAAC and ACCAGATTCGGAGTGTTCATG respectively. For the *inh3-1* genomic PCR, the SALK_044593 LP and SALK_044593 RP primers bond to the INH3 gene, and the primer sequences were GTTTGCTTCTTCGTCGATTG and ACCAGATTCGGAGTGTTCATG respectively.

Pollen tube phenotyping

Pollen grains were collected from young flowers until there was obvious yellow pollen seen by human eyes on the cellophane of the pollen media plate. The protocol was adapted from Rodriguez-Enriquez et al. (2013). The media contained 10% sucrose, 0.01% boric acid, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% myo-inositol, 0.1 mM spermidine, 10 mM γ-aminobutyric acid and 500 µM methyl jasmonate. After that, the pH was adjusted to 8.0. Low-melting temperature agarose was added and heated on a hot plate until the solid was fully dissolved. The media was then transferred into Petri plates. The pollen tube was incubated in a wet and dark box and allowed for incubation for 1-3 hours depending on the experiment. The pollen tube length measurement experiment had pollen tube grow for at least 2 hours, while the pollen bursting frequency experiment had pollen tube grow for 1 hour at most. Bursting was observed when there was a burst or explosion at the end of the pollen tube. The pollen tube length was measured from the end of the pollen seed to the end of the pollen tube. Pictures were then taken using DIC microscopy. Phenotype measurement was measured using ImageJ.

Seed counting

Arabidopsis thaliana siliques were collected into a 1.5ml tube when the siliques were nearly mature. At least 1ml of 70% ethanol was added to completely clear the siliques. Ethanol was changed daily until the silique coat became transparent and seeds could be seen from the outside without breaking the silique valve. The seeds were counted under a dissecting microscope for the number per silique.

Results

The *inh3-3* mutation suppresses *hpat1/3* by improving pollen transmission and pollen tube growth in the *hpat1/3* background

Since the *inh3-3* mutation was identified as a candidate suppressor mutation through whole-genome sequencing, we first wanted to confirm its presence in plants with the suppressor phenotype in the *hpat1/3* background. We designed dCAPS primers to genotype plants for the *inh3-3* SNP mutation. The mutation creates a BspHI restriction enzyme recognition site which is absent in the WT. The *INH3/INH3* plants had a full-length PCR product of 430 bp of the targeted gene sequence. The *INH3* allele of the *INH3/inh3-3* plant would also have this 430 bp gene sequence, while digestion of the allele with *inh3-3* with BspHI would result in two fragments of 297 and 133 bp (Figure 1A). The result showed that all plants with an *inh3-3* mutation were suppressors while all plants without *inh3-3* mutation were WT, which indicated that the suppression of *hpat1/3* fertility defects co-segregated with *inh3-3* mutation (Figure 1A, 1D). This confirmed that the previous suppressor genome sequencing did work and that we could genotype for the *inh3-3* mutation in the following experiments.

We then used genotyping as a tool to analyze how the *inh3-3* mutation affected *hpat1/3* transmission. If *inh3-3* did not affect the transmission of *hpat1/3*, according to Mendel's law of independent assortment, there would be a 1:2:1 ratio of *INH3-3/INH3-3* to *INH3-3/inh3-3* to *inh3-3/inh3-3* plants. Because the *inh3-3/inh3-3* homozygous mutants do not germinate under normal conditions, we would expect to see an *INH3-3/INH3-3* to the *INH3-3/inh3-3* ratio of 1:2. However, our result did not follow the 1:2 ratio. To determine if the influence of *hpat1/3* was significant in plants with *inh3-3*, we performed a chi-squared test. The observed total number of plants was 48, so if there was no *inh3-3* influence, we would expect 16 *INH3-3/INH3-3* and 32 *INH3-3/inh3-3* plants in the progenies (Figure 1B). Comparing the expected ratio to the observed value, we observed a p-value of $3.564*10^{-5}$, less than 0.05, which proved that there was a strong transmission bias favoring inheritance of the *inh3-3* allele in *hpat1/3* plants.

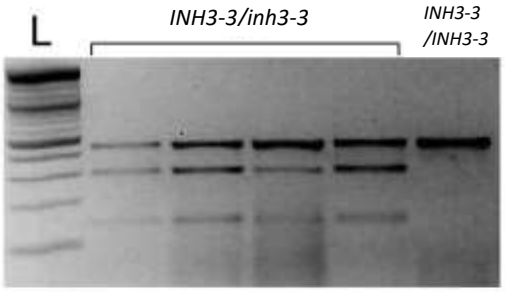
Since the improvement of seed production was the basis of identifying the *inh3-3* mutation as the suppressor by the MacAlister lab when screening for the suppressor, we wanted to find out if *inh3-3* could improve the seed counts of the *hpat1/3* plant. Counting the number of seeds in each silique, we found out that it was statistically significant that the plant with *inh3-3* mutation had a higher seed production per silique compared to the *INH3-3/INH3-3* plant, where the p-value was less than 0.001 (Figure 1C, 1D). As a result, the seed production phenotype co-segregated with the *inh3-3* mutation. Seed production was tightly linked to and could be improved by *inh3-3* mutation in the *hpat1/3* plant.

After finding out that the higher transmission and seed production in *hpat1/3* with *inh3-3* mutation, we looked at the phenotype of pollen tube, which played an important role for delivering the pollen to the ovule for fertilization, for a possible explanation of the phenotypic improvement in seed production. We evaluated the phenotype of pollen tube by looking out its pollen tube length and pollen tube bursting frequency, since pollen tubes were shorter and burst at an abnormally high frequency in *hpat1/3* pollen (Beuder et al., 2020). We measured the pollen tube length under the DIC after letting the pollen grow on a pollen growth media plate for two hours. The pollen tube length in the *hpat1/3* plant shifted towards the longer 100-300um length interval with the *inh3-3* mutation while the pollen tube was mainly less than 100um with the *INH3-3/INH3-3* pollen (Figure 1C, 1D, 1F), which indicated that the pollen tube were longer in *hpat1/3* plants with *inh3-3* mutation.

For the pollen tube bursting experiment, we incubated the pollen on the media plate for 1 hour, in *INH3-3/ INH3-3* pollen tubes, we noticed that there was a 36.7% of the pollen tube burst at its tip (Figure 1E). Since the total number of *inh3-3* heterozygous pollen tubes we imaged was 258, there should have

been 94.7 bursting pollen tubes and 163.3 non-bursting pollen tubes expected like the *INH3-3/INH3-3* plants if *inh3-3* did not affect bursting frequency. In reality, we noticed that the observed bursting vs non-bursting directly from our experiment was 46 to 212. Comparing the expected number of ruptured tubes to the observed number, we found out that there was a significant decrease in pollen tube rupture in the *inh3-3* heterozygous plants than observed in the *hpat1/3* background (P value= $2*10^{-10}$), indicating that the *inh3-3* helped to rescue the pollen tube bursting frequency in the *hpat1/3* plant effectively. In conclusion, *inh3-3* could improve the pollen tube length and decrease pollen tube bursting in *hpat1/3* plants.

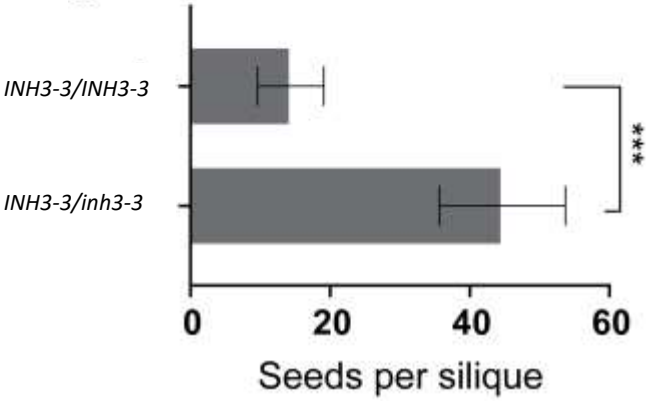
A



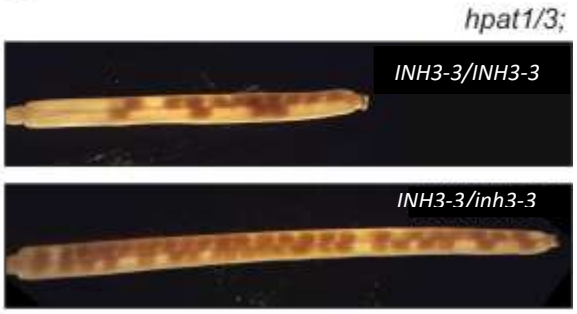
B

Hpat1/3	Number of progeny observed	Percent of progeny observed
INH3-3/INH3-3	3	6.25
INH3-3/inh3-3	45	93.75
p-value	3.54E-05	

C



D



E

INH3-3/inh3-3 progeny Self-fertilization	Number of bursting pollen tube	Number of non-bursting pollen tube
Observed *INH3-3/inh3-3* progeny	46	212
Expected INH3-3/inh3-3 progeny	94.795392	163.2046
Chi-square value	2.95196E-10	

F

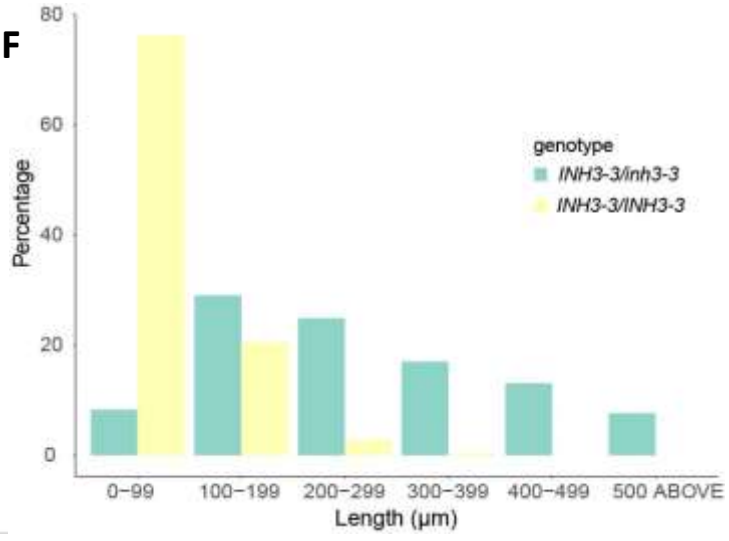


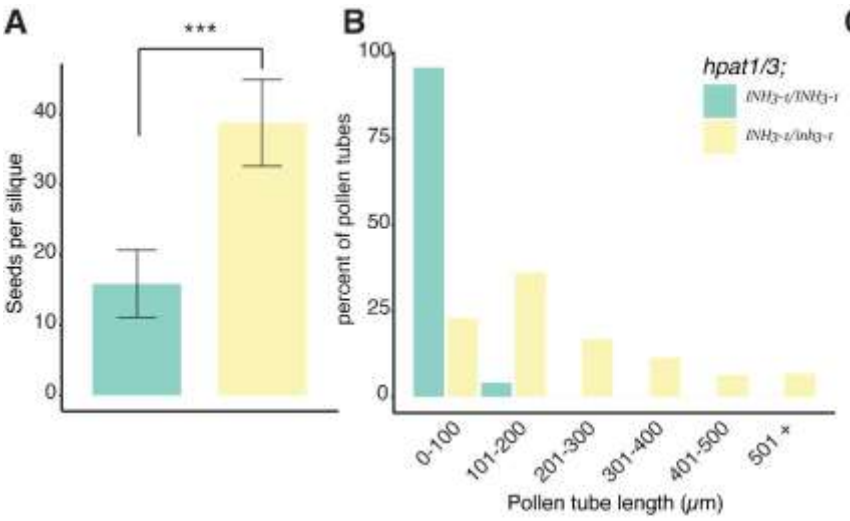
Figure 1. The genotype and phenotype analysis of hpat1/3 plants with inh3-3 mutation. *A) Confirmation of the genotype of phenotypically defected and suppressed hapt1/3 plants. PCR products were treated with BspHI restriction enzymes and run on 2% agarose gel. Full length PCR product = 430 bp. One of the alleles in the PCR product with inh3-3 mutation was cleaved resulting in 297 and 133 bp bands. L stood for the ladder bands. B) Segregation ratios of INH3-3/inh3-3 self-fertilization (BC5F2) individuals. PCR was performed using primers that amplify the inh3-3 mutation sequence, and gel electrophoresis was run. The number and ratio of INH3-3/INH3-3 and INH3-3/inh3-3 were collected. Statistics were performed using the chi-squared test. P-value < 0.001. The bias towards INH3-3/inh3-3 was significant. C) Average seed counts grouped by INH3 genotype. Error bars represent standard deviation. N ≥ 30 siliques per genotype. Statistics performed using Student's t-test; *** indicates p-value < 0.001. D) Representative siliques of each INH3 genotype in the hpat1/3 genetic background. Mature siliques located at the middle location of each branch were collected and cleared with 70% ethanol. E) The expected vs. observed pollen tube bursting frequency in heterozygous INH3-3/inh3-3 genotype plants. The observed INH3-3/inh3-3 was counted directly under the DIC after growing the pollen tube on pollen growth media for one hour. The expected value was calculated with the assumption that inh3-3 could not affect pollen tube bursting frequency, so it would have the same bursting frequency as INH3-3/INH3-3 plants with a percentage of bursting in 36.5%. The difference between these two genotypes were significant, p-value < 0.001. F) The difference in pollen tube length between INH3-3/INH3-3 and INH3-3/inh3-3 genotype after 2.5-hour of growth N>300.*

The *inh3-1* mutation suppresses *hpat1/3* in the same way as the *inh3-3* mutation

After proving that *inh3-3* mutation could suppress the phenotypic defect of *hpat1/3* plants, we looked at if the *inh3-1,* another mutation in the *INH3* gene, could also rescue the transmission, pollen tube, and seed production defect caused by *hpat1/3* like *inh3-3*.

We crossed *INH3-3/inh3-1* with *hpat1/3*; I*NH3-1/INH3-1* plants and allowed its F1 progeny to self-fertilize. We first looked at if the *inh3-1* could improve the pollen tube growth and seed production in *hpat1/3* plants. The average pollen tube length in the *hpat1/3* plants had a significant increase in the presence of the *inh3-1* mutation (Figure 2D). With the *INH3-3/INH3-3* genotype, more than 90% of the pollen tubes were between 0-100 um long. However, in the *INH3-1/inh3-1* plant, the overall pollen tube length shifted to the longer side, having 40% of the pollen tube between 100-200um long and 35% of the pollen tube longer than 300um, which increased the pollen tube growth of *hpat1/3* plants (Figure 2B). The pollen was a mix of *INH3-1* and *inh3-1* pollens. Our result showed that pollens with *inh3-1* mutation, although were only 50% of the total pollen produced in numbers, could bring advantageous factors to the plant thus outcompete the *INH3-1* pollen to fertilize the female gametes. We also found that there were more seeds produced in the *INIH3-1/inh3-1* plants, which stated that *inh3-1* significantly increased the average seeds in each silique in the *hpat1/3* plant (Figure 2A, 2E). As a result, we inferred that the *inh3-1* initially improved the pollen tube quality by having longer pollen tubes, which made the transfer of pollen more efficiently. Since more better pollens could be delivered, more female gametes could be fertilized, thus more seeds would be produced.

However, although there was indeed longer and nicer pollen tubes produced, we were not sure if the transmission of *inh3-1* pollen was successful in *hpat1/3* plants, which was necessary for fertilization and seed production in the downstream. To prove this, we allowed the *INH3-1/inh3-1* parents to self-fertilize and then compared the segregation ratio between *INH3-1/INH3-1* and heterozygous *INH3-1/ inh3-1* progenies. Since *inh3-1* was a T-DNA insertion mutation, we developed primers for both the genomic and the TDNA fragment for *inh3-1*. We would expect to see a band in the genomic product in both *INH3-3/INH3-3* and *INH3-1/inh3-1*, while only the *inh3-1* allele in *INH3-1/inh3-1* would contain the transgene, which was showed as a band for the transgene product. After analyzing the genotype of the progenies, we noticed that there were significant more *INH3-1/ inh3-1* than *INH3-1/INH3-1* plant. This showed a significant transmission bias towards the *inh3-1* mutation (Figure 2C, top). To figure out if this transmission bias was occurring through the pollen, we crossed a *INH3-1/INH3-1* female with *hpat1/3; inh3-1/*+ male, where the *inh3-1* was only expressed in pollen, and analyzed the transmission frequency of the *inh3-1* mutation in progenies. Our data showed that there was a significant bias towards progenies with the transgene, which was carried by *inh3-1* (Figure 2C, bottom). Since *inh3-1* was only carried by the male pollen in this cross, this indicated that the suppression of *inh3-1* on *hpat1/3* occurred when the *inh3-1* mutation was in the pollen. In conclusion, with *inh3-1* mutation, the rescued pollen tube growth delivered *inh3-1* pollen with significant transmission bias to fertilize the female gamete, which eventually led to a higher seed production in *hpat1/3* plants. The suppression brought by *inh3-1* was very similar to that by *inh3-3*, so we inferred that *inh3-3,* like *inh3-1,* was a loss-of-function mutation that involved in the *hpat1/3* fertility pathway as well.



C

Self-fertilize hpat1/3; INH3-3/inh3-1	Number of progeny observed	Percent of progeny observed
INH3-1/INH3-1	3	13.04
INH3-1/inh3-1	20	86.96
p-value	0.039	

WT x hpat1/3; INH3-1/inh3-1	Number of progeny observed	Percent of progeny observed
INH3-1/INH3-1	4	4.7
INH3-1/inh3-1	81	95.3
p-value	6.72E-17	

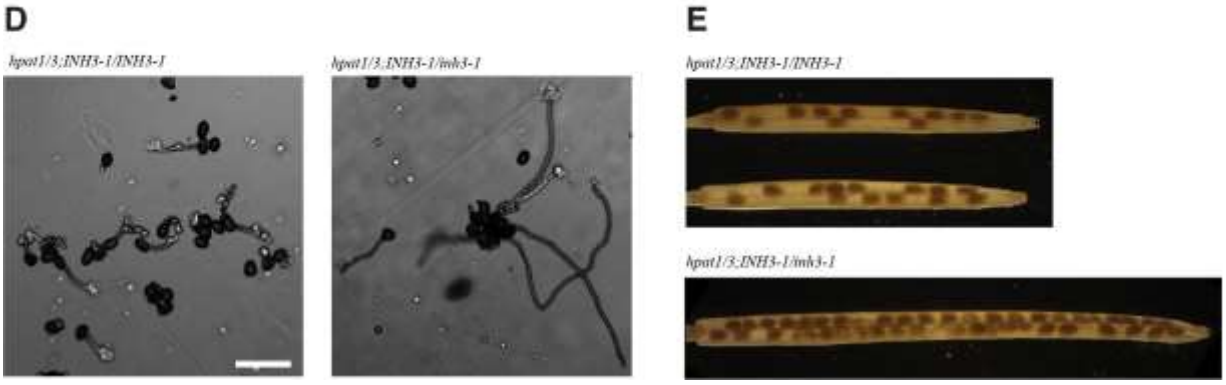


Figure 2. inh3-1 mutation improves transmission, pollen tube growth, and seed count phenotypes in hpat1/3. *A) Seed counts for each genotype. Seeds in each silique were collected after clearing the siliques in the ethanol until the seeds were clear to see from the outside without breaking the siliques. The seed number were counted under the DIC microscope. N ≥ 29 siliques per genotype. Mint column represented INH3-1/INH3-1 while lemon color represented INH3-1/inh3-1. *** indicates that p-value<0.001. B) Pollen tube lengths after 2.5 hours growth in vitro. The pollen was collected and plated on agarose plates. After 2.5 hours incubation at room temperature in a humid and dark environment, the observed length was measured in pollen tubes from INH3-1/INH3-1 and INH3-1/inh3-1 plants. N ≥ 201 pollen tubes per genotype. C) Segregation ratios of progenies with inh3-1 carrying parent(s). Top- Segregation of inh3-1 in self-fertilized progeny of hpat1/3; INH3-3/inh3-1 parents. PCR was amplified using primers that cuts inh3-1 gene sequence and ran on the gel. The number of INH3-1/INH3-1 and INH3-1/inh3-1 was counted. P-value <0.05. Bottom- Segregation of inh3-1 in the progeny of hpat1/3 inh3-1 +/- outcrossed as a male with WT female parent. inh3-1 was expressed only in male pollen/ The number of INH3-1/INH3-1 and INH3-1/inh3-1 progenies were counted. P-value < 0.001. D) Representative INH3-1/INH3-1 (left) and INH3-1/inh3-1(right) pollen tubes imaged in Figure 3A with DIC at 10x magnification; scale bar represents 100 μm. E) Representative INH3-1/INH3-1 (top) and INH3-1/inh3-1(bottom) in the hpat1/3 background siliques cleared with ethanol imaged with DIC.*

inh3-3 leads to the transmission defect in the WT background as *inh3-1*

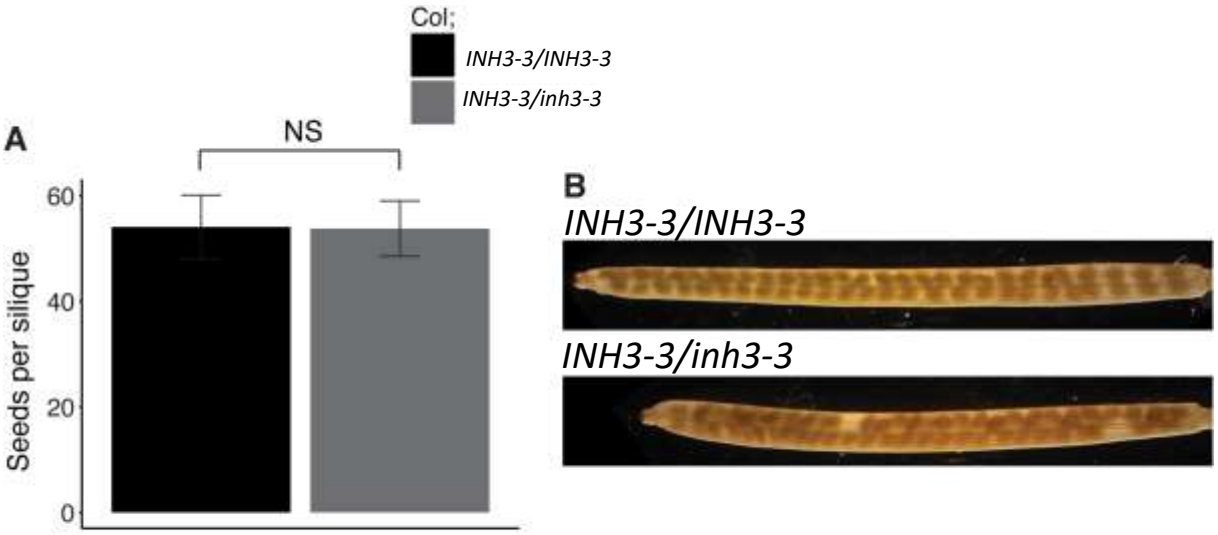
According to Takemiya et al. (2009), *inh3-1*, when expressing in the WT background with no *hpat1/3*, would decrease the pollen transmission and seed production. Since there was a strong similarity between *inh3-1* and *inh3-3* in suppressing the *hpat1/3* mutation, we questioned if the *inh3-3* mutation would also negatively affect the transmission and phenotype of plants in the WT background like *inh3-1*.

Before stepping into our experiment, we needed to get the *hpat1/3* background out of the plants with *inh3-3*. We crossed the WT background plant (*INH3/INH3*) with *hpat1/3; INH3-3/inh3-3*. By genotyping the F2 progeny, we selected *INH3-3/inh3-3* plants without the *hpat1/3* mutation as the *inh3-3* resource plant for the following experiments.

First, we wanted to know how the pollen transmission was affected when there was an *inh3-3* mutation in the WT background. We crossed the *INH3-3/ INH3-3* as female with an *INH3-3/inh3-3* plant as male and tested for the segregation of *inh3-3* in the progenies. We noticed a significantly fewer*INH3-3/inh3-3* compared to the high production of *INH3-1/INH3-1* plants (Figure 3C, bottom). This indicated that since the *inh3-3* was not able to outcompete the *INH3-1* pollen, there was a transmission bias against *inh3-3* pollen in the WT background.

Although the *inh3-3* transmission was not preferred when the mutation was in the pollen, we were curious if the transmission could also decrease when the *inh3-3* mutation was in the female gamete in the WT background. We repeated the same experiment with a reciprocal cross, using an *INH3-3/inh3-3* female and a *INH3-3/INH3-3* male as parents. We found out that, in the progeny, there was a nearly equal separation between the *INH3-3/INH3-3* and *INH3-1/inh3-3*, which meant that no transmission, either positively or negatively, was affected by *inh3-3* in female gamete (Figure 3C, top). These together proved that the transmission of *inh3-3* allele was not favored in the WT background. The *inh3-1* allele was normal through male and female gametes in the WT background (Tekemiya et al., 2009). However, the homozygous mutant *inh3-1* had an embryo arrest phenotype, which might also negatively affect the fertility of the plants.

Finally, we wanted to prove that *inh3-3* was indeed bringing phenotypic defect to the WT background plants by looking the seed production of WT plants with *inh3-3* mutation and compared to the seed production *defect* of *inh3-1* studied by Takemiya et al (2009). We allowed *INH3-3/inh3* plants to self-fertilize and looked at the seed production of their F2 progeny in different genotypes. Our result showed that there was no significant difference between the number of seeds per silique in plants with or without the *inh3-3* mutant (Figure 3A, 3B). The *INH3-3* allele in the *INH3-3/inh3-3* progeny itself outcompetes the *inh3-3* allele so that the seed counts were the same in *INH3-3/INH3-3 a*nd *INH3/inh3-3* plants because it was their “competitive” largely-produced *INH3-3* pollen that fertilized most of the female gametes. These stated that *inh3-3* mutation in the WT background did not affect the seed production. Although we could not see the decrease of seed production in plants with *inh3-3* as it did in *inh3-1*, it was consistent that both *inh3-1* and *inh3-3* were unable to improve the phenotypic character, such as seed production, in the WT background.



C

INH3-3/inh3-3 x INH3-3/INH3-3	Number of progeny observed	Percent of progeny observed
INH3-3/INH3-3	24	51
INH3-3/inh3-3	23	49
p-value	0.884	

INH3-3/INH3-3 x INH3-3/inh3-3	Number of progeny observed	Percent of progeny observed
INH3-3/INH3-3	38	83
INH3-3/inh3-3	8	17
p-value	9.72E-06	

Figure 3. ***inh3-3 causes pollen-specific transmission defects in the WT background.*** *A) The average number of seeds per silique plus and minus standard deviation in INH3-3/INH3-3 and INH3-3/inh3-3 genotype in the WT background. N≥ 37 per genotype. NS represented that there was no significant difference in seed production based on Student's t-test. Standard deviation for INH3-3/INH3-3 is 5.1 while the standard deviation for INH3-3/inh3-3 is 4.7. B) Representative siliques of INH3-3/INH3-3 and INH3-3/inh3-3 genotype after cleared in the 70% ethanol. C) Reciprocal crosses between INH3-3/inh3-3 and INH3-3/INH3-3. Top: INH3-3/inh3-3 as female crossed with INH3-3/INH3-3 as male. The progenies with INH3-3/INH3-3 and INH3-3/inh3-3 in the WT background were counted. P-value > 0.05. No significance. Bottom: INH3-3/inh3-3 as male crossed with INH3-3/INH3-3 as female. The progenies with INH3-3/INH3-3 and INH3-3/inh3-3 in the WT background were counted. P-value <0.001 with great significance. Statistics were performed using the chi-squared test.*

Discussion

This paper proved that both *inh3-3* and *inh3-1* mutants in pollen could increase the transmission in *hpat1/3* pollen and the fertility of *hpat1/3* plants. However, the *inh3-3* pollen actually decreased the transmission through the pollen in the WT background.

The homozygous mutant *inh3-3* in the *hpat1/3* background was unable to grow on the soil under normal growth conditions, while it could germinate but produce abnormal plants with phenotypic defect when germinated in growth media and transferred to the soil. According to Takemiya et al. (2009), the embryo of *inh3-1* aborted and stopped growth at an early developmental stage (the globular stage)., so it was likely that the *inh3-1* would be completely unable to germinate in the *hpat1/3* background. Since it was the germination stage that got disrupted, these defects brought by *INH3* mutation had no connection with the pollen produced. As a result, it was suspected that although *inh3-3* and *inh3-1* might differ in respect to their embryo phenotype, both of their embryos got some levels of defect that made them abnormal comparing to *INH3/INH3* plants. However, the exact mechanism still remained unknown.

For the *inh3-3* mutation in the *hpat1/3* background, we noticed the significant increase in seed production, pollen tube growth, and transmission in *INH3-3/inh3-3* plants. These results proved that, similar to what MacAlister lab observed before, the seed production was rescued in *hpat1/3* plants, so *inh3-3* was indeed an *hpat1/3* suppressor. From our analysis on pollen tube growth and transmission, we suggested that *inh3-3* mutation first improved the quality of pollen tube by forming long-enough and intact (less-bursting) pollen tube to reach the ovule. Because *inh3-3* might better the quality of the pollen, that made the *inh3-3* pollen more competitive and fertilize most of the female gametes. Since more female gametes could be fertilized, it led to the production of more seeds in each silique, which suppressed the low seed production phenotype defect brought by the *hpat1/3* mutations. However, although the pollen tube was indeed growing longer and in better quality, the plants we used could have the *inh3-3* mutation in either the pollen or the female gametes, so we could not 100% confirm that it was the improvement of pollen that rescued the *hpat1/3* phenotypic defect. Additionally, even though the *inh3-3* was specifically benefiting the pollen, we did not prove how the mutation made the pollen itself more competitive. As a result, the *inh3-3* mutant was still a candidate suppressor mutation. We would need to do a reciprocal cross with *inh3-3* in either only male or female parents to locate *inh3-3*'s location of effectiveness and conduct research to evaluating specifically the quality of pollen using phenotypic methods in order to confirm our hypothesis.

We later proved that as another mutation in the *INH3* gene, *inh3-1* could improve pollen tube growth, seed production, and transmission the same as the *inh3-3* mutation in the *hpat1/3* background. Besides these, inspired by our previous *inh3-3* conclusion, we added another experiment with *inh3-1* mutation existing only in the male parent and looked at its impact on pollen tube again. As expected, it confirmed that the *inh3-1* locating in pollen could indeed improve the pollen tube growth. Since *inh3-1* and *inh3-3* were mutations in the same gene and had very similar effect on suppressing the *hpat1/3* phenotypic defect, we speculated that *inh3-3* also located in pollen to rescue the phenotypic defect and was also a full loss-of-function knock-out mutation as *inh3-1* did (Takemiya et al., 2009). Nevertheless, although we proved that the *inh3-1* in pollen suppressed *hpat1/3*, we still needed another reciprocal experiment with *inh3-1* in the female parent only to test if the *inh3* mutations only had effects on male pollen.

To continue verifying our guessing from the previous two experiment, we performed a similar experiment but to study the phenotypic and transmission effect in *inh3-3* plants in the WT background. The reasoning behind it was that Takemiya et al. (2009) noticed the reduce in seed production of WT plants with *inh3-1*; if we could see a similar result in *inh3-3* WT plants, then it could support our conclusion that *inh3-3* and *inh3-1* functioned in a very similar way, which maybe both these two mutations could be

used as suppressors to further study the *hpat1/3* fertility pathway. Additionally, we improved the experiment with a reciprocal cross to locate the effect of *inh3-3* in either pollens or female gametes. Noticeably, *inh3-3* transmission in pollen did decrease in the WT background while there was no significant effect when the *inh3-3* was in the female gametes. As a result, the *inh3-3* was specifically affecting the pollen. However, there was no phenotypic difference in seed production between the *INH3-3/INH3-3* and the *INH3-3/inh3-3*, progenies of *inh3-3* male and WT female, in the WT background, with both of them having a normal average of 50 seeds per silique. Although we did not see the seed production decrease in *inh3-3*, like *inh3-1,* in the WT background, the result still proved that *inh3-3* brought phenotypic defect though in the pollen. The reason behind that was since *INH3-3* pollens were in better quality and could lead to better phenotypic traits to the plant, the high number of *INH3-3* pollen would outcompete and leave less chance for the pollen with *inh3-3* mutation to fertilize the egg. Together with our previous experiment, we could conclude that *inh3-3* and *inh3-1* were similar mutations that improved and decreased the phenotypic defect in the *hpat1/3* pathway and WT background respectively.

To further study the question raised in this paper, the following experiment is being conducted in progress or will be done in the future. First, we would like to know where the INH3 protein is expressed and where it localizes. We generated a reporter construct using the native promoter and the coding sequence of *INH3* and carried out the cloning. We will later transform the plasmids we built into *INH3/INH3* and *inh3* mutant plants to screen for transformants and analyze the localization of the INH3 protein. We will also test for the functionality of the fusion by reducing the *inh3* mutant phenotype. Second, to more directly analyze the phenotype of the whole plant, we will need to take high-quality pictures of the overall morphology of the plant, flowers, and pollen. Lastly, since *inh3* homozygous mutant seedlings often grow as twins, we are curious if the insertion alleles could also cause this “twinning” and vegetative effect. This experiment is currently conducted in the lab.

Although these above experiments still need time to provide further information on the *INH3* mutation, we could prove the validity of our results and speculate the mechanism of suppressor from effect of two *hpat1/3* suppressors discovered previously, *exo70a2* and *sec15a* in the *hapt1/3* plants. Beuder et al. (2020) found out that *exo70a2* and *sec15a* were all full loss-of-function mutants of the exocyst, which could suppress the *hpat1/3* pollen fertility phenotype. It was the *EXO70A2* and/or *SEC15A* that reduced in the key exocyst cargo Hyp-Ara secretion that rescued defect of *hpat1/3* pollen tubes (Beuder et al, 2020). Additionally, the suppressors rescued the pollen tube cell wall organization, proved by a reduction of the dme-HG and callose accumulation at the tip of pollen tube tip and an increase in pollen tube growth rate (Beuder et al, 2020).

It is hypothesized that *INH3* mutations might also affect the *hpat1/3* pollen tube in a similar mechanism. We knew that *INH3* protein is the inhibitor of PP1, the protein required for cell wall integrity maintenance (Houston et al., 2016). Mutating *INH3* allows PP1 to function properly and fix and rescue the defect of the pollen tube cell wall by reducing Hyp-Ara caused by hpat1/3, which improves the pollen tube growth and the fertility of the pollen tube.

Overall, this thesis shows that knocking out *INH3* with either *inh3-1* or the *inh3-3* allele will suppress *hpat1/3* pollen fertility defects. However, the exact mechanism of how the loss-of-function *INH3* interacts with the hpat1/3 mutant protein in the pollen tube fertility pathway at the cellular level is still unknown and requires further investigation.

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