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The effects of PPAL-1 in Arabidopsis gamete development.

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The Effects of PPAL-1 in *Arabidopsis* Gamete Development

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And

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Abstract

Prenylation is a type of post-translational modification in which a 15- or 20-carbon lipid is added to the carboxyl (C) terminus of the protein. *Arabidopsis thaliana* contains the *PROTEIN PRENYLTRANSFERASE ALPHA SUBUNIT-LIKE* (*PPAL*) gene, which encodes a protein with homology to the α-subunits of the three known prenylation enzymes, PFT, PGGT, and Rab-GGT. We previously identified two mutations in *PPAL*, one of which is *ppal-1*, which contains a T-DNA insertion in the fourth intron. We have previously observed that self-fertilizing heterozygous *ppal-1* plants produce progeny in which homozygous *ppal-1* is underrepresented. This project attempts to ascertain possible affects of ppal-1 in gametophyte growth and development that might cause this underrepresented homozygous *ppal-1* population.

Crosses were performed between homozygous *ppal-1* and wild-type (WT) plants. Both F_0 and F_1 generations were genotyped. The results indicated that there was WT contamination of the *ppal-1* F_0 population. The data also indicated the *ppal-1* primers were nonfunctional. Additionally, a pollen germination test was performed for both *ppal-1* and WT plants. The results indicated that *ppal-1* pollen had developmental delays for germination, but upon germination, they could form pollen tubules of equal length to the WT pollen. However, due to the likely WT contamination in the *ppal-1* population used, these experiments must be replicated in further studies.

Introduction

Most proteins undergo post-translational modification, which impacts their function. One important type of post-translational modification is prenylation: the addition of a 15- or 20-carbon lipid to the carboxyl (C) terminus of the protein. As this process adds a hydrophobic moiety to the protein, it facilitates membrane association and protein-protein interactions [1].

Protein prenylation is a conserved process in all eukaryotes [1,2]. This process involves the addition of a single 15-carbon farnesyl or the addition of a single or double 20-carbon geranylgeranyl moieties to either one or two Cysteines near the C-terminus of the target protein. The three known heterodimeric proteins that perform prenylation are: farnesyltransferase (PFT), geranylgeranyltransferase (PGGT), and Rab geranylgeranyltransferase (Rab-GGT). PFT and PGGT have the same α-subunit, and different, but related, β-subunits that determine their respective substrate specificities. Both PFT and PGGT recognize a C-terminal CaaX box, in which C is the prenylated Cysteine; a is usually an aliphatic amino acid, meaning that it is non-polar and non-aromatic; and X varies for each enzyme. For PFT, X is usually alanine, cysteine, glutamine, methionine, or serine. For PGGT, X is almost always leucine. Rab-GGT also consists of an α and a β subunit, which are distantly related to their PFT/PGGT counterparts. Additionally, Rab-GGT requires Rab escort protein (REP) to function properly. Rab-GGT can also perform prenylation on a wider variety of C-terminal target sequences, such as CC, CXC, CCX, CCXX, CCXXX, and CXXX [1,3]. It is also worth noting that in *Arabidopsis*, there is only one copy of the α and β subunits of PFT and PGGT, but there are two putative Rab-GGT α and β subunits in the genome, along with a REP homolog [4,5,6].

The target proteins affected by PFT, PGGT, and Rab-GGT are diverse. PFT and PGGT prenylate a wide variety of signaling proteins, such as members of the Ras superfamily of small

GTPases, protein kinases, and heterotrimeric G protein γ subunits. Ras mutations have been implicated in about a third of human cancers, particularly overactivation, and therefore, chemotherapies may be developed by inhibiting PFT [7]. Mammalian and yeast Rab-GGT prenylate Rab-GTPases, which are involved in organelle biosynthesis and vesicle transport. Rab-GGT mutations have also been implicated in several human diseases [8]. Rab-GGT has not been as well-characterized in plants as in mammals and yeast; however, recent evidence from our lab suggests that Arabidopsis Rab-GGT has broader substrate specificity and can prenylate select non-Rab small GTPases [9].

Mutations in the three prenylation enzymes have shown the importance of this process in plant growth and development. For example, mutations in the PFT β subunit in *Arabidopsis*, called *ENHANCED RESPONSE TO ABSCISIC ACID1* (*ERA1*), cause an increased sensitivity to abscisic acid [10]. These *era1* mutants also have a variety of developmental defects including increased size of shoot meristems, wider floral meristems, and extra organs, especially sepals and petals [11]. Interestingly, PGGT β subunit (*GGB*) mutations do not cause developmental defects [12]. Mutations in the common *Arabidopsis* PFT/PGGT α subunit (*PLURIPETALA* or *PLP*) cause extreme developmental traits, including large shoot meristems, extra floral organs, and stem fasciation [11]. Rab-GGT β subunit 1 (*RGTB1*) mutants have shown many interesting phenotypes including extreme branching, smaller, epinastic leaves, infertility, and shoot gravitropic defects [6]. In *Arabidopsis*, Rab-GGT β subunit 2 (*RGTB2*) and *RGTB1* mutations have been shown to affect cells involved in intense vesicle transport, such as pollen tubes and root hairs [13]. No mutations have been reported in either α subunit, *RGTA1* and *RGTA2*.

Recently, the Running lab has discovered a previously uncharacterized protein called *PROTEIN PRENYLTRANSFERASE ALPHA SUBUNIT-LIKE* (*PPAL*), which is related in amino

acid sequence to the α subunit of PFT/PGGT and the α subunits of Rab-GGT [14]. *PPAL* is thought to play a role in *plp* viability. However, the function of *PPAL* remains relatively unknown.

Preliminary research in the Running lab with two *Arabidopsis ppal* mutant alleles has shown that it plays a role in sugar metabolism. *ppal-1* contains a T-DNA insertion in the fourth intron and appears to be a partial loss of function allele. When grown with sugar, it hyperaccumulates that sugar to the point of death. Even when grown without an external sugar source, *ppal-1* over-accumulates internally made sugars. This sugar hyperaccumulation could potentially have implications as a bioethanol source because plants used for bioethanol would ideally have a relatively large cellular sugar concentration [15]. Although *ppal-1* develops slower than WT, it is still able to grow as tall as WT. In contrast, *ppal-2* contains a T-DNA insertion in the third exon and appears to be a complete knockout (KO). This mutation results in a small plant with very slow growth, and nearly completely male infertility.

The potential of *ppal-1* as a bioethanol source has interested the Running lab in further studying this mutation. However, it has been difficult to identify homozygous *ppal-1 Arabidopsis* mutants from self-fertilizing heterozygous plants. This project is specifically interested in the cause of why homozygous *ppal-1 Arabidopsis* plants are underrepresented in progeny of heterozygous plants.

The homozygous *ppal-1* progeny of self-fertilizing heterozygous plants is lower in frequency than expected. Interestingly, when homozygous *ppal-1* plants are created, they produce seeds at a normal rate. This indicates a possible competition between the WT and mutant gametophytes during the reproductive and/or developmental process, in which the WT gametophytes are more successful than the *ppal-1* gametophytes.

Plant gametophytes are haploid, meaning they only have one copy of their genes. Whereas the heterozygous diploid plant cells have a copy of both WT and *ppal-1*, the haploid gametophytes only have a single copy of either the WT *PPAL* allele or the *ppal-1* allele. Gametophytes are also multicellular; the male gametophyte of *Arabidopsis thaliana* has three cells, and the female gametophyte has seven cells. The seven cells of the female gametophyte include: the homo-diploid central cell nucleus, the egg cell, two synergid cells, and three antipodal cells. The three cells of the male gametophyte include: the vegetative cell and two generative (sperm) cells [16].

I hypothesize that the difficulty in producing homozygous *ppal-1 Arabidopsis thaliana* comes from a defect in male or female gametophytes, or both. Specifically, these defects could be the inability of the *ppal-1* male gametophyte (pollen) to germinate as well, grow as fast, and/or fertilize as well as a WT gamete, and/or defects in the development or receptivity of the female gametophyte (embryo sac). Our reasoning is that, in heterozygous plants that self-fertilize, *ppal-1* gametophytes are outcompeted by WT gametophytes, resulting in an underrepresentation of the *ppal-1* allele in diploid progeny. A second possibility, that early embryo development is severely affected in *ppal-1*, will also be examined.

Materials and Methods

Arabidopsis Growth Conditions

All *Arabidopsis* plants, whether *ppal-1* or WT, were planted in the same manner. Sungro Horticulture Propagation Mix soil was de-clumped and used to fill 3x4 inch pots. A tray was filled with 3 rows of 6 pots. 3 L of 0.265% Gnatrol (*w/v*), a solution of *Bacillus thuringiensis* which make the natural larvicide bacillus toxin, was added to the tray to control fungus gnat larvae. These sat overnight to promote absorption of the solution. Four seeds per pot were placed on top of the moist soil, one per corner. The trays were then incubated at $4^{o}C$ for six days. On the sixth day, trays were transferred to an environmental chamber at $23.0^{o}C$, with a relative humidity of 50%. Plants grew until the ages specified in the subsequent experiments.

Crosses

Arabidopsis in the F_0 generation were grown until flowers appeared (approximately 3 weeks). Plants considered for crossing were genotyped as described in the Genotyping *Arabidopsis* section. Two groups of crosses were generated: *ppal-1* ovules with WT pollen (*ppal-1* x WT) and WT ovules with *ppal-1* pollen (WT x *ppal-1*). For each cross, an unopened flower was chosen, as flowers at this stage are likely to have not yet released pollen. The flower was opened and inspected to ensure the stamens were a green color – an indication that pollen had not been released yet. These stamens were then removed. The flower was allowed to continue growth for 24 hours. After 24 hours, the pistil was inspected to ensure it had stigma that would be receptive the pollen from the cross. Pollen from the opposite plant type (WT pollen for a *ppal-1* ovule or *ppal-1* pollen for a WT ovule) was dusted onto the exposed pistil. This flower was then marked with a thread. A cross was considered successful if the ovule formed a seed

pod, marked with the thread. The seeds were collected when the pods were completely ripe (brown). These seeds represent the F_1 generation. They were planted as described in the *Arabidopsis* Growth Conditions section. They were genotyped as described in the Genotyping *Arabidopsis* section.

Genotyping *Arabidopsis*

DNA extraction

10 mL of DNA extraction solution was prepared with 2 mL of 1 M Tris-HCl (pH 7.5), 0.5 mL of 5 M sodium chloride, 0.5 mL of EDTA (pH 8.0), 0.5 mL of 10% SDS, and 6.5 mL of deionoized water. Leaf tissues from three-week old plants were collected in individual sterile Eppendorf tubes. The tissues were ground to a paste with a small plastic pestle in the Eppendorf tubes. 400 µL of extraction solution was added and the tubes were vortexed for 5 minutes at 14000 rpm. 300 µL of the supernatant was transferred to a new microcentrifuge tube. 300 µL of isopropanol was added to the supernatant and the tubes were gently inverted a few times to allow thorough mixing. The tubes were incubated for 2 minutes at room temperature, and then centrifuged at 14000 rpm for 10 minutes at room temperature. The supernatant was removed with long-tipped pipettes without disturbing the pellets. 400 µL of 70% ethanol was added to the tubes without disturbing the pellets. The ethanol was quickly removed with long-tipped pipettes to remove salts from the pellets. The pellets were allowed to dry and 50 µL of sterile DI water was added to the tubes to dissolve the pellet. The tubes were heated at 95°C for 3 minutes to destroy any remaining DNases or kill any remaining microorganisms that could harm/degrade the extracted DNA. A Nanodrop was used to judge the quality and concentration of the extracted DNA, as a

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minimum of 10 ng/μL were needed for PCR, and a 260/280 value of about 2.00 showed a decent DNA peak.

Polymerase Chain Reaction (PCR)

Samples for PCR were prepared using the reagents described in Table 1. Table 1 shows the reagents necessary for 1 sample of DNA. For more than one sample, the amount of reagent used was multiplied by the number of samples present. For both WT and *ppal-1* DNA amplification, the R-primer, RP, was the same. However, the F-primers differed for WT and *ppal-1*. The F-primer for wild type was LP, whereas the F-primer for *ppal-1* was LBal. All three primers are shown in Table 2.

Samples were then run in the PCR machine using the program shown in Figure 1. Briefly, Stage 1 of the cycle occurred at $95.0^{\circ}C$ for 30 seconds and occurred only once. Stage 2 cycled through three steps, and cycled 30 times. Step 1, the denaturation step, ran at $95.0^{\circ}C$ for 30 seconds. Step 2, the annealing step, ran at $58.0^{\circ}C$ for 35 seconds. Step 3, the extension step, ran at $68.0^{\circ}C$ for 1 minute and 30 seconds. Stage 3 of the cycle occurred at $68.0^{\circ}C$ for 5 minutes. Stage 4 ran until the samples were taken out of the PCR machine, and it kept the samples at $4.0^{\circ}C$. After the cycle was run, the DNA samples were transferred to a $-20^{\circ}C$ freezer until they were needed further.

Table 1: PCR Reagents

Reagent	Concentration	Amount (μL)
Sterile H_2O	N/A	17.375
Ex taq buffer	10X	2.5
dNTPs	2.5 mM	2.0
F-Primer	10 μM	0.5
R-Primer	10 μM	0.5
DNA	>10 ng/μL	2.0
taq DNA Polymerase	5000 U/mL	0.125

Table 1 shows the list of reagents used for PCR amplification per sample of DNA. For a greater number of samples for PCR, the amount of each reagent was multiplied by the number of samples used.

Table 2: PCR Primers

Primer	Sequence
RP	5'-ACTATCGGCTACATGAAGCCC-3'
LP	5'-TGTATTCCCGAGAGTGACGTC-3'
LBal	5'-TGGTTCACGTAGTGGGCCATCG-3'

Table 2 shows the list of primers used for PCR. RP was the R-primer common to both WT and *ppal-1*, LP was the F-primer for WT, and LBal was the F-primer for *ppal-1*.
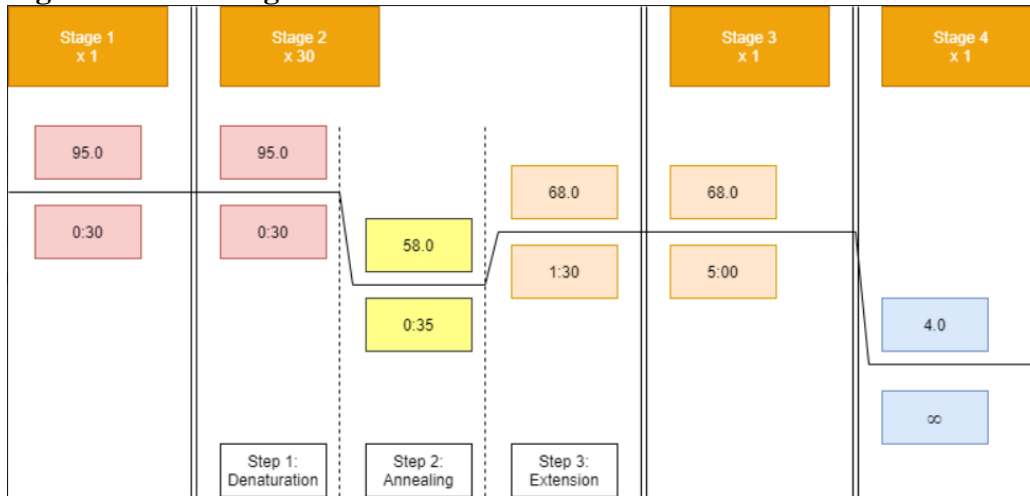
Figure 1: PCR Program

Figure 1 shows the program settings for PCR. Stage 1 ran at 95.0°C for 30 seconds. Stage 2 included the denaturation, annealing, and extension steps, each with their own temperatures and time periods. Stage 3 ran at 68°C for 5 minutes. Stage 4 let the finished product sit at 4.0°C until the products were taken out of the machine.

Gel Electrophoresis

To check the results of PCR, gel electrophoresis through 1.2% agarose was performed (0.6 g of agarose was mixed with about 50 mL of 0.5X TBE). This mixture was heated in a microwave for 1 minute and 30 seconds. 1 drop of ethidium bromide was added. The solution was poured into a sealed gel holder, and a 14-well comb was added. The solution hardened for 30 minutes away from light. While the gel hardened, the DNA samples were prepared. 2 μL of 6X bromophenol blue dye was added to 3 μL of each DNA sample in PCR tubes. Once the gel solidified, the wells were loaded with each DNA sample. The ladder used was the 100 base pairs (bp) ladder. Gels were run for 30 minutes at 75 V, and then observed under a UV lamp.

In Vitro Pollen Germination Test

The pollen germination test was adapted from [17] and performed on both *ppal-1* and WT. Briefly, an agar medium for the pollen germination test was made with 18% sucrose (*w/v*), 0.01% boric acid (*w/v*), 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 0.5% agar (*w/v*), pH 7.0. The agar medium was autoclaved, allowed to cool, and transferred into 10 sterile petri dishes, 5 for each plant type. Pollen from 25 open flowers was transferred onto the medium of each plate, totaling 125 flowers for the 5 *ppal-1* plates and 125 flowers for the 5 WT plates. The plates were then incubated for 24 hours at room temperature. After incubation, pollen was transferred from the plate to a microscope slide to examine under a Nikon Eclipse TE200 compound microscope. Pictures of 40 *ppal-1* pollen tubules and 42 WT tubules were taken with the digital camera. Germinated and non-germinated pollen of both *ppal-1* and WT was counted to find the percent germination for each plant type. A chi-square test was performed on the percent germination data to determine statistical significance. Additionally, the length of the

tubules of both *ppal-1* and WT germinated pollen was recorded. A two-sample t-test was performed on the tubule length data to test for statistical significance.

Ovule Staining and Microscopy

Ovule staining was done as described [18]. The only modification was the dissection step. Carpels were removed from various life stages of unopened flowers and mounted into immersion oil on a new microscope slide. However, this is an ongoing process, and the data have not yet been recorded.

Results

Genotyping the Crosses

<u>Gel of WT Primers on F_0 and F_1 Generations</u>

DNA extraction was done for 2 WT plants in the F_0 generation and 18 plants in the F_1 generation. PCR amplification was run with WT primers, and gel electrophoresis was performed on these PCR products. The rationale for genotyping is as follows. Because of the large T-DNA insert in the *ppal-1* allele, WT genomic primers that are on either side of the insertion will not amplify the *ppal-1* allele, since our extension time is limited. Meanwhile, *ppal-1* primers, one of which corresponds to the sequence of the inserted T-DNA, will not amplify a WT allele, since the T-DNA insertion does not exist in WT. Both sets of primers would be expected to amplify DNA in a plant heterozygous for *ppal-1*, since one chromosome has the T-DNA insert, and the other does not.

Figure 2 shows the gel from this WT PCR amplification. Lanes 1, 14, 15, and 24 all had the 100 bp ladder. Lanes 2 and 3 included the WT samples from the F_0 plants. Lanes 4-9 included DNA from the *ppal-1* x WT F_1 plants. Lanes 10-13 and 17-23 included DNA from the WT x *ppal-1* F_1 plants. Every sample, except for those in lanes 3, 11, and 17 showed a band around 1500 bps, corresponding to the wild type *PPAL* gene amplicon.

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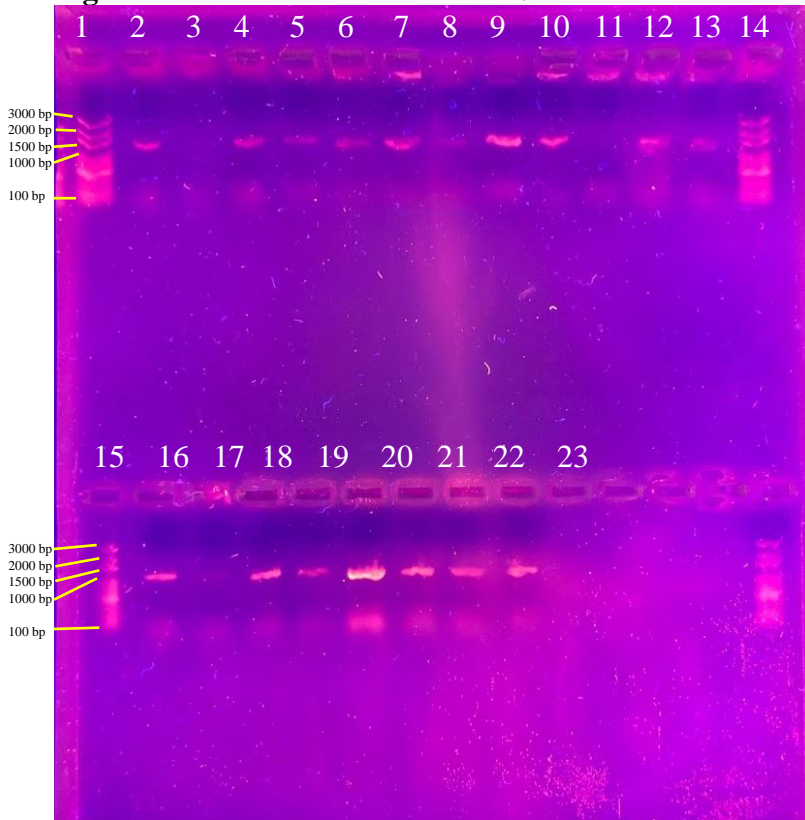**Figure 2: Gel of WT Primers on F₀ and F₁ Generations**

Figure 2 shows the gel of the DNA that was amplified using WT primers in the F₀ and F₁ generations. Lanes 1, 14, 15, and 24 were the 100 bp ladders. Lanes 2 and 3 were the 2 WT DNA samples from the F₀ generation. Lanes 4-13 and 16-23 were the DNA samples from the F₁ plants.

Gel of *ppal-1* Primers on F₀ and F₁ Generations

DNA extraction was done for 9 *ppal-1* plants in the F₀ generation and 15 plants in the F₁ generation. PCR amplification was run with *ppal-1* primers, and gel electrophoresis was performed on these PCR products. Figure 3 shows the gel from this *ppal-1* PCR amplification. Lanes 1, 14, 15, and 28 all had the 100 bp ladder. Lanes 2-6 and 16-19 included the *ppal-1* DNA samples from the F₀ plants. Lanes 7-12 included DNA from *ppal-1* x WT F₁ plants. Lanes 13, and 20-27 included DNA from WT x *ppal-1* F₁ plants. No sample showed any bands within the .
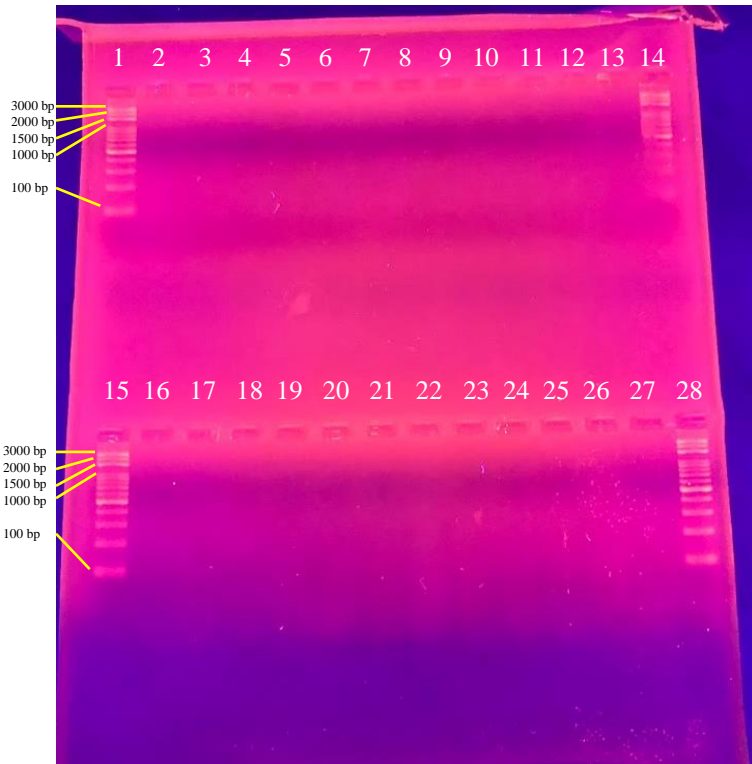
Figure 3: Gel of *ppal-1* Primers on F₀ and F₁ Generations

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Figure 3 shows the gel of the DNA that was amplified using *ppal-1* primers in the F_0 and F_1 generations. Lanes 1, 14, 15, and 28 were the 100 bp ladders. Lanes 2-6 and 16-19 all contained the *ppal-1* DNA samples from the F_0 generation. Lanes 7-13 and 20-27 all contained the DNA samples from the F_1 plants.

Gel for Troubleshooting *ppal-1* PCR

DNA extracted from 1 WT F_0 plant and 3 *ppal-1* F_0 plants were PCR amplified using WT primers. Additionally, DNA extracted from 2 *ppal-1* F_0 plants and 2 F_1 plants were PCR amplified using *ppal-1* primers. Two different working primers (working primer 1 and working primer 2) of LBal were used to amplify these 4 DNA samples, resulting in 8 PCR products. Figure 4 shows the gel from these PCR products. Lanes 1 and 14 contained the 100 bp ladder. Lanes 2-5 were all amplified with WT primers. Lane 2 was a DNA sample from a WT F_0 plant. Lanes 3-5 were all DNA samples from *ppal-1* F_0 plants. Lanes 6-13 were all amplified with *ppal-1* primers. However, lanes 6-9 were amplified with working primer 1 and lanes 10-13 were amplified with working primer 2. Lanes 6, 7, 10, and 11 were all DNA samples from the *ppal-1* F_0 plants. Lanes 8 and 12 used a *ppal-1* x WT sample. Lanes 9 and 13 used a WT x *ppal-1* sample. Lanes 2, 4, and 5 all had bands at about 1500 bps.

Figure 4: Gel for Troubleshooting *ppal-1* PCR

Figure 4 shows the gel for troubleshooting the *ppal-1* PCR. Lanes 1 and 14 included the 100 bp ladder. Lane 2 included the WT DNA sample from the F_0 generation that was amplified with WT primers. Lanes 3-5 included the *ppal-1* DNA samples from the F_0 generation that were amplified with WT primers. Lanes 6-9 were amplified with *ppal-1* primers, including working LBal primer 1. Lanes 10 -13 were amplified with *ppal-1* primers, including working LBal primer 2. Lanes 6, 7, 10, and 11 were *ppal-1* DNA samples from F_0 plants. Lanes 8 and 12 used a *ppal-1* x WT sample. Lanes 9 and 13 used a WT x *ppal-1* sample.

In Vitro Pollen Germination Test

Table 3 summarizes the results of the pollen germination test. In order to calculate the percent germination, 239 *ppal-1* pollen samples and 253 WT pollen samples were counted. Of the 239 *ppal-1* samples, 10 germinated, which gave a 4.18% pollen germination rate. Of the 253 WT samples, 25 germinated, which gave a 9.88% germination rate. A chi-square test of independence was performed to examine the relation between percent pollen germination and *ppal-1*/WT. The relation between these variables was significant X^2 (1, N=492) = 6.04, p = .014.

Average tubule length (μm) of 40 *ppal-1* pollen samples (M=31.05, SD=40.28) was compared to the average tubule length (μm) of 42 WT pollen samples (M=51.11, SD=100.95), and was found to be not significant t(80) = -1.156, p = .251. However, the data for *ppal-1* pollen included two outliers, and the data for WT pollen included three outliers, where outliers were defined as having a Z-value with an absolute value of greater than 2.5. When these outliers were

removed from the data analysis, average tubule length (μm) of the *ppal-1* samples (M=22.71,

SD=13.15) were again compared to the average tubule length (μm) of the WT samples

(M=24.44, SD=15.26), and was again found to be not significant $t(75) = -0.528$, $p = 0.599$.

Figure 5 shows the tubules of the germinated *ppal-1* pollen. Figure 6 shows the tubules of the

germinated WT pollen.

Table 3: Pollen Germination Test Summary

	*Percent Germinated	Average tubule length (μm) (outliers included)	Average tubule length (μm) (outliers excluded)
ppal-1	4.18	31.05±40.28	22.71±13.15
WT	9.88	51.11±100.95	24.44±15.26

*Statistically significant

Table 3 shows the summary of the pollen germination test. Whereas the percent germination difference between *ppal-1* and WT were statistically significant, the average tubule length was not, regardless of the inclusion of outliers.
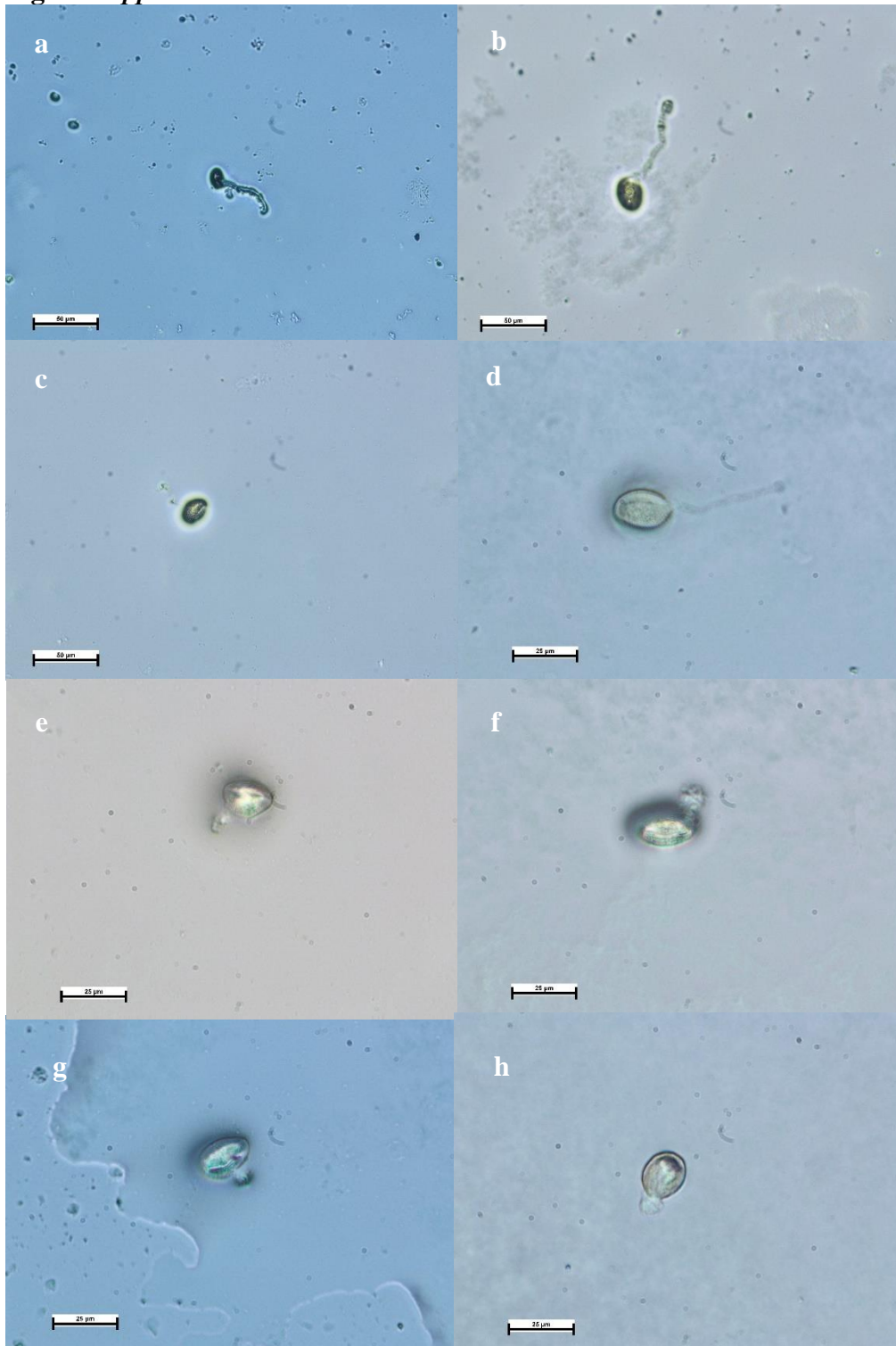
Figure 5: *ppal-1* Pollen Tubules

Figure 5 shows the tubules of germinated *ppal-1* pollen. a-d have a scale of 50 μm, while d-f have a scale of 25 μm. a-c show pollen that generated longer tubules, whereas e-h show pollen that generated shorter tubules.

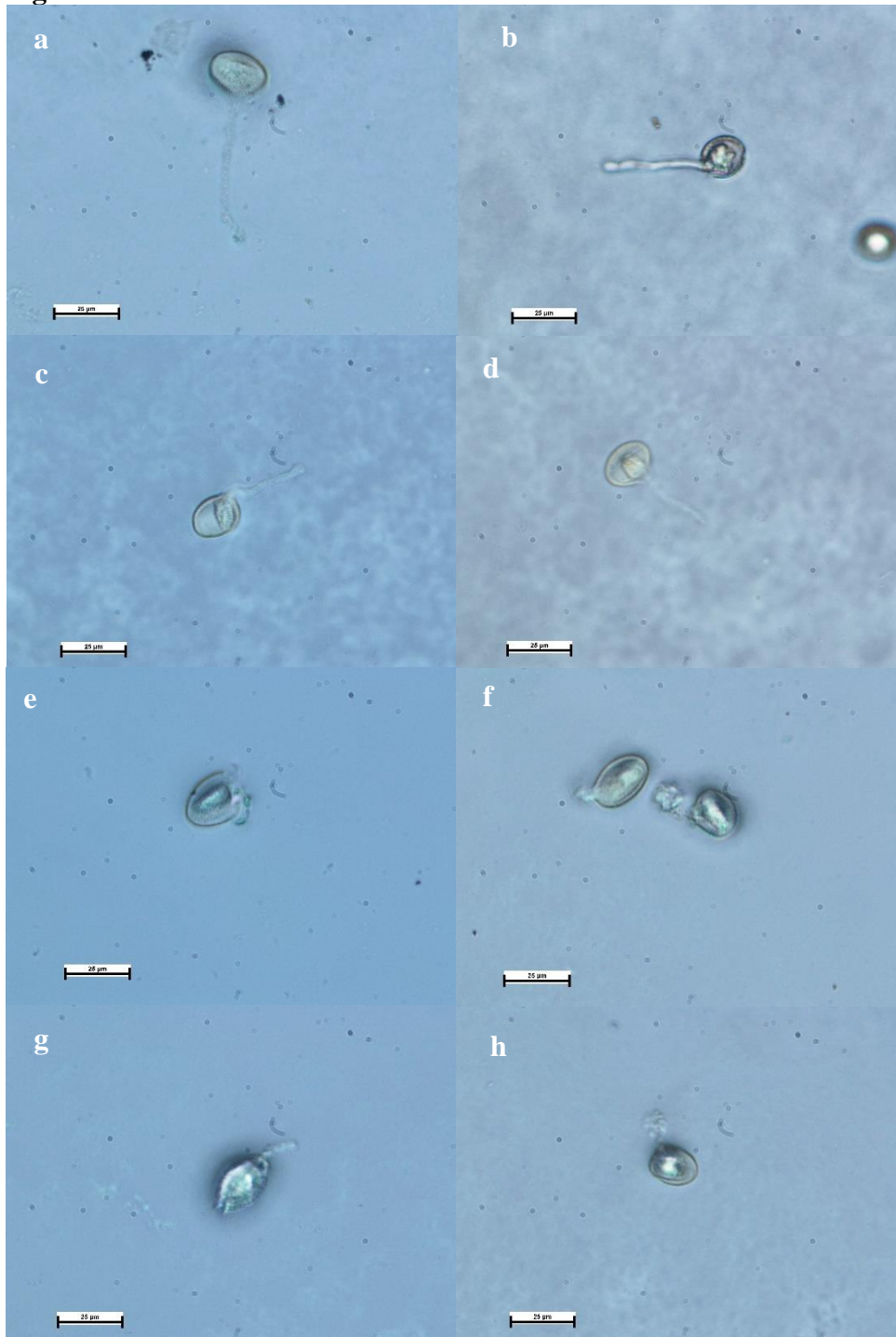**Figure 6: WT Pollen Tubules**

Figure 6 shows the tubules of germinated WT pollen. a-d show pollen that generated longer tubules, whereas e-h show pollen that generated shorter tubules.

Discussion

Developing Crosses

Genotyping of the crosses revealed that nearly all contained WT DNA. Using WT F_0 DNA as a control, the WT *ppal* gene was shown to occur at 1500 bp. Nearly all of these crosses had this same band in their lanes, which indicated that they had at least one WT *ppal* allele within their genome. However, the WT F_0 control in Lane 3, as well as the WT x *ppal-1* DNA in lanes 11 and 17 did not show a band around 1500 bp. This was likely due to some sort of issue in the PCR process.

While genotyping the crosses showed WT DNA, there were no conclusive results regarding *ppal-1* DNA. As seen in the gel of DNA amplified with *ppal-1* primers, there were no bands in either the F_0 controls or in any of the F_1 generation. This had two probable causes, which were further tested: WT contamination or expired *ppal-1* primers. Both causes were tested in the troubleshooting gel. This gel showed the PCR products of three different sets of primers.

WT contamination was tested by amplifying 1 WT F_0 plant and 3 *ppal-1* F_0 plants with WT primers. Lanes 2-5 included these PCR products. Lane 2 was the WT DNA from the F_0 generation that showed a band in Lane 2 of the WT gel. It was used as a control because it had already been shown to have a band around 1500 bp, indicating WT DNA. Lanes 3-5 included *ppal-1* DNA from the F_0 plants. Lanes 4 and 5 showed bands at 1500 bp when amplified with the WT primers, which indicated that these plants were WT contaminants rather than being purely *ppal-1*. However, lane 3 did not have a band at 1500 bp, which indicated that this F_0 plant was not a WT contaminant. As only 4 F_0 plants were tested, it was not conclusive as to how many of the entire F_0 plant population were WT contaminants. Additionally, it had been shown that not

all of these F_0 plants were WT, so it was likely that even though there were contaminants, there were still enough *ppal-1* plants that data could be analyzed.

Additionally, *ppal-1* primer functionality was tested by using two different sets of premade working primers (working primers 1 and 2). Each working primer was used to amplify the DNA of two *ppal-1* F_0 plants, 1 *ppal-1* x WT F_1 plant, and 1 WT x *ppal-1* F_1 plant. Lanes 6-9 showed these four PCR products for working primer 1, and lanes 10-13 showed these four PCR products for working primer 2. As can be seen, none of these lanes showed any bands. This could have been caused by WT contamination, as had previously been shown, or it could still have been caused by expired primers. Interestingly, Lane 3, 6, and 10 used the same *ppal-1* F_0 sample, and yet it did not show a band for either WT or *ppal-1* primers. This *ppal-1* sample was unlikely to be a WT contaminant, as previously explained. Therefore, the cause of these missing bands was likely due to primer nonviability.

Overall, this indicated that not only were there WT contaminants within the *ppal-1* F_0 generation, but also the *ppal-1* primers were likely expired.

In Vitro Pollen Germination Test

The pollen germination test was done to test the pollen viability of *ppal*-1 versus that of WT. This test showed significant results in the percent germination of *ppal-1* versus that of WT. It did not, however, show significant results in the length of the tubules of the germinated pollen in *ppal-1* versus those of WT. This indicates a possible developmental delay in forming the pollen tubule for *ppal-1* pollen, but once the tubule has started to form, there is no difference in the elongation between *ppal-1* pollen and WT pollen. This, in turn, lends support to the idea that WT pollen can outcompete *ppal-1* pollen in self-fertilizing heterozygotes, which could be a

cause as to why homozygous *ppal-1* plants are underrepresented in the progeny of self-fertilizing heterozygotes.

It is important to note, however, that the pollen used for this test came from the same seed sample as the F_0 plants. The F_0 plants were shown to have WT contamination within the *ppal-1* population. Therefore, these results possibly overestimate the real percent germination value of *ppal-1*.

Conclusions

There are two ways in which this project could be continued. Firstly, the ovule staining test must be completed to have data on if there are any differences in ovule development between *ppal-1* and WT. Even though the staining was complete, microscopy could not be performed in the timespan of this project. Secondly, this project needs to be carried out to the F_2 generation to show evidence of non-Mendelian inheritance. In other words, homozygous *ppal-1* will not form in 25% of the F_2 generation, as would be predicted in Mendelian inheritance.

While these two continuations would improve the results of this project, they probably cannot be continued with the data that has already been acquired. In other words, the contamination shown in the F_0 generation is a limitation to these future studies. The seeds used for the F_0 generation were from the same pool used for the plants that produced the ovules for ovule staining. Additionally, the contamination in the F_0 generation in and of itself could affect the study of Mendelian inheritance in the F_2 generation. By starting with an impure sample, any evidence of non-Mendelian inheritance could be caused by the impure F_0 generation rather than gametophyte development issues in *ppal-1* plants.

Finally, there were several limitations throughout the course of this project. Primarily, the RP primer used for both WT and *ppal-1* PCR ran out, and there was limited time to order it and try genotyping again. This was the reason that the troubleshooting gel only used a few samples from both the F_0 and F_1 generations, rather than all samples from both. Additionally, growing time was a limitation. It took about one to two months for the plants to be usable for various experiments, and once they were ready, there was a limited time in which they were usable. Therefore, only short periods of time were available to get data for this project.

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