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## **FERONIA-RELATED RECEPTOR KINASE 7 AND FERONIA AND THEIR ROLE IN RECEIVING AND TRANSDUCING SIGNALS**

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FERONIA-RELATED RECEPTOR KINASE 7 AND FERONIA AND THEIR ROLE IN  
RECEIVING AND TRANSDUCING SIGNALS

A Thesis Presented

by

DAVID VYSHEDSKY

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Biochemistry

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## DEDICATION

To all who have helped, help, and will help me in my pursuits.

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I want to also acknowledge Anthony Federico, Amy Kwong, my roommates Jay Ming Wong and Stepan Houtchens, and all my friends for providing me with a lot of fun in my life.

## ABSTRACT

### FERONIA-RELATED RECEPTOR KINASE 7 AND FERONIA AND THEIR ROLE IN RECEIVING AND TRANSDUCING SIGNALS

SEPTEMBER 2018

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Receptor kinases (RKs) are transmembrane proteins that have been shown to regulate an array of important processes in *A. thaliana*, including polar cell growth, plant reproduction, and many other plant growth processes. In this thesis, I examine RECEPTOR KINASE 7 (RK7) and FERONIA (FER), two closely related transmembrane RKs, and their effects on plant reproduction. The RK7 gene when knocked out (*rk7*) in conjunction with FER resulted in delayed plant growth, decreased seed yield, and a lower percentage of the seeds germinating as compared to the single FER knockout. Transgenic plants with GUS reporter driven by RK7 promoter and RK7 promoter expressed GFP-tagged RK7 (RK7-GFP) were generated to study, respectively, the expression property of the RK7 gene and characterize the location of the RK7 protein. RK7 expression increased in the papillary cells as a direct result of pollination. Transgenic plants with RK7-GFP showed that RK7 protein localizes to the plasma membrane of

stigma cells and pollination induces prominent internalization of this protein. RK7 is also expressed during seedling growth. *rk7* mutant seedlings had a much weaker physiological response to brassinosteroids than wild type plants, implicating an involvement of RK7 in brassinosteroid signaling. Taken together this data point to the importance of RK7 in plant growth and reproduction through its ability to receive and transduce signals.

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## INTRODUCTION

### SEXUAL PLANT REPRODUCTION

The reproductive cycle of *Arabidopsis thaliana* begins when the pollen meets the stigma. The stigmatic region is at the apex of the pistil, and has finger-like structures called papillary cells to which the pollen attaches. The pollen is subsequently hydrated through small fissures that appear in the papillary cells of the stigma. Hydration is noticeable within 5 minutes and complete within 20 minutes (Fiebig et al., 2000). In order for hydration to occur in plants with dry stigmas, like *A. thaliana*, the pollen grain must have a pollen coat composed of lipids, carbohydrates, and proteins; and it must have direct contact with the stigma. The lipids somehow allow for the pollen grains to absorb water from the stigma. The pollen coat must have certain species specificity in order for the stigma to distinguish compatible pollen grains from pollen generated by foreign plant species. *A. thaliana* is a self-compatible plant allowing pollen generated by the plant to be used for self-pollination in addition to pollen generated by other *A. thaliana* plants (Preuss et al., 1993; Wolters-Arts et al., 1998; Shimizu, K. K., 2008).

Once hydrated by the papillary cell, the pollen tube begins to grow. It elongates through the transmitting tissue to the ovules as demonstrated by (Fig.1). The transmitting tissue provides the pollen tube with an extracellular matrix full of polysaccharides, glycosylated proteins, and lipids (Knox, 1984). Some proteins in the transmitting tissue are believed to play a big part in pollen recognition, specifically arabinogalactan proteins (Reger et al., 1988; Pereira, et al. 2016). One of these guide proteins is called transmitting tissue-specific (TTS) protein found in the style of tobacco plants (Wu et al., 1995; Cheung et al., 1995) and *A. thaliana* (Ming-Che Liu PhD).

TTS guides the pollen tube through the transmitting tissue. TTS is produced in the extracellular matrix of the transmitting tissue and is a glycoprotein. The pollen tube deglycosylates TTS as it incorporates it into the pollen tube wall. TTS is also used for directional guidance in a gradient-dependent manner and as a nutrient source for the pollen tube.

The pollen tubes when proximate to the ovule are guided by a cysteine-rich diffusible protein called LURE. LURE is a defensin-like protein that is derived from the synergid cells of *Torenia fournieri* and in *A. thaliana* (Okuda et al., 1993). When the pollen tubes are near the ovule, the synergid cell uses the LURE protein, in a gradient-dependent manner to attract them. LURE binds to male discoverer 1 (MDIS1) and MDIS2 which bind to MDIS1-interacting RLK 1 (MIK1) and MIK2, and to pollen-specific receptor kinase 6 (PRK6) on the pollen tube (Wang et al., 2016; Takeuchi et al., 2016). LURE's binding targets interact with various, but yet to be identified downstream effectors which in turn regulate the direction in which the pollen tube grows.

Polar pollen tube growth is supported by a dynamic actin cytoskeleton and a membrane trafficking system that focuses  $\text{Ca}^{2+}$  and  $\text{H}^+$  to the pollen tube tip. The  $\text{Ca}^{2+}$  and  $\text{H}^+$  are maintained in a tip-focused concentration gradient when the pollen tube is elongating. The tip-focused  $\text{Ca}^{2+}$  concentrations and actin dynamics in the pollen tube are maintained by small signaling G proteins called RAC/ROPs, the Rho GTPases of plants (Anderhag et al., 2000; Cresti & Hepler, 1994; Cheung and Wu, 2008).

The pollen tube elongates by a continuous addition of cell wall and cell membrane material at the tube's tip. The materials are synthesized by organelles throughout the tube and are transported through secretory vesicles to the tube tip. The pollen tubes continue to grow, leaving behind a trail of cell wall material. The pollen tubes grow until they reach the female

gametophyte located inside an ovule. Upon reaching its target, the pollen tube ruptures releasing the two sperm cells fertilizing the egg and the central cell. (Cresti & Hepler, 1994; Cheung et al., 2008, Duan et al., 2014).

#### FERONIA, FER-related Receptor kinases and RHO GTPases in plant reproduction

RKs play an important part in the development and growth of pollen tubes and other vegetative plant tissues. *A. thaliana* has about 600 RKs (Torri et al., 2004; Shiu and Bleeker some years PP paper). Receptor kinases in plants comprise a large family of proteins with the potential to receive extracellular signals and mediate cellular responses. The *A. thaliana* subfamily of *Catharanthus roseus* RLK1-like kinases (CrRLK1Ls) consists of 17 members with an extracellular malectin domain, capable of binding carbohydrates, and an intracellular kinase domain (Fig. 2)(Lindner et al., 2012; Cheung and Wu, 2011). Of the 17 proteins in the family, many of them have been implicated in coordinating cell growth, cell to cell communication, and cell wall remodeling during both vegetative and reproductive development (Lindner et al., 2012; Boisson-Dernier, Kessler, and Grossniklaus, 2011).

FERONIA (FER), HERCULES1 (HERK1) and THESEUS1 (THE1) RKs are all part of the 17-member CsRLK1Ls-family and are closely related in structure and function -- all three play a part in controlling cell elongation during vegetative development (Lindner et al., 2012). All three proteins have been shown to be plasma membrane-localized and the genes are expressed in the leaves, stems, and roots with increased expression in elongating cells. Additionally, these three genes are upregulated after brassinosteroid (BR) reception (Nemhauser et al., 2004; Gou et al. 2009). THE1 is responsible for hypocotyl elongation by inhibiting cell

expansion until an adequate amount of lignin is produced, ensuring healthy plant growth. When THE1 is knocked out, the hypocotyl continues to elongate even in the absence of proper levels of nutrients that are needed to produce lignin (Hématy et al., 2007). When both THE1 and HERK1 are knocked out there is noticeable growth inhibition (Gou et al., 2009). RECEPTOR KINASE 7 (RK7) is a previously not studied receptor kinases, part of the FER family. Based on a microarray assay it has been shown to be heavily expressed in the stigmatic region of the pistil along with FER.

FER is a regulator of reproductive and developmental processes. It is able to do this by acting upstream of RAC/ROPs. RAC/ROPs are RHO-type GTPases and they play a major role in cell signaling (Foreman et al., 2003). *A. thaliana* has 11 RAC/ROP genes that are vital regulators of plant development and growth (Nibau et al., 2006). A yeast two-hybrid assay has shown that FER and ROPGEF interact which then allows ROPGEF to act on ROP2. In a pull-down assay, FER was pulled down by ROP2 GTPase bait, suggesting complex formation is formed with their shared interaction with ROPGEF (Duan et al., 2010). The ROPGEF is known to preferentially bind GDP-bound RAC/ROPs. Once bound it exchanges GDP for GTP which then activates the small GTPases to interact with downstream effectors (Berken et al., 2005; Gu et al., 2006).

FER's closest homologs are, ANXUR1 (ANX1) and ANX2. The transmembrane RKs ANX1 and ANX2 modulate pollen tube polarity through their effect on RAC/ROPs which release GTPases that regulate the NADPH oxidase. The NADPH oxidase then modulates reactive oxygen species (ROS) concentrations in growing pollen tubes, with increasing concentrations at the tube tip (Potocký et al., 2007). The ROS-induced pollen tube rupture is done in a Ca<sup>2+</sup>-dependent process involving Ca<sup>2+</sup> channel activation (Duan et al., 2014).

Both ANX1 and ANX2 are expressed heavily in pollen tubes while FER is not. The main function of ANX1 and ANX2 is to maintain cell wall integrity while the pollen tube is elongating (Boisson-Dernier et al., 2009; Linder et al., 2012). ANX1, and 2 also work with Buddha's Paper Seal 1 (BUPS1) and BUPS2. BUPS1 and 2 are both pollen tube receptors that can bind RALFs. BUPS1 and 2 interact with receptors ANX1 and ANX2 by their ectodomains (Ge et al., 2017). When ANX1 and ANX2 are over expressed pollen tubes have an increased rate of exocytosis, increased cell wall accumulation, decreased chance of bursting, and inhibition of pollen grain germination (Boisson-Dernier et al., 2013). In pollen tubes, the RAC/ROP complexes insure that the pollen tube elongates in a polarized fashion with all the growth happening at the apex of the tube tip (Fig. 3) (Cheung and Wu, 2008). As the pollen tube grows, the ROPGEF at the tip exchanges the GDP bound to RAC/ROPs to GTP bound to the RAC/ROPs (Fig. 3) (Klahre et al., 2006). This process ensures that ROS is only produced at the tip and the pollen tube only grows from the tip (Fig. 2). Up regulation of RAC/ROPs arrests pollen tube growth resulting in a balloon-like tip (Cheung et al., 2003; Zou et al., 2011). Down-regulation of RAC/ROPs creates wider pollen tubes with little to no polarized growth. Thus, proper function of the RKs is of vital importance for pollen tube growth.

ROS is essential in polar root hair growth, polar pollen tube growth, and pollen tube rupture (Forman et al., 2003; Duan et al., 2010; Duan et al., 2014). Pollen tube rupture is imperative for proper fertilization to occur. In the female gametophyte, FER mediates ROS production which in turn induce pollen tube rupture. This rupture allows for the release of the sperm from the pollen tube. *fer* mutants lack the upstream regulator of the NADPH oxidase and thus produce insufficient levels of ROS in the filiform apparatus. The filiform apparatus is thus unable to induce the pollen tube to rupture (Duan et al., 2014). Similar to pollen tubes, root hairs

have a similar mechanism of growth and with insufficient levels of ROS resulting in poor root hair growth (Duan et al., 2010). ROS species also underlie the immune system, increasing plant's resistance to pathogens (Fujiwara et al., 2006).

To gain a more complete understanding of the FER protein, four extracellular domain (ECD) point mutants and three intracellular kinase domain (KD) truncations were generated in our lab to determine the specific function of a number of regions and residues (Fig. 4). The first point mutation studied was glycine 41 in the malectin domain (MD), converted to aspartic acid (G41D). The malectin domain has a similar homology to that of malectin from animal endoplasmic reticulum, which binds to di-glucose. Other RLK have lectin domains which bind to pectins. FER and ERULUS another member of the malectin receptor family have been shown to respond to rapid alkalization factor 1 (RALF1) (Lindner et al., 2012; Haruta et al., 2014). Previous work on a closely related protein, THE1, has shown that the amino acid (AA) substitution of glycine to aspartic acid in the MD has resulted in a knockout of the gene function (Hématy et al., 2007). The three FER kinase domain (KD) truncations generated ranged from a full removal of the KD, to just the tail end of the KD. the KD truncations demonstrate the effect of the KD on downstream regulation.

FER is trafficked to the cell membrane of the female gametophyte and tethered there by LORELEI (LRE) a glycosylphosphatidylinositol-anchored protein (GPI-AP) and LORELEI-like GPI-AP 1(LLG1) (Li, Wu, and Cheung, 2016). GPI-APs are cell surface proteins that play a role in regulating among other things growth, morphogenesis, and reproduction (Lingwood & Simons, 2010; Fujita & Kinoshita, 2012; Yu et al., 2013). LRE is exclusively expressed in the ovule, so in the seedling, LLG1 performs the function of binding FER and trafficking it to the plasma membrane. LLG1 binds FER in the endoplasmic reticulum (ER) and then brings FER to

the plasma membrane in seedlings (Chao et al., 2015). The *llg1* mutant has a similar phenotype to the *fer* mutant, meaning that LLG1 is needed for proper FER localization, which then allows FER to function properly.

FER has been shown to be the binding target of RALF1 (Haruta et al., 2014). RALF a 5-kD peptide, initiates downstream effectors that inhibit plasma membrane H<sup>+</sup>-ATPase activity, increases apoplastic pH, and reduces cell elongation. Cells prefer to elongate in acidic environments; by inhibiting the H<sup>+</sup>-ATPase activity RALF effectively arrests cell growth. RALF1 acts on FER by binding to it and inducing phosphorylation of FER residues Serine 871, 874, and 875 (Haruta et al., 2014). In WT background RALF has a shortening effect on roots which is absent to a large degree in *fer* background.

THE1, FER, and HERK1 are responsible for regulating hypocotyl elongation by interacting with brassinosteroids and ethylene (Deslauriers et al., 2012). The brassinosteroids function by binding the BRI1 receptor which then undergoes heterodimerization with RLK BAK1 (Li, 2002; Russinova, 2004). The heterodimer is then endocytosed which results in the degradation of BIN2. BIN2 is a kinase that negatively regulates brassinosteroid function. BIN2 negatively regulates brassinosteroid responses by inhibiting transcription factors that positively regulate brassinosteroid function from entering the nucleus (He, et al., 2002). In the *fer* background, the seedlings have shown insensitivity to brassinosteroids consistent with a FER linkage with brassinosteroids (Deslauriers et al., 2012).

Brassinosteroids have also been shown to regulate pollen tube germination. Pollen grains germinated on media with higher concentrations of brassinosteroids, had higher levels of germination compared to media containing a smaller concentration of brassinosteroids.

Germination rates increase due to brassinosteroids, indicating that they are an important part of pollen germination (Vogler et al., 2014).

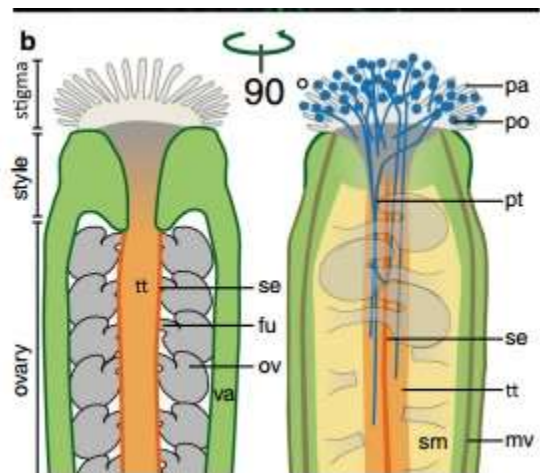
Pollen tube reception and fungal invasion share the *FER*-dependent pathway. Fungal growth is analogous to pollen tube growth. The fungal spore lands on the stigmatic region. It is then hydrated and begins to grow in a tip-dependent manner. Fungal invasions have shown to modulate the transcript levels of the CrRLK1Ls gene family (Lindner et al., 2012). *FER* has been shown to play a part in the plant immune system. *FER* induces ROS production, which activates the mitogen-activated protein kinase (MAPK) and stomatal closure (Kessler, 2010; Keinath, 2010).

Plants have a pattern-triggered immunity (PTI) to pathogen-associated molecular patterns (PAMPs). The most widely studied substrate that causes a PTI response is flg22, a 22-amino acid peptide derived from *Pseudomonas syringae* flagellin. Flg22 is recognized by *Arabidopsis* Flagellin sensing 2 (FLS2), FLS2 then binds to its coreceptor BAK1, also a receptor kinase which is also a coreceptor for BRI1, the brassinosteroid receptor. This complex then initiates the microbe-associated molecular patterns (MAMPs) response which includes ROS production, callose formation on the leaves, and a mitogen-activated protein kinase MAPK cascade (Smith et al., 2014; Chinchilla et al., 2007; Daudi et al., 2012). Callose is a plant polysaccharide produced in response to infection or wounding. MAPK cascades work like a domino effect, a select few MAP3K are activated which phosphorylate a greater amount of MAP2K which then phosphorylates an even greater amount of MAPK thereby increasing the severity of the signal.

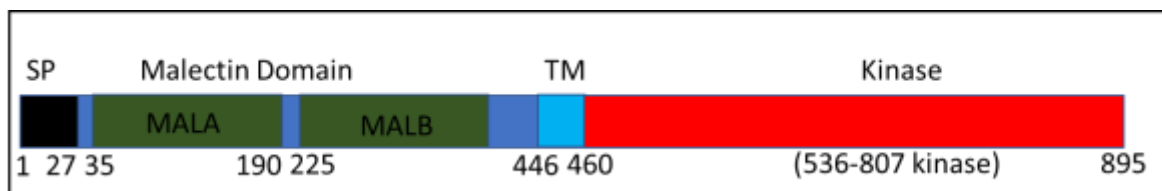
ROS bursting and callose formation on the leaf prevents pathogens from gaining entry to the plant and sequestering the pathogens that have already entered. After treating *Arabidopsis thaliana* leaf disks with flg22 the ROS burst is observed in the time interval of 10-20 minutes

after treatments, reaching peak production at 15 minutes. The MAPK response to flg22 has a very similar time frame, with the production of MAPK6, MAPK3, and MAPK4 15 minutes after treatments (Cheng et al., 2015). Taken together this evidence demonstrates that plants have very quick and efficient pathways and responses for reacting to flg22.

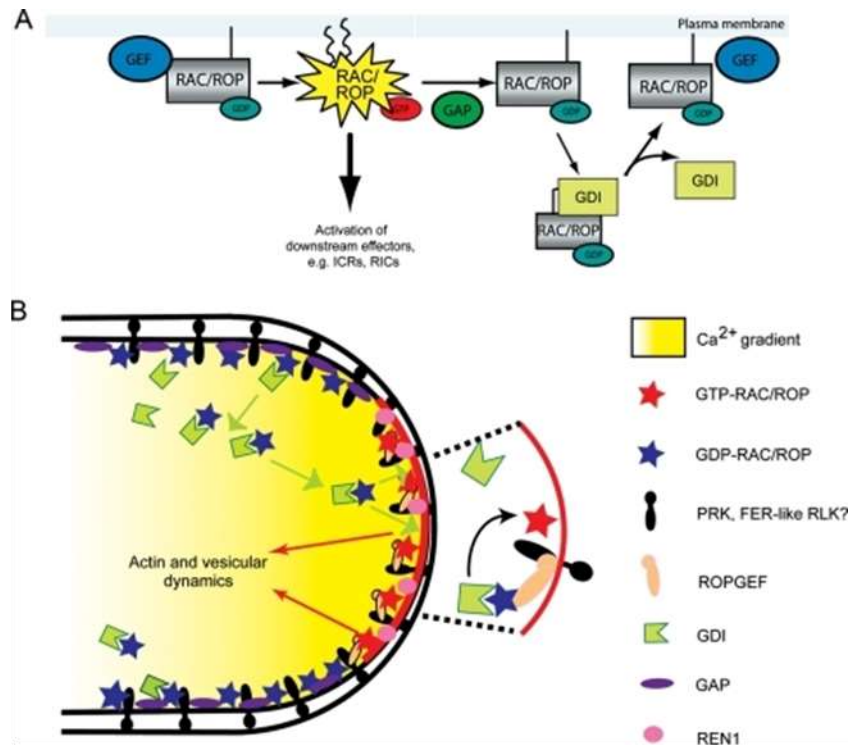
### Figures



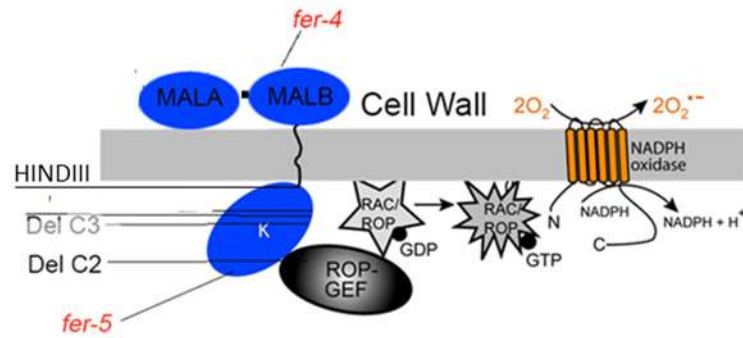
**Figure 1. The structure of the pistil before and after pollination, with the pollen in blue and the ovules in gray.** Appended from (Vogler et al., 2014).



**Figure 2. Domain map of the FERONIA protein.** SP, signal peptide; MAL, Malectin; TM, transmembrane domain.



**Figure 3. RAC/ROP GTPase signaling system directing polarized pollen tube growth.** (A) Regulation of the RAC/ROP molecular switch (modified from Nibau et al., 2006). (B) RAC/ROP signaling complex located at the pollen tube tip mediating polar growth (modified from Cheung and Wu 2008).



**Figure 4. FERONIA and downstream interactions.**

FERONIA protein shown in blue with malectin A domain point mutant labeled on top, kinase domain point mutants labeled on the kinase domain and areas of kinase domain truncations labeled HindIII, C3, and C2. This figure demonstrates an activated FERONIA protein and its subsequent activation of the downstream pathway resulting in the activation of the NADPH oxidase.

## CHAPTER 1

### DISSECTION OF THE N-TERMINAL AND KINASE DOMAIN OF FERONIA FOR A DEEPER UNDERSTANDING OF SPECIFIC FUNCTION OF EACH DOMAIN

#### Results

Observing relative abundance of protein expressed by *fer* mutant, single amino-acid complementation lines

To get a more in-depth understanding of the FER protein, various FER mutants were generated. This was done by others in the laboratory; they generating transgenic lines containing different versions of the *fer* mutant protein in *fer* loss-of-function mutants. The constructs were driven by an endogenous promoter and contained the mutated protein, kanamycin resistance, and a GFP tag.

Three FER cytoplasmic domain truncation mutants. The cytoplasmic domain contains several residues that have been shown to get phosphorylated after treatment with RALF1. RALF phosphorylates FER and initiates a downstream response that turns on the proton pump. The WT and promoter-FERONIA: FERONIA-GFP were used as controls and compared to other complementation mutants to determine the relative amounts of transgenic protein produced.

Four point mutants were generated in the FER N-term, specifically the extracellular malectin A binding domain of the FER gene. The mutants were developed and screened to be homogenous for transgenic protein levels and homozygous *fer* background by others in our laboratory. To ensure that all the mutants could be compared a western blot was performed with an anti-GFP antibody that would demonstrate that the various transgenic lines expressed comparable amounts of protein. Equal amounts of plant material were used for the western blot

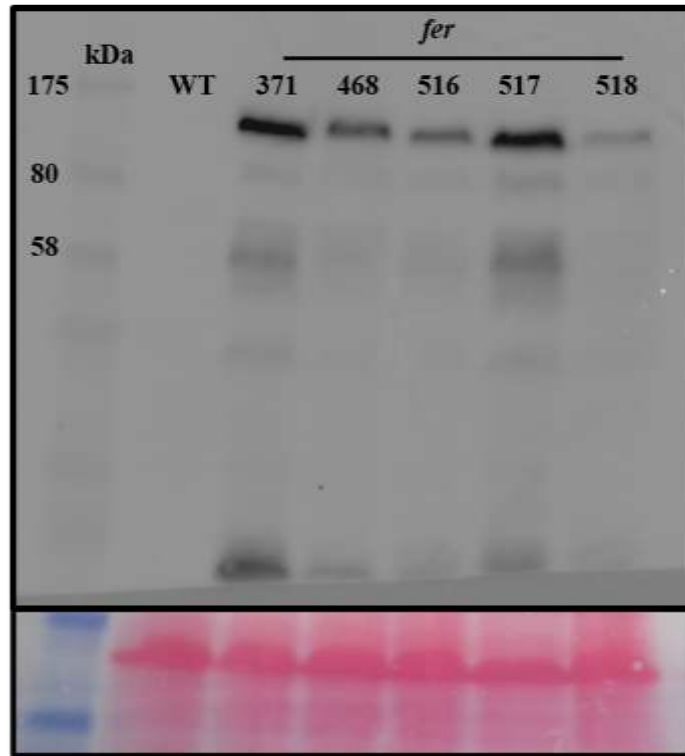
and the results demonstrated that all the transgenic complementation mutants were expressing the transgenic gene well (Fig. 5)

## Discussion

### Generation of *fer* mutant complement

The transgenic N-terminal single amino-acid substitution *fer* complementation mutants can be used in future studies to decipher the role specific amino acids play during ligand binding. The kinase domain deletion complementation mutants can be used to study how the kinase domain of FER affect downstream elements. Studies have shown that ROS is important for root elongation, and thus these mutants can be tested to see their effect on levels of ROS. The generated complementation mutants can be used for further studies of FER and the findings can be used to model specific amino-acid function in related RLKs.

Figures



**Figure 5. GFP tagged FERONIA malectin A domain point mutant protein expression levels.** (A) protein expression levels of *fer* plants complemented with GFP tagged FER malectin A domain point mutant. FER-GFP has a weight of 100 kDa and is localized between the 175 and 80 kDa molecular weight band. Numbers at the top indicate mutant plant lines.

## Materials and Methods

### Generation of *fer* mutant complement

#### GFP expression level Assay

Grow seedlings for 10 days. Grind 10 seedlings in protein extraction buffer until the solution is green and no seedlings are visible. Immediately boil the sample for 5 minutes. Spin down sample, add loading dye and load onto gel. The gel should be 15% acrylamide and run at 100 volts band separation. After transfer onto membrane, block with 3% milk for 1 hour. Then added primary antibody GFP 1:1000 in 5 % milk and keep in primary antibody for 1 hour. Secondary antibody can be used in 3% milk.

Protein extraction buffer:

0.4M Tris-HCl pH 7.5, 4% SDS

1% beta-mercaptoethanol.

#### Transforming the plants

Mutations in FER had been generated and introduced into *fer-4* via the *Agrobacterium tumefaciens* floral dip transformation method (Clough and Bent, 1998).

Transgene screening process for transgene homogeneity and background homogeneousness

#### Part I

To generate the transgenic lines containing desired transgenes in both WT and *fer* background, plants with Heterozygous FER/*fer* background were used. The plants were dipped into agrobacterium containing the transgenic plasmid using the (Bechtold,1993) protocol.

Generated Transgenic generation zero (T0) seeds were then screened on B5 plates with 50µg/mL of Kanamycin (Kanamycin Plates) The hypothesized genetic background of the FERONIA (FER) gene in the T1 plants based on Mendelian genetics is 25% WT, 50% FER/*fer*, and 25% *fer*. However the expected genetic background of the Feronia gene in the T1 plants based on previous screens with Feronia mutant plants is 50% WT and 50% FER/*fer* background. This is because the plants in *fer* background have a much harder time surviving on the kanamycin plates. The plants that survive the Kanamycin Plates are transferred to soil and allowed to grow.

## Part II

Once the T1 plants have senesced the plants are left to dry. The T1 seeds are then collected and treated in -80 degrees Celsius for >1Hour. The T1 seeds are then screened in parallel on Kanamycin plates and on B5 plates containing 5.25µg/mL of sulfadiazine (sulfadiazine plates) to identify the introduced transgene and the *fer-4* genotype.

The Sulfadiazine plates will demonstrate only FER gene background because the *fer* plants contain resistance to Sulfadiazine plates. The Percentage of green plants on the Sulfadiazine plates have the following genetic background 100% green means all the plants are from a parent plant that is in *fer* background, 75% green means the parent plant had Fer/*fer* background, and no green plants means that the parent is in Feronia background. The surviving plants from a sulfadiazine screen, derived from a T1 lines with Heterozygous Feronia background, will have the following breakdown of the Feronia gene 33% will have *fer* background and 66% will have Fer/*fer* background.

The seedlings generated from the previously mentioned sulfadiazine screen will then be transferred to soil. To confirm that they contain the transgene fluorescence microscopy will be used to identify which plants contain GFP and which do not. The *Feronia* gene is heavily expressed in the Ovule thus to identify *Feronia* related gene transformants the ovules are observed. To identify transformant in the *fer* background PCR is used in conjunction with pollen tube ovule entry assays (Duan, 2014). The plants in *Feronia* background will have very few pollen tube over growth and multiple pollen tube entrances phenotypes. The plants in *feronia* background have 80% of the ovules with pollen tubes over growing and 40% of the ovules with multiple pollen tube entrances (Duan, 2014). The seeds are collected plant by plant.

### Part III

The T2 seeds generated from the Sulfadiazine screen with previously confirmed background are again screened in parallel on Kanamycin and sulfadiazine plates. The sulfadiazine screen will confirm the FER gene background. This time, however, the plants from the Kanamycin plate will be transferred to soil and the pollen tube ovule entry assays will be repeated. Other physiological assays can also be performed on the T2 seeds to get preliminary data. The seeds generated by the T3 plants should be collected plant by plant. Seeds generated by the T3 plants are used for physiological assays to get publishable data.

## CHAPTER 2

### STUDY OF RK7 AND ITS ROLE IN PLANT DEVELOPMENTS

#### Results

##### RK7 and its role in plant developments

A phenotypic study was performed to determine the role of RK7 during plant development. A previous postdoc and graduate student received a *rk7/fer* double knockout and *rk7* single knockout. The *rk7/fer* double knockout has a complete knockout of both the RK7 and FER genes. The *rk7* single knockout only has the RK7 gene knocked out. Since this line was provided to me, I first carried out a genotypic analysis to ensure their background. The *rk7/fer* double knockout plant was missing the RK7 and FER genomic DNA, meaning that the DNA encoding the RK7 and FER protein was not accessible to the RNA polymerase, and no RK7 and FER protein could be made. Additionally, the RK7 and FER T-DNA were present leading to the conclusion that the plant is a double knockout (Fig. 6). The *rk7* knockout was missing the RK7 genomic and had RK7 TDNA confirming its background (Fig. 6).

The first phenotypic study done was the comparison of one-month old WT, *fer*, *rk7*, and *fer/rk7* *A. thaliana* plants. The *rk7* single mutant does not confer any significant growth and developmental defects on the plant and was comparable to the WT. At the one-month time point the Col. WT, and *rk7* mutant grew significantly taller than the *fer* mutant. The *fer* mutant grew significantly more than the *rk7/fer* double mutant (Fig. 7). The defects compounded by the *rk7/fer* double mutation is also manifested in seed production. Seed yield is drastically reduced in the *fer* mutant and even more so in the *rk7/fer* double mutant. In the *fer* single knockout

background many ovules have pollen tube over growth and others have multiple pollen tubes (Duan et al., 2014). These irregularities caused by the absence of FER, result in improper fertilization and a decrease in seeds (Fig. 8). The *rk7/fer* double mutant has fewer seeds than the *fer* single knockout, indicating that the knockout of RK7 results in the decrease in seed yield (Fig. 8). In addition to producing a reduced seed yield, *fer* plants produce larger seeds. The *rk7*, *rk7/fer*, *lre*, and *llg1* mutant's seeds were also measured and compared to the WT (Fig. 9, Fig. 10). All the mutants except the *rk7* mutants had enlarged seeds. The largest belonging to the *rk7/fer* mutant. These studies remain preliminary and results require confirmation.

To ascertain the reason for the enlarged seed phenotype two hypotheses were tested. The first hypothesis was that the seeds grow larger in the *fer* mutant because the *fer* mutants have many ovules with multiple pollen tubes and ovules with pollen tube overgrowth. These developmental irregularities allow for fewer healthy gametes, leading to a reduction in seed production. The extra room in the siliques results in more space for the remaining seeds to grow. The second hypothesis was that FER regulates seed growth, and arrests growth when the seeds reach a certain size. To test the first hypothesis a limited pollination assay was performed, WT pistils were emasculated and pollinated using only 50 pollen grains. The average pistil has 60 ovules and the limited pollination ensures that not all the ovules are fertilized. The limited pollination resulted in an average of 19.8 seeds forming per silique with a standard deviation of 12.6, which compared to *fer*'s self-pollination of 17.9 seeds per silique. The seeds were then collected and measured in length and width. The width of the seeds was not significantly different between the WT self-pollination, WT limited-pollination, and *fer* (Fig 11). Both the limited pollination seeds and *fer* seeds were significantly longer than the WT self-pollination

seeds. Based on this result seed size is in part directed by spatial cues indicating room to grow (Fig. 12).

The generated seeds of WT, *fer*, *rk7*, and *fer/rk7* were collected and germinated to compare the germination rates. Two seed germination assays performed on WT, *fer*, *rk7*, and *fer/rk7* seeds grown on B5 medium with 1% sucrose and on ½ MS medium has shown that while the WT, *fer*, and *rk7* have germination rates of above 90%, the *fer/rk7* double mutant has a 60% and 50% germination rate on the two different B5 and ½ MS medium respectively (Fig. 13). Taken together with seed yield results this data indicates that the addition of the *rk7* knockout to the *fer* mutant adds a seed germination phenotype to the already present seed yield difficulties.

To further understand the significance of RK7, the initial step of pollen-stigma adhesion was examined. Based on microarray data RK7 and FER are heavily expressed by the stigma. WT pollen grains were used to pollinate WT and *rk7/fer* stigmas, rinsed with a detergent and observed for pollen grains. The WT stigma was able to bind and hold far more pollen grains than the *fer/rk7* stigma (Fig. 14).

### Study of RK7 Localization and Expression

RK7 promotor::*RK7*-GFP transgenic lines were generated to study the localization of RK7. RK7 was most heavily localized on the stigma, specifically the papillary cells. The WT control had high auto fluorescence of the papillary cells but when compared to RK7 promotor::*RK7*-GFP transgenic plant it is evident that the transgenic lines had stronger GFP fluorescence than the control (Fig. 15). From the RK7 promotor::*RK7*-GFP transgenic line it is evident that RK7 localized to the cell surface of the papillary cells. Limited assays performed by pollinating the RK7 promotor::*RK7*-GFP transgenic line with WT pollen have suggested that

after pollination RK7-GFP might undergo internalization (Fig. 16). As the pollen grains hydrate and germinate on the papillary cells the RK7-GFP gets displaced from the cell surface and can be observed inside the papillary cell.

Additionally, RK7 promoter::GUS transgenic lines were generated to study RK7 expression patterns. The seedlings were transferred from the plates that they were grown on, to wells containing H<sub>2</sub>O using tweezers to grab the leaves. The seedlings were then treated with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc). Seven-day old RK7 promoter::GUS lines were studied and RK7 promoter::GUS was active in the leaf vasculature, the root tip, the hypocotyl, and locations where lateral root formations had begun (Fig. 17). The locations on the leaf where the tweezers had bruised the leaf had an increased expression of RK7 as compared to the surrounding tissue indicating that RK7 might be expressed in response to the wound (Fig. 18). Taken together it is evident that RK7 plays a role in plant growth and development.

The RK7 promoter::RK7-GFP lines showed that RK7 is localized on the papillary cells. To better understand the temporal expression of the RK7 protein an inflorescence from an RK7 promoter::GUS line was taken and stained with X-gluc. The results show a clear trend that the stigma has a high level of RK7 expression and that as the pistil matures and prepares for pollen germinations the levels of RK7 expression increase (Fig. 19). Within one hour of pollination the levels of RK7 expression in the stigma increase drastically (Fig. 20). As observed in (Fig. 20) the pistil that is not pollinated has very low level of RK7 expression as compared to the pistil that has been pollinated for one hour. This assay has been performed using hand pollination, but the same exact result can be observed from pistils that have undergone self-pollination as compared to emasculated un-pollinated pistils.

## RK7 hormonal studies

The RK7 promoter::GUS plants were additionally treated with auxin and brassinosteroids. RK7 promoter::GUS seedlings had significantly increased expression of RK7 after 1 $\mu$ M Auxin treatment (Fig. 21). RK7 promoter::GUS seedlings also had increased expression of RK7 after treatment with brassinosteroids (Fig. 22 ). A dose dependent brassinosteroid treatment of RK7 promoter::GUS seedlings was performed and a dose as low as 10mM brassinosteroid would increase RK7 expression in the hypocotyl. A brassinosteroid sensitivity assay was performed on the *rk7* knockout to determine if RK7 was important for brassinosteroid activated hypocotyl elongation and root truncation. The *rk7* knockout was, in fact, less sensitive to brassinosteroid treatment. The *rk7* mutant did not exhibit hypocotyl elongation and root truncation to the same degree as the WT control (Fig. 23). The untreated *rk7* mutant had a longer root and hypocotyl as compared to the WT control so additional data manipulation was performed to calculate the percent change of the hypocotyl and root with brassinosteroid treatment. The data showed that *rk7* had a smaller percentage change in both metrics which confirmed that the *rk7* is less sensitive to brassinosteroids (Fig. 24a and 24b).

## Discussion

### Analysis of RK7 protein and its role in plant developments

FER and RK7 are both important in the development and growth of *A. thaliana*. Although RK7 by itself does not confer any significant defects, it becomes important when other related transmembrane receptor kinases are knocked out. The *rk7/fer* plants develop at a slower

rate than WT, *fer*, and *rk7* plants, indicating that RK7 is important in development and may play a role in cell expansion (Fig. 7). The decrease in seed production present in the *fer* and *rk7/fer* indicate that both are important in seed production. The low germination rate of the *rk7/fer* seeds suggests that the knockout of *rk7* is important for seed development when *fer* is knocked out (Fig. 13), indicating that RK7 might play a role in pollen grain reception.

The increase in seed size visible in *lre*, *llg1*, and *fer* mutant backgrounds indicates that these RLKs play an important part in facilitating proper interactions between the male and female gametes. The *lre* mutant had larger seeds than the *llg1* mutant (Fig. 9). This can be attributed to the fact that LRE is expressed in ovule while LLG1 is not. The special cues hypothesis directing the increase in seed size was tested with a partial pollination assay, and the outcome showed that size was directly correlated with room to grow in the silique. This finding suggests that the mutants affect the large seed phenotype upstream of seed production.

The seed reduction in the *rk7/fer* double mutant compared to the *fer* single mutant might be to a defect in the stigma conferred by the absence of RK7. In the pollen adhesion assay far fewer pollen grains attached themselves to the stigma of the *rk7/fer* as compared to the WT (Fig. 14), indicating a possible defect of the stigma confirmed by the double knockout of the RK7 and FER.

### Study of RK7 Localization and Expression

RK7 is localized on the papillary cells and may be important in the process of pollen reception. RK7 is also expressed near wounding areas, hypocotyl, lateral root formations, and root tip. RK7 is displaced from the surface of the papillary cells as pollen grains germinate,

indicating that RK7 plays a part in pollen grain reception. The part it plays is not yet evident but evidence from the hormonal study suggests that RK7 is assisting in brassinosteroid reception, thus facilitating proper pollen germination. The *rk7/fer* double mutant develops fewer healthy seeds that are able to germinate, adding additional evidence that RK7 in one fashion or other assists the reproductive process.

The pistils of the RK7 promotor::*GUS* line were further analyzed showing an increase of RK7 expression in the pistil the more mature it was. The older pistils that were mature and ready to be pollinated exhibited higher levels of RK7 expression. The pollinated pistils expressed RK7 promotor::*GUS* to a much greater extent than unpollinated controls.

The fact that RK7 is expressed in wounded areas and areas experiencing growth indicates that RK7 might function as a cell surface receptor for interruptions to cell wall integrity. This function becomes important and helpful with pathogens attempting to enter through the stigma, bugs eating the leaves, and general cell destruction because of new growth.

#### RK7 hormonal studies

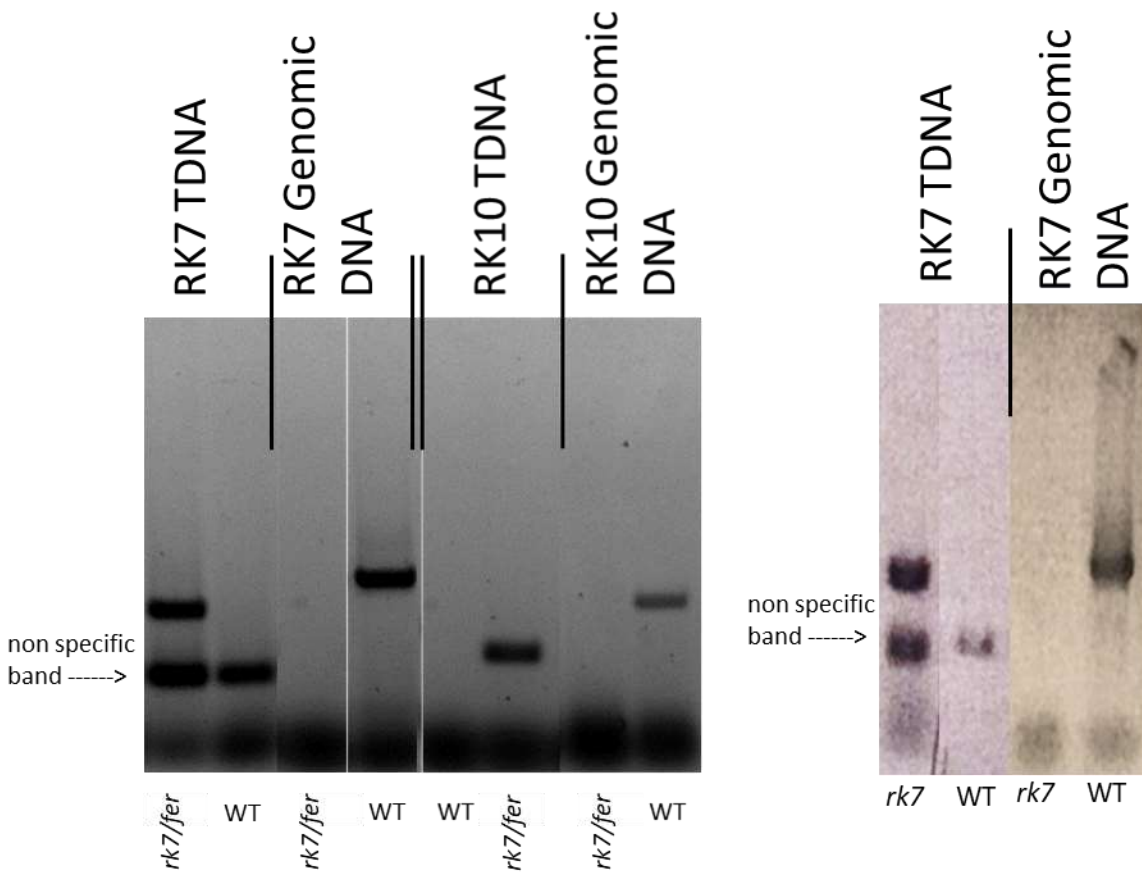
The RK7 promotor::*GUS* lines increased RK7 expression when treated with brassinosteroid. Suggesting that RK7 may be a co-receptor for brassinosteroids. The *rk7* knockout mutant also exhibited a reduced effect when treated with brassinosteroids as compared to the WT control. The fact that the *rk7* plants still had an increase in hypocotyl length and a decrease in root length when treated with brassinosteroids suggest that RK7 participates in brassinosteroid signaling. The hypothesis that RK7 is important in pollen grain reception at the stigma is strengthened by this data. Pollen grains germinate more readily in the presence of

brassinosteroid and produce it themselves. The presence of RK7 thus facilitates proper pollen grain germination.

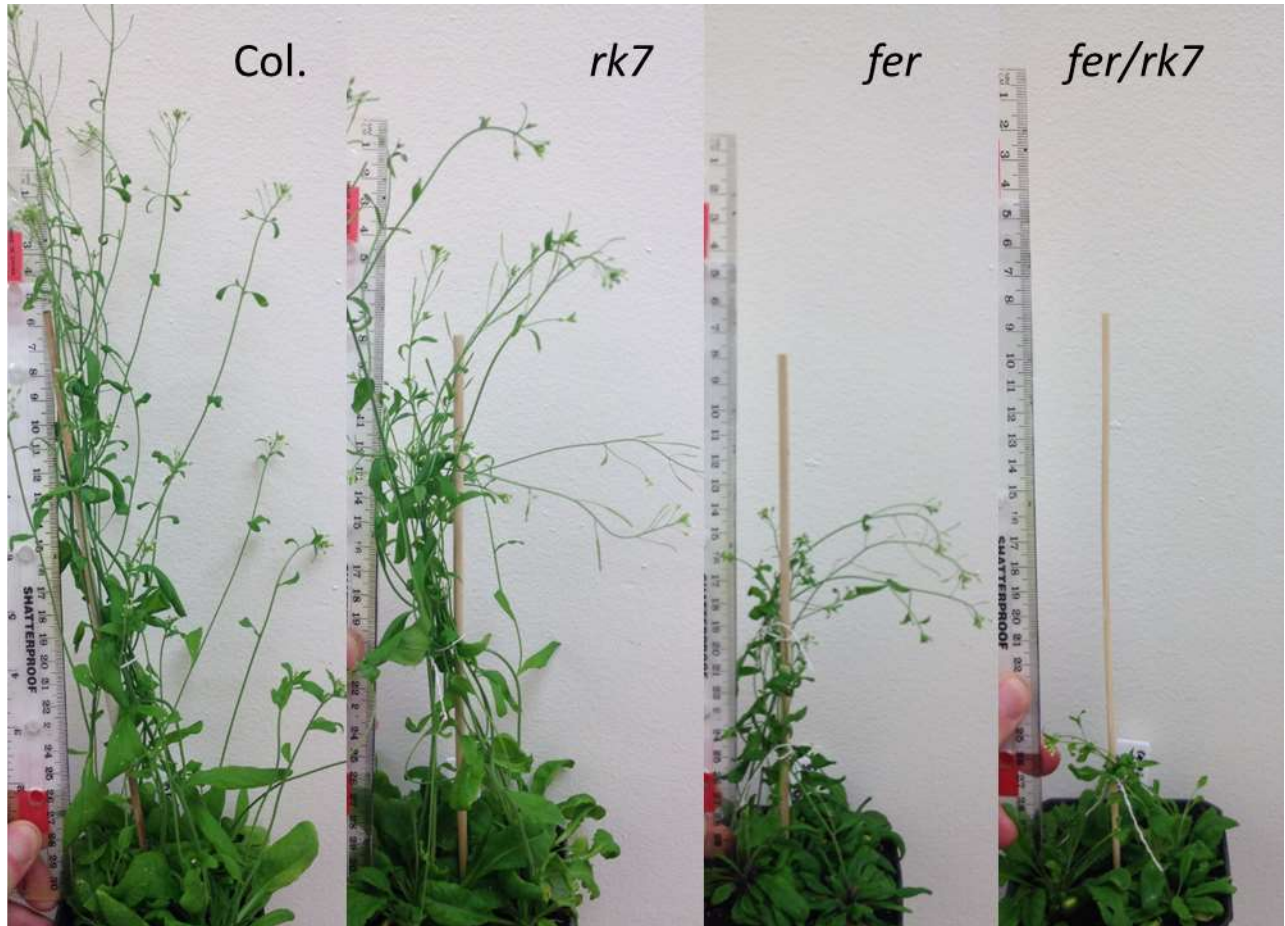
The hormone auxin-induced RK7 expression in RK7 promoter::GUS lines indicating that RK7 is also important in some part of the auxin pathway. More work needs to be done as to understand how RK7 and auxin interact.

In conclusion, while observations reported here are all very interesting, they are preliminary and warrant repeating before firm conclusions can be made.

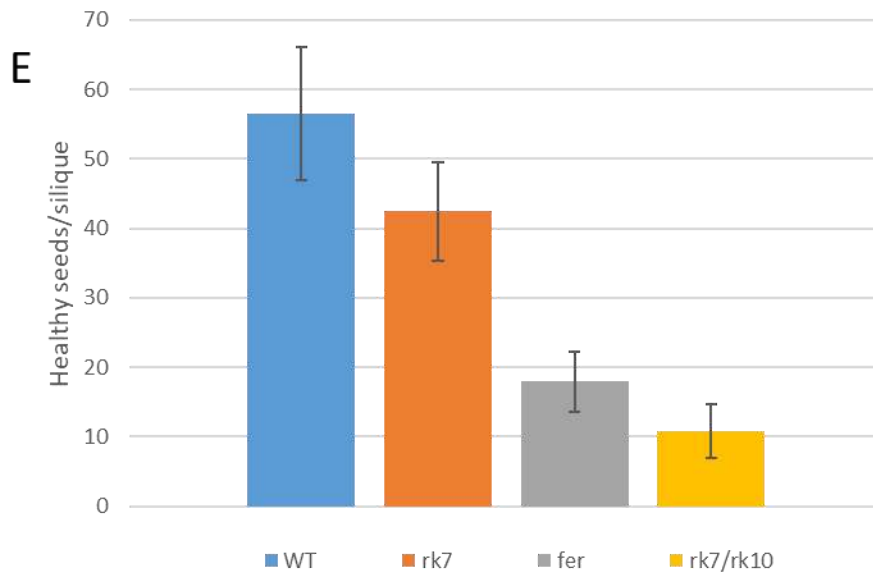
Figures



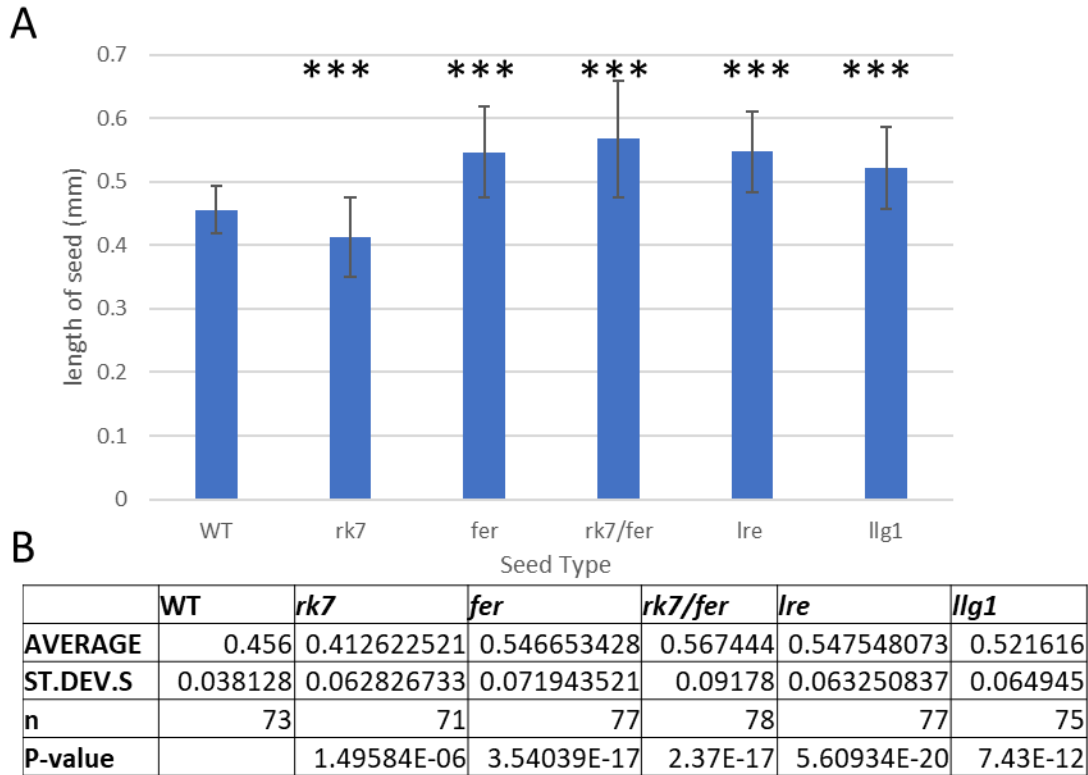
**Figure 6. Homozygosity test.** (A) A *rk7/fer* was tested for homozygosity of the *rk7* and *rk10* genes. A WT plant was used as a control. RK7 TDNA has a nonspecific band. (B) A known *rk7* plant was tested for homozygosity of the *rk7* gene. A WT plant was used as a control.



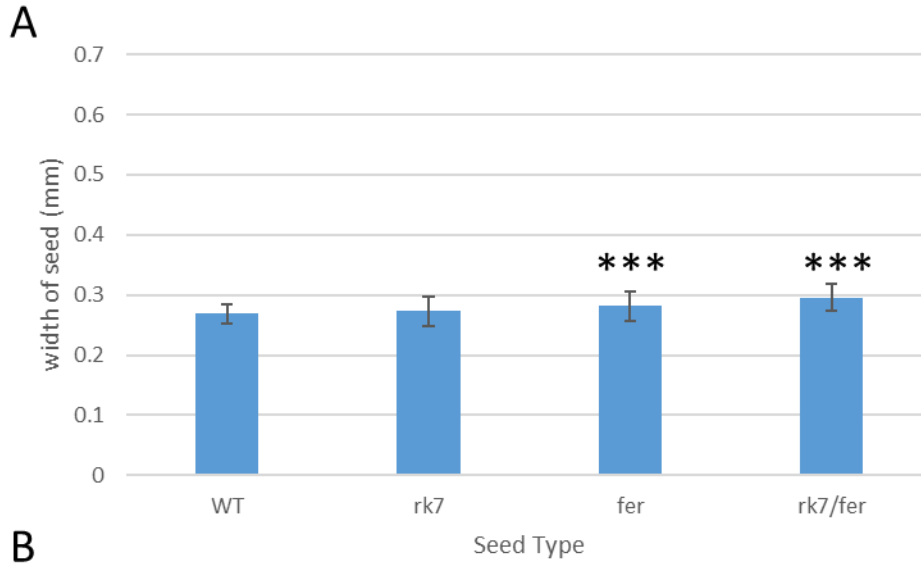
**Figure 7. One month old Arabidopsis plants. (A)Col., (B) *rk7* mutant, (C) *fer* mutant, (D) *rk7/fer* mutant.**



**Figure 8. Silique analyses.** (A) Col. Had an average of 56 healthy seeds with an SD of 9 (n=16 siliques counted), (B) *rk7* mutant had an average of 42 healthy seeds with an SD of 7 (n=16 siliques counted), (C) *fer* mutant had an average of 18 healthy seeds with an SD of 4 (n=16 siliques counted), (D) *rk7/fer* mutant had an average of 11 healthy seeds with an SD of 4 (n=19 siliques counted) (E) Quantification of healthy seeds. Siliques are not represented at same magnification



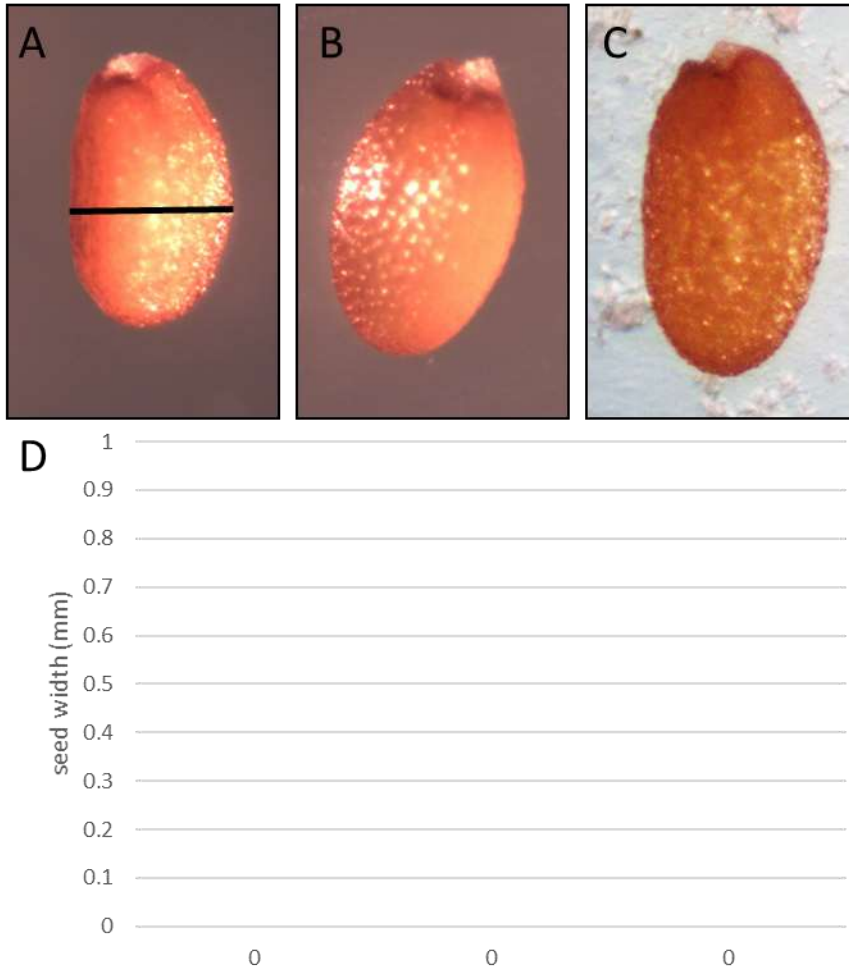
**Figure 9. Seed length comparison.** (A) Measurements in mm of length of the seeds. (B) Table of the seed length average, standard deviation, number of seeds measured, and P-value.



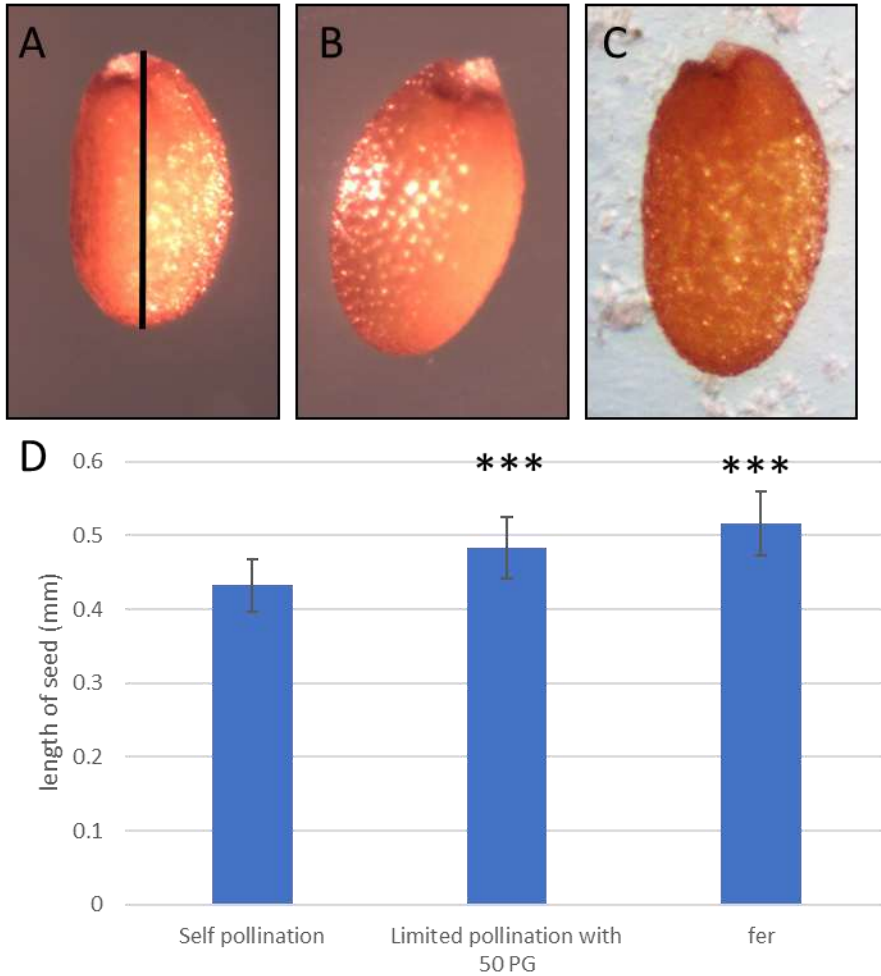
**B**

	WT	<i>rk7</i>	<i>fer</i>	<i>rk7/fer</i>
<b>AVERAGE</b>	0.269088	0.273327	0.281304	0.296037
<b>ST.DEV.S</b>	0.01625	0.024832	0.024583	0.022605
<b>n</b>	73	71	77	78
<b>P-value</b>		0.226241	0.000482	4.05E-14

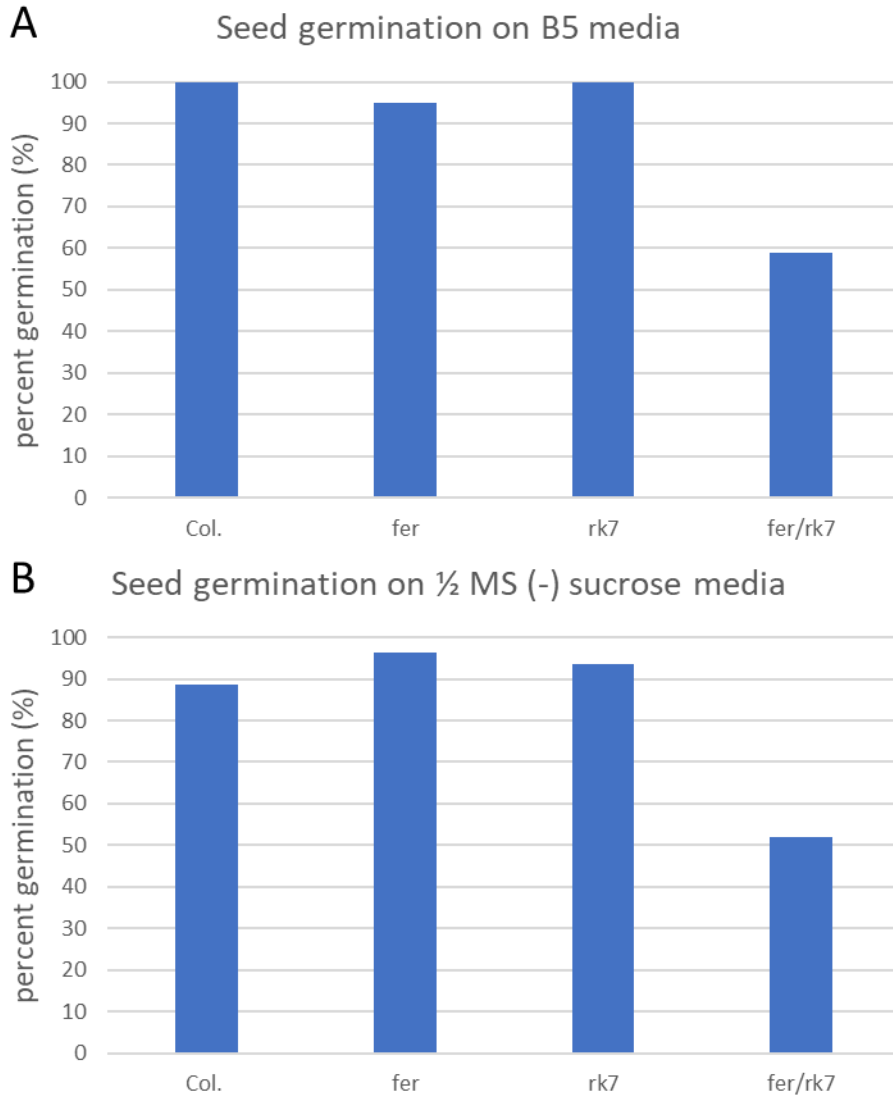
**Figure 10. Seed width comparison.** (A) Measurements in mm of width of the seeds. (B) Table of the seed length average, standard deviation, number of seeds measured, and P-value.



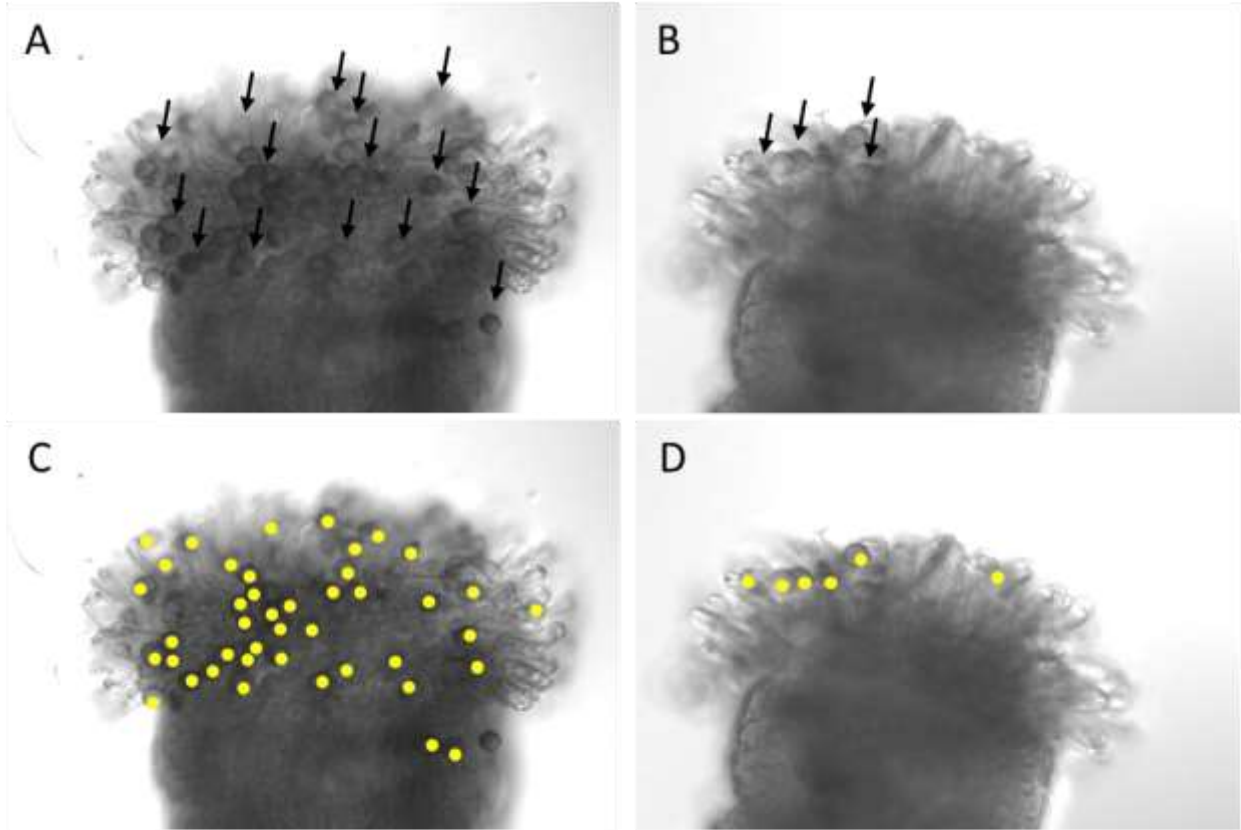
**Figure 11. limited pollination siliques and *fer* siliques do not grow wider seeds.** (A) Col. Seed from self pollination pistil. The black line indicates where the width measurement for the seed was measure. 88 seeds measured for Self pollination siliques with an average of 56 seeds per silique. (B) Seed developed from stigma that was pollinated with only 50 pollen grains. 368 seeds were measured for this condition with the average number of seeds per silique at 19.8 with a standard deviation of 12.6. (C) *fer* seed developed from self pollination. 25 seeds measured with 17.9 seeds per silique on average. (D) Bar graph of the average seed width with limited pollination and *fer* seeds being significantly larger.



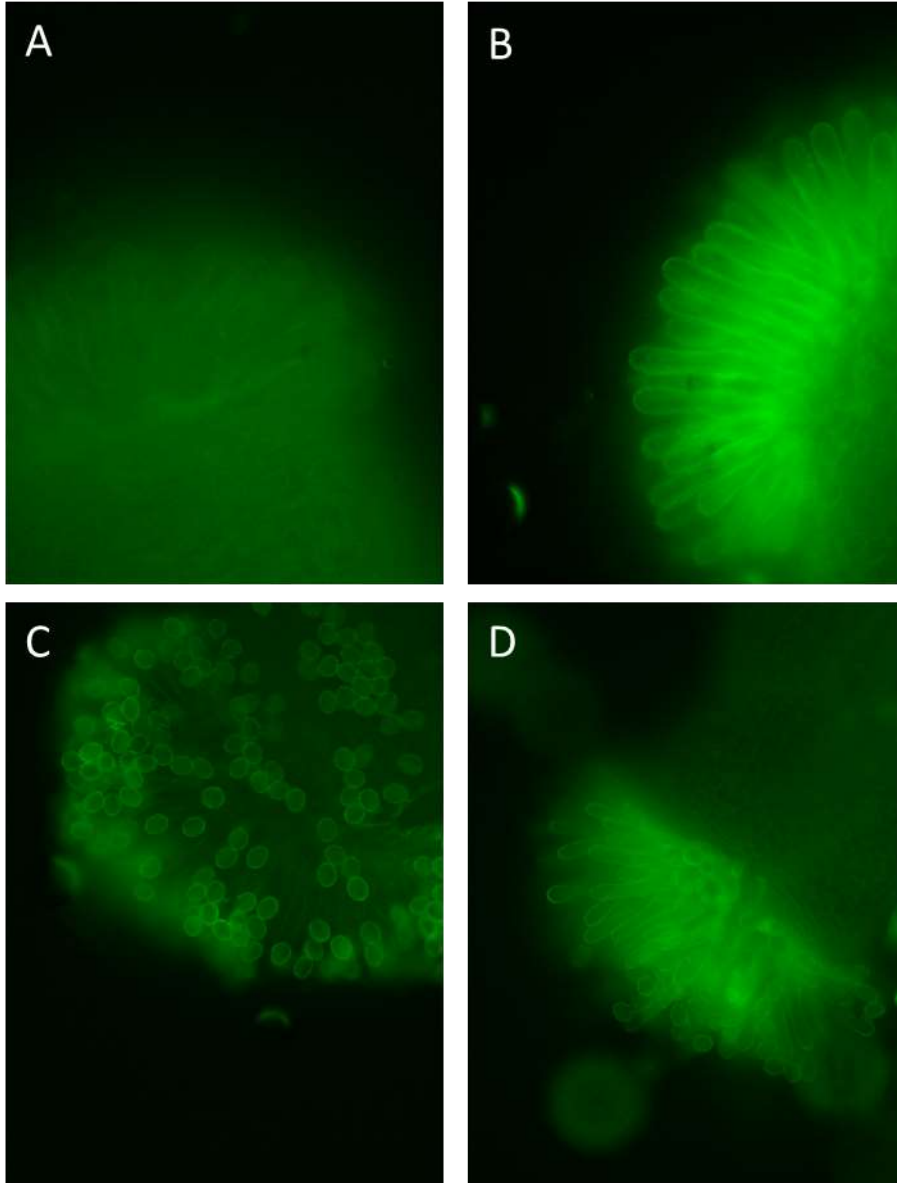
**Figure 12. limited pollination siliques and *fer* siliques grow longer seeds.** (A) Col. Seed from self pollination pistil. The black line indicates where the length measurement for the seed was measure. 196 seeds measured for Self pollination siliques with an average of 56 seeds per silique. (B) Seed developed from stigma that was pollinated with only 50 pollen grains. 935 seeds were measured for this condition with the average number of seeds per silique at 19.8 with a standard deviation of 12.6. (C) *fer* seed developed from self pollination. 34 seeds measured with 17.9 seeds per silique on average. (D) Bar graph of the average seed lengths with limited pollination and *fer* seeds being significantly larger.



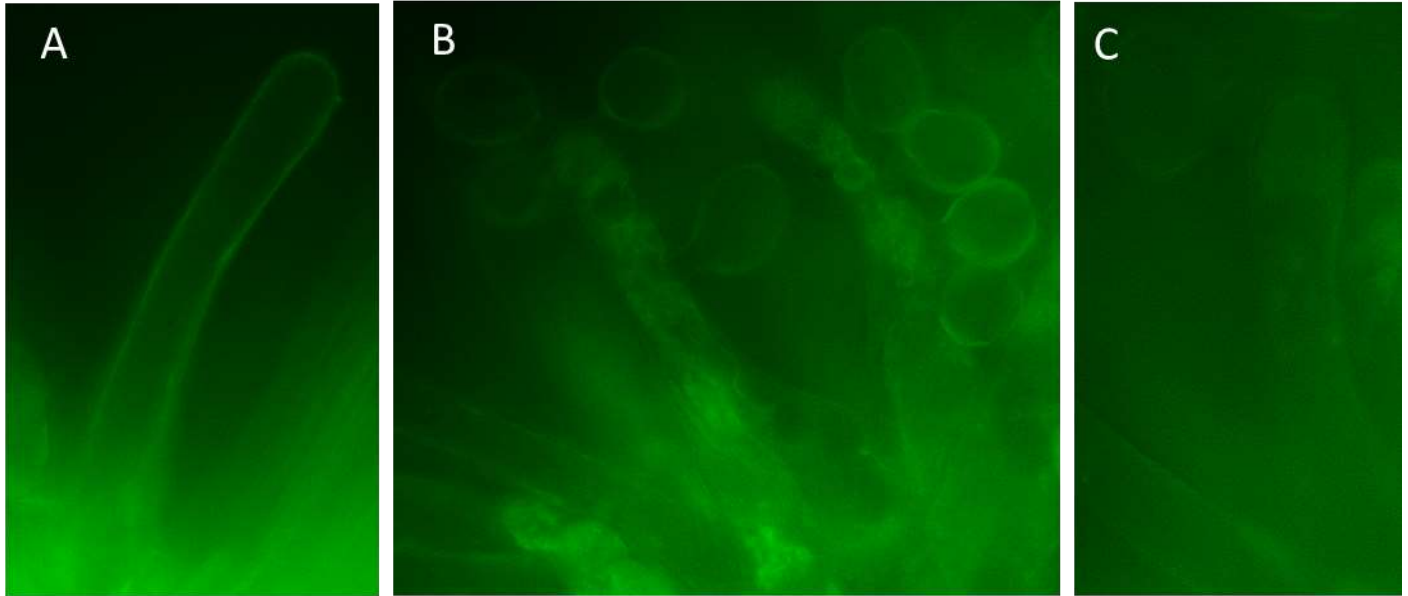
**Figure 13. Seed germination rate.** (A) Seeds germinated on B5 medium observed after 7 days for root growth. 37, 39, 40, and 34 seeds counted for Col., *fer*, *rk7*, and *fer/rk7* respectively. (B) Seeds germinated on ½ MS medium without sucrose and observed after 7 days for root growth. 44, 56, 47, and 77 seeds counted for Col., *fer*, *rk7*, and *fer/rk7* respectively.



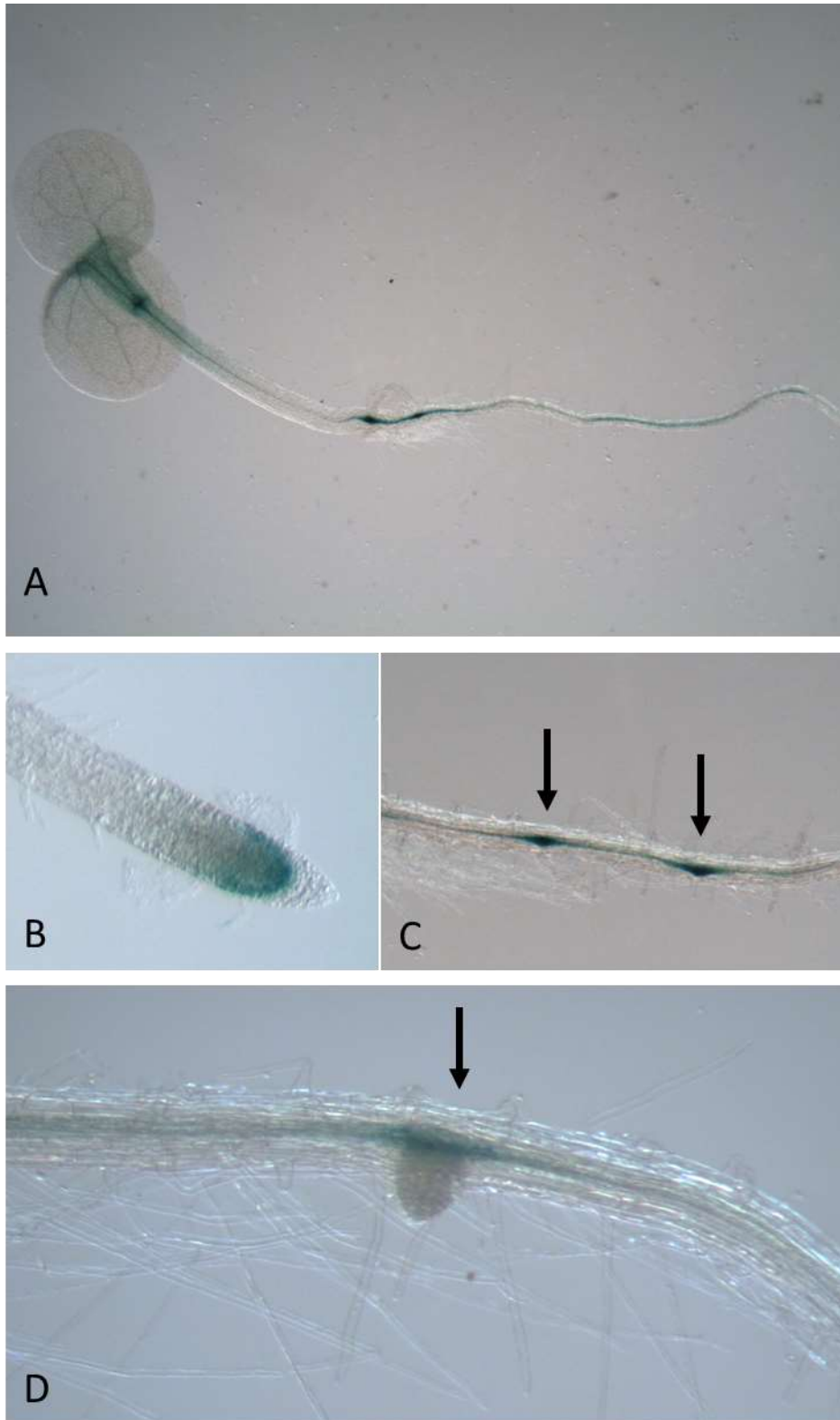
**Figure 14. Pollen-Stigma interaction.** Pistils pollinated, washed with detergent after 1 minute, of germination, and fixed. (A) WT stigma with many pollen grains adhering to the stigma. (B) Shows an *rk7/fer* mutant with very few pollen grains adhering to the stigma. (C and B) Are copies of the top panel with all observable pollen grains marked with a yellow dot.



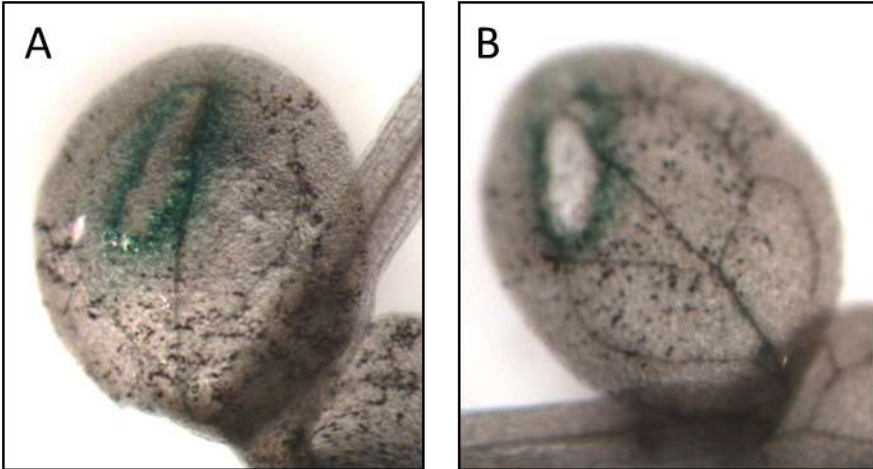
**Figure 15. Localization of RK7 promoter-RK7-GFP.** (A) Col. stigma. (B) RK7 promotor-RK7-GFP transgenic stigma. (C) Col. stigma one hour after pollination. (D) RK7 promotor-RK7-GFP transgenic stigma 1 hour after pollination



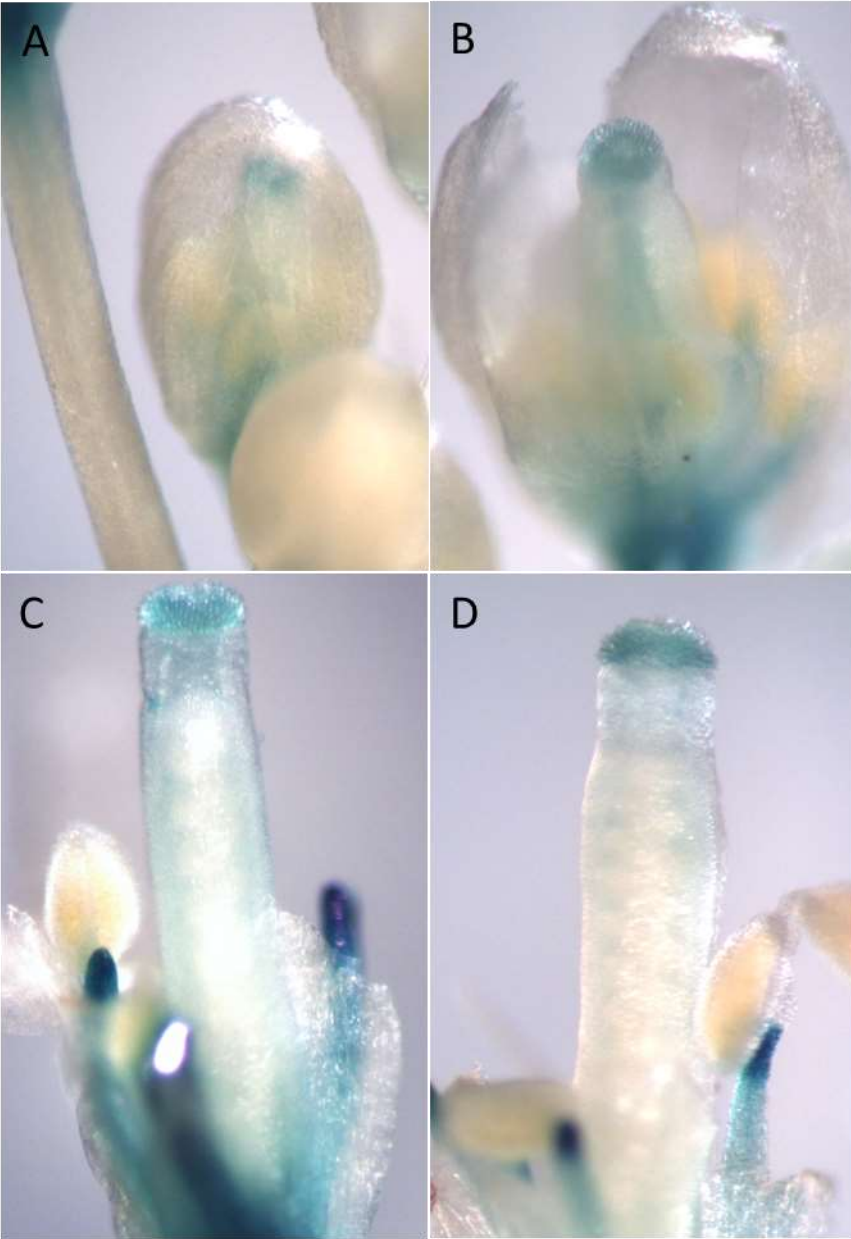
**Figure 16. Endocytosis of RK7 promotor-RK7-GFP after pollination.** (A) Papillary cell with RK7 promotor-RK7-GFP on the cell surface. (B) Papillary cell with RK7 promotor-RK7-GFP two hours post pollination. (C) Papillary cell with RK7 promotor-RK7-GFP three hours post pollination.



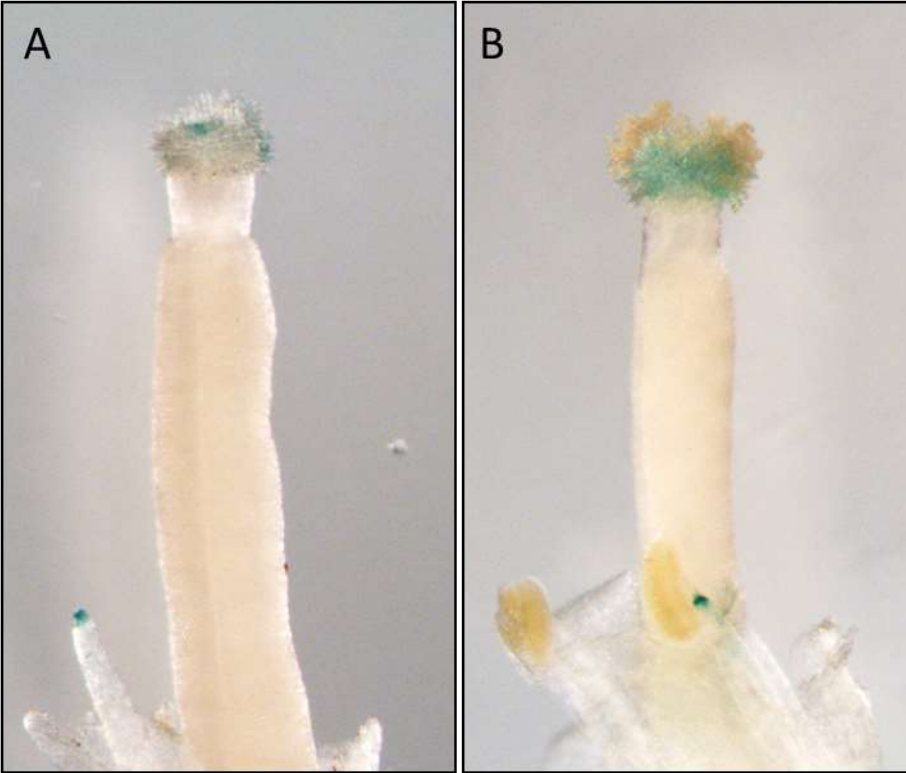
**Figure 17. RK7 promoter: GUS distribution in development of seedling.** Seven day old day old seedlings were placed in placed in X-gluc 0.1mg/mL solution for 2 days. (A) Seven day old seedling. (B) Root tip. (C) Lateral root formation has increased RK7 promoter : GUS expression. (D) Lateral root formation



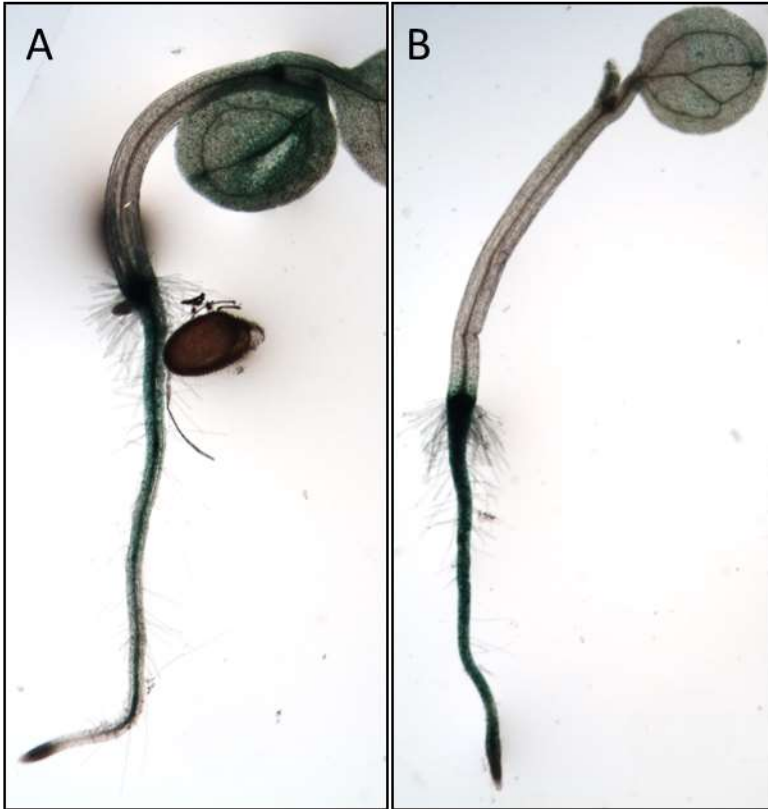
**Figure 18. RK7 promoter: GUS distribution in wounded leaf.** Five day old day old seedlings were placed in placed in H<sub>2</sub>O for two days and then into X-gluc 0.1mg/mL solution for 19 hours. (A) and (B) demonstrate elevated RK7 promotor: GUS expression where the tweezers bruised the leaf



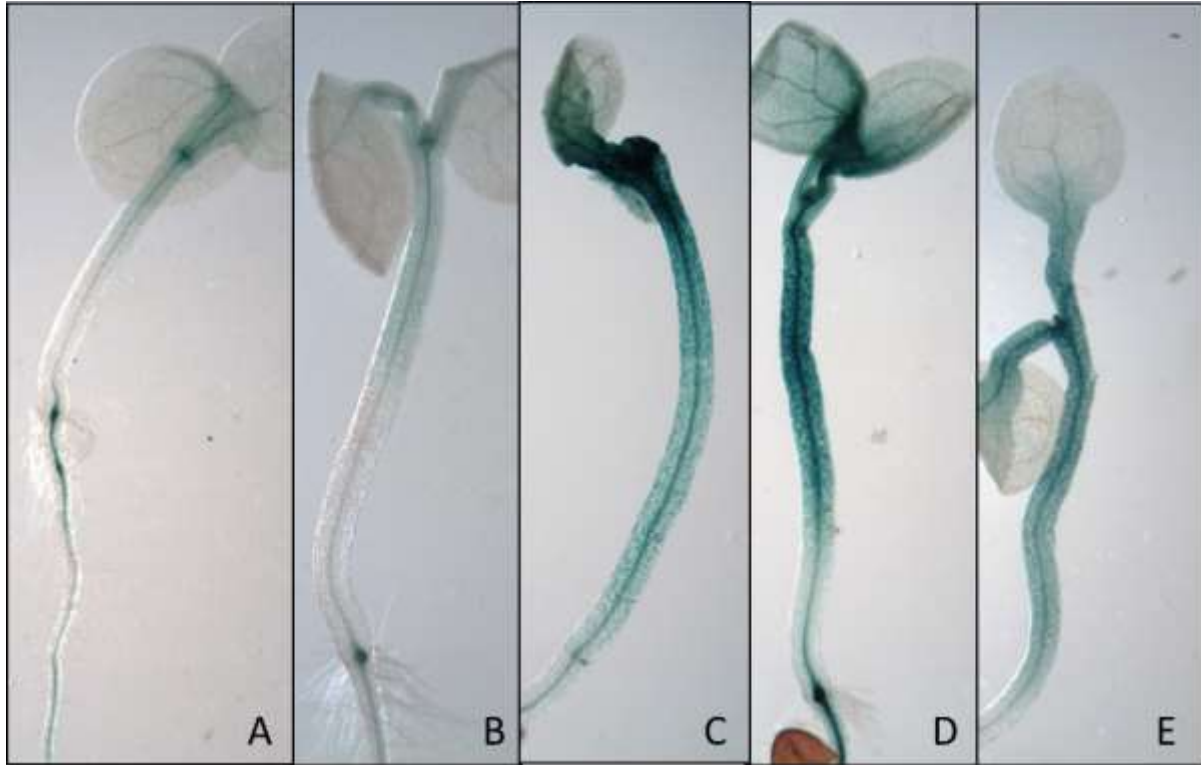
**Figure 19. Analysis of RK7 promoter: GUS expression in developing flowers from transformed Arabidopsis.** As the flower matures the RK7 promoter becomes more active in the stigma (A) The flower is stage 8 and the stigma has not yet formed. (B) The flower is around stage 9 and limited papillary cells have formed. (C) The flower is stage 10. (D) The flower is stage 12.



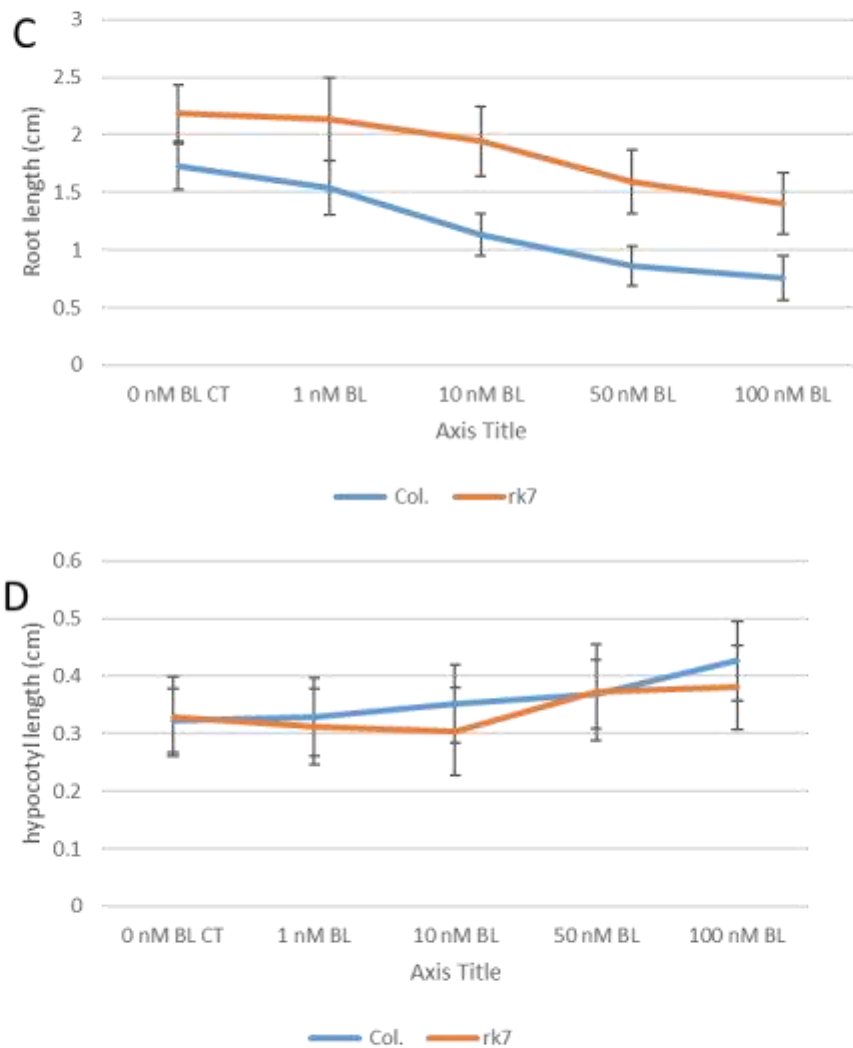
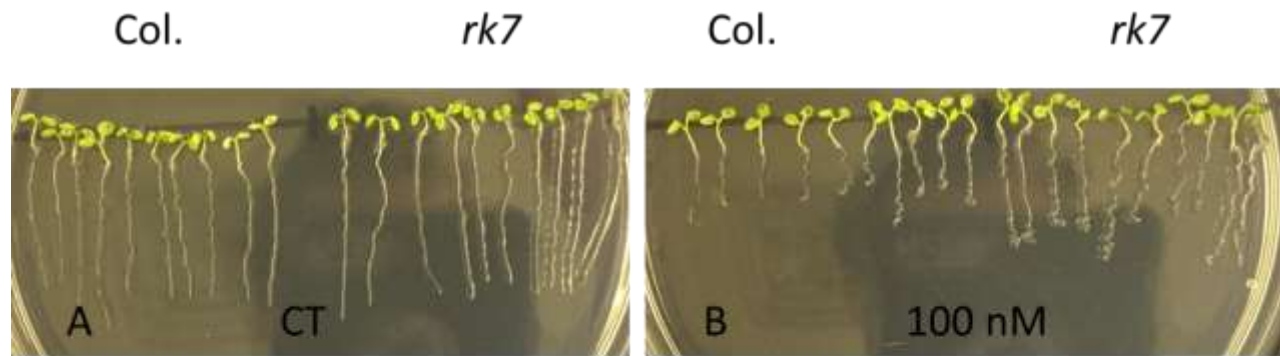
**Figure 20. Pollination induced RK7 promoter: GUS .** Mature emasculated pistils are pollinated and collected one hour post pollination. (A) Not pollinated pistil. (B) One hour post pollination pistil. Both pistils were collected at the same time and put in X-gluc 0.1mg/mL solution for 9 hours.



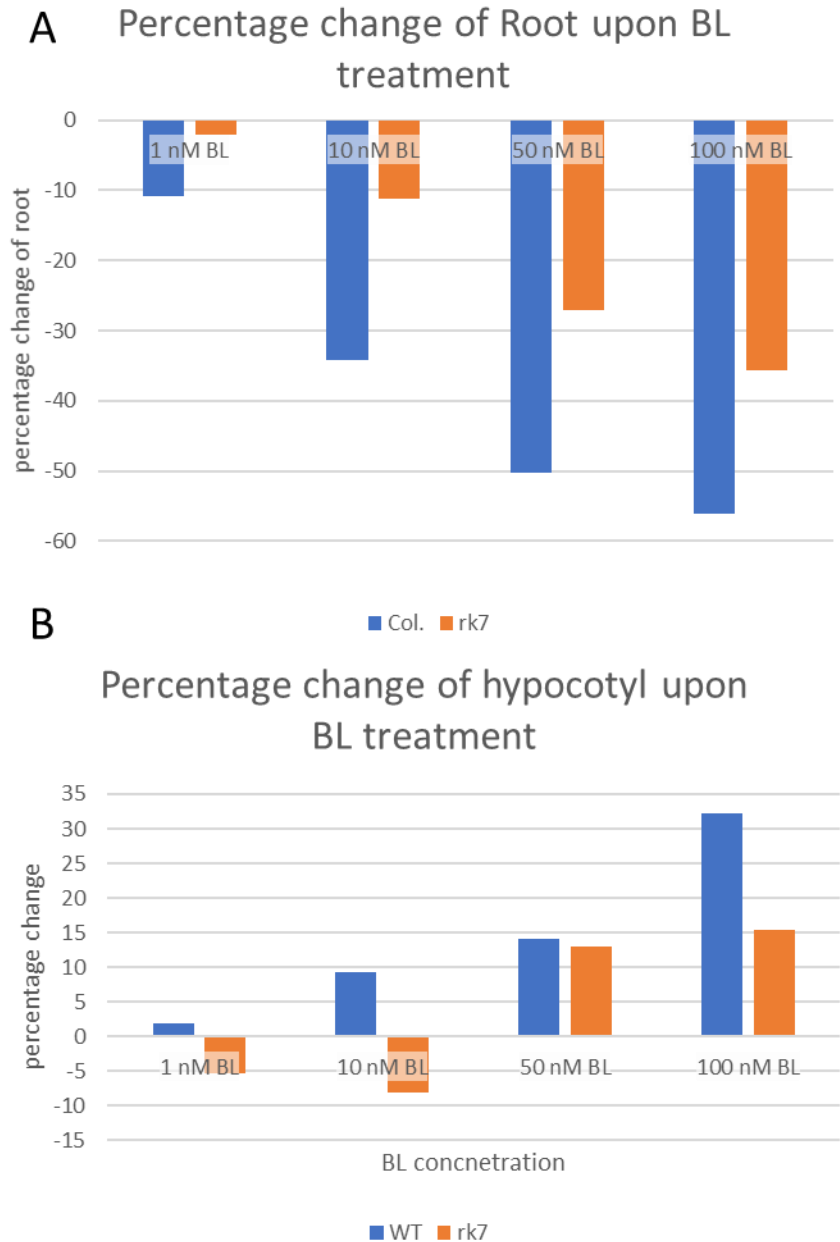
**Figure 21. Auxin induces RK7 promoter: GUS.** Five day old day old seedlings were placed in H<sub>2</sub>O containing 1 μM Auxin for two days. After treatment seedlings placed in X-gluc 0.1mg/mL solution for 19 hours. (A) Control seedling. (B) Seedling treated with 1 μM Auxin.



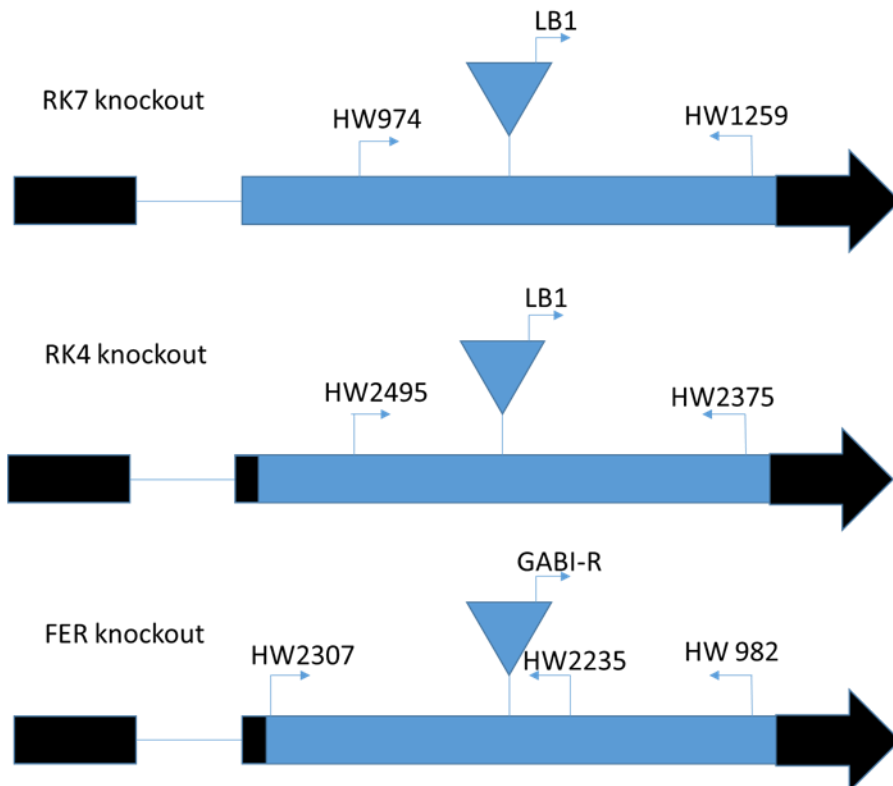
**Figure 22. Brassinosteroid induces RK7 promoter: GUS.** Five day old day old seedlings were placed in H<sub>2</sub>O containing varying amounts of BL for two days. After treatment seedlings placed in X-gluc 0.1mg/mL solution for 24 hours. (A) Control seedling. (B) Seedling treated with 1mM BL. (C) Seedling treated with 10mM BL. (D) Seedling treated with 50mM BL. (E) Seedling treated with 100mM BL.



**Figure 23. *rk7* shows a decreased sensitivity to Brassinosteroids.** Plants were grown on B5 plates containing varying amounts of Brassinosteroids (BL). (A) B5 plate containing no BL with Col. on the right and *rk7* on the left. (B) B5 plate containing 100 nM BL with Col. on the right and *rk7* on the left. (C) Root length of Col. and *rk7* grown on various concentrations of BL. (D) Hypocotyl length of Col. and *rk7* grown on various concentrations of BL.



**Figure 24. *rk7* shows a decreased sensitivity to Brassinosteroids.** (A) Root truncation percentage change calculated by comparing various BL treatment concentrations to the untreated controls. (B) Hypocotyl elongation percentage change calculated by comparing various BL treatment concentrations to the untreated controls.



**Figure 25. RK7, RK4, FER knockout gene maps with primers used for detection.**

## Materials and Methods

### Analysis of RK7 protein and its role in plant developments

#### Transforming the plants

Transgenes inserted into WT plants via the *Agrobacterium tumefaciens* floral dip transformation method (Clough and Bent, 1998).

#### Plant growth and genotyping

WT and mutant plants were all *Arabidopsis thaliana*. Plants were grown as described in (Duan et al, 2010).

DNA was extracted from 2 weeks old seedlings. A 1 cm<sup>2</sup> sector was taken from an expanding leaf of the seedlings. 400µL of DNA Extraction buffer (200 mM Tris-HCl pH8, 250 mM NaCl, 25 mM EDTA pH8, and 0.5% SDS) was added. The sample was then centrifuged for 5 minutes at top speed in a microfuge. 300 µL of supernatant was moved to another tube and equal volume of isopropanol was added. Sample was then mixed well by inverting several times and centrifuged again. Supernatant are removed and 70% ethanol was added. Ethanol was then removed and the sample is dried in a speed-vacuum. Once the sample is dry, 50 µL of TE (10mM Tris-HCl pH8, 1mM EDTA) was added and the sample is resuspended.

To genotype the *fer*, *rk7*, and *rk4* plants PCR procedure was used under normal conditions. The denaturing temperature is 94° C, annealing temperature was 60 °C, and extension temperature was 72 ° C. The denaturing step was 40 seconds, the annealing time was 1 minute, and the extension time was 1 minute. The primers used for RK7 TDNA were LB1 and HW 1259. Primers used for RK7 Genomic DNA were HW974 and HW1259. Primers used for T-DNA insert at *FER* were HW 982 and HW GABI-R. Primers used for *FER* genomic DNA were HW2307 and HW2235. Primers

used for RK4 TDNA were LB1 and HW2375. Primers used for RK4 genomic DNA were HW2235 and HW2459.

All primers written in 5 to 3 prime direction.

(LB1) HW 69 - GCG TGG ACC GCT TGC AAC T

HW 982 - CGGATCC GCT GAT TAC TCT CCA ACA GAG

HW 974 - CGGATCC ATG GGT GGT GAA AAG TTT GGA TTT TTG

HW1259 - CCCATGGC CTT TGA ATT ACC ATC TTG GTG TCG

(GABI-R)HW 1288 - GTGGATT GATGTG ATA TCT CC

HW 2235 - GTCT AACAAAG GTA AGT GAA ACC

HW 2307 - GAG AAG TGC TCT TGA TCG ATG AAG

HW 2459 - CGGATCC TTT GAC GAG AGC CGC AAC ATC

HW 2375 – CCTCGAG TCT TCC TTC AGA TTT CAC CAG

### Transforming the plants

*Arabidopsis* plants were transformed via *Agrobacterium tumefaciens* using the floral dip method (Clough and Bent, 1998). The constructs that were transformed into the WT plant were RK7-promoter-GUS (RK7p::GUS) and RK7-promotor::RK7-GFP (RK7p::RK7-GFP)

### Analysis of transformed plants for RK7 expression and localization

WT plants transformed with RK7 promoter-GUS were screened on kanamycin plates to determine which WT plants were transformed. The transformed plants were then stained with 5-bromo-4-chloro-3-indolyl glucuronide (0.1mg/mL) from five hours to 2.5 days and the RK7 promoter activity was observed from T3 plants. Furthermore, other WT plants transformed with

RK7-GFP. Observing these plants under GFP channel light will allow the GFP protein to be seen. Observing the GFP protein will show where the RK7 protein is targeted to.

Knockout plants used

RK4 knockout At3g46290 - T423 SALK\_008043(C)

RK7 knockout At5g59700 - T692 SALK\_118242

FER knockout At3g51550 - T415 GK\_106A06

Genes written in 5' to 3' direction with black indicating 5' and 3' UTR and the thin blue line indicating introns

### Root treatment assays

Seedlings are grown on B5 media with 1% sucrose for five days. The seedlings are then transferred to 24 well plate, 10 seedlings per well containing 1mL of solution. The solution contains various concentrations of brassinosteroids, Auxin, or RALF1 diluted in H<sub>2</sub>O. The seedlings are grown in the wells for two days at room temperature with 16 hours of light and eight hours of dark.

### Brassinosteroid sensitivity assay

WT and *rk7* seeds were germinated on B5 media with 1% sucrose plates and various concentrations of brassinosteroids. The seedlings were grown vertically for six days in the growth chambers with 16 hours of light and eight hours of dark in 22-23 degrees Celsius. To measure the hypocotyl and root, the seedlings were picked up by the leaf and places on fresh B5 plates with extra care to straighten the seedling. Pictures were taken with iphone 6, imported into

ImageJ and measured. Each picture had a ruler on the side to maintain accurate pixel to cm conversion.

#### Limited pollination assay

WT pistils were emasculated at stage 12 and allowed to mature for 18 hours to stage 14. Pollen was placed on glass slide, 50 pollen grains were placed on the stigma by lightly dabbing it into the pollen. Silique allowed to mature until seeds are fully developed. The number of seeds per silique was counted under the microscope. Pictures of the seeds were taken under 4.5 times magnification with Nikon camera. Seeds measured in length and width in imageJ.

#### Pollination induced RK7 promoter: GUS

RK7 promoter::GUS pistils were emasculated at stage 12 and allowed to mature to for 18 hours to stage 14. The pistils were then pollinated and left for one hour. Both un-pollinated and pollinated pistils were collected at the same time and put in X-gluc 0.1mg/mL solution for 9 hours in incubation chamber at 37 degrees Celsius. Pictures of the pistils with Nikon camera.

#### Pollination of RK7 promotor::RK7-GFP

RK7 promotor::RK7-GFP were emasculated at stage 12 and allowed to mature to stage 14. The pistils were then pollinated and left for different amounts of time. Observing these plants under Nikon E800 GFP channel light will allow the GFP protein to be seen. Observing the GFP protein will show where the RK7 protein is targeted to.

#### Pollen-stigma adhesion assay

In this assay the WT pistils were emasculated at stage 12 and allowed to mature to stage 14 the stigma was pollinated with WT pollen. One anther was used, and it was rubbed onto the pistil until pollen grains ceased to come off the anther. After waiting ~1 minute the pistil was rinsed in the detergent solution (.05M Sodium Phosphate, 1% tween, pH7.4). The pistil was then placed in acetone to fix the pistils till observation by Differential interference contrast (DIC) microscopy. The pollen grains remained stuck on the stigma were then counted and compared amongst the different mutants and the WT plants.

#### Analysis of transformed plants for RK7 expression and localization

WT plants transformed with RK7 promoter-GUS were screened on kanamycin plates to determine which WT plants were transformed. The transformed plants were then stained with 5-bromo-4-chloro-3-indolyl glucuronide (0.1mg/mL) from five hours to 2.5 days at 37 degrees Celsius and the RK7 promoter activity was observed from T3 plants. Observing these plants under GFP channel light will allow the GFP protein to be seen. Observing the GFP protein will show where the RK7 protein is targeted to.

## CHAPTER 3

### RAPID ALKALINIZATION FACTOR 1, POLYGALACTURONIC ACID, AND SMALL PECTIN FRAGMENT INDUCE CELLULAR RESPONSES THROUGH FERONIA

#### Results

##### Innate immunity response in reaction to RALF and flg22

FER and other related RKs have been implicated in *Arabidopsis*'s innate immune response. To test the innate immune response, flg22, a 22-amino acid bacterial derived peptide was used to trigger the innate immune response. Oxidative burst assay and mitogen-activated protein kinase (MAPK) assay have both been used to assay flg22 triggered innate immunity (Asai et al., 2002). To test innate immunity responses, we performed an oxidative burst assay where leaf disks were treated with varying concentrations flg22 (Fig. 26). As previously reported, the more flg22 used the more ROS species was released causing a stronger luminescence (Daudi et al., 2012). Additionally, we used flg22 to elicit a MAPK cascade and test the efficacy of our system (Fig. 27). Like previously published results the Col. WT showed a relatively smaller production of MAPKs when treated with 100nM flg22 in comparison to *fer* and *llg1*. The *fer* and *llg1* seedlings showed a strong response with with the activation of MAPK6, MAPK3, and MAPK4. The antibody used in this assay binds to MAPK6, MAPK3, and MAPK4 thus the more of these proteins produced the stronger the bands on the Western blot.

Using the MAPK reporter system, we wanted to test if RALF1 would induce the MAPK cascade. To ensure that RALF1 was biologically active, an activity assay was performed. The assay confirmed the activity of the RALF1, the seedlings treated with 1 $\mu$ M of RALF1 had inhibited root growth compared to the untreated, and the 3 $\mu$ M RALF1 treatment inhibited root

growth even more than the 1 $\mu$ M concentration, thus confirming a concentration dependent root growth inhibition (Fig. 28). Seedlings were then treated with RALF1 in concentrations varying from 250nM to 2 $\mu$ M that produced a MAPK response with the higher concentration of RALF1 activating a stronger MAPK response (Fig. 29). To test the efficacy of the oxidative burst assay 1 $\mu$ M of flg22 was added to WT leaf discs and oxidative burst was observed comparable to previously published data (Fig. 27).

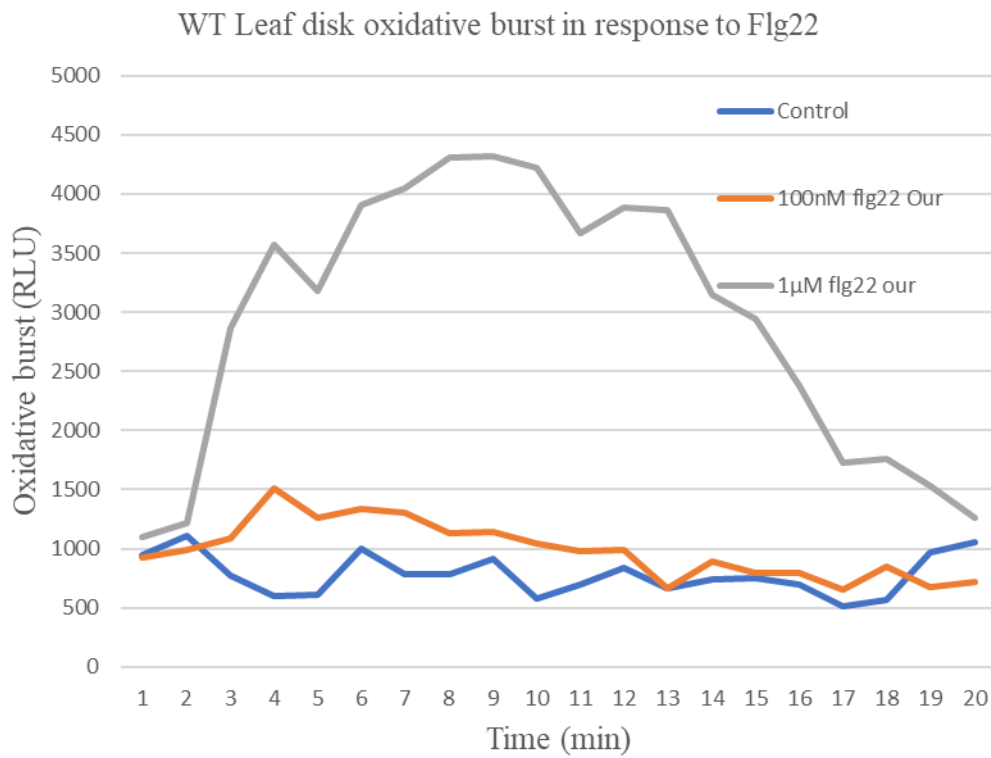
## Discussion

### Innate immunity response in reaction to RALF and flg22

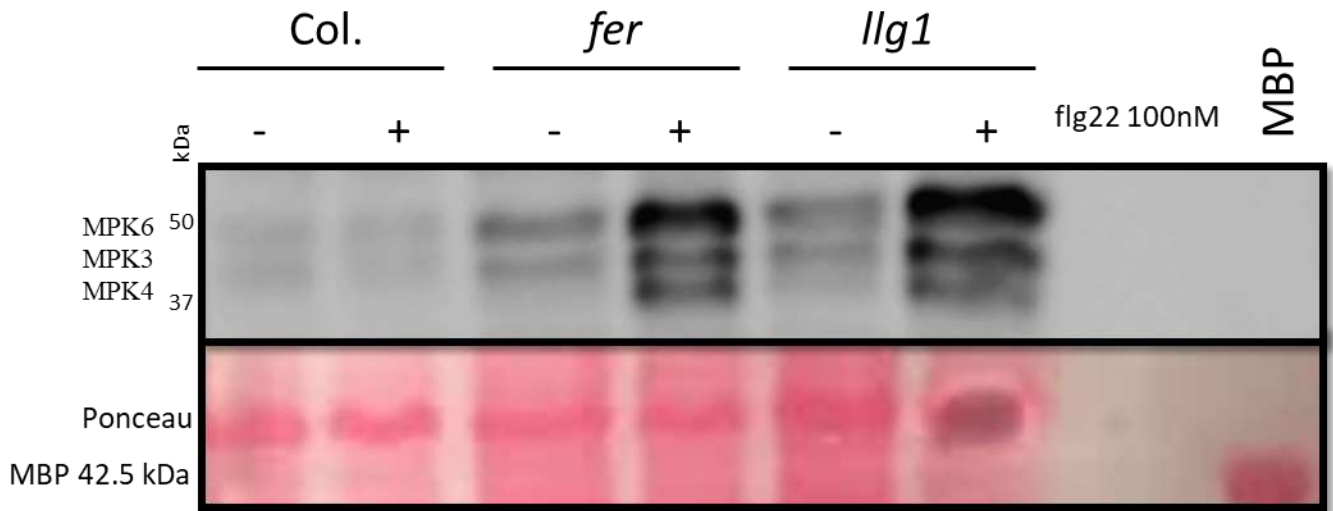
The innate immunity MAPK reporter was tested in seedlings treated with flg22 to establish a system, to test if RALF utilizes this system as well. Flg22 caused the phosphorylation of MAPK cascade indicating that the system was effective. A RALF activity assay was performed on seedlings where RALF was able to arrest growth demonstrating that it was active. The RALF was then used in increasing concentrations on seedlings evoking a proportionally stronger response with increasing RALF concentrations. This indicates that RALF uses the MAPK cascade to transduce its effects. Future studies can test *fer* and *llg1* mutants to test if RALF works through them. The *fer* mutant lacks the FER protein and the *llg1* mutants do not localize the FER protein properly thus they are good candidates to test and see if the MAPK cascade is triggered when treated with RALF. The efficacy of the oxidative burst assay was also established and can be used to test whether other substrates such as PGA can induce burst activity. PGA has been shown to bind to the malectin A domain of FER by members of our

laboratory. With the help of the oxidative burst reporter system we will be able to further demonstrate that PGA acts through FER and initiates downstream effectors.

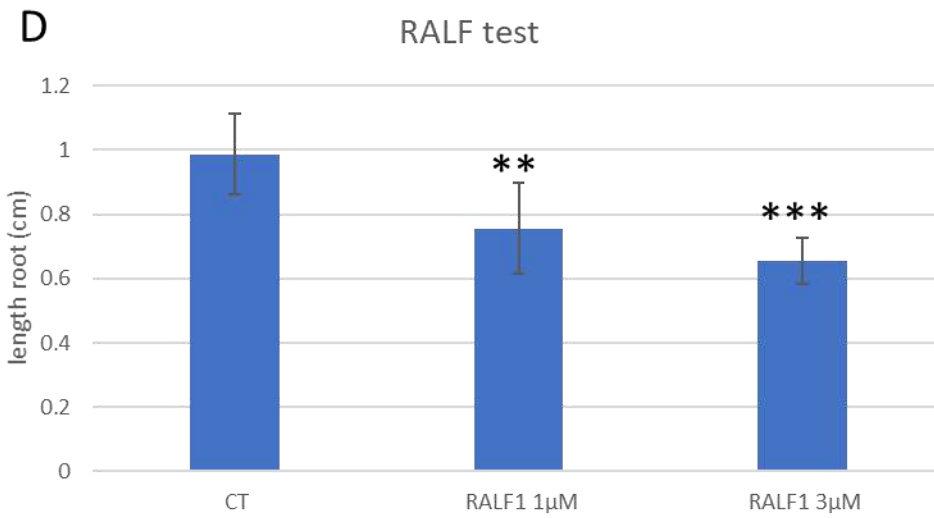
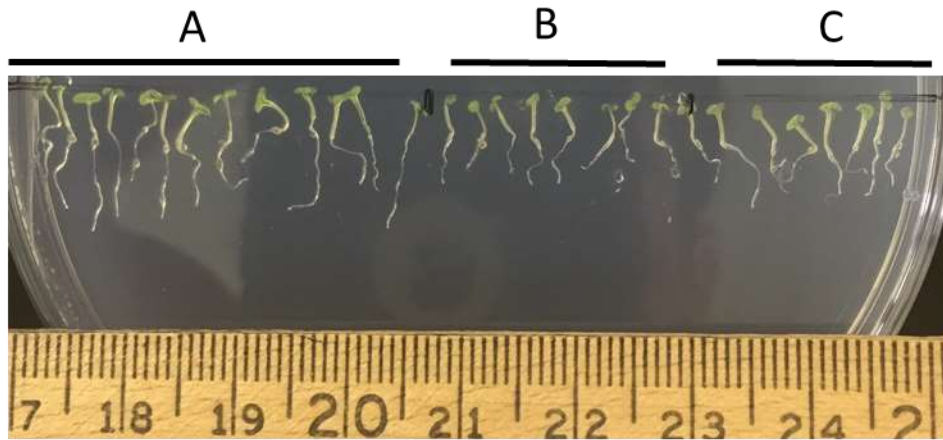
## Figures



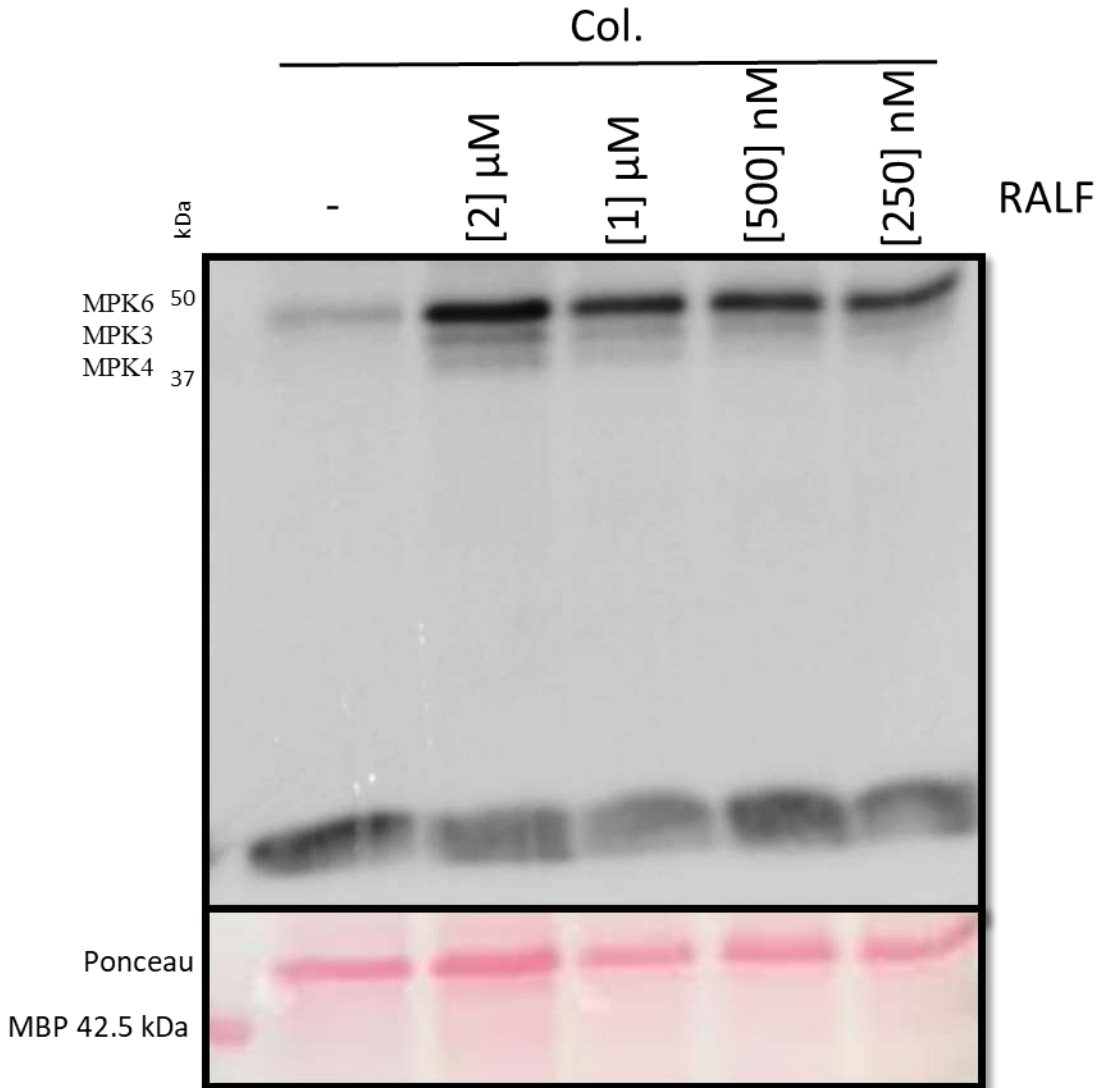
**Figure 26. Oxidative burst triggered by varying concentrations of Flg22.** Chemiluminescence assay showing of elicitation of an oxidative burst by Flg22. Relative light units (RLU)



**Figure 27. flg22 triggered innate immunity.** Ten day old seedlings treated with flg22. The Western blot depicting activation of MAPKs by flg22. Numbers on the left indicate the size. Ponceau indicating protein concentrations and MBP marker.



**Figure 28. Rapid alkalization factor 1 (RALF1) activity assay.** Five day old seedlings treated with varying concentrations of RALF1 for two days. (A) Control seedlings grown in H<sub>2</sub>O. (B) Seedling grown in 3 $\mu$ M RALF1 for two days. (C) Seedlings grown 1 $\mu$ M RALF1 for two days. (D) Average root length calculated after RALF1 treatment. For this assay n is 13, 7, and 9 for CT, RALF1 1 $\mu$ M, and RALF1 3 $\mu$ M respectively. The average length of the roots was 0.99, 0.76, and 0.66 (cm) with a standard deviation of 0.13, 0.14, and 0.07 (cm) for CT, RALF1 1 $\mu$ M, and RALF1 3 $\mu$ M respectively.



**Figure 29. Rapid alkalization factor 1 (RALF1) concentration dependent triggered signaling cascade.** Ten day old seedlings treated with RALF. The Western blot depicting activation of MAPKs by RALF. Numbers on the top indicating RALF concentration. Numbers on the left indicate the size. Ponceau indicating protein concentrations and MBP marker.

## Methods and Materials

### Innate immunity response in reaction to RALF and flg22

#### Map Kinase Assay

Grow seedlings for 10 days. Transfer 10-15 seedlings (usually 12 used) to 1 mL of H<sub>2</sub>O overnight. Remove all the H<sub>2</sub>O and treat seedlings with 1 mL of solution (RALF and PGA dissolved in water). Pipet up and down 2-3 times to ensure the seedlings have contacted the added solution. Treatment should take 12 minutes. Waiting shorter or longer time will affect severity MAPK response. After treatment remove seedling from solution, quickly dry the seedlings and put seedlings directly in the protein extraction solution. Used pestle to grind seedlings until solution is green and no seedlings are visible. Immediately boil the sample for 5 minutes. Spin down sample, add loading dye and load onto gel. The gel should be 12.5% acrylamide and run at 80 volts for best MAPK band separation. After transfer onto membrane, block with 5% BSA for 1 hour. Then added primary antibody ERK (Thr 202, Tyr 204) 1:2000 in 5 % BSA and keep in primary antibody overnight. The secondary antibody used was anti-mouse and can be used in 3% milk.

Protein extraction buffer:

0.4M Tris-HCl pH 7.5, 4% SDS

1% beta-mercaptoethanol, NaF 1mM, Na<sub>3</sub>VO<sub>4</sub> 1mM

### Oxidative burst

The oxidative burst assay was performed with leaves from 4-5 week old plants. The center of the base of the leaf was hole-punched. The leaf disk was excised from the leaf then cut with a razor perpendicular to the mid vein. The half leaf disk was placed in a 96 well plate into 200  $\mu$ L of H<sub>2</sub>O covered and left in the darkness overnight. Luminol (dissolved in dimethyl sulfoxide), HRP, and the elicitor were premixed. The H<sub>2</sub>O was removed from the wells replaced with the pre-mixed solution and placed into the plate reader. The plate reader performed a top read for 30 cycles, with one cycle per minute. Highest available gain (230) was used.

Final concentrations:

Luminal: 225  $\mu$ M

HRP: 5  $\mu$ M

### Root treatment assays

Seedlings are grown on B5 media with 1% sucrose for five days. The seedlings are then transferred to 24 well plate, 10 seedlings per well containing 1mL of solution. The solution contains various concentrations of brassinosteroids, Auxin, or RALF1 diluted in H<sub>2</sub>O. The seedlings are grown in the wells for two days at room temperature with 16 hours of light and eight hours of dark.

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