

Three MYB transcription factors coordinate expression of an array of secreted proteins that promote pollen tube identity and reproductive cell signaling.

By

Alexander Rivinus Leydon

Sc.B., Brown University, 2007

Thesis

**Submitted in partial fulfillment of the requirements for the
Degree of Doctorate in the Department of Molecular Biology, Cellular Biology, and
Biochemistry at Brown University**

PROVIDENCE, RHODE ISLAND

May 2015

Copyright © 2015 by Alexander R Leydon

Signature Page

This thesis by Alexander Rivinus Leydon is accepted in its present form by the Department of Molecular Biology, Cellular Biology, and Biochemistry as satisfying the thesis requirements for the degree of Doctor of Philosophy

Date _____

Mark A. Johnson, Ph.D., Thesis Advisor

Date _____

Judith Bender, Ph. D., Committee Chair

Date _____

Alison DeLong, Ph.D., Committee Member

Date _____

Erica Larschan, Ph. D., Committee Member

Date _____

Sharon Kessler, Ph. D., Outside Reader

Date _____

Peter M. Webber, Ph. D., Dean of Graduate School

Abstract of (Three MYB transcription factors coordinate expression of an array of secreted proteins that promote pollen tube identity and reproductive cell signaling), by Alexander Rivinus Leydon, Ph.D., Brown University, May 2015.

Successful plant reproduction is the lynchpin of agricultural food production. With high susceptibility to climatic variations and other stress conditions, plant reproduction demands further investigation into its mechanisms, especially pollen and pistil signaling pathways that can be optimized for seed production. During pollination a series of pollen tube-pistil interactions ensure that the right sperm get to the right egg to deliver sperm for fertilization. In *Arabidopsis*, the reception of a pollen tube into an ovule initiates with a cell:cell interaction between the pollen tube and the synergid cell, which culminates in sperm release.

We define a trio of R2R3-MYB transcription factors (*MYB97*, *MYB101*, *MYB120*) that are expressed in pollen tubes and are essential for pollen tube reception. *myb* triple-mutant pollen tubes fail to stop growing and burst within the female gametophyte, accordingly the genes regulated by these transcription factors are candidates for pollen tube components of the pollen tube reception mechanism. We developed a novel SNP-informed RNA-sequencing method to simultaneously identify pollen and pistil gene expression profiles from inter-ecotype crosses between Cape Verde Island (Cvi) and Columbia (Col) *Arabidopsis* accessions. We applied this method to comprehensively define genes regulated by MYBs in the pollen tube, which were enriched in three categories: carbohydrate active proteins, transmembrane transporters, and small

secreted proteins. We demonstrate that two MYB-regulated small secreted proteins promote pollen tube reception; a Thionin (CRP2460), and an S protein homolog (SPH). Thionins are secreted from the pollen tube to promote synergid degeneration during reception. One SPH protein is sufficient to increase *myb* triple-mutant fertility, which is the first pollen gene described that controls pollen tube reception.

Our studies have, for the first time, identified pollen tube genes that create pollen tube identity via extracellular molecular patterns recognized by synergid cells. MYB-regulated pollen tube genes uncover reception signaling pathways that are surprisingly akin to plant defense responses. Both the pollen tube and synergid cells convey bits of information to each other upon cell-cell contact, which constitute what we term a reproduction-associated molecular pattern or RAMP.

Curriculum Vitae
Alexander R. Leydon
Alexander_Leydon@brown.edu
165 Waterman Street, Apt. 1, Providence, RI, 02906
(215) 518-4664

EDUCATION:

Ph.D April 2015 (expected) – Brown University. Department of Molecular Biology, Cellular Biology and Biochemistry. Advisor: Dr. Mark Johnson.

ScB. May 2007 – Brown University. Concentration: Human Biology. *Honors*. Advisor: Alison DeLong.

RESEARCH EXPERIENCE:

2010-2015 Brown University PhD – Doctoral Dissertation: Three MYB transcription factors coordinate expression of an array of secreted proteins that promote pollen tube identity and reproductive cell signaling.

- Live-cell imaging using confocal and fluorescence microscopy to visualize MYB transcription factor mutant pollen tube reception defects within the ovule.
- Transcriptional profiling of MYB transcription factor-mutant pollen by microarray and RNA-Seq and bioinformatics analysis of differential expression. RNASeq-SNP identification and read cell-type origin using inter-ecotype crosses.
- Heterologous expression & purification of MYB-regulated small proteins in *E. coli* & the methylotrophic yeast *P. pastoris* to test protein activity against cultured ovules in vitro.

2007-2010 Brown University Pollen Genetics Lab - Research Assistant:

Identification and characterization of genes required for pollen development and function. Advisor: Mark Johnson

- Medium through-put T-DNA mutant screening (~200) for pollen grain developmental defects using distorted segregation, and microscopy.
- Identified five genes with altered growth phenotypes observable during in vitro pollen tube growth and two genes required for pollen tube growth and guidance in the pistil.

2006-2007 Brown University, Undergraduate Independent Research: Protein Phosphorylation controls reproductive development in an isoform specific manner. Advisor: Alison DeLong

- Discovered roles for PP2A A-regulatory isoforms in gametophyte and early embryo development.

TECHNIQUES:

- Standard cloning, primer design, PCR, qPCR, Fusion PCR, Site-directed mutagenesis, RNA/DNA isolation, RNA sequencing and bioinformatics analysis, protein isolation & quantification, Western blotting, Northern blotting, genetic analysis of insertion mutations, production of transgenic plants, confocal and fluorescence microscopy, CRISPR/Cas9 mutagenesis, drosophila cell culture, RNAi.

PUBLICATIONS:

- **Leydon AR**, Chaibang A, Johnson MA. "Interactions between pollen tube and pistil control pollen tube identity and sperm release in the Arabidopsis female gametophyte." *Biochemical Society Transactions*. 2014.
- Soruco MM, Chery J, Bishop EP, Siggers T, Tolstorukov MY, **Leydon AR**, Sugden AU, Goebel K, Feng J, Xia P, Vedenko A, Bulyk ML, Park PJ, Larschan E. "The CLAMP protein links the MSL complex to the X chromosome during Drosophila dosage compensation". *Genes and Development*. 2013.
- **Leydon, AR**, Beale, KM, Woroniecka, K, Castner, E, Chen, J, Horgan, C, Palanivelu, RS, Johnson, MA. "Three MYB transcription factors control pollen tube differentiation required for sperm release". *Current Biology*. 2013.
- Kristin M. Beale, **Alexander R Leydon**, Mark A Johnson. "A block to polytubey is triggered by gamete fusion in Arabidopsis thaliana". *Current Biology*. 2012.
- Wong JL, **Leydon AR**, Johnson MA. "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain". *PLoS Genetics*. 2010.
- Qin Y, **Leydon AR**, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R.. "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil". *PLoS Genetics*. 2009.

PUBLICATIONS (In preparation):

- **Leydon AR**, Johnson MA. "Arginine biosynthesis is localized to a novel chloroplast-attached cell body and is required for gamete development and function in Arabidopsis". To be submitted to *Plant Physiology* 2015.
- **Leydon AR**, Tsukamoto T, Johnson MA, Palanivelu R. "The relationship

between synergid degeneration and pollen tube discharge". To be submitted to *Plant Reproduction* 2015.

- **Leydon AR**, Johnson MA. "Identifying Sex Specific gene expression using SNP-informed RNA-Sequencing". 2015.
- **Leydon AR**, Johnson MA. "A family of small secreted thionin proteins control synergid degeneration during pollen tube reception in Arabidopsis". 2015.

AWARDS:

- Best Oral Communication: Regulation of Fertilization and Early Seed Development, University of Bath, UK, September 2013.
- Best Poster Presentation: Northeast Regional Meeting of the Society for Developmental Biology. Woods Hole, MA. 2011.
- Best Graduate Student Poster Award: Molecular Biology, Cellular Biology, and Biochemistry Annual Retreat. 2011.

INVITED TALKS:

- Plants in New England, Whitehead Institute, MA. "Decoding Reproductive Dialog: Small secreted proteins control pollen tube-synergid interactions". 2014.
- 23rd International Congress on Sexual Plant Reproduction, Porto, Portugal. "Untangling pollen tube and pistil gene expression using SNP-informed deep sequencing". 2014.
- Biochemical Society, Regulation of Fertilization and Early Seed Development, University of Bath, UK. "Three MYB transcription factors control interactions with synergid cells required for reception and sperm release in Arabidopsis". 2013.
- Brown University Department of Molecular Biology, Cell Biology, and Biochemistry Annual Retreat. "Penetration of the stigma and style elicits a novel transcriptome in Pollen Tubes, Indicating Potentiating Transcriptional Networks". 2011.
- Pollen Research Coordination Network Meeting, Minneapolis, MI. "Identifying transcriptional regulators essential for pollen tube growth and guidance". 2011.

POSTER PRESENTATIONS:

- American Society for Plant Biology, Providence, RI. "A pollen tube transcriptional network controls interactions with synergid cells required for reception and sperm release in Arabidopsis". 2013.

- XXII International Congress on Sexual Plant Reproduction Meeting. Melbourne, Australia. “A family of pollen tube-expressed transcription factors is essential for pollen tube reception”. 2012.
- American Society for Plant Biology, Minneapolis, MI. “A pair of pollen-specific beta-galactosidases is essential for pollen tube growth”. 2011.
- Northeast Regional Meeting of the Society for Developmental Biology, Woods Hole, MA. “Identifying transcriptional regulators essential for capacitating pollen tube growth and guidance”. 2011.
- Frontiers in Sexual Plant Reproduction III, Tucson, Arizona. “*hapless* mutations affecting sperm development and the paternal contribution to embryogenesis”. 2008.

TEACHING EXPERIENCE:

- Cold Spring Harbor Frontiers and Techniques in Plant Science. Teaching Assistant. Responsible for planning and running laboratory sessions. 2013.
- Howard Hughes Medical Institute Undergraduate Summer Course. Teaching Assistant. Responsible for leading and planning laboratory sessions. 8-week course. 2012.
- Brown University Plant Organism Course (BIOL 0440). Teaching Assistant. Responsible for leading and planning laboratory sections. 2012.
- International Pollen Genetics Course. Teaching Assistant. Responsible for leading and planning laboratory sessions. 2-week intensive laboratory course for graduate students, postdocs, and young professors. 2011.
- University of Rhode Island Department of Chemistry Laboratory for Chemistry 101 (General Chemistry Lecture) CHM 102. Instructor (Part-Time Faculty). Providence, RI. 2007 – 2008.
- Brown University, Department of Molecular Biology, Cell Biology, and Biochemistry. Undergraduate Senior Honor Biology Thesis Mentor: 2010-2015 (six students).

Acknowledgements

To my advisor:

Mark Johnson. We got to Brown University the same year, both freshman; you a freshman Professor and me a college freshman. It has been more than a decade since, and we have managed to get weirder, grumpier and hopefully a lot wiser. Best of all is the progress we have made in understanding pollen biology. We have come a long way since we first started thinking about pollen tube transcription, and I'm grateful I got to be a part of it. You have given me near complete independence to pursue my scientific curiosity and for that I thank you. As a pollen biologist, I am excited when I imagine what you'll discover in the years to come.

To my committee members:

Alison DeLong. You took a chance, and gave me an opportunity to work in your laboratory and that was the best thing that happened to me during my undergraduate years. That was such a formative experience that I can honestly say it set me on a better path. You taught me how to think critically and communicate effectively. You have been like family to me during my time at Brown.

Erica Larschan. You took me in to your lab for an amazing rotation. Everything I learned from you about sequencing technology, gene regulation and dosage compensation has greatly informed my own research. More importantly, you taught to me how to investigate and conceptualize biological systems in the

broader context of the genome and its physical regulation. Your advice and enthusiasm during the course of my graduate work has been invaluable.

Judith Bender. You have been an excellent committee member, and my go-to person for any ideas I have about MYBs. I have always felt that you have critically engaged the full breadth of my research and helped me to discover the intricacies of MYB regulation of the pollen tube transcriptome. What's more, I've appreciated your personal help designing and running experiments for my many tangential projects.

I would also like to thank my outside reader, Dr. Sharon Kessler for traveling from Oklahoma to participate in my final defense and contribute her valuable expertise.

To my friends.

Kristin. You have been my friend through so many years of research joys and woes. We worked together, grew together, laughed and cried together, and even traveled to distant lands together on our scientific adventures. Our lab hours, devious exploits, and glorious party-times are priceless memories to me.

Andrea. My dear friend, I have valued your thoughts, your laugh and your love. You have given me support at so many stages of graduate school, and cheered me on when I thought I might explode. Thank you for keeping me rooted and giving me perspective.

Mary. My partner in crime, my other self, you have more than supported me in my grad school career. I have only love for you, and I thank you for everything, you dark-sided chicken.

To my family.

Ariadne. You are my big-pea, the only other pea in our pod of two. Thank you for your special brand of support. I love you, you crazy, spinning top.

Mom and Dad, I dedicate my doctoral thesis to you. You are the best parents I could ever have asked for. From the beginning of my childhood you fostered my scientific curiosity, even when it was completely bizarre. You have unconditionally supported me in my successes and many failures. You approach life with grace and strength, and I can only hope I grow up as well as you. There is no way to begin to thank you for everything, nor would a simple thank you ever be enough. You gave me life, and you made me into who I am today.

Table of Contents

Title Page	i
Copyright Page	ii
Signature Page	iii
Abstract	iv
Curriculum Vitae	vi
Acknowledgements	x
Table of Contents	xiii
List of Figures and Tables	xix
Chapter 1: Introduction. Pollen tubes: an adaptive sperm delivery device	1
Gametophytes control the success of plant reproduction: Male gametophyte development	2
Female gametophyte development	3
Pollen tube growth and guidance in the pistil	4
Pollen tube reception in the ovule	5
What is the role of transcription in the pollen tube?	7
What is the pollen tube transcriptional network	9
Self-recognition and species-recognition in pollination	12
Peptide signaling: small secreted proteins involved in pollen-pistil interactions	16
References	19

Chapter 2: Interactions between pollen tube and pistil control pollen tube identity and sperm-release in the female gametophyte 25

Abstract 26

Introduction 27

Pollen tubes differentiate in response to growth through the pistil 29

Pollen tube differentiation for sperm release is controlled by three MYBS 31

Pollen tube reception signaling by the female gametophyte 32

Do MYB target genes interact with the pollen tube reception signaling components? 34

MYB-mediated pollen tube differentiation is important for species recognition during pollen tube reception. 37

References 40

Chapter 3: Pollen tube discharge completes the process of synergid degeneration that is initiated by pollen tube – synergid interaction in Arabidopsis 42

Abstract 43

Introduction 44

Results 49

Discussion 67

Materials and Methods 74

References 79

Chapter 4: Three MYB transcription factors control pollen tube differentiation required for sperm release	82
Summary	83
Results and Discussion	84
Conclusions	95
Experimental Procedures	97
References	99
Supplemental Inventory	102
Supplemental Experimental Procedures	109
Supplemental References	115

Chapter 5: Identifying Sex-Specific gene expression using SNP-informed RNASequencing	117
Abstract	118
Introduction	119
Results	122
Discussion	149
Supplemental Information	154
Materials and Methods	157
Supplemental File Inventory	161
References	164

Chapter 6: A family of small, secreted Thionin proteins control synergid degeneration during pollen tube reception in <i>Arabidopsis</i>	169
Abstract	170
Introduction	171
Results	176
Discussion	202
Future Directions	205
Materials and Methods	211
Supplemental Material	216
References	224
 Chapter 7: A novel role for Self-incompatibility protein S1 homologs in pollen tube reception.	 230
Abstract	231
Introduction	232
Results	238
Discussion	252
Materials and Methods	256
References	259
 Chapter 8: Discussion. MYB97, MYB101, and MYB120 produce the molecular pattern of the pollen tubes perceived by synergids during reception	 263

Pollen tube reception in flowering plants is required for seed production	264
1. Three R2R3 MYB transcription factors are required for pollen tube reception with the synergid	265
2. MYB transcription factors positively regulate a subset of pollen tube genes that are specifically induced by growth in the pistil.	269
3. Small secreted MYB-regulated Thionin proteins promote synergid degeneration.	278
4. A small secreted MYB-regulated Self-incompatibility protein homolog (SPH) promotes pollen tube reception.	282
Identification of a reproduction-associated molecular pattern or RAMP in pollen tube reception	285
Pollen tube reception: the final barrier to species non-specific reproduction in flowering plants	294
References	296

Appendices

Appendix 1: SUC A family of MYB-regulated Sucrose/Proton symporters and their role in pollen tube reception	302
--	------------

Appendix 2: Alpha carbonic anhydrases ACA4/ACA6 positively regulated pollen tube guidance to ovules and synergid degeneration	332
--	------------

Appendix 3: A dominant mutation in the AUX/IAA protein IAA31 uncovers a role for Auxin signaling in pollen tube guidance	358
---	------------

Appendix 4: Gamete fusion prevents multiple pollen tubes from entering a single *Arabidopsis* ovule 396

Appendix 5: Argininosuccinate lyase (ASL) is localized to a novel chloroplast-attached structure and is required for gamete development and function in *Arabidopsis* 416

Appendix 6: Nortia (NTA) localization in ovules receiving *myb* triple-mutant pollen tubes 450

List of Figures and Tables	xix
Chapter 1: Introduction	
Figure 1. Gametophyte development in <i>Arabidopsis</i>	3
Figure 2. Pollen tube growth in the <i>Arabidopsis</i> pistil	5
Figure 3. Pollen tube characteristics are altered by interaction with female tissues	8
Figure 4. Mechanisms of SI involving small secreted ligands	12
 Chapter 2	
Figure1. Pollen tube S18 MYB transcription factors respond to growth through the pistil	29
Figure 2. S18 MYB regulated gene classes and their putative roles during pollen tube receptions	34
Figure 3. Pollen tube reception is defective in inter-accession and inter-species crosses.	39
 Chapter 3:	
Figure 1. Two patterns of synergid degeneration are observed in wild-type ovules accessed by <i>aca9-1</i> pollen tubes.	50
Figure. 2. Synergid degeneration is observed in unpollinated ovules, but is enhanced by presence of the pollen tube.	55
Figure 3. Synergid degeneration is higher in ovules that attract <i>aca9</i> pollen tubes that discharge relative to those that enter the micropyle, but do not discharge.	58

Figure 4. Pollen tube discharge is defective in <i>gfa2</i> female gametophytes.	61
Figure 5. Pollen tube discharge and synergid degeneration are detectable in <i>gfa2</i> female gametophytes and synergid degeneration is increased in <i>gfa2</i> female gametophytes containing a discharged pollen tube relative to those containing an arrested pollen tube.	64
Figure 6. Synergid degeneration occurs more frequently in <i>lre</i> female gametophytes containing a discharged pollen tube relative to those containing an arrested or a coiled pollen tube.	66
Supplemental Figure 1. Confocal analysis of synergid degeneration in <i>gfa2/GFA2</i> .	77

Chapter 4

Figure 1. MYB97, MYB101, and MYB120 localize to the growing pollen tube nucleus.	85-86
Figure 2. MYB97, MYB101, and MYB120 are required for pollen tube burst, sperm release and synergid degeneration.	90
Figure 3. MYB97, MYB101, and MYB120 regulate gene expression during pollen pistil interactions.	94
Figure S1. A Summary of T-DNA Insertion Lines Used in This Study and Their Impact on RNA Abundance, Seed Set, and Pollen Tube Function.	103
Figure S2. MYB:GFP Fusion Constructs Are Capable of Rescuing the <i>myb</i> Triple-Mutant Phenotype.	105
Figure S3. RT-qPCR Analysis of MYB-Regulated Genes.	106

Table S1. Differentially Regulated Genes and Small Peptides Identified by Microarray Analysis (table legend)	107
Oligonucleotides used in this study	112-113

Chapter 5:

Figure 1. SNPs are distributed across genomic features and are abundant in coding sequences.	123
Figure 2. Experimental design employed to identify ecotype-specific reads originating from pollen or pistil.	126
Figure 3. SNP-informed RNA-seq accurately represents gene expression.	127
Figure 4. Inter-ecotype pollinations enable identification of sex-specific gene expression during pollination.	129
Figure 5. Sex-specific gene expression profiles correspond to known reproductive transcriptional profiles	133
Figure 6. MYB regulated gene expression in SNP-dissected transcriptomes.	135
Figure 7. MYB-regulated gene expression becomes visible after Sex-specific read dissection.	137
Figure 8. Pollen specific gene expression of 3 MYB-regulated genes detected by SNP-sequencing.	140
Figure 9. Reproductive gene regulation is highly divergent between Col and Cvi.	142
Figure 10. Pollen genotype effect on pistil expression in inter-ecotype crosses.	145

Table 1. SNP rates in reproductively expressed genes.	147
Table 2. SNPs causing missense mutations are enriched in reproductively expressed genes.	148
Supplemental Figure 1. Quantitative analysis on reads identified from SNPs ensures an accurate representation of total gene expression.	154
Supplemental Figure 2. Correlation between SNP-specific sex identification and published microarray analysis.	155
Supplemental Figure 3. Comparison of differential expression callers on RNA-sequencing results for wild-type and <i>myb</i> triple-mutant pollinations.	156
Supplemental Figure 4. Cis-Regulatory element analysis on cell-type specific MYB-regulated gene promoters.	156

Chapter 6:

Figure 1. MYB transcription factors control expression of Thionin CRPs (CRP2460)	177
Figure 2. A phylogenetic tree of paralogous Thionin (CRP2460) proteins of <i>A. thaliana</i> , <i>A. lyrata</i> , <i>C. rubella</i> , <i>B. rapa</i> , and <i>T. halophila</i> .	179
Figure 3. Transient expression of Thionin proteins provides subcellular localization data	181
Figure 4. Thionins are secreted from the pollen tube to the synergid during pollen tube reception.	183
Figure 5. Genetic strategy to identify the function of Thionin proteins.	186

Figure 6. Thionin expression constructs in <i>myb</i> triple-mutant pollen increase synergid degeneration without affecting pollen tube burst.	189
Figure 7. Thionin:GFP proteins increase synergid degeneration by <i>myb</i> triple-mutant pollen tubes.	191
Figure 8. Thionin proteins are not sufficient to rescue <i>myb</i> triple-mutant seed set defects.	194
Figure 9. Thionin protein primary sequence and secondary structure prediction.	196
Figure 10. Expression of Thionin Protein in the methylotrophic yeast <i>Pichia pastoris</i> .	198
Figure 11. Purified Thionin (At5g36720) promotes synergid degeneration <i>in vitro</i> .	201
Figure 12. CRISPR mutagenesis of Thionins in the CRP2460 clade.	207
Figure 13. Purification of related Thionin proteins.	208
Supplemental Figure 1. Protein alignment of CRP2460 from <i>A. thaliana</i> .	216
Supplemental Figure 2. Phylogenetic analysis of all Thionin and Thionin-related CRPs in <i>Arabidopsis thaliana</i> (CRP2200-2550).	217
Supplemental Figure 3. The location of CRP2460 genes in the <i>Arabidopsis</i> Genome.	218
Supplemental Figure 4. Expression of Thionin protein in <i>E. coli</i> cytoplasm.	221
Supplemental Figure 5. Expression and purification of Thionin protein in <i>N. benthamiana</i> leaf infections.	223

Chapter 7:

Figure 1. MYB transcription factors control expression of S protein homologs (SPH)	239
Figure 2. Molecular phylogenetic analysis of the SPH gene family	242
Figure 3. Genomic distribution of SPH genes	244
Figure 4. Pollen tube phenotypes 12 hours after pollination	245
Figure 5. SPH:GFP rescues pollen tube burst and fertilization	247
Figure 6. SPH:GFP add back partially rescues <i>myb</i> triple-mutant seed set defects.	248
Figure 7. Amino acid sequence of AtSPH and Papaver S proteins reveals regions of conservation.	250
Figure 8. SPH protein expression in <i>P. pastoris</i> .	251

Chapter 8: Discussion

Figure 1. A model for MYB-regulated secreted proteins in pollen tube reception compared to fungal infection.	293
---	-----

Chapter 1: Introduction

Pollen tubes: an adaptive sperm delivery device

Cell-cell and extracellular environmental signals regulate differentiation, morphology, and development of multicellular organisms. Cellular differentiation begins with perception of signaling molecules at the cell membrane transduced through the cytoplasm to the nucleus to induce transcriptional changes (Bishopp et al. 2006). The pollen grain provides an exceptional model to study cell-cell interactions, for it is a single cell that grows through the pistil, a cellular environment comprised of many cell types (Sessions et al. 1995; Johnson et al. 2004). The pollen tube must interact with cells throughout the pistil and respond correctly in order to find and fertilize the female gametes (Johnson et al. 2004; Okuda et al. 2009). Despite this rich network of cell-cell interactions we know surprisingly little about the signal transduction cascades and their transcriptional response in either pollen tube or pistil cells. In fact, early work suggested that pollen contained sufficient protein and mRNA stores to make transcriptional changes negligible (Mascarenhas 1966), yet recent studies have demonstrated dynamic regulation of the pollen tube transcriptome (Qin et al. 2009; Wei et al. 2010). Understanding the mechanisms that control reproduction in flowering plants is of utmost importance for human agriculture, as successful plant reproduction produces seed crops. Seed crop creation and improvement may well be a key to human survival in the coming decades of agricultural productivity

under global economic instability (Levin et al. 2013), climate variability (Enete 2010), and loss of species diversity (Rusch et al. 2010; Mendenhall et al. 2014).

Gametophytes control the success of plant reproduction

Male gametophyte development

Flowering plants develop through two phases of their life cycle, the diploid sporophyte phase and the haploid gametophyte stage. Male and female gametophyte development initiates when sporophytic cells go through meiosis to create haploid spores, which then divide to form the gametophyte (Borg et al. 2009). Male gametophytes develop within a specialized sporophytic structure called the anther (McCormick 2004) and female gametophytes develop in sporophyte structures known as ovules, buried inside the larger tissue of the pistil (Christensen et al. 1997; Yadegari et al. 2004) (Figure 1). After meiosis, male microspores are released from a tetrad by digestion of thick encasing walls of callose. Microspores then undergo an asymmetric division to produce a large vegetative cell that quickly envelops the smaller generative cell (Borg et al. 2009) (Figure 1). This generative cell divides again to produce two sperm cells which are physically linked to the vegetative nucleus (McCue et al. 2011; Zhou et al. 2014), which together comprise the male germ unit (Borg et al. 2009).

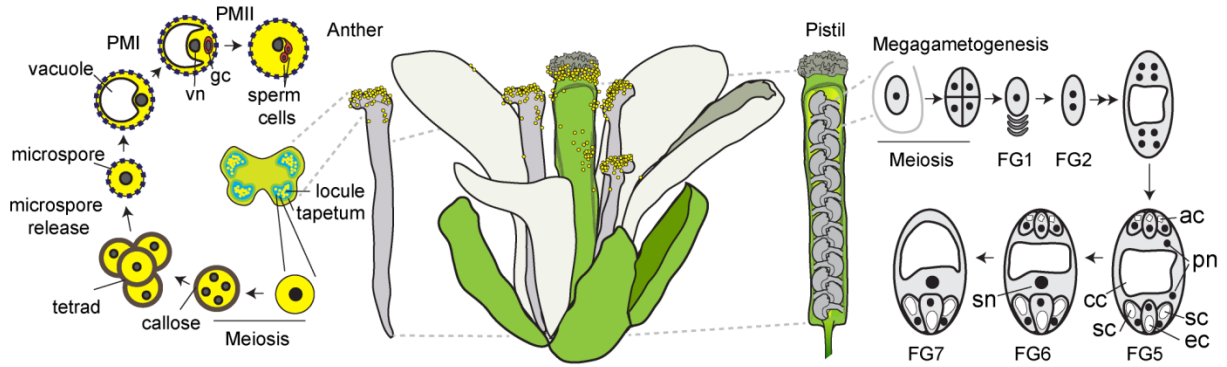


Figure 1. Gametophyte development in *Arabidopsis*.

The *Arabidopsis* flower is framed by male (left) and female (right) gametophyte development. Male gametophyte development is adapted from (Twell et al. 2006). vn – vegetative nucleus, gc – generative cell, PMI – pollen mitosis I, PMII – pollen mitosis II. Female gametophyte development is adapted from (Yadegari et al. 2004). ac, antipodal cells; cc, central cell; ec, egg cell; fm, functional megaspore; m, megaspore; pn, polar nuclei; sc, synergid cell; sn, secondary nucleus.

Female gametophyte development

Arabidopsis female gametophyte development follows a well characterized developmental plan (reviewed in Cucinotta et al. 2014) and results in a polar arrangement of four cell types: synergid, egg, central cell, and antipodals (Figure 1). At the entrance of the ovule, known as the micropyle, two genetically identical synergid cells form a cell layer that separates the egg cell from the micropylar opening. The two synergid cells produce a specialized cell wall at the micropyle known as the filiform apparatus (Drews et al. 2002). The synergid cells produce rich folds of plasma membrane at the filiform apparatus, providing an extensive deposition site for the ovule to secrete signals to the extracellular tissue (Punwani et al. 2007). Above the egg is the large central cell, a diploid cell formed from the fusion of the two polar nuclei. At the most chalazal end of the

female gametophyte are three antipodal cells, which persist through fertilization and early development (Song et al. 2014).

Pollen tube growth and guidance in the pistil

When released from the anther, mature pollen grains are deposited on the stigma of the pistil. Here, long filamentous papilla cells create the first cell:cell contact the pollen grain makes with the pistil tissue. Mature pollen grains are desiccated and metabolically inactive, yet once they contact the stigma they rapidly undergo hydration, and germinate a pollen tube that penetrates the stigma cell wall before entering the extracellular matrix of the style and transmitting tract (Elleman et al. 1992; Johnson et al. 2002). The pollen tube extends by polar deposition of vesicles bearing new cell wall material along an actin-myosin cytoskeleton (Cardenas et al. 2008; Cheung et al. 2008). The apex of the pollen tube forms new cell-cell contacts with pistil tissues as it navigates towards the ovules following a gradient of long and short range attractants (Reviewed in Higashiyama 2010). The male germ unit migrates sub-apically through cytoskeletal attachments (Figure 2, Zhou et al. 2014). Pollen tubes exit the transmitting tract in response to LURE proteins secreted from the synergid cells (Takeuchi et al. 2012), grow up the funiculus, and into the female gametophyte.

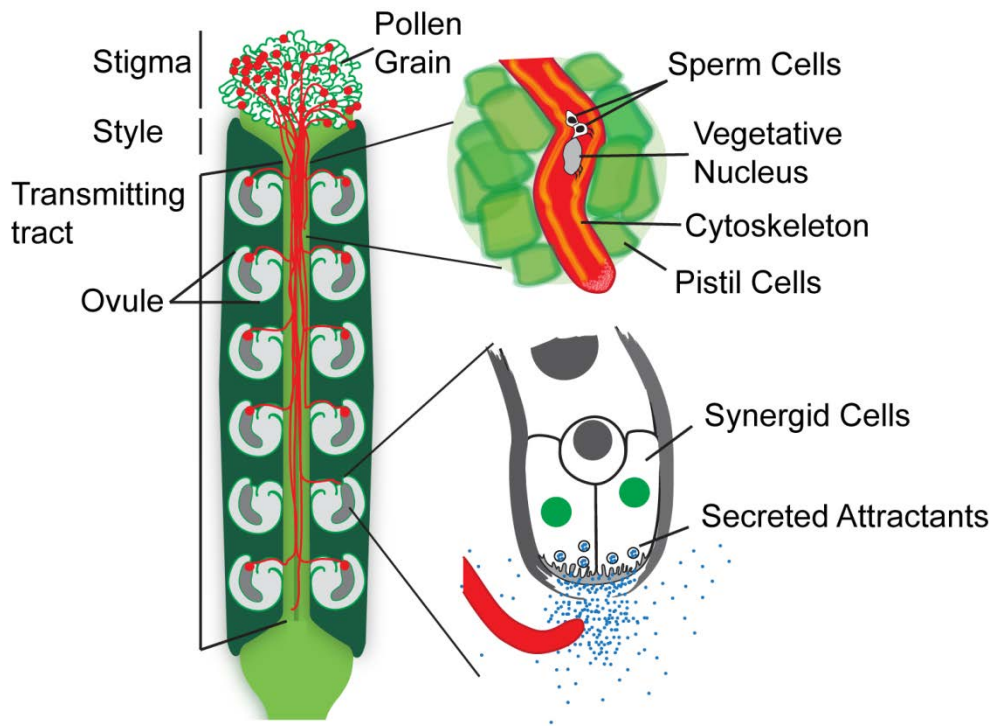


Figure 2. Pollen tube growth in the *Arabidopsis* pistil.

The pollen grain and tube (red circle, line) is depicted during its journey through the pistil en route to the ovules. The upper inset depicts the relative orientation of the vegetative nucleus and sperm cells as they are carried on the cytoskeleton (yellow). The lower inset depicts the secretion of small secreted attractants (such as LURE proteins) by the synergid cells (green nuclei) that diffuse along the funiculus (not depicted) and reorient the growth of the pollen tube towards the micropyle.

Pollen tube reception in the ovule

Pollen tube reception is used to describe the process of pollen tube arrival and arrest within the ovule, synergid interaction, and subsequent pollen tube burst. The pollen tube tip contacts the synergid cells where it arrests its growth, prompting a dialogue illuminated by a flurry of dynamic calcium signaling (Ngo et al. 2014). Several signaling components are synergid-expressed, membrane-associated proteins such as FERONIA (Escobar-Restrepo et al. 2007), LORELEI

(Capron et al. 2008; Tsukamoto et al. 2010), and NORTIA (Kessler et al. 2010; Kessler et al. 2011). Pending successful communication between the pollen tube and the synergid, one synergid (termed the 'receptive synergid') undergoes a programmed cell death (Sandaklie-Nikolova et al. 2007). This is followed by rapid extension of the pollen tube tip and pollen tube burst, the cytoplasm of which is projected into the receptive synergid (Ngo et al. 2014). The degeneration of the synergid and pollen tube creates a mixture of synergid and pollen tube cytoplasm into which the male germ unit is projected. The relative position of the vegetative nucleus and sperm flips during projection, with the vegetative nucleus flung behind the two sperm cells, which rapidly attach to their fusion partners: the egg and central cell (Hamamura et al. 2011). Gamete fusion is induced by secreted egg cell cysteine rich proteins (EC1) interacting with sperm cells to relocalize the sperm protein (HAP2) to the sperm surface (Wong et al. 2010; Sprunck et al. 2012). Successful fertilization of both the egg and central cell is required for seed development, and the non-receptive synergid persists to monitor fertilization state and attract subsequent pollen tubes in event of a defective pollen tube or sperm donor (Beale et al. 2012; Kasahara et al. 2012). The interaction of the pollen tube with the ovule is the focus of chapters two and three, where known molecular effectors and recent advances in imaging the reception event are described respectively.

What is the role of transcription in the pollen tube?

Early investigation into the functions of the pollen tube examined the morphology of the vegetative nuclei and sperm cells nuclei using microscopy, and highlighted the cytological disparity between these nuclei. While the sperm cells are compact and easily visible after DNA staining, the vegetative nucleus is large and de-condensed (Figure 3A). This led early researchers to conclude that “the vegetative nucleus seems to begin disintegration even before the pollen tube is formed and apparently plays little part in further gametophytic development” (Sax 1933). However, later studies clearly showed that the vegetative nucleus was biochemically active: RNA synthesis occurs during pollen tube germination, and was clearly inhibited by the transcriptional inhibitor actinomycin-D (Mascarenhas 1966). In these experiments pollen was still able to germinate a pollen tube even in the absence of transcription, suggesting that ample long-lived RNA stores are present in the mature pollen grain (Mascarenhas 1966).

Experimentation on pollen transcription benefitted from the ease of in vitro pollen germination and growth, and yet, this advantage also posed a limitation because pollen never made contact with its natural environment, the pistil. Therefore, the role of transcription in the pollen tube was relegated to replenishment of transcripts pre-existing in the pollen grain that are required for pollen tube growth (i.e. Actin, Figure 3B), as opposed to pollen tube differentiation. Several experiments pointed to a more complex model of pollen tube function. First was the discovery that pollen tubes behaved differently during in vitro growth as compared to growth in the pistil. Pollen tubes grown in vitro grew more slowly

than pollen tubes in vivo (Taylor et al. 1997), and could not perceive or locate ovules (Higashiyama et al. 1998; Palanivelu et al. 2006) (Figure 3C). Second, advances in microarray technology demonstrated differential gene expression landscapes in pollen grains, pollen tubes, and pollen tubes that were grown through pistil explants (Wang et al. 2008; Qin et al. 2009). In fact, the transcriptome of pollen tubes responds dramatically to growth in the pistil (Figure 3D), which begged the question: What have we missed by studying pollen tubes growing in vitro?

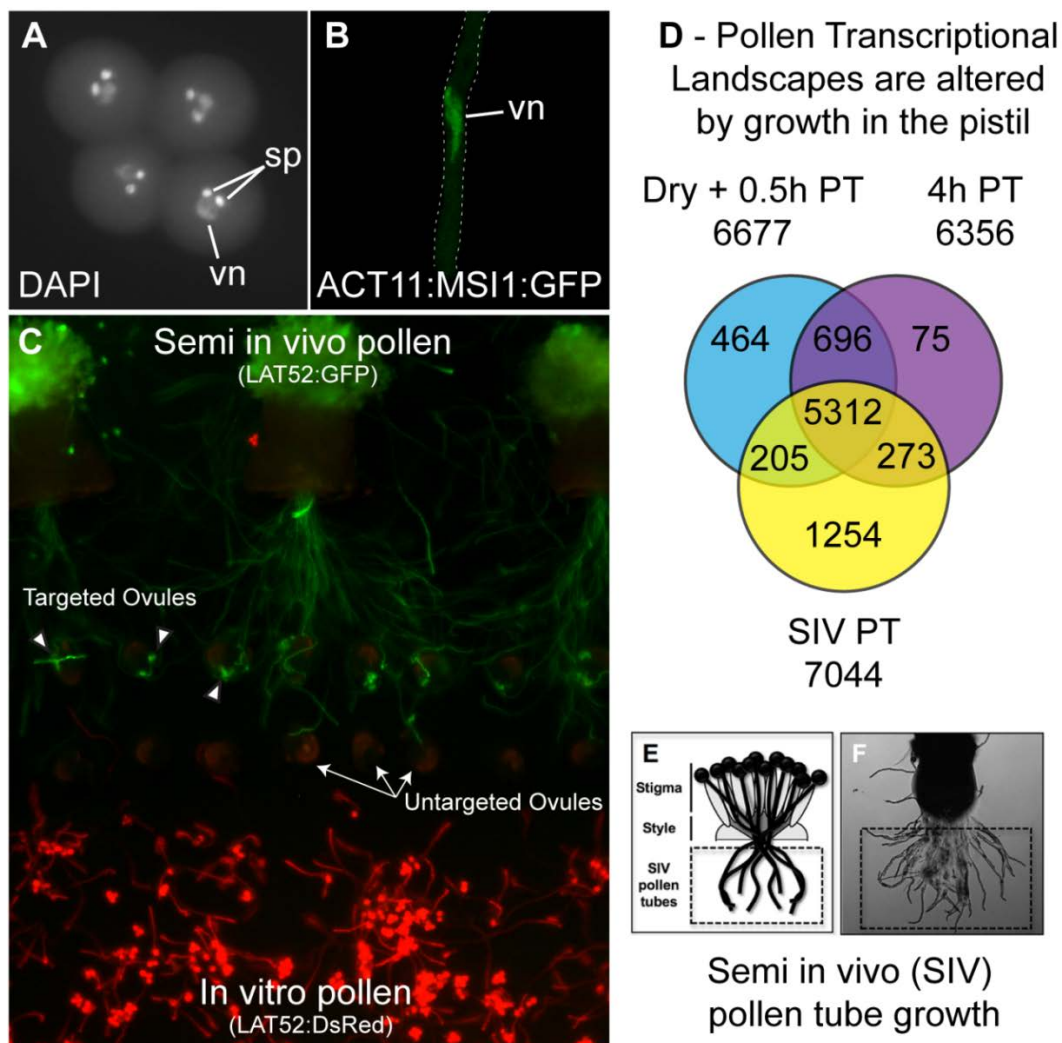


Figure 3. Pollen tube characteristics are altered by interaction with female tissues. **A.** DAPI DNA staining of the pollen nuclei in a *quartet* of pollen grains (Preuss et al. 1994). vn – vegetative nucleus, sp – sperm cells. **B.** In vitro grown pollen tube expressing nuclear localized GFP under the *ACTIN11* promoter (confocal 3d stack). vn – vegetative nucleus, dotted line indicates the cell wall of the pollen tube. **C.** Example of pollen tube targeting excised ovules (center row, faint red autofluorescence). Pollen carrying a *LAT52:GFP* T-DNA were crossed onto a pistil explant and allowed to grow onto solid media (SIV). Pollen carrying a *LAT52:DsRed* T-DNA were placed directly onto solid media. SIV pollen tubes target ovules, while in vitro pollen tubes do not. **D.** Genes that had an expression value >100 in Dry pollen + 0.5h in vitro PT, 4h in vitro PT, and SIV grown PT are compared, and the numbers of shared or unique genes are shown in Venn diagram form. **E.** Diagram of semi in vivo pollen tube growth. **F.** DIC image of semi in vivo pollen tubes (**D-F** are adapted from Qin et al. 2009).

What is the pollen tube transcriptional network?

In preliminary gene ontology analyses of the pollen tube transcriptomes, several gene categories were shown to respond dynamically during growth in the pistil. Families of transmembrane transporters, antiporters and symporters along with cell wall modifying enzymes are enriched in pollen tube transcriptomes and their expression fluctuates during growth (Qin et al. 2009). Considering the invasive process of pollen tube growth through the pistil, transport of extracellular components and alteration the cell wall is mechanistically straightforward to explain (Sanati Nezhad et al. 2013). However, the transcriptional network that controls the dynamic expression of these gene families was only a hypothetical supposition; 237 transcription factors are expressed in pollen, and approximately 20 semi-in-vivo specific pollen tube genes are predicted to be transcription factors. Building a transcriptional network of pollen gene expression therefore requires the identification of components in both the pistil and the pollen tube.

Chapter four presents our work in identifying a pollen tube expressed family of MYB transcription factors that control expression of genes required for pollen tube reception, demonstrating a functional role for gene transcription in the vegetative nucleus during pollen tube growth in vivo. In chapter five I present a SNP-informed RNA deep sequencing approach to discovering pollen and pistil-specific transcriptional landscapes from inter-ecotype pollinations. However, both of these chapters bring to light the expansive genetic redundancy of genes expressed in the pollen tube. Taking the MYB-regulated transcripts as an example subset of pollen tube genes, we observe families of carbohydrate modifying enzymes (10 pectate lyases, 4 pectin methylesterase inhibitors, 4 α -glycosylhydrolases), transmembrane transporters (8 major facilitator type carbohydrate transporters [Thesis Appendix 1], 9 CHX Na^+/H^+ antiporters), and a diverse group of small secreted proteins (30 Thionin-like CRPs [Thesis Chapter 6], 8 rapid alkalization factors [RALF], 12 self-incompatibility protein homologs [SPH, Thesis Chapter 7], 8 GDSL-like lipase/acylhydrolase family proteins). Coordinated expression of gene families may account for the dearth of information of pollen-expressed factors with defined roles in pollen tube reception, because single gene knockouts of multigene families would display no mutant phenotype due to genetic redundancy. We hypothesized that MYB-regulated gene expression is required for proper pollen tube differentiation, regulating the identity of the pollen tube cell, secreted protein profile, and ability to respond to the extracellular environment. We explore this hypothesis in Chapter 2, where we examined the growth and function of pollen tubes from

varying genetic distances within the *Arabidopsis thaliana* pistil. Pollen from inter-ecotype crosses are capable of normal interaction with ovules from the *Arabidopsis thaliana* reference strain, while pollen from related species of *Brassica* frequently display reception defects similar to *myb* triple mutant pollen tubes. This effect was also observed previously using *Arabidopsis lyrata* pollen (Escobar-Restrepo et al. 2007), and in interspecific crosses with *Rhododendron* (Williams et al. 1986). Taken with the relatively normal growth of the *myb* triple mutant pollen tubes within the pistil and the similarity to loss of reception in interspecific crosses, the MYB-regulated transcriptome appears to promote pollen tube identity for species-specific interaction with the ovule. There is strong evidence for rapid evolution of many components of animal fertilization that generate species-specific interactions between the sperm and the egg such as the sea urchin ZP glycoproteins (Rankin et al. 2003) reviewed in (Clark et al. 2006). In plant reproduction species-specific interactions are functionally assumed by the gametophytes: the pollen tube and the ovule. Therefore we looked critically at mechanisms regulating recognition of gametophyte identity such as self-incompatibility and universal incompatibility.

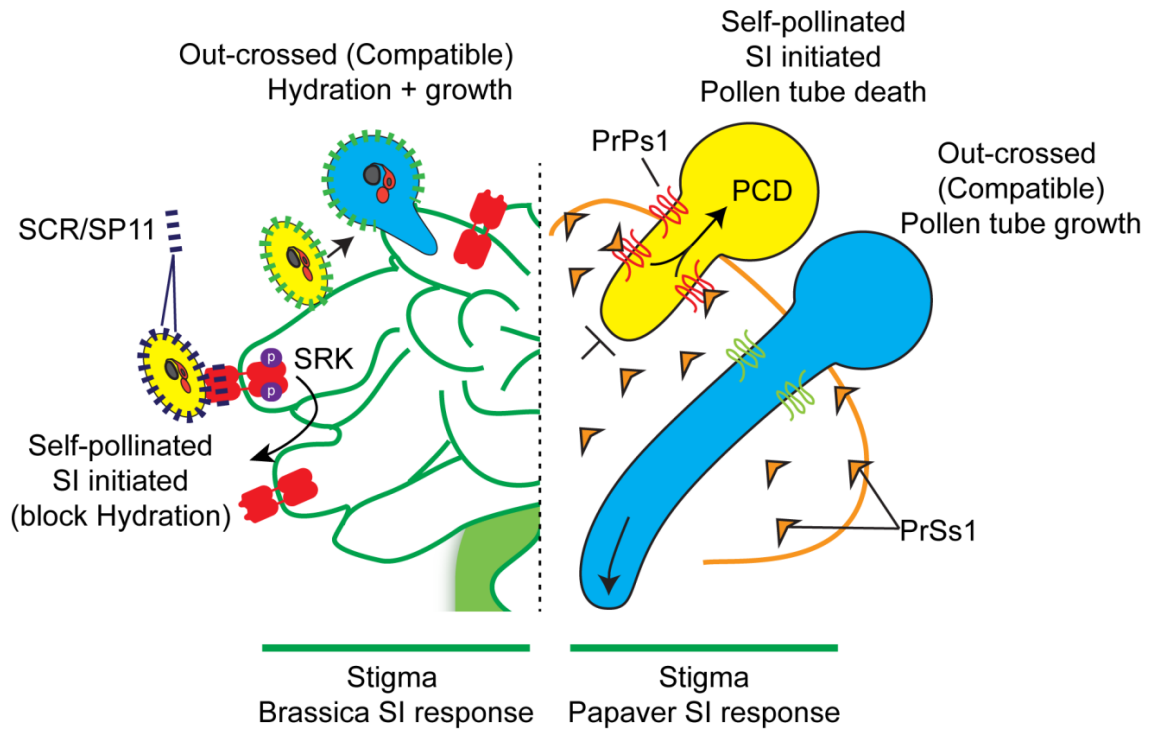


Figure 4. Mechanisms of SI involving small secreted ligands.

Two modes of Self-incompatibility are shown. On the left is the Brassica-type SI involving a pistil expressed receptor kinase (SRK – Red Protein) that binds the pollen coat SCR protein (SP11 – Dashes). Binding of pollen carrying the SP11 from self-pollen induces a signal transduction cascade through SRK phosphorylation blocking pollen hydration. On the right is the mode of *Papaver rhoeas* Self-incompatibility. Here, the small secreted SI ligand (PrSs1) is produced from the pistil, and is recognized by a transmembrane receptor on the pollen tube (PrPs1), which causes programmed cell death in self-pollen tubes.

Self-Recognition and Species-Recognition in pollination

Self-incompatibility (SI) is a system to block self-reproduction and promote outcrossing, a system employed by roughly half of documented Angiosperms (Raduski et al. 2012). The three well defined mechanisms of angiosperm self-incompatibility evolved independently, and are each controlled by a single polymorphic locus, the S-locus (Takayama et al. 2005; Wheeler et al. 2009). In

each defined mechanism, one SI effector is secreted and diffuses to cells from the opposing sex.

Species in the *Brassicaceae* family express a small secreted cysteine rich protein (S-locus Cysteine-Rich (SCR)/SP11) from the tapetum of the anther, which is secreted into the locule and incorporated into the pollen grain coat (Schopfer et al. 1999). This pollen coat SCR is recognized by the female effector of self-incompatibility, a stigma expressed transmembrane receptor (SRK) that binds the SCR protein and transduces the signal to block pollen hydration and growth of self-pollen (Stein et al. 1991; Schopfer et al. 1999; Takayama et al. 2001) (Figure 4). The small secreted SCR/SP11 is a defensin-like protein stabilized by four disulfide bonds into an alpha-beta sandwich structure (Mishima et al. 2003; Chookajorn et al. 2004).

In the *Papaveraceae* family, the small secreted S-protein (*Papaver rhoeas* stigma S-protein 1, PrSs1) is produced in the stigma. PrSs proteins are stabilized by two cysteine bonds, and may be glycosylated (Foote et al. 1994). The male determinant was recently described, and was found to be a polymorphic pollen-specific transmembrane protein, without significant sequence homology to proteins of known function (PrPs) (Wheeler et al. 2009). Interestingly, the *Arabidopsis thaliana* genome contains a large family (92 members) of *Papaver rhoeas* PrSs protein homologues (Ride et al. 1999; Zhou et al. 2013). These diverse homologs are not confined to a single locus and perform as yet unknown

functions. However, recent research will be presented in Chapter 6 that suggests at least one subclade of these *Arabidopsis* S-protein homologs (SPH) has a role in promoting pollen tube reception at the ovule.

The remaining mechanism of self-incompatibility is utilized by the Solanaceae, Rosaceae, and Scrophulariaceae, where the female effector is an S-RNase expressed in the style. The pollen tubes of both self- and non-self-pollen take up this S-RNase, which is recognized by an S-locus specific F-box protein which targets the S-RNase for protein degradation (McClure et al. 1989; Lai et al. 2002). In an as yet uncharacterized mechanism, only self-pollen selectively block the degradation of the S-RNase, which degrades RNA thereby inhibiting pollen tube growth. This mechanism does not utilize a small signaling molecule, and is thought to require high concentrations of the S-RNase to be particularly effective in blocking self-rejection (Qin et al. 2006).

Species-specific pollen-pistil interactions are less well mechanistically characterized than self-incompatibility. Inter-species hybridization can be blocked either by pollen-pistil interactions (prezygotic) or by developmental incompatibility of hybridized genomes (postzygotic). Prezygotic barriers have been observed to affect different stages of pollen tube growth in the pistil. In *Trifolium*, self-incompatibility is gametophytically controlled by multiple 's' alleles at a single locus (Atwood 1942), which fascinatingly is broken only by a rare and dominant self-compatible 'Sf' allele (Yamada et al. 1989). Interspecies *Trifolium* crosses cause pollen tubes to swell, coil and burst in the style (Chen et al. 1972).

Heterospecific pollinations using Louisiana Irises exhibited normal growth at the top of the style that became less competitive in the lower portion of the style (Emms et al. 1996). Other studies have identified a role for the self-incompatibility machinery in inter-specific compatibility. In general, between a self-fertile and self-incompatible plant, inter-specific compatibility is only possible in one direction; only the self-compatible pistil is competent to produce hybrid seeds (Pandey 1981). This unilateral interspecific incompatibility in flowering plants has been observed in two of the SI systems: Brassica -SCR/SRK (Hiscock et al. 1993), and Tobacco – S-RNase/F-BOX (Hancock et al. 2003). Interestingly, in *Solanum* there are examples of strains without a functional S-RNase where unilateral interspecific incompatibility still occurs, suggesting an alternative mechanism (Bedinger et al. 2011). The mechanism by which cells in the pistil escape S-RNase activity is undetermined, however it is suggested to occur either by specific exclusion from endocytic re-uptake into pistil cells, or a pistil-specific mechanism of inactivating intracellular S-RNase. Finally, inter-species pollen rejection at pollen tube reception in the ovule is observed for *Rhododendron* (Williams et al. 1986), *A. thaliana* x *A. lyrata* or x *C. flexuosa* (Escobar-Restrepo et al. 2007), and *A. thaliana* x *A. korshinskyi* or *Olimarabidopsis pumila* (Thesis Chapter 2, Leydon et al. 2014). Together, these studies indicate multiple steps at which pistils and female gametophytes may control interspecific pollination.

Peptide signaling: small secreted proteins involved in pollen-pistil interactions

Families of secreted signaling peptides have been identified that play roles in development of plant tissues, such as at the shoot apical meristem where secreted CLAVATA3/ESR peptides control the size of the meristematic domain (Clark et al. 1995). Additionally, an endosperm secreted CRP family (ESF1) controls early embryo patterning of the apical-basal axis (Costa et al. 2014). Increasingly, small secreted CRPs appear to control reproductive processes as well. In addition to the small proteins utilized in SI responses discussed previously, tomato pollen tube germination is positively regulated by LAT52 (CRP – 4 Cysteines, Muschietti et al. 1994; Tang et al. 2002) and LeSTIG1 (CRP – 16 Cysteines, Goldman et al. 1994). Pollen tube growth in the Lily pistil is promoted by the lipid transfer protein SCA (CRP – 8 Cysteines, Jauh et al. 1997), which promotes adhesion of pistil proteins to the pollen tube surface (Kim et al. 2003). Pollen tube guidance to the micropyle of the ovule depends on the secreted protein LURE attractants (CRP – 6 Cysteines) in *Torenia* (Okuda et al. 2009) and *Arabidopsis* (Takeuchi et al. 2012), and ZmEA1 (CRP – 2 Cysteines) in *Maize* (Marton et al. 2005). One group of CRPs (ZmES1-4) has been implicated in the molecular communication between the pollen tube and ovule during reception. ZmES4 is secreted from the synergid and via species-preferential interaction with the pollen tube expressed potassium channel KZM1 induces pollen tube burst (Amien et al. 2010).

While the remaining cast of extracellular effectors in pollen tube reception remains unknown, several observations suggest putative identities. First, the MYB98 transcription factor controls synergid development and positively regulates a large number of secreted CRPs (at least 48 CRPs, Punwani et al. 2007; Punwani et al. 2008), and *myb98* mutants fail to attract pollen tubes (Kasahara et al. 2005). While pollen tube attraction likely involves AtLURE proteins, the roles of synergid-expressed CRPs must be tested to uncover functions in pollen tube reception. Second, calcium imaging of pollen and synergid during pollen tube reception reveals a rich dialogue between these two cells likely to involve secreted signals from both partners (Iwano et al. 2012; Ngo et al. 2014).

Analysis of pollen tube expressed genes identified enriched expression of many families of secreted proteins (Qin et al. 2009). Families of transmembrane transporters, carbohydrate active proteins, and small secreted proteins are positively regulated by MYB transcription factors in pollen tubes growing in vivo, as described in Chapter 4. Deep sequencing of the pollen and pistil transcriptomes demonstrated the breadth and redundancy of MYB gene regulation: an entire CRP2460 family of genes is positively regulated by MYB97, MYB101, and MYB120. We delve into the pollen tube and pistil transcriptional landscape in Chapter 5, highlighting the evolutionary divergence of reproductively expressed genes. Because CRPs have potential signaling functions, we investigated the functional role of the Thionin-like CRP2460 genes

in pollen tube reception in Chapter 6; where we demonstrate a role for this CRP family in promoting synergid degeneration, using both genetic and biochemical approaches. We extended our research to another small MYB-regulated secreted protein family, the *Arabidopsis* PrSs1 protein homologues (SPH). We describe in Chapter 7 the first known role for SPH proteins in *Arabidopsis*, where expression of one MYB-regulated SPH is sufficient to rescue *myb* triple-mutant pollen tube reception. Pollen tube reception acts as the final opportunity for the female to guarantee that the correct species of sperm are delivered to the ovule, and here we present the MYB-regulated secreted proteome of the pollen tube as a molecular pattern that creates a species-specific identity. This identity acts as a reproductive-associated molecular pattern, or RAMP, which is perceived by the synergid cells of the female gametophyte in a final species recognition barrier to reproduction.

References

- Amien, S., I. Klier, et al. (2010). "Defensin-Like ZmES4 Mediates Pollen Tube Burst in Maize via Opening of the Potassium Channel KZM1." Plos Biology **8**(6).
- Atwood, S. S. (1942). "Oppositional alleles causing cross-incompatibility in *trifolium repens*." Genetics **27**(3): 0333-0338.
- Beale, K. M., A. R. Leydon, et al. (2012). "Gamete Fusion Is Required to Block Multiple Pollen Tubes from Entering an Arabidopsis Ovule." Curr Biol.
- Bedinger, P. A., R. T. Chetelat, et al. (2011). "Interspecific reproductive barriers in the tomato clade: opportunities to decipher mechanisms of reproductive isolation." Sexual Plant Reproduction **24**(3): 171-187.
- Bishopp, A., A. P. Mahonen, et al. (2006). "Signs of change: hormone receptors that regulate plant development." Development **133**(10): 1857-1869.
- Borg, M., L. Brownfield, et al. (2009). "Male gametophyte development: a molecular perspective." Journal of Experimental Botany **60**(5): 1465-1478.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Cardenas, L., A. Lavy-Wheeler, et al. (2008). "Pollen tube growth oscillations and intracellular calcium levels are reversibly modulated by actin polymerization." Plant Physiol **146**(4): 1611-1621.
- Chen, C. C. and P. B. Gibson (1972). "Barriers to Hybridization of *Trifolium Repens* with Related Species." Canadian Journal of Genetics and Cytology **14**(2): 381-+.
- Cheung, A. Y. and H. M. Wu (2008). "Structural and signaling networks for the polar cell growth machinery in pollen tubes." Annu Rev Plant Biol **59**: 547-572.
- Chookajorn, T., A. Kachroo, et al. (2004). "Specificity determinants and diversification of the Brassica self-incompatibility pollen ligand." Proc Natl Acad Sci U S A **101**(4): 911-917.
- Christensen, C. A., E. J. King, et al. (1997). "Megagametogenesis in Arabidopsis wild type and the Gf mutant." Sexual Plant Reproduction **10**(1): 49-64.
- Clark, N. L., J. E. Aagaard, et al. (2006). "Evolution of reproductive proteins from animals and plants." Reproduction **131**(1): 11-22.
- Clark, S. E., M. P. Running, et al. (1995). "Clavata3 Is a Specific Regulator of Shoot and Floral Meristem Development Affecting the Same Processes as Clavata1." Development **121**(7): 2057-2067.
- Costa, L. M., E. Marshall, et al. (2014). "Central cell-derived peptides regulate early embryo patterning in flowering plants." Science **344**(6180): 168-172.
- Cucinotta, M., L. Colombo, et al. (2014). "Ovule development, a new model for lateral organ formation." Front Plant Sci **5**: 117.
- Drews, G. N. and R. Yadegari (2002). "Development and function of the angiosperm female gametophyte." Annual Review of Genetics **36**: 99-124.
- Elleman, C. J., V. Franklinton, et al. (1992). "Pollination in Species with Dry Stigmas - the Nature of the Early Stigmatic Response and the Pathway Taken by Pollen Tubes." New Phytologist **121**(3): 413-424.

- Emms, S. K., S. A. Hodges, et al. (1996). "Pollen-tube competition, siring success, and consistent asymmetric hybridization in Louisiana irises." Evolution **50**(6): 2201-2206.
- Enete, A. A. A., Taofeeq A. (2010). "Challenges of Agricultural Adaptation to Climate Change in Nigeria: a Synthesis from the Literature." Field Actions Science Reports **4**.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Foot, H. C., J. P. Ride, et al. (1994). "Cloning and expression of a distinctive class of self-incompatibility (S) gene from *Papaver rhoeas* L." Proc Natl Acad Sci U S A **91**(6): 2265-2269.
- Goldman, M. H. S., R. B. Goldberg, et al. (1994). "Female Sterile Tobacco Plants Are Produced by Stigma-Specific Cell Ablation." Embo Journal **13**(13): 2976-2984.
- Hamamura, Y., C. Saito, et al. (2011). "Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in *Arabidopsis thaliana*." Curr Biol **21**(6): 497-502.
- Hancock, C. N., K. Kondo, et al. (2003). "The S-locus and unilateral incompatibility." Philosophical Transactions of the Royal Society of London Series B-Biological Sciences **358**(1434): 1133-1140.
- Higashiyama, T. (2010). "Peptide signaling in pollen-pistil interactions." Plant Cell Physiol **51**(2): 177-189.
- Higashiyama, T., H. Kuroiwa, et al. (1998). "Guidance in vitro of the pollen tube to the naked embryo sac of *torenia fournieri*." Plant Cell **10**(12): 2019-2032.
- Hiscock, S. J. and H. G. Dickinson (1993). "Unilateral Incompatibility within the Brassicaceae - Further Evidence for the Involvement of the Self-Incompatibility (S)-Locus." Theoretical and Applied Genetics **86**(6): 744-753.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Jauh, G. Y., K. J. Eckard, et al. (1997). "Adhesion of lily pollen tubes on an artificial matrix." Sexual Plant Reproduction **10**(3): 173-180.
- Johnson, M. A. and D. Preuss (2002). "Plotting a course: Multiple signals guide pollen tubes to their targets." Developmental Cell **2**(3): 273-281.
- Johnson, M. A., K. von Besser, et al. (2004). "*Arabidopsis* *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Kasahara, R. D., D. Maruyama, et al. (2012). "Fertilization recovery after defective sperm cell release in *Arabidopsis*." Curr Biol **22**(12): 1084-1089.
- Kasahara, R. D., M. F. Portereiko, et al. (2005). "MYB98 is required for pollen tube guidance and synergid cell differentiation in *Arabidopsis*." Plant Cell **17**(11): 2981-2992.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.

- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Kim, S., J. C. Mollet, et al. (2003). "Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism." Proc Natl Acad Sci U S A **100**(26): 16125-16130.
- Lai, Z., W. Ma, et al. (2002). "An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum." Plant Mol Biol **50**(1): 29-42.
- Levin, S., T. Xepapadeas, et al. (2013). "Social-ecological systems as complex adaptive systems: modeling and policy implications." Environment and Development Economics **18**(2): 111-132.
- Leydon, A. R., A. Chaibang, et al. (2014). "Interactions between pollen tube and pistil control pollen tube identity and sperm release in the *Arabidopsis* female gametophyte." Biochem Soc Trans **42**: 340-345.
- Marton, M. L., S. Cordts, et al. (2005). "Micropylar pollen tube guidance by egg apparatus 1 of maize." Science **307**(5709): 573-576.
- Mascarenhas, J. P. (1966). "Pollen Tube Growth and Ribonucleic Acid Synthesis by Vegetative and Generative Nuclei of *Tradescantia*." Am J Bot **53**(6p1): 563-&.
- McClure, B. A., V. Haring, et al. (1989). "Style self-incompatibility gene products of *Nicotiana glauca* are ribonucleases." Nature **342**(6252): 955-957.
- McCormick, S. (2004). "Control of male gametophyte development." Plant Cell **16**: S142-S153.
- McCue, A. D., M. Cresti, et al. (2011). "Cytoplasmic connection of sperm cells to the pollen vegetative cell nucleus: potential roles of the male germ unit revisited." Journal of Experimental Botany **62**(5): 1621-1631.
- Mendenhall, C. D., D. S. Karp, et al. (2014). "Predicting biodiversity change and averting collapse in agricultural landscapes." Nature **509**(7499): 213-+.
- Mishima, M., S. Takayama, et al. (2003). "Structure of the male determinant factor for *Brassica* self-incompatibility." Journal of Biological Chemistry **278**(38): 36389-36395.
- Muschiatti, J., L. Dircks, et al. (1994). "LAT52 protein is essential for tomato pollen development: pollen expressing antisense LAT52 RNA hydrates and germinates abnormally and cannot achieve fertilization." Plant J **6**(3): 321-338.
- Ngo, Q. A., H. Vogler, et al. (2014). "A calcium dialog mediated by the FERONIA signal transduction pathway controls plant sperm delivery." Developmental Cell **29**(4): 491-500.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Pandey, K. K. (1981). "Evolution of Unilateral Incompatibility in Flowering Plants - Further Evidence in Favor of Twin Specificities Controlling Intraspecific and Interspecific Incompatibility." New Phytologist **89**(4): 705-728.

- Preuss, D., S. Y. Rhee, et al. (1994). "Tetrad analysis possible in Arabidopsis with mutation of the QUARTET (QRT) genes." Science **264**(5164): 1458-1460.
- Punwani, J. A., D. S. Rabiger, et al. (2007). "MYB98 positively regulates a battery of synergid-expressed genes encoding filiform apparatus-localized proteins." Plant Cell **19**(8): 2557-2568.
- Punwani, J. A., D. S. Rabiger, et al. (2007). "MYB98 positively regulates a battery of synergid-expressed genes encoding filiform apparatus localized proteins." Plant Cell **19**(8): 2557-2568.
- Punwani, J. A., D. S. Rabiger, et al. (2008). "The MYB98 subcircuit of the synergid gene regulatory network includes genes directly and indirectly regulated by MYB98." Plant Journal **55**(3): 406-414.
- Qin, X., B. Liu, et al. (2006). "Style-by-style analysis of two sporadic self-compatible *Solanum chacoense* lines supports a primary role for S-RNases in determining pollen rejection thresholds." Journal of Experimental Botany **57**(9): 2001-2013.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Raduski, A. R., E. B. Haney, et al. (2012). "The Expression of Self-Incompatibility in Angiosperms Is Bimodal." Evolution **66**(4): 1275-1283.
- Rankin, T. L., J. S. Coleman, et al. (2003). "Fertility and taxon-specific sperm binding persist after replacement of mouse sperm receptors with human homologs." Developmental Cell **5**(1): 33-43.
- Ride, J. P., E. M. Davies, et al. (1999). "Analysis of Arabidopsis genome sequence reveals a large new gene family in plants." Plant Mol Biol **39**(5): 927-932.
- Rusch, A., M. Valantin-Morison, et al. (2010). "Biological Control of Insect Pests in Agroecosystems: Effects of Crop Management, Farming Systems, and Seminatural Habitats at the Landscape Scale: A Review." Advances in Agronomy, Vol 109 **109**: 219-259.
- Sanati Nezhad, A. and A. Geitmann (2013). "The cellular mechanics of an invasive lifestyle." Journal of Experimental Botany **64**(15): 4709-4728.
- Sandaklie-Nikolova, L., R. Palanivelu, et al. (2007). "Synergid cell death in Arabidopsis is triggered following direct interaction with the pollen tube." Plant Physiol **144**(4): 1753-1762.
- Sax, K. E., H. W. (1933). "Development of the male gametophyte in *Tradescantia*." Botanical Gazette **95**(1): 156-163.
- Schopfer, C. R., M. E. Nasrallah, et al. (1999). "The male determinant of self-incompatibility in Brassica." Science **286**(5445): 1697-1700.
- Schopfer, C. R., M. E. Nasrallah, et al. (1999). "The male determinant of self-incompatibility in Brassica." Science **286**(5445): 1697-1700.
- Sessions, R. A. and P. C. Zambryski (1995). "Arabidopsis gynoecium structure in the wild and in etlin mutants." Development **121**(5): 1519-1532.

- Song, X. Y., L. Yuan, et al. (2014). "Antipodal cells persist through fertilization in the female gametophyte of Arabidopsis." Plant Reproduction **27**(4): 197-203.
- Sprunck, S., S. Rademacher, et al. (2012). "Egg cell-secreted EC1 triggers sperm cell activation during double fertilization." Science **338**(6110): 1093-1097.
- Stein, J. C., B. Howlett, et al. (1991). "Molecular-Cloning of a Putative Receptor Protein-Kinase Gene Encoded at the Self-Incompatibility Locus of Brassica-Oleracea." Proc Natl Acad Sci U S A **88**(19): 8816-8820.
- Takayama, S. and A. Isogai (2005). "Self-incompatibility in plants." Annu Rev Plant Biol **56**: 467-489.
- Takayama, S., H. Shimosato, et al. (2001). "Direct ligand-receptor complex interaction controls Brassica self-incompatibility." Nature **413**(6855): 534-538.
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis." PLoS Biol **10**(12): e1001449.
- Takeuchi, H. and T. Higashiyama (2012). "A Species-Specific Cluster of Defensin-Like Genes Encodes Diffusible Pollen Tube Attractants in Arabidopsis." Plos Biology **10**(12).
- Tang, W., I. Ezcurra, et al. (2002). "A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2." Plant Cell **14**(9): 2277-2287.
- Taylor, L. P. and P. K. Hepler (1997). "Pollen Germination and Tube Growth." Annu Rev Plant Physiol Plant Mol Biol **48**: 461-491.
- Tsukamoto, T., Y. Qin, et al. (2010). "A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development." Plant J **62**(4): 571-588.
- Twell, D., S.-A. Oh, et al. (2006). Pollen Development, a Genetic and Transcriptomic View. The Pollen Tube. R. Malhó, Springer Berlin Heidelberg. **3**: 15-45.
- Wang, Y., W. Z. Zhang, et al. (2008). "Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in Arabidopsis." Plant Physiol **148**(3): 1201-1211.
- Wei, L. Q., W. Y. Xu, et al. (2010). "Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in Oryza sativa." BMC Genomics **11**(1): 338.
- Wheeler, M. J., B. H. de Graaf, et al. (2009). "Identification of the pollen self-incompatibility determinant in Papaver rhoeas." Nature **459**(7249): 992-995.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of Rhododendron Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.

- Yadegari, R. and G. N. Drews (2004). "Female gametophyte development." Plant Cell **16 Suppl**: S133-141.
- Yamada, T., H. Fukuoka, et al. (1989). "Recurrent Selection Programs for White Clover (*Trifolium-Repens* L) Using Self-Compatible Plants .1. Selection of Self-Compatible Plants and Inheritance of a Self-Compatibility Factor." Euphytica **44**(1-2): 167-172.
- Zhou, P., K. A. T. Silverstein, et al. (2013). "Detecting small plant peptides using SPADA (Small Peptide Alignment Discovery Application)." Bmc Bioinformatics **14**.
- Zhou, X. and I. Meier (2014). "Efficient plant male fertility depends on vegetative nuclear movement mediated by two families of plant outer nuclear membrane proteins." Proc Natl Acad Sci U S A **111**(32): 11900-11905.

Chapter 2:
**Interactions between pollen tube and pistil control pollen tube identity and
sperm-release in the female gametophyte**

Alexander R. Leydon*, Adisorn Chaibang*[†], and Mark A. Johnson*¹

* Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, U.S.A. [†]Current Address: Energy Biosciences Institute, University of California Berkeley, California 94720, USA

This chapter was published as a mini-review in Biochemical Society Transactions (2014) 42, (340–345).

Abstract

Flowering plants have immotile sperm that develop within the pollen cytoplasm and are delivered to female gametes by a pollen tube, a highly polarized extension of the pollen cell. In many flowering plant species, including seed crop plants, hundreds of pollen tubes grow toward a limited number of ovules. This system should ensure maximal fertilization of ovules and seed production, however we know very little about how signaling between the critical cells is integrated to orchestrate delivery of two functional sperm to each ovule. Recent studies suggest that the pollen tube changes its gene expression program in response to growth through pistil tissue and that this differentiation process is critical for pollen tube attraction by the female gametophyte and for release of sperm. Interestingly, these two signaling systems, called pollen tube guidance and pollen tube reception are also species preferential. This review focuses on *Arabidopsis* pollen tube differentiation within the pistil and addresses the idea that pollen tube differentiation defines pollen tube identity and recognition by female cells. We review recent identification of genes that may control pollen tube:female gametophyte recognition and discuss how these may be involved in blocking interspecific hybridization.

Introduction

The pollinated pistil is a system that integrates hundreds of individual cell:cell interactions to achieve maximal seed production. Consider the journey of a single pollen tube: it interacts with several female cell types as it germinates on the stigma, enters the transmitting tissue of the style, turns out onto the ovary surface, grows up a funiculus, enters an ovule micropyle, contacts a synergid cell, and bursts to release its cargo of two sperm (Johnson et al. 2002; Dresselhaus et al. 2013) (Figure 1, Figure 2A). The two sperm cells then interact with the degenerated synergid cell before fusing with the female gametes (the egg and central cell) to produce an embryo and endosperm within a developing seed (Hamamura et al. 2011).

The complexity of the pollen-pistil system becomes apparent when you consider a model like the *Arabidopsis thaliana* (At) that contains 50-60 ovules and hundreds of pollen tubes (Figure 1). It is likely that each of the cell:cell interactions listed above (and others) is mediated by multiple pollen tube and female cell proteins and other molecules (e.g. carbohydrates). It is also becoming clear that these signaling systems are not discrete or linear, but feedback on each other to optimize reproductive success. For example, it was recently shown that the fusion of sperm cells with female gametes prevents attraction of more pollen tubes to an already fertilized ovule (Beale et al. 2012; Kasahara et al. 2012; Maruyama et al. 2013). Integration of gamete fusion and pollen tube

attraction systems ensures that only a single pair of sperm cells is delivered to the female gametophyte and that gamete fusion occurs successfully before an ovule becomes incapable of attracting another pollen tube. These findings suggest the interesting possibility that steps in the flowering plant reproductive process that were thought to be distinct from each other are integrated in ways that significantly enhance reproductive success.

Pollen tube reception is the cell:cell signaling system that results in cessation of pollen tube growth in the female gametophyte, synergid degeneration, and pollen tube rupture/sperm release. Substantial progress has been made in *At* toward identifying female components of this signaling system (reviewed in Kessler et al. 2011), however, relatively little is known about how the female gametophyte senses pollen tube arrival or about how the pollen tube prepares for rupture and sperm release. We review recent studies that suggest the pistil induces a specific pollen tube gene expression program that is critical for pollen tube reception. We discuss this as an example of how distinct cell:cell interaction systems are integrated to maximize reproductive success and how differentiation of the pollen tube within the pistil may be essential for species-preferential recognition of the pollen tube by the female gametophyte.

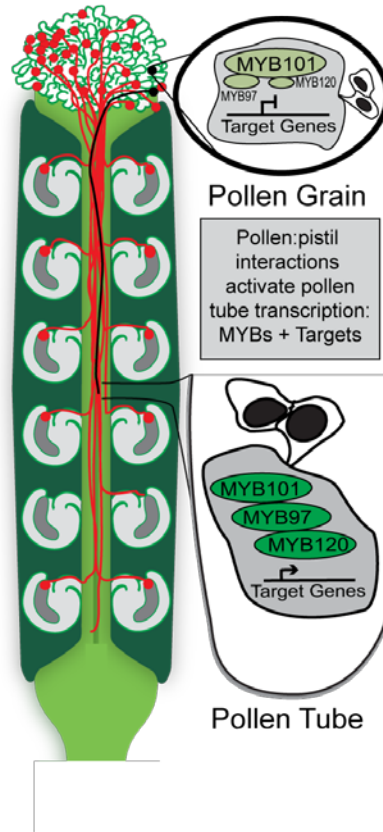


Figure 1. Pollen tube S18 MYB transcription factors respond to growth through the pistil A schematic of an *Arabidopsis thaliana* ovary with 12 ovules (instead of actual 50-60). Pollen grains (red) are germinating pollen tubes on the stigma. Pollen tubes target the female gametophyte (gray). Mature pollen grains (represented in black with two sperm and a nucleus (gray) express MYB101, and lower levels of both MYB97 and MYB120. Transcription of MYB targets is low or absent in pollen grains. Growth through the stigma and style activate transcription of MYB97 and MYB120; the three MYBS activate target genes in the pollen tube. The pollen tube factors that sense the pistil environment and activate MYB expression are not yet known.

Pollen tubes differentiate in response to growth through the pistil

Pollen tube physiology changes when it grows through floral tissue. For example, pollen tubes must grow through pistil explants to be able to respond to attractants in vitro (Higashiyama et al. 1998; Palanivelu et al. 2006; Palanivelu et al. 2010). These experiments use a semi-in vitro (SIV) pollen tube guidance assay in which pollen tubes grow through the stigma and a portion of style before growing onto

the surface of pollen growth media toward ovules (Palanivelu et al. 2006). In *Torenia*, where LURE pollen tube attractants were first described (Okuda et al. 2009), the ability to respond to attractants in vitro increases with prolonged growth through pistil tissue (Okuda et al. 2013). However, when LURE binding sites were assessed by incubating pollen tubes with LUREs followed by detection with anti-LURE antibodies, it was observed that, while growth the pistil was required to generate LURE binding sites, prolonged exposure to the pistil did not increase detection of binding sites further (Okuda et al. 2013). These data suggest that the pollen tube senses the pistil environment, that LURE receptor (not yet identified) expression increases as a consequence, and that prolonged exposure to the pistil environment may activate the receptor.

Interestingly, a pair of *At* pollen tube expressed membrane-associated receptor-like cytoplasmic kinases (LIP1 and LIP2) were recently shown to be induced by growth in the pistil and required for the full response to purified LURE proteins in the SIV assay and for pollen tube guidance in the pistil (Liu et al. 2013). It is not yet clear whether LIP1 and LIP are directly involved in LURE perception, however, these findings underscore the idea that pollen tubes differentiate during growth in the pistil resulting in expression of proteins required to respond to pollen tube guidance cues.

Pollen tube differentiation for sperm release is controlled by three MYBS

Over 1000 genes were detected in the transcriptome of pollen tubes grown in the SIV assay that were not detected in pollen tubes grown in vitro (Qin et al. 2009). To identify regulators that control pollen tube gene expression in response to the pistil, 26 SIV-induced transcription factors were identified (Qin et al. 2009). This list included three closely related R2R3-MYB type transcription factors (Subgroup18 (S18): *MYB97*, *MYB101* and *MYB120*) (Qin et al. 2009); single and double *myb* mutants did not display seed set defects, however triple mutants had a ~70% reduction in seed production (Leydon et al. 2013). *myb* triple mutant pollen grains and tubes develop normally, grow through the pistil, and target ovules nearly as efficiently as wild type. These data suggest that *MYB97*, *MYB101* and *MYB120* do not regulate expression of the LURE receptors. However, upon interaction with the female gametophyte, *myb* triple mutants fail to arrest their growth and ~72% of ovules contain coils of pollen tubes within the female gametophyte (Leydon et al. 2013). Synergid cells targeted by these mutant pollen tubes fail to degenerate normally suggesting a loss of communication between pollen tube and synergid cells during pollen tube reception (Leydon et al. 2013). Furthermore, *myb* triple-mutant pollen tubes fail to burst and release sperm. These data suggest that *MYB97*, *MYB101* and *MYB120* are critical for the pollen tube to exchange signals with the female gametophyte required for successful fertilization.

Pollen tube reception signaling by the female gametophyte

Pollen tube reception requires a number of synergid-expressed genes including *FERONIA* (*FER*) (Escobar-Restrepo et al. 2007), *NORTIA* (*NTA*) (Kessler et al. 2010) and *LORELEI* (*LRE*) (Capron et al. 2008) (Figure 2A). Loss-of-function mutations in each of these genes cause the same phenotype: wild type pollen tubes coil in mutant ovules and fail to release sperm. *FER* is a transmembrane receptor-like kinase of the CrRLK1L family predicted to have pollen tube or female-gametophyte ligand(s) important for pollen tube reception. *FER* has a malectin-like extracellular domain, so its ligand could be a carbohydrate or glycoprotein (Lindner et al. 2012) (Fig 2A). *FER* is localized to the filiform apparatus, and is required for *NTA*, a 7-pass transmembrane Mildew Resistance Locus O family protein, to be re-localized from the secretory system to the filiform apparatus upon pollen tube arrival (Kessler et al. 2010) (Figure 2A-2B). *NTA* may interact with synergid- or pollen tube-expressed proteins upon relocalization and also contains a calmodulin-binding domain in its cytoplasmic C-terminus, possibly allowing synergid cell perception of calcium oscillations during reception (Iwano et al. 2012) (Fig 2B-2D). *LRE* encodes a glycosylphosphatidylinositol-anchored protein predicted to be associated with the synergid membrane (Capron et al. 2008) (Figure 2A). The mechanisms by which *FER*, *NTA*, and *LRE* direct pollen tube reception are unknown and it will be important to define how they sense pollen tube arrival to initiate synergid degeneration and pollen tube burst.

Relatively little is known about the pollen tube-expressed genes involved in pollen tube reception and no direct connections between pollen tube and synergid genes have been made. Interestingly, a pair of pollen tube-expressed members of the *FER* family members [CrRLK receptor-like kinases, *ANXUR1* (*ANX1*) and *ANXUR2* (*ANX2*)] may be negative regulators of pollen tube burst. *anx1*, *anx2* double mutants produce pollen tubes that burst very soon after germination (Boisson-Dernier et al. 2009; Miyazaki et al. 2009). Like *FER*, the ligands for *ANX1* and *2* are not yet known and the nature of *ANX* contribution to pollen tube reception is unclear because the double mutant phenotype is not informative about their role during synergid interactions.

Auto-inhibited Calcium ATPase9 (*ACA9*) encodes a pollen-specific calmodulin-binding calcium pump localized to the pollen tube plasma membrane (Schiott et al. 2004). *aca9* pollen tubes have growth defects, however, they can reach ovules in the upper portion of the pistil and ~50% of these enter the ovule micropyle and arrest, but fail to burst (Schiott et al. 2004). This result suggests that *ACA9* is important for regulating pollen tube calcium dynamics required for pollen tube burst. Recent live-imaging experiments have shown that calcium concentrations spike in the pollen tube and the synergids as pollen tube burst occurs (Iwano et al. 2012) (Figure 2B-D). It will be very interesting to determine how *aca9* pollen and *nta* (which contains a predicted calmodulin-binding domain) synergid mutants affect these dynamics.

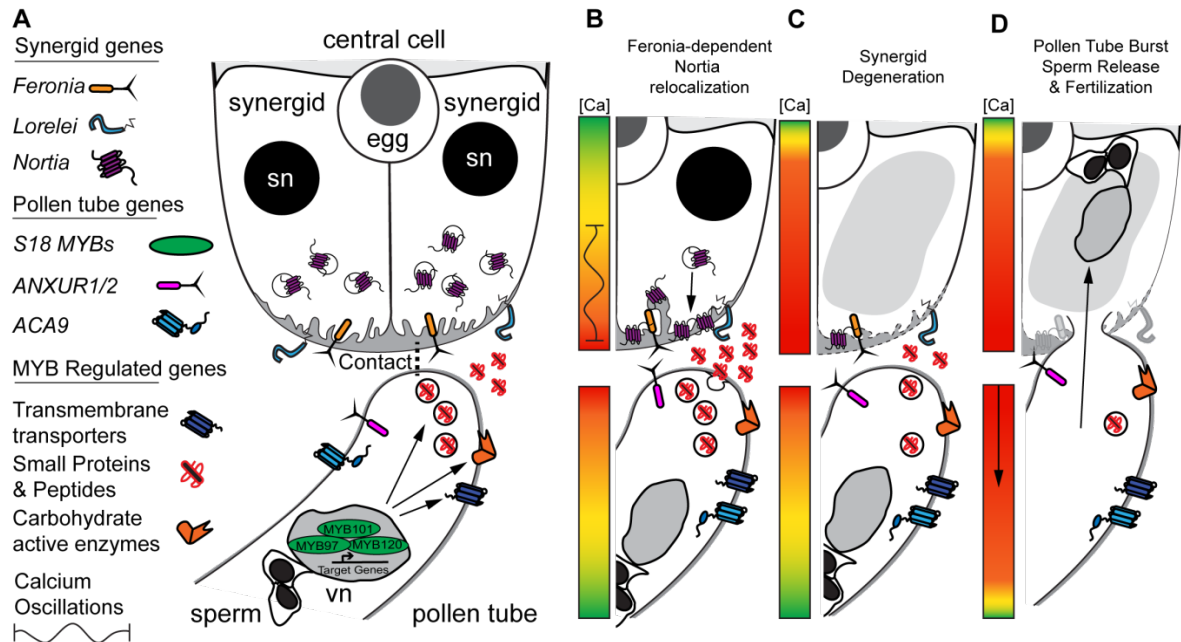


Figure 2. S18 MYB regulated gene classes and their putative roles during pollen tube receptions

(A) Schematic of the pollen tube and female gametophyte at the beginning of pollen tube reception. Pollen tube contact with one of the synergid cells is indicated (dotted line). Proteins with roles in pollen tube reception and MYB-regulated gene products are highlighted. Abbreviations: sn - synergid nucleus, vn - vegetative nucleus. (B-C) Schematic shows pollen tube reception between a pollen tube and the synergid that will degenerate. Calcium concentrations are symbolized with a color spectrum (red is high calcium, green is low calcium). (B) FER dependent-NTA relocalization occurs after pollen tube arrival. (C) Synergid degeneration is accompanied by loss of synergid nuclear integrity. (D) Pollen tube burst into the synergid (arrow), Sperm release and double fertilization.

Do MYB target genes interact with the pollen tube reception signaling components?

myb97, *myb101*, *myb120* triple-mutant pollen tubes fail to stop growing and burst within the female gametophyte, so the genes regulated by these transcription factors are candidates for pollen tube components of the pollen tube reception mechanism. By comparing the transcriptomes of pistils pollinated with either wild-

type or *myb* triple-mutant pollen, three main categories of MYB-regulated genes were identified: transporters, small proteins and peptides, and carbohydrate active enzymes (Leydon et al. 2013) (Figure 2A). Many of these genes were found to be in large gene families, potentially explaining why extensive genetic screening in *At* has not identified male pollen tube reception mutants.

Several sugar/proton symporters in the major facilitator family (Reinders et al. 2012) were identified as MYB regulated. *AtSUC7*, *AtSUC8*, and *AtSUC9* are highly induced in wild-type pollen tubes grown in SIV conditions, but are absent in *myb* triple-mutants (Leydon et al. 2013). *myb* triple-mutants have no pollen tube growth defect in vitro or in the pistil, suggesting these sucrose transporters are not required to import sugars to fuel growth, but may have a specialized function in regulating the pollen tube osmotic state during pollen tube reception.

Multiple small proteins and peptides were found to be potential targets of MYB activation during pollen tube growth (Figure 2A). One of these is a thionin, an 87 amino acid, secreted, cysteine-rich protein (CRP2460; Silverstein, 2007). Thionins have been shown to nucleate membrane pores in artificial lipid bilayers and rat neuronal cells (Hughes et al. 2000); this is an intriguing potential function for pollen tube-expressed thionins given that *myb* triple-mutant pollen tubes fail to express these peptides and also have defects in pollen tube burst and initiating synergid degeneration (Leydon et al. 2013). Interestingly, small (130-152 aa) secreted proteins with domains homologous to stigmatic *Papaver rhoeas* self-

incompatibility (SI) protein PrsS1 (Foote et al. 1994) were also found to require pollen tube MYBs for expression in the pollen tube (Leydon et al. 2013). In *Papaver* PrsS1 is secreted from the stigma and is bound by PrpS, its pollen-expressed receptor (Wheeler et al. 2009). Ligand:receptor interaction blocks germination of self pollen through a mechanism involving calcium as a second messenger and programmed cell death (PCD) (Poulter et al. 2010). The function of *At* S-protein homologues (SPHs) is unclear (Ride et al. 1999) and it is surprising to find that they are expressed in pollen tubes in response to growth through the pistil. It will be interesting to test whether a signaling module similar to that described in *Papaver* is involved in gametophyte interactions that lead to synergid degeneration and pollen tube burst.

The third category of pollen tube MYB-regulated genes encode proteins that interact with carbohydrates, many of which are many are predicted to be extracellular (Figure 2A). These include hydrolases (i.e. Glycoamylase, O-glycosylhydrolase, β -1,3-glucanase, Pectin lyase-like), and pectin methylesterase inhibitors (Leydon et al. 2013). These proteins may be expressed by the pollen tube to modify the cell wall in preparation for pollen tube reception. Alternatively, these proteins may modify the cell wall or other pollen-derived carbohydrates for recognition by the female gametophyte. As mentioned above, FER contains an extracellular lectin domain that could interact with a pollen tube-derived carbohydrate and since *myb* triple mutant pollen tubes behave as if FER

signaling is defective, it will be interesting to determine whether pollen tube MYBs regulate production of FER ligands.

MYB-mediated pollen tube differentiation is important for species recognition during pollen tube reception.

Pollen tube reception fails in interspecific crosses of *Rhododendron* (Williams et al. 1986) or *At* (Escobar-Restrepo et al. 2007); pollen tubes of one species overgrow without releasing sperm in ovules of the exotic species. These data suggest that pollen tube reception is an important pre-zygotic barrier to interspecific hybridization. When *At myb* triple-mutant pollen tubes enter a wild-type *At* ovule, they overgrow and fail to release sperm (Leydon et al. 2013). These observations lead to the hypothesis that pollen tube MYBs may control expression of the determinants of pollen tube identity during interspecific recognition.

To begin to assess the role of pollen tube MYBs in determining pollen tube recognition, pollen tube reception was investigated in inter-accession and interspecies crosses (Figure 3). The rates of pollen tube overgrowth when *myb* triple and *myb* double mutants were used to pollinate *At* pistils were higher than in any of the inter-accession crosses tested (Figure 3M). *Arabidopsis korshinskyi* (*Ak*) and *Olimarabidopsis pumila* (*Op*) pollen tubes overgrew in *At* ovules more frequently than any of the accessions tested, but the *At myb* triple-mutant phenotype was even more pronounced (Figure 3M). These results are consistent

with the idea that pollen tube MYBs control pollen tube identity as recognized by the female gametophyte.

When *Ak* or *Op* were used as females in crosses with *At* pollen, increased rates of pollen tube overgrowth were not observed because the percentage of untargeted ovules (likely due to incongruity in pollen tube attraction) was so high that pollen tube reception could not be adequately assessed (Figure 3I,J,M). However, crosses of *At myb* triple mutant pollen onto *Ak* or *Op* pistils showed increases in rates of pollen tube coiling as well as increases in the number of untargeted ovules (Figure 3K-L, M). This finding suggests that MYBs are required for two levels of pollen tube identity factors: 1) core pollen tube identity recognized by exotic female gametophytes and 2) species-preferential identity recognized by the native female gametophyte.

Future work will need to address the mechanisms by which individual MYB target genes confer these identities. One possibility is that MYB-mediated pollen tube differentiation fails when pollen tubes grow through an exotic pistil. Alternatively, MYBs may be properly activated by the exotic pistil and may in turn activate their targets, but the products of these target genes are not recognized by the exotic female gametophyte. In either case, exploration of how the pistil induces MYB97, MYB101, and MYB120 and how the products of their target genes mediate gametophyte interactions provides a path to understand how suitable mates are identified during flowering plant reproduction.

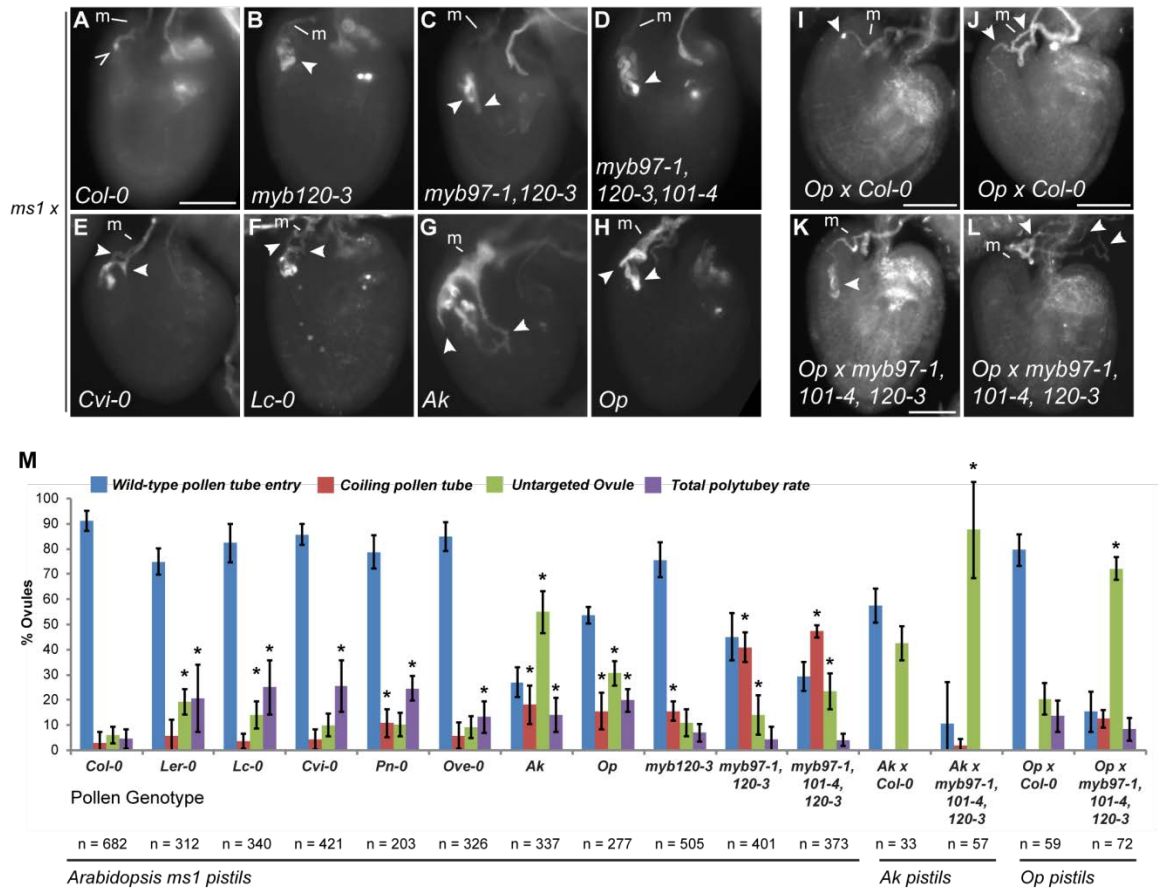


Figure 3. Pollen tube reception is defective in inter-accession and inter-species crosses.

(A-H) *Arabidopsis thaliana* (*At*) *male sterile 1* (*ms1*) pistils from the *Landsberg* accession were pollinated with various pollen donors for 24 hours before imaging pollen tube growth patterns using aniline blue staining and fluorescence microscopy (as in Mori et al. 2006). m - micropyle, arrowheads indicate pollen tubes. (A) *At Col-0* accession, normal entry. (B) *At myb120-3*, coiling. (C) *At myb97-1,120-3*, coiling and attraction of two pollen tubes. (D) *At myb97-1,101-4,120-3*, coiling. (E) *At Cvi-0* accession, two pollen tubes. (F) *At Lc-0* accession, coiling, two pollen tubes. (G) *Ak*, coiling, two pollen tubes. (H) *Op*, coiling, two pollen tubes. (I-L) Emasculated *Ak* pistils were crossed with *At Col-0* (I-J) or *myb* triple mutant (K-L). *At Col-0* tubes show (I) wild-type pollen tube entry, and (J) wild-type entry & one tube failing to enter the micropyle; the majority of ovules were untargeted in interspecific crosses (M). *myb* triple mutants show (K) pollen tube coiling and (L) failure to enter the micropyle (3 tubes) in interspecific crosses. (M) Quantification of pollen tube reception. *Arabidopsis* accessions: *Col-0* (CS1092), *Ler-0* (CS20), *Lc-0* (CS28443), *Cvi-0* (CS902), *Pn-0* (CS28645), *Ove-0* (CS28590). Related species: *Arabidopsis korshinskyi* (*Ak*) (CS4653) or *Olimarabidopsis pumila* (*Op*) (CS3700). Asterisks denote statistically significant differences from controls by a students t-test, p-value <0.05.

References

- Beale, K. M., A. R. Leydon, et al. (2012). "Gamete Fusion Is Required to Block Multiple Pollen Tubes from Entering an Arabidopsis Ovule." Curr Biol.
- Boisson-Dernier, A., S. Roy, et al. (2009). "Disruption of the pollen-expressed FERONIA homologs ANXUR1 and ANXUR2 triggers pollen tube discharge." Development **136**(19): 3279-3288.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Dresselhaus, T. and N. Franklin-Tong (2013). "Male-Female Crosstalk during Pollen Germination, Tube Growth and Guidance, and Double Fertilization." Mol Plant **6**(4): 1018-1036.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Foot, H. C., J. P. Ride, et al. (1994). "Cloning and expression of a distinctive class of self-incompatibility (S) gene from *Papaver rhoeas* L." Proc Natl Acad Sci U S A **91**(6): 2265-2269.
- Hamamura, Y., C. Saito, et al. (2011). "Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in Arabidopsis thaliana." Curr Biol **21**(6): 497-502.
- Higashiyama, T., H. Kuroiwa, et al. (1998). "Guidance in vitro of the pollen tube to the naked embryo sac of *Torenia fournieri*." Plant Cell **10**(12): 2019-2032.
- Hughes, P., E. Dennis, et al. (2000). "The cytotoxic plant protein, beta-purothionin, forms ion channels in lipid membranes." J Biol Chem **275**(2): 823-827.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Johnson, M. A. and D. Preuss (2002). "Plotting a course: multiple signals guide pollen tubes to their targets." Dev Cell **2**(3): 273-281.
- Kasahara, R. D., D. Maruyama, et al. (2012). "Fertilization recovery after defective sperm cell release in Arabidopsis." Curr Biol **22**(12): 1084-1089.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.
- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Lindner, H., L. M. Muller, et al. (2012). "CrRLK1L receptor-like kinases: not just another brick in the wall." Curr Opin Plant Biol **15**(6): 659-669.

- Liu, J., S. Zhong, et al. (2013). "Membrane-bound RLCKs LIP1 and LIP2 are essential male factors controlling male-female attraction in Arabidopsis." Curr Biol **23**(11): 993-998.
- Maruyama, D., Y. Hamamura, et al. (2013). "Independent control by each female gamete prevents the attraction of multiple pollen tubes." Dev Cell **25**(3): 317-323.
- Miyazaki, S., T. Murata, et al. (2009). "ANXUR1 and 2, sister genes to FERONIA/SIRENE, are male factors for coordinated fertilization." Curr Biol **19**(15): 1327-1331.
- Mori, T., H. Kuroiwa, et al. (2006). "GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization." Nat Cell Biol **8**(1): 64-71.
- Okuda, S., T. Suzuki, et al. (2013). "Acquisition of LURE-Binding Activity at the Pollen Tube Tip of *Torenia fournieri*." Mol Plant **6**(4): 1074-1090.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Palanivelu, R. and M. A. Johnson (2010). "Functional genomics of pollen tube-pistil interactions in Arabidopsis." Biochem Soc Trans **38**(2): 593-597.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Poulter, N. S., M. J. Wheeler, et al. (2010). "Self-incompatibility in Papaver: identification of the pollen S-determinant PrpS." Biochem Soc Trans **38**(2): 588-592.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Reinders, A., A. B. Sivitz, et al. (2012). "Evolution of plant sucrose uptake transporters." Front Plant Sci **3**: 22.
- Ride, J. P., E. M. Davies, et al. (1999). "Analysis of Arabidopsis genome sequence reveals a large new gene family in plants." Plant Mol Biol **39**(5): 927-932.
- Schiott, M., S. M. Romanowsky, et al. (2004). "A plant plasma membrane Ca²⁺ pump is required for normal pollen tube growth and fertilization." Proc Natl Acad Sci U S A **101**(25): 9502-9507.
- Wheeler, M. J., B. H. de Graaf, et al. (2009). "Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*." Nature **459**(7249): 992-995.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of *Rhododendron* Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.

Chapter 3:

Pollen tube discharge completes the process of synergid degeneration that is initiated by pollen tube – synergid interaction in Arabidopsis

Alexander R. Leydon², Tatsuya Tsukamoto¹, Damayanthi Dunatunga¹, Yuan Qin³, Mark A. Johnson^{*2}, Ravishankar Palanivelu^{*1}

¹School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

²MCB Department, Brown University, Providence, RI 02912, USA

³Center for Genomics and Biotechnology, College of Crop Science, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian Province, China

^{*}Corresponding Authors

Alexander Leydon performed the experiments in Figures 2,3,5, and 6. Tatsuya Tsukamoto, Yuan Qin, and Damayanthi Dunatunga performed the experiments in Figures 1, 4, and the supplement.

Abstract

Flowering plant reproduction relies on precise integration of multiple signaling exchanges between the male gametophyte (the pollen tube), the female gametophyte, and the sporophyte. Pollen tube reception is the signaling system that results in pollen tube discharge, sperm release, synergid degeneration, and successful delivery of male gametes (two sperm cells) to the site where they can fuse with female gametes (egg cell, and central cell). Some of the molecules required for this complex and essential signaling exchange have been identified, however, there are fundamental questions about the nature of the interactions between the pollen tube and the synergid cells that need to be clarified. In this study, we monitor pollen tube arrival, pollen tube discharge, and synergid degeneration in wild type and in male and female mutants that disrupt gametophyte interactions. We combine assays used previously to study these interactions and a new assay that facilitates simultaneous analysis of pollen tube discharge and synergid degeneration. We find that pollen tube – synergid interaction is sufficient to promote frequent synergid degeneration even when the pollen tube fails to burst and discharge its contents of two sperm. However, we also note that when pollen tubes successfully burst, they always discharge into a degenerated synergid. Our data suggest the possibility that pollen tube – synergid contact, or signaling via secreted molecules, initiates synergid degeneration and pollen tube rupture completes this process.

Introduction

Flowering plant reproduction requires that development of the male and female gametophytes is coordinated so that gametes can be brought together to fuse and initiate seed development. A critical phase of this coordination occurs when the two gametophytes encounter each other to complete double fertilization: each gametophyte directly influences the development and function of the other. The male gametophyte develops in the anther and differentiates into a pollen cell with two sperm cells in its cytoplasm (Berger et al. 2011). The female gametophyte develops within the ovule and is more complex, comprising seven cells in *Arabidopsis* - six of which are haploid (two synergids, an egg, and three antipodals) and one homodiploid central cell (Drews et al. 2002). When a pollen grain lands on a receptive stigma it germinates a highly polar pollen tube that extends without cell division through stigma and stylar tissue before entering an ovule micropyle (Palanivelu et al. 2012). The first female gametophytic cell encountered by the pollen tube in the ovule is one of the two synergids and it is clear that synergids directly control pollen tube functions: they secrete attractant proteins that guide the pollen tube into the micropyle (Takeuchi et al. 2012) and induce pollen tube discharge (Huck et al. 2003; Kessler et al. 2011). After pollen tube arrival, one of the two synergid cells degenerates (called the receptive synergid) and receives the pollen tube contents to provide access for sperm to the female gametes, the egg and central cell (Christensen et al. 1997; Drews et al. 2002; Sandaklie-Nikolova et al. 2007; Hamamura et al. 2011; Hamamura et al. 2014).

Live imaging of Arabidopsis pollen tubes interacting with female gametophytes in a semi-in vitro system (Palanivelu et al. 2006) has increased the resolution of the timing of these events (Hamamura et al. 2012) and showed that cytoplasmic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyto}}$) is a key second messenger that can be used to track communication between the gametophytes (Iwano et al. 2012; Denninger et al. 2014; Hamamura et al. 2014; Ngo et al. 2014). These studies show that synergids sense pollen tube arrival and their response can be observed in the form of increases in $[\text{Ca}^{2+}]_{\text{cyto}}$. The interaction phase begins when the pollen tube enters the micropyle, appears prolonged ($\sim 30 \pm 15$ minutes), and involves a reduction in pollen tube growth rate. This phase is followed by an increase in pollen tube growth rate and a large increase in pollen tube $[\text{Ca}^{2+}]_{\text{cyto}}$ (Ngo et al. 2014); this dramatic increase in pollen tube $[\text{Ca}^{2+}]_{\text{cyto}}$ is likely followed shortly thereafter by pollen tube discharge/sperm release (Denninger et al. 2014). Interestingly, the apex of $[\text{Ca}^{2+}]_{\text{cyto}}$ in the receptive synergid may precede sperm release by 15 minutes (Denninger et al. 2014), during which time synergids may degenerate to accommodate pollen tube discharge. Although the mechanistic relationships between $[\text{Ca}^{2+}]_{\text{cyto}}$ oscillations, pollen tube rupture, and synergid degeneration remain unknown, these studies nevertheless provide a strong basis for future efforts to understand the molecular nature of the molecules that signal pollen tube arrival to the synergid and trigger the pollen tube to discharge its cargo.

Completion of double fertilization, fusion between one sperm with the egg and the other with the central cell, activates an ethylene-response cascade that results in degeneration of the remaining synergid (persistent synergid cell) (Volz et al. 2013). Degeneration of the persistent synergid cell removes a source of pollen tube attractants and prevents polytubey (additional pollen tubes accessing a fertilized ovule), thereby reducing the chances of polyspermy (Beale et al. 2012; Kasahara et al. 2012).

In *Arabidopsis*, a signaling system that coordinates sperm release, termed pollen tube reception, was first appreciated when a female gametophyte mutant (*feronia*) was identified that failed to induce the pollen tube to discharge its cargo of two sperm cells (Huck et al. 2003; Rotman et al. 2003). Subsequent studies identified *lre* and *nta* female gametophyte mutants with a similar loss-of-function phenotype (Capron et al. 2008; Kessler et al. 2010; Tsukamoto et al. 2010). It has been proposed that these genes, which encode a receptor-like kinase (FER, Escobar-Restrepo et al. 2007), a membrane anchored protein (LRE, Capron et al. 2008; Tsukamoto et al. 2010), and a 7-pass membrane protein in the MLO family (NTA, Kessler et al. 2010) are central to a signaling mechanism that senses pollen tube arrival and is required for pollen tube discharge. When pollen tubes enter ovules lacking this signaling system they continue to grow, forming a coil. A triple mutant lacking three *MYB* transcription factors (*MYB97*, *MYB101*, and *MYB120*) is the only pollen mutant described with the complementary loss of function phenotype (failure to stop growing and discharge sperm upon contact

with the female gametophyte) (Leydon et al. 2013; Liang et al. 2013). It has been proposed that *myb* triple-mutant pollen tubes fail to produce signaling molecules sensed by the female *FER*, *LRE*, *NTA* system; however, this has not yet been demonstrated. Many questions remain about pollen tube reception signaling and how the final acts of pollen tube and synergid cell development, their synchronized destruction, are coordinated to ensure that sperm will be released into a female gametophyte prepared to complete double fertilization.

Analysis of pollen tube reception in wild-type ovules targeted by *myb* triple-mutant pollen tubes revealed a complex relationship between pollen tube arrival, pollen tube discharge, and synergid degeneration (Leydon et al. 2013). 10% of ovules were targeted by *myb* triple-mutant pollen tubes that entered the micropyle and burst like wild type. These ovules all had at least one degenerated synergid suggesting that the receptive synergid had always degenerated and that in many cases the persistent synergid had degenerated as well. However, in 90% of ovules targeted by *myb* triple-mutant pollen tubes, the pollen tube failed to discharge, and formed a coil within the female gametophyte. 58% of ovules containing a coiling *myb* triple-mutant pollen tube had at least one degenerated synergid. These data suggested that synergid degeneration could be initiated without pollen tube discharge. Conversely, the remaining 42% of ovules containing coiling *myb* triple-mutant pollen tubes had two intact synergids. Targeted ovules with intact synergids could indicate that this subset of *myb* triple-mutant pollen tubes is deficient in a signaling process that normally promotes

synergid degeneration. Taken together, these findings suggest that a combination of signaling and physical interactions can result in synergid degeneration without pollen tube discharge, but that when pollen tube discharge is successful, the receptive synergid always degenerates.

Here we explore the spatio-temporal relationship between pollen tube discharge and synergid degeneration using male (*aca9*, Schiott et al. 2004) and female (*gfa2*, Christensen et al. 2002; *lre*, Capron et al. 2008; Tsukamoto et al. 2010) gametophyte mutants that disrupt pollen tube synergid interactions. We find 1) that pollen tube arrival without PT discharge triggers synergid degeneration above levels observed in unpollinated pistils. 2) Pollen tube overgrowth is associated with higher levels of synergid degeneration compared to arrested or unpollinated ovules, but does not cause synergid degeneration at levels found in ovules with successful pollen tube discharge. 3) Pollen tube discharge is always associated with synergid degeneration. Our findings are consistent with the model that pollen tube arrival and interaction with the receptive synergid cell initiates synergid degeneration through signaling via direct contact between pollen tube and synergid cells or by exchange of secreted factors. This interaction is completed when the pollen tube discharges, which is always associated with synergid degeneration.

Results

Two patterns of synergid cell degeneration are observed in *aca9-1* pollinations. To further understand the relationship between pollen tube discharge and synergid degeneration in the female gametophyte, we analyzed pollen tube arrival and synergid degeneration in ovules targeted by *aca9-1* mutant pollen tubes. *aca9-1* pollen tubes are only able to target the ovules proximal to the stigma and frequently fail to discharge in the ovules they reach; consequently seed set is dramatically reduced [(Schiott et al. 2004) and Supplementary Table 1]. We analyzed *aca9-1* mutant pollen tube growth by pollinating wild-type pistils with *aca9-1* mutant pollen carrying the *LAT52:GUS* transgene and scoring pollen tube behavior in wild-type ovules (Twell et al. 1989; Johnson et al. 2004). As expected, but unlike wild-type pollen tubes (Figs. 1A, 1B, and 1E), the *aca9-1* GUS⁺ pollen tubes showed two types of behaviors: About 32% of wild-type ovules contained *aca9-1* pollen tubes that exhibited wild-type behavior as evident from the normal pollen tube discharge (Figs. 1C and 1E). In the remainder (67%), *aca9* GUS⁺ pollen tubes ceased growth in the region of the ovule that contained the synergid cell (Figs. 1D-1E); however, these tubes did not show the characteristic discharge pattern found in wild-type ovules accessed by wild-type pollen tubes (note lack of a bolus of GUS staining in Figure 1D compared to Figure 1C). These results are consistent with the previous report that *aca9* pollen tubes fail to discharge (Schiott et al. 2004).

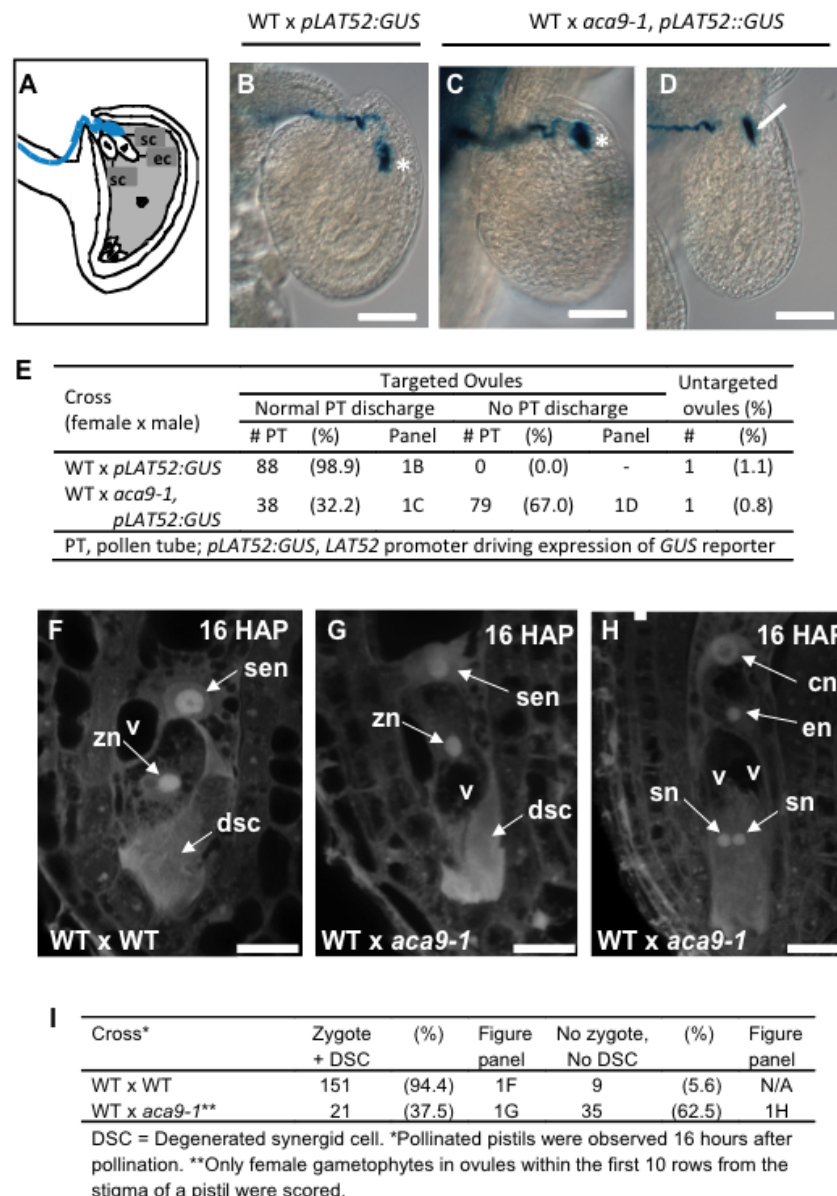


Figure. 1. Two patterns of synergid degeneration are observed in wild-type ovules accessed by *aca9-1* pollen tubes. (A-E) Pollen tube discharge is defective in wild-type ovules accessed by *aca9-1* pollen tubes. (A) Diagram depicting pollen tube discharge in the receptive synergid cell (sc) flanking an egg cell (ec) in a female gametophyte. (B-D) Micrographs of *LAT52::GUS* pollen tube interaction with ovules. (B) Normal discharge (white asterisk) of *LAT52::GUS* pollen tube in a wild-type ovule. (C) Normal discharge (white asterisk) of *aca9-1, LAT52::GUS* pollen tube in a wild-type ovule. (D) Failure of pollen tube discharge (white arrow) by a *aca9-1, LAT52::GUS* pollen tube in a wild-type ovule. (E) Quantification of pollen tube discharge in wild-type ovules accessed by wild-type and *aca9-1* pollen tubes shown in (B-D). Letters within parentheses refer to

panels B-D in this figure. Scale bars, 50 μ m. (F-H). CLSM images of embryo sacs of wild-type ovules isolated from pistils 16 hours after pollinating them with either wild-type or *aca9-1* pollen. Only the micropylar end of an ovule is shown and each image is a projection of several 1 μ m optical sections. (F) Wild-type ovule from pistils pollinated with wild-type pollen tube containing an embryo sac with a highly autofluorescent region denoted as degenerating synergid cell (dsc). (G, H) Wild-type ovules from pistils pollinated with *aca9-1* pollen containing an embryo sac with (G) or without (H) a highly autofluorescent region denoted as degenerating synergid cell (dsc). (I) Quantification of ovules from pollinated pistils and analyzed by CLSM. Letters within parentheses refer to panels F-H in this figure. cn, central cell nucleus, en, egg cell nucleus, sen, secondary endosperm nucleus; sn, synergid cell nucleus; v, vacuole; zn, zygote nucleus. Scale bars, 10 μ m.

Since a significant fraction of *aca9-1* pollen tubes enter the micropyle, but do not discharge, we analyzed how pollen tube arrival without discharge affects synergid degeneration. For this analysis we used a confocal laser scanning microscopy (CLSM) assay that takes advantage of autofluorescence from fixed tissues to image female gametophyte cells and nuclei. This assay has been used extensively to analyze synergid development and degeneration (Christensen et al. 1997; Christensen et al. 1998; Christensen et al. 2002; Faure et al. 2002; Sandaklie-Nikolova et al. 2007). We pollinated wild-type pistils with wild-type or *aca9-1* pollen and scored synergid degeneration in ~20 stigma-proximal ovules/pistil. Unlike in wild-type ovules targeted by wild-type pollen tubes, 94% of which showed a highly autofluorescent region in the portion of the ovule where a synergid cell is located (Fig. 1F), we observed two types of ovules in *aca9-1* pollinations. 37.5% of ovules appeared like wild type and had a highly autofluorescent region in the portion of the ovule where a synergid cell is located (Figs. 1G and 1I). The remaining 62.5% of ovules contained what appeared to be

two intact synergid cells, suggesting that synergid cells in these ovules did not undergo degeneration (Figs. 1H and 1I). The ratio of ovules with and without synergid degeneration is similar to the ratio of *aca9-1*-pollinated ovules with and without pollen tube discharge (Figs. 1E and 1I), indicating that lack of discharge by *aca9-1* pollen tubes is correlated with failure of synergid degeneration.

These results showed that synergid degeneration occurs at a reduced frequency in *aca9-1*-pollinated ovules. However, the CLSM assay for synergid degeneration has two shortcomings. First, it is not clear whether the highly autofluorescent signal that occupies the position of one of the two synergid cells is from the synergid alone, or whether it represents a combination of cell contents from the discharged pollen tube and the degenerated synergid. If autofluorescent signal is due to latter, it would not be possible to use the CLSM assay to determine whether synergid degeneration could occur in the absence of pollen tube discharge. Second, the CLSM assay does not allow one to determine whether a given ovule has been targeted by a pollen tube or whether the pollen tube has discharged. An assay that simultaneously analyzes the state of the pollen tube and the synergid cell in individual ovules would be preferable.

Simultaneous analysis of pollen tube and synergid cells shows that synergid degeneration occurs without pollination, is promoted by *aca9-1* pollen tube arrival, and is always associated with *aca9-1* pollen tube discharge.

We used a previously developed assay (Leydon et al. 2013) that simultaneously determines the state of the pollen tube and the synergid using the *LAT52:DsRED* (Francis et al. 2007) pollen tube cytoplasm marker and the *ACT11:MSI1:GFP* nuclear marker (Ingouff et al. 2007; Beale et al. 2012). *ACT11:MSI1:GFP* accumulates in the nuclei of female gametophyte cells. Importantly, upon synergid degeneration, GFP fluorescence is distorted and dissipates in the synergid nuclei, and becomes localized diffusely in the cytoplasm (Ingouff et al. 2007; Beale et al. 2012; Leydon et al. 2013). Analysis of nuclear morphology using 4',6-diamidino-2-phenylindole (DAPI)-staining of *Triticum aestivum* was used previously to characterize synergid degeneration (An et al. 2004). When pistils expressing this marker are pollinated with *LAT52:DsRED*, confocal microscopy can be used to determine in each ovule whether synergid degeneration has occurred and whether a pollen tube has arrived and discharged.

To establish the timeframe for synergid degeneration in the absence of pollen tube arrival, we first analyzed synergid degeneration in unpollinated pistils at 12, 16, 24, and 48 hours after the time when we perform hand-pollinations (methods and experiments below). In unpollinated pistils, synergid degeneration is rare 12 hours after when hand-pollinations would have been performed and nearly 95% of ovules have two intact synergid nuclei (Figs. 2A and 2J). At 16 hours after the time when hand-pollinations would have been performed, synergid degeneration is still rare (5% of ovules have one degenerated synergid and 5% of ovules have

two degenerated synergids, Fig. 2J). However, synergid degeneration, as indicated by distortion and dissipation of nuclear GFP signal specific to synergids, is progressively observed in unpollinated pistils (Figs. 2B 2C and 2J) and roughly 60% of ovules have either one or two degenerated synergids by 48 hours after the time when pistils would have been hand-pollinated (Fig. 2J).

Wild-type pistils were pollinated with wild-type *LAT52:DsRED* pollen and analyzed 12 and 16 hours after the time when pistils were hand-pollinated. At either of these two time points, all pollen tubes that had entered ovules had discharged and all of the targeted ovules had at least one degenerated synergid (Figs. 2D-J). The fraction of ovules with two degenerated synergids increased from 66% at 12 hours to 86% at 16 hours after pollination (Fig. 2J). In ovules with one synergid remaining (Figs. 2D-F), the receptive synergid has degenerated and received DsRED from the pollen tube; the persistent synergid remains and has an intact nucleus.

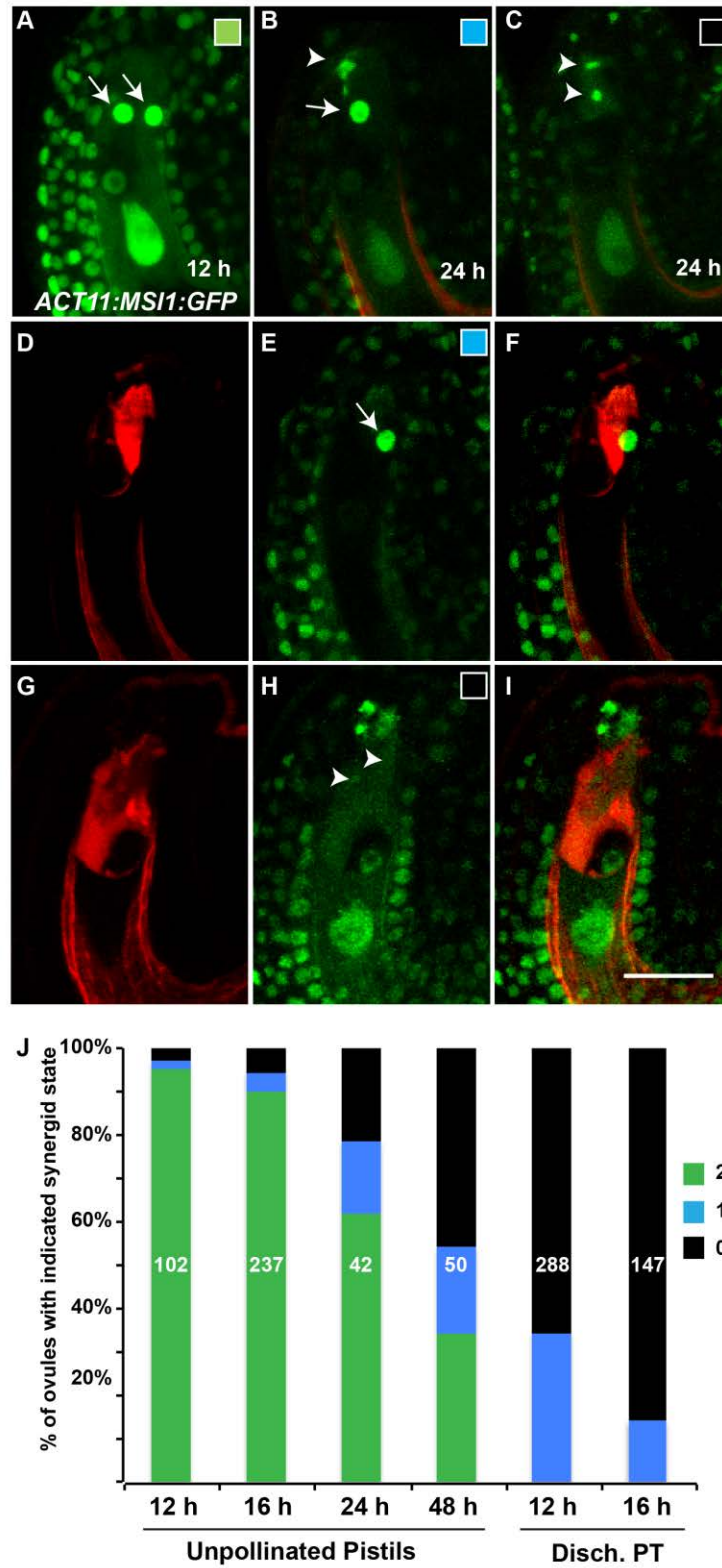


Figure. 2. Synergid degeneration is observed in unpollinated ovules, but is enhanced by presence of the pollen tube. (A-F) Confocal micrographs of

female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated (A-C) or pollinated pistils (D-I). (A-C) Unpollinated emasculated pistils were analyzed 12, 16, 24, and 48 hours (h) after the time when pistils would have been hand pollinated (see methods for explanation of time course). (D-I) Ovules were imaged 16 hours after pollination with *LAT52:DsRED* pollen. The red channel (D, G) indicates that a pollen tube has discharged within a synergid. The green channel reveals the synergid state (E, H). The overlay shows the discharged pollen tube and the synergid nuclei. Images show two intact synergids (A, green square), one intact and one degenerated synergid (B, E, blue square), or two degenerated synergids (C, H black square). Synergid nuclei are round and brightly fluorescent (arrow), upon degeneration GFP signal becomes diffuse and/or irregular (arrowhead). J) Quantification of synergid degeneration using the color code described above at indicated time points in ovules in unpollinated pistils compared with ovules that have received a pollen tube that has discharged (Disch. PT). The number of ovules analyzed in each experiment is given in the center of each bar. Scale bar, 20 μ m.

These data show that synergid degeneration can occur in the absence of pollination. However, it does not occur at significant levels during the time frame when pollen tubes arrive at ovules following of self- or hand-pollination (8 – 16 hours, see 12 and 16h unpollinated, Fig. 2J). In contrast, synergid degeneration had always occurred in ovules in which a pollen tube discharged (see 12 and 16h discharged PT Fig. 2J). We next used this simultaneous assay to ask whether pollen tube arrival, without discharge, is associated with synergid degeneration using *aca9-1* pollen tubes and whether ovules with degenerated synergids also contained a successfully discharged pollen tube.

When we pollinated wild-type pistils expressing *ACT11:MSI1:GFP* with *aca9-1*, *LAT52:DsRED*, we found that 37% (50/135) of the targeted ovules had a discharged pollen tube (Fig. 3E). The remaining 63% (85/135) had one (Figs. 3B, 3C) or more (Fig. 3D) pollen tubes that had entered the micropyle, but had not

discharged. These fractions are in agreement with data using *LAT52:GUS* as a marker for pollen tube arrival (Figs. 1C-E). Simultaneous analysis of synergid degeneration using the *ACT11:MSI1:GFP* marker showed that ovules receiving *aca9-1* pollen tubes that discharged always had at least one degenerated synergid and the majority had two degenerated synergids (Fig. 3E). Interestingly, we also found that 49% of ovules that had attracted an *aca9-1* pollen tube but failed to discharge had at least one degenerated synergid (Fig. 3E, arrested PT). This is significantly higher than the rate of synergid degeneration observed in unpollinated pistils 16 hours after the time of pollination and suggests that pollen tube arrival promotes synergid degeneration even when discharge does not occur. In addition, nearly 51% of ovules that had attracted an *aca9-1* pollen tube but failed to discharge had both synergid cells intact (Fig. 3E). These observations corroborate those made with *myb* triple-mutant (Leydon et al. 2013) and *aca9-1* (Fig. 2) pollen tubes and prompted us to investigate if the same patterns are detected in female gametophyte mutants that alter pollen tube-synergid interactions.

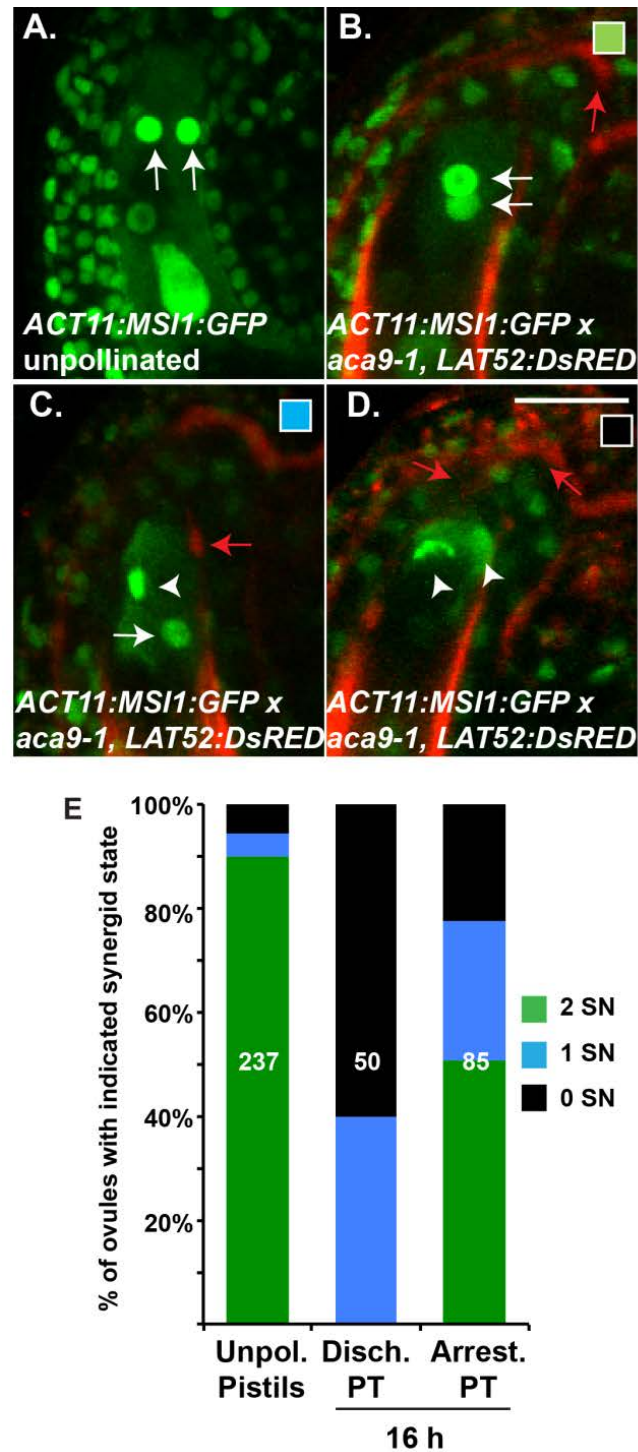


Figure 3. Synergid degeneration is higher in ovules that attract *aca9* pollen tubes that discharge relative to those that enter the micropyle, but do not discharge. (A-D) Confocal micrographs of wild-type female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated pistils (A) or pistils pollinated with *aca9-1, LAT52:DsRED* pollen and imaged 16 hours after pollination (B-D). (A) Unpollinated pistils were analyzed 12 hours after the time when pistils would

have been hand pollinated. Two intact synergid nuclei are present (arrows). B-D) Images show pollen tubes that have arrived in the micropyle, but have not discharged. The pollen tube tip is intact (red arrow). Images show two intact synergids (B, green square, white arrows), one intact synergid (C, blue square, arrow; white arrowhead is pointing to the degenerating nucleus), or two degenerated synergids (D, black square, arrowheads). E. Quantification of synergid degeneration using the color code described above in unpollinated pistils or pistils pollinated with *aca9-1*, *LAT52:dsRED* and observed 16 hours after pollination (16 h). The synergid state was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (discharged, arrested) is given in the center of each bar. Scale bar, 20 μ m.

Pollen tube discharge and synergid degeneration occur at decreased frequencies in *gfa2* mutant ovules

gfa2 is a gametophytic mutation that arrests female gametophyte development and results in reduced seed set (Christensen et al. 2002). Using the autofluorescence-based CLSM assay, it was shown that, as expected for a fully penetrant gametophytic mutation, ~50% of ovules in unpollinated *gfa2/GFA2* pistils contain a female gametophyte with two polar nuclei that fail to fuse and two intact synergid cell nuclei (Christensen et al. 2002); mature wild type female gametophytes have a single fused central cell nucleus and two synergid nuclei. Our analysis of *gfa2/GFA2* (Table S1) using this CLSM assay (Supplemental Figs. 1A-D) yielded similar results. Also consistent with a previous report (Christensen et al. 2002), analysis of wild type female gametophytes 16 hours after pollination using this technique showed that ~94% of ovules have a brightly autofluorescent signal in the position of one of the synergids (denoted as degenerated, Supplemental Fig. 1E); while ~50% of *gfa2/GFA2* female gametophytes contained unfused polar nuclei and two synergids with intact synergid nuclei (Supplemental Figs. 1G, J).

Although it was reported that *gfa2* female gametophytes attracted pollen tubes at wild type levels (Christensen et al. 2002), it is not known if pollen tube discharge occurs normally in a *gfa2* female gametophyte. We therefore characterized pollen tube discharge in *gfa2* ovules using two approaches. First, we imaged pollen tube arrival in real-time using a semi *in vivo* pollen tube guidance assay that recapitulates much of the *in vivo* pollen tube behavior in ovules (Palanivelu et al. 2006). For this assay, pollen tubes were marked with *LAT52:DsRED* (Francis et al. 2007). Similar to the wild type behavior (Movie S1; n=89), in *gfa2* ovules, *LAT52:DsRed* pollen tubes always navigated to the synergids (Movies S2-S3). After reaching the synergids in *gfa2/GFA2* ovules, some *LAT52:DsRed* pollen tubes discharged normally (Movie S2; n=67) while others arrested growth in a synergid but did not discharge (Movie S3). In the latter, the pollen tube appears to arrest its growth and following a brief pause, the tube appears to then grow over the synergid cell but not discharge. These results showed that pollen tube discharge is defective in *gfa2* female gametophytes despite normal entry into the ovule micropyle and successful navigation to the synergid cells.

Second, we examined pollen tube interaction with a female gametophyte *in vivo* by pollinating pistils with wild-type *LAT52:GUS* pollen and scoring pollen tube behavior in ovules of fixed pistils (Twell et al. 1989; Johnson et al. 2004). In wild type, the *GUS*⁺ pollen tubes entered an ovule, stopped growth and discharged in the region of the ovule that contained the synergid cell (Fig. 4A). In contrast, in *gfa2/GFA2* pistils, we observed two types of ovules. About 48% of ovules

contained pollen tubes that exhibited wild-type behavior (Fig. 4B). In the remainder, GUS⁺ pollen tubes ceased growth in the region of the ovule that contained the synergid cell; however, these tubes did not show the characteristic discharge observed in wild type ovules (note lack of bolus of GUS staining in Fig. 2C compared to Figs. 2A or 2B). The ratio of ovules with and without pollen tube discharge in *gfa2* (Fig. 2G) is consistent with the ratio of pollinated *gfa2* ovules with and without synergid autofluorescence in the position of one of the two synergids (Supplemental Fig. 1H), indicating that pollen tube discharge and synergid degeneration are disrupted in *gfa2* ovules and that lack of discharge by wild-type pollen tube is correlated with failure of synergid degeneration in *gfa2* ovules.

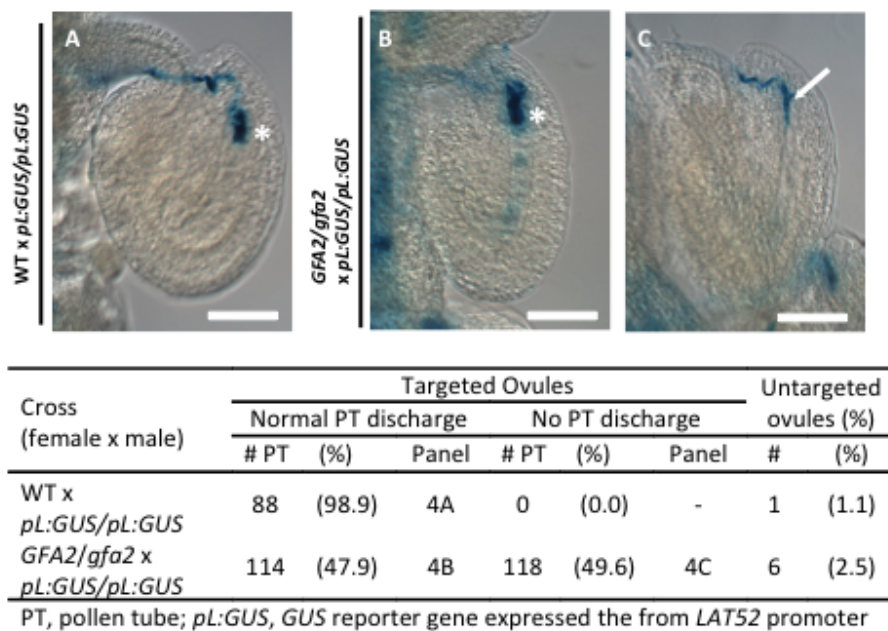


Figure 4. Pollen tube discharge is defective in *gfa2* female gametophytes. (A-C) Micrographs of *LAT52:GUS* pollen tube growth in ovules. (A) Normal discharge (white asterisk) of *LAT52:GUS* pollen tube in a wild-type ovule. (B) Normal discharge (white asterisk) of a *LAT52:GUS* pollen tube in a *gfa2/GFA2* ovule. (C) Failure of pollen tube discharge (white arrow) by a *LAT52:GUS* pollen tube in a *gfa2/GFA2* ovule. (D) Quantification of pollen tube discharge in wild-

type and *gfa2/GFA2* ovules accessed by wild-type pollen tubes shown in (A-C). Letters within parentheses refer to panels A-C. Scale bars, 50µm.

Simultaneous analysis of synergid degeneration and pollen tube discharge in *gfa2* ovules indicates that pollen tube arrival promotes synergid degeneration

Analysis of pollen tube-synergid interactions in *gfa2* female gametophytes has relied on assays that separately analyze either pollen tube discharge (e.g. Fig. 4) or synergid state (e.g. Supplemental Fig. 1). Consequently, it has not been possible to analyze the state of pollen tube and synergid degeneration simultaneously in individual female gametophytes. We therefore pollinated *gfa2/GFA2* pistils expressing *ACT11:MSI1:GFP* with wild-type *LAT52:DsRED* pollen to analyze pollen tube and synergid states simultaneously. In *gfa2/GFA2* pistils, we can distinguish wild type from *gfa2* mutant female gametophytes by determining whether there is a single central cell nucleus (*GFA2*, Fig. 5A) or two unfused polar nuclei (Figs. 5B-E).

Synergid degeneration was similar between *gfa2* and *GFA2* (WT in Fig. 5) female gametophytes in unpollinated pistils (Fig. 5F), and was comparable to wild type (Fig. 2J). A total of 224 ovules were analyzed in pollinated *gfa2/GFA2* pistils. The vast majority of *gfa2/GFA2* ovules that attracted a pollen tube were wild type (*GFA2*, polar nuclei fused) and all of these had at least one degenerated synergid (Fig. 5F). We also observed a fraction of targeted ovules that were *gfa2* mutant (polar nuclei not fused), but that contained a discharged

pollen tube (Figs. 5C, D, F). As in wild type ovules, synergid degeneration always accompanied pollen tube discharge in *gfa2* ovules (Fig. 5F). We also observed a notable fraction of targeted *gfa2* ovules that attracted a pollen tube that had failed to discharge (as observed in Figure 4C by GUS assay). Synergid degeneration was reduced in these ovules, but increased over unpollinated pistils (Compare Figure 5F *gfa2* arrested PT with Figure 2J unpollinated 16 hours). In addition, nearly 55% of *gfa2* ovules that had an arrested pollen tube had both synergid cells intact (Fig. 5F). These data are consistent with those from male mutants that enter ovules without discharging [*myb* triple-mutant (Leydon et al. 2013) and *aca9-1* (Fig. 2)] showing two types of ovules: one in which pollen tube arrival promotes synergid degeneration and another in which both synergid cells remain intact. These findings prompted us to analyze a female mutant, *lre*, in which pollen tubes enter and continue to grow without discharging so that we could determine the impact on synergid degeneration following prolonged physical interaction with a pollen tube that has failed to discharge.

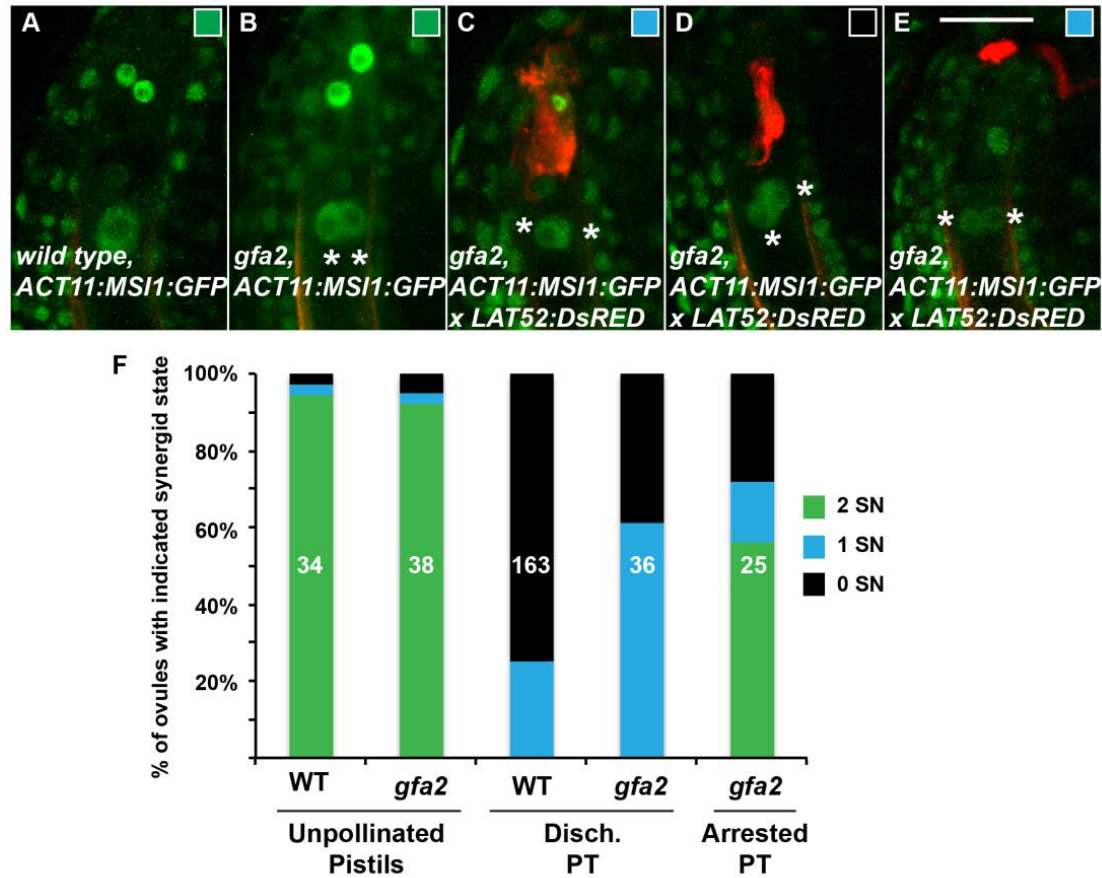


Figure 5. Pollen tube discharge and synergid degeneration are detectable in *gfa2* female gametophytes and synergid degeneration is increased in *gfa2* female gametophytes containing a discharged pollen tube relative to those containing an arrested pollen tube. (A-E) Confocal micrographs of female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated (A-B) or pollinated pistils (C-E). (A-B) Unpollinated *gfa2/GFA2* pistils were analyzed 16 hours after the time when pistils would have been hand pollinated. Wild-type (*GFA2*) female gametophytes have a single central cell nucleus (34/72 of ovules analyzed); *gfa2* mutant female gametophytes have two unfused polar nuclei (**, 38/72 of ovules analyzed). (C-E) Ovules were imaged 16 hours after pollination with *LAT52:DsRED* pollen; overlays of the red (*LAT52:DsRED*) and green (*ACT11:MSI1:GFP*) channels are shown. Images show a single pollen tube that has discharged within a *gfa2* mutant ovule (C, D); or a pollen tube that has arrested at the *gfa2* micropyle (E). Images show two intact synergids (A, B, green square), one intact synergid (C, E, blue square), or two degenerated synergids (D, black square). (F) Quantification of synergid degeneration using the color code described above in unpollinated pistils compare with ovules that have received a pollen tube that has either discharged or arrested. The synergid state and the state of polar nuclear fusion (*gfa2*, two unfused polar nuclei **; wild-type CCN, one fused central cell nucleus) was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (arrested, discharged) is given in the center of each bar. Scale bar, 20 μ m.

Synergid degeneration occurs in *lre* female gametophytes containing an overgrown (coiled) pollen tube that has not discharged

Prior analysis of pollen tube-synergid interactions in *lre* mutants was based on two assays, one that analyzed synergid degeneration and one that measured pollen tube discharge (Capron et al. 2008; Tsukamoto et al. 2010). These reports revealed that wild type pollen tubes fail to stop growing and discharge within *lre* ovules and that pollen tubes often form extended coils within *lre* ovules. In addition, CLSM autofluorescence patterns suggested that synergid degeneration was significantly reduced in *lre* ovules (Tsukamoto et al. 2010). We analyzed pollen tube discharge and synergid degeneration simultaneously in *lre* ovules using the assay described in Figs. 2, 3, and 5. As expected, *lre-5* female gametophytes displayed wild-type female gametophyte development (Fig. 6A) and the vast majority of ovules in unpollinated pistils contained two intact synergids (Figs. 6A, 6E). However, unlike wild-type ovules, which attract pollen tubes that discharge upon entering the micropyle (Figs. 1C, 2D, and 4A), at 16 hours after pollination, 88% (147/167) of *lre* ovules contained an overgrown and coiling pollen tube. Interestingly, the majority (123/147, 84%) of *lre-5* ovules containing an overgrown pollen tube that failed to discharge had either 1 or 2 degenerated synergids. This is a higher proportion of synergid degeneration than observed in male or female mutants that attracted pollen tubes that arrested without overgrowth in the ovule (compare Fig. 6 coil PT with Fig. 5 arrest PT or Fig. 3 arrest PT). The remaining *lre* ovules with a coiling pollen tube (16%) had two intact synergids, pointing to another instance of a subset of ovules in which

synergid cells remain intact when pollen tubes do not discharge. Finally, and consistent with all observations made in wild-type and male or female mutants reported in this study, all *lre-5* ovules that attracted a discharged pollen tube (12% of targeted *lre-5* ovules) had at least one degenerated synergid (Figure 6E).

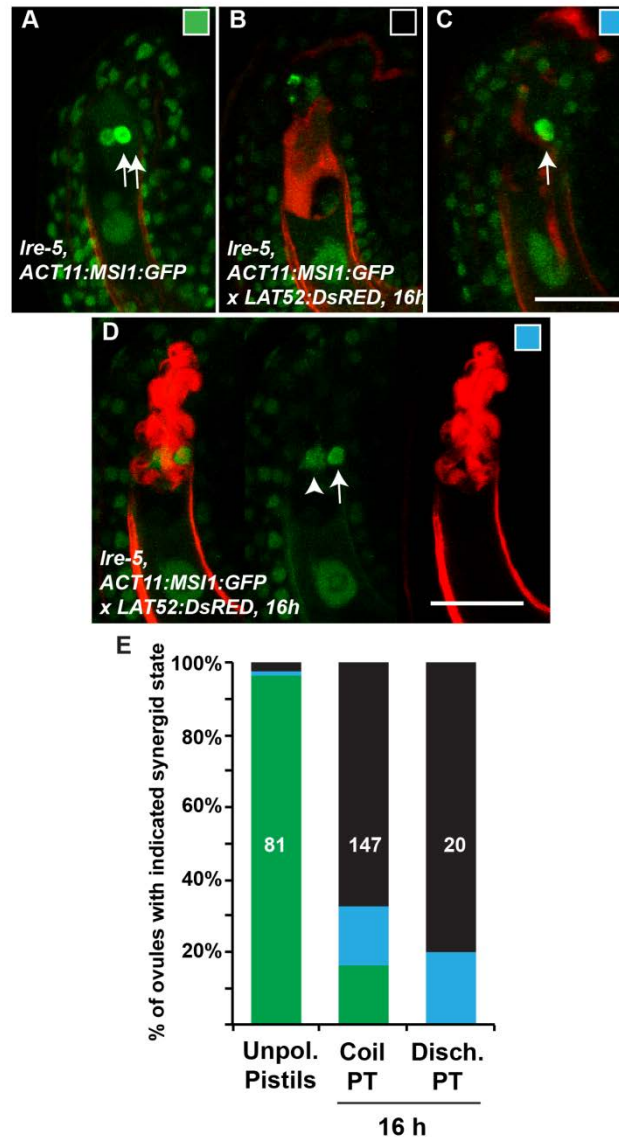


Figure 6. Synergid degeneration occurs more frequently in *lre* female gametophytes containing a discharged pollen tube relative to those containing an arrested or a coiled pollen tube. (A-D) Confocal micrographs of *lre-5* female gametophytes expressing *ACT11:MSI1:GFP* in unpollinated pistils

(A) or pistils pollinated with *LAT52:DsRED* pollen and observed 16 hours after pollination (B-D). (A) Unpollinated *lre-5/lre-5* pistils were analyzed 12 hours after the time when pistils were hand pollinated. Two intact synergid nuclei are present (arrows). (B-D) *lre-5* ovules Images show discharged pollen tubes (B), or pollen tubes that are overgrowing (coiling) within the female gametophyte (C,D). Images show two intact synergids (A, green square), one intact synergid (C, D, blue square), or two degenerated synergids (B, black square). All images are overlays of green (nuclei of female cells) and red (pollen tube) channels except for D, which shows an overlay on the left along with green (center) and red (right) channels. Synergid nuclei are round and brightly fluorescent (arrow), upon degeneration GFP signal becomes diffuse and/or irregular (arrowhead). In D the middle panel (green channel) shows that one synergid has degenerated; this is difficult to appreciate in the overlay (left panel) E) Quantification of synergid degeneration using the color code described above in *lre-5* ovules from unpollinated pistils or pistils pollinated with *LAT52:DsRED* and observed 16 hours (16 h) after hand pollination. The synergid state was determined in all ovules that had attracted a pollen tube; the number with the indicated pollen tube state (coiled, burst) is given in the center of each bar. Scale bar, 20 μ m.

Discussion

A basal pathway mediates synergid degeneration in the absence of pollination.

Previous analyses focused on whether the pollen tube induces synergid degeneration and did not define the kinetics of the basal synergid cell death program in the absence of pollination (Christensen et al. 2002; Faure et al. 2002; Hamamura et al. 2011). Our results (Fig. 2) show that synergid degeneration occurs in *Arabidopsis* ovules in the absence of pollination and that degeneration rates increase over time. This points to a basal cell death mechanism in a synergid cell that is gradually activated, but that is much slower than degeneration induced by the pollen tube. For example, at the 12 hour time point, >95% of ovules in unpollinated pistils had two intact synergids compared to zero with two intact synergids at the same time point after pollen tubes had discharged (Fig. 2F). Furthermore, even after remaining unpollinated for 48 hours

(72 hours after emasculation), both synergids remained intact in 35% of ovules in unpollinated pistils (Fig. 2F). Thus, our results are consistent with the long-held view that the presence of a pollen tube in the female gametophyte promotes synergid degeneration in *Arabidopsis* (Christensen et al. 2002; Faure et al. 2002; Hamamura et al. 2011). However, whether pollen tube discharge is absolutely required for synergid degeneration had not been fully evaluated.

Pollen tube discharge is not required for synergid degeneration.

The temporal sequence of events in pollen tube-female gametophyte interactions was analyzed in multiple studies in *Arabidopsis* (Christensen et al. 1997; Faure et al. 2002; Sandaklie-Nikolova et al. 2007). Using transmission electron microscopy and real-time observation of synergids marked with a cytoplasmic marker in a semi *in vivo* assay, it was proposed that receptive synergid cell death initiates after the pollen tube arrives at the female gametophyte but before pollen tube discharge (Sandaklie-Nikolova et al. 2007). A subsequent study using the same semi *in vivo* assay and a synergid cell marked with a nuclear marker showed that receptive synergid cell degeneration occurs after pollen tube arrival at the synergid cell, but upon pollen tube discharge (Hamamura et al. 2011). These studies, however, did not address a fundamental question about pollen tube reception: is pollen tube discharge required for synergid degeneration?

Insights into this question first came from a recent study that simultaneously analyzed synergid degeneration and pollen tube discharge in wild-type ovules

accessed either by wild-type pollen tubes that discharge or *myb* triple-mutant pollen tubes, which fail to discharge (Leydon et al. 2013). By directly observing the states of pollen tube discharge and synergid degeneration in an ovule, it was shown that 58% of ovules containing an overgrown (coiling) *myb* triple-mutant pollen tube had at least one degenerated synergid. These data suggested that synergid degeneration could be initiated without pollen tube discharge. However, this could have been a consequence of this specific set of male mutations, so we tested an additional male mutant and two female mutants. Consistent with the results obtained for *myb* triple-mutant pollinations, we identified ovules that contained at least one degenerated synergid despite having a pollen tube that had not discharged. This was found in a male mutant (*aca9*, Fig. 3) and two female mutants (*gfa2* (Fig. 5; *lre*, Fig. 6). Additionally, pollen tube arrival without discharge promoted synergid degeneration much more rapidly than the basal pathway (Figs 3, 5, 6 compared to Fig. 2J). Our results confirm that pollen tube discharge is not essential for synergid degeneration and that uncoupling of synergid degeneration from pollen tube discharge is not unique to ovules accessed by *myb* triple-mutant tubes.

Pollen tube discharge is always associated with synergid degeneration and synergid degeneration rates are lower in ovules that attract pollen tubes that fail discharge.

Forty-two percent of ovules containing coiling *myb* triple-mutant pollen tube (fail to discharge) had two intact synergids. Conversely, at least one synergid had

always degenerated in the 10% of ovules that attracted a *myb* triple-mutant pollen tube that discharged (Leydon et al. 2013). These results suggest that pollen tube discharge is associated with synergid degeneration. Combined with our other findings, this means that pollen tube arrival can induce synergid degeneration, but at lower rates than pollen tube discharge. This pattern held when additional female (*gfa2*, Fig. 5; *lre*, Fig. 6) and male (*aca9*, Fig. 3) mutants were analyzed. We found that i) pollen tube arrival and arrest without PT discharge triggers synergid degeneration above levels observed in unpollinated pistils (Figs. 1, 3, 5). ii) pollen tube overgrowth, without PT discharge, is associated with higher levels of synergid degeneration compared to arrested or unpollinated ovules, but does not cause synergid degeneration at levels found in ovules with successful pollen tube discharge (Figs. 2, 3, 5, 6).

Our results suggest a spectrum of synergid degeneration associated with different pollen tube behaviors: synergid degeneration was always associated with pollen tube discharge (Fig. 2, 3,5,6), this was followed by lower rates of degeneration induced by pollen tubes that overgrow in the female gametophyte but not discharge [*myb* triple mutant (leydon et al), *lre* (Fig. 5)], followed by pollen tubes that arrived and ceased growth (*aca9-1* mutant pollen tubes, *gfa2* mutant ovules), followed by basal synergid degeneration that occurs in the absence of pollination (Fig. 2).

Synergid degeneration occurs through three modes.

We have provided evidence for a basal pathway of synergid degeneration that occurs in the absence of pollination (Fig. 2). Previous studies (Christensen et al. 2002; Faure et al. 2002; Hamamura et al. 2011), and work reported here (Figs. 1-6), provide evidence for a second pathway that leads to degeneration of the receptive synergid upon pollen tube arrival. Finally, a third, ethylene-mediated signaling pathway is activated by successful double fertilization and results in degeneration of the persistent synergid (Volz et al. 2013). It is not yet known whether these modes of synergid degeneration share common mechanistic components. We found that *gfa2* mutant female gametophytes, which were known to have a defect in synergid degeneration (Christensen et al. 2002), do not affect the basal pathway. In unpollinated *gfa2/GFA2* pistils, the frequency of synergid degeneration after 16 hours is comparable to wild type (Fig. 5F). Future experiments will be aimed at extending this observation and determining whether each mode of synergid degeneration is altered by mutations known to affect one.

Conclusions and future perspectives

A defining feature of sexual reproduction in flowering plants is the interaction between the male and female gametophytes, which results in fusion of a pair of female gametes with a pair of male gametes. Both male and female gametophytes are multicellular, consisting of the gametes and accessory cells, which are critical for successful double fertilization. The pollen tube is male accessory cell; the female gametophyte has a pair of synergid cells and three

antipodal cells. The role of antipodals is not known (Song et al. 2014). However, it is clear that the pollen tube and synergids function to coordinate their mutual demise and in so doing facilitate gamete delivery for double fertilization (Kessler et al. 2011).

We show that *Arabidopsis* pollen tube discharge is associated with synergid degeneration and that when pollen tube discharge is successful, at least one synergid always degenerates. Based on these findings, we propose that while pollen tube discharge is not required to initiate synergid degeneration, pollen tube discharge may ensure that synergid degeneration is completed in all ovules that receive sperm. Live imaging (Hamamura et al. 2011) suggests that degeneration of the receptive synergid is required so that the sperm cells can be projected by the force of pollen tube discharge to the site where they will fuse with female gametes. Synergid degeneration is likely required to make this site available to sperm cells. By promoting completion of synergid degeneration in all ovules, signaling interactions between the pollen tube and synergid coupled with pollen tube discharge ensure successful double fertilization between the sperm cells and the female gametes and increases the odds of successful seed formation.

Our findings are consistent with the emerging idea (Boisson-Dernier et al. 2011; Cheung et al. 2011; Kessler et al. 2011; Lindner et al. 2012) that a complex set of interactions between the pollen tube and synergid cells result in synergid

degeneration. We conclude that signaling from the pollen tube, either by direct physical contact and/or secreted factors, to the receptive synergid induce degeneration and that these interactions are always completed when the pollen tube discharges. Many interesting questions need to be addressed, including: How is the receptive synergid selected? What is the nature of the signaling molecules that do so? How do these molecules affect changes in $\text{Ca}^{2+}_{\text{cyto}}$ that accompany pollen tube synergid interactions and mark the receptive synergid (Denninger et al. 2014; Hamamura et al. 2014; Ngo et al. 2014)? Genomic and imaging resources for the study of flowering plant reproduction have improved dramatically in recent years and should enable rapid progress toward addressing these questions.

Materials and Methods

Growth conditions and plant materials Wild type (Columbia ecotype), *LAT52:DsRED*, *LAT52:GUS*, *gfa2/GFA2*, and *aca9-1* seeds were plated on Murashige and Skoog media (MS, Carolina Biological Supply Company, Catalog # 195703) in growth chamber maintained at 20°C and 24 hours illumination. 7-10 day seedlings were moved from plates to soil (SUNSHINE MIX #1, Crop Production Services, INC., Product # 1000188678), which was mixed with vermiculite (Vermiculite #2, Crop Production Services, Product # 1000190472) in a ratio of 5:1 and were grown in chambers maintained at 20°C and 24 hours illumination. *ACT11:MS1:GFP* and *gfa2/GFA2* and *aca9-1* mutations in the *ACT11:MS1:GFP* background were grown as described (Johnson et al. 2004; von Besser et al. 2006; Wong et al. 2010).

***In vivo* analysis of pollen tube discharge in ovules** *LAT52:GUS* or *aca9-1*, *LAT52:GUS* pollen was crossed to wild-type or *gfa2/GFA2* pistils, harvested 16 hours after hand pollination, ovary walls were removed as previously described (Johnson et al. 2010), stained for GUS activity (Johnson et al. 2004), and imaged using a Zeiss Axiovert 200 DIC microscope (Carl Zeiss, Germany). For emasculation, 12b or 12c stage buds (Smyth et al. 1990) were emasculated and waited for 24 hours before pollinated with indicated pollen. For each cross, reported in this study, at least three emasculated pistils were stained for GUS activity.

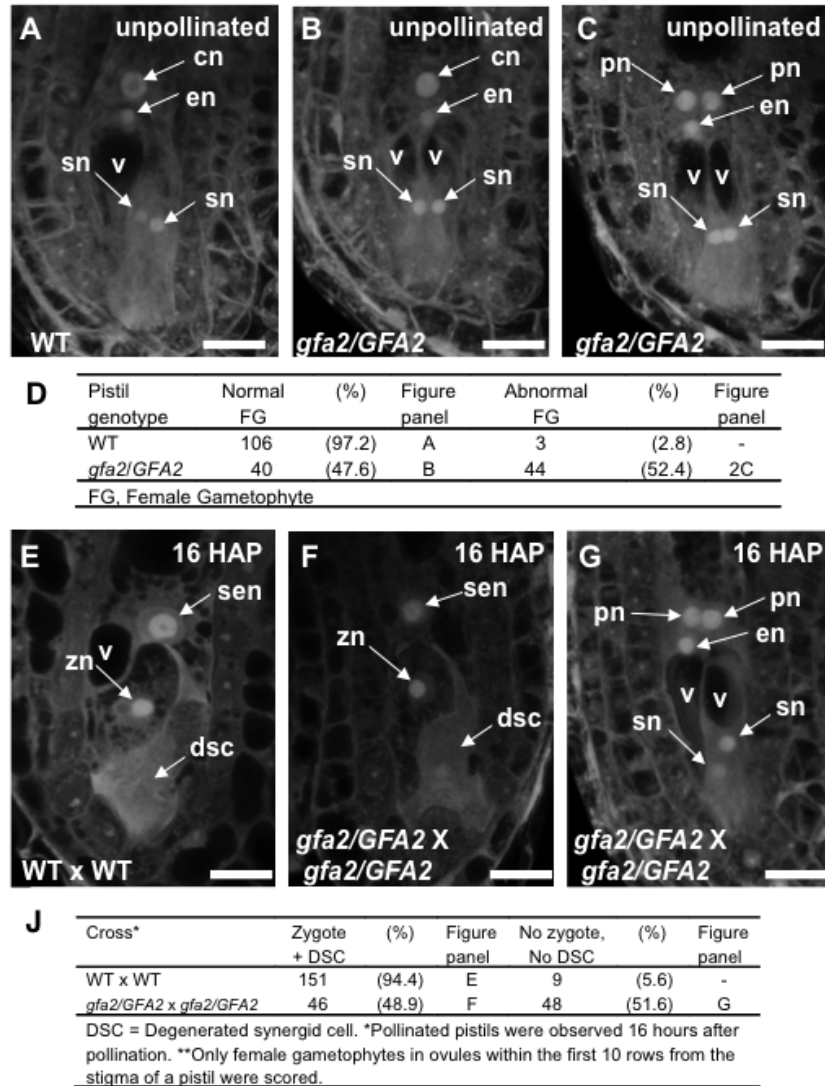
CLSM analysis of female gametophytes and double fertilization Stage12b or 12c buds (Smyth et al. 1990) were emasculated and 24 hours later, pistils were either observed directly (unfertilized ovules) or were hand-pollinated and analyzed 16 hours afterward, prepared for CLSM to score unfertilized female gametophytes (Christensen et al. 1997) or double fertilization (Faure et al. 2002). For each genotype, ovules in six pistils were observed using a Leica TCS SP5 confocal microscope.

Simultaneous assay of pollen tube discharge and synergid degeneration

Stage12b or 12c buds (Smyth et al. 1990) were emasculated and 24 hours later they were hand pollinated. Twenty-four hours after emasculation marks Time = 0 hours in all of the experiments reported here (unpollinated or pollinated). Hand pollinations were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating emasculated *ACT11:MSI1:GFP* stigmas (Smyth et al. 1990) with pollen from male donor plants carrying *LAT52:DsRED* (Francis et al. 2007). 16 hours after hand pollinations were performed, pistils were harvested and ovary walls were removed as previously described (Johnson et al. 2010). Unpollinated pistils were analyzed 12, 16, 24, and 48 hours after Time = 0 (the time point when hand-pollinations were performed, 24 hours after emasculation). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV)

and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks).

Time-lapse imaging of pollen tube discharge in ovules using a semi *in vivo* pollen tube guidance assay To directly observe pollen tube discharge in ovules, time-lapse imaging of pollen tube-ovule interactions was performed essentially as described in Palanivelu and Preuss 2006. Pistils (*ms1*) were pollinated with *LAT52:DsRed* pollen and time-lapse imaged using a Zeiss Axiovert 100 DIC microscope (Carl Zeiss, Germany) equipped with an automated X, Y, Z stages and shutter to generate multiple time-lapse movies using bright light plus red fluorescent laser lights. A Retiga CCD digital camera was used to capture images and Metamorph and ImageJ image software were used to analyze these images.



Supplemental Figure 1. Confocal analysis of synergid degeneration in *gfa2/GFA2*. (A-C) CLSM images of unfertilized stage7 wild-type and *gfa2* female gametophytes. Only the micropylar end of an ovule is shown and each image is a projection of several 1µm optical sections. (A) Confocal image of wild-type female gametophytes containing synergid, egg, and central cells. The gametophytic cell nuclei appear as bright fluorescent spots (arrows). (B, C) In a *gfa2GFA2* pistil, two types of female gametophytes were detected. One resembled the wild type (B), with the normal compliment of female gametophytic cells. The second type (C) had a female gametophyte in which the two polar nuclei remain unfused. (D) Quantification of unfertilized stage7 wild-type and *gfa2* female gametophytes shown in A-C. Letters within parentheses refer to panels A-C of this figure. (E-H) CLSM images of embryo sacs of wild-type and *gfa2/GFA2* ovules isolated from pistils 16 hours after pollinating them with wild-type pollen. Only the micropylar end of an ovule is shown and each image is a

projection of several 1µm optical sections. (E) Wild-type ovule isolated from pistils pollinated with wild-type pollen containing an embryo sac with a highly autofluorescent region denoted as degenerating synergid cell (dsc). (F, G) *gfa2/GFA2* ovules from pistils pollinated with wild type pollen containing an embryo sac with (F) or without (G) a highly autofluorescent region denoted as degenerating synergid cell (dsc). (H) Quantification of pollinated ovules analyzed by CLSM. Letters within parentheses refer to panels E-G of this figure. cn, central cell nucleus, en, egg cell nucleus, pn, polar nucleus, sen, secondary endosperm nucleus; sn, synergid cell nucleus; v, vacuole; zn, zygote nucleus. Scale bars, 10µm.

Supplemental Movie 1. A semi *in vivo* pollen tube guidance assay to monitor pollen tube discharge in wild-type ovules. Time-lapse imaging of DsRed-tagged pollen tubes, entering an ovule and reaching the portion of the ovule where synergid is expected to be located. Within the ovule, pollen tube discharge occurs presumably in one of the synergids, leaving a visible fluorescent spot. Time elapsed between each frame = 10 minutes.

Supplemental Movie 2. A semi *in vivo* pollen tube guidance assay to monitor pollen tube discharge in *gfa2/GFA2* ovules. Time-lapse imaging of DsRed-tagged pollen tubes, entering an ovule and reaching the portion of the ovule where synergid is expected to be located. Within the ovule, apparently normal pollen tube discharge occurs presumably in one of the synergids, leaving a visible fluorescent spot. This is a representative movie of instances in a *gfa2/GFA2* ovule in which there is an apparent normal pollen tube discharge. Time elapsed between each frame = 10 minutes.

Supplemental Movie 3. A semi *in vivo* pollen tube guidance assay to monitor pollen tube discharge in *gfa2/GFA2* ovules. Time-lapse imaging of DsRed-tagged pollen tubes, entering an ovule and reaching the portion of the ovule where synergid is expected to be located. Within the ovule, the pollen tube remains without discharge, as evident from the lack of a visible fluorescent spot. This is a representative movie of instances in a *gfa2/GFA2* ovule in which the pollen tube did not discharge. Time elapsed between each frame = 10 minutes.

ACKNOWLEDGEMENTS

We thank Dr. Gary Drews for *gfa2* seeds and CLSM protocol; Dr. Gregory Copenhaver for the *LAT52:DsRED* line; Dr. Jeffery Harper for *aca9-1* seeds; Dr. Fred Berger for *ACT11:MSI1:GFP* line. NSF grants to MJ (IOS-1353798) and RP (IOS-1146090) supported this study.

REFERENCES

- An, L. H. and R. L. You (2004). "Studies on nuclear degeneration during programmed cell death of synergid and antipodal cells in *Triticum aestivum*." Sexual Plant Reproduction **17**(4): 195-201.
- Beale, K. M., A. R. Leydon, et al. (2012). "Gamete Fusion Is Required to Block Multiple Pollen Tubes from Entering an Arabidopsis Ovule." Curr Biol.
- Berger, F. and D. Twell (2011). "Germline specification and function in plants." Annu Rev Plant Biol **62**: 461-484.
- Boisson-Dernier, A., S. A. Kessler, et al. (2011). "The walls have ears: the role of plant CrRLK1Ls in sensing and transducing extracellular signals." J Exp Bot **62**(5): 1581-1591.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Cheung, A. Y. and H. M. Wu (2011). "THESEUS 1, FERONIA and relatives: a family of cell wall-sensing receptor kinases?" Curr Opin Plant Biol **14**(6): 632-641.
- Christensen, C. A., S. W. Gorsich, et al. (2002). "Mitochondrial GFA2 is required for synergid cell death in Arabidopsis." Plant Cell **14**(9): 2215-2232.
- Christensen, C. A., E. J. King, et al. (1997). "Megagametogenesis in Arabidopsis wild type and the Gf mutant." Sexual Plant Reproduction **10**(1): 49-64.
- Christensen, C. A., S. Subramanian, et al. (1998). "Identification of gametophytic mutations affecting female gametophyte development in Arabidopsis." Dev Biol **202**(1): 136-151.
- Denninger, P., A. Bleckmann, et al. (2014). "Male-female communication triggers calcium signatures during fertilization in Arabidopsis." Nat Commun **5**: 4645.
- Drews, G. N. and R. Yadegari (2002). "Development and function of the angiosperm female gametophyte." Annual Review of Genetics **36**: 99-124.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Faure, J. E., N. Rotman, et al. (2002). "Fertilization in Arabidopsis thaliana wild type: developmental stages and time course." Plant J **30**(4): 481-488.
- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Hamamura, Y., S. Nagahara, et al. (2012). "Double fertilization on the move." Curr Opin Plant Biol **15**(1): 70-77.

- Hamamura, Y., M. Nishimaki, et al. (2014). "Live imaging of calcium spikes during double fertilization in Arabidopsis." Nat Commun **5**: 4722.
- Hamamura, Y., C. Saito, et al. (2011). "Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in Arabidopsis thaliana." Curr Biol **21**(6): 497-502.
- Huck, N., J. M. Moore, et al. (2003). "The Arabidopsis mutant *feronia* disrupts the female gametophytic control of pollen tube reception." Development **130**(10): 2149-2159.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Kasahara, R. D., D. Maruyama, et al. (2012). "Fertilization recovery after defective sperm cell release in Arabidopsis." Curr Biol **22**(12): 1084-1089.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.
- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 function as male factors that control pollen tube-synergid interaction in Arabidopsis thaliana fertilization." PLoS Genet **9**(11): e1003933.
- Lindner, H., L. M. Muller, et al. (2012). "CrRLK1L receptor-like kinases: not just another brick in the wall." Curr Opin Plant Biol **15**(6): 659-669.
- Ngo, Q. A., H. Vogler, et al. (2014). "A calcium dialog mediated by the FERONIA signal transduction pathway controls plant sperm delivery." Dev Cell **29**(4): 491-500.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro." BMC Plant Biol **6**: 7.

- Palanivelu, R. and T. Tsukamoto (2012). "Pathfinding in angiosperm reproduction: pollen tube guidance by pistils ensures successful double fertilization." Wiley Interdiscip Rev Dev Biol **1**(1): 96-113.
- Rotman, N., F. Rozier, et al. (2003). "Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*." Current Biology **13**(5): 432-436.
- Sandaklie-Nikolova, L., R. Palanivelu, et al. (2007). "Synergid cell death in *Arabidopsis* is triggered following direct interaction with the pollen tube." Plant Physiol **144**(4): 1753-1762.
- Schiott, M., S. M. Romanowsky, et al. (2004). "A plant plasma membrane Ca^{2+} pump is required for normal pollen tube growth and fertilization." Proc Natl Acad Sci U S A **101**(25): 9502-9507.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in *Arabidopsis*." Plant Cell **2**(8): 755-767.
- Song, X., L. Yuan, et al. (2014). "Antipodal cells persist through fertilization in the female gametophyte of *Arabidopsis*." Plant Reprod **27**(4): 197-203.
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*." PLoS Biol **10**(12): e1001449.
- Tsukamoto, T., Y. Qin, et al. (2010). "A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in *Arabidopsis thaliana* double fertilization and early seed development." Plant J **62**(4): 571-588.
- Twell, D., T. M. Klein, et al. (1989). "Transient Expression of Chimeric Genes Delivered into Pollen by Microprojectile Bombardment." Plant Physiol **91**(4): 1270-1274.
- Volz, R., J. Heydlauff, et al. (2013). "Ethylene signaling is required for synergid degeneration and the establishment of a pollen tube block." Dev Cell **25**(3): 310-316.
- von Besser, K., A. C. Frank, et al. (2006). "*Arabidopsis* *HAP2(GCS1)* is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.

Chapter 4:
Three MYB transcription factors control pollen tube differentiation
required for sperm release

Alexander R. Leydon¹, Kristin M. Beale¹, Karolina Woroniecka¹, Elizabeth Castner¹, Jefferson Chen¹, Casie Horgan¹, Ravishankar Palanivelu², and Mark A. Johnson¹

¹Department of Molecular Biology, Cell Biology, and Biochemistry, Brown University, Providence, RI 02912, USA.

²School of Plant Sciences, University of Arizona, Tucson, AZ, 85721, USA.

This study was published in Current Biology (2013) Jul 8;23(13):1209-14

Summary

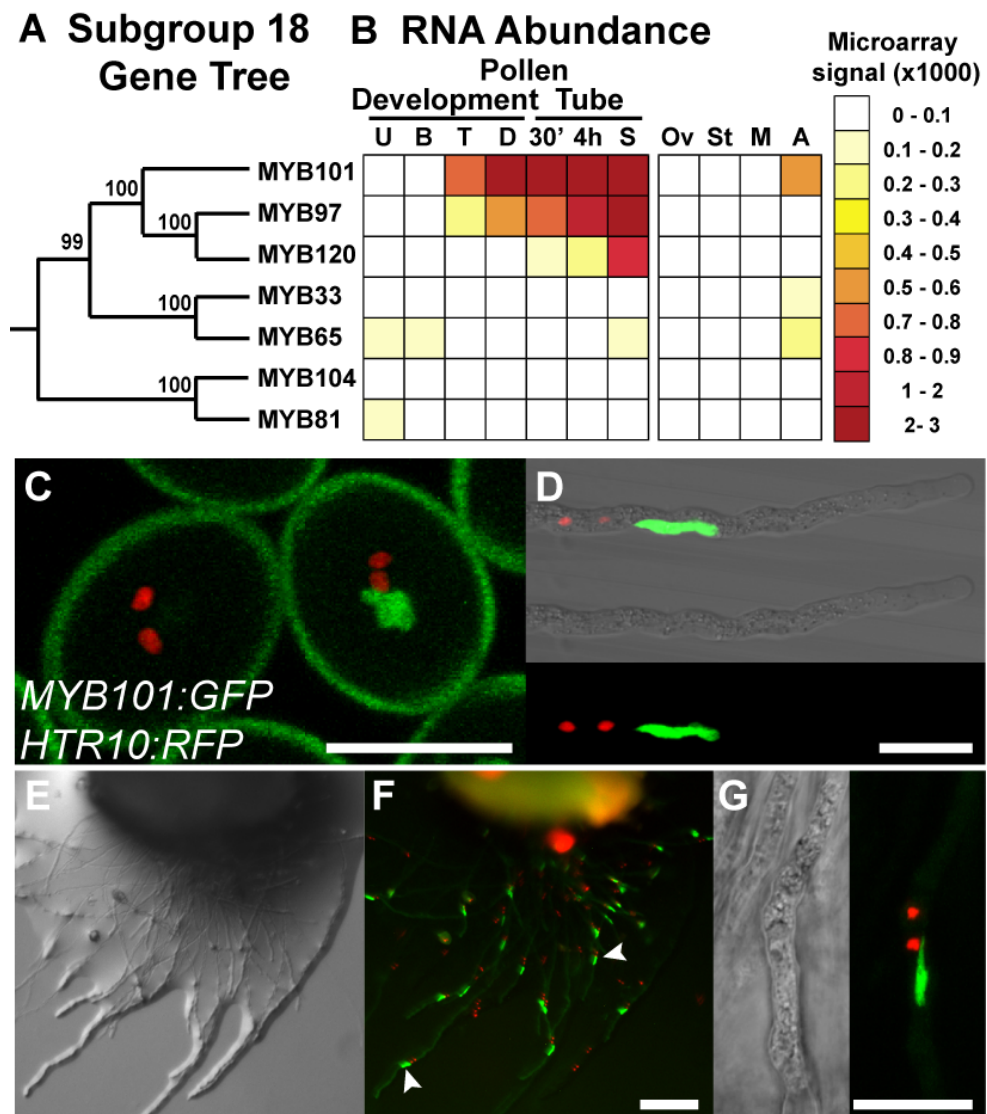
In flowering plants, immotile sperm cells develop within the pollen grain and are delivered to female gametes by a pollen tube (Kim et al. 2003; Berger et al. 2011). Upon arrival at the female gametophyte, the pollen tube stops growing and releases sperm cells for successful fertilization (Kessler et al. 2011). Several female signaling components essential for pollen tube reception have been identified (Huck et al. 2003; Rotman et al. 2003; Escobar-Restrepo et al. 2007; Boisson-Dernier et al. 2008; Capron et al. 2008; Rotman et al. 2008; Kessler et al. 2010; Tsukamoto et al. 2010); however, male components remain unknown. We show that the expression of three closely related MYB transcription factors is induced in pollen tubes by growth in the pistil. Pollen tubes lacking these three transcriptional regulators fail to stop growing in synergids, specialized cells flanking the egg cell that attract pollen tubes (Higashiyama et al. 2001; Kasahara et al. 2005; Marton et al. 2005; Okuda et al. 2009; Takeuchi et al. 2012) and degenerate upon pollen tube arrival (Faure et al. 2002; Sandaklie-Nikolova et al. 2007). *myb* triple mutant pollen tubes also fail to release their sperm cargo. We define a suite of pollen tube-expressed genes regulated by these critical MYBs and identify transporters, carbohydrate active enzymes, and small peptides as candidate molecular mediators of pollen-female interactions necessary for flowering plant reproduction. Our data indicate that de novo transcription in the pollen tube nucleus during growth in the pistil leads to pollen tube differentiation required for release of sperm cells.

Results and Discussion

MYB97, 101, and 120 are localized to the pollen tube nucleus. Microarray comparisons of the transcriptomes of pollen tubes grown in vitro with those grown through pistil tissue showed the pollen tube transcriptome is dramatically remodeled in response to the pistil and that multiple transcription factors are induced (Qin et al. 2009). We proposed that these transcription factors control expression of genes required for pollen tube - pistil interactions (Qin et al. 2009). We focused our attention on *MYB120* because its expression was higher in pollen tubes grown through pistil tissue relative to pollen tubes grown in vitro (Qin et al. 2009). *MYB120* is a member of a subgroup of the >130 Arabidopsis R2-R3 MYBs called S18 (Dubos et al. 2010). We integrated the phylogeny of the Arabidopsis S18 MYB family with the expression pattern of these genes during pollen development and pollen tube growth and found that *MYB97*, *MYB101*, and *MYB120* formed a closely related, pollen tube-expressed sub-clade (Figure 1A,B).

MYB97:GFP, *MYB101:GFP*, and *MYB120:GFP* fusion proteins, expressed from their native promoters in transgenic plants accumulated in the pollen tube nucleus during pollen tube growth through the pistil (Figure 1C - 1R). *MYB101:GFP* was detected in the vegetative nucleus of mature pollen grains and pollen tubes (Figures 1C – 1G and 1R). *MYB97:GFP* was detected only in the vegetative nucleus of pollen tubes grown in vitro or through pistil tissue (Figures 1H-1L); the *MYB120:GFP* signal was the weakest and only detected in

the vegetative nucleus of pollen tubes that had grown through pistil tissue (Figures 1M-1Q). These patterns of protein accumulation in the pollen tube nucleus reflect mRNA accumulation patterns and are consistent with the hypothesis that this group of MYBS regulates pollen tube gene expression in response to growth through the pistil.



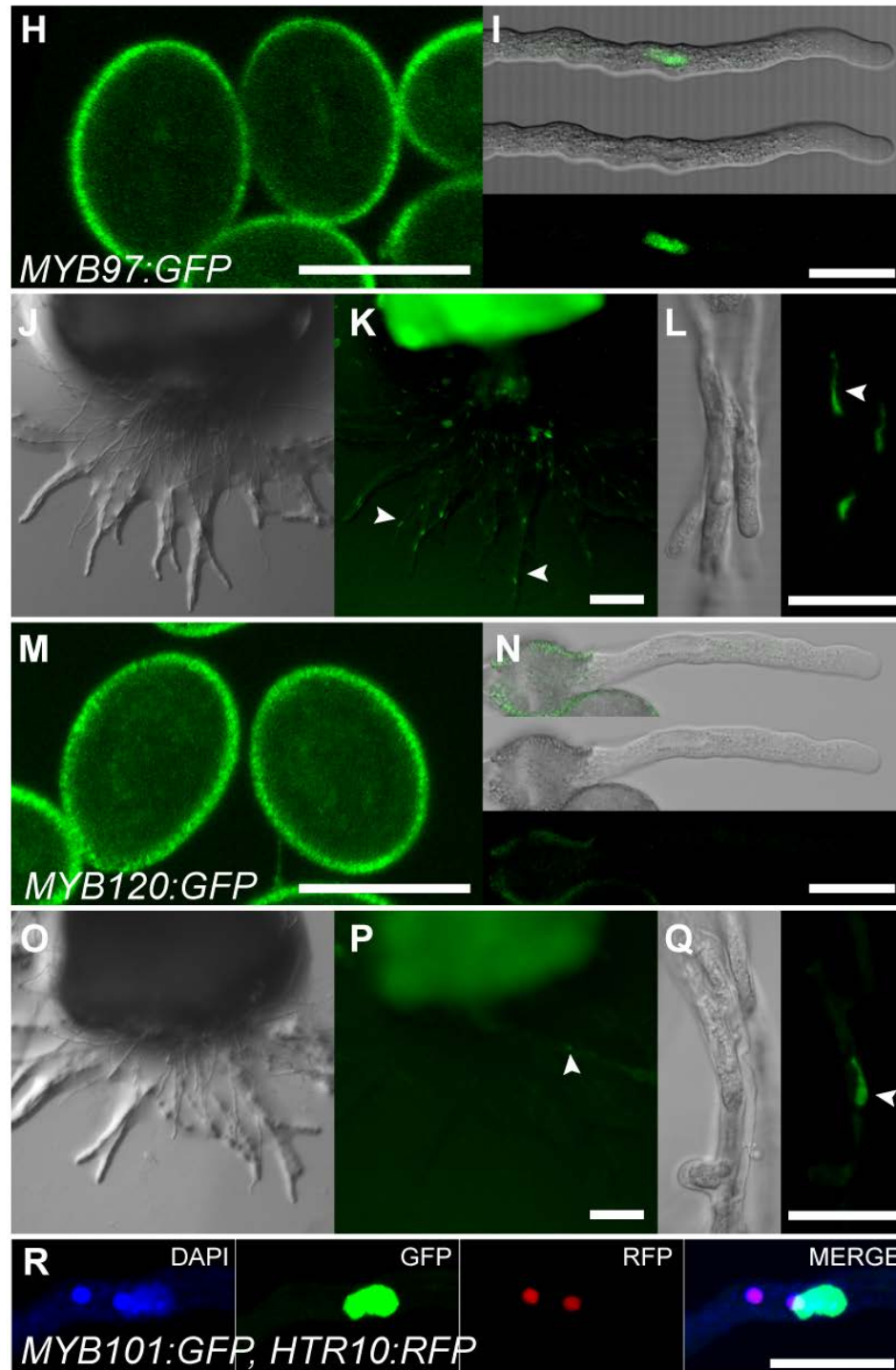


Figure 1. MYB97, MYB101, and MYB120 localize to the growing pollen tube nucleus. (A) Phylogeny of the S18 group of MYBs (bootstrap values at branches). (B) Expression heatmap of S18 MYBs. Pollen development: U, Unicellular; B, Bicellular, T, Tricellular(Honys et al. 2004) ; D, Dry Pollen. Pollen tube: 30', 30' in vitro grown pollen; 4h - 4 hour in vitro grown pollen; S, Semi in vivo grown pollen (Qin et al. 2009). Ov, Ovary; St, Stigma(Swanson et al. 2005),

M, maximum sporophytic expression from 7 tissues; A, Anther (Schmid et al. 2005). (C, H, M) Hydrated pollen grains of plants hemizygous for *MYB:GFP* reporter constructs. *HTR10:RFP* homozygous plants were transformed with *MYB101:GFP*, *qrt1-2* plants were transformed with *MYB97:GFP* or *MYB120:GFP*. (D, I, N) Pollen tubes grown for 6 hours in vitro. (E, J, O) DIC and (F, K, P) Epifluorescence images of semi-in vivo grown *MYB:GFP* pollen. (G, L, Q) Confocal micrographs of semi-in vivo grown *MYB:GFP* pollen tubes. (R) DAPI staining of *MYB101:GFP*, *HTR10:RFP* pollen tubes grown in vitro.

***myb* triple mutant pollen tubes reach ovules, but fail to burst and do not release sperm.** We identified T-DNA insertions (Sessions et al. 2002; Alonso et al. 2003) in *MYB97*, *MYB101*, and *MYB120* that disrupted mRNA accumulation (Figure S1A,B). Analysis of seed production in wild type, single, and multiple mutants showed that only plants lacking expression of *MYB97*, *MYB101* and *MYB120* (*myb97-1*, *myb101-4*, *myb120-3*; *myb* triple mutant hereafter) had reduced seed set and this reduction is pollen specific (Figure S1C-F). Expression of either *MYB97:GFP*, *MYB101:GFP*, *MYB120:GFP*, or a *MYB120* genomic clone rescued the *myb* triple mutant seed set defect (Figure S2A), indicating the seed set defect is the result of loss of *MYB* function and that GFP fusion constructs (Figure 1) are functional. *myb* triple mutant pollen development is normal and pollen tube growth is comparable to wild type either in vitro or in a semi-in vivo assay (Figure S1H-K); however *myb* triple mutant pollen tubes appeared to overgrow in wild-type ovules (Figure S1G). These results point to a role for these three MYBs in regulating interactions between the pollen tube and the ovule or female gametophyte.

To quantitatively assess pollen tube - ovule interactions using confocal microscopy, we produced *myb* triple mutants that express green fluorescent protein (GFP) in the pollen tube cytoplasm (*LAT52:GFP*, (Twell et al. 1990) and crossed this pollen to *male sterile1 (ms1)* pistils. When ovules were targeted by wild-type pollen tubes expressing GFP, a diffuse green fluorescence pattern, indicative of pollen tube burst, was observed in one of the two synergids (Figure 2A). However, approximately 70% of ovules targeted by *myb* triple mutant pollen tubes contained intact (unburst) coiling pollen tubes and lacked the diffuse green fluorescence pattern observed following pollen tube burst (Figures 2B,C).

We used a sperm cell-specific histone variant fused to RFP (*HTR10:HTR10:mRFP*) to examine sperm cell release by *myb* triple mutant pollen tubes (Ingouff et al. 2007; Hamamura et al. 2011; Beale et al. 2012). We crossed wild-type or *myb* triple mutant pollen expressing *HTR10:HTR10:mRFP* to females carrying a nuclear localized GFP reporter (*ACT11:MSI1:GFP*, (Ingouff et al. 2007) that marks female gametophyte nuclei (Figure 2D). When triple mutant pollen was used, ~70% of ovules contained sperm nuclei that were compact, close together, and did not co-localize with either female gamete nucleus (Figures 2F-H). In contrast, nearly all wild-type sperm nuclei had separated from each other and fused with female gamete nuclei (Figure 2E,H). These data suggest that gamete fusion does not occur in ovules targeted by *myb* triple mutants because sperm are not released from *myb* triple mutant pollen tubes and therefore cannot fuse with their female partners. We propose that

pollen tube genes regulated by MYB97, MYB101, and MYB120 are essential for pollen tube growth arrest and sperm cell release in the female gametophyte.

We observed the presence of multiple un-fused sperm cell pairs in ovules targeted by *myb* triple mutant pollen tubes (Figure 2G), and multiple aniline blue-stained pollen tubes entering a single ovule (Figure S1G). This is consistent with our observation that sperm cells are not released in these ovules and the recent findings that fusion between male and female gametes is required to prevent multiple pollen tubes from targeting a single ovule (Beale et al. 2012; Kasahara et al. 2012).

To analyze the relationship between entry and growth of *myb* triple mutant pollen tubes and synergid degeneration, we examined interactions between *myb* triple mutant pollen tubes expressing a red fluorescent protein (*LAT52:DsRed*, Francis et al. 2007) and female gametophytes expressing *ACT11:MSI1:GFP*. Synergid degeneration was defined as loss of discrete nuclear *ACT11:MSI1:GFP* signal (Figure 2I, Ingouff et al. 2007; Beale et al. 2012).

wild-type *LAT52:GFP* or *myb* triple mutant *LAT52:GFP* pollen 12 hours prior to dissection and imaging by confocal microscopy. (A) Wild-type GFP+ pollen tubes target the micropyle (m) and burst in the female gametophyte yielding a diffuse green signal (arrow). (B) *myb* triple mutant GFP+ target ovules, overgrow (arrowhead) and fail to burst, with a contained GFP signal inside the pollen tube (inset). Scale bars, 50µm. (C) Quantitative analysis of pollen tube burst by confocal microscopy. The percentage of ovules receiving a pollen tube with a normal burst or a coiling phenotype is shown as a percentage (+/-sd). P-value <0.005, student's t-test. (D) Wild-type *ACT11:MSI1:GFP* ovule with position of micropyle (m) and funiculus (fn) indicated. Inset: sn, synergid nucleus; ecn, egg cell nucleus; ccn, central cell nucleus. (E-G) First panel, GFP and DsRed channels merged; middle panel, DsRed channel; last panel, GFP Channel. (E) Wild-type RFP+ sperm nuclei (arrows) undergo double fertilization. (F,G) *myb* triple mutant RFP+ sperm nuclei remain condensed and associated (arrowheads). (G) Multiple pairs of unfused sperm (arrowheads) were observed *myb* triple mutant pollinations. Scale bars, 50µm. (H) Quantitative analysis of double fertilization. 1 UFP, ovules with one pair of unfused sperm; 2 UFP, two pairs of unfused sperm; 1 UFP 1 FP, one unfused pair and one fused pair of sperm; 1 FP, one fused pair of sperm. (I-L) Wild-type *ACT11:MSI1:GFP* pistils were pollinated with wild-type *LAT52:DsRed* or *myb* triple mutant, *LAT52:DsRed* and allowed to grow for 12h. (I) Wild-type, *LAT52:DsRed* pollen tubes targeting wild-type, *ACT11:MSI1:GFP* ovules (dsn, degenerated synergid; sn, persisting synergid nucleus), Inset is GFP channel alone. (J) Coiling *myb* triple pollen tubes with two intact synergid nuclei, (K) one intact synergid or, (L) two degenerated synergids. Scale bars, 50µm. (M) Quantitative analysis of synergid degeneration. Percent of ovules targeted by pollen tubes with; 2 SN, two persisting synergid nuclei; 1 SN, one persisting synergid nucleus; 0 SN, no persisting synergid nuclei; N, total targeted ovules.

We consistently observed two GFP-positive synergid nuclei in untargeted *ACT11:MSI1:GFP* ovules (Figure 2D). However, we observed degeneration of one synergid nucleus that co-localized with a diffuse *DsRed* signal from the pollen tube cytoplasm when wild-type *LAT52:DsRed* pollen tubes were received by *ACT11:MSI1:GFP* ovules. The discharged wild-type pollen tube cytoplasm filled the position vacated by the degenerated synergid and formed a characteristic hook around the egg cell (Figure 2I). Importantly, we never observed synergid degeneration without pollen tube burst in crosses with wild

type, supporting the hypothesis that a synergid cell degenerates soon after pollen tube arrival. In contrast, we observed a dramatic increase in the frequency of ovules (38%) containing two intact synergid nuclei in the presence of a coiling *myb* triple mutant pollen tube (Figure 2J,M). Interestingly, ~50% of ovules contained a coiling *myb* triple mutant pollen tube(s) that had triggered synergid degeneration, apparently without pollen tube burst (Figures 2K-M). It is possible that mechanical stress from pollen tube overgrowth may induce degeneration of one or both synergids (Figure 2K,L). However, a mechanical stress hypothesis for synergid degeneration is not consistent with the finding that two synergids frequently persist in the presence of a coiling pollen tube(s) (Figure 2J,M). These data suggest the possibility that signaling from the pollen tube, potentially disrupted in *myb* triple mutants, triggers synergid degeneration.

MYB97, MYB101, and MYB120 regulate pollen tube gene expression.

To identify the pollen tube-expressed genes regulated by *MYB97*, *MYB101*, and *MYB120* as tubes grow through the pistil, we performed microarray analysis (Affymetrix ATH1, three biological replicates) on *ms1* pistils pollinated with either wild-type or *myb* triple mutant pollen. We identified differentially expressed genes eight hours after pollination and compared these patterns with those observed in unpollinated pistils. This approach facilitates analysis of pollen tube gene expression in the context of an intact pistil. We identified 48 genes with significantly different transcript abundance in pistils pollinated with *myb* triple mutant compared to pistils pollinated with wild type (45 were lower in *myb* triple

mutants [down]; 3 were higher [up]) (Table S1). Of 45 genes that were down in pistils pollinated by *myb* triple mutants, only three had obvious expression in unpollinated pistils (Table S1), indicating that microarray analysis of pollinated pistils detected primarily pollen tube-expressed genes that are potentially regulated by *MYB97*, *MYB101*, and *MYB120* during pollen tube growth through the pistil.

We analyzed expression patterns during pollen development and tube growth for the 11 genes that were statistically different and at least four-fold lower in *myb* triple mutant pollinations versus wild type (Figure 3A). We compared our data with previously published pollen (Honys and Twell 2004; Qin et al. 2009), sperm cell (Borges et al. 2008), and sporophytic (Schmid et al. 2005; Swanson et al. 2005) microarray data. All 11 genes have their peak expression in pollen tubes that have grown through pistil tissue (Figure 3A). Reverse-transcription followed by real time quantitative PCR (RT-qPCR) analysis indicated that MYB activity is required for mRNA accumulation in pollinated pistils for each of these 11 genes (Figure S3). We conclude these 11 genes are either direct or indirect targets of MYB activation in the pollen tube as it grows through the pistil.

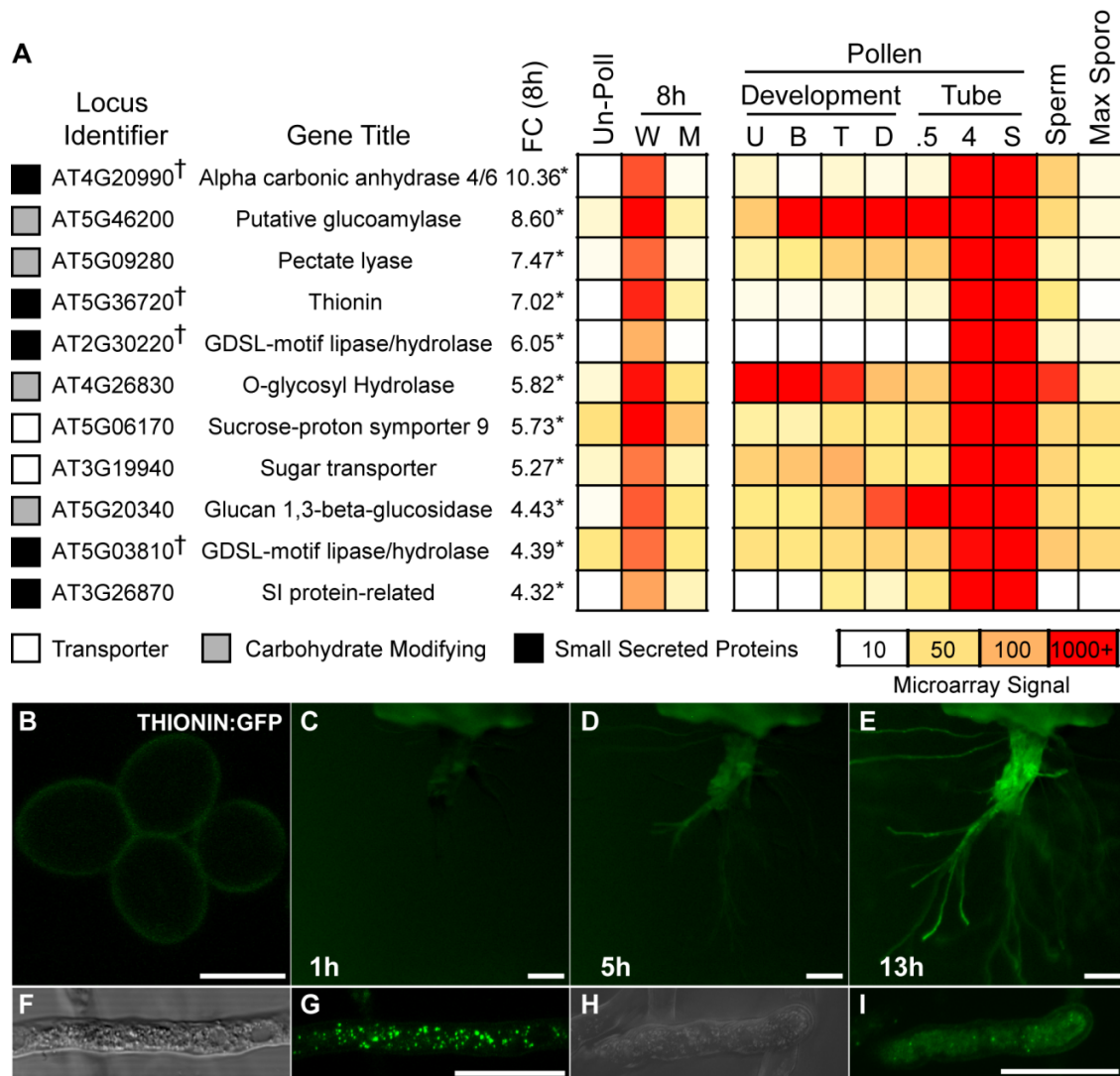


Figure 3. MYB97, MYB101, and MYB120 regulate gene expression during pollen pistil interactions. (A) Expression heat map of genes identified as differentially expressed by microarray analysis. *ms1* pistils were left un-pollinated (Un-Poll), or pollinated by either wild-type (W) or *myb* triple mutant (M) pollen and grown for 8 hours. The genes identified (Locus Identifier) are indicated with the gene name or predicted function (Gene Title). [†], Array ID identifies two. The fold change (FC) between wild-type and *myb* triple mutant pollinations is shown, with asterisks (*) indicating fold changes that were statistically significant ($P < 0.05$). Previously published data from pollen grain development (Honys and Twell 2004), pollen tube growth (Qin et al. 2009), sperm cells (Borges et al. 2008), and sporophytic tissues (Schmid et al. 2005) are included for comparison. Pollen development: U, Unicellular; B, Bicellular; T, Tricellular; D, Dry Pollen. Pollen tube: 0.5, 30' in vitro grown pollen; 4, 4 hour in vitro grown pollen; S, Semi in vivo grown pollen tubes; Sperm, Isolated sperm cells, M - Max sporophytic expression from 7 tissue types.

Genes regulated by pollen tube MYBs include transporters, carbohydrate active enzymes, and small proteins.

The 11 potentially MYB-regulated genes identified above were enriched for transmembrane transporters (2/11), carbohydrate active enzymes (4/11), and small, secreted proteins (5/11, black squares Figure 3A). These gene categories are also enriched across all 48 differentially expressed genes (transmembrane transporters 7/48, carbohydrate active enzymes 6/48, and small secreted proteins 17/48, Table S1). One of these small secreted proteins is a cysteine-rich thionin (AT5G36720, CRP 2460, Silverstein et al. 2007) that is specifically expressed in pollen tubes and requires *MYB97*, *MYB101*, and *MYB120* for expression (Figures 3A, S3). Thionins have been shown to have antimicrobial properties attributed in part to their ability to form membrane pores (Florack et al. 1994; Stec 2006; Oard 2011), a function that could be involved in mechanisms required for pollen tube burst. THIONIN:GFP signal was undetectable in pollen grains (Figure 3B); however, we detected strong GFP signal in pollen tubes grown in the semi in vivo assay for at least 5 hours (Figures 3C-E). When we examined semi in vivo grown pollen tubes using confocal microscopy we observed THIONIN:GFP in puncta throughout the body of the pollen tube cytoplasm, suggesting localization in the secretory pathway (Figures 3F-I).

Conclusions

Loss of *MYB97*, *MYB101*, and *MYB120* function results in lack of expression of a suite of pollen tube expressed genes (Figure 3 and Table S1) and disrupts the

ability of the pollen tube to stop growing and burst once it reaches a synergid cell (Figure 2). We also found that *MYB97*, *MYB101*, and *MYB120* expression is induced by growth through pistil tissue (Figure 1). These findings suggest that the ability of pollen tubes to respond to female signals required for sperm cell release is activated by growth through the pistil and controlled by this closely related group of transcription factors. MYB-regulated genes could be required for any or all of the following critical activities: pollen tube perception of female reception signals, recognition of pollen tube arrival by the female, or the ability of the pollen tube to respond to these signals and release sperm cells. It has been demonstrated that pollen tube - female gametophyte recognition fails in interspecific crosses of *Rhododendron* (Williams et al. 1986) or *Arabidopsis* (Escobar-Restrepo et al. 2007), resulting in pollen tube coiling within the exotic ovule. Further analysis of pollen tube genes regulated by *MYB97*, *MYB101*, and *MYB120* will provide insight into the determinants of species specificity in flowering plant mating and the male components of pollen tube - female gametophyte signaling required to release sperm cells.

Experimental Procedures

Plant Growth

Procedures for selection of antibiotic and/or herbicide resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; von Besser et al. 2006; Qin et al. 2009; Wong et al. 2010).

Genetic Analysis

Full-length *MYB101* and *MYB97* cDNA clones were available (NM_128805, NM_118827), but *MYB120* cDNAs had not been cloned (Genbank Accession #: KC544014). *ACT11:MSI1:GFP*, *HTR10:HTR10:mRFP*, and *LAT52:GFP* plants were obtained from Frederic Berger (National University of Singapore, (Ingouff et al. 2007). *LAT52:DsRed* (Francis et al. 2007) plants were obtained from Greg Copenhaver (UNC, Chapel Hill). SALK and SAIL lines (*Col-0* accession) and *ms1/MS1* (CS75, Landsberg ecotype) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

Imaging

Procedures for imaging pistils containing aniline blue-stained pollen tubes (Mori et al. 2006) or pollen tubes expressing fluorescent proteins (Beale et al. 2012) have been described.

Accessing Microarray Data

Raw data (.CEL and CHP files) from 9 microarrays reported in this study have been deposited in Gene Expression Omnibus public repository (Accession #: GSE44405).

Acknowledgements

NSF Grant IOS-1021917 funded this work. We thank Frederic Berger, Gregory Copenhaver, and Daphne Preuss for generously providing transgenic plants expressing markers. JC, KW, EC were funded by the HHMI-Brown Summer Scholars program. The Brown University Genomics Core Facility is supported by grant: 8 P30 GM10103410.

References

- Alonso, J. M., A. N. Stepanova, et al. (2003). "Genome-wide insertional mutagenesis of *Arabidopsis thaliana*." Science **301**(5633): 653-657.
- Beale, K. M., A. R. Leydon, et al. (2012). "Gamete Fusion Is Required to Block Multiple Pollen Tubes from Entering an *Arabidopsis* Ovule." Curr Biol.
- Berger, F. and D. Twell (2011). "Germline specification and function in plants." Annu Rev Plant Biol **62**: 461-484.
- Boisson-Dernier, A., S. Frietsch, et al. (2008). "The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition." Curr Biol **18**(1): 63-68.
- Borges, F., G. Gomes, et al. (2008). "Comparative transcriptomics of *Arabidopsis* sperm cells." Plant Physiol **148**(2): 1168-1181.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in *Arabidopsis* Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Dubos, C., R. Stracke, et al. (2010). "MYB transcription factors in *Arabidopsis*." Trends Plant Sci **15**(10): 573-581.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Faure, J. E., N. Rotman, et al. (2002). "Fertilization in *Arabidopsis thaliana* wild type: developmental stages and time course." Plant J **30**(4): 481-488.
- Florack, D. E. and W. J. Stiekema (1994). "Thionins: properties, possible biological roles and mechanisms of action." Plant Mol Biol **26**(1): 25-37.
- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Hamamura, Y., C. Saito, et al. (2011). "Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in *Arabidopsis thaliana*." Curr Biol **21**(6): 497-502.
- Higashiyama, T., S. Yabe, et al. (2001). "Pollen tube attraction by the synergid cell." Science **293**(5534): 1480-1483.
- Honys, D. and D. Twell (2004). "Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*." Genome Biol **5**(11): R85.
- Huck, N., J. M. Moore, et al. (2003). "The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception." Development **130**(10): 2149-2159.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.

- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Kasahara, R. D., D. Maruyama, et al. (2012). "Fertilization recovery after defective sperm cell release in Arabidopsis." Curr Biol **22**(12): 1084-1089.
- Kasahara, R. D., M. F. Portereiko, et al. (2005). "MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis." Plant Cell **17**(11): 2981-2992.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.
- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Kim, S., J. C. Mollet, et al. (2003). "Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism." Proc Natl Acad Sci U S A **100**(26): 16125-16130.
- Marton, M. L., S. Cordts, et al. (2005). "Micropylar pollen tube guidance by egg apparatus 1 of maize." Science **307**(5709): 573-576.
- Mori, T., H. Kuroiwa, et al. (2006). "GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization." Nat Cell Biol **8**(1): 64-71.
- Oard, S. V. (2011). "Deciphering a mechanism of membrane permeabilization by alpha-hordothionin peptide." Biochim Biophys Acta **1808**(6): 1737-1745.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Rotman, N., M. Gourgues, et al. (2008). "A dialogue between the SIRENE pathway in synergids and the fertilization independent seed pathway in the central cell controls male gamete release during double fertilization in Arabidopsis." Mol Plant **1**(4): 659-666.
- Rotman, N., F. Rozier, et al. (2003). "Female control of male gamete delivery during fertilization in Arabidopsis thaliana." Current Biology **13**(5): 432-436.
- Sandaklie-Nikolova, L., R. Palanivelu, et al. (2007). "Synergid cell death in Arabidopsis is triggered following direct interaction with the pollen tube." Plant Physiol **144**(4): 1753-1762.
- Schmid, M., T. S. Davison, et al. (2005). "A gene expression map of Arabidopsis thaliana development." Nat Genet **37**(5): 501-506.
- Sessions, A., E. Burke, et al. (2002). "A high-throughput Arabidopsis reverse genetics system." Plant Cell **14**(12): 2985-2994.

- Silverstein, K. A., W. A. Moskal, Jr., et al. (2007). "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants." Plant J **51**(2): 262-280.
- Stec, B. (2006). "Plant thionins--the structural perspective." Cell Mol Life Sci **63**(12): 1370-1385.
- Swanson, R., T. Clark, et al. (2005). "Expression profiling of Arabidopsis stigma tissue identifies stigma-specific genes." Sexual Plant Reproduction **18**(4): 163-171.
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis." PLoS Biol **10**(12): e1001449.
- Tsukamoto, T., Y. Qin, et al. (2010). "A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development." Plant J **62**(4): 571-588.
- Twell, D., J. Yamaguchi, et al. (1990). "Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis." Development **109**(3): 705-713.
- von Besser, K., A. C. Frank, et al. (2006). "Arabidopsis *HAP2(GCS1)* is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of Rhododendron Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.

Supplemental Inventory

Figure S1. A summary of T-DNA insertion lines used in this study and their impact on RNA abundance, seed set, and pollen tube function related to Figure 2.

Figure S2. MYB:GFP fusion constructs are capable of rescuing the *myb* triple-mutant phenotype, related to Figure 1.

Figure S3. RT-qPCR analysis of MYB-regulated target genes, related to Figure 3.

Table S1. (*See separate Excel workbook.*) Differentially regulated genes and small peptides identified by Microarray Analysis, related to Figure 3.

Supplemental Experimental Procedures

Supplemental References

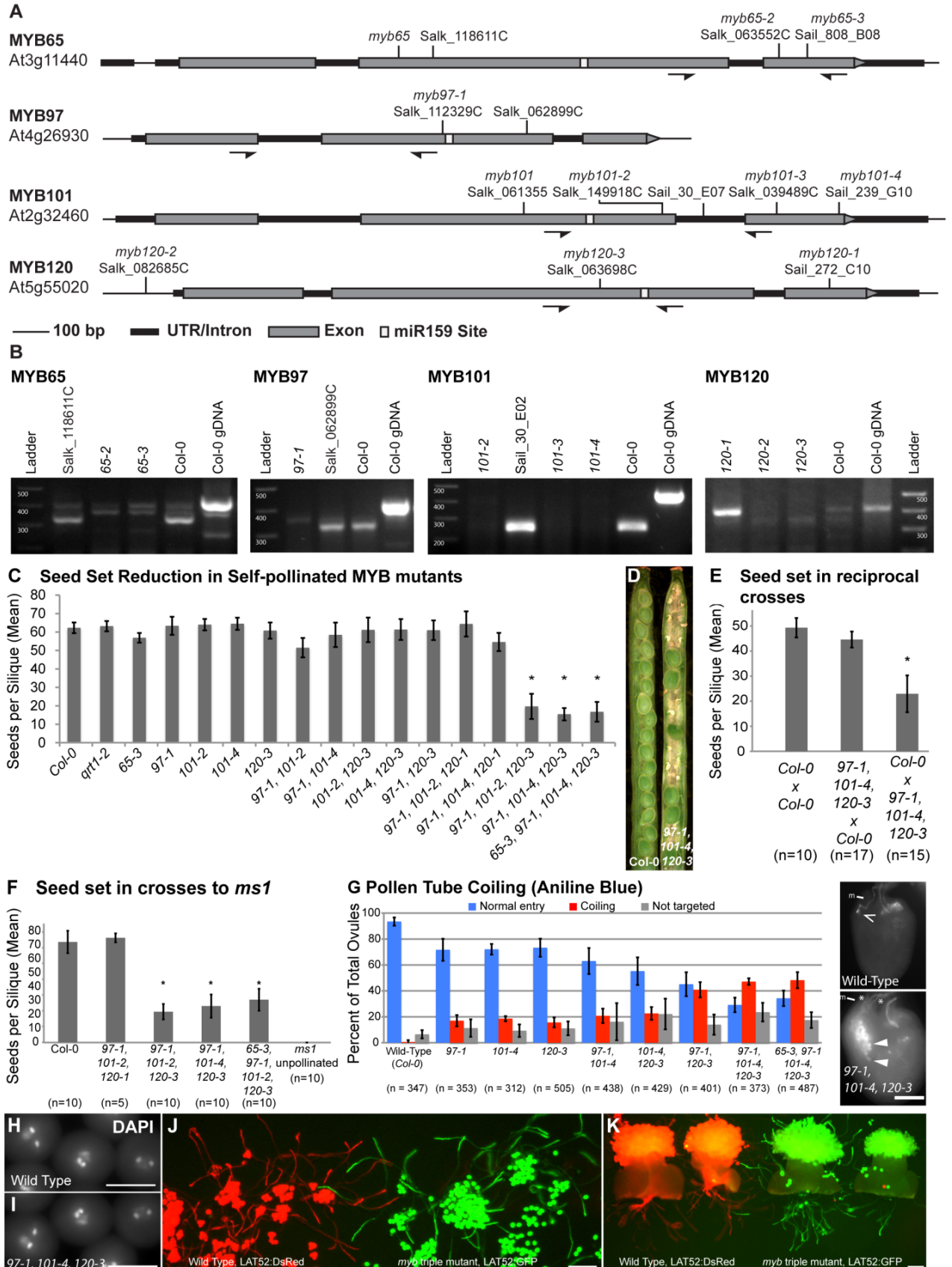


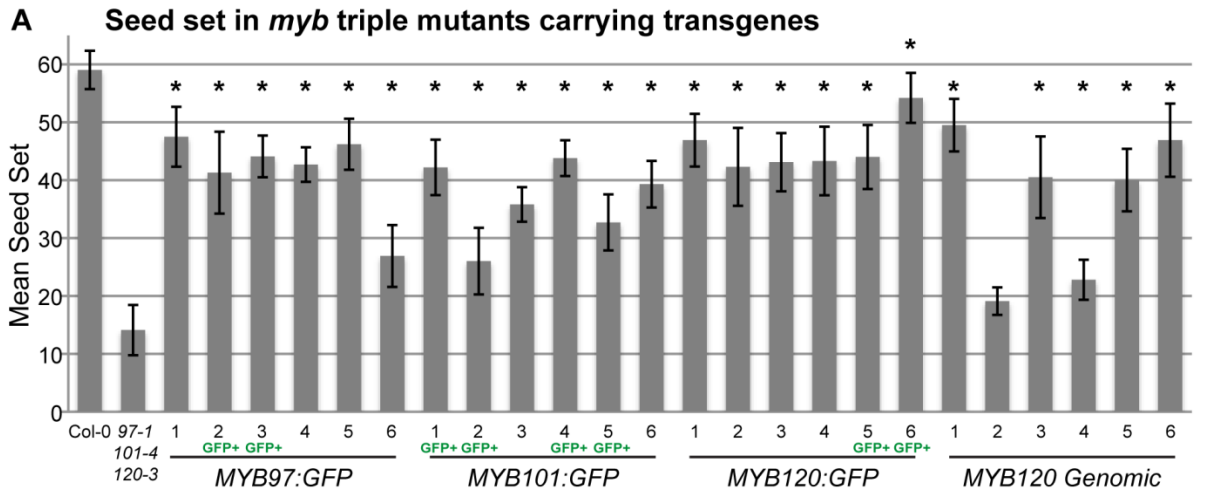
Figure S1.

Figure S1. A Summary of T-DNA Insertion Lines Used in This Study and Their Impact on RNA Abundance, Seed Set, and Pollen Tube Function, Related to Figure 2

(A) Gene maps utilized in this study. Allele designations were given only to insertion lines for which a PCR product could confirm the insertion location in the gene of interest. Arrows denote the location of primers used for RT-PCR analysis in B. Untranslated regions (black bars) are based on previously published full length cDNAs or our experimental results (MYB120). Previously published MYB alleles: *myb65* (Millar et al. 2005), *myb101* (Alonso-Peral et al. 2010).

(B) RT-PCR analysis for floral cDNA. The gene being analyzed is in bold type. Genotypes being analyzed are directly above each lane. **(C)** Seed set from wild-type (Col-0, *qrt1-2*) and *myb* single, double, triple and quadruple mutants. Seed set analysis is reported as the average seed number in ten consecutive siliques on the primary bolt, excluding the first five flowers (+/- standard deviation). Asterisks indicate seed set reductions significantly different than wild-type controls (p-value < 0.001, students t-test). **(D)** Representative images of seed set in dissected siliques from wild-type (Col-0) and *myb97-1*, *myb101-4*, *myb120-3*.

(E) Reciprocal crosses using wild-type (Col-0 accession) and *myb* triple-mutant flowers identify male-specific defects in seed production. All flowers were emasculated a minimum of 12 hours before being manually pollinated and allowed to set seed. **(F)** *myb* triple mutants display drastic reductions in seed production when used as a pollen donor. *ms1* pistils were pollinated with wild-type or mutant pollen and allowed to set seeds before seeds were counted in each silique. Asterisks (*) indicate significantly different quantities of seeds produced per silique by a students t-test (P<0.001). n, number of crosses performed. **(G)** Quantitative analysis of coiling events and targeting measured by aniline blue staining. *ms1* pistils were pollinated with wild-type or *myb* triple-mutant pollen 24 hours prior to dissection and staining with aniline blue for fluorescence microscopy. Insets: Wild-type pollen tubes grow into the micropyle (m) and arrest growth (arrow) at the entrance to the female gametophyte. Multiple *myb* triple-mutant pollen tubes (asterisk) grow into the micropyle (m) and overgrow (coil) in the embryo sac (solid arrowhead). **(H and I)** Wild-type (Col-0) pollen grains (H) and *myb* triple-mutant pollen grains (I) each develop two compact sperm nuclei and a diffuse vegetative nucleus. **(J and K)** *myb* triple mutants express *LAT52:GFP* and grow normally in vitro (2 hours) and in a semi in vivo growth assay (4 hours). *LAT52:GFP* was introgressed into the *myb* triple mutant and was grown next to wild-type (*qrt1-2*, *LAT52:dsRed*) pollen in vitro (J), and in the semi in vivo condition (K).



B The *myb* triple mutant genotype is transmitted through the male germline at reduced frequency

Genotype		% Transmission	N
Single Mutants	<i>myb65-3/MYB65</i>	48.78	123 (B)
	<i>myb97-1/MYB97</i>	51.85	189 (P)
	<i>myb101-2/MYB101</i>	57.29	192 (P)
	<i>myb101-4/MYB101</i>	48.09	445 (B)
	<i>myb120-1/MYB120</i>	46.83	568 (B)
	<i>myb120-3/MYB120</i>	54.42	190 (P)
	Control Heterozygote	50.74	1078 (B)
Double Mutants	<i>myb97-1, myb101-4/MYB101</i>	49.56	428 (B)
	<i>myb120-3, myb101-4/MYB101</i>	43.49	347 (B)
	<i>myb120-3, myb97-1/MYB97</i>	45.50	189 (P)
	<i>myb97-1, myb120-1/MYB120</i>	46.09	473 (B)
	<i>myb120-3, myb65-3/MYB65</i>	46.45	394 (B)
Triple Mutants	<i>myb120-3, myb97-1, myb101-4/MYB101</i>	15.45*	261 (B)
	<i>myb120-3, myb97-1, myb101-2/MYB101</i>	17.18*	192 (P)
	<i>myb97-1, myb101-2, myb120-1/MYB120</i>	43.60	270 (B)
Quadruple Mutant	<i>myb65-3, myb101-4, myb120-3, myb97-1/MYB97</i>	17.86*	84 (P)
Rescue Lines	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB101:GFP/+ (1-1)</i>	45.17	292 (B)
	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB101:GFP/+ (1-2)†</i>	38.36	753 (B)
	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB97:GFP/+ (1-4)</i>	37.61	435 (B)
	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB97:GFP/+ (3-1)</i>	36.29	566 (B)
	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB120:GFP/+ (5-2)</i>	39.27	950 (B)
	<i>myb97-1, myb120-3, myb101-4/MYB101, MYB120:GFP/+ (1-1)</i>	40.05	565 (B)

Figure S2. MYB:GFP Fusion Constructs Are Capable of Rescuing the *myb* Triple-Mutant Phenotype, Related to Figure 1

(A) Seed set was analyzed in self-pollinated primary transformants (carrying indicated constructs) and compared to the parental *myb* triple mutant and wild type (Col-0). All values are the averages (+/- sd) of at least 10 consecutive siliques on the primary inflorescence. Data from six representative primary transformants (of 23-24 analyzed) are shown for each construct. *MYB101:GFP* lines were assayed for GFP accumulation in the vegetative nucleus of mature pollen grains. *MYB97:GFP* and *MYB120:GFP* were assayed for GFP accumulation in the vegetative nucleus of pollen tubes grown in the semi in vivo

assay. Lines that were GFP⁺ are indicated. Asterisks (*) indicate seed set increases that are statistically significantly different from the *myb* triple-mutant parent by a students t-test ($P < 0.001$).

(B) *MYB:GFP* constructs rescue transmission of the *myb* triple-mutant genotype to progeny. % Mutant transmission is the percentage of heterozygous F₁ progeny following crosses of the indicated genotype with *ms1* females; determined either by selection on Basta plates ('B') or by PCR ('P') genotyping. Transmission through the female gametophyte is not affected (1:1, *myb120-3*, *myb97-1*, *myb101-4* : *myb120-3*, *myb97-1*, *MYB101*, 50.93% triple mutant, $n = 540$). Asterisks (*) indicate transmission ratios that differ significantly from 50% transmission expected for alleles that do not affect male gametophyte function (students t-test, P -value < 0.001). †, indicates constructs that were first transformed into wild-type and then crossed into the *myb* triple-mutant background.

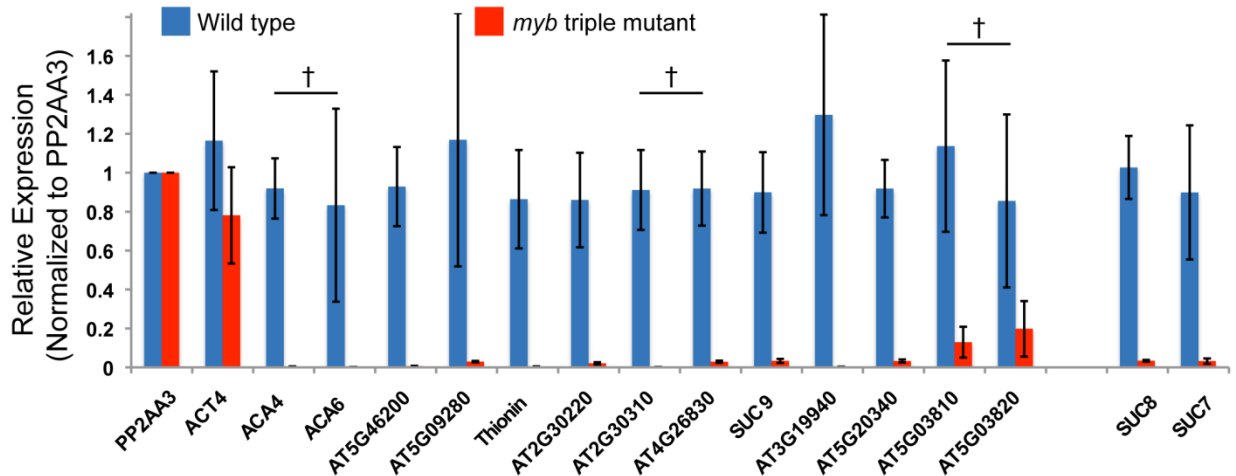


Figure S3. RT-qPCR Analysis of MYB-Regulated Genes, Related to Figure 3

RT-qPCR analysis of the 14 genes found to have the lowest mRNA abundance in *myb* triple mutants compared to wild type by microarray analysis. *SUC8* and *SUC9* were also analyzed. Three biological replicates, normalized to *PP2AA3* (AT1G13320); standard deviation is shown for normalized expression values. *ACT4* (AT5G59370) is a pollen specific actin, which was not differentially expressed between *myb* triple-mutant pollinations and wild-type pollinations. For analysis of related gene pairs gene-specific primers were designed where possible; this is denoted by a bar and † symbol above expression data.

Table S1. Differentially Regulated Genes and Small Peptides Identified by Microarray Analysis, Related to Figure 3

(See separate Excel workbook.)

Average normalized expression data are given for *ms1* unpollinated pistils, *ms1* x wild type (Col-0) 8 hours, *ms1* x *myb* triple mutant 8 hours, and selected experiments from publically available sources. Only genes with a p-value less than 0.01 are shown (Column H). Genes that are down regulated in the *myb* triple mutant are shown in rows 3-40, while genes upregulated in *myb* triple mutant are shown in rows 42-43. The table has been sorted by fold change (Wild type / *myb* triple mutant) (Column I, descending). Affymetrix probe sets associated with a single nuclear gene do not contain '_s_' in their Array Element name (Column A). Array Elements that identify multiple genes have multiple Locus identifiers (Columns C and D). RefSeq Transcript IDs are given in Column G, multiple genes are separated by ///. Publically available microarray data were renormalized and incorporated for comparison. Uninucleate microspores, Bicellular pollen, and Tricellular pollen(Honys et al. 2004). Dry Pollen, 0.5 Hour Pollen Tubes, 4 Hour Pollen Tubes, Semi in vivo(Qin et al. 2009), Sperm cells(Borges et al. 2008) Max sporophyte (Column W) represents the maximum value from the tissue types in columns Y through AF. Ovules and Stigma(Swanson et al. 2005), 7 Day Root, 17 Day Root, 8 Day Seedling, 21 Day Seedling, Rosette 17 Day(Schmid et al. 2005). Regulatory sequence analysis of differentially regulated genes was performed on the RSAT server (<http://rsat.ulb.ac.be/>) using the HvGAMYB position weight matrix(Gubler et al. 1999). The presence of a predicted site within -3000 upstream of the differentially regulated gene is indicated (HvGAMYB core site identified (Y/N)). For each predicted site the locus is shown (Locus Identifier), followed by the strand (Strand) and position relative to the start site (start, and end). Sequences were analyzed in two rounds. The first round used the default settings where the regulatory sequence was searched from -3000 to -1 bp of the start site or to the closest upstream ORF, the second searched from -3000 to -1 regardless of the inclusion of upstream ORFs. The location of the predicted HvGAMYB site with respect to the upstream ORF is indicated (before upstream ORF (Y/N)). The sequences identified are listed (sequence), followed by the weight, and measures of significance (Pval, ln_Pval, sig).

Sheet 2. S18MYB-regulated small peptides. The number of amino acids encoded by the gene model is listed (#aa, Column E), followed by the predicted localization listed on TAIR 10 (Column F). The amino acid sequence including any putative signal peptides (aa seq, Column H), was used to independently predict subcellular localization using the SignalP 4.1 server (<http://www.cbs.dtu.dk/services/SignalP/>). If the protein was predicted to contain a signal peptide this is noted (Signal peptide?, Column H, where T = true, and F

= false). The position of the signal peptide cleavage site is also noted (Cleavage site after aa#, column I). Any known Gene Ontology information from TAIR 10 have been included (GO Molecular Function, Columns J and K).

Supplemental Experimental Procedures

Seedling Selection and Plant Growth

Seeds were grown on solid Murashige and Skoog (MS) medium (MP Biomedicals, LLC, Solon, OH, USA). SAIL (Syngenta Arabidopsis Insertion Library, Sessions et al. 2002) lines were grown on solid media supplemented with 25µg/ml glufosinate ammonium (Basta; Chem service, INC, West Chester, PA, USA). *MYB:GFP* constructs were selected on solid media supplemented with 25µg/ml Hygromycin B (Research Products International, Mount Prospect, IL USA). Arabidopsis plants were grown at 21°C and received 100 µmol/m²/sec illumination for 16 hours each day (Environmental growth chambers, Chagrin Falls, OH, USA). Seedlings were transplanted to sterile 2MIX potting media (www.fafard.com) between 7 and 10 days after plating. In vitro pollen tube growth was performed as previously described (Boavida et al. 2007). Semi in vivo pollinations were performed as previously described (Palanivelu et al. 2006; Qin et al. 2009).

MYB120 cDNA Cloning

We obtained multiple identical cDNAs following amplification with RT-PCR primers (see Oligonucleotides used in this study, below) complementary to predicted 3' and 5' untranslated regions. These clones (Genbank Accession #: KC544014) contained an open reading frame (Figure S1) encoding a different C-terminus from that predicted (TAIR 10, <http://www.arabidopsis.org/>).

GFP Fusion Constructs and Genomic Clones

To generate *MYB101:GFP* and *MYB97:GFP*, genomic fragments containing the promoter (*MYB101*, 2.3kb upstream of the start codon; *MYB97*, 1.2kb), introns and exons (excluding the stop codon) were cloned into pCR2.1 (Invitrogen). These were subcloned into a pCAMBIA1300 (www.cambia.org) variant containing *GFP* and a *NOS* terminator. The *MYB120:GFP* genomic fragment was based on the incorrect TAIR 10 annotation and consequently did not include the experimentally determined third exon. *MYB120:GFP* contains the promoter (1 kb upstream of start codon) and the experimentally determined first two introns and exons and was cloned into the pENTR-D vector (Invitrogen). The *MYB120:GFP* vector was created by recombination into pMDC107 (Curtis et al. 2003) using LR Clonase II (Invitrogen). *qrt1-2*; Col-0, *HTR10:HTR10:RFP*; or *myb97-1*, *myb101-4*, *myb120-3* (Col-0) plants were transformed as previously described (Clough et al. 1998). For each construct at least 20 primary transformants were recovered by selecting for hygromycin resistance. GFP was detectable by epifluorescence microscopy in pollen grains from *MYB101:GFP* primary transformants (26/27), but not for *MYB97:GFP* (0/12) or *MYB120:GFP* (0/20).

To generate *THIONIN:GFP*, a genomic fragment containing the promoter (1 kb upstream of the start codon of *AT5G36720*) and a single exon were cloned into the pENTR-D vector (Invitrogen) and transferred to pMDC107 (Curtis and Grossniklaus 2003) using LR Clonase II (Invitrogen). The current Arabidopsis genome annotation, (TAIR10, <http://www.arabidopsis.org/>) states that

AT5G36720 is perfectly duplicated as *AT5G36805*. We suspect this to be an annotation error based on analysis of the genome sequences of other *Arabidopsis* accessions (Cao et al. 2011).

PCR to Identify T-DNA Insertion Sites

T-DNA insertion sites were identified using left border (SAIL - LB3, LB2, and/or LB1, SALK - LBb1.3) and gene-specific flanking primers (see Oligonucleotides used in this study, above), designed at <http://signal.salk.edu/tdnaprimers.2.html>) in PCR reactions. The PCR program used for this reaction was: 94°C for 5 min followed by 36 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 2 min, and a final elongation step of 72°C for 4 min.

Imaging of Pollen Tube Growth

Aniline blue analysis was performed on crosses dissected 24 hours after pollination as reported previously (Mori et al. 2006). We made the following adjustments; all fixative and exchange steps were extended to 12 hours, and aniline blue incubation was extended from overnight to a period of 4-12 days before analysis by epifluorescence microscopy on a Zeiss Axiovert 200M Fluorescence Microscope with an ultraviolet filter. For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated *ACT11:MSI1:GFP* stage 14 stigmas (Smyth et al. 1990) with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al. 2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

RNA Extraction, RT-PCR, and RT-qPCR

RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis and PCR was performed using the Access RT-PCR System (Promega). For RT-PCR, RNA was DNase treated with RNase-Free DNase (Qiagen). cDNA libraries were constructed from 2.5ug of total RNA using Super Script VILO Master Mix (random hexamer and oligo dT, Super Script III), (Invitrogen). For Real-time RT-qPCR, RNA was DNase treated using the Ambion TURBO DNA-free kit from Invitrogen (AM1907). Primers were designed at Primer3Plus (primer3plus.com/), using qPCR-optimized settings. All primers were tested to assure that they have near 100% efficiency and linearity prior to use with an ABI 7000 real time PCR

machine. RT-qPCR was performed in triplicate. The comparative Ct method (User Bulletin #2, Applied Biosystems) was used for quantification. Expression was normalized to the ubiquitous PP2AA3 gene(Czechowski et al. 2005). RT-qPCR was performed with cDNA libraries using primers of equivalent efficiency in triplicate reactions using SYBR Green qPCR Master Mix (Invitrogen) on an ABI 7000 PRISM Sequence Detection System (Applied Biosystems).

Microarray Analysis

Total RNA was extracted from unpollinated *ms1* pistils and *ms1* pistils pollinated with wild-type pollen or *myb* triple-mutant pollen and grown for 8 hours. Total RNA was extracted using the RNeasy kit (Qiagen). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific). RNA integrity was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). Hybridization and post hybridization processing was performed as per the manufacturer's instructions (Affymetrix, ATH1) by the Brown University Genomics Core Facility (www.brown.edu/Research/CGP/core/). Analysis of differential expression was performed on the Partek Genomics Suite 6.6 beta (www.partek.com). Renormalization of publicly available microarray data was performed with RMAExpress (rmaexpress.bmbolstad.com/), and compared using Microsoft Excel.

Bioinformatic Analysis

MYB97, MYB101, MYB120-regulated small peptides were analyzed for predicted signal peptides using the SignalP 4.1 server(Petersen et al. 2011). Analysis of microarray differential expression was performed on the Partek Genomics Suite 6.6 beta (www.partek.com). Renormalization of publicly available microarray data (Figure 3, Table S1) was performed with RMAExpress (rmaexpress.bmbolstad.com/).

Oligonucleotides Used in This Study			
	Gene	Primer Name	Sequence
T-DNA Identification	MYB101	SAIL 30 E07 LP	TCTCAACCATCAAAAAGGCTG
	MYB101	SAIL 30 E07 RP	ATGGGCAGGTTCCAAACTAAC
	MYB101	SALK 039489C LP	TCATCCTCGTCTAACGAGGTG
	MYB101	SALK 039489C RP	TGCAAAATTAGGTTTTGTCCC
	MYB120	SALK 063698C LP	AACTCCATCCACATCCACATC
	MYB120	SALK 063698C RP	TGTTGTGGTTCGGAAGAGAAG
	MYB120	SALK 082685C LP	GACAAAAGAGCATTGTTGCCAC
	MYB120	SALK 082685C RP	ACGTGTAACGCAGGTAACACC
	MYB97	SALK 112329C LP	TGGCAGGTATATTCCACTTGC
	MYB97	SALK 112329C RP	GTATCCTCAACAGCCCTCCTC
	MYB65	SAIL 808 B08 LP	AAAACGATGAACCAATGCAAG
	MYB65	SAIL 808 B08 RP	ACTCAGAATGTACCACGTGGC
	MYB65	SALK 063552C LP	CCAATGCAAGAAGAAGTTTGC
	MYB65	SALK 063552C RP	CATCGCAGGTAGAGGAGTCAG
	SALK LB	LBb1.3	ATTTTGCCGATTTCGGAAC
	SAIL LB	LB3	GCCTTTTCAGAAATGGATAAATAGCCT
	SAIL LB	LB2	GCTTCCTATTATATCTTCCCAAATTACC
	SAIL LB	LB1	TAGCATCTGAATTTTCATAACCAATCTC
MYB120 cDNA	MYB120	120 5pRTF2	CTCTTCTTGAATCGGCAAGG
	MYB120	1203pUTRR5	AGTTAACAGATGATTTGCAACAAT
RT-PCR analysis of mutants	MYB101	101 3pRTF	AGGGTTTCCTCCAGTGACCT
	MYB101	101 3pRTR	TGCATTTCTGTCACCCGATA
	MYB120	120 3pRTF	CCGCTCAAACCGCTACTTAC
	MYB120	SALK 063698C RP	AACTCCATCCACATCCACATC
	MYB97	97 ex1F	CCACTCTCAGCTAGGCAACA
	MYB97	97 ex2R1	GTCGGAGACGAGACAAGGAA
	MYB65	65 3pRTF2	CGCTCCGAGATTACAAGTCC
	MYB65	65 cDNAR	CAGCGACCAAACAGGAGGCAT
Genetic Constructs	MYB101:	BamHI-MYB101F1	GGATCC AGGTCCGACTCACAGTCACA
	MYB101:GFP	BamHI-MYB101F2	GGATCC GTCAACACAATGGGCTGAAG
	MYB101:GFP	Sall-Myb101R	GTCGACT GCACAGATGCTAGGCATGT
	MYB97:GFP	BamHI-MYB97F	GGATCC AGCGCTAGCGGTTTGACTAA

RT-qPCR Primers	MYB97:GFP	Sall-MYB97R	GTCGACT GCGCAGATCCCTGGCAAGT
	MYB120:GFP	Myb120gatewayGe	CACCTTT GGCCGAGTCTTCAAGTT
	MYB120:GFP	Myb120forGFPR	ATGTTTATGATCAAAACGCAGATT
	MYB120	Myb120gatewayGe	GCAGTTCTCCACCGTTGATT
	THIONIN:GFP	AT5G36720Genom	CACCCGGT CAAAATTGTTTTTGGAA
	THIONIN:GFP	AT5G36720NoStop	GGTATCACATAGAAACCAGAATAATCC
	PP2AA3	PP2AA3qPCRF	GAGTTTGGTCCTGAATGGGCAATG
	PP2AA3	PP2AA3qPCRR	ACTGGAGCGAGAAGCGATACTG
	ACTIN4	ACT4F1	GATTGCGAAAGCAGAGTACG
	ACTIN4	ACT4R1	ACTTCCGTCTTCGTTTGGTG
	AT4G20990	AT4G20990 F2	CTCTTAGGCAAGCCGTTGAC
	AT4G20990	AT4G20990 R2	TCTTCCCTTTGAGTCTTGAACC
	AT4G21000	AT4G21000 F2	ATCGTGGAAAGGAGATGGTG
	AT4G21000	AT4G21000 R2	TCGTGTGAACCATGTGAAGC
	AT5G46200	AT5G46200 F2	ATTGGCCACGTCAAAGTCAG
	AT5G46200	AT5G46200 R2	AGACGATGCAGTGTTCCTG
	AT5G09280	AT5G09280 F4	CAGTCGCAAACGCTAAACAG
	AT5G09280	AT5G09280 R4	AGACGGAGTTTCTGGTGCAG
	AT5G36720	AT5G36720 F1	GCAGAGTTTTGTGCAAGCAG
	AT5G36720	AT5G36720 R1	ATACGCGAGTACGGTGGAAAG
	AT2G30310	AT2G30310 F3	TTGCGGAACAGGATACTTGG
	AT2G30310	AT2G30310 R1	ATCGGAGTGATTCCGGACAAG
	AT2G30220	AT2G30220 F1	GATGGACATGATCCGAAACC
	AT2G30220	AT2G30220 R1	TATCCTGTTCCGCAACATCC
	AT4G26830	AT4G26830 F1	TGGTACGTGCTTCTTTGGTG
	AT4G26830	AT4G26830 R1	AATGTCCGGTGGGAAACTC
	AT5G06170	AT5G06170 F2	ATTCCATTTGCATTAGCTTCC
	AT5G06170	AT5G06170 R2	CCACCTCCAAATAACGCATC
	AT3G19940	AT3G19940 F4	TGCGATCATGACGGTTTTTC
	AT3G19940	AT3G19940 R4	GCTTCCAAACTCTTCCCATC
	AT5G20340	AT5G20340 F1	ACCCATCTAGCATCCGTTTG
	AT5G20340	AT5G20340 R1	TAGCCCAAGTGATCCATCTTG
	AT5G03810	AT5G03810 F4	TCAAGAAGGGCTTGTTGTGG
	AT5G03810	AT5G03810 R3	TAGCATTCGAGCATGTACCC
	AT5G03820	AT5G03820 F3	GTGGGTACATGTTCTGAATGC
	AT5G03820	AT5G03820 R3	GAGATGAGGGGTATTCCTTGG

SUC7	SUC7 F2	CGTTTGGAGTTGGTCCTATTG
SUC7	SUC7 R2	TGCCACAATACTGCTGACTG
SUC8	SUC8 F2	CGCTTTTTTGGAGGTGGAAAC
SUC8	SUC8 R2	CACGGTAAATGCCACAACAC

Supplemental References

- Salonso-Peral, M. M., J. Li, et al. (2010). "The microRNA159-regulated GAMYB-like genes inhibit growth and promote programmed cell death in *Arabidopsis*." Plant Physiol **154**(2): 757-771.
- Boavida, L. C. and S. McCormick (2007). "Temperature as a determinant factor for increased and reproducible in vitro pollen germination in *Arabidopsis thaliana*." Plant J **52**(3): 570-582.
- Borges, F., G. Gomes, et al. (2008). "Comparative transcriptomics of *Arabidopsis* sperm cells." Plant Physiol **148**(2): 1168-1181.
- Cao, J., K. Schneeberger, et al. (2011). "Whole-genome sequencing of multiple *Arabidopsis thaliana* populations." Nat Genet **43**(10): 956-963.
- Clough, S. J. and A. F. Bent (1998). "Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*." Plant J **16**(6): 735-743.
- Curtis, M. D. and U. Grossniklaus (2003). "A gateway cloning vector set for high-throughput functional analysis of genes in planta." Plant Physiol **133**(2): 462-469.
- Czechowski, T., M. Stitt, et al. (2005). "Genome-wide identification and testing of superior reference genes for transcript normalization in *Arabidopsis*." Plant Physiol **139**(1): 5-17.
- Gubler, F., D. Raventos, et al. (1999). "Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone." Plant J **17**(1): 1-9.
- Honys, D. and D. Twell (2004). "Transcriptome analysis of haploid male gametophyte development in *Arabidopsis*." Genome Biol **5**(11): R85.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Millar, A. A. and F. Gubler (2005). "The *Arabidopsis* GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development." Plant Cell **17**(3): 705-721.
- Mori, T., H. Kuroiwa, et al. (2006). "GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization." Nat Cell Biol **8**(1): 64-71.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Petersen, T. N., S. Brunak, et al. (2011). "SignalP 4.0: discriminating signal peptides from transmembrane regions." Nat Methods **8**(10): 785-786.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.

- Schmid, M., T. S. Davison, et al. (2005). "A gene expression map of Arabidopsis thaliana development." Nat Genet **37**(5): 501-506.
- Sessions, A., E. Burke, et al. (2002). "A high-throughput Arabidopsis reverse genetics system." Plant Cell **14**(12): 2985-2994.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in Arabidopsis." Plant Cell **2**(8): 755-767.
- Swanson, R., T. Clark, et al. (2005). "Expression profiling of Arabidopsis stigma tissue identifies stigma-specific genes." Sexual Plant Reproduction **18**(4): 163-171.

Chapter 5:
Identifying Sex-Specific gene expression using SNP-informed RNA-
Sequencing

Alexander R. Leydon, Elena Venable, Caleb Weinreb, Mark A. Johnson

I performed the majority of experiments in this study. Two students EV and CW performed invaluable bioinformatics analyses and contributed their work. EV performed analysis of SNP distributions between Col and Cvi, and contributed Figure 1 and Tables 1 & 2. CW contributed all scatterplots in this manuscript, performed correlation analyses (Figure 5 & Supplemental Figure 2), SNP-read representation (Supplemental Figure 1), and SNP-read graphs (Figures 3 & 8).

Abstract

In flowering plants single-celled pollen tubes deliver sperm to female gametes via polarized growth through complex pistil tissue. Consequently, the transcriptome of the pollen tube is challenging to directly ascertain. Transcriptional differentiation of the male gametophyte requires *MYB97*, *MYB101*, and *MYB120* as the pollen tube grows in the pistil and is required for fertility. We sought to identify the complete MYB-dependent differentiation in response to growth in the pistil. We achieved accurate identification of sex-specific gene expression by RNA-sequencing pollinations between polymorphic *Arabidopsis* ecotypes, Col-0 and Cvi-0. Sex-specific transcriptomes were extracted from RNA-sequencing data using SNPs in individual reads based upon SNPs present in the Col-0 and Cvi-0 ecotypes. We identified pollen and pistil genes that are dysregulated in pollinations with MYB transcription factor (*MYB97*, *MYB101*, *MYB120*) loss of function mutants. Sex specific read analysis identifies a group of MYB-regulated genes that are otherwise obscured by pistil gene expression. This analysis identifies a subset of the pollen tube transcriptome that is expressed during pollen tube growth in vitro, and reveals MYB-dependent transcription that contributes to pollen tube reception. Genes specifically induced during pollen tube – pistil interactions were found to have elevated SNP frequencies between Cvi-0 and Col-0, suggesting adaptive evolution of sex-biased genes. Furthermore, we demonstrate a promising method to analyze pollen- and pistil-specific gene expression in reproductive tissues from any plant species.

Introduction

Plant seed production results from successful fertilization. Flowering plant reproduction occurs inside pistil tissues during distinct spatio-temporal windows. Pollen grains are deposited on the stigma tissues of the pistil where they undergo adhesion, hydration and initiate formation of a pollen tube in a rapid process spanning only 15 minutes (Elleman et al. 1986). Over the next 8 hours, pollen tubes rapidly extend into the style and transmitting tract, growing through a complex extracellular matrix and forming hundreds of cell-cell contacts on route to the female gametes. Pistil signals induce pollen tube gene expression of species determinants that allow proper pollen tube guidance, reception with the ovule and fertilization (Leydon et al. 2013). Pistil tissues provide a physical scaffold and nutritional resource for pollen tube growth, yet they also create an environment that actively signals the pollen tube to properly differentiate (Leydon et al. 2014) and selects against non-compatible mates (Sawada et al. 2014). How the pollen tube senses the pistil environment and transduces these signals to alter transcriptional regulation is unknown.

Extensive progress in whole genome sequencing techniques has rapidly expanded our understanding of transcriptional networks across cell types and tissues. Tissues containing mixed cell types, however, still pose a challenge to identification of cell-type specific transcriptomes. Individual cell types have been transcriptionally profiled and have provided key insights into the transcriptomes of pollen (Becker et al. 2003; Honys et al. 2003; Pina et al. 2005), embryo sac

(Yu et al. 2005; Johnston et al. 2007) (Jones-Rhoades et al. 2007; Steffen et al. 2007), pistil (Scutt et al. 2003; Tung et al. 2005), sperm (Borges et al. 2008), and isolated egg and central cells (Dresselhaus et al. 1994; Wuest et al. 2010). Additionally, determining the transcriptomes of pollen tubes grown *in vitro* (Wang et al. 2008) and pollen tubes grown in a semi-*in vivo* condition (SIV) (Qin et al. 2009) identified dynamic regulation of pollen tube transcription during growth through the pistil. However, none of these studies provides a comprehensive and integrated analysis of the molecular timing and relationships between these cell types *in vivo*. Notably, recent microarray transcriptional profiling of pollinated pistils unveiled dynamic changes over 8 hours of pollen tube growth *in vivo*, however in this study it was impossible to distinguish pollen tube from pistil gene expression (Boavida et al. 2011).

Previous cell-type specific cell-sorting and gene expression analyses have been utilized in the *Arabidopsis* root (Brady et al. 2007). Mechanical methods such as fluorescence assisted cell sorting or manual dissection allow purification of cell types. However these methods are invasive and require extensive optimization and specialized equipment. In *Arabidopsis* and other flowering plants with perfect flowers both sexes, the male sex (pollen) and the female sex (pistil), are present in one flower. The sex-specific cell types are separate from each other and distinct until pollen grains germinate on the stigma and penetrate the pistil. We set out to identify sex-specific (pollen tube vs. pistil) gene expression patterns during pollination, when pollen tube and pistil tissues are intertwined. We took

advantage of the modular nature of pollen; any ecotype or mutant can be freely applied to a desired pistil recipient. We utilized an in-silico dissection of RNA-sequencing reads from crosses between two *Arabidopsis thaliana* accessions, Col-0 (Col) and Cvi-0 (Cvi). This strategy allowed us to distinguish pollen tube and pistil transcripts containing Col/Cvi single nucleotide polymorphisms (SNPs). We used de novo SNP calling on our laboratory Col and Cvi strains to ensure accurate representation of all available SNPs between Col and Cvi. Comparison between un-pollinated and pollinated pistils (8 hours after pollination) clearly defines a complex landscape of gene expression changes in pollen tubes and in pistils. We have previously demonstrated activation of pollen tube-specific genes by three MYB transcription factors during growth in the pistil by microarray analysis (Leydon et al. 2013). We utilized inter-ecotype pollinations with *myb* triple-mutant pollen tubes in the Col accession to define the patterns of pollen tube and pistil gene expression that are dependent on MYB genes during growth in vivo. Finally, we tested whether the frequency of SNPs was elevated in reproductively expressed coding sequences between Cvi and Col. We observed that the polymorphism rates in the coding sequence of pollen tube and pistil genes was higher than the background rates of SNPs.

Results

Variant analysis and refinement of a SNP database for sex-specific read determination. In order to comprehensively utilize SNPs to determine sex-specific transcript derivation, we chose two *Arabidopsis* ecotypes, Col-0 (Col) and Cvi-0 (Cvi) that have high rates of single nucleotide polymorphisms (SNPs) between them (approximately 1 SNP every 200bp) (Schmid et al. 2003; Nordborg et al. 2005). We utilized a database of SNPs present in Cvi that were validated against next generation sequencing results (Gehring et al. 2011; Nodine et al. 2012; Pignatta et al. 2014). In order to ensure that we captured the greatest number of informative SNPs in expressed genes from Col and Cvi, we incorporated SNPs from sequencing libraries from unpollinated Cvi pistils and Cvi self-pollinated for 8 hours (three biological replicates) into an existing Col/Cvi SNP database (Pignatta et al. 2014). Reads were mapped to the Col genome (TAIR10) using the tuxedo suite (Kim et al. 2013). We performed variant analysis using the Genome Analysis ToolKit (GATK, see Methods, McKenna et al. 2010) to identify variants that were either previously un-annotated (Gehring et al. 2011; Nodine et al. 2012; Pignatta et al. 2014) or specific to our lab strain of Cvi. Variant analysis of unpollinated and self-pollinated Cvi pistils added 15,995 novel SNPs to the previously identified 576,722 Cvi SNP database (Pignatta et al. 2014). Newly identified SNPs fell in 237 genes that previously lacked documented SNPs, and 5,217 genes that had at least one previously known SNP. Novel SNPs and previously identified SNPs were compiled into a master

SNP database (1 SNP every 201bp; Supplemental File S1), that was used in subsequent analyses.

Only SNPs that occur in genomic sequences that are detectable by RNA-seq are used in our approach (exons and UTRs). Analysis of our combined data set of Col/Cvi SNPs demonstrates that 36.2% of SNPs occur in exons and UTRs (Figure 1A/B). 23% of SNPs in the database fall in transposable elements (TEs), which allow identification of pollen-specific and ecotype-specific activation of TEs. Prediction of SNP effects on gene function revealed that SNPs in Cvi were most likely to cause a silent change in coding sequence, followed by missense mutations. SNPs predicted to effect splicing patterns and translation start and stop sites were observed, but at lower frequencies (Figure 1B).

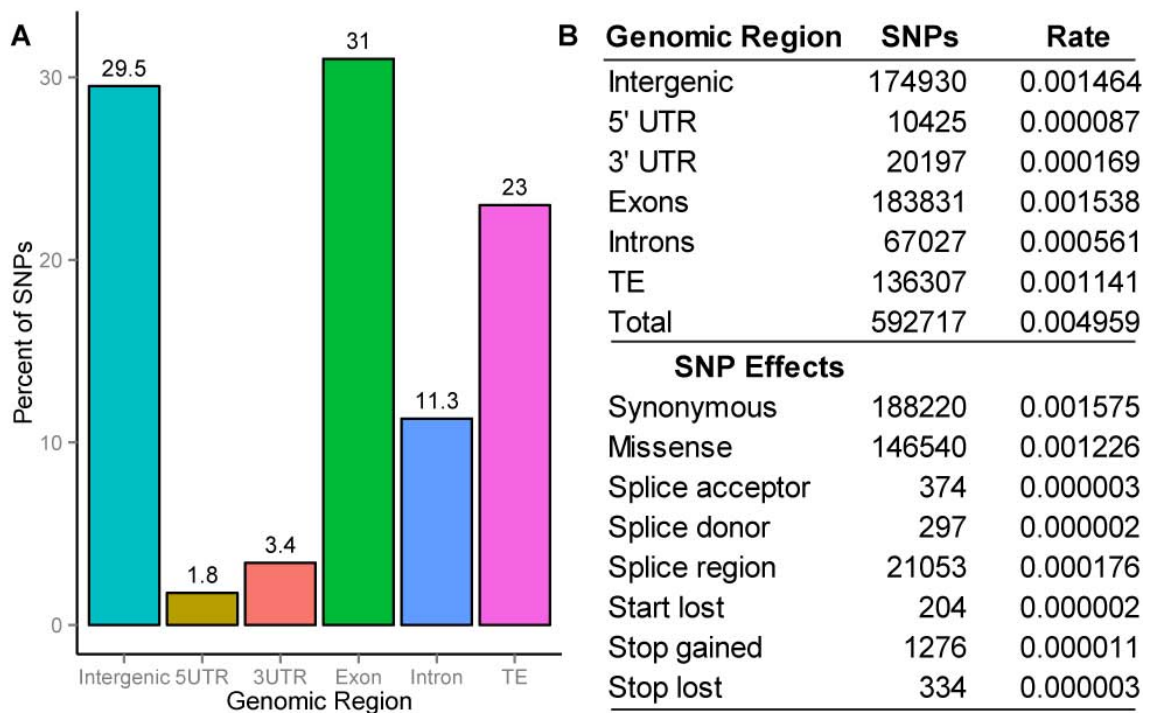


Figure 1. SNPs are distributed across genomic features and are abundant in coding sequences. **A.** The distribution of SNPs across indicated gene regions in the final database (% is given above bars). **B.** SNP counts and frequencies (SNP/1000bp) in our final database. Frequency of predicted Cvi SNP effects in data set (SnpEff, Cingolani et al. 2012).

Sex-specific gene expression analysis detects and separates 60% of the pollen and pistil transcriptome

We applied a methodology previously used to define allele-specific gene expression in developing embryo and endosperm (Gehring et al. 2011; Pignatta et al. 2014), to identify sex-specific gene expression in inter-ecotype crosses between Cvi and Col (Figure 2A). Our RNA-seq methodology followed a standard pipeline to identify differential gene expression that we modified to determine whether transcripts originated from pollen or pistil tissues (Figure 2A). To determine the relative contributions of pollen tube and pistil to the pollinated pistil transcriptome, we compared the total number of reads from pollinated pistils with Col-0 or Cvi-0 SNPs. There are ~100–200 pollen tube cells and thousands of pistils cells in a pollinated pistil, so we estimated that about 1% of the transcriptome would be pollen-derived. Quantification of our RNA-Seq libraries revealed that 92% of reads containing SNPs corresponded to the Cvi allele while 8% had the Col allele (Figure 2B).

We analyzed the distribution of Cvi (pistil) and Col (pollen) gene expression in order to define useful thresholds in determining pollen and pistil transcript specificity. For all genes that contained SNPs, we plotted mean Cvi reads/gene versus Col reads/gene from Cvi x Col pollinations (three replicates). Pistil cells

outnumber pollen cells resulting in a skewed distribution biased towards pistil enrichment (Figure 2C, dotted gray line, see difference in axis scale). Most genes display significantly greater numbers of Cvi reads than of Col reads. However there are genes that have significantly more Col reads than Cvi reads (Figure 2C, dotted blue line). These genes are likely to be preferentially or specifically expressed by pollen tubes. Therefore, for future analyses we defined pollen-specific genes as those with more than 10 Col reads and fewer than 10 Cvi reads (Figure 2C, dotted blue line, Supplemental File S2), and pistil-specific genes as those with fewer than 10 Col reads and greater than 10 Cvi reads (Figure 2C, dotted red line, Supplemental File S3).

We performed a quantitative analysis on reads identified from SNPs to ensure an accurate representation of total gene expression. We observed highly accurate representation of gene expression, with only slight bias towards reads over 5' ends of genes (Supplemental Figure 1A-C, E). This bias co-correlated with increased SNP density between Col and Cvi at the 5' coding sequencing of genes (Supplemental Figure 1D), indicating that our method produces high fidelity representation of gene expression. To illustrate how SNPs were used to call sex-specific gene expression, we chose two genes representative of pollen-specific or pollen and pistil gene expression (Figure 3A).

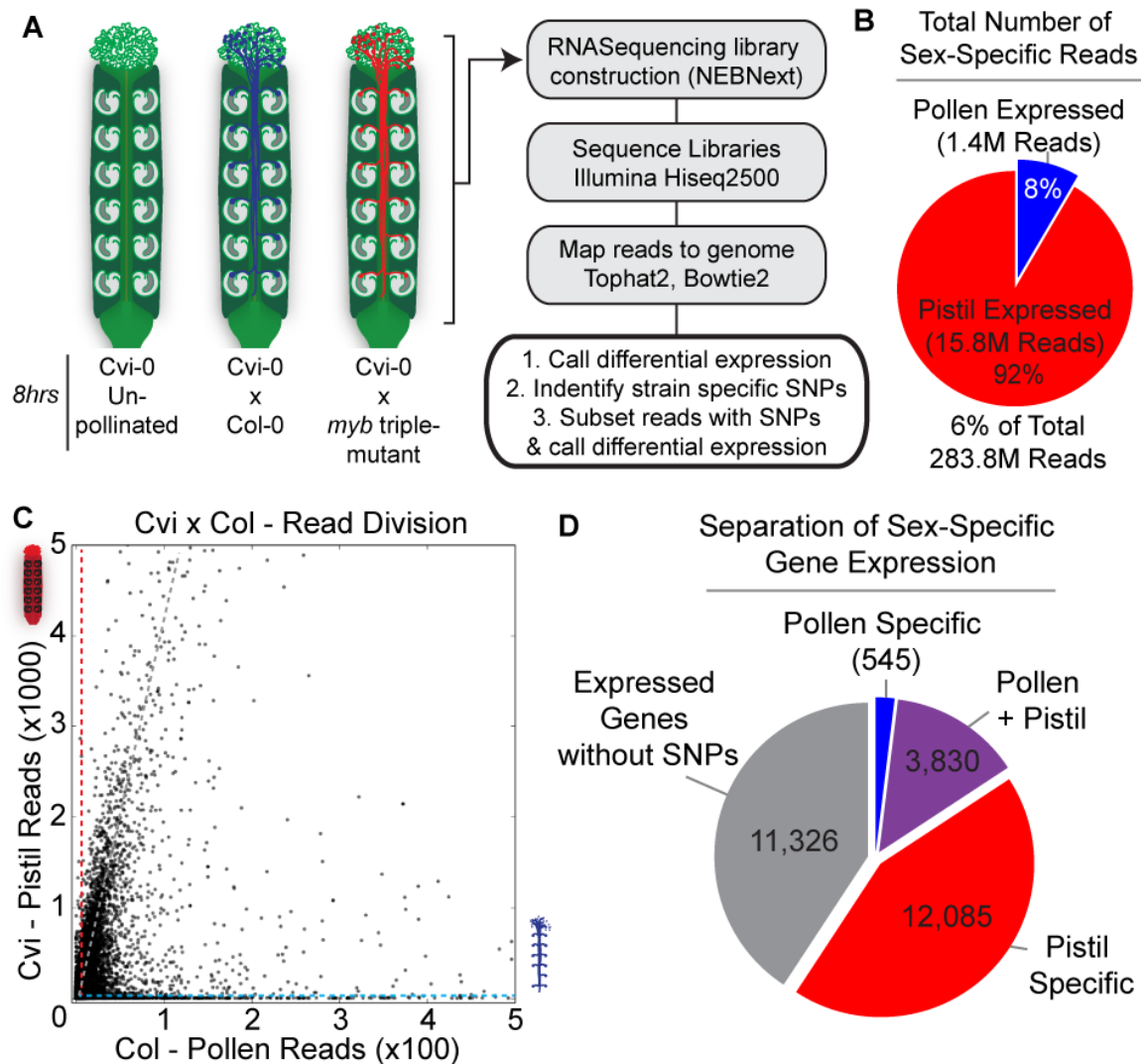


Figure 2. Experimental design employed to identify ecotype-specific reads originating from pollen or pistil. **A.** Experimental samples prepared for RNA-sequencing. **B.** Total Reads were subset over the presence of a SNP between Col-0 and Cv-0. Total reads over SNPs are compared to the total number of reads (sum of three biological replicates). **C.** Scatterplot of sex-specific reads divided by ecotype SNP. Genes with detectable SNPs in Col and Cvi are plotted with mean of all reads over each gene, y-axis = number of reads with Cvi SNP, x-axis number of reads with Col SNP. Pistil expression bias is indicated with gray dotted line. Sex-specific cutoffs are shown for Cvi (Pistil - Red dotted line) and Col (Pollen - Blue dotted line) expressed transcripts. **D.** Gene enrichment in pollen and pistil tissues, quantification data from (C) is color coded, and incorporated with reads without SNPs (Gray slice). This graph incorporates expressed genes only. (Genes were considered specific if they met a cutoff of >10 in on tissue and < 10 reads in the other, mean of three replicates).

We graphed SNP-dissected sequencing coverage over the genomic loci of these two genes and the SNPs (green lines) contained in their coding sequence (black arrows, Figure 3A). *AT4G25040* is a pollen-specific gene; all reads over SNPs contain the Col SNP (Figure 3A, blue peaks). *AT2G18440* is expressed in both the pollen tube (Figure 3B, blue peaks) and the pistil (Figure 3B, red peaks). This quantitative method unambiguously determines sex-specific expression values.

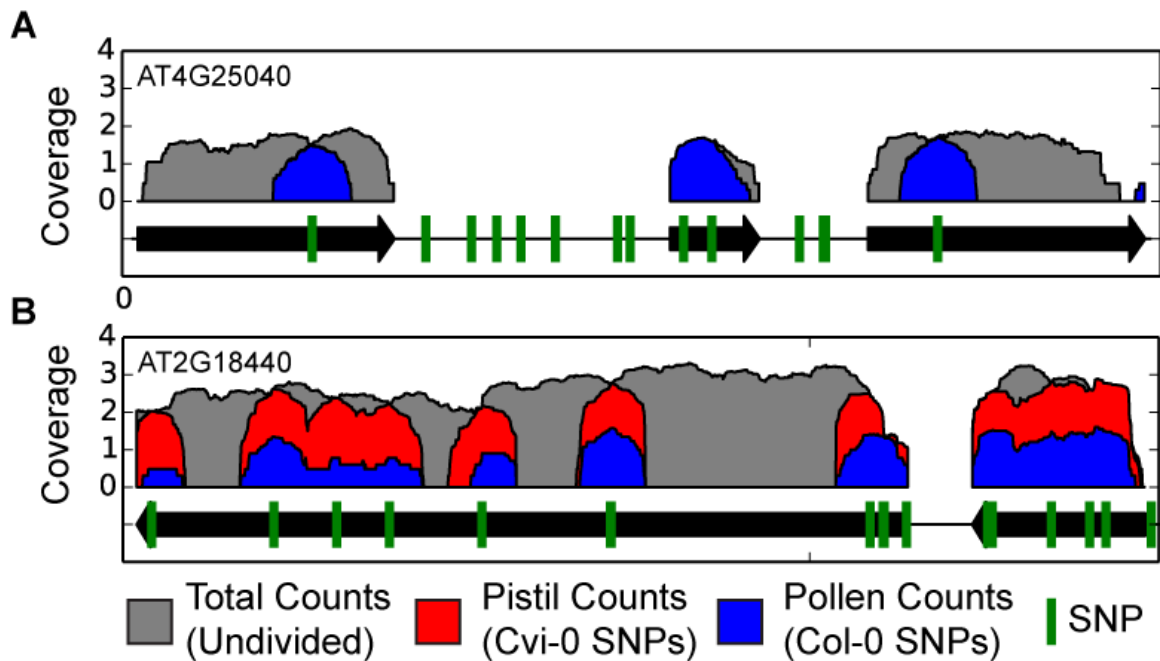


Figure 3. SNP-informed RNA-seq accurately represents gene expression. A plot of SNP-reads over gene models at two representative loci; **A.** *AT4G25040* - a pollen specific gene, and **B.** *AT2G18440* a gene expressed from both pollen and pistil. Coverage = Reads/nucleotide, log10. Grey color represents reads without SNPs. In regions with SNPs reads are divided into sex-specific components. Red color represents reads with the Cvi SNP (from the pistil parent), Blue color represents reads with the Col SNP (from the pollen parent). Reads from sex-specific alignments (Red and Blue) add up to total read alignments (Grey).

Sex-specific transcript analysis can be used to untangle differential gene expression during pollination

Comparisons between unpollinated Cvi pistils and Cvi x Col pollinated pistils identifies differentially expressed genes (reads/gene, Figure 4A), specifically a number of genes that are expressed in pollinated pistils that are absent from unpollinated pistils (Figure 4A, grouping of genes along the x-axis). We hypothesized that this pattern is produced by a number of pollen-expressed genes that are absent from the unpollinated pistil. We tested this hypothesis by applying our criteria for sex-specific expression (threshold = mean of 10 reads, see Figure 2C) to this dataset (Figure 4A), where pollen-specific reads are shaded blue, pistil-specific reads are shaded red, and genes expressed in both tissues are assigned a color in-between based upon the ratio of Cvi to Col (Figure 4B, see legend). As hypothesized, pollen-specific gene expression is primarily responsible for the group of genes that are highly induced during pollination (Figure 4B, blue dots). The majority of pistil-expressed genes remain unchanged by pollination (Figure 4B, red dots, diagonal line). However, it is clear that a number of pistil-expressed genes are differentially expressed in response to pollination (476 genes, Supplemental file S4), and could play roles in responding to pollen tube growth in the pistil.

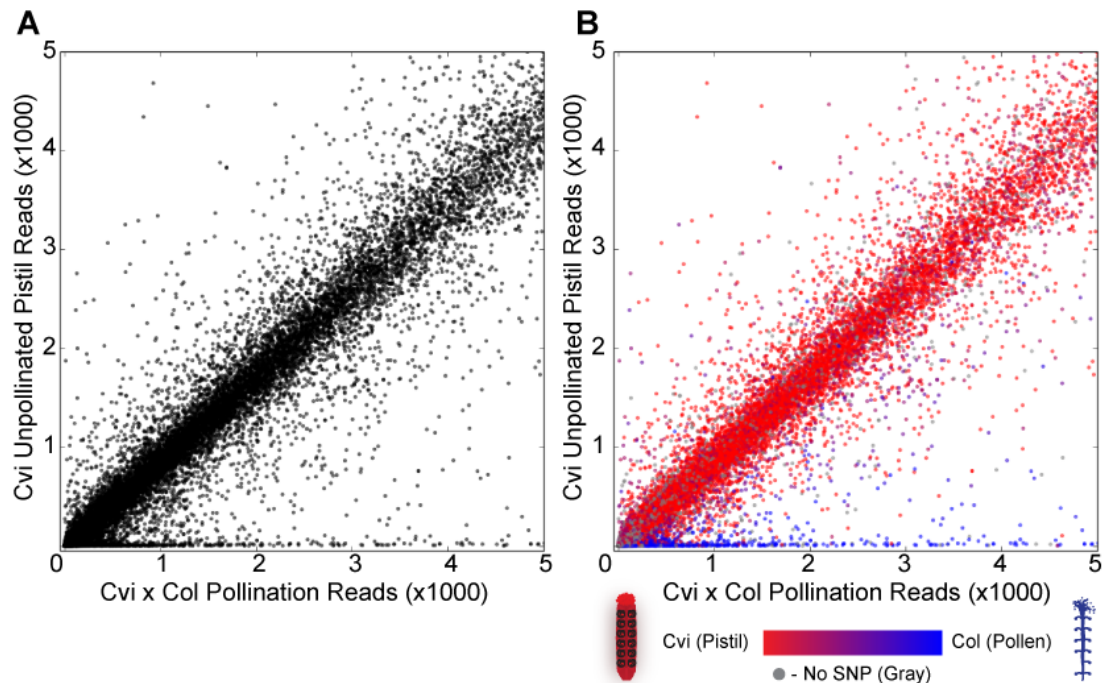


Figure 4. Inter-ecotype pollinations enable identification of sex-specific gene expression during pollination. A-B. Scatterplots of mean # of reads/gene from three RNA-seq replicates. **A.** Gene expression from Unpollinated Cvi pistils and Cvi x Col pollinations. All genes are plotted as black dots. **B.** Data from B, color coded to indicate sex specific expression. An arbitrary cutoff was utilized to set sex-specific thresholds (mean # Col reads >10, <10 Cvi reads– Blue, pollen specific; mean # Col reads <10, >10 Cvi reads – Red, pistil specific; See Figure 2C).

The pollen-specific component of the pollination transcriptome comprises distinct functional categories

We tested whether pollen tube-specific genes were enriched for functional annotations indicative of specific functions. Gene Ontology (GO) terms in Col-specific reads identified enrichment in biological processes associated with pollen tubes: cell-cell signaling (12.7-fold; p value=2.07E-5), pollen tube growth (11.1-fold; p value=1.59E-5), reproductive cellular process (6.7-fold; p value=1.17E-5), pollination (6.0-fold; p value=1.17E-5), and cation transport (3.5-fold; p value 1.78E-5). Analysis of GO cellular component annotations

revealed 25% of transcripts encoded genes predicted to enter the endomembrane system ($n = 127$, $p = 3.21E-6$) (Supplemental File S5). Enrichment in membrane-localized and secreted proteins has been observed in pollen tube transcriptomes by microarray (Wang et al. 2008; Qin et al. 2009), supporting the identity of Col-specific reads as representative of pollen-specific genes.

SNP-informed sequencing identifies previously defined pollen-expressed genes and provides a refinement over the semi *in vivo* assay

We assayed the characteristics of our pollen-expressed (Col-SNPs) transcriptome to determine whether it shared features with previously identified pollen gene expression studies. First we compared the transcriptional profile of SIV pollen tubes to pollen-expressed genes defined by our method (Qin et al. 2009). SIV pollen tubes were grown through a pistil explant and onto solid pollen growth media before RNA analysis. The SIV transcriptome included 7,275 expressed genes, whereas we identified 4,375 expressed genes with Col SNPs (Cvi x Col pollinations). Intersection of these genes reveals 2,540 genes that are detected by both methods (Figure 5A). Interestingly, 3,157 genes expressed in SIV grown pollen tubes were not detected in our analysis (Figure 5A, red text). This is not due to an absence of SNPs in these genes; 1,578 SIV expressed genes do not have SNPs between Col and Cvi, rendering them undetectable by our analysis (Figure 5A, grey shading). SIV pollen tube growth involves pollen tube outgrowth onto agar media, which may introduce gene expression changes

unique to this condition such as stress response genes (Qin et al. 2009). This may account for the 3,157 genes that are not observed with SNP-sequencing approaches. Conversely, a set of 1,835 genes are detected in our analysis were not identified by SIV microarray analysis (Figure 5A). These genes may have been missed due absence from microarrays, or because they require extended periods of growth in pistil tissues for induction.

Sex-specific gene expression profiles correlate well with relevant microarray experiments

In order to assess the utility of our sex-specific transcriptomes we compared our data set to microarray data sets that analyzed isolated tissues of the pollinated pistil. We performed a Spearman's correlation analysis between our sex-specific dataset and relevant previously published microarray data sets. We procured and re-normalized (methods) a set of reproductive and somatic microarray studies. These included microarray analysis of the stages of pollen development and pollen tube growth in vitro: uninucleate microspores, tricellular pollen, mature pollen grains - (Honys et al. 2004), dry pollen, pollen grown in vitro 0.5h, 4h and SIV pollen tubes - (Qin et al. 2009). We also compared our results to microarray analysis of unpollinated pistils, pollinated pistils, ovules - (Boavida et al. 2011), stigma, and ovary (Swanson et al. 2005). We also compared our results to unrelated sporophytic tissues: roots, seedlings, and rosettes - (Schmid et al. 2005). We identified genes that are identified by one unambiguous array ID, and correlated expression values between our RNA-seq results and microarray

databases. We found that Col-specific genes demonstrated a high correlation with pollen transcriptomes, especially SIV grown pollen (Figure 5B, dark red is the highest Pearson coefficient observed). No correlation was identified between these data and pistil or vegetative microarray data (Figure 5B, left column).

Conversely, genes from Cvi x Col pollinations identified as Cvi-specific demonstrated a high correlation with pistil transcriptomes, especially pistils pollinated for 3.5-8 hours (Figure 5B, right column). Correlations were absent (blue color) or very weak (yellow color) with previously published data sets analyzing pollen development, tube growth, or vegetative tissues (Figure 5B, right column, Supplemental Figure 2). Therefore, SNP-informed RNA-sequencing identifies sex-specific expression and, because it is noninvasive and analyzes interactions between pollen tube and pistil, it represents a more biologically relevant view of the pollination transcriptome.

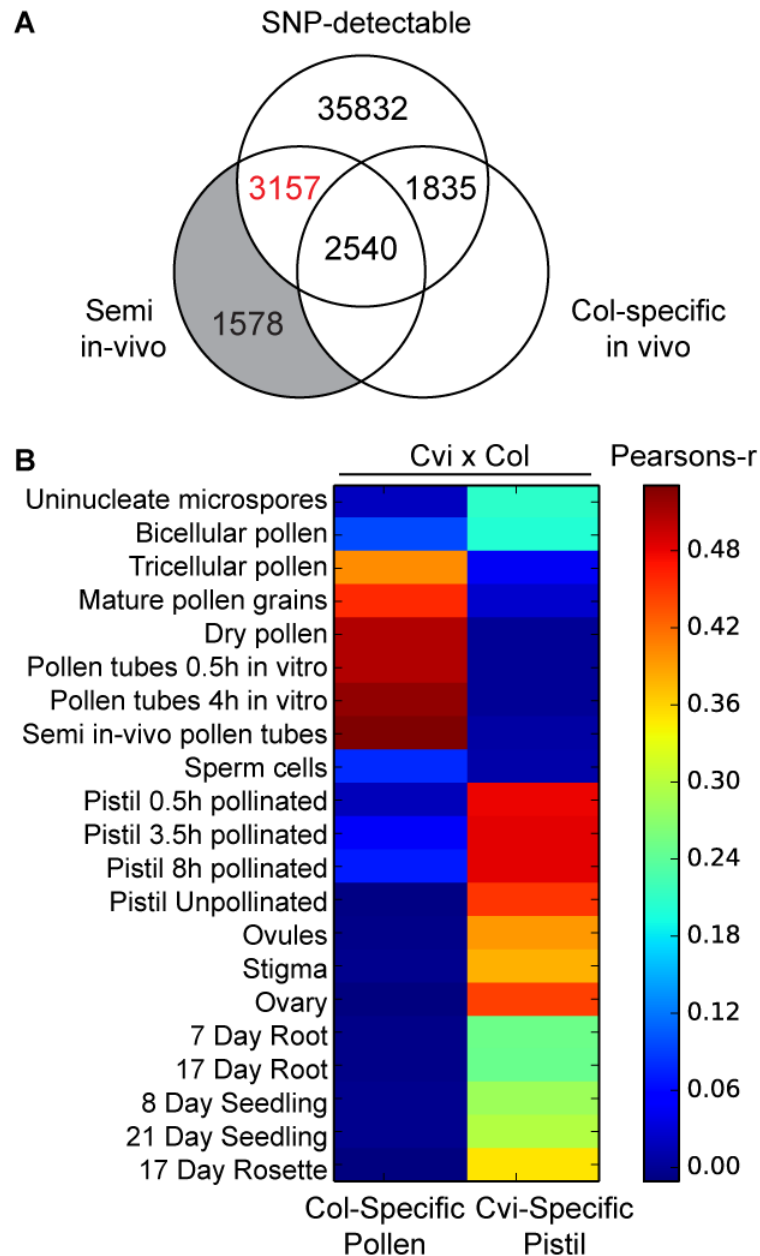


Figure 5. Sex-specific gene expression profiles correspond to known reproductive transcriptional profiles. A. Intersection of SIV gene expression with Col to Cvi SNP-detectable reads. Red text indicates the set of genes expressed in SIV analysis that is absent from our dataset. **B.** Correlation between SNP-specific sex identification and published microarray analysis. In this analysis Col-specific and Cvi-specific indicates all genes defined as expressed based on reads containing Col or Cvi SNPs, respectively. Microarray data sources are as follows. Uninucleate microspores, Tricellular pollen, Mature pollen grains - (Honys et al. 2004). Dry pollen, 0.5h, 4h and Semi in vivo pollen tubes - (Qin et al. 2009). Unpollinated & pollinated pistils, Ovules - (Boavida et al. 2011). Stigma, Ovary (Swanson et al. 2005). Root, Seedling, Rosette - (Schmid et al. 2005).

SNP-informed RNA-seq analysis can be used to define the role of pollen tube transcriptional regulators

Subgroup 18 MYB genes *MYB97*, *MYB101*, and *MYB120* regulate genes involved in pollen tube reception either directly or indirectly (Leydon et al. 2013; Liang et al. 2013). Previous microarray studies identified genes that were differentially expressed in *myb* triple-mutant pollen grains (Liang et al. 2013) and *myb* triple-mutant pollinated pistils (Leydon et al. 2013). SNP-informed RNA-sequencing presents an excellent method to simultaneously analyze MYB-responsive gene expression in the pollen tube and pistil. First, we compared Cvi x Col pollinations to Cvi x *myb* triple-mutant pollinations and analyzed differential expression using all mapped reads, which ignores transcript parent of origin. We used three differential expression callers, DESeq, DESeq2, and EdgeR (Anders et al. 2010; Robinson et al. 2010; Love et al. 2014), and compared the sum of these lists with MYB-regulated genes previously identified by microarray analysis. We observed a significant overlap in differentially regulated genes: RNA-seq identified 89% (43/48) of previously identified genes (Supplemental Figure 2, Supplemental File S6). We identified a core set of differentially expressed genes common to all differential expression analyses and chose a conservative differential expression caller for further analysis. DESeq2 (Love et al. 2014) discovers 317 differentially regulated genes (Figure 6A, purple dots) and captures 62.5% of known MYB-regulated genes- 30/48.

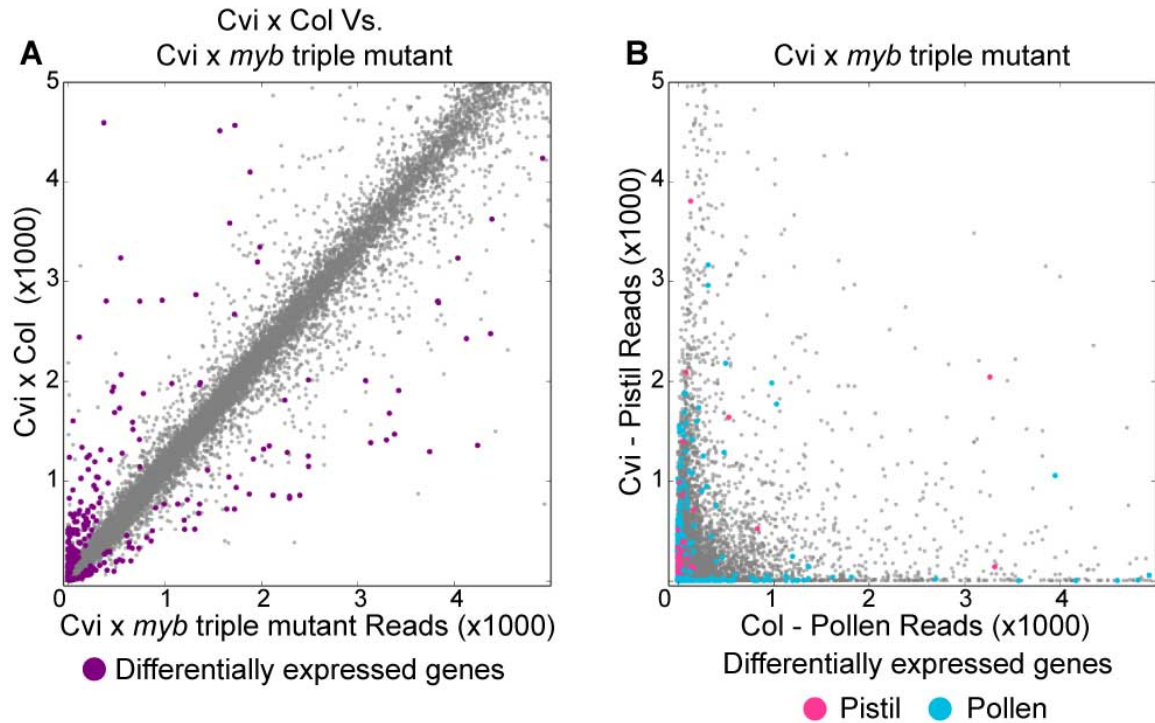


Figure 6. MYB regulated gene expression in SNP-dissected transcriptomes.
A. Scatterplot of gene expression in wild type and *myb* triple-mutant pollinations. All mapped reads (pre-division) with differentially expressed genes colored purple (317 genes; adjusted p-value<0.05). **B.** Scatterplot of sex-specific gene expression in *myb* triple-mutant pollinations. Genes that are differentially expressed compared to wild type pollinations are colored Pink if Pistil expression changes (N=34, adjusted p-value <0.05, or Light Blue if pollen expression changes (N=278, p-value<0.05).

Next, we implemented sex-specific read identification on RNA-seq libraries from Cvi pollinations with *myb* triple-mutant pollen (Col ecotype). This allows analysis of differential expression that results from loss of *MYB97*, *MYB101* and *MYB120* in pollen tubes and pistil tissues independently. If some number of MYB-regulated pollen genes are expressed in both pollen and pistil tissues, SNP-informed read division will be uniquely capable of identifying differential expression occurring only in the pollen tube, which may be otherwise masked by pistil derived transcripts. Bulk (before sex-specific read division) differential gene

expression defines 317 (Purple dots, Figure 6A, Supplemental File S6). Sex-specific read division and differential expression analysis identified 278 pollen-expressed genes and 34 pistil-expressed genes differentially expressed in *myb* triple-mutant pollinations (Supplemental File S7). Plotting the number of pistil (Cvi) reads/gene versus the number of pollen (Col) reads/gene for Cvi x *myb* triple-mutant pollinations (Figure 6B) shows that, like wild-type pollinations (Figure 2C), the majority of genes have many more pistil reads than pollen reads and the distribution is heavily pistil-biased. When this distribution is color-coded to highlight MYB-regulated pollen genes (light blue, Figure 6B, Supplemental File S7), it is clear that a subset of these genes are also expressed in pistil tissues. A smaller number of MYB-regulated pistil-expressed genes were also identified (pink, Figure 6B). These may represent pistil-expressed genes that respond to growth of *myb* triple-mutant pollen tubes.

Differential expression analysis on pollen- and pistil-specific reads identifies an additional 223 genes with MYB-dependent gene expression that is masked by expression of the same gene in the opposite sex. 210 pollen-specific genes (Figure 7A, Venn Diagram, Red shading) and 13 pistil-specific genes (Figure 7A, Yellow shading) are detected as differentially expressed after SNP-specific division, while 86 pollen-specific genes (Figure 7A, Purple shading) and 21 pistil specific genes (Figure 6A, Green shading) are identified both by sex-specific read division, and by the remaining reads that do not cover SNPs. 267 genes were found to be differentially expressed in *myb* triple-mutant pollinations (Figure

6A, Blue), which is due to the absence of SNPs in these genes between Col and Cvi.

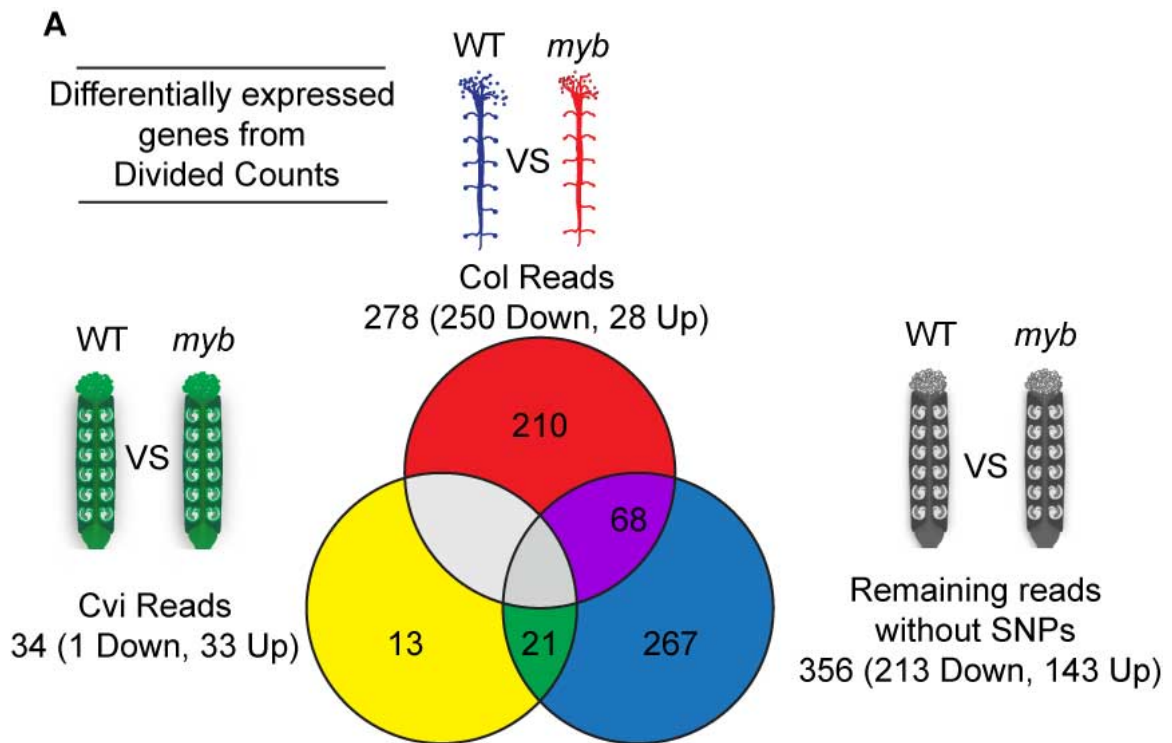


Figure 7. MYB-regulated gene expression becomes visible after Sex-specific read dissection. A. Differentially expressed genes identified by sex-specific read division and intersected. Pistil-specific Cvi reads (Adjusted p-value <0.05). Pollen-specific Col reads (p-value<0.05). Remaining reads without SNPs (Adjusted p-value <0.05). Total number of differentially expressed genes is shown beneath each category, along with the number of down-regulated (Down) and up-regulated (Up).

Cis-regulatory prediction in promoters of pollen expressed genes

Previous microarray analyses identified predicted MYB binding sites in differentially expressed genes from microarray analysis of pollen grains (Liang et al. 2013) and pollinated pistils (Leydon et al. 2013) using the GAMYB binding motif (Skriver et al. 1991; Gubler et al. 1992; Lanahan et al. 1992). We utilized the regulatory sequence analysis toolset (RSAT, van Helden 2003; Thomas-

Chollier et al. 2008) to examine whether genes with decreased mRNA abundance in *myb* triple-mutants were likely to contain MYB binding sites. We analyzed DNA sequence preceding each MYB-dependent gene's start codon from total (undivided) reads (excluding TEs & TE genes = 272 Sequences, Supplemental File S8) and pollen specific reads (Excluding TEs & TE genes = 264 Sequences, Supplemental File S9), and found that the GAMYB binding sequence was enriched in promoters from total reads (66/272) and from Pollen-specific (Col) reads (136/264). We also found that the MYB TAACAA core sequence was further enriched in targets identified by total reads (218/272) or by pollen-specific reads (219/264) (Supplemental Figure 4). The ability to identify pollen-specific genes that may be directly regulated by *MYB97*, *MYB101* and *MYB120 in vivo* allows prediction of MYB DNA binding motif with greater confidence.

Genetic reporter constructs corroborate pollen-specific gene expression.

Three MYB-regulated genes that expressed only the Col-0 allele in reads over SNPs were deemed pollen tube-specific and chosen for transgenic reporter analysis (Figure 8A,D,G). Full genomic loci containing the predicted promoter regions and coding sequences of *SUC9* (*AT5G06170*), *Pectate Lyase* (*AT5G09280*), and *ACA4* (*AT4G20990*) were cloned in-frame with GFP to produce translational fusions. We introduced these reporter constructs into wild-type *Arabidopsis* carrying the pollen expressed *LAT52:DsRed* marker to test pollen tube expression (Twell et al. 1989; Francis et al. 2007). Pollen from

primary transformants was analyzed for GFP expression in mature pollen and semi in vivo pollen tubes. Mature pollen from each reporter was analyzed by confocal microscopy, and GFP was undetectable (data not shown). However, each reporter was detectable in pollen tubes after semi in-vivo pollen tube growth. We analyzed at least 21 independent primary transformants (T_1) for each construct and found that pollen-tube specific expression was observed in the vast majority of transformants (*SUC9:GFP*, 36/36; *Pectate Lyase:GFP (PL:GFP)*, 18/21; *ACA4:GFP*, 32/36; Figure 8B,E,H). We determined the subcellular localization of each GFP fusion protein in growing pollen tubes and each displayed a localization pattern consistent with the predicted localization. *SUC9:GFP* appeared to localize predominantly to the plasma membrane, and to vesicles in the pollen tube tip (Figure 8C, Supplemental Movie 1). *PL:GFP* was observed in the endomembrane system (puncta) and what is likely the pollen tube cell wall (Figure 8F, Supplemental Movie 2). *ACA4:GFP* was observed in the endomembrane system (puncta, Figure 8I, Supplemental Movie 3). In all cases where GFP fluorescence was observed (86/93), it was pollen-tube specific, indicating that SNP-informed sequence analysis of pollination successfully identified these MYB-regulated genes as pollen-expressed.

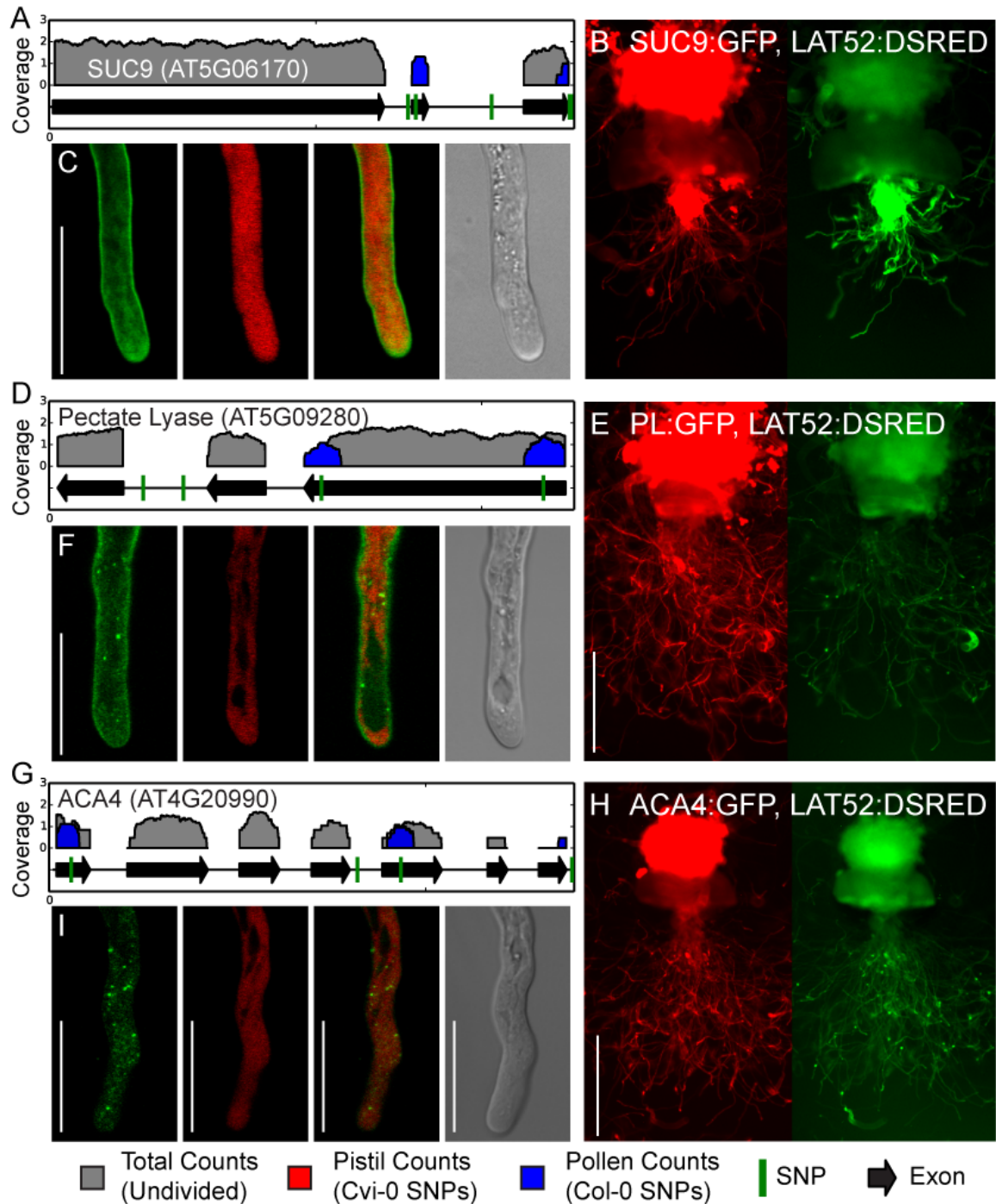


Figure 8. Pollen specific gene expression of 3 MYB-regulated genes detected by SNP-sequencing. A,D,G. Sequencing reads over SNPs identifying target genes as pollen-specific, as in Figure 3C. B,E,H. Representative semi in vivo pollen tube growth experiments on wild-type pistils, epifluorescent micrographs, scale bar = 500um. In each, the left image is the Red channel (LAT52:DsRed) signal, which is present in the pollen grain and the tube. In each, the right image is the Green channel. The autofluorescence of the stigma explant is visible in each panel. C,F,I. Pollen tube subcellular localization experiments,

confocal micrographs, scale bar = 20um. **B,C.** SUC9:GFP (*AT5G06170*), LAT52:DsRed. **E,F.** Pectate Lyase or PL:GFP (*AT5G09280*), LAT52:DsRed. **H,I.** ACA4:GFP (*AT4G20990*), LAT52:DsRed.

Analysis of gene expression changes induced by inter-ecotype pollination

Pollination induces a broad transcriptional change, in both the pistil (Boavida et al. 2011) and the pollen tube (Qin et al. 2009; Leydon et al. 2013). Col and Cvi are inter-fertile, however inter-ecotype crosses frequently show subtle defects in pollen tube reception that resemble pollen tube overgrowth defects in *fer*, *lre*, or *myb* triple-mutant pollinations (Escobar-Restrepo et al. 2007; Capron et al. 2008; Leydon et al. 2014). We therefore compared gene expression profiles in Col and Cvi self-pollinated pistils. First, we used hierarchical clustering to assess whether inter-ecotype pollinations were distinct from unpollinated pistils and intra-ecotype pollinations. As expected, hierarchical clustering of RNA sequencing demonstrates that pollinated pistils cluster distinctly from unpollinated Cvi pistils (Figure 9A). We compared the transcriptomes of self-pollinations (Cvi x Cvi, Col x Col) to inter-ecotype pollinations (Cvi x Col), and found a core set of 16,838 genes that are expressed at detectable levels (Figure 9B). However, differential expression analysis identified hundreds of genes with different expression patterns between Col and Cvi self-pollinations (Figure 9C, Supplemental File S10).

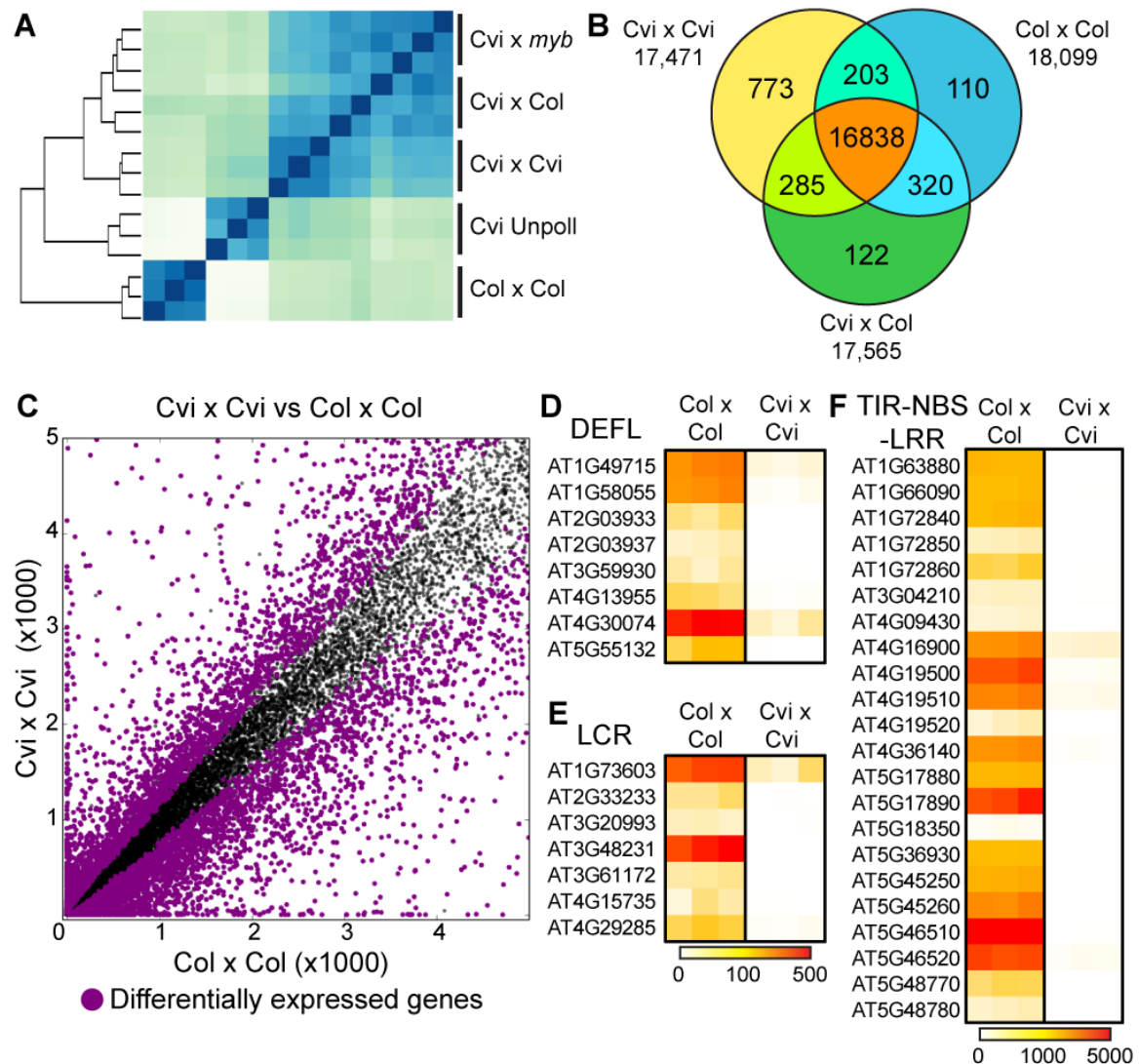


Figure 9. Reproductive gene regulation is highly divergent between Col and Cvi. **A.** Hierarchical clustering of all RNA-sequencing samples identifies Ecotype-specific clusters, DESeq2. **B.** Intersection of all expressed genes (Mean expression >100 reads) from self and inter-ecotype crosses. **C.** Differential gene expression observed between Col and Cvi 8h self-pollinations. Differentially expressed genes are colored purple for visualization, (adjusted p-value<0.005), DESeq2. **D-F.** Heat maps of Col-specific gene expression, colors represent read number, with scales below indicating the number of reads for color value. **D.** Defensin-like genes. **E.** Low-Molecular-Weight Cysteine-Rich genes. **F.** Toll-Interleukin receptor - Nucleotide Binding Site - Leucine Rich Region (TIR-NBS-LRR) type disease resistance proteins.

Ecotype-specific transcriptional responses to cold stress (Barah et al. 2013) and heat stress (Barah et al. 2013) have been previously observed, so it is perhaps

unsurprising that Col and Cvi ecotypes display variation in reproductive transcriptional profiles. However, there were categories of genes that were enriched in the Col pollination transcriptome that were absent from Cvi pollinations such as small secreted defensin-like proteins (DEFL, Figure 9D), Low-Molecular-Weight Cysteine-Rich proteins (LCR, Figure 9E), and NBS-LRR genes (TIR-NBS-LRR, Figure 9F). Additionally a Thionin-related clade of CRPs (CRP2460) is expressed in Col pollinations that is absent in Cvi (Thesis Chapter 6). These genes are expressed in Col pollinated pistils yet are absent in Cvi pistils, and may contribute to Col-specific signaling pathways during pollination. Cvi-specific transcripts displayed low levels of GO-enriched terms, in part due to the absence of assignable GO-terminology to the genes that are Cvi-specific. However, further analysis revealed that 44% of Cvi-specific pollination expressed genes were predicted to contain signal peptides and enter the secretory pathway, similar to ~31% of Col-specific pollination expressed genes entering the secretory system (SignalP).

We used SNP-informed RNA sequencing to define pollen tube and pistil changes in gene expression that occur during intra-(Cvi x Cvi) versus inter-ecotype (Cvi x Col) pollinations. First we ascertained genes that were differentially expressed between self-crosses (Cvi x Cvi) and inter-ecotype crosses (Cvi x Col) (Figure 10A, Supplemental File S10). We then used SNP-informed RNA sequencing to define pollen tube and pistil changes in gene expression that occur during inter-ecotype (Cvi-0 x Col-0) pollinations. This analysis identified 2,877 differentially

expressed genes; 1,238 are significantly more abundant in intra-ecotypic pollinations, 1,640 are significantly higher in inter-ecotypic pollinations (632 up in inter-ecotype pollinations, 418 up in intra-ecotype pollinations). We then defined the pistil-specific genes as those with expression biased in favor of the Cvi SNPs in inter-ecotype crosses (Figure 10B). The overlap of these sets identified 1,050 pistil-expressed genes that are differentially expressed depending on the pollen genotype. The majority of detected pistil transcriptional changes were subtle (< 4-fold). The 632 pistil genes that were induced in inter-ecotype pollinations were enriched in a cluster of responses (22.38 - enrichment score): response to chitin (n = 37, p – 2.6E-25), response to carbohydrate stimulus (n = 39, p – 2.26E-20), and response to organic substance (n = 83, p – 1.2E-15), as well as transcription factors (n = 61, p – 1.6E-6). Interestingly, the differentially expressed pistil transcriptome included 14 Ethylene-responsive transcription factors (ERFs) that are implicated in pathogenesis-related transcriptional response (n = 14, p – 4.7E-3, Supplemental File S11). The 418 pistil-expressed genes that were enriched in intra-ecotype crosses (Cvi x Cvi) were not strongly enriched for these or other functional categories (highest enrichment was plastid thylakoid, n = 26, p – 3.3E-7). This subtle difference in transcriptional profiles indicates that pollen genotype, in this case ecotype, is perceived by the pistil, activating genes associated with defense responses.

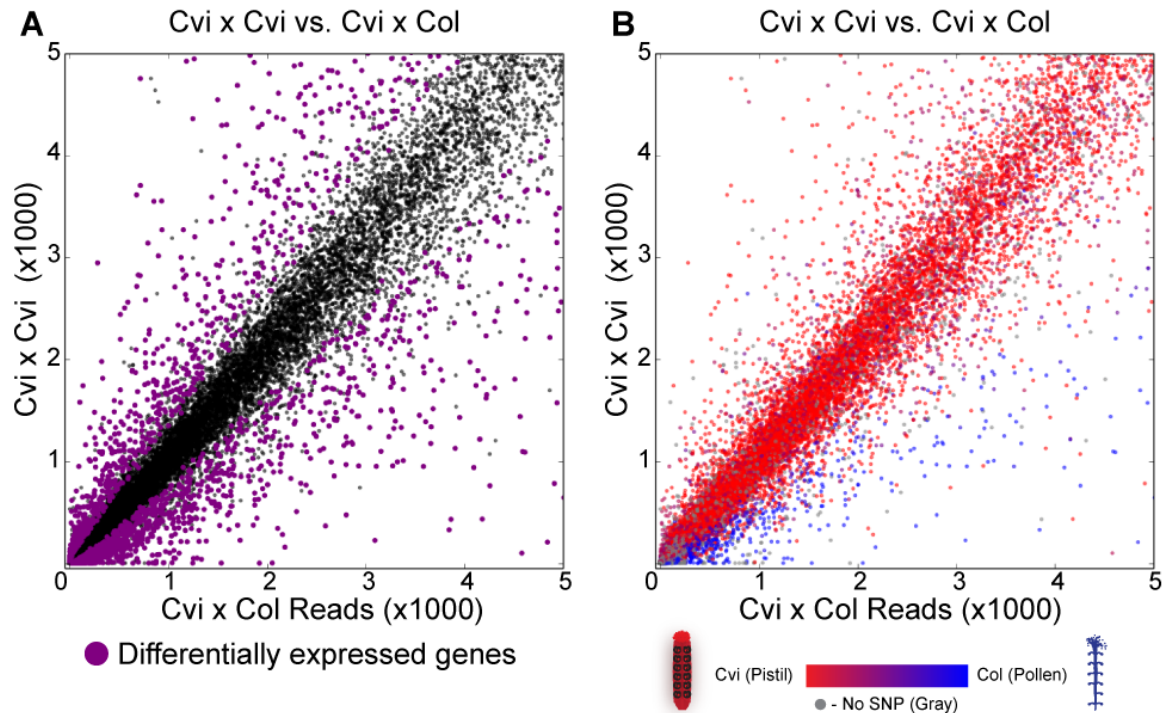


Figure 10. Pollen genotype effect on pistil expression in inter-ecotype crosses. **A.** Differential gene expression between self-pollinated Col and Cvi pistils. 1,640 transcripts are higher in inter-ecotype (Cvi x Col) pollinations, 1,238 transcripts are higher in self-pollinations (Cvi x Cvi), for a total of 2,877 differentially expressed genes, adjusted p-value < 0.005. **B.** Sex specific transcript identity of A. Red dots - Cvi-specific reads, Blue dots - Col-specific reads, and Gray dots do not have SNPs for assignment.

Reproductive gene evolution in *Arabidopsis* ecotypes

Genes involved in reproductive processes evolve more rapidly than other genes, with some of the most rapidly evolving proteins found in sperm from marine gastropods (Metz et al. 1998). Rapid evolution of reproductive genes contributes to species-specific gamete interactions and fertilization (Clark et al. 2006). Cvi is phylogenetically distant from Col (Schmitz et al. 2013), providing the most distal within-species comparison to look for hotspots of reproductive gene evolution. We tested whether SNP frequency per nucleotide was greater within exonic regions of genes involved in reproductive processes (Table 1). We focused on

genes that were expressed in reproductive tissues, using NBS-LRR genes as a control group, which were previously described to have high rates of major effect SNPs in *Arabidopsis* ecotypes (Clark et al. 2007; Schmitz et al. 2013). We used our RNA-sequencing data to define lists of genes that are enriched in the pollinated pistil (>1000 mean reads in Cvi x Col pistils), pollen expressed (>10 mean reads overlapping Col SNPs), pistil expressed (>10 mean reads overlapping Cvi SNPs), or specific to pollen or pistil (Cutoff of mean 10 reads, as previously defined). In order to capture genes that might be specifically expressed during pollination, we also defined a set of genes that are induced by pollination (enriched in Cvi x Col vs Cvi unpollinated samples). Because MYB-regulated genes putatively define pollen tube species-identity we defined lists of MYB-regulated genes from undivided and SNP-specific data sets. We additionally compiled a list of small cysteine-rich proteins (CRPs) (Silverstein et al. 2007), that are related to Thionin proteins (CRP2200-2610). Finally, our RNA-sequencing uncovered MYB-dependent regulation of 15 S protein homolog (SPH) genes (Ride et al. 1999), which led us to create a list of the entire SPH family in *Arabidopsis*. For each list we identified SNP rates per base pair. We compared the SNP rates in these to the average rates calculated from 100 randomly selected control gene sets of equal size. We observed elevated SNP rates in pollen expressed genes, MYB-regulated genes and pistil genes that are strongly induced by pollination (Table 1). Pollen-specific genes that require *MYB97*, *MYB101*, and *MYB120* for expression showed the highest ratio of SNPs to control gene sets (1.72-fold).

Table 1. Col/Cvi Whole Genome SNP Rates	Simulated Rate	True List Rates	0 1 2  TR/SR
Pollinated Pistil Enriched	4.36E-03	3.07E-03	0.70
Pollen Expressed (Col Reads)	4.34E-03	6.62E-03	1.53
Pistil Expressed (Cvi Reads)	4.35E-03	4.48E-03	1.03
Pollen Specific (Col Reads)	4.34E-03	5.86E-03	1.35
Pistil Specific (Cvi Reads)	4.35E-03	3.72E-03	0.85
Pollination Induced (All Reads)	4.34E-03	6.24E-03	1.44
Pistil Specific Pollination Induced (Cvi Reads)	4.38E-03	6.97E-03	1.59
MYB-Regulated (All Reads)	4.33E-03	4.21E-03	0.97
Pollen Specific MYB-Regulated (Col Reads)	4.36E-03	7.44E-03	1.71
CRPs	4.35E-03	5.11E-03	1.17
CRP2200-2610 (Thionin)	4.40E-03	4.87E-03	1.11
SPH	4.54E-03	6.16E-03	1.36
NBS-LRR	4.24E-03	5.27E-03	1.24

Table 1. SNP rates in reproductively expressed genes. Pollinated pistil enriched – Mean reads > 1000 in pollinated pistils (Cvi x Col). Pollen expressed Mean reads >10 in pollen (Col reads). Pistil expressed – Mean reads >10 in pistil (Cvi reads). Pollen Specific – Mean reads >10 in pollen and <10 in pistil. Pistil Specific – Mean reads <10 in pollen, >10 in pistil. Pollination induced pistil genes –All reads, differentially expressed genes (Cvi UP vs Cvi x Col), DESeq2 adjusted p-value <0.005. Pistil specific pollination induced – Pistil specific genes that are differentially expressed during pollination (Unpollinated Cvi vs Cvi x Col DESeq2), adjusted p-value <0.005. MYB-regulated (All reads) – pre-read-division MYB-regulated genes, adjusted p-value<0.005. Pollen Specific MYB-regulated genes (Col Reads) – After read-division, p-value<0.05. CRPs – all CRPs as defined by (Silverstein et al. 2007). CRP2200-2610 subset of only Thionin related CRPs. SPH – S protein homologs (Ride et al. 1999). NBS-LRR genes – TAIR annotations including “NBS-LRR”. Simulated lists (100 randomly chosen gene lists) were used to create a ratio of list rates to demonstrate SNP enrichments – see color scale. TR/SR – True list rate/simulated gene list rate. Ratios that are in bold are statistically significant, p<0.01, Poisson distribution.

Reproductive proteins that mediate species-preferential or specific interactions are expected to show signs of rapid evolution (Clark et al. 2006). We reasoned that these genes with a high frequency of SNPs could result in amino acid substitutions. Therefore, we defined the effects of SNPs on coding capacity in reproductive proteins. To predict the effects of SNPs in expressed genes in the pistil before and during pollination we used SnpEff and SnpSift (Cingolani et al.

2012). DNA variants in Cvi contain a range of predicted effects on protein function, such as strong effects (start site loss, splice site mutations, nonsense mutations – Supplemental File S13). We focused on SNPs that create missense mutations and have the possibility to directly alter protein function. We quantified the frequency of missense mutations in reproductively expressed genes for Cvi and for five other *Arabidopsis* ecotypes. Missense mutations were enriched in reproductively expressed genes across these selected ecotypes. Reproductively expressed genes identified in Col and Cvi have elevated missense mutation rates across other ecotypes (Table 2). CRPs, especially those with homology to Thionins (CRP2200-2610), showed elevated missense mutation rates compared to background rates (Table 2). Indeed, the rate of amino acid substitution-causing SNPs was over seven times the genomic average in Cvi. Elevated missense mutations are not by themselves indicative of rapid evolution; however they identify the potential raw genetic diversity of *Arabidopsis* ecotypes that can be selected upon during the evolution of reproductive processes within the species.

Table 2.	Cvi	Ratio	Kondara	Ratio	Qar	Ratio	RRs	Ratio	Ws	Ratio	Ler	Ratio
Background Rate	0.91	1.00	0.30	1.00	0.40	1.00	0.28	1.00	0.26	1.00	0.01	1.00
Pollinated Pistil Enriched	1.99	2.19	0.71	2.35	0.87	2.19	0.69	2.47	0.63	2.44	0.01	1.75
Pollen Expressed	1.65	1.81	0.59	1.95	0.71	1.79	0.53	1.90	0.49	1.91	0.01	1.37
Pistil Expressed	1.83	2.01	0.61	2.03	0.76	1.93	0.58	2.10	0.54	2.10	0.01	1.15
Pollen Specific	1.51	1.66	0.64	2.12	0.65	1.64	0.41	1.46	0.50	1.92	0.02	1.94
Pistil Specific	1.88	2.07	0.62	2.06	0.78	1.97	0.60	2.15	0.56	2.16	0.01	1.10
Pollination Induced (All Reads)	1.96	2.15	0.74	2.43	0.85	2.14	0.58	2.08	0.55	2.13	0.01	1.57
Pistil Specific Pollination Induced (Cvi Reads)	1.27	1.39	0.31	1.03	0.47	1.18	0.26	0.95	0.33	1.29	0.02	2.34
MYB-Regulated (All Reads)	0.73	0.80	0.17	0.58	0.31	0.79	0.24	0.87	0.23	0.91	0.00	0.00
Pollen Specific MYB-Regulated (Col Reads)	1.16	1.28	0.39	1.29	0.48	1.21	0.44	1.59	0.30	1.17	0.00	0.00
CRPs	1.48	1.63	0.50	1.65	0.73	1.84	0.47	1.68	0.32	1.25	0.00	0.00
CRP2200-2610 (Thionin)	7.22	7.94	1.51	4.99	1.73	4.38	0.17	0.60	0.00	0.00	0.00	0.00
SPH	1.24	1.36	0.67	2.22	0.46	1.16	0.50	1.78	0.50	1.92	0.00	0.00
NBS-LRR	0.14	0.16	0.02	0.07	0.04	0.09	0.03	0.10	0.04	0.16	0.00	0.00

Table 2. SNPs causing missense mutations are enriched in reproductively expressed genes. Gene lists are the same as in Table 1. Missense mutations were called using SnpEff. Background rates - total missense mutation rate (Missense mutation rate/100bp x 1000) across the whole genome. The missense

mutation rate for each gene list (defined in Table 1) was calculated in each Ecotype (Missense mutation rate/100bp x 1000). Ratio – List rate/Background Rate. Values above one are colored red, values below one are colored blue. Ecotype data was accessed from the Salk 1001 genomes database and SNP effects are called using Col-0 TAIR10 as a reference genome. Ecotypes: Cvi-0 – Cape Verde Island (CD28198), Kondara – Kondara (CS76532), Qar – Qar_8a, (CS76581), RRs – RRs_10 (CS76592), Ws – Ws-2 (CS76631), Ler – Ler-0 (CS77020).

Discussion

Successful plant reproduction is the lynchpin of agricultural food production. With high susceptibility to climatic variations and other stress conditions, plant reproduction demands further investigation into its mechanisms, especially pollen and pistil signaling pathways that can be optimized for seed production. Pollen tube growth and differentiation within the complex tissues of the pistil has posed a barrier to direct evaluation of the pollen tube transcriptome. We present a novel method to circumvent the challenges of accurately detecting pollen specific gene expression during growth in the pistil. SNP-based discrimination of pollen versus pistil RNA-seq reads allows a direct view of sex specific transcriptomes without any separation or purification steps that pose challenges because they are technically not feasible and may alter gene expression profiles. While epitope-based purification such as TRAP-seq (Kim et al. 2015) or INTACT (Deal et al. 2011) could provide cell type specificity, they require extensive construction of transgenic lines for reproductive cell expression and development of purification procedures. For example, a recent pollen tube TRAP-seq approach reported low correlation with SIV expressed genes (Lin et al. 2014), which may be in part due to competition for ribosome binding of the epitope tagged somatically-expressed

RPL18 and the pollen tube specific RPL18A isoforms. Therefore, the naturally occurring ecotypes are of great utility, as pollen and pistil can be crossed in any combination without any experimental investment— such is the modular nature of pollen. Furthermore, the method we have developed here is easily transferable to crop plants with sequenced genomes.

Sequencing mixed ecotype samples followed by SNP-based read division has been used to examine allele-specific gene expression patterns due to imprinting with high fidelity (Gehring et al. 2011; Pignatta et al. 2014) and here we have applied this concept to discover cell-type specific gene expression. Our analysis identified pollen- and pistil-specific gene expression profile in wild-type crosses that are well correlated with previous microarray studies of reproductive tissues (Rutley et al. 2015). Many known pollen and pistil genes were identified, as well as many new and exciting gene candidates for further genetic analysis (Figures 6,7). We focused on pollen tube-expressed genes that were also regulated by *MYB97*, *MYB101* and *MYB120*, as they represent pollen tube genes that are likely to have a role in pollen tube reception (Leydon et al. 2013; Liang et al. 2013). SNP-informed sequencing has revealed a new component of the MYB-regulated transcriptome that was previously undetectable due to dilution by RNA from the surrounding pistil tissue. For example, our method was able to identify specific changes in pollen tube expression due to loss of MYB function for genes that are expressed highly in the pistil (Figure 7). These genes are not detected as differentially expressed in *myb* triple mutant pollen tubes when total pistil RNA is analyzed (Leydon et al. 2013). RNA sequencing has also revealed the full extent

of genetic redundancy in MYB-dependent genes in large gene families. Previous microarray analysis ignored many members of gene families because hybridization-based probes could not differentiate them or because they were not annotated at the time of microarray production.

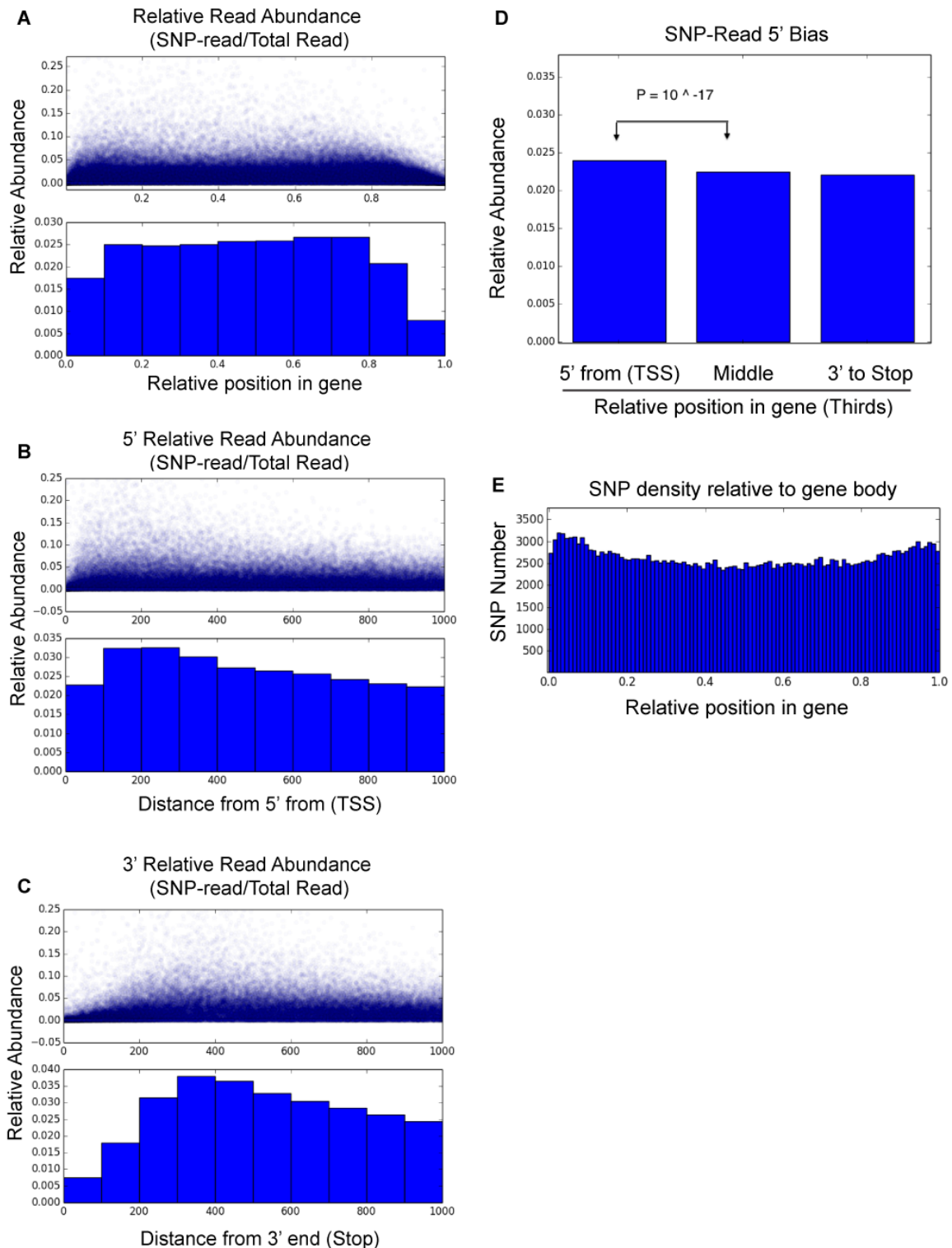
A diverse transcriptional response to pollination was observed when we compared the Cvi and Col self-pollinated transcriptomes. Col and Cvi were collected from different climates: Col from temperate Germany, Cvi from tropical Cape Verde. Geography and temperature most likely played a role in the evolutionary life-history of these ecotypes. Indeed ecotype-specific responses to abiotic stresses such as temperature have been reported before (Barah et al. 2013; Barah et al. 2013). We report divergence in the transcriptional profiles between Col and Cvi self-pollinations, as well as a core set of reproductively expressed genes. Separating the ecotype-specific influences from core pollen tube identity genes will be informative in evaluating the role of conserved and rapidly evolving mechanisms of pollen tube growth, guidance and reception. Specifically, we observed a small group of Cvi pistil-specific genes that are activated by Col pollen tube growth in the pistil and are functionally implicated in defense responses. Given the abundance of pollen tube secreted proteins we observed in *Arabidopsis*, and that have been discovered in Tobacco (Hafidh et al. 2014), it is probable that pollen tube cell wall constituents play a role in pollen tube-pistil signaling. Indeed, the pollen transcriptome is overrepresented by carbohydrate active enzymes involved in cell wall synthesis (Honys et al. 2004;

Pina et al. 2005; Qin et al. 2009), an enzymatic function that was enriched in pollen tube-expressed genes from our RNA-seq experiments (Annotation clusters 4 & 6, Supplemental File 11). It is interesting to speculate that pollen tube secreted carbohydrate active enzymes such as Pectate Lyase (Figure 8D-E) determine a cell wall carbohydrate profile that is species-unique. Added into this unique extracellular matrix are secreted proteins such as the Thionin proteins (Leydon et al. 2013) that decorate the pollen tube cell and may diffuse from the pollen tube tip to the pistil tissues.

We defined patterns of SNP occurrence and their effects on coding sequences in reproductive genes (Table 1) defined by our study of pollination (Figures 1-4). Reproductive genes have been shown to be rapidly evolving in many species. Sperm proteins from species with motile aquatic sperm highlight this characteristic, with high rates of non-synonymous amino acid substitutions (Metz et al. 1998). It has been proposed that rapid co-evolution between sperm surface proteins and their egg binding partners provides a barrier to inter-specific gamete fusion for marine organisms that spawn at the same time and place. In flowering plants, the pollen tube is motile and performs the required cell-cell interactions with female tissues (the pistil) that determine male reproductive recognition and fitness – not the sperm as in mammals. Indeed, SNPs are 1.5x more likely to fall in pollen-expressed genes (Col to Cvi), and these SNPs are 1.7-1.9x more likely to cause a missense mutation (6 ecotypes, Table 2). Pistil specific genes that are pollination responsive also display increased SNP-rate and missense

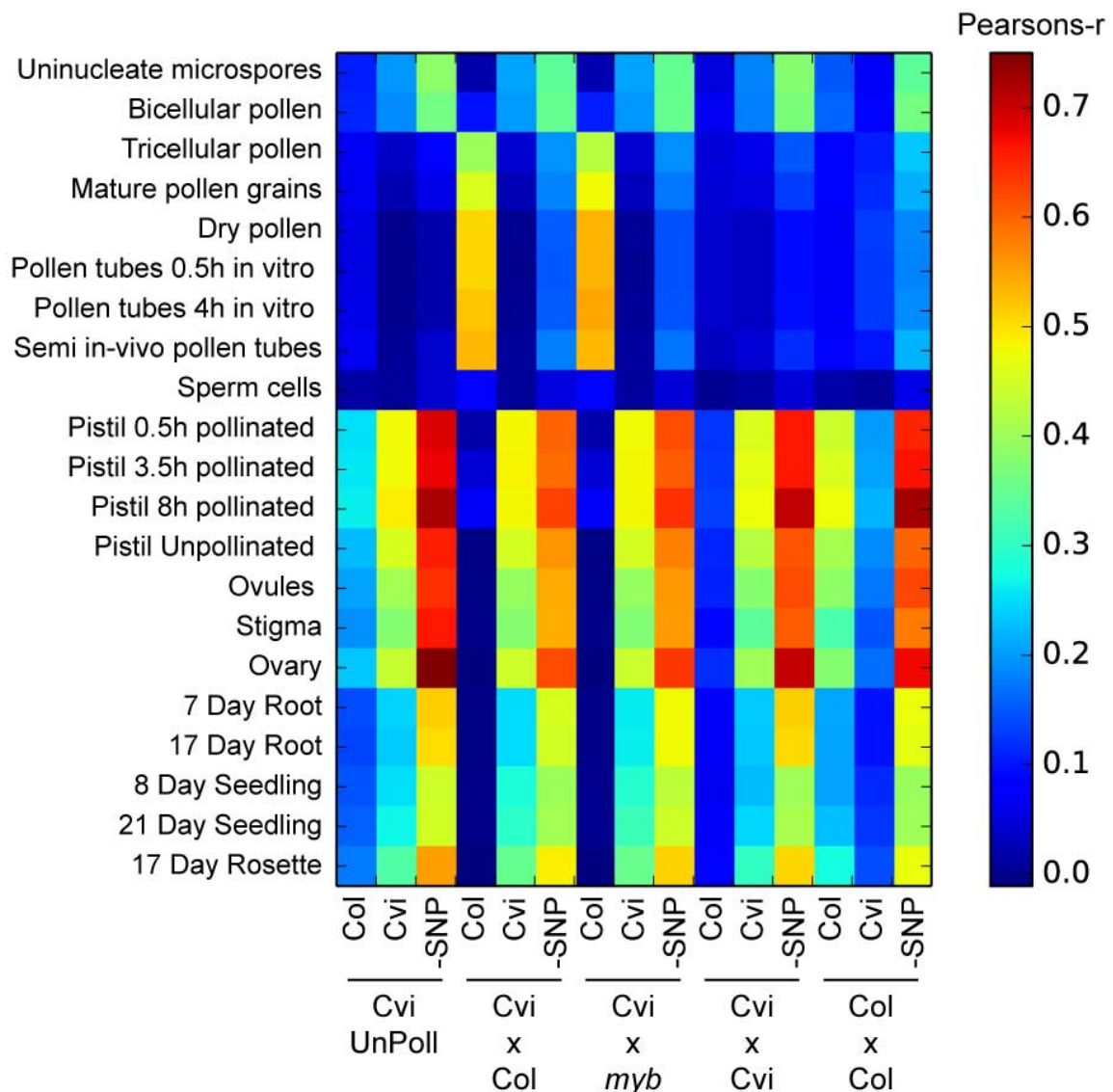
frequencies. These results are exciting and require further evolutionary analysis on individual pollen expressed gene families, such as the small secreted cysteine rich proteins (CRPs), and the Self-incompatibility protein homologs (SPHs).

This study provides a snapshot of pollen tube-pistil interaction at one time point (8 hours of pollen tube growth), and captures a wealth of information on pollen tube and pistil gene expression. We have described comprehensive profiles of genes expressed in the pollinated pistil, and applied sex-specific determination for all available SNPs. This methodology provides a powerful method to quantify pollen-pistil interactions over any time-course to catch transcriptional responses. We have used this method to further define the set of genes controlled by pollen tube MYB transcription factors *MYB97*, *MYB101*, and *MYB120*. However, this method could be applied to any pollen tube or pistil mutant proposed to affect pollen-pistil interactions. Moreover, this method lends itself beautifully to analysis of the impact of environmental change on the reproductive transcriptome of crop plants and holds the potential to uncover how pollen tube – pistil interactions change in response to stress.



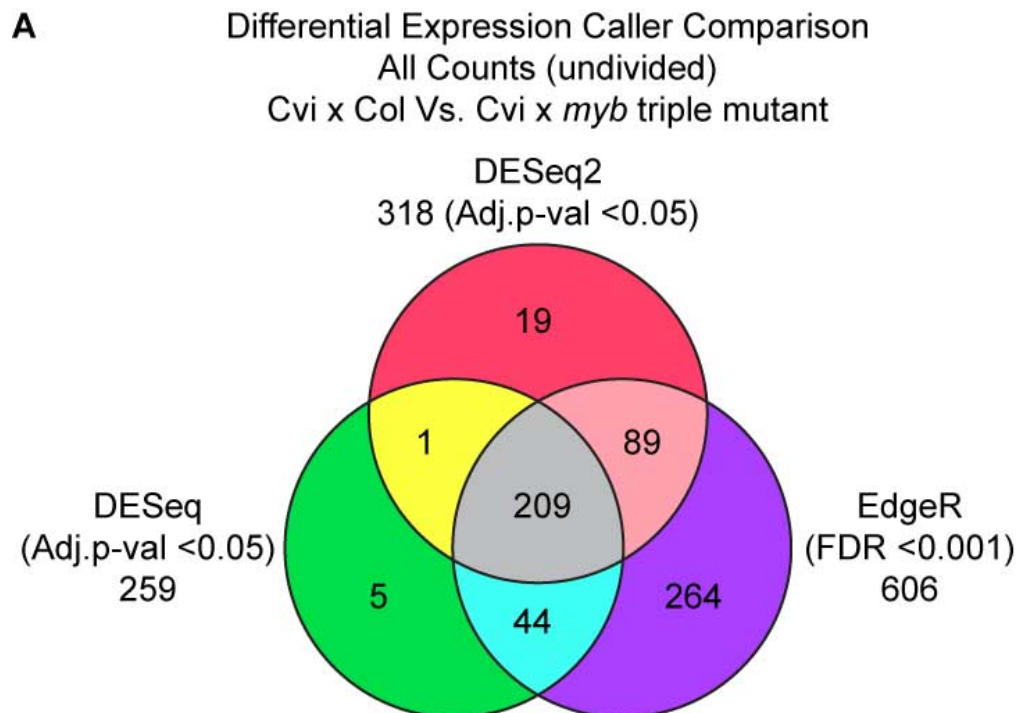
Supplemental Figure 1. Quantitative analysis on reads identified from SNPs ensures an accurate representation of total gene expression. A-C. Relative read abundance of SNP-reads to total reads (SNP-spanning reads/total reads). A. Relative read abundance over total gene bodies, normalized to a meta-gene body (x-axis). B. Relative SNP-read abundance at 5' ends of genes, aligned by

position of the transcription start site (TSS). C. Relative SNP-read abundance at 3' ends of genes, aligned by position of the translation stop site (Stop). **D.** Relative abundance of SNP-reads to total reads was divided and binned into thirds (TSS/Middle/STOP) and plotted side-by-side. A slight but significant enrichment was seen in 5' ends of genes (t-test, $p=10^{-17}$). **E.** Genome wide analysis of SNP distribution within coding sequences reveals slight bias towards 5' ends of genes. Total number of SNPs falling in coding sequences distributed along a meta-gene profile (all gene lengths normalized to 1, x-axis).



Supplemental Figure 2. Correlation between SNP-specific sex identification and published microarray analysis. Colors are assigned based upon Pearson's-R correlation value. Microarray data sources are as follows. Uninucleate microspores, tricellular pollen, mature pollen grains - (Honys et al. 2004). Dry pollen, 0.5h, 4h and SIV pollen tubes - (Qin et al. 2009). Unpollinated &

pollinated pistils, ovules - (Boavida et al. 2011). stigma, ovary (Swanson et al. 2005). Root, seedling, rosette - (Schmid et al. 2005).



Supplemental Figure 3. Comparison of differential expression callers on RNA-sequencing results for wild-type and *myb* triple-mutant pollinations. Differential expression was determined following the default pipeline as described by the respective vignettes available for DESeq, DESeq2, and EdgeR. (Supplemental File S6).

A Promoters from MYB-regulated Targets:	Word Search	
	GAMYB	TAACAA
Total Reads	66/272	218/272
Pollen specific	136/264	219/264

Supplemental Figure 4. Cis-Regulatory element analysis on cell-type specific MYB-regulated gene promoters. Analysis was performed in RSAT (van Helden 2003) using the GAMYB PWM (Skriver et al. 1991; Gubler et al. 1992; Lanahan et al. 1992), or the TAACAA core sequence.

Materials and Methods

Plant Material

Seeds were grown on solid Murashige and Skoog (MS) medium (Sigma Aldrich, St. Louis, MO). *Arabidopsis* plants were grown in chambers at 21°C under illumination (100 mmol m⁻² s⁻¹ with a 16-hour photoperiod). Seedlings were transplanted to sterile 2MIX potting media (www.fafard.com) between 7 and 10 days after plating. All plants were grown at 20°C, 50-60% humidity, on a 16day/8hour night/light cycle in growth chambers (Environmental growth chambers, Chagrin Falls, OH, USA). SIV pollinations were performed as previously described (Palanivelu et al. 2006; Qin et al. 2009).

mRNA-seq library construction

RNA was isolated from excised pistil tissues either unpollinated or 8 hours after pollination using the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). At least 10ug of total RNA was oligo-dT-bead purified and RNA-sequencing libraries were prepared according to the NEBNext protocol. Primers 4, 6, and 12 were used from NEBNext® Multiplex Oligos for Illumina (New England Biolabs, Ipswich, MA). Amplification was for 15 cycles. Libraries were tested by qPCR after amplification to validate amplification.

mRNA-seq data analysis

Pistils were collected with and without pollination in triplicate (15 samples). Single-end sequencing was performed with mRNA-seq libraries on an Illumina HiSeq machine. Read length was 50 bp. Sequencing quality was analyzed using

fastqc. Reads were aligned to the Arabidopsis genome (TAIR10) using Tophat v2.0.13 (Kim et al. 2013). Tophat options were --segment-length 22 --segment-mismatches 1 --max-segment-intron 11000. Aligned reads were parsed using a PERL script released by the Ghering Lab in (Pignatta et al. 2014). We quantified read counts per gene using EasyRNASeq (Delhomme et al. 2012), we allowed multi-mapping reads to be counted because many pollen expressed genes were observed to be in redundant gene families due to gene duplication. Differential expression was performed using DESeq2 (Love et al. 2014).

GO analysis

GO analysis was performed using the DAVID bioinformatics resource version 6.7 (Huang et al. 2009). Reported p-values are corrected using the Benjamini method.

Calling variants

RNA sequencing reads from unpollinated Cvi-0 and Cvi-0 self-pollinated for 8 hours was analyzed for novel SNP variants following the GATK Best Practices workflow (McKenna et al. 2010). Briefly, mapped reads (Tophat2) were prepared for variant calling: Read group addition, mark duplicates, Split'N'Trim, ReassignMappingQuality, Indel Realignment, Base Recalibration. Variant calling was performed using the GATK HaplotypeCaller. Variant filtration was performed (FS>30, QD<2.0). SNPs supported with less than 10 reads, or that were not homozygous were discarded. Any SNPs in common with previously established Cvi-0 SNPs (Pignatta et al. 2014) were considered validated and set aside from

the list of novel SNPs. Unpollinated Cvi-0 and Cvi-0 self-pollinated RNAseq library reads were then ran against this novel SNP list using a previously published Perl pipeline (Pignatta et al. 2014). Any SNPs locations that returned a Col-0 SNP, were discarded from the BED file. This eliminated 2000 SNPs, and created a final additional 15995 SNPs (in 237 genes previously without documented SNPs, and 5217 genes that had one or more previously described SNP).

Variant Analysis

Effect of SNPs were predicted using the SnpEff and SnpSift software (Cingolani et al. 2012; Cingolani et al. 2012). Master SNP lists were correlated to the TAIR10 gene annotation file to identify the distribution of SNPs in genomic elements using a range overlap function via the IRanges package (Lawrence et al. 2013) and a custom R script - R Core Team (2013), R: A language and environment for statistical computing; R Foundation for Statistical Computing, Vienna, Austria (<http://www.R-project.org/>). The SNP rate was computed for each gene, and the average SNP rate of Individual gene lists were compared to background frequencies simulated using randomly generated lists of the same size as input lists. Each comparison background frequency was the average of 100 simulations. Similarly, the rate of missense mutations were calculated for each gene and averaged for the individual gene lists. These frequencies were compared to the rate of missense mutations across the entire genome. R scripts

and data for analysis are part of the package SexSpecificExpression, which is available on GitHub at <https://github.com/evenable/SexSpecificExpression.git>.

Transcriptional profile correlation analysis

Pairwise correlations were generated between our RNA-seq data and microarray data from previous studies: Uninucleate microspores, tricellular pollen, Mature pollen grains - (Honys et al. 2004). Dry pollen, 0.5h, 4h and Semi in vivo pollen tubes - (Qin et al. 2009). Unpollinated & pollinated pistils, Ovules - (Boavida et al. 2011). Stigma, Ovary (Swanson et al. 2005). Root, Seedling, Rosette - (Schmid et al. 2005). For each pair, we computed pearson-r over all genes with a unique SNP, using mean reads from three biological replicates for RNA-seq and mean absolute intensity for multiple microarray replicates.

Graphing Sequencing data

Scatterplots were generated from sequence data using Python's Matplotlib module.

Acknowledgements

We thank the Brown University Core Facility, especially Christoph Schorl for his expertise and help with RNA-seq. We thank Dr. Mary Gehring for help utilizing SNP-based read analysis pipelines developed by her Laboratory. We also thank Dr. William Fairbrother and John Urban for assistance with scripting.

Supplemental Files

Supplemental File S1. Master SNP database file. A BED format file of all Col/Cvi SNPs used in our study. This file is a tab delimited, 5-column file: Chr, ChrStart, ChrEnd, RefBase, AltBase, where Ref=Col, Alt=Cvi.

Supplemental File S2. Excel spreadsheet of Genes/TEs expressed in Pollen based upon the presence of Col SNPs in coding sequences. Column A – AGI. K – Average counts over indicated gene with a Col SNP (Avg Col SNP, average of A2-A4). Average counts over indicated gene with a Cvi SNP (Avg Cvi SNP, average of A5-A7). Average counts over indicated gene with a No SNP (Avg no SNP, average of A8-A10). Columns Q-S are respective standard deviations of counts. Column AG – Male expressed genes (>10 average reads) are marked (“ME” = male exp). Column AH – Female expressed genes (>10 average reads) are marked (FE = female Exp). Column AH – Genes defined as Male specific are marked TRUE/FALSE (Male specific (>10 male, <10 female)). Column AJ - Genes defined as Male specific are marked TRUE/FALSE (Female specific (<10 male, >10 female)). Column AK – Genes that are expressed in both tissues are marked TRUE/FALSE (>10 in both).

Supplemental File S3. Excel spreadsheet of Genes/TEs expressed in Pistils based upon the presence of Cvi SNPs in coding sequences. Column A – AGI. K – Average counts over indicated gene with a Col SNP (Avg Col SNP, average of A2-A4). Average counts over indicated gene with a Cvi SNP (Avg Cvi SNP, average of A5-A7). Average counts over indicated gene with a No SNP (Avg no SNP, average of A8-A10). Columns Q-S are respective standard deviations of counts. Column AG – Male expressed genes (>10 average reads) are marked (“ME” = male exp). Column AH – Female expressed genes (>10 average reads) are marked (FE = female Exp). Column AH – Genes defined as Male specific are marked TRUE/FALSE (Male specific (>10 male, <10 female)). Column AJ - Genes defined as Male specific are marked TRUE/FALSE (Female specific (<10 male, >10 female)). Column AK – Genes that are expressed in both tissues are marked TRUE/FALSE (>10 in both).

Supplemental File S4. Pistil-specific Genes/TEs differentially expressed during pollen tube growth in the pistil. Sex-specific read division was performed on unpollinated Cvi pistils and Cvi pistils pollinated with Col pollen. Alignments were then analyzed for differential expression using DESeq2. Column A - Gene/TE identifier (AGI). Column B - mean of normalized counts for all samples (baseMean). Column C - log2 fold change: Cvi unpollinated vs Cvi x Col pollinated. Column D – Standard error. Column E – Wald statistic. Column F – Wald test p-value. Column G – BH adjusted p-values. (Trimmed DESeq2 Results).

Supplemental File S5. Pollen Specific GO Analysis using the DAVID server. This excel spreadsheet has four tabs. The first is a clustering analysis of GO terminology to identify enriched clusters of genes and their

functions/localizations. The three tabs following are for Cellular Component, Biological Process, and Molecular Function, respectively.

Supplemental File S6. Differential gene expression in *myb* triple-mutant pollinations from mapped reads prior to SNP-division (Cvi x Col vs Cvi x *myb* triple-mutant). This excel file contains the differential gene expression analysis using three differential expression callers: DESeq, DESeq2, and edgeR. There are three tabs in this file. The first tab contains a comparison of the genes identified by each differential expression caller and relates directly to Supplemental Figure 3. To the right of this table, are the overlap between genes identified by microarray and sequencing.

Supplemental File S7. Differential gene expression in *myb* triple-mutant pollinations from mapped reads after SNP-division (Cvi x Col vs Cvi x *myb* triple-mutant). This excel file contains three different differential expression comparisons in three separate tabs. The first tab – “Pollen_Specific” contains differentially expressed Genes/TEs based upon divided reads that contained the Col SNP. The second tab – “Pistil_Specific” contains differentially expressed Genes/TEs based upon divided reads that contained the Cvi SNP. The third tab – “Remaining_reads” contains differentially expressed Genes/TEs based upon divided reads that contained No SNPs attributable to Col or Cvi. For each tab: Column A - Gene/TE identifier (AGI). Column B - mean of normalized counts for all samples (baseMean). Column C - log2 fold change: Cvi x Col vs Cvi x *myb*. Column D – Standard error. Column E – Wald statistic. Column F – Wald test p-value. Column G – BH adjusted p-values. (Trimmed DESeq2 Results).

Supplemental File S8. Promoter sequences from genes that were differentially expressed in total undivided reads (Supplemental File S6). This is a FASTA format text file pulled from the *Arabidopsis* genome using RSAT retrieve-seq, 1997-98 by Jacques van Helden (<http://plants.rsat.eu/>).

Supplemental File S9. Promoter sequences from genes that were differentially expressed in pollen reads (Supplemental File S7). This is a FASTA format text file pulled from the *Arabidopsis* genome using RSAT retrieve-seq, 1997-98 by Jacques van Helden (<http://plants.rsat.eu/>).

Supplemental File S10. Differentially expressed Genes/TEs between Col x Col and Cvi x Cvi pollinations. DESeq2, padj < 0.005. Here, log2 values with a negative value indicate high expression in Col ecotype. Conversely, log2 values with a positive value indicate high expression in the Cvi ecotype. Therefore, for ease of viewing they have been sorted for Log2FC (Column C). Column A - Gene/TE identifier (AGI). Column B - mean of normalized counts for all samples (baseMean). Column C - log2 fold change: Col x Col vs Cvi x Cvi. Column D – Standard error. Column E – Wald statistic. Column F – Wald test p-value. Column G – BH adjusted p-values. (Trimmed DESeq2 Results).

Supplemental File 11. GO analysis on Cvi Pistil-specific genes that are induced by growth of Col pollen tubes (Based on Cvi x Cvi vs Cvi x Col differential expression). This excel spreadsheet has one tab: a clustering analysis of GO terminology to identify enriched clusters of genes and their functions/localizations.

Supplemental File S12. Gene lists used in SNP rate analyses.

Supplemental File S13. Table of SNP Effects predicted to have strong impacts on protein coding or splicing discovered in the Cvi genome. SnpEff results subset using SnpSift for “HIGH” impact effects. Data is in a headerless VCF format.

Supplemental Movies.

Supplemental Movie 1 - SUC9:GFP, SIV PT, Confocal Time-course, 7 frames/sec, each frame is 30 seconds apart.

Supplemental Movie 2 - PL:GFP, SIV PT, Confocal Time-course, 7 frames/sec, each frame is 30 seconds apart.

Supplemental_Movie_3 - ACA4:GFP, SIV PT, Confocal Time-course, 7 frames/sec, each frame is 30 seconds apart.

References

- Anders, S. and W. Huber (2010). "Differential expression analysis for sequence count data." Genome Biol **11**(10): R106.
- Barah, P., N. D. Jayavelu, et al. (2013). "Genome scale transcriptional response diversity among ten ecotypes of *Arabidopsis thaliana* during heat stress." Front Plant Sci **4**.
- Barah, P., N. D. Jayavelu, et al. (2013). "Genome-scale cold stress response regulatory networks in ten *Arabidopsis thaliana* ecotypes." BMC Genomics **14**: 722.
- Becker, J. D., L. C. Boavida, et al. (2003). "Transcriptional profiling of *Arabidopsis* tissues reveals the unique characteristics of the pollen transcriptome." Plant Physiol **133**(2): 713-725.
- Boavida, L. C., F. Borges, et al. (2011). "Whole genome analysis of gene expression reveals coordinated activation of signaling and metabolic pathways during pollen-pistil interactions in *Arabidopsis*." Plant Physiol **155**(4): 2066-2080.
- Borges, F., G. Gomes, et al. (2008). "Comparative transcriptomics of *Arabidopsis* sperm cells." Plant Physiol **148**(2): 1168-1181.
- Brady, S. M., D. A. Orlando, et al. (2007). "A high-resolution root spatiotemporal map reveals dominant expression patterns." Science **318**(5851): 801-806.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in *Arabidopsis* Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Cingolani, P., V. M. Patel, et al. (2012). "Using *Drosophila melanogaster* as a Model for Genotoxic Chemical Mutational Studies with a New Program, SnpSift." Front Genet **3**: 35.
- Cingolani, P., A. Platts, et al. (2012). "A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3." Fly (Austin) **6**(2): 80-92.
- Clark, N. L., J. E. Aagaard, et al. (2006). "Evolution of reproductive proteins from animals and plants." Reproduction **131**(1): 11-22.
- Clark, R. M., G. Schweikert, et al. (2007). "Common sequence polymorphisms shaping genetic diversity in *Arabidopsis thaliana*." Science **317**(5836): 338-342.
- Deal, R. B. and S. Henikoff (2011). "The INTACT method for cell type-specific gene expression and chromatin profiling in *Arabidopsis thaliana*." Nat Protoc **6**(1): 56-68.
- Delhomme, N., I. Padioleau, et al. (2012). "easyRNASeq: a bioconductor package for processing RNA-Seq data." Bioinformatics **28**(19): 2532-2533.
- Dresselhaus, T., H. Lorz, et al. (1994). "Representative cDNA libraries from few plant cells." Plant J **5**(4): 605-610.

- Elleman, C. J. and H. G. Dickinson (1986). "Pollen-stigma interactions in Brassica. IV. Structural reorganization in the pollen grains during hydration." J Cell Sci **80**: 141-157.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Gehring, M., V. Missirian, et al. (2011). "Genomic analysis of parent-of-origin allelic expression in Arabidopsis thaliana seeds." PLoS One **6**(8): e23687.
- Gubler, F. and J. V. Jacobsen (1992). "Gibberellin-responsive elements in the promoter of a barley high-pI alpha-amylase gene." Plant Cell **4**(11): 1435-1441.
- Hafidh, S., D. Potesil, et al. (2014). "In search of ligands and receptors of the pollen tube: the missing link in pollen tube perception." Biochem Soc Trans **42**(2): 388-394.
- Honys, D. and D. Twell (2003). "Comparative analysis of the Arabidopsis pollen transcriptome." Plant Physiol **132**(2): 640-652.
- Honys, D. and D. Twell (2004). "Transcriptome analysis of haploid male gametophyte development in Arabidopsis." Genome Biol **5**(11): R85.
- Huang, D. W., B. T. Sherman, et al. (2009). "Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources." Nat Protoc **4**(1): 44-57.
- Johnston, A. J., P. Meier, et al. (2007). "Genetic subtraction profiling identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte." Genome Biol **8**(10): R204.
- Jones-Rhoades, M. W., J. O. Borevitz, et al. (2007). "Genome-wide expression profiling of the Arabidopsis female gametophyte identifies families of small, secreted proteins." PLoS Genet **3**(10): 1848-1861.
- Kim, D., G. Pertea, et al. (2013). "TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions." Genome Biol **14**(4): R36.
- Kim, T., C. S. Lim, et al. (2015). "Cell type-specific gene expression profiling in brain tissue: Comparison among TRAP, LCM and RNA-seq." BMB Rep.
- Lanahan, M. B., T. H. Ho, et al. (1992). "A gibberellin response complex in cereal alpha-amylase gene promoters." Plant Cell **4**(2): 203-211.
- Lawrence, M., W. Huber, et al. (2013). "Software for computing and annotating genomic ranges." PLoS Comput Biol **9**(8): e1003118.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.

- Leydon, A. R., A. Chaibang, et al. (2014). "Interactions between pollen tube and pistil control pollen tube identity and sperm release in the Arabidopsis female gametophyte." Biochem Soc Trans **42**: 340-345.
- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 Function as Male Factors That Control Pollen Tube-Synergid Interaction in Arabidopsis thaliana Fertilization." PLoS Genet **9**(11).
- Lin, S. Y., P. W. Chen, et al. (2014). "Profiling of transcriptomes of in vivo-grown pollen tubes reveals genes with roles in micropylar guidance during pollination in Arabidopsis." Plant Cell **26**(2): 602-618.
- Love, M. I., W. Huber, et al. (2014). "Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2." Genome Biol **15**(12): 550.
- McKenna, A., M. Hanna, et al. (2010). "The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data." Genome Res **20**(9): 1297-1303.
- Metz, E. C., R. Robles-Sikisaka, et al. (1998). "Nonsynonymous substitution in abalone sperm fertilization genes exceeds substitution in introns and mitochondrial DNA." Proc Natl Acad Sci U S A **95**(18): 10676-10681.
- Nodine, M. D. and D. P. Bartel (2012). "Maternal and paternal genomes contribute equally to the transcriptome of early plant embryos." Nature **482**(7383): 94-97.
- Nordborg, M., T. T. Hu, et al. (2005). "The pattern of polymorphism in Arabidopsis thaliana." Plos Biology **3**(7): e196.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Pignatta, D., R. M. Erdmann, et al. (2014). "Natural epigenetic polymorphisms lead to intraspecific variation in Arabidopsis gene imprinting." Elife **3**: e03198.
- Pina, C., F. Pinto, et al. (2005). "Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation." Plant Physiol **138**(2): 744-756.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Ride, J. P., E. M. Davies, et al. (1999). "Analysis of Arabidopsis genome sequence reveals a large new gene family in plants." Plant Mol Biol **39**(5): 927-932.
- Robinson, M. D., D. J. McCarthy, et al. (2010). "edgeR: a Bioconductor package for differential expression analysis of digital gene expression data." Bioinformatics **26**(1): 139-140.
- Rutley, N. and D. Twell (2015). "A decade of pollen transcriptomics." Plant Reproduction.
- Sawada, H., M. Morita, et al. (2014). "Self/non-self recognition mechanisms in sexual reproduction: New insight into the self-incompatibility system

- shared by flowering plants and hermaphroditic animals." Biochem Biophys Res Commun **450**(3): 1142-1148.
- Schmid, K. J., T. R. Sorensen, et al. (2003). "Large-scale identification and analysis of genome-wide single-nucleotide polymorphisms for mapping in *Arabidopsis thaliana*." Genome Res **13**(6A): 1250-1257.
- Schmid, M., T. S. Davison, et al. (2005). "A gene expression map of *Arabidopsis thaliana* development." Nat Genet **37**(5): 501-506.
- Schmitz, R. J., M. D. Schultz, et al. (2013). "Patterns of population epigenomic diversity." Nature **495**(7440): 193-198.
- Scutt, C. P., M. Vinauger-Douard, et al. (2003). "The identification of candidate genes for a reverse genetic analysis of development and function in the *Arabidopsis gynoecium*." Plant Physiol **132**(2): 653-665.
- Silverstein, K. A., W. A. Moskal, Jr., et al. (2007). "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants." Plant J **51**(2): 262-280.
- Silverstein, K. A. T., W. A. Moskal, et al. (2007). "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants." Plant Journal **51**(2): 262-280.
- Skriver, K., F. L. Olsen, et al. (1991). "cis-acting DNA elements responsive to gibberellin and its antagonist abscisic acid." Proc Natl Acad Sci U S A **88**(16): 7266-7270.
- Steffen, J. G., I. H. Kang, et al. (2007). "Identification of genes expressed in the *Arabidopsis* female gametophyte." Plant J **51**(2): 281-292.
- Swanson, R., T. Clark, et al. (2005). "Expression profiling of *Arabidopsis* stigma tissue identifies stigma-specific genes." Sexual Plant Reproduction **18**(4): 163-171.
- Thomas-Chollier, M., O. Sand, et al. (2008). "RSAT: regulatory sequence analysis tools." Nucleic Acids Res **36**(Web Server issue): W119-127.
- Tung, C. W., K. G. Dwyer, et al. (2005). "Genome-wide identification of genes expressed in *Arabidopsis* pistils specifically along the path of pollen tube growth." Plant Physiol **138**(2): 977-989.
- Twell, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.
- van Helden, J. (2003). "Regulatory sequence analysis tools." Nucleic Acids Res **31**(13): 3593-3596.
- Wang, Y., W. Z. Zhang, et al. (2008). "Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in *Arabidopsis*." Plant Physiol **148**(3): 1201-1211.
- Wang, Y., W. Z. Zhang, et al. (2008). "Transcriptome analyses show changes in gene expression to accompany pollen germination and tube growth in *Arabidopsis*." Plant Physiol **148**(3): 1201-1211.
- Wuest, S. E., K. Vijverberg, et al. (2010). "*Arabidopsis* Female Gametophyte Gene Expression Map Reveals Similarities between Plant and Animal Gametes." Current Biology **20**(6): 506-512.

Yu, H. J., P. Hogan, et al. (2005). "Analysis of the female gametophyte transcriptome of Arabidopsis by comparative expression profiling." Plant Physiol **139**(4): 1853-1869.

Chapter 6:

A family of small, secreted Thionin proteins control synergid degeneration during pollen tube reception in *Arabidopsis*

Alexander R. Leydon, and Mark A. Johnson

I performed all the experiments contained in this chapter.

Abstract

Successful plant reproduction requires species-specific interaction of the male and female gametophytes. The male gametophyte, the pollen tube, navigates the extracellular environment of the pistil, extending via polar tip growth, and following guidance cues to the female gametophyte, within the ovule. Gametophyte:gametophyte physical interaction is species specific, and requires correct interaction between the pollen tube cell and the synergid cells of the female gametophyte. Factors that determine pollen tube identity in these interactions are as yet unknown, however they are presumed to signal through the extracellular environment. Here we define a family of small cysteine rich proteins (CRPs) in the Thionin protein family that are specifically expressed in the pollen tube growing in the pistil, and are secreted into the shared pollen tube-synergid apoplast. These proteins are unique to *Brassica*, demonstrate species-specific gene family duplications, and coordinated gene regulation in the pollen tube. We demonstrate using genetic and biochemical approaches that these pollen tube Thionins are sufficient to trigger synergid degeneration. Thionins describe the first pollen tube secreted factors that act as determinants of gametophytic recognition in *Brassica*.

Introduction

Sexual reproduction requires mechanisms that determine correct gamete interaction, whether they are pre-mating behaviors that eliminate unproductive couplings, or post-mating molecular interactions that block exotic species sperm from reaching the egg. Plant reproduction utilizes both mechanisms, using behaviors such as flowering time and pollinator-specific interactions as well molecular interactions between the pollen and the pistil to ensure correct species sperm are chosen for fertilization (Clark et al. 2006). In fact, species-specific interactions are observed at every point of pollen tube interaction with the pistil, even gametophytic interactions between the pollen tube and the synergid such as in *Brassica* (Escobar-Restrepo et al. 2007). Despite the observation of species-specific interactions with the synergid cells of the female gametophyte we know surprisingly little about the pollen tube determinants of species identity. Furthermore, we know little about core pollen tube identity and the types of proteins that are involved in creating this identity.

During pollen tube reception in the ovule, both the pollen tube and the synergid undergo precisely controlled cell death events in order to deliver sperm for fertilization (Christensen et al. 1998; Berger et al. 2008). Pollen tubes coordinate gene expression during growth in the pistil through activation of three MYB transcription factors (*MYB97*, *MYB101*, *MYB120*). When *myb* triple-mutant pollen tubes interact with the female gametophyte, both the pollen tube and synergid frequently fail to undergo cell death, which results in pollen tube overgrowth in

the ovule (Leydon et al. 2013; Liang et al. 2013). This trio of MYB transcription factors positively regulates a subset of pollen tube genes, either directly or indirectly, which are putative identity determinants in the pollen tube during reception. One group of genes that is positively regulated by MYBs is a family of cysteine-rich proteins (CRPs), CRP2460 (Silverstein et al. 2007).

Small secreted CRPs were missed in original annotations of the *Arabidopsis* genome (Silverstein et al. 2007; Zhou et al. 2013), and are increasingly found as signaling components in the dialogue between cells. Peptide signals regulate meristem identity through CLV peptides (Clark et al. 1995), embryo patterning through ESF (Costa et al. 2014, Thesis Chapter 1). In reproductive processes, CRPs include secreted micropylar attractants such as the LURE defensin proteins in *Torenia* and *Arabidopsis* (Okuda et al. 2009; Takeuchi et al. 2012). Membrane localization of the sperm fusion protein HAP2/GCS1 results from egg cell secretion of the EC1 CRPs, which activates plasmogamy (Sprunck et al. 2012). Reproductive-associated CRPs demonstrate several key characteristics. First, they are cell-type specific, as in the case of the synergid-expressed pollen tube attractants (LUREs), where local gradients are established from the micropyle. Second, species-specific phylogenetic groupings of reproductive CRPs have been observed, as in the case of the AtLURE proteins (Takeuchi et al. 2012).

CRPs are characterized by their cysteine-rich composition, usually containing an even number of between 4-16 cysteines, which are involved in disulfide bond formation. These intramolecular bonds stabilize CRPs in the extracellular environment, and render them resistant to proteolysis (Vila-Perello et al. 2005). High protein stability has been evaluated in other cysteine-rich toxin proteins as an adaptive quality for the evolvability of diverse biological activities upon a stable protein framework (Zhu et al. 2005). Thionins are a plant-specific family of CRPs (Silverstein et al. 2007), which have been described in many species as components of the immune response, protecting against infection and herbivory through their toxic effects (Reviewed in Stec 2006). Furthermore, in *Arabidopsis*, the PR-13 subclass of Thionins (Thi2.1-2.4) protect against pathogens (Loeza-Angeles et al. 2008; Asano et al. 2013).

The first indications of Thionins biological activity was described by Jago & Jago in 1885 when wheat flour exerted a lethal effect on brewer's yeast, which was later identified as purothionin (Balls AK 1942). Purothionin exerts a toxic effect on bacteria (Fernandez de Caley et al. 1972), fungi (Reimann-Philipp et al. 1989), insect larvae (Kramer et al. 1979), mammalian cells in culture (Carrasco et al. 1981), as well as whole animals (Coulson 1942). A number of homologs in various plant species have been discovered in the endoseperm of barley (Hernandezlucas et al. 1986), maize (Jones et al. 1980), and oats (Bekes et al. 1981). Purothionins and related Thionins Viscotoxin (Samuelss.G 1973) and

Crambin (Teeter et al. 1979) have also been structurally characterized, allowing insight into the molecular mode of Thionin toxicity (Stec 2006).

Founding members of the Thionin family were identified as small proteins (~5kDa), yet cloning and sequencing of their corresponding full length cDNAs indicated cleavage from a pro-protein (Ponz et al. 1986). The pro-protein precursor consists of an N-terminal signal peptide, the Thionin domain (corresponding to the mature Thionin peptide), and an acidic C-terminal domain (Figure 9A). There have been limited studies on the role of the C-terminal acidic domain, which may have some anti-microbial activity (Abbas et al. 2013). Thionins interact with cell membranes (Osorio e Castro et al. 1989), and through an as yet undetermined mechanism permeate membranes (Florack et al. 1994; Hughes et al. 2000). It has been proposed that their pore-forming abilities are due to direct interaction with phospholipid head groups, as they are able to form proteolipid complexes (Stec 2006).

One attractive hypothesis is that pollen expressed-CRPs in the Thionin family are acting on synergid cells to promote cell death during pollen tube reception. Here we evaluate the MYB-regulated Thionins (CRP2460), to determine what role they play in pollen tube reception. Transcriptional profiling of pollinated pistils revealed MYB-regulated transcripts that are expressed only during growth in the pistil (Thesis Chapter 4, (Leydon et al. 2013). Deep sequencing revealed that many MYB-regulated genes were members of large gene families that displayed similar

expression patterns and required MYB transcription factors for expression (Thesis Chapter 5). Reporter fusions show Thionins enter the pollen endomembrane system, and are secreted from the pollen tube to the synergid during reception. We describe a genetic strategy to test the ability of Thionin proteins to restore pollen tube reception to *myb* triple-mutant pollen tubes. We demonstrate that Thionin proteins induce synergid degeneration when introduced into *myb* triple-mutant pollen tubes, and that purified Thionin proteins induce synergid degeneration in vitro. This work identifies a putative component of the pollen tube signaling machinery during reception, and identifies a role for Thionin proteins in defining pollen tube identity during reproduction.

Results

Thionin-like CRPs are regulated by pollen-specific MYB transcription factors

AT5G36720 is a MYB-regulated CRP, which is expressed only in pollen tubes grown through pistil tissues (Qin et al. 2009; Leydon et al. 2013). This MYB-regulated CRP is a member of a large CRP family (28 genes - CRP2460) described to contain a motif of 8 invariant cysteines predicted to form 4 disulfide bonds (Figure 1A, Supplemental Figure 1) (Silverstein et al. 2007). In order to determine the breadth of CRPs controlled by *MYB97*, *MYB101* and *MYB120*, we plotted the expression of each family member (RNA-seq Data, Thesis Chapter 5) alongside the CRP2460 phylogenetic tree (Figure 1B). Differential expression analysis identifies 22/28 CRP2460 transcript levels as lower in pollinations with *myb* triple-mutant pollen tubes (Figure 1B, asterisk) compared to the wild type. We compared the expression of CRP2460 genes in two wild-type *Arabidopsis* ecotypes Col and Cvi. Surprisingly, CRP2460 Thionin proteins were expressed ecotype-specifically: high expression was observed in Col pollen tubes, while Cvi pollen tubes expressed fewer members and at lower levels (Figure 1B). These Thionin proteins therefore describe a MYB-dependent reproductive expression pattern that differentiates *Arabidopsis* ecotypes from each other (Thesis Chapter 5).

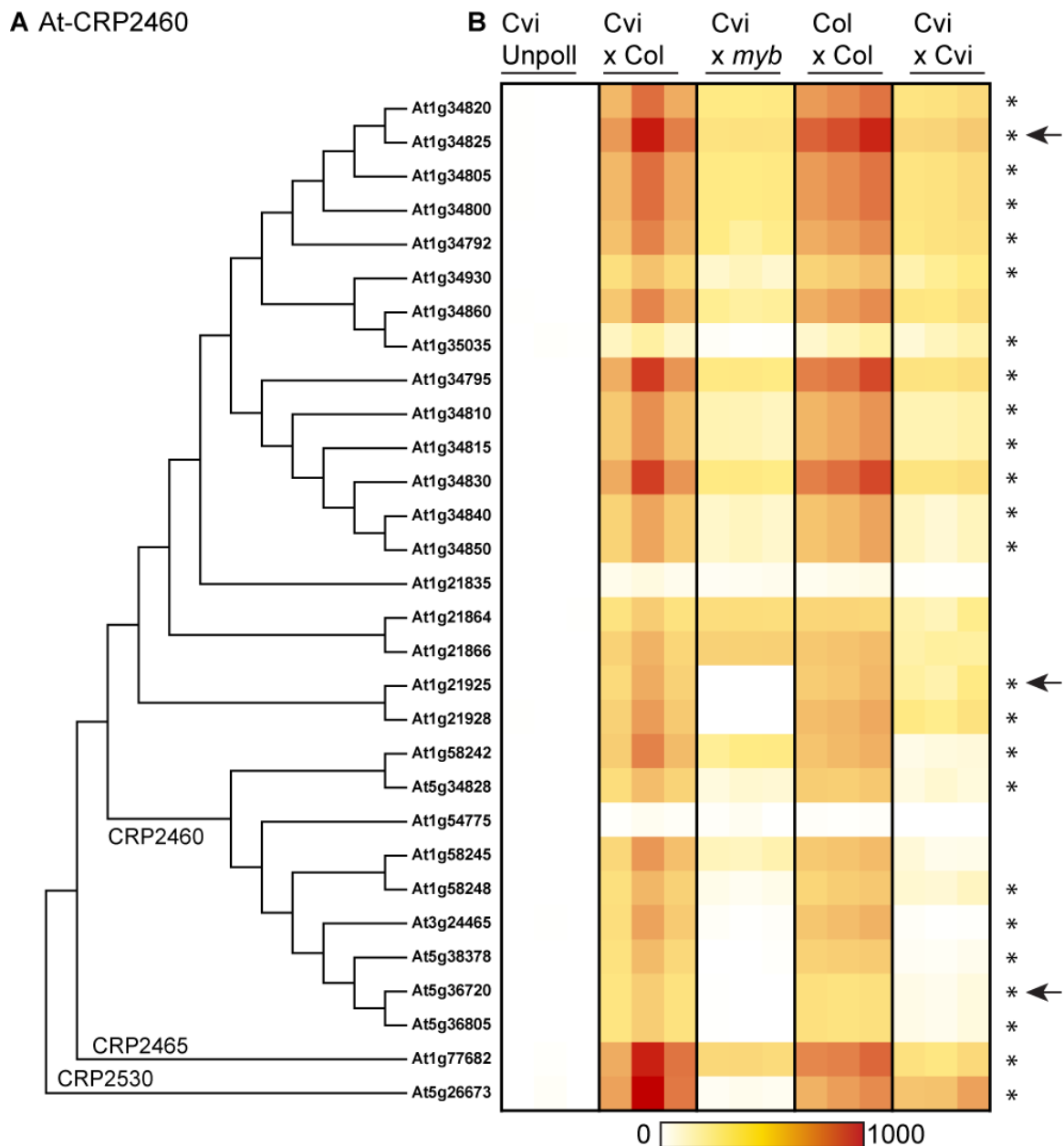


Figure 1. MYB transcription factors control expression of Thionin CRPs (CRP2460). **A.** A phylogenetic tree of the CRP2460 protein coding sequences (Neighbor-joining, jukes-cantor). **B.** Expression heatmap of the CRP2460 transcripts in pistils. Cvi Unpoll – Unpollinated Cvi pistils (mock pollinated 8 hours), Cvi x Col – Cvi pistils pollinated with Col pollen for 8 hours, Cvi x *myb* – Cvi pistils pollinated with *myb* triple-mutant pollen for 8 hours, Col x Col – Col pistils pollinated with Col pollen for 8 hours, Cvi x Cvi – Cvi pistils pollinated with Cvi pollen for 8 hours. Sequencing data is shown in three columns per condition, one column per biological replicate, color intensity is based on read number - scale (See Thesis Chapter 5). At1g77682 and At5g26673 are MYB-regulated CRPs from neighboring CRP Thionin-related families CRP2465 and CRP2530 respectively. Asterisks indicate significant gene expression differences between

Cvi x Col and Cvi x *myb* triple mutant pollinations (adjusted p-value <0.005). Arrows indicate Thionin genes investigated by expression analysis, see below.

CRP2460 genes are *Brassicaceae*-specific and are enriched by gene duplication events in *Arabidopsis thaliana*

While proteins with homology to the α/β Thionin genes have been identified across many flowering plant lineages, Thionin-related genes similar to CRP2460 were identified only in *Brassicaceae* (Silverstein et al. 2007). To survey species-specific expansions in the *Brassicaceae* lineage, we identified all homologous genes to *A. thaliana* CRP2460 genes in the closely related *A. lyrata*, and more distantly related *Capsella rubella*, *Brassica rubella*, and *Thelungiella halophila* families (Supplemental File 1). A total of 67 sequences were collected and a phylogenetic tree was constructed using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). This analysis reveals expansions of this gene family in specific lineages. The *A. thaliana* CRP2460 gene family underwent multiple gene duplication events at one locus on chromosome 1 (Figure 2, bottom right). Furthermore, these duplicated CRP genes do not have one clear ortholog in *Arabidopsis lyrata*, which exhibits its own history of gene expansions of this family. Similar trends are seen in *C. rubella* and *T. halophila*, which have individually expanded clades of CRP2460 (Figure 2, purple triangle, aqua diamond). *Brassica rapa* retains only two members of the CRP2460 clade in its genome, with little evidence of gene expansion in its lineage (Figure 2, green triangle).

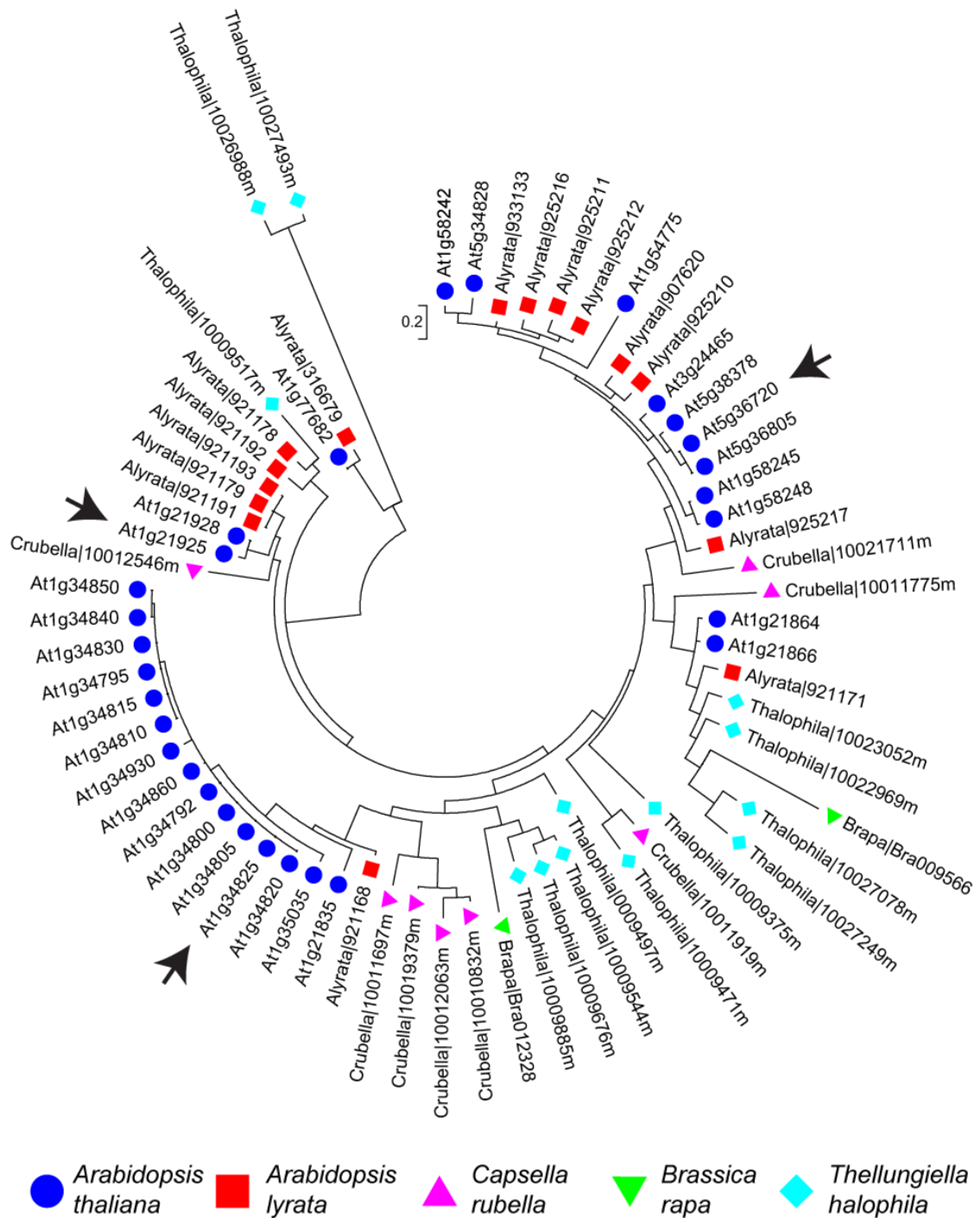


Figure 2: A phylogenetic tree of paralogous Thionin (CRP2460) proteins of *A. thaliana*, *A. lyrata*, *C. rubella*, *B. rapa*, and *T. halophila*. The tree includes only paralogous CRP2460 members from *A. thaliana* (blue circles) and their orthologs from *A. lyrata* (red square), *C. rubella* (pink triangle), *B. rapa* (green triangle), and *T. halophila* (aqua diamond). The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (-3860.1344) is

shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 67 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 72 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013).

Thionin proteins enter the secretory system

CRP2460s contain a signal sequence and are predicted to transit the secretory system and enter the extracellular environment (Silverstein et al. 2007). We tested the subcellular localization of the Thionin protein AT5G36720 using transient expression in *Nicotiana benthamiana*. Soluble GFP can be observed throughout the cytoplasm and nucleus of *N. benthamiana* epidermal cells (Figure 3A-D). A translational fusion of the Thionin protein and GFP was expressed in *N. benthamiana*, and was observed in a punctate pattern, suggestive of endomembrane localization (Figure 3E-H). Co-localization with Red fluorescent protein subcellular markers (Nelson et al. 2007), demonstrates that Thionin proteins enter the secretory system, and are observed co-localized with the golgi apparatus (Figure 3K, Yellow overlapping signal).

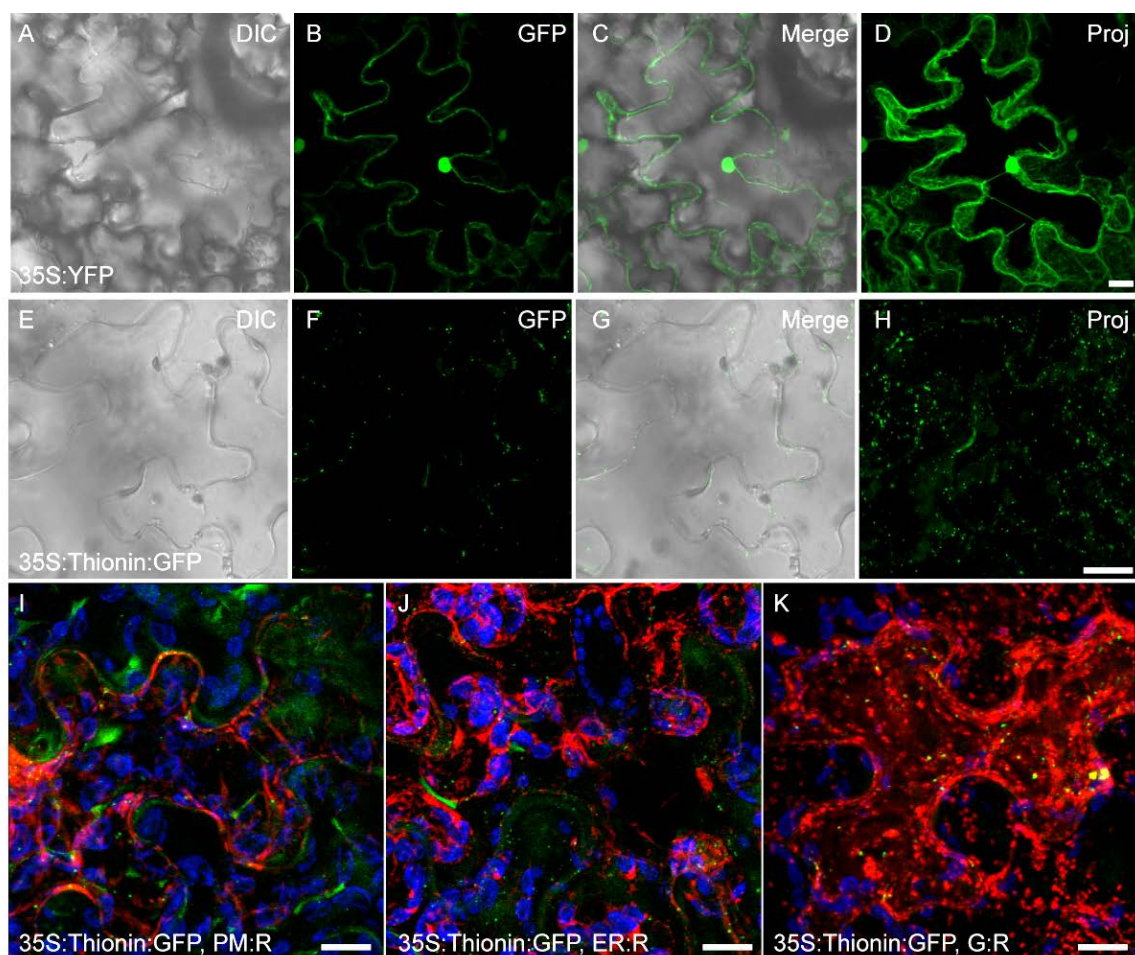


Figure 3. Transient expression of Thionin proteins provides subcellular localization data. **A-D.** Soluble YFP overexpression in *Nicotiana benthamiana*. **E-H.** Thionin:GFP expression in *Nicotiana benthamiana*. **I.** Co-expression of Thionin:GFP with plasma membrane marker PM:R (35S:AtPIP2A:mCherry). **J.** Co-expression of Thionin:GFP with ER:R (the ER marker was created by combining the signal peptide of AtWAK2 (*Arabidopsis thaliana* wall-associated kinase 2) at the N-terminus of the FP and the ER retention signal His-Asp-Glu-Leu at its C-terminus). **K.** Co-expression of Thionin:GFP with the golgi marker G:R (utilizes the cytoplasmic tail and transmembrane domain (first 49 aa) of GmMan1, soybean α -1,2-mannosidase I). Yellow indicates colocalization of Thionin:GFP and G-RK. Documentations on markers can be found in (Nelson et al. 2007). All infections were co-infected with P19 (Lombardi et al. 2009). Scale bars = 20um.

To test Thionin subcellular localization in the pollen tube, the CRP2460's native expression domain, we created a Thionin:GFP transgene under the control of the native Thionin promoter (Thionin:GFP) (Leydon et al. 2013). Thionin:GFP was

undetectable in pollen grains and in vitro grown pollen tubes, but was induced in semi in-vivo grown pollen tubes (Figure 4A), where it accumulates in puncta in the pollen tube (Figure 4E), similar in size to the golgi apparatus compartments as visualized by the RAB2:GFP reporter construct (Figure 4C). Thionin:GFP puncta are much larger than the small secretory vesicles that ferry pollen tube membrane proteins to the pollen tube tip as visualized by ACA9:GFP or SUC9:GFP (Figure 4B,D). Thionin:GFP is visible in the ovule following pollen tube burst (Figure 4G). Thionin:GFP expression and localization to the pollen tube throughout pollen tube reception and burst indicates a possible role in pollen tube:synergid interactions.

Thionins are secreted from the pollen tube to the synergid

Thionin proteins are expressed at the right time and place to be involved in signaling the synergid during pollen tube reception. Pollen tube reception takes place rapidly after pollen tube arrival and is therefore difficult to catch in action. We pollinated *ms1* pistils (*male sterile 1*) with pollen carrying a pollen tube specific red fluorescent protein (*LAT52:DsRed*) and *Thionin:GFP* for 8 hours, to maximize the chances of catching pollen tube reception in the act. Live imaging using confocal microscopy allowed us to image a small number of pollen tube reception events where the pollen tube had not yet burst, yet was clearly inside the female gametophyte, as determined by position and DsRed signal. Excitingly, Thionin:GFP signal was visible in a region outside of the pollen tube cytoplasm, extending into the ovule region at the approximate position of the synergid cells

(Figure 4G,H). We suggest that when the pollen tube arrives at the micropyle and temporarily arrests its growth (Iwano et al. 2012; Hamamura et al. 2014; Ngo et al. 2014), Thionin proteins are secreted into the apoplastic region of the filiform apparatus (Figure 4I,J).

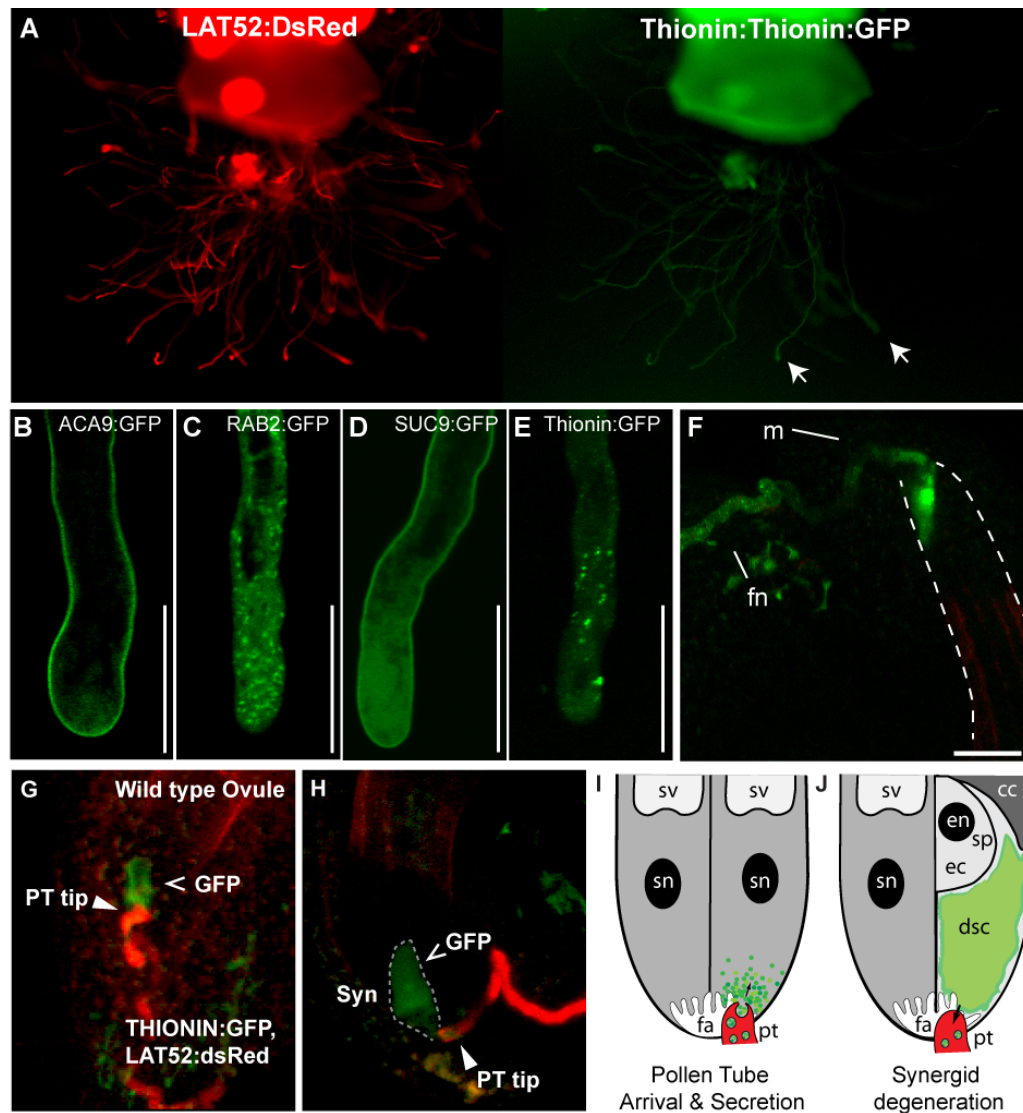


Figure 4. Thionins are secreted from the pollen tube to the synergid during pollen tube reception. **A.** Semi in-vivo grown pollen tubes from T1 *LAT52:DsRed*, *Thionin:GFP* plants. Arrows indicate pollen tube tips, enriched for Thionin:GFP expression. Epifluorescence. **B-F.** Semi in vivo grown pollen tube tips, confocal microscopy, scale bar – 10um **B.** Control membrane reporter – *ACA9:GFP*. **C.** Control golgi marker – *RAB2:GFP*. **D.** MYB-regulated membrane marker *SUC9:GFP*. **E.** *Thionin:GFP*. **F.** *Thionin:GFP* pollen tube burst in a wild type *ms1* ovule. Scale bar – 20um. **G.** Pollen tube reception event. PT tip - pollen

tube tip (LAT52:DsRed), GFP – Thionin:GFP extends into the micropylar end of the embryo sac. **H.** Pollen tube reception, same as in G, Thionin:GFP extends into the synergid cell region. **I.** Diagram of Thionin secretion during pollen tube reception. Fa – filiform apparatus, pt – pollen tube, sn – synergid nucleus. **J.** Thionins may trigger synergid degeneration preceding pollen tube burst. dsc – degenerating synergid cell, en - egg nuclei, ec – egg cell, sp – site of projection of sperm.

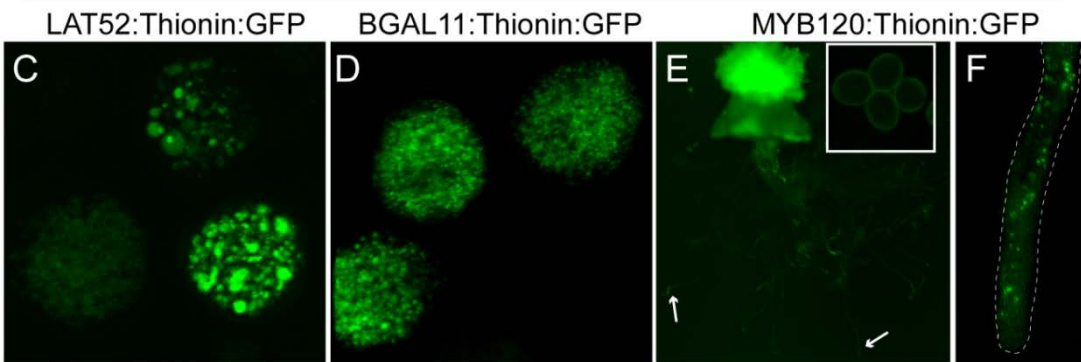
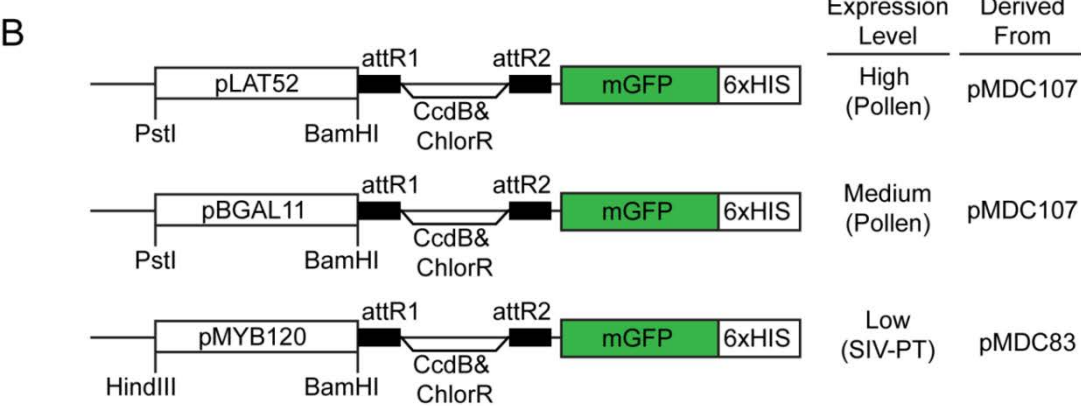
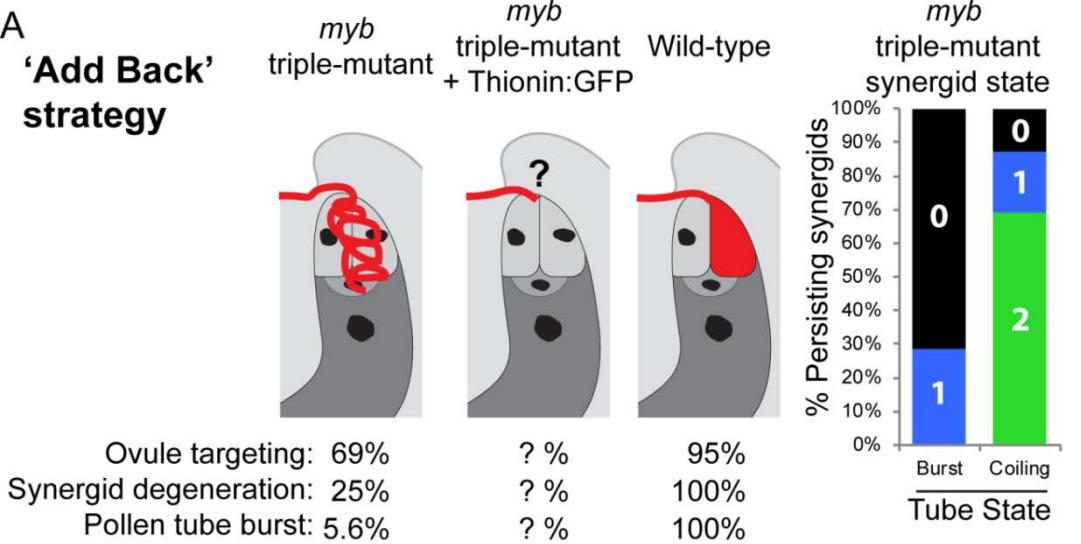
A genetic strategy to identify functions of MYB-regulated genes

We have shown that Thionins are expressed by the pollen tube and secreted to the synergid, yet it is unclear whether they directly influence pollen tube reception. The CRP2460 family is especially intractable for reverse genetic approaches as it is comprised of many tightly linked and duplicated loci. 15/28 members are encoded within a short, tandemly repeated region on chromosome 1 (Figure 1A, Supplementary Figure 3). CRP2460s are encoded by small single exon genes, reducing the frequency of recovering T-DNA insertions, and all tested insertions had no reception phenotype (Data not shown). Therefore we designed an alternative approach to testing Thionin protein function during pollen tube reception. We utilized *myb* triple-mutant as a pollen donor, which is essentially a loss of function for genes that require MYBs for their expression, with several distinct phenotypes. We designed a methodology to express MYB-regulated genes, such as the Thionins, in the *myb* triple-mutant background. This method allows us to test whether a gene is sufficient in rescuing *myb* triple-mutant phenotypes. The MYB loss of function phenotype results in an increase in pollen tube coiling, and a failure to release sperm. Synergids receiving *myb* triple-mutant pollen tubes fail to undergo synergid degeneration. Additionally pollen tube guidance to ovules is reduced in *myb* triple-mutant pollinations. All of

these phenotypes contribute to reduce the total seeds produced in the *myb* triple-mutant pollinations. We tested whether expression of any one MYB-regulated Thionin in *myb* triple-mutant pollen is capable of modifying these distinct phenotypes (Figure 5A).

We designed three destination vectors to rapidly introduce MYB-target genes into the *myb* triple-mutant under transcriptional control of a non MYB-regulated promoter. *LAT52* drives high levels of expression in pollen grains and tubes (Twell et al. 1989), *BGAL11* drives a moderate level of expression in pollen grains and tubes (Unpublished Data), while *MYB120* drives low levels of expression, only in pollen tubes growing in the pistil (Figure 5, Leydon et al. 2013). Thionin:GFP expression level and subcellular localization was characterized for multiple independent transformants (Figure 5C-F). We consistently observed over-expression phenotypes in *LAT52:Thionin:GFP* transformants (Figure 5C), with large GFP positive bodies of uneven size. In vitro pollen tube growth also revealed that *LAT52:Thionin:GFP* failed to enter the pollen tube, remaining in the evacuated pollen grain. *BGAL11:Thionin:GFP* was detectable in the mature pollen grain, and was observed in small evenly-sized puncta that were visible throughout pollen tube growth in vitro (Figure 5D). *MYB120:Thionin:GFP* was detectable only after semi in vivo pollen tube growth localized to small puncta (Figure 5E-F), similar to expression under the native promoter (Figure 4E). We selected the *BGAL11* promoter destination vector for our expression strategy due to its consistent expression levels in mature pollen

grains, which allowed efficient identification of GFP positive primary transformants by fluorescence microscopy. Furthermore, the *BGAL11* promoter is less likely to cause over-expression phenotypes that could pose technical challenges to our experimental design.



(Thionin cDNA - AT5G36720)

Figure 5. Genetic strategy to identify the function of Thionin proteins. **A.** *myb* triple mutant pollen tubes have reduced ovule targeting, and disrupted synergid degeneration, and low rates of pollen tube burst in targeted ovules compared to wild type. Expression of Thionins may shift the distribution of any of these phenotypes towards wild type. To the right is an example distribution of synergid status in burst and coiling tubes in pistils pollinated with *myb* triple-mutant pollen. **B.** Diagram of destination vector construction. Expression level is based on microarray analysis (Qin et al. 2009) and reflects the known, relative expression levels of the selected promoters. Derived from – indicates the destination vectors from (Curtis et al. 2003) modified for our add-back libraries. **C,D,F** are confocal micrographs, **E** is a epifluorescent micrograph, with an inset confocal micrograph. **C.** *LAT52:Thionin:GFP* T₁ pollen, with large overexpression puncta. **D.** *BGAL11:Thionin:GFP* T₁ pollen. **E.** *MYB120:Thionin:GFP* T₁ pollen tubes grown in a semi in vivo growth assay. Arrows indicate pollen tube tips. Inset shows absence of expression in mature pollen. **F.** *MYB120:Thionin:GFP* subcellular localization in semi in vivo grown pollen tubes.

Thionins restore the ability of *myb* triple-mutant pollen tubes to induce synergid degeneration

We selected four MYB-regulated Thionin proteins to test in our expression strategy, three that span the CRP2460 clade (*AT1G34825*, *AT1G21928*, and *AT5G36720*) and one from a neighboring Thionin clade (*AT5G26673*, CRP2530). We cloned the coding sequences from these genes into our *BGAL11* destination vector and introduced them into the *myb* triple-mutant, *LAT52:DsRed* genetic background (See Methods). We isolated 36 primary transformants (T₁) for each construct, screened for expression of Thionin:GFP in mature pollen grains (Figure 7A), and confirmed the *myb* triple-mutant background by PCR. We chose three representative T₁ lines with consistent Thionin:GFP expression to perform live imaging of pollen tube reception. To analyze synergid cells during reception we used pistils from plants expressing a ubiquitous nuclear GFP reporter (*ACT11:MSI1:GFP*) (Ingouff et al. 2007). *ACT11:MSI1:GFP* nuclear signal in the

synergid disappears after synergid degeneration allowing efficient quantification of synergid status (Thesis Chapter 3&4, Leydon et al. 2013). We emasculated *ACT11:MSI1:GFP* pistils 12 hours before pollination with *BGAL11:Thionin:GFP* T₁ hemizygous pollen. After 12 hours of pollination, we dissected and imaged crosses by confocal microscopy to quantify pollen tube targeting and pollen tube reception as previously described (Thesis Chapters 3&4, Leydon et al. 2013). We did not observe differences in the overall distribution of pollen tube burst, pollen tube coiling and ovules targeted by a pollen tube (Figure 6, left hand graph). However, when we analyzed synergid degeneration in ovules with a coiling pollen tube, we observed increases in synergid degeneration compared to the *myb* triple-mutant parental line (Figure 6, right hand graph). Thionins in the CRP2460 clade, *AT1G34825*, *AT1G21928*, and *AT5G36720*, were able to promote synergid degeneration without affecting pollen tube burst. There were equivalent changes in synergid degeneration rates between *pBGAL11* and *pMYB120* driven constructs, indicating that the *BGAL11* promoter was sufficient to drive gene expression tube during pollen tube growth in vivo. Pollen from *LAT52:Thionin:GFP* transgenic plants were statistically indistinguishable from controls, consistent with our observations that Thionin:GFP in these lines failed to enter the pollen tube during growth (Figure 6). Interestingly, *AT5G26673:GFP* showed higher synergid degeneration than background levels (p-value <0.05), yet lower than Thionins in the CRP2460 clade, indicating that *AT5G26673* may have low levels of biological activity.

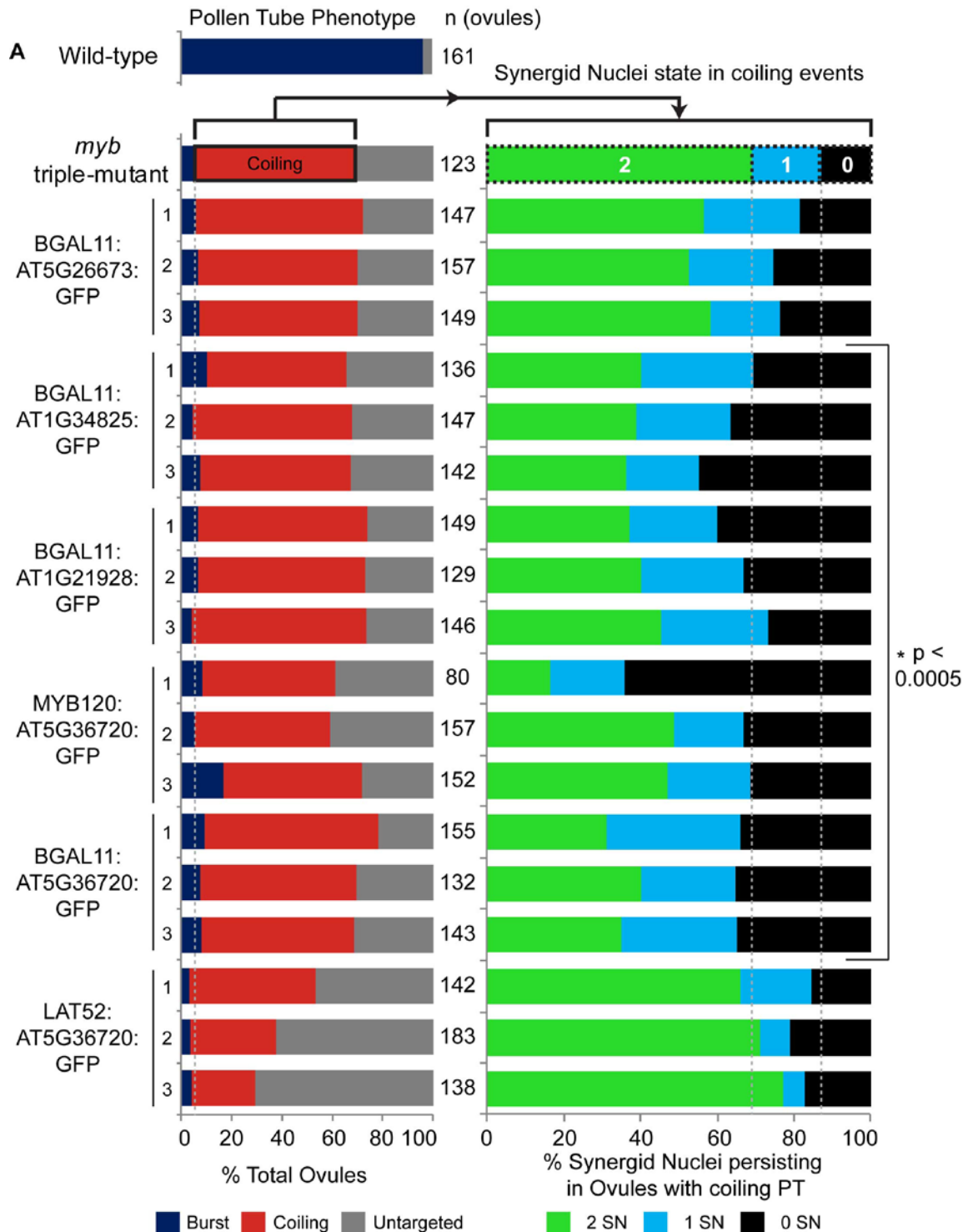


Figure 6. Thionin expression constructs in *myb* triple-mutant pollen increase synergid degeneration without affecting pollen tube burst. A. Pollen tube phenotypes 12 hours after pollination. *ACT11:MSI1:GFP* pistils were emasculated and crossed with *myb* triple-mutant pollen for 12 hours before dissection and quantification by confocal microscopy. Data on the right is paired with the red “Coiling” fraction shown on the left. Synergid numbers are indicated

by position and by color, from left to right, Green – 2 synergid nuclei, Blue – 1 synergid nucleus, Black – 0 synergid nuclei. Data are shown for three T_1 lines that were tested for expression of Thionin:GFP. n (ovules) – refers to total number of ovules, 3 pistils per experiment. 1-3 – indicate three independent transformant lines, with Thionin:GFP expression. P-value <0.0005 – chi-squared test (Includes three conditions: Burst, Coiling, Untargeted).

Co-localization of Thionin:GFP with pollen tube cytoplasm via LAT52:DsRed, allows simultaneous observation of pollen tube interaction with the synergid and localization of Thionin:GFP. Visualization of *BGAL11:Thionin:GFP* pollen tubes during reception revealed Thionin:GFP localization in the pollen tube tip during reception (Figure 7C-E). From examining multiple reception events, we observe an enrichment of Thionin:GFP in and around the pollen tube at the micropylar end of the ovule (Figure 7I-K, arrow). In our expression analyses, the fluorescence intensity of Thionin:GFP is lower in the tips of overgrown pollen tubes than it is in the tube that is closest to the entrance of the ovule. This localization suggests that Thionin proteins are enriched at the pollen tube:synergid interface while the pollen tube is growing slowly or arrested (Iwano et al. 2012; Ngo et al. 2014). When wild-type pollen tubes reinitiate rapid growth just prior to burst (Iwano et al. 2012), *myb* triple-mutants fail to burst, continuing to coil inside of the ovule. Alternatively, secreted Thionin:GFP may lose fluorescence intensity compared to intracellular Thionin:GFP in this assay.

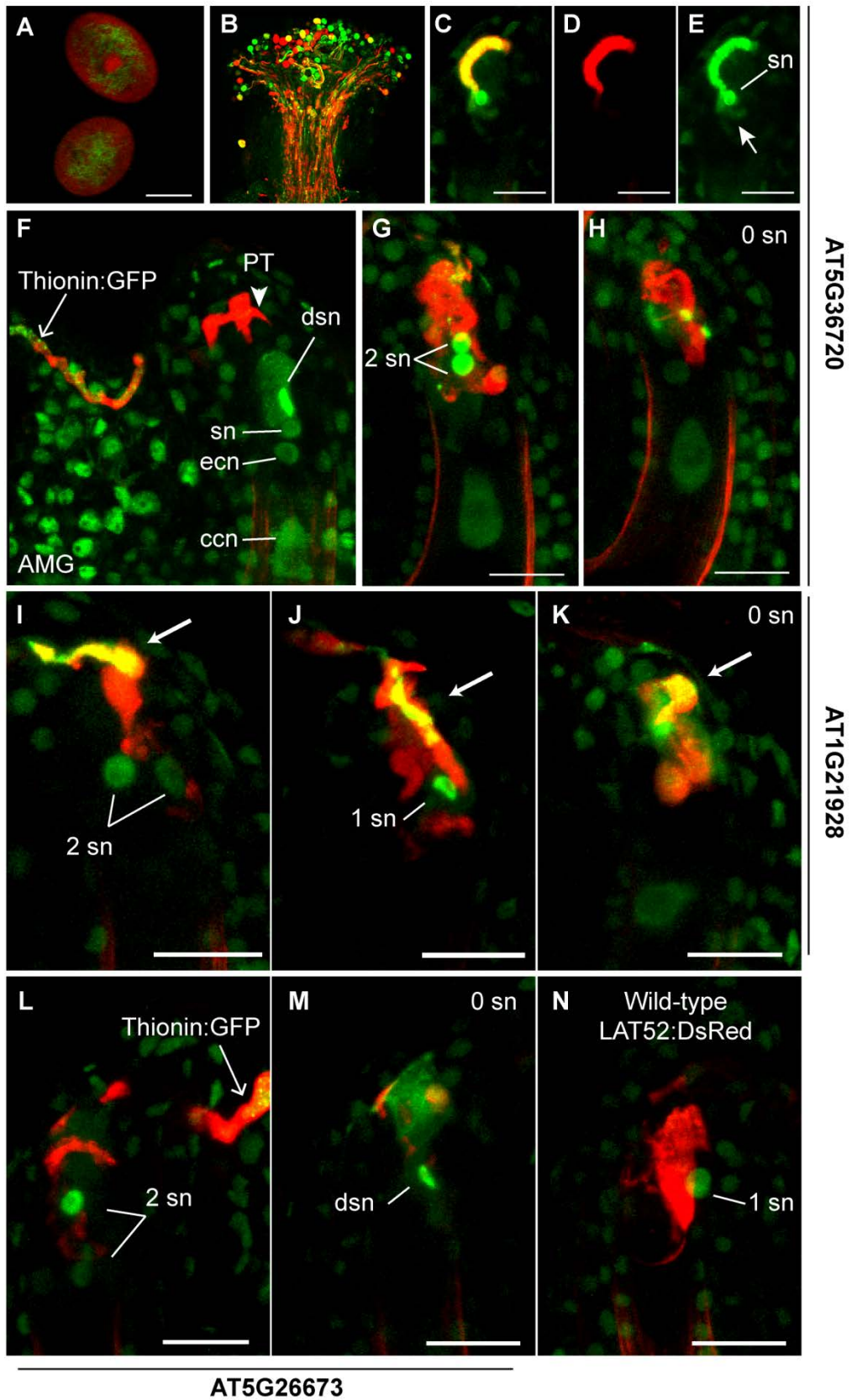


Figure 7. Thionin:GFP proteins increase synergid degeneration by *myb* triple-mutant pollen tubes. **A.** *BGAL11:Thionin:GFP* (At5g36720) expression in *myb* triple-mutant, *LAT52:DsRed* mature pollen. Confocal micrograph, scale bar – 10um. **B.** *BGAL11:Thionin:GFP* positive pollen tubes growing in the stigma and style. **C-E.** *BGAL11:Thionin:GFP* localization in an *ACT11:MSI1:GFP* ovule. C – merge, D – Red channel, *LAT52:DsRed*, E – Green channel, *BGAL11:Thionin:GFP*. **F-N.** Confocal micrographs of pollen tube reception in *ACT11:MSI1:GFP* pistils, 12 hours after pollination. **F.** *BGAL11:AT5G36720:GFP* pollen tube reception with one degenerated synergid, puncta are visible in the pollen tube, PT arrowhead indicates the entry of the pollen tube in the embryo sac. Dsn – degenerated synergid nucleus, sn – synergid nucleus, ecn – egg cell nucleus, ccn – central cell nucleus. **G.** Two persisting synergid nuclei. **H.** Both synergids degenerated. **I-K.** *BGAL11:AT1G21928:GFP* pollen reception, with (I) 2 intact synergid nuclei, (J) 1 intact synergid nuclei and (K), both synergid nuclei degenerated. **L-M.** *BGAL11:AT5G26673:GFP* pollen tube reception (L) 2 intact synergid nuclei (M), both synergid nuclei degenerated (N), wild-type *LAT52:DsRed* pollen tube reception in a wild-type ovule – pollen tube burst, one persisting synergid nucleus.

We used quantitative confocal microscopy to analyze multiple aspects of pollen tube reception 12 hours after pollination, a time point when pollen tubes have reached ovules (Figure 6). However, we considered the possibility that *myb* triple-mutants expressing Thionin proteins may exhibit altered timing, taking longer to complete pollen tube reception. In this case, sperm release would be delayed, but successful fertilization and seed production would be expected. To test whether *myb* triple-mutant pollen tubes expressing *BGAL11:Thionin:GFP* result in pollen tube burst at time-points after 12 hours, we performed crosses to examine seed set. Seed production was quantified for self-pollinated siliques as well as female, and male-specific crosses (Figure 8A). All Thionin transformants exhibited seed production rates equivalent to the *myb* triple-mutant background, in self-pollinated siliques as well as male- and female-specific crosses. These results indicate that expression of *Thionin:GFP* is not sufficient to restore sperm

release and fertility to *myb* triple-mutant pollen tubes even though synergid degeneration is increased.

To further test the impact of *Thionin:GFP* expression in *myb* triple-mutant fertility, we determined whether expression of *Thionin:GFP* affects genetic transmission in primary *myb* triple-mutant transformants hemizygous for one or more insertions of a *Thionin:GFP* transgene. We calculated the ratio of male to female transmission to normalize differences in transgene copy number between individual primary transformants (Figure 8C). Values less than one indicate a pollen specific defect. Values above one indicate increases in pollen tube success, which would indicate that *Thionin:GFP* conferred a competitive advantage to pollen. Expression of *Thionin:GFP* had no effect on pollen tube transmission in the primary transformants we analyzed (four *Thionin:GFP* constructs, 6 transformants each). The ratio of male to female transmission was near one for all transformants, indicating that *Thionin:GFP* does not confer an advantage to *myb* triple-mutant pollen (Figure 8C). These results indicate that out of all the MYB-regulated genes, Thionins play a distinct role in synergid degeneration without affecting sperm release or fertility.

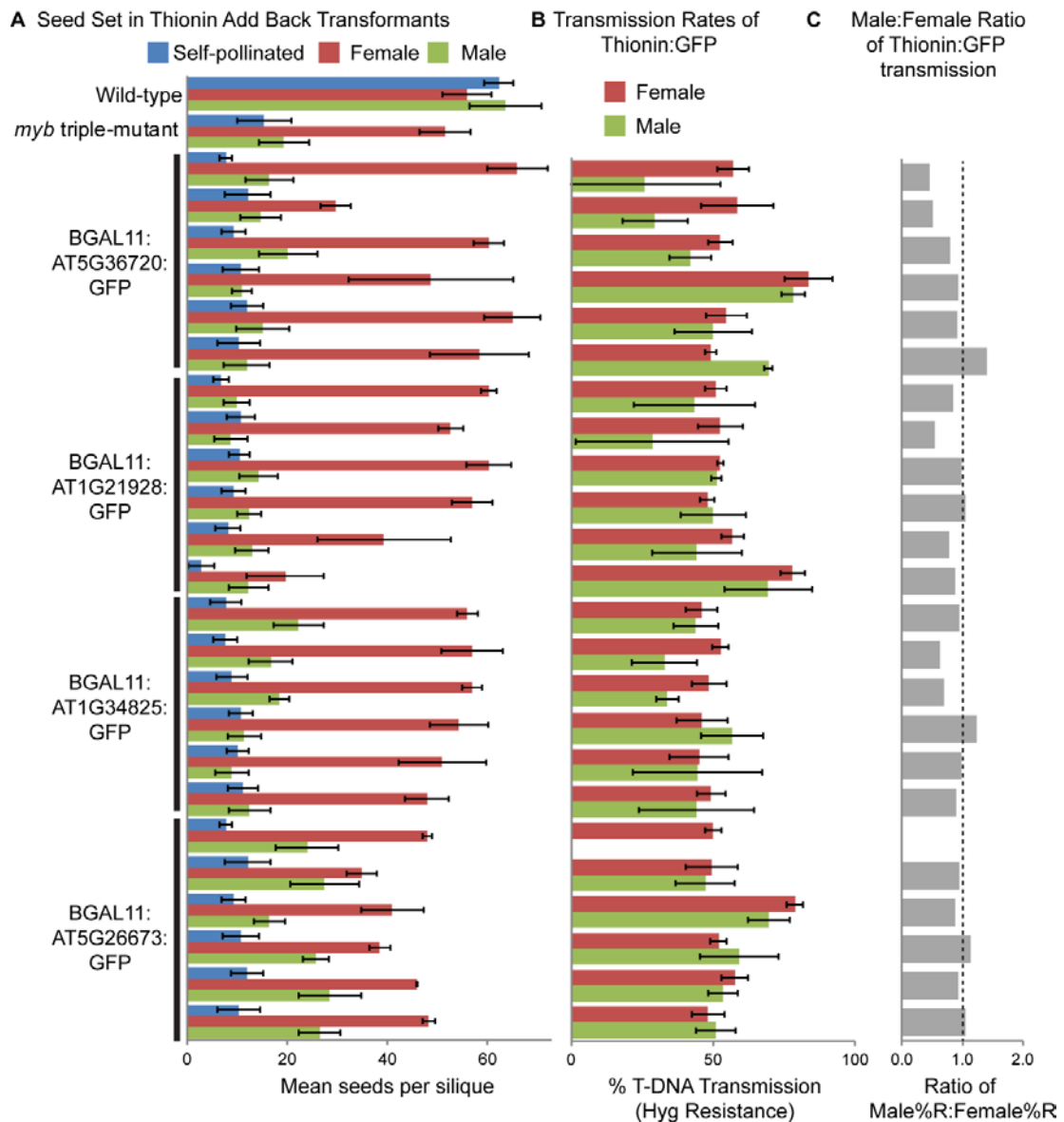


Figure 8. Thionin proteins are not sufficient to rescue *myb* triple-mutant seed set defects. **A.** Mean seed set in *Thionin:GFP* crosses, 6 T1s per construct. All bars are mean values $\pm$ std dev. Blue bars are mean seed set in self-pollinated siliques. Red bars are emasculated T1 *Thionin:GFP* pistils pollinated with Col-0 pollen. Green bars are *ms1* pistils pollinated with T1 *Thionin:GFP* add back pollen. **B.** Transmission ratios, paired to data in A, from reciprocal crosses with T1 hemizygous plants, Red bars – female crosses, green bars – male crosses. Each value shown is the average of at least 6 crosses $\pm$ std dev. **C.** Gray bars are the ratios of the averages in B, (Male%R/Female%R), with a dotted line at 1.

Heterologous expression of Thionin proteins

Our expression strategy uses a genetic approach, treating the pollen tube as a biological micro-injector of Thionin proteins. This approach is powerful, as it produces concentrations of Thionin proteins at or above endogenous levels. Furthermore, it takes advantage of the pollen tube secretory system to fold and secrete proteins of interest in its native environment. We wanted to complement our observations in this system with an alternative approach that would be capable of independently testing the biological activity of the Thionin proteins. However, Thionins possess physical characteristics that limit the ease by which they can be purified from heterologous expression systems.

First, Thionins contain between 6 and 8 cysteine residues, which stabilize the secreted protein through disulfide 3-4 bridges (Bohlmann 1994). Unlike the founding Thionins, the CRP2460 protein family does not appear contain C-terminal acidic domain (Figure 9B). Thionins are usually basic proteins (Stec 2006) and the average predicted isoelectric point of CRP2460 Thionins is 8.4 (Supplemental Table 1). Any expression system for Thionins requires production in cell type capable of proper disulfide bond formation; otherwise the purified protein would require refolding in vitro. Second, the majority of described Thionin proteins are toxins, which may limit heterologous expression. The lack of sequence conservation between proteins in CRP2460 and the founding Thionin proteins may suggest divergence in biochemical activity, however it is also possible that they retain their ability to bind and permeabilize membranes. It was

critical to carefully test multiple heterologous expression systems and conditions to identify the characteristics of CRP2460 Thionin related proteins. We tested expression constructs in *E. coli* and *N. benthamiana*. Expression of Thionin from *E. coli* was sub-optimal due to low yields of the mature protein. Additionally expression from *N. Benthamiana* was sub-optimal due to inclusion of the relatively large GFP tag (See Supplemental Expression section).

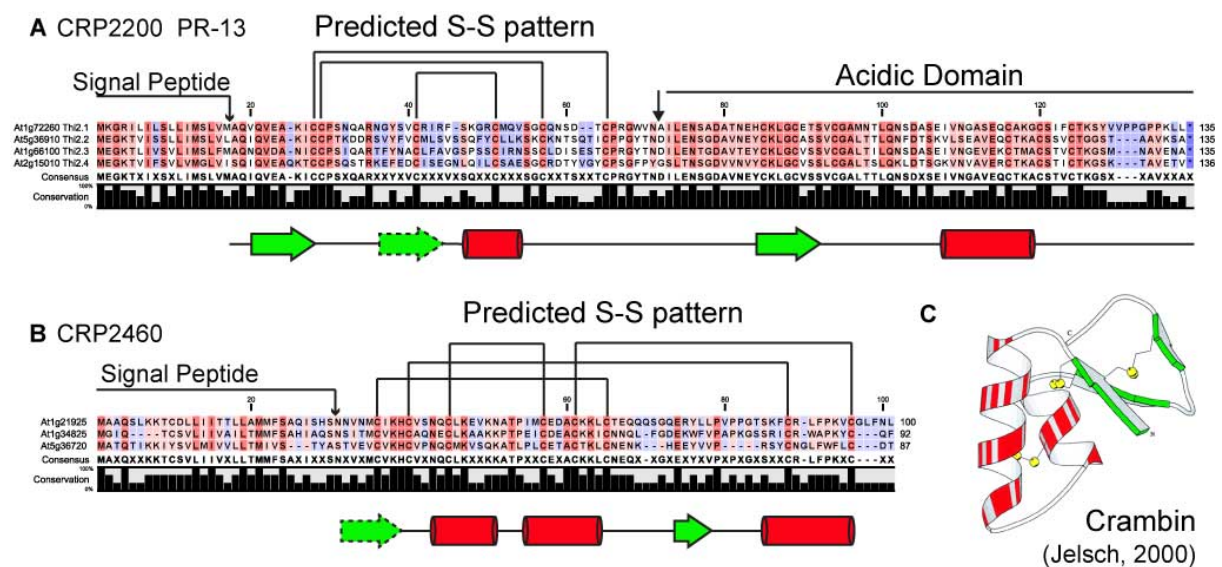


Figure 9. Thionin protein primary sequence and secondary structure prediction. **A.** The *A. thaliana* PR-13, or Thi2.1-2.4 subgroup. This group is most highly related to Crambin, and retains a clear Acidic domain. The disulfide bond pattern of the PR-13 Thionins has not been directly tested. The arrow before the Acidic Domain indicates the cleavage point to release the mature Thionin protein. **B.** Three CRP2460 members *AT1G34825*, *AT1G21928*, and *AT5G36720*. The disulfide bond pattern was predicted (Ceroni et al. 2006). The cleavage point of the signal peptide is shown with an arrow. Predicted secondary structure is described by a green arrow (Beta sheet) and a red tube (Alpha helix). **C.** The crystal structure of Crambin is shown for comparison from (Jelsch et al. 2000); lines with yellow dots represent disulfide bonds.

Heterologous expression of Thionin in *P. pastoris*

We developed an expression protocol using the methylotrophic yeast *Pichia pastoris*. We cloned the coding sequence of AT5G36720 into a vector that produces a secreted Thionin-Myc-His protein (Figure 10A,B). We selected the isolate that exhibited the highest levels of secretion into the media (905-4), and performed purifications of the secreted Thionin-Myc-His protein from the media after 24-hour induction (See Methods). We obtained eluates free of contaminating endogenous *P. pastoris* secreted proteins (Figure 10C). We obtained approximately 100µg of purified Thionin protein from 100mls of culture, which was desalted and re-suspended for further analysis. SDS-PAGE analysis of the Thionin-Myc-His protein from *Pichia* revealed a prominent band at approximately 9.8kDa, consistent with the predicted molecular weight of the secreted protein without the alpha factor signal peptide (Figure 10C). Secretion of Thionin through the *Pichia* secretory system allows the formation of disulfide bonds and permits glycosylation events that are unlikely to occur properly during heterologous expression in *E. coli* (Moir et al. 1990; Daly et al. 2005). However, we wanted to test whether our Thionin-Myc-His protein was forming disulfide bond formation homogenously. Therefore we analyzed purified peptides by Electrospray Ionization-Mass spectrometry to determine their molecular weight. We observed three distinct protein peaks, corresponding to the three possible alpha factor signal peptide cleavage sites that are cleaved by the Kex2 and Ste13 proteases (Figure 10A). We calculated the predicted molecular weight of each peptide with each possible disulfide bond (Figure 10E), and determined that

each purified Thionin has a molecular weight consistent with an intact protein with 4 disulfide bonds (Figure 10D-E).

A

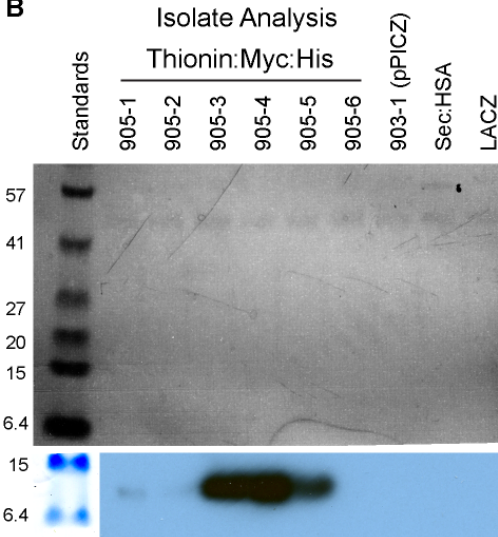
Pichia pastoris Thionin Expression - PGL905, αFss:Thionin:Myc:His

α-factor signal sequence † - Kex2 signal cleavage * - Ste13 signal cleavage

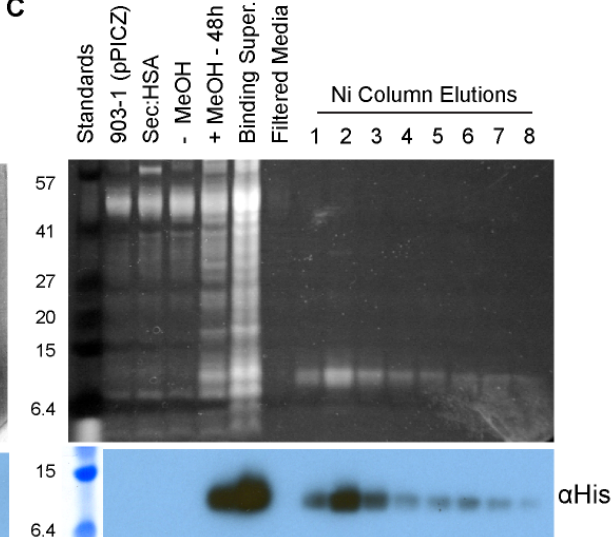
M R F P S I F T A V L F A A S S A L A A P V N T T T E D E T A Q I P A E
A V I G Y S D L E G D F D V A V L P F S N S T N N G L L F I N T T I A S
I A A K E E G V S L E K R † E A * E A * E F S T V E V C V K H C V P N Q C
M K V S Q K A T L P L C E T A C T K L C N E N K H E E Y V V P R S Y
C N G L F W F L C D T G L E Q K L I S E E D L N S A V D H H H H H H

MYC-Tag 6xHIS-Tag

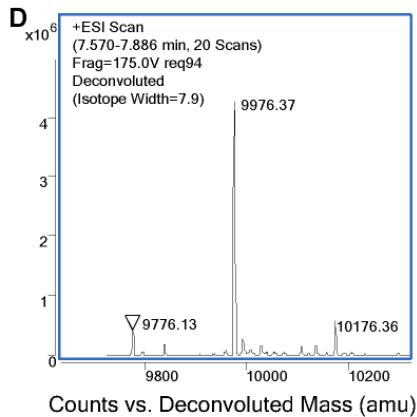
B



C



D



E

Protein size predictions MW (MS)					
Disulfide bond number:	0	1	2	3	4
† EAEAEFST...	10185	10183	10181	10179	10177
* EAEFST...	9984	9982	9980	9978	9976
* EFST...	9784	9782	9780	9778	9776

† - Kex2 signal cleavage
* - Ste13 signal cleavage

Figure 10. Expression of Thionin Protein in the methylotrophic yeast *Pichia pastoris*. **A.** Sequence map of the SS-Thionin-Myc-His construct. The alpha factor signal sequence is shown in light blue. The signal sequence cleavage sites are shown with enzyme specific symbols for Kex2, and Ste13. The Thionin

coding sequence is colored black with cysteines colored purple. The Myc epitope tag is shown in Green, and the 6xHIS tag is shown in Red. **B.** Screening of individual isolates for expression (905-1,6) and controls. Top panel – coomassie staining of SDS-PAGE. Lower panel – immunoblot probed with anti-His antibody. 903-1 – pPICZalpha empty construct, Sec:HSA – secreted human serine albumin control (dot to right~58kDa), and LACZ – intracellular LacZ control. **C.** Upper panel – SYPRO Ruby protein staining, Lower Panel – anti-His antibody. All lanes under the Thionin:Myc:His label are from isolate 905-4. -MeOH (pre-induction media). +MeOH (Media 24 hours after induction). Binding super – media concentrated by Amicon column. Filtered media – Amicon column flow through (3Kd cutoff). 1-6 - Nickel column elutions from concentrated media. **D.** ESI-MS results from concentrated Thionin-Myc-His proteins. The major fraction of secreted Thionin peptides are cleaved at the first Ste13 cleavage site. **E.** Table of predicted molecular weights for Thionin proteins with all possible numbers of disulfide bonds.

Purified Thionin proteins induce synergid degeneration

In order to test the hypothesis that Thionin proteins secreted by the pollen tube are directly involved in synergid degeneration, we determined whether heterologously expressed, purified thionin induced synergid degeneration in vitro. Seed- and leaf-specific Thionins have well characterized toxic effects on cell growth in cell culture systems (Stec et al. 2004), but synergid cells are buried within the ovule tissue, which makes them challenging material for bioassays. However, the genetic reporter employed in observing pollen tube reception in vivo, *ACT11:MSI1:GFP*, provides an method by which to observe the action of Thionins on synergid cells specifically. We performed ovule incubation assays using each of our purified Thionin proteins, from each of the three heterologous expression experiments. *ACT11:MSI1:GFP* pistils were dissected to remove the pistil walls, exposing the ovules, and placed in ovule incubation media with or without purified Thionin proteins. Synergid nuclear degeneration was quantified after 3 hours of incubation by confocal microscopy (Figure 11). We observed

increases in synergid degeneration when Thionin proteins were incubated with ovules, greater than incubation with buffer alone (Figure 11A,B). MBP-Thionin produced in *E. coli* (Supplemental Figure 4A-D) exhibited low activity without cleavage with TEV protease. Despite incomplete TEV cleavage (<50%), cleaved MBP-Thionin demonstrated higher synergid degeneration rates than un-cleaved fusions (Figure 11A). His-Thionin produced in Shuffle C303 host strains (Supplemental Figure 4E- G) contained *E. coli* protein contamination, but also induced synergid degeneration over background levels. Thionin-GFP-His purified from *N. Benthamiana* (Supplemental Figure 5) also demonstrated increased synergid degeneration levels, suggesting that the Thionin peptide is active in this fusion protein (Figure 11A,C). Finally, Thionin-Myc-His fusions purified from *P. pastoris* were capable of inducing synergid degeneration, even at low concentrations. We asked whether longer incubations (6h) would induce higher degeneration rates; however, the degeneration rate remained unchanged. This indicates that Thionin may act rapidly and may be consumed in the process. For example, based upon the predicted molecular action of Thionin proteins in binding membrane head groups, CRP2460 proteins may bind irreversibly to synergid membranes. Future studies will address the concentration-dependent activity of purified Thionin proteins on synergid degeneration.

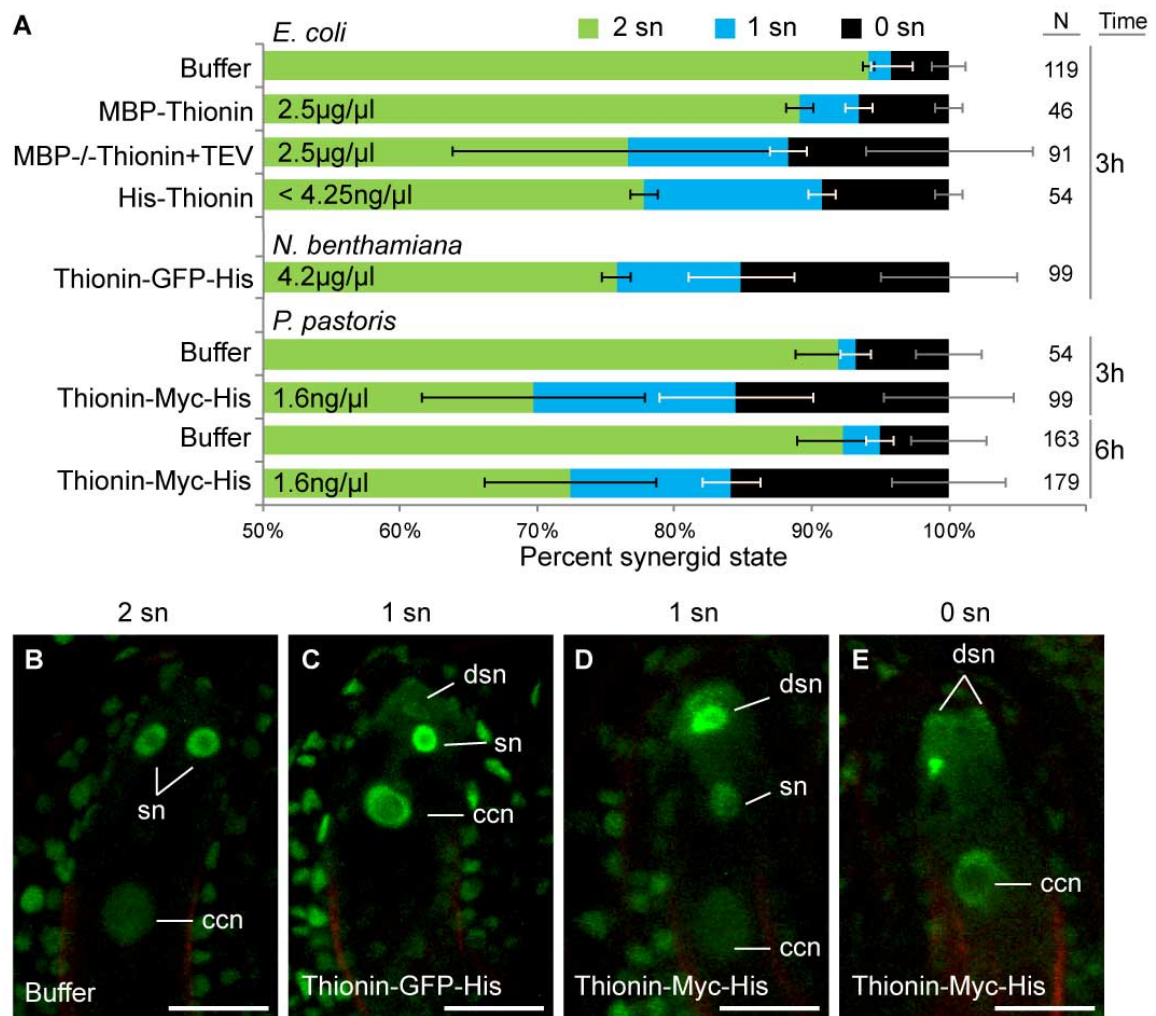


Figure 11. Purified Thionin (At5g36720) promotes synergid degeneration in vitro. **A.** Synergid nuclear degeneration after incubation with purified Thionin proteins. All data are presented as the mean of at least two replicates $\pm$ std dev. Green – 2 intact synergid nuclei, Blue – 1 intact synergid nucleus, Black – 0 intact synergid nuclei. **B.** Buffer only control, 2 intact synergid nuclei. **C.** Thionin-GFP-His protein purified from Tobacco leaf tissue, with 1 intact synergid nucleus (sn). The degenerated synergid nucleus (dsn) is indicated. **D-E.** Ovules incubated with Thionin-Myc-His purified from *Pichia*. **D.** 1 degenerated synergid, 1 intact synergid. **E.** 0 intact synergids.

Discussion

Plant reproduction requires species specific interaction of pollen and pistil to ensure sperm delivery and double fertilization. A number of small secreted proteins act as self- determinants during pollen binding and hydration in *Brassica* (Mishima et al. 2003; Chookajorn et al. 2004). However, pollen tube interactions with the synergid are cell-identity interactions that we know surprisingly little about. Pollen tubes deliver sperm cells to the female gametophyte after a cell:cell interaction with the synergid cell. If this interaction is successful, near-simultaneous synergid degeneration and pollen tube burst create a mixed cytoplasm, into which the sperm cells are projected for fertilization (Thesis Chapter 2). These steps are both cell death events that are required for successful reproduction (Heydlauff et al. 2014). CRP2460 genes are not expressed in *myb* triple-mutant pollen tubes, which fail to elicit a cell death response in synergids. Therefore, CRP2460 proteins are attractive candidates as inducers of synergid degeneration.

There is evidence that pollen tube reception is species-specific in *Brassica* (Escobar-Restrepo et al. 2007), and in *Rhododendron* (Williams et al. 1986). Factors involved in species-specific gametic interactions are often rapidly evolving and highly diversified (Clark et al. 2006). The CRP2460 family of Thionin proteins appears to be diversified in both gene number and amino acid sequence even between closely related species such as *Arabidopsis thaliana* and *lyrata* (Figure 2). However, if we consider the biological activity of Thionins such as

alpha Hordothionin, which is capable of binding to the membrane (Oard 2011), we are presented with a dilemma. If pollen tube Thionins indiscriminately bind to membranes, then we would expect cell death in cells other than the synergid in our ovule incubation assays, which we did not observe (Figure 13C-E). Therefore there remains some mechanism by which pollen expressed Thionin proteins exert a cell type specific activity. This could be achieved through at least three mechanisms. First, the membrane permeation activity of the Thionin family could be maintained, but only activate upon interaction with distinct phospholipid head groups. These phospholipid head groups would by necessity be specific to or enriched at the synergid membrane, where the pollen tube makes contact during reception. Second, Thionin activity could be controlled by factors in the synergid extracellular environment. Purothionin activity is antagonized by high concentrations of calcium and other bivalent cations (Okada et al. 1973). Other Thionins may have evolved a mechanism for ion-mediated activation. Dynamic fluctuations of calcium at the synergid membrane may indeed be the direct result of Thionin-induced pore formation (Iwano et al. 2012; Ngo et al. 2014). Alternatively, the C-terminal region of CRP2460 proteins may be a non-acidic inhibitory pro-protein domain that is cleaved by an extracellular protease secreted by the synergid cell.

The third explanation for Thionin cell specificity would require that CRP2460 Thionins have a function distinct from membrane permeation. Indeed, Thionins have been implicated in many roles outside of membrane degeneration. For

example, the well-studied Thionin protein Crambin is a seed storage protein that lacks any known membrane permeabilizing activity. Thionins are basic proteins and they can directly bind nucleic acids (Woynarowski et al. 1980), inhibit translation (Li et al. 2002), and inhibit enzyme activities (Diaz et al. 1992), just to name a few of the observed effects. While these effects may magnify the toxic effects of already toxic Thionins (such as Purothionin), they also highlight the versatility of the Thionin structure. Indeed, *Arabidopsis* Thi2.1 protein retains cytotoxic activity on cells presumably via membrane binding (Loeza-Angeles et al. 2008), however Thi2.4 has been demonstrated to bind a fungal fruit body lectin domain (FFBL) of *F. graminearum* (Asano et al. 2013). While that study also observed Thi2.4 membrane localization on fungal membranes, it highlights the possibility that Thionin CRP2460 proteins could act as ligands, as is the case for CRP proteins LAT52, LUREs or SCR/SP11 (Shiba et al. 2001; Tang et al. 2002; Takeuchi et al. 2012).

It was recently demonstrated that a rapid alkalization factor (RALF) was a ligand for *Feronia* in somatic tissues (Haruta et al. 2014). Therefore we do not anticipate that Thionins are the ligand for *Feronia*, though *Feronia* is predicted to bind carbohydrates in the cell wall (Kessler et al. 2015). If Thionins competitively bind cell wall constituents away from the *Feronia* ecto-domain, it could amplify the binding effect of RALF ligands. Whatever their mode of action, Thionins may act synergistically with *Feronia* and *Lorelei* to rapidly induce synergid degeneration during normal pollen tube reception (Kessler et al. 2011). The

combination of multiple pollen tube signals, for example multiple CRPs, would provide an information-rich signature by which the female gametophyte can judge the sperm donor species and fitness before committing to fertilization. Thus, Thionins may constitute novel bits of information in the reproduction-associated molecular pattern that the pollen tube presents to the female gametophyte.

Future Directions

We have described the first insights into the CRP2460 gene family, however many questions still remain. By expressing Thionins in the *myb* triple-mutant background we were able to show that Thionins are sufficient to increase synergid degeneration when pollen tubes are interacting with synergids. However, the *myb* triple-mutant phenotype is complex and our re-introduction experiment does not directly address the function of Thionin proteins. Ideally, we would generate mutant pollen tubes that only lack Thionin genes expressed in pollen tubes so that we could define their loss of function phenotype. However, this is a significant challenge because Thionins are arranged in multiple, likely redundant, clusters in the genome (Figure S3). Therefore, we have begun a CRISPR/CAS9 mutagenesis pipeline to completely remove the CRP2460 family from the *Arabidopsis* genome through targeted genomic deletions (Feng et al. 2014). We have generated CRISPR mutagenesis constructs for all members of the CRP2460 clade, utilizing their repetitive sequences to our advantage. In brief, we targeted duplicated loci by designing a guide RNA that recognizes multiple

repeated sequences at one locus (Figure 12A). This guide was cloned and introduced into *LAT52:DsRed Arabidopsis* plants on a T-DNA also carrying the CAS9 gene (Jiang et al. 2013). Primary transformants are analyzed by PCR analysis with gene loci specific primer pairs that report on the presence of gene deletions (Figure 12A, lower gel). T₁ lines with detectable deletions were prioritized, and PCR-products spanning the deletion locus were cloned and sequenced. We identified multiple deletion and insertion mutations that interrupted the coding sequence of the Thionin genes. These plants have been carried through another generation (T₂) to identify homozygous mutations that can be evaluated for pollen tube reception defects using the *LAT52:DsRed* marker as in previous experiments (i.e. Figure 7). Characterizing the Thionin loss of function phenotype will require deletion of multiple duplicated Thionin loci in one mutant strain, which we can evaluate for effects on pollen tube reception using established assays (described in Figures 5,6, and 8).

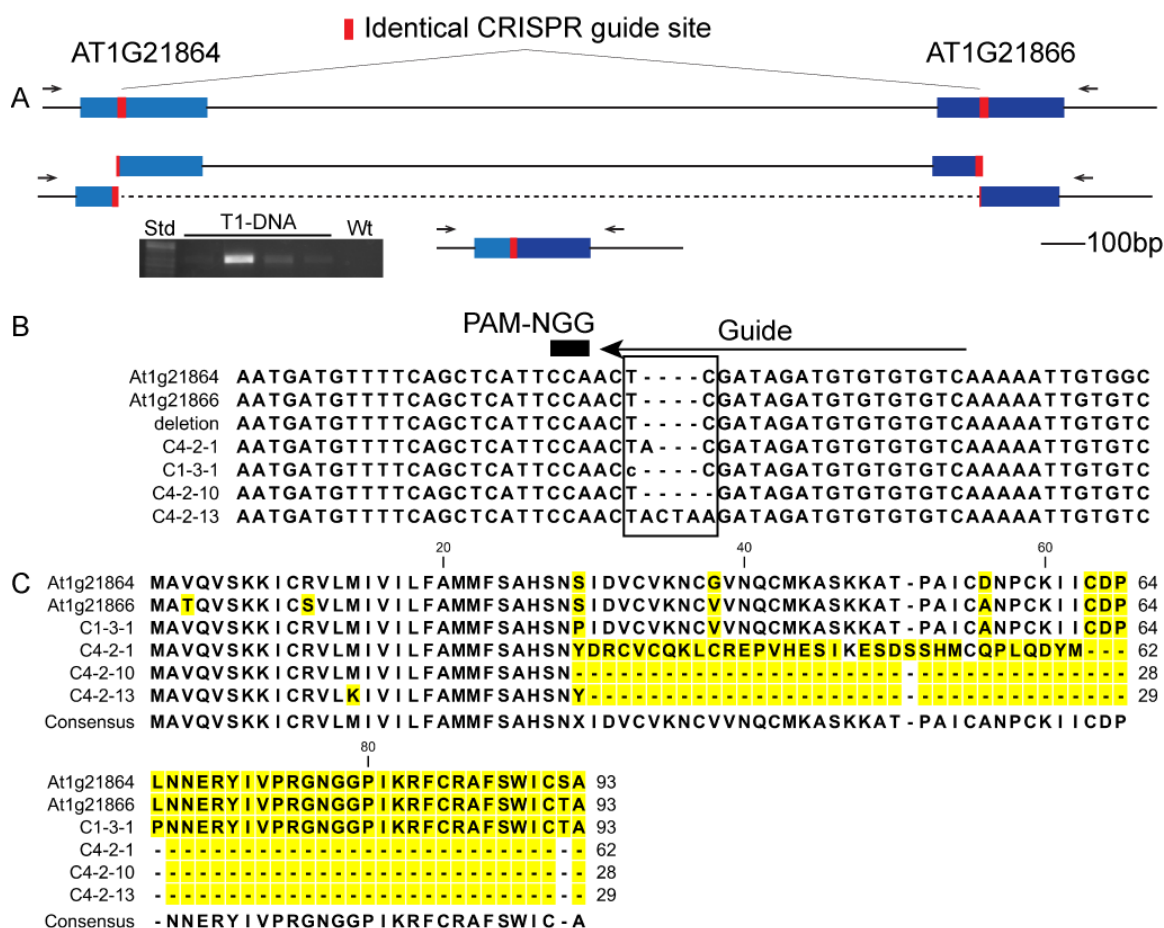


Figure 12. CRISPR mutagenesis of Thionins in the CRP2460 clade. A. A representative Thionin locus carrying a tandem duplication AT1G21864 (light blue) and AT1G21866 (dark blue). Arrows indicate primer pairs that are unable to amplify PCR products in the wild-type locus but produce a PCR product only in the case of CRISPR-CAS9-mediated deletions (inset gel at bottom). Red bar indicates location of homology to the CRISPR guide sequence. **B.** Alignment of the duplicated Thionin pair DNA sequence with the predicted perfect deletion and 4 observed CRISPR mutations. The guide that recognizes this locus is shown with an arrow, and the PAM site (CCA, TGG on reverse strand) is marked with a black block. The black box delineates the region in which we detected CRISPR mutations in T1 plants. **C.** CRISPR mutations interrupt the coding sequence of the deleted hybrid locus. C1-3-1 isolate is a deletion and creates a chimera between these Thionins, C4-2-1 and C4-2-10 produce a frameshift and early stop, and C4-2-13 is an insertion that also causes an early stop.

Biochemical purification of other Thionin proteins and control proteins from *Pichia* is also under way (Figure 13). It will be critical to test whether individual Thionin proteins have high specific activities, which will lead to hypotheses about

residues and domains that are integral to function. Furthermore, pollen tubes express combinations of Thionin proteins, which we can simulate using ovule incubation assays with mixtures of related Thionin proteins. We plan on looking at the dynamics of Thionin interactions as homo- or hetero-Thionin complexes using these purified proteins.

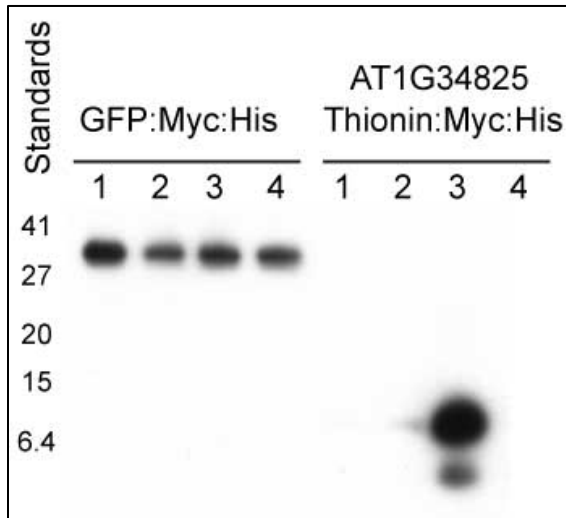


Figure 13. Purification of related Thionin proteins. Secreted GFP and CRP2460-AT1G34825 proteins were constructed and expression optimized for future studies. GFP:Myc:His lanes 1-4, and AT1G34825:Myc:His – lanes 1-4 are screening of individual isolates for expression. Immunoblot of SDS-PAGE probed with anti-His antibodies.

While CRP2460 Thionins are not predicted to have an acidic C-terminal domain, they do have a C-terminal extension with a predicted alpha helical secondary structure (Figure 9B). At this point, none of our constructs rule out cleavage of this C-terminal domain. Thionin-GFP proteins expressed in pollen tubes may be cleaved either in the secretory pathway or in the extracellular region between the pollen tube and the synergid cell. To test this possibility we will perform western blotting of extracts from semi in-vivo pollen tubes to determine whether the C-

terminal GFP remains attached to the Thionin protein in pollen tube secretory network. We can determine the size of this His or GFP reactive C-terminal fragment compared to the purified fragment from Tobacco, which we suspect is intact (Supplemental Figure 5). To test cleavage in the extracellular region of the pollen-synergid apoplast, we will introduce the Myc epitope tag to the N-Terminus of the Thionin-GFP-His construct. If we observe a Myc-Thionin fragment from pollinated pistil extracts that migrates at a lower protein size than the GFP-His reactive band, then the Thionin C-terminus is cleaved during reception. This would provide an ideal mechanism to regulate Thionin protein activity, as Thionin-specific synergid secreted proteases could activate Thionin proteins and contribute to species specificity.

Finally, we will test the disulfide-linkage pattern of the pollen tube Thionin proteins by partial reduction followed by sulfonylation and MADLI-TOF mass spectrometry. We have observed that the Thionin-Myc-His protein purified from *Pichia* is bioactive (Figure 11). We plan to identify the linkage pattern of the 4 stabilizing disulfide bonds to create structural predictions about the folding pattern of the CRP2460 Thionin protein family. If we determine that the Thionin fold, described as a gamma (Γ) shaped structure (Stec et al. 1995), is conserved, it would provide a framework for predicting the effects of Thionin charge and electrostatic interactions with membranes.

Acknowledgements

We thank Rebecca Page for donation of *E. coli* expression constructs and advice on expressing MBP-protein fusions. We would like to acknowledge Tun-Li Shen for his ESI-MS analysis of the Purified Thionin-Myc-His protein expressed in yeast.

Materials and Methods

Plant growth

Procedures for selection of antibiotic and/or herbicide resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; von Besser et al. 2006; Qin et al. 2009; Wong et al. 2010).

Crossing Methods

Expression constructs carry an antibiotic selection cassette (Hygromycin, Hyg). We performed reciprocal crosses to wild type (Col-0 pollen for female crosses, and *male sterile 1*, *ms1* for male crosses), and collected individual siliques. We quantified F1 Hygromycin resistance, and plotted male and female transmission ratios side-by-side. We then took the ratio of male transmission to female transmission, which normalizes transmission to insert number, critical for evaluating mixed populations of primary transformants that may contain more than one T-DNA insert site and therefore multiple unlinked hygromycin resistance genes.

Cloning

For cDNA cloning RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis was performed with Superscript III (Invitrogen), and PCR used Phusion Polymerase (NEB). cDNA sequences were cloned into entry vectors pENTR-D or

pCR8 (Invitrogen), and LR cloned into destination vectors with LR clonase II (Invitrogen). Promoters were cloned into Gateway Destination vectors (Curtis et al. 2003). Destination vectors promoter sequences were swapped after the ccDb/CAM cassette was replaced with a placeholder DNA sequence (LR reaction) before being replaced with the ccDb/CAM cassette from pDONRZEO using BP clonase II (Invitrogen). All clones were sequenced before introduction to plants (Genewiz).

Bioinformatic Analysis

MYB-regulated CRPs were analyzed for predicted signal peptides using the SignalP 4.1 server (Petersen et al. 2011) and PI was calculated using Compute pI/Mw tool (Expasy). Phylogenetic relationships were analyzed using MEGA software (Tamura et al. 2013).

Transient Expression in *N. benthamiana*

We followed a standard transient expression protocols (Cournoyer et al. 2011). Briefly, 7ml LB+Drug cultures of Agrobacterium carry desired constructs were grown overnight, spun down, and re-suspended in Infiltration media (10mM MgCl₂, 10mM MES, 200uM acetosyringone), and then shaken at 25C for at least three hours. Cultures were spun down and re-suspended at an OD600 of one before injection into *N. Benthamiana* leaves, and allowed to express for three days to visualize protein localization by confocal microscopy. At least 4 leaves of Thionin-GFP-His with strong GFP expression were used for purification. Briefly, leaves were ground in liquid nitrogen, suspended in Native purification buffer with

1% NP-40, vortexed for ten minutes and then spun down. Supernatants were analyzed by SDS-PAGE and nickel column purification (ProBond manual – Invitrogen).

Pollen tube reception microscopy

For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated *ACT11:MSI1:GFP* stage 14 stigmas (Smyth et al. 1990) with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al. 2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

Heterologous expression in *E. coli*

pET derived plasmids were generous gifts of Dr. Rebecca Page (Brown University, Providence, RI). Thionin fragments from (AT5G36720) cDNA without the signal peptide were cloned into pET vectors and sequenced (Genewiz). BL21, Rosetta-gami 2(DE3) and Shuffle C3030 were procured and respective

plasmids were chemically transformed in to host strains, and grown on Kan selection plates. Precultures were grown at 37C overnight at 200rpm (30C for C3030). For production of Tagged protein, cultures were expanded to 100 mls, and grown to an OD600 of 1, before cooling to 30C, and addition of IPTG to a final concentration of 1mM. Cultures were induced for 4 hours at 37C (BL21, RG2) or 30C (C3030). Purification of His-Tagged proteins: Cultures were harvested by centrifugation at 3,000rpm for 15 minutes at 4C and frozen in liquid nitrogen. Pellets were thawed on ice, then re-suspended in 2 mls of Native lysis buffer (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, pH8.0). Eight mg of lysozyme was added, and then tubes were incubated on ice for 30min before sonication. DNase and RNase were added (8ul), lysis buffer added to 8mls, and extractions were passed through a 27.5G needle, before centrifugation at 3,000rpm for 15 minutes at 4C. Supernatant was separated from insoluble pellets before SDS-PAGE and nickel column purification (as per ProBond manual – Invitrogen).

Heterologous expression in Pichia

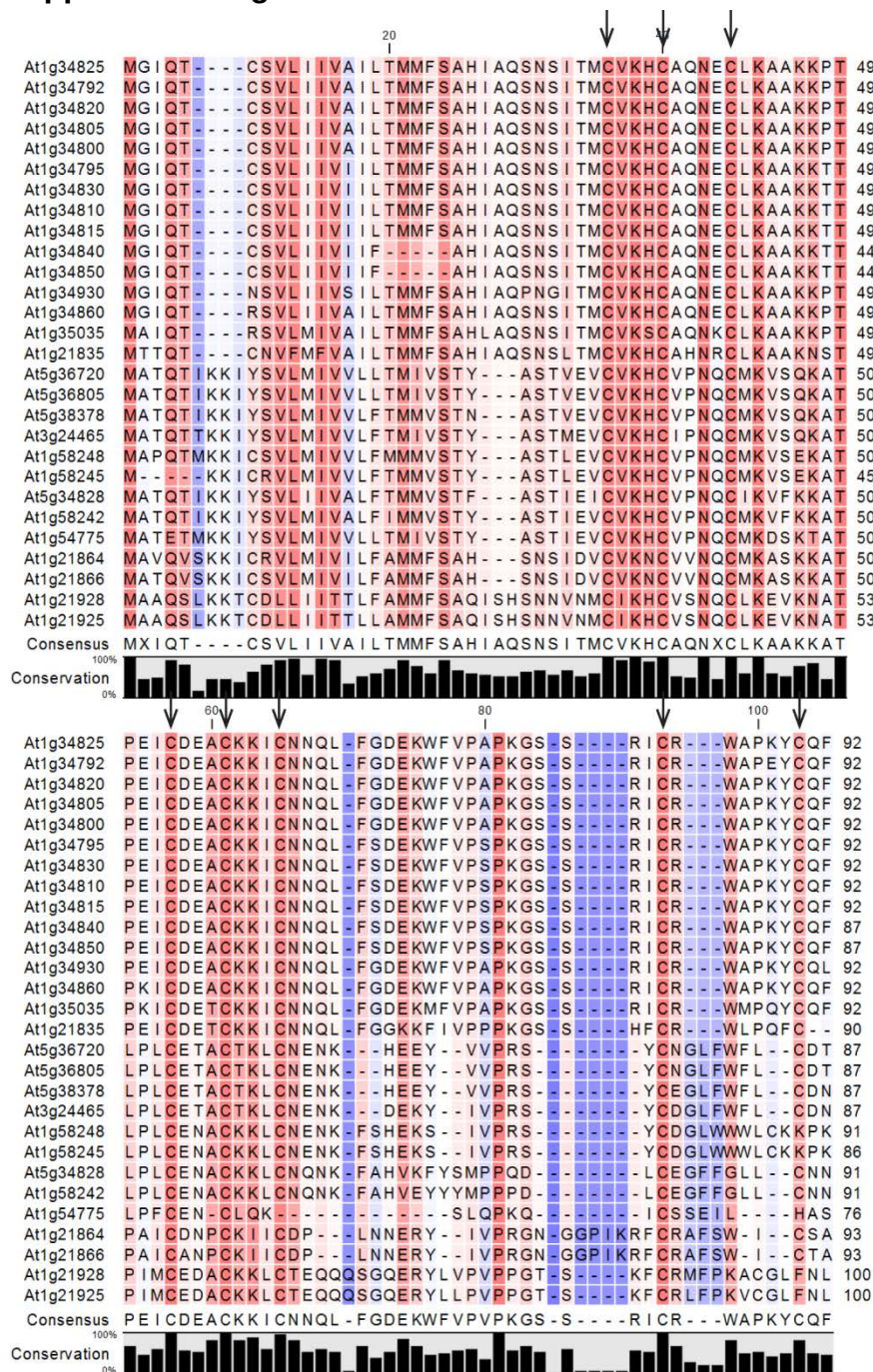
Pichia expression clones, wild-type and control strains were purchased from Invitrogen. Thionin cDNAs were cloned into pPICZ alpha as an EcoRI-Thionin-XbaI fragment from a TA cloned intermediate (pCR2.1, Invitrogen). Plasmids were midi-prepped, linearized with SacI, ethanol precipitated and concentrated. Wild-type X-33 *Pichia pastoris* was made electrocompetent, and 5-10ug of linearized DNA was transformed into 80ul of competent cells. Transformants were identified by outgrowth on YPDS+ Zeocin for 2-3 days and re-streaked onto

YPD+Zeocin. Isolates were tested for the Mut phenotype, and Mut⁺ strains were selected for isolate testing in small cultures (25mls in 50ml conical tubes). Upscaled Thionin-Myc-His purifications from isolate 905-4 were grown o/n in 50ml BMGY, before dilution to OD₆₀₀ of 1 (~200ml) in BMMY and grown for 48 hours. Cultures were spiked with MeOH at 24hours to maintain induction. Cultures were spun down (3,000rpm, 15min, 4°C) and supernatant was concentrated over a 3K Amicon column (Millipore), and diluted with 7mls of Native binding buffer (50mM NaH₂PO₄, 500mM NaCl, 10mM imidazole, pH8.0) before nickel column purification (as per ProBond manual – Invitrogen) and analysis by SDS-PAGE, Western blotting or subsequent experimentation.

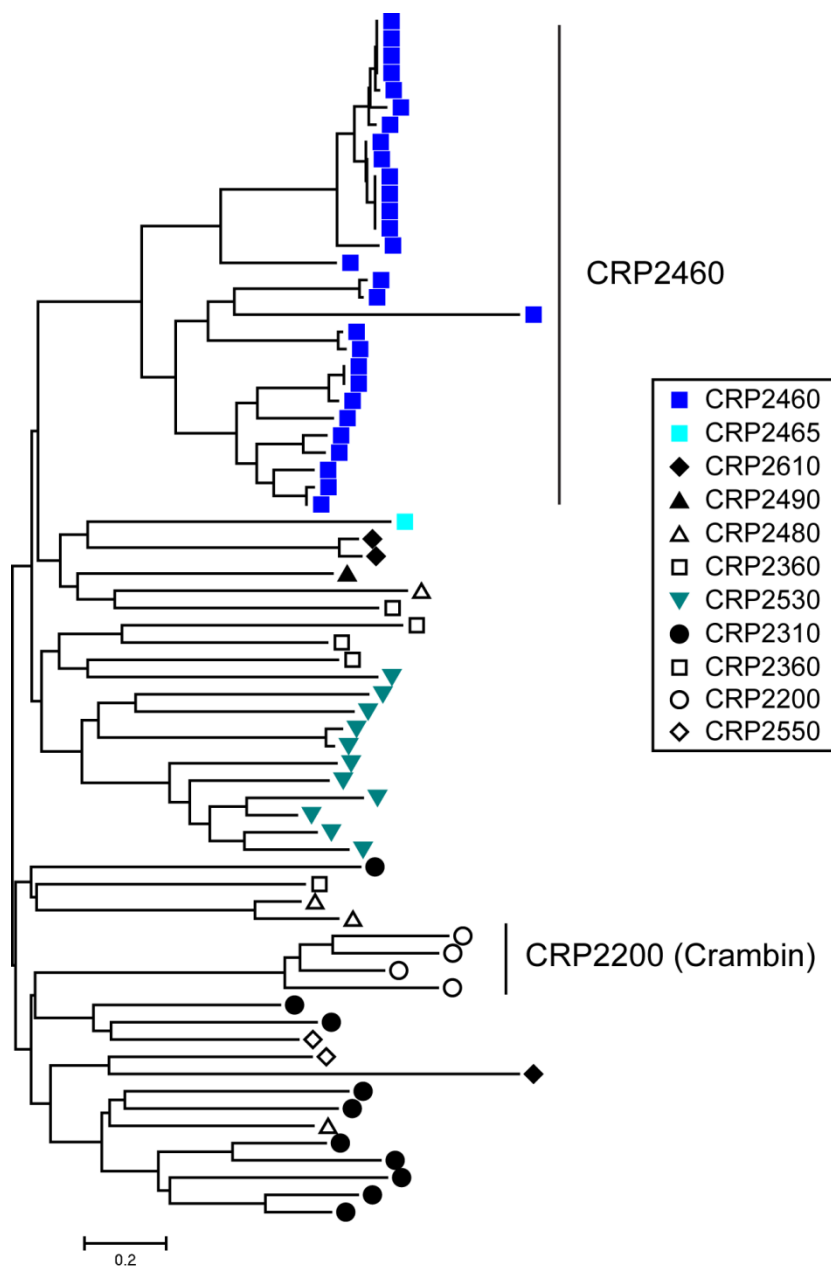
Ovule incubation assays

Ovule assay conditions were modified from a prior report (Costa et al. 2014). Incubation media (2% Sucrose, 2.2g/L Murashige and Skoog media, 400mg/L Glutamine, 0.5g/L MES pH5.6), was supplemented with extraction buffer (50mM NaH₂PO₄, 500mM NaCl, 250mM imidazole, pH8.0) or with purified Thionin protein in extraction buffer. *ACT11:MSI1:GFP* pistils were emasculated 12 hours prior to dissection and removal of the carpel walls, and then submerged into liquid medium for 3 or more hours in a Percival growth chamber shelf without illumination (22°C). Pistils were removed and mounted for visualization and quantification by confocal microscopy.

Supplemental Figures

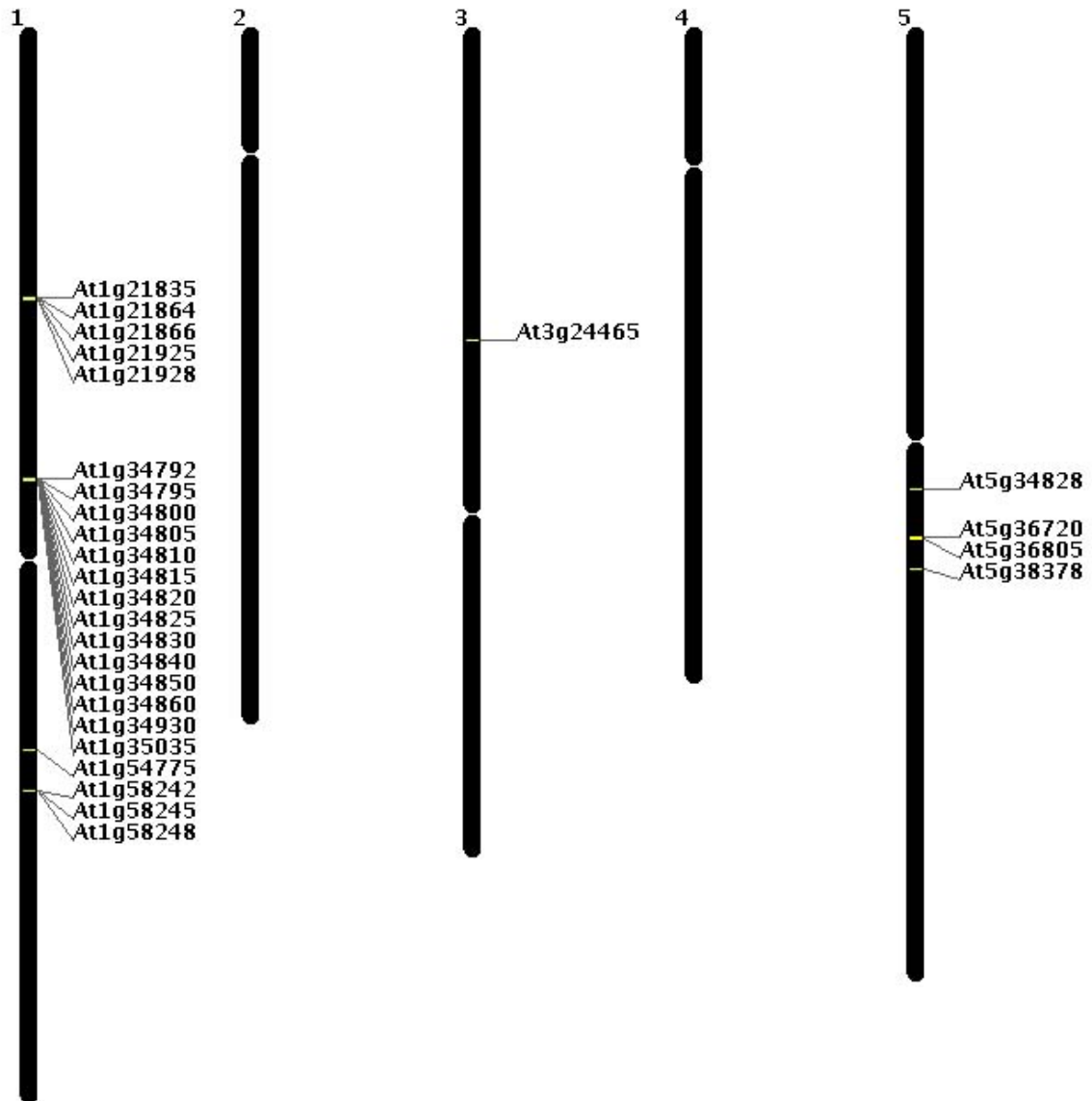


Supplemental Figure 1. Protein alignment of CRP2460 from *A. thaliana*. Amino acids with higher conservation are shaded darker red, low conservation – purple. Arrows indicate conserved cysteines. Alignment performed in CLC Sequence Viewer 6.



Supplemental Figure 2. Phylogenetic analysis of all Thionin and Thionin-related CRPs in *Arabidopsis thaliana* (CRP2200-2550). The evolutionary history was inferred using the Neighbor-Joining method (Saitou et al. 1987). The optimal tree with the sum of branch length = 22.19256209 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 70 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 39 positions in the final dataset.

Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Nodes with colors indicate genes highlighted in our experiments (light blue – AT1G77682 was used as outgroup in Figure1), (blue-green triangles are CRP2530, within which AT5G26673 is found – Figure 1, Add-back).



Supplemental Figure 3. The location of CRP2460 genes in the *Arabidopsis* Genome. Generated with the Chromosome map tool from TAIR.

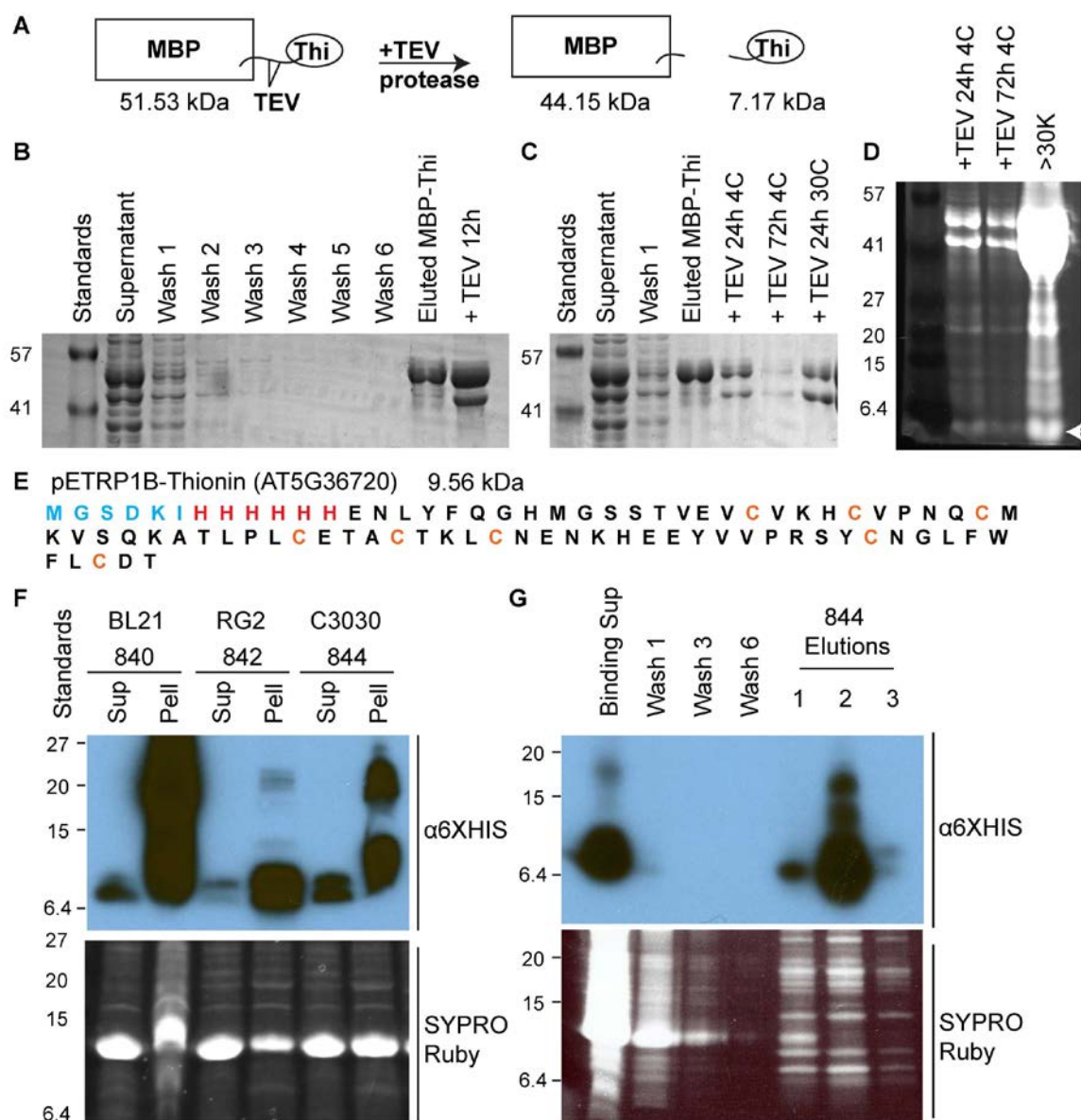
Supplemental Section: Heterologous expression of Thionin proteins

Heterologous expression of Thionin in *E. coli*

While many seed specific Thionins have been purified from their tissue of origin, the CRP2460 family is only expressed in the pollen tube, and only after growth in the pistil. Therefore, to produce large quantities of Thionin protein we evaluated several different species for protein expression. Heterologous expression of *Arabidopsis thaliana* Thionin proteins (Thi2.1-2.4, Figure9A) has been documented in *E. coli* (Abbas et al. 2013), which increased soluble expression with the linked solubility tags Thioredoxin (TRX) and Maltose Binding Protein (MBP). We therefore constructed a similar fusion construct that fuses the MBP protein with the Thionin protein AT5G36720 coding sequence lacking the signal peptide. MBP can be expressed at very high levels in *E. coli* and may reduce toxic effects of the Thionin proteins (Romero et al. 1997). This construct includes a Tobacco Etch Virus protease cleavage site (TEV) allowing removal of the HIS-MBP solubility tag after purification from *E. coli* (pET-M30-MBP, Supplemental Figure 4A) (O'Connell et al. 2012). We expressed this IPTG inducible construct in the Rosetta-gami 2 host strain (Novagen) that includes the *trxB* and *gor* mutations that facilitate cytoplasmic disulfide bond formation. We observed high induction levels of the ~51kDa fusion protein, which was purified using nickel column chromatography (Supplemental Figure 4, Methods). HIS-MBP-TEV-Thionin protein was then digested with TEV protease, however we observed low efficiency TEV protease cleavage (<50%), even after optimization of cleavage conditions (Supplemental Figure 4C). Furthermore, the cleaved Thionin protein

failed to elute from the nickel purification column after TEV proteolysis, complicating the purification process (Supplemental Figure 4D, white arrowhead).

We next expressed the Thionin protein AT5G36720 without a solubility tag to test whether its expression would be toxic. We cloned the coding sequence lacking the signal peptide behind an MGSDKIHHHHHH- leader, which combines the 6xHIS tag with a 6 amino acid sequence designed to increase translational output (Peti et al. 2007). We expressed this Thionin construct (His-Thionin) in BL21, Rosetta-gami 2, and Shuffle C3030 host strains, and compared the soluble fraction of the His-Thionin peptide (Supplemental Figure 4F). We observed that the bulk of the His-Thionin protein was insoluble, with only a small amount in the soluble fraction. Despite the observed low levels, we observed soluble His-Thionin, which did not appear to be toxic to *E. coli* growth rates of our induction cultures. Unfortunately, test nickel column purifications revealed that there were not sufficiently high concentrations in soluble fractions to purify pure His-Thionin proteins, as judged by the large number of *E. coli* proteins that remained bound to nickel columns and were eluted by Imidazole elution steps (Supplemental Figure 4G).

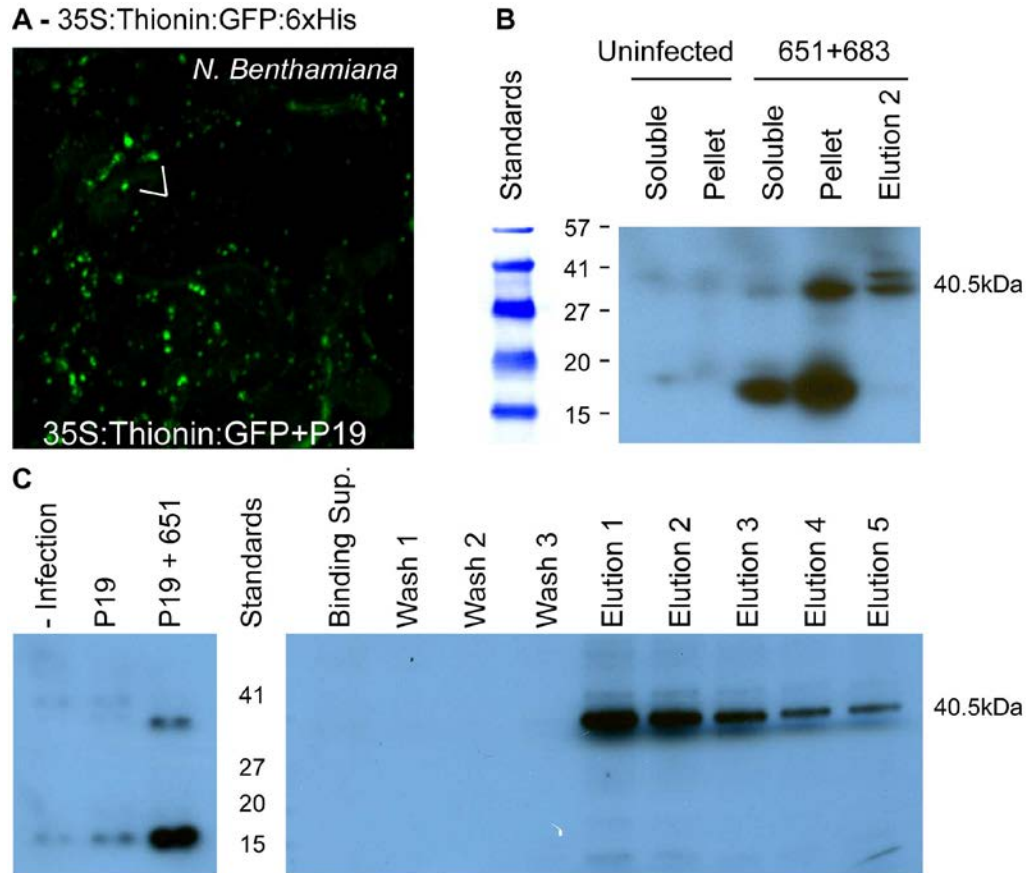


Supplemental Figure 4. Expression of Thionin protein in *E. coli* cytoplasm.

A. Expression strategy for His-MBP-TEV-Thionin in *E. coli*. **B.** Purification of His-MBP-TEV-Thionin, Supernatant – soluble protein extracted from 24hour IPTG inductions in RG2, Wash 1-6 – Nickel column washes, Eluted MBP-Thi – Eluted His-MBP-TEV-Thionin protein, +TEV 12h – 12 hour TEV treatment at 37C. **C.** TEV cleavage optimization. **D.** SYPRO Ruby protein staining of TEV cleavage products. >30K indicates the eluted His-MBP fraction passed through a 30K cutoff centricon column, White arrow indicates the Thionin fragment that failed to wash off the nickel column. **E.** His-Thionin coding sequence, translation tag is in Blue, His tag is in Red, and the conserved cysteines are in orange. **F.** Comparison of His-Thionin expression in various host strains. BL21 – BL21(DE3), RG2 – Rosetta-gami 2(DE3), C3030 – Shuffle C3030. **G.** Purifications of His-Thionin from C3030 (PGL844). **F-G** the antibody is listed on the right (anti-6xHis), and the lower gel is SYPRO Ruby protein staining.

Heterologous expression of Thionin in *N. Benthamiana*

We previously demonstrated expression of *AT5G36720:GFP* in *N. benthamiana* leaf cells by *Agrobacterium* infiltration (Figure 3). This construct is driven by the viral 35S promoter and retains the endogenous signal peptide, and accumulates visible Thionin:GFP puncta in the golgi. These expression conditions were therefore potentially attractive for purification of Thionin-GFP-His protein. We co-infected *N. Benthamiana* leaves with *35S:Thionin:GFP:His* and *P19* (Hsieh et al. 2009; Lombardi et al. 2009) and ensured that we observed high levels of Thionin-GFP accumulation by confocal microscopy (Supplemental Figure 5A, green puncta). We performed a trial protein extraction and purification of Thionin-GFP, and found that the majority of the fusion protein was in the insoluble fraction (Supplemental Figure 5B). We subsequently optimized the extraction procedure to allow increased solubility of Thionin-GFP through addition of detergents (Supplemental Figure 5C, Methods). This allowed purification of Thionin-GFP from the endomembrane system of the Tobacco leaf. Purified Thionin-GFP fractions were free of contaminating proteins (Data not shown) and at reasonably high concentrations given the low levels of input tissue (~800ug).



Supplemental Figure 5. Expression and purification of Thionin protein in *N. benthamiana* leaf infections. **A.** Detection of Thionin-GFP in leaves prior to harvest. **B.** Detection and purification of Thionin-GFP in leaves using extraction buffer without detergents. **C.** Purification of Thionin-GFP with 1% NP-40.

References

- Abbas, A., S. Plattner, et al. (2013). "Comparison of periplasmic and intracellular expression of Arabidopsis thionin proproteins in *E. coli*." Biotechnol Lett **35**(7): 1085-1091.
- Asano, T., A. Miwa, et al. (2013). "The secreted antifungal protein thionin 2.4 in *Arabidopsis thaliana* suppresses the toxicity of a fungal fruit body lectin from *Fusarium graminearum*." PLoS Pathog **9**(8): e1003581.
- Balls AK, H. W., Harris TH (1942). "A crystalline protein obtained from a lipoprotein of wheat flour." Cereal Chemistry **19**: 279-288.
- Bekes, F. and R. Lasztity (1981). "Isolation and Determination of Amino-Acid-Sequence of Avenothionin, a New Purothionin Analog from Oat." Cereal Chemistry **58**(4): 360-361.
- Berger, F., Y. Hamamura, et al. (2008). "Double fertilization - caught in the act." Trends Plant Sci **13**(8): 437-443.
- Bohlmann, H. (1994). "The Role of Thionins in Plant-Protection." Critical Reviews in Plant Sciences **13**(1): 1-16.
- Carrasco, L., D. Vazquez, et al. (1981). "Thionins - Plant Peptides That Modify Membrane-Permeability in Cultured Mammalian-Cells." European Journal of Biochemistry **116**(1): 185-189.
- Ceroni, A., A. Passerini, et al. (2006). "DISULFIND: a disulfide bonding state and cysteine connectivity prediction server." Nucleic Acids Res **34**(Web Server issue): W177-181.
- Chookajorn, T., A. Kachroo, et al. (2004). "Specificity determinants and diversification of the Brassica self-incompatibility pollen ligand." Proc Natl Acad Sci U S A **101**(4): 911-917.
- Christensen, C. A., S. Subramanian, et al. (1998). "Identification of gametophytic mutations affecting female gametophyte development in *Arabidopsis*." Dev Biol **202**(1): 136-151.
- Clark, N. L., J. E. Aagaard, et al. (2006). "Evolution of reproductive proteins from animals and plants." Reproduction **131**(1): 11-22.
- Clark, S. E., M. P. Running, et al. (1995). "Clavata3 Is a Specific Regulator of Shoot and Floral Meristem Development Affecting the Same Processes as Clavata1." Development **121**(7): 2057-2067.
- Costa, L. M., E. Marshall, et al. (2014). "Central cell-derived peptides regulate early embryo patterning in flowering plants." Science **344**(6180): 168-172.
- Coulson, E. J., Harris, T. H., Axelrod, B. (1942). "Effect on small laboratory animals of the injection of a sulfur protein from wheat flour." Cereal Chemistry **19**: 301.
- Cournoyer, P. and S. P. Dinesh-Kumar (2011). "Studying NB-LRR immune receptor localization by agroinfiltration transient expression." Methods Mol Biol **712**: 1-8.
- Curtis, M. D. and U. Grossniklaus (2003). "A gateway cloning vector set for high-throughput functional analysis of genes in planta." Plant Physiol **133**(2): 462-469.

- Daly, R. and M. T. Hearn (2005). "Expression of heterologous proteins in *Pichia pastoris*: a useful experimental tool in protein engineering and production." J Mol Recognit **18**(2): 119-138.
- Diaz, I., M. J. Carmona, et al. (1992). "Effects of Thionins on Beta-Glucuronidase In vitro and in Plant-Protoplasts." Febs Letters **296**(3): 279-282.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Feng, Z., Y. Mao, et al. (2014). "Multigeneration analysis reveals the inheritance, specificity, and patterns of CRISPR/Cas-induced gene modifications in *Arabidopsis*." Proc Natl Acad Sci U S A **111**(12): 4632-4637.
- Fernandez de Caley, R., B. Gonzalez-Pascual, et al. (1972). "Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro." Appl Microbiol **23**(5): 998-1000.
- Florack, D. E. and W. J. Stiekema (1994). "Thionins: properties, possible biological roles and mechanisms of action." Plant Mol Biol **26**(1): 25-37.
- Hamamura, Y., M. Nishimaki, et al. (2014). "Live imaging of calcium spikes during double fertilization in *Arabidopsis*." Nat Commun **5**: 4722.
- Haruta, M., G. Sabat, et al. (2014). "A Peptide Hormone and Its Receptor Protein Kinase Regulate Plant Cell Expansion." Science **343**(6169): 408-411.
- Hernandezlucas, C., J. Royo, et al. (1986). "Polyadenylation Site Heterogeneity in Messenger-Rna Encoding the Precursor of the Barley Toxin Beta-Hordothionin." Febs Letters **200**(1): 103-106.
- Heydlauff, J. and R. Gross-Hardt (2014). "Love is a battlefield: programmed cell death during fertilization." Journal of Experimental Botany **65**(5): 1323-1330.
- Hsieh, Y. C., R. T. Omarov, et al. (2009). "Diverse and Newly Recognized Effects Associated with Short Interfering RNA Binding Site Modifications on the Tomato Bushy Stunt Virus P19 Silencing Suppressor." Journal of Virology **83**(5): 2188-2200.
- Hughes, P., E. Dennis, et al. (2000). "The cytotoxic plant protein, beta-purothionin, forms ion channels in lipid membranes." J Biol Chem **275**(2): 823-827.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Jelsch, C., M. M. Teeter, et al. (2000). "Accurate protein crystallography at ultra-high resolution: Valence electron distribution in crambin." Proceedings of the National Academy of Sciences of the United States of America **97**(7): 3171-3176.
- Jiang, W., H. Zhou, et al. (2013). "Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in *Arabidopsis*, tobacco, sorghum and rice." Nucleic Acids Res **41**(20): e188.

- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Jones, B. L. and D. B. Cooper (1980). "Purification and Characterization of a Corn (*Zea-Mays*) Protein Similar to Purothionins." J Agric Food Chem **28**(5): 904-908.
- Jones, D. T., W. R. Taylor, et al. (1992). "The rapid generation of mutation data matrices from protein sequences." Comput Appl Biosci **8**(3): 275-282.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.
- Kessler, S. A., H. Lindner, et al. (2015). "Functional analysis of related CrRLK1L receptor-like kinases in pollen tube reception." EMBO Rep **16**(1): 107-115.
- Kramer, K. J., L. W. Klassen, et al. (1979). "Toxicity of purothionin and its homologues to the tobacco hornworm, *Manduca sexta* (L.) (Lepidoptera: Sphingidae)." Toxicol Appl Pharmacol **48**(1 Pt 1): 179-183.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Li, S. S., J. Gullbo, et al. (2002). "Ligatoxin B, a new cytotoxic protein with a novel helix-turn-helix DNA-binding domain from the mistletoe *Phoradendron liga*." Biochemical Journal **366**: 405-413.
- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 Function as Male Factors That Control Pollen Tube-Synergid Interaction in *Arabidopsis thaliana* Fertilization." PLoS Genet **9**(11).
- Loeza-Angeles, H., E. Sagrero-Cisneros, et al. (2008). "Thionin Thi2.1 from *Arabidopsis thaliana* expressed in endothelial cells shows antibacterial, antifungal and cytotoxic activity." Biotechnol Lett **30**(10): 1713-1719.
- Lombardi, R., P. Circelli, et al. (2009). "High-level HIV-1 Nef transient expression in *Nicotiana benthamiana* using the P19 gene silencing suppressor protein of Artichoke Mottled Crinckle Virus." Bmc Biotechnology **9**.
- Mishima, M., S. Takayama, et al. (2003). "Structure of the male determinant factor for Brassica self-incompatibility." Journal of Biological Chemistry **278**(38): 36389-36395.
- Moir, D. T. and J. I. Mao (1990). "Protein secretion systems in microbial and mammalian cells." Bioprocess Technol **9**: 67-94.
- Nelson, B. K., X. Cai, et al. (2007). "A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants." Plant J **51**(6): 1126-1136.
- Ngo, Q. A., H. Vogler, et al. (2014). "A calcium dialog mediated by the FERONIA signal transduction pathway controls plant sperm delivery." Developmental Cell **29**(4): 491-500.

- O'Connell, N., S. R. Nichols, et al. (2012). "The Molecular Basis for Substrate Specificity of the Nuclear NIPP1:PP1 Holoenzyme." Structure **20**(10): 1746-1756.
- Oard, S. V. (2011). "Deciphering a mechanism of membrane permeabilization by alpha-hordothionin peptide." Biochim Biophys Acta **1808**(6): 1737-1745.
- Okada, T. and Yoshizum.H (1973). "Lethal Toxic Substance for Brewing Yeast in Wheat and Barley .3. Mode of Action of Toxic Protein in Wheat and Barley on Brewing Yeast." Agricultural and Biological Chemistry **37**(10): 2289-2294.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Osorio e Castro, V. R. and L. P. Vernon (1989). "Hemolytic activity of thionin from *Pyricularia pubera* nuts and snake venom toxins of *Naja naja* species: *Pyricularia* thionin and snake venom cardiotoxin compete for the same membrane site." Toxicon **27**(5): 511-517.
- Petersen, T. N., S. Brunak, et al. (2011). "SignalP 4.0: discriminating signal peptides from transmembrane regions." Nat Methods **8**(10): 785-786.
- Peti, W. and R. Page (2007). "Strategies to maximize heterologous protein expression in *Escherichia coli* with minimal cost." Protein Expression and Purification **51**(1): 1-10.
- Ponz, F., J. Paz-Ares, et al. (1986). "Cloning and nucleotide sequence of a cDNA encoding the precursor of the barley toxin alpha-hordothionin." Eur J Biochem **156**(1): 131-135.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Reimann-Philipp, U., G. Schrader, et al. (1989). "Intracellular thionins of barley. A second group of leaf thionins closely related to but distinct from cell wall-bound thionins." J Biol Chem **264**(15): 8978-8984.
- Romero, A., J. M. Alamillo, et al. (1997). "Processing of thionin precursors in barley leaves by a vacuolar proteinase." European Journal of Biochemistry **243**(1-2): 202-208.
- Saitou, N. and M. Nei (1987). "The neighbor-joining method: a new method for reconstructing phylogenetic trees." Mol Biol Evol **4**(4): 406-425.
- Samuelss.G (1973). "Mistletoe Toxins." Systematic Zoology **22**(4): 566-569.
- Shiba, H., S. Takayama, et al. (2001). "A pollen coat protein, SP11/SCR, determines the pollen S-specificity in the self-incompatibility of *Brassica* species." Plant Physiol **125**(4): 2095-2103.
- Silverstein, K. A., W. A. Moskal, Jr., et al. (2007). "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants." Plant J **51**(2): 262-280.

- Silverstein, K. A. T., W. A. Moskal, et al. (2007). "Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants." Plant Journal **51**(2): 262-280.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in *Arabidopsis*." Plant Cell **2**(8): 755-767.
- Sprunck, S., S. Rademacher, et al. (2012). "Egg cell-secreted EC1 triggers sperm cell activation during double fertilization." Science **338**(6110): 1093-1097.
- Stec, B. (2006). "Plant thionins--the structural perspective." Cell Mol Life Sci **63**(12): 1370-1385.
- Stec, B., O. Markman, et al. (2004). "Proposal for molecular mechanism of thionins deduced from physico-chemical studies of plant toxins." J Pept Res **64**(6): 210-224.
- Stec, B., R. Zhou, et al. (1995). "Full-matrix refinement of the protein crambin at 0.83 Å and 130 K." Acta Crystallogr D Biol Crystallogr **51**(Pt 5): 663-681.
- Takeuchi, H. and T. Higashiyama (2012). "A Species-Specific Cluster of Defensin-Like Genes Encodes Diffusible Pollen Tube Attractants in *Arabidopsis*." Plos Biology **10**(12).
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*." PLoS Biol **10**(12): e1001449.
- Tamura, K., G. Stecher, et al. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol **30**(12): 2725-2729.
- Tang, W. H., I. Ezcurra, et al. (2002). "A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2." Plant Cell **14**(9): 2277-2287.
- Teeter, M. M. and W. A. Hendrickson (1979). "Highly Ordered Crystals of the Plant Seed Protein Crambin." Journal of Molecular Biology **127**(2): 219-223.
- Twell, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.
- Vila-Perello, M. and D. Andreu (2005). "Characterization and structural role of disulfide bonds in a highly knotted thionin from *Pyrularia pubera*." Biopolymers **80**(5): 697-707.
- von Besser, K., A. C. Frank, et al. (2006). "*Arabidopsis* HAP2(GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of *Rhododendron* Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.
- Wojnarowski, J. M. and J. Konopa (1980). "Interaction between DNA and Viscotoxins - Cyto-Toxic Basic Polypeptides from *Viscum-Album* L."

- Hoppe-Seylers Zeitschrift Fur Physiologische Chemie **361**(10): 1535-1545.
- Zhou, P., K. A. T. Silverstein, et al. (2013). "Detecting small plant peptides using SPADA (Small Peptide Alignment Discovery Application)." Bmc Bioinformatics **14**.
- Zhu, S., B. Gao, et al. (2005). "Phylogenetic distribution, functional epitopes and evolution of the CS alpha beta superfamily." Cellular and Molecular Life Sciences **62**(19-20): 2257-2269.

Chapter 7:

A novel role for Self-incompatibility protein S1 homologs in pollen tube reception.

Alexander R. Leydon, Mark A. Johnson

I performed all the experiments in this study.

Abstract

Pollen tube-pistil interactions determine the success of reproduction in flowering plants, blocking both inter-species and self-incompatible reproduction. Pollen tube delivery of sperm can be regulated by the pistil at nearly every stage of pollen tube growth, including pollen tube reception in the ovule. The molecular mechanisms that control pollen tube reception, especially pollen expressed genes, are not well understood. Recent discovery that a trio of pollen tube-specific MYB transcription factors is required for normal pollen tube reception has uncovered pollen tube genes that may create pollen tube identity during reception by the female gametophyte. Here we define a role for small secreted proteins related to Self-incompatibility S1 genes from *P. rhoeas* known as S-protein homologs (SPH), in pollen tube reception. A subset of SPH genes requires *MYB97*, *MYB101*, and *MYB120* for expression in the pollen tube. Reintroduction of one SPH protein into *myb* triple-mutant is sufficient to partially restore pollen tube burst and seed set, suggesting that at least one SPH gene functions in pollen tube reception.

Introduction

In flowering plant species, pistil compatibility with self-pollen is controlled by Self-Incompatibility systems (SI). SI acts as an important genetic system in hermaphroditic Angiosperms that limits the success of self-pollination in favor of out-crossing, in order to maximize genetic diversity. SI has long been considered an evolutionary advantage, in part due to the large distribution of self-incompatible species, and the negative impact of genetic inbreeding in self-compatibility (SC) systems. These factors have led to the interpretation that self-compatible systems are an evolutionary dead end (SEDE hypothesis, Lande et al. 1985), and that SC systems result exclusively from breakdown in SI species, as has been observed for the model plant *Arabidopsis* where the pollen SI component SCR has been functionally lost and nearly fixed at the population level (Tsuchimatsu et al. 2010). However, the genetic benefit of SI is balanced by the observation of large numbers of self-compatible species, which may be favored when pollinator systems break down (Darwin 1878; Goodwillie et al. 2005). Furthermore, self-compatible plants gain an outcrossing advantage in relative transmission when they can self-pollinate and outcross simultaneously (Goodwillie et al. 2005). Therefore both SI and SC comprise relevant reproductive modalities in the evolution of plant reproduction.

Self-incompatibility is not monolithic in its genetic makeup, with at least three genetic systems. The most evolutionarily widespread mechanism of SI utilizes a pistil expressed S-RNase and an S-locus specific F-box protein. S-RNase is

expressed at very high levels and secreted into the pistil tissues, then taken up by growing pollen tubes where it interacts with the F-Box protein. In the case of self-interaction, the S-RNase is specifically protected from proteolysis, allowing it to degrade pollen tube RNA (McClure et al. 1989; Lai et al. 2002). The molecular mechanism of the SI system in *Brassicaceae* has also been well characterized; it is controlled by *SCR* and *SRK*. *SCR* is the male determinant and *SRK* is the female determinant, both encoded by the S-locus (Takayama et al. 2005). *SCR* is a low molecular weight cysteine-rich pollen protein which decorates the pollen grain coat and acts as a ligand for *SRK*. The transmembrane Serine/Threonine kinase receptor *SRK* is expressed in the stigma and mediates pollen hydration and germination (Schopfer et al. 1999; Takasaki et al. 2000; Takayama et al. 2001).

The third SI mechanism understood in molecular detail was described in *Papaver rhoeas*, where a small secreted protein is produced in the stigma (*Papaver rhoeas* stigma S-protein - PrsS) and acts as the female SI determinant (Foote et al. 1994). PrsS proteins act as ligands for the pollen expressed transmembrane protein (*Papaver rhoeas* pollen S-protein – PrpS). Both components are encoded by the *Papaver* S-locus (Wheeler et al. 2009). Specific interaction of the pistil PrsS ligand with the pollen PrpS receptor triggers a signal transduction event that induces programmed cell death of the self-pollen tube (Foote et al. 1994). The mechanisms controlling the programmed cell death (PCD) of the pollen tube have been extensively characterized due to the ease of in vitro manipulation of

the SI response in *Papaver* pollen tubes. Primary response to PrsS-PrpS interaction in incompatible pollen tubes is a rapid increase in cytosolic free Ca^{2+} (Calder et al. 1997) and K^{+} (Wu et al. 2011), indicating activation of nonspecific cation channels. Subsequent increases in reactive oxygen species and pollen tube acidification follow sequentially (Wilkins et al. 2011; Wilkins et al. 2015). F-Actin depolymerization soon follows (Geitmann et al. 2000) through the calcium-dependent activities of Profilin and PrABP80, a putative gelsolin (Huang et al. 2004). Co-localization of actin depolymerizing factor (ADF/cofilin) and cyclase-associated protein (CAP) with the depolymerized F-actin foci are thought to positively influence subsequent PCD. SI triggers the activation of a MAPK, p56 (Rudd et al. 1996), and the phosphorylation of two inorganic pyrophosphatases, Pr-p26.1a/b, (de Graaf et al. 2006). Downstream activation of caspase-3-like/DEVDase and caspase-6-like/VEIDase activity likely depends on activation of p56 (Bosch et al. 2008; Li et al. 2011), and results in pollen tube PCD and DNA fragmentation (Jordan et al. 1999; Wilkins et al. 2014).

Pollen tube perception of the stigma PrsS protein is specifically mediated by PrpS, and PrpS can be introduced to *Arabidopsis thaliana*, where it retains SI activity (de Graaf et al. 2012). The ability to transfer *Papaver* SI to *Arabidopsis* indicates that the signaling machinery that interacts with PrpS is present and functional in the *Arabidopsis* pollen tube. However, the pollen S-protein, the receptor for PrsS, is unique and has no sequence homology to any proteins in the *Arabidopsis* genome (Wheeler et al. 2009; Wheeler et al. 2010). Therefore,

the specific recognition of PrsS proteins by PrpS proteins does not implicate a conserved transmembrane receptor family as the putative receptors for *Arabidopsis* SPHs (PrsS homologs). One additional poppy protein has been observed to bind PrsS proteins in an isoform-nonspecific manner, termed the S protein binding protein (SBP) (Hearn et al. 1996; Jordan et al. 1999). SBP is expressed in the pollen tube membrane, and is hypothesized to act as an accessory receptor for PrsS proteins, however the gene remains to be cloned and phylogenetically characterized. The roles of SPH proteins outside of *Papaver* SI systems remain to be discovered, as do their molecular mode of signal perception.

Like SI, pollen-pistil interactions in self-compatible pollinations involve recognition and discrimination of self and non-self as has been observed for the breakdown of pollination in inter-species crosses (Chen et al. 1972; Williams et al. 1986; Escobar-Restrepo et al. 2007). Breakdown in pollen tube-pistil interaction in exotic crosses to self-compatible species has been observed in every step of pollination, including at pollen tube reception, the final stage of gametophyte-gametophyte interaction (Leydon et al. 2014). During pollen tube reception, the pollen tube grows into the ovule where it interacts with the receptive synergid prompting dynamic fluctuations of intracellular calcium concentrations in both pollen tube and synergid cells (Iwano et al. 2012; Hamamura et al. 2014; Ngo et al. 2014). If arriving pollen tubes are compatible, such as intra-species pollen in the self-compatible *Arabidopsis thaliana*, the receptive synergid undergoes cell

death. Synergid cell death prompts the pollen tube to re-initiate extension followed by pollen tube burst (Iwano et al. 2012). The cell death of the receptive synergid and the pollen tube are near simultaneous during wild type reception, and each is required to deliver sperm to female gametes for fertilization. The signaling pathway that coordinates these tightly regulated cell death events is unknown.

Arabidopsis pollen tubes that do not express three MYB transcription factors, *MYB97*, *MYB101*, and *MYB120*, fail to activate gene expression and differentiate properly, resulting in pollen tube coiling in the female gametophyte (Thesis Chapters 2&4, Leydon et al. 2013; Leydon et al. 2014). Here we define a novel role for S-protein homologs (SPH) in *Arabidopsis* pollen tube reception. 15 MYB-regulated SPH genes were identified through SNP-informed RNA-sequencing (Thesis Chapter 5). SPH proteins are conceptually attractive as signaling components for signal transduction in pollen tube reception, which culminates in pollen tube and synergid cell death. In the *Papaver* SI system, they are secreted proteins that act in a haplotype-specific manner to induce death of cells carrying the cognate receptor. SPH genes are found throughout the flowering plants, and even as basally as *Physcomitrella patens*. The MYB-regulated SPH protein encoded by *AT3G26870* is localized to the pollen tube endomembrane system and partially restores synergid degeneration, pollen tube burst, and seed production when expressed in *myb* triple-mutant pollen tubes. This is the only MYB-regulated gene tested so far (n = 12) that increases fertility when expressed

in *myb* mutant pollen tubes. Our results define a function for SPH proteins outside of the *Papaver* SI system, and suggest that these proteins may be involved in reproductive signaling during gametophyte interactions.

Results

Multiple *Arabidopsis* self-incompatibility S1-like proteins are MYB-regulated

The MYB transcription factors *MYB97*, *MYB101*, and *MYB120* coordinate expression of a large number of pollen tube genes that are required for proper pollen tube differentiation and successful reception (Leydon et al. 2013; Liang et al. 2013). MYB-regulated genes fall in many gene categories, notably small secreted proteins such as the Thionin proteins (Thesis Chapter 6). However, an additional group of small secreted proteins was also enriched within the MYB-regulated transcriptome, which contain a conserved amino acid sequence that places them in the self-incompatibility S1-like family (PFAM05938) (Foote et al. 1994). These genes were therefore named the S-Protein Homologs (SPH) in *Arabidopsis* (Ride et al. 1999). *Arabidopsis* genome sequencing revealed a large expansion of SPH genes, however they are not found at loci with transmembrane proteins homologous to the pollen expressed S-protein receptor (PrpS), as PrpS is in *Papaver* (Wheeler et al. 2009). In fact, no homologs to the PrpS protein are found in the *Arabidopsis* genome. The *Arabidopsis* genome encodes 64 predicted SPH genes, and in RNA-sequencing of pollinated pistils we detected expression of 53 separate SPH genes (Figure 1). 15 SPH genes require *MYB97*, *MYB101*, and *MYB120* for expression (compare Cvi x Col to Cvi x *myb*, Figure 1B, asterisks).

A At-SPH

B

Cvi Cvi Cvi Col Cvi
Unpoll x Col x *myb* x Col x Cvi

C

Cvi x Col
Col- Cvi-
Pollen Pistil

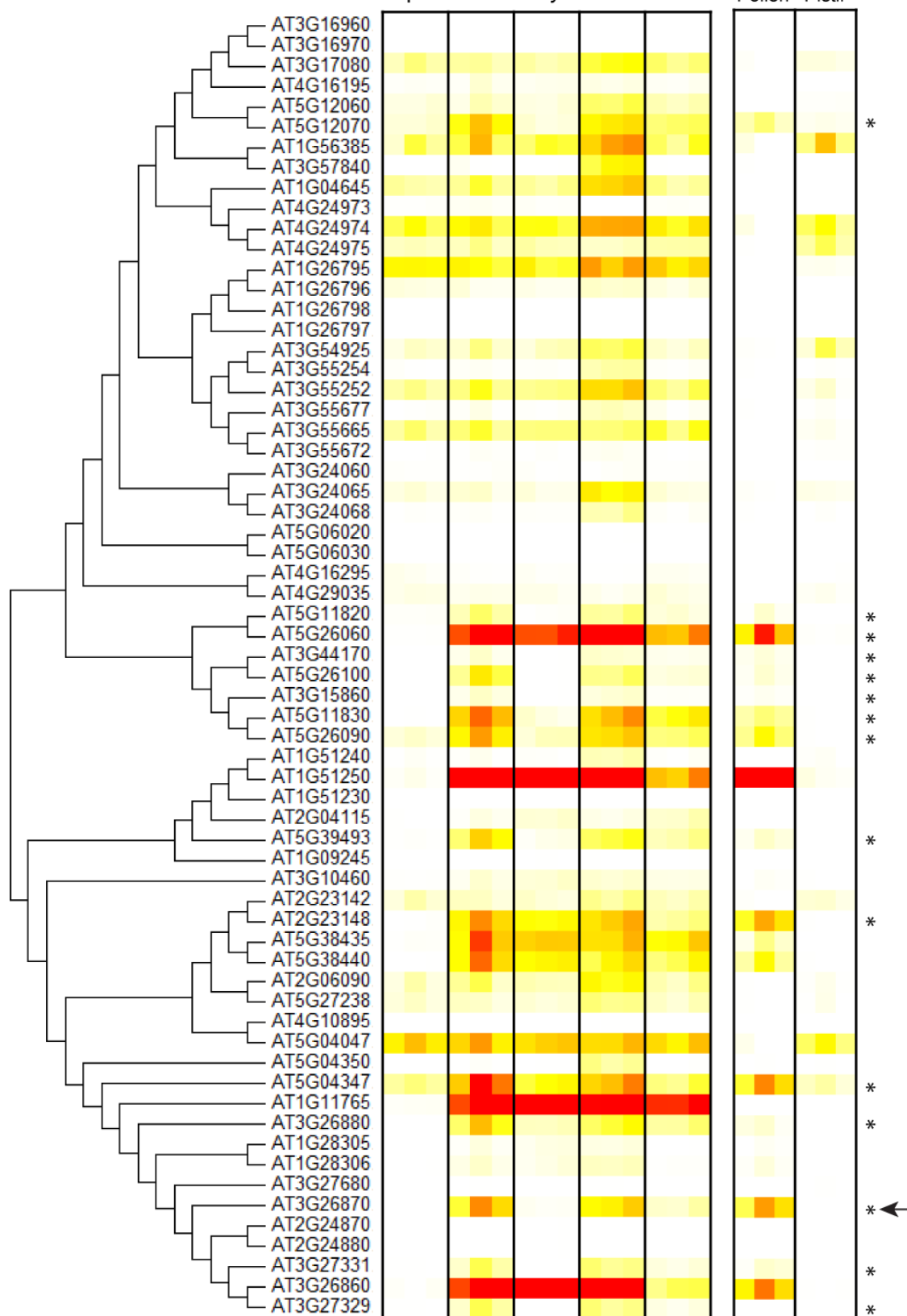


Figure 1. MYB transcription factors control expression of S protein homologs (SPH). **A.** A gene tree of the AtSPH protein coding sequences (Neighbor-joining, jukes-cantor, bootstrap values are at nodes of tree). **B.** Expression heatmap of the SPH transcripts in pistils. Cvi Unpoll – Unpollinated Cvi pistils (mock pollinated 8 hours), Cvi x Col – Cvi pistils pollinated with Col pollen for 8 hours, Cvi x *myb* – Cvi pistils pollinated with *myb* triple-mutant pollen for 8 hours, Col x Col – Col pistils pollinated with Col pollen for 8 hours, Cvi x Cvi – Cvi pistils pollinated with Cvi pollen for 8 hours. **C.** SNP-dissected reads from inter-ecotype crosses (Cvi x Col). Col reads correspond to pollen expressed transcripts and Cvi reads correspond to pistil expressed transcripts. Sequencing data is shown in three columns per condition, one column per biological replicate (Thesis Chapter 5). Asterisks indicate significant gene expression differences between Cvi x Col and Cvi x *myb* triple-mutant pollinations (adjusted p-value <0.05). Arrow indicates SPH gene identified previously by microarray (Leydon et al. 2013) and investigated by expression analysis (Figures 4-6).

Unlike some families of small secreted proteins such as the Thionins, which are expressed in the Columbia (Col) ecotype but not the Cape Verde Island (Cvi) ecotype (Thesis Chapter 6), the SPH genes are expressed in pollinated pistils from both ecotypes (compare Col x Col to Cvi x Cvi, Figure 1). SNP-informed dissection of RNA-sequencing data allowed us to confidently identify whether SPH transcripts were produced in pollen tubes or pistil tissues. SNPs are present between Col/Cvi genomes in 53/64 SPH genes, and 33/53 SPH genes that are expressed in pollinated pistils. SNP-informed sequencing identified 18/53 expressed SPH genes with assignable reads to the pollen tube (Col-pollen, Figure 1C), and 16/53 SPH genes with assignable reads to the pistil (Cvi-pistil, Figure 1C). The MYB-regulated SPH genes are not strictly phylogenetically isolated into one subclade of the SPH family tree.

Analysis of the SPH gene family in *Arabidopsis thaliana* and *lyrata* reveals one-to-one gene orthology and a lack of recent expansion

The defensin-like LURE genes that control species-specific pollen tube guidance have undergone a lineage-specific expansion in *Arabidopsis thaliana* and *lyrata* (Takeuchi et al. 2012). Additionally, the Thionin proteins that promote synergid degeneration display evidence of lineage-specific gene expansion (Thesis Chapter 6). If SPH genes help to create species identity in pollen tubes, they may show similar signs of unique molecular evolution. Rapid gene expansion and gene turnover may provide signaling diversity as has been seen in defense genes from *Drosophila* (Sackton et al. 2007). To explore the phylogenetic relationship of SPH proteins within the *Arabidopsis* lineage we identified *Arabidopsis lyrata* homologs of AtSPHs and performed protein sequence alignments. Based upon phylogenetic analysis with *Arabidopsis lyrata*, two clades appear to be enriched for MYB-regulated SPH genes, which we termed group 1 and 2 (Figure 2). We examined the structure of the family tree and observed that most SPH genes have clear orthologs or ortholog pairs with *Arabidopsis lyrata*, indicating that SPH proteins are not expanded in a species-specific cluster as was observed for the Thionins (Thesis Chapter 6) or the defensin AtLURE proteins (Takeuchi et al. 2012). Therefore, phylogenetic analysis suggests that the SPH genes are unlikely to act as pollen tube factors that create species identity.

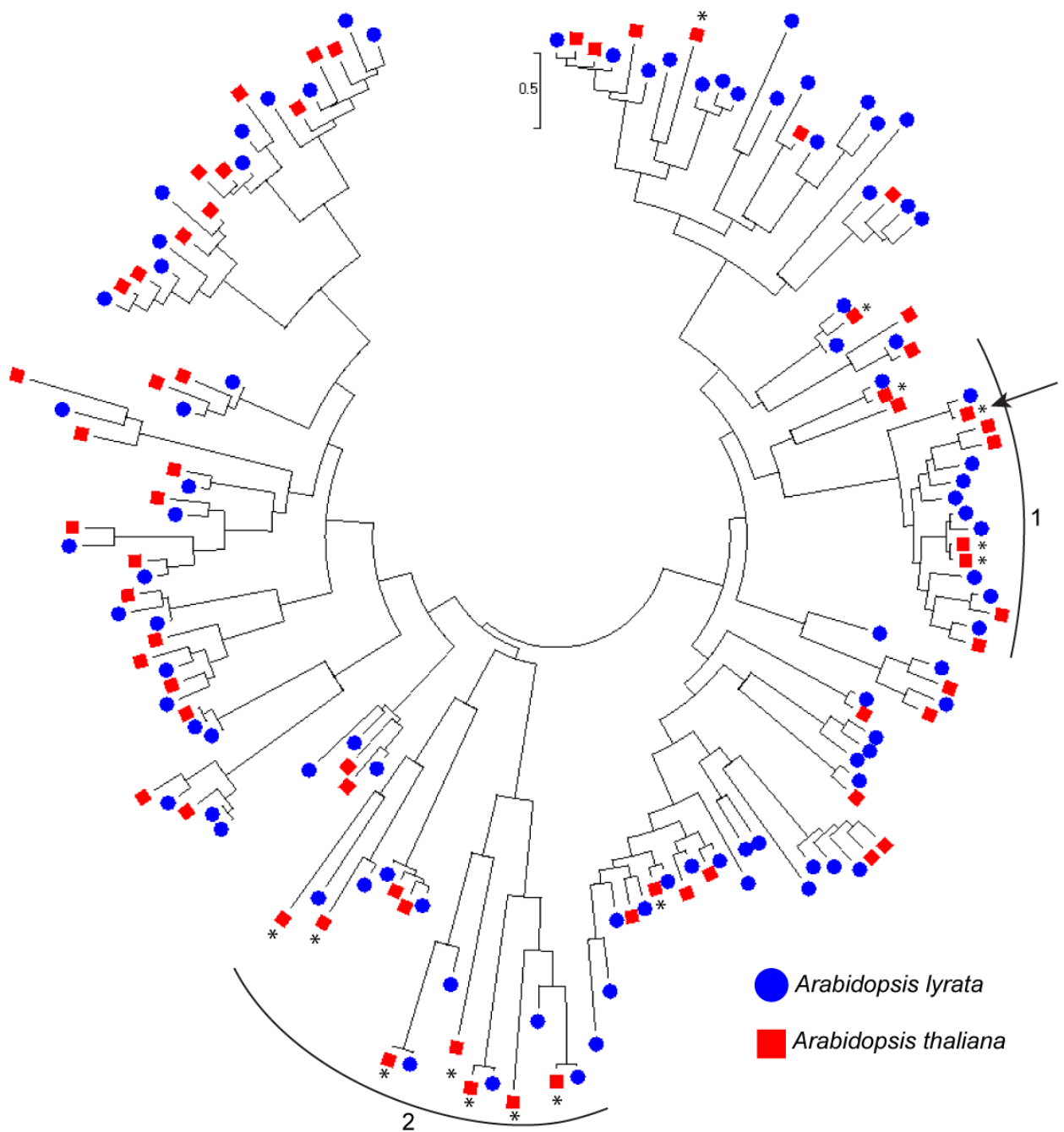


Figure 2. Molecular phylogenetic analysis of the SPH gene family.

The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (-28996.1071) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 154 amino acid sequences. There were a total of 433 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). The MYB-regulated SPH (AT3G26870) that was further tested for activity

is indicated with an arrow. Clades 1 and 2 are labelled, as they are slightly enriched for MYB-regulated SPH genes.

A single SHP is sufficient to partially restore *myb* triple-mutant fertility

SPH genes are short and localized to small genomic clusters (Figure 3), which makes loss of function analyses challenging. Furthermore, *myb* triple-mutant pollen tubes lack expression of many genes including SPHs, which results in a complex phenotype: reduced pollen tube guidance to ovules and a failure in pollen tube reception. In order to identify whether SPHs play a role in *myb*-regulated pollen tube processes we expressed the SPH gene (*AT3G26870*) in *myb* triple-mutant pollen tubes to determine whether it was sufficient to restore aspects of pollen tube reception. We used an expression strategy described previously (Thesis Chapter 6). Briefly, we cloned the cDNA of the MYB-regulated SPH gene (*AT3G26870*) for expression under the *BGAL11* promoter and introduced this construct into *myb* triple-mutant plants. 36 primary transformants were tested for expression of the SPH:GFP fusion protein in the *myb* triple-mutant, *LAT52:DsRed* background (Twell et al. 1989; Francis et al. 2007; Leydon et al. 2013).

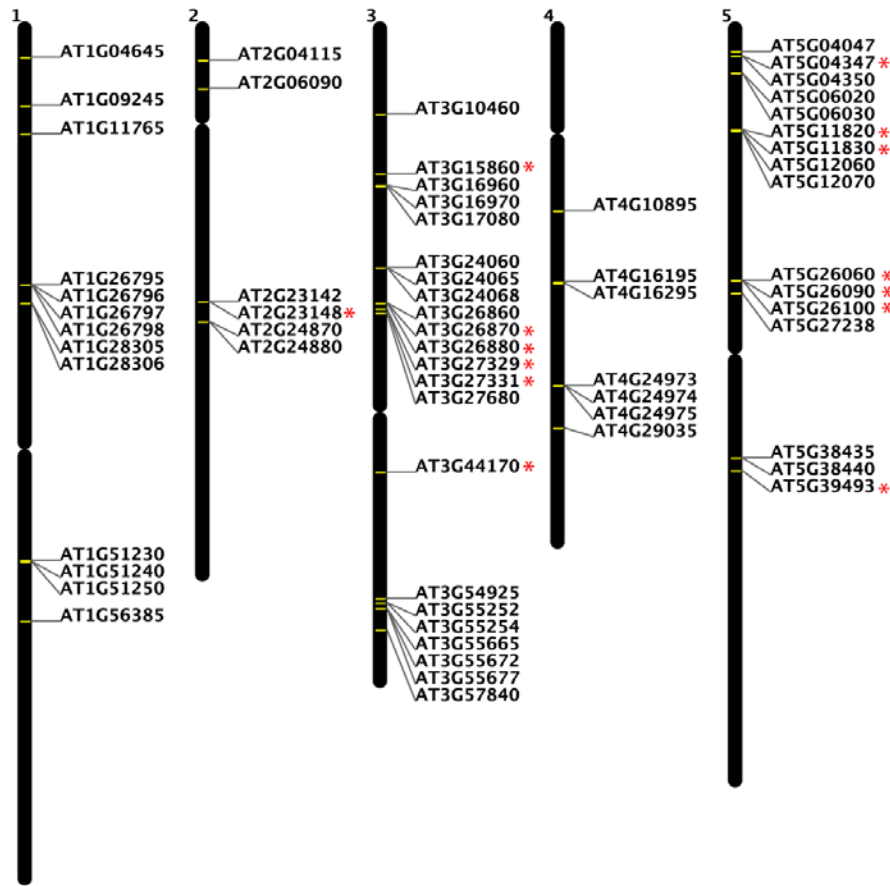


Figure 3. Genomic distribution of SPH genes. Red asterisks indicates MYB-regulated.

All primary transformants (24/24) had detectable SPH:GFP signal (Figure 5B) and three representative lines were chosen for analysis of pollen tube reception via live imaging. Our crossing method utilized pistils expressing *ACT11:MSI:GFP*, which allows visualization of synergid degeneration during reception (Ingouff et al. 2007; Leydon et al. 2013). *ACT11:MSI:GFP* pistils were emasculated 12 hours prior to pollination with pollen hemizygous for *BGAL11:SPH:GFP* constructs. We then dissected and imaged pistils by confocal microscopy to quantify pollen tube targeting and pollen tube reception within the ovule.

We observed significant increases in the frequency of ovules that contained a burst pollen tube compared to the *myb* triple-mutant background (Figure 4, dark blue bars). T₂ homozygous pollen exhibited similar increases in pollen tube burst, with an average pollen tube burst rate of 30%, a 3-fold increase over *myb* triple-mutants. Furthermore, we observed increased rates of synergid degeneration in ovules that had received a coiling pollen tube (Figure 4, green, blue, and black bars).

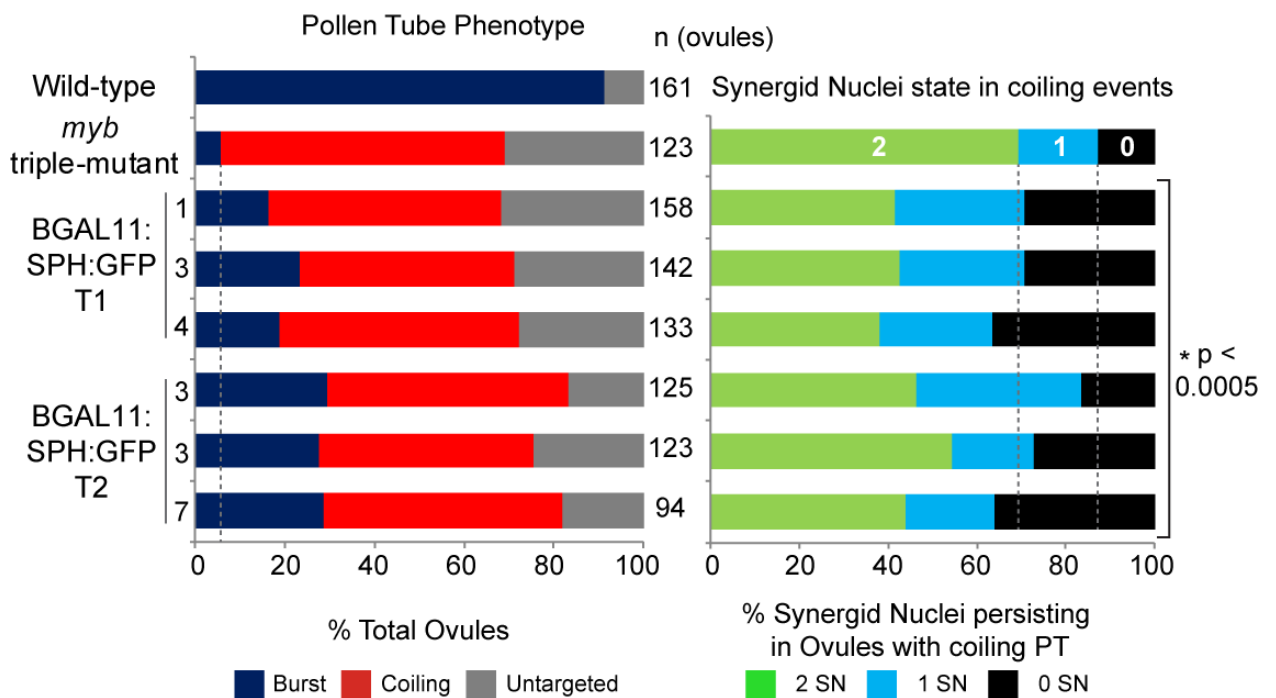


Figure 4. Pollen tube phenotypes 12 hours after pollination. *ACT11:MSI1:GFP* pistils were emasculated and crossed with *myb* triple-mutant pollen for 12 hours before dissection and quantification by confocal microscopy. Data on the right are paired with the red “Coiling” fraction shown on the left. Synergid numbers are indicated by position and by color, from left to right, Green – 2 synergid nuclei, Blue – 1 synergid nuclei, Black – 0 synergid nuclei. Data are shown for three T1 lines that were tested for expression of SPH:GFP. n (ovules) – refers to total number of ovules, 3 pistils per experiment. 1-3 – indicate three independent transformant lines, with SPH:GFP expression. Three T2 plants homozygous for SPH:GFP were chosen to repeat the reception microscopy experiments. P-value <0.0005 – chi-squared test.

Expression of the *AT3G26870* coding sequence partially rescued the *myb* triple-mutant coiling phenotype. We still observed pollen tube coiling in pollinations of *ACT11:MSI1:GFP*, yet at reduced levels (Figure 4, red bars; Figure 5E,I). When *BGAL11:SPH:GFP* pollen tubes burst, they appeared morphologically similar to wild type pollen tube burst (Figure 5F,G,H), however it is uncertain whether the kinetics of pollen tube reception was altered. In order to visualize whether fertilization was likely to follow *BGAL11:SPH:GFP* pollen tube burst normally, we used the reporter line *FGR7.0* as a pistil parent. *FGR7.0* combines cell-specific marker genes for synergids, egg cell, and central cell (Volz et al. 2013). We could clearly observe early divisions of the endosperm (Figure 5K-M), indicating that *myb* triple-mutant pollen tubes expressing *BGAL11:SPH:GFP* burst, and release sperm for double fertilization and subsequent seed development.

Live imaging of pollen tube reception demonstrated that the SPH *AT3G26870* partially rescues the *myb* triple-mutant pollen tube coiling defect, allowing pollen tube burst and sperm release. In order to test whether expression of SPH *AT3G26870* results in increased fertility, we performed crosses to examine seed set. We quantified seed set in self-pollinated siliques and observed increased seed set in 5/8 T1 hemizygous lines (Figure 6A). Generation of T2 homozygous lines demonstrated that both self-pollinations and male crosses to *male sterile one (ms1)*, produced an intermediate seed set compared to wild type (Figure 6B).

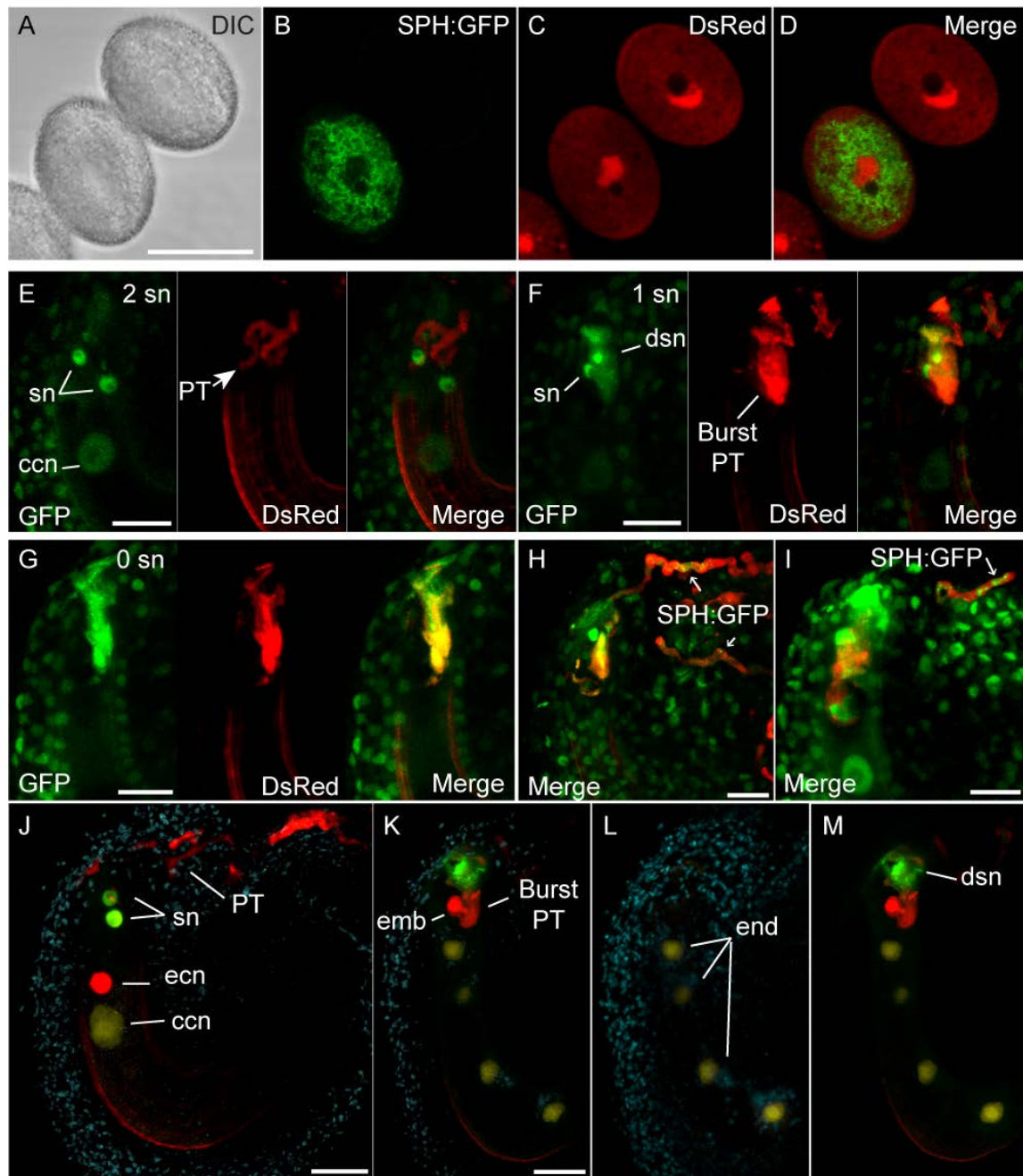


Figure 5. SPH:GFP rescues pollen tube burst and fertilization A-D. *BGAL11:SPH:GFP* (AT3G26870) expression in *myb* triple-mutant, *LAT52:DsRed* mature pollen. Confocal micrograph, scale bar – 20um. A.DIC, B. GFP, C. DsRed, D. Merge. E. Synergids persisting in a *ACT11:MSI1:GFP* ovule that has received a *myb* triple-mutant, *LAT52:DsRed* pollen tube, scale bar – 20um. F. One persisting synergid is partially obscured by the mixed cytoplasm of the degenerated synergid and the burst pollen tube in an *ACT11:MSI1:GFP* ovule that has been targeted by an *SPH:GFP* pollen tube. G. No synergids persisting in an ovule where an *SPH:GFP* pollen tube has burst. H. SPH:GFP is visible in the shank of a pollen tube that has burst into an ovule (arrows) and in I, where the

pollen tube is coiling. **J-M.** FGR7.0 pistils were emasculated before pollination for 12 hours with *BGAL11:SPH:GFP*, *myb* triple-mutant, *LAT52:DsRed* pollen. YFP – central cell nucleus, RFP – egg cell nucleus, GFP – synergid nuclei, DsRed – pollen tube. **J.** An FGR ovule that has received a pollen tube that has begun to coil (red overlapping synergid nuclei is pollen tube tip). **K-M.** FGR ovule that received SPH:GFP pollen tube that burst (red swoosh), and has undergone endosperm divisions characteristic of early development. **K.** Full merge, **L.** Endosperm and plastid only. **M.** Endosperm, Embryo, Synergids, and pollen tube merge.

This indicates that *AT3G26870* is not fully sufficient to rescue the entire *myb* triple-mutant phenotype and works together with as yet unknown MYB-regulated genes to promote pollen tube reception. There are many MYB-regulated candidate genes (>300) that could co-operate with *AT3G26870*, such as other SPH genes or Thionins (Thesis Chapter 6), however it is quite surprising that any one MYB-regulated gene is capable of rescuing pollen tube burst at all. For this reason the SPH gene *AT3G26870* represents an exciting lead in the search for pollen tube-specific effectors of pollen tube reception.

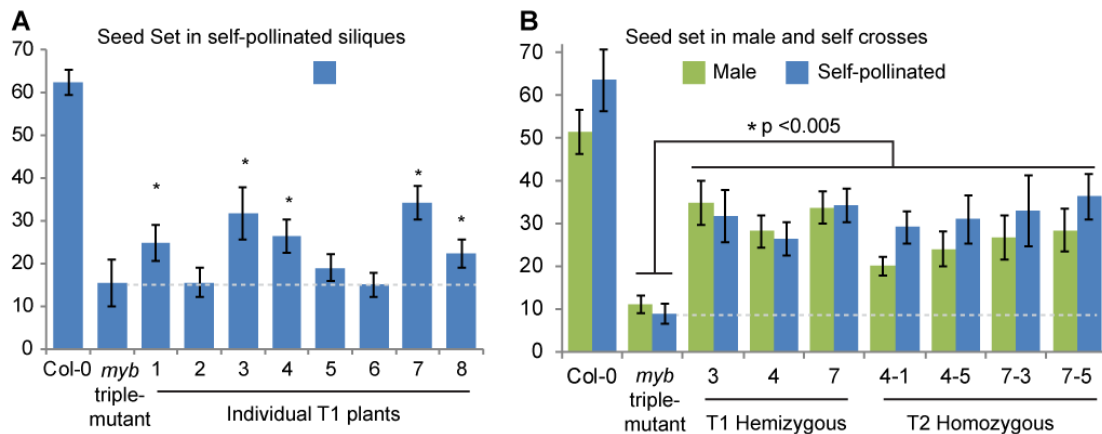


Figure 6. SPH:GFP add back partially rescues *myb* triple-mutant seed set defects. **A.** Mean seed set from individual hemizygous T1 plants +/- stdev. Each data point is the average of at least 10 siliques. **B.** Mean seed set in male and self-crosses. Male crosses were performed manually onto *ms1* pistils and are mean of at least 6 crosses +/- stdev.

Toward structure-function analysis of MYB-regulated SPH proteins

Sequence analysis of the *Arabidopsis* SPH and *Papaver* S proteins identifies the conservation of key residues including two of the four cysteines that are conserved within *Papaver* S proteins (Walker et al. 1996). Based on this observation, it was previously hypothesized that the two most highly conserved cysteine residues (PrsS1-C24 and C57) form a disulfide bond linkage (Kakeda et al. 1998). PrsS1 protein has been successfully expressed in *E. coli* for use in pollen inhibition bioassays (Foote et al. 1994). Heterologous expression of S1 from *E. coli* required refolding for activity, indicating that disulfide bond formation is critical to stabilize the signaling competent S1 protein. *Arabidopsis* SPH proteins within clade 1 display high levels of sequence divergence in their C-terminal region, as well as interspersed divergent residues (Figure 7) as was observed for S protein variation within *Papaver* (Kurup et al. 1998). Secondary structure predictions are congruent between SPH and PrsS1 amino acid sequences, suggesting that the protein structure is comprised of 5-6 beta sheets with hydrophilic interlinking surface loops (Kakeda et al. 1998). Mutagenesis of residues in PrsS1 loop 6 (RDDD) ablates SI activity of S1, however these residues are not conserved in AT3G26870 (Figure 7) (Kakeda et al. 1998). Lack of loop 6 conservation suggest that there is either more than one critical hydrophilic loop, or that AtSPH proteins utilize an alternate region for interface with their receptor.

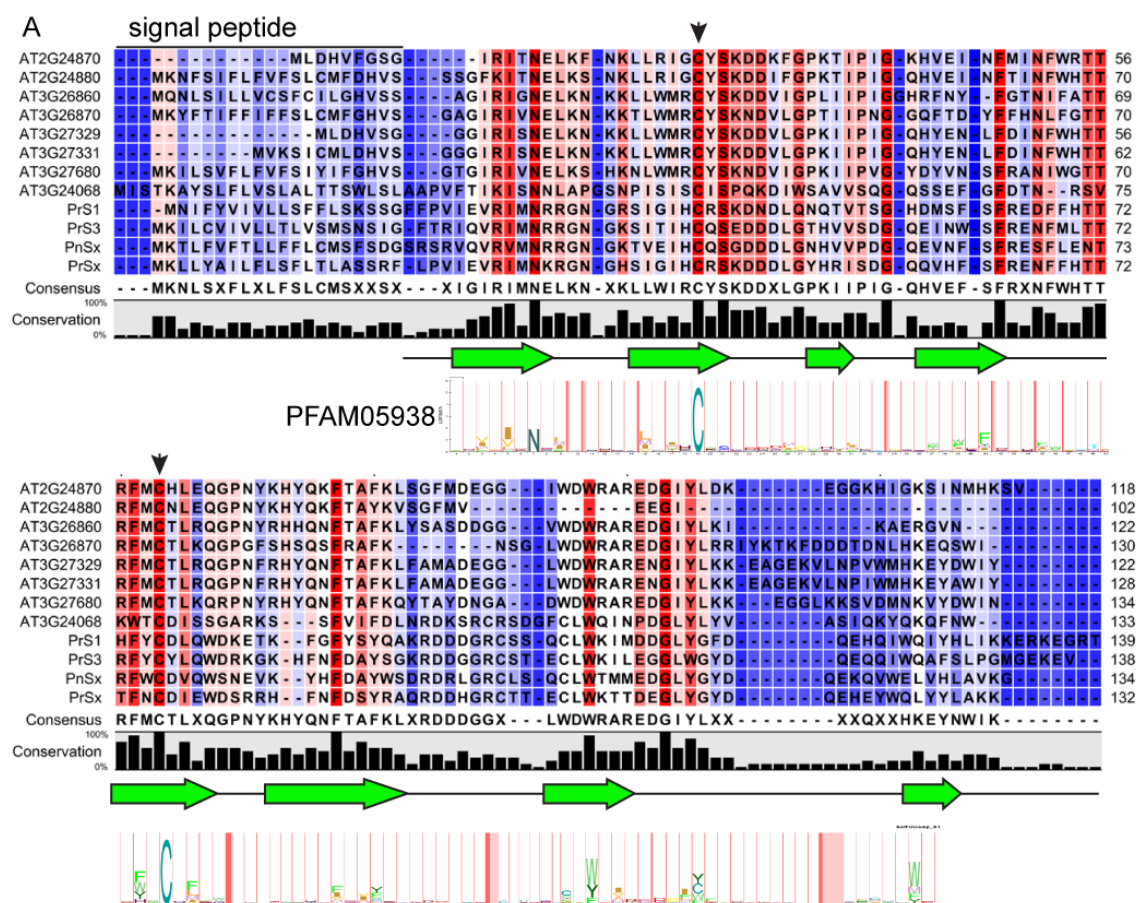


Figure 7. Amino acid sequence of AtSPH and Papaver S proteins reveals regions of conservation. A. The amino acid sequence from AtSPH genes in clade 1, and AT3G24068 (closest sequence match to PrsS1), were aligned to PrsS1, PrsS2, PrsS3, and PnS. Conservation is highlighted by the background color of the sequence, and the conservation percent is graphed below. Predicted secondary structure of PrsS1 is shown below conservation graph, green arrows indicate predicted beta sheet structure (Cole et al. 2008).

In order to test the biological activity of the SPH protein *AT3G26870* on ovules and pollen tubes, we chose to heterologously express *AT3G26870*, using the methylotrophic yeast *Pichia pastoris*. Expression in *Pichia* is advantageous because SPH will transit a eukaryotic secretion pathway, which facilitates purification without the requirement for in vitro refolding and disulfide bond formation. We cloned the SPH *AT3G26870* into the pPICZalpha expression vector and recombined this methanol-inducible construct into wild type (X-33)

Pichia. The pPICZalpha vector fuses the alpha factor signal peptide to the coding sequence of AT3G26870 and incorporates a C-terminal Myc epitope tag and a 6X-Histidine sequence for nickel column purification (Figure 8A). Initial expression of transformants (Figure 8B) and time-course expression analysis (Figure 8C) indicates that the epitope-tagged SPH protein is induced and secreted properly in our expression system. Future analysis will focus on developing bioassays to test the activity of purified AT3G26870 protein towards promoting synergid degeneration and/or pollen tube burst.

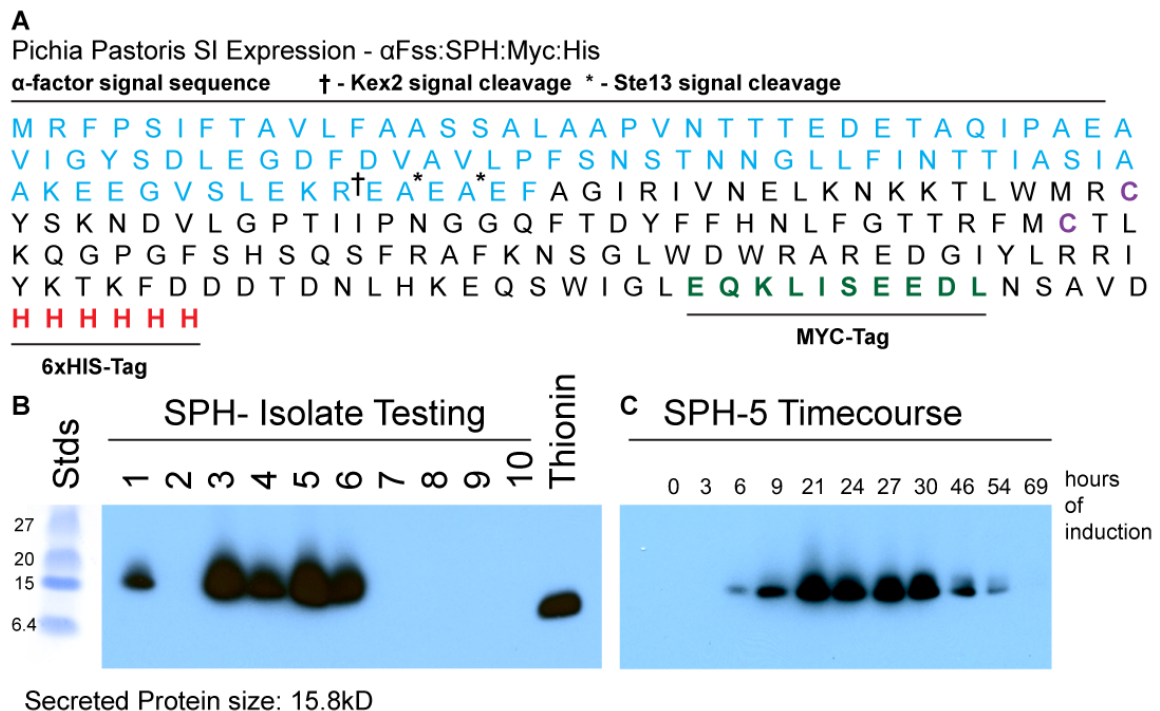


Figure 8. SPH protein expression in *P. pastoris*. **A.** Sequence map of the SS-SPH-Myc-His construct (using the coding sequence of AT3G26870). The alpha factor signal sequence is shown in light blue. The signal sequence cleavage sites are shown with enzyme specific symbols for Kex2, and Ste13. The SPH coding sequence is colored black with the 2 conserved cysteines colored purple. The Myc epitope tag is shown in Green, and the 6xHIS tag is shown in Red. **B.** Isolate testing media from 10 representative isolates (1-10) and a Thionin-Myc-His control. **C.** Time-course expression of isolate SPH-5 to identify the optimum time-point for protein purification.

Discussion

SI systems are widely employed genetic systems that allow control over outcrossing to ensure maximal diversity within a population. The *Papaver* SI system represents a model SI system incorporating a small secreted identity determinant (PrsS) that interacts with its cognate transmembrane receptor (PrpS) to induce programmed cell death. Synergid degeneration follows a cell:cell interaction with the pollen tube, and is thought to require pollen-tube secreted signaling components that influence the calcium oscillations preceding wild-type pollen tube reception and synergid cell death (Iwano et al. 2012; Ngo et al. 2014). Therefore it is attractive to hypothesize that the MYB-regulated SPH proteins are acting in a manner similar to their *Papaver* homologs: interacting with synergid-specific transmembrane receptors to induce synergid cell death. To this end, we have purified heterologously produced SPH proteins in order to perform bioassays to identify the signaling competency of AtSPH AT3G26870. Additionally, purified SPH facilitates biochemical approaches to identifying the SPH receptor(s).

While it is unknown what the receptors for AtSPH proteins are, it is possible that another class of transmembrane receptors that do not display sequence similarity to PrpS is involved in binding SPH genes. It will be critical to mine expression databases to identify the transmembrane proteins that are synergid expressed and whether any possess similar predicted membrane topologies to the *Papaver* pollen S determinant (PrpS) (Wheeler et al. 2009). Alternatively, the identity of

the *Arabidopsis* SPH receptor may be a homolog to the *PrSBP* membrane glycoprotein, which has not yet been cloned and characterized (Kurup et al. 1998; Jordan et al. 1999). If purified SPH protein demonstrates biological activity, it would be possible to design experiments to biochemically identify synergid-expressed SPH binding proteins.

We define here the first single gene that is capable of restoring *myb* triple-mutant fertility. Furthermore, we report the first biological role for an individual SPH gene, *AT3G26870*. Phylogenetic analysis indicates that *AT3G26870* has a clear homolog in the closely related *Arabidopsis lyrata*, and retains conserved amino acid sequences that are suggested to be structurally important for tertiary structure (Kakeda et al. 1998). *AT3G26870* may perform a conserved, as opposed to species-specific, function during pollen tube reception. Initial reports identified SPH genes and observed gene expression in reproductive tissues (Ride et al. 1999), and suggested possible roles in reproduction. Our initial findings support a role for *AT3G26870* in promoting pollen tube burst during pollen tube reception. Given the role of PrsS proteins as ligands in Papaver SI systems, it is exciting to hypothesize that SPH *AT3G26870* is secreted from the pollen tube to the receptive synergid during reception. Could *AT3G26870* bind a synergid receptor and activate a signal transduction cascade that allows synergids to induce pollen tube rupture?

Three synergid expressed genes, *LORELEI* (*LRE*) (Capron et al. 2008), *NORTIA* (*NTA*) (Kessler et al. 2010), *FERONIA* (*FER*) (Escobar-Restrepo et al. 2007) are required for pollen tube reception: mutants in these result in pollen tube coiling. *NTA* requires *FER* for localization to the point of pollen tube interaction with the synergid (Kessler et al. 2010). During reception, pollen tube arrival and contact with the synergid (phase I) induces oscillations in synergid intracellular calcium concentrations, which requires *FER* (Ngo et al. 2014). After a period of interaction wherein *NTA* is presumably secreted to the synergid membrane, the pollen tube begins to grow rapidly and receptive synergid calcium levels are uniformly elevated (phase II), which requires *NTA* (Ngo et al. 2014). SPH AT3G26870 may act at either of these two stages before pollen tube burst (stage III). However, *myb* triple-mutant pollen tubes were found to activate *FER*-mediated signaling normally: phase I calcium oscillations are normal in ovules targeted by *myb* triple-mutant pollen tubes (Unpublished data, Q. Ngo, personal communication). Second, we observed *NTA*:GFP localization in response to *myb* triple-mutant pollen tube arrival at synergids. This indicates that *FER*-dependent *NTA* localization does not require MYB-regulated genes to be present in the pollen tube (Thesis Appendix 6). We therefore hypothesize that the potential SPH receptor acts during phase II of pollen tube reception. The SPH receptor may be re-localized to the synergid membrane with *NTA* to effect pollen tube burst.

Future experiments will focus on the biological activity of other MYB-regulated SPH genes in clade 1 and clade 2, and their contributions during pollen tube reception. Additionally, purified SPH protein will allow development of biochemical methods to identify SPH receptors from synergid tissue.

Methods

Plant growth

Procedures for selection of antibiotic and/or herbicide resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; von Besser et al. 2006; Qin et al. 2009; Wong et al. 2010).

Cloning

For cDNA cloning RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis was performed with Superscript III (Invitrogen), and PCR used Phusion Polymerase (NEB). cDNA sequences were cloned into entry vectors pENTR-D or pCR8 (Invitrogen), and LR cloned into destination vectors with LR clonase II (Invitrogen). All clones were sequenced before introduction to plants (Genewiz).

Phylogenetic analysis

Phylogenetic relationships were analyzed using MEGA software (Tamura et al. 2013). Heat trees were constructed from sequencing data (Thesis Chapter 5)

Reception Microscopy

For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated *ACT11:MSI1:GFP*, *FGR7.0* stage 14 stigmas (Smyth et al. 1990) with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al.

2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

Heterologous expression in *P. pastoris*

Pichia expression clones, wild-type and control strains were procured from Invitrogen. SPH cDNA was cloned into pPICZ alpha as an EcoRI-Thionin-XbaI fragment from a TA cloned intermediate (pCR2.1, Invitrogen). Plasmids were midi-prepped, linearized with SacI, ethanol precipitated and concentrated. Wild-type X-33 *Pichia* was made electrocompetent, and 5-10ug of linearized DNA was transformed into 80ul of competent cells. Transformants were identified by outgrowth on YPDS+ Zeocin for 2-3 days, re-streaked onto YPD+Zeocin. Isolates were tested for the Mut phenotype, and Mut+ strains were selected for isolate testing in small cultures (25mls in 50ml conical tubes). Upscaled SPH-Myc-His purifications from isolate 5 were grown o/n in 50ml BMGY, before dilution to OD600 of 1 (~200ml) in BMMY and grown for 48 hours, spiking with MeOH at 24hours to maintain induction. Cultures were spun down (3,000rpm, 15min, 4C)

and supernatant was concentrated over a 3K Amicon column (Millipore), and diluted with 7mls of Native binding buffer (50mM NaH₂PO₄, 500mM NaCl, 10mM imidazole, pH8.0) before nickel column purification (as per ProBond manual – Invitrogen) and analysis by SDS-PAGE, Western blotting or subsequent experimentation.

References

- Bosch, M. and V. E. Franklin-Tong (2008). "Self-incompatibility in Papaver: signalling to trigger PCD in incompatible pollen." Journal of Experimental Botany **59**(3): 481-490.
- Calder, G. M., V. E. Franklin-Tong, et al. (1997). "Ca²⁺ oscillations in plant cells: initiation by rapid elevation in cytosolic free Ca²⁺ levels." Biochem Biophys Res Commun **234**(3): 690-694.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Chen, C. C. and P. B. Gibson (1972). "Barriers to Hybridization of Trifolium-Repens with Related Species." Canadian Journal of Genetics and Cytology **14**(2): 381-+.
- Cole, C., J. D. Barber, et al. (2008). "The Jpred 3 secondary structure prediction server." Nucleic Acids Res **36**: W197-W201.
- Darwin, C. (1878). The effects of cross and self fertilisation in the vegetable kingdom. London,, J. Murray.
- de Graaf, B. H., J. J. Rudd, et al. (2006). "Self-incompatibility in Papaver targets soluble inorganic pyrophosphatases in pollen." Nature **444**(7118): 490-493.
- de Graaf, B. H., S. Vatovec, et al. (2012). "The Papaver self-incompatibility pollen S-determinant, PrpS, functions in Arabidopsis thaliana." Curr Biol **22**(2): 154-159.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Foot, H. C., J. P. Ride, et al. (1994). "Cloning and expression of a distinctive class of self-incompatibility (S) gene from Papaver rhoeas L." Proc Natl Acad Sci U S A **91**(6): 2265-2269.
- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Geitmann, A., B. N. Snowman, et al. (2000). "Alterations in the actin cytoskeleton of pollen tubes are induced by the self-incompatibility reaction in Papaver rhoeas." Plant Cell **12**(7): 1239-1251.
- Goodwillie, C., S. Kalisz, et al. (2005). "The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence." Annual Review of Ecology Evolution and Systematics **36**: 47-79.
- Hamamura, Y., M. Nishimaki, et al. (2014). "Live imaging of calcium spikes during double fertilization in Arabidopsis." Nat Commun **5**: 4722.
- Hearn, M. J., F. C. H. Franklin, et al. (1996). "Identification of a membrane glycoprotein in pollen of Papaver rhoeas which binds stigmatic self-incompatibility (S-) proteins." Plant Journal **9**(4): 467-475.

- Huang, S., L. Blanchoin, et al. (2004). "A gelsolin-like protein from *Papaver rhoeas* pollen (PrABP80) stimulates calcium-regulated severing and depolymerization of actin filaments." J Biol Chem **279**(22): 23364-23375.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Jones, D. T., W. R. Taylor, et al. (1992). "The rapid generation of mutation data matrices from protein sequences." Comput Appl Biosci **8**(3): 275-282.
- Jordan, N. D., K. Kakeda, et al. (1999). "S-protein mutants indicate a functional role for SBP in the self-incompatibility reaction of *Papaver rhoeas*." Plant J **20**(1): 119-125.
- Kakeda, K., N. D. Jordan, et al. (1998). "Identification of residues in a hydrophilic loop of the *Papaver rhoeas* S protein that play a crucial role in recognition of incompatible pollen." Plant Cell **10**(10): 1723-1732.
- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Kurup, S., J. P. Ride, et al. (1998). "Identification and cloning of related self-incompatibility S-genes in *Papaver rhoeas* and *Papaver nudicaule*." Sexual Plant Reproduction **11**(4): 192-198.
- Lai, Z., W. Ma, et al. (2002). "An F-box gene linked to the self-incompatibility (S) locus of *Antirrhinum* is expressed specifically in pollen and tapetum." Plant Mol Biol **50**(1): 29-42.
- Lande, R. and D. W. Schemske (1985). "The Evolution of Self-Fertilization and Inbreeding Depression in Plants .1. Genetic Models." Evolution **39**(1): 24-40.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Leydon, A. R., A. Chaibang, et al. (2014). "Interactions between pollen tube and pistil control pollen tube identity and sperm release in the Arabidopsis female gametophyte." Biochem Soc Trans **42**: 340-345.
- Li, S. and V. E. Franklin-Tong (2011). "Modulating and monitoring MAPK activity during programmed cell death in pollen." Methods Mol Biol **779**: 165-183.

- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 Function as Male Factors That Control Pollen Tube-Synergid Interaction in *Arabidopsis thaliana* Fertilization." PLoS Genet **9**(11).
- McClure, B. A., V. Haring, et al. (1989). "Style self-incompatibility gene products of *Nicotiana glauca* are ribonucleases." Nature **342**(6252): 955-957.
- Ngo, Q. A., H. Vogler, et al. (2014). "A calcium dialog mediated by the FERONIA signal transduction pathway controls plant sperm delivery." Developmental Cell **29**(4): 491-500.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Ride, J. P., E. M. Davies, et al. (1999). "Analysis of *Arabidopsis* genome sequence reveals a large new gene family in plants." Plant Mol Biol **39**(5): 927-932.
- Rudd, J. J., F. C. H. Franklin, et al. (1996). "Increased phosphorylation of a 26-kD pollen protein is induced by the self-incompatibility response in *Papaver rhoeas*." Plant Cell **8**: 713-724.
- Sackton, T. B., B. P. Lazzaro, et al. (2007). "Dynamic evolution of the innate immune system in *Drosophila*." Nat Genet **39**(12): 1461-1468.
- Schopfer, C. R., M. E. Nasrallah, et al. (1999). "The male determinant of self-incompatibility in *Brassica*." Science **286**(5445): 1697-1700.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in *Arabidopsis*." Plant Cell **2**(8): 755-767.
- Takasaki, T., K. Hatakeyama, et al. (2000). "The S receptor kinase determines self-incompatibility in *Brassica* stigma." Nature **403**(6772): 913-916.
- Takayama, S. and A. Isogai (2005). "Self-incompatibility in plants." Annu Rev Plant Biol **56**: 467-489.
- Takayama, S., H. Shimamoto, et al. (2001). "Direct ligand-receptor complex interaction controls *Brassica* self-incompatibility." Nature **413**(6855): 534-538.
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in *Arabidopsis*." PLoS Biol **10**(12): e1001449.
- Tamura, K., G. Stecher, et al. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol **30**(12): 2725-2729.
- Tsuchimatsu, T., K. Suwabe, et al. (2010). "Evolution of self-compatibility in *Arabidopsis* by a mutation in the male specificity gene." Nature **464**(7293): 1342-1346.
- Twiss, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.
- Volz, R., J. Heydlauff, et al. (2013). "Ethylene Signaling Is Required for Synergid Degeneration and the Establishment of a Pollen Tube Block." Developmental Cell **25**(3): 310-316.
- von Besser, K., A. C. Frank, et al. (2006). "*Arabidopsis* HAP2(GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.

- Walker, E. A., J. P. Ride, et al. (1996). "Molecular analysis of two functional homologues of the S3 allele of the *Papaver rhoeas* self-incompatibility gene isolated from different populations." Plant Mol Biol **30**(5): 983-994.
- Wheeler, M. J., B. H. de Graaf, et al. (2009). "Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*." Nature **459**(7249): 992-995.
- Wheeler, M. J., S. Vatovec, et al. (2010). "The pollen S-determinant in *Papaver*: comparisons with known plant receptors and protein ligand partners." Journal of Experimental Botany **61**(7): 2015-2025.
- Wilkins, K. A., J. Bancroft, et al. (2011). "Reactive oxygen species and nitric oxide mediate actin reorganization and programmed cell death in the self-incompatibility response of *papaver*." Plant Physiol **156**(1): 404-416.
- Wilkins, K. A., M. Bosch, et al. (2015). "Self-incompatibility-induced programmed cell death in field poppy pollen involves dramatic acidification of the incompatible pollen tube cytosol." Plant Physiol **167**(3): 766-779.
- Wilkins, K. A., N. S. Poulter, et al. (2014). "Taking one for the team: self-recognition and cell suicide in pollen." Journal of Experimental Botany **65**(5): 1331-1342.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of *Rhododendron* Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.
- Wu, J., S. Wang, et al. (2011). "Self-incompatibility in *Papaver rhoeas* activates nonspecific cation conductance permeable to Ca^{2+} and K^{+} ." Plant Physiol **155**(2): 963-973.

DISCUSSION

Chapter 8:

***MYB97, MYB101, and MYB120* produce the molecular pattern of the pollen tubes perceived by synergids during reception**

Pollen tube reception in flowering plants is required for seed production

Flowering plants have achieved evolutionary success, as can be observed from their incredible species diversity (Estimated at 250,000-400,000 species, Joppa et al. 2011). This diversity is in part due to the evolution of the flower, which houses the female gametes, and provides a microenvironment that promotes successful fertilization and seed development. Two sperm cells develop within the male gametophyte, or pollen grain, and are delivered to the female gametophyte, inside the ovule, by the pollen tube (Berger et al. 2011). Pollen tube growth in the pistil is coordinated by cell-cell interactions between pollen tube and pistil cells (Thesis Chapter 1). The pollen tube is a highly polarized cell, which grows through the complex pistil environment following a gradient of extracellular signals to the female gametophyte (Okuda et al. 2009; Takeuchi et al. 2011). Pollen tube reception at the female gametophyte involves a cell-cell dialogue between the pollen tube and synergid cell (Kessler et al. 2011). Successful pollen tube reception culminates in the degeneration of the synergid cell and near-simultaneous pollen tube burst, which projects the sperm cells to the site of fusion (Berger et al. 2008). Once the two sperm cells arrive at the female gametes, they rapidly undergo fusion, one with the egg cell to generate the embryo, and the other with the central cell to generate endosperm. Fertilization of both female gametes is required for development of a seed.

While there have been recent advances in our understanding of pollen tube reception in the ovule, especially synergid-expressed factors that control pollen

tube reception, there has been little progress into the pollen tube expressed factors that coordinate reception. This thesis provides novel insight into flowering plant reproduction, specifically the role of pollen tube differentiation during growth in the pistil and how pollen tube identity is established during reception in the ovule. Here we discuss how three MYB transcription factors determine pollen tube molecular identity through the creation of patterns recognized by synergid cells during reception and compare this pattern of recognition to mechanisms employed during pathogen defense. We suggest that the gametophytes reciprocally detect a reproduction-associated molecular pattern, or RAMP, that determines gametophyte identity, species-specificity and reproductive success. We will first review the major findings of our research with respect to the establishment of pollen tube RAMPs, which will be synthesized into a model in the final section. This thesis presents four main findings: 1) Three R2R3 MYB transcription factors are required for pollen tube reception by the synergid. 2) MYB transcription factors positively regulate a subset of pollen tube genes that are specifically induced by growth in the pistil. 3) Small secreted MYB-regulated Thionin proteins promote synergid degeneration. 4) A small secreted MYB-regulated Self-incompatibility protein homolog (SPH) promotes pollen tube reception.

1) Three R2R3 MYB transcription factors are required for pollen tube reception with the synergid

Pollen tube transcriptional differentiation is induced by growth in the pistil

Pollen tubes make cell contacts with pistil cells when growing towards ovules. These cell-cell contacts are constantly changing as the pollen tube migrates, which can bring it new information on its position and proximity to ovules. This information is critical to the pollen tube polar growth machinery, for example in sensing gradients of secreted attractants and reorganizing the direction of growth (Takeuchi et al. 2012). Signal transduction between pistil and pollen tube is not limited to the reorganization of pollen tube growth direction. Pollen tubes that are grown in vitro fail to respond to LURE proteins (Okuda et al. 2009; Takeuchi et al. 2012) and target ovules at very low frequencies (Palanivelu et al. 2006). Pollen tubes that have grown through an explant of stigma and style in the 'semi in vivo' assay (SIV) gain the capacity to bind LUREs (Okuda et al. 2013) and target ovules with high efficiency (Palanivelu et al. 2006). Furthermore, pollen tube growth through the stigma and style (SIV) elicits a novel transcriptional landscape in pollen tubes (Qin et al. 2009). Compared to transcription factors that pattern pollen grain development (Twell 2011), genes controlling transcriptional responses to pollen tube growth in the pistil have not been well studied. We hypothesized that transcription factors that are specific to the SIV pollen tube growth condition may play roles as master regulators of the pollen tube differentiation program during growth in the pistil. Therefore, we analyzed the transcriptome of pollen tubes grown in SIV conditions and identified three related R2R3-MYB transcription factors that were induced in SIV pollen tube conditions; *MYB97*, *MYB101*, and *MYB120* (Thesis Chapter 4, Figure 1).

We used reverse genetic analysis to determine whether MYB transcription factors regulate critical pollen tube functions. Single and double RNA-null mutations had no obvious effect on reproductive success. However, the triple mutant (*myb97-1*, *myb101-4*, *myb120-3*) had dramatically reduced seed set (Thesis Chapter 1, Supplemental Figure 1). Analysis of pollen function using aniline blue staining and a series of pollen, sperm, and female gametophyte marker genes shows very clearly that loss of *MYB97*, *MYB101*, and *MYB120* results in pollen tubes that enter the ovule, but fail to stop growing, burst, and release sperm (Thesis Chapter 4, Figure 2, Leydon et al. 2013). *myb* triple-mutant pollen tubes do not release sperm in ~75% of the ovules they enter, explaining the reduction in seed set. We propose that growth through the pistil induces *MYB97*, *MYB101*, and *MYB120* expression to levels required for activation of a set of pollen tube genes that promote differentiation and sperm release. This was the first indication that the pollen tube plays an active signaling role in pollen tube reception.

Pollen tube signaling leads to synergid degeneration.

Synergid cells produce pollen tube attractants that guide the pollen tube into the ovule (reviewed in (Takeuchi et al. 2011). They also express signaling molecules required for the pollen tube to stop growing and release sperm (see below, reviewed in Kessler et al. 2011) and upon pollen tube arrival, one of the two synergids degenerates (Faure et al. 2002). By marking the pollen tube cytoplasm and the synergid nucleus with distinct fluorescent proteins, we found that when

wild-type pollen tubes enter an ovule they burst; these ovules contained at least one degenerated synergid (Thesis Chapters 3,4). However, entry and overgrowth of *myb* triple-mutant pollen tubes frequently (~38% of ovules, Chapter 4, Figure 2) failed to initiate synergid degeneration. This finding suggests that synergid degeneration is initiated either by MYB-regulated extracellular signals from the pollen tube, or by the physical force generated by pollen tube rupture. Analysis of *sirene* (*srn*, a *fer* allele) (Rotman et al. 2003) and *amc* (Boisson-Dernier et al. 2008) found that pollen tube overgrowth in mutant ovules failed to initiate synergid degeneration. This finding is consistent with the idea that pollen tube rupture initiates synergid degeneration. However, independent analysis of *fer*, showed that one of two synergids was degenerated in ovules with coiling pollen tubes (Huck et al. 2003). Analysis of *lre* suggested two phenotypic classes: *lre* ovules with coiling pollen tubes and two intact synergids, and *lre* ovules with coiling pollen tubes and a degenerated or displaced synergid (Tsukamoto et al. 2010). Each of these previous studies relied on techniques that could not differentiate between the pollen tube cytoplasm and synergids. Our studies took advantage of pollen, sperm, and female gametophyte fluorescent marker proteins that enabled quantitative, high-resolution phenotypic analysis. We observed loss of synergid nuclear GFP signal in ovules with *myb* triple-mutant pollen tubes that had not burst in ~50% of the ovules we analyzed (n=191). These data suggest that synergid degeneration is not the consequence of pollen tube rupture. We favor the proposal that synergid degeneration is initiated by signals from the pollen tube that are regulated in part by MYB transcription

factors. This proposal is supported by our observations of synergid status in male (*aca9*, Schiott et al. 2004) and female (*gfa2*, Christensen et al. 2002; *lre*, Capron et al. 2008; Tsukamoto et al. 2010) mutants known to affect pollen tube reception. We found that synergid degeneration increased in the presence of a pollen tube, even if that pollen tube was unable to burst (Thesis Chapter 3). These findings are consistent with a model where synergid degeneration is induced by direct interaction with pollen tubes or secreted signaling components that present a distinct molecular pattern to synergid cells.

2) MYB transcription factors positively regulate a subset of pollen tube genes that are specifically induced by growth in the pistil.

MYB-regulated genes point to functions required for pollen-female gametophyte recognition and sperm release.

Our first approach was to use microarray analysis to analyze the global pattern of gene expression in pistils pollinated by either wild type or *myb* triple-mutant pollen (Thesis Chapter 4, Figure 3). Analysis of growth in the pistil allows discovery of transcriptional changes that occur within the pollen tube in response to the pistil. Our goal was to discover pollen tube expressed genes regulated by *MYB97*, *MYB101*, and *MYB120* as the pollen tube grew through the pistil. We identified 48 genes with significantly different transcript abundance in *myb* triple-mutant pollinations (45 were lower in *myb* triple-mutant pollinations, and 3 were higher, Thesis Chapter 4). Of the 45 genes that were lower in *myb* triple-mutant pollinations, only 3 had detectable expression in unpollinated pistils. This

observation suggested that we were primarily detecting gene expression changes dependent upon *MYB97*, *MYB102*, and *MYB120* in pollen tubes as they grew in the pistil. Furthermore, the observation that most MYB-regulated genes were positively regulated by *MYB97*, *MYB101*, and *MYB120* suggests that these genes act as transcriptional activators, which is consistent with observations of homologous MYBs in other plant species. *MYB97*, *MYB101*, and *MYB120* are evolutionarily evolved from the Gibberellin (GA) responsive R2R3-MYB transcription factor *GAMYB*, which is encoded by a single homolog in the basal plant relatives *Selaginella mollendorffii* and *Physcomitrella patens* (Aya et al. 2011). In *Selaginella* and *Physcomitrella* male reproductive structures, *Oryza* tapetum (Aya et al. 2011), and *Hordeum* aluerone layer (Gubler et al. 1995; Gubler et al. 1999) *GAMYB* homologs act as transcriptional activators. Additionally, the related *Arabidopsis* *MYB33* and *MYB65* genes (Thesis Chapter 4, Figure 1A) activate gene expression during anther tapetum development (Millar et al. 2005). In fact, the *Arabidopsis* *MYB33*, *MYB65*, and *MYB101* genes can activate *HvGAMYB* target promoters in the *Hordeum* aluerone layer (Gocal et al. 2001). Additionally, the C-terminal domains of *MYB101* and *MYB97* function as activators in yeast (Liang et al. 2013), supporting the hypothesis that these transcription factors are transcriptional activators with a conserved DNA binding motif. Therefore, *myb* triple-mutant pollen tubes lack expression of factors that may be responsible for creating molecular patterns that may define pollen tube identity during reproductive interactions.

Three general characteristics emerged from analysis of genes that were down-regulated in *myb* triple-mutant pollinations. The first was an enrichment of transmembrane symporters (7 sugar/proton symporters) including 3/10 members of the *Arabidopsis* sucrose uptake transporter family (Reinders et al. 2012). The traditional role of sugar transporters in somatic tissues has been considered to be the movement of sugars from source to sink tissues for organismal metabolism (Reinders et al. 2012). However, *myb* triple-mutant pollen tubes grow normally *in vitro* and *in vivo* (Thesis Chapter 4, Supplemental Figure 1), suggesting that these sugar/proton transporters may adopt a novel function during pollen tube reception (Thesis Appendix 1).

Second, 22/48 MYB-regulated genes (48.9%) were predicted to enter the endomembrane system and/or be secreted. Of these secreted proteins we observed several carbohydrate active proteins, which may modify the cell wall of the pollen tube to prepare it for interactions with female cells and rupture. For example, we identified two genes (*AT5G09280*, pectate lyase, PLL3, (Palusa et al. 2007); *AT1G02550*, pectin methylesterase inhibitor) predicted to modify pectin, a major component of the pollen tube wall. Regulation of pectin structure at the pollen tube tip has been shown to be essential for tube growth; mutants lacking pectin methylesterases (enzymes facilitating pectin cross-linking), rupture *in vitro* and in the pistil (Jiang et al. 2005; Bosch et al. 2006).

The remaining secreted proteins were diverse and included two identical small cysteine rich Thionin genes (*AT5G36720*, *AT5G36805*), four GDSL Lipase hydrolase genes, and 5 genes in the S protein homolog family (SPH, Ride et al. 1999) – all of which may act as signaling molecules. In addition, two highly similar alpha carbonic anhydrase genes (*ACA4/6*) were the most differentially expressed genes between *myb* triple-mutants and wild type (Thesis Chapter 4, Figure 3). These enzymes interconvert water and carbon dioxide to bicarbonate and protons. It is interesting to speculate that these enzymes may also be involved in regulating pollen tube ion dynamics (Thesis Appendix 2).

This initial study of the MYB-dependent transcriptional regulation of pollen genes within the pistil was informative; we defined an initial set of MYB-regulated genes with promising roles in pollen tube reception. However, microarray analysis of the pollinated pistil suffers from at least two distinct drawbacks. First, the entire genome is not contained on the microarray; so some percentage of pollen tube expressed genes are not detectable. This is most evident when looking at the MYB-regulated Thionin gene in the CRP2460 gene family, which is made up of 28 genes. Only one duplicated pair of these genes, *AT5G36720/AT5G36805*, has a probe on the Affymetrix ATH1 microarray (Thesis Chapter 6, Figure 1, Leydon et al. 2013), making phylogenetic correlation to gene expression impossible. Second, microarray analysis cannot identify the cell-type of a transcript in a mixed tissue. This limitation is compounded by the low number of

pollen cells compared to pistil cells in pollinated pistils. Therefore, we sought an alternative method to capture the entire MYB-regulated transcriptome.

We set out to identify pollen tube- and pistil-specific transcriptomes from pollinated pistils using an RNA-sequencing approach. We performed an in-silico dissection of RNA-sequencing alignments from crosses between two *Arabidopsis* ecotypes, Col-0 (Col) and Cvi-0 (Cvi), a technique which has been highly successful in identifying gene expression bias due to imprinting (Gehring et al. 2011; Nodine et al. 2012; Pignatta et al. 2014). We used known and new single nucleotide polymorphisms (SNPs) that are specific to Col and Cvi ecotypes to distinguish pollen and pistil transcripts (Thesis Chapter 5, Figures 1-3). We accurately defined pollen- and pistil-specific gene expression in wild type pollinations between Cvi pistils and Col pollen, and determined for the first time that 8% of reads from pollinated pistils arise from pollen tubes (Reads containing SNPs, Thesis Chapter 5, Figure 2B). We classified 59.2% of expressed genes and TEs based upon available SNPs between Col and Cvi (Thesis Chapter 5, Figure 2C,D), and found that sex-specific transcriptomes were highly correlated with previous microarray analyses of reproductive tissues (Thesis Chapter 5, Figure 5). Using this approach we have described comprehensive profiles of genes expressed in the pollinated pistil, and applied sex-specific determination for all available SNPs between Col and Cvi. This methodology provides a powerful method to quantify pollen-pistil interactions over any time-course to

catch transcriptional responses in any plant species with an available genome sequence.

In our analysis of the Col and Cvi pistil transcriptomes we uncovered differential expression of genes in self-pollinated pistils from intra-ecotype crosses (Col x Col, and Cvi x Cvi, Thesis Chapter 5, Figure 9). Hundreds of genes displayed ecotype-bias in their expression during reproduction. These genes may underlie the subtle defects in inter-ecotype pollinations (Col x Cvi) that we have previously observed (Thesis Chapter 2, Leydon et al. 2014). For example, genes that could play roles in cell signaling such as the cysteine rich proteins (CRPs) in the defensin-like family (DEFL, Thesis Chapter 5, Figure 9D), and Thionin related family (CRP2460, Thesis Chapter 6) are expressed in Col self-pollinations, but absent in Cvi self-pollinations. Additionally a number of NBS-LRR genes (TIR-NBS-LRR, Thesis Chapter 5, Figure 9F) display the same ecotype-specific bias, and could play roles in intracellular signal transduction during reproduction. Our analysis has identified reproductive expression patterns in the two most distal yet within-species *Arabidopsis thaliana* ecotypes (Col/Cvi), which may identify gene families that influence molecular patterns or signaling systems during reproduction that are under evolutionary selection.

We applied this SNP-informed RNA-seq methodology to the study of the pollen expressed *MYB97*, *MYB101*, and *MYB120* genes to comprehensively define the MYB-regulated transcriptome during pollination. We performed RNA-sequencing

on crosses between Cvi pistils and *myb* triple-mutant pollen, which is in the Col-0 ecotype. First, we compared the differential gene expression changes in our RNA-seq experiment to previous microarray experiments on *myb* triple-mutant pollen grains (Liang et al. 2013) and *myb* triple-mutant pollinated pistils (Leydon et al. 2013). We observed that 89% (43/48) of MYB-regulated genes identified by microarray were also detected by RNA-seq, which also increased the number of differentially expressed genes (317 genes, Thesis Chapter 5, Figure 6A). Subsequently, we analyzed differential expression in reads that were specific to pollen or pistil. Differential expression analysis on pollen- and pistil-specific reads identified an additional 223 genes with MYB-dependent gene expression that is masked by expression of the same gene in the opposite sex. 210 pollen-specific and 13 pistil-specific genes are revealed by SNP-informed RNA-seq to require MYBs for wild-type transcript expression levels (Thesis Chapter 5, Figure 7).

RNA-Sequencing provided the first comprehensive analysis of MYB-dependent gene expression during growth in the pistil. The initial functional categories we defined using microarray analysis were corroborated by RNA-sequencing, and the level of genetic redundancy in MYB-regulated gene expression became visible. First, transmembrane transporters are highly enriched in the MYB-dependent transcriptome (23 transport genes), including 7 Na⁺/H⁺ transporters, 8 carbohydrate/proton symporters in the major facilitator family, four of which are sucrose/proton symporters in the SUC family, *SUC6-9* (Thesis Chapter 5, Figure 8A-C, Thesis Appendix 1). Second, we observed an increase in the number of

carbohydrate active genes (19 genes), including 2 pectin methylesterase inhibitors, and 10 genes with predicted pectate lyase activity. Finally, RNA-sequencing has revealed several families of small secreted proteins that require pollen MYBs for expression: 22/28 Thionins in the CRP2460 gene family (Thesis Chapter 6), 15/64 SPH genes (Thesis Chapter 7), 2 Cysteine-rich secretory, Antigen 5, and Pathogenesis-related 1 proteins (CAP; *AT3G09590*, *AT5G02730*), and 4 rapid alkalinization factors (RALFs; *AT2G32835*, *AT2G32788*, *AT2G32785*, *AT1G60815*). This analysis provides a definitive improvement over microarray analysis, which failed to detect many members of gene families due to probe hybridization limitations or an absence of gene annotation at the time of microarray synthesis. Our results also explain the difficulty in identifying pollen tube genes involved in pollen tube reception by reverse genetic analysis. Numerous mutant screens identified only female mutants that disrupt reception (Kessler et al. 2011). Most MYB-regulated genes are found in large gene families that are co-expressed; they are redundantly regulated (three MYBs) and are themselves likely to be redundant. This redundancy allows maintenance of reception signaling integrity, and may confer an advantage in the case of mutations in any single component (Morris et al. 2003).

Gene expression specific to male or female reproductive tissues, or Sex-biased gene expression, has been observed in many animal species, and frequently sex-biased genes undergo rapid evolution due to selective pressures (Swanson

et al. 2002). This has been observed animal sperm proteins where sperm-egg interaction utilizes a 'lock and key' model to ensure species-specific gamete interactions (Metz et al. 1998). However, in plants the pollen tube is responsible for the motility and reproductive interactions with female cells, making it the more likely cell to express genes that determine species-specific interactions. Therefore, we asked whether pollen tube and pistil specific genes displayed higher SNP rates than background SNP rates across the genome, and whether those SNPs were more likely to cause a missense mutation. We found that, SNPs are 1.5x more likely to occur in pollen-expressed genes, and these SNPs are 1.7-1.9x more likely to cause a missense mutation (Thesis Chapter 5, Tables 1&2). Pistil-specific genes that are pollination responsive also display increased SNP-rate and missense frequencies. These results require further evolutionary analysis on individual pollen-expressed gene families, especially MYB-regulated pollen specific genes (1.71x more likely to contains SNPs), which may be responsible for presenting the molecular identity of the pollen tube to the synergid cell. Two recent studies have presented support for the sexually driven evolution of pollen-expressed genes (Arunkumar et al. 2013; Gossmann et al. 2014) compared to sporophytic or sperm-expressed genes, and it will be exciting to test this observation using our SNP-informed RNA-seq dataset, which should be more comprehensive than gene lists compiled from microarray analyses.

MYB-regulated genes are enriched in three general categories, 1) transmembrane transporters, 2) secreted carbohydrate active proteins, and 3)

small secreted proteins and enzymes. Taken together with the observation that *myb* triple-mutant pollen tubes coil during pollen tube reception, we hypothesized that genes in each of these three categories may contribute to pollen tube reception by the synergid cells. In order to test whether these genes play roles in pollen tube reception, we developed an expression system whereby we could re-introduce MYB-regulated genes one-by-one into the *myb* triple-mutant genotype. This method allowed us to test whether any individual gene is sufficient in modifying or rescuing pollen tube reception phenotypes. We tested 12 MYB-regulated genes for their ability to modulate the *myb* triple-mutant phenotype: 2 sucrose/proton transporters in the SUC family (*SUC7* – *AT1G66570*, *SUC9* – *AT5G06170*, Appendix 1), 2 carbohydrate active genes (*PMEI* – *AT1G55770*, *O-glycosyl hydrolase* – *AT4G26830*, no statistical difference, data not shown), and 8 small secreted proteins: 4 Thionin genes, 1 *SPH*, 1 *GDSSL*, 1 RALF (*RALFL16*), and 1 ACA (*ACA4*). We discovered that *ACA4* completely rescued the *myb* triple-mutant pollen tube guidance defect (Thesis Appendix 2). Excitingly, we found that two families of small secreted proteins modulated the *myb* triple-mutant phenotype, the cysteine rich proteins in the Thionin family (CRP2460, Thesis Chapter 6) and the small excreted proteins in the S protein homolog family (*SPH*, Thesis Chapter 7).

3) Small secreted MYB-regulated Thionin proteins promote synergid degeneration.

We identified a family of small secreted CRPs (CRP2460) that contain sequence homology to Thionin proteins and require *MYB97*, *MYB101* and *MYB120* for expression in the pollen tube (Thesis Chapter 6, Figure 1). Thionin expression is induced by pollen tube growth through pistil tissues, where Thionin proteins localize to the pollen tube endomembrane system and are secreted during pollen tube reception (Thesis Chapter 6, Figure 4). Expression of *BGAL11:Thionin:GFP* in the *myb* triple-mutant background promotes synergid degeneration in ovules targeted by coiling pollen tubes at rates higher than the *myb* triple-mutant (Thesis Chapter 6, Figures 6,7). Purified Thionin proteins also promoted synergid degeneration during in vitro ovule incubation assays (Thesis Chapter 6, Figure 11). Taken together, these complementary approaches suggest that the CRP2460 Thionin proteins act during pollen tube reception to promote synergid degeneration.

The mechanism by which MYB-regulated Thionin proteins (CRP2460) promote synergid degeneration is unknown. Thionin proteins such as Purothionin bind to lipids on soil pathogen cell membranes and create membrane pores (Florack et al. 1994; Hughes et al. 2000; Stec 2006). Our experiments detected degeneration of only synergid nuclei, and no other cell types in mature ovules (Thesis Chapter 6). If CRP2460 genes retain membrane pore-forming activities, how might they be regulated such that they only permeate the synergid cell? One mechanism by which Thionin activity could be synergid-specific might involve lipid-specificity or membrane micro-domain specificity. The filiform apparatus

region of synergid cells is enriched in highly invaginated plasma membrane folds, where lipid raft environments may exist not found in any other cell types. Alternatively, the extracellular environment outside the filiform apparatus may contain an optimal environment for Thionin activity. Based upon our observation that purified Thionin proteins are able to trigger degeneration in synergid cells in isolated ovules without the presence a pollen tube, it suggests that Thionin proteins act on mature synergid cells. Given this observation, we considered whether pollen tube secreted factors such as the Thionin proteins direct the synergid chosen for pollen tube receptivity based upon a local protein gradient. For example, during pollen tube reception calcium concentrations are dynamic, and can be broken into phases (Figure 1) (Iwano et al. 2012; Denninger et al. 2014; Ngo et al. 2014). The first phase of pollen tube reception “slow PT growth” (phase I) occurs when the pollen tube arrives at the synergid and calcium signaling is dynamic. The calcium signaling in phase I depends on the synergid receptor *FERONIA* (*FER*) (Ngo et al. 2014). The synergid expressed MLO protein *NORTIA* (*NTA*) requires *FER* for localization to the site of pollen tube entry into the synergid (Kessler et al. 2010), and is itself required to maintain proper calcium signaling during phase II of calcium signaling (Ngo et al. 2014). Future experiments can now be designed to test the effects of purified Thionin proteins on 1) Calcium signaling in the synergid cell, and 2) *FER* activity on the localization of *NTA* to the synergid micropylar membrane (Kessler et al. 2010). If we observe calcium oscillations or *NTA* re-localization, it would suggest Thionins

are involved in phase I, and act as a RAMP signal that initiates pollen tube reception.

If Thionins determine the pollen tube identity during interaction with the synergid, it is possible that it does so in a species-specific manner. It is interesting to note that the CRP2460 Thionin genes are found only in *Brassica*, suggesting that they have only recently evolved (Thesis Chapter 6, Figure 2). Comparatively, plant pathogens are frequently uniquely adapted to their host species and cannot successfully infect non-host species (Stam et al. 2014), utilizing rapidly evolving families of effector proteins to escape the host immune system (de Jonge et al. 2013) . If the recent expansion of Thionin proteins has been selected for as a mechanism to discern pollen tube species, it will be useful to determine whether Thionin synergid degeneration activity is specific to *Arabidopsis thaliana*, or can act on other members of the *Brassica* clade. Future experiments will utilize genetic reporters such as *ACT11:MSI1:GFP* introduced into the related *Brassica* species *Olimarabidopsis pumila* and *Arabidopsis lyrata* to test the ability of purified Thionin proteins to initiate synergid degeneration in vitro assays (as in Thesis Chapter 6, Figure 11). In a complementary approach, expression of CRP2460 homologs from *O. pumila* and *A. lyrata* in *myb* triple-mutant pollen will determine the functional conservation between these genes within the *Brassica* clade. Finally, it will be critical to generate loss of function mutations in all members of the CRP2460 family in *Arabidopsis* to determine whether these genes are required for pollen tube reception. We have designed a

CRISPR/CAS9 methodology to target multiple Thionin genes for deletion from the genome in parallel, which should increase the efficiency of multiple-mutant construction (Thesis Chapter 6, Figure 12).

4) A small secreted MYB-regulated Self-incompatibility protein homolog (SPH) promotes pollen tube reception.

Our SNP-informed RNA-sequencing of *myb* triple-mutant pollen tubes growing in vivo has uncovered a wealth of genes that require *MYB97*, *MYB101*, and *MYB120* for expression. One group of MYB-regulated genes was a family of small secreted proteins with sequence homology to a self-incompatibility determinant identified in *Papaver rhoeas* (PrsS1) (Foote et al. 1994). In *Arabidopsis*, there are 64 S-Protein Homologs (SPH, (Foote et al. 1994), and 53 were detected in pollinated pistil tissues in our RNA-sequencing samples; 15 require MYBs for expression (Thesis Chapter 7, Figure 1). The majority of SPH genes contain a signal peptide that is predicted to target them to the secretory system. We tested the ability of one MYB-regulated SPH gene, *AT3G26870*, to modulate the *myb* triple-mutant phenotype. When we expressed a *BGAL11:SPH:GFP* construct in *myb* triple-mutant pollen, we observed a localization pattern consistent with the endomembrane system. Surprisingly, this SPH:GFP fusion protein was capable of partially rescuing the *myb* triple-mutant pollen tube defect, significantly increasing the frequency of pollen tube burst during reception in the ovule and total number of seeds produced (Thesis

Chapter 7, Figures 4,5). This marks the first pollen gene with a role in pollen tube reception in the ovule, and the first known SPH gene activity in *Arabidopsis* (Ride et al. 1999).

In the self-incompatible species *Papaver rhoeas* PrsS1 is secreted from the stigma cells (Foote et al. 1994), where it interacts with a receptor on the pollen tube membrane (PrpS1) (Wheeler et al. 2009). In the event of self-pollination, a signal transduction cascade induces pollen tube programmed cell death (Reviewed in, Eaves et al. 2014). Given the high level of SPH (AT3G26870) sequence homology to PrsS genes (Thesis Chapter 7, Figure 7), and the absence of any additional protein domains, it is likely that *Arabidopsis* SPH genes act as secreted signaling molecules as they do in *P. rhoeas*. It is attractive to hypothesize that SPH genes are secreted from the pollen tube to the synergid cell during pollen tube during reception, where they bind to a synergid expressed receptor protein and activate a signal transduction cascade that promotes synergid degeneration. Both cascades are coordinated by dynamic calcium signaling that result in a programmed cell death event. However, there are no genes with strong sequence homology to PrpS receptors in the *Arabidopsis* genome, which means that putative SPH receptors are not readily identifiable based upon protein sequences alone.

We have created an expression system that allows purification of epitope-tagged SPH protein (AT3G26870) from *P. pastoris* (Thesis Chapter 7, Figure 8). This

resource will allow us to perform *in vitro* activity tests as has been previously described for PrsS proteins activity on pollen tubes (Foote et al. 1994) and Thionin protein activity on synergid cells (Thesis Chapter 6, Figure 11). In preliminary experiments, purified SPH protein produced no change in synergid nuclear status in *ACT11:MSI1:GFP* ovules (Data not shown). This experiment must be replicated with varying protein concentrations; however it may also suggest that SPH activity requires the contact between pollen tube and synergid in order to be active. For example, the SPH receptor may not be localized to the synergid cell membrane until *FER* signaling pathways are activated (Figure 1B, phase II), as is observed for *NTA* (Kessler et al. 2010). In this situation, SPH activity on synergid cells *in vitro* would not be observed without co-incubation of *FER* ligands such as RALFs (Haruta et al. 2014), which would allow localization of the SPH receptor to the synergid membrane.

Phylogenetic analysis of the SPH gene family reveals that genes with homology to PrsS (S1-like) are found throughout the entire plant lineage (Thesis Chapter 7), however the only known PrsS function that has been described previously is in self-incompatibility in *Papaver* species (Kurup et al. 1998). Interestingly, PrpS is not found in *Arabidopsis* genomes and is also absent from all other sequenced genomes (BLAST (in 2015), Wheeler et al. 2010). This suggests that the S1-like proteins original molecular function may have been co-opted for self-incompatibility functions in *Papaver*. It has been predicted that small, stable proteins tolerate mutations without disrupting tertiary structure making them

highly evolvable for new gene functions (Zhu et al. 2005; Bloom et al. 2006). If this is the case for SPH proteins, it could help explain diverse signaling activity in different processes (i.e. SI and reception). It will be interesting to determine whether other plant species express S1-like proteins during reproduction, and whether one of the ancestral functions of this gene family was in coordinating self-nonself recognition between gametophytes during pollen tube reception.

Identification of a reproduction-associated molecular pattern or RAMP in pollen tube reception

In examining the molecular interaction between the pollen tube and the synergid cell we must take into account a number of biological observations. First, the observation that pollen tube reception is disrupted in interspecific interactions as is seen in *Brassica* (Escobar-Restrepo et al. 2007; Leydon et al. 2014) and *Rhododendron* (Williams et al. 1986), indicates that the physical arrival of any pollen tube into an ovule is not sufficient to induce synergid degeneration and pollen tube burst. Second, the synergid coordinates pollen tube burst through at least three signaling components (*LORELEI* (*LRE*), Capron et al. 2008, *FER* Escobar-Restrepo et al. 2007, and *NTA*, Kessler et al. 2010). On the pollen tube side, approximately three hundred genes are activated by a trio of MYB transcription factors (*MYB97*, *MYB101*, *MYB120*) which coordinate pollen tube burst and synergid degeneration. Ablation of synergid components (*LRE*, *FER*, *NTA*) and male components (*MYB97*, *MYB101*, *MYB120*) results in a loss of cell-cell recognition and pollen tube coiling identical to exotic species pollen tube

reception. Therefore, genetic factors expressed by the pollen tube and synergid cells are required to generate the molecular identity of the pollen tube (Leydon et al. 2013). The molecular identity specific to the pollen tube and the synergid cells is similar to the molecular identity of pathogens to their hosts. In fact, the pollen tube growth in the pistil was initially thought to be parasitic growths in the pistil, so much does the invasive lifestyle of the pollen tube mirror fungal infection (Amici 1847 as cited by Kessler et al. 2010). Initial analysis of the pollen tube and synergid transcriptomes revealed large numbers of secreted proteins that were characterized as defense genes (Punwani et al. 2007; Qin et al. 2009; Schmid et al. 2012), and, identification of the defensin-like LURE proteins as pollen tube attractants sparked new interest in the molecular overlap between defense and reproduction (Dresselhaus et al. 2009). Furthermore, reception components share molecular functions with powdery mildew infection defense responses (*NTA* and *FER*, Kessler et al. 2010), prompting our question: could there be a basis for a reproduction-associated molecular pattern, or RAMP?

Animals and plants discriminate between self- and non-self cellular interactions, which are required in the defense against pathogens. The first line of defense involves the recognition of pathogen or microbe-associated patterns (PAMPs/MAMPs, Figure 1A, light red dots), that are not expressed in host cells (Nurnberger et al. 2004; Ausubel 2005; Akira et al. 2006; Boller et al. 2009; Macho et al. 2014). PAMPs and MAMPs are recognized by pattern recognition receptors (PRRs, Figure 1A, blue and orange lines) localized to the host plasma

membrane. PRRs recognize pathogen-expressed components such as flagellin from bacterial flagella, or chitin from fungal cell walls, to effect a state of PAMP/MAMP triggered immunity (PTI/MTI, Figure 1A) (Jones et al. 2006). Accordingly, the PAMP/MAMP interaction with PRRs activates a signal transduction cascade that acts through mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), to generate reactive oxygen species (ROS, Figure 1A), immune-related gene expression and callose deposition at the cell wall (Boller et al. 2009; Tena et al. 2011).

In plants PRRs belong to leucine rich repeat receptor-like kinases or receptor-like proteins (LRR-RLKs/LRR-RLPs) or lysine motif containing proteins (LysM/RLKs/LysM-RLPs) (Boller et al. 2009; Macho et al. 2014). Another source of extracellular signals that feed into pathogen triggered immunity is the production of peptides and carbohydrates that are produced by wounding or damage of plant tissue. As the pattern is produced from the pathogen invading the cellular structure of the host, this is known as a damage-associated molecular pattern (DAMP, Figure 1A, light green dots). DAMP elicitors include carbohydrates, lipids, glycopeptides that are released by the pathogen cell wall or by interaction of secreted pathogen enzymes with the host cell wall (Vidal et al. 1998; Boudart et al. 2003).

After PAMP/MAMP/DAMP interaction with PRRs, the host cells activates production of anti-microbial metabolites (phytoalexins) and proteins

(Pathogenesis-Related proteins, Figure 1A, light blue dots - defense proteins), which together with ROS and callose production, block the ability of the pathogen to grow into the host cell, creating PTI. If the pathogen is adapted to its host, effector proteins expressed by the pathogen are used to evade PTI. If effector proteins are adapted to the selected host, the pathogen achieves effector triggered susceptibility (ETS) (Boller et al. 2009). Alternatively, host cells may detect pathogen effector proteins by direct or indirect interaction with R genes to produce effector triggered immunity (ETI). ETI typically leads to a hypersensitive response, characterized by reactive oxygen species (ROS) production, and cell death (Figure 1A, yellow cell on far right, (Matzinger 2002; Nimchuk et al. 2003). The majority of described R genes are in the NB-LRR class of intracellular receptors (Figure 1A, yellow boomerangs - NBS-LRR, (Meyers et al. 2003; Caplan et al. 2008; Li et al. 2013).

Effectors are diverse, depending upon which pathogen is considered, however they typically target and manipulate host proteins that are involved in establishing PTI. For example, several gram-negative bacterial pathogens utilize type III-secretion systems to inject effector proteins into the host cytoplasm. These effectors are commonly referred to as type III effectors (T3Es), and they have diverse host targets including all known steps of the PTI signaling pathway (Macho et al. 2015). Other well studied effectors include the RXLR and crinkling and necrosis (CNR) effectors from the oomycete *Phytophthora* (Haas et al. 2009; Shen et al. 2013; Stam et al. 2013). RXLR and CNR effectors encode N-terminal

peptide sequences that allow translocation into host cells allowing cytoplasmic effector activity without an injector system (Schornack et al. 2010; Tyler et al. 2013). Effectors may also be secreted to target host extracellular proteins involved in the establishment of PTI, such as the *P. infestans* protease inhibitors EPI1 & EPI10 (Tian et al. 2004; Tian et al. 2005).

Comparing pathogen-host interactions to pollen tube reception, it is interesting to posit the pollen tube as a 'pathogen' and the synergid cell as the 'host'. Mechanically, the pollen tube penetrates the pistil tissue and determines which cell is likely to be receptive to 'infection', or reception (Figure 1A). Just as an adapted fungal pathogen would depend on the extracellular environment of its normal host to present cues to induce successful infection (Mendgen et al. 2002), the pollen tube responds to the extracellular environment of the pistil to properly grow and target an ovule and the synergid cell within (Higashiyama et al. 2008). Upon entry of the pollen tube into the micropyle, the synergid cell and the pollen tube make physical contact – similar to the initiation of fungal penetration hyphae (Grenville-Briggs et al. 2005). Pathogens that are adapted to their hosts will present PAMPs, but will be able to evade PTI through the production of co-evolved effectors. Likewise, pollen tubes from the same species will present RAMPs to synergid cells, which determine their receptivity and control pollen tube burst.

The first interactions between the pollen tube and the synergid cell are likely to involve signaling between RAMPs from the pollen tube to PRRs on the synergid. The most likely PRR candidate at this time is *FER*, which is highly enriched at the micropylar membrane of the synergid cell (Escobar-Restrepo et al. 2007). The *FER* ligand in synergids is not yet known, however a rapid alkalization factor (RALF) activates vegetatively expressed *FER* (Haruta et al. 2014). It is possible that a pollen tube-expressed RALF acts as a ligand for *FER* in pollen tube reception; there are several highly expressed pollen tube-specific RALFs, and 4 MYB-regulated RALF genes (Thesis Chapter 5, Supplemental information). Alternatively, the extracellular carbohydrate-binding (Malectin-Like) domain of *FER* may detect the arrival of the pollen tube and an altered cell wall, as CrRLKs (*FER* is a member of this family of RLKs) are implicated in sensing the cell wall (Boisson-Dernier et al. 2011). Sensing pollen tube arrival may involve the production of carbohydrates that act as DAMPs; carbohydrate active proteins secreted from the pollen tube may modify the cell wall of the filiform apparatus or pollen tube to release or expose carbohydrates as ligands for *FER* (Trouvelot et al. 2014). It is interesting to note that Pectin Lyase genes are MYB-dependent, because they are well known as bacterially secreted effector proteins that aid the depolymerization of cell walls during *Erwinia* infection (Barras et al. 1994). Pectate lyases cleave the pectin backbone, resulting in the loosening of the cell wall and the release of oligogalacturonides, which function as elicitors of the defense response (Cabrera et al. 2008). Alternatively, the pollen tube may damage the synergid cell wall, indirectly activating *FER* by disrupting binding to

its carbohydrate ligand. *FER* activity is required for synergid ROS production (Duan et al. 2014), normal phase I calcium signaling in the synergid cell (Figure 1B, Ngo et al. 2014), and for the re-localization of *NTA* to the synergid micropylar membrane (Kessler et al. 2010). These steps parallel the steps of Powdery Mildew (PM) infection.

NTA homolog MLO proteins are normally localized to the plasma membrane, however upon PM infection MLO is re-localized to the site of infection, and is required for host entry into the cell (Buschges et al. 1997; Devoto et al. 1999). MLO proteins appear to cluster into microdomains at the penetration point, and may be involved in lipid raft formation or establishment of defense component secretion. In non-adapted pathogen infections, MLO and PEN1 (PEN1 is a syntaxin containing a SNARE domain, Collins et al. 2003) proteins are re-localized beneath fungal appressoria and block infection spread. However, adapted PM species are presumably able to block this activity, possibly by targeting MLO proteins using effector proteins (Bhat et al. 2005). If *MYB97*, *MYB101*, and *MYB120* activate genes functionally similar to RAMPs, then we would expect that *FER* would not be activated in *myb* triple-mutant pollinations. We would hypothesize that synergid Ca²⁺ oscillations would be dampened or absent, and *NTA* would fail to localize to the site of pollen tube reception.

However, we tested the localization pattern of *NTA:GFP* in ovules targeted by *myb* triple-mutant pollen tubes, and found that *NTA:GFP* re-localization occurred

normally in all observed cases (Appendix 6). Therefore it seems more likely that the secreted MYB-regulated genes are involved in the cellular interaction that follows NTA localization to the site of pollen tube reception. NTA localization to the micropylar membrane of the synergid may enrich the membrane with either a distinct lipid domain, or membrane protein with which secreted MYB-target genes such as the SPH genes interact. Furthermore, Ca^{2+} dynamics in ovules targeted by *myb* triple-mutant pollen tubes were reported to be normal during phase I interactions, but disrupted at phase II (Figure 1B, Personal communication from Q. Ngo, unpublished data). This pattern is similar to what is observed in *nta* mutant synergids (Figure 1B, Ngo et al. 2014), and suggests that MYB targets promote normal phase II interactions with synergids during pollen tube reception. These findings, taken together suggest that the *MYB97*, *MYB101*, and *MYB120* dependent transcriptome acts during a step similar to fungal effector proteins, after PRRs (*FER*) have identified the presence of a pollen tube RAMP.

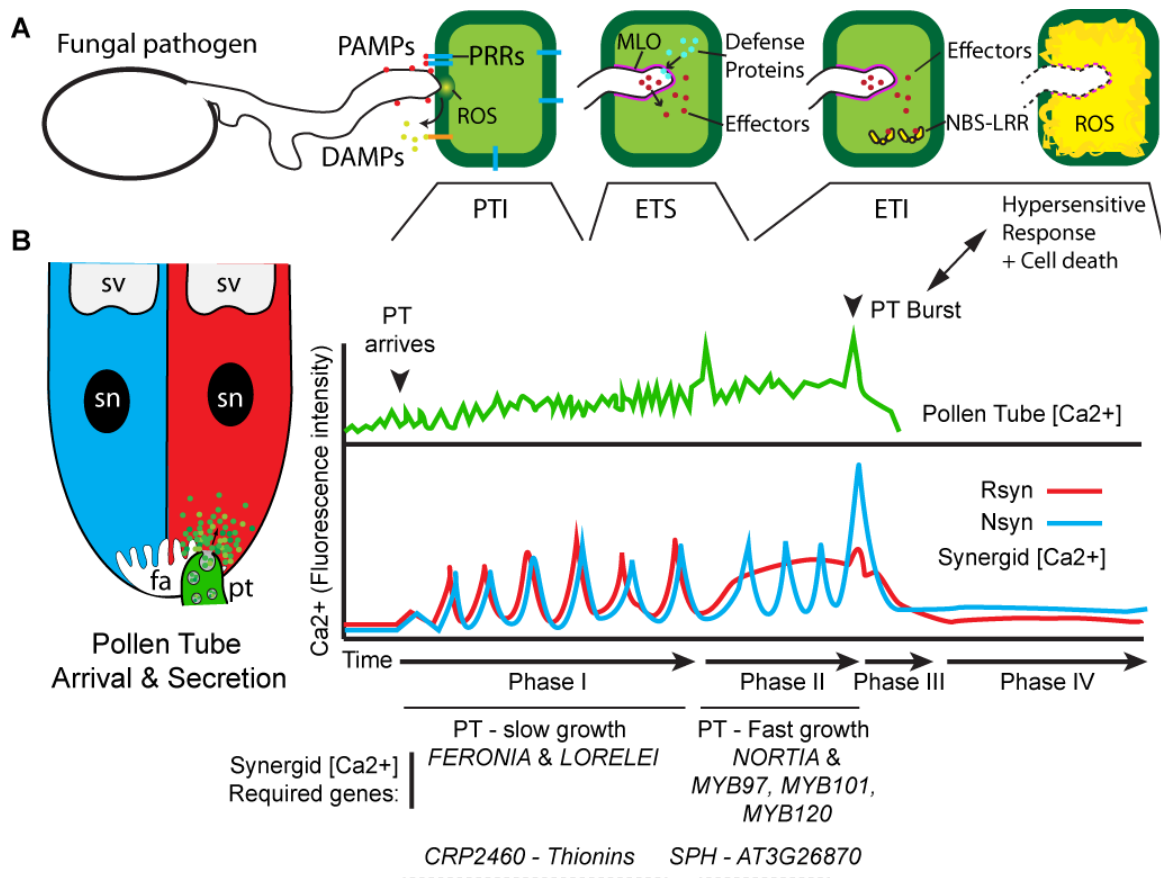


Figure 1. A model for MYB-regulated secreted proteins in pollen tube reception compared to fungal infection. A. A simplified model demonstrating a linear representation of PAMP recognition and effector action leading to a hypersensitive response. Lines below the represented steps are roughly correlated to steps of pollen tube reception, culminating in hypersensitive response, which we posit as an endpoint similar to pollen tube burst. **B.** The synergids receive a pollen tube that has entered the micropylar end of the ovule. The Non-receptive synergid is colored blue (Nsyn), the Receptive synergid red (Rsyn), and the pollen tube green (pt). To the right is a cartoon interpretation of synergid and pollen tube calcium signalling (Denninger et al. 2014; Ngo et al. 2014). Peaks indicate increases in cytoplasmic calcium concentrations. The pollen tube reception phases and the genes required for Ca²⁺ dynamics are shown below the x-axis. The hypothesized time periods of Thionin and SPH gene activity are plotted at the bottom of the pollen tube graph (dotted lines).

While it can be conceptually useful to place the pollen tube in the role of a fungal pathogen, it is important to note that the pollen tube also receives information from the synergid, which is programmed to promote pollen tube entry and burst.

In fact, the GPI anchored protein *LRE* may function as a synergid RAMP detected by an as yet unknown pollen tube PRR. When *lre-5* mutant ovules carrying an *ACT11:MSI1:GFP* nuclear reporter were pollinated with wild type pollen, synergid degeneration was observed in the absence of pollen tube burst (Thesis Chapter 3, Figure 6). *LRE* is required for phase I calcium oscillations (Figure 1B, Ngo et al. 2014), and it may activate secretion of pollen tube RAMPs. Therefore, the pollen tube requires signaling components from the synergid in order to undergo pollen burst, making the pollen and synergid cells perhaps functionally more similar to each other than to pathogen and host. Furthermore, four NBS-LRR genes are specific to pistil-activated pollen tubes, which may be involved in perceiving pistil or synergid secreted effector-like proteins during reproduction (Qin et al. 2009).

Pollen tube reception: the final barrier to species non-specific reproduction in flowering plants

During plant reproduction a series of pollen tube-pistil interactions ensures that the right sperm gets to the right egg. At every stage of pollen tube growth, from hydration and germination through tube growth and guidance to ovules, unfit or inter-species pollen is selected against. Pollen tube reception represents the final barrier to inter-species fertilization. Accordingly, the signaling mechanisms that control pollen tube reception are capable of identifying non-productive pollen tubes and blocking reception (Williams et al. 1986; Escobar-Restrepo et al. 2007; Leydon et al. 2014). Pollen tube reception mechanisms share conserved

components with pathogen defense signaling pathways (Dresselhaus et al. 2009; Kessler et al. 2010), that involve a dynamic calcium signaling dialogue (Iwano et al. 2012; Denninger et al. 2014; Hamamura et al. 2014; Ngo et al. 2014). Both the pollen tube and synergid cells convey bits of information to each other upon cell-cell contact, which constitute a reproduction-associated molecular pattern (RAMP). Pollen tube RAMPs likely signal through *FER* to initiate a second phase of pollen tube reception that requires NTA microdomains and MYB-dependent secreted effector-like proteins (i.e. Thionins, SPHs) to trigger synergid degeneration and ultimately pollen tube burst. As in pathogen:host interactions where hosts evolve mechanisms to detect pathogen identity, pollen tube:synergid interactions sense the species identity of their interaction partner. By using a two-step identification system, synergid cells ensure that egg cells are only fertilized by pollen tubes of the correct species or fitness level. This is critical, because while pollen is abundant, eggs are in limited supply and require maternal investment during seed development.

Our studies have, for the first time, identified pollen tube genes that control pollen tube identity and function during reception at the ovule. The gene families we have discovered through studies of the MYB transcriptional regulators are poised to inform our understanding of the pollen tube-synergid cellular dialogue. In the future, MYB-regulated species barriers can be deconstructed and resynthesized at will to allow inter-species crosses to produce hybrid seedlings in agriculturally relevant crops.

References

- Akira, S., S. Uematsu, et al. (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.
- Amici, J. B. (1847). Annales des Sciences Naturelles **193**
- Arunkumar, R., E. B. Josephs, et al. (2013). "Pollen-Specific, but Not Sperm-Specific, Genes Show Stronger Purifying Selection and Higher Rates of Positive Selection Than Sporophytic Genes in *Capsella grandiflora*." Mol Biol Evol **30**(11): 2475-2486.
- Ausubel, F. M. (2005). "Are innate immune signaling pathways in plants and animals conserved?" Nat Immunol **6**(10): 973-979.
- Aya, K., Y. Hiwatashi, et al. (2011). "The Gibberellin perception system evolved to regulate a pre-existing GAMYB-mediated system during land plant evolution." Nat Commun **2**: 544.
- Barras, F., F. Vangijsegem, et al. (1994). "Extracellular Enzymes and Pathogenesis of Soft-Rot *Erwinia*." Annual Review of Phytopathology **32**: 201-234.
- Berger, F., Y. Hamamura, et al. (2008). "Double fertilization - caught in the act." Trends Plant Sci **13**(8): 437-443.
- Berger, F. and D. Twell (2011). "Germline specification and function in plants." Annu Rev Plant Biol **62**: 461-484.
- Bhat, R. A., M. Miklis, et al. (2005). "Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain." Proc Natl Acad Sci U S A **102**(8): 3135-3140.
- Bloom, J. D., S. T. Labthavikul, et al. (2006). "Protein stability promotes evolvability." Proc Natl Acad Sci U S A **103**(15): 5869-5874.
- Boisson-Dernier, A., S. Frietsch, et al. (2008). "The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition." Curr Biol **18**(1): 63-68.
- Boisson-Dernier, A., S. A. Kessler, et al. (2011). "The walls have ears: the role of plant CrRLK1Ls in sensing and transducing extracellular signals." J Exp Bot **62**(5): 1581-1591.
- Boller, T. and G. Felix (2009). "A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors." Annu Rev Plant Biol **60**: 379-406.
- Bosch, M. and P. K. Hepler (2006). "Silencing of the tobacco pollen pectin methylesterase NtPPME1 results in retarded in vivo pollen tube growth." Planta **223**(4): 736-745.
- Boudart, G., M. Charpentier, et al. (2003). "Elicitor activity of a fungal endopolygalacturonase in tobacco requires a functional catalytic site and cell wall localization." Plant Physiol **131**(1): 93-101.
- Buschges, R., K. Hollricher, et al. (1997). "The barley Mlo gene: a novel control element of plant pathogen resistance." Cell **88**(5): 695-705.
- Cabrera, J. C., A. Boland, et al. (2008). "Egg box conformation of oligogalacturonides: the time-dependent stabilization of the elicitor-active

- conformation increases its biological activity." Glycobiology **18**(6): 473-482.
- Caplan, J., M. Padmanabhan, et al. (2008). "Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming." Cell Host Microbe **3**(3): 126-135.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Christensen, C. A., S. W. Gorsich, et al. (2002). "Mitochondrial GFA2 is required for synergid cell death in Arabidopsis." Plant Cell **14**(9): 2215-2232.
- Collins, N. C., H. Thordal-Christensen, et al. (2003). "SNARE-protein-mediated disease resistance at the plant cell wall." Nature **425**(6961): 973-977.
- de Jonge, R., M. D. Bolton, et al. (2013). "Extensive chromosomal reshuffling drives evolution of virulence in an asexual pathogen." Genome Res **23**(8): 1271-1282.
- Denninger, P., A. Bleckmann, et al. (2014). "Male-female communication triggers calcium signatures during fertilization in Arabidopsis." Nat Commun **5**: 4645.
- Devoto, A., P. Piffanelli, et al. (1999). "Topology, subcellular localization, and sequence diversity of the Mlo family in plants." J Biol Chem **274**(49): 34993-35004.
- Dresselhaus, T. and M. L. Marton (2009). "Micropylar pollen tube guidance and burst: adapted from defense mechanisms?" Curr Opin Plant Biol **12**(6): 773-780.
- Duan, Q., D. Kita, et al. (2014). "Reactive oxygen species mediate pollen tube rupture to release sperm for fertilization in Arabidopsis." Nat Commun **5**: 3129.
- Eaves, D. J., C. Flores-Ortiz, et al. (2014). "Self-incompatibility in Papaver: advances in integrating the signalling network." Biochem Soc Trans **42**(2): 370-376.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Faure, J. E., N. Rotman, et al. (2002). "Fertilization in Arabidopsis thaliana wild type: developmental stages and time course." Plant J **30**(4): 481-488.
- Florack, D. E. and W. J. Stiekema (1994). "Thionins: properties, possible biological roles and mechanisms of action." Plant Mol Biol **26**(1): 25-37.
- Foote, H. C., J. P. Ride, et al. (1994). "Cloning and expression of a distinctive class of self-incompatibility (S) gene from Papaver rhoeas L." Proc Natl Acad Sci U S A **91**(6): 2265-2269.
- Gehring, M., V. Missirlian, et al. (2011). "Genomic analysis of parent-of-origin allelic expression in Arabidopsis thaliana seeds." PLoS One **6**(8): e23687.
- Gocal, G. F., C. C. Sheldon, et al. (2001). "GAMYB-like genes, flowering, and gibberellin signaling in Arabidopsis." Plant Physiol **127**(4): 1682-1693.

- Gossmann, T. I., M. W. Schmid, et al. (2014). "Selection-driven evolution of sex-biased genes is consistent with sexual selection in *Arabidopsis thaliana*." Mol Biol Evol **31**(3): 574-583.
- Grenville-Briggs, L. J. and P. van West (2005). "The biotrophic stages of oomycete-plant interactions." Adv Appl Microbiol **57**: 217-243.
- Gubler, F., R. Kalla, et al. (1995). "Gibberellin-regulated expression of a myb gene in barley aleurone cells: evidence for Myb transactivation of a high-pI alpha-amylase gene promoter." Plant Cell **7**(11): 1879-1891.
- Gubler, F., D. Raventos, et al. (1999). "Target genes and regulatory domains of the GAMYB transcriptional activator in cereal aleurone." Plant J **17**(1): 1-9.
- Haas, B. J., S. Kamoun, et al. (2009). "Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*." Nature **461**(7262): 393-398.
- Hamamura, Y., M. Nishimaki, et al. (2014). "Live imaging of calcium spikes during double fertilization in *Arabidopsis*." Nat Commun **5**: 4722.
- Haruta, M., G. Sabat, et al. (2014). "A Peptide Hormone and Its Receptor Protein Kinase Regulate Plant Cell Expansion." Science **343**(6169): 408-411.
- Higashiyama, T. and Y. Hamamura (2008). "Gametophytic pollen tube guidance." Sexual Plant Reproduction **21**(1): 17-26.
- Huck, N., J. M. Moore, et al. (2003). "The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception." Development **130**(10): 2149-2159.
- Hughes, P., E. Dennis, et al. (2000). "The cytotoxic plant protein, beta-purothionin, forms ion channels in lipid membranes." J Biol Chem **275**(2): 823-827.
- Iwano, M., Q. A. Ngo, et al. (2012). "Cytoplasmic Ca²⁺ changes dynamically during the interaction of the pollen tube with synergid cells." Development **139**(22): 4202-4209.
- Jiang, L., S. L. Yang, et al. (2005). "VANGUARD1 encodes a pectin methylesterase that enhances pollen tube growth in the *Arabidopsis* style and transmitting tract." Plant Cell **17**(2): 584-596.
- Jones, J. D. and J. L. Dangl (2006). "The plant immune system." Nature **444**(7117): 323-329.
- Joppa, L. N., D. L. Roberts, et al. (2011). "How many species of flowering plants are there?" Proceedings of the Royal Society B-Biological Sciences **278**(1705): 554-559.
- Kessler, S. A. and U. Grossniklaus (2011). "She's the boss: signaling in pollen tube reception." Curr Opin Plant Biol.
- Kessler, S. A., H. Shimamoto-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Kurup, S., J. P. Ride, et al. (1998). "Identification and cloning of related self-incompatibility S-genes in *Papaver rhoeas* and *Papaver nudicaule*." Sexual Plant Reproduction **11**(4): 192-198.

- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Leydon, A. R., A. Chaibang, et al. (2014). "Interactions between pollen tube and pistil control pollen tube identity and sperm release in the Arabidopsis female gametophyte." Biochem Soc Trans **42**: 340-345.
- Li, J., Q. Y. Zhang, et al. (2013). "Genome-wide identification and comparative expression analysis of NBS-LRR-encoding genes upon Colletotrichum gloeosporioides infection in two ecotypes of Fragaria vesca." Gene **527**(1): 215-227.
- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 Function as Male Factors That Control Pollen Tube-Synergid Interaction in Arabidopsis thaliana Fertilization." PLoS Genet **9**(11).
- Macho, A. P. and C. Zipfel (2014). "Plant PRRs and the activation of innate immune signaling." Mol Cell **54**(2): 263-272.
- Macho, A. P. and C. Zipfel (2015). "Targeting of plant pattern recognition receptor-triggered immunity by bacterial type-III secretion system effectors." Curr Opin Microbiol **23**: 14-22.
- Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.
- Mendgen, K. and M. Hahn (2002). "Plant infection and the establishment of fungal biotrophy." Trends Plant Sci **7**(8): 352-356.
- Metz, E. C., R. Robles-Sikisaka, et al. (1998). "Nonsynonymous substitution in abalone sperm fertilization genes exceeds substitution in introns and mitochondrial DNA." Proc Natl Acad Sci U S A **95**(18): 10676-10681.
- Meyers, B. C., A. Kozik, et al. (2003). "Genome-wide analysis of NBS-LRR-encoding genes in Arabidopsis." Plant Cell **15**(4): 809-834.
- Millar, A. A. and F. Gubler (2005). "The Arabidopsis GAMBY-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development." Plant Cell **17**(3): 705-721.
- Morris, J. A. and R. D. Morris (2003). "The conservation of redundancy in genetic systems: effects of sexual and asexual reproduction." J Biosci **28**(6): 671-681.
- Ngo, Q. A., H. Vogler, et al. (2014). "A calcium dialog mediated by the FERONIA signal transduction pathway controls plant sperm delivery." Developmental Cell **29**(4): 491-500.
- Nimchuk, Z., T. Eulgem, et al. (2003). "Recognition and response in the plant immune system." Annual Review of Genetics **37**: 579-609.
- Nodine, M. D. and D. P. Bartel (2012). "Maternal and paternal genomes contribute equally to the transcriptome of early plant embryos." Nature **482**(7383): 94-97.
- Nurnberger, T., F. Brunner, et al. (2004). "Innate immunity in plants and animals: striking similarities and obvious differences." Immunol Rev **198**: 249-266.

- Okuda, S., T. Suzuki, et al. (2013). "Acquisition of LURE-Binding Activity at the Pollen Tube Tip of *Torenia fournieri*." Mol Plant **6**(4): 1074-1090.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Palusa, S. G., M. Golovkin, et al. (2007). "Organ-specific, developmental, hormonal and stress regulation of expression of putative pectate lyase genes in *Arabidopsis*." New Phytologist **174**(3): 537-550.
- Pignatta, D., R. M. Erdmann, et al. (2014). "Natural epigenetic polymorphisms lead to intraspecific variation in *Arabidopsis* gene imprinting." Elife **3**: e03198.
- Punwani, J. A., D. S. Rabiger, et al. (2007). "MYB98 positively regulates a battery of synergid-expressed genes encoding filiform apparatus localized proteins." Plant Cell **19**(8): 2557-2568.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Reinders, A., A. B. Sivitz, et al. (2012). "Evolution of plant sucrose uptake transporters." Front Plant Sci **3**: 22.
- Ride, J. P., E. M. Davies, et al. (1999). "Analysis of *Arabidopsis* genome sequence reveals a large new gene family in plants." Plant Mol Biol **39**(5): 927-932.
- Rotman, N., F. Rozier, et al. (2003). "Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*." Current Biology **13**(5): 432-436.
- Schiott, M., S. M. Romanowsky, et al. (2004). "A plant plasma membrane Ca²⁺ pump is required for normal pollen tube growth and fertilization." Proc Natl Acad Sci U S A **101**(25): 9502-9507.
- Schmid, M. W., A. Schmidt, et al. (2012). "A powerful method for transcriptional profiling of specific cell types in eukaryotes: laser-assisted microdissection and RNA sequencing." PLoS One **7**(1): e29685.
- Schornack, S., M. van Damme, et al. (2010). "Ancient class of translocated oomycete effectors targets the host nucleus." Proceedings of the National Academy of Sciences of the United States of America **107**(40): 17421-17426.
- Shen, D., T. Liu, et al. (2013). "Gene duplication and fragment recombination drive functional diversification of a superfamily of cytoplasmic effectors in *Phytophthora sojae*." PLoS One **8**(7): e70036.
- Stam, R., J. Jupe, et al. (2013). "Identification and Characterisation CRN Effectors in *Phytophthora capsici* Shows Modularity and Functional Diversity." PLoS One **8**(3): e59517.
- Stam, R., S. Mantelin, et al. (2014). "The role of effectors in nonhost resistance to filamentous plant pathogens." Front Plant Sci **5**: 582.
- Stec, B. (2006). "Plant thionins--the structural perspective." Cell Mol Life Sci **63**(12): 1370-1385.

- Swanson, W. J. and V. D. Vacquier (2002). "Reproductive protein evolution." Annual Review of Ecology and Systematics **33**: 161-179.
- Takeuchi, H. and T. Higashiyama (2011). "Attraction of tip-growing pollen tubes by the female gametophyte." Curr Opin Plant Biol **14**(5): 614-621.
- Takeuchi, H. and T. Higashiyama (2012). "A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis." PLoS Biol **10**(12): e1001449.
- Tena, G., M. Boudsocq, et al. (2011). "Protein kinase signaling networks in plant innate immunity." Curr Opin Plant Biol **14**(5): 519-529.
- Tian, M., E. Huitema, et al. (2004). "A Kazal-like extracellular serine protease inhibitor from *Phytophthora infestans* targets the tomato pathogenesis-related protease P69B." J Biol Chem **279**(25): 26370-26377.
- Tian, M. Y., B. Benedetti, et al. (2005). "A second kazal-like protease inhibitor from *Phytophthora infestans* inhibits and interacts with the apoplastic pathogenesis-related protease P69B of tomato." Plant Physiol **138**(3): 1785-1793.
- Trouvelot, S., M. C. Heloir, et al. (2014). "Carbohydrates in plant immunity and plant protection: roles and potential application as foliar sprays." Front Plant Sci **5**: 592.
- Tsukamoto, T., Y. Qin, et al. (2010). "A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development." Plant J **62**(4): 571-588.
- Twell, D. (2011). "Male gametogenesis and germline specification in flowering plants." Sexual Plant Reproduction **24**(2): 149-160.
- Tyler, B. M., S. D. Kale, et al. (2013). "Microbe-Independent Entry of Oomycete RxLR Effectors and Fungal RxLR-Like Effectors Into Plant and Animal Cells Is Specific and Reproducible." Molecular Plant-Microbe Interactions **26**(6): 611-616.
- Vidal, S., A. R. B. Eriksson, et al. (1998). "Cell wall-degrading enzymes from *Erwinia carotovora* cooperate in the salicylic acid-independent induction of a plant defense response." Molecular Plant-Microbe Interactions **11**(1): 23-32.
- Wheeler, M. J., B. H. de Graaf, et al. (2009). "Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*." Nature **459**(7249): 992-995.
- Wheeler, M. J., S. Vatovec, et al. (2010). "The pollen S-determinant in *Papaver*: comparisons with known plant receptors and protein ligand partners." Journal of Experimental Botany **61**(7): 2015-2025.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of *Rhododendron* Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.
- Zhu, S., B. Gao, et al. (2005). "Phylogenetic distribution, functional epitopes and evolution of the CSalpbeta superfamily." Cell Mol Life Sci **62**(19-20): 2257-2269.

Appendix 1:

A family of MYB-regulated Sucrose/Proton symporters and their role in pollen tube reception

Alexander R. Leydon, Jennifer J. Forcina, Samantha K. Paul, Alicia B. Sivitz,
Anke Reinders, Mark A. Johnson

Abstract

Pollen tubes navigate a complex extracellular environment in order to deliver sperm to the female gametes for fertilization. During growth in the pistil pollen tubes express three MYB transcription factors (*MYB97*, *MYB101*, and *MYB120*) that promote expression of a group of genes that promote pollen tube reception with the synergid cells. A group of transmembrane carbohydrate/proton transporters in the SUC family (*SUC6*, *SUC7*, *SUC8*, and *SUC9*) requires MYBs for their transcriptional activation in the pollen tube. We demonstrate here that *SUC9* and *SUC7* are expressed in the pollen tube, where they localize to the plasma membrane and ER, respectively. We also demonstrate that they are expressed in the pollen tube during reception in the ovule, and that *SUC9* may promote synergid degeneration in an as yet undetermined manner.

Introduction

Pollen tubes deliver sperm to female gametes over a long cellular distance by extending through a polarized tip-growth mechanism. The dramatic migration of the pollen tube cytoplasm requires coordinated secretion of new cell wall and cell membrane material at the tip (Chebli et al. 2013). Coordinated secretion of receptors through the endoplasmic reticulum (ER) and the trans-golgi network (TGN) to the pollen tube is thought to be critical for perception of extracellular guidance cues such as the *LURE* proteins (Okuda et al. 2009). The secretion of pollen tube guidance receptors is likely coordinated by the ER protein *POD1* (Li et al. 2011). Two cation/proton exchangers (CHX), *CHX21* and *CHX23*, were also demonstrated to be essential for pollen tube guidance in *Arabidopsis*, indicating that potassium (K⁺) transport activity is a critical component of the pollen tube membrane. When pollen tubes reach the ovule they undergo a dynamic cell-cell interaction with the synergid cells that results in pollen tube burst and sperm delivery for double fertilization. The synergid cells mediate this interaction, blocking the pollen tube reception of exotic species in the ovule (Williams et al. 1986; Escobar-Restrepo et al. 2007). The synergid expressed single-pass transmembrane receptor-like kinase protein *FERONIA* (*FER*) (Escobar-Restrepo et al. 2007), seven-pass transmembrane MLO protein *NORTIA* (*NTA*) (Kessler et al. 2010), and the extracellular GPI-linked protein *LORELEI* (*LRE*) (Capron et al. 2008; Tsukamoto et al. 2010) promote the reception of pollen tubes with the synergid. Mutations in any of these three

components results in a loss of pollen tube recognition and subsequent pollen tube coiling within the female gametophyte.

The molecular components that encode the identity of the pollen tube that *FER*, *NTA* and *LRE* recognize in *Arabidopsis* are completely unknown. In Maize, a small synergid-secreted defensin-like cysteine-rich protein ZmES4 induces pollen tube rupture through activation of the inward rectifying K⁺ channel KZM1 on the pollen tube membrane (Cordts et al. 2001; Amien et al. 2010). This action is species-preferential indicating that if the inward rectifying K⁺ channels in *Arabidopsis* pollen tubes are performing a similar role, the ligand for their activity is not conserved. The discovery that three R2R3-MYB transcription factors, *MYB97*, *MYB101*, *MYB120*, control pollen tube reception has opened an experimental inroad into the identity of pollen tube identity genes in *Brassica* (Leydon et al. 2013; Liang et al. 2013). In *myb97-1*, *myb101-4*, *myb120-3* pollen tube reception, there is a marked increase in pollen tube coiling within the ovule, and the synergid cells within ovules receiving a coiling pollen tube frequently fail to degenerate. These MYB transcription factors regulate gene transcription, either directly or indirectly, to promote the transcription of hundreds of genes (Thesis Chapter 5). These MYB-regulated genes provide insight into the classes of genes that create the identity of the pollen tube that the synergid cells perceive. MYB-regulated genes are enriched in three categories: small secreted proteins, extracellular carbohydrate active proteins, and transmembrane proteins with predicted transporter activity (Thesis Chapters 4,5; Leydon et al. 2013).

Among the MYB-regulated transmembrane transporter genes are eight genes in the major facilitator (MF) superfamily involved in carbohydrate/proton symport, including four sucrose/proton symporters (SUC6-9). Sugar-transport genes, such as the SUCs, are predominantly considered to be involved in the cell-to-cell and long-distance distribution of sugars throughout the plant, in a source-to-sink model (Williams et al. 2000; Sivitz et al. 2007). However, there are examples where sugar transport plays a signaling role, as in sensing extracellular sucrose concentrations (Smeekens et al. 1997; Lalonde et al. 1999). Certain sugar transporters are induced during plant-microbe interactions such as *Arabidopsis AtSTP4* during infections with *Alternaria brassicola* or *Fusarium oxysporum* (Truernit et al. 1996). Therefore, the role of sugar transporters SUC6-9 may not be limited to cell metabolism of sugars by the pollen tube. Previous loss of function mutants in SUC9 identified a role for SUC9 in regulating flowering time (Sivitz et al. 2007). The related SUC1 gene is required for pollen tube growth *in vitro* and *in vivo* (Sivitz et al. 2008), yet is not transcriptionally dependent on MYBs in pollen tubes, which display wild type germination and pollen tube growth rates (Leydon et al. 2013). However, the sucrose transporters SUC8 and SUC9 both have well described sucrose transport activity in *Xenopus* oocyte expression systems (Sauer et al. 2004; Sivitz et al. 2007). The role of the SUC genes in pollen tubes remains to be identified.

Here we tested the role of the SUC genes in pollen tube reception. We identified mutations in *SUC7*, *SUC8* and *SUC9* and determined their impact on gene expression. We determined that existing *SUC8* and *SUC7* mutants did not ablate RNA expression and designed alternative approaches to create loss-of-function mutations. We report that the regulation of *SUC7* and *SUC9* requires MYB genes for transcriptional activation. We determined that the *SUC7* gene is not a pseudogene in the pollen tube, and that based on RNA-seq and GFP reporter analysis *SUC7* is expressed. While *SUC9:GFP* is strongly localized to the plasma membrane, we observed co-localization of *SUC7:GFP* with the endoplasmic reticulum. Finally, expression of *SUC7:GFP* and *SUC9:GFP* in *myb* triple-mutant pollen tubes did not rescue pollen tube coiling or fertilization; however *SUC9:GFP* promoted synergid degeneration, the mechanism of which remains to be identified.

Results

Pollen tube growth in the pistil induces a unique transcriptional response (Qin et al. 2009), including a trio of MYB transcription factors required for pollen tube differentiation and proper pollen tube reception (Leydon et al. 2013). *MYB97*, *MYB101* and *MYB120* activate expression of a subset of the pollen tube transcriptome that is specifically enriched in transmembrane localized transport proteins such as Sodium/Hydrogen (Na⁺/H⁺) antiporters (7 CHX genes, p-value=1.3E-5), and carbohydrate/proton symporters in the major facilitator family (8 MF genes, p-value=8.7E-6). Four sucrose/proton symporters in the SUC family, *SUC6*, *SUC7*, *SUC8*, and *SUC9* require *MYB97*, *MYB101*, and *MYB120* for expression (Figure 1). SNP-informed RNA-sequencing between Col and Cvi ecotypes identifies *SUC7* and *SUC9* as pollen specific genes (Thesis Chapter 5). *SUC6-9* are expressed in self-pollinations of both Col and Cvi ecotypes (Figure 1), and are therefore more likely to be involved in conserved pollen tube processes. *myb* triple-mutant pollen tubes are unable to correctly interact with the synergid cells of the female gametophyte, which we hypothesize is due to the ablated expression of pollen tube genes such as *SUC6-9*. Sucrose/H⁺ symporters in the SUC family share a high level of amino acid conservation (Sauer et al. 2004), suggesting that there may be genetic redundancy between family members. We identified available T-DNA insertions in *SUC7-9* and confirmed the location of insertion by PCR (Figure 2A). We excluded *SUC6* from this analysis based upon its minimal change in transcript levels in *myb* mutants (Figure 1) and the absence of available insertions for this gene.

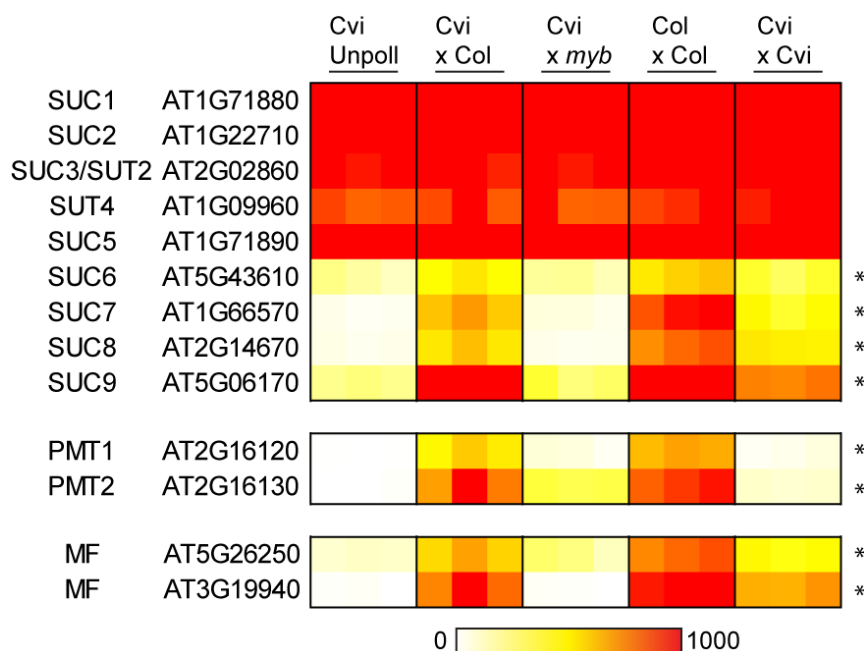


Figure 1. Carbohydrate/H⁺ symporters in the Major Facilitator family are enriched in MYB-regulated target genes. Expression heatmap of selected Major facilitator family transcripts in pistils. Cvi Unpoll – Unpollinated Cvi pistils (mock pollinated 8 hours), Cvi x Col – Cvi pistils pollinated with Col pollen for 8 hours, Cvi x *myb* - Cvi pistils pollinated with *myb* triple-mutant pollen for 8 hours, Col x Col – Col pistils pollinated with Col pollen for 8hours, Cvi x Cvi – Cvi pistils pollinated with Cvi pollen for 8 hours. Sequencing data is shown in three columns per condition, one column per biological replicate (See Thesis Chapter 5). Asterisks indicate significant gene expression differences between Cvi x Col and Cvi x *myb* triple-mutant pollinations (adjusted p-value <0.05). PMT – Polyol/monosaccharide transporter. MF – Major facilitator, no known substrate.

One insertion line in SUC7 was identified, where the T-DNA (SAIL_221_C05) falls 110 base pairs before the start codon. Previous studies of the SUC7 RNA transcript in *Arabidopsis* ecotypes (Col-0, C24, Ler, Ws) found that SUC7 splicing was aberrant, causing a loss of functional transporter activity (Sauer et al. 2004). We observed two splice-forms of SUC7 in RT-PCR analysis of wild-type floral tissue (Figure 2B). The SUC7 RT-PCR product of larger size is consistent with inclusion of exon 2, which was confirmed by full length cDNA cloning and sequencing. Surprisingly, the *suc7-1* insertion promoted the inclusion of exon 2,

indicating that as opposed to reducing functional mRNA transcripts, *suc7-1* increases the total functional pool of SUC7 mRNA. This allele is therefore not ideal for loss of function analysis, and we have designed CRISPR guide RNAs targeting SUC7 for targeted gene deletion and future analysis.

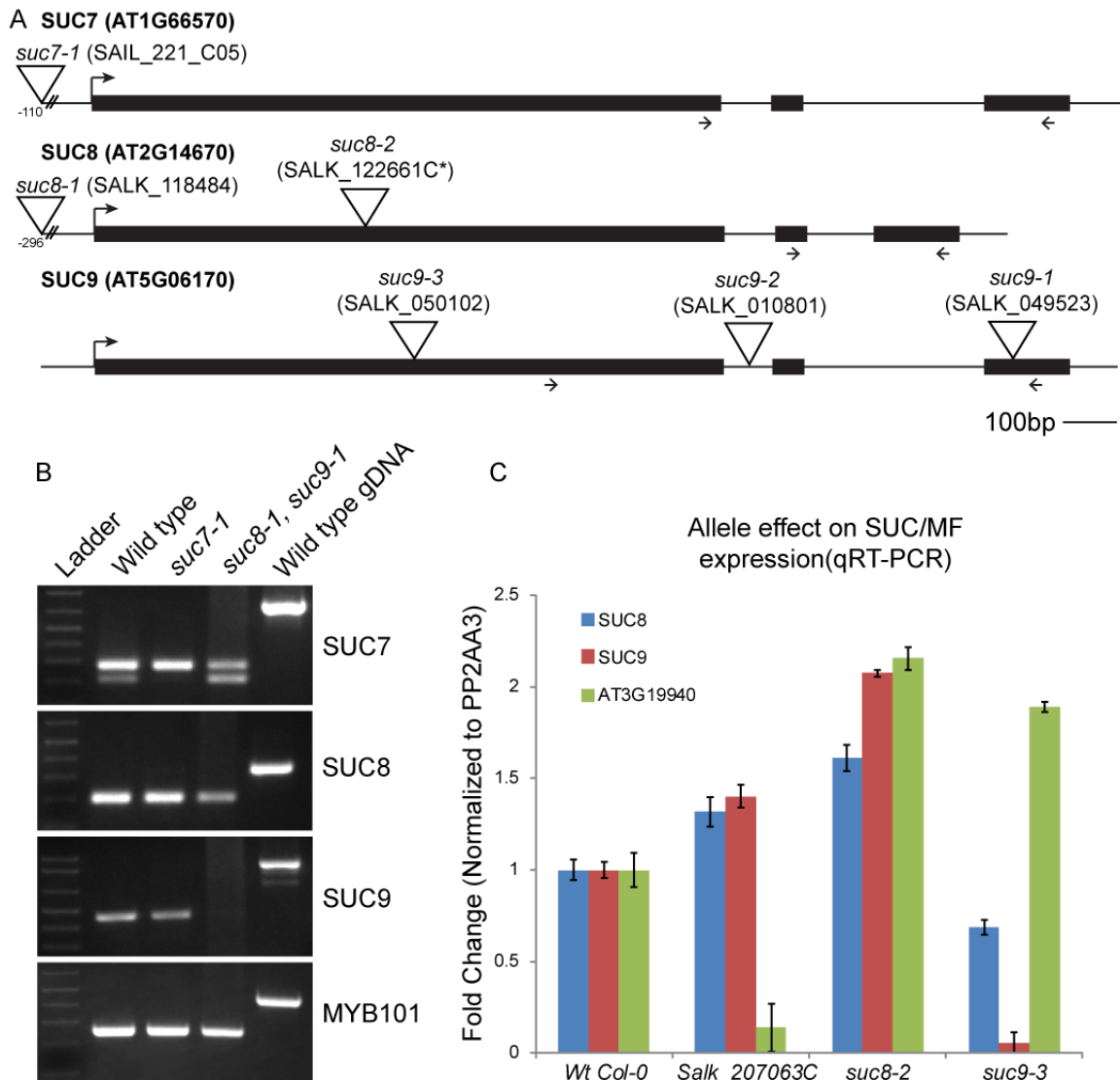


Figure 2. Insertional mutants in MYB-regulated SUC genes, SUC7, SUC8, and SUC9, and their effect on RNA accumulation. A. Gene loci of SUC7, SUC8, and SUC9 with T-DNA insertion positions (white triangle) Arrows indicate the position of primer pairs used in B. The asterisk by SALK_122661C indicates that this allele was identified as a second site mutation in an existing SALK insert line (T-DNA-Seq, O'Malley, R.C., 2014 - Unpublished). **B.** RT-PCR analysis of *suc* mutants. Control RT-PCR primers span the last intron of the MYB101 gene.

C. RT-qPCR analysis of SALK_207063C – AT3G19940, *suc8-2*, and *suc9-3*. Expression levels were normalized to PP2AA3 transcript levels.

In the *SUC8* genomic locus, we tested the effect of two T-DNAs, *suc8-1*, and *suc8-2*. Like, *suc7-1*, *suc8-1* falls in the upstream promoter region of the *SUC8* gene (Figure 2A), and only slightly affects the accumulation of the *SUC8* mRNA (Figure 2B). The *suc8-2* insertion was identified as a second site mutation in an existing SALK line, (T-DNA-seq) and we confirmed the left border interrupts the 1st exon of *SUC8* by DNA sequencing. This allele introduces five amino acids (EQIDA) and early stop codon after the fourth transmembrane domain, which is predicted to render any protein product non-functional. Despite its position early in the first exon, mRNA is detected downstream of the *suc8-2* insert site (Figure 2C), which may result from introduction of the T-DNA right border sequence into the *SUC8* locus. Each of the three *suc9* mutants tested were found to be RNA null (Figure 2, Sivitz et al. 2007).

Expression analysis of *SUC8* and *SUC9* using GUS reporter fusions had previously identified low levels of expression in plant vasculature (Sauer et al. 2004; Sivitz et al. 2007). We tested the expression pattern of *SUC9* in pollen using a *SUC9:GFP* reporter that incorporates the endogenous promoter, and genomic locus fused to the coding sequence of GFP.

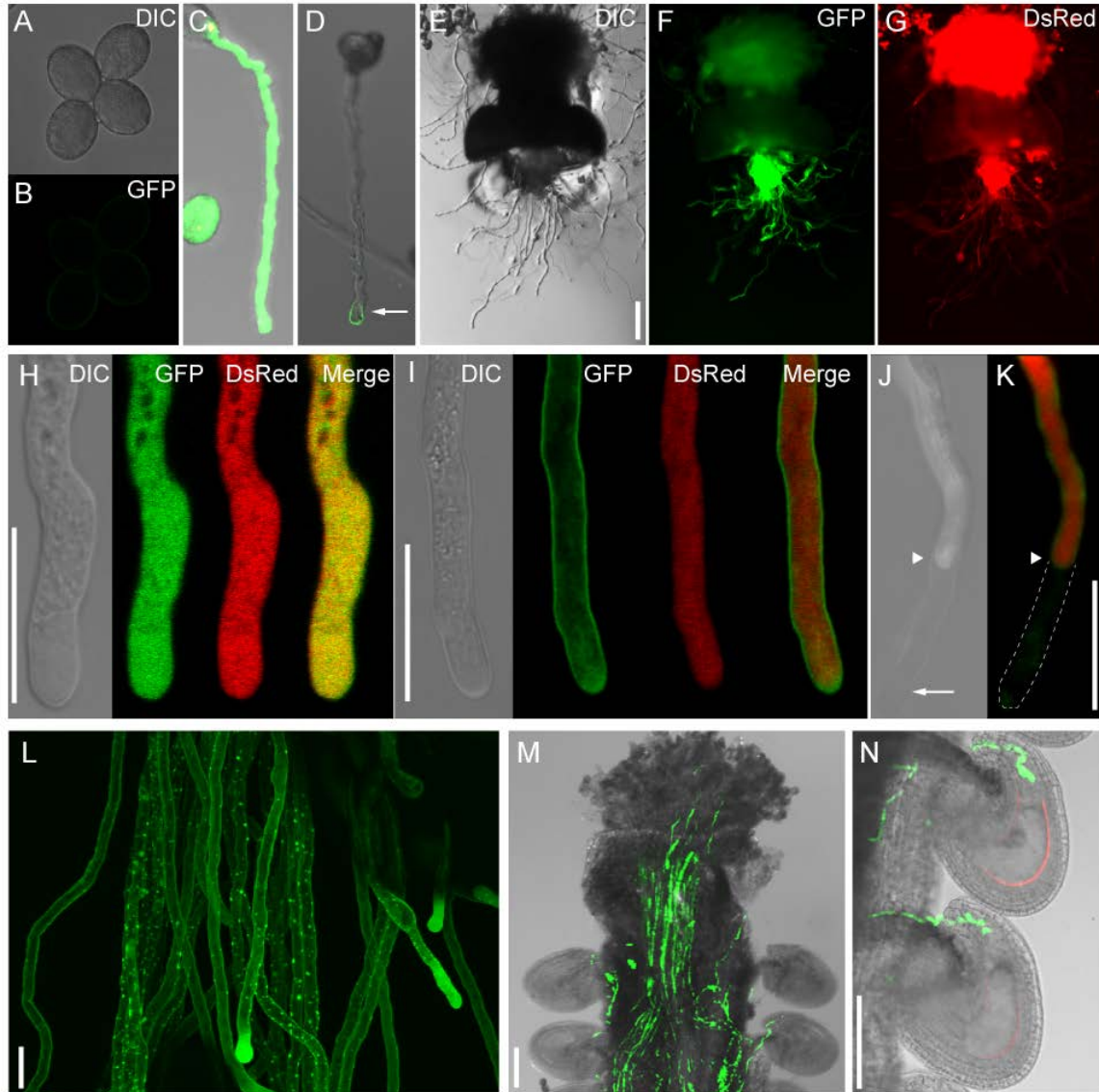


Figure 3. *SUC9:GFP* is induced by pollen tube growth in the pistil. A,B. *SUC9:GFP* is not expressed in mature pollen. **C,D.** *SUC9:GFP* is rarely detected in pollen tubes grown in vitro for 6 hours. **C.** *LAT52:DsRed*, *HTR10:RFP* pollen tubes **D.** *SUC9:GFP* in vitro grown pollen tubes, arrowhead indicates *SUC9:GFP* expression. **E-G.** *SUC9:GFP* is induced by semi in vivo pollen tube growth (SIV-PT), Scale bar = 100um. **H.** *pSUC9:GFP* – *SUC9* promoter is sufficient to drive SIV PT expression (*pSUC9:GFP*, *LAT52:DsRed*, SIV-PT). **I.** *SUC9:GFP* is localized to the plasma membrane (*SUC9:GFP*, *LAT52:DsRed*, SIV-PT). **J,K.** Plasmolysis of SIV-PT *SUC9:GFP*, *LAT52:DsRed*. H-K – scale bars = 20um. Arrow – tube tip, arrow head - cytoplasm. **L.** 3d projection of *SUC9:GFP*, SIV-PTs exhibiting plasma membrane and tip localization, as well as punctate expression patterns, scale bar = 20um. **M.** *SUC9:GFP* pollen tube growth in the pistil, 3d projection of confocal micrographs, scale – 100um. **N.** *SUC9:GFP* expression in pollen tubes that have targeted wild-type (*ms1*) ovules, 12 hours after pollination, scale bar = 100um.

We observed no detectable expression in mature pollen grains (Figure 3A,B), and only rarely did we detect low levels of GFP signal in pollen tubes grown *in vitro* (Figure 3C,D). However, growth through the stigma and style in semi *in vivo* pollen tube growth assays (SIV) strongly induced *SUC9:GFP* expression in every transgenic line tested (Figure 3E-G, 56/56). We tested the *SUC9* promoter sequence alone and observed the same expression domain, indicating that *SUC9* expression in the male gametophyte is driven by the Promoter sequence and does not require the genic sequences as was observed for sporophytic expression of the *SUC9:GUS* constructs (Sivitz et al. 2007). *SUC9:GFP* expression in pollen tubes is clearly localized to the plasma membrane, and is also observed in secretory vesicles at the pollen tube tip (Figure I). Plasmolysis of SIV pollen tubes reveals the contracted pollen tube cytoplasm (Red) and membrane *SUC9:GFP* signal (Figure 3K, Green). As pollen tubes grow out from the bottom of the style in SIV assays, *SUC9:GFP* can be seen enriched at pollen tube tips, and remaining in the pollen tube shank, post callose plug formation (punctate signal in central tube bundle, Figure 3L). Pollen tubes growing *in vitro* synthesize high levels of *SUC9:GFP*, allowing visualization of pollen tube growth in the style, transmitting tract (Figure 3M), and even pollen tube burst in the ovule (Figure 3N).

We tested the expression of the *SUC9:GFP* construct in wild-type and *myb* triple-mutant genotypes. *SUC9:GFP* was introduced by floral dip into *myb* triple-mutant plants and primary transformants were analyzed for GFP signal in self-pollinated T1 SIV explants. *SUC9:GFP* expression was observed in all primary

transformants (56/56), while no transformants in the *myb97-1*, *myb101-4*, *myb120-3* background expressed *SUC9:GFP* (21/21, Figure 4A-F). Introduction of wild type MYB genes by crossing reactivated *SUC9:GFP* expression (4/4 lines, Figure 4G-H). This observation provides a complementary method that corroborates MYB-regulation of *SUC9* transcription.

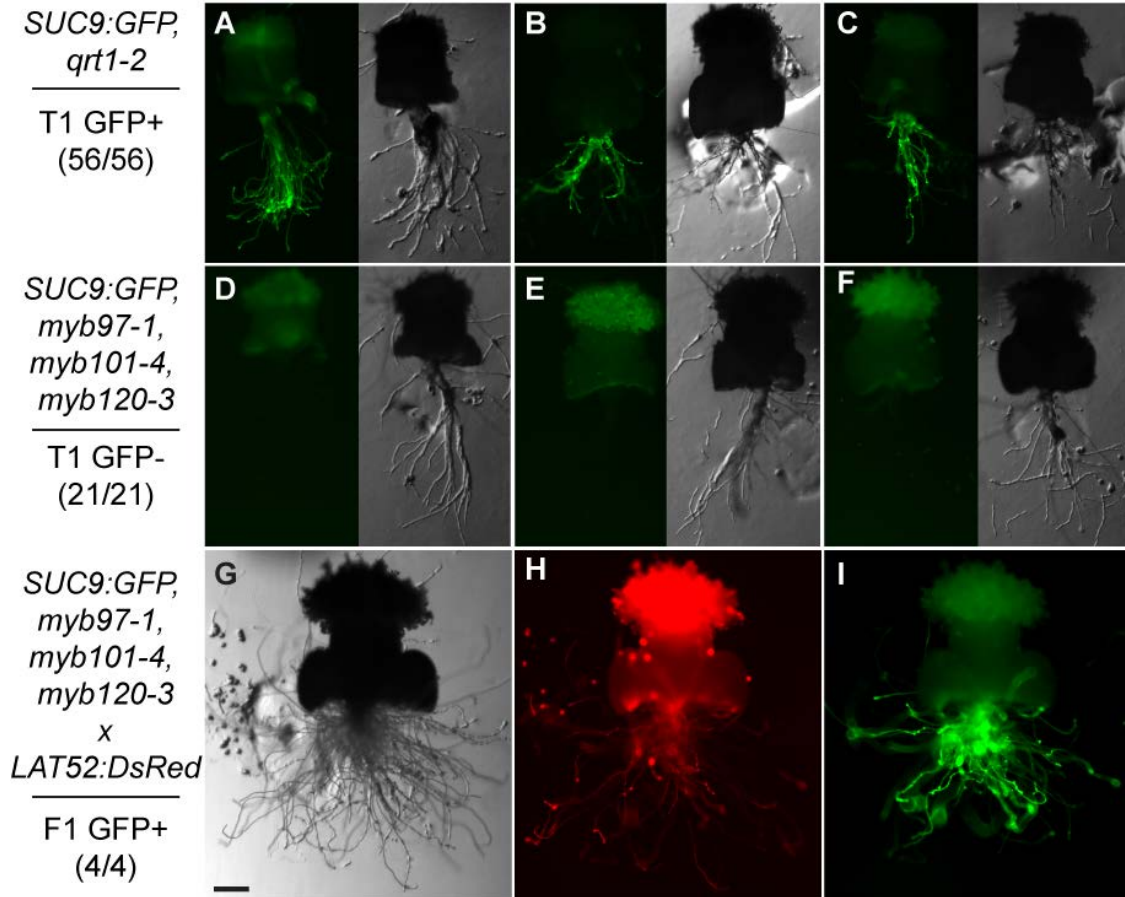


Figure 4. *SUC9:GFP* requires *MYB97*, *MYB101*, *MYB120* for expression in pollen tubes. **A-C.** Three individual *SUC9:GFP* primary transformants in wild type, *qrt1-2* background. **D-F.** Three individual *SUC9:GFP* primary transformants in *myb* triple-mutant background. Each panel is a side-by-side (GFP/DIC) epifluorescent micrograph of 1 SIV experiment. **G-I.** Representative image of SIV from 1 F1 (*SUC9:GFP*, *myb97-1*, *myb101-4*, *myb120-3* x *LAT52:DsRed*, *qrt1-2*) plant. Panels are G - DIC, H - DsRed, I - GFP. Summaries of total T1 expression numbers is shown at left of each set of panels.

SUC7 was previously reported to be a pseudogene due to observations of aberrant splicing in sporophytic tissues (Sauer et al. 2004). We cloned full length cDNAs that represented two populations of SUC7, one with exon 2, and one without exon 2. We therefore examined our RNA-sequencing from wild-type pistils (Cvi x Col) and observed reads that demonstrated inclusion of exon 2, and correct splicing (Figure 5A, B). Therefore, we hypothesized that some fraction of the total *SUC7* mRNA pool contains the second exon, which is required to form a functional transporter (Sauer et al. 2004). In order to directly visualize the production of correctly spliced SUC7, we cloned the SUC7 genomic locus with ~1Kb of presumed promoter sequence into frame with GFP (Figure 5C). This construct will only be visible if exon 2 is spliced correctly, as exon exclusion results in an early stop codon before the GFP coding sequence. Furthermore, this construct allows us to test the subcellular localization of SUC7 in pollen tubes.

We transformed wild type and *myb* triple-mutant plants with the genomic SUC7:GFP reporter construct and quantified the expression of SUC7:GFP in SIV grown pollen tubes. We observed GFP positive pollen tubes in wild type primary transformants (10/18) but never in *myb* triple-mutant primary transformants (26/26). The average signal intensity of *SUC7:GFP* lines was considerably lower than *SUC9:GFP* lines. To our surprise, the subcellular localization of *SUC7:GFP* was not consistent with membrane localization. Unlike *SUC9:GFP*, *SUC7:GFP* is

found in cytoplasmic strands, that are excluded from the pollen tube tip (Figure 6G,H).

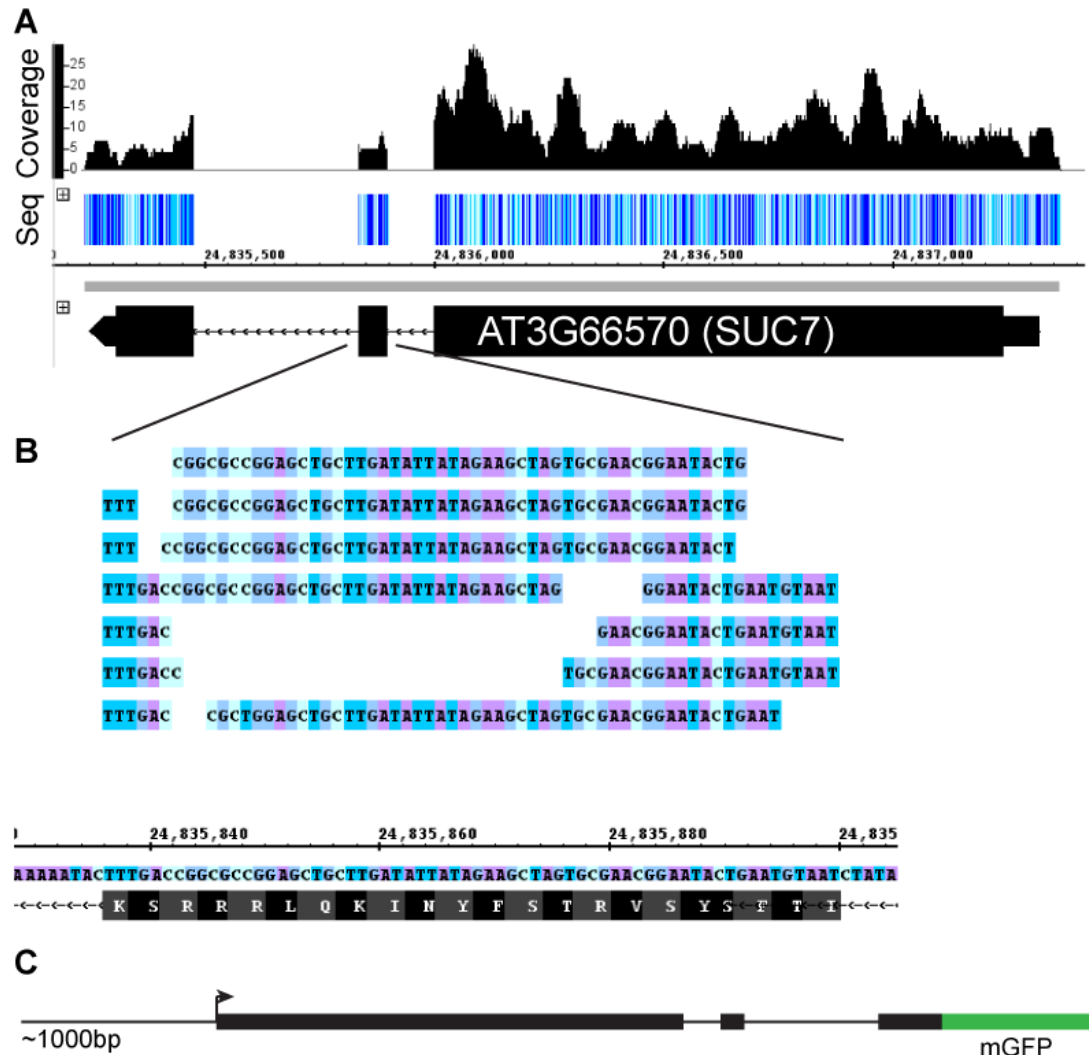


Figure 5. RNA-sequencing reveals that *SUC7* is correctly spliced in reproductive tissues. **A.** *SUC7* gene locus with RNA sequencing data from Cvi x Col 8 hour crosses. Coverage was calculated based on a representative sequencing library in IGB. ‘Seq’ track represents the nucleotide sequence in blue, and the TAIR10 gene locus for *SUC7* is shown below. **B.** Mapped reads are shown for exon 2, with spliced-reads at junctions. Genomic sequence is shown below the sequence line, as is the amino acid sequence encoded by exon 2. **C.** Gene map of the genomic *SUC7*:GFP reporter.

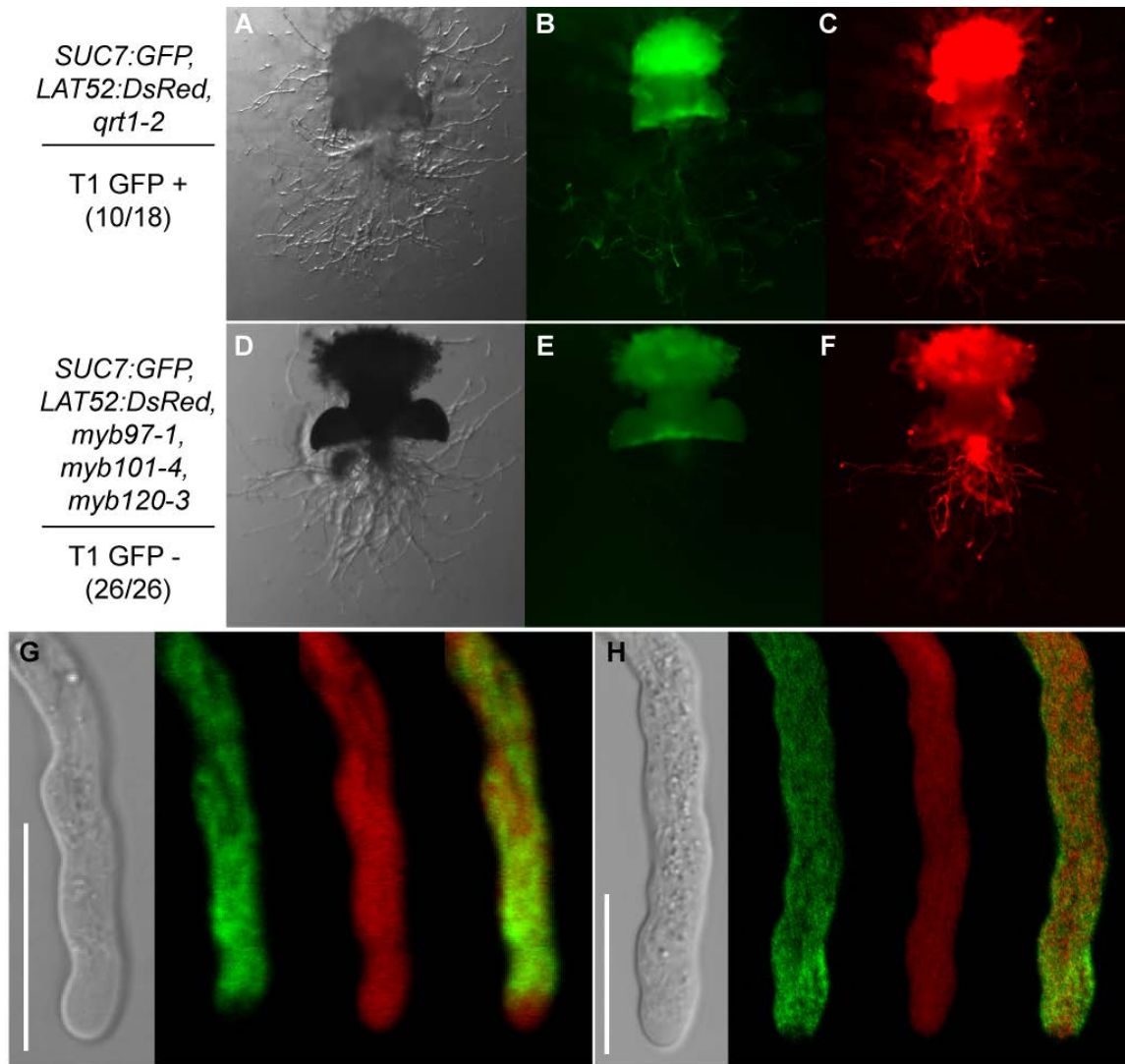


Figure 6. SUC7:GFP is not localized to the plasma membrane, and requires MYB97, MYB101, MYB120 for expression. A-F. Representative SIV PT assays of *SUC7:GFP*, *LAT52:DsRed* in wild type (A-C), and *myb* triple-mutant (D-F). Quantification of T1 lines is displayed at left. **G,H.** Confocal micrograph of *SUC7:GFP* pollen tubes, panels are DIC, GFP, DsRed, and Merge, scale bar – 20μm. **G.** Genomic *SUC7:GFP*, *LAT52:DsRed*, *qrt1-2* SIV PT. **H.** BGAL11:*SUC7:GFP*, *LAT52:DsRed*, *myb97-1*, *myb101-4*, *myb120-3* in vitro PT.

Due to the disparity between the *SUC9:GFP* and *SUC7:GFP* subcellular localization that we observed in SIV-PTs, we tested the subcellular localization of *SUC9* and *SUC7* in a complementary system. We cloned cDNA sequences of each *SUC* into an expression vector under a strong viral promoter (35S) and

performed transient expression in *N. Benthamiana*. Leaves were co-infiltrated with known markers that localize to discrete subcellular locations and then analyzed by confocal microscopy. SUC9:GFP was observed to localize to the plasma membrane (Figure 7A), and not with ER or Golgi markers (Figure 7B,C).

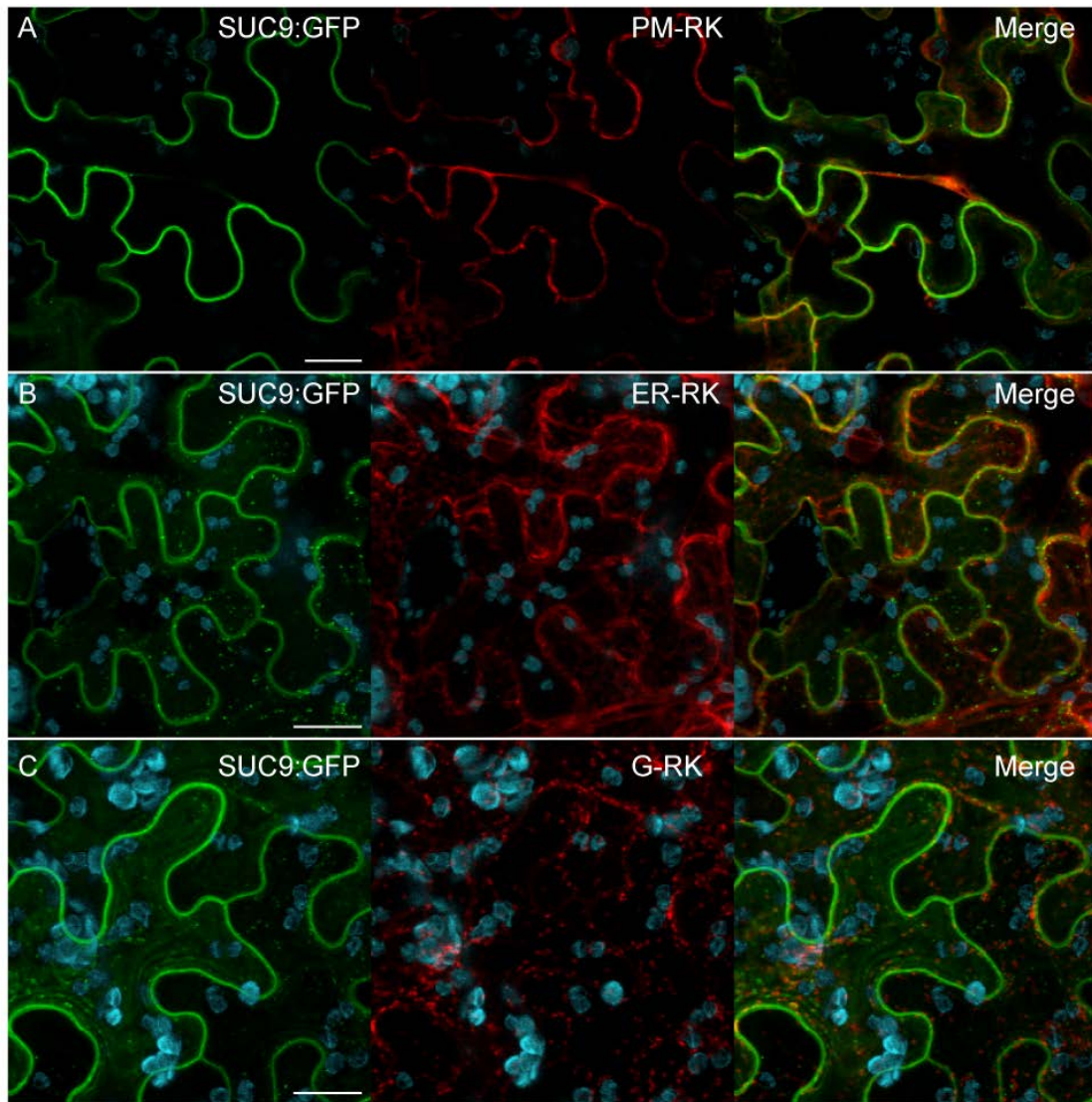


Figure 7. SUC9:GFP localizes to the plasma membrane. A-C. 3d projections of confocal micrographs, Panels are GFP, DsRed, Merge from left to right. Scale bars are 20um. **A.** 35S:SUC9:GFP + red fluorescent plasma membrane marker PM-RK. **B.** 35S:SUC9:GFP + red fluorescent endoplasmic reticulum marker ER-RK. **C.** 35S:SUC9:GFP + red fluorescent golgi marker G-RK. Markers are described in (Nelson et al. 2007).

SUC7:GFP displayed strong co-localization with ER markers in transient transformations (Figure 8A). Additionally, in leaf infiltrations that were tested after long periods of infection, large masses of over-expressed SUC7:GFP was observed surrounding the nucleus (Figure 8B). Closer analysis of cells over-expressing SUC7:GFP revealed bubbles of ER-RK reporter coated with SUC7:GFP, supporting the interpretation that SUC7 is ER-localized (Figure 8C).

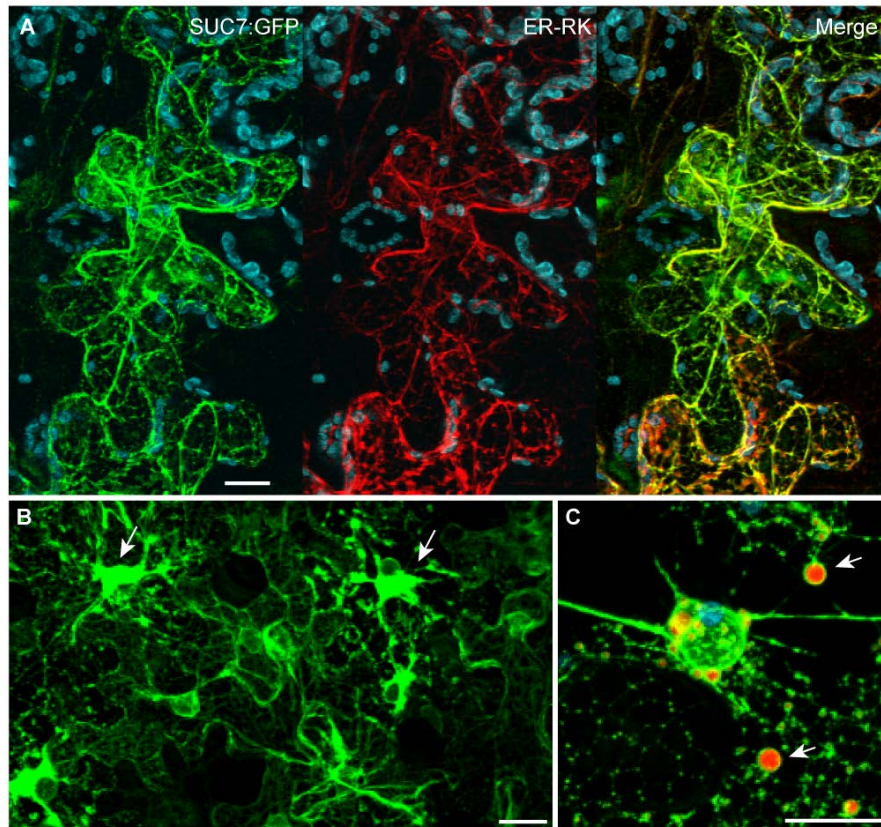


Figure 8. SUC7:GFP localizes to the ER. **A.** SUC7:GFP co-localization with red fluorescent endoplasmic reticulum marker, ER-RK. **B.** SUC7:GFP overexpression localized around nuclei. **C.** Close-up view of expanded SUC7:GFP coated, ER bubbles. All images are 3d projection of confocal micrographs. Scale bars – 20um.

Expression of *SUC9:GFP* in pollen tubes allowed the observation of pollen tube morphology after pollen tube growth, as SUC9:GFP strongly labels the pollen

tube membrane. While no conclusive dominant effect can be correlated to expression of *SUC9:GFP* at this time, we observed qualitative defects in *SUC9:GFP* pollen tube morphology after extended periods of *in vitro* pollen tube growth (>12 hours) growth. We observed two main types of pollen tube morphological defects. First, the production of secondary and distorted pollen tube tips (Figure 9A,B). Second, membrane blebs that escaped the pollen tube cell wall (Figure 9C-E). These defects were not observed as severely in pollen tubes grown in the SIV-PT growth assay. This may indicate that the process of pollen tube growth in the pistil has a protective effect on pollen tubes. Alternatively, *in vitro* pollen tube growth for extended periods may be detrimental to pollen tube physiology. Future work is required to address the role that *SUC9* plays in controlling membrane dynamics in the pollen tube.

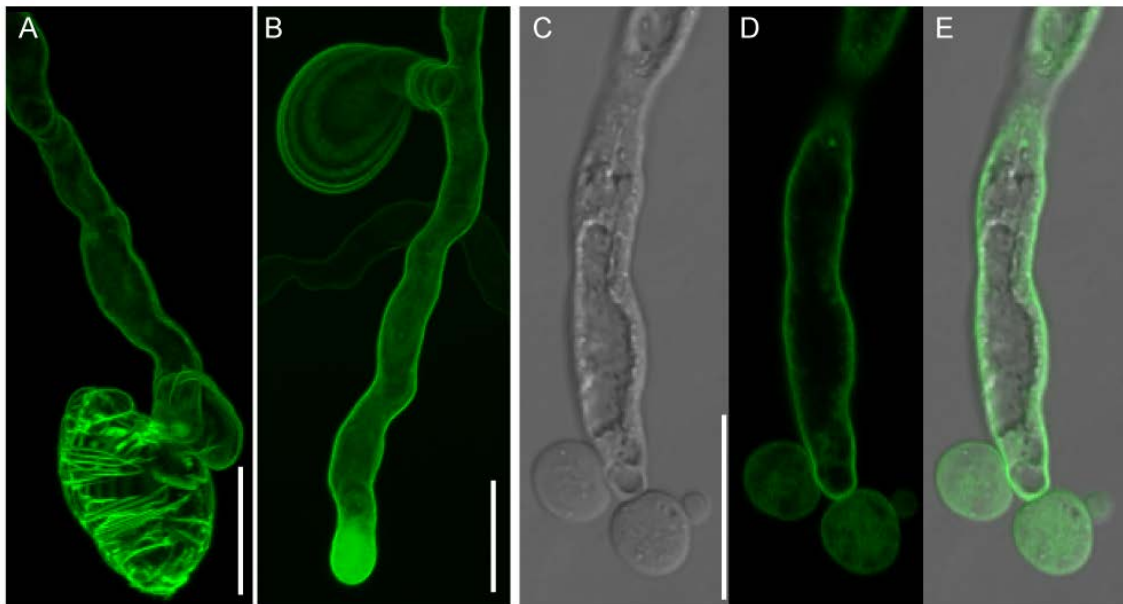


Figure 9. Pollen tube defects in *SUC9:GFP* pollen tubes. A-E. Genomic *SUC9:GFP* pollen tubes imaged by confocal microscopy after extended pollen tube growth *in vitro*. **A.** Distorted pollen tube tip. **B.** Branched pollen tube, with a swollen second tip. **C-E.** Pollen cytoplasmic blebs protrude through the cell wall. Scale bars = 20um.

As genetic analysis of the SUC genes is still in preliminary stages, we performed an alternative method of genetic analysis to identify the role of the SUCs in pollen tube reception. We utilized an 'Add back' strategy, which has been previously described (Thesis Chapters 6-7). Briefly, we cloned cDNAs from SUC7 and SUC8 into an expression vector that does not require MYB97, MYB101, MYB120 for expression, which were introduced into *myb* triple-mutant, *LAT52:DsRed* plants. The ability to rescue or modify the *myb* triple-mutant phenotype was quantified with pollen from T1 hemizygous plants and *ACT11:MSI1:GFP* pistils (Ingouff et al. 2007). SUC add back constructs were not capable of modifying the *myb* triple-mutant phenotype with respect to pollen tube targeting, or pollen tube coiling rates (Figure 10A). However, SUC9:GFP expression in *myb* triple-mutant pollen tubes did promote slightly higher synergid degeneration rates, which was only barely evident in SUC7:GFP add back (Figure 10A). Consistent with this observation, no SUC add back constructs were capable of rescuing pollen tube burst or seed production (Figure 10B).

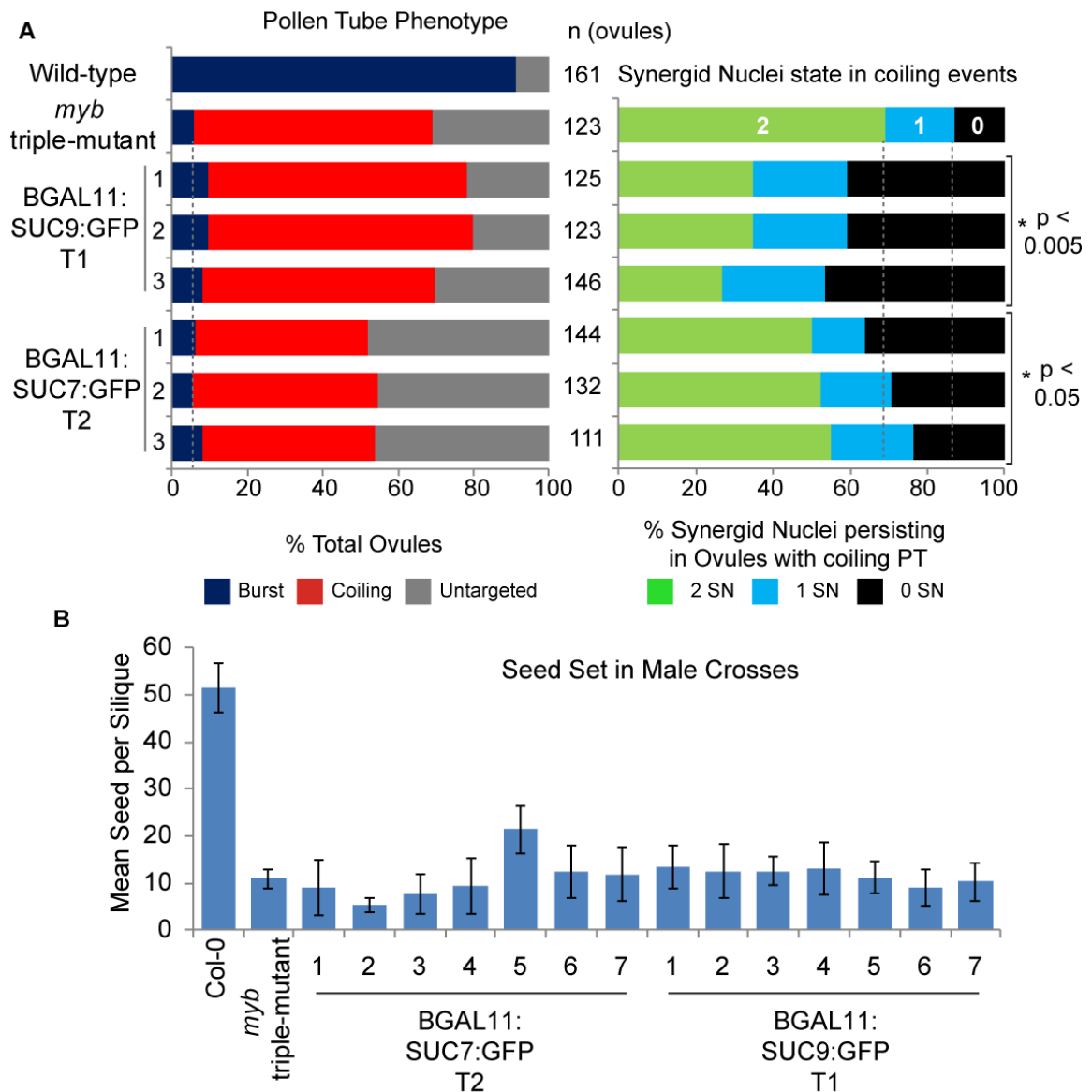


Figure 10. SUC:GFP expression in *myb* triple-mutant pollen tubes increase synergid degeneration without affecting pollen tube burst. A. Pollen tube phenotypes 12 hours after pollination. *ACT11:MSI1:GFP* pistils were emasculated and crossed with *SUC:GFP*. *myb* triple-mutant pollen for 12 hours before dissection and quantification by confocal microscopy. Data on the right is paired with the red “Coiling” fraction shown on the left. Synergid numbers are indicated by position and by color, from left to right, Green – 2 synergid nuclei, Blue – 1 synergid nuclei, Black – 0 synergid nuclei. Data is shown for three T1 lines that were tested for expression of *SUC:GFP*. n (ovules) – refers to total number of ovules, 3 pistils per experiment. 1-3 – indicate three independent transformant lines, with *SUC:GFP* expression. P-values – chi-squared test.

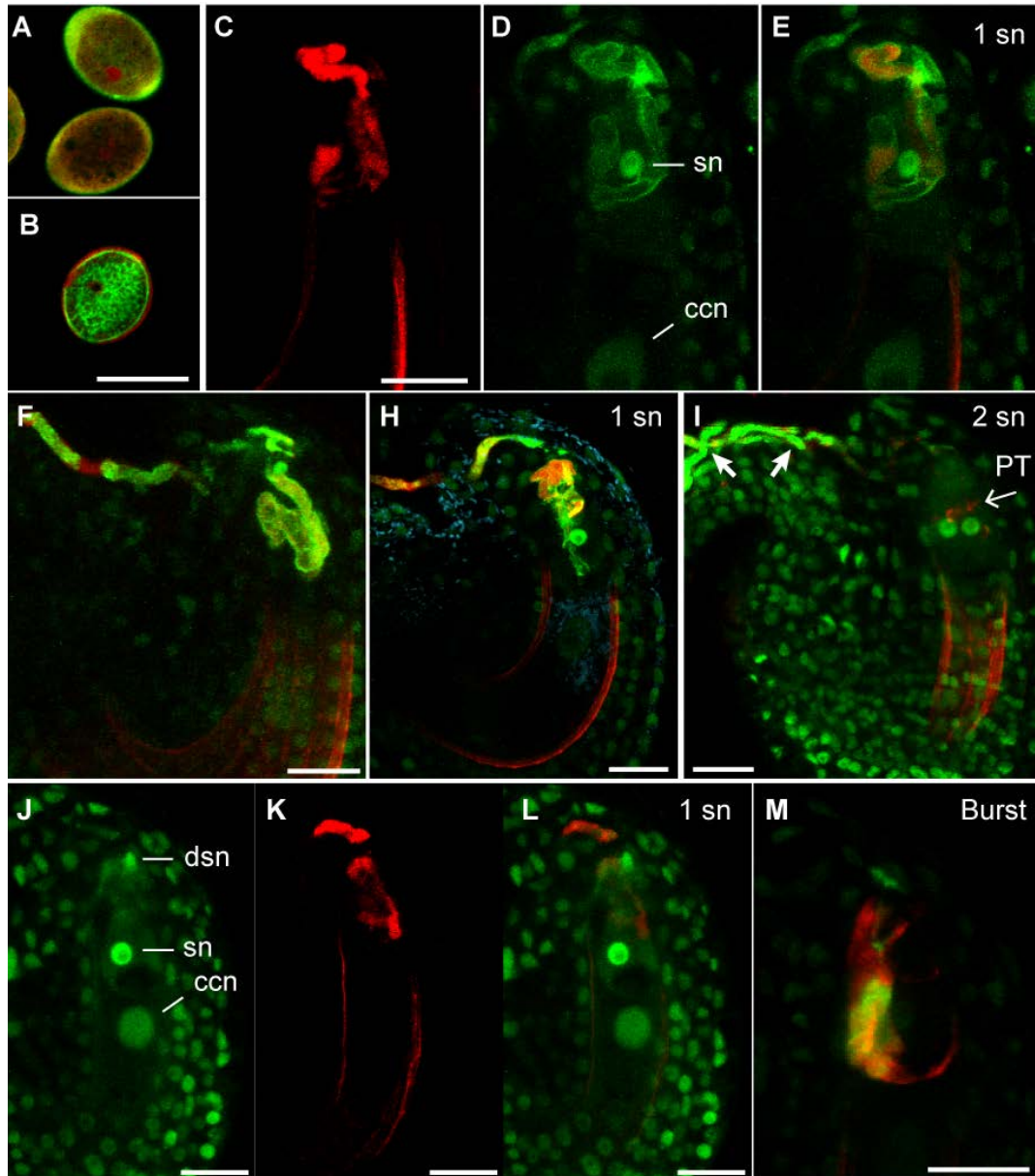


Figure 11. SUC:GFP proteins influence synergid degeneration. **A.** *BGAL11:SUC9:GFP* and **B** *BGAL11:SUC7:GFP* expression in *myb* triple-mutant, *LAT52:DsRed* mature pollen. **C-E.** *BGAL11:SUC9:GFP* pollen tube coiling in an *ACT11:MSI1:GFP* ovule, where 1 synergid has degenerated. **C** – DsRed, **D** – SUC9:GFP, **E** – Merge. **F,H.** Additional representative images of pollen SUC9:GFP pollen tube coiling. **I-M.** *BGAL11:SUC7:GFP* pollen tube reception images. **I.** SUC7:GFP positive pollen tubes (arrows) targeting an ovule that has received a GFP negative PT. **J-L.** SUC7:GFP pollen tube that is coiling in an ovule with 1 degenerated synergid. **M.** SUC7:GFP pollen tube that has burst into a degenerated synergid. All images are 3d projections of confocal micrographs, 20um, dsn – degenerated synergid nucleus, sn – synergid nucleus, ccn – central cell nucleus.

Discussion

The major facilitator superfamily is a highly diverse family found throughout every kingdom, capable of transporting a wide range of compounds (Pao et al. 1998). The sucrose/proton symporter family includes diversely expressed members, which catalyze the symport of sucrose and protons. *Arabidopsis* SUC1 is localized to the pollen grain where it is required for pollen tube germination and growth (Sivitz et al. 2008). SUC2 is required for loading sucrose into phloem tissues (Gottwald et al. 2000). SUC4 and TMT1/2 localize to the vacuole where they control loading of glucose and sucrose (Schulz et al. 2011). SUC5 is expressed in the endosperm (Baud et al. 2005). SUC9 localizes to the membrane of pollen tubes, where it is most abundantly expressed (Figure 3) and in *N. benthamiana* leaves (Figure 8). Surprisingly, SUC7 is not a pseudogene as was previously predicted based upon cloned splicing results (Sauer et al. 2004). Furthermore, SUC7 is localized to the ER, not the plasma membrane in both pollen tubes (Figure 6G,H) and in *N. benthamiana* leaves (Figure 8). Preliminary analysis of a SUC8:GFP reporter displayed a clear membrane localization in primary transformant SIV pollen tubes (Data not shown). Given the high level of sequence similarity between SUC7 and SUC8 (473/492 identical residues - 96%) there are a limited number of amino acids that direct SUC7 ER localization, which in the future could be tested in sugar uptake assays.

The pollen tube growth and morphology of SUC9:GFP has not indicated a strong dominant phenotype in pollen tube growth. After we determine a loss of function

mutant phenotype in the SUC7-9 genes, we will test whether these constructs are functional in pollen tubes. We did frequently observe pollen tube blebbing and membrane swelling in SUC9:GFP pollen tubes (Figure 9). It is possible that this represents a gain of function phenotype, but that wild-type pollen tubes are highly resistant to fluctuations in SUC activity, rendering the effects marginal. We would like to test this hypothesis in future experiments using sugar feeding assays on pollen tubes that overexpress SUC:GFP fusion proteins. Pollen tube morphology defects were observed more frequently in pollen tubes that had grown for extended periods *in vitro*, as compared to SIV grown pollen tubes. Pollen tubes grown *in vitro* express different genes than pollen tubes that have penetrated the stigma and style (Qin et al. 2009). Therefore, the transcriptional induction of pollen tube growth in the pistil may induce gene expression that promotes normal pollen tube physiology.

We have identified a likely loss of function mutation in SUC8, however there are no available mutations in SUC7. Therefore we have designed an alternative method to genetically engineer strains lacking SUC7 by CRISPR/CAS9 mutagenesis, which we will introduce into *suc8*, *suc9* double mutants to create a triple mutant strain. We will analyze the ability of *suc7*, *suc8*, *suc9* triple-mutant pollen to grown pollen tubes and interact with wild type ovules. Expression of SUC7:GFP and SUC9:GFP in *myb* triple-mutant pollen tubes demonstrated that neither fusion protein was capable of rescuing *myb* triple-mutant pollen tube targeting or coiling rates (Figures 10,11). However SUC9:GFP expression in the *myb* triple-mutant pollen tube did increase the frequency that synergid cells

degenerated in the presence of a coiling pollen tube (Figure 10). It is possible that membrane sucrose/proton transport signals the exocytosis of pollen tube components to the synergid cells to promote degeneration, such as is observed with Thionin proteins (Thesis Chapter 6). Alternatively, the pollen tube may swell to larger sizes in the presence of SUC9, increasing the physical interaction of the pollen tube and the synergid cell, inducing higher rates of degeneration. These models will be investigated in the future through time-lapse microscopy of *BGAL11:SUC9:GFP*, *myb* triple-mutant tubes in *ACT11:MSI1:GFP* ovules. Imaging pollen tube membrane dynamics in *suc7*, *suc8*, *suc9* triple-mutant pollen tubes may also reveal disruptions of membrane identity or secretion zones.

Methods

Plant growth

Procedures for selection of antibiotic and/or herbicide resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; von Besser et al. 2006; Qin et al. 2009; Wong et al. 2010).

Cloning

For cDNA cloning RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis was performed with Superscript III (Invitrogen), and PCR used Phusion Polymerase (NEB). cDNA sequences were cloned into entry vectors pENTR-D or pCR8 (Invitrogen), and LR cloned into destination vectors with LR clonase II (Invitrogen). All clones were sequenced before introduction to plants (Genewiz).

Phylogenetic analysis

Phylogenetic relationships were analyzed using MEGA software (Tamura et al. 2013). Heat trees were constructed from sequencing data (Thesis Chapter 5)

Reception Microscopy

For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated *ACT11:MSI1:GFP*, *FGR7.0* stage 14 stigmas (Smyth et al. 1990) with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al.

2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

References

- Amien, S., I. Kliwer, et al. (2010). "Defensin-like ZmES4 mediates pollen tube burst in maize via opening of the potassium channel KZM1." PLoS Biol **8**(6): e1000388.
- Baud, S., S. Wullemme, et al. (2005). "The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis." Plant Journal **43**(6): 824-836.
- Capron, A., M. Gourgues, et al. (2008). "Maternal Control of Male-Gamete Delivery in Arabidopsis Involves a Putative GPI-Anchored Protein Encoded by the LORELEI Gene." Plant Cell **20**(11): 3038-3049.
- Chebli, Y., J. Kroeger, et al. (2013). "Transport logistics in pollen tubes." Mol Plant **6**(4): 1037-1052.
- Cordts, S., J. Bantin, et al. (2001). "ZmES genes encode peptides with structural homology to defensins and are specifically expressed in the female gametophyte of maize." Plant J **25**(1): 103-114.
- Escobar-Restrepo, J. M., N. Huck, et al. (2007). "The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception." Science **317**(5838): 656-660.
- Gottwald, J. R., P. J. Krysan, et al. (2000). "Genetic evidence for the in planta role of phloem-specific plasma membrane sucrose transporters." Proc Natl Acad Sci U S A **97**(25): 13979-13984.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Kessler, S. A., H. Shimamoto-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Lalonde, S., E. Boles, et al. (1999). "The dual function of sugar carriers. Transport and sugar sensing." Plant Cell **11**(4): 707-726.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Li, H. J., Y. Xue, et al. (2011). "POD1 Regulates Pollen Tube Guidance in Response to Micropylar Female Signaling and Acts in Early Embryo Patterning in Arabidopsis." Plant Cell **23**(9): 3288-3302.
- Liang, Y., Z. M. Tan, et al. (2013). "MYB97, MYB101 and MYB120 Function as Male Factors That Control Pollen Tube-Synergid Interaction in Arabidopsis thaliana Fertilization." PLoS Genet **9**(11).

- Nelson, B. K., X. Cai, et al. (2007). "A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants." Plant J **51**(6): 1126-1136.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Pao, S. S., I. T. Paulsen, et al. (1998). "Major facilitator superfamily." Microbiol Mol Biol Rev **62**(1): 1-34.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Sauer, N., A. Ludwig, et al. (2004). "AtSUC8 and AtSUC9 encode functional sucrose transporters, but the closely related AtSUC6 and AtSUC7 genes encode aberrant proteins in different Arabidopsis ecotypes." Plant J **40**(1): 120-130.
- Schulz, A., D. Beyhl, et al. (2011). "Proton-driven sucrose symport and antiport are provided by the vacuolar transporters SUC4 and TMT1/2." Plant Journal **68**(1): 129-136.
- Sivitz, A. B., A. Reinders, et al. (2007). "Arabidopsis sucrose transporter AtSUC9. High-affinity transport activity, intragenic control of expression, and early flowering mutant phenotype." Plant Physiol **143**(1): 188-198.
- Sivitz, A. B., A. Reinders, et al. (2008). "Arabidopsis sucrose transporter AtSUC1 is important for pollen germination and sucrose-induced anthocyanin accumulation." Plant Physiol **147**(1): 92-100.
- Smeekeens, S. and F. Rook (1997). "Sugar Sensing and Sugar-Mediated Signal Transduction in Plants." Plant Physiol **115**(1): 7-13.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in Arabidopsis." Plant Cell **2**(8): 755-767.
- Tamura, K., G. Stecher, et al. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol **30**(12): 2725-2729.
- Truernit, E., J. Schmid, et al. (1996). "The sink-specific and stress-regulated Arabidopsis STP4 gene: enhanced expression of a gene encoding a monosaccharide transporter by wounding, elicitors, and pathogen challenge." Plant Cell **8**(12): 2169-2182.
- Tsukamoto, T., Y. Qin, et al. (2010). "A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development." Plant J **62**(4): 571-588.
- von Besser, K., A. C. Frank, et al. (2006). "Arabidopsis HAP2(GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Williams, E. G., V. Kaul, et al. (1986). "Overgrowth of Pollen Tubes in Embryo Sacs of Rhododendron Following Interspecific Pollinations." Australian Journal of Botany **34**(4): 413-423.

- Williams, L. E., R. Lemoine, et al. (2000). "Sugar transporters in higher plants--a diversity of roles and complex regulation." Trends Plant Sci **5**(7): 283-290.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.

Appendix 2:

Alpha carbonic anhydrases ACA4/ACA6 positively regulated pollen tube guidance to ovules and synergid degeneration

Alexander R. Leydon, Mark A. Johnson

I performed all the experiments in this study.

Abstract

In flowering plants, sperm are delivered to female gametes for double fertilization by pollen tubes, which transport sperm through the complex tissue of the pistil. Pollen tubes navigate the pistil environment through the detection of guidance cues that orient pollen tube growth into ovules. Guidance is controlled by pollen tube membrane receptors which perceive attractants and transduce the pollen tubes relative location in the pistil to the growth machinery. While recent research has informed the identity of pollen tube attractants, the signaling components that perceive these cues are not yet known at this time. Here, we describe a pair of alpha carbonic anhydrases (ACAs) that play a role in pollen tube guidance to ovules.

Introduction

In flowering plants, sperm develop within pollen grains, which produce pollen tubes to carry sperm to ovules for double fertilization. During growth in the pistil pollen tubes perceive their relative location in the pistil in order to target the micropyle of the ovule, where female the female gametes are housed. This process of pollen tube targeting is known as pollen tube guidance (Reviewed in Higashiyama et al. 2008). Pollen tube guidance involves sporophytic guidance and gametophytic guidance. Sporophytic guidance involved pollen tube guidance in the stigma, style and transmitting tract and pistil signals in this process are derived from sporophytic cell types. Gametophytic pollen tube guidance is controlled by factors produced in the female gametophyte and primarily control pollen tube guidance into the micropyle.

A number of gametophytic pollen tube guidance factors are produced from gametophytic cells in the embryo sac, such as Central Cell Guidance in the central cell (Chen et al. 2007), GEX3 in the egg cell (Alandete-Saez et al. 2008). However, the synergid cells control secretion of a number of small diffusible protein ligands such as the LUREs that are likely to control the bulk of gametophytic pollen tube guidance (Punwani et al. 2007; Okuda et al. 2009; Takeuchi et al. 2012). The mechanism by which these attractants are perceived by the pollen tube are not yet understood, however a handful of mutations have been identified that disrupt pollen tube guidance in vivo. It was shown that a Glu receptor-like protein acts as a D-serine receptor to modulate calcium levels within

the pollen tube (Michard et al. 2011). Two endoplasmic reticulum (ER) localized potassium (K⁺) transporters are required for micropylar pollen tube guidance (Lu et al. 2011). Additionally, the pollen expressed ER protein POD1 is required for pollen tube guidance (Li et al. 2011). POD1 interacts with Calreticulin3 (CRT3) and likely controls secretion of guidance receptors to the pollen tube plasma membrane. These genetic factors indicate a role for cation and pH dynamics in controlling pollen tube guidance and secretion of guidance-receptors.

In order to perceive guidance cues pollen tubes must first growth through a portion of the stigma and style (Palanivelu et al. 2006; Okuda et al. 2013), which is accompanied by an induction of pollen tube transcription (Qin et al. 2009). Despite the apparent importance pistil induced activation of pollen tube receptor secretion, the majority of the pollen tube signaling machinery that perceives and controls guidance cues is unknown. We have identified a trio of pollen specific MYB transcription factors (*MYB97*, *MYB101*, and *MYB120*) that control pollen tube reception and to a lesser extent, pollen tube guidance (Thesis Chapter 4, Leydon et al. 2013). MYB transcription factors regulate expression of ~300 genes, two of which are a pair of alpha carbonic anhydrases (*ACA4/ACA6*). Carbonic Anhydrases are Zn-containing metallo-enzymes that catalyze the reversible hydration of CO₂ (Fabre et al. 2007). Here, we demonstrate that *ACA4* is localized to the pollen endomembrane system, and may be secreted to the extracellular environment. *ACA4* is capable of fully rescuing *myb* triple-mutant pollen tube guidance defects, and also promotes synergid degeneration. *ACA4/6*

single mutants display subtle defects in pollen tube guidance and reception, in a manner consistent with partial genetic redundancy. This work demonstrates a role for Alpha Carbonic Anhydrase activity in pollen tube reception.

Results

***ACA4* & *ACA6*: Two alpha carbonic anhydrases controlled by *MYB97*, *MYB101*, and *MYB120* during pollination**

Pollen tube growth in the pistil induces a unique transcriptional response (Qin et al. 2009), including a trio of MYB transcription factors required for pollen tube differentiation and proper pollen tube reception (Leydon et al. 2013). *MYB97*, *MYB101* and *MYB120* activate expression of a subset of the pollen tube transcriptome that is specifically enriched proteins predicted to enter the pollen endomembrane system (Thesis Chapters 4,5). We discovered two small secreted proteins *ACA4* and *ACA6* that require *MYB97*, *MYB101*, and *MYB120* for expression in the pollen tube. The *ACA4/6* genes are members of the alpha carbonic anhydrase (ACA) gene family with 8 gene members encoded in the *Arabidopsis* genome (Figure 1A, Fabre et al. 2007). There are three carbonic anhydrase enzyme families: alpha, beta, and gamma carbonic anhydrases. These enzymes have no sequence homology, and evolved from independent origins (Liljas et al. 2000), and we have included the beta carbonic anhydrases as an outgroup in our phylogenetic analysis (Figure 1A, Fabre et al. 2007). Only two alpha carbonic anhydrases *ACA4* and *ACA6* require *MYB97*, *MYB101*, and *MYB120* for expression during reproduction, however a closely related gene,

ACA3 is also highly expressed in pollen grains and tubes (Figure 1B, Qin et al. 2009).

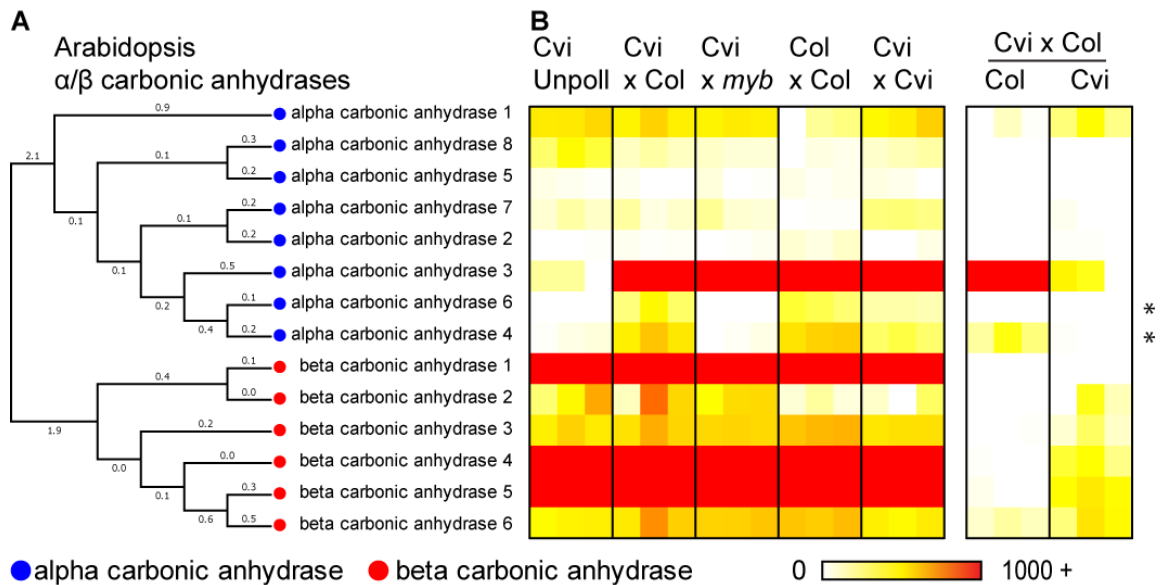


Figure 1. Arabidopsis carbonic anhydrase heat-tree **A.** Molecular Phylogenetic analysis of the Arabidopsis α/β carbonic anhydrases by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al. 1992). The tree with the highest log likelihood (-3655.1785) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 14 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 152 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). **B.** Expression heatmap of carbonic anhydrase transcripts in pistils. Cvi Unpoll – Unpollinated Cvi pistils (mock pollinated 8 hours), Cvi x Col – Cvi pistils pollinated with Col pollen for 8 hours, Cvi x *myb* – Cvi pistils pollinated with *myb* triple-mutant pollen for 8 hours, Col x Col – Col pistils pollinated with Col pollen for 8 hours, Cvi x Cvi – Cvi pistils pollinated with Cvi pollen for 8 hours. The far right columns are SNP-dissected reads from Cvi x Col, where Col reads are pollen specific and Cvi reads are pistil specific. Sequencing data is shown in three columns per condition, one column per biological replicate (See Thesis Chapter 5). Asterisks indicate significant gene expression differences between Cvi x Col and Cvi x *myb* triple-mutant pollinations (adjusted p-value < 0.05).

Our RNA-sequencing experiments (Thesis Chapter 5) revealed that *ACA4* was pollen tube specific based upon the presence of SNPs in inter-ecotype cross pollinations. In order to test *ACA4* expression patterns, we cloned the *ACA4* genomic locus including 967bp of upstream sequence in frame with GFP to create a promoter-gene-GFP fusion (*ACA4:GFP*). We introduced this expression construct into wild type, *LAT52:DsRed* plants (Twell et al. 1989; Francis et al. 2007) and grew pollen tubes in semi-in vivo pollen tube growth assays. We consistently saw *ACA4:GFP* expression in pollen tubes (Figure 2A), but not in mature pollen grains (Data not shown), indicating that *ACA4* requires interaction with the pistil tissues for expression as SIV-PT microarrays had indicated (Qin et al. 2009).

We introduced *ACA4:GFP* into *myb* triple-mutant, *LAT52:DsRed* plants to test the regulation of *ACA4* gene expression by MYBs. In all transformants we could observe pollen tubes growing in SIV-PT experiments (Figure 2B, Red channel), which consistently lacked *ACA4:GFP* expression; 11/11 independent primary (T_1) lines (Figure 2B, Green channel). We chose 4 of these T_1 lines and crossed them with wild type, *LAT52:DsRed* pollen and collected F1 progeny. to introduce wild type MYB gene expression. We grew F1 pollen in SIV-PT growth assays and in each F1 cross (4/4) we detected *ACA4:GFP* expression, which was reactivated due to the introduction of *MYB97*, *MYB101*, and *MYB120* (Figure 4C,D).

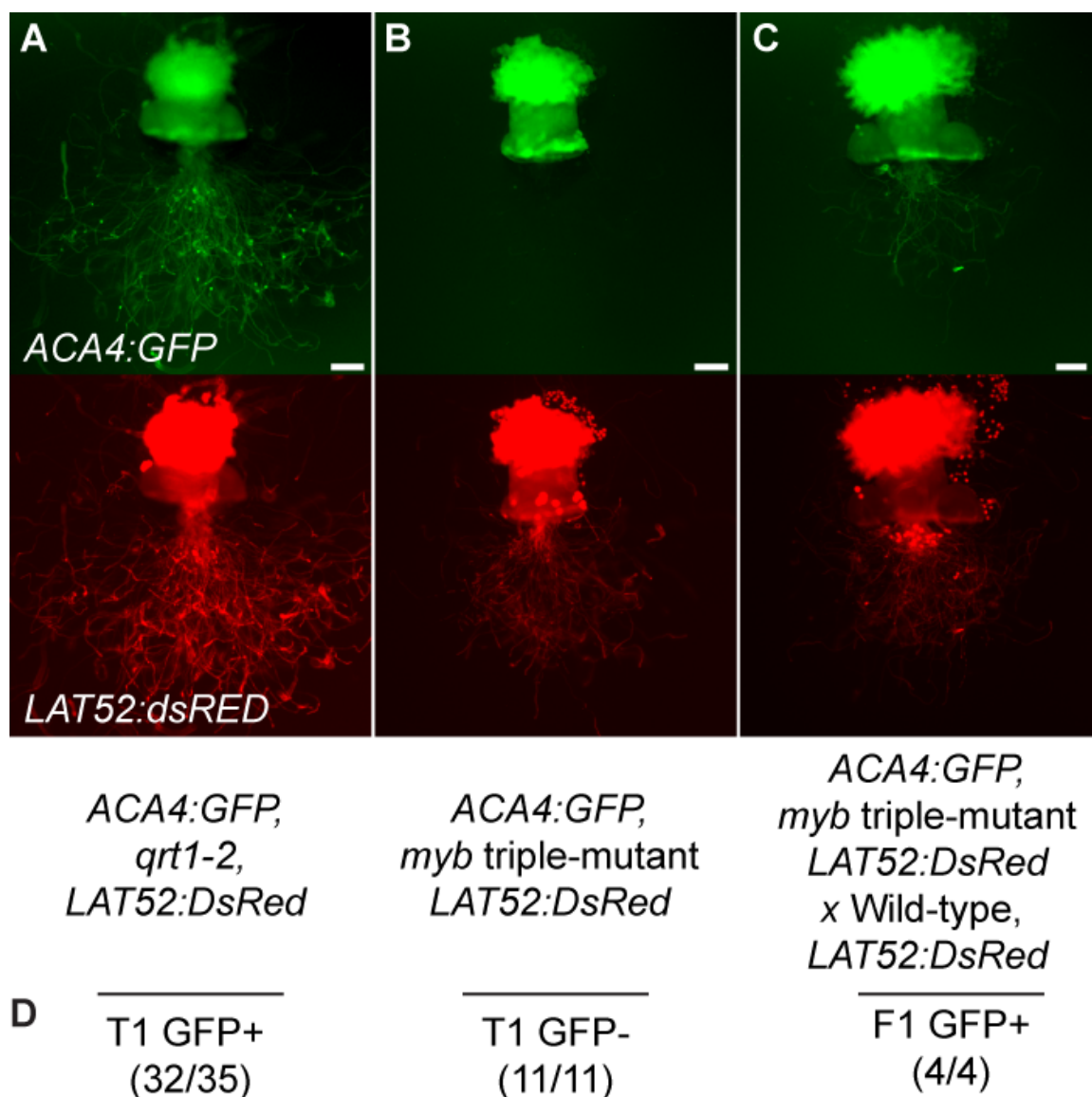


Figure 4. *ACA4:GFP* requires *MYB97*, *MYB101*, *MYB120* for expression in pollen tubes. **A.** One representative SIV-PT growth experiment with *pACA4:ACA4:GFP* primary transformant in the wild type, *qrt1-2*, *LAT52:DsRed* background. **B.** One representative *pACA4:ACA4:GFP* primary transformant in the *myb* triple-mutant *LAT52:DsRed* background. **C.** One representative SIV-PT growth experiment with F1 pollen from an *ACA4:GFP*, *myb* triple-mutant, *LAT52:DsRed* x Wild-type, *qrt1-2*, *LAT52:DsRed* cross. Each panel is a vertical side-by-side (GFP/DsRed) epifluorescent micrograph of 1 SIV experiment. **D** Summaries of total T1 expression numbers is shown at the bottom of each set of panels.

ACA4 is localized to the pollen tube endomembrane system

While two pollen expressed ACAs (ACA3 and ACA4) contain a signal peptide which predicts their localization to the endomembrane system (SignalP), ACA6 is predicted to contain two transmembrane domains, one at each terminal portion of the protein, which may anchor it to the extracellular face of the plasma membrane (TMHMM, Supplemental Figure 1). This topology has been observed for the human ACAXIV (Fujikawa-Adachi et al. 1999). In order to observe the subcellular localization of ACA4:GFP, we analyzed SIV-PT by confocal microscopy (Figure 3A). In SIV-PT ACA4:GFP is localized to small puncta most likely due to localization to an endomembrane compartment (Figure 3B-E). ACA4:GFP signal is also observed around the periphery of the pollen tube cell forming a line around the cytoplasm that can be visualized by the *LAT52:DsRed* reporter (Figure 3D,E). SIV-PTs grown for extended periods have high levels of GFP fluorescence, particularly enriched in the exterior of the cell (Figure 3F). These results suggest that ACA4 does indeed enter the endomembrane system of the pollen tube, and may be secreted to the extracellular environment. We also observed pollen tube morphological defects after extended SIV-PT growth (Figure 3F). It is unclear whether these defects were due to extensive incubation times and natural decay of pollen tube physiology, or that they are due to an ACA4 gain of function phenotype due to the fusion of the GFP protein.

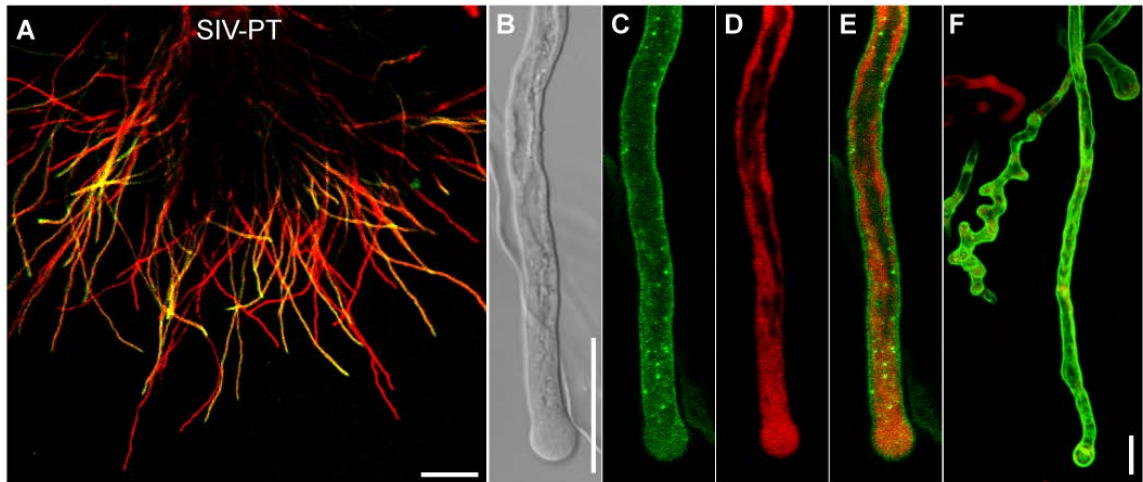


Figure 3. *ACA4:GFP* is induced by pollen tube growth in the pistil. **A.** *pACA4:ACA4:GFP* is induced by semi in vivo pollen tube growth (SIV-PT), Scale bar = 100um. **B-E.** *pACA4:ACA4:GFP* is localized to the endomembrane system and putatively the cell membrane/extracellular region (*pACA4:ACA4:GFP*, *LAT52:DsRed*, SIV-PT). (B) – DIC, (C) – *pACA4:ACA4:GFP*, (D) – *LAT52:DsRed*, (E) – Merge Green & Red Channels. **F.** SIV pollen tubes grown for extended periods (12 hours) display strong *ACA4:GFP* expression and localization to the cell periphery. All images are confocal micrographs. (B-F) scale bars are 20um.

***ACA4* expression in *myb* triple-mutant pollen rescues pollen tube guidance to the ovule**

ACA4 expression during growth in the pistil requires *MYB97*, *MYB101*, and *MYB120*. However, these MYB transcription factors also directly or indirectly regulate ~300 genes, and have multiple mutant phenotypes: reduced pollen tube guidance, failure to burst, and failure to induce synergid degeneration (Thesis Chapter 4, Leydon et al. 2013). In order to determine the role that *ACA4* plays in controlling these MYB-regulated, pollen tube functions, we utilized a genetic system whereby *ACA4:GFP* is expressed in the *myb* triple-mutant to test its ability to complement *myb* mutant defects (Described in Thesis Chapters 6,7). We cloned the *ACA4* cDNA into an expression vector that does not require

MYB97, *MYB101*, *MYB120* for expression, which were introduced into *myb* triple-mutant, *LAT52:DsRed* plants. We isolated transformants where *BGAL11:ACA4:GFP* expression was detected in mature pollen grains (Figure 5A-D). The ability to rescue or modify the *myb* triple-mutant phenotype was quantified with pollen from T1 hemizygous plants and *ACT11:MSI1:GFP* pistils (Ingouff et al. 2007). *ACA4:GFP* expression in *myb* triple-mutant pollen completely rescued pollen tube guidance back to wild type levels (Figure 4A, Untargeted – Grey bars). We tested three primary transformants and three homozygous T2 lines, which consistently rescued *myb* triple-mutant pollen tube guidance to *ACT11:MSI1:GFP* ovules. Once they reached ovules, pollen tubes still failed to undergo pollen tube burst, instead a larger proportion coiled in ovules, indicating that *ACA4* does not capable of rescuing *myb* triple-mutant pollen tube burst defects (Figure 4A, Red and Dark Blue bars).

***ACA4* expression in *myb* triple mutants promotes synergid degeneration**

The *ACT11:MSI1:GFP* nuclear GFP marker (Ingouff et al. 2007) allows clear visualization of synergid degeneration during pollen tube reception when the pollen tube carries the pollen specific red fluorescent reporter (*LAT52:DsRed*, (Francis et al. 2007), Thesis Chapter 3,4).

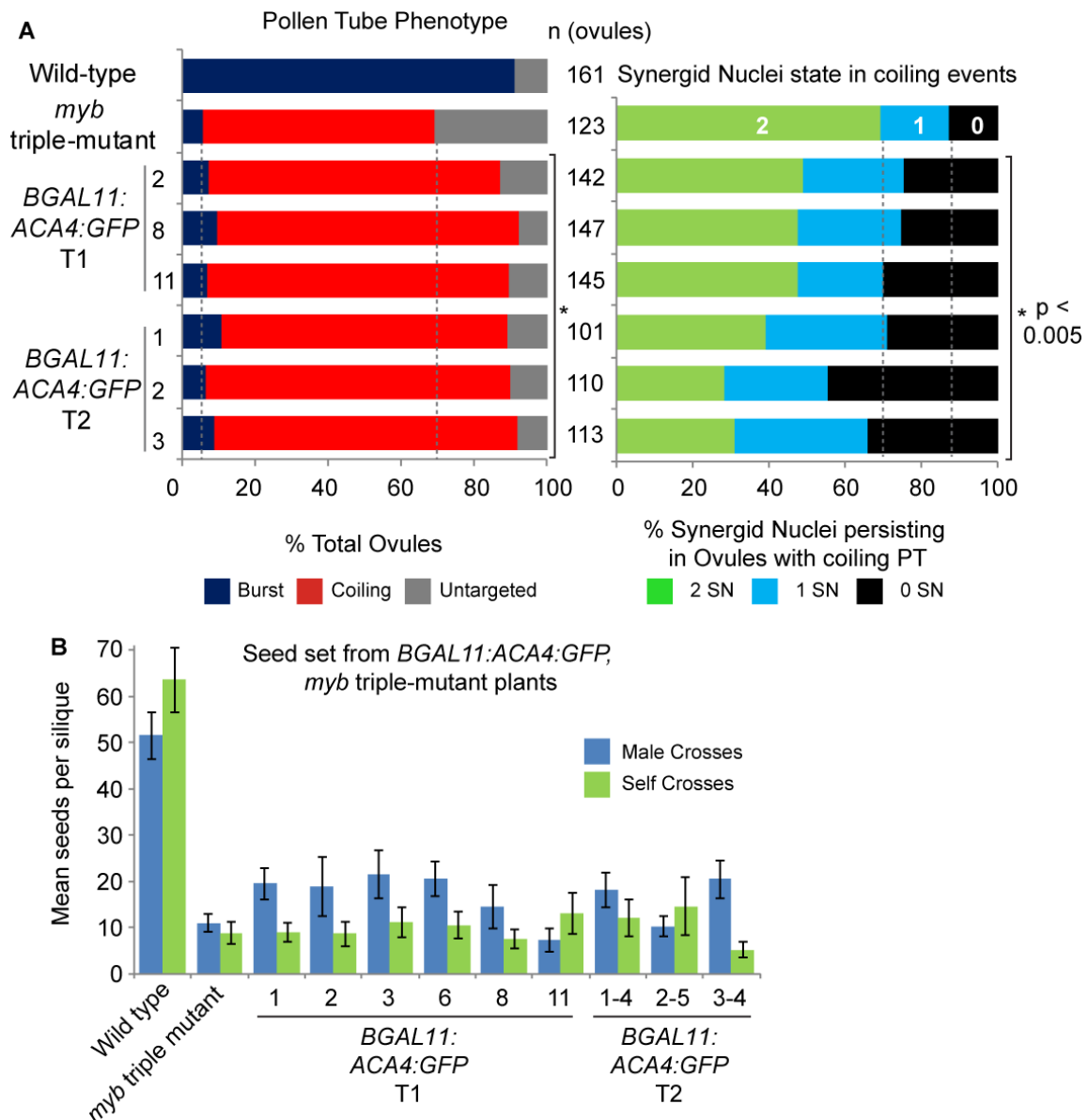


Figure 4. ACA4 expression constructs in *myb* triple-mutant pollen rescue pollen tube guidance and promote synergid degeneration. A. Pollen tube phenotypes 12 hours after pollination. *ACT11:MSI1:GFP* pistils were emasculated and crossed with *myb* triple-mutant pollen for 12 hours before dissection and quantification by confocal microscopy. Data on the right is paired with the red “Coiling” fraction shown on the left. Synergid numbers are indicated by position and by color, from left to right, Green – 2 synergid nuclei, Blue – 1 synergid nucleus, Black – 0 synergid nuclei. Data are shown for three T₁ lines and three T₂ that were tested for expression of ACA4:GFP. n (ovules) – refers to total number of ovules, 3 pistils per experiment. (2,8,11 & 1,2,3) – indicate the founding independent transformant lines, with ACA4:GFP expression. P-value <0.0005 – chi-squared test (Includes three conditions: Burst, Coiling, Untargeted). **B.** Mean seed set in ACA:GFP crosses. All bars are mean values +/- std dev. Blue bars are mean seed set in self-pollinated siliques. Green bars

are seed set from male crosses to *ms1* pistils pollinated with T₁ or T₂ *ACA4:GFP* add back pollen.

We quantified the rate of synergid degeneration in ovules receiving a coiling pollen tube in lines hemi and homozygous for the *BGAL11:ACA4:GFP* expression construct (Figure 4A, Right graph, Figure 5E-G). We observed increased synergid degeneration rates in ovules receiving a coiling *myb* triple-mutant pollen tube when *ACA4:GFP* was expressed (Figure 4A, Black and Blue bars, Figure 5G). This trend increased when pollen donors were homozygous for the *BGAL11:ACA4:GFP* expression construct (Figure 4A, T2 lines). These results demonstrate a role for *ACA4* in pollen tube guidance in addition to promoting synergid degeneration during the pollen tube:synergid interaction during reception.

In order to ascertain whether pollen tube coiling events were resolved in pollen tube burst, we quantified the seeds produced from crosses with *BGAL11:ACA4:GFP*, *myb* triple-mutant plants. In self crosses (Figure 4B, Green Bars) we saw no statistical difference between *myb* triple-mutant plants with or without the *BGAL11:ACA4:GFP* expression construct. However, in male crosses to *male sterile 1* (*ms1*) pistils, we saw slight increases in seed production (Figure 4B, Blue Bars).

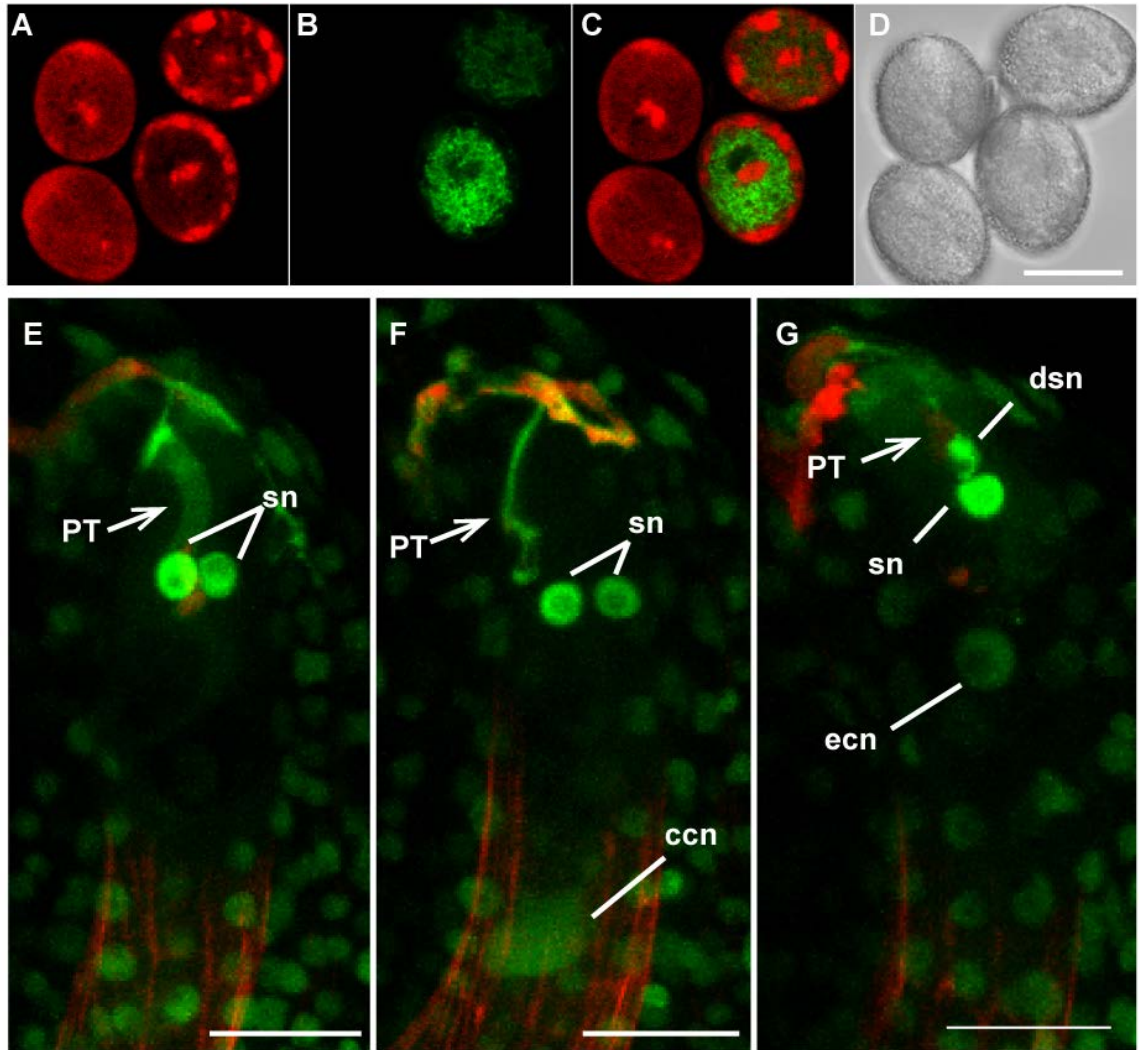


Figure 5. *ACA4:GFP* proteins increase ovule targeting and synergid degeneration by *myb* triple-mutant pollen tubes. A-D. *BGAL11:ACA4:GFP* expression in *myb* triple-mutant, *LAT52:DsRed* mature pollen. Confocal micrograph, scale bar – 20um. E-G. Confocal micrographs of pollen tube reception in *ACT11:MSI1:GFP* pistils, 12 hours after pollination, scale bars = 20um. E-F. *BGAL11:ACA4:GFP* pollen tube reception with two intact synergid nuclei, *ACA4:GFP* is visible in the pollen tube, PT arrow indicates the entry of the pollen tube in the embryo sac. G. One degenerated synergid nuclei. Dsn – degenerated synergid nucleus, sn – synergid nucleus, ecn – egg cell nucleus, ccn – central cell nucleus.

The slight increase in seeds produced by male crosses could be explained by an increase in pollen grain deposition during hand pollination as opposed to self-pollination. This would increase the amount of pollen tube competition and slightly raise the chances of producing seeds in the *myb* triple-mutant background.

ACA4/6 loss of function mutants have subtle defect in pollen tube guidance

The *ACA4* (AT4G20990) and *ACA6* (AT4G21000) genes are encoded at a duplicated region of the *Arabidopsis* fourth chromosome and are separated by only 1401 base pairs (Figure 6A). The tight linkage between these two genes renders mutational analysis challenging. Despite their close proximity, they share only 71% amino acid identity. Therefore, it is possible that they may have distinct or only partially overlapping molecular function. Furthermore, *ACA4* transcripts accumulate at higher levels than *ACA6* transcripts in every RNA-seq experiment (Supplemental Figure 2). In order to test whether either these genes are redundant, we isolated T-DNAs in *ACA4* and *ACA6*. We identified two SALK insertions in *ACA4*, and named them *aca4-1* and *aca4-2*, and one SAIL insertion in *ACA6*, which we named *aca6-1* (Figure 6A). We were able to identify homozygous mutants for each mutant line, and there were no obvious defects in seed set (Data not shown). In order to determine whether pollen tube growth and guidance in the pistil was abnormal or delayed, we quantified pollen tube guidance and reception defects using the pollen tube specific stain, Aniline Blue (Mori et al. 2006). In crosses with wild type pollen 24 hours after pollination

approximately 95% ovules were targeted by one pollen tube, with low rates of polytubey (~3%) and untargeted ovules (~5%) (Figure 6B, Blue bars). As previously observed, *myb* triple-mutant pollen tubes reach ovules less frequently (30% untargeted), and when they do arrive in ovules they coil and fail to burst (~60%, Figure 6B, Red bars).

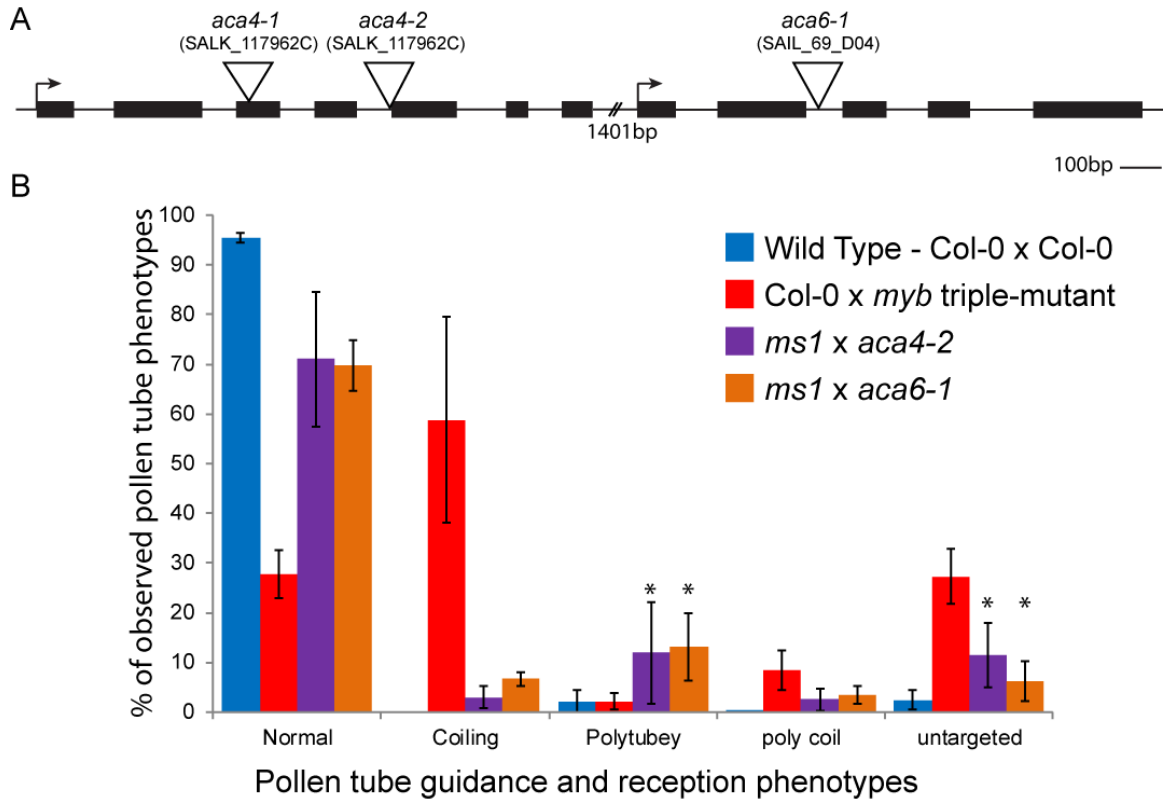


Figure 6. ACA4/6 mutants diminish pollen tube targeting. **A.** Gene loci of *ACA4* and *ACA6* with T-DNA insertion positions (white triangles). Double hash marks indicate the intergenic space between *ACA4* and *ACA6*. **B.** Aniline Blue staining of pollen tubes grown in vivo for 24h, reveals subtle ovule targeting defects in *ACA* single mutants. Ovule phenotypes: Normal – wild type pollen tube position in the ovule with a single tube, Coiling – one single pollen tube coiling in the ovule, Polytubey – multiple pollen tubes entering one ovule, normal position within the ovule, poly coil – multiple pollen tubes entering the ovule, with at least one coiling pollen tube, untargeted – ovule with no detectable pollen tube entry. Data is presented as the Mean of 5-6 pistils, +/- the standard deviation.

Pollen from *aca4-2* and *aca6-1* mutants displayed subtle defects in pollen tube guidance and reception. Pistils pollinated with single mutant pollen exhibit elevated rates ovules that had failed to receive a pollen tube (Figure 6B, Purple and Orange bars, untargeted). Additionally, the polytubey rate was significantly higher than wild type pollinations (Figure 6B, Polytubey). Polytubey occurs when ovules have failed to receive sperm, which can be due to failure in pollen tube burst (Beale et al. 2012; Kasahara et al. 2012). The elevated rate of untargeted ovules (6-11%) taken with the observation of high polytubey rates (12-13%) suggests that *ACA4* and *ACA6* have partially overlapping molecular functions in pollen tube guidance to the ovule. We also observed increases in pollen tube coiling in the ovule, at rates that are similar to inter-ecotype pollinations (Thesis Chapter 2, Leydon et al. 2013). The observed increase in pollen tube coiling in the ovule suggests that pollen tube-synergid interactions are delayed in *aca4/6* mutants, allowing larger windows of time for additional pollen tubes to target the same ovule before fertilization is achieved (Beale et al. 2012).

Discussion

We have identified a pair of related alpha carbonic anhydrase genes that require the *MYB97*, *MYB101*, and *MYB120* transcription factors for expression. The *ACA4* gene is expressed specifically in the pollen tube and enters the endomembrane system. *ACA4* expression in *myb* triple-mutant pollen tube completely rescues pollen tube guidance to the ovule, and promotes synergid degeneration upon pollen tube reception. The *ACA4/6* gene pair is almost completely functionally redundant, with single mutants displaying only subtle

defects in pollen tube guidance and reception. Our initial findings suggest that ACA4 and ACA6 perform an overlapping role in coordinating pollen tube guidance to ovule during growth in the pistil.

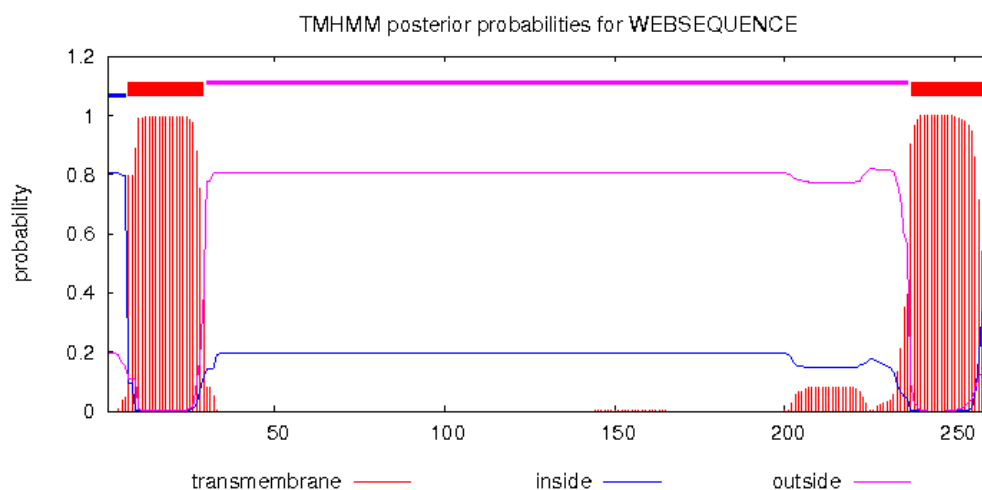
Alpha Carbonic Anhydrases: Diverse roles in photosynthetic organisms

Carbonic anhydrases (CAs) are Zn-containing enzymes that catalyze the reversible conversion of H_2O and CO_2 into bicarbonate (H_2CO_3). In environments with low CO_2 concentrations the conversion of HCO_3^- and H^+ into CO_2 and H_2O is favored. This enzymatic activity drives high concentrations of CO_2 for photosynthesis as has been characterized for CAH3 in the green alga *Chlamydomonas* (Karlsson et al. 1995; Funke et al. 1997). CAs are thought to modulate CO_2 concentrations across many subcellular compartments to enrich CO_2 at the sites of photosynthesis (Moroney et al. 2011). However, CAs are involved in a wide range of cellular processes such as pH regulation, inorganic carbon transport, ion transport, and water balance (Tashian 1989; Henry 1996; Smith et al. 2000).

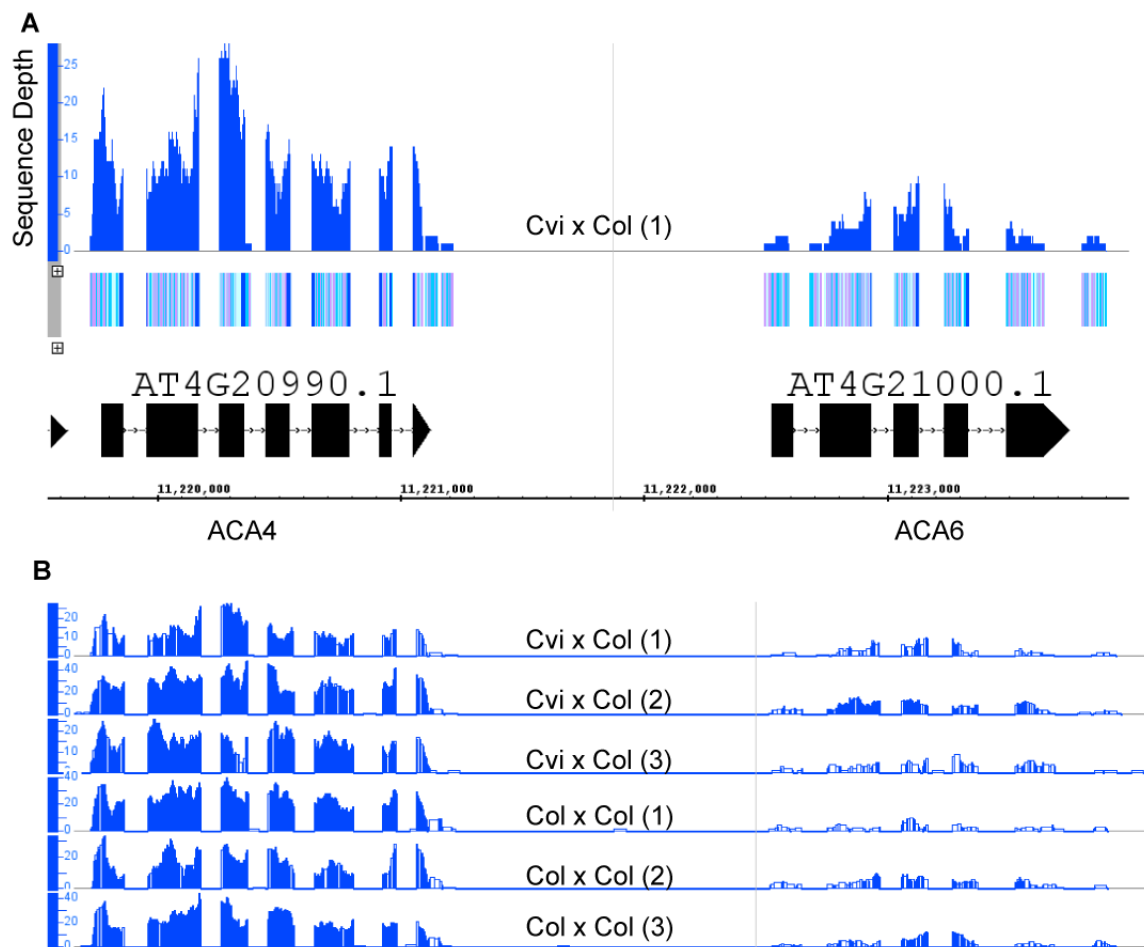
Recent research has also discovered a role for two beta carbonic anhydrases in sensing and responding to CO_2 levels. The CAs, CA1 and CA4, control short-term CO_2 induced stomatal movement (Hu et al. 2010). The CA1 enzyme is localized to the chloroplast, while the CA4 enzyme is localized to the plasma membrane (Fabre et al. 2007; Hu et al. 2010). CA1 and CA4 are required for activation of signaling pathway that induces expression of *EPIDERMAL PATTERNING FACTOR 2 (EPF2)* and an extracellular protease *CRSP* (CO_2

RESPONSE SECRETED PROTEASE), which activates *EPF2* by cleavage of its pro-peptide domain (Engineer et al. 2014). Together, these genes control stomatal development in response to CO₂ concentrations. These results point to a putative role for CAs in sensing and responding to CO₂ concentrations. The CO₂ concentration of pistil tissues is unknown, and it is as yet unclear whether alpha carbonic anhydrases could perform a similar function.

The MYB-regulated *ACA4* and *ACA6* CAs are expressed at lower levels than *ACA3*, which is by far the most highly expressed pollen tube ACA (Figure 1). It is possible that the *ACA3/4/6* perform semi-redundant roles during pollen tube guidance. To test this hypothesis, *ACA3* loss of function mutants should be identified and tested for pollen tube guidance defects. It is possible that the *myb* triple-mutant pollen tube guidance defect is subtle due to the maintained expression of *ACA3*. If this is the case, then an *ACA3* loss of function mutant would be expected to have a strong pollen tube guidance, which we will test in future studies.



Supplemental Figure 1. ACA6 predicted membrane topology as predicted by TMHMM Server 2.0



Supplemental Figure 2. Relative transcript abundance of ACA4 and ACA6 across multiple sequencing libraries. A. Sequencing depth is plotted (Blue graphs) above collapsed mapped reads at the *ACA4* (*AT4G20990.1*) and *ACA6* (*AT4G21000.1*) gene loci. **B.** Depth plots for each replicate (using wild type pollen donors) reveals consistent bias in expression favoring *ACA4* transcripts.

Methods

Plant growth

Procedures for selection of antibiotic and/or herbicide resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; von Besser et al. 2006; Qin et al. 2009; Wong et al. 2010). SALK and SAIL lines (*Col-0* accession) and *ms1/MS1* (CS75, Landsberg ecotype) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH).

PCR to Identify T-DNA Insertion Sites

T-DNA insertion sites were identified using left border (SAIL - LB3, LB2, and/or LB1, SALK - LBb1.3) and gene-specific flanking primers (see Oligonucleotides used in this study, above), designed at <http://signal.salk.edu/tdnaprimers.2.html>) in PCR reactions. The PCR program used for this reaction was: 94°C for 5 min followed by 36 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 2 min, and a final elongation step of 72°C for 4 min.

Cloning

For cDNA cloning RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis was performed with Superscript III (Invitrogen), and PCR used Phusion Polymerase (NEB). cDNA sequences were cloned into entry vectors pENTR-D or pCR8 (Invitrogen), and LR cloned into destination vectors with LR clonase II (Invitrogen). All clones were sequenced before introduction to plants (Genewiz).

Phylogenetic analysis

Phylogenetic relationships were analyzed using MEGA software (Tamura et al. 2013). Heat trees were constructed from sequencing data (Thesis Chapter 5)

Reception Microscopy

For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated *ACT11:MSI1:GFP*, *FGR7.0* stage 14 stigmas (Smyth et al. 1990) with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al. 2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

Aniline Blue Staining and Imaging

Aniline blue analysis was performed on crosses dissected 24 hours after pollination as reported previously (Mori et al. 2006). We made the following adjustments; all fixative and exchange steps were extended to 12 hours, and aniline blue incubation was extended from overnight to a period of 4-12 days before analysis by epifluorescence microscopy on a Zeiss Axiovert 200M Fluorescence Microscope with an ultraviolet filter.

References

- Alandete-Saez, M., M. Ron, et al. (2008). "GEX3, expressed in the male gametophyte and in the egg cell of *Arabidopsis thaliana*, is essential for micropylar pollen tube guidance and plays a role during early embryogenesis." Mol Plant **1**(4): 586-598.
- Beale, K. M., A. R. Leydon, et al. (2012). "Gamete Fusion Is Required to Block Multiple Pollen Tubes from Entering an *Arabidopsis* Ovule." Curr Biol.
- Chen, Y. H., H. J. Li, et al. (2007). "The central cell plays a critical role in pollen tube guidance in *Arabidopsis*." Plant Cell **19**(11): 3563-3577.
- Engineer, C. B., M. Ghassemian, et al. (2014). "Carbonic anhydrases, EPF2 and a novel protease mediate CO₂ control of stomatal development." Nature **513**(7517): 246-250.
- Fabre, N., I. M. Reiter, et al. (2007). "Characterization and expression analysis of genes encoding alpha and beta carbonic anhydrases in *Arabidopsis*." Plant Cell Environ **30**(5): 617-629.
- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Fujikawa-Adachi, K., I. Nishimori, et al. (1999). "Human carbonic anhydrase XIV (CA14): cDNA cloning, mRNA expression, and mapping to chromosome 1." Genomics **61**(1): 74-81.
- Funke, R. P., J. L. Kovar, et al. (1997). "Intracellular carbonic anhydrase is essential to photosynthesis in *Chlamydomonas reinhardtii* at atmospheric levels of CO₂ - Demonstration via genomic complementation of the high-CO₂-requiring mutant *ca-1*." Plant Physiol **114**(1): 237-244.
- Henry, R. P. (1996). "Multiple roles of carbonic anhydrase in cellular transport and metabolism." Annual Review of Physiology **58**: 523-538.
- Higashiyama, T. and Y. Hamamura (2008). "Gametophytic pollen tube guidance." Sexual Plant Reproduction **21**(1): 17-26.
- Hu, H., A. Boisson-Dernier, et al. (2010). "Carbonic anhydrases are upstream regulators of CO₂-controlled stomatal movements in guard cells." Nature Cell Biology **12**(1): 87-93; sup pp 81-18.
- Hu, H. H., A. Boisson-Dernier, et al. (2010). "Carbonic anhydrases are upstream regulators of CO₂-controlled stomatal movements in guard cells." Nature Cell Biology **12**(1): 87-U234.
- Ingouff, M., Y. Hamamura, et al. (2007). "Distinct dynamics of HISTONE3 variants between the two fertilization products in plants." Curr Biol **17**(12): 1032-1037.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "*Arabidopsis hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Jones, D. T., W. R. Taylor, et al. (1992). "The rapid generation of mutation data matrices from protein sequences." Comput Appl Biosci **8**(3): 275-282.
- Karlsson, J., T. Hilttonen, et al. (1995). "Intracellular Carbonic-Anhydrase of *Chlamydomonas-Reinhardtii*." Plant Physiol **109**(2): 533-539.

- Kasahara, R. D., D. Maruyama, et al. (2012). "Fertilization recovery after defective sperm cell release in Arabidopsis." Curr Biol **22**(12): 1084-1089.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Li, H. J., Y. Xue, et al. (2011). "POD1 Regulates Pollen Tube Guidance in Response to Micropylar Female Signaling and Acts in Early Embryo Patterning in Arabidopsis." Plant Cell **23**(9): 3288-3302.
- Liljas, A. and M. Laurberg (2000). "A wheel invented three times. The molecular structures of the three carbonic anhydrases." EMBO Rep **1**(1): 16-17.
- Lu, Y., S. Chanroj, et al. (2011). "Pollen tubes lacking a pair of K⁺ transporters fail to target ovules in Arabidopsis." Plant Cell **23**(1): 81-93.
- Michard, E., P. T. Lima, et al. (2011). "Glutamate receptor-like genes form Ca²⁺ channels in pollen tubes and are regulated by pistil D-serine." Science **332**(6028): 434-437.
- Mori, T., H. Kuroiwa, et al. (2006). "GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization." Nat Cell Biol **8**(1): 64-71.
- Moroney, J. V., Y. B. Ma, et al. (2011). "The carbonic anhydrase isoforms of *Chlamydomonas reinhardtii*: intracellular location, expression, and physiological roles." Photosynthesis Research **109**(1-3): 133-149.
- Okuda, S., T. Suzuki, et al. (2013). "Acquisition of LURE-binding activity at the pollen tube tip of *Torenia fournieri*." Mol Plant **6**(4): 1074-1090.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Punwani, J. A., D. S. Rabiger, et al. (2007). "MYB98 positively regulates a battery of synergid-expressed genes encoding filiform apparatus-localized proteins." Plant Cell **19**(8): 2557-2568.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Smith, K. S. and J. G. Ferry (2000). "Prokaryotic carbonic anhydrases." Fems Microbiology Reviews **24**(4): 335-366.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in *Arabidopsis*." Plant Cell **2**(8): 755-767.
- Takeuchi, H. and T. Higashiyama (2012). "A Species-Specific Cluster of Defensin-Like Genes Encodes Diffusible Pollen Tube Attractants in *Arabidopsis*." Plos Biology **10**(12).

- Tamura, K., G. Stecher, et al. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol **30**(12): 2725-2729.
- Tashian, R. E. (1989). "The Carbonic Anhydrases - Widening Perspectives on Their Evolution, Expression and Function." Bioessays **10**(6): 186-192.
- Twell, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.
- von Besser, K., A. C. Frank, et al. (2006). "Arabidopsis *HAP2(GCS1)* is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Wong, J. L., A. R. Leydon, et al. (2010). "HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain." PLoS Genet **6**(3): e1000882.

Appendix 3:

A dominant mutation in the AUX/IAA protein *IAA31* uncovers a role for Auxin signaling in pollen tube guidance

Alexander R Leydon, Trenton Woodham, Mark A. Johnson

I performed the following experiments, with the exception of TW's contributions – *IAA31-2* developmental timecourse, basal stability westerns, optimization of liquid culture growth, identification of qPCR gene targets and qPCR analysis of gene expression in siliques.

Abstract

Auxin is a plant hormone central to plant growth and development. Auxin signaling is controlled by the Auxin co-receptors Aux/IAA proteins and TIR, which in turn regulate a transcriptional response pathway governed by ARF transcription factors. Plant reproductive tissues are patterned by hormone signaling networks, including the Auxin response pathway. Here, we present a role for Auxin signaling in pistils that coordinates pollen tube guidance to the ovule. We report a new allele of the naturally dominant-negative IAA31 Aux/IAA factor, which disrupts pollen tube guidance in the pistil. We propose a model where Auxin signaling is desensitized by reduction of available Auxin receptor complex components such as TIR1, which are effectively sponged by a dominant fragment of the IAA31 protein containing domain I & II. Auxin signal transduction in the pistil during pollen tube growth is required for correct attraction of pollen tubes to ovules and successful fertilization.

Introduction

Pollination induces broad changes in the pistil transcriptional landscape, including many genes involved in hormone signaling (Boavida et al. 2011). Amongst this dynamic pollen-pistil signaling landscape, genes involved in the Auxin response pathway have emerged that suggest Auxin signaling cascades are modulated during pollination. Auxin signaling is involved in cell-cell signaling in a myriad of plant processes (Peer 2013) and recent advances have demonstrated the molecular mechanism by which it is perceived (Dharmasiri et al. 2005; Kepinski et al. 2005). The Auxin receptor is a co-receptor complex formed from an Aux/IAA protein's conserved domain II and the F-box protein Transport Inhibitor Response 1 (TIR1), which together create an Auxin binding pocket. TIR1 incorporates into an SKP1-Cullin-F-box E3 ligase complex that induces Aux/IAA protein degradation in the presence of Auxin (Dharmasiri et al. 2005; Kepinski et al. 2005). IAA proteins interact with Auxin Response Factors (ARFs) through conserved domains III and IV in both proteins, and repress ARF transcriptional activity. In the presence of Auxin, ARFs dissociate from IAAs and activate gene expression. (Chapman et al. 2009). IAA domain I recruits the transcriptional repressor Topless (TPL) to maintain repression of ARF targets in the absence of Auxin (Szemenyei et al. 2008).

The *Arabidopsis* genome contains 29 IAA proteins, many of which have been analyzed for loss of function analysis (Overvoorde et al. 2005). The *Arabidopsis* IAA gene family is highly diverse, and can be divided into clades based upon the

identity of residues within the highly conserved domains I-IV (Overvoorde et al. 2005). Three of these IAA proteins, IAA20, IAA30 and IAA31 deviate from the conserved domain II: IAA20 and IAA30 completely lack domain II, whereas IAA31 contains a degenerate domain II. Additionally, IAA31 lacks a Lysine-Arginine (KR) residue pair that is required for protein destabilization. Accordingly, IAA20 and IAA31 are highly stable in the absence of Auxin (Dreher et al. 2006). In the presence of Auxin, IAA20 is completely insensitive, whereas IAA31 is slightly responsive, indicating that IAA31 retains Auxin responsiveness through auxin binding activity. IAA31 is expressed at low levels *in vivo*, yet over-expression induces dominant Auxin-related phenotypes, consistent with the interpretation that IAA31 acts as a repressor of Auxin signaling (Sato et al. 2008).

Development of the gynoecium depends on proper Auxin signaling, which creates a polar gradient controlling both the apical-basal and abaxial-adaxial identity of cells within the pistil (Larsson et al. 2013). However, the role of Auxin in pollination after pistil development is unclear. We identified up-regulated expression of *IAA31* transcripts in pistils pollinated by wild-type pollen for 8 hours, a time-point when pollen tubes are navigating the pistil tissue (Leydon et al. 2013) (Thesis Chapter 4). We were intrigued to find that pistils pollinated with pollen lacking three MYB transcription factors (*MYB97*, *MYB101*, *MYB120*) failed to induce *IAA31* transcript levels. Because pollen tube identity is controlled by these transcription factors, we hypothesized that pollen tube differentiation is sensed by the pistil and in turn activates gene expression programs in part

through IAA31. If IAA31 is acting as a dominant negative regulator of Auxin signaling, then pollination may induce a down-regulation of Auxin signaling in the pistil as part of normal pollination responses.

In this study we identified insertion mutations in the *IAA31* genomic locus and characterized their impact on Arabidopsis pollen tube function in the pistil. We describe two mutations, one previously described RNA null mutation with no gross mutant phenotype (*iaa31-1*, Overvoorde et al. 2005), and a novel gain-of-function mutation that severely disrupts reproduction, in addition to affecting many stages of plant development (*iaa31-2*). Molecular characterization of *iaa31-2* reveals a hybrid protein containing IAA31 domains I & II translationally fused to a random amino acid sequence from the insertional DNA. This protein, hereafter termed IAA31-2, is highly expressed and exhibits hyperstability even in the presence of Auxin. Pistils expressing IAA31-2 display severely reduced pollen tube targeting in a genetically dominant fashion: IAA31-2 exerts its effect on pollen tube guidance even in the presence of a wild type copy of *IAA31*. Our preliminary analysis of Auxin regulated genes in *iaa31-2* tissues suggest that the Auxin-responsive transcriptional landscape is altered. Finally, we present a model where IAA31-2 siphons Auxin-TIR1 complex away from pistil expressed IAA proteins, thereby reducing IAA protein sensitivity to Auxin.

Results

IAA proteins are expressed in female tissues during pollination

Auxin responses are controlled by proteins in the AUX/IAA family and their interaction with ARFs and their Auxin co-receptor TIR1. To uncover putative roles for Auxin in pollination responsive gene expression we examined RNA expression levels of the AUX/IAA gene family in publically available microarray analyses (Figure 1). IAA proteins are lowly expressed in the male gametophyte, but several members (*IAA8*, *IAA9*, *IAA7*, and *IAA17*) are enriched in female tissues such as the stigma and ovary (Figure 1B). Mutations in *IAA8* that abrogate auxin binding produces dominant phenotypes including flower morphology defects and seed set reductions (Wang et al. 2013). The *IAA8* dominant phenotype is suggested to be caused by disrupted Auxin signaling in the developing pistil. Conversely, the *myb97*, *myb101*, *myb120* transcription factor triple mutant reduces seed set (Thesis Chapter 4), presumably through a male-specific loss of MYB regulated gene expression. Loss of seed production in *myb* triple-mutants is due to a distinct pollen tube reception defect compounded by reductions in pollen tube-ovule targeting. However, in both microarray and RNA-sequencing analysis, *myb* triple-mutant pollen tubes fail to induce the AUX/IAA gene *IAA31* (Thesis Chapters 4 & 5, Leydon et al. 2013). *IAA31* expression is not detected in the pollen tube, either in purified pollen cells (Qin et al. 2009) or in SNP-specific RNA sequencing of pollen tubes growing in vivo (Thesis Chapter 5). Therefore, *IAA31* up-regulation during pollination is likely localized to female tissues. The observation that *IAA31* is induced in wild-type pollinations, yet lost in *myb* triple-mutant pollinations suggest a specific cue originating from the pollen that is sensed by the pistil.

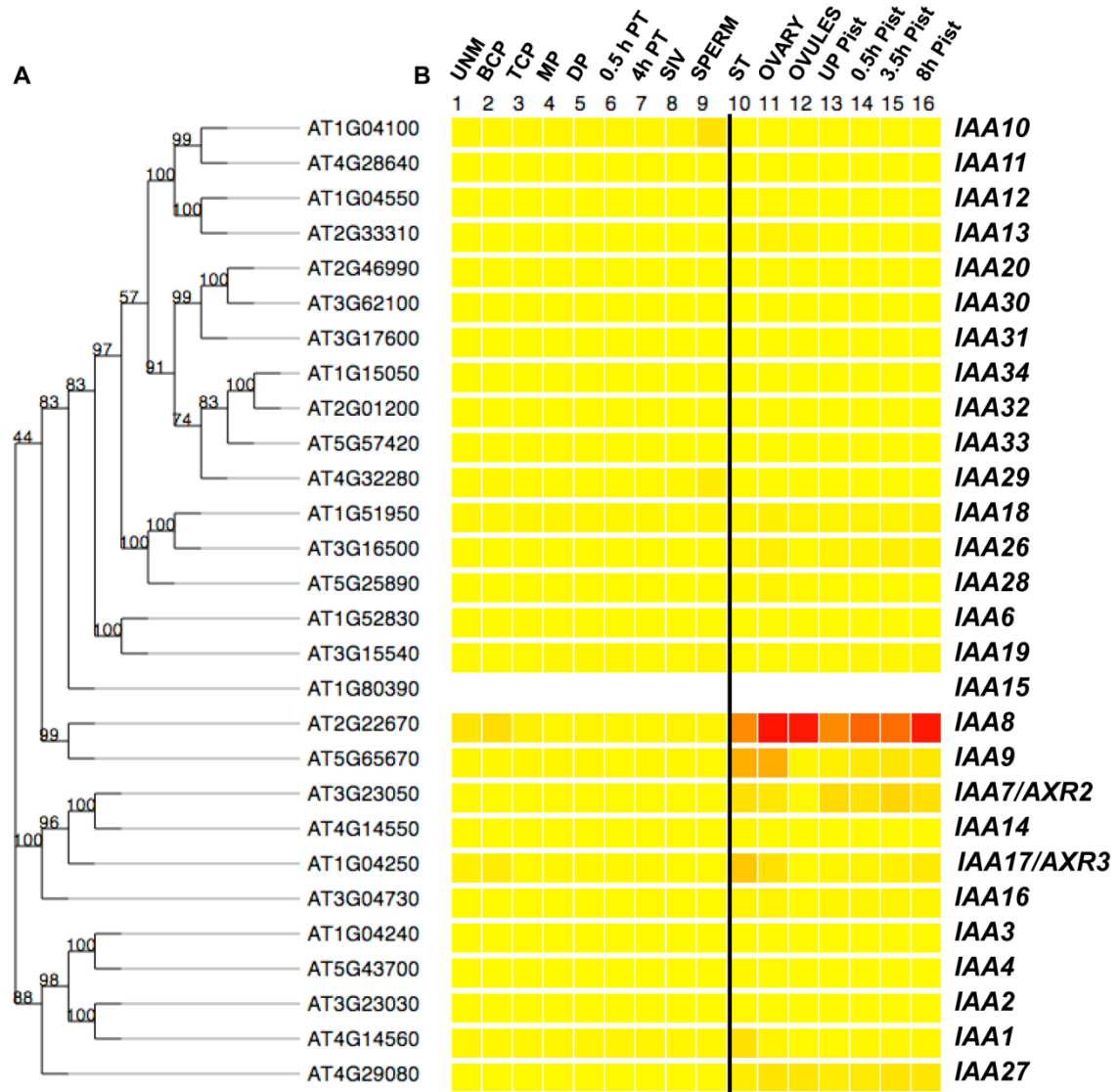


Figure 1. AUX/IAA gene expression in reproductive tissues. **A.** Arabidopsis IAA tree based on TAIR10 IAA protein sequences (ClustalW). **B.** IAA gene expression in reproductive tissues. *IAA31* exhibits low RNA expression levels compared to other IAA family members. UNM – uninucleate microspores, BCP – bicellular pollen, TCP – tricellular pollen, MP – mature pollen, DP – Dry pollen, 0.5h PT – 0.5 hour grown in vitro pollen tubes, 4h PT – 4 hour grown in vitro pollen tubes, SIV – semi in vivo grown pollen tubes, SPERM – isolated sperm cells, ST – stigma, OVARY – ovary tissue, UP Pist – unpollinated pistils, 0.5h Pist – pistils pollinated for 0.5 hours, 3.5h Pist – pistils pollinated for 3.5 hours, 8h Pist – pistils pollinated for 8 hours.

IAA	AGI	Mean Col reads	FC (log2)	p-value	p-adj
IAA1	AT4G14560	1,162	0.235	0.071	1.000
IAA2	AT3G23030	2,701	0.190	0.187	1.000
IAA3	AT1G04240	805	-0.173	0.183	1.000
IAA4	AT5G43700	1,597	-0.289	0.049	0.989
IAA5	AT1G15580	80	0.006	0.969	1.000
IAA6	AT1G52830	25	0.195	0.175	1.000
IAA7	AT3G23050	3,743	0.041	0.761	1.000
IAA8	AT2G22670	13,649	-0.130	0.042	0.907
IAA9	AT5G65670	15,553	-0.138	0.066	1.000
IAA10	AT1G04100	182	0.007	0.954	1.000
IAA11	AT4G28640	303	-0.055	0.628	1.000
IAA12	AT1G04550	1,370	-0.098	0.254	1.000
IAA13	AT2G33310	1,380	-0.060	0.518	1.000
IAA14	AT4G14550	21	-0.001	0.995	1.000
IAA15	AT1G80390	13	-0.072	0.561	1.000
IAA16	AT3G04730	3,636	0.025	0.753	1.000
IAA17	AT1G04250	3,254	0.010	0.934	1.000
IAA18	AT1G51950	1,363	0.330	0.001	0.082
IAA19	AT3G15540	421	0.119	0.405	1.000
IAA20	AT2G46990	62	0.053	0.729	1.000
IAA26	AT3G16500	1,366	0.081	0.422	1.000
IAA27	AT4G29080	3,352	-0.051	0.655	1.000
IAA28	AT5G25890	776	-0.027	0.835	1.000
IAA29	AT4G32280	4	-0.030	0.732	1.000
IAA30	AT3G62100	148	-0.048	0.727	1.000
IAA31	AT3G17600	79	-0.465	0.001	0.079
IAA32	AT2G01200	355	-0.188	0.175	1.000
IAA33	AT5G57420	12	-0.228	0.083	1.000
IAA34	AT1G15050	7	0.067	0.501	1.000

Table 1. IAA31 is downregulated in *myb* triple-mutant pollinations. IAA – Arabidopsis IAA family member name. AGI – Arabidopsis IAA gene locus identifier. Mean Col read – Mean count number in wild type pollinations (Cvi-0 x Col-0 8 hours after pollination). FC (log2) – fold change between Col-0 and *myb* triple-mutant pollinations (log2). p-value – p-value (preadjusted, DESeq2). p-adj – Benjamini adjusted p-value (DESeq2) Red – lowest values.

Identification of two IAA31 mutant alleles that display distinct phenotypes

In order to test whether IAA31 plays a role in pollen tube:pistil interactions during pollination, we identified available mutations in the *IAA31* gene. The *iaa31-1* insertion falls early in the second exon of IAA31 and was previously described by (Overvoorde et al. 2005) as a null allele. We procured a second allele with an annotated insertion site slightly downstream of *iaa31-1* and named this allele *iaa31-2* (FLAG173D10) (Figure 2A). We could recover plants homozygous for each mutation by genotyping, indicating that these alleles were not embryo lethal, which was a concern as *IAA31* expression reaches a maxima in the early embryo (Schmid et al. 2005). To test the affect these insertional alleles had on IAA31 mRNA accumulation we performed Northern blotting on RNA harvested from siliques 2-4 days after pollination (Figure 2B). *IAA31* wild-type transcripts were detected at very low levels and required long exposures (>7 days) to effectively visualize, while *iaa31-1* produced no detectable IAA31 mRNA. On the other hand, *iaa31-2* plants expressed an abundant transcript labelled by an *IAA31* full length cDNA probe. This *iaa31-2* RNA migrated at a size much larger than the wild type IAA31 transcript, suggesting that a chimeric mRNA, produced by read-through of the T-DNA, may be accumulating in *iaa31-2* plants. To examine the mRNA levels in these two alleles we performed RT-qPCR using primers specific to the 5' and 3' regions of the *IAA31* transcript (Figure 2C). Expression of the IAA31 gene is essentially undetectable in *iaa31-1*, while *iaa31-2* expresses an RNA containing only the sequence 5' to the T-DNA insertion.

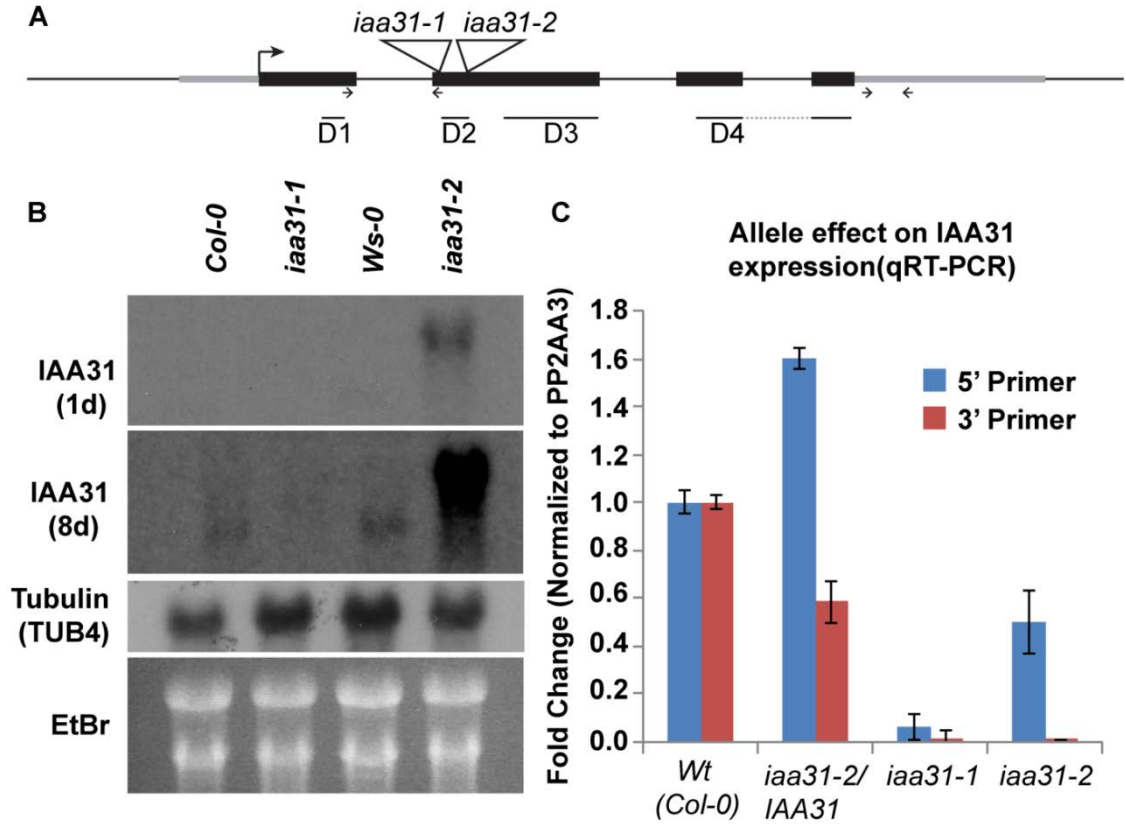


Figure 2. Two IAA31 mutant alleles and their effect on the IAA31 mRNA transcript. **A.** Gene map of the IAA31 genomic locus. Insertional alleles are displayed as white triangles. D1-D3 indicate the positions of the conserved AUX/IAA domains I-IV in the coding sequence of IAA31. Gray lines – UTRs, black rectangles – exons, line – intergenic regions and introns. Primers used in qPCR are indicated as arrows below gene body. 100bp = 33.9 pixels. **B.** Northern analysis of IAA31 mutants and wild type floral tissue (unopened buds, flowers and siliques). IAA31 was probed with a full-length cDNA. Tubulin4 was probed as a loading control, and EtBr staining of the pre-transferred gel is at bottom. **C.** RT-qPCR analysis of *iaa31-1*, and *iaa31-2* floral tissues. Gene expression (delta/delta CT) was normalized to PP2AA3 transcripts. **D.**

In *iaa31-2*, the coding sequence of the gene:T-DNA chimeric RNA includes the first two conserved domains of IAA31 fused to the left border of the T-DNA. When translated, this mRNA is predicted to encode a 14 KDa protein. However, we also observed accumulation of a larger, less-abundant cDNA which, when sequenced, was shown to retain the first intron. Retention of this intron

introduces an early stop codon after domain I, and would eliminate Auxin-TIR1 interaction, which requires an intact domain II (Dreher et al. 2006) (Figure 3A).

***iaa31-2* mutant plants express a chimeric AUX/IAA protein**

To determine which, if any, of these *iaa31-2* mRNA isoforms are translated in vivo, we designed three custom antibodies to specific epitopes in wild type IAA31 protein (Figure 3A). Two of these antibodies (α IAA31-2, α IAA31-3) were raised against synthesized peptides that fall before domain III while the third (α IAA31-1) is the most c-terminal region portion of domain IV. We tested protein extracted from wild type and mutant siliques 2-4 days post pollination by SDS-PAGE and Western Blotting (Figures 3B, 4). Wild type IAA31 was not detected in either ecotype or with any antibody (Figures 3-4). Strikingly, a novel band of approximately 20KDa was observed in *iaa31-2* protein extracts that was undetectable in wild type and distinct from non-specific bands (Figure 3B). This *iaa31-2*-specific band is only detectable with α IAA31-2, which is the most N-terminal epitope and spans the border of domain II.

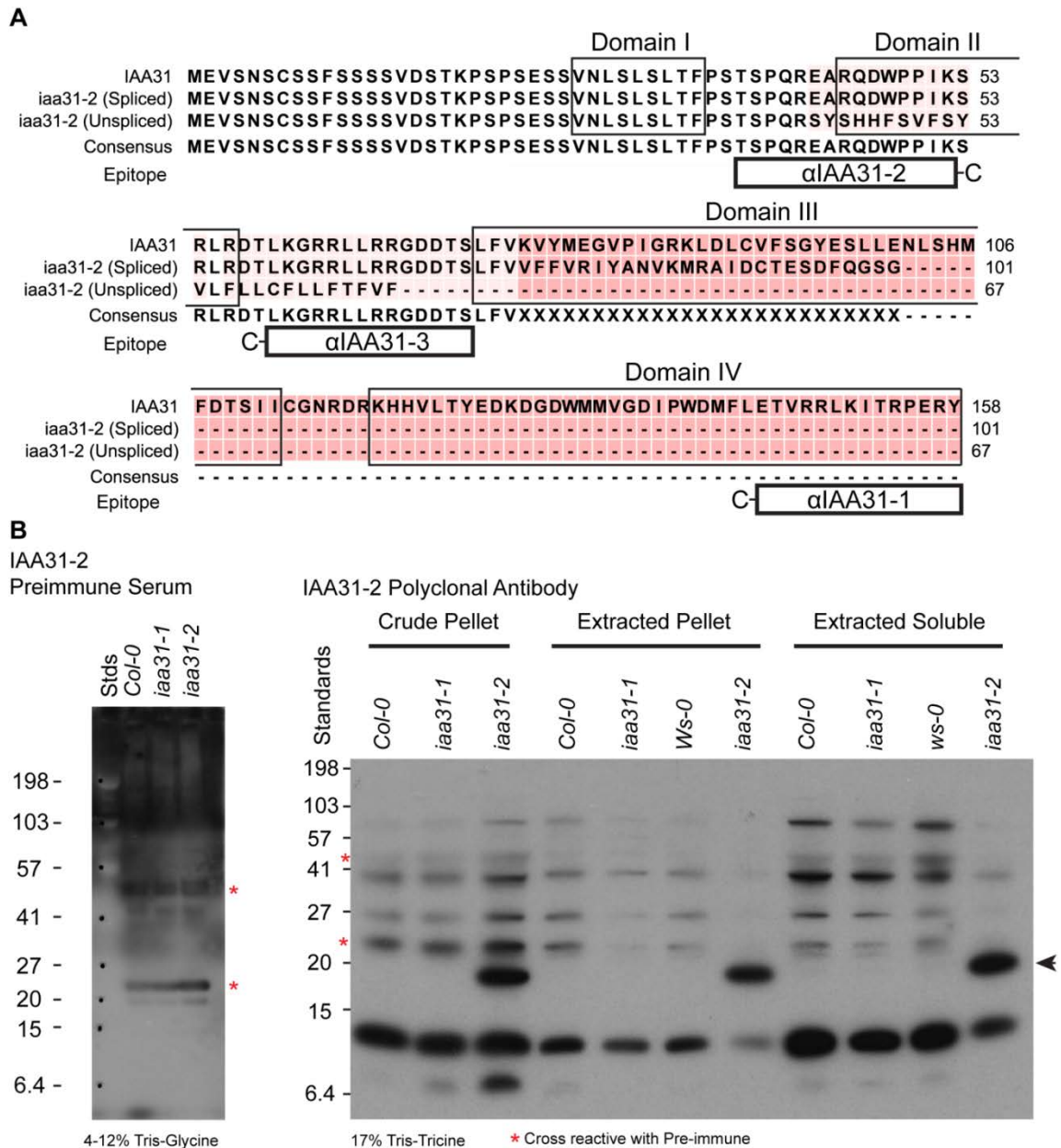


Figure 3. *iaa31-2* mutant plants express a protein that immuno-reacts to antibodies produced against IAA31 protein peptides. A. Protein alignment of wild type IAA31 and the translated reading frames from *iaa31-2* cDNA sequences. Domains I-IV are boxed. Loss of homology is highlighted with red shading, and epitopes are indicated below the consensus sequence. **B.** Western blotting analysis of wild type and *iaa31* mutant siliques. Pre-immune is displayed on the left with two cross-reactive bands indicated with red asterisks. The black arrow indicates the protein band detected only in *iaa31-2* mutant plants.

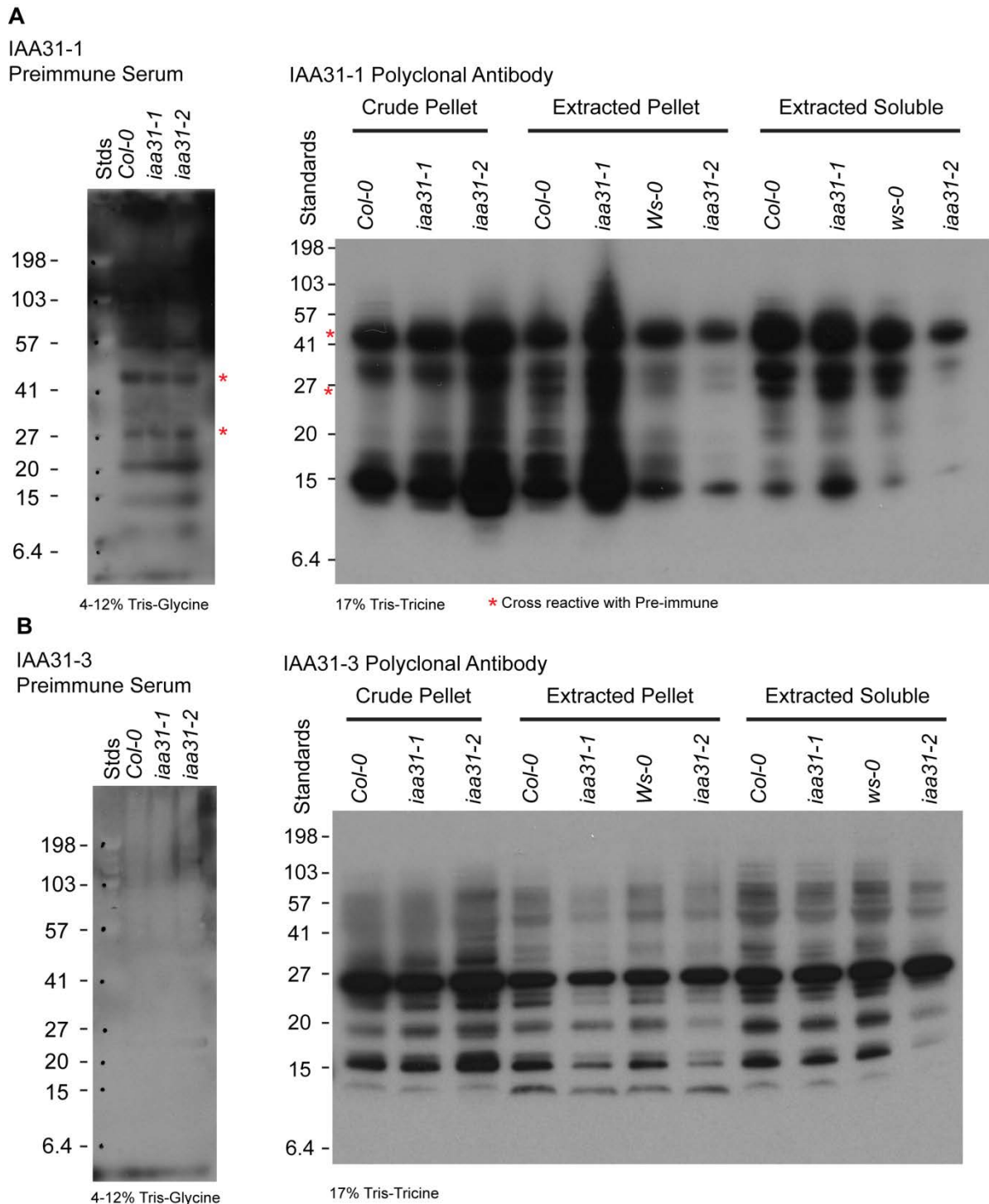


Figure 4. Wild-type protein IAA31 is not readily detectable. A. IAA31-1 antibody optimization. **B.** IAA31-3 antibody optimization. Western blotting analysis of wild type and *iaa31* mutant siliques. Preimmune is displayed on the left with two cross-reactive bands indicated with red asterisks.

The *iaa31-2* protein is expressed throughout growth and reproduction

IAA31 transcript levels are uniformly low throughout plant growth and development (cite), consistent with our observation that IAA31 protein accumulation is undetectable (Figures 3-4). However, IAA31-2 chimeric protein is easily detectable in siliques, suggesting an altered domain of *iaa31-2* protein expression. Therefore we tested *iaa31-2* protein accumulation in other tissues (Figure 5A). We observed varying levels of IAA31-2 protein in all tissues, with a clear induction of expression in the pistil following pollination that peaked at 3-4 days after pollination.

AUX/IAA proteins are unstable in the presence of Auxin (Dharmasiri et al. 2005; Kepinski et al. 2005), which targets them for proteosomal degradation. In turn ARF transcriptional cascades replenish pools of IAA proteins to reset the balance of Auxin response (Ulmasov et al. 1999; Hagen et al. 2002; Paponov et al. 2008; Middleton et al. 2010). We tested whether proteosomal inhibition with the molecule MG132 would stabilize IAA31 protein in wild type and our chimeric *iaa31-2* mutant. We observed no accumulation of wild-type IAA31, and minimal stabilization of IAA31-2 compared to mock treated seedlings (Figure 5B). Interestingly, IAA31-2 protein levels were essentially unaffected by addition of the Auxin analogue Dichlorophenoxyacetic acid (2,4-D) (Figure 5B). Wild type IAA31 was not inducible by seedling incubation with either MG132 or 2,4-D, which is consistent with published microarray studies on Auxin inducible gene expression in seedlings (Schmid et al. 2005). Furthermore, the IAA31-2 protein's apparent

insensitivity to Auxin treatment complements previous protein stability analyses of IAA31, which is highly stable in the presence of Auxin when over-expressed (Dreher et al. 2006). Surprisingly, our loading control protein of choice, PEPC, was consistently higher in all *iaa31-2* conditions. Auxin treatment reduces two PEPC isoform expression levels in wild type seedlings (Supplemental Figure 1). Observation of overexpressed PEPC isoforms suggests that Auxin responses may be tuned down in *iaa31-2* seedlings. While this complicates use of PEPC as a protein loading control, it suggests that IAA31-2 is antagonizing Auxin signaling.

***IAA31-2* protein exhibits low protein turnover in the presence of Auxin**

In order to test basal IAA31-2 protein stability, we grew *iaa31-2* seedlings in liquid culture with or without the transcriptional inhibitor cyclohexamide (CHX, Figure 6A). We collected tissue at 0 and 10 hours, and compared the abundance of IAA31-2 by Western blotting, and found IAA31-2 levels to be essentially unchanged. We then tested IAA31-2 protein stability in the presence of the synthetic Auxin 2,4-D. IAA31-2 protein levels were only slightly reduced even after 10 hours of incubation with 2,4-D, indicating a very high stability in the presence of Auxin (Figure 6B).

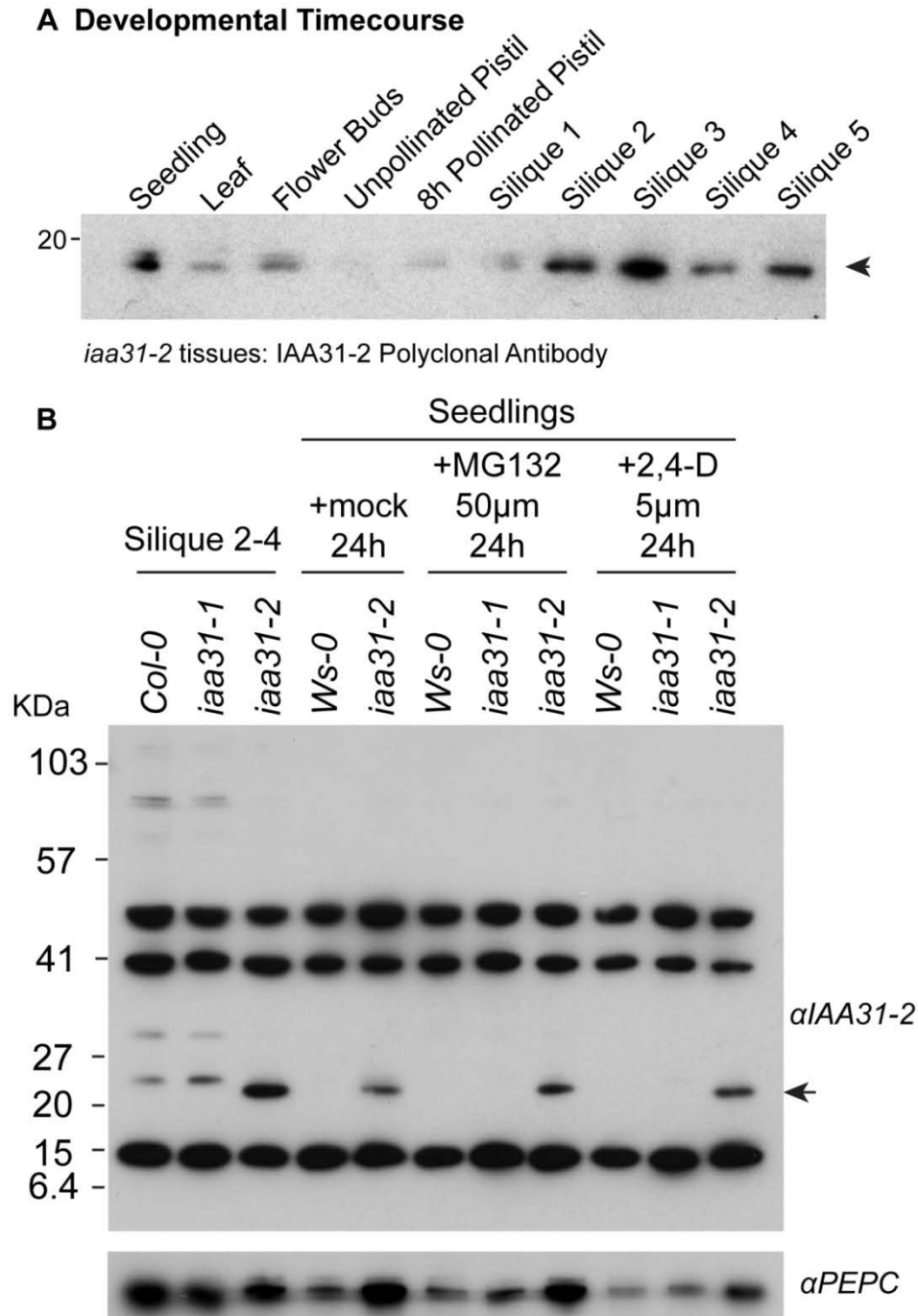


Figure 5. The *iaa31-2* mutant protein is detectable at varying levels in a range of plant tissues. A. A range of tissues and timepoints during the reproductive process were analyzed in *iaa31-2/iaa31-2* homozygous plants for expression of the *iaa31-2* mutant protein. Seedlings – 7 day old whole seedlings. Leaf – rosette leaf tissue, Unpollinated pistils – pistils emasculated 12 hours before collection, 8h Pollinated pistils - pistils emasculated 12 hours before collection and pollinated with Ws-0 wild type pollen for 8 hours, Silique 1 – 1-2 days after pollination (dap), Silique 2 – 3-4dap, Silique 3, 4-5dap, Silique 4, 6-

7dap, Silique 5 – 8-9dap. **B.** Seedling expression of the *iaa31-2* mutant protein is highly stable even in the presence of the auxin analog 2,4-D. 7 day old seedlings were transferred to MS0 liquid media and incubated in the presence of MG132, 2,4-D, or mock with agitation for 24 hours. Silique tissue spanning timepoints 2-4 tissue is provided as a positive control.

***iaa31-2* is a dominant gain-of-function allele**

IAA31-2 retains AUX/IAA protein domain II, which is sufficient to form the Auxin-TIR1 binding complex in other IAA proteins (dreher), and over-expression of wild type IAA31 protein causes dominant Auxin related phenotypes (Sato, 2008). The *iaa31-1* mutation is an RNA null allele with no observable phenotypes. However, we observed sporophytic developmental phenotypes in *iaa31-2* seedlings and plants. Seedlings from self-pollinated *iaa31-2*/IAA31 plants were grown on MS media with Basta displayed defects in seedling development; a large number of seedlings germinate yet cease to grow, remaining small. Roots from small *iaa31-2* seedlings frequently failed to develop, instead growing large numbers root hairs. We observed a buildup of anthocyanin at the apical hook regions, and often the seed coat remained on cotyledons (Figure 7A-D). The frequency of small seedlings suggested a non-mendelian segregation: less than one quarter of seedlings exhibited the small phenotype (Figure 7E).

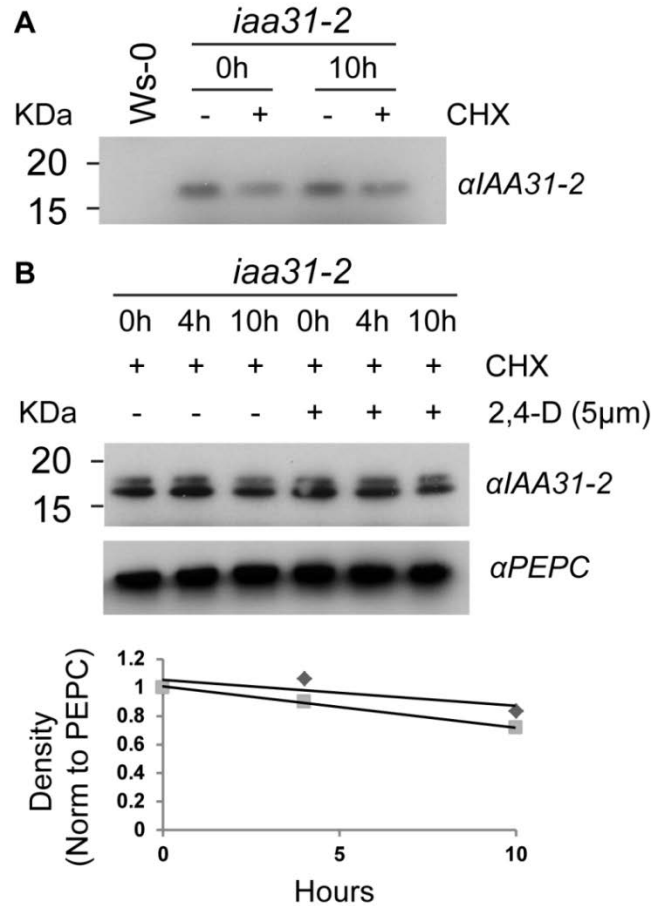


Figure 6. *iaa31-2* mutant protein exhibits low rates of protein turnover, even in the presence of Auxin analogs. **A.** Homozygous *iaa31-2* 7d old seedlings were incubated with mock treatment or cyclohexamide (CHX) for 0h and 10h and analyzed by western blotting. Ws-0 seedlings were mock treated as a negative control. **B.** Homozygous *iaa31-2* 7d old seedlings were pre-incubated with 2,4-D or mock for two hours before the addition of mock solution or CHX. Seedlings were collected at 0h, 4h, and 10h, before protein expression analysis by Western Blotting. Membranes were stripped and re-probed with α PEPC as a loading control. Densitometry was performed and protein density was quantified normalized to PEPC density (ImageJ).

Homozygous seedlings with a normal seedling phenotype develop leaf morphology and floral defects as adult plants, however these defects were not observed in the heterozygous *iaa31-2/IAA31* plants (Data not shown).

Both heterozygous and homozygous *iaa31-2* plants displayed reduction in seed set in siliques from self-pollinations (Figure 8A). We tested whether *iaa31-2* seed set disruption was due to male or female gametophytic defects using reciprocal crosses to wild-type (Figure 8B), and found normal transmission of both male and female gametes. Normal gamete transmission by both sexes suggested a role for the IAA31-2 protein in the sporophytic tissues of the pistil. Seed set in crosses to pistils carrying the *iaa31-2* locus is reduced, both when heterozygous and homozygous, regardless of pollen genotype (Figure 8C). Therefore we suggest that IAA31-2 is acting dominantly in sporophytic pistil tissues, such as the stigma, style and transmitting tract.

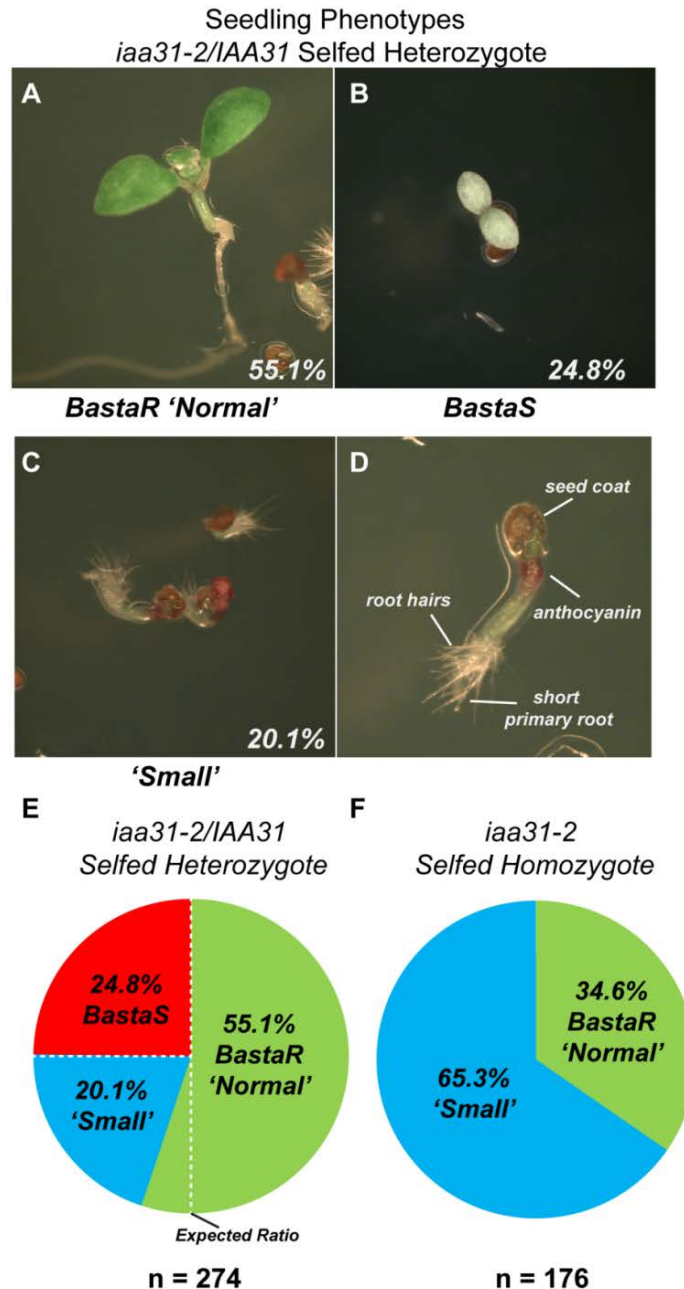


Figure 7. *iaa31-2* seedlings frequently fail to develop normally. A-D. Seedling phenotypes in seeds from selfed-heterozygous plants. **A.** *Basta* Resistant plants exhibiting a wild-type seedlings morphology. **B.** *Basta* sensitive plants not carrying the *iaa31-2* T-DNA. **C.** "Small" *iaa31-2* homozygous seedlings exhibiting severe growth phenotypes. **D.** Close-up image of "Small" seedlings that frequently fail to develop a long root, develop high levels of anthocyanin at the apical hook, and fail to fully shed the seed coat. **E.** Frequency of phenotypes (A-C) observed in selfed-heterozygous seedling populations. **F.** Frequency of phenotypes observed in selfed-homozygous seedling populations.

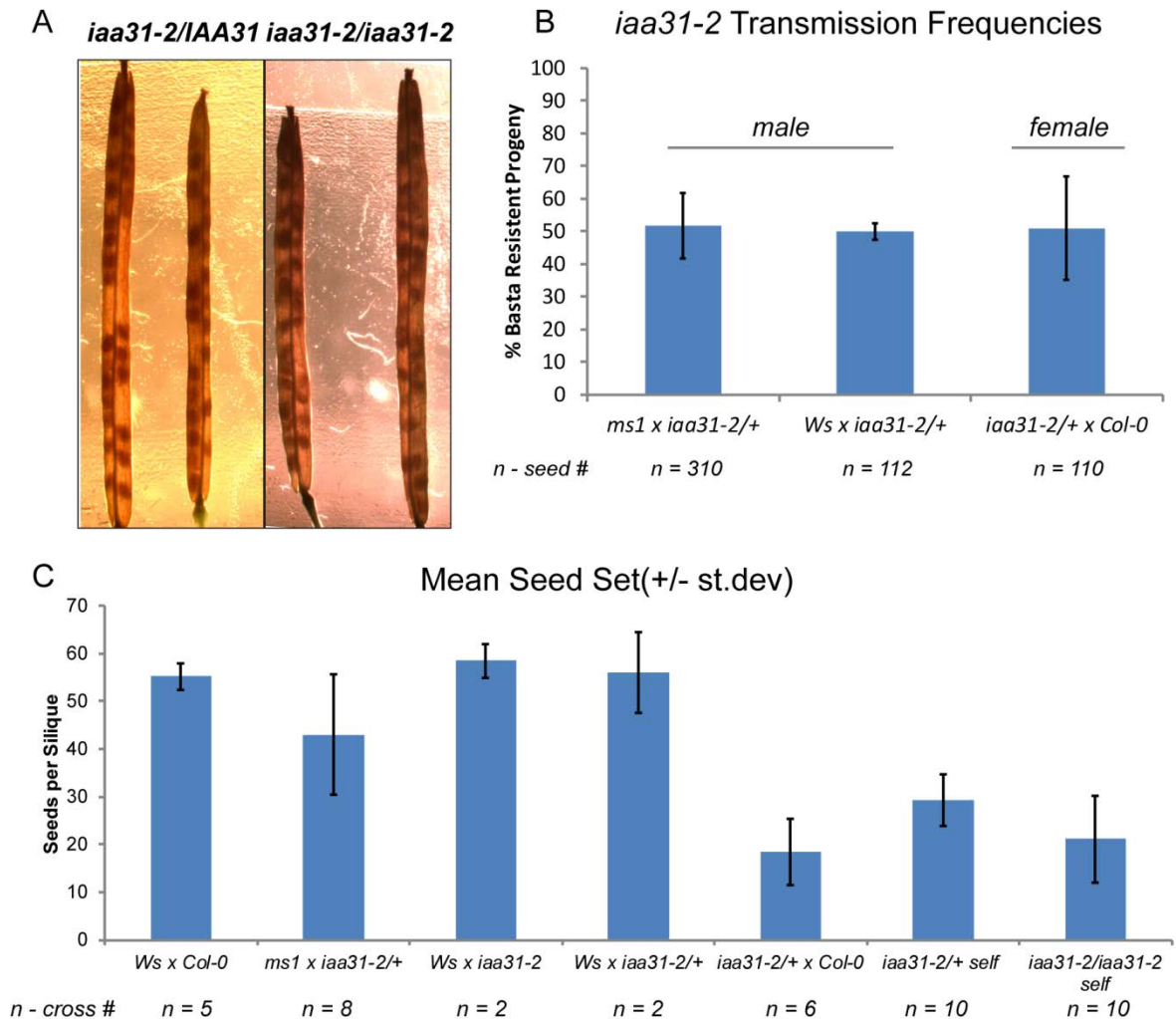


Figure 8. The *iaa31-2* mutation is a dominant mutation that disrupts seed production. **A.** Seed set defects in selfed heterozygous and homozygous siliques. **B.** Transmission of *iaa31-2* gametes is not disrupted in either sex. *ms1* – male sterile 1. Data is the average transmission rate of at least 6 independent crosses +/- Std Dev. **C.** Mean seed set from *iaa31-2* mutant crosses +/- Std Dev.

Pollen tube guidance is disrupted in *iaa31-2* mutant pistils

We observed seed set reduction in *iaa31-2* mutant pistils, yet transmission of the *iaa31-2* allele through female gametes was normal. We therefore hypothesized that *iaa31-2*'s dominant activity acts in steps subsequent to pollen tube reception and double fertilization. We performed crosses using *iaa31-2* mutant pistils and wild-type carrying a pollen specific dsRed protein (*LAT52:dsRed*) for live imaging

of pollen tube growth and guidance in the pistil using confocal microscopy (Figure 9A-G). We observed disrupted pollen tube guidance in all crosses using *iaa31-2* pistils 12 hours after pollination. Specifically, pollen tubes growing in wild type pistils find more than 80% ovules, while pollen tubes growing in *iaa31-2* pistils arrive at only 30-40% of ovules, even when one wild-type copy of *IAA31* is present (Figure 9G). We frequently observed ovules that failed to attract pollen tubes (Figure 9A,B), and intriguingly, we observed a subset of ovules with pollen tubes growing on the *iaa31-2* funiculus (Funicular wandering, Figure 9D,E). In order to examine whether ovules in *iaa31-2* pistils that received pollen tubes underwent double fertilization and began development, we crossed *iaa31-2* pistils with pollen carrying a sperm specific histone fused to RFP (*HTR10:HTR10:RFP*) and performed live imaging via confocal microscopy 12 hours after pollination. We clearly observed double fertilization and karyogamy (Figure 9H), and subsequent nuclear divisions indicative of early seed development (Figure 9I-J). These results indicate *iaa31-2* pistils cause a distinct loss of pollen tube targeting in the pistil, which is not due to the genotype of the female gametophyte.

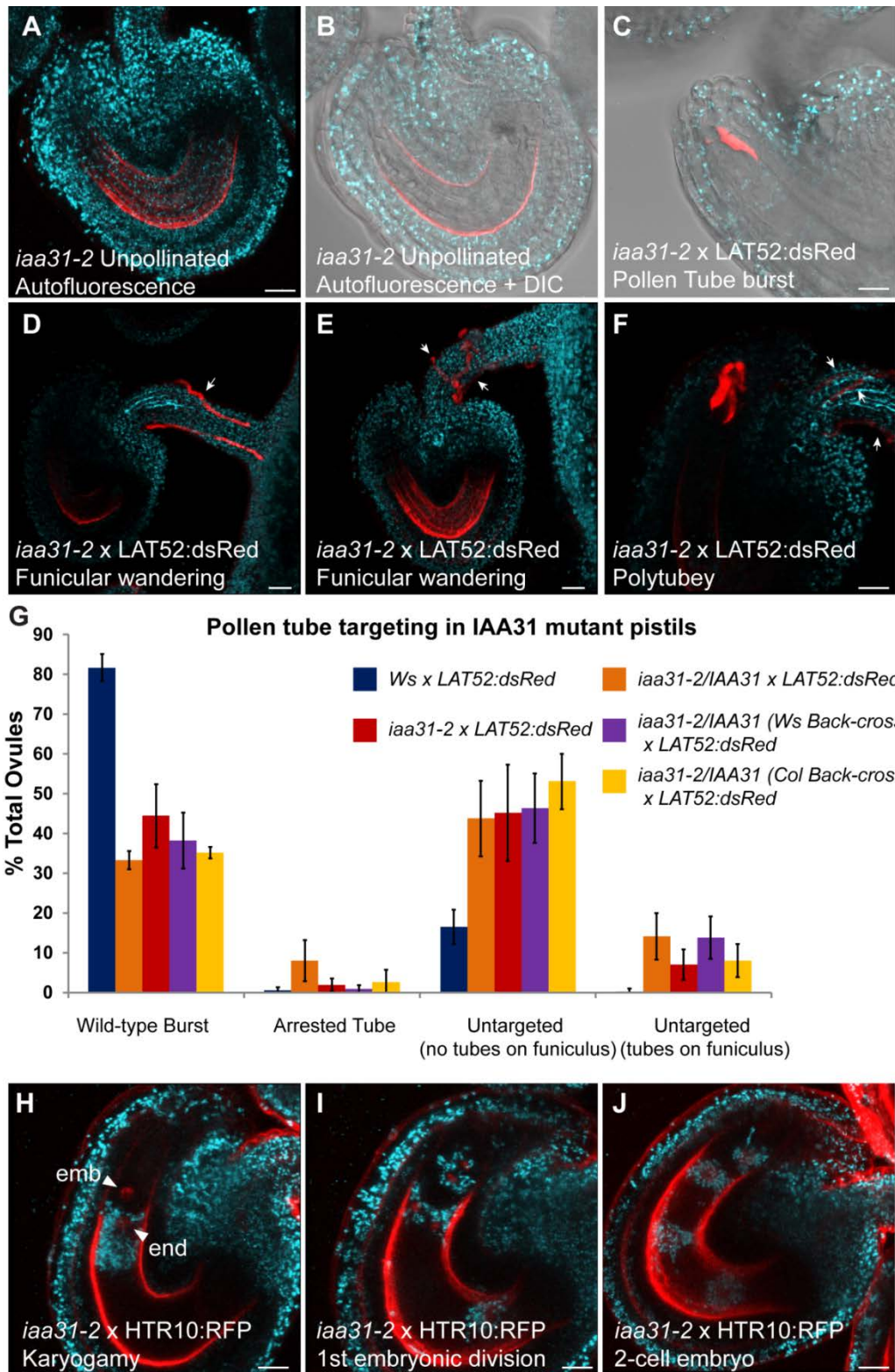


Figure 9. Pollen tube guidance is crippled in *iaa31-2* pistils. A-F. Ovules in *iaa31-2* homozygous pistils pollinated with wild type pollen carrying a LAT52:DsRed marker (12hour pollinations). Chloroplast autofluorescence is

shown in cyan. **A+B.** Untargeted ovule with no pollen tubes on funiculus. **C.** Ovule successfully targeted by a pollen tube that has burst (Red hook in ovule). **D+E.** Ovules that have pollen tubes 'wandering' (arrows) on the funiculi that have failed to enter the micropyle. **F.** An ovule that has been targeted by three pollen tubes (arrows), at least one of which has burst in the ovule (red hook). **G.** Quantification of pollen tube guidance defects in *iaa31-2* mutant pistils. Mean phenotype rate from three biological replicates, +/- Std Dev (>100 ovules each exp). **H-J.** Ovules from *iaa31-2* pistils pollinated with pollen carrying HTR10:RFP, 12hap. **H.** *iaa31-2* ovule at karyogamy. **I.** 1st division. **J.** 2-cell stage. Chloroplasts autofluorescence provides corroboration of early divisions (cyan). Scale bars = 20um.

***iaa31-2* pollen tube guidance is rescued by semi in vivo pollen tube growth conditions**

Pollen tubes emerge from the transmitting tract in response to gradients of attractants. While there are likely multiple attractants that act at both long and short range, one class of defensin related cysteine rich proteins known as as LUREs are known to be secreted from the synergid cells in Arabidopsis ovules (Takeuchi). These small LURE proteins are pollen tube attractants that diffuse out of the micropyle of the ovule, coating the funiculus in a gradient with a maximum concentration at the ovule. Pollen tubes grown in vitro are unable to perceive LURE proteins, and require growth through the pistil to acquire LURE binding activity (Cite). We hypothesized that *iaa31-2* pistils failed to signal to pollen tubes, rendering them unable to fully perceive LURE proteins secreted from the ovule. To test this hypothesis, we performed semi in vivo pollen tube targeting assays using wild type and *iaa31-2* mutant pistil explants (Figure 10).

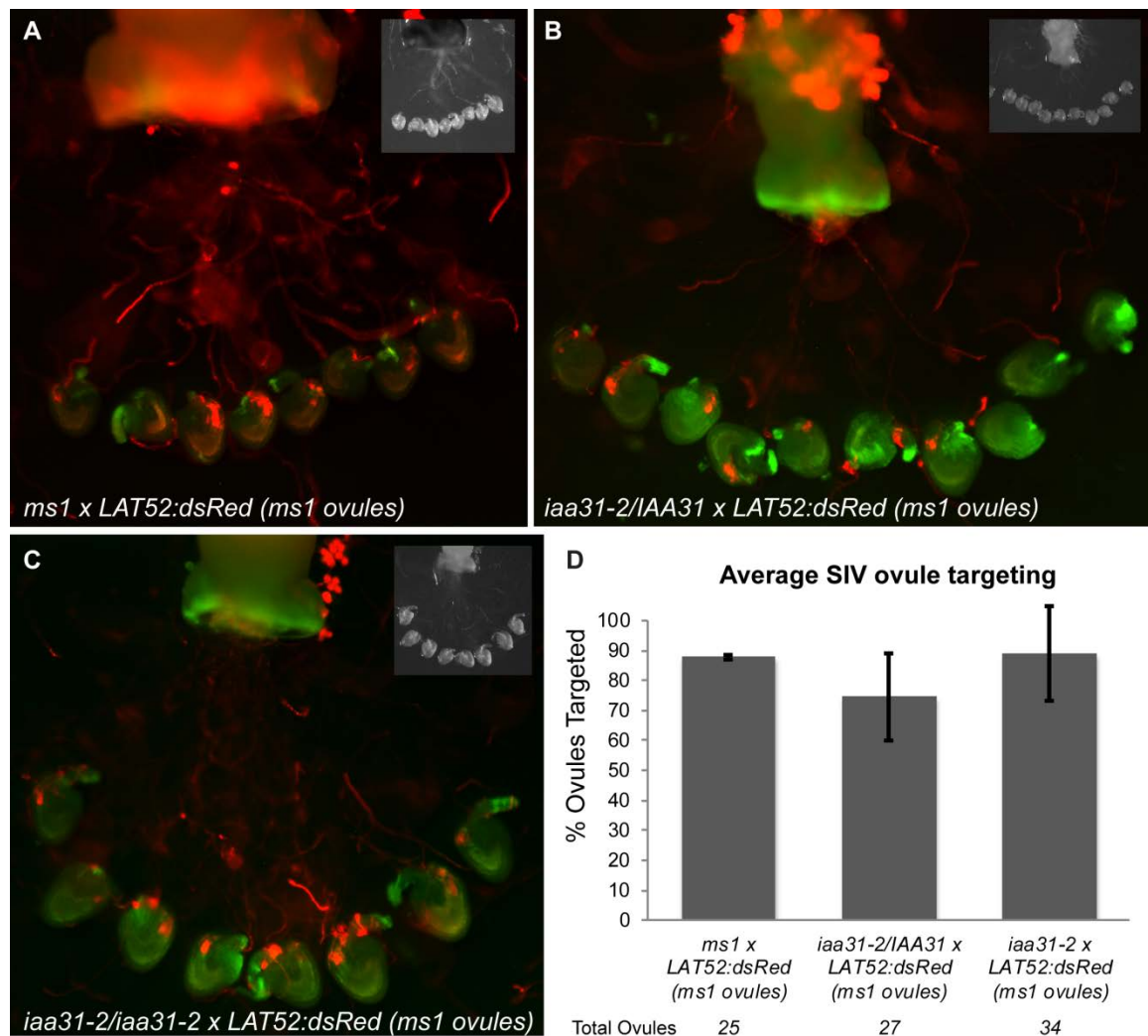


Figure 10. Semi in-vivo pollen tube growth through *iaa31-2* pistils to wild-type ovules is normal. A-C. Epifluorescent micrographs of SIVs using *iaa31-2* stigma explants, wild-type LAT52:DsRed pollen, and wild type *ms1* ovules. One representative replicate from each genetic combination is shown with DIC inset at top right. D. Quantification of targeting success is shown as the average of three replicates +/- Std Dev.

However, we observed normal pollen tube targeting in all experiments, indicating pollen tube perception of LURE proteins unaffected by the *iaa31-2* mutation.

An alternative hypothesis for pollen tube guidance defects in *iaa31-2* pistils is that LURE proteins are not released from ovules from *iaa31-2* ovaries. If IAA31-2 interferes with LURE protein secretion, pollen tubes should exhibit low rates of targeting to ovules. We performed semi in vivo pollen tube targeting assays using

wild type and *iaa31-2* pistil explants and ovules from *iaa31-2* pistils (Figure 11). Again, pollen tube guidance to ovules was unaffected by the pistil or ovule genotype. Together, these results suggest that *iaa31-2* affects pollen tube guidance in vivo through a mechanism that is as yet, elusive.

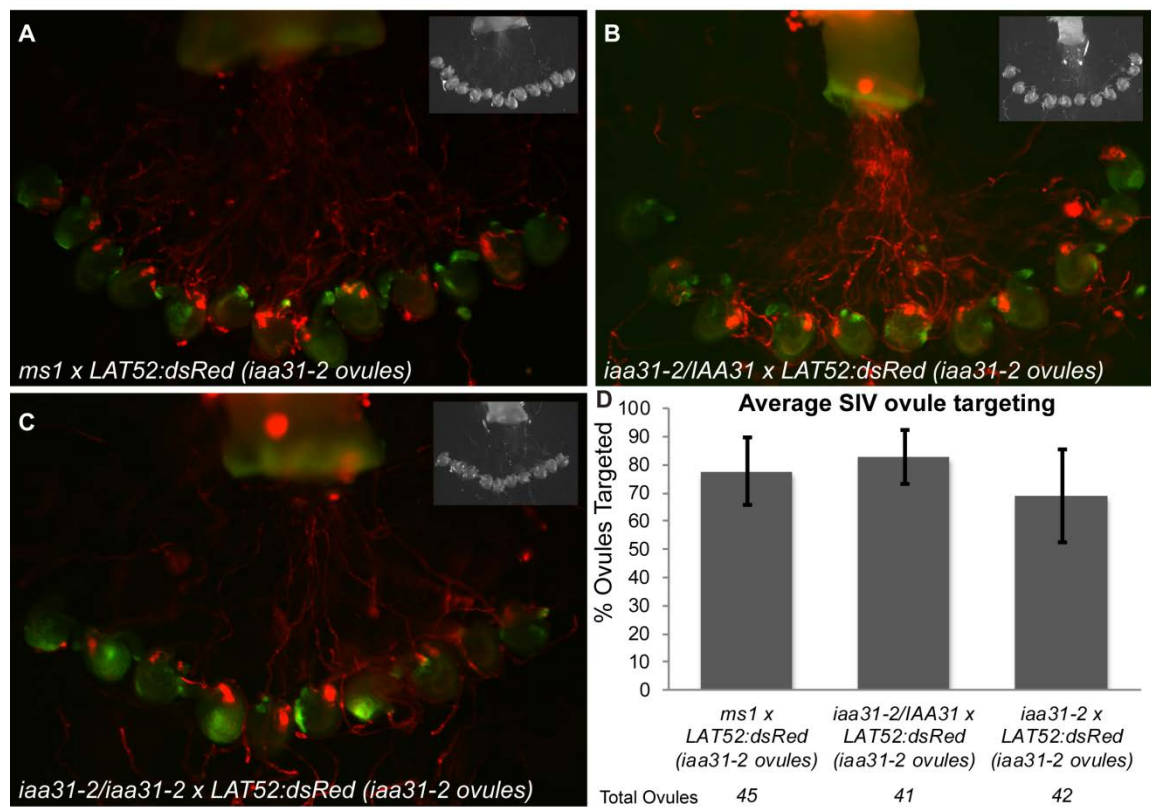


Figure 11. Semi in-vivo pollen tube growth through *iaa31-2* pistils to *iaa31-2* ovules is normal. A-C. Epifluorescent micrographs of SIVs using *iaa31-2* stigma explants, wild-type LAT52:DsRed pollen, and *iaa31-2* ovules. One representative replicate from each genetic combination is shown with DIC inset at top right. D. Quantification of targeting success is shown as the average of three replicates +/- Std Dev.

***iaa31-2* disrupts Auxin signaling networks**

AUX/IAA proteins regulate Auxin transcriptional responses through direct binding with ARF proteins, where IAA/ARF complexes typically repress ARF target gene

expression. We performed preliminary experiments to identify Auxin inducible genes that change expression levels in *iaa31* mutant plants.

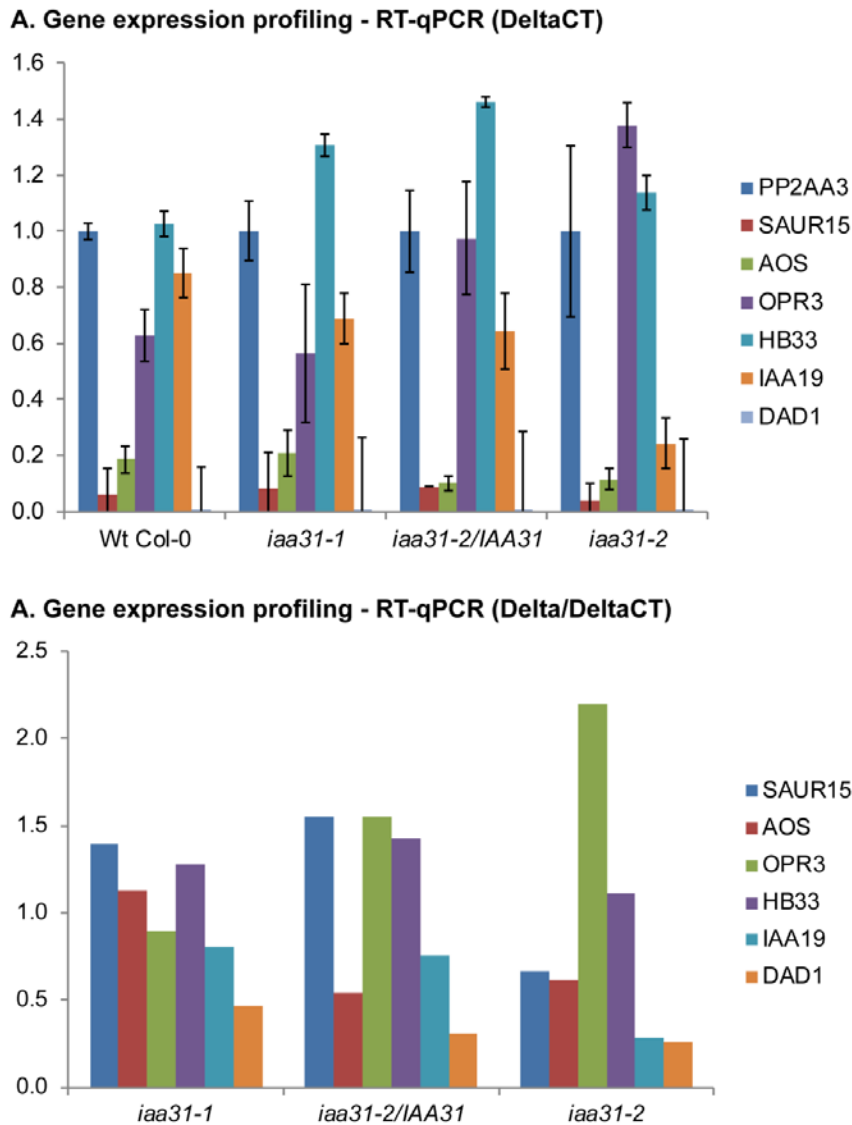


Figure 12. RT-qPCR gene expression profiling of Auxin-responsive genes in *IAA31* mutant siliques. RNA from siliques 2-4 days after pollinated was pooled and converted to cDNA for analysis by qRT-PCR. **A.** Gene expression quantified by normalization to PP2AA3 transcript levels – Delta CT method. **B.** Data in **A** normalized to wild-type – Delta/Delta CT method.

We identified Auxin responsive target genes that contain the conserved Auxin response element in their promoters, and have previously published evidence for

transcription in the pistil (Wang et al. 2013). We performed RT-qPCR on RNA extracted from siliques 2-4 days after pollination, which represents a wide window of early seed development. While still a very preliminary analysis, we did observe several genes that show differential regulation in *iaa31-2* siliques, especially OPR3, DAD1 and IAA19.

Auxin signaling homeostasis may be disturbed by *IAA31-2*

Our results suggest that *IAA31* is normally expressed at very low levels in floral tissues, and loss of *IAA31* does not affect pollen tube guidance or fertilization. However, the *IAA31-2* protein is highly expressed in pistil tissues, and exerts a dominant effect on pollen tube guidance. Therefore we present the following model. *IAA31-2* protein contains an intact domain II, allowing interaction with TIR1 and Auxin, but not with ARF proteins. This truncated IAA protein acts as a TIR1 sponge, occupying Auxin-TIR1 complexes and blocking other IAA/ARF complexes from being degraded efficiently. Effective reduction of TIR1 protein activity reduces overall Auxin signaling output, altering the expression of ARF regulated genes.

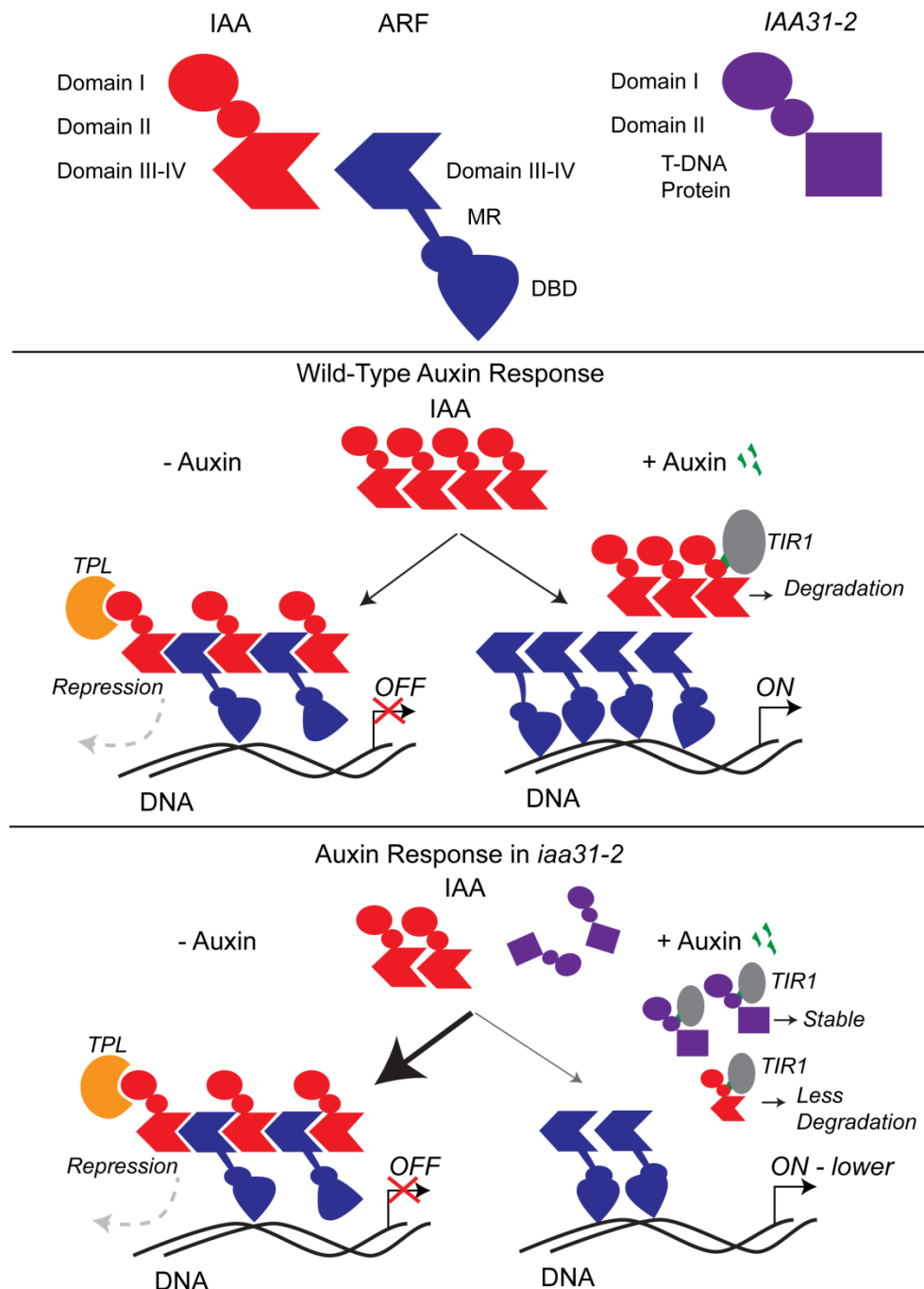


Figure 13. Summary of proposed mechanism of *IAA31-2* dominant suppression of Auxin signaling. IAA – Aux/IAA protein, ARF – Auxin response factor. MR – Middle Region, DBD – DNA binding Domain. Domains I-IV refer to the conserved domains I-IV found in IAA and ARF proteins. T-DNA Protein – refers to the chimeric portion of IAA31-2 protein formed from translational fusion of IAA31 and the *iaa31-2* T-DNA. TPL – Topless, TIR1 – Transport Inhibitor Response I. Cartoon is adapted from (Han et al. 2014)

Discussion

A novel role for Auxin in pollen tube guidance

Auxin has occupied a central role in plant hormone signaling, there are myriad publications about its role in sporophytic development and function. Reproductive involvement of Auxin has been far less elucidated, possibly due to the complex tissue structure of the pistil – especially during the dynamic window of pollen tube growth and guidance. However, many IAA and ARF gene family members are expressed in the pistil and are poised to respond to any fluctuations of Auxin and Auxin signaling components. Many IAA proteins have been discovered and described by mutations in domain II, where ablation of Auxin-TIR1 binding renders IAA degradation defunct and ARF proteins repressed (cite review of mutations). IAA domain II mutations by nature inhibit their ARF protein-binding partners specifically, reducing transcription of specific target types. For example, targeted mutation of IAA8 Domain II (Pro170 to Leu170) ablated proteosomal degradation and reduced ARF6/8 transcriptional activation of target genes (DAD1, AOS, OPR3) (Wang et al. 2013). Dominant IAA8 expression disrupts floral morphology and reduces seed set, however post-pollination defects were not characterized. We have described an *IAA31* mutation, *iaa31-2*, which produces an identical seed set reduction, specifically through loss of pollen tube targeting. Unlike typical IAA mutations that disrupt interaction between domain II-Auxin-TIR1, IAA31-2 preserves the Auxin receptor domain yet separates it from ARF binding. This unique mutation provides a method for subtly diminishing the Auxin response of all IAA proteins in a given tissue.

Future directions

Identifying IAA31 expression domain

At present we have been unable to detect wild-type IAA31 protein localization using custom polyclonal antibodies that span its amino acid sequence. We suggest that IAA31 gene expression is tightly regulated during development functions as an ARF-specific Auxin “brake”. IAA31-2 protein appears to retain tissue specific transcriptional activation, yet accumulates to higher levels. Therefore we propose performing immunolocalization of IAA31-2 protein in *iaa31-2* pistils. Using the IAA31-2 polyclonal antibody on wild-type and *iaa31-2* pistils will identify the pattern of expression in the pistil. If we observe localization and/or enrichment of IAA31-2 protein to specific cell types in the gynoecium, we may better focus our analysis of pollen tube targeting. In a complementary approach, we have designed genetic constructs carrying the presumed IAA31 promoter region driving GFP, or IAA31 coding sequence fused to GFP. These constructs will help to identify the expression domain of IAA31 within reproductive tissues.

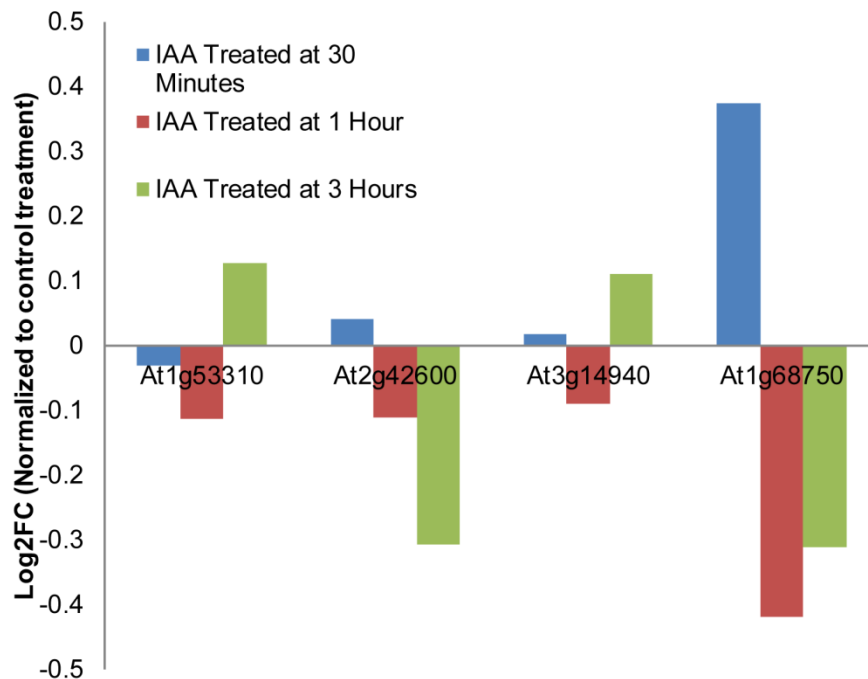
Transcriptional regulation by IAA31 and IAA31-2

Our preliminary experiments uncovered tantalizing changes in gene expression in *iaa31-2* tissues. These experiments were performed with a collection of tissues along a broad range of developmental stages. As such, it is impossible to make correct quantitative interpretations of our results. However, general trends are

established in these qPCR analyses that warrant further exploration. We are performing RNA-sequencing to identify differential gene expression at a specific window of pollination. Buds from wild type, *iaa31-1*, and *iaa31-2* pistils were emasculated 12 hours before manual self-pollination. Pistils were dissected from the remaining floral tissue after 8 hours and RNA-sequencing libraries were generated. We predict that a unique subset of genes will show differential expression between wild type and *iaa31-1* pollinations. These genes are most likely specific to ARFs that interact with wild-type IAA31. Genes differentially expressed between wild type and *iaa31-2* pollinations represent targets of multiple ARFs regulated by all IAAs expressed in pistil tissue. Finally, genes that change expression in both mutants represent high confidence target genes regulated by ARFs specifically in complex with IAA31. This sequencing experiment will reveal both the specific role of IAA31 and simultaneously uncover the full breadth of Auxin signaling in pollen tube growth and guidance.

Supplemental Figures

A



B	Control at 30 Minutes	IAA Treated at 30 Minutes	Control at 1 Hour	IAA Treated at 1 Hour	Control at 3 Hours	IAA Treated at 3 Hours
PEPCs 1 - 3 all 110 kDa						
At1g53310	803.44	786.59	837.49	774.18	825.91	902.64
stdev	91.41	93.34	71.07	68.86	9.58	180.58
At2g42600	679.48	698.83	700.83	649.13	746.04	603.21
stdev	19.16	12.77	2.7	50.13	33.33	18.23
At3g14940	280.61	283.93	293.89	276.22	290.45	313.57
stdev	5.74	35.09	18.66	0.82	14.51	67.77
PEPC4 116.5 kDa						
At1g68750	15.69	20.32	20.78	15.55	23.29	18.77
stdev	0.45	2.9	2.57	1.98	1.49	0.91

Supplemental Figure 1. A. All Arabidopsis PEPC isoforms show Auxin responsive transcription. Publically available Auxin response data provided by the Shimada lab, accessed through eBAR. **B.** Normalized microarray data and Gene information used to plot column graphs in A.

Methods

Seedling Selection and Plant Growth

Seeds were grown on solid Murashige and Skoog (MS) medium (MP Biomedicals, LLC, Solon, OH, USA). Arabidopsis plants were grown at 21°C and received 100 $\mu\text{mol}/\text{m}^2/\text{sec}$ illumination for 16 hours each day (Environmental growth chambers, Chagrin Falls, OH, USA). Seedlings were transplanted to sterile 2MIX potting media (www.fafard.com) between 7 and 10 days after plating. In vitro pollen tube growth was performed as previously described (Boavida et al. 2007). Semi in vivo pollinations were performed as previously described (Palanivelu et al. 2006; Qin et al. 2009).

Northern Blotting

For IAA31 probe, full length cDNA was ordered from ABRC (PYAT3G17600), Tubulin control probe is a cDNA of *AT5G44340* (TUB4). Probe was labelled with 32P (Amersham MegaPrime, GE), and Northern blotting was performed as previously described (Melquist et al. 2004).

Cloning

T-DNA borders were cloned using Phusion Polymerase (NEB, Ipswich, MA), dATP was added using GoTaq (Promega, Madison, WI), and subsequently cloned into pCR2.1 (Invitrogen) and sequenced (Genewiz, Cambridge, MA). The *iaa31-2* cDNA was cloned using primers that tiled successively into the left border sequence of the FLAG T-DNA.

RNA Extraction, RT-PCR, and RT-qPCR

RNA was purified using the Qiagen RNeasy kit (<http://www.qiagen.com>). The yield and RNA purity were determined by Nano-Drop (Thermo Scientific, Wilmington, DE, USA). Gene-specific first strand synthesis and PCR was performed using the Access RT-PCR System (Promega). For RT-PCR, RNA was DNase treated with RNase-Free DNase (Qiagen). cDNA libraries were constructed from 2.5ug of total RNA using Super Script VILO Master Mix

(random hexamer and oligo dT, Super Script III), (Invitrogen). For Real-time RT-qPCR, RNA was DNase treated using the Ambion TURBO DNA-free kit from Invitrogen (AM1907). Primers were designed at Primer3Plus (primer3plus.com/), using qPCR-optimized settings. All primers were tested to assure that they have near 100% efficiency and linearity prior to use with an ABI 7000 real time PCR machine. RT-qPCR was performed in triplicate. The comparative Ct method (User Bulletin #2, Applied Biosystems) was used for quantification. Expression was normalized to the ubiquitous PP2AA3 gene (Czechowski et al. 2005). RT-qPCR was performed with cDNA libraries using primers of equivalent efficiency in triplicate reactions using SYBR Green qPCR Master Mix (Invitrogen) on an ABI 7000 PRISM Sequence Detection System (Applied Biosystems).

Polyclonal Antibody production

Custom antibodies were produced by GenScript (Piscataway, NJ). Blotting was performed with 1ug/ml of primary polyclonal antibody in 5% Milk, in PBS-T.

Pollinations and Microscopy

Unopened buds were emasculated 12-18 hours 1 day previous to manual pollinations. For live imaging, crosses were performed under a dissecting microscope (Zeiss Stemi 2000C) by pollinating emasculated stage 14 stigmas with pollen from male donors. Ovary walls were removed 12 hours post-pollination as previously described (Johnson et al. 2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). Semi in vivo pollen tube imaging was performed on a Zeiss Lumar V12 Fluorescence Stereomicroscope.

Oligonucleotides used in this study

At3g17600qF1	tgtgattgcgcagttacgag
At3g17600qR1	cttcttcaaggcctttgttgc
IAA31_5pqF1	TCTTCATCCTCTGTCGACAGTAC
IAA31_5pqR1	GGCCAATCTTGTCTTGCTTCTC
iaa31-2_cdnar1	TACGGCAATGTACCAGCTGA
iaa31-2_cdnar2	TGCCTTTTCTTATCGACCATGT
iaa31-2_cdnar3	ACACTGGGATTCGTCTTGGA
iaa31-2_cdnar4	AGCGTAGACAACCCTCAACT
PP2AA3_qPCR_F	GAGTTTGGTCCTGAATGGGCAATG
PP2AA3_qPCR_R	ACTGGAGCGAGAAGCGATACTG
qHB33_F	GGACAATCAAGCGGAGAAGGAGC
qHB33_R	CTCCGATCTCGCCGAGAATCTC
qSAUR15_F	ATGGCTTTTTTGAGGAGTTTCTTGGG
qSAUR15_R	TCATTGTATCTGAGATGTGACTGTG
qIAA19_F	CTCGGGCTTGAGATAACGGA
qIAA19_R	CCACATCTCTCCCCGGAA
qDAD1_F	TAAGGAGCTTCGGCTGAGTAGCCGT
qDAD1_R	GAGAACTCTCCGAGCTGTTTCTCTG
qAOS_F	ACTACGGTTTACCAATCGTAGGAC
qAOS_R	TCTGTACACCGTGGAGTTGTATTT
qOPR3_F	TTCACGCTAAGGGAGGTTTCA
qOPR3_R	CAAACTCGCCACCTGTTTTTC

References

- Boavida, L. C., F. Borges, et al. (2011). "Whole genome analysis of gene expression reveals coordinated activation of signaling and metabolic pathways during pollen-pistil interactions in Arabidopsis." Plant Physiol **155**(4): 2066-2080.
- Boavida, L. C. and S. McCormick (2007). "Temperature as a determinant factor for increased and reproducible in vitro pollen germination in Arabidopsis thaliana." Plant J **52**(3): 570-582.
- Chapman, E. J. and M. Estelle (2009). "Mechanism of Auxin-Regulated Gene Expression in Plants." Annual Review of Genetics **43**: 265-285.
- Czechowski, T., M. Stitt, et al. (2005). "Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis." Plant Physiol **139**(1): 5-17.
- Dharmasiri, N., S. Dharmasiri, et al. (2005). "The F-box protein TIR1 is an auxin receptor." Nature **435**(7041): 441-445.
- Dreher, K. A., J. Brown, et al. (2006). "The Arabidopsis Aux/IAA protein family has diversified in degradation and auxin responsiveness." Plant Cell **18**(3): 699-714.
- Hagen, G. and T. Guilfoyle (2002). "Auxin-responsive gene expression: genes, promoters and regulatory factors." Plant Mol Biol **49**(3-4): 373-385.
- Han, M., Y. Park, et al. (2014). "Structural basis for the auxin-induced transcriptional regulation by Aux/IAA17." Proc Natl Acad Sci U S A **111**(52): 18613-18618.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Kepinski, S. and O. Leyser (2005). "The Arabidopsis F-box protein TIR1 is an auxin receptor." Nature **435**(7041): 446-451.
- Kepinski, S. and O. Leyser (2005). "The Arabidopsis F-box protein TIR1 is an auxin receptor." Nature **435**(7041): 446-451.
- Larsson, E., R. G. Franks, et al. (2013). "Auxin and the Arabidopsis thaliana gynoecium." Journal of Experimental Botany **64**(9): 2619-2627.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Curr Biol **23**(13): 1209-1214.
- Melquist, S. and J. Bender (2004). "An internal rearrangement in an Arabidopsis inverted repeat locus impairs DNA methylation triggered by the locus." Genetics **166**(1): 437-448.
- Middleton, A. M., J. R. King, et al. (2010). "Mathematical modelling of the Aux/IAA negative feedback loop." Bull Math Biol **72**(6): 1383-1407.
- Overvoorde, P. J., Y. Okushima, et al. (2005). "Functional genomic analysis of the AUXIN/INDOLE-3-ACETIC ACID gene family members in Arabidopsis thaliana." Plant Cell **17**(12): 3282-3300.
- Palanivelu, R. and D. Preuss (2006). "Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro." BMC Plant Biol **6**: 7.
- Paponov, I. A., M. Paponov, et al. (2008). "Comprehensive transcriptome analysis of auxin responses in Arabidopsis." Mol Plant **1**(2): 321-337.

- Peer, W. A. (2013). "From perception to attenuation: auxin signalling and responses." Curr Opin Plant Biol **16**(5): 561-568.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Sato, A. and K. T. Yamamoto (2008). "Overexpression of the non-canonical Aux/IAA genes causes auxin-related aberrant phenotypes in Arabidopsis." Physiologia Plantarum **133**(2): 397-405.
- Schmid, M., T. S. Davison, et al. (2005). "A gene expression map of Arabidopsis thaliana development." Nat Genet **37**(5): 501-506.
- Szemenyei, H., M. Hannon, et al. (2008). "TOPLESS mediates auxin-dependent transcriptional repression during Arabidopsis embryogenesis." Science **319**(5868): 1384-1386.
- Ulmasov, T., G. Hagen, et al. (1999). "Dimerization and DNA binding of auxin response factors." Plant J **19**(3): 309-319.
- Wang, J., D. W. Yan, et al. (2013). "A gain-of-function mutation in IAA8 alters Arabidopsis floral organ development by change of jasmonic acid level." Plant Mol Biol **82**(1-2): 71-83.

Appendix 4:
Gamete Fusion Prevents Multiple Pollen Tubes from Entering a Single
Arabidopsis Ovule

Beale, KM, **Leydon, AR** and Johnson, MA. Curr Biol. 2012 Jun 19;22(12):1090-4.

This manuscript was submitted in a Letter format to Current Biology. I performed Aniline blue staining of pollen tubes in *duo1-3/+* mutants (Figure 1B, ARL), and performed preliminary quantification of polytubey events and editing of the manuscript.

Summary

In double fertilization, a reproductive system unique to flowering plants, two immotile sperm are delivered to an ovule by a pollen tube. One sperm fuses with the egg to generate a zygote, the other with the central cell to produce endosperm (Berger et al., 2008). A mechanism preventing multiple pollen tubes from entering an ovule would ensure that only two sperm are delivered to female gametes. We use live-cell imaging (Berger et al., 2008; Hamamura et al., 2011) and a novel mixed-pollination assay that can detect multiple pollen tubes and multiple sets of sperm within a single ovule to show that *Arabidopsis* has a robust 'block to polytubey'. We find that when gamete-fusion defective *hap2* or *duo1* sperm are delivered to ovules as many as three additional pollen tubes are attracted. When gamete fusion fails, one of two pollen tube-attracting synergid cell persists, enabling the ovule to attract more pollen tubes for successful fertilization. This mechanism prevents the delivery of more than one pair of sperm to an ovule and ensures maximum reproductive success by distributing pollen tubes to all ovules.

Results and Discussion

‘Polytubey’ increases when sperm incapable of gamete fusion are delivered to ovules

We use the term ‘polytubey’ (many pollen tubes) to describe the attraction of multiple pollen tubes to a single ovule. We chose this term to facilitate comparison with ‘polyspermy’ (the fusion of more than one sperm with a female gamete), which is distinct and also needs to be prevented to maximize reproductive success (Wong and Wessel, 2006). We developed a mixed pollination assay that unambiguously detects polytubey (Figure 1A). Half of a *male sterile1-1* (*ms1-1*) stigma was hand-pollinated with pollen expressing GFP from the pollen-specific LAT52 promoter (Twell et al., 1990) (*LAT52:GFP*); the other half with pollen expressing *DsRed* (Francis et al., 2007) (*LAT52:DsRed*). These markers define two nearly isogenic populations of pollen tubes and allow pollen tube growth, guidance, and burst to be monitored in the pistil. Twenty-one hours after pollination 48% of ovules had been targeted by a *LAT52:DsRed* pollen tube (Figure 1B, left) and 51% by a *LAT52:GFP* pollen tube (Figure 1B, middle). This slight discrepancy from equality reflects the difficulty of placing an equal load of pollen from each genotype on the stigma. Nonetheless, the ~1% of ovules with both the GFP and DsRed signal were obvious and unambiguously indicate that these ovules had been targeted by two pollen tubes (Figure 1B, right).

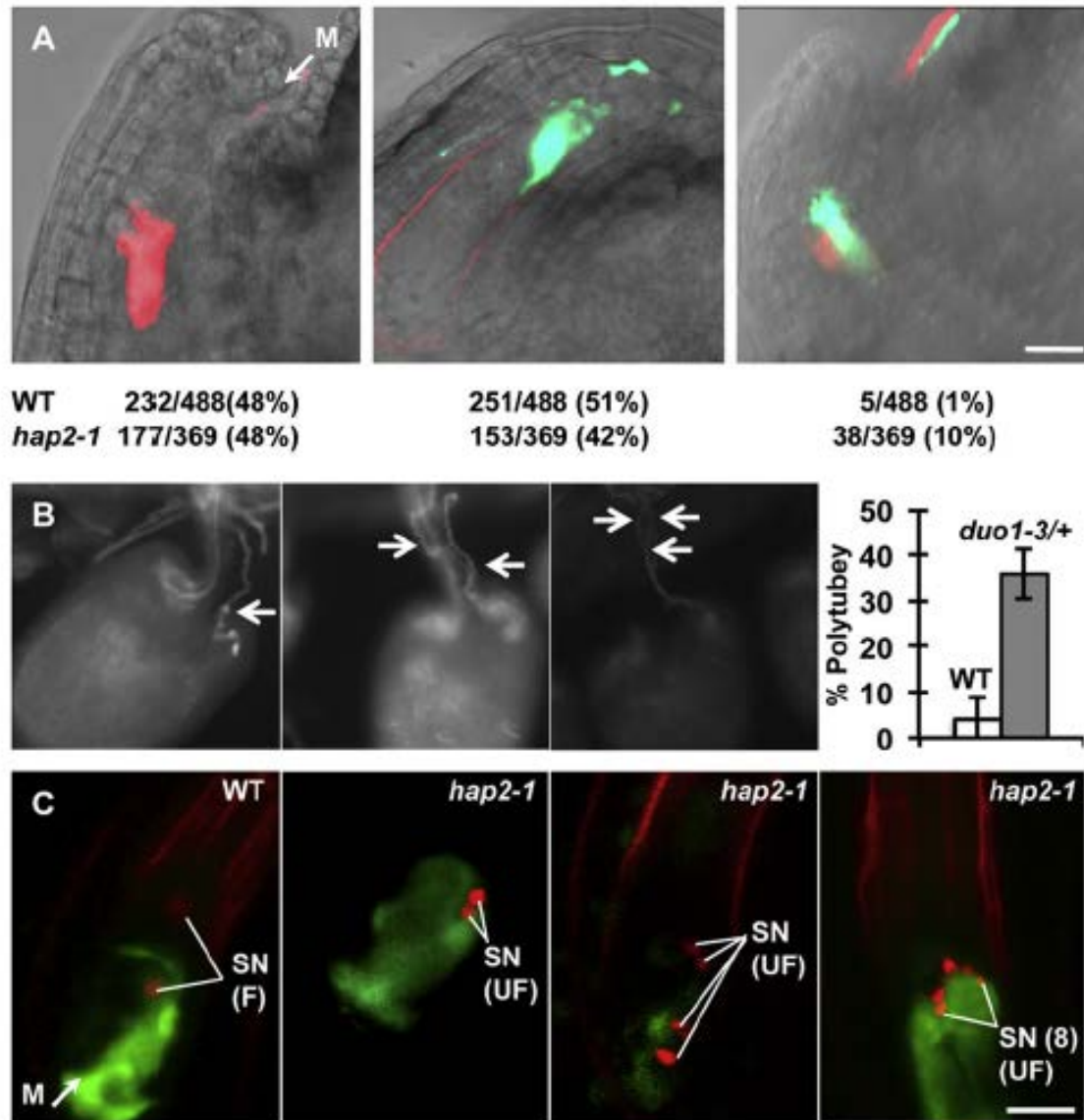


Figure 1. Polyubey is rare in wild type, but increases significantly when gamete fusion fails. (A) *hap2(gcs1)* enhances polyubey. Differentially marked pollen tubes can be tracked as they grow into an ovule at the micropylar end (M). Representative images of ovules targeted by a DsRed+ pollen tube (left), a GFP+ pollen tube (center), or by two different pollen tubes (right). The number of each type of targeting event observed is also shown (bottom). WT, *LAT52:GFP* pollen mixed with *LAT52:DsRed* (both types of pollen are wild type) or *hap2-1* (*hap2-1/HAP2(GCS1)*, *LAT52:GFP* mixed with wild-type, *LAT52:DsRed* pollen). Scale bars, 20µm. **(B) *duo1* enhances polyubey.** *duo1-3/+* heterozygous plants were manually self-pollinated and pollen tubes were analyzed 24 hours later using aniline blue staining(Mori et al., 2006). Ovules are shown that have attracted a single pollen tube (left panel), two pollen tubes (center panel), or

three pollen tubes (right panel). The percentage of ovules with two or more pollen tubes is plotted for No-0 (wild type, 9 pistils, 260 targeted ovules) and *duo1-3/+* (8 pistils, 275 ovules targeted). Error bars represent standard deviation (each pistil is one trial). Rates of polytubey are higher in the No-0 accession (4%) than in *ms-1* (Landsberg accession, 1%, Figure 1A) and are significantly enhanced by *duo1-3* (36%). **(C)** Multiple *hap2-1* sperm are released into a single ovule. The first panel shows an ovule targeted by a wild-type *HTR10:HTR10:mRFP*, *LAT52:GFP* pollen tube. Green signal is released from the burst pollen tube; sperm nuclei (SN, red signal) have fused (F) with the egg cell and central cell nuclei. The second panel shows an ovule targeted by a *hap2-1*, *HTR10:HTR10:mRFP*, *LAT52:GFP* pollen tube. The pollen tube has burst (green signal), but sperm cell nuclei remain unfused (UF). The third panel shows an ovule containing four unfused *hap2-1*, *HTR10:HTR10:mRFP* sperm. The last panel shows an ovule containing eight unfused *hap2-1*, *HTR10:HTR10:mRFP* sperm. Five sperm nuclei are clearly visible, the others are outside the plane of this image. In this ovule, pollen tube cytoplasm (GFP) appears to fill the area occupied by both synergids. Scale bars, 10µm.

Analysis of pollen tube growth to ovules in vitro showed that late-arriving additional pollen tubes were actively repelled (Palanivelu and Preuss, 2006). Furthermore, analysis of several female gametophyte mutants (e.g. *feronia*) that fail to signal to the pollen tube to stop growing, burst, and release sperm indicated that these all attract multiple pollen tubes (Boisson-Dernier et al., 2008; Capron et al., 2008; Huck et al., 2003; Rotman et al., 2003; Tsukamoto et al., 2010). These studies suggested an active mechanism that blocks polytubey and indicate that any of the steps in double fertilization that occur after the pollen tube stops growing and burst could trigger the block. We tested whether gamete fusion is a potential trigger using Arabidopsis *hap2-1* mutants (Johnson et al., 2004; von Besser et al., 2006). Arabidopsis *hap2* mutant pollen tubes target ovules, burst, and release sperm, but mutant sperm do not fuse with female gametes (Hamamura et al., 2011; Mori et al., 2006). We performed mixed

pollination assays using pollen homozygous for *LAT52:GFP* and heterozygous for *hap2-1/HAP2* and pollen homozygous for *LAT52:DsRed* and *HAP2*. In this experiment, half of the GFP-expressing pollen tubes carry wild-type sperm and half carry *hap2-1* mutant sperm. All DsRed-expressing pollen tubes carry wild-type sperm. The rate of polytubey increased 10 fold over wild-type (Figure 1A) even though only $\frac{1}{4}$ of the pollen on the stigma carried *hap2-1* mutant sperm.

We found that like *hap2-1* mutants, *duo1-3* (Figure S1) mutants enhance polytubey ~10 fold over levels observed in the No-0 accession (wild type, genetic background of *duo1-3*, Figure 1B). *duo1* mutant pollen tubes carry a single sperm-like cell that is incapable of fertilization (Brownfield et al., 2009; Rotman et al., 2005). These data support the hypothesis that gamete fusion, or an event shortly thereafter is the trigger that prevents multiple pollen tubes from entering one ovule.

To closely monitor pollen tube burst and sperm release, we generated *hap2-1* and wild-type pollen tubes expressing *LAT52:GFP* along with the sperm nuclear marker *HTR10:HTR10:mRFP*. Twenty-one hours after pollination, wild-type pollen tubes had burst and sperm nuclei had fused with the egg and central cell nuclei (Figure 1C, left panel). The *HTR10:mRFP* signal diffuses after nuclear fusion as it incorporates into the egg or central cell nucleus (Ingouff et al., 2007). *hap2-1* mutant pollen tubes also burst, but sperm nuclei remained compact at the site where gamete plasma membrane fusion would normally occur (Figure 1C

second panel, Hamamura et al., 2011). In many cases, we observed multiple pairs of sperm nuclei (Figure 1C, third and fourth panels) and as many as four pairs of sperm were detected indicating that four pollen tubes had been attracted to one ovule (1 pair of unfused sperm, n=118; 2 pairs of unfused sperm, n=10; 3 pairs of unfused sperm, n=2; four pairs of unfused sperm, n=1). These data suggest that when gametes do not fuse, the block to polytubey fails and ovules continue to attract multiple pollen tubes.

The gamete-fusion triggered block to polytubey enhances reproductive success

To determine whether fertilization can be salvaged in an ovule that has attracted a pollen tube with defective sperm, we performed a mixed pollination experiment to analyze interactions between sperm and female gametes. Two types of differentially marked pollen were placed on the stigma at the same time: 1) *hap2-1/HAP2* pollen marked with *HTR10:HTR10:mRFP*; 2) wild-type pollen marked with *LAT52:GFP*. In addition to the expected cases where a single pollen tube was attracted (Table 1, Figure S2), a significant number of polytubey events were observed (Table 1, 39/432 targeted ovules). These included ovules containing multiple pairs of non-functional sperm (Figure S2). Importantly, these also included ovules that contained a pair of unfused RFP⁺ sperm nuclei and a wild-type, GFP⁺ pollen tube; and ovules containing a pair of non-functional RFP⁺ sperm (*hap2-1*) along with a pair of RFP⁺ wild-type sperm that had fused with female nuclei (Figure S2). We did not observe ovules that were targeted by two

pollen tubes carrying wild-type sperm (e.g. wild-type GFP+ pollen tube and functional RFP+ sperm). As expected, when this mixed pollination experiment was conducted with wild-type pollen, polytubey was very rare (2/411 targeted ovules, Table 1). These data indicate that ovules first targeted by defective sperm can attract additional pollen tubes; but when wild-type sperm are attracted subsequent pollen tubes are blocked.

Table 1. Ovules that have attracted defective sperm can attract functional sperm and polytubey increases if pistils are first pollinated with <i>hap2-1/HAP2</i> pollen before wild-type pollen is added										
<u>Simultaneous Mixed Pollinations</u>		Single pollen tube (Signal observed)			Polytubey (Signals observed)					Polytubey
		RFP + F	RFP + U	GFP+	RFP+ F GFP +	RFP+ U GFP +	RFP+ U RFP+ U	RFP+ U RFP +F	N	
<i>hap2-1/HAP2, HTR10:RFP</i>	Wild type, LAT52:GFP	34	70	289	0	33	3	3	432	39/432 (9%)
Wild type, HTR10:RFP	Wild type, LAT52:GFP	178	0	231	1	1	0	0	411	2/411 (0.4%)
<u>Staggered Mixed Pollinations</u>										
Added First	Added 1.5hr later									
<i>hap2-1/HAP2, HTR10:RFP</i>	Wild type, LAT52:GFP	87	58	93	0	51	7	14	310	73/310 (23%)
Wild type, LAT52:GFP	<i>hap2-1/HAP2, HTR10:RFP</i>	12	4	353	0	1	0	0	370	1/370 (0.1%)
Pollen genotypes for mixed pollinations are given in the first two columns.(h), time in hours of pollination; <i>HTR10:RFP</i> , <i>HTR10:HTR10:RFP</i> ; RFP+F, ovule contains RFP+ sperm nuclei that have undergone karyogamy; RFP+U, RFP positive sperm that remain unfused; GFP+, GFP positive pollen tube has burst in the ovule; N, total number of ovules targeted.										

If gamete fusion is required to trigger the block to polytubey, we would predict that polytubey would be exacerbated if *hap2-1/HAP2* pollen tubes were allowed to begin growth before addition of wild-type (GFP+) pollen to the stigma. In this scenario, the number of ovules that receive defective sperm would be increased. When mixed pollinations were staggered by 1.5 hours and *hap2-1/HAP2* pollen was applied first, the rate of polytubey further increased to 23% (Table 1). However, if wild type pollen was applied first, polytubey decreased to well below

1% and the vast majority of ovules were targeted by a wild-type GFP⁺ pollen tube.

Pollen tube-attracting synergid cells persist in ovules that have received defective sperm

We observed that ovules receiving defective sperm attracted up to four pollen tubes (Figure 1C). In *Torenia fournieri*, synergid cells secrete pollen tube attractants called LUREs (Okuda et al., 2009) and at least one intact synergid is required for pollen tube attraction (Higashiyama et al., 2001). In *Arabidopsis*, synergids are also required for pollen tube attraction (Kasahara et al., 2005) and degeneration of one synergid is correlated with arrival of a pollen tube (Faure et al., 2002). We propose that in the absence of gamete fusion, ovules are able to continue to attract pollen tubes because one of the synergids persists and continues to secrete attractants. We determined the relationship between synergid degeneration, gamete fusion, and pollen tube attraction in ovules receiving wild type or *hap2* mutant sperm.

Upon synergid degeneration, *ACT11:MSI1:GFP* signal is no longer concentrated in the synergid nucleus and becomes diffuse throughout the cytoplasm, providing a clear marker for this important event (Ingouff et al., 2007). We found that all ovules that had received either wild-type sperm (fused *HTR10:HTR10:mRFP* pattern, Fig 4C,D) or *hap2-1* mutant sperm (unfused, Figure 2E,F) had one degenerated synergid (Figure 2C, E), or degeneration of both synergids (Figure

2D,F). Therefore, arrival and burst of a *hap2-1* mutant pollen tube triggers degeneration of one synergid cell as in wild type. Ovules that had attracted multiple pairs of sperm also had either one or two degenerated synergids. This result shows that arrival of a second pollen tube does not trigger degeneration of the persistent synergid and suggests that a downstream event like gamete fusion may trigger degeneration of the remaining synergid.

To begin to address this hypothesis, we analyzed synergid degeneration over time. At four hours after pollination, no ovules had been targeted and all ovules contained two intact synergid cells (Figure 2I). At each subsequent time point (8, 12, 16, 20 hours), we found that ovules with unfused sperm were more likely to have an intact second synergid than ovules with fused sperm (Figure 2I U, unfused *hap2-1*, v. F, fused wild type). Twelve hours after pollination, 97% of ovules with unfused sperm had a persistent synergid compared with 48% that had been fertilized by wild-type sperm. By 20 hours after pollination both synergids had degenerated in all ovules with fused sperm, whereas 50% of ovules with unfused sperm still had one intact synergid. These data show that if gamete fusion fails, a pollen tube-attracting synergid cell persists and attracts multiple pollen tubes.

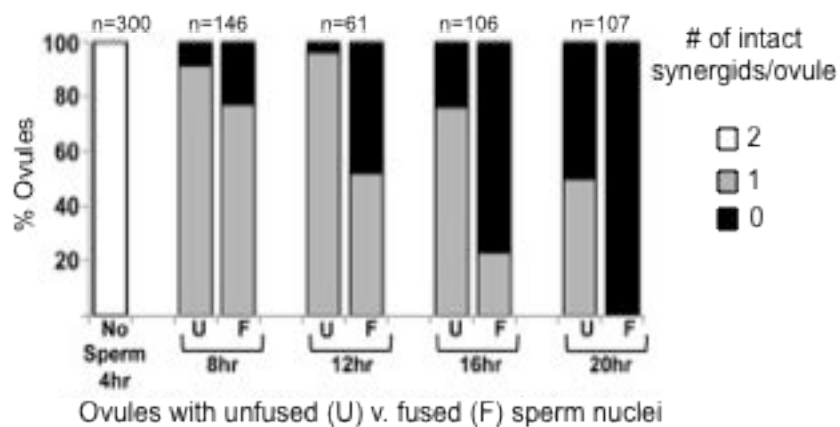
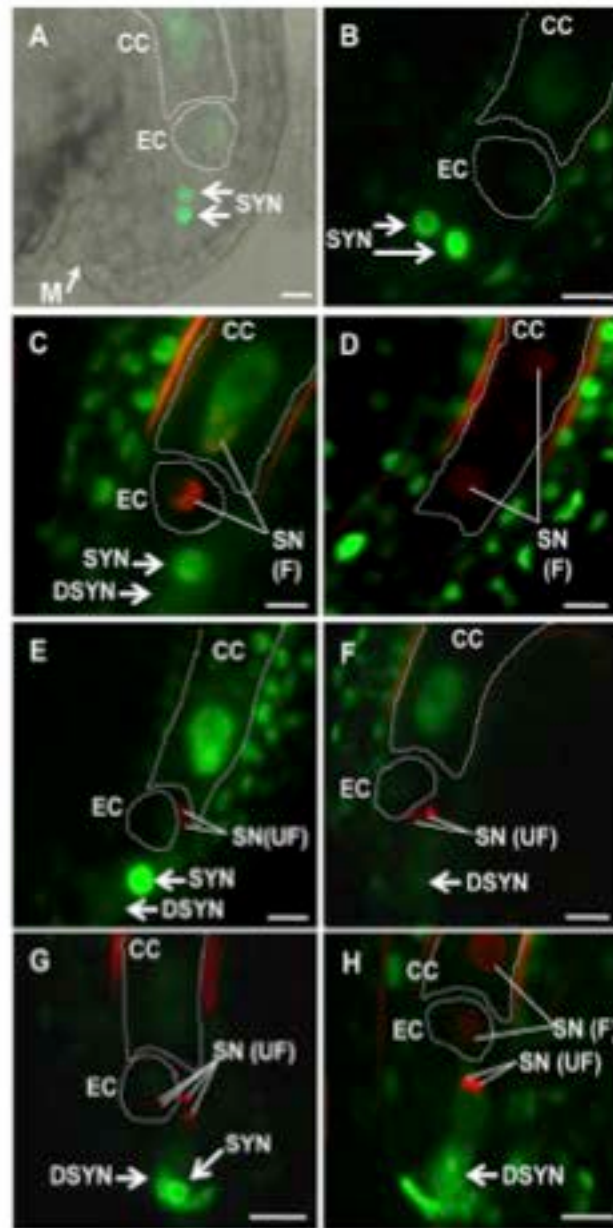


Figure 2. The remaining synergid cell persists longer in ovules targeted by non-functional sperm. (A) An unfertilized ovule expressing *ACT11:MSI1:GFP*. DIC and confocal images are overlaid to highlight the position of the micropyle (M, same in all panels) and GFP accumulation in the egg (EC), central (CC) and synergid (SYN) nuclei. **(B-H)** Pistils were pollinated with *hap2-1/HAP2(GCS1)*, *HTR10:HTR10:mRFP*. Representative confocal micrographs are shown of an unfertilized ovule **(B)**, ovules that have received sperm and have a persistent synergid (SYN) **(C,E,G)**, or ovules that have received sperm and both synergids have degenerated (DSYN) **(D,F,H)**. **(D)** *ACT11:MSI1:GFP* signal was not detectable in synergids because this image was obtained later in development (note dividing primary endosperm) and significantly after synergid degeneration. **(I)** Ovules were scored for the number of synergids that remain intact (2, white bar; 1 gray bar; 0 black bar) and whether they had received sperm whose nuclei fused with female nuclei (fused, F) or sperm that remained unfused (U). Data are reported as the percentage of the total number of ovules analyzed (n) at each time point following pollination (hours, hr). Scale bar, 10µm.

Discussion

We have shown that the attraction of multiple pollen tubes to one ovule is rare in *Arabidopsis* (Figure 1B), but polytubey increases dramatically when sperm incapable of gamete fusion are deposited (Figure 1, Table 1). Interestingly, in wild type, one synergid cell persists well after gamete fusion (faure), but multiple pollen tubes are rarely attracted. This suggests that gamete fusion itself, or an event soon thereafter (e.g. nuclear fusion, initiation of development), triggers a mechanism that can overcome attraction by the persistent synergid and prevent entry of additional pollen tubes. We found that synergids persist longer when defective sperm are deposited, which provides an explanation for why gamete fusion defects lead to polytubey. However, synergid degeneration alone does not account for the rapid and efficient block to polytubey that follows deposition of functional sperm. Future work will aimed at defining the components of this important mechanism.

The cases of polytubey observed in *Arabidopsis* mutants can all be accounted for by defects in a block to polytubey that requires gamete fusion. Female mutants defective in pollen tube reception (e.g. *feronia*) fail to instruct pollen tubes to burst, sperm are not released, gamete fusion does not occur, and consequently, they attract multiple pollen tubes (Boisson-Dernier et al., 2008; Capron et al., 2008; Huck et al., 2003; Rotman et al., 2003; Tsukamoto et al., 2010). We predict that any mutation disrupting gamete fusion will result in polytubey.

The block to polytubey was likely a significant evolutionary innovation allowing flowering plants to maximize reproductive success by promoting fertilization of all ovules and ensuring that only two sperm are delivered to each ovule. Pollen tubes bypassing fertilized ovules can continue to grow to unfertilized ovules. If the block to polytubey were initiated by any earlier step in the process (pollen tube entry, pollen tube contact with the female gametophyte, pollen tube burst), then ovules would go unfertilized in cases where pollen tubes failed to burst or sperm were defective. The block to polytubey is therefore a final opportunity for the flower to scrutinize pollen, reject unworthy suitors and replace them with productive mates.

Experimental Methods

Plants used for analysis

ms1-1/MS1 plants were obtained from the Arabidopsis Biological Resource Center (stock #CS261) and maintained as a heterozygous line. *ACT11:MSI1:GFP* and *HTR10:HTR10:mRFP* plants were obtained from Frederic Berger (National University of Singapore, Ingouff et al., 2007). *LAT52:GFP* plants were obtained from Daphne Preuss (University of Chicago) and *LAT52:DsRed*(Francis et al., 2007) plants were obtained from Greg Copenhaver (UNC, Chapel Hill). *hap2-1/HAP2* (ABRC stock#CS16308) plants or *hap2-1/hap2-1*; *hap2^{hypo}/hap2^{hypo}*(Wong et al., 2010) were crossed with *LAT52:GFP/LAT52:GFP* and *HTR10:HTR10:mRFP/HTR10:HTR10:mRFP* plants(Ingouff et al., 2007) to obtain *genotypes* used for mutant analysis of pollen tube and sperm cell nuclei behavior.

Maintenance of plants

Seeds were plated on solid Murashige and Skoog (MS) medium (MP Biomedicals LLC, Solon, OH, USA) supplemented with 25µg/ml glufosinate ammonium (Basta; Chem service, INC, West Chester, PA, USA) to select *hap2-1/HAP2* mutant seedlings or with 25µg/ml glufosinate ammonium and 50µg/ml kanamycin sulfate (Agri-bio, Bay Harbour, FL, USA) to select *hap2^{hypo}* seedlings. Seedlings were transplanted to sterile 2MIX potting medium (Conrad Fafard INC, Agawam, MA, USA) 7 days after plating. All plants were grown at 20°C, 50-60%

humidity, on a 16day/8hour night light cycle in growth chambers (Environmental growth chambers, Chagrin Falls, OH, USA).

Analysis of double fertilization using live imaging

Standard crosses were performed under a dissecting microscope by pollinating *ms1-1* or *ACT11:MSI1:GFP* (emasculated one day prior to crossing) stage 14(Smyth et al., 1990) stigmas with dehiscent pollen from male donors. All crosses were collected 21 hours post-pollination and ovary walls were removed as previously described(Johnson and Kost, 2010). Dissected pistils were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope, LeDuc Bioimaging facility, Brown University, Providence, RI, USA). Zeiss-recommended emission and detection wavelength settings were used for each fluorophore (GFP; argon laser 458/477/488/514nm at 30mW, DsRed and mRFP; helium neon laser 633nm at 3mW). Signal intensity was optimized for each fluorophore so that signals could be shown individually and in overlay (Figure 1-4, S1).

Analysis of synergid cell degeneration

Stage 14(Smyth et al., 1990) *ACT11:MSI1:GFP* flowers were emasculated and hand pollinated with *hap2-1/HAP2*; *HTR10:HTR10:RFP*/ *HTR10:HTR10:RFP* pollen one day after emasculatation. At each time point after pollination (4,8,12,16,20 hrs) pistils were dissected and ovary walls were removed as

previously described(Johnson and Kost, 2010). Six to eight crosses were performed for each experiment. Dissected pistils were mounted for confocal microscopy (as described above). Targeted ovules were scored for the number of intact synergid cell nuclei and whether sperm nuclei had fused with egg and central cell nuclei or remained unfused (*hap2-1*).

Mixed pollinations Assays

Mixed pollinations were performed under a dissecting microscope by saturating one half of a stage 14(Smyth et al., 1990) *ms1-1* stigma with pollen of one genotype and the other half of the stigma with a second genotype of pollen. In simultaneous mixed pollinations, the genotype added first was varied from cross to cross and the second genotype was added immediately after the first. Six to eight crosses were performed for each experiment. In staggered mixed pollinations one genotype was added 1.5 hours before the other. Plants were placed in the growth chamber during this interval. All crosses were collected 21 hours after the addition of the second pollen donor. Samples were prepared for confocal microscopy as described above.

Acknowledgements

We thank Frederic Berger, Gregory Copenhaver, and Daphne Preuss for generously providing transgenic plants expressing markers. This work was supported by the National Science Foundation through grant no. IOS-1021917 to MAJ and a Graduate Research Fellowship to KMB. KMB was initially supported by a National Institute of Health Brown University Initiative to Maximize Student Development grant (NIGMS Grant:R25GM083270 & R25GM083270-S1).

Supplemental Information

Contains 2 Figures.

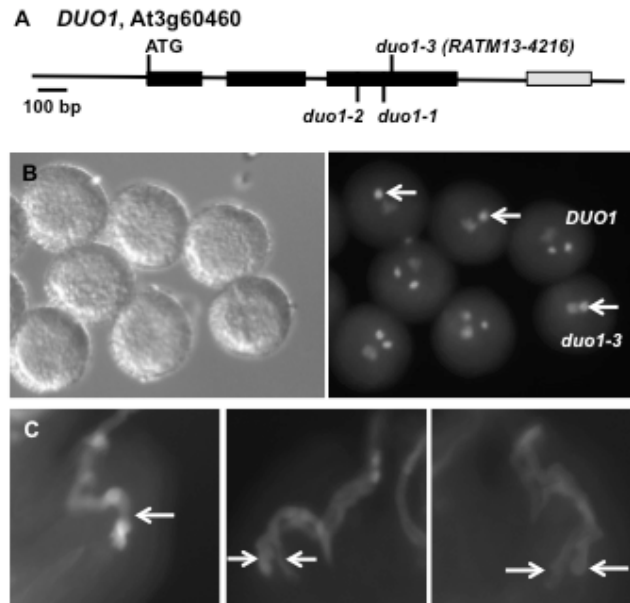


Figure S1. Analysis of *duo1-3* polytubey. Loss of *DUO1* function results in pollen that carry a single sperm-like cell that is deposited in the female gametophyte, but cannot fertilize either the egg or the central cell (Brownfield et al., 2009; Rotman et al., 2005). **(A)** A transposon insertion (RATM13-4216) in the third exon of *DUO1* (15 base-pairs downstream of *duo1-1* (Rotman et al., 2005) was obtained (Ito et al., 2005). *DUO1* exons are shown as black bars (the position of the start codon is indicated), the 3' exon of the nearest gene is shown as a gray bar. The positions of *duo1-1* and *duo1-2* alleles (Rotman et al., 2005) are shown, as is the position of the *duo1-3* (RATM13-4216) transposon insertion. The *duo1-3* insertion site position was confirmed by PCR with gene-specific (Duo1Sac1F, AGAAGAGCTCGTCCGAAGTTTCCCTCTTGG) and transposon-specific (DS3-4, CCGTCCCGCAAGTTAAATATG) primers. **(B)** *duo1-3/+* heterozygous plants produce equal numbers of wild-type pollen with two normal sperm (*DUO1*, 108/200, 54%) and mutant pollen with a single sperm-like cell (*duo1-3*, arrow, 92/200, 46%). This is similar to *duo1-1* and *duo1-2* (Rotman et al., 2005) and like these alleles, *duo1-3* is not transmitted to progeny by pollen (not shown). A light micrograph of pollen is shown in the left panel, a fluorescence micrograph of 4',6-diamidino-2-phenyl-indole (DAPI)-stained pollen (Park et al., 1998) is shown on the right panel. Sperm and sperm-like cells (arrows) are bright staining and compact, the pollen nucleus is larger and has diffuse staining. **(C)** Higher magnification examples of ovules that have received either one pollen tube (left) or two pollen tubes (center, right). Ovules targeted by

multiple sperm were smaller indicating that they had not yet received functional sperm and had not begun to expand following fertilization.

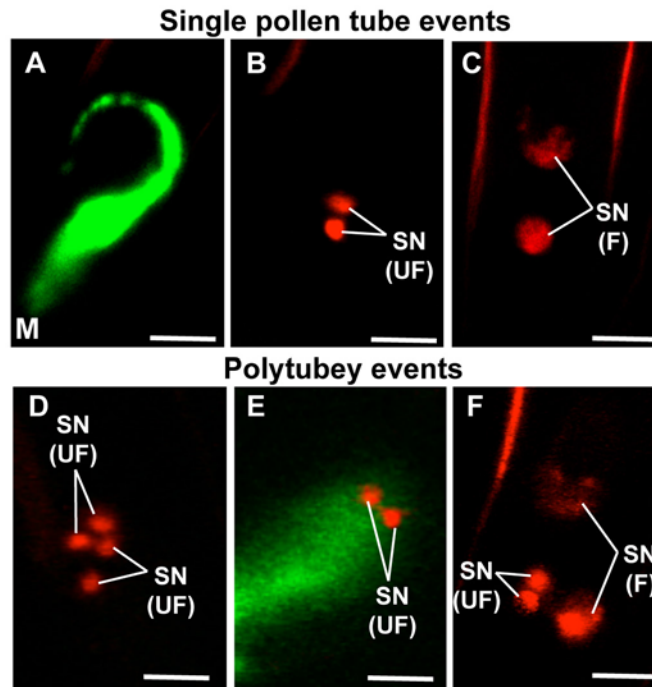


Figure S2. Ovules first targeted by pollen tubes carrying non-functional sperm are able to attract subsequent pollen tubes carrying functional sperm that initiate fertilization. Stigmas were mix-pollinated with half *hap2-1/HAP2*, *HTR10:HTR10:mRFP* and half wild-type, *LAT52:GFP* pollen. Representative images of ovules targeted by one pollen tube (**A-C**) or two pollen tubes (**D-F**) are shown. Ovules are shown that have been targeted by: a GFP+ pollen tube (**A**), a pollen tube carrying non-functional (unfused, UF) RFP+ sperm (**B**), or a pollen tube carrying functional (fused, F) RFP+ sperm(**C**), two pollen tubes each carrying non-functional sperm (**D**), a pollen tube carrying non-functional RFP+ sperm and a GFP+ pollen tube (**E**), a pollen tube carrying non-functional RFP+ sperm and a pollen tube carrying a pair of functional RFP+ sperm (**F**). The frequency of each event is recorded in Table 1. The position of the micropyle (M) in each image is indicated in panel A. SN, Sperm nuclei, fused(F) unfused(UF). Scale bars, 10µm.

References

- Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. *Trends in plant science* 13:437-443.
- Boisson-Dernier, A., Frietsch, S., Kim, T.H., Dizon, M.B., and Schroeder, J.I. (2008). The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. *Current biology : CB* 18:63-68.
- Brownfield, L., Hafidh, S., Borg, M., Sidorova, A., Mori, T., and Twell, D. (2009). A plant germline-specific integrator of sperm specification and cell cycle progression. *PLoS genetics* 5:e1000430.
- Capron, A., et al. (2008). Maternal control of male-gamete delivery in *Arabidopsis* involves a putative GPI-anchored protein encoded by the LORELEI gene. *The Plant cell* 20:3038-3049.
- Faure, J.E., Rotman, N., Fortune, P., and Dumas, C. (2002). Fertilization in *Arabidopsis thaliana* wild type: developmental stages and time course. *The Plant journal : for cell and molecular biology* 30:481-488.
- Francis, K.E., Lam, S.Y., Harrison, B.D., Bey, A.L., Berchowitz, L.E., and Copenhaver, G.P. (2007). Pollen tetrad-based visual assay for meiotic recombination in *Arabidopsis*. *Proc Natl Acad Sci U S A* 104:3913-3918.
- Hamamura, Y., et al. (2011). Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in *Arabidopsis thaliana*. *Curr Biol* 21:497-502.
- Higashiyama, T., et al. (2001). Pollen tube attraction by the synergid cell. *Science (New York, N.Y.)* 293:1480-1483.
- Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The *Arabidopsis* mutant *feronia* disrupts the female gametophytic control of pollen tube reception. *Development (Cambridge, England)* 130:2149-2159.
- Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. *Current biology : CB* 17:1032-1037.
- Ito, T., et al. (2005). A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of *Arabidopsis* transposed from start loci on chromosome 5. *Plant Cell Physiol* 46:1149-1153.
- Johnson, M.A., and Kost, B. (2010). Pollen tube development. *Methods Mol Biol* 655:155-176.
- Johnson, M.A., et al. (2004). *Arabidopsis* hapless mutations define essential gametophytic functions. *Genetics* 168:971-982.

- Kasahara, R.D., Portereiko, M.F., Sandaklie-Nikolova, L., Rabiger, D.S., and Drews, G.N. (2005). MYB98 is required for pollen tube guidance and synergid cell differentiation in *Arabidopsis*. *The Plant cell* 17:2981-2992.
- Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. *Nature cell biology* 8:64-71.
- Okuda, S., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. *Nature* 458:357-361.
- Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro. *BMC plant biology* 6:7.
- Park, S.K., Howden, R., and Twell, D. (1998). The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. *Development* 125:3789-3799.
- Rotman, N., et al. (2005). A novel class of MYB factors controls sperm-cell formation in plants. *Current biology* : CB 15:244-248.
- Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F., and Faure, J.E. (2003). Female control of male gamete delivery during fertilization in *Arabidopsis thaliana*. *Current Biology* 13:432-436.
- Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. *The Plant cell* 2:755-767.
- Tsukamoto, T., Qin, Y., Huang, Y., Dunatunga, D., and Palanivelu, R. (2010). A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in *Arabidopsis thaliana* double fertilization and early seed development. *The Plant journal : for cell and molecular biology* 62:571-588.
- Twell, D., Yamaguchi, J., and McCormick, S. (1990). Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. *Development* 109:705-713.
- von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). *Arabidopsis* HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. *Development (Cambridge, England)* 133:4761-4769.
- Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. *PLoS genetics* 6:e1000882.
- Wong, J.L., and Wessel, G.M. (2006). Defending the zygote: search for the ancestral animal block to polyspermy. *Current topics in developmental biology* 72:1-151.

Appendix 5:

Argininosuccinate lyase (ASL) is localized to a novel chloroplast-attached structure and is required for gamete development and function in *Arabidopsis*

Alexander R Leydon, Mark A Johnson

I performed all the experiments in this manuscript.

Abstract

Pollen tubes deliver two sperm cells to the egg cell and central cell in the ovule for double fertilization and seed development. Sperm development depends on pollen grain biosynthetic resources, including production of amino acids. Synthesis of amino acids is central to cellular physiology, and here we characterize the final enzyme in the Arginine biosynthesis pathway, Argininosuccinate lyase (ASL), which is required for normal sperm development. *Arabidopsis thaliana* pollen mutant for ASL frequently fail to undergo pollen mitosis II, producing single sperm-like cells. ASL mutants display broad defects in male and female gametophyte fitness, and homozygous ASL mutants are never recovered due to embryo lethality. *Arabidopsis* ASL protein is observed in novel chloroplast-attached bodies. We report a novel cellular structure in plants that is coupled to the chloroplast and is responsible for catalyzing the final step of L-Arginine biosynthesis.

Introduction

Arginine biosynthesis is a critical component of amino acid production in plants. Arginine is required for protein synthesis, and downstream derivatives can be utilized to create secondary metabolites. Arginine can be converted to polyamines, which are involved in a range of cellular processes including stress response (Minocha et al. 2014), biotic interactions (Del Duca et al. 2014) and plastid development (Sobieszczuk-Nowicka et al. 2014). Genes responsible for Arginine biosynthesis have been well characterized in other kingdoms (Cunin et al. 1986; Davis 1986), and to a certain extent in plants (Slocum 2005). While arginine biosynthesis genes demonstrate conservation of sequence and enzymatic function across kingdoms, the subcellular localization of these genes are variable (Slocum 2005). Amino acid biosynthesis loss of function mutations are difficult to study as they can be homozygotic lethal. However, amino acid biosynthesis genes are well suited for gametophytic studies as they can be carried and analyzed as heterozygotes.

Flowering plants produce haploid male gametophytes (pollen) and female gametophytes (ovules) through well-characterized differentiation programs (Jones-Rhoades et al. 2007; Borg et al. 2011; Rabiger et al. 2013). Pollen cells develop within an anther and upon maturity they contain a vegetative nucleus and two sperm cells that are engulfed within the pollen grain cell, these three together are termed the male germ unit (MGU) (Twell 1994). The female gametophyte is comprised of 7 cells, 3 antipodals, 1 central cell, 1 egg cell and 2

synergid cells (Hamamura et al. 2011). Pollen grains are shed from the dehiscent anther onto the stigma of the pistil where they undergo hydration metabolic activation (cite). Pollen grains germinate a pollen tube which grows through the stigma and style, responding to pistil cues to induce a transcriptional activation of pollen genes (Qin et al. 2009). The ovule secretes LUREs, protein attractants that guide the pollen tube to the synergid cells within the ovule (Okuda et al. 2009). Upon pollen tube-synergid interaction, the synergid and pollen tube undergo tightly controlled degeneration events resulting in both sperm being projected to the site of fusion (Hamamura et al. 2011). Both sperm must undergo plasmogamy and karyogamy with the egg cell and central cell for proper embryo and endosperm development (von Besser et al. 2006; Aw et al. 2010).

Many genes have been discovered that control the development and function of the male gametophyte. The R2-R3 MYB transcription factor *DUO1* is specifically induced in the sperm lineage and is required for the division of the generative cell that gives rise to the two sperm cells, as *duo1* mutant pollen grains contain one single sperm-like cell (SSLC) as opposed to two sperm (Borg et al. 2011). Study of transcriptional pathways underlying pollen formation have helped to describe gene networks controlling pollen development, however, the requirement of pollen biosynthesis of amino acids is less well understood. It is also unclear which amino acids can be transported from the extracellular environment into the pollen tube during growth in the pistil (Lalanne et al. 1997; Schwacke et al. 1999). While His, Lys, and Trp synthesis in the pollen tube can be ablated with

minimal effect on the pollen tube (Niyogi et al. 1993; Song et al. 2004; Hudson et al. 2006; Muralla et al. 2007) Cys biosynthesis is required for pollen tube function (Birke et al. 2013). Broadly inhibiting aromatic or branched chain amino biosynthesis blocks tobacco pollen tube growth (Grabe et al. 1997). Further work is required to determine the required amino acid biosynthesis genes.

In this work we utilized an existing collection of mutations, the hapless collection produced using T-DNA mutagenesis in the *quartet* (*qrt1-2*) mutant background that maintains the four products of meiosis in a physically linked tetrad of pollen grains and allows the zygoty of the linked mutation to be easily determined (Preuss et al. 1994). The T-DNA bears a glyphosphate herbicide resistance gene and co-segregates with mutant gametes. The T-DNA also carries a pollen specific β -Glucuronidase gene (*LAT52:GUS*) allowing histochemical staining of mutant pollen grains and tubes (Twell et al. 1989; Johnson et al. 2004). *Hapless* mutations were identified via distorted segregation, and here we re-examined these mutants for pollen developmental phenotypes, especially the development of the MGU. The hapless mutant *hap7* failed to produce normal sperm cells, instead frequently producing a single sperm-like cell (SSLC) similar the *duo1* mutant pollen (Borg et al. 2011). Pollen carrying these SSLCs fertilize female gametes rarely, resulting in single fertilization events (Frank et al. 2009). The HAP7 gene was identified as Argininosuccinate Lyase (ASL), the final enzyme in the Arginine biosynthesis pathway. Additionally, we demonstrate that in

Arabidopsis this enzyme is localized to an unidentified cell body, which is closely associated with the chloroplast or plastid.

Results

HAP7 is required for male gametophyte development

Pollen from the hapless mutant collection was screened for pollen viability using a differential stain (Alexander Stain) to identify mutants that fail to develop an intact cell wall or cytoplasm at maturity (Alexander 1969). We observed seven hapless mutants where pollen failed to develop intact cytoplasm and frequently exhibited collapsed cell walls (*hap5*, *hap7-1*, *hap8*, *hap12*, *hap14*, *hap16*, *hap21*, and *hap27*, Supplemental Table S1, Supplemental Figure S1). DAPI staining DNA identified defects in the formation of the MGU of hapless mutants, specifically elevated frequencies of single sperm-like cells (SSLCs) (*hap7-1*, *hap18*, *hap23*, Supplemental Table S1, Supplemental Figure S1). Pollen from the hapless mutant *hap7-1* had increased rates of unviable pollen by Alexander Staining (27.8% lacking intact cytoplasm), and frequent failure of the generative cell to divide into sperm cells (SSLC – 13.8%, Figure 1A-F, Supplemental Table S1). *HAP7* mutant pollen tubes are identifiable during in vitro pollen growth experiments using the linked *LAT52:GUS* reporter, where they germinate less frequently and grow to shorter lengths than their wild-type counterparts (Figure 1J). In high competition crosses (high pollen volume) pollen tube growth of *hap7-*

1 mutant pollen tubes in the pistil was drastically reduced, with only 1.2% of *hap7-1* mutant pollen tubes reaching ovules (Figure 11,K). Similarly, mutant pollen tube targeting rates were higher (16%) when self-pollinated *hap7-1/HAP7* pistils were tested. Self-pollinated pistils deposit less pollen on the pistil, allowing the less competitive pollen genotypes to target ovules more frequently. Together, these results demonstrate *HAP7* is required for normal pollen grain and MGU development, pollen tube growth and guidance to the female gametophyte.

HAP7 is L-argininosuccinate lyase (ASL)

In order to identify the gene responsible for the *hap7* phenotype TAIL-PCR was employed to amplify and sequence the left border of the *hap7/HAP7* T-DNA (Liu et al. 1995). The *hap7* T-DNA interrupts the second exon of *AT5G10920*, the sole L-Argininosuccinate Lyase (ASL) in the *Arabidopsis* genome. ASL is the final enzyme in the arginine biosynthesis pathway, converting its substrate Argininosuccinate into L-arginine (Arg) and fumarate (Reviewed in (Slocum 2005), Figure 2E). The ASL enzyme, also known as ARGH in bacteria, or ARG4 in *Saccharomyces*, is deeply conserved across all kingdoms, with three domains of highest conservation (C1-C3) that form the active site (Figure 2B) (Sampaleanu et al. 2001). The ASL enzyme functions as a homotetramer of two pairs of asymmetrically bound ASL dimers (Figure 2C) (Sampaleanu et al. 2001). The *hap7* T-DNA is inserted in the second exon of ASL, and is predicted to introduce six novel amino acids (HQTIV) and a premature stop codon shortly after the C2 region (Figure 2A).

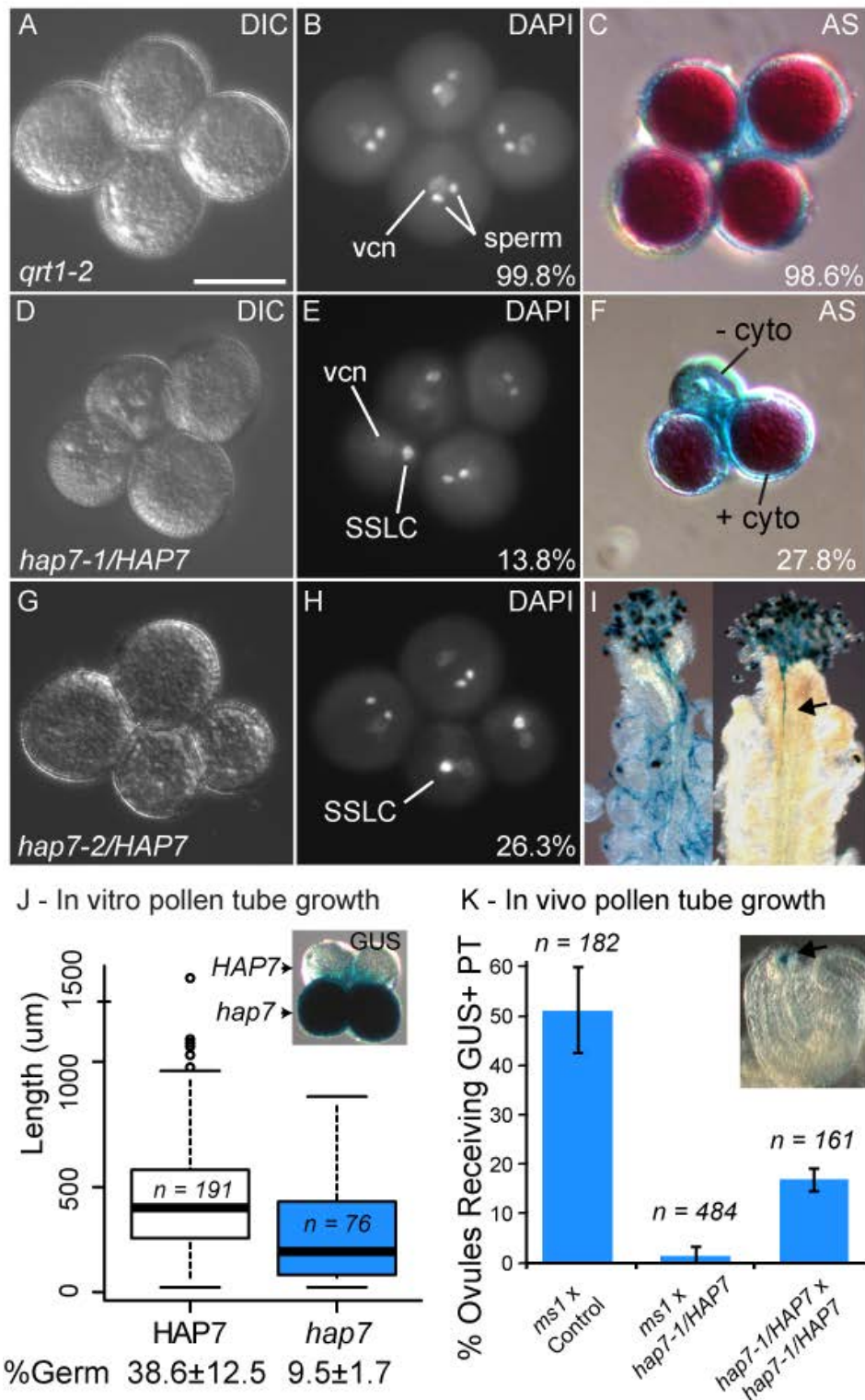


Figure 1. A-C. Wild type *qrt1-2* pollen. **D-F.** *hap7-1/HAP7*, *qrt1-2* pollen. **G,H.** *hap7-2/HAP7*, *qrt1-2* pollen. **A,D,G.** DIC micrograph. **B,E,H.** DAPI micrograph, % of quartets exhibiting SSLC phenotype shown in image is in bottom right corner. **C,F.** Alexander Staining, % of quartets exhibiting viability shown in image is in

bottom right. **I.** Light image of GUS+ pollen tubes growing in the pistil. Left panel - wild-type, *LAT52:GUS* pollen. Right panel – *hap7-1/HAP7*, *LAT52:GUS* pollen. **J.** In vitro pollen tube growth assay from heterozygous *hap7-1/HAP7* pollen. Quantified data is in box whisker plot format, n = number of pollen tubes counted. Data is pooled from 3 replicates. Average % germination is shown below each plot. Inset at top right depicts one quartet of pollen grains, GUS stained to visualize *hap7* mutant pollen grains. **K.** In vivo pollen tube targeting rates in wild-type *ms1* pistils. Mean % ovules receiving a GUS+ pollen tube +/- stdev. n = total number ovules in each experiment. Inset at top right depicts a GUS+ targeted ovule. A heterozygous control *LAT52:GUS* pollen donor is provided for comparison – *ms1* x control.

As there are 16 solved structures for ASL, we performed protein structural prediction using the *Arabidopsis* ASL (*AtASL*) protein sequence and the Phyre2.0 protein prediction server (Kelley et al. 2009). The predicted structure of the *Arabidopsis* ASL protein closely was predicted to be very similar to existing ASL structures, with the exception of the N-terminal signal sequence, which is predicted to target ASL to the chloroplast (ChloroP 1.1) (Emanuelsson et al. 1999). We also identified the wild type protein domains predicted to be retained in the *hap7-1* mutant isoform if expressed, namely regions domains 1, C1, C2 and a residual portion of Domain 2 (figure 2C).

To independently test the identity of *HAP7* we examined the existing T-DNA insertion lines in the ASL gene region and identified a second insertion line in the 5'-UTR of ASL. This allele, which we named *hap7-2*, exhibited negligible rates of unviable pollen (data not shown) but high rates of single sperm-like cells in mature pollen grains (26.3%, Figure 1G,H). Both *hap7-1* & *hap7-2* gametes are transmitted to progeny less frequently, with male transmission of *hap7* gametes at exceptionally low rates compared to wild type (<1%, Table 1, Johnson et al.

2004). Homozygous mutants for either allele were never recovered, suggesting that these mutations are homozygous lethal. We tested the ability of the wild type *AtASL* gene to complement both *hap7* mutant alleles by introducing a T-DNA carrying the wild type HAP7 locus (*gHAP7*) into heterozygous *hap7* mutants. This construct includes the endogenous regulatory regions of the HAP7 gene and rescues both male and female transmission, yet fails to rescue the *hap7/hap7* homozygous genotype suggesting regulatory domains missing from this construct required for embryogenesis (Table 1). Despite this incomplete rescue of the *gHAP7* construct, sperm development and transmission was rescued (Table 1). Therefore, we infer that ASL/HAP7 is required during MGU development, and that arginine biosynthesis is critical to the formation of the two sperm cells.

Arginine biosynthesis is required for generative cell division

To better understand the SSLC phenotype of *hap7-1/HAP7* pollen grains we used TEM microscopy to study the effects of arginine deprivation on the maturing pollen grain. Pollen heterozygous for *hap7-1* carrying the *qrt1-2* mutation allows easy identification of mature tetrads in TEM analysis (Figure 3A). Wild type grains are identifiable with clear development of two sperm cells (sc), a vegetative nucleus (vn), and numerous small vacuoles, and small storage bodies (Figure 3A, Owen et al. 1995). The *hap7-1* mutant pollen nuclei are arrested before generative cell division, with many large cellular bodies accumulating in the cytoplasm (Figure 3A,B). Interestingly, the mitochondria in *hap7-1* pollen are

elongated and their cristae are disorganized compared to wild type (Figure 3C). TEM analysis demonstrates that the *hap7-1* SSLCs are structurally arrested at the bicellular stage.

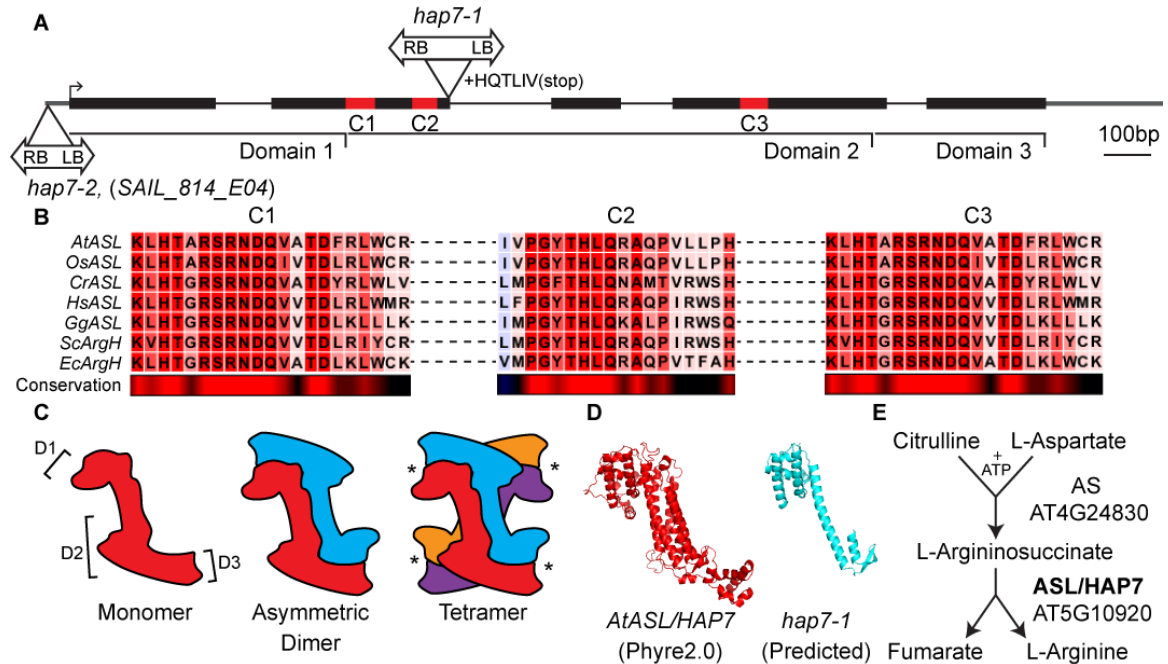


Figure 2. HAP7 is encoded by Argininosuccinate Lyase (ASL). **A.** ASL gene locus. Conserved domains C1, C2 and C3 are highlighted with Red. Positions of T-TNAs are indicated, as is their relative insertion orientation. **B.** Alignment of conserved domains C1, C2, C3. AtASL – *Arabidopsis thaliana*, OsASL – *Oryza sativa*, CrASL – *Chlamydomonas reinhardtii*, HsASL – *Homo sapiens*, GgASL – *Gallus gallus*, ScArgH – *Saccharomyces cerevisiae*, EcArgH – *Escherichia coli*. Conservation is indicated by background shading - High (red) to Low (blue), and in a conservation graph below - High (red) to Low (Black). **C.** Cartoon model of ASL tetramer formation, asterisks indicate active sites. **D.** Left, predicted structure of AtASL, based upon existing ASL structures (Phyre2.0). Right, region predicted to be retained in *hap7* coding sequence. **E.** Enzymatic reaction converting L-Argininosuccinate into Fumarate and L-Arginine catalyzed by ASL/HAP7.

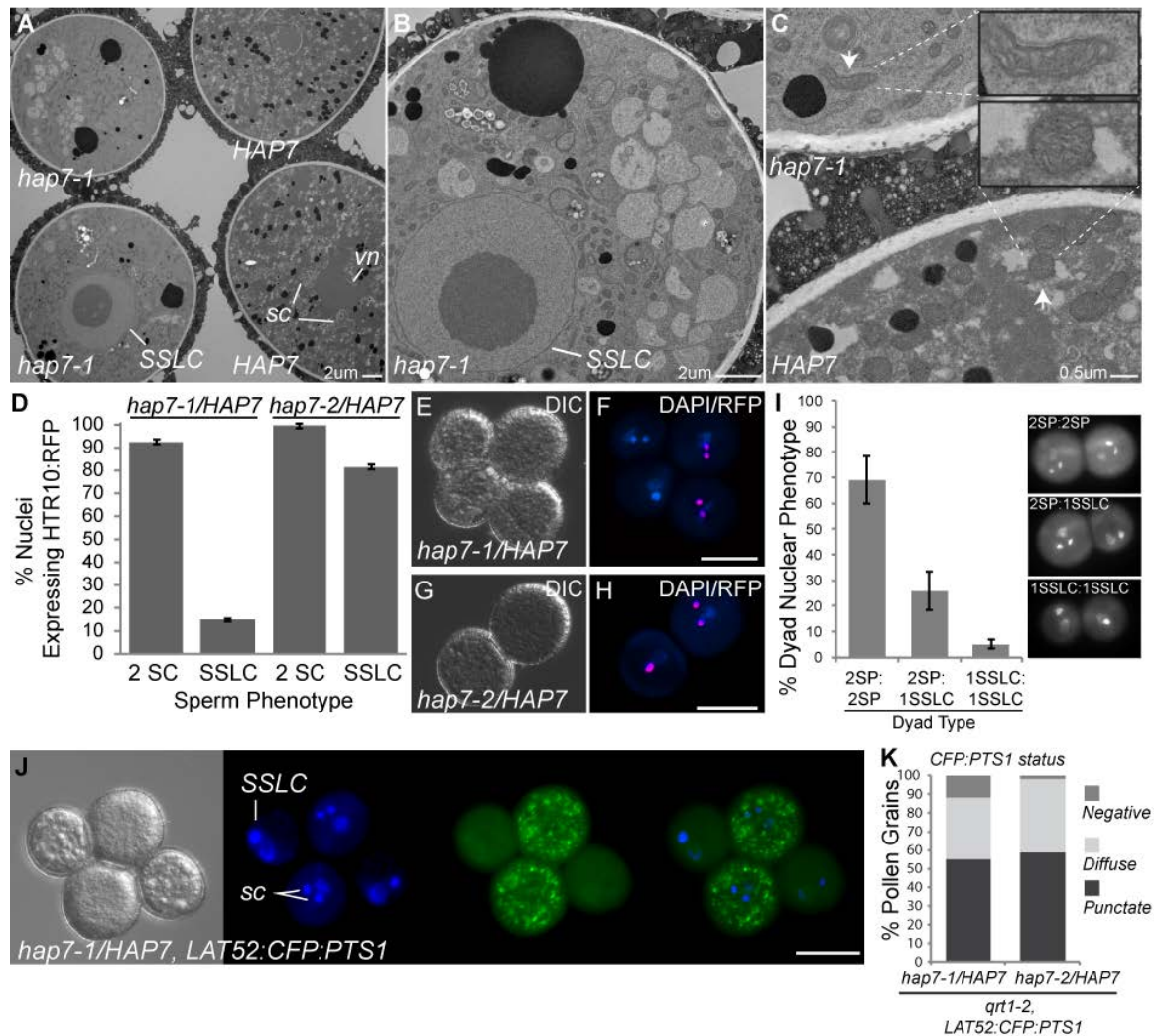


Figure 3. ASL is required for generative cell division and sperm cell development. **A-C.** Transmission electron micrographs of *hap7-1/HAP7*, *qrt1-2* pollen grains. **A.** Tetrad in mature anthers, with two *hap7-1* mutant grains (left) and two wild-type grains (right). **B.** Close up of a *hap7-1* SSLC. **C.** Side-by-side view of the *hap7-1* and *HAP7* pollen cytoplasm, with insets of the mitochondrial structure (arrows indicate inset focus). **D.** Quantification of HTR10:RFP in *hap7/HAP7* SSLC/Sperm cells. 2 SC – 2 sperm cells, SSLC – single sperm-like cell. Data is mean of two replicates from individual F2s homozygous for HTR10:RFP. **E-H.** Representative images of HTR10:RFP in *hap7/HAP7* SSLC/Sperm cells, scale bar = 20µm. **E,G.** DIC micrographs of **F,H.** DAPI/RFP merged images. **I.** Dominance analysis of *hap7-1* in *atps1-1*, *qrt1-2* mutants. Dyads were scored for 2 sperm (2SP) or one SSLC (1SSLC). Data is the mean of three *hap7-1/HAP7*, *atps1-1/atps1-1*, *qrt1-2/qrt1-2* plants (F3). Representative epifluorescent micrographs with DAPI staining are depicted to the right. **J.** Expression of the peroxisomal localized CFP protein (*LAT52:CFP:PTS1*) in *hap7-1/HAP7* pollen grains (Nowak et al. 2004). Panels from left to right, DIC, DAPI, CFP, Merge. Scale bar = 20µm. **K.** CFP:PTS1 expression quantified in *hap7-1* and *hap7-2* mutant pollen.

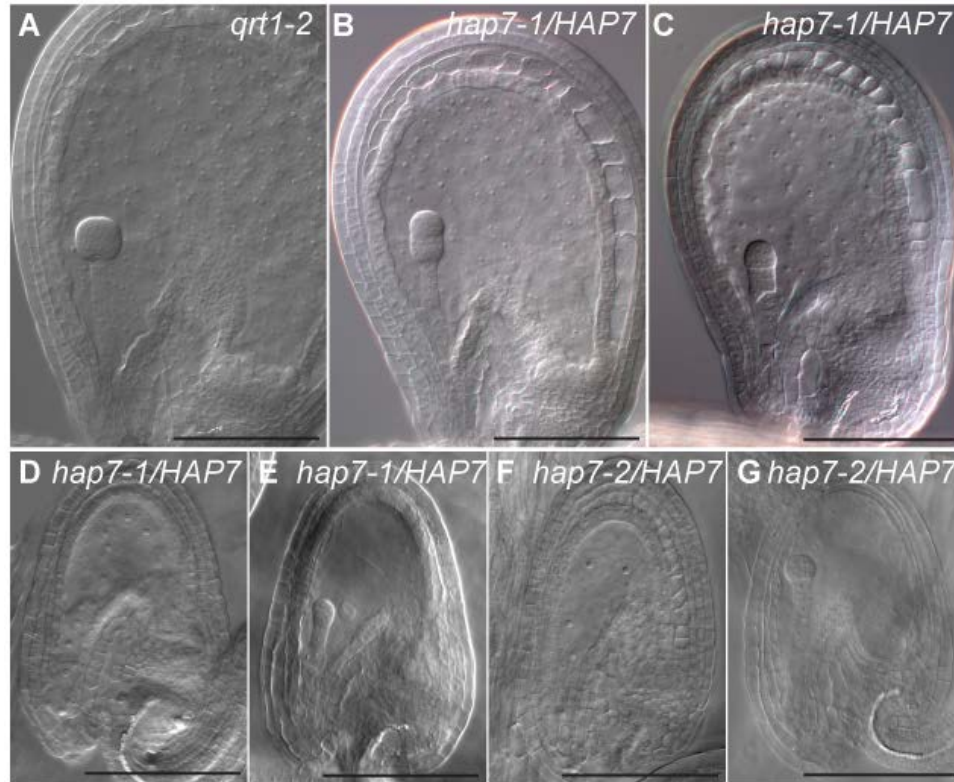
Next, we tested *hap7* SSLC expression of the sperm identity gene HTR10, a sperm specific histone variant specific to mature sperm cells. The genetic construct *HTR10:RFP* was introgressed by crossing into each *hap7/HAP7* heterozygote, and nuclear expression of the histone variant was quantified. Expression of *HTR10:RFP* in *hap7-1* SSLCs was significantly lower than in *HAP7* sperm cells, and interestingly, significantly lower than *hap7-2* SSLCs (Figure 3D,E). We even observed *hap7-1* pollen with two sperm cells lacking *HTR10:RFP* expression, which was never observed in *hap7-2* pollen (Figure 3D-H). The insertion site of the *hap7-1* T-DNA creates a putative truncation mutant (Figure 1A,D) that may act in a semi-dominant manner, explaining greater penetrance of the *hap7-1* allele. To test the hypothesis that *hap7-1* is semi-dominant, we took advantage of the *atps1-1* mutation, which produces diploid pollen grains, in conjugation with *qrt1-2* to produce diploid pollen dyads (d'Erfurth et al. 2008). We crossed *atps1-1* into *hap7-1/HAP7*, *qrt1-2* and scored SSLC rates in diploid dyads in the first generation of *atps1-1* homozygosity. If *hap7-1* is recessive, then diploid dyads containing two SSLCs should never be detected, whereas if *hap7-1* is dominant, then doubly SSLC-bearing diploid dyads will make up $\frac{3}{4}$ of the population. We observed an intermediate number of dyads where each grain contained a SSLC (5%), indicating that *hap7-1* acts in a semi-dominant manner (Figure 3G).

While the source of nitric oxide (NO) has been contentious in plants, it is unlikely that a nitric oxide synthase (NOS) that generates NO directly from L-arginine is the primary source of NO (Baudouin 2011). Despite the source, pollen grain peroxisomes carry high levels of NO and peroxisomes are required for proper pollen tube reception (Boisson-Dernier et al. 2008). We examined status of peroxisomes in *hap7* mutant pollen (Figure 3J) and found that localization of a pollen tube specific peroxisome targeted CFP, *LAT52:CFP:PTS1* (Nowak et al. 2004), frequently failed to be expressed and localized to peroxisomes (Figure 3I). These results suggest that gametophyte development is impaired in ASL loss of function mutants. Therefore, the male defects in *hap7* mutants combine pollen and sperm defects, which drastically reduce the fitness of *hap7* pollen.

ASL is required for early embryo development

Single sperm-like cells from *hap7* mutant pollen grains arrest before sperm cell division, yet express the sperm gene HTR10, which suggest they may be competent to undergo fertilization. To test fusion of *hap7-1* SSLCs with female gametes, we performed crosses to wild type *ms1* pistils under low competition conditions (2-4 anthers as donors) and examined embryo and endosperm development 4 days after pollination. In wild type (*qrt1-2*) crosses nearly all ovules had undergone double fertilization and embryos had developed to the globular stage (Figure 4A). Pistils pollinated with *hap7/HAP7* mutant pollen displayed embryo development defects and single fertilization events. Developing seeds with abnormal embryos contained both an embryo and an

endosperm indicative of successful double fertilization (and therefore two sperm), yet displayed abnormalities in the structure of the embryo proper (Figure 4C,D). Embryonic defects in heterozygous *hap7/HAP7* embryos indicate a haplo-insufficiency for ASL activity during early development. Furthermore, the *hap7-1* allele produces significantly more abnormal embryo phenotypes than the *hap7-2* allele (Figure 4H). Pistils pollinated with both *hap7-1* and *hap7-2* pollen produce single fertilization events (Figure 4D-E). Quantification of single fertilization events reveals that *hap7* SSLCs produce more single fertilization events than other SSLC producing *hapless* mutants, and have no preference between fusion with the egg or the central cell (Figure 4H).



H. Single fertilization events & abnormal embryo development caused by *hapless* mutants.

Paternal Genotype	Seed development in <i>ms1</i> ovules			
	Normal	Abnormal Embryo	Embryo-Only	Endosperm-Only
<i>qrt1-2</i>	123	0	0	0
<i>hap2-1/HAP2</i>	59	0	0	0
<i>hap5/HAP5</i>	115	3	0	0
<i>hap7-1/HAP7</i>	469	19	5	4
<i>hap7-2/HAP7</i>	197	3	4	2
<i>hap23/HAP23</i>	149	0	0	0
<i>hap30/HAP30</i>	107	1	2	0

Figure 4. ASL is required for early embryo development. A-G. Cleared seeds from crosses onto *ms1* pistils 4 days after pollination, scale bars = 100um. **A.** Wild-type, *qrt1-2* pollen. **B,C.** *hap7-1/HAP7*, *qrt1-2* pollen, double fertilized ovules, with embryos exhibiting developmental abnormalities (Abnormal Embryo, in H). **D-G.** Single fertilization events caused by delivery of SSLCs. **D,F.** Endosperm only. **E,G.** Embryo only. **H.** Quantification of seed development in ovules fertilized by *hapless* mutants sperm/SSLCs. Additional *hapless* mutants were chosen for comparison that exhibited significant rates of SSLC formation.

Wild-type *qrt1-2* and *hap2-1/HAP2* plants were used as controls that do not exhibit single fertilization events.

ASL localizes to a novel cell compartment attached to the plastid

Plant ASLs have been localized to the cytoplasm (PsASL, Taylor et al. 1981) and the chloroplast (OsASL, Xia et al. 2014), yet the localization of the *Arabidopsis* ASL protein has not been determined experimentally. The N-terminal sequence (amino acids 1-45) of *AtASL* is predicted to direct the mature ASL protein to the lumen of the chloroplast through the canonical chloroplast-targeting pathway (ChlorP-Sort, Emanuelsson et al. 1999). We cloned the full length ASL cDNA into a Gateway destination vector under the control of the ubiquitous 35S promoter and performed transient expression in the dicot *Nicotiana benthamiana*. We analyzed transient expression experiments using confocal microscopy, and to our surprise ASL:GFP was always observed in puncta of even size, and always in tight association with what appeared to be the chloroplast (Figure 5A). Therefore we used the natural autofluorescence of the chloroplast and found that the ASL:GFP signal was always found to be in tight association with the chloroplast (Figure 5B,C).

To definitively test the identity of the chloroplast, we co-infected a series of subcellular compartment reporter constructs linked to the red fluorescent protein mCherry and performed co-localization (Nelson et al. 2007). Again, the ASL:GFP signal was observed to co-localize only with the plastid signal, which confirmed the identity of chloroplast autofluorescence signal (Figure 5D-F, Supplemental Figure 2). In transient experiment experiments we always observed localization

of ASL:GFP in tight co-localization with the chloroplast, except in the instances where expression was performed for extended periods (5-7days) and large collections of ASL:GFP were detected in close proximity to membrane blebs inside the cell (Supplemental Figure 2).

We then tested the subcellular localization pattern in *Arabidopsis*. We transferred the ASL coding sequence into a Gateway destination vector that produces ASL:GFP under the transcriptional control of a fragment of the RCN1 promoter region, which drives expression in the root and leaves, especially stomata. We imaged stomata and roots from primary *Arabidopsis* transformants using confocal microscopy. ASL:GFP was found to be associated with the chloroplast in stomata (Figure 5G), and with the amyloplast in the root (Figure 5H).

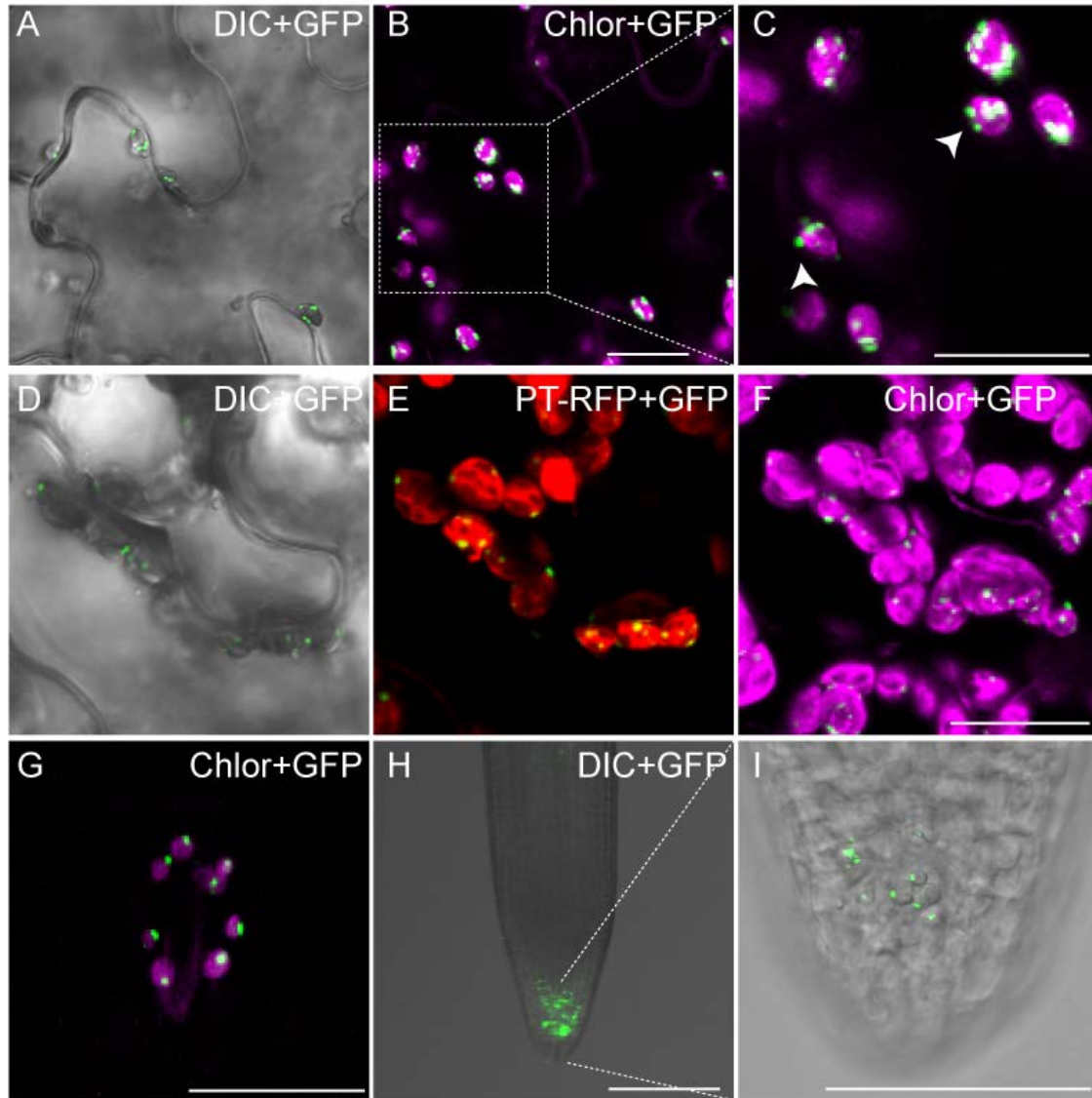


Figure 5. ASL:GFP localizes to a subcellular structure that is associated with the chloroplast. A-I. Confocal micrographs. **A-C.** *N. benthamiana* infiltrated with ASL:GFP and P19. **A.** 1 optical slice of epidermal cell expressing ASL, DIC and GFP channel. **B.** Same epidermal cell as in A, 3d projection of GFP and chloroplast autofluorescence. **C.** Inset of dotted line area in B, Arrow heads highlight the positioning of the ASL:GFP puncta at the presumed surface of the chloroplast. **D-F.** *N. benthamiana* infiltrated with ASL:GFP, Plastid-mCherry (pt-rk), and P19 (Nelson et al. 2007; Garabagi et al. 2012). **D.** 1 optical slice with ASL:GFP and P19. **E.** 3d projection of GFP and chloroplast mCherry marker. **F.** 3d projection of GFP and chloroplast autofluorescence. **G-I.** *RCN1:ASL:GFP* expression in T1 hemizygous *Arabidopsis* tissues. **G.** 3d projection of ASL:GFP and chloroplast autofluorescence around a leaf stomata. **H.** ASL:GFP expression in the root tip. **I.** Single optical slice of ASL:GFP association with amyloplasts in the root cap. (Inset from H). A-G. Scale = 20μm, H,I, scale = 50μm.

ASL adopts a punctate localization in pollen similar to plastids

We tested the localization of ASL in pollen. We transferred the ASL coding sequence into a Gateway destination vector containing an promoter that confers pollen specific expression (BGAL11, Unpublished Data, Thesis Chapter 6), and fuses the ASL coding sequence to mGFP. We transformed *BGAL11:ASL:GFP* into wild type plants and examined ASL:GFP localization in pollen using confocal microscopy. ASL:GFP was observed in puncta of similar size and quality as seen in the sporophytic tissues (Figure 6A,B). These puncta are visible in pollen tubes growing in vitro and in vivo, and can even be seen in the mixed ovule after pollen tube burst (Figure 6C). The pollen-specific *BGAL11:ASL:GFP* was capable of rescuing the sperm development of *hap7* mutant pollen grains, indicating that the C-terminal GFP fusion protein is active (Table 1, Figure 6D-F). Pollen does not have mature chloroplasts, however it does contain plastids (Matsushima et al. 2008; Tang et al. 2009). We performed co-localization with the Mitotracker Red fluorescent dye, and the peroxisomal reporter *PEX:mCherry* (J. Harper, Unpublished), to identify whether pollen ASL subcellular localization was drastically altered in pollen. We observed no co-localization with mitochondria (Figure 6G) or peroxisomes (Figure 6H) in pollen. Further studies with plastid specific markers are in progress to positively localize the ASL protein to the pollen plastid.

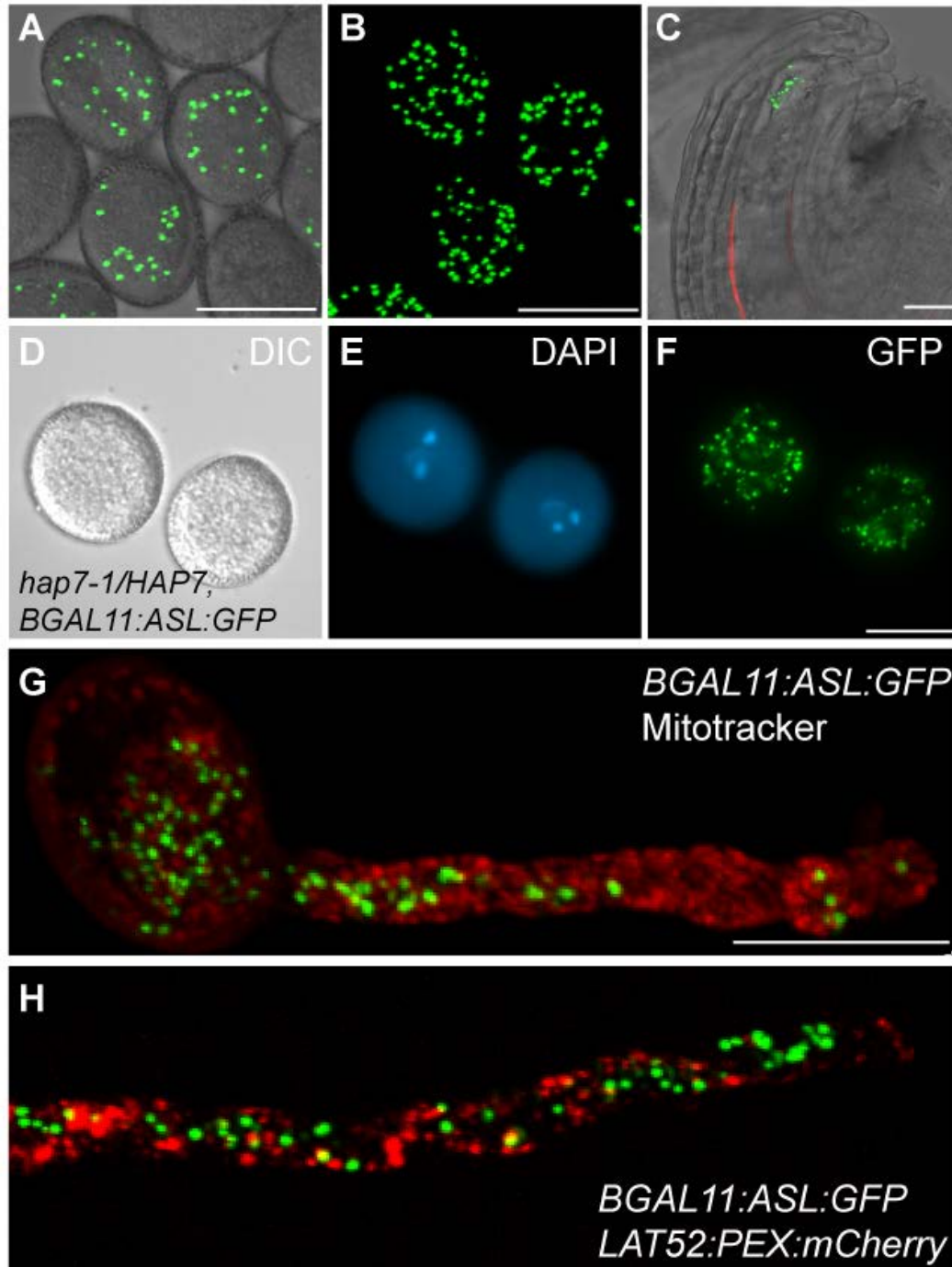


Figure 6. ASL:GFP pollen expression rescues *hap7* sperm development. A-B. *BGAL11:ASL:GFP* expression in wild-type pollen grains. Confocal micrographs, scale bar = 20 μm. A. Single optical slice of ASL:GFP and DIC. B. 3d projection of GFP channel (same field as A). C. ASL:GFP is detectable in ovules targeted by pollen tubes. D-F. Representative epifluorescent images of *hap7-1/HAP7*, *BGAL11:ASL:GFP* pollen grains where sperm development has been rescued by expression of ASL:GFP (see table 1). D – DIC, E – DAPI, F – GFP. G. *BGAL11:ASL:GFP* pollen tubes incubated with Mitotracker Red, 3d

projection. **H.** *BGAL11:ASL:GFP, LAT52:PEX19:mCherry* pollen tube, 3d projection. **G&H** are at the same magnification, scale bar = 20um.

Discussion

Amino acid biosynthesis is a fundamental component of cellular physiology, and the cellular pathways that produce amino acids are conserved across all kingdoms. Arginine biosynthesis is intricately linked to the citric acid cycle as well as many downstream biosynthetic pathways. Despite its central importance, Arginine biosynthesis has not been comprehensively studied in a plant genetic system (Slocum 2005). Here we present a genetic characterization of ASL mutants and their effect on development and function of the male gametophyte. ASL deficiency results in a range of gametophyte defects, and a striking loss of male germline development at pollen mitosis II, resulting in the production of single sperm-like cells (SSLC). It is as yet unknown what role arginine concentrations play in sperm development, however, it is unlikely that any one downstream pathway is solely responsible. Instead, sperm development most likely represents a cellular bottle-neck through which gametophytes lacking ample cellular biosynthetic resources may continue.

Plant ASL subcellular localization was investigated in Pea (*Pisum sativum*, PsASL) through biochemical purification (Taylor et al. 1981) and recently the rice ASL (*Oryza sativa* OsASL) using immunofluorescence and transient expression (Xia et al. 2014). Biochemical fractionation suggested a cytoplasmic localization of PsASL, while transient expression of OsASL in onion epidermal cells co-localizes with chloroplast markers, suggesting OsASL may localize to the

chloroplast in a species-specific manner. Our results clearly demonstrate that the *Arabidopsis* ASL protein is localized to a small cellular structure that is in tight association with the chloroplast. Tight localization of the ASL punctate structure with the chloroplast would allow the chloroplast tight spatial control over Arginine biosynthesis. Future experiments are focused on the subcellular localization of the ASL protein in pollen tubes, where we hypothesize we will observe co-localization with known plastid markers.

Table 1. Transmission of *hap7* gametes

	Line	Self-fertilized		Male		Female			
		% BastaR	<i>n</i>	% BastaR	<i>n</i>	% BastaR	<i>n</i>	%SSLCs	<i>n</i>
<i>hap7-1/HAP7</i>		6.75	756	0.12	1606	5.95	908	13.8	304
<i>hap7-2/HAP7</i>		18.11	646	0.14	729	12.32	341	26.3	152
Control Heterozygote (76224)				49	400			0.2	520
<i>hap7-1/HAP7 gHAP7/gHAP7</i> (F3)	G1	51.93 ± 4.86	1787	13.4 ± 5.99	786	ND	ND	6.05	2367
	G4	52.86 ± 3.13	2100	17.3 ± 2.5	912	ND	ND	3.61	2008
<i>hap7-1/HAP7 gHAP7/gHAP7</i> (F4)	G1	55.28 ± 1.59	8261	24.81 ± 7.26	2789	45.10 ± 4.17	577	ND	ND
	G4	55.02 ± 5.16	9119	17.44 ± 7.36	3129	46.03 ± 8.52	606	ND	ND
<i>hap7-2/HAP7 gHAP7/gHAP7</i> (T3)	G1	37.87 ± 11.82	1058	32.53 ± 6.03	1469	ND	ND	2.15	1972
	G4	42.19 ± 2.26	301	24.33 ± 8.74	859	ND	ND	2.56	2027
<i>hap7-2/HAP7 gHAP7/gHAP7</i> (T4)	G1	52.56 ± 2.9	3023	45.36 ± 6.26	3668	42.75 ± 3.97	1483	ND	ND
	G4	56.03 ± 5.23	2537	20.25 ± 6.13	1412	45.93 ± 5.02	812	ND	ND
<i>hap7-1/HAP7 35S:cHAP7/cHAP7</i> (F3)	C1	45.59	174	8.93 ± 6.2	112	48.59 ± 5.98	142	ND	ND
	C2	37.77	122	7.39 ± 8.6	176	35.43 ± 5.26	127	ND	ND
<i>hap7-1/HAP7 BGAL11:cHAP7/cHAP7</i> (F3)	P1	ND	ND	46.26 ± 3.29	227	ND	ND	0.96	311
	P2	ND	ND	34.67 ± 5.16	199	ND	ND	0.00	217
<i>hap7-2/HAP7 BGAL11:cHAP7/cHAP7</i> (F3)	P3	ND	ND	20.51 ± 5.16	117	ND	ND	1.19	252
	P4	ND	ND	16.17 ± 7.18	266	ND	ND	2.64	265

Self-fertilized: *hap+/-* × *hap+/-*, data are shown for the T5 generation. Male: *ms1* × *hap+/-*, *ms1* females were hand pollinated using *hap+/-* anthers. Female: *hap+/-* × *qrt1*, *hap+/-* females were emasculated and hand pollinated using *qrt1-2* anthers. % BastaR, the percentage of BastaR F1 progeny from the indicated cross. gHAP7 - genomic locus of AT5G10920. Data is presented as the pooled percentage of at least 4 crosses ± standard deviation. The gHAP7 construct was transformed into *hap7-2/HAP7*, and then introgressed into *hap7-1/HAP7* to create near isogenic lines.

Methods

Plant Growth

Procedures for selection of antibiotic and/or herbicide-resistant seedlings and plant growth conditions have been described previously (Johnson et al. 2004; Qin et al. 2009). *LAT52:PEX:mCherry* was a gift from the laboratory of Dr. Jeff Harper. *LAT52:CFP:PTS1* was a gift from (Nowak et al. 2004).

Bioinformatic analysis

Phylogenetic relationships were analyzed using MEGA software (Tamura et al. 2013) and visualized with CLC sequence viewer (CLCbio, Boston, MA). Structural prediction was performed with Phyre2.0 (Kelley et al. 2009).

Genetic Analysis

Mutants from the *hapless* collection were grown from in house seed stocks for re-analysis of developmental phenotypes. The *hap7-2* allele (*SAIL_814_E04*, *Col-0* ecotype) was obtained from the Arabidopsis Biological Resource Center. Pollen tube growth assays and ovule targeting assays were performed as previously described (Qin et al. 2009).

Cloning

A full length *AtASL* cDNA clone (DKLAT5G10920) in a gateway vector was ordered from ABRC and used for subsequent cDNA analysis. The genomic complementation clone (gHAP7) included 880bp of upstream promoter

sequence, and 995bp of downstream sequence. The fragment amplified from genomic Col-0 DNA using Extaq polymerase (Takara,), cloned into the pCR2.1 TA cloning vector (Invitrogen) and sub-cloned into the pCAMBIA2300 plant transformation vector using the restriction site SbfI, which was engineered into the cloning primer (see primer table). Plants were transformed by the floral dip method and T1 transformants were selected using Kanamycin resistance. For Gateway vector expression: cDNA sequences were cloned into entry vectors pENTR-D or pCR8 (Invitrogen), and LR cloned into destination vectors with LR clonase II (Invitrogen). Destination vectors promoter sequences were swapped after the ccDb/CAM cassette was replaced with a placeholder DNA sequence (LR reaction) before being replaced with the ccDb/CAM cassette from pDONRZEO using BP clonase II (Invitrogen). All clones were sequenced before introduction to plants (Genewiz, Boston, MA).

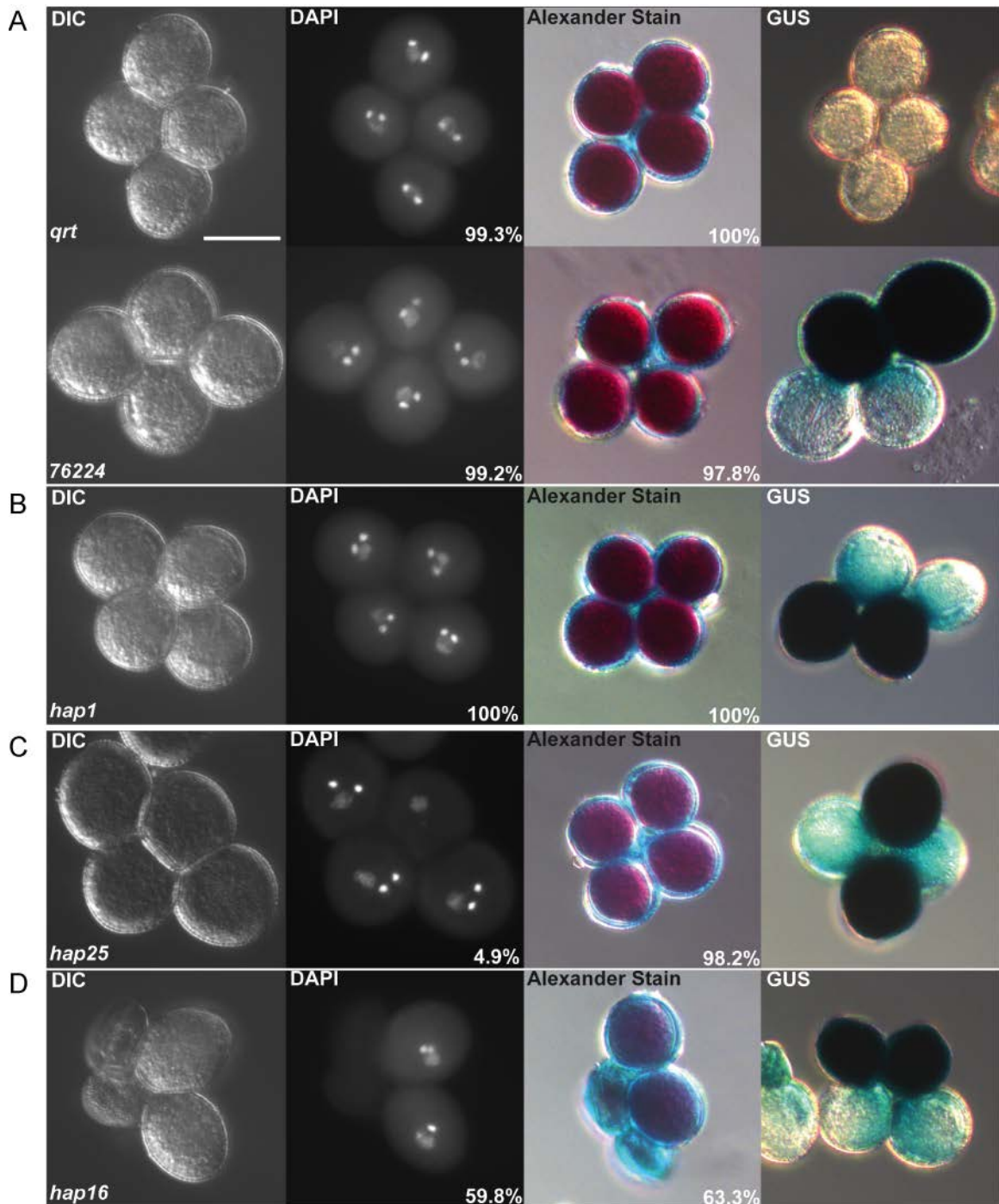
Transient Expression in *N. benthamiana*

Transient expression followed protocols from (Cournoyer et al. 2011). Briefly, 7ml LB+Drug cultures of *Agrobacterium* carry desired constructs were grown overnight, spun down, and re-suspended in Infiltration media (10mM MgCl₂, 10mM MES, 200uM acetosyringone), and then shaken at 25C for at least three hours. Cultures were spun down and re-suspended at an OD₆₀₀ of one before injection into *N. Benthamiana* leaves, and allowed to express for 3 days to visualize protein localization by confocal microscopy.

Microscopy

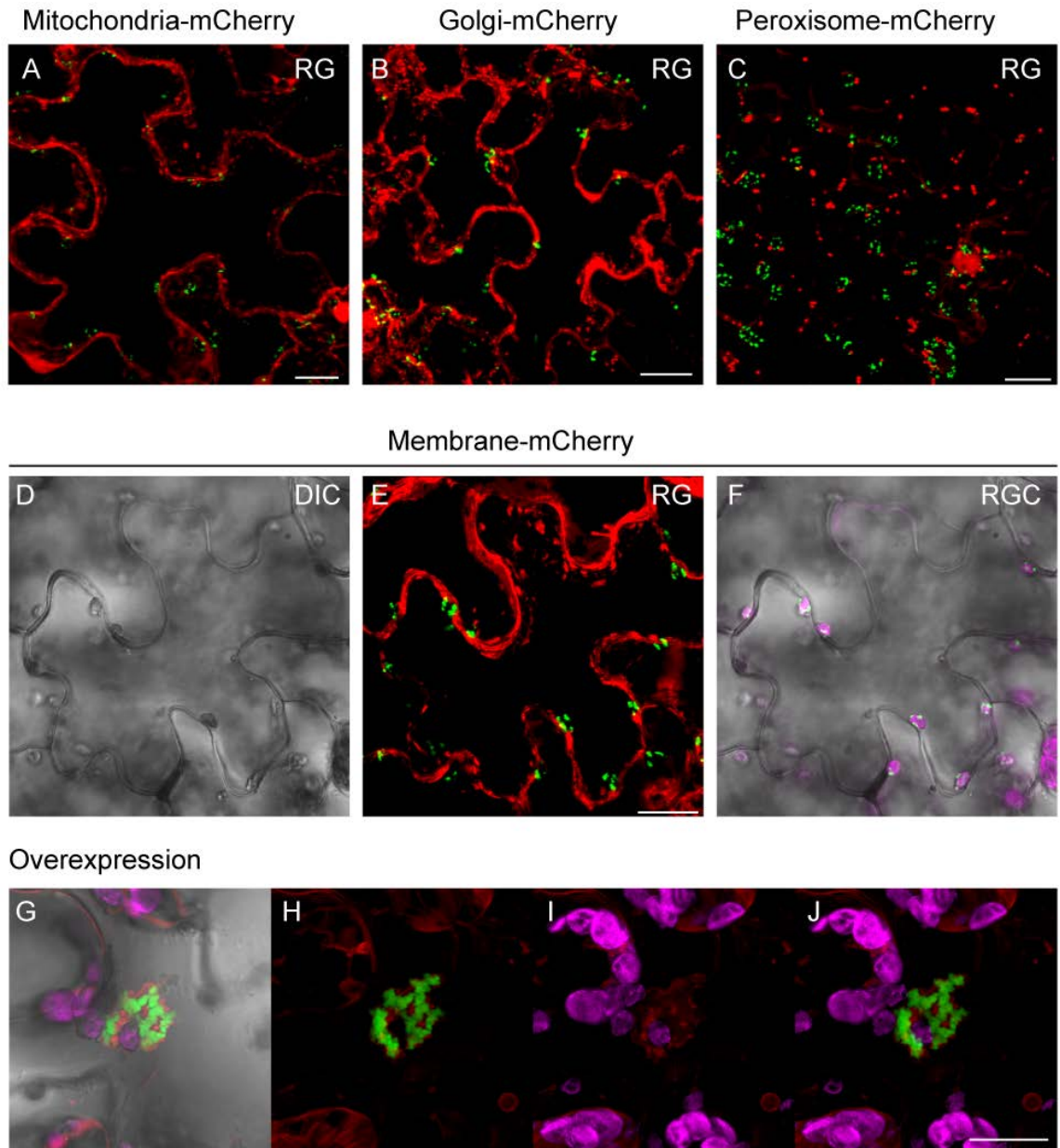
Alexander Staining was adapted from (Alexander 1969). Alexander staining and DAPI DNA staining was analyzed with a Zeiss Axiovert 200M Fluorescence Microscope, with either an AxioCam MRC5 (color) or Hamamatsu ORCA-ER monochrome (epifluorescence) camera. For live imaging, crosses and in vitro pollen growth experiments were prepared under a dissecting microscope (Zeiss Stemi 2000C) by pollinating *ms1* or emasculated wild-type stage 14 stigmas (Smyth et al. 1990) with pollen from male donors, or incubating pollen tubes with in vitro pollen growth media (Boavida et al. 2007). For live imaging of in vivo growth ovary walls were removed 12 hours post-pollination as previously described (Johnson et al. 2010). Dissected pistil tissues were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope). Standard emission and detection wavelength settings were used to image GFP expression (argon laser 458/477/488/514nm at 30mV) and dsRed/mRFP/Chloroplast autofluorescence expression (helium neon laser 633nm at 3mV). Signal intensities were optimized for each individual fluorophore and then combined in overlay. Final images represent a merge of single planes at varying depths (z stacks). MitoTracker® Red FM was purchased from Invitrogen (M22425), and used at a final concentration of 100nm in pollen growth media.

Supplemental Figures and Tables.



Supplemental Figure S1. Representative images of pollen and MGU developmental defects. In each panel the first two images are paired DIC and DAPI micrographs of the same quartet of pollen grains. The third panel is an Alexander stained tetrad (AS), and the fourth panel is a GUS stained tetrad. In DAPI, AS, and GUS panels the percentage of tetrads that displayed the phenotype presented is shown in white text at the bottom right. **A.** Wild type control lines of *qrt1-2*, and 76224 (*qrt1-2*, *LAT52:GUS*). **B.** Pollen from a *hapless*

mutant displaying no developmental defects (*hap1/HAP1*). **C.** Pollen from a *hapless* mutant demonstrating MGU defects, specifically *hap25*, which demonstrates loss of sperm cells from the MGU at low rates. **D.** Pollen from a *hapless* mutant demonstrating grain development defects, specifically *hap16*, which frequently produces non-viable grains.



Supplementary Figure S2. Transient transformations of *35S:ASL:GFP* in *N. benthamiana*. Expression of ASL:GFP with **A.** Mitochondrial-mCherry, **B.** Golgi-mCherry, **C.** Peroxisomal-mCherry, and **D-J.** Membrane-mCherry (Nelson et al. 2007). **A-C,E.** 3d projections of Red and Green Channels (RG). **D.** DIC image of **E.** **F.** GFP, DIC and chloroplast autofluorescence signal in one optical slice. **G-J.** Overexpression of *35S:ASL:GFP* after 5 days of transient expression in *N. benthamiana*. **G.** Red, Green, Autofluorescence and DIC merge in one optical slice. **H.** 3d projection of Red and Green Channel. **I.** 3d projection of chloroplast autofluorescence and Red channel. **J.** 3d projection of Red, Green, and chloroplast autofluorescent channels.

Supplementary Table 1. Pollen developmental defects in the hapless mutant collection

Pollen viability - Alexander Stain for intact cell wall and pollen cytoplasm				DAPI Staining of the Male Germ Unit (MGU)			
Mutant	% Viable	% Abnormal	N (Grains)	% 2 Sperm Cells	% 1 SSLC	% MGU Absent	N (Grains)
<i>hap1</i>	100.0	0.0	160	100.0	0.0	0.0	284
<i>hap2</i>	99.1	0.9	216	98.9	0.6	0.6	176
<i>hap3</i>	99.1	0.9	232	100.0	0.0	0.0	160
<i>hap4</i>	100.0	0.0	232	97.8	1.7	0.6	180
<i>hap5</i>	60.8	39.2	232	64.9	0.9	34.2	228
<i>hap6</i>	98.6	1.4	216	98.4	0.0	1.6	380
<i>hap7</i>	72.2	27.8	176	59.6	13.8	26.6	304
<i>hap8</i>	60.6	39.4	180	59.1	0.0	40.9	296
<i>hap9</i>	99.6	0.4	256	100.0	0.0	0.0	200
<i>hap10</i>	98.0	2.0	204	97.8	0.0	2.2	232
<i>hap11</i>	97.4	2.6	268	100.0	0.0	0.0	252
<i>hap12</i>	75.5	24.5	208	88.9	0.0	11.1	244
<i>hap13</i>	98.3	1.7	232	100.0	0.0	0.0	196
<i>hap14</i>	87.5	12.5	152	86.2	0.5	13.3	196
<i>hap15</i>	99.4	0.6	176	97.6	0.5	1.9	412
<i>hap16</i>	47.5	52.5	316	63.5	0.0	36.5	348
<i>hap18</i>	98.6	1.4	208	95.2	4.8	0.0	272
<i>hap19</i>	98.9	1.1	276	100.0	0.0	0.0	272
<i>hap20</i>	98.3	1.7	408	98.1	1.4	0.5	208
<i>hap21</i>	64.6	35.4	460	85.2	1.2	13.5	244
<i>hap22</i>	98.4	1.6	364	100.0	0.0	0.0	304
<i>hap23</i>	97.1	2.9	272	81.3	14.3	4.5	336
<i>hap24</i>	97.1	2.9	208	98.4	1.1	0.5	184
<i>hap25</i>	99.6	0.4	224	98.4	0.4	1.2	244
<i>hap26</i>	98.9	1.1	356	97.1	0.8	2.1	240
<i>hap27</i>	77.5	22.5	236	92.0	0.9	7.1	212
<i>hap28</i>	98.6	1.4	296	99.3	0.4	0.4	276
<i>hap29</i>	98.7	1.3	392	99.6	0.0	0.4	284
<i>hap30</i>	99.3	0.7	268	96.0	3.5	0.5	200
<i>hap31</i>	98.3	1.7	292	98.8	1.3	0.0	320
<i>qrt1-2</i>	98.6	1.4	296	99.8	0.2	0.0	544
<i>76224</i>	99.2	0.8	356	99.8	0.2	0.0	520

Supplementary Table 1. All values in each section are the % of total pollen grains in each column N.

References

- Alexander, M. P. (1969). "Differential staining of aborted and nonaborted pollen." Stain Technol **44**(3): 117-122.
- Aw, S. J., Y. Hamamura, et al. (2010). "Sperm entry is sufficient to trigger division of the central cell but the paternal genome is required for endosperm development in Arabidopsis." Development **137**(16): 2683-2690.
- Baudouin, E. (2011). "The language of nitric oxide signalling." Plant Biol (Stuttg) **13**(2): 233-242.
- Birke, H., C. Heeg, et al. (2013). "Successful fertilization requires the presence of at least one major O-acetylserine(thiol)lyase for cysteine synthesis in pollen of Arabidopsis." Plant Physiol **163**(2): 959-972.
- Boavida, L. C. and S. McCormick (2007). "Temperature as a determinant factor for increased and reproducible in vitro pollen germination in Arabidopsis thaliana." Plant J **52**(3): 570-582.
- Boisson-Dernier, A., S. Frietsch, et al. (2008). "The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition." Curr Biol **18**(1): 63-68.
- Borg, M., L. Brownfield, et al. (2011). "The R2R3 MYB transcription factor DUO1 activates a male germline-specific regulon essential for sperm cell differentiation in Arabidopsis." Plant Cell **23**(2): 534-549.
- Cournoyer, P. and S. P. Dinesh-Kumar (2011). "Studying NB-LRR immune receptor localization by agroinfiltration transient expression." Methods Mol Biol **712**: 1-8.
- Cunin, R., N. Glansdorff, et al. (1986). "Biosynthesis and metabolism of arginine in bacteria." Microbiol Rev **50**(3): 314-352.
- d'Erfurth, I., S. Jolivet, et al. (2008). "Mutations in AtPS1 (Arabidopsis thaliana parallel spindle 1) lead to the production of diploid pollen grains." PLoS Genet **4**(11): e1000274.
- Davis, R. H. (1986). "Compartmental and regulatory mechanisms in the arginine pathways of Neurospora crassa and Saccharomyces cerevisiae." Microbiol Rev **50**(3): 280-313.
- Del Duca, S., D. Serafini-Fracassini, et al. (2014). "Senescence and programmed cell death in plants: polyamine action mediated by transglutaminase." Front Plant Sci **5**: 120.
- Emanuelsson, O., H. Nielsen, et al. (1999). "ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites." Protein Sci **8**(5): 978-984.
- Frank, A. C. and M. A. Johnson (2009). "Expressing the diphtheria toxin A subunit from the HAP2(GCS1) promoter blocks sperm maturation and produces single sperm-like cells capable of fertilization." Plant Physiol **151**(3): 1390-1400.
- Garabagi, F., E. Gilbert, et al. (2012). "Utility of the P19 suppressor of gene-silencing protein for production of therapeutic antibodies in Nicotiana expression hosts." Plant Biotechnol J **10**(9): 1118-1128.

- Grabe, A. and U. Kristen (1997). "Growth inhibition of pollen tubes by blocking the aromatic or branched-chain amino acid pathways of biosynthesis." Physiologia Plantarum **101**(3): 577-582.
- Hamamura, Y., C. Saito, et al. (2011). "Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in *Arabidopsis thaliana*." Curr Biol **21**(6): 497-502.
- Hudson, A. O., B. K. Singh, et al. (2006). "An LL-diaminopimelate aminotransferase defines a novel variant of the lysine biosynthesis pathway in plants." Plant Physiol **140**(1): 292-301.
- Johnson, M. A. and B. Kost (2010). "Pollen tube development." Methods Mol Biol **655**: 155-176.
- Johnson, M. A., K. von Besser, et al. (2004). "Arabidopsis *hapless* mutations define essential gametophytic functions." Genetics **168**(2): 971-982.
- Jones-Rhoades, M. W., J. O. Borevitz, et al. (2007). "Genome-wide expression profiling of the Arabidopsis female gametophyte identifies families of small, secreted proteins." PLoS Genet **3**(10): 1848-1861.
- Kelley, L. A. and M. J. Sternberg (2009). "Protein structure prediction on the Web: a case study using the Phyre server." Nat Protoc **4**(3): 363-371.
- Lalanne, E., C. Mathieu, et al. (1997). "Structure and specific expression of a *Nicotiana sylvestris* putative amino-acid transporter gene in mature and in vitro germinating pollen." Plant Mol Biol **35**(6): 855-864.
- Liu, Y. G., N. Mitsukawa, et al. (1995). "Efficient isolation and mapping of Arabidopsis *thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR." Plant J **8**(3): 457-463.
- Matsushima, R., Y. Hamamura, et al. (2008). "Mitochondrial dynamics in plant male gametophyte visualized by fluorescent live imaging." Plant Cell Physiol **49**(7): 1074-1083.
- Minocha, R., R. Majumdar, et al. (2014). "Polyamines and abiotic stress in plants: a complex relationship." Front Plant Sci **5**: 175.
- Muralla, R., C. Sweeney, et al. (2007). "Genetic dissection of histidine biosynthesis in Arabidopsis." Plant Physiol **144**(2): 890-903.
- Nelson, B. K., X. Cai, et al. (2007). "A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants." Plant J **51**(6): 1126-1136.
- Niyogi, K. K., R. L. Last, et al. (1993). "Suppressors of *trp1* fluorescence identify a new arabidopsis gene, TRP4, encoding the anthranilate synthase beta subunit." Plant Cell **5**(9): 1011-1027.
- Nowak, K., N. Luniak, et al. (2004). "Fluorescent proteins in poplar: a useful tool to study promoter function and protein localization." Plant Biol (Stuttg) **6**(1): 65-73.
- Okuda, S., H. Tsutsui, et al. (2009). "Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells." Nature **458**(7236): 357-361.
- Preuss, D., S. Y. Rhee, et al. (1994). "Tetrad analysis possible in Arabidopsis with mutation of the *QUARTET* (*QRT*) genes." Science **264**(5164): 1458-1460.

- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the stigma and style elicits a novel transcriptome in pollen tubes, pointing to genes critical for growth in a pistil." PLoS Genet **5**(8): e1000621.
- Qin, Y., A. R. Leydon, et al. (2009). "Penetration of the Stigma and Style Elicits a Novel Transcriptome in Pollen Tubes, Pointing to Genes Critical for Growth in a Pistil." PLoS Genet **5**(8).
- Rabiger, D. S. and G. N. Drews (2013). "MYB64 and MYB119 are required for cellularization and differentiation during female gametogenesis in *Arabidopsis thaliana*." PLoS Genet **9**(9): e1003783.
- Sampaleanu, L. M., F. Vallee, et al. (2001). "Structural studies of duck delta 1 and delta 2 crystallin suggest conformational changes occur during catalysis." Biochemistry **40**(9): 2732-2742.
- Schwacke, R., S. Grallath, et al. (1999). "LeProT1, a transporter for proline, glycine betaine, and gamma-amino butyric acid in tomato pollen." Plant Cell **11**(3): 377-392.
- Slocum, R. D. (2005). "Genes, enzymes and regulation of arginine biosynthesis in plants." Plant Physiol Biochem **43**(8): 729-745.
- Smyth, D. R., J. L. Bowman, et al. (1990). "Early flower development in *Arabidopsis*." Plant Cell **2**(8): 755-767.
- Sobieszczuk-Nowicka, E. and J. Legocka (2014). "Plastid-associated polyamines: their role in differentiation, structure, functioning, stress response and senescence." Plant Biol (Stuttg) **16**(2): 297-305.
- Song, J. T., H. Lu, et al. (2004). "Divergent roles in *Arabidopsis thaliana* development and defense of two homologous genes, aberrant growth and death2 and AGD2-LIKE DEFENSE RESPONSE PROTEIN1, encoding novel aminotransferases." Plant Cell **16**(2): 353-366.
- Tamura, K., G. Stecher, et al. (2013). "MEGA6: Molecular Evolutionary Genetics Analysis version 6.0." Mol Biol Evol **30**(12): 2725-2729.
- Tang, L. Y., N. Nagata, et al. (2009). "Visualization of plastids in pollen grains: involvement of FtsZ1 in pollen plastid division." Plant Cell Physiol **50**(4): 904-908.
- Taylor, A. A. and G. R. Stewart (1981). "Tissue and subcellular localization of enzymes of arginine metabolism in *Pisum sativum*." Biochem Biophys Res Commun **101**(4): 1281-1289.
- Twell, D. (1994). The diversity and regulation of gene expression in the pathway of male gametophyte development, Cambridge University Press.
- Twell, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.
- von Besser, K., A. C. Frank, et al. (2006). "*Arabidopsis* HAP2(GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization." Development **133**(23): 4761-4769.
- Xia, J., N. Yamaji, et al. (2014). "Normal root elongation requires arginine produced by argininosuccinate lyase in rice." Plant J **78**(2): 215-226.

Appendix 6:

NTA localization in ovules receiving *myb* triple-mutant pollen tubes

We designed an experiment to test whether NTA:GFP localization depends on MYB-target gene expression. NTA:GFP pistils were crossed by wild type and *myb* triple-mutant pollen tubes carrying *LAT52:dsRed* (Twell et al. 1989; Francis et al. 2007). Crosses were analyzed by confocal microscopy (Leydon et al. 2013) at 8 hours after pollination to catch early pollen tube reception interactions. We observed NTA:GFP in a uniform pattern in ovules where pollen tubes had not grown into the embryo sac (Figure 1A,B). This pattern was consistent with previous reports on NTA:GFP localization (Kessler et al. 2010). Upon the entry of a wild type, *LAT52:dsRed* pollen tube into the embryo sac, NTA:GFP could be observed re-localizing to the micropylar region of the ovule (Figure 1C), as had been previously observed (Kessler et al. 2010). When ovules received a *myb* triple-mutant, *LAT52:dsRed* pollen tube NTA was always observed to re-localize to the point of pollen tube contact at the micropylar region of the ovule (13/13 events) (Figure 1D-H). After pollen tube coiling began in *myb* triple-mutant pollen tubes, it became difficult to image distinct NTA:GFP dynamics due to the complex pollen tube coils, however enrichment of NTA:GFP was somewhat discernable (Figure 1I). Future work needs to be performed to analyze this localization pattern in ovules receiving a *myb* triple-mutant pollen tube using time-lapse microscopy. However, these preliminary experiments suggest that NTA re-localization to the site of pollen tube:synergid contact occurs normally in the absence of genes activated by *MYB97*, *MYB101*, and *MYB120*.

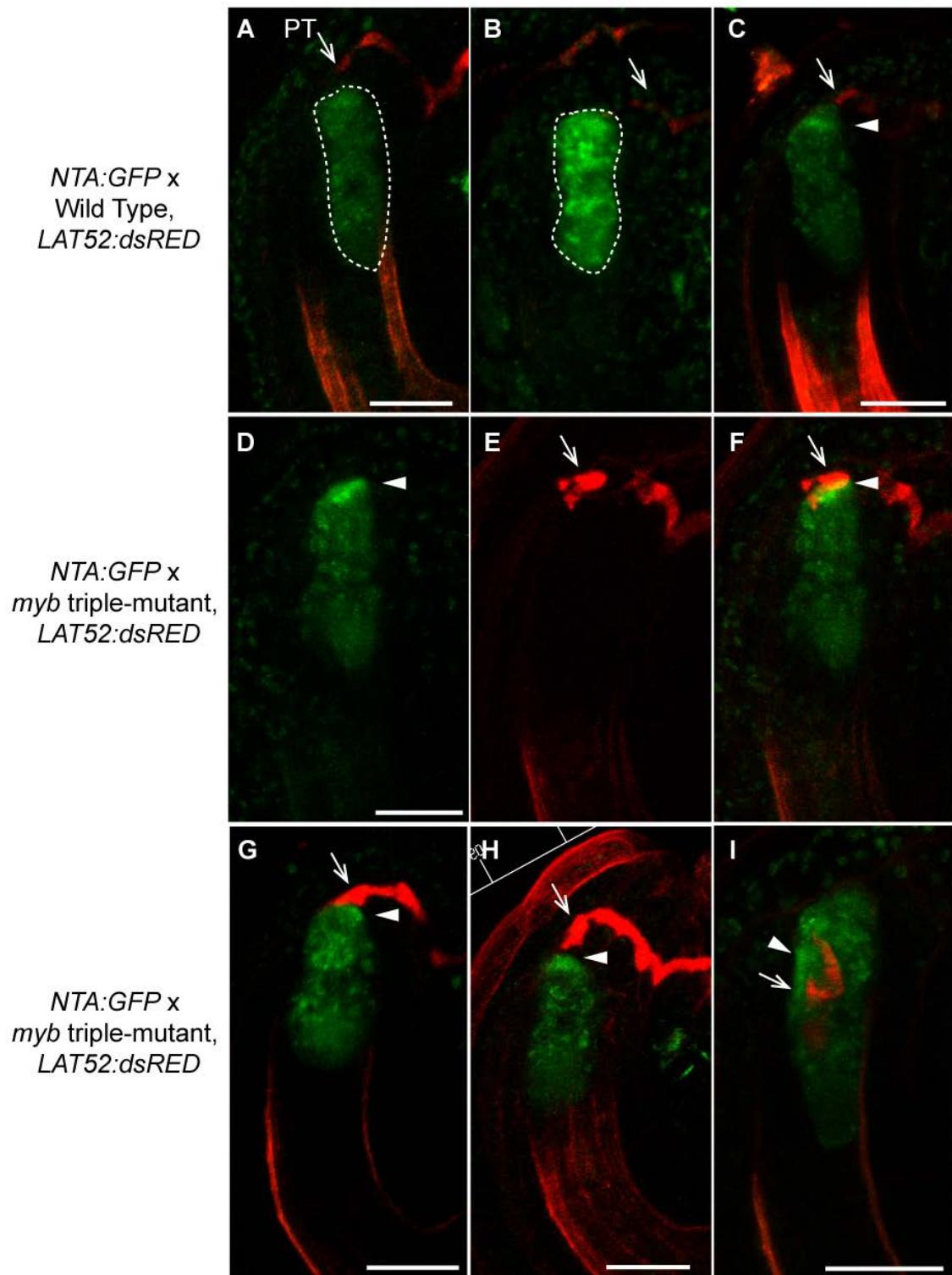


Figure 1. NTA:GFP localization in ovules receiving *myb* triple-mutant pollen tubes. A-B. NTA:GFP localization in ovules just prior to pollen tube contact with synergid cells. C. One NTA:GFP ovule receiving a wild type, LAT52:dsRed pollen tube. NTA:GFP is visible localizing to the contact point between the pollen tube

(PT) (Red – white arrow) and the synergid cell (GFP maxima - white arrowhead). **D-I.** NTA:GFP localization occurs normally in ovules targeted by *myb* triple-mutant pollen tubes. D-F. One NTA:GFP ovule receiving a *myb* triple-mutant, LAT52:dsRed pollen tube. D – NTA:GFP, E – *myb* triple-mutant, LAT52:dsRED pollen tube, F – merge. . All scale bars are 20um. **G & H.** Early stages of pollen tube reception, before *myb* triple-mutant pollen has undergone massive overgrowth in the embryo sac as in **K**.

References

- Francis, K. E., S. Y. Lam, et al. (2007). "Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis." Proc Natl Acad Sci U S A **104**(10): 3913-3918.
- Kessler, S. A., H. Shimosato-Asano, et al. (2010). "Conserved molecular components for pollen tube reception and fungal invasion." Science **330**(6006): 968-971.
- Leydon, A. R., K. M. Beale, et al. (2013). "Three MYB Transcription Factors Control Pollen Tube Differentiation Required for Sperm Release." Current Biology **23**(13): 1209-1214.
- Twell, D., R. Wing, et al. (1989). "Isolation and expression of an anther-specific gene from tomato." Mol Gen Genet **217**(2-3): 240-245.