AN ABSTRACT OF THE DISSERTATION OF

Johanna C. Smyth for the degree of Doctor of Philosophy in Botany and Plant Pathology presented on August 18, 2016.

Title: Characterization of the Transition to the Progamic Phase in *Zea mays* Pollen

Abstract approved:

_____

John E. Fowler

The transition of mature pollen to the progamic phase represents an important step in the regulation of plant sexual reproduction. The progamic phase encompasses the initiation of pollen tube germination, growth of the pollen tube through the stigma (the silk in maize) and concludes with fertilization of the female gametophyte. This action involves interactions between the male gametophyte and female sporophyte and represents possible sites for regulation of reproduction. Due to its location within the sporophytic tissue, analysis of gene expression of the actively growing pollen tube is difficult to conduct *in vivo*. Two broad, complementary experimental approaches were used to identify genes important in the progamic phase of maize pollen development and to investigate how their products are regulated. The work presented here represents an initial characterization of the transition to the progamic phase of *Zea mays* (maize) pollen through the use of proteomic and transcriptomic analyses.

The first approach was to analyze and characterize changes to the protein profile of maize pollen before and after the initiation of pollen tube germination. Unlike in many plants, germination of the pollen tube in maize occurs rapidly, with no significant changes detected in the transcriptome. This supports a hypothesis where the modulation of protein levels and/or post-transcriptional modification of protein

activity, rather than transcription, play critical roles in the developmental transition to the progamic phase in maize. Tests of germination in the presence of drugs that inhibit protein synthesis or degradation indicate that *de novo* translation and ubiquitin-mediated protein degradation are crucial to successful germination in maize. Consistent with these findings, SDS-PAGE gel silver staining of total protein extracts found evidence of particular proteins increasing and decreasing in abundance at 30 minutes post-germination. Quantitative profiling of the proteome and phosphoproteome identified 393 differentially abundant proteins between mature and germinated pollen. In addition, 103 proteins are associated with a significant change in phosphorylation state largely independent of changes to the total proteome upon germination. Functional analysis by gene ontology of differentially abundant proteins revealed enrichment for several terms associated with translation in the total proteome, as well as significantly increased phosphorylation in several kinases and components of protein degradation machinery. Genes functioning in the jasmonic acid biosynthesis pathway were found to be enriched among those proteins decreasing in abundance with germination suggesting that this pathway is down regulated with the transition to the progamic phase. Male transmission defect analysis (MTDA) was used as a genetic test for biological significance of the results from the quantitative proteome profiling by testing for reduced inheritance of insertions in genes predicted to be important for pollen function. Ten insertions located in exons and with robust PCR genotyping reactions were tested for transmission of the insertions through the male and female. Of these ten, five were found to exhibit reduced transmission though the male supporting the use of proteomic data to identify biologically relevant components affecting pollen function.

The second approach presents a pilot study utilizing single nucleotide polymorphisms (SNPs) to distinguish RNA derived from the progamic male gametophyte from that of the female sporophyte during growth of the pollen tube through the silk. This approach takes advantage of the high level of genetic variation found in maize. By using different inbred lines for the pollen (B73) and silk (W22), SNPs known to exist between the two lines were able to identify pollen-derived alleles from those of the

silk by RNA-seq. An initial analysis focused on chromosome 8 found 222 genes with alignment of reads to the B73 allele; these putatively represent gene expression of the male gametophyte. Supporting this conclusion is the presence of 23 genes previously found to be associated with enrichment of expression in mature pollen. CAPS (Cleaved Amplified Polymorphic Sequence) can validate the presence of inbred specific transcripts by allele specific endonuclease restriction digest of RT-PCR products. An initial CAPS analysis of the transcripts from one gene was able to detect reads containing the B73 allele in the pollinated W22 silk sample, though the results from the unpollinated W22 silks were inconclusive.

Characterization of the Transition to the Progamic Phase in *Zea mays* Pollen

by
Johanna C. Smyth

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented August 18, 2016
Commencement June 2017

Doctor of Philosophy dissertation of Johanna C. Smyth presented on August 18, 2016

APPROVED:

Major Professor, Representing Botany and Plant Pathology

Head of the Department of Botany and Plant Pathology

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Johanna C. Smyth, Author

ACKNOWLEDGEMENTS

I would like to thank my major professor John Fowler for being an amazing mentor and an eternal optimist. A special thanks to Zuzana Vejlupkova and Dianne Simpson, I am not sure how I could have done without your help, guidance and friendship during my PhD. I would like to acknowledge and thank each of my committee members for pushing me to become a better scientist and writer: Carol Riven, Jeff Change, Aaron Liston, and my graduate student representative Walt Ream.

Lastly, I am so grateful for all the support from my friends and family who have encouraged me to study what I love. To my Mom, Dad, Sister, Husband and Virginia Family: Thank you.

The research presented here was funded through the National Science Foundation (Grants #IOS-0924023 and #IOS-1340050).

CONTRIBUTION OF AUTHORS

John Fowler assisted with funding, experimental design, data interpretation and editing of this manuscript. Carol Riven, Jeff Change and Aaron Liston contributed useful critiques, advice and helpful discussions for improving this manuscript.

TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF FIGURES (Continued)

Figure Page

LIST OF FIGURES (Continued)

Figure Page

LIST OF TABLES

Table Pages

LIST OF APPENDIX FIGURES

Figure Page

LIST OF APPENDIX TABLES

Table Page

LIST OF APPENDIX SCRIPTS

Script Page

DEDICATION

To my wonderful sister who encouraged me to be all I could. I am still laughing and crying about it.

Characterization of the Transition to the Progamic Phase in *Zea mays* Pollen

Chapter 1 : General Introduction

Much of biological research is focused on transitions, where a change in the state of a cell or organism is triggered in response to either internal cues or external stimuli. Examples of such transitions include the stepwise progress to maturation of cells in a leaf (Facette *et al.*, 2013), the cyclic expression of circadian rhythm genes (Harmer, 2009; McClung, 2006) or cellular response to external stressors like hypoxia (Van Ven *et al.*, 2016). These events are not necessarily rapid or discrete, and can occur as a slow or continuous change between two states. As the cells within a maize leaf mature, they transition over several days through four stages: division, differentiation, elongation and finally, to maturity (Sylvester *et al.*, 1990). During each of these stages the cells exhibit unique compositions of expressed genes, reflecting the demands of that developmental stage (Facette *et al.*, 2013, Sekhon *et al.*, 2011). Thus, the transitions in this developmental progression must include regulatory mechanisms that control the change from one stage-specific gene expression program to another. Unlike the linear transition observed during leaf development, the cyclical response of circadian rhythm genes represents a repeat set of transitions between multiple states, tied to the day/night cycle. Responses to external stressors usually represent a rapid and acute transition in the state of a cell or organism, and are characterized by a return to a base state once the stressor has been resolved. The mechanisms that control the cellular responses to these different transitions vary and may include transcriptional, post-transcriptional, translational and/or post-translational regulation. For example, a proteomic profiling study of maze leaf development suggests the importance of post-translational regulation in transitions between stages (Facette *et al.*, 2013). The central hypothesis of this work is that the initial steps of the transition from mature pollen to the progamic phase in maize pollen are under the control of mechanisms regulating protein abundance directly, for example translation and protein degradation, and not primarily through transcriptomic means.

Regulation of developmental transitions

Given that developmental biology focuses on change in an organism over time, understanding the mechanisms orchestrating transitions between different developmental states is of great interest. Recent research has largely been focused on

the changes to gene expression, specifically changes to the transcriptome and transcription factors. However, while regulation of transcription has been shown to be important in many transitions (Qin *et al.*, 2009; Wei *et al.*, 2010; Sekhon *et al.*, 2011; Digel *et al.*, 2015; Xiao *et al.*, 2011; Chen *et al.*, 2014), it is has also been observed that, mainly due to varying translation rates (Piques *et al.*, 2009), transcript levels do not always correlate with protein abundance (Gygi *et al.*, 1999; Greenbaum *et al.*, 2003; Schwanhäusser *et al.*, 2011; Walley *et al.*, 2016). Therefore, to develop a clearer model of a developmental transition and the mechanisms underlying that transition, proteomic data must be analyzed in addition to transcriptomic data.

Evidence suggests that in cases where a rapid response is required, regulation occurs through more direct control of protein abundance and utilization of stored RNA and proteins rather than *de novo* transcription (Dure and Waters, 1965; Raff *et al.*, 1972; Grosfeld and Littauer, 1975; Muthukrishnan *et al.*, 1975; Amaldi *et al.*, 1977; Capco and Jeffer, 1979; Davidson, 1986; Richter and Smith, 1984; Hughes and Galau, 1989, 1991; Rosenthal *et al.*, 1993; Curtis *et al.*, 1995; Hart and Wolniak, 1998; Nakabayashi *et al.*, 2005; Malatesta *et al.*, 2009; Boothby *et al.*, 2013). One mechanism of posttranscriptional regulation observed in many eukaryotes is the retention of introns to prevent the premature translation of specific transcripts. During rapid developmental transitions these stored transcripts are then processed and translated (Dure and Waters, 1965; Muthukrishnan *et al.*, 1975; Amaldi *et al.*, 1977; Nakabayashi *et al.*, 2005). For example, this mechanism has been described in the fern *Marsilea vestita* (Boothby *et al.*, 2013) as controlling spermatogenesis of the male gametophyte. Another regulatory mechanism observed in plants during a transition is the rapid shift of translation of mRNAs from single ribosomes to polysomes (Linskens *et al.*, 1970; Tupy, 1977; Hoekstra and Bruinsma, 1978, 1979; Piques *et al.*, 2009; Juntawong *et al.*, 2013). Polysomes are composed of multiple ribosomes all attached to a single mRNA strand and all simultaneously translating the protein product (Staehelin *et al.*, 1963; Warner *et al.*, 1963; Rich *et al.*, 1963). This permits a swift increase in abundance of specific proteins (Beilharz and Preiss, 2004; Brockmann *et al.*, 2007). The rapid accumulation of mRNA into polysomes has also

been observed in the moss *Tortula ruralis* in response to rehydration and is characterized by the translation of a set of peptides unique to the transition from desiccation to rehydration (Scott *et al.*, 1994; Oliver *et al.*, 2004).

In addition to polysomes, mRNA has also been demonstrated to accumulate in to mRNA-protein complexes (mRNPs) or particles (Pramanik *et al.*, 1992; Honys *et al.*, 2009). Often these particles also include components of translational machinery (Jensen, 2001; Carson and Barbarese, 2005), leading to precise control of storage and expression (Honys *et al.*, 2009). Stored mRNPs have been implicated in the development of transcriptionally quiescent tissues and in directing polarized development in some cells (Honys *et al.*, 2009). Some of the initial evidence of developmental regulation at the level of protein synthesis occurred in studies of sea urchin eggs. These studies found nonnucleated eggs could be stimulated to form blastulas (Harvey, 1936, 1940) and that this formation was dependent on translation but not *de novo* transcription (Gross and Cousineau, 1963, 1964). In plants, cell-free translational and northern blot analysis of early somatic development of *Medicago sativa* (alfalfa) found accumulation of specific storage protein transcripts in mRNPs during early stages of embryogenesis that were then transcribed during later cotyledon development (Pramanik *et al.*, 1992). In tobacco, mRNPs have been observed to form in the immature male gametophyte, and are held inactive until the beginning of the progamic phase, when, the previously ‘silent’ transcripts are heavily translated (Honys *et al.*, 2009).

Elucidating the molecular mechanism regulating the transition from mature to germinated pollen is fundamental to the understanding of fertility and for the development of future methods for manipulating fertility, such as limiting potential cross pollination between GMO and non-GMO crops. As maize is an important food crop worldwide, understanding this phase in reproduction could have positive impacts in food production, both in maize and in other grasses (such as wheat or rice) for which maize can be used as a research model. Recent work with maize (*Zea mays*) pollen suggests translational control rather than transcriptional, as a possible

mechanism coordinating the transition from mature to germinated pollen in maize (Figure 1.1) (Fowler, Vejlupkova, Cooper, Watrud, unpublished). Maize pollen is an excellent model for the study of plant sexual reproduction, which starts with meiosis and ends with fertilization, as it is produced in large, developmentally synchronized quantities and can be harvested and evaluated easily. Additionally it germinates relatively rapidly *in vitro*: pollen tubes are seen to develop after only 5 minutes (Heslop-Harrison, 1979a) in maize as compared to 45 minutes in *Arabidopsis* (Wang *et al.*, 2008).

The release of the maize genome sequence (Schnable *et al.*, 2009) has spurred the development of several resources for the study of maize and provided a reference for the evaluation of RNA-seq data. Previous studies have used RNA-seq data to characterize mature pollen in maize (Davidson *et al.*, 2011; Chettoor *et al.*, 2014) as well as a number of other plant species (Li *et al.*, 2016; Shen *et al.*, 2011; Honys and Twell, 2004; Grennan, 2007; Wang *et al.*, 2008; Hafidh *et al.*, 2012). Interestingly, transcriptomic analysis via microarray comparing mature and *in vitro* germinated maize pollen found no significant differences in transcript abundance between the two stages (Fowler, Vejlupkova, Cooper, Watrud, unpublished). Moreover, application of the drug actinomycin D, which blocks *de novo* transcription, only affects pollen tube germination at the highest concentrations of the drug (Figure 1.1) where it appears to induce non-specific rupture of the pollen grain (Z. Vejlupkova, J. Fowler, unpublished). Together, these imply that the transition from mature to germinated pollen in maize is not controlled primarily through *de novo* transcription, but is orchestrated through more direct control of protein levels and/or activity.

General characteristics of different categories of pollen and factors impacting germination

Several different characteristics may be used to categorize pollen. However, there does not appear to be a single feature that can be used to fully predict the activity within a pollen grain at germination. At dehiscence, pollen may be bi- or trinucleate depending on if it has undergone the second pollen mitotic event. Approximately

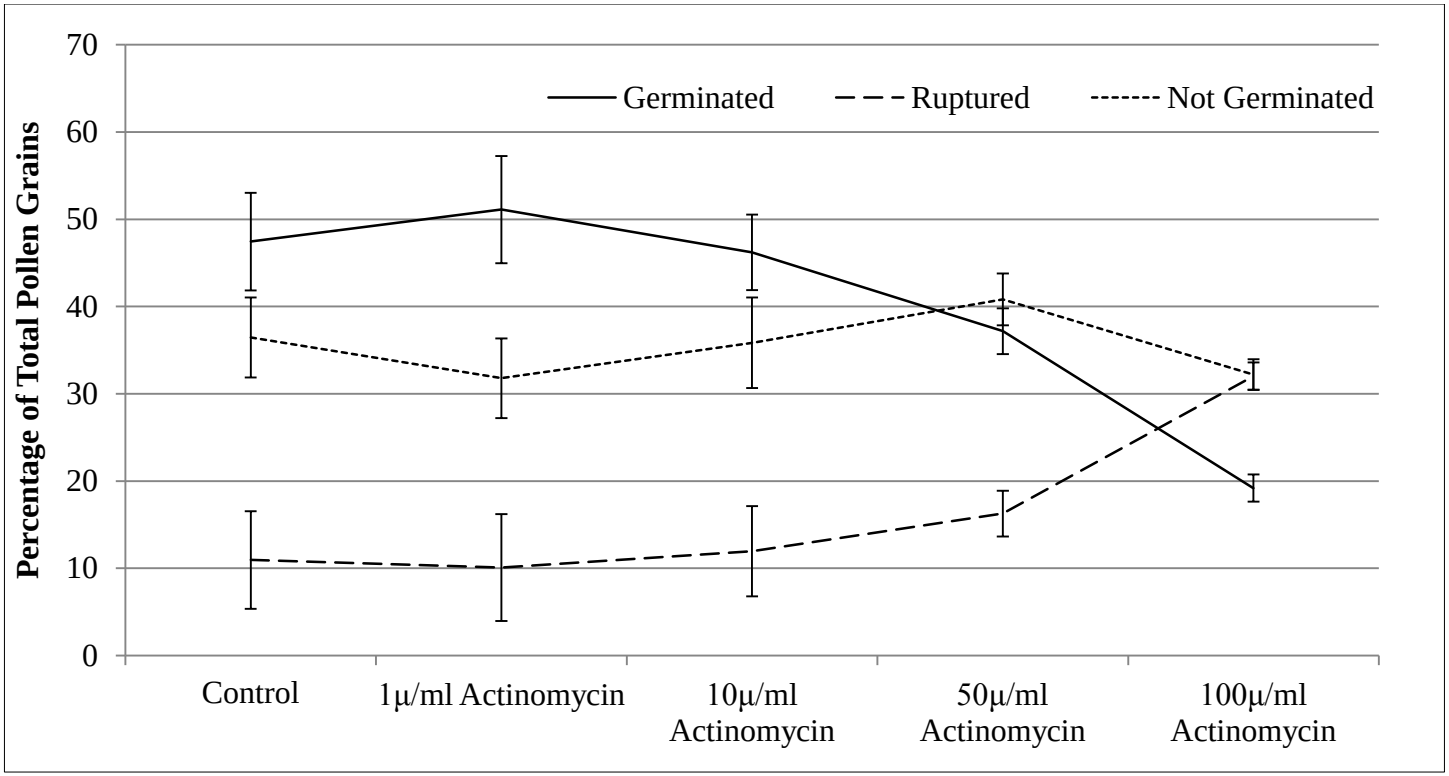


Figure 1. 0.1: Pollen tube germination in maize is insensitive to all but the highest concentrations of actinomycin D. Reduced germination at 50 and 100 µg/ml is associated with increased rupture of the pollen grain, but not an increase in the percentage of non-germinated pollen grains. This rupturing appears to be a non-specific effect, as an inactive analog, 7-actinomycin D, also significantly increases rupture at the same concentrations (data not shown). Bars represent 95% confidence intervals, n= 3-7. (Data from Z. Vejlupkova, J. Fowler, unpublished.)

30% of species are trinucleate at pollen shed (Williams *et al.*, 2014) and they are found in both monocots and dicots (Brewbaker, 1967). Though it was originally thought that pollen solely evolved from a bi- to trinucleate state (Schürhoff, 1926; Schnarf, 1939; Rudenko, 1959), studies have shown that species can transition between either state, though the development of a trinuclear state appears more rapidly than the reverse (Brewbaker, 1967; Williams *et al.*, 2014).

Additionally, the level of water content at pollen shed may be used to define pollen. Grains have been classified as 'partially hydrated' if their water content is above 30%, or 'partially dehydrated' if it is below that level (Nepi *et al.* 2001; Franchi *et al.*, 2002). While the majority of Angiosperms and Gymnosperms have partially dehydrated pollen, partially hydrated species are found in both groups and even within the same genus as partially dehydrated species (Pacini *et al.* 1999). Nucleate state does not appear to highly correlate with hydration state, as bi- and trinucleate species are found in each hydration category, though there is a trend towards partially hydrated pollen developing trinucleate pollen (Brewbaker, 1967; Williams *et al.*, 2014). There does not appear to be a single evolutionary trend towards the development of either hydration strategy, though there is a slight correlation between the development of partially hydrated pollen in environments with high relative humidity or in cases of high competition between pollen grains for fertilization (Nepi and Pacini 1993, 1999; Pandolfi and Pacini 1995; Franchi *et al.* 1996; Mulcahy *et al.* 1996; Speranza *et al.* 1997; Nepi *et al.* 2001). Common, though not ubiquitous, features of partially hydrated pollen species are a spherical form without furrows, rapid emergence of the pollen tube (i.e. germination) and a shorter window of viability after dehiscence (Lisci *et al.* 1994; Nepi and Pacini 1993, 1999; Nepi *et al.* 2001; Pacini 1990, 1996; Pacini *et al.* 1997; Speranza *et al.* 1997). Partially dehydrated pollen are generally smaller, with a higher sucrose content, and increased longevity and tolerance of low humidity (Franchi *et al.*, 2002).

Other characteristics used to categorize pollen during germination (Williams, 2012) include the number of apertures or pores (Dajoz *et al.* 1991; Furness and Rudall

2004), the thickness of the pollen wall (Heslop-Harrison 1979b; Nepi *et al.* 2001), the starch or oil content of the grain (Baker and Baker 1979; Franchi *et al.* 1996), the level of mitochondria development at dehiscence (Hoekstra and Bruinsma 1978, 1979; Hoekstra 1979; Rounds *et al.* 2011) and metabolic rate (Hoekstra and Bruinsma 1975; Hoekstra 1979). Generally, germination is observed to occur faster in pollen with multiple pores, thin walls, starchy grains, mature mitochondria, high metabolic rates, or are partially hydrated or trinucleate (Williams, 2012). These characteristics are not universally predictive of rapid germination. For example, maize under goes rapid germination, consistent with the observations above maize pollen is trinucleate, partially hydrated, starchy and processes a thin wall; however, it is also contains only a single pore.

A study investigating the role protein synthesis plays during germination in bi- and trinucleate pollen found that dependence on protein synthesis appeared to correlate with metabolic development rather than the nucleate state (Hoekstra and Bruinsma, 1979). Plants in which mitochondria have fully formed at dehiscence were more likely to have undergone large amounts of protein synthesis prior to pollen shed and were not reliant on translation for pollen tube germination. These developmentally advanced plants were characterized by rapid respiration, large amounts of polysomes or a lack of ribosomal content and a relatively short lag period prior to germination. Polysomes or high levels of ribosomal content in trinucleate pollen were not observed in their study, however trinucleate maize pollen was known to possess high levels of polysomes. Hoekstra and Bruinsma hypothesized that this was in response to the long style of maize (located within the silk), which may require the pollen to undergo protein synthesis in order to traverse the length of the silk.

Abiotic stress has been shown to have varying effects on germination rates between different species. In maize, high temperatures have been shown to decrease the viability of mature pollen and lead to reduced pollen tube growth *in vitro* (Lyakh *et al.*, 1991). However, in peach an increase in temperature was correlated with an increase in pollen germination rate and speed of tube growth through the style

(Hedhly *et al.*, 2005). The molecular response to heat stress is not fully characterized in pollen, heat shock proteins have been found to be expressed in some species (Volkov *et al.*, 2005) but not all (Mascarenhas and Altschuler, 1983; Mascarenhas and Crone, 1996). Water loss due to low humidity has also been shown to affect pollen germination and viability in plants such as maize and *Cucurbita* (Barnabas, 1985; Franchi *et al.*, 2002). This response is most often observed in partially hydrated pollen, which generally do not contain mechanisms for controlling water loss, rather than in partially dehydrated pollen where water loss is under regulation (Lisci *et al.* 1994; Nepi and Pacini 1993, 1999; Nepi *et al.* 2001; Pacini 1990, 1996; Pacini *et al.* 1997; Speranza *et al.* 1997).

Effects of inhibition of de novo transcription, de novo translation and protein degradation on pollen tube germination

The effect of the transcriptional inhibitor actinomycin D on the germination of pollen is varied across species (Table 1.1). In *Arabidopsis* it has been shown to inhibit pollen germination even at low concentration (0.001 μg/ml) (Honys and Twell, 2004; Wang *et al.*, 2008). This is in contrast to maize (Z. Vejlupkova, J. Fowler, unpublished.) where germination is not inhibited except at higher concentrations (Figure 1.1), lacebark pine (Hao *et al.*, 2005) where there is only a slight inhibition, and petunia (Linskens *et al.*, 1971) where no inhibition of germination rates is observed. Additionally, biochemical analysis has shown that the tRNA, ribosomes and mRNA required for germination are synthesized in many species during the maturation of the pollen grain and are stored until the germination process requires translation (Steffensen, 1966; Mascarenhas and Bell, 1970; Peddada and Mascarenhas, 1975; Mascarenhas, 1975; Mascarenhas, 1989). These results support the hypothesis that transcription is not a requirement during the initiation pollen tube germination in maize. As maize pollen is known to germinate more rapidly than Arabidopsis pollen (Heslop-Harrison, 1979a; Wang *et al.*, 2008), the differences in their response to transcriptional inhibition may be a reflection of this as it has been observed that rapidly germinating pollen correlate with a swift initiation of protein synthesis (Hoekstra and Bruinsma, 1979). It is interesting to note that in many species (for

Table 1. 1: Summary of the results from studies investigating the effects of actinomycin D, cycloheximide and MG-132 on pollen tube germination and length.

		Affects on:		
Drug	**Species**	**Germination**	**Pollen Tube length**	**Reference**
Actinomycin D Inhibits transcription	Maize (*Zea mays*)	Slight inhibition at high concentrations		Z. Vejlupkova, J. Fowler, unpublished, Figure 1.1
	Arabidopsis (*Arabidopsis thaliana*)	Inhibition at low concentrations		Honys and Twell, 2004
	Petunia (*Petunia hybrida*)	No effect	Reduces growth	Linskens *et al.*, 1971
	Lacebark pine (*Pinus bungeana*)	Slight inhibition	Significant reduction	Hao *et al.*, 2005
	Tobacco (*Nicotiana tabacum*)	No effect	Reduces growth	Capkova *et al.*, 1983
	Confederate spiderwort (*Tradescantia paludosa*)	Delayed inhibition	Delayed reduction of length	Mascarenhas, 1965, 1966
	Crinum lily (*Crinum asiaticum*)		No effect until higher concentrations	Iwanami, 1968
	Common Climbing Aloe (*Aloe ciliaris*)		No effect	Iwanami 1968
	Snapdragon (*Antirrhinum majus* L.)	No effect	Reduces growth	Mascarenhas, 1975
	Amaryllis (*Amaryllis belladonna* L.)	No effect	Reduces growth	Mascarenhas, 1975

Table 1.1: Continued

		Affects on:		
Drug	**Species**	**Germination**	**Pollen Tube length**	**Reference**
Cycloheximide Inhibits translation	Arabidopsis (*Arabidopsis thaliana*)	Inhibits germination		Honys and Twell, 2004
	Lacebark pine (*Pinus bungeana*)	Inhibits germination	Reduces growth and causes abnormal morphology	Hao *et al.*, 2005
	Confederate spiderwort (*Tradescantia paludosa*)	No effect	Reduces growth	Mascarenhas, 1971, 1978
	Impatiens (*Impatiens balsamina*)	No effect	No effect	Shivanna*et al.*, 1974
	Lily (*Lilium longiflorum*)	Inhibits germination		Franke *et al.*, 1972
	Chia (*Salvia hispanica*)	Inhibits germination		Franke *et al.*, 1972
	Broadleaf Cattail (*Typha latifolia*)	Inhibits germination		Hoekstra and Bruinsma, 1979
	Virginia spiderwort (*Tradescantia virginiana*)		Delayed reduction of length	Picton and Steer, 1983
	Snapdragon (*Antirrhinum majus* L.)	Inhibits germination		Whipple and Mascarenhas, 1975
	Amaryllis (*Amaryllis belladonna* L.)	Inhibits germination		Whipple and Mascarenhas, 1975
MG-132 Inhibits protein degradation	Wilson spruce (*Picea wilsonii*)		Reduces growth	Sheng *et al.*, 2006
	Kiwi (*Actinidia deliciosa*)	Inhibits germination	Reduces growth	Speranza *et al.*, 2001

example Petunia, Tobacco, Snapdragon and Amaryllis) where there is no inhibition to the rates of germination in response to transcription inhibition, they do experience a delayed response to the actinomycin D and produce shorter pollen tubes (Linskens *et al.*, 1971; Mascarenhas, 1975; Capkova *et al.*, 1983)(Table 1.1). This suggests that while *de novo* transcription is not required for germination *per se*, it appears to be needed to maintain rapid pollen tube growth.

Regulation of the transition of mature pollen to the progamic phase through direct control of protein levels is not a new or recent hypothesis (Capkova *et al*., 1988; Franklin-Tong, 1999). The role protein synthesis may play in this transition, however, appears to vary across plant species (Mascarenhas, 1975, 1993). To investigate the possible role post-transcriptional control plays in the transition to the progamic phase, previous studies have analyzed the effects of translational or proteasome inhibitors on pollen germination rates. Exposure to cycloheximide, a translation elongation inhibitor, has been shown to decrease pollen tube germination rates in several plant species across a wide evolutionary range, such as *Arabidopsis*, lacebark pine and lily (Franke *et al.*, 1972; Honys and Twell, 2004; Hao *et al.*, 2005; Table 1.1 for full list). In *Tradescantia paludosa* (Mascarenhas and Goraluick, 1971) and *Impatiens balsamina* (Shivanna *et al.*, 1974), however, no effect on germination was observed after exposure to cycloheximide except for a reduction in pollen tube length in *Tradescantia paludosa*. The role of protein synthesis during germination does not appear to correlate with any specific type of pollen. It has been observed play a part in germination of species with bi- or trinucleate pollen, as well as species with partially hydrated or partially dehydrated pollen.

Although less well characterized than protein synthesis, there have been a few investigations of the role of protein degradation in pollen germination. In eukaryotic cells, the majority of protein degradation occurs in the 26S proteasome, which is comprised of two subunits: the 19S and 20S (Rock *et al.*, 1994; Craiu *et al.*, 1997; Sorokin *et al.*, 2009). Selection and targeting of proteins into the 20S core for degradation is reliant on polyubiquitin labeling of substrates and regulation by the

19S subunit (Glickman *et al.*, 1998; Elsasser *et al.*, 2004; Sorokin *et al.*, 2005). Thus, this process is designated 'ubiquitin-dependent degradation of proteins' (Sorokin *et al.*, 2009). The drug MG-132 acts as a transition-state analog (Lowe *et al.*, 1995) and inhibits protein degradation by the proteasome (Rock *et al.*, 1994; Tsubuki *et al.*, 1996). Addition of MG-132 to germination media has been shown to inhibit pollen tube growth and cause morphological abnormalities in Wilson spruce (*Picea wilsonii*) (Sheng *et al.*, 2006) and reduce pollen tube emergence and growth in kiwifruit (*Actinidia deliciosa*) (Speranza *et al.*, 2001). The presence of high levels of free and conjugated ubiquitin in the mature pollen of some maize inbreds suggests a possible post-transcriptional regulatory mechanism that utilizes the ubiquitin and proteasome pathway (Muschiette *et al.*, 1994; Kulikauskas *et al.*, 1995). These studies are thus consistent with the hypothesis that the transition to germination in maize pollen is controlled, at least partially, through a mechanism regulating protein levels directly rather than through transcription.

The progamic phase in Zea mays pollen

At maturity maize pollen is trinucleate, the generative cell already having undergone the second pollen mitosis event creating two sperm cells alongside the single vegetative cell (Bedinger and Fowler, 2009) (Figure 1.2A and 1.2B). After dehiscence, pollen grains are shed in a partially hydrated and largely quiescent state. The male gametophytes land on silks (female sporophyte), where the pollen tube rapidly germinates through a pore in the pollen coat following a regulated process of rehydration (Lush *et al.*, 1998) (Figure 1.2C). The pollen tube is an extension of the vegetative cell and extends by directed tip growth. As it lengthens, the sperm cells and vegetative nucleus move into and through the pollen tube. This is the start of the progamic phase of pollen.

Unlike many other plants were the stigma is located at the apex of the style, the maize silk is unusual in that its entire length functions as the stigma, and this stigmatic surface is present around the entire style, which contains the transmitting tracts. Following the initial germination of the pollen grain, the infiltration and growth of the

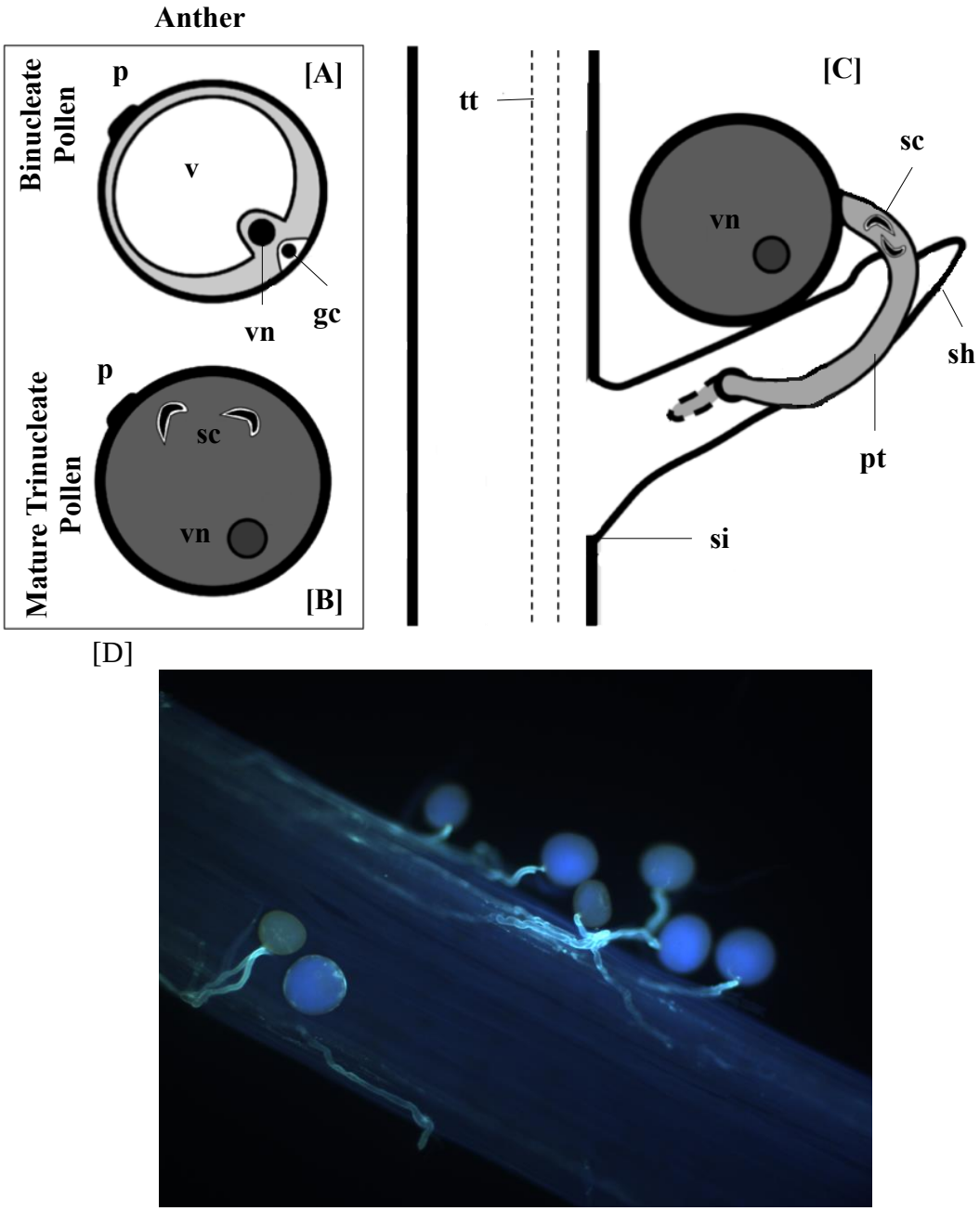


Figure 1. 0.2: Last steps of male gametophyte development and transition to the progamic phase.

[A] Binucleate gametophyte after the first round of pollen mitosis, [B] Trinucleate mature pollen after the second round of pollen mitosis, [C] Germination of the pollen tube on the silk, [D] Aniline blue stained pollinated silk where the pollen tubes have grown intro the silk and entered the transmitting tract. Labels: (p) pollen pore; (vn) vegetative nucleus; (gc) generative cell; (sc) sperm cells; (tt) transmitting tract; (sh) silk hair; (pt) pollen tube; (si) silk. Modified from Bedinger and Fowler, 2009.

pollen tube through the stigma into the transmitting tract is the next important step during pollination (Jansen, 2001; Bedinger and Fowler, 2009) (Figure 1.2D), involving interaction between the male gametophyte and the female sporophyte. Directed growth and competition of the pollen tubes characterize this stage of the progamic phase. During growth though the silk, a callose plug is periodically produced to separate the actively growing tip containing the cytoplasm, sperm cells and vegetative nucleus from the proximal end of the pollen tube (Lord and Russell, 2002).

Large-scale analyses of pollen and the stigma during pollination support the roles of directed growth and metabolism during this phase

During the past decade there has been progress in characterizing the changes to the transcriptome of the pollen tube and the stigma during pollination. Though general shared functional groups, such as central metabolism and directed cell growth, have been found across species, the fundamental molecular components regulating pollen-pistil interactions are still unknown (Hiscock *et al.*, 2008; Qin *et al.*, 2009; Li *et al.*, 2016). This may be due to the tendency of genes regulating reproduction to diversify and evolve more rapidly than those controlling more centralized cellular functions (Swanson and Vacquier, 2002), making proteins involved in male-female cross-talk recalcitrant to identification via homology. In closely related species, sequences for genes that function during sexual reproduction exhibit higher rates of divergence than those genes expressed in non-reproductive tissues (Singh and Kulathinal, 2000; Vacquier, 1998). This may be the product of the high level of competition for fertilization between male gametes or the interaction of the male gametophyte with the female sporophyte and gametophyte defining species boundaries. These situations would be anticipated to exhibit a high level of diversity as these processes have been demonstrated to be under rapid adaptive evolution and positive selection (Swanson *et al.*, 2004). Thus, ‘omics-scale studies have been used as a method to help define these interactions, by identifying genes and proteins specific to this developmental stage.

Recent studies have investigated changes to gene expression in the transcriptome of pollinated stigmas; however, the contribution of RNA from pollen in the analyzed transcripts is not always clear (Yue *et al.*, 2014; Li *et al.*, 2016). An *Arabidopsis* study employing a semi-*in vivo* method found the transcriptome of pollen grown through stigmas to be distinct from pollen germinated on media alone (Qin *et al.*, 2009). A dual transcriptomic and proteomic study of pollinated rice stigmas found evidence of only 15 out of 952 genes previously demonstrated to have enriched expression in pollen and the pollen tube (Li *et al.*, 2016). The authors concluded that the majority of the RNA contribution from the pollen may be too diluted by stigma transcripts to be detected by RNA-seq. A similar study in maize found significant changes to the transcriptome of silks following pollination (Xu *et al.*, 2013). While they uncovered evidence increased gene expression related to processes such as microtubule based movement and transport, the study was unable to determine the contribution of the germinating pollen to the expression observed. An investigation of the transcriptomes of *Arabidopsis* and maize pollinated stigmas found evidence of multiple metabolic pathways, such as glycolysis, activated upon pollination (Yue *et al.*, 2014). As the maize pollen grain contains only enough resources for 2 cm of growth through the silk (Heslop-Harrison *et al.*, 1984), it is hypothesized that, unlike the transition from mature to germinated pollen, transcription plays an active role in the transition from germination initiation to growth through the silk.

A semi-*in vivo* proteomics analysis of the secretome of growing tobacco pollen tubes found a number of novel proteins, the majority of which were predicted to be secreted by unconventional mechanisms, given that they lacked the N-terminal signal peptide characteristic of conventional secretion (Hafidh *et al.*, 2016). These proteins were found to be overrepresented in the GO categories previously correlated with growth of the pollen tube and fertilization, such as cellulose activity, ATP binding and defense response (Takeuchi *et al.*, 2011; Dresselhaus *et al.*, 2013).

Interaction of the pollen tube and the female sporophyte during the progamic phase

Characterization of the progamic phase in maize has largely focused on cross-incompatibility barriers and the contributions of the female sporophyte and gametophyte in supporting and signaling the male gametophyte. The growth of the pollen tube into the silk and guidance to the transmitting tract represents a possible hybridization barrier (Heslop-Harrison *et al.*, 1982) as it is hypothesized that the transmitting tract releases species specific attractants to guide compatible pollen tubes to the transmitting tract. Work with *Tripsacum dactyloides* (eastern gamagrass) pollen (Lausser *et al.*, 2010) found that, unlike maize pollen, a large percentage of the pollen tubes were directed to the stigma cortex of the maize silk rather than the transmitting tract supporting this hypothesis. Identifying any secreted attractants for the maize pollen tube has proven difficult (Lausser and Dresselhaus, 2010), though two have been found in lily (Kim *et al.*, 2003; Park and Lord, 2003). While the transmitting tract may provide a barrier to non-maize pollen tubes, there are a few cross-incompatibility systems known to exist between maize lines and between its ancestor teosinte (Demerec, 1929; Nelson, 1952; Lausser *et al.*, 2010; Evans and Kermicle, 2001; Kermicle and Evans, 2010; Lu *et al.*, 2014). This includes the *Ga1* system, which prevents pollen tube growth in incompatible crosses (Lausser *et al.*, 2010), and the *Teosinte crossing barrier1* (*Tcb1*) and *Gametophytic factor2* (*Ga2*) systems, which inhibit pollination between *Zea mays* pollen and the silks of some populations of teosinte (*Z. mays* ssp. *mexicana*) (Evans and Kermicle, 2001; Kermicle and Evans, 2010). The evidence from these studies indicates that the genes involved are good candidates for speciation genes between maize and teosinte.

The maize pollen grain contains only enough resources for about 2 cm of pollen tube growth through the silk (Heslop-Harrison *et al.*, 1984); however, as the silk may reach 20 cm or more, any growth beyond 2 cm is reliant on support from the female sporophyte. Activation of this growth support appears to be in response to pollen-generated flavonols (Pollack *et al.*, 1995), as pollen deficient in this phytochemical display slow growth through the silk unless rescued by exogenous application of

flavonols. Growth of the pollen tube through the transmitting tract appears to be mainly under mechanical control, as the tract physically guides the tube towards the female gametophyte rather than relying on long-range factors, like is found in *Arabidopsis* (Dresselhaus *et al.*, 2011). This hypothesis is supported by observation of some tubes growing towards the silk tip rather than towards the silk base and ovule. It is hypothesized that the growth from the transmitting tract to the micropyle is under sporophytic control (Lausser *et al.*, 2010) and there is at least one short-range pollen tube attractant factor expressed by the egg and synerid cells, ZmEA1 (*Zea mays Egg Apparatus 1)* (Marton *et al.*, 2005).

Reduced Recovery of Mutations (RRM) and Gametophyte Transmission Defect Assessment (GTDA)

Analysis of the proteomes of mature and germinated pollen is expected to uncover proteins putatively important functionally to pollen development and pollen tube germination. Validation of predictions from quantitative proteomic profiling, however, can be difficult (eg. western blotting) and time consuming (eg. cloning and creating loss-of-function mutants) to assess as several hundred candidate proteins are identified. The initial validation of predictions of functional importance to the male gametophyte can be indirectly tested through a variant of an analysis developed by the Maize Gametophyte Project (http://maizegametophyte.org/, Chettoor *et al.*, 2014); we are designating this analysis the Reduced Recovery of Mutations (RRM) test. This test compares genes known to be expressed in gametophyte tissue with the presence of mutations in those genes from large transposon-mutagenized populations of maize.

The RRM test makes use of the three large sequence-indexed transposon insertion populations available in maize: *UniformMu* (Settles *et al.*, 2007), *Mu-Illumina* (Williams-Carrier *et al.*, 2010) and *DsMutagenesis* (Volbrecht *et al.*, 2010). These populations utilize *Mutator* and *Activator/Dissociation* transposons to produce lines with new insertions. The locations of these insertions are then determined though flanking sequence tags. The RRM test is based on the prediction that in the three available insertion populations, genes important for gametophyte function will

contain fewer insertions in exons than those genes important for sporophyte function. This prediction is based on the differing selective pressure applied to genes important for gametophyte versus sporophyte function. As gametophytes are haploid, their genes are under increased selective pressure against insertions that abrogate function. In contrast, the presence of a wild-type allele in diploid sporophytic tissue prevents a loss-of-function insertion in a gene from expressing a full deleterious effect, and thus important sporophytic genes would be under less selective pressure against retaining the insertion in a population

The RRM test initially showed that genes whose expression was enriched preferentially in the transcriptome of maize mature pollen were associated with fewer mutations located in exons in both the *UniformMu* and *Mu-Illumina* populations than a comparable set of genes whose expression is enriched in the sporophytic (seedling) transcriptome (Chettoor *et al.*, 2014). However, this trend was not present in the *DsMutagenesis* population (Chettoor *et al.*, 2014). This disagreement reflects how the *Mu* and *Ds* lines are predominately propagated: the *UniformMu* and *Mu-Illumina* populations are propagated through both the male and the female (with the initial mutagenic events in *UniformMu* primarily generated through the male), whereas the *DsMutagenesis* population is exclusively propagated through the female. This means that the *Mu* populations are undergoing selection against insertions negatively affecting male gametophyte function whereas the *DsMutagenesis* population is not.

A more direct application of the principles acting in the RRM test focuses on reduced transmission of specific insertions through the male or the female gametophyte (Gametophytic Transmission Defect Assessment, GTDA). Lines from the three transposon insertion populations are obtained, containing insertions for specific predicted genes of interest and are outcrossed though the male and through the female to a tester homozygous for the wild type allele. Presence of the insertion in the progeny of these crosses is then scored to determine if there is evidence of reduced transmission to the offspring via the male or female gametophyte. In the GTDA, a mutation occurring in a gene integral to pollen function will impair the ability of that

grain to compete against wild type grains for fertilization of the female gametophyte. The result is reduced transmission of that mutation to the progeny of the affected male germ line. At the species level, this would be reflected in reduced recovery of mutations that negatively affect important male gametophyte genes. However, if the function of the gene is specific to the male gametophyte, there will be no similar reduction of transmission or recovery of such mutations through a cross where the insertion is present in female parent but not the male. Use of GTDA as a follow-up to the RRM analysis in the Chettoor *et al.* study found evidence of reduced transmission of *Ds* insertion mutations in four out of 17 genes that are highly expressed in either male or female gametophytes, with the transmission defect concordant with the gametophyte associated with high expression (Chettoor *et al.*, 2014). In contrast, only one out of 16 insertions tested in genes expressed highly in seedling tissue showed reduced transmission (and this insertion showed only a borderline significant effect).

Central Hypotheses:

The central hypothesis of this work is that the initial transition to the progamic phase in maize pollen is regulated through more direct control mechanisms of proteins abundance, such as translation and protein degradation, and not through transcriptomic means. Changes to the transcriptome do, however, play a role in later stages of the progamic phase as the pollen tube grows through the silk. While an initial characterization of mature maize pollen proteome has been published (Chao *et al.*, 2016), there has not been a quantitative study of changes to the proteome occurring with germination with multiple replicates. To investigate the mechanisms regulating pollen germination we have taken a proteomics approach and followed up with a male transmission defect test. Additionally we present a new approach using RNA-seq that takes advantage of the genetic variation present in maize to identify genes expressed in pollen tubes grown *in vivo* inside female sporophytic tissue.

Literature Cited

Amaldi PP, Felicetti L, Campioni N (1977) Flow of information RNA from cytoplasmic poly(A)-containing particles to polyribosomes in *Artemia salina* cysts at early stages of development. Dev. Biol. **59**: 49–61

Baker HG, Baker I (1979) Starch in angiosperm pollen grains and its evolutionary significance. American Journal of Botany **66**: 591 – 600

Barnabas B (1985) Effect of water loss on germination ability of Maize (*Zea mays* L.) pollen. Annals of Botany **55**: 201–204

Bedinger P, Fowler J (2009) The maize male gametophyte. In: Hake S, Bennetzen J (eds) Handbook of maize: its biology. Springer, New York, pp 57–77

Beilharz TH, Preiss T (2004) Translational profiling: the genome-wide measure of the nascent proteome. Brief Funct Genomic Proteomic **3**: 103–111

Boothby TC, Zipper RS, van der Weele CM, Wolniak SM (2013) Removal of Retained Introns Regulates Translation in the Rapidly Developing Gametophyte of Marsilea vestita. Developmental Cell **24**: 517–529

Brewbaker JL (1967) The Distribution and Phylogenetic Significance of Binucleate and Trinucleate Pollen Grains in Angiosperms. American Journal of Botany **54**: 1069–1083

Brockmann R, Beyer A, Heinisch JJ, Wilhelm T (2007) Post- transcriptional expression regulation: what determines translation rates? PLoS Comp Biol **3**: 0531–0539

Capco DG, Jeffery WR (1979) Origin and spatial distribution of maternal messenger RNA during oogenesis of an insect, *Oncopeltus fasciatus*. J. Cell Sci. **39**: 63–76

Čapková V, Hrabětová E, Tupý J, Říhová L (1983) Amino acid uptake and protein synthesis in cultured tobacco pollen. Biochem. Physiol. Pflanzen **178**: 511-520

Čapková V, Hrabětová E, Tupý J (1988) Protein synthesis in pollen tubes: preferential formation of new species independent of transcription. Sexual Plant Reproduction **1**: 150–155

Carson JH, Barbarese, E (2005) Systems analysis of RNA trafficking in neural cells. Biol. Cell **97** (1): 51–62

Chao Q, Gao Z-F, Wang Y-F, Li Z, Huang X-H, Wang Y-C, Mei Y-C, Zhao B-G, Li L, Jiang Y-B, et al (2016) The proteome and phosphoproteome of maize pollen uncovers fertility candidate proteins. Plant Mol Biol 1–18

Chen J, Zeng B, Zhang M, Xie S, Wang G, Hauck A, Lai J (2014) Dynamic Transcriptome Landscape of Maize Embryo and Endosperm Development. Plant

Physiology **166**: 252–264

Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MMS (2014) Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. Genome Biol **15**: 414

Craiu A, Gaczynska M, Akopian T, Gramm CF, Fenteany G, Goldberg AL, Rock KL (1997) Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. J. Biol. Chem. **272**: 13437-13445

Curtis D, Lehmann R, Zamore PD (1995) Translational regulation in development. Cell **81**: 171–178

Dajoz I, Till-Bottraud I, Gouyon PH (1991) Evolution of pollen morphology. Science **253**: 66–68

Davidson EH (1986) Gene Activity in Early Development, Third Edition (New York: Academic Press)

Davidson RM, Hansey CN, Gowda M, Childs KL, Lin H, Vaillancourt B, Sekhon RS, de Leon N, Kaeppler SM, Jiang N, et al (2011) Utility of RNA Sequencing for Analysis of Maize Reproductive Transcriptomes. The Plant Genome Journal **4**: 191

Demerec M (1929) Cross-sterility in maize. Zeitschrift fur Induktive- und Abstammungslehre **50**: 281–291

Digel B, Pankin A, Korff von M (2015) Global Transcriptome Profiling of Developing Leaf and Shoot Apices Reveals Distinct Genetic and Environmental Control of Floral Transition and Inflorescence Development in Barley. THE PLANT CELL ONLINE **27**: 2318–2334

Dresselhaus T, Lausser A, Marton ML (2011) Using maize as a model to study pollen tube growth and guidance, cross-incompatibility and sperm delivery in grasses. Annals of Botany **108**: 727–737

Dresselhaus T, Franklin-Tong N (2013) Male-Female Crosstalk during Pollen Germination, Tube Growth and Guidance, and Double Fertilization. Molecular Plant **6**: 1018–1036

Dure L, Waters L (1965) Long-Lived Messenger RNA: Evidence from Cotton Seed Germination. Science **147**: 410–412

Elsasser S, Chandler-Militello D, Muller B, Hanna J, Finley D (2004) Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. J. Biol. Chem. **279**: 26817-26822

Evans MMS, Kermicle JL (2001) Teosinte crossing barrier1, a locus governing hybridization of teosinte with maize. Theoretical and Applied Genetics **103**: 259–265

Facette MR, Shen Z, Bjornsdottir FR, Briggs SP, Smith LG (2013) Parallel Proteomic and Phosphoproteomic Analyses of Successive Stages of Maize Leaf Development. THE PLANT CELL ONLINE **25**: 2798–2812

Franchi GG, Bellani L, Nepi M, Pacini E (1996) Types of carbohydrate reserves in pollen: localization, systematic distribution and ecophysiological significance. Flora **191**: 143–159

Franchi GG, Nepi M, Dafni A, Pacini E (2002) Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. Plant Syst Evol **234**: 211–227

Franke WW, Herth W, Vanderwoude WJ, Morré DJ (1972) Tubular and filamentous structures in pollen tubes: Possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta **105**(4): 317-341

Franklin-Tong VE (1999) Signaling and the Modulation of Pollen Tube Growth. The Plant Cell **11**: 727–738

Furness CA, Rudall PJ (2004) Pollen aperture evolution—a crucial factor in eudicot success? Trends in Plant Science **9**: 154–158

Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA, Finley D (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94**: 615-623

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol **4**: 117

Grennan AK (2007) An Analysis of the Arabidopsis Pollen Transcriptome. Plant Physiology **145**: 3–4

Grosfeld H, Littauer UZ (1975) Cryptic form of mRNA in dormant *Artemia salina* cysts. Biochem. Biophys. Res. Commun. **67**: 176–181

Gross PR, Cousineau GH (1963) Effects of actinomycin D on macro- molecule synthesis and early development in sea urchin eggs. Biochem. Biophys. Res. Commun. **10**: 321–326

Gross PR, Cousineau GH (1964) Macromolecule Synthesis and the Influence of Actinomycin on Early Development. Exp. Cell Res. **33**: 368–395

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity

tags. Nature Biotechnology **17**: 994–999

Hafidh S, Breznenová K, Ruzicka P, Feciková J, Capkova V, Honys D (2012) Comprehensive analysis of tobacco pollen transcriptome unveils common pathways in polar cell expansion and underlying heterochronic shift during spermatogenesis. BMC Plant Biol **12**: 24

Hafidh S, Potěšil D, Fíla J, Capkova V, Zdráhal Z, Honys D (2016) Quantitative proteomics of the tobacco pollen tube secretome identifies novel pollen tube guidance proteins important for fertilization. Genome Biol 1–29

Hao H, Li Y, Hu Y, Lin J (2005) Inhibition of RNA and protein synthesis in pollen tube development of Pinus bungeana by actinomycin D and cycloheximide. New Phytologist **165**: 721–729

Harmer SL (2009) The Circadian System in Higher Plants. Annu Rev Plant Biol **60**: 357–377

Hart PE, Wolniak SM (1998) Spermiogenesis in Marsilea vestita: a temporal correlation between centrin expression and blepharoplast differentiation. Cell Motil. Cytoskeleton **41**: 39–48

Harvey EB (1936). Parthenogenetic merogony or cleavage, without nuclei in *Arbacia punctulata*. Biol. Bull. **71**: 101–121

Harvey EB (1940) A Comparison of the Development of Nucleate and Non-Nucleate Eggs of *Arbacia punctulata*. Biol. Bull. **79**: 166–187

Hedhly A, Hormaza JI, Herrero M (2005) The Effect of Temperature on Pollen Germination, Pollen Tube Growth, and Stigmatic Receptivity in Peach. **7**: 476–483

Heslop-Harrison J (1979a) Pollen–stigma interaction in grasses: a brief review. New Zealand Journal of Botany **17**: 537–546

Heslop-Harrison J (1979b) Pollen walls as adaptive systems. Annals of the Missouri Botanical Garden **66**: 813–829

Heslop-Harrison J (1982) Pollen-Stigma Interaction and Cross-Incompatibility in the Grasses. Science **215**: 1358–1364

Heslop-Harrison Y, Reger BJ, Heslop-Harrison J (1984) The pollen–stigma interaction in the grasses. 6. The stigma (silk) of *Zea mays* L. as host to the pollens of Sorghum bicolor (L.) Moench and Pennisetum americanum (L.) Leeke. Acta Botanica Neerlandica **33**: 205–227

Hiscock SJ, Allen AM (2008) Diverse cell signaling pathways regulate pollen-stigma interactions: the search for consensus. New Phytologist **179**: 286–317

Hoekstra FA (1979) Mitochondrial development and activity of bi- nucleate and

trinucleate pollen during germination in vitro. Planta **145**: 25–36

Hoekstra FA, Bruinsma J (1975) Respiration and vitality of binucleate and trinucleate pollen. Physiologia Plantarum **34**: 221 – 225

Hoekstra FA, Bruinsma J (1978) Reduced independence of the male gametophyte in angiosperm development. Ann. Bot. **42**: 759-762

Hoekstra FA, Bruinsma J (1979) Protein synthesis of binucleate and trinucleate pollen and its relationship to tube emergence and growth. Planta **146**: 559-566

Honys D, Reňák D, Feciková J, Jedelský PL, Nebesářová J, Dobrev P, Capkova V (2009) Cytoskeleton-Associated Large RNP Complexes in Tobacco Male Gametophyte (EPPs) Are Associated with Ribosomes and Are Involved in Protein Synthesis, Processing, and Localization. J Proteome Res **8**: 2015–2031

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5**: R85.1–R85.13

Hughes DW, Galau GA (1989) Temporally modular gene expression during cotyledon development. Genes Dev. **3**: 358–369

Hughes DW, Galau GA (1991) Developmental and environmental induction of Lea and Lea A mRNAs and the post abscission program during embryo culture. Plant Cell **3**: 605–618

Iwanami, Y (1968).Physiological researches of pollen. XVII. On the resistance of pollen to radial rays and chemical inhibitors. J. Yoko- hama City Univ. 175 (2-55, Biol. 24), 1-31

Jansen RP (2001) mRNA localization: message on the move. Nat. Rev. Mol. Cell Biol. **2** (4): 247–256

Juntawong P, Girke T, Bazin J, Bailey-Serres J (2013) Translational dynamics revealed by genome-wide profiling of ribosome footprints in Arabidopsis. PNAS 203–212

Kermicle JL, Evans MMS (2010) The Zea mays Sexual Compatibility Gene ga2: Naturally Occurring Alleles, Their Distribution, and Role in Reproductive Isolation. Journal of Heredity **101**: 737–749

Kim M, Ahn JW, Jin UH, Choi D, Paek KH, Pai HS (2003) Activation of the Programmed Cell Death Pathway by Inhibition of Proteasome Function in Plants. Journal of Biological Chemistry **278**: 19406–19415

Kulikauskas R, Hou A, Muschietti J, McCormick S (1995) Comparisons of diverse plant species reveal that only grasses show drastically reduced levels of ubiquitin monomer in mature pollen. Sex Plant Reprod **8**: 326-332

Lausser A, Kliwer I, Srilunchang K-O, Dresselhaus T (2010) Sporophytic control

of pollen tube growth and guidance in maize. Journal of Experimental Botany **61**: 673–682

Lausser A, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in grasses. Biochemical Society Transactions **38**: 631–634

Li M, Wang K, Li S, Yang P (2016) Exploration of rice pistil responses during early post-pollination through a combination proteomic and transcriptomic analysis. Journal of Proteomics **131**: 214–226

Linskens HF, Schrauwen JAM, Konings RNH (1970) Cell-free protein synthesis with polysomes from germinating Petunia pollen grains. Planta **90**: 153-152

Linskens HF, Van der Donk JAWM, Schrauwen J (1971) RNA Synthesis during Pollen Germination. Planta **97**: 290–298

Lisci M, Tanda C, Pacini E (1994) Pollination ecophysiology of *Mercurialis annua* L. (*Euphorbiaceae*), an anemophilous species flowering all year round. Ann Bot 74: 125–135

Lord EM and Russell SD (2002) The mechanisms of pollination and fertilization in plants. Annu Rev Cell Dev Biol **18**: 81-105

Lowe J, Stock D, Jap B, Zwickl P, Baumeister W, Huber R (1995) Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4A resolution. Science **268**: 533-539

Lu Y, Kermicle JL, Evans MMS (2014) Genetic and cellular analysis of cross-incompatibility in Zea mays. Plant Reproduction **27**(1): 19-29

Lush WM, Grieser F, Wolters-Arts M (1998) Directional Guidance of Nicotiana alata Pollen Tubes *in Vitro* and on the Stigma. Plant Physiology **118**: 733–741

Lyakh VA, Kravchenko AN, Soroka AI, Dryuchina EN (1991) Effects of high temperatures on mature pollen grains in wild and cultivated maize accessions. Euphytica **55**: 203–207

Malatesta M, Perdoni F, Battistelli S, Muller S, Zancanaro C (2009) The cell nuclei of skeletal muscle cells are transcriptionally active in hibernating edible dormice. BMC Cell Biol. **10**: 19

Marton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. Science **307**: 573 – 576

Mascarenhas JP (1975) The Biochemistry of Angiosperm Pollen Development. Botanical Review **41**: 259–314

Mascarenhas JP (1989) The Male Gametophyte of Flowering Plants. The Plant Cell **1**: 657–664

Mascarenhas JP (1993) Molecular Mechanisms of Pollen Tube Growth and

Differentiation. The Plant Cell **5**: 1303–1314

Mascarenhas JP, Altschuler M (1983) The response of pollen to high temperatures and its potential applications. In: Mulcahy DL, Ottoviano E (eds) Pollen: biology and implications for plant breeding. Elsevier, Amsterdam, pp 3–8

Mascarenhas JP and Bell E (1970) RNA synthesis during development of the male gametophyte of *Tradescantia*. Dev Bio **21**(4): 475-490

Mascarenhas JP, Crone DE (1996) Pollen and the heat shock response. Sexual Plant Reproduction **9**(6): 370-374

Mascarenhas JP and Goralnick RD (1971) Synthesis of small molecular weight RNA in the pollen tube of *Tradescantia paludosa*. Biochem. Biophys. Acta **240**: 56-61

McClung CR (2006) Plant Circadian Rhythms. The Plant Cell **18**: 792–803

Mulcahy DL, Sari-Gorla M, Bergamini Mulcahy G (1996) Pollen selection – past, present and future. Sex. Plant Reprod. **9**: 353–356

Muschietti J, Dircks L, Vancanneyt G, McCormick S (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

Muthukrishnan S, Filipowicz W, Sierra JM, Both GW, Shatkin AJ, Ochoa S (1975) mRNA methylation and protein synthesis in extracts from embryos of brine shrimp, *Artemia salina*. J. Biol. Chem. **250**: 9336–9341

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in Arabidopsis thaliana seed germination: epigenetic and genetic regulation of transcription in seed. Plant J. **41**: 697–709

Nelson OE (1952) Non-reciprocal cross-sterility in maize. Genetics **37**: 101–124

Nepi M, Pacini E (1993) Pollination, pollen viability and pistil receptivity in *Cucurbita pepo*. Ann Bot **72**: 527–536

Nepi M, Pacini E (1999) What could be the significance of polysiphony in *Lavatera arborea*? In: Clement C, Pacini E, Audran JC (eds.) Anther and pollen: from biology to biotechnology. Springer, Berlin, pp. 13–20

Nepi M, Franchi GG, Pacini E (2001) Pollen hydration status at dispersal: cytophysiological features and strategies. Protoplasma **216**: 171–180

Oliver MJ, Dowd SE, Zaragoza J, Mauget SA, Payton RR (2004) The rehydration transcriptome of the desiccation-tolerant bryophyte *Tortula ruralis*: transcript classification and analysis. BMC Genomics **5**: 1–19

Pacini E (1990) Harmomegathic characters of Pteridophyta spores and

Spermatophyta pollen. Plant Syst Evol [Suppl.] **5**: 53–69.

Pacini E (1996) Types and meaning of pollen carbohydrate reserves. Sex Plant Reprod **9**: 362–366

Pacini E, Franchi GG, Ripaccioli M (1999) Ripe pollen structure and histochemistry of some gymnosperms. Plant Syst Evol **217**: 81–99

Pacini E, Franchi GG, Lisci M, Nepi M (1997) Pollen viability related to type of pollination in six angiosperm species. Ann. Bot. **80**: 83–87

Pandolfi T, Pacini E (1995) The pollinium of Loroglossum hircinum (L.) C. Rich. (Orchidaceae) between pollination and pollen tube emission. Plant. Syst. Evol. **196**: 141–151

Park SY, Lord EM (2003) Expression studies of SCA in lily and confirmation of its role in pollen tube adhesion. Plant Molecular Biology **51**: 183 – 189

Peddada L, Mascarenhas JP (1975) 5S ribosomal RNA synthesis during pollen development. Develop Growth Diff **17**: 1-8

Picton JM and Steer MW (1983) Membrane recycling and the control of secretory activity in pollen tubes. J Cell Sci **63**: 303-310

Piques M, Schulze WX, Hohne MHO, Usadel BOR, Gibon Y, Rohwer J, Stitt M (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in Arabidopsis. Molecular Systems Biology **5**: 1–17

Pollak PE, Hansen K, Astwood JD, Taylor LP (1995) Conditional male-fertility in maize. Sexual Plant Reproduction **8**: 231–241

Pramanik SK, Krochko JE, Bewley JD (1992) Distribution of Cytosolic mRNAs Between Polysomal and Ribonucleoprotein Complex Fractions in Alalfa Embryos. Plant Physiology **99**: 1590–1596

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Raff RA, Colot HV, Selvig SE, Gross PR (1972) Oogenetic origin of messenger RNA for embryonic synthesis of microtubule proteins. Nature **235**: 211–214

Rich A, Warner JR, Goodman HM (1963) The structure and function of polyribosomes. Cold Spring Harbor Symp. Quant Biol **28**:269–285

Richter JD, Smith LD (1984) Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. Nature **309**: 378–380

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cell

proteins and the generation of peptides presented on MHC class I molecules. Cell **78**(5): 761-71

Rosenthal ET, Bahr M, Wilt FH (1993) Regulation of maternal messenger RNA translation during oogenesis and embryogenesis in *Urechis caupo*. Dev. Biol. **155**: 297–306

Rounds CM, Winship LJ, Hepler PK (2011) Pollen tube energetics: respiration, fermentation and the race to the ovule. AoB PLANTS doi:10.1093/aobpla/plr019

Rudenko FE (1959) The significance of the male gametophyte for the taxonomy of Angiospermae. Botanicheskii Zhurnal **44**: 1467–1475

Schnable PS, Ware DH, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton LA, Graves TA, et al (2009) The B73 Maize Genome: Complexity, Diversity, and Dynamics. Science **326**: 1112–1115

Schnarf K (1939) Variation im bau des pollenkornes der angiospermen. T abulae Biologicae **17**: 72–89

Schürhoff PN (1926) Die zytologie der blütenpflanzen. Enke, Stuttgart, Germany

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature **473**: 337–342

Scott II HB, Oliver MJ (1994) Accumulation and polysomal recruitment of transcripts in response to desiccation and rehydration of the moss *Tortula ruralis*. J. Exp. Bot. **45**: 577–583

Sekhon RS, Lin H, Childs KL, Hansey CN, Buell CR, de Leon N, Kaeppler SM (2011) Genome-wide atlas of transcription during maize development. The Plant Journal **66**: 553–563

Settles AM, Holding DR, Tan B, Latshaw SP, Liu J, Suzuki M, Li L, O'Brien BA, Fajardo DS, Wroclawska E, et al (2007) Sequence-indexed mutations in maize using the UniformMu transposon-tagging population. BMC Genomics **8**: 116

Shen Y, Venu RC, Nobuta K, Wu X, Notibala V, Demirci C, Meyers BC, Wang GL, Ji G, Li QQ (2011) Transcriptome dynamics through alternative polyadenylation in developmental and environmental responses in plants revealed by deep sequencing. Genome Research **21**: 1478–1486

Sheng X, Hu Z, Lu H, Wang X, Baluska F, Samaj J, Lin J (2006) Roles of the Ubiquitin/Proteasome Pathway in Pollen Tube Growth with Emphasis on MG132-Induced Alterations in Ultrastructure, Cytoskeleton, and Cell Wall Components. Plant Physiology **141**: 1578–1590

Shivanna KR, Jaiswal VS, Mohan Ram HY (1974) Inhibition of gamete formation

by cycloheximide in pollen tubes of Impatiens balsamina. Planta **117**(2): 173-177

Singh RS, Kulathinal R J (2000) Sex gene pool evolution and speciation: a new paradigm. Genes Genet. Syst. **75**: 119–130

Sorokin AV, Selyutina AA, Skabkin MA, Guryanov SG, Nazimov IV, Richard C, Th'ng J, Yau J, Sorensen PH, Ovchinnikov LP, Evdokimova V (2005) Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response. EMBO J. **24**: 3602-3612

Sorokin AV, Kim ER, Ovchinnikov LP (2010) Proteasome system of protein degradation and processing. Biochemistry Moscow **74**: 1411–1442

Speranza A, Calzoni GL, Pacini E (1997) Occurrence of mono- or disaccharides and polysaccharide reserves in mature pollen grains. Sex Plant Reprod **10**: 110–115

Speranza A, Scoccianti V, Crinelli R, Calzoni GL, Magnani M (2001) Inhibition of Proteasome Activity Strongly Affects Kiwifruit Pollen Germination. Involvement of the Ubiquitin/Proteasome Pathway as a Major Regulator. Plant Physiology **126**: 1150–1161

Staehelin T, Brinton CC, Wettstein FO, Noll H (1963) Structure and Function of E. coli Ergosomes. Nature **199**: 865–870

Steffensen DM (1966) Synthesis of ribosomal RNA during growth and division in Lilium. Exp Cell Res **44**(1): 1-12

Swanson WJ, Vacquier VD (2002) The Rapid Evolution of Reproductive Proteins. Nature Reviews Genetics **3**: 137–144

Swanson WJ, Wong A, Wolfner MF, Aquadro CF (2004) Evolutionary Expressed Sequence Tag Analysis of Drosophila Female Reproductive Tracts Identifies Genes Subjected to Positive Selection. Genetics **168**: 1457–1465

Sylvester AW, Smith LG (2009) Cell Biology of Maize Leaf Development. *In* JL Bennetzen, SC Hake, eds, Handbook of Maize: Its Biology. Springer New York, New York, NY, pp 179–203

Takeuchi H, Higashiyama T (2011) Attraction of tip-growing pollen tubes by the female gametophyte. Current Opinion in Plant Biology **14**: 614–621

Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S (1996) Differential Inhibition of Calpain and Proteasome Activities by Peptidyl Aldehydes of Di-Leucine and Tri-Leucine. Journal of Biochemistry **119**: 572–576

Tupy J (1977) RNAsynthesis and polysome formation in pollen tubes. Biol. Planta. **19**: 300-309

Vacquier VD (1998) Evolution of gamete recognition proteins. Science **281**: 1995–

1998

Van Veen H, Vashisht D, Akman M, Girke T, Mustroph A, Reinen E, Hartman S, Kooiker M, van Tienderen P, Schranz ME, et al (2016) Transcriptomes of eight Arabidopsis thaliana accessions reveal core conserved, genotype- and organ-specific responses to flooding stress. Plant Physiology pp.00472.2016

Volkov RA, Panchuk II, Schoffl F (2005) Small heat shock proteins are differentially regulated during pollen development and following heat stress in tobacco. Plant Mol Biol **57**(4): 487-502

Vollbrecht E, Duvick J, Schares JP, Ahern KR, Deewatthanawong P, Xu L, Conrad LJ, Kikuchi K, Kubinec TA, Hall BD, et al (2010) Genome-Wide Distribution of Transposed Dissociation Elements in Maize. THE PLANT CELL ONLINE **22**: 1667–1685

Walley JW, Sartor R, Shen Z, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR, et al (2016) Integration of omic networks in a developmental atlas of maize. Science **353**: 814–818

Wang Y, Zhang W-Z, Song L-F, Zou J-J, Su Z, Wu W-H (2008) Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in Arabidopsis. Plant Physiology **148**: 1201–1211

Warner JR, Knopf PM, Rich A (1963) A Multiple Ribosomal Structure in Protein Synthesis. PNAS **49**: 122–129

Wei LQ, Xu WY, Deng ZY, Su Z, Xue Y, Wang T (2010) Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in Oryza sativa. BMC Genomics **11**: 338

Whipple AP, Mascarenhas JP (1978) Lipid synthesis in germinating Tradescantia pollen. Phytochemistry **17**: 1273-1274

Williams JH (2012) The evolution of pollen germination timing in flowering plants: Austrobaileya scandens (Austrobaileyaceae). AoB Plants pls010–pls010

Williams JH, Taylor ML, O'Meara BC (2014) Repeated evolution of tricellular (and bicellular) pollen. American Journal of Botany **101**: 559–571

Xiao L, Wang H, Wan P, Kuang T, He Y (2011) Genome-wide transcriptome analysis of gametophyte development in *Physcomitrella patens*. BMC Plant Biol **11**: 177

Xu XH, Wang F, Chen H, Sun W, Zhang XS (2013) Transcript Profile Analyses of Maize Silks Reveal Effective Activation of Genes Involved in Microtubule-Based Movement, Ubiquitin-Dependent Protein Degradation, and Transport in the Pollination Process. PLoS ONE **8**: e53545

Yue X, Gao X-Q, Wang F, Dong Y, Li X, Zhang XS (2014) Transcriptional

Evidence for Inferred Pattern of Pollen Tube-Stigma Metabolic Coupling during Pollination. PLoS ONE **9**: e107046

Chapter 2 : Proteomic Profiling of Mature and Germinated Maize Pollen Suggests that Control of Translation and Protein Degradation are Crucial for the Transition to the Progamic Phase

Abstract

Quantitative profiling of the proteome and phosphoproteome was used to directly assess changes in abundance of specific proteins during the transition from mature to germinated pollen. Results from inhibitor studies indicate that *de novo* translation of polypeptides and protein degradation, rather than activation of transcription, are key facets in the control of pollen tube germination. Consistent with the inhibitor studies, combined output of three statistical packages identified 393 proteins significantly (FDR of 0.05) increasing and decreasing in abundance (up to ~150-fold) by 30 minutes post-germination. Gene ontology revealed several terms associated with translation as significantly enriched among proteins increasing in abundance. Proteins decreasing in abundance were significantly enriched in jasmonic acid biosynthesis suggesting that down regulation of this pathway. Largely independent of changes in proteins abundance, 103 proteins were also associated with a significant change in phosphorylation state, suggesting that control of protein phosphorylation is also a feature of germination. As a genetic test for biological relevance of the proteomic analysis, transposon insertion mutants were obtained in 12 genes identified based on the proteomic profiling and transcriptomic characterization of mature pollen (Chettoor *et al.*, 2014). Five insertions were determined to exhibit decreased transmission through the haploid male gametophyte but not through the female, representing genes that may be important for pollen function. Further characterization of the insertion mutants was conducted through phenotyping studies of pollen germination. The findings presented here support the use of quantitative proteomic profiling to successfully identify components putatively important for pollen function in the progamic phase.

Introduction

The transition from mature to germinated pollen represents an important developmental step in the process of fertilization, as the start of the progamic phase of the male gametophyte life cycle. Regulation of transcription has been shown to be important in many transitions (Qin *et al.*, 2009; Wei *et al.*, 2010; Sekhon *et al.*, 2011; Digel *et al.*, 2015; Xiao *et al.*, 2011; Chen *et al.*, 2014). However, it has also been

observed that transcript levels do not always correlate with protein abundance (Gygi *et al.*, 1999; Greenbaum *et al.*, 2003), mainly due to varying translation rates (Piques *et al.*, 2009). Where a rapid response is required, evidence suggests regulation occurs through more direct control of protein abundance (eg. translation or protein degradation) rather than *de novo* transcription (Dure and Waters, 1965; Raff *et al.*, 1972; Grosfeld and Littauer, 1975; Muthukrishnan *et al.*, 1975; Amaldi *et al.*, 1977; Capco and Jeffer, 1979; Davidson, 1986; Richter and Smith, 1984; Hughes and Galau, 1989, 1991; Rosenthal *et al.*, 1993; Curtis *et al.*, 1995; Hart and Wolniak, 1998; Nakabayashi *et al.*, 2005; Malatesta *et al.*, 2009).

Characterization by microarray of the transcriptomes of mature and *in vitro* germinated maize pollen found no significant differences in gene expression (Fowler, Vejlupkova, Cooper, Watrud, unpublished). Additionally, germination of maize pollen grains is resistant to the transcription inhibitor actinomycin D, with germination inhibited at only the highest concentration of the drug (Figure 1.1; Data Vejlupkova and Fowler, unpublished.). This result suggests that maize pollen germination does not rely on *de novo* transcription for pollen germination, but may rely on more direct control of protein levels (eg. translation or protein degradation). The control of germination through protein synthesis is not a new hypothesis (Capkova *et al.*, 1988; Franklin-Tong, 1999). However, the importance of this mechanism during pollen tube germination appears to differ, as the effects of inhibition of translation vary across pollen types and plant families (Mascarenhas and Goraluick, 1971; Franke *et al.*, 1972; Honys and Twell, 2004; Hao *et al.*, 2005) (Table 1.1). Though less well characterized, inhibition of protein degradation as also been shown to reduce germination in at least two species (kiwi and Wilson spruce) (Speranza *et al.*, 2001; Sheng *et al.*, 2006).

Pollen may be categorized using several different characteristics; however, there does not appear to be a single feature that can be used to fully predict the activity within a pollen grain during the transition to the progamic phase (germination). At maturity, pollen may be bi- or trinucleate depending on if it has undergone the second pollen

mitotic event. Found in both monocots and eudicots, approximately 30% of species are trinucleate at pollen shed (Brewbaker, 1967; Williams *et al.*, 2014). A study investigating the role protein synthesis in the transition to the progamic phase found that dependence on protein synthesis during germination appeared to correlate with metabolic development rather than the nucleate state (Hoekstra and Bruinsma, 1979). Additionally, pollen grains can be classified as 'partially hydrated' if their water content is above 30%, or 'partially dehydrated' if it is below that level (Nepi *et al.* 2001; Franchi *et al.*, 2002). Hydration levels can vary even within the same genus (Pacini *et al.* 1999) and, generally, partially hydrate species germinate more rapidly than those which are partially dehydrated but this is not a universal characteristic (Franchi *et al*., 2002). Maize pollen is trinucleate and partially hydrated at maturity and, like many other pollen species with these two characteristics (Hoekstra and Bruinsma, 1979; Franchi *et al.*, 2002), germination of the pollen tube occurs rapidly (Figure 2.1). After it is shed from the male flowers, it lands on the silks and germination of the pollen tube is initiated to start the progamic phase.

When characterizing transitions in gametophytes, it is important to remember that they are haploid, unlike sporophytic tissues, and that the male gametophytes (pollen grains) often function under high levels of competition for fertilization of the female. Therefor, genes important for gametophytic function are expected to be under strong selective pressure. This difference in ploidy between gametophytes and sporophyte has consequences for the representation and rates of genetic mutations present in plant populations. A survey, designated here as the Reduced Recovery of Mutations (RRM) test, of both the *UniformMu* (Settles et al., 2007) and *Mu-Illumina* (Williams-Carrier et al., 2010) populations, found fewer transposon insertions in the exons of genes whose expression was enriched in maize mature pollen than a comparable set of genes whose expression was enriched in the transcriptome of sporophytic tissue (Chettoor *et al.*, 2014). Also consistent with this idea, the Chettoor et al. study notes "pollen-specific genes in an out- crossing relative of *Arabidopsis* (*Capsella grandiflora)* are associated with stronger purifying selection and greater proportion of

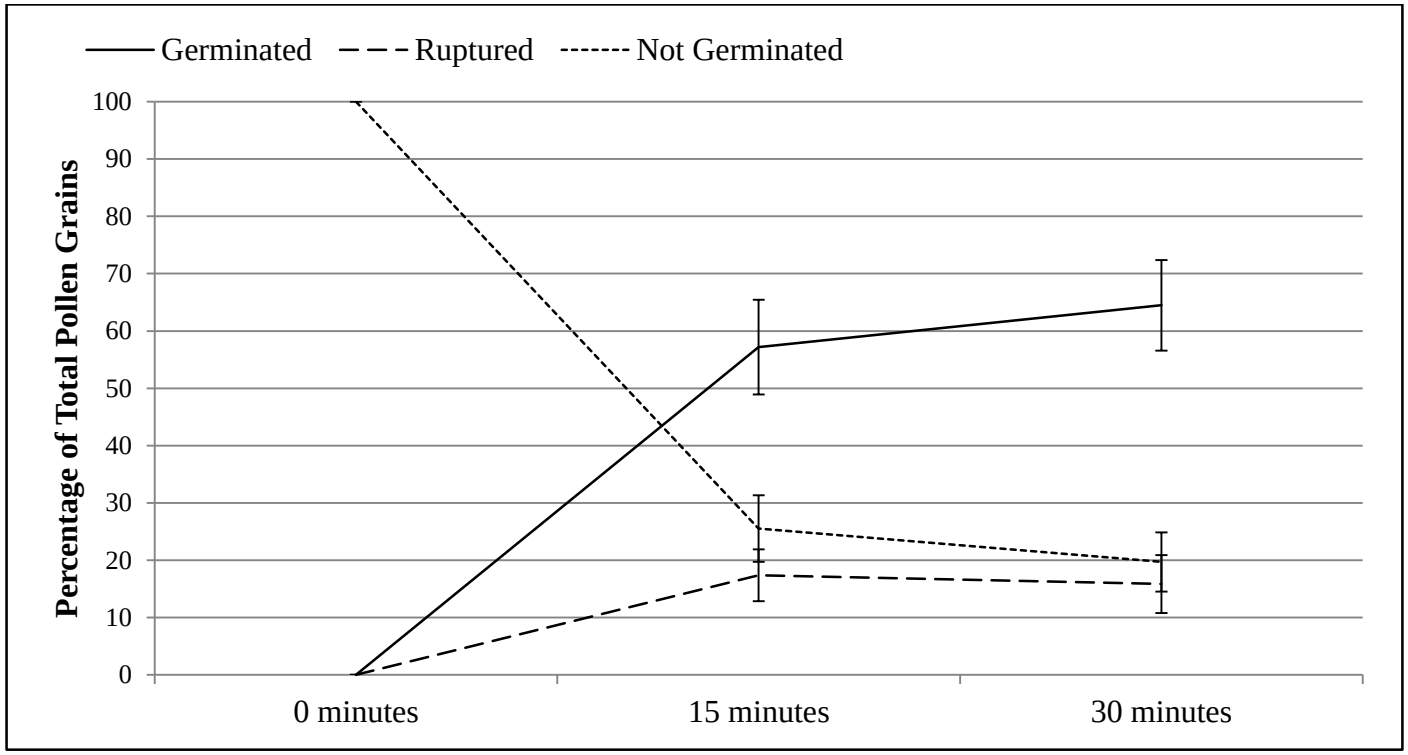


Figure 2.1: Time course of pollen germination in media over 30 minutes.
By 15 minutes, 91 percent of grains have germinated that will do so by 30 minutes. Error bars represent 95% confidence interval, n=10 with at least 112 pollen grains categorized per replicate.

adaptive substitutions than sporophyte- specific genes (Arunkumar et al., 2013)" (Chettoor *et al*., 2014, pg. 16).

Recent advances in technology have improved label free shotgun proteomics, increasing the depth of proteomic profiles and allowing for quantitative comparisons between the total non-modified proteome and the phosphoproteome of different tissues (Zhu *et al.*, 2010; Walley *et al*., 2013; Facette *et al.*, 2013; Marcon *et al.*, 2015). Using these methods, the proteome and phosphoproteome of mature and germinated pollen can be used to help infer models of the mechanisms acting at the transition from mature pollen to the progamic phase. An initial study characterizing the proteome and phosphoproteome of mature B73 pollen, found candidate male fertility proteins and evidence of regulation by phosphorylation (Chao *et al.*, 2016), but did not assess the transition to growing pollen tubes.

The hypothesis of this study is that regulation of the transition from mature to germinated pollen is through direct control of protein abundance (translation and protein degradation) rather than a transcriptional control mechanism. In this study, we present results from quantitative proteomic profiling of mature and germinated maize pollen, and genetic validation of the observations from the proteome analysis. Our findings illustrate changes in protein abundance during the transition to the progamic phase, including down regulation of a pathway not previously linked to maize pollen tube germination, and the identification of several proteins that appear to be important for pollen function and development.

Methods

Plant Material

Mass spectrometry (MS) analyses of mature and germinated B73 pollen, was conducted on four sample replicates that were harvested from field grown plants in San Diego, CA, each replicate representing a pool of plants, with a total of 1-2 grams of tissue. Mature pollen samples were flash frozen in liquid nitrogen. For the germinated samples, pollen was mixed with germination media (Schreiber and

Dresselhaus, 2003; 10% sucrose, 0.0005% H_3BO_3, 10mM $CaCl_2$, 0.05mM KH_2PO_4, 6% PEG 4000) and incubated at RT for 30 minutes in glass petri plates; adequate germination was confirmed by microscopy. Samples in media were flash-frozen with liquid nitrogen poured over the sample and harvested by scraping the frozen material. Samples for confirmatory protein gel and western blot analyses were harvested in a similar fashion from field-grown plants in Corvallis, OR. For the mature leaf samples, B73 plants were grown for 43 d in the greenhouse, without supplemental lighting, until leaf 8 was at least 50 cm and leaf 10 was starting to emerge from the whorl. Blade tissue, minus the midrib, was harvested from leaf 8 for a total of 6 replicates (Facette *et al*., 2013). Mature pollen from W23 was harvested in a similar manner to that of B73.

Results from the proteomic analysis were used to identify candidate genes putatively important for pollen tube germination. Candidates were identified from proteins increasing in abundance or changed in phosphorylation state with germination, or were highly expressed in both the mature pollen proteome and transcriptome. Lists of candidates were cross-indexed with insertions in the maize genome, using a custom database mapping location of publically available insertions to specific genic regions (e.g. exons) to identify candidate insertions for characterization. Insertions were obtained from the *UniformMu* (Settles *et al.*, 2007), *Mu-Illumina* (Williams-Carrier *et al.*, 2010) and *DsMutagenesis* (Vollbrecht *et al.*, 2010) projects. Reciprocal pollinations were performed on field grown plants in Corvallis, OR between heterozygous insertions lines and homozygous wild type tester plants to generate transmission test crosses. To encourage competition between pollen grains carrying the insertion and wild type, pollinations were made using heavy pollen density.

From each of the male and female transmission crosses, approximately 36-48 kernels were randomly selected and grown on germination paper prior to DNA extraction from seedling leaf tissue for PCR genotyping. For phenotyping, heterozygous and homozygous wild-type siblings were grown in 3 gallon pots in a greenhouse with 14-hour light period and night temperatures of 21-26°C.

Protein extraction and preparation

For both mass spectrometry and SDS-PAGE analyses, proteins were extracted as described (Walley *et al.*, 2013; Facette *et al.*, 2013). Briefly, frozen samples were ground in liquid nitrogen prior to protein precipitation for 30 minutes in methanol (EMD Millipore Corporation) with 0.2mM Na_3VO_4 (Enzo Life Sciences). Samples were washed two times more in the methanol solution and three times in acetone (EMD Millipore Corporation). Precipitation and washes were conducted at -20°C. Proteins were dried in a SpeedVac at 4°C and stored at -80° C.

Processing for mass spectrometry was as described (Walley *et al.*, 2013; Facette at al., 2013), including reduction and alkylation of cysteines, two trypsin digests, and quantification via Bradford assay (Pierce). Samples were either re-suspended in 1% formic acid for direct MS analysis of non-modified peptides, or in 3% trifluoroacetic acid for enrichment of phosphopeptides. Phophopeptide enrichment was via Ce02 affinity capture, followed by MS analysis.

Mass spectrometry, peptide identification and database search

Mass spectrometry (MS) and peptide identification were conducted as previously described (Walley *et al.*, 2013; Facette at al., 2013). In brief, the MS analysis was performed on an Agilent 1100 HPLC system (Agilent Technologies). Peptides were eluted using 0 to 80% acetonitrile gradient. Fractionation using a 29 step (non-modified proteome) or 19 step (phosphoproteome) series of salt gradients was followed by high-resolution reverse phase separation using an acetonitrile gradient. A LTQ Velos linear ion trap tandem mass spectrometer (Thermo Electron) was used to acquire spectra using automated, data-dependent acquisition.

Spectrum Mill v3.03 (Agilent Technologies) was used to extract and search the raw data. MS/MS spectra were discarded if they did not have at least three fragment ions with the mass differences between the ions matching two amino acids. The filtered MS/MS spectra were then searched against the maize B73 RefGen_v2 5a Working

Gene Set downloaded from www.maizesequence.org with the enzyme parameter limited to full tryptic peptides with a maximum miscleavage of 1 and other parameters set to the Spectrum Mill default settings. For the phosphoproteome data, Ox-Met, n-term pyro-Gln and phosphorylation on Ser, Thr or Tyr were defined as variable modifications with a maximum of two modifications per peptide.

To calculate the false discovery rate (FDR), a 1:1 concatenated forward-reverse database was constructed and compared to the forward database with reshuffling of any tryptic peptides that matched. To maintain an FDR of 0.1% at the peptide level for non-modified proteins and 1% for phosphopeptides, peptide cutoff scores were assigned dynamically to each of the databases. To account for protein database redundancy, parsimony was used to group proteins that share common peptides with a single protein designator as the "group leader".

Statistical Analysis

To minimize the effect of noise, distribution of the log counts across replicates were modeled to select a minimum count threshold. Thresholds were selected as the lowest count cut off that when modeled in a histogram resulted in an approximate normal distribution (Appendix Figure A.1). Based on this analysis, proteins were included in the analysis if, across replicates, there were at least 25 counts in mature versus germinated B73 pollen, 20 counts in mature pollen versus mature leaf and 25 counts in mature B73 pollen versus mature W23 pollen. For the phosphoproteome, a cutoff of 10 counts was used for the mature versus germinated B73 pollen.

The raw filtered counts were analyzed using three different statistical packages available in R (www.r-project.org): edgeR (Robinson *et al.*, 2010), IBB (Pham *et al.*, 2010) and PLGEM (Pavelka *et al.*, 2008). The output from these different packages was then run through the R package qvalue (Storey, 2015) to calculate FDR values for the proteins. Proteins were designated as differentially abundant if at least one package identified them as such with a FDR of 0.05.

Heatmaps were generated in R using the native package ‘heatmap’. Counts for the heatmap were normalized between replicates to one million. These normalized counts were used to generate Pearson’s correlation coefficients between the proteomes of mature pollen, germinated pollen, mature leaf and the three leaf developmental zones. Correlation between mature pollen transcriptome and the proteomes of mature and germinated pollen were calculated by Pearson’s correlation of normalized counts for groups containing only a single protein in the proteomic data. Scripts available upon request.

Bioinformatics

Functional enrichment of gene ontology terms was conducted by submitting lists of gene identifiers for proteins determined to exhibit significant changes in abundance with germination in this study to the online AgriGO Analysis Toolkit and Database for Agricultural Community (Du *et al*., 2010; http://bioinfo.cau.edu.cn/agriGO/). Lists were uploaded to the Singular Enrichment analysis (SEA) tool using the suggested *Zea mays* background reference with the Fisher’s exact test and a FDR of 0.05.

Identification of proteome enrichment in metabolic pathways was conducted using the PlantReactome (http://plantreactome.gramene.org). Manual curation of enrichment in the jasmonic pathway was conducted by comparison of proteins found in the mature and germinated pollen proteomes to the genes annotated by MaizeCyc (maizecyc.maizegdb.org*)* to be in the jasmonic acid biosynthesis pathway.

SDS-PAGE gel, Coomassie and silver staining

Proteins were extracted using the same method as that for MS analysis and suspended in 0.1X Wes sample buffer (ProteinSimple, 042-195). Protein concentration was quantified using a Qubit X.0 Fluorometer and Quant-iT Protein Assay Kit (Life Technologies). Samples were diluted to 2mg/ml of total protein with 1x SDS gel-loading buffer (50mM Tris*Cl (pH 6.8), 100mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Proteins were separated on polyacrylamide gels (8-

15%) prior to staining with Coomassie Brilliant Blue R250. Gels were destained for at least 24 hours in 90% methanol:H_2O (1:1 v/v) and 10% glacial acetic acid. Gels were imaged on an Aticio MP 5001 (RICOH).

After imaging, gels were washed in ddH_2O prior to silver staining. The gels were incubated for 2 hours (0.5M Sodium acetate, 0.1% Sodium thiosulfate, 30% Ethanol, 0.5% Glutaraldehyde) then washed again in ddH_2O prior to staining in silver nitrate (0.1% Silver nitrate, 0.01% Formalin). Gels were flushed in 2.5% Sodium carbonate, followed by developing in 2.5% Sodium carbonate and 0.01% Formalin. Development was stopped using Coomassie destain solution and 2 washes with ddH_2O. Gels were imaged on an Aticio MP 5001 (RICOH).

Pollen Germination Studies

Pollen was harvested from B73 plants greenhouse grown in 3 gallon pots with 14 hour light period and night temperatures of 21-26°C. Pollen was pooled from available plants (n=3-6) and then added to tubes containing 1x pollen germination media (Schreiber and Dresselhaus, 2003) mixed with cycloheximide (SIGMA Life Sciences; at concentrations of 150, 225, 300 and 450μM), MG-132 (SIGMA Life Sciences; at concentrations of 5, 15, 20 and 80μM), E-64 (Calbiochem; at concentrations of 5, 20 and 80μM) or methyl jasmonate (meJA; ALDRICH; at concentrations of 4, 20, 100 and 500μM). The addition of the DMSO solvent to germination media was used as a control for all of chemicals save meJA, which was dissolved in water. The media and pollen was spread thinly across a glass petri dish and at least 3 images were recorded at 15 and 30 minutes after mixing at 2.5x magnification using a dissecting microscope (ZEISS) and LEICA DFC 280 microscope camera. Images were then scored for germination rates using ImagePro software (Image-Pro Plus 6.0 software; Media Cybernetics, Bethesda, MD). Statistical significance was determined using 95% confidence intervals.

DNA Extraction

DNA for PCR genotyping was extracted using a rapid prep method (Vejlupkova and Fowler, 2003). Leaf tissue from seedlings was ground using a drill-mounted plastic pestle in the presence of extraction buffer (2M NaCl; 200mM Tris-HCl, pH 8.0; 70mM EDTA, pH 8.0; 20 mM sodium meta-bisulfite) and 5% Sarkosyl. After incubation at 60°C, samples were centrifuged and the supernatant was removed and added to 5M ammonium acetate and isopropanol for DNA precipitation. Samples were centrifuged again to form a pellet and were washed with cold 70% ethanol. DNA was suspended in TE buffer, pH 8.0 with a brief incubation at 65°C.

Transmission Assessment

Assessment of transmission was conducted by PCR genotyping of offspring from male and female crosses of transposon insertion heterozygotes and wild-type tester plants. Primers were designed to flank candidate insertions and were used in conjunction with an internal *AcDs* or *Mu* primer (Table 2.1 and Figure 2.2). The number of offspring containing the insertion was recorded separately for male and female crosses (Appendix Table A.8). The presence of a transmission defect was tested using a Chi-square statistic to determine if inheritance of the insertion differed significantly from the 50% expected from Mendelian inheritance.

Male Transmission Defect Phenotyping

Pollen was harvested from heterozygous plants genotyped to contained insertions identified from the transmission study as exhibiting a male transmission defect and from wild type siblings. For each insertion, five plants were selected for each condition and two replicates were collected from each plant on different days. Pollen was mixed with 1x pollen germination media (Schreiber and Dresselhaus, 2003, 10% sucrose, 0.0005% H_3BO_3, 10mM $CaCl_2$, 0.05mM KH_2PO_4, 6% PEG 4000) and allowed to germinate in glass petri dishes. Images were recorded at 15 and 30minutes after addition to media using a dissecting microscope (ZEISS) set at 2.5X magnification and LEICA DFC 280 microscope camera. A minimum of three images was taken of each replicate at each time point. Germination rates, pollen tube lengths

Table 2.1: Primers for male transmission defect study.
All primer pairs for Mu insertions were used with the additional third primer 'Mu_End'.

Insertion	Gene Designation (Insertion ID)	Protein Prediction	Primers	Sequence 5'-3'
AcDs	GRMZM2G173289 (I.S07.2815)	7TM Receptor	I.S07.2815	GCTCCACGAACCACTGGTCCTC
			AcDc_JGp3	ACCCGACCGGATCGTATCGG
AcDs	GRMZM2G030265 (I.S06.0796)	Polygalacturonase	030265_F1	GCAAGTAGGTTCAGACGCAGGC
			030265_R1	TAGACAAGGTGACGATCAAGGC
			AcDs_JSR01	GTTCGAAATCGATCGGGATA
Mu	GRMZM2G102404 (mu1008434, mu1045886)	Acid Phosphate Hydrolyase, UNKNOWN	Mu_09R	TGCTCTAGCCGATCACAATG
			Mu_09L	CCTGCTTTGGTACCGTGATT
			Mu_10R	GCTTTAGCGCCTGTGAAATC
			Mu_10L	TTCCCCAAATATGTGCAGGT
Mu	GRMZM2G133282 (mu-illumina_226847.3)	Signaling - ARO1 Ortholog	Mu_04R	ATCTGCTCCCAGATGAGGAA
			Mu_04L	ATGGACGACCTCAAGCAGAT
Mu	GRMZM2G156365 (mu1044656)	Pectinacetylesterase Family Protein	Mu1044656_F1	ACGAGTCGCAGAAGGAGAAAAAC
			Mu1044656_R1	GGATCCTCCAGTGATCTGATTGA
Mu	GRMZM2G301647 (mu-illumina_250175.3)	Serine/Threonine protein kinase	Mu_03R	ACCCGATCGAACATTGTGAT
			Mu_03L	GTACACGAGGCAGCCGTACT
Mu	GRMZM2G318992 (mu1041512)	KIP1 - NET actin-binding	Mu1041512_F1	TCAGCAACTTCAAATTCCCTACG
			Mu1041512_R1	TTTTTCTGGTCCTGCAAGTTCAC
Mu	GRMZM2G389462 (mu1057062)	Ubiquitin Ligase	Mu1057062_F1	TATATGGGAAACGCACAACGAAG
			Mu1057062_R1	GGAAATGGCACCCCACTCTATTA
Mu	GRMZM2G422641 (mu1042381)	KIP1 - NET actin-binding	Mu1042381_F1	GACGCCACCACGATTTTCTC
			Mu1042381_R1	TAGAGCTCTGCCTTCTTGCTGAA

Table 2.1: Continued

Insertion	Gene Designation	Protein Prediction	Primers	Sequence 5'-3'
Mu	GRMZM2G066024 (mu1069861)	Fructose-Bisphosphate Aldolase Cytoplasmic Isozyme	066024_F1	CTCCGTGAGCTCCTGTTCTG
			066024_R1	GTTGGGCTTCAGGAGGGTAC
Mu	GRMZM2G017334 (mu1047209)	2-Phosphoglycerate Kinase-Related	017334_F1	CCGTCCGCCTCTACATCTTC
			017334_R1	ATGCTACCTTGGCGTGTTCA
Mu	GRMZM2G457370 (mu1013491)	Male Gametophyte Specific Argonaute	457370_F1	AAACTTCTACGTGAGCGCCA
			457370_R1	CTGGTTCTCGGACAACAGCT
Used with all Mu Primers			Mu_End	AGAGAAGCCAACGCCAWSGCCTCYATTTCGTC
Internal Control Primers			Rop7_F5	AGCTTTAACGCCGGTCAGTTGAG
			Rop7_R5	TGACCAACCAACTGTATACGGTGGAA

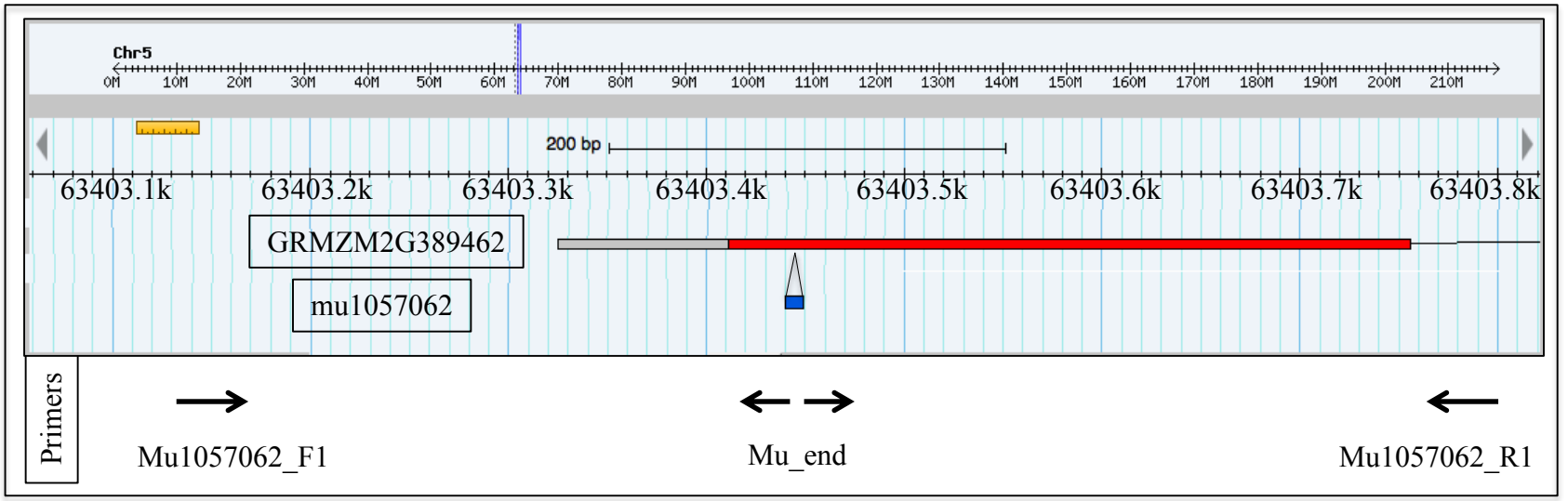


Figure 2.2: Conceptual model of the arrangement of PCR primers for MTDA genotyping.
Figure displays the genomic region around the first exon of GRMZM2G389462 (Table3.1) and the location of its associated insertion, designated mu1057062. Two primers are designed to flank the region of a selected insertion with a third primer located within the insertion (Mu_End). This third primer anneals to the terminal inverted repeats (TIRs) found at both ends of the *Mu* insertion permitting it to amplify out of the insertion into the exon towards the two gene specific primers. Without the insertion, the only PCR product detected is that between Mu1057062_F1 and Mu1057062_R1. Presence of the insertion will result in smaller bands.

and diameters of germinated and non-germinated pollen grains were collected using ImagePro software (Image-Pro Plus 6.0 software; Media Cybernetics, Bethesda, MD).

Results

De novo translation and proteasome activity are critical for pollen germination

As an initial test of the hypothesis that the transition to the progamic stage is regulated through more direct control of protein levels rather than transcription, total protein extracts from mature and germinated pollen were run on SDS-PAGE gels and stained with Coomassie followed by silver staining to visualize large-scale changes to the proteome between the mature, non-germinated pollen and germinated pollen (Figure 2.3). Consistent with the hypothesis, comparison of the banding in the mature and geminated pollen samples found evidence of both increases (Figure 2.3, right side), and decreases in specific protein levels (Figure 2.3, left side), occurring along with the transition to pollen tube germination.

To establish a baseline time course for maize pollen tube germination, images were captured at 15 and 30 minutes after the addition of pollen to germination media (Figure 2.1). Pollen grains were then categorized as not germinated, germinated or ruptured – where the grain bursts at the pore releasing its contents into the media (Figure 2.4). By 30 minutes of incubation in liquid germination media, B73 pollen produced viable pollen tubes at frequencies of 60% to 63% (Figure 2.1). Overall, 91% of grains that will develop tubes by 30 minutes had a recognizable pollen tube after only 15 minutes. Thus, in maize, pollen tube germination is a very rapid response. Rupture may be a result of germination that fails to maintain cell wall integrity at the initiating or growing pollen tube tip, as the percent of ruptured grains was negatively correlated with the percent of germinating grains in each experiment (Pearson's $r = -0.86$).

Previous work with actinomycin D indicated that *de novo* transcription was not a central requirement for the transition of mature to germinated pollen (Figure 1.1).

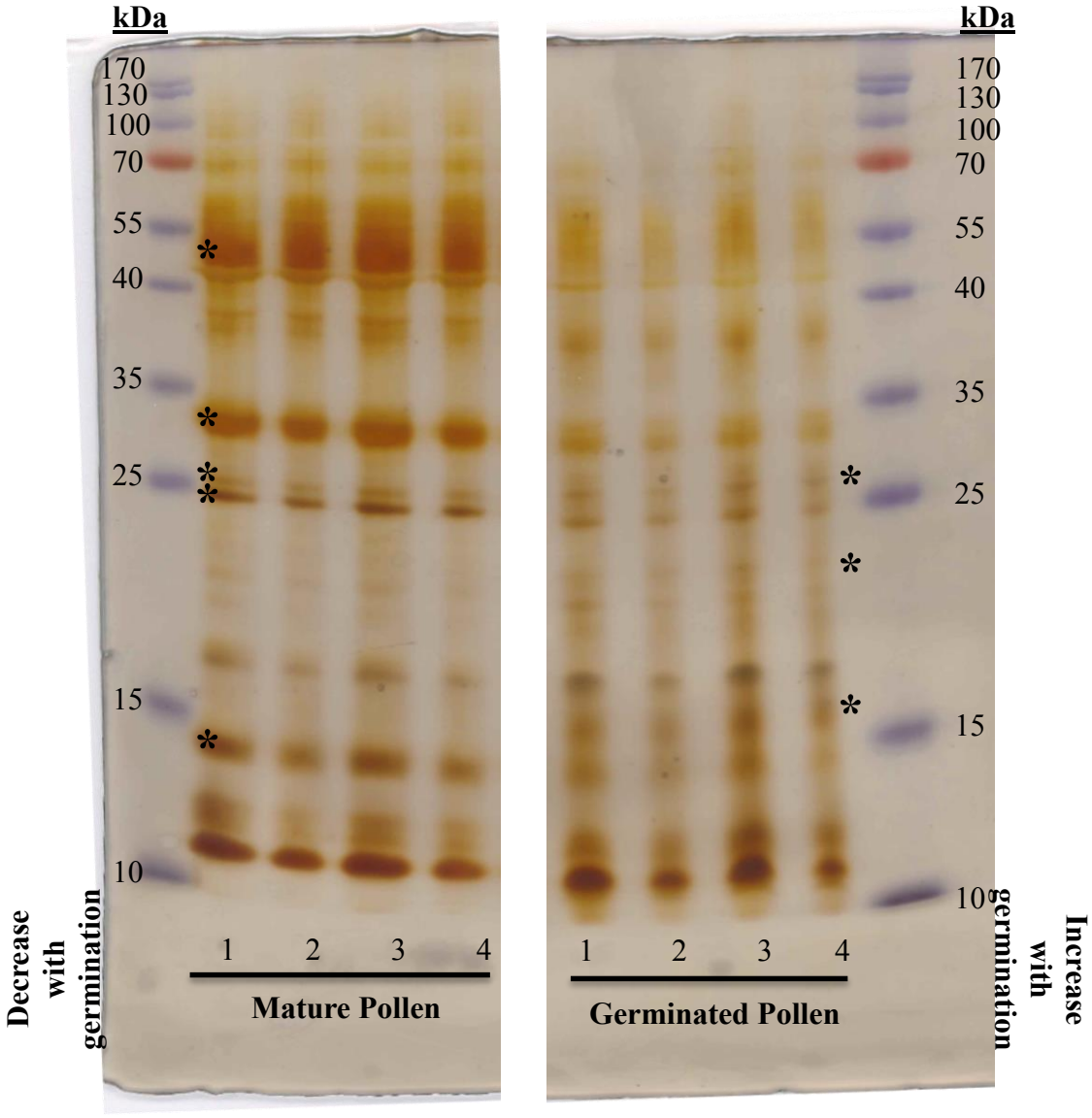


Figure 2.3: Silver staining of a 15% SDS-PAGE gel containing the total protein extracts from mature and germinated pollen.
Several groups (asterisks) of specific proteins decreasing and increasing in abundance with germination were distinguishable from total protein extracts. Lanes represent 4 replicates of 4 paired mature and germinated pollen samples.

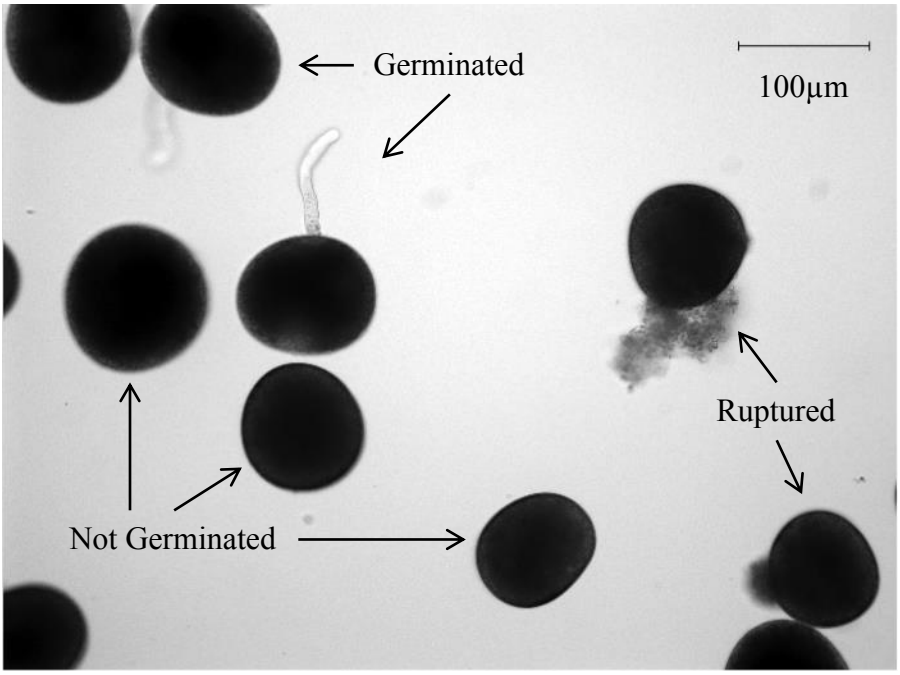


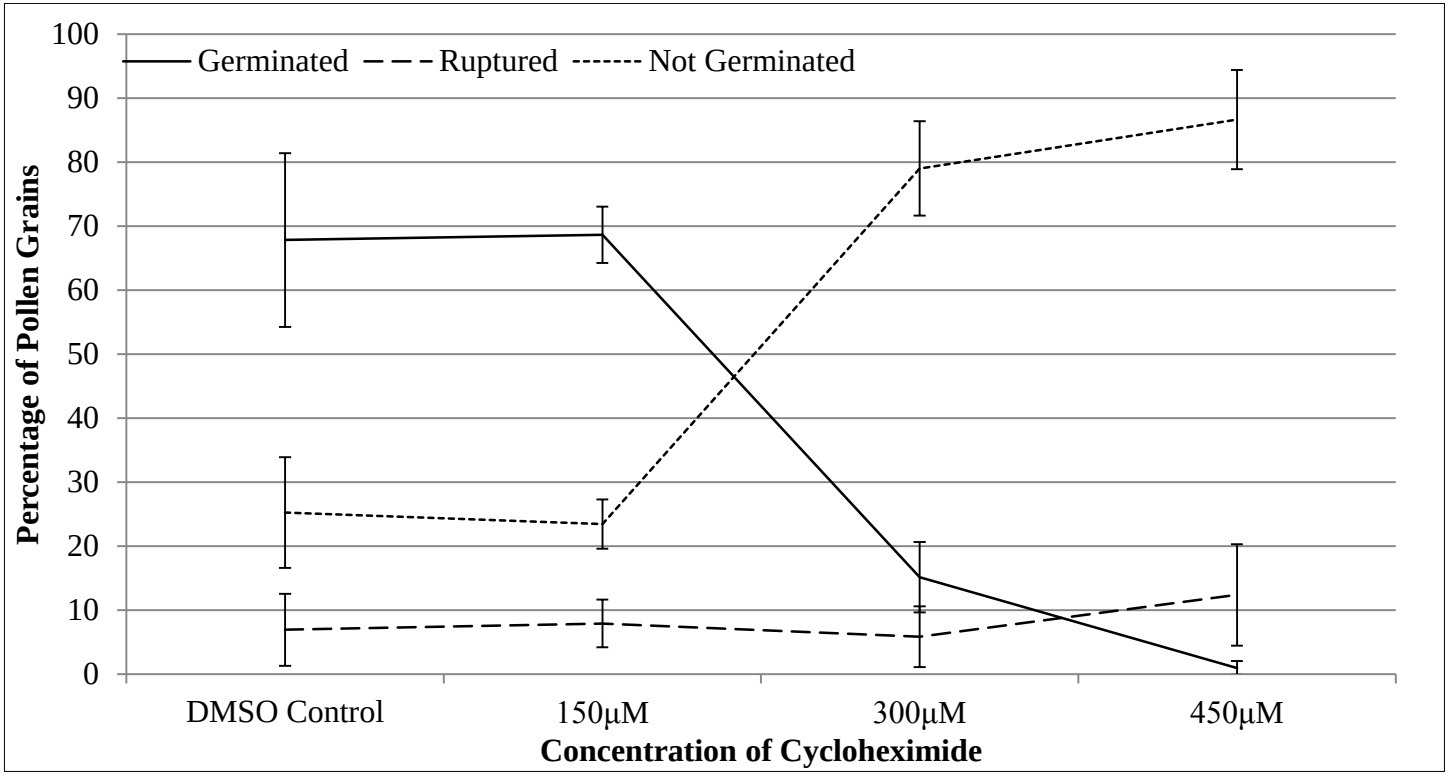
Figure 2.4:Examples of pollen grain categories.
After 15 or 30 minutes, pollen grains were categorized as germinated, ruptured or not germinated. Magnification at 20x, scale bar represents 100µm..

This suggests an alternative where the transition is controlled though post-transcriptional mechanisms. To test two such possibilities, *de novo* translation and ubiquitin-mediated protein degradation, increasing concentrations of cycloheximide and MG-132 were added to germination media with freshly harvested maize pollen. Concentration ranges were comparable to previous studies in plants (Lee and Goldberg, 1996; Genschik *et al.*, 1998; Woffenden *et al.*, 1998; Speranza *et al.*, 2001, Sheng *et al.*, 2006; Honys and Twell, 2004; Hao *et al.*, 2005; Mascarenhas, 1971).

Pollen incubated with cycloheximide, a translation inhibitor, exhibited a significant reduction in germination rates from the control (67.8%) at the higher concentrations tested of 300μM and 450μM (Figure 2.5A), 15.1% and 1% respectively. This decrease in germination was matched with an increase in grains that failed to germinate. There were no significant changes to the percentage of pollen grains rupturing, suggesting that blocking translation does not lead to a reduction in cell wall integrity. This indicates that *de novo* translation is an important process for successful pollen tube germination.

The drug MG-132 inhibits the proteasome (Rock *et al.*, 1994; Tsubuki *et al.*, 1996), acting as a transition-state analog (Lowe *et al.*, 1995). The addition of MG-132 to the germination media resulted in a significant decrease in the percentage of successfully germinated pollen tubes, with only 6% visible in 80μM of MG-132 (Figure 2.5B). This reduction is similar to the effects of MG-132 observed in kiwifruit, where the addition of 80μM MG-132 resulted in only a 17% germination rate (Speranza *et al.*, 2001), though there was no reported increase in pollen grain rupture. In contrast to the cycloheximide results, the reduction in germination in the presence of MG-132 is correlated with an increase in the percentage of pollen grains rupturing, indicating that proteasome activity and translation are impacting different cellular processes and leading to different outcomes when inhibited. One possibility is that the proteasome may be required during germination to maintain the stability of the growing pollen tube cell wall.

[A]


Germinated
Ruptured
Not Germinated
Percentage of Pollen Grains
100
90
80
70
60
50
40
30
20
10
0
DMSO Control
150µM
300µM
450µM
Concentration of Cycloheximide

[B]

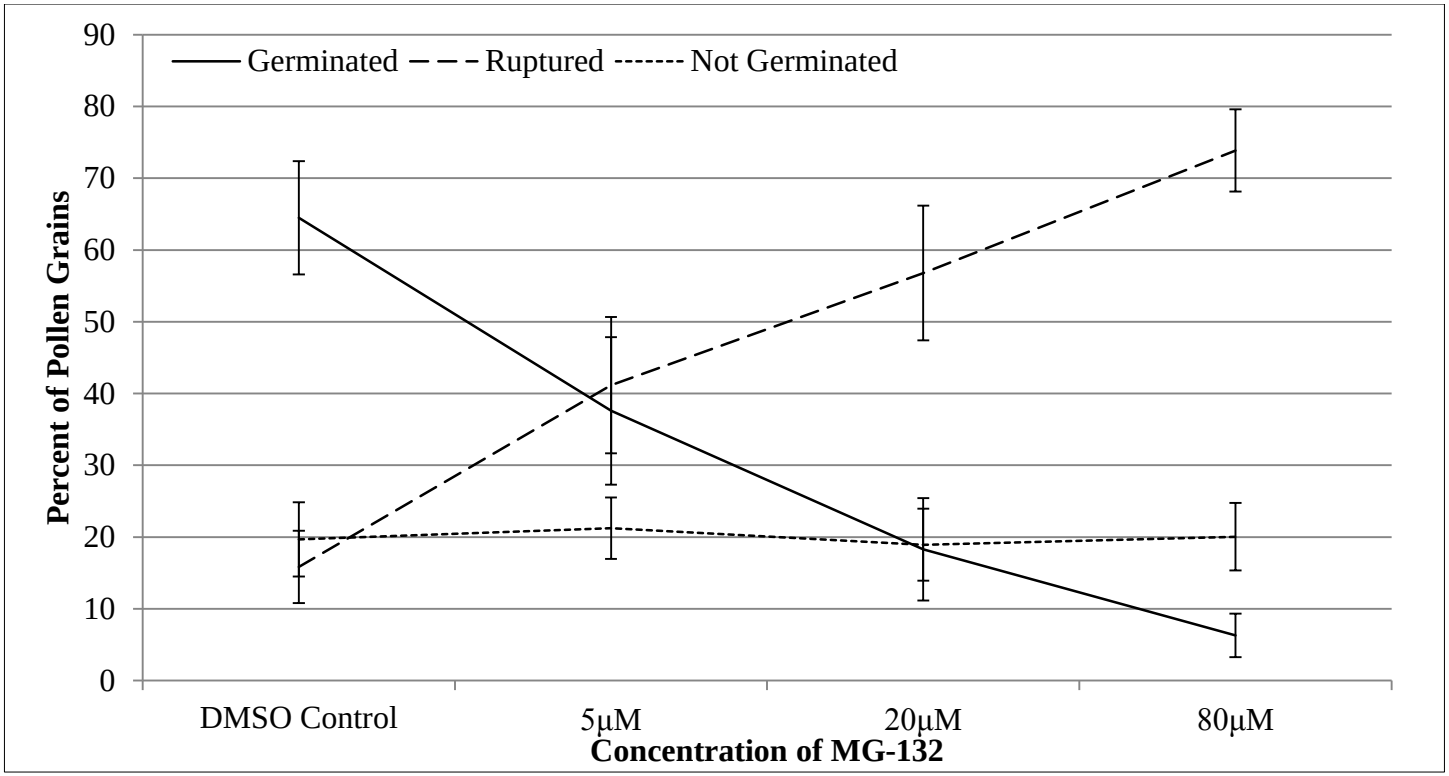


Figure 2.5:Pollen germination response to cycloheximide and MG-132 after 30 minutes.
[A] Decreased germination with increasing cycloheximide suggests that *de novo* translation is required for germination. [B] Increased rupturing with inhibition of protease activity suggests that protein degradation is required for pollen/pollen tube stability during germination. Error bars represent 95% confidence intervals, n=4-10; at least 75 pollen grains were categorized in each treatment.

In addition to its effect on the proteasome, MG-132 can also inhibit calpains (Rock *et al.*, 1994). To control for this possibility, the cell permeable calpain inhibitor -E64 (transepoxy succinyl-L-leucylamido-(4-guanido) butane ester) was also tested and was not found to have a significant effect on germination rates (Figure 2.6). This strongly suggests that the effects observed of MG-132 on germination rates are due to its proteasome inhibition and not its inhibition of calpains.

Two additional studies were conducted using a combination of cycloheximide and MG-132 to determine if translation or protein degradation constitutes the earlier step in pollen tube germination. Our hypothesis is that pollen rupturing in the presence of MG-132 were grains that hydrated and initiated germination, but were then unable to continue growth to inhibited protein degradation resulting in cell wall instability and eventual rupture. In other words, translation starts the process but protein degradation is required to continue successful pollen tube growth (and thus germination).

For the first experiment, mature pollen samples in germination media were exposed to 15μM of MG132 along with concentrations of cycloheximide ranging from 150 to 450μM. In the reciprocal experiment, a constant concentration of 300μM cycloheximide was used in combination with concentrations of MG-132 ranging from 5 to 80μM. If translation occurs prior to protein degradation during germination, the first experiment should exhibit a decrease in rupture with increasing cycloheximide, as fewer grains initiate germination; in the second experiment rupture should remain constant across increasing concentrations of MG-132. If protein degradation occurs first, then a constant rate of pollen grain rupture will be observed in the first experiment with increasing cycloheximide, whereas increasing rupture in the second experiment should be observed.

The results from both experiments were consistent with the idea that translation is required for sensitivity to MG-132 (and its likely effect on proteasome activity) during pollen germination. In the first experiment (Figure 2.7A), a significant reduction in MG-132-induced pollen grain rupture was observed when the concentration of cycloheximide was increased from150μM (72.0% +/- 9.0% 95%CI)

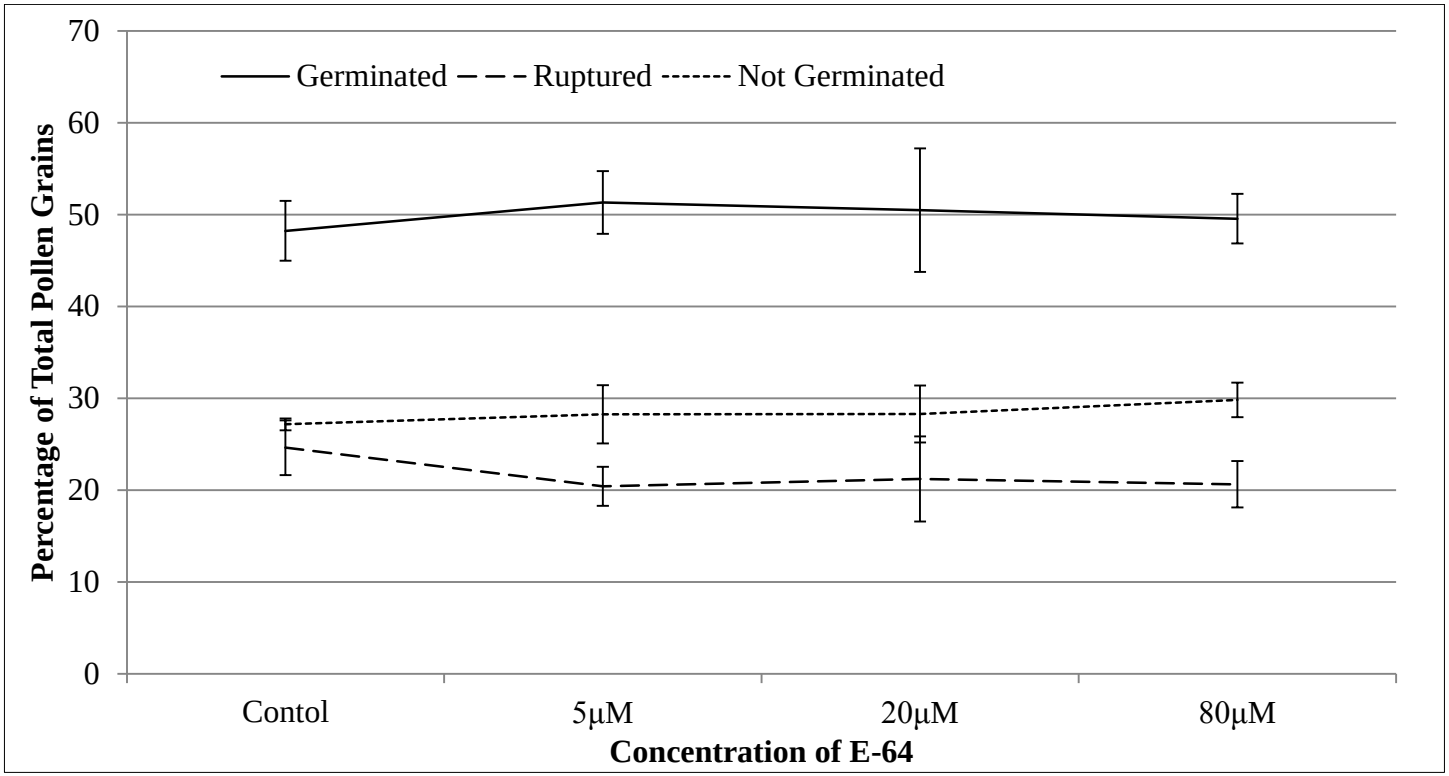
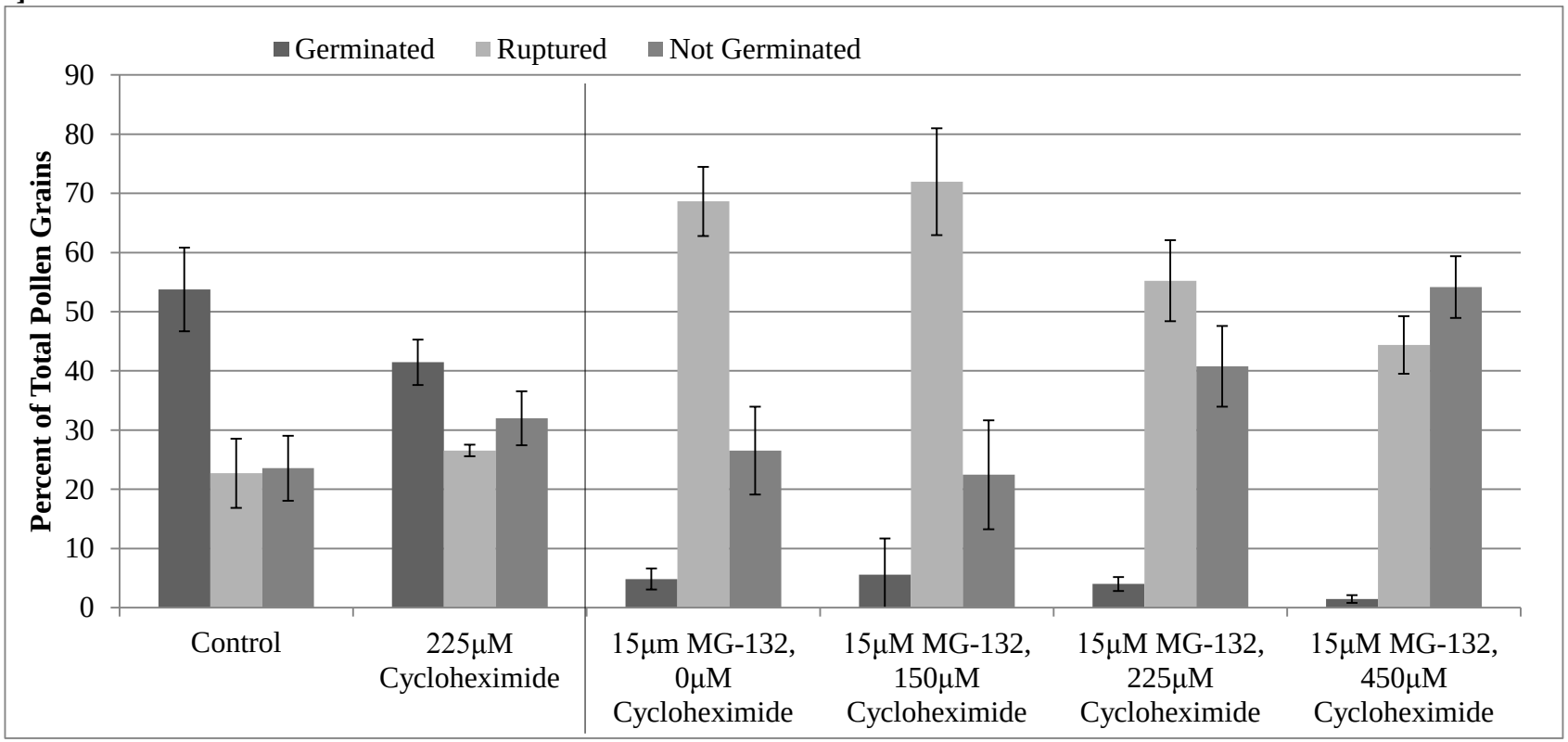


Figure 2.6:Pollen tube germination rate response the addition of the calpain inhibitor E-64 after 30 minutes.
The inhibitor E-64 did not exhibit a significant effect on germination or rupturing suggesting the effect observed in response to MG-132 is due to MG-132 inhibition of the proteasome and not calpain inhibition. Error bars represent 95% confidence intervals, n=4; at least 264 pollen grains categorized per treatment.

[A]


Germinated
Ruptured
Not Germinated
Percent of Total Pollen Grains
90
80
70
60
50
40
30
20
10
0
Control
225µM Cycloheximide
15µm MG-132, 0µM Cycloheximide
15µM MG-132, 150µM Cycloheximide
15µM MG-132, 225µM Cycloheximide
15µM MG-132, 450µM Cycloheximide

[B]

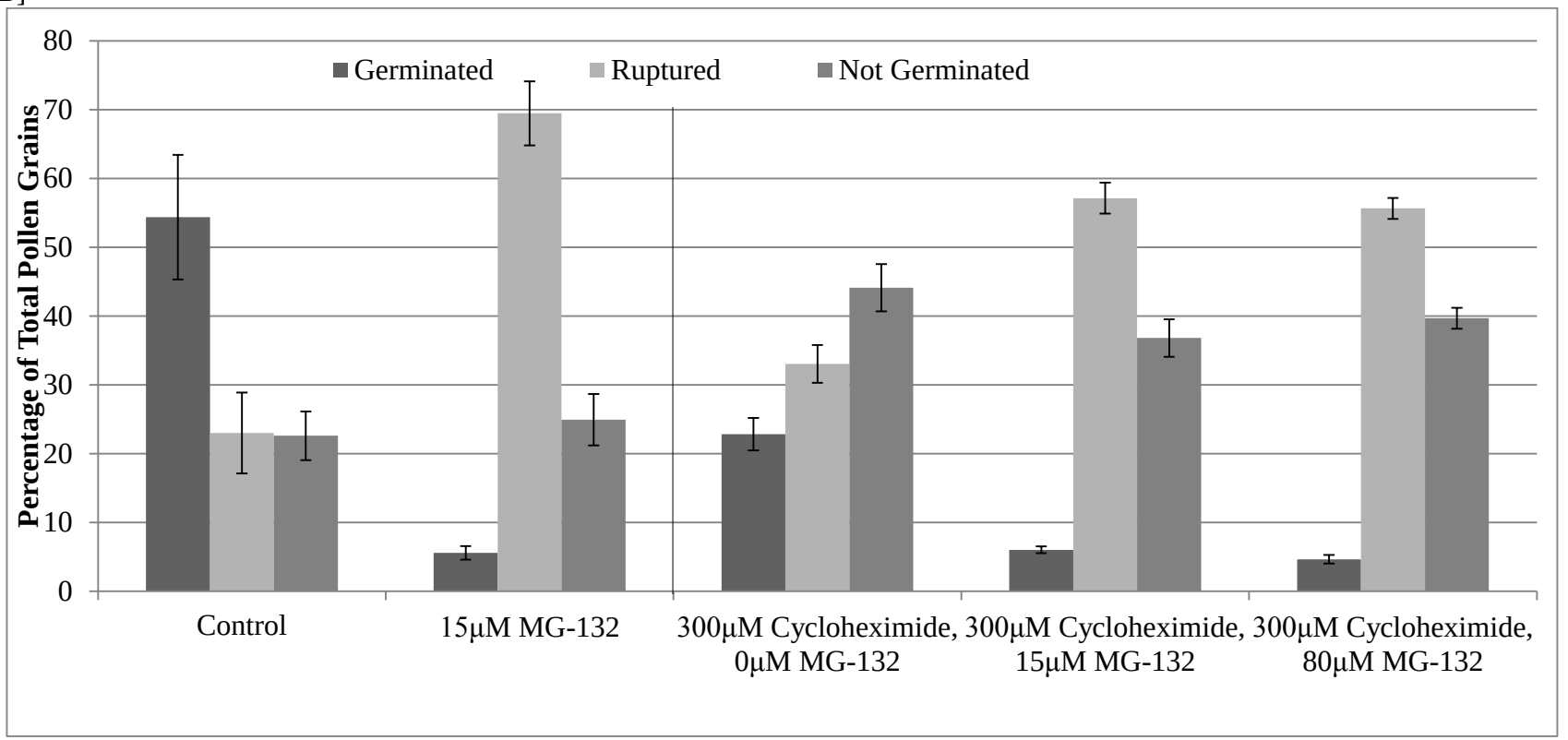


Figure 2.7:Pollen germination response to a mixture of cycloheximide and MG-132 added to the germination media suggests that translation occurs prior to protein degradation.

[A] Increasing concentrations of cycloheximide added to a constant concentration of MG-132 exhibited a decrease in rates of rupturing matched with an increase in grains that fail to germinate. [B] Increasing concentrations of MG-132 added to a consistent concentration of cycloheximide does not appear to affect the rates of germination, rupturing or failure to germinate. Bars represent 95% confidence intervals; n=4; at least 121 pollen grains categorized per replicate.

to 450μM (44.4% +/- 4.9% 95%CI). This was matched with an increase in the percentage of pollen grains that failed to germinate, and no significant change in the rates of successful germination. In the second study, there was a slight increase in rupturing with the addition of MG-132 to cycloheximide-treated pollen grains (Figure 2.7B). This likely represents the rupturing of the grains that were still able to germinate at 300μM cycloheximide, as this increase in rupture is matched with a decrease in observed germination. However, increasing the MG-132 concentration from 5μM to 80μM did not lead to any further significant differences in rupturing or germination, again consistent with the idea that translation is a crucial component of the initial germination response.

HPLC-MS/MS identified 6,624 non-modified proteins in mature and germinated pollen

Results from microarray and inhibitor studies (Figure 1.1 and Figure 2.5) suggest that the transition to the progamic phase in maize pollen is regulated through more direct control of proteins rather than transcription. To directly test for evidence of this regulation, quantitative proteome profiling was conducted on mature and germinated pollen. As described in Methods, total protein was extracted from mature pollen and pollen grains left in germination media for 30 minutes. Tryptic peptides from these samples were analyzed by HPLC-MS/MS and spectral counts were assigned to B73RefGenv2 Working Gene Set proteins. The counts and matched peptides are listed in Appendix Table A.2. To limited overestimation of the number of proteins represented in the samples due to peptides mapping to more than one protein, proteins that share peptides were grouped together. For each group a "group leader" was assigned based on the largest number of spectral counts or longest protein in case of ties. Using these criteria a total of 26,706 peptides were identified, representing 6,624 protein groups (Table 2.2). For this Chapter, the term "protein" will be used to refer to the designated leader and group members collectively. Supporting the accuracy and reproducibility of our biological replicates, average Pearson correlations were found to be 0.94 and 0.85 between the replicates in the total proteome and phosphoproteome, respectively.

Table 2.2: HPLC-MS/MS identified 393 non-modified proteins and 103 phosphoproteins differentially abundant in mature and germinated pollen.
Filters used consisted of a minimum of 25 spectral counts for the total proteome and 10 for the phosphoproteome.

	Total Proteome	Phosphoproteome
Proteins Detected	6,624	1,584
Distinct Peptides	26,706	6,364
Proteins Detected Above Filter	3,506	842
Differentially Abundant, p-value = 0.05	1,658	294
Differentially Abundant, FDR = 0.05	393	103

To visualize large-scale expression patterns between the mature pollen and germinated pollen, a heatmap was constructed using spectral counts normalized to one million (Figure 2.8). In addition the pollen proteomes, a mature leaf proteome was included as a comparator, from a larger project that also analyzed the pollen samples (Facette *et al.*, 2013, Walley *et al.* 2013). This comparison helps highlight large sets of proteins specific to the pollen (or leaf) samples. It also showed evidence of changes in protein abundance between the two pollen samples, indicating control of particular protein levels during this transition. Though the transition of the mature to germinated pollen in our samples represents only 30 minutes of elapsed time, the correlation coefficient of normalized spectral counts for our pollen samples (0.93) represents a similar magnitude of change as observed between different stages of leaf development occurring over several days (Appendix Table A.1)(Facette *et al.*, 2013).

Different statistical packages identify distinct sets of differentially abundant proteins from HPLC-MS/MS data

To identify specific proteins that significantly change in abundance upon germination, we looked for statistical approaches that were publically available to analyze spectral count data. The differences between these packages are largely due to the statistical model used and the input of either raw or normalized spectral count data. We found little to no consensus as to which packages, methods or models are the most appropriate for determining differential abundance of proteins. Many of the available packages were developed for analysis of read counts in RNA-seq datasets. A few recent studies have compared different packages' capacity to analyze spectral counts (Mueller *et al.*, 2008; Choi *et al*., 2008; Ning *et al.*, 2012; Gonzalez-Galarza *et al.*, 2012; Kannaste *et al.*, 2014), with differing conclusions. We chose to select some of the best-ranked packages from these studies to determine what method might be the best to model our HPLC-MS/MS data. We limited our search to packages available in R (www.r-project.org), which were capable of interpreting raw spectral counts without normalization.

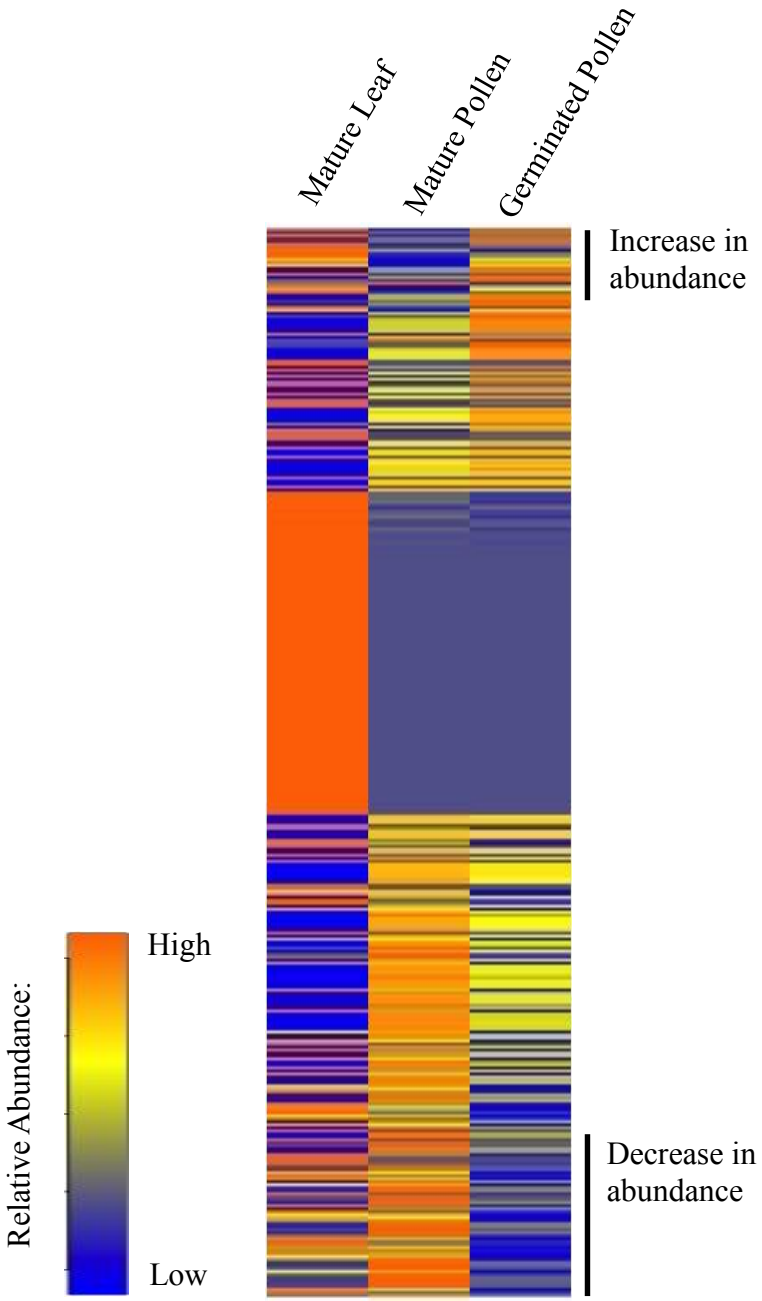


Figure 2.8: Heat map comparing the relative abundance of proteins found to be expresses in mature leaves, mature pollen and germinated pollen.
Counts were normalized to 1 million with orange indicating relatively higher abundance and blue lower. Comparison of the mature and germinated pollen highlights two groups of proteins those that increase (approximately 3.7% of proteins) with germination and those that decrease (approximately 7.6% of proteins), groups indicated above.

The three packages chosen were edgeR (Robinson *et al.*, 2010), IBB (Pham *et al.*, 2010) and PLGEM (Pavelka *et al.*, 2008). While initially developed for RNA-seq reads, edgeR uses a Negative Binomial Model and can test the data using either an exact test (Robinson and Smyth, 2007) or a generalized linear model. A Beta Binomial Test is used in IBB with variation for each protein tested separately. PLGEM is built on the Power Law Global Error Model and unlike IBB, it does not calculate variation independently for each protein.

Raw spectral counts for mature and germinated B73 pollen were processed though the three packages using parameters recommended by the package creators. The Bioconductor package 'qvalue' (Storey, 2015) was then used to determine a FDR cutoff of 0.05 to determine differential abundance between the two tissues, details in the Material and Methods. Spectral counts were not normalized against protein length as the focus of this study was on the relative change of abundance in proteins between the two developmental stages (i.e. the length of a protein will have the same effect on the spectral counts in both tissues so it does not affect the determination of differential abundance.) However, they were filtered to reduce the effect of noise (See Methods), a minimum threshold of spectral counts was determined by modeling the log of the total spectral counts and selecting a cutoff that provided an approximate normal distribution (Appendix Figure A.1). A cutoff of 25 counts was identified as the best for the comparison of the two pollen samples. This reduced the number of protein groups from 6,624 to 3,506 (Table 2.2).

There was little overlap in the proteins identified as differentially abundant between the two stages by the three packages (Figure 2.9A), with 42 percent of significant proteins selected by only one package. Additionally, only edgeR and IBB were able to identify any differentially abundant proteins in the phosphoproteome (Figure 2.9B). The 12 proteins from the total proteome identified by all three of the packages all display fold changes of 2.8x or higher and have small coefficients of variance; however, these are not traits unique to this set of proteins. When comparing fold

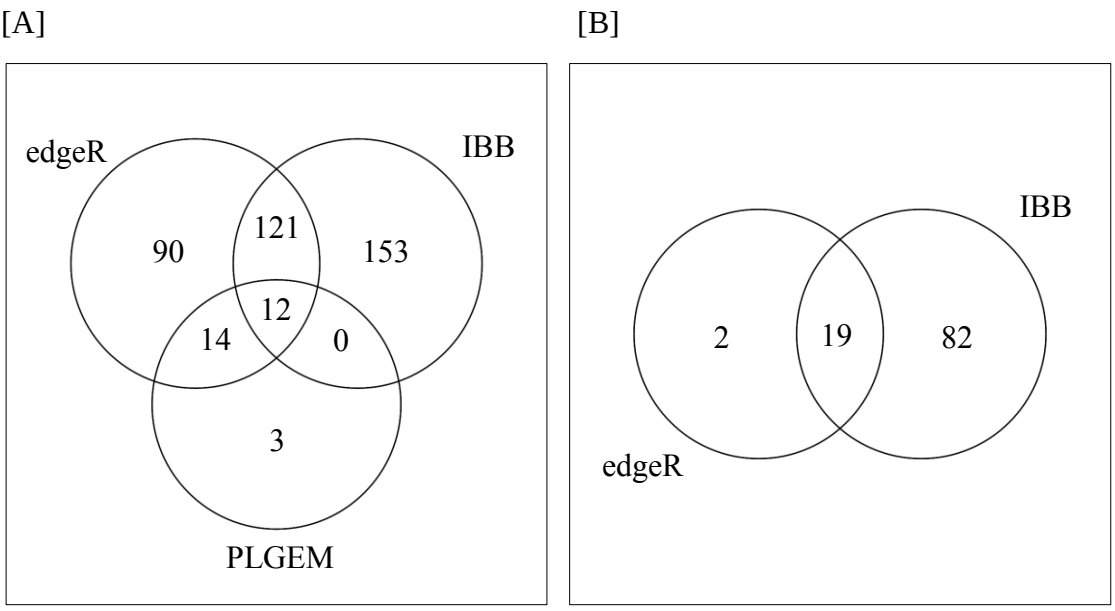


Figure 2.9: Overlap of protein designators identified as differentially abundant between mature and germinated B73 pollen by different statistical packages, FDR 0.05 in the [A] Total proteome and [B] Phosphoproteome.
The different statistical packages display low levels of agreement in the identification of differentially abundant proteins.

change, variance, standard deviation and coefficient of variance, there does not seem to be a constant pattern to the proteins selected by a single package except that the proteins identified by only edgeR or PLGEM are observed to have higher coefficients of variance in general than those found only by IBB. Overall, PLGEM appears to be more conservative in calling differential abundance than edgeR or IBB though this does not necessarily indicate that it is more successful at highlighting biologically important differences between the two tissues.

The different outcomes from the three packages serve to emphasize the point that package choice for statistical analysis can have a large effect on the interpretation of a proteomic study and some algorithms may miss potentially important proteins. For our study, we decided to use the results from all three of the packages to identify the largest pool of candidate proteins important to pollen germination. At least 393 protein groups were found to be differentially abundant, at an FDR of 0.05, by at least one package (Table 2.2). This represents 11.2% of the proteins above the threshold for statistical analysis. Of these protein groups, roughly 67.5% (265/393) exhibited decreased abundance with germination, roughly two-fold more than showed an increase. Thus, these data are consistent with the effect that could be attributed to either translational control or target protein degradation.

Comparison of mature pollen proteomes from the two maize inbred lines B73 and W23

Maize is known to exhibit large genetic variability between its different varieties (Chia *et al.* 2012), including presence/ absence variation (PAV) and differential expression. Due to its haploid state, genes important for pollen development and germination are expected to be under increased selective pressure relative to other genes. A comparison of mature pollen proteomes from different inbred lines could identify a “core set” of proteins important for male gametophyte development, as one would expect such a “core set” of proteins to be highly expressed in many, or all lines.

Spectral counts were obtained for mature pollen of the W23 inbred using the same methods as the B73 pollen samples and compared to the spectra from the B73 mature pollen samples. A majority of the proteins detected above the defined threshold were present in both tissues (2711 proteins), with 795 found to be in B73 only vs. 218 only in the W23 samples. A Spearman's rank correlation of protein abundance within the two inbreds found a value of 0.83. Using the same parameters and statistical packages as the comparison between mature and germinated pollen, 707 proteins were found to be significantly differentially abundant between B73 and W23 mature pollen. The larger number of differentially expressed proteins between B73 and W23 relative to mature versus germinated pollen may be due to the use of the B73 reference genome for assigning an identity to each peptide. As there is no currently available W23 reference genome, W23 spectra were searched against B73 protein models, however, due to polymorphisms that are present between the two lines, W23 spectra will not match all B73 models. The result is that W23 peptides are less likely to be identified and assigned to a protein.

Overall, 2,473 proteins above threshold were not found to be significantly differentially abundant between mature B73 and W23 pollen, at an FDR of 0.05. This large set likely includes proteins important to pollen function, and thus under evolutionary pressure that maintains their expression in mature pollen. Analysis of GO term enrichment in AgriGO found 362 terms to be over represented among these shared proteins. These included terms related to translation and ribosome function, protein localization and transport, GTPase activity and ubiquitin-dependent protein degradation. These enriched GO terms match functions predicted to be important to germination by our hypothesis, as well as other functions previously indicated to be important for germination and pollen tube growth.

Functional analysis of differentially abundant non-modified proteins

Lists of the proteins exhibiting significant increases or decreases in abundance with germination were uploaded to the AgriGO Analysis Toolkit and Database for Agricultural Community (Du *et al*., 2010) for functional enrichment of gene ontology

terms. Analysis of proteins increasing in abundance with germination (UP) found 38 GO terms to be enriched (Appendix Table A.4); among those proteins decreasing in abundance with germination (DOWN) 39 terms were determined to be ,enriched (Appendix Table A.5).

Consistent with our hypothesis, terms associated with the ribosome and translation were enriched in the UP proteins, as well as terms linked to the cell wall (such as pectinesterase activity) and various carbohydrate and nitrogen compound metabolism. GO terms related to transport in the nucleus and nucleotide binding were found to be enriched among the DOWN proteins. Interestingly, cell wall modification, cellular protein localization and pectinesterase activity were also enriched among the proteins decreasing in abundance with germination. Analysis with AgriGO of proteins not found to change in abundance with germination, found 355 terms to be enriched. Among these are several terms related to protein transport and localization, translation (e.g. GO translational initiation and elongation), ribosome function and components, and the proteasome complex. This suggests that some of the components that may be important to the control of germination through translation and protein degradation are already present in the mature pollen.

Characterization of the phosphoproteome

Phosphorylation of proteins occurs post-translationally and can function to target proteins for degradation (eg., by the ubiquitin dependent protein degradation pathway) or activate, deactivate, or modify the function of specific proteins (Bonifacino 2003). Phosphopeptides from mature and germinated pollen were similarly analyzed by HPLC-MS/MS following enrichment by cerium oxide. A total of 6,364 peptides were identified, representing 1,584 protein groups (Table 2.2). These peptides were filtered using the same method as the non-modified spectral counts. A cutoff of 10 counts gave an approximately normal distribution (Appendix Figure A.1) leaving 842 protein groups above filter. Of these, use of both IBB and edgeR identified 103 proteins as differentially abundant with an FDR of 0.05 (Table 2.2), with only 19 proteins identified by both packages. Comparing this to the

analyses of the transition to germination with the non-modified proteome, the PLGEM package again identified the fewest significant differences (as it gave no proteins), whereas the IBB package identified a much larger number than edgeR (Figure 2.9B).

Comparison of the changes to protein levels in the phosphoproteome to those in the total proteome found them to be largely independent of each other (Pearson's r=0.29). Among those proteins expressed in both the total and phosphoproteome, little overlap was identified in differentially abundant proteins (Figure 2.10). Of the four proteins differentially abundant in both proteomes, three were found to change in the same direction though there was little agreement in the magnitude of change in protein level. This suggests some regulation at the level of phosphorylation, and that the changes in abundance observed in the phosphoproteome were not an artifact of changes in total proteome abundance.

Functional analysis using AgriGO of the phosphoproteome identified several functional terms related to nucleotide and ribonucleotide binding, though no processes were highlighted as over represented among those proteins significantly changing in abundance. However, there were some hints of potential biological significance in the phosphoproteome. For example, several kinases and a proteasome subunit member were identified among the proteins corresponding to the functional GO terms selected by AgriGO. This raises the possibility that both kinase activity and protein degradation at germination could be regulated at least partially by phosphorylation.

Components of the jasmonic acid biosynthesis pathway were found to be enriched among proteins decreasing in abundance with germination

A list of the proteins found to change in abundance with germination was uploaded to the PlantReactome site hosted on Gramene (http://plantreactome.gramene.org) to highlight metabolic pathways that may be enriched in the changes to the proteome. Among the pathways highlighted was the jasmonic acid biosynthesis pathway. As the

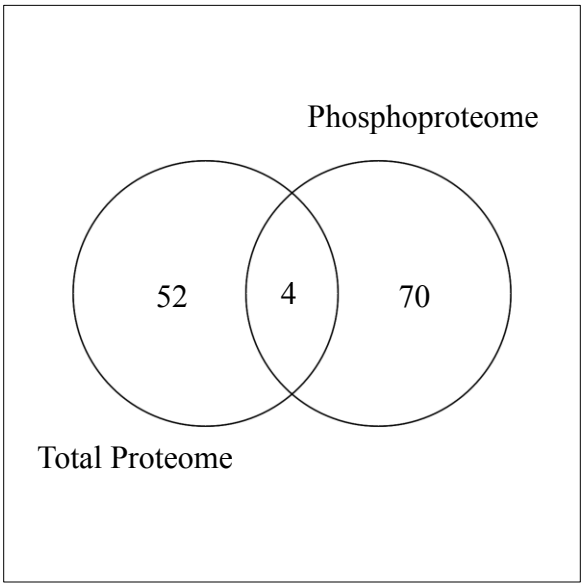


Figure 2.10: Changes in abundance of phosphorylated proteins are largely independent of changes to the total proteome.
Comparison of proteins identified as significantly changing in abundance with germination between the total proteome and the phosphoproteome from proteins found in both data sets.

full pathway is not currently available on the PlantReactome, MaizeCyc (http://maizecyc.maizegdb.org/, version 2.2) was used to manually curate the remainder of the pathway.

Of the 50 genes annotated in the jasmonic acid biosynthesis pathway, 13 were present in the proteomes of mature and germinated pollen, and three of these are proteins found to significantly decrease in abundance with germination (Table 2.3, Figure 2.11, Appendix Table A.7). Using a chi square contingency table, this represents a significant enrichment of down regulation in this pathway (p-value = 0.034). Each step in the pathway is annotated with between one to eleven genes, and several of these genes appear more than once during jasmonic acid biosynthesis. All of the steps of this pathway are represented in our proteomic dataset save for one (Reaction EC 4.2.1.92). Additionally, the majority of the annotated reactions and enzymes are unique to the jasmonic acid biosynthesis pathway (http://maizecyc.maizegdb.org/). This suggests that jasmonic acid may play a role in pollen development or germination in maize.

To test the response of maize, pollen was *in vitro* germinated in media containing increasing concentrations of methyl jasmonate (meJA) (Figure 2.12). Concentrations were selected based on similar experiments performed in other species. The meJA does reduce successful pollen tube germination in maize; however, this is due to an increase in rupturing of pollen grains similar to the effect observed by the addition of MG-132 to germination media. The length of pollen tubes that did successfully germinate were shorter at the higher meJA concentration than the control (Figure 2.13). It is unclear whether meJA-induced germination inhibition in other species (Yamane *et al*., 1981, 1982; Sembdner and Gross, 1986; Yildiz and Yilmaz, 2002) is also due to an increase in pollen grain rupture, or to a rise in the number of grains that fail to germinate.

Table 2.3: List of genes identified as present or changing in abundance with germination and annotated by MaizeCyc to participate in the biosynthesis of jasmonic acid.
Of the 50 genes included in the pathway, 13 were present in the mature and germinated pollen proteomes and 3 of these were found to significantly decrease in abundance with germination (FDR of 0.05). See Appendix Table A.7 for full list of genes annotated in the jasmonic acid biosynthesis pathway.

Gene Accession	Reaction EC	Enzymatic activity	Change in Abundance with Germination (Fold Change)
GRMZM2G102760	1.13.11.12	lipoxygenase5	No significant change (-3.1x)
GRMZM2G109056	1.13.11.12	lipoxygenase4	Significantly decrease (-3.1x)
GRMZM2G415793	5.3.99.6	Allene-oxide cyclase	No significant change (4.3x)
GRMZM2G082087	1.3.1.42	12-oxophytodienoate reductase	Significantly decrease (-2x)
GRMZM2G148281	1.3.1.42	12-oxophytodienoate reductase	No significant change (-1.4x)
GRMZM2G052389	1.3.3.6	Acyl-CoA oxidase	No significant change (-1.4x)
GRMZM2G014136	1.3.3.6	Acyl-CoA oxidase	Significantly decrease (-6.6x)
GRMZM2G445791	1.3.3.6	Acyl-CoA oxidase	No significant change (-3.5x)
GRMZM2G132903	4.2.1.17, 1.1.1.35	Enoyl-CoA hydratase 2	No significant change (1.7x)
GRMZM2G459755	4.2.1.17, 1.1.1.35	Long-chain-3-hydroxyacyl-CoA dehydrogenase	No significant change (-1.6x)
GRMZM5G854613	4.2.1.17, 1.1.1.35	Long-chain-3-hydroxyacyl-CoA dehydrogenase	No significant change (-1.6x)
GRMZM2G033491	4.2.1.17	Enoyl-CoA hydratase 2	No significant change (-3.7x)
GRMZM2G110201	2.3.1.16	Acetyl-CoA C-acyltransferase	No significant change (2.6x)

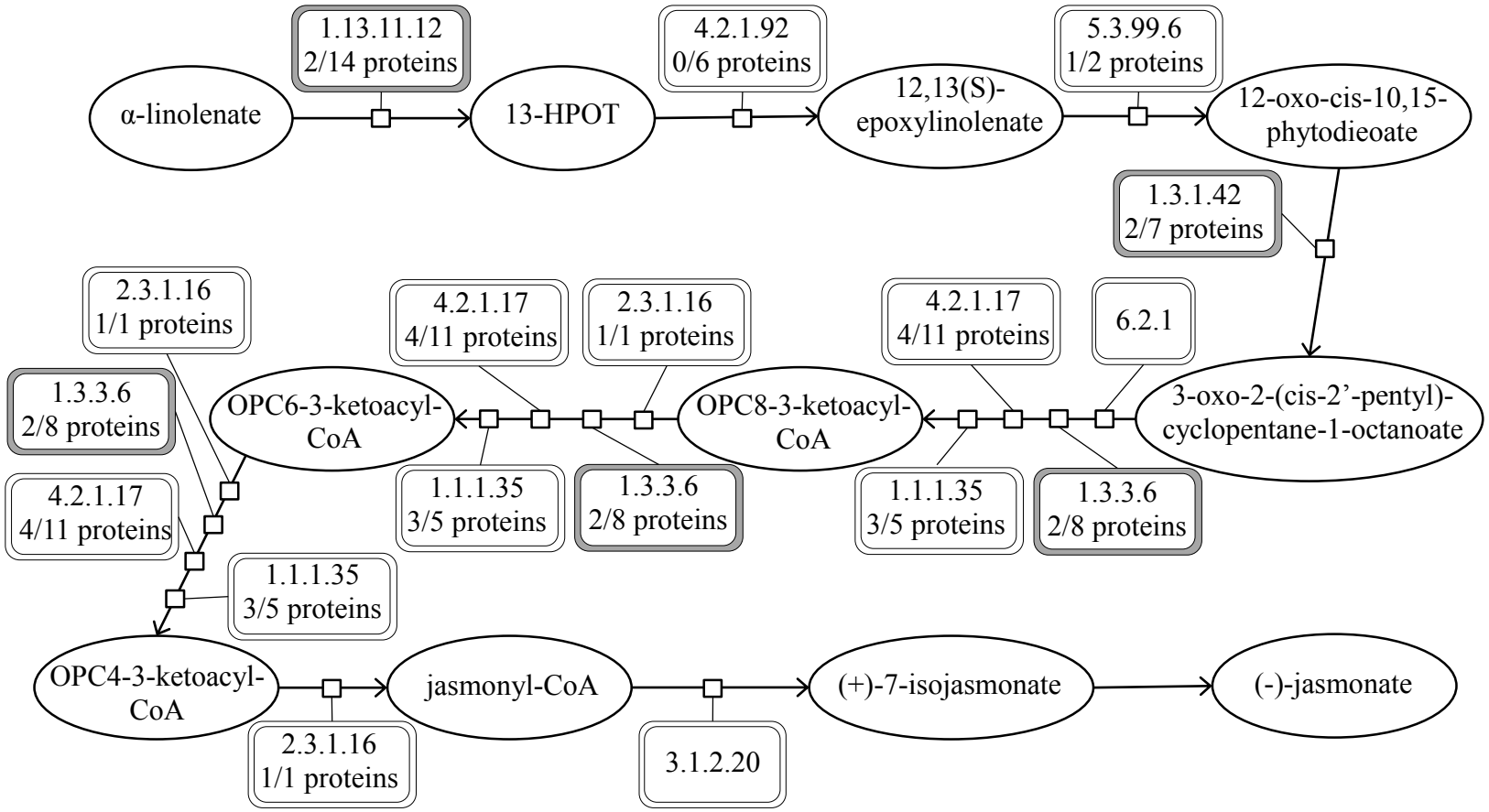


Figure 2.11: Reaction pathway for the biosynthesis of jasmonic acid.
Enzymes are indicated by boxes with double borders and the number of proteins found in the pollen proteomes out of the total proteins annotated in maize are listed below the reaction EC for that step; shaded borders indicate steps where at least one protein was found to significantly decrease in abundance with germination.

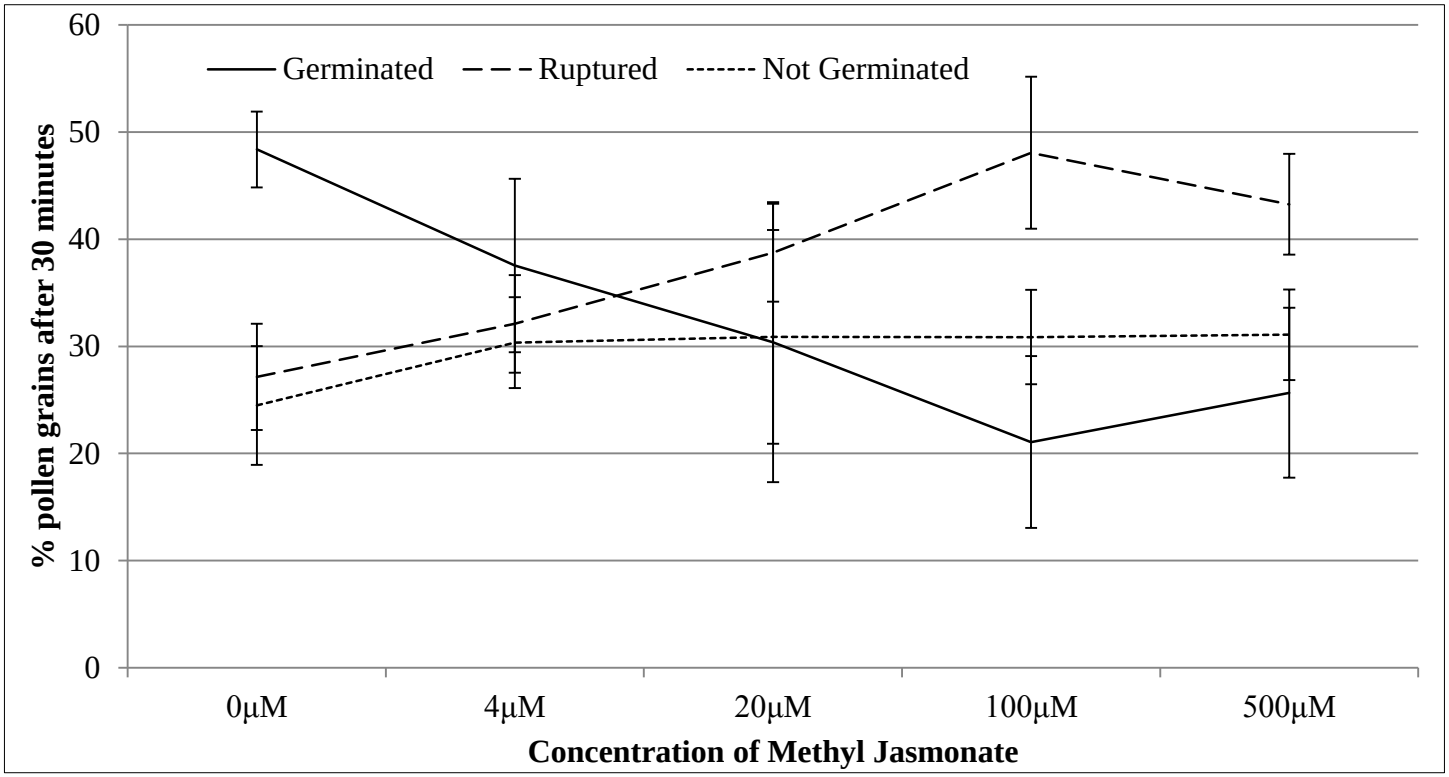


Figure 2.12: Pollen germination rates in response to increasing concentrations of methyl jasmonate after 30 minutes. Addition of methyl jasmonate to pollen germination media is correlated with a reduction in germination of pollen grains and an increase in rupturing. Error bars indicate 95% confidence intervals, n=4; at least 252 pollen grain categorized per replicate.

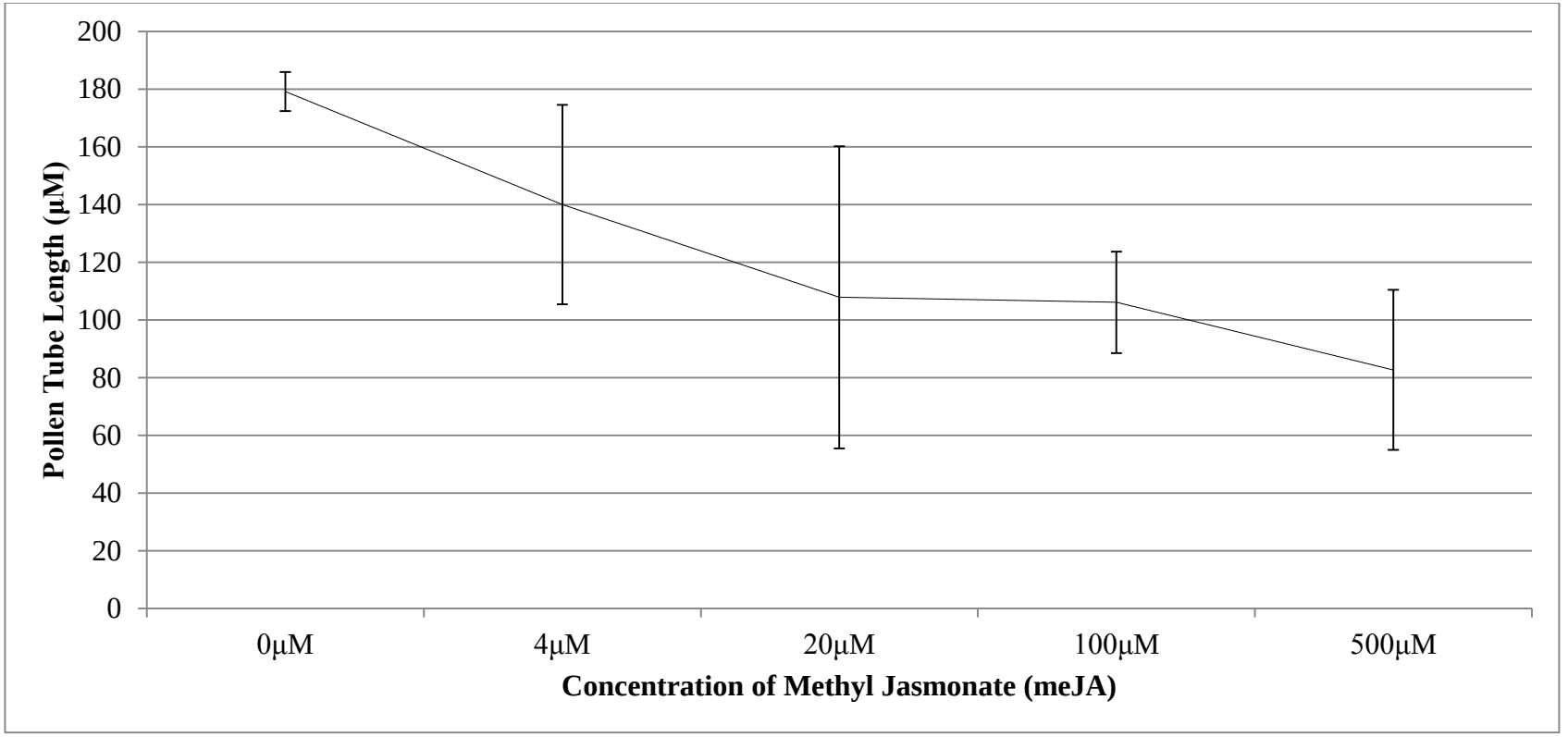


Figure 2.13: Pollen tube lengths in response to increasing concentrations of methyl jasmonate after 30 minutes.
Shortening of pollen tube length is correlated with increasing concentrations of methyl jasmonate. Error bars indicate 95% confidence intervals, n=4; at least 50 pollen tubes were measured per replicate.

Comparison of transcriptomic and proteomic datasets from mature and germinated pollen

Previous work has shown that transcript levels exhibit poor correlation with protein abundance (Gygi *et al.*, 1999; Greenbaum *et al.*, 2003; Schwanhäusser *et al.*, 2011), often due to variation in translation rates (Piques *et al.*, 2009). Comparison of the normalized counts of proteins from the mature pollen sample to a recently described transcriptome of mature pollen (Chettoor *et al.*, 2014) also reflects this poor correlation (Table 2.4). The low correlation between the mature pollen transcriptome and proteome may be partially due to the presence of proteins derived from tapetal cells that secrete pollen cell wall components in early pollen development (Bedinger and Fowler, 2009). As the tapetal cells are part of the sporophyte, protein contributions from these cells to the pollen are unrelated to the mature pollen transcriptome. Based on our hypothesis that translation plays a key role in the regulation of germination, one would expect the correlation of the mature pollen transcriptome to the germinated pollen proteome to be higher than that of the mature pollen proteome, as it should play a predictive role in indicating the proteins that will increase in abundance with germination. In agreement with our hypothesis, we see a slight increase in correlation to transcript abundance in the mature pollen transcriptome in the germinated pollen proteome relative to the mature pollen proteome, especially among those proteins increasing in abundance with germination (Table 2.4).

The GO terms identified as enriched by this proteomic study were compared with the terms reported in a previous transcriptomic study of mature pollen (Chettoor *et al.* 2014). While there was overlap in our findings, functional analysis of the proteomic data was also able to identify enrichment of terms related to stages of translation, translation factors, ribosome activity and aspects of the proteasome consistent with our hypothesis of control of germination through the regulation of protein levels. Additionally terms related to signaling and vesicle transport were highlighted, functions important for the directed cell growth of germination.

Table 2.4: Table of Person's correlation coefficients between the transcriptome of mature pollen and the proteomes of mature and germinated pollen.
Correlation coefficient was calculated using normalized counts from proteins where all reported peptides could be uniquely assigned to that protein.

	Mature Pollen Transcriptome vs.	
	Mature Pollen Proteome	**Germinated Pollen Proteome**
All Proteins	0.176	0.217
Increase in Abundance with Germination	0.216	0.265
Decrease in Abundance with Germination	0.087	0.087

Intron retention does not appear to play a role in the regulation of protein abundance during the transition from mature to germinated pollen

If intron retention were a factor in the increases in protein abundance in the germinating maize pollen tube (as it is in the microspore of *Marsilea vestita*, (Boothby *et al*., 2013)), than one would expect to see an enrichment of intron retention in the mature pollen transcriptome of genes whose protein products are significantly up regulated with germination. Transcriptomic data for mature B73 pollen was retrieved from a published data set (Chettoor *et al.*, 2014) for transcripts coding for proteins significantly increasing in abundance with germination, for those that significantly decreased in abundance and for a random selection of highly expressed genes. Reads were aligned using the B73 RefGen_v2 genome and .gff file. Counts of reads aligning to exons and introns were gathered for each gene, and percentages of genes containing reads in introns was then calculated for each of the three groups (Table 2.5). Both a manual inspection and this more comprehensive look failed to find evidence of a correlation between intron retention and increased protein abundance as similar percentages of genes were observed to retain introns irrespective of protein abundance. This argues against intron retention functioning as a mechanism regulating protein abundance during the transition to the progamic phase in maize.

Genes identified by quantitative proteomic show reduced rates of insertions relative to proteins whose abundance is enriched in sporophytic tissue

The Reduced Recovery of Mutation test uses the three large sequence-indexed transposon insertion populations available in maize (*UniformMu* (Settles *et al.*, 2007), *Mu-Illumina* (Williams-Carrier *et al.*, 2010) and *DsMutagenesis* (Volbrecht *et al.*, 2010)) to screen for reduced rates of transposon insertion in genes predicted to be important for gametophyte function (Chettoor *et al*., 2014). This transmission assay is based on three tenets: the functional haploidy of gametophytes, the assumed functional interference of insertions in coding regions of genes and competition between pollen grains for fertilization in male crosses. The prediction derived from

Table 2.5: Retention of introns is not enriched in transcripts coding for proteins found to increase in abundance upon germination.
Percentages of proteins with intron retaining transcripts are similar between proteins increases in abundance with germination, those decreasing in abundance with germination and a random selections of transcripts highly expressed in mature pollen.

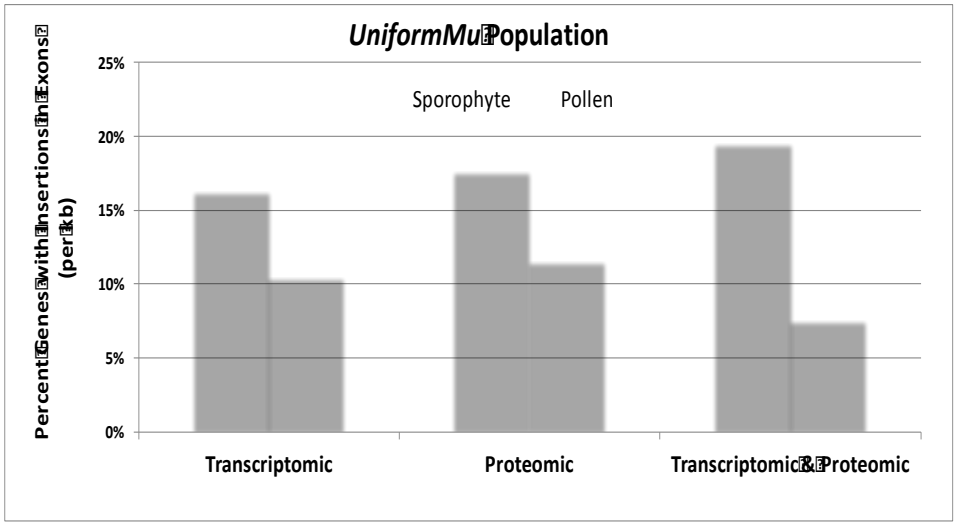
	Total in Group	Do Not Retain Introns	Retain Introns
Proteins Increasing in Abundance	125	79 (63.2%)	46 (36.8%)
Proteins Decreasing in Abundance	258	175 (67.8%)	83 (32.2%)
Random Selection of High Abundance Proteins	115	70 (60.9%)	45 (39.1%)

these is that genes critical for gametophyte function will harbor fewer transposon insertion mutations relative to genes expressed in sporophytic tissues.

Given that the analysis of the proteomic dataset in this study identified a different (but overlapping) set of expressed genes (protein rather transcript) in maize mature pollen relative to the Chettoor *et al.* 2014 study, it was important to determine if the proteomics-defined gene sets showed similar trends to the transcriptomic-defined sets in insertion frequency when tested by RRM. The Chettoor *et al.* 2014 study found genes whose expression where enriched in gametophytic tissue were significantly less likely to contain a transposon insertion relative to genes expressed in sporophyte tissue. As the previous analysis showed that the most significant effect was apparent with insertions in exons (consistent with the prediction, as these insertions are most likely to abrogate gene function) (Chettoor *et al.* 2014), we focused only on the frequencies of insertions located in exons.

A 'pollen-enriched' gene set was defined using proteins that were significantly more abundant in Mature Pollen relative to Mature Leaf, and a comparator 'sporophyte-enriched' set was determined using the converse. The resulting two gene sets were compared to previously-defined transcriptome-based predicted gene sets for pollen and seedling and divided into three subsets of genes with enriched expression: those identified solely by transcriptomic data; those identified solely by proteomic data; and those identified by both the transcriptomic and proteomic data. When cross-referenced to a database of sequenced-indexed insertion sites, the gene sets provided by the proteomic data gave very similar trends to those seen previously with transcriptomic data (Figure 2.14). That is, in the *UniformMu* population (propagated through male) (Figure 2.14A) but not the *DsMutagenesis* population (propagated through the female) (Figure 2.14B), a significantly reduced frequency of mutations was observed in pollen-enriched genes relative to sporophyte-enriched genes. The different percentages in the analysis are due to the size of the populations: 73,392 insertions have been mapped in *UniformMu*, whereas only 1,969 have been mapped in *DsMutagenesis* leading to overall fewer insertions in exons. Notably, the greatest

[A]



[B]

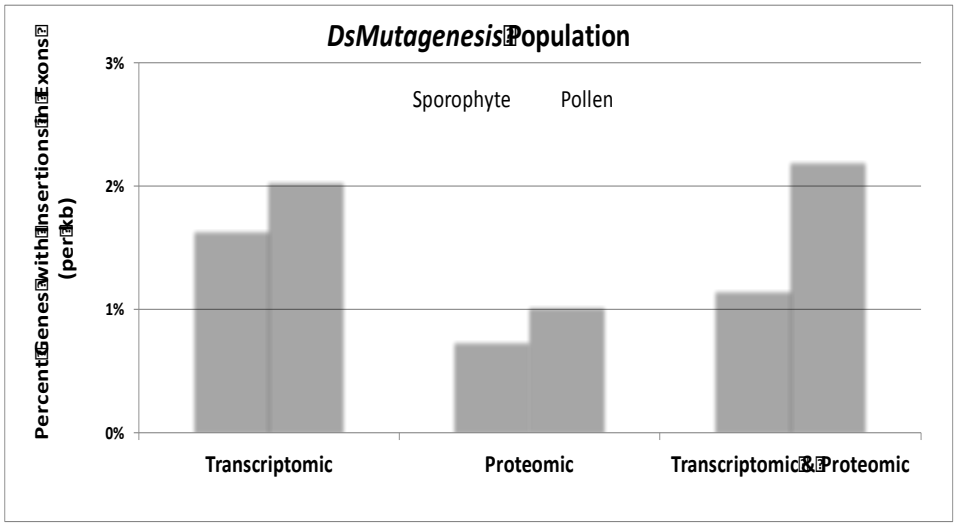


Figure 2.14: A reduced frequency of insertions in 'pollen-enriched' genes (those expressed highly in the mature pollen transcriptome or proteome) relative to 'sporophyte-enriched' is observed in the [A] the UniformMu population but not in [B] the DsMutagenesis population.
This reflects the propagation of insertions through the male in [A] and through the female in [B] and is seen in gene sets predicted from both transcriptomic and proteomic studies.

effect in the *UniformMu* population was associated with genes enriched in both the transcriptomic and proteomic datasets, which showed a 2.6-fold decrease in insertion frequency (7.4% per kb of exon in pollen-enriched genes vs. 19.5% per kb of exon in sporophyte-enriched genes). As expected, similar trends were seen in the *Mu-Illumina* population as in the *UniformMu* population (10,877 insertions mapped; data not shown). This analysis suggests that enrichment in the proteomic dataset can successfully identify genes whose proteins impact the development or function of the male gametophyte. Further extrapolation of the results suggests that genes associated with high levels of both transcript and protein at the mature pollen stage, are the most likely set important for pollen function, as they exhibit greatest reduction in the frequency of insertions.

The RRM test was also used to compare insertion frequencies in proteins determined to exhibit differential abundance in either the total proteome or phosphoproteome between mature and germinated pollen (Chapter 2). Because these protein sets are relatively small, we used only the largest population, *UniformMu* (Figure 2.15). As a comparator, we used gene sets in which the associated protein was above the detection threshold in mature pollen, but for which there was no statistical support for differential protein abundance between mature and germinated pollen, i.e., those not meeting the threshold of either FDR <0.05 or the less stringent $p<0.05$. Genes associated with decreased protein abundance in germinated pollen (GP) had a similar insertion frequency as those with no change detected (13.3% and 12.9% per kb of exon, respectively). However, the frequency was much lower (7.8% per kb of exon) for those genes associated with increased abundance in germinated pollen, suggesting that these 125 genes are likely important for pollen function. For proteins assessed in the phosphoproteomic analysis, the analysis did not support the idea that a change in phosphorylation status was associated with reduced recovery of mutations, relative to other phosphorylated proteins that did not change significantly upon germination (11.1% vs. 9.8%, respectively). However, given that both these frequencies are lower than those for proteins that either decrease or remain unchanged in abundance, there

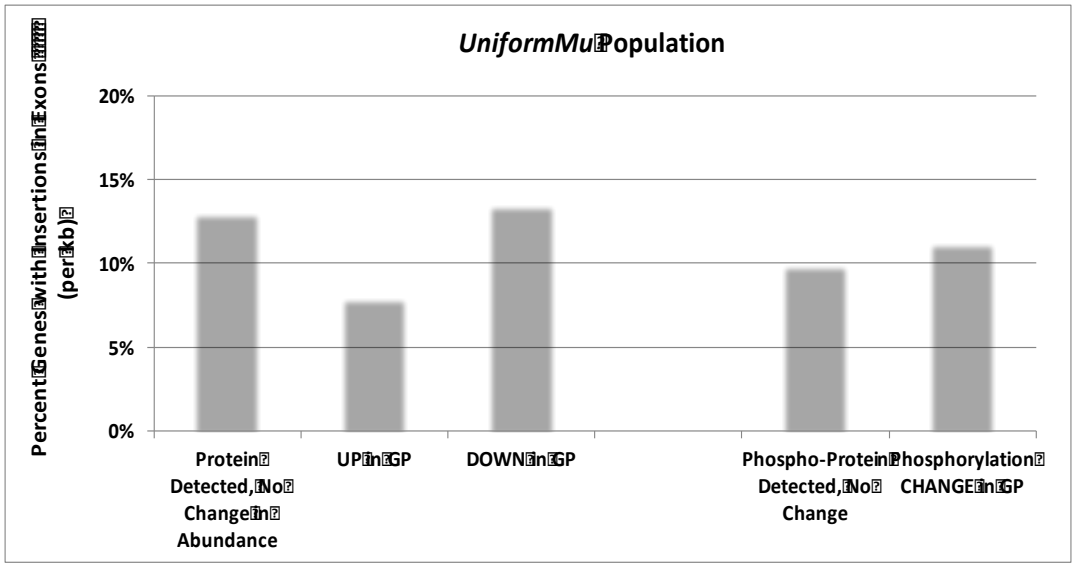


Figure 2.15: Genes associated with increased total proteome abundance in germinated pollen are less likely to contain insertions in exons and phosphorylated proteins exhibit lower overall rates of exon insertions. The lower insertions rates in these two groups suggest a higher likelihood of functional importance in the germinating pollen grain.

is a possibility that phosphoproteins in general may be associated with a higher likelihood of functional importance in pollen.

Mutation in five of ten genes selected by proteomic profiling are associated with male transmission defect

As a validation of our approach to study the developmental transition from mature to germinated pollen by characterizing changes to the pollen proteome, a transmission study was performed on *Ds* and *Mu* insertions in genes encoding proteins identified by the proteomic analysis (Table 2.6). This is similar in concept to the RRM test, except it focuses on assessing transmission of specific insertions through the male and the female gametophyte, with the prediction that genes identified by proteomic profiling should be associated with male-specific transmission defects. Lines containing insertions for specific predicted genes of interest were selected from the three insertion populations (*UniformMu, Mu-Illumina* and *DsMutagenesis*), and individuals heterozygous for each insertion were reciprocally outcrossed as a male and as a female to a homozygous wild-type tester. Presence of the insertion in the progeny of these crosses was then scored to determine if there is evidence of reduced transmission of the transposon insertion to the offspring via the male or female gametophyte. As a haploid, the pollen grain will carry only one copy of the gene, either of the wild type or of the insertion (mutant) allele. If there is no defect in the transmission of the insertion allele of the gene, about 50% of the offspring should carry the insertion through both the male and female crosses in accordance with Mendelian inheritance (Figure 2.16B). However, if the identified protein is important for germination or other pollen function, an insertion in the gene for that protein will inhibit the ability of a pollen grain carrying it to successfully fertilize the female gametophyte. Conversely, the insertion should have little or no effect on the ability of a female gametophyte to pass the insertion on to its offspring. This will lead to an observed transmission of less than 50% in the progeny from the male cross but a transmission of about half for the female (Figure 2.16A).

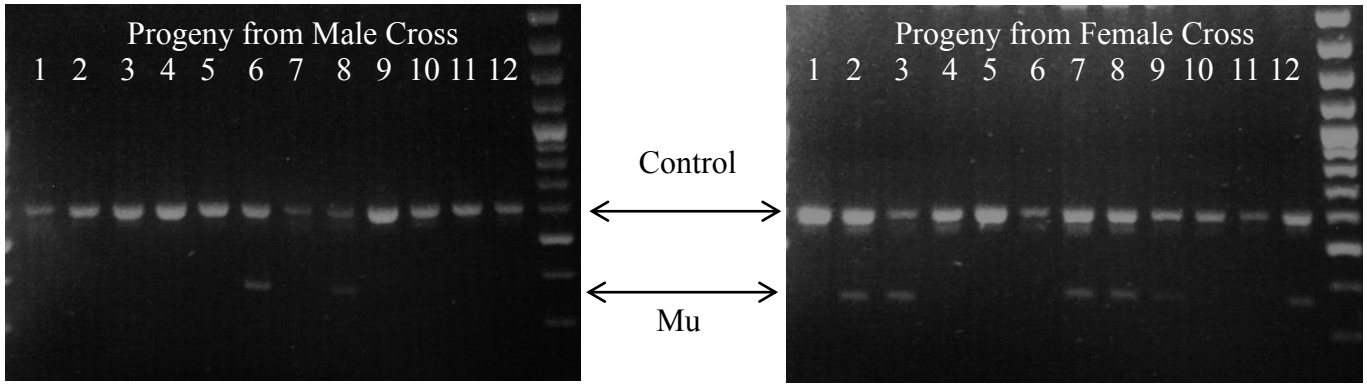
Table 2.6: **Male-specific transmission defects were observed in 5 out of the 10 insertions tested via PCR genotyping.** Crosses represent 29-73 progeny. For the 10 insertions, female transmission rates ranged from 44.8-57.1%. Insertions were selected from candidate genes displaying increased protein abundance with germination (GPP UP), a change in phosphorylation state with germination (Phos. change) or high expression in the mature pollen transcriptome and proteome (High MPT&MPP). The last two insertions in this table (below the triple line border) were tested for transmission but omitted from the final tally as they were found to not be located in exons.

Gene ID	Predicted Function	Insertion Pop.	Character	Defect? (χ^2)	Male Transmission Rates
GRMZM2G173289	7 Transmembrane receptor	AcDs	GPP UP	Unclear (0.004 & 0.465)	28% and 45%
GRMZM2G030265	Polygalacturonase	AcDs	GPP UP	Yes (0.01)	27%
GRMZM2G066024	Fructose-Bisphosphate Aldolase Cytoplasmic Isozyme	UniformMu	GPP UP	Yes (1.97E-09)	0%
GRMZM2G133282	Armadillo repeat protein	Mu-illumina	High MPT&MPP	Yes (0.045)	33%
GRMZM2G301647	Serine/Threonine protein kinase	Mu-illumina	High MPT&MPP	Yes (2.67E-07)	6%
GRMZM2G389462	Ubiquitin ligase	UniformMu	High MPT&MPP	Yes (0.0001)	17%
GRMZM2G318992	KIP1 - NET actin-binding	UniformMu	High MPT&MPP	No (0.18)	59%
GRMZM2G422641	KIP1 - NET actin-binding	UniformMu	High MPT&MPP	No (0.61)	53%

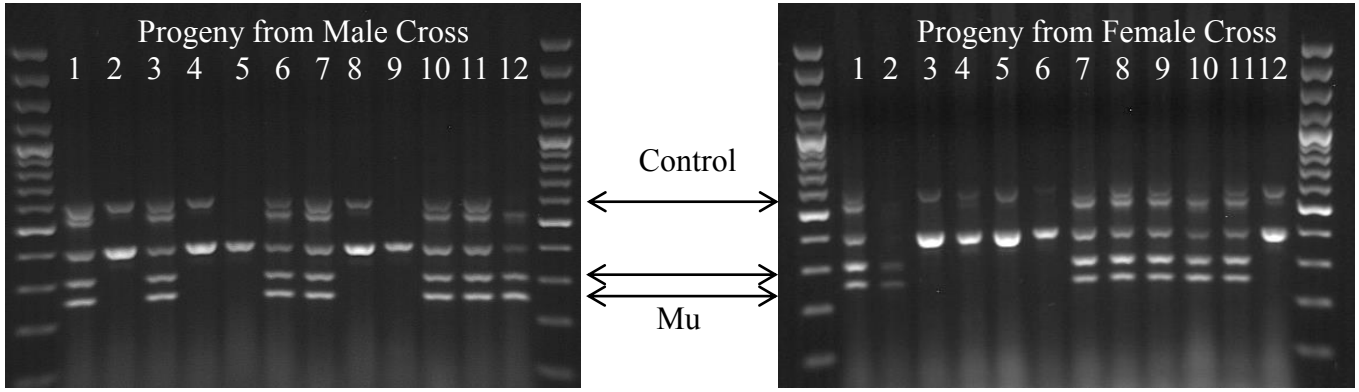
Table 2.6: Continued

Gene ID	Predicted Function	Insertion Pop.	Character	Defect? (χ^2)	Male Transmission Rates
GRMZM2G457370	Male Gametophyte Specific Argonaute	UniformMu	Phos. Change	No (0.31)	40%
GRMZM2G017334	2-phosphoglycerate kinase-related	UniformMu	Phos. Change	No (0.09)	66%
GRMZM2G102404	Acid Phosphate Hydrolyase, UNKNOWN	UniformMu	GPP UP	No (0.37, 0.38)	58%,58%
GRMZM2G156365	Pectinacetylesterase Family Protein	UniformMu	High MPT&MPP	No (0.51)	51%

[A]



[B]



Caption on next page.

Figure 2.16: Examples of insertions that exhibited and did not exhibit reduced transmission through the male.
[A] The *Mu* insertion mu1057062 in GRMZM2G389462 shows reduced transmission through the male (2/12), transmission in the female is at 50% (6/12). [B] The *Mu* insertion mu1042381 in GRMZM2G422641 shows no reduction in transmission through the male (7/12) relative to the female (7/12). The band characteristic of the insertion is indicated, along with an internal control for an allele found in both the wild type and *Mu* containing siblings to ensure the PCR was working.

Twelve candidate genes (Table 2.6) were chosen based on availability of insertion alleles from public *UniformMu* (Settles *et al.*, 2007), *Mu-Illumina* (Williams-Carrier *et al.*, 2010) and *DsMutagenesis* (Vollbrecht *et al.*, 2010) populations and robust PCR genotyping reactions. These candidates were proteins that were identified as either (1) increasing in abundance with germination, (2) exhibiting a change in phosphorylation state at this transition, or (3) highly expressed in both the mature pollen transcriptome and proteome. Our initial goal was to limit our choice to alleles in which insertions were within an exon, as these would be most likely to interrupt protein function. However, upon closer inspection and updated data, we found that three alleles assessed, mu1008434 and mu1045886 in GRMZM2G102404, and mu1044656 in GRMZM2G156365, were likely not exon insertions, but rather located in either the 5' UTR or an intron (Table 2.6). Though these three alleles were not found to cause a transmission defect in the male or female crosses, the two genes were removed from further analysis until verified exon insertions in these genes can be identified for testing.

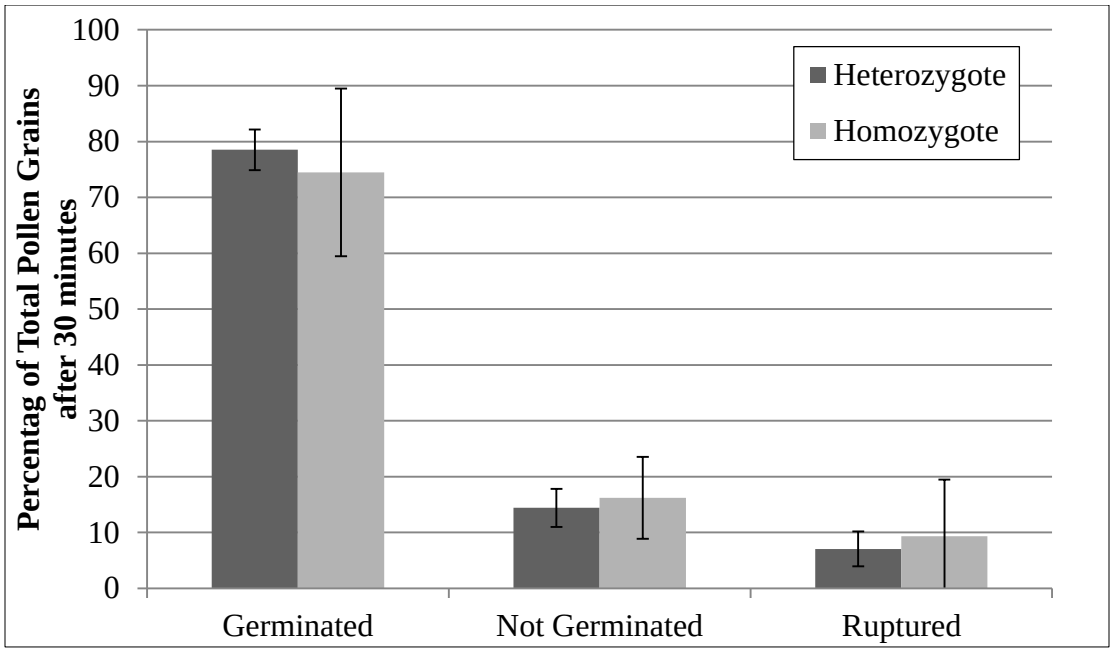
Comparison of allele transmission using a chi-square test with a cut-off of p-value = 0.05 (Table 2.6; Appendix Table A.7), found five out of the final ten tested insertions to exhibit a male specific transmission defect (Table 2.6, Appendix Table A.8). These five proteins include an ubiquitin ligase (GRMZM2G389462) providing a potential link to proteasome-mediated degradation; a protein involved in glycolysis (GRMZM2G066024); and proteins relating to signal transduction (GRMZM2G133282 and GRMZM2G301647) and the cell wall (GRMZM2G030265). An additional gene, GRMZM2G173289, displayed a male transmission defect in one test cross (X^2=0.004); however, the test of a second cross did not replicate this defect (X^2=0.465) (Table 2.6) leaving the effect of an insertion in this gene on pollen function currently unclear.

The percentage of defect inducing insertions identified by this study is higher than both a control study of insertions in sporophyte expressed genes, which found only one insertion out of 16 to have a possible defect, and a similar study that was done based on predictions solely from pollen transcriptome data (Chettoor *et al.*, 2014). The transcriptome study used nine A*cDs* insertions in genes highly expressed in mature pollen and found reduced transmission in two cases. This supports the use of proteomic data to identify biologically important components controlling the transition to pollen tube germination. It is important to note that the five insertions found to have transmission defects in this study are from single alleles, and thus we cannot yet definitively conclude that the insertions we tracked are causal for the defects observed.

Phenotyping of pollen from insertions displaying a male specific transmission defect reveals trends towards reduced germination rates and an insertion with significantly smaller non-germinated pollen grains

Collecting phenotype information for insertions displaying reduced transmission through the male can give indications of the functional activity of the identified proteins during pollen development or germination. Pollen from four of the insertions exhibiting a male specific transmission defect (GRMZM2G133282, GRMZM2G030265, GRMZM2G301647, GRMZM2G389462) and the unclear GRMZM2G173289 insertion were *in vitro* germinated in media in an attempt to determine the phenotype underlying the associated defects. The study was performed on heterozygous and homozygous wild-type siblings for the identified insertions and images were scored for germination rate, pollen tube length and pollen grain diameter. Two of the insertions exhibited no discernable phenotype: GRMZM2G173289 (a predicted seven transmembrane receptor) and GRMZM2G133282 (an armadillo repeat protein) (Figure 2.17 and 2.18) as 95% confidence intervals for germination rates, pollen tube length and pollen grain width were not significantly different did not overlap between siblings containing the insertion and ones containing the wild type allele. For siblings heterozygous for the insertion in the GRMZM2G030265 (a polygalacturonase) and GRMZM2G389462 (a

[A]



[B]

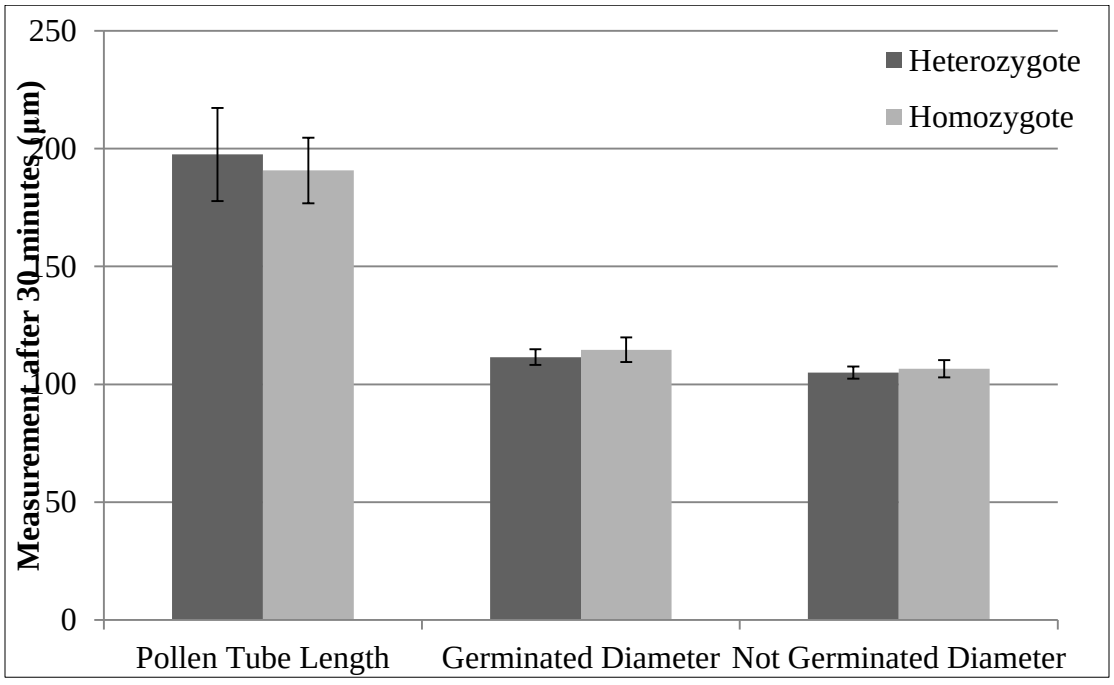
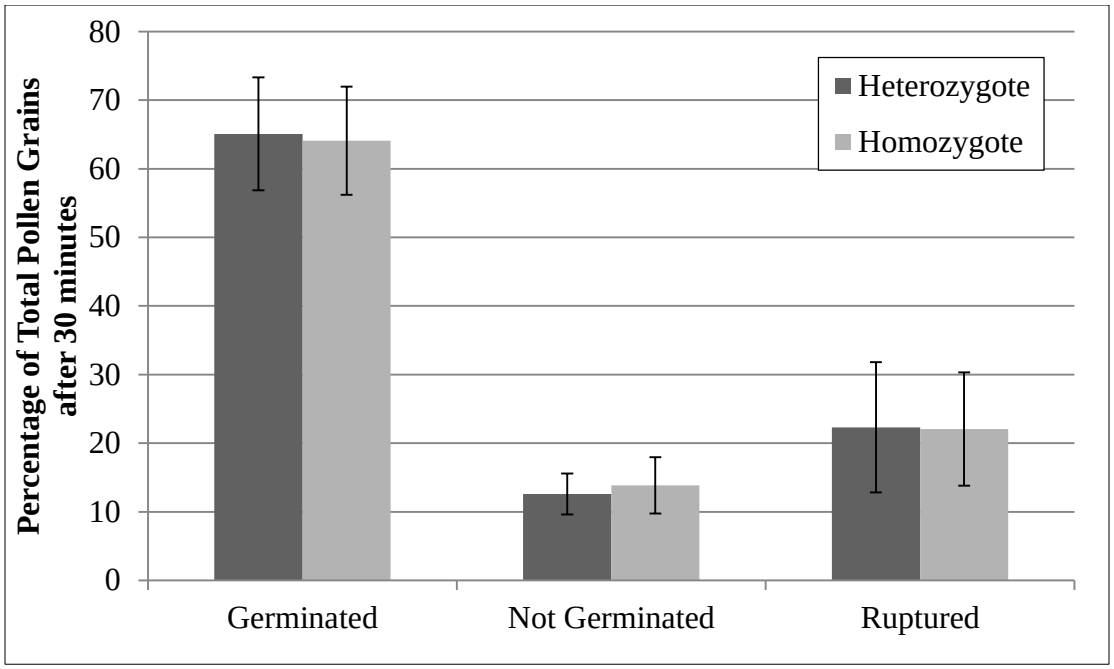


Figure 2.17: Germination rates, pollen tube lengths and pollen grain diameters from phenotyping of GRMZM2G173289, a predicted seven transmembrane receptor.

No significant difference in germination rates [A] or pollen tube lengths and pollen diameters [B] was detected in between wild type siblings and siblings with the insertion. Bars represent 95% confidence intervals, n=5-6.

[A]



[B]

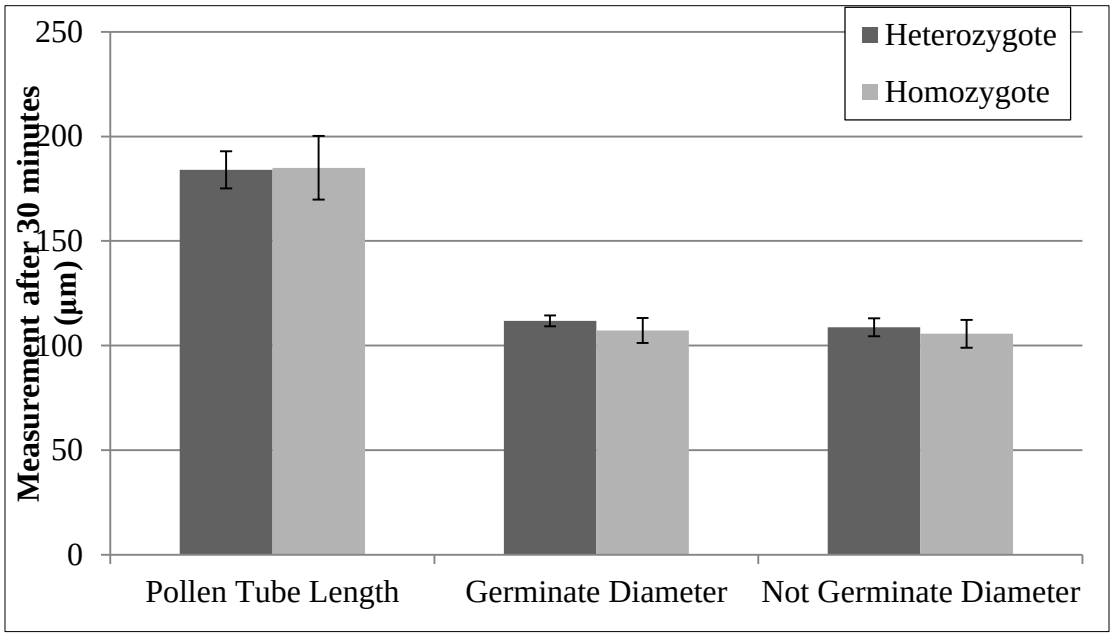


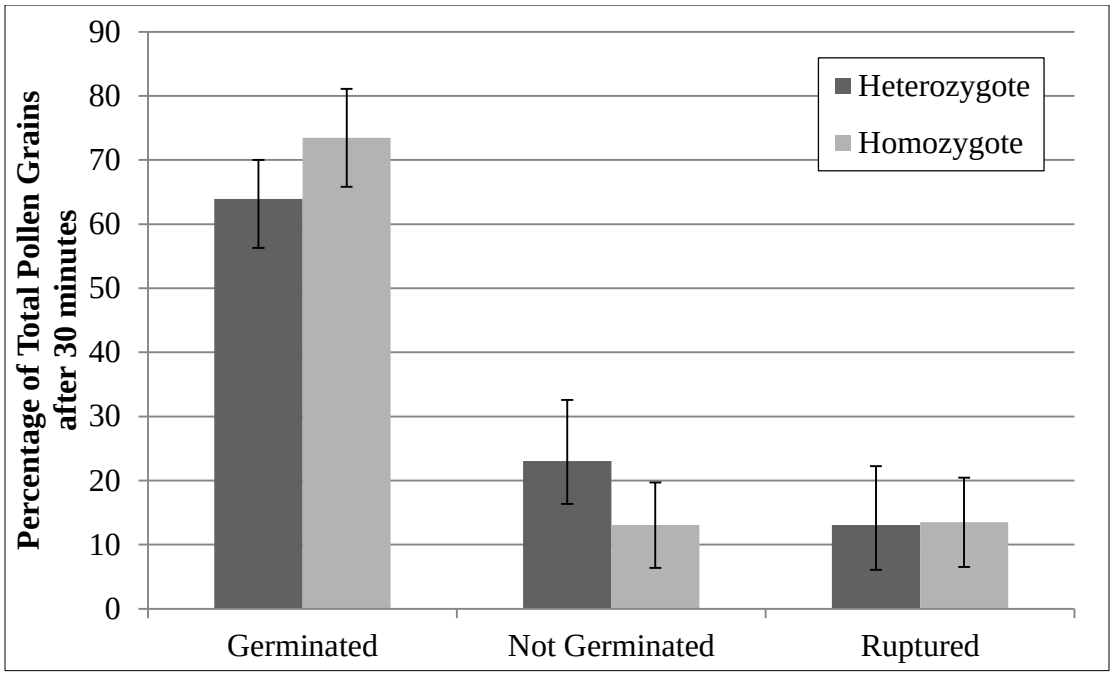
Figure 2.18: Germination rates, pollen tube lengths and pollen grain diameters from phenotyping of GRMZM2G133282, an armadillo repeat protein.

No significant difference in germination rates [A] or pollen tube lengths and pollen diameters [B] was detected in between wild type siblings and siblings with the insertion. Bars represent 95% confidence intervals, n=5-6.

ubiquitin ligase), there was a trend towards reduced germination and shorter pollen tubes, however there was a slight overlap in 95% confidence intervals (Figure 2.19 and Figure 2.20). The diameters of non-germinated pollen grains from plants heterozygous for an insertion in GRMZM2G301647 (a serine/threonine protein kinase) were smaller than the diameters of their wild type siblings' (Figure 2.21B) and their 95% confidence intervals did not overlap. There was also a trend towards reduced germination and shorter pollen tubes (Figure 2.21A and B).

Given that the parent plants are heterozygous for the insertions, their pollen represents a mixed population with half of the pollen grains harboring the insertion mutation and the other half the wild type allele. Therefore, plotting the diameters from all pollen grains from individuals heterozygous for the insertion in the GRMZM2G301647 serine/threonine protein kinase should exhibit evidence of these two pollen types. This was the observed result, as a histogram of pollen grain diameters from populations derived from heterozygotes covered a broader range than those from populations derived from their wild type siblings (Figure 2.22A and B). The larger diameters in this population overlap with those from the wild type siblings, suggesting these are the diameters of the grains carrying the wild type allele. The diameters in the lower range are presumably the diameters of the pollen grains containing the insertion allele. Similarly, histograms of pollen tube lengths from the pollen populations derived from plants heterozygous for the GRMZM2G030265 polygalacturonase and their wild type siblings were also plotted. Again, evidence of two populations of pollen grains was detectable in the pollen derived from heterozygotes (Figure 2.23), suggesting a link between loss of function in this gene and defects in pollen tube growth.

[A]



[B]

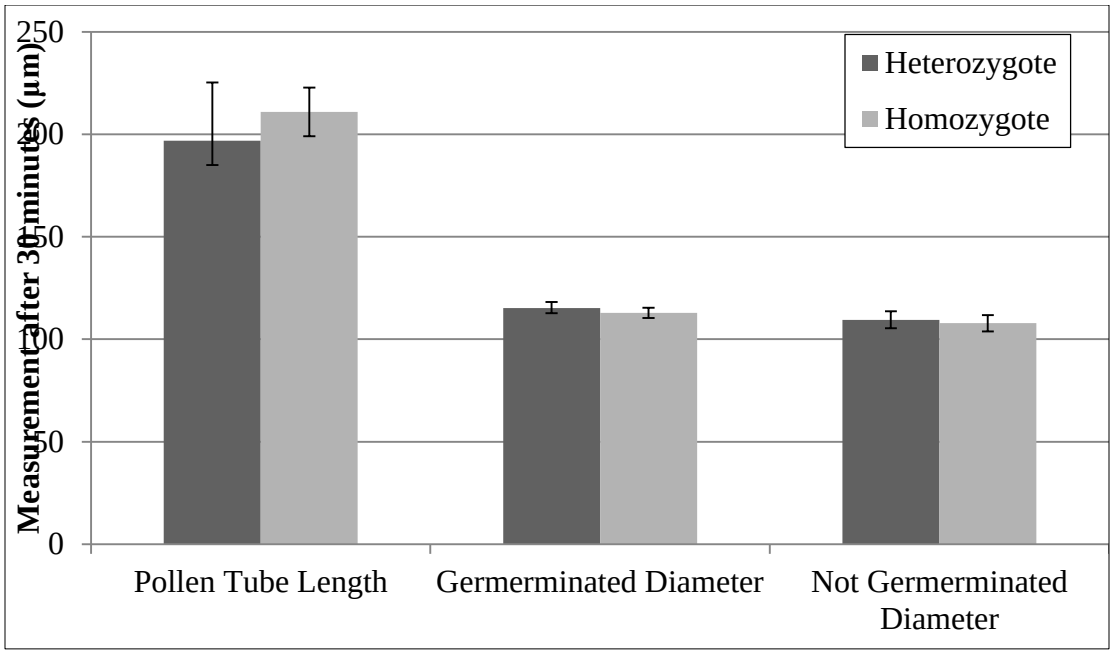
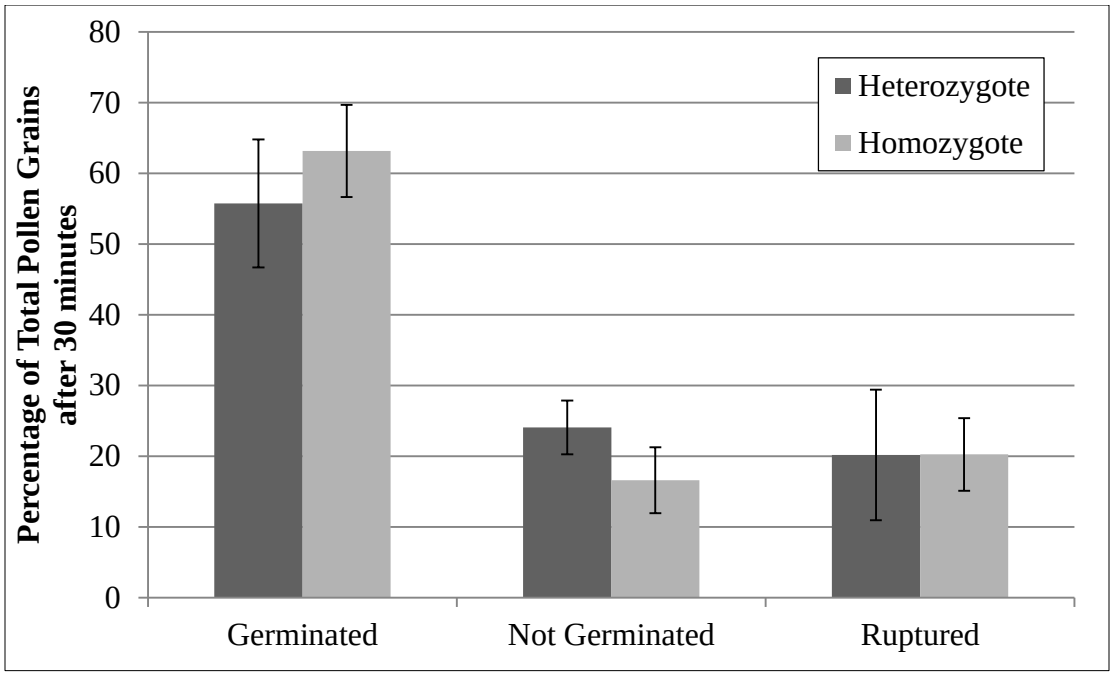


Figure 2.19:Germination rates, pollen tube lengths and pollen grain widths from phenotyping of GRMZM2G030265, a polygalacturonase.
A trend towards reduced germination [A] and shorter pollen tubes [B] was observed in individuals with the insertion in GRMZM2G030265.No significant difference was detected in diameters of germinated and non-germinated pollen grains [B]. Bars represent 95% confidence intervals, n=5-6.

[A]



[B]

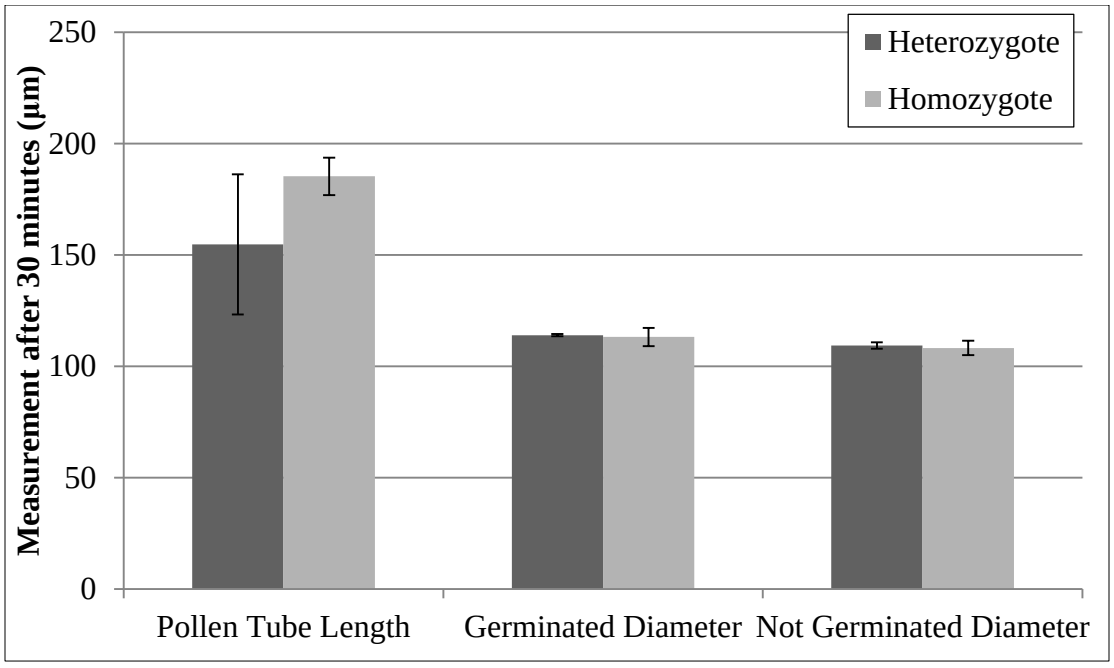
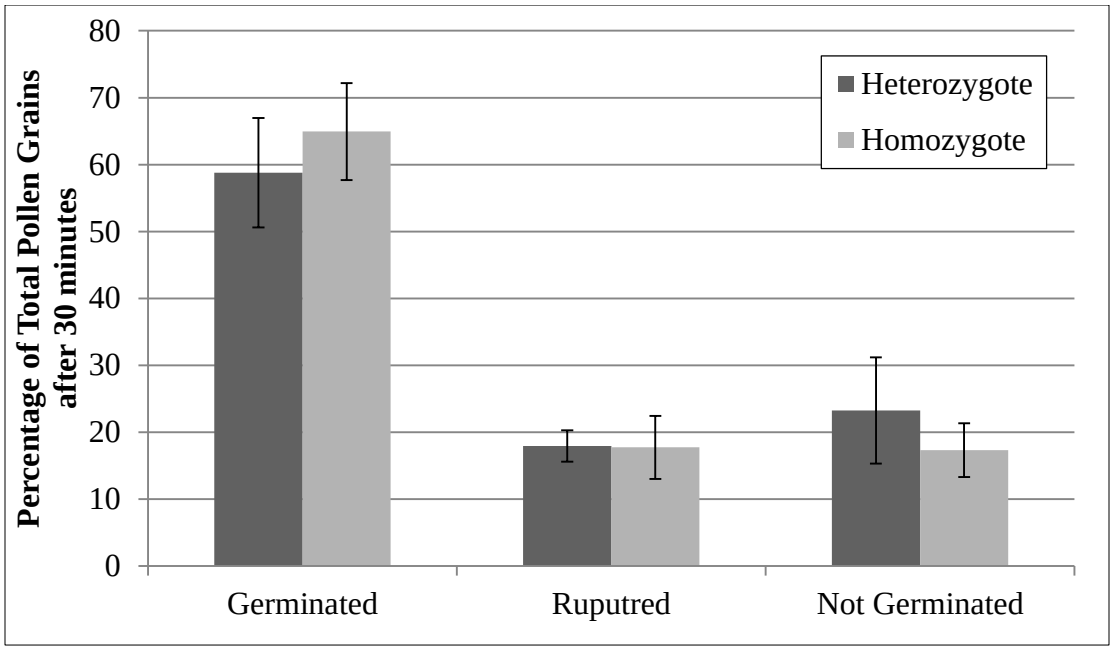


Figure 2.20:Germination rates, pollen tube lengths and pollen grain widths from phenotyping of GRMZM2G389462, a ubiquitin ligase.
A trend towards reduced germination [A] and shorter pollen tubes [B] was observed in individuals with the insertion in GRMZM2G389462. No significant difference was detected in diameters of germinated and non-germinated pollen grains [B]. Bars represent 95% confidence intervals, n=5-6.

[A]



[B]

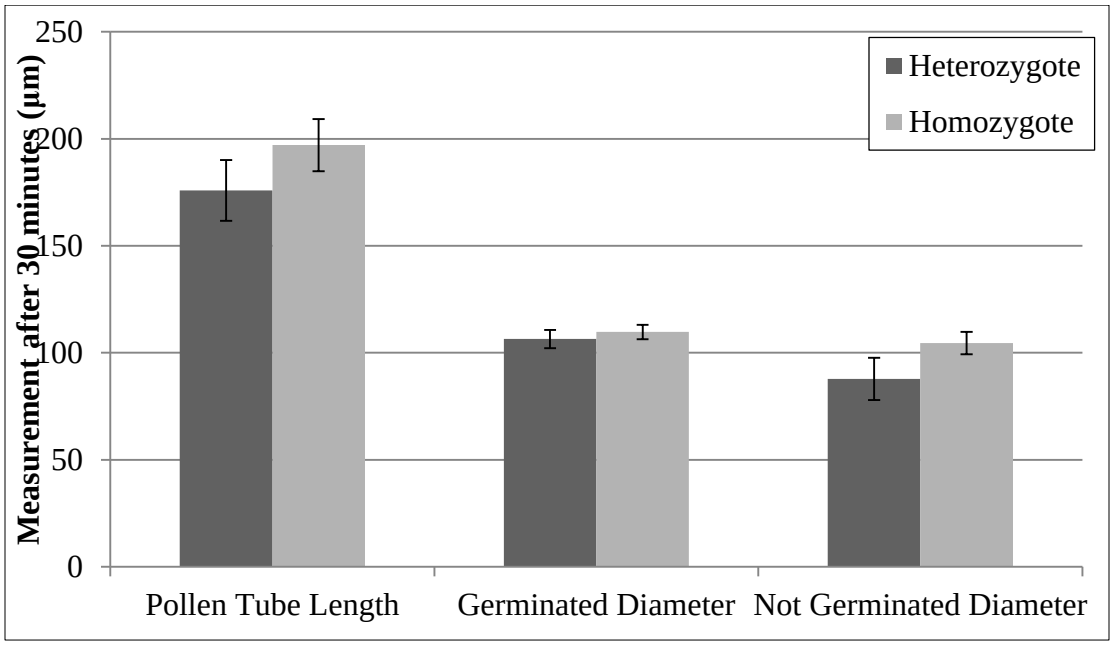
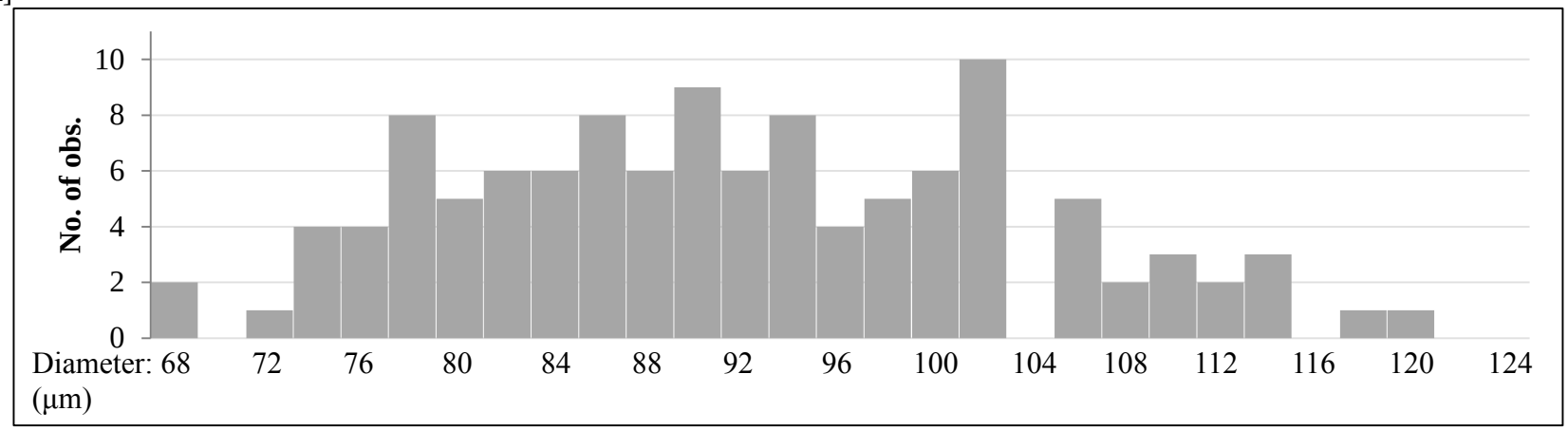


Figure 2.21:Germination rates, pollen tube lengths and pollen grain widths from phenotyping of GRMZM2G301647, serine/threonine protein kinase.
A trend towards reduced germination [A] and shorter pollen tubes [B] was observed in individuals with the insertion in GRMZM2G301647. A significant reduction in pollen grain diameters was detected in the non-germinated pollen grains of the heterozygous mutants. Bars represent 95% confidence intervals, n=5-6.

[A]



[B]

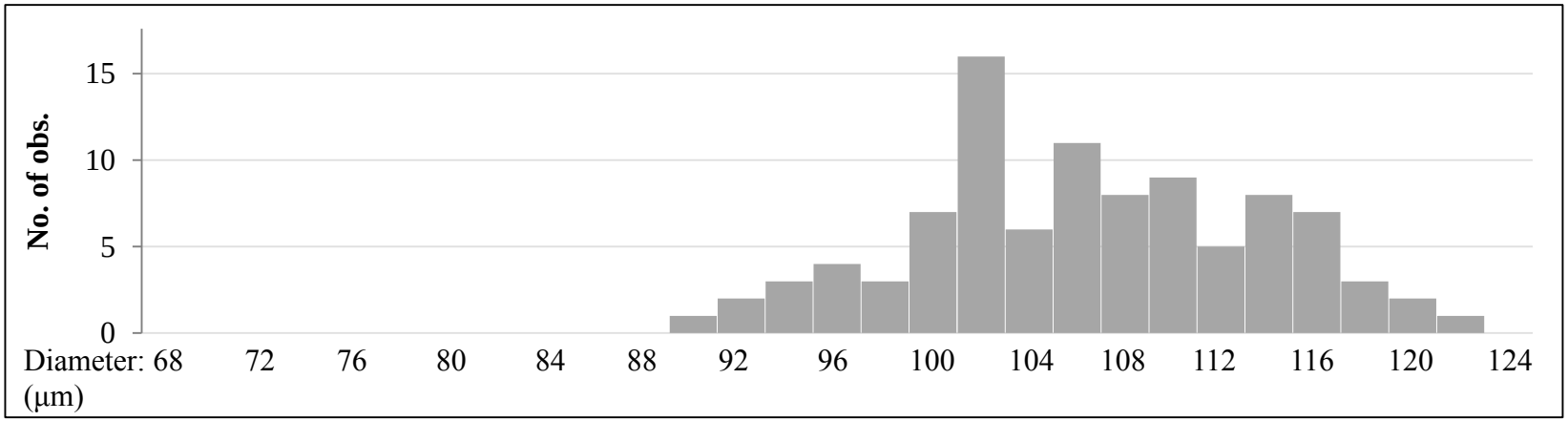
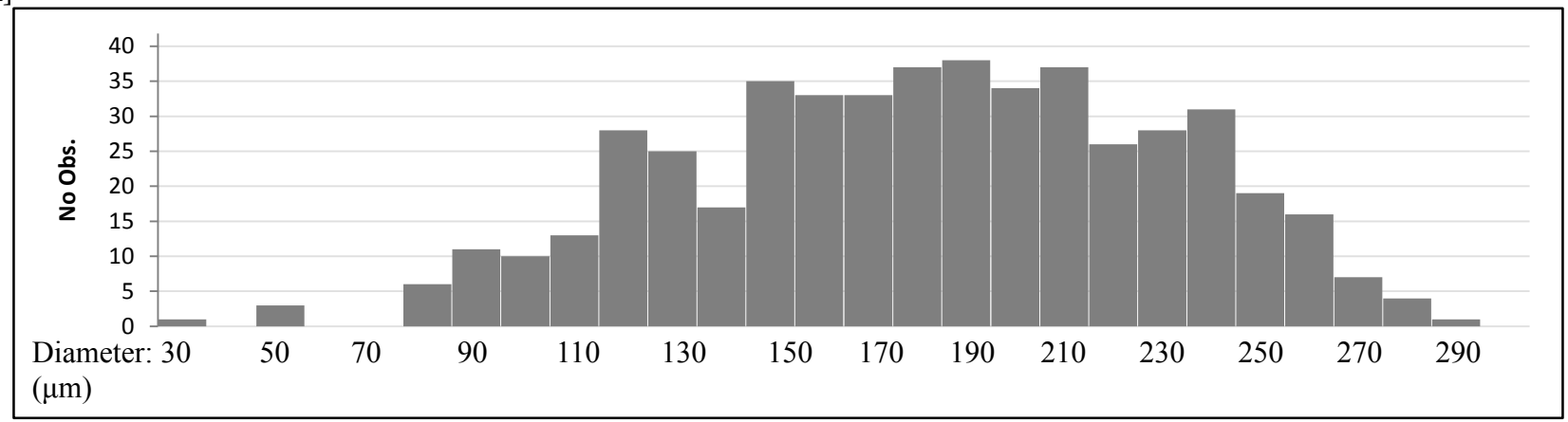


Figure 2.22: Histograms of non-germinated pollen grains diameter from individuals heterozygous for an insertion in GRMZM2G301647 [A] and their wild type siblings [B].

The broader range of diameters from plants containing the insertion relative to their wild type siblings supports the presence of pollen with and without the insertion in the grains from the heterozygous individuals [A].

[A]



[B]

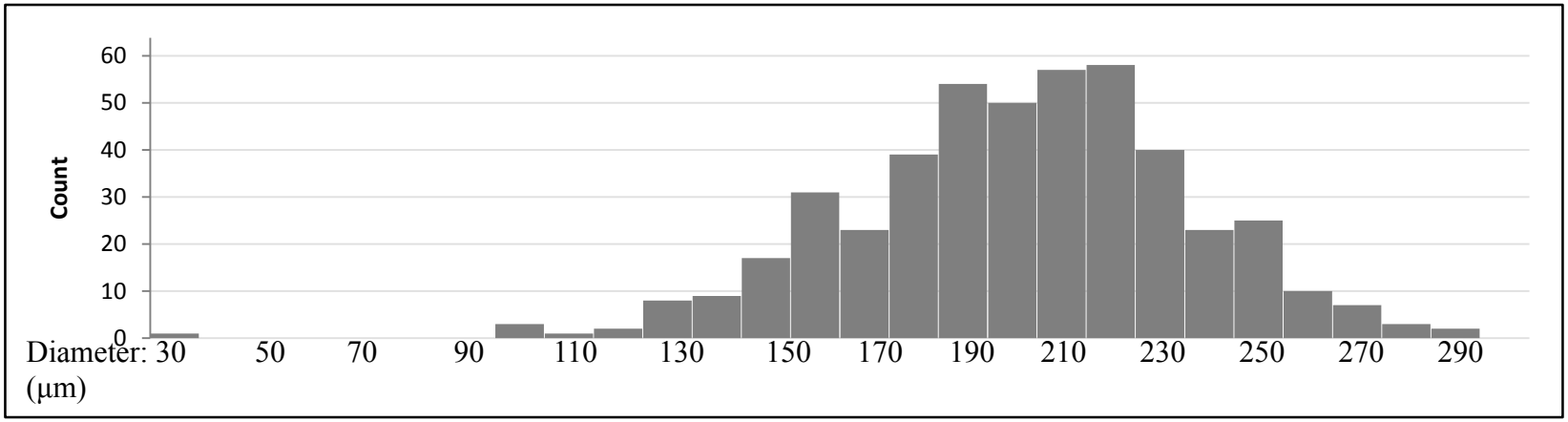


Figure 2.23: **Histograms of pollen tube lengths from individuals heterozygous for an insertion in GRMZM2G2G302265 [A] and their wild type siblings [B].**

Pollen tube lengths in individuals with the insertion overlap with those of their wild type siblings, but also covers a broader range supporting the presence of a mixed population of pollen grains in the heterozygous individuals [A].

Discussion

Germination studies using the translation inhibitor cycloheximide and the proteasome inhibitor MG-132 supported the hypothesis that the transition to the progamic phase of maize pollen is regulated through post-transcriptional control of protein levels. Analysis of HPLC-MS/MS mass spectrometry counts for mature and germinated pollen by three different statistical packages found evidence of both active translation and protein degradation during this shift. This confirms findings from silver staining of total protein extracts from mature and germinated pollen, which displayed several bands of proteins to be present in one tissue but absent in the other. Of 6,624 proteins detected in the total proteome, 393 of them were found to be differentially abundant with the majority of these decreasing in abundance with germination. Functional analysis found enrichment of GO terms related to translation, ribosomal activity and protein degradation in addition to enrichment of proteins found in the jasmonic acid biosynthesis pathway. The results presented in this chapter provide support for the hypothesis that the transition from mature to germinated pollen is orchestrated through post-transcriptional control, potentially by translation and protein degradation, rather than transcriptional means.

Twelve insertion lines were obtained for genes putatively predicted to be important to germination in *Zea mays* pollen based on proteomic and transcriptomic data analysis. The proteins represented in this study were ones found to increase in abundance with germination, exhibit a change in phosphorylation state with germination or were highly expressed in the mature pollen proteome and as transcripts in the mature pollen transcriptome. Of the twelve insertion lines, two insertions were later found to not be located in exons and so were removed from the overall analysis. Of the remaining ten, five displayed reduced transmission of the insertion allele through a male parent but not through a female parent supporting the hypothesis that these proteins are integral for pollen development or germination. Four of the remaining insertions did not display reduced transmission. It is important to note that while candidate proteins were identified from the data of mature and germinated pollen, it is

possible that the effects of the insertions in the genes for these proteins may be expressed earlier in pollen development. Of the five insertions *in vitro* phenotyped, two displayed no significant differences between the pollen of wild type siblings and sibling's heterozygous for the insertion. The three other insertion mutations exhibited a trend towards reduced germination and one also displayed reduced diameters in non-germinated pollen in the heterozygote relative to the homozygote wild type.

Comparison of the results of quantitative proteomic analysis from this study to other recent omics studies

In this study, 6210 proteins were detected by MS in B37 mature pollen. A similar study detected a similar number of proteins in B37 pollen (Chao *et al.* 2016), but there is only a 40% overlap in the identities of these polypeptides. Several factors may be contributing to the differences and make direct comparisons difficult, including differing extraction protocols, the replicated nature (and thus larger dataset) of this study, and the use of differing proteins groups leaders between our two studies. In both studies, in cases where peptides could not be assigned to a specific protein, proteins were combined into protein groups with a designated protein group leader to identify the group. Proteins with the largest number of peptide counts or the longest peptide sequence were assigned as group leaders in our study leaders in our study, the method for assignment in the Chao *et al.*, 2016 study is unclear. In addition, although the published study did not provide data for germinated pollen, the majority of the proteins identified here as exhibiting differential abundance with germination were also detected (67%).

The Chao *et al.*, 2016 study also detected 55 non-modified proteins and 37 phosphoproteins homologous to published male sterility genes characterized in *Arabidopsis* phosphoproteome studies. These published male sterility genes relate to pollen development (Bernal *et al.*, 2008), pollen tube germination and growth (Ariizumi *et al.*, 2004; Deeks *et al.*, 2007; Egli *et al.*, 2010; Golokin and Reddy, 2003; Kang *et al.*, 2003; Bou Daher *et al.*, 2011; Cole *et al.*, 2006; Mouline *et al.*, 2002; Schiott *et al.*, 2004; Zhang *et al.*, 2009) and sperm cell delivery (Lu *et al.*,

2011). Out of the *Arabidopsis* homologs found in Chao *et al.* 2016, 31 of the 55 total proteins and 14 of the 37 phosphoproteins were represented in our mature pollen data. In the total proteome, the majority of these shared proteins were among those decreasing in abundance (20 out of 32) and only five (four decreasing, one increasing) proteins from our differentially abundance set. In the phosphoproteome, there was an overlap of six decreasing and eight increasing in abundance with germination); though only a single protein (decreasing in abundance) was from our differential set.

A recent study of the secretome of tobacco pollen during germination through the stigma using semi-*in vivo* and *in silico* methods found 801 proteins to be secreted by the pollen tube, the majority of which were predicted to be secreted by unconventional pathways as they lacked the short N-terminal signal peptide indicative of the conventional ER-trans-Golgi network pathway (Hafidh *et al.*, 2016). Comparison of *Arabidopsis* orthologs of the tobacco pollen secretome and the 393 proteins found to be differentially abundant in this study between mature and germinated pollen, found a total of 24 proteins to be shared (8 increasing and 16 decreasing in abundance with germination) out of 286 orthologs (identified by UniProt). This suggests that maize pollen may also employ its secretome for directed growth of the pollen tube and in metabolism.

Possible mechanisms regulating the increases in protein abundance exhibited during germination

There are several models that can account for the increase in abundance of certain proteins with the initiation of germination, including [1] intron retention, [2] RNA particles, [3] polysomes and [4] phosphorylation of ribosomes. Intron retention is a mechanism (originally described by Richter and Smith, 1984) in which the precocious translation of transcripts is controlled through the maintenance of introns in transcripts until translation is required. In the fern *Marsilea vestita*, intron retention appears to regulate translation during rapid spermatogenesis (development of the male gametophyte) (Boothby *et al.*, 2013), as there is an enrichment of intron-

retention among transcripts encoding proteins essential for development of the gametophyte.

If intron retention were a factor in the increases in protein abundance during germination, one would expect to see an enrichment of intron retention among transcripts in the mature pollen transcriptome whose protein products are significantly up regulated with germination. However, inspection of the mature pollen transcriptome failed to find evidence of a correlation between intron retention and increased protein abundance as similar percentages of genes were observed to retain introns irrespective of protein abundance. This finding agrees with an earlier study (Chettoor *et al.*, 2014), which found fewer intron aligning reads in the maize pollen transcriptome relative to the transcriptome of a maize seedling, suggesting that intron retention does not function in maize pollen as a major regulator of the transition to the progamic phase.

The second hypothesis is the sequestration of translationally inactive mRNA in to particles (mRNPs) in an association with a number of proteins (Honys *et al.*, 2009). This mechanism was originally described in animal cells as a form of translational regulation of gene expression (Spirin *et al.*, 1965; Kleene *et al.*, 2003; Carson *et al.*, 2005). In plants, mRNPs have been identified in wheat and alfalfa embryos (Ajtkhozhin *et al.*, 1976; Krochko *et al.*, 1992; Pramanik *et al.*, 1992) and in the male gametophyte of tobacco (Honys *et al.*, 2009). While evidence of mRNPs has not currently been found in mature maize pollen, mRNPs have been described in the similarly quiescent maize embryonic axes (Rincon-Guzman *et al.*, 1998). Isolation of possible mRNPs can be achieved through the use of sucrose gradients (Rincon-Guzman *et al.*, 1998; Honys *et al.*, 2000) to determine if they play a role in controlling protein synthesis during the transition to germination in maize pollen as they appear to do in tobacco pollen (Honys *et al.*, 2009).

Polysomes are comprised of transcripts loaded with multiple ribosomes. Previous work in several different species of both bi- and tri-nucleate pollen have observed an

increase in polysomes with germination correlated with a rapid increase in translation (Mascarenhas and Bell, 1969; Tupy, 1977; Hoekstra and Bruinsma, 1979; Piques *et al.*, 2009). Polysome absorbance profile analyses of sucrose gradient fractionation isolated polysomes and ribosomes (Mustroph *et al.*, 2009) were obtained from samples of mature and germinated pollen to test these two scenarios. A larger proportion of polysomes were detected in the mature pollen sample relative to germinated pollen. Additionally there appears to be a shift to monosomes occurring with germination. To further test the role of polysomes in germination, sequencing of the translatome or ribosomal foot printing should be conducted to determine if the transcripts associated with polysomes are correlated with proteins significantly increasing in abundance with germination.

The final hypothesis of the mechanism regulating protein abundance is the phosphorylation of ribosomal proteins, which has been shown to modulate translation of specific proteins (Beltran-Pena *et al.*, 2002; Turkina *et al.*, 2011). Protein translation in eukaryotes is often controlled at the level of initiation (Turkina *et al.*, 2011), which is comprised of multiple events of phosphorylation of proteins (Jackson *et al.*, 2010). This is an attractive explanation, as it would not require a specialized particle like mRNPs or polysomes. In maize embryonic axes, increased selectivity of protein synthesis was observed in correlation with increased phosphorylation of the S6 protein on the 40S ribosomal subunit (Beltran-Pena *et al.*, 2002).

A possible role for methyl jasmonate (meJA) or other jasmonates in maize pollen during development and transition to the progamic phase

Proteomic profiling of mature and germinated pollen revealed the presence of several jasmonic acid (JA) biosynthetic enzymes, with three of these proteins exhibiting significant decreases in abundance with germination. Treatment of pollen with methyl jasmonate (meJA) resulted in decreased germination rates and pollen tube lengths. Together, these observations suggest that down-regulation of JA synthesis is part of the developmental control over the transition to the progamic phase.

A review of literature found evidence linking jasmonic acid with pollen germination in other plant species. Previous studies have found jasmonic acid to regulate the maturation of pollen (Ishiguro *et al.*, 2001; Browse, 2009; Drobritzsch *et al.*, 2015) and to inhibit germination in several species (Yildiz and Yilmaz, 2002; Yamane *et al.* 1982; Sembdner and Gross, 1986; Yamane *et al.*, 1981). However, there have not been any observations connecting jasmonates and the development of maize pollen prior to this study though it has been shown to inhibit seed germination (Norastehnia *et al.*, 2008). The reduction in germination rates observed in this study is similar to the effects described in pollen from strawberry (*Fragaria x ananasa* Duch.), lilly (*Lilium formosanum*), impatiens (*Impatiens balsamina*), and tea plants (*Camellia* spp.) (Yildiz *et al.*, 2002; Yamane *et al.* 1981; Yamane *et al.*, 1982) when exposed to methyl jasmonate. In contrast, the presence of JA appears to be part of the progamic phase regulatory network in *Arabidopsis* by encouraging successful germination when the pollen grain is exposed to moist environments (Ju *et al.*, 2016).

Interestingly the enrichment of the jasmonic acid biosynthesis pathway among proteins decreasing in abundance with germination in maize pollen does not fit with some of the current knowledge regarding jasmonic acid's mechanism of action in the cell. JA is characterized to mostly affect transcription factors (Chini *et al.*, 2007; Thines *et al.*, 2007), so even as JA biosynthesis appears to be reduced, this should have implications for the content of the transcriptome during germination. However, no significant changes to the transcriptome were detected by microarray during germination (Fowler, Vejlupkova, Cooper, Watrud, unpublished). This incongruity maybe explained by looking at another plant hormone known for its effects on transcription: auxin. While the effects of auxin mediated through the ARF family of transcription factors are well characterized (Calderón-Villalobos *et al.*, 2010; Hayashi, 2012), auxin can trigger a quick transcription-independent response as well (Sauer and Kleine-Vehn, 2011; Scherer, 2011) affecting various developmental pathways. It is possible that there is a similar response distinct from transcriptional interaction in jasmonic acid signaling. Methyl jasmonate has been shown to induce proteasome inhibitors (Farmer and Ryan, 1990) similar to the MG-132 used in this study

to inhibit protein degradation. While the similarities in the response of pollen grains to meJA and MG-132 are intriguing, it is not conclusive of a common mechanism.

The response of the pollen grains to rupture in the presence of methyl jasmonate (meJA) and to reduce pollen tube length, suggests that there is some effect on cell wall integrity and the pollen tube tip. It would be useful to determine if there is a difference in the diameter of the pollen tube tip with exposure to meJA, which may explain some of the pollen response. However, as we do not have a negative analog to test for meJA, we are not completely confident of the effect it has on the pollen grains. There are two possibilities: [1] the meJA does induce the JA signaling pathway and leads to the effects we observe, or [2] it does not directly interact with the hormone signaling pathway but either meJA or a metabolite interferes in a nonspecific way with pollen function. It is interesting to note that a recent study in Arabidopsis (Ju *et al.*, 2016) found that the addition of methyl jasmonate to germination media resulted in improved germination at concentrations demonstrated here to inhibit maize pollen germination (500μM, Figure 2.12 and 2.13). This emphasizes the finding that the behavior, biological factors and mechanisms orchestrating pollen development vary between species.

A combination of methyl jasmonate with cycloheximide or MG-132 may be useful in defining the response of the pollen grains. If meJA does indeed function in a similar manner as MG-132, then we should see a comparable response with the addition of cycloheximide as we saw in the two double drug experiments (Figure 2.7A and B). Additionally, a low concentration of MG-132 should reduce the effective concentration of meJA required to observe increased pollen rupture and reduced tube length. A germination study using the toxin coronatine (COR) which functions as a positive analog of meJA may help to uncover if the effects observed from the addition of meJA to germination are due to direct or indirect effects of the hormone. The inhibitor of endogenous jasmonic acid biosynthesis diethyldithiocarbamic acid (DIECA) could also be an interesting tool for investigating the effects of jasmonic acid on germination. If down regulation of jasmonic acid is a requirement for

successful germination, then addition of this inhibitor should either improve germination rates in maize pollen or have very little effect if jasmonic acid levels are already sufficiently low enough in the pollen.

Proteomic analysis, in addition to transcriptomic, provides useful evidence in the characterization of developmental transitions

The characterization of the proteome and phosphoproteome in this study has led to the discovery of additional information not covered by transcriptomic analysis. This additional information includes GO terms not previously found to be enriched and identification of proteins exhibiting reduced transmission when challenged with insertion mutations. These findings include evidence supporting the role control of protein abundance plays in germination of maize pollen. The detection of expressed genes and functional terms not found in the transcriptome is not surprising as transcript levels have been found to correlate poorly with protein abundance (Gygi *et al.*, 1999; Greenbaum *et al.*, 2003; Schwanhäusser *et al.*, 2011). Our results are also concordant with a recent study comparing maize developmental regulatory networks derived from transcriptomic and proteomic data (Walley *et al.*, 2016). The authors found that approximately 85% of the network hubs were not shared between the two data sets and concluded that integration of both protein and transcript data improved the predictive power of their regulatory models.

Results from transmission testing support the use of proteomic data to identify candidate genes, however more crosses and validation of insertions are required

In the transmission assay, the insertion present in GRMZM2G173289 was placed in a third "Unclear" category as the original test cross suggested reduced transmission though the male (X^2=0.004, 40 progeny tested) but a subsequent test did not display inheritance statistically different from the Mendelian null hypothesis (X^2=0.466, 36 progeny tested). This inconsistency could be due to the possible presence of *Ac* in the heterozygous parent for this *Ds* insertion line, as the progenitor of this line was developed as part of the *DsMutagenesis* population that harbored an active *Ac*. The initial populations tested from the reciprocal crosses may have still harbored *Ac*,

whereas the second population tested (with no apparent defect) was from a male parent in which the absence of active *Ac* had been confirmed. The potentially active *Ac* in the first cross could lead to an artificially low presence of the *Ds* allele, as it could have been induced to transpose out of the reported location, leading to a decreased frequency in the offspring that was unrelated to a pollen defect. Presence of the insertion in the gene or its footprint if it has moved, will need to be verified using PCR. The effects of the insertion in this gene are unclear, however, as it is also possible that the conflicting results are due to a defect that does not always affect transmission (e.g., a defect enhanced at high temperature). Additional tests will be conducted on population derived from reciprocal outcrosses of siblings of the individuals already tested.

The identification of as many as five and possibly six transmission defect-inducing insertions is a little surprising but does support the utility of proteomic data in characterizing important components of developmental transitions. The surprise is due to the origin of some of the insertion lines used in this study. The *Mu* lines were initially generated in both the *UniformMu* and *MuIllumina* populations, which were primarily derived via mutagenesis through the male and which are clearly under selection against maintaining insertions in genes integral for pollen development and germination (Figure 2.14A). The two *Ds* insertion lines, however, was generated through the female and so is not under pressure to select against pollen specific genes (Figure 2.14B). The importance of using proteomic data to select potential genes for RRM testing is highlighted by the results from a similar study using only transcriptomic data (Chettoor *et al.*, 2014). This other study was able to identify two out of nine tested insertions as exhibiting a defect; however, only *Ds* insertions were used (i.e. insertion lines not already under selective pressure to reduce transmission of male gametophyte specific genes with insertions). In the study presented here both *Ds* and *Mu* insertion lines were used and identified as containing transmission defect.

With the exception of the insertion with the unclear defect, the transmission test for the other insertions and genes have only been conducted with a single cross to date.

Additional crosses are needed to be able to conclusively determine if a line is exhibiting a true transmission defect through the male. To address this, 45 families, including the *Mu* insertions tested in this study, have been processed for Mu-seq analysis (McCarty *et al.*, 2013). Mu-seq permits the genotyping of multiple families of *Mu* insertions at a single time using a high throughput NextGen sequencing method and multiplexing, provided that no two families share an insertion. Finally, validation by sequencing is needed to confirm the exact genomic locations of the insertions tested for transmission to ensure their positioning in exons.

Utility of using multiple statistical packages in proteomic analysis

Interestingly, four out of the five proteins exhibiting a male specific transmission defect and the unclear GRMZM2G173289 were identified as differentially abundant between mature and germinated pollen in the proteomic study by either edgeR or IBB statistical packages only. This emphasizes the advantage of using multiple packages to analyze spectral counts, as some of these proteins would not have been identified if only a single statistical package had been used. For two out of the three of the insertions in gene from the GPP UP category (Table 2.6) there is a clear transmission defect, the remaining insertion in GRMZM2G173289 may exhibit a defect but another cross will need to finalize this possibility.

Limitations of the germination phenotyping assay of insertion mutants

Collection of phenotypic information from plants heterozygous for transposon insertions can aid in determining the functional activity of the candidate proteins during pollen development or germination. The transition from mature pollen to the progamic phase to fertilization is reliant on multiple events (Bedinger and Fowler, 2009), each with the potential to cause the observed reduction in transmission. Rates of pollen germination and rupturing may indicate involvement in germination initiation or cell wall stability of the pollen grain. The stigma of maize (silk) may be up to 20cm in length (Heslop-Harrison *et al.*, 1984) and the pollen tube must successfully grow and navigate the distance to achieve fertilization. Characterization of pollen tube length could reveal if the candidate genes are important for growth of

the pollen tube (relative speed to wild type grains) and its ability to compete against other pollen for fertilization. Wild type pollen grains contain enough resources for the initial 2cm of growth through the silk before they are then reliant on the female sporophyte for energy (Heslop-Harrison *et al.*, 1984): an observed reduction in pollen grain diameter could signal decreased stored energy or an earlier disruption in pollen grain development.

Phenotyping of germination to characterize the functional importance of candidate proteins, while very useful, is a difficult assay to conduct. This test relies on heterozygous individuals resulting in a mixed sample of pollen, with only 50% of the pollen containing the insertion. If an insertion exerts an effect early in pollen development, this percentage may be even lower. The genotype of a specific pollen grain cannot be distinguished by independent means in the current setup for this experiment. As such, the measurements from wild type and mutant are averaged with in the sample and this could result in the obscuring of a subtle phenotypic. For example, the polygalacturonase phentotyped in this study did exhibit a trend towards reduced germination in the pollen of siblings carrying the insertion, however, germination rates were not statically different from each other. This could be the results of the wild type pollen all germinating at a high rate and the pollen carrying the insertion allele germinating at a much lower rate with the reported germination as an average between them. The percentage of germination in the pollen with the allele could be significantly lower than their wild type siblings but undetectable using the current method.

These germination tests also represent an artificial situation with no incorporation of pollen competition found in the style during *in vivo* pollen tube growth, and may be the reason why we do not observe defects in germination rates (Figure 2.17 A and B). Additionally, the effects of the insertions may not be felt until later in the progamic phase than was evaluated here and, due to the insertion populations the families are from, many different aspects of pollen development and function may be affected. An improved way to assess the phenotype of these insertion candidates may be through

the use of a linked *waxy1* (*wx*) marker (Bedinger and Fowler, 2009). This marker can be used to distinguish wild type from insertion positive pollen grains *in vitro* in germination media or *in vivo* in silk by staining with iodine. This approach is especially useful in the characterization of mild or inconsistent defects. The limitation of this method is in establishing the lines containing the insertion and the linked *waxy1* marker.

How the proteins identified in the male transmission defect assay may play a role in the progamic phase

The five genes with a clear defect and GRMZM2G030265 can be grouped into four functional categories. There are three that function in signaling (GRMZM2G173289, GRMZM2G133282 and GRMZM2G301647), and one protein each in cell wall function (GRMZM2G030265), ubiquitin dependent protein degradation (GRMZM2G389462) and glycolysis (GRMZM2G066024). These four functional groups all fit into a hypothesis of germination and fit with some of what is already known to occur during this transition.

Previous work has shown signaling to play an important part in successful germination (Mascarenhas, 1993; Dresselhaus *et al.*, 2011; Li *et al.*, 2013; Yue *et al.*, 2014; Li *et al.*, 2016). Work with pollinated maize silks have shown kinases, like GRMZM2G301647 presented here, to play an important role in the interaction between the male gametophyte and female sporophyte (Yu e al., 2014). While long-range signaling between the female gametophyte and the pollen tube does not appear to occur in maize (Lausser *et al.*, 2010; Dresselhaus *et al.*, 2011), there is evidence supporting signaling from the transmitting tract (Heslop-Harrison *et al.*, 1985; Lausser *et al.*, 2010) and short-range factors to guide the pollen tube to the micropyle (Lausser and Dresselhaus, 2010). Signaling along with functions related to the cell wall are thought to play an important role in the directed tip growth of the pollen tube (Labarca *et al.*, 1973; Pina *et al.*, 2005; Wang *et al.* 2008; Obermeyer *et al.*, 2013; Chettoor *et al.*, 2014). Polygalacturonase (e.g. GRMZM2G030265) has been known to play an important role in the elongation of pollen tubes in maize and other plants as

its pectin hydrolase activity permits rearrangement of the pectin the in the pollen tube cell wall allowing for pollen tube growth (Pressey and Reger, 1983; Allen and Lonsdale, 1993; Dearnaley *et al.*, 2001; Kim *et al.*, 2006).

The presence of an ubiquitin ligase (GRMZM2G389462) in this study provides a potential link to protein degradation during germination and matches the predictions from the proteasome inhibitor MG-132 germination study (Figure 2.6). Based on the findings from the drug study, the insertion in GRMZM2G389462 is predicted to results in a similar pollen phenotype with increased rupturing in the insertion carrying siblings. The Arabidopsis ortholog (AT5G42340) is classified by plant ontology (PO) to be enriched in mature and germinated pollen and in pollen tubes (Liu *et al.*, 2012) and to function in protein ubiquitination. This supports the hypothesis that protein degradation plays an important role in pollen germination, and suggests that this role is conserved across species boundaries.

A recent study of pollinated maize and Arabidopsis stigmas found evidence for activation of glycolysis in the stigmas, which the authors hypothesized, was to provide the growing pollen tube with energy (Yue *et al.*, 2016). The silk in maize represents a hypoxic environment, as such metabolism is expect to be mainly anaerobic and employing the glycolysis pathway to produce ATP for energy. The defect we observe in transmission of the insertion located in the fructose-bisphosphate aldolase GRMZM2G066024 may reflect the dependence on glycolysis for growth in the pollen tube. If this is true, than the *in vitro* germination study presented here to phenotype the defect may not reflect a reduction in growth or germination rate as *in vitro* germination does not occur in a an oxygen limited environment. This may be a case where use of the *waxy1* marker and *in vivo* germination may uncover a clear description of the insertion phenotype and function of GRMZM2G066024 in pollen function.

Model of the transition to the progamic phase in Zea mays pollen

The results from the translation and protein degradation studies and the germination

phenotyping of the insertions exhibiting a male transmission defect can be combined into a model of pollen germination (Figure 2.24). Results from the double inhibitor studies, using both cycloheximide and MG-132, supported the hypothesis that translation occurs prior to protein degradation during the transition to the progamic phase. The results were also consistent with the hypothesis that protein degradation occurs after initiation of germination and that it is these initiated pollen grains that rupture when protein degradation is inhibited.

The insertion mutants with male transmission defects and measurable phenotypes can be added to this working model (Figure 2.24). The insertion in the serine/threonine kinase (GRMZM2G301647) resulted in smaller diameters of non-germinated pollen grains suggesting that it acts earlier in pollen development, the reduced pollen tube lengths indicates that its effects may extend into germination. The trend towards reduced germination rates without increased rupture, and shorter pollen tube lengths suggest that the polygalacturonase (GRMZM2G030265) and the ubiquitin ligase (GRMZM2G389462) influence the initiation of germination and possibly the elongation of the pollen tube.

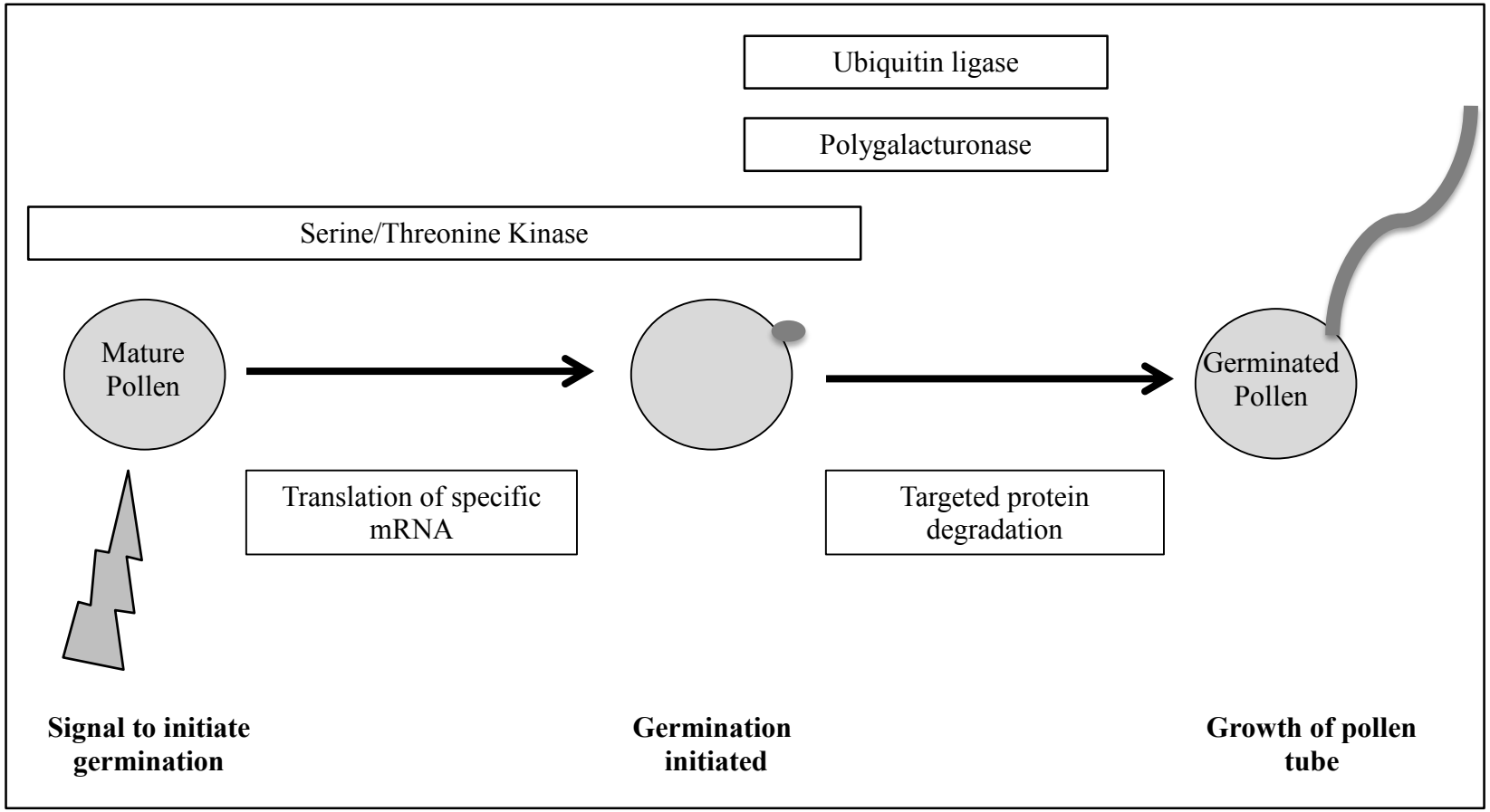


Figure 2.24: Working model of the transition to the progamic phase in *Zea mays* pollen.

Literature Cited

Ajtkhozhin MA, Doschanov KJ, Akhanov AU (1976) Informosomes as a stored form of mRNA in wheat embryos. FEBS Lett. **66**(1): 124–126

Allen RL1, Lonsdale DM (1993) Molecular characterization of one of the maize polygalacturonase gene family members which are expressed during late pollen development. Plant J **3**(2): 261-271

Amaldi PP, Felicetti L, Campioni N (1977) Flow of information RNA from cytoplasmic poly(A)-containing particles to polyribosomes in Artemia salina cysts at early stages of development. Dev. Biol. **59**: 49–61

Ariizumi T, Hatakeyama K, Hinata K, Inatsugi R, Nishida I, Sato S, Kato T, Tabata S, Toriyama K (2004) Disruption of the novel plant protein NEF1 affects lipid accumulation in the plastids of the tapetum and exine formation of pollen, resulting in male sterility in Arabidopsis thaliana. Plant J **39**(2): 170-181

Arunkumar R, Josephs EB, Williamson RJ, Wright SI (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**: 2475–2486

Bedinger P, Fowler J (2009) The maize male gametophyte. In: Hake S, Bennetzen J (eds) Handbook of maize: its biology. Springer, New York, pp 57–77

Beltran-Pena E, Aguilar R, Ortiz-Lopez A, Dinkova TD, De Jimenez ES (2002) Auxin stimulates S6 ribosomal protein phosphorylation in maize thereby affecting protein synthesis regulation. Physiol Plant **115**: 291–297

Bernal AJ, Yoo C-M, Mutwil M, Jensen JK, Hou G, Blaukopf C, Sørensen I, Blancaflor EB, Scheller HV, Willats WGT (2008) Functional analysis of the cellulose synthase-like genes CSLD1, CSLD2, and CSLD4 in tip-growing Arabidopsis cells. Plant Physiol **148**(3):1238–1253

Bonifacino JS, Traub LM (2003) Signals for Sorting of Transmembrane Proteins to Endosomes and Lysosomes. Annual Review of Biochemistry **72**: 395–447

Boothby TC, Zipper RS, van der Weele CM, Wolniak SM (2013) Removal of Retained Introns Regulates Translation in the Rapidly Developing Gametophyte of Marsilea vestita. Developmental Cell **24**: 517–529

Bou Daher F, Geitmann A (2011) Actin is Involved in Pollen Tube Tropism Through Redefining the Spatial Targeting of Secretory Vesicles. Traffic **12**: 1537–1551

Brewbaker JL (1967) The Distribution and Phylogenetic Significance of Binucleate and Trinucleate Pollen Grains in Angiosperms. American Journal of Botany **54**: 1069–1083

Browse J (2009) Jasmonate: preventing the maize tassel from getting in touch with his feminine side. Sci Signal **2**(59): pe9

Calderon-Villalobos LI, Tan X, Zheng N, Estelle M (2010) Auxin perception--structural insights. Cold Spring Harb Perspect Biol. **2**(7): a005546

Capco DG, Jeffery WR (1979) Origin and spatial distribution of maternal messenger RNA during oogenesis of an insect, Oncopeltus fasciatus. J. Cell Sci. **39**: 63–76

Capkova V, Hrabetova E, Tupy J (1988) Protein synthesis in pollen tubes: preferential formation of new species independent of transcription. Sexual Plant Reproduction **1**: 150–155

Carson JH, Barbarese E (2005) Systems analysis of RNA trafficking in neural cells. Biol. Cell **97**(1): 51–62

Chao Q, Gao Z-F, Wang Y-F, Li Z, Huang X-H, Wang Y-C, Mei Y-C, Zhao B-G, Li L, Jiang Y-B, et al (2016) The proteome and phosphoproteome of maize pollen uncovers fertility candidate proteins. Plant Mol Biol 1–18

Chen J, Zeng B, Zhang M, Xie S, Wang G, Hauck A, Lai J (2014) Dynamic Transcriptome Landscape of Maize Embryo and Endosperm Development. Plant Physiology **166**: 252–264

Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MMS (2014) Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. Genome Biol **15**: 414

Chia J-M, Song C, (null), (null), de Leon N, Doebley JF, Elshire RJ, Gaut BS, Geller L, Glaubitz JC, et al (2012) Maize HapMap2 identifies extant variation from a genome in flux. Nature Genetics **44**: 803–807

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signaling. Nature **448**(7154): 666-671

Choi H, Fermin D, Nesvizhskii AI (2008) Significance Analysis of Spectral Count Data in Label-free Shotgun Proteomics. Molecular and Cellular Proteomics **7**: 2373–2385

Cole RA, Fowler JE (2006) Polarized growth: maintaining focus on the tip. Current Opinion in Plant Biology **9**: 579–588

Curtis D, Lehmann R, Zamore PD (1995) Translational regulation in development. Cell **81**: 171–178

Davidson EH (1986) Gene Activity in Early Development, Third Edition (New York: Academic Press)

Dearnaley JDW, Daggard GA (2001) Expression of a polygalacturonase enzyme in germinating pollen of *Brassica napus*. Sexual Plant Reproduction **14**: 27–34

Deeks MJ, Rodrigues C, Dimmock S, Ketelaar T, Maciver SK, Malhó R, Hussey PJ (2007) Arabidopsis CAP1 - a key regulator of actin organization and development. J Cell Sci **120**(Pt 15): 2609-2618

Digel B, Pankin A, Korff von M (2015) Global Transcriptome Profiling of Developing Leaf and Shoot Apices Reveals Distinct Genetic and Environmental Control of Floral Transition and Inflorescence Development in Barley. THE PLANT CELL ONLINE **27**: 2318–2334

Dobritzsch S, Weyhe M, Schubert R, Dindas J, Hause G, Kopka J, Hause B (2015) Dissection of jasmonate functions in tomato stamen development by transcriptome and metabolome analyses. BMC Biology **13**: 1–18

Dresselhaus T, Lausser A, Marton ML (2011) Using maize as a model to study pollen tube growth and guidance, cross-incompatibility and sperm delivery in grasses. Annals of Botany **108**: 727–737

Du Z, Zhou X, Ling Y, Zhang Z, Su Z (2010) agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res **38**: W64-70

Dure L, Waters L (1965) Long-Lived Messenger RNA: Evidence from Cotton Seed Germination. Science **147**: 410–412

Egli B, Kölling K, Köhler C, Zeeman SC, Streb S (2010) Loss of cytosolic phosphoglucomutase compromises gametophyte development in Arabidopsis. Plant Physiol 154(4): 1659-1671

Facette MR, Shen Z, Bjornsdottir FR, Briggs SP, Smith LG (2013) Parallel Proteomic and Phosphoproteomic Analyses of Successive Stages of Maize Leaf Development. THE PLANT CELL ONLINE **25**: 2798–2812

Farmer EE, Ryan CA (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. PNAS **87**: 7713–7716

Franchi GG, Nepi M, Dafni A, Pacini E (2002) Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. Plant Syst Evol **234**: 211–227

Franke WW, Herth W, Vanderwoude WJ, Morré DJ (1972) Tubular and filamentous structures in pollen tubes: Possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta **105**(4): 317-341

Franklin-Tong VE (1999) Signaling and the Modulation of Pollen Tube Growth. The Plant Cell **11**: 727–738

Genschik P, Criqui MC, Parmentier Y, Derevier A, Fleck J (1998) Cell cycle

dependent proteolysis in plants. Identification Of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor MG-132. Plant Cell **10**(12): 2063-2076

Golovkin M, Reddy AS (2003) A calmodulin-binding protein from Arabidopsis has an essential role in pollen germination. PNAS **100**(18): 10558-10568

Gonzalez-Galarza FF, Lawless C, Hubbard SJ, Fan J, Bessant C, Hermjakob H, Jones AR (2012) A Critical Appraisal of Techniques, Software Packages, and Standards for Quantitative Proteomic Analysis. OMICS: A Journal of Integrative Biology **16**: 431–442

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol **4**: 117

Grosfeld H, Littauer UZ (1975) Cryptic form of mRNA in dormant Artemia salina cysts. Biochem. Biophys. Res. Commun. **67**: 176–181

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology **17**: 994–999

Hafidh S, Potěšil D, Fíla J, Capkova V, Zdráhal Z, Honys D (2016) Quantitative proteomics of the tobacco pollen tube secretome identifies novel pollen tube guidance proteins important for fertilization. Genome Biol 1–29

Hao H, Li Y, Hu Y, Lin J (2005) Inhibition of RNA and protein synthesis in pollen tube development of Pinus bungeana by actinomycin D and cycloheximide. New Phytologist **165**: 721–729

Hart PE, Wolniak SM (1998) Spermiogenesis in Marsilea vestita: a temporal correlation between centrin expression and blepharoplast differentiation. Cell Motil. Cytoskeleton **41**: 39–48

Hayashi K (2012) The interaction and integration of auxin signaling components. Plant Cell Physiol **53**(6): 965-975

Heslop-Harrison Y, Reger BJ, Heslop-Harrison J (1984) The pollen–stigma interaction in the grasses. 6. The stigma (silk) of *Zea mays* L. as host to the pollens of Sorghum bicolor (L.) Moench and Pennisetum americanum (L.) Leeke. Acta Botanica Neerlandica **33**: 205–227

Heslop-Harrison Y, Heslop-Harrison J, Reger BJ (1985) The pollen–stigma interaction in the grasses. 7. Pollen-tube guidance and the regulation of tube number in *Zea mays* L. Acta Botanica Neerlandica **34**: 193–211

Hoekstra FA, Bruinsma J (1979) Protein Synthesis of Binucleate and Trinucleate Pollen and Its Relationship to Tube Emergence and Growth. Planta **146**: 559–566

Honys D, Combe JP, Twell D, Capkova V (2000) The translationally repressed

pollen-specific ntp303 mRNA is stored in non-polysomal mRNPs during pollen maturation. Sexual Plant Reproduction **13**: 135–144

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5**: R85.1–R85.13

Honys D, Reňák D, Feciková J, Jedelský PL, Nebesářová J, Dobrev P, Capkova V (2009) Cytoskeleton-Associated Large RNP Complexes in Tobacco Male Gametophyte (EPPs) Are Associated with Ribosomes and Are Involved in Protein Synthesis, Processing, and Localization. J Proteome Res **8**: 2015–2031

Hughes DW, Galau GA (1989) Temporally modular gene expression during cotyledon development. Genes Dev. **3**: 358–369

Hughes DW, Galau GA (1991) Developmental and environmental induction of Lea and Lea A mRNAs and the post abscission program during embryo culture. Plant Cell **3**: 605–618

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCENCE1 Gene Encodes a Novel Phospholipase A1 Catalyzing the Initial Step of Jasmonic Acid Biosynthesis, Which Synchronizes Pollen Maturation, Anther Dehiscence, and Flower Opening in Arabidopsis. The Plant Cell **13**: 2191–2209

Jackson RJ, Hellen CU, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol **11**: 113–127

Ju Y, Guo L, Cai Q, Ma F, Zhu Q-Y, Zhang Q, Sodmergen S (2016) Arabidopsis JINGUBANG is a Negative Regulator of Pollen Germination that Prevents Pollination in Moist Environments. THE PLANT CELL ONLINE tpc.00401.2016

Kang BH, Rancour DM, Bednarek SY (2003) The dynamin-like protein ADL1C is essential for plasma membrane maintenance during pollen maturation. Plant J **35**(1): 1-15

Kannaste O, Suomi T, Salmi J, Uusipaikka E, Nevalainen O, Corthals GL (2014) Cross-correlation of spectral count ranking to validate quantitative proteome measurements. J Proteome Res **13**: 1957–1968

Kim J, Shiu SH, Thoma S, Li WH, Patterson SE (2006) Patterns of expansion and expression divergence in the plant polygalacturonase gene family. Genome Biology **7:** 87

Kleene KC (2003) Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. Cytogenet. Genome Res. **103**(3-4): 217–224

Krochko JE, Pramanik SK, Bewley JD (1992) Contrasting storage protein synthesis and messenger RNA accumulation during development of zygotic and

somatic embryos of alfalfa (Medicago sativa L.). Plant Physiol. **99**(1): 46–53

Labarca C, Loewus F (1973) The nutritional role of pistil exudate in pollen tube wall formation in Lilium longiflorum. Plant Physiol **52**: 87–92

Lausser A, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in grasses. Biochemical Society Transactions **38**: 631–634

Lausser A, Kliwer I, Srilunchang K-O, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in maize. Journal of Experimental Botany **61**: 673–682

Lee DH, Goldberg AL (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae.* J Biol Chem **271**(44): 27280-27284

Li L, Petsch K, Shimizu R, Liu S, Xu WW, Ying K, (null), Scanlon MJ, Schnable PS, Timmermans MCP, et al (2013) Mendelian and Non-Mendelian Regulation of Gene Expression in Maize. PLoS Genet **9**: e1003202

Li M, Wang K, Li S, Yang P (2016) Exploration of rice pistil responses during early post-pollination through a combination proteomic and transcriptomic analysis. Journal of Proteomics **131**: 214–226

Liu J , Jung C , Xu J , Wang H , Deng S , Bernad L , Arenas-Huertero C , Chua NH (2012) Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. The Plant Cell **24**(11): 4333-4345

Lowe J, Stock D, Jap B, Zwickl P, Baumeister W, Huber R (1995) Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4A resolution. Science **268**: 533-539

Lu YX, Chanroj S, Zulkifli L, Johnson MA, Uozumi N, Cheung A, Sze H (2011) Pollen tubes lacking a pair of K? transporters fail to target ovules in Arabidopsis. Plant Cell **23**(1):81–93

Malatesta M, Perdoni F, Battistelli S, Muller S, Zancanaro C (2009) The cell nuclei of skeletal muscle cells are transcriptionally active in hibernating edible dormice. BMC Cell Biol. **10**: 19

Marcon C, Malik WA, Walley JW, Shen Z, Paschold A, Smith LG, Piepho H-P, Briggs SP, Hochholdinger F (2015) A High-Resolution Tissue-Specific Proteome and Phosphoproteome Atlas of Maize Primary Roots Reveals Functional Gradients along the Root Axes. Plant Physiology **168**: 233–246

Mascarenhas JP and Bell E (1969) Protein synthesis during germination of pollen: studies on polyribosome formation. Biochem Biophys Acta **179**(1): 199-203

Mascarenhas JP and Goralnick RD (1971) Synthesis of small molecular weight RNA in the pollen tube of Tradescantia paludosa. Biochem. Biophys. Acta **240**:

56-61

Mascarenhas JP (1975) The Biochemistry of Angiosperm Pollen Development. Botanical Review **41**: 259–314

Mascarenhas JP (1993) Molecular Mechanisms of Pollen Tube Growth and Differentiation. The Plant Cell **5**: 1303–1314

McCarty DR, Latshaw S, Wu S, Suzuki M, Hunter CT, Avigne WT, Koch KE (2013) Mu-seq: Sequence-Based Mapping and Identification of Transposon Induced Mutations. PLoS ONE **8**: e77172

Mouline K, Very A-A, Gaymard F, Boucherez J, Pilot G, Devic M, Bouchez M, Thibaud J-B, Sentenac H (2002) Pollen tube development and competitive ability are impaired by disruption of a Shaker K+ channel in *Arabidopsis*. Genes & Development **16**: 339–350

Mueller LN, Brusniak M-Y, Mani DR, Aebersold R (2008) An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. J Proteome Res **7**: 51–61

Mustroph A, Juntawong P, Bailey-Serres J (2009) Isolation of Plant Polysomal mRNA by Differential Centrifugation and Ribosome Immunopurification Methods. *In* DA Belostotsky, ed, Plant Systems Biology. Humana Press, pp 109–126

Muthukrishnan S, Filipowicz W, Sierra JM, Both GW, Shatkin AJ, Ochoa S (1975) mRNA methylation and protein synthesis in extracts from embryos of brine shrimp, Artemia salina. J. Biol. Chem. **250**: 9336–9341

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in Arabidopsis thaliana seed germination: epigenetic and genetic regulation of transcription in seed. Plant J. **41**: 697–709

Nepi M, Franchi GG, Pacini E (2001) Pollen hydration status at dispersal: cytophysiological features and strategies. Protoplasma **216**: 171–180

Ning K, Fermin D, Nesvizhskii AI (2012) Comparative analysis of different label-free mass spectrometry based protein abundance estimates and their correlation with RNA-Seq gene expression data. J Proteome Res **11**: 2261–2271

Norastehnia A, Sajedi RH, Nojavan-Asghari M (2008) Inhibitory Effects of Methyl Jasmonate on Seed Germination in Maize (*Zea mays*): Effect on alpha-amylase Activity and Ethylene Production. Gen Appl Plant Physiology **33**: 13–23

Obermeyer G, Fragner L, Lang V, Weckwerth W (2013) Dynamic Adaption of Metabolic Pathways during Germination and Growth of Lily Pollen Tubes after Inhibition of the Electron Transport Chain. Plant Physiology **162**: 1822–1833

Pacini E, Franchi GG, Ripaccioli M (1999) Ripe pollen structure and

histochemistry of some gymnosperms. Plant Syst Evol **217**: 81–99

Pavelka N, Fournier ML, Swanson SK, Pelizzola M, Ricciardi-Castagnoli P, Florens L, Washburn MP (2008) Statistical similarities between transcriptomics and quantitative shotgun proteomics data. Molecular and Cellular Proteomics **7**: 631–644

Pham TV, Piersma SR, Warmoes M, Jimenez CR (2010) On the beta-binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Bioinformatics **26**: 363–369

Pina C, Pinto F, Feijo JA, Becker JD (2005) Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiol **138**: 744-756

Piques M, Schulze WX, Hohne M, Usadel B, Gibon Y, Rohwer J, Stitt M (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in *Arabidopsis*. Molecular Systems Biology **5**: 1–17

Pramanik SK, Krochko JE, Bewley JD (1992) Distribution of cytosolic mRNAs between polysomal and ribonucleoprotein complex fractions in alfalfa embryos: stage-specific translational repression of storage protein synthesis during early somatic embryo development. Plant Physiol. **99**(4): 1590–1596

Pressey R, Reger B (1989) Polygalacturonase in pollen from corn and other grasses. Plant Science **59**(1): 57-62

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Raff RA, Colot HV, Selvig SE, Gross PR (1972) Oogenetic origin of messenger RNA for embryonic synthesis of microtubule proteins. Nature **235**: 211–214

Richter JD, and Smith LD (1984). Reversible inhibition of translation by Xenopus oocyte-specific proteins. Nature **309**: 378–380

Rincon-Guzman A, Beltran-Pena E, Ortiz-Lopez A, Sanchez de Jimenez E (1998) Ribonucleoprotein particles of quiescent maize embryonic axes. Plant Mol Biol **38**: 357–364

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**: 139–140

Robinson MD, Smyth GK (2007) Moderated statistical tests for assessing differences in tag abundance. Bioinformatics **23**: 2881–2887

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg

AL (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78**(5): 761-71

Rosenthal ET, Bahr M, Wilt FH (1993) Regulation of maternal messenger RNA translation during oogenesis and embryogenesis in Urechis caupo. Dev. Biol. **155**: 297–306

Sauer M, Kleine-Vehn J (2011) AUXIN BINDING PROTEIN1: the outsider. Plant Cell **23**(6): 2033-2043

Scherer GF (2011) AUXIN-BINDING-PROTEIN1, the second auxin receptor: what is the significance of a two-receptor concept in plant signal transduction? J Exp Bot **62**(10): 3339-3357

Schiøtt M, Romanowsky SM, Baekgaard L, Jakobsen MK, Palmgren MG, Harper JF (2004) A plant plasma membrane Ca2+ pump is required for normal pollen tube growth and fertilization. PNAS **101**(25): 9502-9507

Schreiber DN, Dresselhaus T (2003) *In Vitro* Pollen Germination and Transient Transformation of *Zea mays* and Other Plant Species. Plant Mol Biol Rep **21**: 31–41

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature **473**: 337–342

Sekhon RS, Lin H, Childs KL, Hansey CN, Buell CR, de Leon N, Kaeppler SM (2011)Genome-wide atlas of transcription during maize development. The Plant Journal **66**: 553–563

Sembdner G, Gross D (1986) Plant growth substances of plant and microbial origin. In: Bopp M (ed) Plant growth substances 1985. Springer, Berlin, Heidelberg, New York, Tokyo, pp 139–147

Settles AM, Holding DR, Tan B, Latshaw SP, Liu J, Suzuki M, Li L, O'Brien BA, Fajardo DS, Wroclawska E, et al (2007) Sequence-indexed mutations in maize using the UniformMu transposon-tagging population. BMC Genomics **8**: 116

Sheng X, Hu Z, Lu H, Wang X, Baluska F, Samaj J, Lin J (2006) Roles of the Ubiquitin/Proteasome Pathway in Pollen Tube Growth with Emphasis on MG132-Induced Alterations in Ultrastructure, Cytoskeleton, and Cell Wall Components. Plant Physiology **141**: 1578–1590

Speranza A, Scoccianti V, Crinelli R, Calzoni GL, Magnani M (2001) Inhibition of Proteasome Activity Strongly Affects Kiwifruit Pollen Germination. Involvement of the Ubiquitin/Proteasome Pathway as a Major Regulator. Plant Physiology **126**: 1150–1161

Spirin, AS, Nemer M (1965) Messenger RNA in early sea urchin embryos: Cytoplasmic particles. Science **150**: 214–216

Storey J (2015) qvalue: Q-value estimation for false discovery rate control. R package version 2.0.0. http://qvalue.princeton.edu/, http://github.com/jdstorey/qvalue

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448**(7154): 661-665

Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S (1996) Differential Inhibition of Calpain and Proteasome Activities by Peptidyl Aldehydes of Di-Leucine and Tri-Leucine. Journal of Biochemistry **119**: 572–576

Tupy J (1977) RNAsynthesis and polysome formation in pollen tubes. Biol. Planta. **19**: 300-309

Turkina MV, Arstrand HK, Vener AV (2011) Differential Phosphorylation of Ribosomal Proteins in Arabidopsis thaliana Plants during Day and Night. **6**(12): e29307

Vejlupkova Z, Fowler JE (2003) Maize DNA preps for undergraduate students: a robust method for PCR genotyping. Maize Genetics Cooperation Newsletter

Vollbrecht E, Duvick J, Schares JP, Ahern KR, Deewatthanawong P, Xu L, Conrad LJ, Kikuchi K, Kubinec TA, Hall BD, et al (2010) Genome-Wide Distribution of Transposed Dissociation Elements in Maize. THE PLANT CELL ONLINE **22**: 1667–1685

Walley JW, Shen Z, Sartor R, Wu KJ, Osborn J, Smith LG, Briggs SP (2013) Reconstruction of protein networks from an atlas of maize seed proteotypes. PNAS 1–20

Walley JW, Sartor R, Shen Z, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR, et al (2016) Integration of omic networks in a developmental atlas of maize. Science **353**: 814–818

Wang Y, Zhang W-Z, Song L-F, Zou J-J, Su Z, Wu W-H (2008) Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in Arabidopsis. Plant Physiology **148**: 1201–1211

Wei LQ, Xu WY, Deng ZY, Su Z, Xue Y, Wang T (2010) Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in Oryza sativa. BMC Genomics **11**: 338

Williams JH, Taylor ML, O'Meara BC (2014) Repeated evolution of tricellular (and bicellular) pollen. American Journal of Botany **101**: 559–571

Williams-Carrier R, Stiffler N, Belcher S, Kroeger T, Stern DB, Monde RA, Coalter R, Barkan A (2010) Use of Illumina sequencing to identify transposon insertions underlying mutant phenotypes in high-copy Mutator lines of maize. Plant J **63**(1): 167-177

Woffenden BJ, Freeman TB, Beers EP (1998) Proteasome Inhibitors Prevent Tracheary Element Differentiation in Zinnia Mesophyll Cell Cultures. Plant Physiology **118**: 419–430

Xiao L, Wang H, Wan P, Kuang T, He Y (2011) Genome-wide transcriptome analysis of gametophyte development in Physcomitrella patens. BMC Plant Biol **11**: 177

Yamane H, Takagi H, Abe H, Yokota T, Takahashi N (1981) Identification of jasmonic acid in three species of higher plants and its biological activities. Plant Cell Physiol. **22**: 689–697

Yamane H, Abe H, Takahashi N (1982) Jasmonic Acid and Methyl Jasmonate in Pollens and Anthers of Three *Camellia* Species. Plant and Cell Physiology **23**: 1125–1127

Yildiz K, Yilmaz H (2002) Effect of jasmonic acid, ACC and ethephon on pollen germination in strawberry. Plant Growth Regeneration **38**: 145–148

Yue X, Gao X-Q, Wang F, Dong Y, Li X, Zhang XS (2014) Transcriptional Evidence for Inferred Pattern of Pollen Tube-Stigma Metabolic Coupling during Pollination. PLoS ONE **9**: e107046

Zhang L, Chia J-M, Kumari S, Stein JC, Liu Z, Narechania A, Maher CA, Guill KE, McMullen MD, Ware DH (2009) A Genome-Wide Characterization of MicroRNA Genes in Maize. PLoS Genet **5**: e1000716

Zhu W, Smith JW, Huang C-M (2010) Mass Spectrometry-Based Label-Free Quantitative Proteomics. Journal of Biomedicine and Biotechnology **2010**: 1–6

Acknowledgements

The quantitative proteomic profiling study was conducted in collaboration with the Briggs and Smith labs at University of California, San Diego. The mass spectral count data presented here are from a larger data set generated by these two labs. Justin Walley provided advice on the statistical analysis of the mass spectral counts. Sam Hokin provided assistance with the intron retention analysis and transcriptomic work. John Fowler conducted the Reduced Recovery of Mutations (RMM) analysis presented in this study. The Author would like to thank Zuzana Vejloupkova, Quinn DeYoung, Matthew Borcher and Peter de Roos for help with sample preparation and data collection. A special thanks also goes to the Wolpert, Anderson and Chang labs at Oregon State University for loaning supplies, equipment and expertise.

DsMutagenesis, UniformMu and Mu-Illumina Projects provided the insertion populations used in the male transmission defect analysis.

Chapter 3 : Teasing Out the Transcriptome of *in vivo* Germinated Pollen

Abstract

Growth of the pollen tube through the silk during pollination, which involves interaction between the male gametophyte and the female sporophyte, represents an important step in *Zea mays* reproduction. While advances have been made documenting the transcriptomes of mature pollen and silk, gene expression in the growing pollen tube is difficult to assess due to the location of the pollen tube within sporophytic tissue. The novel approach presented here employs the use of RNA-seq technology and the large number of single nucleotide polymorphisms (SNPs) present in maize. These polymorphisms are used to assign reads to the transcriptomes of the male gametophyte and the female sporophyte within a mixed RNA sample from pollinated silk. As the only published reference genome for maize at the time of analysis was for the inbred line B73, SNP polymorphisms identified by the HapMapv2 study (Gore *et al.* 2009, Chia *et al.* 2012, www.panzea.org) were inserted into the B73 reference genome to create a "pseudogenome" for the inbred line W22. Comparison of the differential alignment of reads against the male and female parent genomes permits assignment of the subset of reads encompassing known SNPs to either the pollen or the silk. The advantage of this method is one can uncover the *in vivo* expression of pollen-specific and silk-specific genes, and thus identify genetic components potentially related to their interaction. Initial analysis of 23.6 million reads aligning to chromosome 8 from a "mixed" sample of W22 silks with growing B73 pollen tubes detected expression of the B73 allele for 222 genes. These are genes whose expression putatively originates in the pollen tube nucleus. Consistent with this, alignment of reads from the W22 silk with B73 pollen tubes sample to the whole genome found a 24.2% to 25.9% overlap of genes with B73 allele-specific reads and genes previously associated with enrichment of expression in mature pollen. An initial validation by CAPS (Cleaved Amplified Polymorphic Sequence) analysis of RT-PCR products from a gene found to be expressed in the pollinated silk supports the detection of B73 allele expression in the pollinated W22 silk sample.

Introduction

An important step in plant reproduction is the growth of the pollen tube through the stigma during pollination, involving interaction between the male gametophyte and the female sporophyte. This progamic phase is marked by directed growth and competition of the pollen tubes and is a point where regulation of self-compatibility or self-incompatibility can be enforced. Self-incompatibility, or the inability to self fertilize, leads to an increased gene pool by promoting outcrossing and avoidance of homozygosity (Dresselhaus *et al.*, 2011). In many plant families, self-incompatibility is controlled through a single polymorphic locus (Rea and Nasrallah, 2008) regulating a series of complex molecular interactions between the male gametophyte and female sporophyte (McCubbin and Kao, 2000; Silva and Goring, 2001). One assumption in this study is that, unlike the earliest stages of the progamic phase discussed in Chapter 2, the growth of the pollen tube through the silk will elicit and require changes to the transcriptome of the male gametophyte.

During the past decade there has been some progress in the investigation of changes to the transcriptome of the pollen tube and the stigma during this stage of sexual reproduction (Wang *et al.*, 2008; Qin *et al.*, 2009; Lausser *et al.*, 2010; Boavida *et al.*, 2011; Dresselhaus and Franklin-Tong, 2013). However, no general consensus has been reached as to the central components regulating pollen-pistil interactions (Hiscock *et al.*, 2008), though general functional categories do appear to be shared across species. This may be because genes that regulate reproductive processes tend to diversify and evolve more rapidly than those enabling more central cellular functions ("housekeeping genes") (Swanson and Vacquier, 2002), or because the gene products engaged in the interaction of the pollen tube and the stigma help define the boundary between species and so would be expected to exhibit diversity (Swanson *et al.*, 2004).

Previous work using a semi-*in vivo* method has demonstrated that the transcriptome of Arabidopsis pollen tubes grown through stigmas is distinct from that of pollen germinated on media (Qin *et al.* 2009). Additionally, the authors found evidence of

enrichment in genes putatively involved in signal transduction, transcription and pollen tube growth, indicating a possible transcription regulatory network triggered in response to interaction with the pistil. A microarray study investigating changes the transcriptome of Arabidopsis pistils post-pollination found over 1300 genes differentially expressed during pollen-stigma interaction and exhibiting distinct clusters of temporal or spatial regulation (Boavida *et al.*, 2011). Transcripts identified in this study included genes encoding predicted secreted proteins, genes involved in signaling and proteins related to the cell wall. A combination transcriptomic and proteomic analysis of pollinated rice pistils found a change in expression of 32 receptor kinases and evidence of up-regulation of genes involved in central metabolism (providing energy for pollination), protein ubiquitination (cell signaling transduction), vesicle trafficking and phytohormone signaling (Li *et al.* 2016). The method used in this study, though, was unable to differentiate pollen reads from those of the pistil. The authors concluded that the contribution of the pollen RNA to the pollinated samples was minimal due to a low number of pollen enriched genes in their dataset.

A recent RNA-seq study focused on characterizing transcripts induced in the maize silk upon pollination (Xu *et al.*, 2013). The authors found evidence of 172 genes induced upon pollination with activation of genes related to signal transduction, lipid metabolism, microtubule based movement, ubiquitin-dependent protein degradation and transport. Their analysis made the assumption that all of the reads detected were derived from silk tissue only, and that any mRNA contribution from pollen would not be detectable. However, it is possible that some of the functional groups they reported as enriched in their data set could be the results of pollen-derived reads, such as the microtubule-based movement which, though not essential for pollen tube growth, aids in movement of organelles to and directed growth at the pollen tube tip (Ehrhardt *et al.*, 2006; Mourino-Perez *et al.*, 2006; Krichevsky *et al.*, 2007; Qin *et al.*, 2011).

Gene expression within the *in vivo* growing pollen tube is difficult to assess due to its location within sporophytic tissue. The approach presented here represents a pilot

study using RNA-seq technology and single nucleotide polymorphisms (SNPs) to isolate the transcriptomes of the male gametophyte and the female sporophyte from two different inbred lines within a "mixed" RNA sample of silk with growing pollen tubes. Polymorphisms identified by the HapMapv2 study (Gore *et al.* 2009, Chia *et al.* 2012) for the inbred W22, available through www.panzea.org, were inserted into the reference genome of the inbred B73, replacing the B73 allele specific SNPS, to create a "W22 pseudogenome". Although not all genes contain SNPs, a large percentage of maize genes have been shown to contain polymorphisms; for example, between B73 and Mo17, 70% of annotated gene models contain at least 1 polymorphism and 58% of the filtered gene set (FGS) contain 3 SNPs (Waters *et al.* 2011).

Differential alignment of reads against the male and female parent genomes permits assignment of a subset of reads encompassing SNPs specific to the inbred line of either the pollen or the silk. The advantage of this method is one can uncover the *in vivo* expression of pollen-specific and silk-specific genes, and thus identify genetic components potentially related to their interaction. This approach would not have been possible with the study reported earlier of pollinated maize silks (Xu *et al.*, 2013), as they did not use two different inbred lines in their samples, making separation of the pollen and silk derived reads impossible. The hypothesis of this study is that detection of male gametophyte transcript expression can be achieved in an *in vivo* pollinated silk sample using polymorphisms to assign reads.

Materials and methods

Plant Materials

Samples were harvested from field grown plants in Corvallis, OR. W22 silks were saturated with B73 pollen and left for three hours to allow for germination and pollen tube growth. A portion of the B73 pollen was frozen in liquid nitrogen for RNA extraction. At three hours post-pollination, an approximately 2 cm portion of silks just below the site of pollination was collected for a "mixed W22 silk/B73 pollen" sample; a similar portion of silks was collected from unpollinated W22 ear. Half of

each sample was frozen in liquid nitrogen for RNA isolation and the other half was fixed in FAA fixative (50% ethanol, 10% formalin, 5% glacial acetic acid) for staining. Fixed samples were brought down to 70% ethanol from FAA fix for storage by processing through an ethanol series (70%, 95%, 95%, 70%).

Aniline blue staining

Fixed silk samples were stained with aniline blue to evaluate the presence of pollen tubes in the pollinated sample. Samples were hydrated and brought down to water from 70% ethanol through a series of washes (50% ethanol, 30% ethanol, ddH_2O 2x). Samples were then cleared in 8M NaOH for 2-6 hours and rinsed 4x in ddH2O. Silks were stained overnight in a 0.1% aniline blue solution in a 0.1 M K_3PO_4 buffer. Stained pollen tubes were then visualized under a UV-excitation filter on a Zeiss Axiovert S100 microscope using transmitted light and epifluorescence

RNA isolation, Poly-A selection and sequencing

RNA isolation for sequencing and CAPs analysis was performed using a combination of Trizol and Qiagen RNAeasy columns. Frozen samples were ground using a mortar and pestle in the presence of Trizol containing High Molecular Weight (HMW) PEG (20,000 MW) at 2% (20mg/ml). After centrifugation to remove insoluble material, chloroform was added to the supernatant and followed by a three-minute incubation period and centrifugation. The aqueous phase was removed and added to isopropanol and High Salt Precipitation Solution (0.8 M Sodium citrate, 1.2 M NaCl) to preferentially precipitate RNA and minimize polysaccharide contaminants. Samples were centrifuged and the resultant pellets were washed in 75% ethanol and dissolved in nuclease-free water. Qiagen RLT buffer containing 3.3% HMW PEG and 100% ethanol were added to the samples prior placement in a Qiagen MinElute spin column. Columns were washed with 80% ethanol to remove residual guanidine salts and samples were eluted with 30ul of heated nuclease-free water.

The quality of RNA extractions for sequencing were assessed by Bioanalyzer 2100 (Agilent) prior to Poly-A selection using the Poly(A)Purist MAG kit and following

the manufacture recommended protocol. Poly-A enrichment of an additional W22 silk sample was performed by the Center for Genome Research and Biocomputing (CGRB) in Corvallis, Oregon. TruSeq stranded RNA library prep and 100bp single end sequencing on an Illumina HiSeq 2000 (Illumina) was performed by the CGRB. The W22 silk with B73 pollen library was sequenced twice in two separate runs.

Construction of W22 genome

A fasta file of version 2 the B73 reference genome was downloaded from the FTP server hosted on MaizeGDB (www.maizegdb.org). HapMapv2 data containing the published SNPs between B73 and W22 were downloaded from Panzea (www.panzea.org). A custom Python script (Python Software Foundation. Python Language Reference, version 2.7, http://www.python.org) was developed in collaboration with Taj Morton of the Megraw Lab at Oregon State University to use the coordinates from the HapMapv2 data to replace B73 bases in the published reference genome file with W22 SNPs (Morton and Megraw, personal communication, Appendix Script A.1).

Alignment and read assignment

Raw RNA-seq reads were trimmed using Trimmomatic V0.32 (Bolger *et al.*, 2014), removing 6 bases from the start of each read and 5 from the end. Samples were then aligned to both the Version 2 B73 reference genome and to the W22 pseudo-genome using CASHX (Fahlgren *et al.*, 2009; Version 2.3) with parameters set to no mismatches and alignment of up to three locations (Figure 3.1). After alignment, reads were separated into two categories: those that aligned uniquely to one location, and those that aligned up to three times to either genome. Comparison of B73 allele specific reads in these two categories was conducted during alignment parameter optimization. Using the ‘sort’ and ‘uniq’ function on the command line, a comparison of headers from the B73- and W22-aligned reads resulted in the categorization of headers. Headers were determined to be either [A] B73 allele specific (Figure 3.2, blue), from reads that aligned to the B73 genome sequence but not the W22 pseudo-genome sequence and potentially derived from pollen, [B] W22 allele specific

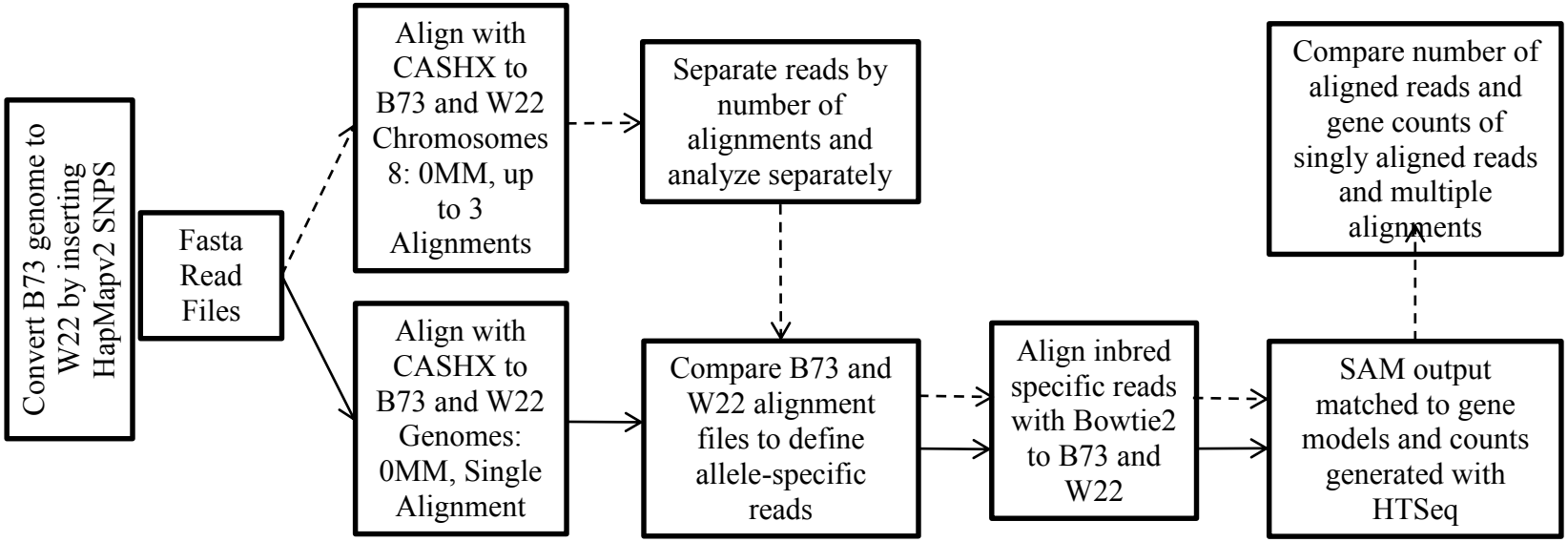


Figure 3.1: Workflow for the alignment and assignment of the RNA-seq reads to B73 or W22.
The top pathway (dashed line) details the steps used to test the possible advantages of allowing reads that align multiple times to the genome. The bottom pathway (solid line) outlines the method used for the whole genome alignment using the final parameters of 0 mismatches and no multiple alignments. MM = Mismatches.

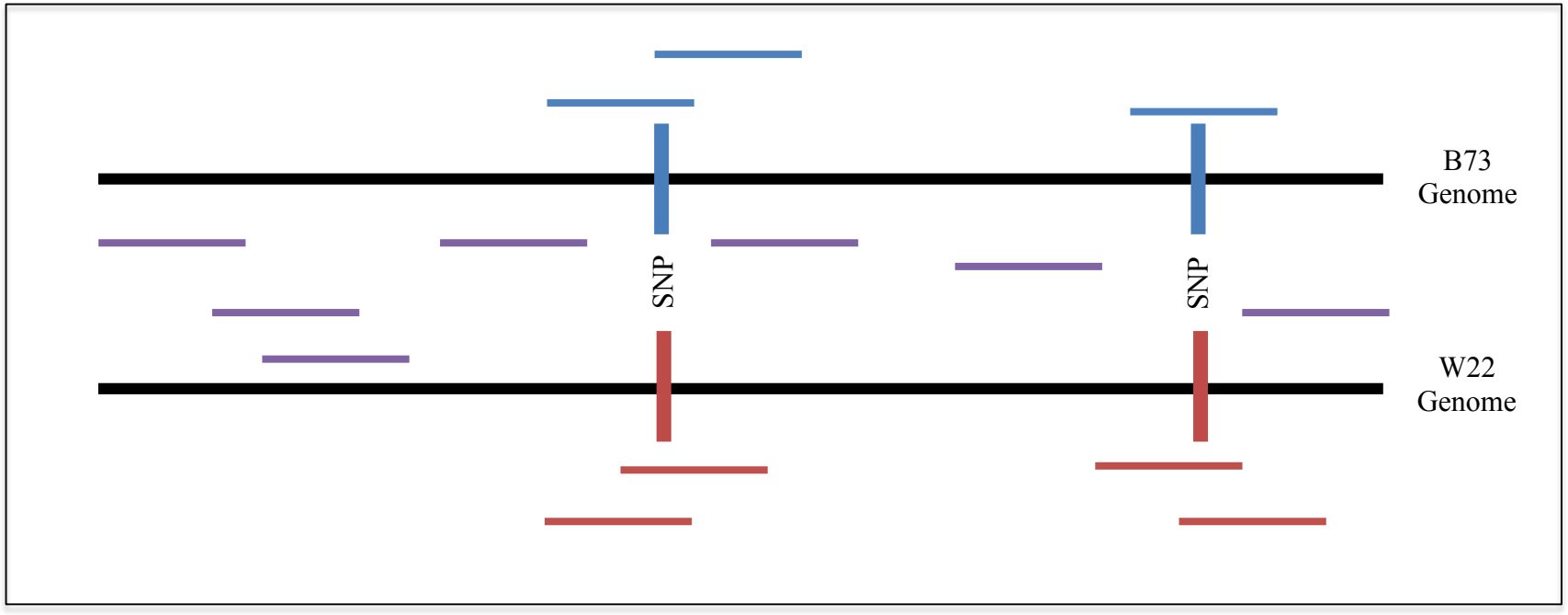


Figure 3.2: Three types of RNA-seq reads derived from B73 (blue) and W22 (red) versions of a gene, differentiated by two SNPs.
Blue and red reads overlap a SNP, and can be associated specifically with B73 or W22; purple reads are associated with the gene, but can not be differentiated between genomes.

(Figure 3.2, red) and aligning to the W22 pseudo-genome sequence only and potentially derived from the silk tissue, or [C] unknown (Figure 3.2, purple) and aligning to sequences in both genomes. The headers present in each of these groups were used to generate a fasta file of the corresponding reads and aligned using Bowtie2 (Langmead and Salzberg, 2012) to the B73 genome and the W22 pseudo-genome. Using the resultant SAM alignment file, gene counts were generated using HTSeq (Anders *et al.*, 2015). Initial testing of parameters was conducted only on reads aligning to chromosome 8 prior to alignment of reads to the whole genome sequence.

The software Geneious (www.geneious.com) was used to visually confirmation the presence of reads containing B73 specific SNPs in the mixed sample of B73 pollen and W22 silk. W22 versions of select genes were manually curated using the B73 reference genome and available HapMapv2 data. Using the native Geneious aligner, reads from the mixed sample were aligned to the curated genes to test for the presence of B73 specific SNPs which would be revealed as mismatches to the W22 version of the gene. Highlighted mismatches were compared to the HapMapv2 data to confirm the presence of a B73 SNP.

cDNA generation, genomic DNA extraction and CAPS analysis

Samples of B73 pollen, W22 silk and W22 silks pollinated with B73 pollen were processed for cDNA and genomic DNA (gDNA). Using manufacturers instructions, SuperScript III First-Strand Synthesis kit for RT-PCR (Invitrogen) was used to generate cDNA from RNA isolated from the above samples. Tissues for genomic gDNA extraction were ground using a drill-mounted plastic pestle in the presence of extraction buffer (2M NaCl; 200mM Tris-HCl, pH 8.0; 70mM EDTA, pH 8.0; 20 mM sodium meta-bisulfite) and 5% Sarkosyl. After incubation at 60°C, samples were centrifuged and the supernatant was removed and added to 5M ammonium acetate and isopropanol for DNA precipitation. Samples were centrifuged again to form a pellet and were washed with cold 70% ethanol. DNA was suspended in TE buffer, pH 8.0 with a brief incubation at 65°C.

The presence of W22 and B73 specific alleles was validated using cleaved amplified polymorphism sequence (CAPS) analysis. Candidate alleles were selected from genes expressing a high level of B73 allele specific reads and whose SNP was located within an available restriction endonuclease recognition site so that digestion should cleave the product from one allele but not the other. Primers (Table 3.1) were designed within 500bp of either side of the recognition cut site. To differentiate between cDNA and gDNA, the 5' and 3' primers for a restriction site were located on different. As the gDNA will still contain the introns, the product from this reaction will be longer than that of the cDNA. PCR was performed on samples of gDNA and cDNA from B73 pollen, W22 silks and W22 silks pollinated with B73 pollen. This was followed by enzyme digestion by Nco1, EcoR1 or Bcl1 and using manufacture's recommended buffers, additives and incubation period (Table 3.1).

Results

Validation of pollen tube growth within the pollinated W22 silk sample

To validate the presence of pollen tubes in the pollinated silk samples, paired tissue samples fixed in FAA were stained with aniline blue. Evidence of pollen tubes was found in the pollinated W22 silk samples (Figure 3.3A) but not in the non-pollinated silk samples (Figure 3.3B). Additionally, evidence of pollen tube tips was found in the pollinated silk samples. As pollen tubes grow through the silk, walls are built along the length sealing the cytoplasm, vegetative cell and sperm cells close to the tip of the pollen tube. The present of the tips in the pollinated silks support the presence of cytoplasm, and thus RNA for generating RNA-seq reads from the pollen tubes in the samples.

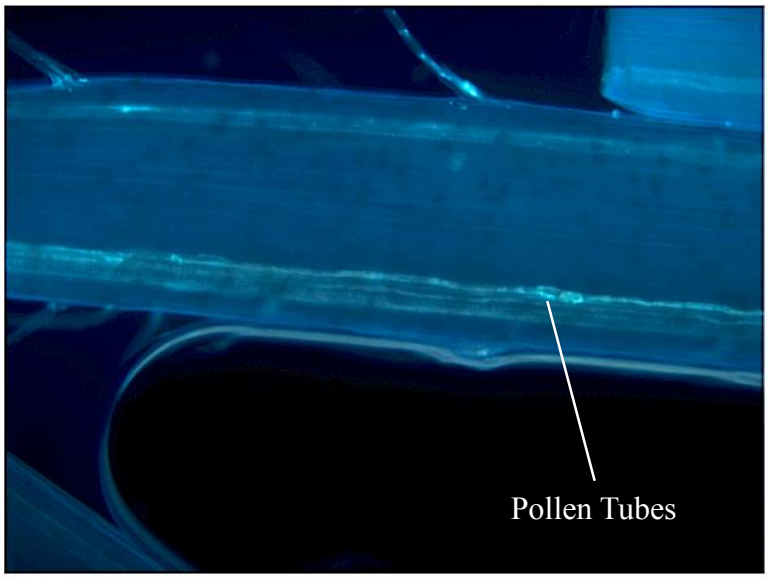
Building of the W22 genome

At the time of alignment of the RNA-seq reads, there was not an available reference genome for the W22 inbred line. However, there was a published reference genome for B73 and a library of known SNPs between the two inbred lines available from the HapMapv2 dataset (panzea.org, Gore *et al.* 2009, Chia *et al.* 2012). Using the B73 genome as a template, a W22 pseudo-genome sequence was created by replacing B73

Table 3.1: Primers for CAPs analysis.
Forward and reverse primers were designed to be located in different exons within 500 bp of the restriction endonuclease cut sit containing an inbred specific SNP.

Gene	Primers	Sequence	Product [Cut] (bp)	Enzyme	Inbred Cut
GRMZM2G433959	GRMZM2G433959_R1	CATGAGACGCCATCCTTCCC	732 [289,443]	Nco1	W22
	GRMZM2G433959_L1	GGCCACTGAACCTAAGCCAT			
	GRMZM2G433959_R2	CATCCTTCCCGACCAACCAC	757 [324,433]	Nco1	W22
	GRMZM2G433959_L2	GATCAAGTTGTGCCAAGCGG			
GRMZM2G139223	GRMZM2G139223_R1	CTACAGCACCGACTCCAACC	706 [229,477]	EcoR1	B73
	GRMZM2G139223_L1	CCTCCTGCAAGCACATGGA			
	GRMZM2G139223_R2	TGAAGCAGCTGGACAGGAAC	721 [332,388]	EcoR1	B73
	GRMZM2G139223_L2	CATGAACCTCCGCCTGTCTC			
GRMZM2G054123	GRMZM2G054123_R1	CAGAGCAGCCAGTCACTGAT	732 [254,478]	Bcl1	W22
	GRMZM2G054123_L1	CACACCGTGCTCATCTCCAC			

A]



[B]

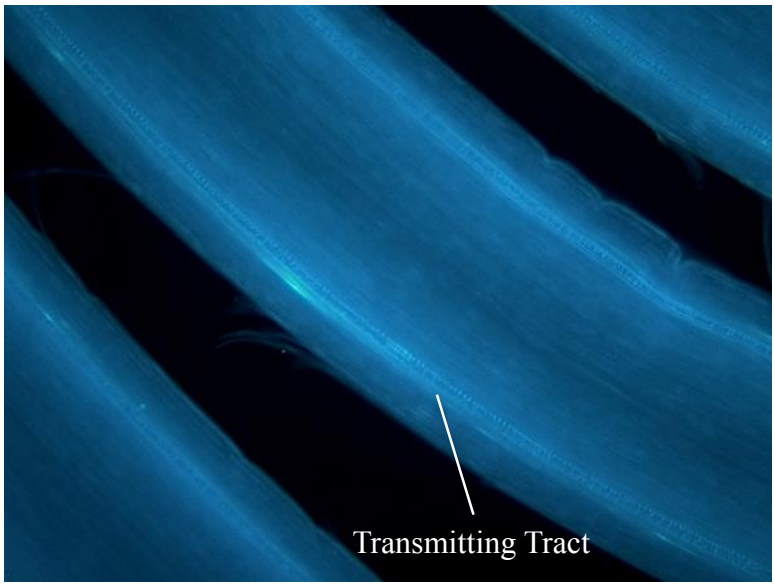


Figure 3.3: **Aniline blue staining of [A] W22 silks with B73 pollen and pollen tubes and [B] W22 silk that has not been pollinated.**
In both the pollinated and unpollinated samples, the two bright transmitting tracks may be seen running down the length of the silks. Images taken at 10x magnification .

specific bases with their W22 counterpart (Morton and Megraw, personal communication, Appendix Script A.1). This method accounted only for single base polymorphisms between the two lines and did not account for any PAV polymorphisms or indels. Of the approximately 39,000 genes in maize, 36.47% do not contain the B73 reference allele in W22 based on known SNP content (Gore *et al.* 2009, Chia *et al.* 2012) and thus were potentially distinguishable by this analysis.

Testing alignment and genome assignment of reads to Chromosome 8

To identify inbred-specific reads from the RNA-seq data by pulling out reads containing inbred-specific SNPs, dual alignment to the two genomes was followed by a comparison to find those reads that aligned to one genome but not the other. Samples were aligned separately to both the B73 genome and the W22 pseudo-genome using CASHX (Fahlgren *et al.*, 2009) allowing for 0 mismatches (Figure 3.1). The stringent cutoff of 0 mismatches was used to prevent alignment to the genome of one inbred of reads containing a SNP corresponding to the other inbred – these reads are interpreted as containing a mismatch to one genome but not the other and this was used later to categorize reads.

An initial test of the parameters was conducted on chromosome 8 as it is one of the shorter chromosomes and was known to contain genes with expression in pollen (Chettoor *et al.*, 2014). A comparison of reads aligning uniquely to a genome location versus those aligning to up to three locations on chromosome 8 was conducted to determine which parameter would provide the most sensitivity in detecting genes associated with B73-specific (i.e., pollen tube-derived) reads (Figure 3.2 and Table 3.2) in the pollinated silk sample. Multiple alignments were considered as maize is a degenerate allotetraploid and contains a large number of duplicate genes. By increasing the number of acceptable alignments we increased the likelihood of detecting expression from these duplicates. The analysis suggested that increasing the threshold of alignment locations does not increase the sensitivity of detecting B73-specific reads, as similar numbers of allele-specific reads (Table 3.2) and genes are identified in each instance (Figure 3.4A), though it may increase the stringency with

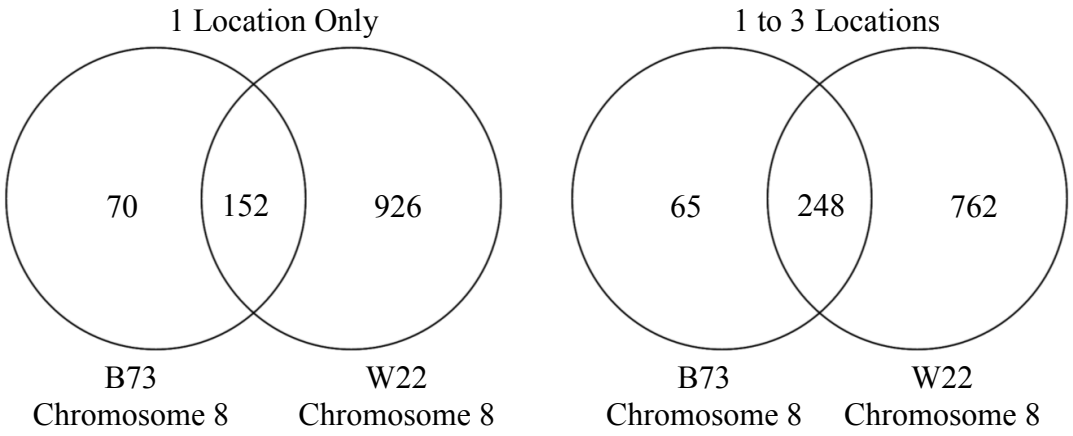
Table 3.2: **Numbers of reads aligning to the B73 genome or W22 pseudogenome sequence for chromosome 8, allowing alignment to a single location or up to three locations.**
In parentheses are the numbers of reads that align to only the W22 or B73 allele. The reads for the pollinated W22 silk sample and the W22 silk only sample come from the initial sequencing run.

	Allele	1 Location Only	1 to 3 Locations
W22 silk and B73 Pollen	W22	364,685 (54,443)	23,600,984 (54,405)
	B73	311,970 (1,728)	23,548,127 (1,548)
W22 Silk	W22	743,134 (105,725)	19,191,970 (105,553)
	B73	640,283 (2,874)	19,088,629 (2,212)
B73 Pollen	W22	1,178,247 (1,916)	18,964,948 (873)
	B73	1,623,069 (446,738)	19,410,783 (446,708)

Table 3.3: Percentage of chromosome 8 genes associated with B73- or W22- allele-specific reads from pollinated W22 silks also enriched in mature pollen transcriptomes.

Allele	1 Location Only	1 to 3 Locations
W22	19.2%	2.3%
B73	24.2%	25.9%

[A]



[B]

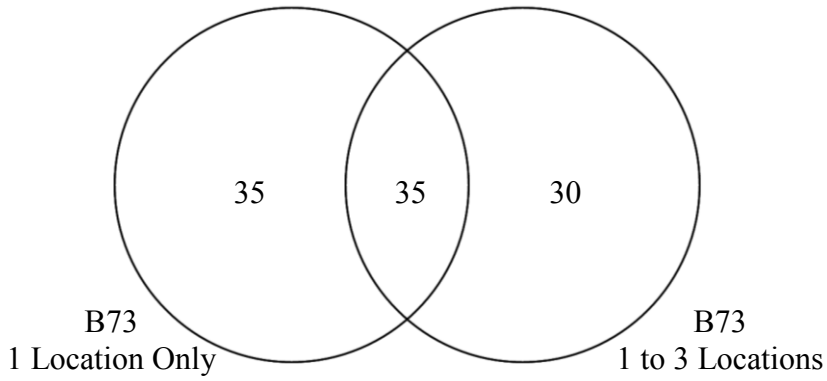


Figure 3.4: Comparison of the number of genes detected in the mixed W22 silk/B73 pollen sample with reads aligning to either the B73 or W22 chromosome 8, allowing for a single alignment or up to three alignments.
[A] Increasing the number of allowable alignments did not result in a large change in the number of genes identified. [B] Sets of genes identified with B73 alleles only from single and multiple alignments exhibit an overlap of about 50%.

which the allele of origin is determined. Though similar numbers of genes were highlighted by the two different parameters, the specific genes identified only overlap by about 50% (Figure 3.4B).

The use of the increased stringency is supported by the comparison of genes containing reads specific to either the W22 or B73 allele from chromosome 8 to a list of genes previously demonstrated to have enriched expression in mature pollen (Table 3.3, Chettoor *et al.* 2014; "MP genes"). The assumption is that an increase in the stringency of an alignment will correlate with an increase in the percentage of genes containing B73 allele specific reads (pollen derived) and a decrease in the percentage of W22 allele containing genes (silk derived) overlapping with the set of "MP genes". Increasing the number of allowable alignments locations did demonstrate an agreement with our assumption, suggesting that using this parameter did increase the stringency in accurately identifying pollen-derived genes.

Estimating the contribution of sequencing errors and erroneous HapMap SNP calls

Requiring a perfect match during alignment to either the B73 or W22 genome was intended to remove reads containing sequencing errors from analysis. If a sequencing error were to occur at a published SNP location, it may lead to the false detection and assignment of an allele-specific read. Incorrect SNP calls in the HapMap data are another source of error, which may lead to the incorrect assignment of reads to an allele. To estimate the contribution of these two errors to allele assignment in this study a ratio of false allele detection was calculated using reads from chromosome 8 determined to be W22 allele specific from the B73 pollen sample, as there should be no W22 specific reads in this sample.

This ratio was calculated for each gene on chromosome 8 by dividing the ratio of W22-assigned reads to total number of reads for a gene by the ratio of gene length to number of SNPs in that gene (Equation 1). Genes were separated into two classes, [1] those associated with both W22 and B73 allele specific reads (Figure 3.2, red) and [2] those with only W22 allele specific reads (Figure 3.2, blue). This second class is

predominately comprised of genes where there appears to be a disagreement between the published B73 genome sequence and the reported HapMapv2 nucleotide for the SNP present in that gene.

There appears to be a low level of false allele assignment due to sequencing error in the B73 pollen sample (Figure 3.5, red); the presence of only a few errors results in a smaller numerator in the ratio calculation (Equation 1) and a log value towards the left of the distribution (Figure 3.5). This is concordant with an estimation of the chance of a false positive SNP containing read, calculated by raising the chance of an erroneous SNP to the power of the read length (0.25^89). Genes where the possible error originates from uncertainty in the reference genome sequence display a larger average error ratio (Figure 3.5, blue) as this will increases the number of W22-specific reads detected in the sample and increase the size of the numerator in the error ratio (Equation 1).

$$Error\ ratio\ for\ a\ gene = Log(\frac{W22\ specfic\ reads/Total\ aligned\ reads}{Length\ of\ gene/SNPs\ in\ gene}) \qquad [1]$$

The error analysis suggests that the level of sequencing errors, resulting in false positives, is low enough to not obscure detection of true SNPs.

Alignment and whole genome assignment of reads

Based on the findings from parameter tests on chromosome 8, alignment of the W22 silk sample and the two replicates of the pollinated W22 silk with B73 pollen was conducted to the whole genome sequences of the B73 reference genome and the W22 pseudogenome, permitting 0 mismatches and alignments to only a single location (Table 3.4). The number of detected B73 allele specific reads in the pollinated silk sample suggest that sensitivity is very low, but that it is possible to detect some pollen expression. Comparison of the 50 genes with the highest expression of B73 allele specific reads (Appendix Table A.9) and the 50 genes with the highest expression of W22 allele specific reads (Appendix Table A.10) from the pollinated sample were compared to the published list of genes known to be enriched in pollen relative to seedling tissue (Chettoor *et al.,* 2014), similar to the analysis done with chromosome

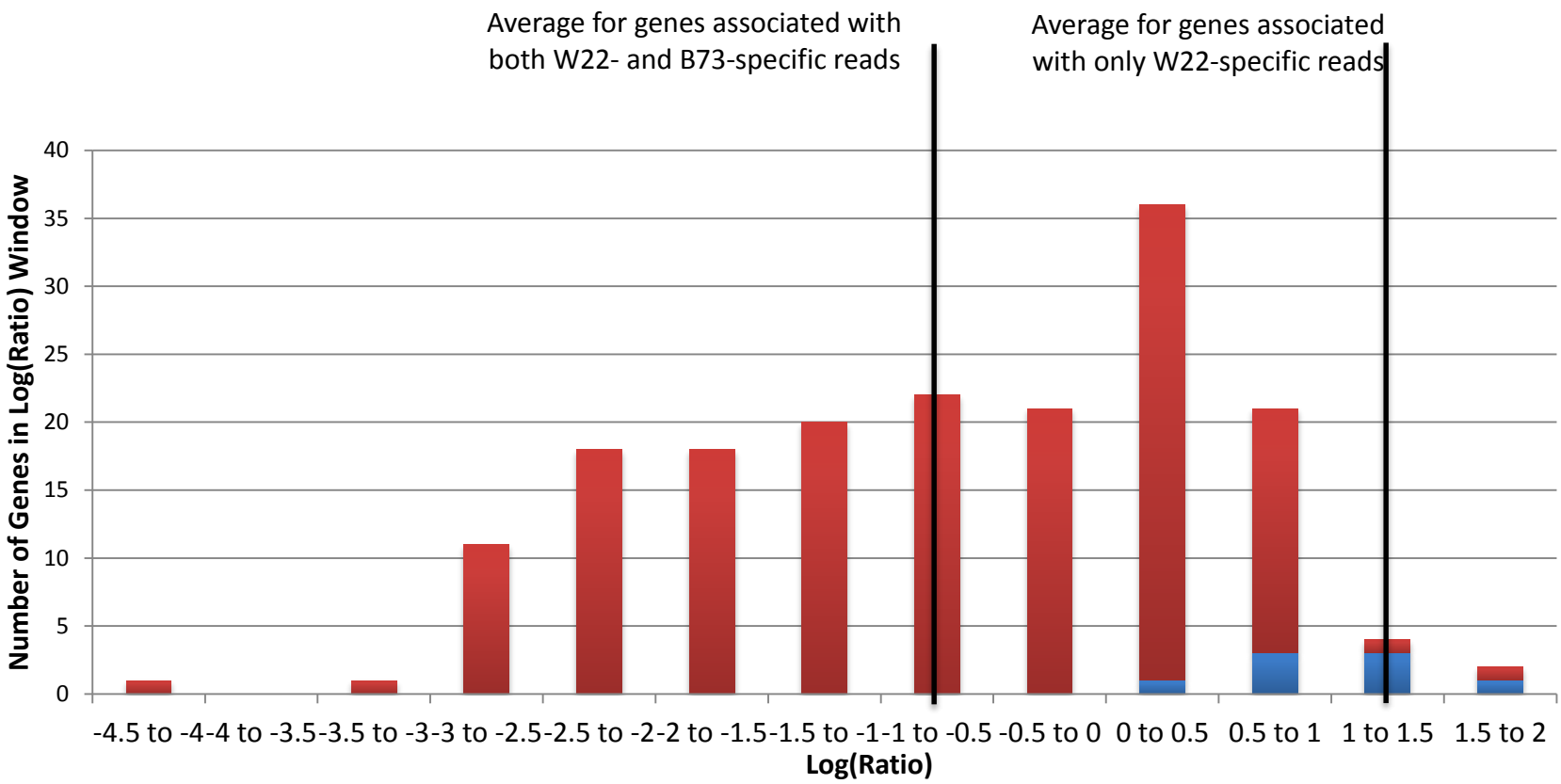


Figure 3.5: Distribution of the Log transformed ratio of aligned W22-specific reads to total reads, accounting for SNPs and gene length in a B73 pollen sample (aligned to chromosome 8).
Genes are separated into two classes, those with B73 and W22 specific reads (red) and those with W22 specific only reads (blue).

Table 3.4: Summary of reads aligned to the whole W22 and B73 genomes from W22 silk only sample and the mixed sample of W22 silk and germinated B73 pollen.
Numbers represent the total number of reads aligning to the specified genome ("Aligned") or the number of reads containing SNPs specific to the specified genome ("Specific") for both reads that align to a single location in the genome. The W22 silk with B73 pollen is the combined totals from the two RNA-seq runs of the same sample.

		Aligned to the W22 Genome		Aligned to the B73 Genome	
	Reads in Sample	**Aligned**	**W22 Specific**	**Aligned**	**B73 Specific**
W22 silk	62,120,276	20,884,612	3,894,783	17,257,711	267,882
W22 silk with B73 Pollen	116,300,999	9,770,135	1,714,276	8,169,986	114,127

8 (Table 3.3). Showing the extended trend, of the 50 top B73 genes, 18 overlapped with the MP gene list, whereas only five genes from the top W22 genes did. Additionally, the results from this study were compared to the findings of a similar study conducted using the maize inbred line Zheng58 (Xu *et al.*, 2013) focusing on changes to the silk's transcriptome during germination. Again, as expected, the 50 highest expressed genes reported by the Xu *et al.* study were found to have no overlap with the top 50 B73 (pollen derived) genes reported, whereas 11 were found to be present in the top W22 (silk derived) genes reported here.

The presence of a large number of B73 allele specific reads in the W22 silk sample was a major concern as this sample should theoretically only contain a small number B73 allele specific reads, due to sequencing error or incorrect SNP assignment from the HapMapv2 data. While evidence does support the presence of B73 pollen derived reads in the pollinated silk samples, the unresolved presence of B73 SNPs in the W22 silk sample and lack of a well-curated W22 reference genome at the time of this analysis suggest revisiting this study at a later date. Similar work currently being conducted using the soon-to-be published W22 genome provide some support that the initial observations made by this study were correct and B73 pollen derived reads are indeed detectable in the pollinated silk samples (Hokin and Fowler, personal communication).

Validation of the presence of B73 pollen reads in the pollinated W22 silk RNA-seq sample

To further confirm the presence of B73 allele specific SNPs in the mixed pollen and silk sample, using the software Geneious (www.geneious.com) reads from the mixed sample were aligned to a selection of W22 allele gene sequences (Figure 3.6). Genes selected were previously identified to be highly expressed in mature pollen (Chettoor *et al.*, 2014) and therefor thought to putatively contain B73 specific reads. Any mismatch highlighted by the software between the RNA reads and the W22 gene models were compared to available B73 SNPs and found to correspond to known polymorphisms. A total of eight genes were tested via this approach, five were

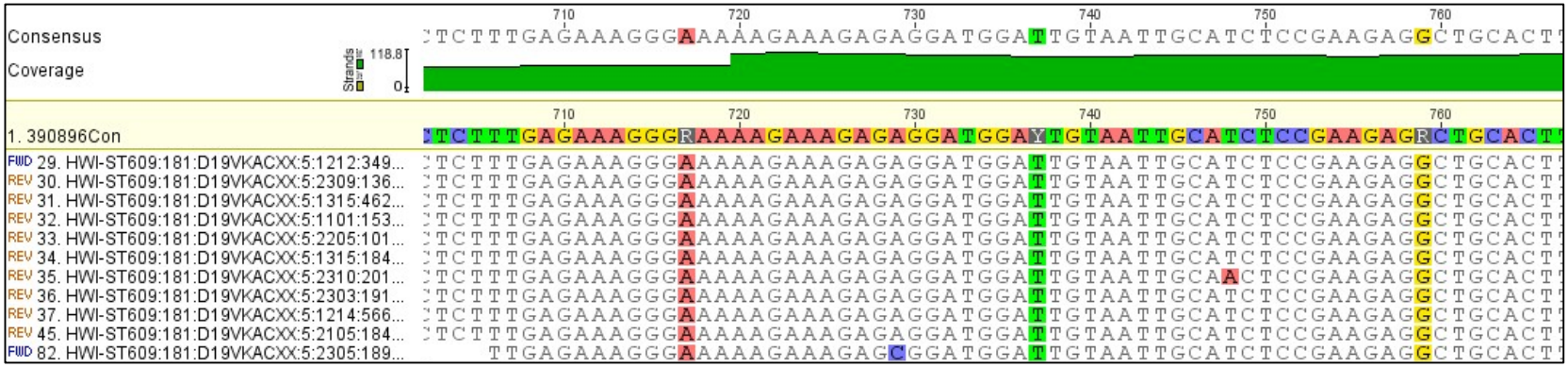


Figure 3.6: **Image from Geneious (www.geneious.com) of a 60 bp section of GRMZM2G390896 with aligned reads from a mixed sample of W22 silks and B73 pollen.**
All reads display B73 SNPs at the three positions located in this region, indicating the presence of pollen-derived transcripts (highlighted bases).

confirmed to contain B73 SNPs (Table 3.5). The three genes without B73 specific SNPs were ones selected as highly expressed in silk tissue and were not expected to contain any B73 allele specific reads.

Cleaved amplified polymorphism (CAPs) analysis was conducted to confirm the presence of B73 allele specific transcripts in independent silk and pollen cDNA samples (Figure 3.7). This analysis takes advantage of SNPs located in endonuclease restriction sites, creating allele-specific digestion products. PCR is used to amplify the region flanking one of these sites and is followed by enzyme digestion to determine the allele present (Table 3.1). Three candidates genes (GRMZM2G433959, GRMZM139223 and GRMZM2G054123) were identified from genes found to contain high numbers of B73 allele specific reads in the whole genome alignment of the mixed W22 silk and B73 pollen sample. However, PCR was only robust for a single gene, GRMZM2G139223. The results from that one gene support the presence of a B73 allele in the cDNA from the pollinated W22 silk sample and in the B73 pollen cDNA and genomic DNA, but not in the unpollinated W22 silk sample (Figure 3.7). This provides independent evidence that the RNA-seq approach investigated here can be successful in identification of transcripts originating from the male gametophyte in pollinated silks.

Table 3.5: **Manual comparison of pollinated silk reads to B73 and W22 allele models of selected genes.**
Models of W22 genes were created by manually inserting described W22 SNPs (HapMapv2) in to B73 gene alleles and reads from the pollinated W22 silks were aligned using Geneious to identify reads containing B73 and W22 allele specific reads.

Characteristic	Gene	Number of SNPs	Total number of Reads Aligned to Gene	B73 Allele Reads	W22 Allele Reads
Randomly Selected	GRMZM2G390896	13	712	485	21
Highly Expressed in Pollen	GRMZM2G378547	1	672	15	2
	GRMZM2G175243	2	148	32	0
	GRMZM2G137037	3	241	72	2
	GRMZM2G027782	5	185	74	0
Highly expressed in silk	GRMZM2G026143	6	2757	0	2636
	GRMZM2G057514	1	539	0	27
	GRMZM2G079616	1	804	0	15

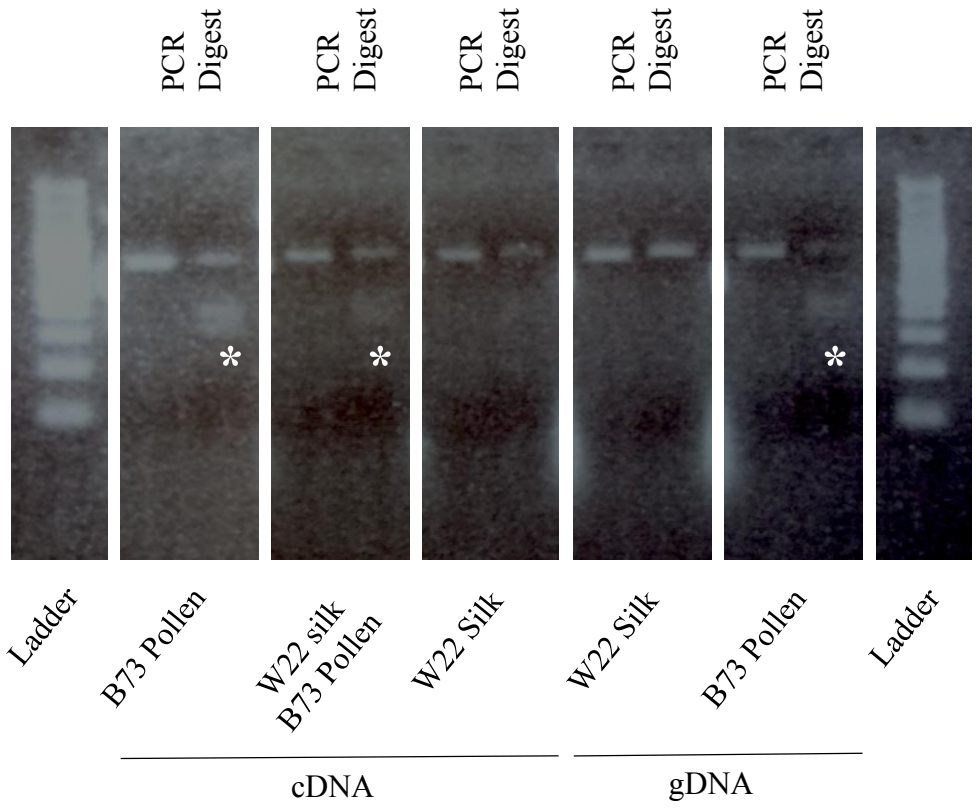


Figure 3.7: **CAPs analysis of GRMZM2G139223.**
Digestion of cDNA and genomic DNA PCR products by EcoR1 cleaves the B73 allele in the samples containing B73 pollen but not in the W22 silk only samples. Asterisks indicate digested cleaved products.

Discussion

Using allele-specific SNPs to assign allele origin, alignment of pollinated W22 silk transcripts identified B73 allele-specific reads. These B73 allele-specific reads putatively represent transcripts originating from the B73 pollen tube. This is least partially supported by the results from CAPS analysis and corresponds with known pollen enriched genes, suggesting that the protocol presented here is sensitive enough to detect pollen reads from a mixed sample. Our analysis of aligned reads from a B73 pollen sample indicate that false allele assignment due to sequencing error only occurs at a low level. Testing of alignment parameters on chromosome 8 revealed that allowing multiple read alignments did not significantly increase the number of pollen derived reads, detected though it did increase the percentage of genes overlapping with genes demonstrated to be enriched in pollen. Using similar parameters to those established in this study, a realignment of the "Mixed" library reads to the updated and improved W22 genome is providing some encouraging results (Hokin and Fowler, personal communication). Notably, 25 out of the top 50 B73 genes identified here (Appendix Table A.9) were identified in a "Good Pollen Tube Candidates" set (defined by B73 allele specific reads giving >0.1 FPKM, and an FPRKM at least 2x greater than the W22 silk only background.).

Increasing alignment parameter from one to three locations increases the enrichment of expressed pollen specific genes detected

The increase in pollen-enriched genes detected after allowing more than a single-alignment location in the pollinated silk sample was expected (Table 3.3), as the pollen genome is biased towards retaining duplicates of genes from the maize allotetraploidy event (Chettoor *et al.*, 2014). This phenomenon may be due to selective pressure acting on the haploid male gametes to maintain gene expression ratios created during the whole genome duplication; the disruption of these ratios by gene loss may have more severe consequences in the male gametophyte than in diploid sporophytic tissue as competition between pollen grains during the progamic phase is high. Genes expressed in the sporophyte appear to be more likely to retain only a single copy of a gene and so single alignments may favor sporophyte tissues,

and multiple alignments may favor those expressed in gametophytes. While the increased sensitivity for pollen-enriched genes is desirable, the inclusion of multiple aligning reads can interfere with the interpretation of the gene counts for the reads.

Detection of reads containing the pollen allele in the unpollinated silk sample may be due to sample bleeding

The presence of a large number of B73 allele specific reads in the W22 silk sample was a major concern, as this sample should theoretically only contain a small number B73 allele specific reads, due to sequencing error or incorrect SNP assignment from the HapMapv2 data. Therefore, the presence of these reads led to postponing complete analysis of the samples. Two possible explanations for the detection of B73 reads are [1] limitations to the available polymorphism information and [2] sample bleeding during sequencing. Though the HapMapv2 data does describe much of the variation present between many different lines of maize, it is not a comprehensive list of all the SNPs present. Additionally, manual inspection of selected SNPs found disagreements between the reported HapMapv2 B73 allele and the sequence from the B73 reference genome (data not shown).

Sample bleeding may occur by two different means, the first is caused by mis-binning during de-multiplexing where the index for the read is attributed to the incorrect sample due to a perceived mismatch in the index. The second scenario is caused by sequencing clusters on a flowcell being in too close proximity, leading to the assignment of the index from one cluster to the other (Kircher *et al.*, 2012). Samples which are most affected by sample bleeding are those intended to detect rare or novel sequences, and this is precisely the type of reads being used in the analysis. If our samples are affected by sample bleeding, it is likely due to the second means, as the number of differences between the indexes used was greater than could be misinterpreted by mismatches.

If sample bleeding has occurred in these datasets, it can be addressed in two ways. The first is to conduct more independent gene validations of genes shown to have

pollen derived SNPs in the silk only sample, such as the CAPs analysis presented here (Figure 3.7). The second method for addressing this read contamination takes advantage of the tissues that were sequenced along with the silk only and pollinated silk samples. During the first sequencing run, both B73 seedling and pollen sample libraries were sequenced in the same flowcell, and thus if bleeding occurred, the majority of the B73 read contamination would be derived from these. In the second run, only the B73 pollen library was included. If sample bleeding occurred, there should be a set of B73-specifc reads from the seedling library that are enriched in the silk only sample from the initial run that are absent or reduced in the second. Additionally, we can use the absence of the seedling samples in the second sequencing run to calculate an estimation of the magnitude of sample bleeding that may be affecting the pollinated silk sample. By focusing on those genes with expression in the first sequencing run but have almost no expression in the second run, a ratio of the aligned reads in these two samples should give an indication of the B73 SNP contamination from the seedling and pollen sample libraries.

Issues affecting alignment of reads from the mixed pollen and silk sample

There are several conditions affecting the interpretation of read alignment. The first is the large number of duplicate genes occurring in the maize genome due to a whole genome duplication. These duplicates introduce an uncertainty when assigning reads to a gene and could lead to the interpretation of gene expression in genes that are only duplicates of expressed genes and are not actively expressed themselves. Between the different inbred lines, expression of duplicates may be different but will appear to be the same if reads align to both copies. While this could be addressed by allowing only reads aligning to single location, the enrichment of duplicate retention in pollen-enriched genes raises the possibility of missing pollen derived gene expression. Another issue impacting the decision of allowable locations for alignment is the observation that some genes are only detected by single alignments while others are only detected with multiple. These could represent genes which occur as a singleton in one inbred but has retained its duplicate in another.

The number of mismatches permitted during alignment represents another parameter that can create a potential problem for the detection of the pollen reads in the mix sample. This study used the stringent cutoff of 0 mismatches, reducing the number of usable reads (for example, over 173k reads were dropped from alignment to chromosome 8). Allowing mismatches could increase the read depth of the samples, especially important for the pollen derived reads which occur at a low concentration in the pollinated silk sample. However, all SNPs specific to an inbred are detected as mismatches in other line and so would align to both genomes. The development of an "allele filter", which can incorporate mismatch information, could permit the increased read depth from allowing mismatches while preventing the loss of inbred specificity. Additionally, the inclusion of a "duplicate filter" which employs ratios of allele specific reads aligning to SNP locations within duplicate genes to assign reads aligning to both could be used to increase the accuracy of read counts assignment.

Future goals for this approach are to incorporate present/absence variation and indels into the pseudo genomes. While there now is an available W22 genome, there also is a published genome for the Mo17 inbred. The relative quality of the two new genomes will determine if the study continues with a mix of B73 and W22 or moves to Mo17.

Literature Cited

Anders S, Pyl PT, Huber W (2015) HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics **31**: 166–169

Boavida LC, Borges F, Becker JD, Feijo JA (2011) Whole Genome Analysis of Gene Expression Reveals Coordinated Activation of Signaling and Metabolic Pathways during Pollen-Pistil Interactions in Arabidopsis. Plant Physiology **155**: 2066–2080

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**(15): 2114-21120

Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MMS (2014) Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. Genome Biol **15**: 414

Chia J-M, Song C, de Leon N, Doebley JF, Elshire RJ, Gaut BS, Geller L, Glaubitz JC, et al (2012) Maize HapMap2 identifies extant variation from a genome in flux. Nature Genetics **44**: 803–807

Dresselhaus T, Lausser A, Marton ML (2011) Using maize as a model to study pollen tube growth and guidance, cross-incompatibility and sperm delivery in grasses. Annals of Botany **108**: 727–737

Dresselhaus T, Franklin-Tong N (2013) Male-Female Crosstalk during Pollen Germination, Tube Growth and Guidance, and Double Fertilization. Molecular Plant **6**: 1018–1036

Ehrhardt DW, Shaw SL (2006) Microtubule dynamics and organization in the plant cortical array. Annu Rev Plant Biol **57**: 859–75

Fahlgren N, Sullivan CM, Kasschau KD, Chapman EJ, Cumbie JS, Montgomery TA, Gilbert SD, Dasenko M, Backman TWH, Givan SA, et al (2009) Computational and analytical framework for small RNA profiling by high-throughput sequencing. RNA **15**: 992–1002

Gore MA, Chia J-M, Elshire RJ, Sun Q, Ersoz ES, Hurwitz BL, Peiffer JA, McMullen MD, Grills GS, Ross-Ibarra J, et al (2009) A First-Generation Haplotype Map of Maize. Science **326**: 1115–1117

Hiscock SJ, Allen AM (2008) Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. New Phytologist **179**: 286–317

Kircher M, Sawyer S, Meyer M (2012) Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res **40**(1): e3

Krichevsky A, Kozlovsky SV, Tian GW, Chen MH, Zaltsman A, Citovsky V

(2007)How pollen tubes grow. Dev Biol **303**(2): 405–20

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Meth **9**: 357–359

Lausser A, Kliwer I, Srilunchang K-O, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in maize. Journal of Experimental Botany **61**: 673–682

Li M, Wang K, Li S, Yang P (2016) Exploration of rice pistil responses during early post-pollination through a combination proteomic and transcriptomic analysis. Journal of Proteomics **131**: 214–226

McCubbin AG and Kao T-H (2000) Molecular recognition and response in pollen and pistil interactions. Annu Rev Cell Dev Biol **16**: 333 – 364

Mourino-Perez RR, Roberson RW, Bartnicki-Garcia S (2006) Microtubule dynamics and organization during hyphal growth and branching in Neurospora crassa. Fungal Genet Biol **43**(6): 389–400

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Qin Y, Yang Z (2011) Rapid tip growth: Insights from pollen tubes. Seminars in Cell and Developmental Biology **22**: 816–824

Rea AC, Nasrallah JB (2008) Self-incompatibility systems: barriers to self-fertilization in flowering plants. International Journal of Developmental Biology **52**: 627–636

Silva NF, Goring DR (2001) Mechanisms of self-incompatibility in flowering plants. Cell Mol Life Sci **58**: 1988–2007

Swanson WJ, Vacquier VD (2002) The Rapid Evolution of Reproductive Proteins. Nature Reviews Genetics **3**: 137–144

Swanson WJ, Wong A, Wolfner MF, Aquadro CF (2004) Evolutionary Expressed Sequence Tag Analysis of Drosophila Female Reproductive Tracts Identifies Genes Subjected to Positive Selection. Genetics **168**: 1457–1465

Wang Y, Zhang W-Z, Song L-F, Zou J-J, Su Z, Wu W-H (2008) Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in Arabidopsis. Plant Physiology **148**: 1201–1211

Waters AJ, Makarevitch I, Eichten SR, Swanson-Wagner RA, Yeh C-T, Xu WW, Schnable PS, Vaughn MW, Gehring M, Springer NM (2011) Parent-of-Origin Effects on Gene Expression and DNA Methylation in the Maize Endosperm. THE PLANT CELL ONLINE **23**: 4221–4233

Xu XH, Wang F, Chen H, Sun W, Zhang XS (2013) Transcript Profile Analyses of Maize Silks Reveal Effective Activation of Genes Involved in Microtubule-Based Movement, Ubiquitin-Dependent Protein Degradation, and Transport in the Pollination Process. PLoS ONE **8**: e53545

Acknowledgements
John Fowler helped with the initial sample collection for this study and the error analysis presented here. Christopher Sullivan, Sam Hokin and the Center for Genome Research and Biocomputing provided bioinformatic support and advice. The author would like to thank Taj Morton for this assistance in creating the W22 “pseudogenome”.

Chapter 4 : General Conclusions

The model of the progamic phase proposed in this work is that the initial rapid transition from mature to germinated pollen in maize (*Zea mays*) is orchestrated through mechanisms of translation and proteasome-mediated protein degradation rather than transcription. After this early step in the progamic phase, transcription is initiated in response to the growth into and through the female sporophyte (Qin *et al.*, 2009). Supporting this model is evidence from SDS-PAGE silver staining of total protein extracts and quantitative proteomic profiling of mature and germinated pollen after 30 minutes. These analyses confirmed changes in abundance of several proteins occurring upon germination. Functional analysis of the proteomic data found components of translation and protein degradation supporting the hypothesis that these two mechanism regulated germination in some fashion. Additionally, an enrichment of proteins decreasing in abundance with germination as found in the jasmonic acid biosynthesis pathway suggesting that this pathway is specifically down regulated in the transition to the progamic phase. RNA-seq of pollinated silks was able to detect reads putatively derived from the male gametophyte but it is unclear if this is the result of *de novo* translation or from transcripts already present in the pollen grain.

Why take a proteomics approach?

Much of the research into developmental transitions has focused on the transcriptome to characterize the mechanisms regulating the developmental stage studied. However, the study presented here provides evidence for using proteomic analyses in addition to transcriptomics. Through the use of quantitative proteomic profiling we were able to detect changes in gene expression occurring with germination that were not reflected in the transcriptome of mature and germinated pollen (Fowler, Vejlupkova, Cooper, Watrud, unpublished). Additionally proteomic profiling was able to uncover a possible role of jasmonic acid biosynthesis pathway in maize pollen development and identify several proteins, putatively important to pollen function, which exhibit defects in transmission when challenged with insertion mutations.

Of the ten genes with exon insertions tested, five were found to exhibit a clear transmission defect. This supports the use of proteomic profiling, in combination with transcriptome data, to identify candidates for male transmission defect testing as a similar study conducted with predictions from transcriptomic data alone found only two out of nine to display a transmission defect (Chettoor *et al.*, 2014). While the phenotypes for the five proteins identified in this study are not currently clear, all appear to link with functions previously determined to be important for pollen function or were predicted to be important in this study including the cell wall, signaling protein degradation and glycolysis.

Future work for characterizing the progamic phase in Zea mays pollen

Going forward, the next step for the proteomic analysis to determine the specific mechanisms regulating protein abundance. Initial testing of three of the hypotheses mentioned above (intron retention, RNA particles (mRNPs), polysomes) has indicated that intron retention probably does not play a large role in coordinating rapid protein translation in the male maize gametophyte. No evidence of enrichment of introns was found among the transcripts in mature pollen for proteins significantly increasing in abundance with germination (Table 2.5). There is, however, some evidence supporting the other two hypotheses. mRNPs, while not yet identified in maize pollen, have been previously studied in tobacco pollen (Honys *et al.*, 2009) where they were observed to be associated with ribosomes and involved with protein synthesis and to function as storage of mRNA, including transcripts for pollen specific genes.

Similar to mRNPs are polysomes, which are formed when a single transcript is loaded with multiple ribosomes. Polysomes may be included as components of mRNPs but they also are found on their own (Honys *et al.*, 2009; Mustroph *et al.*, 2009). Previous studies have demonstrated increases in the number of polysomes occurring with germination in several different species (Mascarenhas and Bell, 1969; Hoekstra and Bruinsma, 1979). This is correlated with a rapid increase in translation in the pollen grain. A pilot study using sucrose gradient fractionation and samples of mature and

germinated pollen found an enrichment of polysomes in the mature pollen which was followed by a shift to monosomes with germination (Data not shown). This suggests that polysomes may play an important role in increases in protein abundance during germination in maize pollen. To test this, one would have to determine if the transcripts associated with the shifting polysomes are correlated with specific proteins increasing with germination. Ribosomal foot printing or profiling (Ingolia *et al.*, 2009) can be used to determine which mRNA are actively being translated (translatome). Digestion of RNA by nucleases will leave only those sections of transcripts protected by ribosomes (the footprint), which can be aligned following sequencing to determine ribosome load and placement on the mRNA. Phosporylation of ribosomes hypothesis may be tested by isolating ribosomal proteins using a sucrose cushion followed by chracterication of the proteins and mapping of phosphorylation site by nano-LC-MS/MS (Turkina *et al.*, 2011).

The results from the germination tests with methyl jasmonate (meJA), rupture of pollen grains and reduced pollen tube length (Figure 2.12 and 2.13), suggest that there is some effect on cell wall integrity and the pollen tube tip in response to the phytohormone. Currently the exact affect the meJA has on the pollen grain is unclear. Further tests with combinations of meJA with translational and proteasomal inhibitors may be useful in characterizing the meJA response. Additionally coronatine (COR), a positive analog for meJA, and diethyldithiocarbamic acid (DIECA), an inhibitor of JA biosynthesis, could be used to determine if the response we are observing are due to direct or indirect effects of the meJA on the pollen grain.

Phenotyping of the insertions demonstrated to exhibit a male transmission defect, while very useful, is a difficult to conduct effectively. Some defects only exhibit a subtle phenotypic effect that may be obscured by measurements of wild type pollen in heterozygous individuals. The functional importance of the affected proteins may also not come into play until later in the progamic phase than was tested in this study. The germination test is also conducted *in vitro* which may ameliorate the effects of the insertion on pollen function. The use of the linked *waxy1* (*wx*) marker (Bedinger and

Fowler, 2009) may represent a more effective approach to the phenotyping of the defect inducing insertions as it allows one to phenotype and genotype the germinating pollen *in vivo*. In addition to improving the phenotyping assay, a second cross to test for transmission should be conducted in the candidate identified. The use of Mu-seq will allow for high throughput genotyping of the progeny of these crosses where the insertion used is *Mutator*. Currently 45 insertions have been submitted for testing by Mu-seq from this project. The locations of the insertions demonstrated to elicit a transmission defect will need to be validated through sequencing.

The transcriptome analysis of the pollinated silk represented a pilot study to determine if the approach was successful in identifying pollen derived reads. While there are some concerns that need to be addressed regarding the method and possible sample bleeding, it does appear to be sensitive enough to detect pollen expression in the silk. To improve the outcome of this study filters should be incorporated into the alignment pipeline to accounts for alignment to gene duplicates and to allow for the alignment of reads with mismatches without causing an increase in false positives for SNPs. The results from the combination of the proteomic profiling and this transcriptomic analysis can be used in the development of a complete model of the progamic phase of *Zea mays* pollen.

Literature Cited

Bedinger P, Fowler J (2009) The maize male gametophyte. In: Hake S, Bennetzen J (eds) Handbook of maize: its biology. Springer, New York, pp 57–77

Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MMS (2014) Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. Genome Biol **15**: 414

Hoekstra FA, Bruinsma J (1979) Protein Synthesis of Binucleate and Trinucleate Pollen and Its Relationship to Tube Emergence and Growth. Planta **146**: 559–566

Honys D, Reňák D, Feciková J, Jedelský PL, Nebesářová J, Dobrev P, Capkova V (2009) Cytoskeleton-Associated Large RNP Complexes in Tobacco Male Gametophyte (EPPs) Are Associated with Ribosomes and Are Involved in Protein Synthesis, Processing, and Localization. J Proteome Res **8**: 2015–2031

Ingolia NT, Ghaemmaghami S, Newman JRS, Weissman JS (2009) Genome-Wide Analysis in Vivo of Translation with Nucleotide Resolution Using Ribosome Profiling. Science **324**(5924): 218–223

Mascarenhas JP and Bell E (1969) Protein synthesis during germination of pollen: studies on polyribosome formation. Biochem Biophys Acta **179**(1): 199-203

Mustroph A, Juntawong P, Bailey-Serres J (2009) Isolation of Plant Polysomal mRNA by Differential Centrifugation and Ribosome Immunopurification Methods. *In* DA Belostotsky, ed, Methods in Molecular Biology. Humana Press, pp 1–21

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Turkina MV, Klang Årstrand H, Vener AV (2011) Differential Phosphorylation of Ribosomal Proteins in Arabidopsis thaliana Plants during Day and Night. PLoS ONE **6**: e29307

References

Ajtkhozhin MA, Doschanov KJ, Akhanov AU (1976) Informosomes as a stored form of mRNA in wheat embryos. FEBS Lett. **66**(1): 124–126

Allen RL1, Lonsdale DM (1993) Molecular characterization of one of the maize polygalacturonase gene family members which are expressed during late pollen development. Plant J **3**(2): 261-271

Amaldi PP, Felicetti L, Campioni N (1977) Flow of information RNA from cytoplasmic poly(A)-containing particles to polyribosomes in *Artemia salina* cysts at early stages of development. Dev. Biol. **59**: 49–61

Anders S, Pyl PT, Huber W (2015) HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics **31**: 166–169

Ariizumi T, Hatakeyama K, Hinata K, Inatsugi R, Nishida I, Sato S, Kato T, Tabata S, Toriyama K (2004) Disruption of the novel plant protein NEF1 affects lipid accumulation in the plastids of the tapetum and exine formation of pollen, resulting in male sterility in Arabidopsis thaliana. Plant J **39**(2): 170-181

Arunkumar R, Josephs EB, Williamson RJ, Wright SI (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**: 2475–2486

Baker HG, Baker I (1979) Starch in angiosperm pollen grains and its evolutionary significance. American Journal of Botany **66**: 591 – 600

Barnabas B (1985) Effect of water loss on germination ability of Maize (*Zea mays* L.) pollen. Annals of Botany **55**: 201–204

Bedinger P, Fowler J (2009) The maize male gametophyte. In: Hake S, Bennetzen J (eds) Handbook of maize: its biology. Springer, New York, pp 57–77

Beilharz TH, Preiss T (2004) Translational profiling: the genome-wide measure of the nascent proteome. Brief Funct Genomic Proteomic **3**: 103–111

Beltran-Pena E, Aguilar R, Ortiz-Lopez A, Dinkova TD, De Jimenez ES (2002) Auxin stimulates S6 ribosomal protein phosphorylation in maize thereby affecting protein synthesis regulation. Physiol Plant **115**: 291–297

Bernal AJ, Yoo C-M, Mutwil M, Jensen JK, Hou G, Blaukopf C, Sørensen I, Blancaflor EB, Scheller HV, Willats WGT (2008) Functional analysis of the cellulose synthase-like genes CSLD1, CSLD2, and CSLD4 in tip-growing Arabidopsis cells. Plant Physiol **148**(3):1238–1253

Boavida LC, Borges F, Becker JD, Feijo JA (2011) Whole Genome Analysis of Gene Expression Reveals Coordinated Activation of Signaling and Metabolic Pathways during Pollen-Pistil Interactions in Arabidopsis. Plant Physiology **155**: 2066–2080

Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**(15): 2114-21120

Bonifacino JS, Traub LM (2003) Signals for Sorting of Transmembrane Proteins to Endosomes and Lysosomes. Annual Review of Biochemistry **72**: 395–447

Boothby TC, Zipper RS, van der Weele CM, Wolniak SM (2013) Removal of Retained Introns Regulates Translation in the Rapidly Developing Gametophyte of Marsilea vestita. Developmental Cell **24**: 517–529

Bou Daher F, Geitmann A (2011) Actin is Involved in Pollen Tube Tropism Through Redefining the Spatial Targeting of Secretory Vesicles. Traffic **12**: 1537–1551

Browse J (2009) Jasmonate: preventing the maize tassel from getting in touch with his feminine side. Sci Signal **2**(59): pe9

Brewbaker JL (1967) The Distribution and Phylogenetic Significance of Binucleate and Trinucleate Pollen Grains in Angiosperms. American Journal of Botany **54**: 1069–1083

Brockmann R, Beyer A, Heinisch JJ, Wilhelm T (2007) Post- transcriptional expression regulation: what determines translation rates? PLoS Comp Biol **3**: 0531–0539

Calderon-Villalobos LI, Tan X, Zheng N, Estelle M (2010) Auxin perception--structural insights. Cold Spring Harb Perspect Biol. **2**(7): a005546

Capco DG, Jeffery WR (1979) Origin and spatial distribution of maternal messenger RNA during oogenesis of an insect, *Oncopeltus fasciatus*. J. Cell Sci. **39**: 63–76

Čapková V, Hrabětová E, Tupý J, Říhová L (1983) Amino acid uptake and protein synthesis in cultured tobacco pollen. Biochem. Physiol. Pflanzen **178**: 511-520

Čapková V, Hrabětová E, Tupý J (1988) Protein synthesis in pollen tubes: preferential formation of new species independant of transcription. Sexual Plant Reproduction **1**: 150–155

Carson JH, Barbarese, E (2005) Systems analysis of RNA trafficking in neural cells. Biol. Cell **97** (1): 51–62

Chao Q, Gao Z-F, Wang Y-F, Li Z, Huang X-H, Wang Y-C, Mei Y-C, Zhao B-G, Li L, Jiang Y-B, et al (2016) The proteome and phosphoproteome of maize pollen uncovers fertility candidate proteins. Plant Mol Biol 1–18

Chen J, Zeng B, Zhang M, Xie S, Wang G, Hauck A, Lai J (2014) Dynamic Transcriptome Landscape of Maize Embryo and Endosperm Development. Plant Physiology **166**: 252–264

Chettoor AM, Givan SA, Cole RA, Coker CT, Unger-Wallace E, Vejlupkova Z, Vollbrecht E, Fowler JE, Evans MMS (2014) Discovery of novel transcripts and gametophytic functions via RNA-seq analysis of maize gametophytic transcriptomes. Genome Biol **15**: 414

Chia J-M, Song C, de Leon N, Doebley JF, Elshire RJ, Gaut BS, Geller L, Glaubitz JC, et al (2012) Maize HapMap2 identifies extant variation from a genome in flux. Nature Genetics **44**: 803–807

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, Micol JL, Solano R (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448**(7154): 666-671

Choi H, Fermin D, Nesvizhskii AI (2008) Significance Analysis of Spectral Count Data in Label-free Shotgun Proteomics. Molecular and Cellular Proteomics **7**: 2373–2385

Cole RA, Fowler JE (2006) Polarized growth: maintaining focus on the tip. Current Opinion in Plant Biology **9**: 579–588

Craiu A, Gaczynska M, Akopian T, Gramm CF, Fenteany G, Goldberg AL, Rock KL (1997) Lactacystin and clasto-lactacystin beta-lactone modify multiple proteasome beta-subunits and inhibit intracellular protein degradation and major histocompatibility complex class I antigen presentation. J. Biol. Chem. **272**: 13437-13445

Curtis D, Lehmann R, Zamore PD (1995) Translational regulation in development. Cell **81**: 171–178

Dajoz I, Till-Bottraud I, Gouyon PH (1991) Evolution of pollen morphology. Science **253**: 66–68

Davidson EH (1986) Gene Activity in Early Development, Third Edition (New York: Academic Press)

Davidson RM, Hansey CN, Gowda M, Childs KL, Lin H, Vaillancourt B, Sekhon RS, de Leon N, Kaeppler SM, Jiang N, et al (2011) Utility of RNA Sequencing for Analysis of Maize Reproductive Transcriptomes. The Plant Genome Journal **4**: 191

Dearnaley JDW, Daggard GA (2001) Expression of a polygalacturonase enzyme in germinating pollen of *Brassica napus*. Sexual Plant Reproduction **14**: 27–34

Deeks MJ, Rodrigues C, Dimmock S, Ketelaar T, Maciver SK, Malhó R, Hussey PJ (2007) Arabidopsis CAP1 - a key regulator of actin organization and development. J Cell Sci **120**(Pt 15): 2609-2618

Demerec M. (1929) Cross-sterility in maize. Zeitschrift fur Induktive- und Abstammungslehre **50:** 281–291.

Digel B, Pankin A, Korff von M (2015) Global Transcriptome Profiling of Developing Leaf and Shoot Apices Reveals Distinct Genetic and Environmental Control of Floral Transition and Inflorescence Development in Barley. THE PLANT CELL ONLINE **27**: 2318–2334

Dobritzsch S, Weyhe M, Schubert R, Dindas J, Hause G, Kopka J, Hause B (2015) Dissection of jasmonate functions in tomato stamen development by transcriptome and metabolome analyses. BMC Biology **13**: 1–18

Dresselhaus T, Lausser A, Marton ML (2011) Using maize as a model to study pollen tube growth and guidance, cross-incompatibility and sperm delivery in grasses. Annals of Botany **108**: 727–737

Dresselhaus T, Franklin-Tong N (2013) Male-Female Crosstalk during Pollen Germination, Tube Growth and Guidance, and Double Fertilization. Molecular Plant **6**: 1018–1036

Du Z, Zhou X, Ling Y, Zhang Z, Su Z (2010) agriGO: a GO analysis toolkit for the agricultural community. Nucleic Acids Res **38**: W64-70

Dure L, Waters L (1965) Long-Lived Messenger RNA: Evidence from Cotton Seed Germination. Science **147**: 410–412

Egli B, Kölling K, Köhler C, Zeeman SC, Streb S (2010) Loss of cytosolic phosphoglucomutase compromises gametophyte development in Arabidopsis. Plant Physiol 154(4): 1659-1671

Ehrhardt DW, Shaw SL (2006) Microtubule dynamics and organization in the plant cortical array. Annu Rev Plant Biol **57**: 859–75

Elsasser S, Chandler-Militello D, Muller B, Hanna J, Finley D (2004) Rad23 and Rpn10 serve as alternative ubiquitin receptors for the proteasome. J. Biol. Chem. **279**: 26817-26822

Evans MMS, Kermicle JL (2001) Teosinte crossing barrier1, a locus governing hybridization of teosinte with maize. Theoretical and Applied Genetics **103**: 259–265

Facette MR, Shen Z, Bjornsdottir FR, Briggs SP, Smith LG (2013) Parallel Proteomic and Phosphoproteomic Analyses of Successive Stages of Maize Leaf Development. THE PLANT CELL ONLINE **25**: 2798–2812

Fahlgren N, Sullivan CM, Kasschau KD, Chapman EJ, Cumbie JS, Montgomery TA, Gilbert SD, Dasenko M, Backman TWH, Givan SA, et al (2009) Computational and analytical framework for small RNA profiling by high-throughput sequencing. RNA **15**: 992–1002

Farmer EE, Ryan CA (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. PNAS **87**: 7713–7716

Franchi GG, Bellani L, Nepi M, Pacini E (1996) Types of carbohydrate reserves in pollen: localization, systematic distribution and ecophysiological significance. Flora **191**: 143–159

Franchi GG, Nepi M, Dafni A, Pacini E (2002) Partially hydrated pollen: taxonomic distribution, ecological and evolutionary significance. Plant Syst Evol **234**: 211–227

Franke WW, Herth W, Vanderwoude WJ, Morré DJ (1972) Tubular and filamentous structures in pollen tubes: Possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta **105**(4): 317-341

Franklin-Tong VE (1999) Signaling and the Modulation of Pollen Tube Growth. The Plant Cell **11**: 727–738

Furness CA, Rudall PJ (2004) Pollen aperture evolution—a crucial factor in eudicot success? Trends in Plant Science **9**: 154–158

Genschik P, Criqui MC, Parmentier Y, Derevier A, Fleck J (1998) Cell cycle dependent proteolysis in plants. Identification Of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor MG-132. Plant Cell **10**(12): 2063-2076

Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, Baumeister W, Fried VA, Finley D (1998) A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell **94**: 615-623

Golovkin M, Reddy AS (2003) A calmodulin-binding protein from Arabidopsis has an essential role in pollen germination. PNAS **100**(18): 10558-10568

Gonzalez-Galarza FF, Lawless C, Hubbard SJ, Fan J, Bessant C, Hermjakob H, Jones AR (2012) A Critical Appraisal of Techniques, Software Packages, and Standards for Quantitative Proteomic Analysis. OMICS: A Journal of Integrative Biology **16**: 431–442

Gore MA, Chia J-M, Elshire RJ, Sun Q, Ersoz ES, Hurwitz BL, Peiffer JA, McMullen MD, Grills GS, Ross-Ibarra J, et al (2009) A First-Generation Haplotype Map of Maize. Science **326**: 1115–1117

Greenbaum D, Colangelo C, Williams K, Gerstein M (2003) Comparing protein abundance and mRNA expression levels on a genomic scale. Genome Biol **4**: 117

Grennan AK (2007) An Analysis of the Arabidopsis Pollen Transcriptome. Plant Physiology **145**: 3–4

Grosfeld H, Littauer UZ (1975) Cryptic form of mRNA in dormant *Artemia salina* cysts. Biochem. Biophys. Res. Commun. **67**: 176–181

Gross PR, Cousineau GH (1963) Effects of actinomycin D on macro- molecule synthesis and early development in sea urchin eggs. Biochem. Biophys. Res. Commun. **10**: 321–326

Gross PR, Cousineau GH (1964) Macromolecule Synthesis and the Influence of Actinomycin on Early Development. Exp. Cell Res. **33**: 368–395

Gygi SP, Rist B, Gerber SA, Turecek F, Gelb MH, Aebersold R (1999) Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. Nature Biotechnology **17**: 994–999

Hafidh S, Breznenová K, Ruzicka P, Feciková J, Capkova V, Honys D (2012) Comprehensive analysis of tobacco pollen transcriptome unveils common pathways in polar cell expansion and underlying heterochronic shift during spermatogenesis. BMC Plant Biol **12**: 24

Hafidh S, Potěšil D, Fíla J, Capkova V, Zdráhal Z, Honys D (2016) Quantitative proteomics of the tobacco pollen tube secretome identifies novel pollen tube guidance proteins important for fertilization. Genome Biol 1–29

Hao H, Li Y, Hu Y, Lin J (2005) Inhibition of RNA and protein synthesis in pollen tube development of Pinus bungeana by actinomycin D and cycloheximide. New Phytologist **165**: 721–729

Harmer SL (2009) The Circadian System in Higher Plants. Annu Rev Plant Biol **60**: 357–377

Hart PE, Wolniak SM (1998) Spermiogenesis in Marsilea vestita: a temporal correlation between centrin expression and blepharoplast differentiation. Cell Motil. Cytoskeleton **41**: 39–48

Harvey EB (1936). Parthenogenetic merogony or cleavage, without nuclei in *Arbacia punctulata*. Biol. Bull. **71**: 101–121

Harvey EB (1940) A Comparison of the Development of Nucleate and Non-Nucleate Eggs of *Arbacia punctulata*. Biol. Bull. **79**: 166–187

Hayashi K (2012) The interaction and integration of auxin signaling components. Plant Cell Physiol **53**(6): 965-975

Hedhly A, Hormaza JI, Herrero M (2005) The Effect of Temperature on Pollen Germination, Pollen Tube Growth, and Stigmatic Receptivity in Peach. **7**: 476–483

Heslop-Harrison J (1979a) Pollen–stigma interaction in grasses: a brief review. New Zealand Journal of Botany **17**: 537–546

Heslop-Harrison J (1979b) Pollen walls as adaptive systems. Annals of the Missouri Botanical Garden **66**: 813–829

Heslop-Harrison J (1982) Pollen-Stigma Interaction and Cross-Incompatibility in the Grasses. Science **215**: 1358–1364

Heslop-Harrison Y, Reger BJ, Heslop-Harrison J (1984) The pollen–stigma interaction in the grasses. 6. The stigma (silk) of *Zea mays* L. as host to the pollens of Sorghum bicolor (L.) Moench and Pennisetum americanum (L.) Leeke. Acta Botanica Neerlandica **33**: 205–227

Heslop-Harrison Y, Heslop-Harrison J, Reger BJ (1985) The pollen–stigma interaction in the grasses. 7. Pollen-tube guidance and the regulation of tube number in *Zea mays* L. Acta Botanica Neerlandica **34**: 193–211

Hiscock SJ, Allen AM (2008) Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. New Phytologist **179**: 286–317

Hoekstra FA (1979) Mitochondrial development and activity of bi- nucleate and trinucleate pollen during germination in vitro. Planta **145**: 25–36

Hoekstra FA, Bruinsma J (1975) Respiration and vitality of binucleate and trinucleate pollen. Physiologia Plantarum **34**: 221 – 225

Hoekstra FA, Bruinsma J (1978) Reduced independence of the male gametophyte in angiosperm development. Ann. Bot. **42**: 759-762

Hoekstra FA, Bruinsma J (1979) Protein synthesis of binucleate and trinucleate pollen and its relationship to tube emergence and growth. Planta **146**: 559-566

Honys D, Combe JP, Twell D, Capkova V (2000) The translationally repressed pollen-specific ntp303 mRNA is stored in non-polysomal mRNPs during pollen maturation. Sexual Plant Reproduction **13**: 135–144

Honys D, Twell D (2004) Transcriptome analysis of haploid male gametophyte development in Arabidopsis. Genome Biol **5**: R85.1–R85.13

Honys D, Reňák D, Feciková J, Jedelský PL, Nebesářová J, Dobrev P, Capkova V (2009) Cytoskeleton-Associated Large RNP Complexes in Tobacco Male Gametophyte (EPPs) Are Associated with Ribosomes and Are Involved in Protein Synthesis, Processing, and Localization. J Proteome Res **8**: 2015–2031

Hughes DW, Galau GA (1989) Temporally modular gene expression during cotyledon development. Genes Dev. **3**: 358–369

Hughes DW, Galau GA (1991) Developmental and environmental induction of Lea and Lea A mRNAs and the post abscission program during embryo culture. Plant Cell **3**: 605–618

Ingolia NT, Ghaemmaghami S, Newman JRS, Weissman JS (2009) Genome-Wide Analysis in Vivo of Translation with Nucleotide Resolution Using Ribosome Profiling. Science **324**(5924): 218–223

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCENCE1 Gene Encodes a Novel Phospholipase A1 Catalyzing the Initial Step of Jasmonic Acid Biosynthesis, Which Synchronizes Pollen Maturation, Anther Dehiscence, and Flower Opening in Arabidopsis. The Plant Cell **13**: 2191–2209

Iwanami, Y (1968).Physiological researches of pollen. XVII. On the resistance of pollen to radial rays and chemical inhibitors. J. Yokohama City Univ. 175 (2-55, Biol. 24), 1-31

Jackson RJ, Hellen CU, Pestova TV (2010) The mechanism of eukaryotic translation initiation and principles of its regulation. Nat Rev Mol Cell Biol **11**: 113–127

Jansen RP (2001) mRNA localization: message on the move. Nat. Rev. Mol. Cell Biol. **2** (4): 247–256

Ju Y, Guo L, Cai Q, Ma F, Zhu Q-Y, Zhang Q, Sodmergen S (2016) Arabidopsis JINGUBANG is a Negative Regulator of Pollen Germination that Prevents Pollination in Moist Environments. THE PLANT CELL ONLINE tpc.00401.2016

Juntawong P, Girke T, Bazin J, Bailey-Serres J (2013) Translational dynamics revealed by genome-wide profiling of ribosome footprints in Arabidopsis. PNAS 203–212

Kang BH, Rancour DM, Bednarek SY (2003) The dynamin-like protein ADL1C is essential for plasma membrane maintenance during pollen maturation. Plant J **35**(1): 1-15

Kannaste O, Suomi T, Salmi J, Uusipaikka E, Nevalainen O, Corthals GL (2014) Cross-correlation of spectral count ranking to validate quantitative proteome measurements. J Proteome Res **13**: 1957–1968

Kermicle JL, Evans MMS (2010) The *Zea mays* Sexual Compatibility Gene ga2: Naturally Occurring Alleles, Their Distribution, and Role in Reproductive Isolation. Journal of Heredity **101**: 737–749

Kim M, Ahn JW, Jin UH, Choi D, Paek KH, Pai HS (2003) Activation of the Programmed Cell Death Pathway by Inhibition of Proteasome Function in Plants. Journal of Biological Chemistry **278**: 19406–19415

Kim J, Shiu SH, Thoma S, Li WH, Patterson SE (2006) Patterns of expansion and expression divergence in the plant polygalacturonase gene family. Genome Biology **7:** 87

Kircher M, Sawyer S, Meyer M (2012) Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. Nucleic Acids Res **40**(1): e3

Kleene KC (2003) Patterns, mechanisms, and functions of translation regulation in

mammalian spermatogenic cells. Cytogenet. Genome Res. **103**(3-4): 217–224

Kliwer I, Dresselhaus T (2010) Establishment of the male germline and sperm cell movement during pollen germination and tube growth. Plant Signal Behav **5**: 885–889

Krichevsky A, Kozlovsky SV, Tian GW, Chen MH, Zaltsman A, Citovsky V (2007)How pollen tubes grow. Dev Biol **303**(2): 405–20

Krochko JE, Pramanik SK, Bewley JD (1992) Contrasting storage protein synthesis and messenger RNA accumulation during development of zygotic and somatic embryos of alfalfa (Medicago sativa L.). Plant Physiol. **99**(1): 46–53

Kulikauskas R, Hou A, Muschietti J, McCormick S (1995) Comparisons of diverse plant species reveal that only grasses show drastically reduced levels of ubiquitin monomer in mature pollen. Sex Plant Reprod **8**: 326-332

Labarca C, Loewus F (1973) The nutritional role of pistil exudate in pollen tube wall formation in Lilium longiflorum. Plant Physiol **52**: 87–92

Langmead B, Salzberg SL (2012) Fast gapped-read alignment with Bowtie 2. Nat Meth **9**: 357–359

Lausser A, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in grasses. Biochemical Society Transactions **38**: 631–634

Lausser A, Kliwer I, Srilunchang K-O, Dresselhaus T (2010) Sporophytic control of pollen tube growth and guidance in maize. Journal of Experimental Botany **61**: 673–682

Lee DH, Goldberg AL (1996) Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. J Biol Chem **271**(44): 27280-27284

Li L, Petsch K, Shimizu R, Liu S, Xu WW, Ying K, (null), Scanlon MJ, Schnable PS, Timmermans MCP, et al (2013) Mendelian and Non-Mendelian Regulation of Gene Expression in Maize. PLoS Genet **9**: e1003202

Li M, Wang K, Li S, Yang P (2016) Exploration of rice pistil responses during early post-pollination through a combination proteomic and transcriptomic analysis. Journal of Proteomics **131**: 214–226

Linskens HF, Schrauwen JAM, Konings RNH (1970) Cell-free protein synthesis with polysomes from germinating Petunia pollen grains. Planta **90**: 153-152

Linskens HF, Van der Donk JAWM, Schrauwen J (1971) RNA Synthesis during Pollen Germination. Planta **97**: 290–298

Lisci M, Tanda C, Pacini E (1994) Pollination ecophysiology of *Mercurialis annua* L. (*Euphorbiaceae*), an anemophilous species flowering all year round. Ann Bot

74: 125–135

Liu J , Jung C , Xu J , Wang H , Deng S , Bernad L , Arenas-Huertero C , Chua NH (2012) Genome-wide analysis uncovers regulation of long intergenic noncoding RNAs in Arabidopsis. The Plant Cell **24**(11): 4333-4345

Lord EM and Russell SD (2002) The mechanisms of pollination and fertilization in plants. Annu Rev Cell Dev Biol **18**: 81-105

Lowe J, Stock D, Jap B, Zwickl P, Baumeister W, Huber R (1995) Crystal structure of the 20S proteasome from the archaeon *T. acidophilum* at 3.4A resolution. Science **268**: 533-539

Lu YX, Chanroj S, Zulkifli L, Johnson MA, Uozumi N, Cheung A, Sze H (2011) Pollen tubes lacking a pair of K? transporters fail to target ovules in Arabidopsis. Plant Cell **23**(1):81–93

Lu Y, Kermicle JL, Evans MMS (2014) Genetic and cellular analysis of cross-incompatibility in *Zea mays*. Plant Reproduction **27**(1): 19-29

Lush WM, Grieser F, Wolters-Arts M (1998) Directional Guidance of Nicotiana alata Pollen Tubes *in Vitro* and on the Stigma. Plant Physiology **118**: 733–741

Lyakh VA, Kravchenko AN, Soroka AI, Dryuchina EN (1991) Effects of high temperatures on mature pollen grains in wild and cultivated maize accessions. Euphytica **55**: 203–207

Malatesta M, Perdoni F, Battistelli S, Muller S, Zancanaro C (2009) The cell nuclei of skeletal muscle cells are transcriptionally active in hibernating edible dormice. BMC Cell Biol. **10**: 19

Marcon C, Malik WA, Walley JW, Shen Z, Paschold A, Smith LG, Piepho H-P, Briggs SP, Hochholdinger F (2015) A High-Resolution Tissue-Specific Proteome and Phosphoproteome Atlas of Maize Primary Roots Reveals Functional Gradients along the Root Axes. Plant Physiology **168**: 233–246

Marton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. Science **307**: 573 – 576

Mascarenhas JP and Bell E (1969) Protein synthesis during germination of pollen: studies on polyribosome formation. Biochem Biophys Acta **179**(1): 199-203

Mascarenhas JP and Bell E (1970) RNA synthesis during development of the male gametophyte of Tradescantia. Dev Bio **21**(4): 475-490

Mascarenhas JP and Goralnick RD (1971) Synthesis of small molecular weight RNA in the pollen tube of Tradescantia paludosa. Biochem. Biophys. Acta **240**: 56-61

Mascarenhas JP (1975) The Biochemistry of Angiosperm Pollen Development.

Botanical Review **41**: 259–314

Mascarenhas JP, Altschuler M (1983) The response of pollen to high temperatures and its potential applications. In: Mulcahy DL, Ottoviano E (eds) Pollen: biology and implications for plant breeding. Elsevier, Amsterdam, pp 3–8

Mascarenhas JP (1989) The Male Gametophyte of Flowering Plants. The Plant Cell **1**: 657–664

Mascarenhas JP (1993) Molecular Mechanisms of Pollen Tube Growth and Differentiation. The Plant Cell **5**: 1303–1314

Mascarenhas JP, Crone DE (1996) Pollen and the heat shock response. Sexual Plant Reproduction **9**(6): 370-374

McCarty DR, Latshaw S, Wu S, Suzuki M, Hunter CT, Avigne WT, Koch KE (2013) Mu-seq: Sequence-Based Mapping and Identification of Transposon Induced Mutations. PLoS ONE **8**: e77172

McClung CR (2006) Plant Circadian Rhythms. The Plant Cell **18**: 792–803

McCubbin AG and Kao T-H (2000) Molecular recognition and response in pollen and pistil interactions. Annu Rev Cell Dev Biol **16**: 333 – 364

Mouline K, Very A-A, Gaymard F, Boucherez J, Pilot G, Devic M, Bouchez M, Thibaud J-B, Sentenac H (2002) Pollen tube development and competitive ability are impaired by disruption of a Shaker K+ channel in *Arabidopsis*. Genes & Development **16**: 339–350

Mourino-Perez RR, Roberson RW, Bartnicki-Garcia S (2006) Microtubule dynamics and organization during hyphal growth and branching in Neurospora crassa. Fungal Genet Biol **43**(6): 389–400

Mueller LN, Brusniak M-Y, Mani DR, Aebersold R (2008) An assessment of software solutions for the analysis of mass spectrometry based quantitative proteomics data. J Proteome Res **7**: 51–61

Mulcahy DL, Sari-Gorla M, Bergamini Mulcahy G (1996) Pollen selection – past, present and future. Sex. Plant Reprod. **9**: 353–356

Muschietti J, Dircks L, Vancanneyt G, McCormick S (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

Mustroph A, Juntawong P, Bailey-Serres J (2009) Isolation of Plant Polysomal mRNA by Differential Centrifugation and Ribosome Immunopurification Methods. *In* DA Belostotsky, ed, Plant Systems Biology. Humana Press, pp 109–126

Muthukrishnan S, Filipowicz W, Sierra JM, Both GW, Shatkin AJ, Ochoa S (1975) mRNA methylation and protein synthesis in extracts from embryos of brine shrimp, *Artemia salina*. J. Biol. Chem. **250**: 9336–9341

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in Arabidopsis thaliana seed germination: epigenetic and genetic regulation of transcription in seed. Plant J. **41**: 697–709

Nakamura N (1978) Physiological studies on the pollen growth of Camellia japonica L. in vitro. J. Yokohama City Univ. Biol. Ser. **5**: 1-00

Nelson OE (1952) Non-reciprocal cross-sterility in maize. Genetics **37**: 101 – 124

Nepi M, Pacini E (1993) Pollination, pollen viability and pistil receptivity in *Cucurbita pepo*. Ann Bot **72**: 527–536

Nepi M, Pacini E (1999) What could be the significance of polysiphony in *Lavatera arborea*? In: Clement C, Pacini E, Audran JC (eds.) Anther and pollen: from biology to biotechnology. Springer, Berlin, pp. 13–20

Nepi M, Franchi GG, Pacini E (2001) Pollen hydration status at dispersal: cytophysiological features and strategies. Protoplasma **216**: 171–180

Ning K, Fermin D, Nesvizhskii AI (2012) Comparative analysis of different label-free mass spectrometry based protein abundance estimates and their correlation with RNA-Seq gene expression data. J Proteome Res **11**: 2261–2271

Norastehnia A, Sajedi RH, Nojavan-Asghari M (2008) Inhibitory Effects of Methyl Jasmonate on Seed Germination in Maize (*Zea mays*): Effect on alpha-amylase Activity and Ethylene Production. Gen Appl Plant Physiology **33**: 13–23

Obermeyer G, Fragner L, Lang V, Weckwerth W (2013) Dynamic Adaption of Metabolic Pathways during Germination and Growth of Lily Pollen Tubes after Inhibition of the Electron Transport Chain. Plant Physiology **162**: 1822–1833

Oliver MJ, Dowd SE, Zaragoza J, Mauget SA, Payton RR (2004) The rehydration transcriptome of the desiccation-tolerant bryophyte *Tortula ruralis*: transcript classification and analysis. BMC Genomics **5**: 1–19

Pacini E (1990) Harmomegathic characters of Pteridophyta spores and Spermatophyta pollen. Plant Syst Evol [Suppl.] **5**: 53–69.

Pacini E (1996) Types and meaning of pollen carbohydrate reserves. Sex Plant Reprod **9**: 362–366

Pacini E, Franchi GG, Lisci M, Nepi M (1997) Pollen viability related to type of pollination in six angiosperm species. Ann. Bot. **80**: 83–87

Pacini E, Franchi GG, Ripaccioli M (1999) Ripe pollen structure and histochemistry of some gymnosperms. Plant Syst Evol **217**: 81–99

Pandolfi T, Pacini E (1995) The pollinium of Loroglossum hircinum (L.) C. Rich. (Orchidaceae) between pollination and pollen tube emission. Plant. Syst. Evol. **196**: 141–151

Park SY, Lord EM (2003) Expression studies of SCA in lily and confirmation of its role in pollen tube adhesion. Plant Molecular Biology **51**: 183 – 189

Pavelka N, Fournier ML, Swanson SK, Pelizzola M, Ricciardi-Castagnoli P, Florens L, Washburn MP (2008) Statistical similarities between transcriptomics and quantitative shotgun proteomics data. Molecular and Cellular Proteomics **7**: 631–644

Peddada L, Mascarenhas JP (1975) 5S ribosomal RNA synthesis during pollen development. Develop Growth Diff **17**: 1-8

Pham TV, Piersma SR, Warmoes M, Jimenez CR (2010) On the beta-binomial model for analysis of spectral count data in label-free tandem mass spectrometry-based proteomics. Bioinformatics **26**: 363–369

Pina C, Pinto F, Feijo JA, Becker JD (2005) Gene family analysis of the Arabidopsis pollen transcriptome reveals biological implications for cell growth, division control, and gene expression regulation. Plant Physiol **138**: 744-756

Picton JM and Steer MW (1983) Membrane recycling and the control of secretory activity in pollen tubes. J Cell Sci **63**: 303-310

Piques M, Schulze WX, Hohne MHO, Usadel BOR, Gibon Y, Rohwer J, Stitt M (2009) Ribosome and transcript copy numbers, polysome occupancy and enzyme dynamics in Arabidopsis. Molecular Systems Biology **5**: 1–17

Pollak PE, Hansen K, Astwood JD, Taylor LP (1995) Conditional male-fertility in maize. Sexual Plant Reproduction **8**: 231–241

Pramanik SK, Krochko JE, Bewley JD (1992) Distribution of Cytosolic mRNAs Between Polysomal and Ribonucleoprotein Complex Fractions in Alalfa Embryos. Plant Physiology **99**: 1590–1596

Pressey R, Reger B (1989) Polygalacturonase in pollen from corn and other grasses. Plant Science **59**(1): 57-62

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Qin Y, Yang Z (2011) Rapid tip growth: Insights from pollen tubes. Seminars in Cell and Developmental Biology **22**: 816–824

Qin Y, Leydon AR, Manziello A, Pandey R, Mount D, Denic S, Vasic B, Johnson MA, Palanivelu R (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**: e1000621

Raff RA, Colot HV, Selvig SE, Gross PR (1972) Oogenetic origin of messenger RNA for embryonic synthesis of microtubule proteins. Nature **235**: 211–214

Rea AC, Nasrallah JB (2008) Self-incompatibility systems: barriers to self-fertilization in flowering plants. International Journal of Developmental Biology **52**: 627–636

Rich A, Warner JR, Goodman HM (1963) The structure and function of polyribosomes. Cold Spring Harbor Symp. Quant Biol **28**:269–285

Richter JD, Smith LD (1984) Reversible inhibition of translation by *Xenopus* oocyte-specific proteins. Nature **309**: 378–380

Rincon-Guzman A, Beltran-Pena E, Ortiz-Lopez A, Sanchez de Jimenez E (1998) Ribonucleoprotein particles of quiescent maize embryonic axes. Plant Mol Biol **38**: 357–364

Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26**: 139–140

Robinson MD, Smyth GK (2007) Moderated statistical tests for assessing differences in tag abundance. Bioinformatics **23**: 2881–2887

Rock KL, Gramm C, Rothstein L, Clark K, Stein R, Dick L, Hwang D, Goldberg AL (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78**(5): 761-71

Rosenthal ET, Bahr M, Wilt FH (1993) Regulation of maternal messenger RNA translation during oogenesis and embryogenesis in *Urechis caupo*. Dev. Biol. **155**: 297–306

Rounds CM, Winship LJ, Hepler PK (2011) Pollen tube energetics: respiration, fermentation and the race to the ovule. AoB PLANTS doi:10.1093/aobpla/plr019

Rudenko FE (1959) The significance of the male gametophyte for the taxonomy of Angiospermae. Botanicheskii Zhurnal **44**: 1467–1475

Sauer M, Kleine-Vehn J (2011) AUXIN BINDING PROTEIN1: the outsider. Plant Cell **23**(6): 2033-2043

Scherer GF (2011) AUXIN-BINDING-PROTEIN1, the second auxin receptor: what is the significance of a two-receptor concept in plant signal transduction? J Exp Bot **62**(10): 3339-3357

Schiøtt M, Romanowsky SM, Baekgaard L, Jakobsen MK, Palmgren MG,

Harper JF (2004) A plant plasma membrane Ca2+ pump is required for normal pollen tube growth and fertilization. PNAS **101**(25): 9502-9507

Schnable PS, Ware DH, Fulton RS, Stein JC, Wei F, Pasternak S, Liang C, Zhang J, Fulton LA, Graves TA, et al (2009) The B73 Maize Genome: Complexity, Diversity, and Dynamics. Science **326**: 1112–1115

Schnarf K (1939) Variation im bau des pollenkornes der angiospermen. T abulae Biologicae **17**: 72–89

Schreiber DN, Dresselhaus T (2003) *In Vitro* Pollen Germination and Transient Transformation of *Zea mays* and Other Plant Species. Plant Mol Biol Rep **21**: 31–41

Schürhoff PN (1926) Die zytologie der blütenpflanzen. Enke, Stuttgart, Germany

Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, Chen W, Selbach M (2011) Global quantification of mammalian gene expression control. Nature **473**: 337–342

Scott II HB, Oliver MJ (1994) Accumulation and polysomal recruitment of transcripts in response to desiccation and rehydration of the moss *Tortula ruralis*. J. Exp. Bot. **45**: 577–583

Sekhon RS, Lin H, Childs KL, Hansey CN, Buell CR, de Leon N, Kaeppler SM (2011) Genome-wide atlas of transcription during maize development. The Plant Journal **66**: 553–563

Sembdner G, Gross D (1986) Plant growth substances of plant and microbial origin. In: Bopp M (ed) Plant growth substances 1985. Springer, Berlin, Heidelberg, New York, Tokyo, pp 139–147

Settles AM, Holding DR, Tan B, Latshaw SP, Liu J, Suzuki M, Li L, O'Brien BA, Fajardo DS, Wroclawska E, et al (2007) Sequence-indexed mutations in maize using the UniformMu transposon-tagging population. BMC Genomics **8**: 116

Shen Y, Venu RC, Nobuta K, Wu X, Notibala V, Demirci C, Meyers BC, Wang GL, Ji G, Li QQ (2011) Transcriptome dynamics through alternative polyadenylation in developmental and environmental responses in plants revealed by deep sequencing. Genome Research **21**: 1478–1486

Sheng X, Hu Z, Lu H, Wang X, Baluska F, Samaj J, Lin J (2006) Roles of the Ubiquitin/Proteasome Pathway in Pollen Tube Growth with Emphasis on MG132-Induced Alterations in Ultrastructure, Cytoskeleton, and Cell Wall Components. Plant Physiology **141**: 1578–1590

Shivanna KR, Jaiswal VS, Mohan Ram HY (1974) Inhibition of gamete formation by cycloheximide in pollen tubes of Impatiens balsamina. Planta **117**(2): 173-177

Silva NF, Goring DR (2001) Mechanisms of self-incompatibility in flowering plants. Cell Mol Life Sci **58**: 1988–2007

Singh RS, Kulathinal R J (2000) Sex gene pool evolution and speciation: a new paradigm. Genes Genet. Syst. **75**: 119–130

Sondheimer E, and Linskens HF (1974) Control of *in vitro* germination and tube extension of Petunia hybrida pollen. Proc K Ned Akad Wet **77 C**: 116-124

Sorokin AV, Selyutina AA, Skabkin MA, Guryanov SG, Nazimov IV, Richard C, Th'ng J, Yau J, Sorensen PH, Ovchinnikov LP, Evdokimova V (2005) Proteasome-mediated cleavage of the Y-box-binding protein 1 is linked to DNA-damage stress response. EMBO J. **24**: 3602-3612

Sorokin AV, Kim ER, Ovchinnikov LP (2010) Proteasome system of protein degradation and processing. Biochemistry Moscow **74**: 1411–1442

Speranza A, Calzoni GL, Pacini E (1997) Occurrence of mono- or disaccharides and polysaccharide reserves in mature pollen grains. Sex Plant Reprod **10**: 110–115

Speranza A, Scoccianti V, Crinelli R, Calzoni GL, Magnani M (2001) Inhibition of Proteasome Activity Strongly Affects Kiwifruit Pollen Germination. Involvement of the Ubiquitin/Proteasome Pathway as a Major Regulator. Plant Physiology **126**: 1150–1161

Staehelin T, Brinton CC, Wettstein FO, Noll H (1963) Structure and Function of E. coli Ergosomes. Nature **199**: 865–870

Steffensen DM (1966) Synthesis of ribosomal RNA during growth and division in Lilium. Exp Cell Res **44**(1): 1-12

Storey J (2015) qvalue: Q-value estimation for false discovery rate control. R package version 2.0.0. http://qvalue.princeton.edu/, http://github.com/jdstorey/qvalue

Swanson WJ, Vacquier VD (2002) The Rapid Evolution of Reproductive Proteins. Nature Reviews Genetics **3**: 137–144

Swanson WJ, Wong A, Wolfner MF, Aquadro CF (2004) Evolutionary Expressed Sequence Tag Analysis of Drosophila Female Reproductive Tracts Identifies Genes Subjected to Positive Selection. Genetics **168**: 1457–1465

Sylvester AW, Smith LG (2009) Cell Biology of Maize Leaf Development. *In* JL Bennetzen, SC Hake, eds, Handbook of Maize: Its Biology. Springer New York, New York, NY, pp 179–203

Takeuchi H, Higashiyama T (2011) Attraction of tip-growing pollen tubes by the female gametophyte. Current Opinion in Plant Biology **14**: 614–621

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448**(7154): 661-665

Tsubuki S, Saito Y, Tomioka M, Ito H, Kawashima S (1996) Differential Inhibition of Calpain and Proteasome Activities by Peptidyl Aldehydes of Di-Leucine and Tri-Leucine. Journal of Biochemistry **119**: 572–576

Tupy J (1977) RNAsynthesis and polysome formation in pollen tubes. Biol. Planta. **19**: 300-309

Turkina MV, Arstrand HK, Vener AV (2011) Differential Phosphorylation of Ribosomal Proteins in Arabidopsis thaliana Plants during Day and Night. **6**(12): e29307

Vacquier VD (1998) Evolution of gamete recognition proteins. Science **281**: 1995–1998

Van Veen H, Vashisht D, Akman M, Girke T, Mustroph A, Reinen E, Hartman S, Kooiker M, van Tienderen P, Schranz ME, et al (2016) Transcriptomes of eight Arabidopsis thaliana accessions reveal core conserved, genotype- and organ-specific responses to flooding stress. Plant Physiology pp.00472.2016

Vejlupkova Z, Fowler JE (2003) Maize DNA preps for undergraduate students: a robust method for PCR genotyping. Maize Genetics Cooperation Newsletter

Volkov RA, Panchuk II, Schoffl F (2005) Small heat shock proteins are differentially regulated during pollen development and following heat stress in tobacco. Plant Mol Biol **57**(4): 487-502

Vollbrecht E, Duvick J, Schares JP, Ahern KR, Deewatthanawong P, Xu L, Conrad LJ, Kikuchi K, Kubinec TA, Hall BD, et al (2010) Genome-Wide Distribution of Transposed Dissociation Elements in Maize. THE PLANT CELL ONLINE **22**: 1667–1685

Walley JW, Shen Z, Sartor R, Wu KJ, Osborn J, Smith LG, Briggs SP (2013) Reconstruction of protein networks from an atlas of maize seed proteotypes. PNAS 1–20

Walley JW, Sartor R, Shen Z, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR, et al (2016) Integration of omic networks in a developmental atlas of maize. Science **353**: 814–818

Wang Y, Zhang W-Z, Song L-F, Zou J-J, Su Z, Wu W-H (2008) Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in Arabidopsis. Plant Physiology **148**: 1201–1211

Warner JR, Knopf PM, Rich A (1963) A Multiple Ribosomal Structure in Protein Synthesis. PNAS **49**: 122–129

Waters AJ, Makarevitch I, Eichten SR, Swanson-Wagner RA, Yeh C-T, Xu WW, Schnable PS, Vaughn MW, Gehring M, Springer NM (2011) Parent-of-Origin Effects on Gene Expression and DNA Methylation in the Maize Endosperm. THE PLANT CELL ONLINE **23**: 4221–4233

Wei LQ, Xu WY, Deng ZY, Su Z, Xue Y, Wang T (2010) Genome-scale analysis and comparison of gene expression profiles in developing and germinated pollen in Oryza sativa. BMC Genomics **11**: 338

Whipple AP, Mascarenhas JP (1978) Lipid synthesis in germinating Tradescantia pollen. Phytochemistry **17**: 1273-1274

Williams JH (2012) The evolution of pollen germination timing in flowering plants: Austrobaileya scandens (Austrobaileyaceae). AoB Plants 2012: pls010–pls010

Williams JH, Taylor ML, O'Meara BC (2014) Repeated evolution of tricellular (and bicellular) pollen. American Journal of Botany **101**: 559–571

Williams-Carrier R, Stiffler N, Belcher S, Kroeger T, Stern DB, Monde RA, Coalter R, Barkan A (2010) Use of Illumina sequencing to identify transposon insertions underlying mutant phenotypes in high-copy Mutator lines of maize. Plant J **63**(1): 167-177

Woffenden BJ, Freeman TB, Beers EP (1998) Proteasome Inhibitors Prevent Tracheary Element Differentiation in Zinnia Mesophyll Cell Cultures. Plant Physiology **118**: 419–430

Xiao L, Wang H, Wan P, Kuang T, He Y (2011) Genome-wide transcriptome analysis of gametophyte development in *Physcomitrella patens*. BMC Plant Biol **11**: 177

Xu XH, Wang F, Chen H, Sun W, Zhang XS (2013) Transcript Profile Analyses of Maize Silks Reveal Effective Activation of Genes Involved in Microtubule-Based Movement, Ubiquitin-Dependent Protein Degradation, and Transport in the Pollination Process. PLoS ONE **8**: e53545

Yamane H, Takagi H, Abe H, Yokota T, Takahashi N (1981) Identification of jasmonic acid in three species of higher plants and its biological activities. Plant Cell Physiol. **22**: 689–697

Yamane H, Abe H, Takahashi N (1982) Jasmonic Acid and Methyl Jasmonate in Pollens and Anthers of Three *Camellia* Species. Plant and Cell Physiology **23**: 1125–1127

Yildiz K, Yilmaz H (2002) Effect of jasmonic acid, ACC and ethephon on pollen germination in strawberry. Plant Growth Regeneration **38**: 145–148

Yue X, Gao X-Q, Wang F, Dong Y, Li X, Zhang XS (2014) Transcriptional Evidence for Inferred Pattern of Pollen Tube-Stigma Metabolic Coupling during Pollination. PLoS ONE **9**: e107046

Zhang L, Chia J-M, Kumari S, Stein JC, Liu Z, Narechania A, Maher CA, Guill KE, McMullen MD, Ware DH (2009) A Genome-Wide Characterization of MicroRNA Genes in Maize. PLoS Genet **5**: e1000716

Zhu W, Smith JW, Huang C-M (2010) Mass Spectrometry-Based Label-Free Quantitative Proteomics. Journal of Biomedicine and Biotechnology **2010**: 1–6

Appendix

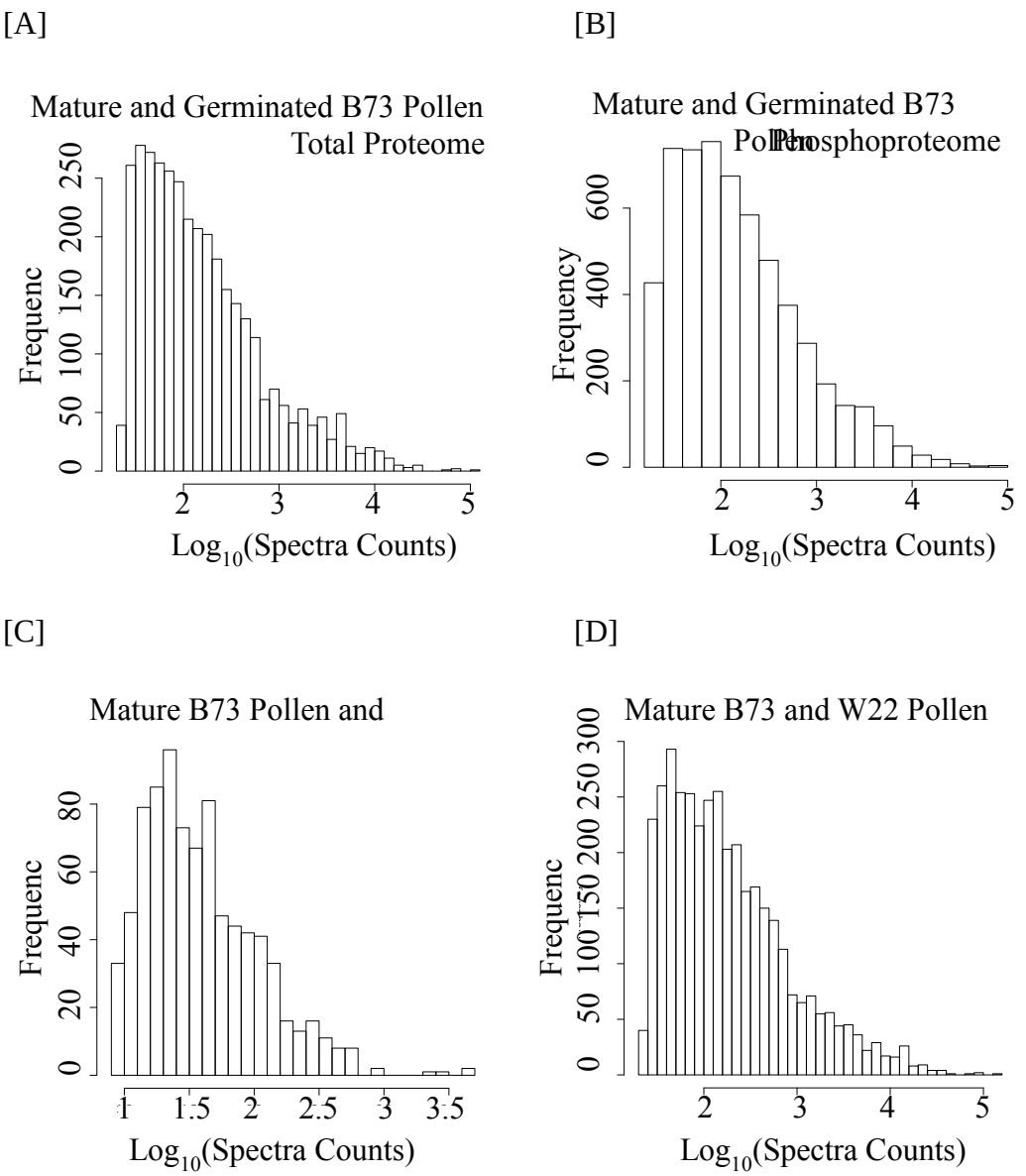


Figure A. 1: Histograms of the distribution of log counts for proteomic analysis used to determine a cutoff of spectra counts that approximates a normal distribution.

[A] Mature and germinated pollen total proteome uses a cutoff of 25 counts; [B] Mature and germinated pollen phosphoproteome uses a cutoff of 10 spectra counts; [C] For the comparison of mature B73 pollen and mature B73 leaf a cutoff of 20 spectra counts was used; [D] Mature B73 and W23 pollen used a cutoff of 25 spectra counts.

Table A. 1: Comparison of total proteome and phosphoproteome between mature pollen, germinated pollen and mature leaf.
(A-B) Matrix of Pearson's correlation coefficients from normalized counts with 25 count cutoff.

[A]

		1	**Mature Pollen**
	1	0.93	**Germinated Pollen**
1	0.06	0.08	**Mature Leaf**
Mature Leaf	**Germinated Pollen**	**Mature Pollen**	

[B]

			1	**Zone 1**
		1	0.97	**Zone 2**
	1	0.99	0.96	**Zone 3**
1	0.16	0.16	0.15	**Mature Leaf**
Mature Leaf	**Zone 3**	**Zone 2**	**Zone 1**	

Table A. 2: 393 proteins found to be differentially abundant by at least one package.
Spectra counts represent the sum of four replicates for each tissue type. LogFC indicates the log base 2 fold change of protein abundance in germinated pollen relative to mature pollen; sign indicates increase or decrease in abundance.

			Statistical Output						
	Spectral counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
AC159612.1_FGP007	37	1	0.0004	0.0137	-4.08	0.0013	0.0298	0.0047	0.0581
AC191412.3_FGP002	132	21	0.0016	0.0301	-1.95	0.0018	0.0344	0.0029	0.0562
AC192244.3_FGP001	53	68	0.0163	0.1377	1.19	0.0034	0.0460	0.4527	0.5342
AC203294.3_FGP001	133	11	0.0002	0.0079	-2.73	0.0187	0.1039	0.0009	0.0562
AC203754.4_FGP008	196	61	0.0490	0.2574	-0.90	0.0038	0.0480	0.0108	0.0673
AC207890.3_FGP002	392	574	0.0016	0.0310	1.37	0.0096	0.0726	0.1702	0.2570
AC213600.3_FGP002	78	19	0.0338	0.2107	-1.29	0.0019	0.0344	0.0109	0.0673
AC214350.3_FGP007	318	68	0.0148	0.1298	-1.31	0.0018	0.0344	0.0023	0.0562
EF517601.1_FGP012	335	109	0.0376	0.2226	-0.84	0.0008	0.0244	0.0082	0.0643
GRMZM2G000645_P01	142	49	0.1017	0.3751	-0.76	0.0016	0.0321	0.0184	0.0805
GRMZM2G000777_P01	36	5	0.0172	0.1416	-2.06	0.0015	0.0319	0.0091	0.0652
GRMZM2G001803_P01	126	146	0.0240	0.1707	0.98	0.0013	0.0298	0.5988	0.6669
GRMZM2G001887_P01	294	30	0.0015	0.0293	-2.40	0.0610	0.2112	0.0005	0.0533
GRMZM2G004799_P01	33	64	0.0008	0.0214	1.76	0.0002	0.0131	0.1218	0.2036

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G005080_P01	7	29	0.0030	0.0456	2.71	0.0229	0.1180	0.0373	0.1089
GRMZM2G005990_P01	26	2	0.0146	0.1282	-2.69	0.0034	0.0460	0.0103	0.0672
GRMZM2G006178_P01	21	76	0.0000	0.0014	2.69	0.0006	0.0203	0.0238	0.0884
GRMZM2G006953_P02	33	3	0.0107	0.1048	-2.44	0.0029	0.0420	0.0083	0.0643
GRMZM2G008047_P01	749	1324	0.0001	0.0045	1.56	0.0010	0.0261	0.0496	0.1244
GRMZM2G009638_P01	187	416	0.0002	0.0083	1.93	0.0113	0.0802	0.0335	0.1028
GRMZM2G010823_P01	516	221	0.2347	0.5812	-0.47	0.0025	0.0377	0.0173	0.0779
GRMZM2G010991_P01	14	44	0.0012	0.0258	2.43	0.0035	0.0467	0.0476	0.1211
GRMZM2G011951_P03	98	0	0.0000	0.0000	-7.40	0.0000	0.0053	0.0006	0.0562
GRMZM2G012863_P02	217	430	0.0002	0.0069	1.72	0.0012	0.0288	0.0516	0.1263
GRMZM2G014136_P01	23	2	0.0096	0.0994	-2.71	0.0038	0.0485	0.0198	0.0824
GRMZM2G014382_P01	45	99	0.0006	0.0172	1.84	0.0045	0.0529	0.0706	0.1483
GRMZM2G014397_P02	138	41	0.0336	0.2101	-1.00	0.0006	0.0210	0.0119	0.0689
GRMZM2G015159_P01	46	1	0.0007	0.0198	-4.53	0.0040	0.0495	0.0029	0.0562
GRMZM2G016189_P01	142	203	0.0023	0.0372	1.36	0.0026	0.0397	0.2472	0.3388
GRMZM2G017110_P02	270	798	0.0000	0.0002	2.45	0.0016	0.0324	0.0064	0.0613
GRMZM2G017170_P01	273	32	0.0001	0.0068	-2.41	0.0092	0.0712	0.0006	0.0562
GRMZM2G018006_P01	25	0	0.0009	0.0222	-5.31	0.0004	0.0185	0.0112	0.0673
GRMZM2G018082_P01	82	21	0.0306	0.1998	-1.27	0.0037	0.0480	0.0117	0.0685
GRMZM2G019121_P01	129	137	0.0365	0.2189	0.89	0.0025	0.0383	0.8155	0.8466

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G019999_P01	627	128	0.0016	0.0310	-1.49	0.0010	0.0264	0.0009	0.0562
GRMZM2G020096_P01	73	6	0.0002	0.0081	-2.72	0.0001	0.0120	0.0021	0.0562
GRMZM2G020852_P01	5662	15644	0.0000	0.0000	2.47	0.0003	0.0165	0.0003	0.0425
GRMZM2G020915_P01	37	108	0.0008	0.0215	2.28	0.1090	0.2946	0.0300	0.0969
GRMZM2G022904_P01	38	1	0.0002	0.0076	-4.04	0.0001	0.0104	0.0044	0.0568
GRMZM2G023194_P01	343	83	0.0226	0.1640	-1.12	0.0013	0.0293	0.0030	0.0562
GRMZM2G024933_P02	87	14	0.0042	0.0570	-1.94	0.0037	0.0480	0.0041	0.0564
GRMZM2G025833_P01	1	26	0.0000	0.0011	4.71	0.0002	0.0134	0.0142	0.0726
GRMZM2G026182_P01	322	32	0.0001	0.0065	-2.43	0.0408	0.1700	0.0004	0.0462
GRMZM2G027835_P01	27	2	0.0060	0.0739	-2.82	0.0004	0.0189	0.0095	0.0656
GRMZM2G027891_P01	117	24	0.0063	0.0752	-1.51	0.0034	0.0460	0.0053	0.0583
GRMZM2G028247_P01	212	63	0.0254	0.1784	-1.00	0.0001	0.0121	0.0088	0.0648
GRMZM2G028905_P01	29	2	0.0093	0.0978	-2.94	0.0021	0.0355	0.0113	0.0673
GRMZM2G029530_P01	31	5	0.0377	0.2229	-1.79	0.0024	0.0373	0.0174	0.0782
GRMZM2G030265_P01	204	419	0.0019	0.0331	1.85	0.0886	0.2608	0.0454	0.1186
GRMZM2G030531_P01	469	990	0.0004	0.0143	1.82	0.0183	0.1026	0.0248	0.0890
GRMZM2G030971_P01	233	32	0.0005	0.0161	-2.33	0.0243	0.1231	0.0011	0.0562
GRMZM2G032049_P01	54	0	0.0000	0.0028	-6.63	0.0008	0.0237	0.0021	0.0562
GRMZM2G032348_P04	77	3	0.0007	0.0202	-3.59	0.0011	0.0272	0.0015	0.0562
GRMZM2G033208_P03	3229	2698	0.1712	0.4949	0.49	0.0011	0.0275	0.2922	0.3810

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G033829_P02	148	38	0.0160	0.1377	-1.28	0.0035	0.0467	0.0076	0.0643
GRMZM2G033846_P01	36	0	0.0001	0.0068	-5.81	0.0000	0.0054	0.0050	0.0583
GRMZM2G036609_P01	96	251	0.0015	0.0293	2.06	0.1239	0.3212	0.0251	0.0895
GRMZM2G037212_P02	33	3	0.0062	0.0752	-2.71	0.0008	0.0248	0.0083	0.0643
GRMZM2G037272_P02	773	2051	0.0001	0.0039	2.04	0.0350	0.1556	0.0045	0.0573
GRMZM2G040397_P01	180	35	0.0011	0.0244	-1.78	0.0005	0.0195	0.0031	0.0562
GRMZM2G041181_P01	96	14	0.0034	0.0500	-2.00	0.0038	0.0480	0.0035	0.0564
GRMZM2G043310_P01	161	47	0.0539	0.2684	-0.99	0.0029	0.0417	0.0104	0.0672
GRMZM2G045278_P01	1930	745	0.1185	0.4022	-0.59	0.0000	0.0065	0.0038	0.0564
GRMZM2G045714_P01	40	60	0.0049	0.0653	1.43	0.0012	0.0288	0.2754	0.3666
GRMZM2G045908_P03	34	3	0.0085	0.0923	-2.60	0.0009	0.0254	0.0106	0.0672
GRMZM2G046436_P01	1088	1497	0.0017	0.0318	1.41	0.0061	0.0594	0.1752	0.2622
GRMZM2G046804_P02	7594	1321	0.0023	0.0373	-1.58	0.0004	0.0185	0.0000	0.0000
GRMZM2G047095_P01	130	17	0.0008	0.0214	-2.14	0.0004	0.0187	0.0019	0.0562
GRMZM2G047274_P01	374	112	0.0181	0.1450	-1.11	0.0028	0.0414	0.0057	0.0595
GRMZM2G047476_P03	33	3	0.0101	0.1031	-2.56	0.0008	0.0248	0.0113	0.0673
GRMZM2G047592_P02	7	45	0.0007	0.0208	3.36	0.0420	0.1729	0.0140	0.0720
GRMZM2G047732_P02	143	321	0.0000	0.0010	1.94	0.0005	0.0200	0.0370	0.1085
GRMZM2G048012_P01	50	0	0.0000	0.0024	-6.54	0.0004	0.0191	0.0025	0.0562
GRMZM2G048846_P02	89	6	0.0018	0.0319	-2.90	0.0009	0.0259	0.0019	0.0562

Table A.2: Continued

			Statistical Output						
	Spectral counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G050193_P01	250	66	0.0101	0.1031	-1.19	0.0002	0.0128	0.0054	0.0583
GRMZM2G050364_P01	202	351	0.0022	0.0366	1.50	0.0218	0.1149	0.0955	0.1765
GRMZM2G051004_P01	762	189	0.0128	0.1182	-1.21	0.0037	0.0480	0.0015	0.0562
GRMZM2G051491_P01	52	0	0.0000	0.0033	-6.21	0.0010	0.0260	0.0023	0.0562
GRMZM2G051630_P01	47	81	0.0046	0.0621	1.53	0.0012	0.0284	0.1637	0.2495
GRMZM2G051677_P02	1164	304	0.0012	0.0255	-1.25	0.0000	0.0033	0.0011	0.0562
GRMZM2G051771_P01	78	11	0.0025	0.0392	-2.15	0.0004	0.0185	0.0041	0.0564
GRMZM2G053637_P01	512	153	0.0209	0.1576	-0.96	0.0001	0.0121	0.0043	0.0564
GRMZM2G055857_P01	60	2	0.0000	0.0014	-3.95	0.0000	0.0104	0.0017	0.0562
GRMZM2G057823_P01	7066	7541	0.0222	0.1631	0.89	0.0023	0.0371	0.6547	0.7169
GRMZM2G058276_P01	385	107	0.0105	0.1048	-1.10	0.0009	0.0252	0.0043	0.0564
GRMZM2G058491_P01	260	98	0.1147	0.3973	-0.67	0.0020	0.0352	0.0167	0.0773
GRMZM2G058948_P01	48	95	0.0017	0.0318	1.70	0.0019	0.0349	0.0991	0.1805
GRMZM2G059445_P04	751	157	0.0011	0.0244	-1.45	0.0086	0.0686	0.0008	0.0562
GRMZM2G060054_P03	34	3	0.0085	0.0923	-2.60	0.0009	0.0254	0.0106	0.0672
GRMZM2G061764_P01	147	154	0.0269	0.1845	0.89	0.0035	0.0467	0.8484	0.8728
GRMZM2G063340_P01	297	103	0.0622	0.2916	-0.78	0.0016	0.0322	0.0113	0.0673
GRMZM2G063544_P01	88	122	0.0062	0.0752	1.23	0.0009	0.0254	0.3118	0.3998
GRMZM2G063617_P01	11	54	0.0000	0.0006	3.08	0.0001	0.0121	0.0161	0.0762
GRMZM2G063909_P03	35	2	0.0018	0.0319	-3.10	0.0016	0.0324	0.0073	0.0635

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G064133_P01	338	46	0.0001	0.0047	-2.02	0.0001	0.0106	0.0006	0.0562
GRMZM2G064163_P01	65	12	0.0127	0.1178	-1.80	0.0024	0.0372	0.0081	0.0643
GRMZM2G064193_P01	25	1	0.0039	0.0544	-3.48	0.0005	0.0203	0.0112	0.0673
GRMZM2G064896_P01	50	78	0.0032	0.0475	1.56	0.0069	0.0628	0.2281	0.3206
GRMZM2G066024_P01	5699	6636	0.0129	0.1187	0.99	0.0011	0.0279	0.3530	0.4404
GRMZM2G066041_P01	106	7	0.0007	0.0198	-3.05	0.0002	0.0131	0.0014	0.0562
GRMZM2G066111_P01	239	608	0.0001	0.0065	2.46	0.0294	0.1402	0.0152	0.0745
GRMZM2G066615_P01	26	0	0.0008	0.0210	-5.29	0.0008	0.0244	0.0103	0.0672
GRMZM2G066902_P01	822	103	0.0000	0.0024	-2.38	0.0070	0.0628	0.0001	0.0273
GRMZM2G067242_P01	28	0	0.0004	0.0142	-5.40	0.0007	0.0228	0.0087	0.0648
GRMZM2G067277_P01	204	45	0.0025	0.0392	-1.51	0.0005	0.0203	0.0038	0.0564
GRMZM2G067417_P02	9	34	0.0028	0.0436	2.55	0.0046	0.0533	0.0479	0.1215
GRMZM2G067511_P01	1807	579	0.0338	0.2107	-0.93	0.0016	0.0324	0.0017	0.0562
GRMZM2G071877_P02	33	6	0.0413	0.2342	-1.70	0.0031	0.0431	0.0194	0.0817
GRMZM2G072089_P01	1748	2307	0.0026	0.0414	1.10	0.0020	0.0349	0.1963	0.2844
GRMZM2G072448_P01	1035	164	0.0001	0.0057	-1.74	0.0010	0.0259	0.0002	0.0367
GRMZM2G072744_P01	11	132	0.0000	0.0006	4.00	0.0057	0.0578	0.0015	0.0562
GRMZM2G072886_P02	3001	9501	0.0000	0.0019	2.38	0.0007	0.0225	0.0002	0.0391
GRMZM2G073888_P01	34	1	0.0023	0.0373	-3.83	0.0322	0.1479	0.0053	0.0583
GRMZM2G075100_P01	16	444	0.0000	0.0000	5.35	0.0092	0.0712	0.0001	0.0183

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G075470_P02	83	22	0.0401	0.2299	-1.18	0.0023	0.0369	0.0127	0.0694
GRMZM2G075496_P01	3	32	0.0010	0.0244	3.92	0.0349	0.1556	0.0116	0.0683
GRMZM2G075680_P01	64	19	0.0686	0.3039	-1.09	0.0035	0.0467	0.0230	0.0873
GRMZM2G077673_P01	41	3	0.0018	0.0320	-2.88	0.0020	0.0352	0.0051	0.0583
GRMZM2G078804_P01	22797	6890	0.0274	0.1868	-1.19	0.0316	0.1464	0.0000	0.0056
GRMZM2G078886_P01	36	137	0.0016	0.0310	2.47	0.0569	0.2045	0.0131	0.0703
GRMZM2G078933_P01	79	21	0.0450	0.2460	-1.14	0.0009	0.0252	0.0131	0.0704
GRMZM2G079236_P01	473	51	0.0018	0.0319	-2.15	0.0460	0.1840	0.0002	0.0391
GRMZM2G079348_P01	0	40	0.0000	0.0000	6.77	0.0000	0.0037	0.0054	0.0586
GRMZM2G079387_P01	241	30	0.0039	0.0542	-2.00	0.0005	0.0203	0.0009	0.0562
GRMZM2G079422_P01	228	4	0.0000	0.0018	-4.73	0.0622	0.2127	0.0001	0.0197
GRMZM2G080839_P01	55	3	0.0011	0.0244	-3.32	0.0050	0.0548	0.0020	0.0562
GRMZM2G081102_P01	95	17	0.0068	0.0789	-1.90	0.0023	0.0371	0.0053	0.0583
GRMZM2G081155_P02	66	13	0.0287	0.1935	-1.56	0.0012	0.0282	0.0090	0.0648
GRMZM2G081682_P01	181	16	0.0005	0.0161	-2.77	0.0019	0.0344	0.0007	0.0562
GRMZM2G082087_P01	444	140	0.0161	0.1377	-0.99	0.0003	0.0151	0.0059	0.0599
GRMZM2G082087_P02	471	170	0.0521	0.2633	-0.78	0.0006	0.0220	0.0093	0.0654
GRMZM2G082185_P01	34	73	0.0017	0.0318	1.85	0.0018	0.0343	0.0871	0.1689
GRMZM2G082517_P01	731	2089	0.0001	0.0068	2.02	0.0302	0.1428	0.0030	0.0562
GRMZM2G082581_P01	217	56	0.0106	0.1048	-1.23	0.0007	0.0225	0.0057	0.0594

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G082792_P01	455	57	0.0003	0.0112	-2.50	0.0072	0.0633	0.0004	0.0462
GRMZM2G082792_P02	410	44	0.0001	0.0065	-2.72	0.0088	0.0694	0.0003	0.0462
GRMZM2G083095_P01	302	97	0.0334	0.2100	-0.98	0.0019	0.0349	0.0085	0.0643
GRMZM2G083317_P02	65	9	0.0053	0.0681	-2.11	0.0028	0.0412	0.0058	0.0597
GRMZM2G083836_P01	240	45	0.0011	0.0250	-1.54	0.0001	0.0120	0.0022	0.0562
GRMZM2G083912_P01	24	57	0.0021	0.0358	2.06	0.0161	0.0965	0.0806	0.1612
GRMZM2G085145_P01	155	17	0.0001	0.0068	-2.65	0.0009	0.0254	0.0012	0.0562
GRMZM2G085260_P01	107	9	0.0004	0.0147	-2.91	0.0058	0.0579	0.0013	0.0562
GRMZM2G086656_P03	32	2	0.0078	0.0863	-3.12	0.0024	0.0372	0.0090	0.0648
GRMZM2G086971_P06	136	47	0.0932	0.3576	-0.76	0.0019	0.0349	0.0192	0.0814
GRMZM2G087245_P01	460	3082	0.0011	0.0244	3.31	0.0800	0.2470	0.0000	0.0100
GRMZM2G088397_P01	34	6	0.0320	0.2052	-1.69	0.0030	0.0427	0.0137	0.0716
GRMZM2G088753_P02	180	36	0.0011	0.0244	-1.67	0.0002	0.0131	0.0033	0.0564
GRMZM2G088847_P04	0	63	0.0000	0.0000	7.39	0.0000	0.0047	0.0021	0.0562
GRMZM2G088847_P07	0	63	0.0000	0.0000	7.39	0.0000	0.0047	0.0021	0.0562
GRMZM2G088995_P01	25	0	0.0014	0.0280	-5.52	0.0014	0.0301	0.0112	0.0673
GRMZM2G091383_P01	107	9	0.0012	0.0258	-2.65	0.0004	0.0185	0.0016	0.0562
GRMZM2G091456_P02	39	0	0.0001	0.0068	-5.80	0.0007	0.0225	0.0042	0.0564
GRMZM2G091715_P03	32	126	0.0008	0.0215	2.67	0.0014	0.0304	0.0138	0.0716
GRMZM2G092672_P01	1374	569	0.1890	0.5209	-0.51	0.0021	0.0355	0.0073	0.0635

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G093175_P02	53	0	0.0001	0.0068	-6.74	0.0095	0.0722	0.0021	0.0562
GRMZM2G094309_P01	80	7	0.0011	0.0247	-2.77	0.0104	0.0767	0.0020	0.0562
GRMZM2G094497_P03	513	167	0.0841	0.3387	-0.78	0.0034	0.0460	0.0058	0.0599
GRMZM2G095348_P03	144	39	0.0299	0.1962	-1.07	0.0015	0.0319	0.0090	0.0648
GRMZM2G096662_P01	232	31	0.0014	0.0280	-1.99	0.0006	0.0203	0.0010	0.0562
GRMZM2G096897_P01	61	342	0.0000	0.0035	3.08	0.0243	0.1231	0.0017	0.0562
GRMZM2G097122_P01	183	355	0.0001	0.0057	1.74	0.0018	0.0344	0.0616	0.1365
GRMZM2G097856_P01	1090	1931	0.0022	0.0364	1.38	0.0113	0.0802	0.0402	0.1114
GRMZM2G098237_P01	31	47	0.0103	0.1036	1.41	0.0040	0.0493	0.2813	0.3719
GRMZM2G100650_P01	82	1	0.0000	0.0014	-5.03	0.0006	0.0207	0.0009	0.0562
GRMZM2G101446_P01	176	210	0.0166	0.1386	0.98	0.0021	0.0355	0.5138	0.5912
GRMZM2G102347_P03	59	6	0.0024	0.0387	-2.78	0.0095	0.0722	0.0043	0.0564
GRMZM2G102404_P01	142	581	0.0000	0.0001	2.79	0.0016	0.0319	0.0025	0.0562
GRMZM2G104632_P01	833	206	0.0136	0.1223	-1.19	0.0030	0.0427	0.0013	0.0562
GRMZM2G105787_P01	39	0	0.0001	0.0068	-5.80	0.0007	0.0225	0.0042	0.0564
GRMZM2G106429_P01	901	186	0.0007	0.0200	-1.66	0.0056	0.0574	0.0006	0.0562
GRMZM2G106899_P01	154	250	0.0006	0.0172	1.54	0.0021	0.0355	0.1418	0.2261
GRMZM2G109056_P01	63	12	0.0122	0.1140	-1.63	0.0020	0.0351	0.0088	0.0648
GRMZM2G109121_P03	26	70	0.0001	0.0065	2.32	0.0013	0.0293	0.0517	0.1263
GRMZM2G110141_P01	48	2	0.0019	0.0331	-3.47	0.0053	0.0558	0.0035	0.0564

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G111355_P02	316	86	0.0189	0.1496	-1.08	0.0012	0.0285	0.0048	0.0582
GRMZM2G111579_P01	73	9	0.0005	0.0155	-2.24	0.0001	0.0120	0.0036	0.0564
GRMZM2G112176_P02	28	0	0.0022	0.0361	-5.35	0.0082	0.0665	0.0080	0.0643
GRMZM2G112232_P01	8	41	0.0018	0.0320	2.98	0.0075	0.0652	0.0239	0.0885
GRMZM2G115065_P01	56	12	0.0441	0.2445	-1.38	0.0024	0.0373	0.0105	0.0672
GRMZM2G115598_P01	144	218	0.0015	0.0300	1.38	0.0022	0.0356	0.1939	0.2814
GRMZM2G115628_P01	73	11	0.0019	0.0329	-1.91	0.0001	0.0120	0.0042	0.0564
GRMZM2G115901_P01	4	29	0.0008	0.0218	3.36	0.0026	0.0391	0.0219	0.0860
GRMZM2G116427_P01	10	36	0.0002	0.0088	2.57	0.0004	0.0185	0.0337	0.1031
GRMZM2G116981_P01	92	5	0.0003	0.0113	-3.19	0.0117	0.0821	0.0010	0.0562
GRMZM2G117603_P02	294	881	0.0004	0.0141	2.23	0.0243	0.1231	0.0057	0.0594
GRMZM2G118286_P01	25	2	0.0163	0.1377	-2.62	0.0017	0.0332	0.0112	0.0673
GRMZM2G118637_P05	143	321	0.0000	0.0010	1.94	0.0005	0.0200	0.0370	0.1085
GRMZM2G118731_P01	21	40	0.0066	0.0777	1.73	0.0015	0.0317	0.1506	0.2358
GRMZM2G119698_P01	228	4	0.0000	0.0018	-4.73	0.0622	0.2127	0.0001	0.0197
GRMZM2G119852_P01	193	24	0.0000	0.0034	-2.29	0.0001	0.0117	0.0012	0.0562
GRMZM2G120047_P01	7	29	0.0030	0.0456	2.71	0.0229	0.1180	0.0373	0.1089
GRMZM2G120517_P01	72	11	0.0043	0.0575	-1.94	0.0010	0.0259	0.0044	0.0564
GRMZM2G121236_P01	34	0	0.0001	0.0057	-5.78	0.0000	0.0047	0.0057	0.0594
GRMZM2G121743_P01	3	41	0.0001	0.0068	4.05	0.0044	0.0527	0.0092	0.0654

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G122230_P01	503	982	0.0009	0.0224	1.62	0.0006	0.0220	0.0360	0.1066
GRMZM2G122479_P02	195	481	0.0023	0.0373	2.17	0.0076	0.0655	0.0207	0.0841
GRMZM2G123633_P01	123	29	0.0283	0.1914	-1.23	0.0025	0.0377	0.0071	0.0628
GRMZM2G125669_P01	222	36	0.0004	0.0140	-1.82	0.0077	0.0656	0.0016	0.0562
GRMZM2G125838_P01	53	1	0.0002	0.0080	-4.39	0.0022	0.0358	0.0022	0.0562
GRMZM2G127404_P01	132	205	0.0026	0.0411	1.37	0.0063	0.0610	0.1796	0.2675
GRMZM2G127581_P01	61	4	0.0029	0.0440	-2.84	0.0038	0.0485	0.0025	0.0562
GRMZM2G127798_P02	382	99	0.0076	0.0857	-1.13	0.0007	0.0225	0.0034	0.0564
GRMZM2G128399_P01	30	1	0.0006	0.0172	-3.76	0.0002	0.0128	0.0075	0.0641
GRMZM2G128549_P01	5739	2642	0.3215	0.6749	-0.35	0.0006	0.0203	0.0039	0.0564
GRMZM2G128995_P01	253	97	0.2337	0.5807	-0.52	0.0014	0.0302	0.0179	0.0791
GRMZM2G129155_P01	248	76	0.0298	0.1962	-0.93	0.0001	0.0115	0.0085	0.0643
GRMZM2G130121_P01	97	24	0.0186	0.1481	-1.28	0.0003	0.0151	0.0096	0.0657
GRMZM2G131939_P01	58	6	0.0017	0.0318	-2.63	0.0013	0.0293	0.0045	0.0572
GRMZM2G132929_P02	288	96	0.0523	0.2637	-0.83	0.0014	0.0308	0.0100	0.0664
GRMZM2G133282_P01	69	19	0.0777	0.3265	-1.05	0.0027	0.0404	0.0159	0.0758
GRMZM2G133937_P01	194	55	0.0166	0.1386	-1.06	0.0002	0.0131	0.0081	0.0643
GRMZM2G134045_P01	158	42	0.0136	0.1223	-1.15	0.0007	0.0225	0.0079	0.0643
GRMZM2G134054_P01	1	27	0.0020	0.0350	4.53	0.0314	0.1459	0.0120	0.0692
GRMZM2G134256_P01	129	23	0.0038	0.0540	-1.76	0.0033	0.0454	0.0035	0.0564

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G134738_P01	498	498	0.0446	0.2453	0.75	0.0028	0.0414	0.9530	0.9530
GRMZM2G135470_P01	46	5	0.0022	0.0363	-2.42	0.0002	0.0134	0.0065	0.0613
GRMZM2G135498_P01	165	352	0.0002	0.0099	1.83	0.0039	0.0489	0.0432	0.1156
GRMZM2G135949_P01	221	45	0.0018	0.0320	-1.75	0.0024	0.0376	0.0029	0.0562
GRMZM2G136143_P01	27	0	0.0001	0.0068	-5.40	0.0001	0.0116	0.0095	0.0656
GRMZM2G136170_P01	170	34	0.0042	0.0572	-1.49	0.0026	0.0389	0.0035	0.0564
GRMZM2G136455_P01	136	47	0.0935	0.3576	-0.76	0.0019	0.0349	0.0192	0.0814
GRMZM2G137528_P01	85	23	0.0643	0.2958	-1.11	0.0035	0.0464	0.0131	0.0703
GRMZM2G138523_P01	35	2	0.0018	0.0318	-3.12	0.0002	0.0147	0.0073	0.0635
GRMZM2G138800_P01	1015	118	0.0001	0.0057	-2.41	0.0124	0.0842	0.0001	0.0183
GRMZM2G139093_P02	36	0	0.0001	0.0068	-5.83	0.0001	0.0106	0.0050	0.0583
GRMZM2G139695_P01	457	990	0.0005	0.0154	1.86	0.0172	0.0997	0.0222	0.0865
GRMZM2G140667_P01	1342	425	0.0485	0.2557	-0.83	0.0005	0.0203	0.0022	0.0562
GRMZM2G141034_P01	1116	1768	0.0009	0.0222	1.45	0.0001	0.0121	0.0769	0.1564
GRMZM2G142661_P01	114	6	0.0001	0.0047	-3.27	0.0165	0.0973	0.0008	0.0562
GRMZM2G144008_P01	180	26	0.0008	0.0209	-2.03	0.0043	0.0520	0.0016	0.0562
GRMZM2G144372_P01	278	110	0.2677	0.6237	-0.47	0.0012	0.0285	0.0191	0.0814
GRMZM2G145061_P02	34	5	0.0132	0.1205	-1.98	0.0018	0.0343	0.0137	0.0716
GRMZM2G145242_P02	1074	343	0.0176	0.1430	-0.91	0.0011	0.0282	0.0028	0.0562
GRMZM2G145280_P01	107	22	0.0164	0.1379	-1.67	0.0040	0.0495	0.0064	0.0613

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G145396_P03	52	107	0.0010	0.0244	1.91	0.0090	0.0703	0.0841	0.1655
GRMZM2G145460_P01	180	35	0.0011	0.0244	-1.78	0.0005	0.0195	0.0031	0.0562
GRMZM2G145552_P01	45	0	0.0000	0.0011	-6.18	0.0000	0.0033	0.0030	0.0562
GRMZM2G145595_P01	131	31	0.0124	0.1152	-1.29	0.0031	0.0439	0.0068	0.0621
GRMZM2G145854_P01	667	271	0.1377	0.4374	-0.59	0.0013	0.0293	0.0116	0.0683
GRMZM2G145870_P01	351	91	0.0059	0.0737	-1.18	0.0000	0.0041	0.0037	0.0564
GRMZM2G145870_P02	356	93	0.0066	0.0774	-1.15	0.0000	0.0041	0.0038	0.0564
GRMZM2G146358_P01	142	39	0.0155	0.1338	-1.14	0.0004	0.0187	0.0095	0.0657
GRMZM2G146358_P04	135	32	0.0056	0.0715	-1.36	0.0001	0.0117	0.0067	0.0620
GRMZM2G146551_P02	4412	18400	0.0000	0.0000	2.93	0.0002	0.0150	0.0000	0.0010
GRMZM2G146670_P01	35	5	0.0202	0.1547	-1.96	0.0021	0.0355	0.0098	0.0657
GRMZM2G146951_P01	226	384	0.0012	0.0262	1.53	0.0182	0.1022	0.1023	0.1837
GRMZM2G147145_P03	32	3	0.0230	0.1654	-2.39	0.0015	0.0315	0.0090	0.0648
GRMZM2G147726_P02	76	0	0.0000	0.0006	-7.08	0.0001	0.0121	0.0010	0.0562
GRMZM2G148323_P01	34	0	0.0013	0.0270	-5.60	0.0100	0.0748	0.0053	0.0583
GRMZM2G148769_P01	60	679	0.0000	0.0000	4.32	0.0000	0.0024	0.0001	0.0220
GRMZM2G148925_P01	138	9	0.0005	0.0158	-3.38	0.0190	0.1051	0.0008	0.0562
GRMZM2G150193_P02	78	4	0.0002	0.0100	-3.53	0.0027	0.0399	0.0015	0.0562
GRMZM2G151252_P01	135	30	0.0161	0.1377	-1.39	0.0008	0.0246	0.0057	0.0594
GRMZM2G152925_P01	278	349	0.0024	0.0386	1.27	0.0059	0.0579	0.3837	0.4695

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G153162_P02	39	79	0.0010	0.0244	1.85	0.0047	0.0543	0.0990	0.1804
GRMZM2G153815_P01	578	130	0.0011	0.0244	-1.53	0.0004	0.0185	0.0014	0.0562
GRMZM2G153984_P01	117	30	0.0169	0.1400	-1.24	0.0003	0.0158	0.0092	0.0652
GRMZM2G153990_P01	73	0	0.0000	0.0018	-6.73	0.0006	0.0203	0.0011	0.0562
GRMZM2G154960_P01	208	36	0.0011	0.0244	-1.83	0.0018	0.0344	0.0021	0.0562
GRMZM2G155183_P01	248	404	0.0008	0.0215	1.44	0.0087	0.0689	0.1185	0.2009
GRMZM2G155260_P01	67	7	0.0014	0.0280	-2.38	0.0006	0.0210	0.0025	0.0562
GRMZM2G155931_P03	4	26	0.0013	0.0269	3.24	0.0021	0.0355	0.0283	0.0937
GRMZM2G156848_P01	3	43	0.0001	0.0068	4.11	0.0048	0.0546	0.0082	0.0643
GRMZM2G157588_P01	50	5	0.0031	0.0460	-2.53	0.0010	0.0265	0.0041	0.0564
GRMZM2G158034_P01	199	49	0.0208	0.1576	-1.17	0.0020	0.0351	0.0053	0.0583
GRMZM2G158130_P01	34	1	0.0005	0.0158	-3.88	0.0001	0.0115	0.0057	0.0594
GRMZM2G158188_P01	124	167	0.0038	0.0540	1.22	0.0008	0.0244	0.3269	0.4148
GRMZM2G159013_P01	125	14	0.0010	0.0244	-2.38	0.0013	0.0293	0.0017	0.0562
GRMZM2G159643_P01	698	51	0.0000	0.0006	-3.06	0.0033	0.0455	0.0000	0.0150
GRMZM2G160184_P03	106	37	0.1217	0.4088	-0.77	0.0037	0.0480	0.0230	0.0873
GRMZM2G160184_P04	111	39	0.1450	0.4528	-0.76	0.0038	0.0483	0.0228	0.0871
GRMZM2G162384_P01	78	128	0.0013	0.0265	1.46	0.0019	0.0344	0.1680	0.2546
GRMZM2G162968_P01	66	18	0.0834	0.3378	-1.10	0.0038	0.0481	0.0160	0.0761
GRMZM2G163081_P01	340	540	0.0005	0.0155	1.60	0.0185	0.1031	0.1192	0.2016

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G163081_P02	307	518	0.0003	0.0108	1.72	0.0165	0.0973	0.0934	0.1747
GRMZM2G163081_P05	319	531	0.0003	0.0120	1.66	0.0169	0.0983	0.0974	0.1787
GRMZM2G163233_P04	744	150	0.0030	0.0458	-1.41	0.0015	0.0319	0.0008	0.0562
GRMZM2G163242_P01	66	15	0.0289	0.1936	-1.33	0.0015	0.0317	0.0106	0.0672
GRMZM2G164352_P02	875	885	0.0395	0.2278	0.82	0.0030	0.0427	0.9331	0.9434
GRMZM2G164418_P01	36	6	0.0342	0.2125	-1.71	0.0022	0.0358	0.0118	0.0687
GRMZM2G165433_P01	263	79	0.0243	0.1718	-1.03	0.0002	0.0131	0.0077	0.0643
GRMZM2G166035_P01	180	49	0.0136	0.1223	-1.13	0.0002	0.0128	0.0077	0.0643
GRMZM2G166044_P01	0	25	0.0000	0.0018	6.33	0.0002	0.0128	0.0155	0.0753
GRMZM2G166345_P02	38	7	0.0288	0.1936	-1.66	0.0021	0.0355	0.0132	0.0705
GRMZM2G166897_P01	35	0	0.0005	0.0161	-5.72	0.0055	0.0573	0.0050	0.0583
GRMZM2G167149_P01	832	1457	0.0000	0.0030	1.55	0.0001	0.0121	0.0496	0.1244
GRMZM2G167505_P01	24	1	0.0064	0.0764	-3.39	0.0003	0.0165	0.0122	0.0693
GRMZM2G168257_P01	0	35	0.0016	0.0310	6.19	0.0445	0.1799	0.0065	0.0613
GRMZM2G170839_P02	1603	317	0.0001	0.0041	-1.62	0.0005	0.0200	0.0002	0.0391
GRMZM2G171229_P01	761	158	0.0009	0.0222	-1.46	0.0074	0.0649	0.0008	0.0562
GRMZM2G171664_P01	325	63	0.0345	0.2131	-1.37	0.0032	0.0451	0.0017	0.0562
GRMZM2G173105_P01	55	3	0.0011	0.0244	-3.32	0.0050	0.0548	0.0020	0.0562
GRMZM2G173289_P01	11	444	0.0000	0.0000	5.92	0.0076	0.0656	0.0000	0.0160
GRMZM2G173341_P03	565	854	0.0007	0.0205	1.57	0.0290	0.1394	0.1294	0.2123

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G173649_P02	15	32	0.0075	0.0846	1.97	0.0036	0.0469	0.1193	0.2016
GRMZM2G174263_P01	45	114	0.0005	0.0154	1.97	0.0037	0.0480	0.0437	0.1163
GRMZM2G175779_P01	1078	1232	0.0302	0.1980	0.88	0.0021	0.0355	0.5102	0.5890
GRMZM2G175818_P02	43	144	0.0013	0.0269	2.45	0.0025	0.0377	0.0178	0.0788
GRMZM2G176396_P01	64	15	0.0292	0.1951	-1.43	0.0024	0.0376	0.0131	0.0705
GRMZM2G177142_P01	97	2	0.0000	0.0001	-4.60	0.0004	0.0189	0.0008	0.0562
GRMZM2G177912_P01	219	60	0.0255	0.1791	-1.01	0.0001	0.0120	0.0068	0.0620
GRMZM2G178618_P01	458	161	0.0770	0.3248	-0.70	0.0014	0.0300	0.0085	0.0643
GRMZM2G178892_P01	335	47	0.0003	0.0126	-1.91	0.0027	0.0399	0.0007	0.0562
GRMZM2G180071_P01	68	8	0.0032	0.0480	-2.24	0.0049	0.0546	0.0030	0.0562
GRMZM2G180172_P02	57	2	0.0016	0.0301	-3.63	0.0111	0.0798	0.0024	0.0562
GRMZM2G180335_P01	207	49	0.0101	0.1031	-1.27	0.0001	0.0120	0.0046	0.0573
GRMZM2G180575_P01	101	152	0.0031	0.0461	1.52	0.0150	0.0935	0.2182	0.3110
GRMZM2G180625_P01	7874	1449	0.0021	0.0358	-1.53	0.0003	0.0183	0.0000	0.0010
GRMZM2G180625_P03	7892	1451	0.0021	0.0358	-1.53	0.0003	0.0181	0.0000	0.0010
GRMZM2G301513_P01	43	3	0.0049	0.0653	-2.79	0.0029	0.0416	0.0045	0.0573
GRMZM2G301647_P01	299	77	0.0094	0.0981	-1.14	0.0030	0.0427	0.0042	0.0564
GRMZM2G301647_P02	352	95	0.0072	0.0818	-1.11	0.0001	0.0117	0.0042	0.0564
GRMZM2G301904_P01	48	6	0.0089	0.0946	-2.11	0.0004	0.0187	0.0058	0.0597
GRMZM2G305254_P05	33	4	0.0115	0.1089	-2.23	0.0009	0.0252	0.0113	0.0673

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G305851_P01	31	1	0.0013	0.0271	-3.86	0.0053	0.0558	0.0069	0.0621
GRMZM2G306374_P01	121	30	0.0114	0.1089	-1.25	0.0001	0.0116	0.0081	0.0643
GRMZM2G312438_P02	304	58	0.0011	0.0244	-1.49	0.0012	0.0282	0.0018	0.0562
GRMZM2G314434_P01	14	36	0.0011	0.0244	2.25	0.0021	0.0355	0.0851	0.1668
GRMZM2G321725_P03	255	433	0.0011	0.0244	1.54	0.0225	0.1171	0.0964	0.1779
GRMZM2G323558_P01	400	2726	0.0000	0.0034	3.29	0.0440	0.1781	0.0000	0.0150
GRMZM2G325285_P01	235	45	0.0029	0.0450	-1.62	0.0016	0.0324	0.0023	0.0562
GRMZM2G329584_P01	782	252	0.0151	0.1318	-0.99	0.0008	0.0247	0.0038	0.0564
GRMZM2G331368_P01	110	35	0.1358	0.4348	-0.80	0.0022	0.0361	0.0171	0.0775
GRMZM2G332259_P01	22	108	0.0000	0.0020	3.06	0.0215	0.1137	0.0076	0.0643
GRMZM2G334409_P01	78	18	0.0173	0.1416	-1.39	0.0006	0.0220	0.0096	0.0657
GRMZM2G342493_P01	769	2048	0.0001	0.0039	2.04	0.0353	0.1557	0.0044	0.0564
GRMZM2G349655_P01	5749	1833	0.0567	0.2762	-0.84	0.0122	0.0838	0.0004	0.0462
GRMZM2G354558_P01	128	46	0.1245	0.4127	-0.72	0.0040	0.0495	0.0224	0.0868
GRMZM2G355906_P01	36	625	0.0000	0.0000	5.04	0.0002	0.0128	0.0001	0.0176
GRMZM2G356938_P01	114	6	0.0001	0.0047	-3.27	0.0165	0.0973	0.0008	0.0562
GRMZM2G357296_P01	143	321	0.0000	0.0010	1.94	0.0005	0.0200	0.0370	0.1085
GRMZM2G359566_P01	1403	529	0.1120	0.3919	-0.63	0.0004	0.0188	0.0046	0.0573
GRMZM2G362088_P02	28	2	0.0022	0.0361	-2.91	0.0005	0.0203	0.0122	0.0693
GRMZM2G363557_P01	75	19	0.0519	0.2632	-1.14	0.0040	0.0491	0.0122	0.0693

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G367051_P01	41	2	0.0017	0.0318	-3.38	0.0008	0.0244	0.0051	0.0583
GRMZM2G367113_P01	106	365	0.0006	0.0175	2.64	0.0277	0.1356	0.0073	0.0635
GRMZM2G368799_P01	28	1	0.0005	0.0163	-3.69	0.0001	0.0121	0.0087	0.0648
GRMZM2G369803_P01	55	3	0.0011	0.0244	-3.32	0.0050	0.0548	0.0020	0.0562
GRMZM2G379758_P01	146	245	0.0003	0.0108	1.52	0.0002	0.0143	0.1249	0.2076
GRMZM2G380014_P01	74	13	0.0034	0.0500	-1.81	0.0002	0.0150	0.0066	0.0620
GRMZM2G381744_P01	322	102	0.0239	0.1707	-1.02	0.0010	0.0264	0.0078	0.0643
GRMZM2G390678_P01	846	132	0.0003	0.0126	-2.04	0.0052	0.0556	0.0003	0.0410
GRMZM2G394259_P01	6096	10922	0.0001	0.0057	1.57	0.0034	0.0460	0.0124	0.0693
GRMZM2G401511_P01	149	16	0.0001	0.0045	-2.53	0.0011	0.0282	0.0011	0.0562
GRMZM2G402211_P01	129	20	0.0009	0.0225	-1.98	0.0056	0.0576	0.0026	0.0562
GRMZM2G409407_P02	98	19	0.0096	0.0994	-1.80	0.0039	0.0491	0.0061	0.0605
GRMZM2G411084_P01	9	36	0.0010	0.0244	2.57	0.0020	0.0351	0.0273	0.0925
GRMZM2G412296_P01	71	11	0.0082	0.0903	-1.80	0.0002	0.0128	0.0046	0.0573
GRMZM2G413226_P01	121	20	0.0012	0.0258	-1.88	0.0070	0.0628	0.0031	0.0562
GRMZM2G413709_P02	263	27	0.0000	0.0009	-2.48	0.0020	0.0351	0.0005	0.0562
GRMZM2G418644_P01	6368	11296	0.0001	0.0057	1.56	0.0030	0.0427	0.0130	0.0703
GRMZM2G419452_P01	556	65	0.0506	0.2618	-2.24	0.0149	0.0933	0.0002	0.0391
GRMZM2G420988_P01	100	480	0.0001	0.0047	3.10	0.0238	0.1214	0.0018	0.0562
GRMZM2G426953_P01	36	0	0.0001	0.0042	-5.97	0.0001	0.0121	0.0050	0.0583

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	**Mature B73 Pollen**	**Germinated B73 Pollen**	**p-value**	**q-value**	**LogFC**	**p-value**	**q-value**	**p-value**	**q-value**
GRMZM2G429540_P01	55	3	0.0011	0.0244	-3.32	0.0050	0.0548	0.0020	0.0562
GRMZM2G430600_P02	1948	580	0.0193	0.1514	-1.02	0.0010	0.0264	0.0011	0.0562
GRMZM2G431821_P01	143	321	0.0000	0.0010	1.94	0.0005	0.0200	0.0370	0.1085
GRMZM2G431856_P01	698	109	0.0027	0.0415	-1.72	0.0013	0.0293	0.0004	0.0462
GRMZM2G435373_P01	445	639	0.0006	0.0183	1.37	0.0031	0.0439	0.1805	0.2682
GRMZM2G438378_P01	6302	6316	0.0456	0.2473	0.77	0.0013	0.0293	0.9515	0.9530
GRMZM2G439306_P01	219	59	0.0150	0.1314	-1.10	0.0027	0.0404	0.0064	0.0613
GRMZM2G439908_P01	221	594	0.0008	0.0219	1.96	0.0607	0.2110	0.0124	0.0693
GRMZM2G439950_P03	68	2	0.0004	0.0138	-3.97	0.0145	0.0924	0.0016	0.0562
GRMZM2G442008_P01	2489	4296	0.0008	0.0215	1.48	0.0004	0.0189	0.0302	0.0971
GRMZM2G443740_P01	1332	1604	0.0037	0.0537	1.07	0.0000	0.0033	0.3649	0.4521
GRMZM2G443985_P01	292	91	0.0267	0.1840	-0.97	0.0020	0.0349	0.0079	0.0643
GRMZM2G450125_P01	199	67	0.0622	0.2916	-0.82	0.0006	0.0203	0.0136	0.0714
GRMZM2G453474_P01	152	256	0.0020	0.0340	1.47	0.0235	0.1203	0.1212	0.2032
GRMZM2G453855_P03	48	1	0.0017	0.0318	-4.16	0.0095	0.0722	0.0027	0.0562
GRMZM2G455828_P04	142	19	0.0001	0.0050	-2.28	0.0002	0.0128	0.0018	0.0562
GRMZM2G456387_P01	228	4	0.0000	0.0018	-4.73	0.0622	0.2127	0.0001	0.0197
GRMZM2G458208_P01	59	12	0.0305	0.1995	-1.48	0.0010	0.0259	0.0090	0.0648
GRMZM2G459291_P01	67	3	0.0004	0.0144	-3.61	0.0019	0.0344	0.0017	0.0562
GRMZM2G460406_P01	188	76	0.2183	0.5620	-0.55	0.0023	0.0364	0.0260	0.0902

Table A.2: Continued

	Spectral counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G460701_P01	217	381	0.0003	0.0128	1.72	0.0133	0.0880	0.0889	0.1701
GRMZM2G466667_P01	129	24	0.0028	0.0428	-1.63	0.0000	0.0104	0.0038	0.0564
GRMZM2G466833_P01	878	2410	0.0000	0.0023	2.01	0.0005	0.0200	0.0032	0.0564
GRMZM2G473906_P05	176	30	0.0017	0.0318	-1.85	0.0070	0.0628	0.0023	0.0562
GRMZM2G479423_P01	65	86	0.0087	0.0929	1.18	0.0039	0.0491	0.3921	0.4777
GRMZM2G482245_P01	26	140	0.0002	0.0071	3.00	0.0308	0.1446	0.0054	0.0583
GRMZM2G701193_P01	54	4	0.0015	0.0300	-2.78	0.0009	0.0252	0.0034	0.0564
GRMZM5G801031_P02	265	50	0.0036	0.0518	-1.83	0.0018	0.0343	0.0021	0.0562
GRMZM5G808899_P01	48	2	0.0019	0.0331	-3.47	0.0053	0.0558	0.0035	0.0564
GRMZM5G815839_P02	415	571	0.0013	0.0265	1.30	0.0058	0.0579	0.2272	0.3200
GRMZM5G825609_P02	152	19	0.0031	0.0470	-2.02	0.0367	0.1591	0.0015	0.0562
GRMZM5G829894_P01	105	12	0.0001	0.0042	-2.41	0.0000	0.0049	0.0019	0.0562
GRMZM5G836674_P01	27	0	0.0025	0.0392	-5.27	0.0074	0.0649	0.0087	0.0648
GRMZM5G858094_P01	406	180	0.3026	0.6585	-0.41	0.0038	0.0480	0.0233	0.0873
GRMZM5G861756_P01	61	11	0.0162	0.1377	-1.66	0.0034	0.0460	0.0069	0.0621
GRMZM5G866758_P02	403	170	0.1797	0.5047	-0.53	0.0020	0.0349	0.0192	0.0814
GRMZM5G873271_P01	2	23	0.0005	0.0161	3.99	0.0042	0.0517	0.0276	0.0926
GRMZM5G874500_P03	54	6	0.0059	0.0736	-2.21	0.0014	0.0301	0.0043	0.0564
GRMZM5G878322_P01	0	32	0.0000	0.004	6.35	0.0018	0.0344	0.0083	0.064

Table A. 3: 103 proteins found to be differentially abundant by at least one package in the phosphoproteome.
Spectra counts represent the sum of three replicates for each tissue type. LogFC indicates the log base 2 fold change of protein abundance in germinated pollen relative to mature pollen; sign indicates increase or decrease in abundance.

			Statistical Output						
	Spectra Counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
AC195340.3_FGP001	32	297	0.0010	0.0392	3.2298	0.1661	0.3722	0.0008	0.3235
AC205471.4_FGP002	0	15	0.0052	0.0901	5.5472	0.0003	0.0131	0.0738	0.3290
AC205703.4_FGP010	14	0	0.0253	0.2153	-5.0166	0.0007	0.0168	0.0824	0.3291
AC207890.3_FGP002	54	100	0.0772	0.3536	1.1451	0.0018	0.0271	0.1570	0.3792
AC209705.3_FGP004	0	18	0.0054	0.0901	5.7640	0.0027	0.0313	0.0529	0.3290
AC213884.3_FGP001	2	11	0.0712	0.3400	2.6542	0.0045	0.0426	0.1956	0.4122
AC218972.3_FGP004	23	4	0.0477	0.3007	-2.2472	0.0053	0.0466	0.0612	0.3290
AC233853.1_FGP002	20	0	0.0022	0.0607	-5.7306	0.0002	0.0121	0.0422	0.3290
GRMZM2G003059_P01	0	27	0.0016	0.0568	6.1524	0.0014	0.0243	0.0231	0.3235
GRMZM2G005753_P03	2	11	0.0738	0.3438	2.6340	0.0047	0.0433	0.1956	0.4122
GRMZM2G009014_P01	7	48	0.0181	0.1709	2.7472	0.0029	0.0328	0.0252	0.3235
GRMZM2G012631_P01	2	26	0.0016	0.0568	3.7910	0.0002	0.0121	0.0342	0.3290
GRMZM2G012958_P03	15	0	0.0143	0.1442	-5.1970	0.0004	0.0142	0.0732	0.3290
GRMZM2G014004_P01	0	10	0.0239	0.2134	4.9878	0.0009	0.0202	0.1573	0.3792
GRMZM2G017334_P01	0	13	0.0261	0.2153	5.2882	0.0052	0.0466	0.0967	0.3379
GRMZM2G017856_P01	25	68	0.0260	0.2153	1.9133	0.0053	0.0466	0.0802	0.3291
GRMZM2G020034_P01	0	28	0.0001	0.0102	6.4130	0.0001	0.0114	0.0219	0.3235

Table A.3: Continued

			Statistical Output						
	Spectra Counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G022061_P01	0	15	0.0110	0.1287	5.4216	0.0007	0.0170	0.0738	0.3290
GRMZM2G027891_P01	43	5	0.0070	0.1073	-2.7893	0.0020	0.0271	0.0192	0.3235
GRMZM2G035839_P02	15	1	0.0398	0.2739	-3.3434	0.0055	0.0467	0.0732	0.3290
GRMZM2G036837_P03	98	25	0.0077	0.1108	-1.8276	0.0055	0.0466	0.0224	0.3235
GRMZM2G037193_P01	11	0	0.0405	0.2769	-4.7645	0.0008	0.0188	0.1265	0.3594
GRMZM2G038988_P01	0	17	0.0025	0.0608	5.6682	0.0001	0.0114	0.0586	0.3290
GRMZM2G040750_P01	106	4	0.0000	0.0102	-4.2995	0.0029	0.0328	0.0019	0.3235
GRMZM2G044398_P05	10	31	0.0497	0.3007	1.9090	0.0020	0.0271	0.1130	0.3466
GRMZM2G044681_P01	9	33	0.0485	0.3007	2.0102	0.0034	0.0350	0.0809	0.3291
GRMZM2G046658_P01	5	24	0.0109	0.1287	2.5602	0.0009	0.0202	0.0719	0.3290
GRMZM2G047223_P03	21	0	0.0026	0.0608	-5.8390	0.0010	0.0209	0.0395	0.3290
GRMZM2G057674_P01	3	15	0.0508	0.3007	2.4347	0.0030	0.0332	0.1119	0.3466
GRMZM2G058870_P01	108	220	0.0435	0.2945	1.2017	0.0007	0.0176	0.0713	0.3290
GRMZM2G060567_P01	12	52	0.0079	0.1113	2.6948	0.0017	0.0271	0.0443	0.3290
GRMZM2G062391_P01	10	0	0.0634	0.3278	-4.5786	0.0017	0.0271	0.1455	0.3769
GRMZM2G062844_P01	2	13	0.0354	0.2588	2.9673	0.0036	0.0358	0.1468	0.3769
GRMZM2G063478_P01	119	49	0.1039	0.3981	-1.0962	0.0031	0.0332	0.0622	0.3290
GRMZM2G073045_P02	17	0	0.0054	0.0901	-5.5957	0.0012	0.0221	0.0594	0.3290
GRMZM2G074233_P01	2	39	0.0007	0.0312	4.2876	0.0031	0.0332	0.0136	0.3235
GRMZM2G077757_P01	14	0	0.0344	0.2558	-4.9664	0.0032	0.0335	0.0824	0.3291

Table A.3: Continued

			Statistical Output						
	Spectra Counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G082167_P01	6	35	0.0031	0.0656	3.0490	0.0041	0.0398	0.0454	0.3290
GRMZM2G082529_P01	18	0	0.0063	0.1005	-5.7013	0.0021	0.0271	0.0529	0.3290
GRMZM2G083475_P01	0	18	0.0020	0.0575	5.7653	0.0001	0.0114	0.0529	0.3290
GRMZM2G084181_P01	26	0	0.0026	0.0608	-5.8577	0.0005	0.0150	0.0253	0.3235
GRMZM2G088245_P01	109	207	0.0444	0.2952	1.2919	0.0019	0.0271	0.0955	0.3379
GRMZM2G094712_P02	53	4	0.0014	0.0522	-3.3477	0.0021	0.0271	0.0109	0.3235
GRMZM2G095308_P02	36	0	0.0000	0.0102	-6.5956	0.0001	0.0114	0.0123	0.3235
GRMZM2G095763_P01	88	17	0.0107	0.1287	-2.1816	0.0032	0.0335	0.0157	0.3235
GRMZM2G096249_P01	45	3	0.0005	0.0266	-3.5549	0.0001	0.0114	0.0099	0.3235
GRMZM2G098900_P01	2	19	0.0076	0.1108	3.4459	0.0020	0.0271	0.0685	0.3290
GRMZM2G099352_P05	33	0	0.0001	0.0102	-6.5241	0.0004	0.0140	0.0156	0.3235
GRMZM2G099980_P02	24	2	0.0115	0.1295	-3.1327	0.0014	0.0243	0.0411	0.3290
GRMZM2G104907_P01	32	76	0.0495	0.3007	1.6661	0.0057	0.0481	0.1046	0.3466
GRMZM2G107867_P04	1	18	0.0017	0.0568	4.1003	0.0006	0.0167	0.0529	0.3290
GRMZM2G112377_P01	3	49	0.0001	0.0105	4.2933	0.0019	0.0271	0.0106	0.3235
GRMZM2G114093_P01	36	3	0.0034	0.0681	-3.3362	0.0005	0.0158	0.0226	0.3235
GRMZM2G121683_P01	83	36	0.1974	0.5631	-0.8705	0.0047	0.0433	0.0878	0.3306
GRMZM2G122873_P01	37	4	0.0184	0.1711	-2.6761	0.0023	0.0284	0.0218	0.3235
GRMZM2G124317_P07	26	5	0.0633	0.3278	-2.1265	0.0047	0.0433	0.0742	0.3290
GRMZM2G124739_P01	200	56	0.0134	0.1377	-1.5162	0.0029	0.0328	0.0119	0.3235

Table A.3: Continued

	Spectra Counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G126922_P01	285	24	0.0000	0.0102	-3.5065	0.0043	0.0415	0.0008	0.3235
GRMZM2G135808_P01	10	26	0.1036	0.3981	1.6491	0.0035	0.0350	0.1673	0.3851
GRMZM2G138410_P01	13	121	0.0001	0.0105	3.2979	0.0013	0.0221	0.0031	0.3235
GRMZM2G143354_P01	94	8	0.0004	0.0248	-3.3426	0.0003	0.0131	0.0053	0.3235
GRMZM2G149751_P01	17	0	0.0089	0.1198	-5.3483	0.0003	0.0131	0.0594	0.3290
GRMZM2G150166_P01	0	13	0.0083	0.1139	5.3384	0.0003	0.0131	0.0967	0.3379
GRMZM2G150862_P01	3	14	0.0635	0.3278	2.5769	0.0035	0.0350	0.1680	0.3856
GRMZM2G151268_P03	32	2	0.0044	0.0798	-3.4666	0.0021	0.0271	0.0224	0.3235
GRMZM2G151387_P01	30	4	0.0123	0.1351	-2.7055	0.0019	0.0271	0.0431	0.3290
GRMZM2G154165_P04	1	16	0.0051	0.0897	3.8744	0.0007	0.0168	0.0660	0.3290
GRMZM2G154328_P01	3	32	0.0042	0.0779	3.3566	0.0011	0.0209	0.0290	0.3290
GRMZM2G156145_P01	8	34	0.0194	0.1784	2.2991	0.0053	0.0466	0.0636	0.3290
GRMZM2G157803_P01	191	46	0.0038	0.0735	-1.7924	0.0000	0.0096	0.0085	0.3235
GRMZM2G157929_P01	21	0	0.0026	0.0608	-5.7968	0.0003	0.0131	0.0395	0.3290
GRMZM2G158918_P01	33	4	0.0106	0.1287	-2.7742	0.0011	0.0209	0.0344	0.3290
GRMZM2G161913_P01	21	0	0.0018	0.0568	-5.7326	0.0001	0.0114	0.0395	0.3290
GRMZM2G162688_P01	3	19	0.0151	0.1505	2.8154	0.0011	0.0209	0.0685	0.3290
GRMZM2G163277_P01	24	1	0.0031	0.0656	-3.9738	0.0005	0.0154	0.0297	0.3290
GRMZM2G166044_P01	8	59	0.0002	0.0173	3.3522	0.0005	0.0150	0.0175	0.3235
GRMZM2G169782_P01	3	27	0.0017	0.0568	3.3137	0.0002	0.0121	0.0316	0.3290

Table A.3: Continued

	Spectra Counts		Statistical Output						
			edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G171187_P02	14	0	0.0131	0.1377	-5.3060	0.0012	0.0221	0.0824	0.3291
GRMZM2G171442_P01	27	0	0.0007	0.0312	-5.9887	0.0001	0.0114	0.0242	0.3235
GRMZM2G173270_P01	14	66	0.0020	0.0575	2.5247	0.0002	0.0121	0.0256	0.3235
GRMZM2G178576_P03	2	13	0.0450	0.2952	2.8522	0.0024	0.0298	0.1468	0.3769
GRMZM2G178892_P01	5	34	0.0075	0.1108	3.0711	0.0031	0.0332	0.0406	0.3290
GRMZM2G179976_P01	64	2	0.0003	0.0224	-4.3621	0.0068	0.0542	0.0038	0.3235
GRMZM2G316113_P04	26	0	0.0004	0.0248	-6.0745	0.0001	0.0114	0.0253	0.3235
GRMZM2G342327_P03	2	14	0.0251	0.2153	3.0242	0.0016	0.0267	0.1254	0.3594
GRMZM2G359746_P01	124	62	0.1974	0.5631	-0.8049	0.0048	0.0433	0.1046	0.3466
GRMZM2G406177_P01	18	85	0.0027	0.0608	2.5345	0.0006	0.0168	0.0202	0.3235
GRMZM2G428391_P01	12	132	0.0000	0.0004	3.7097	0.0000	0.0014	0.0023	0.3235
GRMZM2G429241_P01	33	1	0.0005	0.0266	-4.4451	0.0011	0.0209	0.0156	0.3235
GRMZM2G432566_P01	14	1	0.0549	0.3099	-3.1588	0.0021	0.0271	0.0824	0.3291
GRMZM2G446515_P01	38	164	0.0001	0.0117	2.4951	0.0000	0.0060	0.0090	0.3235
GRMZM2G457370_P02	0	18	0.0042	0.0779	5.6646	0.0004	0.0140	0.0529	0.3290
GRMZM2G462613_P01	34	84	0.0126	0.1354	1.7424	0.0025	0.0299	0.0801	0.3291
GRMZM2G464363_P01	122	17	0.0010	0.0392	-2.6829	0.0017	0.0271	0.0059	0.3235
GRMZM2G471115_P01	0	18	0.0025	0.0608	5.8687	0.0009	0.0204	0.0529	0.3290
GRMZM2G472231_P01	36	10	0.0534	0.3061	-1.6664	0.0054	0.0466	0.0625	0.3290
GRMZM2G563190_P02	47	0	0.0001	0.0102	-6.8936	0.0020	0.0271	0.0072	0.3235

Table A.3: Continued

			Statistical Output						
	Spectra Counts		edgeR			IBB		PLGEM	
Group Leader	Mature B73 Pollen	Germinated B73 Pollen	p-value	q-value	LogFC	p-value	q-value	p-value	q-value
GRMZM2G701193_P04	0	21	0.0006	0.0307	6.0419	0.0003	0.0131	0.0377	0.3290
GRMZM5G815894_P03	2	13	0.0438	0.2945	2.8707	0.0025	0.0300	0.1468	0.3769
GRMZM5G836990_P01	14	0	0.0332	0.2535	-4.9396	0.0020	0.0271	0.0824	0.3291
GRMZM5G881464_P01	15	0	0.0105	0.1287	-5.4848	0.0027	0.0313	0.0732	0.3290

Table A. 4: List of the 38 GO terms enriched in the proteins found to increase in abundance with germination in the total proteome

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0019752	P	carboxylic acid metabolic process	13	742	1.20E-05	0.00058
GO:0043436	P	oxoacid metabolic process	13	742	1.20E-05	0.00058
GO:0005975	P	carbohydrate metabolic process	16	1097	1.00E-05	0.00058
GO:0042180	P	cellular ketone metabolic process	13	749	1.30E-05	0.00058
GO:0006082	P	organic acid metabolic process	13	744	1.20E-05	0.00058
GO:0044265	P	cellular macromolecule catabolic process	8	355	0.00012	0.0044
GO:0034641	P	cellular nitrogen compound metabolic process	8	373	0.00016	0.0053
GO:0016053	P	organic acid biosynthetic process	7	304	0.00028	0.007
GO:0046394	P	carboxylic acid biosynthetic process	7	304	0.00028	0.007
GO:0006412	P	translation	13	1071	0.00044	0.01
GO:0044248	P	cellular catabolic process	8	451	0.00057	0.012
GO:0030599	F	pectinesterase activity	9	129	5.20E-09	8.10E-07
GO:0004091	F	carboxylesterase activity	10	217	3.10E-08	2.40E-06
GO:0004857	F	enzyme inhibitor activity	9	201	2.00E-07	1.00E-05
GO:0030234	F	enzyme regulator activity	11	388	6.90E-07	2.70E-05
GO:0004650	F	polygalacturonase activity	5	52	3.50E-06	0.00011
GO:0015078	F	hydrogen ion transmembrane transporter activity	7	228	5.00E-05	0.0013
GO:0015077	F	monovalent inorganic cation transmembrane transporter activity	7	263	0.00012	0.0026
GO:0016788	F	hydrolase activity, acting on ester bonds	12	879	0.00026	0.0051
GO:0022890	F	inorganic cation transmembrane transporter activity	7	334	0.00049	0.0084
GO:0003735	F	structural constituent of ribosome	10	772	0.0013	0.02
GO:0019842	F	vitamin binding	5	196	0.0014	0.02

Table A.4: Continued

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0005215	F	transporter activity	16	1726	0.0017	0.022
GO:0016787	F	hydrolase activity	28	3943	0.0019	0.023
GO:0022892	F	substrate-specific transporter activity	12	1166	0.0029	0.031
GO:0008324	F	cation transmembrane transporter activity	9	724	0.003	0.031
GO:0004553	F	hydrolase activity, hydrolyzing O-glycosyl compounds	7	479	0.0037	0.036
GO:0016798	F	hydrolase activity, acting on glycosyl bonds	7	515	0.0054	0.05
GO:0044444	C	cytoplasmic part	23	1518	4.20E-08	4.70E-06
GO:0005739	C	mitochondrion	9	187	1.10E-07	6.00E-06
GO:0005737	C	cytoplasm	25	2005	3.90E-07	1.40E-05
GO:0005740	C	mitochondrial envelope	7	153	4.10E-06	0.00011
GO:0044429	C	mitochondrial part	7	174	9.30E-06	0.0002
GO:0031967	C	organelle envelope	7	186	1.40E-05	0.00026
GO:0031975	C	envelope	7	228	5.00E-05	0.00078
GO:0032991	C	macromolecular complex	22	2352	0.00018	0.0025
GO:0005840	C	ribosome	10	779	0.0014	0.017
GO:0030529	C	ribonucleoprotein complex	10	847	0.0025	0.028

Table A. 5: **List of the 39 GO terms enriched in the proteins found to decrease in abundance with germination in the total proteome.**

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0034641	P	cellular nitrogen compound metabolic process	15	373	4.50E-07	0.00033
GO:0019318	P	hexose metabolic process	12	262	1.80E-06	0.00062
GO:0005996	P	monosaccharide metabolic process	13	321	2.50E-06	0.00062
GO:0006066	P	alcohol metabolic process	15	455	4.80E-06	0.00089
GO:0044262	P	cellular carbohydrate metabolic process	15	493	1.20E-05	0.0018
GO:0051169	P	nuclear transport	6	86	8.60E-05	0.0092
GO:0006913	P	nucleocytoplasmic transport	6	86	8.60E-05	0.0092
GO:0070727	P	cellular macromolecule localization	10	320	0.00029	0.016
GO:0055114	P	oxidation reduction	10	309	0.00022	0.016
GO:0046483	P	heterocycle metabolic process	14	591	0.00031	0.016
GO:0006886	P	intracellular protein transport	10	315	0.00025	0.016
GO:0046907	P	intracellular transport	11	372	0.00023	0.016
GO:0034613	P	cellular protein localization	10	320	0.00029	0.016
GO:0005975	P	carbohydrate metabolic process	21	1097	0.00019	0.016
GO:0051649	P	establishment of localization in cell	12	456	0.00033	0.017
GO:0042545	P	cell wall modification	5	74	0.00039	0.018
GO:0051641	P	cellular localization	12	470	0.00043	0.019
GO:0006006	P	glucose metabolic process	8	240	0.00077	0.032
GO:0007264	P	small GTPase mediated signal transduction	8	244	0.00086	0.034
GO:0006575	P	cellular amino acid derivative metabolic process	5	93	0.001	0.039
GO:0006519	P	cellular amino acid and derivative metabolic process	13	600	0.0011	0.04
GO:0003824	F	catalytic activity	136	11249	1.20E-11	4.30E-09

Table A.5: Continued

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0016491	F	oxidoreductase activity	44	2115	2.80E-09	3.50E-07
GO:0050662	F	coenzyme binding	20	473	2.20E-09	3.50E-07
GO:0048037	F	cofactor binding	22	634	1.10E-08	1.00E-06
GO:0003924	F	GTPase activity	11	195	7.20E-07	5.40E-05
GO:0000166	F	nucleotide binding	58	3970	1.40E-06	8.80E-05
GO:0050660	F	FAD binding	9	189	2.70E-05	0.0014
GO:0005509	F	calcium ion binding	12	352	3.20E-05	0.0015
GO:0005525	F	GTP binding	13	441	6.30E-05	0.0024
GO:0032561	F	guanyl ribonucleotide binding	13	441	6.30E-05	0.0024
GO:0019001	F	guanyl nucleotide binding	13	451	7.90E-05	0.0027
GO:0017076	F	purine nucleotide binding	50	3767	0.00012	0.0037
GO:0030599	F	pectinesterase activity	6	129	0.00069	0.02
GO:0016817	F	hydrolase activity, acting on acid anhydrides	21	1287	0.0014	0.038
GO:0005737	C	cytoplasm	35	2005	8.10E-06	0.00086
GO:0033176	C	proton-transporting V-type ATPase complex	5	39	2.30E-05	0.0012
GO:0044444	C	cytoplasmic part	26	1518	0.00018	0.0064
GO:0033178	C	proton-transporting two-sector ATPase complex, catalytic domain	5	82	0.00061	0.016

Table A. 6:**List of the 9 GO terms enriched in the proteins found to increase in abundance with germination in the phosphoproteome**

GO term	Ontology	Description	Number in input list	Number in BG/Ref	p-value	FDR
GO:0001883	F	purine nucleoside binding	9	3384	0.0067	0.04
GO:0001882	F	nucleoside binding	9	3385	0.0067	0.04
GO:0000166	F	nucleotide binding	11	3970	0.0016	0.04
GO:0017076	F	purine nucleotide binding	10	3767	0.004	0.04
GO:0005524	F	ATP binding	9	3193	0.0045	0.04
GO:0030554	F	adenyl nucleotide binding	9	3384	0.0067	0.04
GO:0032559	F	adenyl ribonucleotide binding	9	3197	0.0046	0.04
GO:0032555	F	purine ribonucleotide binding	10	3572	0.0027	0.04
GO:0032553	F	ribonucleotide binding	10	3572	0.0027	0.04

.

Table A. 7: List of genes annotated by MaizeCyc to participate in the biosynthesis of jasmonic acid.
Of the 50 genes included in the pathway, 13 were present in the mature and germinated pollen proteomes and 3 of these were found to significantly decrease in abundance with germination (FDR of 0.05). NC = No sig. change , SD = Sig. difference

Gene Accession	Reaction EC	Enzymatic activity	Change in Abundance (FC)
GRMZM2G070092	1.13.11.12	lipoxygenase7	
GRMZM2G117321	1.13.11.12	Lipoxygenase	
GRMZM2G312997	1.13.11.12	Peroxidase	
GRMZM2G102760	1.13.11.12	lipoxygenase5	NC (-3.1x)
GRMZM2G009479	1.13.11.12	Lipoxygenase	
GRMZM2G015419	1.13.11.12	Lipoxygenase	
GRMZM2G106748	1.13.11.12	Lipoxygenase	
GRMZM2G156861	1.13.11.12	lipoxygenase1	
GRMZM2G040095	1.13.11.12	LOX5; lipoxygenase	
GRMZM2G104843	1.13.11.12	Lipoxygenase	
GRMZM2G017616	1.13.11.12	lipoxygenase	
GRMZM5G822593	1.13.11.12	Lipoxygenase	
GRMZM2G109130	1.13.11.12	lipoxygenase3	
GRMZM2G109056	1.13.11.12	lipoxygenase4	SD (-3.1x)
GRMZM2G067225	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G033098	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G168404	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G002178	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G072653	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G376661	4.2.1.92	Hydroperoxide dehydratase	
GRMZM2G415793	5.3.99.6	Allene-oxide cyclase	NC (4.3x)
GRMZM2G077316	5.3.99.6	Allene-oxide cyclase	
GRMZM2G000236	1.3.1.42	12-oxophytodienoate reductase	
GRMZM2G106303	1.3.1.42	12-oxophytodienoate reductase	
GRMZM2G156712	1.3.1.42	12-oxophytodienoate reductase	
GRMZM2G082087	1.3.1.42	12-oxophytodienoate reductase	SD (-2X)

Table A.6: Continued

Gene Accession	Reaction EC	Enzymatic activity	Change in Abundance (FC)
GRMZM2G068947	1.3.1.42	12-oxophytodienoate reductase	
GRMZM2G087192	1.3.1.42	12-oxophytodienoate reductase	
GRMZM2G148281	1.3.1.42	12-oxophytodienoate reductase	NC (-1.4x)
GRMZM2G052389	1.3.3.6	Acyl-CoA oxidase	NC (-1.4x)
GRMZM2G002959	1.3.3.6	Acyl-CoA oxidase	
GRMZM2G099666	1.3.3.6	Acyl-CoA oxidase	
GRMZM2G419782	1.3.3.6	Acyl-CoA oxidase	
GRMZM5G862219	1.3.3.6	Acyl-CoA oxidase	
GRMZM2G014136	1.3.3.6	Acyl-CoA oxidase	SD (-6.6x)
GRMZM5G820287	1.3.3.6	Acyl-CoA oxidase	
GRMZM2G445791	1.3.3.6	Acyl-CoA oxidase	NC (-3.5x)
GRMZM2G181266	4.2.1.17	Enoyl-CoA hydratase 2	
GRMZM2G132903	4.2.1.17, 1.1.1.35	Enoyl-CoA hydratase 2	NC (1.7x)
GRMZM2G146885	4.2.1.17	Enoyl-CoA hydratase	
GRMZM2G117357	4.2.1.17	Enoyl-CoA hydratase	
GRMZM2G177404	4.2.1.17, 1.1.1.35	Long-chain-3-hydroxyacyl-CoA dehydrogenase	
GRMZM2G459755	4.2.1.17, 1.1.1.35	Long-chain-3-hydroxyacyl-CoA dehydrogenase	NC(-1.6x)
GRMZM2G398500	4.2.1.17	Enoyl-CoA hydratase 2	
GRMZM2G094655	4.2.1.17	Enoyl-CoA hydratase 2	
GRMZM5G854613	4.2.1.17, 1.1.1.35	Long-chain-3-hydroxyacyl-CoA dehydrogenase	NC (-1.6x)
GRMZM2G151087	4.2.1.17	Enoyl-CoA hydratase 2	
GRMZM2G033491	4.2.1.17	Enoyl-CoA hydratase 2	NC (-3.7x)
GRMZM2G106250	1.1.1.35	3-hydroxyacyl-CoA dehydrogenase	
GRMZM2G110201	2.3.1.16	Acetyl-CoA C-acyltransferase	NC (2.6x)

Table A. 7: Chi-square results from male transmission defect study.
Numbers reported represent the genotype information from offspring of reciprocal crosses of male and female parents heterozygous for the listed insertions with wild type testers. Progeny are expected to be heterozygous for the insertion or homozygous wild type.

Gene ID	Insertion Pop.	Insertion ID	Cross	With insertion	Without Insertion	Defect? (χ^2)
GRMZM2G173289	AcDs	I.S07.2815	Male	11	29	0.004
			Female	21	18	0.631
			2nd Male	21	26	0.465
			2nd Female	26	27	0.889
GRMZM2G133282	Mu-illumina	mu-illumina_226847.3	Male	12	24	0.045
			Female	20	20	1
GRMZM2G030265	AcDs	I.S06.0796	Male	8	22	0.011
			Female	15	17	0.724
GRMZM2G301647	Mu-illumina	mu-illumina_250175.3	Male	2	32	2.67E-07
			Female	13	16	0.577
GRMZM2G389462	UniformMu	mu1057062	Male	6	29	0.0001
			Female	17	14	0.59
GRMZM2G066024	UniformMu	mu1069861	Male	0	36	1.97E-09
			Female	22	14	0.182
GRMZM2G318992	UniformMu	mu1041512	Male	33	23	0.181
			Female	32	24	0.285

Table A.7: Continued

Gene ID	Insertion Pop.	Insertion ID	Cross	With insertion	Without Insertion	Defect? (χ^2)
GRMZM2G422641	UniformMu	mu1042381	Male	32	28	0.605
			Female	39	34	0.558
GRMZM2G017334	UniformMu	mu1047209	Male	19	10	0.095
			Female	19	16	0.612
GRMZM2G457370	UniformMu	mu1013491	Male	10	15	0.31
			Female	16	13	0.577
GRMZM2G156365	UniformMu	mu1044656	Male	17	2	0.51
			Female	28	7	0.0004
GRMZM2G102404	UniformMu	mu1008434	Male	18	13	0.369
			Female	17	12	0.353
		mu1045886	Male	19	14	0.384
			Female	17	12	0.353

Script A. 1: Python script developed to replaced bases in the B73 reference genome sequence with their W22 SNP counterpart.
Script written in collaboration with Taj Morton and the McGraw lab.

```
#!/usr/bin/python
import sys

sys.path.append("biopython/biopython-1.62b")
from Bio import SeqIO
from Bio.Seq import MutableSeq
from Bio.Alphabet import DNAAlphabet

# Need to use an iterator because the HapMap file is too large
# to load into memory all at once
class HapMapIterator:
    def __init__(self, filename):
        self.filename = filename
        self.snp_file = open(filename, "rU")
        self.snp_file.readline() # skip header

    def __iter__(self):
        return self

    def next(self):
        try:
            line = next(self.snp_file)
            line_parts = line.strip().split()

            if len(line_parts) < 13:
                print >> sys.stderr, "Error reading line '%s'. Not enough fields (expected 13,
got %d). Skipping." % (line, len(line_parts))
                return self.next()

            snp_info = {}
            snp_info['snp_name'] = line_parts[0]
            snp_info['alleles'] = line_parts[1]
            snp_info['chrom'] = line_parts[2]
            snp_info['position'] = int(line_parts[3]) - 1 # HapMap `pos` field appears to be
1-based
            snp_info['strand'] = line_parts[4]
            snp_info['b73_mz'] = line_parts[11]
            snp_info['w22_mz'] = line_parts[12]
            snp_info['is_indel'] = False if (snp_info['b73_mz'] in ('A','G','C','T', 'N') and
snp_info['w22_mz'] in ('A','G','C','T',)) else True

            return snp_info
```

Script A.1: Continued

```
        except StopIteration: # end of the file
            self.snp_file.close()
            raise StopIteration


def load_fasta_file(filename):
    with open(filename, "rU") as handle:
        record = SeqIO.read(handle, "fasta") # only expect a single FASTA record in
this file (the entire chromosome)
        if record is None:
            print >> sys.stderr, "Error opening FASTA file. Are you sure it contains only
1 record?"
            sys.exit(1)

        # create a sequence we can mutate:
        mut_seq = MutableSeq(record.seq.tostring(), DNAAlphabet())
        record.seq = mut_seq

        return record

    # if we didn't return by now, there was an error:
    print >> sys.stderr, "Error opening FASTA file. Are you sure it exists?"
    sys.exit(1)

def transform_genome(sequence, snps):
    for snp_info in snps:
        snp_name = snp_info['snp_name']
        if '-' in snp_info['alleles'] or '+' in snp_info['alleles'] or snp_info['is_indel']:
            continue

        if sequence[snp_info['position']] != snp_info['b73_mz']:
            print "Mismatch between B73:MZ SNP and ref sequence on %s (%s
vs %s)" % (snp_name, sequence[snp_info['position']], snp_info['b73_mz'])

        # apply SNP
        sequence[snp_info['position']] = snp_info['w22_mz']

    return sequence

if __name__ == "__main__":
    if len(sys.argv) != 4:
        print >> sys.stderr, "Usage: ./hapmap_snp.py [input.fa] [hapmap_file]
[output.fa]"
        sys.exit(1)
```

Script A.1: Continued

```
record = load_fasta_file(sys.argv[1])
#snps = parse_hapmap_file(sys.argv[2])
snps = HapMapIterator(sys.argv[2])

transform_genome(record.seq, snps)

with open(sys.argv[3], "w") as output_file:
    SeqIO.write(record, output_file, "fasta")
```

Table A. 8: The 50 genes with the highest number of B73 specific alleles (pollen derived) in the pollinated silk samples.

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G125635	5010	6	9493	4489	2044	461	2743	1160	2968	651	4059	1742
GRMZM5G830436	9603	7017	2602	16	331	144	995	808	469	219	1436	1186
GRMZM5G840582	336	104	937	705	136	11	862	737	229	11	1842	1624
GRMZM2G077197	12908	3047	9863	2	533	234	958	659	834	415	1410	991
GRMZM2G013619	3171	1703	1471	3	506	39	1033	566	805	67	1628	890
GRMZM2G352129	4722	1657	3851	786	1542	282	1538	278	2227	365	2291	429
GRMZM2G369939	2902	0	2961	59	571	0	822	251	796	0	1222	426
GRMZM2G113073	1397	75	1743	421	287	26	509	248	485	49	823	387
GRMZM2G156145	1995	107	2333	445	454	0	693	239	608	2	994	388
GRMZM2G104542	11402	542	11499	639	2177	108	2285	216	3089	176	3250	337
GRMZM2G421240	30	23	165	158	8	0	211	203	7	2	337	332
GRMZM2G173878	188	1	574	387	17	0	219	202	14	1	368	355
GRMZM2G390896	143	34	230	121	120	10	310	200	227	21	691	485
GRMZM2G018837	768	0	1878	1110	386	0	584	198	546	0	860	314
GRMZM2G165679	1271	892	379	0	260	0	455	195	366	3	615	252
GRMZM2G148411	221	0	1273	1052	56	0	240	184	86	0	288	202
GRMZM2G003762	516	0	1258	742	361	1	528	168	492	1	748	257
GRMZM2G065757	2561	1276	1290	5	93	19	239	165	128	33	342	247
GRMZM2G000264	2274	20	3999	1745	151	0	312	161	246	0	451	205
GRMZM2G314546	1409	6	2950	1547	506	0	650	144	767	1	980	214
GRMZM2G128914	418	49	892	523	77	14	207	144	115	30	314	229

Table A.8: Continued

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G041770	7654	4970	2893	209	1630	933	841	144	2431	1439	1192	200
AC210193.4_FG002	1779	320	2104	645	222	0	365	143	416	0	593	177
GRMZM2G104325	27132	6586	29002	8456	5925	3722	2343	140	9127	5828	3549	250
GRMZM5G872256	86	16	72	2	422	199	359	136	576	265	488	177
GRMZM2G004534	468	1	1052	585	23	0	145	122	36	0	208	172
GRMZM2G030305	481	397	84	0	20	0	142	122	27	1	238	212
GRMZM2G017414	195	55	142	2	127	15	230	118	177	39	329	191
GRMZM2G171559	19	4	87	72	11	0	125	114	34	0	217	183
GRMZM2G006042	1326	41	1769	484	300	16	394	110	415	23	508	116
GRMZM2G095895	26	0	85	59	48	0	158	110	96	5	361	270
GRMZM2G176397	4270	933	3568	231	835	182	761	108	1099	277	994	172
GRMZM2G009808	3506	1236	2349	79	335	114	327	106	499	175	494	170
GRMZM2G172664	4	0	68	64	16	1	121	106	14	0	327	313
GRMZM2G001327	11135	773	10776	414	3545	1181	2459	101	5233	1736	3606	117
GRMZM2G059965	1484	923	582	21	138	0	239	101	180	3	330	153
GRMZM2G407347	56	9	252	205	50	32	115	97	63	40	169	146
GRMZM2G139431	13	4	59	50	15	3	109	97	49	15	226	192
GRMZM2G335521	2408	471	1937	0	371	0	462	91	519	0	640	121
GRMZM5G841015	7	0	13	6	76	1	163	88	115	2	226	113
GRMZM2G069694	18	6	71	59	27	1	113	87	48	0	191	143
GRMZM2G177631	20849	4814	16384	349	1156	316	926	86	1827	446	1512	131

Table A.8: Continued

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G046952	61	0	307	246	415	0	500	85	566	1	682	117
GRMZM2G397242	49	0	99	50	91	0	173	82	199	1	402	204
GRMZM2G327357	74	0	115	41	148	0	230	82	392	0	578	186
GRMZM2G152862	23203	6594	17497	888	1388	360	1106	78	1962	512	1562	112
GRMZM2G115342	1669	29	2165	525	83	10	151	78	137	14	299	176
GRMZM2G154397	38976	14110	24908	42	7576	1817	5837	78	11089	2746	8479	136
GRMZM2G031053	1758	575	1201	18	454	145	390	77	624	151	599	125
GRMZM2G435338	809	557	253	1	50	23	104	77	100	42	176	118

Table A.9: The 50 genes with the highest number of W22 specific alleles (silk derived) in the pollinated silk samples.

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G168651	4637	2486	2151	0	19919	11651	8297	29	25646	17082	8609	45
GRMZM2G080603	54129	51573	2566	10	11325	9518	1845	38	15851	12962	2900	11
GRMZM2G146308	10012	3338	6674	0	11339	8404	2940	5	17367	13127	4249	9
GRMZM2G409726	26621	15681	10983	43	9697	5658	4113	74	14544	8579	6078	113
GRMZM2G136910	7186	14	25975	18803	4899	4199	702	2	7263	6193	1114	44
GRMZM2G333980	18471	7499	10987	15	5539	4362	1182	5	6925	5242	1692	9
GRMZM2G104325	27132	6586	29002	8456	5925	3722	2343	140	9127	5828	3549	250
GRMZM2G075255	42420	29521	12913	14	6181	3528	2654	1	9417	5414	4006	3
GRMZM2G116273	40098	17821	22308	31	8979	3300	5682	3	13001	4846	8159	4
GRMZM5G851266	114272	51072	63241	41	20581	2996	17588	3	30458	4421	26037	0
GRMZM5G822237	167	167	0	0	3159	2960	199	0	4410	4197	215	2
GRMZM2G165917	14248	7854	6396	2	4442	2780	1670	8	6732	4245	2491	4
GRMZM2G153292	146044	60074	86031	61	15629	2711	12944	26	23114	3897	19272	55
GRMZM2G382914	27509	26882	650	23	2763	2646	127	10	4084	3907	181	4
GRMZM2G159397	6564	1218	5346	0	5128	2590	2538	0	7637	3804	3833	0
GRMZM2G343543	30602	16656	13973	27	3971	2608	1372	9	5835	3744	2092	1
GRMZM2G094742	2688	1075	1624	11	2683	2340	350	7	4333	3820	526	13
GRMZM2G053299	20841	6059	14790	8	4052	2071	1982	1	5923	2983	2945	5
GRMZM2G153541	28623	7456	21173	6	6949	1935	5026	12	9735	2904	6835	4
GRMZM2G164229	1300	425	876	1	3096	1713	1388	5	5100	3100	2000	0
GRMZM2G079348	50284	17678	32615	9	11227	1824	9404	1	16668	2820	13848	0

Table A.9: Continued

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G079348	50284	17678	32615	9	11227	1824	9404	1	16668	2820	13848	0
GRMZM2G154397	38976	14110	24908	42	7576	1817	5837	78	11089	2746	8479	136
GRMZM2G049915	365	298	67	0	2674	1892	796	14	3797	2639	1158	0
GRMZM2G009465	3387	1896	1492	1	3147	1879	1285	17	4388	2631	1761	4
GRMZM2G026143	1322	1179	143	0	1824	1736	88	0	2757	2636	121	0
GRMZM2G152466	58555	2061	56510	16	5407	1607	3824	24	7949	2359	5641	51
GRMZM2G039639	228	207	21	0	1814	1636	179	1	2389	2098	294	3
GRMZM2G142705	8203	4540	3663	0	1958	1434	525	1	2989	2202	787	0
GRMZM2G122937	4379	1736	2645	2	8186	1459	6728	1	12091	2153	9940	2
GRMZM2G015295	25004	9843	15165	4	5784	1312	4485	13	8403	1973	6433	3
GRMZM2G457003	12369	9058	3315	4	1909	1352	558	1	2829	1908	922	1
GRMZM2G064302	9166	3647	5524	5	1427	1255	183	11	2129	1874	264	9
GRMZM2G056039	14366	8258	6206	98	2383	1215	1179	11	3616	1880	1738	2
GRMZM2G101875	9340	4912	4428	0	2686	1207	1482	3	3857	1821	2036	0
GRMZM2G151992	800	685	116	1	1508	1168	343	3	2355	1853	506	4
GRMZM2G701082	4011	283	3728	0	2196	1148	1050	2	3436	1859	1579	2
GRMZM2G155532	6197	1917	4280	0	2057	1225	832	0	3053	1748	1305	0
GRMZM2G044498	738	164	574	0	1766	1173	597	4	2805	1792	1013	0
GRMZM2G053206	12277	2746	9532	1	2828	1060	1771	3	4296	1898	2399	1
GRMZM2G012434	8336	5242	3096	2	1615	1131	485	1	2422	1810	616	4
GRMZM2G001327	11135	773	10776	414	3545	1181	2459	101	5233	1736	3606	117

Table A.9: Continued

	W22silk				W22B73				W22B73 Second Run			
	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific	W22	W22 specific	B73	B73 specific
GRMZM2G092447	16145	4805	11357	17	2772	1183	1605	16	3905	1668	2260	23
GRMZM2G310431	613	434	180	1	1803	1131	672	0	2680	1690	991	1
GRMZM2G048324	9686	2953	6734	1	4095	1132	2963	0	5764	1667	4097	0
GRMZM2G128491	6099	714	5395	10	1858	1039	828	9	3066	1628	1462	24
GRMZM2G032807	880	119	769	8	1919	1104	815	0	2744	1522	1222	0
GRMZM5G858094	13231	5323	7913	5	2690	1020	1673	3	3999	1589	2410	0
GRMZM2G181028	9644	2256	7390	2	2668	1080	1590	2	3863	1525	2339	1
GRMZM2G104876	1637	0	1736	99	3084	1046	2038	0	4607	1557	3052	2
GRMZM2G367834	1965	1206	761	2	1149	987	162	0	1839	1570	269	0