Coupling Gamete Fusion with Sperm Cell Delivery Maximizes Reproductive Success in Flowering Plants

By

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This thesis by Kristin M. Beale is accepted in its present form by the Department of Molecular Biology, Cellular Biology, and Biochemistry as satisfying the thesis requirements for the degree of Doctor of Philosophy

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- Optimization of microsomal membrane preparations to quantify HAP2 protein accumulation in sperm cell membranes using Western Blot analysis.
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Table of Contents

List of Tables

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 1: Introduction

"Speed Dating, Rejection, and Finding the Perfect Mate: Advice from *Arabidosis thaliana*"

Beale, KM and Johnson, MA. Current opinions in Plant Biology. Invited Review. May 2013.

This manuscript was prepared for submission to Current Opinions in Plant Biology as an invited review. I present this review as the introduction to my thesis with modifications to the "conclusions and perspectives" section. I wrote this manuscript independently with comments from MAJ.

Abstract

Angiosperm pollen tubes extend through pistil tissue to deliver a pair of immotile sperm to female gametes for double fertilization. The extended journey of the pollen tube requires extensive cell:cell interactions that guide the pollen tube to its destination and instruct it to stop growing and burst. Once sperm are released the molecular exchanges between male and female continue, resulting in sperm activation and gamete fusion. Finally, there is evidence that gamete fusion can feedback on the pollen tube attraction mechanism so that additional pollen tubes can be attracted only if the first pair of sperm fail to fertilize. We review progress toward defining the molecules mediating each of these exchanges and find that small cysteine-rich proteins are a major mode of cellular communication.

Double Fertilization: Speed dating for flowering plants

Angiosperms have evolved a sexual reproduction system that requires multiple cell-cell interactions between the male gametophyte (Box 1), and the pistil, ovule, and female gametophyte (Box 1). The modes of molecular exchange between male and female are beginning to be revealed and it is becoming clear that this complex series of interactions facilitates optimum reproductive success by ensuring that all female gametes are paired with functional male gametes. Angiosperm reproduction resembles a human 'speed dating' event where a limited number of seated females attract individual males for an interview. The male must perceive the attractive cue and respond; if the interview goes well a successful union forms. On the other hand, if the interview goes poorly, the male is rejected and another suitor is considered. Given enough males and enough opportunity for scrutiny, 'speed dating', will result in suitable match for each of the females in the room. We discuss recent progress toward understanding the molecular exchanges between male and female cells in Angiosperms and focus mainly on Zea mays (maize, monocot) and Arabidopsis thaliana (Arabidopsis, dicot), two self-compatible model species representing Angiosperm diversity.

Attracting Mr. Right: Diverse small proteins mediate pollen tube chemotaxis

Flowering plant sperm cells are immotile and develop within pollen grains. They are transported to the female gametophyte by an extension of the pollen cell called a pollen tube (PT, Figure 1A). It is likely that multiple attractive cues guide the lengthy journey (~2mm in Arabidopsis; ~180mm in maize) of the PT as it interacts with several cell types along the stigma, style, and ovary (Dresselhaus et al., 2011; Johnson and Preuss, 2002) (Box 1). Chemocyanin, a small cysteine-rich peptide (CRP) purified from lily stigma and style, is a PT attractant that functions early in the process, attracting germinating PTs into the lily style (Kim et al., 2003).

PTs of both monocots and dicots complete PT guidance by following a gradient of small proteins secreted by the female gametophyte (Figure 1A). Laser ablation studies in the dicot *Torenia fournieri* (Torenia) established that synergid cells are the source of the attractant (Higashiyama et al., 2001) and genetic and genomic studies in Arabidopsis showed synergids express unique genes critical for PT attraction (Johnston et al., 2007; Jones-Rhoades et al., 2007; Kasahara et al., 2005; Shimizu and Okada, 2000). LUREs were identified as micropylar attractants in Torenia and are encoded by highly abundant and synergid-specific transcripts (Okuda et al., 2009). The two LURE proteins analyzed have six cysteine residues at conserved positions within the predicted mature protein of ~60-70 amino acids and have features typical of Defensin-like proteins (Okuda et

al., 2009). Recombinant LURE1 and LURE2 attract Torenia PTs in vitro; but they fail to attract PTs from the closely related genus Lindernia (Okuda et al., 2009). This finding is consistent with prior work showing that PT attraction is species preferential (Higashiyama et al., 2006).

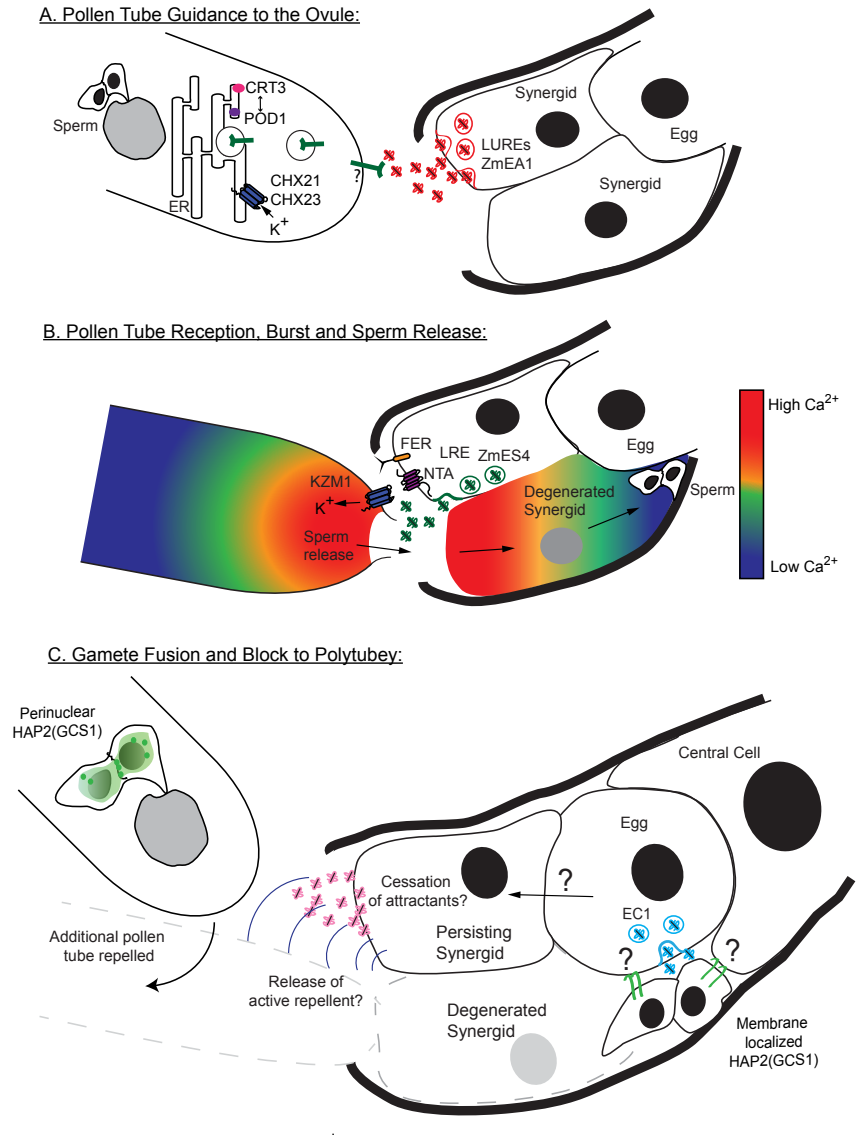

Figure 1. Double fertilization requires coordinated cell:cell communication to maximize reproductive success. (A) Pollen tubes are attracted into the micropyle by Defensin-like LURE CRPs (Arabidopsis/Torenia) or ZmEA1 (maize,

both are red). The pollen tube ER protein POD1 (purple) interacts with the ER membrane folding protein CRT3 (pink) may be involved in folding of the unknown receptor (?, Green) which is drawn here as a transmembrane protein transiting the secretory system. The K^+ channels, CHX21 and CHX23 (blue) may be required for cation balance during PT guidance, as double mutants produce PTs with a micropylar guidance defect. **(B)** Pollen tube reception is accompanied by a Ca2+ spike in the PT as it approaches the ovule; a Ca2+ spike occurs in the synergid as the PT bursts. FER, NTA, and LORELEI (LRE) are expressed in synergid cells and are required for initiating PT burst. In Maize, ZmES4 (green) is secreted from vesicles during PT reception and is required for PT burst. ZmES4 triggers the K+ channel, KZM1 (blue, at PT tip). **(C)** Sperm cells that have been deposited at the site of gamete fusion show a re-localization of HAP2(GCS1) (green) to the sperm cell membranes in response to EC1 (light blue) secretion from the egg cell. HAP2(GCS1) is perinuclear in the PT. Question marks at egg and central cell indicate unknown proteins potentially involved in gamete fusion. Two mechanisms are considered for the block to polytubey 1) cessation of attractants (pink) and 2) production of PT repellents (blue lines).

The recent discovery of Arabidopsis LURE (AtLURE) proteins suggests that this type of CRP might be used broadly by dicots to attract PTs into the ovule (Takeuchi and Higashiyama, 2012). There are over 800 CRPs encoded by the Arabidopsis genome; more than 300 are Defensin-like (Silverstein et al., 2007). Six AtLUREs (one a pseudogene) were found in a unique subclade that could be divided into distinct Arabidopsis thaliana and Arabidopsis lyrata groups, consistent with the hypothesis that the PT attraction system is species-preferential (Takeuchi and Higashiyama, 2012). Immunofluorescence experiments provided evidence that LUREs are secreted from the synergids and onto the funiculus, the structure that bridges the ovary and ovule (Takeuchi and Higashiyama, 2012). These exciting data suggest that LUREs may have long-range activity, perhaps beckoning PTs to turn out of the transmitting tract and attracting them into the micropyle (Figure 1A). It will be difficult to test this

genetically because AtLUREs are clustered in the genome, however evidence from RNAi experiments reducing LURE expression suggest they are required only for the final step in PT guidance, entering the micropyle (Takeuchi and Higashiyama, 2012).

ZmEA1, a small (ZmEA1) peptide that is not a CRP, was identified as a micropylar attractant in maize (Marton et al., 2005). Reduction of ZmEA1 expression resulted in micropylar guidance defects very similar to those observed in Arabidopsis (Takeuchi and Higashiyama, 2012) or Torenia (Okuda et al., 2009) suggesting ZmEA1 is the micropylar attractant in Maize. It appears that maize uses a completely different type of small peptide to attract pollen tubes into the micropyle than Arabidopsis and Torenia. This conclusion was underscored when it was shown that expression of ZmEA1 from the Arabidopsis synergid was sufficient to attract maize PTs to an Arabidopsis ovule in vitro (Marton et al., 2012). These recent data warrant broad comparative analysis of PT attractants to determine the forces that have shaped evolution of multiple PT attraction systems.

Extensive genetic analysis of the male gametophyte has yet to yield a potential PT receptor for micropylar guidance cues. *pod1* and *chx21,chx23* PT mutants (Li et al., 2011; Lu et al., 2011) have very specific micropylar guidance defects that phenocopy loss of pollen tube attraction by female gametophytes (Takeuchi and

Higashiyama, 2012). The proteins identified by these mutations may regulate receptor function by chaperoning it through the ER (POD1) or modifying cation balance within the ER (CHX), but are not likely to bind attractants (Li et al., 2011; Lu et al., 2011). The recent discovery of ZmEA1 and LUREs should facilitate identification of their PT receptors, which in turn will allow understanding of how attractant:receptor binding interfaces with the well-described intracellular signaling apparatus that defines the PT tip and controls directionality of tube extension (Cheung and Wu, 2008).

The interview begins: PT reception results in sperm release and requires signaling between gametophytes

Analysis of female gametophyte mutants in Arabidopsis defined a potentially complex signaling system called PT reception (Box 1) that orchestrates recognition of PT arrival, cessation of PT growth, PT burst, and degeneration of one of the two synergid cells (Kessler and Grossniklaus, 2011). When wild-type PTs enter mutant ovules, instead of stopping immediately (Hamamura et al., 2012; Palanivelu and Preuss, 2006) they continue to extend without bursting and form extensive coils within the mutant female gametophyte (Huck et al., 2003; Rotman et al., 2008) (Figure 1B). *FERONIA* (*FER*) encodes a receptor-like kinase that is abundant at the synergid surface proximal to the PT entry point (filiform apparatus) and has an extracellular carbohydrate-binding domain, suggesting that its ligand could be in the cell wall of the PT or a female gametophyte cell (Boisson-Dernier et al., 2008; Cheung and Wu, 2011; Escobar-

Restrepo et al., 2007). *nortia* (*nta*) female gametophytes have an identical, though less penetrant phenotype (Kessler et al., 2010). Interestingly, NTA (mildew resistance locus O-like protein) relocalizes to the filiform apparatus upon PT arrival, but molecular exchange between male and female gametophytes is blocked in *fer* mutants (Figure 1B). It is not yet known whether FER/NTA sense PT arrival and, if they do, how recognition of PT arrival is converted to another signal that triggers PT burst. Insight could come by studying interactions between FER/NTA and other potential signaling molecules like LORELEI (Capron et al., 2008; Tsukamoto et al., 2010) (Figure 1B). PT reception is also species preferential; further highlighting the level of scrutiny that is being applied to potential mates. For example, experiments analyzing interspecific crosses with either Arabidopsis (Escobar-Restrepo et al., 2007) or Rhodedendron (Williams et al., 1986) reveal that PTs from closely related species that manage to target ovules continue to grow in the female gametophyte without releasing sperm.

Male counterparts to FER signaling have not been identified in Arabidopsis (Figure 1B). However, pollen grains and tubes express a pair of FER paralogs called ANXUR1(ANX1) and 2(ANX2) (Boisson-Dernier et al., 2009; Miyazaki et al., 2009); double loss of function results in PTs that rupture as they germinate in vitro and that fail to reach ovules in the pistil. This early PT growth phenotype suggests a role for this type of receptor-like kinase (*Catharanthus roseus* RLK1-like), CrRLK1L (Amien et al., 2010; Iwano et al., 2012) in regulating cellular integrity, which may provide insight into FER function in the synergid cell. Further

insight into how Arabidopsis gametophytes signal their presence to each other came from live imaging experiments of pollen tubes and synergids expressing a GFP-based Ca^{2+} sensor that indicated that spikes in Ca^{2+} mark contact between the gametophytes (Iwano et al., 2012) (Figure 1B).

CrRLK1Ls are deeply conserved in plants (Boisson-Dernier et al., 2009), however, there isn't yet evidence in other species that they function in PT reception and studies in maize highlight the role of yet another small family of Defensin-like CRPs, ZmES1-4, which are secreted from the synergid cells and were shown to activate a PT expressed K^+ channel KZM1 (Amien et al., 2010) (Figure 1B). Reduction of ZmES4 expression via RNAi led to an apparent maize phenocopy of the *fer* phenotype discussed above. Moreover, synthetic ZmES4 peptides triggered rapid rupture of maize PTs in vitro, but slower rupture of closely related *Tripsacum dactyloides* PTs. These results suggest direct molecular communication between signaling peptides secreted by the female gametophyte and a channel on the PT potentially mediating PT burst. It will be very interesting to see whether this molecular dialogue is part of the pollen tube reception pathway emerging from genetic studies in Arabidopsis (Figure 1B).

Is he the one, or is it time to move on? Gamete fusion and attraction of multiple PTs

Live-imaging of gamete interactions in Arabidopsis revealed that PT burst propels the sperm pair to a site in the female gametophyte where one can fuse with the egg and the other with the central cell (Hamamura et al., 2011) (Figure 1B and Box1). These two plasma membrane fusion events, which initiate embryo and endosperm development and are essential for seed formation, happen about eight minutes after PT burst, which occurs almost immediately after PT arrival (Hamamura et al., 2011). HAP2(GCS1) (herein referred to as HAP2) is a sperm-specific membrane protein of unknown function that is absolutely essential for both of the these gamete fusion events (Beale et al., 2012; Hamamura et al., 2011; Mori et al., 2006; von Besser et al., 2006). *hap2* sperm pairs are propelled to the site where they should fuse, but because plasma membrane fusion does not occur, sperm nuclei cannot migrate to fuse with either the egg or central cell nucleus (Hamamura et al., 2011) . In contrast to many of the unique features of the flowering plant sexual reproduction system, HAP2 appears commonplace; it is conserved broadly across eukaryotes (Wong and Johnson, 2010), and is essential for gamete fusion in such diverse organisms as the green alga *Chlamydomonas reinhardtii* (Liu et al., 2008) and the protozoan malaria parasite *Plasmodium berghei* (Liu et al., 2008; Mori et al., 2010).

Proteins that may participate with HAP2 in fusion with partner gametes have not been identified in any organism. However, it was recently demonstrated that still

another, distinct, family of closely related CRPs (EGG CELL 1, EC1) is critical for HAP2 function (Sprunck et al., 2012). When EC1 expression was significantly reduced in female gametophytes by combining insertion mutants in three EC1 genes with RNAi targeting the two others, wild-type sperm behaved exactly like *hap2* mutant sperm; they failed to fuse. Incubation of synthetic EC1 peptides with sperm expressing a HAP2:YFP fusion protein demonstrated that EC1 activates sperm by shifting the localization of HAP2 from the interior endomembrane system to the surface (Sprunck et al., 2012) (Figure 1C). These results show that before sperm can fuse with female gametes they must receive license from the female; these findings underscore the exquisite regulation of gamete fusion and demonstrate another intriguing molecular exchange between male and female.

In diverse Angiosperms including Arabidopsis (Shimizu and Okada, 2000) and maize (Lausser et al., 2010), wild-type ovules almost never attract more than one PT. Distribution of PTs in the pistil such that each ovule receives only one is advantageous because it can maximize ovule fertilization and minimize polyspermy (Box 1). Analysis of Arabidopsis PT attraction in vitro showed that late-arriving PTs are repelled from the micropyle of a previously targeted ovule, consistent with the hypothesis that an active mechanism prevents multiple PTs from entering an ovule (Palanivelu and Preuss, 2006). In contrast to wild type, Arabidopsis female gametophytes defective in pollen tube reception frequently attract multiple PTs (Boisson-Dernier et al., 2008; Capron et al., 2008; Huck et al., 2003; Tsukamoto et al., 2010). These mutants are apparently unable to

prevent polytubey (Box 1) and suggest that PT burst or a later step in gamete:gamete interactions triggers the mechanism that wards off late arriving PTs. Analysis of *hap2* (Beale et al., 2012; Kasahara et al., 2012; Mori et al., 2006) and *ec1* (Sprunck et al., 2012) shows that when gamete fusion fails, multiple sperm pairs are deposited within ovules. These results suggest a sophisticated system that ensures successful gamete fusion before PT attraction is blocked. Hand pollination with equal amounts of two populations of PTs expressing different fluorescent proteins unambiguously indentifies polytubey because both fluorescent proteins are readily detectable in the female gametophyte (Beale et al., 2012). Using this assay, polytubey was only detected in 1% of Arabidopsis ovules, but this rate went up 10 fold when 1/4 of the PTs carried defective *hap2* sperm (Beale et al., 2012). Analysis of other mutants with defective sperm showed that in all cases, multiple PTs are attracted to ovules (Beale et al., 2012; Kasahara et al., 2012). These results indicate that gamete fusion is required to trigger the block to polytubey (Box 1).

Fertilization recovery (Box 1) (Kasahara et al., 2012) , the ability of a female gametophyte to attract an additional PT if the sperm released by the first PT fail to fertilize, is also expected to enhance reproductive success. Direct analysis of sperm nuclei showed that when defective sperm are deposited, wild-type sperm delivered by a second PT can fuse with female gametes and produce viable seeds (Beale et al., 2012; Kasahara et al., 2012). Analysis of synergid degeneration in ovules that had attracted defective sperm showed that the

remaining synergid (one of two synergids degenerates upon arrival of the first PT (Faure et al., 2002) persists hours longer than when gamete fusion is successful (Beale et al., 2012). It was proposed that this remaining synergid can continue to attract PTs until gamete fusion is successful and as many as four pairs of sperm were observed (Beale et al., 2012). These findings contrasted with analysis of an in vitro system that found a maximum of two PTs, one for each synergid cell (Kasahara et al., 2012). It will be interesting to determine whether, in the absence of successful gamete fusion, LURE expression from the remaining synergid cell can attract multiple PTs into the female gametophyte.

How are late-arriving PTs prevented from entering an already targeted ovule? Since gamete fusion occurs within eight minutes of PT entry (Hamamura et al., 2011), it could trigger a rapid mechanism that prevents other PTs from entering (Figure 1C). The low number of polytubey events observed could then be explained by rare cases when two PTs arrived contemporaneously at one micropyle. Several potential mechanisms for this fast block to polytubey can be imagined. Gamete fusion could trigger cessation of LURE expression and rapid LURE deactivation, rendering the ovule unable to attract PTs even when the remaining synergid has yet to degenerate (Figure 1C). Alternatively, a PT repellent could be released upon gamete fusion (Figure 1C). Axons, which extend by tip-growth like PTs, have well characterized negative growth responses to repulsive proteins during development of the nervous system (Evans and Bashaw, 2010). However, the kinetics of this type of system are not

likely to be fast enough to achieve a rapid block to polytubey. Nitric oxide is an attractive candidate molecule because its release and diffusion could be rapid and because it has been shown to be produced by ovules and to repel PTs in vitro (Marshall et al., 2011; Prado et al., 2008; Prado et al., 2004).

Conclusions and Perspectives

The molecules exchanged between the PT, pistil, and female gametophyte that coordinate PT growth and guidance, PT reception, and double fertilization are just beginning to be catalogued. Like amorous words used in speed dating, small CRPs are the major unit of molecular communication for Angiosperm reproduction. We have discussed distinct CRPs secreted by the pistil to direct early pollen tube extension (Kim et al., 2003), by the female gametophyte to attract pollen tubes into the micropyle (Okuda et al., 2009), or to stop growing and burst (Amien et al., 2010) (Figure 1). CRPs are also secreted by the egg cell to activate sperm cells for gamete fusion (Sprunck et al., 2012). Many more examples have been described (Marshall et al., 2011), however, a challenge for future work will be to explore the PT's CRP lexicon. LAT52 is a pollen-expressed CRP promoting PT extension in tomato (Tang et al., 2002) and SCR/SP11 is a pollen coat CRP that defines pollen identity in self-incompatible Brassica species (Schopfer et al., 1999; Takayama et al., 2000) but PT CRPs signaling to female cells have not been defined. CRPs are dense with information that can define

self v. non-self (Kachroo et al., 2001) and potentially species v. non-species during pollen tube attraction and reception (Figure 1). One of the most pressing goals is to discover how each distinct CRP-type is recognized and how recognition is transduced into cellular responses such as changes in PT direction, PT rupture, and activation of sperm for gamete fusion.

Novel insights into *HAP2*-mediated gamete fusion

The following thesis chapters focus on providing novel insights into flowering plant reproduction, specifically focusing on *HAP2* mechanisms for gamete fusion. We have shown for the first time that *HAP2*-mediated gamete fusion is required for the regulation of sperm cell delivery in Arabidopsis (Chapter 2). This mechanism prevents multiple pollen tubes from targeting a single ovule and ensures that only the two sperm cells required for double fertilization are deposited. We found that when defective *hap2* sperm are delivered to ovules, this block to polytubey fails and additional pollen tubes are attracted to the same ovule (Beale et al., 2012). We also show for the first time that an ovule is capable of attracting as many as four pollen tubes, and that synergid cells persist longer in ovules that have received non-functional sperm. This observation suggests that the second synergid cell continues to secrete attractants in an effort to attract additional pollen tubes that might be carrying functional sperm. Only after successful gamete fusion has occurred is the block to polytubey created,

revealing that pollen tube burst alone is not sufficient for this block as previously described. By coupling the regulation of sperm cell delivery to successful gamete fusion, flowering plants have evolved an elegant mechanism for evenly distributing pollen tubes within the transmitting tract and ensuring that every ovule receives functional sperm capable of gamete fusion, essentially increasing seed production.

Although it has been shown that *HAP2* is required for this successful gamete fusion in Arabidopsis, as well as in several eukaryotes (Blagborough and Sinden, 2009; Liu et al., 2008; Mori et al., 2006; von Besser et al., 2006), it is currently unknown whether *HAP2* is sufficient for driving gamete fusion. In an effort to test this we expressed *HAP2* in *duo1* mutant sperm (Thesis Chapter 3). *DUO1* encodes a MYB transcription factor that controls sperm development and function and as a result, *duo1* mutant sperm fail to undergo the final sperm cell division; producing pollen grains that contain a single sperm-like cell instead of two distinct sperm cells (Rotman et al., 2005). Importantly, these *duo1* mutant sperm lack expression of ~63 sperm expressed genes, including *HAP2*, and are incapable of gamete fusion (Brownfield et al., 2009). We show for the first time that when only *HAP2* is added back to these single sperm we see a rescue of the fusion defect. Analysis of fertilization events two days post-pollination revealed that although gamete fusion was rescued in these *duo1* sperm expressing *HAP2,* early development was halted at, or shortly after karyogamy. This suggests that *DUO1* may regulate sperm-expressed genes that are required for initiation of

early embryo and endosperm development. The ability of *HAP2* to rescue the fusion defect of *duo1* mutant sperm indicates that *HAP2* is the only *DUO1*-regulated gene required for gamete fusion, however, it is possible that other non-*DUO1*-regulated genes are also required for *HAP2* function.

It is also currently unknown whether HAP2 interacts with female cell binding partners, or whether HAP2 requires additional male components that might not be regulated by *DUO1* for gamete fusion. In an effort to identify novel components of the *HAP2* fusion mechanism, we have designed and implemented a novel forward genetic modifier screen using a hypomorphic version of HAP2 that is capable of fusion at a low rate (Thesis chapter 4). This hypomorph produces only 2-6 seeds/silique compared to ~50 seeds/silique in wild type plants. By screening for suppressors of this small seed set phenotype, we have been able to characterize 6 mutations in genes that might encode for proteins that interact with HAP2 to initiate gamete fusion. Interestingly, one of these genes is a mutation in a female-expressed gene and four are within male-expressed genes. We have also identified one mutation that requires expression in both the male and female to increase fertilization. These data suggest HAP2 is interacting with some female-expressed factor and may also require additional sperm components for fusion. Identification of these novel genes will provide new insight into how *HAP2* is mediating gamete fusion.

Finally, we provide insight into components of the HAP2 sequence and structure that might contribute to fusion function (Chapter 5). We identified a highly conserved region within the HAP2 N-terminal region, the HAP2/GCS1 domain (HGD) and mutated specific amino acids within this region to test for effects of fusion ability. We find that theses amino acids are not required for the fusion function of HAP2, however, using a bioinformatics approach we have identified additional regions outside of the HGD that might be required. Interestingly, a region located N-terminal to the HGD is most structurally similar to the structure of known viral fusion proteins (Bressanelli et al., 2004; Rey et al., 1995). This structural comparison and analysis of sequence within the HGD suggest that fusion function might lie outside of the most highly conserved domain, and that the HGD may be required for some level of species specificity.

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Glossary Box 1: Definitions

Male Gametophyte: Develops in the anther following meiosis and is released for pollination. Comprises three haploid cells, two immotile sperm develop within the pollen cytoplasm. PT nucleus and two sperm cells migrate as a unit toward the tip of the extending PT.

Female Gametophyte: Develops in the ovule following meiosis. In the polygonum type (e.g. Arabidopsis, Maize), three mitotic divisions produce 8 haploid nuclei, which cellularize to form the egg cell, two synergid cells, the central cell (diploid, fusion of two polar nuclei), and three antipodal cells.

PT Attraction: Directed growth of PTs up a gradient of chemoattractants secreted by female cells.

PT Reception: PT growth arrest and burst within the synergid cell leading to PT burst, sperm cell release, and degeneration of one synergid cell.

Gamete Fusion: Plasma membrane fusion of gametes. Double fertilization occurs when the plasma membrane of one sperm cell fuses with the egg cell and the plasma membrane of another sperm cell fuses with the central cell.

Block to Polytubey: Prevention of multiple PTs from entering a single ovule. Polytubey is also known as polysiphonogamy.

Fertilization Recovery: The attraction of an additional PT when the first to enter the ovule does not result in gamete fusion

Block to Polyspermy: Prevention of multiple sperm cells from fusing with a single egg or central cell. Polyspermy can lead to embryo abortion and endosperm development defects.

References

Amien, S., et al. (2010). Defensin-like ZmES4 mediates pollen tube burst in maize via opening of the potassium channel KZM1. PLoS biology 8:e1000388.

Beale, K.M., Leydon, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule. Current biology : CB 22:1090-1094.

Blagborough, A.M., and Sinden, R.E. (2009). Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187-5194.

Boisson-Dernier, A., Frietsch, S., Kim, T.H., Dizon, M.B., and Schroeder, J.I. (2008). The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. Current biology : CB 18:63-68.

Boisson-Dernier, A., et al. (2009). Disruption of the pollen-expressed FERONIA homologs ANXUR1 and ANXUR2 triggers pollen tube discharge. Development 136:3279-3288.

Bressanelli, S., et al. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. The EMBO journal 23:728-738.

Brownfield, L., Hafidh, S., Borg, M., Sidorova, A., Mori, T., and Twell, D. (2009). A plant germline-specific integrator of sperm specification and cell cycle progression. PLoS genetics 5:e1000430.

Capron, A., et al. (2008). Maternal control of male-gamete delivery in Arabidopsis involves a putative GPI-anchored protein encoded by the LORELEI gene. The Plant cell 20:3038-3049.

Cheung, A.Y., and Wu, H.M. (2008). Structural and signaling networks for the polar cell growth machinery in pollen tubes. Annual review of plant biology 59:547-572.

Cheung, A.Y., and Wu, H.M. (2011). THESEUS 1, FERONIA and relatives: a family of cell wall-sensing receptor kinases? Current opinion in plant biology 14:632-641.

Dresselhaus, T., Lausser, A., and Marton, M.L. (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.

Escobar-Restrepo, J.M., et al. (2007). The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. Science (New York, N.Y.) 317:656-660.

Evans, T.A., and Bashaw, G.J. (2010). Axon guidance at the midline: of mice and flies. Curr Opin Neurobiol 20:79-85.

Faure, J.E., Rotman, N., Fortune, P., and Dumas, C. (2002). Fertilization in Arabidopsis thaliana wild type: developmental stages and time course. The Plant journal : for cell and molecular biology 30:481-488.

Hamamura, Y., Nagahara, S., and Higashiyama, T. (2012). Double fertilization on the move. Current opinion in plant biology 15:70-77.

Hamamura, Y., et al. (2011). Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in Arabidopsis thaliana. Curr Biol 21:497-502.

Higashiyama, T., et al. (2006). Species preferentiality of the pollen tube attractant derived from the synergid cell of Torenia fournieri. Plant Physiol 142:481-491.

Higashiyama, T., et al. (2001). Pollen tube attraction by the synergid cell. Science (New York, N.Y.) 293:1480-1483.

Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development (Cambridge, England) 130:2149-2159.

Iwano, M., et al. (2012). Cytoplasmic Ca2+ changes dynamically during the interaction of the pollen tube with synergid cells. Development (Cambridge, England) 139:4202-4209.

Johnson, M.A., and Preuss, D. (2002). Plotting a course: multiple signals guide pollen tubes to their targets. Developmental cell 2:273-281.

Johnston, A.J., et al. (2007). Genetic subtraction profiling identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. Genome biology 8:R204.

Jones-Rhoades, M.W., Borevitz, J.O., and Preuss, D. (2007). Genome-wide expression profiling of the Arabidopsis female gametophyte identifies families of small, secreted proteins. PLoS genetics 3:1848-1861.

Kachroo, A., Schopfer, C.R., Nasrallah, M.E., and Nasrallah, J.B. (2001). Allele-specific receptor-ligand interactions in Brassica self- incompatibility. Science (New York, N.Y.) 293:1824-1826.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in Arabidopsis. Current biology : CB 22:1084-1089.

Kasahara, R.D., Portereiko, M.F., Sandaklie-Nikolova, L., Rabiger, D.S., and Drews, G.N. (2005). MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. The Plant cell 17:2981-2992.

Kessler, S.A., and Grossniklaus, U. (2011). She's the boss: signaling in pollen tube reception. Curr Opin Plant Biol.

Kessler, S.A., et al. (2010). Conserved molecular components for pollen tube reception and fungal invasion. Science (New York, N.Y.) 330:968-971.

Kim, S., Mollet, J.C., Dong, J., Zhang, K., Park, S.Y., and Lord, E.M. (2003). Chemocyanin, a small basic protein from the lily stigma, induces pollen tube chemotropism. Proceedings of the National Academy of Sciences of the United States of America 100:16125-16130.

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

Li, H.J., et al. (2011). POD1 regulates pollen tube guidance in response to micropylar female signaling and acts in early embryo patterning in Arabidopsis. The Plant cell 23:3288-3302.
Liu, Y., et al. (2008). The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes & development 22:1051-1068.
Lu, Y., et al. (2011). Pollen tubes lacking a pair of K+ transporters fail to target ovules in Arabidopsis. The Plant cell 23:81-93.
Marshall, E., Costa, L.M., and Gutierrez-Marcos, J. (2011). Cysteine-rich peptides (CRPs) mediate diverse aspects of cell-cell communication in plant reproduction and development. J Exp Bot 62:1677-1686.
Marton, M.L., Cordts, S., Broadhvest, J., and Dresselhaus, T. (2005). Micropylar pollen tube guidance by egg apparatus 1 of maize. Science (New York, N.Y.) 307:573-576.
Marton, M.L., Fastner, A., Uebler, S., and Dresselhaus, T. (2012). Overcoming hybridization barriers by the secretion of the maize pollen tube attractant ZmEA1 from Arabidopsis ovules. Curr Biol 22:1194-1198.
Miyazaki, S., et al. (2009). ANXUR1 and 2, sister genes to FERONIA/SIRENE, are male factors for coordinated fertilization. Current biology : CB 19:1327-1331.
Mori, T., Hirai, M., Kuroiwa, T., and Miyagishima, S.Y. (2010). The functional domain of GCS1-based gamete fusion resides in the amino terminus in plant and parasite species. PloS one 5:e15957.
Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.
Okuda, S., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357-361.
Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro. BMC plant biology 6:7.
Prado, A.M., Colaco, R., Moreno, N., Silva, A.C., and Feijo, J.A. (2008). Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signaling. Molecular plant 1:703-714.
Prado, A.M., Porterfield, D.M., and Feijo, J.A. (2004). Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development (Cambridge, England) 131:2707-2714.
Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., and Harrison, S.C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375:291-298.
Rotman, N., et al. (2005). A novel class of MYB factors controls sperm-cell formation in plants. Current biology : CB 15:244-248.
Rotman, N., Gourgues, M., Guitton, A.E., Faure, J.E., and Berger, F. (2008). A dialogue between the SIRENE pathway in synergids and the fertilization independent seed pathway in the central cell controls male gamete release during double fertilization in Arabidopsis. Molecular plant 1:659-666.

Schopfer, C.R., Nasrallah, M.E., and Nasrallah, J.B. (1999). The male determinant of self-incompatibility in Brassica. Science (New York, N.Y.) 286:1697-1700.

Shimizu, K.K., and Okada, K. (2000). Attractive and repulsive interactions between female and male gametophytes in Arabidopsis pollen tube guidance. Development (Cambridge, England) 127:4511-4518.

Silverstein, K.A., et al. (2007). Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. Plant J 51:262-280.

Sprunck, S., Rademacher, S., Vogler, F., Gheyselinck, J., Grossniklaus, U., and Dresselhaus, T. (2012). Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science (New York, N.Y.) 338:1093-1097.

Takayama, S., et al. (2000). The pollen determinant of self-incompatibility in Brassica campestris. Proceedings of the National Academy of Sciences of the United States of America 97:1920-1925.

Takeuchi, H., and Higashiyama, T. (2012). A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis. PLoS biology 10:e1001449.

Tang, W., Ezcurra, I., Muschietti, J., and McCormick, S. (2002). A cysteine-rich extracellular protein, LAT52, interacts with the extracellular domain of the pollen receptor kinase LePRK2. The Plant cell 14:2277-2287.

Tsukamoto, T., Qin, Y., Huang, Y., Dunatunga, D., and Palanivelu, R. (2010). A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development. The Plant journal : for cell and molecular biology 62:571-588.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.

Williams, E.G., Kaul, V., Rouse, J.L., and Palser, B.F. (1986). Overgrowth of Pollen Tubes in Embryo Sacs of Rhododendron Following Interspecific Pollinations. Aust J Bot 34:413-423.

Wong, J.L., and Johnson, M.A. (2010). Is HAP2-GCS1 an ancestral gamete fusogen? Trends in cell biology 20:134-141.

Chapter 2: Gamete Fusion Prevents Multiple Pollen Tubes from Entering a Single Arabidopsis Ovule

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This manuscript was submitted in a Letter format. I performed all experiments for this manuscript except for Analine blue staining of pollen tubes in *duo1-3/+* mutants (Figure 1B, ARL). ARL also helped with preliminary quantification of polytubey events and editing of the manuscript.

Summary

In double fertilization, a reproductive system unique to flowering plants, two immotile sperm are delivered to an ovule by a pollen tube. One sperm fuses with the egg to generate a zygote, the other with the central cell to produce endosperm(Berger et al., 2008). A mechanism preventing multiple pollen tubes from entering an ovule would ensure that only two sperm are delivered to female gametes. We use live-cell imaging(Berger et al., 2008; Hamamura et al., 2011) and a novel mixed-pollination assay that can detect multiple pollen tubes and multiple sets of sperm within a single ovule to show that Arabidopsis has a robust 'block to polytubey'. We find that when gamete-fusion defective *hap2* or *duo1* sperm are delivered to ovules as many as three additional pollen tubes are attracted. When gamete fusion fails, one of two pollen tube-attracting synergid cell persists, enabling the ovule to attract more pollen tubes for successful fertilization. This mechanism prevents the delivery of more than one pair of sperm to an ovule and ensures maximum reproductive success by distributing pollen tubes to all ovules.

Results and Discussion

'Polytubey' increases when sperm incapable of gamete fusion are delivered to ovules

We use the term 'polytubey' (many pollen tubes) to describe the attraction of multiple pollen tubes to a single ovule. We chose this term to facilitate comparison with 'polyspermy' (the fusion of more than one sperm with a female gamete), which is distinct and also needs to be prevented to maximize reproductive success (Wong and Wessel, 2006). We developed a mixed pollination assay that unambiguously detects polytubey (Figure 1A). Half of a *male sterile1-1* (*ms1-1*) stigma was hand-pollinated with pollen expressing GFP from the pollen-specific LAT52 promoter(Twell et al., 1990) *(LAT52:GFP)*; the other half with pollen expressing *DsRed*(Francis et al., 2007) *(LAT52:DsRed).* These markers define two nearly isogenic populations of pollen tubes and allow pollen tube growth, guidance, and burst to be monitored in the pistil. Twenty-one hours after pollination 48% of ovules had been targeted by a *LAT52:DsRed* pollen tube (Figure 1B, left) and 51% by a *LAT52:GFP* pollen tube (Figure 1B, middle). This slight discrepancy from equality reflects the difficulty of placing an equal load of pollen from each genotype on the stigma. Nonetheless, the ~1% of ovules with both the GFP and DsRed signal were obvious and unambiguously indicate that these ovules had been targeted by two pollen tubes (Figure 1B, right).

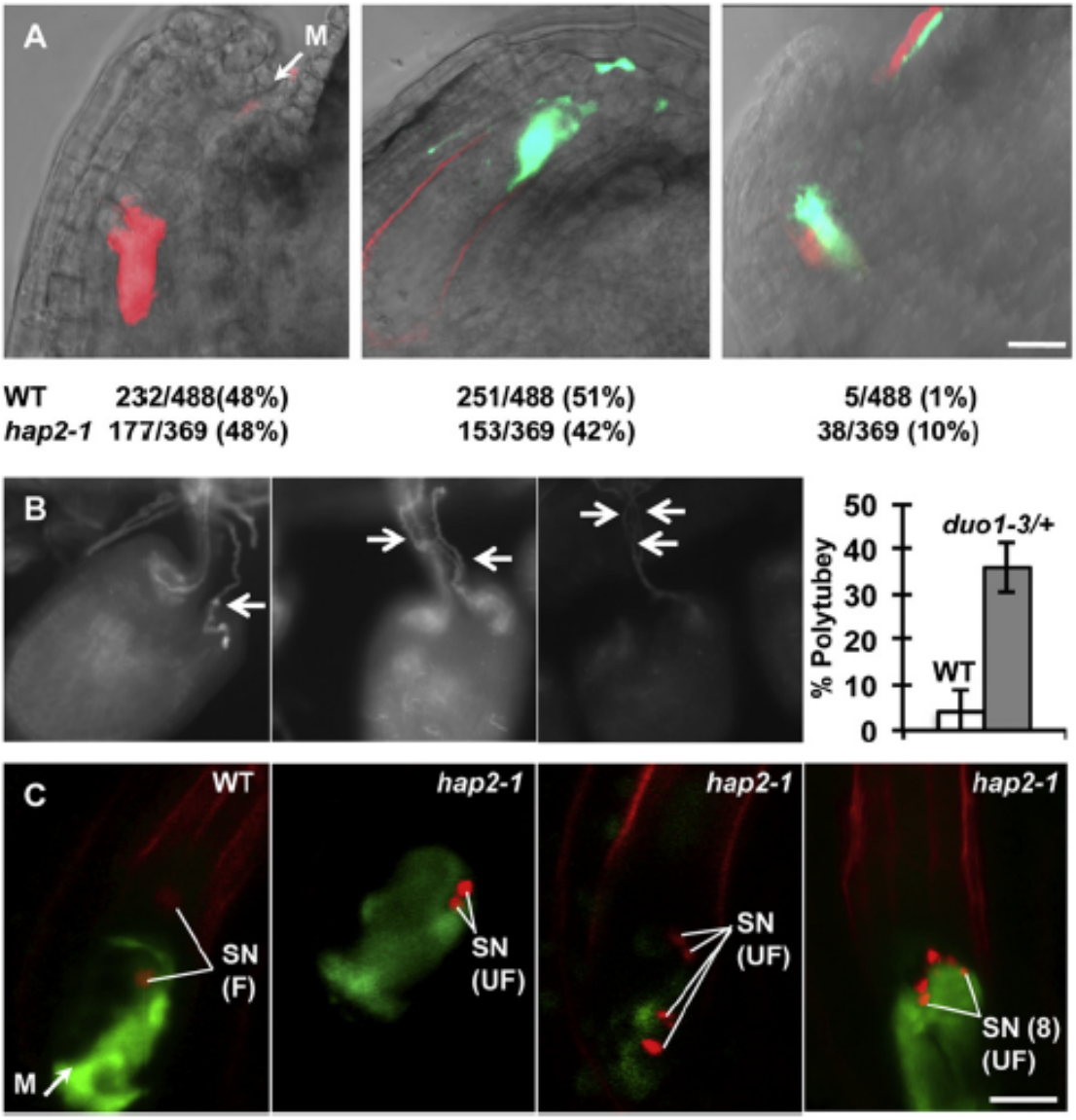


Figure 1. Polytubey is rare in wild type, but increases significantly when gamete fusion fails. (A) *hap2(gcs1)* enhances polytubey. Differentially marked pollen tubes can be tracked as they grow into an ovule at the micropylar end (M). Representative images of ovules targeted by a DsRed+ pollen tube (left), a GFP+ pollen tube (center), or by two different pollen tubes (right). The number of each type of targeting event observed is also shown (bottom). WT, *LAT52:GFP* pollen mixed with *LAT52:DsRed* (both types of pollen are wild type) or *hap2-1* (*hap2-1/HAP2(GCS1), LAT52:GFP* mixed with wild-type, *LAT52:DsRed* pollen). Scale bars, 20μm. **(B)** *duo1* enhances polytubey. *duo1-3/+* heterozygous plants were manually self-pollinated and pollen tubes were analyzed 24 hours later using aniline blue staining(Mori et al., 2006). Ovules are shown that have

attracted a single pollen tube (left panel), two pollen tubes (center panel), or three pollen tubes (right panel). The percentage of ovules with two or more pollen tubes is plotted for No-0 (wild type, 9 pistils, 260 targeted ovules) and *duo1-3/+* (8 pistils, 275 ovules targeted). Error bars represent standard deviation (each pistil is one trial). Rates of polytubey are higher in the No-0 accession (4%) than in *ms-1* (Landsberg accession, 1%, Figure 1A) and are significantly enhanced by *duo1-3* (36%). **(C)** Multiple *hap2-1* sperm are released into a single ovule. The first panel shows an ovule targeted by a wild-type *HTR10:HTR10:mRFP, LAT52:GFP* pollen tube. Green signal is released from the burst pollen tube; sperm nuclei (SN, red signal) have fused (F) with the egg cell and central cell nuclei. The second panel shows an ovule targeted by a *hap2-1, HTR10:HTR10:mRFP, LAT52:GFP* pollen tube. The pollen tube has burst (green signal), but sperm cell nuclei remain unfused (UF). The third panel shows an ovule containing four unfused *hap2-1, HTR10:HTR10:mRFP* sperm. The last panel shows an ovule containing eight unfused *hap2-1, HTR10:HTR10:mRFP* sperm. Five sperm nuclei are clearly visible, the others are outside the plane of this image. In this ovule, pollen tube cytoplasm (GFP) appears to fill the area occupied by both synergids. Scale bars, 10μm.

Analysis of pollen tube growth to ovules in vitro showed that late-arriving additional pollen tubes were actively repelled(Palanivelu and Preuss, 2006). Furthermore, analysis of several female gametophyte mutants (e.g. *feronia*) that fail to signal to the pollen tube to stop growing, burst, and release sperm indicated that these all attract multiple pollen tubes(Boisson-Dernier et al., 2008; Capron et al., 2008; Huck et al., 2003; Rotman et al., 2003; Tsukamoto et al., 2010). These studies suggested an active mechanism that blocks polytubey and indicate that any of the steps in double fertilization that occur after the pollen tube stops growing and burst could trigger the block. We tested whether gamete fusion is a potential trigger using Arabidopsis *hap2-1* mutants (Johnson et al., 2004; von Besser et al., 2006). Arabidopsis *hap2* mutant pollen tubes target ovules, burst, and release sperm, but mutant sperm do not fuse with female gametes (Hamamura et al., 2011; Mori et al., 2006). We performed mixed

pollination assays using pollen homozygous for *LAT52:GFP* and heterozygous for *hap2-1/HAP2* and pollen homozygous for *LAT52:DsRed* and *HAP2.* In this experiment, half of the GFP-expressing pollen tubes carry wild-type sperm and half carry *hap2-1* mutant sperm. All DsRed-expressing pollen tubes carry wild-type sperm. The rate of polytubey increased 10 fold over wild-type (Figure 1A) even though only ¼ of the pollen on the stigma carried *hap2-1* mutant sperm.

We found that like *hap2-1* mutants, *duo1-3* (Figure S1) mutants enhance polytubey ~10 fold over levels observed in the No-0 accession (wild type, genetic background of *duo1-3,* Figure 1B). *duo1* mutant pollen tubes carry a single sperm-like cell that is incapable of fertilization(Brownfield et al., 2009; Rotman et al., 2005). These data support the hypothesis that gamete fusion, or an event shortly thereafter is the trigger that prevents multiple pollen tubes from entering one ovule.

To closely monitor pollen tube burst and sperm release, we generated *hap2-1* and wild-type pollen tubes expressing *LAT52:GFP* along with the sperm nuclear marker *HTR10:HTR10:mRFP*. Twenty-one hours after pollination, wild-type pollen tubes had burst and sperm nuclei had fused with the egg and central cell nuclei (Figure 1C, left panel). The *HTR10:mRFP* signal diffuses after nuclear fusion as it incorporates into the egg or central cell nucleus(Ingouff et al., 2007). *hap2-1* mutant pollen tubes also burst, but sperm nuclei remained compact at the

site where gamete plasma membrane fusion would normally occur (Figure 1C second panel, (Hamamura et al., 2011)). In many cases, we observed multiple pairs of sperm nuclei (Figure 1C, third and fourth panels) and as many as four pairs of sperm were detected indicating that four pollen tubes had been attracted to one ovule (1 pair of unfused sperm, n=118; 2 pairs of unfused sperm, n=10; 3 pairs of unfused sperm, n=2; four pairs of unfused sperm, n=1). These data suggest that when gametes do not fuse, the block to polytubey fails and ovules continue to attract multiple pollen tubes.

The gamete-fusion triggered block to polytubey enhances reproductive success

To determine whether fertilization can be salvaged in an ovule that has attracted a pollen tube with defective sperm, we performed a mixed pollination experiment to analyze interactions between sperm and female gametes. Two types of differentially marked pollen were placed on the stigma at the same time: 1) *hap2-1/HAP2* pollen marked with *HTR10:HTR10:mRFP*; 2) wild-type pollen marked with *LAT52:GFP*. In addition to the expected cases where a single pollen tube was attracted (Table 1, Figure S2), a significant number of polytubey events were observed (Table 1, 39/432 targeted ovules). These included ovules containing multiple pairs of non-functional sperm (Figure S2). Importantly, these also included ovules that contained a pair of unfused RFP+ sperm nuclei and a wild-type, GFP+ pollen tube; and ovules containing a pair of non-functional RFP+

sperm (*hap2-1*) along with a pair of RFP+ wild-type sperm that had fused with female nuclei (Figure S2). We did not observe ovules that were targeted by two pollen tubes carrying wild-type sperm (e.g. wild-type GFP+ pollen tube and functional RFP+ sperm). As expected, when this mixed pollination experiment was conducted with wild-type pollen, polytubey was very rare (2/411 targeted ovules, Table 1). These data indicate that ovules first targeted by defective sperm can attract additional pollen tubes; but when wild-type sperm are attracted subsequent pollen tubes are blocked.

Table 1. Ovules that have attracted defective sperm can attract functional sperm and polytubey increases if pistils are first pollinated with *hap2-1/HAP2* pollen before wild-type pollen is added

		Single pollen tube (Signal observed)			**Polytubey (Signals observed)**					
Simultaneous Mixed Pollinations		RFP + F	RFP + U	GFP+	RFP+ F GFP +	RFP+ U GFP +	RFP+ U RFP+ U	RFP+ U RFP +F	N	Polytubey
hap2-1/HAP2, HTR10:RFP	Wild type, *LAT52:GFP*	34	70	289	0	33	3	3	432	39/432 (9%)
Wild type, *HTR10:RFP*	Wild type, *LAT52:GFP*	178	0	231	1	1	0	0	411	2/411 (0.4%)
Staggered Mixed Pollinations										
Added First	Added 1.5hr later									
hap2-1/HAP2, HTR10:RFP	Wild type, *LAT52:GFP*	87	58	93	0	51	7	14	310	73/310 (23%)
Wild type, *LAT52:GFP*	*hap2-1/HAP2, HTR10:RFP*	12	4	353	0	1	0	0	370	1/370 (0.1%)

Pollen genotypes for mixed pollinations are given in the first two columns.(h), time in hours of pollination; *HTR10:RFP, HTR10:HTR10:RFP;* RFP+F, ovule contains RFP+ sperm nuclei that have undergone karyogamy; RFP+U, RFP positive sperm that remain unfused; GFP+, GFP positive pollen tube has burst in the ovule; N, total number of ovules targeted.

If gamete fusion is required to trigger the block to polytubey, we would predict that polytubey would by exacerbated if *hap2-1/HAP2* pollen tubes were allowed to begin growth before addition of wild-type (GFP+) pollen to the stigma. In this scenario, the number of ovules that receive defective sperm would be increased.

When mixed pollinations were staggered by 1.5 hours and *hap2-1/HAP2* pollen was applied first, the rate of polytubey further increased to 23% (Table 1). However, if wild type pollen was applied first, polytubey decreased to well below 1% and the vast majority of ovules were targeted by a wild-type GFP+ pollen tube.

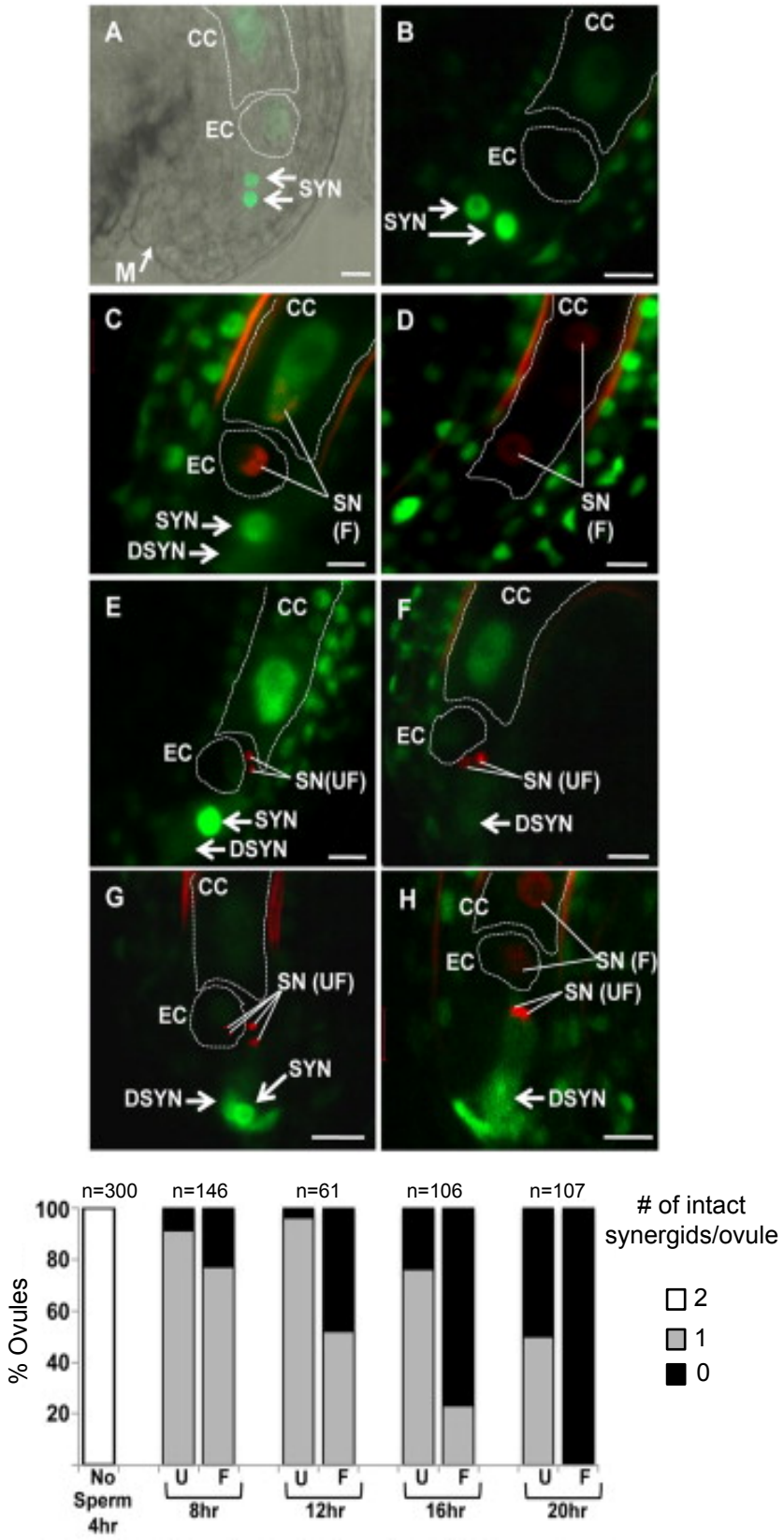
Pollen tube-attracting synergid cells persist in ovules that have received defective sperm

We observed that ovules receiving defective sperm attracted up to four pollen tubes (Figure 1C). In *Torenia fournieri*, synergid cells secrete pollen tube attractants called LUREs (Okuda et al., 2009) and at least one intact synergid is required for pollen tube attraction (Higashiyama et al., 2001). In Arabidopsis, synergids are also required for pollen tube attraction (Kasahara et al., 2005) and degeneration of one synergid is correlated with arrival of a pollen tube (Faure et al., 2002). We propose that in the absence of gamete fusion, ovules are able to continue to attract pollen tubes because one of the synergids persists and continues to secrete attractants. We determined the relationship between synergid degeneration, gamete fusion, and pollen tube attraction in ovules receiving wild type or *hap2* mutant sperm.

Upon synergid degeneration, *ACT11:MSI1:GFP* signal is no longer concentrated in the synergid nucleus and becomes diffuse throughout the cytoplasm, providing

a clear marker for this important event (Ingouff et al., 2007). We found that all ovules that had received either wild-type sperm (fused *HTR10:HTR10:mRFP* pattern, Fig 4C,D) or *hap2-1* mutant sperm (unfused, Figure 2E,F) had one degenerated synergid (Figure 2C, E), or degeneration of both synergids (Figure 2D,F). Therefore, arrival and burst of a *hap2-1* mutant pollen tube triggers degeneration of one synergid cell as in wild type. Ovules that had attracted multiple pairs of sperm also had either one or two degenerated synergids. This result shows that arrival of a second pollen tube does not trigger degeneration of the persistent synergid and suggests that a downstream event like gamete fusion may trigger degeneration of the remaining synergid.

To begin to address this hypothesis, we analyzed synergid degeneration over time. At four hours after pollination, no ovules had been targeted and all ovules contained two intact synergid cells (Figure 2I). At each subsequent time point (8, 12, 16, 20 hours), we found that ovules with unfused sperm were more likely to have an intact second synergid than ovules with fused sperm (Figure 2I U, unfused *hap2-1*, v. F, fused wild type). Twelve hours after pollination, 97% of ovules with unfused sperm had a persistent synergid compared with 48% that had been fertilized by wild-type sperm. By 20 hours after pollination both synergids had degenerated in all ovules with fused sperm, whereas 50% of ovules with unfused sperm still had one intact synergid. These data show that if gamete fusion fails, a pollen tube-attracting synergid cell persists and attracts multiple pollen tubes.



Ovules with unfused (U) v. fused (F) sperm nuclei

Figure 2. The remaining synergid cell persists longer in ovules targeted by non-functional sperm. (A) An unfertilized ovule expressing *ACT11:MSI1:GFP.* DIC and confocal images are overlaid to highlight the position of the micropyle (M, same in all panels) and GFP accumulation in the egg (EC), central (CC) and synergid (SYN) nuclei. (**B-H**) Pistils were pollinated with *hap2-1/HAP2(GCS1), HTR10:HTR10:mRFP*. Representative confocal micrographs are shown of an unfertilized ovule **(B)**, ovules that have received sperm and have a persistent synergid (SYN) **(C,E,G)**, or ovules that have received sperm and both synergids have degenerated (DSYN) **(D,F,H)**. **(D)** *ACT11:MSI1:GFP* signal was not detectable in synergids because this image was obtained later in development (note dividing primary endosperm) and significantly after synergid degeneration. (**I**) Ovules were scored for the number of synergids that remain intact (2, white bar; 1 gray bar; 0 black bar) and whether they had received sperm whose nuclei fused with female nuclei (fused, F) or sperm that remained unfused (U). Data are reported as the percentage of the total number of ovules analyzed (n) at each time point following pollination (hours, hr). Scale bar, 10μm.

Discussion

We have shown that the attraction of multiple pollen tubes to one ovule is rare in Arabidopsis (Figure 1B), but polytubey increases dramatically when sperm incapable of gamete fusion are deposited (Figure 1, Table 1). Interestingly, in wild type, one synergid cell persists well after gamete fusion (faure), but multiple pollen tubes are rarely attracted. This suggests that gamete fusion itself, or an event soon thereafter (e.g. nuclear fusion, initiation of development), triggers a mechanism that can overcome attraction by the persistent synergid and prevent entry of additional pollen tubes. We found that synergids persist longer when defective sperm are deposited, which provides an explanation for why gamete fusion defects lead to polytubey. However, synergid degeneration alone does not account for the rapid and efficient block to polytubey that follows deposition of

functional sperm. Future work will aimed at defining the components of this important mechanism.

The cases of polytubey observed in Arabidopsis mutants can all be accounted for by defects in a block to polytubey that requires gamete fusion. Female mutants defective in pollen tube reception (e.g. *feronia*) fail to instruct pollen tubes to burst, sperm are not released, gamete fusion does not occur, and consequently, they attract multiple pollen tubes (Boisson-Dernier et al., 2008; Capron et al., 2008; Huck et al., 2003; Rotman et al., 2003; Tsukamoto et al., 2010). We predict that any mutation disrupting gamete fusion will result in polytubey.

The block to polytubey was likely a significant evolutionary innovation allowing flowering plants to maximize reproductive success by promoting fertilization of all ovules and ensuring that only two sperm are delivered to each ovule. Pollen tubes bypassing fertilized ovules can continue to grow to unfertilized ovules. If the block to polytubey were initiated by any earlier step in the process (pollen tube entry, pollen tube contact with the female gametophyte, pollen tube burst), then ovules would go unfertilized in cases where pollen tubes failed to burst or sperm were defective. The block to polytubey is therefore a final opportunity for the flower to scrutinize pollen, reject unworthy suitors and replace them with productive mates.

Acknowledgements

We thank Frederic Berger, Gregory Copenhaver, and Daphne Preuss for generously providing transgenic plants expressing markers. This work was supported by the National Science Foundation through grant no. IOS-1021917 to MAJ and a Graduate Research Fellowship to KMB. KMB was initially supported by a National Institute of Health Brown University Initiative to Maximize Student Development grant (NIGMS Grant:R25GM083270 & R25GM083270-S1).

Experimental Methods

Plants used for analysis

ms1-1/MS1 plants were obtained from the Arabidopsis Biological Resource Center (stock #CS261) and maintained as a heterozygous line. *ACT11:MSI1:GFP* and *HTR10:HTR10:mRFP* plants were obtained from Frederic Berger (National University of Singapore(Ingouff et al., 2007)). *LAT52:GFP* plants were obtained from Daphne Preuss (University of Chicago) and *LAT52:DsRed*(Francis et al., 2007) plants were obtained from Greg Copenhaver (UNC, Chapel Hill). *hap2-1/HAP2* (ABRC stock#CS16308) plants or *hap2-1/hap2-1*; $hap2^{hypo}/hap2^{hypo}$(Wong et al., 2010) were crossed with *LAT52:GFP/LAT52:GFP* and *HTR10:HTR10:mRFP/HTR10:HTR10:mRFP* plants(Ingouff et al., 2007) to obtain *genotypes* used for mutant analysis of pollen tube and sperm cell nuclei behavior.

Maintenance of plants

Seeds were plated on solid Murashige and Skoog (MS) medium (MP Biomedicals LLC, Solon, OH, USA) supplemented with 25µg/ml glufosinate ammonium (Basta; Chem service, INC, West Chester, PA, USA) to select *hap2-1/HAP2* mutant seedlings or with 25µg/ml glufosinate ammonium and 50µg/ml kanamycin sulfate (Agri-bio, Bay Harbour, FL, USA) to select $hap2^{hypo}$ seedlings. Seedlings were transplanted to sterile 2MIX potting medium (Conrad Fafard INC,

Agawam, MA, USA) 7 days after plating. All plants were grown at 20°C, 50-60% humidity, on a 16day/8hour night light cycle in growth chambers (Environmental growth chambers, Chagrin Falls, OH, USA).

Analysis of double fertilization using live imaging

Standard crosses were performed under a dissecting microscope by pollinating *ms1-1* or *ACT11:MSI1:GFP* (emasculated one day prior to crossing) stage 14(Smyth et al., 1990) stigmas with dehiscent pollen from male donors. All crosses were collected 21 hours post-pollination and ovary walls were removed as previously described(Johnson and Kost, 2010). Dissected pistils were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope, LeDuc Bioimaging facility, Brown University, Providence, RI, USA). Zeiss-recommended emission and detection wavelength settings were used for each fluorophore (GFP; argon laser 458/477/488/514nm at 30mW, DsRed and mRFP; helium neon laser 633nm at 3mW). Signal intensity was optimized for each fluorophore so that signals could be shown individually and in overlay (Figure 1-4, S1).

Analysis of synergid cell degeneration

Stage 14(Smyth et al., 1990) *ACT11:MSI1:GFP* flowers were emasculated and hand pollinated with *hap2-1/HAP2; HTR10:HTR10:RFP/ HTR10:HTR10:RFP* pollen one day after emasculation. At each time point after pollination (4,8,12,16,20 hrs) pistils were dissected and ovary walls were removed as previously described(Johnson and Kost, 2010). Six to eight crosses were performed for each experiment. Dissected pistils were mounted for confocal microscopy (as described above). Targeted ovules were scored for the number of intact synergid cell nuclei and whether sperm nuclei had fused with egg and central cell nuclei or remained unfused (*hap2-1*).

Mixed pollinations Assays

Mixed pollinations were performed under a dissecting microscope by saturating one half of a stage 14(Smyth et al., 1990) *ms1-1* stigma with pollen of one genotype and the other half of the stigma with a second genotype of pollen. In simultaneous mixed pollinations, the genotype added first was varied from cross to cross and the second genotype was added immediately after the first. Six to eight crosses were performed for each experiment. In staggered mixed pollinations one genotype was added 1.5 hours before the other. Plants were placed in the growth chamber during this interval. All crosses were collected 21

hours after the addition of the second pollen donor. Samples were prepared for confocal microscopy as described above.

Supplemental Information

Contains 2 Figures.

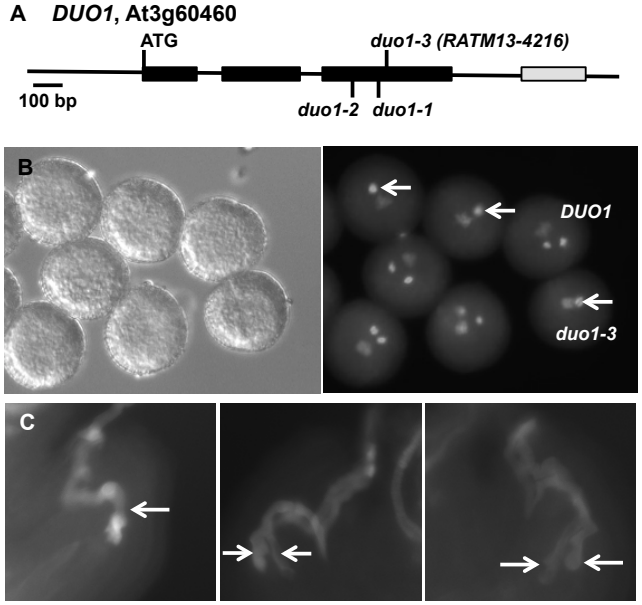


Figure S1. Analysis of *duo1-3* polytubey. Loss of *DUO1* function results in pollen that carry a single sperm-like cell that is deposited in the female gametophyte, but cannot fertilize either the egg or the central cell (Brownfield et al., 2009; Rotman et al., 2005). **(A)** A transposon insertion (RATM13-4216) in the third exon of *DUO1* (15 base-pairs downstream of *duo1-1* (Rotman et al., 2005) was obtained (Ito et al., 2005). *DUO1* exons are shown as black bars (the position of the start codon is indicated), the 3' exon of the nearest gene is show as a gray bar. The positions of *duo1-1* and *duo1-2* alleles (Rotman et al., 2005) are shown, as is the position of the *duo1-3* (RATM13-4216) transposon insertion. The *duo1-3* insertion site position was confirmed by PCR with gene- (Duo1Sac1F, AGAAGAGCTCGTCCGAAGTTTCCCTCTTGG) and transposon-specific (DS3-4, CCGTCCCGCAAGTTAAATATG) primers. **(B)** *duo1-3/+* heterozygous plants produce equal numbers of wild-type pollen with two normal sperm (*DUO1*, 108/200, 54%) and mutant pollen with a single sperm-like cell (*duo1-3*, arrow, 92/200, 46%). This is similar to *duo1-1* and *duo1-2* (Rotman et al., 2005) and like these alleles, *duo1-3* is not transmitted to progeny by pollen (not shown). A light micrograph of pollen is shown in the left panel, a fluorescence micrograph of 4',6-diamidino-2-phenyl-indole (DAPI)-stained pollen(Park et al., 1998) is shown on the right panel. Sperm and sperm-like cells (arrows) are bright staining and compact, the pollen nucleus is larger and has diffuse staining. **(C)** Higher magnification examples of ovules that have received either one pollen tube (left) or two pollen tubes (center, right). Ovules targeted by multiple sperm were smaller indicating that they had not yet received functional sperm and had not begun to expand following fertilization.

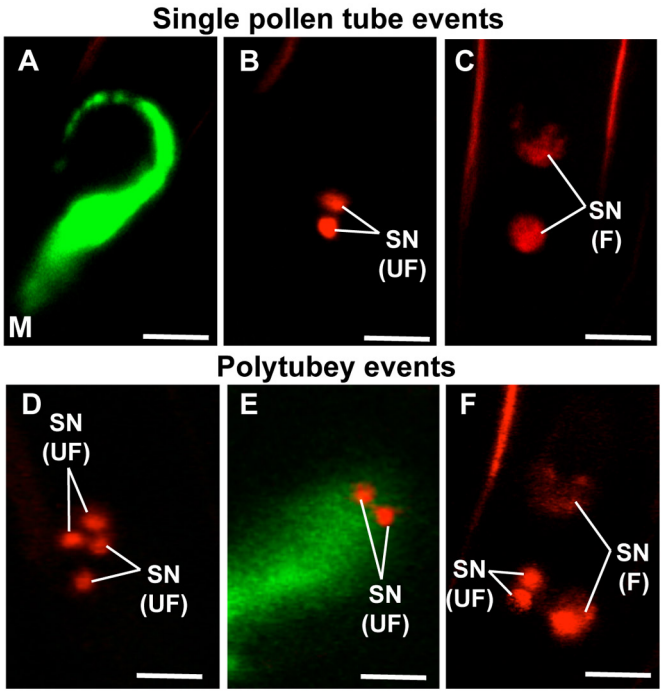


Figure S2. Ovules first targeted by pollen tubes carrying non-functional sperm are able to attract subsequent pollen tubes carrying functional sperm that initiate fertilization. Stigmas were mix-pollinated with half *hap2-1/HAP2, HTR10:HTR10:mRFP* and half wild- type*, LAT52:GFP* pollen. Representative images of ovules targeted by one pollen tube **(A-C)** or two pollen tubes **(D-F)** are shown. Ovules are shown that have been targeted by: a GFP+ pollen tube **(A)**, a pollen tube carrying non-functional (unfused, UF) RFP+ sperm **(B)**, or a pollen tube carrying functional (fused, F) RFP+ sperm**(C)**, two pollen tubes each carrying non-functional sperm **(D),** a pollen tube carrying non-functional RFP+ sperm and a GFP+ pollen tube **(E),** a pollen tube carrying non-functional RFP+ sperm and a pollen tube carrying a pair of functional RFP+ sperm **(F)**. The frequency of each event is recorded in Table 1. The position of the micropyle (M) in each image is indicated in panel A. SN, Sperm nuclei, fused(F) unfused(UF). Scale bars, 10μm.

References

Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in plant science 13:437-443.

Boisson-Dernier, A., Frietsch, S., Kim, T.H., Dizon, M.B., and Schroeder, J.I. (2008). The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. Current biology : CB 18:63-68.

Brownfield, L., Hafidh, S., Borg, M., Sidorova, A., Mori, T., and Twell, D. (2009). A plant germline-specific integrator of sperm specification and cell cycle progression. PLoS genetics 5:e1000430.

Capron, A., et al. (2008). Maternal control of male-gamete delivery in Arabidopsis involves a putative GPI-anchored protein encoded by the LORELEI gene. The Plant cell 20:3038-3049.

Faure, J.E., Rotman, N., Fortune, P., and Dumas, C. (2002). Fertilization in Arabidopsis thaliana wild type: developmental stages and time course. The Plant journal : for cell and molecular biology 30:481-488.

Francis, K.E., Lam, S.Y., Harrison, B.D., Bey, A.L., Berchowitz, L.E., and Copenhaver, G.P. (2007). Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis. Proc Natl Acad Sci U S A 104:3913-3918.

Hamamura, Y., et al. (2011). Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in Arabidopsis thaliana. Curr Biol 21:497-502.

Higashiyama, T., et al. (2001). Pollen tube attraction by the synergid cell. Science (New York, N.Y.) 293:1480-1483.

Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development (Cambridge, England) 130:2149-2159.

Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Current biology : CB 17:1032-1037.

Ito, T., et al. (2005). A resource of 5,814 dissociation transposon-tagged and sequence-indexed lines of Arabidopsis transposed from start loci on chromosome 5. Plant Cell Physiol 46:1149-1153.

Johnson, M.A., and Kost, B. (2010). Pollen tube development. Methods Mol Biol 655:155-176.

Johnson, M.A., et al. (2004). Arabidopsis hapless mutations define essential gametophytic functions. Genetics 168:971-982.

Kasahara, R.D., Portereiko, M.F., Sandaklie-Nikolova, L., Rabiger, D.S., and Drews, G.N. (2005). MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. The Plant cell 17:2981-2992.

Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.

Okuda, S., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357-361.

Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro. BMC plant biology 6:7.

Park, S.K., Howden, R., and Twell, D. (1998). The *Arabidopsis thaliana* gametophytic mutation *gemini pollen1* disrupts microspore polarity, division asymmetry and pollen cell fate. Development 125:3789-3799.

Rotman, N., et al. (2005). A novel class of MYB factors controls sperm-cell formation in plants. Current biology : CB 15:244-248.

Rotman, N., Rozier, F., Boavida, L., Dumas, C., Berger, F., and Faure, J.E. (2003). Female control of male gamete delivery during fertilization in Arabidopsis thaliana. Current Biology 13:432-436.

Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in Arabidopsis. The Plant cell 2:755-767.

Tsukamoto, T., Qin, Y., Huang, Y., Dunatunga, D., and Palanivelu, R. (2010). A role for LORELEI, a putative glycosylphosphatidylinositol-anchored protein, in Arabidopsis thaliana double fertilization and early seed development. The Plant journal : for cell and molecular biology 62:571-588.

Twell, D., Yamaguchi, J., and McCormick, S. (1990). Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. Development 109:705-713.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.

Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS genetics 6:e1000882.

Wong, J.L., and Wessel, G.M. (2006). Defending the zygote: search for the ancestral animal block to polyspermy. Current topics in developmental biology 72:1-151.

Chapter 3: *HAP2* is the only *DUO1*-regulated gene required for gamete fusion in *Arabidopsis thaliana.*

Beale, KM and Johnson, MA. Prepared for Molecular Plant, 2013.

This manuscript was submitted Molecular Plant and is in the revision process. I performed all experiments for this manuscript.

Abstract

The fusion of gamete plasma membranes is an essential step in all sexual reproduction systems. However, the molecules involved in melding distinct gamete membranes have not been described in any system and no conserved mechanism has been proposed. *HAP2* is broadly conserved in eukaryotes and has been shown to be essential for gamete fusion in the flowering plant *Arabidopsis thaliana*, the green alga *Chlamydomonas reinhardtii* and the protozoan *Plasmodium bergheii*. Furthermore, HAP2 is a membrane protein that is only expressed in the male (or -) gamete in this diverse group of organisms. It is not yet known what molecules function with HAP2 to mediate sperm gamete fusion. We set out to determine whether other sperm-expressed genes are required for gamete fusion in Arabidopsis by determining whether expression of *HAP2* is sufficient to restore fertility to *duo1* mutant sperm. *DUO1* is a critical regulator of sperm identity and is required for *HAP2* expression along with several other sperm-expressed genes. We found that *HAP2* is sufficient to rescue the gamete plasma membrane fusion defect of *duo1* mutant sperm, but that later events required for subsequent development require *DUO1* expression.

Introduction

Angiosperms provide a unique model for the study of fertilization because successful reproduction requires two gamete fusion events. Two immotile sperm develop within a pollen grain and are delivered to the female gametophyte by a pollen tube, a highly polar extension of the pollen cell (Berger et al., 2008). Female gametophytes develop within ovules and attract pollen tubes by secreting small cysteine-rich proteins from their synergid cells (Marton et al., 2005; Okuda et al., 2009; Takeuchi and Higashiyama, 2012). Pollen tubes burst in a degenerating synergid, the sperm are propelled to a site where one sperm membrane can fuse with the egg and the other can fuse with the central cell membrane, and fertilization is complete within 15 minutes of pollen tube arrival (Hamamura et al., 2012). These membrane fusion events are both essential for seed development, giving rise to the embryo and endosperm, respectively.

HAP2 is essential for both fertilization events (Mori et al., 2006; von Besser et al., 2006). *HAP2* loss-of-function alleles are not transmitted by pollen (Mori et al., 2006; von Besser et al., 2006) and when *HAP2* sperm are released from the pollen tube they remain at the site of plasma membrane fusion; their nuclei do not migrate to either the egg or central cell nucleus (Beale et al., 2012; Hamamura et al., 2011; Kasahara et al., 2012). *HAP2* is broadly conserved in eukaryotes and encodes an ~700 amino acid protein with a single

transmembrane domain that divides the primary amino acid sequence into a large extracellular N-terminal region and a small C-terminal intracellular region. The protein has no domains of known function, however the N-terminal region has been defined as critical for species-specificity in a sequence-dependent manner while the C-terminal region has been shown to rely on positive charge as opposed to sequence specificity for full function (Mori et al., 2010; Wong et al., 2010). In Arabidopsis, *HAP2* is only expressed in the generative cell and the two sperm. In the green alga *Chlamydomonas reinhardtii*, *HAP2* is only expressed in minus gametes and is essential for fusion with plus gametes (Liu et al., 2008). Interestingly, this expression pattern and function is conserved in protozoans as well; *HAP2* is also essential for gamete fusion in plasmodium, the organism that causes malaria (Liu et al., 2008; Mori et al., 2010). The *hap2* loss-of-function phenotype, expression pattern, and localization to membranes has led to the speculation that the protein may function directly in gamete plasma membrane fusion, but the function of HAP2 and the identity of any sperm- or egg-expressed partner proteins remain unknown (Wong and Johnson, 2010).

We have begun to analyze *duo1* mutants to define the scope of sperm-expressed proteins that may function with HAP2 in gamete fusion. *DUO1* encodes a MYB transcription factor that controls sperm development, identity, and function. Loss of *DUO1* function results in pollen grains with one sperm-like cell rather than two sperm (Rotman et al., 2005). The *duo1* mutant allele is never transmitted by pollen to progeny and pollination with *duo1/DUO1* pollen results in

a high rate of ovule abortion (Rotman et al., 2005); consistent with a lack of fertilization of either the egg or the central cell by the single *duo1* sperm. Interestingly, several other mutants that produce a single sperm-like cell have been shown to be able to mediate single fertilization (Aw et al., 2010; Chen et al., 2008; Frank and Johnson, 2009; Gusti et al., 2009). *DUO1*, however, is required for *HAP2* expression and ectopic expression of *DUO1* is sufficient to activate *HAP2* expression in vegetative tissue (Brownfield et al., 2009). These data suggest that *duo1* sperm could be infertile because they fail to express *HAP2*. *DUO1* also activates expression of many other genes in the male germline (Borg et al., 2011). For example, *DUO1* activates cyclin-B1 expression in the generative cell, which promotes completion of the cell cycle to produce two sperm (Brownfield et al., 2009). *DUO1* is also required to activate expression of several sperm-specific genes including *GEX2* and the sperm-specific histone variant *HTR10* (Brownfield et al., 2009). Recent transcriptome analysis suggests that *DUO1* regulates a broad network of sperm-expressed genes (Borg et al., 2011). Indeed, *DUO1* is required for the expression of all the currently available sperm marker genes except *DUO1* itself and *DUO3* (Brownfield et al., 2009).

We asked whether *HAP2* is the only *DUO1*-regulated gene required for gamete fusion. To address this question we expressed *HAP2:YFP* along with the sperm cell nuclear marker *H2B:RFP* in *duo1* mutant sperm and directly analyzed fertilization capabilities of these single sperm-like cells *in vivo*. We found that expression of *HAP2* was sufficient to render *duo1* single sperm capable of both

plasmogamy and karyogamy. We also found that when *duo1* mutant sperm express *HAP2:YFP* downstream development is halted at, or sometime close after, initial karyogamy, as early embryo and endosperm are not detected in ovules receiving *duo1* mutant sperm expressing *HAP2*. We interpret our results to indicate that *HAP2* is the only *DUO1*-regulated gene required for the specific step of gamete fusion, and that other *DUO1*-regulated genes are required for initiation of development. Our data suggest for the first time that *HAP2* is sufficient for gamete fusion. Our results also suggest that other *DUO1* regulated genes are required from the sperm to initiate early embryo and endosperm development.

Results

DUO1::HAP2:YFP is able to rescue the *hap2-2* fertilization defect

Since *HAP2* promoter activity requires *DUO1*, we made a *DUO1::HAP2:YFP* construct to express *HAP2* from the *DUO1* promoter, which is active in *duo1* mutant sperm (Brownfield et al., 2009). Pollen from two independent hemizygous transgenic lines was crossed to *male sterile 1-1 (ms1-1)* females and 50% of progeny inherited the *DUO1::HAP2:YFP* transgene (Table 1). This experiment indicated that pollen carrying sperm expressing *HAP2* from the *DUO1* promoter were as likely as wild-type pollen to fertilize an ovule.

To determine whether *DUO1::HAP2:YFP* encoded functional HAP2, we transformed *hap2-2/HAP2* plants and analyzed transmission of the *hap2-2* mutant allele. *hap2-2* was generated by insertion of a T-DNA carrying a kanamycin resistance gene and is not transmitted through the male germline (Table 1, (von Besser et al., 2006). Two independent hemizygous *DUO1::HAP2:YFP* transgenic lines that were heterozygous for *hap2-2/HAP2* were crossed to *ms1* and progeny were scored for kanamycin and basta resistance. Both lines showed significant transmission of kanamycin resistance to progeny, consistent with complete rescue of *hap2-2* by *DUO1::HAP2:YFP.* In both cases, the percentage of *hap2-2/HAP2* F_1 progeny was higher than expected (expected, 33%), indicating the possibility that these lines carry multiple insertions of the *DUO1::HAP2:YFP* transgene.

Table 1. Transmission analysis shows that expression of *DUO1::HAP2:YFP* does not affect male fertility and restores fertility to *hap2-2*

Female	Male	Line	% Het.	n
ms1-1	*DUO1::HAP2:YFP*/∅; *HAP2/HAP2*	A	54[a]	59
		B	49[a]	77
ms1-1	*DUO1::HAP2:YFP*/∅; *hap2-2/HAP2*	A	41[b]	51
		B	49[b]	105
ms1-1	*hap2-2/HAP2*		0*	348

* previously published data (von Besser et al., 2006)
[a]percentage of Basta resistant (heterozygous) F_1 seedlings; the *DUO1::HAP2:YFP* T-DNA carries a Basta resistance gene.
[b]percentage of kanamycin/basta resistant (heterozygous) F_1 seedlings; the hap2-2 T-DNA carries a kanamycin resistance gene.
Two independent transgenic lines (A or B) were analyzed.
n, the total number of resistant and sensitive seedlings analyzed

DUO1::HAP2:YFP is expressed in *duo1-3* single sperm cells

To determine whether *DUO1::HAP2:YFP* can restore fertility to *duo1* mutant sperm, we crossed the transgenic lines from the previous experiment with *duo1-3/DUO1* (Beale et al., 2012) heterozygous plants. We took this approach to ensure that the same functional transgenes established by rescue of *hap2-2* would be used in determining whether *HAP2* expression could restore fertility to *duo1* mutant sperm.

We analyzed expression of *DUO1::HAP2:YFP* in F_2 segregants from this cross using confocal microscopy (Figure 1). 50% of pollen from *duo1-3*/DUO1 plants show a single sperm nucleus and YFP signal is not detectable in sperm; only autofluorescence of the pollen wall is observed (Figure 1A). Segregants that were wild type for *DUO1* and *HAP2* and homozygous for *DUO1::HAP2:YFP* produce pollen grains with two sperm that both express HAP2:YFP in a characteristic perinuclear pattern (Figure 1B, see Sprunck et al., 2012; von Besser et al., 2006). We also analyzed segregants that were wild type for *HAP2, duo1-3/DUO1,* and homozygous for *DUO1::HAP2:YFP.* These plants produced pollen grains that contained either one (*duo1-3*) or two (*DUO1*) sperm that expressed HAP2:YFP in the characteristic pattern (Figure 1C). These data suggested that we had constructed *duo1* mutant sperm that express functional HAP2.

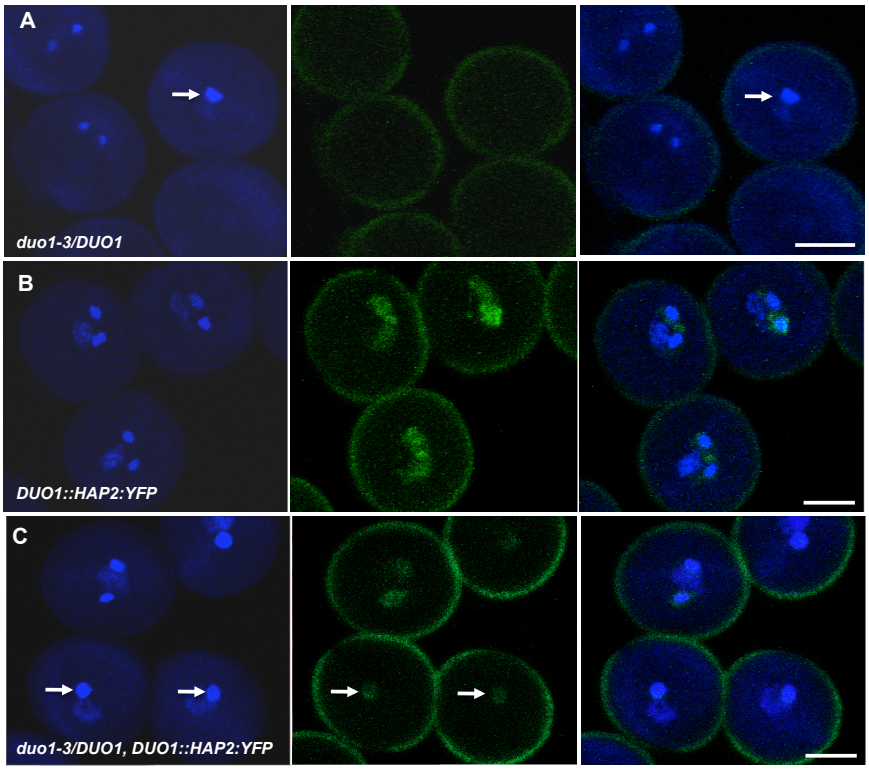


Figure 1. *DUO1::HAP2:YFP* is expressed in wild type and *duo1-3* sperm cells. The left column shows 4',6-diamidino-2-phenyl-indole (DAPI)-stained pollen grains, the middle columns shows YFP expression in the same pollen grains, the right column is an overlay. **(A)** *duo1-3/DUO1* pollen grains containing either *duo1-3* single sperm or pairs of DUO1 sperms. Apparent YFP signal is autofluorescence of the pollen grain. **(B)** Wild-type pollen grains homozygous for *DUO1::HAP2:YFP* **(C)** *duo1-3/DUO1, DUO1::HAP2:YFP/DUO1::HAP2:YFP* pollen grains. White arrows indicate *duo1-3* single sperm nuclei. Scale bars, 10µm**.**

Direct analysis of *duo1-3, DUO1:HAP2:YFP, DUO1:H2B:RFP* sperm cells *in vivo* suggests that *HAP2* is sufficient to rescue the gamete fusion defect of *duo1* sperm

To directly test whether *HAP2* was sufficient to rescue the gamete fusion defect of *duo1* mutants we analyzed sperm cell behavior *in vivo* by labeling *duo1* sperm expressing *DUO1::HAP2:YFP* with *DUO1::H2B:RFP,* a sperm-specific Histone 2B nuclear maker (Brownfield et al., 2009). This marker labels sperm cell nuclei similar to the well established *HTR10::HTR10:mRFP* marker (Figure 2A, Beale et

al., 2012; Hamamura et al., 2012; Ingouff et al., 2007). We were unable to use this very bright marker because the *HTR10* promoter is activated by *DUO1.* Plants were identified that were heterozygous for *duo1,* and homozygous for both *DUO1::HAP2:YFP* and *DUO1::H2B:RFP.* This results in 50% of pollen grains containing a single sperm and 50% of grains containing two sperm, all of which are positive for *DUO1::HAP2:YFP* and *DUO1::H2B:RFP* (Figure 2B).

We predicted that if *HAP2* expression was able to rescue gamete fusion of these *duo1* single sperm that we would be able to see one of two events; 1) Fusion of the sperm cell nucleus with the nucleus of the egg or the central cell, resulting in a diffuse RFP signal that overlaps with the female cell nucleus (reference Thesis Chapter 2). This would indicate successful plasmogamy *and* karyogamy. 2) Migration of the sperm cell nucleus to that of the egg or central cell (successful gamete fusion), but a compact RFP signal indicative of unsuccessful karyogamy (Reference (Aw et al., 2010). In either case, this would indicate that these *duo1* sperm expressing *HAP2* are actually capable of gamete fusion, but karyogamy or a downstream event to development is halted. If *HAP2* expression was unable to rescue gamete fusion, or only able to do so at very low levels, we would observe single, compact nuclei that remain at the egg or central cell periphery without migrating to the nucleus, similar to the *hap2* mutant defect.

We crossed *duo1/+, DUO1::HAP2:YFP/-, DUO1::H2B::RFP*/- pollen onto *ACT11:MSI1:GFP* (labels female nuclei) females and imaged ovules 11 hours

after pollination. Since these plants are heterozygous for *DUO1* we identified ovules that had received two functional sperm capable of double fertilization and exhibiting the diffuse RFP signal representing karyogamy (Figure 2C). Interestingly, we also saw several (6/11) single gamete fusion events using this assay. We identified ovules that contained *duo1, DUO1::HAP2:YFP* single sperm that were able to fuse with the egg cell nucleus and exhibit a diffuse RFP signal (Figure 2D), consistent with successful plasmogamy and karyogamy. We did not detect any ovules that contained *duo1* sperm that fused with the central cell, however, this could have been due to the relatively small sample size (6 ovules containing single sperm were analyzed) of this experiment. Further analysis will be required to determine whether there is a significant difference in the ability of *duo1, DUO1:HAP2:YFP* sperm to fuse with the egg compared to the central cell.

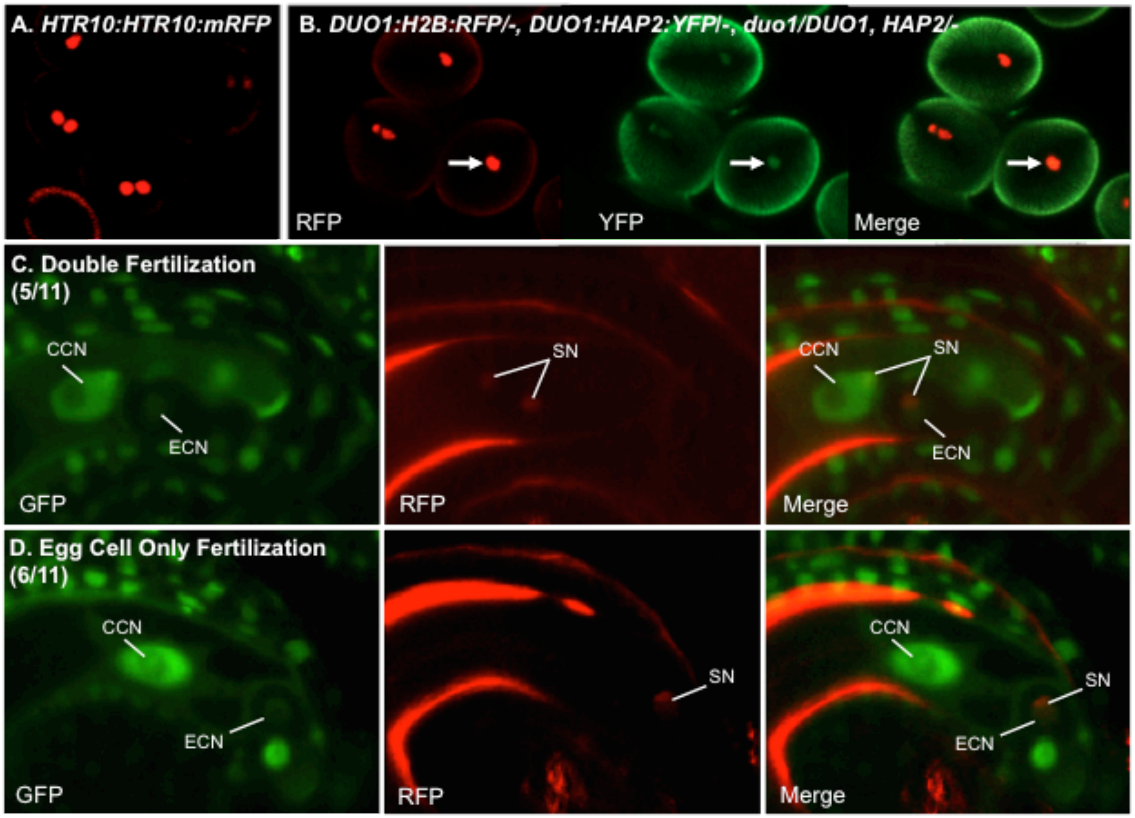


Figure 2. *In vivo* analysis of *duo1* sperm cells expressing *DUO1::HAP2:YFP* and *DUO1::H2B:RFP.* **(A)** Pollen grains expressing the sperm cell nuclei marker *HTR10::HTR10:mRFP* generally used for analysis of fertilization *in vivo*. Expression is brighter than *DUO1::H2B:RFP,* as can be seen by decreased autofluorescense in the pollen grain wall. **(B)** Pollen grains that are heterozygous for *duo1* (single sperm indicated with white arrow) and are homozygous for both *DUO1::HAP2:YFP* and *DUO1:H2B:RFP.* **(C)** An ovule that has received sperm that are wild type for *DUO1* and capable of fertilizing both the egg and central cell. Sperm cell nuclei have migrated to overlap with the egg and central cell nuclei and exhibit a diffuse RFP pattern. **(D)** An ovule that has received a *duo1* mutant sperm that expresses *DUO1::HAP2:YFP* and is capable of fusing with the egg cell. A diffuse RFP pattern is seen co-localizing with the egg cell nuclei and not the central cell. CCN: Central Cell Nucleus, ECN: Egg Cell Nucleus, SN: Sperm Nuclei. N=total number of events quantified.

duo1-3 single sperm do not initiate embryo or endosperm development

We determined that expression of *HAP2* was able to rescue plasmogamy and karyogamy when expressed in *duo1* mutant sperm, but it was unclear whether these products of fertilization continued to early development. If *HAP2* expression in *duo1* mutant sperm is sufficient to initiate early embryo and endosperm development, then we would expect to see a high frequency of single fertilization events (50%) when analyzing the products of fertilization at later developmental time points compared to *duo1* mutants (0%). This would indicate that *HAP2* is sufficient to rescue gamete fusion and that *duo1* mutant sperm contain all necessary components to initiate development once fusion has occurred successfully.

We first analyzed *ms1-1* pistils two days after pollination with either wild-type, or *duo1-3/DUO1* pollen to determine whether double fertilization, single fertilization, or no fertilization had occurred in ovules. When wild type (Col-0) is used as a pollen donor 96% of ovules show embryo and endosperm development indicating double fertilization; remaining ovules are unfertilized. A small subset of ovules are aborted in every cross but not included in our quantifications of fertilization events (Table 2, Figure 3A,B).

Table 2. Analysis of fertilization events within ovules

Female	Male	DF (%)	Emb. (%)	Endo. (%)	UF (%)	n
ms1-1	Col-O	473 (96.3)	0	0	18 (3.7)	491
ms1-1	*duo1-3/DUO1*	1194 (71.8)	0	0	469 (28.2)	1663
ms1-1	*duo1-3/DUO1, DUO1::HAP2:YFP/DUO1::HAP2:YFP*	1412 (81.6)	7 (0.5)	14 (0.8)	297 (17.2)	1730

Fertilization events (double fertilization, DF; embryo-only, Emb.; endosperm-only, Endo.; unfertilized, UF;) were quantified by clearing ovules two days after pollination. n, total number of ovules analyzed

When *duo1-3/DUO1* is used as the pollen donor, ~72% of ovules were found to have an embryo and endosperm; the remaining ovules (~28%) had neither embryo development, nor endosperm development, despite the presence of egg and central cell nuclei (Table 2, Figure 3C,D). It is likely that these ovules have received a single *duo1* sperm that is incapable of fusing with either the egg or the central cell. However, since 50% of *duo1-3/DUO1* carry a single sperm (Beale et al., 2012), the observed rate of double fertilization is higher than expected. It was recently reported that pollination with *duo1/DUO1* leads to an increase in the rate at which multiple pollen tubes enter a single ovule (polytubey). It has been proposed that polytubey results when gamete fusion fails, thus allowing a pair of wild-type sperm to be attracted to ovules that have received non-functional sperm (Beale et al., 2012; Kasahara et al., 2012). This increases seed set above expected levels for mutants that block sperm function.

Expression of *HAP2* in *duo1-3/+* plants results in a low frequency of single fertilization events

If *HAP2* is sufficient to rescue fertility of *duo1-3* single sperm, then we expected to observe single fertilization events when *duo1-3/+, DUO1::HAP2:YFP/DUO1::HAP2:YFP* pollen was crossed onto *ms1-1*. If *HAP2* expression in *duo1-3* mutant sperm is only sufficient to rescue gamete fusion, but these sperm are incapable of initiating development, then we would expect a very low rate of single fertilization events. Due to the rate of polytubey, these rare single fertilization events would result from ovules that had first been targeted by a *duo1, DUO1:HAP2:YFP* sperm cell that initiated gamete fusion with either the egg cell or the central cell, but halted early development and failed to trigger the block to polytubey. It has recently been shown that a single fertilization event is not sufficient to initiate the block to polytubey (Maruyama et al., 2013). Should a second pollen tube enter this same ovule and deposit functional sperm, only the other available female cell would undergo gamete fusion and successful development, resulting in detection of early embryo or endosperm production using ovule-clearing methods.

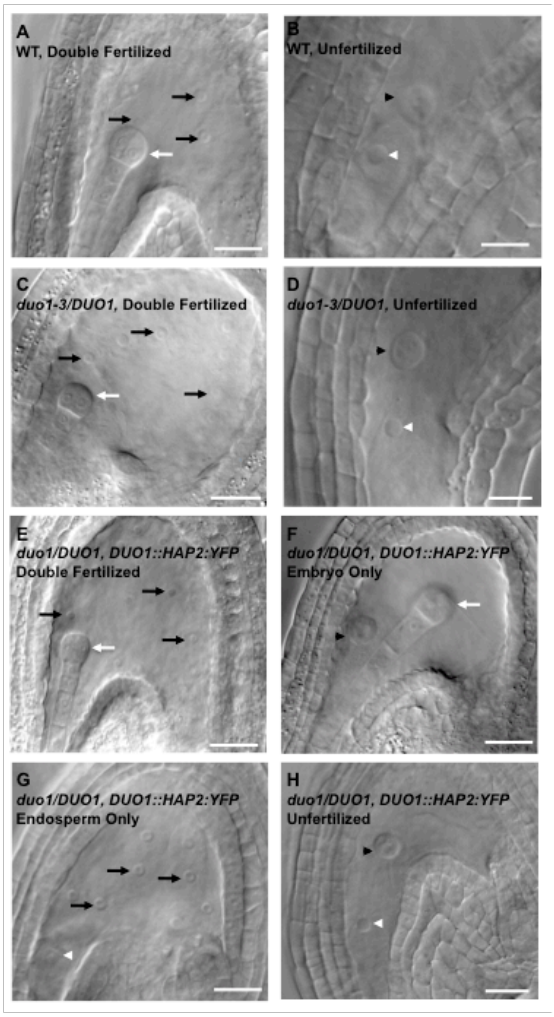


Figure 3. *DUO1::HAP2:YFP* expression in *duo1-3* SSLCs result in single fertilization events. Chloral hydrate was used to clear *ms1-1* ovules two days after pollination; differential interference contrast microscopy was used to determine whether the egg or central cell was fertilized. **(A,B)** Pollination with wild type (WT, Col-O) results in double fertilized ovules **(A)**; occasionally *ms1-1* ovules contain aborted ovules **(B)**. **(C,D)** Pollination with *duo1-1/DUO1* results in double fertilization **(C)** or ovules with unfertilized egg and central cell nuclei. **(E-H)** Pollination with *duo1/DUO1*, *DUO1::HAP2:YFP/DUO1::HAP2:YFP* results in double fertilization **(E)**, fertilization of the egg, but not the central cell **(F)**, fertilizaiotn of the central cell, but not the egg **(G)**, or ovules with unfertilized egg and central cell nuclei **(H)**. White arrowheads indicate an unfertilized egg nuclei; white arrows indicate developing embryos. Black arrowheads indicate an unfertilized central cell nuclei; black arrows indicate proliferating endosperm nuclei. Scale bars, 100μm.

When *duo1-3/+, DUO1::HAP2:YFP/DUO1::HAP2:YFP* pollen was crossed onto *ms1-1* the rates of double fertilized and unfertilized ovules were not significantly changed compared to when *duo1-3/DUO1* pollen was used (Table 2, Figure 3 E, H). However, we observed clear instances of single fertilization (Figure 3 F, G). Single fertilization of the egg cell (n=7) was less common than single fertilization of the central cell (n=14, Table 2). Importantly, the overall rate of single fertilization (1%) was much lower than expected (50%), indicating that other *DUO1*-regulated factors are required for making sperm capable of initiating downstream events like embryo and endosperm development.

Discussion

Fusion of one sperm cell with the egg cell and another with the central cell is essential to produce a viable seed. Our knowledge of the mechanisms required to merge gamete membranes is currently rudimentary in even the best-characterized systems (Wong and Johnson, 2010). Understanding of gamete fusion mechanisms in flowering plants has progressed recently, however, HAP2 remains the only gamete surface protein thought to play a direct role. A family of five small, cysteine-rich, egg cell-expressed proteins (EC1) was recently shown to activate HAP2 by sending it to the sperm surface (Sprunck et al., 2012). EC1 proteins are secreted in response to sperm arrival in the female gametophyte and experiments with synthetic peptides showed that HAP2 could be rapidly relocalized from the perinuclear cytoplasm to the plasma membrane as a

consequence (Sprunck et al., 2012). This work suggests that HAP2 activity is very carefully regulated; perhaps to ensure that plasma membrane fusion activity is limited until the sperm membrane contacts female gamete membranes. Here, we have attempted to determine whether *HAP2* is the central male component of a gamete fusion mechanism. We found that expression of *HAP2* in *duo1* mutant sperm was sufficient to render these single sperm capable of gamete fusion (Figure 2D). However, the rates of single fertilization of *duo1-3* sperm expressing functional *HAP2:YFP* were very low (Figure 2, Table 2). We propose that *HAP2* is the only *DUO1*-regulated gene required for the step of gamete plasma membrane fusion, but that other *DUO1*-regulated genes might also be required for steps beyond gamete fusion, like karyogamy or initiation of development of the embryo and endosperm.

Live cell imaging of sperm cell nuclei revealed that nuclei of single *duo1* sperm expressing *HAP2* and *DUO1:H2B:RFP* were able to migrate to either the egg or central cell nucleus and fuse (Figure 2). Migration of sperm nuclei indicates successful plasmogamy, while diffusion of the RFP signal indicates karyogamy. Preliminary analysis of only 11 ovules revealed that 6 of these ovules (55%) had received single sperm where the sperm nucleus had migrated to the egg nucleus and become diffuse. This result suggests that plasmogamy and karyogamy are occurring at the expected rate for fusion rescue (Figure 2, Table 2). These experiments need to be extended substantially to make firm conclusions, but these preliminary data lead us to propose that *HAP2* may be sufficient to restore

the ability of *duo1* mutant sperm to fuse with female gametes and suggest that other *DUO1*-activated sperm genes are required for activation of embryo and endosperm development.

If further experiments confirm that *HAP2* is sufficient to restore ability of *duo1* sperm to fuse with female gametes, we would conclude that *DUO1*-activated sperm genes might be required for karyogamy or initiation of development of the embryo and endosperm. Precedence for the idea that sperm-expressed genes may be required for embryo development comes from analysis of *SHORT SUSPENSOR (SSR). SSR* is transcribed in the sperm and translated in the zygote where it is required for patterning embryo development (Bayer et al., 2009). However, sperm-expressed genes required to activate zygotic and endosperm divisions have not yet been identified. We showed with preliminary experiments looking directly at gamete fusion *in vivo* that *duo1* mutant sperm expressing both *DUO1::HAP2:YFP* and *DUO1::H2B:RFP* fuse with the egg at rates much higher than the observed rates of initiation of embryo or endosperm development. We also observed that these sperm cell nuclei exhibited a diffuse RFP pattern that overlapped with that of the egg cell nuclei (Figure 2D). This suggests that these sperm undergo karyogamy normally. Taken together, these observations imply that *HAP2* may be sufficient to rescue the fusion defect of *duo1* mutant sperm but other *DUO1*-regulated genes are required for steps beyond nuclear fusion. Future experiments will focus on repeating these crosses in an attempt to quantify many more fertilization events, including the predicted

central cell only single fertilization events that may have not been detected due to the low number of ovules quantified.

Polytubey, which occurs when ovules first attract a pollen tube carrying defective sperm (Beale et al., 2012; Kasahara et al., 2012), may have contributed to the low rates of single fertilization observed when detecting early development events (Figure 3). We previously showed that gamete fusion triggered a mechanism that prevents multiple pollen tubes from targeting an ovule. Pollen tubes carrying *hap2, duo1,* or *duo3* mutant sperm all show high rates of polytubey (Beale et al., 2012; Kasahara et al., 2012). It is possible that when *duo1* sperm expressing *HAP2* arrive in the female gametophyte and fuse with either the egg or the central cell, a second pollen tube is attracted, delivering additional sperm. In our assay, these would have appeared as single fertilized, as the female cell first targeted by the *duo1* sperm expressing *HAP2* would fail to initiate early development, and the second tube containing function sperm would fuse with the remaining female cell and initiate normal development (Figure 3). Indeed, recently published results show that a single fertilization event is not sufficient to trigger the block to polytubey (Maruyama et al., 2013).

In conclusion, we have shown that HAP2 is sufficient to rescue the gamete fusion defect of *duo1* sperm. Since *DUO1* is a critical regulator of sperm cell identity, these results raise the possibility that HAP2 is the sole sperm-expressed protein directly involved in fusion with the egg and the central cell. However, additional

quantitative analysis of sperm nuclear behavior is required before firm conclusions can be made. Our results also indicate that additional *DUO1*-regulated sperm genes are required for the initiation of early development, as *duo1* sperm expressing *HAP2* fail to develop into embryo or endosperm.

Acknowledgements

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Experimental Methods

Growth of plants

ms1/MS1 plants were obtained from the Arabidopsis Biological Resource Center (stock #CS261) and maintained as a heterozygous line. *hap2-2/HAP2* (von Besser et al., 2006)contains a T-DNA which confers Kanamycin resistance and is also available at the ABRC (stock #N652706). *duo1-3/DUO1* contains a transposon insertion in the third exon of *DUO1* (RATM13-4216) (Beale et al.). Insertion site was confirmed using PCR with gene- and transposon-specific primers and pollen was confirmed to contain SSLCs via 4',6-diamidino-2-phenyl-indole (DAPI) staining of pollen grains {(Beale et al., 2012)Sup FigS1}. All seeds were plated on solid Murashige and Skoog (MS) medium (MP Biomedicals LLC, Solon, OH, USA). *ms1/MS1* seeds were plated on no drug media, *hap2-2/HAP2* seeds were plated on medium containing 50µg/ml kanamycin sulfate (Agri-bio, Bay Harbour, FL, USA), and *duo1-3/DUO1* seeds were plated on 25µg/ml Hygromyscin sulfate (Agri-bio, Bay Harbour, FL, USA). Seedlings were transported to sterile 2MIX potting medium 7 days after plating. All plants were grown at 20°C, 50-60% humidity, on a 16day/8hour night/light cycle in growth chambers.

Generation of *DUO1::HAP2:YFP*

The *DUO1p* was amplified by PCR (DUO1pSAC1F: AGAAGAGCTCGTCCGAAGTTTCCCTCTTGG and DUO1pXma_R:TCCCCCCGGGCGCTAATCGATCTCTCTCTCG) and inserted into the *LAT52p:HAP2cDNA:YFP* (BastaR) (Frank and Johnson, 2009) construct in which the *LAT52p* had been excised with Sac1 and Xma1. This construct was transformed into *qrt* and *hap2-2/HAP2* plants using agrobacterium floral dip (Clough and Bent, 1998) and plated on either Basta or Kan/Basta plates, respectively. Pollen from T1 plants were screened for positive YFP expression around the sperm cell nucleus using fluorescence microscopy (Hamamatsu ORCA-ER, YFP cube, 3-6sec exposure). T1 lines with the strongest YFP expression were allowed to self. T2 individuals were identified that were homozygous for YFP expression and were then crossed onto *ms1/MS1* for transmission analysis.

Generation of *duo1-3/DUO1;HAP2/HAP2;DUO1::HAP2:YFP/DUO1::HAP2:YFP* plant lines

T2 lines homozygous for *DUO1::HAP2:YFP/DUO1::HAP2:YFP* with strongest YFP expression and PCR genotyped as *HAP2/HAP2* were crossed onto *duo1-3/DUO1* females. Crosses were plated on Hyg/Basta plates. Resistant seedlings were then PCR genotyped for *HAP2* and *DUO1* to confirm they were

HAP2/HAP2 and *duo1-3/DUO1.* Pollen grains were also screened for YFP expression and SSLCs via DAPI staining.

Chloral Hydrate analysis of single fertilization events

Standard crosses were performed under a dissecting microscope by pollinating *ms1-1* stage 14 stigmas with dehiscent *duo1-3/DUO1;HAP2/HAP2;DUO1::HAP2:YFP/DUO1::HAP2:YFP* pollen. Crosses were dissected 2 days post pollination and ovary walls removed (Capron et al., 2008). Dissected pistils were transferred to 9:1 ethanol:acetic acid and incubated at room temperature overnight. Pistils were then washed with 90% ethanol for 30min and then 70% ethanol for 30min. Pistils were then transferred to Chloral hydrate solution for 1.5hrs. Pistils were then mounted in chloral hydrate on microscope slides and analyzed using DIC microscopy (Zeiss Axiovert 200 M fluorescence microscope and AxioCam MRc5 camera).

Generation of *duo1, DUO1:HAP2:YFP, DUO1:H2B:RFP* plants

T3 plants that were heterozygous for *duo1* (Hyg^R) and homozygous for *DUO1:HAP:YFP* ($Basta^R$) were crossed with plants that were homozygous for *DUO1:H2B:RFP* (Kan^R, gift from David Twell). F1 plants were screened for YFP and RFP in pollen grains. Plants that were positive for RFP and YFP in *duo1* sperm were allowed to self-cross. Crosses were then selected on Kan/Hyg/Basta plates and plants were identified that were heterozygous for *duo1* and

homozygous for both *DUO1:HAP2:YFP* and *DUO1:H2B:RFP.* This was confirmed by screening of pollen using fluorescence microscopy.

Direct analysis of *duo1, DUO1:HAP2:YFP, DUO1:H2B:RFP* sperm cells *in vivo*

duo1, DUO1:HAP2:YFP, DUO1:H2B:RFP pollen was crossed onto *ACT11:MS11:GFP* (emasculated one day prior to crossing) stage 14 stigmas using standard crossing methods. All crosses were collected 11 hours post-pollination and ovary walls were removed using previously established methods (Johnson and Kost, 2010). Dissected pistils were mounted in 80mM sorbitol for analysis by confocal laser scanning microscopy using a 40x objective with DIC capability (Zeiss LSM510 Meta inverted microscope, LeDuc Bioimaging facility, Brown University, Providence, RI, USA). Zeiss-recommended emission and detection wavelength settings were used for each fluorophore (GFP; argon laser 458/477/488/514nm at 30mW, and RFP; helium neon laser 633nm at 3mW). Signal intensity was optimized for each fluorophore so that signals could be shown individually and in overlay.

References

Aw, S.J., Hamamura, Y., Chen, Z., Schnittger, A., and Berger, F. (2010). Sperm entry is sufficient to trigger division of the central cell but the paternal genome is required for endosperm development in Arabidopsis. Development (Cambridge, England) 137:2683-2690.

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T., and Lukowitz, W. (2009). Paternal control of embryonic patterning in Arabidopsis thaliana. Science (New York, N.Y.) 323:1485-1488.

Beale, K.M., Leydon, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule. Current biology : CB 22:1090-1094.

Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in plant science 13:437-443.

Borg, M., Brownfield, L., Khatab, H., Sidorova, A., Lingaya, M., and Twell, D. (2011). The R2R3 MYB transcription factor DUO1 activates a male germline-specific regulon essential for sperm cell differentiation in Arabidopsis. The Plant cell 23:534-549.

Brownfield, L., Hafidh, S., Borg, M., Sidorova, A., Mori, T., and Twell, D. (2009). A plant germline-specific integrator of sperm specification and cell cycle progression. PLoS genetics 5:e1000430.

Capron, A., et al. (2008). Maternal control of male-gamete delivery in Arabidopsis involves a putative GPI-anchored protein encoded by the LORELEI gene. The Plant cell 20:3038-3049.

Chen, Z., Tan, J.L., Ingouff, M., Sundaresan, V., and Berger, F. (2008). Chromatin assembly factor 1 regulates the cell cycle but not cell fate during male gametogenesis in Arabidopsis thaliana. Development (Cambridge, England) 135:65-73.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant journal : for cell and molecular biology 16:735-743.

Frank, A.C., and Johnson, M.A. (2009). Expressing the diphtheria toxin A subunit from the HAP2(GCS1) promoter blocks sperm maturation and produces single sperm-like cells capable of fertilization. Plant physiology 151:1390-1400.

Gusti, A., et al. (2009). The Arabidopsis thaliana F-box protein FBL17 is essential for progression through the second mitosis during pollen development. PloS one 4:e4780.

Hamamura, Y., Nagahara, S., and Higashiyama, T. (2012). Double fertilization on the move. Current opinion in plant biology 15:70-77.

Hamamura, Y., et al. (2011). Live-cell imaging reveals the dynamics of two sperm cells during double fertilization in Arabidopsis thaliana. Current biology : CB 21:497-502.

Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Current biology : CB 17:1032-1037.

Johnson, M.A., and Kost, B. (2010). Pollen tube development. Methods in molecular biology (Clifton, N.J.) 655:155-176.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in Arabidopsis. Current biology : CB 22:1084-1089.

Liu, Y., et al. (2008). The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes & development 22:1051-1068.

Marton, M.L., Cordts, S., Broadhvest, J., and Dresselhaus, T. (2005). Micropylar pollen tube guidance by egg apparatus 1 of maize. Science (New York, N.Y.) 307:573-576.

Maruyama, D., et al. (2013). Independent control by each female gamete prevents the attraction of multiple pollen tubes. Developmental cell 25:317-323.

Mori, T., Hirai, M., Kuroiwa, T., and Miyagishima, S.Y. (2010). The functional domain of GCS1-based gamete fusion resides in the amino terminus in plant and parasite species. PloS one 5:e15957.

Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.

Okuda, S., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357-361.

Rotman, N., et al. (2005). A novel class of MYB factors controls sperm-cell formation in plants. Current biology : CB 15:244-248.

Sprunck, S., Rademacher, S., Vogler, F., Gheyselinck, J., Grossniklaus, U., and Dresselhaus, T. (2012). Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science (New York, N.Y.) 338:1093-1097.

Takeuchi, H., and Higashiyama, T. (2012). A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis. PLoS biology 10:e1001449.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.

Wong, J.L., and Johnson, M.A. (2010). Is HAP2-GCS1 an ancestral gamete fusogen? Trends in cell biology 20:134-141.

Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS genetics 6:e1000882.

Chapter 4: A Forward Genetic Modifier Screen Identifies Potential Genes that Interact with *HAP2* to Mediate Gamete Fusion

Beale, KM, Khadraoui, A, Mislick, KV, Leydon, AR, and Johnson, MA. This chapter is currently unpublished and I performed all experiments with the aid of AK. KVM generated *HAP2::HAP2^{hypo}:YFP* and some of the chloral hydrate analysis of fertilization events. ARL aided in initial screening.

Abstract

The merger of sperm and egg membranes is an essential step in fertilization, but in spite of the conservation of this process across sexually reproducing eukaryotes, a mechanism for gamete fusion has not been elucidated in any system. An experimental inroad into the process of gamete fusion is *HAP2*, a sperm-specific gene required for gamete fusion in several diverged species. Recent studies have suggested that HAP2 may require additional proteins to initiate gamete fusion, however, these proteins are currently unknown. It is also currently unknown whether HAP2 interacts with the egg and central cell via protein:protein interactions as no female interacting partners have been identified. We performed a forward genetic modifier screen using a hypomorphic version of HAP2 that is capable of initiating fertilization at a low rate, resulting in only 2-6 seeds and shorter siliques compared to wild type plants. By screening for suppressors of this small seed set phenotype, we have been able to identify several potential mutations in genes that might interact with HAP2 to create gamete fusion. Four of these mutations are in male-expressed genes and one is in a female-expressed gene. These genes might be some of the first genes identified that work with *HAP2* to fuse the membranes of gametes leading to fertilization and seed production.

Introduction

The merger of sperm and egg membranes is an essential step in fertilization, but in spite of the conservation of this process across sexually reproducing eukaryotes, a mechanism for gamete fusion has not been fully elucidated (Wong and Wessel, 2006). One major contributor to the difficulty of identifying critical factors in the process is that many of the proteins involved evolve very rapidly (Clark et al., 2006; Clark et al., 2009; Vacquier and Swanson, 2011). Biochemical assays are also difficult to perform as gamete fusion occurs very quickly (Hamamura et al., 2012) and isolation of high quantities of proteins can be difficult (especially in flowering plants).

Studying the process of gamete fusion in flowering plants is of great importance, as the products of fertilization generate a seed (Berger et al., 2008). In this system, two sperm cells must fuse with two female cells; the egg and the central cell. These two fusion events generate the embryo and the endosperm, respectively and is referred to as “Double Fertilization”. The endosperm is the primary constituent of seeds and contains the major nutritional components required in the human diet, and the diet of main livestock animals (Li and Berger, 2012). Any insight into gamete fusion in flowering plants therefore has great potential applied significance to seed production and agriculture.

Arabidopsis thaliana represents an excellent genetic model system for studying the process of gamete fusion. Although little success has come from biochemical assays in this system, genetic studies in Arabidopsis have identified the only known gene shown to be directly required for gamete fusion in flowering plants (von Besser et al., 2006). *HAP2* was identified in a forward genetic screen looking for factors that resulted in distorted mendelian segregation (Johnson et al., 2004) and has further been characterized as a sperm-specific gene required for male transmission and gamete fusion in Arabidopsis (Mori et al., 2006; von Besser et al., 2006). *HAP2* is also highly conserved and required for gamete fusion in the green algae *Chlamydomas reinhardtii* and the malaria-causing parasite *Plasmodium berhei* (Blagborough and Sinden, 2009; Liu et al., 2008; Mori et al., 2010). Despite the discovery of *HAP2* several years ago and this high conservation, mechanisms of how *HAP2* is mediating gamete fusion are not known. It is currently unknown whether *HAP2* is sufficient for gamete fusion or whether there are other unidentified factors that interact with *HAP2* to initiate gamete fusion. It is also unknown whether HAP2 is interacting with a protein(s) on the surface of the egg and central cell to initiate gamete fusion.

Recent data suggests that there are additional components of the *HAP2* mechanism required for gamete fusion. Egg Cell 1 (EC1) is a small, cysteine-rich peptide that is required for the activation of HAP2 (Sprunck et al., 2012). When EC1 expression is significantly reduced wild type sperm exhibit a *hap2*-like membrane fusion defect and are unable to fuse with either the egg cell or central

cell. Furthermore, incubation of sperm cells expressing HAP2:HAP2:YFP with synthetic EC1 peptide revealed that EC1 functions to localize HAP2:YFP to the sperm cell membrane (Sprunck et al., 2012). This observation suggests that HAP2 secretion to the membrane must be activated by EC1 upon contact with female gametes. Recent experiments also suggest that HAP2 may require additional components for fusion. When *HAP2* was expressed in *duo1* mutant sperm cells, which are incapable of fusion and lack *HAP2* expression, we saw that *HAP2* expression, the ability to fuse with female gametes was restored. However, this does not exclude the involvement of other sperm-expressed, non-*DUO1*-regulated genes (Thesis Chapter 3). *duo1* mutants lack expression of *HAP2* and ~63 other sperm-expressed genes (Borg et al., 2011), but sperm cells have been shown to express ~6000 unique genes, many of which could be interacting with HAP2 to initiate or enhance fusion function. Similar mechanisms exist in mammalian systems, where the testis-specific serine kinase (Tssk6) is required for the localization of the gamete-fusion protein IZUMO (Sosnik et al., 2009). It is possible that activators of HAP2 are present in Arabidopsis; however, additional sperm components of the *HAP2* fusion mechanism have not been identified.

We took a genetic approach to attempt to identify novel genes require for gamete fusion in Arabidopsis. Forward genetic screens have been used in Arabidopsis to identify many novel genes, including *HAP2* (Johnson et al., 2004; Page and Grossniklaus, 2002; Peters et al., 2003). We decided to utilize a hypomorphic

version of *HAP2* for the starting phenotype of this screen (*HAP2^{hypo})*. HAP2^{hypo} contains a neutralized C-terminal region that was generated by mutating positively charged amino acids to lysine or arginine (Wong et al., 2010). HAP2^{hypo} is able to rescue the *hap2-1* fusion defect at very low frequencies (9-14%), which indicates that *HAP2^{hypo}* encodes a partially functional HAP2. Self-fertilization of plants that were heterozygous for *hap2-1* and homozygous for *HAP2^{hypo}* produced rare progeny that were homozygous for *hap2-1* and *hap2^{hypo}* (*hap2-1/hap2-1, hap2^{hypo}/hap2^{hypo}:* herein referred to as *hap2^{hypo}*). Importantly, these plants have no wild type *HAP2* and only express *HAP2^{hypo}*. This genotype results in sperm that rarely fuse with female gametes, producing siliques (seed pods) that contain only 2-6 seeds instead of the normal 50-60 seeds/silique in wild type pollinations. These plants show no female defects and are able to produce full seed set when hand pollinated with wild type pollen (Wong et al., 2010). Pollen tubes from these plants are also able to target ovules successfully, burst, and release sperm cells as normal. Quantitative analysis of fertilization events also revealed that *hap2^{hypo}* generated both single (fail to develop into seeds) and double fertilization events (produces seeds), a pattern never seen in wild type or *hap2* mutants (Wong et al., 2010). This observation suggests that full HAP2 activity or amount at the sperm membrane is required for full gamete fusion and seed set (Figure 1).

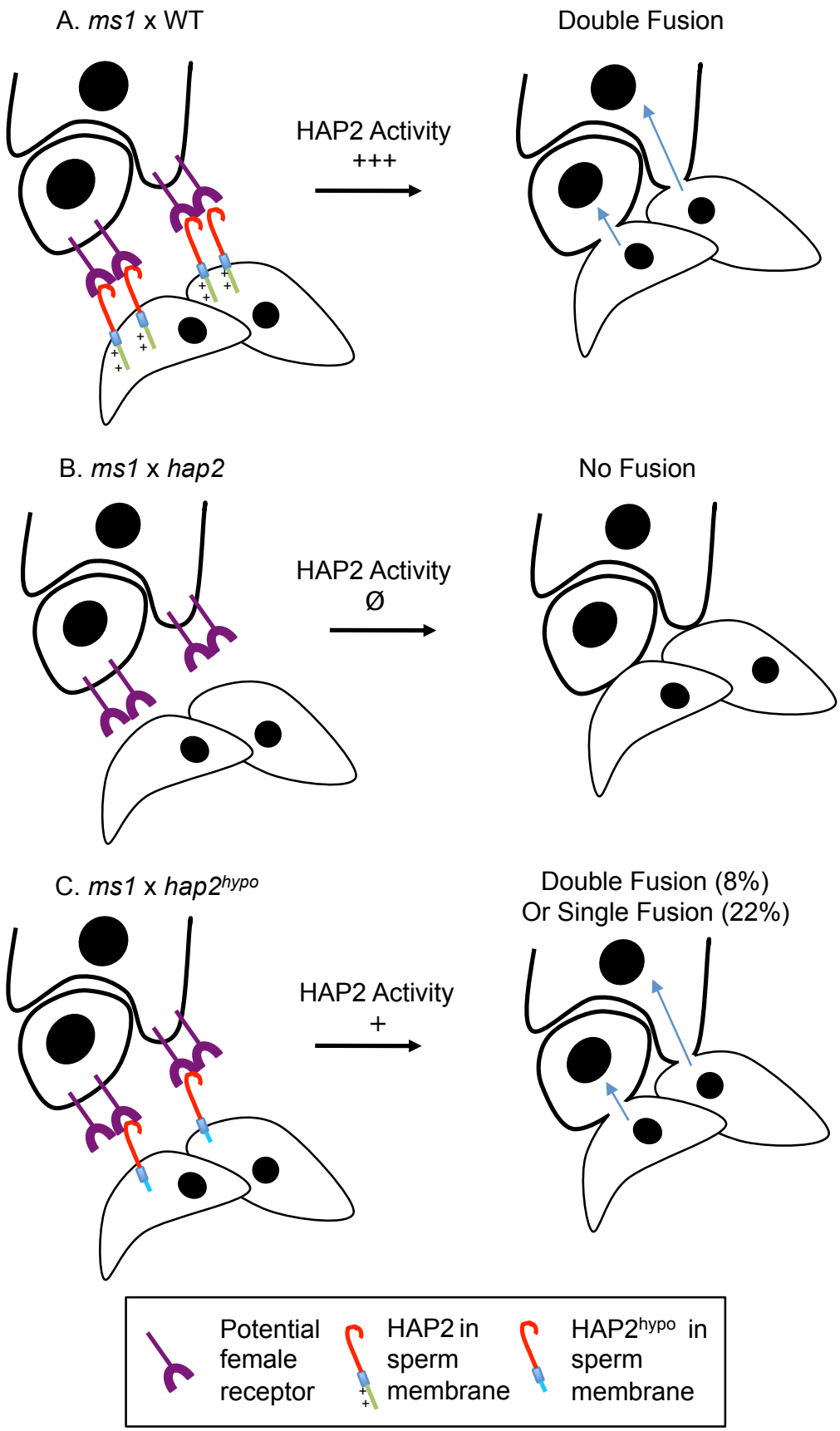

A. ms1 x WT
Double Fusion
HAP2 Activity
+++
B. ms1 x hap2
No Fusion
HAP2 Activity
Ø
C. ms1 x hap2hypo
Double Fusion (8%)
Or Single Fusion (22%)
HAP2 Activity
+
Potential female receptor
HAP2 in sperm membrane
HAP2hypo in sperm membrane

Figure 1. Schematic representing gamete fusion events in Wild type, *hap2* and *hap2^{hypo}*. *(A)* *male sterile 1 (ms1)* crossed with wild type (WT) pollen has full HAP2 activity (+++) and initiates double fertilization events, resulting in 50-60 seeds/silique. HAP2 on the sperm membrane in WT contain a longer C-terminus (CTR) that is highly positively charged (Green, +). WT HAP2 is shown interacting with a potential egg and central cell receptor (purple). Blue arrows indicate that sperm cell nuclei will migrate to female cell nuclei for karyogamy. **(B)** *ms1* crossed with *hap2* mutant pollen. *hap2* mutant sperm have no expression of HAP2 and therefore HAP2 is depicted as absent from the membrane. Lack of HAP2 results in sperm that are unable to fuse with either the egg cell or the central cell, resulting in no seeds. **(C)** *ms1* crossed with *hap2^{hypo}* pollen. HAP2^{hypo} is shown in the sperm membrane with a truncated, neutralized CTR (blue, no + symbols). There may be less HAP2^{hypo} at the sperm membrane, or HAP2^{hypo} may have a decrease in HAP2 activity compared to WT (activity + compared to +++). This results in single fertilization events or rare double fertilization events that produce 2-6 seeds/silique compared to WT.

We mutagenized *hap2^{hypo}* seeds and visually screened for plants that had an increase in silique length. Plants that are able to produce more seeds, and therefore have an increase in silique length would potentially have mutations in genes that interact with *HAP2* to initiate gamete fusion and suppress the *hap2^{hypo}* mutant phenotype (Suppressors of *hap2^{hypo}, sohos*). We have identified ~20 *sohos* and characterized six with the greatest increased seed number. We find that these *sohos* produce more seeds than *hap2^{hypo}* and that four likely effect male genes, while one is likely female-specific. Although map-based cloning is still in progress to identify these genes, this characterization suggests for the first time that there are both male and female expressed genes that are interacting with *HAP2* to initiate gamete fusion, perhaps by increasing activity of a female binding partner, or increasing the amount of HAP2^{hypo} at the sperm cell membrane.

Results

Live cell imaging of *hap2*^{hypo} sperm cell nuclei show single and double fertilization events within ovules

In order to use *hap2*^{hypo} as a basis for a suppressor screen, we first wanted to analyze its effect on gamete fusion. Previous studies showed that *hap2*^{hypo} sperm were capable of producing single fertilization events when products of fertilization were quantified using an ovule clearing method (Wong et al., 2010). In this assay, early embryo and endosperm development can be detected in ovules using DIC microscopy, however, sperm cell behavior at the site of gamete fusion cannot be visualized. *hap2*^{hypo} sperm were labeled using the nuclear sperm cell marker *HTR10::HTR10:mRFP* (Ingouff et al., 2007). The *HTR10:mRFP* signal diffuses after nuclear fusion with the egg and central cell nucleus, a step after gamete fusion (Figure 2A). *hap2*^{hypo}*, HTR10:*RFP pollen was crossed onto *ACT11:MSI1:GFP* female plants. *ACT11:MSI1:GFP* is an actin marker with a nuclear localization signal that marks the egg cell and central cell nuclei. Analysis of fertilization using confocal microscopy 21 hours after pollination revealed that the majority of *hap2*^{hypo}*,HTR10:RFP* sperm (74%, n=174) were incapable of gamete fusion, a phenotype identical to *hap2-1:RFP* (Figure 2B, C). A small percentage of ovules also contained sperm that only fused with the egg cell (Figure 2D, 11%), indicative of a single fertilization event. Only 0.6% of ovules contained a *hap2*^{hypo}*. HTR10:RFP* sperm that fused with only the central cell.

Both of these percentages are lower than the chloral hydrate quantifications (Wong et al., 2010). Finally, a small percentage of ovules contained *hap2*^{hypo}*,HTR10:RFP* sperm that were capable of double fertilization (Figure 2E, 8%). This analysis confirmed that *hap2*^{hypo} sperm are capable of fertilizing the egg and central cell at a low rate.

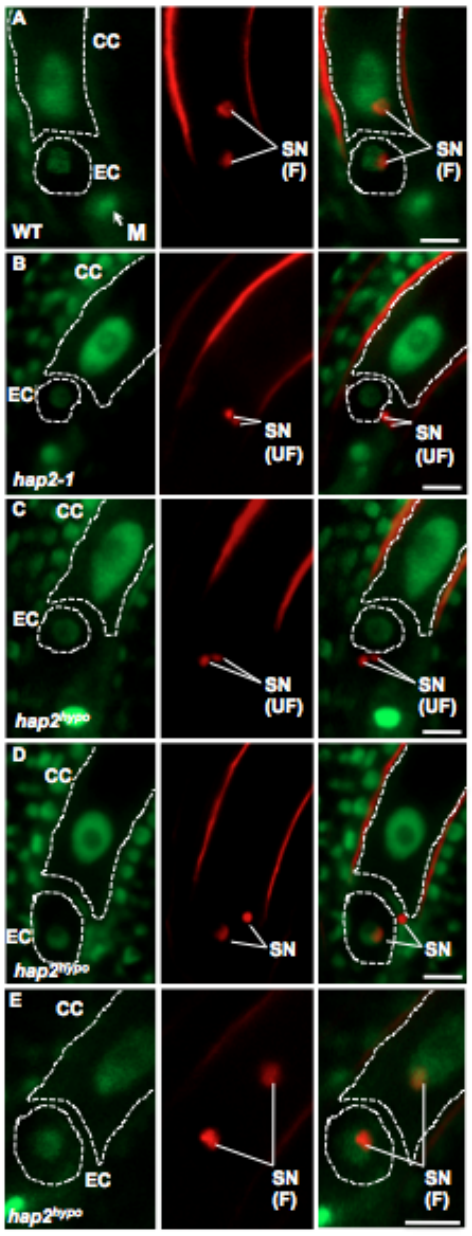


Figure 2. *hap2*^{hypo}*, HTR10:RFP* sperm cell nuclei are capable of both single and double fertilization events. CC: Central cell, EC: Egg Cell, M: Micropyle (entrance to ovule), SN: Sperm nuclei, F: Fused sperm nuclei, UF: Unfused sperm nuclei, WT: Wild type. **(A)** *HAP2, HTR10:RFP* pollen was crossed onto *ACT11:GFP* females and analyzed 21 hours after pollination using confocal microscopy. Sperm cell nuclei have migrated to overlap with both the egg cell nucleus and the central cell nucleus, indicative of successful membrane fusion. **(B)** *hap2-1, HTR10:RFP* sperm cell nuclei do not overlap with female cell nuclei and are incapable of gamete fusion. **(C)** *hap2*^{hypo}*, HTR10:RFP* sperm incapable of gamete fusion. **(D)** *hap2*^{hypo}*, HTR10:RFP* sperm single fertilization event. One sperm cell nuclei has fused with the egg cell nucleus but not the central cell nucleus. **(E)** *hap2*^{hypo}*, HTR10:RFP* sperm capable of double fertilization. RFP signal overlaps with the egg and central cell nuclei as WT (panel A). Scale bar: 100µm.

HAP2^{hypo} is localized to sperm cells similar to HAP2:YFP in pollen grains

It is possible that the single fertilization events observed using *hap2*^{hypo} are due to *hap2*^{hypo} sperm being less active than WT or potentially due to less functional HAP2 being present at the sperm cell membrane. To begin to analyze localization of HAP2^{hypo} we generated a *HAP2:HAP2*^{hypo}*:YFP* construct (Figure 3A).

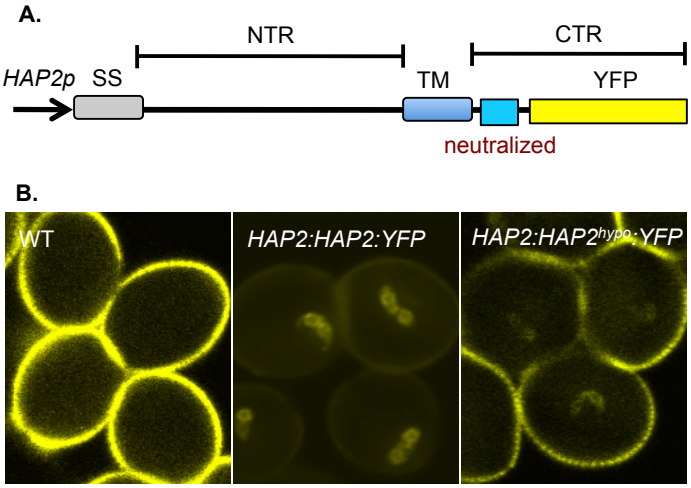


Figure3. *HAP2:HAP2^{hypo}:YFP* expression in mature pollen grains. (A) Schematic of construct used to generate *hap2^{hypo}:YFP*. YFP was added to the end of the truncated, neutralized CTR. **(B)** WT: Wild type pollen grains (Left) show autofluorescense in the pollen grain wall. Grains were over-exposed to ensure no YFP expression in sperm. *HAP2:HAP2:YFP* shows a perinuclear YFP expression in sperm cells (Center). *HAP2:HAP2^{hypo}:YFP* expression in pollen grains is similar to that of *HAP2:HAP2:YFP* (Right). All images were taken at the same exposure time.

YFP expression in mature pollen grains of *HAP2:HAP2^{hypo}:YFP* was similar to that of wild type *HAP2:HAP2:YFP,* showing a perinuclear pattern extending into the putative sperm plasma membranes and absence of signal in the pollen grain nucleus (Figure 3B). However, we do not yet know whether HAP2^{hypo} relocalizes to the sperm surface in a similar pattern to HAP2:YFP in response to EC1 secretion form the egg cell at the site of gamete fusion (Sprunck et al., 2012). .

Using *hap2^{hypo}* as the basis for a forward genetic modifier screen

Together, these data suggest that *hap2^{hypo}* may produce a partially functional version of HAP2. This results in pollinations using *hap2^{hypo}* as the male donor that produce only 2-6 seeds per silique, averaging ~ 3mm compared to 50-60 seeds in a silique measuring ~15mm (Figure 4A). We decided that this visual phenotype could be used as the basis for a forward genetic screen looking for *soho* mutations (Figure 4B).

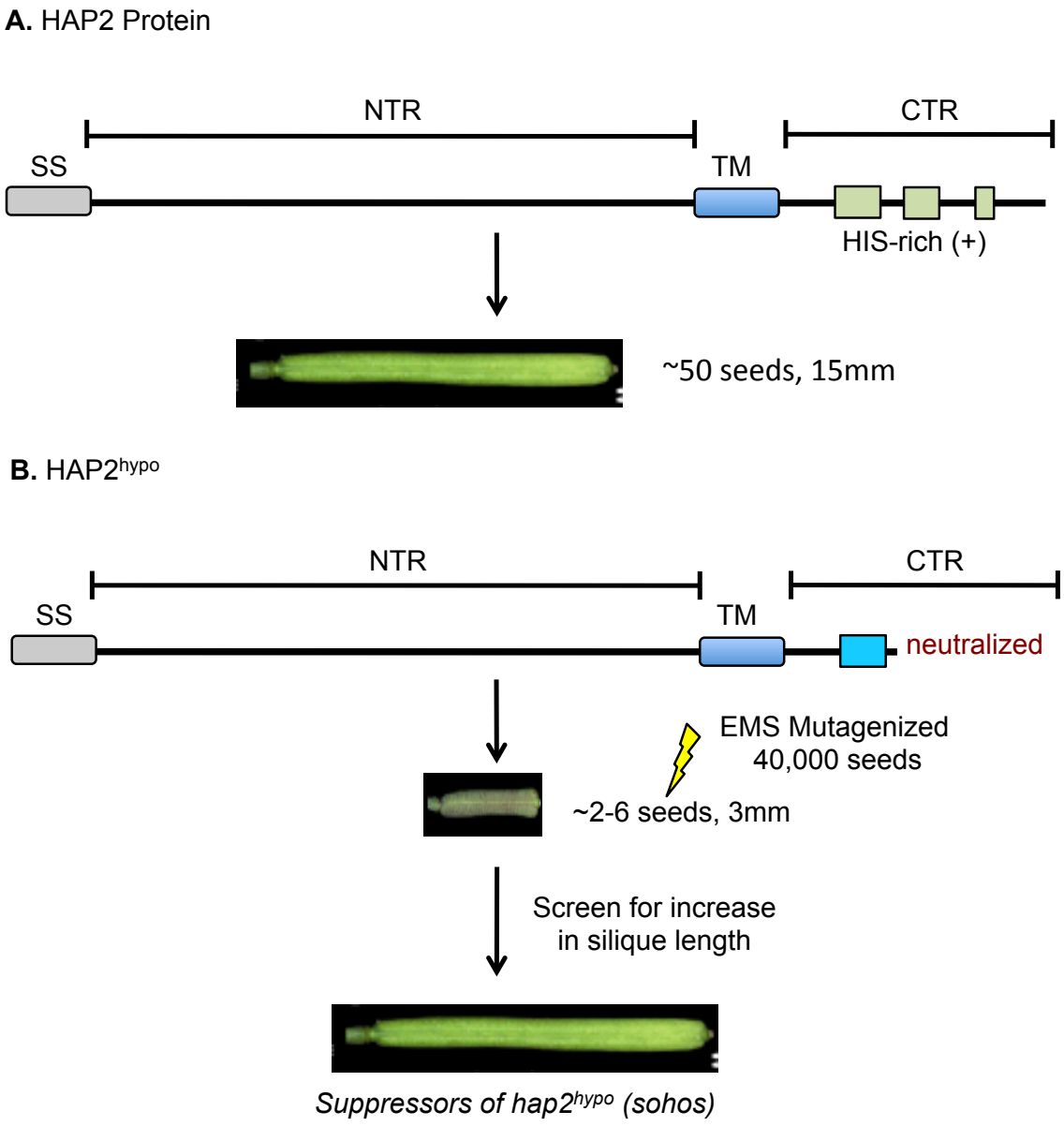


Figure 4. Protocol for forward genetic modifier screen using *hap2^{hypo}*. (A) The HAP2 CTR is composed of three histidine-rich regions (+, green boxes), making the CTR highly positively charged. Functional HAP2 produces ~50 seeds/silique with an average length of 15mm. **(B)** HAP2^{hypo} was generated by truncation and neutralization of the CTR (blue box). This alteration results in a partially functional HAP2 which generates only 2-6 seeds/silique and a shorter silique averaging 3mm. Approximately 40,000 *hap2^{hypo}* seeds were EMS mutagenized and M1 plants were screened for an increase in silique length, a visual tool for identifying potential mutants that may produce more seeds, suppressing the *hap2^{hypo}* mutant phenotype.

Approximately 40,000 *hap2*^{hypo} seeds were mutagenized by soaking in Ethyl methanesulfonate (EMS) and plated on selective media (Figure 4B). The first generation of mutagenized plants (M1) were screened for an increase in silique length over 8mm. False positives were identified as either wild-type contamination in mutagenized seed pools by genotyping, or as plants that had an increase in silique length but not actual seed production. M1 plants that contained more than 10 seeds/silique on average were then allowed to self-pollinate for two additional generations and seed set for each potential mutant was determined (Figure 5). We identified ~20 potential *sohos* from screening.

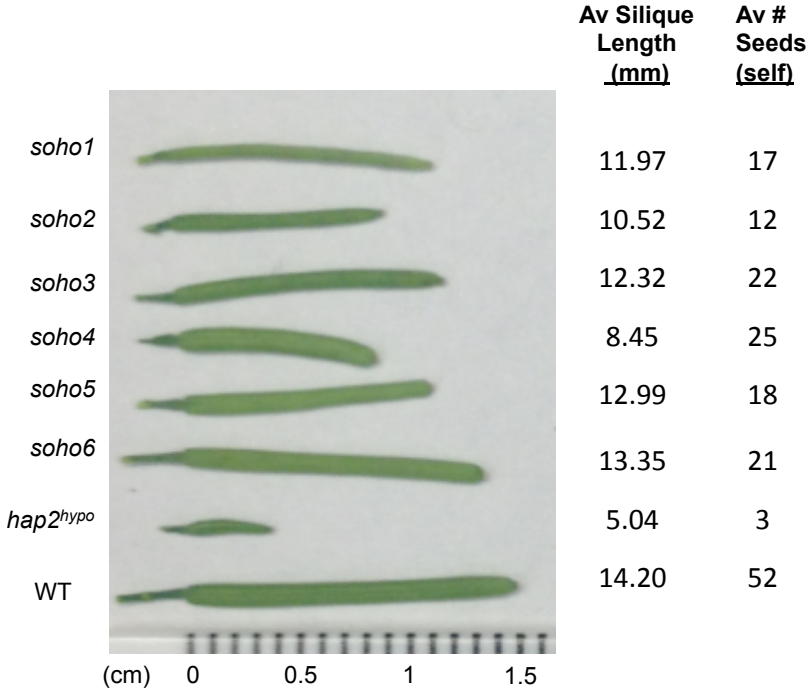


Figure 5. *sohos* have longer siliques and produce more seeds than *hap2*^{hypo}. Mature siliques from the top 6 *sohos* were measured (M3 generation, 10 siliques averaged) and compared to both Wild type (WT) and *hap2*^{hypo}. Average numbers of seeds per self-silique were also quantified (10 siliques averaged).

sohos contain mutations in male and/or female-expressed genes leading to an increase fertilization and seed set

Currently, there are no other male or female-specific genes known to function with *HAP2* in gamete fusion. Increased seed set for the top six *sohos* indicate that this screen has successfully identified additional genes that work to enhance fusion of $hap2^{hypo}$. In addition, these mutations are inherited, as the phenotype has returned in successive generations (we have analyzed the M3 generations for each *soho*). To determine whether these *sohos* were the result of a mutation in a male or female-expressed gene, we performed a series of crosses.

First, *sohos* were crossed onto *ms1* females. If this mutation is in a male-expressed gene, then this cross should generated an increase seed set greater than that of $hap2^{hypo}$ alone, which produces 2-6 seeds when crossed onto *ms1.* (Table 1). To determine if the *soho* contained a mutation in a female-expressed gene, we crossed un-mutated $hap2^{hypo}$ pollen onto each *soho*. If the *soho* x $hap2^{hypo}$ cross produces an increase in seeds, then this *soho* mutation is in a female-expressed gene. We determined that *soho2, soho3, soho4,* and *soho6* were male-expressed genes, and *soho5* contained a mutation in a female-expressed gene. Interestingly, *soho1* did not show an increased seed set in either of the mentioned crosses, but seed set from self-crosses showed increased seed production. This result indicates that *soho1* may only produce more seeds when mutations are present in both a male and a female-expressed gene.

Table 1. Seed set data for reciprocal crosses of *sohos* show mutations in both male and female expressed genes.

Female Cross	Seed Set	Male Cross	Seed Set	Self Cross	Seed set
soho1 x hap2^{hypo}	1.4 ±1[B]	*ms1 x soho1*	3.2 ±1.7[B]	*soho1 x soho1*	*16.9±5.6
soho2 x hap2^{hypo}	*5.6 ±1.5	*ms1 x soho2*	*20.9 ±8[M]	*soho2 x soho2*	*11.8±4.8
soho3 x hap2^{hypo}	2.6 ±1	*ms1 x soho3*	*16.5 ±4.8[M]	*soho3 x soho3*	*22.1±5.4
soho4 x hap2^{hypo}	3.7 ±1.7	*ms1 x soho4*	*29.0 ±10.2[M]	*soho4x soho4*	*25.0±3.5
soho5 x hap2^{hypo}	*15.5 ±5.7[F]	*ms1 x soho5*	*5.0 ±3.2	*soho5 x soho5*	*18.4±4.9
soho6 x hap2^{hypo}	*0.7 ±1	*ms1 x soho6*	*14.2 ±4[M]	*soho6 x soho6*	*20.7±3.2
*hap2^{hypo}*x *hap2^{hypo}*	2.6 ±1.6	*ms1 x HAP2^{hypo}*	3.2±2.1	*hap2^{hypo} x hap2^{hypo}*	*2.6±1.6
WT x WT	52.2±3.5	*ms1 x WT*	54.3±3.7	*WT x WT*	52.2±3.5

[M] Cross that indicates a *soho* that shows an increased seed set only when the *soho* is used as the male.

[F] Cross that indicates a *soho* that shows an increased seed set only when the *soho* is used as the female.

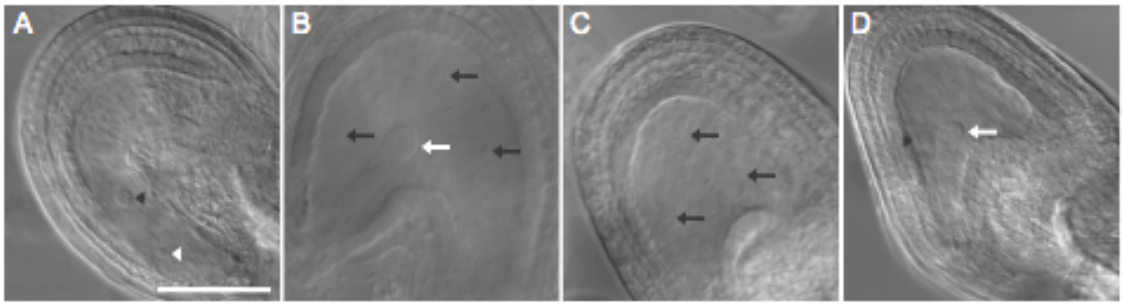
[B] *soho1* only showed increased seed set in self-crosses, requires both.

* p-value <0.05 shows statistical significance compared to corresponding control seed set. Student's t-test was used to calculate statistical significance.

Direct analysis of *soho1* fertilization reveals an increase in central cell only single fertilization events

Crossing data suggests that individual *sohos* are increasing double fertilization events and producing an increased number of seeds. However, *hap2^{hypo}* has been shown to also produce single fertilization events. It is possible that single fusion events are also increasing in the *sohos*, suggesting an increase in gamete fusion that cannot be quantified using seed set alone. To test this directly we

performed chloral hydrate analysis on a subset of *sohos* to determine whether there is an increase in egg cell or central cell only fusion events (Figure 6).



Cross	Unfertilized	Endosperm Only	Embryo Only	Double Fertilized
***ms1* x WT**	**3.7**	**0**	**0**	**92.7**
***ms1* x *hap2*^{hypo}**	**50.5**	**9.4**	**24.2**	**8.2**
ms1* x *soho1	**43.0**	**22.1**	**24.4**	**4.6**

Figure 6. Single fertilization events of the central cell increase in *soho1.* Chloral hydrate was used to clear *ms1* ovules two days after pollination; differential interference microscopy was used to determine whether the egg or central cell was fertilized. **(A)** An unfertilized ovule with egg cell and central cell nucleus. **(B)** A fertilized ovule with embryo development and endosperm proliferation. **(C)** Endosperm only fertilization event. Embryo development is absent but endosperm is proliferating normally. **(D)** Embryo only fertilization event. Distinct embryo is developing and the central cell remains unfertilized and not dividing. White arrowheads indicate unfertilized egg nuclei; white arrows indicate developing embryos. Black arrowheads indicate an unfertilized central cell nuclei; black arrows indicate proliferating endosperm nuclei. Scale bars, 50μm.

Chloral hydrate was used to clear *ms1* ovules that had been pollinated with *soho1* pollen two days after pollination. Ovules were quantified that were unfertilized (Figure 6A), successfully double fertilized (Figure 6B), or exhibiting single fertilization events where only the central cell was fertilized (Figure 6C) or only the egg cell was fertilized (Figure 6D). Interestingly, *soho1* showed a

significant increase in central cell only fertilization events compared to *hap2*^{hypo} pollen crossed onto *ms1* (22% vs. 9%). Although all other *sohos* showed a clear male or female gene mutation in reciprocal crosses that resulted in an increased seed set, it is possible that these *sohos* would also show an increase in embryo or endosperm only fertilization events. In the future, *sohos2-6* will also be tested using this chloral hydrate method.

Discussion

Currently, *HAP2* is the only sperm-specific gene shown to be required for the step of plasma membrane fusion of gametes during fertilization. Truncation and neutralization of the HAP2 CTR results in a hypomorphic version of HAP2 (HAP2^{hypo}*)* that can fuse in rare instances and produce both single and double fertilization events (Wong et al., 2010). These rare double fusion events produce an average of 3 seeds/silique, resulting in a shorter silique compared to wild type (Figure 5). Using this visual phenotype as the basis for a forward genetic modifier screen has identified several potential mutations in genes that may interact with *HAP2* to create gamete fusion. We have identified mutations in both male and female specific genes that suppress the *hap2*^{hypo} phenotype, resulting in enhanced seed production compared to unmutagenized *hap2*^{hypo} (Table 1).

A series of crosses revealed that *soho2, 3, 4* and *6* only showed an increase in seed set when used as the male (Table 1: *ms1* x *soho*). It is possible that mutations in some of these genes are resulting in more HAP2^{hypo} getting to the sperm cell membrane. This increase in HAP2^{hypo} at the membrane could enhance the fusion capabilities of these sperm (Figure 7). For example, if HAP2^{hypo} were not exiting the ER efficiently, then perhaps a mutation in an ER gene required for ER protein retention would result in more HAP2^{hypo} at the membrane. This mechanism has been seen in the analysis of the brassinosteroid receptor 1 (BR1), which is required for processing of brassinolide, a hormone required for cell elongation and division (Gonzalez et al., 2010; Li and Chory, 1997). Point mutations in *BR1* result in structurally defective, but biochemically functional BR1 receptor being partially retained in the ER (Wang et al., 2001). Since the response to brassinolide depends on the amount of BR1 at the membrane the altered amount of BR1 receptor produces dwarfed plants (Wang et al., 2001). A genetic screen using this *br1* mutant identified a suppressor of this dwarf phenotype caused by a point mutation within *EBS1* (*EMS-mutagenized bri1 suppressor 1*). Loss of *EBS1* activity actually prevents *bri1* from interacting with CNX (Calnexin, ER quality control protein), essentially reducing the strictness of the ER quality control checkpoint, and allowing more of this structurally faulty, but biochemically active *bri1* to reach the membrane (Jin et al., 2007). Perhaps one of the male *sohos* will encode a gene like *EBS1.*

It is also possible that HAP2^{hypo} is at the membrane at WT levels, but it is less active. In this case, *sohos* could encode for co-activators of *HAP2.* It has been shown in mouse that mitochondrial fusion increases when the nuclear co-activator PGC-1β is over-expressed (Liesa et al., 2008). Perhaps mutations in a *HAP2* co-activator would result in over-expression of such a co-factor and an increase in HAP2^{hypo} activity that would be sufficient to drive gamete fusion. It could also be possible that one of these male *sohos* codes for the receptor for the *EC1* activation of HAP2. *EC1* has recently been shown to be required for HAP2 localization to the sperm membrane and activation of fusion (Sprunck et al., 2012). Perhaps mutations in the sperm-expressed EC1-receptor would result in a constitutively active form, rendering *hap2^{hypo}* sperm more likely to fuse with the egg and central cells because more HAP2^{hypo} is present at the sperm membrane.

It will also be interesting to see if any of these male *sohos* are *DUO1*-regulated. Experiments have shown that expression of *HAP2* in *duo1* mutant sperm, which normally lack *HAP2* and fail to fuse, may be fully sufficient to rescue the fusion defect (Thesis Chapter 3). However, these results do not rule out the possibility that non-*DUO1*-regulated genes are functioning with *HAP2* to initiate gamete fusion. Once mapped, we would predict that all male candidate *sohos* would be non-*DUO1*-regulated genes.

This screen also identified a potential mutation in a female-specific gene, *soho5* (Table 1). This is an exciting finding, as a direct female binding partner for HAP2 has not been identified. Previous data suggests that HAP2 may participate in protein:protein interactions with the egg and central cell via the NTR (Wong et al., 2010). Analysis of transgenic plants expressing chimeric constructs revealed that if the entire NTR of Arabidopsis HAP2 is replaced with that of the closely related *Sysimbrium irio*, this chimera is still functional in Arabidopsis. However, if the NTR is replaced with that of distantly related Rice, fusion function is lost. This result suggests that there is sequence/species specificity that is required for interaction with the egg and central cell and that there may be a co-evolving female binding partner for HAP2. Perhaps *soho5* encodes for this receptor and the mutation is producing an over-active receptor on the female cells that is capable of compensating for the low fusion activity of HAP2^{hypo} (Figure 7).

Interestingly, *soho1* only exhibited an increased seed set in self-crosses, suggesting that this mutation might occur in a gene that is expressed in both male and female cells (Table 1). *soho1* also showed an increase in central cell only single fertilization events compared to unmutagenized *hap2*^{hypo} when crossed onto *ms1* (Figure 6). It would be interesting to see if an increase in egg cell only single fertilization events occurred when *soho1* is crossed with *hap2*^{hypo} pollen (used as the female). An increase would suggest that *soho1* regulates fusion from the male side with the central cell, and fusion with the egg cell is dependent on *soho1* expression from the female, or egg cell.

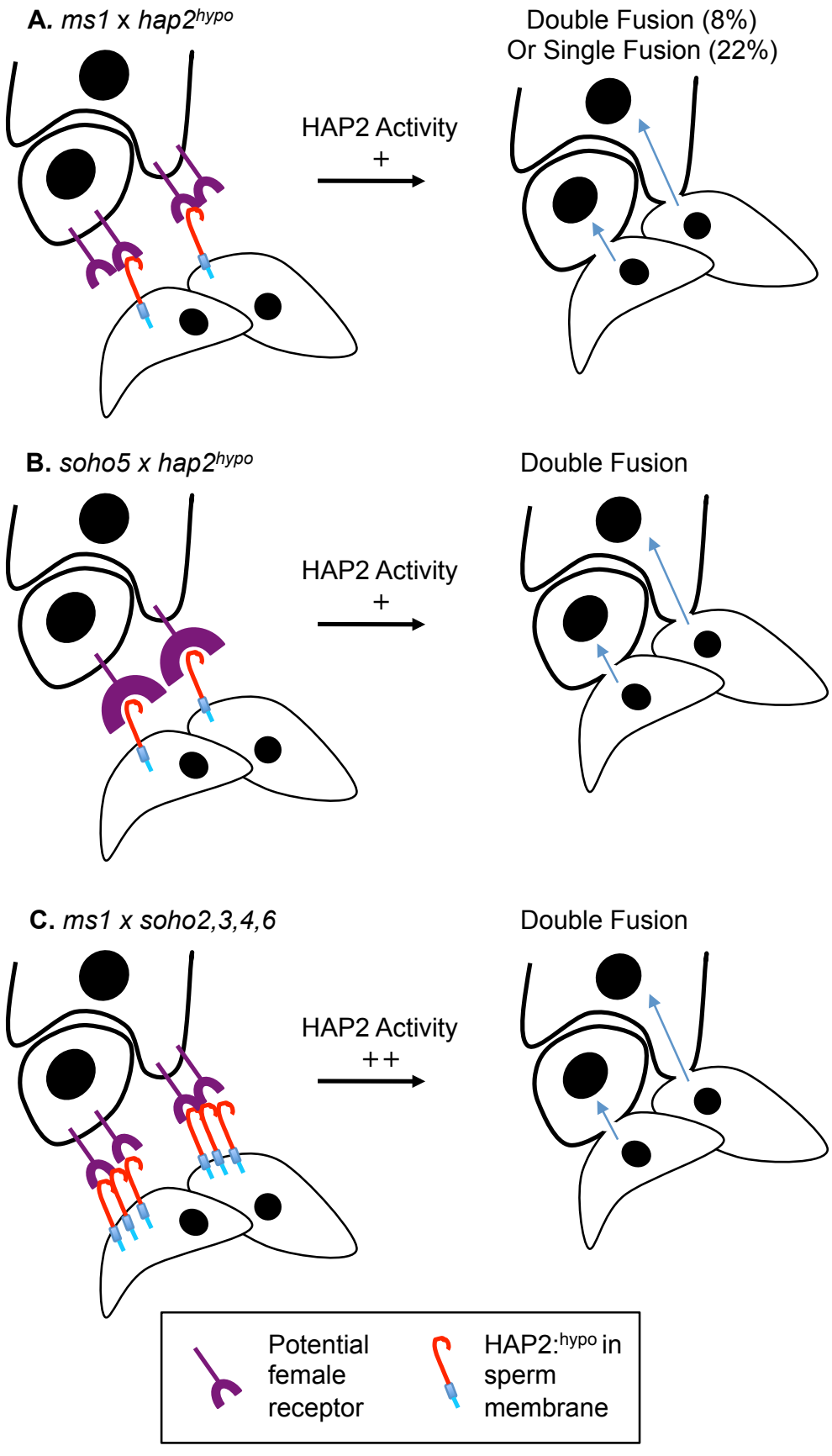

A. ms1 x hap2hypo
Double Fusion (8%)
Or Single Fusion (22%)
HAP2 Activity
+
B. soho5 x hap2hypo
Double Fusion
HAP2 Activity
+
C. ms1 x soho2,3,4,6
Double Fusion
HAP2 Activity
++
Potential female receptor
HAP2:hypo in sperm membrane

Figure 7. Schematic representing potential effects of *sohos* on gamete fusion. (A) *ms1* crossed with *hap2*^{hypo} pollen. HAP2^{hypo} is shown in the sperm membrane with a truncated, neutralized CTR (blue, no + symbols). There may be less HAP2^{hypo} at the sperm membrane, or HAP2^{hypo} may have a decrease in HAP2 activity compared to WT (activity + compared to +++). This results in single fertilization events or rare double fertilization events that produce 2-6 seeds/silique compared to WT. **(B)** *soho5* x *hap2*^{hypo} results in an increase in double fertilization events and an increase in seed set. This indicates that *soho5* may be a mutation in a female-expressed gene that encodes for a theoretical female HAP2 receptor (purple). Over-expression of this receptor may compensate for HAP2^{hypo} and initiate gamete fusion. **(C)** *ms1 x soho2, 3, 4,* and *6* results in increased double fertilization and seed set. These *sohos* may be the result of mutations in a male-expressed gene that bring more HAP2^{hypo} to the membrane, or increases HAP2^{hypo} activity at the membrane.

Although this screen has identified mutations that result in suppression of the *hap2*^{hypo} mutant phenotype, the identity of these genes is still unknown. Future experiments will prioritize positional cloning strategies to identify the gene disrupted by the suppressor mutation (Lukowitz et al., 2000), see experimental methods). To do this, a strain has been created that is polymorphic (in the Landsberg accession) to *hap2*^{hypo} (in the Columbia accession). This line will be homozygous Columbia only at the *hap2-1* and *HAP2*^{hypo} loci after several generations of introgression. All *sohos* will be crossed onto this polymorphic line and mapped to a region that is also homozygous for Columbia. This gene will then be sequenced. Future experiments will use reverse genetic analysis of these genes to determine whether they are required for gamete fusion.

Future experiments should also focus on further characterization of the *sohos* while the mapping line is being generated. For example, the sperm cell nuclear

marker *HTR10:RFP* (Ingouff et al., 2007) could be crossed into each *soho* for direct analysis of sperm cell behavior (Figure 2). Analysis of sperm cell nuclei during fertilization with these *sohos* may reveal more single fertilization events than are detected using chloral hydrate. This analysis might also reveal increases in polytubey (Reference Thesis Chapter 2). If a *soho* contains a mutation in a gene required for something like the prevention of polytubey or polyspermy, we would see an increase in the number of sperm contained within these ovules. Deposition of more sperm cells via polytubey would increase the chances of an ovule receiving *hap2*^{hypo} sperm that were capable of fusing, resulting in an increase in double fertilization through indirect mechanisms.

Finally, experiments should be conducted to determine the amount of HAP2^{hypo} that is present on the sperm cell membrane. This would provide insights into how these *soho* mutations are effecting the fusion of *hap2*^{hypo} sperm as discussed earlier. Western blots could be performed using *hap2*^{hypo} pollen to determine if there is less HAP2^{hypo} associated with membrane fractions than wild type. Furthermore, analysis of *HAP2:HAP2*^{hypo}*:YFP* (Figure 3) within ovules could provide insight into how HAP2^{hypo} localization might change after exposure to female factors like EC1. All of these experiments will help build a model for how *HAP2* is working with these novel genes to promote gamete fusion.

Acknowledgements

This work was supported by the National Science Foundation through grant no. IOS-1021917 to M.A.J. and a Graduate Research Fellowship to K.M.B. K.M.B was initially supported by a National Institute of Health Brown University Initiative to Maximize Student Development grant (NIGMS Grants: R25GM083270 and R25GM083270-S1). We would also like to thank Dr. Julian Wong for construction and previous analysis of *HAP2*^{hypo} , as this was the basis for the screen.

Experimental Methods

Growth of plants

ms1/MS1 plants were obtained from the Arabidopsis Biological Resource Center (stock #CS261) and maintained as a heterozygous line. *hap2*^{hypo} plants were maintained as homozygous lines and grown on Kanamycin (50µg/ml) and Basta (50µg/ml) plates. Seedlings were transported to sterile 2MIX potting medium 7 days after plating. All plants were grown at 20°C, 50-60% humidity, on a 16day/8hour night/light cycle in growth chambers.

Mutagenesis of *hap2*^{hypo} seeds

Seeds were collected in bulk from plants that were confirmed to be homozygous for *hap2-1* and *hap2*^{hypo}. Approximately 5,000 seeds (approximated by weight of 300 seeds) were soaked in Ethyl methanesulfonate (EMS, Sigma-Aldrich) for 16hrs, covered in tin foil, at room temperature. The concentration of EMS to use was determined by calculating a death curve using wild type seedlings. The concentration that resulted in 50% death of seedlings was used for the screen (0.4%EMS added to 12.5ml of H2O). Seeds were washed 3-4x with H20 and EMS waste was collected into an equal volume of 1M NaOH to neutralize. Seeds were then resuspended in 0.1% sterile agar and plated on large Kan/Basta media plates. Resistant seedlings were then transferred to flats containing soil in

a 98-grid pattern. 5,000 seeds were mutagenized at a time; several rounds of this screening method were employed to approximate 40,000 seeds.

Screening of plants

Seedlings that had been transplanted to soil were allowed to grow for several weeks until ~10 siliques per plant could be measured. Plants were visually screened for an increase in silique length. Plants that appeared to have an increase in silique length had their siliques measured using digital calipers for accuracy. If 10 siliques in a row had a silique length >8mm these plants were then transplanted to individual pots. Once siliques were dry, actual seed set was quantified by removing seeds from siliques. Plants that produced more seeds than *hap2*^{hypo} plants were saved for further analysis and allowed to self-pollinate for several generations. M3 plants were then screened for increase in silique length and increased seed production.

Performing crosses to determine whether a *soho* was the result of a mutation in a male or female gene

sohos were emasculated one day prior to crossing with *hap2*^{hypo} pollen (~10 crosses/*soho*). Stage 14 *ms1/+* stigmas were pollinated with each *soho* (~10 crosses/*soho*). All crosses were allowed to mature fully and dry. Once dry, siliques were collected and seed set was quantified.

Generation of mapping population for positional cloning of *sohos*

Unmutagenized *hap2*^{hypo} plants (in the Columbia; Col background) were crossed with Landsberg erecta (Ler) pollen. Progeny from this cross are heterozygous for Col polymorphisms. F1 plants were then backcrossed to Ler over several generations (~6) to generate plants that were only homozygous for Col at the *hap2-1* and *hap2*^{hypo} loci. Each generation was checked with specific marker SSLP PCR primers that detect size differences between Col and Ler at 25 different locations across the 5 Arabidopsis chromosomes (Jander et al., 2002). DNA samples from this PCR reaction were run on 3% agarose gels (2% NuSieve™ GTG™ low melting agarose (Lonza, catalog #50084), 1% ultra-pure agarose (Invitrogen, catalog # 16500500) at 80v for 1 hr and compared against a low molecular weight DNA ladder (NEB, catalog #N3233L). Differences in size fragments between Col and Ler were compared against known marker primer products. Lines that were the most Ler were continued in the backcrossing. Eventually, each *soho* will be crossed onto this line for position based cloning (Lukowitz et al., 2000).

References

Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in plant science 13:437-443.

Blagborough, A.M., and Sinden, R.E. (2009). Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187-5194.

Borg, M., Brownfield, L., Khatab, H., Sidorova, A., Lingaya, M., and Twell, D. (2011). The R2R3 MYB transcription factor DUO1 activates a male germline-specific regulon essential for sperm cell differentiation in Arabidopsis. The Plant cell 23:534-549.

Clark, N.L., Aagaard, J.E., and Swanson, W.J. (2006). Evolution of reproductive proteins from animals and plants. Reproduction (Cambridge, England) 131:11-22.

Clark, N.L., Gasper, J., Sekino, M., Springer, S.A., Aquadro, C.F., and Swanson, W.J. (2009). Coevolution of interacting fertilization proteins. PLoS genetics 5:e1000570.

Gonzalez, N., et al. (2010). Increased leaf size: different means to an end. Plant physiology 153:1261-1279.

Hamamura, Y., Nagahara, S., and Higashiyama, T. (2012). Double fertilization on the move. Current opinion in plant biology 15:70-77.

Ingouff, M., Hamamura, Y., Gourgues, M., Higashiyama, T., and Berger, F. (2007). Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Current biology : CB 17:1032-1037.

Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L. (2002). Arabidopsis map-based cloning in the post-genome era. Plant physiology 129:440-450.

Jin, H., Yan, Z., Nam, K.H., and Li, J. (2007). Allele-specific suppression of a defective brassinosteroid receptor reveals a physiological role of UGGT in ER quality control. Molecular cell 26:821-830.

Johnson, M.A., et al. (2004). Arabidopsis hapless mutations define essential gametophytic functions. Genetics 168:971-982.

Li, J., and Berger, F. (2012). Endosperm: food for humankind and fodder for scientific discoveries. The New phytologist 195:290-305.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90:929-938.

Liesa, M., et al. (2008). Mitochondrial fusion is increased by the nuclear coactivator PGC-1beta. PloS one 3:e3613.

Liu, Y., et al. (2008). The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes & development 22:1051-1068.

Lukowitz, W., Gillmor, C.S., and Scheible, W.R. (2000). Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. Plant physiology 123:795-805.

Mori, T., Hirai, M., Kuroiwa, T., and Miyagishima, S.Y. (2010). The functional domain of GCS1-based gamete fusion resides in the amino terminus in plant and parasite species. PloS one 5:e15957.

Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.

Page, D.R., and Grossniklaus, U. (2002). The art and design of genetic screens: Arabidopsis thaliana. Nature reviews. Genetics 3:124-136.

Peters, J.L., Cnudde, F., and Gerats, T. (2003). Forward genetics and map-based cloning approaches. Trends in plant science 8:484-491.

Sosnik, J., et al. (2009). Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. Journal of cell science 122:2741-2749.

Sprunck, S., Rademacher, S., Vogler, F., Gheyselinck, J., Grossniklaus, U., and Dresselhaus, T. (2012). Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science (New York, N.Y.) 338:1093-1097.

Vacquier, V.D., and Swanson, W.J. (2011). Selection in the rapid evolution of gamete recognition proteins in marine invertebrates. Cold Spring Harbor perspectives in biology 3:a002931.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S., and Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. Nature 410:380-383.

Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS genetics 6:e1000882.

Wong, J.L., and Wessel, G.M. (2006). Defending the zygote: search for the ancestral animal block to polyspermy. Current topics in developmental biology 72:1-151.

Chapter 5: Determining Critical Regions within the HAP2 N-terminus Required for Fusion Function

Beale, KM, Leydon, ARL and Johnson, MA. This chapter is currently unpublished and I performed all experiments presented. ARL aided with western blots of pollen membrane fractions.

Abstract

The fusion of membranes is essential for several processes such as viral entry into host cells, vesicular fusion, and gamete fusion. However, the mechanisms by which membranes overcome the electrostatic barriers to fusion remain unknown. HAP2 is a broadly conserved membrane protein that is required for gamete fusion in the flowering plant Arabidopsis, the green alga *Chlamydomonas reinhardtii* and the malaria-causing parasite *Plasmodium*. Previous work showed that swapping the Arabidopsis N-terminal region (NTR) with that of the closely related *Sysimbrium irio* NTR was able to rescue the fusion defect of *hap2* mutant sperm when expressed in Arabidopsis. On the other hand, swapping the NTR with that of the distantly related *Oryza sativa* resulted in a disruption of fusion function. Although the NTR of HAP2 has been shown to be required for this class-level gamete fusion specificity, the protein currently lacks any defined domains of function. We identified a highly conserved domain within the N-terminus of HAP2, the HAP2/GCS1 domain, and mutated amino acids within this region that potentially contribute to gamete fusion specificity. We found that these amino acids are not required for the fusion function of HAP2. Using a bioinformatics approach, we also show that HAP2 is most structurally similar to known viral fusogens, providing important insights into how HAP2 might be mediating gamete fusion.

Introduction

Cell to cell fusion is essential for several key developmental processes such as gamete fusion, the development of the epidermis, muscle, bone and placenta and occurs in a wide variety of organisms (Chen and Olson, 2005). Mechanisms of plasma membrane fusion between cells are currently best understood in the context of viral entry into host cells and in vesicle fusion, but much less is known about how gamete membranes fuse. For example, in order for a virus to successfully release its genome into the host cell, the virus must provide sufficient amount of energy to overcome the electrostatic barriers that normally prevent fusion of two negatively charged lipid bi-layers. This process is driven by viral fusion proteins like the HIV envelope protein, the influenza virus HA protein, and the paramyxovirus F proteins (Dutch et al., 2000). Although diverse, these proteins share common characteristics such as heavy glycosylation, requirement of proteolytic cleavage for activation, and presence of a hydrophobic fusion peptide that is inserted into the target membrane (Dutch et al., 2000). Often, decreases in pH and acidification mediate a conformational change that exposes the normally buried fusion peptide for membrane fusion with the host cell (Hamilton et al., 2012).

Membrane fusion is also required for intracellular processes such as the fusion of membranous organelles (Sudhof and Rothman, 2009). Vesicular and target-localized SNARE proteins fuse opposing membranes by interacting and

“zippering” up into an α-helical bundle (McNew et al., 2000). This generates an inward force vector that pulls the two membranes together by shear mechanical force (Min et al., 2013). In spite of the extensive research on plasma membrane fusion in the context of virus-host cell fusion and vesicular transport, the mechanisms mediating *cell-cell* fusion, including the mechanisms mediating gamete fusion during fertilization, have not been fully elucidated in any system.

With the evolution of sexual reproduction, cell-cell fusion, or more specifically fusion of male and female gametes, became required for genetic diversity in the next generation (Barrett, 2010). In flowering plants, the fusion of plasma membranes between sperm and egg cell (gives rise to the zygote) and sperm and central cell (gives rise to the endosperm) is required for the development of a seed (Berger et al., 2008). An experimental inroad into the process of gamete fusion is the sperm-specific gene *HAP2* (Mori et al., 2006; von Besser et al., 2006). *hap2* mutant sperm are released from pollen tubes, but their nuclei never migrate to either the egg cell or central cell nuclei, indicative of a gamete fusion defect (Beale et al., 2012; Hamamura et al., 2011; Kasahara et al., 2012). *HAP2* orthologs have also been identified in plant, animal, and protozoan eukaryotic clades, including the green algae Chlamydomonas and the malaria-causing parasite Plasmodium (Blagborough and Sinden, 2009; Liu et al., 2008). Fusion defects caused by loss of *HAP2* function have been observed in three species and expression thus far has been shown to be specific to one gamete (Wong and Johnson, 2010). The conservation of gamete specificity and fusion defects

in these extremely divergent eukaryotes highlights the importance of *HAP2* in fertilization and points toward a conserved mechanism in plasma membrane fusion.

Arabidopsis *HAP2* encodes a 705 amino acid protein containing a signal sequence and a single predicted transmembrane domain that divides the protein into a large extracelluar N-terminal region (NTR) and a smaller intracellular, highly positively charged C-terminal region (CTR). The NTR also contains 16 highly conserved cysteines residues, and six predicted glycosylation sites. However, the NTR and CTR lack characterized functional motifs. Several HAP2 variants were tested for their ability to rescue the *hap2* fusion defect and it was determined that replacement of the Arabidopsis (*At*) HAP2 CTR either with the HAP2 CTR from the same family (*Sysimbrium irio,* London Rocket, *Si*) or with the HAP2 CTR from a distantly related species (*Oryza sativa,* Rice, *Os)* resulted in a functional HAP2 protein in Arabidopsis (Wong et al., 2010). Subsequent experiments showed that positive charge, not primary amino acid sequence is the important feature of the CTR (Wong et al., 2010). Exchange of the Arabidopsis NTR with *Si,* which is 89% identical with *At*, was also fully functional. However, when the NTR from *Os*, which is 59% identical with *At* was exchanged with the *At* NTR, the chimera did not rescue the *hap2* mutant defect (Wong et al., 2010). These experiments indicate that primary sequence is critical for NTR function and that perhaps HAP2 interacts with the egg and central cell via a “lock and key” mechanism (Figure 1).

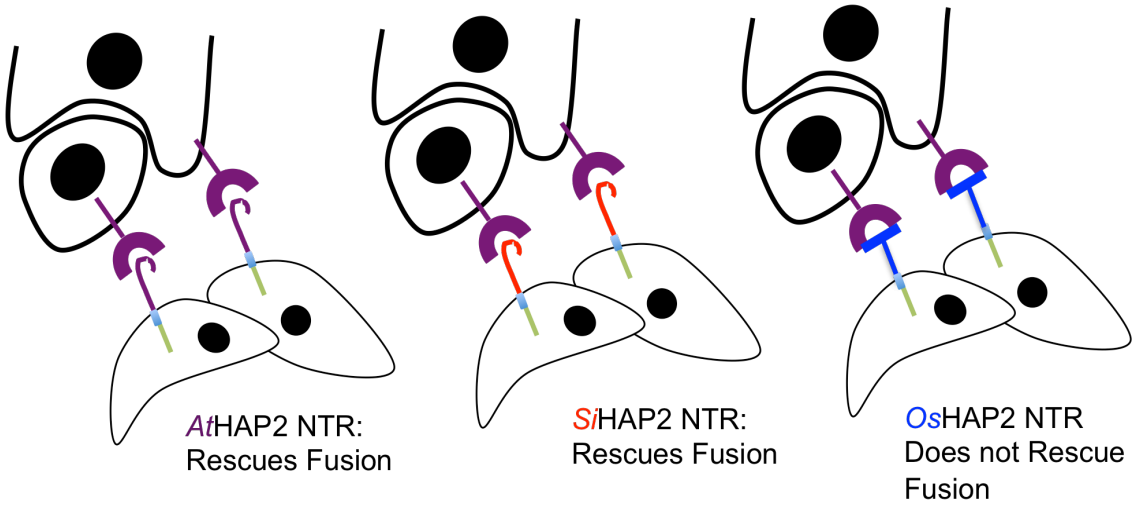


Figure 1. Replacement of the Arabidopsis NTR with that of *Si* or *Os* shows class-level specificity. Functional *At*HAP2 NTR (in sperm; purple) is able to rescue the fusion defect when transformed into *hap2* mutant plants, resulting in sperm that are now able to fuse with the egg and central cell (via interactions with a potential egg and central cell receptor (purple). Replacement with the *Si* NTR (red), which is within the same class as *At*, is also able to rescue the fusion defect of *hap2.* Replacement with the *Os*NTR (blue) results in a non-functional HAP2 that cannot rescue the fusion defect of *hap2. Os*HAP2 is shown no longer being able to interact with the female cell receptors, representing disruption of the "lock and key" model. HAP2 CTR: green, intracellular. TM: light blue, in sperm cell membrane.

The NTR also contains a region of high conservation, the HAP2/GCS1 domain (HGD). Experiments in which this domain is disrupted with insertion of GFP results in a non-functional HAP2 (Mori et al., 2010), however specific amino acids within the NTR that are required for function are currently unknown.

We sought to determine whether there were specific amino acids within this highly conserved HGD that were required for the species specific fusion of HAP2. By aligning the HGD between *At, Si,* and *Os* we determined that there

were only four amino acids that were the same in *At* and *Si* but different in *Os* within this region. We mutated these variant amino acids to either the *Os* equivalent or Alanine and tested for the ability of this construct to rescue the *hap2* fusion defect. We found that changing these amino acids to the *Os* equivalent or Alanine did not disrupt HAP2 function, suggesting that these amino acids were not responsible for the class-level specificity of HAP2. This result suggests that other residues within the HGD are critical for function, or that specificity of fusion function resides outside of the HGD. Furthermore, predicted structural comparisons between HAP2 and solved protein structures suggest that the fusion function of HAP2 may be located N-terminal to the HGD, as this region is most structurally similar to known viral fusogens. These results highlight the importance of HAP2 structure in addition to conserved primary sequence.

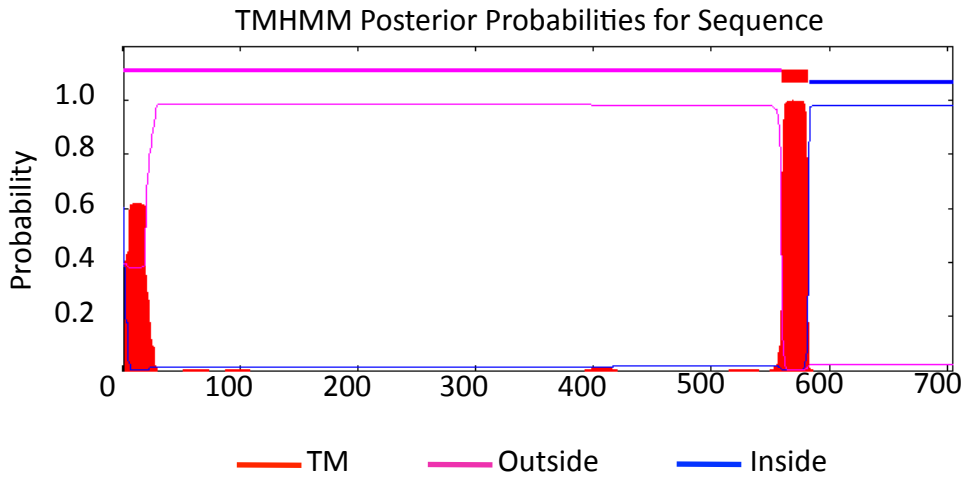
Results

HAP2 is a highly conserved membrane protein

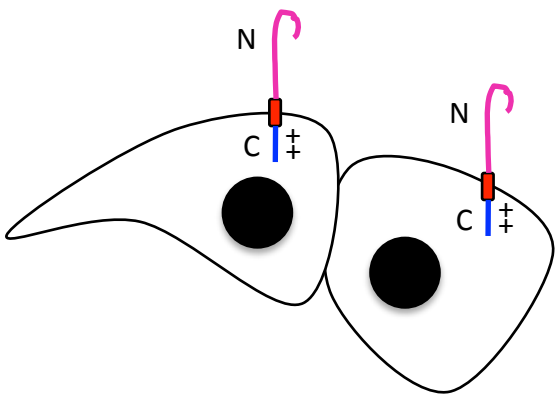
Loss of function of *HAP2* has revealed its requirement for fertilization in Arabidopsis, Chlamydomonas, and Plasmodium, however, mechanisms for fusion function are still unknown. Initial detection of *HAP2* expression using a

HAP2 promoter driving a HAP2:YFP fusion protein showed that HAP2 was only present in mature sperm and was expressed in the perinuclear space with the signal extending into the cell periphery (von Besser et al., 2006). We set out to determine if HAP2 was a membrane protein in Arabidopsis. Sequence analysis of HAP2 using the transmembrane prediction software, TMHMM Server v.2.0 (Krogh et al., 2001), predicts that this protein contains a single transmembrane domain with an extracellular NTR and intracellular CTR (Figure 2A). This suggests that the NTR would be located outside of the sperm cell membrane and the part of the protein that might interact with the female cell membranes (Figure 2B).

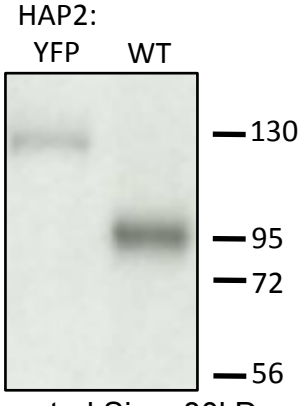
A.



B.



C.



Expected Size: 80kD

Figure 2. HAP2 is a predicted membrane protein that is present in membrane fractions from pollen. (A) Topology prediction for HAP2 generated using the TMHMM 2.0 server for predicting transmembrane (TM, red) helices in proteins. This software predicts a single TM domain from amino acids 560-583. Sequence analysis predicts the HAP2 N-terminal region (N, outside, pink) is extracellular to the membrane and the C-terminal region (C, inside, blue) is intracellular. The signal from amino acids 1-25 is a predicted signal sequence. **(B)** Schematic of predicted HAP2 within the sperm cell membrane. Colors correspond to A. **(C)** Western blot of pollen membrane fractions for Wild type (WT, right lane) and a *hap2-1/hap2-1* homozygous line that contains a HAP2::HAP2:YFP rescue construct (HAP2:YFP, left lane). A HAP2 α–NTR antibody detects HAP2 in WT pollen membrane fractionations and in HAP2:YFP samples. HAP2:YFP is ~35 kD larger due to addition of YFP.

In order to test whether HAP2 was a membrane-associated protein, we performed western blots on pollen membrane fractions. Although this assay cannot distinguish between sperm cell plasma membranes and ER membranes, detection of HAP2 in this membrane fraction would suggest this protein is processed as a membrane-associated protein. Localization of *HAP2:HAP2:YFP* already excludes HAP2 from being associated with pollen grain membranes, which could also be present in this sample preparation. Both wild type HAP2 and HAP2::HAP2:YFP was successfully detected in membrane fractions from Arabidopsis pollen preparations using a HAP2 α–NTR antibody (Figure 2C). The HAP2:YFP fraction was isolated from plants in which *HAP2::HAP2:YFP* was expressed in *hap2-1* homozygous plants lacking *HAP2* expression. This construct was shown previously to rescue the *hap2* fertilization defect, and is detected in pollen membrane fractions like wild type HAP2 (Figure 2C, left). This blot also shows for the first time that *hap2-1* is a null allele, as the endogenous HAP2 band is not detected in the *hap2-1/hap2-1, HAP2::HAP2:YFP* sample. Based on primary sequence HAP2 is predicted to be ~80kD and is migrating

higher than expected in both pollen samples (HAP2:YFP is predicted to migrate at 107kD due to YFP addition) suggesting potential post-translational modifications. Together, these analyses suggest that HAP2 is a membrane-associated protein.

HAP2 contains a highly conserved HGD that may contain regions required for fusion class-level specificity

Orthologs of *HAP2* are present in the green algae *Chlamydomonas reinhardtii,* the malaria-causing parasite *Plasmodium berghei* and the animals *Hydra magnipapillata* and *Nematostella vectensis.* These orthologs all share the common predicted primary structure, with a larger NTR, single TM domain, and smaller CTR (Figure 3A). In order to test specific amino acids that might be required for the species-specificity observed using the chimeric HAP2 constructs, we decided to perform HAP2 alignments to determine regions of highest conservation (Figure 3B). Sequence alignments between *At*HAP2 and several diverged eukaryotes revealed a region of highest conservation, the HAP2/GCS1 domain (HGD). In Arabidopsis, this region is located between amino acids 280-328 (Figure 3B).

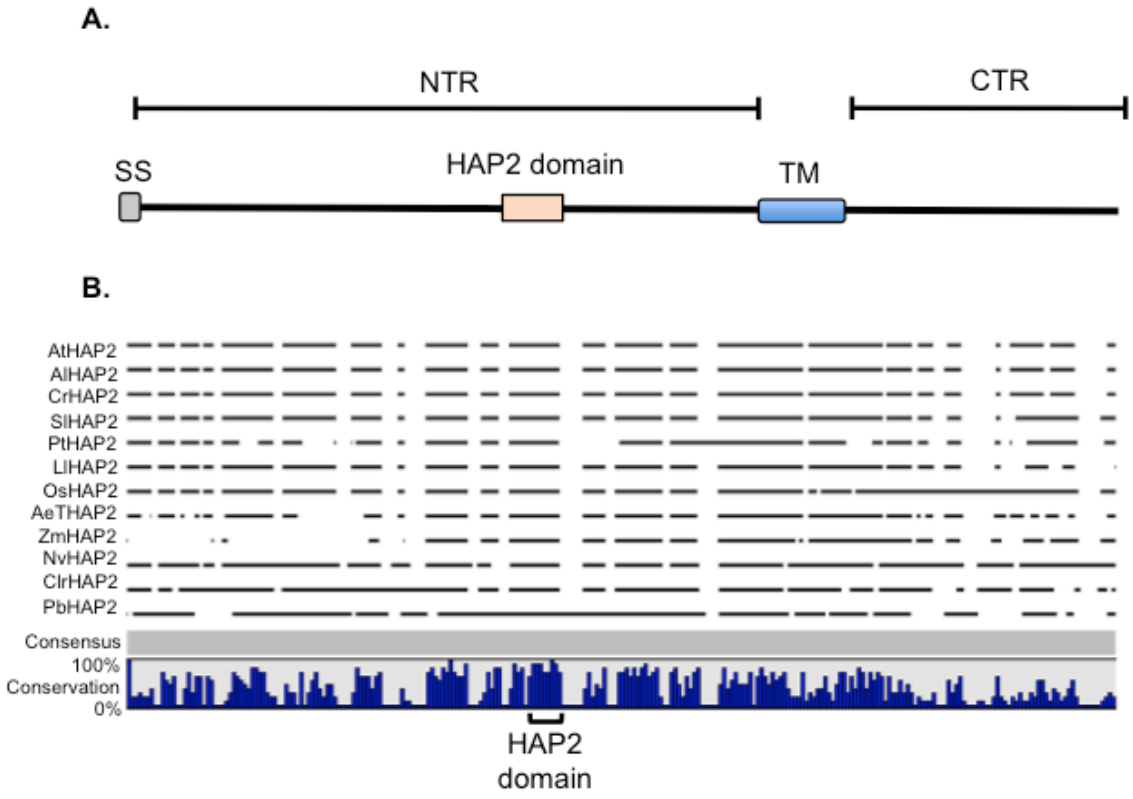


Figure 3. The HGD is the region of highest conservation across various eukaryotes. (A) Predicted HAP2 structure for Arabidopsis. SS: Signal sequence. NTR: N-terminal region, extracellular, TM: Transmembrane domain, CTR: C-terminal region, intracellular. The "HAP2 domain" in Arabidopsis is located in the NTR from amino acids 280-328 (orange box). **(B)** Corresponding protein sequence alignments across various eukaryotes. Lines denote regions of sequence alignment, gaps indicate no sequence alignment. Conservation is ranked from 0-100% (blue bars). The HAP2 domain (indicated with black brackets) represents the region of highest conservation. Numbers on the right indicate amino acid sequence length. At: *Arabidopsis thaliana*, Al: *Arabidopsis lyrata*, Cr: *Capsella rubella*, Sl: *Solamun lycopersicum* (Tomato), Pt: *Populus tricocarpa* (Poplar), Ll: *Lilium longiflorum* (Lily), Os: *Oryza sativa* (Rice), AeT: *Aegilops tauschii* (Goatgrass), Zm: *Zea mays* (Corn), Nv: *Nematostella vectensis* (Sea Anemone), Clr: *Chlamydomonas reinhardtii* (Green Algae), Pb: *Plasmodium berghei* (Malaria Parasite).

Since this region is conserved, it was hypothesized that this region would be critical for function. We set out to determine whether specific amino acids within the HGD were involved in determining class-level specificity of HAP2 function.

Determining specific amino acids within the HGD required for gamete fusion

In order to choose which amino acids to mutate within the HGD that might be important for putative interactions with the egg and central cell we used sequence comparisons between Arabidopsis (*At*), the closely related *Sysumbrium irio* (*Si,* London Rocket) and the distantly related *Oryza sativa* (*Os,* Rice). Since replacement of the *At*NTR with the *Os*NTR disrupted fusion function but the *Si*NTR did not, we hypothesized that there might be specific amino acid differences within the NTR that are required for class-specific fusion function that would be the same in *At* and *Si*, but different in *Os*. Alignments of these primary sequences revealed that there were 111 such differences that would result in non-conservative changes throughout the entire NTR (Sup Figure1). Importantly, this alignment also highlighted only 4 such differences within the actual HGD (Figure 4).

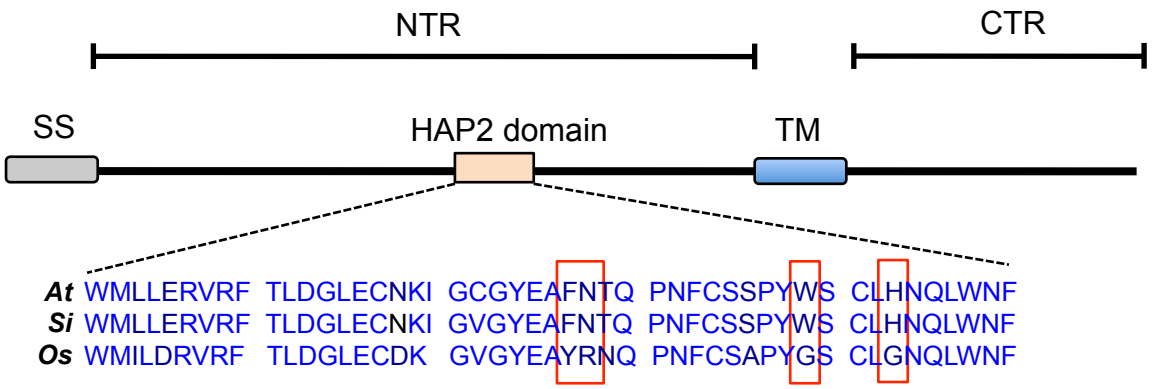


Figure 4. Mutations of amino acids within the Arabidopsis NTR to the *Os* equivalent or Alanine. Arabidopsis HAP2 predicted structure. SS: Signal Sequence, TM: Transmembrane domain, NTR: N-terminal region, CTR: C-terminal region. The highly conserved HAP2 domain is expanded to show amino acid differences between *At*, *Si*, and *Os*. Amino acids that were mutated are highlighted with a red box. Light blue amino acids indicate conservation.

Mutating *At* amino acids within the HGD to the *Os* equivalent has no effect on fusion function

We first tested whether changing all four of the *At* amino acids to the *Os* equivalent (OsQuad) simultaneously would recapitulate the results seen when the entire NTR of *At* was replaced with that of *Os;* inability to rescue the fusion defect of *hap2-2* mutant sperm. We used the *HAP2:HAP2:YFP* construct that has previously been shown to rescue the *hap2-2* fusion defect, and changed only the 4 amino acids within the HGD to the *Os* equivalent (Figure 4). These mutations were confirmed by sequencing, and transformed into *hap2-2* plants. Normally the *hap2-2* allele (generated by insertion of a T-DNA carrying a kanamycin resistance marker) leads to a complete block of male transmission identical to the *hap2-1* allele (von Besser et al., 2006). Therefore, when wild type

females are pollinated with *hap2-2/+* pollen no progeny inherit the *hap2-2* allele (0% transmission) and no progeny will grow on media containing kanamycin.

Multiple primary transformants, which are hemizygous for the OsQuad mutation, were crossed onto *ms1* (male sterile) females and progeny were plated on Kan plates. All crosses using transformed males (5 individual lines, T1-5) resulted in kanamycin resistant progeny around the expected percentage indicative of restoration of normal segregation (33%, Table 1, Sup Figure 2). This segregation indicates that changing these amino acids to the *Os* amino acid has no effect on fusion function, enabling rescue the *hap2-2* fusion defect. Some of these lines exhibit a higher than 33% rescue, perhaps indicating multiple inserts of the construct. These results indicate that changing these amino acids within the HGD does not affect the fusion function of HAP2 even though these are the only four positions within this domain that are the same between *Si* and *At* but different in *Os.*

Table 1. OsQuad mutations rescue the *hap2-2/+* fusion defect.

ms1 x	**# Resistant**	**# Sensitive**	**% Resistant**	**n-value**
OsQuad T1	179	213	**46**	392
OsQuad T2	73	60	**55**	133
OsQuad T3	78	125	**38**	203
OsQuad T4	83	142	**37**	225
OsQuad T5	148	164	**47**	312
hap2-2/+	0	100	**0**	100

% Transmission was determined by crossing each T1 line (3-13 crosses/line averaged) onto *ms1/+* females and counting resistant progeny on Kan plates. Expected % transmission for a rescue of the *hap2* defect is 33%.
n= total number of resistant and sensitive progeny.

Mutating *At* amino acids within the HGD to Alanine has no effect on fusion function

It is possible that mutating these 4 amino acids to the *Os* equivalent did not affect the fusion function of HAP2 because they are not important for the species-specific fusion function previously observed. However, based on conservation, these 4 amino acids might be important for the general fusion function of HAP2. To test this, we also mutated these 4 amino acids within the HGD simultaneously to Alanine (AlaQuad). If these amino acids were important for the fusion function of *At*HAP2, then we would predict that mutating them to Alanine would disrupt the ability to rescue the *hap2-2* fusion defect. Four independent transformed lines (T1-4) were crossed onto *ms1* and plated on Kan plates. Again, Kan resistant progeny were recovered at a percentage indicative of rescue of the *hap2-2* transmission defect (33%, Table 2), suggesting that these 4 amino acids are not important for the fusion of HAP2 or the class-level specificity.

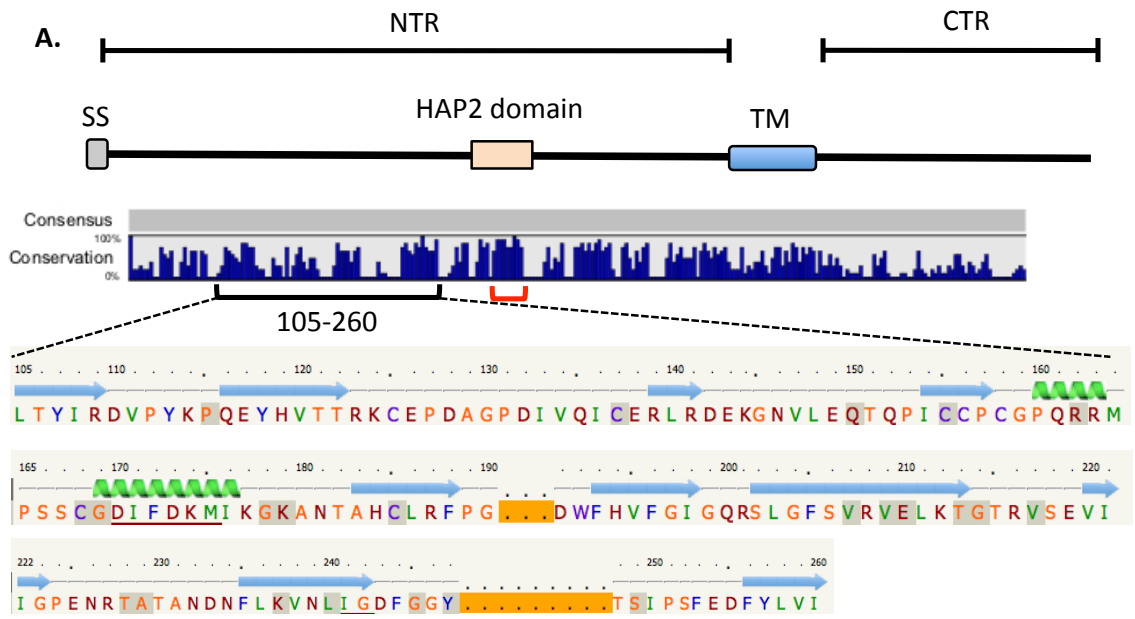
Table 2. AlaQuad mutations rescue the *hap2-2/+* fusion defect.

ms1 x	# Resistant	# Sensitive	% Resistant	n-value
AlaQuadT1	80	132	**38**	212
AlaQuadT2	52	63	**45**	115
AlaQuadT3	46	74	**38**	120
AlaQuadT4	79	168	**32**	247
hap2-2/+	0	100	**0**	100

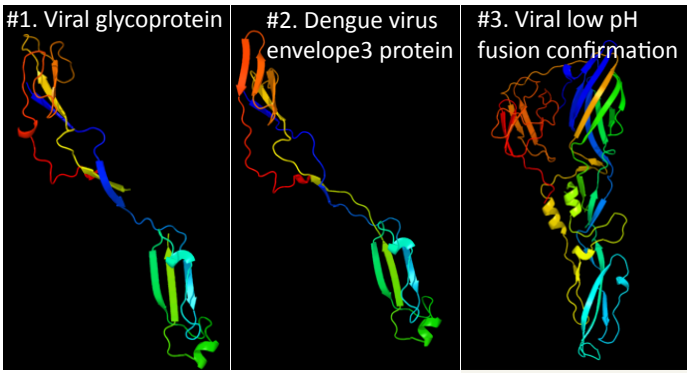
% Transmission was determined by crossing each T1 line (3-13 crosses/line averaged) onto *ms1/+* females and counting resistant progeny on Kan plates. Expected % transmission for a rescue of the *hap2* defect is 33%.
n= total number of resistant and sensitive progeny.

Structural analysis comparisons reveal that the HAP2 NTR is most structurally similar to known viral fusion proteins

Given the results of our mutagenesis within the HGD, we decided to look for other regions outside of the HGD within the NTR that might contribute to gamete fusion. We hypothesized that analysis of the predicted structure of HAP2 would provide additional insight beyond the primary sequence. Conservation plots of HAP2 orthologs across various eukaryotes show that there are additional conserved regions located N-terminally to the HGD that could contribute to function (Figure 5). Interestingly, it was determined using the protein homology program Phyre[2] (Kelley and Sternberg, 2009) that amino acids 105-230 in *At*HAP2 are most *structurally* similar to known structures of viral fusion proteins (Figure 5).



B.



Rank	% Id	% Con
1	16	80.0
2	13	70.9
3	12	68.0

Figure 5. The HAP2 NTR is most structurally similar to viral fusion proteins. **(A)** Predicted HAP2 secondary structure is shown as in previous figures for orientation purposes. The HAP2 domain is highlighted with an orange box. The consensus sequence for HAP2 across the various eukaryotes in Figure 2 was also used for this figure. The HAP2 domain in the bar graph consensus sequence is indicated with a red bracket. The region that is most structurally similar to viral proteins is indicated with a black bracket and corresponds to amino acids 105-230 of the Arabidopsis HAP2. The predicted structure of this region is shown below in an expanded graphic. Blue arrows: β-sheets. Green helices: α-helices. Orange: No prediction. **(B)** Top three structural hits (1-3, Rank#) for HAP2. 1: envelope glycoprotein from the tick-borne encephalitis virus. 2: type-3 envelope glycoprotein of dengue virus. 3: low-pH-induced membrane fusion confirmation of the flavivirus envelope3 glycoprotein. %Id: sequence identity. % Con: confidence in structure comparison.

Specifically, this region has highest predicted structural similarity to the envelope glycoprotein from the tick-borne encephalitis virus (Rey et al., 1995), the type-3 envelope glycoprotein of dengue virus (Modis et al., 2005), and the low-pH-induced membrane fusion confirmation of the flavivirus envelope glycoprotein (Bressanelli et al., 2004). All of these viruses are in the flavivirus genus of viruses and are all enveloped proteins that infect host cells via receptor-mediated endocytosis (Smit et al., 2011). The Phyre^2 algorithm is un-biased and searches all known protein structures using a structural prediction of the target protein. Only amino acids 105-230 of *At*HAP2 yielded a significant match. The finding that this region matches proteins known to be directly involved in virus:host fusion is striking given the HAP2 loss-of-function phenotype.

Comparison between HAP2 and the top match, the envelope protein from the encephalitis virus, shows most structural similarity with domain I of the virus (amino acids 137-189) (Rey et al., 1995). This domain is an 8-stranded up and down β-barrel that lies parallel to the viral membrane. Two of the β-sheets in this region face each other across a tightly packed hydrophobic interior, is stabilized by two disulfide bonds, and contains one conserved glycosylation site (Rey et al., 1995). HAP2 also is most structurally similar to a region that is fully conserved across all of these viruses, the Cd loop region (amino acids 98-113), or fusion peptide. The Cd loop region is a glycine-rich, tightly folded, hydrophobic region that has been shown to give viruses fusogenic properties (Allison et al., 2001;

Roehrig et al., 1989). This analysis suggests that regions N-terminal to the actual HGD might be the regions of HAP2 required for fusion.

Discussion

Plasma membrane fusion is essential for several key development processes including fertilization: however our knowledge of the mechanisms required to merge membranes is rudimentary. Currently, highly conserved HAP2 is the only known surface membrane protein in plants thought to play a direct role in gamete fusion. Sequence analysis revealed that the extracellular NTR of HAP2 is the most conserved part of the protein, containing a specific region of highest conservation, the HGD (Figure 3). Previous experiments indicated that the NTR might also be required for species-specific fusion in a sequence dependent manner, as replacement of the *At*NTR only with the distantly related *Os*NTR disrupted fusion function (Wong et al., 2010). We identified four specific non-conservative changes within the HGD when comparing *At* and *Os* sequences and hypothesized that these changes might account for the inability of the *Os*NTR to rescue the fusion defect of *hap2-2.* Here, we showed that mutating these specific amino acids within this conserved region to either Alanine or the *Os* equivalent did not affect HAP2 function (Table 1 and 2).

These results suggested that amino acids required for gamete fusion might lie elsewhere within the HGD, or outside of the HGD. We turned to structure-based bioinformatic analysis to try to identify other potentially important regions. These structural comparisons provide exciting insight into the HAP2 protein, suggesting that regions outside of the HGD might be responsible for the fusion function of HAP2. Future experiments could be targeted at mutagenizing specific amino acids within this region, or swapping the *At* region for that of the virus equivalent and determining whether this is sufficient for fusion.

Another interesting result from the Phyre^2 analysis was that the HGD does not show structural similarity to any known solved structure. This suggests that the HGD might be highly specific to gamete fusion mediated by HAP2, perhaps interacting with a protein on the egg and central cell surface. It is likely that mutations in the most conserved residues within this sequence would disrupt HAP2 function, but this remains to be tested. Here, only amino acids that were common to *At* and *Si,* but different in *Os* were analyzed. The reason *Os*NTR fails to rescue the *hap2-2* fusion defect also remains to be determined, but it could be due to amino acid changes outside of the HGD or differences in post-translational modification. Western blot analysis showed that wild type HAP2 and HAP2:HAP2:YFP were both migrating at a higher than expected molecular weight (80kD predicted vs observed 95kD), suggesting potential post-translational modifications. Glycosylation has been shown to play a role in cleavage-dependent activation of the fusion peptide (F protein) in several viruses (Alkhatib et al.,

1990). Glycosylation can also affect binding of viruses to host cells. For example, the envelope protein gp120 of the HIV-1 virus is the most glycosylated protein to be characterized thus far in nature, and loss of glycosylation results in diminished binding of the virus to the CD4 receptor of host cells (Vigerust and Shepherd, 2007). The interaction between viral glycosylated proteins and their host cell receptor points toward a model in which both virus and host cells use glycosylation modification as evolutionary mechanisms for increased entry by the virus, and increased resistance for the host cell. Sperm:egg recognition, in flowering plant reproduction could also rely on specific patterns of HAP2 glycosylation. *At* and *Si* HAP2 are predicted to contain six N-linked glycosylation sites at invariant locations. However, *Os*HAP2 is predicted to contain only three N-linked glycosylation sites, only one of which is in the same position as *At*HAP2. Perhaps this putative difference in glycosylation patterns could account for the inability of the *Os*NTR to restore fusion function. Future experiments could focus on determining whether these predicted glycosylation sites are present using deglycosylating enzymes that remove sugars from the protein and comparing membrane fractions treated with these enzymes verses wild type HAP2 via western blot. These predicted sites could also be mutagenized to see if these sites are required for HAP2 fusion function.

Finally, in an effort to further narrow down specific amino acids within the NTR that might be required for gamete fusion, we could create further chimeric constructs in which we replace the NTR with the NTR of a species that lies

between *Si* and *Os.* For example, the Tomato HAP2 (*Solanum lycopersicum, Sl)* shares 73% homology with the *At*NTR (compared to 89% in *Si* and 53% in *Os*) and the Poplar HAP2 (*Poplulus trichocarpa, Pt)* shares 78% homology. If replacement of the *At* NTR with the *Sl* NTR resulted in a rescue of the fusion defect, then there would be 72 amino acids that are the same between *At, Si* and *Sl*, but different in *Os.* If this construct did not rescue the fusion defect, then there are only 39 amino acids that are the same in *At* and *Si,* but different in *Sl* and *Os.* This would narrow down potential amino acids from 111 (Sup Figure 1) down to 39. These amino acids could be mutated to test for their role in fusion function. Furthermore, replacement of the *At* NTR with *Sl* or *Pt* could provide further insight into the role of glycosylation in fusion function. *Sl* is predicted to contain the same glycosylation sites at *At*, whereas *Pt* is predicted to contain three of the same sites as *At* and two that are unique to *Pt.* If the *Sl* construct rescues the *hap2* fusion defect, but the *Pt* construct does not, then it is possible that the differences is predicted glycosylation sites could contribute to this result given that their sequence homology is similar.

Furthermore, we could use these alignments to identify additional regions with the HAP2 NTR that are most highly conserved and mutate these amino acids. For example, alignments of HAP2 sequence shows that there are 16 highly conserved cysteines within the NTR, three of which are located within the HGD (Figure 6).

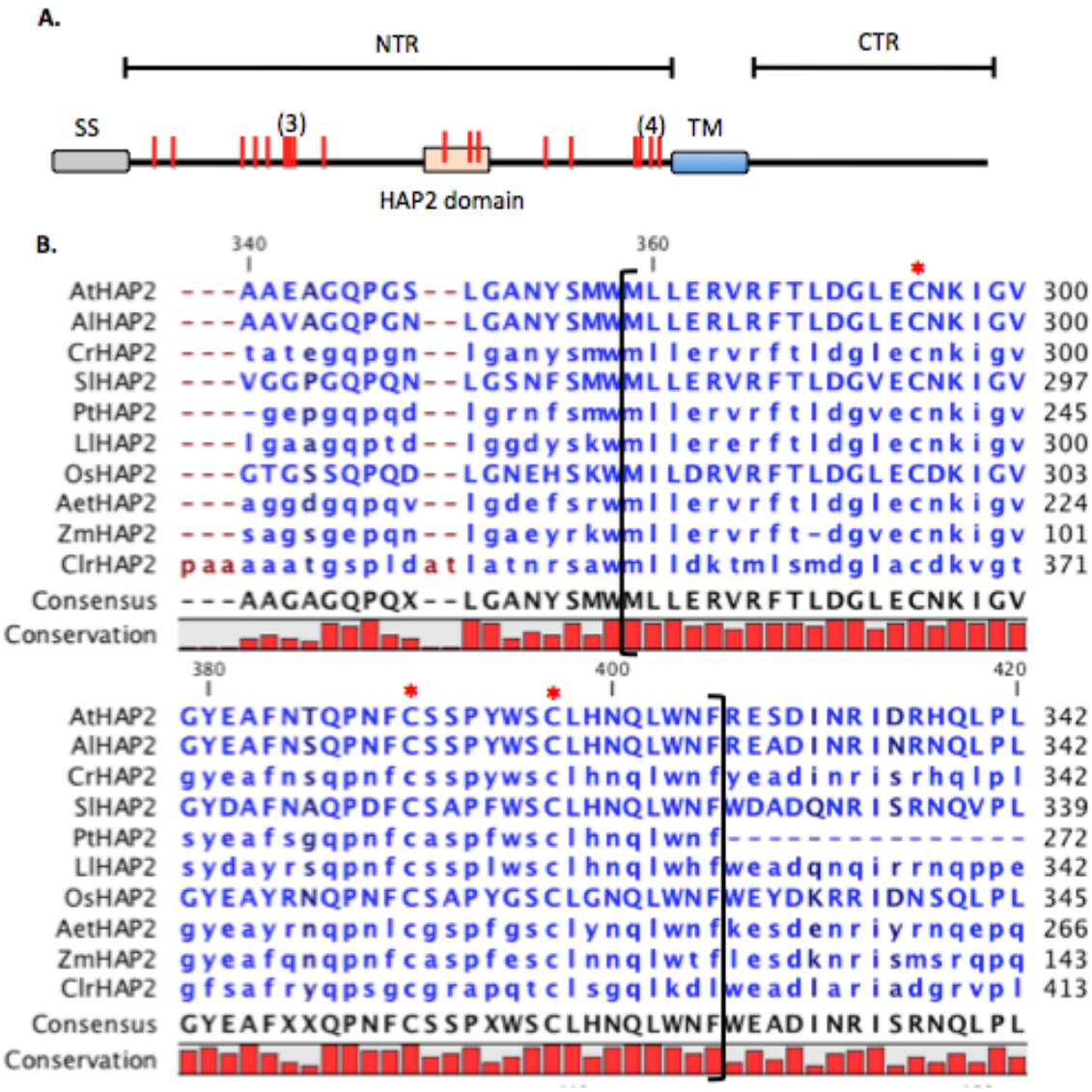


Figure 6. Conserved cysteines within the NTR and the HGD. (A) Arabidopsis HAP2 predicted structure showing conserved cysteines within the NTR (red hash marks) and the positive charge within the CTR. **(B)** Expanded sequence alignment of the HAP2 domain between Arabidopsis and several other eukaryotes highlighting cysteine conservation among all of these species. At: *Arabidopsis thaliana*, Al: *Arabidopsis lyrata*, Cr: *Capsella rubella*, Sl: *Solamun lycopersicum* (Tomato), Pt: *Populus tricocarpa* (Poplar), Ll: *Lilium longiflorum* (Lily), Os: *Oryza sativa* (Rice), AeT: *Aegilops tauschii* (Goatgrass), Zm: *Zea mays* (Corn), Clr: *Chlamydomonas reinhardtii* (Green Algae). Brackets indicate HGD. Red asterisks indicate conserved cysteines.

In extracellular proteins, like the NTR of HAP2, cysteines are frequently involved in disulfide bonds, which lend stability to the protein structure (Narayan, 2012). In addition, cysteines have been shown to play a role in the ability of viruses to fuse with host cells (Wahid et al., 2013) (Syu et al., 1991). We would therefore hypothesize that mutation of these cysteines would alter the function of HAP2.

In conclusion, we have shown that the four variant amino acids within the HGD between *Os* and *At* do not contribute to the fusion function of HAP2. However, we have identified several regions within the NTR that might contribute to HAP2 function, including the region located N-terminally to the HGD that is most structurally similar to viral fusion proteins, and several conserved cysteines within the HDG and NTR. Future experiments will focus on determining if these regions contribute to the fusion function of HAP2.

Acknowledgements

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Experimental Methods

Growth of plants

ms1/MS1 plants were obtained from the Arabidopsis Biological Resource Center (stock #CS261) and maintained as a heterozygous line. *hap2-2/+* (von Besser et al., 2006) contains a T-DNA which confers Kanamycin resistance and is also available at the ABRC (stock #N652706). All seeds were plated on solid Murashige and Skoog (MS) medium (MP Biomedicals LLC, Solon, OH, USA). *ms1/MS1* seeds were plated on no drug media, *hap2-2/HAP2* seeds were plated on medium containing 50µg/ml kanamycin sulfate (Agri-bio, Bay Harbour, FL, USA). T1 lines were plated on medium containing 50µg/ml kanamycin sulfate and 50µg/ml glufosinate ammonium (Basta). Seedlings were transplanted to sterile 2MIX potting medium (Conrad Fafard INC, Agawam, MA, USA) 7 days after plating. All plants were grown at 20°C, 50-60% humidity, on a 16day/8hour night light cycle in growth chambers (Environmental growth chambers, Chagrin Falls, OH, USA).

Generation of mutated *HAP2:HAP2:YFP*

Original HAP2:HAP2:YFP was made previously (von Besser et al., 2006) and specific amino acids within the HAP2 domain were mutated using Multi-site Directed Mutagenesis (Agilent Technologies, Catalog #200514). All constructs were sequenced to confirm mutation. Constructs were transformed into Agrobacterium strain GV3101 and resultant colonies expanded for floral dipping into *hap2-2/+* plants (Barton and Chilton, 1983; Clough and Bent, 1998). All T1 lines were checked for construct expression using PCR primers. A detailed schematic of determinacy of transmission is provided in Supplemental Figure 2.

Pollen collection for membrane preparations

Pollen was collected from freshly opened flowers (~10 flats worth/~100 plants) by cutting inflorescences and placing them into 2-4L of cold (4°C) pollen growth media (Mayfield et al., 2001). Pollen was released via manual agitation with gloved hands. Plant debris were removed from the media and liquid was filtered using cheesecloth. Pollen was collected via centrifugation at 3,750g, 5 minutes. Pollen was concentrated by washing with small volume of PGM (45ml) and collected by centrifugation in a 50ml conical tube. Pollen can be stored at -80°C or used immediately for membrane fractionation.

Purification of microsomal membrane fractions from pollen

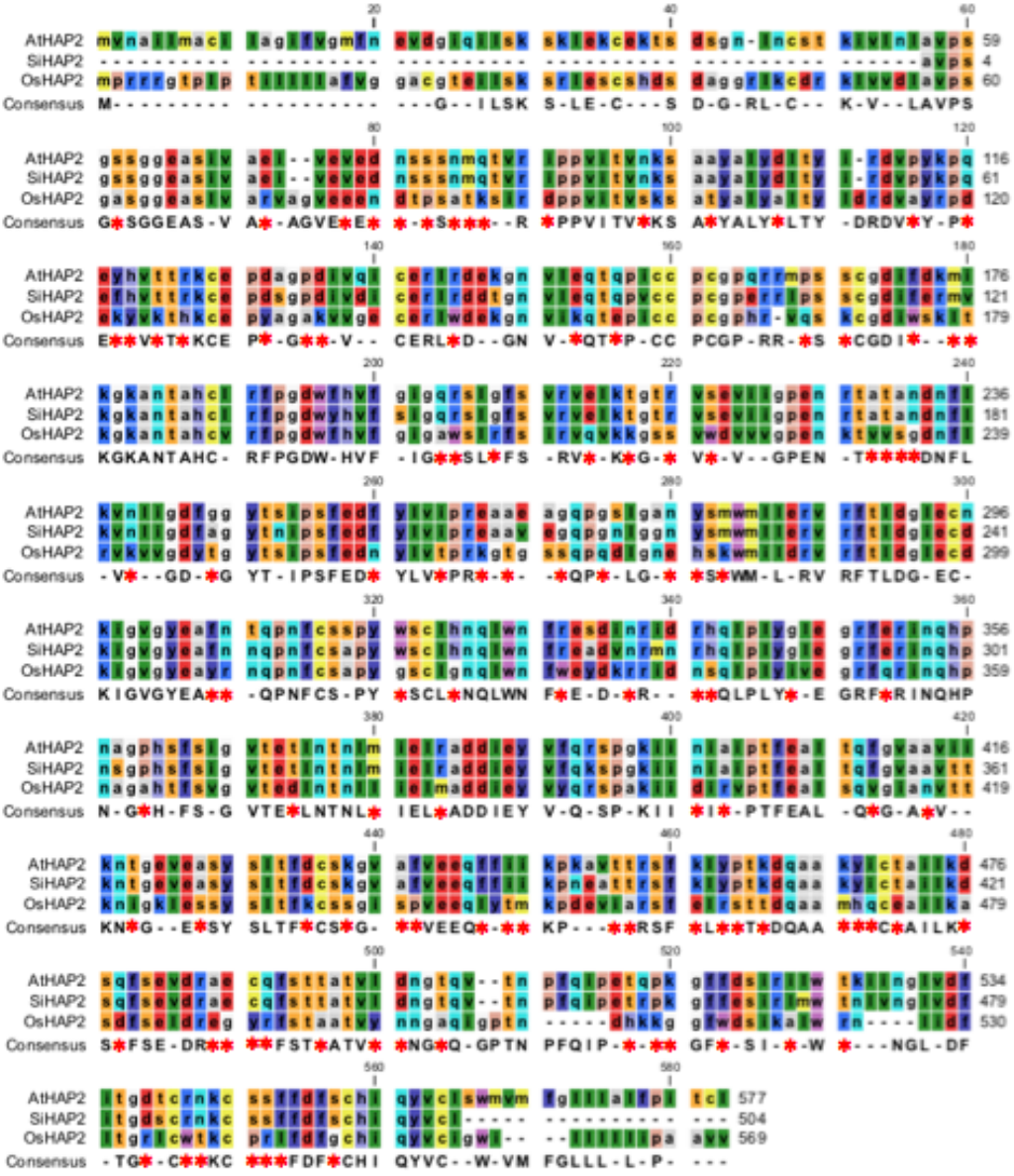
Pollen pellet was homogenized in grinding buffer (For 100ml: 0.29M sucrose, 25mM HEPES (pH 8.5), 20mM EDTA (FA), 250 µM PVP, pH to 8.5, refrigerate. Just before use add 3mM DTT, 200ng leupeptin, PMSF (100mM in EtOH), 200µM benzamide) using a glass tube mortar and pestle. Solution was cleared of unwanted organelles by spinning sample at 10,000g, 30min, at 4°C. Supernatant (contains sample) was transferred to a new tube and centrifuged using an ultra centrifuge at 100,000g, 50min, 4°C. After this step, the pellet contains the membrane fraction. Pellet was resuspended with 2ml of resuspension buffer (10mM BTP-MES, pH 7.8, 250mM sucrose, 20% glycerol. Just before use add 1mM DTT, 200ng/ml leupeptin, PMSF (100mM in EtOH), 200mM benzamide). Sample was stored in liquid nitrogen until ready for western blot.

Western blot for HAP2 in membrane fractions

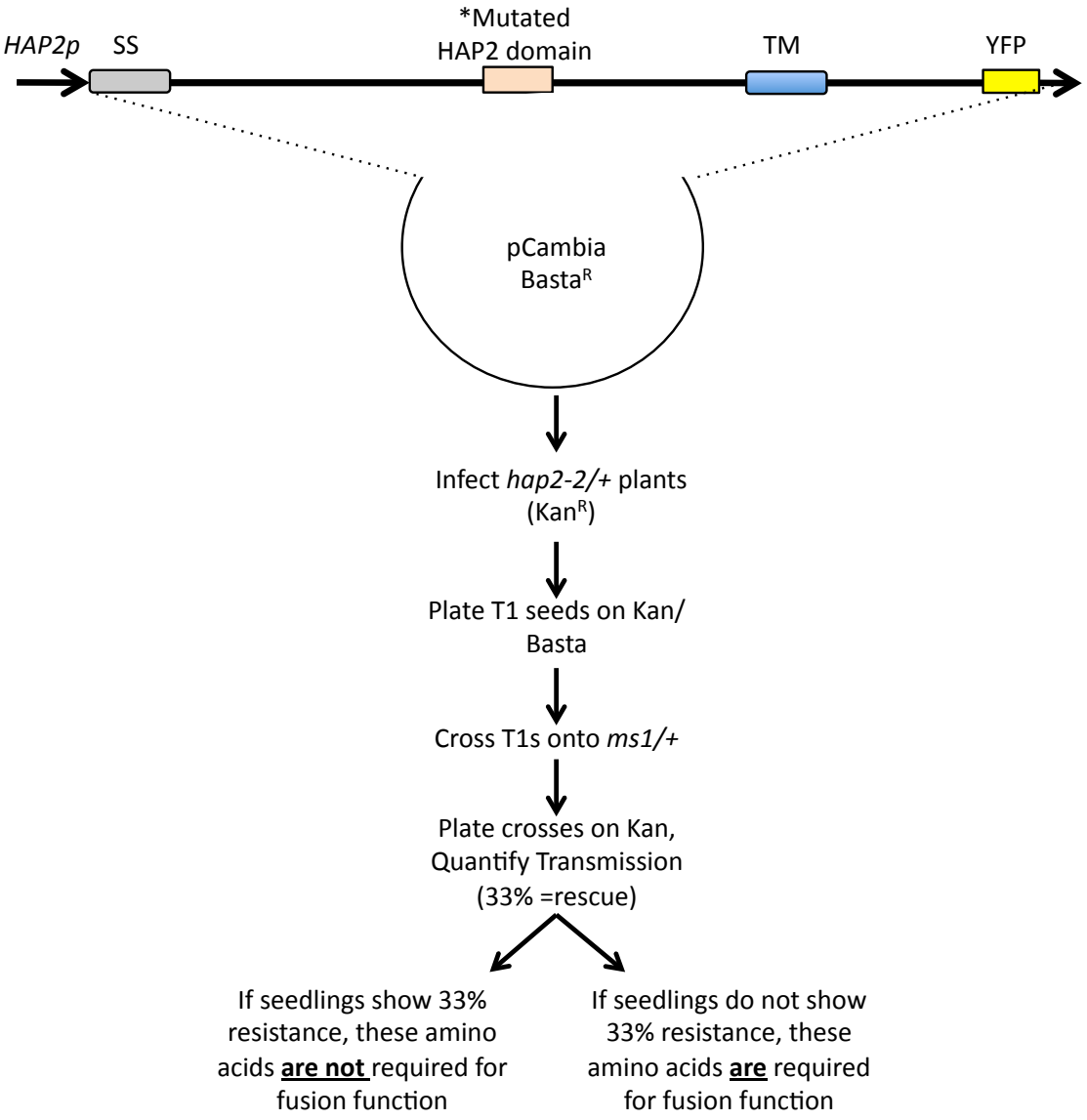
Standard western blots were performed on membrane fractions as previously described (Kaur and Bachhawat, 2009). Samples were loaded by volume and run on a pre-cast sucrose SDS-gradient gel (Invitrogen, catalog #NP0322BOX).

Resolved protein samples were transferred onto nitrocellulose membrane using Tris-glycine buffer (48mM Tris, 39mM glycine, 0.037% SDS, and 20% methanol, pH 9.2). The blot was probed with a polyclonal 1° α-NTR HAP2 antibody at a 1:1000 dilution overnight (gift from Julian Wong), followed by wash and incubation with a 2° -Goat α-Rabbit antibody conjugated to horseradish peroxidase at a 1:5000 dilution. Membrane was washed 3x with PBST at room temperature. Signal detection was done using ECL detection spray (HyGLO, Denville Scientific, catalog #E2400) and developed using standard procedures.

Supplemental Information



Sup Figure 1. Sequence alignment of the N-terminal region of HAP2 between *Arabidopsis thaliana, Sysimbrium irio, and Oryza sativa.* The N-terminal region of Arabidopsis (amino acids 1-559) was aligned to the corresponding *Sysimbrium irio* (*Si*HAP2) and *Oryza sativa* (*Os*HAP2) sequence. Colors denote amino acid properties. Consensus represents 100% similarity between all three sequences. Dashes indicate differences in sequence. Amino acids that are the same between *At* and *Si*, but different in *Os* and would constitute a non-conservative change are marked with a red asterisk.



Sup Figure 2. Protocol for analyzing rescue of the *hap2-2* fusion defect using mutations within the Arabidopsis HGD. Schematic of the HAP2::HAP2:YFP sequence that was mutated within the pCambia plant vector that confers Basta resistance. HAP2p represents the 1.5kb promoter included within the plasmid. HAP2 domain contains either *Os* mutations or Alanine mutations. TM: Transmembrane domain. YFP is located within the CTR. Mutated plasmid was transformed into agrobacteria and dipped into *hap2-2/+* plants. T1 (transformants) seeds are selected on Kan/Basta plates as *hap2-2* confers Kan resistance. Several T1 plants were selected and pollen was crossed onto *ms1/+* females. Crosses were plated on Kan plates and sensitive and resistant seedlings were quantified.

References

Alkhatib, G., Richardson, C., and Shen, S.H. (1990). Intracellular processing, glycosylation, and cell-surface expression of the measles virus fusion protein (F) encoded by a recombinant adenovirus. Virology 175:262-270.

Allison, S.L., Schalich, J., Stiasny, K., Mandl, C.W., and Heinz, F.X. (2001). Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. Journal of virology 75:4268-4275.

Barrett, S.C. (2010). Darwin's legacy: the forms, function and sexual diversity of flowers. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 365:351-368.

Barton, K.A., and Chilton, M.D. (1983). Agrobacterium Ti plasmids as vectors for plant genetic engineering. Methods in enzymology 101:527-539.

Beale, K.M., Leydon, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule. Current biology : CB 22:1090-1094.

Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in plant science 13:437-443.

Blagborough, A.M., and Sinden, R.E. (2009). Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187-5194.

Bressanelli, S., et al. (2004). Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. The EMBO journal 23:728-738.

Chen, E.H., and Olson, E.N. (2005). Unveiling the mechanisms of cell-cell fusion. Science (New York, N.Y.) 308:369-373.

Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant journal : for cell and molecular biology 16:735-743.

Dutch, R.E., Jardetzky, T.S., and Lamb, R.A. (2000). Virus membrane fusion proteins: biological machines that undergo a metamorphosis. Bioscience reports 20:597-612.

Hamamura, Y., et al. (2011). Live-Cell Imaging Reveals the Dynamics of Two Sperm Cells during Double Fertilization in Arabidopsis thaliana. Curr Biol 21:497-502.

Hamilton, B.S., Whittaker, G.R., and Daniel, S. (2012). Influenza virus-mediated membrane fusion: determinants of hemagglutinin fusogenic activity and experimental approaches for assessing virus fusion. Viruses 4:1144-1168.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in Arabidopsis. Current biology : CB 22:1084-1089.

Kaur, J., and Bachhawat, A.K. (2009). A modified Western blot protocol for enhanced sensitivity in the detection of a membrane protein. Analytical biochemistry 384:348-349.

Kelley, L.A., and Sternberg, M.J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. Nature protocols 4:363-371.
Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of molecular biology 305:567-580.
Liu, Y., et al. (2008). The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes & development 22:1051-1068.
Mayfield, J.A., Fiebig, A., Johnstone, S.E., and Preuss, D. (2001). Gene families from the Arabidopsis thaliana pollen coat proteome. Science (New York, N.Y.) 292:2482-2485.
McNew, J.A., et al. (2000). Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. The Journal of cell biology 150:105-117.
Min, D., Kim, K., Hyeon, C., Hoon Cho, Y., Shin, Y.K., and Yoon, T.Y. (2013). Mechanical unzipping and rezipping of a single SNARE complex reveals hysteresis as a force-generating mechanism. Nature communications 4:1705.
Modis, Y., Ogata, S., Clements, D., and Harrison, S.C. (2005). Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. Journal of virology 79:1223-1231.
Mori, T., Hirai, M., Kuroiwa, T., and Miyagishima, S.Y. (2010). The functional domain of GCS1-based gamete fusion resides in the amino terminus in plant and parasite species. PloS one 5:e15957.
Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.
Narayan, M. (2012). Disulfide bonds: protein folding and subcellular protein trafficking. The FEBS journal 279:2272-2282.
Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., and Harrison, S.C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375:291-298.
Roehrig, J.T., Hunt, A.R., Johnson, A.J., and Hawkes, R.A. (1989). Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology 171:49-60.
Smit, J.M., Moesker, B., Rodenhuis-Zybert, I., and Wilschut, J. (2011). Flavivirus cell entry and membrane fusion. Viruses 3:160-171.
Sudhof, T.C., and Rothman, J.E. (2009). Membrane fusion: grappling with SNARE and SM proteins. Science (New York, N.Y.) 323:474-477.
Syu, W.J., Lee, W.R., Du, B., Yu, Q.C., Essex, M., and Lee, T.H. (1991). Role of conserved gp41 cysteine residues in the processing of human immunodeficiency virus envelope precursor and viral infectivity. Journal of virology 65:6349-6352.
Vigerust, D.J., and Shepherd, V.L. (2007). Virus glycosylation: role in virulence and immune interactions. Trends in microbiology 15:211-218.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.
Wahid, A., Helle, F., Descamps, V., Duverlie, G., Penin, F., and Dubuisson, J. (2013). Disulfide bonds in hepatitis C virus glycoprotein E1 control the assembly and entry functions of E2 glycoprotein. Journal of virology 87:1605-1617.
Wong, J.L., and Johnson, M.A. (2010). Is HAP2-GCS1 an ancestral gamete fusogen? Trends in cell biology 20:134-141.
Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS genetics 6:e1000882.

Chapter 6: Discussion

Analysis of *HAP2* Function Provides Insights into Gamete Fusion Mechanisms in Flowering Plants

Double fertilization in flowering plants is required for seed production

Flowering plants have come to dominate our planet, with a predicted 250,00-400,000 different species, they have become arguably one of the most successful sexually reproducing organisms (Joppa et al., 2011). In these organisms, the ultimate product of fertilization is a seed, and it is this product that provides the major nutritional components that are required for the human diet, and propagates the next generation of plants (Li and Berger, 2012; Li, 2009). These species have evolved a complex fertilization mechanism that involves two male gametes (haploid sperm) fertilizing two female cells (haploid egg cell and diploid central cell), a process referred to as double fertilization (Berger et al., 2008). In this system, two immotile sperm cells develop within the male gametophyte, or pollen grain, which must be delivered to female gametophytes that are buried deep within floral tissue (Twell, 2011). Every step of double fertilization requires coordinated cell-cell communication between the male and female cells (Thesis Chapter 1). To accomplish sperm delivery mature pollen that has landed on a receptive stigma will germinate a highly polarized cellular outgrowth called a pollen tube (Qin and Yang, 2011). The pollen tube must then grow through the transmitting tract of the pistil and respond to signals from the female that enables proper targeting, PT burst and sperm cell delivery at the site of gamete fusion within an ovule (Kessler and Grossniklaus, 2011). Once delivered, one sperm cell must fuse with the egg cell generating the embryo and

the other sperm cell must fuse with the central cell generating the endosperm. Both of these gamete fusion events are required for successful development of a seed.

Although advances in the study of double fertilization have occurred quite recently due to live cell imaging, genomics, and genetic analysis, the mechanisms for pollen tube reception and burst, prevention of polyspermy, and mechanisms mediating gamete fusion remain largely unknown. This thesis provides novel insights into flowering plant reproduction specifically by focusing on *HAP2*-mediated mechanisms for gamete fusion. This thesis contains three main findings: 1) *HAP2* requires additional factors for full gamete fusion. 2) Structural comparisons suggest that HAP2 contains regions within its N-Terminal region (NTR) that bare resemblance to viral fusion proteins. 3) Flowering plants have coupled *HAP2*-mediated gamete fusion with sperm cell delivery to maximize reproductive success.

I) *HAP2* requires additional factors for full gamete fusion function

HAP2 is sufficient for rescuing the fusion defect of *duo1* mutant sperm

Analysis of *HAP2* loss of function mutants in Arabidopsis, Chlamydomonas, and Plasmodium has shown that *HAP2* is required for successful gamete fusion (Blagborough and Sinden, 2009; Liu et al., 2010; Liu et al., 2008; von Besser et al., 2006). However, it was unknown whether *HAP2* was sufficient for driving gamete fusion, or whether other male and female-expressed genes were also required. The first approach we took to test whether *HAP2* is the central male component of gamete fusion was to express *HAP2* in a naïve sperm-like cell that is incapable of gamete fusion and test whether the fusion defect was rescued (Thesis Chapter 3). We utilized *duo1* mutant sperm for this purpose. *duo1* mutants lack expression of *HAP2* and ~63 other sperm-expressed genes (Brownfield et al., 2009) and produce single-sperm like cells that are incapable of gamete fusion (Rotman et al., 2005). We hypothesized that if we expressed *HAP2* in these mutant sperm, and we saw a rescue of fertilization and generation of single fertilization events, that this would indicate that *HAP2* was the only *DUO1*-regulated gene required for gamete fusion. Recently obtained data generated by analyzing fertilization directly by imaging *duo1* sperm expressing *HAP2,* and *H2B:RFP in vivo* suggest that the rescue of gamete fusion is occurring at ~50% (n=11), the predicted rate of rescue if *HAP2* is sufficient. These data suggest that expression of *HAP2* is sufficient to restore gamete

fusion to *duo1* sperm, however, these results do not rule out that other non-*DUO1*-regulated genes are also required for *HAP2* fusion function. In order to draw more definitive conclusions from this data (given the low n-value), many more fertilization events need to be quantified. Since we never detected central cell only events using this method, it is possible that *H2B:RFP* is being degraded much faster in the central cell than in the egg cell, making detection of this type of event difficult. However, since we are able to detect overlap of *H2B:RFP* with the central cell nucleus when two functional sperm cells are deposited (Chapter 3, Figure 2C), we suspect that we should be able to see this type of fusion event with single *duo1* sperm expressing *HAP2* as well.

Analysis of *duo1, DUO1:HAP2:YFP* fertilization events using ovule clearing methods revealed that although we are detecting nuclear fusion when directly visualizing sperm cell nuclei, we are not able to detect single fertilization events using this assay at the 50% expected frequency. Instead, single fertilization events were detected at a rate of 1%. This could be the result of polytubey. For example, it has been recently shown that a single fertilization event is not sufficient to initiate the block to polytubey (Maruyama et al., 2013). It is possible that the first pollen tube to target these ovules containing only an embryo or endosperm carried *duo1, DUO1:HAP2:YFP* sperm that initiated gamete fusion with either the egg cell or the central cell. Both plasma membrane fusion and karyogamy would occur normally, however early development would be halted due to the lack of expression of other *DUO1*-regulated genes. Fusion of this

sperm would initiate a block to polyspermy on this targeted female cell even though early development had not occurred. This fusion event however, would not initiate the block to polytubey. Should a pollen tube carrying functional sperm target this same ovule, then only one of the female cells would be available for a fusion event (the one that did not fuse with the *duo1, DUO1:HAP2:YFP* sperm). Since this sperm is fully functional, this fusion event would result in a viable embryo or endosperm, appearing as a single fertilization event using this assay. This type of polytubey event would be very rare, as it would require a single ovule being targeted first by a pollen tube carrying *duo1, DUO1:HAP2:YFP* sperm, and then by a second pollen tube carrying wild type, functional sperm, which is consistent with our very low occurrence of single fertilization events (Chapter 3, Figure 3).

These results indicate that *HAP2 is* sufficient for rescuing the gamete fusion defect of *duo1* mutant sperm. We would predict that other *DUO1*-regulated genes are required for an event that occurs immediately after karyogamy and before detectable embryo and endosperm development. Sperm-expressed genes required to activate zygotic and endosperm divisions have not yet been identified, however *SHORT SUSPENSOR (SSR)* has been shown to be transcribed in the sperm and translated in the zygote where it is required for embryo patterning (Bayer et al., 2009). We could also use this *duo1* system to express potential development genes and test whether they are required for early

embryo and endosperm divisions. It is also possible that other non-*DUO1*-regulated genes are required for *HAP2*-mediated gamete fusion.

The forward genetic modifier screen identified mutations in genes that might interact with *HAP2* to generate gamete fusion

Another piece of evidence that suggests *HAP2* requires additional components for fusion function is the identification of several mutations that suppress the *hap2*^{hypo} phenotype (*sohos,* Chapter 4). HAP2^{hypo} was generated by truncation and neutralization of the CTR, generating a version of HAP2 that was only capable of double fertilization in ~8% of ovules (producing ~3seeds/silique) and single fertilization events (either the egg or the central cell) occurred in ~24% of ovules (Wong et al., 2010). These single fertilization events indicated that there might be less HAP2^{hypo} reaching the sperm cell membrane, or this mutation produced a less active version of HAP2. The *sohos* identified in the screen produce a significantly greater number of seeds than *hap2*^{hypo} alone. We identified one-female expressed *soho*, five male-expressed *sohos* and one *soho* that required expression in both males and females.

As previously hypothesized (Chapter 4), it is possible that the *sohos* encode for a gene that is required for transporting HAP2 to the sperm cell membrane. An increase in the amount of HAP2 at the site of fusion could enhance fusion

capabilities of these sperm. For example, if HAP2^{hypo} were being retained in the ER due to neutralization of the CTR, then perhaps a mutation in an ER gene required for this retention of mis-folded proteins would enable escape of more HAP2^{hypo} to the membrane. This mechanism has been previously seen in the analysis of suppressors of the BR1 receptor small leaf phenotype (Gonzalez et al., 2010; Li and Chory, 1997). It is also possible that additional mutations occurred in *HAP2^{hypo}.* If positive charge were restored to the CTR, then more *HAP2^{hypo}* might travel to the sperm membrane. However, this explanation is highly unlikely as EMS generates point mutations and there are several residues within the remaining CTR that would have to be mutated to restore positive charge.

HAP2^{hypo} might also produce a less active form of HAP2. In this case, a *soho* mutation might result in over-expression of a co-activator of *HAP2* (similar to the co-activator PGC-1β that increases mitochondrial fusion (Liesa et al., 2008) or a constitutively active form of the receptor for ECI. *EC1* has recently been shown to be required for HAP2 localization to the sperm membrane and activation of fusion (Sprunck et al., 2012).

A *soho* mutation could also result in an increase in polytubey. Polytubey has been shown to occur in ovules that receive non-functional *hap2* and *hap2^{hypo}* sperm cells that are incapable of gamete fusion (Beale et al., 2012; Kasahara et

al., 2012). An increase in polytubey would increase the chances that multiple pollen tubes are targeting an ovule that has received non-functional *hap2*^{hypo} sperm, resulting in an increased chance of the second pollen tube containing functional sperm capable of initiating double fertilization. This type of *soho* mutation might result from mutations in a gene that is required for the pollen tube to respond to the block to polytubey. Currently the mechanisms for the block to polytubey are unknown, but if this block involves release of a repellent from the female that must be perceived by the pollen tube in order for it to turn away from an already targeted ovule, then mutations in such a pollen tube receptor could bypass this block. It is also possible that the female *soho* is the result of a mutation in a gene that is required for *release* of the signal required for the block to polytubey from the egg cell, central cell, or synergid cell. If the block to polytubey were never created due to such a mutation, then pollen tubes would continue to target a single ovule. In order to test for this possibility, the pollen tube cytoplasm marker *LAT52:DsRED* (Francis et al., 2007) could be introduced into each *soho* so that mixed pollinations with wild type pollen tubes expressing cytoplasmic GFP could be performed to directly quantify rates of polytubey as in Chapter 2.

Given the results from the *duo1* rescue experiments, it might also be beneficial to label the sperm cell nuclei of each male *soho* and look directly at gamete fusion within the ovule. It is possible that some of these *sohos* might exhibit an increase in single fertilization events, an event that would indicate an increase in gamete

fusion, but one that would not be detected using seed set as the output for the screen. We might also see a preference for fusion with either the egg cell or the central cell, indicating a mutation in a gene that might be required for fusion of only the egg cell, but not the central cell. Previous analysis of *hap2*^{hypo} fertilization events did indicate a preference for fusion with the egg cell (Wong et al., 2010).

We also identified a mutation in a female-expressed gene. This is an exciting finding, as female HAP2 binding partners have not been identified and previous data suggests that HAP2 may participate in protein:protein interactions with the egg (Wong et al., 2010). Perhaps *soho5* encodes for this binding partner and the mutation is producing an over-active receptor on the female cells that is capable of compensating for the low fusion activity of HAP2^{hypo}. *HAP2* does require *EC1* for fusion function; a recent finding that indicates *HAP2* does interact with female-expressed components for fusion (Sprunck et al., 2012). However, EC1 encodes for a small, secreted peptide and is not likely the female binding partner of HAP2 that might be localized to the egg and central cell membrane. Continued screening should be performed to identify additional *sohos* that may encode for a female-expressed gene.

Important future work will be to identify these novel genes using a mapping population that is currently being generated in the lab. Once we have identified

these genes using map-based cloning techniques, we can perform several additional experiments. First, we can perform reverse genetics on these genes and determine whether mutations in these genes directly effect the fusion of gametes. We can also determine whether these genes are dominant or recessive mutations. The suppressor allele will be cloned and introduced into *HAP2*^{hypo} plants and screened for an increase in seed set. If seed set increases, then the mutation is dominant. Conversely, if the WT allele is cloned and introduced into the *hap2*^{hypo}*, soho* plant and an increased seed set is observed, then the mutation is recessive.

II) Structural analysis of the HAP2 N-terminal region provides insights into possible fusion mechanism

Variant amino acids within the HGD are not required for the class-level specificity of HAP2 gamete fusion

Previous experiments indicated that the NTR of HAP2 might be required for species-specific fusion in a sequence dependent manner, as replacement of the *At*NTR only with the distantly related *Os*NTR disrupted fusion function (Wong et al., 2010). We identified four variant amino acids within the region of highest conservation, the HGD, when comparing *At, Si* and *Os* sequences and hypothesized that these changes might account for the inability of the *Os*NTR to rescue the fusion defect of *hap2-2.* It was proposed that these changes would result in a non-functional HAP2 if these amino acids were responsible for the

class-level specificity we observed previously (Wong et al., 2010). However, when we mutated these amino acids to the *Os* equivalent or to Alanine, there was no affect on fusion function of *At*HAP2. These results suggested that amino acids required for gamete fusion might lie elsewhere within the HGD, or outside of the HGD. Outside of the HGD there are 111 such differences between the *Os*NTR and the *At*NTR. As that is a lot of mutations to make, we could further prioritize amino acids by creating several additional chimeric constructs in which we exchange the *At*NTR with that of species that lie evolutionarily between *Si* and *Os.* Two good candidate sequences for this exchange would be that of Tomato (73% homology) and Poplar (78% homology).

We focused on variant amino acids that were the same between *At* and *Si*, but different in *Os* because we knew that the *Os*NTR disrupted HAP2 function, and focused on the HGD specifically because it was the region of highest conservation. Another approach will be to mutate amino acids within this region that are the most highly conserved. For example there are 12/45 amino acids within the HGD that are completely conserved between plant, animal, and protozoan orthologs of HAP2 (Chapter 5, Figure 6). There are also three highly conserved cysteines within this region that might be required for lending stability to HAP2. Cysteines have been shown to play a role in the ability of viruses to fuse with host cells due to decreased viral stability (Wahid et al., 2013).

It is also interesting to consider whether post-translational modifications are required for the class-level specificity of gamete fusion. Detection of HAP2 and HAP2:YFP in pollen membrane fractions indicated that HAP2 was migrating ~15kD above expected size in both samples (Chapter 5, Figure 1). *At* and *Si* HAP2 is predicted to contain 6 invariant N-linked glycosylation sites within the NTR. *Os*HAP2 is predicted to contain only three N-linked glycosylations sites, only one of which is at the same position in *At* and *Si.* Glycosylation has been shown to be important for the ability of virus cells to fuse with host cells via receptors like CD4 on the host cell and the envelope protein gp120 of the HIV-1 virus (Vigerust and Shepherd, 2007). Glycosylation has also recently been shown to be important for sexual reproduction in the social amoeba *Dictyostelium discoideum* (Araki et al., 2012). In this system, complementary mating-type cells fuse under dark conditions. MacA, encodes a heavily glycosylated, membrane protein that is required for fusion of these cells during mating. The overall similarity of MacA currently is restricted to proteins of unknown function, but macA encodes the exact same protein in both mating types, only it is expressed at a significantly lower lever in one of the mating types (Araki et al., 2012). This suggests that proteins required for mating fusion in this system rely on adhesion of two heavily glycosylated proteins. Although HAP2 has only been identified as being expressed in one gamete (sperm cells only in Arabidopsis, or the – gamete in Chlamydomonas) this might indicate that the female binding protein on the egg and central cell might also be heavily glycosylated. On the other hand, N-linked glycosylation has been shown to *not* be required for the sperm fusion function of

IZUMO in mice (Inoue et al., 2008). Still, the differences in glycosylation between *Os* and *At* might account for the inability of the *Os*NTR to rescue the fusion defect in *hap2-2* mutants. We can test this by performing the mentioned NTR swaps between *At* and Tomato and Poplar. Tomato is predicted to have the same glycosylation sites as *At* and *Si*, but Poplar is predicted to only contain three invariant glycosylation sites and two unique sites. If the Tomato NTR rescues, but the Poplar NTR does not, this could lend further support to the importance of glycosylation patterns in gamete fusion.

Structural analysis of HAP2 reveals that a region located N-terminally to the HGD is most structurally similar to known viral fusion proteins

It is also possible that the regions required for gamete fusion are located outside of the HGD. When we compared the HAP2 predicted structure to proteins with solved structures, we discovered that the HAP2 NTR located N-terminally to the HGD was most structurally similar to known virus fusion proteins. Specifically, this region was most structurally similar to the actual fusion peptide part of the virus, which lends viruses their fusion capabilities (Allison et al., 2001; Rey et al., 1995; Roehrig et al., 1989). The HGD did not match structurally with any known protein structures. These results suggest that the HGD could be required for species-specific gamete recognition but not the potential fusogenic properties of HAP2. The region located N-terminally to the HGD might be required for initiation of fusion once gamete recognition and adhesion has occurred, and not

be under positive selection like the HGD. Future experiments in which the *At*HAP2 NTR corresponding to this fusogenic region of viral fusion proteins is replaced with the viral equivalent sequence could determine whether this region is structurally conserved enough to initiate gamete fusion in sperm cells. Alternatively, the native fusion protein of the virus could be replaced with that of HAP2, or the fusogenic portion of HAP2 could be expressed in a heterologous system. This has been done recently to show that expression of the parasitic nematode *Trichinella spiralis* FF protein (related to the C. elegans *EPITHELIAL FUSION FAILURE*-1; a known membrane fusogen) is capable of causing hampster kidney cells to spontaneously fuse (Avinoam et al., 2011). In addition, expression of AFF-1 (a related fusogen, Sapir et al., 2007) in place of viral fusogens on the viral envelope was capable of initiating fusion between these AFF-1 expressing viral cells and normal mammalian host cells (Avinoam et al., 2011). We could take a similar approach to express HAP2 in place of the native viral glycoprotein.

It could also be interesting to use this structural comparison method to look for potential female proteins required for gamete fusion. Perhaps these female proteins would structurally resemble known receptors on host cells that viral glycoproteins bind to, like CD4 (Kassler and Sticht, 2013; Klasse, 2012). Potential candidate female proteins, which could include membrane proteins that are only expressed in the egg and central cell and potentially co-evolved with

HAP2, or female *sohos* identified in the suppressor screen, could be analyzed using the Phyre^2 program for structural comparisons.

III) Flowering plants have coupled *HAP2*-required gamete fusion with sperm cell delivery to maximize reproductive success.

Gamete fusion is required for the block to polytubey in Arabidopsis

In order to accomplish maximum seed production, every ovule within the pistil must receive two functional sperm cells, no more, no less. This requires tightly regulated communication between the pollen tubes that deliver these sperm cells and the ovules, and between the sperm cells and the egg and central cell. Like in animal systems (Wong and Wessel, 2006), mechanisms have evolved in flowering plants to prevent multiple sperm cells from fusing with a single egg cell (prevention of polyspermy), however, this block to polyspermy starts at the upstream event of pollen tube targeting and sperm cell delivery. Instead of hundreds of sperm cells competing for a single egg cell like in organisms such as the mouse (Sun, 2003), hundreds of pollen tubes are competing for a limited number of ovules. Targeting of each ovule by only one pollen tube ensures that only the required two sperm cells are delivered for gamete fusion.

We have shown that polytubey occurs very rarely in Arabidopsis, but increases significantly when sperm incapable of gamete fusion are deposited (Beale et al.,

2012). We also show that in wild type, one synergid cell persists well after gamete fusion (Faure et al., 2002) but that multiple pollen tubes are rarely attracted. This observation suggested that gamete fusion, or an event soon thereafter, triggers a mechanism that can overcome attraction by the persistent synergid and prevent entry of additional pollen tubes. We found that synergids persist longer in ovules that have received non-functional sperm compared to those that had received functional sperm, which provides some insights into why gamete fusion defects (Beale et al., 2012; Kasahara et al., 2012; Mori et al., 2006), or pollen tube burst defects (Boisson-Dernier et al., 2008; Huck et al., 2003; Rotman et al., 2008), lead to polytubey.

Potential mechanisms for the block to polytubey

It has been well established that synergid cells are required for pollen tube attraction in flowering plants. Laser ablation studies in Torenia established that synergid cells are the source of pollen tube attractants (Higashiyama et al., 2001; Okuda and Higashiyama, 2010) and genomic and genetic studies in Arabidopsis showed that synergids express unique genes critical for pollen tube attraction (Johnston et al., 2007; Jones-Rhoades et al., 2007; Kasahara et al., 2005; Shimizu and Okada, 2000). LUREs were identified as micropylar pollen tube attractants in Torenia (Okuda et al., 2009) and recently Arabidopsis LUREs were also identified (Takeuchi and Higashiyama, 2012). These LUREs are secreted

from both synergid cells and synthesized LURE peptides can attract pollen tubes *in vitro* (Okuda et al., 2009; Takeuchi and Higashiyama, 2012).

In the process of double fertilization, one of these synergid cells degenerate upon pollen tube burst whether the pollen tube is carrying functional, or non-functional sperm (Kessler and Grossniklaus, 2011). In wild type ovules that receive functional sperm, the synergid cell was observed to persist well after gamete fusion had occurred (Beale et al., 2012; Faure et al., 2002). We observed that the remaining synergid cell persisted even longer in ovules that had received *hap2* mutant sperm incapable of gamete fusion. It was also observed that an ovule was capable of attracting up to four pollen tubes (Beale et al., 2012). We therefore predicted that the non-degenerated synergid cell was still capable of secreting pollen tube attractants, allowing for targeting of more pollen tubes. It is also interesting that the remaining synergid cell persists after successful fertilization in wild type.

We propose that after successful double fertilization, a rapid or fast-block to polytubey is created, much like a rapid block to polyspermy observed in mammals, fish, amphibians, and salamanders (Jaffe and Cross, 1986; Jaffe et al., 1983; Nuccitelli, 1980). The “rapid block to polyspermy” or the “electrical block to polyspermy” occurs when the membrane of the egg cell becomes rapidly depolarized after sperm fusion. In *Xenopus* this depolarization occurs due to an

efflux of Cl^- ions followed by an efflux of Na^+, leading to changes in Ca^+ channel activity (Glahn and Nuccitelli, 2003). The rapid block to polytubey in Arabidopsis would have to be triggered by something that was fast, and easily diffusible within the ovule. A potential candidate in Arabidopsis is Nitric Oxide (NO). When pollen tubes grown in vitro are exposed to NO, growth is slowed and a sharp reorientation in growth direction is observed (Prado et al., 2004). It is possible that upon fertilization the concentration of NO is significantly increased, leading to pollen tubes turning away from this already targeted ovule. However, it has also been shown that NO is already present at very high levels within the female gametophyte and required for pollen tube targeting (Prado et al., 2008). Perhaps a NO increases significantly above this basal level after fertilization, however, this needs to be tested directly.

Eventually, the remaining synergid cells does degenerate after successful fertilization, but persists significantly longer in ovules that have received *hap2* mutant sperm. This suggests that there might also be a permanent, or “slow” block to polytubey upon successful fertilization, that is established by degeneration of the remaining synergid cell. This would abolish secretion of the LURE peptides required for pollen tube attraction permanently. Recently, it was discovered that ethylene (another gas) is required for synergid degeneration and establishment of a pollen tube block (Volz et al., 2013). Microinjection of the ethylene precursor ACC into the female gametophyte resulted in premature degeneration of the synergid cell and inability of pollen tube attraction (Volz et al.,

2013). Perhaps after successful fertilization ethylene is released, leading to degeneration of the persisting synergid cell and a permanent block to polytubey.

It would be interesting to know whether the remaining synergid cell in ovules that have received *hap2* mutant sperm are still actively secreting LUREs to attract additional pollen tubes containing functional sperm. Now that LUREs have been identified in Arabidopsis, experiments should be performed in which their activity is monitored after deposition of *hap2* mutant sperm. This could be accomplished be looking of expression of LUREs tagged with GFP in the ovule after *hap2, HTR10:HTR10:mRFP* sperm are delivered. We would predict that LUREs continue to be secreted until functional sperm have been deposited, at which point both the slow and fast block to polytubey would be created.

Potential mechanisms for the block to polyspermy

The observation that there is a basal rate of polytubey in wild type ~1% suggests that additional mechanisms could be in place to prevent polyspermy. Should four functional sperm be delivered to an ovule, these mechanisms would ensure that both the egg and central cell were fertilized by only a single sperm cell, and that even if only two sperm are deposited, one fertilizes the egg cell and the other fertilizes the central cell. Evidence in Arabidopsis suggests that there is a block to

polyspermy on the egg cell but not the central cell, however there is no known mechanism ensuring that each sperm cell fuses with only one gamete (Scott et al., 2008). It was hypothesized that the first sperm cell to leave the PT would fertilize the egg cell, and the remaining sperm by default would then fertilize the central cell. This hypothesis was supported by the observation that *cdc2a* mutants, which produce only a single sperm-like cell (SSLC) capable of fusion, always fertilized the egg cell (Nowack et al., 2006). However, it was later discovered that *cdc2* mutants undergo division before fertilization, producing two functional sperm that are actually capable of double fertilization (Aw et al., 2010).

More recent studies have found that other SSLCs capable of fusion (DTA:HAP2 and *hap2*^{hypo}, Thesis Chapter 4*)* have a preference to fertilize the central cell (Frank and Johnson, 2009; Wong et al., 2010) or exhibit no preference at all (*msi1,* (Chen et al., 2008). We see in our preliminary analysis of *duo1* sperm expressing *HAP2* and *H2B:RFP* a distinct preference for fertilization with the egg ell. Furthermore, live cell imaging of double fertilization has shown by individual sperm tracking that there is no preference for the first sperm cell to exit the pollen tube to fertilize the egg (Hamamura et al., 2012; Hamamura et al., 2011).

It is possible that different factors are expressed in each sperm cell, but testing this directly would be extremely challenging. In our genetic screen we saw that *soho1* required a mutation that was expressed in both the male and female

(Thesis Chapter 4, Table 1). Analysis of the products of fertilization using *soho1* as a male-donor, showed a significant increase in the amount of central cell only fertilization events compared to *hap2*^{hypo} (These Chapter 4, Figure 6). If we performed this analysis using *soho1* as the female, as we saw an increase in egg cell only fertilization events, this would suggest that there are factors like *soho1* that regulate fusion from the male side with the central cell, and fusion with the egg cell from the female side.

It is also possible that upon release one sperm cell membrane always touches the egg cell membrane and the other with the central cell membrane at the exact site of fusion (more of a physical placement model) and that when only one sperm cell capable of fusion is released this placement is random depending on where the sperm cell "lands". Development of sperm and female cell membrane markers that are easily visualized within the ovule at the time of fusion would greatly aid in determining these models.

Coupling gamete fusion with the block to polytubey maximizes reproductive success and seed development in Arabidopsis

When hundreds of pollen tubes are growing through the transmitting tract competing for a limited number of ovules tightly regulated signals are required to ensure that every ovule is targeted by functional sperm. Genes like *FER* are then

required to trigger a signaling cascade that initiates both PT rupture and synergid cell degeneration (Escobar-Restrepo et al., 2007; Huck et al., 2003). This synergid degeneration makes room for the pollen tube contents and the sperm cells are shot to the site of fusion where gamete fusion occurs ~8min after discharge (Hamamura et al., 2011). If this pollen tube contains functional sperm, then double fertilization occurs, and the remaining synergid cell eventually degenerates after initiation of embryo development (Beale et al., 2012; Faure et al., 2002). The time from pollen tube arrival to gamete fusion has been approximated to take ~15min (Hamamura et al., 2011) and pollen tubes arriving at an ovule up to 80min after initial targeting by the first pollen tube have been shown to be actively repelled *semi in vivo* (Palanivelu and Preuss, 2006). We hypothesize that when instances of polytubey occur in wild type the two tubes that enter the ovule may have been traveling within ~15min of one another, at which point they could enter the same ovule since the block to polytubey would not yet have been initiated. In these cases, it would be important for additional mechanisms to be in place that would prevent actual polyspermy. A potential 'fast block' to polytubey would then be created to deter the persisting synergid cell attractants from attracting additional pollen tubes. Then, a permanent or 'slow block' to polytubey would then be created via degeneration of the persisting synergid cell. This permanent block would ensure that additional pollen tubes that might have been growing towards this ovule now turn away to target a neighboring ovule that has yet to be targeted.

However, if this first pollen tube contains non-functional sperm like *hap2* or *duo1* sperm, then the block to polytubey is not created, the remaining synergid cell persists for a longer time period, and presumably continues to secrete pollen tube attractants in an effort to attract an additional pollen tube carrying functional sperm. Once the ovule receives functional sperm, the block to polytubey is then created, and the persisting synergid cell eventually degenerates. This block then again, redirects additional pollen tubes to ovules that have not yet been targeted.

This elegant system of coupling pollen targeting with successful *HAP2*-mediated gamete fusion would enhance reproductive success by ensuring that every ovule receives functional sperm, and that additional pollen tubes are evenly distributed within the pistil to other available ovules, leading to maximum seed production.

References

Allison, S.L., Schalich, J., Stiasny, K., Mandl, C.W., and Heinz, F.X. (2001). Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. Journal of virology 75:4268-4275.

Araki, Y., et al. (2012). A surface glycoprotein indispensable for gamete fusion in the social amoeba Dictyostelium discoideum. Eukaryotic cell 11:638-644.

Asquith, K.L., Baleato, R.M., McLaughlin, E.A., Nixon, B., and Aitken, R.J. (2004). Tyrosine phosphorylation activates surface chaperones facilitating sperm-zona recognition. Journal of cell science 117:3645-3657.

Avinoam, O., et al. (2011). Conserved eukaryotic fusogens can fuse viral envelopes to cells. Science (New York, N.Y.) 332:589-592.

Aw, S.J., Hamamura, Y., Chen, Z., Schnittger, A., and Berger, F. (2010). Sperm entry is sufficient to trigger division of the central cell but the paternal genome is required for endosperm development in Arabidopsis. Development (Cambridge, England) 137:2683-2690.

Bayer, M., Nawy, T., Giglione, C., Galli, M., Meinnel, T., and Lukowitz, W. (2009). Paternal control of embryonic patterning in Arabidopsis thaliana. Science (New York, N.Y.) 323:1485-1488.

Beale, K.M., Leydon, A.R., and Johnson, M.A. (2012). Gamete fusion is required to block multiple pollen tubes from entering an Arabidopsis ovule. Current biology : CB 22:1090-1094.

Berger, F., Hamamura, Y., Ingouff, M., and Higashiyama, T. (2008). Double fertilization - caught in the act. Trends in plant science 13:437-443.

Blagborough, A.M., and Sinden, R.E. (2009). Plasmodium berghei HAP2 induces strong malaria transmission-blocking immunity in vivo and in vitro. Vaccine 27:5187-5194.

Boisson-Dernier, A., Frietsch, S., Kim, T.H., Dizon, M.B., and Schroeder, J.I. (2008). The peroxin loss-of-function mutation abstinence by mutual consent disrupts male-female gametophyte recognition. Current biology : CB 18:63-68.

Borg, M., Brownfield, L., Khatab, H., Sidorova, A., Lingaya, M., and Twell, D. (2011). The R2R3 MYB transcription factor DUO1 activates a male germline-specific regulon essential for sperm cell differentiation in Arabidopsis. The Plant cell 23:534-549.

Brownfield, L., Hafidh, S., Borg, M., Sidorova, A., Mori, T., and Twell, D. (2009). A plant germline-specific integrator of sperm specification and cell cycle progression. PLoS genetics 5:e1000430.

Chen, Z., Tan, J.L., Ingouff, M., Sundaresan, V., and Berger, F. (2008). Chromatin assembly factor 1 regulates the cell cycle but not cell fate during male gametogenesis in Arabidopsis thaliana. Development (Cambridge, England) 135:65-73.

Escobar-Restrepo, J.M., et al. (2007). The FERONIA receptor-like kinase mediates male-female interactions during pollen tube reception. Science (New York, N.Y.) 317:656-660.

Faure, J.E., Rotman, N., Fortune, P., and Dumas, C. (2002). Fertilization in Arabidopsis thaliana wild type: developmental stages and time course. The Plant journal : for cell and molecular biology 30:481-488.

Francis, K.E., Lam, S.Y., Harrison, B.D., Bey, A.L., Berchowitz, L.E., and Copenhaver, G.P. (2007). Pollen tetrad-based visual assay for meiotic recombination in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 104:3913-3918.

Frank, A.C., and Johnson, M.A. (2009). Expressing the diphtheria toxin A subunit from the HAP2(GCS1) promoter blocks sperm maturation and produces single sperm-like cells capable of fertilization. Plant physiology 151:1390-1400.

Glahn, D., and Nuccitelli, R. (2003). Voltage-clamp study of the activation currents and fast block to polyspermy in the egg of Xenopus laevis. Development, growth & differentiation 45:187-197.

Gonzalez, N., et al. (2010). Increased leaf size: different means to an end. Plant physiology 153:1261-1279.

Hamamura, Y., Nagahara, S., and Higashiyama, T. (2012). Double fertilization on the move. Current opinion in plant biology 15:70-77.

Hamamura, Y., et al. (2011). Live-cell imaging reveals the dynamics of two sperm cells during double fertilization in Arabidopsis thaliana. Current biology : CB 21:497-502.

Higashiyama, T., et al. (2001). Pollen tube attraction by the synergid cell. Science (New York, N.Y.) 293:1480-1483.

Huck, N., Moore, J.M., Federer, M., and Grossniklaus, U. (2003). The Arabidopsis mutant feronia disrupts the female gametophytic control of pollen tube reception. Development (Cambridge, England) 130:2149-2159.

Ikawa, M., et al. (1997). The putative chaperone calmegin is required for sperm fertility. Nature 387:607-611.

Inoue, N., Ikawa, M., and Okabe, M. (2008). Putative sperm fusion protein IZUMO and the role of N-glycosylation. Biochemical and biophysical research communications 377:910-914.

Jaffe, L.A., and Cross, N.L. (1986). Electrical regulation of sperm-egg fusion. Annual review of physiology 48:191-200.

Jaffe, L.A., Cross, N.L., and Picheral, B. (1983). Studies of the voltage-dependent polyspermy block using cross-species fertilization of amphibians. Developmental biology 98:319-326.

Johnston, A.J., et al. (2007). Genetic subtraction profiling identifies genes essential for Arabidopsis reproduction and reveals interaction between the female gametophyte and the maternal sporophyte. Genome biology 8:R204.

Jones-Rhoades, M.W., Borevitz, J.O., and Preuss, D. (2007). Genome-wide expression profiling of the Arabidopsis female gametophyte identifies families of small, secreted proteins. PLoS genetics 3:1848-1861.

Joppa, L.N., Roberts, D.L., and Pimm, S.L. (2011). How many species of flowering plants are there? Proceedings. Biological sciences / The Royal Society 278:554-559.

Kasahara, R.D., Maruyama, D., Hamamura, Y., Sakakibara, T., Twell, D., and Higashiyama, T. (2012). Fertilization recovery after defective sperm cell release in Arabidopsis. Current biology : CB 22:1084-1089.

Kasahara, R.D., Portereiko, M.F., Sandaklie-Nikolova, L., Rabiger, D.S., and Drews, G.N. (2005). MYB98 is required for pollen tube guidance and synergid cell differentiation in Arabidopsis. The Plant cell 17:2981-2992.

Kassler, K., and Sticht, H. (2013). Molecular mechanism of HIV-1 gp120 mutations that reduce CD4 binding affinity. Journal of biomolecular structure & dynamics.

Kessler, S.A., and Grossniklaus, U. (2011). She's the boss: signaling in pollen tube reception. Current opinion in plant biology 14:622-627.

Klasse, P.J. (2012). The molecular basis of HIV entry. Cellular microbiology 14:1183-1192.

Lefevre, B., Wolf, J.P., and Ziyyat, A. (2010). Sperm-egg interaction: is there a link between tetraspanin(s) and GPI-anchored protein(s)? BioEssays : news and reviews in molecular, cellular and developmental biology 32:143-152.

Li, J., and Berger, F. (2012). Endosperm: food for humankind and fodder for scientific discoveries. The New phytologist 195:290-305.

Li, J., and Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90:929-938.

Li, X.Q. (2009). Developmental and environmental variation in genomes. Heredity 102:323-329.

Liesa, M., et al. (2008). Mitochondrial fusion is increased by the nuclear coactivator PGC-1beta. PloS one 3:e3613.

Liu, Y., Misamore, M.J., and Snell, W.J. (2010). Membrane fusion triggers rapid degradation of two gamete-specific, fusion-essential proteins in a membrane block to polygamy in Chlamydomonas. Development (Cambridge, England) 137:1473-1481.

Liu, Y., et al. (2008). The conserved plant sterility gene HAP2 functions after attachment of fusogenic membranes in Chlamydomonas and Plasmodium gametes. Genes & development 22:1051-1068.

Mori, T., Kuroiwa, H., Higashiyama, T., and Kuroiwa, T. (2006). GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nature cell biology 8:64-71.

Nowack, M.K., Grini, P.E., Jakoby, M.J., Lafos, M., Koncz, C., and Schnittger, A. (2006). A positive signal from the fertilization of the egg cell sets off endosperm proliferation in angiosperm embryogenesis. Nature genetics 38:63-67.

Nuccitelli, R. (1980). The fertilization potential is not necessary for the block to polyspermy or the activation of development in the medaka egg. Developmental biology 76:499-504.

Okuda, S., and Higashiyama, T. (2010). Pollen tube guidance by attractant molecules: LUREs. Cell structure and function 35:45-52.
Okuda, S., et al. (2009). Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature 458:357-361.
Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel Arabidopsis thaliana pollen tubes in vitro. BMC plant biology 6:7.
Prado, A.M., Colaco, R., Moreno, N., Silva, A.C., and Feijo, J.A. (2008). Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signaling. Molecular plant 1:703-714.
Prado, A.M., Porterfield, D.M., and Feijo, J.A. (2004). Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development (Cambridge, England) 131:2707-2714.
Qin, Y., and Yang, Z. (2011). Rapid tip growth: insights from pollen tubes. Seminars in cell & developmental biology 22:816-824.
Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C., and Harrison, S.C. (1995). The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature 375:291-298.
Roehrig, J.T., Hunt, A.R., Johnson, A.J., and Hawkes, R.A. (1989). Synthetic peptides derived from the deduced amino acid sequence of the E-glycoprotein of Murray Valley encephalitis virus elicit antiviral antibody. Virology 171:49-60.
Rotman, N., et al. (2005). A novel class of MYB factors controls sperm-cell formation in plants. Current biology : CB 15:244-248.
Rotman, N., Gourgues, M., Guitton, A.E., Faure, J.E., and Berger, F. (2008). A dialogue between the SIRENE pathway in synergids and the fertilization independent seed pathway in the central cell controls male gamete release during double fertilization in Arabidopsis. Molecular plant 1:659-666.
Sapir, A., et al. (2007). AFF-1, a FOS-1-regulated fusogen, mediates fusion of the anchor cell in C. elegans. Developmental cell 12:683-698.
Scott, R.J., Armstrong, S.J., Doughty, J., and Spielman, M. (2008). Double fertilization in Arabidopsis thaliana involves a polyspermy block on the egg but not the central cell. Molecular plant 1:611-619.
Shimizu, K.K., and Okada, K. (2000). Attractive and repulsive interactions between female and male gametophytes in Arabidopsis pollen tube guidance. Development (Cambridge, England) 127:4511-4518.
Sosnik, J., et al. (2009). Tssk6 is required for Izumo relocalization and gamete fusion in the mouse. Journal of cell science 122:2741-2749.
Sprunck, S., Rademacher, S., Vogler, F., Gheyselinck, J., Grossniklaus, U., and Dresselhaus, T. (2012). Egg cell-secreted EC1 triggers sperm cell activation during double fertilization. Science (New York, N.Y.) 338:1093-1097.
Sun, Q.Y. (2003). Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. Microscopy research and technique 61:342-348.

Takeuchi, H., and Higashiyama, T. (2012). A species-specific cluster of defensin-like genes encodes diffusible pollen tube attractants in Arabidopsis. PLoS biology 10:e1001449.

Twell, D. (2011). Male gametogenesis and germline specification in flowering plants. Sexual plant reproduction 24:149-160.

Vigerust, D.J., and Shepherd, V.L. (2007). Virus glycosylation: role in virulence and immune interactions. Trends in microbiology 15:211-218.

Volz, R., Heydlauff, J., Ripper, D., von Lyncker, L., and Gross-Hardt, R. (2013). Ethylene signaling is required for synergid degeneration and the establishment of a pollen tube block. Developmental cell 25:310-316.

von Besser, K., Frank, A.C., Johnson, M.A., and Preuss, D. (2006). Arabidopsis HAP2 (GCS1) is a sperm-specific gene required for pollen tube guidance and fertilization. Development (Cambridge, England) 133:4761-4769.

Wahid, A., Helle, F., Descamps, V., Duverlie, G., Penin, F., and Dubuisson, J. (2013). Disulfide bonds in hepatitis C virus glycoprotein E1 control the assembly and entry functions of E2 glycoprotein. Journal of virology 87:1605-1617.

Wong, J.L., Leydon, A.R., and Johnson, M.A. (2010). HAP2(GCS1)-dependent gamete fusion requires a positively charged carboxy-terminal domain. PLoS genetics 6:e1000882.

Wong, J.L., and Wessel, G.M. (2006). Defending the zygote: search for the ancestral animal block to polyspermy. Current topics in developmental biology 72:1-151.