

***HAP2(GCS1)* is a conserved sperm-expressed gene
required for fertilization**

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Dissertation submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the Division of Biology and Medicine at

Brown University

Providence, Rhode Island

May 2010

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Acknowledgments:

There are many people I wish to thank who have helped me along the way to this ultimate thesis. First and foremost I wish to thank my parents who have been supportive throughout my life of my insatiable curiosity and taught me so many traits and habits that have helped through all of my schooling and extra-curricular activities. I would also like to thank those friends who have helped me through horrible teachers and tests to ultimately keep me sane and persevering: Maggie Byrkit who I would not have passed high school chemistry without, Adrienne Aiona, Heather McGivney, Amy Ackerman and Sarah Matson who showed me that my evenings could be just as useful not doing homework, and Jennifer Carini without whom I would never have attempted or completed Graduate School.

Then I would like to thank those teachers and professors who have pushed me to keep questioning, keep working and keep learning, even in the face of difficult situations. Judy Stone was kind enough to take me on for a research project before she had barely met me, Arthur Champlin who pushed me to go abroad, and Timothy O'Brien who hired me just out of college and who taught me so much about how science research should be conducted. My thesis committee has been truly amazing in their dedication to making me a better writer and scientist and I am grateful for their comments, suggestions and never ending enthusiasm. Alison DeLong has even helped with specific experiments, Gary Wessel and Wolfgang Peti have been a breath of fresh air to help me think outside of plants. And I would like to put in a special thank you to my outside reader William Snell who has traveled a fair piece to come help me complete my thesis and whose discussions about HAP2 even before this have been amazingly insightful, helpful and

enjoyable.

I would most certainly like to commiserate with my fellow lab mates in the Johnson Lab without whom cloning and plant dissections would not be nearly as much fun. I would like to wish them my heartfelt thanks and good luck with all the work that I am leaving behind. Althea Moore, Alex Leydon, Kristin Beale, Julian Wong and all the undergraduates who have listened to my rantings, ravings, and countless lab meeting presentations, thank you for all your kindness, support and pleasant company.

As to my advisor Mark Johnson, this entire thesis would not have been possible without him and I would be very sad if I had not found my way into his lab. He has been a constant source of encouragement, scientific insight, joyful mirth, and gentle prodding, all of which have made my graduate career enjoyable and enlightening. He has taught me so much about plants, science and writing that I am truly indebted and grateful.

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Abstract:

In flowering plants, two sperm cells develop in the pollen cytoplasm and are transported through floral tissues to an ovule by a pollen tube, a highly polarized cellular extension. After targeting an ovule, the pollen tube bursts, releasing two sperm that fertilize the egg and central cell producing the embryo and endosperm respectively. The mechanisms responsible for fusion of the two sperm with female cells are unknown. This thesis presents the initial characterization of the *Arabidopsis HAP2* gene, demonstrating that it is essential for fertilization and probably directly involved in a deeply conserved gamete fusion mechanism. We also show that HAP2 is required for pollen tube guidance. We searched for proteins that interact with HAP2 through a yeast two-hybrid screen and identified proteins that regulate disulfide bond formation, these data indicate several invariant cysteine residues within the N-terminal region of HAP2 are likely critical for its structure and function. We utilized the tightly regulated sperm-specific expression pattern of *HAP2* to disrupt sperm development by expressing the Diphtheria toxin A subunit. By disrupting sperm translation in this manner, single sperm-like cells (SSLCs) were generated that preferentially fertilized the central cell. This finding is contrary to the previously held idea that SSLCs like those created in the *cdc2* mutant have a default targeting to the egg. We were able to ectopically express HAP2 in the pollen tube, but were unable to find transformants that express HAP2 in the leaves when expressed from the 35S promoter. These experiments begin to elucidate the expression pattern, proper folding, and function of HAP2 during fertilization.

Chapter 1: Reproduction in flowering plants

Sexual reproduction is one of the most critical functions of life. Although seemingly simple, with two cells finding and fusing with one another, it is a very complex process to make the specialized gamete cells and coordinate the interaction between cells to bring the gametes in contact with one another and fuse. Even though reproduction is so important the mechanisms of specifying and differentiating gamete cells, bringing these cells together, and fusion are not well understood. Fertilization is the most difficult of these to study not only because it is greatly variable between different organisms, but also because it is a very fleeting event and efficiently executed.

Plants specify their germ line after maturity

Unlike animals, plants specify the cells that will become gametes after the plant is mature. Animals perform this vital task during embryo development and keep the germ cells separate from other stem cell populations in anticipation of reproductive maturity (Juliano et al., 2010). The germ cells of animals are also completely differentiated and after meiosis cannot divide further. Flowering plants (angiosperms), produce their gametes from the same stem cell population that makes all the other cells of the plant and the cells that produce gametes replicate mitotically after meiosis, before the cells are specialized as gametes.

Plants oscillate between two life stages: that of sporophyte and gametophyte. Sporophytic tissue is diploid and makes the leaves, roots and all the tissues of the adult flowering plant. Gametophytic tissues make the gametes and are haploid; in flowering plants these cells reside within the flowers. Sporophyte and gametophyte are considered different life stages because both the diploid and haploid cells divide mitotically and some species spend the majority of their life as either haploid or diploid organisms.

The process of fertilization in flowering plants requires that the pollen, which contains two sperm cells, travels through the pistil tissue to find the female cells that the two sperm will fuse with, within an ovule. When the pollen reaches an ovule it releases the two sperm cells which each fuse with one of the receptive female cells, either the egg or the central cell to produce the embryo and endosperm respectively. This is referred to as double fertilization because both sperm fertilize a female cell and each of these events is critical to make a mature and viable seed. An ovule will not mature into a seed without the presence of both the embryo and endosperm (Frank and Johnson, 2009; Nowack et al., 2006). The endosperm feeds the growing embryo somewhat akin to the avian yolk or mammalian placenta.

The flowers of angiosperm plants house both female and male gametes as the gametophytic generation of the plant.

Each flower arises from the shoot apical meristem, a collection of cells that can differentiate into most tissues of the adult plant. Under the correct conditions of temperature, light intensity and color, and day length the plant is signaled to flower (reviewed by Sablowski, 2009). Each flower is arranged in a pattern of concentric circles where each whorl is a different tissue type. These whorls are determined by the expression and overlap of transcription factors (reviewed by Tanaka, 1997). The outermost layer is the sepals, which look very much like leaves, and the next layer is comprised of the petals. The anthers and carpels make up the inner two whorls and undergo more specific cell differentiation to produce the male and female gametophytes respectively. The timing of anther dehiscence, which releases the pollen, is carefully controlled. In the self-fertile *Arabidopsis* this manifests itself such that when the ovules

and pistil are receptive, the pollen is mature and released from the anthers (Faure et al., 2002).

The male gametophyte is made of two different cell types.

After meiosis of the microspore mother cell, the four meiotic products dissociate from each other and each will become a pollen grain. This microspore then divides asymmetrically to produce the large vegetative cell and the smaller generative cell, which is engulfed by the vegetative cell to make bicellular pollen (Tanaka, 1997). While the vegetative cell does not divide again, the generative cell undergoes another cell cycle and divides into two sperm cells. This second mitosis occurs in some species after the pollen reaches a female pistil, but in *Arabidopsis* the pollen has three cells (tricellular) at anthesis, when it is released from the anther. The second pollen mitosis to produce two sperm was studied in detail by mutations that disrupt the division of the generative cell. Many of these are mutations in cell cycle control proteins such as cyclin dependent kinase A (*cdc2*, Iwakawa et al., 2006) and *Fbl17*, part of the ubiquitin complex that regulates CDKA and cyclin B (Gusti et al., 2009; Kim et al., 2008). When either of these is lacking, the cell cycle does not progress and pollen is left at the bicellular stage.

The vegetative and generative cells have very different functions. The vegetative cell forms the pollen tube, which travels to an ovule to deliver the two sperm, while the generative cell is the precursor of the two sperm. The two sperm each fertilize a female cell within the ovule. The gene expression patterns of the vegetative and generative cell types are different for genes specific to the functions of each of the cell types. Pollen grains or the vegetative cells express genes such as *LAT52* that mediate the interaction with female cells and proteins required for their travel through the pistil towards ovules

(Twell et al., 1990; Muschietti et al., 1994; Tang et al., 2002). The sperm express their own set of germline-specific proteins. Although the list of these genes is not extensive nor complete the few that are known are an interesting set under the regulation of germline-specific transcription factors DUO1 and DUO3 (Rotman et al., 2005; Brownfield et al., 2009b). The known sperm specific genes are a histone variant of H3 known as H3.3 (Okada et al., 2005), GEX2 of unknown function (Engel et al., 2005), and the fusion dependent protein HAP2(GCS1) (Johnson et al., 2004; Mori et al., 2006; von Besser et al., 2006).

The female gametophyte cells divide from one meiotic product into a multi-celled ovule

The female gametophyte is made up of seven haploid cells. After meiosis of the mother cell, only one daughter cell is retained and it goes through three mitotic divisions to create eight nuclei that form the seven-celled female gametophyte. Initially there are two groups of four uncellularized nuclei located at either pole of the gametophyte. Two of these polar nuclei migrate to the center and fuse to form the diploid central cell. At one pole three nuclei are specified to become the egg and two synergid cells. At the other pole, the three remaining nuclei become the antipodal cells whose function is unknown. The female gametophyte (Figure 2) is surrounded by diploid maternal tissue to make an ovule.

The mechanism that determines the fate of each female cell was recently shown to be mediated by the hormone auxin (Pagnussat et al., 2007). The mutant *eostre* produces multiple egg cells by specifying that one of the synergids will be an egg instead (Pagnussat et al., 2007). By making double mutants it was discovered that an auxin

gradient establishes which cells within the ovule would be which type of cell. It was determined that the gradient originates outside of the ovule near the micropyle from the pistil tissue and those cells that receive the greatest amount of auxin become the synergids. Regions of the female gametophyte farther from the micropyle become the egg and central cell, but if the gradient is increased or decreased other cells can take on the fate of an egg or central cell.

Pollen tubes grow through the female pistil guided by cues to reach an ovule

When pollen is deposited on a female pistil of the same plant or species, it is hydrated by the papilla cells at the top of the pistil (Dickinson et al., 1998) and Figure 1). The pollen grain then quickly breaks its dormancy and begins to extend a pollen tube projection into the papilla cell and through the style of the pistil. The pollen tube travels through the cell files of the transmitting tract of the pistil utilizing sugars, cell wall material and proteins from the pistil to grow, increasing from the tip.

The cytoplasm of the pollen grain along with the pollen nucleus and the two sperm (known as the male germ unit, Lalanne and Twell, 2002), move along at the front of the pollen tube (Taylor et al., 1991). Within the older part of the tube, plugs of callose are deposited that block off the back end of the pollen tube such that a hollow shell is left on the stigma and in the transmitting tract. The pollen tubes of some species, including maize, can grow as much as a hundred times the original pollen grain's length to reach through very long pistils. The pollen cytoplasm is visibly busy with cytoplasmic streaming and vesicle movement to deposit membrane and cell wall components, but not at the very tip which is considered the clear zone because it is devoid of visible structures (Krichevsky, 2007).

Signals and interactions between the pollen grain and female tissue are constantly occurring as soon as pollen lands on the pistil (Johnson and Preuss, 2002). If the pollen is from the same flower in a self-infertile plant, enzymes recognize it as having the same genotype and will stop its further growth to allow for outcrossing (Murfett et al., 1994). Signals such as Transmitting Tissue-Specific in tobacco, GABA and Chemocyanin in *Arabidopsis* guide the pollen tube out of the transmitting tract and along the funiculus to an ovule (McCormick and Yang, 2005); Palanivelu et al., 2003; Kim et al., 2003). As the pollen tube grows through the style near the top of the pistil, it receives a signal from the female tissue that allows it to ultimately find an ovule. Palanivelu and Preuss found that *Arabidopsis* pollen tubes grown in vitro are unable to properly find an ovule unless they travel through the style of a pistil (Palanivelu and Preuss, 2006). They were able to isolate the female compound that capacitates pollen so they are able to perceive later guidance cues. The fact that this process is similar in function to that of animal sperm capacitation is interesting and could lead to a better understanding of how closely related the processes of plant and animal fertilization are.

Other signals from within the ovule are released through the filiform apparatus of the synergid cells and guide the pollen tube into the micropylar opening (Swanson et al., 2004). When synergid cells are ablated either by laser (Higashiyama et al., 2001), or disrupted through mutating the Myb98 transcription factor (Kasahara et al., 2005), signals attracting pollen tubes were not able to leave the ovule and pollen tube guidance was depleted. Cysteine-rich defensin-like molecules were isolated from synergid cells of *Torenia fournieri*. These species specific proteins had the ability to guide pollen tubes in vitro (Okuda 2009).

When a pollen tube enters an ovule at the micropyle it is signaled to stop growing and burst. In the female mutants *feronia* and *sirene* this signal is absent and without this signal the pollen tube continues to grow in the ovule disrupting the female cells (Huck et al., 2003; Escobar-Restrepo et al., 2007). Normally the pollen tube will burst open after growth stops in the degenerated synergid space and the sperm are released.

To ensure that an ovule is only fertilized once, only one pollen tube is allowed to enter a given ovule. A signal is sent out from the ovule when a pollen tube is received to block further pollen tubes from entering (Palanivelu and Preuss, 2006). In the *feronia* mutant, multiple pollen can tubes enter an ovule (Huck et al., 2003). Not only is there a signal to stay away, but when the synergid cell degenerates it stops sending guidance cues to attract pollen tubes (Faure et al., 2002).

Double fertilization occurs when the sperm are delivered to an ovule

Once the pollen tube is received in the ovule and stops growing, it bursts open at the tip, releasing the two sperm into the ovule. It is not yet known if the sperm are propelled by the force of the pollen tube bursting (Berger et al., 2008) towards the female gametes or if they travel there in some other manner. Possibly they utilize the cytoskeletal remains of the pollen tube and degenerated synergid (Faure et al., 2002; Fu et al., 2000). The sperm reach the female gametes and fuse quickly after the pollen tube bursts (Berger et al., 2008). Whether the sperm dissociate from one another or are only separated through the action of fusing with a female gamete and whether one of the fertilization events occurs before the other are details still under debate.

Upon fertilization and the fusion of nuclei the central cell starts dividing to make the endosperm, which will go on to feed the developing embryo, and the zygote divides

beginning the embryo. Recently it was found that the embryo and endosperm communicate with one another during embryogenesis (Nowack et al., 2006). Development of both structures is necessary to create a viable seed and only when both are developing normally does the maternal tissue make the seed coat (Ingouff et al., 2006). The endosperm of most dicot plants is absorbed by the embryo and is not stored in the mature seed, while in monocot plants there is a store of endosperm within the seed when mature and released to make a new seedling.

The two sperm of flowering plants are visibly distinct or dimorphic in some species and seem identical in others

All three cells of the pollen grain have the same haploid genotype, but the vegetative and sperm cells differ greatly in their morphology and function. The two sperm of *Arabidopsis* appear identical (Twell et al., 1998), but it is an open question as to whether they are equal. In the species *Plumbago zeylanica* (Russell, 1985; Russell and Cass, 1981) and *Torenia fournieri* (Chen, 2006) the two sperm are different in their morphology. One of the *Plumbago* sperm has almost all the mitochondria and the other almost all the plastids, and they each have a different female gamete target (Russell, 1985). The larger sperm, which contains most of the mitochondria more often fuses with the central cell rather than the egg. The two sperm in both *Plumbago* and *Torenia* species were seen to associate with the vegetative nucleus differently, with the larger sperm attached to the vegetative nucleus and the smaller sperm only attached to the larger sperm.

Germ-line gene expression

It is difficult to study fertilization in flowering plants because the ovule is often buried deep within maternal tissue and few in vitro techniques exist (Khalequzzaman and Haq, 2005; Kranz and Lorz, 1994). However, mutants offer a useful tool to identify genes involved in this process. Because this is such an important aspect of biology, many researchers have observed fertilization, but none has found any possible mechanism or been able to determine the molecular players (Russell, 1980; Jensen, 1964; Faure et al., 2002).

In a mutagenesis screen for male factors affecting reproduction, Dr. Johnson and colleagues found the HAP2 protein which blocks fertilization completely when absent from the pollen. Although the HAP2 protein has been found in multiple organisms (Johnson et al., 2004; von Besser et al., 2006; Mori et al., 2006; Liu et al., 2008) it is still a novel protein and little is known about it. The conserved nature of the protein sequence raises many questions about evolution and the conservation of cell fusion methods. Many proteins involved in gamete recognition are rapidly evolving and may enhance speciation by making gamete:gamete interactions species specific (Ferris and Goodenough, 1997; Swanson and Vacquier, 2002). However, the conservation of HAP2 argues that this evolutionary force has not acted on HAP2. It also raises the question of why we cannot find this protein in some other organisms, especially the very well known model organisms *Drosophila* and *Strongylocentrotus* (fruit fly and sea urchin)?

There are only three cell fusion events known in plants; the fusion between a sperm and an egg, the fusion between a sperm and the central cell, and when the two polar nuclei fuse to create the central cell nucleus. The first two require *HAP2*, while the third does not require *HAP2* expression, possibly because it is nuclear fusion only and

does not have the fusion of a plasma membrane like the other two. This leads to our hypothesis that HAP2 acts as a fusogenic protein and is located on the outside of the sperm plasma membrane.

This thesis focuses on characterizing the defects caused when *HAP2* is disrupted and the normal expression, structure and functions of HAP2. Experiments aimed at determining the expression pattern of HAP2 during pollen development and pollen tube growth, finding interacting proteins through a yeast two hybrid screen, ectopically expressing the HAP2 cDNA in sperm and vegetative cells, and using the HAP2 promoter to express the Diphtheria Toxin A subunit to disrupt sperm development and gene expression. From this research we learned that HAP2 is turned on just after DNA synthesis during the second pollen mitosis, producing two sperm cells from the generative cell. There is also a small amount of expression within the female gametophyte, but HAP2 is tightly regulated in all other tissues besides the gametophytes.

Since HAP2 shares a conserved protein sequence between species and has a conserved fertilization function in three species it is likely that HAP2 has been evolutionarily maintained as a membrane fusion factor. It is also likely that the conserved cysteine residues within the amino-terminus are forming disulfide bonds to fold the HAP2 protein or to mediate function either through binding with other proteins or conformational changing to interact with the egg and central cell membranes.

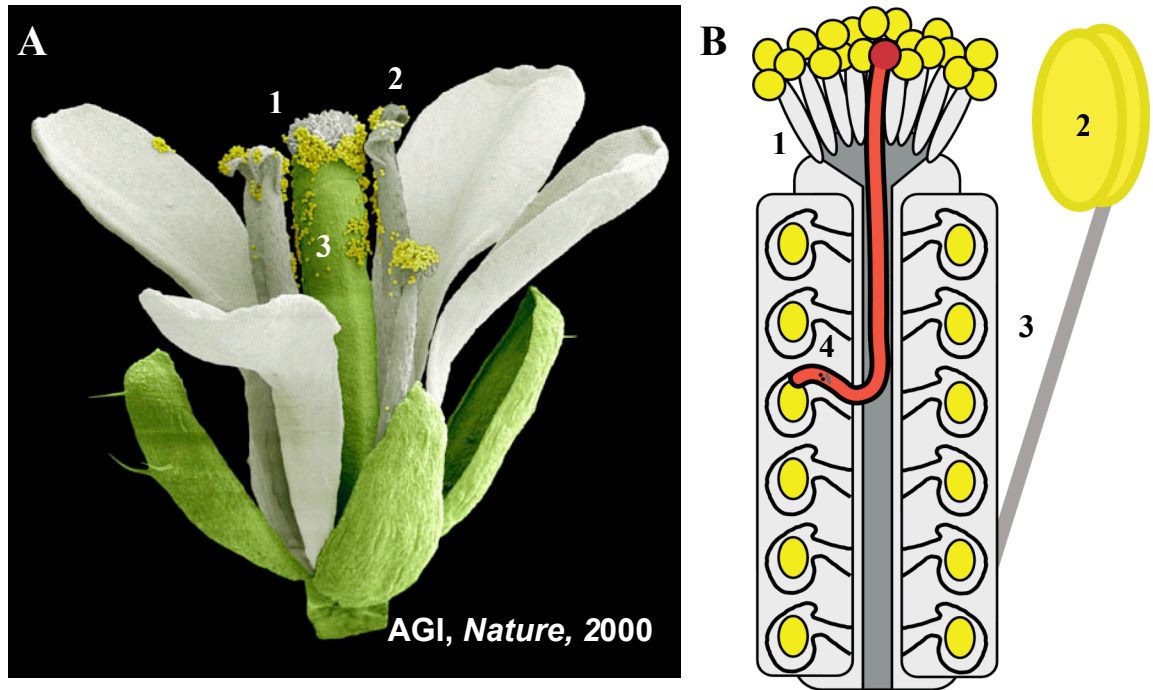


Figure 1: The Flower of *Arabidopsis thaliana*

A.) False colored scanning electron micrograph of an *Arabidopsis* flower, showing the top of the pistil and the papilla cells (1), the anther where the yellow pollen develops (2), and the pistil or ovary (3). B.) Cut away model of how the pollen tube (red) travels through the pistil from the papillae cells (1) through the transmitting tract (3) to and ovule (4). The anther is labeled 2.

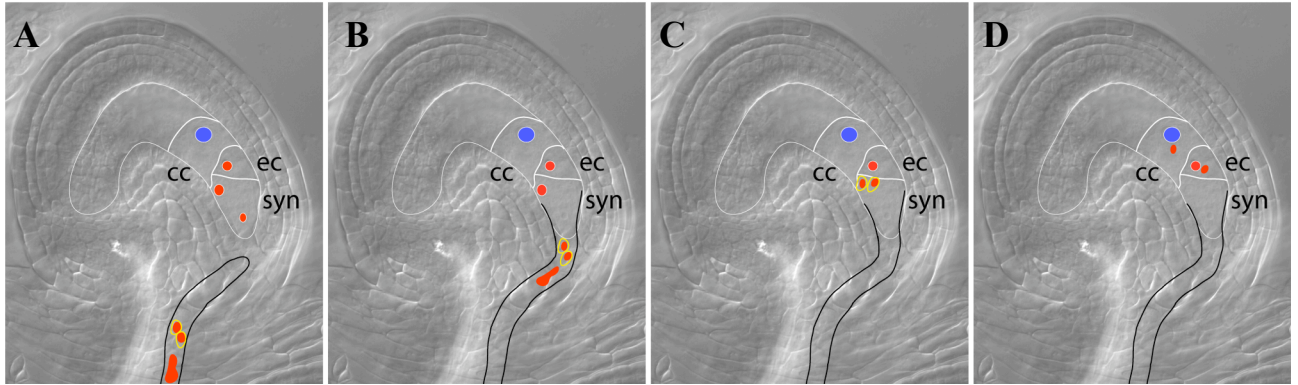


Figure 2: Double Fertilization

When the pollen tube reaches an ovule, it enters through the micropyle opening (A). When the pollen receives a signal from the ovule that it has arrived in one of the two synergids it burst and releases the two sperm (B). These two sperm each fuse with a female cell, either the egg or central cell through plasmogamy (C), and then the nuclei fuse through karyogamy (D).

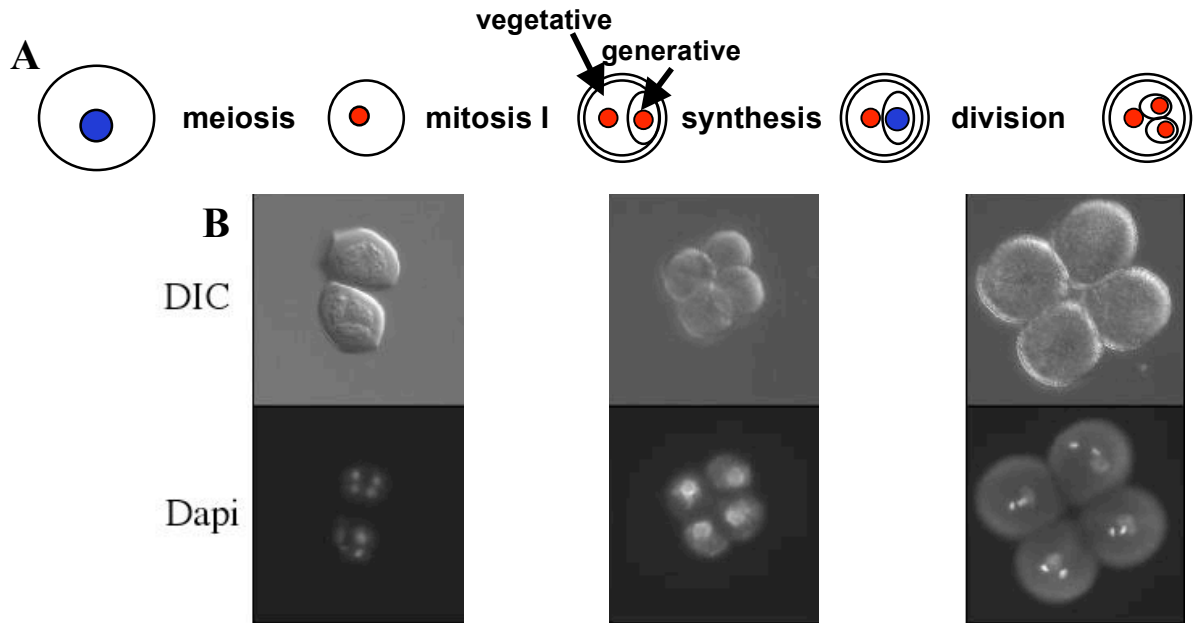


Figure 3: Pollen development from meiosis through two rounds of mitosis to make the pollen grain and two sperm cells.

A) Microspore mother cells within the anthers, shown with a diploid nucleus (blue) on the far left of the cartoon undergo meiosis and the developing pollen grains are haploid (red nucleus). Each pollen grain divides asymmetrically to produce the vegetative cell that will become the pollen grain and the generative cell, which divides once more to produce the two sperm cells. A mature pollen grain is shown on the right. B) Images of developing pollen in the *qrt* mutant where the four meiotic products do not dissociate and remain as a tetrad. DAPI staining shows the nuclei below DIC images.

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Chapter 2: *Arabidopsis HAP2 (GCS1)* is a sperm-specific gene required for pollen tube guidance and fertilization

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This chapter is a reformatted version of the article published in *Development* 2006. I defined the HAP2(GCS1) expression pattern using transgenic *Arabidopsis* plants. Images that I took are included in Figures 5 and 6 as well as Supplemental Figure 1.

Abstract:

In flowering plants, sperm cells develop in the pollen cytoplasm and are transported through floral tissues to an ovule by a pollen tube, a highly polarized cellular extension. After targeting an ovule, the pollen tube bursts, releasing two sperm that fertilize an egg and a central cell. Here, we identified the gene encoding *Arabidopsis* HAP2, demonstrating that it is allelic to *GCSI*. *HAP2* is expressed only in the haploid sperm and is required for efficient pollen tube guidance to ovules. We identified an insertion (*hap2-1*) that disrupts the C-terminal portion of the protein and tags mutant pollen grains with the β -glucuronidase reporter. By monitoring reporter expression, we showed that *hap2-1* does not diminish pollen tube length in vitro or in the pistil, but it reduces ovule targeting by twofold. In addition, we show that the *hap2* sperm that are delivered to ovules fail to initiate fertilization. *HAP2* is predicted to encode a protein with an N-terminal secretion signal, a single transmembrane domain and a C-terminal histidine-rich domain. These results point to a dual role for HAP2, functioning in both pollen tube guidance and in fertilization. Moreover, our findings suggest that sperm, long considered to be passive cargo, are involved in directing the pollen tube to its target.

Introduction:

Reproduction in flowering plants is mediated by pollen tubes – polarized cellular extensions of pollen grains that germinate at the stigma surface, invade the pistil and migrate to an ovule where they deliver two immotile sperm to the female gametophyte (FG). A pollen grain (male gametophyte, MG) comprises three genetically identical haploid cells derived from one meiotic product: a vegetative cell (pollen tube cell) and two sperm that reside in the cytoplasm of the vegetative cell (McCormick, 2004).

The pollen tube interacts with many female sporophytic cells on its journey to the FG (Johnson and Preuss, 2002). The stigma surface binds pollen from a select set of species and subsequently perceives pollen signals that trigger the controlled hydration of the pollen grain, enabling tube germination (Swanson et al., 2004). Guidance cues provided by the female sporophytic tissues determine the initial polarity of pollen tube extension (Kandasamy et al., 1994; Kim et al., 2003; Lord, 2003; Park and Lord, 2003; Wolters-Arts et al., 1998), directing its growth through the extracellular matrix of the style and transmitting tract (Lord, 2003; Wu et al., 2000). After a pollen tube exits the transmitting tract, it emerges onto the septum and grows towards an ovule, navigating up the funiculus and into the micropyle, which provides access to a FG. The FG is a seven-celled haploid structure comprising an egg and two synergid cells at the micropylar pole, a large central cell, and three antipodal cells (Yadegari and Drews, 2004). Pollen tube growth arrests within a synergid and the pollen tube tip bursts, releasing two sperm (Russell, 1992). This process, called pollen tube reception, is accompanied by the degeneration of the receptive synergid and is rapidly followed by the fusion of one sperm with the egg and one sperm with the central cell to produce the zygote and endosperm,

respectively (reviewed in Faure and Dumas, 2001; Weterings and Russell, 2004).

Pollen tube guidance in the ovary is controlled by factors expressed by diploid floral cells and by haploid FG cells (Christensen et al., 2002; Hülskamp et al., 1995; Pagnussat et al., 2005; Palanivelu et al., 2003; Ray et al., 1997; Shimizu and Okada, 2000). Genetic experiments in *Arabidopsis* show that pollen tubes bypass incompletely formed ovules or those that lack a FG (Hülskamp et al., 1995; Ray et al., 1997). When ovules carry *maal* or *maa3*, mutations that delay FG development (Shimizu and Okada, 2000), pollen tubes grow up the ovule funiculus but fail to enter the micropyle, suggesting that the FG produces distinct signals for funicular and micropylar guidance. Laserablation studies combined with an elegant in vitro pollen tube guidance assay using *Torenia fournieri*, showed that the synergids produce a short-range micropylar attractant (Higashiyama et al., 2001). A candidate FG-derived pollen tube attractant has recently been identified; *Zea mays egg apparatus1* (*ZmEAI*) is expressed exclusively in egg and synergid cells, and encodes a 94 amino acid hydrophobic protein that is required for efficient micropylar guidance in a maize in vitro guidance system (Marton et al., 2005).

FG signals may act in concert with sporophytic pollen tube attractants produced by ovule integuments or by the funiculus. γ -amino butyric acid (GABA) may be one such signal; *Arabidopsis pop2* mutants disrupt the GABA gradient near the micropyle, resulting in random pollen tube growth (Palanivelu et al., 2003). Additional signaling events take place after the pollen tube enters the micropyle. When pollen tubes enter ovules bearing *feronia* or *sirene* mutant FGs, the synergid degenerates, but the pollen tube does not stop growing and does not burst, suggesting that the FG produces a signal that controls pollen tube reception (Huck et al., 2003; Rotman et al., 2003). These results

represent tremendous progress in understanding the female signals that guide the pollen tube to the ovule; however, there is little information on how the pollen tube perceives these signals and transmits them into changes in the direction of its growth.

To identify pollen-expressed genes that are important for pollen tube growth and guidance, we isolated a series of MG mutants by screening for distorted inheritance (Johnson et al., 2004). Mutants were induced by random genomic insertions of a T-DNA carrying an herbicide-resistance gene (Basta resistance, BastaR), as well as a cell autonomous histochemical marker (β -glucuronidase, GUS) under the control of the post-meiotic pollen-specific promoter LAT52 (Twell et al., 1989). LAT52 is active during pollen tube growth, marking mutant pollen tubes from their initial interactions with stigmatic papillae cells to their arrival at an FG. Mendelian inheritance predicts that plants with one copy of the T-DNA insertion will produce 75% BastaR offspring when self-fertilized. By contrast, self-fertilization of *hap2/HAP2* plants yielded ~50% BastaR progeny and no *hap2/hap2* homozygotes were obtained (Johnson et al., 2004). This transmission defect was male-specific; reciprocal crosses of *hap2/HAP2* with wild type generated BastaR progeny at 0.7% or 47.0% when *hap2/HAP2* was used as the male or female, respectively (Johnson et al., 2004). Initial phenotypic characterization of *hap2* pollen tube growth suggested that *hap2* pollen tubes do not follow the wild-type path, growing chaotically within the ovary. We identified the *hap2* T-DNA insertion site within a hypothetical gene of unknown function (At4g11720) (Johnson et al., 2004).

Recently, a generative cell-specific protein (GCS1) from lily was characterized, and a T-DNA insertion into its *Arabidopsis* homolog, At4g11720 (*HAP2*), indicated a role in fertilization. GCS1 is localized to sperm cells, and we confirm here that a HAP2-

fusion protein is sperm-expressed. Importantly, with the LAT52-GUS insertion into *HAP2*, we were able to look at phenotypes prior to gamete fusion, showing that HAP2 is also required for pollen tube guidance. *hap2* pollen tube length is not affected. However, *hap2* pollen tubes are half as likely as wild type to target ovules; after leaving the septum, *hap2* tubes meander over ovule surfaces where wild-type pollen tubes do not typically grow. In the cases where *hap2* pollen tubes reach ovules, *hap2* completely blocks fertilization. This study thus provides genetic evidence for the active participation of sperm cells in their delivery to female gametes.

Results:

***hap2* pollen tubes are defective in ovule targeting**

We used the LAT52:GUS pollen-specific reporter gene, which highlights the cytoplasm of every *hap2-1* pollen tube, to refine our analysis of the *hap2* pollen tube-growth and pollen tube-guidance phenotype. To determine whether *hap2* pollen tubes have inherent growth defects, we germinated *hap2-1/HAP2* pollen in vitro and stained them for GUS activity to differentiate between mutant and wild-type pollen (Fig. 1A). *hap2* pollen tube germination was not affected (*hap2-1*, 85%, $n=80$; *HAP2*, 88%, $n=81$) and, after 3 hours of growth in vitro, *hap2-1* pollen tubes were as long as those of wild type (*hap2-1*, mean 418 μ m, s.d. 217 μ m, $n=62$; *HAP2*, mean 372 μ m, s.d. 195 μ m, $n=62$).

ms1 stigmas were pollinated with *hap2-1/HAP2* pollen to analyze pollen tube growth in the pistil. At 5 hours after pollination, the longest *hap2-1* pollen tubes had extended through 58% of the ovary (s.d. 12%), whereas the longest *HAP2* pollen tubes had grown 35% of the length of the ovary (s.d. 4%; $n=4$ pistils). At 10 hours after

pollination, *hap2* and wild-type tubes had traveled through 81% (s.d. %) and 75% (s.d. 10%) of the pistil, respectively ($n=4$ pistils). These data indicate that *hap2-1* does not limit pollen tube growth and that the inability to target ovules is attributable to a defect in the perception of pollen tube-guidance cues.

To define the stage at which *hap2* pollen tubes fail, we monitored the path of *hap2-1* pollen tubes in the ovary and determined the frequency with which *hap2-1* pollen tubes successfully enter a micropyle and burst. At 14 hours after hand-pollinating *msl* pistils with control anthers heterozygous for the LAT52:GUS reporter, GUS activity was observed in the synergid cells of approximately half of the ovules, as expected (116/234, 50%, Fig. 1B,C). By contrast, when *hap2-1/HAP2* anthers were used, a smaller proportion of GUS+ pollen tubes entered a micropyle and burst (115/505, 23%; Fig. 1B,D). Furthermore, *hap2-1* pollen tubes were observed growing on portions of the ovule where wild-type pollen tubes do not grow (Fig. 1E) and also stalled on the funiculus (Fig. 1F). These data indicate that *hap2* pollen tubes have a diminished capacity to enter the micropyle and burst within synergids (~50% reduction from wild type) because of defects in funicular and/or micropylar guidance.

The *hap2* ovule-targeting defect was not suppressed when competition from wild-type pollen tubes was decreased. We pollinated wild-type pistils with single *hap2-1/HAP2* tetrads and, after 14 hours, fixed and stained the pistils with Aniline Blue to follow the route of *hap2-1* and *HAP2* pollen tubes. With a control tetrad donor, 100% of the pollen tubes that germinated were able to target and enter an ovule ($n=35$). By contrast, 69/89 (78%) of the pollen tubes from single *hap2-1/HAP2* tetrads targeted an ovule, a significant difference from wild type ($P<0.05$, χ^2). These experiments indicate

that only half of *hap2* pollen tubes reached their target – the same result as that obtained with excess pollinations. Thus, the observed *hap2* pollen tube-guidance defect is not caused by an inability to compete with wild-type pollen tubes for access to ovules, but rather reflects an inherent pollen tube-guidance defect.

***hap2* completely blocks fertilization**

When *hap2-1* or *hap2-2* pollen were used to pollinate wild-type pistils, no mutant progeny were recovered (*hap2-1*, 363 F1 tested; *hap2-2*, 348 F1 tested), indicating that *hap2* completely blocks transmission of the mutant allele through the male. Because *hap2* disrupts pollen tube guidance, we addressed whether competition for available ovules from *HAP2* pollen tubes masks rare *hap2* fertilization events by performing limiting pollinations of wild-type pistils. We obtained 87 F1 seeds from ten crosses: none inherited the *hap2-1* allele. This absolute block in transmission through pollen cannot be explained by an approximately 50% reduction in the ability of *hap2* pollen tubes to target ovules. Therefore, *hap2* must also disrupt a step in reproduction that occurs after the contents of the pollen tube are released into the synergid cell of the FG. We examined the development of seeds in self-fertilized *hap2/HAP2* flowers and found that, *hap2-1/HAP2* plants averaged 37 ± 7 seeds (742 seeds in total, 20 siliques), whereas *HAP2/HAP2* plants average 56 ± 8 seeds (564 seeds in total, ten siliques). *hap2-1/HAP2* siliques had gaps (Fig. 2A,B) where ovules failed to develop into seeds, suggesting that the FGs in ovules targeted by *hap2* pollen tubes do not get fertilized.

To test this hypothesis, we pollinated wild-type pistils with *hap2-1/HAP2* pollen, allowed 48 hours for fertilization, early embryo and endosperm development, and then stained them for GUS activity to identify ovules that had been targeted by *hap2-1* pollen

tubes.

Ovules targeted by *HAP2* pollen tubes (no GUS activity) contained early globular embryos and obvious proliferation of endosperm nuclei (122/132 ovules analyzed were fertilized; Fig. 2D). By contrast, ovules targeted by *hap2-1* pollen tubes (GUS activity in the synergid cell indicating that the pollen tube cytoplasm and sperm had been deposited) showed no indication of embryo or endosperm development (0/26 ovules analyzed were fertilized; Fig. 2F). Instead, we consistently observed only the unfertilized central cell nucleus in FGs targeted by *hap2-1* pollen tubes. We analyzed 112 unstained ovules in wild-type pistils pollinated with *hap2-1/HAP2* pollen and found that 18% contained only one central cell nucleus (Fig. 2G), and that the remaining 82% contained an embryo and several endosperm nuclei (Fig. 2E); these values are consistent with the rate of *hap2-1* ovule targeting and, when combined with the data from GUS-stained ovules, suggest that when *hap2* sperm are released into the FG, they fail to fertilize the egg and central cell.

One of the two synergid cells degenerates either just before or at the same time as the pollen tube enters the micropyle (Faure et al., 2002). In ovules targeted by *hap2* pollen tubes, the central cell nucleus is the only FG nucleus that remains 48 hours after pollination. We hypothesized that, in the absence of fertilization, the egg nucleus degenerates and that the second synergid degenerates shortly after the pollen tube bursts. Analysis of embryo and endosperm development at an earlier time point (24 hours after pollination) showed that both the egg and central cell remain in ovules targeted by *hap2* pollen tubes (Fig. 2H), whereas an embryo and endosperm nuclei are clearly present in ovules targeted by *HAP2* pollen tubes (Fig. 2I). These results confirm that *hap2* sperm are incapable of fertilizing the egg or central cell and suggest that, in the absence of

fertilization, the egg cell degenerates following pollen tube reception. Similarly, Mori et al. found that *gcs1* sperm fail to fertilize and that they persist in the degenerating synergid 16 hours after pollination, whereas wild-type sperm do not (Mori et al., 2006). Presumably, wild-type sperm were not observed because they are rapidly transported to the egg and central cell where they bind these target cells and undergo plasmogamy.

***hap2* sperm develop normally and migrate to the pollen tube tip**

To determine whether the *hap2* fertilization defect is due to a defect in sperm development or in migration of sperm within the pollen tube, we analyzed *hap2* pollen grains and tubes throughout their development. DAPI-staining revealed morphologically normal sperm and vegetative nuclei ($n > 500$, Fig. 3A-D). Staining *hap2/HAP2* pollen tubes either with Aniline Blue, which binds callose (β -1-3-glucan) in the pollen tube walls, or FM 4-64, which becomes incorporated into the membrane architecture of growing tubes, showed that *hap2* pollen tubes were indistinguishable from wild type (data not shown). In newly germinated tubes, the vegetative nucleus consistently exited the pollen grain before the two sperm (*hap2/HAP2*, $n=24$; control, $n=19$), and, as tubes elongated, the sperm and vegetative nuclei were always in the subapical region of the tube (*hap2/HAP2*, $n=138$; control, $n=117$). Within 1 hour after pollination, *hap2* vegetative nuclei emerged from the pollen grain ahead of the two sperm ($n=11$); this normal male germ-unit organization was maintained as the tubes migrated through the transmitting tract ($n=20$). These results indicate that defects of *hap2* pollen tube guidance and fertilization are not the result of aberrant pollen tube structure or sperm transport within the pollen tube.

***HAP2* encodes a predicted membrane protein with a histidine-rich C-terminus**

Previously, we used TAIL PCR to map the *hap2-1* T-DNA insertion to the twelfth of 14 exons in the single-copy uncharacterized gene At4g11720 (Fig. 4A) (Johnson et al., 2004). No cDNAs or ESTs corresponding to this gene were present in public databases, so we used 5' and 3' RACE to generate a full-length cDNA and to annotate At4g11720, determining that the gene comprises 17 exons instead of the 14 predicted by the initial annotation of the *Arabidopsis* genome (GenBank DQ022676 and DQ022375; Fig. 4A). With PCR, we confirmed both *hap2-1* T-DNA-genome junctions, as well as a 10 bp genomic deletion at the insertion site (exon 15). A second allele, *hap2-2*, with a T-DNA insertion in exon 9 (Fig. 4A) was identified from the SIGNAL collection (Alonso et al., 2003); this allele also caused distorted T-DNA segregation (46.2% KanR progeny from self-fertilization, $n=600$) and completely blocked transmission through pollen (0 KanR progeny when crossed with wild type, $n=348$). The location of the *gcs1* allele is also indicated (Fig. 4A) (Mori et al., 2006).

To confirm that At4g11720 is indeed *HAP2*, we complemented the *hap2* defect by transforming plants heterozygous for *hap2-1* with a T-DNA carrying NPTII (conferring KanR) and a wild-type *HAP2* transgene (*HAP2tr*, a genomic fragment from 983 bp upstream to 277 bp downstream of the open reading frame; Fig. 4A). The progeny of primary *hap2/HAP2* transformants were collected and BastaR and KanR were analyzed in ten transgenic families with a single-locus insertion of *HAP2tr*. The average rate of BastaR among these families was 68.6% ($n=2362$) and of KanR was 83.1% ($n=2525$). Complementation of both ovule-targeting and fertilization defects is expected to yield T2 progeny that segregate 67% (8/12) BastaR and 83% (10/12) KanR; failure

would lead to approximately 50% BastaR, as observed in the progeny of self-fertilizing *hap2-1/HAP2* plants. These results indicate that wild-type At4g11720 rescues the *hap2-1* pollen tube-guidance and fertilization defects. Self-fertilization of *hap2/HAP2;HAP2tr* plants yielded *hap2/hap2* homozygous progeny (all four members of tetrad are GUS+), the progeny of which were 100% BastaR; homozygous progeny were never observed when *hap2/HAP2* is self-fertilized.

The *HAP2* open reading frame is predicted to encode a 705 amino acid protein with a N-terminal signal sequence (amino acid 1-24), a single transmembrane domain (amino acid 560-582) and a C-terminal histidine-rich domain (Fig. 4B). *HAP2* is not similar to any proteins with known functions and has no obvious functional motifs, although several genes in flowering plants and in more distantly related organisms have been described (Mori et al., 2006). Pair-wise comparison of amino acid identity between the *Arabidopsis* *HAP2* and *HAP2* from other flowering plants showed that the N-terminal region is conserved (mean identity 64%), whereas the C-terminal region is more divergent (mean identity 35%). An alignment of the C-terminal histidine-rich regions showed that the presence of histidine is conserved among these proteins, but that amino acid sequence is not (Fig. 4C). Interestingly, these histidine-rich domains are present in *HAP2* from angiosperms, but not from other organisms (Fig. 4D).

***HAP2* is only expressed in sperm**

RT-PCR and northern blot analysis showed that *HAP2*mRNA is only detected in tissue samples that contain mature pollen (Fig. 5A,B). This specific expression pattern is confirmed by hundreds of publicly available microarray experiments showing that *HAP2* mRNA only accumulates in pollen (<https://www.genevestigator.ethz.ch/>). To determine

the precise location of *HAP2* expression, we fused a DNA fragment corresponding to 983 bp upstream of the *HAP2* start codon (Fig. 4A) to the yellow fluorescent protein (YFP) coding sequence (*HAP2promoter:YFP* fusion protein) and generated transgenic plants expressing this construct. Accumulation of YFP was only observed in the two sperm cells contained within the cytoplasm of mature pollen grains (Fig. 5C,D); YFP was not observed in uninucleate microspores or in bicellular pollen (Fig. 5D), nor was it observed in other floral or vegetative cells (data not shown).

Protein localization algorithms predict that HAP2 is localized to the plasma membrane and/or the endoplasmic reticulum (ER). To determine where HAP2 is localized within sperm, we fused YFP to the penultimate codon of *HAP2* exon 17 (Fig. 4A, *HAP2protein:YFP* fusion protein) and generated transgenic plants expressing this construct. Three independent transgenic lines were crossed with *hap2-1/HAP2* plants and homozygous *hap2-1* lines were obtained in the F2 generation of each cross, indicating that *HAP2protein:YFP* complemented *hap2* pollen tube-guidance and fertilization defects, and suggesting that *HAP2protein:YFP* forms a functional and properly localized protein. We detected the fusion protein only in the sperm cells (Fig. 6A-D). Unlike the transcriptional fusion, which produced YFP distributed throughout the elongated, spindle-shaped sperm cytoplasm (Fig. 5C), the protein fusion was excluded from the nucleus and was predominantly perinuclear, forming a ring of fluorescence around sperm nuclei (Fig. 6B). However, fluorescence was not limited to the perinuclear region and in many sperm cells, fluorescence extended to the sperm plasma membrane (Fig. S1 in the supplementary material). These results, in combination with protein-localization predictions, suggest that HAP2 is predominantly localized to sperm ER membranes and

that HAP2 may also reside in other endomembranes, including the plasma membrane.

Discussion:

***HAP2* is necessary for targeting of the ovule micropyle by the pollen tube**

In addition to the previously reported role of *HAP2* in fertilization (Mori et al., 2006), we have shown that *hap2-1* pollen tubes grow the entire length of the pistil, yet grow aberrantly on ovule surfaces and often fail to enter the micropyle, resulting in a twofold reduction in ovule targeting. In vitro, *hap2-1* pollen tubes grow slightly longer and, in the pistil, *hap2-1* pollen tubes grow slightly faster than wild type pollen tubes.

This mild enhancement of pollen tube extension could reflect the lack of a tube-growth behavior that is required for optimum guidance. Interestingly, the ability of *hap2* pollen tubes to target ovules does not improve when pistils are underpollinated, indicating that the *hap2* pollen-tube-guidance defect is not a consequence of an inability of *hap2* pollen tubes to compete with *HAP2* pollen tubes for a limited number of ovules. Thus, *hap2* specifically disrupts pollen tube guidance without diminishing pollen tube growth, making this mutant a unique resource for understanding how pollen tubes perceive and respond to guidance cues.

hap2-1 pollen tubes were tagged with the LAT52:GUS reporter gene, allowing quantitative analysis of ovule targeting success (Fig. 1B); this feature was crucial for detecting the pollen tube-guidance defect. By contrast, previous studies of the *gcs1* allele of *HAP2* did not detect a pollen tube-guidance defect (Mori et al., 2006). *gcs1* pollen tubes are not tagged and analysis of pollen tube guidance by staining mutant and wild-type tubes with Aniline Blue, particularly when many pollen tubes are present, is not

sufficiently sensitive to detect *hap2* guidance differences. Nonetheless, consistent with our observations, pollinations of wild-type pistils with *gcs1/GCS1* pollen always yielded a greater number of fully formed (targeted by *GCS1* pollen) than aborted seeds (targeted by *gcs1* pollen). These data suggest that the *gcs1* allele may also disrupt pollen tube guidance.

HAP2 is a sperm-specific protein

HAP2 mRNA only accumulates in mature pollen (Fig. 5A,B), *HAP2* promoter activity is only detected in mature sperm (Fig. 5C,D), and HAP2 protein is only detected in the sperm during pollen development and tube growth (Fig. 6). In lily, expression of the *HAP2* ortholog *GCS1* is first apparent in the generative cell and persists after the generative cell divides during pollen tube growth to produce two sperm (Mori et al., 2006). This earlier onset of *HAP2* expression probably reflects a difference in pollen development between these species. In *Arabidopsis* (which has tricellular pollen grains), the generative cell divides to produce two sperm before anthesis, whereas, in lily and other species with bicellular pollen grains, the generative cell does not divide until after the pollen tube has germinated. These expression studies, combined with the *hap2*-mutant phenotype and the finding that *HAP2* is conserved among a diverse set of angiosperms (Fig. 4D) (Mori et al., 2006), support the hypothesis that *HAP2* is a sperm-specific gene that is universally essential for double fertilization.

HAP2 is predicted to encode a 705 amino acid protein that shares no similarity with proteins of known function. However, it has three features that offer clues to its biochemical function; HAP2 has an N-terminal cleavable signal sequence, a single transmembrane domain and a C-terminal histidine-rich domain (Fig. 4B). Multiple

algorithms predict that HAP2 is an integral ER or plasma-membrane protein and that the C-terminus is cytoplasmic (type 1a membrane protein). Lily GCS1 (HAP2 ortholog) was shown to be associated with membranes by cellular fractionation studies (Mori et al., 2006).

The *HAP2protein:YFP* fusion protein we generated complements *hap2-1*, suggesting that it encodes a fully functional protein and that its localization pattern is that of the endogenous protein. *HAP2protein:YFP* is predominantly localized in a perinuclear ring (Fig. 6B) (consistent with ER localization), with extensions of *HAP2protein:YFP* to the plasma membrane (Fig. S1 in the supplementary material). These results are consistent with those recently obtained by immunofluorescence in both lily and *Arabidopsis* (Mori et al., 2006), and suggest that HAP2 is associated with membranes of the perinuclear ER and, perhaps, with secretory vesicles bound for the plasma membrane. Further studies using higher-resolution techniques will be required to determine the precise HAP2 localization pattern within sperm cells.

Potential roles for sperm-expressed HAP2 in pollen tube guidance

We have shown that HAP2 is required for pollen tube guidance and that it is sperm-specific. These results point to a previously unrecognized role for sperm in directing the growth of the pollen tube. The ability of a sperm protein to alter the efficiency of pollen tube guidance may suggest a checkpoint or sperm quality-control mechanism, such that pollen tubes carrying defective sperm could not efficiently target ovules.

HAP2 may be directly involved in transducing a pollen tube guidance cue (Johnson and Lord, 2006). The sperm are associated with the pollen tube cytoskeleton and migrate near the tip of the pollen tube as it grows to the ovule (McCormick, 2004);

they are well positioned to mediate events within the pollen tube cytoplasm that result in changes in the direction of tube extension. Interestingly, because sperm develop within the pollen tube cytoplasm, the majority of the predicted N-terminal portion of plasma membrane localized HAP2 would reside in the pollen tube cytoplasm and could interact directly with pollen tube cytoplasmic factors involved in directing tube extension.

Multiple, overlapping chemotropic factors may guide the pollen tube to ensure optimal efficiency of ovule targeting and fertilization (Johnson and Lord, 2006). Therefore, mutations that diminish perception of a single pollen tube-guidance factor are not expected to completely block ovule targeting. This may explain why *hap2-1* results in a reduction rather than a complete loss of ovule targeting. The finding that perturbations in the production of individual female guidance cues such as GABA (Palanivelu et al., 2003), ZmEA1 (Marton et al., 2005) and plantacyanin (Dong et al., 2005) do not completely block pollen tube guidance or seed production, supports this view. It will be interesting to test this hypothesis in the future by constructing plants with defects in production and/or perception of multiple pollen tube-guidance signals.

HAP2-mediated interactions between sperm and FG are required for fertilization

Approximately half of *hap2-1* pollen tubes reach ovules, enter the micropyle and burst, releasing GUS activity and two sperm into the degenerating synergid (Fig. 1B). However, *hap2* mutant progeny are never recovered when either *hap2-1* or *hap2-2* pollen is used to pollinate a wild-type pistil. This complete transmission block was also observed when the *gcs1* allele was characterized (Mori et al., 2006). These results indicate that HAP2 is essential for a step in fertilization that occurs after sperm have been delivered to the FG by a pollen tube. Initiation of embryo development and initiation of endosperm

development are both completely blocked in ovules targeted by *hap2-1* pollen tubes (Fig. 2F-H). Furthermore, *gcs1* sperm persist within the degenerating synergid, whereas wild-type sperm immediately migrate to their egg and central cell target membranes and fuse (Mori et al., 2006).

Taken together, these results indicate that HAP2 is probably required for either sperm migration within the FG, or for binding/fusion of sperm to egg and of sperm to the central cell. Further analysis of the *hap2* fertilization defect using transmission electron microscopy or live imaging in a system in which *hap2* sperm and target membranes are tagged with fluorescent proteins offers an opportunity to determine the precise role for *HAP2* in fertilization.

Plasma membrane-localized HAP2 could directly mediate gamete-gamete interactions through its extracellular domain. There is substantial precedence in animal reproduction for similar sperm-egg interactions; for example, a sperm-expressed type 1a membrane protein is essential for sperm-egg fusion in mice (Inoue et al., 2005; Rubinstein et al., 2006). Alternatively, ER-localized HAP2 may indirectly mediate fertilization by regulating the processing or secretion of plasma-membrane proteins or by regulating calcium levels in sperm.

Insights into the mechanisms of double fertilization

hap2/gsc1 are the only *Arabidopsis* mutants described so far in which two sperm are released into the degenerating synergid but fertilization does not occur. This affords a unique opportunity to clarify some of the basic mechanisms of double fertilization. Because *hap2-1* blocks initiation of both embryo and endosperm development, there must be a single system in *Arabidopsis* that mediates interactions between sperm and egg and

between sperm and central cell. Sperm are dimorphic in some flowering plants, with one sperm type preferentially fertilizing the egg and the other preferentially fertilizing the central cell (Roman, 1948; Russell, 1985). It will be interesting to determine whether these plants have an additional system to direct sperm of one type to a specific target or whether HAP2 functions to mediate specific gamete interactions in these systems.

In wild-type *Arabidopsis*, only one pollen tube is attracted to each ovule; attraction of multiple pollen tubes to a single FG would presumably decrease female fitness and could lead to polyspermy (Shimizu and Okada, 2000). Evidence for a repulsive cue that directs supernumerary pollen tubes away from an ovule that has already been targeted was recently obtained in vitro (Palanivelu and Preuss, 2006). Interestingly, the FG mutants *feronia* and *sirene* attract supernumerary pollen tubes; in these mutants, pollen tubes enter the micropyle but fail to stop growing and burst (Huck et al., 2003; Rotman et al., 2003). This shows that pollen tube entry into the micropyle is not sufficient to trigger production of a repellent. We do not observe supernumerary pollen tubes on ovules targeted by *hap2-1* pollen tubes. Furthermore, we analyzed 26 ovules targeted by *hap2-1* pollen tubes by staining for GUS activity in synergid cells (Fig. 4F) and did not find any that initiated seed development, indicating that supernumerary wild-type pollen tubes do not fertilize an ovule already targeted by a *hap2-1* pollen tube. Taken together, these results indicate that the pollen tube repellent is produced after the pollen tube bursts but before fertilization, and suggest that production of a pollen tube repellent may be initiated by FG perception of a factor present either on the sperm surface or in the pollen tube cytoplasm.

A new view of sperm cells: no longer passive cargo

The data presented here challenge the assumption that sperm cells are passive cargo delivered to the FG by the pollen tube. We show that a sperm-specific gene, *HAP2*, is required for optimal ovule targeting by the pollen tube, suggesting that sperm function may impact the growth of the pollen tube. Recently, it has become clear that despite their compact chromatin structure, sperm are dynamic cells that express many genes (Engel et al., 2003; Engel et al., 2005; Xu et al., 1999); *HAP2* is the first such gene with a demonstrated function in reproduction (Mori et al., 2006). It is likely that more sperm-expressed genes will be identified that play roles in pollen tube guidance and in fertilization. The identification of *HAP2* presents an opportunity to identify FG-expressed interacting factors that, together with *HAP2*, mediate double fertilization, a process that is crucial for agriculture, but about which we know very little at the molecular level.

Materials and Methods:

Plant growth and *HAP2* cloning

hap2-1, *hap2-2* (Salk_152706) (Alonso et al., 2003) and control lines in the Col-0 background were grown at 21°C in 24-hour 100 E fluorescent lighting or in ambient greenhouse conditions. Kanamycin resistance (KanR; *hap2-2*) was determined on MS salts with 25 g/ml kanamycin, and BastaR (*hap2-1*) was determined as by Johnson et al. (Johnson et al., 2004). Plant transformation was performed as described (Clough and Bent, 1998), selecting for growth on 50 g/ml kanamycin or on soil with 1:2000 Liberty herbicide (AgrEvo, Pikeville NC). Limiting pollinations were performed with male sterile (*ms1*) pistils and *hap2/HAP2* anthers; crosses producing 25 or fewer seeds were analyzed. The *hap2-1* T-DNA-gene junction was confirmed with the PCR primers LB3 (McElver et al., 2001), *HAP2* upstream (5'-GGCCTCACTCGTTCTCAATTGGAG-3') and *HAP2*

downstream (5'-GTGAGAGTCGCTGTGGTCACGTTC-3'). The *hap2-2* T-DNA-gene junction was confirmed with the PCR primers LBa1 (Alonso et al., 2003) and HAP2seqTR3 (5'-CAATCAAAGTGCAGGAAGGAAGC-3'). Full-length *HAP2* cDNA (GenBank DQ022375) was amplified from 200 ng total pollen RNA using primers F, 5'-AAACAATTTTCAATTCGCGTCTCCG-3' and R, 5'-AACTCGGATATATTTTGC-3'. For complementation, a genomic *HAP2* fragment was PCR amplified with F, 5'-GCCCTGCAGGTTCTGATCCTAACAACAACGGCGGC-3', and R, 5'-CGCCTGCAGGATATCTTCGAGAGAATCACCAGTCGCC-3', inserted into the *SbfI* site of pCAMBIA2300 (GenBank AF234315), sequenced, introduced into *Agrobacterium* (strain GV3101), and transformed into *hap2-1/HAP2* plants, selecting KanR.

Protein sequence analysis

The *Arabidopsis HAP2* coding sequence guided annotation of the Poplar ortholog from genomic sequence (Poptr1:63588; <http://genome.jgi-psf.org>). Other sequences were obtained from GenBank (accession number): *Lilium longiflorum* (AB206810), *Oryza sativa* (AK072871), *Chlamydomonas reinhardtii* (AB206813), *Cyanidioschyzon merolae* (AP006493), *Plasmodium falciparum* (AAN35337), *Physarum polycephalum* (AB206812), *Leishmania major* (CP000081) (Mori et al., 2006). Predicted proteins were aligned using ClustalW (MegAlign, DNASTAR, Madison, WI), and structure and subcellular localization was predicted with PSORT (Nakai and Kanehisa, 1992), TargetP (Emanuelsson et al., 2000), Genoplante Predator (<http://www.genoplante.com/content.php?idcontent=bioinfotools&lg=en>), Prosite (<http://us.expasy.org>) and TMHMM (<http://www.cbs.dtu.dk>).

Phenotypic analysis

Pollen tube growth in pistils was analyzed as previously described (Johnson et al., 2004). For in vitro analyses, pollen from stage-14 flowers (Smyth et al., 1990) was germinated for 3 hours in an inverted drop of medium (Hicks et al., 2004). Pollen tubes or grains were transferred to polylysine-coated slides and stained with DAPI (Park et al., 1998), Aniline Blue (0.01% in 50 mM KPO₄, 50% glycerol), FM 4-64 (3.4 μ M in germination medium) or X-Gluc (for GUS activity) (Johnson et al., 2004). All images were captured on a Zeiss Axioskop (Carl Zeiss, Germany); pollen tube lengths were measured using ImageJ (<http://rsb.info.nih.gov/ij/>). Fertilization and embryo development was analyzed as previously described (Yadegari et al., 1994); indicated samples were first stained for GUS activity (Johnson et al., 2004).

HAP2 expression

Northern blotting was performed on 20 μ g of total pollen RNA probed with *HAP2* cDNA. RT-PCR was performed on 1 μ g total pollen RNA using the *HAP2* primers F, 5' - TTAATGGCTTGTATACTCGCCGG-3' and R, 5' - ACGAAGGCAATGCGCGGTATTTGCC-3', and *EF-1* controls F, 5' - GCCCCTTCGTCTCCCACTTC-3' and R, 5' - CACTTCGCACCCTTCTTGACG-3'; products were analyzed after 25, 30, 35 and 40 PCR cycles. The *HAP2promoter:YFP* fusion contained 983 bp upstream of the *HAP2* start codon, amplified using *HAP2SacIF*, 5' - GGCGAGCTCAATTTCCCTGATACAATCCCGAGGC-3' and *HAP2BamHIR*, 5' - GCGGATCCTTTCTCTCTCACGGAGACGCG-3'; *HAP2protein:YFP* fusion contained the *HAP2* promoter and all exons and introns, and was amplified using primers *HAP2SacIF* and *HAP2NcoR*, 5' -

GGCCCATGGTACTCTCACGTAGTCTTTGTTTCCTC-3 . Amplified products were digested with *SacI* and *BamHI*, or *SacI* and *NcoI*, respectively, and introduced upstream of eYFP (Clontech) followed by the CaMV polyadenylation sequence, and incorporated into the binary vector, pGREENII02229 (Hellens et al., 2000). Transgenic plants were analyzed using a Zeiss Axioplan 200 equipped with a Photometrics cooled CCD camera (CoolSNAP fxHQ, Roper Scientific, Tuscon, AZ), or a confocal fluorescence microscope (SP2 A OBS, Leica Microsystems AG, Wetzlar, Germany) and analyzed with Openlab software (Improvision, Lexington, MA). For analysis of YFP fluorescence during pollen development, uninucleate, bicellular and tricellular pollen grains were released from anthers and stained with DAPI (Park et al., 1998). They were then analyzed using a Leica DMIRE2 Confocal microscope with a 63X water objective using Leica software. DAPI was excited with a 405 nm laser and detected at 410-581 nm, whereas the YFP was excited with a 514 nm laser and detected at 517-597 nm. Pollen tubes were grown for up to 6 hours in liquid media on an inverted microscope slide (Hicks et al., 2004).

Acknowledgments:

We thank A. DeLong, A. Dobritsa, K. Vigh and J. Wong for critical reading of the manuscript; S. Luo for advice; and M. Root for technical assistance. This work was supported in part by the Department of Energy (DE-FG02- 96ER20240) and the Howard Hughes Medical Institute. M.A.J. was supported by an NIH, Ruth L. Kirschstein National Research Service Award and Brown University. A.F. was supported by an NIH pre-doctoral training grant in Molecular and Cell Biology and Biochemistry.

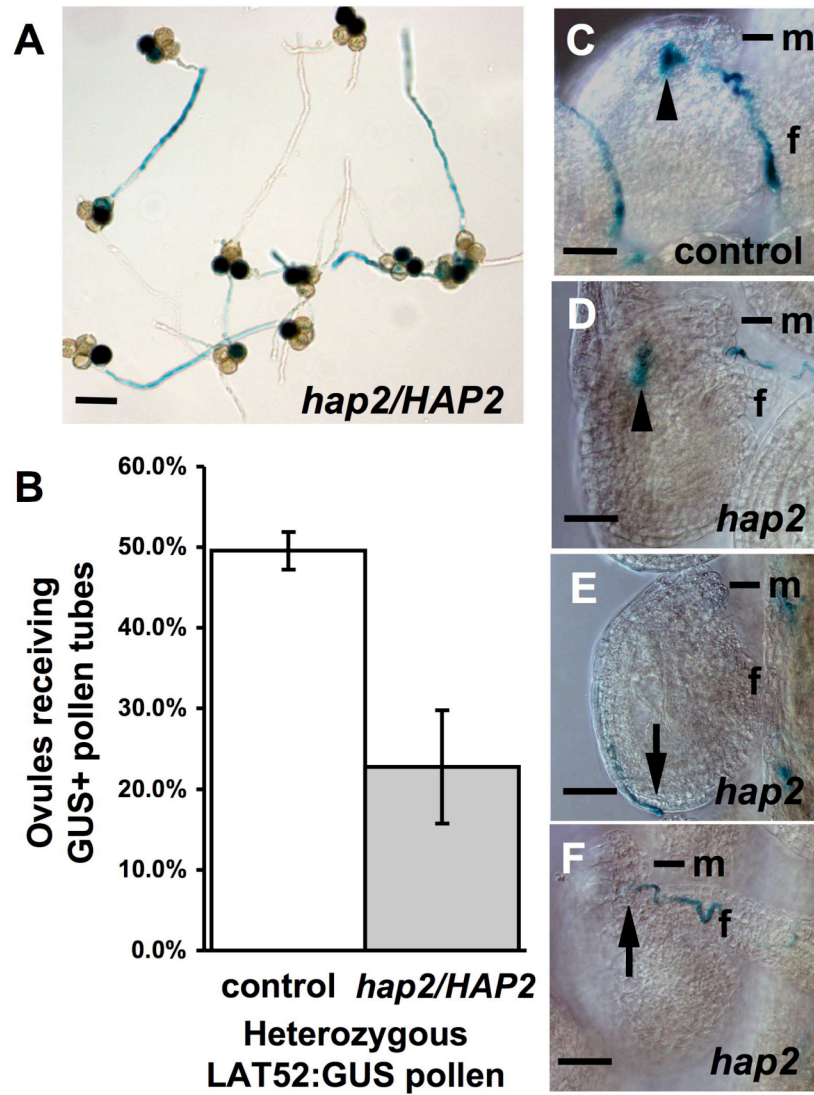


Figure 1: *hap2* disrupts pollen tube guidance.

(A) In vitro grown *hap2/HAP2* pollen tubes stained to reveal GUS expression in *hap2* pollen tubes. (B) Quantitative analysis of ovule targeting. *ms1* pistils were hand-pollinated with *hap2/HAP2* or control pollen heterozygous for LAT52:GUS. The number of ovules receiving GUS activity from the pollen tube was counted for each and is plotted as a percentage ($\pm$ s.d.) of the total number of ovules. (C-F) *ms1* pistils stained for GUS activity 14 hours after pollination. (C) An ovule that has received a GUS+ control pollen tube. (D) An ovule that has received a GUS+ *hap2* pollen tube. GUS is released into synergids from tubes that successfully enter the micropyle and burst (arrowheads in C and D). (E,F) *hap2* pollen tube tips that fail to enter the micropyle (arrows). m, micropyle; f, funiculus. Scale bars: 40 μ m in A; 20 μ m in C-F.

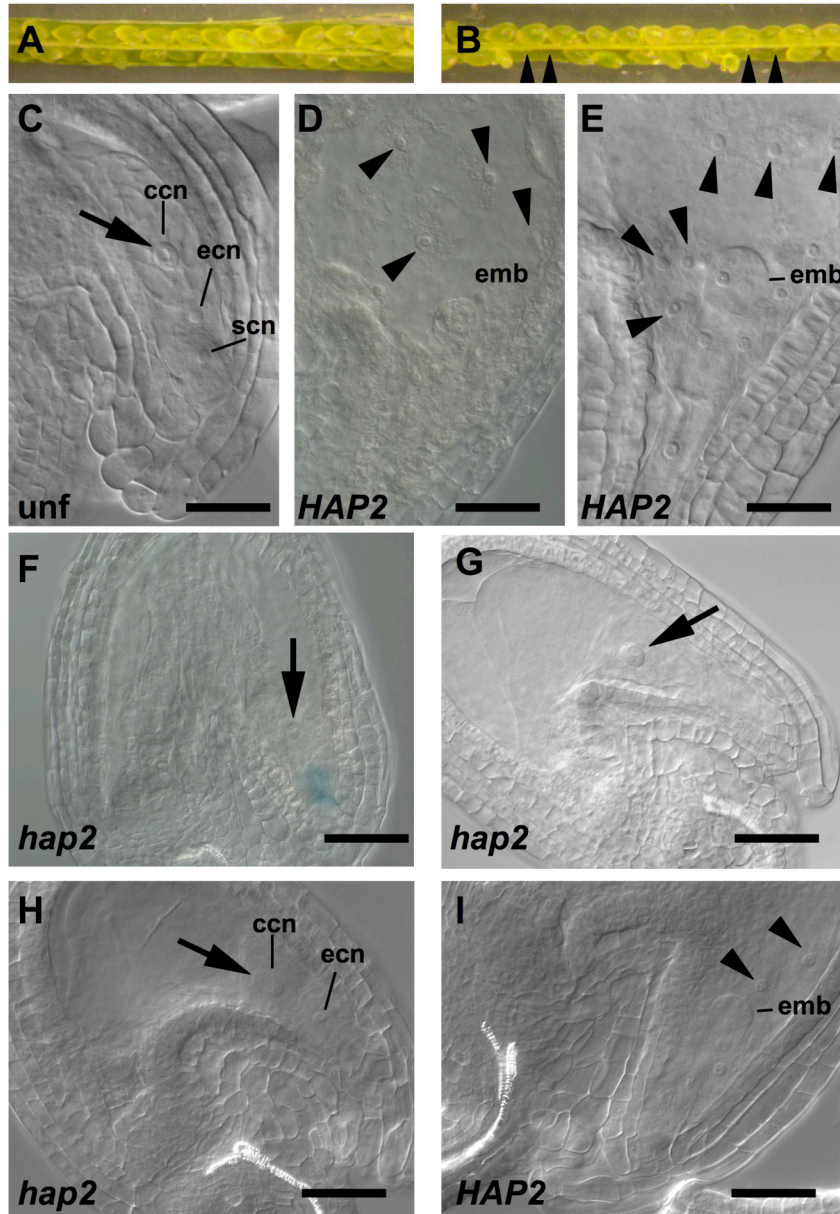


Figure 2: *hap2* blocks egg fertilization and central cell fertilization.

Siliques from self-fertilized plants heterozygous for a control T-DNA insertion (A) or *hap2/HAP2*, which contain aborted ovules (B, arrowheads). (C) An unfertilized ovule showing nuclei of FG cells. (D-I) Ovules following targeting by *HAP2* (D,E,I) or *hap2* (F,G,H) pollen tubes at either 48 (D-G) or 24 hours (H,I) after pollination. Early embryo and endosperm development are apparent in ovules targeted by *HAP2* (D,E,I), but not *hap2* (F,G,H) pollen tubes. Ovules stained for GUS activity (D,F) show that they have been targeted by either *HAP2* (D, no GUS activity) or *hap2* (F, GUS activity present in synergid) pollen tubes and that *hap2* pollen tubes have burst in the targeted ovule. ccn, central cell nucleus; ecn, egg cell nucleus; scn, synergid cell nucleus; emb, embryo; arrowheads, endosperm nuclei. Arrows (C,F,G,H) denote an unfertilized central cell. Scale bars: 20 μ m.

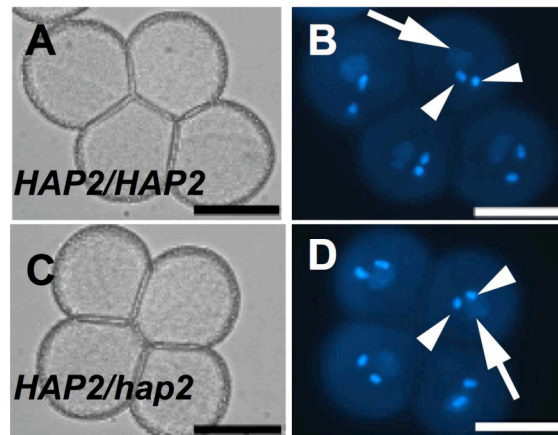


Figure 3: *hap2* pollen contains a normal male germ unit.

(A-D) Pollen tetrads imaged with bright field (A,C) or epifluorescence (B,D) following DAPI staining. The morphology of pollen grains, vegetative nuclei (arrows) and sperm nuclei (arrowheads) were indistinguishable in tetrads from wild-type (A,B) and *hap2/HAP2* plants (C,D). Scale bars: 20 μ m.

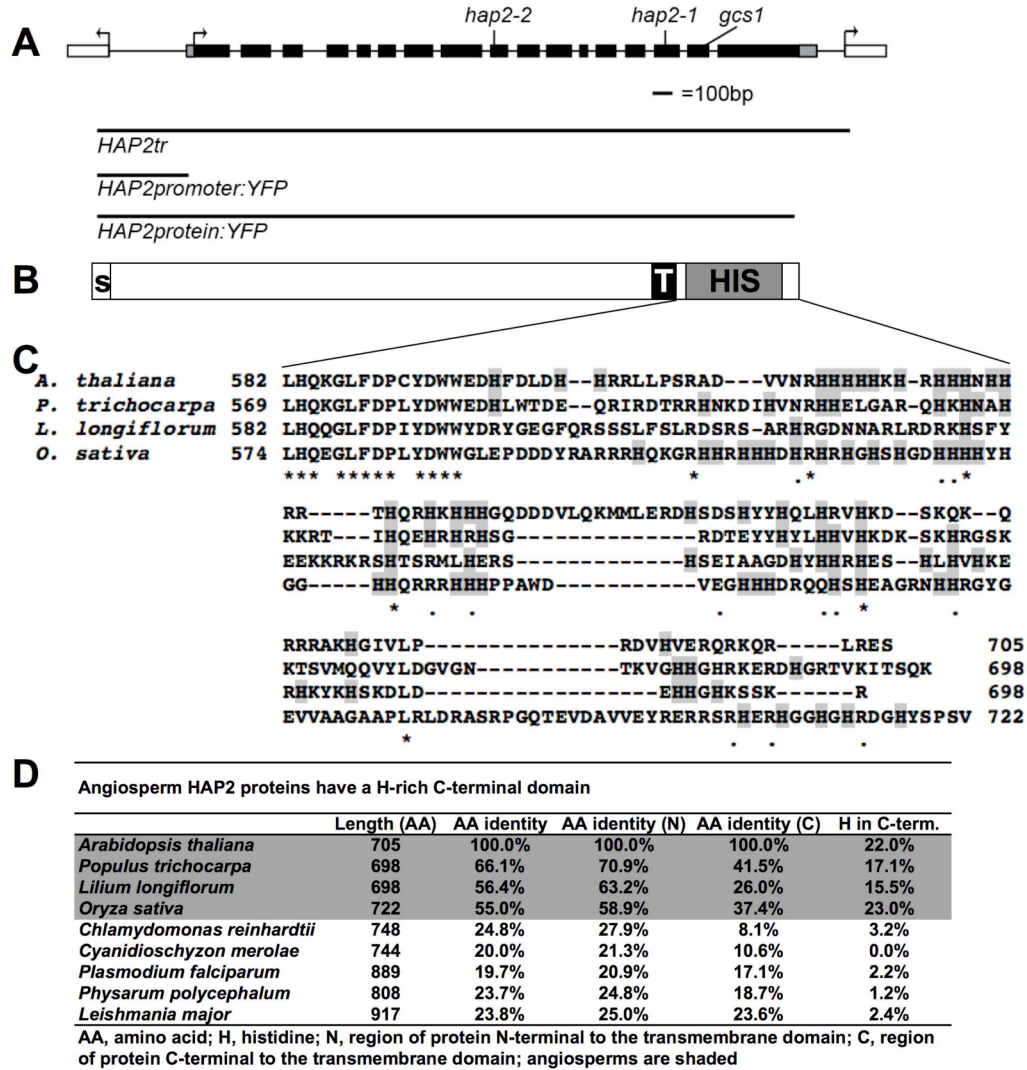


Figure 4: HAP2 and its predicted protein structure.

(A) HAP2 has 17 exons (black rectangles, untranslated regions are gray). *hap2-1*, *hap2-2* and *gcs1* (Mori et al., 2006) are T-DNA insertions in exons 15, 9 and 16, respectively. Black lines under the gene schematic indicate regions used for molecular complementation (*HAP2tr*), analysis of expression pattern (*HAP2promoter:YFP*) and subcellular localization (*HAP2protein:YFP*). (B) HAP2 encodes a 705 amino acid protein with a predicted 19 amino acid N-terminal signal sequence (S), a 23 amino acid transmembrane domain (T) and a C-terminus containing a 96 amino acid histidine-rich domain (HIS). (C) An alignment of the HAP2 HIS-rich region from *Arabidopsis* (*A. thaliana*), poplar (*P. trichocarpa*), lily (*L. longiflorum*) (Mori et al., 2006) and rice (*O. sativa*). The alignment includes the region of the HAP2 C terminal to the transmembrane domain. HIS residues within the HIS-rich region are shaded gray. Conserved residues are indicated with a star. Conservative amino acid substitutions are indicated with a dot. (D) Pairwise analysis of amino acid identity between *Arabidopsis* HAP2 and HAP2 sequences from other organisms; only HAP2 sequences from angiosperms have a C-terminal HIS-rich region.

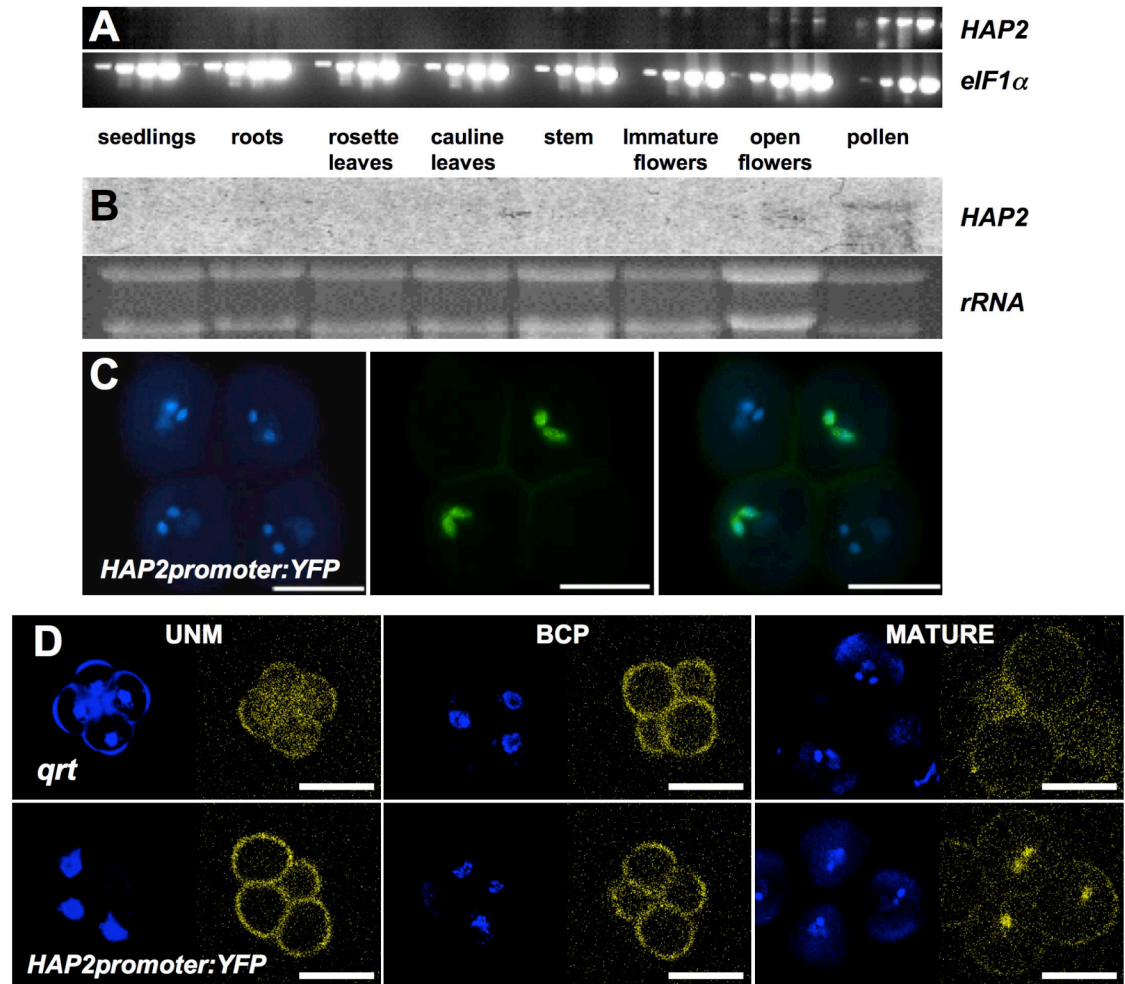


Figure 5: *HAP2* expression is sperm specific.

(A) RT-PCR of wild-type tissues, using primers spanning *HAP2* exon 2 (25- 40 cycles). (B) Northern blot of wild-type RNA probed with full-length *HAP2* cDNA; ethidium bromide stained gel before blotting shows relative RNA amounts in each lane. The source of each RNA sample is indicated between A and B. (C) A pollen tetrad from a plant heterozygous for the *HAP2promoter:YFP* fusion; DAPI fluorescence (left), YFP fluorescence (center), merged image (right). (D) DAPI (left) and YFP (right) fluorescence in pollen tetrads from *qrt* and homozygous *HAP2promoter:YFP* transgenic plants. Uninucleate microspores (UNM, left panels), bicellular pollen (BCP, center panels) and mature pollen grains (right panels) were analyzed. Autofluorescence from the pollen surface is observed in wild-type and transgenic pollen; signal from YFP is only observed in the sperm of mature pollen grains from transgenic plants. Scale bars: 20 μ m.

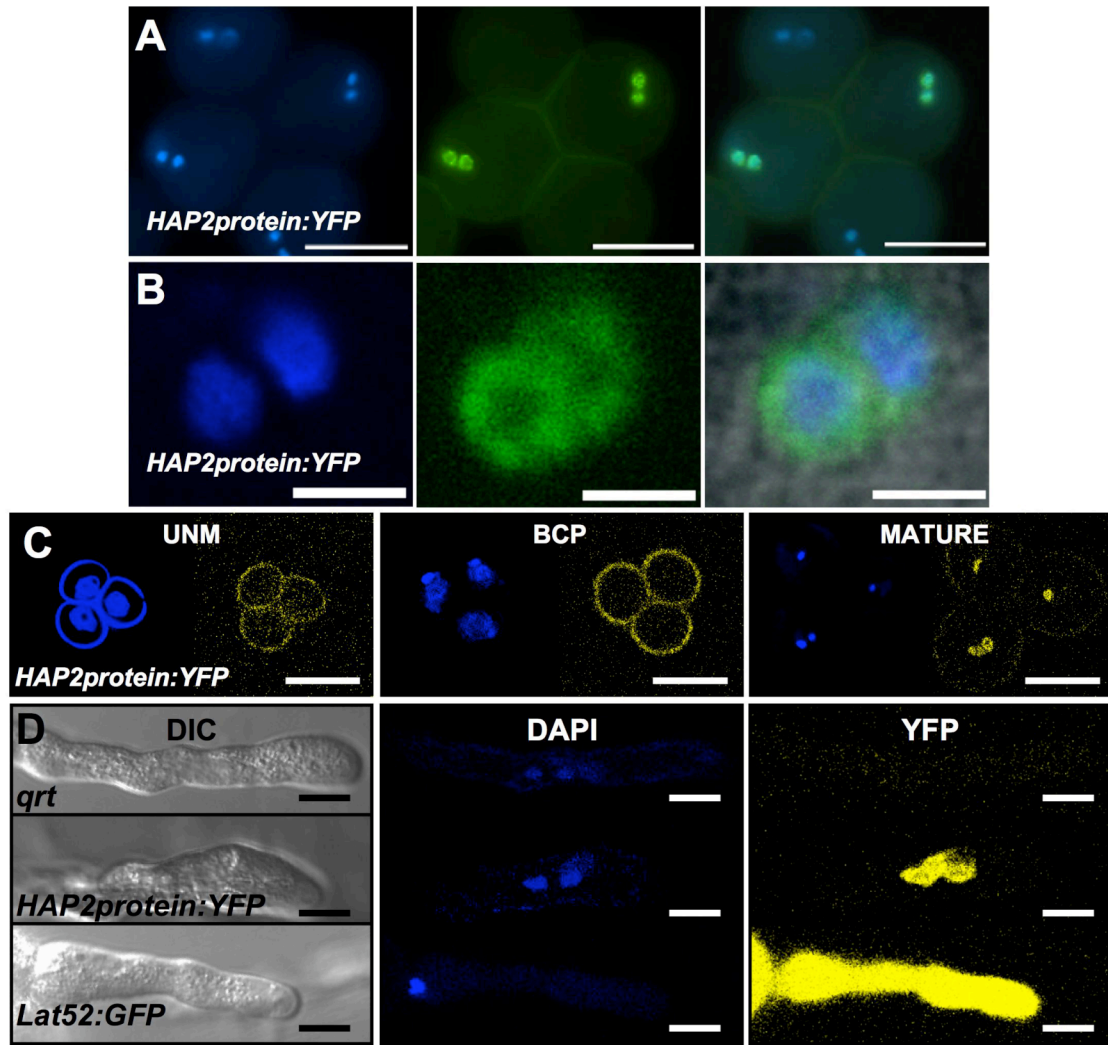
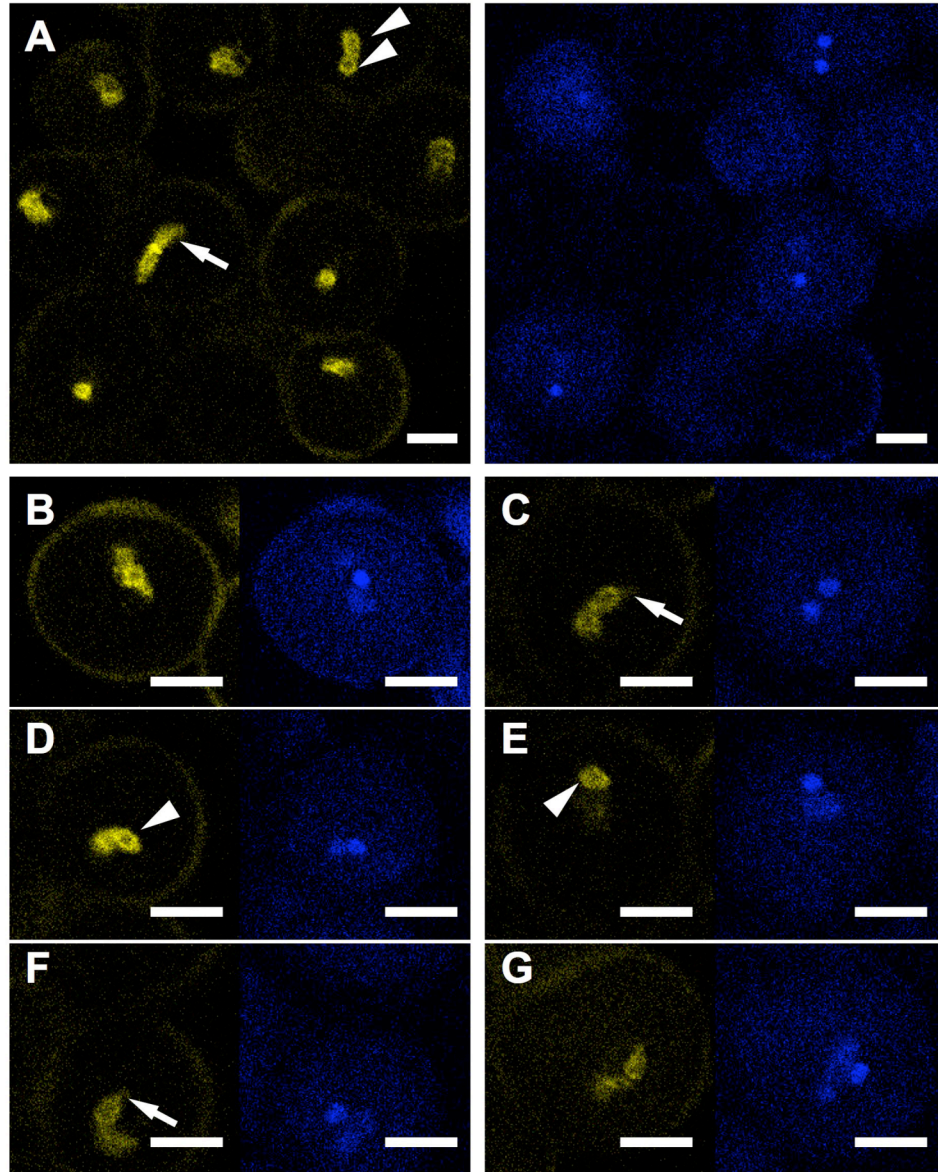


Figure 6: HAP2 protein is sperm-localized during pollen development and tube growth.

(A) A pollen tetrad and (B) sperm nuclei from a plant heterozygous for the *HAP2protein:YFP* fusion; DAPI fluorescence (left), YFP fluorescence (center), merged image (right). (C) DAPI (left) and YFP (right) fluorescence in pollen tetrads from homozygous *HAP2protein:YFP* transgenic plants at three stages of pollen development; see Fig. 5D for comparison with *qrt*. (D) DAPI (center) and YFP (right) fluorescence in growing pollen tubes from *qrt*, *HAP2protein:YFP* and *LAT52:GFP* transgenic plants. DIC images of growing pollen tubes (left). Signal from YFP is observed only in the sperm (*HAP2protein:YFP*) or throughout the entire pollen tube cytoplasm (*LAT52:GFP*). Scale bars: 20 μm A and C; 1 μm in B; 5 μm in C.



Supplemental Figure 1: HAP2 subcellular localization.

(A) Field of pollen grains expressing *HAP2protein:YFP* (left, YFP; right, DAPI). Fluorescence is often observed in a perinuclear ring (arrowhead) but also in the cytoplasm, extending to the likely position of the sperm plasma membrane (arrow). (B-G) Individual pollen grains expressing *HAP2protein:YFP* (left, YFP; right, DAPI). The arrows in C and F indicate fluorescence in cytoplasmic tails; arrowheads indicate perinuclear fluorescence. Scale bars: 10 μ m.

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Chapter 3: Yeast two hybrid analysis of HAP2(GCS1)

identifies possible interacting proteins as well as

characteristics of the HAP2 protein

I performed all the experiments discussed in this chapter, I designed the bioinformatics pipeline and analyzed the data. I was assisted by Alexander Chang who wrote the bioinformatics program and by Adisorn Chaibang who helped with the initial molecular cloning of the HAP2 fragments used for the yeast two hybrid screen.

Abstract:

HAP2 (also known as GCS1) was shown to be critical for fertilization in flowering plants, its role in promoting gamete fusion is unknown. One possible means of promoting membrane fusion is by binding to a protein localized to the membrane of the egg and central cell, thus linking the two membranes and allowing fusion. Although no female-specific fertilization mutant has been found to affect fusion, suggesting a HAP2 binding partner, it might be possible to find this partner or other proteins involved in fertilization by screening from proteins that bind with HAP2. In an attempt to identify the proteins that interact with HAP2 within the pollen and/or female cells we undertook a yeast two hybrid screen. We screened a library of flower-expressed cDNA fragments with part of the amino terminal region of HAP2 (lacking the signal sequence and the transmembrane domain). We found possible interacting proteins, where the strongest interactions and greatest number of interactions were with disulfide bond altering proteins. By comparing our list of interacting proteins to currently available microarray data we determined that none are expressed in the female gametophyte, but many are pollen or sperm specific genes. The overrepresentation of disulfide bond altering proteins suggests the possibility that this type of enzymes interact with HAP2, perhaps to ensure proper folding and disulfide bond formation between the deeply conserved cysteine residues in the amino terminus of HAP2. Through the yeast two hybrid screening with HAP2 we have identified 32 possible interacting proteins and will work to determine if they have a role in fertilization by binding HAP2. The evolutionary conservation and the likely binding of proteins to the cysteines in the amino terminus of HAP2 indicates that these are important for either the structure or function of HAP2.

Introduction:

HAP2 is critical for fertilization and is sperm specific

HAP2(GCS1) is a conserved protein that is expressed only by the male gametes (von Besser et al., 2006; Wong and Johnson, in review). The gene was shown critical for fertilization in three evolutionarily distant species: *Arabidopsis thaliana* (von Besser et al., 2006), *Chlamydomonas reinhardtii* (Liu et al., 2008), and *Plasmodium falciparum* (Liu et al., 2008). HAP2 was also shown to be expressed in the male gametes of the basal animal Hydra, although a fertilization function has not been confirmed (Steele, 2009). Each organism where the *HAP2* gene is found has only one copy of the gene, which is predicted to encode a single transmembrane domain, and have a region of conserved amino acids. The protein has no easily identifiable domains for binding and the biochemical function is completely unknown (Mori et al., 2006; von Besser et al., 2006).

HAP2 was shown to be critical for the fusion of the sperm with female gametes. In *Arabidopsis* pollen that lacks *HAP2*, transmission to the next generation is completely blocked and pollen tube guidance is reduced such that only half of the *hap2-1* (mutant) pollen reach a female ovule (Mori et al., 2006; von Besser et al., 2006). *Chlamydomonas* minus gametes lacking *HAP2* are able to come into close proximity with plus gametes, but the cells are unable to fuse with one another (Liu et al., 2008). Lack of this single protein thus blocks all fertilization events, indicating a vital role for this protein in the fusion of male gametes to the female gametes. The ability of a sperm expressed protein to influence the guidance of the separate cell in which they reside is novel. The two defects of *hap2-1* mutants indicates the possibility of multiple interactions mediated by HAP2 in the pollen and ovule.

HAP2 is hypothesized to be in the male gamete plasma membrane with the amino terminus outside the cell (Liu et al., 2008; Wong and Johnson, in review). The predicted transmembrane domain is diagramed in Figure 1 and the strongly conserved domain is shown with an alignment of ten species. Despite the ability of prediction programs to find domains within amino acid sequences, no other motifs or secondary structure could be determined from the amino acid sequence of HAP2. The signal sequence at the beginning of the protein is predicted (by the TargetP, www.cbs.dtu.dk/services/TargetP/, and PSORT, www.psort.org programs) to target HAP2 to the ER, where it is then brought through the Golgi and vesicle transport to the plasma membrane. HAP2 was specifically localized to the mating projection of *Chlamydomonas minus* gametes, when in close proximity to plus gametes. A HAP2 mutant in *Chlamydomonas* showed that the minus and plus gametes come into direct contact, but do not fuse, indicating a role for HAP2 in plasmogamy, or the fusion of the two plasma membranes (Liu et al., 2008).

The mechanism by which HAP2 mediates fusion is a critical question within the field (Liu et al., 2008; Mori et al., 2006; von Besser et al., 2006; Wong and Johnson, in review) and we propose that it interacts with a protein or proteins on the female gametes to induce fusion. This hypothesis is born from the observations that the two sperm are in very close proximity in *Arabidopsis* and both express HAP2, but they do not fuse until they reach a female gamete, so the HAP2 protein is unlikely to act alone to induce fusion. It is also likely that HAP2 interacts with pollen tube proteins since the lack of HAP2 reduces pollen tube guidance by half (Mori et al., 2006; von Besser et al., 2006; Wong and Johnson, in review). The proteins that interact with HAP2 are completely unknown.

Identifying proteins that interact with HAP2 should lead to a better understanding of HAP2 function during fertilization.

Biochemical approaches for isolating HAP2 and its interacting proteins are technically challenging. Not only is there very little HAP2 expressed in vivo since only the sperm express HAP2 (Liu et al., 2008; von Besser et al., 2006), but in Arabidopsis the sperm are contained within the pollen tube, making isolation difficult. The interaction between the male and female gametes is extremely fleeting (Faure et al., 2002; Matsushima R, 2008) and an attempt to isolate HAP2 bound to proteins on the female gamete is unlikely. When a full-length recombinant protein was expressed in bacteria in an attempt to crystallize the protein for X-ray crystallography the protein was not stable and would not form crystals (unpublished data). Thus HAP2 is resistant to traditional biochemical analysis.

Genetic analysis has been more fruitful in determining functionally important domains in the HAP2 protein. By swapping amino and carboxy domains of the HAP2 protein of Arabidopsis with those of Rice, Wong and Johnson were able to identify regions implicated in species specificity and to show that the carboxy terminus requires a positive charge to function (Wong and Johnson, in revision). Interestingly the amino terminus of the closely related plant *Sysimbrium irio*, was able to rescue the *hap2-1* fertilization defect, while the amino terminus of the more distantly related rice, *Oryza sativa*, was not able to rescue. The authors came to the conclusion that the species specificity may be due to the amino terminus on the outside of the sperm interacting with a protein on the egg and central cell that changed enough over evolution that HAP2 from the other species cannot be recognized (Wong and Johnson, in revision). Unfortunately

these studies cannot provide the identity or function of the egg and central cell expressed protein(s) implicated in this interaction.

One approach to quickly and efficiently identify protein(s) that interact with HAP2 and which has the potential to find transient interactions is a yeast two hybrid screen. Since HAP2 is membrane bound, performing a yeast two hybrid screen with the full-length gene is not easily accomplished, but breaking the HAP2 protein into regions lacking the transmembrane domain should allow those fragments to enter the yeast nucleus, which is a requirement for a traditional yeast two hybrid screen. There is precedence that a yeast two hybrid screen does work for transmembrane proteins when each section of the protein is expressed in the yeast separately (Harada Y, 2007; Tang et al., 2002).

Saccharomyces cerevisiae does not have a HAP2 homologue, and none has been found in any fungal genomes (Wong and Johnson,). The lack of a HAP2 homologue or pathway in yeast is important so that we would not deplete the HAP2 protein by binding endogenous yeast proteins. Especially since there is a species specific nature to the fertilization mechanism such that Rice HAP2 cannot complement Arabidopsis HAP2 (Wong and Johnson, in prep.) we did not want to reduce the potential binding with flower library proteins.

The HAP2 protein has regions of high conservation within plants (Wong and Johnson, in review). The amino-terminal domain of HAP2, which also makes up the bulk of the protein is highly conserved in plants. Eighteen of the nineteen cysteines found in Arabidopsis HAP2 are conserved throughout all the plant species where HAP2 mRNA has been sequenced. Ten cysteines are conserved throughout all species where

HAP2 is found (Figure 1). They are spread through the entire amino terminus and do not clump in any one location. Of the seventeen amino acids conserved through all 12 species in this alignment, ten of them are cysteines.

The yeast two hybrid screening method takes advantage of the fact that the yeast transcription factor Gal4 has two functions, carried out by two separate modular domains. When these domains are brought into close proximity they promote the expression of reporter genes through the UAS promoter. The Gal4 activation domain is linked with the proteins of a flower library and the Gal4 DNA binding domain (DBD) is attached to a region of HAP2 as the bait. The amino terminal domain and the carboxy terminal domain of HAP2 were fused to the GAL4 DBD and expressed in yeast.

Through this yeast two hybrid screen we have been able to find proteins that potentially interact with the amino terminus of HAP2. Although this list of 32 genes is likely not all the proteins that interact with HAP2 it indicates a role for various amino acids within the amino terminus of HAP2 for structure and/or function. It also provides new avenues of investigation into how HAP2 might mediate both fusion and pollen tube guidance.

Results:

Strategy to identify HAP2 interacting proteins by yeast two hybrid screening

We utilized a yeast two hybrid system that makes use of multiple layers of stringency to evaluate the strength of an interaction between two proteins within the yeast nucleus. The bait transcript, in our case, regions of the *HAP2* cDNA, was cloned in frame with the DNA binding domain of the Gal4 transcription factor. This plasmid, pGBK-T7, confers the ability to grow on media lacking Tryptophan (Trp) and has a c-Myc protein tag in the Y187 yeast strain. A library of prey constructs were cloned in frame with the Gal4 activation domain, in our case obtained from, and made by the Yadegari lab from a collection of flower transcripts (Wang et al., 2006). This plasmid library was made using the pGAD-T7 backbone, which confers the ability to grown on media lacking Leucine (Leu) and was transformed into the yeast strain AH109 (a mating type).

The regions of HAP2 cloned into the bait plasmid are depicted in cartoon form in Figure 2, where the N-terminal domain is the full length N from the signal sequence to the transmembrane domain, and C-terminal is everything after the transmembrane domain. The short N-terminal (SNT) construct is the part of the amino terminus that includes the region of greatest conservation and what is now being called in Genbank the “HAP2-GCS1” domain and the male gamete fusion factor (At 280-328). The HAP2 containing plasmids were transformed into yeast of the opposite mating type and which have a series of reporter genes under the control of a Gal4 upstream activating sequence (UAS). Expression was checked by Western blot using antibodies specific for the Myc

tag that was also fused to HAP2 (Figure 2). All three constructs resulted in robust HAP2 expression in yeast.

This yeast strain has three reporter genes under control of the Gal4 UAS system: 1) ADE2 2) HIS3 and 3) LACZ. When activated, through the interaction of the DNA binding domain and activation domains of Gal4 coming into close contact through the association of a bait with a prey protein, they allow for growth on plates lacking Histidine (His) and Adenine (Ade). The strength of the interaction between the bait and prey constructs is variable and the output expression of the reporter constructs is therefore also variable. The LACZ expression is the most stringent and so the blue color that each colony possesses can be used as an indicator of the strength of the interaction between bait and prey proteins. Colonies that possess both a bait and prey plasmid, but which do not interact will only grow on media lacking Trp and Leu, indicating a successful mating of the two cells. If a colony is able to grow on media lacking the four amino acids Trp, Leu, His and Ade (-Quad) then the UAS response genes are active and there is an interaction between the prey and bait proteins.

The regions of HAP2 chosen as bait (Figure 1 above the full length HAP2 protein) represent the amino terminus of HAP2 (NT), a shortened region of the amino terminus which contains the highly conserved HAP2/GCS1 region (SNT), and the short carboxy terminus (CT). The HAP2 fragment containing plasmids were transformed into yeast strain Y187 and individual colonies were confirmed to carry the correct plasmid. Expression of the HAP2 fragments was confirmed by Western Blot on yeast protein extracts by the anti c-Myc antibody (Figure 3). Each is the expected size for the specific fragment along with the Gal4 DNA binding domain. Although the Western Blot was not

quantitative it was encouraging that the HAP2 protein fragments were expressed at a similar level to the p53 control fragment from an equivalent amount of yeast cells.

Mating and screening for interactions with the short amino terminus of HAP2

Initially the mating efficiency of the Y187 yeast carrying the HAP2 fragments to the AH109 strain containing the flower cDNA library was not high enough to ensure full coverage of the flower library. Efficiency for all matings was similar to the full length amino terminal construct shown in Table 1, with an efficiency below 10% which was not acceptable. Once the mating efficiency reached above 15% (see methods) we proceeded to plate the entire SNT mating with the flower library on selection plates lacking Trp, Leu, His and Ade, and containing X-Gal to screen for beta-galactosidase expression.

Although the –Quad plates also included X-Gal to identify colonies resulting from strong interactions which would be evident by the expression of beta-galactosidase and a blue color, only one colony was actually blue when it grew on these screening plates. The rest of the colonies were either white or pink in color (Figure 3). Since these colonies were able to grow on the stringent media lacking both Histidine and Adenine, which could only be expressed when the Gal4 activation and DNA binding domains come into close contact, these were picked for further characterization. About 300 colonies were picked, archived and further characterized.

As a control for mating and screening, we were provided with yeast strains containing the pGBK-T7 p53 and pGAD-T. The proteins p53 and T are known to interact with one another in this yeast two hybrid system. Although the mating efficiency between these two stains was low (6.4%), even at a thousand fold dilution there were six colonies that turned blue on the most stringent –Quad media with X-Gal (Table 1). This

indicated that our regents were functional and that we could find interactions if they occurred.

Colonies were characterized for library clone and to confirm an interaction with HAP2 by beta-galactosidase expression

Two hundred and eighteen colonies that grew on –Quad gave good PCR products by high throughput colony PCR (methods). Products were amplified from the plasmid with primers that spanned the cDNA library insert and included some of the GAL4 activation domain. These products were sequenced and the sequence tags manipulated by a PERL computer program written by Alexander Chang. The sequencing read length was often long enough to provide the entire gene within the plasmid and always enough to identify the gene it came from. The computer program that was developed for our screening process, and is diagramed in Figure 4, used BLAST to search the Arabidopsis genome and determine the gene the sequence tag came from and the endogenous amino acid sequence of the gene product. Then the computer program aligned the amino acid sequences of the endogenous protein with the protein made in the yeast by the transcript in frame with the Gal4 activation domain and determined if these matched. These were considered “in frame” and amounted to 127 colonies. Any clone that produced an amino acid sequence not endogenously produced by a plant was marked as “not in frame” and culled from further characterization.

To further prioritize colonies, we used a more sensitive LACZ assay than on media plates. Colonies were lifted from a patch plate (Figure 3D) and broken open by freezing, before exposing the contents to X-Gal and looking for blue color over time (Figure 3E). The clone that showed blue on a plate and a positive control showed strong

blue quickly, while 86 (Figure 4) developed color more slowly. This information along with the results of the computational comparisons with genomic sequence and protein products was compiled in a database along with the gene ontology (GO) classifications for these genes.

We designed a computational routine to analyze the sequence data from the PCR products (Figure 4). This routine, written and implemented by Alex Chang performed multiple database queries and searches to analyze the sequence tags: 1) it searched by BLAST to identify what gene the sequence tag came from; 2) translated the sequence tag to determine what protein was produced from the yeast plasmid and compared this with the endogenous Arabidopsis protein to determine if the protein was “in frame”; 3) downloaded from databases the gene expression profile, gene ontology (GO) information and predicted subcellular localization of the endogenous gene; 4) and compared sequence tags to the list of all colony sequence tags to determine their uniqueness and frequency. A “unique clone” was designated as one that has a different sequence tag from others of the same gene, often times a sequence tag would begin with a different amino acid as another. We also determined whether the endogenous genes were predicted to encode a transmembrane domain using the TMHMM algorithm (www.cbs.dtu.dk/services/TMHMM-2.0/). Seven of the 32 genes that passed all our computational and experimental confirmations, have a predicted transmembrane domain, one gene, AT4G33520 a metal-transporter, has multiple transmembrane domains.

Genes found to interact with the short amino terminus of HAP2 can be grouped into seven functional categories

The 32 genes that passed our rigorous characterization pipeline (Figure 4) can be grouped into eight categories based on their predicted function, GO classification, and predicted subcellular localization. The seven classes, in no particular order, are: 1) the thioredoxin or disulfide isomerases, 2) genes involved in transcription, 3) those with a chloroplast function or localization, 4) proteins with the capability to bind metals, 5) proteins that have a membrane or cell wall modifying activity, 6) those with protein modifying enzymatic activity, 7) and proteins of unknown function. These are shown in Figure 5 as either the number of unique clones (Figure 5A) within each category or by the number of different genes per category (Figure 5B).

The greatest proportion of unique clones was from thioredoxin and disulfide bond modifying genes (Figure 5A and Table 2). These genes were not only the greatest number of genes represented (Table 2), they also showed the highest beta-galactosidase activity and the only colony that turned blue on the initial screening plates was within this group (not shown). Thioredoxins make and break disulfide bonds between cysteines (Meyer, 2005). Some thioredoxin proteins show an amino acid motif similar to a region of conserved amino acids in HAP2 seen in Figure 6 (Houston et al., 2005).

Discussion:

Strengths and Weaknesses of a yeast two hybrid screen in the case of HAP2

Because so little HAP2 protein is made by a plant in the small sperm cells and only at a specific developmental time point and within the pollen grain cell, it is difficult to obtain enough protein to perform biochemical assays. By expressing HAP2 in yeast and screening in the yeast for interacting proteins we were more rapidly and easily able to find potential binding partners. Since HAP2 is a transmembrane protein we split the protein and utilized an available flower library. The fact that yeast does not have a HAP2 homologue also reduces the folding ability if there are any chaperones or modifying enzymes that need to act on HAP2 to reach a proper fold. It seems quite likely that since there were so many disulfide modifying enzymes found in the yeast two hybrid screen with the short amino terminus (SNT) of HAP2, that the cysteine residues in the yeast are not in their native form and they are sticking to more than they might when in the sperm.

Proteins that are pollen- and sperm-specific were pulled out of the yeast two hybrid screen.

The ability to find proteins encoded by rare mRNAs was a concern going into this study. Ideally we would have used specific libraries from pollen, sperm, and the female gametes, but these were not available and would be difficult to make, since the cells are not easily dissociated from other tissue. Furthermore this would have required multiple screens whereas all of these cell types could be screened simultaneously using a flower library. The fact that some of the proteins that interacted with the short amino terminus (SNT) of HAP2 are pollen specific or sperm enriched shows that we were able to find those rare proteins from the library (Table 3). We did not identify many proteins that are

female specific or expected to be ovary enriched, possibly due to the fleeting nature of an interaction between HAP2 and such proteins. Another possibility of why we failed to identify female specific proteins is that HAP2 requires a conformational change or protein modification before it can interact with the female gamete proteins.

The second largest category of proteins found to interact with the SNT fragment of HAP2 were those with a localization or function within the chloroplast. This was unexpected because HAP2 is predicted to be a secreted protein and has been shown to be associated with membranes (Mori et al., 2006; von Besser et al., 2006). Three of these proteins have only predicted localization and two others have disulfide or reducing enzymatic activity that might bind with the cysteines of the SNT. Although it is possible that HAP2 interacts with chloroplast associated proteins, HAP2 puncta when ectopically expressed in the pollen tube could be localizing to plastids (Chapter 4 Figure 8), it is unlikely that this is their only localization especially since sperm have few plastids.

The yeast nucleus is likely not the same environment as the sperm membrane

Although the short amino terminus of HAP2 was able to pull out interacting proteins in this yeast two hybrid system it seems quite possible that this region was not properly folded or processed by yeast cells. The yeast nucleus is a reducing environment, while most extracellular locations are oxidizing environments (De Jaeger, 2000). It is possible that the cysteines of the HAP2 SNT were not in the correct state. This may be one reason why so many of the putative HAP2-interactors identified in the yeast two hybrid assay were disulfide modifying proteins. It is possible that the disulfide modifying enzymes found in the yeast two hybrid screen are binding with the SNT fragment because it is misfolded in the yeast and the cysteines are binding anything that

they can. This is certainly possible, but it still lets us know that these cysteine residues are important for the HAP2 protein to fold correctly and that there might be a disulfide modifying enzyme expressed in the sperm that interacts with HAP2, but which we might not have found yet.

The same reasoning could explain why so many HAP2 interacting proteins are predicted to be localized to the chloroplasts, while HAP2 has no known function or expression in the chloroplasts. The proteins that interact with HAP2 SNT in the yeast two hybrid assay could be interacting with the misfolded cysteine residues which act as a sticky binding location. The chloroplast proteins that we identified in the yeast two hybrid screen are some of the most abundant proteins in the entire plant and could be over represented in the flower library. Perhaps there are less abundant proteins that perform similar functions within the sperm secretory pathway that do interact with HAP2 that were not found in this screen. These might act as chaperones to ensure that the disulfide bonds are formed properly.

The third largest category was comprised of proteins with proposed roles in membrane and cell wall synthesis or modification. This seems a likely class of proteins that would interact with HAP2 since the process of fertilization and pollen tube guidance both require cell membrane and wall modification. One of the genes in this group is Actin 4, a pollen-specific actin (Huang, 1996). It seems interesting that HAP2 might bind with a cytoskeletal protein like actin because the sperm are thought to move with the vegetative nucleus in the pollen tube and one hypothesis states that sperm utilize the cytoskeleton of the pollen or synergid cells to move to the egg and central cell (Faure et al., 2002). It would be interesting to know if HAP2 has a role in sperm motility.

The eight functional categories that putative interactors were placed in were frequently based on the predicted function of a gene and further information or testing is needed to determine if these genes are required for HAP2 function or have a role in fertilization themselves. To validate these candidate interactions, mutants in the genes found through this screen can be characterized for fertilization defects. The interactions between HAP2 and these candidate genes must be further corroborated with immunoprecipitation if enough HAP2 protein could be made by ectopically expressing HAP2 in the plant (Chapter 4).

Materials and Methods:

Cloning HAP2 pieces into yeast expression plasmids and transforming yeast

The amino (NT), short amino (SNT), and carboxy (CT) regions of HAP2 were amplified by PCR from a HAP2 cDNA containing plasmid (pgl) and cloned into the yeast expression vector pGBK-T7 with the enzymes EcoRI and XmaI. These regions of HAP2 were in frame with the Gal4 DNA binding domain.

Each of these plasmids was transformed into the yeast strain Y187 by normal calcium chloride techniques (see manual). The pGBK-T7 vector includes a c-Myc epitope tag between the Gal4 DNA binding domain and the HAP2 region, which allowed for confirmation by Western blot that the protein was being expressed to high levels within the yeast cells (figure 3) with the commercial antibody E10 for c-Myc.

Yeast two hybrid mating

500ml cultures of the HAP2 expressing yeast were grown up (as indicated by the manual) and mated with the frozen stock of library in liquid culture and slow rotation at 30°C for

12 hours without selection. These were then plated on yeast media plates including X-gal and lacking Trp, Leu, His, and Ade (-Quad) and grown for up to 10 days at 30°C. Colonies that grew on these selection plates were picked, re-tested on -Quad with X-gal for whether they turn blue, indicating expression of beta-galactosidase. These were each replica plated to continue screening.

Mating efficiency was determined after liquid culture mating by diluting 100µl by 100, 1000, and 10,000 and plated on plates lacking Trp, and Leu separately and Trp/Leu together. After two days of growth at 30°C colonies were counted on each of these plates and the efficiency that the parental strains conferring Trp or Leu growth combined to produce progeny that are able to grow on the double drop-out media Trp/Leu (Table 1).

After initial trials we determined that slow (30 RPM) shaking was critical to allow efficient mating and the efficiency increased to 17% for the SNT plasmid containing yeast. The Yadegari lab that made the library suggested that they had 15% mating efficiency when they performed their assay (personal communication).

Screening colonies for interactions

Colonies that grew on -Quad plates were picked onto patch plates, grown up and archived for future use. High throughput colony PCR was performed by picking a small amount of the yeast into a PCR tube, letting it incubate at 30°C for 15 minutes with 2ul of Zymolyase (1mg/ml) before a PCR mixture with the primers (5' BD vector screening primer F CTATTCGATGATGAAGATACCCACCAAACCC and 3' BD insert screening primer R GTGAACTTGCGGGGTTTTTCAGTATCTACGATT) was added and run with normal conditions and a melting temperature of 65°C. Half the PCR reaction was run on an agarose gel and if it showed a good banding pattern the other half

of that reaction exonuclease I and alkaline phosphatase treated to degrade the primers from the PCR (McElver et al. 2001). These samples were then sent for sequencing by the Marine BioLabs Sequencing Core Facility with the T7 sequencing primer. This gave us a sequence tag for 218 of the plasmids encoding proteins with potential interactions with the SNT HAP2/GCS1 fragment.

The sequence tag was then run through a Perl bioinformatics program (created by Alex Chang) that compared the sequence with the Arabidopsis genome by BLAST to find which gene the colony contained. Then the protein product was determined and compared to the known protein of that gene to establish if the fusion protein from the flower library was in frame to make the endogenous protein. From comparing colonies within the dataset we counted how many of the yeast colonies were unique clones of that gene. The database also pulled information from the literature and online databases to determine whether the endogenous gene has a predicted transmembrane domain, what the gene ontology is, and what the expression profile in Arabidopsis is.

Analysis of beta-galactosidase expression in colonies

Beta-galactosidase expression was determined by lifting a bit of the colonies from patch plates onto filter paper and exposing the proteins within these cells to the X-Gal substrate to determine if there was even a small amount of beta-galactosidase being produced (Mockli and Auerbach 2004). In brief, after colonies were transferred to filter paper, they were soaked in liquid nitrogen for 10 seconds, then thawed to room temperature. These were then layed on a second filter pretreated with a solution of 60 mM Na₂HPO₄, 40 mM NaH₂PO₄ H₂O, 10 mM KCl, 1 mM MgSO₄ 7H₂O, 39 mM 2-mercaptoethanol and

1 mg/ml X-gal at pH 7.0. These were then covered and were checked periodically for five hours to see which colonies turned a blue color.

Acknowledgments:

We are indebted to the Yadegari lab for their generous gift of the flower library and the yeast strains used in this assay. We also wish to thank the members of the Serio and Laney labs of Brown University for their yeast expertise, helpful hints and gift of reagents.

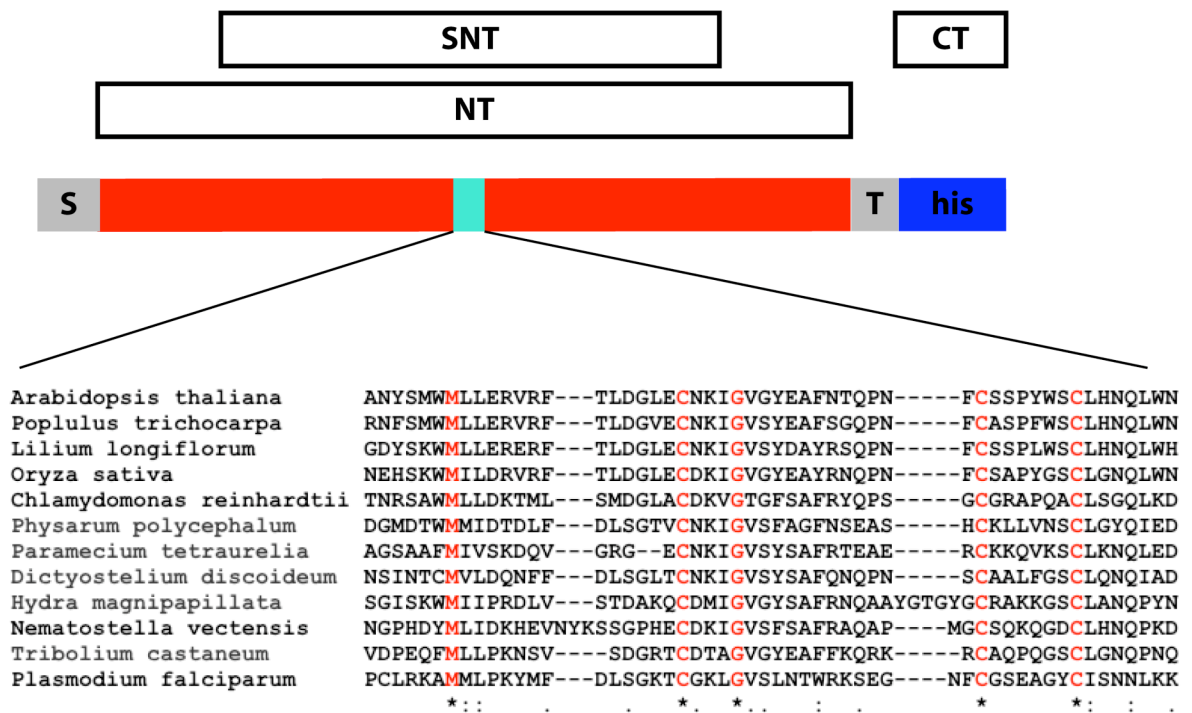


Figure 1: Pictorial representation of the HAP2 protein of Arabidopsis.

This image shows the signal sequence (S), transmembrane domain (T), histidine rich carboxy terminus in blue (his), and the HAP2/GCS1 domain in light blue. This amino acid sequence of this highly conserved region is shown below as an alignment from 12 species. The domains of HAP2 that were cloned for the yeast two hybrid screen are shown above the Arabidopsis HAP2 protein, the full amino terminus (NT), the carboxy terminus (CT), and the short amino terminal domain (SNT).

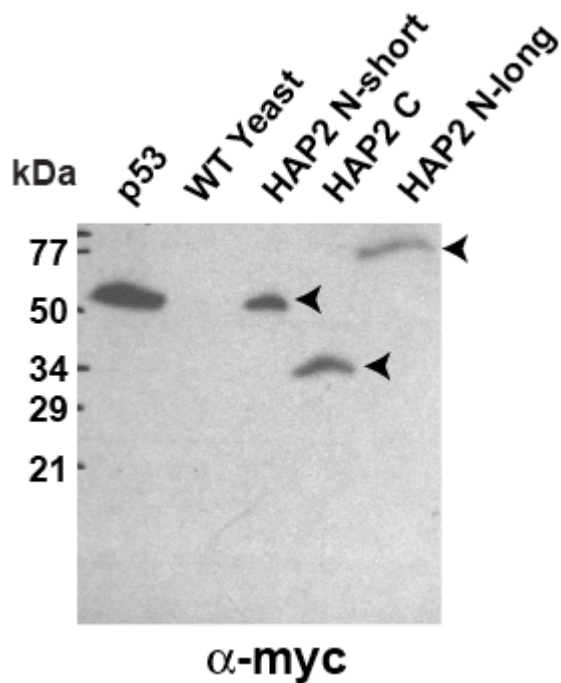


Figure 2: Immuno-blot analysis of yeast strains expressing portions of HAP2 fused to the GAL4 DNA binding domain.

Protein from a control pGBK-T7 p53 plasmid-containing yeast strain (p53), from non-transformed yeast (WT yeast) and from the three HAP2 plasmid-containing yeast strains (HAP2 N-short, HAP2C, HAP2 N-long) was isolated, and analyzed using the anti-Myc antibody E10 which detects a Myc epitope tag. Each protein is the expected size for the fusion of the HAP2 fragment to the Gal4 DNA binding domain.

Table 1: Mating efficiency for Yeast Two Hybrid assay.

Strains mated	Media	# of colonies at indicated dilution			Efficiency
		10^{-4}	10^{-3}	10^{-2}	
p53 X T	-Trp	28	>500	>500	6.4%
	-Leu	20	263	>500	
	-Trp/Leu	0	17	116	
	-Quad	0	6	105	
SNT X Library	-Trp	26	200	>500	17.0%
	-Leu	76	>500	>500	
	-Trp/Leu	0	34	212	
	-Quad	0	0	0	
NT X Library	-Trp	10	105	500	3.8%
	-Leu	38	337	>500	
	-Trp/Leu	0	4	40	
	-Quad	0	0	0	

Yeast strains were mated in liquid cultures and plated at various dilutions on media lacking Tryptophan (-Trp), Leucine (-Leu), both Trp and Leu (-Trp/Leu), and quadruple combination of Trp, Leu, Arg, and His (-Quad). Colonies on these plates were counted after two days growth at 30°C. Efficiency of mating was determined by the number of colonies growing on the Trp/Leu and dividing by the lower number of Trp or Leu alone. Any plate that had so many colonies it looked more than 500 or a lawn was not counted.

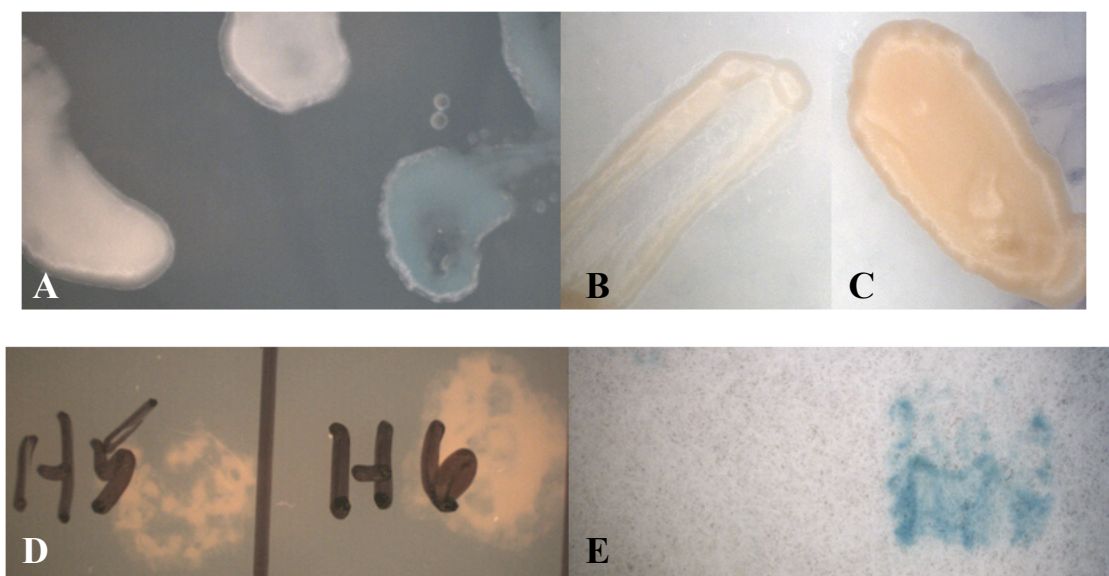


Figure 3: Yeast colonies were picked onto patch plates and characterized for growth and LacZ expression.

Colonies from efficient mating of SNT with flower library were collected from stringent –Quad media plates and characterized for beta-galactosidase activity by colony lifts. Colonies on –Quad showed blue only when the interaction was extremely tight, as in the case of the control interaction between p53 and T seen on the right in (A). Colonies picked from –Quad after mating the HAP2 SNT with the flower library were usually either white (B), or pink (C), in color. After replica plating (D) colonies were lifted onto filter paper and lysed to allow detection of beta-galactosidase expression (E). D and E are the same colonies on the patch plate and after X-Gal staining on filter respectively.

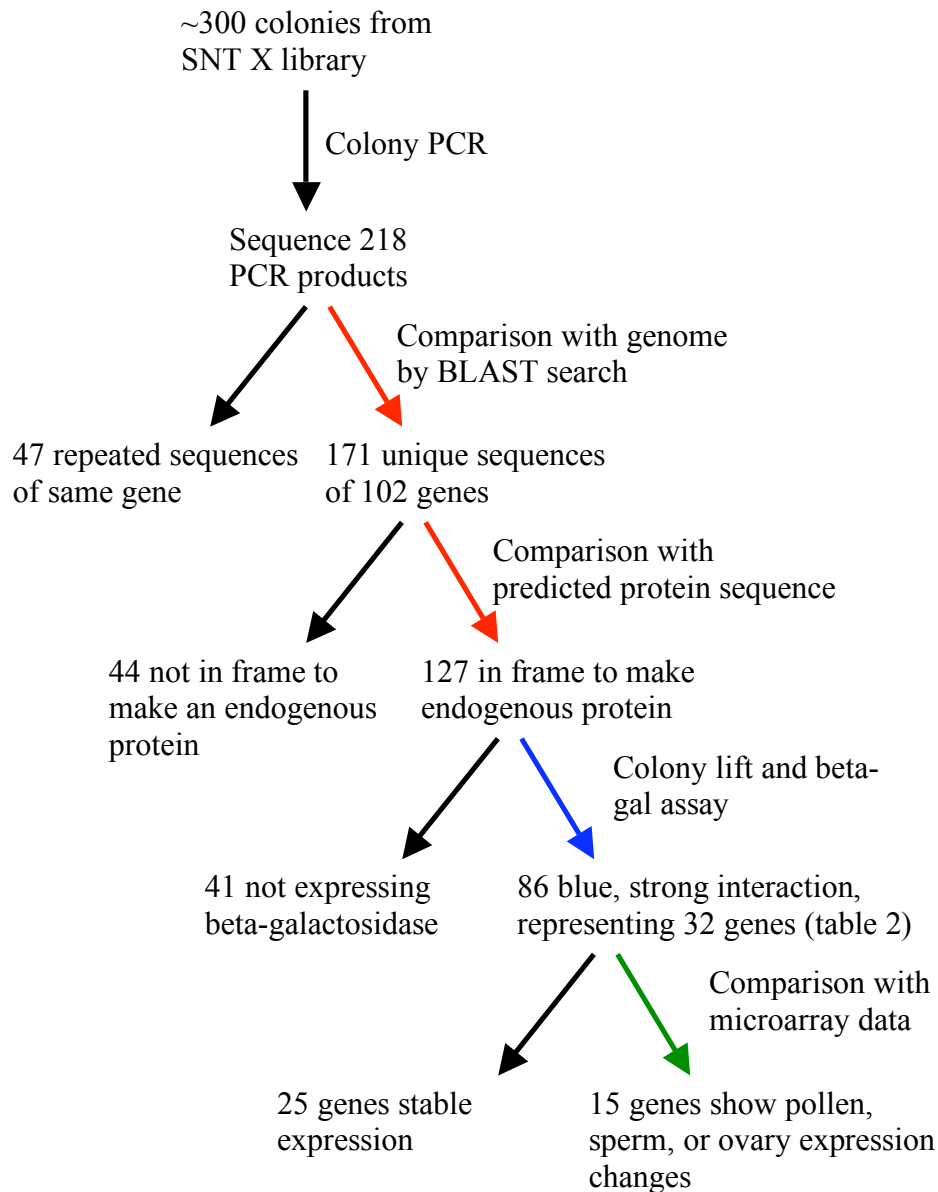


Figure 4: Flow chart of screening strategy for yeast two hybrid positives

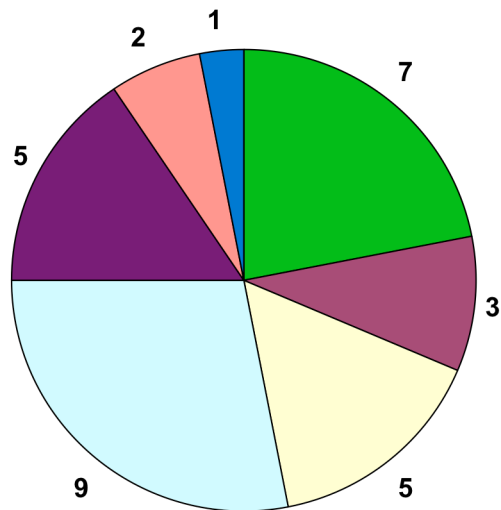
Colonies were sequenced by colony PCR, then compared with the annotated Arabidopsis genome sequence to determine the gene they are part of and if the protein made in the yeast colony is the same as the endogenous protein. Then colonies were re-tested for beta-galactosidase expression and the genes compared against microarray data to determine gametophytic expression. Red arrows indicated methods performed using a computer program, while blue arrows are experiments performed with the yeast, and green arrows were performed manually in excel.

Table 2: Genes identified by Yeast Two Hybrid screening with the Short N terminal fragment of HAP2.

<i>Gene Number</i>	<i>Unique clones</i>	<i>Category</i>	<i>Gene Name</i>
AT4G03520	14	thio	ATHM2 thioredoxin M-type 2; thiol-disulfide exchange mediator
AT2G04700	12	thio	ferredoxin thioredoxin reductase catalytic beta chain family protein
AT5G60390	7	transl	calmodulin binding / translation elongation factor
AT1G67090	4	chloro	ribulose biphosphate carboxylase /RuBisCO small subunit 1A CXIP2 (CAX-INTERACTING PROTEIN 2); electron carrier/
AT2G38270	4	chloro/thio	protein disulfide oxidoreductase
AT1G47128	4	thio	cysteine proteinase (RD21A) / thiol protease
AT4G22240	3	memb	plastid-lipid associated protein PAP, putative
AT4G33520	3	metal	PAA1 (metal-transporting P-type ATPase 1)
AT4G04460	3	protease	aspartyl protease family protein
AT4G21860	3	thio	protein-methionine-S-oxide reductase
AT5G38430	2	chloro	ribulose biphosphate carboxylase/RuBisCO small subunit 1B
AT1G79040	2	chloro	PSBR (photosystem II subunit R)
AT1G50900	2	unkn	similar to unnamed protein product
AT2G27880	2	unkn	argonaute protein, putative / AGO, putative
AT1G11430	1	chloro	plastid developmental protein DAG, putative
AT4G03280	1	chloro	PETC (PHOTOSYNTHETIC ELECTRON TRANSFER C)
AT5G27380	1	chloro	GSH2 (GLUTATHIONE SYNTHETASE 2); glutathione synthase
ATCG00220	1	chloro	PSII low MW protein
AT4G12060	1	chloro	Clp amino terminal domain-containing protein
AT5G45950	1	memb	GDSL-motif lipase/hydrolase family protein
AT2G40610	1	memb	ATEXPA8 (ARABIDOPSIS THALIANA EXPANSIN A8)
AT5G59370	1	memb	ACT4 (ACTIN 4)
AT3G13750	1	memb	BGAL1 (BETA GALACTOSIDASE 1); beta-galactosidase
AT2G28190	1	metal	CSD2 copper, zinc superoxide dismutase 2
AT5G19580	1	thio	glyoxal oxidase-related
AT5G54760	1	transl	eukaryotic translation initiation factor SUI1, putative
AT1G07940	1	transl	calmodulin binding / translation elongation factor
AT4G26530	1	unkn	fructose-bisphosphate aldolase, putative
AT4G39130	1	unkn	dehydrin family protein
AT5G19370	1	unkn	rhodanese-like domain-containing protein
AT1G49170	1	unkn	similar to unknown protein with DUF167
AT3G07090	1	unkn	similar to unknown protein with DUF862

These genes were confirmed to make an in frame endogenous protein fragment and were able to produce beta-galactosidase through the interaction of the activation and DNA binding domains of Gal4. They are listed with the greatest number of unique clone fragments at the top indicating these were found more. Thio=thioredoxin related, memb=membrane or cell wall altering, metal=metal binding, unkn=unknown function, transl=translation, chloro=chloroplast localization, and protease=protein degradation function.

A: Number of genes in each category



B: Number of unique clones in each category

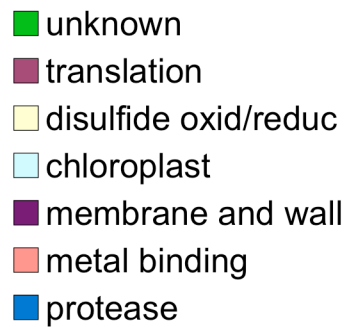
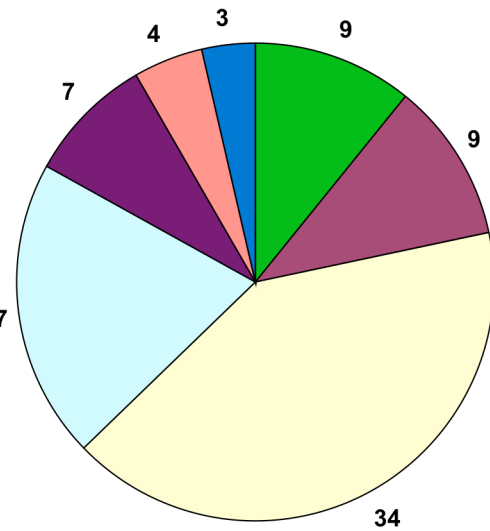


Figure 5: Genes found to interact with the HAP2 amino terminus by category.

A) Graph of number of genes per category found in the yeast two hybrid screen. The greatest number of genes found. B) Graph of number of unique clones found in the yeast two hybrid grouped by category. These are only the clones that are in frame and express beta-galactosidase.

Table 3: Genes found through Yeast Two Hybrid assay that show pollen, sperm or ovary specific expression

<i>Gene number</i>	<i>Expression change</i>	<i>Gene name</i>
AT2G04700	Down in pollen ¹	ferredoxin thioredoxin reductase
AT5G27380	Down in pollen ¹	Encodes a protein with similarity to glutathione synthetases
AT4G21860	Down in pollen ¹	methionine sulfoxide reductase domain-containing protein
AT5G19370	Down in pollen ¹	rhodanese-like domain-containing protein
AT2G28190	Down in pollen ¹	chloroplastic copper/zinc superoxide dismutase
AT5G19580	Up in pollen ¹	glyoxal oxidase-related
AT5G59370	Up in pollen ¹	ACT4 (ACTIN 4), reproductive actin subclass
AT2G27880	Up in ovaries ³ , sperm selective ²	argonaute protein, putative
AT3G07090	Sperm enriched ²	similar to unknown protein

All thirty-two of the yeast two hybrid hits were compared against microarray data from assays on sperm (Borges et al., 2008), pollen (Qin et al., 2009), and female gametophyte (Yu et al., 2005) experiments. Those genes that showed a change in expression from vegetative tissues are indicated in the table along with which microarray study showed the change. Qin et al., 2009 by a ¹; Borges et al, 2008 by a ²; and Yu et al, 2005 by a ³.

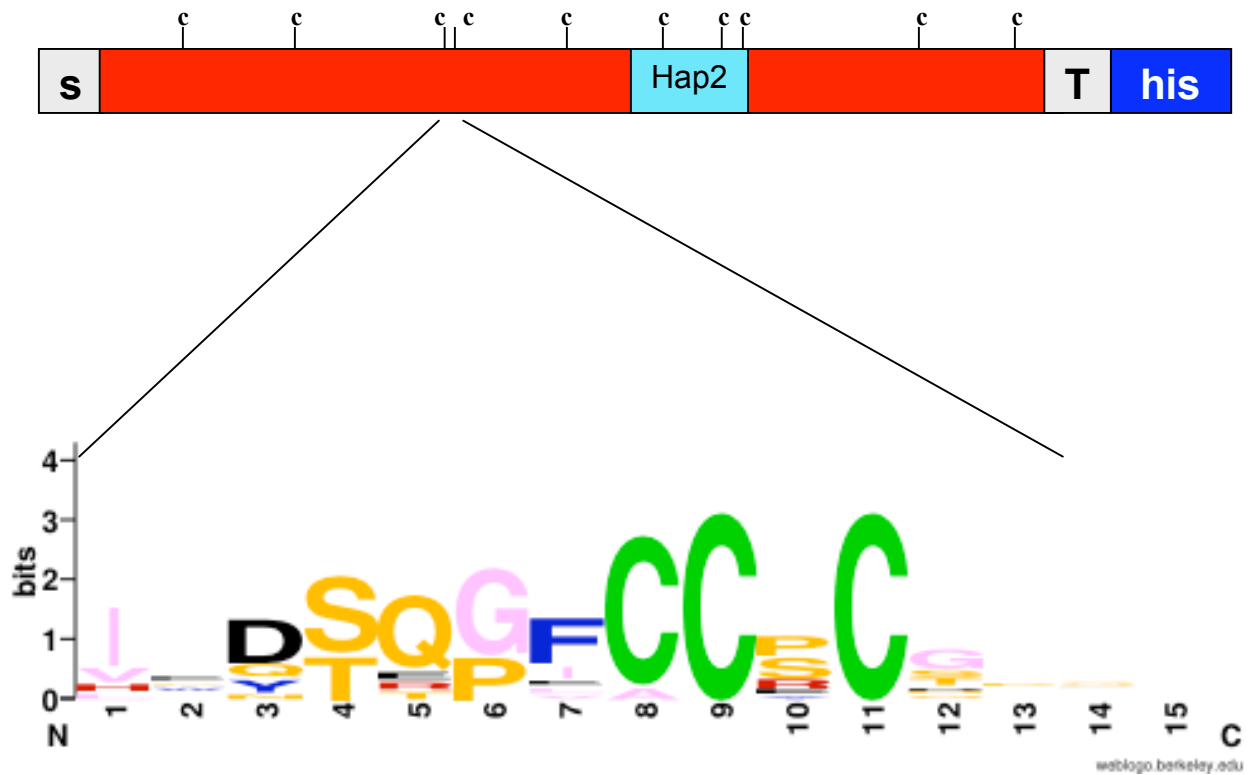


Figure 6: Highly conserved region of HAP2 with cysteine motif.

Pictorial representation of the region of HAP2 that is highly conserved among the 12 species aligned and which has a motif similar to known thioredoxin proteins of CXXC. The CXXC is only conserved in plant species where the CCPC is maintained.

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Chapter 4: Ectopic expression of *HAP2(GCS1)* provides insight into its regulation, localization, and mode of action

I performed all the experiments that make up the data for this chapter. The constructs in this chapter were cloned by Mark Johnson and Sophia Tintori. The Northern blot of Figure 2, Mark Johnson was kind enough to run with me and blot for me. I am very grateful to Alison DeLong for her help with Western blots.

Abstract:

In flowering plants, two sperm are delivered to female gametes by a pollen tube. Sperm develop within the pollen grain and carry the same haploid genome as the pollen cell. Pollen tubes grow by tip growth through female floral tissue and use guidance cues to reach an ovule where the female gametes develop. The mechanisms responsible for fusion of the two sperm with the female egg and central cell are unknown. We propose that HAP2, a sperm-specific transmembrane protein, is directly involved in gamete membrane fusion. To test models for this function of HAP2 we expressed HAP2 in the cytoplasm of the pollen tube, in cells adjacent to the egg and throughout the plant. Preliminary results from these experiments suggest that HAP2 expression and localization may be under a variety of regulatory controls. By expressing HAP2 in the pollen cytoplasm, with the Lat52 promoter, we were able to rescue the fertilization defect of *hap2-2* which lacks endogenous HAP2 in the sperm and which does not transmit through the male.

Introduction:

HAP2 is critical for fertilization by male gametes

The HAP2(GCS1) protein was shown in various species to be critical for fertilization with a proposed role in membrane fusion. *Plasmodium falciparum* and *Arabidopsis thaliana* (Arabidopsis) lacking HAP2 do not fertilize or transmit the mutation to the next generation (Hirai et al., 2008; Liu et al., 2008; Blagborough and Sinden, 2009; von Besser et al., 2006). In *Chlamydomonas reinhardtii* the minus and plus gametes come into close contact through their mating projections, but without HAP2 their plasma membranes do not fuse (Liu et al., 2008). This observation brings up questions of how HAP2 mediates membrane fusion and whether it acts alone or in combination with other proteins either on the female gamete or within the male gamete. It also brings up questions of how the gene expression pattern can remain in the male gamete within multiple species with very different gamete developmental programs.

To study the role of HAP2 in membrane fusion and determine whether HAP2 is sufficient to induce fusion by itself we undertook ectopic expression studies in Arabidopsis (del Campo et al., 2005). We expected that by expressing HAP2 in a different cell type we could induce fusion of neighboring cells if HAP2 were the only protein required for the fusion process. By expressing HAP2 in a cell next to the female gametes we expected that we could induce fusion of these specific cells if factors on the female are also required for fusion.

HAP2 expression is restricted to the sperm and sperm precursor cells

A protein fusion between HAP2 and YFP showed that HAP2 is expressed only in the sperm cells late in development (von Besser et al., 2006 and Figure 4E). This

expression pattern was confirmed by the expression of the potent translation inhibitor Diphtheria Toxin A subunit (DTA) which blocks protein synthesis and leads to apoptosis in most cell types (Day et al., 1995). When DTA was expressed by the *HAP2* promoter it disrupted sperm development after DNA replication in the sperm precursor cell, but had no effect on other cell types within the plant (Frank and Johnson, 2009). This indicates a very tight regulation on the expression pattern on *HAP2* since a single protein of DTA can ablate a large number of cells in culture (Yamaizumi et al., 1978).

In plants the male gamete expression pattern of *HAP2* is regulated by two MYB transcription factors, *DUO1* and *DUO3*, that influence *HAP2* and other sperm specific proteins (Brownfield et al., 2009a; Brownfield et al., 2009b). *DUO1* is regulated by a small RNA-mediated mechanism with miRNA 159. Micro RNA 159 is made from four separate loci in Arabidopsis that produce a similar RNA product that can bind the *DUO1* transcript and degrade it in tissues outside of the male gametophyte. *DUO1* also regulates the expression of other sperm specific genes including the H3 histone variant H3.3 (Ingouff et al., 2007) and *Gex2*, which has an unknown function (Engel et al., 2005).

***HAP2* is involved in pollen tube guidance in Arabidopsis**

When pollen that is heterozygous for the *hap2-1* mutation (50% *HAP2*, 50% *hap2-1*) is used to pollinate a wild-type pistil, mutant pollen tubes are half as likely as wild type to reach an ovule and burst (von Besser et al., 2006). *hap2-1* mutant pollen tubes do not have slower rates of extension in vitro or in vivo, so the difference in ability to target ovules is attributed to a defect in pollen tube guidance (von Besser et al., 2006). *HAP2* expression has so far only been detected in sperm, so these observations suggest

that sperm-resident HAP2 may be interacting with factors within the growing pollen tube as it is navigating female tissue and responding to guidance cues (von Besser et al., 2006). During tube extension, the sperm are located ~20-50 μm behind the tip, where localization and activation of ROP (Rho-like GTPases) determines tube direction (Lin reference). So, it is not yet clear how sperm-resident HAP2 could play a role in signaling events resulting in changes in pollen tube direction in response to guidance signals.

Current goals to explore the function of HAP2 through biochemical experiments are hampered by the low-level expression of HAP2.

Although it is possible to extract sperm from pollen tubes, it is not trivial to isolate enough to perform the types of biochemical experiments that would lead us to an understanding of the function of HAP2. Nor is it yet possible to express full length HAP2 in an exogenous expression system such as bacteria and obtain a large quantity of correctly folded HAP2. If we could express HAP2 to higher abundance in plant cells we might extract sufficient protein to perform studies of HAP2 by 1) immunoblot and 2) immuno-precipitation to identify HAP2 binding partners and in a larger cell type we could perform biological assays to determine HAP2 localization patterns. To obtain a large amount of HAP2 protein, we decided to express HAP2 in the vegetative cells of the plant with the Cauliflower Mosaic Virus 35S promoter in the hope of acquiring a large quantity of protein for biochemical studies and to observe where HAP2 localizes.

Expression of HAP2 within the sperm should rescue the *hap2-1* fertilization defect, but expression in the pollen grain alone is not expected to

DUO1 influences the expression of HAP2 as well as other sperm specific genes (Brownfield et al., 2009a), one of which is the histone variant H3.3, but H3.3

accumulates at a higher level than HAP2. By exchanging the HAP2 promoter for the H3.3 promoter we most likely can rescue the null *HAP2* mutants *hap2-1* and *hap2-2*.

Our model has been that HAP2 must be in the sperm membrane in order for the sperm to fuse with the egg or central cell. Mutants that lack HAP2, such as *hap2-1* and *hap2-2* do not fuse with either female gamete (von Besser et al., 2006), nor do single sperm-like cells that lack HAP2 (Frank and Johnson, 2009). We expect that expression of HAP2 in the pollen grain and tube, outside of the sperm, from the Lat52 promoter would not rescue the fertilization defect of a null *hap2-2* mutant. We also expected based on the signal sequence of HAP2 that it would localize to the pollen tube membrane (Figure 3B). HAP2 in this location might actually cause a dominant negative effect on fertilization under wild type conditions. If the exogenous HAP2 from the pollen tube is able to bind with the proteins on the female gametes it could block the interaction of the endogenous HAP2 and not allow fertilization.

Expression of the amino terminal region of HAP2 in the pollen grain could cause a dominant negative affect

If our hypothesis that the amino terminus of HAP2 (HAP2 N) is on the outside of the sperm cell and interacts with some factor from the female gamete is correct, it is possible that we could disrupt this interaction by expressing soluble HAP2 N. To accomplish this within the ovules, which are hidden deep within the pistil, we expressed the HAP2 N in the pollen tube from the LAT52 promoter. Pollen tubes bust in the ovule and release their cytoplasm along with the sperm within the ovule. If HAP2 N then binds to the female gamete factors that interact with HAP2 normally we might interfere with fertilization.

Expression of HAP2 on cells adjacent to the female gametes might cause membrane fusion between these cells if HAP2 is sufficient to induce fusion with female gametes.

Fusogens are proteins that induce fusion directly and without binding with another protein, such as EFF-1 in *Caenorhabditis elegans*, which fuses epithelial cells during development (Shemer et al., 2004) and which is capable of fusing cells that do not normally fuse when ectopically expressed (Kontani, 2005). Viral fusogens were shown to interact alone or as multimers of the same protein to cause fusion (Shmulevitz and Duncan, 2000). If HAP2 acts alone as a fusogen to cause the sperm to fuse with the female gametes it is possible that fusion could occur between any two cells when HAP2 is present, such as the two sperm next to each other within the pollen grain. Since we do not see this occurring normally, it stands to reason that something on the egg and central cell is required to work with HAP2 to cause fusion. But if HAP2 was expressed in a cell next to female gametes, such as in the synergid cell, it is possible that we could cause fusion of these cells if HAP2 is the only protein required from the male gamete for fusion.

The *MYB98* gene was shown to be specifically expressed in the synergid cells of the female gametophyte (Kasahara et al., 2005), which are adjacent to both cells sperm fuse with, the egg and the central cell. So to express HAP2 next to the female gametes we expressed the full length *HAP2* cDNA from the *MYB98* promoter. We expected that if the female gametophyte of these plants was disrupted by either the synergid cells fusing with each other or with the egg or central cell that transmission would be decreased and that these defects would be visible by microscopy.

By expressing HAP2 outside of the sperm in various cell types we expect to increase our understanding of HAP2 function

Sperm have a specific task and are terminally differentiated cells with a specialized expression pattern. Through ectopic expression of HAP2 we can understand better the function and specific expression of HAP2. It is also our hope that through understanding of HAP2 we can learn about the role of sperm in fertilization and pollen tube guidance. By ectopically expressing HAP2 in vegetative tissue we have determined that the regulation of HAP2 is not simple and that various mechanisms could be acting on gene expression or protein translation to ensure that HAP2 is only found in the male gametophyte. Contrary to our expectations expression of HAP2 or parts of HAP2 in the pollen grain does not block endogenous HAP2 from fertilizing, but full length HAP2 in the pollen tube is able to rescue a lack of HAP2 in the sperm.

Results:

35S:HAP2:YFP transgenic plants do not express HAP2 in vegetative tissues

We attempted to express HAP2 to high levels throughout somatic cells of transgenic *Arabidopsis* plants using the strong cauliflower mosaic virus 35S promoter (Szwacka, 2009) (Benfey B.B.C.). Our goal was to express high levels of the protein to facilitate analysis of HAP2 protein function and to refine our view of the subcellular localization of HAP2. We generated a construct in which HAP2:YFP protein fusion was driven by the 35S promoter (35S:HAP2:YFP). When *qrt* plants were transformed with a control construct consisting of the 35S promoter driving expression of YFP (35S:YFP) the entire vegetative plant tissue fluoresced as expected (Figure 1 A). We analyzed 22 independent 35S:HAP2:YFP transgenic plants; none showed fluorescence (Figure 1) in any of the tissues we analyzed (Table 1).

The sequence of the 35S:HAP2:YFP construct was correct (not shown) and we confirmed that the DNA was present in transgenic plants using PCR (not shown). The only difference between this construct and the abundantly expressed 35S:YFP control construct was insertion of the HAP2 coding sequence in frame with YFP. We then investigated whether the transgenic plants made any protein by running a Western blot on total protein extracts from control and 35S:HAP2:YFP transgenic plants. Blots were probed with antibodies raised against GFP; an abundant protein of the predicted size (~27 Kd) was detected in 35S:YFP extracts, but none of the 35S:HAP2:YFP samples showed any protein (Figure 2).

Since there was no protein visible either by fluorescence or Western blot we assayed for accumulation of HAP2:YFP RNA by Northern blot. RNA was extracted

from whole young seedlings and probed with a full-length YFP probe. As in the immunoblot, 35S:YFP plants showed high expression and all the 35S:HAP2:YFP transgenics had no trace of RNA at the expected size (Figure 2 B). Considering that the full transgene is present, but no transcript or protein is found in any of the 35S:HAP2:YFP transgenic plants analyzed so far, we conclude that the transgene is being silenced at the RNA level.

Many of the primary transgenic plants were single insertion site transgenes based on the transmission frequency (Table 1) where the transgene is inherited by $\frac{3}{4}$ of the progeny as expected for a single locus insert. Although it is possible that there were multiple copies of 35S:HAP2:YFP in some lines, it is unlikely that so many different transgenes would be silenced because of complex T-DNA insertions. This seems especially unlikely given the ease with which we identified 35S:YFP transgenic plants..

HAP2 expression in the sperm from either the HAP2 promoter or the H3.3 promoter shows the same localization

We expressed the full length HAP2 cDNA from the HAP2 promoter to test whether the cDNA is able to complement the *hap2-1* null mutation just as the full length genomic construct can ((von Besser et al., 2006) and Chapter 1). We also expressed the HAP2 cDNA from the strong sperm specific Histone H3.3 promoter to test whether over expression of HAP2 would have a negative effect on fertilization. Expression of the HAP2:HAP2:YFP transgene has the same expression pattern and similar fluorescence levels (Figure 3B and 3F) as a construct characterized previously that carries the full genomic HAP2 gene with promoter and introns, which was shown to be functional by complementation of the *hap2-1* defect (von Besser et al., 2006), Chapter 1 Figure 6. This same expression pattern within the sperm is also seen with the H3.3:HAP2:YFP construct

(Figure 3 F and G), but this promoter is a little stronger. Both the HAP2 and H3.3 promoters are under DUO1 regulation and it seems likely that they would induce a similar expression pattern (Brownfield et al., 2009b). There were areas of brighter fluorescence in the H3.3:HAP2:YFP (Figure 4F) sperm than in the HAP2:HAP2:YFP (Figure 4E), which is consistent with the H3.3 promoter being a stronger promoter (Ingouff et al., 2007). The strength of fluorescence is variable between transgenic lines and between generations (data not shown). The puncta in the H3.3:HAP2:YFP sperm are cytoplasmic (Figure 5E) while there is still a large quantity at the sperm plasma membrane that is not clumped.

Single insertion site transgenic lines for both of these constructs show no defect in transmission of the transgene in the *qrt* background when self pollinated (Table 2). These transgenes were inserted in the *qrt* background, so there is wild-type HAP2 present as well as the transgene. We are currently analyzing these constructs for their ability to rescue the fertilization defect when there is no endogenous HAP2, with both the *hap2-1* or *hap2-2* alleles. The expression profile of either HAP2:HAP2:YFP or H3.3:HAP2:YFP does not change when there is wild type HAP2 present or not (Figure 5E,F compared with Figure 7B,C). From these experiments we can conclude that neither the HAP2:HAP2:YFP or the H3.3:HAP2:YFP constructs interfere with the endogenous HAP2, even when over expressed. We can also see that the H3.3 promoter is stronger than the HAP2 promoter and that when there is extra HAP2 puncta are formed either as protein aggregates or in vesicular structures.

Expression of HAP2 from the pollen-specific LAT52 promoter rescues the *hap2-1* fertilization defect

We attempted to express the HAP2 cDNA in the pollen grain to look at cellular localization in a cell much larger than sperm and to determine if we could rescue the pollen tube guidance defect of sperm lacking HAP2. Expression of the Lat52:YFP construct is restricted to the pollen grain and tube cytoplasm (Figure 4A and 5B) and is restricted from the sperm (Figure 5B). Expression of the Lat52:HAP2:YFP construct produces bright foci of fluorescence unlike the diffuse fluorescence of the Lat52:YFP pollen (Figure 4B compared to 4A). This localization is similar to the puncta seen in H3.3:HAP2:YFP sperm, but there is not the diffuse localization seen in the H3.3:HAP2:YFP. These puncta are either a protein aggregate or due to being localized into a cellular compartment. It is more likely that this is a membrane containing entity since the full length HAP2 has the transmembrane domain and would likely be restricted to a membranous compartment such as an endosome or secretory vesicle. Ectopic HAP2 might still be functional even within these punctuate structures since endogenous HAP2 on the sperm is able to effect pollen tube guidance when sequestered to the male germ unit (von Besser et al., 2006).

The LAT52 promoter is not active in the sperm (McCormick et al., 1991). The exclusion from the sperm can be seen in Figure 5B where the Lat52:YFP construct shows dark spots within the pollen tube that correspond to where the two sperm are located by DAPI staining. This same exclusion from the sperm is seen with the Lat52:HAP2:YFP construct, although in this case fluorescence is not conclusive to say there is a complete lack of ectopic HAP2 in the sperm. It does not seem to be strong expression within these sperm from the Lat52 promoter (Figure 4B).

To identify the organelle to which the puncta of HAP2 are localizing we crossed in the Lat52:CFP-PTS1 marker that localizes to the peroxisomes (Nowak et al., 2004). The fluorescence of HAP2:YFP and CFP-PTS1 overlap to a certain degree in pollen tubes (Figure 8). They are not complete in their co-localization indicating that although some of the HAP2 is localized to peroxisomal organelles, some is in different organelles or in other peroxisomes that the PTS1 domain does not target.

When the Lat52:HAP2:YFP construct was crossed into *hap2-1* and *hap2-2* it was able to rescue the fertilization defect (Table 4) and allow for the *hap2-2* T-DNA to become homozygous (Figure 9). The localization within the pollen remained the same as when endogenous HAP2 was present (Figure 4B) and still seemed to be excluded from the sperm visually (Figure 7). The *hap2-2* allele with a kanamycin resistance gene allowed us to track both the inheritance of the *hap2-2* and Lat52:HAP2:YFP constructs simultaneously (Figure 9). To confirm that these plants contained the *hap2-2* allele and to determine if individuals were homozygous for the mutation we performed PCR on a selection of seedlings and found that all kanamycin resistant seedlings had the *hap2-2* allele and four of the eight F3 generation plants were homozygous by PCR for *hap2-2* (data not shown).

Expression of the amino terminus of HAP2 in the pollen grain results in localized protein accumulation, but does not affect fertilization

By expressing the amino and carboxy domains of HAP2 in the pollen tube we were attempting to determine which regions of the HAP2 protein are involved in pollen tube guidance. When the amino terminus of HAP2 is expressed from the LAT52 promoter in pollen grains it has a punctuate localization that the carboxy terminus of

HAP2 does not show (Figure 4C and D). This was not as expected (Figure 6), but is consistent with the finding that the Lat52:HAP2:YFP fusion is localized in puncta. Figure 4 shows in pollen grains that although not as pronounced as the full length HAP2, the amino terminus has some bright puncta (Figure 4C) and the carboxy terminus has a diffuse fluorescence throughout the pollen grain (Figure 4D). Neither construct has the transmembrane domain nor the signal sequence, but the amino terminus of HAP2 contains many of the completely conserved cysteine amino acids that might cause HAP2 to clump together when not properly localized or folded (Chapter 3). It has yet to be determined if the puncta in the Lat52:HAP2 N:YFP are the same organelle or method of association as the Lat52:HAP2:YFP puncta.

Neither of these constructs causes a dominant negative effect on fertilization. Transmission of the constructs in self crosses causes no reduction in transmission (Table 3) or in seed set (data not shown), indicating that the presence of either region of HAP2 alone is insufficient to block the normal interaction of the sperm and female gametes.

We have not identified transgenic plants that express HAP2 from the synergid cell specific promoter *MYB98*

We attempted to express the HAP2 cDNA in the synergid cells of ovules to test whether the sperm are unique in their ability to fuse with the egg and central cell. If HAP2 is the only factor required from the male germ line to promote fusion with the female gametes, then synergid cells expressing ectopic HAP2 should fuse with the female gametes. Neither the MYB98:YFP nor the MYB98:HAP2:YFP constructs show bright fluorescence in mature ovules in any of the seven single insertion site lines we have tested (data not shown). Controls from Gary Drews were obviously fluorescent in both

synergid cells of ovules before fertilization. Further transformations are needed to determine if these plants have a similar problem expressing as the 35S:HAP2:YFP lines or if there is a problem with the promoter.

Discussion:

Expression of HAP2 throughout the plant is not feasible with the 35S promoter

There are three different possibilities for the issues with expression from the 35S:HAP2:YFP construct in multiple primary transformants. First, the construct itself is corrupted and the promoter is not able to express, but the promoter is the same for both the YFP alone and when fused to HAP2, so this is unlikely. Second, expression of HAP2 within the embryo is lethal or detrimental to growth and so any transformant that was viable had the construct silenced. And third, there is a mechanism in tissues outside of the gametes silencing expression of HAP2, possibly through methylation or RNA degradation. DUO1 is regulated by a small RNA-mediated mechanism with miRNA 159 (Reyes, 2007). HAP2 does not share any sequence similarity with miRNA 159 or any other known small RNA fragment (TAIR, data not shown). It does share sequence identity with a part of the looped region of the miRNA 159 stem-loop structure, although this region is not included in the final miRNA and would likely be degraded during hairpin processing.

Tight regulation of HAP2 might be necessary to ensure that cells near each other do not fuse

The expression of HAP2 is very tightly controlled endogenously. When DTA was expressed from the HAP2 promoter we saw that the expression of HAP2 was limited strictly to the generative cell and its two descendant sperm (Frank and Johnson, 2009). This tight regulation could be in place to ensure that only sperm cells are capable of fusing with other cells. However, this does not answer the question of why the two sperm, which are in close proximity within the pollen grain, do not fuse together or with

the pollen grain. The two sperm are in close proximity within the pollen tube and move as a unit with the pollen grain nucleus, so must be held together in some way. It could be that a protein on the female gametes is required to promote fusion, and this interaction could be the reason we are not finding any transformants expressing either the 35S:HAP2:YFP or the Myb98:HAP2:YFP. If HAP2 is expressed in the female gametophyte when it is delivered by the *Agrobacterium* transformation and the fusion of female cells with one another produces inviable seeds, it is possible that only those transformants which silence the transgene are able to make seeds. It is also possible that HAP2 is prone to silencing, and we have not looked at enough lines to find ones that express the HAP2:YFP constructs. Alternatively it is possible that the transgenes are corrupted and unable to express from the 35S or Myb98 promoters.

HAP2 forms puncta in pollen grains and sperm when over expressed

The puncta seen in the Lat52:HAP2:YFP, Lat52:HAP2 N:YFP and H3.3:HAP2:YFP transformants do not seem to affect function, but are a perplexing phenomenon. It is possible that these are only a consequence of over expression since the H3.3 and Lat52 promoters are stronger than the HAP2 promoter. This is also supported by the variability of puncta between different transgenic lines and different generations. The puncta might be due to the large amount of HAP2 blocking the secretory pathway from delivering the entirety of HAP2 to the plasma membrane. The failure of Lat52:HAP2 C:YFP to make these puncta may indicate that it is the amino terminus that causes puncta formation when in overabundance. It is also possible that the puncta represent the accumulation of proteins destined for degradation because there is too much of the protein as might be the reason some are seen in peroxisomal organelles, the

presence of the bright foci makes visualization of a more diffuse subset of protein more difficult. There is also the possibility that the extra HAP2 is getting trapped in the secretory pathway because it is not properly disulfide bonded. The enzymes that fold HAP2 correctly fold and process the protein might be incapable of processing large amounts of HAP2 and the excess is seen as puncta.

HAP2 expression in pollen rescues the *hap2-1* fertilization defect

Our observation that expression in the pollen tube rescues this defect was unanticipated. The LAT52 promoter is highly active in the pollen grain and pollen tube (Twell et al., 1989), but expression is not thought to occur in the sperm cells (McCormick et al., 1991). Fluorescence visualization seems to corroborate this finding and shows a lack of fluorescence within the sperm cells (Figure 5B). Explanations of how HAP2 in the pollen grain affects the sperm include the following: HAP2 is entering the sperm either as mRNA or protein; the puncta of HAP2 that accumulate in the pollen tube cytoplasm are able to mediate sperm fusion when they are released into the ovule along with sperm; or that HAP2:YFP is cleaved at some stage and the free floating HAP2 fragment released from the punctate structures and delivered by the pollen tube cytoplasm is able to interact with the sperm and female gametes to allow for fertilization.

It is possible that the HAP2 transcript or protein migrates into the sperm during pollen development since the Lat52 promoter is expressed at the two cell stage of pollen development (Twell et al., 1998) before the two sperm divide. It was shown with a small RNA that expression from the Lat52 promoter was able to knock down expression within the sperm, suggesting that the small RNA was transported into the sperm (Slotkin et al., 2009), but the HAP2 transcript is much larger and is not involved in the RNA

interference process that might assist movement between the pollen grain and sperm. However, it is possible that a very small amount of HAP2 is enough to rescue the fertilization defect as the DTA studies showed.

It is also possible that the HAP2 within the puncta structures is released into the ovule when the pollen tube bursts and that this allows an interaction between the sperm, female gametes, and HAP2 from the pollen grain, thus promoting fusion. This release of HAP2 can either be within membrane packets or cleaved in some manner to release a part of the HAP2 protein. It was recently shown that CD9 in mammals can promote fusion in vitro of mutant eggs if wild type eggs were included in the media. The authors suggest this is from CD9 containing vesicles were able to bud off wild type eggs as exosomes and interact between mutant eggs and sperm to mediate fertilization, (Miyado, 2008). Another group was not able to repeat this experiment to the same efficiency (Gupta, 2009). Perhaps HAP2 is able to act in a similar manner to CD9 and interact with the sperm and egg from vesicle like puncta to mediate fusion.

Further experiments are required to determine which of these suppositions is occurring to allow the Lat52:HAP2:YFP construct to rescue the *hap2-1* and *hap2-2* mutations. By introducing the amino terminus of HAP2 (Lat52:HAP2 N:YFP) that makes similar puncta as the full length HAP2 in pollen grains we could test whether this region is enough to induce fertilization and test the hypothesis that HAP2 is being cleaved to allow fertilization. And to test whether the transcript or protein is entering the sperm during development we can introduce the Lat52:HAP2:YFP construct into HAP2:DTA plants ((Frank and Johnson, 2009) and Chapter 5). If the RNA transcript is entering the sperm from the pollen cell and replacing the HAP2 lacking in *hap2-2* sperm,

it is likely that the DTA construct will block the translation of that product and the double transgenic single sperm-like cells (SSLCs) will be just as likely to fertilize as the HAP2:DTA SSLCs alone. If the protein is being transported to sperm from the pollen cell, we would expect to see all of the SSLCs with fluorescence from the HAP2:YFP and that all of these would fertilize. And if the Lat52:HAP2:YFP puncta are mediating fusion without entering the sperm, we would see an increase in the ability of SSLCs to fertilize, but without the fluorescence of HAP2:YFP.

Further work is also needed to determine if the Lat52:HAP2:YFP construct or the amino or carboxy termini of HAP2 can rescue the pollen tube guidance defect in the *hap2-1* and *hap2-2* mutant alleles. My results indicate that the ectopic expression of HAP2 in different tissue types shows promise of elucidating mechanisms that regulate the function of HAP2 in fusion between the sperm and female gametes.

Materials and Methods:

Cloning of constructs

The full HAP2 cDNA was PCR amplified from a plasmid of the Arabidopsis cDNA with primers that included XmaI and SacI restriction sites (5'-atggtgaacgcgattttaatggc-3' Forward and 5' ctcttaactctcacgtagtc-3' Reverse). PCR fragments were cloned directly into the TOPO vector with A overhangs and then the HAP2 cDNA was excised with these restriction enzymes, purified and ligated into the Lat52:YFP construct (von Besser et al., 2006). This plasmid was then used to replace the Lat52 promoter with those of HAP2, H3.3, 35S, and Myb98 that were each PCR amplified with SalI and XmaI restriction sites. Plasmids were confirmed by restriction digest and sequencing to make sure the sequences were not corrupted.

HAP2 fragments were amplified from the same Arabidopsis HAP2 cDNA plasmid with primers for the amino terminus lacking the signal sequence and transmembrane domain or for the carboxy terminus. These were cloned into the Lat52:HAP2:YFP construct replacing the HAP2 with each of the two fragments.

Plant growth and transformation

Plasmids for each of the various promoters were transformed into *qrt* plants along with the pSOUP plasmid with Agrobacteria and primary transformants were selected either on media containing BASTA or on soil sprayed with BASTA. Arabidopsis (*Arabidopsis thaliana*) *qrt1* (Preuss et al., 1994) plants (Col-0 ecotype) were transformed by floral dip (Clough and Bent, 1998) and transformants were selected on MS (Murashige and Skoog) plates containing 50mg/ml BASTA (Johnson et al., 2004). Plants were grown in growth

chambers (Environmental Growth Chambers, Chagrin Falls, Ohio) at 21°C in 16 hr light (100 μ E), 8 hr dark.

Microscopy

Fluorescence of both pollen and root tips were visualized on a Zeiss Axiovert 200M fluorescent microscope (Carl Zeiss, Germany). Images were acquired with a Hamamatsu ORCA-ER or Zeiss AxioCam MRc5 camera.

Immuno and RNA gel blots

Whole flowers of the genotypes desired were collected and ground in liquid nitrogen and the extracts boiled in Laemli buffer before 10 μ g was loaded onto the 10% SDS-PAGE gel and run.

Acknowledgements:

We wish to thank A. DeLong and J. Bender for their advice on the Western and Northern blots respectively and for their generous contributions of reagents for these experiments. We would like to thank G. Drews for the Myb98:GFP plants, S. Tintori for her work trying to clone pieces of the constructs and to B. Leib and F. Jackson for their greenhouse expertise.

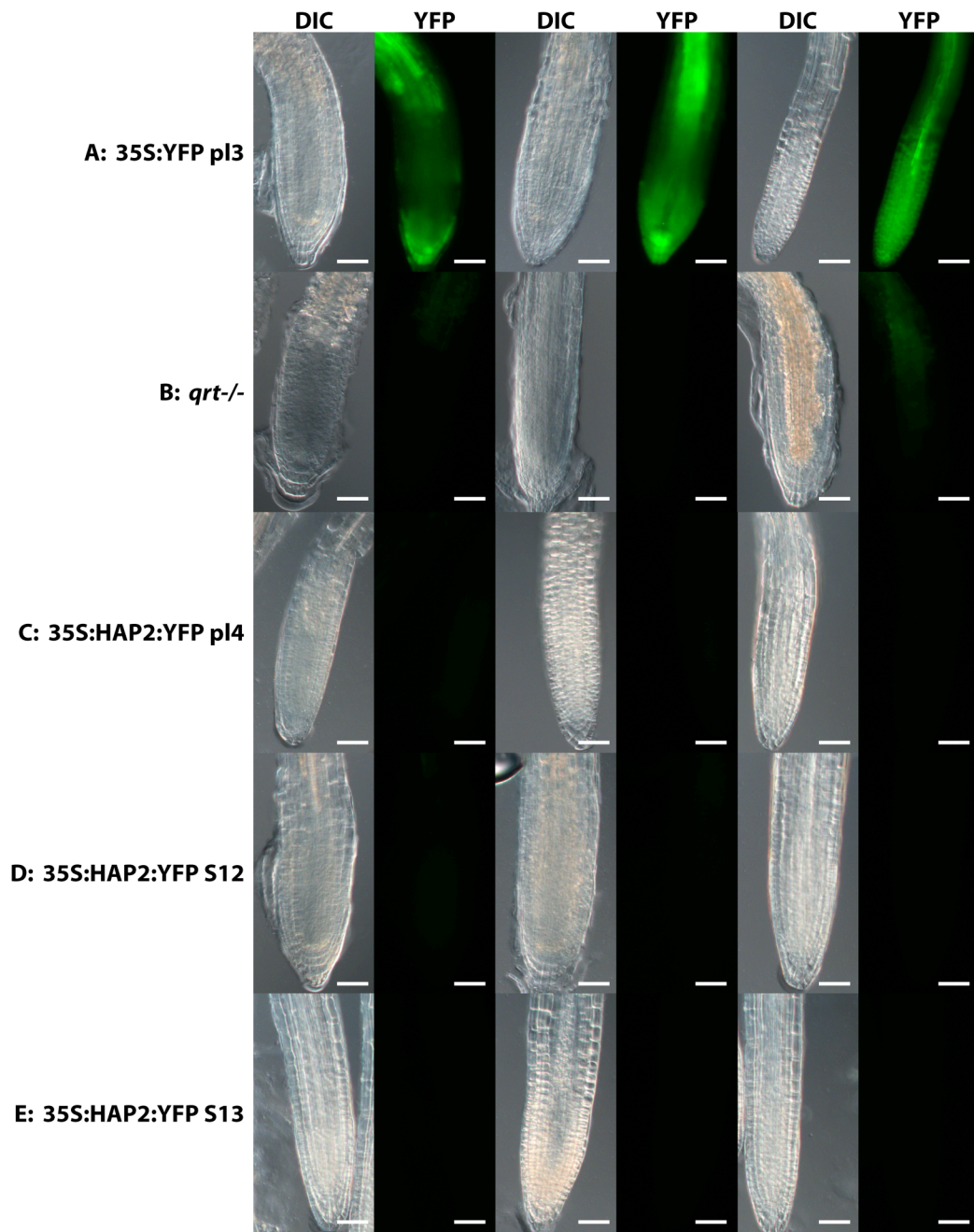


Figure 1: Fluorescent images of 35S:YFP and 35S:HAP2:YFP seedlings.
 Root tips of seedlings grown on plates containing BASTA and visualized on a fluorescent microscope at 20X magnification. Scale bar=50 μ m.

Table 1: Transmission efficiency in self-crosses of constructs with the 35S promoter for multiple lines with a wild-type background.

35S:HAP2:YFP		self cross		
line #	resistant	sensitive	% resistant	total
pl3	124	53	70%	177
pl4	202	66	75%	268
s1	148	36	80%	184
s3	142	51	74%	193
s8	102	110	48%	212
s9	138	41	77%	179
s10	121	54	69%	175
s11	129	55	70%	184

35S:YFP		self cross		
line #	resistant	sensitive	% resistant	total
s3	140	49	74%	189
s4	151	30	83%	181
s8	126	35	78%	161
s9	145	34	81%	179
pl3	78	24	76%	102

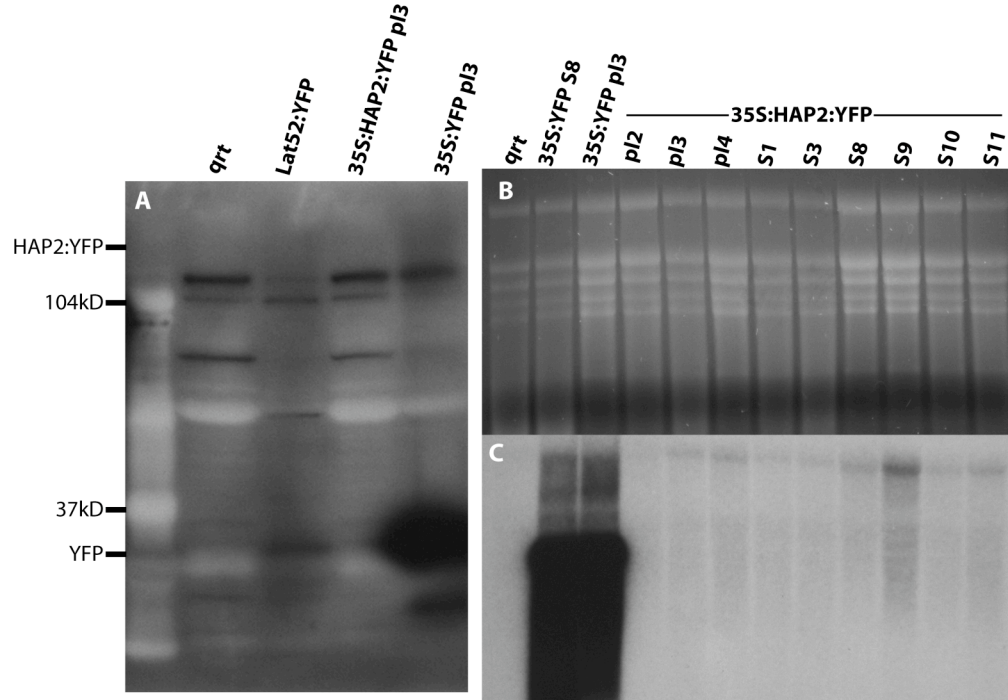


Figure 2: Western and Northern blot analysis of plants with the 35S promoter constructs.

(A) Western blot of protein from whole seedlings and leaf tissue from *qrt*, probed with the commercial GFP antibody ab290 (www.abcam.com). Protein standards are loaded on the left and relevant sizes are marked along with the expected sizes of YFP (~27kD) and the HAP2:YFP (~107kD) fusion. **(B,C)** Ethidium bromide stained and Northern blot of whole seedling RNA extracts from individual 35S:YFP or 35S:HAP2:YFP lines and the negative control of wild-type *qrt*.

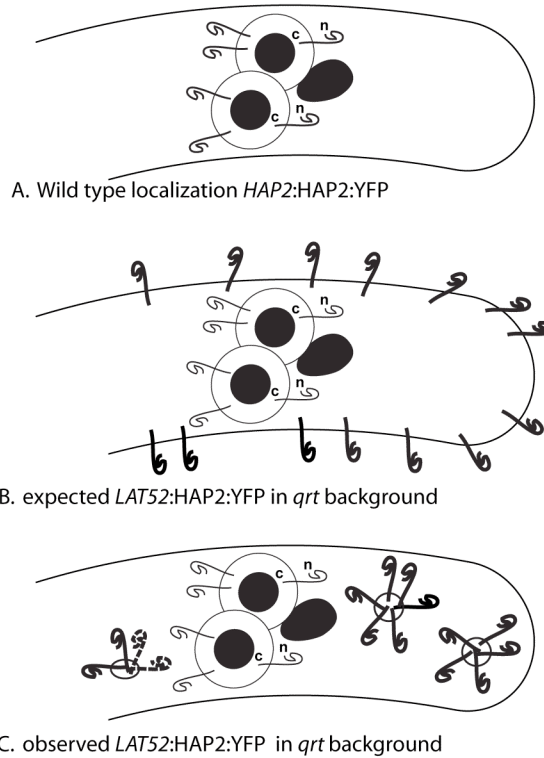


Figure 3: Model for expected and observed localization patterns in pollen tubes expressing Lat52:HAP2:YFP.

(A) Wild type localization of endogenous HAP2 in the sperm membrane as seen by a full genomic clone of HAP2 fused with YFP (von besser 2006 and Chapter 1). (B) Expected localization of Lat52:HAP2:YFP (dark) in the pollen tube membrane of *qrt* plants with endogenous HAP2 in the sperm (light). (C) Observed localization of Lat52:HAP2:YFP (dark) to punctate structures or vesicles within the pollen tube cytoplasm.

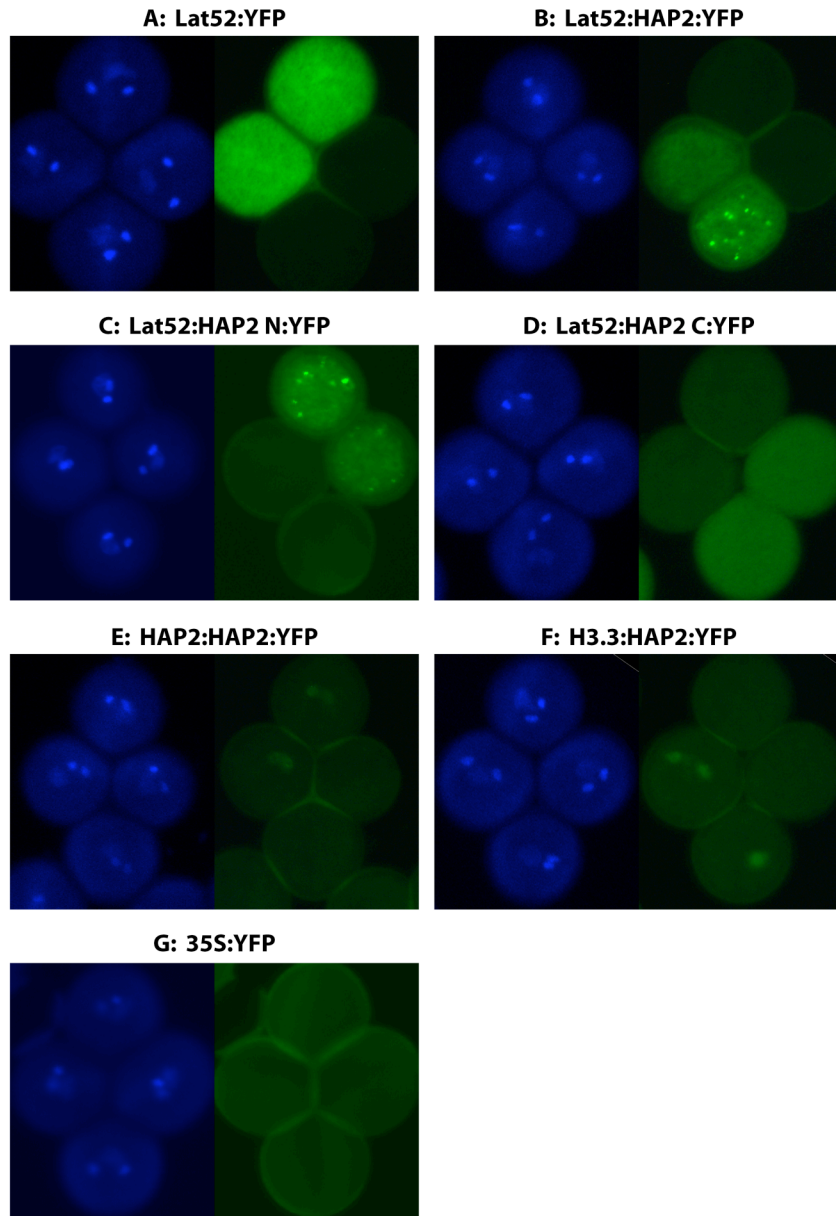


Figure 4: Fluorescence micrographs of pollen grain tetrads from heterozygous plants carrying various constructs expressing HAP2.

DAPI and green fluorescent images are shown for a representative tetrad of pollen grains for each construct. (A) Two pollen grains show fluorescence throughout the cytoplasm when YFP is expressed from the Lat52 promoter. (B) When YFP is expressed tethered to the HAP2 protein from the Lat52 promoter it forms puncta in the pollen grain. (C) Expression of just the amino terminus of HAP2 (HAP2 N) in the pollen cell produces puncta, but this is not seen when the carboxy terminus of HAP2 (HAP2 C) is expressed (D). (E and F) Expression of HAP2:YFP in the sperm by either the HAP2 promoter or H3.3 promoter shows the same localization, but fluorescence with the H3.3 promoter is stronger. (G) Although the 35S:YFP construct shows strong fluorescence in vegetative tissue and leaves, it is absent from pollen. All images are at the same magnification and the same exposure time for each fluorescence excitation.

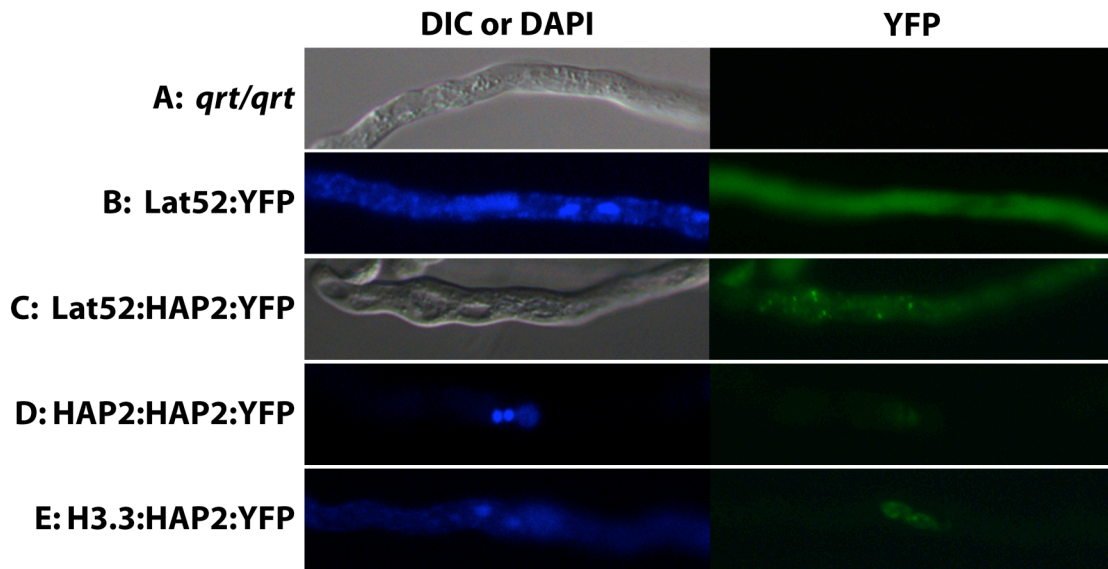


Figure 5: Pollen tubes showing fluorescence of ectopically expressed HAP2.

DIC or DAPI images are shown on the left for each construct depending on whether the sperm were in focus for the region that showed the best YFP fluorescence. All images are at the same magnification and exposure times are consistent across construct type for each fluorescence excitation. Wild type pollen show no autofluorescence at this exposure (A). Lat52:YFP shows fluorescence throughout the pollen tube cytoplasm, but lacks fluorescence where the sperm nuclei are by DAPI (B). Lat52:HAP2:YFP shows punctuate structures of fluorescence in the pollen tube (C). Both HAP2:HAP2:YFP and H3.3:HAP2:YFP show fluorescence in the sperm (D and E), but the H3.3:HAP2:YFP is slightly brighter and more punctuate (E).

Table 2: Transmission of lines that express the HAP2 cDNA in sperm for both self crosses and when ectopic pollen was crossed onto wild type pistils.

HAP2:HAP2:YFP								
self cross					ms1 X HAP2:HAP2:YFP			
line #	resistant	sensitive	% resistant	total	resistant	sensitive	% resistant	total
S6	111	34	77%	145	22	29	43%	51
S12	113	36	74%	189	40	37	52%	77
H3.3:HAP2:YFP								
self cross					ms1 X H3.3:HAP2:YFP			
line #	resistant	sensitive	% resistant	total	resistant	sensitive	% resistant	total
S1	137	46	75%	183	40	37	52%	77
#1	124	24	84%	148	42	39	52%	81

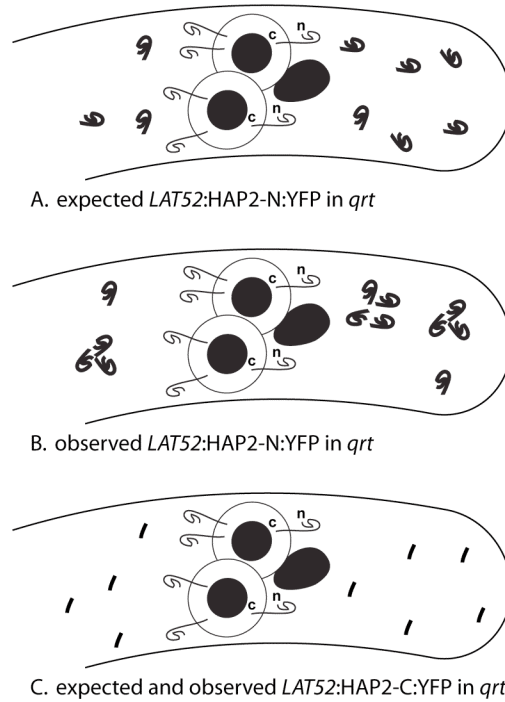


Figure 6: Model of expected and observed localization pattern in pollen of *Lat52:HAP2 N:YFP* and *Lat52:HAP2 C:YFP*.

(A) Expected localization of the amino terminus of HAP2 to the cytoplasm when expressed in the pollen tube cell. Endogenous HAP2 in the sperm is lighter while the ectopic HAP2 N is dark. (B) Observed localization to punctuate structures of the amino terminus of HAP2 in the pollen tube cell. This model also includes the observation that some pollen grains of lines with this construct show fewer puncta and a more diffuse localization. (C) Expected and observed localization of the HAP2 carboxy terminus when expressed in pollen tube cells diffuse throughout the cytoplasm and not punctate.

Table 3: Transmission of ectopic constructs expressing parts of the HAP2 protein within the pollen grain in self crosses

Lat52:HAP2 N:YFP								
self cross					ms1 X Lat52:HAP2N:YFP			
line #	resistant	sensitive	% resistant	total	resistant	sensitive	% resistant	total
S5	200	74	73%	274	59	52	53%	111
S9	198	78	72%	276	41	25	62%	66
S11	119	49	71%	168	N/A			
Lat52:HAP2 C:YFP								
self cross					ms1 X Lat52:HAP2 C:YFP			
line #	resistant	sensitive	% resistant	total	resistant	sensitive	% resistant	total
S6	171	71	71%	242	N/A			
S8	85	29	75%	114	N/A			
S14	133	47	74%	180	N/A			

Crosses that have not been done are denoted by N/A.

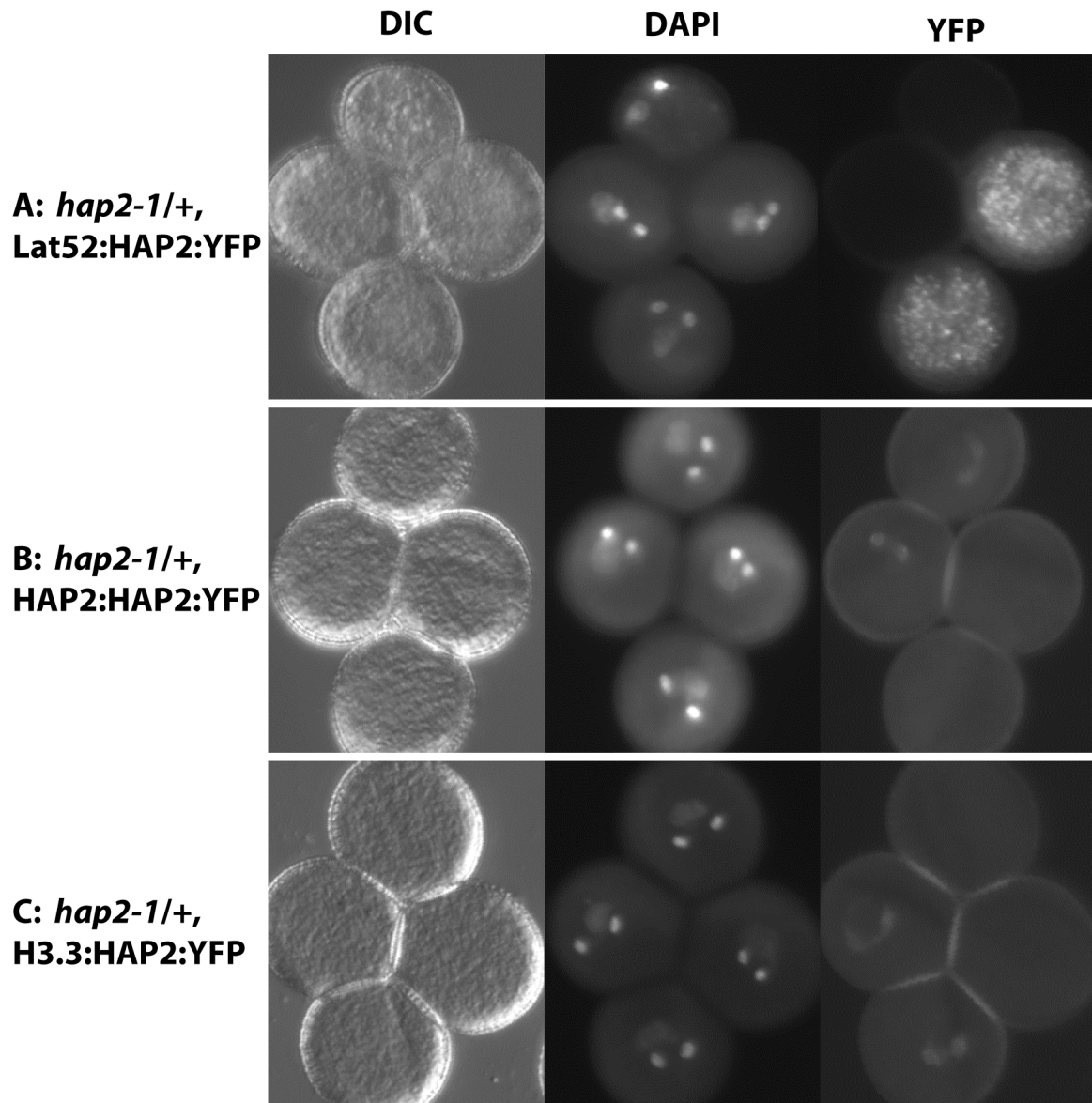


Figure 7: Tetrads of double heterozygous pollen from crosses with *hap2-1* null mutants and expressing HAP2 constructs.

DIC, DAPI and yellow fluorescent images are shown for a tetrad of pollen from each of the three constructs that were crossed into the *hap2-1* null mutant (A) Lat52:HAP2:YFP, (B) HAP2:HAP2:YFP, and (C) H3.3:HAP2:YFP. Each picture is at the same magnification and exposure times are consistent for each fluorescence type. Ectopic HAP2 localization is not altered by the presence of a T-DNA in the HAP2 locus.

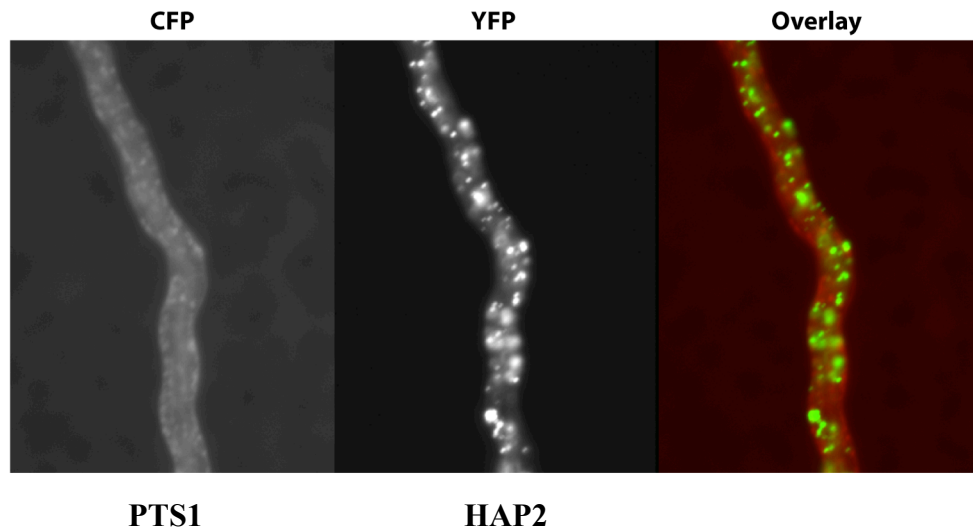


Figure 8: Lat52:HAP2:YFP and Lat52:CFP-PTS1 puncta sometimes colocalize in pollen tubes.

Micrographs and an overlay showing fluorescence in peroxisomes from Lat52:CFP-PTS1 (false-colored red in overlay) and fluorescent puncta from Lat52:HAP2:YFP (false-colored green in overlay) colocalize for some, but not all puncta within a pollen tube expressing both constructs.

	hap2-2, HAP2:YFP	hap2-2, +	+, HAP2:YFP	+, +	♂
hap2-2, HAP2:YFP	hap2-2/hap2-2, HAP2:YFP/HAP2:YFP	hap2-2/hap2-2, HAP2:YFP/+	hap2-2/+, HAP2:YFP/HAP2:YFP	hap2-2/+, HAP2:YFP/+	
hap2-2, +	hap2-2/hap2-2, +/HAP2:YFP	hap2-2/hap2-2, +/+	hap2-2/+, +/HAP2:YFP	hap2-2/+, +/+	
+, HAP2:YFP	+/hap2-2, HAP2:YFP/HAP2:YFP	+/hap2-2, HAP2:YFP/+	+/+, HAP2:YFP/HAP2:YFP	+/+, HAP2:YFP/+	
+, +	+/hap2-2, +/HAP2:YFP	+/hap2-2, +/+	+/+, +/HAP2:YFP	+/+, +/+	
♀					

hap2-2/+, Lat52:HAP2:YFP pl10

self cross plated on BASTA/Kan

resistant	sensitive	% R	total
240	148	62%	388

Figure 9: Lat52:HAP2:YFP strain pl10 rescues the *hap2-2* fertilization defect in double heterozygous plants.

Expected values and genotypes are shown as a Punnet square from a self-cross of a double heterozygous *hap2-2* and Lat52:HAP2:YFP plant. The resistance to BASTA (blue) and Kanamycin (Kan, red), conferred by the Lat52:HAP2:YFP and *hap2-2* constructs respectively, are shown for each genotype. The expected ratio of resistant to sensitive seedlings when plated on BASTA/Kan plates if the Lat52:HAP2:YFP construct rescues the *hap2-2* fertilization defect is 58.3% (7/12) and all genotypes except those with the *hap2-2* and not the HAP2:YFP (the second column of the square) will be represented in the seedling population. If the Lat52:HAP2:YFP does not rescue the *hap2-2* defect, genotypes in the shaded area will be lost, so the expected ratio of resistant to sensitive would be 37.5% (3/8).

Table 4: Transmission of the *hap2-2* allele through the pollen when rescued by the Lat52:HAP2:YFP construct on wild type female pistils.

hap2-2, Lat52:HAP2:YFP pl10					
MS1 X hap2-2, Lat52:HAP2:YFP on Kanamycin					
	pollen			%	
plant	phenotype	resistant	sensitive	resistant	total
#1	HMZ YFP	15	28	35%	43
#4	HMZ YFP	38	62	38%	100
#6	het YFP	56	0	100%	56
#13	het YFP	17	42	29%	59
	expected if rescue			50%	
	expected if no rescue			0%	

Although the pollen fluorescence phenotype was determined before crossing only wild-type ms1 pistils, the hap2-2 genotype was unknown.

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**Chapter 5: Expressing the diphtheria toxin A subunit
from the *HAP2(GCS1)* promoter blocks sperm
maturation and produces single sperm-like cells capable
of fertilization**

Aubrey C. Frank and Mark A. Johnson

This chapter is a reformatted version of the paper published in Plant Physiology (2009) with Aubrey C. Frank and Mark A. Johnson as authors. I performed all the experiments presented in the figures and tables of this chapter except for some images taken by Dr. Johnson in supplemental Figure 1.

Abstract:

After meiosis, the male germline of flowering plants undergoes two mitoses, producing two sperm that are carried within a pollen tube to an ovule. One sperm fuses with the egg to form the zygote and the other fuses with the central cell to form the primary endosperm. The mechanisms that control male germline development and gene expression, and ensure that sperm properly fuse with female gametes are just beginning to be understood. Expression of the potent translation inhibitor, diphtheria toxin A subunit, from the *Arabidopsis thaliana* *HAP2(GCSI)* promoter blocked sperm development before the final cell division, resulting in pollen tubes that carried a single sperm-like cell rather than two sperm. These pollen tubes targeted ovules and fertilized either the egg or the central cell, producing seeds with either endosperm or an embryo, but not both. Endosperm-only seeds significantly outnumbered embryo-only seeds, suggesting that single sperm-like cells preferentially fuse with the central cell. These experiments show that de novo translation is required for completion of sperm development, that the *HAP2(GCSI)* promoter is very tightly controlled, and that disruption of gene expression can result in male germ cells with a bias for gamete fusion.

Introduction:

Flowering plants have a unique reproductive system called double fertilization. Two non-motile sperm cells develop within a pollen grain and are delivered to female gametes by a pollen tube, a highly polar extension of the pollen grain that elongates by tip growth. The pollen tube is attracted to an ovule where it bursts and releases sperm. One sperm fertilizes the egg to produce the zygote, thereby initiating development of the embryo. The other sperm fuses with the central cell to produce the primary endosperm, which develops into endosperm, a tissue that provides nutritional support for the embryo as it develops into a seedling (Berger et al., 2008).

Male germline development occurs in the anther where microspore mother cells undergo meiosis to produce a tetrad of haploid microspores. Each microspore undergoes an asymmetric mitotic division during which a vegetative cell engulfs a generative cell. These cells have very different fates (Twell et al., 1998); the vegetative cell does not divide again, while the generative cell undergoes DNA replication and divides once more to produce two haploid sperm cells within the vegetative cell cytoplasm. The vegetative cell and the two sperm cells each carry the same haploid genome and constitute the three-celled male gametophyte (pollen grain). One question raised by this developmental program and gamete delivery system is whether the two sperm cells are identical and interchangeable, or whether the two sperm have different fates and are programmed to fuse with one or the other female gamete.

In *Plumbago zeylanica*, pollen tubes carry morphologically distinct sperm that have distinct targets for fertilization (Russell and Cass, 1981; Russell, 1984). One sperm is larger than the other, contains many mitochondria but few or no plastids, is associated

with the vegetative nucleus, and is more likely to fuse with the central cell. The smaller sperm contains many plastids but fewer mitochondria and is more likely to fuse with the egg (Russell, 1985). In maize, the egg is fertilized two to three times more frequently by sperm carrying B chromosomes than by sperm carrying only the A complement of chromosomes (Carlson, 1969; Faure et al., 2003; Roman, 1948). Proposed explanations for preferential fusion in these systems have included the order of sperm arrival at the female gametophyte, the morphology/size of the sperm, and differential expression of cell surface factors that mediate specific cellular gamete:gamete interactions leading one sperm to specifically target either the egg or the central cell for fusion (Roman, 1948; Russell, 1985; Spielman and Scott, 2008).

Flowering plant sperm, like mammalian sperm, have compact chromatin, a unique set of histones (Xu et al., 1999; Okada et al., 2005), and were assumed to be transcriptionally quiescent. However, several genes have been described that are male germline-specific (Xu et al., 1999, 1999; Engel et al., 2003; Singh et al., 2003; Durbarry et al., 2005; Engel et al., 2005; Okada et al., 2005; Rotman et al., 2005) and microarray analysis shows that *Arabidopsis* sperm possess a large, diverse, and cell-type specific array of transcripts (Borges et al., 2008). While no genes have yet been identified that are differentially expressed between *Arabidopsis* sperm, the morphologically distinct *Plumbago zeylanica* sperm express different mRNAs (Gou et al., 2009). These findings raise the possibility that flowering plant sperm may express a specific gene expression program leading to differentiation and gamete fusion specificity.

To disrupt gene expression during male germline development, we expressed the translation blocking diphtheria toxin A chain (DTA) from the male germline-specific

HAP2(GCSI) (At4g11720) promoter (von Besser et al., 2006). We anticipated that this approach would result in sperm ablation and/or disruption of sperm gene expression. DTA blocks protein synthesis by ADP-ribosylation of translation elongation factor 2 and has been expressed from various promoters in studies of animal and plant development to determine the consequences of loss of particular cells/tissues within the context of otherwise normal development (Breitman et al., 1987; Thorsness et al., 1993; Day et al., 1995; Twell, 1995; Singh et al., 2003). A single molecule of DTA is sufficient to completely block propagation of mouse cells in vitro (Yamaizumi et al., 1978). Therefore, DTA can reveal very weak promoter activity and has been used to refine understanding of expression patterns of sporophytic (Thorsness et al., 1991) and gametophytic (Thorsness et al., 1991; Twell, 1995; Singh et al., 2003) genes in flowering plants. Furthermore, when DTA is expressed without the diphtheria toxin B chain, it cannot be transported across the plasma membrane, and is therefore cell autonomous (Pappenheimer, 1977). This is a critical feature for our purpose because the sperm cells are housed within the vegetative cell cytoplasm and we wanted to perturb sperm gene expression without interfering directly with gene expression in the vegetative cell.

We show that male germline development is blocked just before the generative cell divides in transgenic plants expressing a *HAP2(GCSI)promoter:DTA* [*HAP2(GCSI):DTA*] construct. This result is consistent with recent data showing that DUO1 and DUO3 are required to activate *HAP2(GCSI)* transcription in the generative cell (Brownfield et al., 2009a; Brownfield et al., 2009b). Furthermore, it demonstrates that de novo translation in the generative cell is necessary for completion of sperm development. *HAP2(GCSI):DTA* pollen tubes are functional and deliver single sperm-

like cells to female gametophytes, resulting in ovules that contain either an embryo or an endosperm, but not both products of double fertilization. We show that these are the products of fertilization and that endosperm-only ovules significantly outnumber embryo-only ovules. We conclude that expression of DTA in the male germline perturbs the normal sperm gene expression program and results in a population of single sperm-like cells that preferentially fuse with the central cell. Recent analysis of Arabidopsis mutants that generate single sperm-like cells have led to models suggesting either that the egg is the default target for sperm (Nowack et al., 2006), or that fusion is random (Chen et al., 2008). Our data indicate that the egg is not the default target for fertilization, and that blocking translation during germline development results in a single sperm-like cell that preferentially fertilizes the central cell.

Results:

Expression of *HAP2(GCS1):DTA* generates mature pollen grains containing a single sperm-like cell.

We transformed Arabidopsis *qrt1* mutants, which release pollen grains as intact meiotic tetrads but do not otherwise affect pollen function (Preuss et al., 1994), with a *HAP2(GCS1):DTA* construct and recovered transgenic plants that had no obvious vegetative or floral developmental defects. However, all transgenic lines had a striking pollen defect; many mature pollen grains contained a vegetative nucleus and a single additional nucleus rather than the two sperm nuclei present in wild-type pollen grains (Fig. 1A). Subsequent analyses showed these single cells with bright staining nuclei shared features with sperm cells (see below), so we called them single sperm-like cells.

We determined the segregation of this defect in tetrads of three transgenic lines (Fig. 1B); for *HAP2(GCSI):DTA B7*, the majority (92/106, Fig. 1A,B) of tetrads comprised two normal pollen grains and two that contained a single sperm-like cell. This 2:2 segregation pattern in tetrads indicated that the plant was hemizygous for the transgene, the transgene caused a post-meiotic defect, and expressivity of the defect was complete in these tetrads. A fraction of tetrads (14/106, Fig. 1B) comprised three normal pollen grains and one containing a single sperm-like cell. The 3:1 (3 normal: 1 defective) segregation observed in these tetrads indicated that the defect was not completely expressed in all pollen grains that carried *HAP2(GCSI):DTA*. The expressivity of pollen developmental defects was lower in two other lines analyzed (*HAP2(GCSI):DTA B1* and *B4*, Fig. 1B).

In reciprocal crosses to wild type, *HAP2(GCSI):DTA* behaved like a mutation that disrupts the development and/or function of the male gametophyte. We used pollen from three independent hemizygous *HAP2(GCSI):DTA* transgenic lines to pollinate *male sterile1 (ms1)* flowers and determined how many of the F₁ progeny inherited the *HAP2(GCSI):DTA* transgene by scoring Basta resistance (BastaR, the *HAP2(GCSI):DTA* T-DNA carries a BastaR gene). When *HAP2(GCSI):DTA B7* was used as a pollen donor, only 1% of progeny inherited the BastaR gene (Table I). T-DNAs that do not disrupt male gametophyte function are inherited by 50% of progeny in such crosses (Johnson et al., 2004; von Besser et al., 2006). This severe reduction in transmission through the male gametophyte indicated that the transgene disrupted pollen development and/or function. Data from two other transgenic lines were consistent with

a reduction in transmission through the male gametophyte, however the expressivity of the defect was lower in these lines (Table I).

Transmission through the female gametophyte was also reduced in the lines with the greatest reduction in transmission through the male gametophyte (Table I). This result suggests that the *HAP2(GCSI)* promoter is active in female cells, consistent with a recent report that *HAP2(GCSI)* mRNA is present in ovule and siliques samples (Borges et al., 2008). *HAP2(GCSI)* expression in sperm/minus gametes has been shown to be essential for fertilization in *Arabidopsis* (Mori et al., 2006; von Besser et al., 2006), *Plasmodium* (Hirai et al., 2008; Liu et al., 2008), and *Chlamydomonas* (Liu et al., 2008); however, expression from female/plus gametes is not required in these organisms, so the role of female-expressed *HAP2(GCSI)* is currently not known. Furthermore, *DUOI*, a critical regulator of *HAP2(GCSI)* expression (Brownfield et al., 2009a), is restricted to the male germline (Rotman et al., 2005), suggesting a distinct control mechanism for expression in female cells.

DNA content of single sperm-like cells.

To determine the stage at which DTA expression disrupted male germline development, we measured the fluorescence intensity of DAPI (4',6-diamidino-2-phenylindole)-stained nuclei of single sperm-like cells and normal sperm in *HAP2(GCSI):DTA B7* and *HAP2(GCSI):DTA B1* pollen. In both lines, the fluorescence intensity of single sperm-like cells was roughly twice that of normal sperm (Fig. 1C). We concluded that the generative cell, which normally divides to produce two sperm cells, completed S-phase of the cell cycle but failed to complete mitosis in pollen grains that expressed *HAP2(GCSI):DTA*.

Expression of sperm identity markers in single sperm-like cells

To explore the identity of single sperm-like cells and to assess the impact of the *HAP2(GCSI):DTA* construct on sperm gene expression, we analyzed four male germline markers in *HAP2(GCSI):DTA B7* transgenic plants: *DUO1:H2B:mRFP1* (Rotman et al., 2005), *HTR10:HTR10:mRFP1* (Ingouff et al., 2007), *HAP2(GCSI):HAP2(GCSI):YFP* (von Besser et al., 2006), and *AtGEX2:eGFP* (Engel et al., 2005)(Fig. 2A-D). *DUO1* is a Myb protein required for expression of other generative and sperm cell-expressed genes including *HAP2(GCSI)*, *AtGEX2*, and *HTR10* (Brownfield et al., 2009a). We analyzed hemizygous *HAP2(GCSI):DTA* plants that were hemizygous for *DUO1:H2B:mRFP1*. In this experiment, a maximum of ~50% of pollen with either normal sperm or a single sperm-like cell carried the reporter construct. We found that ~50% of pollen of either type expressed *DUO1:H2B:mRFP1*, indicating that almost all single sperm-like cells that carried this reporter expressed it (Fig. 2A,E). Consequently, we suggest that the *DUO1:H2B:mRFP1* reporter construct is translated in single sperm-like cells before *DTA* begins to block translation. Similarly, nearly all single sperm-like cells expressed *HTR10:HTR10:mRFP1* (Fig. 2B,E). Microarray analysis of sperm showed that *HTR10* transcripts were among the most abundant and accumulated to significantly higher levels than *HAP2(GCSI)* or *AtGEX2* (Borges et al., 2008). We propose that the *HTR10* promoter is sufficiently active before the onset of translation inhibition by *DTA* to allow the reporter to accumulate to detectable levels.

Analysis of hemizygous *HAP2(GCSI):DTA* pollen that was homozygous for *HAP2(GCSI):HAP2(GCSI):YFP* or *AtGEX2:eGFP* (100% of pollen carry the reporter) showed that many single sperm-like cells expressed these markers, but a significant

fraction did not (Fig. 2C-E). The *HAP2(GCSI)* and *AtGEX2* promoters are expected to be activated contemporaneously with the *HAP2(GCSI):DTA* construct. We propose that in some single-sperm like cells, detectable levels of *HAP2(GCSI):HAP2(GCSI):YFP* or *AtGEX2:eGFP* were translated, whereas in other single sperm-like cells translation of these markers was blocked by DTA. These data suggest that the effect of DTA on sperm gene expression is variable and that some single sperm-like cells express the endogenous *HAP2(GCSI)* and *AtGEX2* genes, while others do not. *HAP2(GCSI)* is essential for fertilization (Mori et al., 2006; von Besser et al., 2006; Hirai et al., 2008; Liu et al., 2008) and it, along with any other sperm-expressed genes required for gamete fusion, would have to be expressed for single sperm-like cells to fertilize female gametes.

Pollen tubes carrying a single sperm-like cell germinate and grow like wild type.

We germinated pollen from *HAP2(GCSI):DTA B7* and *HAP2(GCSI):DTA B1* hemizygous plants in vitro and stained them with DAPI to identify pollen tubes that carried single sperm-like cells and those with two normal sperm (Fig. 3A). In both cases, the number of growing pollen tubes that contain a single sperm-like cell was roughly equivalent to the number containing two sperm (Fig. 3B). This indicated that pollen grains with a single sperm-like cell germinated at the same rate as wild-type pollen grains. Furthermore, after six hours of pollen tube growth, the length of *HAP2(GCSI):DTA B7* and *HAP2(GCSI):DTA B4* pollen tubes with a single sperm-like cell was the same as that of pollen tubes with two sperm (Fig. 3C). Expression of DTA from the *HAP2(GCSI)* promoter does not affect pollen tube growth, suggesting that the effects of DTA are limited to the generative cell contained within the vegetative cell cytoplasm.

We previously proposed that sperm-expressed genes play a role in pollen tube guidance because *hap2-1* mutant pollen tubes were less likely than their wild-type counterparts to target ovules in competitive hand pollinations of *ms1* pistils (von Besser et al., 2006). Recently, *duo3* pollen tubes, which are defective in expression of *HAP2(GCSI)* and other sperm-expressed genes, were also shown to be less likely to target ovules than wild-type pollen tubes (Brownfield et al., 2009b). We could not determine whether *HAP2(GCSI):DTA* expression caused pollen tube guidance defects in competitive crosses because the incomplete expressivity of DTA defects precluded quantitative analysis of ovule targeting.

Single sperm-like cells fertilize the central cell more often than the egg.

We analyzed embryo and endosperm development in pistils of self-fertilized hemizygous *HAP2(GCSI):DTA B7*, *HAP2(GCSI):DTA B1*, and *HAP2(GCSI):DTA B4* transgenic plants approximately five days after pollination. In each independent transgenic line, we observed three classes of developing seeds: 1) normal seeds containing a late globular-stage embryo and proliferating endosperm, 2) abnormal seeds in which endosperm proliferated, but there was no embryo, 3) abnormal seeds in which embryos initiated development, but endosperm was completely absent (Supplemental Fig. S1). We also observed a significant number of unfertilized ovules. We hypothesized that normal seeds were the result of fertilization by wild-type pollen produced by *HAP2(GCSI):DTA* hemizygous plants and that abnormal seeds were the result of fertilization of either the egg or the central cell by a pollen tube carrying a single sperm-like cell. Neither endosperm-only nor embryo-only seeds completed development; they

resulted in aborted ovules that were obvious in developing siliques of self-fertilized *HAP2(GCSI):DTA* hemizygous plants (Supplemental Fig. S1).

In each of these transgenic lines, endosperm-only ovules significantly outnumbered embryo-only ovules, suggesting that single sperm-like cells preferentially fertilize the central cell. We performed a quantitative analysis of seed development by pollinating *msl* pistils with pollen from homozygous *HAP2(GCSI):DTA B7* transgenic plants to determine the relative frequency of embryo- and endosperm-only ovules and to analyze the development of these aberrant seeds over time. This rare homozygous line was likely the progeny of a pollen grain that carried *HAP2(GCSI):DTA*, but was not defective in generative cell division or expression of essential sperm genes (Fig. 1B, Fig. 2). As expected, seed production was significantly reduced in this line; however, it produced a small number of homozygous individuals following self-fertilization. We used this homozygous line for quantitative analysis of seed development because ~80% of its pollen carried single sperm-like cells (Supplemental Fig. S2A,B) and pollen tubes targeted ~90% of *msl* ovules following hand-pollination (Supplemental Fig. S2C). These features increased the number of single fertilization events that could be analyzed.

We analyzed ovule development 1.5, 3, and 4 days after pollination of *msl* (Fig. 4) and found that endosperm-only ovules outnumbered embryo-only ovules at each time point when homozygous *HAP2(GCSI):DTA B7* pollen was used (Fig. 4, Table II). In total, we observed 73 endosperm-only ovules and 8 embryo-only ovules, a ratio of 9:1. These classes of aberrant ovule development were not observed when *qrt1* pollen was used to pollinate *msl* or when no pollen was applied to *msl* (Table II). Significantly, a central cell nucleus is clearly apparent in embryo-only ovules (Fig. 4C,F,I); this

observation is consistent with the hypothesis that embryo-only ovules are the result of fertilization of the egg by a single sperm-like cell. The central cell remains unfertilized and does not proliferate autonomously (Fig. 4C,F,I). This observation distinguished single sperm-like cells produced by expression of *HAP2(GCSI):DTA* from those of *cdc2* or *fb117* mutants (Nowack et al., 2006; Kim et al., 2008; Gusti et al., 2009). In these cases, egg-only fertilization by a single sperm-like cell initiated division of the primary endosperm nucleus.

We analyzed 899 ovules across the three time points in pistils pollinated with homozygous *HAP2(GCSI):DTA B7* pollen. In addition to the 81 single fertilization events observed, there were 32 products of double fertilization that contained an embryo and endosperm (Table II). These ovules were likely targeted by *HAP2(GCSI):DTA B7* pollen tubes that carried two sperm, indicating that DTA was either not expressed in these pollen, or that DTA expression did not affect generative cell division or expression of essential sperm genes. The majority of ovules were unfertilized (Table II; Supplemental Fig. S3). This high rate of unfertilized ovules cannot be explained by failure of *HAP2(GCSI):DTA B7* pollen tubes to target ovules (Supplemental Fig. S2C) and is likely due to lack of expression of *HAP2(GCSI)* (Fig. 2C,E) and/or other essential sperm genes required for fertilization. It is likely that sperm gene expression is also disrupted in pollen grains that contain two sperm, because we observed two sperm in ~20% of pollen grains, but double fertilization was observed in only ~4% of ovules. The observed rates of double fertilization, egg-only fertilization, and central cell-only fertilization increased during the time course we analyzed (Table II). This suggests that

fertilization events mediated by sperm and single sperm-like cells carrying *HAP2(GCSI):DTA* were delayed relative to wild type.

Initiation of endosperm or embryo development by a single sperm-like cell requires fertilization.

We considered the possibility that deposition of a single sperm-like cell in the female gametophyte initiated endosperm or embryo development through an autonomous pathway not requiring gamete fusion. Proliferation of endosperm without fertilization occurs in female gametophyte mutants with loss-of-function of either *FISI-2*, *FIE*, or *MEA*, genes that normally repress endosperm development until fertilization (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999). To address the possibility that single sperm-like cells promoted autonomous endosperm proliferation, we examined whether a single sperm-like cell could transmit *KS117* endosperm marker (Sorensen et al., 2001) expression to a wild-type female. Embryo development and endosperm proliferation were apparent in ovules fertilized by wild-type or *KS117* pollen (Fig. 5A,C); GFP signal was only detectable in ovules fertilized by *KS117* pollen (Fig. 5D compared to Fig. 5B). We generated plants hemizygous for *HAP2(GCSI):DTA B7* and *KS117* and used these to pollinate *msl*. Importantly, in these crosses, we observed endosperm-only ovules (Fig. 5E) and products of double fertilization (Fig. 5G) that express the *KS117* marker (Fig. 5F,H), indicating that endosperm initiation resulted from fertilization by a single sperm-like cell and not autonomous endosperm proliferation.

To determine whether embryos developing in embryo-only ovules were the product of fertilization by a single sperm-like cell we used an *RCN1promoter:GUS* (*RCN1:GUS*) transgene as a paternal marker for embryo gene expression. *RCN1* is a

ubiquitously expressed protein phosphatase 2A regulatory subunit (Deruere et al., 1999; Zhou et al., 2004; Blakeslee et al., 2008). Four days after pollination of *msl* pistils with pollen carrying *RCN1:GUS*, we detected GUS activity in globular embryos (Fig. 5J); GUS activity was not detected in ovules pollinated with *qrt1* (Fig. 5I). When we pollinated *msl* with pollen hemizygous for both *HAP2(GCS1):DTA B7* and *RCN1:GUS* we observed GUS expression in embryos resulting from double fertilization (Fig. 5K) and in embryo-only ovules (Fig. 5L), indicating that embryos developing without endosperm were the product of fertilization by a single sperm-like cell carrying the *RCN1:GUS* reporter gene.

Discussion:

***HAP2(GCS1):DTA* transgenic plants offer a new tool for the dissection of male germline development and fertilization.**

We generated pollen grains that carry a single sperm-like cell, instead of two sperm, by expressing DTA from the *HAP2(GCS1)* promoter in transgenic plants. Single sperm-like cells were capable of fertilization and produced significantly more endosperm-only ovules than embryo-only ovules, suggesting that single sperm-like cells were biased toward fusing with the central cell rather than the egg. *HAP2(GCS1):DTA* transgenic plants can be used to identify proteins that are translated immediately before, or after the final cell division in male germline development, and that may mediate differentiation of sperm and specific gamete:gamete interactions.

***HAP2(GCS1):DTA* expression did not cause vegetative, generative, or sperm cell lethality.**

Complete ablation of the generative cell, a possible outcome of our experiment given that DTA leads to cell death in other systems, would have produced pollen grains (and/or tubes) with a vegetative nucleus but no generative or sperm cell(s). We did not observe this phenotype, which would have been detectable even at low frequencies. It is possible that the brief period of time between onset of *HAP2(GCSI):DTA* expression and release of sperm is insufficient for induction of cell death. Alternatively, the relatively mild and variable phenotypes associated with *HAP2(GCSI):DTA* expression may be due to low and/or sporadic expression of the DTA transgene.

HAP2(GCSI):DTA expression did not affect pollen grain development (Fig. 1) or the ability of pollen tubes to grow in vitro (Fig. 3). In contrast, expression of DTA from either the *lily generative cell 1 (LGCI)* promoter (Singh et al., 2003), or the vegetative nucleus-specific *LAT52* promoter (Twell, 1995), resulted in pollen grain collapse. Since the pollen grain was not affected in *HAP2(GCSI):DTA* plants, we conclude that the *HAP2(GCSI)* promoter is restricted to the generative cell and not active in the vegetative nucleus.

Models for regulation of *HAP2(GCSI)* promoter activity and mitotic arrest of *HAP2(GCSI):DTA* generative cells.

The developmental defects observed in *HAP2(GCSI):DTA* transgenic plants are consistent with the emerging view that the combined activity of DUO1 and DUO3 transcription factors is required for *HAP2(GCSI)* expression (Brownfield et al., 2009a; Brownfield et al., 2009b). Loss of function of either *DUO1*, or *DUO3* blocks *HAP2(GCSI)* expression in the generative and sperm cells (Brownfield et al., 2009a; Brownfield et al., 2009b). DUO1 expression is limited to the male germline (Rotman et

al., 2005), while DUO3 is expressed in the male germline and broadly in sporophytic tissues (Brownfield et al., 2009b). The developmental defects caused by expression of *HAP2(GCSI):DTA* were limited to the male germline, consistent with the hypothesis that *HAP2(GCSI)* promoter activity is restricted by the expression pattern of *DUO1* (Brownfield et al., 2009a).

In addition to regulating *HAP2(GCSI)* expression, DUO1 and DUO3 have overlapping and distinct roles in controlling expression of other male germ-line expressed genes and in promoting generative cell mitosis. *duo1* mutants do not express *AtGEX2*, *HTR10*, or the G2/M regulator, *AtCycB1;1* (Brownfield et al., 2009a). *duo3* mutants are also defective in *AtGEX2* expression, but express *HTR10* and *AtCycB1;1* (Brownfield et al., 2009b). The generative cells of both mutants complete S-phase, but fail to enter M-phase of the cell cycle (Brownfield et al., 2009a; Brownfield et al., 2009b). In the case of *duo1* mutants, failure to complete mitosis is likely due to lack of expression of *AtCycB1;1*. We propose that *HAP2(GCSI):DTA* expression in the generative cell, blocks translation of *AtCycB1;1* and/or other positive regulators of the generative cell mitosis, resulting in mature pollen grains with a single sperm-like cell with approximately twice the DNA content of wild-type sperm.

Regulation of sperm fusion events during double fertilization.

All Arabidopsis sperm-expressed genes studied thus far are expressed in both sperm (Xu et al., 1999; Engel et al., 2005; Rotman et al., 2005; Mori et al., 2006; von Besser et al., 2006; Ingouff et al., 2007), suggesting these cells do not differentiate from each other. Furthermore, when two wild-type sperm are delivered to a mutant female gametophyte that produces two eggs rather than one, both eggs can be fertilized,

suggesting that both sperm have the capacity to fuse with an egg (Pagnussat et al., 2007; Ingouff et al., 2009). These data suggest that Arabidopsis sperm are essentially interchangeable and that either is capable of fertilizing the egg.

On the other hand, experiments analyzing the outcomes of single fertilization events mediated by pollen tubes carrying a single sperm-like cell suggest the potential for preferential fertilization. Loss of function of either of two critical cell cycle regulators, *CDC2A* (*CDKA;1*) or *FBL17*, blocks the generative cell cycle during S-phase and produces a single sperm-like cell that preferentially fertilizes the egg (Iwakawa et al., 2006; Nowack et al., 2006; Kim et al., 2008; Gusti et al., 2009). Three explanations for egg-only fertilization were proposed: 1) the architecture of the female gametophyte dictates fertilization of the egg because sperm are deposited in one of two synergids that are adjacent to the egg cell. 2) There is active signaling by the egg cell that attracts the first sperm cell released by the pollen tube. 3) Each of the two sperm cells has a specific, predetermined target for fertilization (Nowack et al., 2006).

In another study of single fertilization, the fusion target for the single sperm-like cell appeared to be random (Chen et al., 2008). Approximately 6% of pollen grains generated by Arabidopsis *msi1/MSII* mutants contain a single sperm-like cell due to pleiotropic defects in male germline development caused by loss of function of a complex that regulates chromatin assembly (Chen et al., 2008). Interestingly, an equal number of egg-only (18/3600 ovules scored) and central cell-only (20/3600 ovules scored) single fertilization events were observed.

Single sperm-like cells produced by *msi1* or by expression of *HAP2(GCSI):DTA* can bypass the egg and fertilize the central cell. Therefore, female gametophyte

architecture or active signaling by the egg to attract the first sperm deposited can be ruled out as explanations for egg-only fertilization by *cdc2a* or *fb117* mutants. Analysis of single sperm-like cells produced in *msi1* mutants, which were equally likely to fuse with either female gamete, led to the conclusion that the two sperm produced by wild-type pollen are identical (Chen et al., 2008). Our results indicate that expression of a translation inhibitor during sperm development results in a single sperm-like cell that preferentially fuses with the central cell.

We propose that single sperm-like cells produced by *cdc2a*, *fb117*, *msi1* or by expression of *HAP2(GCSI):DTA* show different preferences for fertilization because each of these perturbations has a different effect on male germline gene expression and development. *cdc2a* and *fb117* specifically block the generative cell cycle at S-phase and produce single sperm-like cells that fuse with the egg, but are not expected to directly affect gene expression in the male germline (Iwakawa et al., 2006; Nowack et al., 2006; Kim et al., 2008; Gusti et al., 2009). *msi1* is pleiotropic, sometimes causing arrest immediately after meiosis or after the first pollen mitosis; therefore, it is possible that the population of single sperm-like cells generated by this mutation is heterogeneous, hence the finding that they fertilize either the egg or the central cell (Chen et al., 2008). Expression of *HAP2(GCSI):DTA* blocks generative cell mitosis, but might also directly block translation of mRNAs involved in regulating fusion of the sperm with either the egg or central cell. Therefore, *HAP2(GCSI):DTA* single sperm-like cells may fuse predominantly with central cells because they fail to translate a factor expressed by *cdc2a* and *fb117* single sperm cells that directs or enables sperm to fuse with the egg.

The mechanisms that ensure that one sperm fertilizes the egg and the other fertilizes the central cell are not known, but it is clear that such a mechanism is in place to prevent both sperm from fusing with either the egg or the central cell. Differentiation between the two sperm resulting in specific gamete fusion provides one potential mechanism. An alternative hypothesis is that fusion is random and a mechanism similar to the rapid block to polyspermy in animals (Wong and Wessel, 2006) prevents both sperm from fertilizing either of the female gametes (Scott et al., 2008; Spielman and Scott, 2008). Our studies on pollen carrying a single sperm-like cell suggest that male germline development can be altered to produce a sperm cell with a specific fate and suggest the possibility of an underlying program of differential gene expression that could ensure that one sperm fuses with the egg and the other with the central cell. Support for this hypothesis will require identification of genes that differentially mark the two sperm and direct a specific fertilization preference. Comparison of single sperm-like cells produced by *cdc2a* or *fb117* mutants (egg preference) and by expression of *HAP2(GCS1):DTA* (central cell preference), provides a path toward discovery of these sperm fusion specificity factors.

Materials and Methods:

Generation of *HAP2(GCS1):DTA* plants

The DT-A coding sequence (Palmiter et al., 1987) was amplified by PCR (primers: DTA NcoF, GGCCTCGCCATGGATCCTG; DTA XbaR, GCTCTAGACTTAAAAATTTTATATTACCTTAGAGC) and inserted into *HAP2(GCS1)promoter:YFP* (von Besser et al., 2006), replacing YFP. *Arabidopsis* (*Arabidopsis thaliana*) *qrt1* (Preuss et al., 1994) plants (Col-0 ecotype) were transformed

by floral dip (Clough and Bent, 1998) and transformants were selected on MS (Murushige and Skoog) plates containing 50mg/ml BASTA (Johnson et al., 2004). Plants were grown in growth chambers (Environmental Growth Chambers, Chagrin Falls, Ohio) at 21°C in 16 hr light (100μE), 8 hr dark.

Analysis of pollen and sperm development

Pollen tetrads were stained with DAPI (Park et al., 1998) and visualized on a Zeiss Axiovert 200M fluorescent microscope (Carl Zeiss, Germany). Images were acquired with a Hamamatsu ORCA-ER (Fig. 1) or Zeiss AxioCam MRc5 (Fig. 2). For DNA quantification, 12 bit images were acquired using a Leica (SP2 A OBS, Leica Microsystems AG, Wetzlar, Germany) confocal microscope and analyzed using Metamorph software (www.moleculardevices.com). A circle of uniform size was drawn around single sperm or single sperm-like cells and the fluorescence intensity (minus intensity of a background circle from the same image) was measured. Pollen tubes were grown in vitro on inverted drops of pollen growth medium (Hicks et al., 2004) for 6 hours. The following numbers of cells were analyzed to determine expression of male germ-line markers in *HAP2(GCSI):DTA B7* transgenic plants: *DUO1:H2B:mRFP1* (94 pairs of normal sperm, 63 single sperm-like cells), *HAP2(GCSI):HAP2(GCSI):YFP* (158 pairs of normal sperm, 42 single sperm-like cells), *AtGEX2:eGFP* (211 pairs of normal sperm, 113 single sperm-like cells), *HTR10:HTR10:mRFP1* (118 pairs of normal sperm, 77 single sperm-like cells). Pollen tube growth in *msl* pistils was analyzed using aniline blue staining (Mori et al., 2006).

Analysis of embryo and endosperm development and expression of marker genes

Developing siliques (seed pods) resulting from self-fertilization or hand-pollination of *male sterile1* (*ms1*) were excised and ovary walls were removed (Johnson et al., 2004). To analyze embryo and endosperm development, ovules were cleared using chloral hydrate (Yadegari et al., 1994) and imaged using DIC microscopy. For quantitative analysis of early seed development, *ms1* flowers that had just opened (stage 13, (Smyth et al., 1990) were marked and were either left unpollinated or were pollinated with *qrt1* or *HAP2(GCSI):DTA* pollen. Pollinations were performed under a dissecting microscope with at least two flowers to ensure complete pollination; pistils were removed and processed (as above) 1.5, 3, or 4 days later. To analyze *KS117* (Sorensen et al., 2001) GFP expression in developing endosperm, ovules were mounted in water before DIC and fluorescence imaging. To analyze *RCNI:GUS* (Deruere et al., 1999) expression in developing embryos, ovules were incubated in X-gluc solution (0.5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM NaPO₄, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl-glucuronic acid, 10 mM EDTA, 0.5% Triton X-100) at 37°C for 4 days, incubated in 1:1 ethenol:acetic acid for 16 hours at room temperature, and then incubated in chloral hydrate solution (chloral hydrate:glycerol:water solution, 8:1:2, w:v:v) (Yadegari et al., 1994) for 16 hours at room temperature before mounting in chloral hydrate solution. All images were obtained using a Zeiss Axiovert 200M fluorescence microscope and an AxioCam MRc5 camera (*RCNI:GUS* expression) or a Hamamatsu ORCA-ER camera (DIC, fluorescence).

Acknowledgments:

We thank Kiera von Besser and Daphne Preuss for providing the *HAP2(GCS1)* promoter fragment; Ian Maxwell and June Nasrallah for providing the DTA coding sequence; the Arabidopsis Biological Resource Center for transgenic plants expressing sperm and endosperm markers: *AtGEX2:eGFP* (ABRC# cs6508), and *KS117* (ABRC# cs9341, generated by the Haseloff lab: <http://www.plantsci.cam.ac.uk/Haseloff>); Alison DeLong for *RCN1:GUS* transgenic plants; Frederic Berger for *HTR10:HTR10:mRFP1* and *DUO1:H2B:mRFP1* transgenic plants, Robert Creton and Geoff Williams of the Brown University Leduc Bioimaging Facility; members of the DeLong and Bender labs for helpful discussions; and Ravishankar Palanivelu and Alison DeLong for critical reading of the manuscript.

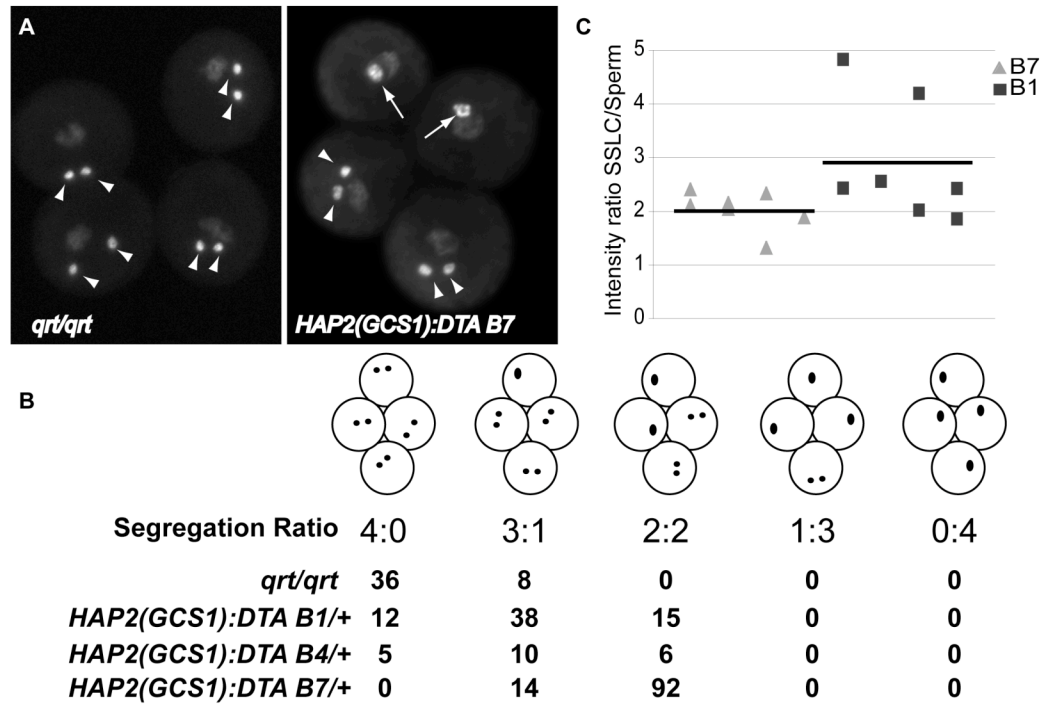


Figure 1. Expression of *HAP2(GCS1):DTA* generates mature pollen grains containing a single sperm-like cell.

A) DAPI-stained pollen grains of *qrt1* show a faint-staining vegetative nucleus and two sperm nuclei (arrow heads) in each of the four grains. Two of the four grains from a hemizygous *HAP2(GCS1):DTA B7* plant have two sperm, the other two grains show a single sperm-like cell (arrow). B) Schematics represent the segregation of sperm developmental defects in pollen tetrads (vegetative nuclei not drawn). *qrt1* tetrads are observed in which one of the pollen grains appears to contain a single sperm nucleus; this is an artifact of imaging overlapping sperm nuclei. C) Ratio of DAPI fluorescence intensity of single sperm-like cells (SSLC)/sperm. Each triangle or rectangle represents the average relative fluorescence intensity of two single sperm-like cells/four sperm nuclei in a single tetrad; black bars represent the mean.

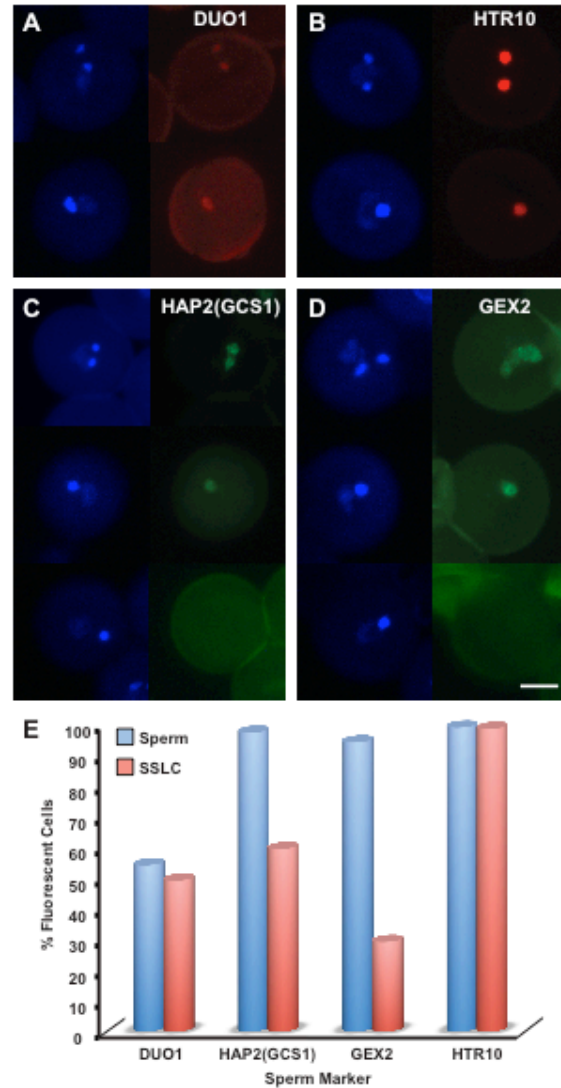


Figure 2. Single sperm-like cells express markers of generative and sperm cell identity.

A-D) DAPI fluorescence (left) and fluorescent protein images (right) of individual pollen grains with normal sperm (top) or single sperm-like cells (bottom). All transgenic plants analyzed were hemizygous for *HAP2(GCS1):DTA B7* and (A) hemizygous for *DUO1:H2B:mRFP1* [DUO1], (B) homozygous for *HTR10:HTR10:mRFP1* [HTR10], (C) homozygous for *HAP2(GCS1):HAP2(GCS1):YFP* [HAP2(GCS1)], (D) homozygous for *AtGEX2:eGFP* [GEX2]. C,D) Bottom pair of images show a single sperm-like cell that does not express the indicated marker gene. Scale bar = 10 μ m. E) Percentage of sperm and single sperm-like cells (SSLC) expressing indicated fluorescent markers (same genotypes as A-D, a minimum of 157 pollen grains were scored for each genotype, see Methods). Because the *DUO1:DUO1:mRFP1* marker was hemizygous, the maximum expected % fluorescent cells = 50.

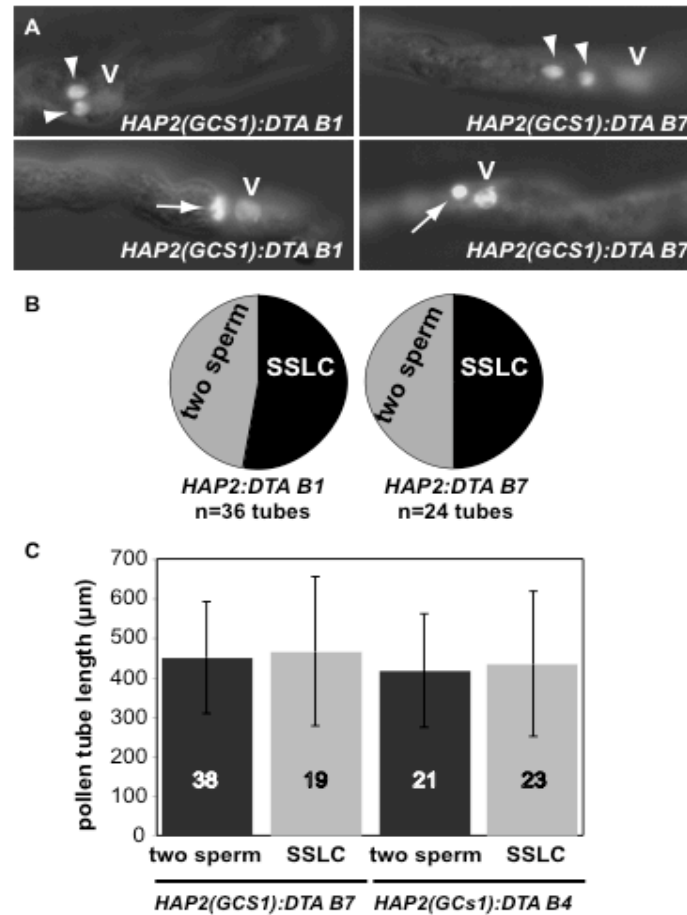


Figure 3. Pollen tubes containing a single sperm-like cell germinate and grow similarly to pollen tubes with two sperm.

A) DAPI-stained pollen tubes with a single sperm-like cell or two normal sperm from two hemizygous *HAP2(GCS1):DTA* lines. Arrowheads denote sperm, arrows single sperm-like cells, and a V points out the vegetative nucleus in each pollen tube. B) Pie charts showing the distribution of pollen tubes that carry two normal sperm versus those that carry a single sperm-like cell (SSLC) in *HAP2(GCS1):DTA B1* and *HAP2(GCS1):DTA B7* hemizygous transgenic plants. C) Length of in vitro grown pollen tubes containing two sperm (black bars) or a single sperm-like cell (SSLC, gray bars); error bars represent standard deviation, numbers of tubes measured is indicated in each bar.

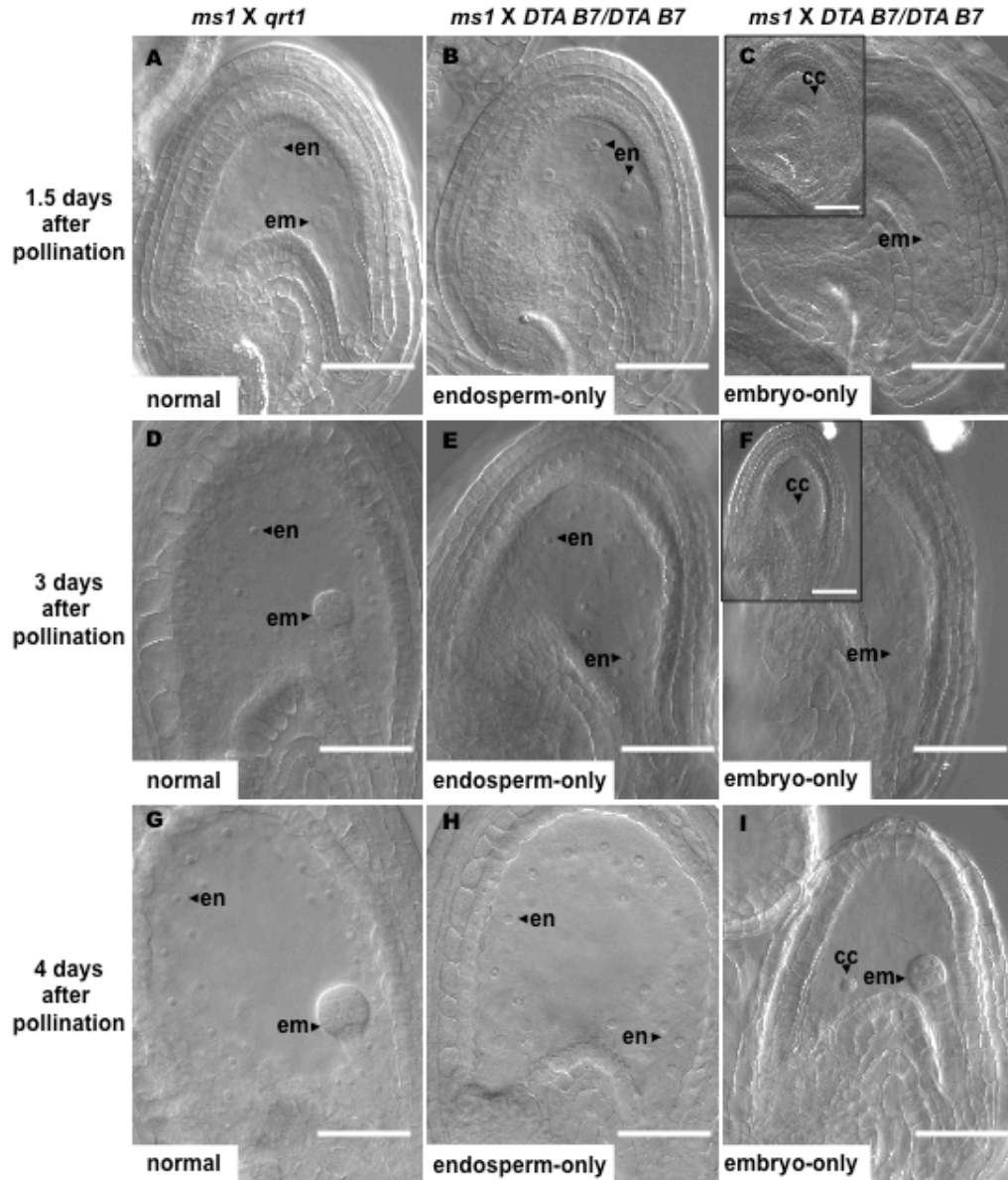


Figure 4. Single sperm-like cells are delivered to ovules and fertilize either the egg or the central cell.

Analysis of early seed development in crosses between *ms1* and *qrt1* pollen (A,D,G), or *ms1* and pollen homozygous for *HAP2(GCS1):DTA B7* (B,C,E,F,H,I) at 1.5, 3, and 4 days after pollination. Normal early seed development (A,D,G), and endosperm-only ovules (B,E,H) or embryo-only ovules (C,F,I) are shown. Insets in C and F show the unfertilized central cell nucleus in a different focal plane. Scale bar = 50 μ m; en, endosperm nuclei; em, embryo; cc, central cell nucleus.

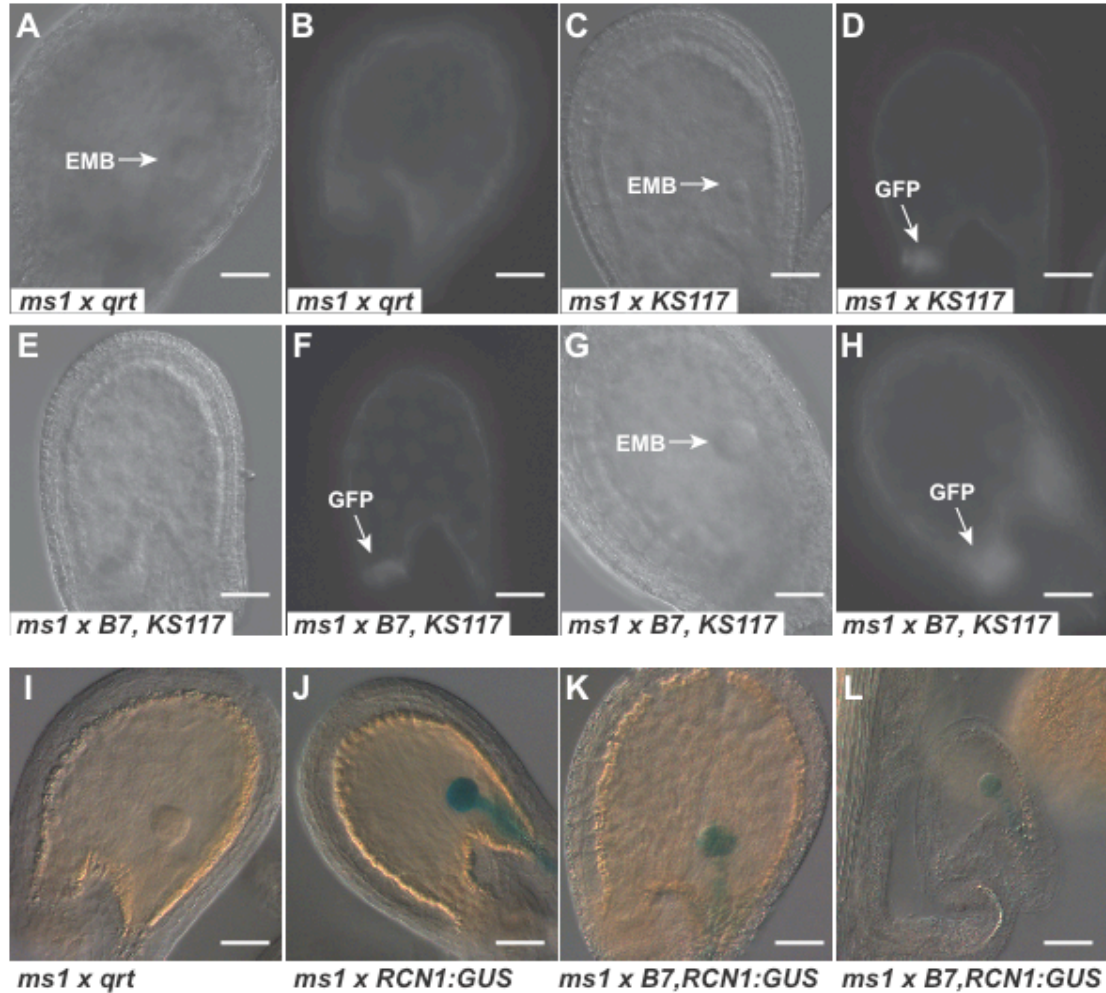
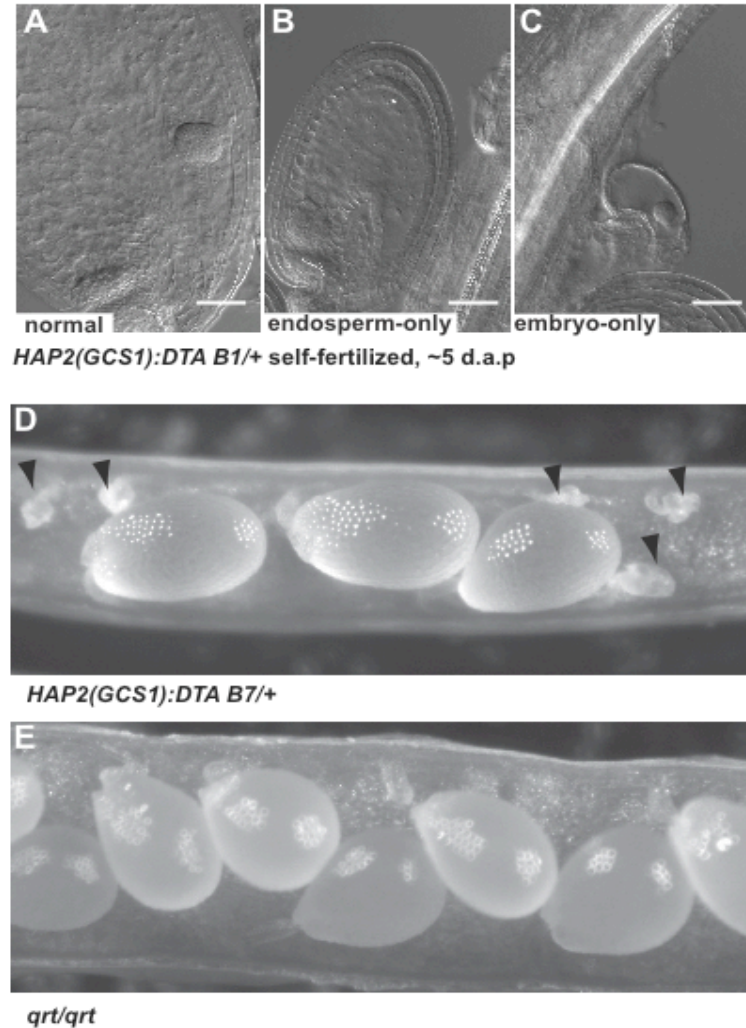
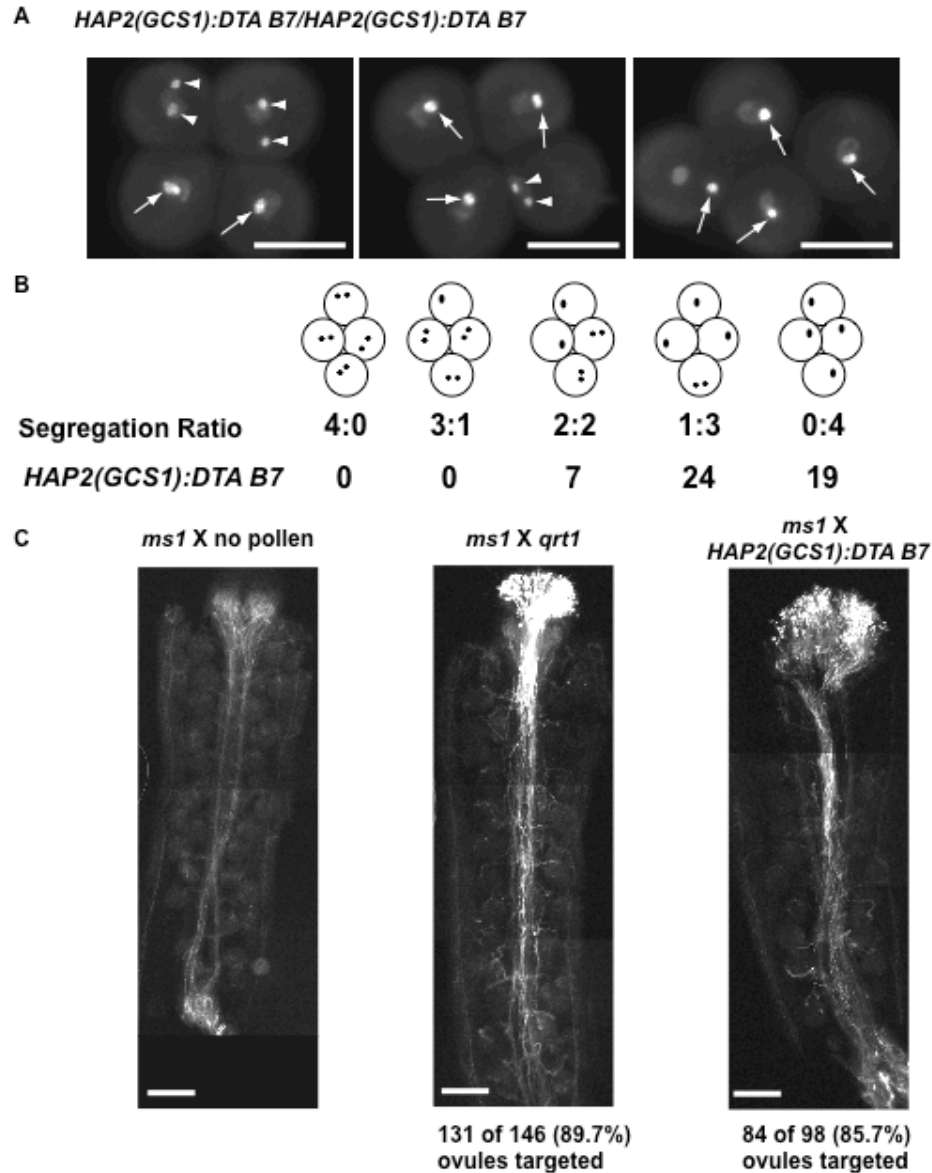


Figure 5. Endosperm-only and embryo-only ovules are the products of fertilization. Analysis of paternal transmission of the *KS117* endosperm marker three days after hand-pollination of *ms1* (A-H) by DIC (A,C,E,G) and fluorescence microscopy (B,D,F,H). Expression of GFP from the *KS117* endosperm marker is highlighted with an arrow (D,F,H) this pattern of fluorescence is present in ovules fertilized by pollen tubes carrying the *KS117* marker alone (D) and when pollen tubes carry *KS117* and *HAP2(GCS1):DTA B7* (F,H). This fluorescence pattern is absent in negative control ovules fertilized by *qrt1* pollen (B). Developing embryos are designated by arrows (A,C,G); endosperm proliferation without embryo development is shown in E. Analysis of paternal transmission of the *RCN1:GUS* embryo marker four days after hand-pollination of *ms1* (I-L). Expression of GUS from the *RCN1* promoter is apparent in ovules fertilized by pollen carrying *RCN1:GUS* alone (J) and when pollen tubes carry *RCN1:GUS* and *HAP2(GCS1):DTA B7* (K,L). GUS activity is absent in control ovules fertilized by *qrt1* pollen (I), and is present in embryo-only ovules (L). Scale bar = 50 μ m.



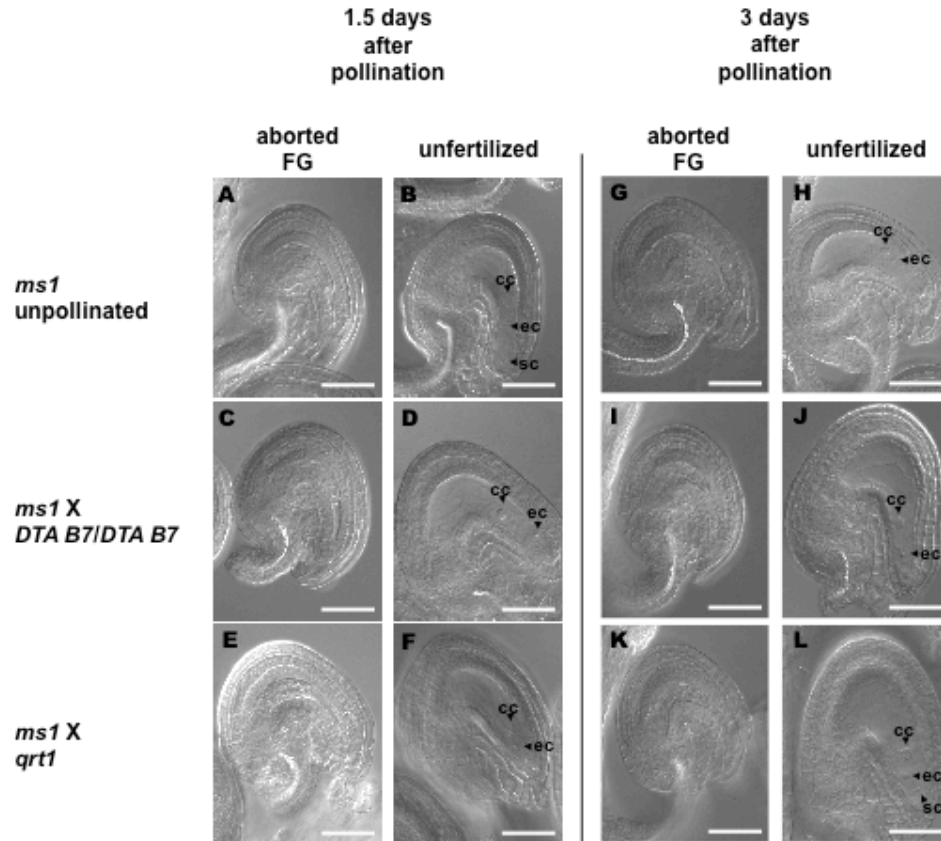
Supplemental Figure S1. *HAP2(GCS1):DTA* causes early paternal effect seed abortion.

Analysis of early seed development in self-fertilized *HAP2:DTA B1* hemizygous plants (A-C). Normal early seed development (A), endosperm-only ovules (B) or embryo-only ovules (C) are observed. Ovules within developing siliques of self-fertilized *HAP2(GCS1):DTA B7* (hemizygous, D) and *qrtI* plants (E). Images (D,E) were obtained using a Zeiss Lumar stereomicroscope with Axiocam MRm camera (Carl Zeiss, Germany). Arrowheads point to aborted ovules containing either the products of single fertilization or unfertilized ovules. Both of these classes of aborted ovules are completely absent in mature siliques. Scale bar = 50 μ m.



Supplemental Figure S2. Analysis of homozygous *HAP2(GCS1):DTA B7* pollen.

A) DAPI stained tetrads of homozygous *HAP2(GCS1):DTA B7* pollen; sperm are indicated by an arrowhead, single sperm-like cells by an arrow. Scale bar = 20µm. B) Schematics represent the segregation of sperm developmental defects in homozygous *HAP2(GCS1):DTA B7* pollen (vegetative nuclei not drawn). C) Pollen tube growth and ovule targeting in *ms1* pistils was analyzed by aniline blue staining 36 hours after pollination. An unpollinated *ms1* pistil is shown as a negative control; fluorescent pollen tubes are obvious in *ms1* pistils pollinated with *qrt1* pollen or with homozygous *HAP2(GCS1):DTA B7* pollen. Individual ovules were scored as targeted if a pollen tube had grown into the micropyle. Scale bar = 200µm.



Supplemental Figure S3. Unfertilized ovules observed in quantitative analysis of early seed development.

Ovule development was analyzed 1.5 days (A-F), 3 days (G-L), and 4 days (micrographs not shown, see Table II) after pollination. Ovules with a female gametophyte (FG) that aborted early in development (A,C,E,G,I,K) occurred regardless pollen. Unfertilized ovules (B,D,F,H,J,L) were observed in which the central cell (cc) and egg (ec) (and occasionally a synergid (sc) as in B and L) nuclei were visible. Scale bar = 50µm.

Tables

Table 1. *HAP2(GCSI):DTA* displays distorted segregation.

	Self-fertilized		Male		Female	
	%Basta ^R	n	%Basta ^R	n	%Basta ^R	n
Transgenic Line						
<i>HAP2(GCSI):DTA B1</i>	57 ^a	72	8 ^a	79	36 ^a	33
<i>HAP2(GCSI):DTA B4</i>	52 ^a	356	17 ^a	207	50	351
<i>HAP2(GCSI):DTA B7</i>	43 ^a	683	1 ^a	446	33 ^a	593
EXPECTED (no defect)	75		50		50	

Self-fertilized, progeny of self-fertilization of primary transformants.

Male: *ms1* x *HAP2(GCSI):DTA*, *ms1* females were hand-pollinated using *HAP2(GCSI):DTA* anthers.

Female: *HAP2(GCSI):DTA* x *qrt1*, *HAP2(GCSI):DTA* females were emasculated and hand-pollinated using *qrt1* anthers.

% Basta^R = the percentage of Basta^R F₁ progeny from the indicated cross.

^a Significantly different from expected value, χ^2 , P<0.01

Table 2. Distribution of ovule development phenotypes following pollination of *ms1* with *HAP2(GCSI):DTA B7* homozygous pollen.

		Fertilized ovules			Unfertilized ovules		Total
		Normal	Endosperm -only	Embryo o-only	Aborted FG	Unfertilized	
	DAP	%	%	%	%	%	
<i>qrt1</i>	1.5	84.1	0.0	0.0	7.2	8.6	511
<i>qrt1</i>	3	88.0	0.0	0.0	9.4	2.6	308
<i>qrt1</i>	4	86.4	0.0	0.0	9.8	3.8	184
no pollen	1.5	0.0	0.0	0.0	13.9	86.0	215
no pollen	3	0.0	0.0	0.0	10.7	89.3	140
no pollen	4	0.0	0.0	0.0	9.8	90.2	132
<i>HAP2(GCSI):DTA B7</i>	1.5	2.9	2.2	1.0	7.5	86.4	412
<i>HAP2(GCSI):DTA B7</i>	3	2.7	9.8	1.0	8.5	78.0	295
<i>HAP2(GCSI):DTA B7</i>	4	6.3	18.2	0.5	12.5	62.5	192

Normal, embryo and endosperm development observed; FG, female gametophyte; DAP, days after pollination

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Chapter 6: Synthesis and Future Directions

In flowering plants, two sperm cells develop in the pollen cytoplasm and are transported through floral tissues to an ovule by a pollen tube, a highly polarized cellular extension (Cheung and Wu, 2008). After targeting an ovule, the pollen tube bursts, releasing two sperm that fertilize an egg and a central cell (Berger et al., 2008). The mechanisms responsible for fusion of the two sperm with respective female cells are unknown.

This thesis presents the initial characterization of the *Arabidopsis HAP2* gene, demonstrating that it is essential for fertilization and probably directly involved in a deeply conserved gamete fusion mechanism. We also show that HAP2 is required for pollen tube guidance, a function that is specific to flowering plants.

Arabidopsis HAP2 was identified in a genetic screen for mutations that blocked the ability of pollen to reproduce (Johnson et al., 2004). A T-DNA insertion was identified (*hap2-1*) that disrupted *HAP2* and tagged mutant pollen grains with the β -glucuronidase (GUS) reporter gene (Johnson et al., 2004). By monitoring GUS activity in growing pollen tubes, we showed that *hap2-1* does not diminish pollen tube length in vitro or in the pistil, but it reduces ovule targeting by two fold (von Besser et al., 2006). In addition, we showed that *hap2-1* sperm that were delivered to ovules failed to initiate fertilization. *HAP2* was predicted to encode a protein with an N-terminal secretion signal, a single transmembrane domain and a C-terminal histidine-rich domain. These results pointed to a dual role for HAP2, functioning in both pollen tube guidance and in fertilization. Moreover, these findings suggested that sperm, long considered to be passive cargo, were involved in directing the pollen tube to its target.

When *HAP2* was disrupted in the green alga *Chlamydomonas*, gametes were able to adhere to one another, but unable to fuse (Liu et al., 2008). These data, combined with the

finding that HAP2 specifically localized to the tip of the minus gamete mating projection (Liu et al., 2008) suggested that HAP2 is required specifically for gamete membrane fusion and that it may be directly involved in the process. The function of HAP2 was also found to be conserved in the malaria parasite, *Plasmodium*, where *HAP2* knockouts were shown to be unable to reproduce even though gametes could bind each other (Hirai et al., 2008; Liu et al., 2008). *HAP2* has been identified in all of the completed flowering plant genomes and in several animal and protozoan genomes (Hirai et al., 2008; Liu et al., 2008; Mori et al., 2006; Steele and Dana, 2009; von Besser et al., 2006). This widespread distribution, coupled with the demonstration of conserved function in three distantly related eukaryotic organisms, suggests that HAP2 may be part of an ancestral mode of membrane fusion. Whether HAP2 is directly involved in mediating membrane fusion or is required to activate a gamete fusion mechanism has not yet been established. Furthermore, the molecular mechanism of HAP2 function is unknown, but remains a critical goal for future research.

One model for the function of HAP2 is that it interacts with a partner protein present on the female gamete. This hypothesis is based on well-characterized protein complexes that fuse membranes through protein:protein interactions that bring membranes into close proximity favoring fusion (reviewed in (Sollner, 2004)). To test this idea, we sought to ectopically express HAP2 at the site of gamete fusion (Chapter 4). If HAP2 functions by a protein:protein interaction, we reasoned that ectopically expressed HAP would disrupt fusion by competing for access to the HAP2 binding partner. The results of these experiments suggested that HAP2 expression and localization may be under a variety of regulatory controls. We were unable to express HAP2 in somatic cells of *Arabidopsis* or in cells near the female gametes. HAP2 was expressed in pollen tubes, but this did not disrupt

gamete fusion. Interestingly, rather than blocking the function of sperm-expressed HAP2, expression of HAP2 from the pollen-specific LAT52 promoter, rescued the *hap2-1* fertilization defect. One explanation for this result is that the LAT52 promoter is active in sperm. A second possibility is that HAP2 function can be supplied to the sperm when it is expressed in the pollen cytoplasm. This latter possibility could have important mechanistic implications because this would mean that HAP2 might be able to function in trans, outside of the sperm membrane as either a signaling molecule, or that HAP2 is cleaved from the sperm membrane before it functions to induce fusion. To test these possibilities it would be ideal to make artificial vesicles that contained functional HAP2 and inject these into growing *hap2-1* pollen tubes and observe if double fertilization is able to proceed normally with the exogenously introduced HAP2.

We set out to find proteins that interact with HAP2 utilizing a yeast two-hybrid approach (Chapter 3). We screened a library of flower-expressed protein coding sequences for interactions with part of the amino terminal region of HAP2 that lacked a signal sequence and the transmembrane domain. The goal was to identify egg- and central-cell expressed proteins that may interact with HAP2 to mediate gamete fusion. The strongest and most numerous potential interactions were with disulfide bond altering proteins. Alignments of HAP2 from even the most distantly related species define at least 10 invariant cysteine residues. We propose that these cysteine residues define the structure of HAP2 by forming disulfide bonds within the extracellular HAP2 N-terminal region. The overrepresentation of disulfide bond altering proteins suggests the possibility that this type of enzyme interacts with HAP2, perhaps to ensure proper folding and disulfide bond formation between the deeply conserved cysteine residues in the amino terminus of HAP2.

Through yeast two hybrid screening with HAP2 we have identified 32 possible interacting proteins. Although we expected to find egg and central cell expressed proteins to interact with HAP2 to promote membrane fusion, these were not found in the yeast two hybrid screen. This lack of female expressed genes could be due to their scarcity within the library or because HAP2 was not folded correctly to interact with these proteins, or that a change occurs within HAP2 when it reaches the ovule and this was not the isoform taken in the yeast two hybrid screen.

Future experiments will be aimed at determining whether any of the proteins found through the yeast two hybrid approach are essential for gamete fusion and whether they interact with HAP2 in vivo. Mutations within these genes can be tested in the plant to find if any of them show a similar fertilization defect as *hap2-1*. To test whether the proteins interact with HAP2 in vivo, split GFP constructs can be cloned, with half within HAP2 near the N-terminus, and half of the GFP on the potential interactor, such that if the two proteins come into close contact a fluorescent signal can be detected.

To test which of the highly conserved cysteine amino acids are required for HAP2 function we can make targeted mutations of these residues and determine if they can rescue the *hap2* fertilization defect. Perhaps through this method of singling out specific cysteine residues we can begin to determine which are essential for the structure of HAP2. It will also be interesting to determine whether Arabidopsis sperm express specific disulfide isomerases responsible for defining the correct structure of HAP2. Microarray analysis of sperm has defined a limited set of these enzymes (Borges et al., 2008), which can now be tested for their role in the regulation of HAP2 function. By determining which cysteines are important for HAP2 function we can know more about which regions are available to interact with

other proteins or with the female cell membranes and if a conformational change is important for HAP2 to mediate membrane fusion.

After meiosis, the male germline of flowering plants undergoes two mitoses, producing two sperm that are carried within a pollen tube to an ovule. One sperm fuses with the egg to form the zygote and the other fuses with the central cell to form the primary endosperm. The mechanisms that control male germline development and gene expression, and ensure that sperm properly fuse with female gametes are just beginning to be understood. Expression of the potent translation inhibitor, diphtheria toxin A subunit, from the *HAP2* promoter blocked sperm development before the final cell division, resulting in pollen tubes that carried a single sperm-like cell rather than two sperm (Frank and Johnson, 2009). These pollen tubes targeted ovules and fertilized either the egg or the central cell, producing seeds with either endosperm or an embryo, but not both. Endosperm-only seeds significantly outnumbered embryo-only seeds, suggesting that single sperm-like cells preferentially fuse with the central cell. These experiments show that de novo translation is required for completion of sperm development, that the *HAP2* promoter is very tightly controlled, and that disruption of gene expression can result in male germ cells with a bias for gamete fusion. Although it was known that gene expression was required to complete sperm development, the requirement for protein translation after DNA synthesis is a novel finding since sperm have long been thought to be quiescent.

There are multiple experiments that can help us determine what proteins are expressed by the sperm to determine female cell preference and fertilization. One of these would be to compare expression profiles of the HAP2:DTA and *cdc2* mutant single sperm-like cells (SSLCs). This experiment might require that the SSLCs be marked with

fluorescence such as Duo1:RFP and isolated SSLCs be FACS sorted before RNA is isolated and a microarray assay being performed.

A recent method for marking sperm nuclei with fluorescence has led to the ability to watch fertilization within the ovule occur in real time (Berger et al., 2008; Ingouff et al., 2007). We have recently adapted these methods and can image single sperm-like cells (SSLCs) after they have been deposited near female gametes (Figure 1 and data not shown). This technique will allow us to image SSLCs as they migrate to, and fuse with a female target. These experiments will allow us to define the mode of sperm transport within ovules that allow sperm to find their female targets. If SSLCs travel to central cells more often than to the egg it is likely that what has been disrupted by the HAP2:DTA to induce a bias for the central cell is a factor for sperm motion or cell recognition. However if SSLCs are found to associate with both female cells with equal frequency it is likely that HAP2:DTA has disrupted other factors required for fusion. Furthermore, these experiments will allow us to determine whether there is a specific site on the female gamete membrane where sperm fuse. If sperm are always found in the same location in relation to the egg and central cell it would show that there is a specific membrane location for fusion, perhaps containing all the proteins required for this mechanism.

We can also use this method to determine at what step in fertilization various *HAP2* mutants are defective. For example, neutralizing the charge of its positively charged carboxy-terminus (Wong et al., 2010) resulted in a hypomorph of HAP2. This hypomorph almost completely eliminates HAP2 function and could be used to determine the precise step in the fertilization process where *hap2* mutant sperm fail. This information would be useful for determining the precise function of HAP2 in flowering plants and whether this function

is the same as that identified in *Chlamydomonas* (Liu et al., 2008) and *Plasmodium* (Hirai et al., 2008; Liu et al., 2008). By knowing how the hypomorph and mutants of HAP2 travel and interact with female cells we can determine how alterations to the protein have an effect on fertilization.

The *hap2-1* pollen tube guidance defect is subtle, reducing the ability to target ovules by about two fold. Furthermore, it is difficult to formulate a hypothesis for how a sperm-resident protein could participate in a mechanism that directs the extension of the pollen tube tip. However, the sperm are located near the pollen tube tip and HAP2 is expressed in sperm as pollen tubes grow through the pistil. So, the extracellular portion of HAP2 could contact proteins within the pollen tube cytoplasm that determine the direction of pollen tube extension. Using a series of HAP2 mutant constructs already established in our lab (Wong et al., 2010), we could determine which regions of HAP2 are required for pollen tube guidance. Furthermore, we can test whether sperm-expressed proteins identified as potential HAP2 interacting proteins are required for efficient pollen tube guidance. We have recently shown that antibodies raised against the HAP2 N-terminus specifically recognize HAP2 and HAP2:YFP in membrane fractions from pollen (Leydon, Beale, Wong, unpublished). These antibodies can be used to immunoprecipitate proteins that interact with pollen tubes to mediate pollen tube guidance.

HAP2 is the first protein directly implicated in gamete fusion mechanisms that is conserved across a broad array of eukaryotic organisms. One hypothesis for why HAP2 has been conserved over a large evolutionary distance states that in organisms that have HAP2, species specificity is determined by other mechanisms and that membrane fusion is most efficient with HAP2. To maintain species specificity they could employ proteins involved

in the targeting of the gametes or in the binding and association of the gametes. Organisms that evolved away from these mechanisms of species specificity might have required membrane fusion proteins that also conferred the ability to discern between closely related gametes and thus these organisms evolved away from utilizing HAP2 for membrane fusion.

By determining the biochemical function of HAP2, we may be able to elucidate a mechanism through which gamete membranes fuse. These mechanisms remain mysterious despite its central importance to the life cycle of all sexually reproducing eukaryotes. Reproduction is a critical biological process that is at the heart of evolution, development, and population dynamics. It is also responsible for the spread of diseases, including malaria and Chagas disease in humans caused by *Plasmodium falciparum* and *Trypanosoma cruzi*, respectively. Reproduction of Plasmodium has been blocked by antibodies against the extracellular portion of HAP2 (Blagborough and Sinden, 2009), and HAP2-derived vaccines could be produced to prevent transmission of these parasites. The ability to control fertilization in plants by blocking HAP2 could be used to generate transgenic crop plants that are incapable of cross-pollinating wild-relatives. This would allow biotechnologists to ensure that genes controlling agriculturally important traits like herbicide resistance are not introduced into wild populations.

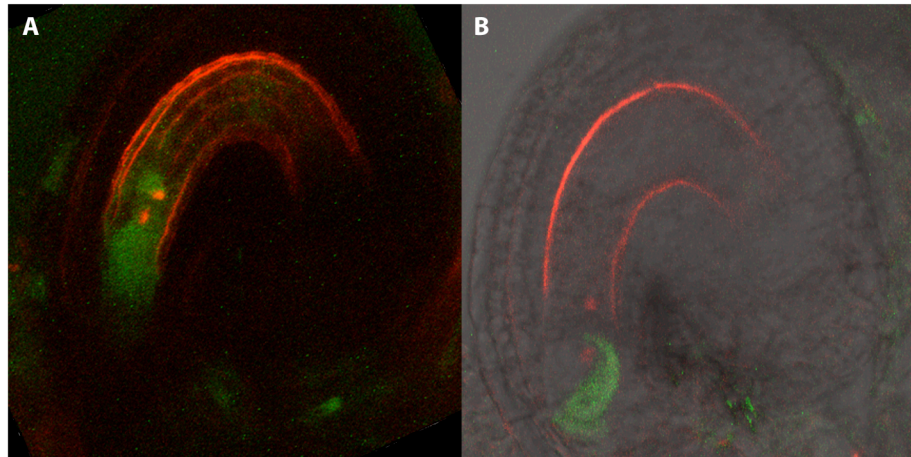


Figure 1: Live imaging of fluorescent sperm entering wild type ovules.

Sperm carrying the H3.3:RFP are marked by red nuclei and the pollen tube cytoplasm is shown by the Lat52:GFP where the pollen tube has burst in the synergid cell space of the ovule. In these images the sperm membranes have fused with the egg and central cell and the nuclei have migrated in anticipation of fusing with the nuclei of the female cells.

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