Development 133, 4761-4769 (2006) doi:10.1242/dev.02683

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

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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 *GCS1*. *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.

KEY WORDS: *Arabidopsis*, Pollen tube guidance, Double fertilization, Sperm, Gamete interactions

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 *maa1* 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. Laser-ablation 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* (*ZmEA1*) 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.

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Accepted 4 October 2006

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, Basta^R), as well as a cell-autonomous histochemical marker (β-glucoronidase, 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% Basta^R offspring when self-fertilized. By contrast, self-fertilization of *hap2/HAP2* plants yielded ~50% Basta^R 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 Basta^R 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.

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 μE fluorescent lighting or in ambient greenhouse conditions. Kanamycin resistance (Kan^R; *hap2-2*) was determined on MS salts with 25 μg/ml kanamycin, and Basta^R (*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 μg/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′-GGCCTCACTCGTTC-TCAATTGGAG-3′) and *HAP2* downstream (5′-GTGAGAGTCGCTGTG-GTCACGTTC-3′). The *hap2-2* T-DNA-gene junction was confirmed with the PCR primers LBa1 (Alonso et al., 2003) and HAP2seqTR3 (5′-CAATCAAACTGCGCAGAAGGAAGC-3′). Full-length *HAP2* cDNA (GenBank DQ022375) was amplified from 200 ng total pollen RNA using primers F, 5′-AAACAATTTTCAATTCGCGTCTCCG-3′ and R, 5′-AACTCGGATATATTTTTGC-3′. For complementation, a genomic *HAP2* fragment was PCR amplified with F, 5′-GGCCCTGCAGGTTCTGAT-CCTAACAACAACGGCGGC-3′, and R, 5′-CCGCCTGCAGGATATC-TTCGAGAGAATCACCAGTCGCC-3′, inserted into the *SbfI* site of pCAMBIA2300 (GenBank AF234315), sequenced, introduced into *Agrobacterium* (strain GV3101), and transformed into *hap2-1/HAP2* plants, selecting Kan^R.

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 μM 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 μg of total pollen RNA probed with *HAP2* cDNA. RT-PCR was performed on 1 μg total pollen RNA using the *HAP2* primers F, 5′-TTAATGGCTTGTATACTCGCCGG-3′ and R, 5′-ACGAAGGCAATGCGCGGTATTTGCC-3′, and *EF-1α* controls F, 5′-GCCCCTTCGTCTCCCACTTC-3′ and R, 5′-CACTTCGCACCCTT-CTTGACG-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′-GGCGAGCTCAATTTCC-CTGATACAATCCCGAGGC-3′ and *HAP2BamHIR*, 5′-GGCGGATCC-TTTCTCTCTCACGGAGACGCG-3′; *HAP2protein:YFP* fusion contained the *HAP2* promoter and all exons and introns, and was amplified using primers *HAP2SacIF* and *HAP2NcoR*, 5′-GGCCCATGGTACTCTCACG-TAGTCTTTGTTTCCTC-3′. Amplified products were digested with *Sac*I and *Bam*HI, or *Sac*I and *Nco*I, respectively, and introduced upstream of eYFP (Clonetech) 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 63× 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).

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

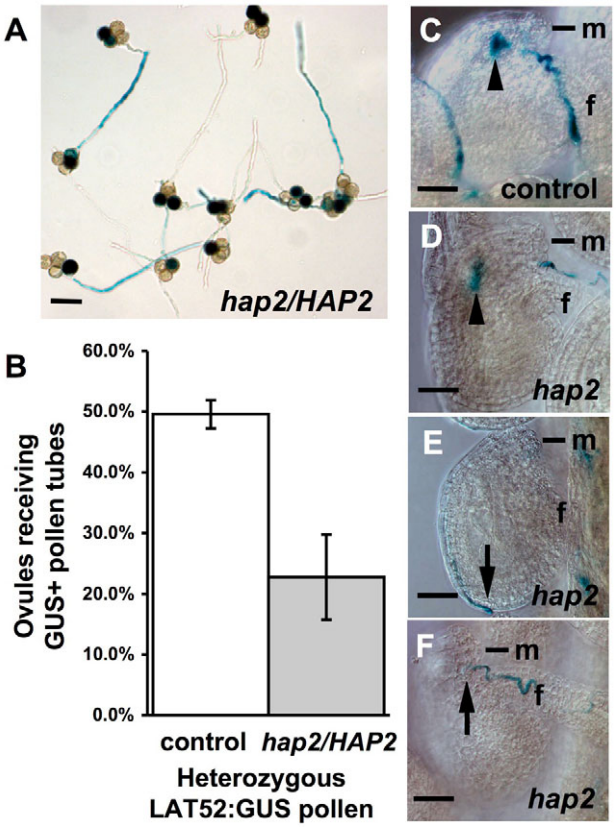


Fig. 1. *hap2* disrupts pollen tube guidance. (**A**) In vitro grown *hap2/HAP2* pollen tubes stained to reveal GUS expression in *hap2* pollen tubes. (**B**) Quantitative analysis of ovule targeting. *ms1* pistils were hand-pollinated with *hap2/HAP2* or control pollen heterozygous for LAT52:GUS. The number of ovules receiving GUS activity from the pollen tube was counted for each and is plotted as a percentage (±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 μm in A; 20 μm in C-F.

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 μm, s.d. 217 μm, *n*=62; *HAP2*, mean 372 μm, s.d. 195 μm, *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. 4%) 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 *ms1* 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 F_1 tested; *hap2-2*, 348 F_1 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 F_1 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),

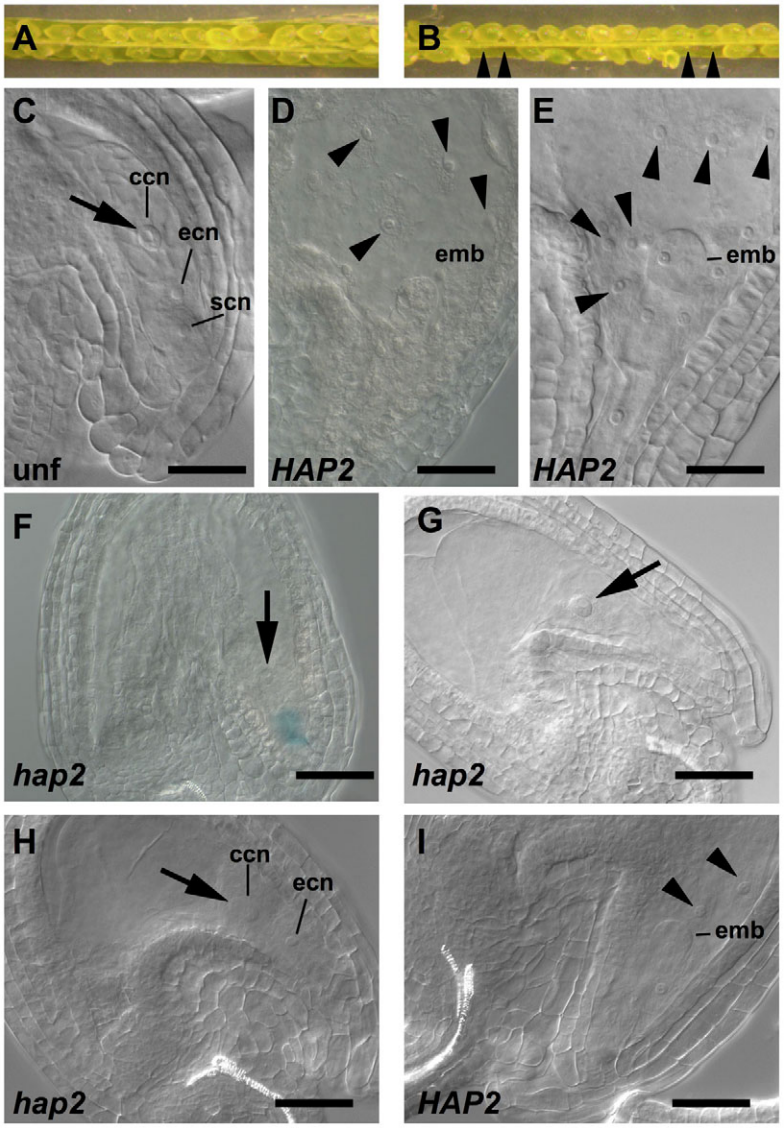


Fig. 2. *hap2* blocks egg fertilization and central cell fertilization. Siliques from self-fertilized plants heterozygous for a control T-DNA insertion (**A**) or *hap2/HAP2*, which contain aborted ovules (**B**, arrowheads). (**C**) An unfertilized ovule showing nuclei of FG cells. (**D-I**) Ovules 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 μm.

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

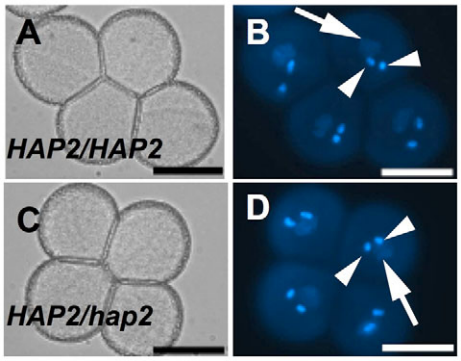


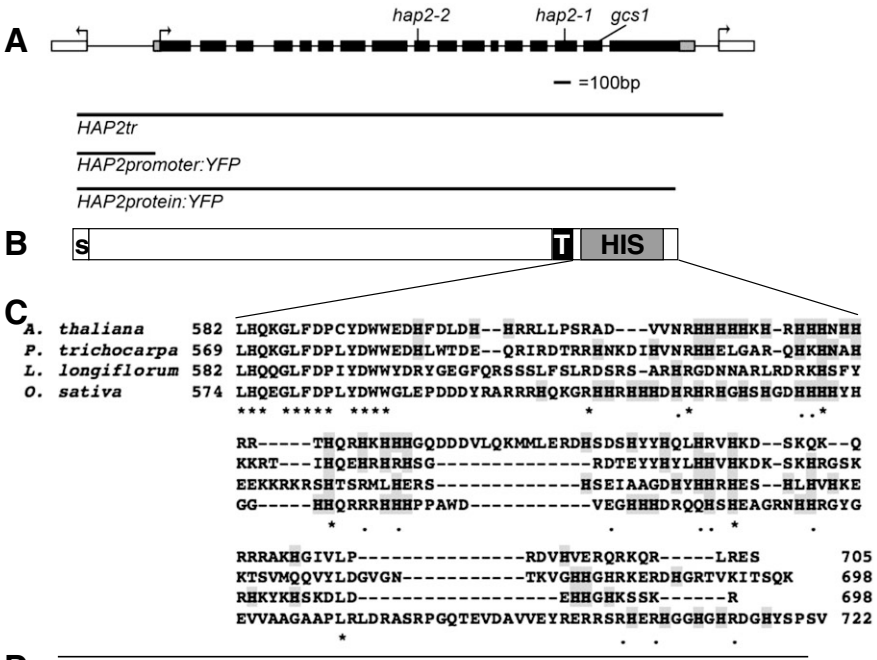
Fig. 3. *hap2* pollen contains a normal male germ unit. (**A-D**) Pollen tetrads imaged with bright field (A,C) or epifluorescence (B,D) following DAPI staining. The morphology of pollen grains, vegetative nuclei (arrows) and sperm nuclei (arrowheads) were indistinguishable in tetrads from wild-type (A,B) and *hap2/HAP2* plants (C,D). Scale bars: 20 μm.

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% Kan^R progeny from self-fertilization, *n*=600) and completely blocked transmission through pollen (0 Kan^R 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 Kan^R) 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 Basta^R and Kan^R were analyzed in ten transgenic families with a single-locus insertion of *HAP2tr*. The average rate of Basta^R among these families was 68.6% (*n*=2362) and of Kan^R was 83.1% (*n*=2525). Complementation of both ovule-targeting and fertilization defects is expected to yield T2 progeny that segregate 67% (8/12) Basta^R and 83% (10/12) Kan^R; failure would lead to approximately 50% Basta^R, 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.



Angiosperm HAP2 proteins have a H-rich C-terminal domain

	Length (AA)	AA identity	AA identity (N)	AA identity (C)	H in C-term.
Arabidopsis thaliana	705	100.0%	100.0%	100.0%	22.0%
Populus trichocarpa	698	66.1%	70.9%	41.5%	17.1%
Lilium longiflorum	698	56.4%	63.2%	26.0%	15.5%
Oryza sativa	722	55.0%	58.9%	37.4%	23.0%
Chlamydomonas reinhardtii	748	24.8%	27.9%	8.1%	3.2%
Cyanidioschyzon merolae	744	20.0%	21.3%	10.6%	0.0%
Plasmodium falciparum	889	19.7%	20.9%	17.1%	2.2%
Physarum polycephalum	808	23.7%	24.8%	18.7%	1.2%
Leishmania major	917	23.8%	25.0%	23.6%	2.4%

AA, amino acid; H, histidine; N, region of protein N-terminal to the transmembrane domain; C, region of protein C-terminal to the transmembrane domain; angiosperms are shaded

Fig. 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.

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% Basta^R; 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 F_2 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

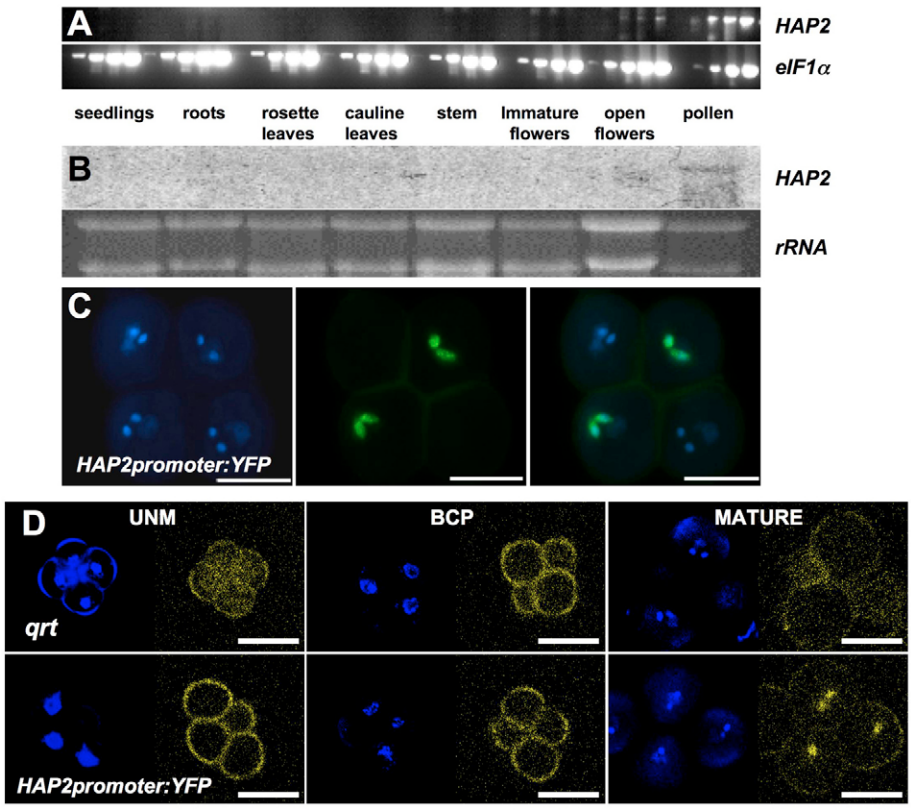


Fig. 5. *HAP2* expression is sperm specific. (**A**) RT-PCR of wild-type tissues, using primers spanning *HAP2* exon 2 (25-40 cycles). (**B**) Northern blot of wild-type RNA probed with full-length *HAP2* cDNA; ethidium bromide stained gel before blotting shows relative RNA amounts in each lane. The 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 μm.

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

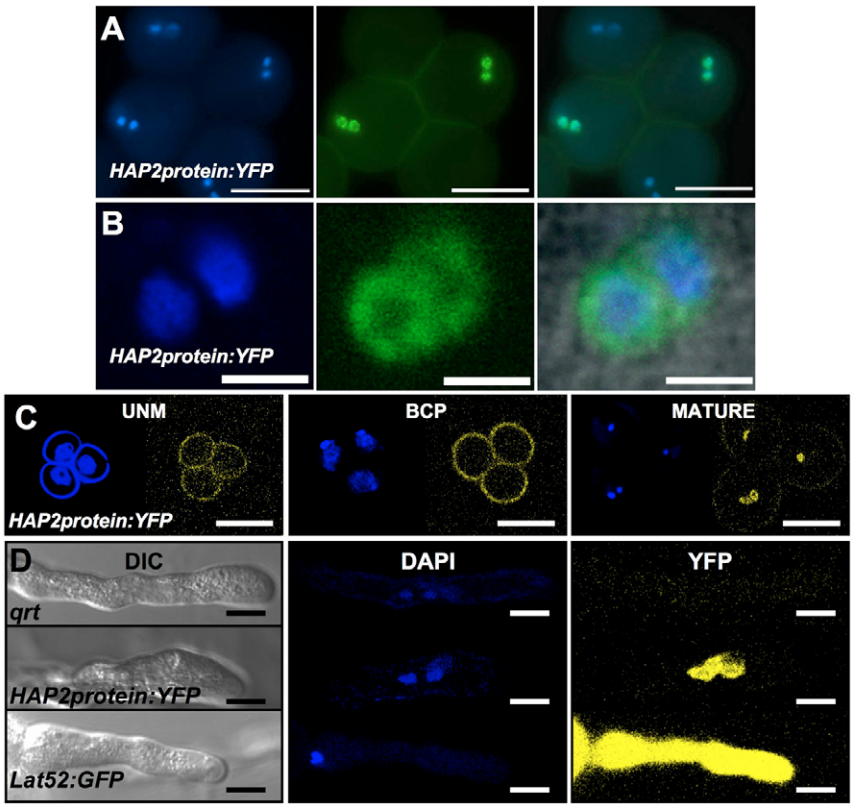


Fig. 6. HAP2 protein is sperm-localized during pollen development and tube growth. (**A**) A pollen tetrad and (**B**) sperm nuclei from a plant heterozygous for the *HAP2protein:YFP* fusion; DAPI fluorescence (left), YFP fluorescence (center), merged image (right). (**C**) DAPI (left) and YFP (right) 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.

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 repellant. 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.

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.

Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/133/23/4761/DC1

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