Expressing the Diphtheria Toxin A Subunit from the HAP2(GCS1) Promoter Blocks Sperm Maturation and Produces Single Sperm-Like Cells Capable of Fertilization1[W][OA]

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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 (Arabidopsis thaliana) HAP2(GCS1) 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(GCS1) promoter is very tightly controlled, and that disruption of gene expression can result in male germ cells with a bias for gamete fusion.

Flowering plants have a unique reproductive system called double fertilization. Two nonmotile 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 (Zea mays), 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 (Roman, 1948; Carlson, 1969; Faure et al., 2003). 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., 1999a; 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., 1999a, 1999b; Engel et al., 2003, 2005; Singh et al., 2003; Durbarry et al., 2005; Okada et al., 2005; Rotman et al., 2005) and microarray analysis shows that Arabidopsis (Arabidopsis thaliana) 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 P. 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 (DTA) chain from the male germline-specific HAP2(GCS1) (for HAPLESS2, GENERATIVE CELL SPECIFIC; A4g11720) 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; Thorsson 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 (Thorsson et al., 1991) and gametophytic (Thorsson 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(GCS1)promoter: DTA [HAP2(GCS1):DTA] construct. This result is consistent with recent data showing that DUO POLLEN1 (DUO1) and DUO3 are required to activate HAP2 (GCS1) transcription in the generative cell (Brownfield et al., 2009a, 2009b). Furthermore, it demonstrates that de novo translation in the generative cell is necessary for completion of sperm development. HAP2(GCS1):

**RESULTS**

Expression of HAP2(GCS1):DTA Generates Mature Pollen Grains Containing a SSLC

We transformed Arabidopsis quartet1 (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 SSLCs.

We determined the segregation of this defect in tetrads of three transgenic lines (Fig. 1B); for HAP2 (GCS1):DTA B7, the majority (92/106; Fig. 1, A and B) of tetrads comprised two normal pollen grains and two that contained a SSLC. This 2:2 segregation pattern in tetrads indicated that the plant was hemizygous for the transgene, the transgene caused a postmeiotic 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 SSLC. 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(GCS1):DTA. The expressivity of pollen developmental defects was lower in two other lines analyzed [HAP2(GCS1):DTA B1 and B4; Fig. 1B].

In reciprocal crosses to wild type, HAP2(GCS1):DTA behaved like a mutation that disrupts the development and/or function of the male gametophyte. We used pollen from three independent hemizygous HAP2(GCS1):DTA transgenic lines to pollinate male
sterile1 (ms1) flowers and determined how many of the F1 progeny inherited the HAP2(GCS1):DTA transgene by scoring Basta resistance [BastaR; the HAP2(GCS1):DTA T-DNA carries a BastaR gene]. When HAP2(GCS1):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(GCS1) promoter is active in female cells, consistent with a recent report that HAP2(GCS1) mRNA is present in ovule and silique samples (Borges et al., 2008). HAP2(GCS1) 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(GCS1) is currently not known. Furthermore, DUO1, a critical regulator of HAP2(GCS1) 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 SSLCs

To determine the stage at which DTA expression disrupted male germline development, we measured the fluorescence intensity of 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei of SSLCs and normal sperm in HAP2(GCS1):DTA B7 and HAP2(GCS1):DTA B1 pollen. In both lines, the fluorescence intensity of

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**Table 1.** HAP2(GCS1):DTA displays distorted segregation  
Self-fertilized, Progeny of self-fertilization of primary transformants. Male: ms1 × HAP2(GCS1):DTA, ms1 females were hand pollinated using HAP2(GCS1):DTA anthers. Female: HAP2(GCS1):DTA × qrt1, HAP2(GCS1):DTA females were emasculated and hand pollinated using qrt1 anthers. %BastaR = the percentage of BastaR F1 progeny from the indicated cross.

| Transgenic Line          | Self-Fertilized | Male | Female |
|--------------------------|-----------------|------|--------|
|                          | %BastaR n       | %BastaR n | %BastaR n |
| HAP2(GCS1):DTA B1        | 57              | 72     | 8       |
| HAP2(GCS1):DTA B4        | 52              | 356    | 17      |
| HAP2(GCS1):DTA B7        | 43              | 683    | 1       |
| EXPECTED (no defect)     | 75              | 50     | 50      |

*Significantly different from expected value, χ², P < 0.01.*
SSLCS 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(GCS1):DTA.

Expression of Sperm Identity Markers in SSLCS

To explore the identity of SSLCS and to assess the impact of the HAP2(GCS1):DTA construct on sperm gene expression, we analyzed four male germline markers in HAP2(GCS1):DTA B7 transgenic plants: DUO1:H2B:mRFP1 (Rotman et al., 2005), HISTONE THREE RELATED10 (HTR10):HTR10:mRFP1 (Ingouff et al., 2007), HAP2(GCS1):HAP2(GCS1):YFP (von Besser et al., 2006), and GAMETE EXPRESSED2 (AtGEX2): eGFP (Engel et al., 2005; Fig. 2, A–D). DUO1 is a Myb protein required for expression of other generative and sperm cell–expressed genes including HAP2(GCS1), AtGEX2, and HTR10 (Brownfield et al., 2009a). We analyzed hemizygous HAP2(GCS1):DTA plants that were hemizygous for DUO1:H2B:mRFP1. In this experiment, a maximum of approximately 50% of pollen with either normal sperm or a SSLC carried the reporter construct. We found that approximately 50% of pollen of either type expressed DUO1:H2B:mRFP1, indicating that almost all SSLCS that carried this reporter expressed it (Fig. 2, A and E). Consequently, we suggest that the DUO1:H2B:mRFP1 reporter construct is translated in SSLCS before DTA begins to block translation. Similarly, nearly all SSLCS expressed HTR10:HTR10:mRFP1 (Fig. 2, B and E). Microarray analysis of sperm showed that HTR10 transcripts were among the most abundant and accumulated to significantly higher levels than HAP2(GCS1) 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(GCS1):DTA pollen that was homozygous for HAP2(GCS1):HAP2(GCS1):YFP or AtGEX2:eGFP (100% of pollen carry the reporter) showed that many SSLCS expressed these markers, but a significant fraction did not (Fig. 2, C–E). The HAP2(GCS1) and AtGEX2 promoters are expected to be activated contemporaneously with the HAP2(GCS1):DTA construct. We propose that in some single-sperm–like cells, detectable levels of HAP2(GCS1):HAP2(GCS1):YFP or AtGEX2:eGFP were translated, whereas in other SSLCS 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 SSLCS express the endogenous HAP2(GCS1) and AtGEX2 genes, while others do not. HAP2(GCS1) 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 SSLCS to fertilize female gametes.
Pollen Tubes Carrying a SSLC Germinate and Grow Like Wild Type

We germinated pollen from HAP2(GCS1):DTA B7 and HAP2(GCS1):DTA B1 hemizygous plants in vitro and stained them with DAPI to identify pollen tubes that carried SSLCs and those with two normal sperm (Fig. 3A). In both cases, the number of growing pollen tubes that contain a SSLC was roughly equivalent to the number containing two sperm (Fig. 3B). This indicated that pollen grains with a SSLC germinated at the same rate as wild-type pollen grains. Furthermore, after 6 h of pollen tube growth, the length of HAP2(GCS1):DTA B7 and HAP2(GCS1):DTA B4 pollen tubes with a SSLC was the same as that of pollen tubes with two sperm (Fig. 3C). Expression of DTA from the HAP2(GCS1) 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(GCS1) 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(GCS1):DTA expression caused pollen tube guidance defects in competitive crosses because the incomplete expressivity of DTA defects precluded quantitative analysis of ovule targeting.

SSLCs Fertilize the Central Cell More Often Than the Egg

We analyzed embryo and endosperm development in pistils of self-fertilized hemizygous HAP2(GCS1):DTA B7, HAP2(GCS1):DTA B1, and HAP2(GCS1):DTA B4 transgenic plants approximately 5 d 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(GCS1):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 SSLC. Neither endosperm-only nor embryo-only seeds completed development; they resulted in aborted ovules that were obvious in developing siliques of self-fertilized HAP2(GCS1):DTA hemizygous plants (Supplemental Fig. S1).

In each of these transgenic lines, endosperm-only ovules significantly outnumbered embryo-only ovules, suggesting that SSLCs preferentially fertilize the central cell. We performed a quantitative analysis of seed development by pollinating ms1 pistils with pollen from homozygous HAP2(GCS1):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(GCS1):DTA, but was not defective in generative cell division or expression of essential sperm genes (Figs. 1B and 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

Figure 3. Pollen tubes containing a SSLC germinate and grow similarly to pollen tubes with two sperm. A, DAPI-stained pollen tubes with a SSLC or two normal sperm from two hemizygous HAP2(GCS1):DTA lines. Arrowheads denote sperm, arrows SSLCs, 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 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 SSLC (gray bars); error bars represent SD; numbers of tubes measured is indicated in each bar.
development because approximately 80% of its pollen carried SSLCs (Supplemental Fig. S2, A and B) and pollen tubes targeted approximately 90% of ms1 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 d after pollination of ms1 (Fig. 4) and found that endosperm-only ovules outnumbered embryo-only ovules at each time point when homozygous HAP2(GCS1):DTA B7 pollen was used (Fig. 4; Table II). In total, we observed 73 endosperm-only ovules and eight embryo-only ovules, a ratio of 9:1. These classes of aberrant ovule development were not observed when qrt1 pollen was used to pollinate ms1 or when no pollen was applied to ms1 (Table II). Significantly, a central cell nucleus is clearly apparent in embryo-only ovules (Fig. 4, C, F, and I); this observation is consistent with the hypothesis that embryo-only ovules are the result of fertilization of the egg by a SSLC. The central cell remains unfertilized and does not proliferate autonomously (Fig. 4, C, F, and I). This observation distinguished SSLCs produced by expression of HAP2(GCS1):DTA from those of cell division cycle2 (cdc2) or f-box-like17 (fbl17) mutants (Nowack et al., 2006; Kim et al., 2008; Gusti et al., 2009). In these cases, egg-only fertilization by a SSLC initiated division of the primary endosperm nucleus.

We analyzed 899 ovules across the three time points in pistils pollinated with homozygous HAP2(GCS1):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(GCS1):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 ovule development confirmed the role of DTA in SSLC development and fertilization.

Figure 4. SSLCs 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, and G), or ms1 and pollen homozygous for HAP2 (GCS1):DTA B7 (B, C, E, F, H, and I) at 1.5, 3, and 4 d after pollination. Normal early seed development (A, D, and G), and endosperm-only ovules (B, E, and H) or embryo-only ovules (C, F, and I) are shown. Insets in C and F show the unfertilized central cell nucleus in a different focal plane. Scale bar = 50 μm; en, Endosperm nuclei; em, embryo; cc, central cell nucleus.
ovules cannot be explained by failure of HAP2(GCS1):DTA B7 pollen tubes to target ovules (Supplemental Fig. S2C) and is likely due to lack of expression of HAP2(GCS1) (Fig. 2, C and 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 approximately 20% of pollen grains, but double fertilization was observed in only approximately 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 SSLCs carrying HAP2(GCS1):DTA were delayed relative to wild type.

**Initiation of Endosperm or Embryo Development by a SSLC Requires Fertilization**

We considered the possibility that deposition of a SSLC 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 FERTILIZATION-INDEPENDENT SEED2, FERTILIZATION-INDEPENDENT ENDOSPERM, or MEDEA 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 SSLCs promoted autonomous endosperm proliferation, we examined whether a SSLC 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. 5, A and C); GFP signal was only detectable in ovules fertilized by KS117 pollen (Fig. 5, D compared to B). We generated plants hemizygous for HAP2(GCS1):DTA B7 and KS117 and used these to pollinate ms1. Importantly, in these crosses, we observed endosperm-only ovules (Fig. 5E) and products of double fertilization (Fig. 5G) that express the KS117 marker (Fig. 5, F and H), indicating that endosperm initiation resulted from fertilization by a SSLC and not autonomous endosperm proliferation.

To determine whether embryos developing in embryo-only ovules were the product of fertilization by a SSLC we used an RCN1 promoter:GUS (RCN1:GUS) transgene as a paternal marker for embryo gene expression. RCN1 (for ROOTS CURL IN NPA1) 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 ms1 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 ms1 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 SSLC carrying the RCN1:GUS reporter gene.

**DISCUSSION**

**HAP2(GCS1):DTA Transgenic Plants Offer a New Tool for the Dissection of Male Germine Development and Fertilization**

We generated pollen grains that carry a SSLC, instead of two sperm, by expressing DTA from the HAP2(GCS1) promoter in transgenic plants. SSLCs were capable of fertilization and produced significantly more endosperm-only ovules than embryo-only ovules, suggesting that SSLCs 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 germine development.

| Pollen Genotype | Fertilized Ovules | Unfertilized Ovules | Total |
|-----------------|-------------------|---------------------|-------|
|                 | Normal Endosperm Only Embryo Only Fertilized Ovules Unfertilized Total |                  |
| DAP % % % % % |                  |                     |       |
| qrt1            | 1.5 84.1 0.0 0.0 0.0 | 7.2 8.6 511         |       |
| qrt1            | 3 88.0 0.0 0.0 0.0 | 9.4 2.6 308         |       |
| qrt1            | 4 86.4 0.0 0.0 0.0 | 9.8 3.8 184         |       |
| No pollen       | 1.5 0.0 0.0 0.0 0.0 | 13.9 86.0 215       |       |
| No pollen       | 3 0.0 0.0 0.0 0.0 | 10.7 89.3 140       |       |
| No pollen       | 4 0.0 0.0 0.0 0.0 | 9.8 90.2 132        |       |
| HAP2(GCS1):DTA B7 | 1.5 2.7 2.2 1.0 1.0 | 7.5 86.4 412       |       |
| HAP2(GCS1):DTA B7 | 3 2.7 9.8 1.0 1.0 | 8.5 78.0 295       |       |
| HAP2(GCS1):DTA B7 | 4 6.3 18.2 0.5 0.5 | 12.5 62.5 192       |       |
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(GCS1):DTA expression and release of sperm is insufficient for induction of cell death. Alternatively, the relatively mild and variable phenotypes associated with HAP2(GCS1):DTA expression may be due to low and/or sporadic expression of the DTA transgene.

**HAP2(GCS1):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 cell1 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(GCS1):DTA plants, we conclude that the HAP2(GCS1) promoter is restricted to the generative cell and not active in the vegetative nucleus.**

**Models for Regulation of HAP2(GCS1) Promoter Activity and Mitotic Arrest of HAP2(GCS1):DTA Generative Cells**

The developmental defects observed in HAP2(GCS1):DTA transgenic plants are consistent with the emerging view that the combined activity of DUO1 and DUO3 transcription factors is required for HAP2(GCS1) expression (Brownfield et al., 2009a, 2009b).
Loss of function of either DUO1 or DUO3 blocks HAP2 (GCS1) expression in the generative and sperm cells (Brownfield et al., 2009a, 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(GCS1):DTA were limited to the male germline, consistent with the hypothesis that HAP2 (GCS1) promoter activity is restricted by the expression pattern of DUO1 (Brownfield et al., 2009a).

In addition to regulating HAP2(GCS1) 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 (for CYCLIN B1; 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, 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(GCS1):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 SSLC 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., 1999a, 1999b; 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 SSLC 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 SSLC 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 SSLC appeared to be random (Chen et al., 2008). Approximately 6% of pollen grains generated by Arabidopsis ms1/MS1 (for MULTICOPY SUPPRESSOR OF IRA1) mutants contain a SSLC 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 (18/3,600 ovules scored) and central cell-only (20/3,600 ovules scored) single fertilization events were observed.

SSLCs produced by ms1 or by expression of HAP2 (GCS1):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 fbl17 mutants. Analysis of SSLCs produced in ms1 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 SSLC that preferentially fuses with the central cell.

We propose that SSLCs produced by cdc2a, fbl17, ms1, or by expression of HAP2(GCS1):DTA show different preferences for fertilization because each of these perturbations has a different effect on male germline gene expression and development. cdc2a and fbl17 specifically block the generative cell cycle at S phase and produce SSLCs 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). ms1 is pleiotropic, sometimes causing arrest immediately after meiosis or after the first pollen mitosis; therefore, it is possible that the population of SSLCs 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(GCS1):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(GCS1):DTA SSLCs may fuse predominantly with central cells because they fail to translate a factor expressed by cdc2a and fbl17 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 SSLC 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 SSLCs produced by cdc2a or fb17 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 DTA coding sequence (Palmiter et al., 1987) was amplified by PCR (primers: DTA NcoI, GGTCTCTGGGATGATCTGCT; DTA XhoI, GCTCTA- GACTTAAAAATTTTAAATCCATTAGACG) and inserted into HAP2(GCS1) promoter/YFP (von Besser et al., 2006), replacing YFP. Arabidopsis (Arabidopsis thaliana) protoplasts were transformed by floral dip (Clough and Bent, 1998) and transformants were removed and processed (as above) 1.5, 3, or 4 d later. To analyze pollen and sperm development, young flowers that had just opened (stage 13; Smyth et al., 1990) were pollinated with at least two flowers to ensure complete pollination; pistils were excised and ovary walls were removed (Johnson et al., 2004). Plants were grown in growth chambers (Environmental Growth Chambers) at 21°C, 16 h light (100 µE), 8 h dark.

Analysis of Pollen and Sperm Development

Pollen tetrads were stained with DAPI (Park et al., 1998) and visualized on a Zeiss Axiosvert 200 m fluorescent microscope (Carl Zeiss). Images were acquired with a Hamamatsu ORCA-ER (Fig. 1) or Zeiss AxiosCam MRc5 (Fig. 2). For DNA quantification, 12-bit images were acquired using a Leica (SP2 A OBS, Leica Microsystems AG) confocal microscope and analyzed using Metamorph software (www.moleculardevices.com). A circle of uniform size was drawn around single sperm or SSLC 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 h. The following numbers of cells were analyzed to determine expression of male germ-line markers in HAP2(GCS1):DTA B7 transgenic plants: DUO1:H2B:mRFP1 (94 pairs of normal sperm, 63 SSLCs), HAP2(GCS1):HAP2(GCS1):YFP (158 pairs of normal sperm, 42 SSLCs), HAP2(GCS1):mRFP1 (211 pairs of normal sperm, 113 SSLCs), HTR10:HTR10:mRFP1 (118 pairs of normal sperm, 77 SSLCs). Pollen tube growth in ms1 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 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 differential interference contrast (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 prot or HAP2(GCS1):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 d later. To analyze KS1T17 (Sorensen et al., 2003) GFP expression in developing endosperm, ovules were mounted in water before DIC and fluorescence imaging. To analyze RCNI1:GUS (Derue et al., 1999) expression in developing embryos, ovules were incubated in 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution (0.5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM NaPO4, 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronic acid solution. (0.5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM NaPO4, 0.5 mg/mL 5-bromo-4chloro-3-indolyl-GlcUA, 10 mM EDTA, 0.5% Triton X-100) at 37°C for 4 d, incubated in 1:1 ethanoleato-acetic acid for 16 h 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 h at room temperature before mounting in chloral hydrate solution. All images were obtained using a Zeiss Axiosvert 200 m fluorescence microscope and an AxiosCam MRc5 camera (RCNI1:GUS expression) or a Hamamatsu ORCA-ER camera (DIC, fluorescence).

Supplemental Data

The following materials are available in the online version of this article.

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

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

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

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LITERATURE CITED

Berger E, Hamamura Y, Ingouff M, Higashiyama T (2008) Double fertilization—caught in the act. Trends Plant Sci 13: 437–443
Blakelock JF, Zhou HW, Heath JT, Skotké KK, Barrios JA, Liu SY, DeLong A (2008) Specificity of RCNI-mediated protein phosphatase 2A regulation in meristem organization and stress response in roots. Plant Physiol 146: 539–553
Borges F, Gomes G, Gardner R, Moreno N, McCormick S, Feijo JA, Becker JD (2008) Comparative transcriptomics of Arabidopsis sperm cells. Plant Physiol 148: 1168–1181
Breitman ML, Clapoff S, Rossant J, Tsui LC, Glode LM, Maxwell HH, Bernstein A (1987) Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. Science 238: 1563–1565
Brownfield L, Hafidh S, Borg M, Sidorova A, Mori T, Twell D (2009a) A plant germ-line-specific integrator of sperm specification and cell cycle progression. PLoS Genet 5: e1000430
Brownfield L, Hafidh S, Durbarry A, Kathab H, Sidorova A, Doerner P, Twell D (2009b) Arabidopsis DUO POLLEN3 is a key regulator of male germline development and embryogenesis. Plant Cell 21: 1940–1956
Carlson WR (1969) Factors affecting preferential fertilization in maize. Genetics 62: 543–554
Chen Z, Tan JL, Ingouff M, Sundaresan V, Berger F (2008) Chromatin assembly factor 1 regulates the cell cycle but not cell fate during male gametogenesis in Arabidopsis thaliana. Development 135: 65–73
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743
Day CD, Galgoci BE, Irish VF (1995) Genetic ablation of petal and stamen primordia to elucidate cell interactions during floral development. Development 121: 2887–2895
Derue E, Jackson K, Garbers C, Soll D, Delong A (1999) The RCNI-
encoded a subunit of protein phosphatase 2A increases phosphatase activity in vivo. Plant J 20: 389–399

Durbarry A, Vizir I, Twell D (2005) Male germ line development in Arabidopsis: double pollen mutants reveal gametophytic regulators of generative cell cycle progression. Plant Physiol 137: 297–307

Engel ML, Chaboud A, Dumas C, McCormick S (2003) Sperm cells of Zea mays have a complex complement of mRNAs. Plant J 34: 697–707

Engel ML, Holmes-Davis R, McCormick S (2005) Green sperm: identification of male gamete promoters in Arabidopsis. Plant Physiol 138: 2124–2133

Faure JE, Rusche ML, Thomas A, Keim P, Dumas C, Mogensen HL, Rougier M, Chaboud A (2003) Double fertilization in maize: The two male gametes from a pollen grain have the ability to fuse with egg cells. Plant J 33: 1051–1062

Gou X, Yuan T, Wei X, Russell SD (2009) Gene expression in the dimorphic sperm cells of Plumbago zeylanica: transcript profiling, diversity, and relationship to type cell. Plant J (in press)

Grossniklaus U, Vielle-Calzada JP, Hoeppner MA, Gagliano WB (1998) Maternal control of embryogenesis by MEDEA, a polycistron gene group in Arabidopsis. Science 280: 446–450

Gusti A, Baumberger N, Nowack M, Pusch S, Eisler H, Potuschak T, De Gou X, Yuan T, Wei X, Russell SD (2009) The conserved plant homologue, controls proliferation of generative cells in male gametogenesis of male gamete promoters in Arabidopsis. Plant Physiol 140: 607–614

Hicks GR, Rojo E, Hong S, Carter DG, Raikhel NV (2004) Geminating pollen has tubular vacuoles, displays highly dynamic vacuole biogenesis, and requires VACUOLESS for proper function. Plant Physiol 134: 1227–1239

Hirai M, Ari M, Mori T, Miyagishima SY, Kawai S, Kita K, Kuroiwa T, Terenius O, Matsuoka H (2008) Male fertility of maize parasites is determined by GCS1, a plant-type reproduction factor. Curr Biol 18: 607–613

Ingouff M, Hamamura Y, Gourgues M, Higashiyama T, Berger F (2007) Distinct dynamics of HISTONE3 variants between the two fertilization products in plants. Curr Biol 17: 1032–1037

Ingouff M, Sakata T, Li J, Sprunck S, Dresselaar T, Berger F (2009) The two male gametes share equal ability to fertilize the egg cell in Arabidopsis thaliana. Curr Biol 19: R19–R20

Iwakawa H, Shinmyo A, Sekine M (2006) Arabidopsis CDK4A1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. Plant J 45: 819–831

Johnson MA, von Besser K, Zhou Q, Smith E, Aux G, Patton D, Levin JZ, Engel ML, Holmes-Davis R, McCormick S (2005) Analysis of the histone H3 gene family in Arabidopsis and identification of the male-gamete-specific variant AMG H3. Plant J 44: 557–568

Pagnussat GC, Yu HJ, Sundaresan V (2007) Cell-fate switch of synergid to egg cell in Arabidopsis eooire mutant embryo sac arises from misexpression of the BEL1-like homeodomain gene BLH1. Plant Cell 19: 3578–3592

Palmriter RD, Behringer RR, Quaife CJ, Maxwell E, Maxwell IH, Brinster RL (1987) Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. Cell 50: 435–447

Pappenheimer AM (1977) Diphtheria toxin. Annu Rev Biochem 46: 69–94

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

Preuss D, Rhee SY, Davis RW (1994) Tetrads analysis possible in Arabidopsis within the question (QRT) genes. Science 264: 1458–1460

Romen H (1948) Directed fertilization in maize. Proc Natl Acad Sci USA 34: 58–59

Rotman N, Durbarry A, Wardle A, Yang WC, Chaboud A, Faure JE, Berger F, Twell D (2005) A novel class of MYB factors controls sperm-cell formation in plants. Curr Biol 15: 244–248

Russell SD (1984) Ultrastructure of the sperm of Plumbago zeylanica. II. Quantitative cytology and 3-dimensional organization. Planta 162: 385–396

Russell SD (1985) Preferential fertilization in Plumbago: Ultrastructural evidence for gamete-level recognition in an angiosperm. Proc Natl Acad Sci USA 82: 619–632

Russell SD, Cass DD (1981) Ultrastructure of the sperms of Plumbago zeylanica. I. Cytology and association with the vegetative nucleus. Protoplasma 107: 85–107

Scott RJ, Armstrong SJ, Doughty J, Spielman M (2008) Double fertilization in Arabidopsis thaliana involves a polysperm blocking on the egg but not the central cell. Mol Plant 1: 611–619

Singh M, Bhalia PL, Xu H, Singh MB (2003) Isolation and characterization of a flowering plant male gametic cell-specific promoter. FEBS Lett 542: 47–52

Smyth DR, Bowman JL, Mayerowitz EM (1990) Early flower development in Arabidopsis. Plant Cell 2: 755–767

Sorensen MR, Chaudhury AM, Robert H, Bancharel E, Berger F (2001) Polycistron group genes control pattern formation in plant seed. Curr Biol 11: 277–281

Spielman M, Scott RJ (2008) Polyspermy barriers in plants: from preventing to promoting fertilization. Sex Plant Reprod 21: 53–65

Thorsness MK, Kandasamy MK, Nasrallah ME, Nasrallah JB (1991) A Brassica S-locus gene promoter targets toxic gene expression and cell death to the pistil and pollen of transgenic Nicotiana. Dev Biol 143: 173–184

Thorsness MK, Kandasamy MK, Nasrallah ME, Nasrallah JB (1993) Genetic ablation of floral cells in Arabidopsis. Plant Cell 5: 253–261

Twell D (1995) Diphtheria toxin-mediated cell ablation in developing pollen—vegetative cell ablation blocks generative cell-migration. Protoplasma 187: 144–154

Twell D, Park SK, Lalanne E (1998) Asymmetric division and cell-fate determination in developing pollen. Trends Plant Sci 3: 305–310

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

Wong JL, Wessel GM (2006) Defending the zygote: search for the ancestral animal block to polyspermy. Curr Top Dev Biol 72: 1–151

Xu H, Swoboda I, Bhalia PL, Singh MB (1999a) Male gametic cell-specific expression of H2A and H3 histone genes. Plant Mol Biol 39: 607–614

Xu H, Swoboda I, Bhalia PL, Singh MB (1999b) Male gametic cell-specific gene expression in flowering plants. Proc Natl Acad Sci USA 96: 2554–2558

Yadegari R, Paiva G, Laux T, Koltunow AM, Aypa N, Zimmerman JL, Fischer RL, Harada JJ, Goldberg RB (1994) Cell differentiation and morphogenesis are uncoupled in Arabidopsis raspberry embryos. Plant Cell 6: 1713–1729

Yamaizumi M, Mekada E, Uchida T, Okada Y (1978) One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. Cell 11: 407–416

Zhou HW, Nussbaumer C, Chao Y, DeLong A (2004) Disparate roles for the regulatory A subunit isoforms in Arabidopsis protein phosphatase 2A. Plant Cell 16: 709–722