

# Chromatin assembly factor 1 regulates the cell cycle but not cell fate during male gametogenesis in *Arabidopsis thaliana*

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The interdependence of cell cycle control, chromatin remodeling and cell fate determination remains unclear in flowering plants. Pollen development provides an interesting model, as it comprises only two cell types produced by two sequential cell divisions. The first division separates the vegetative cell from the generative cell. The generative cell divides and produces the two sperm cells, transported to the female gametes by the pollen tube produced by the vegetative cell. We show in *Arabidopsis thaliana* that loss of activity of the Chromatin assembly factor 1 (CAF1) pathway causes delay and arrest of the cell cycle during pollen development. Prevention of the second pollen mitosis generates a fraction of CAF1-deficient pollen grains comprising a vegetative cell and a single sperm cell, which both express correctly cell fate markers. The single sperm is functional and fertilizes indiscriminately either female gamete. Our results thus suggest that pollen cell fate is independent from cell cycle regulation.

**KEY WORDS:** Pollen, CAF1, Cell cycle, *Arabidopsis thaliana*

## INTRODUCTION

The Chromatin assembly factor 1 (CAF1) (Hennig et al., 2005; Kaya et al., 2001) has a conserved chaperone activity for chromatin assembly at the DNA replication fork during S phase. CAF1 targets acetylated histone H3–H4 dimers to newly synthesized DNA, thus allowing nucleosome assembly (Polo and Almouzni, 2006). In mammals, CAF1 is essential for cell viability and consists of the three proteins p150, p60 and p48. Loss of function of CAF1 activity in mammalian cells leads to activation of a DNA-damage signaling pathway that slows down the S phase and arrests the cell cycle (Haushalter and Kadonaga, 2003). In yeast, the CAF1 assembly complex subunits Cac1, Cac2 and Cac3 are dispensable for cell viability (Haushalter and Kadonaga, 2003; Ridgway and Almouzni, 2001), but loss of function of CAF1 causes increased sensitivity to DNA-damaging stresses (Linger and Tyler, 2005). *Arabidopsis* CAF1 contains a core of three proteins encoded respectively by the p150 homolog *FASCIATA1* (*FAS1*), the p60 homolog *FASCIATA2* (*FAS2*) and the pRbAp48 homolog *MULTICOPY SUPPRESSOR OF IRA1* (*MSI1*) (Kaya et al., 2001). The WD40 domain proteins MSI1 and pRbAp46/48 also bind to Retinoblastoma-related proteins (Ach et al., 1997; Qian and Lee, 1995; Rossi et al., 2003). This presumably regulates activity of the downstream E2F transcription factor, thereby influencing the gene activation required for entrance into S phase (Hennig et al., 2005). In *Arabidopsis*, reduction of *FAS1* and *FAS2* activities upregulates the expression of genes in the DNA-damage response pathway (Schonrock et al., 2006). In the absence of CAF1 activity, cells endoreduplicate to higher levels, suggesting that loss of function of *FAS1* or *FAS2* affects the S–G2 and the G2–M transitions in *Arabidopsis* (Endo et al., 2006; Exner et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007). In parallel to the deregulation of the cell cycle, the loss of CAF1 function causes a reduction of the heterochromatic fraction (Kirik et al., 2006), releases transcriptional gene silencing from endogenous transposons

(Ono et al., 2006) and alters the pattern of histone acetylation and methylation at promoters of genes encoding components and regulators of the cell cycle (Ramirez-Parra and Gutierrez, 2007). It remains unclear whether the vegetative developmental defects in *fas1* and *fas2* mutants (Costa and Shaw, 2006; Exner et al., 2006) are direct consequences of the loss of CAF1 on the cell cycle or result indirectly from the gradual accumulation of epigenetic defects.

In pollen produced from *msi1/+* plants, the loss of MSI1 affects only half of the haploid pollen produced after meiosis. It is thus possible to investigate the immediate developmental consequence of the loss of CAF1 function using male gametogenesis as a model. Reduced paternal transmission of *msi1* was recorded from the null allele *msi1-2* (Guitton et al., 2004), suggesting the alteration of pollen development by removal of CAF1 function. Pollen development is characterized by a relatively simple series of events involving simultaneously cell fate and cell cycle regulation (McCormick, 2004). We report that loss of function of CAF1 complex members affects pollen development. Null alleles of *msi1* prevent division in a fraction of pollen. The effect of *msi1* on pollen arrest is synergistically enhanced when CAF1 activity is further compromised in *fas1* and *fas2* mutants. We further investigate the fate and differentiation of the pollen in the absence of CAF1.

## MATERIALS AND METHODS

### Plant material

The wild-type ecotypes Columbia (Col-0), Landsberg *erecta* (Ler), Enkheim (En) and C24 were provided by the Nottingham *Arabidopsis* Stock Centre. The mutant allele *msi1-1* (Col background) was previously characterized and kindly provided by Lars Hennig (Köhler et al., 2003). The mutant allele *msi1-2* (C24 background) was previously described in the laboratory in F.B. group (Guitton et al., 2004). The mutant allele *msi1-3* (Ler background) was obtained by V.S. The mutant allele *msi1-4* (Col background) was obtained and kindly provided by Gary Drews and Jayson Punwani (University of Utah). Seeds from *qrt1/qrt1* (Ler accession) were provided by D. Preuss. Homozygote *fas1-1* (CS265 En background) *fas1-4* (SAIL\_662\_D10 Col background) and *fas2-4* (SALK\_033228, Col background) seeds were obtained from the *Arabidopsis* Biological Research Center. The KS22 enhancer-trap line (C24 accession) expressing the GFP reporter protein was generated in J. Haseloff's laboratory ([www.plantsci.cam.ac.uk/Haseloff](http://www.plantsci.cam.ac.uk/Haseloff)). Marker lines for cell identity in pollen pDUO1-DUO1-mRFP1 (C24) was obtained by N. Rotman (Rotman et al., 2005), AC26 mRFP (C24)

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was obtained by A. Chaboud (Unite Mixte de Recherche 9004, Lyon, France) from C24 plants transformed with pACTIN11-Histone-mRFP1; E1 GFP (Col) was a kind gift from G. Strompen (Strompen et al., 2005). ATGEX1 marker line was the kind gift from Sheila McCormick (Engel et al., 2005). The marker HTR10-mRFP1 results from a genomic fusion described in Ingouff et al. (Ingouff et al., 2007).

#### MSI1:MSI1-mRFP1 plasmid construction and transformation

The full-length *MSI1* cDNA was amplified by PCR and inserted in frame between the gateway cassette (GW) and the monomeric Red fluorescent protein 1 (*mRFP1*) gene contained in the pGREENII (pGII)-based vector pGIIInK-GW-mRFP1-35ter.

A 3050 bp DNA fragment consisting of the upstream region of *MSI1* corresponding to the putative promoter until the beginning of exon3 was amplified by PCR using the KOD-plus-PCR kit (TOYOBO, Japan) and cloned into pCR2-1 TOPO vector (Invitrogen, Carlsbad, CA). The final vector pGIIInK-promMSI1::MSI1-mRFP1-35S consists of the upstream region of *MSI1*, its first two exons and introns and the remaining *MSI1* cDNA fused to the fluorescent reporter mRFP1. Heterozygous *msi1-1/+* mutant BASTA-selected plants were transformed using the *Agrobacterium*-mediated floral dip method.

Fifteen transgenic lines were obtained, all showing a similar pattern of expression. Maternal transmission of *msi1-1* mutation, which is null in the mutant background was completely restored by expression of *promMSI1:MSI1-mRFP1* (46.6% transmission of *msi1* from seeds produced by *msi1/+*; *promMSI1:MSI1-mRFP1/promMSI1:MSI1-mRFP1* ovules crossed to wild-type pollen,  $n=250$ ). Plants homozygous for *msi1-1* and homozygous for *promMSI1:MSI1-mRFP1* were obtained.

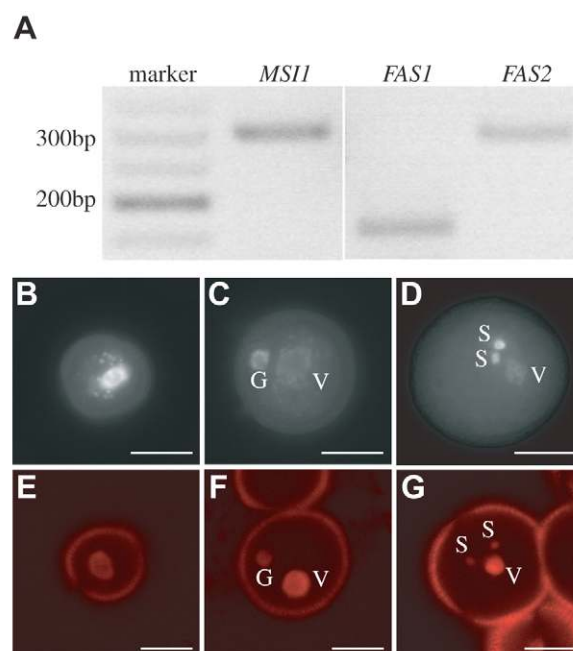
## RESULTS

### Reduced paternal transmission of *msi1* loss-of-function alleles

*MSI1*, *FAS1* and *FAS2* were all expressed in mature pollen (Fig. 1A), in agreement with microarray analyses, which also showed a level of expression comparable to or lower than levels in leaves and roots (see Fig. S1 in the supplementary material). In order to know precisely in which cell types *MSI1* expression takes place during pollen development, we produced a translational reporter line for *MSI1*. We obtained plants that express *MSI1* fused to the monomeric Red fluorescent protein 1 (mRFP1) under the control of *MSI1* 5' regulatory cis-elements (*pMSI1-MSI1-mRFP1*). The expression of this construct complemented the loss-of-function allele *msi1-1*, and thus its expression pattern was likely to reflect the pattern of *MSI1* expression.

We compared our observations of *MSI1-mRFP1* expression with the wild-type pattern of chromatin staining by DAPI. The microspore (Fig. 1B) underwent an asymmetric division (PMI), leading to production of bicellular pollen. This comprises the larger vegetative cell and the smaller generative cell initially positioned at the periphery and later engulfed by the vegetative cell (Fig. 1C). The generative cell further divided (PMII) and produced the two sperm cells (Fig. 1D). In contrast to the sperm cells, the large spherical vegetative cell had a larger, less condensed nucleus positioned at the center of the tricellular pollen.

*MSI1-mRFP1* was expressed at all stages of pollen development in both cell types (Fig. 1E-G), as reported in previous microarray analyses (Honys and Twell, 2004). The level of *MSI1-mRFP1* appeared to increase in the vegetative cell throughout pollen development, whereas the expression of *MSI1* reported by microarrays decreased (see Fig. S1 in the supplementary material). We favor the idea that the increase of *MSI1-mRFP1* signals provides evidence for new synthesis and accumulation of *MSI1-mRFP1* during pollen development. It is thus unlikely that detected *MSI1-mRFP1* was inherited through meiosis. The mRFP1 fluorescence in



**Fig. 1. Expression of CAF1 components in pollen.** (A) RT-PCR was performed from RNAs extracted from mature *Arabidopsis thaliana* pollen grains and shows expression of *MSI1*, *FAS1* and *FAS2*.

(B-D) Wild-type pollen development. Nuclei are stained with DAPI. The uni-nucleate microspore (B) undergoes an asymmetrical mitosis, leading to the bicellular stage (C). At that stage the pollen grain contains a large vegetative cell and a small generative cell with a nucleus showing relatively higher chromatin compaction. (D) The generative cell divides, giving rise to two sperm cells with highly condensed chromatin. (E-G) Expression pattern of *promMSI1-MSI1-mRFP1* in pollen in microspore (E), and developing pollen at bicellular stage (F) and tricellular stage (G). Confocal sections. Scale bars: 10  $\mu$ m. G, generative cell; S, sperm cell; V, vegetative cell.

the generative cell was weaker than in the vegetative cell (Fig. 1F). Although the chromatin of the sperm cells was very compact, the *MSI1-mRFP1* signal was rather low (Fig. 1G). *MSI1* thus appears to be differentially expressed between the vegetative and generative lineage during pollen development.

Reduced paternal transmission is reported for the allele *msi1-2* (Guitton et al., 2004) but not for the allele *msi1-1* (Köhler et al., 2003). This discrepancy may result from the distinct genetic background of each allele (C24 versus Col) or from distinct growth conditions. We isolated two new alleles of *msi1* in Ler (*msi1-3*) and Col (*msi1-4*) genetic backgrounds (see Fig. S2 in the supplementary material). Imperfect insertions of a Ds transposon and of a T-DNA created a stop codon in the first exon of *MSI1* in the alleles *msi1-3* and *msi1-4*. Both alleles do not transmit *msi1* maternally and are presumably null alleles as concluded for the alleles *msi1-1* and *msi1-2* (Guitton et al., 2004; Köhler et al., 2003).

We measured the paternal transmission in each *msi1* allele grown in the same conditions (Table 1). Pollen development takes place after meiosis, and crosses between wild-type ovules and pollen from *msi1/+* plants are expected to transmit *msi1* in 50% of the offspring if there is full transmission. Paternal transmission was reduced to approximately 36% in every allele (Table 1). Reduced transmission could result from a paternal effect causing reduction of seed germination or seedling viability. The germination rate of wild-type

**Table 1. Paternal transmission of *msi1* alleles**

Female × male	% ± s.d. (mutant allele) in F1	n	T.E. %	Penetrance %
<i>msi1-1/+</i> × <i>msi1-1/+</i>	35.7±5.0 ( <i>msi1-1</i> )	206	56	44
Col × <i>msi1-1/+</i>	36.8±4.9 ( <i>msi1-1</i> )	85	57	43
<i>msi1-2/+</i> × <i>msi1-2/+</i>	33.7±5.1 ( <i>msi1-2</i> )	132	50	50
C24 × <i>msi1-2/+</i>	35.9±6.6 ( <i>msi1-2</i> )	206	56	44
<i>msi1-3/+</i> × <i>msi1-3/+</i>	35.8±10.0 ( <i>msi1-3</i> )	96	55	45
Ler × <i>msi1-3/+</i>	34.8±3.4 ( <i>msi1-3</i> )	121	53	47
<i>msi1-4/+</i> × <i>msi1-4/+</i>	37.0±6.1 ( <i>msi1-4</i> )	143	59	41
Col × <i>msi1-4/+</i>	36.6±9.3 ( <i>msi1-4</i> )	72	57	43
Col × <i>msi1-1/+; fas1-1/+</i>	28.8±4.9 ( <i>msi1-1</i> )	255	40	60
Col × <i>msi1-3/+; fas1-4/+</i>	29.3±4.6 ( <i>msi1-3</i> )	389	41	59
Col × <i>msi1-1/+; fas2-4/+</i>	28.5±2.2 ( <i>msi1-1</i> )	84	40	60

The paternal transmission was calculated from the percentage of *msi1* plants in the progeny from crosses between pollen from *msi1/+* plants and ovules of the relevant wild-type ecotype. This transmission is comparable to the transmission of *msi1* from self-pollinated *msi1/+* plants. The effect of *fas1* and *fas2* on *msi1* transmission is indicated in the three last rows.

n, The number of plants scored.

T.E., Transmission efficiency.

seeds was 98.2% ( $n=277$ ,  $s.d.=0.53$ ), hence comparable to the germination rate in seeds from crosses between wild-type ovules and pollen from *msi1-1/+* plants (98.0%,  $n=293$ ,  $s.d.=0.23$ ). Similarly the survival rate of *msi1-1/+* seedlings was not affected (99.6%,  $n=273$ ,  $s.d.=0.07$ ). Hence *msi1* effect on paternal transmission does not originate from an effect on seed development or on seedlings, and must result from a reduction of male fertility. The reduction of paternal transmission of all four *msi1* alleles to 36% indicated a transmission efficiency of 56%. Hence a penetrance of 44% is associated with null mutations in *msi1*, resulting in defective male gametogenesis in *msi1/+* plants. We conclude that *msi1* causes defects during pollen development.

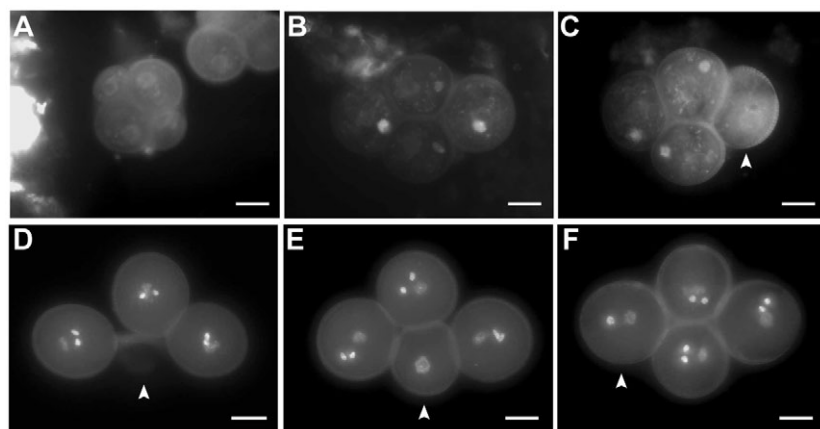
### Reduced paternal transmission of *msi1* is enhanced by further loss of CAF1 function

*MSII* was shown to interact in vitro with the complex containing *FAS1* and *FAS2* (Kaya et al., 2001). We analyzed the paternal transmission associated with the loss-of-function alleles *fas1-4* and *fas2-4* (Exner et al., 2006; Ramirez-Parra and Gutierrez, 2007). Paternal transmission of the *fas1-4* allele analyzed from crosses between wild-type ovules and pollen from *fas1-4/+* plants was 49.6% ( $n=115$ ,  $s.d.=7.4$ ). Similarly paternal transmission of *fas2-4* was 48.6% ( $n=140$ ,  $s.d.=5.2$ ). According to these data, neither *fas1* nor *fas2* appear to affect male gametogenesis. Nevertheless, if we assume that *fas1* and *fas2* affect paternal transmission, the associated transmission efficiency would be of the order of 98%. To test whether the CAF1 pathway is responsible for the reduced transmission of *msi1* null alleles, we obtained double mutant lines

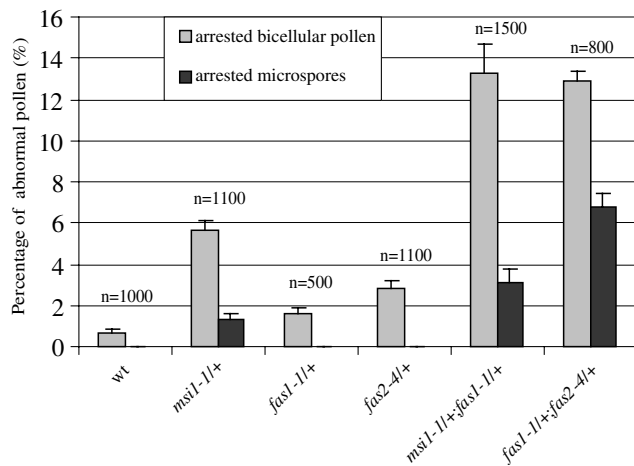
between *msi1/+* and loss-of-function alleles of *fas1/+* and *fas2/+*. Paternal transmission of *msi1* mutant allele was similarly reduced by the introduction of *fas1* and *fas2* (Table 1). An additive interaction between *fas1* or *fas2* and *msi1* is predicted to lead to a decrease of *msi1* transmission efficiency from 56 to 55% in *fas1* or *fas2* background. However, in the *fas1* or the *fas2* background the transmission efficiency of *msi1* was reduced significantly from 56% to 40% (Table 1). In conclusion, the interaction between *msi1* and *fas1* or *fas2* was not additive but synergistic. Additive interactions between null mutations indicate that each mutation affects a distinct pathway. However, a synergistic interaction is observed in combination of partially penetrant alleles of mutations affecting the same genetic pathway. The synergy observed between *msi1* and *fas1* or *fas2* on paternal transmission of *msi1* suggests that *MSII*, *FAS1* and *FAS2* act in a common genetic pathway influencing male gametogenesis.

### Loss of *MSI1* arrests pollen development

We investigated the developmental origin of the reduced male fertility in *msi1/+* plants. In order to compare directly *msi1* and wild-type pollen development, we used the mutant *quartet* (*qrt*), which produces four microspores remaining associated as a tetrad (Preuss et al., 1994). Hence *qrt/qrt; msi1-1/+* plants produce tetrads containing two wild-type and two *msi1* pollen grains. Alexander staining for pollen viability showed 7.3% lethality in *msi1* pollen at maturity ( $s.d.=1.9$ ;  $n=800$ ) in comparison with 1.0% in the wild type ( $s.d.=0.7$ ;  $n=500$ ) (see Fig. S3 in the supplementary material). Tetrads of microspores produced by wild-type and *msi1-1/+* plants



**Fig. 2. Defects in pollen development in *msi1/+* mutants.** Pollen development in meiotic tetrads from *qrt1/qrt1* and *qrt1/qrt1; msi1-1/+ Arabidopsis* plants. At uni-nucleate stage (A) microspore tetrads of wild-type and mutant plants are indistinguishable. In contrast to wild-type tetrads (B), *msi1/+* tetrads at the bicellular stage contain one arrested microspore (C, arrowhead). (D-F) At the tricellular stage, *qrt1/qrt1; msi1-1/+* plants produce tetrads containing three wild-type pollen grains and an abnormal pollen grain (arrowhead). The abnormal pollen is either a dead microspore (D), an arrested microspore (E) or a bicellular pollen grain (F). Epifluorescence micrographs of DAPI-stained pollen. Scale bars: 10  $\mu$ m.

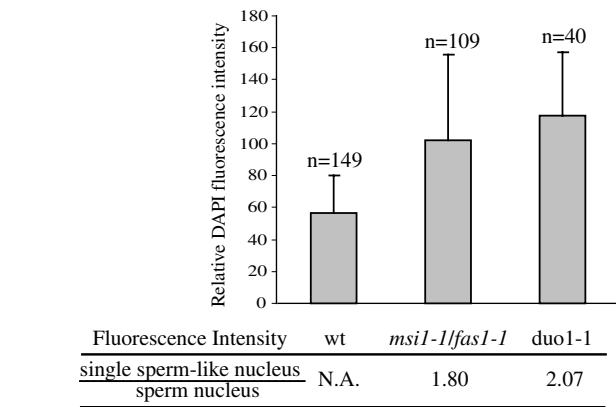


**Fig. 3. Synergistic effects of combination between mutations in members of the CAF1 complex.** Bar chart showing percentage of the two types of abnormal pollen at the tricellular mature stage in *msi1-1/+*, *fas1-1/+*, *fas2-4/+*, *msi1-1/+;fas1-1/+* and *fas1-1/+;fas2-4/+* mutant *Arabidopsis* plants. Gray bars correspond to arrested bicellular pollen and black bars to arrested microspores. Error bars correspond to standard errors calculated on the basis of several samples of 100 pollen grains, and the size of the total population analyzed (*n*) is indicated above each column.

in *qrt/qrt* background could not be distinguished (*n*=120) (Fig. 2A). After PMI, in *qrt/qrt*, all tetrads consisted of four pollen grains with a vegetative cell and a generative cell (Fig. 2B). By contrast, a fraction of tetrads in pollen from *qrt/qrt; msi1-1/+* plants contained an arrested microspore (Fig. 2C). At the mature stage, in contrast to wild-type pollen tetrads, a fraction of tetrads in *qrt/qrt; msi1-1/+* plants contained an aborted microspore (Fig. 2D), or a pollen grain with one (Fig. 2E) or two (Fig. 2F) nuclei. These results suggest that the absence of MSII causes pleiotropic arrest of pollen development before PMI, or PMII. The fraction of each class of arrest was measured in pollen produced by *msi1-1/+* plants (Fig. 3). The percentage of arrested pollen cumulated with the percentage of aborted microspores amounted to 14.7%. This value is lower than the 22% abnormal pollen in a population of pollen from *msi1/+* plants, as predicted from the transmission efficiency of 56%. This suggested that 7.3% of pollen from *msi1/+* plants contain two sperm cells and appear morphologically normal, albeit not functional. These *msi1* tricellular pollen grains probably grow a pollen tube, but the two sperm cells are incompetent for fertilization, causing ovule abortion. In agreement with this prediction, pollination of wild-type plants by *msi1/+* plants caused a 6% increase in ovule abortion (see Fig. S4 in the supplementary material). In conclusion, our observations show that *msi1* causes delays and arrests of pollen development, leading to partial male sterility and reduced paternal transmission of *msi1*.

### Loss of CAF1 activity causes delay and arrest of the cell cycle in pollen

As genetic interactions suggested that impairment of the CAF1 pathway affects male gametogenesis, we expected that loss of function of the other members of the CAF1 pathway would cause pollen arrests similar to *msi1*. A small proportion of pollen arrested at the bicellular stage was observed in *fas1/+* and *fas2/+* mutants (Fig. 3). This very limited penetrance of *fas* mutations was also supported by the very limited reduction of paternal

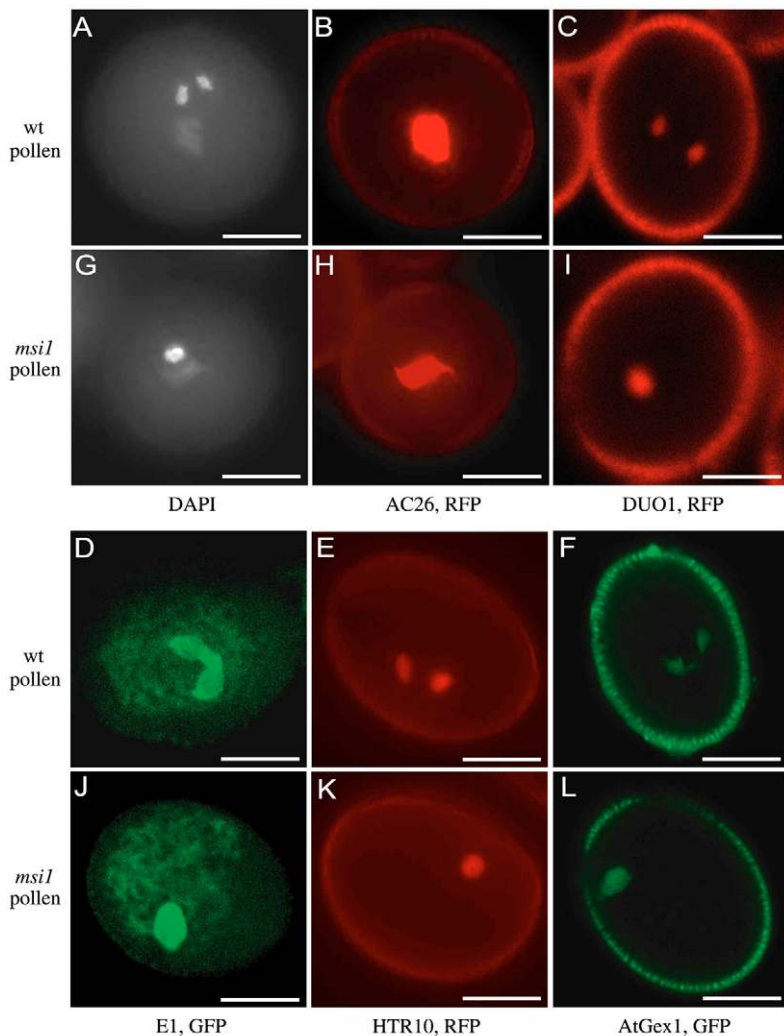


**Fig. 4. Effect of *msi1* on DNA content in sperm cell nuclei.** The DNA content in *Arabidopsis* sperm cells was estimated relative to the fluorescence intensity after DAPI staining. The relative DAPI fluorescence intensity between control wild-type sperm cells and single sperm-like cells in *msi1-1;fas1-1* pollen and in *duo1-1* pollen is indicated under the graph. Error bars correspond to standard deviations, and the size of the sample (*n*) is indicated above each column.

transmission efficiency of *fas1* and *fas2*. By contrast, the combination of *fas1* and *fas2* in double heterozygous mutant plants caused a high proportion of arrest at the bicellular stage and an additional arrest as microspores, which was never observed in the single mutants. This observation demonstrates that the combination of *fas1* and *fas2* does not cause additional, but synergistic, effects, in agreement with the established participation of *fas1* and *fas2* to the CAF1 pathway. Similarly the combination of *fas1* to *msi1* caused a synergistic increase in the proportion of pollen developmental arrests (Fig. 3). A two-way analysis of variation confirmed that an additive phenotype could not explain the extra increase in single sperm pollen in the double mutants ( $P=0.0047$ ). The synergistic effects of the combinations between *fas1*, *fas2* and *msi1* on pollen development and paternal transmission indicate that *msi1* causes pollen developmental arrest through the CAF1 pathway.

FACS analyses in young seedlings have shown that loss-of-function mutants for *MSII*, *FAS1* and *FAS2* lead to a G2/M arrest, presumably as a result of the activation of the DNA repair pathway following stalling of the DNA replication fork (Endo et al., 2006; Exner et al., 2006; Honys and Twell, 2004; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007). To investigate the consequence of loss of activity of CAF1 on cell cycle during pollen development, we measured DNA content at the early tricellular stage. We compared the DNA content in sperm cells of wild-type tricellular pollen with the single sperm-like cell in bicellular pollen from *msi1/+;fas1/+* plants. Both measurements were compared to *duo1* pollen single sperm-like cells, which contain twice the amount of DNA contained in wild-type sperm cells at the early tricellular stage (Rotman et al., 2005). The single sperm-like cell in *msi1/+;fas1/+* also contained approximately twice the amount of DNA in comparison to wild-type sperm cells (Fig. 4). These measurements suggest that the loss of CAF1 function delays the cell cycle pace in the generative cell, preventing the G2-M transition, which leads to PMII in the wild type.





**Fig. 5. Cell identities in bicellular *msi1* pollen.**

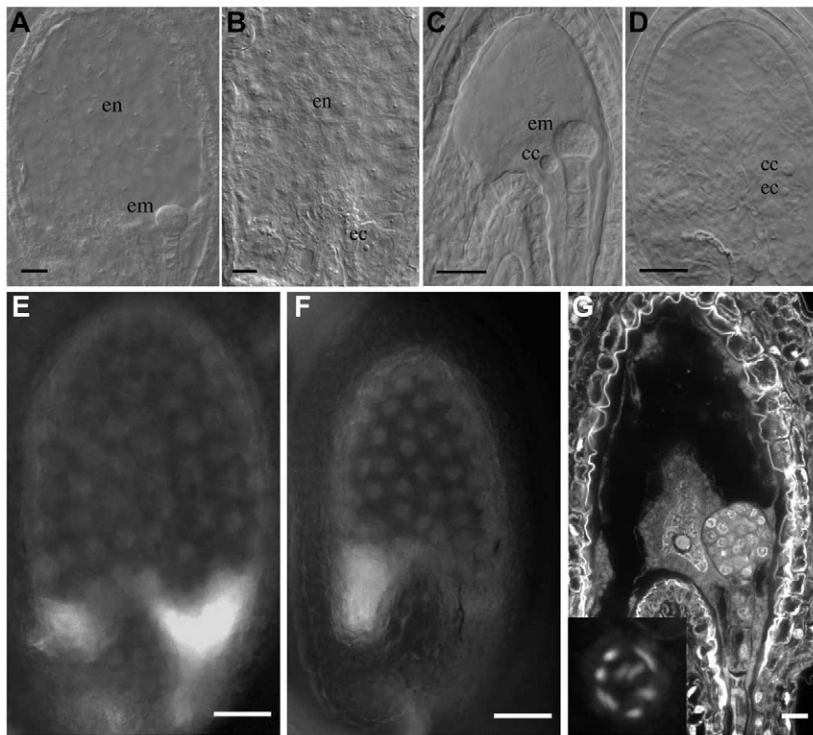
(A-F) Wild-type *Arabidopsis* pollen at the tricellular stage. (G-L) Bicellular and tricellular pollen segregating from *msi1/+* plants. (A,G) Fluorescence images of pollen grains stained with DAPI. (B,H) The same pollen grains as in A,G, respectively, expressing the vegetative cell marker AC26-Histone2B-mRFP1. (C,I) Pollen grains expressing the generative lineage marker pDUO1-DUO1-mRFP1. (D,J) Pollen grains expressing the generative lineage marker E1-GFP. (E,K) Pollen grains expressing the sperm cell marker HTR10-mRFP1. (F,L) Pollen grains expressing the sperm cell marker ATGEX1-GFP. GFP was examined using the 488 nm excitation line of an argon laser and an emission filter long pass of 510 nm. Fluorescence of mRFP1 was examined using the 543 nm excitation line of a HeNe laser and an emission filter of 585-615 nm. Scale bars: 10  $\mu$ m.

### Cell-fate specification and differentiation is normal in CAF1-deficient pollen

*Arabidopsis* PMI is coupled to cell-fate specification, leading to specific expression of vegetative cell (Twell et al., 1991) and generative cell (Engel et al., 2005; Rotman et al., 2005) markers. The second mitosis is coupled with sperm cell differentiation, marked by the onset of DNA synthesis (Durbarry et al., 2005) and expression of specific genes (Engel et al., 2005). To further elucidate whether arrests of cell division in *msi1* pollen grain are associated with cell fate changes, we analyzed cell identities in pollen grains arrested at PMII produced by *msi1/+* plants. These pollen grains contained a large vegetative-like cell nucleus and a sperm-like cell with condensed DNA (Fig. 5G). The marker AC26 is associated with expression of HISTONE 2B-mRFP fusion protein under the control of the *ACTIN-11* promoter and is specifically expressed in wild-type vegetative cell nuclei (Rotman et al., 2005) (Fig. 5A,B). In *msi1* pollen arrested at PMII, AC26 was also expressed in the larger spherical cell in correlation with its vegetative identity (Fig. 5G,H). We never observed any pollen grain expressing AC26 in the condensed nucleus of the sperm-like cell, nor in more than one nucleus, indicating that loss of CAF1 function does not perturb the vegetative cell identity during pollen development. In addition, we performed a germination test to estimate whether the vegetative cell deficient in CAF1 is able to produce a pollen tube. We observed

comparable germination rates between wild-type pollen and pollen from *msi1/+*, *fas1/+*, *fas2/+* and the double mutant combinations, with a slight reduction corresponding to the proportion of dead pollen recorded for each genetic background (see Fig. S5 in the supplementary material). Our results support the argument that the vegetative cell differentiation and function is not affected by the loss of CAF1 function.

In wild-type pollen, the generative cell lineage is marked by pDUO1-DUO1-mRFP1, expressed in the generative cell and in the sperm cells (Rotman et al., 2005) (Fig. 5C). The maturing sperm cells are further marked specifically by the accumulation of the vacuolar V-ATPase E1 GFP on the outer plasma membrane (Strompen et al., 2005) (Fig. 5D) and of the HISTONE3 variant HTR10 in the sperm nucleus (Ingouff et al., 2007) (Fig. 5E). Eventually, the sperm cells specifically express ATGEX1 (Engel et al., 2005) (Fig. 5F). We observed that all the markers of sperm cell maturation and identity were expressed only in the single sperm-like cell in *msi1* pollen arrested at PMII (Fig. 5I-L). The proportion of pollen with a single sperm cell expressing HTR10-mRFP1 (6%,  $n=150$ ; s.d.=2) is similar to the proportion of pollen with a single sperm cell (Fig. 3). We conclude that all *msi1* single sperm cells express sperm cell fate markers. These findings indicate that cell fates are not affected in *msi1* pollen grains arrested at PMII, which appear to comprise a vegetative cell and a single sperm cell.



**Fig. 6. Pollination of wild-type ovules with *msil* pollen leads to single fertilization events.** (A) Wild-type *Arabidopsis* seed with an embryo and an endosperm, 2 days after pollination (2 DAP). (B) Seed with an endosperm but without an embryo, 3 DAP. At the embryo region, the egg cell remains unfertilized. (C) Seed with an embryo but without endosperm, 3 DAP. At the side of the embryo the nucleus of central cell that remains unfertilized is still observed. (D) Fully aborted ovule without embryo and endosperm, 3 DAP. The egg cell and the central cell remain unfertilized. (E) Expression of the fluorescent KS22 GFP endosperm marker in a wild-type seed at 3 DAP. (F) Expression of KS22 GFP in a seed containing only endosperm from a cross between wild-type ovules and *msil-2/+*; KS22/KS22 pollen. (G) Confocal section of a seed containing only an embryo, produced by a cross between a wild-type ovule and *msil-2* pollen. The nucleus of the unfertilized central cell remains associated with the surface of the embryo. Feulgen staining marks brightly the DNA in nuclei as described previously (Garcia et al., 2003). The inset shows ten chromocentres at pre-prophase in the nucleus from the single embryo. The single embryo is diploid and thus originates from a fertilized egg cell. Scale bars: 100  $\mu$ m in A-D; 50  $\mu$ m in E,F; 10  $\mu$ m in G. cc, central cell; ec, egg cell; em, embryo; en, endosperm.

### Pollination with *msil* pollen causes single-fertilization events

In wild-type pollen, the two sperm cells separately fertilize the female egg cell and the central cell producing the embryo and the endosperm, respectively (Fig. 6A). The apparent correct establishment of cell fate in the single sperm-like cell present in *msil* pollen suggested that it was able to perform fertilization as a wild-type sperm cell. We suspected that the single sperm cell in *msil* pollen could fertilize either the egg cell or the central cell. Crossing wild-type ovules to pollen from *msil/+* plants produced a low proportion of seeds containing only endosperm ( $n=20$  out of 3600) (Fig. 6B) or an embryo ( $n=18$  out of 3600) (Fig. 6C). In both cases, these seeds contained residual material from the unfertilized egg cell or central cell (Fig. 6B,C), comparable to those observed in unfertilized ovules (Fig. 6D). We concluded that *msil* pollen with a single sperm cell probably caused single fertilization events. Alternatively, single sperm cells may only fuse with one of the female gametes without the fusion of the parental genetic material (karyogamy), and autonomous development of embryo or endosperm would follow. If this were the case, the paternal genome would be excluded and the single embryo or the single endosperm should not express paternally derived alleles. We tested this hypothesis for the expression of the endosperm marker KS22 (Ingouff et al., 2005) (Fig. 6E), provided by the pollen of *msil/+* plants crossed to wild-type ovules (Fig. 6F). Seeds with single endosperm showed expression of the paternally derived endosperm marker KS22 (Fig. 6F,  $n=7$ ). We conclude that the single endosperm develops from a central cell fertilized by the single gamete in *msil* pollen. To establish whether single sperm cells are able to fertilize the egg cell, we performed ploidy measurements on seeds containing a single embryo. We observed ten chromosomes at pre-prophase in single embryo cells (inset in Fig. 6G,  $n=2$ ). These embryos are thus diploid and have been produced by fertilization of the egg cell by the single sperm from *msil* pollen. We conclude that a fraction of

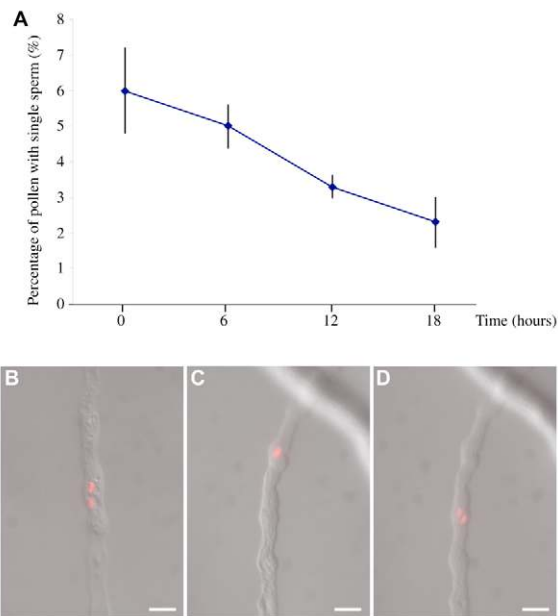
*msil* pollen arrested at PMII delivers a functional single sperm cell equally able to fertilize the egg cell or the central cell. Similarly, pollination with pollen from *fas1/+* and *fas2/+* plants produced seeds containing either an endosperm or an embryo (0.3%,  $n=400$ ; and 0.4%,  $n=360$ ). This result suggests that CAF1-deficient pollen produces functional single sperm cells.

However, the proportion of single fertilization events was of the order of 1%, whereas the proportion of *msil* pollen with a single sperm cell was of the order of 6%. This suggested that either only a fraction of the single sperm cells was transported by the pollen tube or was able to perform single fertilization. Alternatively, a fraction of single sperm cells would divide during the transport by the pollen tube. We measured the proportion of single sperm cell at various times after pollen germination in vitro and observed a reduction of the percentage of pollen harboring a single sperm cell from 6 to 2% (Fig. 7A). Hence, *msil* single sperm cells could be transported into the pollen tube as in wild-type pollen harboring two sperm cells (Fig. 7B,C). However, we could record the division of single sperm cells into two sperm cells during pollen tube growth (Fig. 7C,D;  $n=2$ ). This observation and the decreased proportion of pollen tubes with single sperm cells following germination indicated that a fraction of sperm cells divide during their transport by the pollen tube, leading to a low proportion of pollen tubes delivering a single sperm cell. This low proportion is similar to the proportion of single fertilization events. We thus conclude that single fertilization of the egg cell or of the central cell is likely to result from the delivery of single sperm cells produced as a result of delayed cell cycle in CAF1-deficient pollen.

## DISCUSSION

### Loss of *MSI1* function affects CAF1 function during pollen development

In *Arabidopsis*, MSI1 directly interacts with members of a conserved, endosperm-specific Polycomb group (Pc-G) complex (Guitton et al., 2004; Köhler et al., 2003) and null *msil* alleles cause



**Fig. 7. Fate of the single sperm cell during *msi1* pollen tube growth.** (A) Percentage of pollen tubes transporting a single sperm cell from a *msi1-1/+ Arabidopsis* plant at different time points after pollen germination in vitro. Errors bars correspond to standard errors calculated on the basis of three replicates of 100 pollen grains. (B–D) Transport of sperm cells marked with HTR10-mRFP in pollen tube germinated in vitro from wild-type (B) and *msi1/+* (C,D) plants. (B) At 6 hours after germination, wild-type pollen tubes transport two sperm cells (two fluorescent red dots). (C) By contrast, at 6 hours a *msi1* pollen tube transports a single sperm cell. (D) At 7 hours after germination the *msi1* single gamete had divided into two sperm cells. (B,C,D) Superimposed DIC images of the pollen tube with the mRFP1 fluorescence signal identifying the sperm cells. Scale bars: 20  $\mu$ m.

production of autonomous endosperm and abnormal development of endosperm as the other mutants in genes encoding the subunits of the endosperm Pc-G complex (Guitton et al., 2004). MSI1 is also likely to be part of Pc-G complexes active during plant vegetative development (Berger et al., 2006; Chanvivattana et al., 2004). Accordingly, partial reduction of MSI1 activity during vegetative development phenocopies the effect of mutations in vegetative Pc-G complexes (Hennig et al., 2005; Hennig et al., 2003; Katz et al., 2004; Moon et al., 2003). However, *msi1*-null alleles also produce Pc-G independent phenotypes, including parthenogenesis and sporophytic embryo lethality, not observed in Pc-G mutants (Guitton and Berger, 2005; Guitton et al., 2004).

In this study, we report that *msi1* affects pollen development and is associated with reduced paternal transmission. By contrast, loss-of-function mutations in all members of the Pc-G complex active in endosperm do not show male transmission or pollen defects (Chaudhury et al., 1997; Grossniklaus et al., 1998). Other Pc-G genes are expressed during pollen development (see Fig. S1 in the supplementary material), but their absence does not cause male sterility (Chanvivattana et al., 2004; Gendall et al., 2001; Goodrich et al., 1997; Wang et al., 2006). FIE is essential for Pc-G function (Guitton et al., 2004; Ohad et al., 1996) and is expressed during pollen development (Hony and Twell, 2004). If defects in *msi1* pollen depended on Pc-G function, a similar reduction of paternal transmission should be expected for *msi1* and *fie*-null alleles. However, *fie* mutations are fully transmitted paternally (Guitton et

al., 2004; Ohad et al., 1996), and we could not observe any pollen defects in *fie/+* mutant (transmission efficiency of the null *fie-11* allele from the male is 99.8%;  $n=800$ ). We thus conclude that *fie* null mutations do not affect paternal transmission and that the effect of *msi1* on male transmission does not rely on Pc-G complexes.

Biochemical studies have demonstrated the potential association of MSI1 to the Rb-related protein RBR1 (Ach et al., 1997) but no direct evidence has been provided for a common function of RBR1 and MSI1 in *Arabidopsis*. Loss of RBR1 function alters pollen development. However the phenotypes associated with *rbr1* in pollen are dramatically distinct from *msi1* (C.Z. and F.B., unpublished data) and this difference does not support the association between RBR1 and MSI1 as the origin of pollen developmental defects in *msi1*.

Biochemical evidence has shown that MSI1 is also associated with the two core subunits of the CAF1 complex, FAS1 and FAS2 (Kaya et al., 2001). G2/M arrests have been reported in vegetative tissue of *fas1* and *fas2* homozygous mutants (Ramirez-Parra and Gutierrez, 2007). Our study shows the synergy between *fas1*, *fas2* and *msi1* mutations on paternal transmission of *msi1* and on pollen development, strongly suggesting that the defects of *msi1* pollen development are caused by the loss of CAF1 function.

The limited penetrance of single mutations in *msi1*, *fas1* and *fas2* may originate from inheritance of wild-type proteins or transcripts from the microspore mother cell heterozygous for the mutation. In mammals and *Drosophila*, CAF1-independent histone chaperone activities include HISTONE REGULATORY A (HIRA) and ANTISILENCING FACTOR 1 (ASF1), which are associated directly with the deposition of histones H3 and H4 on newly synthesized chromatin (Polo and Almouzni, 2006). In *Arabidopsis*, the limited penetrance associated with the *fas1*, *fas2* and *msi1* mutations might also result from a redundant activity mediated by the putative HIRA homolog (Phelps-Durr et al., 2005) pathway or by a basic function of putative ASF1 homologs (www.chromdb.org).

### Loss of CAF1 function during pollen development arrests the cell cycle but does not alter cell fate

Loss of CAF1 function causes activation of the DNA repair machinery (Endo et al., 2006; Exner et al., 2006; Kirik et al., 2006; Ramirez-Parra and Gutierrez, 2007) and a decline in CDKA1 activity (Ramirez-Parra and Gutierrez, 2007). Moreover, the expression of FAS1 is activated at the G1-S transition by E2F (Ramirez-Parra and Gutierrez, 2007). Whether the cell cycle and cell fate deregulation observed in *fas1* and *fas2* vegetative tissues (Costa and Shaw, 2006; Exner et al., 2006) originates directly from the deficit in CAF1 or results from more indirect epigenetic deregulations caused by the absence of CAF1 has remained unclear.

Within one to two cell divisions the pollen deficient for *MSI1* or *FAS1* and *FAS2* arrests before the first or the second pollen mitosis. DNA measurements suggested that the cell cycle arrest takes place at the G2-M transition, which is similar to that observed in vegetative tissues in plants and in other species. Hence we propose that loss of CAF1 function causes a cell cycle arrest before the first or the second mitosis during pollen development.

Cell cycle arrests at the G2-M transition would account for the most prominent phenotypes observed in *msi1* pollen. The wild-type sperm cells reach the mid-S phase, when pollen is shed from the anthers (Durberry et al., 2005), and probably reach the G2-M transition, when they are released into the female gametes (Friedman, 1999). The transmission efficiency indicates that an additional fraction of *msi1* pollen is unable to transmit *msi1* (see Fig. S6 in the supplementary material). It is thus possible that *msi1* pollen



in this fraction contain two gametes that do not reach the G2-M transition when they are released into the female gametophyte and are unable to fertilize the female gamete, leading to ovule abortion.

### Cell cycle impairment does not prevent differentiation during male gametogenesis

It has become clear that some regulators of cell division take an active part in cell-fate decisions. In flowering plants, most cells in roots and in shoots are produced by the activity of meristems, which contain dividing stem cells (Benfey and Scheres, 2000; Gegas and Doonan, 2006; Scheres, 2001). In the root meristem, cell fate appears to be specified after the asymmetric division of the stem cell (Castellano and Sablowski, 2005; Wildwater et al., 2005). However, the cell fate is established during the G1 phase (Caro et al., 2007; Costa and Shaw, 2006) and is not fixed until the last meristematic division, after which differentiation is initiated (Berger et al., 1998). Hence it is possible that cell division influences cell-fate commitment. The effects of mild alteration of cell cycle regulation during embryogenesis support the latter hypothesis (Jenik et al., 2005). Alteration of CAF1 causes aberrant morphogenesis of trichomes (Exner et al., 2006) and alters cell fate in root epidermal cells (Costa and Shaw, 2006). In contrast to cell-fate commitment in vegetative tissues, cell-fate establishment in pollen appears to be largely independent of cell-cycle deregulation in the *msi1* mutants, producing a fraction of bicellular pollen grains with a functional vegetative cell that delivers a functional single sperm-like cell. We have shown that *msi1* bicellular pollen correctly expresses cell-fate markers and produces a functional single sperm cell. Single sperm cells able to fertilize the egg cell are also produced by the mutant *cdka1* (Nowack et al., 2006). Hence cell fate and cell differentiation appear to be independent from cell-cycle regulation in pollen development.

### Are male gametes specialized for specific fusion with each type of female gamete?

*Plumbago zeylanica* produces dimorphic sperm cells with specialized organelle content that specifies the target female gamete (Russell, 1983; Russell, 1985). Several degrees of polymorphism have been reported in other species and in maize lines harboring supernumerary B chromosomes (Faure et al., 2003). Interestingly, B chromosomes are transmitted at higher frequency to the egg cell (Roman, 1948). Although such dimorphism was not reported in many species, including *Arabidopsis* (Faure et al., 2003; McCormick, 2004), it is nevertheless possible that isomorphic sperm cells differentiate to fertilize the egg cell or the central cell exclusively. This hypothesis was supported by the apparent preferential fertilization of the egg cell by single sperm cell produced in absence of the cyclin kinase *cdka1* (Iwakawa et al., 2006; Nowack et al., 2006). However, the *cdka1* single sperm cell may be able to fertilize the central cell, but this fertilization product may not be viable and was not detected as such. According to this hypothesis, sperm cell differentiation would rely on cell-cycle regulation by *cdka1*. However, such an event would cause a type of seed abortion, which was not detected in two independent studies (Iwakawa et al., 2006; Nowack et al., 2006). In contrast to *cdka1* pollen, *msi1* pollen causes a low percentage of single fertilization events, leading either to embryo or endosperm development. The proportion of *msi1/+* pollen that delivers single sperm cells is similar to the proportion of single fertilization events, suggesting that *msi1* single sperm cells are responsible for the single fertilization events. In addition we have shown that *msi1* single sperm cells express the terminal differentiation markers ATGEX1, accumulate HTR10 as wild-type

sperms, and are transported by the pollen tube. We thus propose that *msi1/+* plants produce a fraction of bicellular pollen with a single fully differentiated sperm cell able to fertilize either the egg or the central cell.

Thus, the origin of the absence of fertilization of the central cell by *cdka1* pollen remains unclear. The differentiation status of the *cdka1* single sperm cell has not been established and it could be incompletely functional and able to fertilize only the egg cell. Similarly, it is still possible that the two sperm cells produced in some *msi1* pollen may not reach full competence for fertilization, and one fertilization may fail and block further development. This could explain why when a single embryo is produced by *msi1* pollen, no autonomous endosperm development occurs, as reported in cases of single fertilization events by *cdka1* pollen (Nowack et al., 2006).

Whether in the wild type each of the two sperm cells has an equal capacity to fertilize either the egg or the central cell for each sperm cell remains unresolved. Our results rather support the argument that the two sperm cells are identical, in agreement with the identical morphology of the two sperm cells, the identical expression of any molecular marker studied to date in *Arabidopsis*, and the apparent capacity to fertilize either female gamete. The results obtained from the study of *cdka1* pollen rather support the opposite idea, which has been so far clearly shown only in the species *Plumbago zeylanica* (Russell, 1983; Russell, 1985). In vivo observation of double fertilization in *Arabidopsis* has been achieved recently (Ingouff et al., 2007) and might provide an answer to this long-standing problem.

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#### Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/135/1/65/DC1>

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