

Migration of sperm cells during pollen tube elongation in *Arabidopsis thaliana*: behavior during transport, maturation and upon dissociation of male germ unit associations

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Abstract The promoter sequence of sperm-expressed gene, PzIPT isolated from the S_{vn} (sperm associated with the vegetative nucleus) of *Plumbago zeylanica*, was fused to a green fluorescent protein (GFP) reporter sequence and transformed into *Arabidopsis thaliana* to better visualize the live behavior of angiosperm sperm cells. Angiosperm sperm cells are not independently motile, migrating in a unique cell-within-a-cell configuration within the pollen tube. Sperm cells occur in association with the vegetative nucleus forming a male germ unit (MGU). In *Arabidopsis*,

GFP was expressed equally in both sperm cells and was observed using a spinning disk confocal microscope, which allowed long duration observation of cells without bleaching or visible laser radiation damage. Pollen activation is reflected by conspicuous movement of sperm and pollen cytoplasm. Upon pollen germination, sperm cells enter the forming tube and become oriented, typically with a sperm cytoplasmic projection leading the sperm cells in the MGU, which remains intact throughout normal pollen tube elongation. Maturation changes, including vacuolization, general rounding and entry into G2, were observed during in vitro culture. When MGUs were experimentally disrupted by mild temperature elevation, sperm cells no longer tracked the growth of the tube and separated from the MGU, providing critical direct evidence that the MGU is a functional unit required for sperm transmission.

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Abbreviations

CCDs Charge-coupled devices
GFP Green fluorescent protein
GUS β -Glucuronidase
IPT Isopentenyl transferase
MGU Male germ unit
Pz *Plumbago zeylanica*

Introduction

Sperm cells of flowering plants are indeed true cells with a unique cell-within-a-cell relationship with the

surrounding pollen and an outwardly simple cytoplasmic organization. These male gametes are also unusual in being entirely dependent on the surrounding cytoplasm for their nutrition. Unlike the earliest seed plants, angiosperm sperm cells are not independently motile, but are believed to be conveyed by interactions of myosin with dynamic F-actin bundles located in largely isotropic orientation in pollen grains and axially within the sub-apical pollen tube (Hepler et al. 2001). For much of the twentieth century flowering plant sperm cells were believed to consist of bare nuclei, but careful light microscopic observation (Maheshwari 1950) and electron microscopy indicated that sperm, despite their small size, contained organelles and displayed other normal cell functions including transcription, translation and unique gene expression (Singh et al. 2008). The two male gametes also appear to be physically linked, as one sperm cell appears to be intrinsically associated with vegetative nucleus, whereas the other is linked to the first within a common inner pollen plasma membrane (Russell and Cass 1981), forming an assemblage known as the male germ unit (MGU) (Dumas et al. 1984). The role of the MGU in the passage of the male gametes is believed to optimize co-transmission of sperm cells at the time of optimal receptivity in the embryo sac, but its dynamic nature has not been examined before in living pollen tubes.

With the development of molecular biology, cell-specific promoters controlling transcription in the sperm cells have been described and they have been used to direct GUS and fluorescent labeling (Xu et al. 1999; Engel et al. 2005; Kliwer and Dresselhaus 2010) that vividly labels its cytoplasm and clearly delimits the sperm cells. Such constructs have also proved to be an enabling technology for isolating sperm cells through fluorescence activated cell sorting (Borges et al. 2008). However, historical attempts to visualize living sperm cells in pollen grains and tubes have been hampered by poor contrast despite interest in these morphologically dynamic cells. In the current paper, we use a sperm-specific promoter from *Plumbago zeylanica* to drive expression of green fluorescent protein (GFP) in *Arabidopsis*.

Considerable interest has been generated in observing living pollen tubes and directly observing pollen tube interactions (Higashiyama and Hamamura 2008), as well as fertilization and post-fertilization events (Berger et al. 2008), as well as the behavior of the cytoskeleton of sperm cells (Kliwer and Dresselhaus 2010). There have also been technical achievements that address early limitations of confocal microscopic observation, which include laser damage and bleaching. One approach uses infrared wavelengths to reduce the effects of high energy irradiation using two-photon microscopy techniques (Cheung et al.

2010), and in the current study we employ spinning disk confocal microscopy, which has design features that provide greater photon collection efficiency through sensitive CCDs and decreased exposure to the incident laser light. Spinning disk confocal microscopy also allows rapid collection of z-stacks so that the entire depth of the cell may be analyzed and reconstructed three-dimensionally over a relatively long duration (>20 min per session) with minimal bleaching.

In the current study, we employ GFP sperm labeling and newly available confocal microscopy techniques to better define the behavior of the male germ lineage, as well as changes in the organization of the MGU that accompany normal progamic maturation. We also examine the question of whether the MGU represents a “functional unit” required for targeting sperm cells.

Materials and methods

Plant promoter and gene construct

The promoter was isolated from the *PzIPT* gene of *P. zeylanica* L. (Gou et al. 2009) using nested PCR to amplify the 5'-flanking region upstream of the start codon from a *P. zeylanica* genomic DNA library constructed using the Lambda FIX II/*Xho*I Partial Fill-in Vector Kit (Stratagene, <http://www.stratagene.com/>). The vector used in these constructs was modified from pBIB (Becker 1990). pBIB vector was digested by *Bam*HI and *Hind*III, and the *pAnos-NPTII-Pnos* sequences were replaced by *Pmas* promoter and *BASTA* region, which were PCR amplified from pSKI015 (Weigel et al. 2000) and flanked with *Hind*III and *Bgl*II sites. The GFP coding sequence was inserted into *Bam*HI and *Sac*I sites of pBIB-BASTA to make pBIB-BASTA-GFP. For the construct of *pPzIPT1::GFP*, a 1,098 bp promoter region (*pPzIPT1*) was PCR-amplified using primers *PzIPT1p-Hind*III (AGTAAGCTTGCTGC AGAAAATTAACCAAAT) and *PzIPT1p-Sal*I (ACA GTCGACCGCTCGCTCAGTGAGTTACTGT) and then cloned into the *Hind*III and *Sal*I sites of pBIB-BASTA-GFP (Fig. 1a). Transgenic plants were generated using the floral dipping method (Clough and Bent 1998).

Plant growth, pollen tube culture and microscopic observation

Plants of *Arabidopsis thaliana* (DC.) Heynh. cv. Columbia (Col-0, NASC, University of Nottingham, UK) containing the above construct were grown in a growth chamber with 16 h light and 8 h dark at 18–20°C. The growth medium for this study was modified from Boavida and McCormick (2007) and contains 1% ultra low melt agarose (MB grade,

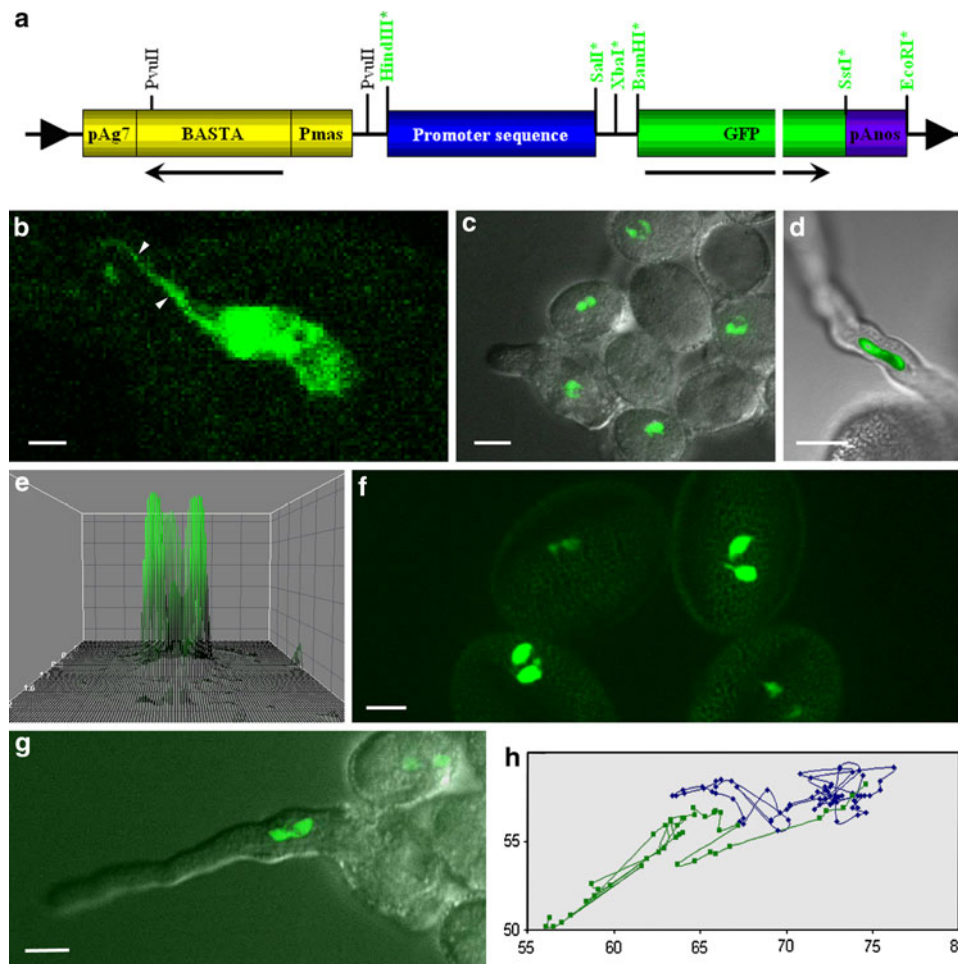


Fig. 1 *pPzIPT:GFP* construct and images of transformed *Arabidopsis* pollen showing GFP-fluorescent sperm cells. **a** Molecular construct of BASTA selection module fused to promoter of *Plumbago zeylanica IPT* gene fused to green fluorescent protein (*GFP*, construct not drawn to scale). **b** Sperm showing cellular projection (unlabeled arrowheads), intense fluorescence in cytoplasm and dense organellar regions at opposite pole that do not appear to accumulate GFP (bar 2 μm). **c** Germinating pollen grains with fluorescent sperm cells (bar 10 μm). **d** Sperm cells elongate upon entering the pollen tube

(bar 10 μm). **e** Z-plot of fluorescence intensity in the two sperm cells, displaying essentially equal GFP labeling. **f** Fluorescent sperm cells showing paired sperm cells and cytoplasmic projections, sometimes with shed cytoplasmic bodies nearby (bar 10 μm). **g** Rapidly elongating pollen tube showing fluorescent sperm cells entering tube (bar 10 μm). **h** Pathway of sperm cells is largely random prior to entry in to the pollen tube (blue lines), after which pathway becomes more linear (green lines). Numbers represent (x, y) locations in micrometers

USB Corporation, <http://www.usbweb.com/>), 0.01% boric acid, 5 mM CaCl₂, 5 mM KCl, 1 mM MgSO₄, and 10% sucrose in deionized water, adjusted to pH 7.5. Fresh pollen of five flowers was spread on a 6 μl drop of solidified medium on a glass microscope slide, to which another drop of liquid medium (without agar) of same size was added. A chamber was formed consisting of four intersecting lines of vaseline which support the coverslip and seals its edges. The glass slide was incubated in a moist chamber to avoid osmotic changes in the medium.

Germinated pollen and pollen tube images were collected after culturing for 0.5, 1, 3, 6, and 8 h using an UltraView ERS spinning disk confocal microscope (Perkin Elmer, Inc., http://www.cellularimaging.com/technology/spinning_disk_

<http://www.usbweb.com/>) or a Leica TCS SP2 AOBs confocal laser scanning microscope (Leica Microsystems, <http://www.leica-microsystems.com/>). Images were collected using the spinning disk confocal microscope using multiple z-planes (usually 15) at approx. 7 s intervals, which were integrated and constructed into movies or serial images. Laser intensity and CCD gain were optimized to minimize fading of the GFP signal, which even after periods of observation of >1 h could be maintained. The z-plot, velocity, cell path and fluorescence measurements were made using ImageJ analysis software, available free from the US National Institutes of Health (<http://rsbweb.nih.gov/ij/>). Velocity was calculated using a point representing the center of mass of the sperm cell in projection,

calculating linear distances and dividing by elapsed time between images.

Ablation of MGU associations

Normally growing pollen tubes were exposed to mild thermal elevation after 0.5–1 h culturing at room temperature. In each experiment, pollen grains of at least five fresh-opened flowers were spread on the medium of a minimum of five glass slides, with the results of prior observations representing the control. The temperature of the heater was adjusted to within 29–33°C for 1 h, adjusting temperature empirically to obtain about half altered MGUs, with the remaining normal MGUs serving as an internal control. Treated pollen grains were, except for MGU effects, typically healthy and displayed a high germination rate (70–80%, similar to Boavida and McCormick 2007).

Results

pPzIPT:GFP reporter gene construct labels sperm cells with high fluorescence yield

The introduced pPzIPT:GFP reporter gene construct provides high fluorescence yield in the sperm cells of transformed *Arabidopsis* plants. Expression of the fluorescent protein in sperm cells provides cellular detail, differentially labeling hyaloplasm, but apparently is excluded from non-porate membrane-bound organelles such as mitochondria and vacuoles (Fig. 1b). A sperm cellular projection is conspicuously labeled in Fig. 1b (unlabeled white arrowheads) and is present to a greater or lesser degree throughout transport of sperm cells in the pollen tube. Sperm cells are labeled in almost all pollen grains when viewed in through-focus series (Fig. 1c). Activation of pollen is evident in the movement of the sperm cells, which display migration even in ungerminated pollen grains, and through organellar movement in the cytoplasm of the pollen vegetative cell. Sperm cell fluorescence by itself is not, however, indicative of activation, as some pollen containing GFP-labeled sperm cells fail to display cytoplasmic movement and do not germinate (see Supplementary Movie 1). Labeling of sperm cells remains intense within elongating pollen tubes, whereas pollen tube cytoplasm (Fig. 1d) and sporophytic tissues (not shown) appear to be unlabeled.

In *Arabidopsis*, fluorescent protein expression is essentially equal in paired sperm cells, as shown by a z-plot of fluorescence intensity (Fig. 1e) and comparable images of other transformed cells using confocal laser scanning microscopy (Fig. 1f). Elongating pollen tubes allowed the

sperm cells to be well resolved using spinning disk confocal fluorescence microscopy since tubes generated no noticeable autofluorescence (Fig. 1g). Movement of sperm cells was evident in intervals as short as 5 s. To compile high-resolution images showing entire sperm cells, 15 planes were collected for each composite image, allowing images to be collected every 7–10 s. When the spinning disk microscope was optimally adjusted, fluorescence decay was not appreciable over 20 min or longer.

Sperm movement, initially circumferential, orients within a single pole in the tube

Movement of the sperm cells within the pollen grain tends to follow a typically circumferential pattern, remaining several micrometers from the intine. As pollen germinates, however, this pattern tends to become less random (Fig. 1h, blue lines), presumably in response to actin cytoskeletal alterations that coincide with tube establishment (Hepler et al. 2001). As the pollen tube reaches a length of 5 μm or longer, sperm cells typically enter tubes displaying vigorous cytoplasmic activity. During initial transport within the tube, the pathway sometimes retains its random nature and the polarity of movement appears to be poorly determined—the cytoplasmic projection may lead or follow and may even rotate within the tube (Supplementary Movie 2). This pattern may remain for several minutes as sperm cell movement within the pollen tube becomes established (as shown), but this is variable. During ensuing elongation of the tube, the sperm cells appear to establish a polarity with preference for the projection leading the sperm cell pair (Fig. 1h; Supplementary Movie 2). Once polarity of the projection is established, sperm cell movement increases in linearity and decreases in randomness (Fig. 1h, green lines).

Movement of sperm cells has both a cyclic and variable component

Figure 2 shows examples of sperm cells in two pollen tubes, one in early (Fig. 2a) and later elongation (Fig. 2b). Within each 200-s period, there are typically ≈ 6 cycles of alternating faster and slower elongation. The velocity of the cells could reach or exceed 0.6 $\mu\text{m}/\text{s}$, but this varied considerably with changes in the tube diameter, age and vigor. The majority of velocity measurements, however, were between 0.01 and 0.1 $\mu\text{m}/\text{s}$ during early stages, increasing by up to 2–3 \times at later stages. The most rapid velocities occurred more than 1 h after pollen germination, when the tube is $>20\times$ the width of the pollen grain. Retrograde movement is also observed, but the net movement is overwhelmingly directed toward the tip. Cellular projections of the sperm cell associated with the vegetative nucleus frequently lead the sperm cells, but the projection

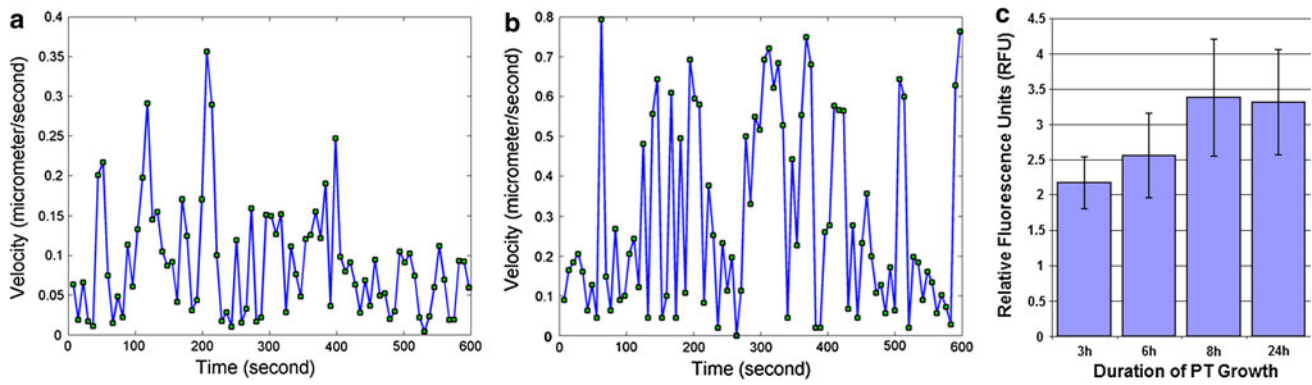
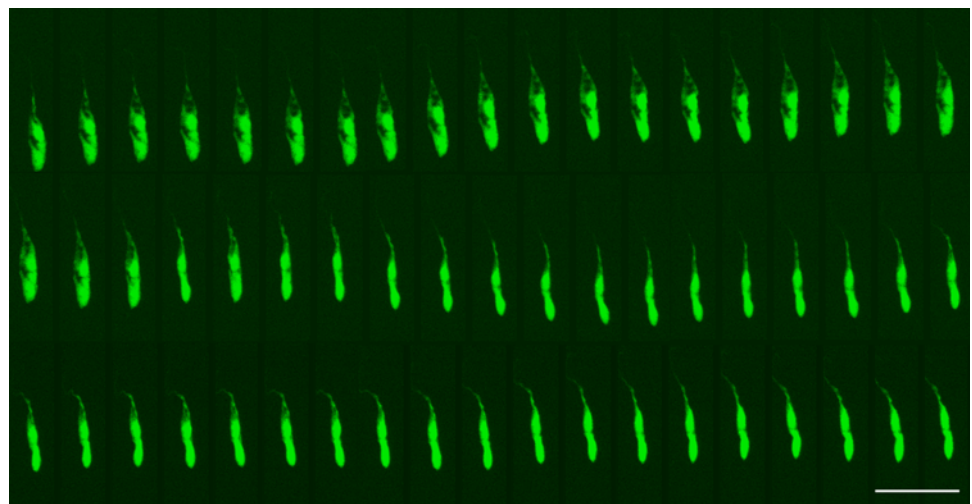


Fig. 2 Charts summarizing velocity of sperm cell movement and cell cycle progression in the pollen tube. **a** Early elongation phase. **b** Later elongation phase. **c** DAPI fluorescence in relative fluorescence units

(RFU) reflects changes in DNA concentration in sperm cells after 3, 6, 8 and 24 h of tube elongation reflects completion of cell cycle

Fig. 3 Panel of images of fluorescent sperm cell pairs taken 7.5 s apart over approximately 7 min during pollen elongation, displaying relative location, pattern of cytoplasmic labeling, vacuolization, flattening of cells, changes in the projection and rotation of the cells. Pollen tube tip is toward the top of the image (bar 10 μ m)



itself often displays movement that is somewhat independent of the overall momentum of the sperm cells. Thus, the cellular projection appears to change length and its association often appears slack and not under tension. Sometimes the projection retracts, while the remainder of the sperm cell proceeds forward (Supplementary Movie 3).

Behavior of sperm cells appear to mimic in vivo behavior throughout tube elongation

To examine whether sperm cells grown in vitro may mimic maturational changes that occur during in vivo growth periods, we compared the progression of the cell cycle in sperm cells over time between our in vitro data and published in vivo data (Friedman 1999). In our in vitro material, DAPI fluorescence intensity in sperm cells increased by nearly 60% during 8 h of pollen tube elongation, after which intensity did not increase any further (Fig. 2c). We believe that this is indicative of completion of the cell cycle within 8 h, which reflects prior in vivo measurements.

In pollen tubes having reached 5–8 h of elongation—a time sufficient to have fertilized the embryo sac—we found that the sperm cells had undergone changes in organization that mimicked the changes reported in ultrastructural studies. Figure 3 provides a panel of sperm cell images from one 5 h pollen tube, shown at 7.5-s intervals over approximately 7 min. First, the paired cells tend to be more elongated and often became flattened as the pollen tube reached 5 h after germination. Also, cells appear to accumulate vacuoles during their time in transit and change form as they penetrate regions of the tube of subtly different diameters. Each change is indicative of prior observed sperm cell behaviors as cells approach fertilization (see also Supplementary Movie 4).

MGU dissociation inhibits sperm transport

To investigate the role of the MGU in sperm translocation, we experimentally exposed elongating pollen tubes to mild temperature elevation above that recommended for

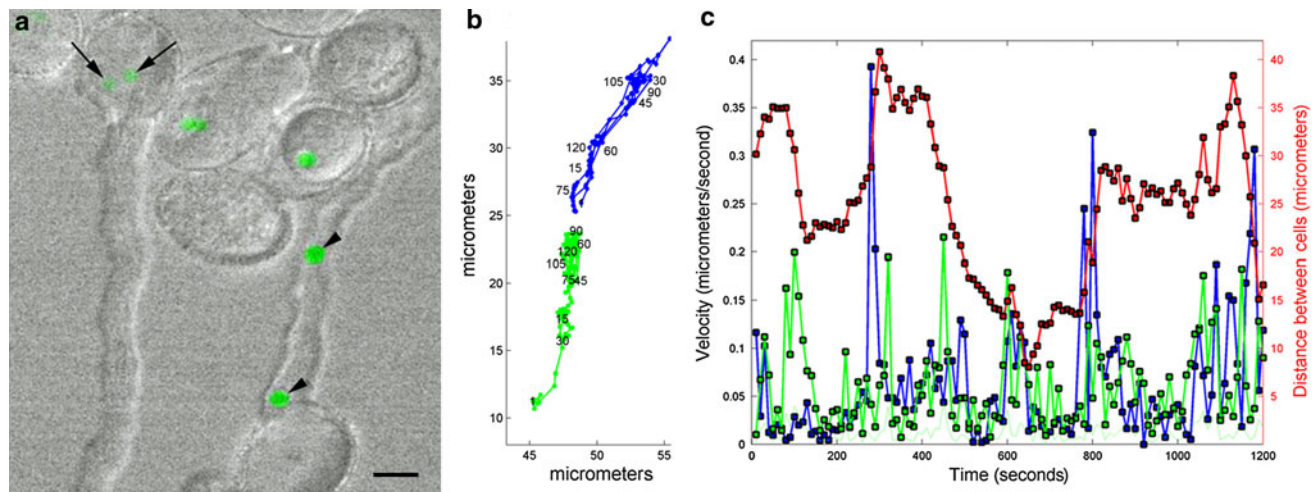


Fig. 4 MGU dissociation after exposure to mild temperature elevation results in failure of sperm cells to maintain pace with tip elongation. **a** Pollen grains with tubes displaying, delayed or absent sperm cell entry into the tube (unlabeled arrows) and dissociated sperm cells after entering the elongating tube (unlabeled arrowheads)

Arabidopsis (Boavida and McCormick 2007). This procedure could be optimized to obtain dissociation of approximately half of the MGUs, such that we could separate the intrinsic effects of temperature from MGU dissociation. Figure 4 illustrates sperm cells from two pairs of such altered MGUs. In one, the sperm cells (unlabeled arrowheads) have become distantly separated, losing their usually stable position in register with the growing pollen tube tip. In a second pollen grain, the two sperm cells (unlabeled arrows) have not left the pollen grain, despite the formation of a normal appearing elongating pollen tube. The two sperm cells have dissociated and remain in random motion, but have not entered the tube; at this length all tubes under normal conditions display active MGUs moving directionally within the tube. The dissociated sperm cells in the pollen tube move randomly relative to each other, whereas those in the pollen tube display highly active but disorganized movement (Supplementary Movie 5). Restoring elongating pollen tubes to normal temperatures did not restore normal sperm cell associations.

Discussion

Sperm promoter function is conserved but does not express dimorphic differences in *Arabidopsis* sperm cells

PzIPT in its native plant, *P. zeylanica*, is strongly transcribed in just one of the two sperm cells within the pollen, as confirmed by real-time RT-PCR and in situ hybridization (Gou et al. 2009). However, in *Arabidopsis*, the pPzIPT:GFP

but failing to track the tip ($bar\ 2\ \mu m$). **b** Chart of sperm cell positions at intervals shown by position numbers. **c** Velocity of sperm cells (blue and green lines, scale on left axis) plotted versus distance between sperm cells (red line, scale on right axis) over time

reporter gene construct is highly expressed in both sperm cells and does not appear to recognize differences that may exist between the two sperm cells. *Arabidopsis* displays a MGU displaying an inborn polarity through the presence of the cellular projection that appears to physically associate one sperm to the vegetative nucleus, which is similar to other angiosperms (Mogensen 1992). This suggests that sperm cells of *Arabidopsis* respond monomorphically, or in any case, do not display dimorphism in the same pattern as *Plumbago*, despite high conservation of essentially sperm-specific expression. Used in concert with spinning disk confocal microscopy in appropriate in vitro and in vivo contexts, this reporter construct provides a powerful tool for exploring sperm behavior.

MGU transport and sperm maturation in the pollen tube

The passage of sperm cells in the pollen tube exceeds rate of pollen tube elongation because of repeated retreats and advances of the MGU during conveyance. In addition to these longitudinal changes in placement, the position of the MGU is also subject to changes, as well as rotation, of the MGU. As the MGU is enclosed by a common pollen membrane that surrounds the sperm cells, this also contributes to dynamism as this system is subjected to stress. The pollen tube cytoplasmic face of this membrane is believed to be the one that is associated with myosin and thus is a focal location of stresses involved in MGU conveyance and is subjected to rapid changes in form that affect the shape of the constrained sperm cells. The cellular projection often leads but frequently appears to move more slowly than the rest of the cell. This indicates a role of the

projection in linking the MGU rather than exerting force on the larger bodies of the sperm cells.

Limited data are available on changes occurring in living sperm cells in the tube during maturation, but ultrastructural studies have indicated that they become more highly vacuolated, more rounded, and have a less conspicuous projection (Russell et al. 1990; Russell 1992; Yu et al. 1994). A similar pattern is observed in MGUs in the current study. Additional evidence that these changes mimic sperm maturation in vivo is that the sperm cells appear to advance in the cell cycle and complete S-phase. *Arabidopsis* sperm cells are reported to have a DNA content of $\sim 1.53 C$ at anthesis. When sperm cells reach the ovules they are at G2, and are ready for fertilization (Friedman 1999), although mature pollen tube expression appears to require passage in the style (Qin et al. 2009). Our observations of sperm cells in vitro appear to mimic cellular changes observed in vivo, indicating that in vitro tube culture may be a convenient experimental system for modeling progamic changes during flowering plant sexual reproduction.

Sperm dissociation from the MGU inhibits sperm transport/fertilization

Thermally induced male sterility in plants has been attributed to many causes, including failures in synthesis, signaling and assembly, and is often associated with the formation of heat shock proteins (Ma 2005). However, this appears to be the first report of sterility from sperm cells failing to be transported in otherwise normally appearing tubes. Although specific molecular causes underlying sperm dissociation are unknown, it is clear that the sperm cells are no longer contained in common within the usual internal pollen plasma membrane, rather they track separately within the tube and their position within the tube becomes largely random. Since this thermal effect occurs at relatively low temperatures, it may be a significant source of targeting failure within the tube which could occur with even modest change in environmental temperature. This may be an unexpected cause of failure that is relatively subtle compared to other predictions (Wan et al. 2002).

That the dissolution of the MGU resulted in failure of the sperm cells to accurately track elongation at the tip, the presence of the association of the sperm cells within the common internal pollen plasma membrane appears to be necessary for function. Thus, it is clear that early statements about this assemblage representing a “functional unit” (Russell and Cass 1981) are supported and the MGU is expected to be generally necessary among angiosperms.

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