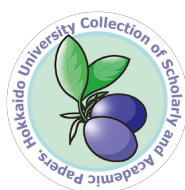




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Abstract

Pollen tubes of *Cyrtanthus mackenii*, a species with bicellular pollen, were cultured in vitro to investigate nuclear phase changes during generative cell division and male germ unit (MGU) formation using flow cytometric analysis. Results revealed that sperm cells were formed after 12 h of culture. During sperm maturation, the nuclei of sperm cells were not associated with the vegetative nucleus (unassociated sperm cells; Sua) and became longer than those of sperm cells associated with the vegetative nucleus (Svn). These findings indicate that the pair of sperm cells in the *C. mackenii* MGU is dimorphic in terms of nuclear shape. Dimorphism coincides with anti- α -tubulin antibody immunofluorescence, which was higher in the Sua than in Svn. Following treatment with oryzalin, triggering microtubule depolymerization, differences between nuclear shape in the two sperm nuclei disappeared, suggesting that microtubule accumulation between sperm cells in the MGU correlates with differences in the nuclear shape.

Keywords: *Cyrtanthus mackenii*, male germ unit, microtubule, pollen tube, sperm dimorphism

Introduction

Double fertilization in flowering plants is a phenomenon through which pollen tubes deliver two sperm cells into female gametes. The generative cell or the pair of sperm cells in pollen tubes or grains is reported to be closely associated with the vegetative nucleus in many species, including both bicellular and tricellular pollen (Mogensen 1992). This association forms the male germ unit (MGU), which is proposed to function as a vehicle for the transmission of male gametes to female gametes and to participate in the fusion with the target female cell during fertilization (Dumas et al. 1984). In *Arabidopsis thaliana* L. Heynh, the position or organization of the MGU in mature pollen has been suggested to influence the efficiency of male gamete delivery (Lalanne and Twell 2002).

In the MGU of several species, sperm cells that are associated with the vegetative nucleus (Svn) and those that are not (unassociated sperm cells; Sua) differ in size, shape, and organelle content (reviewed in Mogensen 1992; Weterings and Russell 2004). In *Plumbago zeylanica* L., the Sua, which contains numerous plastids, preferentially fuses with the egg cell, while the Svn, which contains numerous mitochondria, fuses with the central cell (Russell 1984; Russell 1985). The two sperm cells in the MGU are also reported to differ in terms of polyubiquitin gene expression (Singh et al. 2002), surface charge (Yang et al. 2005; Zhang and Russell 1999). Additionally, numerous genes were highly upregulated in the dimorphic sperm cells of *P. zeylanica*, displaying significantly different expressional profiles (Gou et al. 2009). Such evidence of preferential fertilization and expressional differences between sperm cells suggests the possibility of the presence of a system for controlling male gamete maturation and identity.

Generative cells and sperm cells have many structural features that are distinct from somatic cells (Southworth and Cresti 1997). From an early stage, generative cells become spindle shaped and shed cytoplasm (Russell and Strout 2005). The microtubular cytoskeleton of generative cells and sperm cells differs from that of somatic cells: in maturing male germ cells, microtubules are typically arranged in prominent bundles that are aligned helically or longitudinally relative to the long axis of the cell (Palevitz and Tiezzi 1992; Southworth and Cresti 1997). It has been reported that isolation of generative and sperm cells from pollen grains and tubes results in loss of microtubules or a mesh-like pattern, and the cells acquire an essentially spherical shape (Russell 1991; Thunis et al. 1991). Therefore, microtubules are believed to play a key role in maintaining cell shape.

We previously reported that flow cytometry (FCM) analysis combined with micromanipulation is useful for a rapid evaluation of the general behavior of male gametes during pollen tube growth in *Alstroemeria aurea* Graham (Hirano and Hoshino 2009). However, the applicability of this method to other species has not yet been examined. In the present study, we applied the FCM-based method to *Cyrtanthus mackenii* Hook. f., a species with bicellular pollen, and analyzed male gamete behavior during pollen tube elongation. Furthermore, we cytologically characterized the pairs of sperm cells in the MGUs, which were detected by FCM, and attempted to elucidate morphological differences between the two sperm cells in a pair during maturation.

Materials and methods

Plant materials and pollen culture

Cyrtanthu mackenii Hook. f. (Amaryllidaceae) plants used in this study were greenhouse grown with anthers were collected from the flowers after dehiscence and maintained at -20°C . Pollen grains from two anthers were sown in 2 ml of liquid pollen culture medium (Hirano and Hoshino 2009) and cultured at 25°C in the dark. For microtubule depolymerization, oryzalin (final concentration, $5\text{ }\mu\text{M}$) was added to culture medium after 12-h incubation, and the pollen grains were cultured for an additional 12 h. During 6-36-h culture, pollen tubes were collected at appropriate intervals from the culture medium using a $77\text{-}\mu\text{m}$ nylon mesh and were used for the subsequent male gamete analysis. To visualize the nuclei within the pollen tube, we added 4',6-diamidino-2-phenylindole (DAPI; final concentration, $1\text{ }\mu\text{g ml}^{-1}$) and 0.5% Triton X-100 (final concentration) to the culture medium. After 15 min, nuclei within pollen tubes were observed under an epifluorescence microscope (Axiovert 200; Carl Zeiss, Oberkochen, Germany).

FCM analysis of pollen tubes and isolated cells and nuclei

Pollen tubes and isolated cells and nuclei were analyzed by FCM according to the method described by Hirano and Hoshino (2009). To determine nuclear ploidy level in pollen tubes, we collected pollen tubes as noted above and transferred them to $200\text{ }\mu\text{l}$ of nuclear extraction buffer (CyStain UV Precise P kit; Partec, Münster, Germany), chopped tubes with a sharp razor blade, and incubated then with $800\text{ }\mu\text{l}$ of staining buffer from the CyStain UV Precise P kit. The cell suspension was filtered through a $30\text{-}\mu\text{m}$ nylon mesh and analyzed using a flow cytometer (Ploidy Analyzer PA; Partec) according to our previous protocol (Hirano and Hoshino 2009), repeating the experiment six times.

Vegetative nuclei, generative cells, sperm cells, and MGUs (n=30 each) were individually collected using a microcapillary connected to a micropump (Nano Spuit; Ikeda Scientific Co. Ltd., Tokyo, Japan). Cells and nuclei were identified based on shape and labeling characteristics: vegetative nuclei were filamentous or irregularly shaped, generative cells had oval nuclei, and sperm cells had spherical nuclei. Collected cells and nuclei were transferred to 100 μ l of fresh extraction buffer, to which 400 μ l of staining buffer was added and analyzed by FCM. The 2C DNA value was determined using the leaves of *C. mackenzii* 1C DNA content was estimated. The lowest DNA content in the pollen tubes, corresponding to half the fluorescence peak for the leaf 2C DNA, was regarded as 1C DNA in the FCM histogram.

Immunofluorescence analysis for microtubule detection

Immunocytochemical analysis was performed according to the protocol described by Hoshino et al. (2004) and Kranz et al. (2008) with several modifications. Pollen tubes cultured for 6 or 12 h were transferred onto a polylysine-coated coverslip and fixed for 1 h with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in the microtubule-stabilizing buffer (MSB, 50 mM 1,4-piperazinediethanesulfonic acid, 5 mM ethylene glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1 mM MgCl₂, and 2% (v/v) glycerol, pH 6.9). Pollen tubes were then washed 3 times with MSB and treated with an enzyme solution containing 1% Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% Macerozyme R-10 (Yakult Pharmaceutical), 0.5% hemicellulase (ICN Biomedicals Inc., Costa Mesa, CA, USA), 0.05% potassium dextran sulfate, and 0.22 M sucrose in phosphate-buffered saline (PBS) for 10 min. Thereafter, the tubes were washed 3 times with PBS to remove the enzyme solution and immersed in PBS supplemented with 0.1% polyoxyethylene (20) sorbitan monolaurate

for 15 min.

Pollen tubes were collected at each culture period from 9-36 h into 200 μ l of fixative containing 2% polyoxyethylene (20) sorbitan monolaurate and chopped to extract MGUs. To the isolated MGUs, 800 μ l of the above fixative solution was added and DAPI (final concentration, 1 μ g ml⁻¹) was added to the filtered cells. After 30 min fixation, MGUs in the suspension were transferred onto polylysine-coated coverslips using a microcapillary and rinsed 3 times with MSB.

The enzymatically treated, surface-activated pollen tubes and the fixed MGUs were treated with a blocking agent (Image-iT FX Signal Enhancer; Molecular Probes Inc., Eugene, OR, USA) for 30 min, and incubated with 1 μ g ml⁻¹ of an anti- α -tubulin mouse monoclonal antibody (A11126, Molecular Probes) in PBS supplemented with 0.05% bovine serum albumin (BSA) for 120 min at 25°C. Then pollen tubes and MGUs were washed 3 times in washing buffer (as above) and treated with 5 μ g ml⁻¹ of the secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse antibody, A11001; Molecular Probes) in BSA-supplemented PBS at 25°C for 120 min. Thereafter, MGUs were washed 3 times with washing buffer, labeled with 1 μ g ml⁻¹ DAPI for 15 min, and mounted with coverslips in the presence of the SlowFade Gold antifade reagent (Molecular Probes). Nuclei and immunofluorescence of the sperm cell pairs in the MGUs were observed using an epifluorescence microscope. In the following immunological control experiments, no staining was detected: omission of primary antibody, omission of secondary antibody, omission of both antibodies.

A Nikon TE2000-E inverted microscope equipped with a Nikon C1Si spectral imaging confocal laser scanning system (Nikon Co. Ltd., Tokyo, Japan) was used for confocal microscopy experiments, and the data analyzed using the Nikon EZ-C1 3.70 software. For estimating the intensity of fluorescence arising from Alexa Fluor 488 in

sperm cells in the MGUs, all the sperm cell pairs were imaged at 0.5- μ m intervals. Total fluorescence emitted from each sperm cell was measured by integrating volume and mean fluorescence over the entire image. Net nuclear fluorescence intensity was calculated by subtracting total background fluorescence around each sperm cell from total measured fluorescence of each cell. Intensities from 15 MGUs were compared at each time point, assuming the Svn to be 1.0 units.

Statistical analysis

Proportions of C-values in the pollen tubes were subjected to an analysis of variance after arcsine transformation, and the means were compared using the least significant difference (LSD) test. The values of nuclear size and fluorescence intensity ratio during each culture period between 12-36 h were also analyzed by the LSD test. Nuclear length, width and immunofluorescence intensities of Svn and Sua were compared by Student's *t* test.

Results

Relative 1C, 2C, and 3C DNA content in pollen tubes cultured for 6 h was determined to be 41.4%, 42.7%, and 15.9%, respectively (Fig. 1a; Table 1). Nuclear phase changes were observed between 6 and 12 h of culture: the proportion of nuclei with 2C DNA decreased from 42.7% to 27.9%, whereas that of nuclei with 1C DNA increased from 41.4% to 56.2% (Table 1). The proportion of nuclei with 3C DNA remained unaltered during the culture period from 6 to 24 h but tended to decrease with extended culture (>30 h; Table 1).

Pollen tubes cultured for 6 h displayed longitudinally oriented microtubule bundles in the generative cell with cytoplasmic extensions associated with the vegetative

nucleus (Fig. 2). Pollen tubes cultured for 12 h contained sperm cells (Fig. 2), which contained microtubules distributed in the cytoplasmic region between adjacent sperm cells and the nuclear surface, but cortical microtubules had not developed (Fig. 2).

Suspension of crushed pollen tubes obtained after 6 h of culture contained individual vegetative nuclei associated with the generative cells (Fig. 3a, b), indicating intact MGUs. FCM analysis of these nuclei and cells (n=30) contained 1C and 2C DNA, respectively (Fig. 1b, c), whereas the entire MGUs contained 3C DNA (Fig. 1d). Pollen tubes cultured for 6 h in vitro also showed the presence of a vegetative nucleus and generative cell, but MGUs did not persist (Supplemental Fig. S1). Therefore, we believe that the nuclear ploidy level in the 6h cultured pollen tubes (Table 1) displayed proportionate levels of 1C, 2C, and 3C DNA corresponding to individual vegetative nuclei, generative cells and MGUs, respectively. 18-h suspension cultures of pollen tubes contained vegetative nuclei, sperm cells, pairs of sperm cells (Fig. 3c, d) and MGUs (Fig. 3e, f). FCM confirmed that the vegetative nuclei and sperm cells contained 1C DNA (Fig. 1e, f), and that sperm cells and the MGUs contained 2C and 3C DNA, respectively (Fig. 1g, h).

Overlapping cell boundaries between Svn and Sua in the pollen tubes impeded individual cell measurements (Fig. 2), so we isolated living MGUs from pollen tubes cultured for 12–36 h and measured the nuclear size of the Svn and Sua. Isolated generative cells and sperm cells, however, acquired a spherical shape in the extraction buffer (Fig. 3a–f), so pollen tubes were fixed in a solution containing a surface-active agent and nuclear size measurements were made on fixed material. Among sperm cells isolated from pollen tubes cultured for 12 h, the length and width of the Svn nuclei were 10.3 and 5.9 μm , respectively (Table 2), whereas those of the Sua nuclei were 11.4 and 5.7 μm , respectively; the Sua nuclei were significantly longer than the Svn nuclei (Table

2). In 24-h cultured pollen tubes, the both sperm nuclei elongated, but their width remained unchanged. Svn and Sua nuclei were 10.9 and 13.2 μm in length, respectively. Thus, Sua nuclei were still significantly longer than Svn nuclei, with no change in nuclear parameters from 24 to 36 h culture.

Microtubule distribution in the sperm cell pair was examined to determine its potential relationship with morphological differences between the two sperm in the pair. In MGUs isolated from pollen tubes cultured for 12 h, immunofluorescence arising from the anti- α -tubulin antibody was 1.12-times more intense in the Sua than in the Svn (Table 3). The fluorescence-intensity ratio in the pairs of sperm cells (Sua/Svn) was 1.29 at 24-h culture and 1.34 at 36-h culture. Thus, the fluorescence intensity of the Sua was significantly higher than that of the Svn from 24–36 h of culture, the ratio of immunofluorescence remained unchanged during 12–36 h of culture.

Pollen tubes cultured for 12 h with 5 μM oryzalin were cultured for an additional 12 h to determine its effect on sperm cells. In control sperm cells isolated from 24-h pollen tube culture, abundant microtubules were distributed between the plasma membrane and nucleus (Fig. 4). In contrast, oryzalin-treated pollen tubes contained sperm cells in which microtubule fragmentation was observed, and these cells contained a spherical nucleus, unlike those of untreated sperm cells (Fig 4). The length and width of oryzalin-treated Svn nuclei were 9.5 and 6.3 μm , respectively, and those of the Sua nuclei were 10.0 and 6.3 μm (Table 4). In contrast to wild type, these treated Svn and Sua nuclei did not significantly differ from one another in terms of length and width. Results using oryzalin to experimentally disrupt microtubule polymerization suggest that microtubules in sperm cells were responsible for the dimorphism in the cytoplasm and nuclei of the Svn and Sua.

Since differences were noted in microtubule accumulation patterns of the Svn and

Sua in 12-h pollen tube cultures (Table 3), microtubule distribution was examined earlier, during generative cell division in the MGUs isolated from 9-12 h cultured pollen tubes. We found that generative cells at that stage contained a basket-like array of cortical microtubule bundles (Fig. 5) that was arranged in a pattern similar to that of generative cells in pollen tube (Fig. 2). No preprophase band was observed during generative cell division before mitotic spindle formation (data not shown). Kinetochore microtubule bundles were formed between metaphase and anaphase, but the chromosomes did not align along an equational metaphase plate (Fig. 5). When the daughter chromosomes separated and kinetochore bundles disappeared, phragmoplasts formed toward the center of the daughter cells (Fig. 5). These results indicated that microtubule distribution during generative cell division did not differ markedly between the two spindle poles. In newly-formed sperm cells isolated from 12 h cultured pollen tubes for, the microtubules accumulated from the connected region of the sperm cells to the nucleus (Fig. 5). Immunofluorescence arising from the anti- α -tubulin antibody was significantly higher in the Sua than in the Svn (Table 3).

Discussion

Changes occurring during the maturation of the MGU, particularly in the context of dimorphism are uncommon in the literature. During pollen tube growth *in vivo*, sperm cells become rounded as the pollen tubes approach the ovule (Russell and Cass 1981; Russell et al. 1990). However, in the present study, we observed that the sperm cells in the MGUs did not exhibit changes in nuclear shape or pattern of microtubule accumulation from 24 to 36 h of culture (Table 2, 3). In addition, the distances between vegetative nucleus and Svn nucleus and between nuclei of Svn and Sua were not significantly different from 24 to 36 h culture (Supplemental Table 1), although

distances between Svn and Sua were significantly different in 12-24 h cultures (Supplemental Table 1). That the proportion of 3C DNA did not change (Table 1) suggests that no changes in MGU were detected by flow cytometer from 12 to 36 h of culture. Isolated paired sperm cells from pollen grains often separate in buffer solution (Zhang et al. 1998), and a similar phenomenon occurred in our previous study on FCM analysis of pollen tubes (Hirano and Hoshino 2009). Therefore, some MGUs appear to dissociate into individual cells and nuclei, or into vegetative nucleus and pair of sperm cells during isolation or in the extraction buffer used for FCM. It is possible that some physiological and/or morphological changes occur in the MGUs from 24 to 36 h of culture, and the dissociation of MGUs may be enhanced in pollen tubes or during the FCM procedure.

Generative cells and sperm cells isolated from pollen tubes generally become spherical because the microtubule bundles are lost or reorientated in a mesh-like pattern (Russell 1991; Theunis et al. 1991). In a previous study on *Nicotiana tabacum* L., when pollen grains were squashed in the fixation medium, the isolated generative cells were found to retain their spindle shape and the basket-like cytoskeleton of the microtubule bundle (Theunis et al. 1992), with spindle shapes retained by sperm cells as late as their deposition in the synergid (Tian et al. 2005). Consistent with these report, our results revealed that generative cells and sperm cells became spherical when isolated from pollen tubes in FCM extraction buffer (Fig. 3), whereas fixed cells supplemented with surface-active agent remained spindle shaped and retained microtubule bundles in the same pattern as those in pollen tubes (Fig. 2, 5).

Sperm dimorphism in terms of structure or organelle content has been identified in several angiosperm species (reviewed in Saito et al. 2002, Weterings and Russell 2004). In the present study, we observed that *C. mackenzii* sperm cells also exhibited structural

dimorphism in terms of nuclear shape. Immediately after the formation of sperm cells in pollen tubes cultured for 12 h, the Sua nuclei were found to be significantly longer than the Svn nuclei (Table 2). The difference in nuclear length between Svn and Sua increased with extended culture time from 12 to 24 h. The patterns of dimorphism in sperm cells in the MGUs, in terms of cell shape, have been reported to vary among species: the volume and surface area of Svn are greater than those of Sua in *P. zeylanica* (Russell 1984) and *Torenia fournieri* (Chen et al. 2006), while the volume of the Sua is greater than that of Svn in *N. tabacum* (Tian et al. 2001). Moreover, in the case of *N. tabacum*, dimorphism arises late during sperm development (Tian et al. 2001), whereas in *Plumbago zeylanica*, it progressively advanced during development (Russell and Strout 2005). We propose that sperm dimorphism in *C. mackenii* follows the pattern of *Nicotiana*, as changes occur in Sua characteristics during sperm maturation.

Microtubule bundles in sperm cells are reported to show no arms or bridges to the nuclear envelope; however, a reduction in the cytoplasm volume brings the microtubule bundles closer to the nucleus (Southworth and Cresti 1997). We therefore investigated the effect of microtubule accumulation nuclear shape in sperm cells. The intensity of immunofluorescence arising from the anti- α -tubulin antibody was higher in Sua than in Svn, from immediately after sperm cell formation, to just before the cessation of pollen tube growth (Table 3), indicating that the Sua showed higher microtubule accumulation than Svn and there was sperm dimorphism in terms of microtubule accumulation.

To determine the origin of the difference between Svn and Sua in terms of microtubule accumulation, we examined microtubule distribution during generative cell division in isolated MGUs. Kinetochore microtubule bundles and phragmoplast microtubules were equally distributed in both the hemispheres of the generative cell (Fig. 5). Immediately after sperm formation, the distribution of microtubules from the

connected region of the sperm cells to the nuclear surface (Fig. 5) and the intensity of immunofluorescence arising from the Sua was significantly higher than in the Svn (Table 3). These differences were not enhanced with additional culture (Table 3). Therefore, we concluded that differences in microtubule accumulation between the Svn and Sua mainly arose during the process of cortical microtubule reorganization. Since microtubule depolymerization through oryzalin treatment reduced nuclear differences (Table 4), sperm cell microtubules played a key role in determining and regulating nuclear shape.

In conclusion, FCM could detect sperm cell formation between 6 and 12 h of culture and MGUs both before and after sperm cell formation during pollen tube growth in *C. mackenii*. Since the FCM-based method for analyzing pollen tubes could be applied to *A. aurea* (Hirano and Hoshino 2009) and *C. mackenii*, we believe that this method may be applicable for the analysis of pollen tubes of other species, as well, and has the potential to be used as a high throughput technique for preparing MGU material for molecular approaches in combination with a cell-sorting system. In the present study, we detected two kinds of sperm dimorphisms in terms of nuclear shape and microtubule accumulation and prior work has indicated that microtubules play a role in cell shaping, cell polarity determination, and vesicular transport (Southworth and Cresti 1997). Further, proteins colocalized with microtubules in male gametes may play an important role in the development of the gametes (Lee et al. 2007; Mori et al. 2003; Twell et al. 2002), in mediating the relationship between microtubules and in the plasma membrane (Cai et al. 2005). Microtubule distribution in male gametes and their correlation with the distribution of microtubule-associated proteins may also be demonstrated in *C. mackenii* and are possibly related to the biological roles of sperm cells in double fertilization and in mediating the functions of microtubule-associated proteins. In *P. zeylanica*, a number

of genes were found to be highly upregulated in sperm cells and some genes showed differential expression between Svn and Sua (Gou et al. 2009). We expect that there is a mechanism for controlling microtubule distribution in sperm cells of *C. mackenzii* and this may be present in other angiosperms as well.

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Table 1 Proportion of 1C, 2C, and 3C nuclear DNA in pollen tubes cultured for 36 h, as determined by FCM analysis.

Culture duration (h)	Percentage of nuclei		
	1C	2C	3C
6	41.4 ± 2.6 ^a	42.7 ± 4.2 ^a	15.9 ± 4.9 ^a
12	56.2 ± 3.6 ^b	27.9 ± 5.8 ^b	15.9 ± 4.3 ^a
18	58.8 ± 5.5 ^b	25.8 ± 2.8 ^b	15.4 ± 5.3 ^a
24	59.4 ± 5.0 ^b	24.9 ± 4.3 ^b	15.7 ± 1.8 ^a
30	59.3 ± 4.5 ^b	29.0 ± 3.9 ^b	11.8 ± 2.9 ^{a,b}
36	61.3 ± 2.6 ^b	29.9 ± 3.6 ^b	8.7 ± 1.5 ^b

The data represent mean ± standard deviation of 6 replicates. In each column, values indicated with the same superscript letter are not significantly different at the 0.05 level.

Table 2 Nuclear size of sperm cells in MGUs isolated from the pollen tubes cultured in vitro.

Culture duration (h)	Nuclear size of isolated sperm cells					
	Length (μm)			Width (μm)		
	Svn	Sua	<i>p</i> *	Svn	Sua	<i>p</i> *
12	10.3 ± 1.3 ^a	11.4 ± 1.4 ^a	0.01	5.9 ± 0.7 ^{NS}	5.7 ± 0.6 ^{NS}	NS
24	10.9 ± 1.0 ^b	13.2 ± 1.5 ^b	0.01	5.7 ± 0.9 ^{NS}	5.6 ± 0.7 ^{NS}	NS
36	11.2 ± 1.2 ^b	13.1 ± 1.3 ^b	0.01	5.6 ± 0.6 ^{NS}	5.6 ± 0.7 ^{NS}	NS

The data represent mean ± standard deviation determined for 30 MGUs. In each column, values indicated with the same superscript letter are not significantly different at the 0.05 level. *Student's *t* test was used to compare the data between Svn and Sua at each culture period. NS, not significant.

Table 3 Intensity of immunofluorescence staining with anti- α -tubulin antibody of the pairs of sperm cells in isolated MGUs.

Culture duration (h)	Fluorescence intensity	
	ratio (Sua/Svn)	p^*
12	1.12 ± 0.20^{NS}	0.05
24	1.29 ± 0.33^{NS}	0.01
36	1.34 ± 0.41^{NS}	0.01

The data represent mean $\pm$ standard deviation determined for 15 pairs of sperm cells in isolated MGUs. In each column, values indicated with the same superscript letter are not significantly different at the 0.05 level. NS, not significant. *Student's t test was used to compare the mean fluorescence intensity between Svn and Sua.

Table 4 Effect of oryzalin treatment on the nuclear size of sperm cells isolated from pollen tubes.

Nuclear size (μm)	Untreated controls			Oryzalin-treated cells		
	Svn	Sua	p^*	Svn	Sua	p^*
length	11.2 ± 1.5	13.4 ± 1.5	0.01	9.5 ± 1.5	10.0 ± 1.9	NS
width	5.9 ± 0.8	5.9 ± 0.5	NS	6.3 ± 0.9	6.3 ± 1.1	NS

The data represent mean $\pm$ standard deviation determined for 30 MGUs. *Student's t test was used to compare the data between Svn and Sua at each culture period. NS, not significant.

Figure legends

Fig. 1 FCM analysis of the cells and nuclei isolated using a microcapillary. FCM histogram of pollen tubes cultured for 6 h (a). Vegetative nuclei (b), generative cells (c), and MGUs comprising 1 vegetative nucleus and 1 generative cell (d) were isolated from pollen tubes cultured for 6 h. Vegetative nuclei (e), sperm cells (f), a pair of sperm cells (g), and MGUs comprising 1 vegetative nucleus and a pair of sperm cells (h) were isolated from pollen tubes cultured for 18 h. In all, 30 cells and 30 nuclei of each type were collected using a microcapillary and analyzed using a flow cytometer

Fig. 2 Microtubules of the generative cell and sperm cells in pollen tubes cultured for 3 h and 12 h, respectively. The fluorescence photomicrographs show nuclei stained with DAPI (left column), a microtubule stained with the anti- α -tubulin antibody followed by an Alexa 488-conjugated secondary antibody (middle column), and the merging of the images showing the nuclei and microtubules (right column). GN: generative nucleus, VN: vegetative nucleus, SN: sperm nucleus. Bars = 30 μ m

Fig. 3 MGUs and a pair of sperm cells isolated from pollen tubes. MGU comprising 1 vegetative nucleus and 1 generative cell was isolated from pollen tubes cultured for 6 h by immersing the tubes in extraction buffer and chopping them. MGUs were stained with DAPI and observed under an epifluorescence microscope with variable relief contrast (a) and fluorescence (b). A pair of sperm cells and MGUs comprising 1 vegetative nucleus and a pair of sperm cells were isolated from pollen tubes cultured for 18 h by using the abovementioned procedure and observed under an epifluorescence microscope with variable relief contrast (c, e) and fluorescence (d, f). GN: generative

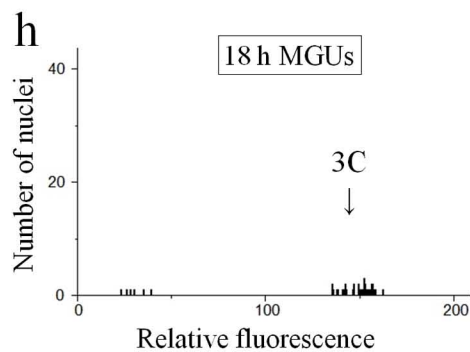
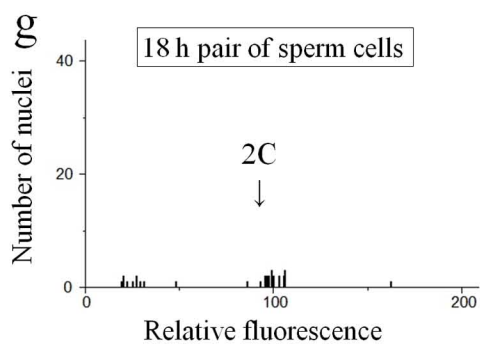
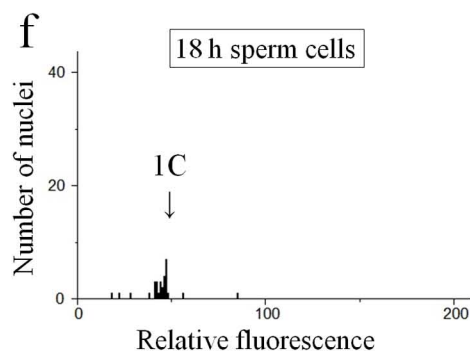
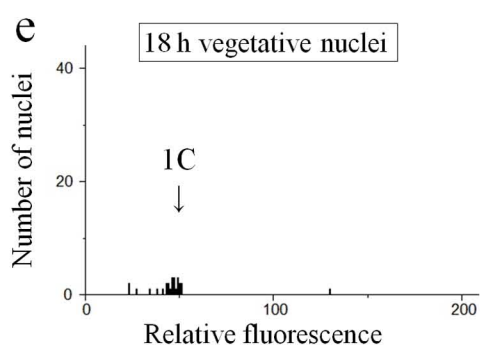
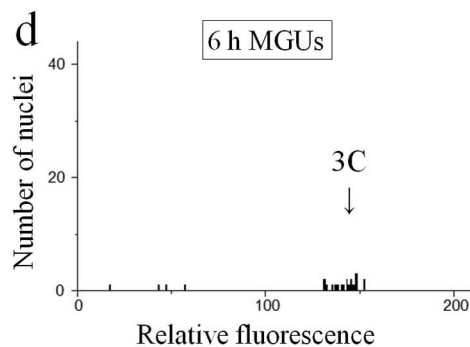
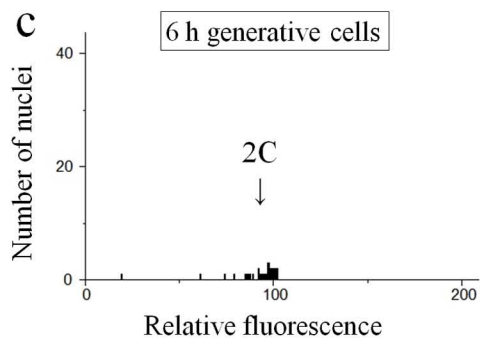
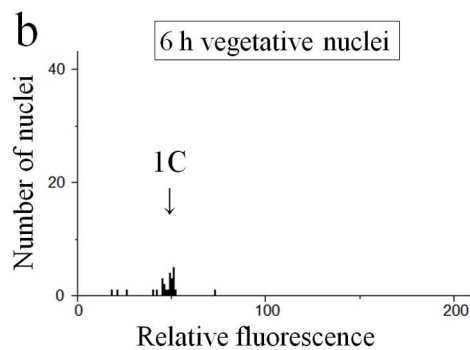
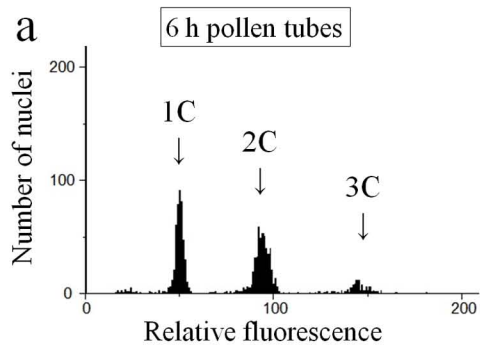
cell, VN: vegetative nucleus, SN: sperm nucleus. Bar = 30 μ m

Fig. 4 Morphological changes in the sperm cells in the oryzalin-treated MGUs. Oryzalin was added to the culture medium after incubation for 12 h, and the pollen tubes were cultured for an additional 12 h. Some pollen tubes were cultured for 24 h in a medium without oryzalin and were used as untreated controls. Images of each MGU isolated from the oryzalin-treated or untreated pollen tubes were obtained with variable relief contrast, DAPI staining, anti- α -tubulin staining, and image merging. VN: vegetative nucleus, Svn: sperm cell associated with the vegetative nucleus, Sua: sperm cell not associated with the vegetative nucleus. Bars = 30 μ m

Fig. 5 Microtubule distribution in the generative cell during mitosis. MGUs comprising 1 generative cell during mitosis were isolated from pollen tubes cultured for 9 h, and MGUs comprising sperm cells were isolated from pollen tubes cultured for 12 h. Each MGU was photographed with variable relief contrast, DAPI staining, anti- α -tubulin staining, and merging of the DAPI staining and anti- α -tubulin staining images. GC: generative cell, SC: sperm cell. Bars = 30 μ m

Supplemental figure legend

Fig. S1 DAPI staining of nuclei in a pollen tube cultured for 6 h. The pollen tube was observed under bright field (a) and fluorescence (b) followed by DAPI staining. Arrowheads and arrows indicate vegetative nucleus and generative cell, respectively. Bar = 100 μ m

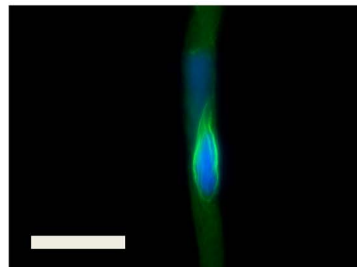
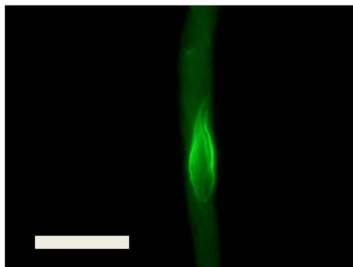
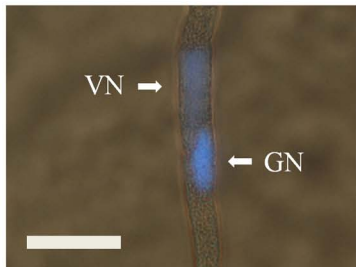


DAPI

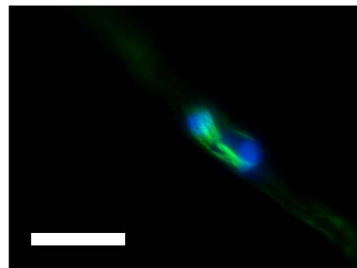
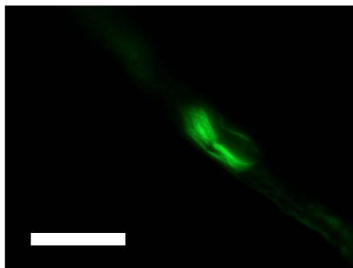
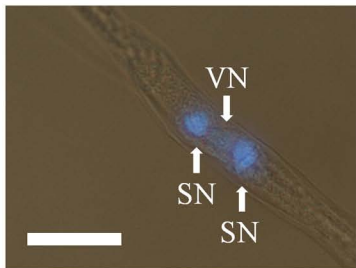
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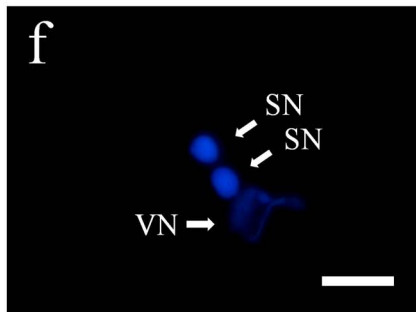
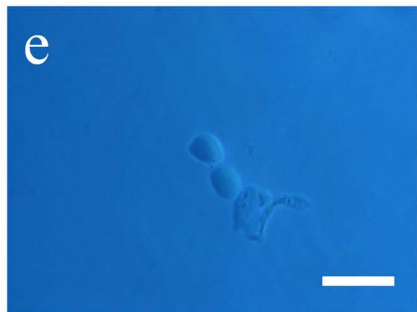
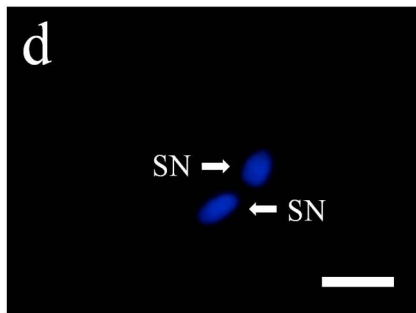
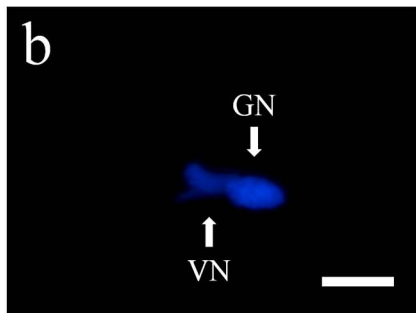
Merge

6 h



12 h





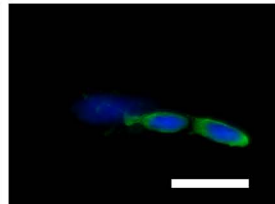
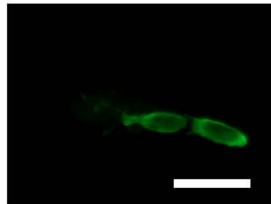
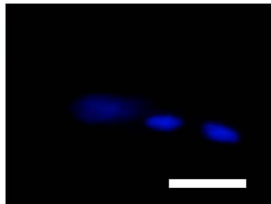
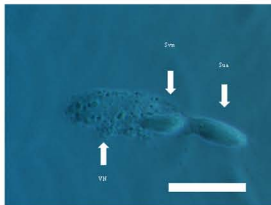
VAEREL
contrast

DAPI

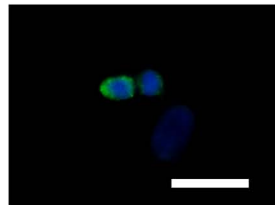
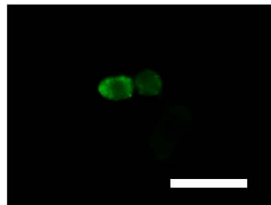
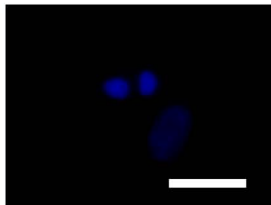
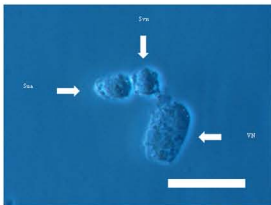
Microtubule

Merge

Untreated
control



Oryzalin
treated



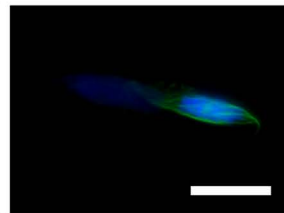
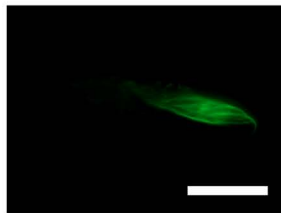
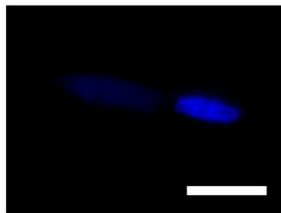
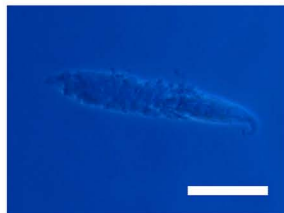
VAEREL
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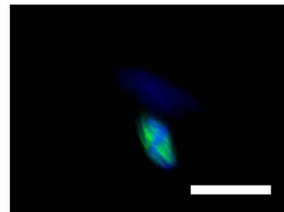
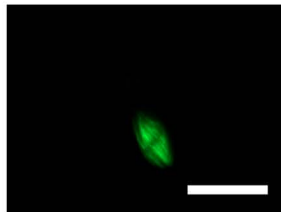
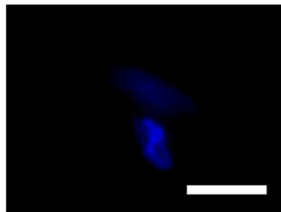
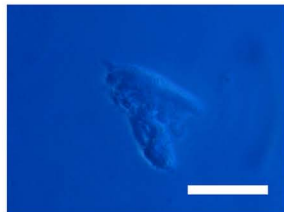
Microtubule

Merge

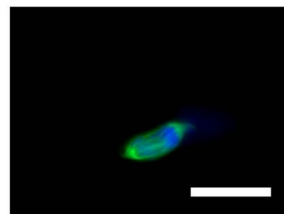
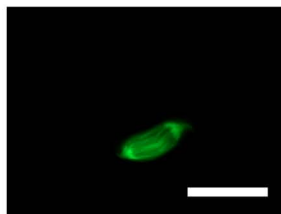
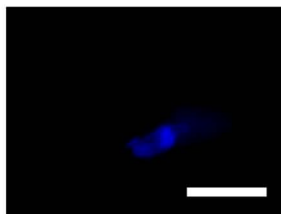
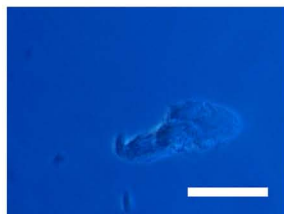
GC



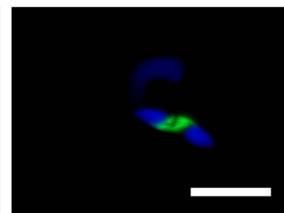
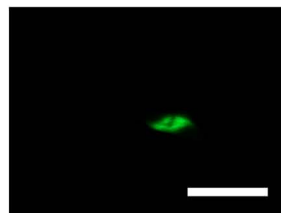
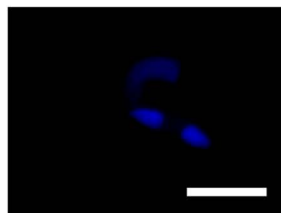
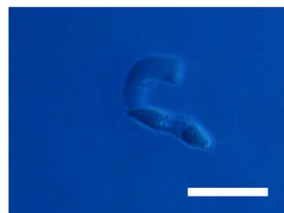
Metaphase



Anaphase



Telophase



SC

