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Title: Detection of changes in the nuclear phase and evaluation of male germ units by flow cytometry during *in vitro* pollen tube growth in *Alstroemeria aurea*.

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Abstract

This study aimed to analyze male gamete behavior from mature pollen to pollen tube growth in the bicellular pollen species *Alstroemeria aurea*. For mature pollen, pollen protoplasts were examined using flow cytometry. The protoplasts showed two peaks of DNA content at 1C and 1.90C. Flow cytometry of pollen tubes at different developmental stages that were cultured *in vitro* revealed changes in the nuclear phase at 9 h and 18 h after culture. Sperm cell formation occurred at 6–9 h after culture, indicating that the first change was due to the division of the generative cells into sperm cells. After sperm cell formation, the number of vegetative nucleus associations with sperm cell showed a tendency to increase. The association was suggested as male germ unit. When sperm cells, vegetative nuclei, and partial male germ units were separately collected from pollen tubes cultured for 18 h and analyzed using a flow cytometer, the sperm cells and vegetative nuclei contained 1C DNA, while the DNA content of partial male germ units was counted as 2C. Therefore, the second change in the nuclear phase, which resulted in an increase in 2C nuclei, was possibly related to the formation of male germ units.

Keywords: flow cytometry ·generative cell · male germ unit ·pollen protoplast sperm cell

Introduction

The genus *Alstroemeria* (Alstroemeriaceae) is native to South America and is found from Venezuela to Chile and Argentina. *Alstroemeria* is a cut flower of increasing commercial importance, and many hybrids that produce large and beautiful flowers have been bred by using interspecific crosses (Hoshino 2008). Cross incompatibility between distantly related species has been observed (Buitendijk et al. 1995) and is one of the problems limiting further progress in *Alstroemeria* breeding.

The *in vitro* fertilization (IVF) technique, in which isolated male and female gametes are directly fused under artificial conditions, has been developed for higher plants (Kranz and Lörz 1993; Kranz et al. 1998; Uchiumi et al. 2007). IVF is considered a useful approach for overcoming cross incompatibility and additionally has potential as an experimental tool for the analysis of fertilization processes or early embryogenesis (Sauter et al. 1998; Scholten et

al. 2002; Faure et al. 2003; Hoshino et al. 2004; Kranz and Scholten 2008). IVF requires the isolation of living male and female gametes. At present, the manipulation of female gametophytes is difficult because these are usually deeply embedded in the ovule tissue. In *Alstroemeria*, however, an efficient method for the isolation of living egg cells and zygotes was recently developed (Hoshino et al. 2006). Thus, an isolation procedure for sperm cells should be developed in order to perform IVF in *Alstroemeria*. Sperm cells are formed by mitosis of generative cells in the pollen or pollen tube. In the latter case, which occurs in bicellular pollen species, including *Alstroemeria*, pollen germination and pollen tube elongation are required for sperm cell isolation, and two methods have been used to isolate sperm cells: those utilizing *in vitro* culture of pollen grains (reviewed in Russell, 1991) and those utilizing a semi-*in vivo* technique (Shivanna et al. 1988).

Understanding the dynamics of male gametes during pollen tube growth is also important for IVF and the elucidation of the double fertilization process. In *Plumbago zeylanica* L., which has tricellular pollen, two sperm cells are joined by common transverse cell wall and enclosed by inner vegetative cell membrane, and one of the two sperm cells is

associated with vegetative nucleus (Russell 1984). On the other hand, bicellular pollen species, such as *Hippeastrum vitatum* Hreb., *Rhododendron* spp, and *Petunia hybrida* Vilm., generative cell and sperm cell were physically associated with vegetative nucleus (Mogensen 1986; Kaul et al. 1987; Wagner and Mogensen 1988). This association was termed the male germ unit (MGU) and was proposed to functional unit as a vehicle for transmission and to participate in fusion with the female target cells during fertilization (Dumas et al. 1984). Sperm dimorphism, which showed difference of sperm cells in size, shape, and organelle content, is also observed in the pollen tube (Russell 1991; Tian et al. 2001; Chen et al. 2006). Moreover, in *Arabidopsis thaliana* (L.) Heynh, sperm cells are in the S phase of the cell cycle and continue to synthesize DNA during pollen tube growth. By the time the pollen tubes reach the ovary and emerge from the septum, the sperm nuclei contain approximately 1.75C DNA (Friedman 1999). Up to now, there is no simple and quick method to evaluate the general tendency of male gamete behavior such as MGU formation or DNA synthesis of sperm cell during pollen tube growth. For analysis of the male gamete behavior, we focused on flow cytometry (FCM) analysis. Since FCM analysis is a very simple procedure that can

rapidly measure DNA in large cell populations, it is widely used to estimate the DNA contents in plant cell nuclei (Doležel and Bartoš 2005). Several ploidy-based studies have been conducted on pollen by using FCM (reviewed in Suda et al. 2007). FCM was only used to study germinated gymnosperm pollen of *Cupressus dupreziana* A. Camus (Pichot and El Maâtaoui 2000), and no study has used FCM to analyze male gamete during pollen tube growth.

In the present study, we established an *in vitro* pollen culture method in a liquid medium for *Alstoemeria aurea* Graham toward isolation of sperm cell from pollen tube and analysis of male gamete. We performed DNA analysis of male gamete during pollen tube growth by novel methods which combine the germination technique with FCM, and attempted to detect changes in the nuclear phase during pollen tube growth in *A. aurea*.

Materials and methods

Plant materials and pollen culture

A. aurea Graham was grown under field conditions in Hokkaido University, and anthers that

had undergone dehiscence were collected from the flowers. To overview the procedures for analysis of pollen grains and pollen tubes, protocols developed in this study were summarized in Fig. 1. Detailed procedures were described as followings.

Pollen grains from an anther were sown in 2 ml liquid culture medium that contained 0.01% (w/v) $CaCl_2$, 0.01% (w/v) H_3BO_3, 0.0007% (w/v) KH_2PO_4, 10% (w/v) sucrose, and 0.01% (w/v) yeast extract at pH 5.8; the culture medium was sterilized by autoclaving at 121°C for 15 min. The pollen grains were then cultured at 25°C under dark condition. After culture in the medium for 3–18 h, 4′, 6-diamidino-2-phenylindole (DAPI; final concentration, 1 μg ml^{-1}) and Triton X-100 (final concentration, 0.5%) were directly added to the culture medium. After staining for 15 min, the nuclei in the pollen tubes cultured 3-18 h were observed through an epifluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany). The nuclei in the pollen tubes were observed for evaluating the rates of sperm cell formation or MGU formation by following conditions after DAPI staining: vegetative nucleus and generative cell entered the pollen tube at 3h, and the pollen tubes elongated over approximately 0.7 mm and 1.5 mm at 6 h and after 9 h culture, respectively.

Isolation and FCM analysis of pollen protoplasts

For the isolation of pollen protoplasts, pollen grains from an anther were directly suspended in 2 ml enzyme solution which contained 2% (w/v) Cellulase Onozuka R-10, 1% (w/v) Macerozyme R-10, 20 mM morpholineethansulphonic acid, and 1 M mannitol in the presence of the following salts: KH_2PO_4 (27.2 mg/l), KNO_3 (100 mg/l), $CaCl_2$ (150mg/l), $MgSO_4$ (250 mg/l), $Fe_2(SO_4)_3 \cdot 6H_2O$ (2.5 mg/l), KI (0.16 mg/l) and $CuSO_4$ (0.00025 mg/l), pH 5.8 (Frearson et al. 1973). After incubation for 1 h in the enzyme solution at 25°C, the solution was removed by centrifugation at 400 × *g* for 3 min. For FCM, the protoplasts were suspended in 100 µl extraction buffer of CyStain UV precise P (Partec, Münster, Germany), and then 400 µl staining buffer of CyStain UV precise P was added. The suspension was filtered through a 30 µm nylon mesh and then analysed using a flow cytometer (Ploidy Analyzer PA, Partec). Partec FloMax software was used for the analysis of FCM data. 2C DNA value in *A. aurea* was determined by leaf, and then used it to estimate the 1C value. The smallest DNA value of pollen protoplast, which showed half of fluorescence value in

leaf 2C peak, was regarded as the 1C DNA value in the FCM histogram. The experiment was repeated 4 times.

FCM analysis for pollen tubes

The nuclear ploidy level in pollen tubes was determined using a flow cytometer. For collecting pollen tubes at different developmental stages, nylon meshes were utilized. Based on the pollen sizes, 65 μm in length and 45 μm in width approximately, the pore sizes of meshes were selected. For FCM analysis, pollen tubes cultured for 3 h were collected from the culture medium using a 30 μm mesh. By this filtration, pollen tubes and pollen grains were recovered and provided for FCM analysis after nucleus extraction and staining processes as described in next paragraph. In this protocol, nuclei from pollen grains were not extracted and only nuclei from pollen tubes could be measured. After culture for 6–18 h, pollen grains and pollen tubes which had burst in the early developmental stage were removed by filtering the culture medium through a 77 μm mesh, and only elongated pollen tubes on the mesh were collected.

The collected pollen tubes were transfered into 200 μl extraction buffer were chopped with a sharp razor blade, and 800 μl staining buffer was added. The suspension was filtered through a 30 μm nylon mesh and immediately analyzed. At least 200 nuclei were counted for each sample. The experiment was repeated 4 times.

FCM and fluorescence intensity measurement for separately collected cells and nuclei

For detailed FCM analysis, 30 generative cells, 30 sperm cells, 30 vegetative nuclei, 20 paired sperm cells, and 20 MGUs, which were isolated from pollen protoplasts or pollen tubes in the extraction buffer with staining buffer, were separately collected using a microcapillary connected to a micropump (Nano Spuit, Ikeda Scientific Co., Ltd, Tokyo, Japan) under an epifluorescence microscope. Collected cells and nuclei were transferred to fresh 100 μl extraction buffer, and 400 μl staining buffer was added. The solution was analyzed using a flow cytometer. The collected cells and nuclei were also observed and photographed through an epifluorescence microscope with a variable relief contrast (VAREL, Carl Zeiss, Oberkochen, Germany) function. In order to estimate the fluorescence

intensity of nuclei in each cell collected from pollen tubes cultured for 18 h, area and mean of fluorescence intensity of DAPI-labeled nuclei were measured using ImageJ (The National Institute of Health, USA), and then total fluorescence of each sample was calculated. A net fluorescence intensity of nuclei was subtracted the background total fluorescence of same area near the nucleus from total fluorescence of each nucleus. Fluorescence intensity was standardized by average of fluorescence intensity of 5 generative nucleus regarded as 200 relative fluorescence intensity.

Statistical analysis

The proportions of nuclear DNA C-values in the pollen tube culture were subjected to analysis of variance after arcsin transformation, and the means were compared using the least significant difference test.

Results

When the pollen grains of *A. aurea* were cultured in liquid medium, approximately 70% of

the pollen grains germinated, and pollen tubes elongated until 18 h of culture. The pollen tubes were used for male gamete analysis by using a procedure developed in this study (Fig. 1).

DAPI staining identified a vegetative nucleus and generative cell in a pollen tube cultured for 6 h (Fig. 2a, b). Sperm cells were observed in pollen tubes cultured for 9 h (Fig. 2c, d). When pollen tubes were stained with DAPI after culture for 18 h, it was observed that one of the two sperm cells was occasionally associated with vegetative nuclei (Fig. 2e, f). This suggested that sperm cells and vegetative nuclei formed MGUs in the pollen tubes. For analyzing the timings and frequencies of generative cell division and MGU formation, sperm cell formation and proportion of male gametes associated with vegetative nucleus were counted at each developmental stage. Sperm cells were observed in pollen tubes cultured after 6 h, and proportion of the pollen tubes contained sperm cells were drastically increased from 32% at 6 h to 79% at 9 h (Table 1). Vegetative nuclei were associated with generative cells or sperm cells in the pollen tubes, and the number of vegetative nucleus associations with sperm cell showed a tendency to increase during pollen culture periods from 12 h to 18

h (Table 1).

For analyzing the DNA value of mature pollen, we isolated protoplasts from pollen grains by enzyme treatment. When pollen protoplasts were transferred to the extraction buffer for FCM, the pollen protoplasts burst, and the vegetative nuclei and generative cells were released into the buffer (Fig. 3a, b). FCM analysis of the suspension revealed peaks of 1C and 1.90C nuclei (Fig. 3c). For the confirmation of the peak sources, vegetative nuclei, which showed filamentous or irregular shape, and generative cells, which had cylindrical shape nuclei, in the suspension were separately collected with a microcapillary connected to a micropump. It was observed that the relative DNA content of the separated vegetative nuclei (Fig. 3d) and generative cells (Fig. 3e) corresponded to the 1C and 1.90C peaks of pollen protoplasts, respectively. This confirmed that the vegetative nuclei were responsible for the 1C DNA content, and the generative cells, for the 1.90C DNA content. The DNA content value of generative cells in pollen grains was regarded as 2C in following pollen tube analysis.

During *in vitro* culture of pollen grains of *A. aurea*, it was observed that the germination

of some pollen grains was delayed. Moreover, some pollen tubes burst during the early developmental stage (data not shown). For the analysis of the male gametes during pollen tube growth, a protocol for removal of contaminated cells and nuclei from bursting pollen grains and tubes is required. Therefore, we filtered the tubes during 6–18 h culture through a 77 μm mesh and collected only elongated pollen tubes (Fig. 1). For the evaluation of male gamete behavior, the filtered pollen tubes at different developmental stages were subjected to FCM analysis. When pollen tubes cultured for 3 h and 6 h were analyzed using FCM, the proportion of 1C and 2C nuclei was the same (Table 2). In pollen tubes cultured for 9 h, the quantity of 1C nuclei increased from 48% to 72%. Further, the proportion of 1C and 2C nuclei did not change between 9 h and 15 h after culture. Extended culture period from 15 h to 18 h significantly increased 2C nuclei from 24% to 32% ($P = 0.05$). The proportion of 3C nuclei remained almost constant during pollen tube growth.

The cells and nuclei isolated from pollen tubes in extraction buffer by chopping were observed under an epifluorescence microscope after DAPI staining. The suspension from pollen tubes cultured 3 h contained vegetative nuclei and generative cells, and it was also

observed that a small number of the vegetative nucleus and generative cell formed the MGU (Fig. 4a, b). In the suspension from pollen tubes cultured 18 h, vegetative nuclei, sperm cells, undivided generative cells, and paired sperm cells (Fig. 4c, d) were observed. Moreover, two types of association between sperm cells and vegetative nuclei were observed in the suspension: those with one of the two sperm cells and one vegetative nucleus (partial MGU; Fig. 4e, f), and those with two sperms cells and one vegetative nucleus (MGU; Fig. 4g, h).

In order to determine the change in the nuclear phase detected with FCM analysis between 15 and 18 h of culture, the sperm cells, vegetative nuclei, paired sperm cells, and partial MGUs from pollen tubes cultured 18 h were separated with a microcapillary connected to micropunp and then analyzed using FCM. Relative DNA content of the sperm cells and vegetative nuclei were same as 1C of pollen tubes cultured 18 h (Fig. 5a, b, c), while the paired sperm cells and partial MGUs showed 2C value (Fig. 5d, e). Relative fluorescence intensities of the separated cells and nuclei were also measured. When fluorescence intensity of generative nucleus (2C) was used as DNA standard corresponding to 200 relative fluorescence intensity, average of the 30 sperm nuclei and 30 vegetative

nuclei showed 96.4 ± 13.5 and 84.6 ± 14.9 relative fluorescence intensity, respectively (Table 3). Relative fluorescence values of the 20 paired sperm cells and 20 partial MGUs were averaged 190.2 ± 13.5 and 184.0 ± 16.5, respectively (Table 3), and the results are consistent with prospect value from the sperm nuclei and vegetative nuclei. These results indicate that 2C nuclei, which were counted in the FCM analysis of pollen tubes grown for 18 h, contained paired sperm cells and partial MGUs.

Discussion

The isolation of nuclei from tissues or organs is indispensable for FCM analysis. Extracting sufficient numbers of intact nuclei from pollen grains is often difficult, and the effectiveness of different methods varies from species to species (Suda et al. 2007). Some methods have successfully used for nuclei isolation of pollen grains, such as chopping method, which was used for pollen tubes in the present study (Bino et al. 1990; Sugiura et al. 1998; Sugiura et al. 2000; van Tuyl et al. 1989), bursting in hypotonic solution (Zhang et al. 1992), crushing or squashing (Jacob et al. 2001; Pichot and El Maâtaoui 2000), and sonication (Pan et al. 2004).

In the case of *A. aurea*, it is difficult to isolate nuclei and cells from pollen grains by using the chopping method (data not shown). We used pollen protoplasts and isolated a sufficient number of vegetative nuclei and generative cells from *A. aurea* pollen grains; this indicated that the new method developed in this study is effective for FCM of pollen.

FCM of generative cells of *A. aurea* pollen stained with DAPI revealed a DNA content value of 1.90C (Fig. 3c). Vegetative, generative, and sperm nuclei can be quite different structurally and morphologically, and as a result, may take up nuclear stains differently (de Paepe et al. 1990). In *Chamerion angustifolium* (L.) Holub, 2*n* generative nuclei show approximately 1.7 times the fluorescence of 1*n* vegetative nuclei when stained with propidium iodide (Suda et al. 2007). Propidium iodide is sensitive to the chromatin structure, whereas DAPI is less influenced by it (Doležel and Bartoš 2005). Therefore, it is suggested that nuclear staining in vegetative or generative nuclei of *A. aurea* with DAPI may be only slightly affected by the structural or morphological state such as chromatin structure. FCM of pollen revealed few 3C nuclei (Fig. 3c), and the association of the vegetative nucleus and generative cell was not observed in suspension (Fig. 3a, b). This suggested that MGUs

consisting of 1 vegetative nucleus and 1 generative cell might be not formed in *A. aurea* pollen grains and formed after pollen germination.

To investigate male gamete behavior, pollen tubes at different developmental stages were subjected to FCM analysis, and the chopping method for nuclei isolation was used. FCM analysis of pollen tubes cultured for 3 and 6 h showed the same proportion of 1C and 2C DNA content in the nuclei (Table 2), suggesting that the 1C content in vegetative nuclei and the 2C content in generative cells were isolated efficiently from pollen tubes. The proportion of 3C nuclei was almost constant during pollen tube growth (Table 2). In pollen tubes, generative cells and sperm cells were associated with vegetative nuclei (Table 1). Moreover, by using isolation technique for male gametes from pollen tubes, MGUs consisting of one vegetative nucleus and one generative cell were observed before sperm cell formation (Fig. 4a, b), and those consisting of two sperm cells and 1 vegetative nucleus were formed after generative cell mitosis (Fig. 4g, h). This suggested that the 3C nuclei detected by FCM were derived from MGUs.

The FCM analysis revealed two changes in the nuclear phase between culture periods 6

h and 9 h and between culture periods 15 h and 18 h (Table 2). Sperm cells were found to form in pollen tubes cultured for 6 h, and most of generative cells divided into sperm cells until 9 h of culture (Fig. 2c, d and Table 1). These results suggest that sperm cell formation was begun at 6 h approximately and completed by 9 h under the culture conditions used in the present study. The first change observed at 6–9 h of culture in FCM analysis was a decrease in 2C nuclei. Further, this period was consistent with the period of sperm formation observed using DAPI staining of the pollen tubes, indicating that the first change in the nuclear phase is due to the division of the generative cell (2C) into sperm cells (1C).

After the second change in the nuclear phase, the number of 2C nuclei in pollen tubes cultured for 18 h was significantly higher than the corresponding number after culture for 15 h. It has reported that sperm cells of *A. thaliana* are in the S phase of the cell cycle and continue to synthesize DNA during pollen tube growth (Friedman 1999) and sperm cells in *Nicotiana tabacum* L. are in the S phase after deposition in the degenerated synergid (Tian et al. 2005). Therefore, one possibility of the cause of the second change in the nuclear phase might be DNA replication in sperm cells. However, sperm cells isolated from pollen tubes

cultured for 18 h contained 1C DNA (Fig. 5b) and paired sperm cells were counted as 2C in FCM analysis (Fig. 5d). Relative fluorescence intensity of sperm nuclei showed about half of generative nuclei, and that of paired sperm nuclei was also confirmed equivalent value to generative nuclei (Table 3). These results suggest the possibility that the sperm cells of *A. aurea* might not synthesize DNA in the pollen tubes at least when cultured in our condition. Population of 2C nuclei counted by the FCM analysis of pollen tubes grown for 18 h were thought to be consisted of undivided generative cells, paired sperm cells, and partial MGUs (Fig. 5d, e and Table 3). Among them, partial MGUs might be increased at the second nuclear phase change as following speculations. In *N. tabacum*, it has been reported that the vegetative nucleus and generative cell form the MGU in the pollen tube (Yu and Russell 1994a). The MGU association loosens when the generative cell undergoes mitosis and enters the prophase; the generative cell separates from the vegetative nucleus in the metaphase, and the MGU is reestablished after sperm cells enter the interphase (Yu and Russell 1994b). In *A. aurea*, although proportion of MGU formation in pollen tube is estimated to approximately same just before and after generative cell mitosis, the association rate between sperm cells

and vegetative nuclei in pollen tube increased from 35% at 12 h to 44% at 18 h (Table 1). These results imply that sperm cells were newly associated with the vegetative nucleus after 12 h culture rather than reestablishment of MGU observed in *N. tabacum*. As sperm cells and vegetative nuclei progressively move toward the tip of the pollen tubes as the culture period increases, the opportunity of MGU formation might continuously increase. In *A. aurea*, isolated sperm cells from pollen tubes cultured 18 h contained paired sperm cells (Fig. 4c, d), indicating that two sperm cells in the pollen tube enclosed by inner vegetative cell membrane and/or connected with each other. If it is assumed that paired sperm cells newly associated with vegetative nucleus, proportion of 3C nuclei increases between culture periods 15 h and 18 h. However, proportion of 3C nuclei was not changed in the culture periods (Table 2). Isolated paired sperm cells generally become spherical shape and loosely connected in the buffer (reviewed in Russell 1991; Theunis et al. 1991), suggesting that one sperm cell came off MGU during isolation procedure or in extraction buffer. One possibility might be that the inner vegetative cell membrane is partly broken but still remains as enclosing one sperm cell. It is also conjectured that some physiological and/or morphological

changes occur in connected sperm cells between culture periods 12 h and 18 h. To clarify the phenomenon involved in the pollen tubes, we are now attempting to analyze in detail the sperm cell changes by comparison of gene expression and cytoskeleton.

The MGU is thought to be related to double fertilization (Dumas et al. 1984). Regarding the MGU of *P. zeylanica*, sperm cells that were not associated with vegetative nuclei preferentially fused with egg cells, and sperm cells associated with vegetative nuclei fused with central cells (Russell 1985). It was also reported that the expression of germ-line specific polyubiquitin gene differed between sperm cells in the MGUs of *P. zeylanica* (Singh et al. 2002). In *A. aurea*, the second change in the nuclear phase occurred just before pollen tube growth stopped, suggesting that the change may be related to sperm cell maturation for the double fertilization process.

In the present study, we developed an *in vitro* pollen culture system for *A. aurea*. The manipulation of pollen tubes for nuclei and cell isolation, DAPI staining, and FCM analysis is facilitated by this method because of the use of a liquid medium. FCM was very useful for grasping male gamete behavior in pollen tube because large populations were rapidly

analyzed, and some changes which occurred during pollen tube growth, such as sperm cell formation and MGU formation, were detected. FCM could also be applied to a small number of targeted sperm cells and MGUs isolated using a microcapillary controlled by a micropump, and confirmed MGU formation and whether DNA synthesis of sperm cells occurs or not. These results of the present study indicate that the FCM based method combined with observation using fluorescence microscopy has potential as a simple and quick tool for analyzing male gamete behavior during pollen tube growth. The time course study provides the necessary time frame for successful isolation of sperm cell that could be potentially used in IVF experiments. Clarifying the reasons of second change in the nuclear phase, which detected in *A. aurea* pollen tubes, are expected to lead to successful IVF in *Alstroemeria*.

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Table 1 Changes of male gametes during pollen tube growth.

culture periods (h)	pollen tube containing generative cell		pollen tube containing sperm cells	
	number of pollen tubes	number of generative cell associated with the vegetative nucleus	number of pollen tubes	number of sperm cell associated with the vegetative nucleus
3	100 (100%)	31 (31.0%)	0 (0%)	0 (0.0%)
6	68 (68%)	25 (36.8%)	32 (32%)	11 (34.4%)
9	21 (21%)	7 (33.3%)	79 (79%)	27 (34.2%)
12	17 (17%)	4 (23.5%)	83 (83%)	29 (34.9%)
15	15 (15%)	6 (40.0%)	85 (85%)	34 (40.0%)
18	11 (11%)	4 (36.4%)	89 (89%)	39 (43.8%)

Pollen tubes were measured 100 individuals at each culture period.

Table 2 Frequency distribution of three C-values of nuclear DNA in pollen tubes during 18 h of culture in liquid medium.

culture	percentage of nuclei		
periods (h)	1C	2C	3C
3	51.6 a	46.4 a	2.0 ns
6	48.4 a	46.4 a	5.2 ns
9	71.8 bc	25.9 bc	2.3 ns
12	73.7 b	23.8 b	2.5 ns
15	72.8 b	24.3 b	2.9 ns
18	64.9 c	32.4 c	2.7 ns

Data represent the mean of 4 replicates. Values in each column followed by the same letter are not significantly different at 0.05 level. ns: not significant.

Table 3 Relative fluorescence intensity of isolated cells and nucli from pollen tubes cultured 18 h.

	n	relative fluorescence intensity
vegetative nucleus	30	84.6 ± 14.9
sperm cell nucleus	30	96.4 ± 13.5
pair of sperm cell nuclei	20	190.2 ± 13.5
partial MGU*	20	184.0 ± 16.5

Data represent mean ± standard deviations of three replicates. Relative fluorescence intensities were standardized by average of fluorescence intensity of generative nucleus regarded as 200. *MGU was consisted of one sperm cell and one vegetative nucleus.

Figure legends

Fig. 1 Schematic flow of the method for male gamete analysis developed in this study. Mature pollen grains were cultured in liquid medium, and elongated pollen tubes at different developmental stages were used for observation of male gamete nuclei stained with DAPI and for FCM analysis. For FCM analysis, liquid medium containing pollen tubes was filtered to remove ungerminated pollen, pollen tubes that burst at an early developmental stage, and discharged pollen tube contents. The collected pollen tubes in extraction buffer were chopped with a sharp razor blade, and the relative DNA content of isolated male gametes and vegetative nuclei was then analyzed using a flow cytometer. For further detailed study, only targeted cells or nuclei were separated from the suspension by using a microcapillary connected to micropump, and small-scale FCM analysis was performed on the separated cells or nuclei.

Fig. 2 DAPI staining of nuclei in pollen tubes of *A. aurea*. Pollen tubes after culture for 6 h (a, b), 9 h (c, d), and 18 h (e, f) were observed under bright field (a, c, e) and fluorescence (b,

d, f) followed by DAPI staining. GN: generative nucleus, VN: vegetative nucleus, SN: sperm nucleus. Bars = 50 μm.

Fig. 3 FCM analysis of pollen protoplasts. Generative cell (arrowhead) and vegetative nucleus (arrow) isolated from pollen protoplasts were stained with DAPI and then observed by an epifluorescence microscope with a variable relief contrast function (a) and under fluorescence (b). Histogram obtained after FCM analysis of pollen protoplasts (c). For detailed study of pollen protoplasts, vegetative nuclei (d) and generative cells (e) were separately collected from the suspension and analyzed using FCM. Bars: (a, b) 100 μm.

Fig. 4 Isolated MGUs and sperm cells from pollen tubes. MGU consisting of one vegetative nucleus and one generative cell were isolated from pollen tubes cultured for 3 h (a, b). In pollen tubes cultured for 18 h, paired sperm cells (c, d), partial MGUs, which consist of one sperm cell and one vegetative nucleus (e, f), and MGUs consisted of two sperms cells and one vegetative nucleus (g, h) were isolated. The MGUs and paired sperm cell isolated in

extraction buffer were stained with DAPI, and then observed by an epifluorescence microscope with a variable relief contrast function (a, c, e, g) and under fluorescence (b, d, f, h). GN: generative cell, VN: vegetative nucleus, SN: sperm nucleus. Bar = 50 μm.

Fig. 5 Flow cytometric analysis of manually separated cells and nuclei from pollen tubes cultured for 18 h. Histogram obtained by FCM of pollen tubes cultured for 18 h (a). Sperm cells (b), vegetative nuclei (c), paired sperm cells (d), and partial MGUs consisting of one sperm cell and one vegetative nucleus (e) were collected using a microcapillary and then analyzed using a flow cytometer.

Fig. 1

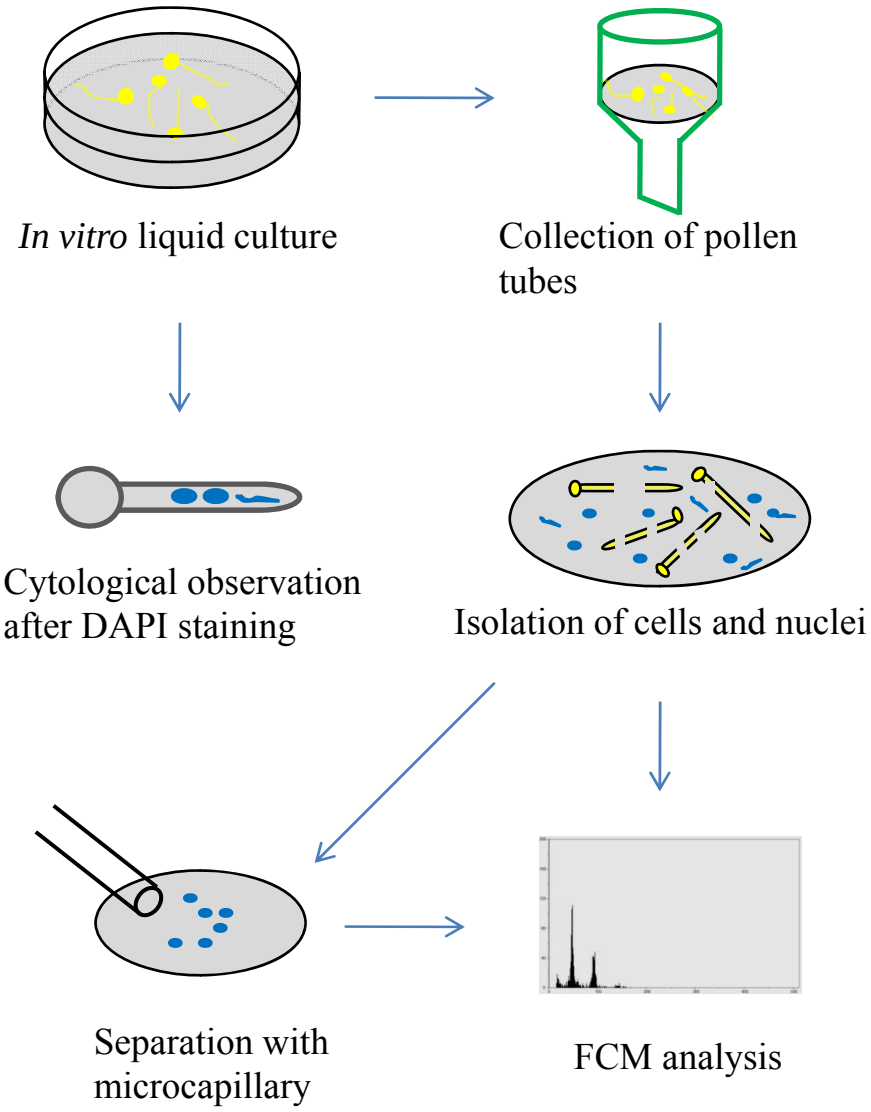

In vitro liquid culture
Collection of pollen tubes
Cytological observation after DAPI staining
Isolation of cells and nuclei
Separation with microcapillary
FCM analysis

Fig. 2

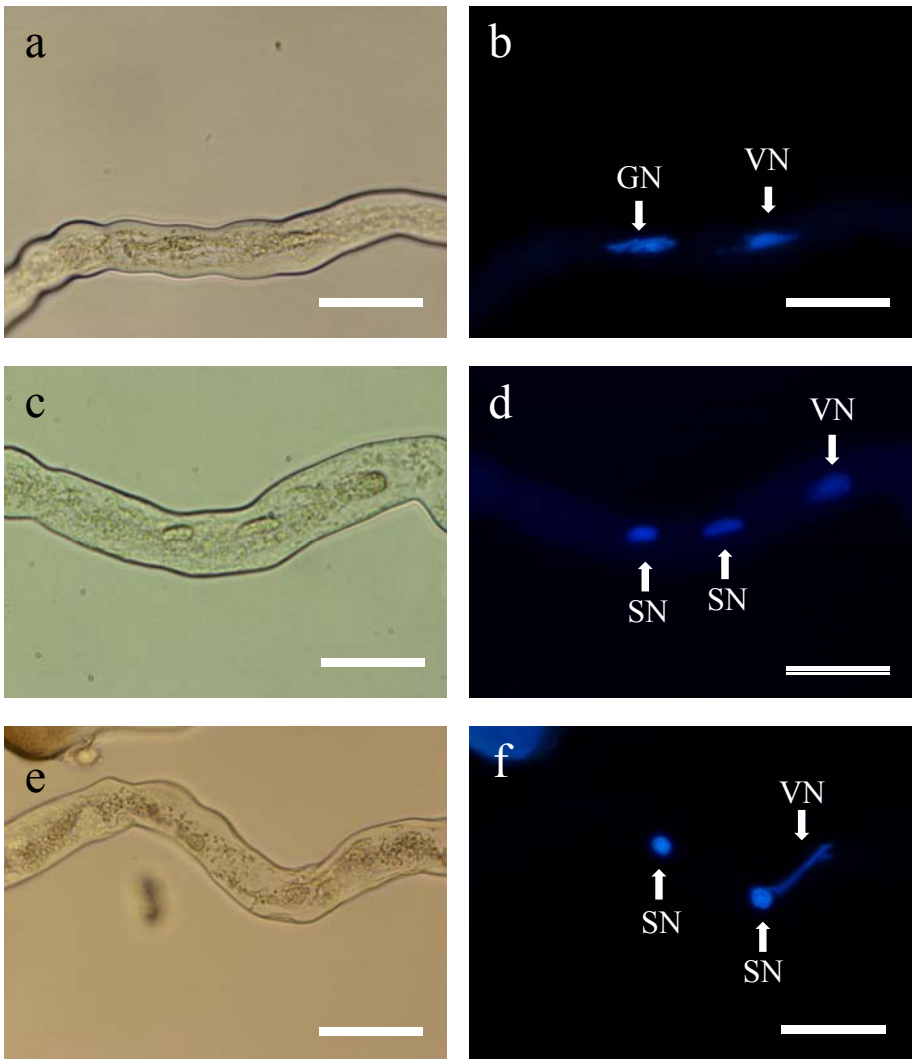


Fig. 3

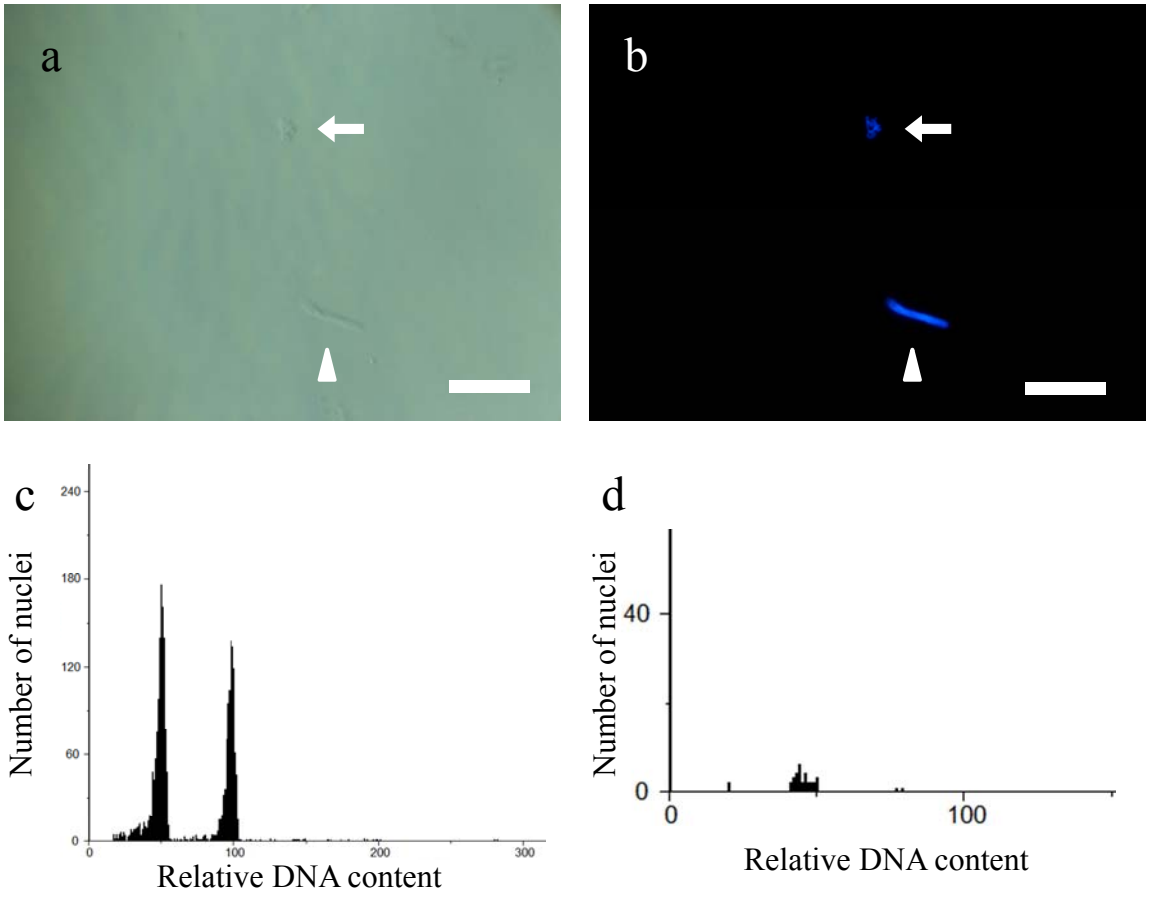

a
b
c
Number of nuclei
240
180
120
60
0
0
100
200
300
Relative DNA content
d
Number of nuclei
40
0
0
100
Relative DNA content
e
Number of nuclei
40
0
0
100
Relative DNA content

Fig. 4

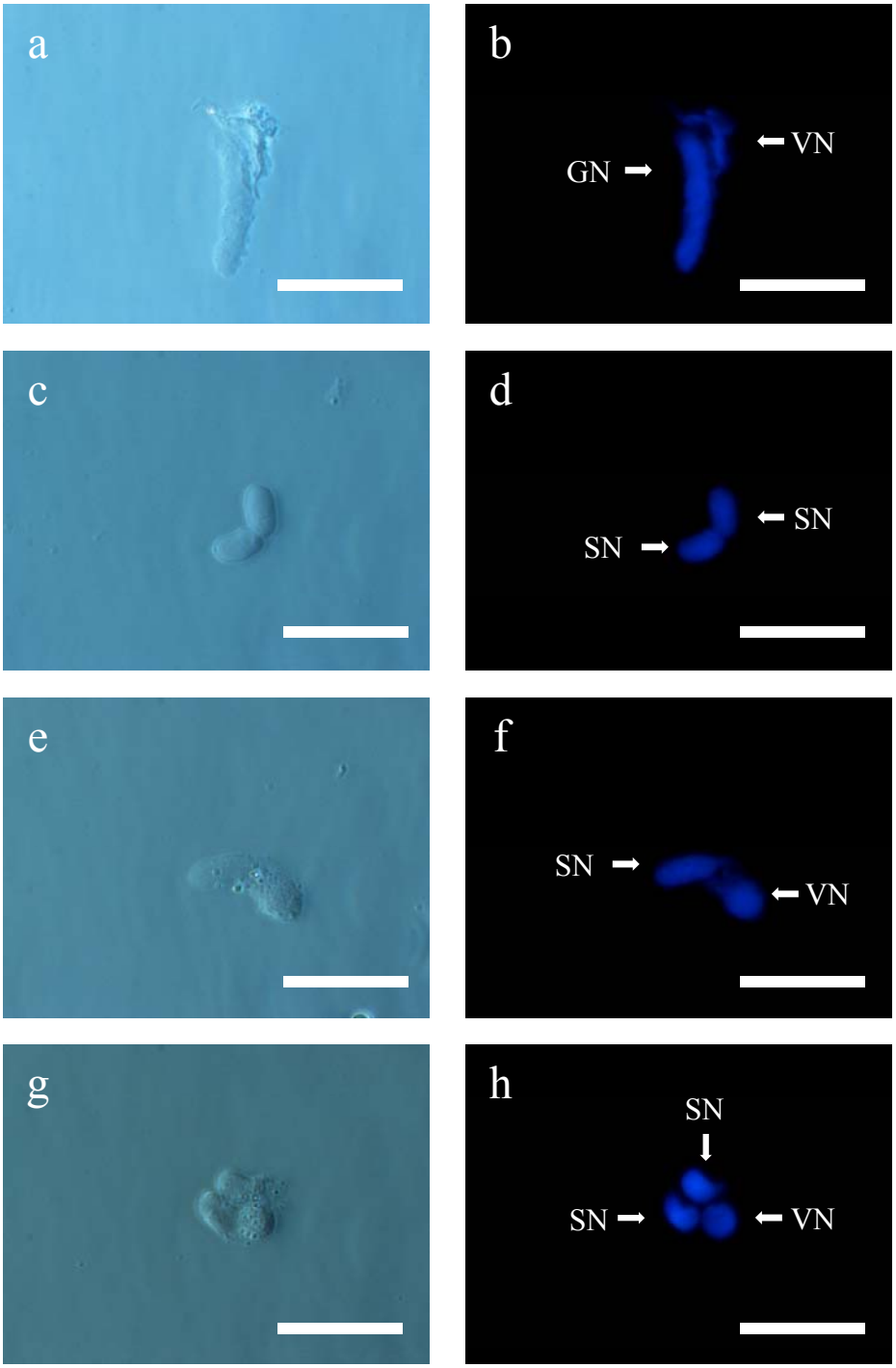
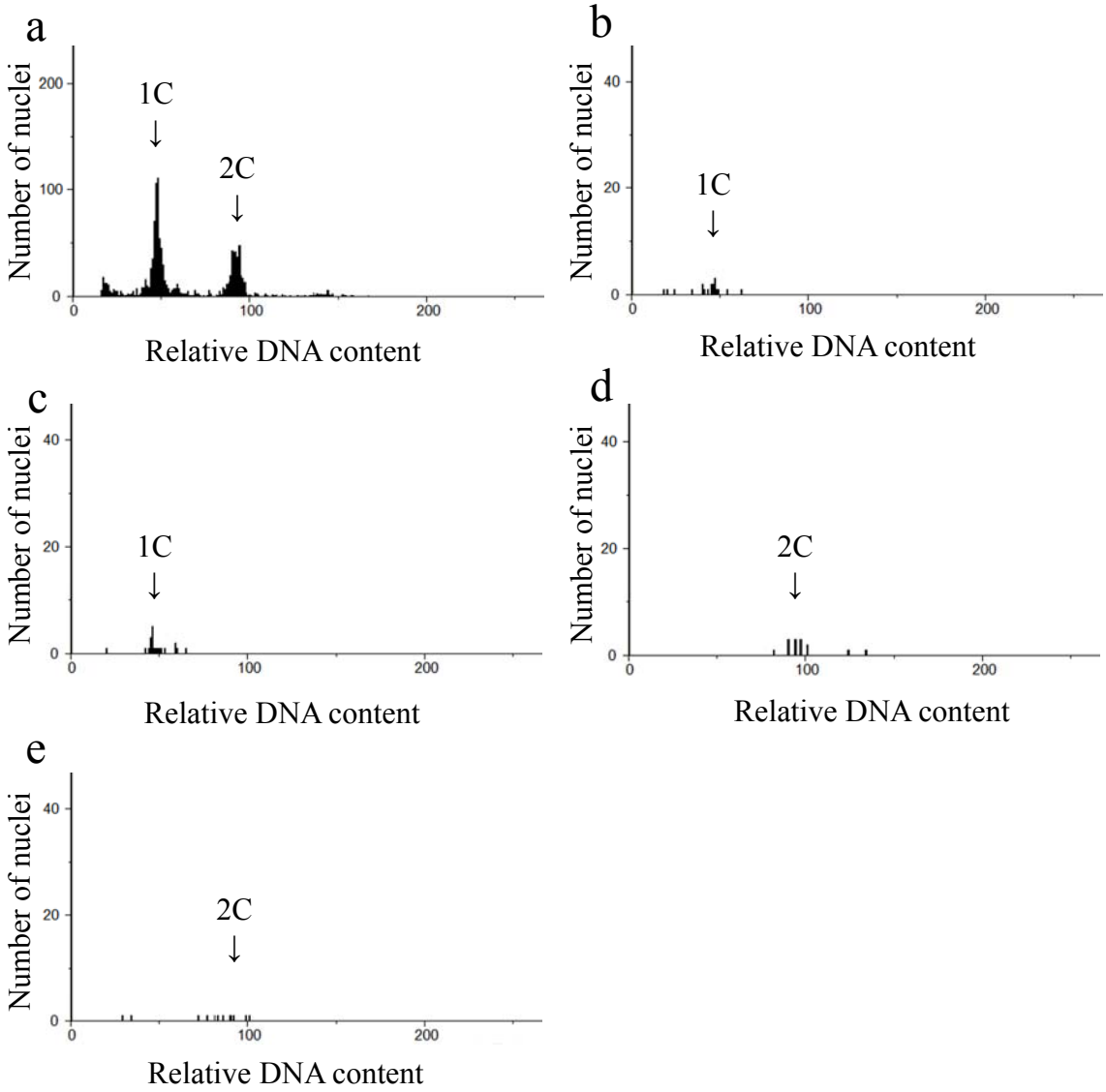


Fig. 5


a
Number of nuclei
1C
2C
Relative DNA content
b
Number of nuclei
1C
Relative DNA content
c
Number of nuclei
1C
Relative DNA content
d
Number of nuclei
2C
Relative DNA content
e
Number of nuclei
2C
Relative DNA content