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Isolation of two populations of sperm cells and microelectrophoresis of pairs of sperm cells from pollen tubes of tobacco (*Nicotiana tabacum*)

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Abstract Prior research has indicated that the two sperm cells of *Nicotiana tabacum* are dimorphic, suggesting that they may participate in preferential fertilization during in vivo fusion with the egg and central cells. To probe the mechanism of potential preferential fertilization in this plant, it will be necessary to use modern sensitive molecular techniques. For this purpose, two individual populations of two sperm cells, constituting the Svn (associated with the vegetative nucleus) and Sua (unassociated with the vegetative nucleus), were isolated in the thousands from tobacco pollen tubes with a micromanipulator as a preliminary step toward research on gametic recognition using molecular techniques. Microelectrophoresis of paired sperm cells from a single pollen tube was conducted at different developmental stages. Sperm cells isolated from 1-, 2-, 3- and 4-cm stylar lengths migrated to the negative pole, with the Sua displaying significantly greater electrophoretic mobility than the Svn, reflecting a more positively charged cell surface on the Sua. The sperm cells isolated from 1-cm style are very sensitive to electron potential in an electrophoretic field, presumably reflecting that they are still in a young state. Differences in cell surface charge between the Sua and Svn may be related with cell fate during fertilization.

Keywords Microelectrophoresis · *Nicotiana tabacum* · Preferential fertilization · Sperm cells · Surface charge

Introduction

In flowering plants, double fertilization involves the fusion of one male gamete with the egg cell, forming an embryo, and the fusion of the second male gamete with the central cell to form the nutritive endosperm. In many flowering plants, the two male gametes are regarded as identical, but some plants have dimorphic sperm cells. The most extreme example may be *Plumbago zeylanica*, in which one sperm cell (Svn) is physically associated with the vegetative nucleus (VN) and contains many mitochondria and rare plastids; in contrast, the other one (Sua) is unassociated with vegetative nucleus and contains fewer mitochondria and many more plastids (Russell 1984). During double fertilization, the plastid-rich sperm cell (Sua) preferentially fuses with the egg cell (over 95%), and the mitochondrion-rich sperm cell (Svn) more likely fuses with the central cell (Russell 1985). This was the first report concerning cytoplasmic markers of non-random fertilization of two brother sperm cells, although nuclear markers were described long before in sperm cells containing an extra B-chromosome, which is more likely to be found in sperm cells fusing with the egg cell (Roman 1948). Since then, differences between brother sperm cells have been reported in a number of plants (Russell 1991; Mogensen 1992). These differences include cell size and content, and also certain molecular markers (Singh et al. 2002). Unfortunately, except for *Plumbago zeylanica*, preferential fertilization has not been confirmed in these species with dimorphism sperm cells because of technical difficulty. The sperm cells of tobacco were discovered to be dimorphic in elongating pollen tubes (Yu and Russell 1994), with divergence becoming most evident late in development (Tian and

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Russell 1998; Tian et al. 2001). In this study, we report a protocol for the individual collection of two populations of sperm cells from pollen tubes of tobacco, a preliminary step for future molecular research. In the current study, microelectrophoresis was done of paired sperm cells from a single pollen tube at different developmental stages to examine the surface charge of these sperm cells, possible differences in charge and their developmental divergence, as part of a larger study of mechanisms of double fertilization in flowering plants.

Materials and methods

Isolation of two populations of sperm cells

Plants of *Nicotiana tabacum* L. were grown in a controlled growth room at 20°C in dark and 27°C in light with 16 h day length at Xiamen University. Tobacco pollen is bicellular, containing a generative cell and a vegetative cell at anthesis. Although pollen tubes can germinate and grow when pollen grains are cultured in a medium in vitro, the generative cell does not divide to form the two sperm cells unless some specialized physiological requirements are met (Read et al. 1993). Generative cell division and formation of the two sperm cells occurs in pollen tubes growing within stylar tissue at about 6–8 h after pollination.

Flowers were emasculated 12 h before anthesis and hand-pollinated at the time of anthesis. The style of tobacco is about 4 cm in length and the pollen tube takes about 40–44 h to reach the ovules. To obtain sperm cells at different developmental stages, stylar excisions were made at four locations (Fig. 1). The youngest stage, collected at 1-cm stylar length, was cut 13 h after pollination. Styles that were 2- and 3-cm long were excised after 20 and 27 h of pollen tube growth, respectively. The most mature stage sampled was the full 4-cm length style, which was cut at 38 h

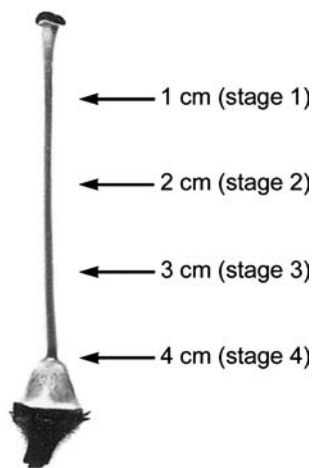


Fig. 1 Tobacco gynoecium showing segments used for stylar excision to obtain sperm cells at four stages of development

after pollination. Styles at the above stages were cut and the cut ends were immersed in a medium containing 15% (w/v) sucrose, 0.01% (w/v) boric acid, 0.01% (w/v) CaCl₂, 0.01% (w/v) KH₂PO₄, at pH 5.0. The osmolality of the medium was 455 mOsmol/Kg H₂O. After about 3 h of incubation in the medium, numerous pollen tubes emerged from the cut end of the style. To release sperm cells, the cut end of the style was transferred and immersed into a bursting solution containing 9% mannitol in ddH₂O, pH 5.0, with an osmolality of 493 mOsmol/kg H₂O. Pollen tubes burst in the latter solution and pairs of sperm cells were reliably released.

Paired sperm cells released from one pollen tube can be selectively harvested using a micromanipulator. Sua and Svn were identified and collected into two individual groups based upon their size differences according to the study of Tian et al. (2001) and their association with the vegetative nucleus (Svn) or not (Sua). Paired sperm cells without a conspicuous size difference were omitted to keep the two groups pure.

Cell microelectrophoresis

Microelectrophoresis was conducted using a 6-cm petri dish onto which two cut slides were glued with super glue to form a 2 mm wide, 5 cm long trough in the center of the dish. Both ends of the trough were sealed with 2% agarose. The electrophoresis trough was filled with low ionic buffer (van Oss and Fike 1979), containing 10 mM NaH₂PO₄, 0.3 mM citric acid and 9% mannitol. Buffer with seven different pH levels (3, 4, 5, 6, 7, 8, 9) were tested to find optimal pH. Electrodes were immersed on opposite ends of the trough into the buffer reservoirs. A pair of sperm cells isolated from one pollen tube was transferred into the buffer in the trough near the center of the optical field using a microinjector. Selected voltages were applied between the electrodes using constant current, and the mobility of the sperm cells was observed using an inverted phase contrast microscope. Images were recorded using a high sensitivity video recording device and a digital camera. The velocity was measured using the recorded images, and electrophoretic mobility was calculated using the equation (van Oss and Fike 1979):

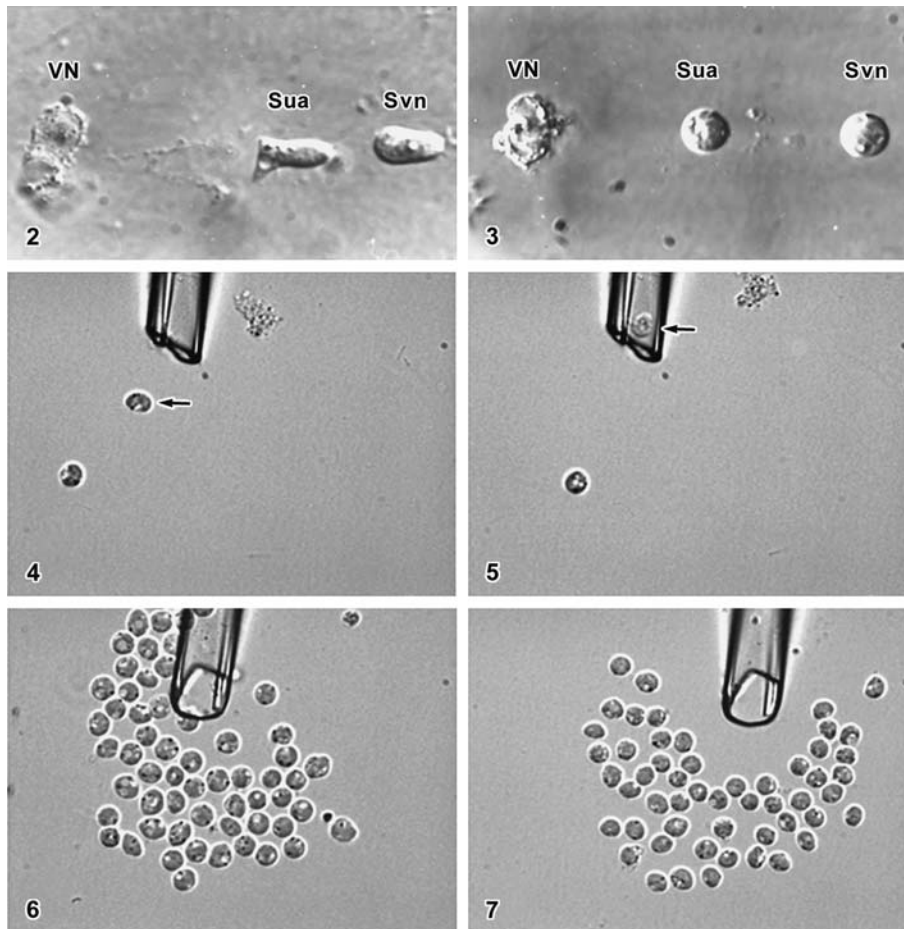
$$\mu = \frac{d/t}{V/D},$$

where μ is electrophoretic mobility, d is distance traveled (in μm), t is time (in s), V is voltage, and D is trough length (in cm).

Surface charge density was calculated using the general formula of the Smoluchowski equation (Levine et al. 1983):

$$\sigma = \mu\eta\kappa,$$

where σ is surface charge density in electrostatic units (esu) of charge per unit area (cm²), η is viscosity coefficient



Figs. 2-7 Collection of tobacco sperm cell populations. *VN* vegetative nucleus; *Sua* sperm cell unassociated with the vegetative nucleus; *Svn* sperm cell associated with the vegetative nucleus. Figures 2-3: $\times 2350$; Figures 4-7: $\times 940$

Fig. 2 Newly-released two sperm cells wrapped by some cytoplasm of pollen tube ($\times 2,350$)

Fig. 3 Twenty minutes later, pollen tube cytoplasm disappears and two sperm cells become round ($\times 2,350$)

Fig. 4 Two sperm cells being separated using a micromanipulator. Arrow indicates sperm cell being drawn into flame-drawn capillary tube ($\times 940$)

Fig. 5 Two sperm cells being separated using a micromanipulator. Arrow indicates sperm cell being drawn into flame-drawn capillary tube ($\times 940$)

Fig. 6 Collection of larger sperm cells, representing the *Sua*, purified by washing twice in bursting solution ($\times 940$)

Fig. 7 Collection of smaller sperm cells representing the *Svn*, ($\times 940$)

cient, and κ is the reciprocal of the Debye-Hückel distance, defined as:

$$\kappa = \frac{10^8 \cdot c^{1/2}}{3.04 \text{ cm}^{-1}},$$

where c is electrolyte concentration (moles). The calculated value of κ for this experiment was $3.5 \times 10^6 \text{ cm}^{-1}$, and the value of η was 1.746×10^{-3} poise, as measured using a Cannon-Fenske viscometer.

Results

Isolation of two populations of sperm cells

The most mature sperm cells that could be practically collected prior to fertilization were those isolated and

collected at stage 4, despite that true cell cycle maturity may not be achieved until immediately prior to fertilization (Tian et al. 2005). High pollen tube quality is critically important for obtaining sperm cells of suitable quality. If pollen tubes are elongating normally, they display uniform size and appear vigorous, with conspicuous cytoplasmic streaming, as evidenced by rapid organelle movement. When styles with pollen tubes were transferred to the bursting solution containing 9% mannitol, the pollen tubes quickly burst at their tips and their cytoplasm was discharged into the bursting solution. In this condition, the two sperm cells were easily identified, as the cytoplasm of the tube soon disperses and only one of the two sperm cells is associated directly with the vegetative nucleus (*VN*). Generally, the sperm associated with the *VN* (*Svn*) is the smaller one (average diameter: $6.79 \mu\text{m}$), whereas the larger one (average

diameter: 7.76 μm) is unassociated with the VN (Sua). Typically, if pollen tubes growing out of the cut end of the style elongate poorly, the tip of the pollen tube swells, and no cytoplasmic movement can be observed. When poorly elongating pollen tubes were transferred into the bursting solution, only a few pollen tubes burst, and the tube required squashing to extrude its cytoplasmic contents. In the latter case, the two sperm cells could rarely be identified because they were surrounded by coagulated pollen tube cytoplasm. Generally, pollen tubes elongating out of the cut end of the style within 4 h of in vitro culture are best. If styles are cultured in vitro over 9 h, the pollen tubes grow too long, and only a few pollen tubes burst.

Sperm cells newly released from the pollen tube are generally ellipsoidal and elongated, and quickly become football shaped in the 9% mannitol bursting solution. Just after release from the tube, some pollen cytoplasm remains around the two sperm cells and the vegetative nucleus (Fig. 2). The two sperm cells and vegetative nucleus are initially associated in a male germ unit (MGU) (Fig. 3). Soon afterwards, the vegetative nucleus swells and dissipates, losing its integrity quickly, but the association between the two sperm cells may remain for at least 30 min, with an enveloping membrane surrounding the two sperm cells. The enveloping membrane remains spindle-shaped, and is divided into two compartments in which the two sperm cells are individually located. This membrane may remain intact for about 20 min after isolation. The cytoplasmic material initially associated with the membrane disperses, but the two sperm cells still remain connected with each other. When the two sperm cells surrounded by their enveloping membrane were transferred to an enzymatic solution containing 0.01% cellulase (Onozuka R-10), 0.008% pectinase (Serva) and 9% mannitol, the connection between the two sperm cells immediately disappeared. The two sperm cells could then be easily separated using a micromanipulator (Fig. 4). Generally, it took approximately 30 min to select 100 sperm cells from two styles. After sperm cells were washed twice in bursting solution, pollen tube organelles were essentially absent from sperm cell isolates (Figs. 5, 6, 7). Bursting solution containing purified sperm cells was then transferred to an Eppendorf microcentrifuge tube that was pre-cooled to liquid nitrogen temperatures. Each tube can store over 1,000 sperm cells. Sperm cells were stored in liquid

nitrogen to pool multiple collections of mRNA or protein isolates.

Developmental and maturational changes were also observed in the sperm cells and their association during their passage in the pollen tube was noted. In stage 1, the association between the two sperm cells appeared weak, and many paired sperm cells spontaneously separated during the first 10 min in the bursting solution. As sperm cells matured, however, the association between sperm cells became stronger, and separation of the sperm cells becomes progressively more difficult. At stage 4, incubation of the sperm cells for 30 min or more was not always sufficient to separate the cells. In this case, 0.01% cellulase and 0.008% pectinase were used.

Mobility of two associated sperm cells

When the two separated sperm cells were introduced into a microelectrophoretic chamber with low ionic buffer at pH 6, both cells migrated toward the negative pole, indicating a positive charge on the surface of both cells. At stage 1, electrophoretic mobility of the larger sperm (Sua) and smaller sperm (Svn) were 0.311 and 0.280 $\mu\text{m/s}$, respectively. As the pollen tube elongated and the sperm cells matured, the movement of the two sperm cells increased and remained directed toward the negative pole of the electrophoretic trough. At stage 2, electrophoretic mobility increased to 0.327 and 0.290 $\mu\text{m/s}$ for the Sua and Svn, respectively; 0.348 and 0.308 $\mu\text{m/s}$ at stage 3, and 0.376 and 0.335 $\mu\text{m/s}$ at stage 4. The difference in electrophoretic mobility between the two sperm cells became increasingly negative during maturation (Table 1; and Figs. 8, 9, 10, 11), presumably reflecting increased surface charge.

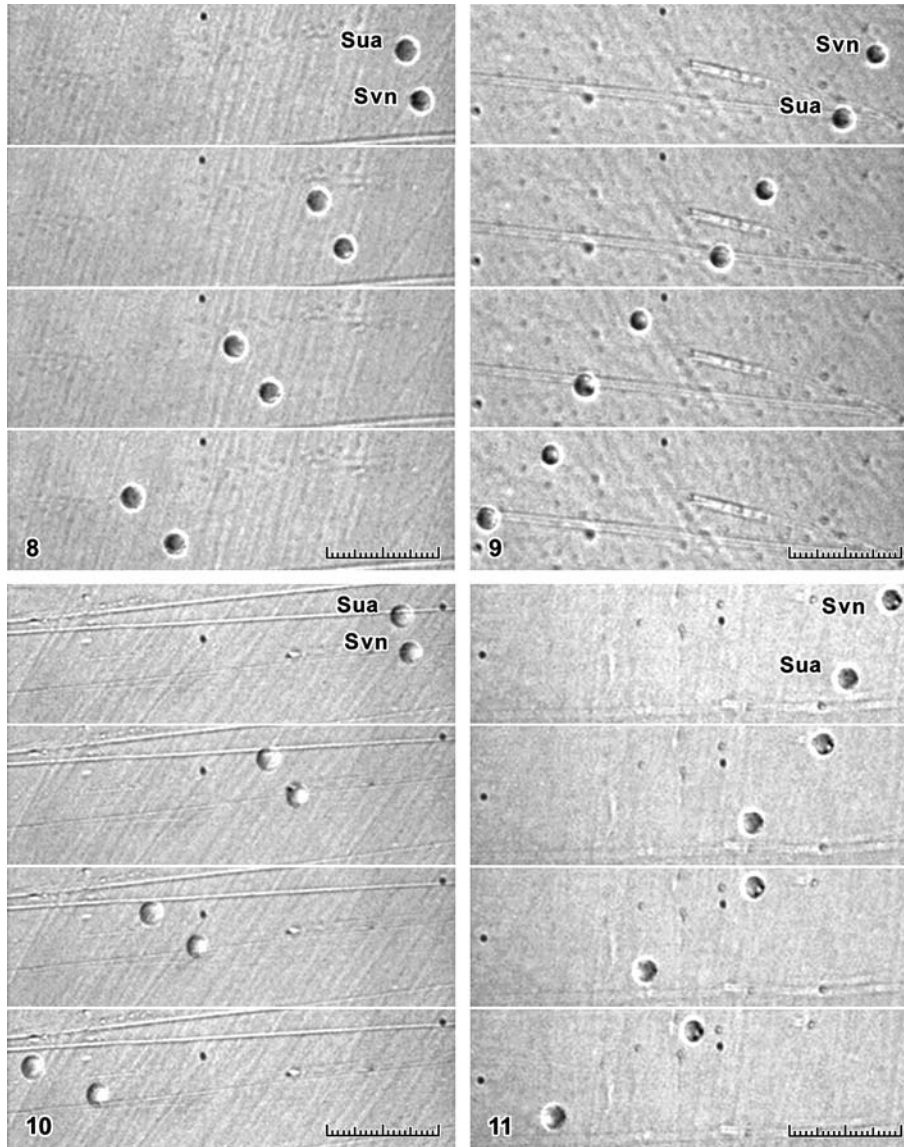
The sperm cells isolated from the 1-cm style are very sensitive to electrical potentials, and some of them burst within 5 min when an electrical potential was applied. Most of the organelles released from pollen tubes were negatively charged and moved faster than both of the sperm cells.

Effect of pH on mobility of sperm cells

The movement of sperm cells in an electrical field depends on surface charge, but the pH of the buffer can

Table 1 Electrophoretic mobility and surface charge of sperm cells at different stages in buffer (pH 6)

Stage	Sample no.	Sperm type	Mobility of sperm cells ($\mu\text{m/s}$)	Surface charge (esu/cm^2) $\times 10^3$
1	$n = 19$	Sua	0.311 ± 0.016	1.90 ± 0.098
		Svn	0.280 ± 0.019	1.71 ± 0.116
2	$n = 16$	Sua	0.327 ± 0.017	2.00 ± 0.103
		Svn	0.290 ± 0.018	1.77 ± 0.110
3	$n = 19$	Sua	0.348 ± 0.010	1.77 ± 0.061
		Svn	0.308 ± 0.016	1.88 ± 0.098
4	$n = 16$	Sua	0.376 ± 0.007	2.30 ± 0.043
		Svn	0.335 ± 0.010	2.05 ± 0.061



Figs. 8-11 Movement of a pair of sperm cells from one pollen tube in an electrophoretic field. (Time interval = 3 s). Ruler shows 1 μm increments *VN* vegetative nucleus; *Sua* sperm cell unassociated with the vegetative nucleus; *Svn* sperm cell associated with the vegetative nucleus. Paired sperm cells are shown at the following stages: *VN*, vegetative nucleus; *Sua*, sperm cell unassociated with

the vegetative nucleus; *Svn*, sperm cell associated with the vegetative nucleus

Fig. 8 Stage 1

Fig. 9 Stage 2

Fig. 10 Stage 3

Fig. 11 Stage 4

change the surface charge of sperm cells, and even cause the sperm cells to break down (Table 2). In buffers of pH 3 and pH 9, the two sperm cells immediately became brown and began to break down as soon as they were released in the buffer. In pH 4 buffer, both sperm cells migrated toward the positive pole, suggesting that their cell surface was negatively charged, but both moved very slowly and were easily broken. In pH 5 buffer, both sperm cells, however, migrated toward negative, suggesting that they acquired a positive surface charge, but the cells broke down later during electrophoresis. In pH 6 buffer, both sperm cells migrated toward the positive pole with the greatest electrophoretic mobility,

retaining their integrity for at least 30 min during electrophoresis. In pH 7 buffer, sperm cells displayed slower electrophoretic mobility than at pH 6, and at pH 8, sperm cells still move toward to negative pole but slower than at pH 7, breaking down early in electrophoresis. Based on these results we concluded that the optimal pH of buffer for electrophoresis of tobacco sperm cells is 6.

Discussion

Since the first report on sperm cell isolation by Cass (1973), sperm isolation has become an active research

Table 2 Effect of buffer pH on sperm cell viability and electrophoretic mobility

pH	Live state	Surface charge	Movement to positive pole
3	Immediately broken		
4	Fragile in current field	–	+
5	Broken sometimes	+	–
6	Migrate rapidly; none broken	+	–
7	Migration slower than at pH 6	+	–
8	Migrate slowly; very fragile	+	–
9	Immediately broken		

field in reproductive biology of higher plants. Using such isolated sperm and generative cells, genes and male germ unit-expressed proteins have been isolated and examined in *Brassica*, *Lilium*, and maize (Southworth and Kwiatkowski 1996; Xu and Tsao 1997; Xu et al. 1999, 2002; Zhang et al. 1993, 1999; Singh et al. 2002, 2003, 2002). Sperm cells isolated from tricellular pollen species have been the most frequently studied, as sperm cells are present in the pollen and can be isolated directly by osmotic shock or physical grinding, which facilitates en masse collection. Isolating sperm cells of bicellular pollen species, in contrast, requires germination and sometimes extended periods of pollen tube culture before sperm cells are formed in sufficient numbers for isolation. Although mass culture is possible, generative cells often fail to divide and distinguishing them from sperm cells is problematical. Using an in vivo-in vitro technique (Shivanna et al. 1988) improves the likelihood that all of the male germ cells isolated are sperm cells, but a major limitation is the quantity of emerging pollen tubes at the excision, which is spatially limited. Sperm cells of tobacco isolated using this method are of sufficiently high quality to be employed in experiments involving in vitro fertilization (Tian and Russell 1997a, b) and for screening expressed genes of male gametes (Xu et al. 2002), but no attempt was made to distinguish the two sperm cells in prior studies. To probe the mechanism of gametic recognition, we isolated two populations of sperm cells, distinguishing between sperm cells from the same pollen tube (Zhang et al. 1998) with the goal of further biochemical and molecular examination. This approach will be used in combination with molecular methods to discover and characterize male germ unit-expressed genes and proteins that may be involved in the mechanism of double fertilization in tobacco.

Since preferential fertilization of dimorphic sperm cells was first reported in *Plumbago zeylanica* (Russell 1985), the phenomenon of sperm dimorphism has been reported in numerous plants studied (Mogensen 1992; Hu and Tian 2002). In addition to differences in size and the number of organelles observed in dimorphic sperm cells, surface charge differences were also reported between the two sperm cells of *Plumbago zeylanica* (Zhang and Russell 1999). This presumably reflects another tier of dimorphism in sperm cells that is initiated at a cellular level in higher plants. Differences between the two sperm cells may also have an influence on the process of double fertilization. Ye et al. (2002) reported

that when the two sperm cells of *Phaius tankervilleae* were released into the cytoplasmic matrix of the degenerating synergid, the migration of sperm cells proceeded on two separate paths. The leading sperm cell migrated toward the central cell, whereas the second cell migrated laterally toward the egg cell. This difference in movement prior to gamete contact with female target cells indicates that preferential migration may contribute to sperm choice at fertilization and that a separate mode of short distance communication and complex targeting may exist within the degenerate synergid. Dimorphism in tobacco sperm cells emerges during pollen tube growth (Yu and Russell 1994) and becomes more significantly dimorphic during development (Tian and Russell 1998; Tian et al. 2001), which may ultimately point to upstream molecular control differences, as occurs in *Plumbago* (Singh et al. 2002). Surface charge represents yet another difference between two sperm cells that could functionally distinguish the two cells during fertilization and may be related with cell fate once sperms are discharged into the embryo sac.

In contrast with a prior study of *Plumbago zeylanica* in which both sperm cells were negatively charged (Zhang and Russell 1999), the sperm cells of *Nicotiana tabacum* are positively charged at optimum migration pH. This difference in surface charge presumably reflects differences in the sperm cells of the two species. In contrast to *Plumbago*, the size of the Sua of *Nicotiana* is larger than that of Svn, but the Sua of both species has higher electrophoretic mobility and thus a higher surface charge than the Svn. Although the specific role of surface charge in sperm cell biology is unknown, it is tempting to speculate that it is related to flowering plant fertilization, and that it could relate to differences in cell fate during double fertilization. To date, microelectrophoretic mobility and surface charge of sperm cells have been examined only in *Plumbago* and *Nicotiana*, but it will be interesting to understand how these differences in plant sperm cell biology relate to our emerging knowledge of gene expression in gametes.

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