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## Sperm dimorphism in *Nicotiana tabacum* L.

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**Abstract** Sperm cells of tobacco have been intensively studied as examples of isomorphic gametes in which major cellular and organellar parameters remain statistically indistinguishable in the two sperm cells. An examination of sperm cells late in maturation, however, displays that the sperm cell associated with the vegetative nucleus becomes statistically significantly smaller than the other sperm cell in tobacco. If late divergence occurs in the two sperms of other angiosperms, sperm dimorphism may be more prevalent than has previously been assumed and dimorphism may have a major influence on the pattern of double fertilization.

**Keywords** Dimorphism · Heterospermy · *Nicotiana* · Preferential fertilization · Sperm cells · Tobacco

### Introduction

Fertilization in angiosperms is the consequence of complex interactions between cells of the sporophyte and gametophyte generations, in which the male gametic cells have a seemingly passive role. Highly dependent on the pollen vegetative cell (and, later, the tube cell) for their physiological needs, the sperm are ultrastructurally simple and appear undifferentiated. Exactly two sperm cells are created by mitosis of the generative cell, either in the pollen (in tricellular species) or in the pollen tube (in bicellular species). The fates of specific sperm cells differ, yet both will fuse with a female gametophyte cell. One

sperm cell fuses with the egg cell to form the zygote (and later embryo), whereas the other sperm fuses with the central cell to form the nutritive endosperm. Whether this developmental divergence in the majority of angiosperms is random or is related to inborn sperm cell differences in any way is of continuing interest in flowering plants. Apparent cases of dimorphism have been reported in numerous species (Mogensen 1992).

Sperm cells may differ in numerous cell parameters, including cell volume, surface area, nuclear volume and surface area, cellular organelles (including DNA-containing heritable organelles) and overall shape (Russell 1991; Mogensen 1992). Regardless of these parameters, the sperm cells have an inborn polarity within the male germ unit (MGU). One sperm cell (the  $S_{vn}$ ) is physically associated with the vegetative nucleus in the MGU, whereas the other sperm cell (the  $S_{ua}$ ) is unassociated with the vegetative nucleus, but usually linked to the  $S_{vn}$ . The most extreme examples of organellar sperm dimorphism are members of the Plumbaginaceae (Plumbaginaceae), according to a survey of plastids in flowering plants by Corriveau and Coleman (1988). In *Plumbago zeylanica*, the most completely described dimorphic species, the  $S_{ua}$  receives most (and frequently all) of the plastids, whereas the  $S_{vn}$  contains few (typically no) plastids and 5 times the number of mitochondria (Russell 1984).

There are, however, examples in which the sperm cells do not statistically differ in any of the measured cellular parameters. Among these, the most completely studied species is tobacco, *Nicotiana tabacum*, in which quantitative cytological studies have suggested that the sperm cells are similar in all parameters during early development (Yu et al. 1992; Yu and Russell 1993, 1994a, b). Loss of cytoplasmic material from the sperm cells occurs in both sperm, and particularly in the  $S_{vn}$ , during development (Yu and Russell 1994b); there was no statistical difference in cells at that time, however. Studies using scanning electron microscopy (Zhang et al. 1999) and DNA epifluorescence microscopy (Tian HQ, Zhang Z, Russell SD, unpublished data) suggest a need to re-

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examine sperm cells in angiosperms to determine whether dimorphism is more common than previously suspected, and how this may be related to cell fate during fertilization.

## Materials and methods

Tobacco plants (*Nicotiana tabacum* L.) were grown in a controlled growth chamber at 20–27°C with 16 h daylength. Flowers were emasculated 12h before anthesis and pollinated at anthesis unless otherwise noted. Sperm cells were collected from semi-vivo cultured pollen tubes. Styles were cut near the growing pollen tubes at predetermined distances from the stigma, and the cut tip of the style was immersed into a culture medium of 0.01% (w/v)  $H_3BO_3$ , 0.01% (w/v)  $KH_2PO_4$ , 0.01% (w/v)  $CaCl_2 \cdot 2H_2O$  and 15% (w/v) sucrose for several hours, until pollen tubes emerged from the cut tip (Tian and Russell 1997; Tian et al. 1998). Five stages were sampled to examine maturational changes in sperm cells over time. Styles with excised lengths of 1, 2, 3, and 4 cm (cut at 13, 20, 27, and 34 h after pollination, respectively) were grown in culture medium for 6–12 h, until numerous pollen tubes emerged from the cut end of the style (Tian and Russell 1998). Data from electron microscopy reconstructions (Yu and Russell 1994b, Yu et al. 1994) were also used. Living sperm cells were collected and examined using a Leitz Dialux 20 photomicroscope. Their diameters were measured using an ocular reticle. Surface area and volume were calculated using formulae for a sphere and statistically analyzed using a G-test for goodness-of-fit compared to a normal distribution. A paired T-test was used for  $S_{ua}$ – $S_{vn}$  sperm pairs.

## Results and discussion

At the earliest stages, the generative cell of angiosperms is an asymmetric lenticular cell. This cell separates from the pollen wall and migrates into the vegetative cytoplasm (Yu and Russell 1994b, Russell et al. 1996). The volume of the generative cell may increase slightly during early development through vacuolization, but the cytoplasmic volume of this cell tends to decrease (Mogensen and Rusche 1985). The production of enucleated cytoplasmic bodies (ECBs), vesicle-containing bodies (VCBs) or both has been implicated in size changes of generative cells (Yu and Russell 1992) and later sperm cells (Russell et al. 1996). Typically, the sperm cells of tobacco form at approximately 8–9 h after pollination in vivo (Yu and Russell 1993), resulting in two sperm cells of similar size and volume.

In this work, at 13 h after pollination, sperm cell volume was 87–321  $\mu m^3$ , with an average of  $138.6 \pm 39.6 \mu m^3$  (Fig. 1A). The distribution initially diverged statistically from a normal distribution according to goodness-of-fit tests, and was skewed toward lower volumes. At 20 h after pollination, sperm cells had an average volume of  $186.3 \pm 50.5 \mu m^3$  (Fig. 1B) and a statistically normal distribution, which was maintained through 26 h after pollination. At 26 h after pollination, the in-vivo sperm cells differed by an average of 4.9%, which was statistically insignificant. The  $S_{ua}$  was  $236.4 \pm 14.2 \mu m^3$  and the  $S_{vn}$  was  $225.3 \pm 13.2 \mu m^3$  (Fig. 2A).

At 34 h (approximately 10 h before fertilization), sperm cell volumes became distinctly bimodal (Fig. 2B).

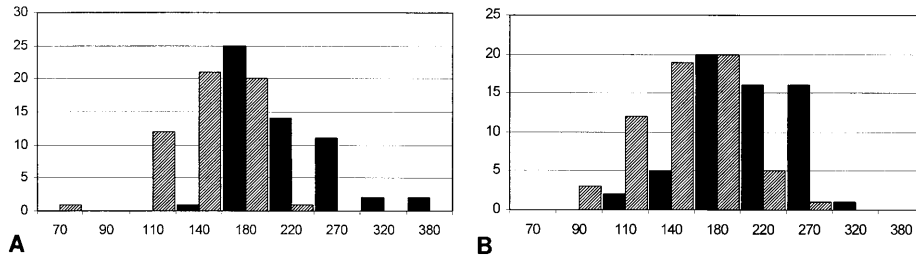
The larger sperm cells had an average volume of 247  $\mu m^3$ , and the smaller, 166  $\mu m^3$ . The average sperm volume was  $207 \pm 56 \mu m^3$ . The larger of the two sperm cells is the  $S_{ua}$ , which averages 81  $\mu m^3$  (30.4%) larger than the  $S_{vn}$ . This difference is very significant statistically ( $P < 0.01$ ). The distribution of sperm volumes diverges very significantly from a simple normal distribution at this stage, according to goodness-of-fit tests.

As the pollen tube approached the ovary, the volume of the two sperm cells diverged, apparently through the differential production of more ECBs and VCBs in the  $S_{vn}$  than in the  $S_{ua}$ . The difference in volume of the sperm cells became statistically very significant ( $P < 0.01$ ), establishing the sperm as dimorphic cells.

Sperm cell differences at fertilization are still difficult to assess using conventional methods. Sperm cells enlarge considerably during the final stages of pollen tube elongation and within the synergid. Based on the only available three-dimensional reconstruction of a pair of sperm cells in the tobacco synergid, the volume of the  $S_{ua}$  was 940.2  $\mu m^3$  and that of the  $S_{vn}$  was 496.3  $\mu m^3$  (Yu and Russell 1994b), an increase of 290% and 120%, respectively. This volume increase is largely the result of vacuolization of sperm cells prior to fertilization, which may also be dramatic in other species (see Russell et al. 1990). Coinciding with this enlargement, sperm cells undergo an increase in the content of DNA to 2C prior to fusion (H.Q. Tian, T. Yuan, S.D. Russell, personal communication). The volume of the nucleus and cytoplasm is maintained at a relatively consistent proportion during development. The volume of the nucleus apparently doubles with the increased nuclear DNA, and so does the overall volume of the cell.

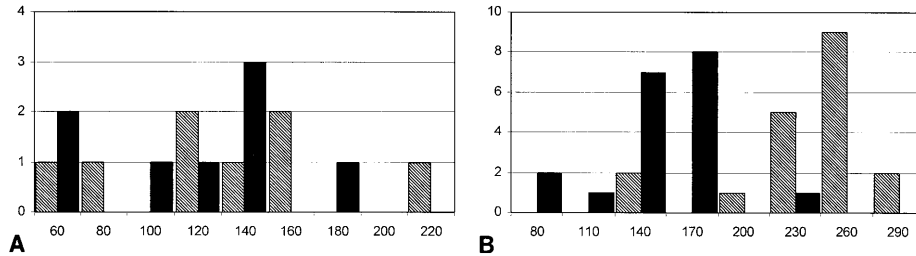
Prior to this study, tobacco could be considered as the best-documented example of sperm isomorphism (Yu et al. 1992; Yu and Russell 1993, 1994a, b); however, the current study clearly indicates late-arising dimorphism in this plant. In prior studies, differences between the  $S_{ua}$  (narrowly the larger of the two cells) and the  $S_{vn}$  were insignificant. The prior study noted that ECB and VCB production seemed to be greater in the  $S_{vn}$  than in the  $S_{ua}$ , but it was unclear that differences between the two sperm cells would diverge. This is the first documentation of such a late divergence of sperm types. This discovery casts doubt on the various other reports of isomorphic sperm cells, which have also tended to characterize early stages of maturation (review: Mogensen 1992; Yu and Russell 1994b). Prior conclusions about the abundance of sperm isomorphism and whether sperm cells of other species differ innately in physiology and behavior may be difficult to assess.

Membrane fusion in lipid vesicle systems is aided by hyperosmotic changes. With osmotic swelling, an equal number of membrane lipids are stretched over an increasing surface area, which favors the relaxation of membrane stress through fusion with a larger membrane-bound structure (Wilschut 1991). Considering the significant differences in surface area and volume of sperm egg and central cells, there may be biophysical reasons



**Fig. 1A, B** Distribution of sperm cell volumes in *Nicotiana tabacum* in  $\mu\text{m}^3$  for sperm cell pairs in which the  $S_{ua}$  and  $S_{vn}$  were not distinguished. *Hatched bars* represent the smaller of the two cells,

*solid bars* represent the larger cells. **(A)** 13 h after pollination in newly formed sperm cells and **(B)** 20 h after pollination, displaying a statistically normal distribution of sperm cell volumes



**Fig. 2A, B** Distribution of sperm cell volumes in *Nicotiana tabacum* in  $\mu\text{m}^3$  for the two types of sperm cells ( $S_{ua}$ , *hatched* and  $S_{vn}$ , *solid black*) at **(A)** 26 h after pollination, showing essentially a random distribution of volumes between the  $S_{ua}$  and  $S_{vn}$ , and **(B)** 34 h after pollination, after the  $S_{ua}$  has become significantly larger than the  $S_{vn}$

for preferential fusion between a specific sperm cell and its particular target female cell based on size and surface differences alone. Alternatively, surface determinants of sperm cells may reflect differences in genetic expression that become evident once the sperm cells are deposited in the embryo sac and during gamete adherence and fusion. The emergence of sperm dimorphism late in sperm development – with potentially differential hypertrophication – may be particularly susceptible to preferential fertilization.

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