Plant Cell Reports (1998) 18: 143–147

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Isolation of the male germ unit: organization and function in tobacco (*Nicotiana tabacum* L.)

Received: 23 February 1998 / Revision received: 21 May 1998 / Accepted: 1 June 1998

Abstract Sperm cells are released from pollen tubes of tobacco as linked cells, associated with the vegetative nucleus in an assemblage known as the male germ unit (MGU). Using light microscopy, the MGU assemblage appears to be ensheathed by cytoplasmic material of the pollen tube, which may stabilize their association. Following their release, the shape of the sperm cells and vegetative nucleus changes from an ellipsoidal to a more spheroidal morphology. When most of the cytoplasmic material is dispersed, a boundary remains around the two sperm cells. Using scanning electron microscopy, the cytoplasmic material surrounding the MGU appears filamentous, sometimes twisted and rope-like. Based on these observations, the function of the MGU of tobacco is discussed.

Key words *Nicotiana tabacum* · Male germ unit · Scanning electron microscopy · Sperm isolation · Angiosperms

Abbreviation *MGU* Male germ unit

Introduction

Since the earliest published electron microscope work, the two sperm cells have been observed to form a linked unit within the pollen tube (Jensen and Fisher 1970), pollen grain (Bannikova and Khvedynich 1974; Chu and Hu 1984) and in isolated cells (Cass 1973). Russell and Cass (1981) were the first to describe a "functional unit" consisting of the two sperm cells associated with the vegetative nucleus in the mature pollen grain and growing pollen tube of flowering plants. Based on these findings, Dumas et al. (1984) termed the linked unit the "male germ unit" (MGU) – a functional assemblage transmitting all DNA of the male gametophyte (cytoplasmic and nuclear) during sexual reproduction in flowering plants (Keijzer et al. 1988; Matthys-Rochon and Dumas 1988).

A neglected aspect of MGU function is that despite their proximity and fusigenic ability, the two sperm cells do not fuse within the small space available in the pollen tube (Tian and Russell 1998). In the present paper, we use light and scanning electron microscopy to characterize the organization of the isolated MGU of tobacco, to further understand MGU function in flowering plants.

Materials and methods

Plants of *Nicotiana tabacum* L. were grown in a controlled growth chamber at 20–27 °C with 16 h daylength. Styles were pollinated and allowed to grow for 34 h in vivo. The excised style was cultured in a medium of 0.01% [wt/vol] H_3BO_3, 0.01% [wt/vol] KH_2PO_4, 0.01% [wt/vol] $CaCl_2 \cdot 2H_2O$ and 15%[wt/vol] sucrose (510 mOsmol/kg) for several hours until pollen tubes emerged from the cut end of the style. The style with numerous emergent pollen tubes was then transferred to a 9% [wt/vol] mannitol solution (524 mOsmol/kg), where the pollen tubes burst, releasing paired intact sperm cells and vegetative nuclei into the solution (Tian and Russell 1997).

For light microscopy, MGUs were transferred from the releasing solution into a low-ionic buffer containing 10 mM Na_2HPO_4, 0.3 mM citric acid (Van Oss and Fike 1979) and 9% mannitol using an inverted phase contrast microscope (Zeiss Axiovert 10) equipped with a microinjector (Eppendorf, type 5242). The MGUs were observed using a research phase contrast microscope (Leitz Dialux 20). For scanning electron microscopy, the contents of the pollen tube were collected by immersing the cut end of the style into 0.5 ml of 9% [wt/vol] mannitol solution in a 1.5-ml microcentrifuge tube containing a Formvar-coated 200-mesh grid in the bottom for 5 min. The microcentrifuge tube was then centrifuged for 10 min at 1500 *g*. Material was fixed using 0.1 M cacodylate-buffered 2% glutaraldehyde (pH 6.8) added dropwise to the tube and fixed for 30 min. Specimens were then dehydrated using a graded ethanol series, critical-point dried, coated with gold-palladium, and examined with an ETEC Autoscan scanning electron microscope.

Communicated by G. Phillips

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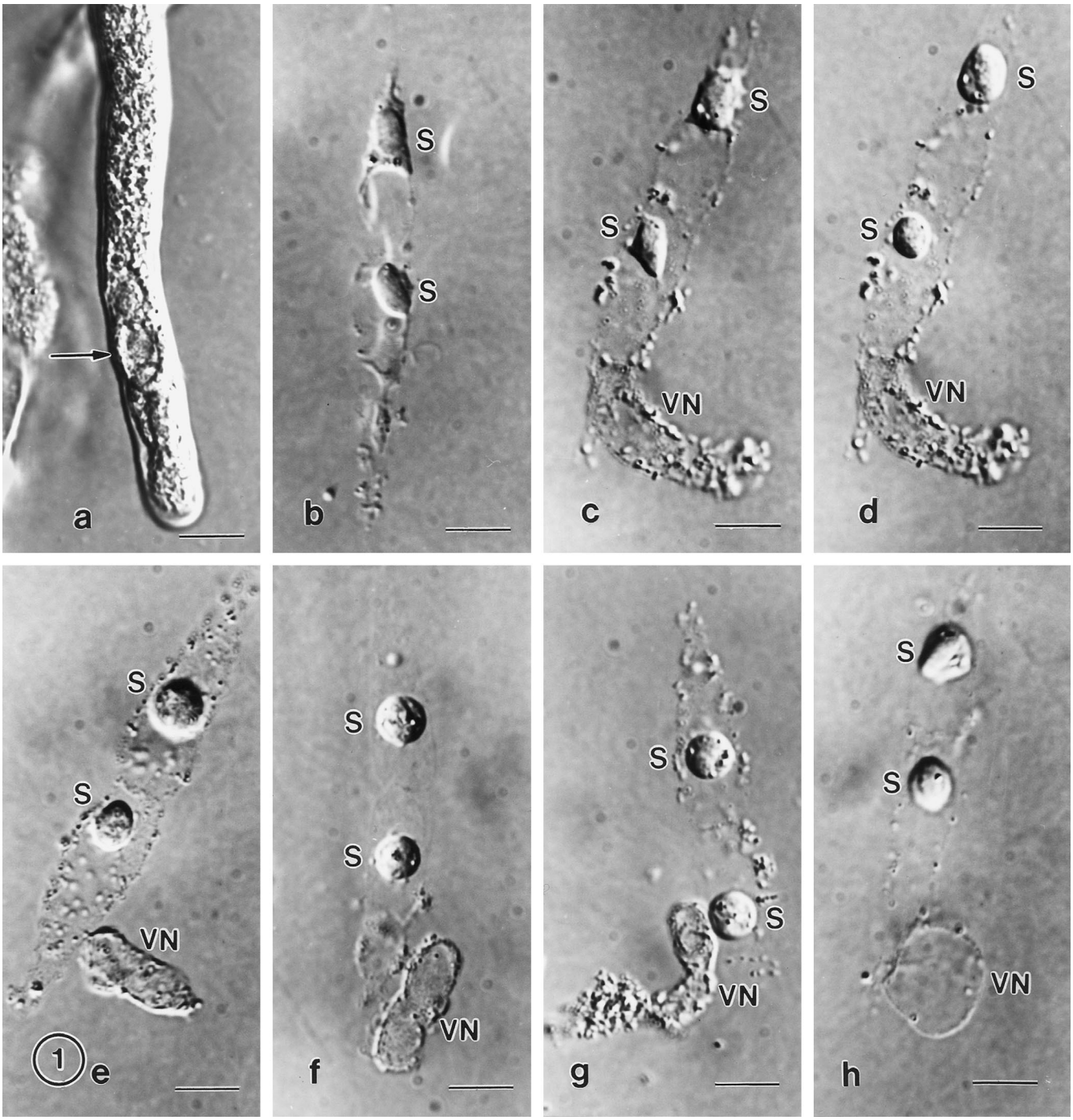
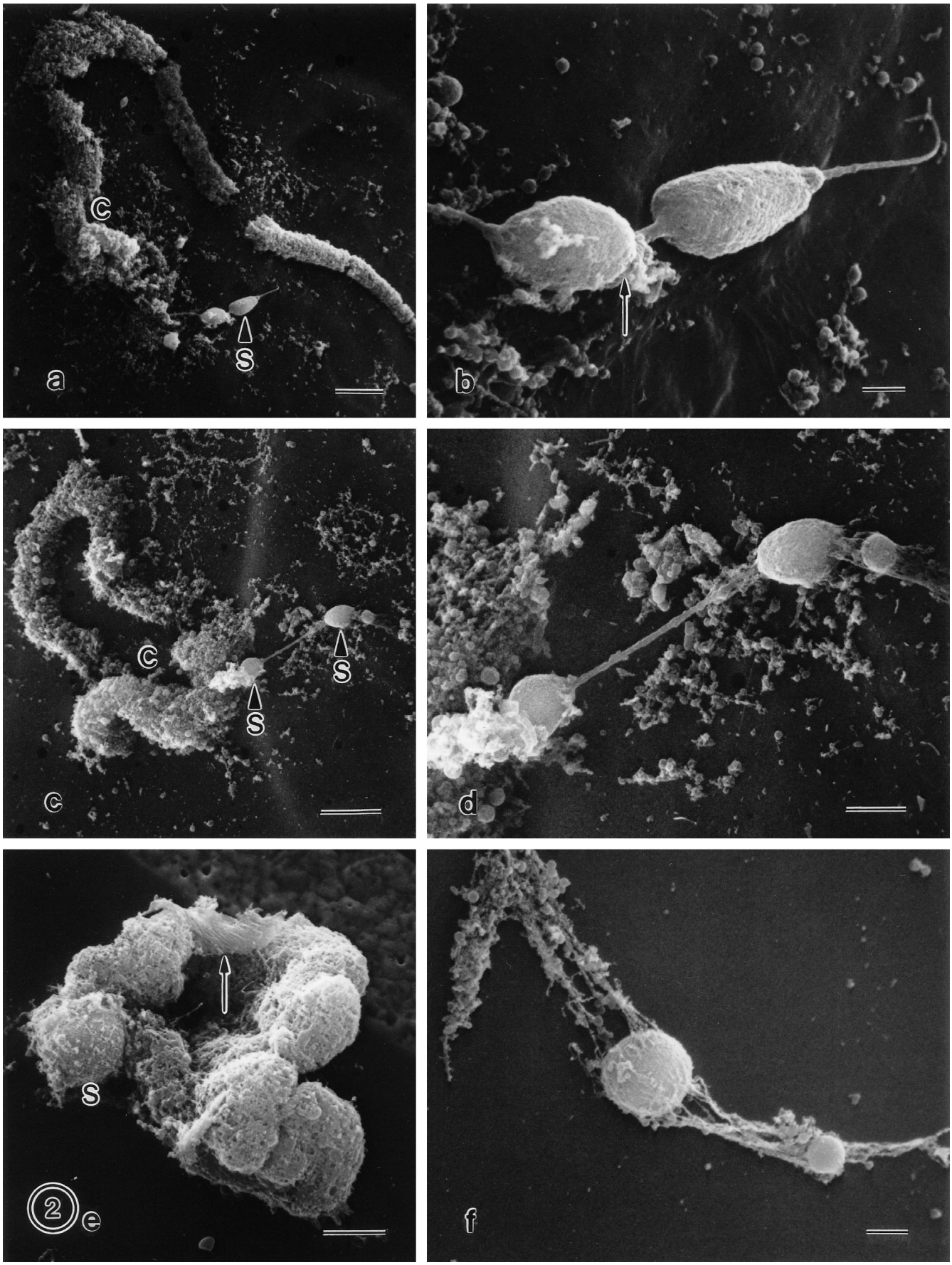


Fig. 1a–h Light microscopy of the MGU of tobacco (*bars* 10 μm; *S* sperm cell, *VN* vegetative nucleus). **a** Pollen tube grown in 15% sucrose medium using the in vivo-in vitro technique. Two sperm cells are located near the tip of the pollen tube (*arrow*). **b** Two connected sperm cells soon after release from a pollen tube. Note the layer of pollen tube cytoplasm surrounding the sperm cells. **c** MGU 5 min after release from pollen tube showing two ellipsoidal sperm cells. **d** Same MGU 30 min after release, showing the two sperm cells becoming essentially spheroidal. **e** MGU, showing a boundary enclosing the two sperm cells in separate compartments. **f** MGU 1 h after release, showing loss of organelles from the surface of the boundary enclosing the sperm cells. **g** MGU during early breakdown of vegetative nucleus. **h** MGU with a rounded vegetative nucleus

Fig. 2a–f Scanning electron microscopy of the MGU of tobacco (*C* pollen tube cytoplasm, *S* sperm cell). **a** Two sperm cells and cytoplasm from the pollen tube (*bar* 10 μm). **b** Higher magnification of two sperm cells in **a**, showing a layer of pollen tube cytoplasm (*arrow*) surrounding sperm cells (*bar* 2 μm). **c** Two sperm cells and pollen tube cytoplasm from another pollen tube (*bar* 10 μm). **d** Higher magnification of two sperm cells in **c**, showing a slender connection between them after loss of the surrounding pollen tube cytoplasm (*bar* 4 μm). **e** Pollen tube cytoplasm surrounding the MGU, showing locally twisted filamentous material (*arrow*) (*bar* 5 μm). **f** Filamentous material still surrounds a sperm cell even after most of the pollen tube cytoplasm is lost (*bar* 2 μm)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Results and discussion

Light microscopy

The diameter of the pollen tube using the in vivo-in vitro technique averaged 9.60 μm (Fig. 1a). The two sperm cells were located near the tip of the tube. When styles with their emergent pollen tubes were transferred into the 9% mannitol solution, many of the tubes broke and released their contents, including the two sperm cells and a vegetative nucleus. The released sperm cells were wrapped in a layer of cytoplasm from the pollen tube, and initially were slender and spindle-shaped for ~5 s, rapidly becoming ellipsoidal (Fig. 1b). As the surrounding cytoplasm dissipated, the two sperm cells became nearly spherical (Fig. 1c–f). The two sperm cells were associated by a common boundary, divided by a central partition. Numerous organelles adhered to the surface of the boundary (Fig. 1c–e), but following isolation, most of them disappeared (Fig. 1f). The vegetative nucleus was associated with the compartment containing the smaller of the two sperm cells (Fig. 1c–f). The vegetative nucleus remained associated and intact for ~30 min in the 9% mannitol low-ionic buffer, but lost its integrity and contents soon thereafter (Fig. 1g). Some vegetative nuclei persisted for a longer time and became nearly spherical (Fig. 1h).

Scanning electron microscopy

After glutaraldehyde fixation and ethanol dehydration, the vegetative nucleus was only infrequently preserved. Sperm cells, however, persisted and were associated with the cytoplasm of the pollen tube (Fig. 2a, c). A layer of cytoplasm containing a filamentous material tightly surrounded the two sperm cells (Fig. 2b). The distance between the two sperm cells became greater with time after isolation, with a slender structure connecting the sperm cells (Fig. 2d). The filamentous nature of this structure sometimes twisted (Fig. 2e), reflecting torsion of the cell assemblage. Even when most of the pollen cytoplasm had dissipated, some filamentous material remained around the sperm cells (Fig. 2f).

Functional significance of the MGU

The concept of the MGU has been discussed for many years (Dumas et al. 1984), and it has been recognized in increasingly more flowering plants (Southworth and Knox 1988; Russell 1991; Theunis et al. 1991). The proposed principal function of the MGU has been the transport of the male gametes. This is based on connections between the two sperm cells and their association with the vegetative nucleus, which appears to facilitate the positioning of the two sperm cells precisely next to their female target cells (Hu 1990; Russell et al. 1990; Mogensen 1992). Another potentially important characteristic of the MGU is that fusions between the two sperm cells in the pollen tube appear to be prevented by materials on their surfaces. Paired sperm cells usually do not fuse spontaneously, but when they are placed in an enzymatic solution containing both cellulase and pectinase, the two sperm cells often fuse with each other (Tian and Russell 1998). In the current study, the two sperm cells were separated into two compartments in isolated MGUs. The separation of these regions may contribute to preventing fusion between brother sperm cells in the pollen tube.

MGUs arise during the development of the male gametophyte, forming specialized structural units that hold the two sperm cells and vegetative nucleus together during their transportation in the pollen tube. However, the MGU has two challenges. First, the two sperm cells and vegetative nucleus are too large to fit into the pollen tube without deformation. As measured in vitro, the average diameter of the two sperm cells was 7.76 μm and 6.79 μm, respectively, and that of the vegetative nucleus was 15.62 μm. The external diameter of the pollen tube, however, was only 9.60 μm. Clearly, the spherical shape of sperm cells and vegetative nucleus is not possible during transportation in the pollen tube. Although sperm cells contain microtubules, their abundance is believed to be insufficient to maintain the shape of the sperm cells (Palevitz and Tiezzi 1992). In addition, although tubulin antibodies label the sperm cell cytoplasm of *Spinacia oleracea*, microtubules were rarely visible in and around the vegetative nucleus (Theunis and Wilms 1988). In addition to the presence of microtubules inside the sperm cells, which may partially control the shape of the two sperm cells, there was evidence that additional cytoplasmic forces were needed to maintain this assemblage. Second, the association of the vegetative nucleus with the sperm cells increases both the volume and surface area of the MGU, which moves as a unit within the small space of the pollen tube. In vivo, the two sperm cells and vegetative nucleus were aligned in a row and slender in shape (Yu and Russell 1992, 1993, 1994). This arrangement of the MGU along with the shape of the two sperm cells and vegetative nucleus appear to facilitate their transmission. Using scanning electron microscopy, a layer of pollen tube cytoplasm was observed tightly surrounding the two ellipsoidal sperm cells (Fig. 2b). The filaments of cytoplasm from the pollen tube formed a mesh-like network of filaments around the sperm cells and vegetative nucleus (Fig. 2f), and displayed localized areas of torsion (Fig. 2e). The filamentous material containing the MGU may be able to apply force to the periphery of the sperm cells and vegetative nucleus, causing the MGU to achieve a more slender shape, thus facilitating their passage in the pollen tube.

Acknowledgements This research was supported by US Department of Agriculture grant 95-37304-2361.

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