

Localization of myosin on sperm-cell-associated membranes of tobacco (*Nicotiana tabacum* L.)

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Summary. Actomyosin interactions are reportedly the principal mechanism for the transport of nonmotile sperm cells of flowering plants inside the pollen tube and inside the embryo sac. Myosin has been demonstrated on the generative cell (the predecessor of sperm cells), although it is unclear from previous studies whether myosin is located directly on the plasma membrane of the male germ cells or on the external plasma membrane of the pollen cell that surrounds them. Immunogold scanning electron microscopy was used to localize myosin on isolated tobacco sperm cells, with and without associated membranes. When present, the pollen tube plasma membrane surrounding the sperm cells was labeled by an antimyosin antibody, as were pollen tube cytoplasmic organelles. Negligible labeling was observed directly on the plasma membrane of the sperm cells.

Keywords: Immunogold; Myosin; *Nicotiana tabacum*; Pollen tube; Scanning electron microscopy; Sperm cell transport.

Introduction

Fertilization in flowering plants involves the transport of sperm cells from the pollen grain, through the pollen tube cell, and ultimately to the female germ cells. Sperm cells are formed either inside the pollen grain (tricellular pollen) or inside the pollen tube (bicellular pollen). Unlike non-seed plants and some gymnosperms, sperm cells in flowering plants are believed to be nonmotile, passive participants during their transport in the pollen tube. Sperm cell transport is composed of two phases: transport inside the pollen

tube and positioning of sperm cells inside the embryo sac after the sperm cells are discharged from the pollen tube (Russell 1996).

Evidence suggests that actomyosin interactions may be involved in sperm cell movement inside the pollen tube. Bundles of actin filaments are present in the cortical cytoplasm of the pollen tube (Tang et al. 1989a, Heslop-Harrison and Heslop-Harrison 1989a), and antimyosin antibody labels the surface of the generative cell, the predecessor of sperm cells (Heslop-Harrison and Heslop-Harrison 1989b; Tang et al. 1989b; Miller et al. 1995; Tirilapur et al. 1995, 1996). Pollen-wall-generating vesicles are also labeled by antibodies to myosin (Heslop-Harrison et al. 1997), as are other cytoplasmically transported pollen tube organelles (Miller et al. 1995). The role of actomyosin interactions in male germ cell movement is supported by depolymerization of actin filaments using cytochalasin and inhibition of ATP replenishment (Lancelle and Hepler 1988; Heslop-Harrison and Heslop-Harrison 1989a, 1996). During male germ cell transport inside the pollen tube, the male germ cells are surrounded by their own plasma membrane and, in turn, by the plasma membrane of the pollen tube; the latter membrane separates the sperm cells from the interior of the tube cell cytoplasm (Yu et al. 1992, Russell 1994, Russell et al. 1996). It was unclear however from prior studies whether myosin was associated directly with male germ cells or with the tube cell plasma membrane.

During migration of the sperm cells following discharge from the pollen tube, the cell in which they

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are deposited, the synergid, is dramatically altered; two actin bands, or "coronas", are formed within the penetrated synergid and between the egg and the central cell (Huang and Russell 1994, Huang and Sheridan 1994). These actin bands appear to trace the pathway taken by the sperm cells during their passage to the egg and central cell. Immunogold labeling of isolated sperm cells of *Plumbago zeylanica*, however, reflects that negligible myosin is present on the plasma membrane of sperm cells (Zhang and Russell 1999).

In the present study, the location of myosin on sperm-cell-associated membranes was determined with isolated sperm cells of tobacco and immunogold scanning electron microscopy.

Material and methods

Isolation of sperm cells

Plants of *Nicotiana tabacum* L. were grown in a controlled growth chamber at 20–27 °C with 16 h daylength. Isolation of sperm cells was based on the method of Tian and Russell (1997). Briefly, flowers were hand pollinated 1 day after emasculation. After 30 h of pollen tube growth, pollinated styles were excised 4 cm from the tip of the stigma. The cut end of the style was immersed in a culture medium containing 0.01% H₃BO₃, 0.01% KH₂PO₄, 0.01% CaCl₂, and 15% sucrose (pH 5.4) and grown for about 9 h at 25 °C. After numerous pollen tubes emerged from the end of the style, the cut end was immersed in a solution containing 9% mannitol (pH 5.4). Most of the pollen tubes burst within 5 min, releasing the sperm cells and the contents of the pollen tubes into the solution.

Scanning electron microscopy

Pollen tubes were burst and sperm cells released from the pollen tubes onto silane-coated coverslips (Büchi and Bächli 1979) placed at the bottom of an Eppendorf microcentrifuge tube. The coverslip was then centrifuged at 1,500 g for 10 min to ensure that the sperm cells were firmly attached. The sample was fixed 40 min with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 6.3), dehydrated in a graded ethanol series, critical-point dried, coated with gold-palladium and examined with an ETEC Autoscan scanning electron microscope at 20 kV.

Immunogold labeling

For immunogold labeling, samples fixed 40 min in 1% glutaraldehyde were blocked with 3% dried milk and 1% fish gelatin in phosphate-buffered saline (PBS) (pH 7.3), incubated in a 1 : 10 dilution of antimyosin antibody (M-7648; Sigma Chemical Co., St. Louis, Mo.) with blocking solution for 1 h, washed three times with blocking solution, and then incubated in a 1 : 5 dilution of 15 nm diameter gold-conjugated goat anti-rabbit IgG (EY Laboratories, Inc., San Mateo, Calif.) with blocking solution for 40 min. The specimen was then washed with PBS, dehydrated in a graded ethanol series, critical-point dried, coated with carbon, and examined with a JEOL JSM-880 scanning electron microscope at 25 kV. Controls were prepared by substituting primary antibody with blocking solution.

Results

Isolated sperm cells appeared ellipsoidal to spherical in scanning electron microscopy (Figs. 1–4). Discharged pollen tube cytoplasm remained largely intact in well-preserved samples (Fig. 1), despite the absence of the outer plasma membrane. The pollen tube plasma membrane around the sperm cells was variably preserved. Figure 2 illustrates a partially preserved segment of pollen membrane surrounding the sperm cell and an associated cytoplasmic body. The plasma membrane of the tube cell degenerated progressively, first forming a broken sheath, then fine threads or tubules associated with the sperm cells (Fig. 3), and ultimately disintegrated (Fig. 4). The increasingly rounded shape of the sperm cells was associated with the loss of the surrounding pollen tube plasma membrane. The underlying sperm plasma membrane, however, remained intact (Figs. 2–4).

Filamentous structures associated with the sperm cells (Fig. 5A) displayed dense immunogold myosin labeling, although the underlying sperm cell surface appeared to be labeled only in conjunction with adherent pollen membrane fragments and organelles (Fig. 5B). In sperm cells that lacked surrounding tube cell plasma membranes or had sparse adherent pollen organelles (Fig. 6A), gold labeling was either absent or restricted to these adherent materials (Fig. 6B) and did not occur directly on the sperm cell surface. In Fig. 7A, a sperm cell without adherent pollen organelles appeared to represent a cell with the surrounding membrane intact (Fig. 7B). Typically, sperm cells that retained their native shapes had intact surrounding membranes, and their entire surface was heavily labeled (Fig. 8). No gold particles were observed in control samples in which the primary antibody was omitted.

Discussion

The movement of angiosperm male germ cells appears to depend on interactions between bundles of filamentous actin and membrane-associated myosin during their transport in the pollen tube (for a review, see Pierson and Cresti 1992). Among the structures labeling with antibodies to myosin in pollen are the surfaces of generative cells, both in the pollen grain (Heslop-Harrison and Heslop-Harrison 1989b) and, later, in the pollen tube (Tirlapur et al. 1995, 1996). From their inception, the generative cell and later sperm cells are

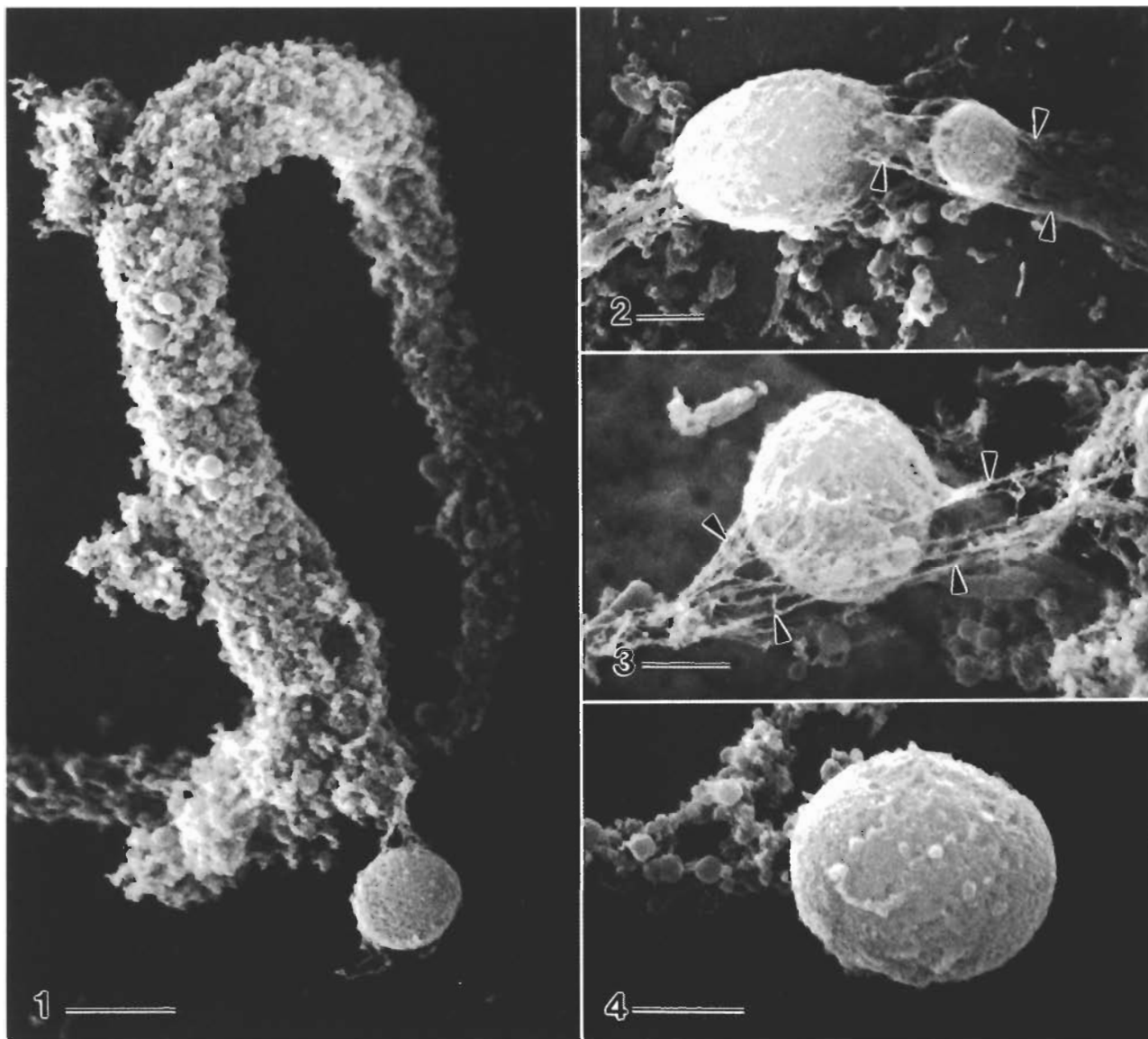
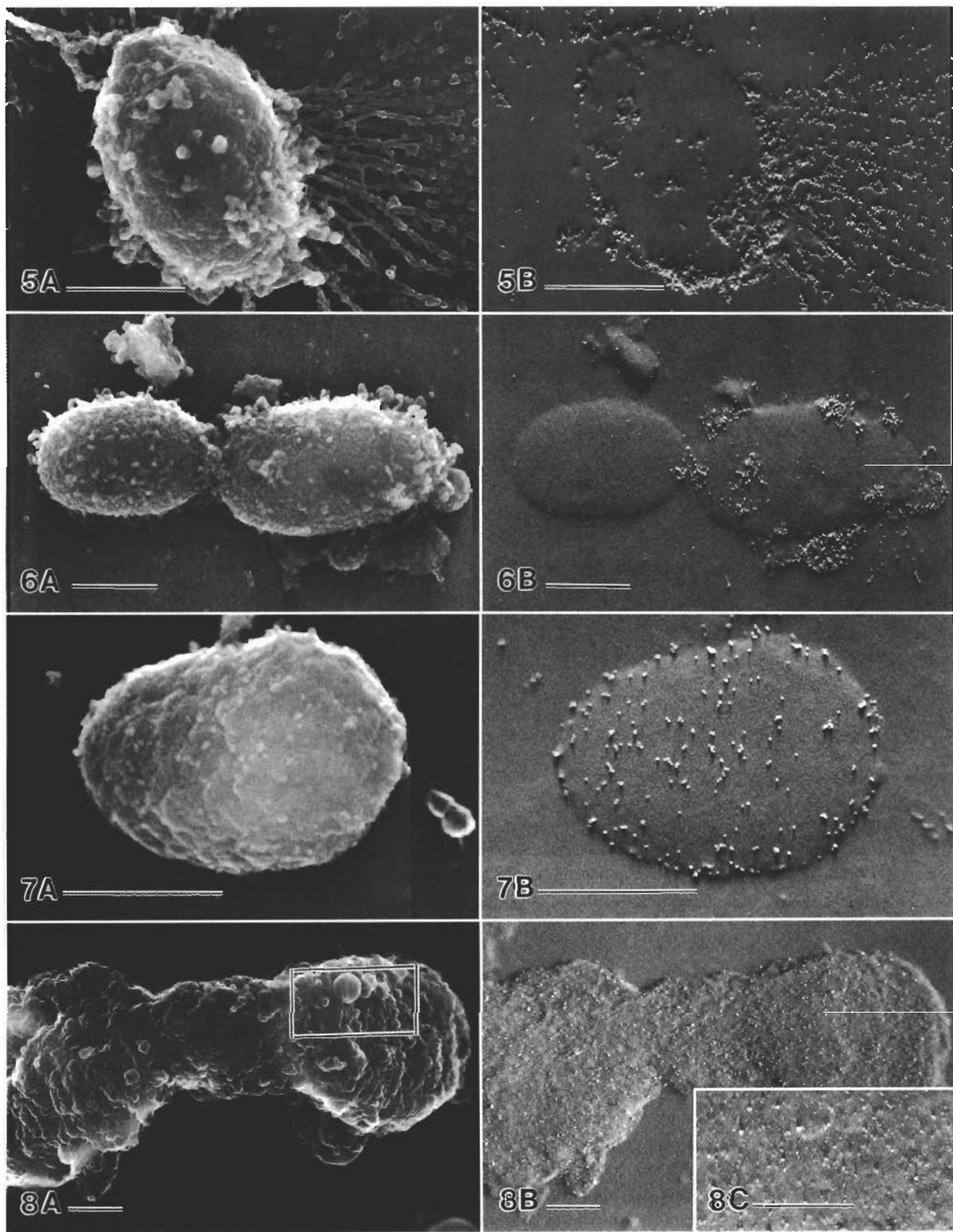


Fig. 1. Scanning electron micrograph of an isolated sperm cell and associated pollen tube cytoplasm. Bar: 5 μ m

Figs. 2–4. Scanning electron micrographs of isolated sperm cells, showing an enveloping membrane (arrowheads) around the sperm cells (Fig. 2). The membrane later may become filament-like (arrowheads) after isolation (Fig. 3), with the membrane eventually disappearing (Fig. 4). Bars: 2 μ m

surrounded by pollen plasma membrane, which originates during pollen formation and encloses the generative cell (Russell et al. 1996). The pollen plasma membrane continues to surround the plasma membranes of the generative and sperm cells during later development (Russell 1994); however, in prior studies the precise location of the antimyosin label could not be distinguished between the pollen and male germ unit membranes. The earliest indication in literature

that myosin was located on the ensheathing pollen plasma membrane was reported by Heslop-Harrison and Heslop-Harrison (1989b), who noted that only some of the generative cells examined were labeled with antimyosin antibodies. They noted that labeled cells appeared to be associated with an external membrane and postulated that generative cells that were unlabeled may have lacked this membrane (Heslop-Harrison and Heslop-Harrison 1989b).



The current study appears to confirm this finding, since antimyosin labeling is localized on pollen cell plasma membranes. Those sperm cells lacking adherent pollen membranes are essentially unlabeled. Immunogold transmission electron microscopic observations of *Plumbago* sperm cells have revealed a similar localization of antimyosin label on the pollen plasma membrane but not on the sperm cell surface (Zhang and Russell 1999).

The loss of the surrounding pollen tube plasma membrane is an expected consequence of pollen tube discharge during normal fertilization (Russell 1994), which also occurs during sperm cell isolation (Russell 1991). Although actomyosin migration of sperm cells to the female target nuclei is still possible, the sperm cells would have to obtain myosin from other sources after their release into the embryo sac (Zhang and Russell 1999, Russell 1996).

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Figs. 5–8. Immunogold labeling of myosin on isolated sperm cells and organelles

Fig. 5. **A** Secondary electron image (SEI), showing a sperm cell and surrounding filamentous structures. **B** Backscattered electron image (BEI) of the same field as in **A**, showing filament-like structures and organelles on the sperm cell labeled with gold particles, but no label on the sperm plasma membrane

Fig. 6. **A** SEI of a sperm cell pair isolated from the same pollen tube. The left sperm cell lacks surrounding pollen membrane. **B** BEI of the same field as in **A**, showing absence of labeling on the plasma membrane of sperm cells, whereas some organelles and adherent membrane fragments on the sperm cells are heavily labeled

Fig. 7. **A** SEI of an isolated sperm cell with associated pollen membranes. **B** BEI of the same field as in **A** shows surrounding membrane labels for myosin

Fig. 8. **A** SEI of two sperm cells surrounded by a pollen plasma membrane. **B** BEI of the same field as **A**, showing the membrane labeled with gold particles. **C** BEI of the field as boxed in **A**, showing gold particle labeling at larger magnification. Bars: 1 µm

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