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Identification and localization of three classes of myosins in pollen tubes of *Lilium longiflorum* and *Nicotiana alata*

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SUMMARY

The presence and localization of actin and myosin have been examined in pollen tubes of *Lilium longiflorum* and *Nicotiana alata*. Immunoblot analysis of pollen tube extracts with antibodies to actin, myosins IA and IB, myosin II, and myosin V reveals the presence of these contractile proteins. Immunofluorescence microscopy using various methods to preserve the pollen tubes; chemical fixation, rapid freeze fixation and freeze substitution (RF-FS) followed by rehydration or by embeddment in a methacrylate mixture, was performed to optimize preservation. Immunocytochemistry reaffirmed that actin is localized longitudinally in the active streaming lanes and near the cortical surface of the pollen tube. Myosin I was localized to the plasma membrane, larger organelles, the surface of the generative cell and the vegetative nucleus, whereas, myosin V was found in the vegetative cytoplasm in a punctate fashion representing smaller organelles. Myosin II subfragment 1 and light meromyosin were localized in a punctate fashion on the larger organelles throughout the vegetative cytoplasm. In addition, isolated generative cells and vegetative nuclei labeled only with the myosin I antibody. Competition studies indicated the specificity of the heterologous antibodies utilized in this study suggesting the presence of three classes of myosins in pollen. These results lead to the following hypothesis: Myosin I may move the generative cell and vegetative nucleus unidirectionally through the pollen tube to the tip, while myosin V moves the smaller organelles and myosins I and II move the larger organelles (bidirectionally) that are involved in growth.

Key words: myosin, actin, pollen, confocal microscopy

INTRODUCTION

In pollen tubes there is a rapid flow of vesicles along the shank of the tube to the tip where they eventually fuse, supporting the extensive elongation exhibited by these cells (Larson, 1965; Steer and Steer, 1989; Kohno and Shimmen, 1988a,b; Heslop-Harrison and Heslop-Harrison, 1990; Emons et al., 1991). In addition to the bidirectional movement of vesicles there is a slower unidirectional acropetal flow of the male germ unit (Mascarenhas, 1975, 1990; Knox and Singh, 1987; Emons et al., 1991) consisting of the vegetative nucleus and generative cell, at about the rate of pollen tube growth (personal observation). Several lines of evidence indicate that these motile processes are generated by an actomyosin system. For example, treatment of pollen tubes with cytochalasin, a drug that modulates actin function, causes complete cessation of streaming (Franke et al., 1972; Mascarenhas and Lafountain, 1972; Perdue and Parthasarathy, 1985; Brawley and Robinson, 1985; Lancelle and Hepler, 1988; Tang et al., 1989a) and the movement of the generative cell and vegetative nucleus (Franke et al., 1972; Picton and Steer, 1981; Lancelle and Hepler, 1988; Heslop-Harrison et al., 1988, 1989a). Furthermore, the movement of the generative cell and vegetative nucleus is not inhibited by microtubule inhibitors (Joos et al., 1994). In addition, isolated lily pollen tube organelles can move along the surface of actin cables in *Chara* (Kohno and Shimmen, 1987, 1988a,b) and these organelles cease moving in the presence of elevated free Ca^{2+} ion concentrations, heat or *N*-ethyl maleimide (NEM), which are all known to inhibit proteins, such as myosin (Kohno and Shimmen, 1988a). Elevated free Ca^{2+} ion concentrations (>10 μM) can also cause the induction of fragmentation of the actin cables as well as inhibit myosin motor function (Kohno and Shimmen, 1988a,b). Likewise, microfilaments have been localized in a longitudinal fashion with rhodamine-conjugated phalloidin in various pollen tubes (Perdue and Parthasarathy, 1985; Pierson et al., 1986; Steer and Steer, 1989; Cresti and Tiezzi, 1990; Emons et al., 1991; Tiezzi, 1991; Pierson and Cresti, 1992; see review by Mascarenhas, 1993); and with electron and fluorescence microscopy in *Nicotiana alata* (Lancelle et al., 1987) and *Lilium longiflorum* (Lancelle and Hepler, 1992).

Despite our understanding of the structure, localization and presumed function of actin, we know much less about the motor molecule myosin in pollen tubes. A few plant myosins have been isolated biochemically (Kato and Tonomura, 1977; Ohsuka and Inoue, 1979; Vahey et al., 1982; Ma and Yen, 1989;

Yokota and Shimmen, 1994), immunolocalized (Tang et al., 1989b; Heslop-Harrison and Heslop-Harrison, 1989b; Qiao et al., 1989; La Claire II, 1991; Liebe and Quader, 1994) or determined through sequence analysis (Knight and Kendrick-Jones, 1993; Moepps et al., 1993; Kinkema and Schiefelbein, 1994; Kinkema et al., 1994). Using antibodies to myosin II, a 175 kDa protein in *Nicotiana alata* has been identified that localized as large punctate spheres thought to represent organelles (Tang et al., 1989b) and organelle localization was reported in the grass families *Alopecurus pratensis* and *Secale cereale* with another myosin II antibody (Heslop-Harrison and Heslop-Harrison, 1989b). Tang et al. (1989b) revealed with epitope mapping that myosin II antibodies to the head and tail regions of the protein both localized to the organelles, whereas only the head antibody could localize to the surface of the male germ unit.

In recent years, our knowledge of myosin has expanded greatly. Whereas there were originally two myosins, labeled I and II, several new classes have now been identified (Warrick and Spudich, 1987; Korn and Hammer, 1988; Cheney and Mooseker, 1992; Cheney et al., 1993b; Hammer, 1994). Of the ten classes of myosins now known, only a few have been studied biochemically or localized. Of particular interest, some of these myosins show specific cellular localizations; for instance, myosins I are found at the leading edge of moving cells (Fukui et al., 1989; Yonemura and Pollard, 1992) and are associated with cellular membranes (Miyata et al., 1989; Adams and Pollard, 1986; Hayden et al., 1990; Zot et al., 1992) whereas myosin II is localized in the tails of locomoting cells (Fukui et al., 1989) and myosin V tends to be associated with vesicles in chicken brain (Espindola et al., 1992; Espreafico et al., 1992) and yeast (Lillie and Brown, 1994). In addition, the phenotype of the yeast MYO2 (Johnston et al., 1991; Govindan et al., 1995) and mouse *dilute* mutants (Mercer et al., 1991) are consistent with vesicle transport defects.

Taking advantage of this new information, and in particular of specific antibodies to some of these myosins, we have probed pollen tubes to determine if these proteins are present and where they are localized. In addition, we have employed several preparative techniques to provide faithful immunolocalization. Briefly, we find that myosin I is localized on the nuclei, on larger organelles and the plasma membrane, myosin II on larger organelles, whereas myosin V is only on smaller organelles.

MATERIALS AND METHODS

Protein extraction

Method 1

Lilium longiflorum cv Nellie White pollen was hydrated in a moist chamber followed by germination in pollen culture medium containing 1.6 mM boric acid, 15 mM 2-[*N*-morpholino]ethanesulfonic acid (MES), 1 mM $CaCl_2$, 1 mM KCl and 10% sucrose, pH 5.5. The *Nicotiana alata* cv beltsville pollen was cultured as above except 2% polyethylene glycol (PEG-400) was used instead of sucrose. The pollen was rotated at room temperature until approximately 200-400 μm in length, collected and the proteins were solubilized by homogenization with a dounce homogenizer and extracted in high ionic strength buffer containing 0.5 M KCl, 15 mM Tris-HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT), including 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg/ml tosyl-*L*-arginine methyl ester (TAME), 10 μg/ml antipain, 10 μg/ml chymostatin A, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml *N*-benzoyl-*L*-arginine ethyl ester hydrochloride (BAEE), 244 μM benazamidine and 10 μg/ml *L*-tosylamide-2-phenyl-ethylchloromethyl ketone (TPCK) as proteolytic enzyme inhibitors for 30 minutes at 4°C. The proteins were precipitated with 3 volumes of acetone and resuspended in SDS gel sample buffer (Laemmli, 1970). Protein concentration was estimated by the Lowry assay (Lowry et al., 1951) to provide equal loading of samples.

Table 1.

1° Antibody	Dilution	Contributor
Polyclonal anti-myosin IA	1:2,000	John Hammer III (NIH)
Polyclonal anti-myosin IB	1:5,000	John Hammer III (NIH)
Polyclonal anti-myosin V	1:1,000	Mark Mooseker (Yale University)
Monoclonal anti-actin	1:1,000	Richard Cyr (Pennsylvania State University)
Monoclonal anti-LMM	1:1,000	Amersham Life

Table 2.

1° Antibodies	Dilution	Reference
Polyclonal anti-myosin IB	1:400	Baines et al., 1992
Polyclonal anti-myosin V	1:200	Espindola et al., 1992
Monoclonal anti-actin	1:200	Andersland et al., 1994
Monoclonal myosin II (S1)	1:10	Tang et al., 1989b
Monoclonal myosin II (LMM)	1:10	Tang et al., 1989b
Dictyostelium myosin II (LMM)	1:100	DeLozanne et al. (in preparation)
2° Antibodies	**Dilution**	**Supplier**
Bodipy goat anti-rabbit IgG	1:200	Molecular Probes, Eugene, OR
Texas Red goat anti-mouse IgG	1:200	Molecular Probes, Eugene, OR
Monoclonal goat anti-mouse IgM (biotin conjugate)	1:50	Sigma Chemical Co.
3° Antibody	**Dilution**	**Supplier**
Streptavidin-Cy3	1:50	Jackson ImmunoResearch Laboratories

Table 3. Competition studies

Antibody	Antibody concentration	Protein concentration	Effectiveness
Myosin IB	~2 μg	2.5, 5 μg	2.5 μg +
Myosin V	0.0066 μg	0.2, 0.4, 0.6, 1.0 μg	0.2 μg +
S1	0.01 μg	0.03, 0.06 μg	0.06 μg
LMM	0.01 μg	0.03, 0.06 μg	0.06 μg
LMM (*Dictyostelium*)	~2 μg	6 μg	6 μg

SDS-PAGE gels and immunoblots

The extracted proteins were electrophoresed on 7.5% or 10% SDS-PAGE slab gels (Laemmli, 1970) and transferred electrophoretically (Towbin et al., 1979) to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA). The PVDF was blocked with 5% blotto (non-fat dry milk) in Tris-buffered saline (TBS) before the addition of primary antibody diluted appropriately in 1% bovine serum albumin (BSA) in TBS-0.05% Tween-20 (Table 1). The secondary antibody, alkaline phosphatase conjugated goat anti-rabbit IgG or goat anti-mouse IgG and IgM, was added. The blot was developed with the NBT/BCIP development system by Pierce.

Western blot competition experiments

The extracted and standard proteins were prepared for immunoblot analyses as above. A mixture of the myosin V protein (2 μg myosin

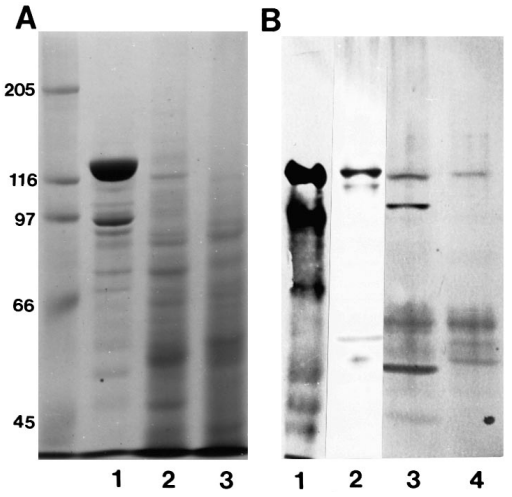

Fig. 1. Myosin IA and IB immunoblot analysis. (A) SDS-PAGE gel of *Acanthamoeba* myosin IB (kindly provided by John Hammer III) (lane 1); *L. longiflorum* extract (lane 2) and *Nicotiana* extract (lane 3). (B) *Acanthamoeba* myosin IA antibody recognized a band about 125 kDa in *L. longiflorum* (lane 2). The polyclonal antibody to *Acanthamoeba* myosin IB recognized *Acanthamoeba* myosin IB (lane 1) and a distinct band at approximately 125 kDa representing a cross-reacting polypeptide in *L. longiflorum* and *N. alata* pollen tube extracts (lanes 3 and 4, respectively) as well as a degradation band at about 97 kDa which is also found in the *Acanthamoeba* myosin IB protein standard used.

V, courtesy of Mark Mooseker, Yale University) and the anti-myosin V antibody (0.33 μg antibody) was prepared and used for immunoblot analysis. The control immunoblot contained only anti-myosin V antibody at the same dilution. A similar competition assay was performed with myosin IB (data not shown) in which a mixture of myosin IB protein (10 μg myosin IB, courtesy of John Hammer, NIH) and anti-myosin IB antibody, (approximately 4-10 μg from whole serum antibody) was prepared. An immunoblot assay was performed with this mixture and decreased labeling of the myosin IB protein (0.6 μg) and a disappearance of the bands in the pollen extracts was observed. Staining of the myosin I band did not decrease in the control immunoblot with the anti-myosin IB antibody alone.

Immunofluorescence assays for *Lilium longiflorum* pollen tubes

Chemically fixed preparation

Hydrated *L. longiflorum* pollen was grown at approximately 3 mg pollen/ml lily pollen culture medium. The tubes were fixed for 1 hour at room temperature with 4% formaldehyde in 50 mM Pipes buffer containing 1 mM $MgSO_4$ and 5 mM EDTA (PME). After washing, the pollen tubes were air dried onto coverslips coated with 1 mg/ml poly-L-lysine. Following air drying, the pollen was digested for 7 minutes with 0.75% macerozyme (Yakult Honsha Co. Ltd, Tokyo, Japan) and 0.75% cellulase (Sigma Chemical Co.) in PME buffer. The pollen tubes were then treated with 0.5% Triton X-100 for 30 min. followed by a –20°C methanol treatment for 10 minutes. The pollen was preblocked with 5% BSA in phosphate buffered saline (PBS; 138 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4 and K_2HPO_4 mixture, pH 7.4; Sigma Chemical Co.) for approximately 1-2 hours, then incubated in the appropriately diluted 1° antibody overnight at room temperature. The pollen was washed with PBS and incubated with 2° antibody for 2 hours at room temperature in the dark and, if needed, 3° antibody for 2 hours (Table 2), then mounted in antifade (90% glycerol, 0.1 M Tris, pH 9.5, 1 mg/ml *p*-phenylenediamine, 1 μg/ml Hoechst 33342 dye (Sigma Chemical Co.) or Vectashield (Vector Laboratories, Inc., Burlingame, CA).

The images were taken on a confocal laser scanning microscope, (Bio-Rad MRC 600) and processed for photography on the Silicon Graphics Iris computer system (SGI).

Isolation of generative cells and vegetative nuclei

Pollen tubes grown in lily pollen culture medium were exchanged for culture medium lacking calcium and sucrose and subsequently dounced until the generative cells and vegetative nuclei were released. The preparation was then fixed in 4% formaldehyde to preserve the overall shape of the generative cells and vegetative nuclei. The cells and nuclei were then air dried onto a coverslip and stained as in the chemically fixed material.

Rapid freeze fixation and freeze substitution followed by rehydration

The pollen tubes were rapidly frozen as described by Lancelle et al. (1986), and placed in 4% formaldehyde in absolute ethanol and substituted for 36 hours. The tubes were removed and rehydrated as described by Raudaskoski et al. (1991). Since the freezing and substitution take place on a loop, the loop was placed in a vial and the pollen tubes on them were treated with 0.75% macerozyme and 0.75%

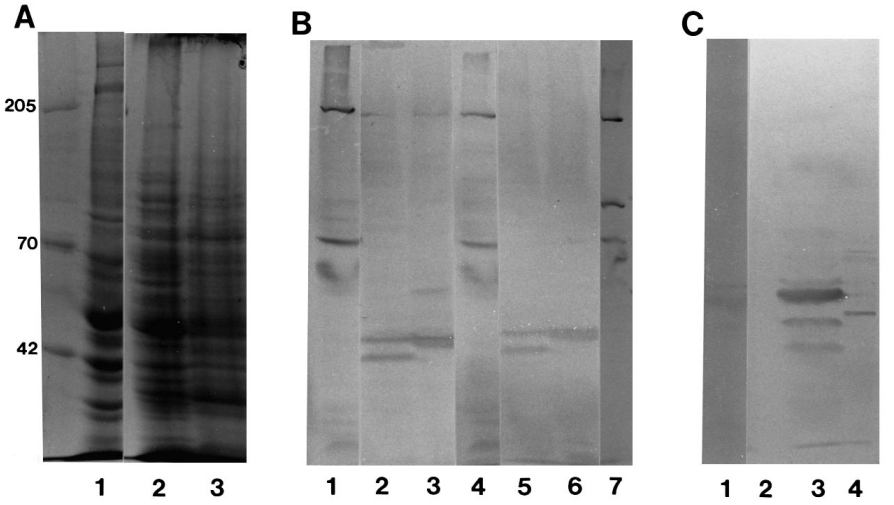

Fig. 2. Myosin V immunoblot analysis. (A) 7.5% SDS-PAGE gel of crude chicken brain extract (lane 1). Lanes 2 and 3 were loaded with *L. longiflorum* and *N. alata* extracts, respectively. (B) Affinity purified myosin V antibody from chicken brain recognizes chicken brain myosin V at about 190 kDa, as well as its proteolytic fragments in chicken brain at about 80 and 65 kDa (lanes 1, 4 and 7). Intact myosin V at approximately 190 kDa in *L. longiflorum* and *N. alata* are shown in lanes 2 and 3, respectively. Under the competition assay conditions where myosin V protein was added to myosin V affinity purified antibody, intact myosin V bands were reduced in the chicken brain standard myosin V (Fig. 2B, lane 4) and disappeared in the pollen extracts (Fig. 2B, lanes 5 and 6). (C) Non-immune rabbit IgG antibody did not react with myosin V (lane 1) or myosin I (lane 2) proteins. Non-specifically staining lower molecular mass bands were observed in *L. longiflorum* and *N. alata* pollen extracts at about 50 and 45 kDa (lanes 3 and 4).

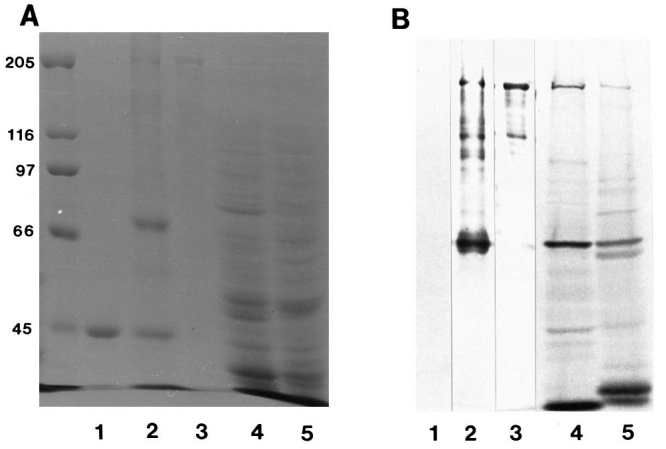

Fig. 3. Myosin II (LMM) immunoblot analysis. (A) 7.5% SDS-PAGE gel was loaded with rabbit skeletal muscle actin (lane 1), standard mixture, containing cardiac myosin II, myosin subfragment 1 (S1), subfragment 2 (S2), LMM and actin (lane 2), rabbit skeletal muscle myosin heavy chain (lane 3), *L. longiflorum* pollen extract (lane 4) and *N. alata* pollen extract (lane 5). (B) Immunoblot analysis with antibody to myosin II LMM did not stain rabbit skeletal muscle actin (lane 1). The LMM antibody did bind to cardiac myosin II and LMM in the standard mixture (lane 2) and rabbit skeletal muscle myosin heavy chain (lane 3). In the *L. longiflorum* and *N. alata* extracts, the LMM antibody recognized intact myosin II (205 kDa band) and a breakdown product at 70 kDa (lanes 4 and 5, respectively).

cellulase followed by Triton X-100 treatment and incubation in antibodies diluted as above.

Rapid freeze fixation and freeze substitution followed by embeddment in methacrylate

The pollen tubes were rapidly frozen and substituted as described by Lancelle et al. (1986), and embedded in methacrylate according to the method of Baskin et al. (1992) except that the stem of the

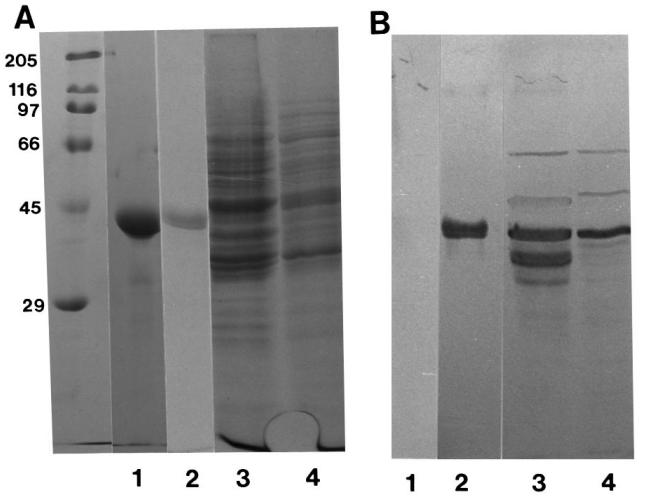

Fig. 4. Actin immunoblot analysis. (A) 10% SDS-PAGE analysis of rabbit skeletal muscle actin (lane 1), maize pollen actin (lane 2), and extracts of *L. longiflorum* and *N. alata* (lanes 3 and 4, respectively). (B) Immunoblot analysis indicated that the antibody made to stabilized F-actin filaments from pea did not recognize rabbit skeletal muscle actin (lane 1) but did recognize maize, *L. longiflorum* and *N. alata* actin (lanes 2, 3, and 4, respectively)

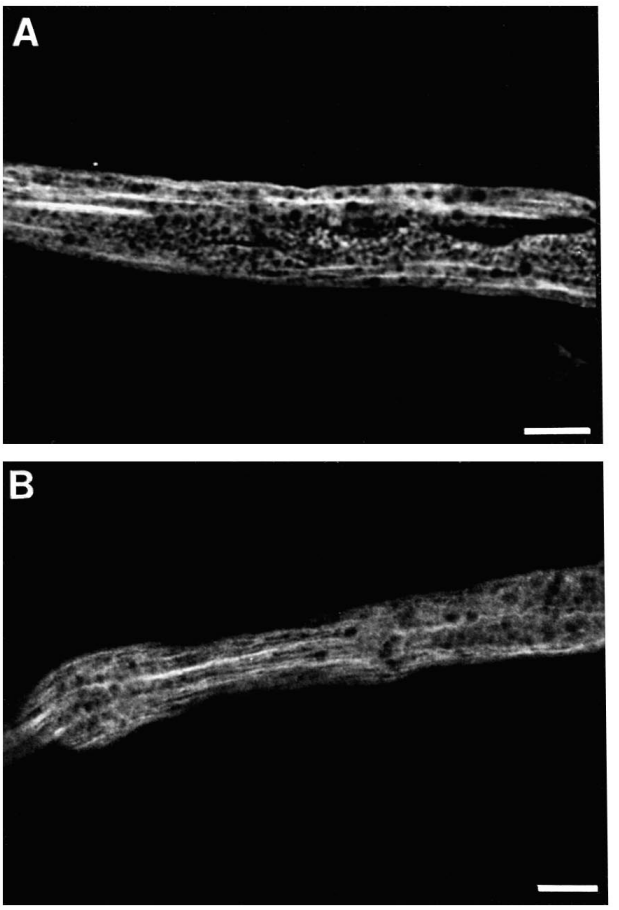

Fig. 5. Actin localization in pollen tubes of *L. longiflorum* pollen tubes. Long, straight actin filaments are more consistently seen in RF-FS pollen tubes (A), as shown in a pollen tube embedded in methacrylate and sectioned, than observed in chemically fixed pollen tubes of *L. longiflorum* albeit they can be seen occasionally (B). Bars, 10 μm.

loop was removed and the loop was placed into a beem capsule. Sections, 4 μm, were cut dry, placed on small drops of water and affixed to silane-coated slides by heating briefly on a slide warmer (2 minutes) at 60°C. The embedding medium was removed from the sections by incubation in acetone for 22 minutes, followed by rehydration in PBS. The sections were blocked with 1% BSA and then incubated in primary antibody (actin, myosin IA, myosin IB, myosin II, myosin V, nonimmune IgG) appropriately diluted (1:10 to 1:1,000) for 2 hours at 37°C, and, after rinsing in PBS, secondary antibody (either anti-mouse IgG or anti-rabbit IgG Cy3 at 1:200 dilution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) incubation occurred for 2 hours at 37°C. The sections were rinsed in PBS, mounted in Vectashield (Vector Laboratories, Burlingame, CA) and observed on a confocal laser scanning microscope (Bio-Rad MRC 600). With the antibodies to myosin II S1 or LMM fragments, a biotinylated anti-mouse IgM secondary diluted 1:50 was followed by streptavidin Cy3 diluted 1:50.

Competition studies

Competition assays were performed as above except that the primary antibody consisted of a mixture of the antibody and the corresponding protein as shown in Table 3. The S1 and LMM proteins utilized for the competition studies were purchased from Sigma Chemical Co. The images were recorded with T-MAX 100 film.

Quantitation of organelle size

The fluorescent spheres in the figures labeled with the different classes of myosins were measured with a micrometer bar. Twenty to thirty measurements were taken for each image and averaged. Determination of the variation in organelle size was analyzed by Tukey's Studentized Range Test.

RESULTS

Immunoblot analysis of actin and of different classes of myosins in pollen tube extracts of *Lilium longiflorum* and *Nicotiana alata*

SDS-PAGE and immunoblot analyses of *L. longiflorum* and *N. alata* pollen tube protein extracts are shown in Figs 1, 2, 3 and 4. A 7.5% gel (Fig. 1A) was loaded with *Acanthamoeba* myosin IB (lane 1), *L. longiflorum* extract (lane 2) and *N. alata* extract (lane 3). These proteins were transferred to PVDF and immunoblotted with polyclonal antibodies to *Acanthamoeba* myosins IA and IB (Fig. 1B). Immunoblot analyses revealed the presence of an approximately 125 kDa band representing a pollen myosin I with *Acanthamoeba* myosin IA antibody probed against *L. longiflorum* extract (Fig. 1B, lane 2) and with *Acanthamoeba* myosin IB antibody probed against *Acanthamoeba* myosin IB protein, *L. longiflorum* extract, and *N. alata* extract (Fig. 1B, lanes 1, 3 and 4, respectively). In the pollen extract of *L. longiflorum* (Fig. 1B, lane 3), a proteolytic fragment at about 97 kDa was observed and a similar band was

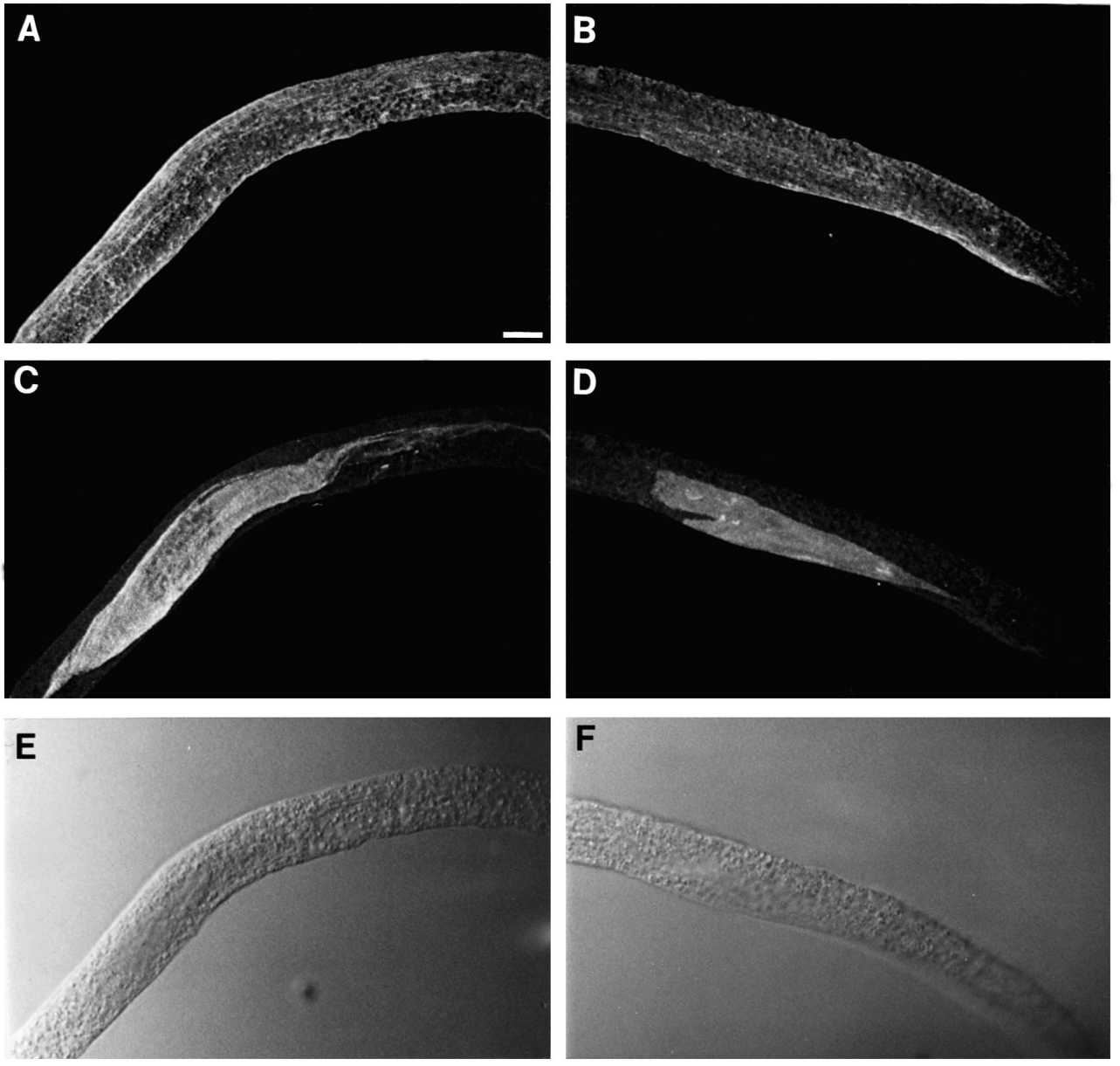

Fig. 6. Immunofluorescence microscopy with myosin IB and actin. Double labeling experiments with myosin IB and actin show distinct actin cables located longitudinally along the axis of the pollen tube (A,B), whereas the myosin IB is located on the surfaces of the generative cell (C) and the vegetative nucleus (D) of the pollen tube. Bright field transmission images of the same pollen tube in A,C and B,D can be seen in E and F, respectively. Bar, 10 μm.

known to be present in the *Acanthamoeba* myosin IB protein provided (John Hammer, personal communication) (Fig. 1B, lane 1). In a competition immunoblot assay, the intact *Acanthamoeba* myosin IB band was reduced in the presence of *Acanthamoeba* myosin IB protein and the bands in the pollen extracts disappeared (data not shown). The 97 kDa band in *N. alata* did not disappear. Since an equal amount of *Acanthamoeba* myosin IB protein was added instead of an excess, this band may represent a higher affinity binding form of myosin I in *N. alata* pollen or a non-specific cross-reaction. Insufficient *Acanthamoeba* myosin IB protein did not allow for further elaboration.

Myosin V immunoblot analysis showed an intact band at about 190 kDa representing a plant homologue (Fig. 2). 7.5% SDS-PAGE (Fig. 2A) shows crude chicken brain extract (Fig. 2A, lane 1), and *L. longiflorum* and *N. alata* extracts (Fig. 2A, lanes 2 and 3, respectively). Immunoblot analysis indicated the presence of intact myosin V in chicken brain extract, *L. longiflorum* and *N. alata* extracts (Fig. 2B, lanes 1, 2 and 3, respectively). In a competition study, intact myosin V bands were reduced in the chicken brain standard myosin V and disappeared in the pollen extracts in the presence of a mixture of the anti-myosin V antibody and myosin V protein (Fig. 2B, lanes 4, 5 and 6, respectively). This demonstrated that the polypeptide in the plant extracts was specific for a myosin V since the anti-myosin V antibody was made to a unique region of the tail portion of this protein (Espreafico et al., 1992). Lower molecular mass bands in pollen were frequently observed at approximately

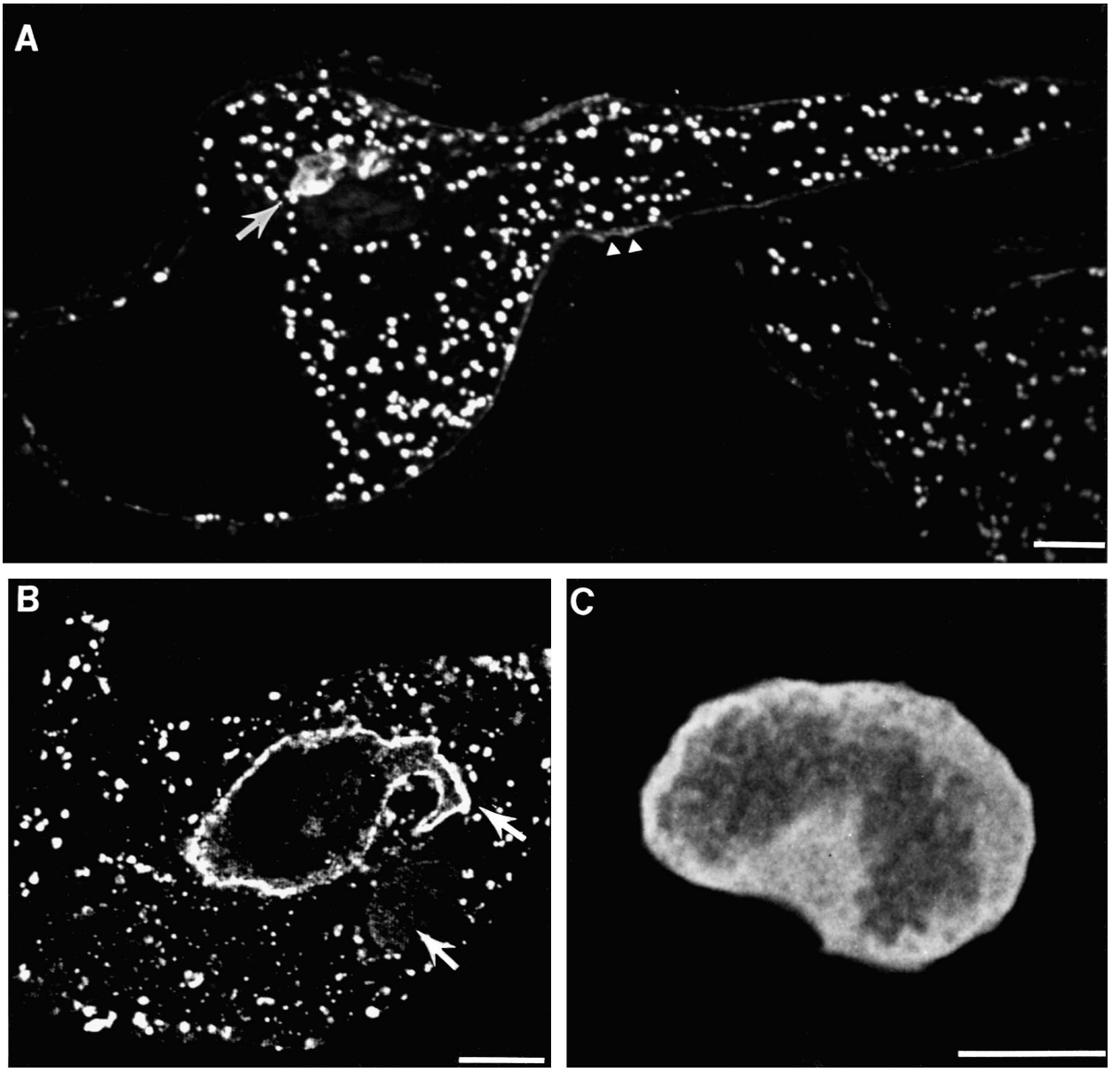

Fig. 7. Immunolocalization of myosin IA and IB antibodies in *L. longiflorum* pollen tubes. (A) Myosin IA antibody labeled the surface of the generative cell (arrow) as well as localized to the plasma membrane of the vegetative cell and the surfaces of organelles. Myosin IB antibody also localized to the same areas as myosin IA (B). The myosin IB antibody was observed at the surface of the generative cell and vegetative nucleus (B, arrows) of sectioned material. (C) Depiction of an isolated generative cell labeled with myosin IB antibody. Bars, 10 μm.

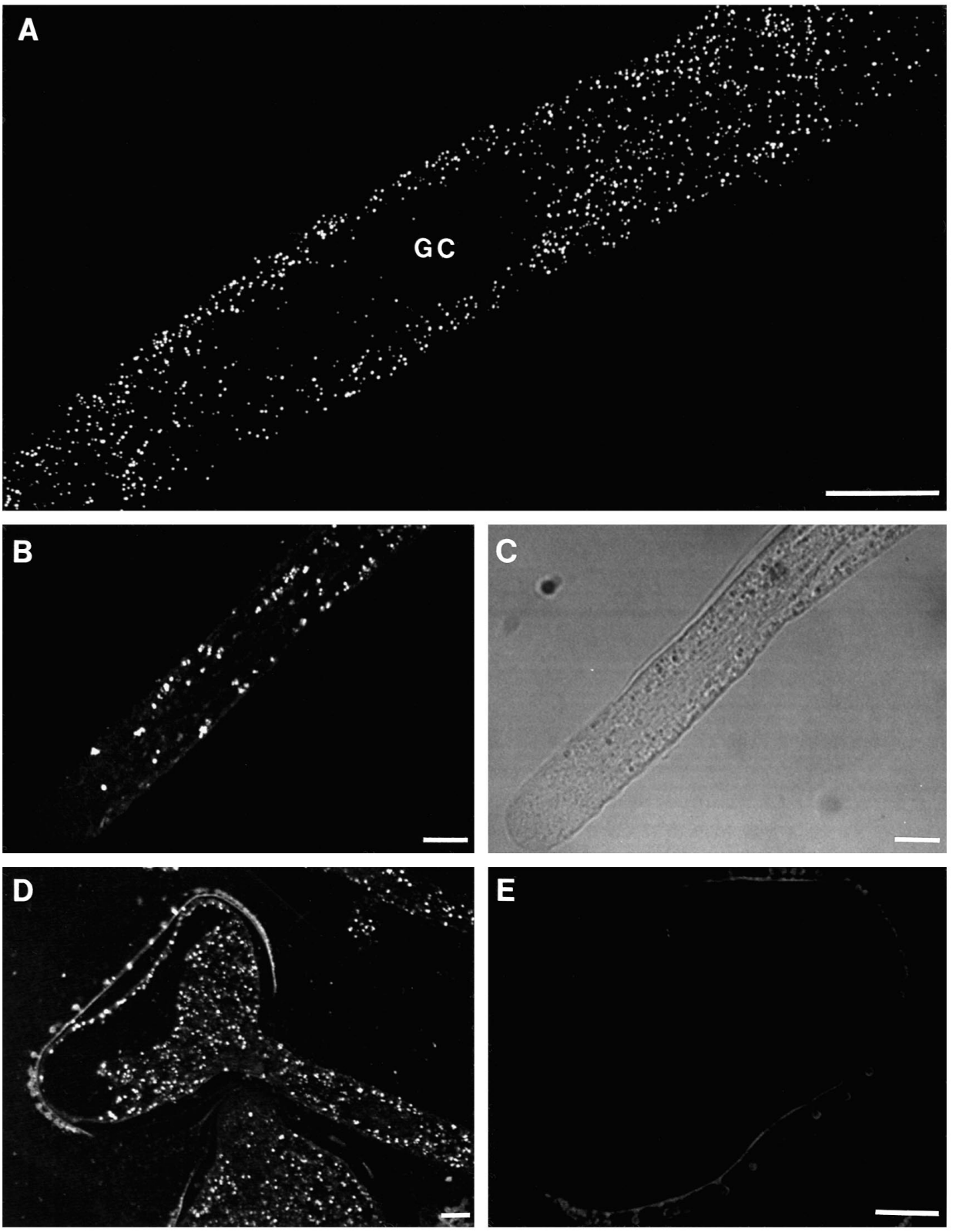

Fig. 8. Myosin V localization in pollen tubes of *L. longiflorum*. Myosin V antibody to the tail region of the protein labels only in a punctate fashion throughout the vegetative cytoplasm (A,B,D). Two different preparations, RF-FS followed by rehydration (A) and RF-FS followed by embeddment in methacrylate (B,D) indicate that the myosin V is associated with organelles. Rows of punctate dots are observed (B), indicating that the myosin runs along tracks of actin. The bright field transmission image in (C) depicts the zonation of the cells after RF-FS and embeddment in methacrylate. Non-immune rabbit IgG does not give a perceptible signal (E) but the autofluorescence of the wall can be observed. Bars, 10 μm.

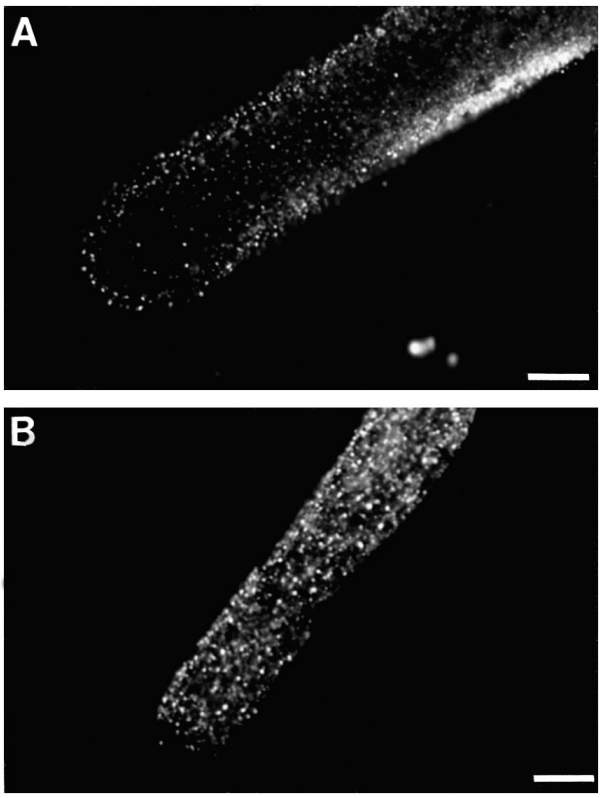

Fig. 9. Myosin II subfragment 1 (S1) and light meromyosin (LMM) localization in *L. longiflorum* pollen tubes. (A) A punctate pattern was observed throughout a chemically fixed specimen of *L. longiflorum* pollen with S1 antibody and a similar pattern was seen with LMM antibody (B). Bars, 10 μm.

80 kDa and 65 kDa (data not shown), which match the proteolytic fragments of the crude chicken brain standards used (Fig. 2B, lane 7). The 80 kDa band correlates with a distinctive calpain cleavage site found in myosin V that is not found in myosin II (Espindola et al., 1992). Observing intact myosin heavy chain proteins in plants is difficult especially since it is well known that many active proteases exist, especially within the vacuole, and therefore proteolytic degradation is a major problem in the isolation of intact plant proteins.

Non-immune rabbit IgG was also tested on myosin IB, myosin V, and *L. longiflorum* and *N. alata* extracts to determine if there was any nonspecific binding (Fig. 2C, lanes 1-4, respectively). The non-immune IgG did not recognize any bands in the isolated myosin proteins (Fig. 2C, lanes 1, 2) but did recognize only the lowest molecular mass bands (Fig. 2C, lane 3, 4) observed here and in the blots of both myosins I (Fig. 1B, lanes 2,3,4) and V (Fig. 2B, lanes 2, 3, 5, 6). The lower molecular mass bands that did not disappear in the competition study previously mentioned are the same as those found in the non-immune rabbit IgG control (Fig. 2B, lanes 5 and 6).

Myosin II antibody to the 70 kDa light meromyosin fragment (LMM) was immunoblotted and shown in Fig. 3. A 7.5% SDS-PAGE gel electrophoresis was performed with rabbit skeletal muscle actin (Fig. 3A, lane 1), standard mixture containing cardiac myosin II, myosin subfragment 1 (S1), subfragment 2 (S2), LMM and actin (Fig. 3A, lane 2), rabbit skeletal muscle myosin heavy chain (Fig. 3A, lane 3), *L. longiflorum* pollen extract (Fig. 3A, lane 4) and *N. alata* pollen extract (Fig. 3A, lane 5). Myosin II LMM antibody immunoblot analysis indicated that the LMM antibody did not react with actin (Fig. 3B, lane 1) but did bind to the intact myosin II and LMM fragment in the standard mixture (Fig. 3B, lane 2) and skeletal muscle myosin heavy chain (Fig. 3B, lane 3). The LMM antibody reacted with a 205 kDa peptide in the *L. longiflorum* and *N. alata* pollen extracts (Fig. 3B, lane 4 and 5, respectively) as well as a breakdown product at 70 kDa in both extracts.

A 10% SDS-PAGE gel electrophoresis (Fig. 4A) was used to separate rabbit skeletal muscle actin (lane 1), carboxyfluorescein-labeled maize pollen actin (lane 2), and extracts of *L. longiflorum* and *N. alata* (lanes 3 and 4, respectively). Immunoblot analysis with the pea anti-actin antibody did not crossreact with the animal actin (Fig. 4B, lane 1) but did stain a 42 kDa band in isolated maize pollen actin (Fig. 4B, lane 2), and *L. longiflorum* and *N. alata* pollen extracts (Fig. 4B, lanes 3 and 4, respectively) as well as degradation products and occasionally two higher molecular mass bands of unknown proteins.

Localization of actin in pollen tubes utilizing different methods of preservation

Three methods have been employed to preserve the cells for immunofluorescence in this study: (a) chemical fixation of whole pollen tubes; (b) rapid freeze fixation and freeze substitution (RF-FS) of whole pollen tubes followed by rehydration; and (c) RF-FS followed by embedment in a methacrylate mixture that was sectioned to 4 μm. Even though chemically fixed pollen tubes showed a staining pattern similar to that of the RF-FS material, the RF-FS material showed additional information and the staining pattern was more consistent throughout the experiments with all the antibodies used. The actin cables in the RF-FS and embedded material were straighter, finer and observed more frequently (Fig. 5A) than in the chemically fixed material of *L. longiflorum*, although an occasional chemical preparation could give a similar result (Fig. 5B).

Myosin I localizes to the generative cell while myosins II and V localize to the organelles

Chemically fixed pollen tubes were doubly labeled with antibodies to pea phalloidin-stablilized actin filaments and *Acanthamoeba* myosin IB (Fig. 6). The images in Fig. 6 are within the same pollen tube but at slightly different regions. The actin cables appear as filaments that run along the tube in a longitudinal fashion (Fig. 6A,B), whereas the myosin IB localizes to the surface of the generative cell (Fig. 6C) and the vegetative nucleus (Fig. 6D). Bright field transmission images of the pollen tube can be seen in Fig. 6E and F. The myosin IA and IB antibody staining pattern on the surface of the generative cell and vegetative nucleus was still observed in the RF-FS material (Fig. 7A, arrow), but, in addition, one could see labeling of the plasma membrane of the vegetative cell (Fig. 7A, triangles), large punctate dots possibly representing larger organelles (Fig. 7A,B).

To ensure that the myosin I antibody staining was specific, generative cells and vegetative nuclei were isolated and labeled with myosin IB or myosin V antibodies. As can be seen in Fig. 7C, the surface of an isolated generative cell is stained with myosin IB, while the condensed chromosomes appear as a dark

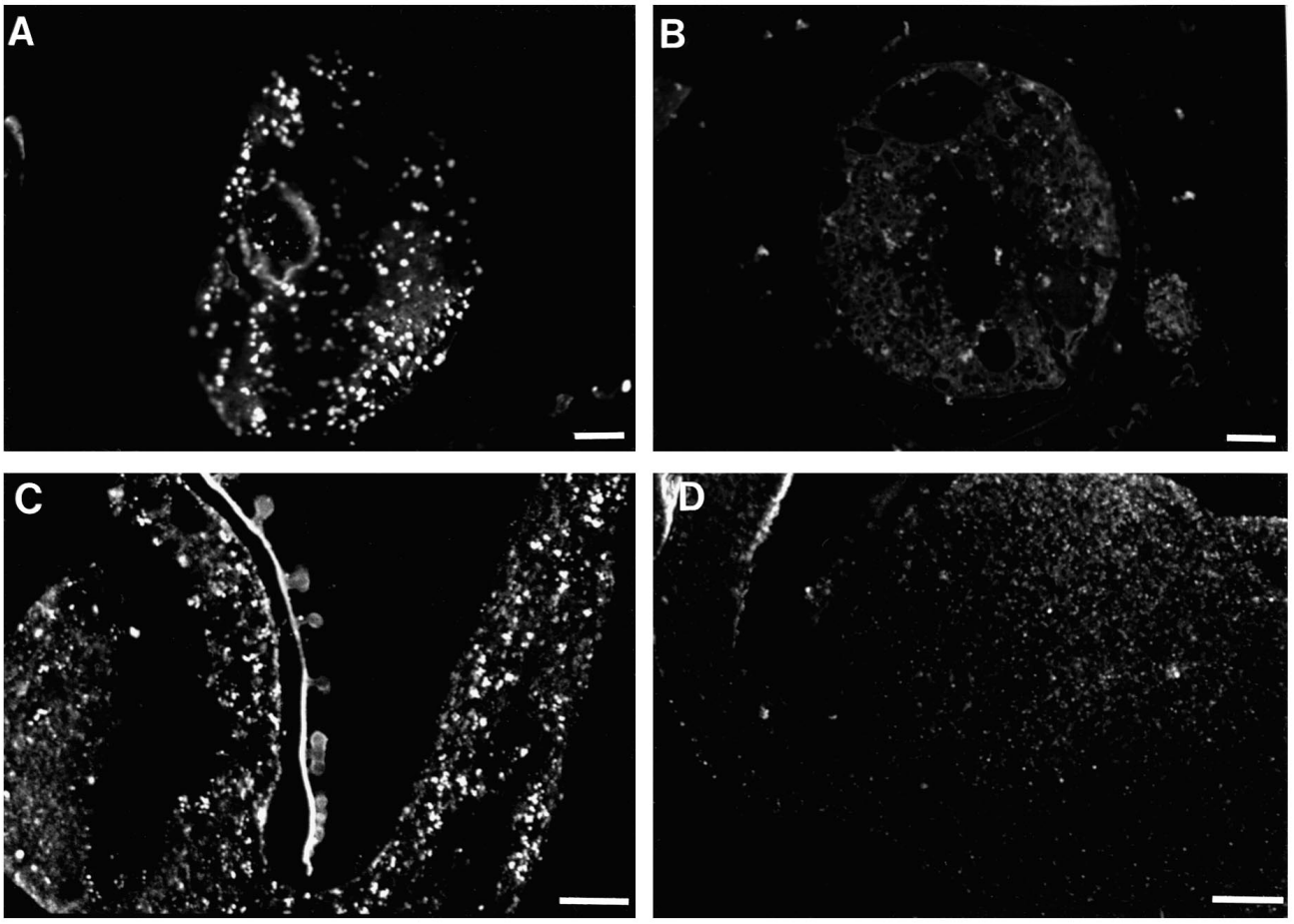

Fig. 10. Immunofluorescence competition studies performed with myosin IB and myosin V on *L. longiflorum* pollen. (A) *L. longiflorum* pollen labeled with myosin IB antibody had a punctate staining pattern while a mixture of myosin IB antibody (~2 μg) and myosin IB protein (5 μg) indicate a diminished staining pattern (B). Myosin V antibody also stained in a punctate fashion (C) but the addition of a myosin V antibody (0.0066 μg) and myosin V protein (0.2 μg) mixture decreased the punctate staining pattern (D). Bars, 10 μm.

crescent within the generative cell. The organelles in this chemically fixed preparation did not stain with the myosin IB antibody but did stain faintly with the myosin V antibody (data not shown), whereas the surface of the generative cell or vegetative nucleus was not stained with the myosin V antibody (data not shown).

Even though the myosin V antibody occasionally gave a punctate staining pattern in the chemically fixed material, it was not as clear as either the actin or myosin IB antibodies. Because the pollen tubes are treated with methanol and Triton X-100 some of the soluble proteins or those possibly associated with organelles could be lost. To ensure better preservation the cells were rapidly frozen. In Fig. 8A, myosin V antibody in RF-FS *L. longiflorum* pollen tubes that had been rehydrated, appeared punctate throughout the vegetative cytoplasm except where the generative cell was located (GC). The generative cell was observed with the DNA stain, Hoechst 33342, with 40× conventional microscopy to verify that indeed this dark region was the generative cell.

An alternative method involved RF-FS followed by embeddment in a methacrylate mixture and serial sectioning. Removal of the methacrylate by acetone treatment permitted staining with antibodies to actin, myosin IA, IB, myosin II or myosin V. The staining pattern was again punctate with the myosin V (Fig. 8B,D) with no staining of the generative cell, vegetative nucleus or the plasma membrane. Unfortunately, the treatment with acetone appears to cause the removal of many organelles. However, in Fig. 8B and Fig. 12C,D, one can see that the punctate staining pattern appears in rows, possibly indicating a rigor complex between myosin and actin that is not susceptible to extraction with the acetone. We speculate that the myosin, attached to organelles, is traveling along tracks of actin. The phase image of this cell is shown in Fig. 8C. Immunofluorescence labeling with a rabbit non-immune IgG did not show any staining whatsoever (Fig. 8E), but indicated the level of autofluorescence within the wall of the pollen grain.

Punctate staining of presumed organelles was observed with antibodies to myosin II subfragment I (S1) (Fig. 9A) and myosin II light meromyosin (LMM) (Fig. 9B) in chemically fixed *L. longiflorum* pollen. This punctate labeling is similar to that observed by Tang et al. (1989) although the staining at the surface of the generative cell and vegetative nucleus was not observed. Upon review, we realize that the staining of the generative cell and vegetative nucleus was not observed often in *N. alata* as believed previously. The new technology (RF-FS embedded material and confocal imaging) presented in this

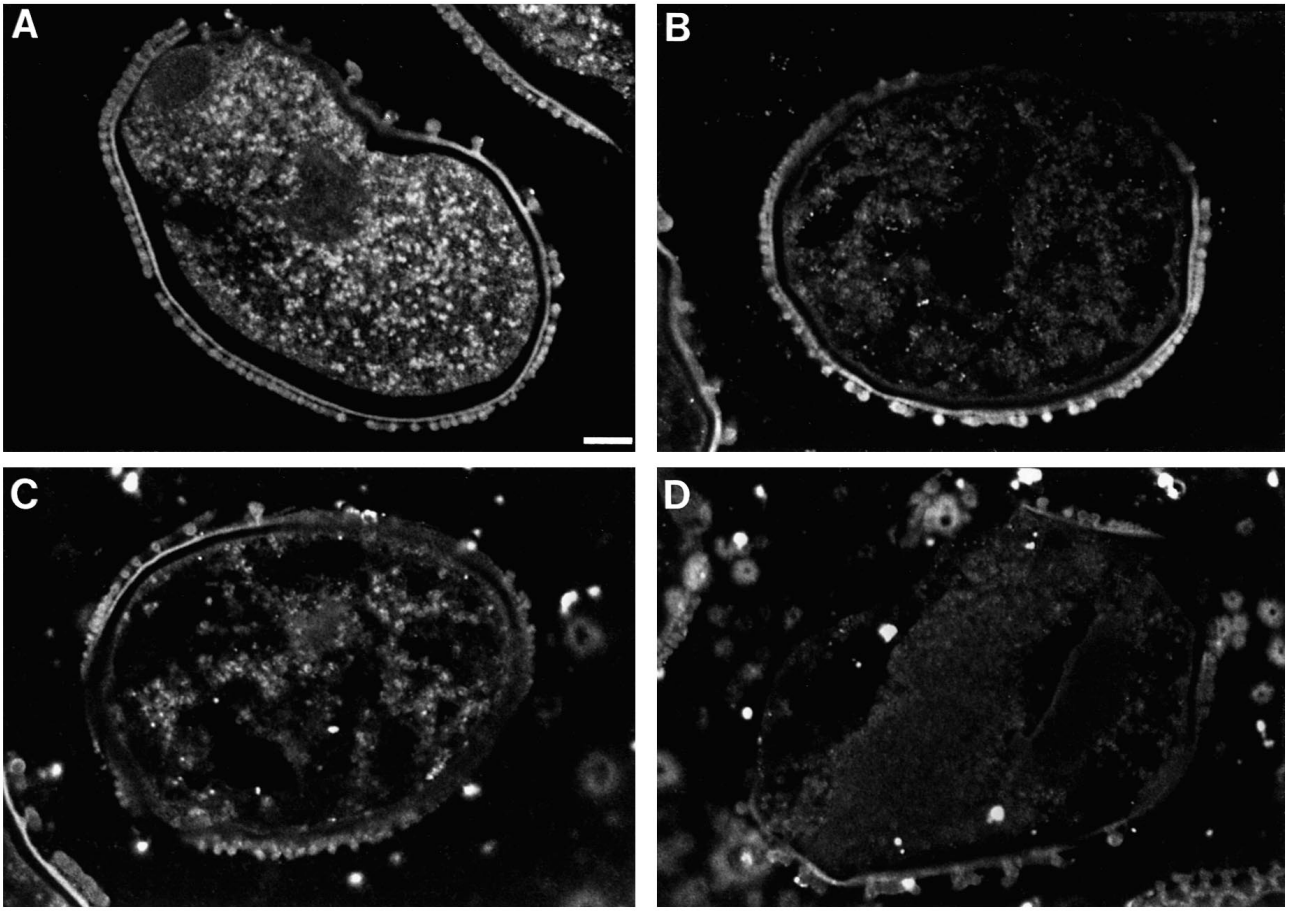

Fig. 11. Immunofluorescence competition studies performed on *L. longiflorum* pollen with myosin II antibodies. (A) S1 antibody (0.01 μg) and S1 protein (0.03 μg) mixture added to *L. longiflorum* pollen still contained a punctate staining pattern when the concentration of S1 was not sufficient to cause complete competition. Increased concentration of S1 protein (0.06 μg) in the S1 antibody/protein mixture allowed adequate competition to occur (B). LMM antibody (0.01 μg) and LMM protein (0.03 μg) mixture showed a diminished staining pattern (C) whereas increasing the concentration of LMM protein (0.06 μg) in the antibody mixture decreased the staining even more (D). Bar, 10 μm.

study has allowed us to clarify more precisely the location of the S1 and LMM antibodies on the organelles of *L. longiflorum* and *N. alata* along with the new classes of myosins.

Competition studies on immunofluorescent assays

Competition studies were performed to elucidate the specificity of the heterologous myosin antibodies used in this study. In Fig. 10A, *L. longiflorum* pollen was treated with myosin IB antibody, while in Fig. 10B the pollen was treated with a mixture of myosin IB protein and myosin IB antibody. The staining diminishes significantly in the competition assay. A competition assay with myosin V antibody (Fig. 10C) and myosin V protein and myosin V antibody (Fig. 10D) also indicate diminished staining.

Presented in Fig. 11 are the myosin II competition studies with antibodies to the head and tail region of chicken skeletal muscle myosin II or an antibody from *Dictyostelium*. In Fig. 11A, the S1 antibody along with the S1 protein (Sigma Chemical Co.) were added to the pollen; loss of staining did not occur at this protein concentration. Addition of more S1 protein to the antibody resulted in the reduction of the punctate staining pattern (Fig. 11B). Likewise, the LMM antibody along with the LMM protein (Sigma Chemical Co.) did not completely inhibit staining of this *L. longiflorum* pollen, whereas increasing the concentration of LMM protein did result in the loss of binding (Fig. 11D).

An antibody from *Dictyostelium* myosin II specific to the LMM region (A. DeLozanne, personal communication) was used to obtain additional information on the staining pattern of LMM in pollen. When applied to sections of *L. longiflorum* pollen, the staining pattern was punctate; the addition of LMM protein to the antibody caused the loss of the punctate staining pattern (data not shown).

Colocalization of myosin V along actin filaments

Initial studies indicate that myosin V labeled organelles can appear in rows suggesting that these organelles traveled along the actin filaments. To establish whether they could indeed be localized to the actin filaments, colocalization studies were performed with myosin V and actin antibodies. Actin filaments can be observed in Fig. 12A,B and myosin V labeled organelles in Fig. 12C,D. A few areas where the two overlap are marked by arrows.

Statistical analysis of organelle size

The sizes of the organelles labeled with the three classes of

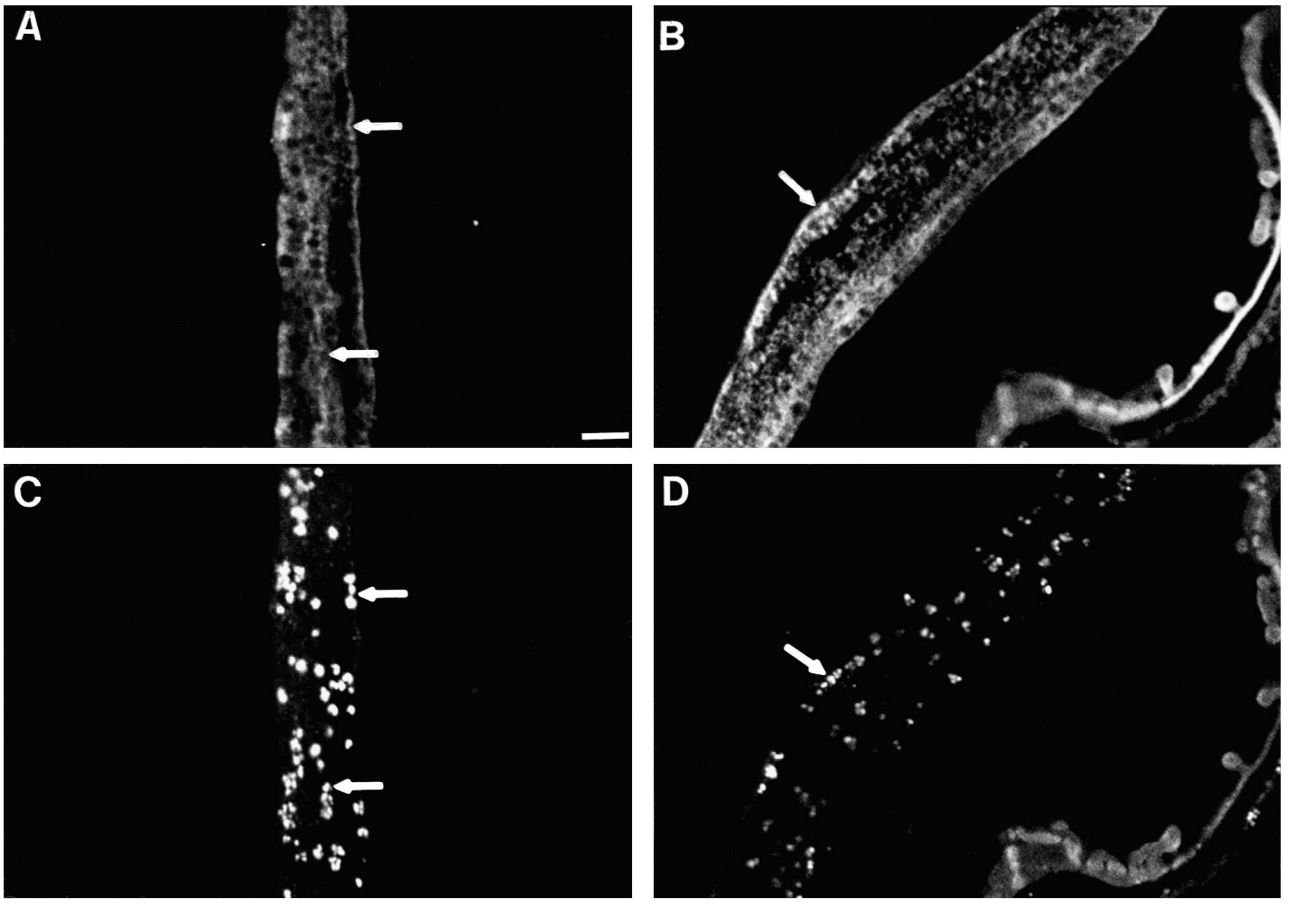

Fig. 12. Colocalization of organelles labeled with myosin V along actin filaments. (A,B) Actin filaments (see arrows) in *L. longiflorum* pollen labeled with anti-actin antibody. Organelles labeled with myosin V antibody (C,D; see arrows) can line up in rows matching regions where actin filaments are located (see arrows). Bar, 10 μm.

myosins appeared different. In Table 4 the average organelle size was measured; the values indicate that there are different populations of organelles labeled with these myosins. The myosin I antibodies recognized organelles of about 1.44 μm diameter whereas the myosin II antibodies recognized ones between 0.68 and 1.07 μm diameter. The myosin V antibodies bound to organelles of about 0.42 μm diameter. In addition, statistical analysis indicated that the three myosin II antibodies used were not significantly different from each other, but there is a significant difference between the three classes of myosin. Thus, the different classes of myosins are potentially located on different subclasses of organelles and possibly have specificity for transporting different cargos.

DISCUSSION

The results of this study indicate the potential presence of at least three classes of myosin in pollen tubes and show that they exhibit different spatial localizations. This study has identified and localized not only myosin II, but two other classes of myosins (I and V) within pollen tubes of *L. longiflorum* and *N. alata*. Immunoblot analysis of crude extracts reveals the presence of the different classes of myosins and immunfluorescence microscopy using various preservation techniques has localized them. On the basis of immunological evidence, we suggest that myosins I, II, and V are present in pollen tubes, however, they are localized in strikingly different patterns. Myosin I associates with the vegetative cell plasma membrane, larger organelles and the surface of the generative cell and vegetative nucleus, whereas, myosins II and V are associated with different classes of organelles. The competition studies suggest that the heterologous antibodies used to probe the pollen system recognize putative plant homologues of myosin I, II and V. Quantitation (Table 4) revealed that myosin V labeled smaller organelles, which are approximately 0.42 μm in diameter, whereas myosin I labels larger organelles which are approximately 1.44 μm in diameter, and myosin II labels organelles between 0.68-1.07 μm. Taking into account the enlargement of the images due to fluorescent flare it is reasonable to suggest that the 0.42 μm organelles correspond to vesicles while the 0.68-1.44 μm organelles represent mitochondria and/or amyloplasts as reported by Pierson et al. (1990).

Myosin associated with the nuclear membrane has been observed in several instances. Generative cell and vegetative nuclear envelope staining has been observed in pollen tubes of *N. alata* with antibody to the head portion of a myosin II along with punctate staining of organelles (Tang et al., 1989b). Likewise, Heslop-Harrison and Heslop-Harrison (1989b)

Table 4. Comparison of organelle size labeled with myosin antibodies

(A) Average organelle size

Myosin antibody	*n*	Mean organelle size (μm)	s.d.
Myosin IB	30	1.44	0.45
Myosin V	30	0.42	0.22
Myosin II (S1)	20	0.68	0.14
Myosin II (LMM)	20	0.86	0.20
Myosin II (*Dictyostelium* LMM)	20	1.07	0.25

(B) Statistical significance between classes of myosins and organelle size

	Myosin I	Myosin V	Myosin II (S1)	Myosin II (LMM)	Myosin II (*Dictyostelium* LMM)
Myosin I	–				
Myosin V	***	–			
Myosin II (S1)	***	***	–		
Myosin II (LMM)	***	***	‡	–	
Myosin II (*Dictyostelium* LMM)	***	***	‡	‡	–

Statistical significance was tested by Tukey's Studentized Range (HSD) Test with 0.05 confidence limit: ***statistically significant difference; ‡statistically insignificant difference.

n, number of individual organelles measured.

s.d., standard deviation.

reported localization of a myosin with a myosin II antibody to the surface of the generative cell and vegetative nuclei from pollen of *Hyacinthus orientalis* and *Helleborus foetidus*. Berrios and Fisher (1986) observed nuclear staining in rat liver cells and *Drosophila* embryos with an ATPase thought to be a form of 188 kDa myosin II heavy chain. Antibodies against this peptide also decorated mammalian nuclei and higher plant cell (onion) nuclei. The present study infrequently reveals staining of the generative cell and vegetative nucleus in *L. longiflorum* or *N. alata* pollen tubes with myosin II antibodies, yet consistent staining of these structures is observed with the myosin I antibody. This staining indicates that a myosin I, rather than a myosin II, may be the driving force for the slow movement of the generative cell and vegetative nucleus through the pollen tube.

The association of myosin I with the vegetative nucleus and generative cell may relate to the relatively slow (100 times slower than organelles) motion of these nuclei. By comparison it is of note that in vitro (*Nitella*) assays show that brush border myosin I (0.01 μm/s; Wolenski et al., 1993) and *Acanthamoeba* myosin I coated beads (0.03 μm/s; Pollard et al., 1991) and crude organelles (0.2 μm/s; Pollard et al., 1991) move 100- to 1,000-fold more slowly than skeletal myosin II coated beads (3-6 μm/s; Sheetz et al., 1984) or myosin-coated pollen tube organelles (5.4-9 μm/s; Kohno and Shimmen, 1988a; Kuroda, 1990). The more current sliding actin filament assays indicate that the movement of the various classes of myosins are faster than that measured by the *Nitella* assay but according to Wolenski et al. (1993) the actin filament assay still shows a 3-fold difference in motility rates between myosin I (0.04-0.66 μm/s) vs myosin V (0.27 μm/s). Additionally, Zot et al. (1992) have shown that myosin I can move at an average rate of 0.2 μm/s. Therefore, it is possible that a myosin I could be moving the generative cell and vegetative nucleus unidirectionally through the tube to the tip, while myosins II and V are quickly moving the different classes of organelles bidirectionally throughout the cytoplasm.

In our pollen system, the myosin IA and IB antibodies reacted with the same 125 kDa isoform of myosin. The localization of the antibodies in pollen is similar to that of *Acan-*

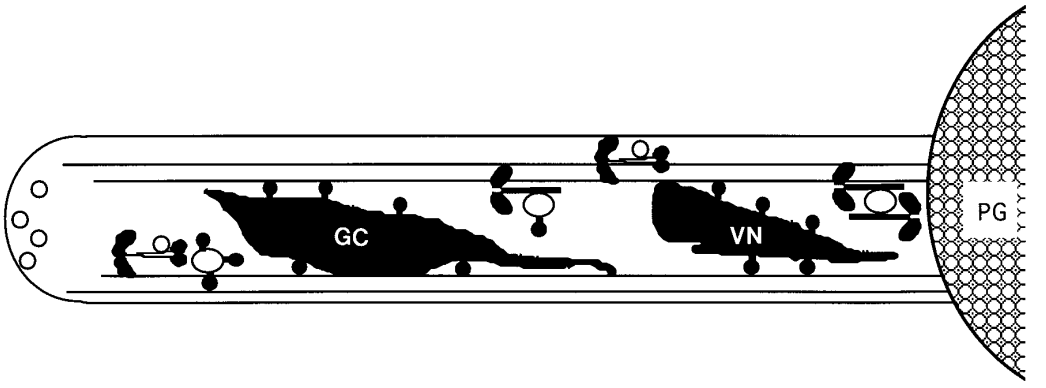

Fig. 13. Model of putative roles of three different classes of myosins in pollen tube growth. Myosin I moves the generative cell (GC) and vegetative nucleus (VN) unidirectionally toward the tube tip; myosin I and II move the larger organelles bidirectionally; and myosin V moves the smaller organelles bidirectionally throughout the pollen tube. (◯) larger organelles (plastids, lipid bodies, mitochondria, dictyosomes), (○) smaller organelles (vesicles), (●) myosin I, (⟶) myosin II, (⟷) myosin V, (═) actin microfilaments; (PG) pollen grain.

*thamoeb*a myosin IA and IB, being found at the surface of organelles and along the membrane. Thus, the pollen tube may contain only one myosin I isoform or only one isoform is recognized by these antibodies. Immunocytochemical data also indicate that the antibodies to myosins I, II and V do not cross-react with each other (see Figs 1, 2, 3, and data not shown). This enhances our ability to distinguish the roles of the different classes of myosins in pollen.

It is known that various classes of myosins have different or overlapping locations within the cell and presumably offer diverse functions. For example, *Acanthamoeba* has three myosin I isoforms (Baines et al., 1992). Myosin IA was found to be located mostly in the vegetative cytoplasm, the cortex beneath phagocytic cups and in association with small cytoplasmic vesicles. Myosins IB and IC, on the other hand, were both found to be associated with the plasma membrane and large vacuole membranes, while only myosin IC was associated with the contractile vacuole. Myosin IA may function in cytoplasmic vesicle transport and myosin I mediated cortical contraction, myosin IB in pseudopod extension and phagocytosis, and myosin IC in contractile vacuole function. Injection of antibodies against myosin IC was reported to inhibit the contractile vacuole function in *Acanthamoeba* (Doberstein et al., 1993), indicating that myosin I is necessary for force generation needed for intracellular membrane movement. Myosin I has been found to be located at the leading edge of the motile cell, *Dictyostelium*, while myosin II is located near the tail (Fukui et al., 1989); this same pattern is also found in fibroblasts albeit less distinctly than in *Dictyostelium* (Conrad et al., 1993). Localization of myosin V in chicken brain is perinuclear and at the periphery of neurons in tissue culture cells (Espreafico et al., 1992; Espindola et al., 1992). The localization of myosin V to the periphery of nerve cells is consistent with the finding that antibodies generated against an enriched synaptic vesicle fraction from chicken brain identified a 190 kDa protein (Sanders et al., 1992). Myosin V is believed to move vesicles in other organisms such as the budding yeast, *Saccharomyces cerevisiae* (Johnston et al., 1991) and mouse melanocytes (Mercer et al., 1991).

Like many unconventional myosins, myosins I and V are calmodulin binding proteins (Cheney and Mooseker, 1992). However, in contrast to other calmodulin binding proteins, the presence of high Ca^{2+} causes the release of the calmodulin on myosins I and V thereby inhibiting motility (Mooseker and Coleman, 1989; Collins et al., 1990; Espreafico et al., 1992; Titus, 1993; Cheney et al., 1993a). Brain myosin V is inhibited in motility assays by increasing the level of Ca^{2+} to 10 μM with optimal motility occurring at resting levels (Cheney et al., 1993a; Wolenski et al., 1993). The increase in Ca^{2+} causes two effects, the immediate inhibition of motility and the gradual decay or inactivation of the myosin V (Wolenski et al., 1993). Addition of exogenous calmodulin alleviates the gradual decline but a drop in filament velocity still occurred, although the replacement of Ca^{2+} with EGTA allowed the motility rate to recover (Cheney et al., 1993a). Of particular importance is the fact that elevated Ca^{2+} inhibits streaming in pollen tubes and plant cells in general. Inhibition of myosins I and V in the presence of high Ca^{2+} is consistent with the lack of streaming at the tip of pollen tubes and the inhibition of streaming when high levels of Ca^{2+} are introduced into pollen tubes. How myosin II could be regulated in pollen tubes is less clear. Most myosin II light chains cause activation of the enzyme in the presence of elevated Ca^{2+}; however, there is evidence that phosphorylation of the heavy chain can inhibit ATPase activity and decrease filament formation (reviewed by Tan et al., 1992).

In conclusion, the results shown herein provide the first identification and localization of three classes of myosins in one plant cell. A putative model (Fig. 13) describing the location and function of actin and different classes of myosins within the pollen tube system includes the following: actin is localized longitudinally along the length of the tube to act as a track along which pollen organelles, vesicles and the male germ unit may move. Different classes of myosins have distinct or overlapping functions; myosins I and II are located on the surface of larger organelles while myosin V is consistently located on the surface of smaller organelles. In addition, myosin I alone is located on the surface of the generative cell and vegetative nucleus, indicating its role in the movement of these nuclei to the tip.

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