Myosin associated with the surfaces of organelles, vegetative nuclei and generative cells in angiosperm pollen grains and tubes

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Summary

Myosin, detected by immunofluorescence using an antibody to bovine skeletal and smooth muscle myosin, has been localised on individual identifiable organelles from the grasses *Alopecurus pratensis* and *Secale cereale*, and on the surfaces of vegetative nuclei and generative cells from pollen and pollen tubes of *Hyacinthus orientalis* and *Helleborus foetidus*. Taken in conjunction with recent evidence showing that the growing pollen tube contains an actin cytoskeleton consisting of numerous mainly longitudinally oriented microfilament bundles, and that isolated pollen-tube organelles show ATP-dependent movement along the actin bundles of the giant cells of the characeous algae, this finding suggests that an actomyosin motility system is present in pollen tubes, and indicates that the movements of the different classes of inclusions are driven by interaction of the surface myosin with the actin fibrils at the zones of contact.

Key words: pollen-tube myosin, organelle movement, vegetative nucleus movement, generative cell movement.

Introduction

Plant myosin, now known from characeous algae (Kato & Tonomura, 1977; Grolig *et al.* 1988) and from diploid somatic tissues of a number of angiosperms (Ohsuka & Inoué, 1979; Vahey & Scordilis, 1980; Turkina *et al.* 1987), is generally considered to be involved in intracellular movement as one component of an ATP-dependent force-generating actomyosin system comparable with that of animal cells. The presence in angiosperm pollen tubes of longitudinally disposed fibrils made up of bundles of uniformly polarised actin microfilaments (Condeelis, 1974; review, Heslop-Harrison, 1988) suggests that in these often greatly extended haploid cells a mechanism of this kind drives the long-range movement of various cytoplasmic inclusions. This view is consistent with the fact that movement in the pollen tube is sensitive to cytochalasins, well known as inhibitors of actomyosin-dependent motility in animal cells (Mascarenhas & Lafountain, 1972), and it receives further support from the reports by Yan *et al.* (1986) and Tang *et al.* (1989) of the extraction of myosin-like molecules from pollen tubes of *Luffa cylindrica* and *Nicotiana alata*.

In most accounts so-called 'cytoplasmic streaming' in the pollen tube relates to the movement of organelles. During growth these circulate throughout the length of the active part of the protoplast along separate acropetal and basipetal lanes. These lanes are readily identifiable in the living tube, and in the more attenuated parts of the protoplast they can be distinguished as individual fibrils (Heslop-Harrison & Heslop-Harrison, 1987, 1988, 1989*a*), corresponding to those identifiable by phalloidin binding as being composed of, or containing, actin (Perdue & Parthasarathy, 1985). The obvious implication of this is that myosin, or a plant equivalent, must be associated with the motile organelles (Heslop-Harrison & Heslop-Harrison, 1987), a conclusion receiving massive support from the work of Kohno & Shimmen (1988*a*,*b*), who have shown that organelles isolated from pollen tubes show ATP-dependent movement along the actin cables of the giant cells of algae of the Characeae.

Vegetative nuclei, generative cells and gametes – the largest bodies moving through the pollen tube – are not involved in the general cyclotic flux; in contrast with the smaller organelles they travel more or less consistently away from the grain, tracking the advancing tube apex. Various authors have suggested that generative cells and gametes have independent powers of movement (e.g. see Steffen, 1953), and this possibility has been considered afresh against the background of new evidence concerning the organisation of the microtubule cytoskeleton in the generative cell (Lancelle *et al.* 1987). However, the cytochalasins block the movements of the larger bodies in the tube, indicating that that they, also, advance in association with the actin fibril system (Heslop-Harrison & Heslop-Harrison, 1989*a*,*b*), and implying that they, like the organelles, must carry surface myosin. In this paper we show that a component cross-reacting with

antibody against bovine myosin is indeed present on the surfaces of individual identifiable organelles and on vegetative nuclei and generative cells.

The methods hitherto in common use for immunofluorescence localisation of antigens in pollen tubes involving aldehyde fixation, enzymic degradation of the wall and exhaustive detergent permeabilisation of the protoplast were unsuitable for the present investigation. Apart from the obvious disadvantages of these quite harsh procedures (Lancelle *et al.* 1987), it became clear during the study that individual structures could not be identified satisfactorily in the congested parts of pollen tubes when virtually every constituent bound the primary antibody. Ideally, for the purpose of tracing surface antigens one would wish to offer free access of antibodies to isolated, unfixed, intact organelles, vegetative nuclei and generative cells. A method comparable with that used by Kohno & Shimmen (1988*a*,*b*) for the extraction of motile organelles from lily pollen tubes proved satisfactory for the isolation of identifiable organelles from the grass pollen tubes, but fractionation procedures involving bulk maceration could not be used for the isolation of the larger constituents. The vegetative nuclei were too readily disrupted or rendered unrecognisable, and the outer membranes of generative cells, derived originally as invaginations of the plasmalemma of the parent vegetative cells, were usually stripped away. We have therefore adopted a simple and rapid method of extracting the contents of small populations of pollen grains and pollen tubes that provides an adequate yield of intact structures.

Materials and methods

The grasses *Alopecurus pratensis* L. and *Secale cereale* L. (rye) were used as the sources of pollen-tube organelles. Exsertion of fresh anthers was promoted as required by exposure to light. Newly shed pollen was germinated on a rotator at 22–24 °C in a medium containing 25 % sucrose and 1 mM-$Ca(NO_3)_2$ and 1 mM-H_3BO_3. The monocotyledon *Hyacinthus orientalis* L. and the dicotyledon *Helleborus foetidus* L. were used for immunofluorescence localisation of myosin on vegetative nuclei and generative cells. Pollen from dehiscing anthers was germinated in the above medium with 15 % sucrose. The onset of movement in the grains as observed by differential interference (DIC) microscopy was taken as an indication of pre-germination activation. To establish the state of the nuclei, samples were withdrawn from the cultures as required, fixed for 15–30 min in 2·5 % glutaraldehyde in 0·05 M-phosphate buffer at pH 6·8, containing 15 % sucrose, and stained with the DNA-specific fluorochrome, 4-6-diamidino-2-phenyl-indole (DAPI) at approximately 0·0005 % (Heslop-Harrison & Heslop-Harrison, 1984).

Organelles were extracted from grass pollen tubes by a procedure comparable with that used by Kohno & Shimmen (1988*a*,*b*) for the preparation of organelle fractions capable of movement along the actin fibrils of characeous algae. Two alternative extraction media were used: the first, similar to that of Kohno & Shimmen (1988*a*), containing 10 % mannitol, 30 mM-piperazine-*N*,*N*′-bis-2-ethane sulphonic acid (Pipes), 6 mM-$MgCl_2$, 5 mM-ethyleneglycol-bis(α-amino-ethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) and 1 % bovine serum albumin (BSA), adjusted to pH 7·0; and the second without EGTA, and with the Pipes buffer replaced by 50 mM-phosphate buffer at pH 6·8. Pollen was germinated as above and, when examination showed that the majority of grains were fully activated with organelles moving vigorously in the emerging tubes, the samples were concentrated by low-speed centrifugation and then washed in the appropriate medium for 5–10 min on the rotator. After a second concentration, the samples were resuspended in smaller volumes of the same medium and ground rapidly in a glass microhomogeniser until inspection showed that a high proportion of the grains and tubes had been disrupted. Cell debris was then removed by low-speed centrifugation. Examination of the supernatant showed that amyloplasts, readily identifiable by size and shape as well as by I/KI staining, formed the most conspicuous component, together with mitochondria, identified by rhodamine 123 staining, and large numbers of the smaller (0·3 μm) polysaccharide-containing wall precursor bodies. Samples were dispersed thinly on poly-L-lysine-coated slides and subjected to the immunofluorescence localisation procedure described below.

The mannitol–phosphate medium was used for the preparation of vegetative nuclei and generative cells from growing pollen tubes because of the damaging effect of EGTA, and the

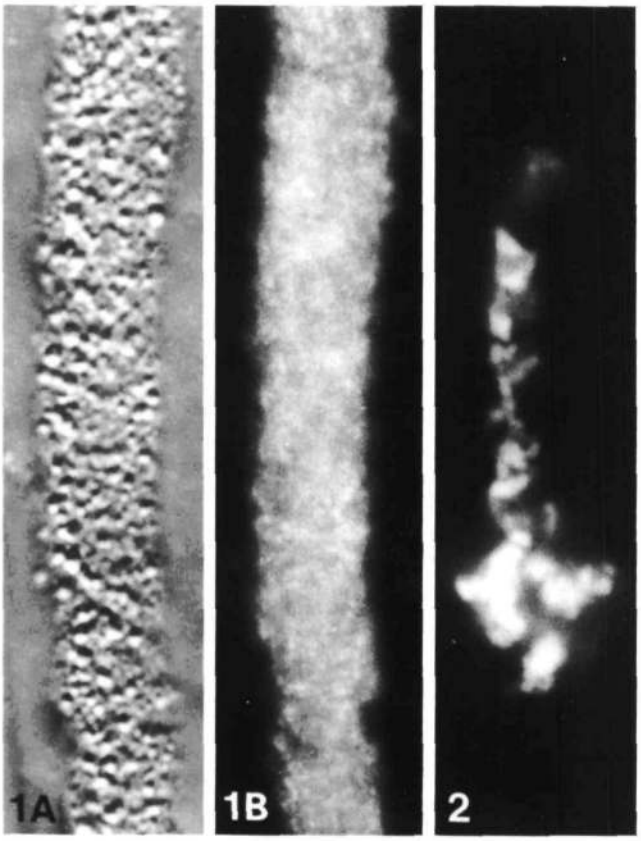
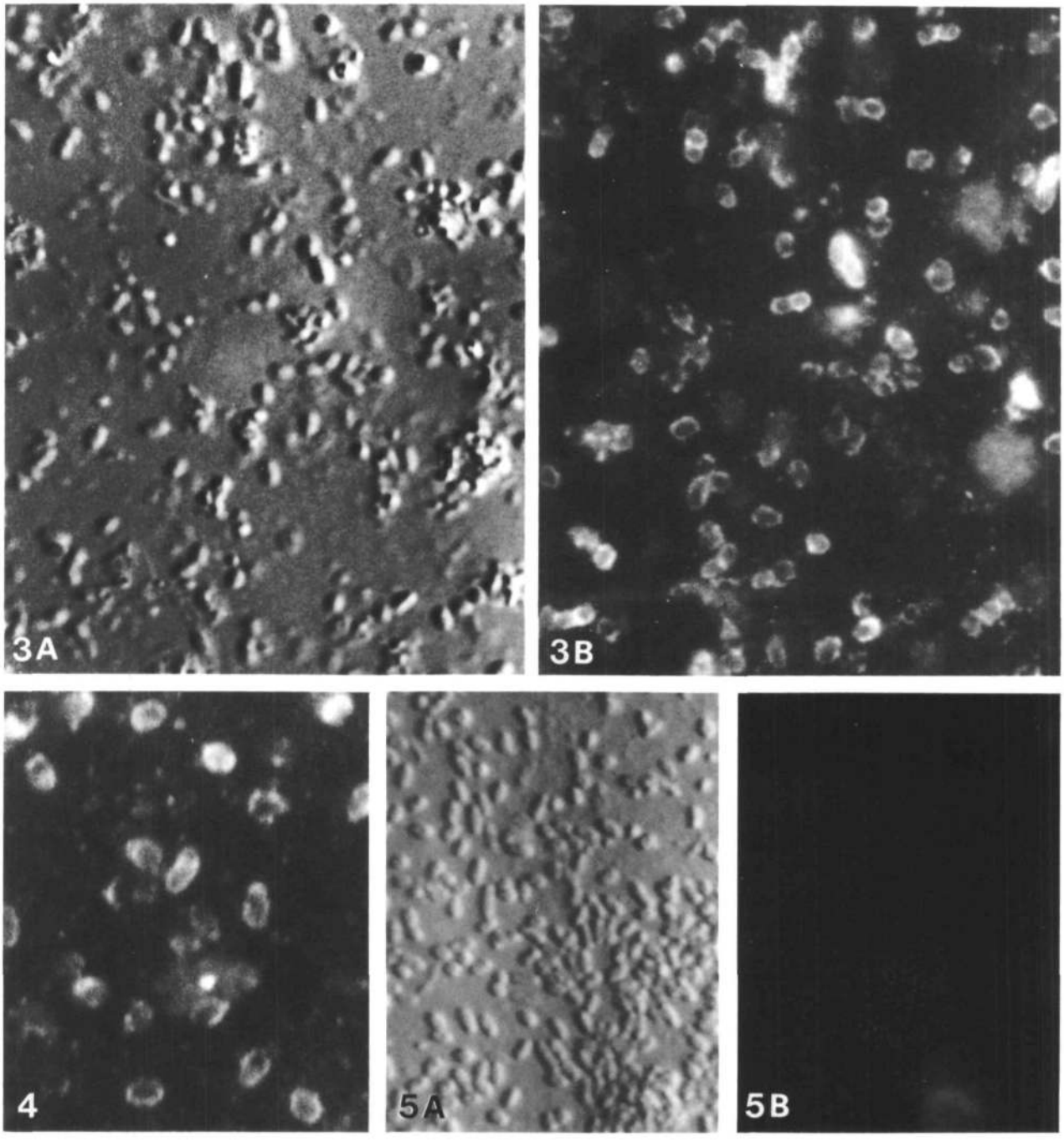


Fig. 1. Intact segment of a pollen tube of *H. orientalis* from a region immediately proximal to the apex, immunofluorescence localisation of myosin. A. DIC micrograph showing the congested state of the cytoplasm in this region. B. The same field, fluorescence micrograph. The ubiquitous binding of the antibody prevents identification of any individual organelles. ~×1000.

Fig. 2. Immunofluorescence localisation of myosin in a coherent column of cytoplasm extruded from a 20-min pollen tube of *A. pratensis*. Individual organelles are heavily labelled, but it is not feasible to identify them without further dispersal. ~×750.

same medium served also as a diluent for the antibodies to maintain an essentially uniform osmotic environment throughout the whole processing period. Preliminary tests showed that pollen tubes transferred to this medium retained a normal form, without the swelling, distortion and bursting often found with EGTA-containing media. For the preparation of samples for immunofluorescence, activated pollen grains or growing pollen tubes were sedimented by low-speed centrifugation, drained completely of germination medium, and washed for 5–10 min in the buffered mannitol medium on the rotator. The samples were concentrated again, and for each preparation about 50 μl of the suspension was dispersed evenly on a poly-L-lysine-coated



Figs 3–5. Immunofluorescence localisation of myosin associated with organelles from pollen tubes of grass species.
Fig. 3. Amyloplasts of *A. pratensis* extracted from 10 min pollen tubes. A. DIC micrograph in the focal plane of the amyloplasts. B. Fluorescence micrograph of the same field. Virtually every organelle is labelled, and in most cases it is possible to distinguish that the fluorescence is associated with the outer surface. ~×1300.
Fig. 4. Organelle fraction from *S. cereale*, fluorescence micrograph, focal plane at the level of the smaller organelles, mainly mitochondria and wall-precursor bodies. ~×1350.
Fig. 5. Control preparation of amyloplasts from *S. cereale* with an inappropriate primary antibody substituted for anti-myosin. A. DIC micrograph of the dispersed amyloplasts. B. Fluorescence micrograph showing the absence of second antibody binding. ~×1000.

slide. Controlled pressure was then applied through a similarly coated slide inverted on the sample until most of the grains or tubes had been disrupted. Roughly equal amounts of material, including severed pollen tube segments, vegetative nuclei, generative cells and organelles, remained firmly attached to each of the slides, and this was then rinsed immediately to remove the residual cytosol and loose debris. One from each pair of slides was used for myosin localisation while the other served as a control.

The antibody used for immunofluorescence localisation of myosin was Sigma Product M 7648 (Biofile Code 1083). This is raised in rabbits against purified bovine smooth muscle myosin preparations that show one heavy-chain band and two light-chain bands on SDS–polyacrylamide gel electrophoresis, and has been shown to stain specifically the A bands of skeletal muscle, and to give a characteristic striated pattern of staining on stress fibres of cultured fibroblasts. For the present use the antiserum was diluted 1:10 in the mannitol-containing buffers immediately before application to the samples. The preparations were incubated in a saturated atmosphere for 30–60 min at 37 °C, rinsed free of the primary antibody in several 2-min changes of buffer, flooded with the second antibody, FITC-conjugated sheep anti-rabbit IgG (Sigma) diluted 1:40 in buffer, and incubated at 37 °C until inspection showed an adequate degree of coupling, when the preparations were mounted for observation.

Controls were run by omitting the primary antibody from the first incubation, and, for the organelle localisations, also by substituting an irrelevant primary antibody, rabbit anti-tubulin (Sigma).

Micrographs were made on Ilford XP1 film developed to give a nominal speed of 1200 ASA.

Results

Organellar myosin

Fluorescence attributable to binding of the antibody was observed in intact segments of pollen tubes of each of the species examined. In the distal parts of the tubes where the organelles tend to be closely packed, individual bodies could not be resolved because of generalised and intense fluorescence throughout the protoplast (Fig. 1A,B); nor was it feasible to distinguish between the bounding membranes of vegetative nuclei, generative cells and – in the case of the grasses – male gametes, all of which are retained in this zone of the tube and are thus masked by the ubiquitous fluorescence of the organelles. In columns of cytoplasm derived from older, less densely populated parts of the tubes individual organelles could be resolved; however, while these invariably showed fluorescence attributable to associated myosin (Fig. 2) it was not feasible to identify them.

No such ambiguity arose in distinguishing amyloplasts in the organelle fractions derived from the grass pollens. The DIC micrograph of Fig. 3A is of a typical preparation from *A. pratensis* after processing for immunofluorescence, and the fluorescence micrograph of Fig. 3B of the same field shows labelling associated with the surfaces of the individual amyloplasts. Smaller organelles in the size range of mitochondria and the wall-precursor bodies were also labelled (Fig. 4), although they could not always be categorised with certainty.

Comparison of the results obtained with organelles isolated in the two media, EGTA-containing and EGTA-free, showed no discernible differences in the distribution or intensity of binding.

Controls were uniformly negative. Fig. 5A is of a control amyloplast sample from *S. cereale*, prepared in a manner identical with that used for the preparation in Fig. 3, but with anti-tubulin substituted for anti-myosin as the primary antibody; Fig. 5B of the same field shows the absence of binding.

Myosin associated with vegetative nuclei and generative cells

Figs 6 and 7 show the distribution of myosin as revealed by immunofluorescence on the surfaces of vegetative nuclei of *H. orientalis* from grains drawn from the culture 15–20 min from the beginning of hydration. The more

Figs 6–11. Immunofluorescence localisation of myosin associated with vegetative nuclei and generative cells.

Fig. 6. Vegetative nucleus from *H. orientalis* extracted from a grain approximately 15 min after the beginning of hydration. A. DIC micrograph. B. Fluorescence micrograph of the same field, showing labelling of the nuclear envelope. The two unidentifiable organelles adherent to the nuclear envelope (arrow) also fluoresce, indicating that they bear a myosin coating. ~×1800.

Fig. 7. Vegetative nucleus from another grain from the same culture as that of Fig. 6. Comparison with the states of nuclei *in situ* suggests that this came from a grain in a later stage of activation. Again the label is dispersed over the whole surface of the nuclear envelope, seen mainly in profile in this focal plane. As in Fig. 6, adherent organelles also fluoresce. ~×1800.

Figs 8, 9. Immunofluorescence localisation of myosin associated with vegetative nuclei extracted from 40 min pollen tubes of *H. orientalis*. ~×2000.

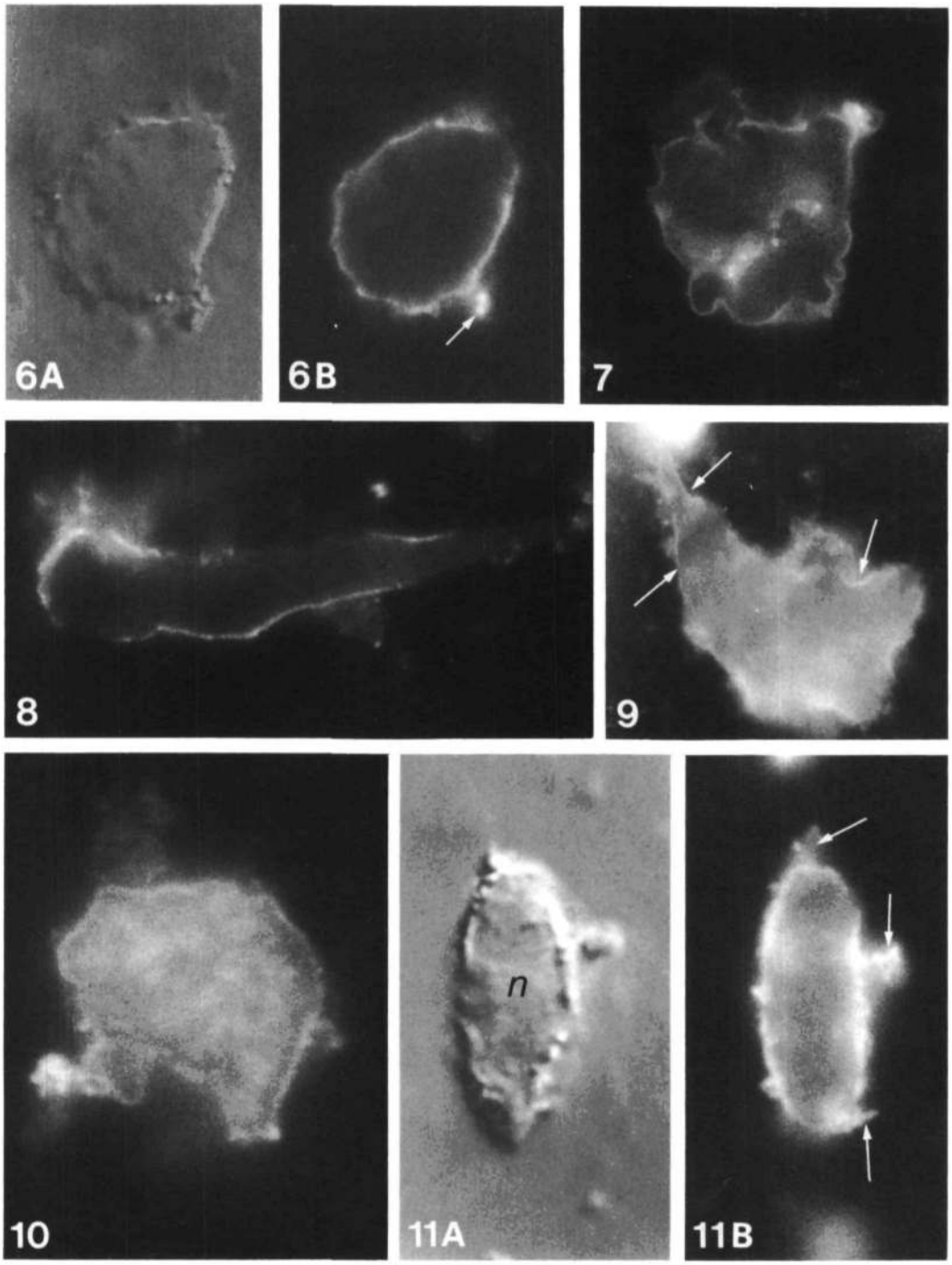
Fig. 8. Nucleus drawn out into a configuration comparable with those seen in actively growing pollen tubes (Heslop-Harrison & Heslop-Harrison, 1989*b*). The label initially extended over the whole surface of the nuclear envelope, but faded rapidly from the more attenuated part during excitation.

Fig. 9. Partly contracted vegetative nucleus from the same culture as that of Fig. 8, focal plane near the proximal surface. The association of myosin with the nuclear envelope is clearest where it is seen in profile (arrows); the more generalised fluorescence is attributable in part to retained organelles.

Figs 10, 11. Immunofluorescence localisation of myosin on the vegetative nucleus and generative cell of *H. foetidus*. ~×2500.

Fig. 10. Slightly flattened vegetative nucleus, fluorescence micrograph. The labelling extends over the whole surface of the nuclear envelope, seen partly in surface view. The mottling may result from the flattening of surface lobes, or may possibly indicate an uneven distribution of the antigen.

Fig. 11. Generative cell, likely to be intact, but with the shape somewhat modified by the rounding up of the pointed terminations during extraction. A. DIC micrograph. The presence of the lipid globuli of the generative cell around the nucleus (*n*) provides one indication that the cell is not disrupted. B. Fluorescence micrograph of the same field, showing the surface labelling. A few organelles (arrows) remain attached to the surface.



pronounced lobing of the nucleus of Fig. 7 may indicate that it came from a grain in a later stage of activation than that from which the nucleus in Fig. 6 was derived. In each the distribution is such as to suggest a uniform dispersal of myosin over the whole surface, the fluorescence attributable to the FITC-labelled second antibody faithfully following the convolutions and lobings. Figs 8 and 9 illustrate the distribution on the surfaces of vegetative nuclei from 40 min pollen tubes of the same species. The envelope of the compact part of the nucleus in Fig. 8 is mostly heavily labelled, while the fainter labelling of the surfaces of the attenuated 'tail' was rapidly lost on excitation, and was not recorded in the micrograph. Presumably the membranes of the attenuated part were more severely stretched, with a consequent dilution of the surface molecules.

Vegetative nuclei of *H. foetidus* gave a similar pattern of surface labelling (Fig. 10). Immunofluorescence localisation showed that myosin was also dispersed over the outer surfaces of presumably intact generative cells (Fig. 11). However, some recognisable generative cells showed no labelling in preparations where the vegetative nuclei were adequately labelled. While the point cannot readily be confirmed by optical microscopy, the likely explanation is that the outer membrane of these cells had been lost during processing, leaving the residue of the

continuous pectic wall (Cresti *et al.* 1987) overlying the plasmalemma of the generative cell itself. The outer membrane of the generative cell is derived originally as an invagination of the vegetative cell plasmalemma (Heslop-Harrison, 1968), and it is readily shed in response to mechanical and osmotic disturbance.

Control preparations in which the primary antibody had been omitted showed no fluorescence attributable to binding of the FITC-conjugated second antibody.

Discussion

Amyloplasts are the most readily identifiable of the organelles circulating in grass pollen tubes. Video analysis of the movement in *S. cereale* has shown that they travel independently along acropetally or basipetally polarised cytoplasmic lanes, achieving velocities up to $2{\cdot}1\,\mu m\,s^{-1}$ in pollen tubes growing *in vitro* (Heslop-Harrison & Heslop-Harrison, 1987). Mitochondria, identified by size and shape in living pollen tubes of the same species, were similarly shown to move independently and unidirectionally in single traffic lanes, achieving measured velocities up to $2{\cdot}58\,\mu m\,s^{-1}$. The pollen-tube traffic lanes correspond to actin fibrils or fibril aggregates (Heslop-Harrison & Heslop-Harrison, 1988), and the results reported in the present paper substantiate the inference drawn from these earlier observations, namely that the motive force is likely to be generated by interaction between the actin fibrils and myosin on the outer membranes of the organelles. They also provide confirmation for the views of Kohno & Shimmen (1987, 1988*a*,*b*), who offered a similar explanation for the capacity of isolated pollen tube organelles for movement along actin bundles when introduced into tonoplast-free characeous cells.

It is instructive to compare the movements of the smaller cytoplasmic inclusions in the pollen tube with those of the native organelles of the giant characeous cells. While organelles in the former seem in general to travel along the actin fibrils independently, in the latter the movement is largely co-ordinated, a circumstance interpreted by Kachar & Reese (1988) as indicating that the motive force is transmitted through investing masses of endoplasmic reticulum. The demonstration by Grolig *et al.* (1988) that myosin detected by immunofluorescence using an antibody to animal myosin heavy chain is associated with tangled endoplasmic strands in *Chara* provides further support for the view that the general movement of the endoplasm is induced by a separate translocator system in the algal cells, but it is noteworthy that the authors also accept that myosin-coated organelles may interact independently with the actin bundles. We consider it likely that some of the generalised fluorescence observed in pollen tube cytoplasm (Fig. 1) is attributable to membrane-held myosin, and that this may be involved in the movement of the lipid globuli and other non-organellar inclusions through the tube.

The present observation of myosin associated with the bounding membranes of vegetative nuclei and generative cells provides an obvious parallel. Taken together with the evidence from cytochalasin responses (Heslop-Harrison & Heslop-Harrison, 1989*a*,*b*) and from direct observation of the association of presumed actin fibrils with the outer surfaces of vegetative nuclei (Raudaskoski *et al.* 1987; Lancelle *et al.* 1987; Heslop-Harrison & Heslop-Harrison, 1988, 1989*b*; Tiwari & Polito, 1988), this finding substantiates the view that the movement of these bodies through pollen tubes is driven by the interaction of actin and myosin at the zones of contact between the surface membranes and the longitudinally disposed microfilament bundles, much as is envisaged for the mass movement of endoplasm in characeous cells. This interpretation explains much of the behaviour in the tube. A striking feature of vegetative nuclei, especially in species where the nucleus is large in relation to the diameter of the tube, is that they undergo continuous change of form during their passage, often elongating greatly along the tube axis. The circulatory pattern of organelle movement affirms that microfilament bundles of both acropetal and basipetal polarities are present in the tube, and the distortions of the vegetative nucleus, which are relaxed when the fibril system is disrupted (Heslop-Harrison & Heslop-Harrison, 1989*a*,*b*), are interpretable as resulting from contacts with actin fibrils of different polarities at the surface sites where the stresses on the nuclear envelope are generated. Generative cells must be exposed to similar conflicting stresses, and they do indeed vary in length during passage through the tube. However, they are considerably less protean, retaining an overall fusiform shape during their passage (Heslop-Harrison *et al.* 1988). This is attributable to the fact that they lack the plasticity of the naked vegetative nuclei, being cells with a continuous wall faced by two plasmalemmas (Cresti *et al.* 1987) and possessing a cage-like peripheral microtubule system likely to impart greater rigidity (Cresti *et al.* 1984).

Vegetative nuclei and generative cells, while not structurally linked together, tend to remain in the denser cytoplasm of the leading part of extending pollen tubes. This implies a net movement away from the parent grain – or, in other terms, movement in an acropetal sense in relation to any fixed point in the tube wall. At first sight it may seem surprising that they should contrive to maintain this apparent forward migration throughout the period of growth if their surfaces are subject simultaneously to acropetally and basipetally directed forces statistically in balance over an interval of time. There is no anomaly, however. During growth the bulk of the protoplast is itself migrating steadily forward into fresh volumes of the tube as new wall is synthesised at the tip. The cyclotic pathways are continuously extending at the apex, and the actin cytoskeleton is thus in no way an immutable framework. Vegetative nuclei and generative cells may therefore simply be remaining more or less near a position of dynamic equilibrium as the system as a whole is propagated forward.

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