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Immunocytochemical localisation of actin and profilin in the generative cell of angiosperm pollen: TEM studies on high-pressure frozen and freeze-substituted *Ledebouria socialis* Roth (Hyacinthaceae)

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Abstract Actin was demonstrated for the first time at the EM level in the generative cell of mature angiosperm pollen by using immuno-gold labelling of high-pressure frozen and freeze-substituted *Ledebouria socialis* Roth anthers. In addition, profilin, an actin-monomer binding protein, is shown to coexist in the generative cell. We attribute the detection of actin and profilin to the applied cryomethods which yield a much better preservation of ultrastructure and antigenicity of delicate cytoskeletal constituents than conventional fixation techniques. Actin labelling was observed within the cytoplasm of the generative cell and became especially clear in close vicinity to microtubular bundles. Filamentous structures congruent with the actin labelling patterns do occur, but are not a frequent feature. Profilin was localised throughout the cytoplasm.

Abbreviations Ac Acetone · BSA bovine serum albumin · CFS calf fetal serum · CGA chicken gizzard actin · EA Epon-Araldite · E-PTA ethanolic phosphotungstic acid · FG fish gelatine · GC generative cell · LR LR white · mAb monoclonal antibody · Os osmium tetroxide · Pb lead-staining · PBS phosphate-buffered saline · UA uranyl acetate

Introduction

In the generative cell (GC) of pollen grains and tubes, bundles of interconnected microtubules represent the most conspicuous cytoskeletal elements (see Palevitz and Tiezzi 1992; Pierson and Cresti 1992; Terasaka and Ni-

itsu 1994 for references). Furthermore, F-actin bundles were recently detected in the gymnosperms *Pinus densiflora* and *Pinus thunbergii* (Terasaka and Niitsu 1994). Concerning the angiosperms, however, it is still unclear whether or not the GC contains actin. Thus, numerous investigations provide indirect evidence for the absence of F-actin (Tiezzi 1991; Palevitz and Liu 1992; Palevitz and Tiezzi 1992; Pierson and Cresti 1992). Only an immunocytochemical study by Taylor et al. (1989) showed patterns of actin fluorescence in the GC and sperm cells of *Rhododendron laetum* suggestive of longitudinally oriented fibrils, but these data were not confirmed by Palevitz and Liu (1992), who investigated the same species. It was, however, proposed that actin may be present in its G-form in the GC (Palevitz and Tiezzi 1992), although definite proof was, to our knowledge, hitherto not available.

Lack of evidence also pertains to the possible occurrence of actin-monomer binding proteins within the GC. The only actin-monomer binding protein identified in plants, as yet, is profilin (Valenta et al. 1991a,b, 1992, 1993; Staiger CJ et al. 1993). This 12- to 15-kDa protein, previously known only from animals and lower eukaryotes, was recently recognized as a common allergen in pollen grains throughout the angiosperms (Valenta et al. 1991a,b, 1992; Mittermann et al. 1995).

We prepared pollen of the monocotyledonous angiosperm *Ledebouria socialis* Roth (Hyacinthaceae) for ultrastructural analysis by using high-pressure freezing followed by freeze substitution and resin embedding (Hess 1993a,b, 1995; Hess and Frosch 1994). The good overall preservation quality obtained allowed localisation of actin and profilin epitopes by immuno-electron microscopy within the GC of mature pollen grains.

Material and methods

Plants and specimen preparation

L. socialis Roth (Hyacinthaceae) was grown in the glasshouses of the Urban Horticultural Centre of Zürich (Stadtgärtnerei Zürich, Switzerland; taxonomical note: *Ledebouria* was formerly included

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within the large genus *Scilla*). Mature, undehiscent anthers were removed from flowers at the stage of anthesis and prepared for high-pressure freezing according to Studer et al. (1989). Freeze substitution of cryoimmobilised anthers was performed at approximately -90°C for at least 8 h in anhydrous acetone, either pure (Ac), or containing 2% osmium tetroxide (OsAc) as fixative/stain as described (Hess 1993a; Hess and Glaser 1993). Samples were embedded in Epon-Araldite (EA) polymerised at 60°C or in LR-white (LR; London Resin, UK) polymerised overnight by UV light at room temperature. [It seems worth briefly discussing here why ambient-temperature resin embedding was chosen instead of low-temperature processing with lowicryls or LR gold. Of course, the latter approach is expected to minimise membrane lipid extraction (e.g. Weibull and Christiansson 1986) and to retain the hydration shell of macromolecules (Kellenberger 1987, 1991) as has been shown for plant tissues as well (tobacco stems; Hippe et al. 1989). With respect to freeze-substituted anthers, however, low-temperature embedding has, to our knowledge, failed as yet. Thus, pollen grains with highly impermeable sporopollenin cell walls, being surrounded by the sap-like locular fluid mixed with tapetal cell remnants, as well as by multiple layers of anther cells, are not adequately infiltrated at subzero temperatures (see Staiger D et al. 1994)].

Thin sections (80 nm) were optionally post-stained with uranyl acetate (UA, 0–40 min 25°C) and/or lead (Pb, 5 min 25°C) or with hot ethanolic phosphotungstic acid (E-PTA, 5 min 60°C ; Locke and Krishnan 1971). The specimens were examined with an electron microscope at 50 and 80 kV using a goniometer stage (EM 900, Zeiss, Oberkochen, Germany).

Immuno-electron microscopy

All immuno-gold labelling experiments including the controls were carried out on sections obtained from three different anthers, and were repeated at least twice; thus, the data presented are based on the examination of more than 150 pollen grains (i.e. approx. 100 GCs). Specimens were substituted in pure acetone and embedded in LR. Incubations were performed in a moist chamber at room temperature by using thin sections collected on formvar-carbon-coated gold grids. The following primary antibodies were employed: (a) mouse monoclonal antibodies (mAbs) against chicken gizzard actin (Boehringer Mannheim, Mannheim, Germany; Amersham, UK), and (b) a rabbit anti-recombinant birch profilin antiserum (see Valenta et al. 1993) and the respective preimmune serum which were affinity purified using mAb traps (Pharmacia,

Uppsala, Sweden); according to the photometric determination the eluents were diluted so that the affinity-purified antisera contained a similar concentration of rabbit immunoglobulins.

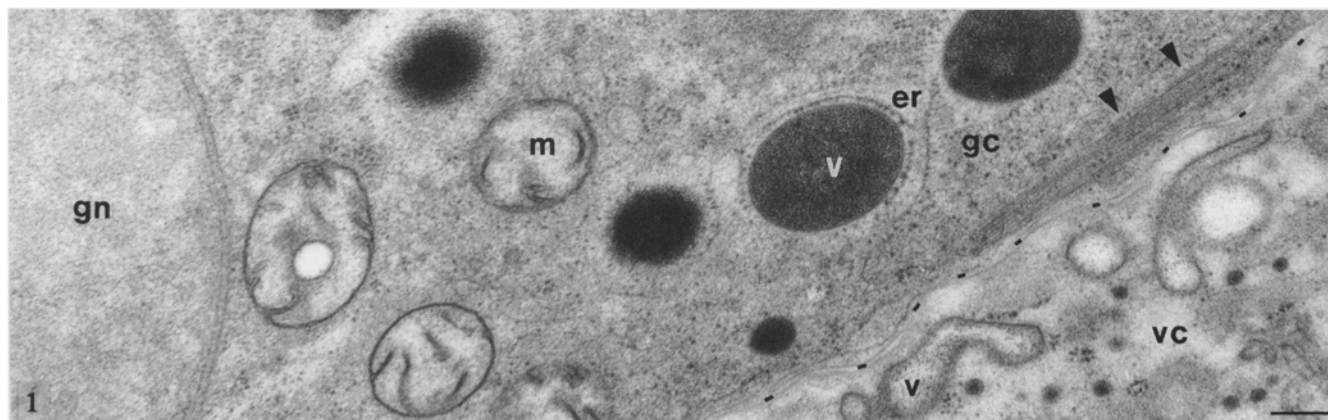
Primary antibodies were diluted to 10 $\mu\text{l}/\text{ml}$ (for actin) or to 50 $\mu\text{g}/\text{ml}$ (for profilin) in 10 mM phosphate-buffered saline (PBS; Sigma, St. Louis, Mo., USA) with 2% bovine serum albumin (BSA; Sigma), 1% fish gelatine (FG; British Biocell Cardiff, UK) and 0.1% Tween (Sigma), pH 7.4. The gold-labelled secondary antibodies (for actin, goat anti-mouse IgG-5 nm gold; for profilin, goat anti-rabbit IgG-10 nm gold; Sigma) were diluted 1:30–1:60 in PBS with 0.2% BSA and 0.01% Tween.

The grids were preincubated for 30–60 min in PBS/BSA/FG/Tween containing 10% newborn calf serum (CFS; Gibco, BRL) followed by incubation in the primary antibody for 90–120 min. After several rinses in PBS (for approx. 5 min) the grids were incubated for 30 min in PBS with 10% normal goat serum (Serva, Heidelberg, Germany), 0.2% BSA and 0.01% Tween followed by treatment with the gold-labelled secondary antibody for 90–120 min. Final rinsing was done in double-distilled water for about 5 min. Sections were optionally post-stained. The specificity of labelling was tested by comparing the density of gold particles per unit area of generative and vegetative pollen cytoplasm with the density of background label on pollen and anther cell walls; gold particles were counted directly on TEM micrographs.

Controls for actin and profilin staining

1. Omission of the primary antibody.
2. Replacing the primary antibodies with (a) normal mouse serum (Amersham, for actin) or (b) the preimmune serum (for profilin).

Fig. 1 High-pressure freezing followed by freeze substitution in anhydrous acetone with 2% osmium tetroxide (OsAc) and Epon-Araldite (EA) embedding excellently preserves the ultrastructural integrity of generative and vegetative cell in mature *Ledebouria socialis* pollen (arrowheads mark a bundle of microtubules). OsAc/EA/uranyl acetate-lead staining (UA-Pb), $\times 38\,500$, bar 0.2 μm . (er Endoplasmic reticulum, gc generative cell, gn generative nucleus, m mitochondrion, v vacuole, vc vegetative cell; outline of generative cell indicated by dashed line)

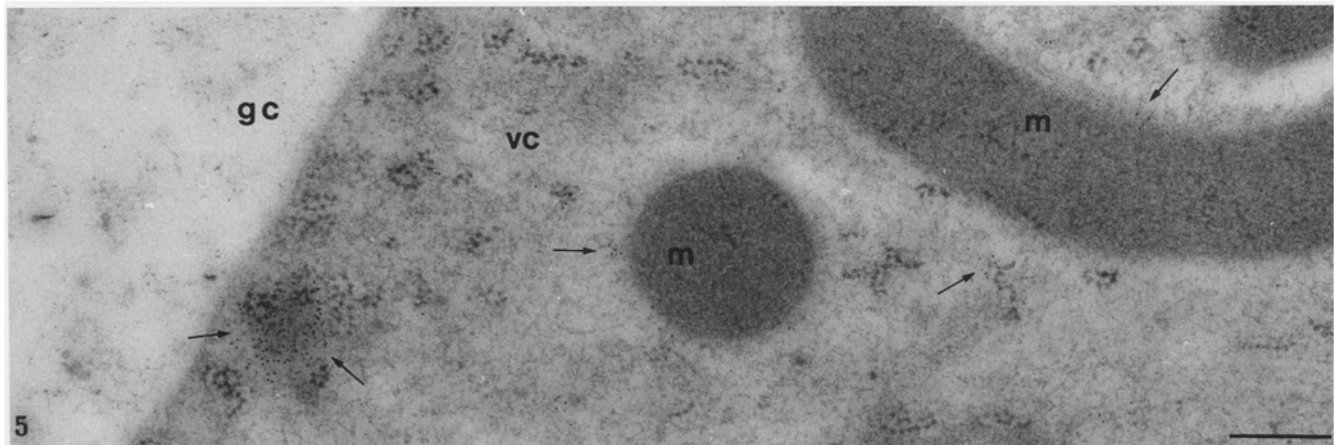
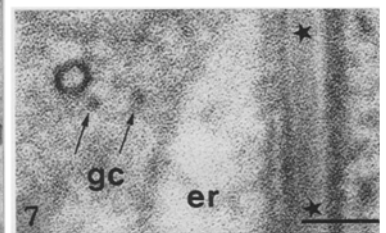
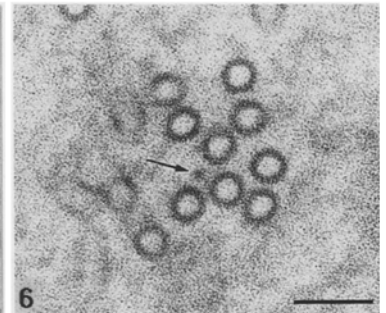
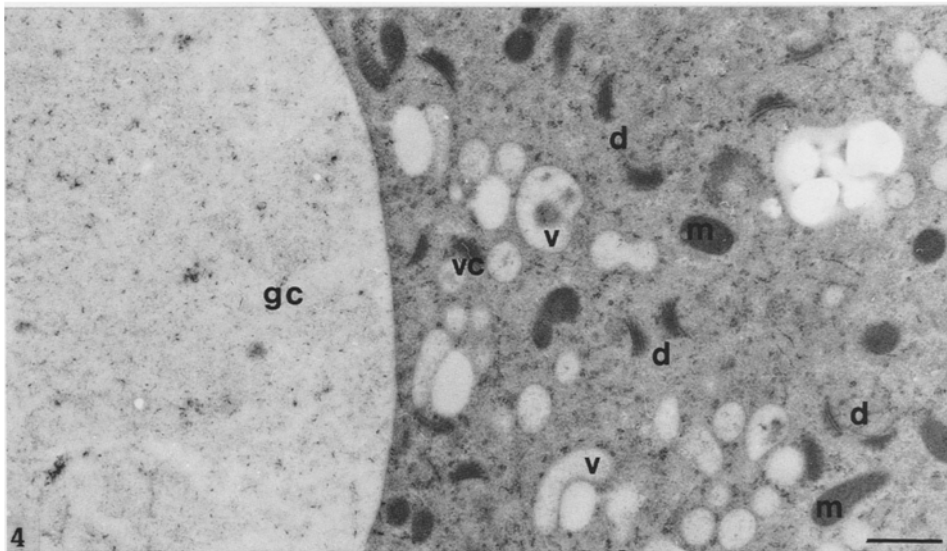
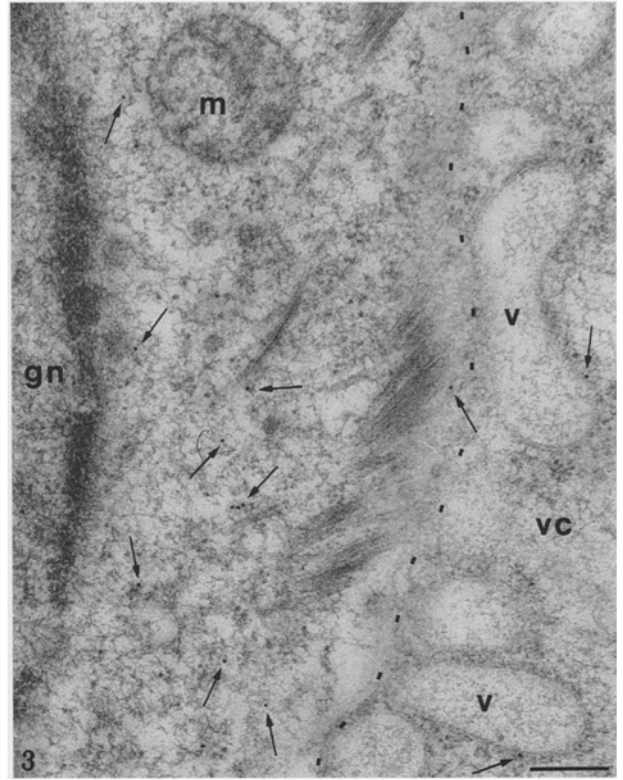
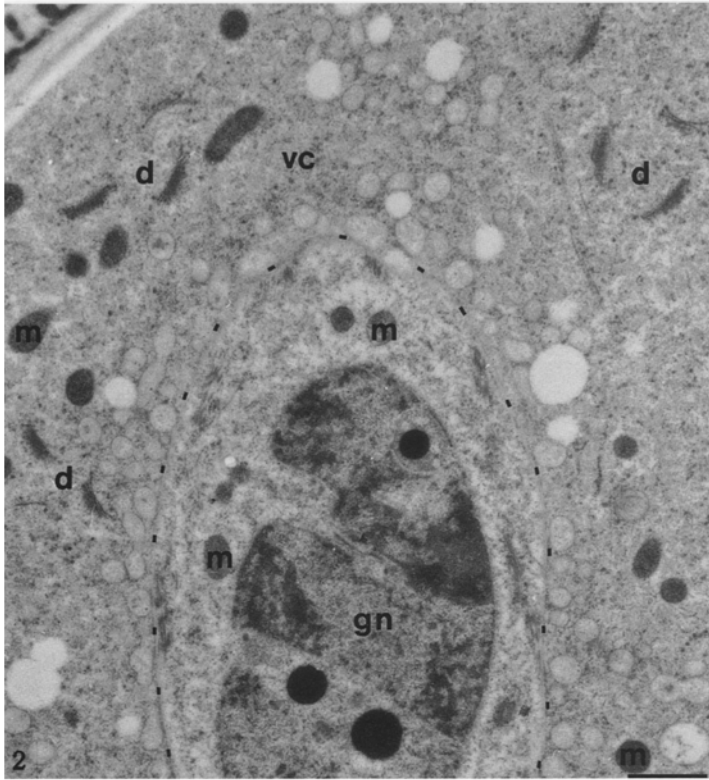


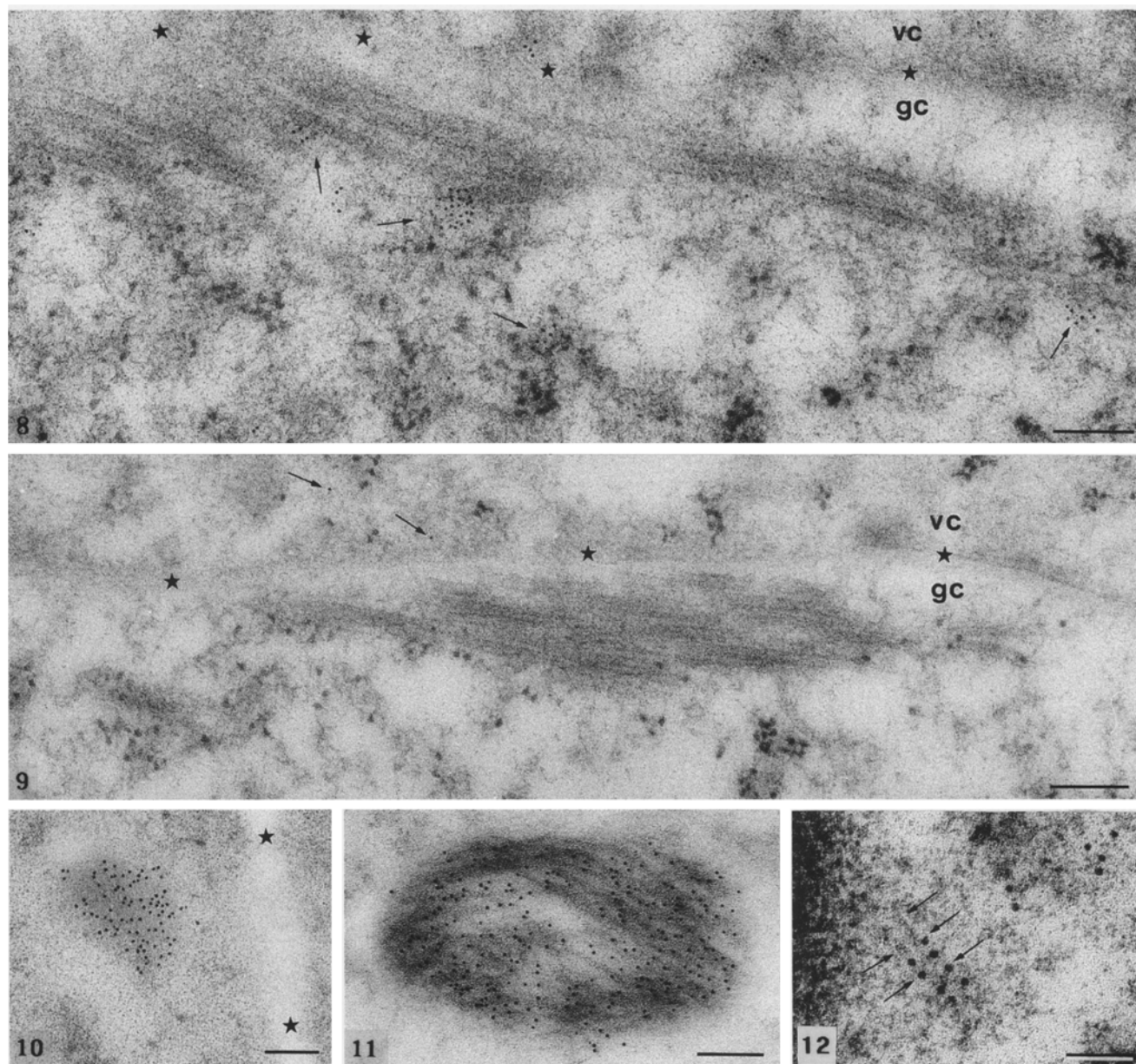
Figs. 2–5 Preservation patterns of generative and vegetative cell in *L. socialis* pollen after freeze substitution in pure acetone (Ac) and LR white (LR) embedding; Ac/LR/UA-Pb

Figs. 2, 3 Adequate ultrastructure preservation of both generative and vegetative cell. Membranes are, if present at all, generally hardly visible in samples subjected to freeze substitution in pure solvents without any (heavy metal) fixative/stain followed by ambient temperature resin embedding. (d Dictyosome; outlines of generative cell indicated by dashed line; for other abbreviations see Fig. 1); arrows in **Fig. 3** indicate profilin labelling. **Fig. 2** $\times 10\,000$, bar 1 μm ; **Fig. 3** $\times 50\,000$, bar 0.2 μm

Figs. 4, 5 Conspicuous extraction of generative cell compounds contrasting with the well-preserved vegetative cell; note in **Fig. 5** distinct actin labelling restricted to the vegetative cell (arrows; mouse mAb against chicken gizzard actin, Boehringer). **Fig. 4** $\times 10\,000$, bar 1 μm ; **Fig. 5** $\times 65\,000$, bar 0.2 μm

Figs. 6, 7 Opaque dots (arrows) in the vicinity of microtubules, as seen in cross section through the generative cell are interpreted as single microfilaments. OsAc/EA/hot ethanolic phosphotungstic acid (E-PTA) $\times 200\,000$, bar 0.05 μm . (outlines of generative cell indicated by stars)





Figs. 8–12 Immuno-gold localisation of actin within the generative cell of high-pressure frozen, freeze-substituted *L. socialis* pollen by using Boehringer mouse mAb against chicken gizzard actin as primary antibody; Ac/LR/UA-Pb

Fig. 8 Actin labelling (arrows) mainly occurs in the vicinity of microtubular bundles; $\times 60\,000$, bar $0.2\,\mu\text{m}$

Fig. 9 Control: preabsorption with $0.1\,\mu\text{mol/ml}$ chicken gizzard actin. The few gold particles observed are marked by arrows; $\times 60\,000$, bar $0.2\,\mu\text{m}$

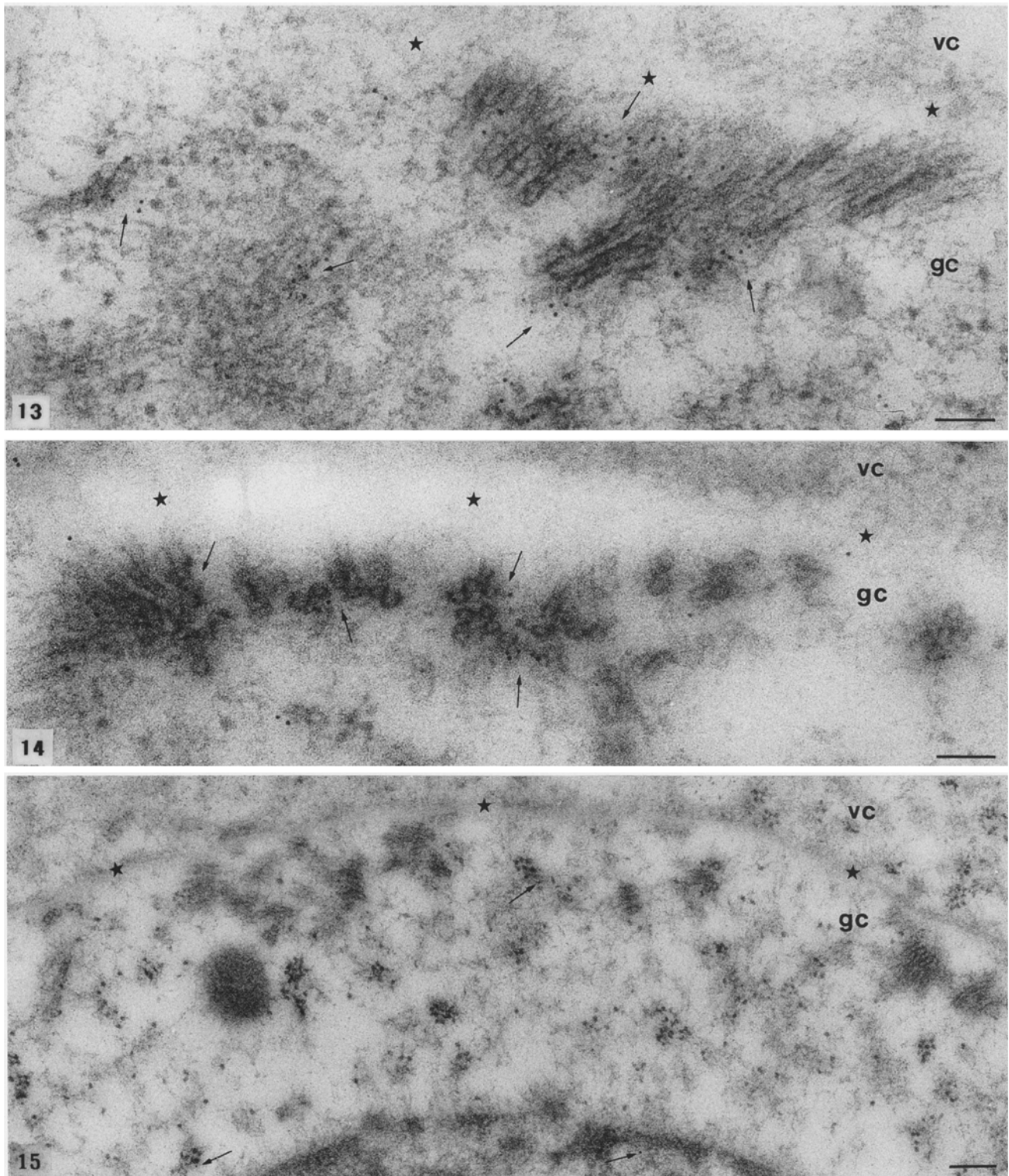
Fig. 10 Amorphous ovoid body intensively labelled by anti-actin mAb; $\times 80\,000$, bar $0.1\,\mu\text{m}$

Fig. 11 Actin labelling of filamentous subunits of ovoid to fusi-form body; $\times 100\,000$, bar $0.1\,\mu\text{m}$

Fig. 12 Actin labelling patterns congruent with microfilament-like structures (arrows). $\times 200\,000$, bar $0.05\,\mu\text{m}$ (Abbreviations, see Figs. 1, 2)

3. Absorption of the primary antibody with $0.1\,\mu\text{mol/ml}$ of the appropriate antigen for 60 min prior to use (actin from chicken gizzard: CGA, Sigma; recombinant birch profilin which was expressed in plasmid pKK223–3 *Escherichia coli* and purified as described (see Valenta et al. 1992)).

4. As an additional control, immunoblotting was used to determine whether the antibodies were indeed binding to actin and profilin, respectively. Proteins from *L. socialis* flowers and leaves were extracted by grinding the tissue in liquid nitrogen and boiling the powder in SDS-sample buffer (Laemmli 1970). After 10 min centrifugation in a bench centrifuge at $10\,000\text{ g}$, supernatants were removed and analysed by SDS-PAGE and subsequent Coomassie Blue staining of the gels. According to the Coomassie staining, comparable amounts of flower and leaf proteins were separated by preparative SDS-PAGE and blotted to nitrocellulose. Anti-actin mAbs (Boehringer, Amersham) were diluted 1:100 and detection was done as described (Grote et al. 1995). Profilin was detected as described (Valenta et al. 1993).



Figs. 13–15 Immuno-gold localisation of actin within the generative cell of *L. socialis* pollen by using Amersham mouse mAb against chicken gizzard actin as primary antibody; Ac/LR/UA-Pb

Figs. 13, 14 Actin labelling marked by *arrows*; $\times 100\,000$, bar $0.1\,\mu\text{m}$

Fig. 15 Control: preabsorption with the pure antigen. The few un-specifically bound gold particles are marked by *arrows*. $\times 40\,000$, bar $0.2\,\mu\text{m}$. (Abbreviations, see Figs. 1, 2)

Results

Technical notes

As regards ultrastructural analysis, Epon-Araldite (EA) embedding, preceded by freeze substitution with OsAc, has proven to be the method of choice (Fig. 1; see also Hess 1993b and references therein).

For on-section immunocytochemistry we used specimens embedded in LR and found that freeze substitution in pure Ac adequately preserved the GC in approximately half of the samples (Figs. 2, 3). The other half suffered from remarkable extraction artefacts (Figs. 4, 5). Extraction could be limited by adding Os as fixative to the substitution medium (not shown), which is, however, generally not recommended for immunocytochemistry (see Bittermann et al. 1992). Surprisingly, the vegetative pollen cytoplasm was uniformly well preserved under any conditions (Figs. 2–5).

Ultrastructure

The most prominent cytoskeletal elements within the spindle shaped GC of mature *L. socialis* pollen are longitudinally oriented bundles of microtubules (Figs. 1, 3, 6). In addition, tilting and rotating cross sections through the GC revealed the regular occurrence of highly opaque dots (diameter approx. 7 nm) in the vicinity of the microtubular bundles. These dots, which become especially clear in sections post-stained with E-PTA, are tentatively interpreted as single microfilaments (Figs. 6, 7; see Ding et al. 1991). In longitudinal sections, however, the tight packing of microtubules and the resulting superimposition of intensely stained material did not allow unequivocal identification of structures to be considered as microtubule-associated microfilaments (not shown).

Sometimes, the GC of *L. socialis* contains ovoid to fusiform bodies which either consist of filamentous subunits or appear amorphous (Figs. 10, 11). These bodies are probably homologous to "crystalline fibrillar bodies" (Cresti et al. 1986) and to rhodamine-phalloidin-binding "fusiform bodies" (Heslop-Harrison et al. 1986) reported so far only from the vegetative cytoplasm of various angiosperm pollen.

Immuno-electron microscopy

Distinct actin and profilin labelling was found in most of the GCs that appeared, according to morphological criteria, to be well preserved by the substitution/embedding schedule applied (i.e. Ac/LR). Both anti-actin mAbs gave similar results: Gold particles are observed in the vicinity of, and/or over, the microtubular bundles as well

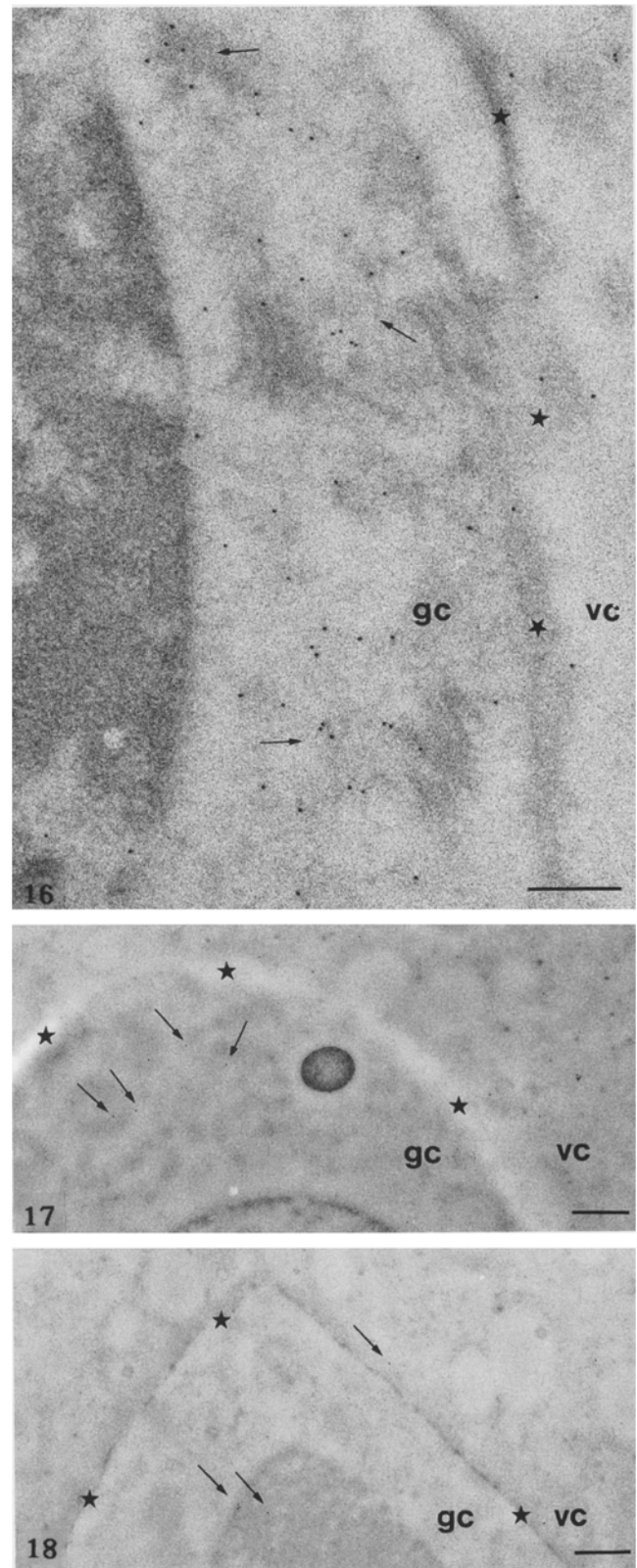
Figs. 16–18 Profilin localisation within the generative cell of *L. socialis* pollen (see also Fig. 3)

Fig. 16 Profilin labelling (arrows) is seen throughout the generative cell cytoplasm; Ac/LR/Pb, $\times 60\,000$, bar $0.2\,\mu\text{m}$

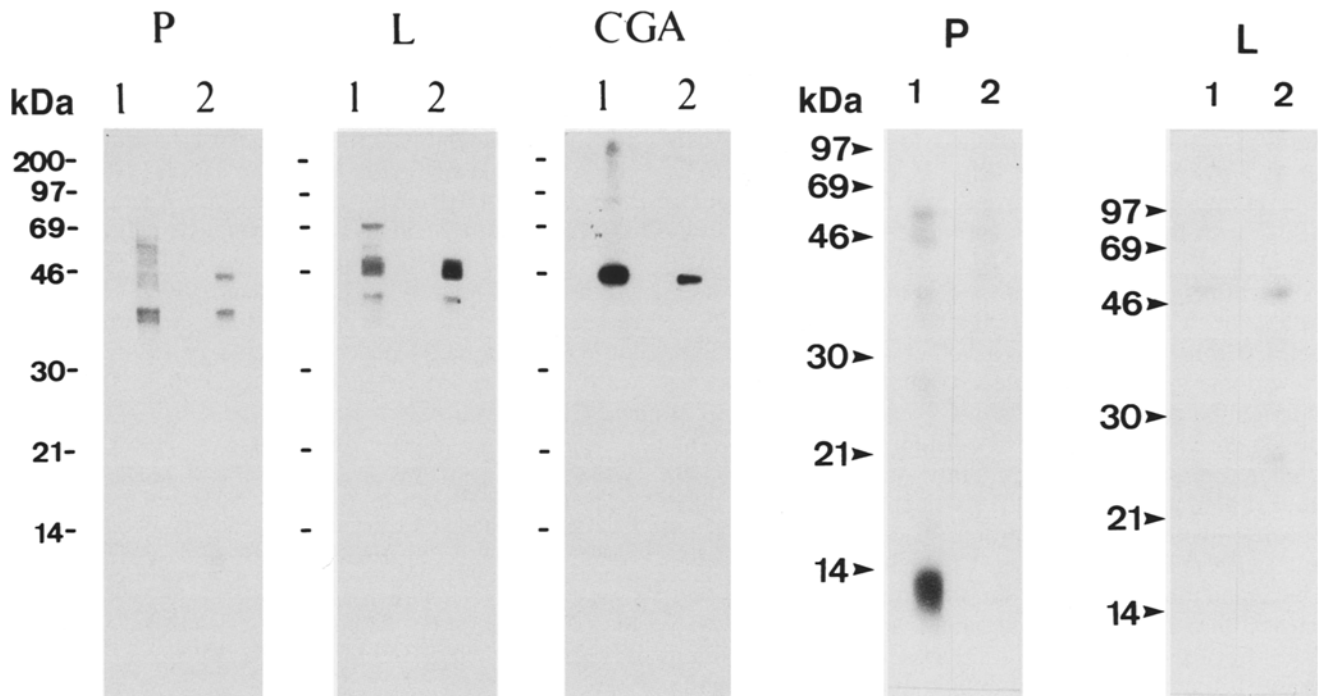
Figs. 17, 18 Negative controls; Ac/LR, no post-staining, $\times 15\,000$, bar $0.2\,\mu\text{m}$; arrows mark low non-specific labelling (single gold particles)

Fig. 17 Replacement of the primary antibody with preimmune serum

Fig. 18 Preabsorption of the primary antibody with excess recombinant birch profilin. (Abbreviations, see Figs. 1, 2)



as throughout the cytoplasm (Figs. 8, 13, 14). The ovoid to fusiform bodies described above are intensely labelled (Figs. 10, 11). Occasionally, immuno-gold labelling patterns could be clearly related to microfilament-like structures (Figs. 11, 12). Binding of profilin antibodies within



A

Fig. 19 Detection of actin (A) and profilin (B) in nitrocellulose blotted protein extracts from *L. socialis* flowers (P) and leaves (L). **A** Actin. Anti-actin mAbs (lane 1 Boehringer, lane 2 Amersham) detected proteins of approximately 46 kDa in *L. socialis* flower (P) and leaf (L) extracts. Both mAbs strongly reacted with nitrocellulose-blotted chicken gizzard actin (CGA). **B** Profilin. A positive reaction was observed in *L. socialis* flower extracts (P, lane 1 anti-profilin antiserum). The controls did not show any reaction (P, lane 2 preimmune serum; L, lane 1 anti-profilin antiserum; L, lane 2 preimmune serum)

the GC occurred more or less evenly all over the cytoplasm (Fig. 16; see also Fig. 3). It is important to note that there is no congruence between actin and profilin labelling patterns since the anti-profilin antiserum employed in this study does not bind to profilactin complexes (Valenta et al. 1993). GCs suffering from extraction artefacts did not show any actin labelling (Fig. 5), and profilin labelling was strongly reduced (not shown).

Controls

Omission of the primary antibodies gave virtually no staining (not shown), nor did replacement with normal mouse serum (actin labelling; not shown) or rabbit preimmune serum (profilin labelling; Fig. 17). Absorption of the primary antibodies with the appropriate antigen completely inhibited any labelling in *L. socialis* pollen grains (Figs. 9, 15, 18).

Immunoblotting showed anti-actin mAbs (Fig. 19A) to recognise proteins of approximately 46 kDa in *L. socialis* flower (P) and leaf (L) extracts. The fact that actin in

B

flowers and leaves migrated at slightly different molecular weight is probably explained by the expression of different actin isoforms in the tissues (McLean et al. 1990). Additional bands at higher molecular weight may be due to the detection of profilactin complexes, whereas binding to components of a lower molecular weight could be due to proteolysis in the extracts (see Koropp and Volkmann 1994 and references therein). Both mAbs strongly reacted with the nitrocellulose-blotted CGA which was used as a positive control. Profilin (Fig. 19B) could be detected in *L. socialis* flower extracts at approximately 14 kDa, whereas the preimmune serum showed no binding. Nitrocellulose strips containing comparable amounts of *L. socialis* leaf proteins showed no profilin signal.

Discussion

Taking advantage of cryopreparation methods, we detected actin and profilin epitopes by postembedding immuno-gold labelling within the GC of an angiosperm, the monocotyledon *L. socialis* Roth (Hyacinthaceae). In order to substantiate our data, the labelling experiments were carried out on three different populations of pollen grains by using two well-characterised antibodies which are widely used for actin localisation in plant and fungal specimens (e.g. Schmit and Lambert 1987; Tang et al. 1989; Kaminskyj and Heath 1994). Preabsorption with purified CGA completely inhibited any antibody binding in GC, vegetative cell (Hess 1995), and other anther cells, indicating that the labelling is genuinely of actin.

Actin has, to our knowledge, hitherto not been unequivocally demonstrated in angiosperm GCs. Thus, all other taxa investigated so far, by using, for instance, immunostains or fluorochrome-tagged phallotoxins, showed actin labelling restricted to the vegetative cell (e.g. Lancelle and Hepler 1991; Palevitz and Liu 1992; Gervais et al. 1994; for review see Palevitz and Tiezzi 1992) with the possible exception of *R. laetum* (cf. Taylor et al. 1989; Palevitz and Liu 1992, in this context). As regards potential causes for this divergence, it cannot be excluded that *L. socialis* is exceptional in containing actin within the GC whereas other species have none. Nevertheless, methodological aspects should, in our opinion, be considered as well. GC and vegetative cell of pollen are known to react very differently to denaturing preparation procedures (e.g. Cresti et al. 1990; Bartosch et al. 1994; Van Aken et al. 1994) apparently due to their specific nucleic acid and protein patterns (e.g. Bartosch et al. 1994; for reviews see Chaboud and Perez 1992; Ueda and Tanaka 1994). In the present study it was shown that even low-temperature solvent dehydration (i.e. freeze substitution) of cryoimmobilised pollen grains followed by ambient-temperature resin embedding may cause more or less conspicuous, selective extraction of GC compounds (see also Cresti et al. 1990), while the vegetative cell always becomes adequately preserved. We therefore suppose that this apparent delicate nature of the GC may inhibit, in particular the preservation of the vulnerable actin cytoskeleton, even when the surrounding vegetative cytoplasm looks intact and well preserved.

Although the immuno-electron microscopic data presented here provide evidence for the occurrence of actin within the GC of *L. socialis*, it is clear that a complete depiction of all kind of actin arrays requires various complementary approaches, as pointed out by Kaminskyj and Heath (1994; see also DuBose and Haugland 1993). Thus, the polymerisation state of actin in the GC of *L. socialis* remains to be examined in detail. Congruence of the immuno-gold labelling patterns with clearly visible microfilaments was only occasionally found; for instance, it was not possible to verify the actin nature of the structures considered to be microtubule-associated single microfilaments. The intensively labelled ovoid to fusiform bodies, some of which consist of 7-nm filaments, probably represent intermediate storage forms of actin, according to an interpretation by Heslop-Harrison et al. (1986) concerning similar structures in the vegetative pollen cytoplasm. Altogether, on the basis of information available, we favour the notion that a considerable amount of actin in the GC of *L. socialis* is non-filamentous and, thus, G-actin. Another question still to be investigated concerns the organisation and changes of the GC actin cytoskeleton during ontogenesis.

Apart from the demonstration of actin epitopes we could localise profilin, an actin-monomer-binding protein, within the GC of *L. socialis*. Multiple interactions of profilin with actin and/or phospholipids are well established for a large number of eucaryotes other than

plants (e.g. Theriot and Mitchison 1993, for review). Recently, microinjection experiments revealed that profilin also acts as a potential regulator of actin organisation in plant cells (Staiger CJ et al. 1994). In the GC of pollen, profilin may inhibit actin polymerisation during interphase, as suggested by Palevitz and Tiezzi (1992). Moreover, any possible involvement of actin filaments in GC division requires conspicuous changes in the organisation of the actin cytoskeleton (Staiger and Schliwa 1987; Staiger and Lloyd 1991; Palevitz and Tiezzi 1992); such reorganisation could be mediated by profilin, which is known to interact with components of signalling pathways (Aderem 1992; Machesky and Pollard 1993; Sohn and Goldschmidt-Clermont 1994).

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