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Cytosolic Proteins From Tobacco Pollen Tubes That Crosslink Microtubules and Actin Filaments In Vitro Are Metabolic Enzymes

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In plant cells, many processes require cooperative action of both microtubules and actin filaments, but proteins mediating interactions between these cytoskeletal members are mostly undiscovered. Here, we attempt to identify such proteins by affinity purification. Cytosol from *Nicotiana tabacum* (tobacco) pollen tubes was incubated first with actin filaments, and then proteins eluted from the actin were incubated with microtubules, and finally those microtubule-binding proteins were pooled in an active fraction. This fraction bundled actin filaments but not microtubules. However, when the fraction was added to both actin and microtubules, large bundles resulted, containing both polymers, regardless of the order of addition of components. Similar results were obtained when the order of affinity purification was reversed. The four most abundant bands from the fractions were identified from peptide fragments analyzed by mass spectrometry. The same four proteins were identified regardless of the order of affinity purification. The proteins are: homocysteine methyltransferase, phosphofructokinase, pyruvate decarboxylase, and glucan protein synthase (reversibly glycosylated protein). These results suggest the importance of structuring metabolism within the confines of the pollen tube cytoplasm. © 2010 Wiley-Liss, Inc.

Key Words: actin-associated protein, cytoskeletal bundling, microtubule-associated protein, *Nicotiana tabacum*, structured metabolism

Introduction

Morphogenesis combines upstream information processing with downstream construction. In constructing cells, the cytoskeleton plays a pre-eminent role. In plants, specific jobs for actin and microtubules have long been recognized, and for the most part, these systems have been studied independently. Nevertheless they are unlikely to function independently in the cell. Actin filaments and microtubules often colocalize, for example in the preprophase band, phragmoplast, cell cortex, and in transvacuolar strands [Collings, 2008]. The use of cytoskeletal inhibitors suggests that the two cytoskeletal tracks interact with each other, insofar as perturbing one type of filament affects the stability of the other [Justus et al., 2004; Bannigan and Baskin, 2005; Collings et al., 2006]. Actin and microtubules are coordinately regulated in the morphogenesis of cells with elaborate shapes, including trichomes and pavement cells, a complex regulatory network that choreographs the behavior of the cytoskeleton with great finesse [Panteris and Galatis, 2005; Smith and Oppenheimer, 2005]. Yet we are largely ignorant of the proteins that integrate these two cytoskeletal systems [Collings, 2008].

Over the past few years, efforts to identify accessory proteins for the plant cytoskeleton have increasingly borne fruit [Sedbrook, 2004; Hussey et al., 2006; Hamada, 2007; Ren and Xiang, 2007]. Such efforts have identified candidates to mediate interactions between actin and microtubules. Perhaps the first to be identified is a 190-kDa polypeptide isolated from tobacco BY-2 cells and potentially active during cell division [Igarashi et al., 2000]. A more recent example is a 30-kDa peptide from wild potato (*Solanum berthaultii*) that is pollen specific [Huang et al., 2007; Liu et al., 2009]. Beyond the demonstrated affinity of these proteins for actin and microtubules, their functions are unknown. An example where function can be assigned are certain kinesins of the KIN14 class (which are minus-end directed microtubule motors) that contain domains homologous to calponin and have been shown to bind actin as well as microtubules [Preuss et al., 2004; Frey et al., 2009; Xu et al., 2009]. The above examples all represent plant-specific clades. Protein families known in animal and yeast cells to bind or bundle actin and microtubules appear to be absent from plants [Gavin, 1997].

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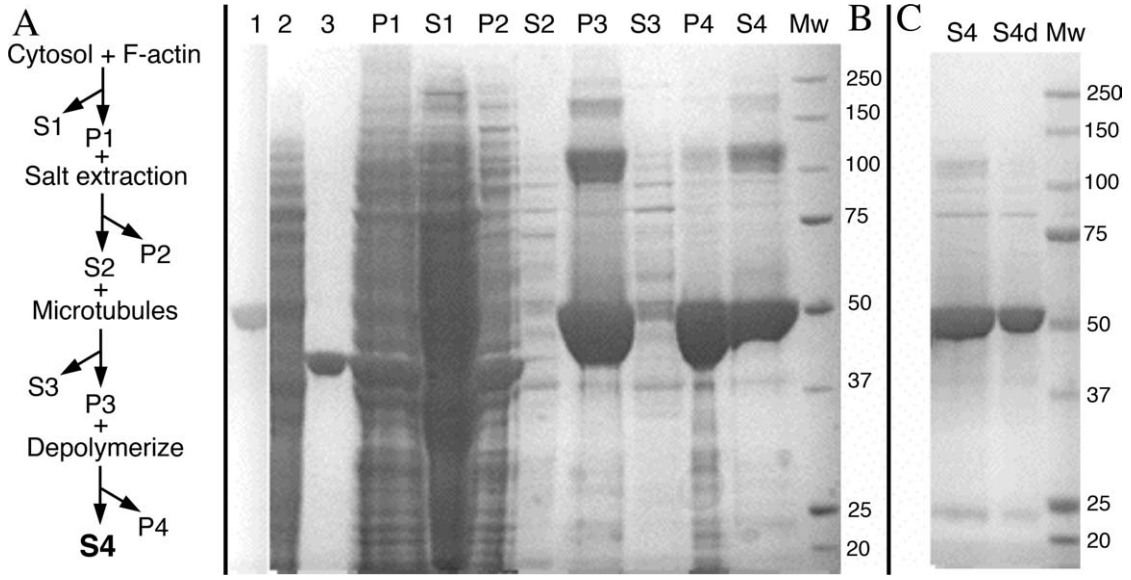

Fig. 1. **Purification of the S4 fraction**. **A:** Schematic of the double affinity steps. S, supernatant; P, pellet. **B:** Coomassie-stained gel of the purification. Lane 1: bovine tubulin standard. Lane 2: tobacco pollen tube cytosol. Lane 3: rabbit muscle actin standard. Mw = molecular weight standards. Other lanes are annotated based on the scheme in A. **C**: Coomassie-stained gel comparing the original S4 fraction to that obtained after desalting (S4d). The desalted preparation was used in the experiments. Relative molecular mass in kDa given to the right of both gels.

A pre-eminent model for studying the plant cytoskeleton is the pollen tube [Hepler et al., 2001; Raudaskoski et al., 2001]. The cells are ideal for imaging and can be obtained in sufficient quantity for biochemistry. The pollen tube grows by tip growth, a mode of growth where expansion is confined to the very apex of the cell, and the absolute value of elemental expansion rates are extraordinarily high. This extreme degree of cell polarization and the rapid expansion rate place significant demands on the cytoskeleton to maintain cell polarity as well as to orchestrate needed secretion and endocytosis of wall precursors and membrane. In pollen tubes potential interactions between actin and microtubule networks have been inferred from inhibitor studies [Justus et al., 2004], colocalization of the cytoskeletal systems [Lancelle and Hepler, 1991], and from assays of organelle motility [Van Gestel et al., 2002; Romagnoli et al., 2007]. Mechanisms mediating any such interaction have yet to be studied systematically.

We hypothesized that the pollen tube cytoplasm should contain proteins mediating interactions between microtubules and actin. To test this hypothesis, we used a biochemical approach. We purified proteins based on affinity to one element (e.g., microtubules) and then further purified those proteins based on affinity to the other (e.g., actin filaments). We identify four proteins that copurify when either actin or tubulin is used in the first step. Surprisingly, the proteins are metabolic enzymes.

Materials and Methods

Plant Material and Reagents

Tobacco (*Nicotiana tabacum*) plants used in this research were grown in the Botanical Garden of Siena University. Pollen was germinated and grown for 3 h, as described in Romagnoli et al. [2003], before being used for extract preparation. Chemical reagents for electrophoresis, including molecular mass standards, were purchased from Bio-Rad (Hercules, CA). Purified bovine brain tubulin (TL238), fluorescein tubulin (T332M), and purified rabbit skeletal muscle actin (AKL99) were purchased from Cytoskeleton (Denver, CO).

Preparation of Cytoskeletal Affinity Probes and Pollen Tube Cytosol

Taxol-stabilized microtubules and cytosol from tobacco pollen tubes were prepared as described previously [Romagnoli et al., 2003]. To prepare actin filaments, monomeric, skeletal muscle actin was diluted to 0.4 mg/mL with buffer A (5 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM DTT, 1 mM ATP) and incubated 1 h on ice. Ionic strength was then adjusted by addition of one-tenth volume of APB buffer (0.5 M KCl, 20 mM $MgCl_2$, 1 mM ATP) and the solution was incubated for 1 h at room temperature.

Cosedimentation Assays

To prepare the S4 fraction (Fig. 1A), purification started against actin. Actin filaments (1 mL) were centrifuged at 127,000 × g for 30 min at 24°C to separate filamentous from monomeric actin. The pellet of actin filaments was resuspended in 2 mL of tobacco pollen tube cytosol and incubated for 1 h at room temperature and then centrifuged at 127,000 × g for 30 min at 24°C. The pellet was resuspended with 400 μL of salt extraction buffer (25 mM PIPES, pH 7.0, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, 2 mM PMSF, 1 M KCl) and incubated for 20 min at room temperature to elute proteins from actin filaments. The

solution was centrifuged at 127,000 × g for 30 min at 24°C. The supernatant was diluted 10-fold with a solution containing 1.3 mL of taxol-stabilized microtubules, 20 μM taxol, in a buffer containing 25 mM PIPES, pH 7.0, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 2 mM GTP, thereby bringing the concentration of KCl to 0.1 M and allowing proteins to bind microtubules. This solution was incubated for 30 min at room temperature and then centrifuged at 98,000 × g for 60 min at 25°C. The pellet was resuspended in 300 μL of microtubule depolymerizing buffer (25 mM PIPES, pH 6.8, 1 mM $CaCl_2$, 1 M KCl, 2 mM GTP, 5 mM DTT, 2 mM PMSF), incubated 1 h on ice, and then centrifuged at 98, 000 × g for 20 min at 4°C. The supernatant (S4) was desalted overnight in a cold room by the use of a disposable dialyzer (Sigma, St. Louis, MO) and used for experiments.

To prepare the S4′ fraction, essentially the same procedure was followed, except that purification was started against microtubules. Microtubules polymerized in vitro (1.3 mL) were centrifuged at 98,000 × g for 30 min at 24°C to eliminate monomeric tubulin. The pellet was then suspended with 2 mL of tobacco pollen tube cytosol brought up to 20 μM taxol, incubated for 30 min at room temperature, and then centrifuged at 98,000 × g for 60 min at 25°C. The pellet was suspended with 400 μL of depolymerization buffer (25 mM PIPES, pH 6.8, 1 mM $CaCl_2$, 1 M KCl, 2 mM GTP, 5 mM DTT, 2 mM PMSF), incubated for 1 h on ice, and then centrifuged at 98,000 × g for 20 min at 4°C. The supernatant was diluted 10-fold with 1 mL of actin filaments in buffer (25 mM PIPES, pH 6.8, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, 1 mM PMSF, 2 mM GTP), incubated for 1 h at room temperature, and centrifuged at 127,000 × g for 30 min at 24°C. The pellet was then suspended with 400 μL of salt extraction buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, 2 mM PMSF, 1 M KCl), incubated for 20 min at room temperature, and centrifuged at 127,000 × g for 30 min at 4°C. The supernatant, named S4′, was desalted overnight at 4°C with a disposable dialyzer and used for fluorescence experiments.

Negative Staining

For negative staining, the protein fraction and cytoskeletal polymers were mixed in a microfuge tube at room temperature, with 25 μL of protein solution, 3 μL of actin filament solution, and 2 μL of microtubule solution, in the order and duration indicated. For observation, grids were coated with a Formvar film followed by a layer of evaporated carbon. The carbon layer was activated by treatment with ultraviolet light prior to use. The sample (3.5 μL) was placed on the prepared grid for 10 min followed by addition of 4 μL of ammonium molybdate (pH 7.3) for 2 min. The grid was dried quickly and carefully and observed immediately through a Philips Morgagni 268D transmission electron microscope (TEM) operating at 80 kV and equipped with a Mega View II CCD camera.

Fluorescence Observation

For fluorescent analysis, perfusion chambers were used, made from two strips of double-sided tape separating a slide and cover slip. The chamber was washed once with washing buffer (100 mM PIPES, pH 6.9, 10 mM $MgCl_2$, 10 mM EGTA), sample components perfused, in the indicated order and duration, and then fixation buffer was added (100 mM PIPES, pH 6.9, 10 mM $MgCl_2$, 10 mM EGTA, 0.1% NaN_3, 1.5% formaldehyde, 0.05% glutaraldehyde). In some experiments, the fixation buffer contained 1 μM alexa-phalloidin (Alexa-fluor 568 phalloidin; Invitrogen; Carlsbad, CA) and samples were incubated for 1 h at room temperature before observation. Fluorescent images were observed through a Zeiss Imager Z1 Apotome optical microscope, equipped with a 63× objective. Images were acquired using a AxioCam MRm with AxioVision software.

Gel Electrophoress

Separation of proteins by 1D electrophoresis was carried out on precast gels (Criterion XT Bis–Tris Precast Gel, 10%, 11 cm, Bio-Rad). Gels were run using the Criterion Cell (Bio-Rad) at 200 V for 1 h and then stained with Bio-Safe Coomassie (Bio-Rad). Because of the small quantity of proteins in the S4 and S4′ fractions, protein concentrations were not quantified and these samples were loaded at the maximum possible volume (30 μL). Gel images were captured with the Fluor-S Multi-Imager (Bio-Rad).

Protein Identification by Mass Spectrometry

Protein bands were excised from a Coomassie blue-stained gel and subjected to in-gel digestion with trypsin as described [Hellman et al., 1995; Soskic et al., 1999). Briefly, following destaining (2.5 mM ammonium bicarbonate, 50% acetonitrile), proteins were reduced (10 mM DTT) and then alkylated (50 mM iodoacetic acid). After drying in acetonitrile, the gel pieces were incubated with modified, porcine trypsin (10 ng/μL; Promega, Sunnyvale, CA) in 50 mM ammonium bicarbonate for 16 h at 37°C. The digest was then directly applied on a MALDI-TOF target together with 0.75 μL of matrix solution, composed of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid (TFA). Protein identification was performed using an Ettan MALDI-TOF Pro mass spectrometer (GE Healthcare Bio-Sciences, Uppsala, Sweden).

After tryptic peptide mass acquisition, mass fingerprint searching was performed using Swiss-Prot/TrEMBL and NCBInr databases with MASCOT software (Matrix Science, London, UK, http://www.matrixscience.com). Each spectrum was internally calibrated with the masses of two trypsin autolysis products. Protein identification was achieved on the basis of corresponding experimental and theoretical peptide-mass-fingerprinting data using a peptide mass tolerance of 100 ppm, carbamylation of cysteine residues, and allowance for a single missed tryptic cleavage. The criteria used to accept identifications included the extent of sequence

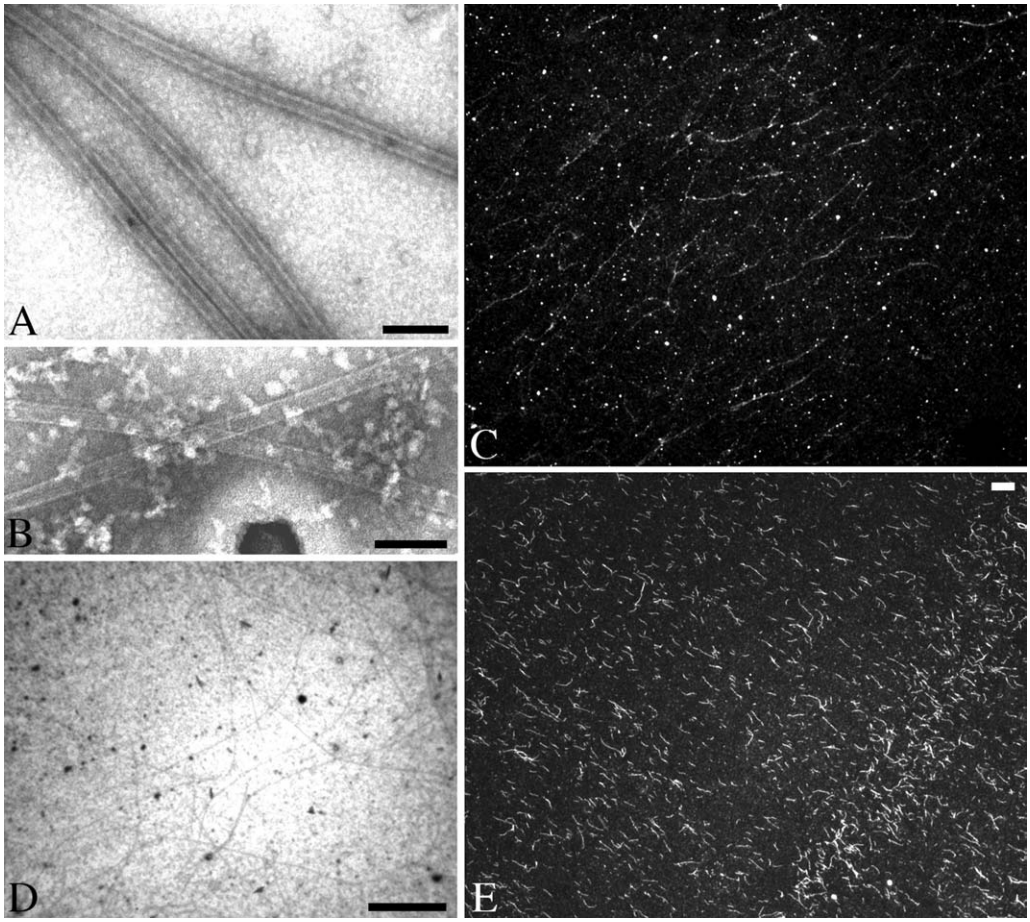

Fig. 2. **Absence of bundles in various conditions**. **A:** Microtubules alone. **B**, **C**: S4 incubated with microtubules alone for 30 min and then assayed by negative stain TEM (B) or fluorescence microscopy (C) based on incorporation of FITC-tubulin into microtubules. **D**, **E**: Actin filaments and microtubules incubated together for 30 min (without S4) and assayed by negative stain TEM (D) and fluorescence from alexa-phalloidin (E). Scale bar in A, B = 100 nm; in D = 200 nm; and in E = 5 μm (same magnification as C).

coverage, number of matched peptides, and probabilistic scores, as reported in Table I.

Identifications were confirmed by ESI–IT MS/MS peptide sequencing on a LCQ DECA Ion Trap mass spectrometer (Thermo Finnigan, San Jose, CA). For this, the tryptic peptide mixture was acidified with 2 μL of 1% TFA, equilibrated in 50% acetonitrile, extensively washed in 0.5% TFA, and then concentrated in ZipTipC18 devices (Millipore, Billerica, MA). Peptide elution was achieved with a 70% methanol, 0.5% formic acid solution. The nanospray method was used to inject 3 μL of the concentrated sample into the spectrometer. MS/MS database searching was performed by TurboSEQUEST (Thermo) and MASCOT MS/MS ion search software.

Results

Tobacco Pollen Tube Cytosolic Proteins Cosediment With Sequential Affinity Purification Against Actin Filaments and Microtubules

From the cytosol of tobacco pollen tubes, we developed a method to isolate a polypeptide fraction that contains proteins able to cosediment with both actin filaments and microtubules based on sequential affinity (Figs. 1A and 1B). After 3 h of germination, tobacco pollen tubes were homogenized and differential centrifugation was used to prepare a cytosolic fraction. The cytosolic fraction was first incubated with polymerized rabbit muscle actin for 1 h at room temperature. Proteins able to bind actin filaments were recovered by sedimentation and then eluted from actin filaments with 1 M KCl. The eluate was then diluted 10-fold into a solution containing microtubules from bovine brain and incubated for 30 min at room temperature. Microtubules were then sedimented and bound proteins again eluted with 1 M KCl. Finally, microtubules were removed from the eluate by centrifugation and the supernatant ("S4") was desalted before use. The S4 fraction appears to contain a significant amount of tubulin and a small amount of actin, as well as a variety of other bands visible by Coomassie staining (Fig. 1C).

Copurified Proteins Bundle Microtubules in the Presence of Actin Filaments

To assay the ability of the S4 fraction to crosslink actin filaments and microtubules, we incubated polymerized actin,

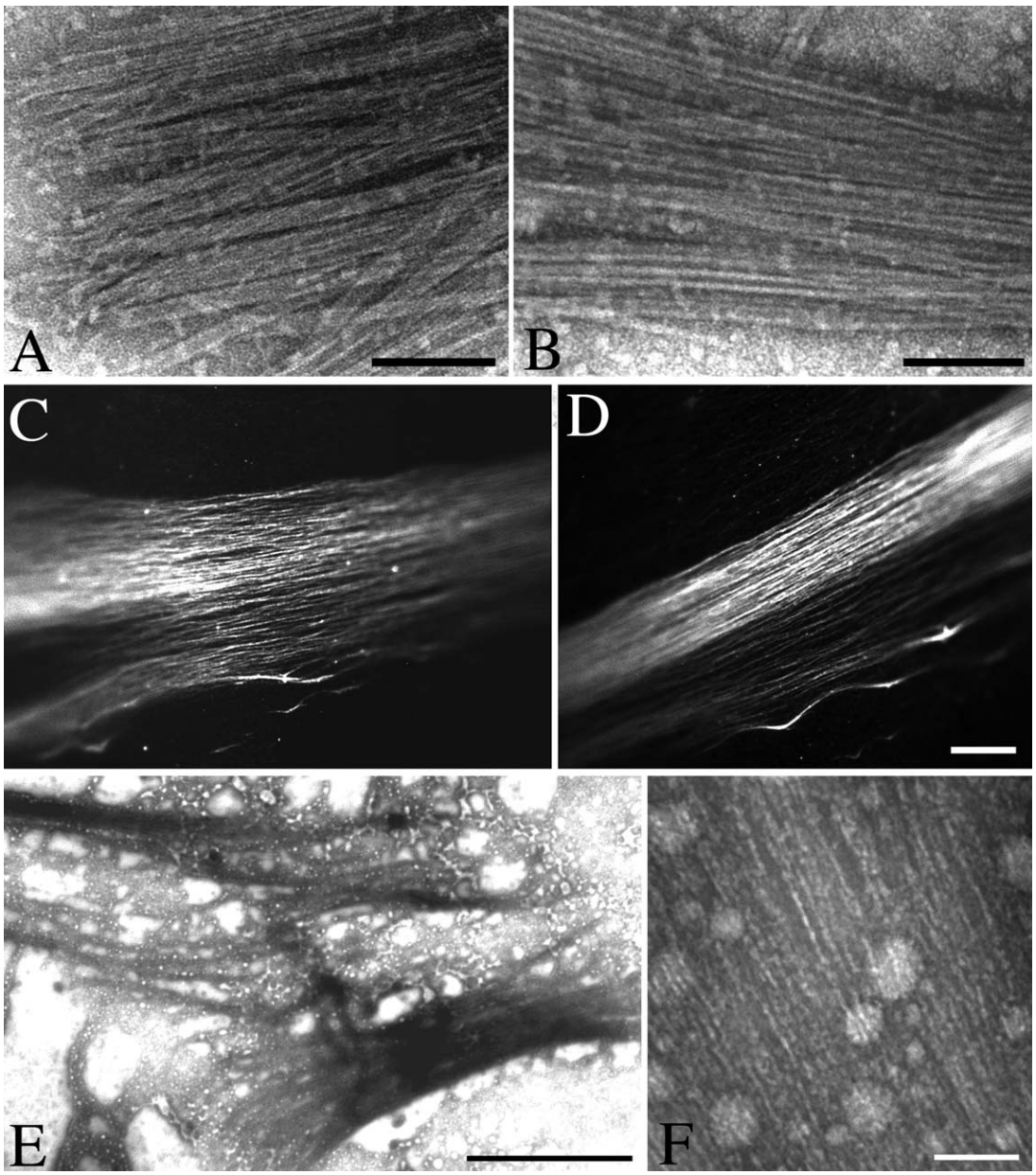

Fig. 3. **Bundles formed in the presence of actin, microtubules, and S4**. The S4 fraction was incubated with actin filaments for 30 min followed by microtubules for 30 min. **A**, **B**: Negative stain TEM. Scale bars = 200 nm. **C**, **D**: Fluorescence microscopy. Tubulin used to make microtubules was derivatized with fluorescein. Scale bar = 10 μm. **E**, **F:** S4 incubated with microtubules for 30 min followed by actin filaments for 30 min. Negative stain TEM. Scale bar = 5 μm in E and 200 nm in F.

taxol-stabilized microtubules, and S4 in various combinations at room temperature. The microtubule preparation alone did not form bundles (Fig. 2A) nor were bundles formed when the S4 fraction was incubated with microtubules for 30 min, as seen both by negative staining in TEM and by fluorescence microscopy when tubulin subunits derivatized with fluorescein were incorporated into the microtubules (Figs. 2B and 2C). Likewise, bundles were absent when actin filaments and microtubules were incubated together but without the S4 fraction (Figs. 2D and 2E).

When the S4 fraction was incubated with actin filaments for 30 min and then taxol-stabilized microtubules added for a further 30 min, strikingly large bundles formed (Figs. 3A and 3B). Microtubules were closely appressed, without clear crosslinking structures. Actin filaments were not visible within the large bundles but were sometimes seen elsewhere on the grid. As an alternative, the experiment was repeated using derivatized tubulin and assayed with fluorescence microscopy. Again, large bundles formed, containing labeled microtubules (Figs. 3C and 3D).

For the bundles shown in Fig. 3 (Figs. 3A–3D), the S4 fraction was incubated with actin for 30 min and then microtubules were added. We also performed the experiment in the reverse order, incubating S4 with microtubules for 30 min and then adding actin filaments. Large bundles again formed (Fig. 3E, note the scale bar = 5 μm). At higher

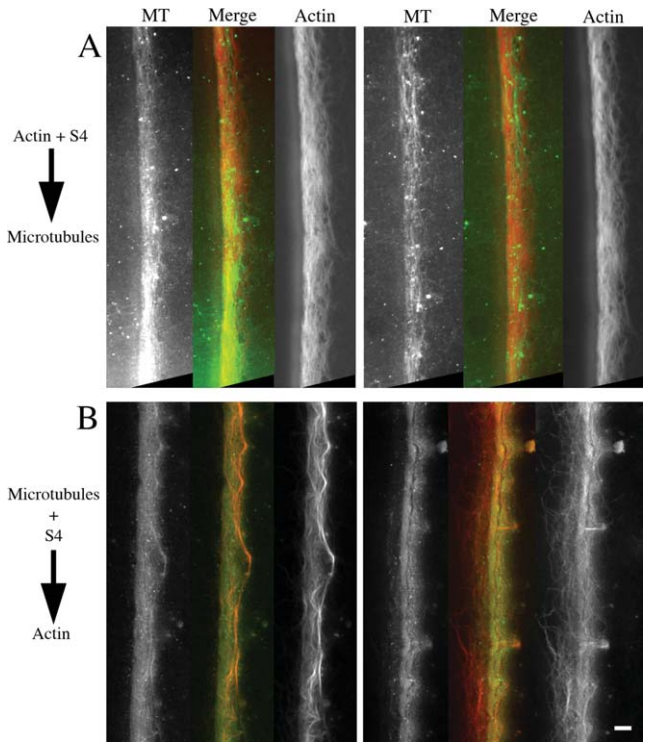

Fig. 4. Colocalization of cytoskeletal polymers. A: S4 incubated for 5 min with actin filaments followed by microtubules for 30 min. **B:** S4 incubated for 5 min with microtubules followed by actin filaments for 30 min. Microtubules incorporated tubulin subunits derivatized with FTIC (green) and actin filaments were stained with alexa-phalloidin (red). Scale bar = 5 μm.

magnification (Fig. 3F), microtubules were not imaged clearly, perhaps because the bundles were too thick.

To determine whether actin filaments and microtubules were present together in the same bundle, we took advantage of double staining with fluorescence microscopy. In these experiments, the S4 fraction was incubated for 5 min at room temperature with actin filaments and then for 30 min with fluorescent microtubules (Fig. 4A); or, S4 was incubated for 5 min with microtubules and then for 30 min with actin filaments (Fig. 4B). To localize actin, fluorescent phalloidin was added after fixation. In whichever order S4 was incubated with microtubules and actin filaments, the bundles that formed contained both polymers.

The S4 fraction induced actin filaments to bundle without microtubules. Actin on its own did not form bundles (Fig. 5A); however, when S4 was incubated with actin filaments for 30 min, bundles formed (Figs. 5B–5D). In negative staining, the separation between neighboring filaments appeared to be somewhat larger than between microtubules (Figs. 3A and 3B) but this was not analyzed quantitatively. That S4 on its own could bundle actin was confirmed with fluorescence (Figs. 5E and 5F). The bundles that formed with S4 and actin filaments were shorter and thinner than those formed when S4 was incubated with both microtubules and actin.

To determine whether the order of affinity purification mattered, we purified a fraction comparable to S4 but starting by incubating cytosol with microtubules instead of with actin filaments, but otherwise prepared in the same way. The resulting fraction, which we call S4′, had a protein profile not unlike that of S4 except that the relative contamination by actin and tubulin was reversed (Fig. 6A). When the S4′ fraction was incubated for 5 min at room temperature with fluorescent microtubules, followed by actin filaments for 30 min, large bundles resulted that again contained both polymers (Fig. 6B).

Taken together, our results show that the pollen tube contains proteins able to bundle actin filaments and to form massive bundles in the presence of both cytoskeletal polymers.

Mass Spectroscopy Identifies Proteins in S4 and S4′

After electrophoretic separation of S4′ and S4 fractions, common bands from both fractions (Fig. 6A, red circles) were excised, destained, digested with trypsin, and subjected to peptide mass fingerprinting (MALDI-TOF MS) and sequencing analyses (ESI-IonTrap MS/MS) followed by database searching. Our MS analysis identified rabbit actin in the S4′ fraction and bovine tubulin in the S4 fraction (Table I), as expected based on probable contamination from the second affinity purification protein. Our analysis allowed the unambiguous identification of four proteins; two of them were clearly visible on the gel for both fractions and two others were faint on the S4′ gel but still detected as peptide fragments (Fig. 6A). The comparison between theoretical and measured molecular weight values contribute to confirming the identifications. Insofar as the complete genome of tobacco is not yet present in UniProt or NCBI databases, the fact that three out of four identifications have been obtained at high confidence from different plant species underscores the potential conservation of these proteins (the fourth was identified based on a deposited sequence from tobacco).

The four identified proteins are associated with metabolism (Table I). The homocysteine methyltransferase (metE) is involved in methionine synthesis and in methyl group homeostasis through regeneration of *S*-adenosyl methionine [Matthews and Goulding, 1997; Ranocha et al., 2001]. The phosphotransferase, better known as phosphofructokinase, is a well characterized glycolytic enzyme. Likewise, pyruvate decarboxylase is well characterized, in this case catalyzing the first step in ethanolic fermentation. Note that pollen tubes appear to carry out ethanolic fermentation even when growing aerobically in culture [Bucher et al., 1995; Mellema et al., 2002; Rounds et al., 2010]. Finally, the glucan protein synthase, which is also known as "reversibly glycosylated protein," is believed to be involved in polysaccharide synthesis in the Golgi apparatus and has recently been shown to be an essential protein for pollen viability [Drakakaki et al., 2006]. Our results suggest that metabolic enzymes associate functionally with the cytoskeleton in the tobacco pollen tube.

Discussion

Crowded as a city street at rush hour, secretory traffic in the pollen tube nevertheless flows rapidly. Traffic in the pollen

Table I. Protein Identification by Mass Spectrometry

	Fraction						Mascot search results		
N°	S4′	S4	Protein name	UniProt	Organism	Theoretical (Exp'l) MW, kDa	Matched peptides	Sequence coverage	Score
1	H[a]	H	5-Methyltetrahydro-pteroyl-triglutamate-homocysteine methyltransferase[b]	P93263	*Mesembryanthemum crystallinum*	85.05 (83)	8	12	82
							AGINVIQIDEAALR[c]		
2	L	H	Pyrophosphate–fructose 6-phosphate 1-phosphotransferase, subunit α[d]	P21342	*Solanum tuberosum*	67.8 (68)	12	25	99
							NPGPLQFDGPGADAK[c]		
3	L	H	Pyruvate decarboxylase isozyme 2	P51846	*Nicotiana tabacum*	68.2 (65)	11	24	123
							TEEELTEAIATATGEK[c]		
4	A	H	Tubulin, β-3 (Bovine)	Q2T9S0	*Bos taurus*	50.8 (50)	17	33	108
5	H	A	Actin, α skeletal muscle (Rabbit)	P68135	*Oryctolagus cuniculus*	42.4 (43)	9	31	138
6	H	H	α-1,4-glucan-protein synthase[e]	P80607	*Zea mays*	41.7 (41)	10	26	107
							GTLFPMCGMNLAFDR[c] YIYTIDDDCFVAK[c]		

Proteins are specified by number (N°), corresponding to the bands shown in Fig. 6A. UniProt lists the accession number from the UniProt database. The Mascot search results show the number of measured peptide masses matching the UniProt entries, the percentage of the protein sequence covered by the matching peptides (sequence coverage), and the probabilistic score. Scores greater than 70 are usually considered significant matches. The highest scoring peptide sequence is shown.

[a]Intensity of band on the gel. H, high; L, low; A, absent.

[b]Also referred to as metE.

[c]Peptide corresponding to MS/MS analysis.

[d]Also known as phophofructokinase, PFK.

[e]Also known as UDP-glucose:protein transglucosylase, UPTG; and as reversibly glycosylated polypeptide, RGP.

tube has long been known to be driven by actin. The actin cytoskeleton mediates cell polarity, the delivery and retrieval of myriad vesicles to and from the tip, and a role has been proposed for actin in the process of expansion itself [Vidali et al., 2001; Cardenas et al., 2008; Cai and Cresti, 2009]. Pollen tubes contain abundant actin, organized into a dense subapical, cortical fringe and into finely divided filaments that fill the shank [Lovy-Wheeler et al., 2005]. Although not as often studied as actin, microtubules are also important for the pollen tube, functioning in the motility of sperm nuclei as well as of organelles [Joos et al., 1994; Cai and Cresti, 2009]. A recently recognized role for microtubules appears to be in forging straight as opposed to wandering growth, a role that has been argued to be more important when the tube is mechanically constrained inside the style than when the tube grows in liquid in a Petri dish [Gossot and Geitmann, 2007].

We hypothesized that, in the pollen tube, the two cytoskeletal systems function together. Indeed, close association between actin and microtubules observed with TEM [Lancelle and Hepler, 1991], and the organization of actin and microtubules appears to be strictly coordinated in the vicinity of the subapical actin fringe [Lovy-Wheeler et al., 2005]. Furthermore, inhibitor experiments have revealed interdependence of the cytoskeletal elements, albeit for conifer pollen tubes [Justus et al., 2004] but not lily [Lovy-Wheeler et al., 2007], and at least one protein from pollen tubes is already known that can bind both actin and microtubules [Huang et al., 2007; Liu et al., 2009]. Finally, mitochondria interact with both microtubules and actin when assayed in vitro within mixed networks [Romagnoli et al., 2007]. Here, we sought to identify proteins that are able to interact with both types of filament, expecting to find motor proteins or other canonical cytoskeletal-associated proteins. To our surprise, the proteins recovered are all metabolic enzymes.

One explanation is that our identification is spurious, based for example on contaminating proteins. We discount this explanation for several reasons. First, the same set of proteins were uncovered when either actin filaments or microtubules was used as the first affinity step. While highly abundant proteins are expected contaminants, none of the identified proteins are known as highly abundant pollen tube proteins. Second, in animal cells, phosphofructokinase has long been known to interact with microtubules [Lehotzky

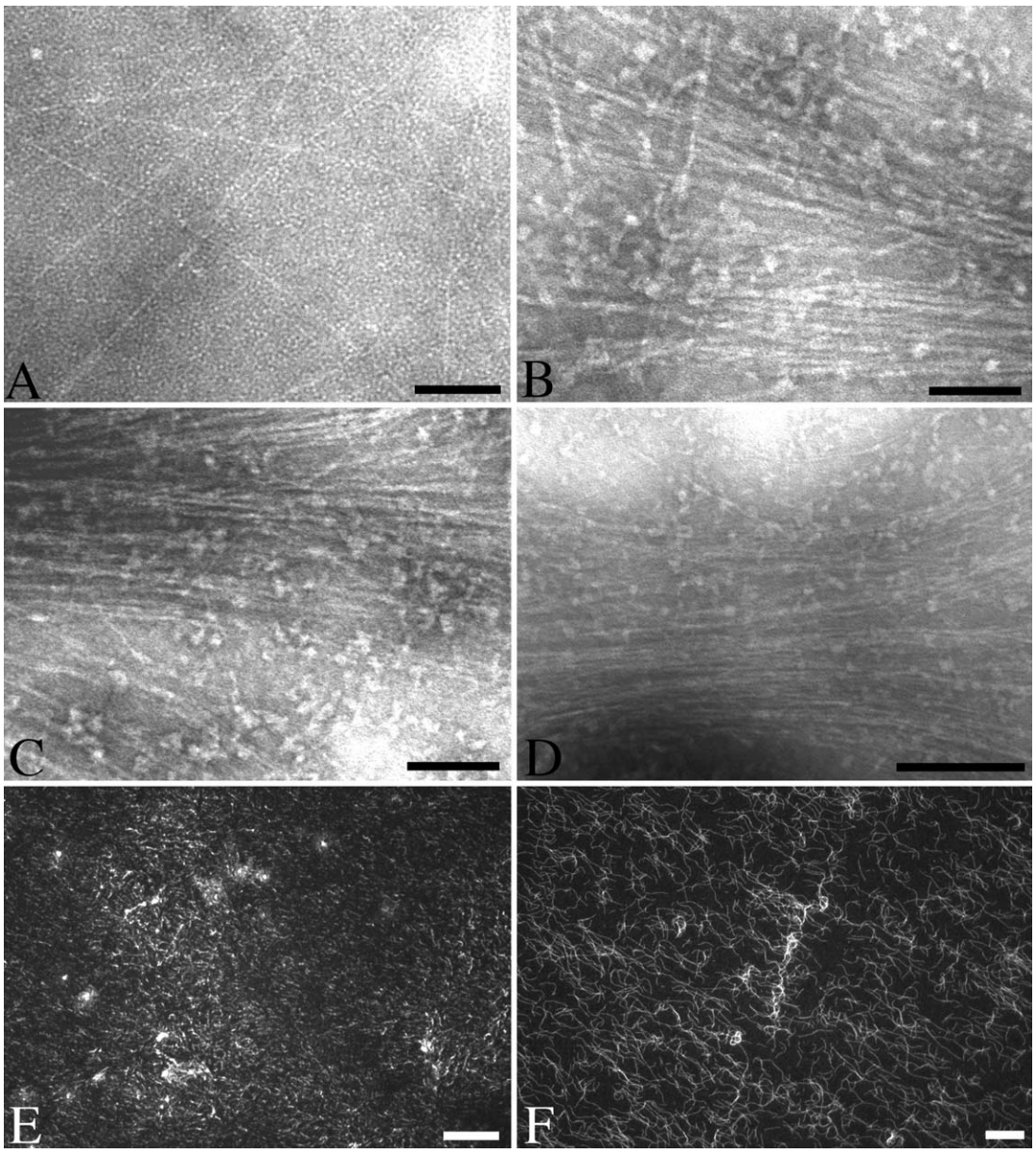

Fig. 5. **The effects of S4 on actin filaments**. **A**, **E:** Actin filaments incubated alone for 30 min. **B–D**, **F:** S4 incubated with actin filaments for 30 min. A–D are negative stain TEM; E, F are fluorescence from alexa-phalloidin added after fixation. Scale bar in A–D = 100 nm and 10 μm in E and F.

et al., 1993]; and in addition, a protein closely related to metE has been identified as being microtubule associated [Sandu et al., 2000]. Third, metE itself has been identified previously in tobacco pollen tube cytosol as putatively colocalizing with microtubules by immunofluorescence [Moscatelli et al., 2005]. Finally, in a characterization of the proteome of microtubule-associated proteins from arabidopsis tissue culture cells, both metE and the α-1,4 glucan protein synthase are among the proteins present in a fraction cosedimenting with microtubules (T. Hamada and T. Hashimoto, Nara Institute of Science and Technology, personal communication).

Unfortunately, neither S4 nor S4′ were prepared in quantities sufficient to allow us to test the activity of single proteins. Therefore it is not clear whether the bundling activities reflect the action of a single protein or of a complex. Insofar as the identified proteins function in distinct pathways, there is no reason to expect these proteins on their own to form a complex.

The most well known function of the cytoskeleton is organelle transport. In this connection, it is noteworthy that both metE [Moscatelli et al., 2005] and protein glucan synthase [Drakakaki et al., 2006] have been localized to membranous compartments of the Golgi apparatus. Therefore, the enzymes recovered here might have acquired a second function, helping to target or move vesicles along cytoskeletal tracks, or in interconnecting the two networks.

Alternatively, cytoskeletal anchoring might aid in the metabolic function of these enzymes. Though less well studied

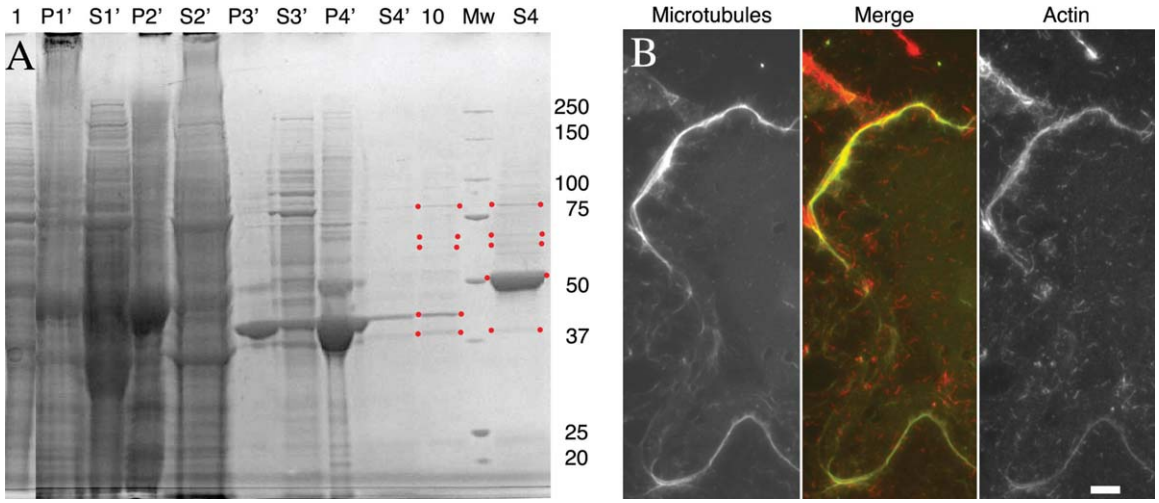

Fig. 6. **Preparation and activity of S4′ fraction**. **A:** Coomassie-stained gel showing the steps of purification. The steps diagrammed in Fig. 1 were followed except that cytosol was incubated with microtubules for the first affinity step and S2′ was incubated with actin filaments for the second affinity step. Lane 1 is starting cytosol fraction from tobacco pollen tubes. Lane 10 is the desalted S4′ fraction, which was used for experiments. Red circles in lanes 10 (S4′) and S4 indicate the position of the bands excised for MS analysis, numbered from 1 (80 kDa) to 6 (40 kDa). Band 4 was absent from S4′ and band 5 absent from S4. Relative molecular mass is shown in kDa to the right of the gel. **B:** The S4′ fraction was incubated with microtubules for 5 min followed by actin filaments for 30 min. Microtubules incorporated tubulin subunits derivatized with FTIC (green) and actin filaments were stained with alexa-phalloidin (red). Scale bar = 5 μm.

than transport, the cytoskeleton is also thought to function in metabolism. Anchoring metabolic enzymes along the cytoskeleton has been shown to be advantageous theoretically and in some cases confirmed experimentally [Ovádi and Srere, 2000; Zhou et al., 2008]. The clearest evidence for this comes from muscle cells or neurons, which are active cells with a highly organized internal structure. Although pollen tubes might not have as structured cytoplasm as muscle or nerve, they are arguably as active, growing at 100s of micrometers per hour, usually for many hours, a rate exceeding that of nerve cells by more than an order of magnitude. Therefore, it is reasonable to suggest that this cell type also exploits thermodynamic benefits from structured metabolism.

The bundles that form in our experiments in vitro are far larger than any structures present in the pollen tube. It was originally thought that actin in the tube shaft formed thick bundles, prominently running up the center of the cell. More recently, with improved fixation [Lovy-Wheeler et al., 2005] and with live cell imaging [Wilsen et al., 2006; Vidali et al., 2009], it has become clear that actin filaments are for the most part fine, and permeate the cytosol within the tube shank rather evenly. Possibly, the double affinity purification used here concentrated the proteins and enabled them to act more strongly than in vivo. In a living cell, if the cytoskeleton is to be a dynamic and flexible framework, then its struts should be linked but not too tightly. We hypothesize that the proteins identified here provide transient crosslinks to integrate actin filaments and microtubules in the pollen tube cytoplasm.

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