

Polarized cell growth, organelle motility, and cytoskeletal organization in conifer pollen tube tips are regulated by KCBP, the calmodulin-binding kinesin

Mark D. Lazzaro · Eric Y. Marom ·
Anireddy S. N. Reddy

Received: 8 May 2013 / Accepted: 11 June 2013 / Published online: 20 June 2013
© Springer-Verlag Berlin Heidelberg 2013

Abstract Kinesin-like calmodulin-binding protein (KCBP), a member of the Kinesin 14 family, is a minus end directed C-terminal motor unique to plants and green algae. Its motor activity is negatively regulated by calcium/calmodulin binding, and its tail region contains a secondary microtubule-binding site. It has been identified but not functionally characterized in the conifer *Picea abies*. Conifer pollen tubes exhibit polarized growth as organelles move into the tip in an unusual fountain pattern directed by microfilaments but uniquely organized by microtubules. We demonstrate here that PaKCBP and calmodulin regulate elongation and motility. PaKCBP is a 140 kDa protein immunolocalized to the elongating tip, coincident with microtubules. This localization is lost when microtubules are disrupted with oryzalin, which also reorganizes microfilaments into bundles. Colocalization of PaKCBP along microtubules is enhanced when microfilaments are

disrupted with latrunculin B, which also disrupts the fine network of microtubules throughout the tip while preserving thicker microtubule bundles. Calmodulin inhibition by W-12 perfusion reversibly slows pollen tube elongation, alters organelle motility, promotes microfilament bundling, and microtubule bundling coincident with increased PaKCBP localization. The constitutive activation of PaKCBP by microinjection of an antibody that displaces calcium/calmodulin and activates microtubule bundling repositions vacuoles in the tip before rapidly stopping organelle streaming and pollen tube elongation. We propose that PaKCBP is one of the target proteins in conifer pollen modulated by calmodulin inhibition leading to microtubule bundling, which alters microtubule and microfilament organization, repositions vacuoles and slows organelle motility and pollen tube elongation.

Electronic supplementary material The online version of this article (doi:10.1007/s00425-013-1919-8) contains supplementary material, which is available to authorized users.

M. D. Lazzaro (✉) · E. Y. Marom
Department of Biology, College of Charleston, Charleston,
SC, USA
e-mail: lazzarom@cofc.edu

E. Y. Marom
e-mail: eymarom@gmail.com

M. D. Lazzaro
School of Biological Sciences, University of Sydney, Sydney,
NSW, Australia

A. S. N. Reddy
Department of Biology, Program in Molecular Plant Biology,
Program in Cell and Molecular Biology, Colorado State
University, Fort Collins, CO, USA
e-mail: reddy@lamar.colostate.edu

Keywords Calmodulin · Cytoskeleton · KCBP · Kinesin
14 · Norway spruce · *Picea abies* · Pollen tube · W-12

Introduction

Pollen tubes are an established model system for examining polarized cell growth. In angiosperms, pollen tube elongation is very fast ($300\text{--}1,500\ \mu\text{m h}^{-1}$) and is localized exclusively to the apex of the cell. These highly polarized cells possess a clear zone at their apex, which contains an inverted cone of secretory vesicles, and surrounding mitochondria, Golgi dictyosomes, ER, but lacking amyloplasts and vacuoles. There is a highly organized array of actin microfilaments as a fringe in the clear zone starting $1\text{--}5\ \mu\text{m}$ back from the tip and confined largely to the cell cortex (Lovy-Wheeler et al. 2005). These microfilaments, together with myosin, drive cytoplasmic streaming and

create the pattern of reverse fountain flow in which vesicles and organelles flow towards the tip along the edge of the cell, and towards the base through the center of the tube (reviewed in Hepler et al. 2001; Cheung and Wu 2008). This cytoplasmic streaming transports secretory vesicles to the apical clear zone, where they eventually fuse with the plasma membrane. Elongation and cytoplasmic streaming are sensitive to alterations in microfilament organization (Heslop-Harrison and Heslop-Harrison 1989; Gibbon et al. 1999). Microtubules, by contrast, are largely absent from the pollen tube apex (Lancelle and Hepler 1992; Pierson et al. 1986; Cheung et al. 2008), and instead are present as a cortical fringe, distal to the actin fringe and thus several microns from the apex (Foissner et al. 2002; He and Wetzstein 1995; Lovy-Wheeler et al. 2005). Their disruption has little effect on elongation in angiosperm pollen tubes (Heslop-Harrison et al. 1988; Åström et al. 1995) but they may influence the direction of growth (Gossot and Geitmann 2007). The control of pollen tube elongation and the underlying motile processes depends on many factors including oscillating intracellular tip-focused calcium (Holdaway-Clarke et al. 1997; Messerli et al. 1999) and pH gradients (Feijó et al. 1999) coordinated by Rop GTPases (Yang 2002). Finally, calmodulin activity exhibits an oscillatory tip-focused gradient (Rato et al. 2004) as it influences elongation of flowering plant pollen tubes (Obermeyer and Weisenseel 1991; Estruch et al. 1994).

Conifer pollen tubes grow much slower than their angiosperm counterparts, with rates in culture of $20 \mu\text{m h}^{-1}$ for *Picea abies* (Anderhag et al. 2000) compared to $300\text{--}1,500 \mu\text{m h}^{-1}$ in culture for flowering plants (Pierson et al. 1996; Parton et al. 2001). The conifer pollen tube must elongate through several millimeters of megagametophyte tissue to reach the egg cell, and since the spermatogenous cells initially remain within the pollen grain, callose plugs cannot form to isolate the elongating apical region of the tube (Dawkins and Owens 1993; Runions and Owens 1999). The elongating conifer pollen tube tip does not contain an inverted cone of secretory vesicles. Instead a clear zone lacking amyloplasts but enriched in mitochondria and the endomembrane system extends $20\text{--}30 \mu\text{m}$ back from the tip. There is a demarcation running perpendicular to the tube axis between this clear zone and the amyloplasts in the rest of the pollen tube (de Win et al. 1996; Lazzaro 1996). Organelles do not typically stream in a reverse fountain pattern in conifer pollen tubes. Instead the dominant pattern in *Pinus sylvestris* and *P. abies* pollen tubes is a fountain, with organelles moving towards the tip in the tube center and away from the tip along the cell cortex (de Win et al. 1996; Justus et al. 2004). This pattern coincides with, and is regulated by an interplay between microtubules and microfilaments (Terasaka and Niitsu 1994; Lazzaro 1996; Lazzaro 1999; Anderhag et al. 2000). Microtubules control the

positioning of organelles into and within the tip and influence the direction of streaming by mediating microfilament organization (Justus et al. 2004). Microfilaments, presumably together with myosin, generate the force for motion, since their disruption completely stops growth (Anderhag et al. 2000) and disrupts vesicle trafficking within the tip altering cell wall construction (Wang et al. 2006; Chen et al. 2007). However, microtubule disruption also stops growth, alters organelle motility within the tip, and alters the organization of actin microfilaments (Anderhag et al. 2000; Justus et al. 2004; Zheng et al. 2010).

It is particularly noteworthy that propyzamide and oryzalin, anti-microtubule agents, cause the accumulation of membrane tubules or vacuoles in the tip that reverse direction and stream in a reverse fountain as microfilaments reorganize into pronounced bundles in the tip cytoplasm (Justus et al. 2004). Elongating conifer pollen tubes exhibit a modest, twofold tip-focused calcium gradient (Lazzaro et al. 2005). Disruption of this gradient by external perfusion of lanthanum or gadolinium to block calcium channels on the plasma membrane stops elongation but does not markedly affect cytoplasmic streaming. During recovery from lanthanum perfusion, as the tip-focused calcium gradient returns, organelle motility initially slows and then switches to a reverse fountain pattern, reminiscent of that seen following microtubule disruption (Lazzaro et al. 2005). Disruption of the tip-focused calcium gradient alters the deposition of cell wall components and causes the accumulation of callose (Chen et al. 2008), which is normally excluded from the apical cell wall of elongating conifer pollen tubes (Lazzaro et al. 2003).

Since microtubules have a functional role in conifer pollen tube elongation (Anderhag et al. 2000; Justus et al. 2004) and calcium influences regulation of the cytoskeleton (reviewed in Cheung and Wu 2008; Dodd et al. 2010), we are interested in proteins that affect microtubule organization and are regulated by calcium. KCBP is a minus end directed motor protein found only in photosynthetic organisms, and a member of the kinesin 14 family (Abdel-Ghany et al. 2005). Calcium and calmodulin, which bind the unique calmodulin-binding domain in KCBP, inhibit the interaction of the motor with microtubules and down regulate its ATPase and motor activity (Narasimhulu et al. 1997; Song et al. 1997; Deavours et al. 1998; Narasimhulu and Reddy 1998). KCBP exhibits microtubule bundling activity within the motor domain and the tail region (Kao et al. 2000).

KCBP is not only involved in cell division (Bowser and Reddy 1997; Smirnova et al. 1998; Vos et al. 2000; Preuss et al. 2003; Dymek et al. 2006) but also functions during interphase in specific cells, including algae flagella and elongating plant cells. KCBP is localized at the base of flagella in *Chlamydomonas reinhardtii* (Dymek et al. 2006)

and in flagella of from *Dunaliella salina* (Shi et al. 2013). In *Gossypium hirsutum* fibers, KCBP co-localizes along the length of microtubule bundles (Preuss et al. 2003). Genetic studies in *Arabidopsis thaliana* indicate that KCBP has a role in cells that exhibit polarized growth, where mutations in the *ZWICHEL* gene (*ZWI*), which codes for KCBP, caused a reduction in trichome height and branching (Krishnakumar and Oppenheimer 1999). KCBP has also been specifically identified (PaKCBP) in the conifer *P. abies* (Abdel-Ghany et al. 2005) but not functionally characterized.

We demonstrate here that polarized cell growth and organelle motility in conifer pollen tubes are both regulated by Ca^{2+} /calmodulin and PaKCBP. Perfusion of the calmodulin antagonist W-12 significantly and reversibly slows pollen tube elongation and disrupts organelle motility, and induces microtubule bundling in the tip. Constitutive activation of PaKCBP stops organelle streaming and pollen tube elongation. PaKCBP immunolocalization is concentrated in the tip, coincident with and dependent on both microtubule and microfilament bundles. We propose that in conifer pollen tubes, PaKCBP bundling activity is normally reduced by Ca^{2+} /calmodulin inhibition. When this inhibition is lost, the microtubule bundling activity of PaKCBP increases to alter the organization of microtubules and microfilaments in the tip, reducing pollen tube elongation and causing the reorganization or cessation of organelle motility within the tip. In conifer pollen tubes, PaKCBP is one of the proteins that influence how the interplay between microtubules and microfilaments governs organelle motility and polarized cell growth.

Materials and methods

Perfusion

Pollen was field collected from *P. abies* trees near Stockholm Sweden and stored at $-20\text{ }^{\circ}\text{C}$. This pollen was germinated for 24 h at $30\text{ }^{\circ}\text{C}$ in liquid media (10 % sucrose, 1 mM CaCl_2 , 1 mM H_3BO_3 , 15 mM MES buffer, pH 4.0). For perfusion and microinjection experiments, aliquots of elongating pollen tubes were embedded on cover slip slides in liquid media plus 1.5 % low melt agarose (type VII) and observed on an inverted microscope. Pollen tubes were continuously perfused with liquid media at $260\text{ }\mu\text{l min}^{-1}$. A subset of pollen tubes was exposed to *N*-(4-aminobutyl)-2-naphthalenesulfonamide hydrochloride (W-12) by changing to a perfusion solution of liquid media plus $100\text{ }\mu\text{M}$ W-12 (Rato et al. 2004) for 10 min, followed by a return to normal liquid media. Pollen tubes were imaged throughout perfusion by capturing digital video at one frame per second with IP Lab software. Elongation was

measured by tracking the pollen tube tip with Image J software.

Protein isolation

A large volume of pollen tubes (30 mls) was grown for 24 h at $30\text{ }^{\circ}\text{C}$ in liquid media (10 % sucrose, 1 mM CaCl_2 , 1 mM H_3BO_3 , 15 mM MES buffer, pH 4.0) and then concentrated by centrifugation at $1,000\times g$ for 2 min. A $200\text{ }\mu\text{l}$ aliquot of a plant cell-specific protease inhibitor cocktail (Sigma #P9599) containing 4-(2-aminoethyl) benzenesulfonyl fluoride, bestatin, pepstatin A, E-64, leupeptin, and 1,10-phenanthroline was mixed with the $1,000\text{ }\mu\text{l}$ pellet. Proteins were extracted by boiling for 5 min in sample buffer (20 % sodium dodecyl sulfate, 10 % glycerol, 5 % beta mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8). Proteins were separated on 12 % acrylamide gels and either stained with Coomassie for total protein or transferred to nitrocellulose and probed with affinity-purified rabbit polyclonal antibody to a calmodulin-binding peptide in AtKCBP (Bowser and Reddy 1997), then goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, followed by detection with Opti 4CN (Bio-Rad).

Confocal microscopy

Pollen tubes growing in liquid culture media were exposed to $100\text{ }\mu\text{M}$ W-12 (Rato et al. 2004), $50\text{ }\mu\text{M}$ oryzalin (Justus et al. 2004) or 5 nM latrunculin B (Justus et al. 2004) for 30 min by diluting stock solutions into liquid media. Pollen tubes were then fixed using a method modified from Lovy-Wheeler et al. (2005), where proteins are cross-linked during fixation with Bis(sulfo-*N*-succinimidyl) ethylene glycol disuccinate (sulfo-EGS). In detail, pollen tubes in liquid culture media were mixed 1:1 with a $2\times$ fixative solution containing 2 mM sulfo-EGS, 3.2 % formaldehyde, 0.1 % glutaraldehyde, 0.1 % TX-100, 1 mM CaCl_2 and 10 mM MgSO_4 in 15 mM PIPES (pH 9.0) such that the final solution contained 1 mM Sulfo-EGS, 1.6 % formaldehyde, 0.05 % glutaraldehyde, 0.05 % TX-100, 5 % sucrose, 1 mM CaCl_2 , 0.5 mM H_3BO_3 , 5 mM MgSO_4 , 7.5 mM MES and 7.5 mM PIPES. Pollen tubes were fixed for 1 h with gentle rotation. To label microfilaments, fixed pollen tubes were rinsed several times in 100 mM PIPES (pH 7.0) containing 0.5 % TX-100, then incubated for at least 1 h in the same buffer plus $0.66\text{ }\mu\text{M}$ Alexa-488 phalloidin (Molecular Probes). To immunolabel microtubules and KCBP, fixed pollen tubes were drop frozen in liquid nitrogen and ground gently with a mortar and pestle to fracture the cell walls. Pollen tubes were then thawed in extraction buffer (phosphate-buffered saline, 5 mM EGTA, 1 % BSA, 1 % TX-100) for 1 h, rinsed in blocking buffer (PBS, 1 % BSA, 0.5 % TX-100) and double labeled with the B512 mouse

monoclonal antibody to alpha-tubulin (Sigma) followed by a fluoronanogold secondary antibody (Nanoprobes) conjugated to Alexa 488, and the rabbit polyclonal antibody to the calmodulin-binding peptide in AtKCPB (Bowser and Reddy 1997) diluted 1:1,000 followed by fluoronanogold secondary antibodies (Nanoprobes) conjugated to Alexa 594. As a control, rabbit pre-immune serum was substituted for AtKCPB antibodies using the same dilution.

Cells were examined by collecting paired optical serial sections with a Zeiss LSM5 Pascal confocal microscope using the 488 Argon laser and the 543 HeNe laser. Confocal settings were identical for all KCBP and preimmune control imaging. Binding of PaKCBP to microtubules in cells was identified and quantitated using the Image J software colocalization plugin which intercalates the 488 and 543 nm sections and identifies colocalized points when labeling intensity is above the threshold values for both channels. Threshold values were determined by evaluating plot profiles of pixel gray values across single line transects through optical sections and were 50 for 488 nm (tubulin) sections and 20 for 543 nm (PaKCBP) sections. These values were chosen to minimize the inclusion of non-specific fluorescence as KCBP labeling and were used for all section analysis. We quantitated experimentally induced changes in PaKCBP labeling as a ratio of the number of colocalized pixels divided by the total pixels above threshold for 488 nm (tubulin) in optical sections. This ratio is an estimate of the percentage of microtubules with attached PaKCBP.

Microinjection

Using a method modified from Vos et al. (2000), actively growing pollen tubes were pressure injected with affinity-purified antibodies for a synthetic 23-amino acid polypeptide containing the Ca^{2+} /calmodulin-binding domain of AtKCBP (Narasimhulu et al. 1997). In the presence of these antibodies, the interaction of KCBP with microtubules is not inhibited by Ca^{2+} /calmodulin, so these antibodies are thought to constitutively activate KCBP (Narasimhulu et al. 1997; Narasimhulu and Reddy 1998; Vos et al. 2000). Antibodies were in a solution of 5 mM HEPES, pH 7.0, 100 mM KCl, and FITC-dextran (4.4 kDa). Controls were injected with pre-immune serum in the same solution. The needle protein concentration was 0.8 mg ml^{-1} for both the antibody and pre-immune serum injections. Needles were inserted about 50 μm back from the tip, just behind the clear zone (Lazzaro et al. 2005). Successful microinjections were verified by briefly observing the localization of FITC-dextran in the cytoplasm. Cells were under continuous perfusion of liquid media ($260 \mu\text{l min}^{-1}$) throughout microinjection and imaged at one frame per second with IP lab software.

Statistics

Changes in elongation rates before, during and after W-12 perfusion and changes in elongation rates before and after antibody microinjection were statistically evaluated with pairwise *t* tests ($p < 0.05$). Experimentally induced changes in the colocalization ratio of PaKCBP immunolabeling to tubulin immunolabeling as an estimate of the percentage of PaKCBP labeling on microtubules were statistically compared with heteroscedastic *t* tests ($p < 0.05$). All data are reported as mean \pm standard error of the mean with sample size and *p* value.

Results

Calmodulin inhibition stops conifer pollen tube elongation

Since active calmodulin is required for pollen tube elongation in lily and tobacco (Rato et al. 2004), we treated elongating *P. abies* pollen tubes with 100 μM W-12, a calmodulin antagonist. Perfusion of W-12 for 10 min effectively stops elongation (Fig. 1a), with average rates significantly dropping from 11.85 to 0.75 $\mu\text{m h}^{-1}$ (Table 1). The thin tubules and vesicles streaming into the tip are also replaced by thicker vacuolar tubules as elongation slows (Fig. 1b, video-Online Resource 1). These effects were not immediate. Since the perfusion rate was $260 \mu\text{l min}^{-1}$ and the chamber volume was 500–750 μl , it took 2–3 min to completely change solutions and reach an effective concentration of 100 μM W-12. Elongation initially slowed after 5 min of W-12 perfusion and completely stopped after another 10–15 min (Fig. 1a). The effect of W-12 was reversible, pollen tubes recovered to elongation rates that did not significantly differ from initial values (Fig. 1a; Table 1). During recovery, the tubules migrated away from the tip as the organization of the original clear zone was recovered (Fig. 1b). Elongation rates were unchanged in pollen tubes treated with control perfusions (Table 1).

PaKCBP concentrates in the elongating tip coincident with microtubules

Affinity-purified antibodies to a 23-amino acid peptide of AtKCBP recognize a 140 kDa protein expressed in *P. abies* pollen tubes (Fig. 2a), which is the predicted molecular weight for PaKCBP based on the DNA sequence (Abdel-Ghany et al. 2005). PaKCBP is detected along the length of the pollen tube, and is concentrated in the elongating tip (Fig. 2b) while control pollen tubes treated with pre-immune serum did not exhibit labeling in epifluorescent images (Fig. 2c). A dense network of microtubules (Fig. 3e, magenta) is concentrated within the tip, coincident with the

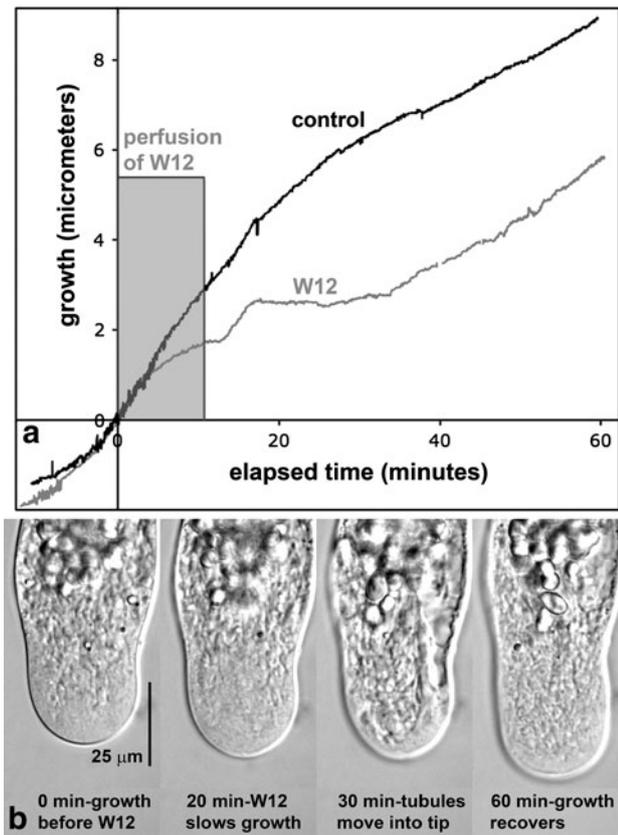


Fig. 1 Active calmodulin is required for pollen tube elongation. **a** Pollen tubes under continuous perfusion were exposed to a 10-min pulse of 100 μ M W-12 in growth media which stopped elongation ($n = 7$ pollen tubes, one representative tube shown in *gray*). Elongation recovered to original rates following the removal of W-12 by perfusion of growth media. Control pollen tubes were perfused continuously with growth media and continued to elongate ($n = 6$ pollen tubes, one representative tube shown in *black*). **b** As elongation stops following W-12 perfusion (30 min) tubules moved into the tip as the clear zone was disrupted. During recovery from calmodulin inhibition (60 min), the clear zone was restored as elongation resumed. See also video-Online Resource 1

microfilaments (Fig. 3a, cyan) responsible for the fountain-streaming pattern observed in conifer pollen tubes (Justus et al. 2004). We note that the inclusion of sulfo-EGS during fixation markedly enhanced the preservation of both microtubules and microfilaments, although we did not observe the actin collar or fringe present in flowering plant pollen tube tips (Lovy-Wheeler et al. 2005).

To evaluate the bundling effects of PaKCBP, we calculated the amount of PaKCBP colocalized to tubulin as a percentage of the total tubulin observed in confocal thin sections (Fig. 3j–m, green). PaKCBP labeling was detected on 3.00 % of observed microtubules (Fig. 3o, white PaKCBP on magenta microtubules, and Table 2). This is significantly greater (based on a heteroscedastic *t* test) than background levels of the percentage of microtubules colabeled with preimmune serum (Fig. 3i, n; Table 2). The localization of PaKCBP is dependent on intact microtubules. When pollen tubes are treated with 50 μ M oryzalin for 30 min, which reduces elongation and causes streaming to switch direction to a reverse fountain as vacuoles move into the tip (Justus et al. 2004), microtubules are reduced to punctate structures (Fig. 3f), and PaKCBP labeling is significantly reduced to background levels observed in pre-immune serum controls (Fig. 3k, p; Table 2). Interestingly, microfilaments form more pronounced bundles following oryzalin treatment (Fig. 3b). The localization of PaKCBP is also indirectly influenced by microfilaments. When pollen tubes are treated with 5 nM latrunculin B for 30 min, which stops elongation and streaming (Justus et al. 2004), microfilaments are disrupted (Fig. 3c). In addition, the network of fine microtubule bundles is lost, while thicker microtubule bundles remain (Fig. 3g). A significantly higher percentage of these microtubule bundles (13.11 %) are colabeled for PaKCBP (Fig. 3l, q; Table 2).

Calmodulin inhibition promotes microtubule and microfilament bundling

Calmodulin is likely to regulate many proteins controlling organelle motility and pollen tube elongation in *P. abies*. Since calmodulin inhibition promotes the microtubule bundling activity of KCBP (Narasimhulu et al. 1997; Narasimhulu and Reddy 1998; Kao et al. 2000), we examined cytoskeletal organization following calmodulin inhibition. A subset of pollen tubes were treated for 30 min with 100 μ M W-12 when elongation has reversibly stopped as vacuolar tubules streamed into the tip (Fig. 1a, b) and then rapidly frozen and prepared for immunolabeling of the cytoskeleton. Treatment with W-12 caused the formation of more pronounced bundles of microtubules in pollen tube tips (Fig. 3h), coincident with significantly increased

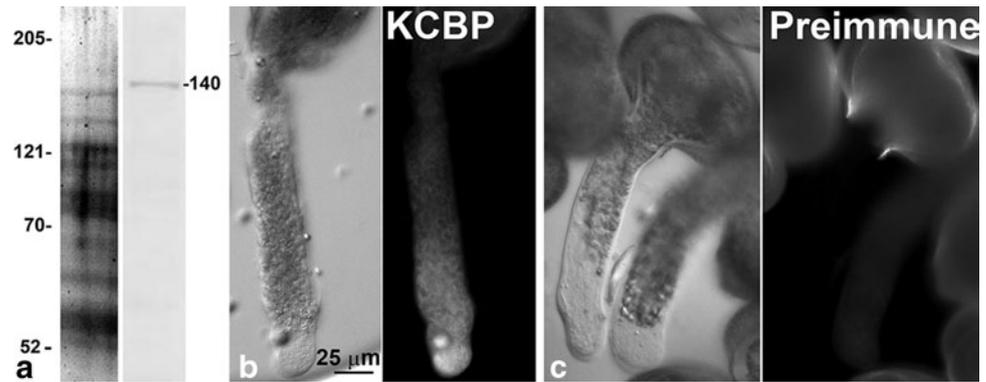
Table 1 Changes in elongation rates during perfusion of calmodulin inhibitor W-12

	Before	During	After	<i>P</i> value before:during	<i>P</i> value before:after	<i>n</i>
W-12	11.85 \pm 2.19	0.75 \pm 0.49*	12.38 \pm 4.89	0.035	0.902	7
Control	11.68 \pm 3.62	12.04 \pm 3.61	12.56 \pm 3.92	0.234	0.118	6

Values are mean \pm standard error (μ m h⁻¹)

* Indicates significant difference based on pairwise *t* test ($p < 0.05$)

Fig. 2 PaKCBP (140 kDa) is expressed in *Picea abies* pollen tubes. **a** Coomassie stain (lane 1) and immunoblot (lane 2) of total protein extracts from germinated pollen tubes. **b** PaKCBP is immunolocalized throughout the tube and concentrated within the elongating tip. **c** Pollen tubes treated with preimmune serum and imaged with the same epifluorescent settings have no background fluorescence



co-localization of PaKCBP on 6.01 % of microtubules (Fig. 3m, r; Table 2). W-12 treatment also enhanced the formation of microfilaments bundles within the tip (Fig. 3d).

Constitutive activation of PaKCBP stops organelle streaming

Since calmodulin inhibition stopped elongation, altered organelle motility, and altered the cytoskeleton coincident with increased PaKCBP localization, we examined how the constitutive activation of PaKCBP affects organelle motility. A subset of pollen tubes was microinjected with affinity-purified antibodies to a regulatory calmodulin-binding peptide of KCBP identified in *A. thaliana* (Narasimhulu et al. 1997). This antibody displaces Ca^{2+} /calmodulin (Bowser and Reddy 1997) and its microinjection constitutively activates KCBP in *Tradescantia virginiana* (Vos et al. 2000). Before microinjection, organelles streamed in a fountain pattern within the elongating tip (Fig. 4a). Microinjection of the antibody ($n = 6$ injected tubes) caused a reorganization of vacuoles into the tip followed by the cessation of organelle motility within the tip (Fig. 4b and video-Online Resource 2). Elongation stopped as the rate significantly dropped ($p = 0.028$) from 11.84 ± 3.42 (mean \pm standard error) to $0 \mu\text{m h}^{-1}$ and there was a visible tensioning in the cytoplasm that differed from Brownian motion. These effects resulted from the constitutive activation of PaKCBP and not from the trauma of microinjection. In controls microinjected with pre-immune serum ($n = 4$ injected tubes), organelles streamed in a fountain pattern before (Fig. 4c) and after microinjection (Fig. 4d, video-Online Resource 3).

Discussion

Microtubules orchestrate microfilament/myosin dependent organelle motility in conifer pollen tubes

Conifer pollen tubes elongate slowly to reach the egg cell and do not form callose plugs since the spermatogenous

cells initially remain in the pollen grain but later migrate towards the tip to deliver sperm cells for fertilization (Dawkins and Owens 1993; Runions and Owens 1999). Pollen tubes in culture also slowly elongate (Anderhag et al. 2000) and this requires a modest, twofold tip-focused calcium gradient (Lazzaro et al. 2005). Within the elongating tip organelles stream in a unique fountain pattern that is directed by microfilaments and myosin (de Win et al. 1996; Justus et al. 2004; Chen et al. 2007) but regulated by microtubules (Justus et al. 2004; Zheng et al. 2010). The disruption of microtubules slows elongation, alters microfilament organization and causes the accumulation of membrane tubules or vacuoles in the tip (Justus et al. 2004) similar to the changes observed in the present study following the generalized inhibition of calmodulin by W-12 and the specific activation of KCBP by displacing bound calmodulin.

In the present study, microfilament disruption by treatment with latrunculin B also reorganized microtubules into pronounced bundles with a significant increase in KCBP colocalization. This result supports the model that the functional interplay between microtubules and microfilaments influences elongation of moss and fern protonema (Schwuchow et al. 1990; Kadota and Wada 1992; Meske et al. 1996; Pressel et al. 2008), conifer pollen tubes (Anderhag et al. 2000; Justus et al. 2004) and more recently flowering plant pollen tubes (Gossot and Geitmann 2007, Poulter et al. 2008). It is possible that PaKCBP is linking microtubules and microfilaments in conifer pollen tubes. KCBP contains the myosin tail homology region 4 (MyTH4) and the talin-like region found in some myosins which have a microtubule-binding site (Narasimhulu and Reddy 1998). However, the search for proteins that crosslink microtubules and microfilaments has yielded a more likely candidate. Kinesins containing the calponin homology domain (KCH), which is a microfilament-binding site, have been identified as crosslinking proteins between microtubules and microfilaments in flowering plants (Preuss et al. 2004; Frey et al. 2009; Xu et al. 2009; Umezu et al. 2011). It is possible that a KCH homolog is involved in the functional interplay between microtubules and microfilaments in

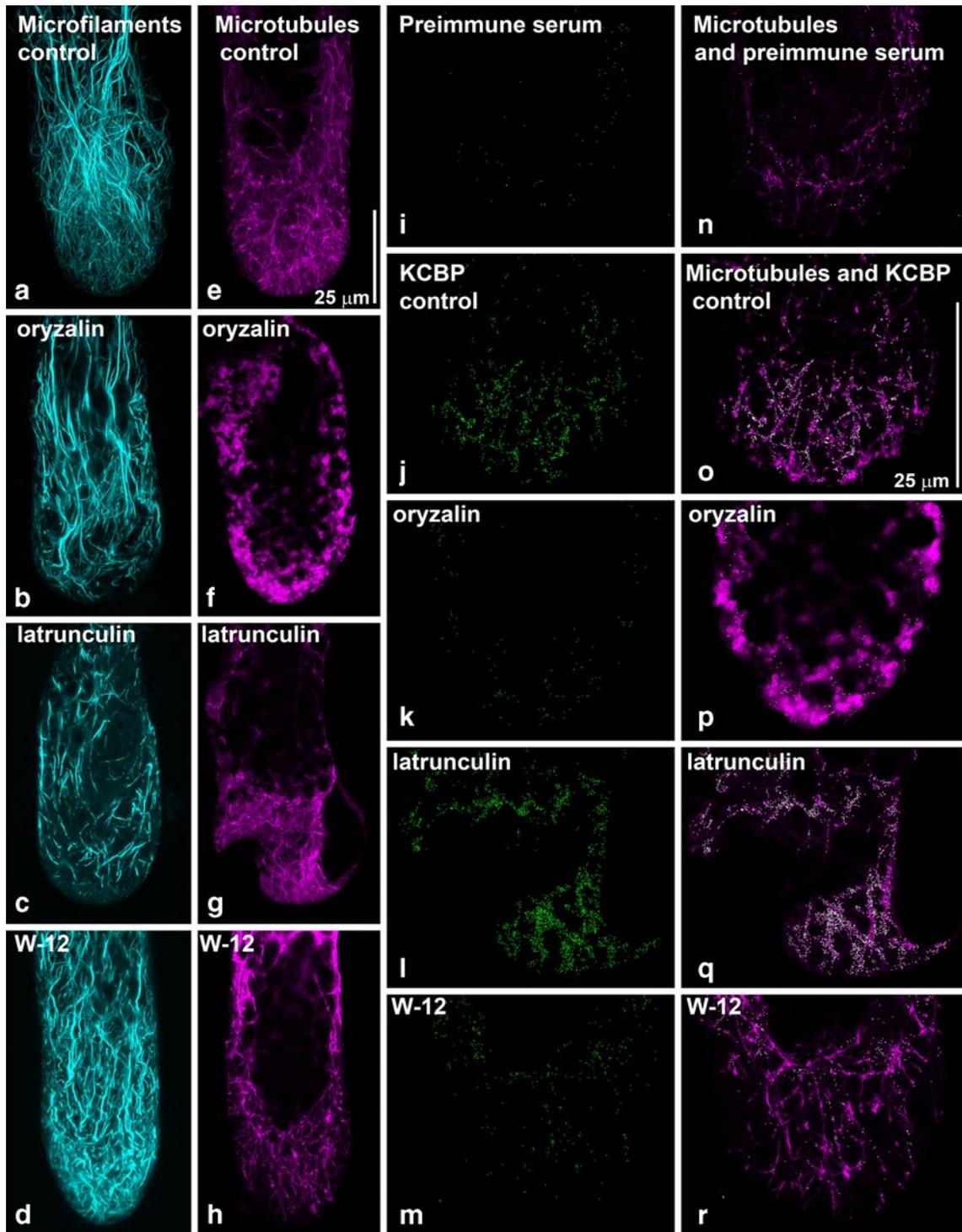


Fig. 3 Microfilaments (a, cyan) and microtubules (e, magenta) form a dense network within the elongating tips of *Picea abies* pollen tubes. PaKCBP (j, green) colocalizes with the microtubules (o, white PaKCBP on magenta microtubules) at the tip while preimmune serum (i, green) has negligible colocalization with microtubules (n, white on magenta). Treatment with 50 μ M oryzalin for 30 min increases bundling of microfilaments (b), disrupts microtubules (f), and reduces PaKCBP colocalization (k and p). Treatment with 5 nM latrunculin

B for 30 min disrupts microfilaments (c) and reorganizes microtubules (g) into thicker bundles with significantly enhanced PaKCBP colocalization (l and q). Treatment with 100 μ M W-12 for 30 min inhibits calmodulin and increases bundling of microfilaments (d) and microtubules (h) with significantly enhanced PaKCBP colocalization (m and r). a–h Confocal projections. i–m Single sections of colocalized PaKCBP. n–r Composites of colocalized PaKCBP (i–m) on microtubules

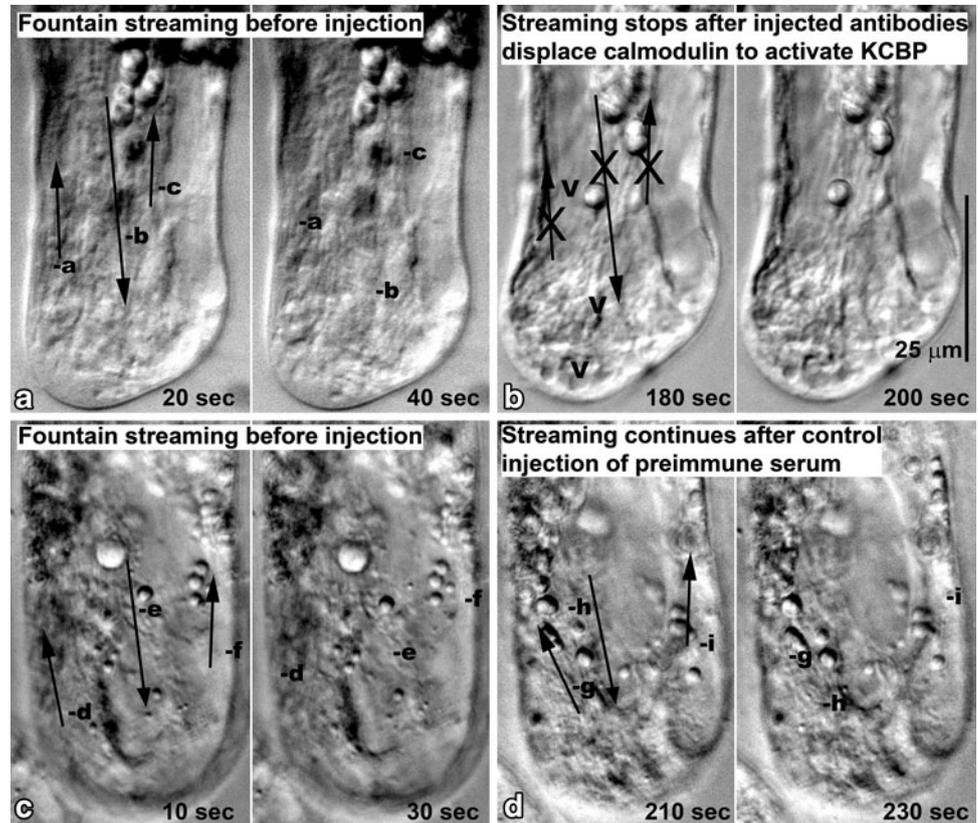
Table 2 Colocalization of antibodies to PaKCBP and tubulin in confocal optical sections

Values are colocalized PaKCPB pixels to tubulin pixels as a percentage of total tubulin pixels

* Significant difference based on heteroscedastic *t* tests ($p < 0.05$)

	Mean %	<i>P</i> values of comparison to labeling with preimmune serum	<i>P</i> values of comparison to control treatment	<i>n</i> (optical sections)
Preimmune	1.35		1.4×10^{-6} *	12
Control treatment	3.00	1.4×10^{-6} *		80
Oryzalin	1.06	1.2×10^{-2} *	6.4×10^{-10} *	55
Latrunculin B	13.11	9.9×10^{-15} *	3.8×10^{-13} *	38
W-12	6.01	4.4×10^{-16} *	2.8×10^{-8} *	75

Fig. 4 **a** Before microinjection, organelles (-a, -b, and -c) streamed in a fountain pattern in the tip. **b** Constitutive activation of PaKCBP by injecting an affinity-purified antibody which displaces calcium/calmodulin caused the migration of vacuoles (V) into the tip and the subsequent cessation of organelle motility with a visible tensing in the cytoplasm ($n = 6$ injected tubes). See also See also video-Online Resource 2. **c** Before control microinjections ($n = 4$ injected tubes), organelles streamed in a fountain pattern (-d, -e and -f). **d** Microinjection of preimmune serum had no effect on organelle positioning as streaming continued (-g, -h and -i). See also See also video-Online Resource 3



conifer pollen tubes. Interestingly, recent evidence indicates that major proteins cross-linking microtubules and microfilaments may not be motor proteins. Metabolic enzymes have been identified as the predominant cross-linking proteins in *Nicotiana tabacum* pollen tubes (Romagnoli et al. 2010).

Calmodulin regulates polarized growth in conifer pollen tubes

Calmodulin antagonists have been used effectively by several labs to elucidate the role of calmodulin in controlling polarized growth in pollen tubes (Obermeyer and Weissenfeld 1991, Estruch et al. 1994, Rato et al. 2004). We find that calmodulin activity is required for conifer pollen tube

elongation since inhibition by W-12 perfusion reversibly stops elongation. Calmodulin activity is also required for the elongation of flowering plant pollen tubes (Estruch et al. 1994) where activity exhibits an oscillatory tip-focused gradient in parallel with the tip-focused calcium gradient (Rato et al. 2004). Calmodulin inactivation dissipates this tip-focused calcium gradient (Obermeyer and Weissenfeld 1991). In the present study, the migration of thicker vacuole/tubules into the tip following calmodulin inhibition by W-12 and by the constitutive activation of PaKCBP is the same phenomenon seen when the tip-focused Ca^{2+} gradient is depleted by blocking Ca^{2+} channels with exogenous application of lanthanides (Lazzaro et al. 2005). This reorganization of vacuoles within the tip is also observed when the microtubule network in live cells is altered by the perfusion

of propyzamide and oryzalin (Justus et al. 2004). We propose that the activation of KCBP by calmodulin inhibition is part of the mechanism causing this reorganization, but may not be the only calmodulin-regulated factor. A proteomic and cytological study in *Picea myeri* pollen tubes identified over 90 proteins whose expression changes following calmodulin inhibition and numerous cytological changes including the slowing of elongation, alteration in calcium dynamics, and reorganization of microfilaments and vacuoles (Chen et al. 2009). Two other $\text{Ca}^{2+}/\text{CaM}$ target proteins promote pollen tube elongation in flowering plants. CNGC-18 is a cyclic nucleotide gated channel that is inhibited by $\text{Ca}^{2+}/\text{CaM}$ (Frietsch et al. 2007) and ACA9 is a Ca^{2+} ATPase that is activated by $\text{Ca}^{2+}/\text{CaM}$ (Schjøtt et al. 2004). In addition NPG1, a pollen-specific calmodulin-binding protein, is also required for pollen germination (Safadi et al. 2000; Golovkin and Reddy 2003).

Role of KCBP in other plant cells

Several studies have found that KCBP is involved in cell division. It has been localized to the pre-prophase band, the mitotic spindle, and the phragmoplast (Bowser and Reddy 1997; Smirnova et al. 1998; Vos et al. 2000; Preuss et al. 2003; Dymek et al. 2006). Constitutive activation of KCBP alters progression through the cell cycle (Vos et al. 2000). This was demonstrated using the same affinity-purified antibodies raised against a synthetic 23-amino acid polypeptide containing the calmodulin-binding domain of AtKCBP as in the present study. These antibodies constitutively activate KCBP by interfering with $\text{Ca}^{2+}/\text{calmodulin}$ regulation but not with the microtubule-binding activity of KCBP. (Narasimhulu et al. 1997; Narasimhulu and Reddy 1998). Microinjection of this antibody into dividing *T. virginiana* stamen hair cells differentially affected phases of cell division, but did not affect cytoplasmic streaming or cell viability (Vos et al. 2000). However, microinjection of the same antibody into elongating conifer pollen tubes stopped cell streaming within the tip. KCBP may have different functional roles in dividing cells compared to those in interphase. KCBP is found during interphase in other elongated plant cells. In *Chlamydomonas reinhardtii* and *Dunaliella salina*, KCBP is concentrated in the flagella (Dymek et al. 2006; Shi et al. 2013). In *Gossypium hirsutum* fibers, KCBP co-localizes along the length of microtubule bundles (Preuss et al. 2003). Genetic studies in *A. thaliana* indicate that KCBP has a role in cells that exhibit polarized growth, where mutations in *ZWI*, which codes for KCBP, caused a reduction in trichome height and branching, and *zwi* mutation together with *suz1*, an allelic-specific suppressor of *zwi*, showed reduced pollen germination, and generated shorter pollen tubes with aberrant morphology

including the accumulation of small vacuoles throughout the tube (Krishnakumar and Oppenheimer 1999). Here we demonstrate that KCBP, which has been identified in *P. abies* (Abdel-Ghany et al. 2005), is concentrated within the elongating tip of conifer pollen tubes and that its constitutive activation alters organelle positioning and subsequently stops cytoplasmic streaming.

Model for PaKCBP's role in conifer pollen tube elongation

Conifer pollen tubes exhibit polarized growth as organelles move into the tip in an unusual fountain pattern that is directed by microfilaments but uniquely organized by microtubules (Anderhag et al. 2000; Justus et al. 2004). Conifer pollen tubes form branches as they slowly grow in vivo (Singh 1978; Dawkins and Owens 1993) and this branching is also induced in vitro by reorganization of microtubules or microfilaments as the original tip stops growing and a new tip emerges (de Win et al. 1996, Anderhag et al. 2000). Since conifer pollen tubes also exhibit a modest twofold tip-focused Ca^{2+} gradient which influences organelle streaming and elongation (Lazzaro et al. 2005), it seems likely that Ca^{2+} -regulated proteins function in the tip to influence motility. Elevated calmodulin activity has also been found in pollen tube tips (Rato et al. 2004). We demonstrate here that elongation and motility are both regulated by $\text{Ca}^{2+}/\text{calmodulin}$ and PaKCBP. Perfusion of the calmodulin antagonist W-12 significantly and reversibly slows pollen tube elongation and disrupts organelle motility, and induces microtubule bundling in the tip. PaKCBP immunolocalization is concentrated in the tip, coincident with and dependent on both microtubule and microfilament bundles. Constitutive activation of PaKCBP alters the position of vacuoles within the tip and subsequently disrupts organelle streaming and pollen tube elongation. We propose that even though PaKCBP is concentrated in the elongating tip, its bundling activity is normally kept at a low level by $\text{Ca}^{2+}/\text{calmodulin}$ inhibition. When this inhibition is lost by reduction of the cytoplasmic calcium concentration or inhibition of calmodulin, then PaKCBP induces microtubule sliding and bundling that alters the organization of microtubules and microfilaments. This alteration repositions organelles and disrupts elongation. PaKCBP activation may be part of the mechanism that reorganizes the cytoskeleton within a disrupted tip in preparation for the resumption of growth in the existing or newly formed tip.

Acknowledgments This work was supported by the College of Charleston through grants to M.D.L. from the Department of Biology, the Office of Research and Creative Activities, and the Faculty Research and Development Committee. E.Y.M. conducted part of this research as an undergraduate. We thank Robyn L. Overall at the University of Sydney for graciously providing bench space and lab

support for M.D.L. Videos accompanying Figs. 1 and 4 are available at our lab website (<http://lazzarom.people.cofc.edu>) and as online resources at the journal website.

References

- Abdel-Ghany SE, Day IS, Simmons MP, Kugrens P, Reddy ASN (2005) Origin and evolution of kinesin-like calmodulin-binding protein. *Plant Physiol* 138:1711–1722
- Anderhag P, Hepler PK, Lazzaro MD (2000) Microtubules and microfilaments are both responsible for pollen tube elongation in the conifer *Picea abies* (Norway spruce). *Protoplasma* 214:141–157
- Åström H, Sorri O, Raudaskoski M (1995) Role of microtubules in the movement of the vegetative nucleus and generative cell in tobacco pollen tubes. *Sex Plant Reprod* 8:61–69
- Bowser J, Reddy ASN (1997) Localization of a kinesin-like calmodulin-binding protein in dividing cells of *Arabidopsis* and tobacco. *Plant J* 12:1429–1437
- Chen T, Teng NJ, Wu XQ, Wang YH, Tang W, Samaj J, Baluska F, Lin JX (2007) Disruption of actin filaments by latrunculin B affects cell wall construction in *Picea meyeri* pollen tube by disturbing vesicle trafficking. *Plant Cell Physiol* 48:19–30
- Chen KM, Wu GL, Wang YH, Tian CT, Samaj J, Baluska F, Lin JX (2008) The block of intracellular calcium release affects the pollen tube development of *Picea wilsonii* by changing the deposition of cell wall components. *Protoplasma* 233:39–49
- Chen T, Wu XQ, Chen YM, Li XJ, Huang M, Zheng MZ, Baluska F, Samaj J, Lin JX (2009) Combined proteomic and cytological analysis of Ca^{2+} /calmodulin regulation in *Picea meyeri* pollen tube growth. *Plant Physiol* 149:1111–1126
- Cheung AY, Wu HM (2008) Structural and signaling networks for the polar cell growth machinery in pollen tubes. *Annu Rev Plant Biol* 59:547–572
- Cheung AY, Duan QH, Costa SS, deGraaf B, DiStilio VS, Feijó J, Wu HM (2008) The dynamic pollen tube cytoskeleton: live cell studies using actin-binding and microtubule-binding reporter proteins. *Mol Plant* 1:686–702
- Dawkins MD, Owens JN (1993) In vitro and in vivo pollen hydration, germination, and pollen tube growth in white spruce, *Picea glauca* (Moench) Voss. *Int J Plant Sc* 154:506–521
- de Win AHN, Knuijman B, Pierson ES, Geurts H, Kengen HMP, Derksen J (1996) Development and cellular organization of *Pinus sylvestris* pollen tubes. *Sex Plant Reprod* 9:93–101
- Deavours BE, Reddy ASN, Walker RA (1998) Ca^{2+} /calmodulin regulation of the *Arabidopsis* kinesin-like calmodulin-binding protein. *Cell Motil Cytoskelet* 40:408–416
- Dodd AN, Kudla J, Sanders D (2010) The language of calcium signaling. *Annu Rev Plant Biol* 61:593–620
- Dymek EE, Goduti D, Kramer T, Smith EF (2006) A kinesin-like calmodulin-binding protein in *Chlamydomonas*: evidence for a role in cell division and flagellar functions. *J Cell Sci* 119:3107–3116
- Estruch JJ, Kadwell S, Merlin E, Crossland L (1994) Cloning and characterization of a maize pollen-specific calcium-dependent calmodulin-independent protein kinase. *Proc Natl Acad Sci USA* 91:8837–8841
- Feijó JA, Sainhas J, Hackett GR, Kunkel JG, Hepler PK (1999) Growing pollen tubes possess a constitutive alkaline band in the clear zone and a growth dependent acidic tip. *J Cell Biol* 144:483–496
- Foissner I, Grolig F, Obermeyer G (2002) Reversible protein phosphorylation regulates the dynamic organization of the pollen tube cytoskeleton: effects of calyculin A and okadaic acid. *Protoplasma* 220:1–15
- Frey N, Klotz J, Nick P (2009) Dynamic bridges- a calponin-domain kinesin from rice links actin filaments and microtubules in both cycling and non-cycling cells. *Plant Cell Physiol* 50:1493–1506
- Frietsch S, Wang YF, Sladek C, Poulsen LR, Romanowsky SM, Schroeder JI, Harper JF (2007) A cyclic nucleotide-gated channel is essential for polarized tip growth of pollen. *Proc Natl Acad Sci USA* 104:14531–14536
- Gibbon BC, Kovar DR, Staiger CJ (1999) Latrunculin B has different effects on pollen germination and tube growth. *Plant Cell* 11:2349–2363
- Golovkin M, Reddy ASN (2003) A calmodulin-binding protein from *Arabidopsis* has an essential role in pollen germination. *Proc Natl Acad Sci USA* 100:10558–10563
- Gossot O, Geitmann A (2007) Pollen tube growth: coping with mechanical obstacles involves the cytoskeleton. *Planta* 226:405–416
- He Y, Wetzstein HY (1995) Fixation induces differential tip morphology and immunolocalization of the cytoskeleton in pollen tubes. *Physiol Plant* 93:757–763
- Hepler PK, Vidali L, Cheung AY (2001) Polarized cell growth in higher plants. *Annu Rev Cell Dev Biol* 17:159–187
- Heslop-Harrison J, Heslop-Harrison Y (1989) Myosin associated with the surfaces of organelles, vegetative nuclei and generative cells in angiosperm pollen grains and tubes. *J Cell Sci* 94:319–325
- Heslop-Harrison J, Heslop-Harrison Y, Cresti M, Tiezzi A, Moscatelli A (1988) Cytoskeletal elements, cell shaping and movement in the angiosperm pollen tube. *J Cell Sci* 91:49–60
- Holdaway-Clarke TL, Feijó JA, Hackett GR, Kunkel JG, Hepler PK (1997) Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *Plant Cell* 9:1999–2010
- Justus CD, Anderhag P, Goins JL, Lazzaro MD (2004) Microtubules and microfilaments coordinate to direct a fountain-streaming pattern in elongating conifer pollen tube tips. *Planta* 219:103–109
- Kadota A, Wada M (1992) Reorganization of the cortical cytoskeleton in tip growing fern protonemal cells during phytochrome mediated phototropism and blue light induced apical swelling. *Protoplasma* 166:35–41
- Kao YL, Deavours BE, Phelps KK, Walker RA, Reddy ASN (2000) Bundling of microtubules by motor and tail domains of a kinesin-like calmodulin-binding protein from *Arabidopsis*: regulation by Ca^{2+} /calmodulin. *Biochem Biophys Res Commun* 267:201–207
- Krishnakumar S, Oppenheimer DG (1999) Extragenic suppressors of the *Arabidopsis* zwi-3 mutation identify new genes that function in trichome branch formation and pollen tube growth. *Development* 126:3079–3088
- Lancelle SA, Hepler PK (1992) Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum*. *Protoplasma* 167:215–230
- Lazzaro MD (1996) The actin microfilament network within elongating pollen tubes of the gymnosperm *Picea abies* (Norway spruce). *Protoplasma* 194:186–194
- Lazzaro MD (1999) Microtubule organization in germinated pollen of the conifer *Picea abies* (Norway spruce, Pinaceae). *Amer J Bot* 86:759–766
- Lazzaro MD, Donohue JM, Soodavar FM (2003) Disruption of cellulose synthesis by isoxaben causes tip swelling and disorganizes cortical microtubules in elongating conifer pollen tubes. *Protoplasma* 220:201–207
- Lazzaro MD, Cardenas L, Bhatt AP, Justus CD, Phillips MS, Holdaway-Clarke TL, Hepler PK (2005) Calcium gradients in conifer pollen tubes; dynamic properties differ from those seen in angiosperms. *J Exp Bot* 56:2619–2628
- Lovy-Wheeler A, Wilson KL, Baskin TI, Hepler PK (2005) Enhanced fixation reveals the apical cortical fringe of actin filaments as a consistent feature of the pollen tube. *Planta* 221:95–104

- Meske V, Ruppert V, Hartmann E (1996) Structural basis for the red light induced repolarization of tip growth in caulonema cells of *Ceratodon purpureas*. *Protoplasma* 192:189–198
- Messerli MA, Danhuser G, Robinson KR (1999) Pulsatile influxes of H^+ , K^+ , and Ca^{2+} lag growth pulses of *Lilium longiflorum* pollen tubes. *J Cell Sci* 12:1497–1509
- Narasimhulu SB, Reddy ASN (1998) Characterization of microtubule binding domains in the Arabidopsis kinesin like calmodulin binding protein. *Plant Cell* 10:957–965
- Narasimhulu SB, Kao YL, Reddy ASN (1997) Interaction of Arabidopsis kinesin-like calmodulin binding protein with tubulin subunits: modulation by Ca^{2+} /calmodulin. *Plant J* 12:1139–1149
- Obermeyer G, Weisenseel MH (1991) Calcium channel blocker and calmodulin antagonists affect the gradient of free calcium ions in lily pollen tubes. *Eur J Cell Biol* 56:319–327
- Parton RM, Fischer-Parton S, Watahiki MK, Trewavas AJ (2001) Dynamics of the apical vesicle accumulation and the rate of growth are related in individual pollen tubes. *J Cell Sci* 114:2685–2695
- Pierson ES, Derksen J, Traas JA (1986) Organization of microfilaments and microtubules in pollen tubes grown in vitro and in vivo in various angiosperms. *Eur J Cell Biol* 41:14–18
- Pierson ES, Miller DD, Callahan DA, van Aken J, Hackett G, Hepler PK (1996) Tip localized calcium entry fluctuates during pollen tube growth. *Dev Biol* 174:160–173
- Poulter NS, Vatovec S, Franklin-Tong VE (2008) Microtubules are a target for self-incompatibility signaling in *Papaver* pollen. *Plant Physiol* 146:1358–1367
- Pressel S, Ligrone R, Duckett JG (2008) Cellular differentiation in moss protonemata: a morphological and experimental study. *Ann Bot* 102:227–245
- Preuss ML, Delmer DP, Liu B (2003) The cotton kinesin-like calmodulin-binding protein associates with cortical microtubules in cotton fibers. *Plant Physiol* 132:154–160
- Preuss ML, Kovar DR, Lee YRJ, Staiger CJ, Delmer DP, Liu B (2004) A plant-specific kinesin binds to actin microfilaments and interacts with cortical microtubules in cotton fibers. *Plant Physiol* 136:3945–3955
- Rato C, Monteiro D, Hepler PK, Malhó R (2004) Calmodulin activity and cAMP signaling modulate growth and apical secretion in pollen tubes. *Plant J* 38:887–897
- Romagnoli S, Faleri C, Bini L, Baskin TI, Cresti M (2010) Cytosolic proteins from tobacco pollen tubes that crosslink microtubules and actin filaments in vitro are metabolic enzymes. *Cytoskeleton* 67:745–754
- Runions CJ, Owens JN (1999) Sexual reproduction of interior spruce (Pinaceae). II. Fertilization to early embryo formation. *Int J Plant Sci* 160:641–652
- Safadi F, Reddy VS, Reddy ASN (2000) A pollen-specific novel calmodulin-binding protein with tetratricopeptide repeats. *J Biol Chem* 275:35457–35470
- Schiøtt M, Romanowsky SM, Baekgaard L, Jakobsen MK, Palmgren MG, Harper JF (2004) A plant plasma membrane Ca^{2+} pump is required for normal pollen tube growth and fertilization. *Proc Natl Acad Sci USA* 101:9502–9507
- Schwuchow J, Sack FD, Hartmann E (1990) Microtubule distribution in gravitropic protonema of the moss *Ceratodon*. *Protoplasma* 159:60–69
- Shi K, Li J, Han K, Jiang H, Xue L (2013) The degradation of kinesin-like calmodulin binding protein of *D. Salina* (DsKCBP) is mediated by the ubiquitin-proteasome system. *Mol Biol Rep* 40:3113–3121
- Singh H (1978) Embryology of gymnosperms. In: *Handbuch der Pflanzenanatomie*. vol 10, Gebrüder Borntraeger, Berlin, p 2
- Smirnova EA, Reddy ASN, Bowser J, Bajer AS (1998) Minus end-directed kinesin-like motor protein, KCBP, localizes to anaphase spindle poles in *Haemanthus* endosperm. *Cell Motil Cytoskeleton* 41:271–280
- Song H, Golovkin M, Reddy ASN, Endow SA (1997) In vitro motility of AtKCBP, a calmodulin-binding kinesin protein of Arabidopsis. *Proc Natl Acad Sci USA* 94:322–327
- Terasaka O, Niitsu T (1994) Differential roles of microtubule and actin-myosin cytoskeleton in the growth of *Pinus* pollen tubes. *Sex Plant Reprod* 7:264–272
- Umezū N, Umeki N, Mitsui T, Kondo K, Maruta S (2011) Characterization of a novel rice kinesin O12 with a calponin homology domain. *J Biochem* 149:91–101
- Vos JW, Safadi F, Reddy ASN, Hepler PK (2000) The kinesin-like calmodulin binding protein is differentially involved in cell division. *Plant Cell* 12:979–990
- Wang X, Teng Y, Wang Q, Li X, Sheng X, Zheng M, Samaj J, Baluska F, Lin J (2006) Imaging dynamic secretory vesicles in living pollen tubes of *Picea meyeri* using evanescent wave microscopy. *Plant Physiol* 141:1591–1603
- Xu T, Qu Z, Yang X, Qin X, Xiong J, Wang Y, Ren D, Liu G (2009) A cotton kinesin GhKCH2 interacts with both microtubules and microfilaments. *Biochem J* 421:171–180
- Yang Z (2002) Small GTPases: versatile signaling switches in plants. *Plant Cell* 14:S375–S388
- Zheng MZ, Wang QL, Teng Y, Wang XH, Wang F, Chen T, Samaj J, Lin JX, Logan DC (2010) The speed of mitochondrial movement is regulated by the cytoskeleton and myosin in *Picea wilsonii* pollen tubes. *Planta* 231:779–791