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ORIGINAL ARTICLE

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

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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.

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

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Introduction

Pollen tubes are an established model system for examining polarized cell growth. In angiosperms, pollen tube elongation is very fast (300–1,500 μm 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 1–5 μm 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 $\mu m\ h^{-1}$ for *Picea abies* (Anderhag et al. 2000) compared to 300–1,500 $\mu m\ h^{-1}$ 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 20–30 μm 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 °C. This pollen was germinated for 24 h at 30 °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 µl 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 µM 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 °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×*g* for 2 min. A 200 µl 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 µl 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 µM W-12 (Rato et al. 2004), 50 µM 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× 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 µM 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 AtKCBP 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 μl 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 ± 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 μm 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 μl 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

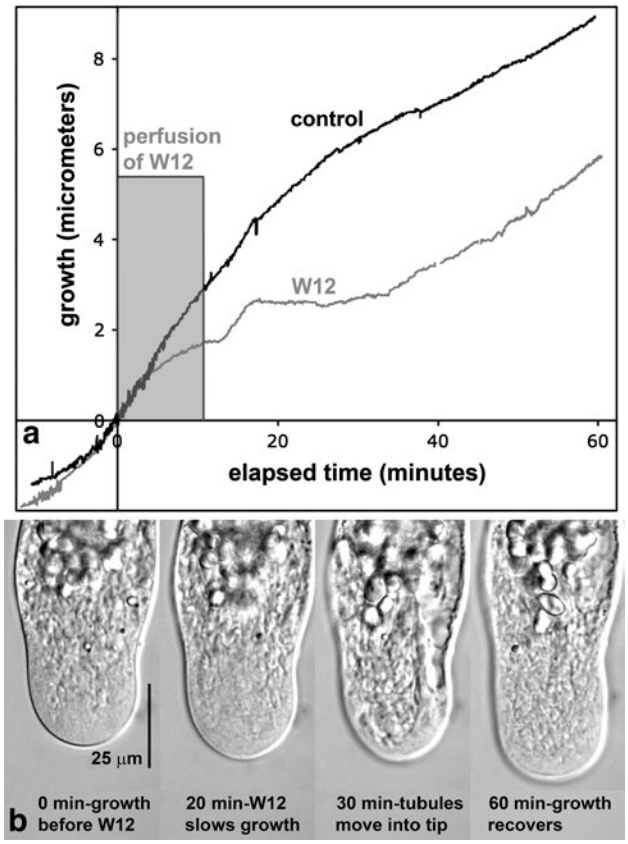

Fig. 1 Active calmodulin is required for pollen tube elongation. **a** Pollen tubes under continuous perfusion were exposed to a 10-min pulse of 100 μM W-12 in growth media which stopped elongation ($n = 7$ pollen tubes, one representative tube shown in *gray*). Elongation recovered to original rates 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 μM 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 preimmune 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 μM 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	*P* value before:during	*P* value before:after	*n*
W-12	11.85 ± 2.19	0.75 ± 0.49*	12.38 ± 4.89	0.035	0.902	7
Control	11.68 ± 3.62	12.04 ± 3.61	12.56 ± 3.92	0.234	0.118	6

Values are mean ± standard error (um h^{-1})

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

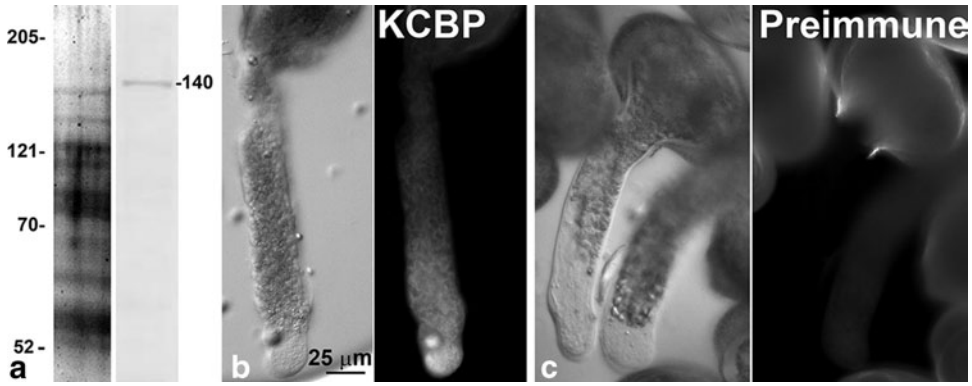

Fig. 2 PaKCBP (140 kDa) is expressed in *Picea abies* pollen tubes. **a** Coomassie stain (*lane 1*) and immunoblot (*lane 2*) of total protein extracts from germinated pollen tubes. **b** PaKCBP is immunolocalized throughout the tube and concentrated within the elongating tip. **c** Pollen tubes treated 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 ± standard error) to 0 µm 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 preimmune 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

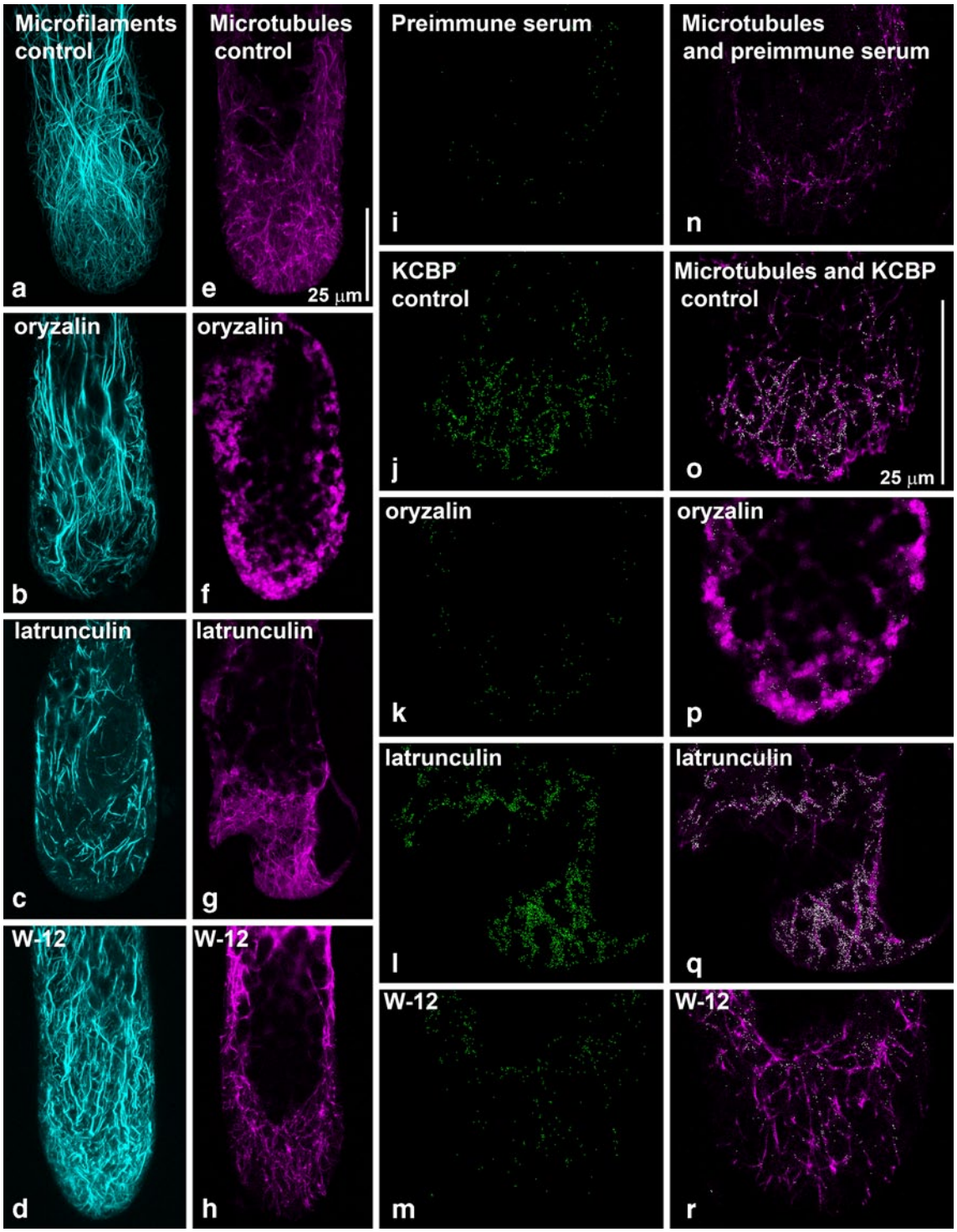

Fig. 3 Microfilaments (**a**, *cyan*) and microtubules (**e**, *magenta*) form a dense network within the elongating tips of *Picea abies* pollen tubes. PaKCBP (**j**, *green*) colocalizes with the microtubules (**o**, *white* PaKCBP on magenta microtubules) at the tip while preimmune serum (**i**, *green*) has negligible colocalization with microtubules (**n**, *white* on *magenta*). Treatment with 50 μM 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 μM 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 %	*P* values of comparison to labeling with preimmune serum	*P* values of comparison to control treatment	*n* (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

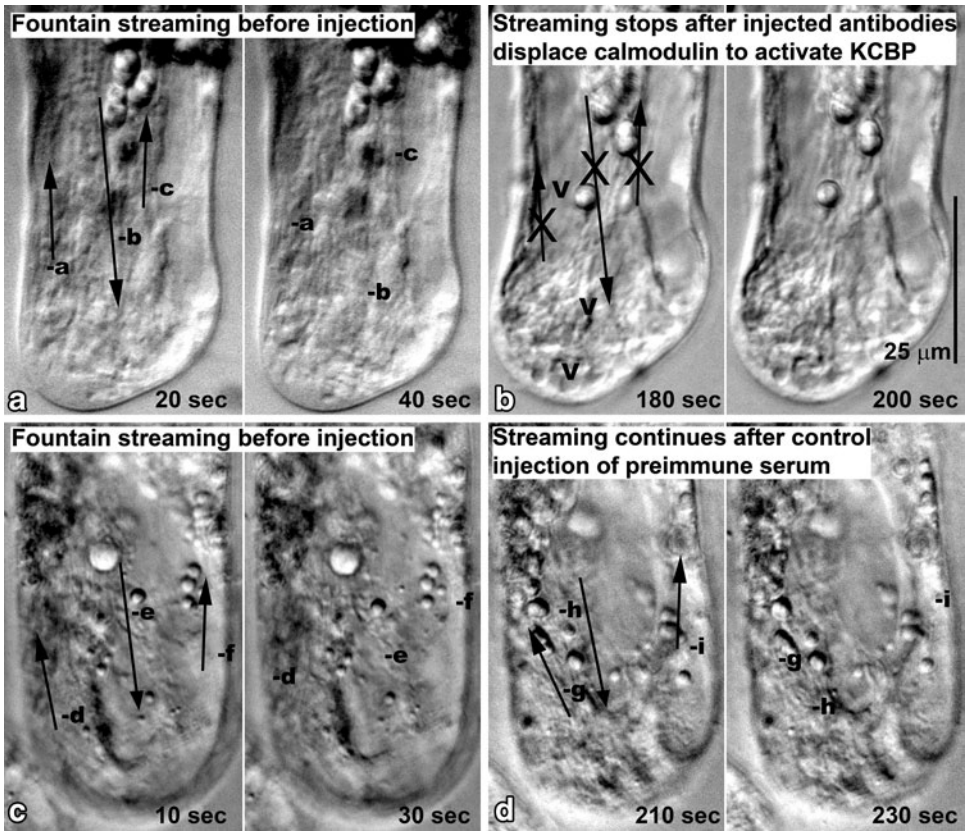

Fig. 4 **a** Before microinjection, organelles (*-a*, *-b*, and *-c*) streamed in a fountain pattern in the tip. **b** Constitutive activation of PaKCBP by injecting an affinity-purified antibody which displaces calcium/calmodulin caused the migration of vacuoles (*V*) into the tip and the subsequent cessation of organelle motility with a visible tensioning 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 Weisenseel 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 Weisenseel 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 Ca^{2+}/CaM target proteins promote pollen tube elongation in flowering plants. CNGC-18 is a cyclic nucleotide gated channel that is inhibited by Ca^{2+}/CaM (Frietsch et al. 2007) and ACA9 is a Ca^{2+} ATPase that is activated by Ca^{2+}/CaM (Schiøtt 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 Ca^{2+}/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 *Chalmydomonas 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 PaKCPB'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 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. 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 Ca^{2+}/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.

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