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Arabidopsis thaliana calmodulin-like protein CML24 regulates pollen tube growth by modulating the actin cytoskeleton and controlling the cytosolic Ca^{2+} concentration

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Abstract Cytosolic free calcium ($[Ca^{2+}]_{cyt}$), which is essential during pollen germination and pollen tube growth, can be sensed by calmodulin-like proteins (CMLs). The *Arabidopsis thaliana* genome encodes over 50 CMLs, the physiological role(s) of most of which are unknown. Here we show that the gene *AtCML24* acts as a regulator of pollen germination and pollen tube extension, since the pollen produced by loss-of-function mutants germinated less rapidly than that of wild-type (WT) plants, the rate of pollen tube extension was slower, and the final length of the pollen tube was shorter. The $[Ca^{2+}]_{cyt}$ within germinated pollen and extending pollen tubes produced by the *cml24* mutant were higher than their equivalents in WT plants, and pollen tube extension was less sensitive to changes in external $[K^+]$ and $[Ca^{2+}]$. The pollen and pollen tubes produced by *cml24* mutants were characterized by a disorganized actin cytoskeleton and lowered sensitivity to the action of latrunculin B. The observations support an interaction between CML24 and $[Ca^{2+}]_{cyt}$ and an involvement of CML24 in actin organization, thereby affecting pollen germination and pollen tube elongation.

Keywords CML24 · Pollen tube · Calcium · Actin · *Arabidopsis thaliana*

Introduction

Once a fully developed pollen grain has successfully adhered to a compatible pistil, it becomes hydrated, germinates, and develops a pollen tube (Taylor and Hepler 1997). Ca^{2+} acts as a prime regulatory signal in pollen germination and pollen tube growth (Jaffe et al. 1975; Schiefelbein et al. 1993). In vitro imaging of extending lily and tobacco pollen tubes has demonstrated the presence of Ca^{2+} concentration gradients and regular oscillations of the cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) at the tip of the tube (Pierson et al. 1996). As both disruption of the endogenous Ca^{2+} gradient and application of high concentrations of exogenous Ca^{2+} are detrimental to pollen tube extension, it is evident that both the intra- and extracellular Ca^{2+} are important for pollen growth (Picton and Steer 1983; Obermeyer and Weisenseel 1991; Iwano et al. 2004; Wang et al. 2004; Lazzaro et al. 2005).

Actin dynamics and F-actin organization represent an additional important feature in pollen germination and tube growth. Actin cables direct the cytoplasmic streaming needed for rapid tube extension (Hepler et al. 2001; Ren and Xiang 2007; Xiang et al. 2007; Cheung and Wu 2008; Zhang et al. 2010). Pollen tube actin filaments are either longitudinally oriented (primarily present in the pollen tube shank), densely clustered at the subapex (the "actin collar"), or behave in a dynamic fashion in the distal region of the pollen tube (Lovy-Wheeler et al. 2005; Cheung et al.

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2008; Chen et al. 2009; Vidali et al. 2009; Staiger et al. 2010). Time-course analyses have established that the actin collar structure alternates with F-actin dynamics, and that the peak of tip F-actin formation precedes tube growth (Fu et al. 2001). The quantity of tip F-actin is inversely related to both the rate of pollen tube elongation and the tip $[Ca^{2+}]_{cyt}$, while the dynamic polymerization and depolymerization of the microfilament cytoskeleton is a key regulator of the pollen grain plasma membrane Ca^{2+} channel (Wang et al. 2004).

Calmodulin-like proteins (CMLs) belong to a plant-specific family of Ca^{2+} sensors. They feature Ca^{2+}-binding EF hands and share at least 16 % amino acid identity with calmodulin (McCormack and Braam 2003; Azimzadeh et al. 2008). The *Arabidopsis thaliana* genome encodes some 50 CML proteins (McCormack and Braam 2003). Calcium-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs) are further members of the Ca^{2+}-binding EF hand superfamily. Calmodulins, CDPKs, and CBLs are all involved in a wide range of physiological processes (DeFalco et al. 2010; Landoni et al. 2010), but the function of most CMLs remains largely unknown. Accumulating data suggest that they have a role in plant growth and signaling (Delk et al. 2005). The *A. thaliana* CML family AtCML24 has been shown to participate in pathogen infection-associated nitric oxide production, as well as having a role in seed germination, the transition from vegetative to reproductive growth, and the sensing of photoperiod (Delk et al. 2005). The *cml24-4* mutant flowers late, and its leaves contain a higher level of nitric oxide than wild type (WT; Tsai et al. 2007); in addition its innate immune response is compromised, as is its pathogen-mediated generation of nitric oxide (Ma et al. 2008). The elevation of $[Ca^{2+}]_{cyt}$ is believed to be linked to nitric oxide generation via the action of CML24 (Tsai et al. 2007), and the protein appears able to bind with a cysteine protease involved in autophagy progression (Tsai et al. 2013). The wide-ranging activity of CML24 suggests that the CMLs may well be associated with many Ca^{2+}-dependent processes. Here, we show that AtCML24 acts as a positive regulator of pollen germination and pollen tube development, and that it influences pollen physiology via an effect exerted on cytoskeleton dynamics signaling.

Materials and methods

Plant materials and growing conditions

A. thaliana ecotype Columbia-0 (WT) and *cml24* mutants were grown in potting mix (Lu-Qing Plant Inc.) under an 8 h photoperiod (140 µmol m^{-2} s^{-1} light), 22/20 °C day/night temperature regime, and 60 % relative humidity. After 3–4 weeks, the photoperiod was lengthened to 16 h to initiate flowering.

cml24 mutants and transcription of CML24

The T-DNA insertion line *cml24-T1* (CS815583) was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info), and homozygous segregants were identified via a genomic polymerase chain reaction (PCR) assay based on the primer pair *cml24-T1* LP/RP, in conjunction with the left border primer LB2 (sequences given in Table S1). Reverse transcription (RT)-PCR was performed to assess the abundance of *CML24* transcript, using two different primer pairs (Table S1). The template for these reactions was RNA extracted from leaves of 21-day-old plants using TriPure isolation reagent (Roche, Basel, Switzerland), followed by complementary DNA (cDNA) synthesis achieved with Moloney murine leukemia virus (MMLV) reverse transcriptase (Thermo, MA, USA). A segment of *AtActin2* was used as the reference sequence. The *cml24-4* mutant was isolated from a targeting-induced local lesions in genome (TILLING) population; it carries a K124E point mutation predicted to alter the functionality of the CML24 protein.

Pollen germination and pollen tube extension measurement

Pollen germination in vitro followed a modified version of the Fan et al. (2001) protocol. In brief, pollen harvested from newly fully opened flowers was placed on the surface of pollen germination medium [1 mM KCl, 10 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 1 % (w/v) agarose, 18 % (w/v) sucrose, 10 mM myo-inositol, 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.8] and incubated at 28 °C for 3–10 h. Pollen grains obtained from wild-type Col-0 and *cml24* mutant plants were germinated for 10 h in vitro. Pollen germination and pollen tube growth were determined from digital micrographs acquired via light microscopy. Three independent replicates were performed, and for each replicate, at least 200 pollen grains or pollen tubes were assessed to obtain a mean value used for drawing the columns and statistical analysis. Student's *t* test was used to test the statistical significance of differences between means.

Aniline blue staining assay

The method used for aniline blue staining of pollen tubes growing within the pistil followed a published protocol, with only minor modifications (Ishiguro et al. 2001). Briefly, pre-emasculated mature WT flowers were

pollinated with either wild-type or *cml24* mutant pollen. After 2, 6, and 10 h, the pollinated pistils were incubated in fixing solution containing ethanol:acetic acid (3:1) for 1 h at room temperature. The fixed pistils were washed three times with distilled water before an 8-h incubation step in 4 M NaOH softening solution. The pistils were washed three times with distilled water before subsequent staining in aniline blue solution (0.05 % aniline blue in 50 mM K_3PO_4 buffer, pH 7.5) for 3–5 h in the dark. After staining, micrographs of the pistils were obtained by fluorescence microscopy. To quantify the seed setting rate, siliques were harvested 10 days after flowering, then decolorized by immersion in 100 % EtOH to allow for seed set to be visualized.

Mutation complementation by transgenesis

A ~1.1-kb upstream sequence, along with a 486-bp genic fragment of *CML24*, was PCR-amplified using the primer pair CML24 fw/rev (Table S1). The fragment was first ligated into the *pEASY*-Blunt Cloning Vector (Transgen, Beijing, China) and subsequently into the *Hin*dIII and *Sac*I cloning sites of the binary pCambia plasmid. The fusion product was introduced into *Agrobacterium tumefaciens* strain EHA105, then transformed into *cml24-T1* plants using the floral dip method (Clough and Bent 1998), followed by hygromycin selection. For detection of the *CML24* expression level in *CML24* gene complementation, total RNA was extracted from mature pollen using TriPure isolation reagent (Roche, Basel, Switzerland) and cDNA was synthesized using MMLV reverse transcriptase (Thermo, MA, USA). Two complementation transgenic lines (C-1 and C-2) were obtained and used for further analyses.

Quantitative RT-PCR (qPCR)

qPCR was performed using the BioRad qPCR system (FastStart Universal SYBR Green Master, Roche) with three biological replicates. An Rn threshold of 0.2 was applied to obtain the threshold cycle number. Relative transcript abundances were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). qPCR primer sequences are listed in Table S1.

Measurement of $[Ca^{2+}]_{cyt}$ in pollen and pollen tube

The fluorescent calcium indicator Fluo-3 AM was loaded into pollen grains following a published protocol (Qu et al. 2012). The estimation of $[Ca^{2+}]_{cyt}$ in the pollen tube required in vitro germination of pollen for 2 h prior to Fluo-3 AM loading. The resulting fluorescence signal was detected by fluorescence microscopy. Three independent

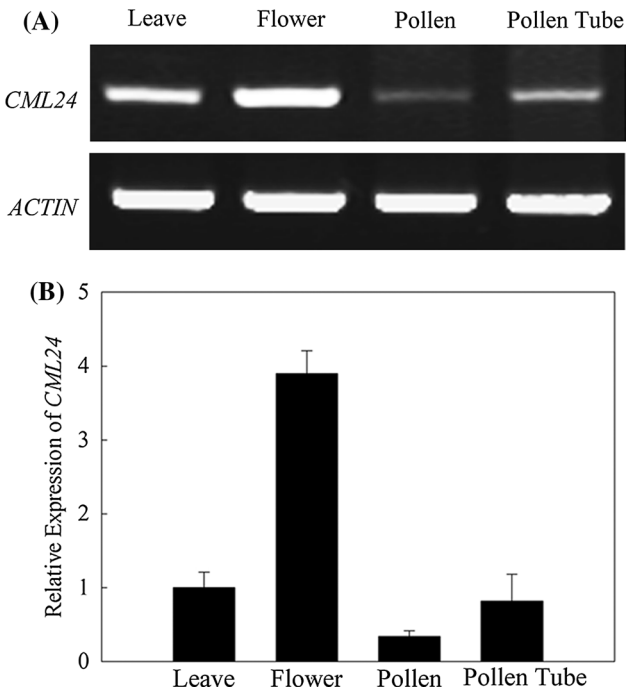

Fig. 1 *CML24* transcript abundance assayed by **a** semiquantitative RT-PCR and **b** qPCR

replicates were performed, and a mean based on at least 100 pollen grains or pollen tubes per replicate was calculated. Student's *t* test was used to test the statistical significance of differences between means.

F-actin staining

The actin cytoskeleton was stained as described elsewhere (Wu et al. 2010). Pollen grains were dispersed over the surface of pollen germination medium (as above) and held at 28 °C for 3 h. The emerged pollen tubes were fixed for 1 h in liquid pollen germination medium supplemented with 300 mM

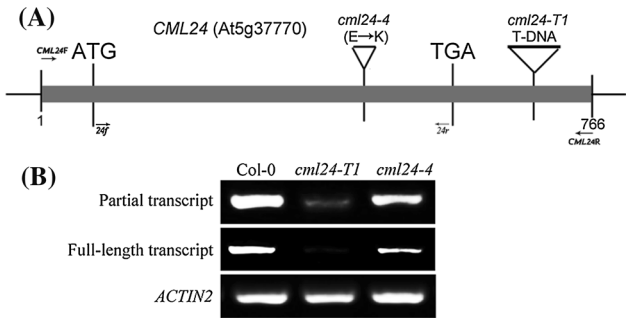

Fig. 2 *CML24* transcript abundance in the *cml24-T1* and *cml24-4* mutants. **a** In the *cml24-T1* mutant, a T-DNA is inserted into the 3′-untranslated region of the gene. **b** *CML24* transcript abundance in WT and the *cml24-T1* and *cml24-4* mutants

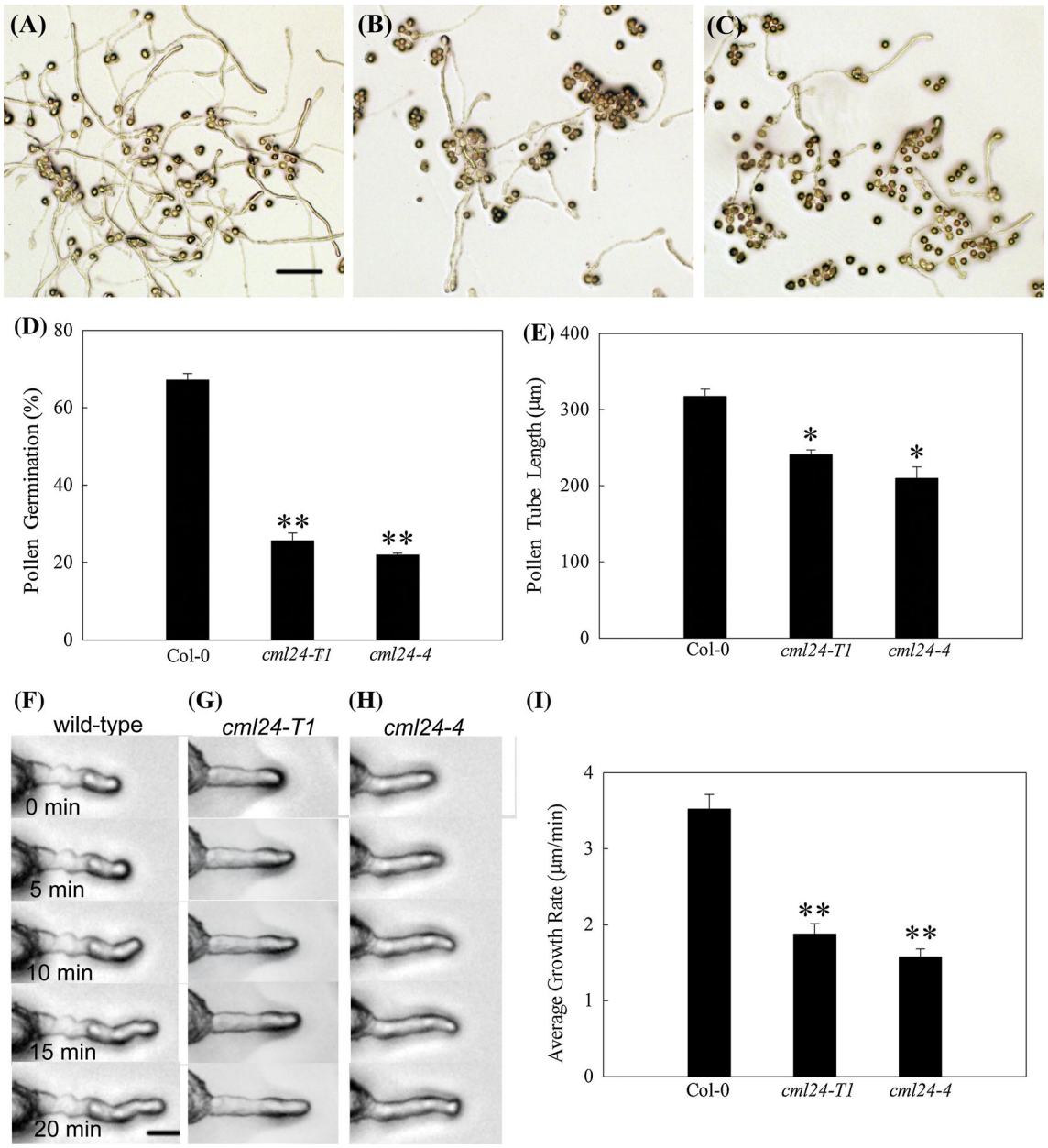

Fig. 3 *cml24* mutants are compromised with respect to both pollen germination and pollen tube extension. **a** WT, **b** *cml24-T1* mutant, and **c** *cml24-4* mutant pollen germinated in vitro for 10 h. *Bar* 100 μm. **d** Pollen germination rate after 10 h. **e** Pollen tube length after 10 h. **f–h** Time-lapse images of WT, *cml24-T1*, and *cml24-4* pollen tubes captured at 5-min intervals. *Bar* 20 μm. **i** Mean growth rate of WT, *cml24-T1*, and *cml24-4* pollen tubes. *, **$P < 0.01, 0.005$; *error bars* show the standard error (SE)

3-maleimidobenzoic acid *N*-hydroxysuccinimide ester, and subsequently extracted in 0.05 % v/v Nonidet P-40 in liquid germination medium for 10 min. After three 10-min washes in TBSS [50 mM Tris-HCl (pH 7.5), 200 mM NaCl, 400 mM sucrose] containing 0.05 % v/v Nonidet P-40, the pollen tubes were stained overnight at 4 °C in 200 nM Alexa-488 phalloidin (Invitrogen, CA, USA) dissolved in the wash solution. Micrographs were obtained using confocal laser scanning microscopy (excitation at 488 nm, emission at 515 nm).

Results

Loss of *CML24* function delayed pollen germination and inhibited pollen tube elongation

Transcription of *CML24* in the WT pollen and pollen tube (but not in the leaf or flower) was confirmed using both semi-quantitative RT-PCR (Fig. 1a) and qPCR (Fig. 1b). In both the T-DNA mutant *cml24-T1* and the K124E point mutant

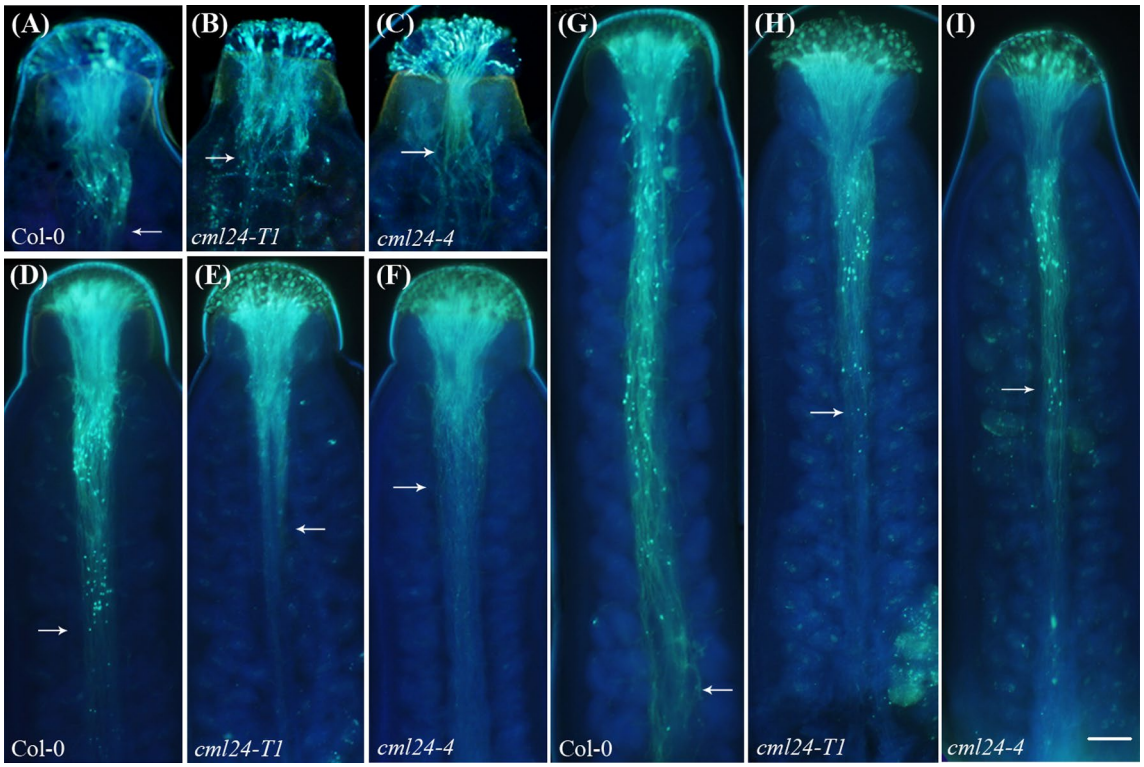

Fig. 4 *cml24* mutation results in slower pollen tube elongation when applied to WT stigma. *Arrows* indicate zones reached by most of the pollen tubes within the transmitting tract. *Bar* 100 μm. **a**–**c** WT, *cml24-T1*, and *cml24-4* pollen germinated 2 h after pollination. **d**–**f** WT, *cml24-T1*, and *cml24-4* pollen germinated 6 h after pollination. **g**–**i** WT, *cml24-T1*, and *cml24-4* pollen germinated 10 h after pollination

cml24-4, the abundance of *CML24* transcript was much lower than in the WT (Fig. 2b). The T-DNA insertion in the 3′-untranslated region (UTR) dramatically decreased the transcript accumulation, suggesting that the disrupted sequence may contain essential regulatory elements for the stability of the messenger RNA (mRNA) (Bashirullah et al. 2001; Winter et al. 2008). Both *cml24-T1* and *cml24-4* pollen germinated less readily in vitro than did WT pollen; the mean germination percentages of the two mutants were 26 % (*cml24-T1*) and 22 % (*cml24-4*), compared with the WT rate of 67 % (Fig. 3d). The mean length of the pollen tube after 10 h was also reduced in both mutants (Fig. 3a–c, e). Quantification of the growth rate of the pollen tube showed a delay in both mutants compared with WT (Fig. 3f–i). Analysis of in vivo germination based on pollinating WT stigma showed that, while WT pollen had successfully penetrated into the style and reached the top of the transmitting tract within 2 h of pollination (Fig. 4a), the mutant pollen tubes had only just begun to penetrate the style by this time (Fig. 4b, c). At 6 h after pollination, WT pollen tubes had grown much more strongly than those of the two mutants (Fig. 4d–f). And by 10 h after pollination, the WT pollen tube had reached the bottom of the transmitting tract (Fig. 4g), while those of the mutants had only reached the halfway point (Fig. 4h, i). Data from the pollination experiments suggest that pollen germination on the stigma is delayed and growth of the pollen tube in the style and transmitting tract is inhibited in *cml24* mutants.

Complementation of the *cml24-T1* mutant

Transforming *cml24-T1* with a copy of *CML24* driven by its native promoter fusion restored the WT level of *CML24* transcription (Fig. 5d). The average pollen germination rate, pollen tube length and growth rate of the transgenic lines C-1 and C-2 did not differ significantly from WT levels (Fig. 5e, f, h). Corresponding photos are shown in Fig. 5a–c, g. In the in vivo germination assay, when measured 6 h after pollination, the length of the pollen tube in C-1 and C-2 was equivalent to that of the WT pollen tube (Fig. 5i). These results confirm that defects in male gametophytes are indeed caused by knock-down expression of *CML24*.

Decreased seed set in *cml24* mutants is caused by abnormal pollen tube growth

Analysis of self-fertilized WT, *cml24-T1* and *cml24-4* mutant plant siliques showed that the seed setting rate of the mutants was about 70 and 67 %, which is lower than

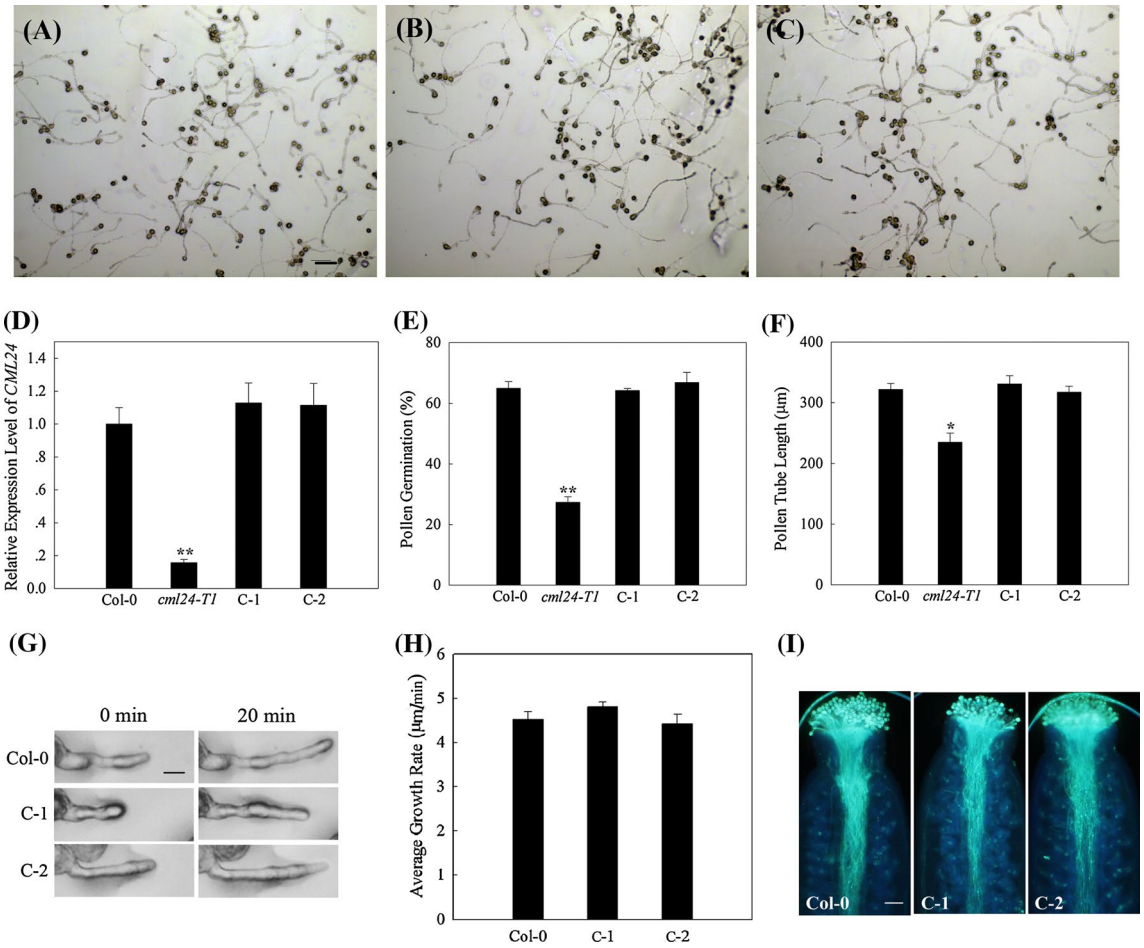

Fig. 5 Complementation of *cml24*. **a** WT pollen germinated for 10 h. **b**, **c** Pollen of complementation lines C-1 and C-2 germinated for 10 h. *Bar* 100 μm. **d** *CML24* transcript abundance assayed by qPCR. **e** Germination rate of pollen and **f** pollen tube elongation in lines C-1 and C-2. **g** Time-lapse images of the growth of WT, C-1, and C-2 pollen tubes. *Bar* 10 μm. **h** Mean growth rate of WT, C-1, and C-2 pollen tubes. *Error bars* indicate the SE. **i** WT, C-1, and C-2 pollen applied to WT stigma, sampled after 6 h. *Bar* 100 μm. *, **$P < 0.01, 0.005$; *error bars* show the SE

the 100 % setting rate of WT (Fig. 6e). Photos of discolored siliques are shown in Fig. 6a, b. The transgenic complemented lines C-1 and C-2 had normal seed settings as for WT (Fig. S3a). The average seed number per silique was equivalent to that of the WT silique (Fig. S3b).

To further clarify if the seed defect in *cml24* mutants was caused by male gametophytes, we performed reciprocal cross-pollinations between wild-type and *cml24* mutant plants. WT stigmas were pollinated with pollen from Col-0 or *cml24* mutants. The average seed setting rate was 72 and 71 % when *cml24-T1* and *cml24-4* was the pollen donor (Fig. 6f), which is consistent with the self-fertilized plants.

cml24 mutants are hyposensitive to extracellular Ca^{2+} and K^+

Extracellular ions such as Ca^{2+} and K^+ are important for pollen germination and tube growth, promoting both processes at low concentration and restraining them at high concentration (Wang et al. 2004; Fan et al. 2001). *CML24* is a candidate downstream effector of calcium, and probably participates in Ca^{2+}-related signal transduction. Also, calcium current by plasma membrane calcium channel is the key trigger in activating Ca^{2+} signal (Pandey et al. 2007; Zhang et al. 2007). A concentration gradient of calcium was employed for pollen germination bioassays. The growth of the WT pollen tube increased as the concentration of exogenous Ca^{2+} supplied rose from 0.1 to 10 mM, but was suppressed at 50 mM. The growth of both the *cml24-T1* and the *cml24-4* pollen tubes behaved quite similarly, although their level of sensitivity to Ca^{2+} was not so great (Fig. 7a). As the effect of extracellular Ca^{2+} on pollen tube growth is regulated by transmembrane cellular signals (Holdaway-Clarke et al. 1997), this suggests that CML24 may participate in the regulation of cellular Ca^{2+} signal.

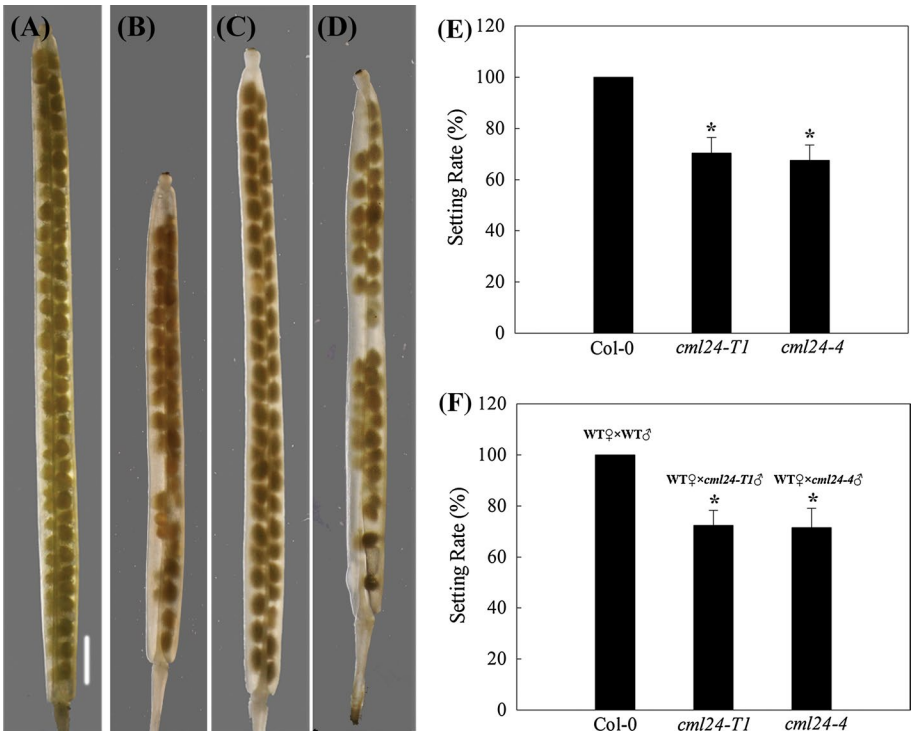

Fig. 6 *cml24* mutation results in a high proportion of nonfertilized ovaries. Siliques of **a** WT, **b** *cml24-T1* mutant, **c** WT♀ × WT♂ hybrid, **d** WT♀ × *cml24-T1*♂ hybrid. *Bar* 0.1 cm. The seed setting rate of siliques in **e** selfed WT, *cml24-T1*, and *cml24-4* plants, **f** pollinated WT, and *cml24* mutant plants. **P* < 0.01. *Error bars* indicate the SE

To keep rapid growth, germinated pollen tubes are equipped with an efficient ion transport system at the plasma membrane to sustain higher cellular osmotic pressure (Zhang et al. 2007). K^+ is a crucial osmotic ion for pollen tube elongation. The extension of the pollen tube in response to exogenous K^+ was also investigated. Supply of up to 10 mM K^+ promoted the elongation of the WT pollen tube, but growth was suppressed once the concentration reached 20 mM. Both *cml24-T1* and *cml24-4* pollen tubes were less sensitive to exogenous K^+ than were WT tubes (Fig. 7b). Pollen tube elongation in the complemented *cml24-T1* lines behaved indistinguishably from WT ones (Fig. S4).

$[Ca^{2+}]_{cyt}$ in the *cml24* mutants was greater than in WT

The effect of extracellular Ca^{2+} and K^+ on pollen tube growth may be mediated by intracellular signals, and then further alter transmembrane transportation of these ions. Intracellular Ca^{2+} has been proved to be one of the most important second messengers and is involved in regulating pollen germination and pollen tube growth (Konrad et al. 2011). Quantification of the Fluo-3 AM fluorescence generated in treated *cml24* mutant pollen and pollen tubes showed that $[Ca^{2+}]_{cyt}$ was higher than in WT (Fig. 8). The level present in the pollen and pollen tubes of the complemented *cml24-T1* lines was the same as in WT plants (Fig. S5).

Disorganized actin cables are a characteristic feature in the *cml24* mutants

Actin dynamics and F-actin patterns play critical roles in regulation of pollen germination and pollen tube growth (Fu et al. 2001), so it is worth checking whether the actin cytoskeleton organization is affected by CML24 mutation. Confocal microscopy imaging showed that, in WT pollen tubes, the actin filaments formed a dense structure at the subapex (Fig. 9a II), but were less abundant in the apical region (Fig. 9a III), and with numerous longitudinal actin cables throughout the shank of the WT pollen tube (Fig. 9a I). However, this normal pattern of actin cytoskeleton in WT pollen tubes was disrupted in the *cml24-T1* pollen tubes (Fig. 9b–f); For example, some of the mutant tubes completely lacked the subapex actin structure (Fig. 9b ii), the actin filaments at the extreme tip also showed abnormal organization (Fig. 9c iii), or the actin cables were not oriented in a strictly longitudinal arrangement parallel to the growth axis (Fig. 9d i, e i). In WT tissue, most (>91 %) of the actin appeared normal, whereas in the *cml24-T1* mutant, this proportion was slightly over 12 %. A similar level of disorganization was a feature of the *cml24-4* pollen tubes as well (Fig. S6b–d). In the complemented *cml24-T1* lines, the actin cytoskeleton was indistinguishable from that of WT (Fig. S6e, f).

cml24 mutant pollen germination and pollen tube extension are insensitive to LatB treatment

The above results suggest that the level of filamentous actin is affected in *cml24* pollen tubes. We sought to determine the effects of the suggested alterations in *cml24-T1* and *cml24-4* actin levels using the actin-depolymerizing drug latrunculin B (LatB), which prevents actin polymerization by binding monomeric actin (Gibbon et al. 1999). A concentration gradient of LatB was employed for pollen germination bioassays. The germination of *cml24* mutant pollen was unaffected by the presence of 0.5–1.5 nM LatB in the medium. In contrast, WT pollen was sensitive to the drug, especially at the highest concentration tested, at which hardly any pollen grains were able to germinate (Fig. 10a). The elongation of both WT and *cml24* mutant pollen tubes was inhibited by LatB in a dose-dependent manner, although the effect was less marked in the mutants' pollen (Fig. 10b). In the presence of 1.5 nM LatB, WT pollen tube growth was stopped, whereas those of *cml24-T1* and *cml24-4* continued to extend (Fig. 10b). Photographs are shown in Fig. S7. Meanwhile, the complemented *cml24-T1* pollen responded in the same way as did WT pollen (Fig. S8).

Discussion

The *A. thaliana CML* genes encode putative Ca^{2+} sensors and feature a conserved Ca^{2+}-binding domain (McCormack and Braam 2003). CML24 is a 161-residue 16-kDa protein which shares 66 % peptide similarity and about 40 % peptide identity with the *A. thaliana* calmodulins 1, 2, 6, and 7. CML24 binds Ca^{2+}, and its conformation is altered following its binding with Ca^{2+} (Delk et al. 2005). The inference is that it probably functions as a Ca^{2+} sensor. Here, it has been demonstrated that CML24 plays a role in regulating pollen germination and pollen tube elongation, since mutations to the gene inhibited both processes both in vitro (Fig. 3) and in vivo (Fig. 4). The abundance of *CML24* transcript appeared to be greater in *cml24-4* than in *cml24-T1* (Fig. 2), even though the phenotype was more severe in *cml24-4*. The assumption is that the K124E point mutation altered functions in addition to its signaling with respect to pollen tube development. 4′,6-Diamidino-2-phenylindole (DAPI) treatment revealed that the nuclei of both *cml24-T1* and *cml24-4* pollen took up as much of the stain as did WT nuclei (Fig. S1), which suggests that the lesion in the *cml24* mutants was not one affecting pollen development, but rather one which compromised pollen germination and tube growth. The mutants also showed lower seed setting rates (Fig. 6), while the outcome of the reciprocal crosses proved that the compromised self-fertility of the mutants was due to a weakness in male gametophytes (Fig. 6).

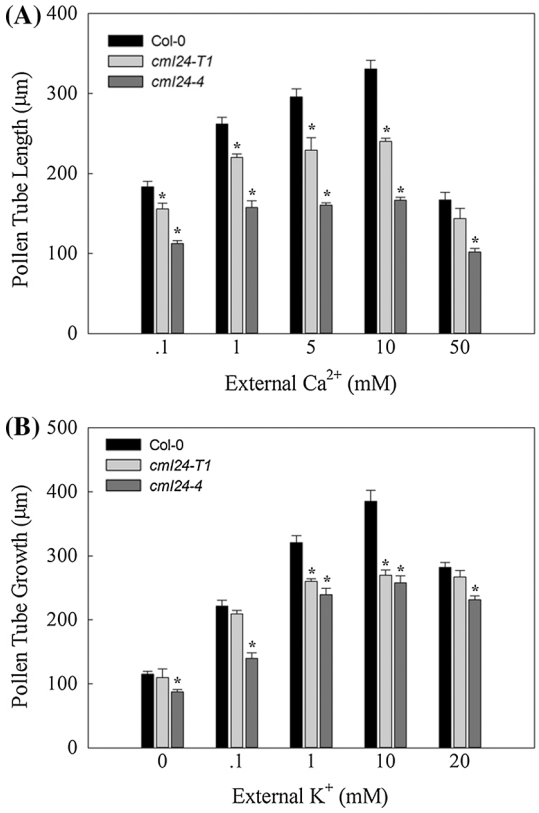

Fig. 7 Effect of externally applied Ca^{2+} and K^+ on pollen tube extension. **a** Externally applied Ca^{2+}. Pollen grains were germinated on solidified medium containing 1 mM K^+ and the indicated concentration of Ca^{2+}. **b** Externally applied K^+. Pollen grains were germinated on solidified medium containing 10 mM Ca^{2+} and the indicated concentration of K^+. *Error bars* indicate the SE. $*P < 0.01$

Cytoplasmic free Ca^{2+} is a ubiquitously exploited messenger during episodes of both biotic and abiotic stress (Batistic and Kudla 2012), while the K^+ ion is used by many plant tissues, including the pollen tube, to balance their osmotic environment and stabilize their membranes (Franklin-Tong 2010). K^+ influx also promotes pollen tube growth (Fan et al. 2001; Mouline et al. 2002; Boavida and McCormick 2007). The expression of CML24-GFP in *Arabidopsis* leaf protoplasts demonstrates that CML24 is deposited in the cytoplasm (Fig. S2). It determines the sensitivity of the pollen and pollen tube to exogenously supplied K^+ and Ca^{2+} (Fig. 7), and it is involved in the regulation of $[Ca^{2+}]_{cyt}$ (Fig. 8). The behavior of the complemented *cml24-T1* lines confirmed the function of CML24 in regulating pollen germination and pollen tube growth (Figs. 5, S4, S5). Raising $[Ca^{2+}]_{cyt}$ has been implicated in the inhibition of inward K^+ currents in the pollen tube (Zhao et al. 2013), so the higher $[Ca^{2+}]_{cyt}$ level in the *cml24* mutants may explain their observed hyposensitivity towards extracellular Ca^{2+} and K^+ during pollen germination and tube growth.

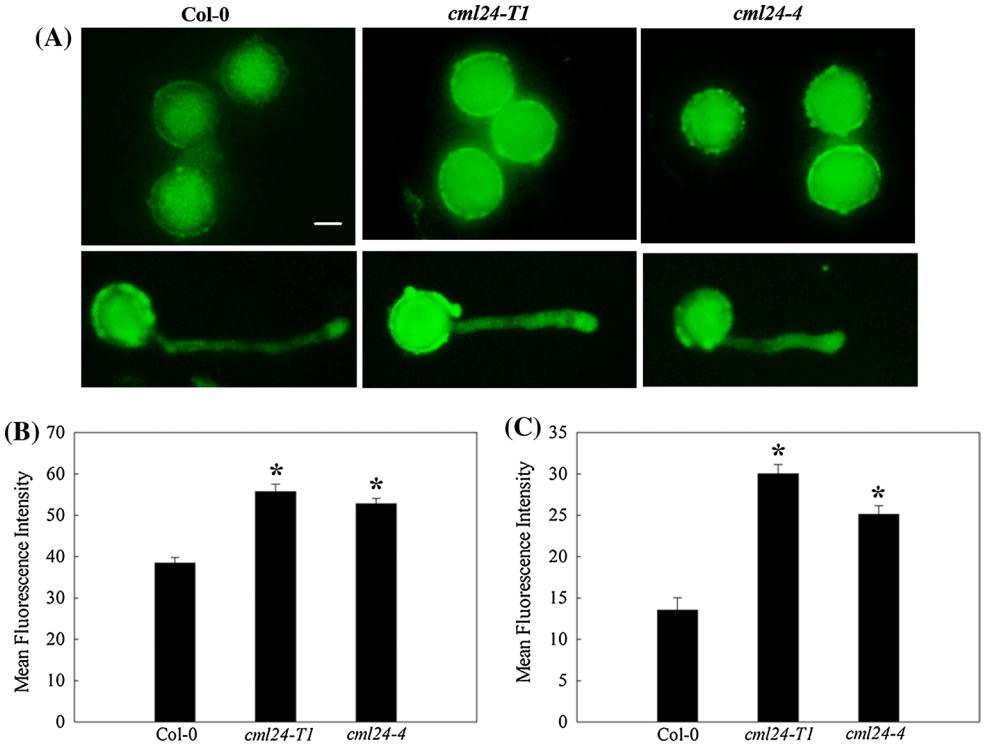

Fig. 8 $[Ca^{2+}]_{cyt}$ in pollen grains and pollen tubes. **a** Fluorescent images of WT, *cml24-T1*, and *cml24-4* pollen grains and pollen tubes treated with Fluo-3 AM. **b** Quantification of $[Ca^{2+}]_{cyt}$ in **c** pollen and **d** pollen tubes. *Scale bar* indicates 10 μm. *Error bars* indicate the SE. $*P < 0.01$

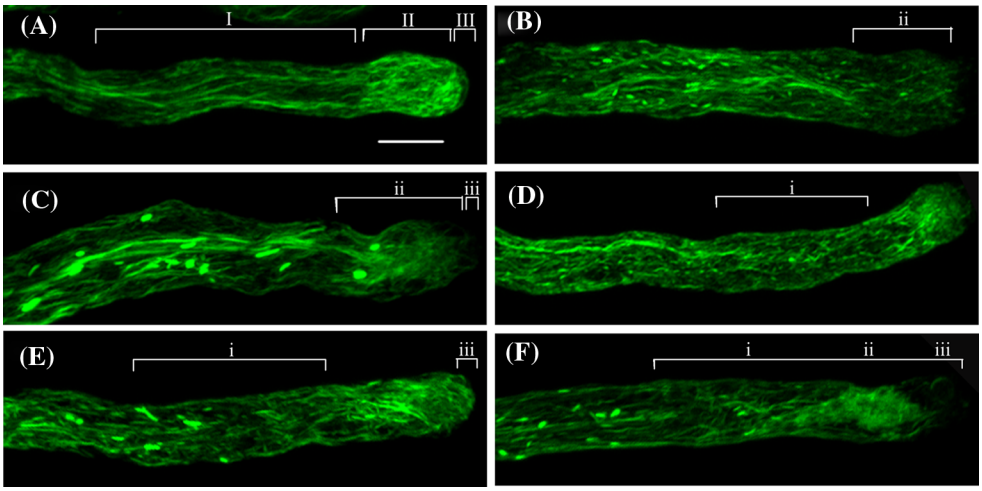

Fig. 9 Disorganization of actin cables in *cml24-T1* pollen tube. **a** WT pollen tube: (*I*) longitudinal actin cables, (*II*) actin fringes at the subapex of WT pollen tube, (*III*) highly dynamic but less abundant actin filaments at the extreme tips. **b–f** Forms of actin disorganization (*square brackets*) in *cml24-T1* pollen tubes. *i*, *ii*, and *iii* show abnormal actin organization compared with normal structures *I*, *II*, and *III* in WT pollen tubes, respectively. *Bar* 10 μm

There is a close interaction between intracellular Ca^{2+} and cytoskeleton in pollen tubes. High levels of $[Ca^{2+}]_{cyt}$ are known to damage the actin cytoskeleton of the lily pollen tube (Cardenas et al. 2008). Here, the actin cables formed in the *cml24* mutant pollen tubes appeared disorganized, especially the actin fringe (Fig. 9). In lily, the actin fringe is essential for the rapid growth of the pollen tube, and its density and length are both determinants of the tubes' growth rate (Dong et al. 2012). The growth rate of the *cml24* pollen tube was much slower than that of the WT tube (Fig. 3), and they exhibited a distinctly abnormal actin fringe. Apical F-actin dynamics are correlated with the $[Ca^{2+}]_{cyt}$ at the distal end of the tube, and it is believed that some cross-talk occurs between actin dynamics and the

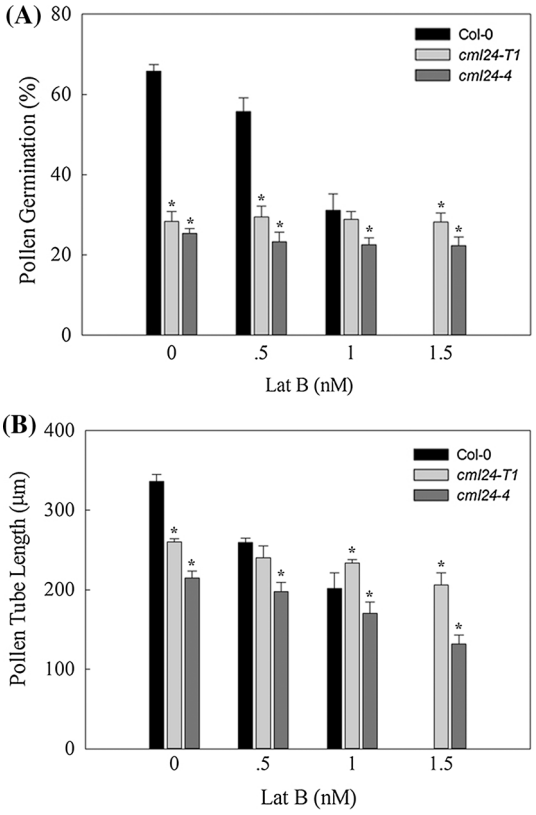

Fig. 10 *cml24* mutation reduces the sensitivity of pollen germination and pollen tube elongation to LatB treatment. **a** WT, *cml24-T1*, and *cml24-4* pollen germinating on a medium containing various concentrations of LatB for 10 h. **b** The insensitivity of pollen tube elongation to LatB treatment in the absence of CML24. $*P < 0.01$

oscillation of the Ca^{2+} gradient in this region during tube growth (Fu et al. 2001; Wang et al. 2004; Gu et al. 2005; Hwang et al. 2005). Ion fluxes regulate the structure and activity of F-actin through the medium of various actin binding proteins (Cardenas et al. 2008). According to Wang et al. (2004), Ca^{2+}-activated actin depolymerization may further raise the $[Ca^{2+}]_{cyt}$, while actin depolymerization-induced elevation of $[Ca^{2+}]_{cyt}$ may in turn accelerate actin depolymerization. Therefore, the disorganized actin filaments noted in the *cml24-T1* and *cml24-4* pollen tubes may reflect the outcome of a higher than normal $[Ca^{2+}]_{cyt}$. A high $[Ca^{2+}]_{cyt}$ can elevate the amount of monomeric actin present, supplying additional targets for LatB to bind to, and thereby preventing an optimal rate of actin polymerization from occurring in the pollen tube. Consequently, the hyposensitivity of the *cml24-T1* and *cml24-4* mutants to LatB may result in a greater frequency of monomeric actin in the pollen tube (Fig. 10). After binding with LatB, the monomeric actin could subsequently be polymerized, in this way supporting the extension of the pollen tube in the *cml24* mutant.

According to Tsai et al. (2013), CML24 interacts with the autophagy-related gene *ATG4b* and affects autophagy progression in *Arabidopsis*. Recycling the contents of the cytoplasm is a marker for cell autophagy, and is a process particularly required for pollen tube extension (Liu and Bassham 2012; Chebli et al. 2013). The *A. thaliana* protein ATG6 co-localizes with ATG8, a symbol of pre-autophagosome formation, and is essential for normal germination of the pollen (Fujiki et al. 2007; Harrison-Lowe and Olsen 2008). The known interaction of CML24 with ATG4b to regulate AGT8 accumulation and autophagy (Tsai et al. 2013) suggests a second possible regulatory pathway in which CML24 could be involved in pollen tube extension.

In line with the proposed function of all *CML* gene products, CML24 is likely to be a downstream component in $[Ca^{2+}]_{cyt}$ signaling. Our hypothesis is that pollen development requires a robust intracellular signaling system to regulate plasma membrane Ca^{2+} channels, generate the appropriate Ca^{2+} concentration, and integrate K^+ influx to modulate the cytoplasmic Ca^{2+} signal. Any blockage of downstream signaling in response to $[Ca^{2+}]_{cyt}$ could activate a feedback response in order to raise the $[Ca^{2+}]_{cyt}$. Since mutations to *CML24* inhibit pollen germination and slow the elongation of the pollen tube, the inference is that CML24 is a positive regulator of both of these processes. Understanding how CML24 modulates $[Ca^{2+}]_{cyt}$ to facilitate K^+ influx will require an electrophysiological approach based on *cml24* mutants and transgenic overexpressors. A major mechanism for the regulation of ion channel activity is protein phosphorylation/dephosphorylation, but CML24 is unlikely to be a protein kinase; so a screen aimed at identifying which proteins interact with CML24 may provide a handle on the relationship between CML24 activity, $[Ca^{2+}]_{cyt}$ regulation of ion channel activity, and pollen germination and tube growth.

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