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ORIGINAL PAPER

PCaP2 regulates nuclear positioning in growing *Arabidopsis thaliana* root hairs by modulating filamentous actin organization

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Abstract
Key message **PCaP2 plays a key role in maintaining the nucleus at a relatively fixed distance from the apex during root hair growth by modulating actin filaments.**
Abstract During root hair growth, the nucleus localizes at a relatively fixed distance from the apex. In *Arabidopsis thaliana*, the position of the nucleus is mainly dependent on the configuration of microfilaments (filamentous actin). However, the mechanisms underlying the regulation of actin dynamics and organization for nuclear positioning are largely unknown. In the present study, we demonstrated that plasma membrane-associated Ca^{2+} binding protein 2 (PCaP2) influences the position of the nucleus during root hair growth. Abnormal expression of *PCaP2* in *pcap2* and *PCaP2 overexpression* plants led to the disorganization of actin filaments, rather than microtubules, in the apex and sub-apical regions of root hairs, which resulted in aberrant root hair growth patterns and misplaced nuclei. Analyses using a PCaP2 mutant protein revealed that actin-severing activity is essential for the function of PCaP2 in root hairs. We demonstrated that PCaP2 plays a key role in maintaining nuclear position in growing root hairs by modulating actin filaments.

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Y. Zhang and E. Kang contributed equally to this work.

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Introduction

Root hairs are tubular protrusions on root epidermal cells. The formation of root hairs expands the root surface area and facilitates the uptake of water and nutrients from the surrounding environment (Dolan et al. 1994; Ketelaar and Emons 2001, 2009; Libault et al. 2010). Root hairs exhibit typical tip growth, which is regulated by a variety of factors, including cytoskeleton elements, vesicular transport, and cell wall synthesis processes (Carol and Dolan 2002; Diet et al. 2006; Ovečka et al. 2005; Pei et al. 2012). Fine filamentous actin in the sub-apical region of growing root hairs likely plays an important role in the regulation of root hair tip growth by delivering new membrane and cell wall material to the growth site (Miller et al. 1999; Fan et al. 2011). A variety of actin-binding proteins (ABPs), such as formins, Arp2/3 complex, profilin, actin depolymerizing factors (ADFs), and villins, are involved in the regulation of filamentous actin organization and dynamics in root hairs (Ketelaar and Emons 2009; Pei et al. 2012). Many upstream signal molecules, such as calcium, small GTPase, reactive oxygen species (ROS), and phospholipids, relay signals to these actin-binding proteins to modulate actin dynamics and organization, as well as root hair growth (Cole and Fowler 2006; Lee and Yang 2008; Pei et al. 2012). The position and migration of nuclei in root hairs is also believed to be closely related to root hair growth. In growing *Arabidopsis thaliana* root hairs, the nucleus migrates during root hair growth and remains at a relatively fixed distance from the growing tip (Miller et al. 1997; Ketelaar et al. 2002). Precise positioning of the nucleus in a

root hair suggests that the nucleus may be involved in the regulation of root hair elongation (Ketelaar et al. 2002; Jones and Smirnoff 2006). Chytilova et al. (2000) observed nuclear localization signal-GFP-β-glucuronidase (GUS) protein in living root hairs after treatment with various cytoskeleton-specific agents. They showed that the rapid and long-distance movement of the nucleus depends on filamentous actin but not on microtubules. Pharmacological analysis has demonstrated that nuclear positioning is dependent on the sub-apical fine filamentous actin located between the nucleus and the root hair apex (Ketelaar et al. 2002). However, the regulatory mechanisms of filamentous actin organization and dynamics for nuclear positioning during root hair tip growth remain unclear.

Initially, plasma membrane-associated Ca^{2+} binding protein2 (PCaP2) was reported to regulate polar cell growth in vegetative tissues by destabilizing microtubules, known as "MICROTUBULE-ASSOCIATED PROTEIN18 (MAP18)" (Wang et al. 2007). Our previous GUS activity analysis and data from the Genevestigator database (www.genevestigator.ethz.ch) indicate that *PCaP2* is widely expressed in the root, hypocotyl, and cotyledon and that it is highly expressed in rapidly elongating cells, such as root hairs and pollen tubes. We previously demonstrated that PCaP2 exhibits calcium-dependent actin filament-severing activity. It regulates pollen tube directional growth by modulating actin dynamics at the apex (Zhu et al. 2013). In addition, Kato et al. (2010, 2013) demonstrated that *PCaP2* was strongly expressed in root hairs and involved in root hair development. N23 domain (the N-terminal basic domain with 23 amino acids) of PCaP2 regulates root hair tip growth negatively through processing Ca^{2+} and phosphatidylinositol phosphates (PtdInsPs) signals on the plasma membrane, while the residual domain plays a role in the polarization of cell expansion. These reports implicate a possible role for PCaP2 in the regulation of root hair growth. However, the underlying mechanism is still unclear.

In this study, we demonstrated that PCaP2 is involved in the regulation of nuclear positioning during root hair growth. Further, its actin filament-severing activity is required to maintain nuclear position with respect to the growing apex.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh. ecotype Columbia (Col) was used for all wild-type and mutant background tissue in this study. Plants were grown in soil at 22 °C with a photoperiod of 16 h light/8 h dark. Seeds were sterilized for 15 min in 5 % sodium hypochlorite and treated in growth medium at 4 °C in the dark for 2 d before their transfer to a growth room. Growth medium contained half-strength Murashige and Skoog salts, 1 % w/v sucrose and 0.9 % plant TC agar (PhytoTechnology Laboratories, Shawnee Mission, KS, USA).

Measurement of root hair length and position of the nucleus in root hairs

Root hairs were observed in 5-day-old seedlings grown on half-strength MS agar plates. The root hairs were selected from tip to 4 mm of each primary root tip to measure. For staining the nucleus, 5-day-old seedlings were incubated in 5 mg/mL PI (propidium iodide, Sigma-Aldrich-P4170) in half-strength MS liquid medium for 3–5 min, then examined under an Olympus SZX16 microscope equipped with a color CCD camera (Olympus DP70, Tokyo, Japan). Images were acquired with ImagePro software (Media Cybernetics Inc., Rockville, MD, USA). ImageJ was used to quantify the pixel intensity in the root hairs (http://rsbweb.nih.gov/ij/; version 1.38).

At least 80 roots grown on three different plates were measured to calculate the means and standard deviations of root hair lengths and the position of the nucleus in root hairs.

Drug treatment

Oryzalin (3,5-dinitro-*N*4,*N*4-dipropylsulfanilamide, Sigma-Aldrich-36182), and latrunculin B (Sigma-Aldrich-L5288) stocks were made in DMSO and diluted at least 1000-fold according to the requirements of the specific experiments.

For calculating the distance between the nucleus and the root hair apex, 4-day-old wild-type seedlings were transferred to other half-strength Murashige and Skoog medium containing the microtubule-specific drug oryzalin at a final concentration of 150 nM for one day.

Microscopic analysis of root hair growth

Seeds of wild type or mutants were germinated on a 48 × 64 mm slide layered with 0.9 % plant TC agar supplemented with half-strength Murashige and Skoog medium and covered by coverslips in a 90-mm petri dish. After 5 days, coverslips containing the seedlings were transferred directly onto the stage of an inverted Olympus IX81 microscope (Olympus) equipped with an spinning disc confocal system (Yokogawa, Tokyo, Japan) and Andor iXon charge-coupled device camera (Andor Technology, Belfast, Northern Ireland) and *a* × 60 1.42 NA Olympus objective.

To examine the growth rate and monitoring nucleus migration simultaneously, root hairs were photographed twenty times within 600 s. The distance between the apex and the nucleus in root hairs was recorded. The increase in the length of each root hair was determined, and mean growth rate was calculated.

Images were acquired using iQ software (Andor Technology). GFP was excited using 488 nm argon lasers, and emission was collected through 525 ± 5.5 nm filters.

To investigate microtubule organization decorated by GFP-MBD, images were acquired using 70 % laser intensity and operating in the mode 512 × 512 with an exposure time of 400 ms.

To observe actin organization, single (for apical region) or Z-stack-projections (for shank region) of scanning laser sections (0.5 μm) of root hairs expressing *GFP-fABD2-GFP* were obtained. Time-lapse images were taken at 5-s intervals over a 10-min period using 200 ms exposure times.

Skewness was measured to quantify the extent of actin bundling and occupancy was measured to quantify the extent of actin filament density in root hair cells as previously described (Zhu et al. 2013). Data were analyzed in ImageJ software.

To quantify the localization of PCaP2-eGFP, mean fluorescence intensity of eGFP signal at the apical plasma membrane (30 μm) and cytosol (40 μm^2) was measured using ImageJ software.

Results

PCaP2 functions in root hair growth

PCaP2 is expressed in various types of cells, tissues, and organs in *A. thaliana*, including roots and root hairs. This suggests that PCaP2 may function in root hair development and growth.

We investigated the function of PCaP2 in root hairs by analyzing previously reported *pcap2* (a T-DNA insertion mutant) and *PCaP2 over-expression* (*PCaP2 OE*, known as *MAP18 OE*) line (Wang et al. 2007). We compared the lengths of root hairs between wild-type, *pcap2*, and *PCaP2 over-expression* plants. We observed that root hairs from *pcap2* seedlings were shorter than those of wild-type seedlings (Fig. 1a). Quantitative analysis showed that root hair of *pcap2* was obviously shorter than the wild type (Fig. 1b) and the mean length of *pcap2* root hairs was a half of the mean length of wild-type root hairs (Fig. 1c). The short root hair phenotype of *pcap2* was rescued by transformation with a *PCaP2promoter:PCaP2-eGFP* construct (line COM#9; described by Zhu et al. 2013)

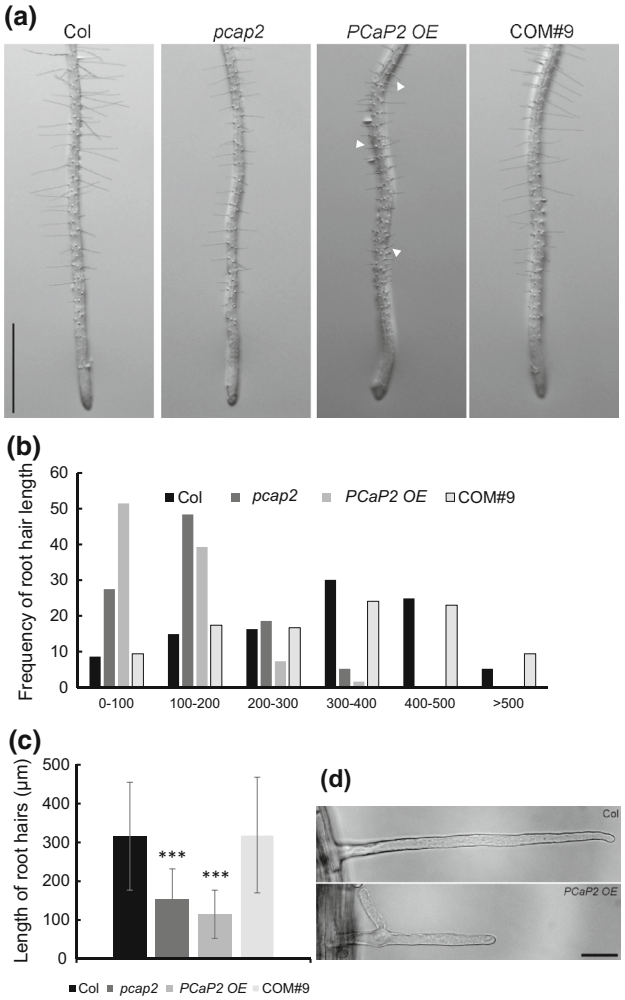


Fig. 1 Root hair morphology of wild-type, *pcap2*, and *PCaP2 over-expression* seedlings. **a** Representative images of root hairs in wild-type, *PCaP2 over-expression (OE)*, *pcap2* mutant, and COM#9 line. *PCaP2 over-expression* and *pcap2* root hairs were obviously shorter than wild-type root hairs. The branching root hairs in the *PCaP2 over-expression* are marked by the *white arrowheads*. *Bar* 1 mm. **b** Frequency distribution of the root hair length of *different lines*. *PCaP2 over-expression* and *pcap2* root hairs were shorter than wild-type root hairs. **c** Average root hair length of root hairs of *different lines*. *PCaP2 over-expression* and *pcap2* root hairs were shorter than wild-type root hairs. There was no significant difference between wild-type and COM#9 root hairs. More than 150 root hairs were measured from each line. Data represent the mean ± SD based on three independent experiments. $***P < 0.001$ by Student's *t* test. **d** The branching phenotype in the *PCaP2 over-expression* root hair. *Bar* 50 μm

(Fig. 1a–c). *PCaP2 over-expression* also exhibited the short root hair phenotype (Fig. 1a). The length of root hairs was significantly reduced in *PCaP2 over-expression* plants compared to the wild type (Fig. 1b, c). Interestingly, some root hairs in *PCaP2 over-expression* plants displayed highly abnormal branched forms (Fig. 1a, white arrowheads and Fig. 1d).

These data indicate that abnormal expression of *PCaP2* leads to abnormal root hair growth and that complementation could rescue the short hair phenotype of a *pcap2* mutant. Together, these findings suggest that PCaP2 has an important function during root hair growth and development.

PCaP2 participates in the positioning of the nucleus in growing root hairs

In growing root hairs, the nucleus normally localizes at a relatively fixed distance from the root hair tip (Ketelaar et al. 2002). However, the nucleus can relocate when root hair growth is arrested. Immobilization of the nucleus by optical trapping increases the distance between the nucleus and the tip and subsequently leads to the growth arrest of root hairs (Ketelaar et al. 2002). We suspected that the growth inhibition observed in *pcap2* and *PCaP2 over-expression* plants could be due to abnormal nuclear positioning in root hairs.

To test our hypothesis, we first observed the location of nuclei in wild-type root hairs from 5-day-old seedlings after staining the nuclei with propidium iodide (PI). In the assay of PI staining, the root hairs at 2–3 mm from each primary root tip were defined as growing root hairs identified by the accumulated cytoplasm at the tip region, and that at 7–8 mm from each primary root tip were defined as fully grown root hairs identified by vacuole at the apex. The distance between the nucleus and the apex in growing root hairs was relatively fixed (Fig. S1a, d), but in fully grown root hairs, the positions of nuclei were scattered (Fig. S1b, e). We then monitored nuclear movement in growing root hairs. A typical time-lapse series of a growing root hair is shown in Fig. S1c. The correlation of nuclear migration with root hair growth was observed and the distance between the nucleus and the root hair apex was fixed (Fig. S1c; Movie S1). These data are consistent with previous reports (Ketelaar et al. 2002; Sliwinska et al. 2012).

Next, we investigated if nuclear positioning was altered in *PCaP2 over-expression* and *pcap2* root hairs. We observed that the average distance between the nucleus and the tip in growing *PCaP2 over-expression* root hairs was greater than that in wild-type root hairs (Table 1). The average distance in growing *pcap2* root hairs was significantly shorter than in wild-type root hairs. The average distance in growing COM#9 hairs was similar to the distance in wild-type root hairs (Table 1). Compared with the wild-type, COM#9 line, and *pcap2* mutant, the positions of nuclei were relatively scattered in growing root hairs of *PCaP2 over-expression* seedlings (Fig. 2a). There were no obvious differences in the distributions of nuclei in fully grown root hairs among these lines, which all displayed scattered patterns of nuclear location (Fig. 2b).

Table 1 Average distance between the apex and the nucleus in root hairs

Type of plant	In growing root hairs (μm)
Col	71 ± 19
pcap2	46 ± 16***
PCaP2 OE	95 ± 47***
COM#9	73 ± 20

Data represent mean ± SD, *** $P < 0.001$ by Student's *t* test

These findings indicate that PCaP2 is involved in the regulation of nuclear positioning in growing root hairs. Both the increase, which was caused by over-expression of *PCaP2*, and the decrease, which resulted from the down-regulation of *PCaP2*, in the distance between the nucleus and the apex in growing root hairs were accompanied by a growth inhibition phenotype. However, abnormal expression of *PCaP2* did not affect the position of the nucleus in fully grown root hairs in which growth had stopped.

The subcellular localization of PCaP2 in root hairs

We observed the localization pattern of PCaP2 in growing root hairs to analyze how PCaP2 regulates nuclear positioning in root hairs. In growing root hairs expressing PCaP2-eGFP (for enhanced green fluorescent protein) in the *pcap2* background, the fluorescence signal was mainly localized to the shank of the plasma membrane and the cytoplasm of the apical region. It appeared throughout the entire plasma membrane, including the apex, in fully grown root hairs (Fig. 3a, left panel). As the control, eGFP signal driven by the *PCaP2* promoter in the *pcap2* background was uniformly distributed throughout the cytoplasm in growing root hairs and not detected localizing to the plasma membrane in fully grown root hairs (Fig. 3a, right panel).

Next, we measured the plasma membrane/cytosol (PM/Cyt) signal ratio of PCaP2-eGFP fluorescence at the apex of growing and fully grown root hairs. The PM/Cyt ratio at the apex was higher in fully grown root hairs than in growing root hairs (Fig. 3b), which indicates that the PCaP2-eGFP fusion protein localizes to the apical cytoplasm of the growing root hair and the apex plasma membrane of the fully grown root hair. Furthermore, typical pixel intensities corresponding to the lines in Fig. 3a, b were plotted in Fig. 3c (signal at growing root hair shank) and Fig. 3d (signal at fully grown root hair apical PM), respectively. These results confirmed that PCaP2-eGFP was located at the apical cytoplasm during hair elongation and moved to the apex plasma membrane when growth stops. As the control, eGFP proteins exhibit cytosolic distribution in growing and fully grown root hairs. The subcellular localization pattern of PCaP2 in root

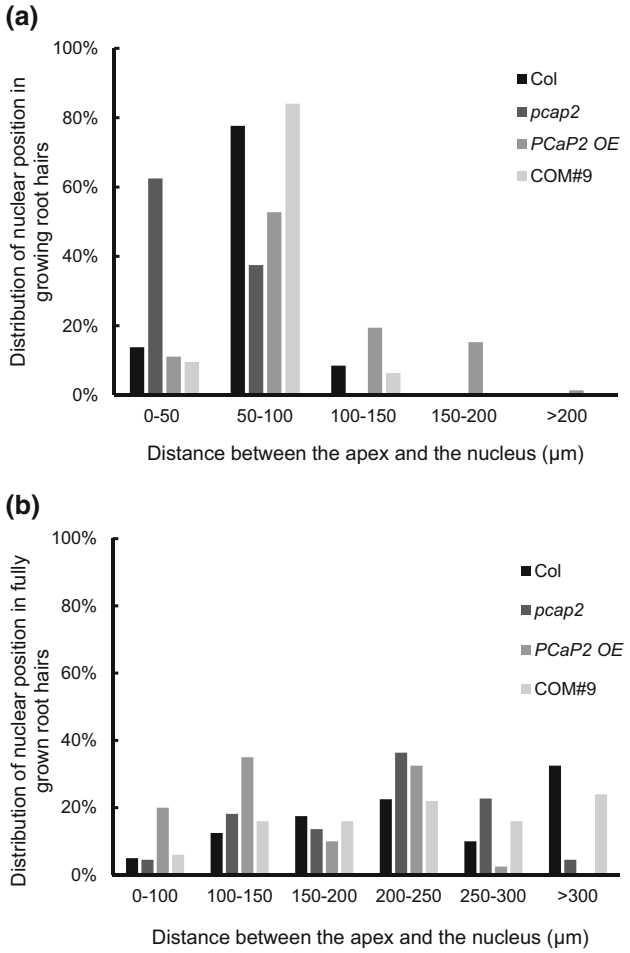


Fig. 2 Nuclear position in growing and fully grown wild-type, *pcap2*, and *PCaP2 over-expression* root hairs. **a** Frequency distribution of the distances between the apex and the nucleus in growing root hairs of wild-type, *PCaP2 over-expression(OE)*, *pcap2* mutant, and COM#9 line. The distance between the nucleus and the apex of growing root hairs in *PCaP2 over-expression* seedlings is longer than the distance in wild-type seedlings, and the distance in COM#9 seedlings is similar to the distance in wild-type seedlings. The distance in *pcap2* mutant seedlings is shorter than the distance in wild-type seedlings. The *X*-axis indicates the ranges of distances between the nucleus and the apex. The *Y*-axis indicates the percentage of plants with a measured distance (μm) between the nucleus and the apex within the given range. **b** Frequency distribution of the distances between the apex and the nucleus in fully grown root hairs of wild-type, *PCaP2 over-expression*, *pcap2* mutant, and COM#9 line. The nuclei are located at scattered positions in the seedlings

hairs was similar to the location pattern of PCaP2 in pollen tubes (Zhu et al. 2013).

Microtubules are not involved in nuclear movement during root hair growth

Previous studies demonstrated that PCaP2 can act on both microtubules and actin filaments (Wang et al. 2007; Zhu et al. 2013). Therefore, we investigated which cytoskeletal elements are modulated by PCaP2 to control nuclear position.

We conducted a pharmacological analysis to determine the role of microtubules and actin in maintaining the distance between the nucleus and the apex. First, we treated 4-day-old wild-type seedlings with 150 nM oryzalin for 1 day (Fig. 4a). This treatment caused complete depolymerization of microtubules. Compared to untreated control seedlings, no obvious difference in the distance between the nucleus and the root hair apex was detected in treated seedlings (Fig. 4b). This showed that depolymerization of microtubules does not obviously affect the position of the nucleus in root hairs, which is consistent with the previous report of Ketelaar et al. (2002) and supports the theory that microtubules do not play a major role in the regulation of nuclear positioning in root hairs.

To further confirm this finding, we observed microtubule organization in root hairs expressing *GFP-MBD* driven by the promoter *UBQ* (Marc et al. 1998). In growing root hairs, microtubules were organized as bundles along the longitudinal axis in the shank region; short microtubule fragments were observed (Fig. 4c, red arrowheads) and only a few microtubules were detected in the apical domain (Fig. 4c). However, there were no microtubule arrays surrounding the nucleus, indicating that the spatial arrangement of microtubules and the position of the nucleus are unrelated. Additionally, 10 min of treatment with 500 nM oryzalin, which depolymerized microtubules, did not significantly affect nuclear position in root hairs (Fig. 4c). We conclude that microtubules are not essential for nuclear positioning in growing *A. thaliana* root hairs.

PCaP2 regulates filamentous actin organization in the apical and sub-apical regions in growing root hairs

In our previous study, we reported that PCaP2 has a role in the calcium-dependent severing of actin filaments and that it modulates filamentous actin dynamics at the pollen tube apex to guide the direction of pollen tube growth (Zhu et al. 2013). Therefore, we speculated that PCaP2 may also be involved in nuclear positioning through the regulation of filamentous actin dynamics during root hair growth.

To test this hypothesis, we observed actin organization in root hairs using *35S::GFP-fABD2-GFP*, an actin reporter described by Wang et al. (2008), under a spinning disc confocal microscope. We detected obvious differences in the apical and sub-apical regions of wild-type, *pcap2,* and *PCaP2 over-expression* growing root hairs. A few highly dynamic fine filamentous actins were observed in the wild-type root hair tips (Fig. 5a, white arrowhead), and the actin cables protruded into the sub-apical regions of *pcap2* root hairs (Fig. 5b, white arrow). Fine actin

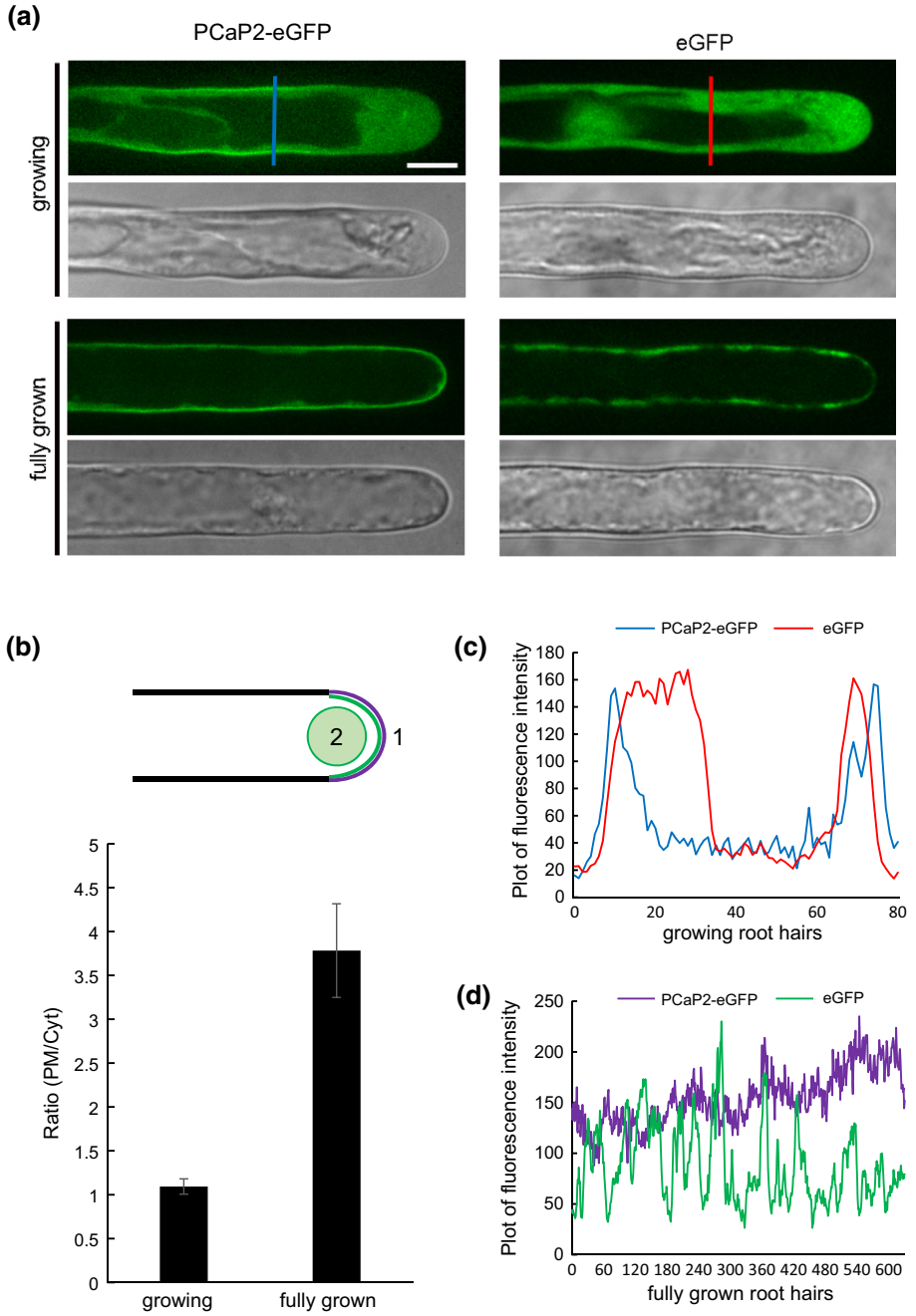
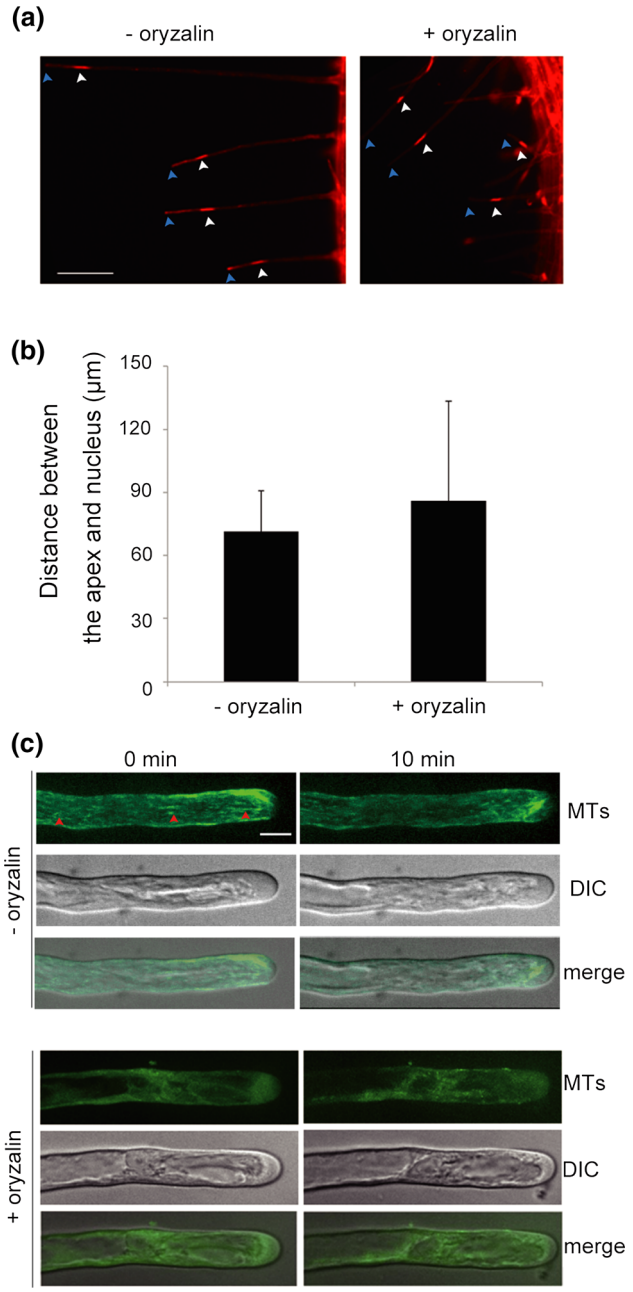


Fig. 3 Location pattern of PCaP2-eGFP in growing and fully grown root hairs. **a** Upper left panel: PCaP2-eGFP localizes to the shank plasma membrane and the cytoplasm of the apical region in a growing root hair. The corresponding DIC images show a large region of cytoplasm at the apex, which indicates that the root hair is still growing. *Bar* 5 μm. *Lower left panel*: The PCaP2-eGFP signal was observed throughout the entire plasma membrane, including the apex, in a fully grown root hair. The corresponding DIC images show a vacuole that has almost protruded to the apex and little cytoplasm at the apex, which indicates that the root hair has stopped growing. *Right panel* the eGFP driven by the *PCaP2* promoter in *pcap2* background exhibits cytosolic distribution in growing and fully grown root hairs. **b** Quantification of the plasma membrane/cytosol (PM/Cyt, marked by 1/2) fluorescence ratio of PCaP2-eGFP at the apex in growing and fully grown root hairs. The PM/Cyt ratio at the apex is higher in fully grown root hairs than in growing root hairs, which indicates that the PCaP2-eGFP fusion protein localizes to the apical cytoplasm of the growing root hair and the apex plasma membrane of the fully grown root hair. Data represent the mean ± SD of at least 20 measurements based on three independent experiments. ***$P < 0.001$ by Student's *t* test. **c** Fluorescence intensities (arbitrary units) across the PCaP2-eGFP growing root hair shank (*blue line*) or across the eGFP growing root hair shank (*red line*) in (a). The PCaP2-eGFP fusion protein localizes to the plasma membrane at the shank of the growing root hair, while eGFP distributes throughout the cytoplasm. **d** Fluorescence intensities (arbitrary units) along the root hair tip region in PCaP2-eGFP fully grown root hair (*purple line*) or eGFP fully grown root hair (*green line*) in (**b**). The PCaP2-eGFP signal's uniform distribution was observed in the entire plasma membrane, including the apex, while eGFP distributed unevenly



◀**Fig. 4** Microtubule arrangement and nuclear position in a growing root hair. **a** The nucleus localizes at a fixed distance from the apex in growing root hairs treated with or without 150 nM oryzalin for 1 day. *White arrowheads* the nuclei in the shanks of the root hairs; *blue arrowheads* the root hair tips. *Bar* 100 μm. **b** Distribution of the distances between the apex and the nucleus in growing root hairs. There is no obvious change in nuclear position in root hairs with or without 150 nM oryzalin. Data represent the mean ± SD from at least ten measurements. $P > 0.5$ by Student's *t* test. **c** Microtubules were visualized in growing root hair cells using expressed *UBQ::GFP-MBD*. *Top panel* microtubules are organized as bundles along the elongation axis of the shank in a growing root hair. *Red arrowheads* the microtubule bundles. No microtubules are located in the apical domains of the root hair. DIC images show the position of the nucleus in the shank of the root hair. No microtubules are surrounding the nucleus. The growth rate of root hair cells expressed *UBQ::GFP-MBD* is 0.9 ± 0.2 μm/min, consistent with that of the wild type. *Bottom panel* microtubules are depolymerized in a root hair treated with 500 nM oryzalin. DIC images show no effect of treatment on the nuclear position in this root hair. The beginning of our observation was defined as time 0 min. More than 50 root hairs were observed for each treatment and representative images are presented. All images are projections of scanning laser sections (0.5 μm) of Z-stacks. *Bar* 10 μm

filaments were also detected in the apical and sub-apical regions of *PCaP2 over-expression* root hairs (Fig. 5c, white arrowheads). The thick longitudinal actin cables were observed in the sub-apical regions of *pcap2* root hairs (Fig. 5e, white arrow), which terminate at the base of the sub-apical regions of wild-type root hairs (Fig. 5d, white arrow) or shank regions of *PCaP2 over-expression* root hairs (Fig. 5f, white arrow). Unlike in the apex, the longitudinal actin cables in the shanks of *pcap2* (Fig. 5e) and *PCaP2 over-expression* (Fig. 5f) root hairs were relatively similar to those in wild-type root hairs (Fig. 5d).

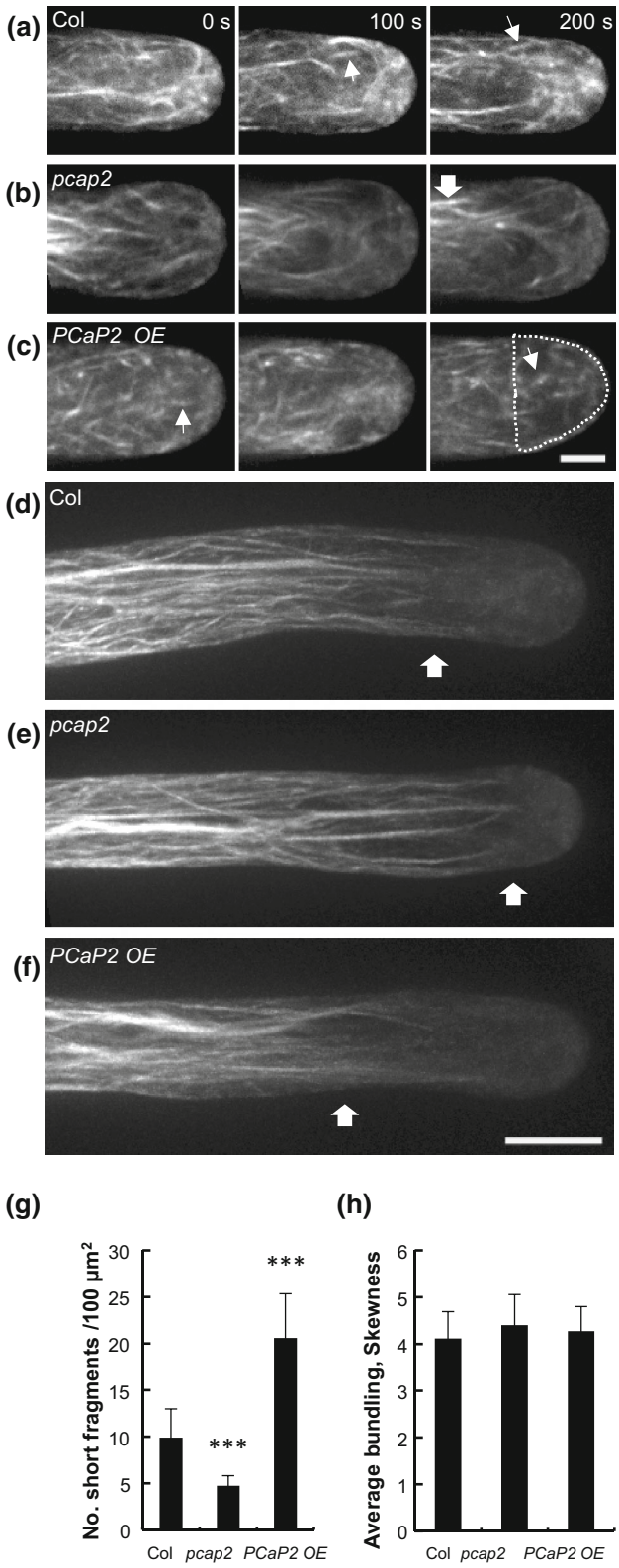
To quantify the defects of actin organization caused by abnormal expression of *PCaP2*, we counted the number of short fragments (length <2 μm) at the apex of growing root hairs. Twice as many short apical actin fragments were displayed in *PCaP2 over-expression* root hairs than in wild-type root hairs, but half the number of short fragments was observed in *pcap2* root hairs compared to wild-type root hairs (Fig. 5g). Next, we measured the skewness in growing root hairs to assess the extent of actin bundling in the shank. As shown in Fig. 5h, the mean skewness values in *pcap2* and *PCaP2 over-expression* root hairs were similar to the skewness value in wild-type root hairs. This suggests that the bundling of actin cables is not affected by either over-expression or down-regulation of *PCaP2*.

Together, these observations indicate that PCaP2 influences actin organization in the apical and sub-apical regions but not in the shank of growing root hairs.

Actin dynamic is essential for maintaining the distance between the nucleus and the hair apex during root hair growth

PCaP2 modulates filamentous actin dynamics in the apical and sub-apical region of growing root hairs. Taken together with the observation that PCaP2 also affects nuclear position in growing root hair, we speculated that PCaP2 plays a role in nuclear positioning by regulating filamentous actin dynamics in the apical and sub-apical region in growing root hairs.

To test this hypothesis, we investigated if apical or sub-apical fine actin dynamics are involved in nuclear positioning during root hair growth. We used latrunculin B



◀**Fig. 5** Actin organization in *PCaP2 over-expression* and *pcap2* growing root hairs. Actin was visualized in growing root hairs using expressed *GFP-fABD2-GFP* and observed using confocal microscopy. Images **a**–**c** are single scanning laser sections (0.5 µm) and images **d**–**f** are projections of scanning laser sections (0.5 µm) of Z-stacks. *Bar* 5 µm in (**c**) for (**a**–**c**), *bar* 10 µm in (**f**) for (**d**–**f**). **a**–**c** Time-lapse images showing actin filaments dynamic in the apical and sub-apical regions of growing root hairs. **a** Short fine actin filaments (*white arrowheads*) exist in the sub-apex of a wild-type root hair. **b** In a *pcap2* root hair, actin cables (*white arrow*) protrude into the tip region. Short fine actin filaments are absent. **c** Short fine actin filaments (*white arrowheads*) exist in the tip region of a *PCaP2 over-expression (OE)* root hair. **d**–**f** Axial actin cables are arranged longitudinally in the shanks of wild-type (**d**), *pcap2* (**e**), and *PCaP2 over-expression* (**f**) growing root hairs. The end of actin cables is indicated by the *white arrows*. **g** Quantitative analysis of the number of short actin fragments in the apex region [the region was defined as indicated by the *dashed line* in (**c**)] of root hairs. *PCaP2 over-expression* root hairs contain more actin fragments that are shorter than 2 µm [e.g., *white arrowheads* in (**c**)] in length than wild-type root hairs. However, fewer short actin fragments are present in *pcap2* root hairs. More than ten growing root hairs from each *line* were measured. *Error bars* ±SD from at least three experiments. $***P < 0.001$ by Student's *t* test. **h** Quantification of actin organization in wild-type, *pcap2*, and *PCaP2 over-expression* root hairs. There is no significant difference in actin bundling (indicated by the average skewness value) between wild-type, *PCaP2 over-expression*, and *pcap2* root hairs. Data represent the mean ± SD from at least ten growing root hairs for each *line*. $P > 0.4$ by Student's *t* test

(LatB), an actin polymerization inhibitor, to disrupt actin configuration; we then monitored the actin dynamics and nucleus behavior in root hairs. Movie S2 shows an untreated root hair, which served as the control. In this root hair, the nucleus remained at a fixed distance from the apex. Additionally, we observed actin bundles wound around the nucleus. The actin bundles were highly dynamic and continued to wind around the nucleus during nuclear migration (Fig. 6a). Movie S3 shows a representative root hair treated with 100 nM LatB for 10 min. This root hair displayed depolymerization of fine actin filaments, but the actin bundles remained relatively intact. Overall, the treatment slowed the dynamics and turnover of apical and sub-apical fine actin based on visual inspection of Movie S3. Further, the nucleus moved back toward the base of the root hair and the growth of the root hair was slow down obviously after treatment with LatB (Fig. 6b, f). These observations are consistent with an important role of actin dynamics and organization in the apical and sub-apical regions during nuclear positioning in root hairs.

To further evaluate the function of PCaP2 in the regulation of nuclear positioning, we monitored nucleus behavior and actin dynamics in *pcap2* and *PCaP2 over-expression* root hairs. We observed that the nucleus remained close to the apex of a slow-growing *pcap2* root hair. The actin cables protrude into the apical region and continued to wind around the nucleus (Fig. 6c, Movie S4). In contrast, there are not obvious actin cables wound around the nucleus, and the nucleus moved away from the apex of growing root hairs in *PCaP2 over-expression*

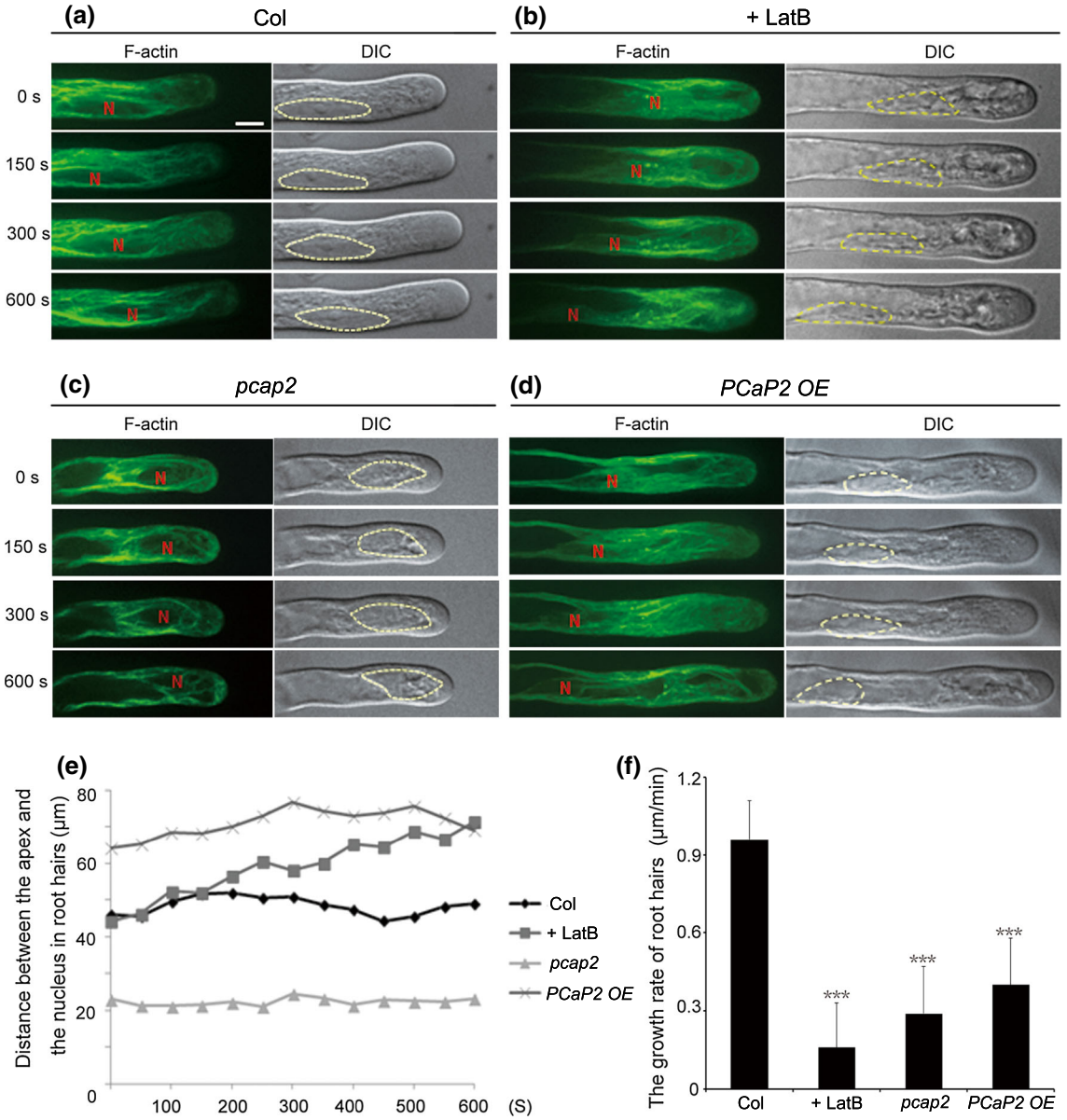


Fig. 6 The position of the nucleus and actin arrangement in growing root hairs. Time-lapse images of root hair growth. More than ten root hairs were observed for each line. All fluorescence images are projections of scanning laser sections (0.5 μm) of Z-stacks and DIC images are single scanning laser sections (0.5 μm). Representative images are presented in (**a**) to (**d**). Bar = 10 μm in (**a**) for (**a**–**d**). **a** Time-lapse images taken from Movie S2 (available online), showing that actin bundles wound around the nucleus during nuclear migration. The nucleus is indicated by the *red letter* "N" in the shank of the root hair in the fluorescence graphs and indicated by the *yellow dashed lines* in the DIC images. The nucleus remains at a fixed distance from the apex. The beginning of our observation was defined as time 0 min. Images were collected every 30 s. **b** Time-lapse images taken from Movie S3 (available online) show that actin is partly depolymerized after treatment with latrunculin B (LatB; 100 nM, 10 min) in a wild-type root hair. The growth of the root hair slows and the nucleus migrates to the base of the root hair. **c** Time-lapse images taken from Movie S4 (available online) show that actin cables protrude into the apical region and wind around the nucleus in a *pcap2* root hair and that the position of nucleus is closer to the apex in the *pcap2* root hair than in the wild-type growing root hair. **d** Time-lapse images taken from Movie S5 (available online) show that many fine actin structures are observed in the tip. There are not obvious actin cables wound around the nucleus in a *PCaP2 over-expression (OE)* root hair, and the position of the nucleus is far away from the apex. **e** Real-time and quantitative analysis of the distance between the apex and the nucleus was tracked in growing root hairs in wild type, wild type after treatment with LatB, *pcap2* mutant, and *PCaP2 over-expression* lines [shown in **a**–**d**]. The nucleus maintains a fixed position in *pcap2* and wild-type root hairs; the nucleus gradually moves away from the apex in the *PCaP2 over-expression* root hair and after treatment with LatB. **f** Quantification of the growth rate of wild type, wild type after LatB treatment, *pcap2* mutant, and *PCaP2 over-expression* root hairs presented in (**a**–**d**). After treatment with LatB, the growth rate of wild-type root hairs decreases compared to the untreated control. *PCaP2 over-expression* and *pcap2* root hair growth significantly slow compared to the wild-type root hairs. More than ten growing root hairs for each line were measured. Error bars represent ± SD. ***$P < 0.001$ by Student's *t* test

(Fig. 6d, Movie S5), which was similar to the behavior of nuclei in wild-type root hairs treated with LatB (Fig. 6b). Quantitative analysis of monitoring nucleus migration showed that the nucleus gradually moved far away from the apex in the *PCaP2 over-expression* root hair, which was similar to the nuclear positioning in the root hair treated with LatB. However, the distance between the nucleus and the root hair tip was fixed in the *pcap2* mutant and the wild-type root hairs. The nucleus was closer to the tip in *pcap2* root hairs than in wild-type root hairs (Fig. 6e). In addition, the growth rate of *pcap2* and *PCaP2 over-expression* was both obviously slower than that of wild type (Fig. 6f); however, the distance between the nucleus and the apex in *PCaP2 over-expression* was greater but that was shorter in *pcap2* growing root hairs. Therefore, we assert that PCaP2 regulates nuclear positioning in growing root hairs by modulating apical and subapical filamentous actin dynamics.

As noted previously, the abnormal expression of PCaP2 does not affect the position of the nucleus in fully grown root hairs. We observed actin arrangement to investigate whether the expression level of PCaP2 influenced actin dynamics and organization in fully grown root hairs. In wild-type, *pcap2*, and *PCaP2 over-expression* fully grown root hairs, actin filaments were organized as bundles along the root hairs and protruded into the apical domain (Fig. S2a–c). We also measured the percentage of occupancy and skewness of the fluorescence intensity distribution of actin filaments in the root hair cells. As shown in Fig. S2d and S2e, actin filament densities (indicated by the occupancy value) and bundling (indicated by the average skewness value) in *pcap2* and *PCaP2 over-expression* fully grown root hairs were similar to those in wild-type root hairs. We conclude that PCaP2 does not significantly impact actin dynamics and organization in fully grown root hairs.

Actin-severing activity is crucial for PCaP2 to function in nuclear positioning

We investigated how PCaP2 regulates filamentous actin dynamics in growing root hairs for proper positioning of the nucleus. PCaP2 has actin-severing activity, which is important for pollen tube directional growth (Zhu et al. 2013) and we expected that such activity would also be important in root hair growth.

We introduced a mutant protein of PCaP2, PCaP2-M1 (MAP18-M1, which lacks actin-severing activity in the presence of calcium) into *pcap2* which is mentioned in Zhu et al. (2013) as *m1* mutant for further investigating the actin-severing function of PCaP2 in root hair.

We used PI staining to compare the cell profiles and nuclear positioning of wild-type and *m1* growing root hairs (Fig. 7a). Statistical analysis revealed that the length of *m1* root hairs was significantly shorter than the length of wild-type root hairs (Fig. 7b). We also detected an obvious difference in the growth rate of root hairs between *m1* and wild-type plants (Fig. 7c). These results suggest that expression of *PCaP2-M1* does not rescue the root hair elongation defect in the *pcap2* mutant. Next, we quantified the distance between the nucleus and the apex of growing root hairs. The distance was much longer in wild-type root hairs than in *m1* root hairs (Fig. 7d). This indicates that expression of the PCaP2 mutant protein that lacked filamentous actin-severing activity does not rescue the phenotype of abnormal nuclear positioning in the *pcap2* mutant background. The COM#9, we previously mentioned, which expresses wild PCaP2 in the *pcap2* mutant could rescue the short hair phenotype and the phenotype of increased distance between the nucleus and the apex in *pcap2* mutants. Taken together, we conclude that the actin-severing activity of PCaP2 is crucial for modulating root hair growth and nuclear positioning in growing root hairs.

Discussion

PCaP2 regulates nuclear positioning in growing root hairs by modulating apical actin dynamics in *A. thaliana*

In most eukaryotic cells, the nucleus moves to a specific location. Both nuclear migration and nuclear anchorage contribute to nuclear positioning. Starr (2007) proposed that communication between the nuclear envelope and the cytoskeleton was the mechanism underlying nuclear migration and anchorage. All three elements of the cytoskeleton—microtubules, actin filaments, and intermediate filaments—are involved in nuclear positioning to varying degrees in different cell types. The actin cytoskeleton is involved in nuclear positioning in yeast, plants, and animals and it is reportedly used as a stable framework to anchor the nucleus and to influence nuclear movement (Bloom 2001; Chytilova et al. 2000; Starr and Han 2002).

AnPcpA, a putative homologue of pericentrin-related protein in filamentous fungus *Aspergillus nidulans*, may affect nuclear positioning by influencing microtubule organization (Chen et al. 2012). Two *Caenorhabditis elegans* proteins and their homologues participate in anchoring and migrating nuclei relative to the actin cytoskeleton. Syne/ANC-1 anchors nuclei by directly tethering the nuclear envelope to the actin cytoskeleton, and UNC-84/SUN functions at the nuclear envelope to recruit Syne/ANC-1 (Starr and Han 2003).

In leaf epidermal and mesophyll cells of *A. thaliana*, blue light-dependent nuclear positioning is regulated by

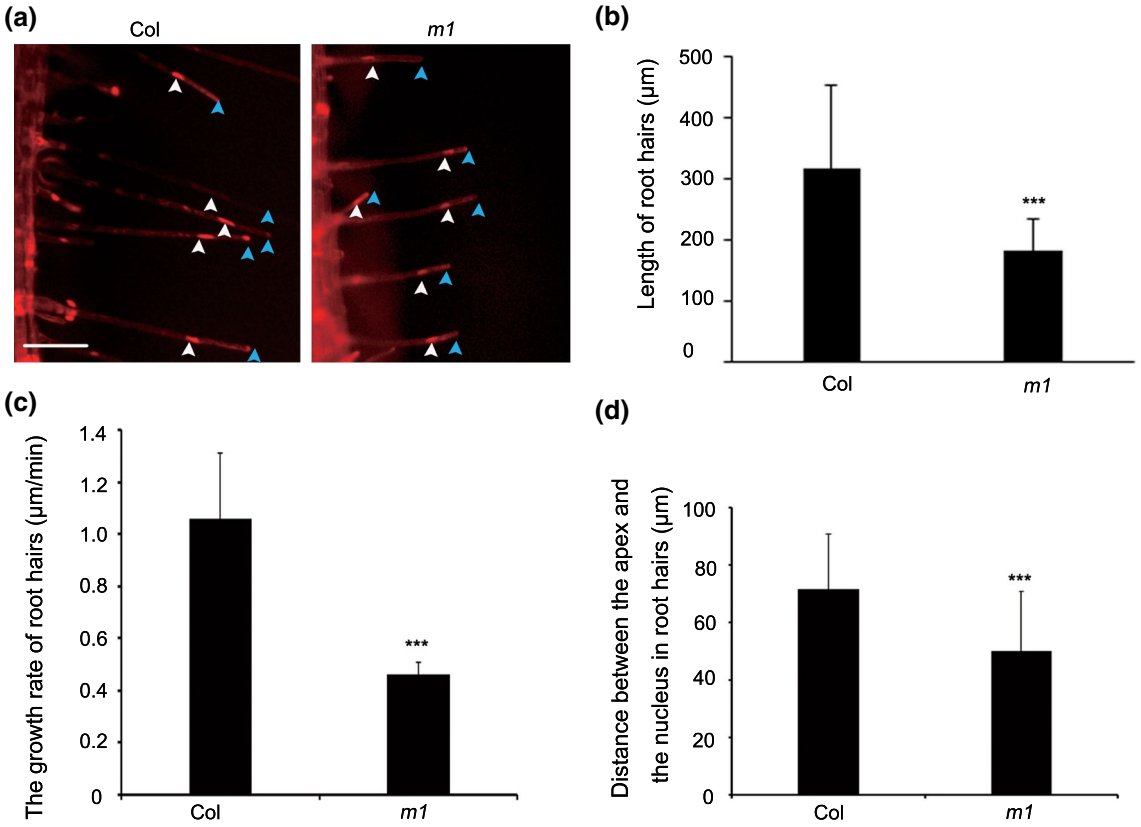


Fig. 7 Phenotype analysis of root hair length and nuclear position in *m1* mutant seedlings. **a** The nuclei stained with propidium iodide located at a relatively fixed distance from the apex in wild-type and *m1* growing root hairs. *White arrowheads* the nuclei in the shanks of the root hairs; *blue arrowheads* the root hair tips. More than 15 seedlings were observed for each *line*. Representative images are presented in (**a**). *Bar* 100 μm. **b** The average length of *m1* root hairs is shorter than the average length of wild-type root hairs. More than 80 growing root hairs were measured from each line. Data represent the mean ± SD. $P < 0.001$ by Student's *t* test. **c** The growth rate of *m1* root hairs is significantly slower than wild-type root hairs. More than ten root hairs were observed for each *line*. Data represent the mean ± SD. $P < 0.001$ by Student's *t* test. **d** The distance between the nucleus and the apex of growing root hairs in *m1* root hairs is shorter than the distance in wild-type root hairs. More than 80 growing root hairs were measured from each *line*. Data represent the mean ± SD. $P < 0.001$ by Student's *t* test

phototropin2-dependent reorganization of the actin cytoskeleton (Iwabuchi et al. 2010). Using immunofluorescence microscopy, Iwabuchi et al. (2010) found that thick actin bundles were associated with the nucleus under dark conditions and were rearranged to a position close to the anticlinal walls under strong blue light. Proper positioning of the nucleus, which is dependent on the actin cytoskeleton but not the tubulin cytoskeleton, was reported to be essential for *A. thaliana* root hair growth (Ketelaar et al. 2002). PCaP2 was previously reported to bind to microtubules (Wang et al. 2007) and PCaP2 exhibited calcium-dependent actin-severing activity in pollen tubes (Zhu et al. 2013). In the current study, we demonstrated that PCaP2 regulates nuclear position in growing root hairs by modulating filamentous actin dynamics in apical and subapical region.

Kato et al. (2013) reported on the role of PCaP2 in root hairs. In their report, the root hair length of a knockdown line of *PCaP2* was longer than wide type; however, we got the opposite result about the root hair length of this knockdown SALK line. We observed that the length of root hairs was significantly reduced in *pcap2* compared with wild type. To investigate the inconsistence caused by the differences in culture conditions, we repeated this assay following the culture conditions (MS salts, 2 % w/v sucrose and 1.2 % agar medium) according to Kato et al. (2013). The root hair length decreased significantly both in *pcap2* and wild type, and root hair length of *pcap2* (124 ± 60 μm, $***P < 0.001$ by Student's *t* test) was shorter than wide type (153 ± 67 μm), all the same. Therefore, we believe that root hair length of *pcap2* is indeed shorter than wild type.

In addition, Kato et al. (2013) reported that over-expressed *PCaP2* using the root hair cell-specific *EXPANSIN A7* promoter showed that *EXPA7::PCaP2–GFP* localized predominantly to the plasma membrane. We also observed

that in root hairs expressing *PCaP2pro:PCaP2-eGFP* (*pcap2* background) the fluorescence signal appeared throughout the entire plasma membrane including apex if the growth of root hairs ceased. This observation is consistent with the report of Kato et al. (2013). However, we further found that the fluorescence signal of *PCaP2pro:PCaP2-eGFP* was mainly detected at the shank of the plasma membrane and the cytoplasm of the apical region in growing root hairs. Therefore, it indicated that the localization of PCaP2 is closely related with the growth status of root hairs.

Ketelaar et al. (2002) reported that the position of the nucleus was not significantly altered after treatment with 1 μM oryzalin, which completely depolymerizes microtubules in *A. thaliana* root hairs within 5 min. However, Siebere et al. (2002) detected that the depolymerization of microtubules with oryzalin decreased the growth rate of root hairs and caused the nucleus to move away from the hair tip in *Medicago truncatula*. Therefore, whether microtubules play a role in nuclear positioning during tip growth appears to be related to species. In this study, we used *UBQ::GFP-MBD* to label microtubules. We observed that no microtubules were located around the nucleus, in the sub-apex, or between the nucleus and the tip regions in growing *A. thaliana* root hairs. Additionally, the nucleus maintained a relatively fixed distance from the root hair tip when microtubules were depolymerized after treatment with oryzalin. These data indicate that microtubules do not play a major role in the regulation of nuclear positioning during root hair growth in *A. thaliana.*

Although PCaP2 affected nuclear positioning independent of microtubules, *PCaP2 over-expression* expressed a branching root hair phenotype. After treatment with high concentration oryzalin (depolymerized microtubules) or taxol (stabilized microtubules), root hairs also showed a branching phenotype (Bibikova et al. 1999; Ketelaar et al. 2003). Therefore, we speculate that PCaP2 affects the arrangement and dynamics of microtubules in root hairs, which regulate the establishment of root hair polarity. However, because PCaP2 also affected the actin cytoskeleton, we cannot rule out the possibility that the branching root hair phenotype of *PCaP2 over-expression* line is due to the effect of PCaP2 on the actin organization.

We also evaluated actin dynamics and organization in root hairs. Actin cables aligned longitudinally in the shank were similar in wild-type, *pcap2*, and *PCaP2 over-expression* root hairs, which was confirmed by the skewness analysis. Knockdown and over-expression of *PCaP2* influenced actin organization in the apical and sub-apical regions of growing root hairs, as well as the positioning of the nucleus (Figs. 5, 6). These results suggest that fine actin dynamics and organization in the apical and sub-apical regions of root hairs play important roles in maintaining nuclear position.

On the other hand, neither knockdown nor over-expression of *PCaP2* significantly influences nuclear position or actin organization in fully grown root hairs (Fig. 2b and Fig. S2). We hypothesize that the fine actin dynamics in the apical and sub-apical regions hold the nucleus in the growing root hair tip until the cessation of growth.

Actin-severing activity and nuclear positioning

Maintaining nuclear position at a fixed distance from the root hair tip is required for root hair growth (Ketelaar et al. 2002). The polymerization and depolymerization of actin are important for maintaining the continuous growth of root hairs (Baluška et al. 2000; Ketelaar et al. 2003; Yoo et al. 2008). It has been reported that the thick actin bundles tethering the nuclear envelope associated with myosin regulate rapid and long-distance nuclear movement (Tamura et al. 2013; Iwabuchi et al. 2010). How actin filaments' dynamics and configuration are involved in nuclear migration in tip growth process is still needed to be investigated.

The polymerization and depolymerization of actin are also responsible for maintaining the dynamics and organization of actin in apical and sub-apical regions of growing root hairs (Baluška et al. 2000; Ketelaar et al. 2003; Yoo et al. 2008). Several actin-binding proteins play a role in regulating the organization and dynamics of actin through severing, capping, bundling, and cross-linking activities (Staiger 2000). Actin-binding proteins with actin-severing activity are involved in regulating the axial expansion of hypocotyl epidermal cells and promoting normal pollen tube growth by maintaining actin dynamics (Chen et al. 2002; Cheung et al. 2008; Staiger et al. 2009). Zhang et al. (2011) demonstrated that AtVLN4 affects root hair growth by regulating actin organization in a calcium-dependent manner. AtVLN4 protein exhibits multiple actions on actin, including calcium-dependent filament severing, actin filament bundling, and barbed-end capping. Loss of *AtVLN4* function results in slowed root hair growth and reduced numbers of both axial and apical actin bundles. Lily villin-like actin-binding protein bundles the sub-apical fine actin filaments into thicker actin bundles in a gradient away from the hair tip in the root hair shank, which appears to prevent the nucleus from approaching the growing root hair tip (Ketelaar et al. 2002). However, it is unclear if severing actin is relevant to the control of nuclear positioning. In this study, considering the actin-severing activity of PCaP2, short fine actin filaments were fewer in sub-apical of *pcap2* root hairs than in wild-type and the thick longitudinal actin cables were protruded into the sub-apical regions (Fig. 5b, e). The area of fine actin filaments in sub-apex reduced and the nucleus was closer to the tip in *pcap2* root hairs than in wild-type root hairs (Fig. 6c). By

contrast, the fine actin filaments were substantially increased in the sub-apical regions of *PCaP2 over-expression* compared to wild-type root hairs (Fig. 5c, g). In addition, we observed that the nucleus was far away from the apex of growing root hairs in *PCaP2 over-expression* which was similar to the behavior of nuclei in wild-type root hairs treated with LatB (Fig. 6b, d). These results suggested that increasing the length of area of sub-apical fine actin filaments may increase the distance between nucleus and the apex, and vice versa. PCaP2 influences actin organization in the apical and sub-apical regions but not the bundling of actin cables, which suggests that the dislocation of nuclei is affected by fine actin filaments dynamic in either over-expression or down-regulation of *PCaP2* growing root hairs.

We speculate that, in growing root hairs, filamentous actin dynamics and configuration in the apical and sub-apical regions make influence the relative position of actin bundles in sub-apical regions through rapidly actin turning over, so the nucleus migrates with the positioning of actin bundles forward or backward. The actin-severing protein, such as PCaP2, plays a role in the position of nuclei through modulating filamentous actin dynamics balance and maintaining actin filaments configuration in the apical and sub-apical regions in growing root hairs.

Furthermore, we analyzed PCaP2 mutants expressing a point mutation to demonstrate that the actin-severing activity of PCaP2 is important for nuclear positioning and appropriate growth patterns of *A. thaliana* root hairs. The *A. thaliana m1* mutant, which expressed a PCaP2 mutant protein lacking the actin-severing activity, did not rescue the abnormal root hair growth phenotypes or the distance between the nucleus and the hair apex (Fig. 7). Therefore, we propose that PCaP2 plays a crucial role in the regulation of nuclear positioning and root hair growth, most likely through its actin-severing activity.

In the future, we plan to investigate if actin-binding proteins with calcium-dependent actin-severing activity other than PCaP2, such as AtVLN4, regulate nuclear positioning.

We proposed a model for the function of PCaP2 in root hair growth: PCaP2 localizes to the shank plasma membrane and apical cytoplasm. Actin cables of the shank run parallel to the direction of growth and surround the nucleus. PCaP2 modulates filamentous actin organization in the apical and sub-apical regions in a growing root hair. This reorganization of actin in the apical and sub-apical regions holds the nucleus in position in the growing root hair tip. PCaP2 regulates the position of the nucleus in growing *A. thaliana* root hairs by modulating filamentous actin organization and affects root hair elongation.

Author contribution statement L.Z. and Y.Z. designed the project. Y.Z., E.K. and L.Z. performed the experiments and analyzed the results. L.Z. wrote the manuscript; Y.F. and M.Y. revised and modified the manuscript.

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Conflict of interest The authors declare that we have no conflict of interest.

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