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Research

Arabidopsis RopGEF4 and RopGEF10 are important for FERONIA-mediated developmental but not environmental regulation of root hair growth

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Summary

- We investigated a genetic pathway in root hair development in *Arabidopsis thaliana*, involving the receptor-like kinase FERONIA (FER), two guanine nucleotide exchange factors for ROPs (RopGEF4 and RopGEF10), and the small GTPase Rho of plants (ROPs).
- Loss- and gain-of-function analyses demonstrated distinct roles of *RopGEF4* and *RopGEF10* such that *RopGEF4* is only important for root hair elongation, while *RopGEF10* mainly contributes to root hair initiation. Domain dissection by truncation and domain-swapping experiments indicated that their functional distinctions were mainly contributed by the noncatalytic domains.
- Using fluorescent ratio imaging, we showed that functional loss of *RopGEF4* and *RopGEF10* additively reduced reactive oxygen species (ROS) production. Bimolecular fluorescence complementation experiments demonstrated that RopGEF4 and RopGEF10 had the same interaction specificity as ROPs, suggesting common downstream components.
- We further showed that the promoting effects of environmental cues such as exogenous auxin and phosphate limitation on root hair development depended on FER. However, although functional loss of *RopGEF4* and *RopGEF10* largely abolished FER-induced ROS production, it did not compromise the responses to FER-mediated environmental cues on root hair development. Our results demonstrated that RopGEF4 and RopGEF10 are genetic components in FER-mediated, developmentally (but not environmentally) regulated, root hair growth.

Introduction

Root hairs are cylindrical outgrowths from root epidermal cells, playing important roles in water uptake, nutrient acquisition and environmental sensing, essential for the success of vascular plants (Grierson & Schiefelbein, 2002). Root hair development provides an excellent system to study fundamental biological questions such as cell fate determination, symmetry breaking, and tip growth, which are exemplified by the three steps leading to a root hair (Schiefelbein, 2003). Transcriptional cascades determine whether a root epidermal cell becomes a hair-forming cell (i.e. a trichoblast) or a nontrichoblast, whose alternative patterns vary among different plant species (Grierson & Schiefelbein, 2002; Schiefelbein, 2003). After a root epidermal cell adopts the trichoblast fate, a small area of its cell wall loosens to form a swelling, that is, the initiation step. It is followed by tip growth, leading to the cylindrical shape of mature root hairs (Grierson & Schiefelbein, 2002). Except for the developmental pathway, root hair initiation and elongation are highly flexible upon environmental factors, such as exogenous hormones and the availability of nutrients (Grierson & Schiefelbein, 2002). Indeed, exogenous auxin and phosphate deficiency are among the most prominent factors promoting root hair initiation and elongation (Gilroy & Jones, 2000; Ma *et al.*, 2001; Zhang *et al.*, 2003; Duan *et al.*, 2010).

Extensive genetic studies in the past decade identified key genes whose mutations resulted in defects in root hair development (Grierson & Schiefelbein, 2002; Kwasniewski *et al.*, 2013). An emerging genetic network controlling root hair development reveals the central role of the small GTPases Rho of plants (ROPs) (Molendijk *et al.*, 2001; Jones *et al.*, 2002; Carol *et al.*, 2005; Bloch *et al.*, 2011; Riely *et al.*, 2011). ROPs are homologous to metazoan Rac GTPases (Winge *et al.*, 2000; Yang, 2002; Vernoud *et al.*, 2003; Nibau *et al.*, 2006). By switching between the GDP-bound 'off' state and the GTP-bound 'on' state, ROPs dynamically regulate diverse cellular activities such as actin microfilament organization, reactive oxygen species (ROS) production and Ca^{2+} gradients (Yang, 2002; Nibau *et al.*, 2006), all of which play essential roles in root hair development (Bibikova *et al.*, 1997, 1998; Foreman *et al.*, 2003; Samaj *et al.*, 2004; Voigt *et al.*, 2005; Jones *et al.*, 2007; Takeda *et al.*, 2008).

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Activation of ROPs is dynamically regulated by several classes of proteins (Yang, 2002). Rho GTPase activating proteins (RopGAPs) promote GTP hydrolysis while Rho guanine nucleotide dissociation inhibitors (RhoGDIs) extract ROPs for cytoplasmic sequestration, whose mutations compromised tip growth in pollen tubes or root hairs (Carol *et al.*, 2005; Klahre & Kost, 2006; Klahre *et al.*, 2006; Hwang *et al.*, 2008). On the other hand, ROPs are activated by the plant-specific guanine nucleotide exchange factors (RopGEFs) (Berken *et al.*, 2005; Gu *et al.*, 2006). RopGEFs typically harbor a conserved central PRONE domain for GTP-GDP exchange and variable N- and C-terminal regions that may play regulatory roles (Berken *et al.*, 2005; Gu *et al.*, 2006; Zhang & McCormick, 2007; Riely *et al.*, 2011). RopGEFs have been shown to regulate pollen tube growth (Gu *et al.*, 2006; Zhang & McCormick, 2007), stomata closure (Li & Liu, 2012), and root development (Chen *et al.*, 2011). Recently, it was reported that functional loss of Arabidopsis *RopGEF4* and its putative homolog in Medicago affected root hair elongation (Won *et al.*, 2009; Riely *et al.*, 2011). However, considering the roles that ROPs play in both hair initiation and hair elongation (Molendijk *et al.*, 2001; Jones *et al.*, 2002), additional RopGEFs may be involved in these processes.

Plant-specific guanine nucleotide exchange factors for ROPs were implicated as cellular components of receptor-like kinase (RLK)-initiated signaling (Zhang & McCormick, 2007; Duan *et al.*, 2010; Chang *et al.*, 2013). RLKs are major cell sensors regulating multiple developmental processes in plants (De Smet *et al.*, 2009). Studies in pollen tubes suggested that intracellular events initiated by RLKs involve RopGEF-mediated ROP activation (Zhang & McCormick, 2007; Chang *et al.*, 2013). Recently, a similar mechanism was proposed for root hair development (Duan *et al.*, 2010). FERONIA (FER) is a leucine-rich repeat RLK initially identified for its roles in pollen tube reception (Escobar-Restrepo *et al.*, 2007). Root hair initiation and elongation require functional FER, either in the default developmental pathway or by exogenous auxin (Duan *et al.*, 2010). FER interacts with several RopGEFs and its overexpression led to increased ROS production (Duan *et al.*, 2010; Yu *et al.*, 2012). However, there was no genetic evidence showing that FER mediates ROP activation through RopGEFs. In addition, it was unclear whether FER is a key factor for plant responses to nutrient limitation during root hair development, such as low environmental phosphate.

We show here that functional loss of the trichoblast-expressed *RopGEF4* and *RopGEF10* resulted in reduced ROP activation and ROS production. Both loss- and gain-of-function analyses indicated that *RopGEF4* and *RopGEF10* play distinct roles in root hair initiation and elongation. By using domain-swapping analyses, we show that the functional distinction between RopGEF4 and RopGEF10 resulted from the noncatalytic domains. Enhanced ROS production by FER overexpression was abolished by functional loss of *RopGEF4* and *RopGEF10*, consistent with their genetic epistasis. FER is critical for enhanced root hair development, induced not only by exogenous auxin as reported (Duan *et al.*, 2010), but also by phosphate limitation. Surprisingly, although *RopGEF4* and *RopGEF10* acted epistatically to *FER* in the developmentally regulated root hair development, their functional loss did not compromise plant responses to these environmental cues, suggesting the presence of an additional pathway for FER-mediated environmental cues during root hair development. Our studies indicate that developmental and environmental cues for root hair development converge upon FER, from which divergent signaling pathways through RopGEFs or other factors are initiated.

Materials and Methods

Plant materials and growth conditions

The T-DNA insertion line, SALK_009456C (*gef10*), was obtained from the Arabidopsis Biological Resource Center (ABRC, http://www.arabidopsis.org). *Arabidopsis thaliana* (L.) Heynh Col-0 ecotype was used as the wildtype. Arabidopsis plants were grown as described (Zhou *et al.*, 2013). For seedlings growing on plates, surface-sterilized Arabidopsis seeds were grown on Murashige and Skoog basal medium with vitamins (MS) (Phytotechlab, http://www.phytotechlab.com/) except where noted. Plates were kept at 4°C in darkness for 4 d before being transferred to a growth chamber with a 16 h light : 8 h dark cycle at 21°C. Transgenic plants were selected on MS medium supplemented with 30 μg ml^{-1} Basta salt (Sigma, http://www.sigmaaldrich.com/). The single or double mutants of *RopGEF4* and *RopGEF10*, *gef4*, *gef10* and *gef4gef10*, were analyzed by genotyping PCR using the following primers: ZP223/ZP224 for *RopGEF4*, ZP5/ZP666 for *gef4*; ZP458/ZP459 for *RopGEF10*, ZP1/ZP226 for *gef10*. *fer-4* was selected on MS medium supplemental with 5.25 mg l^{-1} sulfanilamide. Primers are listed in Supporting Information Table S1.

RNA extraction, RT-PCR and qRT-PCR

Total RNAs from roots at 4 d after germination (DAG) were extracted using the RNeasy Plant miniprep kit according to the manufacturer's instructions (Qiagen). Reverse transcriptions were performed using Superscript™ III Reverse Transcriptase with on-column DNase-I treatment (Invitrogen). Primers used in RT-PCRs are as followed: ZP223/ZP297 for the endogenous *RopGEF4*, ZP12/ZP457 for the exogenous *RopGEF4*, ZP759/ZP760 for the endogenous *RopGEF10* and ZP12/ZP668 for the exogenous *RopGEF10*. Arabidopsis *ACTIN2* was used as the internal control for reverse transcription polymerase chain reactions (RT-PCRs). The quantitative RT-PCR (qRT-PCR) analysis of trichoblast-specific or epidermal-expressed genes was performed as previously described (Zhou *et al.*, 2013). Primers are listed in Table S1.

Plasmid construction

All constructs were generated using the Gateway™ technology (Invitrogen). Entry vectors of the coding sequences of genes or fragments were generated in the pENTRY/SD/D-TOPO vector (Invitrogen). Primers for generating entry vectors were as follows: ZP221/ZP558 for *RopGEF4*, ZP518/ZP519 for *RopGEF10*, and

ZP610/ZP611 for *FER*. Chimeric coding sequences for the domain swapping experiment were generated by a three-step PCR protocol (Tian *et al.*, 2004). Specifically, the following primer pairs were used to amplify the corresponding fragments: ZP221/ZP908 for N_{GEF4}, ZP909/ZP912 for $PRONE_{GEF4}$, ZP753/ZP910 for N_{GEF10}, ZP911/ZP519 for C_{GEF10}, ZP907/ZP754 for $PRONE_{GEF10}$, ZP753/ZP558 for N10-GEF4, ZP221/ZP519 for N4-GEF10 and GEF4-C10, and ZP518/ZP522 for GEF10ΔC. Destination vectors containing either Pro_{E7} or Pro_{LRX1} were generated by replacing Pro_{35S} from a previously described destination vector (Karimi *et al.*, 2002) through a double digestion with *Sac*I/*Spe*I. Expression vectors were generated by LR reactions using LR Clonase II (Invitrogen) with these destination vectors. Primers are listed in Table S1.

Entry vectors for the coding sequences of *ROP2*, *ROP6*, and *ROP7* were generated in the pENTRY/SD/D-TOPO vector using the following primer pairs: ZP492/ZP493 for *ROP2*, ZP498/ZP499 for *ROP6*, and ZP1209/ZP1210 for *ROP7*. Mutant versions of *ROP2* were generated by Phusion site-directional mutagenesis kit (Finnzyme, Waltham, MA, USA). Destination vectors for the Bimolecular Fluorescence Complementation (BiFC) were obtained from ABRC (Martin *et al.*, 2009). ProQuest vectors were used for the yeast hybrid assay (Invitrogen). Primers are listed in Table S1.

All PCR amplifications used Phusion™ hot start high-fidelity DNA polymerase with the annealing temperature and extension times recommended by the manufacturer (Finnzyme). All entry vectors were sequenced using an ABI 3300 sequencer and sequences were analyzed using Vector NTI (Invitrogen). The Bioneer PCR purification kit and the Bioneer Spin miniprep kit were used for PCR product recovery and plasmid DNA extraction, respectively.

Quantification of root hair length, width, and density

For all experiments except the phosphate limitation treatment, the region between 1.5 and 3.5 mm distal from the primary root tip of a 4 DAG seedling was chosen to measure root hair length, width, and density. Images of that region were taken from individual seedling samples using an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a charge-coupled device (CCD) camera. Quantification of root hair length and density for the phosphate limitation experiment were performed as previously described (Zhang *et al.*, 2003). For measurement of root hair density, 33–40 individual images of the 2 mm region from three independent experiments for each genotype were analyzed. For root hair length, all root hairs from the same images were measured using ImageJ (Bethesda, MD, USA). For root hair width, every other root hair from the same images was measured using ImageJ. Results were confirmed by nonbiased double-blind analyses.

Treatment of exogenous auxin and phosphate limitation

For exogenous auxin treatment, seeds of different genotypes were stratified at 4°C for 4 d before being transferred to a controlled growth chamber for 4 d on MS media supplemented with or without 50 nM naphthylacetic acid (NAA) or 100 nM NAA. Measurements of root hair length, width, and density were performed as described earlier.

For phosphate limitation assay, low phosphate (1 μM) and high phosphate (1 mM) media were prepared as described (Ma *et al.*, 2001). Seeds of different genotypes were stratified at 4°C for 4 d before being transferred to a controlled growth chamber for 9 d. Control experiments on MS medium were performed simultaneously as a reference. Measurement of root hair length, width, and density were performed as described earlier.

Measurement of ROS and ROP

Reactive oxygen species concentrations in root hairs were measured using the fluorescent dye H_2DCF-DA (2′,7′-dichlorodihydro-fluorescein diacetate; Sigma) as described (Duan *et al.*, 2010). Fluorescent imaging was performed using an Axio Observer D1 microscope equipped with a CCD camera (Zeiss). The exposure time for all genotypes was set at 30 ms when no signal reached saturation. Fluorescence intensity within a fixed region of interest (ROI) was quantified using ImageJ. Three replicate experiments were conducted for each genotype. Student's *t*-tests were used for statistical analyses.

Analysis of active ROP and total ROPs was done using roots of 4 DAG seedlings according to described (Tao *et al.*, 2002; Duan *et al.*, 2010; Chen *et al.*, 2011).

Protein–protein interaction with bimolecular fluorescent complementation (BiFC) and yeast two hybrid (Y2H) assays

Nicotiana benthamiana plants were grown in the 16 h light : 8 h dark cycle at 22°C for 25 d. The fourth, fifth and sixth leaves were used for infiltration with the Agrobacteria strain GV3101. Agrobacteria suspensions adjusted to an $OD_{600} = 2.0$ in the MES medium (50 mM $MgCl_2$, 50 mM MES, and 200 μM acetosyringone, pH 5.7) were kept at room temperature for 2–4 h before infiltration. Infiltrations were conducted by gently appressing a 1 ml disposable syringe to the abaxial surface of fully expanded leaves with an approximate width of 3 cm at the middle region. Sufficient amounts of Agrobacteria suspension were used to generate a water-soaked appearance, typically requiring four infiltration sites per leaf. Plants were kept in the glasshouse for 24–48 h before being used for imaging. Y2H assays were performed with the ProQuest system according to the manufacturer's instructions.

Fluorescence labeling and microscopy

For double labeling experiments with N-(3-triethylammomium-propyl) 4-(P-diethylaminophenylhexatrienyl) (FM4-64), roots of 4 DAG seedlings were pulse-labeled with 4 μM FM4-64 for 1 min, followed by washing three times with MS liquid medium. Microscopic imaging was performed using either an Axio Observer D1 microscope (Zeiss, www.zeiss.com) with epifluorescence optics equipped with a CCD camera or by confocal imaging using a LSM51 (Zeiss) with a 488 nm argon laser and an LP

505–550 filter. Images were exported and processed using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA).

Accession numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are: At2g45890, *RopGEF4*; At5g19560, *RopGEF10*; At1g20090, *ROP2*; At4g35020, *ROP6*, At5g45970, *ROP7*; At3g51550, *FERONIA*.

Results

RopGEF10 and *RopGEF4* have distinct contributions to root hair initiation and elongation

Because expression patterns seem to be one of the key factors determining the function of *RopGEFs* (Zhang & McCormick, 2007; Chen *et al.*, 2011; Riely *et al.*, 2011), we decided to analyze the contributions of *RopGEF4* and *RopGEF10* in root hair development, both of which showed strong expression in trichoblasts (Won *et al.*, 2009; Li & Liu, 2012), using a reverse genetic approach. We obtained a previously described *RopGEF4* null mutant, *gef4* (SAIL_184_C08)(Li & Liu, 2012), and a T-DNA insertion allele for *RopGEF10*, *gef10* (SALK_009456C) (Fig. 1a), which was confirmed by transcript analysis as a null allele (Fig. 1b).

Although *RopGEF4* and *RopGEF10* belong to the same family (Berken *et al.*, 2005) and are both expressed in root hairs (Won *et al.*, 2009; Li & Liu, 2012), their respective mutants showed different effects in root hair development. As reported previously (Won *et al.*, 2009), functional loss of *RopGEF4* caused a significant reduction in root hair length (Fig. 1c–d). However, no reduction in root hair density, that is, the reduced ability of root hair initiation, was detected for *gef4* (Fig. 1e). In comparison, functional loss of *RopGEF10* resulted in a significant reduction in root hair density (Fig. 1e) and a slight reduction in root hair length (Fig. 1d). *RopGEF10* is expressed in all root epidermal cells (Won *et al.*, 2009) and thus might have affected cell fate determination, that is, fewer epidermal cells differentiate into trichoblasts in *gef10*. To rule out the possibility that reduced root hair number was the result of fewer trichoblast cells in *gef10*, thus reflecting *GEF10* function in cell fate determination rather than in root hair initiation, we analyzed the expression of several genes that were either trichoblast-specific or were expressed in all root epidermal cells. Results of qRT-PCRs showed that expression of the trichoblast-specific genes was not reduced in *gef10* (Fig. S1), suggesting that the reduced root hair density in *gef10* was the result of defects in hair initiation. These results showed that *RopGEF4* and *RopGEF10* perform overlapping but distinct functions in root hair development.

Functional loss of *RopGEF10* and *RopGEF4* compromised root hair initiation and elongation through reduced ROP signaling

Owing to the fact that both *RopGEF10* and *RopGEF4* were involved in root hair development (Fig. 1), we generated a *gef4gef10* double mutant in which both genes were null (Fig. 1b). Functional loss of both genes resulted in reduced root hair length (Fig. 1d) and density (Fig. 1e). However, root hair length (Fig. 1d) and density (Fig. 1e) of the double mutant were not significantly different from those of *gef4* and *gef10*, respectively. These results indicated that *RopGEF10* and *RopGEF4* play distinct roles in root hair development.

Because RopGEFs function through ROP activation (Berken *et al.*, 2005; Gu *et al.*, 2006), we compared the activation status

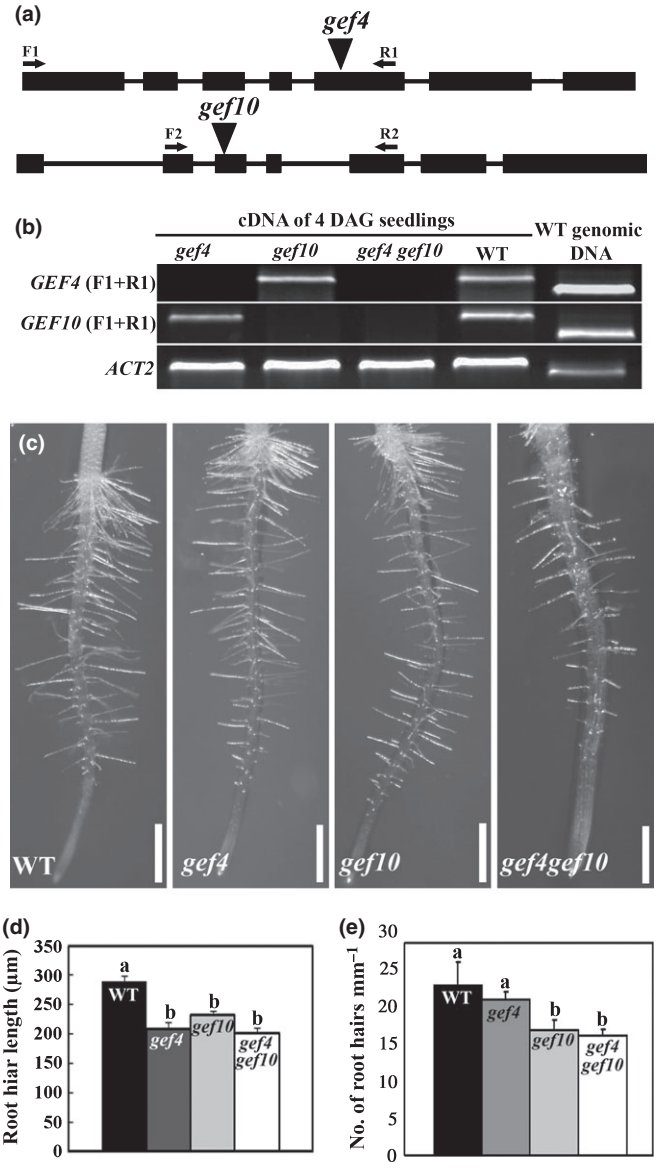


Fig. 1 Functional loss of Arabidopsis *GEF4* and *GEF10* compromised root hair initiation and elongation. (a) Schematic illustration of *GEF4* and *GEF10* loss-of-function alleles used in this study. (b) Transcript analysis in *GEF* single and double mutants. (c) Representative images of wildtype (WT), *gef4*, *gef10* and *gef4gef10* seedlings at 4 d after germination (DAG). Bars, 0.5 mm. (d) Root hair length. (e) Root hair density. For each genetic background, 33–40 seedlings grown under the same conditions in three batches at 4 DAG were analyzed by nonbiased double-blind analysis. Results are given as means ± SE. Means with different letters are significantly different (Student's *t*-test, $P < 0.05$). Bar, 0.5 mm. *ACT2*, *ACTIN2*.

of ROPs in the wildtype and in *gef4gef10*. By a functional pull-down assay that determines the level of activated ROPs and western blot analysis for total ROPs (Tao *et al.*, 2002; Duan *et al.*, 2010; Chen *et al.*, 2011), we discovered that activated ROPs were reduced to an undetectable level in the double mutant, much reduced compared with that in the wildtype (Fig. 2a). A key factor representing enhanced ROP signaling in root hairs is the concentration of ROS such that ROP-dependent activation of NADPH oxidases leads to a localized production of ROS, essential for root hair development (Foreman *et al.*, 2003; Carol *et al.*, 2005; Jones *et al.*, 2007; Takeda *et al.*, 2008). Therefore, to further prove that the reduced hair elongation and initiation in *gef4gef10* were the result of reduced ROP signaling, we analyzed and quantified the production of ROS using the fluorescence ratio imaging dye H_2DCFDA (Duan *et al.*, 2010). Indeed, ROS concentration in root hairs was significantly reduced in the *gef4gef10* double mutant compared with the wildtype (Fig. 2b–f). The effect on ROS production was additive for *gef4* and *gef10* (Fig. 2e,f), suggesting that *RopGEF4* and *RopGEF10* have common downstream components for ROS production. To find out whether this was the case, we tested the interaction between RopGEF4/RopGEF10 and a few ROPs whose enhanced activity either increased or reduced root hair growth (Molendijk *et al.*, 2001; Jones *et al.*, 2002). The results of bimolecular fluorescence complementation showed that both RopGEF4 and RopGEF10 interacted with ROP2 and ROP6 but not ROP7 (Fig. 3), which was confirmed by Y2H assays (Fig. S2). Interestingly, ROP2 and ROP6 promoted hair growth when overexpressed, whereas ROP7 inhibited root hair growth when overexpressed (Molendijk *et al.*, 2001; Jones *et al.*, 2002). These results suggested that, although *RopGEF4* and *RopGEF10* play distinct roles in different steps of root hair development, they share a common set of ROPs for ROS production.

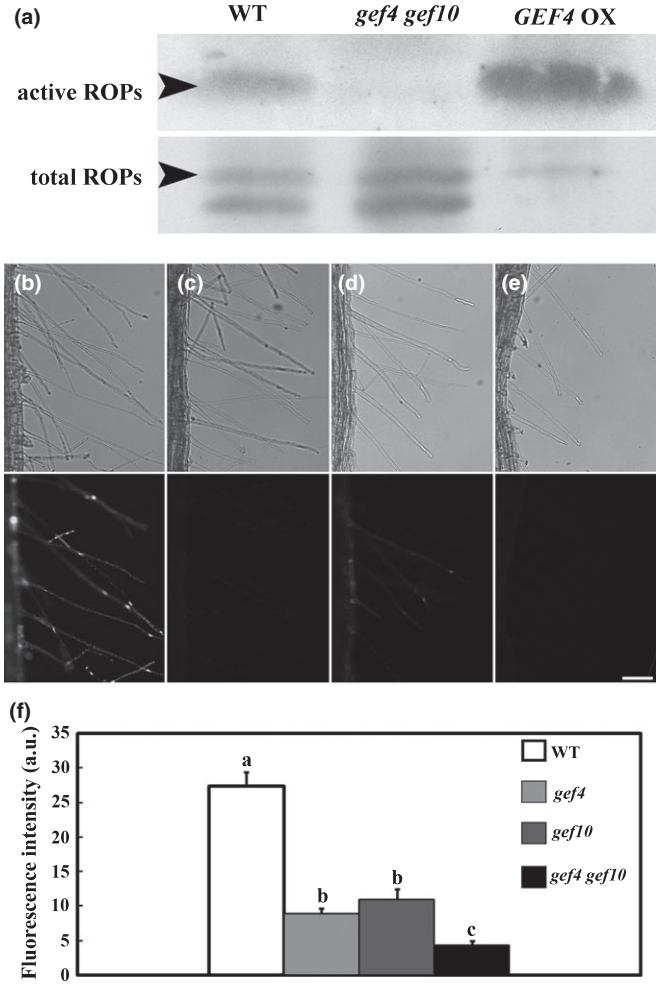


Fig. 2 Functional loss of Arabidopsis *GEF4* and *GEF10* resulted in reduced reactive oxygen species (ROS) production in root hairs and reduced ROP activation. (a) Analysis of active ROPs (ROP-GTP) and total ROPs in root seedlings at 4 d after germination (DAG) in different genetic backgrounds. Arrowheads indicate the correct band for either active ROPs or total ROPs. (b–e) Fluorescent staining of ROS in root hairs of the wildtype (b), *gef4* (c), *gef10* (d) or *gef4gef10* (e). Bright field images are placed on top of their corresponding GFP channel images. Bar, 100 μm. (f) Quantitative analysis of ROS concentration in different genetic backgrounds. a.u., arbitrary fluorescence units. Results are given as means ± SE. Means with different letters are significantly different (Student's *t*-test, $P < 0.05$). Nonbiased double-blind analysis confirmed the result.

Differential gain-of-function effects of *RopGEF4* and *RopGEF10* in root hair development

Characterization of *gef4* and *gef10* indicated that they play distinct function in hair initiation and elongation. To provide further evidence, we generated transgenic plants expressing GFP translational fusions of *RopGEF4* or *RopGEF10* driven by Pro_{E7}, the promoter of *ARABIDOPSIS THALIANA EXPANSIN A7* for trichoblast-specific strong expression (Cho & Cosgrove, 2002; Won *et al.*, 2009) in wild type. The level of active ROPs, that is, GTP-bound ROPs, in relation to the level of total ROPs was significantly increased in lines overexpressing *RopGEF4* and *RopGEF10* (Fig. 2a, data not shown), demonstrating that the overexpression resulted in *RopGEF* gain-of-function. The percentage of root hairs showing abnormal morphology was significantly increased by overexpression of *RopGEF4* and *RopGEF10*, albeit in different categories (Fig. 4). Overexpression of *RopGEF4* induced wavy or undulating growth in almost 30% of root hairs (Fig. 4a,c) without affecting the hair width much (Fig. 4e,f). By contrast, over 30% of root hairs (Fig. 4c) overexpressing *RopGEF10* showed a wider area of the initial bulge (Fig. 4b), which led to short and branched root hairs (Fig. 4e,f). In addition, overexpressing *RopGEF10* significantly increased root hair density but reduced root hair length (Fig. S3). We noticed that overexpression of both *RopGEF4* and *RopGEF10* both induced a basal shift of root-hair emerging sites (Fig. 4d), indicative of reduced auxin signaling (Masucci & Schiefelbein, 1994).

The fluorescent fusions allowed us to dissect the subcellular localization of RopGEF4 and RopGEF10 in detail. At the initiation stage, both proteins accumulated in the apical cytoplasm at the sites of root-hair emergence (Fig. 4a,b). Both proteins remained at the apical region of the growing root hairs starting from the initial formation of a morphologically distinguishable bulge (Fig. 4a,b). Once root hairs ceased growth, RopGEF4 and RopGEF10 became diffusely distributed in the cytoplasm instead of within the apical region (Fig. 4a,b). We also applied the

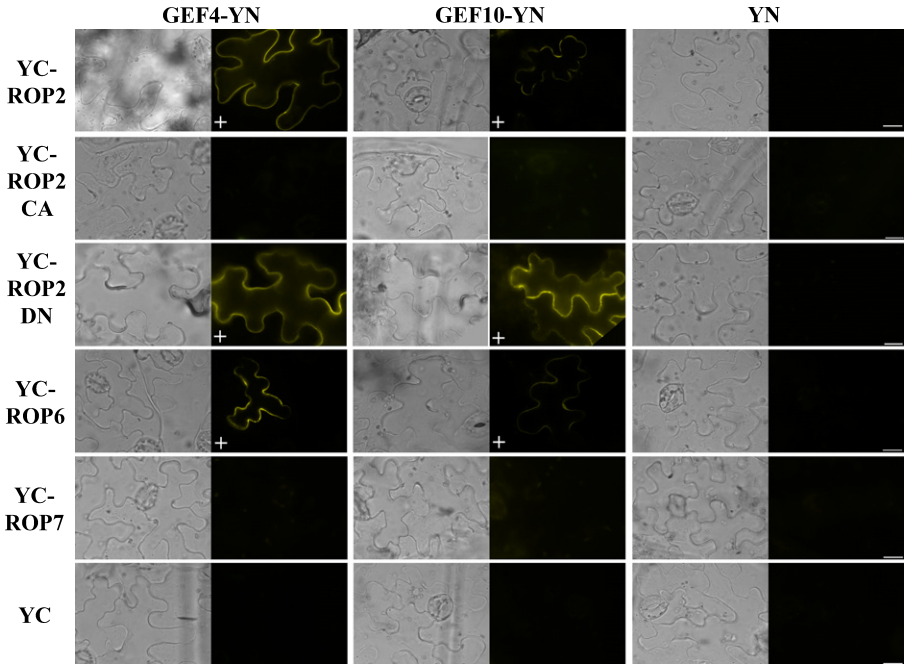


Fig. 3 Arabidopsis RopGEF4 and RopGEF10 interact with two hair growth-promoting Rho of plants (ROPs) but not with the hair growth-inhibiting ROP7 in bimolecular fluorescence complementation (BiFC). Representative images of 100–130 epidermal cells from three biological replicates are shown for each BiFC combination. All fluorescence images were captured with the same setting (300 ms exposure time). Bright field images and corresponding fluorescence images are placed side-by-side. Plus signs indicate positive interaction. Bars, 20 μm.

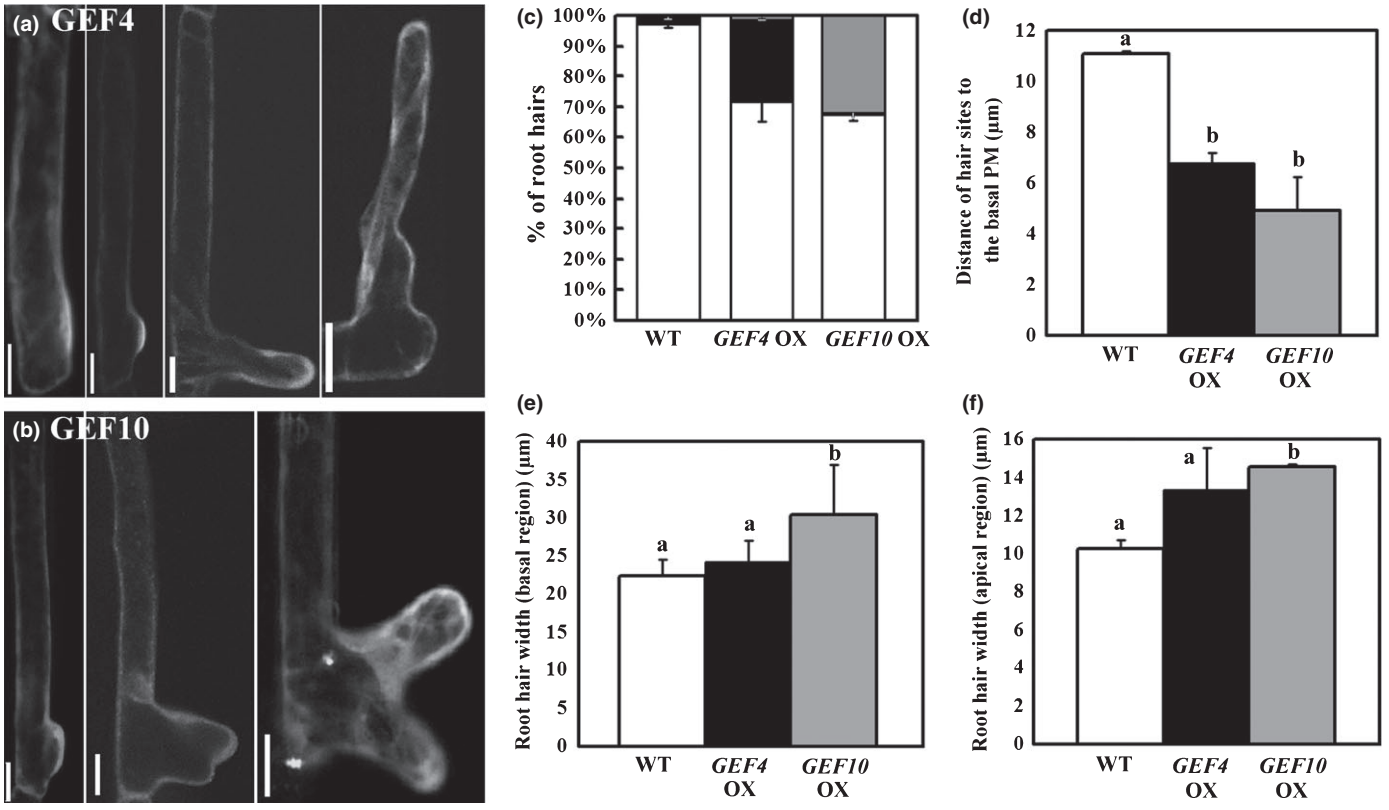


Fig. 4 Distinct effects of Arabidopsis *GEF* overexpression in root hair growth. (a, b) Representative images of root hair initiation, rapid hair elongation, or in growth cessation are shown from left to right. Bars, 20 μm. (c) Percentage of abnormal root hairs in GEF gain-of-function, showing either normal, wavy, or branched morphology. (d) Distance of the root hair initiation site to the basal plasma membrane of its corresponding trichoblast. (e) Root hair width at the basal region. (f) Root hair width at the apical region. Approx. 250 root hairs from 33 to 40 roots were used for (c). Quantification for (d–f) was performed with 180–235 hair cells using ImageJ. Results were confirmed by nonbiased double blind analyses. Results are given as means ± SE. Means with different letters in (d–f) are significantly different (Student's *t*-test, $P < 0.01$).

lipophilic dye FM4-64 transiently to label the apical plasma membrane of growing root hairs (Takeda *et al.*, 2008). Indeed, RopGEF4 and RopGEF10 overlapped with FM4-64 during hair initiation and elongation at the apical plasma membrane (Fig. S4). The results demonstrated the dynamic localization of RopGEF4 and RopGEF10 during root hair development.

Functional divergence between RopGEF10 and RopGEF4 is determined by their noncatalytic domains

Both loss- and gain-of-function analyses demonstrated the distinct function of *RopGEF4* and *RopGEF10* during root hair development. They belong to different homologous groups by a cross-species phylogenetic analysis (Riely *et al.*, 2011). In fact, no C-terminal noncatalytic domain could be detected for RopGEF4 or the other members in the same phylogenetic subgroup (Riely *et al.*, 2011).

To find out the molecular basis for such functional distinction, we carried out a domain dissection analysis by generating deletions and making chimeric proteins (Fig. 5a). Because the C-termini of several RopGEFs were indicated to have regulatory roles (Gu *et al.*, 2006; Zhang & McCormick, 2007; Riely *et al.*, 2011), we first generated a construct expressing a GFP translation fusion of the C-terminal truncated RopGEF10 (GFE10ΔC) and expressed it under Pro_{E7} in root hairs. Unlike the case for a few other RopGEFs, whose C-terminal truncations seemed to induce a higher activity (Gu *et al.*, 2006; Zhang & McCormick, 2007; Chen *et al.*, 2011), the C-terminal deletion largely abolished the overexpression effects of *RopGEF10* (Fig. 5e,o), although transgenic root hairs were slightly wider (Fig. 5j). Also different from the full-length RopGEF10, no clear association of GFE10ΔC was detected at the apical plasma membrane of growing root hairs (Fig. 5i). Adding the C-terminus of RopGEF10 (C10) to the C-terminus of RopGEF4 (GEF4-C10) did not affect the overexpression effects of RopGEF4 much (Fig. 5k), although the number of root hairs with branches increased (Fig. 5o). These results suggested that the C-terminus was critical for RopGEF10 function in a sequence- or context-dependent way.

Except for the C-terminal domains, N-terminal variable region was also hinted for regulatory function (Gu *et al.*, 2006; Zhang & McCormick, 2007; Riely *et al.*, 2011). We therefore carried out a domain-swapping experiment, exchanging the N-terminal domain (N4 and N10) between RopGEF4 and RopGEF10 (Fig. 5a). Replacing N4 with N10 resulted in a dramatic effect in root hair growth such that almost all root hairs ceased growth immediately after a bulge was formed (Fig. 5o). Instead of half-moon-shaped bulges in the wildtype or a few transgenic lines (Fig. 5i,k), overexpression of N10-GEF4 resulted in highly vacuolated bulbs (Fig. 5l), indicating a complete loss of polarity. By contrast, replacing N10 with N4 did not influence the overexpression of RopGEF10, such that a large percentage of root hairs were branched (Fig. 5m–o). In summary, these results suggest that noncatalytic domains were critical but not sufficient for the functional distinction between RopGEF4 and RopGEF10.

Enhanced ROS production by *FER* overexpression depended mainly on *RopGEF4* and *RopGEF10*

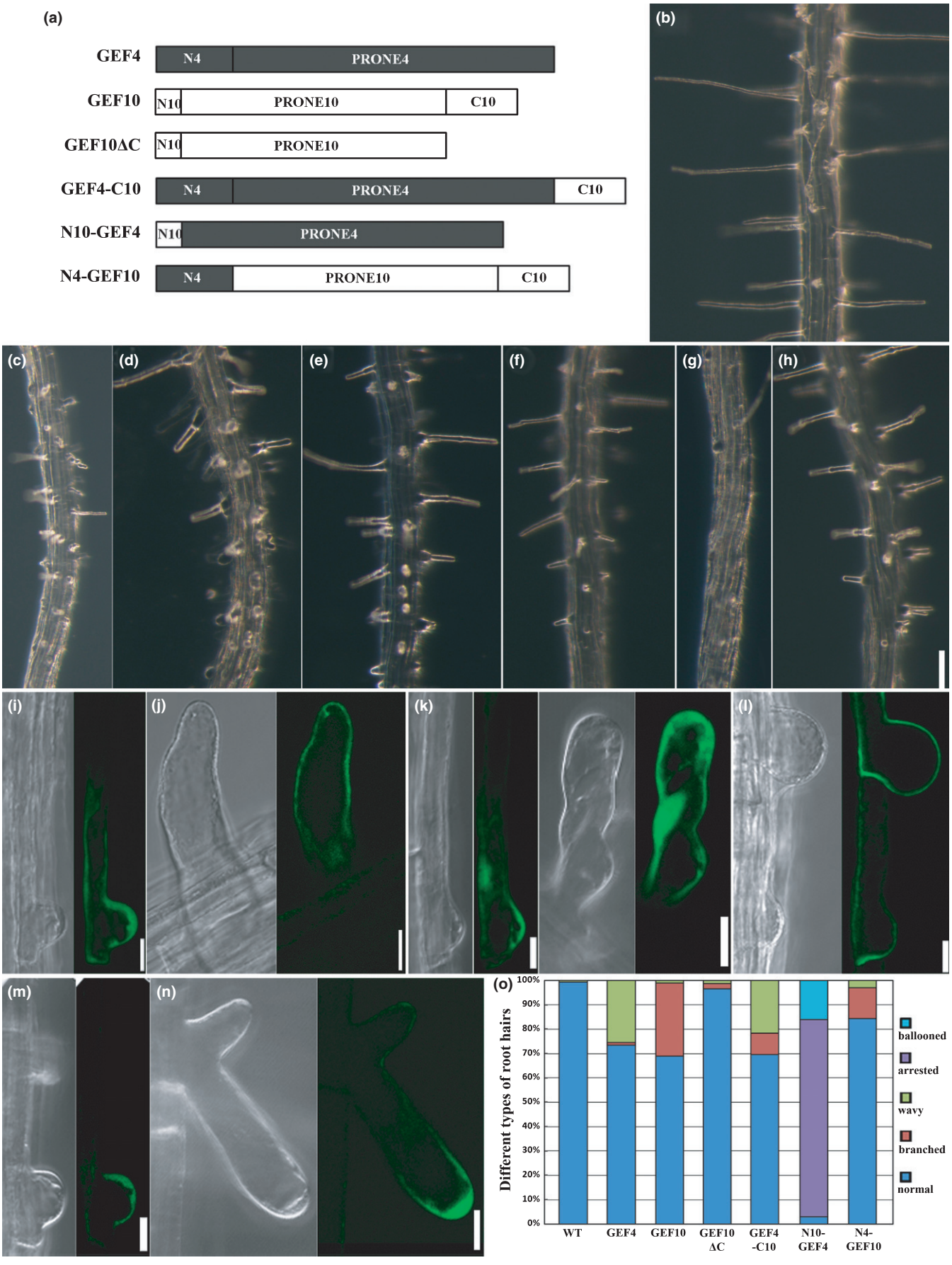
FERONIA was shown to be essential for root hair development and its overexpression promoted ROS production (Duan *et al.*, 2010). In addition, the kinase domain of FER interacts with several RopGEFs, including RopGEF4 and RopGEF10 (Duan *et al.*, 2010; Yu *et al.*, 2012). These lines of data pointed at the exciting possibility that *FER* is in the same genetic pathway with and upstream of *RopGEF4* and *RopGEF10*, mediating root hair development. To test this hypothesis, we generated transgenic plants expressing *FER* driven by Pro_{LRX1}, the promoter of the *LEUCINE-RICH REPEAT/EXTENSIN1* (*LRX1*) that showed trichoblast-specific expression (Baumberger *et al.*, 2001). ROS concentration was significantly increased by *FER* overexpression (Fig. 6a–e), similar to previous reports (Duan *et al.*, 2010). However, the enhanced ROS production by *FER* overexpression was almost abolished by the functional loss of *RopGEF4* and *RopGEF10* (Fig. 6c–e), although not completely (Fig. 6e). The result indicated that *RopGEF4* and *RopGEF10* are important downstream components of FER-mediated ROP signaling.

FER-mediated environmental regulation of root hair development was independent of *RopGEF4* and *RopGEF10*

FERONIA was also shown to be a key component in exogenous auxin-mediated root hair development, except for its role in the default developmental pathway (Duan *et al.*, 2010). We were therefore interested in finding out whether *RopGEF4* and *RopGEF10* were required for root hair development promoted by exogenous auxin. In addition, although phosphate limitation is known to be an important environmental signal promoting root hair initiation and elongation (Gilroy & Jones, 2000; Ma *et al.*, 2001; Zhang *et al.*, 2003), it was not known what plasma membrane components were responsible for sensing and responding to low phosphate in the surroundings. Considering the key role played by FER, we hypothesized that FER might also be crucial for environmental regulation of root hair development.

To find out whether *RopGEF4* and *RopGEF10* were required for root hair initiation and elongation by FER-mediated exogenous auxin, we grew plants of different genetic backgrounds on either control media or media supplemented with different concentrations of NAA. Exogenous auxin induced increased root hair initiation and elongation in a dose-dependent manner in the wildtype (Fig. 7a,b), indicating the effectiveness of the treatments. Hardly any response was shown by *fer-4* (Fig. 7a,b), a confirmed null mutant of *FER* (Duan *et al.*, 2010). However, single and double mutants of *RopGEF4* and *RopGEF10* responded to exogenous auxin as well as, or even more sensitively than, the wildtype to exogenous auxin in both root hair initiation and elongation (Fig. 7a,b). These results indicated that FER-mediated auxin regulation of root hair development was independent of *RopGEF4* and *RopGEF10*.

To find out whether *FER* was crucial for low phosphate-promoted root hair development, we followed previous protocols (Zhang *et al.*, 2003) and grew *fer-4* on either low-phosphate (1 μM) or high-phosphate medium (1 mM). As expected, both root hair density and length were significantly increased by phosphate limitation in the wildtype (Fig. 7e,f). However, *fer-4* was insensitive to phosphate limitation under the same conditions (Fig. 7e,f), suggesting that FER is the essential component in low-phosphate-promoted root hair development. In comparison, *gef4gef10* responded normally to phosphate limitation in root hair density (Fig. 7f) and was slightly hypersensitive in root hair



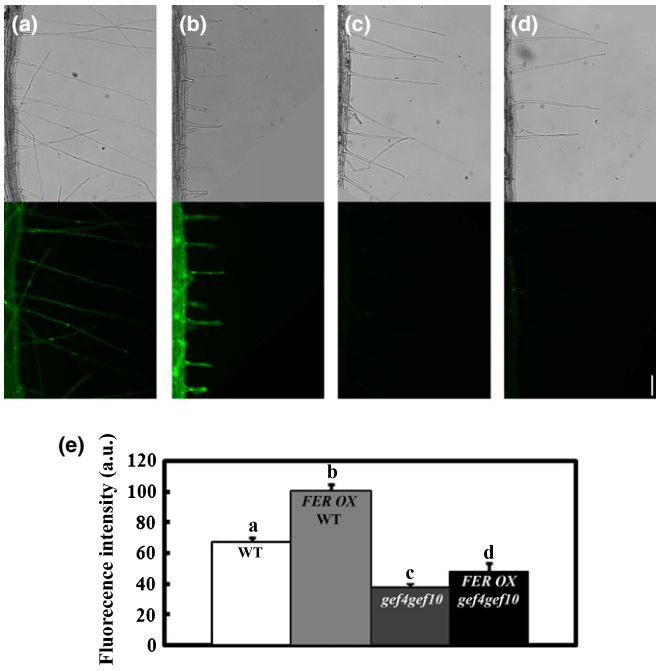


Fig. 6 Enhanced reactive oxygen species (ROS) production by Arabidopsis *FER* overexpression depended on *GEF4*/*GEF10*. (a–d) Fluorescent staining of ROS in root hairs of wildtype (WT) (a), overexpressing *FER* in WT (FER OX/WT) (b), *gef4gef10* (c), and overexpressing *FER* in *gef4gef10* (d). Bright field images are placed on top of their corresponding GFP channel images. Bar in (d), 100 μm for (b–d). (e) Quantitative analysis of ROS concentration in different genetic backgrounds. a.u., arbitrary fluorescence units. Results are given as means ± SE. Means with different letters are significantly different (Student's *t*-test, $P < 0.05$). Nonbiased double-blind analysis confirmed the result.

elongation (Fig. 7e). These results indicated that phosphate-limitation-promoted and FER-mediated regulation of root hair development was independent of *RopGEF4* and *RopGEF10*.

Discussion

Distinct noncatalytic domains of RopGEF4 and RopGEF10 resulted in their different function in root hair initiation and elongation

The function of *RopGEF4* was first hinted at by its preferential expression in trichoblasts (Won *et al.*, 2009). Its functional loss resulted in a 20% reduction of root hairs (Won *et al.*, 2009), consistent with what we have observed (Fig. 1). We suspected at the beginning that the mild phenotype resulted from the presence of *RopGEF10*, also highly expressed in trichoblasts (Won *et al.*, 2009). Functional loss of *RopGEF10* did affect root hair elongation (Fig. 1). However, it had a more significant effect on root hair initiation, which was not observed for *RopGEF4* (Fig. 1). Distinct contributions of *RopGEF4* and *RopGEF10* to root hair growth were also supported by their distinct overexpression effects. A high percentage of root hairs overexpressing *RopGEF4* were undulating or wavy, whereas a high percentage of root hairs overexpressing *RopGEF10* were branched (Fig. 4). Truncation and domain-swapping experiments suggested that such distinct contributions of *RopGEF4* and *RopGEF10* were mainly conferred by their noncatalytic domains. Unlike a few other RopGEFs whose C-termini likely confer autoinhibition to GTP–GDP exchange (Gu *et al.*, 2006; Zhang & McCormick, 2007; Riely *et al.*, 2011), deletion of the C-terminal region of RopGEF10 abolished its overexpression effects (Fig. 5), suggesting a positive role of the C-terminus in RopGEF10. Different roles of the N-termini were reported for distinct members of RopGEFs. The N-terminal region of RopGEF12 was required for ROP activation (Zhang & McCormick, 2007), suggesting a positive role of the N-terminal noncatalytic region. However, overexpression of the N-terminal-deleted MtRopGEF2 induced almost isotropic root hairs (Riely *et al.*, 2011), suggesting autoinhibitory roles of the N-terminus. By replacing N4 with N10 in N4-GEF10, we observed isotropic growth, that is, ballooning root hairs (Fig. 5), similar to those caused by ectopic ROP-GTP (Molendijk *et al.*, 2001; Jones *et al.*, 2002). This result suggests that the N-terminus of GEF4 acts as a cis-inhibitory factor for GEF activity, which could not be fulfilled by N10. Together, these domain-swapping results favor nonuniversal regulatory mechanisms for RopGEFs. Indeed, there might be distinct regulatory mechanisms for subgroups of RopGEFs that have different domain organizations (Riely *et al.*, 2011).

RopGEF-mediated ROP activation and ROS production are intracellular events of FER signaling in root hair development

We showed that RopGEF4 and RopGEF10 interact with ROP2 and ROP6 but not with ROP7 (Fig. 3). Interaction specificity between RopGEFs and ROPs may exist in general. It was previously shown that ROP11 interacts strongly with RopGEF4 and RopGEF1, which are in the same genetic pathway mediating stomata closure, but not so well with genetically irrelevant RopGEFs (Li & Liu, 2012; Yu *et al.*, 2012). Interestingly, both ROP2 and ROP6 promote root hair growth, while ROP7 inhibits it (Molendijk *et al.*, 2001; Jones *et al.*, 2002), suggesting promoting roles of RopGEF4 and RopGEF10 in root hair growth. Loss- and gain-of-function of *RopGEF4* and *RopGEF10* resulted in reduced and enhanced activation of ROPs, respectively (Fig. 2). As a

Fig. 5 Distinct gain-of-function effects of Arabidopsis *GEF4* and *GEF10* were mainly conferred by their noncatalytic C-terminal domains. (a) Schematic illustration of chimeric proteins used in this study. N, N-terminal noncatalytic domain; C, C-terminal noncatalytic domain; PRONE, GEF catalytic domain. (b–h) Representative root images of the wildtype (b), and overexpression of *GEF4* (c), *GEF10* (d), *GEF10ΔC* (e), *GEF4-C10* (f), of *N10-GEF4* (g), and *N4-GEF10* (h). (i–n) Representative images of root hairs overexpressing *GEF10ΔC* at early (i) and later growth stages (j), *GEF4-C10* (k), *N10-GEF4* (l), and those overexpressing *N4-GEF10* at early (m) and later (n) growth stages. Bright field and fluorescent images are placed side-by-side for (i–n). (o) Percentage of abnormal root hairs by overexpressing various proteins, including normal, wavy, branched, ballooned, or arrested classes. Approx. 180–240 hair cells collected from three independent experiments were used for the measurement in (o). Bars: (b–h) 100 μm; (i–n) 20 μm.

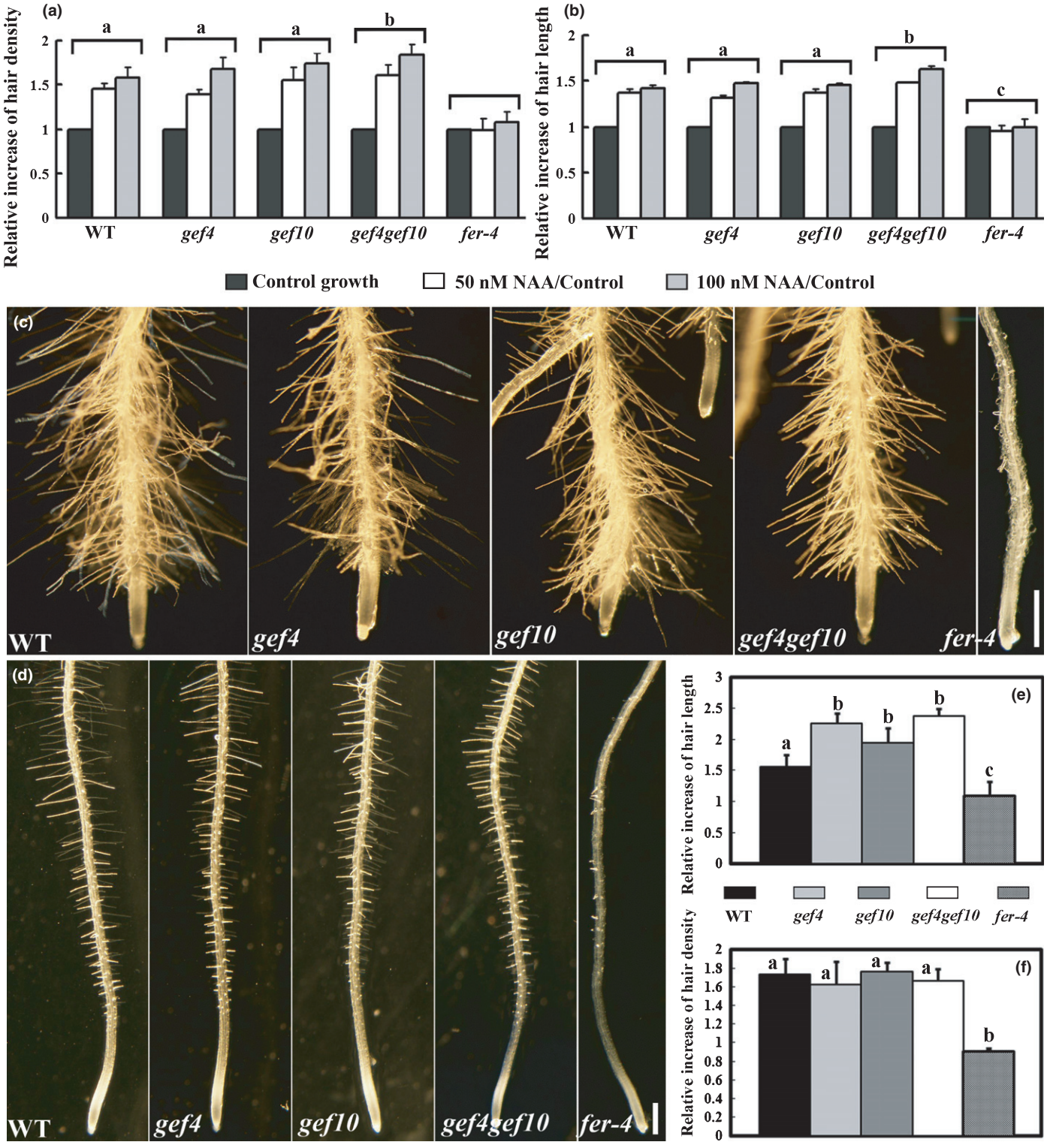


Fig. 7 FERONIA-mediated environmental regulation of root hair growth was independent of *GEF4/GEF10* in *Arabidopsis thaliana*. (a, b) Relative increase of root hair density (a) or length (b) by exogenous auxin (50 or 100 nM naphthylacetic acid (NAA)) in different genetic backgrounds. (c, d) Representative roots of different genetic materials under the phosphate-limiting condition (c, 1 μm) or the nonlimiting condition (d, 1 mM). (e, f) Relative increase of root hair length (e) or density (f) by phosphate limitation in different genetic backgrounds. For root hair density in (a) and (f), 33–40 seedlings grown under the same conditions in four batches were analyzed. Except for *fer-4*, for which few root hairs could be detected, around 180–240 hair cells of other genetic materials were collected in four independent experiments and used for the measurement in (b) and (e). Results are given as means ± SE. Means with different letters are significantly different (Student's *t*-test, $P < 0.05$). Nonbiased double-blind analyses were performed to confirm the results. Bars: (c) 500 μm; (d) 1 mm.

major indicator of ROP signaling in root hairs (Foreman *et al.*, 2003; Carol *et al.*, 2005; Jones *et al.*, 2007; Duan *et al.*, 2010), ROS production was reduced significantly in *gef4gef10* (Figs 2, 6), confirming the roles of RopGEF4 and RopGEF10 in ROP signaling. *FER* was shown to be critical for ROP activation and ROS production in different developmental processes (Duan *et al.*, 2010; Yu *et al.*, 2012). Our results have demonstrated for the first time the genetic epistasis between FER and RopGFEs (Fig. 6). However, considering the mild defects shown by *gef4gef10* (Fig. 1) compared with that of *fer-4*, a hierarchy redundancy as reported for other developmental processes (Zhang & McCormick, 2007; Yu *et al.*, 2012) may exist in which additional RopGEFs play similar roles downstream of FER to mediate root hair development. In fact, functional loss of both *GEF*s did not completely abolish the increased ROS production induced by *FER* overexpression (Fig. 6), suggesting additional GEFs functioning downstream of FER in root hair initiation and elongation. A candidate is *RopGEF11*, which is expressed in the trichoblast by GUS reporter analysis (Li & Liu, 2012) as well as by transcriptome analysis (Won *et al.*, 2009).

FER-mediated environmental regulation of root hair growth is independent of RopGEF4 and RopGEF10

It has long been known that root hair growth is a highly flexible developmental process that can be influenced by hormones and nutrient availability (Gilroy & Jones, 2000). Previously, it was shown that functional loss of *FER* rendered plants insensitive to the promoting effects of exogenous auxin on root hair initiation and elongation (Duan *et al.*, 2010). We have shown here that enhanced root hair initiation and elongation promoted by phosphate limitation in growth media require a functional *FER* (Fig. 7). Considering the genetic epistasis between *FER* and *RopGEF4/10* in root hair development 'by default' (Fig. 6), it was a surprise to see a normal or hypersensitive response of *gef4gef10* in hair initiation and elongation to exogenous auxin or low phosphate (Fig. 7). The possibility of hierarchy functional redundancy of *RopGEF*s, as reported for other systems (Li & Liu, 2012; Yu *et al.*, 2012), was unlikely to be a sufficient explanation here because functional loss of *RopGEF4* and *RopGEF10* almost abolished ROP activation and ROS production in the wildtype (Fig. 2) and by *FER* overexpression (Fig. 6). A more likely scenario is that FER interacts with and activates other cellular pathways upon environmental cues (Fig. 8). The different responses between *fer-4* and *gef4gef10* to exogenous promoting factors, such as low phosphate and high auxin, thus suggested an exciting possibility that FER, as the key factor in root hair development, integrates developmental and environmental signals whereas signals integrated by FER diverge intracellularly either through GEFs or through X factors (Fig. 8). Indeed, overexpression of *ROP2* did not rescue auxin responsiveness of *fer-4* (Duan *et al.*, 2010), suggesting there are ROP-independent pathway(s) for FER-mediated environmental regulation of root hair growth. The double mutant *gef4gef10* was hypersensitive to exogenous auxin or phosphate limitation (Fig. 7). In addition, overexpression of both *RopGEF4* and *RopGEF10* caused a basal shift of root hair bulging sites, suggestive of reduced auxin signaling (Fig. 4). These lines of evidence indicate a feedback mechanism by RopGEF4 and RopGEF10 on FER-mediated, environment-dependent, root hair development (Fig. 8). Thus, the environment-sensitive pathway provides an alternative, though not mutually exclusive, possibility to hierarchy redundancy for the moderate phenotypic defects in *gef4gef10*. The identity of components in the environmentally regulated FER pathway awaits further investigations. Specifically, components in sugar and ethylene signaling might be of interest, owing to the fact that inhibiting sugar or ethylene production or signaling abolished plant responses to the promoting effects of low phosphate in root hair initiation and elongation (Zhang *et al.*, 2003; Lei *et al.*, 2011a,b).

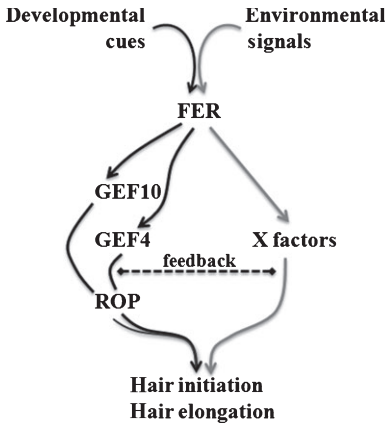


Fig. 8 GEF4 and GEF10 are important for FERONIA (FER)-mediated developmental but not environmental regulation of root hair growth: a working model. FER is the key component in integrating developmental and environmental cues for root hair initiation and growth. GEF-mediated Rho of plant (ROP) activation and reactive oxygen species (ROS) production are intracellular events by developmentally mediated FER signaling (illustrated with black lines), whereas other factors (X) are responsible for environmentally mediated FER signaling (illustrated with gray lines). There may be feedback between these two intracellular pathways to ensure proper root hair development under different environmental conditions.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Reduced root hair density in *gef10* and *gef4gef10* was not the result of abnormal hair cell specification.

Fig. S2 RopGEF4 and RopGEF10 interact with two hair growth-promoting ROPs but not with the hair growth-inhibiting ROP7 in yeasts.

Fig. S3 Overexpression of *RopGEF4* and *RopGEF10* affected root hair initiation and elongation.

Fig. S4 The subcellular localization of RopGEF4 and RopGEF10 by fluorescent double-labeling analysis.

Table S1 Oligos used in this study

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