

# FERONIA regulates auxin-mediated lateral root development and primary root gravitropism

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**The *Arabidopsis* FERONIA (FER) receptor kinase is a key hub of cell signaling networks mediating various hormone, stress, and immune responses. Previous studies have shown that FER functions correlate with auxin responses, but the underlying molecular mechanism is unknown. Here, we demonstrate that the primary root of the *fer-4* mutant displays increased lateral root branching and a delayed gravitropic response, which are associated with polar auxin transport (PAT). Our data suggest that aberrant PIN2 polarity is responsible for the delayed gravitropic response in *fer-4*. Furthermore, the diminished F-actin cytoskeleton in *fer-4* implies that FER modulates F-actin-mediated PIN2 polar localization. Our findings provide new insights into the function of FER in PAT.**

**Keywords:** auxin; FERONIA; gravitropic; lateral root; PIN2; response

Plant receptor-like kinases (RLKs) are a large family of transmembrane proteins that sense and respond to multiple developmental and environmental stimuli. As a member of the *Catharantus roseus* RLK1-like (CrRLK1L) subfamily, the receptor kinase FERONIA (FER) contains an extracellular domain with tandem malectin-like regions that perceives and interacts with diverse ligands, and an intracellular domain that triggers phosphorylation and cellular responses [1,2]. FER, which was initially discovered to regulate female fertility [3,4], was recently demonstrated to be involved in regulating various hormone signaling pathways, including auxin, abscisic acid (ABA), ethylene, and brassinosteroids (BRs) [2,5–8]. FER is required for auxin-induced root hair growth [7] and auxin-dependent transient apoplast alkalization [9]. Cross-talk between FER and the

ABA pathway indicates that FER plays an important role in ABA-mediated stress responses [5,6]. Apart from affecting auxin and ABA responses, FER was shown to inhibit ethylene synthesis and signaling and to control ethylene and BR-regulated hypocotyl elongation [8,10]. Recent studies of the Rapid Alkalinization Factor-FER pathway indicate that FER may modulate immune signaling [11–13]. Additional functions of FER include cell growth, sensing and maintaining cell wall integrity, and salt stress tolerance [14–18].

Several reports have suggested that FER mediates certain auxin-regulated responses. FER was initially linked to auxin-mediated responses to reactive oxygen species (ROS) via the Rho-like GTPases from plants (ROP) signaling pathway [7]. Multiple *fer* mutants exhibit impaired auxin-induced root hair growth, and this

## Abbreviations

ABA, abscisic acid; BFA, Brefeldin A; BRs, brassinosteroids; CrRLK1L, *Catharantus roseus* RLK1-like; FER, FERONIA; Lat B, latrunculin B; LR, lateral root; LRD, lateral root density; NPA, naphthylphthalamic acid; PAT, polar auxin transport; PM, plasma membrane; RLKs, receptor-like kinases; ROP, Rho-like GTPases from plants.

phenotype was rescued by overexpressing the FER binding partner ROP2 [7]. Rho GTPases function as crucial molecular switches in plants [19,20], and several Rho GTPases have been shown to participate in auxin-related gene expression and development [21–23]. Thus, it is possible that FER participates in auxin-regulated processes by activating ROP signaling. In addition, the pavement cells of *fer* mutant plants display defective jigsaw puzzle patterning, which is regulated by auxin; this indicates that FER plays a role in auxin-mediated epidermal morphogenesis [1]. Recently, it was shown that the auxin-dependent cell wall pH is mediated by plasma membrane (PM)-localized FER [9]. Upon gravistimulation, an auxin gradient forms and FER-dependent transient apoplast alkalization inhibits cell elongation at the lower side of the root; this local elongation inhibits epidermal root cell elongation and enables the root to bend [6]. Thus, FER is important for auxin-regulated processes; however, the underlying mechanisms are unknown. We found that *fer-4* mutant seedlings displayed aberrant columella root cell patterns, developed an increased number of lateral root (LR) branches, and showed delayed gravitropic responses in the primary root compared to wild type. All of these phenotypes are associated with defective polar auxin transport (PAT); therefore, we studied the localization and cellular recycling of PIN auxin transporters. Altered PIN2 polarity and shorter actin filaments were observed in *fer-4* mutant plants, indicating that FER controls F-actin-mediated PIN2 polar localization and, ultimately, contributes to PIN2-associated LR development and gravity responses.

## Materials and methods

### Plant materials and growth conditions

Seedlings of the following types were used in this study: *Arabidopsis thaliana* (hereafter, *Arabidopsis*; ecotype Columbia-0 [Col-0]), the *fer-4* mutant [5] and a *fer-4* complemented line (*fer-4* complemented by *pFER::FER-GFP* in a Col-0 background) [5], and three marker lines (*pPIN2::PIN2-GFP* [24], *DR5rev::GFP* [25], and *35S::ABD2-GFP* [26]). Seeds were surface-sterilized and then sown on half-strength Murashige and Skoog (MS) medium supplemented with sucrose (1% [w/v]) and kept in the dark for 2 days at 4 °C. Seedlings were grown in growth chambers under a 16 h of light/8 h of dark cycle at  $22 \pm 2$  °C.

### Gravity response and growth analyses

For the gravity response analysis, 4-day-old seedlings grown vertically under a 16 h/8 h light/dark photoperiod

were rotated 90°. The degree of root curvature was measured 2, 4, 8, 12, and 24 h later using image analysis program IMAGE J (National Institutes of Health, Bethesda, MD, USA). To analyze the LR density (LRD), the root lengths of 7-day-old seedlings were measured using IMAGEJ; meanwhile, the total number of LRs in each seedling was counted under a stereoscopic microscope. LRD is the LR number divided by the primary root length (number/centimeters). About 30 seedlings from three independently repeated experiments were analyzed. Pairwise Student's *t*-tests were used to determine the statistical significance of the results. All analyses were performed using data from at least three separate, repeated experiments.

### Treatment with naphthylphthalamic acid (NPA) or latrunculin B (LatB)

Seedlings were grown on vertical plates containing various concentrations of NPA (Sigma-Aldrich, St. Louis, MO, USA) for 9 days, and then the primary root length was measured using IMAGEJ. Four-day-old seedlings grown on vertical plates were transferred to half-strength MS liquid medium containing 30  $\mu$ M LatB (Sigma-Aldrich) for 2 h; the structure of the cytoskeleton was then observed using a confocal microscope (Zeiss LSM780 META; Oberkochen, Germany).

### Brefeldin A (BFA) treatment and washout analysis

Four-day-old seedlings grown on vertical plates were transferred to half-strength MS medium containing 50  $\mu$ M BFA (Sigma-Aldrich) and then incubated for 0.5 h. Next, the seedlings were washed with half-strength MS medium for 1.5 h. The roots were then observed by confocal microscopy (Zeiss LSM780 META). Cells with visible BFA bodies were counted. Epidermal cells from 15–20 roots were used for a quantitative analysis of BFA bodies.

### $\beta$ -Glucuronidase (GUS) activity assay

$\beta$ -Glucuronidase staining of seedlings was performed as described elsewhere [25] in 0.25 mg·mL<sup>-1</sup> of 5-bromo-4-chloro-3-indolyl-glucuronide at 37 °C for 0.5 h. Photographs were taken using an Olympus BX51 microscope (Tokyo, Japan) equipped with a Ritiga 2000R Digital Camera (QImaging, Surrey, BC, Canada).

### Confocal laser scanning microscopy for marker gene analysis

The marker gene lines *PIN1pro::PIN1-GFP*, *PIN2pro::PIN2-GFP*, *DR5rev::GFP*, and *35Spro::ABD2-GFP* were crossed with *fer-4*, respectively. Homozygotes were identified from

the F2 heterozygote population. F3 seedlings were subjected to further experimentation, with the marker plants as controls. Quantification of the seedling F-actin density was performed as described by Liu *et al.* [27] and according to the methods of Dyachok *et al.* [28] and Higaki *et al.* [29]. The density of F-actin was defined as the ratio of the filament occupancy and cell area, as described by Dyachok *et al.* [28]. At least 50 cells from 15 seedlings were used.

### Quantitative RT-PCR analysis

To analyze *PIN1* and *PIN2* expression, whole-root samples from 7-day-old wild-type (WT) and *fer-4* mutant were collected and subjected to total RNA extraction. cDNA was prepared from 2 µg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio Inc., Otsu, Japan). PCR was performed on a Bio-Rad CFX96 (Bio-Rad Inc., Hercules, CA, USA) system with a SYBR probe (Takara Bio Inc.). *PIN1* and *PIN2* expression was normalized to that of *ACTIN2* and then compared with WT data. The data presented are the averages from at least three biological replicates with standard errors (SEs). Asterisks highlight significant differences from wild type by Student's *t*-test (\* $P < 0.05$ ; \*\* $P < 0.01$ ). The gene-specific primer sets used were as follows: *ACTIN2*, Forward (ATGGCTGAGGCTGATGATATTCAAC) and Reverse (TACAAGGAGAGAACAGCTTGATG); *PIN1*, Forward (TGGAAGACAACCTTTGGAAACT) and Reverse (TGAAGCATTAGAACGACGAACA); and *PIN2*, Forward (CCTCGCCGCACTCTTTCTTT) and Reverse (CGTACATCGCCCTAAGCAAT).

## Results

### FER regulates LR branching and gravitropism

By analyzing *pFER:GUS* and *pFER:GFP-FER*, we found that FER is broadly expressed in roots, including LR primordia (Fig. 1A–F). Although the primary root length in *fer-4* was comparable to that in wild type (Fig. S1A,B), as reported previously [30,31], *fer-4* developed significantly more LRs (Fig. 1G) and the LRD increased by 92% compared to wild type (Fig. 1H). The primary root in *fer-4* showed a delayed gravitropic response as compared to wild type (Fig. 1I), consistent with previously reported data [9]. Columella cells play a critical role in gravity sensing. We thus investigated columella root cell patterns by Lugol staining to detect starch-containing columella root cells. As shown in Fig. 1J–M, aberrant columella root cell patterns were detected in *fer-4*. A typical columella root cap in WT plants consists of four layers of differentiated columella cells (Fig. 1J). However, we noted that in the *fer-4* mutants 38% of the roots

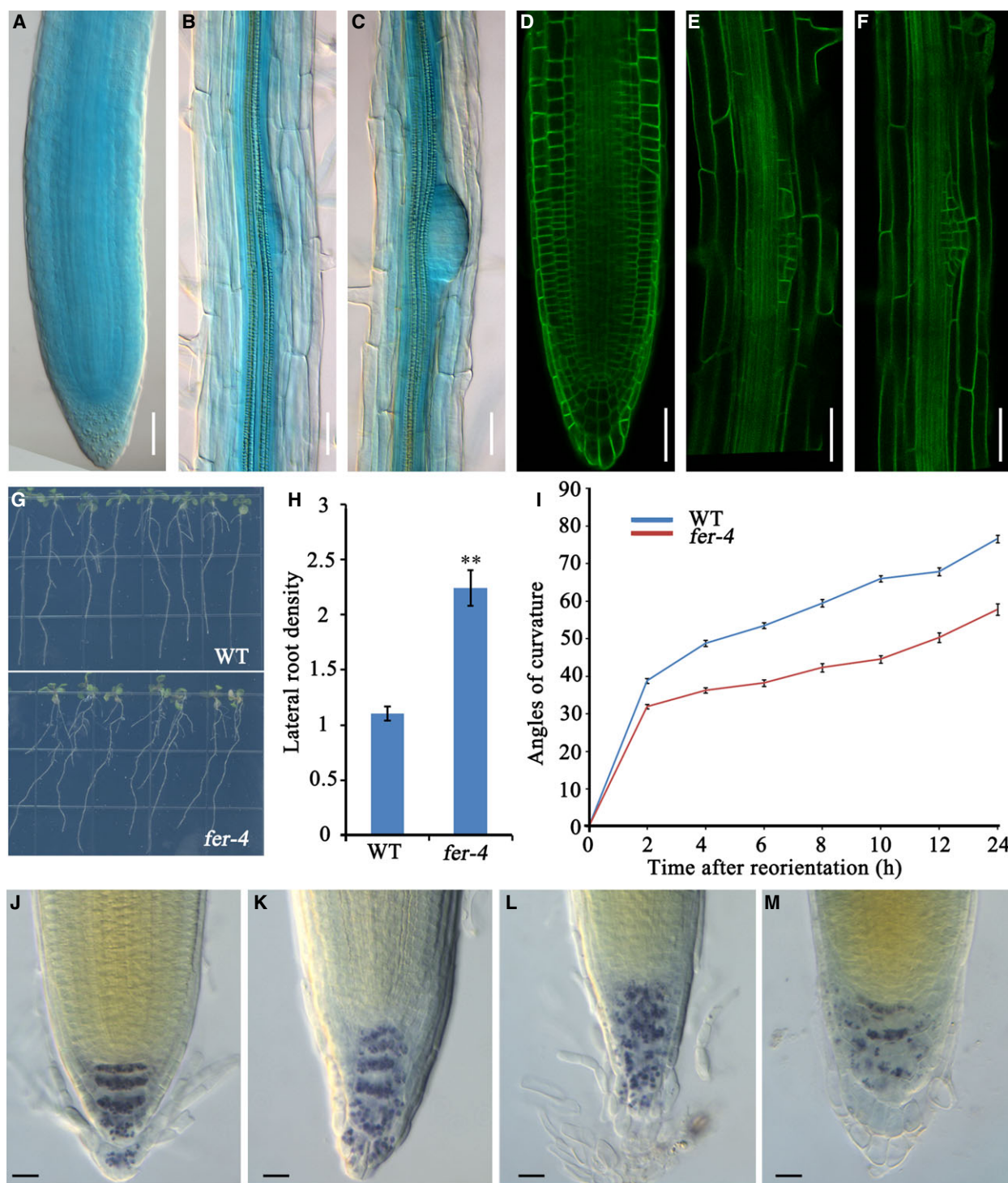
( $n = 40$ ) had more than five tiers of columella cells (Fig. 1K), and nearly 50% ( $n = 40$ ) of the roots showed severely disordered columella cells (Fig. 1L, M). A previous study reported that the root hair defects in the *fer-4* mutant were rescued by a *fer-4* complemented line (*fer-4* complemented by *pFER::FER-GFP*). We found the LRD (Fig. S1C, D) and columella root cells (Fig. S1E) in this complemented line were recovered to normal.

Root gravitropism is an auxin-dependent process. In vertically orientated roots, symmetric auxin gradients form on all sides of the root. However, in horizontally orientated roots, auxin transport is redirected upon gravistimulation and more auxin accumulates at the lower side, forming asymmetrical lateral auxin gradients between the upper and lower sides of the root tips. As a consequence, accumulated auxin at the lower side inhibits local tissue expansion and the root bends towards the gravity vector [32]. The lateral auxin gradient across the root cap can be monitored using the auxin activity reporter *DR5rev:GFP*. To test whether the auxin response was changed in the *fer-4* mutant, we monitored *DR5rev:GFP* fluorescence signals. No significant difference in *DR5rev:GFP* fluorescence was found between vertically cultured WT and *fer-4* seedlings (Fig. 2A, B). However, 3.5 h after being reoriented by 90°, intense *DR5rev:GFP* fluorescence was observed on the lower side of the root tips in wild type (Fig. 2C), but not in *fer-4* (Fig. 2D). This observation indicates that FER contributes to root gravitropic responses by affecting auxin redistribution. We next examined whether auxin transport is impaired in *fer-4*. In the presence of the auxin efflux inhibitor NPA, the root length in *fer-4* was significantly inhibited in a concentration-dependent manner, displaying a hypersensitive response compared to wild type (Fig. 2E, F). Further, the LR branches of *fer-4* were sharply reduced by NPA treatment, revealing that LR development is hypersensitive to NPA (Fig. 2G). Taken together, our results demonstrate that FER regulates LR branching and root gravitropism by contributing to PAT.

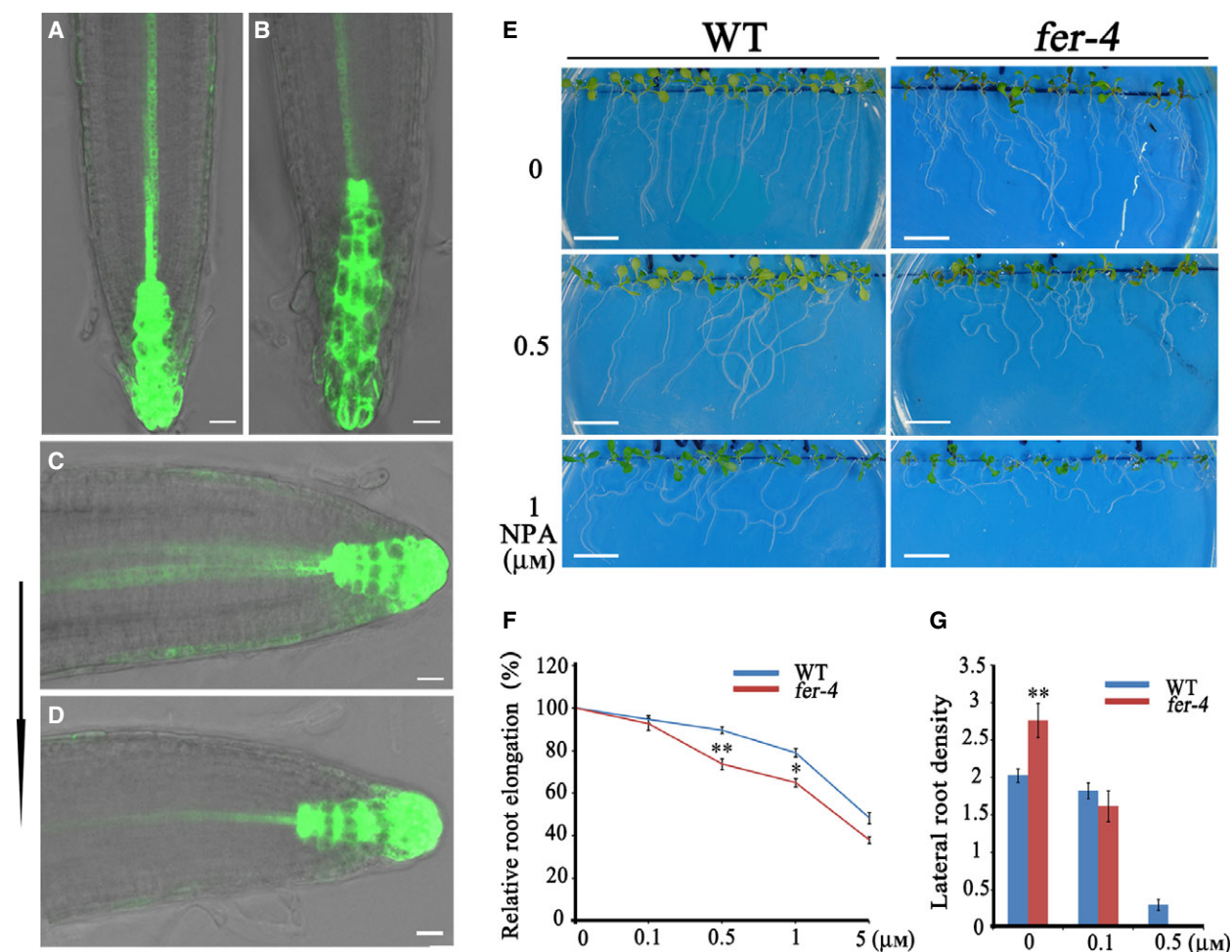
### Polar localization of PIN2 is altered in *fer-4*

It has been reported that PIN2 is necessary (and that its localization is important) for root gravitropic responses [32–34]. To investigate whether PIN2 is involved in the delayed root gravitropic response of *fer-4*, PIN2-GFP was introgressed into the *fer-4* mutant. In WT plants, PIN2 is normally localized to the apical PM of epidermal cells and the basal PM of cortical cells in root tips (Fig. 3A). However, an





**Fig. 1.** *FER* is expressed in roots and affects root development. *pFER:GUS* (A–C) and *pFER:GFP-FER* (D–F) were expressed in 4-day-old roots and LR primordia. (G and H) LRs from 7-day-old *fer-4* and WT seedlings. (I) Comparison of the gravitropic responses of *fer-4* and WT plants. (J–M) Comparison of the columella cell patterns between wild type (J) and *fer-4* (K–M) in 4-day-old roots by Lugol staining. The root columella cells of *fer-4* showed an increased (K) or disordered columella layer (L–M). The data are means  $\pm$  SE from three independent experiments ( $n > 30$ ).  $**P < 0.01$ . Scale bars = 20  $\mu$ m.



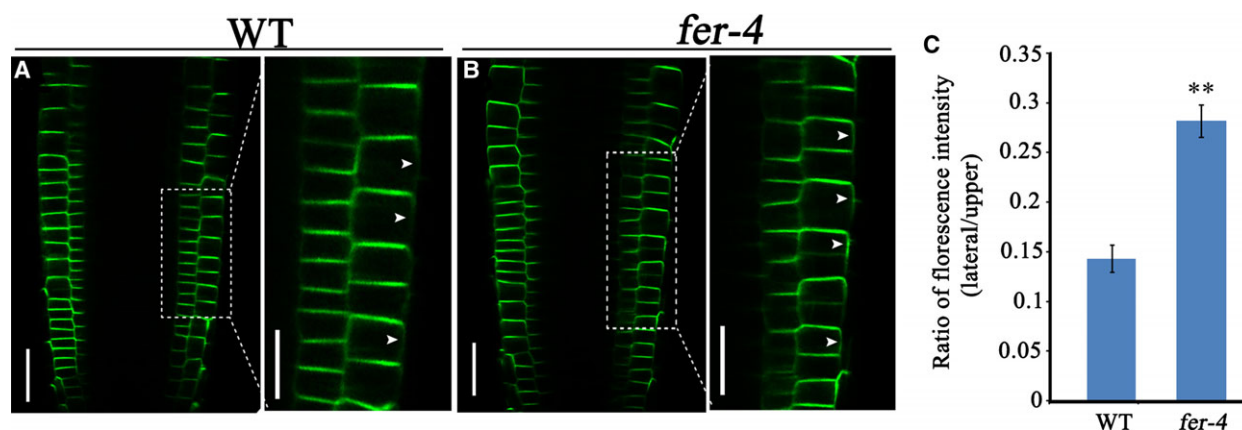
**Fig. 2.** The *fer-4* mutant displays altered auxin redistribution upon gravistimulation. The expression of *DR5rev:GFP* in roots of 4-day-old WT (A) and *fer-4* (B) plants. After the rotation of vertically grown seedlings by 90° for 3.5 h, the expression of *DR5rev:GFP* was detected on the lower side of the root in wild type (C), but not in *fer-4* (D). Scale bars = 20  $\mu$ m. (E) Inhibition of primary and LRs by NPA in 9-day-old *fer-4* and WT plants. Scale bars = 20 mm. (F and G) Quantitative comparison of the relative primary root length and LRD in the presence of NPA in 9-day-old WT and *fer-4* plants. The data are the means  $\pm$  SE from three independent experiments ( $n > 30$ ); \* $P < 0.05$ , \*\* $P < 0.01$ .

analysis of the PM localization of PIN2-GFP in *fer-4* revealed apical-to-lateral membrane diffusion (Fig. 3B). A quantitative analysis of the fluorescence intensity showed markedly enhanced fluorescence on the lateral membranes in *fer-4* (Fig. 3C). These data suggest that the delayed gravitropic response in *fer-4* is due to the abnormal polar localization of PIN2 in epidermal cells. Both auxin influx and efflux transporters were reported to regulate the root gravitropic responses [33,34]. We thus monitored the AUX1-YFP and PIN1-GFP in *fer-4* roots and found that the polar localization or accumulation of AUX1-YFP was not altered (Fig. S2A–C). The polar localization of PIN1-GFP was not changed but the fluorescence ratio of PIN1-GFP was reduced (Fig. S2D–F). We also analyzed the transcription of *PIN1* and *PIN2*, and found

that the transcript level of *PIN1* was unchanged, while that of *PIN2* was reduced, in roots (Fig. S2G). Taken together, our results indicate that *FER* contributes to PAT partly by regulating PIN2 localization.

### PIN2 intracellular cycling is delayed in *fer-4* mutant plants

Brefeldin A is a vesicle trafficking inhibitor that prevents PIN recycling, leading to membrane protein aggregates called BFA compartments [35]. Multiple reports have demonstrated that the root gravitropic response is inhibited by disrupting the BFA-sensitive membrane recycling of PIN2 [26,33,36,37]. We thus examined whether *FER* has an effect on PIN2 recycling. In the presence of 50  $\mu$ M BFA for 0.5 h, visible



**Fig. 3.** The polar localization of PIN2-GFP was altered in the *fer-4* mutant. (A and B) PIN2-GFP localization in the root epidermis of 4-day-old WT and *fer-4* plants. The white arrow denotes the apical-to-lateral membrane diffusion of PIN2-GFP in adjacent epidermal cells of the root. (C) A statistical analysis of the ratio of lateral to apical membrane fluorescence in WT and *fer-4* plants. The data are the means  $\pm$  SE for more than 20 seedlings coming from three independent experiments ( $n > 20$ ). \*\* $P < 0.01$ . Scale bars = 20  $\mu$ m.

BFA bodies of PIN2-GFP appeared in root epidermal cells. There was no significant difference in the number of cells with BFA compartments (represented by PIN2-GFP) between *fer-4* and WT plants (Fig. 4A, B). After the BFA had been washed out, most of the BFA compartments disappeared and PIN2-GFP showed normal polar localization in WT plants (Fig. 4C). By contrast, visible BFA compartments (PIN2-GFP) were retained in the cytosol of epidermis cells in the *fer-4* mutant (Fig. 4D), and a quantitative analysis revealed the presence of a high percentage of cells with BFA compartments in the *fer-4* mutant compared to wild type (Fig. 4E). These results suggest that FER plays a regulatory role in PIN2 recycling.

### FER is required to maintain F-actin stability in roots

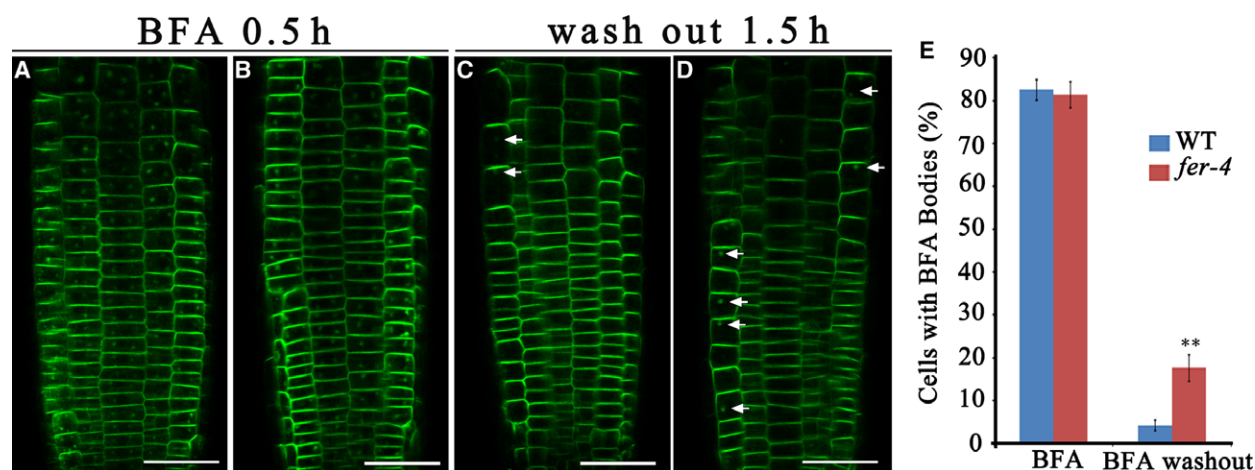
Multiple studies have demonstrated that the constitutive recycling and cellular targeting of auxin transporters rely on the correct organization of actin filaments [38–41]. The actin cytoskeleton is considered to provide primary guidance for vesicle trafficking in interphase plant cells and has been shown to be important for both auxin transport and PIN endocytic recycling [35,41]. Pharmacological disruption of actin filaments was observed to cause PIN2 mislocalization or accumulation in bodies of unknown identity in the root epidermis [39,40]. We therefore examined whether the accumulated BFA compartments (PIN2-GFP) in *fer-4* were caused by a disordered actin cytoskeleton. The second actin-binding domain of Arabidopsis Fimbrin1 (ABD2) is used extensively as an F-actin reporter to show actin dynamics or localization *in vivo*

[42,43]. We introduced a construct expressing an ABD2:GFP fusion protein by crossing into the *fer-4* background, and visualized the actin filaments *in vivo*. We found long and dense fluorescent filaments in epidermal cells from the root elongation and maturation zones in wild type (Fig. 5A), whereas shorter and faint actin filaments were visible in the *fer-4* mutant (Fig. 5B). These data suggest that FER plays a key role in maintaining the integrity of the F-actin cytoskeleton. LatB, which promotes the depolymerization of actin filaments, was used to assess actin organization. After 2 h of treatment with LatB, short fragments of actin filaments were observed in wild type (Fig. 5C). However, in *fer-4*, these actin filaments were degraded into highly punctate or fragmented structures (Fig. 5D), indicating reduced actin stability in *fer-4*. A quantitative analysis of the F-actin density (occupancy) in epidermal cells confirmed this observation (Fig. 5E), indicating that FER plays a key role in maintaining the integrity of the actin cytoskeleton.

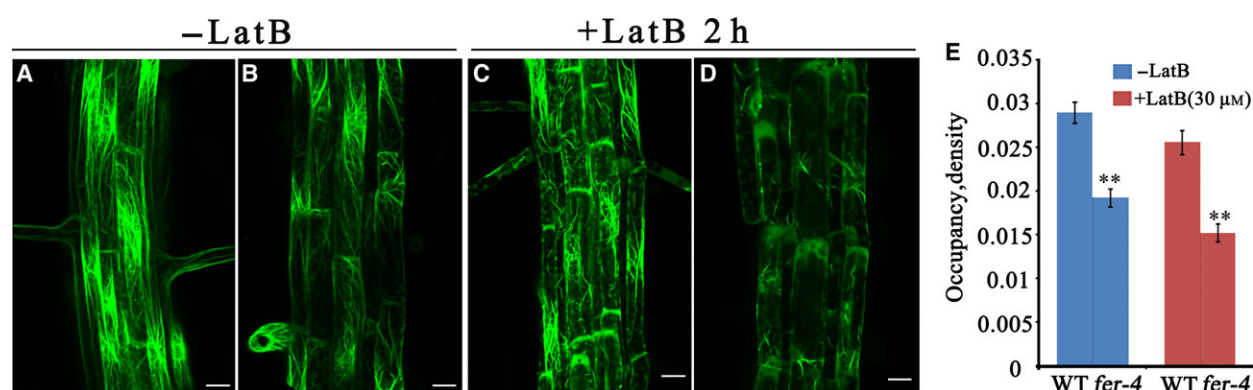
### Discussion

In this study, we found that FER regulates PAT by modulating actin-mediated PIN2 polar localization. We uncovered a prominent role for FER in controlling PAT. Auxin regulates root development and gravitropic responses. The defective root gravitropic responses in *fer-4* seedlings were associated with altered auxin redistribution upon gravistimulation, as visualized with the *DR5rev:GFP* auxin response reporter. Together with the observation that *fer-4* mutant seedlings were hypersensitive to the auxin transport inhibitor NPA, these data indicate that FER regulates





**Fig. 4.** Recycling of PIN2 is delayed in the *fer-4* mutant. (A and B) BFA bodies (PIN2-GFP) in 4-day-old WT (A) and *fer-4* (B) roots following BFA (50  $\mu$ M) treatment for 0.5 h. (C and D) A comparison of cells with BFA compartments between wild type (C) and the *fer-4* mutant (D) after the washout of BFA. The white arrow denotes a BFA compartment. (E) Quantitative comparison of cells with BFA bodies (PIN2-GFP) in WT and *fer-4* plants following BFA treatment and washout. Error bars indicate the SE of measurements made for at least 15 roots in three biological replicates. \*\* $P < 0.01$ . Scale bars = 20  $\mu$ m.



**Fig. 5.** FER affects the stability of actin filaments in roots. (A and B) Actin filaments in the epidermal cells of the root elongation and maturation zones in 4-day-old WT (A) and *fer-4* (B) seedlings. (C and D) Highly fragmented actin filaments in the root epidermal cells of *fer-4* (D) compared to WT (C) plants after treatment with 30  $\mu$ M LatB for 2 h. The images are composed of eleven confocal optical sections. (E) F-actin density (occupancy) in epidermal cells of the root maturation zone. The data are the means  $\pm$  SE from three independent experiments ( $n > 15$ ). \*\* $P < 0.01$ . Scale bars = 20  $\mu$ m.

LR development and gravitropic responses by contributing to PAT.

FER has been revealed to modulate the ROP signaling pathway, which controls ROS-mediated and auxin-regulated root hair development [7]. We previously showed that ROP3 functions to maintain the polarity of PIN proteins at the PM, thus affecting PAT and auxin distribution, and contributing to auxin responses [44]. FER works as an upstream regulator of the RAC/ROP signaling pathway, and RAC/ROP further interact with diverse effectors such as RIC1 and ICR1, which have been linked to auxin signaling [24,45,46]. Therefore, FER may participate in auxin-regulated

processes by activating RAC/ROP effectors. The direction and threshold of auxin transport are determined by the level of activity of influx and efflux transporters such as PINs and AUX [47]. PIN2 and AUX1 are suggested to be major regulators of root gravitropic responses [33,34], but FER seemed to have no impact on the polar localization or accumulation of AUX1-YFP (Fig. S2A–C). In contrast, the polar localization of PIN2 was altered (Fig. 3) and the fluorescence ratio of PIN1-GFP was significantly reduced in *fer-4* roots (Fig. S2D–F). We propose that the defective gravitropic response in *fer-4* mutant plants is largely due to altered PIN2 polar localization (Figs 3 and 4) since

PIN2 is necessary for the transmission of auxin to sites of gravibending [32]. Aberrant polar localization of PIN2 in the epidermis will inevitably disturb the distribution of auxin at the root tip, affecting the root gravitropic response and LR branches. Consistently, the *fer-4* mutant resembled the *pin2* knockout mutant, which exhibits an increased LRD and defective gravitropic response compared to wild type [48]. Our findings establish that FER is involved in gravitropism and LR development by controlling the polarity of PIN2, which is critical for PAT in roots.

Emerging data indicate that the action of the actin cytoskeleton determines PAT and affects auxin responses [35,49,50]. FER plays an important role in the polarized growth of pollen tubes, root hairs, and trichomes, which are heavily controlled by the dynamic organization of the actin cytoskeleton [1,51,52]. Multiple pieces of evidence from pharmacological and genetic studies indicate that changes in actin organization can disturb endocytosis or the recycling of auxin transporters by impairing vesicular trafficking [26,38,39,41]. RAC/ROP signaling appears to play crucial roles in these actin-mediated auxin transporter recycling processes. In leaf pavement cells, the auxin-activated interaction of ROP2 with RIC4 inhibits PIN endocytosis by stabilizing cortical actin filaments, whereas the RIC4-dependent assembly of cortical F-actin promotes lobe outgrowth [53]. In root cells, the SPK1-ROP6-RIC1 pathway inhibits PIN2 internalization by stabilizing actin filaments [26]. Recently, we showed that a Rho of plants (Rop)-guanine nucleotide exchange factor, *RopGEF1*, regulates the polarized localization of AUX1 and affects the distribution of PIN7 and PIN2 [27]. Interestingly, *ropgef1* mutants showed impaired actin filament accumulation in root cells, suggesting that *RopGEF1* participates in PAT by modulating actin filaments [27]. Acting as an upstream regulator of GEF-RAC/ROPs, FER probably affects actin stability through downstream signaling components. Another study revealed the altered configuration of the actin cytoskeleton and a lack of polar PIN2 localization in the *ACTIN2* mutant *act2-5*, supporting the role of the actin cytoskeleton in modulating PIN recycling [38]. We hypothesize that the decreased accumulation of actin into bundles in root cells (Fig. 5) accounts for the disturbed PIN2 polarity in *fer-4* mutant plants. Our findings therefore suggest that FER is involved in regulating the dynamic distribution of PIN2, potentially by controlling F-actin stability. Further work to elucidate how FER exerts its effect on F-actin will be helpful to understand how FER functions in auxin-mediated root responses.

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## Author contributions

LT supervised the study; LT, HL, and QD designed the experiments; QD performed the experiments with assistance from ZZ; YL provided the analytic tools; QD analyzed the data; HL wrote the manuscript; and LT revised the manuscript. All of the authors approve this submission and declare that they have no competing interests.

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## Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Fig. S1.** Phenotypes of the *fer-4* mutant were recovered in *fer-4* complemented *pFER::FER-GFP* seedlings.

**Fig. S2.** The polar localization and accumulation of AUX1 and PIN1 in *fer-4* mutant plants.