FERONIA receptor-like kinase regulates RHO GTPase signaling of root hair development

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Plant RHO GTases (RAC/ROPs) mediate multiple extracellular signals ranging from hormone to stress and regulate diverse cellular processes important for polarized cell growth, differentiation, development, reproduction, and responses to the environment. They shuttle between the GDP-bound inactive state and the GTP-bound activated state and their activation is predominantly mediated by a family of guanine nucleotide exchange factors (GEFs) referred to as ROPGEFs. Using the Arabidopsis ROPGEF1 as bait, we identified members of a receptor-like kinase (RLK) family as potential upstream regulators for RAC/ROP signaling. NADPH oxidase-derived reactive oxygen species (ROS) are emerging as important regulators for growth and development and play a crucial role in mediating RAC/ROP-regulated root hair development, a polarized cell growth process. We therefore screened T-DNA insertion mutants in these RLKs for root hair defects and found that mutations in one of them, At3g51550 encoding the FERONIA (FER) receptor-like kinase, induced severe root hair defects. We show that the fer phenotypes correlated with reduced levels of active RAC/ROPs and NADPH oxidase-dependent, auxin-regulated ROS accumulation in roots and root hairs and that up-regulating RAC/ROP signaling in fer countered the mutant phenotypes. Taken together, these observations strongly support FER as an upstream regulator for the RAC/ROP-signaled pathway that controls ROS-mediated root hair development. Moreover, FER was pulled down by ROP2 GTPase in a guanine nucleotide-regulated manner implying a dynamic signaling complex involving FER, a ROPGEF, and a RAC/ROP.

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RHO GTases are a large family of related monomeric GTP-binding proteins that serve diverse signaling functions (1). Plant RAC/ROPs form a unique clade of RHO-like GTases and play important roles in regulating cell growth and polarity establishment, hormone- and stress-induced responses that underlie growth, development, reproduction, and interactions with the environment (2–4). Several RAC/ROP effectors have been identified (see Fig. S1A). These include a family of CRIB-domain proteins (RICs) and a coiled-coil domain protein (ICR1). RICs are known to mediate intracellular [Ca2+] and actin dynamics in polarized pollen tubes and coordinate actin and microtubule organization in leaf epidermal cells (2), whereas ICR1 regulates secretion and auxin distribution during early embryo development in Arabidopsis (5, 6). Interaction between RAC/ROPs and NADPH oxidase underlies activation of pathogen-elicited reactive oxygen species (ROS)-mediated defense response in rice (7). Upstream of RAC/ROPs, the hormones auxin and abscisic acid can activate and inactivate, respectively, these small GTases, which in turn signal hormone-regulated responses (8–10). Given their involvement in diverse biological processes, RAC/ROPs may act as integration points for crosstalk between multiple signaling pathways (2).

Besides being mediators of stress-induced responses, increasing evidence shows that ROS also serve important regulatory functions in growth and development (11–15). In plants, multiple transmembrane spanning NADPH oxidases, homologous to the catalytic gp91phox subunit of the mammalian enzyme, have been found to underlie several RAC/ROP-mediated, ROS-regulated growth and stress-induced responses. In studies involving the Arabidopsis ROOT HAIR DEFECTIVE2 (RHD2/BRHC) gene encoding a NADPH oxidase and SUPERCENTIPEDE, which encodes a RAC/ROP negative regulator, guanine nucleotide dissociation inhibitor (GDI) (see Fig. SL4), Dolan and colleagues provided compelling evidence for a RAC/ROP-mediated NADPH oxidase-dependent pathway for ROS-regulated root hair development (16, 17). Specifically, they showed that NADPH oxidase-derived ROS is required for polarized root hair growth and regulated RAC/ROP activity controls production and spatially regulated accumulation of ROS at the tip of emerging and elongating root hairs. Similarly, up-regulating RAC/ROP activity by overexpressing a constitutively active RAC/ROP in transgenic Arabidopsis seedlings also induced ectopic accumulation of NADPH oxidase-derived ROS and defective root hairs (18).

Although relying on guanine nucleotide exchange for activation is conserved for RAC/ROPs (Fig. SL4), their activation is largely dependent on a unique guanine exchange factor (GEF) family referred to as ROPGEFs (19–21). ROPGEFs have variable amino- and carboxyl-terminal domains and a conserved centrally located nucleotide exchange activity domain, which exclusively activates RAC/ROPs but not RHO GTPases from other organisms. Little is known about signaling components upstream of ROPGEFs. To date, two pollen-specific leucine-rich repeat receptor-like kinases (LRR RLKs) (22), the tomato LePRK2, and Arabidopsis AtPRK2a are the only surface receptors known to interact with ROPGEFs. These PRKs regulate RAC/ROP-mediated pollen tube growth, another polarized cell type in plant (23–25).

Of the hundreds of RLKs in Arabidopsis (26), several of the 17-membered Catharanthus roseus receptor-like kinase (RLK)-related family have been identified as regulators for various growth-related processes (27). Loss of function in one of these, THESEUS1 (THE1), suppressed the short hypocotyl phenotype in a cellulose synthase mutant, leading to the suggestion that it acts as a sensor for cell wall defects to signal growth inhibition (28). Although single mutations in THE1 and the related HERCULES1 (HERK1) did not induce noticeable phenotype, the1 herk1 double mutant showed inhibited growth (29). Reducing the level of another related RLK, FERONIA (FER), also resulted in growth inhibition and induced altered hormone responses (29, 30). FER was identified as a regulator for female fertility, mediating pollen tube rupture in the female gametophyte to discharge sperm for fertilization and preventing multiple tube entrance into an already fertilized ovule (31–33). The authors declare no conflict of interest.


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pollen-specific ANXUR1 and ANXUR2 are needed for pollen tube growth as double anx1 anx2 mutant pollen tubes rupture precociously and fail to reach the ovules for fertilization (34, 35). Together, these findings suggest that these RLKs play important roles in controlling cell growth and integrity, mediating consequences that could be as diagonally opposed as growth and nongrowth. Here, we show that FER is a ROPGEF-interacting RLK and regulates RAC/ROP-signal and ROS-mediated root hair development, revealing a surface regulator for a signaling pathway that regulates ROS-mediated root hair growth and also broadly impacts growth and development.

Results
FER RLK Is a Broadly Expressed ROPGEF-Interacting RLK. Using ROPGEF1 as bait in a yeast two-hybrid screen, we identified members of the Arabidopsis CrRLK family as potential interacting proteins for ROPGEFs (Fig. 1 A and Fig. S1 B and C). Focusing on one of these, At3g51550, we used bimolecular fluorescence complementation (BiFC) assays (36) in protoplasts to confirm that this RLK also interacted with ROPGEF1 in plant cells (Fig. 1 B). Given the importance of RAC/ROP signaling to polarized cell growth (15–18) and that root hairs are more accessible than pollen tubes for observation, we initially screened T-DNA insertion mutants in these RLKs for root hair defects (see SI Materials and Methods) and found that mutations in At3g51550, which turned out to be identical to FER when its sequence was elucidated (33), uniquely induced severe root hair defect (see below). Hence we adopt the FER nomenclature and called it FER related. We also included in our studies siréne (sn), whose mutation is allelic with fer, the other originally described mutations, and inducing similar female gametophytic defects as fer (31–33).

FER promoter (pFER):GUS analysis in transgenic Arabidopsis indicates that in addition to expression in the ovule (33), FER is broadly expressed (Fig. 1 C) as previously shown by transcript analyses (33, 37). pFER expressed FER–GFP was localized to the plasma membrane of diverse cell types, including emerging and growing root hairs (Fig. 1 D).

FER Is Essential for Root Hair Development. We identified two T-DNA–induced alleles, fer-4 and fer-5 (Fig. 2 A and Fig. S2 A). The originally described fer and sn are both in the vicinity of fer-4 (33) (Fig. 2 A). RT-PCR analysis showed that fer-4, with its T-DNA insertion in the extracellular domain-coding region, is a null mutant, whereas fer-5 harbors truncated transcripts (Fig. 2 B), consistent with its T-DNA insert being closed to the end of its kinase domain-coding region of the gene. fer-4 and sn showed

![Fig. 1.](image)

![Fig. 2.](image)
FER Functions in a RAC/ROP Signaling Pathway and Mediates Auxin-Regulated Root Hair Development. It is well established that auxin plays an important role in regulating root hair development (e.g., refs. 40, 41) and we showed earlier that RAC/ROPs mediate auxin-signalized gene derepression via 26S proteasome-mediated repressor proteolysis (9, 10). We therefore asked whether FER functions in a RAC/ROP signaling pathway and also acts as a regulator for auxin-regulated root hair development. We observed that when grown in the presence of auxin, differences between WT and fer root hairs were further amplified because root hair elongation in WT seedlings was stimulated by auxin, whereas fer-4, -5, and sm root hairs failed to respond and remained short (Fig. 3 A–C and Fig. S2D), pFER::FER-GFP complemented this phenotype, restoring auxin-stimulated root hair growth in fer seedlings (Fig. 3 A and B).

To establish that FER indeed acted upstream of RAC/ROPs, we examined whether increasing RAC/ROP signaling capacity by overexpressing GFP–ROP2 may counteract the root hair defects in fer-4 and fer-5. Indeed fer-5 root hairs were restored to normal, including ability to respond to auxin stimulation (Fig. 3 D and E and see also Fig. S3), consistent with reduced RAC/ROP signaling as underlying the fer-induced root hair defects under normal growth conditions and decreased sensitivity to auxin stimulation of root hair growth. However, a rescued fer-4 was not recovered. Possibly, the higher level of RAC/ROP overexpression required to counteract fer-4’s deficiency could have prevented transgenic plant regeneration. Alternatively, and more pertinent to FER’s multiple functionality as suggested by the multiple phenotypes induced by fer-4 and other null or knockdown mutations (29–33), it is possible that fer-5 is defective only in FER’s RAC/ROP-interacting capacity, whereas fer-4 lacks all of FER’s functions, including those potentially requiring an intact extracellular domain.

To substantiate the FER and RAC/ROP signaling linkage on a biochemical level, we used a functional pulldown assay that specifically targets activated RAC/ROPs (5, 8, 9). Indeed, the level of activated RAC/ROPs was reduced in fer-4, -5 seedlings relative to WT (Fig. 3F and Fig. S2E) consistent with FER being necessary for RAC/ROP activation thus working as an upstream regulator for these small GTPases. Moreover, ROP2 maltose-binding protein (MBP) efficiently pulled down pFER-expressed FER–GFP from seedling microsomal proteins or when FER–HA was overexpressed in mesophyll or root protoplasts (Fig. 3 G–I), suggesting complex formation via their mutual interaction with ROPGEFs, several of which are known to interact with FER (Fig. S1B).

It is known that most ROPGEFs preferentially bind to GDP-bound RAC/ROPs versus the GTP-bound activated form (19, 20), presumably upon nucleotide exchange stimulated by upstream signaling, the activated GTPases would be released to interact with downstream effectors. Therefore the presence of a signaling complex involving an upstream regulator, a ROPGEF and a RAC/ROP should exist but would likely be transient in vivo (see Fig. 3K). To substantiate that the observed pulldown of FER by RAC/ROPs (Fig. 3 G and H Left) was functionally relevant, we included either GDP or GTP in the pulldown reaction to determine whether these guanine nucleotides differentially affect the pulldown efficiency. GDP and GTP would shift RAC/ROPs to the inactive or activated state, respectively, mimicking the action of these guanine nucleotides under physiological conditions. Indeed, pulldown of FER–HA was substantially enhanced by GDP but reduced by GTP (Fig. 3 H Right and I), consistent with the observation that most ROPGEFs (e.g., ROPGEF4, Fig. 3F) are preferentially pulled down by GDP-bound RAC/ROPs but not as efficiently by the GTP-bound form (19, 20). Together with observations that ROPGEF’s interact with FER (Fig. 1 A and B and Fig. S1B) and with RAC/ROPs (19, 20; Fig. S1C), results from these pulldown assays are consistent with a dynamic signaling complex involving interactions between FER, a ROPGEF, and a RAC/ROP, mediating nucleotide exchange and activating the small GTPases (Fig. 3F).

FER Regulates RAC/ROP-Mediated NADPH Oxidase-Dependent ROS Accumulation in Root and Root Hairs. The root hair defects in fer mutants closely resemble those seen in rhd2 (Fig. S4). Together with the FER, RAC/ROP signaling linkage (Fig. 3) and the RAC/ROP linkage to NADPH oxidase-dependent ROS-mediated root hair development (15–18), it seemed highly plausible that FER acted upstream of the RAC/ROP-regulated pathway. We therefore examined whether fer mutants were compromised in RAC/ROP-regulated ROS accumulation.

Roots and root hairs are known to show auxin-regulated diphenylene iodonium (DPI)-sensitive NADPH oxidase-dependent ROS accumulation (e.g., refs. 13, 16, 17, 41, 42; Fig. 4 A and B; Fig. S5A). When treated with H2DCF-DA, a commonly used ROS-detection stain, fer root and root hairs showed that they accumulated significantly lower levels of ROS relative to WT (Fig. 4 C and D–G, 0 nM NAA) and they were also nonresponsive to auxin-stimulated ROS accumulation (Fig. 4D–G, NAA-treated samples). Like the root hair morphological defects, the reduced ROS phenotype in fer was complemented by pFER::FER-GFP (Fig. 4 H and I) and countered by overexpressing ROP2–GFP (Fig. 5A and B and see also Fig. S5B and Fig. S6A and B). The corollary experiment of overexpressing RAC/ROPs, ROPGEF1, or FER also resulted in increased ROS accumulation in transgenic seedling roots (Fig. S5 C and D and Fig. S6 C and D), further supporting that FER, ROPGEFs, and RAC/ROPs act in a common pathway that controls NADPH oxidase-dependent ROS production.

Discussion

In showing that FER regulates RAC/ROP-signaled NADPH oxidase-dependent ROS-mediated root hair growth, our results reveal a surface regulator for a well-established signaling pathway that is not only important for the growth of these polarized cells but also broadly implicated in growth, development, and stress management (11–15). That FER is broadly expressed (Fig. 1) and loss-of-function fer mutants are pleiotropic and severely growth suppressed (Figs. 2–4) suggest FER plays an important and general role in growth promotion throughout development. The presence of ROS in the growing regions of vegetative tissues

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Fig. 3. fer mutants show reduced auxin and RAC/ROP signaling capacity. (A) Four-day-old WT, mutant, and pFER:FER-GFP complemented mutant seedlings grown under standard (0 nM NAA) or auxin-supplemented conditions. Auxin-treated WT and fer-4, -5 mutants are shown magnified on the right. (Scale bar, 2 mm.) (B) Root hair length comparison between WT, fer-5, and complemented fer-5 under standard and auxin-supplemented growth conditions. Data bars represent average of the mean root hair lengths from triplicate samples ± SE. For each replicate, 300 root hairs from at least nine plants were measured. (C) Root hair length comparison between WT and fer-4 (Fig. S2D) shows data for smr. (D and E) Overexpression of GFP-ROP2 rescued fer-5-induced root hair morphological defects (D), restored root hair elongation (E, compare white and black bars), and auxin-stimulated root hair elongation (E, compare 0, 50, and 100 nM NAA data bars). See Fig. S3 for genotyping of these rescued plants. (Scale bar, 100 μm.) (F) Pulldown of active RAC/ROPs. ICR1–MBP was used for fer-4; PBD–GST (8, 9) was used for fer-5. Anti-NtRac1 antibody (9) was used for detection. The fer-4 blot was visualized by chemiluminescence, the fer-5 blot by alkaline phosphatase reaction. Quantity of data is shown in Fig. S2E. (G–J) ROP2–MBP pulldown of pGFP expressed FER–GFP from microsomal proteins from transformed seedlings (G), of FER–HA expressed in transiently transfected mesophyll (H) and root (I) protoplasts, and of GFP–HA expressed in mesophyll protoplasts (J). Lower panels show input bait proteins on Ponceau-stained blots. Anti-GFP (G) and anti-HA (H–J) were used for detection. *band in G was unrelated to the experiment. (K) Sketch showing guanine nucleotide-regulated FER–RAC/ROP interaction as suggested by results shown in G–J. Presumably, in vivo, signal activation-induced changes in the interacting ROPGEF and RAC/ROP would weaken the GEF–RAC/ROP interaction, recycling the ROPGEF, and releasing activated RAC/ROP to interact with effectors for downstream signaling. Data for WT, fer-5, fer-5 + FER–GFP and fer5 + GFP–ROP2 shown in B and E were collected in the same experiments thus the data for WT and fer-5 are used in both panels. Mutant root hair data are overestimations because only hairs with measurable lengths were included in the data set. Brackets and the numbers 1 and 2 in C denote statistical comparisons (1, significant; 2, insignificant) as described in Materials and Methods; these differences are representative for data shown in B and E. and that inhibiting NADPH oxidase-dependent ROS production correlates with impaired growth (e.g., refs. 14, 16, 42) are consistent with the suggestion that FER targets the NADPH oxidase-regulated pathway to promote growth in a broad range of cell types. The fact that ROS both promote and repress growth suggests the functional involvement of ROS in cell growth is complex. Using the diversely functioning RAC/ROPs as regulators for NADPH oxidase would be a versatile strategy that could maximize input regulation of ROS production and output modulation of ROS-mediated responses. Discovery of FER as an upstream regulator for ROS-regulated growth, potentially in a large number of cell types, opens the way to dissect how ROS-mediated signaling may be regulated to meet their roles under diverse conditions. On the other hand, given the multiple downstream pathways that RAC/ROPs are known to regulate (2–6), it is also possible that FER activates other RAC/ROP effectors to control additional target systems, in different or even the same cell types. The fact that FER is required for pollen tube rupture inside the female gametophyte (31–33) suggests cell death, albeit noncell autonomously, as the ultimate target. Therefore, a complete dissection of FER functions throughout growth and development will be needed to fully establish the potentially multiple signal-response pathways that the broadly expressed FER is likely to mediate.

Establishing the FER to RAC/ROP signaling linkage suggests members of the broader FER-like RLK family (27) may also act as surface regulators for signal relay to regulate these molecular switches. Thus far, four other Arabidopsis FER-like RLKs have been implicated in regulating growth, reproduction, and stress-related processes (28, 29, 34, 35). These seemingly diverse pathways may nonetheless share certain functional themes. For instance, with the well-established role of apoplastic ROS in controlling wall stiffness and extensibility (14, 43–45), it is conceivable that FER would mediate a ROS environment that weakens the pollen tube wall, whereas ANXI and ANX2 would do so to maintain its integrity. Given the role of NADPH oxidase-derived ROS in secondary cell wall synthesis and that RAC/ROPs have been implicated as regulating this process (46), it is conceivable for ROS to be involved in the THE1-mediated cell wall integrity-dependent growth regulatory process. Taken together, available information on these FER-related RLKs suggests that the functional roles for at least a subset of these receptors may be irreducible for or impact cell wall integrity and that ROS, with their ability to support cell expansion and induce wall stiffness, could be central to the processes that these RLKs mediate.

Defined signal-receptor linkages for RAC/ROP-regulated pathways remain to be established. The observation of an α-subunit of a heterotrimeric G protein acting upstream of RAC/ROP-
seedlings were treated with H2DCF-fluorescein diacetate for detection (54) (Fig. S6 B). These transgenic plants did not show readily noticeable phenotypes other than mild root hair depolarization (e.g., as seen in GEF1ox seedling shown here), reflecting increased Rac/Rop signaling; their root hair lengths were within the range seen in control seedlings. (Scale bar in C, 200 μm.) 1, significant; 2, insignificant differences.

candidate ligands for LePRK2 could modulate pollen functions differentially before and upon landing on the stigmatic surface and during its growth in the pistil. Elucidating the ligands for FER in root hair development will fill a critical connection to signals that stimulate RAC/ROP regulation of ROS-mediated growth. Multiple hormonal and nutrient factors are known to affect root hair development (e.g., refs. 40, 41, 51, 52). The observations that fer mutants were altered in brassinosteroid and ethylene responses (30) and deficient in auxin-stimulated ROS production and root hair elongation (Figs. 3 and 4) are consistent with the notion that FER-regulated RAC/ROP signaling and auxin signaling may somehow intersect. The identification of FER as a surface regulator for ROS-mediated root hair elongation in this study not only provides a critical discovery, filling in the gap to upstream signal mediation for a well-established RAC/ROP signaling pathway, it also provides a portal to discover surface regulatory mechanisms that link a broad range of signals to multiple cellular responses.

Materials and Methods
Plant growth and transformation, protoplast transfection, molecular and biochemical methods followed standard procedures (see SI Materials and Methods). Arabidopsis thaliana Col-0 was used as control for fer-4, -5 and C24 as control for srn, respectively. Chimeric genes are described in SI Materials and Methods.

Protein-Protein Interaction Analysis. ROPGEF1 was used as bait in yeast two-hybrid to screen a 3-d-old etiolated Arabidopsis seedling cDNA library (CD4-22 from the Arabidopsis Biological Resource Center). BIFC (36) was carried out in transfected root protoplasts using 5–10 μg of one (controls) or both of the split YFP fused with ROPGEF1 or fer.

Root Hair Analysis. Root hairs located between 1.5 and 3.5 mm from the primary root tip of 4-d-old seedlings were observed microscopically (see SI Materials and Methods). Image J was used to measure the length of root hairs observed in the same focal plane. ROS were visualized in the primary root and root hairs by H2DCF-fluorescein diacetate for detection (54) (Fig. S6 A and B). (C and D) Overexpression of CaMV35S-driven FER-HA (Fig. S6C). ROPGEF1 or NtRAC1CA (9) augments ROS production in roots and root hairs. Insets C show DIC images. Quantified comparative ROS levels are shown in D. These transgenic plants did not show readily noticeable phenotypes other than mild root hair depolarization (e.g., as seen in GEF1ox seedling shown here), reflecting increased Rac/Rop signaling; their root hair lengths were within the range seen in control seedlings. (Scale bar in C, 200 μm.) 1, significant; 2, insignificant differences.

mediated pathogen-induced responses in rice (47) implies that members of the seven transmembrane-spanning receptor family could serve as RAC/ROP surface regulators and pathogen-derived elicitor ligands for these receptors. Two cysteine-rich proteins, one pollen and the other stigma produced, interact with LePRK2 (48, 49) and a 3-kDa stylar-secreted peptide specifically dephosphorylates LePRK2 and stimulates pollen tube growth (25, 50). These
Supporting Information

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SI Materials and Methods

Plant Growth and Plant and Protoplast Transformation. Arabidopsis seeds were surface sterilized and germinated on B5 medium supplemented with 1% sucrose and solidified by 0.7% agar. Seeds were cold treated at 4 °C for 2 d for synchronization before transferring to 22 °C for germination. When supplemented with auxin, 1-naphthaleneacetic acid (NAA) was added to concentrations indicated in the figures. For growth to maturity, 10-d-old seedlings were transferred to soil and maintained in a growth chamber at 22 °C under 16/8 h light/dark cycles. Arabidopsis was transformed by floral dip (1) and transgenic plants were selected on kanamycin-containing (50 µg/mL) medium. Mesophyll and root protoplasts were isolated from 3-wk-old seedlings or root tissues of 2- to 3-wk-old plants grown vertically in tissue culture, respectively. Protoplast transformation followed previously described procedures (2).

Molecular, Biochemical, and Histochemical Analysis. All recombinant DNA procedures were performed according to standard and PCR-based methodology. Table S1 shows a lists of primers used for cloning of cDNAs and genomic fragments and PCR amplification. Basic cloning strategies were used; sequences compatible with restriction enzyme recognition sites were included at the end of primers shown in Table S1, indicating cloning strategies. FER promoter (pFER):FER-FGFP containing 2,007 bp upstream of the FER coding region was cloned in Agrobacterium Ti plasmid intermediate vector pAC1532 (3), introduced into Agrobacterium GV2283 (4) and used to transform wild type (WT), fer-4, and fer-5. CaMV35S–FER–GFP, GFP–ROP2 (5), and NtRac1(3C) (2) were similarly transformed into WT and fer plants as indicated in the text. pFER–GUS was derived from pBI121 (Clontech) and transformed into WT Arabidopsis. Plasmids used in protoplast transient transfection assays, 35S–FER–HA and 35S–ROPGEF4–HA were constructed in Bluescript pSK (Strategene) using full-length FER and ROPGEF1 cDNAs. ICR1–MBP and ROP2–MBP were derived from pMALC (NEB) for Escherichia coli expression. Genomic DNA was used for PCR analysis of T-DNA insertions. RNA from 10-d-old seedlings isolated by the PrepEase RNA isolation kit (USB/Affymetrix) was used in RT-PCR analysis for gene expression. The Clontech MATCHMAKER 2 protocol was used for yeast two-hybrid assays (6). ROPGEF1 cDNA was cloned into the BD vector described in ref. 7 except that the gene for ampicillin resistance was replaced by NPTII to facilitate subsequent recovery of kanamycin-resistant plasmids in E. coli. The kinase domain of FER was tested both as fusion with BD and AD with GEF1–AD and GEF1–BD, accordingly. GUS staining of transgenic seedlings followed standard procedure (8) in 0.2 mg/mL X-gluc at 37 °C for 16 h.

Microscopy. Fluorescence and DIC microscopy were carried out on a NIKON Eclipse E800 microscope equipped with a SPOT camera (Molecular Diagnostic). In comparative studies, image acquisition conditions are detailed in the figure legends. An Olympus SZ61 dissection microscope with a Q imaging camera was used to acquire seedling, inflorescence, and trichome images.

Pulldown Assays. For ICR1 and PBD pulldown of activated RAC/ROPs, ICR1–MBP, and PBD–GST (2) was expressed in E. coli BL21 by IPTG (0.5 mM) induction. Cells were resuspended in binding buffer [40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA] and sonicated (six 10-s pulses at 1-min intervals) for protein extraction. After removal of cell debris by centrifugation at 4 °C, proteins were applied to amylose resin (NEB) or GST resin in the same buffer and the ICR1–MBP or PBD–GST-bound resin was used to pull down activated RAC/ROPs. For target proteins, the roots of 7-d-old WT and fer-5 seedlings were homogenized in liquid N2 and 100 mg of frozen powder was extracted in pulldown buffer [binding buffer, plus 5% glycerol, 1 mM PMSF, protease inhibitor mixture (Calbiochem)] supplemented with 0.75% Triton X-100. Proteins were extracted at 4 °C with mixing for 15 min. The debris was removed by centrifugation at top speed in a microcentrifuge for 10 min at 4 °C. A total of 90% of the supernatant was applied to the ICR1–MBP-bound or PBD–GST-bound resin for pulldown assays. The remaining 10% of the supernatant was saved for protein quantification. Pulldown was carried out by binding extracted proteins to the amylose resin at 4 °C for 2 h. The resin was washed five times in binding buffer. Proteins remained bound to the resin were eluted in SDS/PAGE loading buffer, boiled for 5 min, and applied to 15% SDS/PAGE for protein blot analysis. Protein blots were stained by Porceau Red to ensure comparable loading of samples. RAC/ROP detection was carried out by binding with anti-NtRac1 antibodies (2), followed by horseradish peroxidase-conjugated secondary antibodies and chemiluminescence detection (for ICR–MBP pulldown) or alkaline phosphatase-conjugated secondary antibodies and colorimetric detection (for PBP–GST pulldown). Data from immunoblot were quantified by Adobe Photoshop. Mean histogram values were quantified for ROIs of identical area from each band after background subtraction from blank region of the blots.

For ROP2 pulldown of FER and GEF4–HA, ROP2–MBP-bound amylose resin was generated as described above. For protoplast-expressed FER and GEF4, mesophyll or root protoplasts were transfected by 5–10 µg CaMV35S::FER-HA or CaMV35S::GEF4–HA. After overnight culture, protoplasts were collected and sonicated in pulldown buffer. After sonication, Triton X-100 was added to the extract to a final concentration of 0.4%, followed by 15 min of shaking at 4 °C. Pulldown reactions and subsequent analyses were as described above. Five-day-old pFER–FER–GFP seedlings were also used for ROP2–MBP pulldown analysis. Seedlings were ground in liquid nitrogen and resuspended in extraction buffer [40 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% glycerol, 1 mM Na2–EDTA, PMSF, and protease inhibitor mixture (Calbiochem)]. Microsomes were extracted at 4 °C with mixing for 30 min. Cell debris was removed by centrifugation at 10,000 × g at 4 °C and the resulting supernatant was centrifuged at 100,000 × g for 3 h to pellet the microsomal fraction. The pellet was resuspended in extraction buffer supplemented with 0.3% Triton X-100 and then centrifuged again at 2,000 g to remove insoluble material. The microsomal extract was then used for pulldown analysis. HA antibody (Santa Cruz) was used for detection.

Mutant Screening. One to three T-DNA insertion mutants for several vegetative tissue-expressed FER-related genes, At2g23200 (HERK4), At5g24010 (HERK5), At5g54380 (THE1), At3g46290 (HERK1), At5g61350, At3g51550 (FER), At1g30570 (HERK2), At2g39360 (HERK3), At5g39000, At5g38990, At5g39030, and At5g39020 were obtained from the Salk collection and screened for root hair defects in 3- to 4-d-old seedlings. Only insertions in At3g51550 (FER) induced readily noticeable root hair defects and were studied in detail. The bulk of the screening was carried out in 2007 and should be considered preliminary.

Statistics. Fig. 3B shows the difference between WT and fer-5 was significant, P < 0.05, 10−2, 10−4 at 0, 50, and 100 nM NAA.
Difference between WT and complemented fer-5 was insignificant under all conditions \( (P > 0.1) \). Difference between untreated and NAA treated fer-5 was insignificant \( (P > 0.05) \).

Fig. 3C shows the difference between WT and fer-4 was significant, \( P < 10^{-4} \) at 0 and 100 nM NAA. Difference between untreated and NAA-treated WT was significant \( (P < 10^{-4}) \). Difference between untreated and NAA-treated fer-4 was insignificant \( (P > 0.5) \).

Fig. 3E shows the difference between WT and fer-5 was significant, \( P < 0.05 \), \( 10^{-4} \), \( 10^{-2} \) at 0, 50, 100 nM NAA. Difference between WT and ROP2-rescued fer-5 was insignificant under all conditions \( (P > 0.1) \). Difference between untreated and NAA-treated fer-5 was insignificant \( (P > 0.05) \). Difference between untreated and NAA-treated GFP–ROP2-rescued fer-5 was significant \( (P < 10^{-4}) \).


Fig. 4E and G shows the difference between WT and fer-4, -5 and sm was significant \( (P < 10^{-4}) \) under both conditions. Difference between untreated and NAA-treated WT was significant \( (P < 10^{-4}) \). Difference between untreated and NAA-treated fer-4, sm was insignificant \( (P > 0.05) \), but significant for fer-5 \( (P < 0.05) \), reflecting a weaker phenotype than the two null mutants.

Fig. 4F shows the difference between WT and mutants was significant \( (P < 10^{-4}) \), between WT and complemented mutants was insignificant \( (P > 0.1) \).

Fig. 5B shows the difference was significant \( (P < 10^{-4}) \) between WT and fer-5, insignificant \( (P > 0.1) \) between WT and GFP–ROP2-rescued fer-5, and significant \( (P < 10^{-2}) \) between fer-5 and GFP–ROP2-rescued fer-5. Note signal in GFP–ROP2-rescued fer-5 without incubation with the ROS substrate H2DCFDA (fourth data bar) was negligible.
Fig. S1. (A) Schematic summary of the RAC/ROP signaling pathway showing the key elements involved in this study. ROPGEFs stimulate GDP/GTP exchange to activate RAC/ROPs. GDI and GAP (GTPase activating protein) are negative regulators, one inhibiting GDP dissociation and the other accelerating GTP hydrolysis, respectively (10). Activated RAC/ROPs interact directly with downstream effectors RICs and ICR1 to mediate diverse cellular pathways (9–11). RAC/ROP-mediated NADPH-oxidase-dependent ROS production is well established in mediating root hair growth and several biotic and abiotic stress-induced responses (12–16). In rice, NADPH oxidase has also been shown to be an immediate effector for OsRac1 to mediate pathogen-elicited defense responses (16). A large number of surface regulators (designated as ? in the figure) may be involved in mediating diverse signals to RAC/ROP-regulated pathways, but only two are known (17, 18). Hormones, such as auxin (2) and ABA (19) and pathogen elicitors (16) have been shown to regulate RAC/ROP signaling. Using a ROPGEF as a molecular link, we report here identification of a surface regulator, FER, for the RAC/ROP-regulated pathway that stimulates NADPH oxidase-dependent ROS production to mediate polarized root hair growth (20, 21). (B) Yeast two-hybrid assays showing FER kinase domain [FER(K)] interact with multiple Arabidopsis ROPGEFs. The originally isolated ROPGEF1-interacting fragments spanned half the RLKs’ kinase domain till the end of their coding region. (C) Yeast two-hybrid assays showing ROPGEF1 interact with NtRAC1 (2), similar to previously shown for ROPGEFs and RAC/ROPs from Arabidopsis (22, 23).
Additional characterization of fer and complemented fer mutants. (A) Genomic DNA PCR analysis for fer-4 and fer-5. T-DNA insert was present in the fer mutants but not in WT (T-DNA) and genomic FER DNA fragments were present in WT but not in fer mutants (FER(Ex) and FER(K)). For fer-4, primers 1 and GABI(R) were used for the T-DNA insert and primers 1 and 2 were used for the WT FER DNA fragments; for fer-5, primers 3 and LB1 and primers 3 and 4 were used, respectively. See Fig. 2A and SI Materials and Methods for primer information. (B) FER–GFP expression in pFER::FER-GFP transformed fer-4 and fer-5 mutants correlated with complemented root hair phenotype. (Scale bar, 100 μm.) (C) Quantitative data for fer-4–induced trichome defects. Data show the mean % of each category of trichomes averaged from four comparably aged true leaves from 3-wk-old seedlings (a total of ~350 trichomes were counted per sample). Differences between WT and fer-4 are significant, Student’s t-tests showed P < 10^-7 for trichomes with three or fewer branches, P = 1.15 × 10^-3 for those with more than four branches, and P = 4.7 × 10^-3 for collapsed trichomes. (D) Root hair length comparison between WT and srn. Root hair lengths shown for srn were overestimations because only hairs with measurable lengths but not the most severe ones that never elongated were included in the data set. (E) Relative levels of ROP–MBP pulled down fer-4 and ROP–GST pulled down fer-5. Data were averaged from quantification of data obtained from three independent experiments. 1, significant difference (P < 0.05); 2, insignificant difference (P > 0.05).

Analysis of CaMV35S::GFP-ROP2 transformed fer-5. (A) Genomic PCR analysis confirms presence of the original T-DNA insert (fer-5 T-DNA) using primers LB1 and 3 (see Fig. 2A), absence of the WT FER (FER(K)) using primers 3 and 4, confirming homozygosity of the T-DNA insertion locus, and presence of GFP DNA in the CaMV35S::GFP-ROP2 transformed fer-5. (B) Root hairs in fer-5 were restored to normal in GFP–ROP2-expressing CaMV35S::GFP-ROP2 transformed fer-5. (Scale bar, 200 μm.)
Fig. S4. Comparison of root hair phenotypes in fer-4 and rhd2. (A) Root segments from 4-d-old fer-4 and rhd2 mutant seedlings. Arrows point to collapsed root hairs. (Scale bar, 100 μm.) (B) Quantitative comparison of root hair defects. Data show that collapsed root hairs predominated in fer-4, whereas rhd2 root hair defects were more broadly distributed among different severity classes (see Fig. 2D for definition), indicating fer-4 induced more severely defective root hairs. Each data bar represents the mean ± SD where n = 600 root hairs sampled from 12 four-day-old seedlings.

Fig. S5. GFP signal from pFER::FER-GFP and CaMV35S::GFP-ROP2–transformed fer seedlings was negligible under H$_2$DCF–DA-detected ROS imaging condition. (A) pFER::FER-GFP transformed fer-4 seedlings observed with (+) or without (−) H$_2$DCF–DA treatment. The + H$_2$DCF–DA image was acquired by autoexposure (373 ms); when exposed under the same condition, GFP signal from the − H$_2$DCF–DA seedling was not detectable. Autoexposure of the same − H$_2$DCF–DA seedling required a 10-s exposure, suggesting FER–GFP signal could not interfere with ROS detection. (B) CaMV35S::GFP-ROP2 transformed fer-5 seedlings observed with (+) or without (−) H$_2$DCF–DA treatment. (Left) The WT seedling was treated with H$_2$DCF–DA for ROS analysis and imaged by autoexposure (Upper; exposure time was 0.687 s). Lower panel shows a DIC image of the WT seedling. (Right) The fer-5 + GFP–ROP2 seedling was not treated for ROS detection and imaged using 0.687 s exposure time (Upper) to detect contribution to the fluorescence signal by GFP–ROP2, which was negligible. Lower panel shows an autoexposed image of the same fer-5 + GFP–ROP2 seedling root to reveal the GFP signal. The long autoexposure time (9.27 s) signified a very low fluorescence level from GFP–ROP2 under ROS detection condition.
Fig. S6.  (A and B) Dihydroethidium (DHE) detection of ROS shows strong signals in WT (Left) and CaMV35S::GFP-ROP2–rescued fer-5 (Right), whose root hairs showed normal growth, but not in fer-5 mutant, whose root hairs showed aborted growth (Middle). Lower panels show higher magnification images acquired using the WT imaging condition for quantification of signals at root hair tips in boxed ROIs of identical areas. (B) Average ROS level (n = 43 root hairs per sample) quantified from ROI of identical areas (boxes in A). Difference was significant (P < 0.05) between WT and fer-5, between fer-5 and GFP–ROP2-rescued fer-5, and insignificant (P > 0.05) between WT and GFP–ROPs-rescued fer-5. (C) Protein blot analysis for CaMV35S::FER-HA overexpression lines. FERox1 was representative of lines with detectable FER–HA and showed enhanced root ROS accumulation relative to WT (see Fig.5 C and D). FERox2 was representative of the majority of similarly transformed lines; they did not accumulate detectable levels of FER–HA and their ROS levels were not distinguishable from controls. We could not recover transgenic lines expressing levels of FER–HA higher than that observed in FERox1. (D) RT-PCR for FER mRNA levels in WT and FERox1 seedlings (7 d). Primers 1 and 2 (see Fig. 2A) were used for FER DNA amplification.
<table>
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<th>cDNA/genomic DNA</th>
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Primers used for cloning purposes

| Primer 1*       | CGGATCCATGAAGATCACAGAGGGACGATTC |
| Primer 2*       | CGCAGATCTAGCAGAAGAAGACAAACACACAAACCC |
| Primer 3*       | CGGATCCATGGCCTTACCGGAGACGTAAGCGTGG |
| Primer 4*       | CGAATTCAGTCCCTTGGATTGACATCACTCGTG |
| LB1*            | CGGTGGAACGCTTGCTGCAACT |
| GAB1 (R)*       | CGGATCCATGATGCAAGGCGCAACGGGTTC |
| GFP             | CGTACAAACGGCTATTTGCTGG |
| Actin2          | CGTACAAACGGCTATTTGCTGG |

*Restriction enzyme.
†See Fig. 2 for primer designation.