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Functional analysis of related CrRLK1L receptor-like kinases in pollen tube reception

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Abstract

The *Catharanthus roseus* Receptor-Like Kinase 1-like (CrRLK1L) family of 17 receptor-like kinases (RLKs) has been implicated in a variety of signaling pathways in *Arabidopsis*, ranging from pollen tube (PT) reception and tip growth to hormonal responses. The extracellular domains of these RLKs have malectin-like domains predicted to bind carbohydrate moieties. Domain swap analysis showed that the extracellular domains of the three members analyzed (FER, ANX1, HERK1) are not interchangeable, suggesting distinct upstream components, such as ligands and/or co-factors. In contrast, their intercellular domains are functionally equivalent for PT reception, indicating that they have common downstream targets in their signaling pathways. The kinase domain is necessary for FER function, but kinase activity itself is not, indicating that other kinases may be involved in signal transduction during PT reception.

Keywords *Arabidopsis*; *FERONIA*; pollen tube reception; receptor-like kinases; signaling

Subject Categories Plant Biology; Signal Transduction

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Introduction

To accomplish double fertilization in flowering plants (angiosperms), the pollen (male gametophyte) hydrates on the stigma and germinates to form a pollen tube (PT), which transports two nonmotile sperm through the stigma, the style, and the transmitting tract toward the ovule that harbors the embryo sac (female gametophyte). The PT's growth direction is influenced by long- and short-range attractants, ultimately guiding the PT to the female gametophyte, which consists of two female gametes—egg and central cell—and five accessory cells, including two synergids at the micropylar pole (reviewed in [1,2]). The synergids mediate the last step of PT guidance toward the site of PT reception by secreting *LUREs*, small cysteine-rich peptides [3,4]. PT reception occurs at the filiform apparatus, a membrane-rich region at the micropylar end of the synergids, the first point of contact between male and female gametophyte. The PT interacts with the synergid, ruptures, and releases the sperm to effect double fertilization, initiating seed development.

The first evidence that PT reception requires an active signaling process came from the identification of the *Arabidopsis feronia* (*fer*) mutant, whose embryo sacs develop normally but remain unfertilized because the PT continues to grow inside the female gametophyte and does not rupture to release the sperm (Supplementary Fig S1; [5,6]). *FER* encodes a receptor-like kinase (RLK) of the CrRLK1L subfamily [7], which consists of 17 members (Fig 1, reviewed in [8]). FER is expressed throughout the plant except for mature pollen and localizes to the membrane-rich filiform apparatus of the synergids [7].

CrRLK1L proteins have a predicted intracellular S/T kinase domain (ICD) with relatively high conservation, a transmembrane (TM) domain, and a variable extracellular domain (ECD). Boisson-Dernier and colleagues [9] discovered two malectin-like domains in the ECDs of some CrRLK1L proteins. More recent domain searches, however, predict a malectin-like domain in all CrRLK1L members (pfam.sanger.ac.uk). This malectin-like domain has limited homology to the ER-localized, carbohydrate-binding malectin protein of *Xenopus laevis* (reviewed in [9]), suggesting that CrRLK1L ligands may be glycosylated.

The functions of only six of the 17 CrRLK1L family members have been identified: FER, ANXUR1 (ANX1), ANX2, THESEUS1 (THE1), HERCULES1 (HERK1), and HERK2 (reviewed in [8,9]). The two closest homologues of *FER*, *ANX1* and *ANX2*, are only expressed in pollen, where they localize to the plasma membrane (PM) of the growing PT tip. Whereas single *anx1* and *anx2* mutants show no phenotype, *anx1*;*anx2* double mutant PTs burst immediately after germination [10,11]. ANX1/2 modulate the level of NADPH-oxidase-dependent reactive oxygen species (ROS) and the tip-focused Ca^{2+} gradient to sustain secretion of membrane and cell wall material to the PT tip [12]. ANX1/2 function in tip-growing PTs seems to have been adopted by FER in polarly growing root hairs where FER acts upstream of several guanine exchange factors (ROPGEFs), activating Rho-like GTPases (RAC/ROPs) and leading to ROS-mediated root hair development [13–15]. Additionally, FER-RopGEF-RAC/ROP modules seem to negatively regulate abscisic acid responses and positively regulate auxin-promoted root hair initiation and growth [15].

CrRLK1L proteins have also been implicated in cell elongation during vegetative growth. Expression of *FER* and two other CrRLK1L

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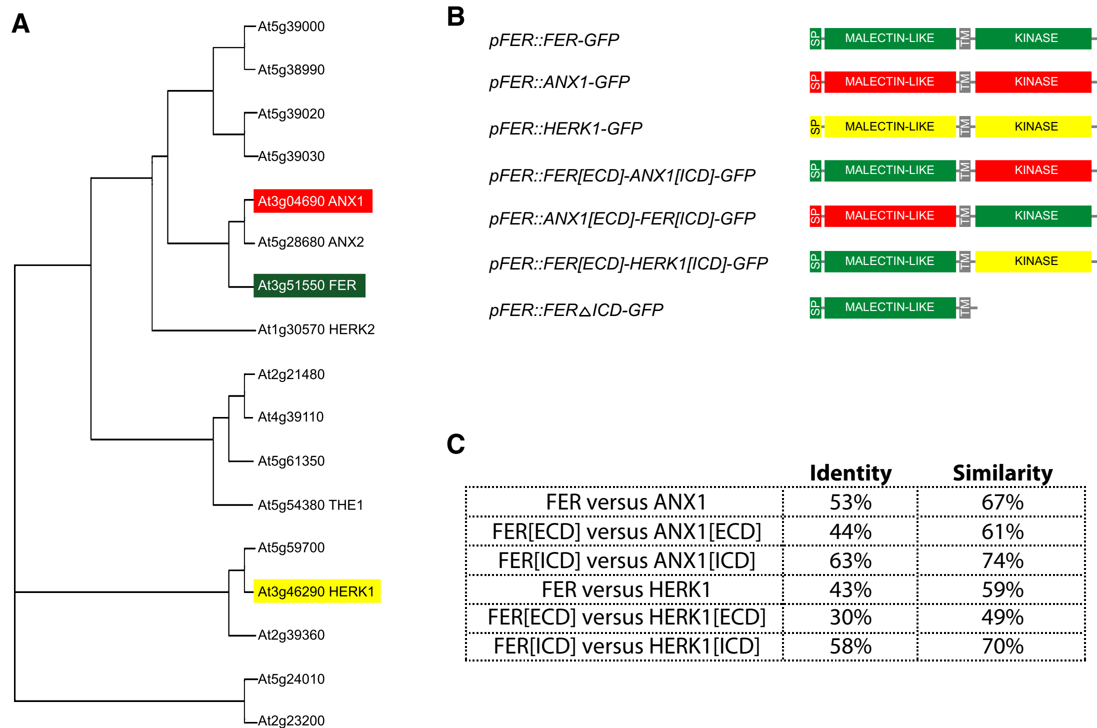

Figure 1. FER is a member of the CrRLK1L family of receptor-like kinases.

A Phylogenetic tree showing the relationships between CrRLK1L proteins (the members highlighted in color were investigated by domain swap experiments).
B Chimeric and truncated proteins assessed in this study. SP refers to the predicted signal peptides.
C Comparisons of amino acid similarity and identity between FER, ANX1, and HERK1 (determined by pairwise blastp alignments at http://blast.ncbi.nlm.nih.gov).

members, *HERK1* and *THE1*, are upregulated in vegetative tissue after brassinosteroid treatment [16]. All three genes are strongly expressed in elongating cells during vegetative growth, where they localize to the plasma membrane (PM). Both *fer* and *the1;herk1* double mutant plants show a vegetative dwarf phenotype, indicating that *FER*, *HERK1*, and *THE1* might act in the same pathway to regulate cell elongation.

In this study, we used the *fer* PT reception phenotype to investigate the functional similarities and differences between three CrRLK1L members (FER, ANX1, and HERK1) in a domain swap analysis. FER proteins with altered kinase domains were used to define the role of kinase activity for FER function. All constructs were expressed under the *pFER* promoter in *fer-1/FER* mutant plants, which normally show ~50% unfertilized ovules due to defects in PT reception [5–7]. Complementation analysis revealed common downstream signaling events but distinct ligand activation. In addition, we show that FER kinase activity is not necessary for FER function in PT reception, indicating that additional kinases may play a role in the FER signal transduction cascade.

Results

Domain swap analysis of FER and related proteins

Like most Receptor-like kinases (RLKs), the main determinant of specificity in the CrRLK1L family is predicted to be receptor–ligand interactions determined by specific amino acids in the ECD. This is supported by the fact that the CrRLK1L family members have highly divergent ECDs (Fig 1). The ICDs of CrRLK1L proteins have highly conserved S/T kinase domains, but divergent C-terminal tails that could play a role in downstream specificity. A third determinant of specificity could be the distinct expression patterns of the various family members in combination with other upstream and downstream regulators. In order to determine the factors controlling CrRLK1L specificity, we tested whether closely related (ANX1) and more distantly related (HERK1) proteins (Fig 1 and Supplementary Fig S2), and/or replacements of the ECD and ICD of FER with the respective domains of ANX1 and HERK1, are able to complement the PT reception phenotype in *fer-1/FER* mutants, when expressed under control of the *pFER* promoter.

We previously showed that a 1.2-kb *pFER* promoter fragment drives the expression of a FER-GFP fusion protein that localizes to the region of the filiform apparatus in synergids and complements the *fer* PT reception phenotype (Fig 2A; [7]). Primary transformants are hemizygous for *pFER::FER-GFP* such that only half of the *fer* embryo sacs carry the complementing construct (Fig 3). We used the same promoter fragment to drive expression of full-length ANX1-GFP and HERK1-GFP fusion proteins in the *fer-1/FER* background. The fusion proteins were detected in the filiform apparatus (Fig 2B, C), and in the PM of transiently transformed onion epidermal cells (Supplementary Fig S3). All primary transformants were either *FER/FER* or *fer-1/FER* and did not show significantly different

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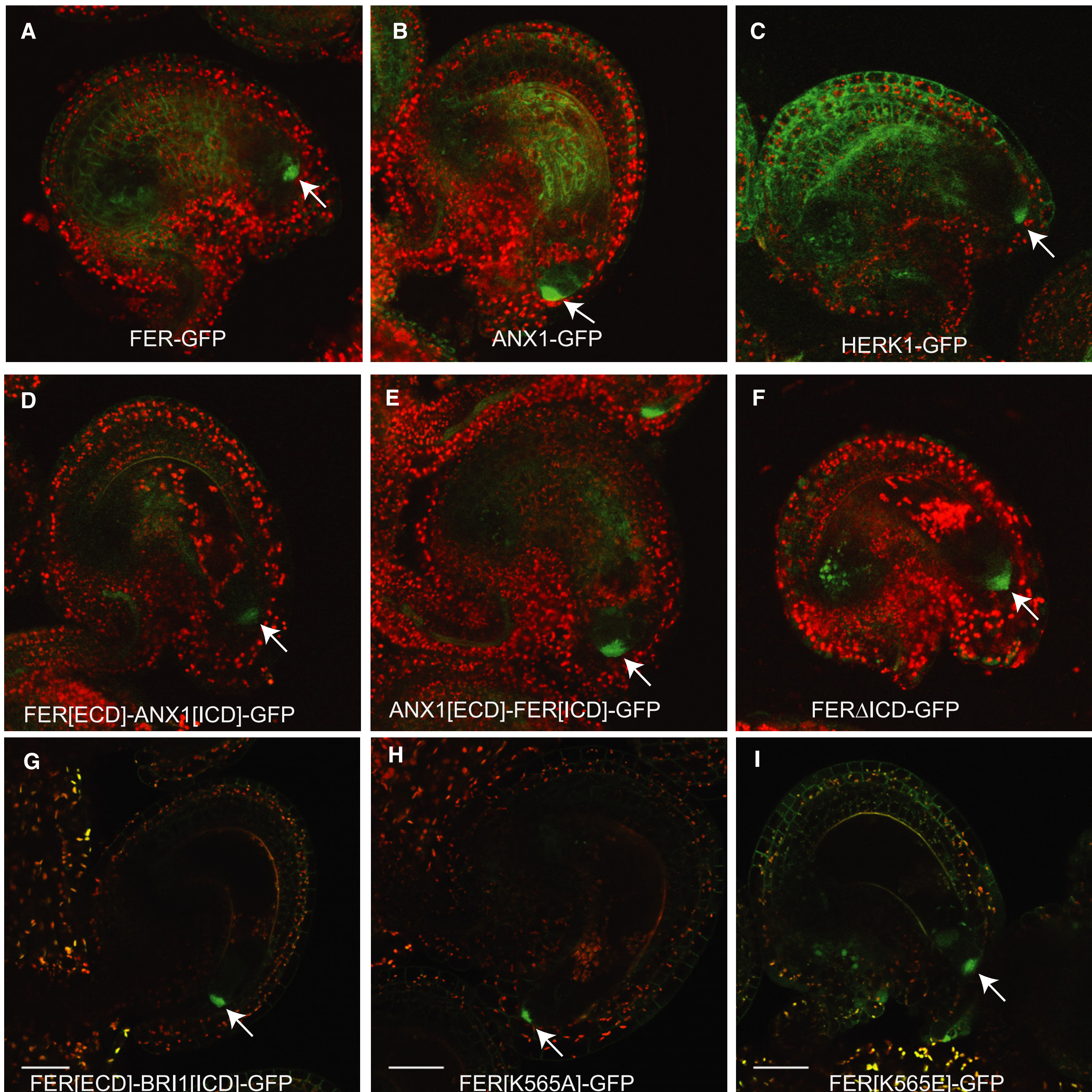

Figure 2. Representative confocal images illustrating that ANX1, HERK1, and domain swap constructs exhibit a FER-like subcellular localization in the filiform apparatus of synergids.

A FER-GFP is localized to the filiform apparatus of synergids (arrow) and can also be detected at the periphery of sporophytic cells of the ovule.

B-I All of the GFP fusion constructs show filiform apparatus localization (arrow). While synergid expression and localization to the filiform apparatus was consistent in all lines examined, sporophytic expression in the ovules varied depending on the transgene insertion site. Scale bars are 30 micrometers.

Data information: For all overlayed images, GFP fusion protein signal is shown in green and chlorophyll autofluorescence is shown in red.

numbers of unfertilized ovules compared to untransformed control plants (Fig 3 and Supplementary Table S1). These results indicate that full-length ANX1-GFP and HERK1-GFP constructs cannot complement the *fer-1* phenotype when expressed in synergids and that no dominant-negative effects on PT reception are conferred by expressing ANX1-GFP and HERK1-GFP.

The failure of ANX1 and HERK1 to complement the *fer* phenotype could be due to a lack of ligand recognition, an inability to perform downstream functions, or a combination of both. In order to distinguish between these possibilities, domain swaps between ANX1 and FER were constructed and driven under the *pFER* promoter as GFP fusion proteins (Fig 1B). Primary transformants

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Figure 3. Complementation of the *fer-1* mutation with related CrRLK1L proteins and domains.

Box plots from complementation assays showing the percentage of unfertilized ovules for control plants and T1 transformants for each of the constructs. **** indicates lines with % unfertilized ovules significantly different from untransformed controls of the same genotype (P-values < 0.0001 in t-tests). At least 3 independent T1 plants ($>$ 300 ovules counted for each) were analyzed for complementing and at least 8 T1 plants for non-complementing lines. See Supplementary Table S1 for raw seed count data.

expressing the ANX1 ECD fused to the FER ICD showed expression of the fusion protein in the filiform apparatus of synergids in multiple transformants (Fig 2E), and in the PM of onion epidermal cells (Supplementary Fig S3). However, only *FER/FER* and *fer-1/FER* plants were recovered. The percentages of unfertilized ovules in the primary transformants were not significantly different from controls (Fig 3), indicating that the ANX1[ECD]-FER[ICD]-GFP protein is not able to complement *fer-1* and that no dominant-negative effect was conferred by the ANX1 ECD. Thus, the FER ECD is indispensible for PT reception in synergids.

We also exchanged the FER ICD with the ICDs of ANX1 or HERK1 and expressed GFP fusion proteins under control of the *pFER* promoter (Fig 1B). Both constructs were able to complement the *fer-1* phenotype (FER[ECD]-ANX1[ICD]-GFP localization is shown in Fig 2D). The percentage of unfertilized ovules was reduced by 50% in *fer-1/FER* and *fer-1/fer-1* primary transformants that were hemizygous for the complementation constructs (Fig 3). These results indicate that either the ICDs of ANX1 or HERK1 proteins are interchangeable with the FER ICD, that the ICD is dispensable for *FER* function, or that any kinase domain is sufficient. In order to distinguish between these possibilities, we deleted the ICD of FER in a GFP fusion construct driven by the *pFER* promoter. The FERΔICD deletion construct was expressed in synergids and localized to the filiform apparatus (Fig 2F) but did not complement *fer-1* (Fig 3), indicating that the ICD is necessary for *FER* function in PT reception. We also exchanged the FER ICD with an ICD from an unrelated RLK, BRASSINOSTEROID INSENSITIVE1 (BRI1). BRI1 is a member of the leucine-rich repeat (LRR) subfamily and is the receptor

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for brassinosteroids [17,18]. The *pFER::FER[ECD]-BRI1[ICD]-GFP* construct localized to the filiform apparatus but was unable to complement *fer-1* (Figs 2G and 3), indicating that a CrRLK1L ICD is necessary for transmitting the signal perceived by the FER ECD in synergids.

Functional analysis of the FER intracellular domain

The ICDs of CrRLK1 proteins contain a typical S/T kinase motif with a K at the active site (Fig 4A and Supplementary Fig S4). In an *in vitro* kinase assay, the kinase domain of FER was capable of autophosphorylation and a point mutation changing K at the active site to R abolished *in vitro* kinase activity [7]. In order to determine whether kinase activity is necessary for *FER* function in PT reception, a *pFER::FER[K-R]-GFP* construct, carrying the K-R change in the active site (K565), was transformed into *fer-1/FER* plants to check for complementation. Surprisingly, this dead kinase version of FER was able to complement the *fer-1* phenotype (Fig 4B and Supplementary Table S2). Two additional non-conserved substitutions, K-A and K-E, were properly localized to the filiform apparatus (Fig 2H and I) and also complemented *fer-1* (Fig 4B), indicating that kinase activity is not necessary for *FER* function in PT reception.

Structures based on X-ray crystallography show that kinases contain an activation segment C-terminal to the catalytic domain [19]. In many kinases, phosphorylation of residues in the activation loop of this segment determines the conformation of the loop, which modulates kinase function. While most structure-function studies have been done with animal kinases, site-directed mutagenesis experiments have shown that activation loop phosphorylation is important for the function of some plant RLKs [20–22]. Each of the CrRLK1L proteins has a predicted activation loop with S and T residues that are targets for phosphorylation (Fig 4A). S695 and T696 of FER were shown to be phosphorylated in a membrane phosphoproteomics study on *Arabidopsis* seedlings, and the corresponding S residues were also found to be phosphorylated in HERK1 [23]. In seedling and mature pollen phosphoproteomic studies [23,24], an activation loop peptide with a phosphorylated S (S701 in FER) was identified; however, this peptide is highly conserved in CrRLK1 family members and could not be assigned to a specific protein. FER function could be modulated through phosphorylation changes at these residues. In order to investigate this possibility, site-directed mutagenesis was used to convert these residues (S695, T696, and S701) to A and D in order to mimic constitutively dephosphorylated and phosphorylated states, respectively. Single and double changes of these residues had no effect on the ability of the construct to complement *fer-1* (Supplementary Table S2). A GFP fusion protein with all three amino acids changed to A (STS-AAA) was able to complement *fer-1* (Fig 4B), indicating that phosphorylation of the activation loop is not necessary for *FER* function. In contrast, the corresponding changes of these residues to D led to a failure to complement *fer-1* (Fig 4B). However, we could not recover any transformants that expressed the *pFER::FER[STS-DDD]-GFP* construct in synergids (12 independent hygromycin-resistant primary transformants were analyzed), indicating that the STS to DDD change in the FER activation loop likely leads to protein instability. Whether this change in protein stability is functionally significant *in planta*, that is whether it mimics what happens to a phosphorylated RLK, remains to be determined.

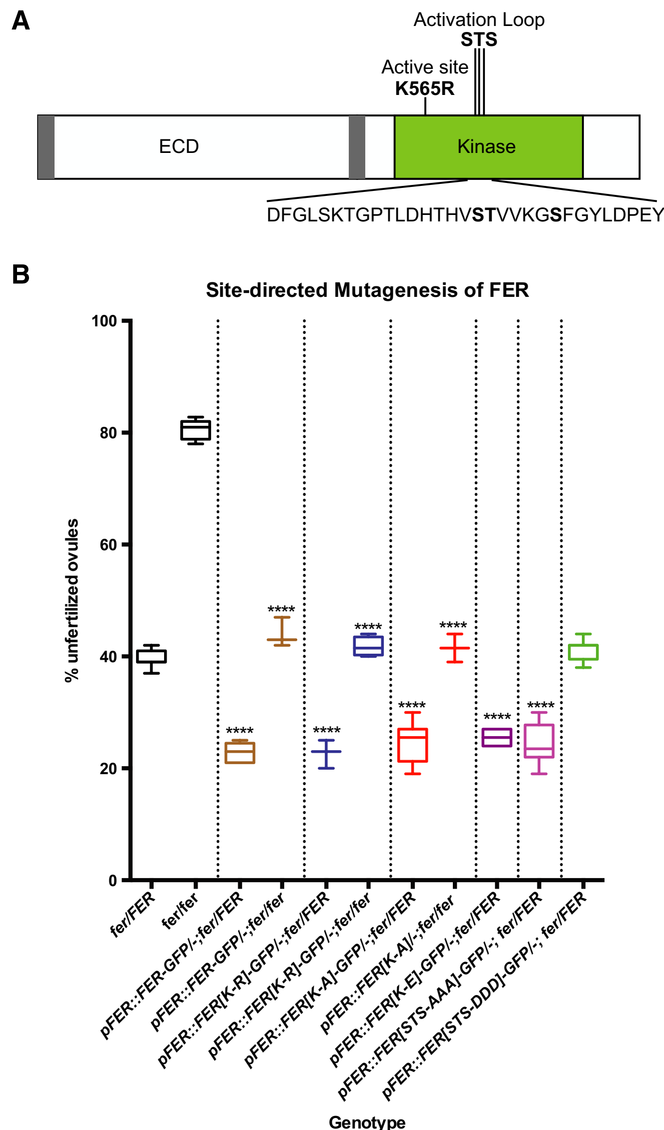

Figure 4. Site-directed mutagenesis of FER kinase domain.

A FER protein domains and positions of the mutations introduced for complementation assays. The bold S, T, and S indicate targets for mutagenesis in the activation segment.

B Box plots from complementation assays showing the percentage of unfertilized ovules for control plants and T1 transformants for each of the site-directed mutagenesis constructs. **** indicates lines with % unfertilized ovules significantly different from untransformed controls of the same genotype (*P*-values < 0.0001 in *t*-tests). See Supplementary Table S2 for raw seed count data.

Discussion

Arabidopsis has over 400 predicted RLKs, but functions are known only for relatively few of these [25]. The majority of plant RLKs are predicted S/T kinases, the largest family being LRR kinases with variable numbers of LRRs in their ECDs that are predicted to bind molecules such as hormones, elicitors, or peptide ligands [26]. The common theme in mutant phenotypes associated with *CrRLK1L* genes is cell wall sensing [8,9,27,28], indicating that these RLKs

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probably bind similar types of ligands. The presence of malectin-like domains in CrRLK1L RLKs indicates that the ligands could be carbohydrates from the cell wall or peptide ligands with specific glycosylation patterns [8]. It was recently shown that, in roots, FER binds to the secreted RALF peptide [29]. However, RALF itself is not expressed in pollen, such that the ligand binding to FER during PT reception is likely another member of the large RALF-like family. RALF and 9 of the 34 RALF-like peptides have potential glycosylation sites (Supplementary Fig S5), including the pollen-expressed RALFL4 and RALFL26, indicating that glycosylation may be important for recognition by the malectin-like domains in the CrRLK1L ECDs. However, these glycosylation sites have not been experimentally verified and, thus, their role in CrRLK1L signaling pathways remains to be determined.

ANX1 and HERK1 are not functionally interchangeable with FER

FER and ANX1/2 perform seemingly opposite functions during pollination, with the ANX1/2 proteins maintaining PT growth and integrity [10,11], while FER signaling leads to cessation of PT tip growth, PT rupture, and release of the sperm to achieve double fertilization [5–7]. One hypothesis for the function of the FER and ANX proteins is that they compete for the same ligand, with FER. This would lead the ligand at higher affinity upon PT arrival at the synergids. This would lead to inactivation of the ANX pathway and bursting of the PT, similar to that seen in *anx1;anx2* double mutant PTs upon germination [10, 11]. If this hypothesis is true, then expressing *ANX1* in synergids should complement the *fer-1* phenotype, since the ANX1 ECD in the filiform apparatus should also compete for the ligand at the PT tip. However, neither the *pFER::ANX1-GFP* nor the *pFER::ANX[ECD]-FER[ICD]-GFP* construct could complement the *fer-1* phenotype, indicating that ANX1 and FER probably bind different ligands and some other mechanism is responsible for inhibiting *ANX* signaling upon PT reception.

As expected, the more distantly related HERK1 RLK was also not able to complement *fer-1* when expressed in synergids. However, both the HERK1-GFP and ANX1-GFP fusion proteins showed a subcellular localization very similar to FER-GFP in synergids. FER is highly enriched in the filiform apparatus, a membrane-rich region where PTs first contact the micropylar end of the synergid. This localization pattern is not seen with all membrane-localized proteins in synergids: the ROP6C protein, for instance, is evenly expressed in synergids [7]; however, the molecular basis for this localization pattern remains to be determined.

The filiform apparatus consists of highly invaginated membranes. FER accumulates in this region of the synergid and the kinase domain is predicted to reside inside the cell [7]. Even though ANX1 and HERK1 localize to the filiform apparatus when expressed in synergids, we cannot exclude the possibility that they are not integral membrane proteins or their topology differs from that of FER. In this case, the failure to complement *fer-1* could reflect a distinct topology rather than a difference in ligand specificity. We consider this unlikely, however, because FER and HERK1 have been detected in membrane phosphoproteomics studies [23,30], and ANX1-GFP is localized to the PM at the tip of growing PTs [10,12] and onion epidermal cells (Supplementary Fig S3), indicating that these three CrRLK1Ls are indeed membrane proteins. Furthermore, GFP is pH sensitive [31] and no signal could be detected in onion epidermal cells when GFP was extracellular in the context of a fusion to a GPI-anchored protein [32]. In contrast, the same protein fused to Citrine, which is less pH sensitive [33], was readily detectable in the filiform apparatus [34]. Given that we can detect a signal in the filiform apparatus from all GFP fusion proteins, it is likely that their ICDs are indeed intracellular and that the proteins have a topology similar to that of FER.

The intracellular domains of CrRLK1L proteins are interchangeable

The CrRLK1L RLKs are predicted to perceive distinct ligands and transmit the signal through signal transduction cascades. Recent publications have shown that FER function in root hair elongation involves binding of ROPGEFs, which transduce a phosphorylation signal to a RAC/ROP, leading to ROS production [13, 14]. In yeast-two-hybrid assays, the FER kinase domain can bind with at least 4 different ROPGEFs [14]. This FER/GEF/ROP pathway has also been implicated in suppression of abscisic acid signaling in roots by activating the ABSCISIC ACID INSENSITIVE2 phosphatase [15]. Furthermore, *ANX1/2* regulate NADPH-oxidase-dependent ROS production during PT elongation, indicating that the same signaling pathway may be shared in tip-growing root hairs and PTs [12]. FER was also shown to control the production of ROS in the filiform apparatus of the synergids [35]. While the exact role of ROPGEF/RAC/ROP signal transduction in FER-mediated PT reception and in other CrRLK1L-mediated processes has yet to be determined, our results, showing that the ANX1 and HERK1 ICDs can complement the *fer-1* PT reception phenotype when linked to the FER ECD, clearly indicate that various CrRLK1L family members share common downstream signaling components.

FER proteins with non-conserved changes in the active site complement the *fer* mutant

Receptor-like kinases are defined by the presence of an ECD potentially involved in ligand binding, a transmembrane domain, and an intracellular kinase domain. Most RLKs are presumed to sense a ligand and phosphorylate another protein to initiate a signal transduction cascade [25]. While FER has kinase activity *in vitro* that can be abolished by a K-R change in the active site [7], the dead kinase version of FER was able to complement *fer-1*, indicating that kinase activity is not necessary for FER function in PT reception. The K565R dead kinase was also able to partially complement the reduced response to root mechanostimulation in *fer* mutants [36]. These results indicate that either a K565R change retains partial kinase function, or that another kinase in the complex is able to substitute for FER's role in signal transduction. Confirmation of the ability of K565 changes to completely abolish kinase activity awaits the identification of endogenous FER targets.

While most plant RLKs that have been studied extensively require kinase activity for their functions (reviewed in [37]), a few do not. Among these are FEI1, a LRR RLK involved in cell wall biosynthesis [38], and *Arabidopsis* CRINKLY4 (ACR4), a CR4-type RLK involved in epidermal development [39]. In addition, ~20% of the more than 400 RLKs in the *Arabidopsis* genome have amino acid substitutions in critical active sites of their kinase domains, leading to the prediction that these RLKs perform their functions without

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kinase activity [40]. Instead of directly phosphorylating downstream components of its signal transduction cascade, FER could function as a part of a complex with another RLK that acts synergistically with FER, and is thus able to complement the lack of kinase activity in the kinase dead version. Plant RLKs often occur in heterodimeric complexes. For example, the BRASSINOSTEROID-INSENSITIVE1-ASSOCIATED KINASE1/SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE3 (BAK1/SERK3) RLK has been shown to act in heterodimeric complexes with other LRR RLKs, such as BRI1 and FLAGELLIN-SENSING2 (FLS2), to enhance their activity [41]. Like BAK1, FER has been shown to be involved in diverse developmental processes [5,7,13–16,42]. It is tempting to speculate that in some of these cases, FER also acts as a co-receptor to enhance the activity of another kinase that transduces the received signal.

FER could also act as a scaffolding protein to bring other components such as ROPGEFs into a complex so that signal transduction can occur. In mice, the EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) family member ErbB3 has a non-functional kinase domain and acts in a complex with other EGFR proteins with active kinase domains. Upon ligand binding, the active EGFR phosphorylates ErbB3 at specific tyrosines, which then serve as docking sites for downstream targets (reviewed in [43]). FER has been shown to directly bind ROPGEF proteins that are involved in downstream signal transduction [13], and our data showing that the kinase domain is necessary for FER function—even though kinase activity is not—indicate that FER may bind ROPGEFs but not be responsible for activating them by phosphorylation. FER might act in a complex with another CrRLK1L protein, a member of a different RLK class, or even a cytoplasmic kinase to provide a docking site for a ROPGEF, which is then phosphorylated by the FER partner. Testing of this model awaits the identification of more FER-interacting proteins.

Taken together, our results suggest that the three members of the CrRLK1L subfamily FER, ANX1, and HERK1 share common downstream signaling targets, but are activated by distinct ligand interactions. Furthermore, the kinase activity of FER may not be essential to execute its function, indicating that FER might act as an important co-receptor recruiting other co-factors or downstream targets to mediate signal transduction during PT reception.

Materials and Methods

Plant material and growth conditions

fer-1 plants (allele described in [7]) were used for all transformations and Landsberg *erecta* was used as the wild-type control for complementation. The FER-GFP line was reported in [7]. The *fer/fer* line was derived from the *fer-1* allele [44]. Plant growth conditions were as described [5]. For transformation, *fer-1* heterozygotes were selected by plating F2 seeds on MS plates supplemented with 50 mg/l kanamycin. After Agrobacterium-mediated transformation, seeds were harvested and plated on MS plates supplemented with 20 mg/l hygromycin to select transformants.

Domain swap and site-directed mutagenesis constructs

PCR with Phusion high-fidelity DNA polymerase (Finnzymes, Espoo, Finland) was used to generate all constructs. The 1.2-kb *pFER* promoter fragment described in [7] was used to drive expression of GFP fusion constructs in synergids as well as sporophytic tissues. Primers described in Supplementary Table S3 were used in the combinations described in Supplementary Table S4 to create overlapping fragments with Gateway attB sites. These PCR products were used to create entry clones in pDONR207 (Life Technologies) and sequenced to ensure that no undesired mutations were introduced by PCR. Entry clones were used in LR reactions with pMDC111 [45] to create in-frame GFP fusions in a plant binary vector. pMDC107 (identical to pMDC111 but with a different reading frame [45]) was used for the ΔICD construct. The site-directed mutagenesis constructs were generated by modifying the *pFER::FER-GFP*/pMDC111 construct that complemented the *fer-1* mutation in [7]. Overlapping primers (Supplementary Table S5) were designed to introduce the desired nucleotide changes between the *Sma*1 and *Xba*1 restriction sites in the FER kinase domain. Restriction digests and ligations were used to replace the native fragments with the mutated PCR fragments, and all constructs were sequenced to verify that the desired mutations had been introduced.

fer-1 complementation assays

Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101, and the resultant strain was used for transformation of *Arabidopsis fer-1/FER* plants using the floral dip method [46]. Progeny were grown on MS plates containing 20 mg/l hygromycin to select transformants. Plants were grown to maturity and genotyped for the *fer-1* mutation [7] and screened for expression of the GFP fusion proteins in synergids. Self-pollinated pistils from synergid expressing plants were collected at 2–4 days after pollination, and counts of fertilized vs. unfertilized ovules were performed to determine whether the constructs complement the *fer-1* PT reception phenotype. For each construct, a minimum of 3 independent T1 transformants (average 6) of each *FER* genotype were used for complementation analysis and 300–500 ovules were counted for each plant. For non-complementing lines, a minimum of 8 primary transformants was analyzed for each genotype.

Confocal microscopy

In order to determine the subcellular localization of GFP fusion proteins, carpel walls were removed from mature pistils and specimens were observed by confocal microscopy. Images 2A-F were captured and processed as described [44]. For images 2G-I, ovules were dissected from pistils 2 days after emasculation and mounted in DI water on standard slides. GFP and autofluorescence were imaged with a Leica TCS SP8 confocal laser scanning microscope, equipped with a 488 notch filter, using a 40× water correction objective (NA = 1.10). Fluorescence was excited using a 488 nm argon laser and detected with HyD detectors set at 489–544 nm (GFP) and 549–669 nm (autofluorescence). Single scan images were processed in ImageJ v.1.48p (NIH, Bethesda MD).

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions

SAK, HL, and UG conceived the experiments; SAK, HL, and DJ performed the experiments; SAK, HL, and UG analyzed the data; SAK, HL, and UG wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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