The BEACH Domain-Containing Protein SPIRRIG Modulates Actin-Dependent Root Hair Development in Coordination with the WAVE/SCAR and ARP2/3 Complexes

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Short Title: Actin-mediated root hair development

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Abstract

Root hairs are single cell protrusions that enable roots to optimize nutrient and water acquisition. They attain their tubular shapes by confining growth to the cell apex, a process called tip growth. The actin cytoskeleton and endomembrane system are essential for tip growth; however, little is known about how these cellular components coordinate their activities during this process. Here, we show that SPIRRIG (SPI), a BEACH domain-containing protein involved in membrane trafficking, and BRK1 and SCAR2, subunits of the WAVE/SCAR (W/SCR) and actin related protein (ARP)2/3 activation complexes, display polarized localizations to root hairs at distinct developmental stages. SPI accumulates at the root hair apex via post-Golgi vesicles and positively regulates tip growth by maintaining tip-focused vesicle secretion and filamentous-actin integrity. BRK1 and SCAR2 on the other hand, mark the root hair initiation domain to specify the position of root hair emergence. Live cell microscopy revealed that BRK1 depletion coincided with SPI accumulation as root hairs transitioned from initiation to tip growth. Furthermore, double mutant studies showed that SPI genetically interacts with BRK1 and ARP2/3. Taken together, our work uncovers a role for SPI in facilitating actin-dependent root hair development through pathways that intersect with the W/SCR and ARP2/3 complexes.
INTRODUCTION

Root hairs are single cell tubular projections that emerge from root epidermal cells. They increase the effective surface area of the root system by extending laterally into soil pores, thus enabling the increased access to nutrients and water (Carminati et al., 2017, Ruiz et al., 2020). Root hairs have been studied extensively by plant biologists for decades because they serve as excellent models to unravel mechanisms by which cell size and shape in plants is regulated (Grierson et al., 2014). To attain their cylindrical shapes, root hairs undergo tip growth, a process in which expansion of the cell is confined to its apical domain. Tip growth involves a balance between the directed delivery of post-Golgi vesicles carrying protein complexes and cell wall building blocks to the cell apex, and localized cell wall loosening and recycling of excess membranes. Besides root hairs, tip growth is exhibited by other cell types such as pollen tubes, fungal hyphae, and rhizoids of mosses, liverworts and algae (Bascom et al., 2018a).

Root epidermal cells called trichoblasts are the cell types that form root hairs. Work in Arabidopsis thaliana has shown that trichoblasts are specified to become root hair-forming cells early during root development through the patterned assembly of protein complexes of transcriptional activators and repressors in different cell files (Schiefelbein et al., 2014, Shibata et al., 2018). Upon establishing their identity, trichoblasts undergo two developmental stages that lead to root hair outgrowth. The first stage is the establishment of a root hair initiation domain (RHID) that eventually leads to a conspicuous root hair bulge at the basal (root-tip oriented) end of the trichoblast (Grierson et al., 2014, Nakamura and Grebe, 2018). Several proteins accumulate at the RHID, most prominently, the Rho of Plants (ROP)/RAC small GTPases and their Guanine Nucleotide Exchange Factor (GEF) activators (Denninger et al., 2019). The second stage is tip growth. In addition to small GTPases, the cytoskeleton, the endomembrane trafficking machinery, cytoplasmic calcium, phosphoinositide lipids, hormones (e.g. auxin and ethylene) and reactive oxygen species are other major players in signaling pathways that modulate root hair development (Bascom et al., 2018a, Nakamura and Grebe, 2018).

The filamentous-actin (F-actin) and microtubule cytoskeletons orchestrate root hair development. The actin cytoskeleton in particular has been studied widely during tip
growth as it functions as tracks for the traffic of cellular cargo to the cell apex (Bascom et al., 2018a, Stephan, 2017, Szymanski and Staiger, 2018). Many insights into the role of the actin cytoskeleton in tip growth have been demonstrated through work with Arabidopsis mutants and pharmacological approaches involving the use of chemicals that disrupt F-actin. For example, the actin-disrupting compound, latrunculin B (LatB), inhibits tip growth, while also inducing the formation of root hairs and pollen tubes with irregular shapes (Bibikova et al., 1999, Gibbon et al., 1999). In this regard, Arabidopsis mutants with defects in the root hair-expressed vegetative ACTIN2 (ACT2) gene are characterized by root hairs that mirror those treated with LatB (Ringli et al., 2002, Yoo et al., 2012). Furthermore, ACT2 and ACT7 mutants display an apical (shoot-ward) shift in the position of the RHID when compared to wild type (Kiefer et al., 2015).

To fulfill its cellular functions, the actin cytoskeleton is organized into higher order networks that correspond to the growth strategy of the cell. This is evident in tip-growing cells, whereby the base and shank of the cell consists mostly of thick, longitudinal F-actin bundles, while the apex contains actin fringes, rings, patches or a fine meshwork depending on the plant species or cell type (Stephan, 2017). To sustain tip growth, the integrity and organization of these tip-focused F-actin arrays must be maintained, a task that is facilitated by a diverse collection of actin-binding proteins and actin nucleators (Li et al., 2015, Paez-Garcia, 2018, Szymanski and Staiger, 2018).

The actin-related protein (ARP2/3) complex and its activator, suppressor of cAMP receptor (SCAR)-WASP family verprolin homologous (WAVE) complex (W/SRC-ARP2/3), is the best characterized actin nucleator in plants (Deeks and Hussey, 2005, Szymanski, 2005, Yanagisawa et al., 2013). The ARP2/3 complex consists of seven subunits that include the proteins ARP2, ARP3, ARPC1, ARPC2, ARPC3, ARPC4, and ARPC5, which were first identified in Acanthamoeba (Machesky et al., 1994). It was shown that the membrane-associated plant ARP2/3 complex has the same assembly mechanisms as the non-plant complexes (Kotchoni et al., 2009). Upon conversion from an inactive open conformation to an inactive closed conformation, the ARP2/3 complex promotes F-actin nucleation from the sides of existing filaments by forming a surface that mimics stable actin dimers (Blanchoin et al., 2000, Robinson et al., 2001, Rodal et al., 2005). Activation of ARP2/3 for efficient nucleation of F-actin requires the W/SCAR
nucleation promoting factor (NPF) complex. In addition to WAVE/SCAR, the NPF consists of the proteins SRA1, NAP1, ABIL and HSPC300/BRICK1 (BRK1) (Deeks et al., 2004, Djakovic, 2006, Frank and Smith, 2002, Jörgens et al., 2010, Le et al., 2006, Saedler et al., 2004). Studies of Arabidopsis trichome development have been most instrumental in revealing insights into the function of W/SCR-ARP2/3 in plants. Plant counterparts to the mammalian W/SCR-ARP2/3 subunits were first uncovered through the cloning of disrupted genes in a set of DISTORTED (DIS) Arabidopsis trichome mutants (Hülskamp et al., 1994, Mathur et al., 2003, Le et al., 2003, El-Assal Sel et al., 2004, Basu et al., 2004, Deeks et al., 2004, Zhang et al., 2005). W/SCR is the only known NPF for ARP2/3, and BRK1 and SCAR mutants display null arp2/3 trichome phenotypes (Le et al., 2006, Zhang et al., 2008). NPF activity of W/SCR to initiate an ARP2/3 actin nucleating response is also known to be positively regulated by ROP/RAC-GTP effector complexes (Basu et al., 2004, Uhrig et al., 2007). In Arabidopsis trichomes, active ROP/RAC-GTPase is coupled to W/SCR by the DOCK (dedicator of cytokinesis)-family GEF, SPIKE1 (SPK1) (Basu et al., 2008) (Yanagisawa et al., 2018).

In addition to trichome development, the W/SRC-ARP2/3 actin filament-nucleating module has been implicated in specifying leaf pavement cell shape, light and auxin-dependent root growth, stomatal gating, gravitropism, responses to salinity stress, and plant immunity (Li et al., 2003, Basu et al., 2005, Zhang et al., 2005, Dyachok et al., 2011, Zhao et al., 2013, Li et al., 2014, Zou et al., 2016, Isner et al., 2017, Badet et al., 2019, Pratap Sahi et al., 2018). The W/SRC-ARP2/3 complex has also been shown to play an essential role in some tip growing cells such as the protonemal cells of the moss Physcomitrella patens. This was shown when RNA interference (RNAi) of transcripts encoding selected subunits of the moss W/SRC-ARP2/3 complex resulted in substantial protonemal tip growth defects (Harries et al., 2005, Perroud and Quatrano, 2006, Finka et al., 2008). The importance of the W/SRC-ARP2/3 complex was further demonstrated by Perroud and Quatrano (2006, 2008) who showed that two of its components, BRK1 and ARPC4, accumulated in tips of moss protonemal cells. By contrast, Arabidopsis plants with mutations in components of the W/SRC-ARP2/3 complex displayed minimal or no defects in root hair and pollen tube growth (Li et al., 2003, Mathur et al., 2003),
and none of the subunits have been conclusively shown to exhibit clear polar localization in these cell types. Therefore, the extent by which the W/SRC-ARP2/3 complex functions in tip growing cells of higher plants remains to be determined.

SPIRRIG (SPI) is one of eight genes that have been considered to be a member of the DIS group, but compared to other DIS mutants, trichome phenotypes of spi are less severe and the mutant does not display early stage cell swelling that is diagnostic of the DIS group (Schwab et al., 2003). SPI was shown to encode a 3571 long amino acid protein with N-terminally located armadillo (ARM) and concanavalin A (ConA)-like lectin domains and C-terminally-located pleckstrin homology (PH), beige and Chediak Higashi (BEACH) and WD40 repeat domains (Saedler et al., 2009). BEACH domain-containing proteins are highly conserved in eukaryotes and are known to function in membrane dynamics, vesicle transport, apoptosis and receptor signaling. This family of proteins are of clinical importance as they have been implicated in a variety of human disorders such as cancer, autoimmunity syndrome and autism (Cullinane et al., 2013).

In addition to mild trichome defects, Arabidopsis SPI mutants have short root hairs characterized by fragmented vacuoles suggesting that SPI, like other eukaryotic BEACH domain-containing proteins, functions in membrane trafficking (Saedler et al., 2009). Additionally, an observation by Steffens et al. (2017) that showed SPI physically interacting with proteins involved in endosomal sorting reinforces its role in membrane remodeling. SPI was also demonstrated to associate with mRNA processing bodies (P-bodies) suggesting a novel role for SPI in post-transcriptional regulation (Steffens et al., 2015). Because SPI is one of the DIS genes, it was suggested that SPI might be involved in actin-mediated cell developmental processes (Saedler et al., 2009), and perhaps function in coordination with W/SCR and ARP2/3 complexes. However, SPI is not a known W/SCR or ARP2/3 subunit; therefore, its relationship to ARP2/3 function is unclear. Moreover, the uncertainty with regard to SPI function is confounded by the fact that its subcellular localization in root hairs, which exhibit the most profound phenotype when SPI is mutated, remains unknown. In this paper, we have addressed these questions by showing that a functional SPI-fluorescent protein fusion accumulated at the tips of rapidly growing root hairs. A SPI-tagged fluorescent protein fusion was not detected in the RHID and in tips of non-growing mature root hairs. We further show that
the W/SCR complex subunits, BRK1 and SCAR2, localized to the RHID and exhibited
temporal dynamics opposite to that of SPI (i.e. functional fluorescent protein-tagged
BRK1 and SCAR2 declined as rapid root hair tip growth commenced). Double mutant
studies revealed that SPI and W/SCR-ARP2/3 genetically interact suggesting that one
function of SPI is to modulate actin-dependent root hair development, in part through
pathways that overlap with W/SCR and ARP2/3 complexes.
RESULTS

Isolation of a New SPIRIG Mutant Allele

We previously described a forward genetic screen that led to the isolation of three non-allelic recessive Arabidopsis mutants that were hypersensitive to the growth inhibitory effects of LatB. The hypersensitive to LatB1 (hlb1) and hlb3 mutants have been described previously (Sparks et al., 2016, Sun et al., 2019). hlb1 was disrupted in a gene encoding a trans-Golgi Network-localized tetratricopeptide repeat protein involved in actin-mediated membrane recycling (Sparks et al., 2016), whereas the genetic lesion in hlb3 was found to encode the class II actin nucleator formin (Sun et al., 2019). Here, we report on hlb2, the third of these recessive mutants. Like hlb1 and hlb3, hlb2 primary root growth was more severely inhibited by LatB when compared to wild type. In the absence of LatB or at low (i.e. 25 nM) LatB concentrations, the primary root length of hlb2 was similar to wild type. Differences in root length between wild type and hlb2 became apparent when seedlings were grown on a concentration of 50 nM LatB and higher (Supplemental Figure 1A and B).

Through map-based cloning, we found that the mutation in hlb2 was confined to a region between the AT1G02740 and AT1G03410 loci. Nucleotide sequencing revealed that hlb2 had a 10 base pair deletion (Chr1 position: 720,152 - 720,161) in exon 14 of the AT1G03060 gene. This 10-base pair deletion led to an open-reading frame shift at the Asp<sup>1526</sup> codon resulting in a truncated protein (Supplemental Figure 2A). AT1G03060 encodes the BEACH domain-containing protein, SPI (Saedler et al., 2009) (Supplemental Figure 2A). Because the first spi mutant alleles were reported to have short root hairs (Saedler et al., 2009), we examined root hairs of hlb2. We found that hlb2 had root hairs that were about 80% shorter than wild type with some root hairs forming only small bulges (Supplemental Figure 2B and C). Moreover, hlb2 had mild trichome defects that were reminiscent of the phenotypes of previously isolated spi mutant alleles (Supplemental Figure 2D; Saedler et al., 2009). To further verify if HLB2 is SPI, we obtained a mutant from the publicly available SALK collection (SALK_065311), which had a T-DNA insertion in the 10<sup>th</sup> exon of the SPI gene.
The SALK_065311 line we obtained is the same as spi-3 mutant allele reported previously in Steffens et al. (2015). In addition to having similar root hair and trichome defects as hlb2, primary roots of spi-3 were hypersensitive to LatB (Supplemental Figure 2E), and a cross between hlb2 and SALK_065311 failed to complement each other in the F1 hybrid. Taken together, these results indicate that hlb2 is a new spi mutant allele. Based on earlier nomenclature (Steffens et al., 2015), we renamed hlb2 to spi-5 (Supplemental Figure 2A). The spi-5 mutant allele was used for all subsequent experiments.

SPIRRIG Localizes to the Tips of Rapidly Elongating Root Hairs

SPI fluorescent protein fusions have been previously demonstrated to associate with P-bodies and endosomes (Steffens et al., 2015, Steffens et al., 2017). However, these constructs have not been shown to complement the defective root hair and trichome phenotypes of spi. Because of the large size of SPI, we were unable to generate native promoter-driven fluorescent protein fusions to the full length SPI complementary DNA or genomic DNA. To overcome this problem, SPI was tagged with the 3x-Yellow Fluorescent Protein (YFP) for energy transfer (YPet; Nguyen and Daugherty (2005)) using a method called recombineering. This method involves tagging the gene of interest in the context of transformation-competent bacterial artificial chromosomes to ensure that all regulatory sequences are included in the fluorescent protein fusion (Brumos et al., 2020, Zhou et al., 2011).

Once spi was transformed with a recombineered SPI-YPet construct, primary root hypersensitivity to LatB, short root hairs and defective trichome phenotypes were rescued, indicating that the construct was functional (Figure 1A; Supplemental Figure 3). Transgenic complementation of spi with SPI-YPet provided additional evidence that HLB2 is SPI. Confocal microscopy revealed that SPI-YPet signal was strongest in the tips of rapidly elongating root hairs (Figure 1B and C; Supplemental Movie 1). SPI-YPet fluorescence was not detected or weak at the RHID and during early root hair bulge formation, but intensified as root hairs transitioned to tip growth (Figure 1C; Supplemental Movie 2). SPI-YPet signal dissipated as root hairs matured and tip growth
rate declined (Figure 1B and C; Supplemental Movie 3). A linear regression analysis showed that the intensity of SPI-YPet fluorescence in root hair tips is significantly positively correlated to rapid root hair growth (Figure 1D). These results indicate that the SPI protein has functions related to root hair elongation and consistent with the short root hair phenotypes of spi.

**SPIRRIG is Transported to the Root Hair Tip via Post-Golgi Vesicles**

The prominent SPI-YPet signal at the tips of elongating root hairs is reminiscent of the localization patterns of post-Golgi markers such as RAB small GTPases, which are known to function in tip-directed secretion (Preuss et al., 2004). We therefore hypothesized that SPI is trafficked to the tips of root hairs via post-Golgi vesicles. To test this hypothesis, seedlings expressing *SPI-YPet* were treated with brefeldin A (BFA). BFA is a fungal toxin that is routinely used to investigate endomembrane dynamics because it prevents vesicle formation for exocytosis by inhibiting ADP ribosylation factor guanine nucleotide exchange factors (ARF-GEFs), while still enabling endocytosis and some retrograde pathways to continue (Baluška, 2002, Doyle et al., 2015). Consequently, BFA treatment causes the formation of endosomal agglomerations called BFA bodies. Treatment of seedlings expressing *SPI-YPet* with 50 μM BFA induced the formation of SPI-YPet agglomerates in root hairs (Figures 2A and B). The formation of SPI-YPet fluorescent agglomerates was also observed in the root elongation zone (Figure 2C). In the absence of BFA, SPI-YPet signal in the elongation zone was barely detectable. However, upon exposure to BFA, bright fluorescent agglomerates of various sizes became visible (Figure 2C). These results indicate that SPI-YPet is associated with endomembranes and is trafficked to the root hair tips via BFA-sensitive post-Golgi vesicles.

Inhibited root hair growth of *spi* and the formation of SPI-YPet agglomerates after BFA treatment, suggest that *spi* may be defective in tip-directed protein secretion and partly explain the short root hair phenotypes of *spi*. To address this question, we expressed the secreted (SEC)-red fluorescent protein (RFP) in *spi* (Faso et al., 2009). Similar to previous reports, rapidly elongating wild-type root hairs had a tip-focused
gradient of SEC-RFP (Sparks et al., 2016) (Figure 2D). By contrast, slow-growing root hairs of spi lacked these tip-focused SEC-RFP gradients (Figure 2E), thereby indicating defects in tip-directed bulk flow exocytosis.

SPIRRIG Maintains Root Hair Tip-Focused F-actin

In a study of other spi mutant alleles, the similarities in phenotypes between spi and w/scr-arp2/3 suggests that SPI could function in actin-dependent cellular processes (Saedler et al., 2009). However, because trichome defects of spi were mild compared to other w/scr-arp2/3 mutants, no obvious actin phenotypes were observed in spi trichomes (Schwab et al., 2003). To clarify the relationship between SPI and actin, we focused on investigating actin organization in root hairs since they displayed the most obvious growth defects in SPI-altered plants.

To study actin organization, we expressed the live F-actin reporter, UBQ10: mGFP-Lifeact, in spi (Vidali et al., 2010). This particular F-actin reporter was selected because it prominently labels the tip-focused F-actin meshwork typically observed in root hairs that are rapidly growing (Sparks et al., 2016). In wild type, fine F-actin networks were observed in the root hair bulge that had a weaker signal compared to the thicker actin bundles in other regions of the trichoblast (Figure 3A). As the root hair bulge expanded and the root hair transitioned to rapid tip growth, the tip-focused F-actin meshwork, which consisted of short filaments and dynamic puncta, became more conspicuous (Figures 3B-E; Supplemental Movie 4; Sparks et al. (2016)). When wild-type root hairs stopped elongating, the tip-focused F-actin meshwork was replaced with thick F-actin cables (Figure 3F).

F-actin organization in the tips of spi root hairs was different from that of wild type. As noted, some root hairs of spi were only able to form small bulges due to premature termination of tip-growth (Supplemental Figure 2). In spi root hairs, the distinct F-actin meshwork observed in wild-type root hairs was unable to form. Instead, F-actin in these spi root hair bulges contained thick F-actin cables that resembled those of wild-type root hairs that had terminated growth (Figure 3G and H). However, the thick F-actin cables in non-growing spi root hair bulges were unstable as they dissipated.
(Figure 3I) and reformed again at a later time (Figure 3J). In the small population of *spi* root hairs that were able to undergo tip growth, a few exhibited the tip-focused F-actin meshwork that resembled those observed in elongating wild-type root hairs (Figure 3K). However, most of these slow growing *spi* root hairs lacked the tip-focused F-actin meshwork (Figure 3L and M; Supplemental Movie 4) or had thick F-actin bundles protruding to the tip, a feature that was reminiscent of non-growing, mature wild-type root hairs (Figure 3N). The disruption of the tip-focused F-actin meshwork in *spi* was quantified by measuring F-actin fluorescence from computer reconstructed transverse sections of the root hair tip and by taking the ratio of the average fluorescence over background signal. A higher ratio indicates a higher signal of tip-focused F-actin (Figure 3O). Our analysis showed that the fluorescence ratio in *spi* root hairs was significantly reduced compared to wild-type root hairs, supporting visual observations that the tip-focused F-actin meshwork in *spi* root hairs is disrupted (Figure 3P).

We next generated plants expressing both *SPI-YPet* and *mRuby-Lifeact* so we could simultaneously observe SPI and F-actin in growing root hairs. In elongating root hairs of dual labeled seedlings, SPI-YPet and the mRuby-labeled F-actin meshwork overlapped at the tip (Figure 3Q; Supplemental Movie 5). This provides support that root hair tip-localized SPI is associated with the tip-focused F-actin meshwork, and as such is involved in sustaining normal root hair elongation in coordination with actin.

**BRK1 and SCAR2 are Molecular Determinants of the Root Hair Initiation Domain**

The identification of *SPI* as one of the genes in the *DIS* group that also included genes encoding subunits of the W/SRC-ARP2/3 complex (Saedler et al., 2009) raises the possibility that SPI might function in root hair developmental pathways mediated by W/SRC-ARP2/3. Furthermore, the observation that SPI-YPET accumulation at the root hair tip (Figure 1) mirrors the enrichment of BRK1-YFP and APRC4-GFP at apex of *Physcomitrella* protonemal cells (Perroud and Quatrano, 2006, Perroud and Quatrano, 2008) raises the possibility that W/SRC-ARP2/3 maybe a root hair tip-localized complex. In an attempt to link SPI with the W/SCR-ARP2/3 complex, we imaged roots of *brk1* complemented with *BRK1promoter:BRK1-YFP* (from here on referred to as
BRK1-YFP) (Figures 4A-C) (Dyachok et al., 2008). Unlike SPI-YPet, we did not observe a BRK1-YFP fluorescence gradient in rapidly elongating root hairs. However, closer examination of trichoblasts revealed prominent BRK1-YFP signal at the plasma membrane of the RHID that mirrored other known early root hair initiation site markers such as ROP (Figure 4A). Unlike ROP, which had a persistent plasma membrane localization throughout root hair development (Jones et al., 2002, Molendijk et al., 2001), BRK1-YFP signal was transient and dissipated as root hairs transitioned to rapid tip growth (Figure 4B; Supplemental Movie 6). This observation was in contrast to the intensification of SPI-YPet fluorescence as root hairs proceeded with rapid tip growth (see Figure 1). In agreement with our visual observations, a linear regression analysis showed that BRK1-YFP fluorescence is inversely proportional to root hair growth rate (Figure 4C).

Given that BRK1 stabilizes the entire family of SCAR proteins and is required for functional W/SRC assembly (Le et al., 2006), we investigated if the SCAR protein localized to the RHID, similarly to BRK1. To address this question, we imaged a recombineered SCAR2-mCherry fusion expressed in the \(\text{scar1 scar2 scar3 scar4} (\text{scar1234})\) quadruple mutant. We found that like BRK1-YFP, SCAR2-mCherry marked the RHID and dissipated when rapid root hair tip growth commenced (Figure 4D).

The accumulation of BRK1-YFP and SCAR2-mCherry at the RHID led us to hypothesize that \(\text{brk1}\) and \(\text{scar1234}\) might have defects in root hair initiation. One parameter that has been studied extensively as an indicator of root hair initiation defects is planar polarity, which is a measure of root hair position along the length of the trichoblast (Nakamura and Grebe, 2018). We found that root hair position of \(\text{brk1}\) and \(\text{scar1234}\) shifted apically (i.e. toward the shoot) when compared to wild type (Figure 4F). The planar polarity defects of \(\text{brk1}\) was rescued by expressing \(\text{BRK1-YFP}\) in \(\text{brk1}\) while partial complementation of \(\text{scar1234}\) was achieved by expressing \(\text{SCAR2-mCherry}\) in \(\text{scar1234}\) (Figures 4E and F). Taken together, our results revealed that BRK1 and SCAR2 are new molecular determinants of the RHID and are required for specifying the position of root hair emergence.

Given that ARP2/3 is the known target of activated W/SRC, we hypothesized that ARP2/3 might also localize to the RHID. To test this hypothesis, we imaged a line
expressing ARPC5-GFP, one of the subunits of the ARP2/3 complex (Yanagisawa et al., 2015). This line has been confirmed to complement the arpc5 trichome defects. As reported previously, ARPC5-GFP labeled the plasma membrane of initiating trichome branches and the apex of elongating trichome branches (Yanagisawa et al. (2015); Supplemental Figure 4A and B). Nevertheless, when observed in root hairs, the ARPC5-GFP signal was distributed uniformly at various stages of development with no distinct accumulation at the RHID or root hair apex (Supplemental Figure 4C and D). Although there was no clear polarized localization of ARPC5-GFP in root hairs, it is possible that ARP2/3 is locally activated in RHID or root hair apex, but that active pool is obscured by a large soluble pool of the complex in this cell type.

**SPI is Required for the Depletion of BRK1 as Root Hairs Transition to Tip Growth**

Live cell microscopy of BRK1-YFP and SPI-YPet revealed contrasting spatial and temporal dynamics, with the former intensifying and the latter dissipating as root hairs elongated (Figure 1 and 4). To observe BRK1 and SPI simultaneously within the same root hair, we generated plants expressing both SPI-YPet and BRK1-mRuby3. Similar to BRK1-YFP, BRK1-mRuby3 labeled the RHID and dissipated as the root hair bulge expanded (Figure 5A). Within the same root hair cell, SPI-YPet fluorescence at the tip intensified as BRK1-mRuby3 dissipated (Figure 5A; Supplemental Movie 7). The depletion of BRK1 coinciding with the accumulation of SPI suggests that these proteins may be antagonistic to each other. To address this question, we expressed BRK1-YFP in *spi* and SPI-YPet in *brk1*. We found that unlike BRK1-YFP in the complemented *brk1* background (Figure 4B), BRK1-YFP signal in *spi* persisted throughout the entire imaging time course. In several cases, BRK1-YFP remained visible in non-growing root hair bulges of *spi* for more than 60 min (Figure 5B). The persistence of BRK1-YFP signal was also observed in short root hair outgrowths of *spi* (Figures 5C; Supplemental Movie 8). By contrast, SPI-YPet in *brk1* exhibited similar dynamics as SPI-YPet in the complemented *spi* (Figure 5D). In *brk1*, SPI-YPet signal was weak during early root hair bulge formation and intensified as root hair tip growth accelerated (Figure 5D). Taken
together, these results indicate that SPI is antagonistic to BRK1 during the transition from root hair initiation to rapid tip growth.

**SPI Genetically Interacts with W/SRC-ARP2/3 in Root Hairs**

The phenotypic overlap between SPI and W/SCR and ARP2/3 mutants suggest that SPI participates in similar biological processes (Saedler et al., 2009). The polarized localization of SPI, BRK1 and SCAR2 in root hairs observed here also indicate potential functional relationships between SPI and W/SCR-ARP2/3 during root hair development. However, it remains to be determined whether SPI genetically interacts with W/SRC-ARP2/3 pathway components. To address this question, we generated double spi brk1 mutants and compared their root hair phenotypes with single mutants. We first investigated the genetic relationship between SPI and BRK1 based on length of mature root hairs. We found that at low magnification, root hair length of brk1 appeared morphologically similar to wild type (Figure 6A). Quantification of root hair length, however, uncovered a small but significant reduction in root hair length in brk1 when compared to wild type (Figure 6B). On the other hand, spi brk1 had short root hairs that were similar to spi (Figure 6A and B). We next examined whether SPI and BRK1 genetically interact in specifying root hair planar polarity. We found that spi displayed a more shoot ward (apical)-shifted root hair position when compared to wild type and brk1. Quantification of root hair planar polarity showed that that root hair position of spi was identical to spi brk1 (Figure 6C). Taken together, our results indicate that SPI is epistatic to BRK1 for root hair planar polarity and tip growth.

Epistasis between SPI and BRK1 prompted us to expand our genetic interaction studies to other genes in the ARP2/3 pathway. We therefore generated spi arp2, spi arp3 and spi arpc5 double mutants and analyzed their root hair phenotypes. Like brk1, root hairs of arp2, arp3 and arpc5 appeared to be morphologically similar to wild type (Figure 7A; Supplemental Figure 5). Quantification of root hair length, however, revealed slightly shorter root hairs in the single arp2/3 mutants when compared to wild type (Figure 7C), an observation consistent with the mild tip growth defects reported for these mutants in the presence of the GFP:TALIN live cell probe for actin (Mathur et al.,
On the other hand, *spi arp2*, *spi arp3* and *spi arpc5* appeared morphologically hairless when viewed at low magnification (Figure 7; Supplemental Figure 5). This observation was in contrast to *spi brk1* and *spi*, which had a number of short, but visible root hair outgrowths spaced between hairless regions (Figures 6A, 7A, and 7BB; Supplemental Figure 5). When viewed at high magnification we found that most of the trichoblasts of *spi arp2*, *spi arp3* and *spi arpc5* had a high number of root hair bulges that aborted tip growth soon after initiation (Figure 7B; Supplemental Figure 5). Quantification of root hair length revealed that the joint contribution to the root hair length phenotype made by combining *SPI* and *ARP2/3* complex mutations was greater than an additive effect of *SPI* or *ARP* single mutations (Figure 7C). The enhancement of the root hair length defect of *spi* by *arp2/3* single mutants indicate a synergistic interaction between *SPI* and *ARP2/3* with regard to root hair tip growth.

We next investigated if *SPI* and *ARP2/3* genetically interact in specifying the position of the RHID. We found a mild but statistically significant shoot ward shift in the position of the RHID in *arp2*, *arp3* and *arpc5* when compared to wild type. The extent to which the position of the RHID shifted towards the shoot in single *arp2/3* complex mutants was less than that of *spi* and *brk1* (compare Figure 6C and 7D). On the other hand, the position of the RHID in *spi arp2/3* was more similar to single *arp2/3* indicating that *ARP2/3* is epistatic to *SPI* for root hair planar polarity (Figure 7C and D). In many cases we observed that the aborted root hair bulges in *spi arp2/3* emerged very close to the basal (root ward) end wall of the trichoblast (Figure 7C). The synergistic and epistatic relationship between *SPI* and *ARP2/3*, for root hair length and planar polarity, respectively, indicate that *SPI* also genetically interacts with *ARP2/3* during root hair development. However, the nature of this interaction appears to differ from that of *BRK1*.

Genetic interaction between *SPI* and *W/SRC-ARP2/3* was also analyzed for other *spi* phenotypes such as primary root hypersensitivity to LatB (Supplemental Figure 1). For LatB sensitivity, we found that *arpc5* primary roots were inhibited to the same extent as wild type when grown on LatB (Supplemental Figure 6B). Although primary roots of *arp2*, *arp3* and *brk1* exhibited some hypersensitivity to LatB, the response of these mutants to the compound was not as severe as *spi* and leaned more
toward wild-type sensitivity. By contrast, the hypersensitivity of primary roots of *spi* arp2, *spi* arp3, *spi* arpc5 and *spi* brk1 to LatB was more similar to *spi* (Supplemental Figure 6A and B) suggesting genetic interactions also occurs between *SPI* and *W/SCR-ARP2/3* for this phenotype.

Genetic interaction between *SPI* and *W/SCR-ARP2/3* was also observed in trichomes (Li et al., 2003, Mathur et al., 2003, Schwab et al., 2003). Consistent with previous reports, *arp2*, *arp3*, *arpc5* and *brk1* had severe trichome shape defects when compared to wild type, while those of *spi* were mild when viewed with a scanning electron microscope. One particular trichome defect is branch length, in which *arp2*, *arp3*, *arpc5* and *brk1* trichomes have distinctively short branches when compared to wild type (Supplemental Figure 7). On the other hand, trichomes of *spi* were wavy and crooked, but not as short as those of *arp2*, *arp3*, *arpc5* and *brk1*. We found that trichomes of all the double mutants resembled those of *spi* (Supplemental Figure 7A). Quantification of branch length of all single and double mutants was consistent with visual observations, especially in showing that *spi* brk1 and *spi* arp2/3 resembled the branch length of *spi* (Supplemental Figures 7A and C). Our observations on trichome branch length provide additional evidence that *SPI* genetically interacts with *W/SCR-ARP2/3*. 
Our work uncovers new insights underlying actin-mediated root hair development. A major result from our studies is the revelation that SPI is a root hair tip-localized protein. Although SPI fused to fluorescent proteins was reported to localize to endosomes and P-bodies, such studies have been limited to transient expression assays in biolistically-bombarded leaves (Steffens et al., 2015; Steffens et al., 2017). As such, a mechanistic link between reported SPI subcellular localization patterns and cell growth phenotypes (i.e. short root hairs and distorted trichome shapes) described in the original spi mutant alleles has never been demonstrated. The large size of SPI and the possibility that it is targeted to discrete cellular domains could have hindered transgenic complementation and subsequent in planta localization efforts. Through our work, we have addressed this knowledge gap and now show that a SPI-YPet fusion generated through recombineering has complemented spi (Brumos et al., 2020, Zhou et al., 2011). In doing so, we provide compelling evidence that SPI is a root hair tip–enriched BEACH domain-containing protein.

Our studies indicate that SPI mediates root hair tip growth by maintaining the tip-focused fine F-actin meshwork, although the precise mechanism by which this is accomplished is unknown. The root hair tip-focused F-actin meshwork is the functional equivalent of domain-specific actin structures found in other tip-growing cells such as cortical actin fringes in pollen tubes and actin spots in moss protonemata (Bascom et al., 2018a). Although the tip-focused F-actin meshwork was able to form in a population of spi root hairs, it often dissipated or was replaced by thick actin cables, a feature of mature wild-type root hairs that are terminating tip growth. The root hair phenotypes and corresponding depletion of tip-focused F-actin in spiis reminiscent of studies of BEACH domain-containing proteins in mammalian cells, particularly in neurons, which have often been compared to tip-growing plant cells (Baluška, 2010). For instance, neuronal dendritic spines are cytoplasmic protrusions that modulate excitatory synaptic transmission in the mammalian nervous system. Actin is a major component of dendritic spines and it plays a central role in driving the dynamic shape changes and secretory activities of these cytoplasmic protrusions during synaptic signaling (Cingolani and
Goda, 2008). In animals, neurobeachin (Nbea) is a BEACH domain-containing protein that has a similar domain architecture as SPI. Cultured neurons of *Nbea* mice knockouts had fewer dendritic spine protrusions and depleted F-actin at the synapse (Niesmann et al., 2011). Another BEACH domain-containing protein called FAN was shown to be crucial for the formation of filopodia, actin-rich plasma membrane extensions that enable motile cells to probe their environment (Mattila and Lappalainen, 2008). FAN deficient fibroblasts had reduced filopodia formation and were unable to reorganize their actin cytoskeleton in response to upstream activation by tumor necrosis factors (Haubert et al., 2007). Taken together, these results indicate that actin-mediated regulation of polarized cell growth by BEACH domain-containing proteins is likely to be conserved across animals and plants.

The formation of SPI-YPet agglomerates in response to BFA indicates that SPI is associated with post-Golgi vesicles. This result is consistent with observations made with BEACH domain-containing proteins in mammals. For example, Nbea was shown to localize to vesicular endomembranes adjacent to the *trans*-Golgi network, and like SPI, the distribution of Nbea-positive post-Golgi vesicles was altered by BFA (Wang et al., 2000). Furthermore, the *Caenorhabditis elegans* Nbea homolog, SEL-2, was demonstrated to function in endomembrane traffic in polarized epithelial cells based on the finding that *sel-2* mutants mistarget proteins normally found in the apical cell surface to the basolateral surface (de Souza et al., 2007). Here, the protein secretory marker SEC-RFP, which is typically trafficked to the tips of elongating wild-type root hairs (Sparks et al., 2016), was uniformly distributed in *spi* root hairs. The absence of tip-directed SEC-RFP gradients in *spi* root hairs shows that, as in mammals, plant BEACH domain-containing proteins are required for proper targeting of molecular cargo to points of polarized cell growth.

Another significant result from our studies is the discovery that BRK1 and SCAR2 are molecular determinants of the RHID. The localization of BRK1 and SCAR2 to the RHID and their dissipation as root hair tip growth commenced, contrasts with findings in *Physcomitrella* in which a functional BRK1-YFP distinctly labeled the tips of caulonemal cells (Perroud and Quatrano, 2008). Like BRK1-YFP, an ARPC4-GFP construct labeled the tips of caulonemal cells (Perroud and Quatrano, 2008) whereas no clear tip-focused
labeling of ARPC5-GFP was observed in root hairs. The observation that BRK1-YFP prominently localized to the RHID, but dissipated during active root hair tip growth was surprising given that the Arabidopsis BRK1 complemented the defective filamentous growth of Physcomitrella BRK1 knockouts (Perroud and Quatrano, 2008). This suggest that seed plants might have evolved a specialized function for the W/SCR-ARP2/3 complex in which it acts predominantly at the site of root hair emergence and early bulge formation with only a minor role in driving actin-dependent tip growth. The fact that Physcomitrella BRK1 and ARPC4 mutants have clear tip growth defects, while Arabidopsis W/SCR-ARP2/3 mutants have only weak to no tip growth abnormalities, supports this possibility (Le et al., 2003, Mathur et al., 2003, Perroud and Quatrano, 2006, Perroud and Quatrano, 2008). By contrast, knockouts of an Arabidopsis SPI orthologue in the liverwort, Marchantia polymorpha, led to short rhizoids indicating conserved functions of SPI across land plants in regulating tip growth (Honkanen et al., 2016). In other single cell types such as diffusely-growing trichomes, W/SCR-ARP2/3 subunits likely play a more prominent role than SPI based on their more severe trichome phenotypes when expression of their encoding genes are suppressed. However, the epistatic relationships between SPI and W/SCR-ARP2/3 for trichome morphology and primary root hypersensitivity to LatB as reported here continue to point to functional relationships between these genes in diffusely-growing cells. The observation that phenotypes of spi w/scr-arp2/3 tilt more toward spi indicate that SPI might negatively regulate an actin pathway that is parallel to ARP2/3.

The weakening of BRK1-mRuby3 fluorescence coinciding with SPI-YPet accumulation, and the persistence of SPI-YPet signal in spi root hair tips, provides indirect evidence that SPI might play a role in mediating BRK1 stability or localized clustering at the plasma membrane of the RHID. Because spi brk1 root hair phenotypes resemble spi, ectopic expression of BRK1 does not appear to contribute to the spi phenotypes. Although, it is unknown why BRK1-YFP signal persists in spi, it is tempting to speculate that SPI might modulate BRK1 via protein degradation pathways. This possibility is supported by studies in mammals pointing to a role for BEACH domain-containing proteins in protein degradation via the ubiquitination pathway. In mouse models for example, the BEACH domain-containing protein, WDR81, was shown to be
essential for removal of autophagy-dependent ubiquitinated proteins (Liu et al., 2017). In this regard, it is worth noting that the W/SCR-ARP2/3 complex was demonstrated to function in stress-induced autophagy (Wang et al., 2016) and proteasome inhibitors stabilized SCAR during dark-induced primary root growth inhibition (Dyachok et al., 2011). It is possible that SPI-mediated proteolytic pathways and BRK1 operate antagonistically to specify the levels of W/SCR at the RHID that enables the transition to actin-dependent rapid tip growth. However, such as scenario is complicated by the observation that the formation of tip-directed SPI-YPet does not appear to require BRK1. This suggest that SPI’s appearance at the root hair tip is regulated by other factors besides W/SCR.

The functional links between SPI and W/SCR-ARP2/3 during root hair development as suggested by live cell imaging is supported by the double mutant studies reported here. We found that SPI is epistatic to BRK1 for RHID and root hair tip growth. For SPI and ARP2/3, synergistic and epistatic genetic interactions were observed for root hair tip growth and root hair planar polarity, respectively. Synergistic genetic interactions can arise when converging pathways are disrupted (Pérez-Pérez et al., 2009), and the severe root hair phenotypes of spi arp2/3 double mutants may reflect parallel pathways that are involved in actin-dependent root hair tip growth. The detection of synergistic genetic interactions in root hairs with ARP2/3 mutants, but not with brk1 is unexpected as brk1 is null for W/SRC-dependent ARP2/3 activation (Le et al., 2006), and W/SRC is the only known ARP2/3 activator in Arabidopsis (Zhang et al., 2008). These genetic data may reflect the existence of an unknown ARP2/3 activator or an NPF-independent function of ARP2/3. Future studies will probe deeper into the regulatory relationships between SPI and W/SCR-ARP2/3 that underlie these unexpected genetic interactions.

In summary, our work provides new data that contribute to our understanding of actin-mediated root hair development. A crucial result from our work is the discovery that SPI and the W/SCR subunits, BRK1 and SCAR2, exhibit polarized localization patterns in root hairs that point to potential functional relationships among these proteins during root hair development. Based on our cell biological studies, we propose a model for which new hypotheses can be tested (Figure 8). We propose that a RHID-
localized ROP/RAC-GTPase (Jones et al., 2002, Molendijk et al., 2001) activates W/SCR during root hair initiation in a manner that mirrors that of the DOCK family GEF, SPK1, which was shown to occur during trichome morphogenesis (Basu et al., 2008). Although the precise ROP-based machinery that facilitates this process during root hair development has yet to be determined, ROP-GEF3 could be the functional equivalent of SPK1 given that it localizes to the RHID (Denninger et al., 2019) in a manner similar to BRK1 and SCAR2. Although epistatic and synergistic genetic interactions presented in our double mutant studies support the occurrence of overlapping pathways between SPI and W/SCR-ARP2/3, the precise nature of such relationships remain unclear. One plausible scenario is that SPI negatively regulates an actin pathway that is parallel to W/SCR-ARP2/3. The persistence of BRK1-YFP signal in spi suggests that one mechanism for negative regulation could occur at a node in which SPI and W/SCR converge. For the future, it will be important to determine whether SPI physically interacts with actin or subunits of the W/SCR-ARP2/3 complex to better explain the functional links between SPI and W/SCR-ARP2/3.
METHODS

Forward-Genetic Screening and Map-Based Cloning

The \textit{hlb} mutants from which the \textit{spi-5} mutant allele was identified were isolated from a population of Transfer-DNA (T-DNA) seed (Arabidopsis Biological Research Center stock CS31100). Plants from which the seeds were derived from were transformed with the activation tagging pSKI015 plasmid (Weigel et al. 2000; Sparks et al., 2016; Sun et al., 2019). Briefly, mutagenized seeds were surface sterilized in 95 % (v/v) ethanol and 20% bleach (v/v), followed by three washes in sterile deionized water. A solution of 0.5 x Murashige and Skoog (MS) basal salt medium with vitamins (PhytoTech Labs, USA) and 1 % (w/v) sucrose was prepared, and the pH of the solution was adjusted to 5.7. After adding 0.5% agar (w/v) (Sigma-Aldrich), the solution was autoclaved and allowed to cool to room temperature. Upon reaching 55ºC, a stock solution of 10 mM LatB (CalBiochem-EMD Chemicals) in 100% Dimethyl Sulfoxide (DMSO) was added to make a final LatB concentration of 100 nM. Sterilized seeds were suspended in the MS-agar-LatB medium and gently swirled to evenly distribute the seed. The seed-MS-agar-LatB mixture was poured to a thickness of 2 mm on the base of 10 cm x 10 cm Petri dishes. After keeping plates at 4ºC for 48 h, they were positioned vertically in a Conviron set to 24ºC with a 14 hours light (120 µmol m\textsuperscript{-2}s\textsuperscript{-1})/ 10 hours dark cycle. Six days after transfer to the Conviron growth chamber, seedlings that exhibited severe growth inhibition were transplanted to LatB-free media and grown to maturity.

For LatB hypersensitivity assays, seed from selected plants and the \textit{hlb2} mutant were planted on the surface of a 3 mm layer of polymerized 0.5 x MS - 1% agar growth media in gridded square 10 cm \times 10 cm Petri plates and grown in the same Conviron used for screening. Four-day-old seedlings with primary roots that were about 1 cm long were selected. Selected seedlings were transplanted to a new set of square Petri dishes containing MS medium supplemented with 50 nM LatB or MS supplemented with the appropriate volume of LatB solvent (i.e. DMSO). During transplant, the tip of the root was positioned at the grid line of the Petri dish and maintained in a vertical orientation. Four days after transplanting, images of the roots were captured with a Nikon Insight.
digital camera mounted on a copy stand. Primary root length was expressed as the
distance between the position of the root tip 4 days after transplant and the grid line
where the root tip was positioned during transplant (Supplemental Figure 1).

To identify the \textit{HLB2} gene, homozygous \textit{hlb2} (Col-0 ecotype) was out-crossed to
the Landsberg \textit{erecta} ecotype to generate seeds for map-based cloning because
attempts to identify the responsible mutation for \textit{hlb2} phenotype using TAIL-PCR had
been unsuccessful. Segregating F2 seedlings were surface sterilized as described
above and grown for three days. These seedlings were then transferred to MS media
containing 50 nM LatB and root lengths were marked on the plates to track root growth.
Transferred seedlings were grown vertically for an additional four days in the growth
chamber and seedlings that showed root hypersensitivity to 50 nM LatB were selected
for mapping. Briefly, DNA was extracted (Edwards et al., 1991) from about 2600 LatB
sensitive seedlings and cloned with simple sequence length polymorphism (SSLP) and
cleavage of amplified polymorphic site (CAPS) markers to chromosome one between
\textit{AT1G02740} and \textit{AT1G03410} loci (Lukowitz et al., 2000), which spanned 248 kb with 77
annotated genes. Primers were then designed to several candidate genes based on
the sequenced 248 kb region. Nucleotide sequencing revealed that \textit{hlb2} mutation had a
10 base pair deletion (Chr1 position 720,152- 720,161) in exon 14 of the \textit{AT1G03060}
gene. In addition, we obtained a T-DNA insertional mutant (SALK_065311) from the
ABRC with predicted insertion in \textit{SPI}. After genotyping, SALK_065311 was subjected to
similar LatB hypersensitivity and growth assays as \textit{hlb2} as aforementioned. Allelism
was determined by examining the F1 progeny from a cross between \textit{hlb2} ×
SALK_065311. Following the nomenclature of Steffens et al. (2015), SALK_065311 and
\textit{hlb2} were named \textit{spi-3} and \textit{spi-5}, respectively.

\textbf{Generation of Fluorescent Protein-Tagged Constructs and Plant Lines}

The \textit{SPI} protein was tagged at the C-terminus with the fluorescent protein 3x-YPet by
the recombineering method (Zhou et al., 2011). \textit{Agrobacterium tumefaciens} UIA143
pMP90 harboring a SPI-YPet recombineering construct was used to transform the \textit{spi-5}
mutant by the floral dip method (Clough and Bent, 1998). Transgenic lines were isolated
based on resistance to kanamycin and restoration of wild-type root length. Presence of the SPI-YPet transgene was amplified with primers (5'-'ATTCCACAAGCAACCAGTCAC-3' and 5'-AACAGAGTTGAGAGTGGCTCG-3') and sequenced to confirm the correct configuration. Final validation of SPI-YPet expression was accomplished by screening selected lines under the confocal microscope for YPet fluorescence and complementation of the short root hair phenotype.

SCAR2-mCherry lines were also generated by recombineering in which an mCherry tag was fused to an internal region of SCAR2 (Sharan et al., 2009). The mCherry tag was inserted after amino acid 585 in SCAR2 to generate SCAR2-i2mCherry. Recombineering primers were designed as follows: the forward primer contained 50 SCAR2 nucleotides upstream from the insertion site of SCAR2 and 18 nucleotides of the 5' end of the mCherry cassette, which had a 5x Glycine, 1 Alanine linker. The reverse primer contained 50 nucleotides after the insert site of SCAR2 and 24 nucleotides of the 3' end of the mCherry cassette. The JAtY75L14 clone was transformed into the recombineering competent E.coli strain SW105 and recombined with the SCAR2-i2mCherry recombineering cassettes. A flippase recombination reaction removed the ampicillin resistance marker. The sequence between the two test primers was verified by DNA sequencing. The clones were transformed into Agrobacterium and then into scar1234 plants (Dyachok et al., 2008). Because both scar1234 plants and the recombineering clones were Basta resistant, plants on MS plates were screened by eye for rescued trichome phenotypes. Rescued plants were genotyped with the recombineering test primers and screened for fluorescence with a confocal microscope.

The BRK1 promoter: BRK1-YFP introduced into the brk1-1 mutant and ARPC5-GFP are described in Dyachok et al. (2008) and Yanagisawa et al. (2015), respectively. For the BRK1-mRuby3 construct, the fluorescent protein 3x-mRUBY3 was tagged with a C-terminal linker (10 Alanine, Glycine) using Thermo Fisher Scientific GeneArt to include BamH1 and XbaI sites at its 5’ and 3’ ends, respectively. Codon optimization for Arabidopsis was performed on the 3x-mRUBY3 and internal linkers prior to synthesis. The 3X-mRuby3 fragment was inserted as a BamH1/XbaI into the plasmid pBRK:YFPpEZRK (Dyachok et al., 2008). The Agrobacterium floral dip method (Clough
and Bent, 1998) was used to transform \( brk1-2 \) plants, and transformation was confirmed when this construct fully restored defective \( brk1-2 \) trichomes.

Plants expressing \( BRK1-YFP \) were crossed with \( spi-5 \) to generate \( BRK1-YFP \) in \( spi-5 \) lines. In parallel, plants expressing \( SPI-YPet \) were crossed with \( brk1 \) to obtain \( SPI-YPet \) in \( brk1 \) lines. \( Spi-5 \) was directly transformed with the \( UBQ10:mGFP-Lifeact \) construct by the floral dip method (Clough and Bent, 1998). \( Spi-5 \) expressing \( SEC-RFP \) was generated by crossing \( spi-5 \) with \( SEC7-RFP \)-expressing wild-type plants and progeny in subsequent generations that exhibited fluorescence and the \( spi \) root hair phenotypes were selected for analysis.

### Generation of Double Mutants and Dual Fluorescent Protein-Labeled Plant Lines

\( Arp2 \) (SALK_077920), \( arp3 \) (SALK_010045), \( arpc5 \) (SALK_123936) and \( brk1-1 \) (Djakovic, 2006, Le et al., 2006, Li et al., 2003) were crossed with \( spi-5 \) to generate double mutants. Single mutants were obtained from the ABRC and genotyped to verify zygosity. Progeny from crosses were genotyped for the presence of the double mutation by PCR-based approaches using the primers: ARP2LP 5’-CGCTTTTTACTCCGTTAAACC-3’ ARP2RP 5’-CATGCAGTAGAAGGAAGGCTGG-3’ ARP3LP 5’-AATTGCTGGCAAGATGTCAC-3’ ARP3RP 5’-AGCTCTTCGTGTATTTGGAAGCTGG-3’ ARP3LP 5’-CTTTTGAAATGGAATGTCAC-3’. The \( spi-5 \) mutation in the double mutants was confirmed using primers SPIF 5’-CAGTTACGAGGATCTTAGTCTT-3’ and SPIR 5’-CCAGTCCAGAAGGACATGTTC-3’. For \( brk1-1 \), which had a single base pair deletion (Djakovic, 2006), PCR products were amplified using BRK1F 5’-TTGAATCGAAAAAGGGCGAAT-3’ and BRK1R 5’-CGAAATCGAGAAAACTCCAAAA-3’ and the resulting fragment was sequenced to ensure deletion using the BigDye Terminator chemistry (v3.1, Thermo Fisher Scientific) on an ABI3730XL DNA sequencer (Thermo Fisher Scientific) following the manufacturer’s instruction.

To generate plant lines co-expressing \( SPI-YPet \) and \( BRK1-mRuby3 \), \( SPI-YPet \) in \( spi-5 \) was crossed with \( BRK1-mRuby3 \) in \( brk1-2 \). Progeny from subsequent generations that exhibited yellow and red fluorescence and rescued root hair and trichome...
phenotypes were selected for analysis. For lines expressing SPI-YPet and mRuby3-Lifeact (Bascom et al., 2018b), SPI-YPet in spi-5 was directly transformed with a UBQ10:Lifeact-mRuby3 construct. Seedlings that showed both YPet and mRuby3 fluorescence were selected under the confocal microscope.

**Evaluation of Root Hair Length and Planar Polarity**

Seeds of mutants were surface-sterilized in ethanol and bleach as described above. To evaluate root hair length and planar polarity, seeds were planted on 48 × 64 mm coverslips coated with 0.5 x MS, 1% sucrose and 0.4 % (w/v) Gelzan™ CM (Sigma Aldrich, USA) according to Dyachok et al. (2016). Coverslips were placed in 9 cm round Petri dishes and stratified in 4°C for 48 h. After stratification, the coverslip system with planted seed were kept in a 24°C growth chamber with 14 hours light (120 μmol m⁻² s⁻¹)/10 hours dark cycle for 5 to 6 days.

To quantify root hair length, fully-grown root hairs from a region of the primary root located between 5 to 15 mm from the root tip were photographed with a Nikon SMZ1500 stereomicroscope. For planar polarity measurements, images of trichoblasts in which the apical and basal end walls were clearly visible were acquired with a Nikon Eclipse TE300 inverted microscope using a 20x objective. Root hair lengths were extracted from digital images using ImageJ (v1.51) software (https://imagej.nih.gov/ij/).

For planar polarity, the distance between the basal trichoblast wall and base of the emerging root hair (a) and the length of the trichoblast (b) were obtained using Image J. The ratio of a/b was used as an indicator of relative root hair position with a value of 0 indicative of root hairs emerging at the basal-most end of the trichoblast and 1 indicative of root hairs emerging at the apical-most end of the trichoblast (Fischer et al., 2006).

Box and violin plots were drawn on R software (R Core Team, 2019) using ggplot function in ggplot2 package (Wickham, 2016). Two-way Analysis of Variance (ANOVA) statistical analysis and Tukey’s post-hoc tests were performed on R software (R Core Team, 2019) using car (Fox and Weisberg, 2019) and agricolae (de Mendiburu, 2020) packages. Pairwise non-parametric Kolmogorov-Smirnov tests were done using ks test function available in the basic package (R Core Team, 2019).
**Microscopy and Image Analysis**

Live cell imaging of root hairs using confocal microscopy was performed on 4 or 5-day-old seedlings grown on the 48 mm × 64 mm coverslip system described above. Coverslips with the seedlings were placed horizontally on the stage of an inverted Leica SPX-8 point scanning confocal microscope (Leica Microsystems, Buffalo Grove, Illinois) or an UltraView ERS spinning-disc confocal microscope (Perkin Elmer Life and Analytical Sciences, Waltham, Massachusetts) equipped with 40 × water (numerical aperture=1.10) or 100 × oil (numerical aperture=1.40) immersion objectives. SPI-YPet and YFP-BRK1 were imaged by illuminating roots growing along the coverslip surface with the 514 nm line of the SPX-8 Argon laser and emission detected at 527 nm. Images of root hairs expressing SCAR2-mCherry, BRK1-mRuby3, Lifeact-mRuby and SEC7-RFP were acquired through illumination with the tunable SP8-X white light laser (560-580 nm) and detecting emission at 610 nm. Excitation and emission parameters for GFP (GFP-ARPC5 and mGFP-Lifeact) were 488 nm and 510 nm, respectively. Time-lapse movies or single time point images were collected using Volocity acquisition version 6.3.5 (Improvision) or SPX-8 LAS software, for the UltraView and Leica SP8-X, respectively.

Quantification of fluorescence from root hair images was conducted on 8-bit confocal images acquired at a single fixed focal plane that spanned the median of the cell (SPI-YPet, BRK1-YFP and SEC-RFP) or from maximum projected images (mGFP-Lifeact). For SPI-YPet and SEC-RFP, an oval region of interest (ROI) at the root hair tip was drawn and mean fluorescence within this area was acquired using Image J. Fluorescence was expressed as the ratio of mean fluorescence within the root tip ROI to background fluorescence. For SPI-YPet, the background used was the region adjacent, but outside the root hair tip (Figure 1C) while for SEC-RFP, the background used was an area on the sub-apical region of the root hair tip (Figure 2F). For BRK1-YFP, a ROI was drawn along the apical-most root hair tip that was about 20 pixels-wide using the selection brush tool of image J. The ratio of fluorescence within this area to background fluorescence was obtained (Figure 2C). Root hair growth rate data was
derived from the same root hair images in which fluorescence images were acquired. For the former, an image of a root hair was taken at time 0 and every 5 minutes thereafter. The growth rate refers to the displacement of the root hair tip in μm divided by time elapsed (min).

To quantify tip-focused F-actin, 25 optical sections were taken at 0.5 μm intervals using the UltraView spinning-disc confocal microscope. Raw Ultraview Z-stacks were exported to Imaris image analysis software version 9.2.0 (Bitplane). Transverse sections of the root hair tip were obtained from Z-stacks using the surpass view interface of the Imaris software and exported as 8-bit TIFF files. From these images, an ROI spanning the circular area of the root hair tip was drawn using image J and mean fluorescence was extracted (Figure 3O). The ratio of the tip fluorescence to background was obtained from 18-21 root hairs.

For SPI-YPet and BRK1-YFP, a scatter plot to determine the relationship between growth rate and tip-focused fluorescence was done in R (R Core Team, 2019) using ggplot function in ggplot2 package (Wickham, 2016). Linear regression analysis were performed using lme4 package on R software (Bates et al., 2015, R Core Team, 2019).

Trichomes were imaged using a Hitachi Table top TM3000 scanning electron microscope or with a Nikon SMZ 1500 stereo microscope. Images of trichomes from newly emerging leaves of 3-week-old plants were collected and branch length was measured using Image J.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SPI (AT5G61350), BRK1 (AT2G22640), ARP2 (AT3G27000), ARP3 (AT1G13180), ARPC5 (AT4G01710), SCAR1 (AT2G34150), SCAR2 (AT2G38440), SCAR3 (AT1G29170), SCAR4 (AT5G01730)

Supplemental Data
Supplemental Figure 1. Primary Root Growth of hlb2 Seedlings is Hypersensitive to LatB.

Supplemental Figure 2. HLB2 Encodes the Beach Domain Containing Protein, SPIRRIIG.

Supplemental Figure 3. SPI-YPet construct complements spi

Supplemental Figure 4. ARPC5-GFP is localized uniformly in the cytoplasm of developing root hairs.

Supplemental Figure 5. Root hair phenotypes of spi arp2/3 mutants

Supplemental Figure 6. SPIpro:SPI-YPet complements the primary root hypersensitivity to LatB, short root hair and distorted trichome phenotypes of spi.

Supplemental Figure 7. Distorted trichome defects of spi arp2, spi arp3, spi arpc5 resemble spi.

Supplemental Movie 1. Time-Lapse Confocal Microscopy of SPI-YPet in a Rapidly Elongating Root Hair.

Supplemental Movie 2. Time-Lapse Confocal Microscopy of SPI-YPet in a Root Hair Bulge Transitioning to Tip Growth.

Supplemental Movie 3. Time-Lapse Confocal Microscopy of SPI-YPet in a Root Hair during Termination of Tip Growth.

Supplemental Movie 4. Spinning-Disc Confocal Microscopy of Lifeact-mGFP in Elongating Root Hairs of Wild type and spi.
Supplemental Movie 5. Spinning-Disc Confocal Microscopy of a Rapidly Elongating Root Hair Co-Expressing SPI-YPet and mRuby3-Lifeact.

Supplemental Movie 6. Time-Lapse Confocal Microscopy of BRK1-YFP Root Hair Bulge Transitioning to Tip Growth.

Supplemental Movie 7. Time-Lapse Confocal Microscopy of a Root Hair Bulge Transitioning to Tip Growth and Co-Expressing SPI-YPet and mRuby3-Lifeact.

Supplemental Movie 8. Time-Lapse Confocal Microscopy of a spi Root Hair Expressing BRK1-YFP.

Supplemental Table 1. Statistical results for non-parametric Kolmogorov-Smirnov test for data in Figure 7C on root hair length in wild-type, spi, arp and spi arp

Supplemental Table 2. Statistical results for non-parametric Kolmogorov-Smirnov test for data in Figure 7D on root hair planar polarity in wild-type, spi, arp and spi arp.

ACKNOWLEDGMENTS
We thank Drs. Jose Alonso and Anna Stepanova (North Carolina State University) for assistance with recombineering and Dr. Magdalena Bezanilla (Dartmouth College) for the Lifeact-mRuby construct. This work was supported by the National Aeronautics and Space Administration (NASA grants 80NSSC19K0129 and 80NSSC18K1462) and the Noble Research Institute to E.B.B.) and by the National Science Foundation (NSF MCB Grant No.1121893) to D.B.S. We also thank Dr. Larry M. York (Noble Research Institute) and Dr. Wayne Versaw (Texas A.M. University) for critical comments on the manuscript.

AUTHOR CONTRIBUTIONS
S.C., T.K, D.B.S and E.B.B. conceptualized and designed the research, and analyzed and interpreted the data. S.C., J.A.S. and E.B.B. conducted root hair, primary root growth and trichome assays, microscopy, image and statistical analysis of data. T.K., J.A.S., B.R.K., E.L.M. and D.B.S. generated various research reagents. All authors contributed to writing the manuscript.

**FIGURE LEGENDS**

**Figure 1.** A Functional SPI-YPet Fusion Localizes to the Tips of Growing Root Hairs.

(A) SPI-YPet rescues the short root hair phenotype of *spi*. Bar = 100 μm.

(B) Low magnification image shows that SPI-YPet signal is most prominent at the tips of rapidly expanding root hairs (arrows). SPI-YPet signal is weak during initiation/early bulge formation and mature root hairs that have terminated tip growth (**). Bar = 50 μm

(C) High magnification images of single root hairs during bulge formation until tip growth termination. SPI-YPet is enriched at the tip of root hairs that are rapidly growing or transitioning to tip growth. Faint SPI-YPet signal is found in bulging root hairs or those that have stopped elongating (**). Bar = 10 μm.

(D) Scatter plot showing correlation analysis of root hair tip SPI-YPet fluorescence and root hair tip growth. The mean fluorescence in the oval in region 1 divided by the oval in region 2 as shown in panel B represents the fluorescence ratio in the Y axis. Line shows linear regression fit with $R^2$ value = 0.308 and $p= 1.72 \times 10^{-5}$. (n=5-7 root hairs per time point).

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(C) SPI-YPet signal is undetectable in the elongation zone of solvent control-treated roots. Within 10 min of BFA application, distinct SPI-YPet fluorescent agglomerates
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(D) and (E) The bulk secretory marker, SEC-RFP, accumulates at the tips of growing wild-type root hairs (arrowhead), but is absent in spi root hairs (**).

(F) Box plot of SEC-RFP root hair tip accumulation expressed as fluorescence ratio. Ratio values were obtained by dividing mean fluorescence in oval region in 1 over region 2 (inset). Box limits indicate 25th percentile and 75th percentile, horizontal line is the mean and whiskers display min and max values. Asterisk (*** Indicates statistical significance (p<0.001) as determined by Student’s T-test. Bars = 10 μm (A, B and D); bars in (C) = 50 μm. Means (n = 8-10 root hairs) ± SE

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(A) to (D) Time course of F-actin organization in a wild-type root hair from bulge formation to rapid tip growth. Weakly fluorescing F-actin networks (**) in the root hair bulge (A) reorganize into prominent tip-focused meshworks as the root hair transitions to rapid tip growth (arrows in B to D).

(E) Tip-focused F-actin meshworks (arrow) remain prominent in a long, rapidly elongating wild-type root hair.

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(K) to (N) F-actin organization in slow-growing spi root hairs. Some spi root hairs show the tip-focused F-actin meshworks typically observed in wild type (arrow, K, L). However, tip-focused F-actin meshworks in slow-growing spi root hairs either dissipate (**, M) or prematurely form thick F-actin bundles that protrude to the tip (arrow, N).

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the root hair tip (oval in 1) to background (oval in 2) was used to quantify tip-focused F-
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(P) Box plot showing tip-focused F-actin fluorescence ratio. Box limits indicate 25<sup>th</sup>
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hair apex (arrows). Bars = 10 μm.

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(A) Maximum projection confocal micrograph of the elongation and maturation zone of
an *Arabidopsis* primary root expressing a functional BRK1-YFP fusion. The image was
generated by merging 50 Z sections taken at 0.5 μm intervals. BRK1-YFP accumulates
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(B) Time course of BRK1-YFP depletion in a developing root hair. BRK1-YFP signal
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dissipates as the root hair undergoes rapid tip growth (35-50 min **). Bar = 20 μm.

(C) A scatter plot showing an inverse relationship between BRK1-YFP signal and root
hair growth rate. Ratio of fluorescence of BRK1-YFP (a) to background (b) (inset) was
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(D) Time course of SCAR2-mCherry in a developing root hair. Like BRK1-YFP, SCAR2-
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showing apical shift in root hair position of *brk1* compared to wild type, and
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the end walls of the trichoblast and asterisks (*** mark the basal wall of the emerged root hair.

(F) Violin plot of root hair planar polarity in wild type, brk1, scar1234, BRK1-YFP in brk1 and SCAR2-mCherry in scar1234 genotypes. Relative root hair position was obtained by taking the ratio of the distance from the basal trichoblast wall (bottom arrows in E) to the basal root hair wall (*** over the total length of the trichoblasts (i.e. length between the two arrows). The plot illustrates kernel probability density in which the width represents distribution of data points. The black dot is the mean and whiskers display min and max values. Statistical significance was determined using non-parametric, two sample Kolmogorov-Smirnov (KS) test. Wild type versus brk1 (***p < 2.2 x 10^{-16}); brk1 versus BRK1-YFP in brk1 (***p=6.812 x 10^{-18}); Wild type versus BRK1-YFP (p=0.202 not significant, ns); Wild type versus scar1234 (***p < 2.2 x 10^{-16}); brk1 versus scar1234 (p= 0.699, ns); scar1234 versus SCAR2-mCherry in scar1234 (**p < 2.2 x 10^{-16}); Wild type versus SCAR2-mCherry in scar1234 (*p=0.012). n = 90-117 root hairs ±S.E.

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(A) Dual imaging of BRK1-mRuby3 (arrow) and SPI-YPet shows that dissipation of BRK1 (**) coincides with accumulation of SPI (arrow) as root hairs transition to rapid tip growth.

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