A Sec14p-nodulin domain phosphatidylinositol transfer protein polarizes membrane growth of Arabidopsis thaliana root hairs

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Phosphatidylinositol (PtdIns) transfer proteins (PITPs) regulate signaling interfaces between lipid metabolism and membrane trafficking. Herein, we demonstrate that AtSfh1p, a member of a large and uncharacterized Arabidopsis thaliana Sec14p-nodulin domain family, is a PITP that regulates a specific stage in root hair development. AtSfh1p localizes along the root hair plasma membrane and is enriched in discrete plasma membrane domains and in the root hair tip cytoplasm. This localization pattern recapitulates that visualized for PtdIns(4,5)P₂ in developing root hairs. Gene ablation experiments show AtSfh1p nullizygosity compromises polarized root hair expansion in a manner that coincides with loss of tip-directed PtdIns(4,5)P₂ dispersion of secretory vesicles from the tip cytoplasm, loss of the tip f-actin network, and manifest disorganization of the root hair microtubule cytoskeleton. Derangement of tip-directed Ca²⁺ gradients is also apparent and results from isotropic influx of Ca²⁺ from the extracellular milieu. We propose AtSfh1p regulates intracellular and plasma membrane phosphoinositide polarity landmarks that focus membrane trafficking, Ca²⁺ signaling, and cytoskeleton functions to the growing root hair apex. We further suggest that Sec14p-nodulin domain proteins represent a family of regulators of polarized membrane growth in plants.

Introduction

Sec14p, the major yeast phosphatidylinositol (PtdIns) transfer protein (PITP), regulates an essential interface between lipid metabolism and protein transport from Golgi membranes to the cell surface (Bankaitis et al., 1990; Cleves et al., 1991a,b; Xie et al., 1998). This regulatory circuit, termed the Sec14p pathway, defines a signaling cascade that involves functionally uncharacterized proteins of the oxysterol binding protein family such as Kes1p (Fang et al., 1996; Li et al., 2002) and ADP-ribosylation factor GTPase activating proteins (Yanagisawa et al., 2002). These regulatory interactions couple lipid signaling to the function of core components of the vesicle trafficking machinery, but precisely how this occurs remains unknown.

Mammals express at least three soluble PITPs: PITPα, PITPβ, and rdgBβ; and all of these share primary sequence homology to each other (for review see Routt and Bankaitis, 2004). The mammalian PITP module is found throughout metazoans and is structurally unrelated to yeast PITPs (Sha et al., 1998; Yoder et al., 2001). Gene ablation experiments in mice, although suggesting an essential housekeeping function for PITPβ, demonstrate that PITPα nullizygosity results in chylomicron retention disorder, severe hypoglycemia, and a fulminating spinocerebellar neurodegenerative disease (Alb et al., 2002, 2003). As at least some forms of human chylomicron retention disease are caused by null mutations in the Sar1b GTPase that regulates coassembly of COPII coat components with ER cargo (Jones et al., 2003), PITPα is suggested to regulate a Sar1b-GTPase activating protein function on the enterocyte ER
surface in the chylomycin biogenic pathway (Bankaitis et al., 2004). Indeed, the hypothesis for PITPs function in chylomycin trafficking shares basic features with that proposed for Sec14p binding of phosphatidylinositol are highly conserved (Phillips et al., 1999). Primary sequence alignment of the COOH-terminal 70 AtSFH nodulin domain residues with those of the Nlj16 nodulin (Szczyglowski et al., 1997). Conserved identities and similarities are highlighted. Schematic illustration of the domain organization of four members of this family from the legume Lotus japonicus is also shown. These LjPITPs exhibit COOH-terminal Nlj16 nodulin domains.

Although PITPs exist in higher plants (Jouannic et al., 1998; Kearns et al., 1998a), there has been no systematic functional analysis of them. Herein, we describe a large and novel family of Sec14p-nodulin domain proteins in Arabidopsis thaliana. We show that the Sec14p domains of these proteins share functional properties consistent with those of Sec14p-like PITPs and report the first analysis of the biological function of any Sec14p-like protein in plants. We demonstrate that loss of AtSfh1p, a membrane-bound Sec14p-nodulin protein, dramatically compromises polarized root hair membrane trafficking. Derangement of polarized membrane growth occurs after the site of root hair emergence has been correctly determined and emergence initiated. The collective data suggest AtSfh1p generates phosphoinositide (PIP) landmarks that focus membrane delivery to the root hair tip plasma membrane in a manner that depends on the actin cytoskeleton. The results further suggest that the polarized secretory pathway establishes a tip-directed Ca\(^{2+}\) gradient that cues microtubule (MT) organization in a manner that further reinforces tip-directed membrane trafficking. The collective data describe the functional characterization of the role for a novel membrane-associated PITP in execution of developmentally regulated polarized membrane trafficking pathway.

### Results

#### A novel family of Sec14p-nodulin domain proteins

A search of the National Center for Biotechnology Information and The Arabidopsis Information Resource databases identified 31 homologous A. thaliana sequences when the Sec14p primary sequence was queried. These sequences each exhibit a 239-residue domain that shares significant primary sequence homology with the Sec14p LBD-like sequences. 11 of these 14 sequences represent proteins where an NH\(_2\)-terminal Sec14p domain is joined to a COOH-terminal Nlj16 nodulin domains.
type morphology to Sec14p-deficient cells (Fig. 2 C). The toroid structures observed in Sec14p-deficient Golgi membranes (Fig. 2 B) and normal or AtSfh6p-LBDs extended to restoration of invertase secretion (Fig. 2 A) and haploid-lethal sec14-1 ts alleles (not depicted). (C) YEp(AtSFH1-LBD) positive control, YEp(AtSFH2-LBD), and YEp(AtSFH2-LBD) were assayed, as indicated. The PtdIns- and PtdCho-transfer assays used 2 and 1 mg of cytosol, respectively. (E) PIP analyses. Isogenic derivatives of the sec14Δ yeast strain CTY303 carrying designated YEp plasmids were radiolabeled for 18 h at 25°C with 20 μCi/ml [3H]inositol. PIPs were extracted, deacylated, and quantified. PtdIns-3-phosphate, PtdIns-4-phosphate, and PtdIns(4,5)P2 are as indicated; n = 6. YEp(URA3) and YEp(SEC14) derivatives served as negative and positive controls (white bars and black bars, respectively), whereas the YEp(AtSFH1-LBD) values are in gray bars. All PIP levels were increased in YEp(SEC14) and YEp(AtSFH1-LBD) derivative strains relative to the YEp(URA3) negative control (P < 0.001).

**Figure 2.** Sec14p-like LBDs exhibit intrinsic PITP activities. (A) Isogenic sec14-1 ts and sec14-1 spo14Δ yeast strains carrying the indicated YEp plasmids were spotted in 10-fold dilution series onto agar plates and incubated at the restrictive temperature of 37°C. YEp(URA3) and YEp(SEC14) derivatives served as negative and positive controls. (B) Invertase secretion indices (secreted invertase/total invertase) are shown for sec14-1 ts strains carrying the designated YEp plasmids at 37°C. YEp(URA3) and YEp(SEC14) derivatives served as negative and positive controls. (C) Electron micrographs of sec14-1 spo14Δ yeast strains carrying the designated YEp plasmids after 37°C challenge for 2 h. Bars = 5 μm. (D) PtdCho- (right; n = 3) and PtdIns-transfer assays (n = 7; Li et al., 2000). Cytosols prepared from the sec14Δ yeast strain CTY303 harboring the YEp(URA3) negative control, the YEp(SEC14) positive control, YEp(AtSFH1-LBD), and YEp(AtSFH2-LBD) were assayed, as indicated. The PtdIns- and PtdCho-transfer assays used 2 and 1 mg of cytosol, respectively. (E) PIP analyses. Isogenic derivatives of the sec14Δ yeast strain CTY303 carrying designated YEp plasmids were radiolabeled for 18 h at 25°C with 20 μCi/ml [3H]inositol. PIPs were extracted, deacylated, and quantified. PtdIns-3-phosphate, PtdIns-4-phosphate, and PtdIns(4,5)P2 are as indicated; n = 6. YEp(URA3) and YEp(SEC14) derivatives served as negative and positive controls (white bars and black bars, respectively), whereas the YEp(AtSFH1-LBD) values are in gray bars. All PIP levels were increased in YEp(SEC14) and YEp(AtSFH1-LBD) derivative strains relative to the YEp(URA3) negative control (P < 0.001).

**AtSfhp-LBDs and Sec14p share functional properties**

Expression in yeast of AtSfh1p-, AtSfh2p-, AtSfh4p-, or AtSfh6p-LBDs rescued growth defects associated with sec14-1 ts (Fig. 2 A) and haploid-lethal sec14Δ alleles (not depicted). These results were scored in phospholipase D (PLD)–proficient (SPO14) or –deficient (spo14Δ) genetic backgrounds. PLD deficiencies exacerbate Sec14p defects, and assessment of rescue in both SPO14 and spo14Δ genetic backgrounds reports quality of rescue. As an example, expression of AtSFH19 (AT5G47730.1) or AtSFH20 (AT1G01630.1) (i.e., representatives of the second Sec14p homology group) rescued sec14-1 ts alleles in SPO14 but not spo14Δ yeast strains (Fig. 2 A). Rescue of sec14 growth defects by AtSfh1p-, AtSfh2p-, AtSfh4p-, or AtSfh6p-LBDs extended to restoration of invertase secretion from Sec14p-deficient Golgi membranes (Fig. 2 B) and normal morphology to Sec14p-deficient cells (Fig. 2 C). The toroid structures observed in sec14-1 ts cells incubated at restrictive temperature represent defective Golgi compartments engorged with secretory cargo. AtSfh1p-LBD expression restores wild-type morphology to >90% of sec14-1 ts cells (Fig. 2 C).

AtSfhp-LBDs exhibit intrinsic PITP biochemical activities. AtSfh1p- and AtSfh2p-LBDs catalyzed phosphatidylinolypeine (PtdCho)- and PtdIns-transfer in vitro (Fig. 2 D). Significance of the PtdIns-transfer activities of AtSfh1p- and AtSfh2p-LBD was confirmed in measurements of PIP synthesis in a Sec14p-deficient yeast strain with basal PIP levels that expressed each LBD. In these experiments, the appropriate yeast strains were radiolabeled to steady-state with [3H]inositol. PIPs were extracted, deacylated, and quantified. PtdIns-3-phosphate, PtdIns-4-phosphate, and PtdIns(4,5)P2 are as indicated; n = 6. YEp(URA3) and YEp(SEC14) derivatives served as negative and positive controls (white bars and black bars, respectively), whereas the YEp(AtSFH1-LBD) values are in gray bars. All PIP levels were increased in YEp(SEC14) and YEp(AtSFH1-LBD) derivative strains relative to the YEp(URA3) negative control (P < 0.001).

**AtSFH1 function is required for proper root hair elongation**

Sec14p-nodulin domain proteins are uncharacterized and expansion of this family suggests tissue-specific functions for its members. AtSfh1p was chosen for detailed analysis because the AtSfh1p-LBD is most homologous to Sec14p. RT-PCR analyses indicated essentially root-specific expression of AtSFH1 (unpublished data), a result in accord with microarray data (http://www.cbs.umn.edu/arabidopsis/). β-Glucuronidase (GUS) histochemical staining confirmed and extended these re-
Hair producing epidermal cell files (arrows) exhibit robust GUS-active trichoblast cell files. (D) Root hydathodes (F), apical shoot meristem (G), and apical cells of the root cap (H). Bars: (A and B) 0.12 cm; (C–H) 50 μm.

Figure 3. Tissue-specific AtSfh1p expression. Otherwise wild-type transgenic plants stained for GUS expressed from a promoterless construct (A) or the AtSFH1 promoter (PAtSFH1::GUS) (B). (C) PAtSFH1::GUS expression is robust in root. Arrows denote GUS-active trichoblast cell files. (D) Root hair producing epidermal cell files (arrows) exhibit robust PAtSFH1::GUS expression. (E) GUS activity is recorded both in trichoblast cell bodies and root hairs. Arrows denote cell plates. PAtSFH1::GUS activity in cotyledon hydathodes (F), apical shoot meristem (G), and apical cells of the root cap (H). Results. AtSFH1 was expressed solely in root trichoblast cell files engaged in root hair growth (Fig. 3, C–E), hydathodes, shoot apical meristem, and apical cells of the root cap (Fig. 3, F–H).

Atsfh1::T-DNA plants (Fig. 4 A) were substantially normal and fertile. However, mutant plants elaborated short root hairs. Mutant single root hairs from 3-d-old seedlings were one-third the length of age-matched wild-type structures (69 ± 13 vs. 224 ± 51 μm, n = 55) and exhibited half the surface area (3494 ± 902 vs. 6924 ± 1045 μm², n = 55). Mutant and wild-type single root hairs exhibited similar volumes (14866 ± 5550 vs. 17113 ± 3510 μm³, n = 55), as did double root hairs (unpublished data). These disparities persisted throughout the lifetime of the plant (Fig. 4 B). In addition, Atsfh1::T-DNA root hairs appeared flaccid. This observation was in contrast to the rigid profiles exhibited by age-matched wild-type root hairs (Fig. 4 C). Although there is robust AtSFH1 expression in apical cells of the root cap, Atsfh1::T-DNA primary roots exhibit no defects in gravitropism (unpublished data).

Reduced lengths of Atsfh1::T-DNA root hairs reflect failures in polarized membrane growth. High frequencies of Atsfh1::T-DNA root hairs with two growing tips were observed and a significant number exhibited three. Wild-type plants rarely elaborated two growing tips, and we never observed any with three (Fig. 4, C and D). Double root hairs exhibited volumes similar to those of wild-type root hairs, but only 50% of the surface area. The data indicate AtSfh1p-deficient root hairs exhibit impaired secretion efficiency and failure in restricting active growing points to a single site that leads to isotropic root hair cell expansion. Yet, Atsfh1::T-DNA plants correctly specified site of root hair emergence from the distal plasma membrane of parent epidermal cells (Fig. 4 E). Moreover, consistent with the tissue restriction of AtSFH1 expression, nullizygous plants did not exhibit obvious defects in other organs (such as leaf trichomes and pollen tubes) whose development requires polarized membrane trafficking (unpublished data).

Four lines of evidence demonstrate the full spectrum of Atsfh1::T-DNA root hair phenotypes results from a single fully penetrant recessive mutation. First, cross of Atsfh1::T-DNA homozygotes to wild-type plants yielded only wild-type progeny. Second, the root hair phenotype cosegregated with Atsfh1::T-DNA through multiple (>3) backcrosses. Third, transgenic Atsfh1::T-DNA plants bearing ectopic AtSFH1 exhibited normal root hairs (Fig. 4 F). Fourth, examination of 2947 F2 progeny from three independent Atsfh1::T-DNA/Atsfh1::T-DNA X AtSFH1/AtSFH1 crosses yielded 701 mutant (23.8%) and 2,246 (76.2%) wild-type phenotypes, respectively. To assess the functional importance of the Sec14p domain, we generated an NH2-terminal GFP-fusion to the AtSfh1p-LBD that inactivates Sec14p domain activities. GFP-AtSfh1p, when placed in the context of full-length AtSfh1p and expressed in plants, fails to complement Atsfh1::T-DNA (Fig. 4 F). Similarly, a COOH-terminal GFP-fusion that preserves Sec14p domain function, but abuts the nodulin domain, also fails to complement Atsfh1::T-DNA (unpublished data). Thus, both functional Sec14p and nodulin domains are critical for AtSfh1p function in plants.

Localization of AtSfh1p in developing root hairs
The AtSfh1p nodulin domain exhibits high primary sequence identity to the Nlj16 nodulin. As Nlj16 functions as a plasma membrane targeting domain (Kapranov et al., 2001), we expected AtSfh1p would also localize to membranes. Consistent with expectation, the GFP-AtSfh1p chimera (with the caveat that it harbors a nonfunctional Sec14p domain) distributed in an apex-directed spiraling arrangement along the root hair cortical plasma membrane in otherwise wild-type plants (Fig. 5 A, top left; and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). Optical cross sections taken through the root hair at positions removed from the apex also indicated a plasma membrane localization for GFP-AtSfh1p (Fig. 5 A, top right). Optical sectioning of the apex plasma membrane at the root hair tip reported a clear enrichment of GFP-AtSfh1p staining on the plasma membrane at that site as well (Fig. 5 A, bottom panel). That this profile reflects plasma membrane staining was confirmed in FM1-43 double label experiments. Under conditions where FM1-43 selectively labels plasma membrane, FM1-43 and GFP-AtSfh1p staining were coincident (unpublished data). Strong enhancement of GFP-AtSfh1p reporter fluorescence was also recorded in the tip cytoplasm (Fig. 5 A, top left and bottom panel; and Video 1). For the reasons detailed in the section Ultrastructure of the Atsfh1 tip cytoplasm, we interpret this staining to reflect an AtSfh1p pool that is localized on post-Golgi vesicles. Expression of GFP or YFP alone gave diffuse staining (Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1).
PtdIns(4,5)P₂ deficiencies in Atsfh1 root hairs

The ability of AtSfh1p-LBD to stimulate PIP synthesis suggests AtSfh1p mediates PIP-dependent regulation of polarized membrane transport in A. thaliana. Because polarized membrane transport in yeast and plants requires involvement of the actin cytoskeleton and actin dynamics are responsive to PtdIns(4,5)P₂, we focused on the role of AtSfh1p in modulating PtdIns(4,5)P₂ homeostasis. Using a phospholipase Cδ1 (PLCδ1) pleckstrin homology (PH) domain–YFP reporter to infer PtdIns(4,5)P₂ status, we found wild-type root hairs exhibited a tip-directed (4,5)P₂ gradient on the root hair plasma membrane. Indeed, PtdIns(4,5)P₂ was distributed in a pattern similar to that recorded for GFP-AtSfh1p in wild-type root hairs. Discrete PtdIns(4,5)P₂–enriched domains were also recorded along the cortical root hair plasma membrane (Fig. 5 B, top left). Pseudocolor rendering of PHPLCδ1–YFP fluorescence revealed a tip-directed spiraling arrangement along the cortical root hair plasma membrane (Fig. 5 B, top right and bottom left; and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1), and optical sectioning of the tip plasma membrane revealed a high concentration of PtdIns(4,5)P₂ at the very apex of the root hair (Fig. 5 B, bottom right). Again, FM1-43 double-labeling experiments confirmed the plasma membrane localization of the PHPLCδ1–YFP reporter fluorescence (see the following paragraph). Strikingly, as was observed for AtSfh1p-GFP, optical cross sections of the root hair apex demonstrated strong enhancement of PHPLCδ1–YFP reporter fluorescence in the tip cytoplasm.

Given the ability of AtSfh1p-LBD to stimulate PIP synthesis, we anticipated Atsfh1::T-DNA root hairs would exhibit PIP deficiencies. We focused on PtdIns(4,5)P₂ as this phospholipid is an established regulator of polarized membrane trafficking. Indeed, the tip-directed PtdIns(4,5)P₂ gradient was compromised, and the prominent cytoplasmic PHPLCδ1–YFP reporter fluorescence was absent from mutant tip cytoplasm (Fig. 5, C and D; and Videos 3 and 4, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). That PtdIns(4,5)P₂ is enriched at the tip plasma membrane of wild-type root hairs, and that this PtdIns(4,5)P₂ enrichment is lost in mutant tip plasma membrane, was indicated by ratiometric imaging of PHPLCδ1–YFP/FM1-43 fluorescence in double-label experiments. From those experiments, we record a threefold tip enrichment of PtdIns(4,5)P₂ in wild-type root hair tip plasma membrane relative to cortical plasma membrane. We detected loss of PtdIns(4,5)P₂ tip enrichment in the Atsfh1 tip plasma membrane relative to wild-type and estimate a 5–10-fold reduction in relative PtdIns(4,5)P₂ in mutant tip plasma membrane (Fig. 5 E). No obvious PtdIns(4,5)P₂ deficiencies were recorded in the cortical plasma membrane of Atsfh1::T-DNA root hairs.

Ultrastructure of the Atsfh1 tip cytoplasm

Loss of PHPLCδ1–YFP fluorescence staining in the tip cytoplasm of AtSfh1p-deficient root hairs was a striking phenotype. Because incubation of metabolically active wild-type root hairs

Figure 4. AtSfh1p function is essential for proper root hair development. (A) Genomic structure of AtSfh1. Sites of T-DNA insertion and the LBD coding regions are indicated. The Atsfh1::T-DNA allele harbors three T-DNA copies at the single site of insertion. (B) Root hair profiles of wild-type (top) and Atsfh1::T-DNA (bottom) plants. Corresponding profiles of 3-d-old seedlings (left) and adult plants (right) are shown. (C) Nomarski images of wild-type [left] and Atsfh1::T-DNA [right] seedlings. Bars, 200 μm. (D) Frequencies of single, double, and triple root hairs in 3-d-old wild-type [black bars] and Atsfh1::T-DNA [gray bars] seedlings as determined by ESEM of 250 root hairs of each genotype [25 root hairs from each of 10 seedlings]. Frequencies of each class of root hair morphology were determined for each individual seedling and the average frequencies and standard errors are given. [E] Nomarski images of wild-type [left] and Atsfh1::T-DNA [right] root epidermal cells initiating root hair growth. Vertical arrows identify cell plates. Horizontal arrows identify direction of primary root growth. Bars = 50 μm. (F) Root hair profiles of 3-d-old wild-type [left] and Atsfh1::T-DNA [right] seedlings. [left] Atsfh1::T-DNA [*] and transgenic derivative bearing an ectopic wild-type gene (TgAtSFH1). [right] Transgenic Atsfh1::T-DNA seedling expressing an NH₂-terminal [TgGFP-AtSFH1] gene fusion.
for 20 min with FM1-43 yielded robust staining of the tip cytoplasm, in a fashion that recapitulated the pattern of GFP-AtSfh1p and PHPLC fluorescence (unpublished data), we interpreted PHPLC fluorescence in tip cytoplasm to reflect the status of small membrane-enclosed structures in this region. In this regard, tip cytoplasm is enriched for post-Golgi secretory vesicles and is referred to as the vesicle-rich zone (VRZ; Braun et al., 1999). Therefore, we inspected wild-type and mutant root hair apices by electron microscopy. Wild-type and Atsfh1::T-DNA Golgi stack ultrastructures were indistinguishable in appearance (Fig. 5 F) and in physical parameters (Table I). The structural integrity of Atsfh1::T-DNA Golgi membranes suggested these systems are not grossly defective in secretory function. Golgi stack distribution throughout the cytoplasm was also unaffected in the mutant root hairs (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). However, two unusual properties of mutant tip cytoplasm were apparent. First, the concentration of vesicles per unit tip cytoplasm was reduced sixfold in mutant relative to wild-type VRZ (Fig. 5, G and H). We interpret this result, and the loss of tip cytoplasm fluorescence as recorded by the GFP-AtSfh1p and PHPLC reporters (see the section Localization of AtSfh1p in developing root hairs), to indicate a dispersal of vesicles from the VRZ throughout the mutant root hair cytoplasm. Second, dramatic vacuolation of the mutant VRZ and tip cytoplasm was observed (Fig. 5 G).

The tip f-actin cytoskeleton is compromised in Atsfh1 root hairs

Vacuolation of Atsfh1::T-DNA root hair tip cytoplasm recapitulated the effects recorded in root hairs where the actin cytoskeleton was disrupted (Čiamporová et al., 2003). To assess f-actin status in Atsfh1::T-DNA root hairs, we used GFP-talin as a reporter for f-actin. These imaging experiments indicated that wild-type root hairs exhibit a discrete cortical actin meshwork and a tip-concentrated f-actin microfilament network in the VRZ (Fig. 6 A and Video 5, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1), as described previously (Baluška et al., 2000; Smith, 2003). However, selective defects in the f-actin cytoskeleton were apparent in mutant root hairs. Although the cortical actin cytoskeleton of mutant root hairs remained intact, the tip-directed f-actin microfilament compartment was lost (Fig. 6 B and C; and Videos 6 and 7, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). As tip f-actin microfilament networks focus transport vesicle de-
livery to the hair apex (Mathur and Hülskamp, 2002; Ketelaar et al., 2003), defects in this actin network are expected to result in dispersal of transport vesicles throughout the root hair cytoplasm. Indeed, loss of the f-actin microfilament network in Atsfh1::T-DNA root hairs coincides with loss of PHPLCδ1-YFP fluorescence in the tip cytoplasm.

**Isotropic influx of Ca^{2+} into Atsfh1 root hairs**

One critical contributing cue that controls polarized root hair growth is a tip-directed Ca^{2+} gradient. Defects in polarized membrane trafficking are expected to randomize ion (e.g., Ca^{2+}) channel delivery to the plasma membrane, with the consequence that spatial regulation of Ca^{2+} signaling will be compromised. To investigate whether or not dysregulation of Ca^{2+} signaling was occurring in Atsfh1::T-DNA root hairs, we used Indo-1 loading strategies to image Ca^{2+} signaling in wild-type and isogenic Atsfh1::T-DNA root hairs. Striking derangements in Ca^{2+} signaling to the growing apex of the root hair plasma membrane were observed (Fig. 7 A). Ca^{2+} imaging recorded a single tip-focused Ca^{2+} gradient in wild-type root hairs, as reported previously (Wymer et al., 1997), with tip cytoplasmic Ca^{2+} reaching concentrations in excess of 600 nM. Cytoplasmic Ca^{2+} fell to concentrations below 100 nM very rapidly away from the root hair tip. In marked contrast, precocious Ca^{2+} signaling was evident along the Atsfh1 root hair cortical plasma membrane with local Ca^{2+} concentrations reaching 600 nM or greater (Fig. 7 A).

One potential mechanism for spatial dysregulation of Ca^{2+} signaling is the isotropic delivery or distribution of active Ca^{2+} channels in the mutant root hair surface. To test this prediction, we used the scanning ion-selective electrode technique (SIET) to monitor Ca^{2+} fluxes along the root hair surface in wild-type and Atsfh1 nullizygous seedlings (see Materials and methods). Inward Ca^{2+} fluxes exceeding 4 pmol cm^{-2} s^{-1} were recorded around the apex region of growing wild-type root hairs, and Ca^{2+} influx decreased dramatically as the self-referencing probe was positioned away from the root hair apex (Fig. 7, B and C). The flux profiles for Atsfh1 nullizygous single root hairs were dramatically altered, however. Large inward Ca^{2+} fluxes were not restricted to the tip region. Rather, robust fluxes exceeding 4 pmol cm^{-2} s^{-1} were detected all along the root hair surface. These flux profiles are completely congruent with the Indo-1 Ca^{2+} imaging data demonstrating well-defined tip-directed cytoplasmic Ca^{2+} gradient in wild-type, but not in Atsfh1 nullizygous, root hairs.

**Atsfh1 root hairs fail to properly organize MTs**

In addition to the f-actin cytoskeleton, MT networks also function in maintaining polarized tip growth (Bibikova et al., 1999; Baluška et al., 2000; Stevenson et al., 2000; Smith, 2003). MTs appear to consolidate the results of polarized membrane deposition, and MT action in supporting tip growth is spatially regulated by Ca^{2+} gradients (Bibikova et al., 1999; Baluška et al., 2000; Smith, 2003). To assess whether or not the derangements in Ca^{2+} signaling observed in Atsfh1::T-DNA root hairs coincided with functional derangement of MT systems, we probed root hair MT organization in Atsfh1p-deficient root hairs by GFP-MAP4 imaging (Marc et al., 1998). As reported by Smith (2003), cortical MTs were organized into discrete filaments. These MT filaments were arranged in spiraling profiles parallel to the longitudinal axis of the cortical plasma membrane in wild-type root hairs (Fig. 8 A and Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). In contrast, Atsfh1 root hairs exhibited only diffuse GFP-MAP4 staining profiles (Fig. 8, B and C; and Videos 9 and 10, available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1). Neither discrete filaments nor obvious spiraling profiles were seen. However, the body of the trichoblast from which the mutant root hair emanates did exhibit organized MTs (Fig. 8 C and Video 10). Thus, Atsfh1 nullizygous root hairs elaborate defects in MT assembly and/or organization that are limited to the growing root hair itself.

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**Table I. Physical dimensions of wild-type and Atsfh1 nullizygous root hair Golgi membranes**

<table>
<thead>
<tr>
<th>Plant genotype</th>
<th>Cisternal length (nm)</th>
<th>Stack width (nm)</th>
<th>Cisternal width (nm)</th>
<th>Cisternae per stack</th>
</tr>
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<tbody>
<tr>
<td>AtSFH1</td>
<td>750 ± 154 (22)</td>
<td>290 ± 81 (19)</td>
<td>30 ± 7 (22)</td>
<td>4.7 ± 0.4 (22)</td>
</tr>
<tr>
<td>Atsfh1::T-DNA</td>
<td>690 ± 162 (26)</td>
<td>321 ± 85 (24)</td>
<td>34 ± 7 (110)</td>
<td>4.4 ± 0.7 (110)</td>
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Cisternal length represents the longest dimension, and width was calculated for each cisterna in the mid-axis of each Golgi stack. Stack width is measured along the cis-trans axis at the midpoint of each stack. Numbers in parentheses represent the number of Golgi stacks analyzed from a total of three independent plants.
AtSfh1p is to generate PIP landmarks that couple to components of the f-actin cytoskeleton, thereby focusing membrane delivery to the root hair tip plasma membrane. The polarized secretory pathway restricts insertion of cargo (e.g., ion channels) to the root hair apex, thereby establishing a tip-directed Ca$^{2+}$ gradient. This gradient cues an organized MT assembly that further reinforces and maintains tip-directed membrane trafficking (Fig. 9).

The data presented emphasize AtSfh1p-mediated regulation of PIP metabolism. What is the mechanism that underlies such regulation? One simple mechanism is that AtSfh1p directly couples its intrinsic AtSfh1p-LBD PtdIns binding/transfer activity to PtdIns kinase action. By this model, AtSfh1p generates PIP signaling pools that regulate the action of multiple effector proteins that couple to actin dynamics and the activities of signaling enzymes (such as PLD). With regard to PLD, an alternative possibility is that AtSfh1p couples its intrinsic PtdCho-binding/transfer activity to PIP synthesis in the plant. In this regard, single or multiple PLD isoforms represent attractive candidates for AtSfh1p effectors. PLDs are PtdIns(4,5)P$_2$-activated PtdCho hydrolases that generate phosphatidic acid (PtdOH), itself a lipid stimulator of PtdIns-4-phosphate 5-kinase. By presenting PtdCho to PLD, AtSfh1p may initiate a robust positive feedback loop that links PLD activity to PIP synthesis via PtdOH signaling. Obviously, PIP signaling mediated by PtdIns-bound AtSfh1p could cooperate with such a regulatory loop. In support of an AtSfh1p-PLD coupling model, Ohashi et al. (2003) found that PLD$\alpha_1$ activity regulates root hair growth, and that, like AtSfh1p and PtdIns(4,5)P$_2$, PLD$\alpha_1$ is enriched in the tip cytoplasm of growing root hairs. Those results suggest a role for PLD$\alpha_1$, and perhaps other PLD isoforms, in root hair morphogenesis. A general precedent for a PTP-PLD coupling also exists in yeast, where nonclassical Sec14p-like PTPs function to optimally activate PLD. In that case, PTPs do so by stimulating PIP synthesis in the absence of direct PTP-regulated PtdCho signaling (Xie et al., 1998; Li et al., 2000).

How might AtSfh1p-stimulated PtdIns(4,5)P$_2$ synthesis interface with the actin cytoskeleton and membrane trafficking? PtdIns(4,5)P$_2$ synthesis may recruit actin to the Golgi surface and modulate its assembly in a polymerization reaction that potentiates vesicle budding. Evidence for an actin involvement in vesicle formation from mammalian Golgi membranes has been reported (Fucini et al., 2000). However, as neither Golgi morphology nor Golgi distribution is perturbed in AtSfh1::T-DNA root hairs, we conclude that the membrane trafficking defects occur at a post-Golgi stage. We speculate AtSfh1p stimulates PLD activity, and ultimately PtdIns(4,5)P$_2$ synthesis, on formed (or forming) secretory vesicles. Such a regulatory loop promotes an on-demand PtdIns(4,5)P$_2$-driven actin polymerization on the transport vesicles and engages nascent vesicles with an f-actin pool that imposes polarized trafficking of those post-Golgi vesicles to the root hair tip plasma membrane. It follows that defects in such a lipid signaling program would compromise a specific f-actin component dedicated to vesicle trafficking. The consequence is imposition of kinetic and polarity defects on membrane trafficking to the

Discussion

Root hair development requires polarized membrane growth from a precise position on the root epidermal cell plasma membrane. Herein, we demonstrate that loss of AtSfh1p, a PtdCho-binding/transfer protein with the ability to regulate PIP metabolism, deranges root hair growth. AtSfh1p dysfunction compromises tip-directed plasma membrane PtdIns(4,5)P$_2$ and Ca$^{2+}$ gradients, elicits tip actin defects, and disorganizes root hair MT networks. The result is a derangement of polarized membrane growth after the site of root hair emergence has been correctly specified. We propose the primary function of
Atsfh1::T-DNA root hair plasma membrane. GTPases of the Rac/Rho/Cdc42 family, and actin binding proteins (e.g., profilin), are attractive candidates as downstream effectors of AtSfh1p-mediated lipid signaling (Braun et al., 1999; Molendijk et al., 2001). It remains possible, perhaps likely, that AtSfh1p sits at the nexus of more complicated lipid signaling cascades. For example, PtdOH modulates PtdIns 3-OH kinase signaling in polar root tip growth (Anthony et al., 2004).

We further suggest that highly polarized membrane deposition at the root hair plasma membrane of wild-type plants sets the tight Ca^{2+} tip gradient by restricting the distribution of functional Ca^{2+} channels to a focused site on the tip plasma membrane. The net effect is a tip-restricted mode of Ca^{2+} entry into the developing root hair from the extracellular milieu. Our demonstration that nullizygous root hairs engage in precocious and isotropic Ca^{2+} entry across the plasma membrane is consistent with delocalized Ca^{2+} channel distribution. We suggest this is a direct consequence of isotropic fusion of post-Golgi vesicles to the root hair plasma membrane. We also note the observed derangements in Ca^{2+} signaling are not consistent with a role for PtdIns(4,5)P_2-specific phospholipase C–mediated generation of IP_3 in the gating of Atsfh1 nullizygous root hair plasma membrane Ca^{2+} channels. Were IP_3-gated Ca^{2+} channels involved in generating the cytoplasmic Ca^{2+} gradients, reductions in Ca^{2+} fluxes would have been expected, not the robust and isotropic Ca^{2+} influxes recorded. As the Ca^{2+} tip gradient then cues appropriate organization of the root hair MT cytoskeleton so that polarized membrane delivery to the root hair apex is further reinforced and consolidated (Bibikova et al., 1999), spatial derangement of Ca^{2+} signaling accounts for the lack of organized cortical MT assembly in mutant root hairs.

Herein, we identify AtSfh1p as a key regulator of polarized membrane trafficking in root hairs. A corollary to these findings is that AtSfh1p, or other members of the Sec14p-nodulin domain family, serve as attractive targets for intervention when root hair membrane growth is naturally reoriented, i.e., as occurs in legumes in response to Rhizobium NOD factors. The regulation of a Sec14p-nodulin domain protein (LjPLP-IVp) during nodulogenesis in the legume Lotus japonicus provides an interesting case in point. LjPLP-IVp is the Lotus orthologue of AtSfh1p (Fig. 1, A and B). During nodulation, the promoter driving full-length LjPLP-IVp transcription is silenced and an internal bidirectional promoter is activated. The result is high level expression, in nodules, of the Nlj16 nodulin and of antisense transcripts directed against the LjPLP-IVp-LBD coding region (Kapranov et al., 2001). We suggest LjPLP-IV transcriptional reprogramming during nodulogenesis is designed to efficiently subvert the normal highly polarized root hair growth program by three converging mechanisms that functionally inactivate a master polarity regulator (LjPLP-IVp). First, formation of new transcripts encoding a functional LjPLP-IVp is terminated. Second, production of antisense RNAs directed against the LjPLP-IVp-LBD coding region silence existing full-length LjPLP-IVp transcripts encoding. Third, residual LjPLP-IVp activity is suppressed via high level expression of a dominant-interfering plasma membrane–targeting module that represents the nodulin itself.

Finally, our functional characterization of AtSfh1p raises the intriguing possibility that Sec14p-nodulin domain proteins define a family of polarized membrane growth regulators in plants. We suggest individual members of this family are dedicated to specific polarity establishment events such as those involving organogenesis and control of intracellular organelle...
morphogenesis. Because the mammalian genome encodes multiple uncharacterized Sec14p domain proteins, Sec14p domain proteins may represent conserved features of lipid-signaling mechanisms that control polarized membrane biogenic programs in eukaryotic cells.

**Materials and methods**

**Bioinformatic methods**

Sequences were analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and at Arabidopsis.org (http://arabidopsis.org). Sequence alignments were generated in ClustalX and multiple alignments were shaded with BOX-SHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

**Yeast strains**

Strains included are as follows: CTY182 ([MATα ura3-52 lys2-801 his3-200], CYT1-1A [CYT182 sec14-11], CYT1079 [CYT1-1A spo4A::HIS3]), and CYT303 ([MATα ura3-52 lys2-801 his3-200 sec14, cki1::HIS3]) (Cleves et al., 1999b; Phillips et al., 1999; Li et al., 2000; Yanagisawa et al., 2002).

**Media, genetic techniques, and PIP determinations**

Yeast media, genetic techniques, invertase assays, and PIP determinations have been described previously (Kearns et al., 1998b; Guo et al., 1999; Phillips et al., 1999; Li et al., 2000; Yanagisawa et al., 2002). Site-directed mutagenesis used Quick-Change (Stratagene). Primers were obtained from the University of North Carolina Lineberger Comprehensive Cancer Center Oligonucleotide Synthesis Core.

**Plant cDNA isolation**

100 μg of total mRNA was prepared from 100 mg of 3-d-old seedlings using the RNAeasy Plant Mini Kit (Qiagen). The 717-bp mRNAs for each AtPsfh1 were amplified for RT-PCR (SuperScript First-Strand Synthesis System; Invitrogen). BamHI and HindIII-restricted cDNAs were cloned into the corresponding sites of a yeast episomal plasmid derived from YepLac195 (http://genome-www2.stanford.edu/vectorb/vecmap/comple/SYPLAC195.SEQ.html). AtPsfh1 expression was driven by a SEC14 promoter and subject to SEC14 termination signals.

**GUS histochemistry**

5-d-old seedlings were stained for GUS activity using standard protocols (Jefferson et al., 1987). The GUS gene was placed under the control of the AtSFH1 promoter (PAtSFH1) and transgenic lines were generated by Agrobacterium-mediated transformation of wild-type plants using the floral dip method. PAtSFH1-GUS expression was recorded after staining under vacuum for 5 min at 25°C followed by 1 h at 37°C. PAtSFH1 represented a 1958-bp DNA fragment directly 5’ to the AtSFH1 initiator codon.

**Imaging and video processing**

Light microscopy was done with a microscope (model MZFLIII; Leica) using a cooled CCD camera (model EODSSD; Canon) interfaced with capture image software (RemoteCapture 1.1; Canon), a dissecting microscope (model SMZU; Nikon) interfaced with a color CCD camera (model DMK12000; Nikon). Pictures were processed in Photoshop 7.0. Environmental scanning EM (ESEM) was used to assayed adult seedlings mounted in 0.8% (wt/vol) agar low-melt agarose in 1× Murashige and Skoog Salt and Vitamin Mixture media (MS; GIBCO BRL). Seedlings were stratified at 4°C for 4 d, and then grown vertically under constant light (90 μM m−2 s−1) at 22°C for 3 d. Adult roots were extracted after 45 d of growth in soil, cleaned in MS medium, and stained with either Ponceau red or Coomassie blue for 30 min. The Atsfh1::T-DNA line of A. thaliana (Brassica family, Columbia ecotype) was obtained from the Arabidopsis Biological Resource Center via TAIR (http://arabidopsis.org; Alonso et al., 2003). The T-DNA insertion was mapped at the Salk Institute Genomic Analysis Laboratory “T-DNA Express” Arabidopsis Gene Mapping Tool (http://signal.salk.edu/cgi-bin/tdnaexpress). Atsfh1::T-DNA mutants were selected for kanamycin resistance (50 μg/mL). Genotypes were confirmed by PCR, Southern blotting, and DNA sequencing of junctional borders. Agrobacterium tumefaciens-mediated transformation floral dip protocols were routinely used to generate transgenic plant lines (Clough and Bent, 1998).

**Plant material and preparation for SIET**

Seeds were incubated for 72 h at 4°C and sterilized in 70% (wt/vol) ethanol for 2 min and 30% (wt/vol) bleach containing 0.01% (wt/vol) Triton X-100 for 25 min. Seeds were placed on sterilized filter paper strips (Fisher Scientific) in Petri dishes (Fisher Scientific) with ~120 seeds per dish (15 seeds per strip on 8 strips). Approximately 3 mL of sterilized liquid growth media was added to each Petri dish, and dishes were sealed with parafilm and positioned vertically on a rack. Seedlings were germinated in a Percival growth chamber at 22°C with a 16:8 h light/dark cycle and 68% relative humidity conditions. Growth medium was sterilized before use and was comprised of 0.1 mM KCl, 0.1 mM CaCl2, 0.1 mM MgCl2, 0.5 mM NaCl, 0.3 mM MES, 0.2 mM Na2SO4, and 6% sucrose, pH 6.0. Filter paper strips containing three to five seedlings were cut off from the Petri dish and glued to the bottom of a measurement Petri dish. Approximately 5 mL of fresh growth medium was added to the chamber and equilibrated for at least 1 h. To avoid acidification of the medium, media were again replaced and the system allowed to stabilize for 15–20 min before Ca2+ measurements.

**Ca2+ flux measurements by SIET**

The SIET (Applicare Electronics, Inc.) determines both static ionic/molecular concentrations and concentration gradients by using ion-selective microelectrodes (Kührerich and Jaffe, 1990; Schieflenhau, 1992). The concentration gradient is measured by moving the electrode repeatedly between two positions in a predefined excursion (5–30 μm) at a fixed frequency in the range of 0.3 to 0.5 Hz. The ion-selective electrode was constructed as follows: glass micropipettes (2 μm aperture) were pulled from 1.5-mm-diam glass capillaries (TW150-4; World Precision Instruments, Inc.) with an electrode puller (P2000; Sutter Instrument Co.) to provide microelectrodes with a 2-μM aperture using a four-step protocol. Micropipettes were silanized with N,N-dimethylamylsilane (Fluka) at 120°C for 50 min, back-filled with 100 mM CaCl2, and then front-filled with LiCl and Li2SO4. A LiCl solution of 80 μM Li2SO4 was used as the reference solution. The Ca2+ selective probe was micropipette-filled into an Ag/AgCl wire holder (WPI), reconditioned every time before measurement with self-constructed 9 V DC...
circuit). The reference was a solid, low leakage electrode (WPI). Ca\(^{2+}\) electrodes were calibrated using a series of 1.0, 0.1, and 0.01-mM CaCl\(_2\) solutions. Only electrodes with Nernstian slopes >25 mV were used. Ca\(^{2+}\) ion flux was calculated from Fick's law of diffusion: $J = -D(d/c/dx)$, where $J$ = ion flux in x direction, $d/c/dx$ = ion concentration gradient, and $D$ = ion diffusion constant. Flux direction was determined by electrode movement with respect to sample and sign of calculated flux.

Online supplemental material

Fig S1 and Video 1 show localization of GFP-AtSH1p to the plasma membrane and VRZ of wild-type root hairs of 3-d-old transgenic plants. Fig S2 and Videos 2–4 show YFP-PHPLC distribution and report the de-arrangement of PtdIns(4,5)\(_{2}\) distribution in nullizygous root hairs. Video 5–7 show GFP-AtTalin imaging in wild-type and nullizygous root hairs and demonstrate loss of the fine tip actin microfilament network in nullizygous root hairs. Videos 8–10 show GFP-MAP4 imaging in root hairs of wild-type and nullizygous root hairs and demonstrate comprehensive loss of organized MTs in nullizygous root hairs. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200412074/DC1.

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Fang, M., A. Gedvilaita, B.G. Kears, S. Kagiwada, M. Kears, M.K.Y. Fung, and V.A. Bankaitis. 1996. Kes1p shares homology with human oxy-


Ohashi, Y., A. Oka, R. Rodrigues-Pousada, M. Possenti, I. Ruberti, G. Mor-

Phillips, S., B. Sha, L. Topalof, Z. Xie, J. Alb, V. Clenchin, P. Swigart, S. Cock-


