Cross species complementation analysis of *Physcomitrella patens* COW1 orthologs: a test for functional equivalence in tip growth

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Abstract

Polarized cell expansion, or “tip growth,” produces cellular morphologies characterized by an outgrowth extending from the main body. Tip growth processes involve membrane trafficking to a specific growing point during cellular morphogenesis, and the resulting structure frequently allows for specialized cellular function. The model bryophyte Physcomitrella patens is an optimal system for tip growth studies due to the ease of its genetic manipulation, and because its life cycle begins with a stage characterized by tip growth and branching. COW1, a Sec14-like PITP in Arabidopsis thaliana, plays an essential role in tip growth that is suspected to involve regulatory phosphoinositides (PIs), particularly PtdIns(4,5)P$_2$. Mutant cow1 individuals manifest a phenotype consistent with a tip growth defect: short, wide root hairs that elongate at an abnormally slow rate from the epidermal root cell (GRIERSON et al. 1997). Three COW1 moss orthologs (Pp1s88, Pp1s169, Pp1s307) were identified in the P. patens genome and experimentally found to be expressed in moss protonemata. A cross-species complementation study was performed to assess the functional equivalence of these orthologs and A. thaliana COW1. Restoration of wild type root hairs in transgenic A. thaliana cow1 mutants expressing P. patens COW1 ortholog coding sequences would signify functional equivalence. In transgenic cow1 expressing Pp1s169 and Pp1s307, only weak rescue of root hair length was observed. However, a gain-of-function phenotype was observed in these transgenic root hairs: 25-37% of root hairs were branched, compared to only 4% in the cow1 mutant. This phenotype is consistent with alterations in PtdIns4P and PtdIns(4,5)P$_2$ landmarks regulated by COW1, suggesting an alternative function of the P. patens ortholog relative to A. thaliana COW1. Characterizing these differences may provide insight into changes in tip growth mechanisms over the history of land plant evolution.
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List of Abbreviations

3' UTR 3' untranslated region  
ABP Actin binding protein  
ADF Actin depolymerizing factor  
ARF ADP ribosylation factor  
DPG Days post germination  
GAP GTPase activating protein  
CDS Coding sequence  
GAP GTPase-activating protein  
GDI Guanine nucleotide dissociation inhibitor  
GEF Guanine nucleotide exchange factor  
LB Luria broth  
Nlj16 Nodulin 16  
Nos-T Nopaline synthetase terminator  
PI Phosphoinositide  
PITP Phosphatidylinositol transfer protein  
PtdCho Phosphatidylcholine  
PtdIns Phosphatidylinositol  
ROPs Rho-like GTPases of Plants  
TGN Trans-Golgi network  
Sec14 Secretory 14
**Introduction**

Specialized cell types are often characterized by a distinct morphology essential for normal cell function. In complex multicellular organisms these specialized morphologies arise from cellular differentiation, which frequently includes the establishment of cell polarity followed by polarized cell expansion, or tip growth. In spite of their evolutionary divergence, members of all eukaryotic kingdoms—protists, fungi, land plants, animals—undergo tip growth during development, and many of the underlying molecular pathways responsible for tip growth have been conserved. In tip-growing cells, new membrane material is trafficked along a dynamic cytoskeletal network and incorporated at a specific growing point, ultimately producing a morphological outgrowth (Nelson 2003). Phosphoinositides, a class of regulatory phospholipids, have emerged as significant players needed to coordinate and execute these steps, enriching our current understanding of tip growth even as they raise new questions about this dynamic process (Ischebeck et al. 2010).

**The role of phosphoinositides in tip growth**

In recent years, phosphoinositides (PIs) have gained recognition for their prominent signaling roles during tip growth (Munnik and Nielsen 2011). PIs are a group of phosphorylated derivatives of phosphatidylinositol (PtdIns) (Fig. 1), a negatively charged phospholipid found on the cytosolic side of eukaryotic cell membranes (Butikofer et al. 1990; Zachowski 1993). Despite their relatively low abundance in cell membranes, PIs are well established as extremely versatile and phylogenetically conserved signaling molecules involved in both cytoskeletal actin remodeling and intracellular vesicle transport—two processes recognized as essential components of the complex tip growth process.
Figure 1. Phosphotidylinositol (PtdIns), the precursor to phosphoinositides (PIs), is a low-abundance phospholipid found in cell membranes. PtdIns can be phosphorylated at the 3, 4, and/or 5 positions (red stars) on the myo-inositol headgroup (bracketed).
A PI’s signaling functions are determined by its headgroup, where PI kinases can phosphorylate the D-3, D-4, and D-5 positions of the inositol ring (Anderson et al. 1999). To deactivate or “turn off” the signal, PI phosphatases serve to remove these phosphate groups; this reversibility allows for fine-tuned control over the spatial and temporal patterns of specific PI accumulation. PtdIns and PIs can also be phosphorylated at multiple sites; for example, PtdIns4P-5 kinase can phosphorylate PtdIns4P to produce phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P$_2$). Various combinations of phosphorylation at the D-3, D-4, and D-5 positions produce up to seven structurally related PIs in animals and six in plants; PtdIns(3,4,5)P$_3$ has not been found in plants (Ischebeck et al. 2010). Efforts towards understanding the role of these PIs in tip growth processes frequently focus on the root hairs of the angiosperm Arabidopsis thaliana, a convenient laboratory model. Root hairs are extreme examples of tip growth that are easy to manipulate genetically, and allow for high-resolution live-cell microscopy (Kost 2008).

Studies of PIs involved in root hair tip growth have identified two PI species, PtdIns4P and PtdIns(4,5)P$_2$, as major players in cytoskeletal organization and polarized membrane trafficking. PtdIns4P pools are maintained along lateral “flanks” adjacent to the growing root hair’s apex (Yoo et al. 2012). Though it was once thought to simply act as a precursor to PtdIns(4,5)P$_2$ (via PtdIns4P-5-kinase phosphorylation), PtdIns4P is now believed to act as a signaling molecule in its own right, and is required for normal root hair tip growth (Munnik and Nielsen 2011; Szumlanski and Nielsen 2010; Vermeer et al. 2009). Evidence for PtdIns4P’s involvement in normal tip growth can be seen in the agd1 mutant. The root hairs of agd1 mutants exhibit wavy or branched phenotypes, and the normal PtdIns4P localization patterns in the subapical tip region are disrupted (Yoo et al. 2008).
While PtdIns4P is found flanking the root hair tip, PtdIns(4,5)P₂ is localized to the apical region of developing root hairs (Kost et al. 1999; Vincent et al. 2005). The PtdIns4P-5-kinase PIP5K3 also localizes to the apical region of root hair cells and catalyzes PtdIns(4,5)P₂ synthesis (Kusano et al. 2008; Stenzel et al. 2008) suggesting that some PtdIns4P is eventually phosphorylated to PtdIns(4,5)P₂, which subsequently migrates to the tip. PIP5K3 mutants exhibit short root hairs indicative of a tip growth defect (Kusano et al. 2008; Stenzel et al. 2008), and the loss of the tip-directed PtdIns(4,5)P₂ gradient has been found to produce short, fat root hairs (Vincent et al. 2005), evidence that PtdIns(4,5)P₂ joins PtdIns4P as a PI species that plays an essential role in root hair elongation.

**Phosphoinositides regulate monomeric GTPase signaling pathways during tip growth.**

Monomeric GTPases or G proteins are molecular switches that control numerous signal transduction cascades in cells; they are bound to GTP in their active state and to GDP in their inactive state. The activation state of monomeric GTPases is subject to exquisite regulation in the cell. Guanine nucleotide exchange factors (GEFs) activate the intrinsic GTPase activity of monomeric GTPases by promoting the exchange of GDP for GTP. In contrast, guanine nucleotide dissociation inhibitors (GDIs) bind to the GDP-bound form and prevent exchange with GTP, thus maintaining the G protein in the inactive state. GTPase-activating proteins (GAPs) hydrolyze GTP to GDP and turn off the GTPase activity (Bos et al. 2007). Several monomeric GTPases play pivotal roles in the regulation of tip growth.

PtdIns(4,5)P₂, the best-studied PI species, plays a central role in regulating the activity of monomeric G proteins of the Rho-like GTPases of Plants (ROPs) Rac-Rop subfamily—key signaling molecules in tip growth initiation. There are 11 ROP GTPases in A. thaliana that
modulate a wide range of processes, often associated with regulation of actin cytoskeletal
dynamics (VERNOUD et al. 2003). During root hair tip growth, ROPs localize to the plasma
membrane of the growing tip and activate PtdIns4P 5-kinase to produce a tip-focused
PtdIns(4,5)P₂ gradient (BLOCH et al. 2005; JONES et al. 2002; KOST et al. 1999; MOLENDIJK et al.
2001; SANTIAGO-TIRADO and BRETSCHER 2011). PtdIns(4,5)P₂ in turn activates ROP-GTPase
signaling pathways by binding to GDIIs that normally block GTPase activation by exchange of
ADP for ATP (DERMARDIROSSIAN and BOKOCH 2005). These GDIIs are required to restrict the
site of tip growth to a single point during root hair development; mutations in GDIIs have been
shown to produce short root hairs with multiple sites of tip growth initiation (CAROL et al. 2005).
ROP-GTPase activities initiate a variety of additional downstream signaling pathways affecting
actin cytoskeletal organization and vesicle trafficking, ROS production, and intracellular tip-
focused Ca²⁺ gradient maintenance—all factors involved in root hair tip growth (ISCHEBECK et al.
2010). By promoting ROP-GTPase activity, PtdIns(4,5)P₂ contributes to a positive feedback loop
that ultimately maintains the tip-focused PtdIns(4,5)P₂ gradient, as well as downstream processes

Monomeric G-proteins have also been found to interact with the PI4-kinase PI-4Kβ1,
thereby regulating PtdIns4P synthesis in developing root hairs. RabA4b GTPase is a member of
the Rab GTPase family that co-localizes to the tip of growing root hairs with PI-4Kβ1,
depending on the presence of a tip-focused Ca²⁺ gradient. Activated RabA4b GTPases have been
found within trans-Golgi network (TGN)-like vesicles carrying complex cell wall
polysaccharides, presumably to be deposited at the tip (KANG et al. 2011). RabA4b recruits PI-
4Kβ1 (PREUSS et al. 2006), an effector protein that generates localized PtdIns4P on the vesicle
membrane; from here, PtdIns4P may be transported to the growing tip, where the previously-
discussed ROP GTPases can recruit PtdIns4-5 kinases to phosphorylate PtdIns4P to
PtdIns(4,5)P_2. Along with RabA4b, the subcellular distribution and enrichment of PtdIns4P is
also regulated by RHD4, a Sac1p-like phosphoinositide phosphatase that localizes to the growing
root hair tip where RabA4b-labeled compartments are found. Mutant *rhd4* root hairs exhibit
abnormal distributions of RabA4b-labeled TGN-like compartments, and PtdIns4P is found in
membrane compartments throughout the root hair, not localized to the tip. This phenotype
suggests that RHD4 functions to limit levels of PI-4Kβ1-generated PtdIns4 in tip-localized
internal membrane compartments tagged with RabA4b (THOLE *et al.* 2008). Together, these
studies indicate that monomeric G protein regulation of PtdIns4P plays an important role in
proper polarized secretion during root hair tip growth.

**PtdIns(4,5)P_2 modulates actin cytoskeletal dynamics**

Tip-focused PtdIns(4,5)P_2 gradients regulate actin cytoskeletal dynamics involved in
polarized cell expansion. Actin filament networks are responsible for polarized protein and
vesicle movement during tip growth in many systems, including root hair development, and,
ultimately, play a central role in cell morphogenesis (WASTENEYS and GALWAY 2003). In
animals, PtdIns(4,5)P_2 regulates actin dynamics by binding directly to actin-binding proteins
(ABPs), which remodel actin filaments by inducing or inhibiting actin polymerization, or by
initiating the branching or breaking of actin strands (ISCHEBECK *et al.* 2010). Homologous
counterparts of several animal ABP’s have been identified in *A. thaliana*, and PtdIns(4,5)P_2 plays
a similar role in regulating plant cell cytoskeletal dynamics via ABP interactions during root hair
tip growth (DRØBAK 2004; WASTENEYS and YANG 2004). For instance, PtdIns(4,5)P_2 can
directly bind to and inhibit the activity of ADF/cofilin, a well-characterized family of ABPs that
binds to actin filaments to mediate actin depolymerization (Bamburg 1999; Ischebeck et al. 2010; Yonezawa et al. 1990). ADF overexpression during in vivo root hair development results in highly disorganized F-actin networks and short root hairs, while underexpression increases the number of actin cables and leads to abnormally long root hairs (Dong et al. 2001). By inhibiting ADF’s depolymerizing activity, PtdIns(4,5)P_2 regulates cytoskeletal dynamics by stimulating actin polymerization in the developing root hair.

COW1, a member of the Sec14 family of PITPs plays an essential role in tip growth

PI transfer proteins (PITPs) have recently gained recognition for their role within the complex network of PI regulation. PITPs are ubiquitous eukaryotic proteins that function in complex ways at the interface between lipid signaling and lipid metabolism (Ghosh and Bankaitis 2011). The PITP Secretory 14 (Sec14), first identified in Saccharomyces cerevisiae, plays an essential role in secretory vesicle formation and protein transport from the Golgi complex (Aitken et al. 1990; Bankaitis et al. 1990; Cleves et al. 1991); sec14 yeast mutants experience a Golgi secretory block that takes place at a late compartment, indicating a defect in vesicle budding from the trans-Golgi complex (Bankaitis et al. 1989; Franzusoff and Schekman 1989). Sec14 binds PtdCho and PtdIns at distinct sites, and its ability to bind both lipids (though not simultaneously) is required for successful Sec14-mediated regulation of PI homeostasis in vivo (SchAAF et al. 2008). Sec14’s precise role in PI regulation is still unknown, but one proposed mechanism suggests that Sec14 is “primed” by PtdCho before presenting the PtdIns substrate to PI kinases (specifically, PI-4 kinase). An alternative model proposes a slow exit of PtdCho from its binding site on Sec14, preventing an invading PtdIns from binding to
Sec14 and leaving it vulnerable to PI kinase activity. In addition to facilitating PtdIns4P synthesis, Sec14 also generates diacylglycerol (DAG) pools essential for TGN vesicle trafficking (KEARNS et al. 1997). DAG is derived from PtdCho breakdown, and DAG levels fall as PtdCho levels rise. Sec14 exchanges PtdCho for PtdIns, stimulating both PtdIns4P synthesis and DAG generation. By generating DAG pools and PtdIns4P, Sec14 is thought to create a membrane lipid environment that recruits downstream effectors required for vesicle formation. (BANKAITIS et al. 2010).

In 2004, the first report of an *A. thaliana* Sec14-like protein’s biological function revealed the role of Sec14-like PITPs in plant tip growth. This protein, COW1, belongs to a large family of Sec14-nodulin domain proteins (Fig. 2) and is essential for normal root hair development (BOHME et al. 2004; VINCENT et al. 2005). Loss-of-function mutations in the COW1 gene result in a phenotype that is consistent with a tip growth-related defect. The *cow1* mutant features root hairs one-quarter the length and 2.5 times the diameter of wild type hairs, and the

Figure 2. The *A. thaliana* COW1 protein is a PITP possessing a Sec14-like domain at the C-terminus and a Nlj16-like domain at the N-terminus.

root hairs of *cow1* individuals were also observed to elongate at a slower rate than wild type. Each short, wide root hair was produced at normal positions along the root, suggesting that COW1 is required for normal root hair elongation but not initiation. The *cow1* mutant also exhibits a "twin hair" phenotype — when two root hairs emerge from a single initiation site — up
to 40 times more frequently than wild type (BOHME et al. 2004; GRIERSON et al. 1997). These twin hairs appear in the same cell files as single hairs, suggesting that they are not the products of positional misinformation. Each of the twin hairs are the same length but narrower than cow1 single root hairs, indicating that they are not simply pairs of mutant hairs. Finally, cow1 plants exhibit signs of compromised tip growth exclusively in root hairs (GRIERSON et al. 1997), and promoter-reporter fusion constructs reveal that COW1 is expressed predominantly in root trichoblast cell files in the zone of elongation (VINCENT et al. 2005). At the subcellular level, Cow1 localizes along the plasma membrane of emerging and growing root hairs (BOHME et al. 2004; VINCENT et al. 2005). Crosses between cow1 mutants and individuals carrying known root hair mutations (rhd1-4, rhd6, and tip1) yield wild-type individuals, evidence supporting the conclusion that COW1 is not part of root hair developmental pathways involved in elongation that had been characterized at the time (GRIERSON et al. 1997).

While COW1’s role in root hair tip growth is not yet fully understood, there is strong evidence to suggest a role in PI regulation (VINCENT et al. 2005). COW1 sequence comparisons reveal that the Cow1 protein contains a lipid-binding Sec14p-like domain and a Nlj16-like domain (Fig. 2). The Sec14 domain of the Cow1 protein exhibits intrinsic PITP biochemical activity, with the ability to catalyze PtdIns transfer in vitro. Furthermore, the Sec14 domain of Cow1 is able to rescue and stimulate PI synthesis in a sec14 yeast mutant, indicating that Cow1 belongs to a large family of Arabidopsis Sec14-like proteins functionally equivalent to yeast Sec14. The Nlj16 domain is thought to play a role in the Cow1 PITP’s localization to the plasma membrane (BOHME et al. 2004; VINCENT et al. 2005). The cow1 mutant does not exhibit the tip-directed PtdIns(4,5)P2 gradient and F-actin network seen in wild type A. thaliana root hairs, causing an accumulation of transport vesicles throughout the root hair cytoplasm. The loss of a
tip-directed \( \text{Ca}^{2+} \) gradient in the \textit{cow1} mutant also suggests that ion channel transport is no longer directed to the tip, presumably due to a disruption in polarized membrane trafficking. These signs of dysregulation collectively implicate COW1 as a key player in the regulation of PI landmarks involved in root hair tip growth. However, the mechanism by which COW1 promotes PI synthesis, helps establish a PtdIns(4,5)P\(_2\) gradient, and organizes F-actin networks remains to be discovered (VINCENT \textit{et al.} 2005).

A proposed model of COW1’s role in root hair development suggests that COW1 generates tip-focused PI “landmarks” that recruit F-actin network components, allowing for tip-directed delivery of new membrane materials (VINCENT \textit{et al.} 2005). COW1’s PI synthesis perhaps involves a direct coupling of COW1-PtdIns with PtdIns kinases, or COW1 may bind and present PtdCho to phospholipase D (PLD), producing phosphatidic acid (PtdOH) that in turn stimulates PtdIns-4-phosphate 5-kinase to produce PtdIns(4,5)P\(_2\). COW1 may initiate PLD activity and subsequent PtdIns(4,5)P\(_2\) synthesis on secretory vesicles that are leaving the Golgi; PtdIns(4,5)P\(_2\) subsequently stimulates actin polymerization and association with the F-actin network that ultimately deposits these vesicles at the root hair tip. Additional players in tip growth processes, including ROP-GTPases and ABPs, may act as downstream effectors of COW1-mediated PI signaling.

Unfortunately, the role of PITPs in tip growth processes—particularly PI regulation—is challenging to study within the network of complex signaling pathways involved in \textit{A. thaliana} tip growth. Since PI-mediated regulation of tip growth appears to date back over 400 million years ago to the common ancestor of fungi, mosses, and angiosperms (RENSING \textit{et al.} 2008), some researchers have begun to shift their focus to simpler models to carry out tip growth studies.
Physcomitrella patens: a convenient moss model for PI and tip growth studies

The bryophyte Physcomitrella patens has emerged as an advantageous model system for studies in PI regulation and tip growth. Bryophytes are descended from a basal lineage of land plants, characterized by relatively simple morphology, the absence of a vascular system, and free-swimming motile sperm. The simplicity of bryophyte morphology is well suited for the study of developmental pathways, which include tip growth. The P. patens life cycle begins with a propagative stage characterized by tip growth and branching. Recent work in P. patens tip growth research highlights the importance of the actin cytoskeleton during this stage (PRIGGE and BEZANILLA 2010), which mirrors the significant role of the actin cytoskeleton in A. thaliana tip growth. In both systems the actin cytoskeleton is dynamic—constantly remodeled and regulated by ABPs to deliver vesicles to the site of tip growth. These parallels can provide new insights on tip growth processes shared between the two systems, despite their evolutionary divergence. For example, ADF/cofilin is known to regulate actin dynamics in A. thaliana, but assessing the extent of ADF’s role is complicated by multiple A. thaliana ADF isoforms (MACIVER and HUSSEY 2002). In P. patens, ADF is the product of a single, intronless gene that RNAi studies have revealed to be essential for moss viability; silencing of ADF results in disorganized F-actin networks, indicating that ADF plays a significant role in P. patens F-actin organization and tip growth processes, and likely shares the same degree of significance in A. thaliana (AUGUSTINE et al. 2008).

Apart from the actin-dependent tip growth that takes place during P. patens development, another advantage to using a P. patens model for tip growth studies is the simplicity of the P. patens PI kinase family. PIs are found in the bryophyte P. patens, but the moss genome encodes only two PtdIns kinases—PpPIPK1 and PpPIPK2—compared to the fifteen A. thaliana genes
coding for proteins with PI kinase catalytic domains (MUELLER-ROEBER and PICAL 2002; SAAVEDRA et al. 2009). Overall, the moss PI kinase gene family is smaller and less redundant than that of *A. thaliana*, and the catalytic domains of the moss kinases are highly conserved when compared to the PI kinases of other organisms. These features make the moss model a promising system for PI investigations, since a major obstacle to studying PI regulation and tip growth in *A. thaliana* is the genomic redundancy of *A. thaliana* PI kinase families. Both moss kinases localize to the plasma membrane, and they each exhibit specific substrate affinities for different PtdIns species: PpPIPK1 binds equally well to PtdIns4P and PtdIns3P, while PpPIPK2 preferentially binds to PtdIns. In the *P. patens* system, PtdIns3P, PtdIns4P, PtdIns(4,5)P$_2$ and PtdIns(3,5)P$_2$ have been detected *in vivo*, while PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ have only been detected *in vitro* (SAAVEDRA et al. 2009).

Finally, it could be argued that the most compelling advantage to working with the *P. patens* model system is the considerable extent to which genetic analyses and manipulations can be performed in the laboratory. Numerous genetic tools are available to manipulate the *P. patens* system, especially since its genome was sequenced in 2006. Since the haploid gametophyte is the dominant life cycle phase, identification and genetic study of mutants is convenient and relatively easy (COVE 2005). In *P. patens*, high-frequency homologous recombination permits precise gene targeting and modification; the transformation procedure itself is relatively simple, involving polyethylene glycol (PEG)-mediated DNA uptake by *P. patens* protoplasts. During the vegetative propagation that follows transformation, protoplasts rebuild their cell walls and develop into protonematal tissue. This vegetative propagation allows for the characterization of mutations affecting early development, as well as indefinite culture maintenance (COVE 2005). After transformation, stable transformants can be detected in as few as 4-6 weeks. RNAi
manipulation of *P. patens* can also be carried out with relative ease, generating mutant phenotypes a week after transformation with the RNAi construct. Reverse genetics studies of moss development are further encouraged by the fact that many moss tissues are only one cell thick, allowing researchers to observe a phenotype’s characteristics at the cellular level (Prigge and Bezanilla 2010), and high-resolution fluorescence microscopy can be used to image F-actin cytoskeletal dynamics during tip growth (Vidalí et al. 2009).

**Do *P. patens* COW1 orthologs function in tip growth?**

The *P. patens* genome contains four COW1 orthologs (Sec14-nodulin domain genes: Pp1s47_303V6.1, Pp1s88_63V6.1, Pp1s169_81V6.1, Pp1s307_16V6.1) (Fig. 3), in contrast to 14 in *A. thaliana* (personal communication K. Peterman). We hypothesized that like *Arabidopsis* COW1, one or more of the *P. patens* COW1 orthologs plays a critical role in tip growth. To test this hypothesis and assess the functional similarities of these genes to *A. thaliana* COW1, a cross-species complementation study was performed. Transgenic lines of the *A. thaliana* cow1
mutant expressing each of the *P. patens* COW1 ortholog coding sequences (CDSs) were observed for restoration of normal root hair tip growth; if the moss COW1 ortholog is an effective substitute for the *A. thaliana* COW1 gene, then the short, fat root hairs of the *cow1* mutant are expected to be replaced by normal wild-type root hairs (Fig. 4). Identification of a functionally equivalent COW1 ortholog in *P. patens* would suggest that the tip growth-specific function arose early in the evolution of land plants, and provide a convenient model for examining the role of Sec14 proteins in plant tip growth.

Homozygous T₃ lines of *cow1* *A. thaliana* expressing Pp1s169 or Pp1s307 exhibited only weak rescue of the *cow1* phenotype. While transgenic root hairs were significantly longer than those of the *cow1* mutant, they were also significantly shorter than wild-type. Interestingly, these lines also exhibited significantly increased incidence of branched root hairs compared to the *cow1* mutant. Our findings suggest that the Pp1s169 or Pp1s307 CDSs are not functionally equivalent to the *A. thaliana* COW1 CDS, and produce an unexpected gain-of-function phenotype—increased root hair branching—that is consistent with derangement of the PI landmarks required for tip growth.
Figure 4. A schematic of a cross-species complementation study investigating whether expression of a *P. patens* COW1 ortholog can restore the wild-type root hair phenotype in transgenic offspring of the *A. thaliana cow1* mutant.
**Materials and Methods**

I. RT-PCR analysis of moss COW1 ortholog (Pp1s47, Pp1s88, Pp1s169, Pp1s307) mRNA expression levels in *P. patens* protonemata

A. *DNase I digestion of genomic DNA contamination in P. patens RNA*

   RNA extracted from 7-day old *P. patens* protonemal tissue (a gift from Yen-Chu Liu and Luis Vidali) was subjected to a DNase I digest (Qiagen RNase-Free DNase Set) to eliminate genomic DNA contamination. The digest mix (≤87.5% RNA solution, 10% Buffer RDD, 6.8 Kunitz units/μL DNase I) was incubated at 25°C for 10 mins. To purify the RNA, 350 μL Buffer RLT and 250 μL of 99% ethanol were added in sequence to 100 μL of digest mix. The diluted RNA solution was applied to an RNeasy MinElute spin column (RNeasy® MinElute® Cleanup Kit) and centrifuged for 15 s at 16,000 x g. After discarding the flow-through, RNA bound to the silica membrane was washed with 500 μL Buffer RPE centrifuged for 15 s at 16,000 x g. A second 500 μL Buffer RPE wash was centrifuged through the column for 2 mins at 16,000 x g. The spin column was transferred to a new collection tube and centrifuged for 5 mins to remove residual Buffer RPE, then transferred to a sterile microfuge tube. The membrane-bound RNA was eluted into 40-50 μL RNase-free H₂O via centrifugation for 1 min at 16,000 x g. RNA concentrations were determined using a NanoDrop2000 spectrophotometer (Thermo Scientific).

B. *cDNA synthesis*

   DNase I-digested RNA was used to synthesize copy DNA (cDNA) using a Phusion® RT-PCR kit according to manufacturer’s directions (Thermo Scientific). An RNA mixture (up to 1 ug RNA, 10 ng/μL oligo(dT) primers, 1 mM dNTPs) was denatured at 65°C for 5 mins and placed on ice. The denatured RNA was used for cDNA synthesis in a reverse transcriptase
reaction (50% denatured RNA mixture, 10% RT enzyme mix, 1X RT Buffer) using a thermal cycler program set to 10 mins at 25°C, 30 mins at 40°C, and 5 mins at 85°C for primer extension, cDNA synthesis, and reaction termination, respectively.

C. RT-PCR primer design

In order to detect mRNA expression levels of Pp1s47, Pp1s88, Pp1s169, and Pp1s307 in *P. patens* protonema, forward primers for polymerase chain reaction (PCR) amplification were designed to span an exon-exon junction, while reverse primers incorporated the 3’ UTR, eliminating the possibility of genomic DNA amplification. Primers for ubiquitin10 amplification were designed to provide a positive control.

Table 1. RT-PCR primers to detect mRNA expression levels of Pp1s47, 88, 169, and 307 in *P. patens* protonema.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence (5’ - 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pp1s47F</td>
<td>GGCTAACACCAAAAAAGACATTGAGATCTG</td>
</tr>
<tr>
<td>Pp1s47R</td>
<td>AAGCCACGCACTTCAAATCCTGT</td>
</tr>
<tr>
<td>Pp1s88F</td>
<td>GGAGTACCGCATGATGAAAAAGAAGC</td>
</tr>
<tr>
<td>Pp1s88R</td>
<td>AGCACCAGCCATGCTCCATGT</td>
</tr>
<tr>
<td>Pp1s169F</td>
<td>GAGAGCTCTGTAAGAAGAAAAAGAAGTCAACCA</td>
</tr>
<tr>
<td>Pp1s169R</td>
<td>TCCACGGCACTGCGCACTTA</td>
</tr>
<tr>
<td>Pp1s307F</td>
<td>GTACCGGAAGATGAAACAGAAGCCA</td>
</tr>
<tr>
<td>Pp1s307R</td>
<td>CTCCAGGTTGGCTGCAAACGA</td>
</tr>
<tr>
<td>UBQF</td>
<td>CAGACAACCTACCCCTGAAGTTGTATAGTTCCGGA</td>
</tr>
<tr>
<td>UBQR</td>
<td>CAAGTCACATTACTTGCCTGCTGATGATGAATACA</td>
</tr>
</tbody>
</table>
D. RT-PCR

A Phusion® RT-PCR kit was used according to manufacturer’s directions (Thermo Scientific) to amplify gene-specific fragments from the P. patens cDNA template. The PCR reaction consisted of 1X Phusion® HF Buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Phusion® Hot Start II enzyme, and 1.5% cDNA template in DNase-free water. A thermal cycler was programmed for 40 cycles of denaturation, annealing, and extension (10 sec at 98°C for denaturation, and 70 sec at 72°C for both annealing and extension), with initial denaturation at 98°C for 30 sec and final extension at 72°C for 5 min.

E. Agarose gel electrophoresis

Amplified PCR products were analyzed via agarose gel electrophoresis (1% agarose in 40 mM Tris, 20 mM acetic acid, 1 mM Na₂EDTA, pH 8.0), and DNA was visualized using SYBR Safe DNA gel stain (1X, Invitrogen). Gel images were taken under UV transillumination using a Kodak Gel Logic 200 Imaging System and Kodak ID software.

II. Creation of transgenic cow1 A. thaliana expressing P. patens COW1 orthologs

A. Construct design

The coding sequence (CDS) of each P. patens COW1 ortholog (Pp1s88_63V6.1, Pp1s169_81V6.1, Pp1s307_16V6.1) was identified from a public database of the P. patens genome (http://www.phytozome.org). In order to express these coding sequences in a spatial and temporal pattern identical to endogenous A. thaliana COW1, the CDS was placed under the control of the A. thaliana COW1 promoter region. This promoter region was identified from a public database of the Arabidopsis genome (http://www.arabidopsis.org). The Agrobacterium
tumefaciens nopaline synthetase terminator sequence (Nos-T) was included to signal the termination of the transgene.

B. Primer design

Primers for PCR amplification of the COW1 promoter were designed with an EcoRI restriction site at the 5’ end of the reverse primer. The forward primer was designed to include a CACC sequence at the 5’ end for eventual TOPO® cloning of the construct into the entry vector. Forward primers for each moss COW1 ortholog CDS were designed with an EcoRI restriction site at the 5’ end, and reverse primers were designed to incorporate a SalI restriction site at the 5’ end. The Nos-T sequence was amplified with a forward primer carrying a SalI restriction site at the 5’ end.

Table 2. PCR primers for amplification of construct components. Pink text denotes EcoRI sites, while orange text denotes SalI sites.

<table>
<thead>
<tr>
<th>Primer</th>
<th>PCR Primer Sequence (5’ -&gt; 3’)</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>COW1PF</td>
<td>CAC CAC AAC TTG GTA AGT ACT CTG AGG CTT T</td>
<td>Genomic A. thaliana DNA</td>
</tr>
<tr>
<td>COW1PR</td>
<td>CTA GGA ATT CGG TTA GAT GAG TTT TAA GCA CTC</td>
<td></td>
</tr>
<tr>
<td>88FRI</td>
<td>ATA TGA ATT CAT GCC CGA CTA TGC GGT G</td>
<td>Genomic P. patens DNA</td>
</tr>
<tr>
<td>88RSI</td>
<td>ATA TGT CGA CTC ACA ACA AAA GCA GCC TGT</td>
<td></td>
</tr>
<tr>
<td>169FRI</td>
<td>ATA TGA ATT CAT GGG AGC TCA AAG TCA AGA ATT</td>
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<tr>
<td>169RSI</td>
<td>ATA TGT CGA CTC ACC AAC AAT GAA GCC TAC T</td>
<td></td>
</tr>
<tr>
<td>307FRI</td>
<td>ATC TGA ATT CAT GGG GGC TTA CAG TCA A</td>
<td></td>
</tr>
<tr>
<td>307RSI</td>
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<td></td>
</tr>
<tr>
<td>NosTF</td>
<td>ATA TGT CGA CAG TAA CAT AGA TGA CAC CGC</td>
<td>pMDC100 vector</td>
</tr>
<tr>
<td>NosTR</td>
<td>GAA TTT CCC CGA TCG TTC AAA CAT TT</td>
<td></td>
</tr>
</tbody>
</table>
C. Construct assembly

The COW1 promoter region, Pp1s88, Pp1s169, and Pp1s307 coding sequences and Nos-T terminator sequence were amplified in a PCR reaction (1X Phusion® HF Buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Phusion® Hot Start II enzyme, and 200 ng DNA template in DNase-free water). A thermal cycler was programmed for 35 cycles of denaturation, annealing, and extension (10 sec at 98°C for denaturation, and 60 sec at 72°C for both annealing and extension), with initial denaturation at 98°C for 60 sec and final extension at 72°C for 10 min.

The PCR products were analyzed by agarose gel electrophoresis and purified using the QIAGen PCR Purification kit according to the manufacturer’s directions (Qiagen). Five volumes of Buffer PB were mixed with one volume of the PCR reaction and centrifuged through a QIAQuick Spin Column at 16,000 x g for 30 sec. DNA bound to the column was washed with 750 μL Buffer PE via centrifugation at 16,000 x g for 30 sec, and residual Buffer PE was removed by centrifuging an empty column for 1 min. Flow-through was discarded after every step. DNA was eluted into 50 μL of Buffer EB (10 mM Tris Cl, pH 8.5) via centrifugation at 16,000 x g for 1 min.

The purified Pp1s88, Pp1s169, and Pp1s307 CDS fragments and the Nos-T fragment were digested with SalI (5 μL purified PCR product, 1X NEB Buffer 3, 10 units SalI) and purified again prior to a Quick Ligation™ reaction (New England Biolabs) consisting of equimolar concentrations of each purified PCR product diluted with water (6.2–9.5 ng/μL), Quick Ligation Reaction Buffer (62.9 mM Tris-HCL, 9.5 mM MgCl₂, 0.95 mM dithiothreitol, 0.95 mM ATP, 7.1% polyethylene glycol at pH 7.6), and 95 units/μL Quick T4 DNA Ligase. The reaction took place at 25°C for 5 mins.
The moss CDS::Nos-T ligation product was amplified via PCR (1X Phusion® HF Buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Phusion® Hot Start II enzyme, and 200 ng DNA template in DNase-free water) using a thermal cycler set to the following program: 35 cycles of denaturation, annealing, and extension (10 sec at 98°C for denaturation, and 60 sec at 72°C for both annealing and extension), with initial denaturation at 98°C for 60 sec and final extension at 72°C for 5 min.

The *A. thaliana* COW1 promoter and *P. patens* CDS::Nos-T fragments were digested with EcoRI (5 μL purified PCR product, 1X NEB Buffer EcoRI, 10 units EcoRI) and purified prior to Quick Ligation™ as described above.

The ligation product was purified and run on an agarose gel. The 4 kb fragment representing the fully assembled COW1 promoter::*P. patens* CDS::Nos-T construct was excised from the agarose gel for QIAquick Gel Extraction (Qiagen). The gel slice was weighed, suspended in 3 volumes of Buffer QG, and incubated at 50°C for 10 mins to dissolve the agarose. One volume of isopropanol was added to the solution and mixed before the solution was applied to a Qiaquick spin column. DNA was bound to the silica membrane via centrifugation for 1 min. Flowthrough was removed, and the column was washed with 750 μL of Buffer PE via 16,000 x g centrifugation for 1 min. After removing the flowthrough, the column was centrifuged again at 16,000 x g for 1 min in order to remove residual ethanol left from Buffer PE. The full-length construct DNA was eluted from the membrane into 50 μL of Buffer EB (10 mM Tris Cl, pH 8.5).

A. Creation of entry clones

The fully assembled COW1 promoter::*P. patens* CDS::Nos-T construct was inserted into the pENTR™/D-TOPO® entry clone vector (Invitrogen) via a TOPO® Cloning reaction (6 μL
mixture of 0.5-4 µL PCR product, 1 µL salt solution, 1 µL TOPO® vector). The reaction was incubated at 25°C for 15 mins prior to transformation of chemically competent *E. coli*.

B. **Transformation of Mach1™ *E. coli* with entry clones**

Chemically competent *E. coli* (Mach1™, F-φ80(lacZ)ΔM15 ΔlacX74 hsdR(rK-mK+) ΔrecA1398 endA1 tonA) cells were transformed with entry clone plasmid DNA according to the manufacturer’s directions (Invitrogen). A mixture of the TOPO® reaction product (2 µL) and 50µL of chemically competent *E. coli* cells were incubated on ice for 20 mins and heat shocked for 30 seconds in a 42°C water bath. The cells were immediately returned to ice and suspended in 250 µL S.O.C. medium (2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose) prior to an hour-long incubation at 37°C on a horizontal shaker set to 200 rpm. Aliquots of each transformation reaction were spread on solid selective LB media (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agarose, 50 µg/µL kanamycin) and incubated overnight at 37°C to yield isolated colonies. Small cultures (5 mL) of selective LB broth (1% Bacto-tryptone, 0.5% yeast extract, 1% NaCl, 50 µg/µL kanamycin) were inoculated with an isolated colony and left on a spinner at 37°C overnight.

C. **Isolation of plasmid DNA from Mach1™ cells transformed with the entry clone**

A QIAPrep Spin Miniprep Kit (Qiagen) was used to extract plasmid DNA from overnight *E. coli* cultures. Approximately 1.5 mL of each culture was centrifuged for 1.5 mins at 16,000 x g; this was done twice to collect pelleted cells from 3 mL of culture. After discarding the supernatant, cells were thoroughly resuspended in 250 µL Buffer P1, lysed with 250 µL of Buffer P2, neutralized with 350 µL Buffer N3, and immediately inverted six times to mix. Cell
debris was pelleted via centrifugation at 16,000 x g. The lysate was decanted into a QIAprep Spin Column in a collection tube and spun at 16,000 x g for 1 minute, binding plasmid DNA to the column’s silica membrane. The membrane was washed with 500 μL of Buffer PB and 750 μL of Buffer PE, discarding the flow-through between steps. All solutions were centrifuged at 16,000 x g. Residual ethanol from Buffer PE was removed by centrifuging an empty column. The spin column was placed into a sterile microcentrifuge tube and plasmid DNA was eluted with 50 μL of Buffer EB (10 mM Tris Cl, pH 8.5). Plasmid DNA concentration was determined using a NanoDrop1000 spectrophotometer (Thermo Scientific).

D. Entry clone plasmid DNA sequence analysis

Plasmid DNA was sequenced by the Massachusetts General Hospital DNA Core Facility (http://https://dnacore.mgh.harvard.edu) using gene-specific primers for each moss COW1 ortholog and primers specific to the pENTR™/D-TOPO® vector (Table 3). Primers were designed for full coverage of the construct and partial coverage of the vector to ensure correct orientation and sequence accuracy.
Table 3. Sequencing primers for Pp1s88, Pp1s169, and Pp1s307 entry clones

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COW1P1</td>
<td>CTC CAT AAC TAA CAT TGG AGT TC</td>
</tr>
<tr>
<td>COW1P2</td>
<td>AAA CTA AAT TCA CTG TAT GTG</td>
</tr>
<tr>
<td>COW1P3</td>
<td>TCG CCG TGT GAA ACA GAG TGC TTA</td>
</tr>
<tr>
<td>COW1P1R</td>
<td>CGG CCA AGA CGC TAC GTT TGA TTT</td>
</tr>
<tr>
<td>88seq</td>
<td>GGA AGT TAC TAC ACT TGA GCG GT</td>
</tr>
<tr>
<td>169seq</td>
<td>AGT TTC CAG CAT GCT CTG TTG CAG</td>
</tr>
<tr>
<td>307seq</td>
<td>AGT TTC CAG CAT GCT CTG TTG CAG</td>
</tr>
<tr>
<td>NOSR2</td>
<td>TAA ATT ATC GCG CGC GGT GTC ATC</td>
</tr>
<tr>
<td>M13F</td>
<td>TGT AAA ACG ACG GCC AGT</td>
</tr>
<tr>
<td>M13R</td>
<td>CAG GAA ACA GCT ATG AC</td>
</tr>
</tbody>
</table>

E. Directional left-right (LR) recombination of the construct from the entry clone into the destination vector

Gateway® LR Clonase™ II Enzyme Mix was used according to manufacturer’s directions (Invitrogen) for site-specific recombination of the construct from the pENTR™/D-TOPO® entry vector into the pMDC123 vector. The pMDC123 plant expression vector belongs to a publicly available set of binary vectors for Agrobacterium-mediated plant transformation, designed by Mark Curtis and Ueli Grossniklaus (CURTIS and GROSSNIKLAUS 2003). A Gateway® recombination reaction mixture (5-15 ng/μL of entry clone plasmid DNA, 15 ng/μL pMDC123 destination vector, 1X LR Clonase™ II Plus Enzyme Mix in TE buffer, pH 8.0) was incubated at 25°C overnight. The reaction was terminated by the addition of Proteinase K to 0.2 μg/μL and incubation at 37°C for 10 mins. Mach1™ E. coli were subsequently transformed with the LR reaction product, and plasmid DNA was isolated from transformants.
F. Transformation of electrocompetent Agrobacterium tumefaciens with the expression clone

Electrocompetent *Agrobacterium tumefaciens* (GV3101::pMP90) was transformed with the moss CDS expression clone via electroporation. GV3101::pMP90 cells (40 μL) were thawed on ice and mixed with 17-20 ng of expression clone plasmid DNA. The mixture of electrocompetent cells and DNA was transferred to a pre-chilled electroporation cuvette, and an Eppendorf electroporator 25110 was used for electroporation (1440V, ~5 msec). The electroporated cells were mixed with 1 mL of LB, incubated at 25°C for an hour, and spread onto selective LB plates with 10 μg/μL gentamicin, 50 μg/μL rifampicin, and 50 μg/μL kanamycin. *Agrobacterium* colonies were grown for two days at 30°C.

G. A. tumefaciens-mediated transformation of mutant cow1 A. thaliana

*A. thaliana cow1* seeds were vernalized for 2 days at 4°C in a mixture of 75% soil (ProMix) and 25% calcine clay. Seeds were grown at 22°C with a 16/8-hour photoperiod for approximately one month. Plants were transformed with the moss COW1 construct according to the floral dip method (*ZHANG et al. 2006*), in which the floral tissues of each plant are exposed to liquid media consisting of 5% sucrose, 0.02% (v/v) Silwet L-77 surfactant, and *Agrobacterium* carrying the expression clone. By infecting *Arabidopsis* through the reproductive tissues, *Agrobacterium* produces a population of transgenic seeds in the transformed plant’s offspring.

Saturated 5 mL liquid cultures (LB, 10 μg/μL gentamicin, 50 μg/mL kanamycin, 50 μg/μL rifampicin) of transformed *A. tumefaciens* were incubated for 48 hours on a roller at 30°C. These small volume cultures were used to inoculate a large (500 mL) liquid culture (LB, 10 μg/μL gentamicin, 50 μg/μL kanamycin, 50 μg/μL rifampicin), which was shaken overnight at 30°C to an A600 or 0.7-1.9. The cells from the large culture were chilled in an ice water bath and
pelleted at 4000 x g for 10 mins at 4°C. The supernatant was discarded, and pelleted cells were resuspended in a 5% sucrose and 0.02% Silwet L-77 solution. The aerial tissues of each A. thaliana cow1 plant were soaked in this suspension with gentle agitation for 10 sec. Four pots of cow1 were transformed for each of the three moss orthologs, signifying four independent transformation events. Plants were laid horizontally on a covered tray overnight at 22°C under continuous light; the next day, plants were righted and the domes removed. After approximately 2-3 weeks, plants were allowed to dry and seed was collected. This first generation of transgenic seed was referred to as the T₁ generation.

III. Phenotypic analysis of transgenic A. thaliana expressing P. patens COW1 orthologs.

A. Screening for transgenic individuals

The T₁ seed collected from transformed cow1 A. thaliana were screened for transgenic individuals, which carry the BASTA resistance marker. T₁ seeds were suspended in a 0.1% agarose solution, sown onto a mixture of 75% soil (ProMix) and 25% calcine clay, and vernalized for 2 days at 4°C. Seeds were grown at 22°C with a 16/8-hour photoperiod for 5-7 days, and sprayed with a 1:1000 dilution of BASTA herbicide every two days. BASTA-sensitive seedlings experienced yellowing cotyledons and stunted growth, while BASTA-resistant seedlings appeared green and healthy. All seedlings were sprayed approximately five times, until the remaining seedlings were clearly unaffected by the herbicide. These transgenic individuals were allowed to grow and produce large quantities of seed for approximately one month. After one month, plants were left to desiccate under continuous light for 2-3 weeks before collection of the T₂ generation of seed.
B. Identification of transgenic lines with a single T-DNA insertion

To identify transgenic T₁ individuals with a single T-DNA insertion, the T₂ generation was scored for segregation of the BASTA-resistance marker. T₂ seeds were first surface sterilized to remove bacterial or fungal contamination. Seeds were washed with a 1% Tween 20 surfactant and 70% ethanol solution before being rinsed with sterile water. Seeds were soaked in 50% bleach for 10 mins, rinsed with sterile water 6-8 times to remove the bleach, and suspended in a 0.1% agarose solution to facilitate sowing onto solid selective growth media (0.5X MS, 1% sucrose, 0.7% plant tissue culture agar, 50 μM BASTA pH 5.5-5.7). These plates were sealed with paper tape and incubated for two days at 4°C, then left upright at 22°C under a 16/8-hour photoperiod.

At approximately 7-10 days post germination, BASTA-sensitive individuals exhibited stunted growth and yellowing cotyledons compared to BASTA-resistant seedlings. All seedlings were scored for BASTA-resistance. Independent segregation of a single T-DNA insertion was reflected by a 3:1 ratio of BASTA-resistant to BASTA-sensitive individuals in the T₂ generation.

C. Growth conditions for phenotypic characterization of cow1 A. thaliana

To characterize the phenotypes of T₂ seedlings, seeds were first surface sterilized as previously described, sown onto solid, nonselective growth media (0.5X MS, 1% sucrose, 0.7% plant tissue culture agar, pH 5.5-5.7), and vernalized for 2 days at 4°C. After five days, roots were observed under a Leica M165 dissecting microscope to characterize the phenotypic effect of Pp1s88, Pp1s169, or Pp1s307 expression.
D. Scoring seedlings for root hair length

Seedlings were scored for root hair length by mounting each root on a slide and digitally measuring the lengths of 10 root hairs using a Nikon80i microscope and NIS-Elements software. Starting from the root tip, sections of the root along which root hair lengths were relatively constant were selected for scoring. When a root hair was branched, the lengths of the hair base (prior to branching) and a single branch were summed.

E. Scoring for root hair branching

Seedlings were scored for root hair branching using a Leica M165 dissecting microscope. Root hairs were scored in regions where they were approximately of equal length, beginning from the root tip. All hairs visible in the microscope field of view were scored for branching.

F. Obtaining T3 homozygous lines for phenotypic characterization

To better characterize the phenotype of transgenic Arabidopsis cow1 plants expressing the moss COW1 orthologs, lines homozygous for the T-DNA were isolated. T2 seeds were first screened on solid selective media (0.5X MS, 1% sucrose, 0.7% plant tissue culture agar, 50 μM BASTA pH 5.5-5.7), isolating individuals homozygous or hemizygous for the T-DNA insertion. After 7-10 days, surviving seedlings were potted into a mixture of 75% soil (ProMix) and 25% calcine clay and allowed to grow for approximately 4 weeks at 22°C under a 16 h/-8-hour photoperiod. Seed was separately collected from each T2 individual, constituting the T3 generation.

To determine the genotypes of T2 individuals, T3 seed was screened on solid selective media (0.5X MS, 1% sucrose, 0.7% plant tissue culture agar, 50 μM BASTA, pH 5.5-5.7), All T3
seed were homozygous and survived on the selective media if the T₂ parent was homozygous, while 25% of T₃ seed died if the T₂ parent was hemizygous. One line of homozygous transgenic cow₁ was isolated for Pp₁s₁₆₉ and for Pp₁s₃₀₇.

IV. RT-PCR analysis of Pp₁s₃₀₇ CDS mRNA expression levels in cow₁ (T₂) A. thaliana

A. RNA collection and DNase I digestion

T₂ generation and control cow₁ seeds were surface-sterilized, vernalized, and sown onto nonselective growth media (0.5X MS, 1% sucrose, pH 5.5-5.7). Seven days post-germination, < 100 mg of whole seedlings were flash-frozen with liquid nitrogen and pulverized with a mortar and pestle. An RNeasy Kit (Qiagen) was used according to manufacturer’s directions to extract RNA. Pulverized plant tissue was suspended in 450 µL Buffer RLT and incubated at 56°C for 1-3 min for tissue lysis. The mixture was centrifuged through a QIAshredder spin column for 2 min at 16,000 x g, and lysate was isolated from the pelleted cell debris. The lysate and a half-volume of isopropanol (96-100%) was mixed via pipetting and centrifuged through an RNeasy Spin column at 16,000 x g for 15 sec. Flow-through was discarded before 700 µL of Buffer RW1 was applied to the column membrane and centrifuged at 16,000 x g for 15 sec. Flowthrough was again discarded, and the column was washed twice with 500 µL of Buffer RPE via centrifugation at 16,000 x g for 15 sec and 2 min for each wash, respectively. Residual Buffer RPE was removed via centrifugation of the empty spin column at 16,000 x g for 1 min. RNA was eluted into 40 µL of RNase-free water applied to the spin column membrane and centrifuged at 16,000 x g for 1 min. Genomic DNA contamination was removed via DNase I digestion as previously described.
B. cDNA synthesis, RT-PCR and agarose gel electrophoresis

RNA digested with DNase I was used to synthesize cDNA using a Phusion® RT-PCR kit as previously described. Gene-specific primers were used to amplify a fragment of the Pp1s307 CDS (Table 4) in a PCR reaction (1X Phusion® HF Buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Phusion® Hot Start II enzyme, and 10% cDNA template in DNase-free water). RNA originating from two independently transformed lines of T₂ seedlings were used for cDNA synthesis and PCR, in addition to DNA templates acting as negative (cDNA synthesized from cow1 RNA and genomic wild-type A. thaliana DNA) and positive (Pp1s307 Sec14 fragment) controls. PCR products were analyzed via agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>307FRI</td>
<td>ATC TGA ATT CAT GGG GGC TTA CAG TCAA</td>
</tr>
<tr>
<td>307HindIII</td>
<td>ATA TAA GCT TTC TAC TAA ATC CAC CGG TGG GTT CT</td>
</tr>
</tbody>
</table>

V. Creation of a hybrid domain-swap CDS (P. patens Sec14::A. thaliana Nlj16) for expression in cow1 A. thaliana

A. Construct design

In order to identify the P. patens Nlj16 domain from the coding sequence of each P. patens COW1 ortholog (Pp1s88_63V6.1, Pp1s169_81V6.1, Pp1s307_16V6.1), the CDS of each ortholog was aligned using the ClustalW program. The Nlj16 domain of A. thaliana was defined according to past studies (BOHME et al. 2004).
B. Primer design for a hybrid P. patens Sec14::A. thaliana Nlj16 CDS

Primers for PCR amplification of the A. thaliana Nlj16 domain were designed with a HindIII site at the 5’ end of the forward primer, and a SalI restriction site at the 5’ end of the reverse primer. The Sec14 domains of Pp1s88, Pp1s169, and Pp1s307 were amplified with gene-specific primers incorporating an EcoRI digestion site at the 5’ end of the forward primer, and a HindIII site at the 5’ end of the reverse primer (Table 5).

Table 5. PCR primers for amplification of P. patens Sec14 and A. thaliana Nlj16 domains. Pink text denotes EcoRI sites, orange denotes SalI sites, blue denotes HindIII sites.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer Sequence (5’-3’)</th>
<th>DNA template</th>
</tr>
</thead>
<tbody>
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<td>88FRI</td>
<td>ATA TGA ATT CAT GCC CGA CTA TGC GGT G</td>
<td>Pp1s88 entry clone</td>
</tr>
<tr>
<td>88HINDIII</td>
<td>ATA TAA GCT TAC CGC ATC CGC TCG TG</td>
<td></td>
</tr>
<tr>
<td>169FRI</td>
<td>ATA TGA ATT CAT GGG AGC TCA AAG TCA AGA ATT</td>
<td>Pp1s169 entry clone</td>
</tr>
<tr>
<td>169HINDIII</td>
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<td></td>
</tr>
<tr>
<td>307FRI</td>
<td>ATC TGA ATT CAT GGG GGTC TTA CAG TCA A</td>
<td>Pp1s307 entry clone</td>
</tr>
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<tr>
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C. Assembly of the hybrid CDS

The A. thaliana COW1 Nlj16 domain and Pp1s88, Pp1s169, and Pp1s307 Sec14 coding sequences were amplified in a PCR reaction (1X Phusion® HF Buffer, 0.2 mM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 1 unit Phusion® Hot Start II enzyme, and 200 ng DNA template in DNase-free water). A thermal cycler was programmed for 35 cycles of denaturation, annealing, and extension (10 sec at 98°C for denaturation, 70 sec at 72°C for both annealing and extension), with initial denaturation at 98°C for 30 sec and final extension at 72°C for 10 min.
Agarose gel electrophoresis was used to analyze PCR products, and DNA was visualized using SYBR Safe DNA gel stain (1X). PCR products were purified using the QIAquick PCR Purification kit. The Pp1s88, Pp1s169, and Pp1s307 Sec14 CDS fragments and the A. thaliana Nlj16 fragment were digested with restriction enzyme HindIII (1X NEB Buffer 2, 5 μL purified PCR product, 10 units HindIII) and purified prior to a Quick Ligation (New England Biolabs) reaction between each P. patens Sec14 and the A. thaliana Nlj16. The fully assembled hybrid CDS was used to generate expression clones and transform cow1 A. thaliana as previously described.
Results

In order to determine if *P. patens* COW1 orthologs are functionally equivalent to *A. thaliana* COW1, a cross-species complementation study was performed (Fig. 4). Four putative *P. patens* orthologs (Pp1s47_303V6.1, Pp1s88_63V6.1, Pp1s169_81V6.1, Pp1s307_16V6.1) carrying Sec14-like and Nlj16-like domains were tested for expression in *P. patens* protonemata, which develop exclusively by tip growth. Reverse transcription PCR (RT-PCR) was used as a semiquantitative assay for mRNA abundance. RNA was extracted from seven day-old moss protonemata and used as a template for cDNA synthesis. Short regions of the cDNA template were amplified with primers specific to each ortholog, and visualized using agarose gel electrophoresis. The number of amplicons reflected the relative abundance of ortholog mRNA. Three (Pp1s88, Pp1s169, Pp1s307) of the four orthologs were strongly expressed in seven-day old moss protonemata, as evidenced by the abundance of amplified cDNA (Fig. 5). These three orthologs were selected for expression in *A. thaliana* cow1 plants.

A moss ortholog expression construct was designed to express the CDS of the three expressed *P. patens* orthologs in *A. thaliana* cow1. This construct was a chimeric gene composed of the moss CDS under the control of the *A. thaliana* COW1 promoter and terminated with a nopaline synthetase sequence. The fully assembled construct was inserted into an entry vector plasmid and proliferated by transforming and culturing chemically competent *E. coli* cells. Copies of the entry clone were isolated from *E. coli*, cut with restriction enzymes to determine successful entry vector cloning, and sequenced to ensure accuracy. After identifying an entry clone with the correct sequence and orientation, the transgene was transferred from the entry vector into a T-DNA expression vector via Gateway® recombination cloning.
Figure 5. Three putative *A. thaliana* COW1 orthologs are strongly expressed in *P. patens* protonematal tissue. Gene-specific primers were used in reverse-transcription PCR (RT-PCR) to assess the relative transcript abundance of four putative *A. thaliana* COW1 orthologs in 7-day old *P. patens* protonemata. Expression levels of Pp1s88 (265 bp), Pp1s169 (118 bp), and Pp1s307 (147 bp) are comparable to the strongly expressed ubiquitin control (250 bp), while Pp1s47’s (95 bp) low level of expression is denoted by its weak amplification. DNA fragment sizes compared to a standard 100bp DNA ladder.
In Gateway® recombination cloning, the transgene is flanked by two sites (L1 and L2) within the entry vector, and the Gateway® LR Clonase II enzyme transfers the transgene between two specific sites (R1 and R2) within the expression vector. The T-DNA expression vector incorporates a transgene into a “T-DNA region,” which is integrated into the A. thaliana genome upon Agrobacterium-mediated transformation. A strain of disarmed, electrically competent A. tumefaciens was transformed with one of three ortholog expression clones (Pp1s88, Pp1s169, Pp1s307) and used to transform cow1 A. thaliana according to the floral dip protocol. The first (T₁), second (T₂), and third (T₃) generations of these transformed cow1 plants were observed to characterize the phenotypes produced by Pp1s88, Pp1s169, or Pp1s307 expression.

I. Creation of a transgene for P. patens ortholog expression in cow1 A. thaliana

To express each of the three putative moss COW1 orthologs in the same spatial and temporal patterns as endogenous A. thaliana COW1, the moss CDS was placed under the control of the A. thaliana COW1 promoter. The construct was terminated with the nopaline synthase terminator (nos-t). The A. thaliana COW1 promoter, each moss CDS, and a nos-t sequence were individually amplified via PCR (Fig. 6) using primers with EcoRI and SalI restriction sites. Restriction enzyme digests with SalI and EcoRI and subsequent ligation reactions yielded a ligation product (Fig. 7A) consisting of the full-length moss COW1 construct for expression in A. thaliana.

The Gateway® TOPO® Cloning system was used to clone the fully assembled construct into the pENTR™/D-TOPO® entry vector. Successful cloning was verified using a NotI and SalI double-digest, which produced two fragments of expected lengths (3.7 kb, 2.9 kb) (Fig. 7B). Entry clone sequences in the correct orientation and exhibiting 100% identity to the expected A.
Figure 6. PCR amplification of the *A. thaliana* COW1 promoter, Pp1s88/Pp1s169/Pp1s307 moss CDS, and the nos-t terminator sequences. (A) Gene-specific primers (red arrows) incorporating restriction sites for eventual digestion and ligation were used to amplify the *A. thaliana* COW1 promoter from *A. thaliana* genomic DNA, each moss CDS (Pp1s88, Pp1s169, and Pp1s307) from *P. patens* genomic DNA, and the nos-t sequence from the pMDC100 vector. (B) Agarose gel electrophoresis of PCR products corresponding to the COW1 promoter (2.0 kb), moss CDS (~1.7 kb) and the nos-t sequence (265 bp). DNA fragment sizes compared to a standard 1kb DNA ladder.
Figure 7. Creation of Pp1s88, Pp1s169, and Pp1s307 moss ortholog expression clones. Individually amplified fragments of the *A. thaliana* COW1 promoter, Pp1s88/Pp1s169/Pp1s307 CDS, and the nos-t terminator were double-digested with SalI and EcoRI, and (A) ligated to generate the full-length construct (~4 kb). The constructs were cloned into the pENTR™/D-TOPO® entry vector. (B) Successful cloning into the entry vector was verified with a restriction double-digest using NotI and SalI, which produced two fragments of expected lengths (3.7 kb, 2.9 kb). (C) Successful recombination of the Pp1s88, Pp1s169, and Pp1s307 entry clones with the pMDC123 expression vector was verified with a SalI restriction digest, producing fragments of expected length (7 kb, 3.9 kb, 1.8 kb).
thaliana COW1 promoter, P. patens Pp1s88, Pp1s169, Pp1s307, and nos-t sequences were recombined into a T-DNA destination vector (pMDC123) to produce ortholog expression clones. Expression clone plasmids were cut with SalI to produce three fragments of expected lengths (7.0 kb, 3.9 kb, 1.8 kb), verifying the accuracy of recombination between the entry and destination vectors (Fig. 7C). Expression clones were successfully generated for each of the three moss orthologs and used to transform Agrobacterium tumefaciens. These transgenic strains of A. tumefaciens were used for Agrobacterium-mediated transformation of cow1 plants.

II. Creation of transgenic cow1 A. thaliana expressing the COW1 moss orthologs

A. thaliana cow1 T-DNA insertion mutants (SALK_002124C) were transformed to express each of the three P. patens COW1 orthologs. The Agrobacterium-mediated transformation method was used, in which a liquid culture of disarmed Agrobacterium tumefaciens (GV3101::pMP90) infects A. thaliana through its reproductive tissues. A small number of transgenic A. thaliana offspring is produced, carrying the T-DNA construct of interest integrated into its chromosomal DNA. The random nature of T-DNA insertion into the A. thaliana genome necessitated at least two independent transformations of each of the three potential moss COW1 constructs.

The first generation of seed (T1) produced by these transformed plants was screened for transgenic individuals using the herbicide BASTA (glufosinate). Transgenic individuals are hemizygous for the construct of interest, which carries a BASTA resistance marker. The number of transgenic T1 seedlings is limited by the relatively low efficiency of Agrobacterium-mediated transformation. Consequently, the offspring produced by T1 plants (the T2 generation) was scored to determine segregation patterns, which reflect whether independently transformed lines
experienced a single T-DNA insertion upon transformation. T₂ seed produced by independently transformed lines of *A. thaliana cow1* carrying the Pp1s88, Pp1s169, or Pp1s307 CDS were surface-sterilized and grown on solid selective media (0.5X MS, 1% sucrose, 50 μM BASTA). At ten days post-germination, T₂ individuals were scored for BASTA resistance or sensitivity; lack of BASTA resistance was evidenced by stunted growth. Chi-square tests (Table 6) indicated independent segregation (P<0.05) of a single T-DNA insertion across all lines aside from 169C, ruling out the possibility of multiple insertions of the T-DNA transgene.

To confirm expression of the moss COW1 ortholog CDS, an RT-PCR was performed using RNA extracted from T₂ lines transformed with the Pp1s307 construct. Expression of the Pp1s307 CDS was confirmed upon successfully amplifying a region of the Pp1s307 CDS from a cDNA template. Two lines of *cow1* independently transformed with the Pp1s307 construct (307B and 307C) were found to express Pp1s307 CDS mRNA (Fig. 8). Experiments to determine Pp1s88 and Pp1s169 mRNA expression in T₂ seedlings are currently underway.

### III. Transgenic cow1 *A. thaliana* expressing Pp1s169 or Pp1s307 exhibit weak rescue of root hair length and increased incidence of branched root hairs

The phenotypic effect of Pp1s169 or Pp1s307 expression in *A. thaliana cow1* mutants was assessed by isolating homozygous lines of transgenic *cow1* plants. Approximately a quarter of the T₂ population is homozygous for the transgene of interest. The seed produced by transgenic T₂ individuals—constituting the T₃ generation—was collected and grown on solid selective media (0.5X MS, 1% sucrose, 50 μM BASTA). The T₃ seed of a single Pp1s169 line (169B) and a single Pp1s307 line (307B) exhibited BASTA resistance across all screened seedlings, identifying these T₃ lines as homozygous for the transgene. Homozygous lines of
Table 6. Transgenic lines (T$_2$) of *cow1* *A. thaliana* Pp1s88, Pp1s169 and Pp1s307 carry a single T-DNA insertion. $\chi^2$ calculated for a 3:1 ratio of BASTA-resistance to BASTA-sensitivity; $p \leq 0.05$ when $\chi^2 \leq 3.841$.

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<td>102 73.9%</td>
<td>36 26.1%</td>
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Figure 8. The Pp1s307 CDS is expressed in independently-transformed lines of *A. thaliana cow1*. RNA was extracted from 7dpg *cow1* seedlings and seedlings (T<sub>2</sub>) from *cow1* plants transformed with the Pp1s307 CDS under the control of the *A. thaliana* COW1 promoter. Gene-specific primers were used to amplify the Sec14 region of Pp1s307 from genomic *A. thaliana* DNA (-), cDNA synthesized from *cow1* RNA, cDNA synthesized from transgenic (T<sub>2</sub>) *cow1* expressing Pp1s307, and the Pp1s307 expression clone used to transform *A. thaliana cow1* (+). B and C represent two independent transformation events.
cow1 expressing the Pp1s88 CDS are still in the process of identification and isolation from a T₉ population.

Homozygous T₃ lines of Pp1s169 and Pp1s307 were grown vertically across standard A. thaliana growth media (0.5X MS, 1% sucrose, 0.7% plant tissue culture agar, pH 5.5-5.7) at 22°C and a 16/8-hour photoperiod. At 4-5 dpg, root hairs were observed under a Leica M165 dissecting microscope (Fig. 9). Transgenic T₃ seedlings expressing either Pp1s169 or Pp1s307 exhibited significantly shorter root hairs than wild type (Fig. 9, 10A), indicating that the Pp1s169 and Pp1s307 CDS do not fully complement the A. thaliana cow1 mutant. Yet compared to control cow1 root hairs, cow1 homozygous for Pp1s169 (student’s t-test, p=0.00018, df=9, α=0.05) and Pp1s307 (student’s t-test, p=0.0057, df=9, α=0.05) exhibited significantly longer root hairs (Fig. 10B). Interestingly, the “twin hair” phenotype described in cow1 mutants—characterized by two root hairs elongating from the same initiation site—was observed at significantly higher frequencies in both Pp1s169 (student’s t-test, p<0.0001, df=8, α=0.05) and Pp1s307 (student’s t-test, p<0.0001, df=3, α=0.05) cow1 T₃ lines (Fig. 11). Approximately 25% and 37% of root hairs were branched in cow1 A. thaliana expressing Pp1s169 (n = 171), and Pp1s307 (n = 90), respectively, compared to 2% of root hairs in the cow1 control.

IV. Creation of cow1 A. thaliana expressing a hybrid P. patens Sec14::A. thaliana Nlj16 CDS

The weak rescue of the cow1 mutant phenotype may be attributed to differences between the Nlj16 membrane localization domains of P. patens and A. thaliana. The A. thaliana Nlj16 domain is involved in plasma membrane targeting at the subcellular level, but the P. patens Nlj16 domain may localize elsewhere in the A. thaliana root hair, potentially preventing the
Figure 9. Representative images of root hairs from wild-type, cow1, and transgenic A. thaliana seedlings. Surface-sterilized seedlings were grown on standard media (0.5X MS, 1% sucrose, pH 5.5-5.7) for five days prior to observation with a Leica M165 dissecting microscope. (A) Wild type, (B) cow1, (C) and homozygous T$_3$ seedlings expressing the Pp1s169 or Pp1s307 CDS were scored for root hair length and frequency of branching.
Figure 10. Root hair lengths of homozygous T<sub>1</sub> cow<sup>1</sup> A. thaliana expressing Pp1s169 or Pp1s307 CDS. Five day old A. thaliana cow<sup>1</sup> seedlings expressing Pp1s169 or Pp1s307 were mounted on slides and observed using a Nikon80i microscope. Nikon80 software was used to measure the lengths of approximately ten root hairs. Homozygous lines of transgenic cow<sup>1</sup> A. thaliana expressing Pp1s169 or Pp1s307 exhibit (A) significantly shorter root hairs compared to Wt, but (B) significantly longer root hairs relative to cow<sup>1</sup>. Error bars = SEM. Brackets and stars represent significantly different groups according to student’s t-test (P<0.05).
Figure 11. Percentage of branched root hairs in homozygous T₃, *cow1* *A. thaliana* expressing Pp1s169 or Pp1s307 CDS. Five day old homozygous (T₃) lines of *cow1* seedlings expressing Pp1s169 or Pp1s307 were observed under a Leica M165 dissecting microscope. Root hairs were scored for branching approximately 30% up from the root tip, the earliest zone where hairs were of equal length in the field of view. Transgenic *A. thaliana* expressing Pp1s169 (n=129 root hairs) or Pp1s307 (n=57) exhibit significantly more frequent incidences of branched root hairs relative to the *cow1* mutant (n=205). Error bars = SEM. Brackets and stars represent significantly different groups according to student’s t-test (P<0.05).
moss Sec14 domain from restoring normal root hair growth. A domain-swap construct was assembled, identical to the original construct apart from a modified moss CDS. Instead of a full-length moss CDS incorporating the *P. patens* ortholog’s Sec14 and Nlj16 domains, a hybrid CDS was designed in which the *P. patens* Nlj16 domain was replaced with the Nlj16 domain of *A. thaliana* COW1.

The moss ortholog’s Sec14 domain and the *A. thaliana* Nlj16 domain were individually amplified (Fig. 12A, B), digested with HindIII, and ligated to produce the full hybrid CDS (Fig. 12C, D). Much like the transgene incorporating the full moss CDS, the hybrid CDS was placed under the control of the *A. thaliana* COW1 promoter, and the transgene was terminated with the nos-t terminator. The full-length transgene carrying a Pp1s307 Sec14 and *A. thaliana* COW1 Nlj16 was ligated (Fig. 13A), cloned into the pENTR™ entry vector (Fig. 13B), and recombined into the pMDC123 expression vector (Fig. 13C). *Agrobacterium*-mediated transformation was again used to create transgenic cow1 *A. thaliana* expressing the hybrid Pp1s307 Sec14::*A. thaliana* COW1 Nlj16 CDS. The T₁ generation of seed is currently being screened for transgenic individuals.
Figure 12. PCR amplification of Pp1s88, Pp1s169, and Pp1s307 Sec14 domains, and the A. thaliana COW1 Nlj16 domain. (A) Gene-specific primers incorporating EcoRI, HindIII, and SalI restriction sites for eventual digestion and ligation were used to amplify each moss CDS (Pp1s88, Pp1s169, and Pp1s307) from P. patens genomic DNA without the P. patens Nlj16 region, and the AtSfh1 (COW1) Nlj16 region of genomic A. thaliana DNA. (B) Successful amplification of each Sec14-containing moss CDS (~1.3 kb) and A. thaliana Nlj16 domain (~0.45 kb). DNA fragment sizes compared to a standard 1kb DNA ladder. (C) HindIII digestion and ligation yields a CDS with a P. patens Sec14 domain and an A. thaliana Nlj16 domain. (D) Re-amplification of the new moss CDS using a forward EcoRI primer and a reverse SalI primer.
Figure 13. Creation of expression clones carrying a hybrid Pp1s307 Sec14 domain::A. thaliana COW1 Nlj16 domain CDS. Individually amplified fragments of the A. thaliana COW1 promoter, the nos-t terminator, and the Pp1s307 Sec14::At Nlj16 hybrid CDS were double-digested with SalI and EcoRI, and (A) ligated to assemble the full-length construct (~4 kb). (B) Successful cloning of these transgenes into the pENTR™/D-TOPO® entry vector was verified with a NotI and SalI restriction double-digest, which produced two fragments of expected lengths (3.7 kb, 2.9 kb). (C) Successful recombination between the Pp1s307 entry clone and the pMDC123 expression vector was verified with a SalI restriction digest, producing three fragments of expected length (7 kb, 3.9 kb, 1.8 kb).
Discussion

In order to determine whether *P. patens* COW1 orthologs are functionally equivalent to the *A. thaliana* COW1 Sec14-like PITP, a construct was designed to express the ortholog CDS in identical spatial and temporal patterns, and at the same levels, as endogenous COW1 in *A. thaliana*. This construct was used to transform *cow1* *A. thaliana* plants, and single T-DNA insertion lines were shown to express ortholog mRNA. Weak rescue of root hair length was observed in homozygous lines of *cow1* individuals expressing moss orthologs, but a striking increase in branched root hairs was also observed in these same lines. This aberrant phenotype may shed light onto how the COW1 PITP modulates root hair polarity and fits into the network of tip growth signaling mechanisms.

*The agd1 cow1 mutant phenotype is characterized by extreme root hair branching*

In a recent study (Yoo et al. 2012), *A. thaliana agd1 cow1* double mutants were found to exhibit a highly branched root hair defect more severe, but still reminiscent of the enhanced branching phenotype seen in our transgenic *cow1* individuals. Understanding the interactions between AGD1 and COW1 that produce this phenotype may shed light onto the similar phenotype found in *cow1* *A. thaliana* expressing moss COW1 orthologs. While the *cow1* single mutant exhibits short root hairs with a relatively low branching frequency of 4-5% (Bohme et al. 2004), approximately 60% of *agd1* root hairs are branched, and hairs that are unbranched exhibit a wavy growth pattern. PtdIns4P localization patterns, Ca\(^{2+}\) gradient, F-actin network organization, and ROP activity are all disrupted in the *agd1* mutant. While the authors did not characterize PtdIns(4,5)P\(_2\) localization patterns in *agd1*, from what is known about PtdIns(4,5)P\(_2\)’s role in regulating actin cytoskeletal dynamics it is likely that this tip-focused
gradient is disturbed as well. In the *cow1* mutant, the PtdIns(4,5)P$_2$ gradient, F-actin organization, and Ca$^{2+}$ gradient are also disrupted, but PtdIns4P localization patterns have not been characterized (Vincent et al. 2005). In light of the *agd1* and *agd1 cow1* mutant phenotypes, as well as PtdIns4P’s role as the precursor to PtdIns(4,5)P$_2$, it is likely that the PtdIns4P gradient is disrupted in *cow1* mutants as well. Comparison of the double *agd1 cow1* mutant—characterized by two to four branches per hair—to each of these single mutant phenotypes suggests that AGD1 and COW1 cooperate to establish and/or maintain the polar axis during tip growth.

*COW1 and AGD1 are involved in PtdIns4P and PtdIns(4,5)P$_2$ regulation*

AGD1 is an ARF-GAP that likely regulates PtdIns4P localization patterns (Yoo et al. 2012; Yoo et al. 2008), and consequently the tip-focused PtdIns(4,5)P$_2$ gradient. It possesses a pleckstrin homology (PH) domain, which are PI-binding domains that generally localize to the plasma membrane, and AGD1 was found to bind PtdIns3P, PtdIns4P, and PtdIns5P. The authors hypothesized that AGD1 plays a part in the PI signaling pathway involved in tip growth (Fig. 14), and investigated its genetic interactions with other known players in PI metabolism to place AGD1’s function within the context of this network. RHD4 (a PtdIns4P phosphatase which converts PtdIns4P to PtdIns, Fig. 14A) is epistatic to AGD1, suggesting that AGD1 functions after RHD4 in root hair tip growth. The *agd1 pip5k3* mutant exhibits significantly shorter root hairs than either the *agd1* or *pip5k3* single mutants, but its branching frequency is identical to the *agd1* mutant, suggesting that AGD1 may function prior to PIP5K3 during tip growth with respect to its branching defect. PIP5K3 is responsible for synthesizing PtdIns(4,5)P$_2$ from PtdIns4P in the subapical region, establishing the PtdIns(4,5)P$_2$ pools seen at the root hair apex (Fig. 14B). The significantly higher frequency of branched root hairs in the *cow1 agd1* double
Figure 14. AGD1 and COW1 are likely involved in the maintenance of PtdIns4P pools in the subapical root tip region. (A) PtdIns is phosphorylated by PI-4Kβ1 at the 4-position to generate PtdIns4P. PtdIns4P localizes to the lateral flanks in the subapical region, where they act as tip growth signaling molecules in their own right, or as the substrate material for (B) PtdIns(4,5)P$_2$ synthesis, catalyzed by PIP5K3. A tip-focused PtdIns(4,5)P$_2$ gradient (C) recruits effector molecules that lead to tip-directed actin cytoskeletal dynamics and vesicle trafficking from the Golgi.
mutant compared to *agd1* strongly suggests that COW1 and AGD1 work synergistically at the level of the PtdIns4P pool. The severity of the *agd1 cow1* branched phenotype suggests that COW1 acts synergistically with AGD1 to stabilize the PtdIns4P pools in the subapical region, and is essential for maintaining PI landmarks that in turn regulate PtdIns(4,5)P₂ pools at the tip (Fig. 14).

Taken in conjunction with what is known about PI regulation of tip growth, AGD1 and COW1’s interactions with PtdIns4P and PtdIns(4,5)P₂ suggest several possible downstream dysregulations responsible for root hair branching. The phenotypes of *agd1 rhd4* and *agd1 pip5k3* double mutants, as well as the deranged PtdIns4P localization patterns in the *agd1* single mutant, are strong evidence for AGD1’s role in establishing and/or stabilizing subapical PtdIns4P pools. PtdIns4P’s role in tip growth as signaling molecules in their own right, or as the substrate molecules for PIP5K3-catalyzed synthesis of PtdIns(4,5)P₂ has been previously discussed. Tip-focused PtdIns(4,5)P₂ gradients recruit effector molecules including ROPs, ABPs, and ADF/cofilin to regulate actin cytoskeletal dynamics and vesicle trafficking.

Though we can only speculate upon the exact nature of interactions between PIs, COW1, and AGD1 in *A. thaliana*, studies in the yeast system may offer some insight into how these two proteins—a PITP and an ARF-GAP—interact with PIs and each other. In yeast, ARF-GAPs are Sec14p effectors that are required for vesicle formation at the TGN. ARF-GAPs and Sec14 PITPs are proposed to belong to a common secretory pathway in which the Sec14 domain creates an appropriate PI environment to stimulate ARF-GAP activity (Mousley *et al.* 2007; Wong *et al.* 2005). The activities of ARF-GAPs Gcs1p and Age2p are stimulated by DAG and PtdIns(4,5)P₂, and inhibited by PtdCho, suggesting that Sec14p plays a role in establishing an appropriate lipid environment for ARF-GAP function (Yanagisawa *et al.* 2002). This model is
reminiscent of the previously proposed role of the COW1 PITP as a PI landmark generator that establishes a tip-focused PtdIns(4,5)P$_2$ gradient, which recruits downstream effectors—ARF-GAPs possibly among them—to stimulate F-actin network formation and vesicle trafficking in the developing root hair (Vincent et al. 2005). However, the Yoo study (2012) suggests that this model of COW1 function may be more complex, involving PtdIns4P activity prior to the establishment of the PtdIns(4,5)P$_2$ gradient. The appropriate Sec14-established lipid environment for downstream activity remains unclear—DAG and PtdCho pools, PtdIns, or both? The activity of some A. thaliana ARF-GAPs are enhanced by or dependent on binding preferentially to PtdIns4P or PtdIns(4,5)P$_2$, warranting further investigation into exactly how the Sec14 domain facilitates downstream activity (Kam et al. 2000; Naramoto et al. 2009).

Yeast studies may also illuminate AGD1 and COW1’s mode of action for establishing PtdIns4P domains in the subapical root tip region. Activated ARF1, a monomeric G protein, recruits a PtdIns 4-kinase to the yeast TGN to increase local concentrations of PtdIns4P. FAPPs (four-phosphate adaptor proteins) are subsequently recruited to the membrane and bind ARF-GAPs that normally inactivate ARF1, producing a positive feedback loop that further enriches PtdIns4P microdomains (Shin and Nakayama 2004). Sec14p may play a role in presenting PtdIns to these PtdIns 4-kinases at the TGN, and in generating DAG pools needed for PtdIns4P synthesis. Within the A. thaliana system, the AGD1 ARF-GAP may serve a similar function in restricting PI4-kinase recruitment to the subapical flanks of the tip, where a FAPP-like protein binds AGD1, recruits PI4-kinases that act upon COW1-established PI landmarks, and ultimately restricts PtdIns4P pools to the subapical flanks.
Implications for the function of Pp1s169 and Pp1s307 CDS in cow1 A. thaliana

If the COW1 P. patens ortholog CDS were simply nonequivalent to the A. thaliana COW1 CDS, a cow1 phenotype would be observed. Instead, A. thaliana cow1 mutants expressing a P. patens ortholog CDS show a clear gain-of-function phenotype, with 25-37% of transgenic root hairs exhibiting twin branches from a common initiation site compared to the cow1 mutant, as well as a modest increase in root hair length. This phenotypic similarity to the cow1 agd1 double mutant phenotype suggests several possibilities regarding moss COW1’s function in A. thaliana root hairs, especially in light of COW1’s probable involvement in PtdIns4P domain maintenance.

First, there is the possibility that expression of the moss Sec14 domain interferes with AGD1 function. Instead of interfacing with PtdIns for presentation to PI4-kinases and subsequent PtdIns4P production, the P. patens Sec14 domain may instead generate a lipid environment of PtdIns3Ps or sterols. These regulatory lipids may interfere with AGD1’s function, or generate their own phenotype upon inappropriate expression. Sterols have only recently gained recognition for their roles in establishing cell polarity cues during root hair tip growth, with altered sterol composition causing defects in clathrin-dependent endocytosis at the tip (BOUTTE et al. 2010). Sterols accumulate at the presumptive site of tip growth in both normal root hairs and hairs with multiple branches, potentially acting as a platform for organizing vesicular recycling and endocytosis. These localized sterol domains disappear once growth stops, indicating that sterols play a role in both tip growth initiation and elongation (OVECKA et al. 2010). Much like the case with sterols, PtdIns3P has also played an underappreciated role in root hair tip growth relative to PtdIns4P and PtdIns(4,5)P_2 (LEE et al. 2008). PtdIns3P is found within endosomal membranes and is responsible for endosome trafficking via ROS production, as well
as late-stage endocytic recycling at the root hair tip. Biochemical characterization of the moss ortholog’s Sec14 domain through yeast rescue experiments or lipid-binding assays for sterols and PtdIns3P will allow us to explore the possibility of alternative binding specificity.

Alternatively, the Nlj16 domain of *P. patens* may not localize to the same site in *A. thaliana* root hairs as it does in *P. patens* protonemata, potentially contributing to PtdIns4P establishment elsewhere in the epidermal root cell. Relatively little is known about the Nlj16-like domain, named after the late nodulin Nlj16 and first identified in a family of *Lotus japonicus* PITP-like proteins with a Sec14 domain at the N-terminus (Kapranov *et al.* 2001). However, several lines of evidence point to the Nlj16 domain’s membrane targeting properties. The Nlj16 domain possesses motifs associated with membrane targeting, centrifugation experiments with an antibody-tagged Nlj16 protein found that the Nlj16 protein cosediments with fractions suggesting association with membrane compartments, and GFP tags with a C-terminal Nlj16 extension were found distributed around the cell periphery (Kapranov *et al.* 2001). Considering the phylogenetic distance between *P. patens* and *A. thaliana*, it is possible that Sec14-like PITPs have different subcellular localization patterns in *P. patens* than in *A. thaliana*. In order to address the potential for membrane mistargeting, the root hairs of *cow1* plants expressing a CDS hybrid composed of *P. patens* Sec14 and *A. thaliana* Nlj16 will be observed for phenotypic differences compared to *cow1* expressing the full moss ortholog CDS. We are currently in the process of screening the T1 generation of seed for transgenic individuals.

*Concluding remarks*

This study has found that *cow1* *A. thaliana* expressing *P. patens* ortholog CDS exhibits a gain-of-function phenotype characterized by an abnormally high frequency of branched root
hairs. The appearance of this phenotype suggests that either the Sec14 domain of *P. patens* orthologs functions differently in the more basal phylogenetic group of bryophytes than in *A. thaliana* angiosperms, or that the Nlj16 domain has a different subcellular membrane localization pattern in the *P. patens* ortholog compared to *A. thaliana* COW1. Both of these possibilities will be explored in future studies, and a Nlj16 domain-swap experiment is already underway. The answer to this question will shed light onto the changes in tip growth mechanisms that have occurred over the evolutionary history of land plants.
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