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RSL genes are sufficient for rhizoid system development in early diverging land plants

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SUMMARY

Land plants are anchored to their substratum from which essential inorganic nutrients are taken up. These functions are carried out by a system of rhizoids in early diverging groups of land plants, such as mosses, liverworts and hornworts. *Physcomitrella patens* RHD SIX-LIKE1 (PpRSL1) and PpRSL2 transcription factors are necessary for rhizoid development in mosses. Similar proteins, AtRHD6 and AtRSL1, control the development of root hairs in *Arabidopsis thaliana*. Auxin positively regulates root hair development independently of *AtRHD6* and *AtRSL1* in *A. thaliana* but the regulatory interactions between auxin and *PpRSL1* and *PpRSL2* are unknown. We show here that co-expression of *PpRSL1* and *PpRSL2* is sufficient for the development of the rhizoid system in the moss *P. patens*; constitutive expression of *PpRSL1* and *PpRSL2* converts developing leafy shoot axes (gametophores) into rhizoids. During wild-type development, *PpRSL1* and *PpRSL2* are expressed in the specialized cells that develop rhizoids, indicating that cell-specific expression of *PpRSL1* and *PpRSL2* is sufficient to promote rhizoid differentiation during wild-type *P. patens* development. In contrast to *A. thaliana*, auxin promotes rhizoid development by positively regulating *PpRSL1* and *PpRSL2* activity in *P. patens*. This indicates that even though the same genes control the development of root hairs and rhizoids, the regulation of this transcriptional network by auxin is different in these two species. This suggests that auxin might have controlled the development of the first land plant soil anchoring systems that evolved 465 million years ago by regulating the expression of RSL genes and that this regulatory network has changed since mosses and angiosperms last shared a common ancestor.

KEY WORDS: Basic helix-loop-helix transcription factor, Rhizoid, Root hair, Root system evolution, *Physcomitrella*, *Arabidopsis*

INTRODUCTION

The development of specialized tip-growing filamentous rhizoids in early diverging groups of land plants was crucial for the establishment of the first continental vegetation sometime before 465 million years ago (Kenrick and Crane, 1997; Bateman et al., 1998; Wellman and Gray, 2000; Gensel and Edwards, 2001; Wellman et al., 2003; Raven and Crane, 2007). The algal ancestors of the land plants lived in water and absorbed nutrients across their entire surface and some developed specialized rhizoids that functioned to anchor these plants in sediments from which nutrients were extracted (Box et al., 1984; Box, 1986; Box, 1987; Karol et al., 2001; Raven and Edwards, 2001). Once plants moved onto land, they developed photosynthetic organs that grew into the air where inorganic mineral nutrients were not available (Niklas, 1997). Systems of filamentous rhizoids and roots with associated filamentous root hairs anchored plants in place and supported growth in height (Raven and Edwards, 2001). The entire anchorage system of these early diverging groups of non-vascular land plants comprised tip-growing filamentous rhizoid cells (Kenrick and Crane, 1997; Bower, 1929). In mosses, rhizoids are multicellular filamentous cells. Two populations of rhizoids in *Physcomitrella patens* can be distinguished by the position on gametophores: basal rhizoids form in basal regions of the gametophore whereas mid-stem rhizoids develop in more apical regions (Sakakibara et al., 2003). The basal rhizoids differentiate from any epidermal cell near the base of the young gametophores whereas mid-stem rhizoids differentiate from the epidermal cells, which are located to the outside of two small leaf trace cells just below adult leaves.

Auxin positively regulates the development of both basal and mid-stem rhizoids in *P. patens*; treatment of wild-type plants with auxin increases the number of rhizoids that develop (Ashton et al., 1979; Sakakibara et al., 2003). Therefore, it is likely that auxin positively regulates the expression of genes required for rhizoid development. One such gene is *PpHb7*, which encodes an HD Zip I subfamily protein required for late rhizoid differentiation; mutants that lack *PpHb7* function develop rhizoids that are indistinguishable from wild type except that they fail to form the red-brown pigment that normally accumulates late in the differentiation of wild-type rhizoids (Sakakibara et al., 2003). PpRSL1 and PpRSL2 are basic-helix-loop-helix (bHLH) transcription factors that are required for the development of rhizoids; mutants that lack *PpRSL1* and *PpRSL2* activity develop few very short basal rhizoids, indicating that *PpRSL1* and *PpRSL2* act early in rhizoid development (Menand et al., 2007). Nevertheless, the regulatory relationship between *PpRSL1* and *PpRSL2* and auxin has not been elucidated.

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As *PpRSL1* and *PpRSL2* are necessary for rhizoid development in *P. patens*, we set out to determine whether the expression of both *PpRSL* genes was sufficient for rhizoid development. We show here that co-expression of *PpRSL1* and *PpRSL2* is sufficient to transform gametophore cells into rhizoid cells, indicating that *PpRSL1* and *PpRSL2* expression is sufficient for rhizoid system development. We also show that auxin positively regulates rhizoid development by promoting cell-specific *PpRSL1* and *PpRSL2* transcription. Together these data suggest that auxin-induced expression of RSL genes controlled the development of early land plant rhizoids systems.

MATERIALS AND METHODS

Plant materials, growth and supplements

The Gransden wild-type strain of *Physcomitrella patens* (Hedw.) Bruch and Schimp was used in this study (Ashton et al., 1979). Cultures were grown at 25°C and illuminated with a light regime of 16/8 hours (light/dark) and a quantum irradiance of 40 μE m^{-2} s^{-1}. For the growth of gametophores, spores were inoculated on a 9 cm Petri dish containing solid minimal media overlaid with a cellophane disk (AA packaging) for 3 weeks (Ashton et al., 1979). Protonemal samples for transformation were grown on minimal media supplemented with 5 mg/l NH_4 and 50 mg/l glucose for 1 week. *P. patens Pprsl1*, *Pprsl2* and *Pprsl1 Pprsl2* double mutants, as well as *A. thaliana* $AtRSL1_{promoter}$*:GFP:AtRSL1* and $AtRHD6_{promoter}$*:GFP:AtRHD6* lines were previously described (Menand et al., 2007). For the phenotypic analysis of plants, small protonemal inocula were placed on solid minimal media overlaid with cellophane disks and grown for 3 weeks. Auxin treatment of *P. patens* was carried out by transferring 3-week-old plants to solid minimal media supplemented with 1 μM α-naphthalene acetic acid (Sigma) and incubating for 1 week.

Construction of constitutively overexpressing vector

For constitutive overexpression of both *PpRSL1* and *PpRSL2* at the same time, the 35S:PpRSL1;35S:PpRSL2 vector was generated. To obtain 35S:PpRSL1:NOS_{Ter} DNA cassette including *Spe*I and *Apa*I enzymatic restriction sites, two different vectors were used: pCAMBIA-35S:PpRSL1 carrying 35S:PpRSL1:NOS_{Ter} cassette (Menand et al., 2007) and pBluescript II SK + (Stratagene) having *Spe*I and *Apa*I sites. First, pCAMBIA-35S:PpRSL1 was excised with *Eco*RI and *Hin*dIII to obtain 35S:PpRSL1:NOS_{Ter} cassette and this cassette was inserted into the pBluescript vector which had been digested with *Eco*RI and *Hin*dIII. pBluescript-35S:PpRSL1 carrying *Spe*I-35S:PpRSL1:NOS_{Ter}-*Apa*I cassette was obtained by ligation. The *Spe*I-35S:PpRSL1:NOS_{Ter}-*Apa*I cassette, which was obtained by digesting pBluescript-35S:PpRSL1 with *Spe*I and *Apa*I, was inserted into pPpRSL1-KO vector carrying partial genomic DNA fragments of *PpRSL1* for homologous recombination (Menand et al., 2007). The vector was named 35S:PpRSL1. To obtain 35S:PpRSL2:NOS_{Ter} cassette carrying *Not*I and *Aap*I sites two different vectors, pCAMBIA-35S:PpRSL2 carrying 35S:PpRSL2:NOS_{Ter} cassette and pGEM-T Easy (Promega) including *Not*I and *Apa*I sites were used. pCAMBIA-35S:PpRSL2 was obtained by amplifying the *PpRSL2* coding sequence from protonema cDNA with 35S:PpRSL2 primers and cloning it into the *Bam*H1 and *Sal*I sites of pCAMBIA 1300 (Menand et al., 2007). 35S:PpRSL2: NOS_{Ter} cassette was obtained by digesting pCAMBIA-35S:PpRSL2 with *Eco*RI and *Sph*I. This cassette was inserted into pGEM-

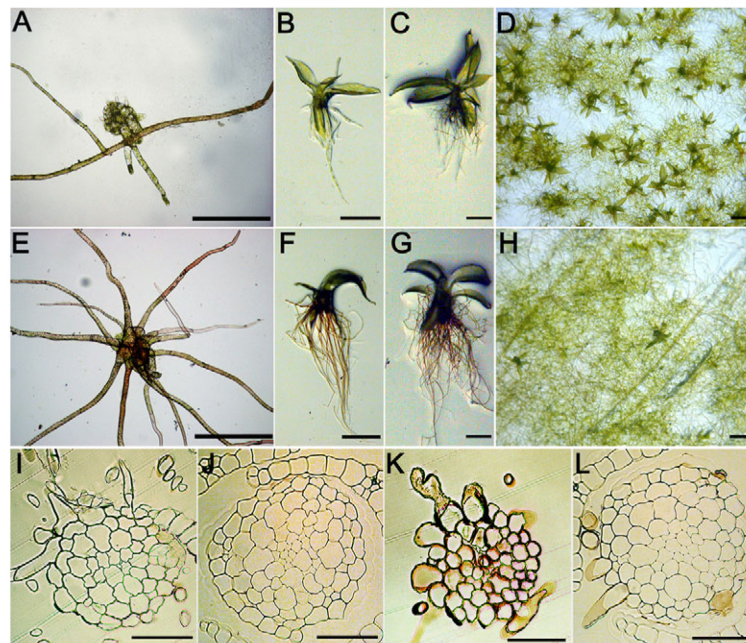

Fig. 1. *PpRSL1* and *PpRSL2* gene expression is sufficient for rhizoid system development in *P. patens*. A-D, I and J show wild-type plants; E-H, K and L show plants transformed with *35S:PpRSL1 35S:PpRSL2*. (**A**) Wild-type gametophore bud that has just started to develop rhizoids but not yet begun to form leaves. (**B**) Young wild-type gametophore with the first juvenile leaves. (**C**) Older gametophore than that shown in B. (**D**) Three-week-old wild-type protonema with developing leafy gametophores on a filamentous network of cells (protonema). (**E**) Gametophore buds are converted to rhizoid masses in plants transformed with both *35S:PpRSL1* and *35S:PpRSL2*. (**F**) One of the rare gametophores that form on protonema transformed with *35S:PpRSL1* and *35S:PpRSL2*. This gametophore produces a mass of rhizoids and is the same age as the wild-type gametophore shown in B. (**G**) Older gametophore than that shown in F. Large numbers of rhizoids develop on the gametophores transformed with both *35S:PpRSL1* and *35S:PpRSL2* compared with wild-type gametophores of the same size and age. (**H**) Fewer gametophores developed in plants transformed with both *35S:PpRSL1* and *35S:PpRSL2* than in wild type (D). (**I**) Transverse section of wild-type gametophores showing the epidermal origin of basal rhizoids. (**J**) Transverse section of wild-type gametophores showing the epidermal origin of mid-stem rhizoids. (**K**) Transverse section showing the epidermal origin of basal rhizoids in the gametophores of plants transformed with both *35S:PpRSL1* and *35S:PpRSL2*. (**L**) Transverse section showing the epidermal origin of mid-stem rhizoids in the gametophores of plants transformed with both *35S:PpRSL1* and *35S:PpRSL2*. Scale bars: 250 μm in A-C,E-G; 1 mm in D,H; 100 μm in I-L.

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Table 1. The number of gametophores in wild type and *PpRSL1*- and *PpRSL2*-overexpressing plants

	Number of gametophores cm^{-2} (± s.d.)	*P*-value (Student's *t*-test)
Wild type	47.8±9.16	
35S:PpRSL1	42.1±5.74	0.34
35S:PpRSL2	45.8±6.97	0.67
35S:PpRSL1;35S:PpRSL2	0.68±0.79	0.0003

T Easy vector which had been self-ligated and digested with *Eco*RI and *Sph*I. *Not*I-35S:PpRSL2:NOS_{Ter}-*Apa*I cassette from pGEM-35S:PpRSL2 vector was inserted into *Not*I and *Apa*I sites of 35S:PpRSL1.

Construction of $PpRSL1_{promoter}$:GUS and $PpRSL2_{promoter}$:GUS vectors

GUS transcriptional reporter vectors were generated by inserting two genomic DNA fragments of *PpRSL1* and *PpRSL2* promoter regions into pBHSNR-GUS vector carrying GUS-$35S_{Ter}$. pBHSNR-GUS vector was constructed by inserting GUS coding sequence and 35S terminator into *Cla*I/*Spe*I sites and *Bgl*II/*Apa*I sites of pBHSNR vector (Menand et al., 2007), respectively. Two genomic DNA fragments of *PpRSL1* gene were cloned by PCR (see Table S1 in the supplementary material). The genomic DNA fragment upstream of *PpRSL1* promoter was inserted into *Asc*I and *Cla*I sites of pBHSNR-GUS and another fragment directly downstream of *PpRSL1* promoter region was inserted into *Bam*HI and *Hin*dIII sites of pBHSNR-GUS. For the $PpRSL2_{promoter}$:GUS vector construction two genomic DNA fragments of *PpRSL2* gene were amplified by PCR. The genomic DNA fragment upstream of *PpRSL2* promoter was inserted into *Asc*I and *Cla*I sites of pBHSNR-GUS and another fragment directly downstream of *PpRSL2* promoter region was inserted into *Sma*I and *Hin*dIII sites.

P. patens transformation

PEG transformation of *P. patens* protoplasts was carried out as described previously (Schaefer and Zrÿd, 1997). The 35S:PpRSL1;35S:PpRSL2 vector and 35S:PpRSL1 vector were linearized with *Avr*II and *Swa*I before protoplast transformation whereas 35S:PpRSL2 vector was linearized with *Swa*I. Transformants for 35S:PpRSL1;35S:PpRSL2 and 35S:PpRSL1 were selected on G418 (50 μl/ml) and transformants for 35S:PpRSL2 were selected on Hygromycin B (25 μl/ml). For the transformations of $PpRSL1_{promoter}$:GUS and $PpRSL2_{promoter}$:GUS, $PpRSL1_{promoter}$:GUS vector was linearized with *Asc*I and *Hin*dIII before protoplast transformation whereas the $PpRSL2_{promoter}$:GUS vector was linearized with *Asc*I and *Bam*HI. Transformants were selected on Hygromycin B (25 μl/ml).

RT-PCR analysis

Total RNA from 4-week-old gametophores was extracted with the RNeasy Plant Mini Kit (Qiagen). One microgram of total RNA was used for cDNA synthesis with the Superscript III reverse transcriptase (Invitrogen) and oligo(dT) primers. PCR was carried out with equivalent amounts of cDNA template for amplification of fragments of *PpRSL1* and *PpRSL2* and *GAPDH* (X72381) (see Table S1 in the supplementary material).

GUS staining analysis, embedding and sectioning

Isolated gametophores were incubated in GUS staining solution [100 mM $NaPO_4$ (pH 7.0), 1 mM 5-bromo-4-chloro-3-indolyl-glucuronide, 1 mM potassium ferricyanide and 0.2% Triton X-100] at 37°C for 2-14 hrs. The samples were then washed with 100 mM $NaPO_4$ (pH 7.0 and incubated in 70% EtOH. Whole-mounted samples were imaged with a Nikon Coolpix 995 camera mounted on a Leica Wild M10 stereomicroscope. For transverse sectioning, gametophores were aligned on a 1 mm layer of solid 1% agarose and covered with 1% molten agarose. After solidification, samples were cut into small blocks and washed twice in double distilled water for 15 minutes, then dehydrated in a graded series of ethanol (25, 50, 75 and 100%), for 30 minutes each. The dehydrated samples were sequentially incubated in a series of Technovit 7100 cold-polymerizing resin [33, 66 and 100% (v/v) in EtOH], for one hour each. Samples were then incubated in 100% Technovit for one day and placed in a plastic moulds. To solidify samples, a 15:1 (v/v) mixture of Technovit and hardener solution II was treated at room temperature for one day. Sections (10 μm) were taken from gametophores with an Ultracut E (Reichert-Jung). Images were captured with a Pixera Pro ES600 camera mounted on Nikon Eclipse 800.

Phylogenetic analysis

The bHLH domain of the RSL proteins were used for the phylogenetic analyses: AtRHD6 (At1g66470), AtRSL1 (At5g37800), AtRSL2 (At4g33880), AtRSL3 (At2g14760), AtRSL4 (At1g27740) and AtRSL5 (At5g43175), PpRSL1 (EF156393), PpRSL2 (EF156394), PpRSL3 (EF156395), PpRSL4 (EF156396), PpRSL5 (EF156397) PpRSL6 (EF156398), PpRSL7 (EF156399). AtIND (At4g00120) was used as an outgroup. The Bayesian analysis was performed with MrBayes version 3.1.2 (http://mrbayes.csit.fsu.edu/): two independent runs were computed for 240,000 generations, at which point the standard deviation of split frequencies was less than 0.01; one tree was saved every 100 generations, and 1800 trees from each run were summarized to give rise to the final cladogram. Trees were visualized using the program Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

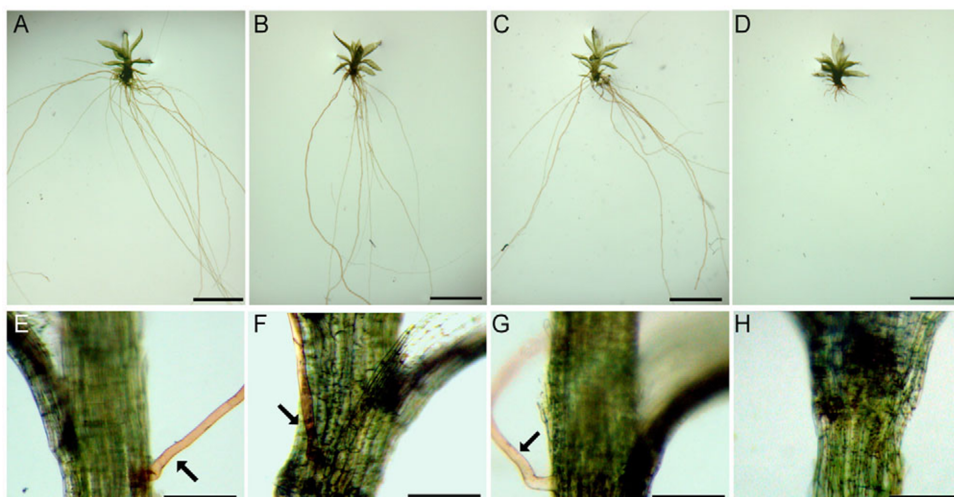

Fig. 2. *PpRSL1* and *PpRSL2* are required for the formation of basal and mid-stem rhizoids in *P. patens*. (**A-D**) Basal rhizoid development in wild type (A), *Pprsl1* mutant (B), *Pprsl2* mutant (C) and *Pprsl1 Pprsl2* double mutant (D). (**E-H**) Mid-stem rhizoid development in wild type (E), *Pprsl1* mutant (F), *Pprsl2* mutant (G) and *Pprsl1 Pprsl2* double mutant (H). Arrows indicate mid-stem rhizoids. Scale bars: 500 μm in A-D; 200 μm in E-H.

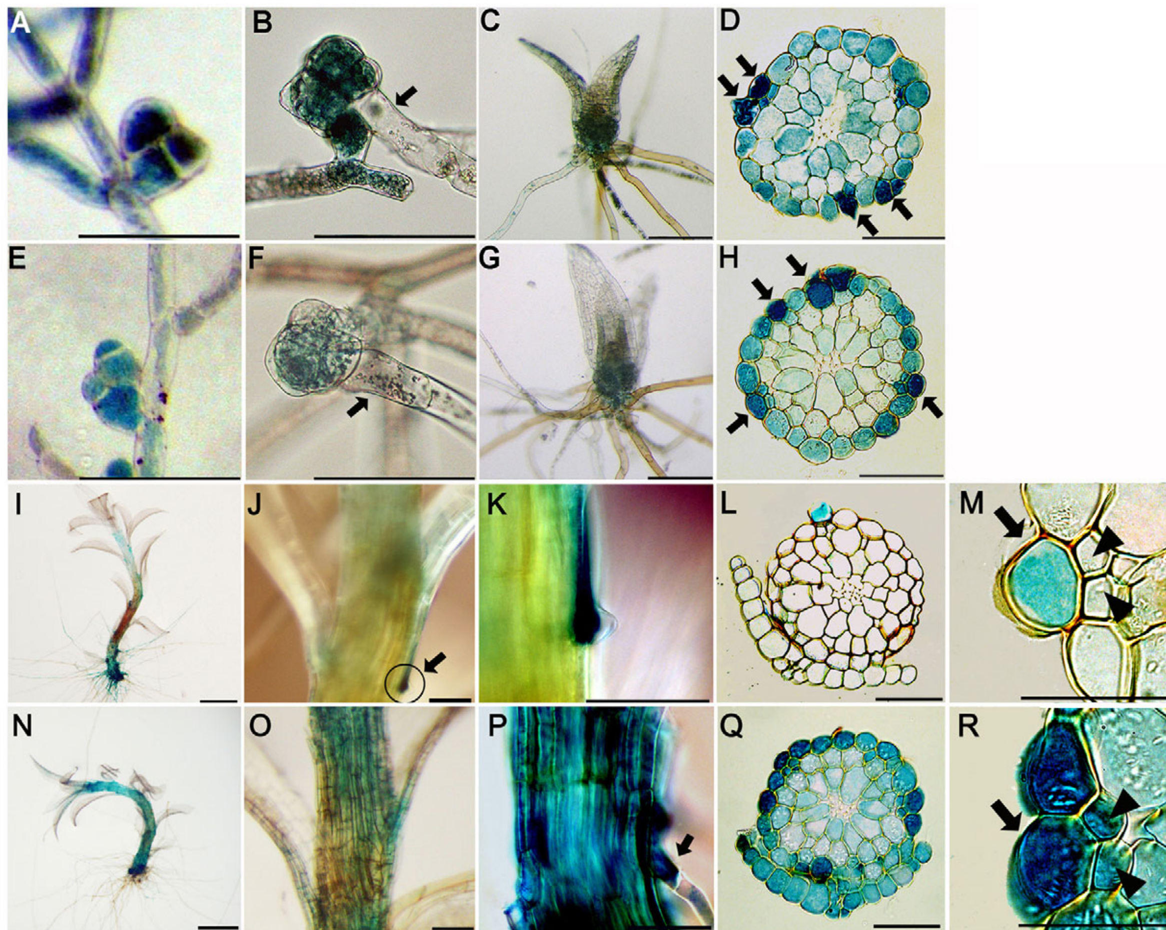

Fig. 3. *PpRSL1* and *PpRSL2* genes are expressed in cells that give rise to rhizoids in *P. patens*. (**A-H**) *PpRSL1$_{promoter}$:GUS* (A-D) and *PpRSL2$_{promoter}$:GUS* (E-H) expression pattern in young gametophores. *PpRSL1* (A) and *PpRSL2* (E) are expressed at the earliest stages of gametophore development before rhizoids differentiate. *PpRSL1* (B) and *PpRSL2* (F) are expressed in young gametophores that have developed their first rhizoids (arrow). *PpRSL1* and *PpRSL2* are expressed in rhizoid progenitor cells but not in rhizoids themselves. *PpRSL1* (C) and *PpRSL2* (G) are specifically expressed in the basal regions of the gametophores where basal rhizoids originate. Transverse sections through the basal region of gametophores demonstrate that *PpRSL1* (D) and *PpRSL2* (H) are strongly expressed throughout the epidermis where any cell can give rise to a rhizoid. Arrows highlight cells with high levels of expression. (**I-R**) *PpRSL1$_{promoter}$:GUS* (I-M) and *PpRSL2$_{promoter}$:GUS* (N-R) expression pattern in adult gametophores with mid-stem rhizoids. *PpRSL1* (I) and *PpRSL2* (N) are expressed in epidermal cells of the gametophore where adult leaves develop. *PpRSL1* (J,K) is expressed in the cells that differentiate as rhizoids whereas *PpRSL2* (O,P) is expressed throughout the gametophore epidermis. Transverse sections through the mid-stem region of gametophores demonstrate that *PpRSL1* (L,M) is specifically expressed in the specialized cells that differentiate as rhizoids and *PpRSL2* (Q,R) is expressed throughout the epidermis. Arrows highlight mid-stem rhizoid forming cells with high levels of expression; arrowheads indicate the positions of the small leaf trace cells. Scale bars: 50 μm in M,R; 100 μm in A-H,J-L,O-Q; 500 μm in I,N.

RESULTS

PpRSL1 and *PpRSL2* expression is sufficient for rhizoid system development in *P. patens*

Given the key function of RSL genes in rhizoid and root hair development in diverse groups of land plants, we tested the hypothesis that *PpRSL1* and *PpRSL2* are master regulators of rhizoid development in *P. patens* (Menand et al., 2007). To determine whether the activity of either *PpRSL1* or *PpRSL2* was sufficient to promote rhizoid development in *P. patens*, we expressed each gene individually at high levels using the *CaMV35S* promoter (Benfey and Chua, 1990). Plants were transformed with either *35S:PpRSL1* or *35S:PpRSL2* constructs. RT-PCR analysis demonstrated that steady state levels of *PpRSL1* and *PpRSL2* transcript was elevated in each transformed line, respectively (see Fig. S1 in the supplementary material). Plants that overexpressed either *PpRSL1* or *PpRSL2* were indistinguishable from wild type (Table 1 and see Fig. S1 in the supplementary material). This suggests that neither expression of *PpRSL1* nor *PpRSL2* individually is sufficient for rhizoid development. To determine whether the combined activities of *PpRSL1* and *PpRSL2* were sufficient for moss rhizoid system development, we generated transgenic plants in which both *PpRSL1* and *PpRSL2* were ectopically expressed at high levels in the same plant. These plants accumulated higher steady state levels of both *PpRSL1* and *PpRSL2* transcript than wild type (see Fig. S1C in the supplementary material). Gametophores are multicellular shoot-like axes with an apical cell and lateral leaf-like appendages that develop from buds that form on filamentous protonema in three-week-old wild type (Fig. 1A) (Sakakibara et al., 2003). By contrast, buds were converted into masses of

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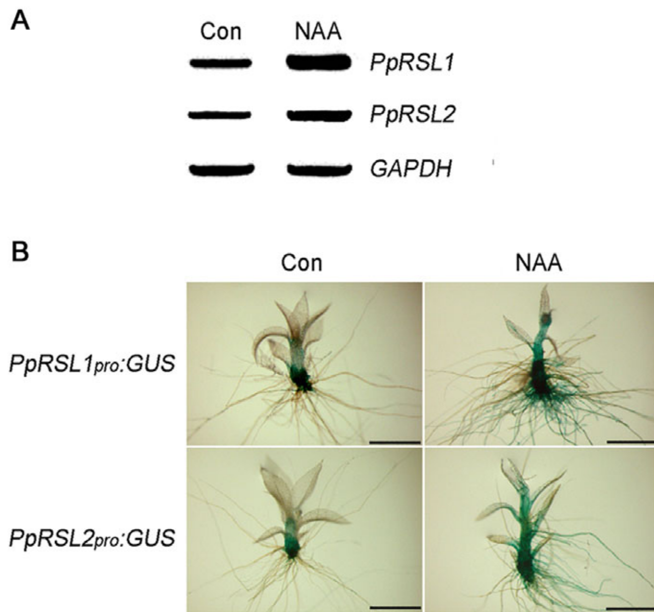

Fig. 4. Auxin positively regulates the transcriptional expression of *PpRSL1* and *PpRSL2* genes in *P. patens*. (**A**) RT-PCR analysis indicates that a one-week auxin treatment (1 μM NAA) increases steady state levels of *PpRSL1* and *PpRSL2* in four-week-old gametophores. (**B**) 1 μM NAA treatment increases the expression levels of $PpRSL1_{promoter}$:*GUS* and $PpRSL2_{promoter}$:*GUS* compared with untreated controls.

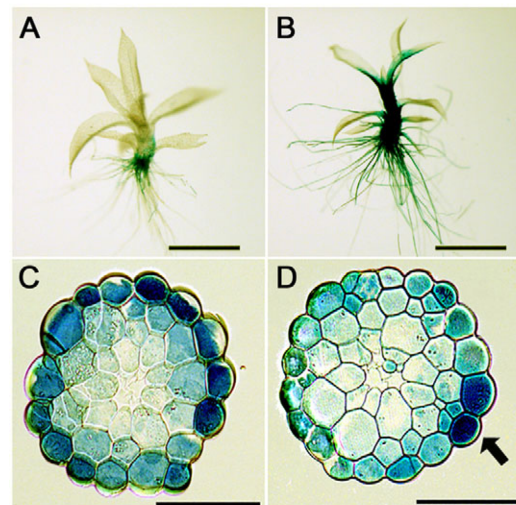

Fig. 5. The spatial pattern of *GmGH3* promoter activity in *P. patens* gametophores. (**A**,**B**) NAA treatment increases *GmGH3* promoter activity in gametophores. *GmGH3* expression pattern in untreated controls (A) and after 1 μM NAA-treatment (B) is shown. (**C**,**D**) The spatial pattern of *GmGH3* promoter activity in basal and mid-stem region of the gametophores. (C) *GmGH3* promoter is active in most cells in the epidermis of basal region. (D) *GmGH3* promoter activity is higher in the epidermal cells that give rise to rhizoids cells located near leaf trace cells (arrow) in the mid-stem region. Scale bars: 500 μm in A,B; 100 μm in C,D.

rhizoids in plants that overexpress both *PpRSL1* and *PpRSL2* (Fig. 1E). Because most cells in the buds that overexpressed both *PpRSL1* and *PpRSL2* differentiated into rhizoids, few or no green gametophores developed in these cultures (Fig. 1D,H); 42.07±5.46 (s.d., *n*=10) gametophores cm^{-2} developed from wild-type protonema whereas 0.68±0.79 (s.d., *n*=12) gametophores cm^{-2} developed from protonema that overexpressed both *PpRSL1* and *PpRSL2* (Table 1). The few gametophores that formed in plants overexpressing both *PpRSL1* and *PpRSL2* developed many more rhizoids than wild type and these rhizoids developed from cells in the epidermis (Fig. 1B,C,F,G,I-L). Together these data indicate that the combined activity of *PpRSL1* and *PpRSL2* is sufficient for the formation of the *P. patens* rhizoid system.

PpRSL1 and *PpRSL2* are required for the development of both basal and mid-stem rhizoids

In wild type, rhizoids differentiate on gametophores. Two populations of morphologically identical rhizoids can be distinguished by the position on the gametophore from which they develop (Sakakibara et al., 2003). The first population of rhizoids form early in gametophore development; they differentiate soon after the gametophore progenitor cell divides to form a bud and continue to develop from epidermal cells below juvenile leaves near the base of the young gametophore (Fig. 2A). When viewed in transverse section, these rhizoids can be seen to grow out from any epidermal cell in the gametophore. Because these rhizoids form at the gametophore base, they are known as basal rhizoids. By contrast, mid-stem rhizoids differentiate from specialized epidermal cells located just below adult leaves on the gametophore axis (leaves 1-5 are considered juvenile and adult leaves are those that form after leaf 5) (Fig. 2E). Only a subset of epidermal cells develop mid-stem rhizoids. When the gametophore is viewed in transverse section, the epidermal cells that give rise to mid-stem rhizoids are located to the outside of two small cells of the leaf trace cells that extend from the stem into the midrib of the adjoining leaf (arrowheads in Fig. 3M,R). Both basal and mid-stem rhizoids developed in *Pprsl1* and *Pprsl2* single mutants (Fig. 2B,C,F,G), but only few very short basal rhizoids and no mid-stem rhizoids developed in *Pprsl1 Pprsl2* double mutants (Fig. 2D,H). The internal cellular anatomy of *Pprsl1 Pprsl2* double mutant gametophores was identical to wild type when viewed in transverse section despite the obvious defects in rhizoid development (see Fig. S2 in the supplementary material). This indicates that *PpRSL1* and *PpRSL2* are required for the development of both basal and mid-stem rhizoids and are not required for the development of other cell types in the gametophore.

PpRSL1 and *PpRSL2* are expressed in cells that form rhizoids

As *PpRSL1* and *PpRSL2* are necessary and sufficient for the development of both classes of rhizoids we hypothesized that *PpRSL1* and *PpRSL2* would be expressed in the cells that give rise to rhizoids. To test this hypothesis, we made $PpRSL1_{promoter}$:*GUS* and $PpRSL2_{promoter}$:*GUS* fusions and introduced these constructs into the endogenous *PpRSL1* and *PpRSL2* loci by homologous recombination (see Fig. S3 in the supplementary material). *PpRSL1* and *PpRSL2* were first expressed in bud cells soon after the division of the initial cell that forms the bud, when the first basal rhizoids develop (Fig. 3A,B,E,F). Expression continued during bud development and when the gametophore developed *PpRSL1* and *PpRSL2* were expressed throughout the epidermis in the basal region, reflecting the fact that all epidermal cells in this region of

the gametophore can develop rhizoids (Fig. 3C,D,G,H). Later in gametophore development, *PpRSL1* expression was restricted to the specialized epidermal cells that give rise to mid-stem rhizoids (Fig. 3I-M). By contrast, *PpRSL2* was expressed throughout the gametophore epidermis in the regions where mid-stem rhizoids develop (Fig. 3N-R). However, no *PpRSL2* expression was detected in other gametophore cell types. Taken together these data indicate that rhizoids develop from cells in which both *PpRSL1* and *PpRSL2* are expressed, where their joint expression is sufficient to promote the rhizoid development programme.

Auxin positively regulates cell specific *PpRSL1* and *PpRSL2* gene expression

Auxin positively regulates rhizoid development (Ashton et al., 1979; Sakakibara et al., 2003). Because the expression of *PpRSL1* and *PpRSL2* is sufficient to promote rhizoid differentiation, we hypothesized that auxin controlled rhizoid development by regulating the expression of *PpRSL1* and *PpRSL2*. To test this hypothesis, we determined the effect of auxin-treatment on *PpRSL1* and *PpRSL2* expression. Auxin-treatment increased *PpRSL1* and *PpRSL2* steady state mRNA levels (Fig. 4A). To verify independently that auxin positively regulates *PpRSL1* and *PpRSL2* expression we determined the effect of auxin-treatment on $PpRSL1_{promoter}$:*GUS* and $PpRSL2_{promoter}$:*GUS* expression. Auxin-treatment increased the expression of each reporter gene (Fig. 4B). Furthermore, whereas expression was restricted to those epidermal cells that gave rise to rhizoids in untreated controls, auxin-treatment induced GUS expression along the entire length of the rhizoid in plants transformed with the $PpRSL1_{promoter}$:*GUS* and $PpRSL2_{promoter}$:*GUS* transgenes. This indicates that not only does auxin-treatment increase *PpRSL1* and *PpRSL2* transcription but it also extends the expression of these genes into the rhizoids. Together these data demonstrate that auxin positively regulates the expression of *PpRSL1* and *PpRSL2*.

Rhizoid-forming cells are sensitive to auxin

Because auxin positively regulates *PpRSL1* and *PpRSL2* expression, we hypothesized that the cell-specific expression of these genes could be, at least in part, determined by cell-specific sensitivity to auxin. Although we cannot directly assay sensitivity of epidermal cells in the gametophore, we can use a promoter that is transcriptionally responsive to auxin to identify cells that are relatively sensitive to auxin. The expression patterns of the *PpRSL1* and *PpRSL2* genes were, therefore, compared with the expression pattern of the auxin sensitive *GmGH3* promoter, which is restricted to the sites of auxin sensitivity or auxin accumulation in *P. patens* (Fig. 5A,B) (Rose and Bopp, 1983; Bierfreund et al., 2003; Ludwig-Müller et al., 2009; Fujita et al., 2008; Eklund et al., 2010a; Eklund et al., 2010b). The expression patterns of $GmGH3_{promoter}$:*GUS* and $PpRSL1_{promoter}$:*GUS* and $PpRSL2_{promoter}$:*GUS* were almost identical. $GmGH3_{promoter}$:*GUS* was expressed early in the development of gametophores when the first rhizoids developed and in the cells that gave rise to basal rhizoids in young gametophores. Later in gametophore development, $GmGH3_{promoter}$:*GUS* was active in the epidermal rhizoid progenitor cells that form mid-stem rhizoids like $PpRSL1_{promoter}$:*GUS* (Fig. 5C,D). These results indicate that *PpRSL1* is most highly expressed in cells that are either relatively sensitive to auxin or accumulate relatively more auxin than surrounding cells. These data are consistent with a model in which the cellular pattern of auxin sensitivity or accumulation regulates the expression of *PpRSL1* and *PpRSL2*, which in turn positively regulates rhizoid development.

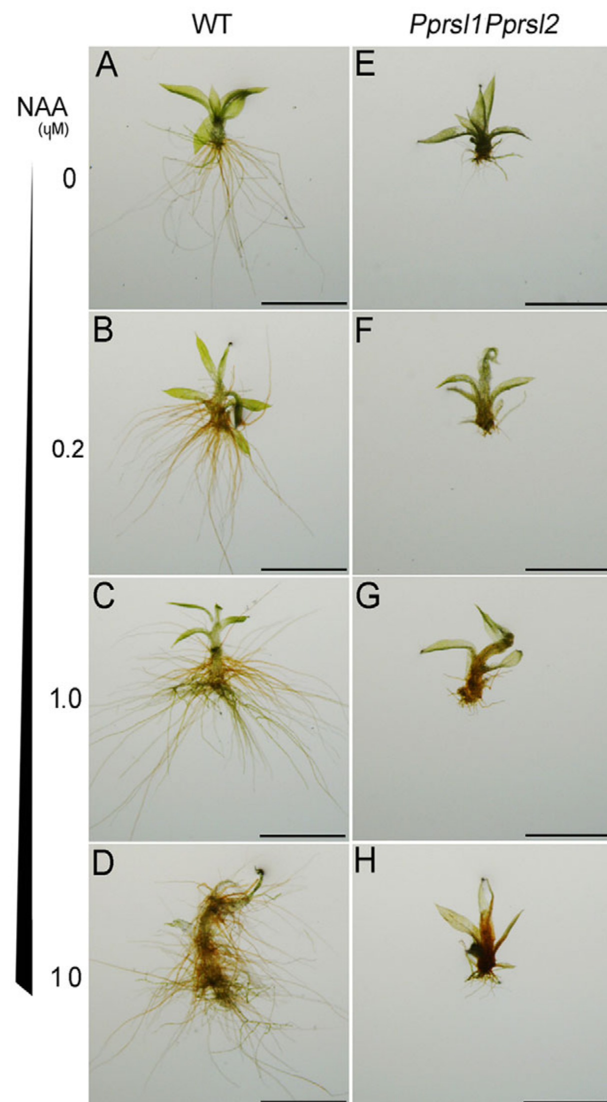

Fig. 6. *PpRSL1* and *PpRSL2* gene activity is required for the induction of rhizoid development by auxin. (**A-H**) NAA treatment (0.2, 1 and 10 μM) for one week enhances rhizoid development in wild-type (WT) *P. patens* (B-D) compared with the untreated control (A), but this is not observed in the *Pprsl1 Pprsl2* double mutant (E-H). Scale bars: 500 μm.

Auxin-induced rhizoid development requires *PpRSL1* and *PpRSL2* activity

To verify that auxin controls the development of rhizoids by regulating *PpRSL1* and *PpRSL2* function, we determined the sensitivity of *Pprsl1* single, *Pprsl2* single and *Pprsl1 Pprsl2* double mutants to auxin-treatment. Auxin-treatment of wild type, *Pprsl1* single and *Pprsl2* single mutants induced the development of large numbers of rhizoids on gametophores compared with untreated controls (Fig. 6 and see Fig. S4 in the supplementary material). By contrast, auxin treatment of *Pprsl1 Pprsl2* double mutants did not increase the number of rhizoids on gametophores compared with untreated controls; the phenotypes of treated and untreated *Pprsl1 Pprsl2* double mutant were identical (Fig. 6). The double mutant is, therefore, resistant to the rhizoid-inducing effect of auxin-treatment. This indicates that auxin controls rhizoid development by positively regulating the activity of *PpRSL1* and *PpRSL2* genes.

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Auxin does not regulate *AtRHD6* and *AtRSL1* during root hair development in *A. thaliana*

Because auxin controls rhizoid development in moss by positively regulating *PpRSL1* and *PpRSL2* activity, we determined whether auxin controlled root hair development in *A. thaliana* by the same mechanism. *AtRHD6* and *AtRSL1* are the *A. thaliana* genes that are most closely related to *PpRSL1* and *PpRSL2* and they positively regulate root hair development; root hairs do not develop in *Atrhd6-3 Atrsl1-1* double mutants (Menand et al., 2007) (Fig. 7A,D). We used RT-PCR to determine whether auxin controlled the expression of *AtRHD6* and *AtRSL1*. Steady state levels of *AtRHD6* and *AtRSL1* mRNA were indistinguishable in NAA-treated and untreated controls (Fig. 7B). To confirm independently that auxin does not positively regulate *AtRHD6* and *AtRSL1* activity, we determined whether NAA-treatment altered the abundance of AtRHD6 and AtRSL1 proteins compared with untreated controls. Plants transformed with $AtRHD6_{promoter}$*:GFP:AtRHD6* and $AtRSL1_{promoter}$*:GFP:AtRSL1* protein fusion constructs were treated with NAA. No distinct change in GFP intensity was observed between NAA-treated and untreated controls (Fig. 7C). These data suggest that auxin-regulated development of root hairs is independent of *AtRHD6* and *AtRSL1*.

If *AtRHD6* and *AtRSL1* act independently to control root hair development, we predicted that NAA-treatment of *Atrhd6-3 Atrsl1-1* would suppress the hairless phenotype characteristic of this double mutant. As predicted, NAA-treatment induced root hairs in the *Atrhd6-3 Atrsl1-1* double mutant (Fig. 7D) (Yi et al., 2010). Furthermore, auxin-treatment of the *Atrhd6-3 Atrsl1-1* double mutant induced the expression of the root hair specific $AtEXP7_{promoter}$*:GFP* gene, which is not expressed in untreated *Atrhd6-3 Atrsl1-1* controls (Fig. 7E). This indicates that the induction of *AtEXP7* expression by auxin is independent of *AtRHD6* and *AtRSL1* activity. Taken together these data indicate that auxin does not act through *AtRHD6* and *AtRSL1* during root hair development in *A. thaliana*, in contrast to *P. patens* where auxin controls rhizoid development by positively regulating *PpRSL1* and *PpRSL2* expression. This suggests that the mechanism by which auxin regulates rhizoid and root hair development is different in *P. patens* and *A. thaliana* and implies that this mechanism has changed since these two species last shared a common ancestor over 400 million years ago.

DISCUSSION

We showed here that *PpRSL1* and *PpRSL2* genes are necessary and sufficient for the development of multicellular rhizoids in *P. patens*. This suggests that *PpRSL1* and *PpRSL2* are key regulators of rhizoid development in early diverging groups of land plants. As RSL genes also control the development of single celled root hairs in angiosperms, we propose that RSL genes were co-opted to control the development of filamentous cells (root hairs) in roots when these specialized axes evolved in later diverging groups of land plants (lycophytes, monilophytes and seed plants). Therefore, it is likely that RSL genes regulate the development of filamentous cells at the plant-soil interface in all groups of land plants. This hypothesis is supported by the observation that RSL genes have been found in all land plants for which genome sequence is available (Pires and Dolan, 2010).

Although auxin positively regulates root hair and rhizoid development in *A. thaliana* and *P. patens*, respectively, our data suggest that the mechanism of auxin action differs between these species in at least two ways (Ashton et al., 1979; Pitts et al., 1998; Sakakibara et al., 2003; Cho et al., 2007; Jones et al., 2009). Comparison of amino acid sequences shows that *PpRSL1* and

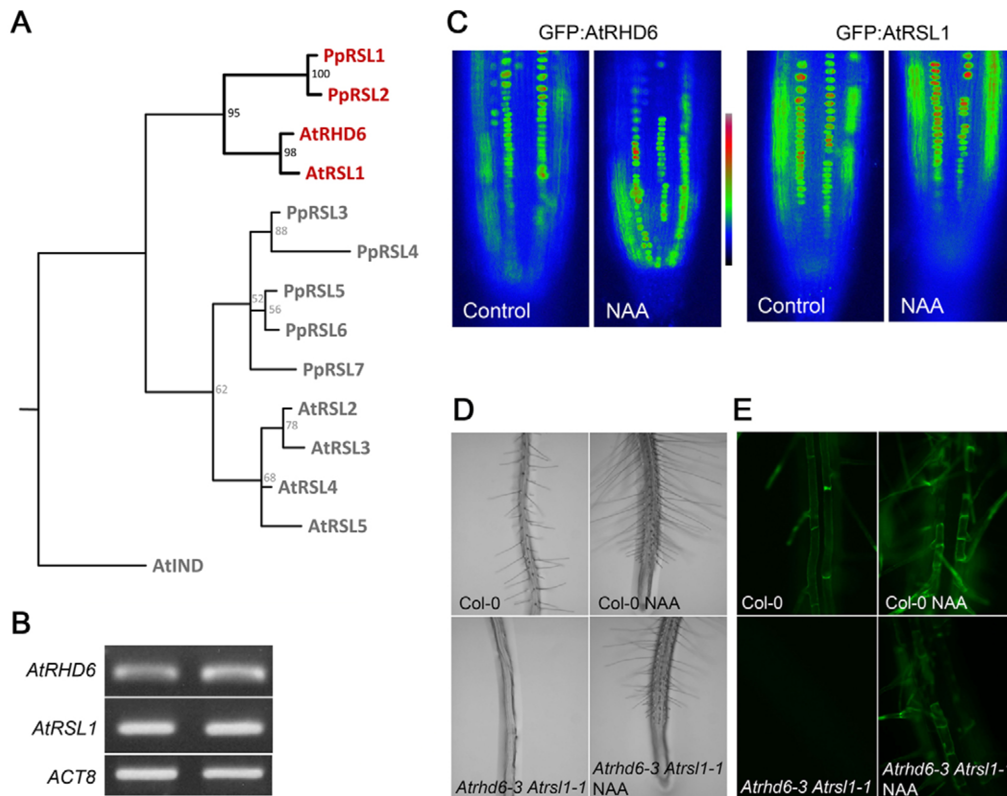

Fig. 7. Auxin-control of root hair development is independent of *AtRHD6* and *AtRSL1*. (**A**) Tree showing the relationships between *A. thaliana* and *P. patens* RSL proteins. AtRHD6 and AtRSL1 are the *A. thaliana* RSL proteins that are most closely related to PpRSL1 and PpRSL2. (**B**) RT-PCR showing that steady state levels of *AtRHD6* and *AtRSL1* mRNA remain unaltered by auxin treatment (150 nM NAA). (**C**) Levels of GFP:AtRHD6 and GFP:AtRSL1 fusion proteins are not regulated by auxin. $AtRSL1_{promoter}$*:GFP:AtRSL1* and $AtRHD6_{promoter}$*:GFP:AtRHD6* are shown with (right) or without (left) treatment with 150 nM NAA. (**D**) Auxin treatment induces root hair development on *Atrhd6-3 Atrsl1-1* double mutants whereas untreated controls remain hairless. (**E**) $AtEXP7_{promoter}$*:GFP* expression is induced by auxin. There is no $AtEXP7_{promoter}$*:GFP* expression in untreated controls.

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PpRSL2 are most closely related to *AtRHD6* and *AtRSL1* in *A. thaliana*, which control the development of root hairs; *Atrhd6 Atrsl1* double mutants do not develop root hairs (Menand et al., 2007). Auxin promotes rhizoid development by positively regulating *PpRSL1* and *PpRSL2* genes in *P. patens*. By contrast, we can find no evidence for the regulation of *AtRHD6* and *AtRSL1* by auxin. Instead, auxin promotes root hair development by positively regulating the expression of a gene from a different clade called *AtRSL4*, which acts downstream of *AtRHD6* and *AtRSL1* (Yi et al., 2010). This indicates that although the mechanisms that control the development of filamentous cells at the land plant-substrate interface is ancient, changes in the regulatory interactions between components of this network have occurred during the course of land plant evolution.

A second difference in auxin-regulated development of root hairs and rhizoids is highlighted by the demonstration that the auxin sensitive reporter gene $GmGH3_{promoter}$:*GUS* is expressed in cells that go on to form rhizoids. This suggests that relatively high levels of auxin might accumulate in these cells compared with the surrounding epidermal cells or that the rhizoid-forming cells are more sensitive to auxin than the surrounding cells that do not form rhizoids. We propose that this relatively high level of auxin or high level of auxin sensitivity then activates the expression of *PpRSL1*, which then positively regulates rhizoid differentiation. This contrasts with *A. thaliana*, in which the auxin sensitive reporter *DR5:GFP* is relatively more active in the non-hair cells and less active in the cells that form root hairs, which has been interpreted to mean that the non-hair cells (N cells) of the *A. thaliana* root are more sensitive to auxin than the hair cells (H cells) (Jones et al., 2009).

Together these data indicate that although auxin regulates RSL networks in *P. patens* and *A. thaliana*, its interaction with these networks is different in each species. If the moss RSL network represents the ancestral state that existed in early diverging groups of land plants, our data suggest that the way in which auxin controls the network changed during land plant evolution. Nevertheless, our results indicate that RSL genes have played a pivotal role in controlling the development of the filamentous cells at the plant-soil interface in gametophytes and sporophytes since plants appeared on land in the Ordovician Period over 460 million years ago (Kenrick and Crane, 1997; Wellman and Gray, 2000; Wellman et al., 2003).

Because auxin-regulated RSL networks control the development of tip growing rhizoid and root hair cells in mosses and flowering plants, which last shared a common ancestor over 420 million years ago, it can be concluded that the RSL-mechanism is ancient. It is, therefore, likely that auxin-regulated RSL genes control the development of rhizoids and root hairs in lycophytes, monilophytes and seed plants. Furthermore, because monilophytes (ferns and horsetails) develop both root hairs and rhizoids in the sporophyte and gametophyte generations, respectively, these data also predict that auxin-regulated RSL genes control the development of these cell types in the two stages of the life cycle of these plants (Bower, 1929). Consistent with these predictions is the demonstration that auxin positively regulates the development of rhizoids and root hairs in the gametophytes and sporophytes, respectively, in ferns (Allsopp, 1952; Bloom and Nichols, 1972; Hickock and Kiruluk, 1984). Nevertheless, RSL genes have yet to be identified in monilophytes. However, this is likely to be due to a lack of genome and expressed sequence tags sequences among this group.

Auxin positively regulates rhizoid development in *Marchantia polymorpha*, an extant representative of the earliest diverging group of land plants, the liverworts (Kaul et al., 1962). Furthermore, land plants and charophycean algae are derived from a common ancestor that existed some time before 450 million years ago. Given that some charophycean algae such as *Nitella* species and *Chara* species develop rhizoids it is possible that the mechanism controlling land plant and algal rhizoid development might be derived from the same ancestral mechanism. Although no genes controlling the development of charophycean rhizoids have been identified, auxin positively regulates rhizoid development in *Chara* species and other green algae (Klämbt et al., 1992; Jacobs, 1951). It is, therefore, possible that RSL-like genes are positively regulated by auxin during the development of algal rhizoids. If this were the case it would suggest that the mechanism controlling the development of filamentous anchoring cells is conserved among land plants and related algae. Alternatively, although auxin positively regulates the development of filamentous cells in land plants and algae, it is possible that auxin activates different downstream regulatory networks that control the development of filamentous anchoring cells in algae and land plants. Distinguishing between these alternatives requires the characterization of the genetic network that controls the development of algal rhizoids.

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Competing interests statement
The authors declare no financial interests.

Supplementary material
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