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.
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.

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 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:NOSter DNA cassette including SpeI and ApaI enzymatic restriction sites, two different vectors were used: pCAMBIA-35S:PpRSL1 carrying 35S:PpRSL1:NOSter cassette (Menand et al., 2007) and pBluescript II SK + (Stratagene) having SpeI and ApaI sites. First, pCAMBIA-35S:PpRSL1 was excised with EcoRI and HindIII to obtain 35S:PpRSL1:NOSter cassette and this cassette was inserted into the pBluescript vector which had been digested with EcoRI and HindIII. pBluescript-35S:PpRSL1 carrying SpeI-35S:PpRSL1:NOSter-ApaI cassette was obtained by ligation. The SpeI-35S:PpRSL1:NOSter-ApaI cassette, which was obtained by digesting pBluescript-35S:PpRSL1 with SpeI and ApaI, 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:NOSter cassette carrying NotI and AapI sites two different vectors, pCAMBIA-35S:PpRSL2 carrying 35S:PpRSL2:NOSter cassette and pGEM-T Easy (Promega) including NotI and AapI 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 BamHI and SalI sites of pCambia 1300 (Menand et al., 2007). 35S:PpRSL2: NOSter cassette was obtained by digesting pCAMBIA-35S:PpRSL2 with EcoRI and SphiI. This cassette was inserted into pGEM-T Easy (Promega) to obtain 35S:PpRSL1;35S:PpRSL2.

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

<table>
<thead>
<tr>
<th></th>
<th>Number of gametophores cm⁻² (± s.d.)</th>
<th>P-value (Student’s t-test)</th>
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<tr>
<td>Wild type</td>
<td>47.8±9.16</td>
<td>0.0003</td>
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<tr>
<td>35S:PpRSL1</td>
<td>42.1±5.74</td>
<td>0.34</td>
</tr>
<tr>
<td>35S:PpRSL2</td>
<td>45.8±6.97</td>
<td>0.67</td>
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<tr>
<td>35S:PpRSL1; 35S:PpRSL2</td>
<td>0.68±0.79</td>
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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_gfr. pBHSNR-GUS vector was constructed by inserting GUS coding sequence and 35S terminator into Clal/SphI sites and BglII/ApaI 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 AscI and Clal sites of pBHSNR-GUS and another fragment directly downstream of PpRSL1 promoter region was inserted into BamHI and HindIII 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 AscI and Clal sites of pBHSNR-GUS and another fragment directly downstream of PpRSL2 promoter region was inserted into SmaI and HindIII sites.

P. patens transformation

PEG transformation of P. patens protoplasts was carried out as described previously (Schaefer and Zry’d, 1997). The 35S:PpRSL1;35S:PpRSL2 vector and 35S:PpRSL1 vector were linearized with AvrII and SmaI before protoplast transformation whereas 35S:PpRSL2 vector was linearized with SmaI. Transformants for 35S:PpRSL1;35S:PpRSL2 and 35S:PpRSL1 were selected on G418 (50 μg/ml) and transformants for 35S:PpRSL2 vector were selected on Hygromycin B (25 μg/ml). For the transformations of PpRSL1 promoter:GUS and PpRSL2 promoter:GUS, PpRSL1 promoter:GUS vector was linearized with AscI and HindIII before protoplast transformation whereas the PpRSL2 promoter:GUS vector was linearized with AscI and BamHI. Transformants were selected on Hygromycin B (25 μg/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 NaPO₄ (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 NaPO₄ (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 (At4g38880), 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/).
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...
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). 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 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 PpRSL1promoter:GUS and PpRSL2promoter: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, \textit{PpRSL1} expression was restricted to the specialized epidermal cells that give rise to mid-stem rhizoids (Fig. 3I-M). By contrast, \textit{PpRSL2} was expressed throughout the gametophore epidermis in the regions where mid-stem rhizoids develop (Fig. 3N-R). However, no \textit{PpRSL2} expression was detected in other gametophore cell types. Taken together these data indicate that rhizoids develop from cells in which both \textit{PpRSL1} and \textit{PpRSL2} are expressed, where their joint expression is sufficient to promote the rhizoid development programme.

**Auxin positively regulates cell specific \textit{PpRSL1} and \textit{PpRSL2} gene expression**

Auxin positively regulates rhizoid development (Ashton et al., 1979; Sakakibara et al., 2003). Because the expression of \textit{PpRSL1} and \textit{PpRSL2} is sufficient to promote rhizoid differentiation, we hypothesized that auxin controlled rhizoid development by regulating the expression of \textit{PpRSL1} and \textit{PpRSL2}. To test this hypothesis, we determined the effect of auxin-treatment on \textit{PpRSL1} and \textit{PpRSL2} expression. Auxin-treatment increased \textit{PpRSL1} and \textit{PpRSL2} steady state mRNA levels (Fig. 4A). To verify independently that auxin positively regulates \textit{PpRSL1} and \textit{PpRSL2} expression we determined the effect of auxin-treatment on \textit{PpRSL1} promoter-GUS and \textit{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 \textit{GUS} expression along the entire length of the rhizoid in plants transformed with the \textit{PpRSL1} promoter-GUS and \textit{PpRSL2} promoter-GUS transgenes. This indicates that not only does auxin-treatment increase \textit{PpRSL1} and \textit{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 \textit{PpRSL1} and \textit{PpRSL2}.

**Rhizoid-forming cells are sensitive to auxin**

Because auxin positively regulates \textit{PpRSL1} and \textit{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 \textit{PpRSL1} and \textit{PpRSL2} genes were, therefore, compared with the expression pattern of the auxin sensitive \textit{GmGH3} promoter, which is restricted to the sites of auxin sensitivity or auxin accumulation in \textit{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 \textit{GmGH3} promoter-GUS and \textit{PpRSL1} promoter-GUS and \textit{PpRSL2} promoter-GUS were almost identical. \textit{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, \textit{GmGH3} promoter-GUS was active in the epidermal rhizoid progenitor cells that form mid-stem rhizoids like \textit{PpRSL1} promoter-GUS (Fig. 5C,D). These results indicate that \textit{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 \textit{PpRSL1} and \textit{PpRSL2}, which in turn positively regulates rhizoid development.

![Fig. 6. \textit{PpRSL1} and \textit{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) \textit{P. patens} (B-D) compared with the untreated control (A), but this is not observed in the \textit{Pprsl1 Pprsl2} double mutant (E-H). Scale bars: 500 µm.](image-url)

**Auxin-induced rhizoid development requires \textit{PpRSL1} and \textit{PpRSL2} activity**

To verify that auxin controls the development of rhizoids by regulating \textit{PpRSL1} and \textit{PpRSL2} function, we determined the sensitivity of \textit{Pprsl1} single, \textit{Pprsl2} single and \textit{Pprsl1 Pprsl2} double mutants to auxin-treatment. Auxin-treatment of wild type, \textit{Pprsl1} single and \textit{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 \textit{Pprsl1 Pprsl2} double mutants did not increase the number of rhizoids on gametophores compared with untreated controls; the phenotypes of treated and untreated \textit{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 \textit{PpRSL1} and \textit{PpRSL2} genes.
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 controls 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 AtRHD6promoter:GFP:AtRHD6 and AtRSL1promoter: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 hair development in Atrhd6-3 Atrsl1-1 (Yi et al., 2010). Furthermore, auxin-treatment of the Atrhd6-3 Atrsl1-1 double mutant induced the expression of the root hair specific AtEXP7promoter: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 PpRSL2 are the RSL proteins shared a common ancestor over 400 million years ago.

**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. AtRSL1promoter:GFP:AtRSL1 and AtRHD6promoter: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) AtEXP7promoter:GFP expression is induced by auxin. There is no AtEXP7promoter:GFP expression in untreated controls.
PpRSL2 are most closely related to AtRHD6 and AtRSL1 in A. thaliana, which control the development of root hairs; AtRhd6 Atrs1 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 (Altsopp, 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 filamentousanchoring cells in algae and land plants. Distinguishing between these alternatives requires the characterization of the genetic network that controls the development of algal rhizoids.

Acknowledgements
We thank John Doonan for technical advice and critical discussions of the data; Thomas Tam, Sourav Datta and Monica Pernas for critical comments on the manuscript. B.M. was funded by the EU-Marie Curie programme (HPMF-CT-2002-01935) and a Natural Environmental Research Council (NERC) responsive mode grant NE/C510732/1 to L.D. N.D.P. was supported by a PhD fellowship from the Portuguese Fundação para a Ciência e a Tecnologia. L.D. was also funded by the PLANTORIGINS Marie Curie Network of the European Union and the EVO500 Advance Grant from the European Research Council. University of Oxford and John Innes Centre also supported this research. GmGH3 promoter GUS fusion lines were kindly provided by Eva Decker.

Competing interests statement
The authors declare no financial interests.

Supplemental material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl;doi:10.1242/dev.060582/-/DC1

References
Early land plant rhizoid development


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