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The *Selaginella* rhizophore has a unique transcriptional identity compared with root and shoot meristems

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Summary

- The genus *Selaginella* resides in an early branch of the land plant lineage that possesses a vasculature and roots. The majority of the *Selaginella* root system is shoot borne and emerges through a distinctive structure known as the rhizophore, the organ identity of which has been a long-debated question.
- The rhizophore of *Selaginella moellendorffii* – a model for the lycophytes – shows plasticity to develop into a root or shoot up until 8 d after angle meristem emergence, after which it is committed to root fate. We subsequently use morphology and plasticity to define the stage of rhizophore identity.
- Transcriptomic analysis of the rhizophore during its plastic stage reveals that, despite some resemblance to the root meristem, rhizophore gene expression patterns are largely distinct from both shoot and root meristems.
- Based on this transcriptomic analysis and on historical anatomical work, we conclude that the rhizophore is a distinct organ with unique features.

Introduction

Extensive radiation of land plants began with the development of vasculature and specialized organs for anchorage and nutrient uptake – roots (Kenrick & Strullu-Derrien, 2014). Although phylogenetic analyses suggest diversification of these first vascular plants into several lineages, only two remain: the euphyllophytes (ferns and seed plants) and the lycophytes. *Selaginella*, or 'spikemoss', represents one extant genus of lycophytes, which appear to have originated 400 Ma (Banks, 2009), making these plants a system of evolutionary interest for over a century (Nägeli & Leitgeb, 1868; Gibson, 1896; Worsdell, 1910). The recent genome sequencing and transcript annotation of one species, *Selaginella moellendorffii*, has revived interest in this genus (Banks *et al.*, 2011; Zhu *et al.*, 2017), and characterization of this model provides an opportunity to examine the early divergence of shoot and root meristems.

Although *Selaginella* spp. have been reported to possess roots that arise from the early sporophyte (Webster, 1967; Karrfalt, 1981), these structures are generally limited in number and, in some species, are insufficient to anchor the plant body. Instead, the majority of the root system is derived much later in development from specialized outgrowths, known as rhizophores, that emerge from meristems located in the angles of shoot branches – hence, angle meristems (Worsdell, 1910; Korall & Kenrick, 2002). The rhizophores then transition to form roots (Nägeli & Leitgeb, 1868; Webster & Steeves, 1963). The rhizophore is particularly interesting from an evolutionary perspective as it could represent a plastic structure in an early land plant lineage that is not committed to either root or shoot fate (Banks, 2015).

Early in their development, rhizophores are pigmentless and exhibit positive gravitropism like mature *Selaginella* roots and the roots of most seed plants but are otherwise smooth and lack root features such as a cap and root hairs (Wochok & Sussex, 1974; Banks, 2015). In subsequent stages, the outgrowth that originated as a rhizophore acquires root characters (root cap and hairs) usually just before or upon reaching the soil. The organ identity of the rhizophore has been debated since its first description in 1868 (Nägeli & Leitgeb, 1868), with botanists claiming that the structure is either a modified shoot (Pfeffer, 1871; Treub, 1874; Bruchmannm, 1905; Worsdell, 1910; Cusick, 1953, 1954), a root throughout development (Webster & Steeves, 1964; Webster, 1969, 1992; Wochok & Sussex, 1974, 1976), or a distinct organ before its transition to a root (Nägeli & Leitgeb, 1868; Bower, 1908; Jernstedt & Mansfield, 1985; Imaichi & Kato, 1991; Lu & Jernstedt, 1996).

In creeping species (e.g. *Selaginella wallacei*), root characters are acquired almost immediately after the rhizophore emerges, showing that this structure can take on morphological root identity early in development and providing some evidence that a root meristem mediates rhizophore growth (Webster & Steeves, 1963; Webster, 1967). However, there are several anatomical differences between the organization of rhizophore tips and the root meristems, such as the superficial apical cell that serves as an initial for rhizophore growth and elongation that is replaced at later stages of development by a newly formed internal apical cell that

gives rise to the root cap (Imaichi & Kato, 1991; Lu & Jernstedt, 1996). These anatomical changes support a unique identity to the rhizophore and its growing tip region, which we will classify here as a meristem. It is important to note that the descriptions of rhizophore development vary among species (Imaichi & Kato, 1991; Lu & Jernstedt, 1996), and no characterizations in the sequenced species, *S. moellendorffii*, have been reported.

The classification of the rhizophore is made further complicated by the great deal of developmental plasticity normally exhibited by *Selaginella* root and shoot meristems. Angle meristems are present in pairs, with one meristem, either dorsal or ventral, giving rise to a rhizophore depending on the species and the remaining angle meristem typically giving rise to a shoot (Cusick, 1954; Korall & Kenrick, 2002; Weststrand & Korall, 2016; Matsunaga *et al.*, 2017). Although the fates adopted by each of these structures is near absolute in each species, it has been reported that a small percentage of angle meristems adopt alternate root or shoot fates under normal developmental conditions (Cusick, 1954; Wochok & Sussex, 1975). Although the factors contributing to the establishment of early root/shoot identity in the genus remain unclear, early studies point towards a putative, conserved role for the phytohormone auxin, which is a well-characterized root polarizing cue in seed plants (Skoog & Miller, 1957; Overvoorde *et al.*, 2010). In *Selaginella*, auxin treatment results in a developmental shift of emerging angle meristems to root fates, whereas inhibition of auxin transport with 2,3,5-triiodobenzoic acid (TIBA) shifts angle meristems towards development of leafy shoots (Wochok & Sussex, 1975). Recent studies have shown that a core conserved set of transcription-mediating auxin response genes evolved in the earliest land plants (Mutte *et al.*, 2018). Although these studies together suggest that early land plant meristems may use a common signaling pathway to specify the root pole, additional studies are needed to confirm what endogenous role, if any, auxin plays in meristem development and identity in *Selaginella*.

Here, we characterize developmental transitions and growth of the rhizophore in *S. moellendorffii* and map the developmental window in which angle meristems retain the plasticity to adopt root or shoot fate. To examine the transcriptional identities of rhizophore, root, and shoot meristems, we performed comparative transcriptional profiling (RNA-seq) on rhizophore, root, and shoot. Rhizophore samples were taken from clearly established rhizophores that were within the window of plasticity and before they developed anatomical root features (cap and root hairs). During this period, the rhizophore possesses a transcriptional profile that shows limited affinity to the mature root and is almost as different from root and shoot meristems as root and shoot meristems are from each other. Thus, the rhizophore appears to be a distinct organ at the transcriptional level.

Materials and Methods

All plant material used in this study was clonally propagated from *S. moellondorffii* plants used for genome sequencing (provided by Jody Banks). Y-shaped cuttings of mature stems that included existing points of angle meristem outgrowths were applied to soil for general propagation. Explants were kept in high humidity (80%) and in a 16 h : 8 h ambient light : dark cycle.

For growth characterizations, a root was defined as a 10- to 20-d-old ventral angle meristem outgrowth that was 5–10 cm in length, as measured from the distalmost tip to the main shoot axis, and displayed a well-developed root cap and root hairs. A rhizophore was defined as a 4- to 7-d-old ventral angle meristem outgrowth 2.5–3.5 cm in length that lacked all of the following features: a root cap, root hairs, green pigmentation, and mature or presumptive microphylls.

Whole-mount imaging of roots and dorsal angle meristem

Clearing was done using a modified version of Ruzin's sodium hydroxide (NaOH) protocol (Ruzin, 1999): samples were incubated in 5% NaOH for several days, then rinsed in water before a 3 min incubation in full-strength household bleach. Samples were then rinsed again with water, followed by incubation in Visikol (Visikol Inc., Whitehouse Station, NJ, USA) for several hours. Staining was done by briefly submerging cleared samples in 25% trypan blue solution before mounting in water on a microscope slide. Images were acquired using a Leica SPE confocal microscope using 635 nm excitation at 100% laser power, pinhole 0.75 AU, with a 20× air objective at 3× zoom.

Auxin treatment of developing angle meristems

A lanolin paste containing either 10 μM of the synthetic auxin analogue 2,4-dichlorophenoxyacteic acid (2,4-D) or 100 μM of the auxin transport inhibitor TIBA was applied to mature stem cuttings in the region proximal to the angle meristems outgrowths at various developmental phases (0, 2, 4, 8, 10, 16 d post-initiation, dpi). The lanolin was applied *c.* 1 cm away from the angle meristem towards the base of the shoot on the ventral surface. For each time point, 20 angle meristem outgrowths not yet showing shoot (green pigmentation or microphylls) or root features (cap or root hairs) were assayed. A 2,4-D concentration of 10 μM was sufficient to elicit fate change without promoting the uncontrolled tissue growth typically associated with treatment with high auxin levels. The morphological identity (root, shoot, or rhizophore) of angle meristem outgrowths was scored 20 d post-treatment under dissecting microscope.

Tissue sampling and generation of RNA-seq libraries

Samples for transcriptional profiling were excised by hand with a 30G dental needle (ExelInt) under a dissecting microscope. For root, shoot, and rhizophore samples, distal tips of each organ were collected. Since we aimed to capture the meristematic region, we severed 30 μm from the distalmost point, a region that contains the apical cells and a small portion of surrounding provascular and tip tissue but excludes mature vascular tissue. Shoot tissue was sampled from the distalmost region of young shoots that were 10–20 d post-branch from main shoot axis, green, and had given rise to microphylls. Leaf tissue was harvested

from whole, 10- to 14-d-old microphylls. Two biological repeats were prepared for each tissue.

For each sample, 40 excised tissues were pooled in 20 µl of RLT Buffer (Qiagen RNeasy Micro Kit), flash frozen, and ground using methods described previously (Sena *et al.*, 2009). RNA yields and quality were assessed by a Bioanalyzer 2100 (RNA Picochip). Illumina libraries for two biological repeats of each tissue were generated using the Nugen Ovation Amp V2 DR kit (Tecan Genomics, Redwood City, CA, USA), with modifications made for multiplexing. Pooled libraries were sequenced using Illumina HiSeq 2500 to generate 50 bp single-end reads.

Expression analysis

Reads were aligned to the *S. moellendorffii* v1.0 genome (including the full genome, i.e. both haplotypes) using BOWTIE 2 (bowtie2 -local). Gene models were obtained from PHYTOZOME, and 2719 new open reading frames were included from a list of 4763 additional gene models from a recent transcriptomic characterization in *Selagenella* (Zhu *et al.*, 2017), which were filtered for duplicates and gene fragments. Gene read counts were obtained using NGSUTILS (bamutil count -library unstranded -multiple partial). EDGER was used to normalize the libraries using the trimmed mean method.

For the correlation analysis with previously generated *Selaginella* root samples (Huang & Schiefelbein, 2015), we set a threshold of > 25 normalized read counts in that dataset to generate a list of genes expressed above a moderate threshold ($n = 1824$). A quasi-linear model was used to derive a list of significantly changed genes across tissues at a false discovery rate (FDR) of < 0.05. Multidimensional scaling was performed in R using the cmdscale function. To identify meristem-specific genes within the subset of genes determined to be significantly regulated by the trimmed mean analysis (1527 genes), we selected the intersection of independent pairwise comparisons using the EDGER exactTest function (taking fold change > 2, FDR < 0.05 for each gene list); root = (root > shoot and root > leaf); shoot = (shoot > root and shoot > rhizophore); rhizophore = (rhizophore > root and rhizophore > shoot). For paired meristem specific genes: shoot/root = (root > rhizo and shoot > rhizo); root/rhizo = (root > shoot and rhizo > shoot); rhizo/shoot = (rhizo > root and shoot > root). Leaf (microphyll) samples were not used in the pairwise meristem analysis, but their expression pattern, which is highly similar to shoot, was included in the heatmaps for comparison.

Sequence and Gene Ontology term enrichment analysis

For genome-wide Gene Ontology (GO) term analysis, *S. moellendorffii* genes (of the two haplotypes) homologous to *Arabidopsis* genes were derived from previously published data (Banks *et al.*, 2011; Zhu *et al.*, 2017) and by performing a reciprocal BLAST of translated sequences against the *Arabidopsis* protein database (obtained from The Arabidopsis Information Resource, Supporting information Table S1) as follows: BLAST hits were ordered by *E*-value, ranked, and the sum of ranks for each gene pair was considered as a pairwise similarity score. Gene pairs with the lowest orthologue score (based on *E*-value) were marked as closest homologues, which was the basis for assigning *S. moellendorffii* GO terms. GO term enrichment was performed using the TOPGO R package using the closest *Arabidopsis* homologue, employing the 'weight01' algorithm, with all annotated *S. moellendorffii* genes used as the background reference in enrichment analysis. Significant GO terms were those with *P*-value < 0.05, without further multiple testing corrections. To construct gene trees for family-specific analyses, CLUSTALOMEGA was used to align *Arabidopsis* genes in a given family and BLAST hits of *Selaginella* transcripts that were expressed in our data (Larkin *et al.*, 2007). Neighbor-joining trees were generated using EMBL-EBI phylogeny tools (Goujon *et al.*, 2010).

Generation and transformation of root protoplasts

Whole root material was added to a cell-wall digestion solution of 2% cellulysin (Sigma), 0.75% macerozyme R-10 (Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan), 1% BSA, and 7 mM calcium chloride and vacuum infiltrated for 30 min. Fresh solution minus enzymes was then added, and protoplasts were isolated as described previously (Bargmann & Birnbaum, 2009). Typical yields from this protocol ranged from 2 million to 10 million cells/1 g of young tissue. Protoplasts were transfected with DR5::GFP (Ulmasov *et al.*, 1997) using the method described previously (Bargmann & Birnbaum, 2009). For 2,4-D treatment assays, 18 h protoplasts were treated with 10 µM 2,4-D, and the number of cells expressing green fluorescent protein (GFP) were counted under a confocal microscope at 6 h post-transfection. GFP reporter activity in 2,4-D-treated cells was compared with mock-treated cells.

Data availability

Raw data are available at the GEO accession: GSE123120.

Results

S. moellendorffii shares developmental features described in other lycophytes

Consistent with other species of *Selaginella*, the majority of the *S. moellendorffii* root system is derived from shoot angle meristem outgrowths (Fig. 1a). Initiation begins by outgrowth from ventral angle meristems located at established or presumptive branch points (Fig. 1b). This initial structure – the rhizophore – is smooth, cylindrical, and lacks a root cap and root hairs, as in other *Selaginella* spp. The root system, once established, expands by successive bifurcations at the apex to form an extensive root system (Fig. 1c). Consistent with some other *Selaginella* species, new shoots in *S. moellendorffii* are formed through bifurcation of existing shoot apices or from outgrowths of dorsal angle meristems (Fig. 1d). In the latter case, initiation of a dorsal outgrowth consistently occurs 6–18 d after the emergence of its ventral,

root-generating counterpart. Under growth conditions used for measurements, most rhizophores acquired mature root characters 10–20 d post-emergence: 6% (3/50) of rhizophores acquired these features at 10 d; 14% (7/50), at 14 d; 46% (23/50), at 16 d; 28% (14/50), at 20 d. Interestingly, a small number of rhizophores (3/50) acquired shoot fates. The rhizophore-to-root transition is marked by the thickening of the distal tip, followed by the formation of a root cap and root hairs (Fig. 1e,f). Although the first roots act as primary anchors, rhizophores continue to emerge along the length of the shoot in *S. moellendorffii*, with the newest rhizophores positioned towards the apex of the growing shoot.

As noted earlier, we observed some natural developmental plasticity of these early angle meristem outgrowths, which was consistent in a second trial, with 10% (2/20) of ventral angle meristems adopting shoot fates. In addition, the dorsal angle meristem also showed plasticity, with 10% (2/20) of dorsal outgrowths adopting root fate after an apparent rhizophore stage.

Our observations of root and shoot growth in *S. moellendorffii* provide a timeline for root and shoot development in this sequenced species.

The rhizophore and early shoot organs of *S. moellendorffii* display developmental plasticity that is influenced by auxin

The rhizophore takes 10–20 d to exhibit root anatomical hallmarks, but the point of transition from rhizophore to committed root fate is unclear. Auxin treatment can trigger meristem identity change in young meristems of *Selaginella* (Wochok & Sussex, 1975). We therefore sought to determine the time window during which auxin perturbations could effectively alter meristem identity, thus identifying when the rhizophore commits to becoming a root.

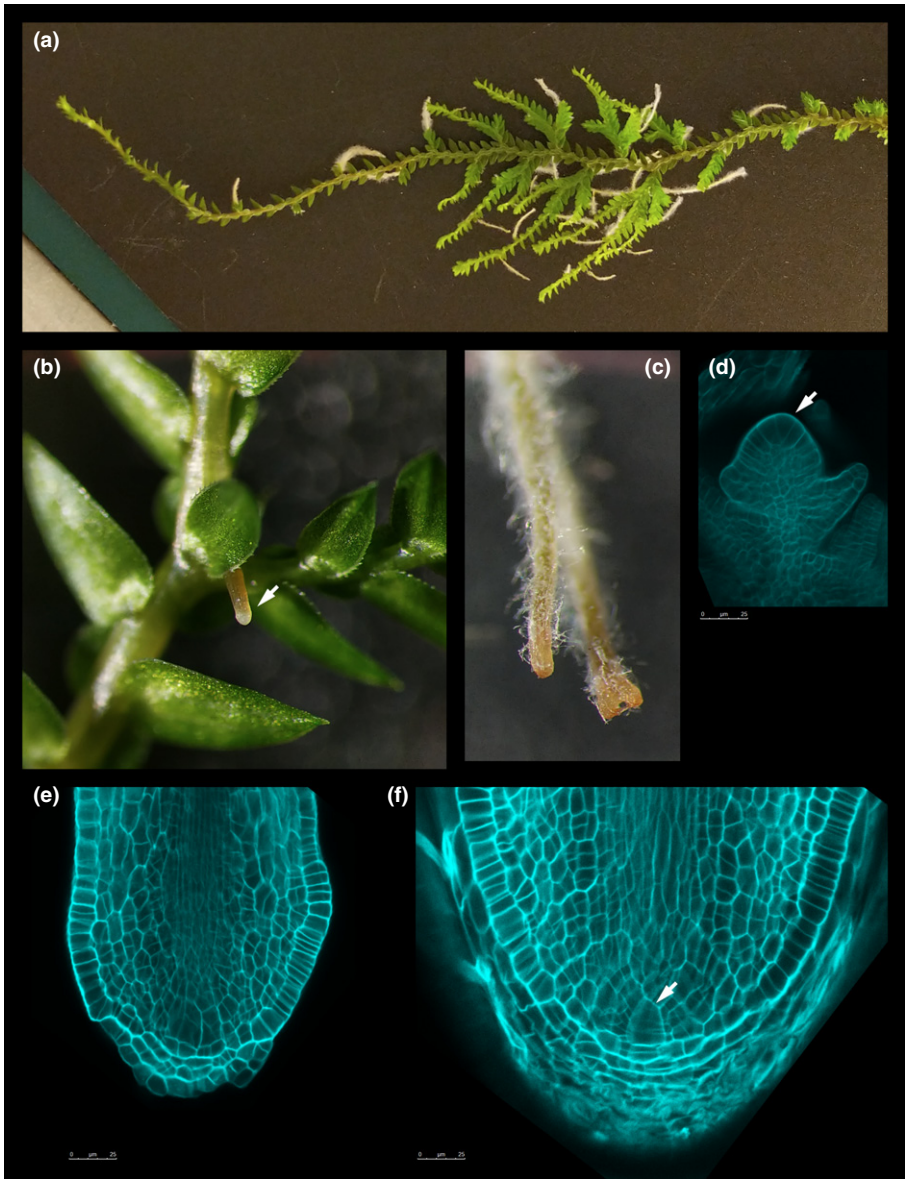

Fig. 1 The shoot-borne rhizophores of *Selaginella moellendorffii* transition to roots. The majority of the *S. moellendorffii* root system is derived from outgrowths in the shoot (a). A ventral meristem becomes a rhizophore (arrow) that transitions to a root (b). This root system expands by successive bifurcations at the root apex (c). Dorsal angle meristems typically give rise to shoots, which maintain an apical cell (arrow) at the surface of the meristem (d). Rhizophores transition to roots when the apical cell becomes internal, giving rise to a nascent cap in early transition stages (e) that forms a multilayered cap at later stages (f). The root in (e) is in the process of transitioning from rhizophore and still lacked root hairs (not shown), whereas the root in (f) had root hairs and a well-defined internal apical cell (arrow). Scale bars are 25 μm.

Lanolin paste containing the synthetic auxin analogue 2,4-D, which we used for its stability over IAA, was applied to mature stem cuttings in the region proximal to the angle meristems outgrowths at various developmental stages (0, 2, 4, 6, 8, 10 and 16 dpi; see Fig. 2a). Consistent with IAA-treatment results in other species, 2,4-D treatment promoted the formation of roots from early dorsal outgrowths (0–8 dpi), indicating that the angle meristem of *S. moellendorffii* is plastic and shows a conserved response to auxin (Fig. 2b vs c). The root-promoting effects of auxin treatment dampen as outgrowths develop, and no effects were seen in dorsal outgrowths treated after 8 dpi, suggesting a loss of developmental plasticity and a commitment to shoot fate at this developmental stage (Fig. 2d).

Within the 0–8 d window of plasticity, 2,4-D treatment led to rapid elongation of the dorsal angle meristem and a more rapid appearance of a root, with a cap and hairs – usually within 3 d post-treatment. Before root features appeared on the treated dorsal meristems, they appeared to take on some rhizophore traits, such as a thickening of the tip and cuticle (although the meristem remained green until transitioning to the root). Thus, auxin promotes root fate in *S. moellendorffii*, possibly through a transient rhizophore stage.

Interestingly, the small percentage of ventral meristems that developed into shoots (Fig. 2e), suggests a noncommitted state in the natural development of the organ. Treatment of ventral meristems with 2,4-D further reduced the low percentage of ventral meristems that adopted shoot fate, suggesting that natural fluctuations in hormone concentrations could play a role the natural plasticity of angle meristems.

To further assess the developmental plasticity of ventral angle meristems and the early rhizophore, we repeated the treatment assay with the auxin transport TIBA (Wochok & Sussex, 1975). Complementary to the 2,4-D results, treatment with 100 μM TIBA promoted the formation of shoots from early developing ventral outgrowths (2–8 dpi), indicating that the ventral angle meristem/early rhizophore of *S. moellendorffii* is indeed plastic and shows conserved responses to auxin inhibition. Similar to dorsal angle meristems, the ventral angle meristems also lost their developmental plasticity by *c.* 8 dpi.

These results show that the early root and shoot organs of *S. moellendorffii* share conserved responses to the root-polarizing hormone auxin characterized in classic studies of *Selaginella* (Wochok & Sussex, 1975). In addition, the time-course treatments also allowed us to establish a developmental window that guided

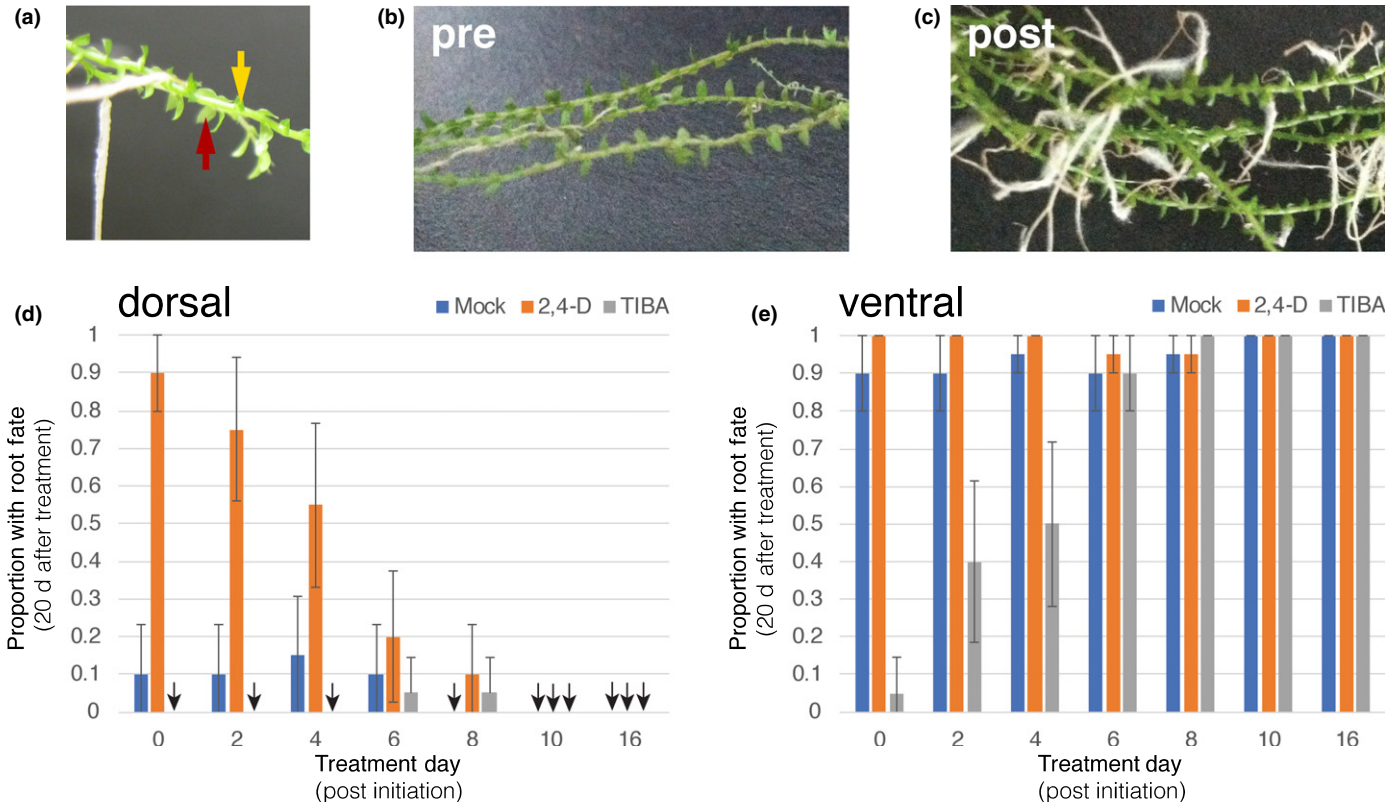

Fig. 2 Window of developmental plasticity in *Selaginella moellendorffii* exhibited through auxin treatment or perturbation. Developing dorsal or ventral angle meristem outgrowths at various developmental stages (0, 2, 4, 6, 8, 10, 16 d post-initiation) were treated with mock solution (blue), 10 μM 2,4-dichlorophenoxyacteic acid (2,4-D; orange) or 100 μM 2,3,5-triiodobenzoic acid (TIBA; gray) and organ identities (root or shoot) were assayed 20 d post-treatment. The location of application of the lanolin paste is shown in (a). Yellow arrow indicates angle meristem location (shoot angle); red arrow indicates the site treatment was applied (on ventral surface). Comparison of overall morphology of pretreatment (b) vs 2,4-D-treated samples (c) shows that auxin treatment greatly enhances root density. Quantitative analysis of the results of either mock, 2,4-D, or TIBA treatment on the dorsal (c) and ventral meristems (d). For each developmental stage, 20 angle meristems outgrowths not yet showing shoot or root features were treated. All outgrowths that did not show root fate by day 20 adopted shoot fates. Error bars show SE of the proportion at 95% confidence limits. Black arrows indicate a frequency of zero.

our sampling strategy for genomic studies to capture a clear rhizophore stage that preceded the transition to root fate.

The rhizophore transcriptome is more similar to root than shoot profiles, but is distinct from both meristems

To address the question of the identity of the rhizophore, we employed global transcriptome analysis. The small genome size and available genome sequence of *S. moellendorffii* (Banks *et al.*, 2011) make this system particularly appealing for such an approach. Although previous transcriptome analyses in *Selaginella* (Frank *et al.*, 2015; Huang & Schiefelbein, 2015; Zhu *et al.*, 2017) provide valuable insights into key evolutionary developments, such as vascular tissue, lignin synthesis, and the transcriptional identities of root and shoot apical subdomains, no comparative transcriptomic analyses of root, rhizophore, and shoot that could identify genes that contribute to their distinct identities has been performed.

We generated RNA-seq libraries for manually dissected root, shoot, and rhizophore meristems (Tables S2, S3). As discussed in greater detail in the Materials and Methods section, a root was defined as a 10- to 20-d-old ventral angle meristem outgrowth that displayed a well-developed root cap and root hairs. The rhizophore was defined as a 4- to 7-d-old ventral angle meristem outgrowth that lacked a root cap and root hairs and corresponded to a stage when rhizophores displayed plasticity for interorgan transformation (Fig. 2). Leaf (microphyll) samples were generated to compare one nonmeristematic organ that emerges from the shoot meristem.

We detected a total of 19 903, 24 924, 18 724 and 19 570 transcripts in leaf, root, shoot, and rhizophore meristems, respectively. To assess our data against independently collected samples, we compared our dataset against previously generated RNA-seq profiles of roots that included *S. moellendorffii* (Huang & Schiefelbein, 2015). Among genes detected above a moderate threshold in the previous *Selaginella* root meristem samples, read counts were most highly correlated to the root and rhizophore ($r = 0.75$ in both) compared with the leaf and shoot samples ($r = 0.06$ and 0.05, respectively), showing reasonable agreement in the root samples between the two experiments and providing a general validation of the data.

Focusing on our dataset, 1527 transcripts were significantly differentially expressed across samples at FDR < 0.05 (Table S4). Hierarchal clustering shows that the root and rhizophore sample shared the most similar expression profiles among the meristems (Fig. 3a). Spearman correlation between the rhizophore and shoot meristem was 0.2, while the correlation between root and rhizophore was 0.57. This indicates that the rhizophore is more closely related to the root than to the shoot at the level of global transcription.

Nonetheless, the overall expression patterns of differentially regulated genes (Fig. 3a) and the relatively weak correlation between root and rhizophore indicated that the rhizophore could exhibit a distinct gene regulatory program. We first applied multidimensional scaling (MDS) based on the list of differentially expressed genes to further break down major expression patterns in the data. Shoot and leaf samples appear in close proximity, again showing they are highly similar (Fig. 3b). However, the root and rhizophore were distinct from the shoot and from one another in the MDS analysis (Fig. 3b), with rhizophore equally distant from root and shoot. Thus, breaking down major patterns of variation in more detail, the rhizophore shows a unique component to its expression profile that is distinct from root and shoot.

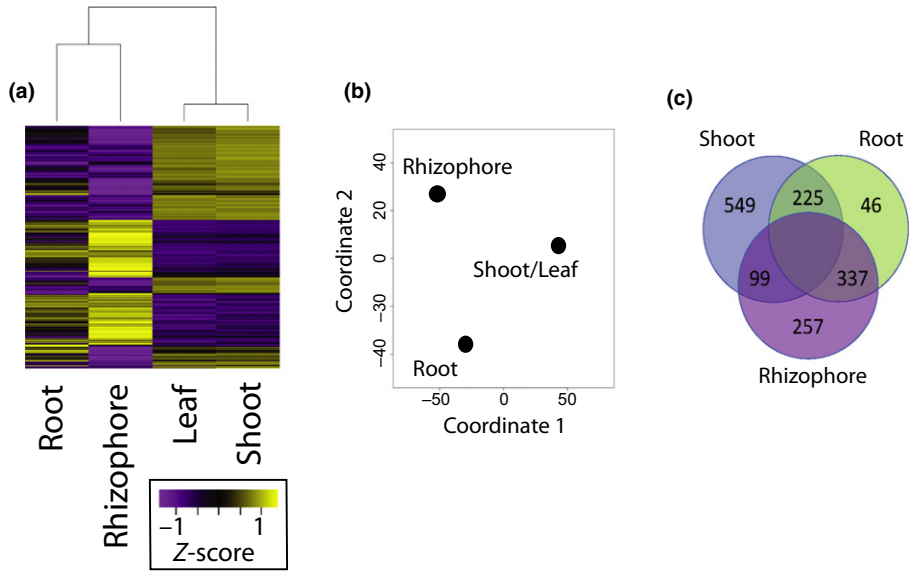

Fig. 3 The rhizophore shows a distinct transcriptional signature with some affinity to root. (a) Row normalized mean expression values of replicate samples showing significantly differentially expressed genes across all sampled tissues. (b) Multidimensional scaling of transcriptional profile of root, rhizophore, shoot meristem, and leaves (performed in R using the cmdscale function). Note that leaf and shoot are merged into one point because they could not be distinguished at the scale of the figure. (c) Venn diagram of the single or paired meristem upregulated transcripts in the root, shoot, and shoot meristematic zones. Only genes with mean reads per kilobase per million values > 0.1 were counted. Color key represents row normalized *Z*-scores.

To further examine the relationship between the root, shoot, and rhizophore meristems, we partitioned the 1527 significantly regulated genes into groups that showed specific enrichment in one or two meristems using pairwise comparisons (see the Materials and Methods section, Fig. 3c, Table S5). The shoot had the most enriched transcripts, with 549 (Fig. 4a), whereas the rhizophore had 257 (Fig. 4b) and the root had only 46 enriched transcripts (Fig. 4c), showing that the rhizophore has a relatively large and distinct transcriptional program. By contrast, the root shared many enriched genes with both the rhizophore (337 transcripts, Fig. 4d) and the shoot (225 transcripts, Fig. 4e). Interestingly, the rhizophore also shared 99 upregulated transcripts with the shoot (Fig. 4f), showing that the rhizophore appears to retain some transcriptional properties of the shoot from which it originated. In addition, the root and rhizophore share a similar number of enriched genes as the root and shoot, which are clearly distinct organs. In the patterns that show unique or shared rhizophore specificity (e.g. Figs 3a, 4b,d,f), gene expression tends to be relatively dramatically upregulated compared with other patterns in the data, showing that markers of rhizophore identity represent a strong signal in the data. Thus, our transcriptional analysis supports a unique identity for the rhizophore despite its relative affinity to the root.

To explore the potential functional role of meristem-enriched transcripts, we performed GO enrichment analyses on each list of the meristem-specific genes using homology to *Arabidopsis* genes. We sought a genome-wide analysis, so we supplemented existing family-specific orthology analyses with reciprocal BLAST analysis to determine the most similar homologous pairs (see the Materials and Methods section, Tables S5, S6).

Interestingly, GO enrichment analysis shows an enrichment for many terms related to protein translation in rhizophore and root–rhizophore sets, with late-stage translation-related terms specific to rhizophore (Fig. 5; e.g. translational elongation), in agreement with earlier biochemical and histological studies that point towards higher levels of translation in the rhizophore just before the root transition in *Selaginella kraussiana* (Jernstedt & Mansfield, 1985).

The GO term enrichment analysis also suggested several functions or pathways that could underlie the affinity of the rhizophore to both root and shoot (Fig. 5). For example, the root–rhizophore shared gene set is enriched for response to the phytohormone cytokinin. The transcripts that contributed to the significant GO term include genes with homologues known to be positive signaling regulators of this hormone (*AHP5*, 267058), which has been implicated in control of root elongation in seed

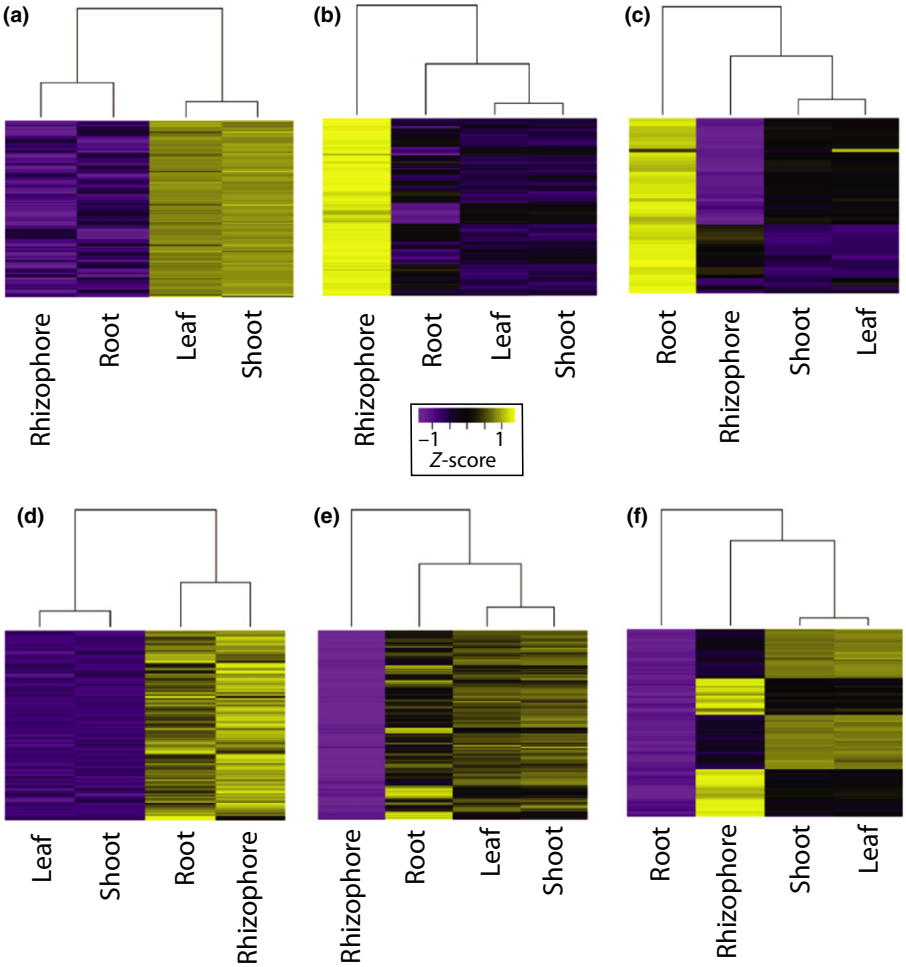

Fig. 4 Heatmaps of meristem-specific and meristem-shared upregulated gene sets show distinct transcriptional programs of shoot and rhizophore. (a) Shoot-, (b) rhizophore-, and (c) root-specific genes were identified as those showing significant upregulation in one meristem compared with both other meristems. Root–rhizophore (d), root–shoot (e), and rhizophore–shoot (f) genes were identified by showing significant upregulation in two meristems compared with the third (see the Materials and Methods section). Note that leaf samples are shown but were left out of the comparisons because analysis shows they are highly similar to the shoot meristem. Color key represents row normalized *Z*-scores.

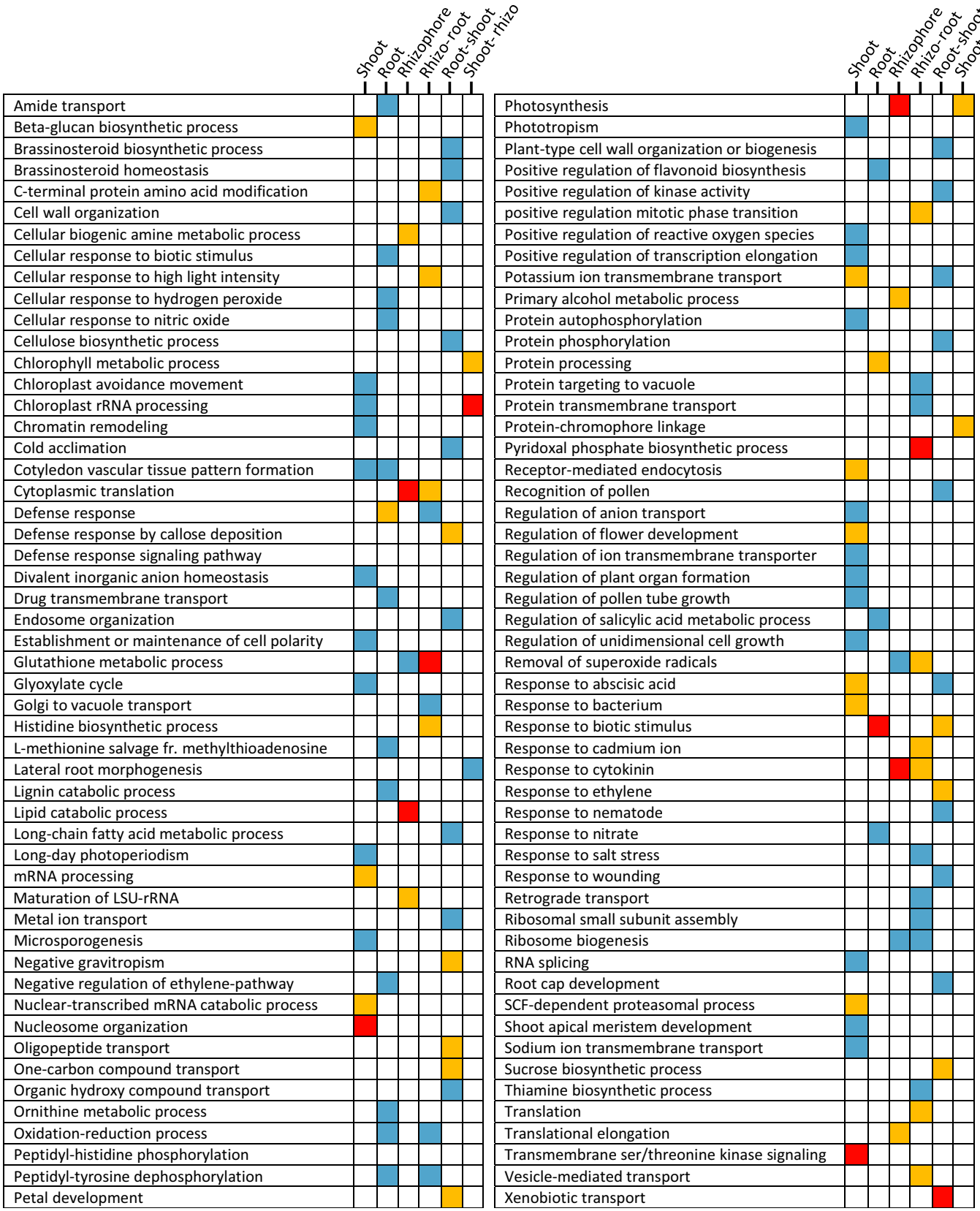

Fig. 5 Gene Ontology term enrichment analysis of meristem-specific and meristem-shared genes. Color coding represents *P*-value of the term's enrichment in a meristem-specific or meristem-shared transcript list, where red is < 0.001, orange is < 0.01, and blue is < 0.05. Note that terms scoring pval > 0.03 are not listed for space considerations. See Supporting Information Table S6 for complete list and other information.

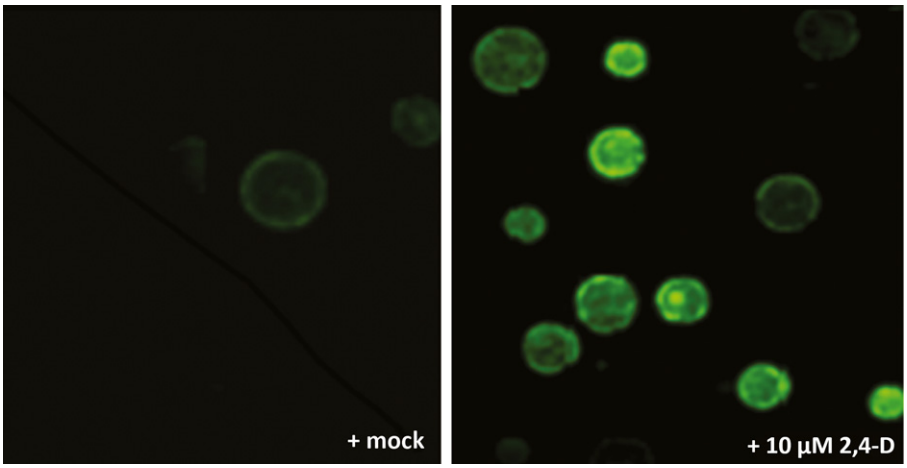

Fig. 6 The DR5 reporter is highly induced in *Selaginella moellendorffii* root cells. Protoplasts derived from whole *S. moellendorffii* root tissue transiently transfected with the DR5::GFP were incubated for 18 h after transfection. The same batch of transfected protoplasts was split and then subjected 10 µM 2,4-dichlorophenoxyacteic acid (2,4-D) or a mock treatment. The two treatments, mock (left) and 2,4-D (right), were visualized under the same excitation and exposure settings 6 h after the treatments.

plants (Hutchison *et al.*, 2006), as well as orthologues to genes involved in cytokinin degradation (*CKX7*, 174721).

On the other hand, biological processes enriched in the rhizophore–shoot shared set included chloroplast ribosomal RNA processing, photosynthesis, and Chl metabolic processes (Fig. 5), suggesting that the rhizophore may retain some photosynthetic properties of the shoot tissue. The class-1 KNOX family (*KNOTTED-LIKE* (*KNAT1*), 159366, 415291) were previously found to be enriched in shoot and rhizophore in *Selaginella utica* (Kawai *et al.*, 2010). Although they did not make our stringent cutoffs for meristem-enriched genes, we detected the same pattern of class-1 KNOX family homologues in *S. moellendorffii* (*KNOTTED-LIKE* (*KNAT1*), 159366, 415291), with higher levels of these two transcripts in rhizophore and shoot meristems and much lower levels in the root (Tables S2, S3).

In the root, we detect an enrichment of genes involved in redox regulation (e.g. oxidation–reduction process). Redox regulation is an important aspect of root meristem size regulation (Tsukagoshi *et al.*, 2010) – one potential shared feature between the root pole in angiosperms and lycophytes. In addition, both the rhizophore- and root–rhizophore-specific lists are enriched for glutathione metabolism, which plays a role in redox regulation. Interestingly, mutants in glutathione synthesis in *Arabidopsis* lead to root-specific defect in cell proliferation that dramatically affects root formation (Cheng *et al.*, 1995; Vernoux *et al.*, 2000).

The shoot meristem-specific list shows strong enrichment for response to ABA, suggesting this phytohormone pathway could have an important role in the early shoot meristem. The list includes homologues to at least one gene implicated in crosstalk between ABA and gibberellic acid (GA), *RGA LIKE1* (102726) – a core GA signaling regulator (Achard *et al.*, 2006; Weiss & Ori, 2007). Other significant terms include mechanisms implicated in various shoot or inflorescence meristems in seed plants, such as shoot apical meristem development, regulation of plant organ formation, and regulation of flower development (Fig. 5). The latter term suggests that mechanisms involved in floral development likely had earlier roles in organ development in nonflowering plants.

Auxin signaling and response pathways are conserved in *S. moellendorffii*

Recent comparative genomic analyses have shown that land plants possess a conserved, core auxin response machinery consisting of orthologues to *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*), *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*), and *AUXIN REPONSE FACTORS* (*ARFs*) (Mutte *et al.*, 2018). Differential accumulation of this hormone in seed plants plays a key role in a number of complex growth and development processes, including the establishment of the root apical meristem in the embryo (Friml *et al.*, 2003) and meristem organization in the postembryonic seed plant root (Sabatini *et al.*, 1999). Indeed, orthologues of *TIR1*, *AUX/IAAs*, *ARFs*, and PIN-FORMED (PIN) auxin transport proteins, have been detected in *Selaginella* transcriptomes (Zhu *et al.*, 2017), and we detect their expression in isolated root, shoot, and rhizophore meristems. To examine the similarity among particular members of gene families detected in our datasets, we generated neighbor-joining trees for *Arabidopsis* auxin-related gene families and their *S. moellendorffii* homologues (see the Materials and Methods section, Fig. S1, Table S7).

Differential distribution and directional transport of auxin are required for several developmental processes, and early biochemical studies have identified directional auxin transport in the emerging rhizophore (Wochok & Sussex, 1974; Matsunaga *et al.*, 2017). Auxin transport is mediated by members of several efflux and influx factors. One such family is the PIN proteins, whose differential expression and localization are well-characterized in *Arabidopsis* (Friml *et al.*, 2002; Benkova *et al.*, 2003; Blilou *et al.*, 2005; Krecek *et al.*, 2009). Previous studies detected expression of several *PIN* orthologues in *Selaginella* (Sanders & Langdale, 2013; Zhu *et al.*, 2017). We detect expression of an expanded set of PIN-like genes (231064, 102666, 268490, Smoe_00006099, Smoe_00028886, 231064) that are most similar to each other but fall into a group with PIN 5/6/8 on our tree. Several PIN-related transcripts show enrichment in the rhizophore and are among a relatively small set of rhizophore–shoot enriched genes (Tables S4, S7).

In all isolated meristems, we detect expression of a *TIR1* homologue (104859) and a set of genes (originally identified as Aux/IAA homologues in Banks *et al.* (2011)), falling into separate branches of the of Aux/IAA family and generally showing higher expression in the shoot (Fig. S1; 116126, 26861, Smoe_00025267, 36236, 85035, 431277, 438333, 405821, 415204, 417391). We also detect a number of homologues of the B and C clades of ARFs (Mutte *et al.*, 2018) that include ARFs 2, 3, 15, 16 (Fig. S1).

In addition, we detect transcripts with specific similarity to the A-type ARFs – a clade that includes a gene in *Arabidopsis* that is necessary for root organogenesis (Hardtke & Berleth, 1998). We detect these A-type ARF homologues (115320, 117217, Smoe_00048009, 181406) in all the samples – showing that positively regulating ARFs are expressed across the different *Selaginella* meristems. Accordingly, we also detected expression of homologues of downstream targets of *MP* involved in provascular development (*TARGET OF MONOPTEROS 5* (*TMO5*), 412681; *TMO5-LIKE1*, 56610) (Schlereth *et al.*, 2010) in all meristems, consistent with previous results that found these transcripts in other *Selaginella* tissues (Zhu *et al.*, 2017).

In seed plants, the *PLETHORA* (*PLT*) family of transcription factors forms gradients in meristematic regions that promote cellular growth and division, in part by mediating auxin signaling (Aida *et al.*, 2004; Galinha *et al.*, 2007; Mahonen *et al.*, 2014). We detect expression of homologues of several PLT family members (e.g. *PLT1*/*PLT2* (17087) and *PLT7* (418551, 17086)) with highest expression in the root.

Prior studies have shown that the synthetic auxin response reporter DR5, whose repeat elements are bound and induced by ARFs (Ulmasov *et al.*, 1999), is responsive to auxin in the moss model *Physcomitrella* (Bierfreund *et al.*, 2003; Lavy *et al.*, 2016). *Physcomitrella* lies on an earlier branch of land plants than *Selaginella*, suggesting a conserved mode of transcriptional response to auxin in early land plants. In addition, *in-silico* protein-folding analysis of ARF sequences from basal land plants showed a similarity in structural topology that also suggests some conservation in the mode of DNA binding (Mutte *et al.*, 2018). Stable transformation methods in *Selaginella* are currently lacking. However, we sought to test whether the transcriptional responses mediated by ARFs could function in *Selaginella* by developing a method to generate protoplasts from *S. moellendorffii* root tissue and transfect cells with a DR5::GFP reporter. Indeed, transfected root protoplasts treated with auxin (10 μM 2,4-D) showed a 520% increase in the number of cells with reporter activity compared with mock-treated cells (Fig. 6). This result, coupled with transcriptional data, supports the model that extant lycophytes possess the core, conserved components of the intracellular auxin response machinery (Mutte *et al.*, 2018), which could mediate the induction of rhizophore and root.

Discussion

Here, we provide a characterization of the developmental transitions and growth of the rhizophore in *S. moellendorffii* and confirm that this sequenced model shows conservation of intriguing root-development features (rhizophore, plasticity, shoot-borne roots) that first made the genus of evolutionary interest a century and a half ago.

To revisit the longstanding debate on the identity of the rhizophore, we provide a comparative transcriptomic analysis of the three apical meristems of *Selaginella*: the root, shoot, and rhizophore. The results indicate that, at the broad transcriptional level, the rhizophore meristem more closely resembles the root than shoot meristem, which may be explained, in part, by these two organs emerging sequentially from the same ventral outgrowth within days of each other. Hierarchal clustering of all significantly differing transcripts among the three meristems groups the rhizophore and the root closest together (Fig. 3a). GO enrichment analysis of transcripts common to rhizophores and roots, but not shoots, reveals an enrichment of homologues for genes involved in response to cytokinin, suggesting some core signaling mechanisms are shared by both meristems.

Despite possessing higher overall transcriptional affinity to the root, the rhizophore shares specific regulation of some genes with the shoot. GO enrichment analyses of transcripts common to the shoot and rhizophore reveal that, despite lacking the green pigmentation, the rhizophore shares gene homologs involved in photosynthesis and Chl metabolism, suggesting that the rhizophore may retain some photosynthetic properties of the shoot tissue from which it was derived or these functions may be induced separately in aerial rhizophores before they reach soil. It is important to note that the rhizophore samples were collected at an early stage, before being committed to a root fate, which may explain some of the residual 'shoot-like' gene expression.

Despite affinity to both other meristems, the rhizophore gene expression program is very clearly distinct from that of either meristem. MDS analysis shows that root and rhizophore are separated by a distance that is similar to the distances that separate the root and shoot or rhizophore and shoot, indicating that the rhizophore has a distinct transcriptional identity when all major expression patterns in the dataset are examined. Additionally, the rhizophore has a distinct set of about 257 genes that are specifically enriched in its meristem and not the root or shoot. In that respect, the rhizophore is nearly as distinct as the shoot.

The divergent transcriptional program of the rhizophore is consistent with a body of work on its distinct anatomy and development in other *Selaginella* species. In its early stages, the rhizophore has a tetrahedral apical meristem cell located at the ventral surface that divides to generate new tissues and elongate away from the main shoot axis. This single or set of superficial apical cells appears to be displaced in the early elongation phases and is replaced by a new apical meristem cell that divides at three faces to support later rhizophore growth. Just before the formation of cap and hairs, a new apical meristem cell emerges from the inner cells of the rhizophore and divides at a fourth face to produce the root cap (Imaichi & Kato, 1991; Lu & Jernstedt, 1996), suggesting that the apical cell that emerges at the termination of the rhizophore phase possesses a different, root-bearing identity than the apical cell that mediates growth in the prior rhizophore stage.

If the rhizophore is a distinct organ, then what developmental and molecular traits characterize its unique identity and functions? In agreement with previous results (Jernstedt & Mansfield, 1985), GO enrichment analysis suggests part of that distinct function may be a preparation for the developmental transition to root by ramping up the translational machinery. It seems feasible that many of properties of the rhizophore are geared towards preparation for growth underground while coping with the aboveground environment. Many of the categories enriched in rhizophore also include metabolic processes that may enable the rhizophore to survive as an aerial structure.

The rhizophore may appear largely distinct from the root, but what developmental features are behind its weak affinity to the root at the transcriptional level? We analyzed the developmental progression of the rhizophore to ensure that we sampled the meristem before the characteristic transitions and internalization of the apical cell that would signify its transition to root. However, the rhizophore had begun to lose plasticity in the later points of our sampling window. This may indicate that the meristem was undergoing initial stages of the transition to root, such that its transcriptional program had already partly transitioned to the root stage. Still, as noted earlier, the rhizophore also shared properties with the shoot that may represent residual shoot identities.

Together, these observations raise the possibility that the distinct rhizophore stage also overlaps with a gradual loss of shoot identity and a gradual gain of root identity. The transition from rhizophore to root may start early when the rhizophore still retains shoot identity. The root then retains but also loses much of the rhizophore-initiated transcriptome as revealed by single and shared meristem analysis in our experiments. In this sense, the rhizophore would represent a distinct developmental stage of root identity that is absent in seed plants.

Alternatively, the affinity of the rhizophore to the root could arise from a common mode of specification. One of the most consistent and conserved mechanisms in land plant development is the role of auxin as a signal that induces the root pole. As with seed plants, auxin treatment can enhance root fate in *Selaginella* (Wochok & Sussex, 1974; Matsunaga *et al.*, 2017; this study). In our study, it appeared that auxin-treated dorsal meristems went through an abbreviated rhizophore stage before the transition to root. This result would suggest that the rhizophore is also induced by auxin. In addition, earlier work showed that the rhizophore transports auxin (and nutrients) in the same direction (acropetal) and at roughly the same rate as mature roots in *Selaginella* and seed plant roots, exhibiting a shared physiological trait with the adult root (Wochok & Sussex, 1974; Matsunaga *et al.*, 2017). Although we did not detect auxin signaling mechanisms shared exclusively by rhizophores and roots, we did detect a shared cytokinin response that could secondarily affect auxin responses in these meristems through auxin–cytokinin crosstalk (Moubayidin *et al.*, 2009).

Lycophyte roots are postulated to have evolved independently from the roots of euphyllophytes (Kenrick & Strullu-Derrien, 2014; Hetherington & Dolan, 2018). Yet, as already noted, our work and that of others shows that auxin can induce roots – and likely rhizophores – in at least one family of lycophytes, Selaginellaceae. In *Marchantia*, which lack roots and vasculature, high auxin induces the root-like rhizoid structure (Kaul *et al.*, 1962). One question is whether the shared rhizophore–root transcriptome represents a core auxin response and whether such a response is part of an ancestral pathway that generates a root or root-like pole. Further studies are needed to clarify if, and to what extent, root and rhizophore share a response to auxin.

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Author contributions

AM and KDB designed experiments. AM performed all experiments. AM and IE performed bioinformatic analyses of the data. RR performed photography and clearing and microscopy of angle meristems. AM, KDB and IE wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Neighbor-joining trees of *Arabidopsis* and *Selaginella moellendorffii* homologs in select gene families.

Table S1. List of most similar homologs between *Selaginella moellendorffii* and *Arabidopsis thaliana*.

Table S2. Raw read counts per gene for all sample replicates.

Table S3. Normalized, average read counts for each meristem or leaf sample.

Table S4. Differentially expressed genes among the three meristem samples.

Table S5. List of single and shared meristem-specific transcripts.

Table S6. GO terms by meristem specific group with statistics and corresponding Arabidopsis homologs.

Table S7. Auxin-related genes and normalized, average read counts of orthologs among samples.

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