1   **Short title:**

2   Selaginella Root Bifurcation

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10   # Cellular and gene expression patterns associated with root

11   # bifurcation in Selaginella

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23   **One-sentence summary:**

24   The root of the spike moss *Selaginella moellendorffii* bifurcates following a cell

25   division of the single stem cell, and the bifurcation involves conserved genetic modules

26   known from angiosperm roots.

27

28   **Footnotes:**

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36 Author Contributions:

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39 and analyzed microscopic data. T.F., H.M. and B.P. performed the RNA-seq
40 experiment. B.P. processed the transcriptomic dataset. H.M. and B.P. analyzed the
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43

44 **Abstract**

45 The roots of lycophytes branch through dichotomy or bifurcation, during which the root

46 apex splits into two daughter roots. This is morphologically distinct from lateral root

47 (LR) branching in the extant euphyllophytes, with LRs developing along the root axis

48 at different distances from the apex. Although the process of root bifurcation is poorly

49 understood, such knowledge can be important, because it may represent an

50 evolutionarily ancient strategy that roots recruited to form new stem cells or meristems.

51 In this study, we examined root bifurcation in the lycophyte *Selaginella moellendorffii*.

52 We characterized an *in vitro* developmental time frame based on repetitive apex

53 bifurcations, allowing us to sample different stages of dichotomous root branching and

54 analyze the root meristem and root branching in *S. moellendorffii* at the microscopic

55 and transcriptomic level. Our results showed that, in contrast to previous assumptions,

56 initial cells in the root meristem are mostly not tetrahedral but rather show an irregular

57 shape. Tracking down the early stages of root branching argues for the occurrence of a

58 symmetric division of the single initial cell, resulting in two apical stem cells that

59 initiate root meristem bifurcation. Moreover, we generated a *S. moellendorffii* root

60 branching transcriptome that resulted in the delineation of a subset of core meristem

61 genes. The occurrence of multiple putative orthologs of meristem genes in this dataset

62 suggests the presence of conserved pathways in the control of meristem and root stem

63 cell establishment or maintenance.

64

65 **Key words:** *Selaginella moellendorffii*, meristem bifurcation, initial cell, evolution, root apical

66 meristem.

67   Roots provide multiple key functions for plants, and their capacity to branch is

68   important to anchor in a substrate and to forage for water and nutrients (Raven and

69   Edwards, 2001; Motte and Beeckman, 2019). Root development and root branching

70   have been in particular studied in angiosperms, e.g. Arabidopsis (*Arabidopsis thaliana*).

71   Here, roots branch via the formation of lateral roots (LRs), which develop in the

72   differentiated part of the root from pluripotent pericycle cells. After a series of well-

73   orchestrated divisions, a new root meristem is formed that grows out of the parent root.

74   Noteworthy, gene expression or anatomical studies of LR formation in different species

75   can be facilitated by employing LR inducible systems, allowing the synchronous

76   induction of many LRs (Himanen et al., 2002; Himanen et al., 2004; Vanneste et al.,

77   2005; De Smet et al., 2008; Jansen et al., 2013; Crombez et al., 2016; Herrbach et al.,

78   2017).

79   In contrast to angiosperms, Selaginella species, members of the lycophyte lineage,

80   do not possess pluripotent cells that give rise to LRs (Fang et al., 2019). Instead, roots

81   dichotomously branch at the tip through bifurcation of the apical meristem (Imaichi and

82   Kato, 1989; Lu and Jernstedt, 1996; Otreba and Gola, 2011; Gola, 2014; Fang et al.,

83   2019; Motte and Beeckman, 2019). Lycophytes were the first plants that acquired roots

84   (Hetherington and Dolan, 2018). Hence, studying their root system can contribute to

85   the understanding of how root developmental mechanisms evolved. Lycophyte roots,

86   however, originated independently from roots of the other land plant lineages

87   (Hetherington and Dolan, 2019), but both molecular and anatomical data suggest a

88   highly convergent evolution, and possibly even a common (partial) recruitment of a

89   genetic program present in the rootless common ancestor of the different lineages

90   (Huang and Schiefelbein, 2015; Motte and Beeckman, 2019). Based on fossil records,

91   dichotomous root branching was found at the base of lycophyte evolution, putting it

92   forward as an early innovation during the evolution of roots (Hao et al., 2010;

93   Matsunaga and Tomescu, 2016; Hetherington and Dolan, 2017; Hetherington et al.,

94   2020).

95   Unfortunately, despite intensive, but almost exclusively histological studies, the

96   development and anatomy of the Selaginella root apical meristem and its bifurcation

97   are still poorly understood. Multiple studies report the presence of one central

98   tetrahedral initial cell (IC) or apical stem cell (Otreba and Gola, 2011 and refs therein)

99   that, in analogy with the apical cell in the root meristem of certain ferns, was suggested

100   to cut off daughter cells in four directions to provide cells for the root cap at the distal

side, and cells for the growing root at the three proximal sides. In some ferns, it is clearly documented that the three proximal daughter cells each divide in an equal pattern, forming merophytes or clonally related groups of cells. Subsequent merophytes are superimposed to form different layers in the root (Hou and Hill, 2004). In contrast to ferns, such division pattern is less obvious in Selaginella roots. Furthermore, the branching process is problematic to study at the microscopic level. Live imaging is difficult because of the lack of biotechnological tools (Motte et al., 2020), and arbitrarily sampled roots rarely represent critical phases in which roots are undergoing bifurcation. Moreover, histological sectioning does not allow a high-throughput approach, and serial sectioning is required to obtain the relevant sections in which the IC is visible. As a consequence, the cell division events associated with root meristem bifurcation remain elusive, and different hypotheses still exist on how the doubling of the meristem might occur (Motte and Beeckman, 2019). One hypothesis suggests the disappearance of the IC as a result of segmentation followed by the recruitment of two new meristematic cells as two new ICs (Otreba and Gola, 2011). Barlow and Lück (2004), on the other hand, suggested that one of the daughter cells of the original IC becomes a new initial. Interestingly, in the Selaginella shoot meristem, a seemingly symmetric division of the apical cell is suggested to initiate dichotomy (Christopher and Andrew, 2009; Jones and Drinnan, 2009). Such a symmetric longitudinal division of the apical cell is also the cause of thallus bifurcation in certain algae (Oltmanns, 1889; von Goebel, 1928; van den Hoek et al., 1995; Gola, 2014).

Being the first lycophyte with a sequenced genome, *Selaginella moellendorffii* became an important model species for evolutionary research (Banks, 2009; Banks et al., 2011; Motte et al., 2020). Moreover, as root bifurcation seems to involve the initiation of a new root stem cell, dichotomous root branching could be used as a unique system to study root stem cell specification in an evolutionary context and to get insight into the complex process of dichotomy in general. Useful datasets and genetic tools start to become available, including several transcriptomic datasets from root samples (Ferrari et al., 2020; Motte et al., 2020; Fang et al., 2021). However, these studies are merely snapshots of the roots and do not allow to study temporal changes associated with the bifurcation process.

S. moellendorffii root branching cannot be induced by hormone treatments and a root branching inducible system, similar as for angiosperms, does not seem to be feasible (Fang et al., 2019), complicating the capturing of early bifurcating root

meristems. Here, as an alternative, we developed a dichotomous branching assay that enriches for samples with bifurcating roots. We applied this assay for histological sectioning and whole-mount confocal microscopy. Based on this, and complementary to the current literature, we advocate that two new root meristems originate from a division of the IC into two ICs. Furthermore, we exploited the assay to sample roots at regular intervals for RNA sequencing (RNA-seq) and revealed as such the *S. moellendorffii* root branching transcriptome. We show that transiently upregulated genes have a meristem signature, supporting the value of this dataset as a resource to identify candidate stem cell or meristem regulators in Selaginella. Moreover, a vast number of differentially expressed genes are homologous to Arabidopsis genes with a known role in stem cell or meristem functioning. This indicates, despite the structural difference in root meristems, a possible conservation in the pathways controlling meristem or stem cell functionality in Selaginella and Arabidopsis.

RESULTS

Developmental Time Course of *S. moellendorffii* Root Bifurcation

The anatomical or transcriptomic study of root bifurcation in *S. moellendorffii* could take advantage of an inducible root branching system like the LR inducible system in Arabidopsis (Himanen et al., 2002), because the initiation of new meristems can be synchronized and thus followed over time. A prerequisite is, however, to obtain a zero point at which no branching is ongoing. We previously reported that auxin transport inhibitors and a synthetic cytokinin were not able to prevent root branching in *S. moellendorffii* without affecting growth (Fang et al., 2019), and as such do not allow to obtain uniform starting material. Moreover, we did not succeed to identify a treatment that could induce meristem bifurcation itself (Fang et al., 2019). As a consequence, a synchronized root branching system that enables the study of root branching initiation does not seem to be feasible.

Therefore, we wondered whether we could predict root branching based on the repetitive apex divisions taking place in *in vitro*-grown *S. moellendorffii* explants as an alternative approach. To characterize root branching, *S. moellendorffii* shoot explants were excised and transferred to fresh medium, which resulted in the spontaneous development of roots. First, rhizophores were initiated, from which the root grew out (Fig. 1, A and B); subsequently, the roots repeatedly bifurcated dichotomously

6

168   (Fig. 1, C and D; Supplemental Movie S1). We followed the origin and bifurcation of

169   in total 983 roots within three independent experiments, and observed all roots on a

170   daily basis. Bifurcation events were macroscopically observed and recognized by the

171   presence of two dome-shaped primordia at the root tip (Fig. 1C). In practice, it was

172   difficult to exactly trace back the time of appearance for all rhizophores, as young

173   rhizophores were almost colorless and mostly covered by the leaves. Still, taking the

174   time point of the shoot explant transfer as the reference, we observed that a majority of

175   the roots underwent the first bifurcation between 10 and 15 d post transfer of the

176   explants (Fig. 1E). We continued to follow a subset of 361 root tips from two

177   experimental repeats, and observed, taking the first bifurcation (Fig. 1C) as a reference

178   point, that the majority of roots bifurcated again between 6 and 9 d after the first

179   bifurcation (Fig. 1F). To study the timing of bifurcation in more detail at the

180   histological level, we took the first bifurcation event as shown in Figure 1C as reference

181   time point 0, and sampled at least ten root tips on a daily basis for microscopic analysis.

182   All root tips were subjected to whole-mount confocal imaging to assess the appearance

183   of new meristems, as shown in Figure 1G. As previously shown, the apices at time point

184   0 never contained two apical meristems (Fang et al., 2019). Similarly, meristem

185   bifurcation was not detected on the first and second day after the first branching

186   (Fig. 1H). The first roots with two clear meristem regions were detected 3 d after the

187   first branching. The number of roots with two meristem regions steadily increased over

188   time and almost all root tips effectively bifurcated after 6 d (Fig. 1H).

189   Thus, in a relatively limited time frame after the first bifurcation event, practically

190   all root tips bifurcated again. Because the presence of two meristems was visible from

191   day 3 onwards, the onset of the bifurcation and related anatomical events could be

192   anticipated to occur early during the time course. Furthermore, because of the steadily

193   increasing percentage of bifurcated meristems, we could anticipate the enrichment of

194   specific developmental stages during consecutive time points. Therefore, we employed

195   this time course starting from newly branched roots to further characterize *S.*

196   *moellendorffii* root branching, both from the histological and transcriptomic point of

197   view.

Meristem Bifurcation Is the Result of a Division of the Irregularly Shaped IC

To characterize the early events in *S. moellendorffii* root branching, we sampled root tips on different days after the first bifurcation event (time point 0) and subjected them to histological sectioning. At time point 0, two root tips are present each with an IC (Fig. 2, A and B). The putative ICs were recognized by their triangular shape, by their central position within the root tip and above the root cap, by their bigger size compared to neighboring cells and/or by the surrounding of apparent merophytic cell clusters (Fig. 2B). The latter illustrates how daughter cells have been cut off from different faces of the IC, and subsequently divide (Fig. 2B). Starting from two days after time point 0, we were able to recognize two similar-sized ICs next to or close to each other in different root tips (Fig. 2, C-G). This challenges the current hypotheses on the initiation of dichotomous root branching in Selaginella, and might point to an IC division as a first stage during root bifurcation. In particular, Figure 2C shows a clear image of two separate basal derivatives from the presumptive ICs that both formed root cap cells. Figure 2D shows a similar image, but here, one of the two presumptive ICs had two nuclei and seemed to be finishing a subsequent cell division. Figure 2E and F show root apices with two presumptive ICs separated by only one cell, whereas Figure 2G shows a separation of the two ICs by more cells. Overall, this indicates that two new ICs originate after a symmetric division (a proliferative division giving rise to two daughter cells with the same cell fate) of the original IC, and continue to develop two new root primordia.

However, in many samples, we were not able to recognize such stages, and designation of the ICs was often not straightforward, possibly because ICs were regularly out of plane in the sections. Therefore, to confirm the observed and seemingly symmetric divisions of the IC, and to increase the workflow in order to analyze more samples at the same time, we decided to use the same approach while performing whole-mount confocal imaging. Supplemental Movie S2 shows a full stack of an unbranched root, and illustrates how this technique enables better designation of the IC by going through the sections. This stack was used to reconstruct the root (Fig. 3A) and clearly presents the organization of the meristem. To have a better view on the structure of the IC, we first used whole stacks for 3D cell segmentation and isolated the IC. Earlier reports with two-dimensional histological sections hypothesized that the root IC

231 is tetrahedral (Imaichi and Kato, 1989; Otreba and Gola, 2011). However, segmentation

232 of the IC from several samples showed that its shape is not uniform and not tetrahedral

233 (Fig. 3B; Supplemental Movie S3 to S7). Most side views, but not all, show triangular

234 faces, while most distal faces are irregularly shaped, and are only triangular-like in a

235 few cases. Hence, the IC in *S. moellendorffii* roots is in general not tetrahedral, and its

236 irregular shape makes it difficult to be recognized, especially in sections that follows

237 only one plane.

238   To confirm this irregular shape of the IC, we also subjected two root samples to

239 serial block face scanning electron microscopy (SBF-SEM), allowing a much higher

240 resolution of the cells and cell walls (Fig. 3, C-J; Supplemental Fig. S1; Supplemental

241 Movie S8 and S9). 3D reconstruction based on the serial sections showed that one IC

242 has five or six faces (face 4 and 5 might be considered as one, see Fig. 3D and

243 Supplemental Fig. S1E), instead of four in a tetrahedron. Furthermore, it has a wedge-

244 like shape with two opposite triangular faces (faces 1 and 3) and a rectangular bottom

245 (face 6) (Fig. 3, D-F; Supplemental Fig. S1, C-E, Supplemental Movie S8). The second

246 IC had three major proximal faces (faces 1-3), all with irregular triangular-like shapes,

247 and a pentagonal distal face (face 4) (Fig. 3, H-J; Supplemental Fig. S1, H-J;

248 Supplemental Movie S9). Hence, the ICs are irregular in shape, and not per se having

249 a triangular face in all longitudinal planes. This is an important observation to take into

250 consideration when looking for ICs in sections, and explains why it is often troublesome

251 to make a correct designation. By using whole stacks, it is possible to reorient the stack

252 in such a way that ICs are clearly recognizable (as illustrated by the 270 degree view in

253 Fig. 3A), as there almost always seems to be two faces with a triangular shape.

254   We next sampled root tips during the root branching time course in independent

255 experiments covering different time points. The roots were subjected to whole-mount

256 confocal imaging and stacks were made and reoriented to select planes with clear ICs

257 (Fig. 4). Again, in some roots, two ICs of apparent similar size were positioned next to

258 each other (Fig. 4, A-D; Supplemental Fig. S2, A-C). This further supports the

259 hypothesis for a symmetric division of the IC.

260   To assess the division spatially, we also performed 3D segmentation and shape

261 analysis of the presumptive ICs in Figure 4. Similar as the ICs in non-bifurcating roots,

262 the ICs were irregular in shape (Supplemental Fig. S3). The neighboring ICs in Figure

263 4, A-D were different in size and shape (Fig. 4, S, U, W, X), indicating that this IC

264 division was, at least spatially, not symmetric. We also observed that some neighboring

265 cells have relatively thin cell walls in common with the presumptive IC, indicative for
266 a recent cell division, and hence a rather fast succession of division events during
267 bifurcation, again hindering the capturing of early stages. Such a thin cell wall can for
268 example be seen in Fig. 4, C-D, where a very faint horizontal line can be observed in
269 the left IC. 3D segmentation showed that the ICs already underwent several divisions.
270 Interestingly, merging the different daughter cells resulted in two groups of cells of
271 similar size and shapes (Fig. 4, T, V, W, X, Supplemental Movie S10-S11). In Figure
272 4, A-B, the right IC probably underwent a recent cell division. Combination of the two
273 daughter cells resulted in a cell size and shape that is similar to the left IC (Fig. 4, T,
274 W, Supplemental Movie S10). The ICs in Figure 4, C-D, likely underwent three to four
275 cell divisions already, but are still positioned next to each other. Also in this case,
276 merging the presumptive daughter cells to one cell resulted in two cells with similar
277 size and shape (Fig. 4, V, X, Supplemental Movie S11). Although this analysis was not
278 able to prove a spatially symmetric cell division of the IC, it does support that, prior to
279 bifurcation, two new ICs originate next to each other, possibly as a result of a spatially
280 and functionally symmetric division of the original IC.

281   In the absence of genetically encoded markers, live imaging to confirm such
282 divisions or to show that this results in the formation of two meristems is unfortunately
283 not possible in *S. moellendorffii*. Still, in multiple samples at early time points, we
284 could, based on the cellular pattern, clearly observe that each of the ICs produced their
285 own distal daughter cells in the root cap (Fig. 4, A and B; Supplemental Fig. S2A),
286 indicating that each of these two cells also underwent at least one additional formative
287 division to cut off a root cap cell. Furthermore, we also observed multiple root tips in
288 which the two ICs were very close to each other and separated by only one or two layers
289 of short cells (Fig. 4, E-L; Supplemental Fig. S2, D-F). These layers seemed to originate
290 from daughter cells of the new ICs. Based on these observations, it is of high likelihood
291 that the two ICs originally neighbored each other, supporting the hypothesis that
292 dichotomous root branching in *S. moellendorffii* is a result of a symmetric division of
293 the original IC. In this scenario, the two new ICs cut off daughter cells in different
294 directions, forming two new meristems (Fig. 4, M-R). These two new meristems or root
295 primordia are initially still embedded in the macroscopically unbranched root, which
296 radially expands because of the doubled meristem (Fig. 4, M-Q), but later on grows out
297 to form two branches as described before (Otreba and Gola, 2011).

298

The *S. moellendorffii* Root Branching Transcriptome

Transient Gene Expression Profiles Support a Return to the Initial Meristem State

Based on the confocal imaging, almost 40% of the root tips formed two clearly distinguishable new meristems 5 d after the first branching (Fig. 1H). Hence, we supposed that programs to initiate dichotomous branching mainly occur before this time point. To identify possible molecular mechanisms with a role in root branching initiation in *S. moellendorffii*, we took advantage of the characterized root branching assay for an RNA-seq experiment and sampled root tips on each day from 0 to 5 d after the first branching (time point 0). More specifically, 300-µm apical parts were sampled to enrich for the meristematic region, while non-meristematic root regions were sampled separately (Fig. 5A).

Considering a fold change (FC) of at least 2 and an adjusted *p*-value (BH FDR corrected) of maximum 0.05, 6,601 differentially expressed genes (DEGs) were retrieved between the meristematic and non-meristematic part. 3,410 genes were more highly expressed in the apical parts and were therefore designated as "meristem-enriched genes". Within the branching time course, however, only 15 genes were differentially expressed with a $|FC| > 2$ (adjusted *p*-value ≤ 0.05; LRT). None of these DEGs had a straightforward annotation (Supplemental Table S1). This low number is not unexpected: the individual samples were most likely only different in a small region where bifurcation was initiated. Differences in gene expression may even only occur in one cell, and hence become diluted within the sampled tips. Still, 1,553 genes were significantly (adjusted *p*-value ≤ 0.05; LRT) affected over the branching time course (Fig. 5, B and C; Supplemental Table S1). In addition, many of these genes were differentially expressed at multiple time points (Fig. 5B; Supplemental Table S1), corroborating their relevance. Interestingly, the number of DEGs at first steadily increased during the time course and peaked on 2 and 3 d after the first branching, and then decreased towards the end of the time course (Fig. 5C). Hence, the final state showed less differential expression and was more similar to the original state.

To get a better insight into the expression profiles of the 1,553 DEGs affected over the branching time course, a cluster analysis via weighted gene co-expression network analysis (WGCNA) was performed (Langfelder and Horvath, 2008). The analysis

revealed 22 main expression profiles (Fig. 5D), which we further grouped according to their shapes. As such, the clusters could be classified into four major groups. We designated the transiently up- and downregulated gene clusters as "dome" (further grouped into early peak, late peak and broad peak subgroups) and "valley", respectively, and the constitutively up- and downregulated clusters were named "up" and "down", respectively. Only a few genes could not be classified into any of these groups (Fig. 5D). Overall, the majority of the 1,553 genes clustered in the dome (490) or the valley (560) groups, which fits the decreased number of DEGs observed towards the end of the time course.

In summary, both the number of DEGs per time point and the clustering of the expression profiles support a transient expression pattern, which might be associated with developmental states at the ontogenetic level: a starting state with a single meristem rapidly initiating bifurcation or evolving into an early bifurcation state and then evolving again into two separated single meristems resembling the initial state. In this respect, a part of the observed differential expression probably reflects a change in the abundance of certain cells or tissues, and not per se differential expression within certain cells.

Root Branching Transcriptome to Identify Core Meristem-Enriched Genes

The Selaginella root branches in association with the development of two root meristems. Therefore, especially at an early stage with two meristems, there should be relatively more meristem cells in the root tip, which should translate into a higher abundance of transcripts of meristem genes. As such, when the meristem bifurcates, transcripts of genes specifically expressed, or repressed, in the IC or in its neighborhood, would be relatively more, or less, abundantly present. Indeed, at an early stage, two young meristems are present within a non-expanded root tip. When the two meristems and the root tip enlarge (Fig. 4), this abundance would get diluted and evolve towards the end of the time course to a relative expression level as in a non-bifurcated meristem. Within the 1,553 DEGs affected over the branching time course, 452 of the 3,410 meristem-enriched genes were present (Supplemental Table S1), and hence, while this is still an overrepresentation (Fig. 6, A-C; Supplemental Table S2), a big portion of the meristem-enriched genes were not differentially expressed during meristem bifurcation. We presume that the group of meristem-enriched genes as such might still encompass many genes that do not associate with the meristem itself, but

364 with another part of the root tip.

365 We next compared the root meristem-enriched genes from our dataset with genes
366 expressed in the shoot apical cell area or within the shoot meristem core (excluding the
367 apical cell), as previously assessed in *S. moellendorffii* via laser capture microdissection
368 by Frank et al. (2015). Interestingly, a high overlap could be observed in particular
369 between the root meristem-enriched genes and the shoot apical cell genes (Table 2),
370 indicative for common mechanisms between the Selaginella root and shoot meristem.
371 The significance of the overlap became higher if only genes with the same directionality
372 in their expression pattern were considered (Table 1), corroborating their similar
373 regulation in the root and shoot meristem. Also the DEGs during the root branching
374 time course showed a high overlap with the shoot meristem genes, which could be
375 almost exclusively attributed to the genes that were upregulated during the time course
376 (Table 1, Supplemental Table S1). Only withholding root meristem-enriched genes that
377 were upregulated during root branching, slightly improved the significance (Table 1).

378 Genes upregulated in both the root meristem and the branching time course could
379 be expected to be associated with the root meristem function and might be of special
380 interest. The set of upregulated root branching meristem-enriched genes (Fig. 6A)
381 includes 173 genes, which are especially present in the early responsive dome clusters
382 (Supplemental Table S1). Likewise, root meristem-enriched genes are overrepresented
383 in such early responsive dome clusters (Fig. 6C). The early upregulation of these genes
384 indicates that two young meristems are formed early, which then mature towards the
385 end of the branching time course.

386 To get insights into the function of the meristem-enriched genes that were
387 differentially regulated during the root branching, GO, InterPro and MapMan term
388 enrichment analyses were performed (Supplemental Table S3 and S4). For the 173
389 genes that were upregulated during root branching, these analyses clearly showed an
390 enrichment in terms related to cell growth, and more particularly, in ribosome- and
391 translation-related terms (Fig. 6D; Supplemental Table S3). Correspondingly, these 173
392 genes included a broad range of ribosomal proteins-encoding genes (Supplemental
393 Table S1). Interestingly, such ribosomal-related genes or terms are typically enriched
394 in young meristematic angiosperm root cells as well. For example, Arabidopsis single-
395 cell transcriptomic studies have shown that ribosomal protein-encoding genes are
396 enriched in meristematic clusters (Ryu et al., 2019; Shulse et al., 2019; Zhang et al.,
397 2019). Hence, the 173 genes seemed to be associated with young meristematic cells,

13

398   underlining and validating the value of the root branching dataset to select core-

399   meristem genes.

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401

402   ***Candidate Regulators of Dichotomous Root Branching***

403   To identify possible regulators in the root branching process, we queried all 1,553

404   DEGs during the root branching time course for transcription factors and orthologs of

405   genes with well-described roles in Arabidopsis root meristem maintenance or LR

406   branching, including auxin and cytokinin signaling genes, and receptor kinases and cell

407   cycle genes (Supplemental Table S5). Transcription factors were collected via

408   PlantTFDB v5 (Tian et al., 2019). As we noticed that not all *AUXIN RESPONSE*

409   *FACTORS* (*ARFs*) and B-type *ARABIDOPSIS RESPONSE REGULATORS* (*ARRs*)

410   transcription factors were recognized in the PlantTFDB, we also looked for their

411   orthologs and included them in the query (Supplemental Table S5).

412   Among these possible regulators, 40 were differentially expressed during the root

413   branching time course (Fig. 7A) and might be involved in the regulation of

414   dichotomous root branching. These included multiple hormone-related genes, such as

415   auxin-responsive *ARF* genes, cytokinin-regulating A-type *ARR* genes, a *PIN-FORMED*

416   (*PIN*) auxin transporter, an *ARABIDOPSIS HISTIDINE KINASE (AHK)* cytokinin

417   receptor, a gibberellic acid signaling *DELLA* gene, ethylene signaling *ETHYLENE-*

418   *INSENSITIVE3-LIKE* (*EIL*) genes and a brassinosteroid signaling *BRI1-EMS-*

419   *SUPPRESSOR1* (*BES1*) gene.

420   We also noticed the presence of putative orthologs of both *TARGET OF*

421   *MONOPTEROS5* (*TMO5*) and *LONESOME HIGHWAY* (*LHW*). In Arabidopsis, the

422   two form a dimer and are together crucial for vasculature initiation (De Rybel et al.,

423   2013; De Rybel et al., 2014).

424   Interestingly, only a few transcription factor-encoding DEGs showed an early

425   upregulation. In particular, a *WUSCHEL RELATED HOMEOBOX* (*WOX*)

426   (SMO239G0078) and a *SCARECROW-LIKE9* (*SCL9*) (SMO358G0343) gene showed

427   a strong early induction (Fig. 7A) and were highly co-expressed (Fig. 7B). In contrast,

428   a *RETINOBLASTOMA* (*RB*) gene (SMO134G0004), in Arabidopsis a negative

429   interactor of *SCR* (Cruz-Ramírez et al., 2012), and an *E2F-DP* gene (SMO133G0001),

430 involved in the same pathway, showed a downregulated valley profile and were
431 inversely associated to the *WOX and SCL9* gene (Fig. 7B).

432

433

434 ***High Cell Division Activity in Young Vasculature***

435 We were intrigued by the overrepresentation of meristem-enriched genes in the

436 downregulated DEGs during the root branching time course (Fig. 6C), which could

437 point to a loss of meristem identity or meristem activity during the bifurcation process.

438 In the GO, InterPro and MapMan term enrichment analyses of this group, we noticed

439 multiple terms related to cell division, including "microtubule binding", "cytokinesis"

440 and "cell cycle" (Supplemental Table S4). Indeed, a lot of cell cycle-related genes could

441 be found in the meristem-enriched genes belonging to the valley or down clusters

442 (Supplemental Table S1). Possibly, cell divisions in the original meristem and the IC

443 stopped or at least decreased, while new meristems only took over towards the end of

444 the time course. To verify this, we performed 5-ethynyl-2′-deoxyuridine (EdU) staining

445 on *S. moellendorffii* root tips, marking all cells that divided during treatment with EdU.

446 In contrast to our presumptions, we did not observe a decrease in cell division activity

447 between different samples over the root branching time course (Fig. 8). The distribution

448 of cell divisions was similar in non-bifurcating roots (Fig. 8, A-C), roots with two ICs

449 neighboring each other (Fig. 8, D-F) or separated by one or two (Fig. 8, G-I) or more

450 cells (Fig. 8, J-L): in all samples, cell division activity was prominent in the region

451 around the IC and in a large region of the vasculature. Also the IC itself, considering

452 an EdU treatment of only 7 h, showed a relative high activity: of 168 analyzed ICs, 55

453 (33%) showed the EdU stain and underwent at least the S-phase during this short

454 treatment. This frequency was similar for the different stages, and could therefore not

455 explain the differential expression in cell division-related gene expression. This may

456 rather be explained by the high cell division activity in the young vasculature (Fig. 8,

457 Supplemental Movie S12-S13), because at 0 d of the branching time course, a relative

458 higher amount of vasculature tissue, and hence dividing cells, is present within the

459 sample compared to later samples. Indeed, in the 0-d samples, two vascular strands are

460 present within one tip (e.g. Fig. 2A). In later samples, despite a possible bifurcation

461 initiation, there is still only one vascular strand present (e.g. Fig. 4 A-P), while at the

462 end of the time course, samples may have again a spliced vasculature (e.g. Fig. 4Q).

463 Hence, the valley and down clusters seem to partially contain genes expressed in the

464 vasculature, and might be used to query vasculature-related genes. For example, the

465 putative *LHW* ortholog, in Arabidopsis known to be expressed in the vasculature (De

466 Rybel et al., 2013; De Rybel et al., 2014) showed downregulation during the branching

467 time course (Fig. 7A). This could also explain why downregulated genes during the

468 branching time course did not show a significant overlap with genes differentially

469 expressed in the shoot apical cell (Table 1).

470

471

472 **DISCUSSION**

473   Lycophyte roots branch dichotomously as a result of bifurcation of the apical

474 meristem. Allthough activities of the IC have been assumed to play a key role in this

475 process, especially in branching initiation, it remained unclear how its cellular activities

476 are associated with root bifurcation in the lycophyte *S. moellendorffii*. Previous reports

477 on Selaginella root bifurcation have suggested that the single root apical IC undergoes

478 segmentation and becomes indistinguishable, which is followed by the initiation of two

479 new ICs at the flanks and the formation of two new root meristems (Imaichi and Kato,

480 1989; Otreba and Gola, 2011). On the other hand, Barlow and Lück (2004)

481 hypothesized that, after a set of rigid cell divisions, a derivative of the IC becomes a

482 new IC to form the second root meristem. In contrast, our results point out that root

483 bifurcation starts with a symmetric division of the original IC, which gives rise to two

484 new ICs. Such a division to initiate dichotomous root branching in Selaginella has never

485 been reported. Additionally, it has been stated that a longitudinal symmetric division of

486 a tetrahedral cell is unlikely to occur, because the surface area of the inserted cell wall

487 is relatively big, which would be inefficient (Cooke and Paolillo Jr, 1980; Gola, 2014).

488 However, we showed that the IC is irregular, in general not tetrahedral and possibly

489 changing in shape over time. This might occur by the spatial arrangement with

490 neighboring cells or after cell divisions. The IC does indeed divide (Fujinami et al.,

491 2017), as we confirmed in this study, and therefore the shape could change after each

492 cell division. As such, ICs might acquire geometries that are better suited to undergo

493 symmetric divisions.

494   Still, there could be other factors controlling the IC to undergo cell division to

initiate dichotomous root branching. Because the stem cell is surrounded by multiple cells in the meristem, interactions with and feedback from the neighboring cells may also contribute to the first division initiating root branching in land plants. In Arabidopsis, the auxin flux from surrounding endodermal cells into the founder cells allows the latter to transition into LR initiation (Dubrovsky et al., 2008; Marhavý et al., 2013; Cavallari et al., 2021), also showing a critical role of auxin in the control of the first asymmetric division. However, we detected a differential downregulation of multiple auxin-responsive *ARF* genes in the branching time course, suggesting a reduction in the auxin response during the process. Supportive of this, we previously found that Selaginella root branching is not induced by auxins (Fang et al., 2019). On the other hand, this lower expression might also be caused by a different abundance of certain tissues or cells during the root branching time course. The abundance of vascular tissues, for example, is lower in the middle of the time course, which could explain the lower expression. Indeed, cell division-related genes showed a similar profile, while we observed that cell divisions were more frequent in young vascular tissue.

During Arabidopsis root growth, (lateral) root branching formation takes place periodically: protoxylem cells in the basal meristem undergo temporal oscillations in auxin concentrations and auxin-responsive gene expression levels, which allows these cells to transport these priming signals to the pericycle cells, which also get primed, forming competent sites to initiate LRs (De Smet et al., 2007; Moreno-Risueno et al., 2010; Van Norman et al., 2014; Xuan et al., 2016; Laskowski and ten Tusscher, 2017). In Selaginella, roots also branch repetitively but dichotomously (Fang et al., 2019). We found that this branching coincides with a transient gene expression profile, and recurrent branching events might therefore show a cyclic expression profile as well. Possibly, this involves priming signals, which may be included in our dataset. However, at least a part of the genes differentially expressed during the branching time course seem to be appear by consequence of the meristem duplication and therefore do not show up by the induction of expression in certain cells but rather by doubling the source cells in the meristem. This was illustrated by the high number of ribosomal protein-encoding genes that showed a higher transcript level during the time course, which, based on single-cell RNA-seq data, typically associates with meristem cells. As such, our root branching dataset can be a valuable resource for candidate genes expressed in the core meristem. Likewise, based on the profiles of genes with a role in cell division and the high abundance of cell divisions in the vasculature, candidate genes associated

17

529 with the vasculature might be retrieved from the downregulated or valley genes, as
530 more vasculature tissue is present at the start and the end of the branching time course.
531 For example, we detected putative *LHW* and *TMO5* orthologs, together crucial for
532 vasculature initiation (De Rybel et al., 2013; De Rybel et al., 2014). The Selaginella
533 genes phylogenetically cluster effectively with the *LHW* and *TMO5* clades (Lu et al.,
534 2020). Their control of vasculature initiation is shown to be highly conserved in all
535 vascular plants, as shown via complementation experiments (Lu et al., 2020). In
536 Arabidopsis, the two transcription factors have a different spatial expression pattern
537 and overlap at the vascular domain (De Rybel et al., 2013), with *TMO5* having a more
538 prominent expression at the vasculature initials, while *LHW* is more equally distributed
539 over the vasculature (Wendrich et al., 2020). This fits our presumption that valley or
540 downregulated genes like *LHW* are expressed in the vasculature, while upregulated
541 genes like *TMO5* have higher expressions close to the IC.

542   It has been hypothesized that lycophyte roots have a shoot origin (Gensel et al.,
543 2001). The high overlap in DEGs between the root meristem in our dataset and the
544 shoot apical meristem apical cell of the dataset of Frank et al. (2015) supports this
545 hypothesis. The common origin could moreover result in shared developmental
546 mechanisms between the shoot and root meristem. This was for instance shown in the
547 lycophyte Lycopodium, where bifurcation of both meristems starts with the emergence
548 of mitotically active cells in a similar quiescent region (Fujinami et al., 2021). In
549 contrast, Selaginella bifurcation of the shoot meristem seems to begin with a symmetric
550 division of the IC (Christopher and Andrew, 2009; Jones and Drinnan, 2009), although
551 it is not sure whether this division event effectively results in two meristems (Harrison
552 and Langdale, 2010). Still, we also observed in the root putative symmetric divisions
553 of the IC, further supporting the shoot origin of the root in lycophytes. It also underlines
554 the possible differences in root anatomy and bifurcation mechanisms within the
555 lycophyte clade, as not all lycophyte subclades have single ICs, and supports that roots
556 originated multiple times during the evolution of lycophytes (Gensel et al., 2001;
557 Fujinami et al., 2017; Fujinami et al., 2020).

558   Although roots originated multiple times during evolution, it is hypothesized,
559 based on comparative transcriptomics studies, that a common rootless ancestor of
560 vascular plants possessed an inheritable genetic tool kit that descendants recruited for
561 similar root meristem-related biological processes, resulting in a highly conserved root
562 developmental program within vascular plants (Huang and Schiefelbein, 2015; Ferrari

563 et al., 2020). In support of this hypothesis, we did find in our dataset multiple homologs
564 of genes with important roles in the Arabidopsis meristem.   For example, we detected
565 type-A *ARRs* and *ARF* genes. Type-A *ARRs*, negatively regulating cytokinin signaling,
566 are important for stem cell specification in the Arabidopsis root meristem (Müller and
567 Sheen, 2008), and several auxin-induced *ARF*s are important to control root stem cell
568 specification (Ding and Friml, 2010) or LR initiation, leading to a new root meristem
569 (Okushima et al., 2005; De Rybel et al., 2010).

570   We furthermore identified homologs of some known root stem cell controllers in
571 Arabidopsis, including *WOX*, *RB* and *E2F-DP* family members, as candidate regulators
572 of dichotomous root branching in Selaginella. These gene families encompass
573 important stem cell regulators. In Arabidopsis, *WOX5* and *RETINOBLASTOMA-*
574 *RELATED1* (*RBR1*) are important for stem cell functioning or fate (Sarkar et al., 2007;
575 Cruz-Ramírez et al., 2012). *RBR1* furthermore interacts with E2F transcription factors
576 (Wildwater et al., 2005). Noteworthy, *RBR1* and *E2FA* show a high degree of co-
577 expression (Magyar et al., 2012), which we also observed for their *S. moellendorffii*
578 homologs during the root branching time course. This and the presence of multiple
579 homologs of known stem cell regulators support as such the existence of a, possibly
580 partial, conserved genetic program in root stem cell or meristem establishment or
581 maintenance.

582   It is important to note, however, that the Selaginella *WOX* gene does
583 phylogenetically not directly cluster with *WOX5*: SMO239G0078 was previously
584 annotated as *SmWOXII*, and sisters the *WOX9/11* intermediate-clade-WOX (IC-WOX)
585 (Nardmann et al., 2009; Nardmann and Werr, 2012). *WOX5*, in contrast, is part of the
586 WUS subclade. As a matter of fact, WOX functioning in angiosperm stem cell control
587 and its action via transcriptional repression is restricted to the WUS subclade,
588 (Dolzblasz et al., 2016; Zhou et al., 2018), which is not present in *S. moellendorffii*
589 (Nardmann et al., 2009; Nardmann and Werr, 2012; Zhang et al., 2017; Wu et al., 2019).
590 Thus, the presence of the Selaginella *WOX* as a branching DEG may imply a different
591 mechanism, possibly as a transcriptional activator (Motte et al., 2020). An IC-WOX
592 gene has also been found to be specifically expressed in the root founder cells in the
593 fern *Ceratopteris richardii* (Nardmann and Werr, 2012), supporting a possible role of
594 this clade in root stem cell establishment as well.

595   Overall, we generated a relevant dataset, in which multiple candidate regulators of
596 root meristem bifurcation in *S. moellendorffii* could be identified that possibly show a

597 conservation with similar pathways in seed plants. In contrast to existing *S.*
598 *moellendorffii* datasets, which mainly focused on tissue-specific expression, we here
599 characterized expression patterns during a developmental process in which new (root)
600 stem cells are established. Although the molecular research using *S. moellendorffii* is
601 still at its infancy, this dataset can be a useful resource to identify possible root
602 meristem- and stem cell-related Selaginella or lycophyte genes. Because of the
603 pioneering evolutionary position of this lineage during colonization of land by plants,
604 this resource can be very useful to identify conserved and early (root) stem cell
605 regulators, and to unravel the early pathways that were at the base of pluripotency.

MATERIALS AND METHODS

Plant Materials

608 *Selaginella moellendorffii* plants were obtained from the lab of Jo Ann Banks,
609 Purdue University. Plants were grown aseptically under a 16-h light/8-h dark
610 photoperiod at 20.25–43.2 μmol/m^2/s in a 24°C growth chamber and roots developed
611 after the transfer of explants to fresh medium as previously reported (Fang et al., 2019).

Microscopy

613 To track root branching events, roots were observed daily starting from their
614 appearance on rhizophores using a Leica S9I stereomicroscope.
615 For whole-mount confocal imaging, *S. moellendorffii* root tips were first fixed in
616 50% (v/v) methanol and 10% (v/v) acetic acid, and then subjected to modified pseudo-
617 Schiff propidium iodide (mPS-PI) staining described by Truernit et al. (2008). Confocal
618 imaging was performed using a Zeiss LSM 5 Exciter microscope (25x water immersion
619 objective lens) with an argon ion laser at 488/505 nm as the excitation/emission
620 wavelengths, or a Leica SP2 confocal microscope [63x water corrected objective (NA
621 1.2) lens and pinhole = 1 AU] with an argon ion laser at 514/600-650 nm. Alternatively,
622 modified staining was performed with a small change in the composition of chloral
623 hydrate solution [8 g chloral hydrate, 3 mL glycerol, and 1 mL water (8:3:1)]. Z-stacks
624 were taken for all samples to allow collective detection of the meristematic region
625 present at different planes. A subset of samples, including the ones used for
626 segmentation, were, after fixation, subjected to amylase treatment in order to digest
627 starch granules. Root tips were washed three times with water and subsequently treated

overnight with α-amylase at a concentration of 300 U/mL in a buffer containing 20 mM phosphate pH 7.0, 2 mM NaCl, 0.25 mM Ca_2Cl. Samples were then washed with water and fixed again in 50% (v/v) methanol and 10% (v/v) acetic acid for 1 h. Samples were subsequently washed two times with 1X PBS, transferred to ClearSee solution (Kurihara et al., 2015) and gently stirred for 1 week. Finally, the samples were stained for 1 h in ClearSee solution containing 0.1% (w/v) Calcofluor White and were washed afterwards for 30 min in ClearSee solution before imaging. Confocal imaging of the Calcofluor White stained roots was conducted using 405 nm excitation and 425-475 nm wavelength detection using a Leica SP8X system. Confocal stacks generated for segmentation were imaged using a 63X water-corrected objective (NA of 1.2) and a pinhole of 0.6 AU. Stacks were further processed using MorphoGraphX (Barbier de Reuille et al., 2015) to identify ICs and to obtain clear sections showing the meristematic organization.

For histological sections, root tip samples were fixed in 1% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde, followed by dehydration and embedding in Technovit 7100 resin (Heraeus Kulzer) according to the manufacturer's instruction. Transparent strips were used to orient tissues for proper orientation (Beeckman and Viane, 2000). 2-µm sample sections were cut using a Leica Reichert-Jung 2040 Autocut Microtome, dried on object glasses using a hot plate (20–40°C), stained for cell walls with 0.05% (w/v) toluidine blue or ruthenium red (Acros Organics) for 10 min and then rinsed with water to wash off excess dyes. The sections were then mounted in Depex medium (Sigma) and covered with cover slips for observation and photography using an Olympus BX53 microscope.

For serial block face scanning electron microscopy (SBF-SEM), samples were fixed using 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M Sodium Cacodylate pH 7.4. Next, samples were processed en-bloc using a ROTOTO staining as described previously (Fendrych et al., 2014). Finally, resin-embedded root tips were mounted on aluminum specimen pins (Melotte) using conductive epoxy (Circuit Works), and coated with 5 nm of Pt in a Quorum sputter coater. Next, samples were placed in the Gatan 3View 2XP in a Zeiss Merlin SEM for imaging at 1.6 kV using the Gatan Digiscan II BSED detector. The system was set up to automatically take 1,000 images at a pixel-size of 15 nm, cutting 100-nm sections of the block-face between each image, resulting in a volume of 150 x 150 x 100 µm.

661   For registration of the 3D image stack, IMOD (http://bio3d.colorado.edu/imod/)

662 was used. Orthogonal views were obtained in Fiji (http://fiji.sc/Fiji). Segmentation was

663 done using Microscopy Image browser (http://mib.helsinki.fi/) and rendering was done

664 using Imaris (Bitplane).

Extraction of Initial Cell Geometry

666   Extraction of IC geometry was performed using MorphoGraphX (Barbier de

667 Reuille et al., 2015). Confocal images were prepared for segmentation using a Gaussian

668 blur of 0.5 in the X, Y and Z directions. Segmentation was performed using the ITK

669 watershed auto seeded algorithm with a threshold of 800 and segmentation errors were

670 manually corrected. 3D cell meshes were constructed for the region of the root

671 containing the IC, using the process marching cubes 3D with a cube size of 0.5, and 50

672 smooth passes at the time of mesh generation to minimize alterations to the mesh

673 structure (Bassel, 2015). All other cells in the mesh were then deleted to isolate the IC,

674 which was subjected to a further 5 smooth passes using the smooth mesh tool to

675 facilitate qualitative visualization. Shape analysis measures were computed in

676 MorphoGraphX using the tools 'Display Shape Axis 3D' (Elongation, Flatness,

677 Eccentricity) or 'Lobeyness Measures 3D' (Compactness, Convexity, Sphericity and

678 Solidity).

EdU Staining

680   To visualize cell division, explants with roots were transferred to medium

681 containing 7 µM EdU. After 7 h, roots were collected and fixed immediately in 4%

682 (w/v) paraformaldehyde for 2 h under vacuum. Subsequently, roots were transferred to

683 a 0.3% (v/v) Triton X-100 in 4% (w/v) paraformaldehyde solution for another 1.5 h

684 under vacuum conditions. The roots were treated overnight with α-amylase at a

685 concentration of 300 U/mL in a buffer containing 20 mM phosphate pH 7.0, 2mM

686 NaCl, 0.25 mM Ca_2Cl, subsequently fixated once more in 4% (w/v) paraformaldehyde

687 for 1 h under vacuum conditions and transferred to Clearsee. In between each treatment,

688 root tips were washed in 1X PBS. After a week of clearing in Clearsee, cell walls were

689 stained using a 0.1% (w/v) Calcofluor white Clearsee solution for 1 h. Following three

690 5-min washes in 1X PBS, the Click-iT EdU staining kit (C10337, Invitrogen, Carlsbad,

691 CA, USA) was used to fluorescently label the incorporated EdU according to the

692 manufacturer's protocol. Finally, the stained root tips were washed three times in 1X

693 PBS and transferred into Clearsee solution for further clearing and preservation prior

694 to confocal analysis.

695   Confocal imaging of EdU-stained roots was performed using a Zeiss LSM710

696 confocal microscope (40x water immersion objective lens, pinhole = 1AU) with an

697 argon ion laser at 488/493-628 nm as the excitation/emission wavelengths to visualize

698 the Alexa Fluor 488-labeled EdU and a diode laser at 405/410-483 nm to visualize the

699 Calcofluor white-stained cell walls.

700

701 **RNA-Seq**

702   Sampled roots in the time course (300 root tips per sample, four biological repeats

703 per time point) were cut off using a pair of stainless microscissors and then stored in

704 RNA*later*® RNA Stabilization Solution (Thermo Fisher Scientific) according to the

705 manufacturer's instruction. Subsequently, microdissection was performed to collect

706 root apices of 0.3 mm long, as well as non-meristematic regions between the apices and

707 branching point for the 5-d post first branching samples. The samples were then

708 collected in 2-mL RNase-free Eppendorf tubes with an RNase-free metal ball

709 [diameter: 5 mm, sprayed by RNaseZap™ RNase Decontamination Solution

710 (Invitrogen™, Thermo Fisher Scientific)] and frozen in liquid nitrogen. After

711 collection, samples were stored at -70°C.

712   For RNA extraction, samples were ground three times using a QIAGEN Retsch

713 Tissuelyser (30 Hz, 30 s). RNA extraction was done via TRIzol™ reagent (Thermo

714 Fisher Scientific) and the RNeasy Plant Mini Kit (Qiagen) according to the provided

715 protocols.

716   RNA concentration and purity were determined spectrophotometrically using a

717 Nanodrop ND-1000 (Nanodrop Technologies), and RNA integrity was assessed using

718 a Bioanalyzer 2100 (Agilent). Per sample, an amount of 250 ng of total RNA was used

719 as input. Using the Illumina TruSeq Stranded mRNA Sample Prep Kit (protocol

720 version: Part # 15031047 Rev. E - October 2013), poly-A containing mRNA molecules

721 were purified from the total RNA input using poly-T oligo-attached magnetic beads. In

722 a reverse transcription reaction using random primers, RNA was converted into first

723 strand cDNA and subsequently converted into double-stranded cDNA in a second

724 strand cDNA synthesis reaction using DNA Polymerase I and RNase H. The cDNA

725 fragments were extended with a single 'A' base to the 3' ends of the blunt-ended cDNA

23

726 fragments, after which multiple indexing adapters were ligated, introducing different
727 barcodes for each sample. Finally, enrichment PCR was carried out to enrich those
728 DNA fragments that have adapter molecules on both ends and to amplify the amount
729 of DNA in the library. Sequence-libraries of each sample were equimolarly pooled and
730 sequenced on Illumina HiSeq 4000 (SBS 300 cycles, Paired End Reads:151-8-8-151)
731 at the VIB Nucleomics Core (www.nucleomics.be).
732

733 **Expression Analysis**

734   We used two versions of *S. moellendorffii* annotated genomes: the NCBI version
735 (Annotation Release 100, released in 2018, containing 33,283 genes) and the JGI
736 version (v1.0, released in 2007, containing 22,285 genes). While the former is the most
737 recent and based on transcriptome data more correctly mapped, the JGI version is more
738 widely used in bioinformatic tools. Therefore, we performed the mapping/alignment
739 based (i) on NCBI if there was a 1:1 BLAST hit between NCBI and JGI annotations
740 (and including the corresponding JGI identifiers), and (ii) on JGI for genes that were
741 uniquely present in the JGI annotation. Counts were quantified using Salmon (Patro et
742 al., 2017) and normalization was done using the DESeq2 R package (Love et al., 2014).
743 To correct for the batch effect, Limma (Ritchie et al., 2015) was applied.

744   Differential analyses of the count data were performed using DESeq2 (Love et al.,
745 2014), only considering normalized read counts ≥ 10. PCA was used to identify and
746 omit outliers within the biological repeats, and differential gene expression was
747 analyzed using a multiple factor analysis. The expression during branching was
748 assessed by a likelihood-ratio test (LRT) (Supplemental Table S1). Differentially
749 expressed genes were selected based on an adjusted *p*-value [Benjamini & Hochberg
750 (BH) (Benjamini and Hochberg, 1995) false discovery rate (FDR) corrected] ≤ 0.05. In
751 addition, meristem DEGs were only retained in case of a $|FC| \geq 2$. Heatmaps showing
752 expression profiles were generated using the R package "pheatmap". A weighted gene
753 co-expression network analysis (WGCNA) was performed according to Langfelder and
754 Horvath (2008) in the pipeline of DESeq2.

755   Statistical significance of the overlap between two groups of genes was calculated
756 via the Fisher exact test. Representation factors were calculated by dividing the number
757 of overlapping genes by the number of expected genes in the overlap.

758   Data for GO, InterPro and Mapman term enrichment were computed using the

759 'Workbench' function of the Dicots PLAZA tool version 4.5:

760 https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_dicots/ (Vandepoele et

761 al., 2013; Van Bel et al., 2018), with default settings.

762   Homology or orthology between Arabidopsis and *S. moellendorffii* genes was

763 inferred based on the gene families in the Dicots PLAZA version 4.5.

764 **Accession Numbers**

765   RNA-seq data are available in the ArrayExpress database

766 (https://www.ebi.ac.uk/arrayexpress) under the accession number E-MTAB-11402.

767 **Supplemental Data**

768 The following supplemental materials are available.

769 **Supplemental Figure S1.** Additional SBF-SEM images and proximal views of the

770   reconstructed root initial cells (ICs) in *S. moellendorffii*.

771 **Supplemental Figure S2.** Additional confocal images taken during the root

772   branching assay in *S. moellendorffii.*

773 **Supplemental Figure S3.** 3D-segmented initial cells (ICs) from confocal stacks

774   in Fig. 4, A-Q.

775 **Supplemental Movie S1.** *S. moellendorffii* shoot explant with root formation and

776   repetitive bifurcation of the roots.

777 **Supplemental Movie S2.** Confocal stack of an unbranched *S. moellendorffii* root.

778 **Supplemental Movie S3.** First IC in Figure 3B.

779 **Supplemental Movie S4.** Second IC in Figure 3B.

780 **Supplemental Movie S5.** Third IC in Figure 3B.

781 **Supplemental Movie S6.** Fourth IC in Figure 3B.

782 **Supplemental Movie S7.** Fifth IC in Figure 3B.

783 **Supplemental Movie S8.** IC in Figure 3D-F.

784 **Supplemental Movie S9.** IC in Figure 3H-J.

785 **Supplemental Movie S10.** ICs and IC daughter cells in Figure 4A.

786 **Supplemental Movie S11.** ICs and IC daughter cells in Figure 4C.

787 **Supplemental Movie S12.** Confocal stack of the root in Figure 8K.

788 **Supplemental Movie S13.** Confocal stack of the root in Figure 8L.

789 **Supplemental Table S1.** Processed RNA-seq gene expression values.

790 **Supplemental Table S2.** Overlap of genes between different subsets or clusters

791   and the meristem-enriched genes.

792   **Supplemental Table S3.** GO, Mapman and Interpro terms associated with the

793   meristem-enriched genes that were downregulated in the root branching course.

794   **Supplemental Table S4.** GO, Mapman and Interpro terms associated with the

795   meristem-enriched genes that were upregulated in the root branching course.

796   **Supplemental Table S5.** Selaginella orthologs or homologs from lateral root- or

797   root stem cell-related gene families as retrieved from PLAZA version 4.5

798   (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_dicots/).

813

Tables

815 **Table 1.** Overlap between DEGs from our root meristem and root branching time
816 course and the shoot apical meristem apical cell and core (meristem without apical
817 cell) datasets from Frank et al., 2015, as indicated by the *p*-value of the Fisher exact
818 test. Bold values indicate $p<0.05$.

	Root meristem	Root meristem-up (meristem-enriched genes)	Root meristem-down	Root branching time course	Root branching time course-up	Root branching time course-down	Root meristem-enriched genes upregulated during root branching
SAM_AC	**2.84E-52**	**2.31E-10**	**5.59E-37**	**1.08E-11**	**4.74E-24**	0.785978169	**1.56E-33**

SAM_AC_UP	**2.73E-31**	**2.09E-66**	1	**8.40E-38**	**2.55E-54**	0.168042067	**2.94E-61**
SAM_AC_DOWN	**9.04E-26**	0.999999983	**1.50E-64**	0.972646833	0.853754181	0.950343777	0.899158476
SAM_core	**1.66E-11**	0.1941765	**4.14E-13**	**3.72E-05**	**1.57E-07**	0.397555177	0.036146755
SAM_CORE_UP	**1.28E-05**	**7.32E-10**	0.965385017	**0.004971335**	**1.09E-05**	0.939146006	**0.001642662**
SAM_CORE_DOWN	**1.69E-07**	0.999955348	**3.18E-19**	**0.00156248**	**0.000857833**	0.17612269	0.733421609

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820

Figure Legends

822

Figure 1. Dichotomous root branching in *S. moellendorffii*. A, A young hairless rhizophore. B, Root emergence from the rhizophore. The transition from the rhizophore to the root is marked by the appearance of a collar of root hairs. Scale bars: 0.1 mm. C and D, Formation of first- (C) and second-order (D) bifurcated roots. Scale bars: 1 mm The insets are magnifications of the squares (Scale bars: 0.1 mm). The arrows indicate newly formed root apices. E, Percentage of root tips that underwent the first bifurcation during 17 d after transfer on 1/2 MS. The bifurcation percentage was calculated as the number of first-order bifurcated roots divided by the total number of roots. Values are averages of three repeats ± SD. F, Percentage of root tips that underwent the second bifurcation during 15 d after the first bifurcation. The bifurcation percentage was calculated as the number of second-order bifurcated root apices divided by the total number of the apices from roots that underwent a first bifurcation. Values are averages of two repeats ± SD. G, Confocal image showing a bifurcated apical meristem: root tip with two new clearly recognizable young meristems (arrowheads). Scale bar: 50 μm. H, Percentage of bifurcated meristems in the root branching time course, i.e. days after the first branching. The percentage was calculated as the number of root apices showing clear apical meristem bifurcation (see Fig. 1G) divided by the total number of sampled root tips. n (daily sample number) ≥ 10.

Figure 2. Histological sections from *S. moellendorffii* roots sampled during the root branching time course. A and B, A newly branched root tip containing two apical meristems sampled on day 0; (B) is a magnification of the indicated area in (A). The inset in (B) shows a magnification of the initial cell (IC) and its neighboring region. The initial and the presumptive most recent daughter cells are indicated by dotted lines.

846   The hollow arrows indicate the expected directions in which the IC cuts off daughter

847   cells from the different faces. Scale bars: 50 μm, except for the inset: 10 μm. C-G,

848   Early-stage bifurcating meristems with two presumptive ICs next or close to each other.

849   The insets show a magnification of the indicated area, the arrows indicate the ICs. Scale

850   bars: 50 μm, except for panel (C), (D) and the insets: 10 μm.

851

852   **Figure 3.** 3D imaging to segment ICs. A, Example of a *S. moellendorffii* root generated

853   from a whole-mount confocal stack (Supplemental Movie S2). Individual cells are

854   randomly colored. Insets show magnifications of the front view or the 270 degree-

855   clockwise turned view. Scale bar: 20 μm. B, Different views of five ICs segmented

856   from confocal stacks. The fifth, blue IC was segmented from the root in panel A. See

857   Supplemental Movies S3-S7 for a full overview of the ICs. Scale bars: 2 μm. C and G,

858   SBF-SEM longitudinal sections (xz-views) showing two ICs. The inset shows a

859   magnification of the indicated area in (C). The asterisks indicate the ICs. Scale bars: 10

860   μm. D-F and H-J, Serial section reconstruction of the ICs. The numbers indicate the

861   corresponding faces of the cells. See Supplemental Movies S8 and S9 for a full

862   overview. The solid and dashed lines in (H-J) separate the different faces. Scale bars: 1

863   μm.

864

865   **Figure 4.** Early stages during *S. moellendorffii* root bifurcation. A-Q, Confocal images

866   of different stages during branching sampled between 1 and 5 d in the root branching

867   time course. B, D, F, H, J, L, N and P show a magnification (scale bars: 20 μm) of the

868   root in A, C, E, G, I, K, M and O (scale bars: 50 μm), respectively. Asterisks indicate

869   the location of the presumptive ICs. A-D, Two ICs are next to each other. See

870   Supplemental Figure S2, A-C for more examples. E-L, Two ICs are only separated by

871   one or two layers of cells. See Supplemental Figure S2, D-F for more examples. M-Q,

872   Further development of the two meristems, including enlargement of the root tip. The

873   root in panel O is the same as in Figure 1G. R, Scheme showing the mechanism of root

874   bifurcation as deduced from independent root samples during the early time points of

875   root branching. Dashed lines indicate possible cell divisions. S-V, 3D-segmented ICs

876   from panel A (S, T) or panel C (U, V). T and V include the presumptive daughter cells

877   of the new ICs. Scale bars: 5 μm. See supplemental Movies S10 and S11 for a full

878   overview. W-X, Cell shape analysis of ICs and IC daughter cells from roots in A or

879   Supplemental Movie S10 and C or Supplemental Movie S11, respectively.

880

Figure 5. *S. moellendorffii* root branching transcriptome. A, Experimental set-up, showing sampled regions (dotted black lines: meristematic region; white: non-meristematic region (NM)). Scale bars: 1 mm. B, Heatmap showing expression [$\log_2$ fold change (FC)] of all root branching differentially expressed genes (DEGs) on the different days in the root branching course versus day 0, or for the meristematic (M) versus non-meristematic (NM) region. Gray values indicate no significance (likelihood-ratio test with adjusted *p*-value (Benjamini & Hochberg false discovery rate corrected) $\geq$ 0.05). C, Overview of numbers of up- and downregulated DEGs at each time point during root branching. Note the decrease in number at the end of the time course, indicating a return to the initial state. D, Profiles of co-expression clusters based on weighted gene co-expression network analysis (WGCNA) of the root branching DEGs. Clusters were grouped according to their shapes: dome and valley groups – the transiently up- and downregulated clusters, respectively; up and down groups – constitutively up- and downregulated clusters, respectively. Based on the peak position and shape, the dome group was further grouped into early peak, late peak and broad peak subgroups. In each cluster, the mangenta curve represents the average expression of all genes in the cluster, the green one represents the eigengene of the cluster.

898

Figure 6. Overview of root branching meristem-enriched genes. A and B, The heatmaps show the expression [$\log_2$ fold change (FC)] of meristem-enriched genes upregulated (A) or downregulated (B) during root branching. C, Representation of the meristem-enriched genes in different expression clusters. The meristem-enriched genes were in particular overrepresented in transiently upregulated (dome) clusters in mangenta frames and constitutively downregulated (down) clusters in blue frames. Orange and green frames indicate constitutively upregulated (up) and transiently downregulated (valley) clusters, respectively. DRBG, Downregulated root branching DEGs; URBG, Upregulated root branching DEGs. D, Enrichment analysis of the meristem-enriched genes that were upregulated during root branching. The graph shows enriched biological process and molecular function gene ontology (GO) terms. Node size is scaled by the *p*-value of the enriched GO term, and node color (from green to red) is determined by the enrichment fold of the term. Negative enrichment folds are faded. The outer green band is determined by the subset ratio. The graph was generated

913 by PLAZA version 4.5

914 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_5_dicots/).

915

916 **Figure 7.** Expression profiles of the transcription factor-encoding DEGs and other

917 genes with their Arabidopsis homologs known to be involved in root meristem control

918 or lateral root (LR) formation. A, Heatmap showing the expression [$\log_2$ fold change

919 (FC)] profiles. The description shows the queried gene family (Supplemental Table S5)

920 or the transcription factor type according to the Plant Transcription Factor Database

921 (PlantTFDB) v5. For the GRAS (including DELLA and SCL) and bHLH transcription

922 factors, subfamilies according to the analyses of Wang et al. (2016) or Pires and Dolan

923 (2009), respectively, are shown. Members of the bHLH Vb/TMO5 and bHLH

924 XIII/LHW subfamilies were verified in the analysis of Lu et al. (2020). B, Relative

925 expression of the *RB* (SMO134G0004), *E2F-DP* (SMO133G0001), *SCL9*

926 (SMO358G0343) and *WOX* (SMO239G0078) gene. Error bars indicate SE (n = 4).

927

928 **Figure 8.** EdU labeling during *S. moellendorffii* root bifurcation. Confocal images of

929 root meristems treated for 7 h with 7.5 µM 5-ethynyl-2′-deoxyuridine (EdU) at different

930 stages during branching are shown. Nuclei of cells that underwent S-phase during these

931 7 h, incorporated EdU (magenta). Cell walls were stained with Calcofluor-white

932 (green). Scale bars: 50 µm. White asterisks indicate the location of the ICs. A-C, A

933 single IC in each root tip. D-F, Two adjacent ICs formed by a symmetrical division. G-

934 I, Two ICs are only separated by one or two layers of cells. J-L, Further development

935 of the two meristems, including enlargement of the root tip. Panels K and L are

936 composed of two different z-stack planes (see Supplemental Movies S12 and S13 for

937 the full stacks), each containing an apical cell.

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