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Smetana, Ondrej

2019-01-24

Smetana , O , Mäkilä , R , Lyu , M , Amiryousefi , A , Rodriguez , F S , Wu , M-F , Sole-Gil , A , Gavarron , M L , Siligato , R , Miyashima , S , Roszak , P , Blomster , T , Reed , J W , Broholm , S & Mähönen , A P 2019 , ' High levels of auxin signalling define the stem-cell organizer of the vascular cambium ' , Nature , vol. 565 , no. 7740 , pp. 485-+ . <https://doi.org/10.1038/s41586-018-0837-0>

<http://hdl.handle.net/10138/325525>

<https://doi.org/10.1038/s41586-018-0837-0>

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High auxin signalling defines stem cell organiser of the vascular cambium

Ondřej Smetana^{1,2}, Riikka Mäkilä^{1,2}, Munan Lyu^{1,2}, Ali Amiryousefi², Filomeno Sánchez Rodríguez^{1,2}, Miin-Feng Wu³, Anna Solé Gil^{1,2}, Marina Leal Gavarrón^{1,2}, Riccardo Siligato^{1,2}, Shunsuke Miyashima^{1,2}, Pawel Roszak^{1,2}, Tiina Blomster^{1,2}, Jason W. Reed³, Suvi Broholm^{1,2} & Ari Pekka Mähönen^{1,2,*}

1. Institute of Biotechnology, HiLIFE, University of Helsinki, Helsinki 00014, Finland

2. Organismal and Evolutionary Biology Research Programme, Faculty of Biological and Environmental Sciences, and Viikki Plant Science Centre, University of Helsinki, Helsinki 00014, Finland

3. Department of Biology, University of North Carolina at Chapel Hill, U.S.A

* Author for correspondence: AriPekka.Mahonen@helsinki.fi

Wood originates from cell proliferation of the vascular cambium. Xylem (i.e. wood) is produced inside and phloem outside of the cambium¹. Morphogenesis in plants is typically coordinated by organiser cells which direct the adjacent stem cells to undergo programmed cell division and differentiation. It is unknown where the vascular cambium stem cells are located and whether the organiser concept applies to the cambium². Here, we combine lineage tracing and molecular genetic studies in *Arabidopsis thaliana* roots to show that cells with xylem identity direct adjacent vascular cambial cells to divide and function as stem cells. Thus, these xylem identity cells constitute an organiser. A local maximum of the phytohormone auxin and consequent expression of *class III homeodomain-leucine zipper (HD-ZIP III)* transcription factors promote xylem identity and cellular quiescence of the organiser cells. Additionally, the organiser maintains phloem identity cell non-autonomously. Consistent with this dual function of the organiser cells, xylem and phloem originate from a single, bifacial stem cell in each radial cell file, thus confirming the classical theory of a uniseriate vascular cambium³. Ectopically activated high auxin signalling clones differentiate as xylem vessels, and induce cell divisions and expression of cambial and phloem markers in the adjacent cells, suggesting that local auxin signalling maximum is sufficient to specify a stem cell organiser. Although vascular cambium has a unique function among plant meristems, its stem cell organiser shares features with the root and shoot meristem organisers.

Plant organs grow axially during primary development, and then radially during secondary development.

In many plant organs, including woody stems, secondary development is organised by two concentric, cylindrical meristems undergoing periclinal cell divisions (i.e., the division plane is parallel to the surface of an organ or a tissue): the inner vascular cambium proliferates to produce conductive xylem and phloem, and the outer phellogen (also called cork cambium) produces protective tissue called phellem⁴. In the *Arabidopsis thaliana* root, secondary meristems originate from parenchymal cells located between primary xylem and phloem, known as procambium, and from cells surrounding the procambium, xylem and phloem, known as the pericycle^{5, 6} (Fig. 1a, Extended Data Fig. 1a, b).

In order to understand at cellular resolution how the vascular cambium and phellogen develop from the procambium and pericycle, we designed a novel CRE//lox recombination system to carry out cell lineage tracing in the *Arabidopsis* root (Extended Data Fig. 2a, b). Clones marked with β -Glucuronidase (GUS) expression were induced during the activation of root secondary growth (Fig. 1a, b), and the subsequent sectors were analysed after 11 days. GUS sectors showed highly repetitive patterns indicating that the secondary development is a tightly controlled process (Fig. 1b, Extended Data Fig. 2c). This enabled us to develop a fate map of procambial and pericycle cells during the morphogenesis of root secondary tissue (Fig. 1b). The fate map showed that phellogen originates from the pericycle (dark and light blue, in Fig. 1b), confirming previous anatomical observations⁴⁻⁶. Pericycle cells adjacent to the primary xylem (xylem pole pericycle (XPP) cells, dark blue in Fig. 1b) are also founder cells of the vascular cambium. XPP cell proliferation thus leads to separation of the two concentric meristems and production of all the secondary tissues from their circumferential position (Extended Data Fig. 2d). Our finding underscores the pluripotency of XPP cells, which are also the source of lateral roots and regenerated tissue (callus)⁷. The fate map also revealed that only those procambial cells that are in physical contact with primary xylem vessels (red, in Fig. 1b) give rise to vascular cambium, and secondary xylem and phloem. Thus, these xylem-adjacent procambial cells together with xylem-adjacent daughters of XPP cells act as the stem cells of vascular cambium during the activation of secondary growth. The fact that procambial and pericycle cells that are not in contact with xylem (pink and light blue, respectively in Fig. 1b) do not form vascular cambium suggests that cellular position (adjacent to xylem) rather than identity during primary development determines the origin of the vascular cambium. This conclusion is further supported by lineage tracing in roots transiently treated with the phytohormone cytokinin, which gives cells in protoxylem position procambium identity. Located adjacent to the remaining xylem, these cells formed only vascular cambium, while cells in the XPP position, no longer adjacent to xylem cells, formed only phellogen and not vascular cambium (Extended Data Fig. 2d-g). Together, these findings suggest that xylem or xylem precursor cells cause neighbouring cells to become vascular cambium stem cells.

At the onset of secondary growth (Fig. 1a), primary xylem cells have already undergone programmed cell death as the final step of their differentiation. We therefore hypothesize that xylem induces stem cell identity in the neighbouring cells earlier, during primary development, when cells with xylem identity are still alive and capable of signal transduction. A family of five redundantly acting HD-ZIP III transcription factors determine xylem identity during the primary development of the *Arabidopsis* root⁸⁻¹⁰. Additionally, an HD-ZIP III orthologue in *Populus* regulates vascular cambium morphogenesis¹¹. HD-ZIP IIIs are thus likely candidates for regulating signals for stem cell identity. HD-ZIP IIIs are expressed in the cambial zone, and at least *AtHB8* is expressed predominantly on the xylem side of the vascular cambium, adjacent to the cells undergoing cell division (highlighted with *AINTEGUMENTA* (*ANT*) expression), thus consistent with the idea that they mark cells with secondary xylem identity (Fig. 2a, Extended Data Fig. 3a, b). Only mutants lacking at least four of the genes show patterning defects and reduced xylem formation in the primary root vasculature⁸. The same mutant combinations also show scattered cambial divisions and xylem patterning during secondary development (Extended Data Fig. 3c, d). To examine whether these severe developmental defects are a direct consequence of the loss of HD-ZIP IIIs or a secondary consequence of defects in primary development, we constructed an inducible¹² *miR165a* line that targets the mRNAs of all five HD-ZIP IIIs¹³ (Extended Data Fig. 4a). Ubiquitous induction of *miR165a* from germination onwards severely inhibited primary xylem differentiation (Extended Data Fig. 4b), similar to the quintuple HD-ZIP III mutant⁸. Induction of *miR165a* during the activation of secondary growth initially blocked vessel differentiation, indicating that the HD-ZIP IIIs are also required for secondary xylem vessel formation (Fig. 2b). Dispersed cell divisions and erratic xylem vessel formation occurred in the remaining undifferentiated tissue, resembling the phenotypes of quadruple HD-ZIP III mutants (Extended Data Fig. 3d). Induction of *miR165a* during secondary growth forced xylem parenchyma cells and cells with xylem identity, which were previously quiescent, to re-enter mitosis, generating a population of dividing cells in the place of xylem (Fig. 2c, Extended Data Fig. 4c). These results indicate that the HD-ZIP IIIs are required for cellular quiescence in xylem cells. *WUSCHEL-RELATED HOMEODOMAIN 4* (*WOX4*)¹⁴ and *PHLOEM INTERCALATED WITH XYLEM/TDIF RECEPTOR* (*PXY/TDR*)^{14, 15} are key cambial regulators. They show expression maxima on the xylem-side of the cambium and weak expression in the adjacent dividing cells (marked with *ANT* expression), thus strongly resembling the *AtHB8* expression pattern (Fig. 2a). Induction of *miR165a* led to downregulation of *WOX4* and *PXY* (Fig. 2d, Extended Data Fig. 4d). These data imply that xylem identity regulators, the HD-ZIP IIIs, define a stem cell organiser which cell non-autonomously confers cambium stem cell identity to the adjacent cells.

Unexpectedly, loss of the HD-ZIP IIIs also caused decreased production of differentiated phloem cells (Extended Data Fig. 4e) and lower expression of early phloem marker *PHLOEM-EARLY-DOF 1* (*PEAR1*)^{16, 17} (Fig. 2a, d, Extended Data Figs 1a, 4d), suggesting that the organiser cells maintain a common stem cell for secondary

93 xylem and phloem. To test this hypothesis, we traced cell lineages during secondary growth. GUS clones were
94 generated in 15-day or 30-day old *Arabidopsis* roots, which contain 2-4 (15-day) or 7-9 (30-day) layers of vascular
95 cambial cells that could be identified histologically (Extended Data Fig. 1b). In each radial cell file, three different
96 types of GUS sectors were observed (Fig. 1c, Extended Data Fig. 5 a-c). GUS clones that originated from the xylem
97 (green) or phloem (yellow) side of the cambium differentiated into the corresponding cell types or divided before
98 differentiation. In contrast, cells located in the centre of the cambium (red) produced broad GUS sectors
99 spanning into the differentiated xylem and phloem (Fig. 1 c, d, Extended Data Fig. 5 c, d). Similarly, induction of
100 clones in the *ANT* expression domain, marking a narrow layer of dividing cells, led to production of GUS sectors
101 spanning to both directions (Fig. 2a, Extended Data Fig. 5e-h). These data demonstrate that the vascular cambium
102 consists of a single, bifacial stem cell in each radial cell file, marked with *ANT* expression. These stem cells produce
103 both xylem and phloem, thus confirming the 145-year-old theory of uniseriate vascular cambium^{3, 4}, at least in
104 the *Arabidopsis* root. This theory states that a single layer of initials (stem cells) produces both xylem and phloem;
105 and was later elaborated by the observation that the position of the 'initials' varied between neighbouring radial
106 files^{1, 4}. Our experimental data support these later modifications; the variable location of the stem cells can be
107 observed as a considerable radial distribution of the GUS sectors originated from stem cells (Fig. 1d, Extended
108 Data Fig. 5d).

109 To test whether the HD-ZIP IIIs are sufficient to induce the formation of an ectopic organiser within the
110 cambium, we inducibly overexpressed an *miR165/166*-resistant version of *AtHB8* (*AtHBd*) in the stem cell domain
111 under the *ANT* promoter¹². Induction of *pANT:XVE>>AtHB8d-YFP* rapidly inhibited cell division in the stem cells,
112 thus confirming the ability of the HD-ZIP IIIs to promote cellular quiescence (Extended Data Fig. 6a-c). This local
113 quiescence was associated with a centrifugal shift (i.e. towards phloem) in the domain of high cell division and
114 organiser marker expression (Extended Data Fig. 6b, d), followed by the formation of ectopic xylem vessels in
115 the cambium (Extended Data Fig. 6e). These data demonstrate that at least *AtHB8* is sufficient to induce organiser
116 function within the vascular cambium.

117 Auxin promotes expression of HD-ZIP IIIs in vascular tissues during primary development, and their
118 expression often correlates with auxin signalling maxima^{18, 19}. Since the auxin maximum peaks on the xylem-side
119 of tree vascular cambia^{20, 21}, we next studied the auxin signalling response pattern in the *Arabidopsis* root
120 cambium. Auxin response markers showed a local maximum in the xylem domain, tapering off towards the
121 cambium stem cells, thus resembling the expression pattern of *AtHB8*, *WOX4* and *PXY* (Figs 2a, 3a; Extended
122 Data Fig. 7a, b). Auxin signalling is mediated by AUXIN RESPONSE FACTOR (ARF) transcription factors²². We
123 focused our expression analysis on class A ARFs, since their activity is preferentially auxin regulated²², and the

124 class A ARF *ARF5/MONOPTEROS (MP)* has been shown to regulate proper vascular formation²³ and cambium cell
125 division by controlling the expression of *WOX4*²⁴. Of the five class A ARFs, three (*ARF5/MP*, *ARF7* and *ARF19*)
126 showed strong and consistent expression in the vascular cambium (Extended Data Fig. 7c, d). Since the *arf7,19*
127 double mutant did not show clear patterning defects (Extended Data Fig. 8a), and strong *mp* mutants failed to
128 initiate roots²⁵, we developed an inducible artificial *microRNA* line targeting *MP* (*amiMP*) in the *arf7,19*
129 background. Ubiquitous induction of *amiMP* led to a reduction in *MP* transcript levels, downregulation of *WOX4*
130 and a subset of HD-ZIP IIIs, and a reduction in secondary vessel formation (Fig. 3b, c; Extended Data Fig. 8b, c),
131 similar to the *miR165a* overexpression phenotype (Fig. 2b). Similarly, ubiquitous induction of an inducible, stable
132 ARF-signalling repressor *axr3-1*²⁶ led to a strong downregulation of the organiser, stem cell and phloem identity
133 genes, as well as the *HD-Zip III* genes, followed by termination of vascular cambium activity (Fig. 3d; Extended
134 Data Fig. 8d-f). These data show that auxin signalling through *MP*, *ARF7* and *ARF19* and perhaps other ARFs
135 promotes the expression of *HD-ZIP III*s, *PXY* and *WOX4* in the stem cell organiser and that this signalling module
136 is required to maintain vascular cambium identity. Since manipulations of HD-ZIP III levels lead to changes in
137 auxin response (Extended data Fig. 7e-g), our data further suggest that auxin, ARFs and HD-ZIP IIIs are under a
138 positive feedback regulation in the vascular cambium.

139 Finally, we tested whether the components determining the stem cell organiser are sufficient to induce
140 vascular cambium formation when ectopically expressed. We generated activation clones in the phloem pole
141 pericycle and surrounding cell files using an inducible S17 promoter^{12, 16} (Extended Data Fig. 9a, b). Even though
142 *AtHB8d* was sufficient to re-specify the organiser when overexpressed in stem cells of the vascular cambium, it
143 was unable to induce xylem vessel formation or expression of cambial markers in the S17 domain (Extended Data
144 Figs 6 and 9c, d). However, a proportion of clones expressing *MPΔ*, a dominant active version of *MP*²⁷, formed
145 ectopic vessel elements and had periclinal cell divisions in the adjacent cells (Fig. 4a, Extended Data Fig. 9e, f).
146 Time-course experiments revealed that *MPΔ* cell-autonomously induced *WOX4*, *PXY* and *AtHB8* expression,
147 followed by differentiation of the clones into vessel elements (Fig. 4a-c). During differentiation into vessels, *MPΔ*
148 clones induced ectopic expression of *WOX4*, *PXY* and *AtHB8* in the adjacent cells, indicating that *MP* can promote
149 organiser cell identity also in a cell non-autonomous manner (Fig. 4b). Additionally, early phloem marker *PEAR1*
150 appeared ectopically at a distance of a few cells from the clone, suggesting that the presence of an *MPΔ* clone is
151 sufficient to promote the formation of a vascular cambium (Fig. 4c). *MPΔ* induces expression of the genes that
152 define the stem cell organiser before xylem vessel formation, suggesting that the cellular identity induced by
153 high auxin signalling rather than the presence of xylem vessels defines the organiser. Supporting this, clonal
154 activation of *VASCULAR-RELATED NAC-DOMAIN 6 (VND6)*, which directly induces terminal differentiation of
155 xylem vessels²⁸, failed to promote organiser gene expression within the clone (Extended Data Figs 1a and 9g). In

156 the longer term, a proportion of *VND6*-induced vessels were surrounded by cells expressing a subset of cambial
157 markers; however, these cells rarely divided (Extended Data Fig. 9e, f). This indicates that the chemical or physical
158 properties of terminally differentiated xylem vessels are not sufficient to induce organiser formation. Together,
159 these data demonstrate that local high auxin signalling within root vascular tissue is sufficient to establish an
160 organiser cell that promotes stem cell-like divisions and identity in the adjacent cells (Fig. 4d). Our data revealed
161 a dynamic nature of the organiser; differentiation of the organiser into a xylem vessel leads to formation of a
162 new organiser in the adjacent cambial stem cell, thus ensuring the maintenance of the vascular cambium
163 (Extended Data Fig. 10).

164 We uncovered a mechanistic framework defining the stem cell organiser of the vascular cambium (Fig.
165 4d). Similarly to the cells in the stem cell organiser (quiescent centre) of the root apical meristem²⁹, the cells in
166 the cambium organiser are quiescent and are defined by an auxin response maximum. However, unlike the root
167 quiescent centre, the stem cell organiser of the vascular cambium is dynamic, since as the root grows thicker,
168 organiser cells differentiate and stem cells acquire organiser identity (Extended Data Fig. 10a). Cells of the
169 organising centre of the shoot apical meristem also are replaced as they differentiate, although in that case the
170 location of the organiser is stable. All three organisers express a *WOX* family member: *WUSCHEL* in the shoot
171 apical meristem, *WOX5* in the root meristem² and *WOX4* in the vascular cambium, underscoring the related
172 identity of cells in the three locations. Future challenges are to identify the cell non-autonomous stem cell factors
173 originating from the organiser cells (Fig. 4d), and to link other known cambial regulators³⁰ to the signalling
174 module defining the organiser (Extended Data Fig. 10).

175

176 SUPPLEMENTARY INFORMATION

177 Supplementary Tables 1 to 8

178

179 ACKNOWLEDGEMENTS

180 We thank T. Berleth, A. Carlsbecker, T. Greb, P. Ramachandran, E. Scarpella, D. Weijers for sharing published
181 material; A.-M. Bågman, M. Herpola and J. Smetana for technical assistance; W. Yang for helping with the *in situ*
182 protocol; S. el-Showk, P. Etchells, Y. Helariutta, S. Otero and B. Scheres for constructive comments. This work
183 was supported by the Academy of Finland (grants #132376, #266431, #271832), University of Helsinki HiLIFE

184 fellowship (O.S., R.M., M.L., A.S.G., F.S.R., M.L.G., R.S., T.B., S.B., A.P.M.), Erasmus+ (F.S.R., M.L.G.), and U.S.
185 National Science Foundation grant IOS-0744874 (M.-F.W., J.W.R.).

186

187 **AUTHOR CONTRIBUTIONS**

188 O.S. and A.P.M. designed the experiments. O.S. carried out all the experiments, except, R.M. and M.L. conducted
189 qRT-PCR, *amiRNA* and inducible *axr3* work; F.S.R. analysed *HD-ZIP III* mutants and S.B. carried out RNA *in situ*
190 hybridisation and lineage tracing with BAP. M.-F.W. and J.W.R. generated and tested *amiRNA* plasmid. A.S.G. and
191 M.L.G. provided data on the inducible miR165a line. A.A. conducted the statistical analyses. R.S., S.M., P.R. and
192 T.B. provided transgenic lines for the study. O.S. and A.P.M. wrote the manuscript with the help of co-authors.

193

194 **COMPETING INTERESTS**

195 The authors declare no competing financial interests.

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197 **CORRESPONDING AUTHOR**

198 Correspondence to Ari Pekka Mähönen: AriPekka.Mahonen@helsinki.fi

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326

327 **FIGURE LEGENDS**

328 **Figure 1 | Lineage tracing revealed the origin of the vascular cambium stem cells.** **a**, The activation of secondary
329 root growth is characterised by the initiation of periclinal cell divisions in procambial cells (black arrowheads),
330 followed by divisions in pericycle (white arrowheads), leading to the formation of vascular cambium and
331 phellogen. Cell divisions are marked only in upper half of the cross section. **b**, Lineage tracing initiated from the
332 primary vasculature (top) showed the importance of an initial xylem connection for the ontogeny of the cambium
333 (yellow dashed line). The bottom panel shows the four major types of clonal sectors obtained. Frames are
334 coloured to match with the corresponding cell lineages in the top panels. The numbers in the fate map (bottom

335 right) refer to the observed number of the sectors. **c, d**, Lineage tracing in active cambium revealed a common
336 stem cell for xylem and phloem. GUS sectors (black bars) plotted based on the position of thinnest cell wall
337 (arrow) in each radial file, representing the most recent periclinal cell division in cambium (y value = 0). Scale
338 bars represent 50 μ m. All experiments were repeated four times.

339

340 **Figure 2 | HD-ZIP IIIs determine the organiser of the vascular cambium. a**, Confocal microscopy of cross sections
341 of 14-day old *ANT* double markers. White arrows indicate pairs of nuclei in recently dividing cells. Insets, merged
342 reporter channels. **b**, Bright field microscopy of root after *miR165a* induction (Ind) for 6 days (in 5-day old plants).
343 Red dots, secondary xylem vessels (SXV); quantification presented on the right. Box-and-whisker plot shows
344 median and interquartile range; whiskers show range, dots outliers. Two-tailed Wilcoxon test. n , independent
345 roots. **c**, Confocal images of EdU staining reveal a centripetal shift of the cell division domain after 2-day *miR165a*
346 induction (in 8-day old plants). Asterisks mark cambium. Histograms showing the relative distances (RD) of EdU-
347 positive nuclei in the radial direction from the root midpoint (xylem axis: x value = 0, periderm: x value = 1), as
348 depicted in Mock. n_n , total number of nuclei. **d**, Confocal microscopy of fluorescent markers after 2-day *miR165a*
349 induction (in 8-day old plants). Dashed lines, borders of the secondary tissues. The numbers in the bottom left
350 corner of subpanels represent the frequency of the observed phenotype. **a, c, d**, Cell wall staining in grey. p,
351 phloem; x, xylem. All experiments were repeated at least three times. Scale bars represent 50 μ m.

352

353 **Figure 3 | Auxin signalling is required for xylem differentiation and organizer identity. a**, Confocal microscopy
354 of *DR5:nYFP* and *cAtHB8-YFP* (heatmap) root cross sections. Nuclear fluorescence signal (circles indicate
355 examples of ROIs) was quantified in three cell layers from the most recent cell division (arrowheads) towards
356 xylem (positions -1, -2, -3) and phloem (positions 1, 2, 3). n_n = total number of nuclei. Cell wall staining in grey.
357 p, phloem; x, xylem. **b**, qRT-PCR analysis of marker gene expression in *amiMP* T1 transformants with the lowest
358 *MP* transcript levels. Each point represents an individual *amiMP* T1 transformant ($n = 10$) or individual *arf7,19*
359 plant ($n = 5$) of the same age. The p -values of a one-sided t-test comparing *amiMP* to *arf7,19* controls are
360 provided under each gene name, and significant values are in bold-face. **c**, Bright field microscopy of
361 *pUBQ10:XVE>>amiMP* transformants in the *arf7,19* background induced for 7 days (in 5-day old plants). Yellow
362 dots, secondary xylem vessels. Experiment was repeated three times with similar results. **d**, qRT-PCR analysis of
363 marker gene expression in inducible *axr3-1* after 24h induction (in 8-day old plants). Data are from three
364 experiments ($n = 16$ roots per treatment). In **a** and **d**, box-and-whisker plots show median and interquartile range;

whiskers show range. One-tailed t-test. In **a**, dots represents independent data points. Scale bars represent 50 μm .

Figure 4 | MP is sufficient for the formation of an ectopic organiser. **a**, Bright field micrograph of an *MPΔ-GUS* clone with periclinal cell divisions induced cell non-autonomously. Red dots, GUS-negative cells dividing periclinally around the *MPΔ-GUS* clone. **b**, Confocal analysis of marker expression within *MPΔ-YFP* clones (top) and around *MPΔ-YFP-induced* ectopic vessels (bottom). Cell wall staining in grey. Insets show YFP channel except for insets of *PXY* and *AtHB8* in bottom panels showing RFP channel. **c**, *PEAR1* is induced a few cell layers from *MPΔ-induced* ectopic vessels (asterisk). An arrow indicates a gap between ectopic vessel and *PEAR1* expression. **d**, A model illustrating how a local auxin maximum and the downstream components define an organiser which positions the stem cell in the adjacent cell. Curved arrows represent bidirectional cell proliferation from the bifacial stem cell. The numbers in the left corner of subpanels represent the frequency of the observed phenotype. Scale bars represent 50 μm unless indicated otherwise.

METHODS

Gene accession numbers

CYCB1;1, AT4G37490; *VND6*, AT5G62380; *WOX4*, AT1G46480; *PXY/TDR*, AT5G61480; *EPM/PEAR1/S29/Dof2.4*, AT2G37590; *ANT*, AT4G37750; *AtHB8*, AT4G32880; *CNA/AtHB15*, AT1G52150; *PHB*, AT2G34710; *PHV*, AT1G30490; *REV*, AT5G60690; *mir165a*, AT1G01183; *MP/ARF5*, AT1G19850; *ARF6*, AT1G30330; *ARF7*, AT5G20730; *ARF8*, AT5G37020; *ARF19*, AT1G19220; *APL*, AT1G79430; *S17*, AT2G22850.

Plant material and cloning

Most of the entry vectors were generated by PCR amplification of the desired DNA sequence with the primers indicated in Table S1 and recombination of the purified PCR product into MultiSite Gateway compatible pDONR entry vectors (Table S2). Obtained PCR products were amplified in two steps (marked with asterisks in Table S2), first with gene-specific primers with partial attB overhangs and then with adaptor primers to generate PCR products with full length attB recombination sequences. The dominant version of *cAtHB8* (*AtHB8d*) has a mutation (CCG→CTG) in the last codon of the *mir165a*-recognition sequence, similar to the dominant *REVOLUTA* mutant *rev-10d*³¹. The entry vector p221z-c *AtHB8d*(-)stop was generated by site-directed mutagenesis using

394 p221z-c AtHB8(-)stop as a template and primers AtHB8dF1 and AtHB8dR1 using the QuikChange II Site-Directed
395 Mutagenesis Kit (Agilent Technologies). The entry vector p221a-CYCB1;1-CRE was made by PCR amplification of
396 CRE recombinase³² from p221z-CRE1 using the CreMfeF and CreMfeR primers, and the resulting PCR product
397 was ligated into the unique *MfeI* restriction site of p221a-CycB1-dbox²⁶. MicroRNA against *MONOPTEROS*
398 (amiMP) was obtained by PCR mutagenising the *MIR167a* precursor sequences (At3g22886)³³ using the following
399 primers: aMIR-MP_1, aMIR-MP_2, aMIR-MP_3 and aMIR-MP_4 (sequences in Table S3). amiMP was then cloned
400 into pENTR/D_TOPO to create pENT-AtMIR167a-MP. To construct *gANT-GFP*, genomic fragment of *ANT* was
401 amplified by using primers ANT(-)5172 and ANT(+)2885R, and cloned into pAN19 (a modified pUC18). GFP was
402 inserted into the C-terminus of *ANT* coding sequence by using inverse PCR. The resultant *gANT-GFP* fragment
403 was re-cloned into pBIN30 (a modified pBIN19) binary vector. pSm43GW was generated from pHm43GW in two
404 steps. First, hygromycin resistance was replaced with a -*SphI*-YFP-*KpnI*- PCR fragment using the YFPfrg_F1 and
405 YFPfrg_R2 primers and the *SphI* and *KpnI* restriction sites to obtain pYm43GW. Next, the YFP fragment was
406 replaced with the sulfadiazine resistance gene amplified from GABI_874H08 line gDNA with primers sulf-*KpnI*F
407 and sulf-*SnaBI*R, and the resulting PCR product was cloned into pYm43GW using the *KpnI* and *StuI* restriction
408 sites.

409 MultiSite Gateway technology (Invitrogen) was used to combine promoter (1st box), gene of interest (2nd box)
410 and tag or terminator (3rd box) Gateway entry clones with Gateway-compatible binary destination vectors^{12, 34} in
411 a MultiSite LR Clonase reaction. All expression vectors generated in this study are listed in Table S4.

412 All constructs were dipped into the Col-0 ecotype, and single insertion lines were screened based on Mendelian
413 segregation of a selection marker. Several lines for each newly made construct were used for phenotype
414 observation. Plant material produced in this study as well as published lines provided by others are indicated in
415 Table S5. The following transgenic and mutant lines have been described elsewhere: *cna-2 phb-13 phv-11*; *athb8-*
416 *11 cna-2 phb-13 phv-11*^{8, 35}; *arf7-1 arf19-1*³⁶; *DR5rev::YFPnuc*³⁷, *DR5rev:erGFP*³⁸, *DR5v2:n3GFP*³⁹, *DR5rev:GUS*⁴⁰,
417 *pWOX4:erYFP*⁴¹, *pMP:MP-GUS*⁴², *pARF7:GUS* & *pARF19:GUS*⁴³, *gARF8-GUS* & *gARF6-GUS*³³, *pG1090:XVE>>axr3-*
418 *1-RFP*²⁶, *pAPL:GUS*⁴⁴, *gVND6-GUS*⁴⁵ and *pAtHB8:GUS*⁴⁶.

419 **Plant growth and chemical treatments**

420 For almost all experiments, seeds were surface-sterilised in 70% ethanol, then in 20% chlorine, washed twice in
421 H₂O, and plated on half strength GM medium (½ GM) containing 0.5 x MS salt mixture with vitamins (Duchefa),
422 1% sucrose, 0,5g/l MES pH 5.8 and 0.8% agar and vernalised in 4°C for 2 days. The age of the seedlings was
423 measured from the moment they were placed in a vertical position in the growth cabinet, where they were

424 grown at 22°C under long day conditions. For experiment with soil-grown plants, seeds were directly placed on
425 soil and grown under the same conditions.

426 17- β -oestradiol (Sigma), a synthetic derivative of oestrogen, and 6-Benzylaminopurine (BAP; Sigma), a cytokinin,
427 were prepared as 20 mM stock solutions in DMSO and stored at -20°C. 5-Ethynyl-2'-deoxyuridine, a thymidine
428 analogue (EdU; Thermo Fisher), was prepared as a 10 mM stock solution in DMSO and stored at 4°C. XVE-based
429 gene induction ('Ind' in figure panels) was carried out by transferring plants to plates supplemented with 5 μ M
430 17- β -oestradiol or an equal volume of DMSO ('Mock' in figure panels) unless otherwise indicated. The BAP
431 working concentration was 0.1 μ M. For EdU treatment, plants were transferred into ½ GM liquid medium
432 supplemented with 10 μ M EdU and, eventually, with inducer or mock for 16 h. Unless stated otherwise, the age
433 of the seedlings at the start of the treatment is indicated in the brackets in the figure legends.

434 **GUS staining, microtome sectioning and histology**

435 GUS detection and histology analysis were performed according to⁴⁷ with slight modifications. First, samples
436 were vacuum-infiltrated with the GUS staining solution for 1 h and then incubated at 37°C until the desired level
437 of GUS staining was obtained (2 h – overnight). Samples were cut on a microtome in 10 μ m sections from 5 mm
438 below the root-hypocotyl junction. For histological analysis of clones, the upper 1-1.5 cm root segment was cut
439 every 200 μ m to avoid repeated analysis of the same cells. Finally, root sections were stained for 5 s in a 0.05%
440 (w/v) Ruthenium Red solution (Fluka Biochemika) or a 0.05% (w/v) Toluidine blue solution in deionised water.
441 To stain phloem companion cells, a 0.5% (w/v) Safranin solution in 50% ethanol and a 1% (w/v) Alcian blue
442 aqueous solution were used. All histological stainings used in each figure are indicated in Table S6.

443

444 **Fluorescent marker analysis, vibratome sectioning and EdU detection**

445 To analyse fluorescent reporters from a lateral view, roots were fixed in 4% paraformaldehyde (PFA, Sigma) in 1
446 x PBS pH 7.2 overnight, washed twice in 1 x PBS and cleared in ClearSee solution⁴⁸ supplemented with 1 μ g/ml
447 Calcofluor white (Sigma) for at least three days.

448 Vibratome sectioning was used for fluorescent marker analysis and EdU detection. Roots were fixed in 4% PFA
449 in 1 x PBS pH 7.2 for 1 h under vacuum, washed in ice-cold 1 x PBS and embedded in 4% agarose in 1x PBS.
450 Samples were cut on a vibratome in 200 μ m sections from 5 mm below the root-hypocotyl junction. Obtained
451 agarose slices containing root cross sections were washed twice in 1 x PBS, cell walls were stained with 1 μ g/ml
452 Calcofluor white (Sigma) in 1 x PBS for 3 h – overnight and finally cleared with ClearSee solution. To visualise EdU

453 positive nuclei with the Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit (Thermo Fisher), fixed cross sections were
454 washed twice in 1 x PBS and then incubated in a modified EdU detection cocktail⁴⁹ at RT for 1 h, washed twice in
455 1 x PBS and stained with 1 µg/ml Calcofluor or DAPI solution in 1 x PBS.

456 **Microscopy and image processing**

457 Histological cross sections were imaged using a Leica 2500 microscope (20× and 40× objectives). Confocal
458 analysis was carried out with Leica SP5 and SP8 microscopes. Confocal images were acquired with the Leica LAS
459 AF Software using ClearSee, 1x PBS or water as an imaging medium. All combinatorial fluorescence analyses were
460 run in sequential scan mode. Confocal settings may have varied between experiments but always remained the
461 same for an experimental sample and its corresponding control. However, to better visualise calcofluor-stained
462 cell walls, the signal was sometimes unevenly adjusted between the sample and the control. EdU images
463 represent maximal projections of five consecutive optical sections captured each 5 µm in the z-direction.

464 Microsoft PowerPoint with image compression mode off was used to compile all the figures and to modify
465 brightness and contrast, which was always identically applied to sample and control pictures. Images in Figure
466 1a were cropped and rotated using Corel PHOTO PAINT X6, which occasionally resulted in empty corners; for
467 aesthetical reasons, these were filled with a triangle of a similar colour as the image background.

468 **Marker expression quantification, EdU and xylem vessel histograms**

469 Changes in marker expression were analysed from the lateral view; optical sections were captured in the middle
470 of the root, which was determined by the root edges being in focus in the transmitted light channel. The data
471 represent the relative width of the fluorescent domain compared with the root diameter. *DR5:nYFP*, *DR5v2:nGFP*
472 and *cAtHB8-YFP* fluorescence were captured as one stack of 16-bit confocal images from 15 cross sections. The
473 signal intensities of fluorescent nuclei were measured in ImageJ using Multi-point tool with mark width set to 15
474 pixels. Relative intensities were determined by comparing the signal with a brightest value in the whole dataset.
475 For EdU and vessel distribution histograms, relative distances of EdU nuclei/vessels were determined by
476 comparing their distance from the centre of xylem axis with the radius of the root. If not indicated otherwise, 25
477 cross sections were analysed. Histograms represent frequencies of EdU nuclei/vessels along the radial axes
478 divided into 10 bins in percentage. Distance measurements were done using Leica Lite or ImageJ.

479 **amiMP analysis**

480 Due to persistent silencing of *pUBQ10:XVE>>amiMP (arf7-1,19-1)* in the T2 and T3 generations, individual plants
481 were analysed in the T1 generation. Transformants in the *arf7*, *19* or wild-type backgrounds were grown on

482 plates with 20 µg/ml phosphinothricin (basta, Duchefa) for 5 days without noticeable phenotypes caused by the
483 selection, and then induced together with the controls for 7 days. Segments of the upper 2 cm of the root were
484 collected for histological analysis, and the remaining (lower) part was used for RNA isolation. Since *arf7-1,19-1*
485 does not produce lateral roots, lower segment contained RNA exclusively from the primary root. Cell divisions
486 were counted as the number of cells in one radial cell file between primary phloem pole and xylem axis.

487 **qRT-PCR**

488 RNA was extracted from the top 2 cm of the main root with lateral roots removed using the GeneJET Plant RNA
489 purification kit (Thermo Fisher) and treated with DNase (Thermo Fisher). cDNA was synthesized from 100 ng
490 (*AtHB8d* and *miR165a* overexpressing plants), 500 ng (*axr3-1* overexpressing plants and *miR165a-ox* for *PXY*) or
491 50 ng (*amiMP* plants) of RNA using MAXIMA H Minus reverse transcriptase (Thermo Fisher) with Oligo(dT)
492 primers (Thermo Fisher). The PCR reaction was done on a Bio-Rad CFX384 cyclor using EvaGreen qPCR mix (Solis
493 Biodyne) and the following program: 10 min at 95 °C, 50 cycles (10 s at 95 °C, 10 s at 60 °C, 30s in 72 °C). All
494 primers used in qRT-PCR are listed in Table S6: primers for *ACT2*, *UBQ10* and *ANT*⁵⁰; *PHB*, *PHV*, *AtHB8* and *CNA*⁸;
495 *PXY*⁵¹; and *TIP41*⁵² were described before. Results were normalized following the method of Vandesompele *et*
496 *al.* (2002)⁵³ and Livak & Schmittgen (2001)⁵⁴ to the reference genes *ACT2*, *UBQ10* and *TIP41*. Three biological
497 replicates were used for each line and time point, as well as three technical replicates, except in the analysis of
498 *amiMP* lines (see above).

499 **RNA *in situ* hybridisation**

500 Primers used for RNA probe synthesis were published previously⁸. Sample preparation, sectioning, probe
501 synthesis using T7 RNA polymerase (Thermo Fisher) and hybridization were performed as described⁵⁵ with slight
502 modifications. The uppermost 1 cm segment of the main root was cut from 21-day old soil-grown plants, and the
503 tissues were vacuum infiltrated in FAA (50 % ethanol; 5 % acetic acid; 3.7 % formaldehyde) for 30 min and then
504 fixed overnight at 4 °C. For paraffin embedding, a Leica ASP200 vacuum tissue processor was used, following the
505 protocol indicated in Table S7.

506 **Clone induction**

507 To induce clones before the onset of secondary growth, plates with 4-day old seedlings grown on ½ GM plates
508 with or without BAP were heat shocked at 37°C for 14 or 17 min, immediately cooled down at 4°C for 15 min and
509 further grown on new ½ GM plates under normal growth conditions until GUS staining and histological analysis.
510 For lineage tracing in active cambium, plants were grown for 15 days prior to heat shock. Clonal activation of
511 *pUAS:MPA-GUS* (Fig. 4a) was carried out in the same way and the clones were analysed 6 days later. For lineage

tracing in one-month old plants, whole 5 cm pots with individual plants were transferred into 37°C water bath for 15 min followed by a cooling step under cold tap water for 1 min. Clone activation in the phloem pole pericycle and surrounding cells (S17 domain) was performed by transferring 5-day old seedlings into liquid ½ GM medium with 17-β-oestradiol for 8 or 16 min followed by four 15 min washes in medium without an inducer. After that, plants were placed back on agar plates and grown under normal conditions. *ANT:XVE>>CRE* transformants in *35S:lox-GUS^{+/+}* background were selected on plates with hygromycin and claforan. After four days, hygromycin-resistant plants were transferred on plates only with claforan and grown further without noticeable phenotype caused by antibiotics. Clones in *ANT* domain were induced in a similar way as S17 clones with slight modifications: 17-day old plants were incubated with an inducer for 5 or 20 min and after washing they were placed on agar plates with claforan. To increase the proportion of clones originating from single cells, roots were pre-screened and only those with a low amount of clones were analysed further.

Fate map and lineage tracing quantification

Because the position of major cell types in the primary root vasculature is invariant, the fate map was generated by counting the frequencies of different GUS sectors and grouping them into three categories: i) the most frequent “basic sectors”, ii) “large sectors” partially sharing clonal boundaries with two or more basic sector types at once and iii) “half sectors” consisting a subdomain of one of the basic sector types. Only “basic sectors” are presented in the fate map (Fig. 1b). BAP-mediated promotion of procambium formation in the place of protoxylem is not a fully penetrant phenotype⁵⁶, and therefore the presence [(+) protoxylem in Extended Data Fig. 2e, f] or absence [(-) protoxylem] of protoxylem was observed before analysing the sectors.

Quantification of GUS sectors in active cambium was carried out by counting the cellular distance of sector boundaries [xylem side ($y < 0$); phloem side ($y > 0$)] from periclinally dividing cambial cells ($y = 0$) in the radial direction. Dividing cambial cells were identified histologically based on the presence of a thin cell wall. In rare cases where two dividing cambial cells were in given cell file, the distance was counted from the cell wall between those cells. Only atypically large GUS sectors, sectors with unclear borders or located far from the vascular cambium (Extended Data Fig. 5a, b, f) were excluded from quantification. Each bar in graphs (Fig. 1d, Extended Data Fig. 5d, h) represents the size and position of an individual sector; bars with extensions denote sectors beginning or ending with a xylem vessel.

Quantification of *MPA* and *VND6* clones

The cell-autonomous effects of *MPA* and *VND6* clones on cambial marker gene induction were measured as the number of independent YFP-positive nuclei showing marker expression within the clones two days after

542 induction. The cell non-autonomous effects were quantified as the number of ectopic vessels showing marker
543 expression in the surrounding cells seven days after induction. At least 10 roots per genotype were quantified.
544 The cell non-autonomous effect of MP on periclinal cell division was analysed by counting the frequency of
545 periclinal cell divisions induced 16 days after germination in the GUS-negative cells surrounding the MPΔ-GUS
546 clones/ectopic vessel. In these experiments (Fig. 4a; Extended Data Fig. 9e), periclinal cell divisions refer to cell
547 division planes parallel to the surface of the neighbouring MPΔ-/VND6-induced vessels/clones. Only clearly
548 ectopic vessels/clones (i.e. outside vascular cambium) were analysed further.

549 **General methodology and statistical analysis**

550 Unless otherwise stated, the number of individual plants, root sections or clones analysed is indicated as “*n*” in
551 Figure panels or legends. The fraction “X/Y” in the corner of certain figure panels denotes the frequency of the
552 presented phenotype. Statistical analyses were all carried out in R version 3.5.0 [<http://www.r-project.org/>]. For
553 the mean comparison analysis, first the Shapiro-Wilk test for normality was performed and, upon failing to reject
554 or rejecting this hypothesis, the appropriate one- or two-sided Student t-test or the nonparametric Wilcoxon
555 rank sum test was performed, respectively. Regression analyses were performed following the affirmation of the
556 normality assumption of the residuals. In general, we considered 0.05 as the significance threshold for *P*-values.
557 The Kullback-Leibler divergent distance was used to quantify the level of dissimilarity between histograms, and,
558 finally, the mean between the two directional values is reported. For all boxplots, the centre line represents the
559 median and the lower and upper limits of boxes indicates the 25th and 75th percentiles, respectively. Whiskers
560 show the maximum or minimum values while empty and filled circles represent the individual samples and
561 outliers, respectively.

562

563 **DATA AVAILABILITY**

564 Gene accession numbers of all the Arabidopsis genes analysed in this study are listed in Methods. Source Data
565 related to Fig 2b, c; Fig 3a, b, d; ED Fig 3d; ED Fig 4a, c, d; ED Fig 6a, b, c, d, e; ED Fig 7b, f, g; ED Fig 8a, b, c, d, e;
566 and ED Fig 9f are provided with the paper. All lines and other data supporting the findings of this study are
567 available from the corresponding author upon request.

568

569

570

571 **EXTENDED DATA FIGURE LEGENDS**

572 **Extended Data Figure 1 | Ontogeny of different cell types after activation of secondary growth in Arabidopsis**
573 **root. a**, Bright field microscopy of root cross sections of an early phloem marker (*PEAR1*), a marker of
574 differentiating phloem expressed in phloem-companion cells (*APL*), an early xylem marker (*AtHB8*) and a marker
575 specific for differentiating xylem vessels (*VND6*) at different times after secondary growth activation. **b**,
576 Histological analysis of different cell types found in 14- and 30-day old root expressing a phloem differentiation
577 marker *pAPL:GUS* (top) and wild-type (bottom). Cambium (red) typically consists of 2-4 cells (14 days) or 7-9 cells
578 (30 days). It is located between fully differentiated phloem elements (purple) and xylem vessels (blue), and is
579 characterized by frequent cell divisions detected histologically as a recently made thin cell wall. Xylem- and
580 phloem-parenchyma cells are quiescent cells located between xylem vessels or rarely dividing cells located
581 between differentiated phloem cells, respectively. Data shown are representative of at least three independent
582 experiments. Scale bars represent 50 μ m.

583

584 **Extended Data Figure 2 | A novel lineage tracing tool for developmental studies in plants. a**, A schematic
585 representation of two clonal activation systems. CRE recombinase was fused to the N-terminal part of *CYCB1*;1⁵⁷
586 to make *CYCB1*;1-CRE stable only in meristematic cells transiting between the early G2 and M phases of the cell
587 cycle. Heat-shock induction of *pHS:CYCB1*;1-CRE causes recombination of two loxP sites which activates
588 expression of *35S:GUS* (i) or *35S:GAL4* (ii), respectively. In the second step, the latter transactivates *pUAS:erGFP*
589 (iii) and, if desired, another gene of interest cloned under *pUAS* in a third construct (iv). **b**, Confocal microscopy
590 of the root (RAM) and shoot (SAM - dashed line, top view) apical meristems, lateral root primordium (LRP) and
591 procambium (PC) containing GFP clones 15 h after induction in 5-day old seedlings harbouring *pHS:CYCB1*;1-CRE
592 and *pCB1*³² constructs. Because of the instability provided by the *CYCB1*;1-CRE fusion and the apparent non-
593 synchronicity of the cell cycle, a high frequency of single-cell descent clones was obtained almost exclusively in
594 the dividing cells in the apical and lateral meristems. **c**, Examples of the three different GUS sector categories:
595 "basic sectors" originate from a single cell clone induced before the activation of secondary growth (red, 75%),
596 "large sectors" originate from a cluster of clones simultaneously induced in several neighbouring cells (black,
597 19%), "half sectors" originate from a single cell clone induced after the activation of secondary growth (purple,
598 6%). A total of 176 sectors were analysed. **d**, A schematic representation of vascular cambium (red) and phellogen
599 (blue) ontogeny. Under normal conditions (top) both meristems originate from xylem pole pericycle (XPP) cells.
600 When roots are pre-treated with cytokinin (CK), cells in the protoxylem position (black arrowheads) remain
601 procambial cells⁵⁶, and are thus capable of contributing to secondary growth (bottom, 'CK'). In these

602 experimental conditions, separation of vascular cambium and phellogen lineage occurs already during the
603 primary growth, and xylem-connection remains the determining factor for the origin of vascular cambium. **e**,
604 Examples of GUS clones 24 h after heat shock in cytokinin (BAP) pre-treated roots. Arrowheads, procambial cells
605 in place of protoxylem; asterisks, pericycle cells which have lost their xylem connection. **f**, Correlation of different
606 GUS sector shapes with the presence (left) or absence (centre, right) of protoxylem (insets, arrowheads). The
607 numbers represent the frequency of these sectors. **g**, XPP cell fate map of BAP-pre-treated roots. XPP cells which
608 lose their xylem connection behave like pericycle cells (light purple, compare with Fig. 1b); cells at the protoxylem
609 position behave like xylem-procambial cells, making wide sectors (red, compare with Fig. 1b). In **b**, **e**, **f**,
610 experiments were repeated at least two times with similar results. Scale bars represent 50 μ m.

611

612 **Extended Data Figure 3 | Transcriptional and mutant analyses of HD-ZIP III transcription factors. a**, *In situ*
613 hybridisation of sense- and anti-sense probes in 21-day old Col-0 cross sections showing HD-Zip III expression in
614 the cambium. Insets are 2x magnifications of the cambial region of the same photo. **b**, Confocal microscopy of
615 cross section of 14-day old *pAtHB8:erYFP* marker line. Cell wall staining in grey. Red arrowheads, recent cell
616 divisions in cambium. **c**, Bright field microscopy of primary and secondary vasculature shows patterning defects
617 in higher order HD-ZIP III mutants 7 days and 10 days after germination. Col-0 and *cna-2 phb-13 phv-11* show
618 normal differentiation of the primary xylem axis and two phloem poles, whereas the majority of *athb8-11 cna-2*
619 *phb-13 phv-11* roots did not have a fully differentiated xylem axis or showed a triarch pattern with three phloem
620 poles. The fraction in upper left corner denotes the frequency of the presented phenotype. Asterisks, primary
621 phloem poles; arrowheads, extremities of the primary xylem. **d**, Confocal images of EdU-stained (green) nuclei
622 in *cna-2 phb-13 phv-11*, *athb8-11 cna-2 phb-13 phv-11* mutants and the *miR165a* overexpressor (top). *miR165a*
623 was induced for 12 days (in 4-day old plants), and the mutants were 14 days old. Inhibition of HD-ZIP III leads to
624 cambium disorganisation (histograms, bottom). Even though *AtHB8*, *CNA*, *PHB* and *PHV*, and perhaps also *REV*
625 are redundantly required for secondary xylem formation and proper cambium organisation, it is possible that
626 individual members of the family have distinct function in the cambial stem cells and their organisers. Histograms
627 of the relative distances (RD) of EdU-positive nuclei and secondary xylem vessels in the radial direction from the
628 root midpoint (xylem axis: x value = 0, periderm: x value = 1, with the scale shown in green in the Col-0 panel).
629 KLD indicates the mean Kullback–Leibler distances between EdU-positive nuclei and vessels. n_c = number of cross
630 sections analysed; n_n = total number of nuclei. In **a** and **b**, data shown are representative of two (**a**) or four (**b**)
631 independent experiments. Scale bars represent 50 μ m.

632

Extended Data Figure 4 | Phenotypic characterization of inducible *miR165a* line. **a**, qRT-PCR analysis of HD-ZIP III expression after *miR165a* induction for 24 h (in 9-day old plants). **b**, *miR165a* overexpressing plants grown on induction medium for 14 days (from germination onwards) resemble the quintuple HD-ZIP III mutant⁸. **c**, Quantification of secondary xylem vessel formation after *miR165a* induction for 2 days (in 8-day old plants). Phenotypic changes on secondary vessel number start to appear after 2 days of the induction. A two-tailed Wilcoxon test ($n = 25$ independent roots). **d**, qRT-PCR analysis for marker gene expression after 2-day *miR165a* induction (in 8-day old plants). **e**, Bright field microscopy of *miR165a* overexpressor double stained with Safranin and Alcian blue to highlight secondary phloem (insets) after *miR165a* induction for 12 days (in 4-day old plants). Red dots, secondary phloem; asterisk, primary phloem; arrowheads, extremities of the primary xylem. **a** and **d** one-tailed t-test. Data are from three experiments ($n = 10$ roots per treatment). In **b** and **e**, the fraction denotes the frequency of the presented phenotype. Box-and-whisker plots show median and interquartile range; whiskers show range, dots outliers. Scale bars represent 50 μm .

Extended Data Figure 5 | Lineage tracing in active cambium using two different lineage tracing systems. GUS sectors obtained after clone induction in 15- (**a**) and 30-day (**b-d**) old plants harbouring *pHS:d-Box-CRE* and *35S:lox-GUS* constructs (see Extended Data Fig. 2a). **a**, **b**, Middle panels are lateral view of roots with GUS sectors before sectioning. **c**, **d**, Lineage tracing in 30-day old cambium revealed that bifacial stem cell is present even in wide cambium where 7 - 9 cell layers could be identified histologically (see Extended Data Fig. 1b). Quantification was performed as in Fig. 1d. **e-h**, Lineage tracing in *ANT* expression domain. **e**, A schematic representation of clonal activation system to induce GUS clones in *ANT* expression domain. After induction, *CRE*-directed recombination leads to clonal activation of GUS reporter. **f**, GUS sectors obtained after clone induction in 17-day old plants harbouring *pANT:XVE>>CRE* and *35S:lox-GUS*. Inset is a 2,5x zoom of the cambial zone of the same picture. **g**, **h**, Lineage tracing in active cambium showed *ANT* as a stem cell marker. Six days after induction (in 17-day old plants), growing GUS sectors had either xylem fate (green) or phloem fate (yellow), but the majority (76 %) had cambial fate (red) based on the position of thinnest cell wall (arrow) in radial direction, representing the most recent periclinal cell division in cambium (y value = 0). **d**, **h**, Each bar represents the size and position of an individual sector ($n = 131$); bars with extensions denote sectors beginning/ending with a xylem vessel. **a**, **b**, **f**, Red arrows, sectors originating from cambium stem cells; yellow arrow, sectors on the phloem side; green arrow, sectors on the xylem side; brown arrow, an example of sectors excluded from the quantification analysis. GUS staining is weak or absent in differentiated xylem vessels. Data represents sectors collected across at least 15 (**a**, **f-h**) or 4 (**b-d**) independent roots. Experiments were repeated at least twice with similar results. Scale bars represent 50 μm unless otherwise specified.

665

666 **Extended Data Figure 6 | Phenotypic characterization of inducible *AtHB8d* line.** **a**, qRT-PCR analysis of *AtHB8*
667 expression after *AtHB8d* induction for 24 h (in 9-day old plants). **b**, Confocal images of EdU staining reveal a
668 centrifugal shift of the cell division domain after 2-day *AtHB8d* induction (in 8-day old plants). Asterisks mark
669 cambium. Histograms showing the relative distances (RD) of EdU-positive nuclei in the radial direction from the
670 root midpoint (xylem axis: x value = 0, periderm: x value = 1), as depicted in Mock. n_n , total nuclei number. **c**,
671 Quantification of secondary xylem vessel formation after *AtHB8d* induction for 2 days (in 8-day old plants). **d**,
672 Confocal microscopy of fluorescent markers after 2-day *AtHB8d* induction (in 8-day old plants). Cell wall staining
673 in grey. Dashed lines, borders of the secondary tissues. Graph is quantification of the relative width of marker
674 expression domains. **e**, Bright field microscopy of roots after *AtHB8* induction for 4 days (in 8-day old plants) and
675 quantification of secondary xylem vessel numbers. Red dots, secondary xylem vessels; yellow dashed line,
676 cambium position in mock-treated roots. **a** and **d**, One-tailed t-test. Data are from three experiments ($n = 10$
677 roots per treatment). **c** and **e**, Two-tailed Wilcoxon test. n , independent roots. Box-and-whisker plots show
678 median and interquartile range; whiskers show range, dots outliers. Scale bars represent 50 μm .

679

680 **Extended Data Figure 7 | Auxin signalling and ARF reporters.** **a**, Bright field microscopy of *DR5rev:GUS* cross
681 section of a 14-day old root. **b**, Confocal microscopy of *DR5v2:nGFP* (heatmap) root cross sections. Nuclear
682 fluorescence signal (examples of ROIs are indicated with a circle) was quantified in three cell layers from the
683 most recent cell division (arrowheads) towards xylem (positions -1, -2, -3) and phloem (positions 1, 2, 3).
684 Fourteen cross sections were analysed; n_n = total number of nuclei. **c**, Bright field microscopy of 17-day old roots
685 expressing class A ARF GUS markers. **d**, Confocal microscopy of 14-day old plant expressing *pAtHB8:erRFP* and
686 *pMP:erYFP*. **e**, Confocal and bright field microscopy of *pMP:erYFP* and *DRrev:GUS* marker width after 2-day
687 *AtHB8d* induction in 8-day old plants. Nuclear localization of *AtHB8d-YFP* is shown in Extended Data Fig. 6d.
688 *pMP:erYFP* roots were stained with calcofluor (grey). The fraction in bottom left corner denotes the frequency
689 of the presented phenotype. White bars, width of the marker domain. Dashed lines, borders of the secondary
690 tissues. **f**, qRT-PCR analysis of *MP* expression after 2-day *miR165a* induction in 8-day old plants. Data are from
691 three experiments ($n = 10$ roots per treatment). **g**, Quantification of the relative width of marker expression
692 domains after *pANT:XVE>>AtHB8d-YFP* induction shown in (**e**) (right panels). Sample size for each group is equal
693 to denominator of the fraction indicated in (**e**). **b**, **f** and **g** one-tailed t-test for the comparison between different
694 positions (**b**) or between mock and induced sample (**f**, **g**). Box-and-whisker plots show median and interquartile
695 range; whiskers show range, dots outliers (**g**) or individual data points (**b**). **a**, **c**, **d**, Representative images of at

696 least three independent observations. **b**, **d** and **e** cell wall staining in grey. p, phloem; x, xylem. Scale bars
697 represent 50 μ m.

698

699 **Extended Data Figure 8 | Phenotypic characterization inducible *amiMP* and *axr3-1* lines.** **a**, The *arf7, 19* double
700 mutant shows no difference in secondary xylem production when compared to wild-type (Col-0) and analysed
701 14 days after germination, ($n_{Col-0} = 17$ and $n_{arf7,19} = 15$ independent roots). **b**, Quantification of secondary xylem
702 vessel formation after *amiMP* induction for 7 days (in 5-day old plants) in wild-type ($n_{Col-0} = 7$ independent roots)
703 and *arf7,19* ($n_{arf7,19} = 34$ independent roots) plants analysed 15 mm below the root-hypocotyl junction. The
704 reduction in xylem vessel formation was less apparent when *amiMP* was induced in the wild-type background,
705 indicating that *ARF7* and/or *ARF19* are also required for this process. **c**, Plots of regressions between *MP*
706 transcript levels and vasculature diameter (left), secondary xylem vessel formation (centre) and cell divisions
707 (right) analysed 5 mm (top) and 15 mm (bottom) below the root hypocotyl junction. Each point represents an
708 individual T1 transformant or *arf7,19* plant ($n_{amiMP} = 33$; $n_{arf7,19} = 6$) grown for 5 days on a basta selection plate or
709 $\frac{1}{2}$ GM and then induced for 7 days. Since the residuals were not normal, a one-way Wilcoxon test for the similarity
710 of the means of the *arf7,19* and *amiMP* groups on *MP* transcription level was performed. A negligible *P*-value of
711 3.065×10^{-7} confirms that the *arf7,19* mean is significantly greater than the *amiMP* mean. In order to assess the
712 linear relationship between *MP* transcript levels and the observed phenotypes, we performed regression
713 analyses. The plotted blue line indicates the fitted regression model to both groups combined, and the *P*-value
714 indicating the goodness of this fit is plotted on the top of each graph, respectively. **d**, qRT-PCR analysis of HD-ZIP
715 IIIs behaviour after 24 and 72h induction of *axr3-1* (in 8-day old plants). Data are from four experiments ($n = 16$
716 roots per treatment). **e**, Bright field microscopy and secondary xylem vessel quantification (bottom) of roots after
717 *axr3-1* induction for 48 and 72h (in 8-day old plants). Insets are 1.5 x magnifications of the cambial region of the
718 same photo. Arrowhead; recent cell division. Sample size for each group is equal to denominator of the fraction
719 indicated in upper panels. **f**, Bright field microscopy of roots after *axr3-1* induction for 7 days (in 5-day old plants).
720 Box-and-whisker plots show median and interquartile range; whiskers show range, dots outliers. **a** and **b** Two-
721 tailed Wilcoxon test. **d**, **e**, One-tailed t-test. In **e**, **f**, the fraction in upper left corner denotes the frequency of the
722 presented phenotype. Scale bars represent 50 μ m.

723

724 **Extended Data Figure 9 | Clonal activation in the S17 expression domain.** **a**, A schematic representation of the
725 clonal activation system to induce clones in phloem pole pericycle cells and surrounding tissue. After induction,
726 *CRE*-directed recombination leads to clonal activation of gene-of-interest (GOI: *AtHB8d*; *MPΔ*; *gVND6*) tagged
727 with a reporter. **b**, Bright field microscopy of GUS sectors in S17 position analysed 1 day after clone induction (in

5-day old plants). Induction of "large" sectors consisting of several single cell GUS clones in the root tip region recapitulated previously described expression pattern of the S17 promoter being specific to phloem-pole pericycle cells (bottom left; Lee et al.). Higher up in the root where secondary growth has already started, GUS clones were obtained in primary phloem and surrounding tissue (right panels) suggesting that after secondary growth activation the S17 expression domain enlarges centripetally. Experiment was repeated twice with similar results. **c**, None of 21 *35S:lox-cATHB8d-GUS* transformants in *pS17:XVE>>CRE* background showed ectopic vessel formation. Confocal (left) and bright field microscopy (right) analysis of *AtHB8-GUS* clones in a cambial marker background. Marker expression was analysed 2 days after clone induction (in 5-day old plants). The bright field and confocal images are from different roots. *n* = number of entire roots screened for ectopic marker expression. **d**, Confocal analysis of *AtHB8d-GUS* clonal activation lines performed 7 days after *AtHB8-GUS* clone induction (in 5-day old plants), with calcofluor staining in grey. *n* = number of entire independent roots screened for ectopic vessel formation. **e, f**, *MPΔ*- and *gVND6*-induced ectopic vessels show different levels of periclinal cell division induction in neighbouring cells. Bright field microscopy of root cross sections stained with toluidine blue showing ectopic vessels with periclinal cell division in neighbouring cells analysed 7 days after induction (in 5-day old plants). Yellow dots, ectopic xylem vessel; red dots, cell which have recently undergone periclinal cell division. *n* = number of xylem vessels examined for each category (0-7 neighbouring cells dividing periclinally). Quantification in **(f)**. Data were collected across 16 independent roots for each genotype. **g**, Confocal analysis of marker expression within *gVND6-YFP* clones (top) and around *gVND6-YFP*-induced ectopic vessels (bottom). The fraction in upper right corner denotes the frequency of the presented phenotype. Cell walls were stained with calcofluor (grey). Insets show merged YFP and RFP channels except for insets of *WOX4* showing only YFP channel. Scale bars represent 50 μ m.

Extended Data Figure 10 | Model describing the dynamic nature of vascular cambium. **a**, When the organiser cells differentiates into a xylem vessel, a new organiser is formed in adjacent cambial stem cell. Stem cells and their xylem side daughters dynamically gain organiser cell gene expression as they mature towards xylem. Only the events leading to xylem production are illustrated in the model: mechanism for phloem production from the bifacial stem cell is unknown and thus not presented in the scheme. Density of the dots corresponds to the level of signalling components shown in **(b)**. **b**, A model presenting a signalling framework defining the stem cell organiser of vascular cambium. Question marks refer to unknown factors that cell non-autonomously promote stem cell or phloem identity. These mobile, stem cell promoting factors could for example be cytokinins, which during the primary development move from primary xylem precursor cells, marked with high auxin signalling, to promote cell division in the neighbouring procambial cells⁵⁸⁻⁶⁰. Supporting this scenario, cytokinins are required

760 for cambial cell proliferation during the secondary development⁶¹. Alternatively, WOX4 or other transcription
761 factors expressed in the organiser could move to the adjacent cell to promote stem cell identity, similar to WUS
762 and WOX5 in the apical meristems².
763

Figure 1

Figure 1 | Lineage tracing revealed the origin of the vascular cambium stem cells. a, The activation of secondary root growth is characterised by the initiation of periclinal cell divisions in procambial cells (black arrowheads), followed by divisions in pericycle (white arrowheads), leading to the formation of vascular cambium and phellogen. Cell divisions are marked only in upper half of the cross section. b, Lineage tracing initiated from the primary vasculature (top) showed the importance of an initial xylem connection for the ontogeny of the cambium (yellow dashed line). The bottom panel shows the four major types of clonal sectors obtained. Frames are coloured to match with the corresponding cell lineages in the top panels. The numbers in the fate map (bottom right) refer to the observed number of the sectors. c, d, Lineage tracing in active cambium revealed a common stem cell for xylem and phloem. GUS sectors (black bars) plotted based on the position of thinnest cell wall (arrow) in each radial file, representing the most recent periclinal cell division in cambium (y value = 0). Scale bars represent 50 μ m. All experiments were repeated four times.

Figure 2

Figure 2 | HD-ZIP III markers determine the organizer of the vascular cambium. a, Confocal microscopy of cross sections of 14-day old ANT double markers. White arrows indicate pairs of nuclei in recently dividing cells. Insets, merged reporter channels. b, Bright field microscopy of root after miR165a induction (Ind) for 6 days (in 5-day old plants). Red dots, secondary xylem vessels (SXV); quantification presented on the right. Box-and-whisker plot shows median and interquartile range; whiskers show range, dots outliers. Two-tailed Wilcoxon test. n , independent roots. c, Confocal images of EdU staining reveal a centripetal shift of the cell division domain after 2-day miR165a induction (in 8-day old plants). Asterisks mark cambium. Histograms showing the relative distances (RD) of EdU-positive nuclei in the radial direction from the root midpoint (xylem axis: x value = 0, periderm: x value = 1), as depicted in Mock. n_n , total number of nuclei. d, Confocal microscopy of fluorescent markers after 2-day miR165a induction (in 8-day old plants). Dashed lines, borders of the secondary tissues. The numbers in the bottom left corner of subpanels represent the frequency of the observed phenotype. a, c, d, Cell wall staining in grey. p, phloem; x, xylem. All experiments were repeated at least three times. Scale bars represent 50 μ m.

Figure 3

Figure 3 | Auxin signalling is required for xylem differentiation and organizer identity. a, Confocal microscopy of DR5:nYFP and cAtHB8-YFP (heatmap) root cross sections. Nuclear fluorescence signal (circles indicate examples of ROIs) was quantified in three cell layers from the most recent cell division (arrow-heads) towards xylem (positions -1, -2, -3) and phloem (positions 1, 2, 3). n_n = total number of nuclei. Cell wall staining in grey. p, phloem; x, xylem. b, qRT-PCR analysis of marker gene expression in amiMP T1 transformants with the lowest MP transcript levels. Each point represents an individual amiMP T1 transformant (n = 10) or individual arf7,19 plant (n = 5) of the same age. The p-values of a one-sided t-test comparing amiMP to arf7,19 controls are provided under each gene name, and significant values are in bold-face. c, Bright field microscopy of pUBQ10:XVE>>amiMP transformants in the arf7,19 background induced for 7 days (in 5-day old plants). Yellow dots, secondary xylem vessels. Experiment was repeated three times with similar results. d, qRT-PCR analysis of marker gene expression in inducible axr3-1 after 24h induction (in 8-day old plants). Data are from three experiments (n = 16 roots per treatment). In a and d, box-and-whisker plots show median and interquartile range; whiskers show range. One-tailed t-test. In a, dots represent independent data points. Scale bars represent 50 μ m.

Figure 4

Figure 4 | MP is sufficient for the formation of an ectopic organiser. a, Bright field micrograph of an *MPΔ-GUS* clone with periclinal cell divisions induced cell non-autonomously. Red dots, GUS-negative cells dividing periclinally around the *MPΔ-GUS* clone. b, Confocal analysis of marker expression within *MPΔ-YFP* clones (top) and around *MPΔ-YFP-induced* ectopic vessels (bottom). Cell wall staining in grey. Insets show YFP channel except for insets of PXY and AtHB8 in bottom panels showing RFP channel. c, PEAR1 is induced a few cell layers from *MPΔ*-induced ectopic vessels (asterisk). An arrow indicates a gap between ectopic vessel and PEAR1 expression. d, A model illustrating how a local auxin maximum and the downstream components define an organiser which positions the stem cell in the adjacent cell. Curved arrows represent bidirectional cell proliferation from the bifacial stem cell. The numbers in the left corner of subpanels represent the frequency of the observed phenotype. Scale bars represent 50 μ m unless indicated otherwise.

Extended Data Figure 1

a

b

- phloem parenchyma
- vascular cambium
- periderm (phelloderm + phellogen + phellem)
- secondary xylem vessels
- xylem parenchyma cells
- phloem sieve elements and neighboring companion cells (not marked)

Extended Data Figure 2

Extended Data Figure 3

Extended Data Figure 4

Extended Data Figure 5

Extended Data Figure 6

Extended Data Figure 7

Extended Data Figure 8

Extended Data Figure 9

Extended Data Figure 10