Progressive maturation of the root apical meristem in *Arabidopsis thaliana* lateral roots

Béatrice Berthet, Lotte Bald, Marion Louveaux and Alexis Maizel*

Center for Organismal Studies (COS), University of Heidelberg, Im Neuenheimer Feld 230,
69120 Heidelberg, Germany.

* to whom correspondence should be addressed: alexis.maizel@cos.uni-heidelberg.de

Emails & ORCID IDs

Béatrice Berthet	beatrice.berthet@hotmail.fr	0000-0003-2651-4854
Lotte Bald	lotte.bald@cos.uni-heidelberg.de	0000-0002-9471-2716
Marion Louveaux	marion.louveaux@gmail.com	0000-0002-1794-3748
Alexis Maizel	alexis.maizel@cos.uni-heidelberg.de	0000-0001-6843-1059

Number of figures: 4

Number of tables: 0

Number of supplemental figures: 4

Running title: Lateral root apical meristem maturation in *Arabidopsis*

23 **Highlight**

24 Analyze of the ontogeny of the quiescent center during lateral root ontogeny reveal its late
25 formation and supports that its emergence results from the establishment of two diverging growth
26 axis.

27

28 **Abstract**

29 Meristems are stem cells niches that support the formation of all plant organs and are either set
30 during embryogenesis and maintained throughout the plant life or specified de novo, post-
31 embryonically. The embryo-derived root apical meristem is organized around a group of
32 infrequently dividing cells, the quiescent centre, that maintains the stem cells, organizes growth
33 along two axes and owing to its resistance to 3ic stress can replace damaged stem cells. In most
34 cases, lateral roots post-embryonically branch off the primary and establish a new root meristem
35 which organization is identical to the primary root one. The cellular and molecular processes
36 underpinning the emergence of new stem cell niches are not well known. Here, we characterize
37 the de novo establishment of the root apical meristem in lateral roots. While the position of the
38 new stem cell niche is set early during morphogenesis, its cellular layout, unique gene expression
39 profile and mitotic quiescence are only acquired after emergence concomitant to the establishment
40 of two diverging growth axis. Our results show that the intertwined attributes of the mature root
41 stem cell niche are progressively acquired during lateral root formation, and support a model in
42 which the position of the stem cell niche emerges from the establishment of diverging growth axis.

43

44 **Keywords**

45 apical meristem; Arabidopsis thaliana; lateral root; maturation; quiescent centre; root; stem cells ;

46 Introduction

47 Above and below ground axial growth of plants is supported by primary meristems that are
 48 established during embryogenesis activated upon germination (Greb and Lohmann, 2016; ten
 49 Hove et al., 2015). Post embryonically, secondary meristems are established and allow branching
 50 and radial growth (Greb and Lohmann, 2016). These secondary meristems have a different
 51 ontogeny from their embryonic counterparts, but their organization and function is very comparable
 52 to the primary ones. They are thus very useful for elucidating the mostly unknown cellular and
 53 molecular processes governing the generation of new meristems (Greb and Lohmann, 2016).
 54 Lateral root (LR) branching from the primary or other root is an example of an organ which growth
 55 is supported by a secondary meristem (Motte *et al.*, 2019). In *Arabidopsis thaliana* (*Arabidopsis*),
 56 LR initiate deep within the primary root, where a few cells of the pericycle adjacent to the xylem
 57 radially expand and divide asymmetrically in response to local accumulation of the phytohormone
 58 auxin. These cells further grow, alternate rounds of anticlinal and periclinal divisions and form a
 59 dome shaped LR primordium (LRP) with a regular cellular layout which allows classification of its
 60 development in seven developmental stages (stage I to stage VII) (Malamy and Benfey, 1997; von
 61 Wangenheim *et al.*, 2016). Ultimately, the new LR forms a new meristem with a cellular and
 62 functional organization quasi identical to the one of the primary root meristem (Tian et al., 2014b).

63
 64 Meristems contain stem cells, also called initials, which activity is maintained by a group of rarely
 65 or non-dividing cells forming the organising centre in the shoot and the quiescent centre (QC) in
 66 the root (Dubrovsky and Ivanov, 2021). In *Arabidopsis*, the cellular organization of the root apical
 67 meristem (RAM) is simple and stereotyped with the central stem cells surrounding the QC. The
 68 stem cells, also called initials, provide, through asymmetric divisions, the progenitors for the root
 69 tissues and the root cap (Dolan *et al.*, 1993). The concept of QC was formulated by F. A. L. Clowes
 70 (Clowes, 1956) and originally referred to both the organizer and the initials (Dubrovsky and Ivanov,
 71 2021). A more restrictive definition of the QC, that only refers to the four to six apically located very
 72 slowly dividing cells was later adopted in *Arabidopsis* (Dolan *et al.*, 1993; Dubrovsky and Ivanov,
 73 2021). Here, we will use this latter definition for the QC. In addition to its low mitotic activity
 74 (Clowes, 1954; Clowes, 1956), the *Arabidopsis* QC is defined by additional features. First, it acts
 75 as an organiser that maintains the stem cell identity of cells in its direct contact (van den Berg et
 76 al., 1995; van den Berg et al., 1997). Second, it is characterized by abundance of auxin, an
 77 oxidizing environment and enrichment for expression of many genes and markers (Brumos et al.,
 78 2018; Haecker et al., 2004; Jiang et al., 2003; Nawy et al., 2005; Petersson et al., 2009; Sabatini
 79 et al., 1999). Third, the QC serves as a reservoir of cells to allow the meristem to recover from
 80 wounding or severe genotoxic damage by replacing compromised or missing cells (Clowes, 1959;
 81 Efroni et al., 2016; Fulcher and Sablowski, 2009; Tsugeki and Fedoroff, 1999). Thus, the QC

82 defines through its specific central location a microenvironment, akin to a niche, that maintains the
83 cellular organization of the meristem (Dubrovsky and Ivanov, 2021). The embryonic origin of the
84 QC in *Arabidopsis* can be traced back to the late globular stage and the division of the hypophysis
85 (Dolan *et al.*, 1993; Scheres *et al.*, 1994; ten Hove *et al.*, 2015). Studies in maize indicate that all
86 cells of the embryonic root proliferate and that quiescence of the QC is only established late during
87 embryogenesis (Clowes, 1978; Dubrovsky and Ivanov, 2021).

88 In LR, seminal works hint at a late formation of the QC in the water cabbage *Pistia*, the
89 water hyacinth *Eichhornia*, bean (*Vicia faba*), mallow (*Malva sylvestris*) and Maize (Byrne, 1973;
90 Clowes, 1958; Clowes, 1978; Macleod, 1977). In *Arabidopsis*, it was proposed that the formation
91 of the RAM in lateral root only occurs after an initial early morphogenesis phase (up to stage IV/V)
92 (Laskowski *et al.*, 1995). Similarly, RAM formation in adventitious roots has been reported to take
93 place late (Della Rovere *et al.*, 2013). Using reporters for QC markers, Goh *et al.* traced back the
94 origin of QC cells to the stage II LRP cells specifically expressing the *SCARECROW* (*SCR*)
95 transcription factor. Disrupting *SCR* function perturbed the formation of these QC precursor cells
96 (Goh *et al.*, 2016). A recent analysis of LR formation at single cell resolution (Serrano-Ron *et al.*,
97 2021) revealed that although expression of QC markers debuts early during LR formation they are
98 distributed amongst different cell populations and a distinct coherent population only emerges at
99 later stages.

100 Thus, whereas the cellular origin of the QC and the expression of QC markers are set early
101 during RAM formation in LR, at which stage a fully functional stem cell niche is established remains
102 elusive. Here, we investigate in detail the establishment of the lateral root stem cell niche. In
103 particular we focus on the ontogeny of the QC, considering when each of its features are
104 established: position, slow proliferation, expression of specific markers, and maintenance of the
105 stem cells. We use a combination of tissue identity reporters, live imaging, lineage tracing and cell
106 proliferation assay to conclude that the maturation of the stem cell niche is a progressive process
107 during LR development with acquisition of QC position, molecular identity, quiescence and
108 function being temporally uncoupled. While the QC position, and its role in proximal (shootward)
109 growth are set early during LR formation, its molecular identity, cellular organization and
110 quiescence are only fixed after LR emergence.

111 Results

112 Classification of lateral root post-emergence

113 Prior to emergence, LR development follows a nomenclature based on standard morphological
 114 stages (Stage I to VII) (Malamy and Benfey, 1997). For the post emergence phases, six types of
 115 lateral roots are defined based on their length and angle to the primary root (Kiss *et al.*, 2002).
 116 Here, we adapted this latter nomenclature to microscopy image, measuring LR from the junction
 117 between the vasculature of the LR and the primary root to the tip of the LR. While we refer to type
 118 1 as pre-emerged LR, type 2 LR have a maximal length of 250 μm ; type 3 a length of up to 1 mm,
 119 type 4 up to 2 mm and type 5, more than 2 mm (Fig. 1A).

120 The position of the lateral root stem cell niche is defined before emergence, its identity after

121 In the primary root, the stem cell niche is positioned by complementary patterns of auxin and
 122 cytokinin accumulation (Bishopp *et al.*, 2011; Müller and Sheen, 2008; Salvi *et al.*, 2020). There,
 123 auxin abundance and auxin signalling are maximal in the QC and the columella cells (Sabatini *et*
 124 *al.*, 1999) while cytokinins signalling is maximal in the root cap, the pro-vasculature above the QC
 125 and the distal region of the differentiation zone (Bielach *et al.*, 2012; Marhavý *et al.*, 2014;
 126 Montesinos *et al.*, 2020). Auxin and cytokinin tightly regulate each phase of LR development
 127 (Nenadić and Vermeer, 2021). We set to see if in the developing LR the position of the new stem
 128 cell niche is defined by the same complementary pattern. We visualized the output of auxin and
 129 cytokinin signalling during LR development using the *pDR5v2::NLS-3xVenus* (*DR5v2::Venus*,
 130 (Liao *et al.*, 2015) and *TCSn::2xVenus-NLS* (*TCSn::Venus*, (Zürcher *et al.*, 2013) reporters (Fig.
 131 1B). Before emergence, *DR5v2::Venus* signal is present throughout the LRP up to stage IV and
 132 after becomes restricted to the tip, as previously observed (Benková *et al.*, 2003). Post emergence,
 133 *DR5v2::Venus* expression is similar to the pattern of the primary root, with highest signal at the
 134 apex of the meristem and the columella. *TCSn::Venus* expression was not detected in the LRP
 135 before emergence. This result confirms the previous observations of low level of cytokinin
 136 response in the LRP before emergence made with the first generation TCS reporter (Bielach *et*
 137 *al.*, 2012; Chang *et al.*, 2015; Müller and Sheen, 2008). In type 2 emerged LR, *TCSn::Venus*
 138 expression is first detected in the LR stele, then in the columella. In longer LR, *TCSn::Venus*
 139 expression is visible in the outermost layers of the LR columella, an expression pattern reminiscent
 140 of the primary root one. Thus while auxin signalling pattern marks the position of the LR stem cell
 141 niche from stage IV onward, the complementary cytokinin signalling pattern is only set post
 142 emergence.

143

Fig. 1 | Position and identity of the stem cell niche during lateral root development.

(A) Classification of emerged LR based on their length measured from the xylem strands of the primary root to the tip of the lateral root. (B-D) Micrographs of mature primary and developing lateral roots meristems expressing reporters for (B) auxin (*DR5v2*) or cytokinin (*TCSn*) signalling, (C) for the niche position (*gWOX5* or *SCR*), (D) for QC specific markers (*AGL4*, *BRAVO*, *QC184*, *QC25^{ET}*, *QC25^{Tg}*). *SCR* is a transcriptional reporter (*pSCR::nGFP*), *gWOX5* is a YFP-tagged genomic clone (*WOX5-YFP*), *AGL42* and *BRAVO* are translational reporters. *QC184* and *QC25^{ET}* are *GUS* enhancer trap lines expressing while in *QC25^{Tg}* erCFP is expressed from the cis regulatory sequence located upstream of the *QC25^{ET}* insertion. Images are confocal sections of fluorescent reporters counterstained with Calcofluor white except for *QC184* and *QC25^{ET}* that are widefield images of *GUS* reporters. The apically located cells are indicated with arrowheads. Scale bars: 10 μ m.

158 In the primary root, the expression of *SCARECROW* (*SCR*) and *WOX5* overlap at the
 159 quiescent centre (Shimotohno *et al.*, 2018). In LRP, an analysis of the expression pattern of *SCR*
 160 and *WOX5* using transcriptional reporters concluded that the LR stem cell niche is established by
 161 stage IV, with *WOX5* specifically expressed in the apically located cells of the second outermost
 162 layer at stage IV/V (Goh *et al.*, 2016). We imaged a *SCR* transcriptional reporter (*pSCR::nGFP*)
 163 and confirmed it was first detected in the outer layer of stage II LRP and in later stages formed the
 164 typical arch marking the stem cell niche and endodermis layer, a profile maintained post
 165 emergence (Fig. 1C). We also imaged a YFP-tagged *WOX5* genomic clone (*WOX5-YFP*, (Pi *et*
 166 *al.*, 2015)) and, unlike what was previously reported (Goh *et al.*, 2016), we readily observed
 167 *WOX5-YFP* expression in all cells of stage I-III primordia. From stage IV onward, *WOX5-YFP* was
 168 restricted to the centre of the primordium (Supplemental Fig. S1 and Fig. 1C). Post emergence,
 169 *WOX5-YFP* expression is progressively restricted to three to five cells at the apex of type 4
 170 emerged LR (Fig. 1C). Together, these results suggest that the position of the LR stem cell niche,
 171 defined by the overlap of an auxin maximum and expression of *WOX5* and *SCR* is defined prior
 172 stage IV of LR development, but is only established with cellular precision after emergence,
 173 suggesting its identity is only set then.

174 To verify that a specific niche identity comparable to the primary root is only acquired post-
 175 emergence, we monitored the expression of several markers specific of the QC in the primary root.
 176 We used two enhancer traps, QC184 and QC25^{ET} (Sabatini *et al.*, 2003) and two transcription
 177 factors, BRAVO (Vilarrasa-Blasi *et al.*, 2014) and AGL42 (Nawy *et al.*, 2005). Before LR
 178 emergence, none of these markers were detected (Fig. 1D). They became visible in type 2
 179 (AGL42, BRAVO, QC184) or type 3 (QC25^{ET}) LR (Fig. 1D). The expression of QC25 was
 180 previously reported in pre-emergence LR, in two apically located cells from stage IV onward (Goh
 181 *et al.*, 2016), much earlier than seen here. However the QC25 reporter used in that study is a
 182 transgenic line (QC25^{Tg}, (ten Hove *et al.*, 2010)) which was constructed by isolation of the
 183 sequence flanking the insertion site of the QC25^{ET} enhancer trap used (QC25^{ET}) here. Whereas
 184 in the primary root both versions mark identically the QC, in the developing LR, QC25^{ET} only marks
 185 the apically located cells of the niche post-emergence, suggesting, that complex genomic
 186 regulation might be at play. Together, these results indicate that the position of the LR root apical
 187 meristem is defined before emergence whereas it only expresses specific QC markers once the
 188 LR have emerged.

189
 190
 191

192 The cellular layout of the LR stem cell niche is set post-emergence

193 We then asked when during LR ontogeny, the typical cellular organization of the meristem is
 194 established. The QC of the primary root consists of six to twelve apically located cells arranged in
 195 a monolayered disk (Dolan *et al.*, 1993; Lu *et al.*, 2021). On sections, two to four apically located
 196 cells are typically visible and located between the two cortex-endodermis initials (Fig. 2A). To
 197 determine at which stage of LR development this organising centre is first visible and how many
 198 cells it has, we imaged by confocal microscopy Calcofluor White stained (Ursache *et al.*, 2018)
 199 LRP of 10-day-old *Arabidopsis*. Prior to emergence, apically located cells between the two cortex-
 200 endodermis initials could only be unambiguously observed from stage V onward (Fig. 2A). Until
 201 emergence, their number was variable, with two cells visible only in 40% of the LRP sections
 202 (n=105, Fig. 2B). Post emergence, this variability progressively diminished with most type 4 LR
 203 having two cells visible, like in the primary root (Fig. 2B). We confirmed this progressive maturation
 204 of the LR stem cell niche by counting the number of QC25^{Tg} forming a disk on transverse section
 205 (Fig. 2C, D). While pre-emergence, less than 5 cells were typically observed, this number
 206 increased as the LR mature post-emergence to reach the values seen in the primary root meristem
 207 (Fig. 2D). Together, these data show the typical cellular layout of the root meristem is only fixed
 208 after emergence and indicates that the cellular organization of the LR organising centre
 209 progressively matures. The primary root meristem has been shown to progressively acquire its
 210 organization post-germination (Salvi *et al.*, 2020). We thus asked whether the maturation of the
 211 LR stem cell niche post-emergence follows a similar path to the primary root meristem post-
 212 germination. We counted the number of apically located cells in the primary root meristem during
 213 germination and observed that on average two cells were already visible on section at 0, 24 or 48h
 214 post imbibition (Fig. S2). Thus the establishment of the cellular layout of the primary and lateral
 215 root stem cell niches follow different path.

Fig. 2 | Cellular layout of developing apical meristem of the lateral root.

(A) Confocal sections of the developing root apical meristem and each class of LR counterstained with Calcofluor white in 10-day-old Arabidopsis. The QC or apically located prospective QC cells (arrowheads) and the cortex-endodermal initial (stars) are indicated. Scale bars: 10µm. (B) Number of QC or apically located prospective QC cells on sections for the different classes of roots. (C) Cross-sections of 10-day-old QC25^{Tg} seedlings counterstained with Calcofluor white. (D) Total number of QC cells on sections for the different classes of roots. In B & D, the size of the circle is proportional to the number of observations (Obs.), and the colour represents the relative proportion of observations within a given class. The total number of observations per given classes is indicated (n).

227 **The LR stem cell niche becomes quiescent shortly after emergence**

228 A distinctive feature of the primary root meristem is the low mitotic activity of the QC cells. We thus
 229 asked when cells become quiescent during LR formation. For this we quantified the proportion of
 230 proliferating apically located cells using the G2-S phase label 5-ethynyl-2'-deoxyuridine (EdU)
 231 during LR ontogeny, before and after emergence. Plants expressing the *pSCR::nGFP* reporter
 232 were incubated with EdU for 8h, fixed, and LR were imaged. We quantified the fraction of *SCR*
 233 positive cells in between the two cortex-endodermis initials (apically located cells) that are EdU
 234 positive (Fig. 3A). Before emergence, >75% of apically located cells of stage IV to VI primordia
 235 incorporated EdU (Fig. 3B) indicating that most cells were proliferating. As the primordia were
 236 incubated for 8h with EdU, we concluded that the cycling time of these cells is comparable to the
 237 one previously measured for the LR (~7.5h, (von Wangenheim *et al.*, 2016)). To directly monitor
 238 the mitotic activity of these apically located cells before emergence, we used the cell cycle
 239 progression reporter, Cytrap (Yin *et al.*, 2014), expressing the S/G2 phase marker
 240 *pHTR2::CDT1a(C3)-RFP* that we crossed to the QC25^{Tg} reporter to mark the position of the future
 241 QC cells from stage IV onward (Goh *et al.*, 2016). We confirmed that most of the cells expressing
 242 the QC25^{Tg} reporter expressed the Cytrap S-phase marker indicating that they were mitotically
 243 active (Supplemental Fig. S3). We then used light sheet live imaging of developing LRP expressing
 244 *SCR::nGFP*. We observed several divisions of *SCR*-expressing apically located cells
 245 (Supplemental Fig. S3). Together, these data indicate that before emergence, apically located
 246 cells are actively proliferating and quiescence must occur once the LR emerges.

247 Emerging LR are covered by a cutin-based cuticle (Berhin *et al.*, 2019) that blocks the
 248 uptake of EdU by the young LR (Supplemental Fig. S4) and consequently prevented us to analyse
 249 EdU incorporation in young emerging LR to pinpoint when post-emergence apically located cells
 250 become quiescent. To circumvent this, we monitored EdU incorporation in the *defective in cuticular*
 251 *ridges (dcr)* mutant with altered cutin biogenesis and enhanced permeability (Berhin *et al.*, 2019).
 252 LR emergence in the *dcr* mutant is slower than wild type and some primordia are mis-shaped
 253 (Berhin *et al.*, 2019), nevertheless as in wild type most apically located cells in *dcr* LRP
 254 incorporated EdU before emergence, indicating that the mutation does not impact the proliferative
 255 behaviour of the primordia (Supplemental Fig. S4). After emergence, the proportion of proliferating
 256 apically located cells dropped to less than 25% already in type 2 LR (<250 µm long), indicating
 257 that quiescence is quickly established after emergence (Fig. 3C). To visualise the moment when
 258 apically located cells stop proliferating, we performed live imaging of LR expressing the
 259 Cytrap/QC25^{Tg} reporters and tracked the position of apically located cells with the QC25^{Tg} marker.
 260 We observed the disappearance of the S-phase Cytrap marker in two apically located cells shortly
 261 after emergence (130µm long, Fig. 3D). Together, these results show that quiescence of the LR
 262 niche cells is established shortly after emergence.

Fig. 3 | Post emergence quiescence of the LR stem cell niche

(A) Confocal sections of a pre-emergence lateral root primordium expressing *pSCR::nGFP* (SCR) counterstained with Calcofluor White and the S-phase label EdU. The open arrowheads indicate apically located cells expressing SCR and positive for EdU (8h incubation), while the filled one shows a SCR positive apical cell with no EdU staining. (B) Proportion of proliferating apically located cells in stage IV-VI lateral root primordia. Each point represents a primordium. The number of observed primordia is indicated. The distributions do not differ from each other according to Student's t test with Bonferroni correction. (C) Confocal sections of primary root and lateral root apical regions at different stages of development in 10-day-old *dcr* plants counterstained with Calcofluor White (grey) and the S-phase label EdU (color). Arrowheads indicate the apically located cells. (D) Quantification of the proportion of proliferating apically located cells in the developing root apical meristem at different stages of LR development. The number of observed primordia is indicated. The level of significance of each comparison is indicated (Student's t test with Bonferroni correction). (E) Time lapse confocal imaging of a type 2 emerged LR expressing the S-G2 phase marker CyTrap and the apical cell marker QC25^{Tg}. The arrowheads track the position of two apically located cells. Note the disappearance of the S-phase marker in these cells between 74h and 82h. Hours indicate the time since induction of LR formation by gravistimulation. Scale bars: A,C: 10 μ m, E: 20 μ m.

282 **Biphasic acquisition of the stem cell niche organiser function in LR**

283 The primary root stem cell niche acts as an organising centre that maintains cells in its direct
284 contact in a stem cell state (Bennett et al., 2014; Pi et al., 2015; van den Berg et al., 1997) and
285 generates tissues in both distal (rootward) and proximal (shootward) directions (Rahni *et al.*, 2016).
286 Whereas the position of the LR stem cell niche is set before emergence, quiescence and the final
287 cellular layout are only established after. This raises the question of when is the niche starting to
288 function like an organising centre able to generates tissues in both distal and proximal directions.

289 We first looked at the expression of markers for the proximal (enhancer trap line Q0990
290 (Radoeva *et al.*, 2016)) or distal (*SOMBRERO*, (Bennett *et al.*, 2010)) axis during LR ontogeny. In
291 the primary root, Q0990 expression marks the vascular cells and the QC (Radoeva *et al.*, 2016).
292 In the developing LR, Q0990 is first detected at stage IV, marking the central elongated cells that
293 will form the LR stele and is excluded from the apically located cells whose position correspond to
294 the future stem cell niche (Fig. 4A). It is only post-emergence, in type 2 LR, that Q0990 expression
295 starts to be visible in these apically located cells in addition to the stele, confirming that these cells
296 only acquire a QC-like identity post-emergence (Fig. 4A). In the primary root meristem,
297 *SOMBRERO* (*SMB*) is expressed in all the differentiated cells of the root cap and excluded from
298 the columella stem cells (Bennett *et al.*, 2010) and is thus a marker for differentiation in the distal
299 direction. We did not detect expression of the *SMB* reporter *pSMB::nGFP* in LR before emergence.
300 In LR that just emerged, the reporter is first detected in the distal layers of the root cap, but
301 excluded from the cells abutting the apically located cells (as previously reported by (Du and
302 Scheres, 2017). As LR grow longer, the number of columella layers increases (see below) and
303 these express *SMB*, but not the cells in direct contact to the apically located cells. Taken together,
304 it appears that proliferation in the proximal (shootward) direction is established before LR
305 emergence, while it is only post-emergence that proliferation in the distal direction is set up (Fig.
306 4A). These observations suggest that it is only post emergence that columella stem cells (CSC)
307 are specified. To verify this, we first quantified the number of columella cells layers. In the primary
308 root four to eight layers of columella cells are homeostatically maintained through the balanced
309 activity of the columella stem cell and the sloughing off of the outermost layer of root cap (Fendrych
310 *et al.*, 2014). Before LR emergence, we observed two layers of columella and this number
311 progressively increased to five layers as LR matured post-emergence (Fig. 4B). We then looked
312 at the distribution of starch granules, a marker of differentiated columella cells typically absent
313 from the CSC (van den Berg *et al.*, 1995), in developing LR. mPS-PI staining (Truernit *et al.*, 2008)
314 of LR showed that starch granules are first formed post-emergence in columella cells, confirming
315 previous observations (Guyomarc'h et al., 2012; Kiss et al., 2002), but also revealed starch in the
316 cells directly abutting the apically located cells. Interestingly, a layer of cells devoid of starch
317 granules next to the apically located cells is formed only in type 5 LR (>2mm) (Fig. 4C). Together

318 these data show that before emergence the apically located cells are able to maintain as stem cell
319 the abutting cells in the proximal (shootward) direction but only acquire this capacity post-
320 emergence in the distal (rootward) direction.

321 To confirm this, we induced clones in the *SCR* domain and checked whether the clones
322 derived from the central cells marked the proximal and/or distal domains. Observation of post-
323 emergence LR expressing the *p35S::lox-Ter-lox:erCFP* / *pSCR::CRE-GR* construct (Efroni *et al.*,
324 2016) and treated with DEX for 38h resulted in erCFP signal in the apically located cells, the CEIs,
325 and some endodermal cells. We also observed erCFP expression in the provascular cells directly
326 adjacent to the apically located cells but none in the cells located distally (Fig. 4D). Thus, the LR
327 stem cell niche acquires its ability to bidirectionally generate tissues in two phases: it contributes
328 to growth in the proximal direction before emergence and in the distal direction only post-
329 emergence, concomitantly to the establishment of its mitotic quiescence and expression of specific
330 marker genes.

331

Fig. 4 | Biphasic acquisition of the stem cell niche organiser function in LR

(A) Confocal sections of calcofluor white counterstained primary root meristem and each class of LR in 10-day-old Arabidopsis expressing the proximal (*Q0990*) and distal (*SMB*) identities reporters. The open arrowheads indicate apically located cells in lateral roots and the QC in the primary root. (B) Number of columella cell layers in the primary root and for the different classes of lateral roots. The size of the circle is proportional to the number of observations (Obs.), and the colour represents the relative proportion of observations within a given class. (C) Confocal sections of mPS-PI stained primary root meristem and each class of LR in 10-day-old Arabidopsis. The open arrowheads indicate apically located cells in lateral roots and the QC in the primary root while the blue arrow point the layer of columella stem cells. (D) Confocal section of a PI counterstained type 2 lateral root (length: 112µm) 38h after generation of clones in the *SCR* domain by dexamethasone treatment. Arrowheads indicate provascular initials resulting from the division of the *SCR*-expressing apically located cells. Scale bars are 10µm (A, D) and 20µm (C).

347 Discussion

348 Here, we characterised the ontogeny of the root apical meristem (RAM) in lateral root, with a
 349 particular emphasis on the QC. We used reporters for tissue identity, live imaging, lineage tracing
 350 and cell proliferation assay and conclude that while the QC position and its role in proximal
 351 (shootward) growth are set early during LR formation, its molecular identity, cellular organization
 352 and quiescence are only fixed after LR emergence. The different attributes of the primary RAM
 353 QC are thus progressively acquired during LR formation, suggesting a progressive maturation
 354 process of this post-embryonic meristem.

355
 356 Despite the similarities in cellular organisation and gene expression profiles between the primary
 357 and LR RAM and the essential role played by the QC in organising and coordinating the primary
 358 RAM (Rahni *et al.*, 2016), there were no comprehensive analysis of the ontogeny of the QC in LR.
 359 Previous reports analysed either the expression patterns of genes of the primary RAM during LR
 360 formation (Tian *et al.*, 2014a) or focused on cell differentiation associated with acquisition of
 361 gravitropic and phototropic responses in LR (Guyomarc'h *et al.*, 2012; Kiss *et al.*, 2002; Waidmann
 362 *et al.*, 2020). Our analysis reveals interesting insights into the mechanisms of LR RAM maturation.

363
 364 Like in the primary RAM, the pattern of auxin signalling and of *SCR* and *WOX5* expression defines
 365 the position of the QC during the early stages of LR development. This result agrees with a prior
 366 report (Goh *et al.*, 2016) and supports the view that a key transition in LR organogenesis occurs
 367 at the stage IV-V transition, when the primordium breaks through the overlying endodermis layer,
 368 becomes radially symmetric (Lucas *et al.*, 2013) and acquires autonomy relative to the auxin fluxes
 369 from the parent root (Laskowski *et al.*, 1995). Yet, our observations nuance the conclusion that the
 370 LR QC is established at this early stage (Goh *et al.*, 2016). Indeed, we observe variation in the
 371 cellular organisation of the LR RAM prior to emergence and a primary root like cellular organisation
 372 of the RAM is only fixed once the LR has emerged. Our analysis of cell proliferation using EdU
 373 incorporation or long term imaging revealed that quiescence of the apically located cells in the LR
 374 RAM is only established after emergence. This quiescence is concurrent to the onset of expression
 375 of most molecular markers associated with the primary RAM (*AGL42*, *BRAVO*, *QC184* and
 376 *QC25^{ET}*). An exception being *QC25^{Tg}* which marks the position of the apically located cells from
 377 stage IV/V albeit a stage when these cells still proliferate. The difference of behaviour between
 378 *QC25^{Tg}* and *QC25^{ET}* reporters suggests that whereas the positional cues responsible for the
 379 localised expression of the *QC25^{Tg}* reporter are present in its promoter sequence, they are
 380 overridden in the enhancer trap line until the cells become quiescent. In addition to revealing the
 381 existence of genomic regulations associated with the quiescence of the apically located cells, this
 382 results support the temporal decoupling between positioning the prospective LR QC cells and their

actual maturation and entry into quiescence. Our results support that the position of the LR QC is set before emergence but only activated post-emergence as it had been suggested earlier (Celenza *et al.*, 1995; Malamy and Benfey, 1997). A recent single cell analysis of the developing LR has shown that expression of genes typically associated with the QC in the primary RAM can be readily detected in stages I to III LR, yet distributed amongst different clusters that only form a distinct coherent population at later stages (Serrano-Ron *et al.*, 2021). Our observation of *WOX5-YFP* expression in most cells of stage II LR supports this notion of a progressive specification of cell identities in the early phases of LR organogenesis. This early expression of *WOX5* is raising questions about the role played by this essential factor for QC identity at early stages. Interestingly, (Goh *et al.*, 2016) reported a very specific onset of *WOX5* expression in four apically located cells at stage IV/V, in apparent contradiction to our and others observations (Ditengou *et al.*, 2008; Du and Scheres, 2017). The absence of essential cis-regulatory element(s) in the transcriptional reporter used by Goh *et al.* (*pWOX5::n3GFP*) compared to the YFP-tagged genomic clone used (Pi *et al.*, 2015) could account for the differences in expression pattern.

Current models assign to the QC an organizer function that signals to the surrounding cells and actively maintains their “stemness”. An alternative view is that the position of the stem cells organizer results from two opposing axis that reciprocally control each other’s differentiation (Rahni *et al.*, 2016). In this so called “stagnation” model, the QC corresponds to the location where cells are not displaced by their neighbours as result of the activity of shootward and rootward gradients of division and differentiation. Where these two gradients meet, cell division is minimal and a stagnation zone is created on either side of which more rapidly dividing cells are bound to displaced their daughter cells in opposite directions (Rahni *et al.*, 2016). In this model, the stemness of stem cells is the result of a passive mechanical process and does not rely on quiescence of the QC. Our results show that the growth axis of the LR are established in distinct phases with the proximal (shootward) growth axis established pre-emergence and the distal (rootward) one after emergence, concomitantly to the stabilisation of number of prospective QC cells, the slowdown of their cell division rate and the activation of QC specific markers. These data support the stagnation model in which the position and maturation of the LR QC cells is fixed once the two opposite gradients are established. Activation of QC specific identities and entry in quiescence would be an emerging property of the slowdown in cell division rate at the position where the two growth axis meet. Quiescence, a late emerging property in LR, would thus not be instrumental in organizing the stem cell niche, but a results of the two diverging growth axis, alike the region of mostly calm weather at the center of a cyclone results from diverging convection flows (Rahni *et al.*, 2016). This shift in the importance of the QC slow mitotic activity is supported by the observation that loss of QC-quiescence has no effect on root growth (Cruz-Ramírez *et al.*, 2013).

420 **Material and Methods**

421 **Plant material and growth conditions**

422 We used the previously described lines: *DR5v2::3xYFP:NLS* (sC111) (Vilches Barro *et al.*, 2019),
 423 *pTCSn::2xVenus:NLS* (El Arbi *et al.*, 2021), *gWOX5::WOX5:YFP* in *wox5-1* (Pi *et al.*, 2015),
 424 *pAGL42::truncAGL42:GFP* (Nawy *et al.*, 2005), *pBRAVO::BRAVO:GFP* (Vilarrasa-Blasi *et al.*,
 425 2014), *pQC184::GUS* (in the *gWOX5::WOX5:YFP* in *wox5-1*) (Pi *et al.*, 2015), *pQC25er::CFP*
 426 (*QC25^{T9}*) (Goh *et al.*, 2016), Cytrap (*pHTR2::CDT1a (C3)-RFP*, *pCYCB1::CYCB1;1-GFP*) (Yin *et*
 427 *al.*, 2014) and *pSCR>>CRE:GR/35S:lox-terminator-lox-CFP* (Efroni *et al.*, 2016) The enhancer
 428 trap lines Q0990 (Radoeva *et al.*, 2016), *QC25^{ET}* (Sabatini *et al.*, 2003) were described previously.
 429 For crosses between lines, F1 generation was used. The *dcr* mutant was previously described
 430 (Berhin *et al.*, 2019). Seeds were surface sterilized (Sodium hypochlorite 5 % and 0.05 % Tween
 431 20 or Ethanol 70 % and 0.05% Triton X-100) and placed on ½ Murashige and Skoog (MS) medium
 432 containing 0.8 % agar (Duchefa). Following stratification (4°C in the dark, > 24 h), seedlings were
 433 grown at 22°C vertically under long day conditions (16 h light / 8 h dark). For the lineage tracking
 434 experiments 15µM dexamethasone were used for 24h.

435

436 **Construction of vectors and plant transformation**

437 The transcriptional reporters *pSCR::nGFP* (*pSCR::H2B-3xGFP::tRBCS*) and *pSMB::nGFP*
 438 (*pSMB::H2B-3xGFP::tRBCS*) reporters were generated using GreenGate assembly
 439 (Lampropoulos *et al.*, 2013). For *pSCR*, 2114bp upstream of ATG were used and 3246bp for
 440 *pSMB*. *Agrobacterium tumefaciens* (Agl-0) based plant transformation was carried out using the
 441 floral dip method (Clough and Bent, 1998). All plant lines examined were homozygous if not
 442 indicated otherwise. Homozygosity was determined by antibiotic resistance and verification of the
 443 fluorescent fusion proteins at the microscope.

444

445 **Microscopy**

446 Unless otherwise specified, imaging was performed on seedlings fixed for 30 min with PFA 4% in
 447 PBS 1X at room temperature, Calco Fluor white staining was performed as previously described
 448 (Ursache *et al.*, 2018). Seedlings were cleared with either 50% glycerol for 24h or ClearSee
 449 solution for up to a week (Kurihara *et al.*, 2015). Root meristems were imaged on medial sections
 450 on a Leica SP8 confocal microscope with 63x, NA = 1.4 oil immersion objective or a 40x, NA = 1.3
 451 oil immersion objectives. Calcofluor White fluorescence was detected using the 405nm excitation
 452 laser line, and 425-475nm emission range. The number of apically located cells is obtained by
 453 counting all cells between the cortex-endodermis initial. For Edu incorporation, Alexa fluor 647nm
 454 detection was achieved using the 638 nm excitation laser line in the 650-695nm range. For the
 455 Cytrap cell cycle progression reporter (Yin *et al.*, 2014), the S and G2 phases marker

456 pHTR2::CDTa (C3)-RFP was detected using the 552nm nm excitation laser line in the 600nm to
457 650nm emission range. The G2/mitosis phases marker was detected using the 488nm excitation
458 laser line in the 500 to 550nm emission range. For live imaging, a Leica SP8 was used with a 20X
459 NA= 1.2 oil immersion objective. Lateral root growth was induced by 52h of gravistimulation (180°)
460 in seedlings of 4 to 6 days after germination (dpg). Seedlings were placed in an imaging chamber
461 and covered with a piece of ½ MS medium for imaging, as described in (Marhavy and Benkova,
462 2015). Stacks were acquired every 2 hours with 1µm z-steps. For live imaging of the SCR reporter,
463 the *pSCR::nGFP* reporter was crossed to a line expressing *pUBQ10::PIP1,4:GFPx3*;
464 *pGATA23::H2B:nCherryx3* (stPVB003, (Vilches Barro *et al.*, 2019) and imaging was done on a
465 MuViSPIM (Luxendo, Bruker, Germany) light sheet microscope equipped with a Nikon NIR Apo
466 40X NA=1.2 water objective for detection. Movies were acquired with a z-step of 0.250µm, and a
467 temporal resolution of 30 min.

468

469

470 **Histochemical analysis**

471 Staining of starch granule in the columella cells was performed as described previously (Truernit
472 *et al.*, 2008). GUS staining was performed as described previously (Maizel and Weigel, 2004)
473 followed by fixation in 4%HCl, 20% methanol solution, for 15 min at 70°C, followed by 7%NaOH
474 and 60% ethanol for 15 min at room temperature. Seedlings were then cleared in successive
475 ethanol baths for 10 mins (40%, 20%, 10%) , followed by 10 min incubation in 25% glycerol and
476 5% ethanol. Finally seedlings are placed in 50% glycerol and stored at 4°C until imaging. Seedlings
477 were mounted in 50% glycerol for imaging with DIC microscopy using an Axio Imager. M1 (Carl
478 Zeiss, Oberkochen, Germany) with a 40X objective.

479

480 **EdU incorporation**

481 For Edu incorporation in LRP before emergence, LR formation was induced by 30h, 36h, or 42h
482 of gravistimulation, and seedling were transferred on plates containing 1µM of EdU in ½ MS plates
483 for the last 8h of the gravistimulation period. For Edu incorporation in emerged lateral root (in *dcr*
484 mutant), LR was induced by 48h, 72h, 96h or 120h of gravistimulation, and EdU incorporation was
485 performed during the last 24h of the gravistimulation using 0.5 µM of EdU in ½ MS plates. EdU
486 detection was performed using a slightly modified version of the previously described protocol
487 (Kotogány *et al.*, 2010). Briefly, seedlings were fixed for 15 min at room temperature in the dark
488 with 4% PFA in PBS, washed 2 times 1 min with 3% BSA in PBS, incubated 20 min with 0.5%
489 Triton X-100 in PBS and washed 2 times 1 min with 3% BSA in PBS, then incubated with the Click-
490 iT™ (Click-iT™ EdU Cell Proliferation Kit for Imaging, Alexa Fluor™ 647™ Invitrogen, Thermofisher
491 , C10340) reaction cocktail for 30 min at room temperature in the dark, rinsed with PBS and
492 washed with 3% BSA in PBS 3 times for 1 min. Seedlings were then washed 2 times with PBS

493 and cell walls were stained with 0.1% Calco fluor white (Fluorescent brightener 28 F3543-1G
494 Sigma-Aldrich) in PBS for 30 min in the dark. After rinsing, seedlings were transferred to 50%
495 glycerol for at least 16h before imaging. Apically located cells showing EdU incorporation or
496 expressing the pHTR2::CDTa (C3)-RFP marker (Cytrap) were considered as proliferative. The
497 proportion of proliferating apically located cells was obtained by dividing the number of proliferative
498 apically located cells divided by the total number of apical cell on that section.

499 **Supplementary data**

500 Supplementary data are available online.

501 Fig. S1. Expression of WOX5 in early lateral root primordia

502 Fig. S2. Expression of SCR during germination

503 Fig. S3. Apically located cells stop to proliferate post-emergence

504 Fig. S4. EdU incorporation and cell proliferation in LR of wild type and dcr mutant

505

506 **Acknowledgements**

507 We thank J. Dubrovsky for critically reading the manuscript, and the Caño-Delgado, Efroni, Greb, Laplaze,
508 Laux, Lohmann, Nawrath and Palatnik labs for sharing resources. The authors gratefully acknowledge the
509 data storage service SDS@hd supported by the Ministry of Science, Research and the Arts Baden-
510 Württemberg (MWK) and the German Research Foundation (DFG) through grant INST 35/1314-1 FUGG
511 and INST 35/1503-1 FUGG.

512

513 **Author contribution**

514 Conceptualization: AM

515 Data Acquisition and curation: BB, AM

516 Formal Analysis: BB, AM

517 Funding Acquisition: AM

518 Methodology: AM, BB, LB, ML

519 Project Administration: AM

520 Resources: BB, LB, ML

521 Supervision: AM

522 Visualization: BB, AM

523 Writing – Original Draft: BB, AM

524 Writing – Review and Editing: BB, AM

525

526

527 **Conflicts of interests**

528 The authors declare no competing interests.

529

530 **Funding**

531 This work was supported by the DFG FOR2581.

532

533 **Data availability**

534 The data supporting the findings of this study are available from the corresponding author upon
535 request.

536 REFERENCES

- 537 **Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G. and**
538 **Friml, J.** (2003). Local, Efflux-Dependent Auxin Gradients as a Common Module for Plant
539 Organ Formation. *Cell* **115**, 591–602.
- 540 **Bennett, T., van den Toorn, A., Sanchez-Perez, G. F., Campilho, A., Willemsen, V., Snel, B.**
541 **and Scheres, B.** (2010). SOMBRERO, BEARSKIN1, and BEARSKIN2 regulate root cap
542 maturation in Arabidopsis. *Plant Cell* **22**, 640–654.
- 543 **Bennett, T., van den Toorn, A., Willemsen, V. and Scheres, B.** (2014). Precise control of plant
544 stem cell activity through parallel regulatory inputs. *Development* **141**, 4055–4064.
- 545 **Berhin, A., de Bellis, D., Franke, R. B., Buono, R. A., Nowack, M. K. and Nawrath, C.** (2019).
546 The Root Cap Cuticle: A Cell Wall Structure for Seedling Establishment and Lateral Root
547 Formation. *Cell* **176**, 1367–1378.e8.
- 548 **Bielach, A., Podlesáková, K., Marhavy, P., Duclercq, J., Cuesta, C., Müller, B., Grunewald,**
549 **W., Tarkowski, P. and Benková, E.** (2012). Spatiotemporal regulation of lateral root
550 organogenesis in Arabidopsis by cytokinin. *Plant Cell* **24**, 3967–3981.
- 551 **Bishopp, A., Benkova, E. and Helariutta, Y.** (2011). Sending mixed messages: auxin-cytokinin
552 crosstalk in roots. *Curr. Opin. Plant Biol.* **14**, 10–16.
- 553 **Brumos, J., Robles, L. M., Yun, J., Vu, T. C., Jackson, S., Alonso, J. M. and Stepanova, A. N.**
554 (2018). Local Auxin Biosynthesis Is a Key Regulator of Plant Development. *Dev. Cell* **47**,
555 306–318.e5.
- 556 **Byrne, J. M.** (1973). The root apex of *Malva sylvestris*. Iii. Lateral root development and the
557 quiescent center. *Am. J. Bot.* **60**, 657–662.
- 558 **Celenza, J. L., Grisafi, P. L. and Fink, G. R.** (1995). A pathway for lateral root formation in
559 *Arabidopsis thaliana*. *Genes Dev.* **9**, 2131–2142.
- 560 **Chang, L., Ramireddy, E. and Schmölling, T.** (2015). Cytokinin as a positional cue regulating
561 lateral root spacing in Arabidopsis. *J. Exp. Bot.* **66**, 4759–4768.
- 562 **Clough, S. J. and Bent, A. F.** (1998). Floral dip: a simplified method for *Agrobacterium*-mediated
563 transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- 564 **Clowes, F. A. L.** (1954). The Promeristem and the Minimal Constructional Centre in Grass Root
565 Apices. *New Phytol.* **53**, 108–116.
- 566 **Clowes, F. A. L.** (1956). Nucleic Acids in Root Apical Meristems of *Zea*. *New Phytol.* **55**, 29–34.
- 567 **Clowes, F. A. L.** (1958). Development of Quiescent Centres in Root Meristems. *New Phytol.* **57**,
568 85–88.
- 569 **Clowes, F. A. L.** (1959). Reorganization of Root Apices after Irradiation. *Ann. Bot.* **23**, 205–210.
- 570 **Clowes, F. A. L.** (1978). Origin of the quiescent centre in *Zea mays*. *New Phytol.* **80**, 409–419.
- 571 **Cruz-Ramírez, A., Díaz-Triviño, S., Wachsman, G., Du, Y., Arteaga-Vázquez, M., Zhang, H.,**
572 **Benjamins, R., Blilou, I., Neef, A. B., Chandler, V., et al.** (2013). A SCARECROW-
573 RETINOBLASTOMA Protein Network Controls Protective Quiescence in the Arabidopsis
574 Root Stem Cell Organizer. *PLoS Biol.* **11**, e1001724.

- 575 **Della Rovere, F., Fattorini, L., D'Angeli, S., Veloccia, A., Falasca, G. and Altamura, M. M.**
576 (2013). Auxin and cytokinin control formation of the quiescent centre in the adventitious
577 root apex of arabidopsis. *Ann. Bot.* **112**, 1395–1407.
- 578 **Ditengou, F. A., Teale, W. D., Kochersperger, P., Flittner, K. A., Kneuper, I., van der Graaff,**
579 **E., Nziengui, H., Pinosa, F., Li, X., Nitschke, R., et al.** (2008). Mechanical induction of
580 lateral root initiation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 18818–
581 18823.
- 582 **Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K. and Scheres, B.**
583 (1993). Cellular organisation of the *Arabidopsis thaliana* root. *Development* **119**, 71–84.
- 584 **Du, Y. and Scheres, B.** (2017). PLETHORA transcription factors orchestrate de novo organ
585 patterning during Arabidopsis lateral root outgrowth. *Proc. Natl. Acad. Sci. U. S. A.* **121**,
586 201714410.
- 587 **Dubrovsky, J. G. and Ivanov, V. B.** (2021). The quiescent centre of the root apical meristem:
588 Conceptual developments from Clowes to modern times. *J. Exp. Bot.*
- 589 **Efroni, I., Mello, A., Nawy, T., Ip, P.-L., Rahni, R., DelRose, N., Powers, A., Satija, R. and**
590 **Birnbaum, K. D.** (2016). Root Regeneration Triggers an Embryo-like Sequence Guided
591 by Hormonal Interactions. *Cell* **165**, 1721–1733.
- 592 **El Arbi, N., Schürholz, A.-K., Schiffner, A., Prados, I. H., Böhme, F., Wenzl, C., Zhao, X.,**
593 **Zeng, J., Lohmann, J. U. and Wolf, S.** (2021). ARGONAUTE10 is required for cell fate
594 specification and the control of formative cell divisions in the *Arabidopsis* root meristem.
595 *Cold Spring Harbor Laboratory* 2021.02.05.429893.
- 596 **Fendrych, M., Van Hautegeem, T., Van Durme, M., Olvera-Carrillo, Y., Huysmans, M., Karimi,**
597 **M., Lippens, S., Guérin, C. J., Krebs, M., Schumacher, K., et al.** (2014). Programmed
598 Cell Death Controlled by ANAC033/SOMBRERO Determines Root Cap Organ Size in
599 *Arabidopsis*. *Curr. Biol.* **24**, 931–940.
- 600 **Fulcher, N. and Sablowski, R.** (2009). Hypersensitivity to DNA damage in plant stem cell niches.
601 *Proc. Natl. Acad. Sci. U. S. A.* **106**, 20984–20988.
- 602 **Goh, T., Toyokura, K., Wells, D. M., Swarup, K., Yamamoto, M., Mimura, T., Weijers, D.,**
603 **Fukaki, H., Laplace, L., Bennett, M. J., et al.** (2016). Quiescent center initiation in the
604 *Arabidopsis* lateral root primordia is dependent on the SCARECROW transcription factor.
605 *Development* **143**, 3363–3371.
- 606 **Greb, T. and Lohmann, J. U.** (2016). Plant Stem Cells. *Curr. Biol.* **26**, R816-21.
- 607 **Guyomarc'h, S., Leran, S., Auzon-Cape, M., Perrine-Walker, F., Lucas, M. and Laplace, L.**
608 (2012). Early development and gravitropic response of lateral roots in *Arabidopsis thaliana*.
609 *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **367**, 1509–1516.
- 610 **Haecker, A., Gross-Hardt, R., Geiges, B., Sarkar, A., Breuninger, H., Herrmann, M. and Laux,**
611 **T.** (2004). Expression dynamics of WOX genes mark cell fate decisions during early
612 embryonic patterning in *Arabidopsis thaliana*. *Development* **131**, 657–668.
- 613 **Jiang, K., Meng, Y. L. and Feldman, L. J.** (2003). Quiescent center formation in maize roots is
614 associated with an auxin-regulated oxidizing environment. *Development* **130**, 1429–1438.
- 615 **Kiss, J. Z., Miller, K. M., Ogden, L. A. and Roth, K. K.** (2002). Phototropism and gravitropism in
616 lateral roots of *Arabidopsis*. *Plant Cell Physiol.* **43**, 35–43.

- 617 **Kotogány, E., Dudits, D., Horváth, G. V. and Ayaydin, F.** (2010). A rapid and robust assay for
618 detection of S-phase cell cycle progression in plant cells and tissues by using ethynyl
619 deoxyuridine. *Plant Methods* **6**, 5.
- 620 **Kurihara, D., Mizuta, Y., Sato, Y. and Higashiyama, T.** (2015). ClearSee: a rapid optical clearing
621 reagent for whole-plant fluorescence imaging. *Development* **142**, 4168–4179.
- 622 **Lampropoulos, A., Sutikovic, Z., Wenzl, C., Maegele, I., Lohmann, J. U. and Forner, J.** (2013).
623 GreenGate - A Novel, Versatile, and Efficient Cloning System for Plant Transgenesis. *PLoS*
624 *One* **8**, e83043.
- 625 **Laskowski, M. J., Williams, M. E., Nusbaum, H. C. and Sussex, I. M.** (1995). Formation of
626 lateral root meristems is a two-stage process. *Development* **121**, 3303–3310.
- 627 **Liao, C.-Y., Smet, W., Brunoud, G., Yoshida, S., Vernoux, T. and Weijers, D.** (2015). Reporters
628 for sensitive and quantitative measurement of auxin response. *Nat. Methods* **12**, 207–210.
- 629 **Lu, R., Canher, B., Bisht, A., Heyman, J. and De Veylder, L.** (2021). Three-dimensional
630 quantitative analysis of the Arabidopsis quiescent centre. *J. Exp. Bot.* **72**, 6789–6800.
- 631 **Lucas, M., Kenobi, K., von Wangenheim, D., VOß, U., Swarup, K., De Smet, I., Van Damme,**
632 **D., Lawrence, T., Péret, B., Moscardi, E., et al.** (2013). Lateral root morphogenesis is
633 dependent on the mechanical properties of the overlaying tissues. *Proc. Natl. Acad. Sci.*
634 *U. S. A.* **110**, 5229–5234.
- 635 **Macleod, R. D.** (1977). Proliferating and Quiescent Cells in the Apical Meristem of Elongating
636 Lateral Roots of *Vicia faba* L. *Ann. Bot.* **41**, 321–329.
- 637 **Maizel, A. and Weigel, D.** (2004). Temporally and spatially controlled induction of gene
638 expression in Arabidopsis thaliana. *Plant J.* **38**, 164–171.
- 639 **Malamy, J. E. and Benfey, P. N.** (1997). Organization and cell differentiation in lateral roots of
640 Arabidopsis thaliana. *Development* **124**, 33–44.
- 641 **Marhavy, P. and Benkova, E.** (2015). Real-time Analysis of Lateral Root Organogenesis in
642 Arabidopsis. *BIO-PROTOCOL* **5**,.
- 643 **Marhavý, P., Duclercq, J., Weller, B., Feraru, E., Bielach, A., Offringa, R., Friml, J.,**
644 **Schwechheimer, C., Murphy, A. and Benková, E.** (2014). Cytokinin Controls Polarity of
645 PIN1-Dependent Auxin Transport during Lateral Root Organogenesis. *Curr. Biol.* **24**,
646 1031–1037.
- 647 **Montesinos, J. C., Abuzeineh, A., Kopf, A., Juanes-Garcia, A., Ötvös, K., Petrášek, J., Sixt,**
648 **M. and Benková, E.** (2020). Phytohormone cytokinin guides microtubule dynamics during
649 cell progression from proliferative to differentiated stage. *EMBO J.* **39**, e104238.
- 650 **Motte, H., Vanneste, S. and Beeckman, T.** (2019). Molecular and Environmental Regulation of
651 Root Development. *Annu. Rev. Plant Biol.* **70**,.
- 652 **Müller, B. and Sheen, J.** (2008). Cytokinin and auxin interaction in root stem-cell specification
653 during early embryogenesis. *Nature* **453**, 1094–1097.
- 654 **Nawy, T., Lee, J.-Y., Colinas, J., Wang, J. Y., Thongrod, S. C., Malamy, J. E., Birnbaum, K.**
655 **and Benfey, P. N.** (2005). Transcriptional profile of the Arabidopsis root quiescent center.
656 *Plant Cell* **17**, 1908–1925.

- 657 **Nenadić, M. and Vermeer, J. E. M.** (2021). Dynamic cytokinin signalling landscapes during lateral
658 root formation in Arabidopsis. *Quantitative Plant Biology* **2**,.
- 659 **Petersson, S. V., Johansson, A. I., Kowalczyk, M., Makoveychuk, A., Wang, J. Y., Moritz, T.,**
660 **Grebe, M., Benfey, P. N., Sandberg, G. and Ljung, K.** (2009). An Auxin Gradient and
661 Maximum in the Arabidopsis Root Apex Shown by High-Resolution Cell-Specific Analysis
662 of IAA Distribution and Synthesis. *Plant Cell* **21**, 1659–1668.
- 663 **Pi, L., Aichinger, E., van der Graaff, E., Llavata Peris, C. I., Weijers, D., Hennig, L., Groot, E.**
664 **and Laux, T.** (2015). Organizer-Derived WOX5 Signal Maintains Root Columella Stem
665 Cells through Chromatin-Mediated Repression of CDF4 Expression. *Dev. Cell* **33**, 576–
666 588.
- 667 **Radoeva, T., Ten Hove, C. A., Saiga, S. and Weijers, D.** (2016). Molecular Characterization of
668 Arabidopsis GAL4/UAS Enhancer Trap Lines Identifies Novel Cell-Type-Specific
669 Promoters. *Plant Physiol.* **171**, 1169–1181.
- 670 **Rahni, R., Efroni, I. and Birnbaum, K. D.** (2016). A Case for Distributed Control of Local Stem
671 Cell Behavior in Plants. *Dev. Cell* **38**, 635–642.
- 672 **Sabatini, S., Beis, D., Wolkenfelt, H., Murfett, J., Guilfoyle, T., Malamy, J., Benfey, P., Leyser,**
673 **O., Bechtold, N., Weisbeek, P., et al.** (1999). An auxin-dependent distal organizer of
674 pattern and polarity in the Arabidopsis root. *Cell* **99**, 463–472.
- 675 **Sabatini, S., Heidstra, R., Wildwater, M. and Scheres, B.** (2003). SCARECROW is involved in
676 positioning the stem cell niche in the Arabidopsis root meristem. *Genes Dev.* **17**, 354–358.
- 677 **Salvi, E., Rutten, J. P., Di Mambro, R., Polverari, L., Licursi, V., Negri, R., Dello Iorio, R.,**
678 **Sabatini, S. and Ten Tusscher, K.** (2020). A Self-Organized PLT/Auxin/ARR-B Network
679 Controls the Dynamics of Root Zonation Development in Arabidopsis thaliana. *Dev. Cell*
680 **53**, 431-443.e23.
- 681 **Scheres, B., Wolkenfelt, H., Willemsen, V., Terlouw, M., Lawson, E., Dean, C. and Weisbeek,**
682 **P.** (1994). Embryonic origin of the Arabidopsis primary root and root meristem initials.
683 *Development* **120**, 2475–2487.
- 684 **Serrano-Ron, L., Perez-Garcia, P., Sanchez-Corrienero, A., Gude, I., Cabrera, J., Ip, P.-L.,**
685 **Birnbaum, K. D. and Moreno-Risueno, M. A.** (2021). Reconstruction of lateral root
686 formation through single-cell RNA sequencing reveals order of tissue initiation. *Mol. Plant*
687 **14**, 1362–1378.
- 688 **Shimotohno, A., Heidstra, R., Blilou, I. and Scheres, B.** (2018). Root stem cell niche organizer
689 specification by molecular convergence of PLETHORA and SCARECROW transcription
690 factor modules. *Genes Dev.* **32**, 1085–1100.
- 691 **ten Hove, C. A., Willemsen, V., de Vries, W. J., van Dijken, A., Scheres, B. and Heidstra, R.**
692 (2010). SCHIZORIZA Encodes a Nuclear Factor Regulating Asymmetry of Stem Cell
693 Divisions in the Arabidopsis Root. *Curr. Biol.* **20**, 452–457.
- 694 **ten Hove, C. A., Lu, K.-J. and Weijers, D.** (2015). Building a plant: cell fate specification in the
695 early Arabidopsis embryo. *Development* **142**, 420–430.
- 696 **Tian, H., Jia, Y., Niu, T., Yu, Q. and Ding, Z.** (2014a). The key players of the primary root growth
697 and development also function in lateral roots in Arabidopsis. *Plant Cell Rep.* **33**, 745–753.
- 698 **Tian, H., De Smet, I. and Ding, Z.** (2014b). Shaping a root system: regulating lateral versus
699 primary root growth. *Trends Plant Sci.* **19**, 426–431.

700 **Truernit, E., Bauby, H., Dubreucq, B., Grandjean, O., Runions, J., Barthelemy, J. and**
701 **Palauqui, J. C.** (2008). High-Resolution Whole-Mount Imaging of Three-Dimensional
702 Tissue Organization and Gene Expression Enables the Study of Phloem Development and
703 Structure in Arabidopsis. *Plant Cell* **20**, 1494–1503.

704 **Tsugeki, R. and Fedoroff, N. V.** (1999). Genetic ablation of root cap cells in Arabidopsis. *Proc.*
705 *Natl. Acad. Sci. U. S. A.* **96**, 12941–12946.

706 **Ursache, R., Andersen, T. G., Marhavý, P. and Geldner, N.** (2018). A protocol for combining
707 fluorescent proteins with histological stains for diverse cell wall components. *Plant J.* **93**,
708 399–412.

709 **van den Berg, C., Willemsen, V., Hage, W., Weisbeek, P. and Scheres, B.** (1995). Cell fate in
710 the Arabidopsis root meristem determined by directional signalling. *Nature* **378**, 62–65.

711 **van den Berg, C., Willemsen, V., Hendriks, G., Weisbeek, P. and Scheres, B.** (1997). Short-
712 range control of cell differentiation in the Arabidopsis root meristem. *Nature* **390**, 287–289.

713 **Vilarrasa-Blasi, J., González-García, M.-P., Frigola, D., Fàbregas, N., Alexiou, K. G., López-**
714 **Bigas, N., Rivas, S., Jauneau, A., Lohmann, J. U., Benfey, P. N., et al.** (2014).
715 Regulation of Plant Stem Cell Quiescence by a Brassinosteroid Signaling Module. *Dev.*
716 *Cell* **30**, 36–47.

717 **Vilches Barro, A., Stöckle, D., Thellmann, M., Ruiz-Duarte, P., Bald, L., Louveaux, M., von**
718 **Born, P., Denninger, P., Goh, T., Fukaki, H., et al.** (2019). Cytoskeleton Dynamics Are
719 Necessary for Early Events of Lateral Root Initiation in Arabidopsis. *Curr. Biol.* **29**, 2443-
720 2454.e5.

721 **von Wangenheim, D., Fangerau, J., Schmitz, A., Smith, R. S., Leitte, H., Stelzer, E. H. K. and**
722 **Maizel, A.** (2016). Rules and Self-Organizing Properties of Post-embryonic Plant Organ
723 Cell Division Patterns. *Curr. Biol.* **26**, 439–449.

724 **Waidmann, S., Sarkel, E. and Kleine-Vehn, J.** (2020). Same same, but different: growth
725 responses of primary and lateral roots. *J. Exp. Bot.* eraa027.

726 **Yin, K., Ueda, M., Takagi, H., Kajihara, T., Sugamata Aki, S., Nobusawa, T., Umeda-Hara, C.**
727 **and Umeda, M.** (2014). A dual-color marker system for in vivovisualization of cell cycle
728 progression in Arabidopsis. *Plant J.* **80**, 541–552.

729 **Zürcher, E., Tavor-Deslex, D., Lituiev, D., Enkerli, K., Tarr, P. T. and Müller, B.** (2013). A robust
730 and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin
731 signaling network in planta. *Plant Physiol.* **161**, 1066–1075.

732

733 **Supplemental material for**
734 **Progressive maturation of the root apical meristem in *Arabidopsis thaliana* lateral**
735 **roots**

736
737 Béatrice Berthet, Lotte Bald, Marion Louveaux and Alexis Maizel

738

739

740 Fig. S1. Expression of WOX5 in early lateral root primordia

741 Fig. S2. Expression of SCR during germination

742 Fig. S3. Apically located cells stop to proliferate post-emergence

743 Fig. S4. EdU incorporation and cell proliferation in LR of wild type and dcr mutant

744

745

746 **Fig. S1 | Expression of *WOX5* in early lateral root primordia**

747 Confocal section of 10-day-old *gWOX5* at stage I, II and IV of lateral root development. Scale bar 10μm.

748

749

750 **Fig. S2 | Expression of SCR during germination**

751 (A) Confocal section SCR (*pSCR::nGFP*) embryos at 0, 24 and 48h post seed imbibition. The QC cells

752 (arrowheads) are indicated. Scale bar 10µm. (B) Number of QC at 0, 24 and 48h post seed imbibition. The

753 size of the circle is proportional to the number of observations (Obs.), and the colour represents the relative

754 proportion of observations within a given class. Total number of observations is indicated.

Fig. S3 | Apically located cells stop to proliferate post-emergence.

(A) Confocal sections of primary root and lateral root apical regions at different stages of development in lines expressing the S-phase marker CyTrap, the apical cell marker QC25^{Tg} and counterstained with Calcofluor White. Arrowheads indicate the apically located cells. (B) Quantification of the proportion of proliferating apically located cells in the primary root meristem and different stages of LR development. The total number of observations and the statistical significance (Wilcoxon test) of the difference between groups are indicated. (C) Time lapse light sheet imaging of a developing LR expressing *SCR* (*pSCR::nGFP*) and a plasma membrane marker (PIP1;4-GFP, see methods). Arrowheads point at apically located cells, while asterisks mark mitosis. Two divisions are observed 360 and 600 minutes after the start of the recording. Scale bars: 10µm.

Fig. S4 | Lack of EdU incorporation in young wild type emerged lateral roots and comparable proliferation of the lateral roots apically located cells in wild type and *dcr*

(A) Confocal section of wild type 10-day-old *SCR* (*pSCR::nGFP*) emerged lateral root counterstained with EdU. (B) Close up on the region boxed in (A) showing cell division despite lack of EdU incorporation. (C) Proportion of proliferating apically located cells in stage IV-VI LRP in wild type (WT) and *dcr* mutant. Each point represents a primordium. The distributions do not differ from each other according to Wilcoxon's test.