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RESEARCH REPORT

Lateral root formation involving cell division in both pericycle, cortex and endodermis is a common and ancestral trait in seed plants

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ABSTRACT

Studies on the model plant *Arabidopsis* have led to the common view that lateral roots are exclusively formed from pericycle cells and that the latter are unique in their ability to be reprogrammed into stem cells. By analysing lateral root formation in an evolutionary context, we show that lateral root primordium formation in which cortex, endodermis and pericycle are mitotically activated, is a common and ancestral trait in seed plants, whereas the exclusive involvement of pericycle evolved in the Brassicaceae. Furthermore, the endodermis can also be reprogrammed into stem cells in some species.

KEY WORDS: *Medicago*, Lateral root development, Primordium, Endodermis, Quiescent centre, Stem cells

INTRODUCTION

Lateral roots are formed post-embryonically and are a major determinant of root architecture. Formation of lateral roots has been best studied in *Arabidopsis*. In this species, lateral roots initiate from pairs of founder cells in the pericycle (Malamy and Benfey, 1997). These founder cells form a primordium, by division, in which a new stem cell niche is created that is composed of the quiescent centre (QC) surrounded by stem cells (Bennett et al., 2014; Ding and Friml, 2010; van den Berg et al., 1997). These findings have led to textbook knowledge that lateral roots of seed plants are exclusively formed from the pericycle and that pericycle cells are unique in their ability to obtain a stem cell fate (Eshel and Beeckman, 2013). However, some studies suggest that endodermis-derived cells become an integral part of lateral root primordia in the model legume *Medicago* (Herrbach et al., 2014) as well as in some other species (Bell and Mccully, 1970; Bonnett, 1969; Esau, 1977; Karas and McCully, 1973; Orman-Ligeza et al., 2013). As lateral root formation has only been studied in a few species, it is unclear whether this pattern is more widespread than suggested by the current paradigm based on *Arabidopsis*. It is also unknown whether endodermal cells can be reprogrammed to a stem cell fate. To answer these questions, we analysed the contribution of pericycle, endodermis and cortex to lateral root formation in *Medicago* and in additional plant species that represent an evolutionary spectrum across land plants.

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RESULTS AND DISCUSSION

We studied the role of endodermis in *Medicago* lateral root formation using cytological methods and molecular markers. We will name the tissues of the root on which a lateral root is formed 'parental'. First, we used *AtCASP1::GUS* (Xiao et al., 2014) to trace cell layers derived from the parental endodermis. This reporter construct is expressed in mature Medicago root endodermis cells and its expression is maintained when these parental endodermis cells divide and lose their casparian strips. Consequently, it allows us to distinguish cells derived from parental pericycle, endodermis and cortex in lateral root primordia. During *Medicago* lateral root development, seven stages can be identified (Fig. S1). At stage V, cells derived from the parental pericycle form a central cone, on top of which two layers derived from parental endodermis (expressing *AtCASP1::GUS)* and three or four layers derived from parental cortex are present (Fig. 1A). At stage VI, cells derived from the parental endodermis are located on top of the central cone and form four cell layers (Fig. 1B). At this stage, *AtCASP1::GUS* is also expressed in a ring of cells that are derived from the parental pericycle surrounding the central core at the base of the primordium and where the new lateral root endodermis develops. At stage VII, the new lateral root endodermis is formed (Fig. 1C, Fig. S2A-D). It is a single cell layer that contains casparian strips and has high *AtCASP1::GUS* expression.

We then identified putative QC cells based on their connection with the newly formed endodermis cell file (Di Laurenzio et al., 1996). At stages VI and VII, these putative QC cells form two cell layers that are formed from the outer-most cells derived from the parental pericycle, located at the tip of the cone (Fig. 1C). To confirm the putative position of the QC in stage VI/VII primordia, we used *AtSCR::GUS* (Di Laurenzio et al., 1996). This reporter construct has previously been shown to be expressed in QC and lateral root endodermis cells of *Medicago* lateral roots (Herrbach et al., 2014). As expected, at stage VI, *AtSCR::GUS* is highly expressed in the group of cells in which the endodermis will be formed, as well as in the putative QC cells, confirming their identity (Fig. 1D). We also performed an *in situ* hybridization with a *Medicago* SCR probe and this further confirmed the position of the QC in stage VI lateral root primordium and in a young lateral root (Fig. S3). Thus, the QC cells are derived from the outer-most layers of the parental pericycle cells. These findings suggest that the cells at the position of the lateral root columella stem cells are derived from the parental endodermis. To confirm the stem cell identity of these putative columella stem cells, we visualized starch, which is a marker for differentiated columella cells and is absent in stem cells (Bennett et al., 2014; Ding and Friml, 2010; van den Berg et al., 1997). Indeed, at stage VII, starch is present at the apex of the primordium in four or five cell layers that are derived from the parental cortex and endodermis (Fig. 1E,F and Fig. S2E,F). This

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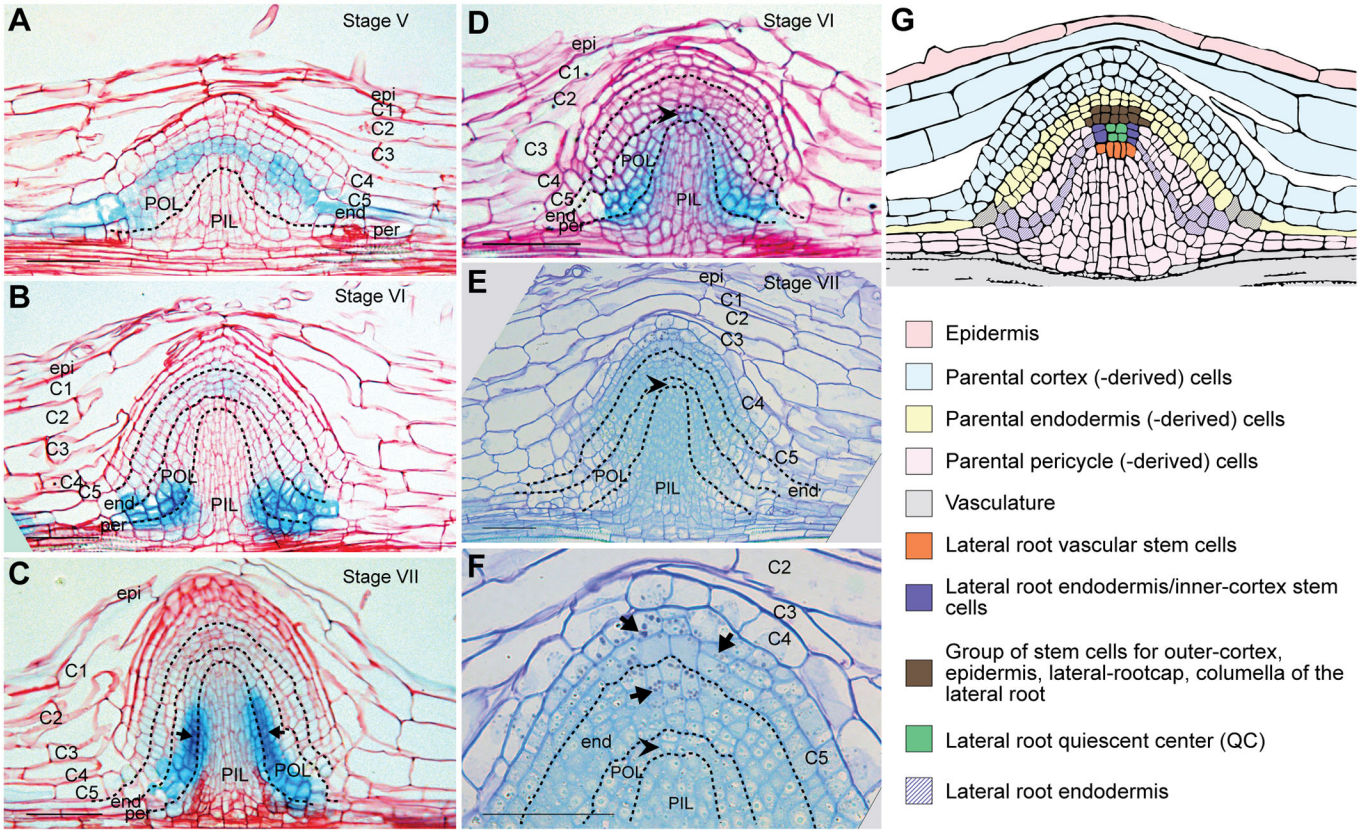

Fig. 1. Endodermis-derived cells form (part of the) lateral root stem cells in *Medicago*. (A-F) Longitudinal sections of *Medicago* lateral root primordia, at different stages, stained with Ruthenium Red and expressing either the endodermal marker construct *AtCASP::GUS* (A-C) or the endodermal and quiescent centre (QC) marker construct *AtSCR::GUS* (D), or stained with Toluidine Blue and Lugol to visualize starch granules (E,F). (A) Stage V lateral root primordium showing *AtCASP::GUS* expression in two cell layers derived from the parental endodermis located between three and four cell layers derived from the fourth and the fifth parental cortical cell layers (C4 and C5), and a cone comprising a core of parental pericycle-derived inner cell layers (PILs) surrounded by parental pericycle-derived outer cell layers (POLs). (B) Stage VI primordium showing *AtCASP::GUS* expression at the base of the POLs. These cells will form the new lateral root endodermis. Parental endodermis-derived cells no longer express *AtCASP::GUS* and form four cell layers surrounding the cone derived from the parental pericycle cells. (C) Stage VII primordium showing *AtCASP::GUS* expression in newly formed endodermis at the most inner cell layer of the POLs (arrows). (D) Stage VI showing *AtSCR::GUS* expression in the newly formed endodermis at the most inner cell layer of the POLs and in the QC (arrowhead) in the POLs at the tip of the cone. (E) Overview of stage VII (QC marked with an arrowhead). (F) Magnification of the apex of the section shown in E showing starch granules (arrows) formed in cells at the tip of the lateral root primordium that are derived from parental cortex and two outer cell layers derived from the parental endodermis, but not in the two inner parental endodermis-derived cell layers. The last comprise the putative columella stem cells (QC marked with an arrowhead). (G) Schematic outline of the ontogeny of the stem cell niche and of cell types in *Medicago* lateral root primordia. epi, epidermis; C1-C5, cortical cell layers 1-5; end, endodermis; per, pericycle. Dashed lines indicate borders between cells derived from the PIL, pericycle outer layer, endodermis and C5. Scale bars: 75 µm.

suggests that inner cortical cells and a subset of cells derived from the parental endodermis have transdifferentiated to lateral root cap cells. Importantly, the putative lateral root columella stem cells, which are derived from the parental endodermis, are devoid of starch granules, supporting the conclusion that they are stem cells. Consequently, given the structure of the *Medicago* stem cell niche (Fig. S4), the lateral root stem cells that give rise to epidermis and outer cortex are also formed from parental endodermis cells. We therefore conclude that, in *Medicago* lateral root primordia, cells derived from parental endodermis and cortex transdifferentiate into columella cells. Furthermore, stem cells are not exclusively formed from pericycle cells but also from a subset of cells derived from parental endodermis (Fig. 1G). Our data show that, in *Medicago*, fully differentiated endodermis cells that contain casparian strips (Figs S1-S2) can be reprogrammed into stem cells.

To place the roles of pericycle, endodermis and cortex during lateral root formation in an evolutionary context, we compared 37 seed plant species from different orders. These included 23 eudicots, eight monocots, three early-diverging angiosperms and three gymnosperms. Two pterophytes were included as outgroups (Fig. 2). For 25 species, we examined lateral root primordium formation directly (Figs S5,S6); for an additional 14 species, we re-examined published data (Table S1). We found that in 33 out of 37 seed plants, both endodermis and pericycle are mitotically activated (Fig. 2). The only exceptions are *Arabidopsis* and its close relatives such as Brussels sprout, black mustard and radish, where exclusively the pericycle is mitotically activated.

Cell division in the parental endodermis and cortex most likely facilitates passage of lateral root primordia through the overlaying tissues of the parental root. When cells derived from the parental endodermis and cortex become part of the lateral root primordium, these layers do not have to be crossed at all. In case they do not become integrated into the primordium the recently divided cells can most likely be passed more easily. For example, upon division of endodermal cells, casparian strips are lost, which possibly reduces the rigidity of the endodermis cell layer. This will facilitate the primordium, which is made only from pericycle cells (as in *Senna* and *Taxus*), to cross this layer. The importance of a

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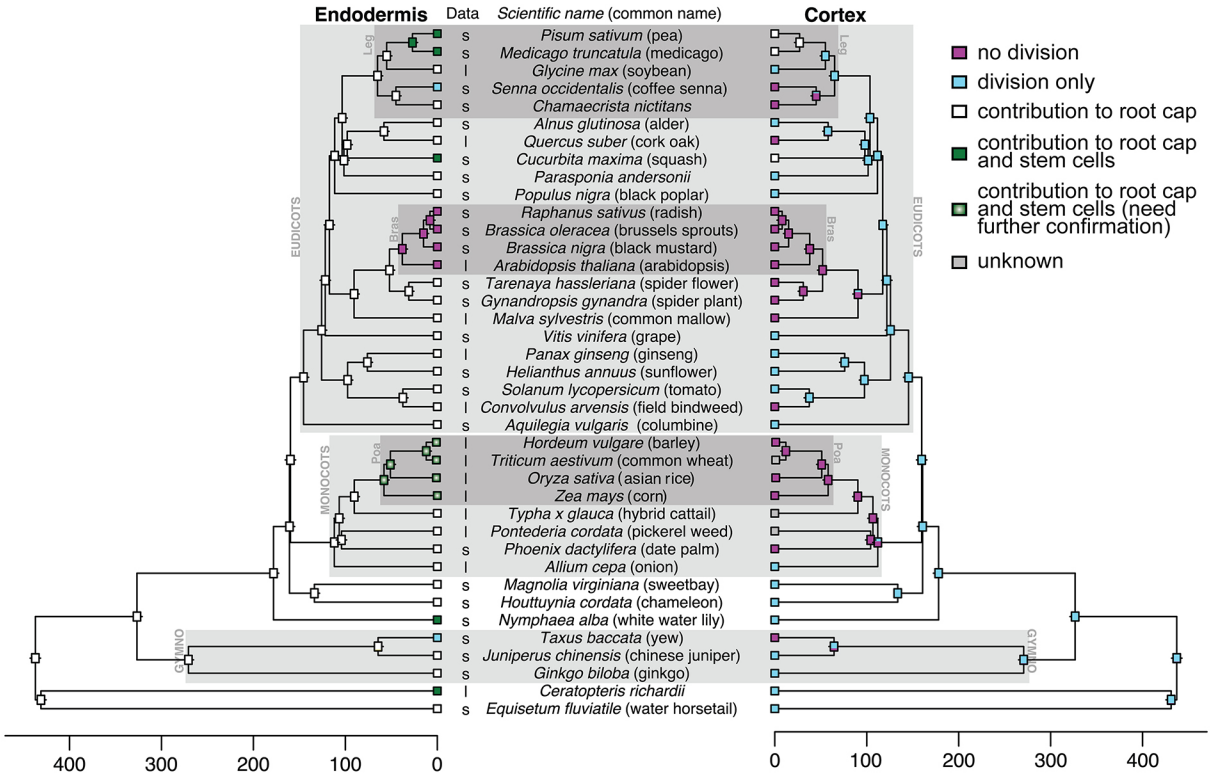

Fig. 2. Comparative analyses of lateral root development in land plants. Ancestral state reconstructions of the contribution of pericycle, endodermis and cortex to lateral root primordia. Scales show divergence times in millions of years ago. Data are based on sections (s) (Figs S5,S6) or on the literature (l) (Table S1). Leg, legumes; Bras, Brassicaceae; Poa, Poaceae.

mechanism that allows pericycle-derived primordia to pass the endodermis is underlined by studies on *Arabidopsis* in which parental endodermal cells on top of the lateral root primordium do not divide but reduce their turgor pressure to allow the primordium to pass (Vermeer and Geldner, 2015). Other Brassicaceae species probably share this mechanism. In most of these 33 species, parental endodermis and pericycle both contribute cells to the lateral root primordia, with the exception of *Taxus* and Senna where the endodermis is mitotically activated, but lateral root primordia are exclusively derived from pericycle cells (Fig. 2). Cortical cell divisions during lateral root formation occur in the majority of the species we analysed (Fig. 2). However, contribution of cortex-derived cells to the lateral root primordia was only observed in Medicago, pea and pumpkin (Fig. 2). We therefore conclude that parental endodermis cells contribute to the formation of part of the lateral root primordium in most seed plants.

We next studied in which plants the parental endodermis forms a subset of the lateral root primordial stem cells based on cell layer counts. Cells derived from the parental cortex and endodermis form the most apical/distal part of the lateral root primordium and can transdifferentiate into root columella cells. When the number of cell layers derived from parental cortex and endodermis is equal to or smaller than the number of primordial columella cell layers, they can only contribute to the formation of the columella. However, when the number of cell layers derived from the parental cortex and endodermis exceeds that of columella, cells derived from parental endodermis can also contribute to the stem cell niche. For example, in *Medicago*, cells derived from the parental inner cortex and endodermis form about seven or eight layers at the top of the primordium. As the primordial columella has only about five or six cell layers, the endodermis can contribute two layers to the stem cell niche. Based on this rationale, we found that cells derived from the parental endodermis contribute to lateral root stem cells in *Medicago*, pea, pumpkin and waterlily, and perhaps also in rice, maize, wheat and barley (Orman-Ligeza et al., 2013). In all other plants the number of parental cortex/endodermis-derived cell layers is smaller than or equal to the number of primordial columella cell layers and therefore most likely cannot contribute to the formation of the stem cell niche. Therefore, in most species the cells derived from the parental endodermis only transdifferentiate into columella cells and do not contribute to the formation of the stem cells.

We then reconstructed ancestral states of the roles of the parental endodermis and cortex in lateral root formation based on a phylogenetic tree (Fig. 2). This suggested that the parental endodermis contributed to lateral root formation in the last common ancestor of all seed plants and that this ancestral state was conserved in most of the descendant species that we examined. Lack of mitotic activation of endodermis in lateral root formation seems to have evolved exclusively in Brassicaceae. The parental cortex most likely divided during lateral root formation in the last common ancestor, but these divisions did not contribute to the formation of root cap. This property most likely evolved independently in legumes and Cucurbitales. Parental cortex cell division during lateral root primordium formation was lost in the grass-like monocots, in a branch including the Brassicaceae and in a few other species.

Based on our results, we conclude that lateral root primordium formation during which the pericycle as well as the cortex and endodermis are activated is a common and ancestral trait in seed plants, and the mitotic activation of endodermis as well as cortex appears to be specifically lost in the Brassicaceae lineage. The formation of stem cells from endodermal-derived cells may have

evolved at least four times within the angiosperms. So the ability to form stem cells during lateral root formation is not unique to pericycle cells in seed plants as commonly assumed based on the *Arabidopsis* paradigm. In some species at least it involves the reprogramming of fully differentiated endodermis cells.

MATERIALS AND METHODS

Plant material and constructs

M. truncatula accession Jemalong A17 wild-type plants were used to study root cytology. This accession was also used to generate *AtCASP1::GUS*- and *AtSCR::GUS Agrobacterium rhizogenes* (strain MSU440)-mediated transgenic roots, as previously described by Limpens et al. (2004). The formation of lateral root primordia in R108 is similar as described for A17. The surface-sterilization and germination of *Medicago* seeds were performed as previously described by Limpens et al. (2004).

Other plant materials were provided by Tuincentrum De Oude Tol and by Marijke Hartog (Wageningen University, The Netherlands). *Juniperus chinensis* and *Equisetum fluviatile* were collected in the Sysselt, a forest near Wageningen and Wageningen University Campus. The *AtCASP1::GUS* and *AtSCR::GUS* constructs have been described by Xiao et al. (2014).

Histochemical β-glucuronidase (GUS) staining

Transgenic plant material containing GUS constructs were incubated in GUS buffer [3% sucrose, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$, 10 mM EDTA and 1 mg/ml X-Gluc salt in 100 mM phosphate buffer solution, pH 7.0] under vacuum for 30 min and then at 37°C for 3 to 24 h as described by Xiao et al. (2014).

Tissue embedding, sectioning and section staining

Root segments were fixed at 4°C overnight with 4% paraformaldehyde (w/v), 5% glutaraldehyde (v/v) in 0.05 M sodium phosphate buffer (pH 7.2). The fixed material was dehydrated in an ethanol series and subsequently embedded in Technovit 7100 (Heraeus Kulzer) according to the manufacturer's protocol. Sections (7 µm) were cut with a RJ2035 microtome (Leica Microsystems), stained for 5 min in 0.05% Toluidine Blue O for wild-type material and for 15 min in 0.1% Ruthenium Red for transgenic GUS material. Sections were analysed using a DM5500B microscope equipped with a DFC425C camera (Leica Microsystems).

RNA *in situ* hybridization

RNA *in situ* hybridization was performed by using Invitrogen ViewRNA ISH Tissue 1-Plex Assay kits (Thermo Fisher Scientific) according to the user manual (assets.thermofisher.com/TFS-Assets/LSG/manuals/UM17400-ViewRNA-ISH-Tissue-1-Plex-Assay.pdf). RNA *in situ* hybridization probe sets cover the 1534-2563 bp region of Medicago SCR gene MTR_7g074650 and were designed and synthesized on request by Thermo Fisher Scientific. Images were taken using an AU5500B microscope equipped with a DFC425c camera (Leica).

Lugol staining

Root segments were stained with Lugol solution (Merck, Germany) to visualize starch grains and tissues were cleared in chloral hydrate solution, which contains 2 ml water, 1 ml glycerol and 8 g chloral hydrate (VWR BDH). Whole-mount root segments were analysed using an Axio Imager A1 microscope (Zeiss) supplied with Nomarski optics.

Ancestral state reconstructions

A time-calibrated phylogenetic tree for seed plants was compiled based on results from Harris and Davies (2016), supplemented with data for legumes from the Legume Phylogeny Working Group (2017), for Brassicaceae from Cardinal-McTeague et al. (2016) and for Poaceae from Bernhardt et al. (2017). Terminals representing taxa for which no data on lateral root primordia were available were removed. Ancestral states were reconstructed based on maximum parsimony, as implemented in the R package ape (Popescu et al., 2012) as well as on the Mk-n maximum likelihood models implemented in the R package diversitree (FitzJohn, 2012). For each cell layer involved (i.e. pericycle, endodermis and cortex), we assessed different models of state change (i.e. equal rates, stepwise symmetrical rates and all rates different) and determined the best-fitting model based on Akaike Information Criterion (AIC). Results based on parsimony and likelihood analyses were congruent.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.T.X., T.B.; Methodology: T.T.X., R.v.V., T.B.; Software: T.T.X., R.v.V.; Validation: R.v.V., O.K.; Formal analysis: T.T.X., R.v.V.; Investigation: T.T.X., O.K., C.F.; Resources: T.T.X.; Data curation: T.T.X., O.K., C.F.; Writing - original draft: T.T.X.; Writing - review & editing: T.T.X., R.v.V., T.B.; Visualization: T.T.X.; Supervision: T.B.; Project administration: T.B.; Funding acquisition: T.B.

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Supplementary information

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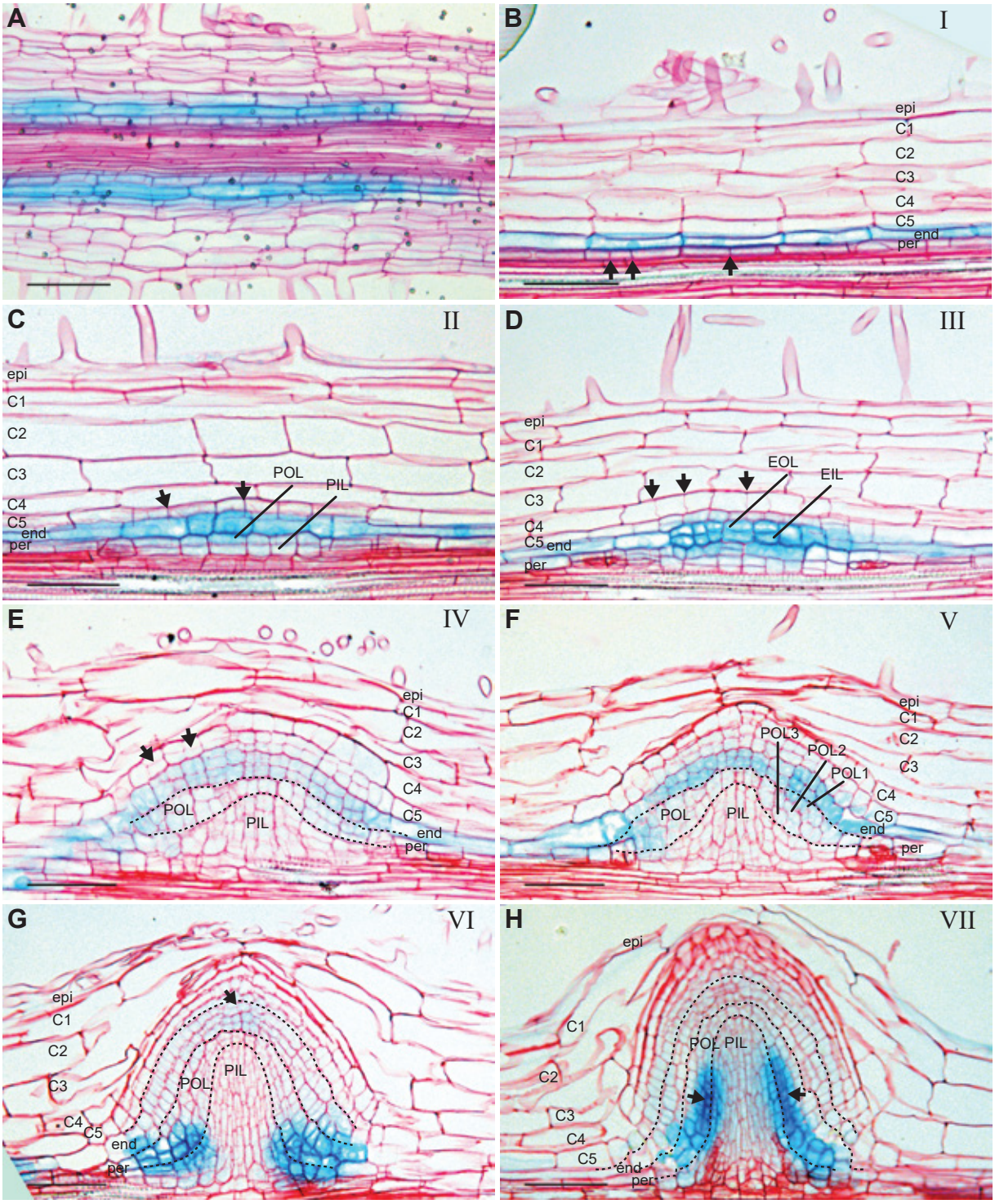


Fig. S1. Subsequent stages of Medicago lateral root development.
AtCASP::GUS, which is used to trace cells derived from parental endodermis. It is expressed in the endodermis of a Medicago root (A). At stage I (B), anticlinal divisions are induced in the parental pericycle (arrows). At stage II (C), anticlinal divisions continued in parental endodermis and are induced in the most inner parental cortical layer (C5, arrows); periclinal divisions are induced in parental pericycle by which 2 cell layers are formed. This are the parental pericycle derived inner layer (PIL) and outer layer (POL). At stage III (D), periclinal divisions continued in PIL and POL and are induced in parental endodermis. The latter results in parental endodermis derived inner layer (EIL) and outer layer (EOL). *AtCASP::GUS* remains active in EIL and EOL. Further, anticlinal divisions are induced in C4 (arrows). At stage IV (E), periclinal division are induced in C5 (arrows) and parental endodermis and cell division continued in PIL forms a cone. At stage V (F), periclinal divisions continued in PIL. An addition periclinal division occur in one of the POL derived cell layers. At this stage 3 POL layers are formed; POL1, POL2 and POL3. C1, C2 and C3 collapse. At stage VI (G), Periclinal divisions induced in EIL and EOL form 4 cell layers (arrow). *AtCASP::GUS* expression is reduced in EIL and EOL and is induced in POL at the base of the primordium (arrows). At stage VII (H), primordium emerges through the epidermis and cells derived from C4/5 at the apex of the primordium start to enlarge (arrow).
In E, F, G and H black lines indicate the border between cells derived from parental PIL, POL, endodermis and C5, respectively. Panels F-H are same with Fig 1A-C.
Bars, 75 µm.

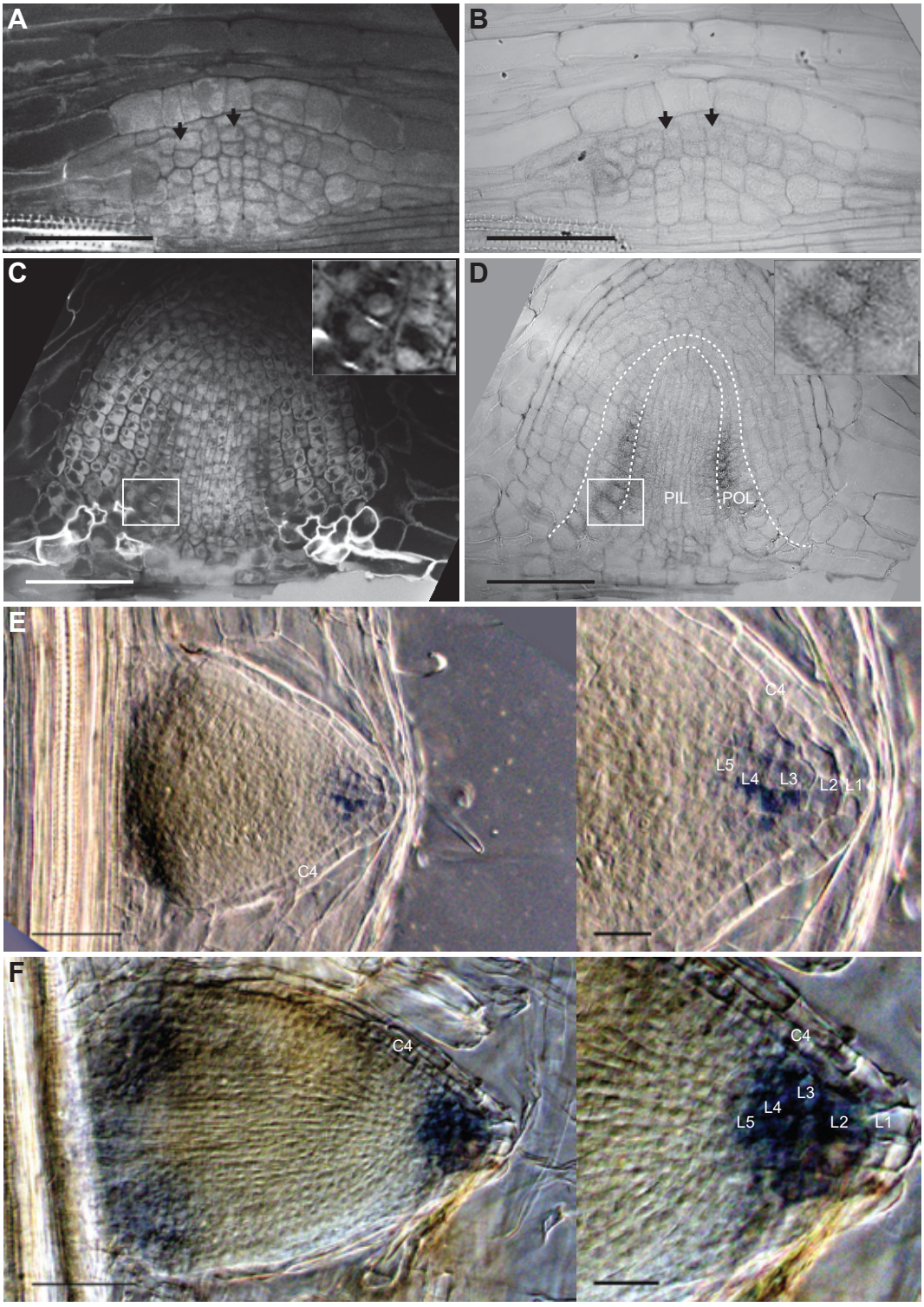


Fig. S2. Parental endodermis derived cells form stem cells for outer-cortex, epidermis, lateral-root cap and columella.

(A) At stage III-IV, casparian strips are absent in dividing parental endodermis cells, although AtCASP::GUS marker is still expressed in these cells (B). (C) At stage VII, casparian strips are formed in cells derived from the parental pericycle outer cell layer (POL3). (D) In this layer *AtCASP::GUS* is highest expressed. Magnifications of cells with casparian strips are shown on the top right corners of C. Casparian strips are detected by autofluorescence under UV light.

(E-F) Lugol staining of the primordia shows starch granules are present in 4-5 cell layers (L1-L5) in the columella of the primordium at stage VI-VII (E). Parental C4 derived cells only contribute to the lateral root cap (F). Magnifications of apex of the primordium for figure E and F are shown on the right.

A-D were captured by a Leica SP8 confocal microscope with DAPI filter. A and B and C and D are shown the same section. In D black lines indicate the border between cells derived from parental PIL, POL and endodermis, respectively.

Bars equal to 75 µm; in magnifications in E and F equal to 25 µm.

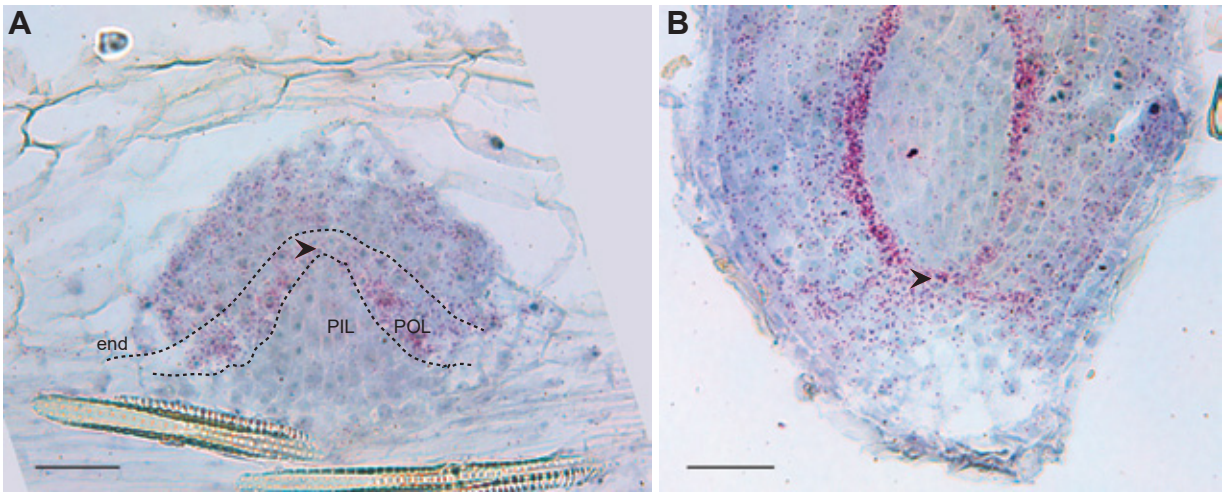


Fig. S3. Medicago *SCR* gene expressed in the endodermis cell layer and the lateral root QC.

RNA *in situ* on the stage VI lateral root primordia (A) and a young lateral root (B) by using probes specifically target Medicago *SCR* mRNAs show Medicago *SCR* gene start to express from lateral root primordia stage VI and highly expressed in lateral root endodermis and QC cells (arrowheads). Red dots are signals.
In A black lines indicate the border between cells derived from parental endodermis, POL and PIL, respectively.
Bars equal to 75 μm.

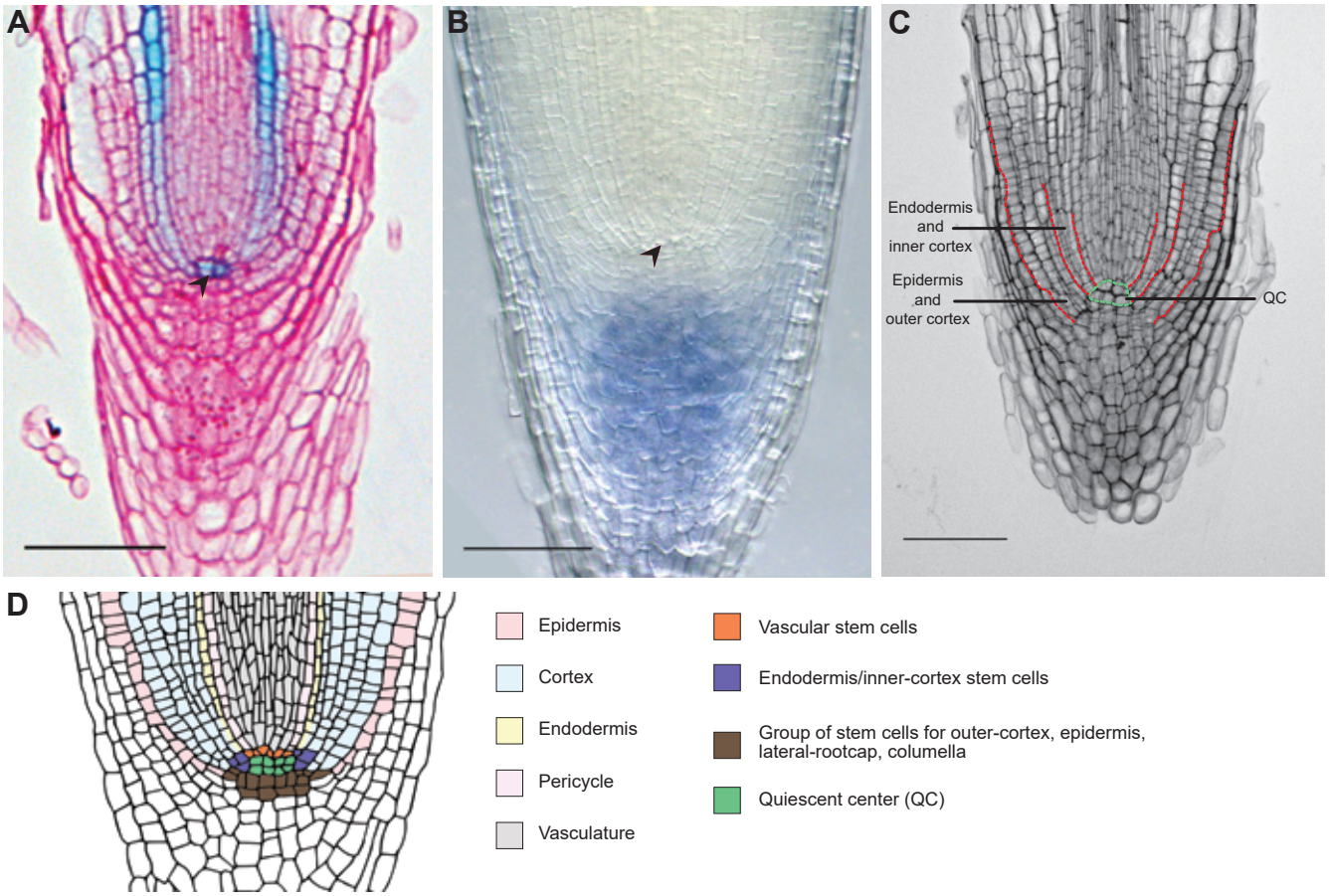
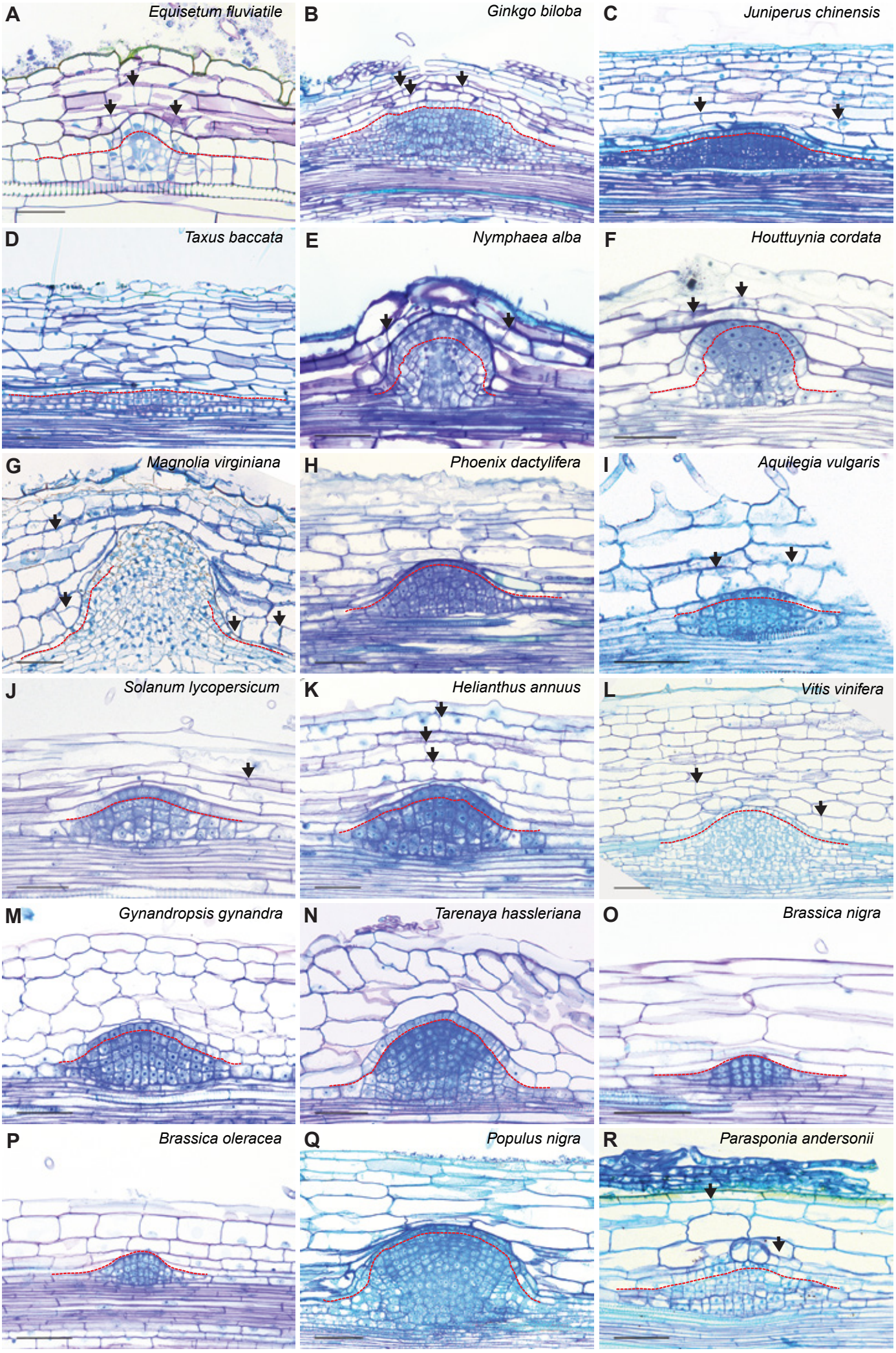


Fig. S4. Characterization of the Medicago root stem cells niche.

(A) *AtSCR::GUS* expression in Medicago, which is a reporter construct highly expressed in QC (arrow head) and the endodermis of the Medicago root. (B) Lugol staining of the Medicago root. About 3 cell layers below the QC lack starch which indicates that these are columella stem cells. (C) Median longitudinal section of a Medicago root tip. The QC is indicated with a green dashed line. The cell files of endodermis and inner cortex end at the QC. Files of the outer cortex and epidermis convolve to the 3rd cell layer below QC. Red lines indicate the border between tissues that are indicated in the figure. (D) Schematic outline of cell types in the Medicago root apical meristem based on cell specific markers (A-C) and the literature.
Bars, 75 µm.



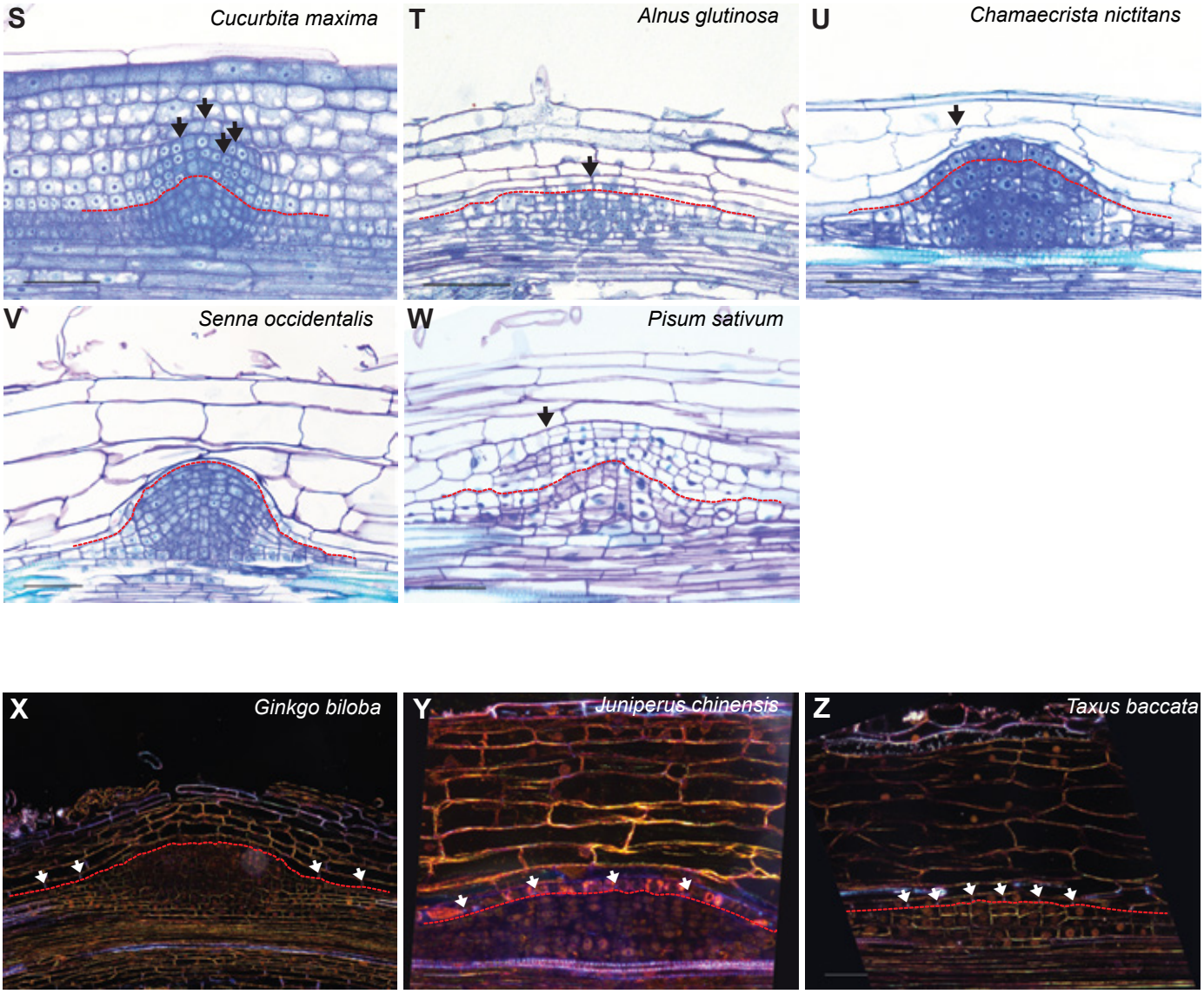


Fig. S5. Lateral root formation involving mitotic activation is common in seed plants; root primordia of 25 additional species.

(A-W) Section of young lateral root primordia from 25 different plant species. Divided cortical cells are indicated by arrows.
(X-Z) Casparian strips disappear in the dividing endodermis cells during lateral root primordium formation. Casparian strips (arrows) are visualized by dark field microscopy.
In A-Z red line indicates the border between cells derived from parental pericycle and endodermis.
Bars, 75 µm.
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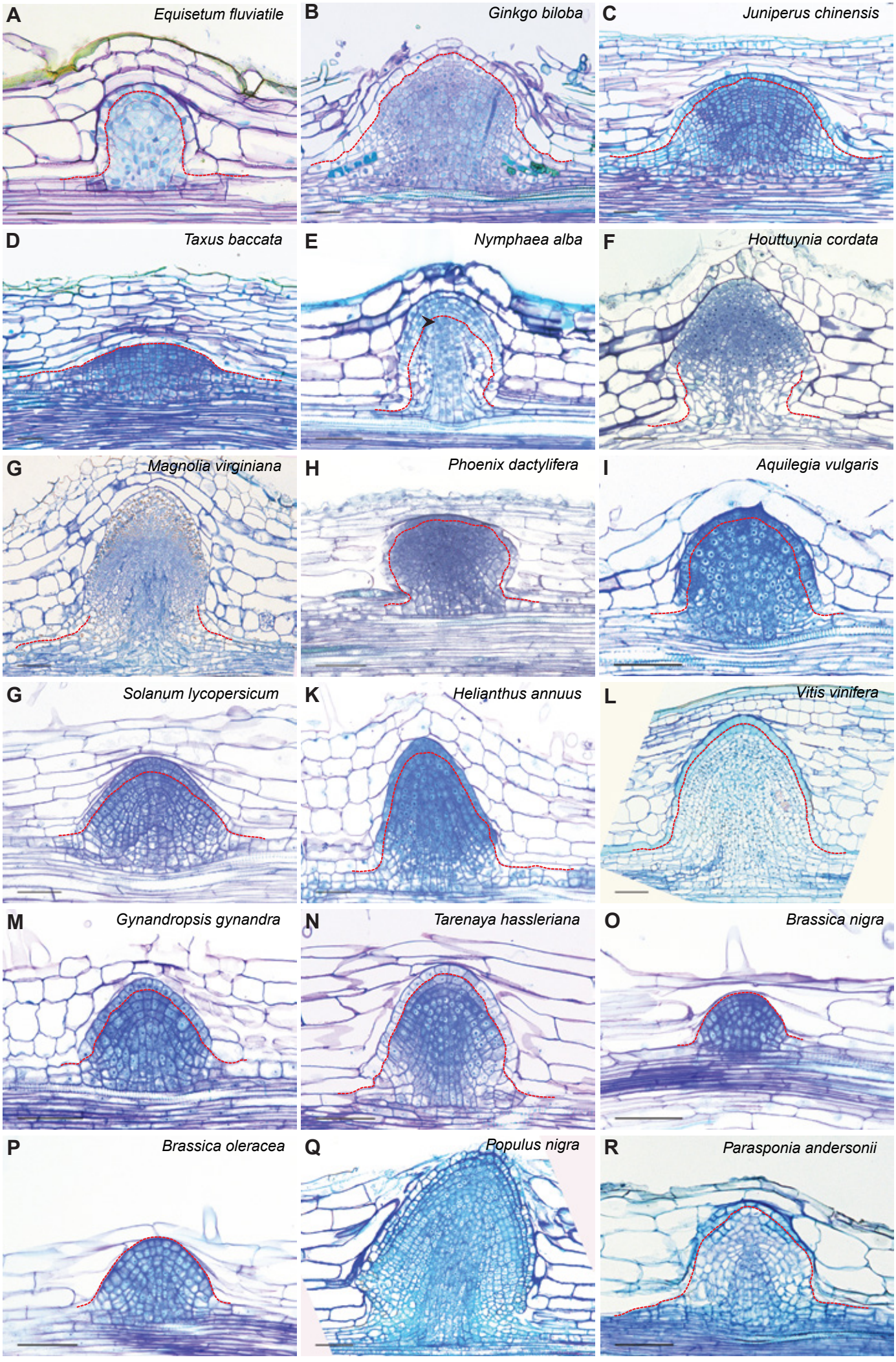

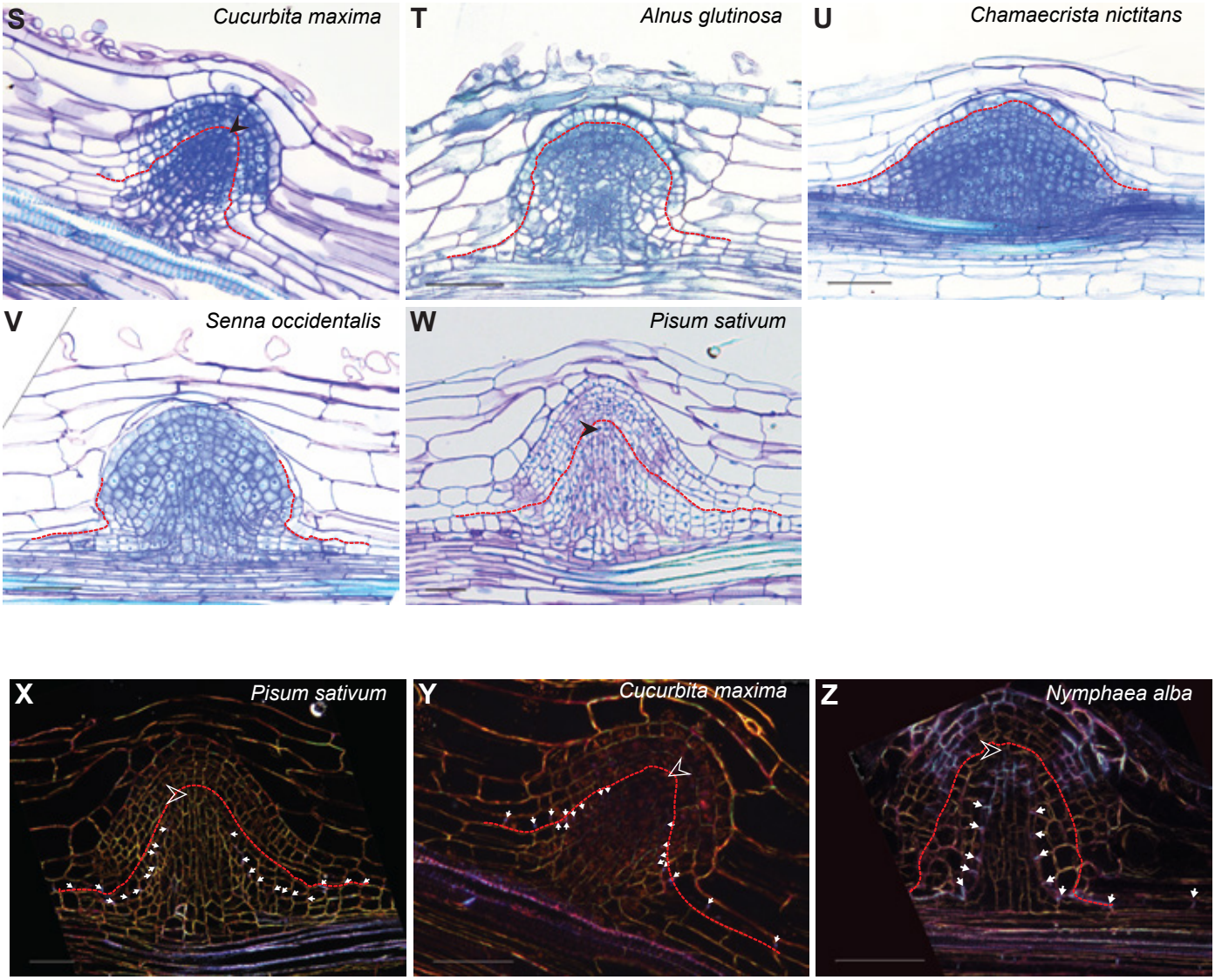

Fig. S6. The ability of endodermis derived cells to form stem cell occurs in several seed plant species.

(A-W) Section of lateral root primordia from different plant species.
(X-Z) Tracing the original root endodermis cell file and the newly formed endodermis in the lateral root primordia by visualizing casparian strips (arrows). In Pea (X), pumpkin (Y) and waterlily (Z), QC (arrowhead) is adjacent to cells derived from endodermis.
Predicted QC in E, S, W and X-Z is marked by arrowhead.
In A-Z red line indicates the border between cells derived from parental pericycle and endodermis.
Bars, 75 μm.

Table S1. Other Species from literature.

Scientific name	*Common name*	*Literature*
Oryza sativa	Asian rice	(Ni et al., 2014)
Hordeum vulgare	barley	(Orman-Ligeza et al., 2013)
Zea mays	corn	(Bell and Mccully, 1970)
Triticum aestivum	common wheat	(Foard et al., 1965)
Convolvulus arvensis	field bindweed	(Foard et al., 1965)
Quercus suber L.	cork oak	(Verdaguer et al., 2000)
Malva sylvestris	common mallow	(Byrne, 1973)
Glycine max (L.)	soybean	(Byrne et al., 1977)
Raphanus sativus	radish	(Blakely et al., 1982)
Allium cepa	onion	(Casero et al., 1993; Casero et al., 1984)
Pontederia cordata	pickerel weed	(Charlton, 1975)
Panax Ginseng	ginseng	(Kim et al., 2003)
Typha X glauca	hybrid cattail	(Seago and Marsh, 1990)
Ceratopteris richardii		(Hou et al., 2004)

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