Specification and evolution of lateral root stem cells

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Plants have evolved a remarkable capacity to develop new organs post-embryonically throughout their lifespan. A prime example of this is root branching. Root branching occurs in two types: dichotomous and lateral branching. The dichotomous branching is the result of the division of the root apical meristem into two daughter meristems likely through symmetric cell divisions of the root apical cell as has recently been illustrated in the extant lycophyte *Selaginella moellendorffii* (Figure 1). The lateral root (LR) branching relies on the *de novo* specification of a subset of founder cells (hereinafter referred to as LR stem cells) located in the internal tissues of an existing root. This step is followed by initiation, in which the specified cells re-enter to cell cycle, and subsequently by primordium formation and emergence. In this primer, we summarize recent advances describing the molecular bases underlying LR stem cell specification in angiosperms and highlight the important positional signals that fine tune this process. By delving into the evolutionary origins of root branching, we point out that positional control on LR stem cell specification has not been the prevailing mechanism and discuss the process in ferns (i.e., a sister group of seed plants), where it seems to be under the control of lineage-dependent mechanisms.

Auxin signaling and homeostasis control LR stem cell specification

In seed plants, LRs initiate acropetally from the pericycle, which is a single cell layer surrounding vascular tissues along the primary root axis. More precisely, LRs initiate from xylem pole pericycle (XPP) cells in eudicot species (e.g. *Arabidopsis thaliana* (Arabidopsis)), while they initiate from the phloem pole pericycle (PPP) cells in monocot species (e.g. *Oryza sativa* (rice), *Zea mays* (maize)). In the model plant Arabidopsis, the earliest events of LR stem cell specification are determined by a root clock mechanism, which can be visualized by periodic activity of the auxin response promoter *DIRECT REPEAT5* (*DR5*) fused to the reporter gene *LUCEFERASE* (*DR5:LUC*). The root clock specifies a subset of XPP cells to become LR stem cells in a periodic manner (i.e. approximately every 6 hours) through oscillation in gene expression both in phase and antiphase with *DR5:LUC*. This oscillation in gene expression occurs in the elongation zone of the primary root (Figure 2A-C). Since the *DR5:LUC* reporter exhibits low spatial resolution, the exact cell type wherein auxin response oscillates is still unclear, but it likely occurs in the protoxylem cells as indicated in root cross sections marked by *DR5: β-glucuronidase*. This suggests that there is positional information transmitted from the protoxylem to the abutting pericycle cells to specify them as LR stem cells. It is also possible that gene expression oscillation occurs in the XPP cells themselves that will be specified to form LR stem cells. At the molecular level, once auxin accumulates in the targeted cells, it will be perceived by the nuclear receptor TRANSPORT INHIBITOR1/AUXIN-SIGNALLING F-BOX (TIR1/AFB) proteins, and which will trigger the ubiquitylation and subsequent degradation of the AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) repressor proteins. The degradation of Aux/IAA repressors releases AUXIN RESPONSE FACTOR (ARF) transcription factors to induce or repress their downstream targets.

Specifically, recent studies in Arabidopsis identified AtTIR1 and AtAFB2 receptors, AtARF7 and its repressor protein AtPOTENT/IAA18 as key components that regulate auxin response oscillation. Indeed, these genes are expressed in the root elongation zone and *AtARF7* transcripts oscillate in anti-phase with *DR5:LUC* (Figure 2B). In line with this, the loss of function mutants *tir1afb2* and *arf7* as well as the gain of function mutant *potent/iaa18* are impaired in *DR5:LUC* oscillation, and are thereby defective in LR stem cell specification. These data confirm that auxin signaling is required for a proper root clock functioning, and thus LR stem cell pre-patterning. Chemical and cell biology approaches uncovered the source of auxin that fine tunes the root clock. It has been shown that recurrent programmed cell death (PCD) in the lateral root cap cells generates auxin pulses through conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA). The generated IAA in dying lateral root cap cells is then transported to internal tissues to regulate the amplitude of the *DR5:LUC* oscillation, and hence LR stem cell specification (Figure 2B).

Recently, computational simulations have generated an alternative model trying to explain the cause of periodic oscillation in auxin response in the elongation zone of the primary root. According to this model, the oscillation of *DR5:LUC* is, at least partly, a consequence of the interplay between auxin-reflux loop and growth dynamics of the primary roots. This conclusion is based on the assumption that the root tip produces cells with a different potential in loading auxin depending on their relative size (smaller or larger cells) (Figure 2B). Regardless of its source, it will be exciting to find how IAA is transported through epidermis, endodermis, and cortex tissues towards the pericycle and/or the protoxylem to trigger the root clock required for the pre-patterning of LR stem cells.

Environmental cues control LR stem cell specification

Recent findings point to major roles of positional signals (e.g. temperature, light and water availability) in controlling LR stem cell specification.

Light is a crucial factor that controls a plethora of growth and developmental processes. Arabidopsis seedlings grown under dark conditions produce fewer LRs compared to their light grown counterparts. Of note, sucrose was supplemented in both conditions (i.e. dark and light) refuting the possibility of carbohydrate starvation. At the molecular level, it has been demonstrated that light signals modulate auxin response oscillation in the elongation zone, promoting LR stem cell specification. Light signals possibly promote LR stem cell specification by inducing the expression of *LONG HYPOCOTYL1 (AtHY1). AtHY1*, which encodes a plastid heme oxygenase required for phytochrome chromophore biosynthesis*,* is expressed in XPP, distal lateral root cap, columella cells and LR primordia. Interestingly, AtHY1 seems to control LR stem cell specification by inducing the expression of two transcription factors *ELONGATED HYPOCOTYL5 (AtHY5)* and its closely related paralog *HY5-HOMOLOG (AtHYH)*. Indeed, the double mutant *hy5hyh* produces fewer LRs compared to the wild type under light conditions (Figure 2B). Moreover, light-induced HY1 also controls the cell-to-cell auxin transport required for LR stem cell specification by regulating the abundance of the auxin efflux carriers PIN-FORMED1 (AtPIN1), AtPIN2 and the auxin influx carrier AUXIN RESISTANT1 (AtAUX1) at the plasma membrane in the root tip. How *AtHY1* ensures this multifunctionality to control LR stem cell specification remains unknown.

Water is a crucial and limiting resource for plant development and survival. Recently, X-ray computed tomography imaging showed that plants rapidly stop producing LRs when their root tips lose contact with water (i.e. air conditions). This adaptive response

is termed xerobranching. Xerobranching strongly blocks *DR5:LUC* oscillation in the elongation zone of the primary root, suggesting that water is an important positional cue that controls LR stem cell specification. The inhibition of auxin response coincides with a transient up regulation of the stress-induced hormone abscisic acid (ABA) response in the elongation zone as marked by the sensor nlsABACUS2. This observation suggests that ABA might control IAA signaling or transport to control LR stem specification. Genetic and cell biology approaches demonstrated that ABA and water co-mobilize from the phloem to the epidermis through plasmodesmata when the root tip loses contact with water. The movement of ABA to the outer tissues triggers the closure of the plasmodesmata. Consequently, plasmodesmata closure blocks the symplastic radial movement of auxin from the epidermis towards pericycle, and thus inhibits LR stem cell specification (Figure 2B). This adaptive strategy is apparently conserved among angiosperms as it has been observed in a number of species (e.g. rice, maize, tomato and Arabidopsis). In conclusion, the xerobranching response allows the plant to accordingly shape root architecture in heterogeneous soil conditions.

Retinal controls LR stem cell specification

Recent advances revealed new molecular players acting upstream of the root clock such as pectin modifications and trafficking as well as metabolites. Blocking the biosynthesis of carotenoids strongly perturbs *DR5:LUC* oscillations in Arabidopsis. Metabolite profiling in the Arabidopsis root revealed the presence of several carotenoid-related metabolites among which, retinal and 14’-apo-b-carotenal. Exogenous applications of these two metabolites increase the number of LR stem cells. This is of particular interest because it has previously been shown that blocking retinoic acid biosynthesis perturbs the clock oscillation controlling the somitogenesis

and left-right patterning in vertebrates. It is tempting to hypothesize that there might be conserved mechanisms underlying root patterning in plants and somitogensis in vertebrates since both mechanisms are controlled by clock oscillation mechanisms. Amino acid sequence homology search for putative retinal-binding proteins in Arabidopsis genome identified *TEMPERATURE INDUCED LIPOCALIN (AtTIL)* gene. AtTIL protein has 25% amino acid sequence identity compared to the vertebrate lipocalin RETINOL-BINDING PROTEIN 4. Remarkably, *AtTIL* is expressed in the root tip and *til* mutants are affected both in *DR5:LUC* oscillation and in LR branching. Moreover, the *til* mutant has decreased sensitivity to retinal-induced LR branching. These results suggest that AtTIL is a retinal-binding protein that acts upstream of the root clock to control LR stem cell specification (Figure 2B). Whether the AtTIL-mediated pathway requires a functional auxin signaling to control LR stem cell specification remains to be discovered.

Root axis evolved in a stepwise manner in lycophytes

The appearance of roots in tracheophytes (vascular plant lineage) was one of the key developmental innovations that contributed to their successful radiation and spread in various ecosystems. They provided plants with the ability to efficiently explore soil for water, nutrients as well as symbiosis with beneficial microorganisms compared to their rootless rhizoid-bearing ancestors (Figure 1). The genetic variations and selective pressure (s) that drove the evolution of roots are still not unveiled, but direct fossil evidence indicated that the earliest roots appeared independently in lycophytes and euphyllophytes (ferns, horsetails and seed plants) and then evolved in a stepwise manner in each lineage. The earliest root axes originated from dichotomous branching from shoot-bearing axes during the Early Devonian period. These roots were lacking root caps and root hairs as indicated by imaging and 3D reconstructions of fossils of

the extinct lycopsid *Asteroxylon mackiei*. Homologies in cellular anatomies, tissue patterning and gene expression profiles observed among roots of the extant lycophytes and euphyllophytes are likely a consequence of convergent evolution. It is possible that the two lineages independently invented roots as an adaptation to the same ecological challenge by employing the same gene toolkit that had been inherited from their last common (rootless) ancestor.

Root branching in euphyllophytes have multiple origins

Another crucial developmental innovation that occurred in the root axis is its ability to branch out. Dichotomous branching originated independently both in the early diverging lycophytes and euphyllophytes lineages, but it has only been preserved in the extant lycophytes (Figure 1). Based on the available fossil records, LR branching evolved seemingly in different times, but only, in the euphyllophyte lineage. It first appeared in the lignophyte lineage during the Middle Devonian period. Later, it evolved in ferns and horsetails during the Late Carboniferous period. Lateral root branching is characterized by high diversity in term of initiation origins (e.g., endodermis, pericycle) and complexity (Figure1-3). We speculate that compared to dichotomous branching, lateral rooting might confer high plasticity, and thus adaptation to various environments.

Root stem cell specification and patterning in ferns

Despite some phenotypic similarities among roots of ferns and seed plants, the ontogeny of their root apical meristems is different. The fern root apical meristem contains a single four-sided pyramidal root apical cell (RAC), with exception of some species that possess a group of RACs as is the case for instance for *Osmunda regalis*. The fern RAC is mitotically active and divides asymmetrically and sequentially from the

three proximal division planes to give rise to daughter merophytes (Figure 3A-C). Each daughter merophyte divides eight times asymmetrically to produce different cell lineages of the root. The fourth distal plane divides asymmetrically to give rise to merophytes that produce root cap (Figure 3B-C). The highly ordered cell divisions of the RAC make fern roots an interesting model to study cell lineage specification, tissue patterning and differentiation as well as their evolutionary trajectories and origins. Recently, *de novo* transcriptome assembly of *Ceratopteris richardii* (C-fern) root tip points to the conservation of several stem cell regulators such as *SHORT ROOT (CrSHR)*, *SCARECROW* (*CrSCR*) and *RETINOBLASTOMA-RELATED* (*CrRBR)* transcription factors. These transcription factors are required for asymmetric cell division of root stem cell initials that give rise to the endodermis and cortex tissues in seed plants. However, whether the SCR-SHR-RBR bistable circuit is conserved in ferns remains to be addressed.

Depending on the species, LRs in ferns develop endogenously either from pericycle (e.g. *Angiopteris, Botrychium, Equisetum, Osmunda*) or endodermis (e.g. *Adiantum, Ceratopteris*, *Marsilea*, *Pteris)*. In the model C-fern, LR development begins with the specification of one endodermal cell within a merophyte. The specified cell expands until it reaches a certain volume then it undergoes four successive asymmetrical divisions to establish a pyramidal LR apical cell in the center and four flanking daughter cells (Figure 3B-C). The subsequent divisions of these cells mark the beginning of the LR primordium development (Figure 3D). The spatial arrangement of LR stem cells along the longitudinal axis of the root is particularly obvious under the microscope. In addition, only two in every three successive merophytes produce LR stem cells, making LR stem cell establishment in C-fern predictable in space and time. It is noteworthy to mention that the water fern *Azolla filiculoides* does not form LRs. The

absence of LRs in *Azolla* might be a unique evolutionary scenario that does not necessarily represent an ancestral state.

Do lineage-dependent cues control LR stem cell specification in ferns?

The relatively simple developmental patterning of LRs in C-fern suggests the presence of a minimal or ancestral molecular machinery that guides this process. The high predictability of LR stem cell establishment is due to the periodic and invariant division patterns of the RAC. It is possible that the RAC produces daughter merophytes with pre-determined cell fates that will follow formative cell divisions in a lineage-dependent manner and therefore positional cues might play a minor role, if any. In line with this assumption, exogenous applications of auxins (e. g. IBA or IAA) or blocking its polar transport in C-fern sporophytes do not affect LR stem cell establishment, whereas they inhibit shoot-borne root elongation in a dose-dependent manner. These data suggest that auxin is neither sufficient nor needed for LR development in C-fern. Notably, the C-fern genome contains all the molecular components required to wire the canonical auxin signaling, including CrTIR1/AFB receptors, CrAux/IAA repressors, and CrARF transcription factors.

The auxin signaling pathway plays a major role in LR development in the model seed plant Arabidopsis. Mutating genes involved in auxin perception or signaling causes strong defects in LR development. For example, knocking out two transcription factors *(arf7arf19)* completely blocks the LR development process in Arabidopsis seedlings grown on agar plates. However, the double mutant *arf7arf19* produces LRs when grown under soil conditions, arguing for the existence of an auxin-independent pathway that controls LR development. This pathway seems to rely on the homeobox transcription factor WUSCHEL-RELATED HOMEOBOX 11 (AtWOX11), which directly activates the expression of the key LR regulator *LATERAL ORGAN BOUNDARIES*

DOMAIN 16 (AtLBD16) transcription factor, and thereby induces LR initiation. This is interesting because *AtWOX11* is an ortholog of the C-fern *CrWOXA*, which is expressed specifically in LR stem cells during the four asymmetric cell divisions and its expression stops after the fourth division. Although the function of *CrWOXA* in LR development is still not known, its closely related paralog *CrWOXB* is also expressed in LR primordia and seems to control LR development. Based on these preliminary observations, we propose a hypothesis that auxin-independent WOX-mediated LR development may represent a conserved alternative pathway that controls LR development in euphyllophytes when grown in natural (soil) conditions. This pathway is possibly masked in seed plants by a dominating effect of positional cues when the seedlings are grown in agar plates.

Concluding remarks and perspectives

Recent findings provide detailed single cell transcriptional maps of different root cell types. This knowledge reinforced our understanding about the molecular bases underlying identity specification and tissue patterning in angiosperms. However, It is still unclear how the pericycle lineage is specified at the root apical meristem, and whether XPP and PPP have different identities. If they do, we wonder how, and when their cell fates are determined. It would also be interesting to unravel the epigenetic landscape and dynamics during LR stem cell specification at the single cell resolution. LR stem cell specification in angiosperm is dominated by the effect of positional cues, especially those steered by auxin, but whether lineage-dependent cues are also involved in this process remains to be addressed. Tackling LR stem cell specification from an evo-devo perspective would likely provide answers to this question and help to unravel the underlying mechanisms. The recent availability of genome sequences of ferns and lycophytes as well as the rapid development of spatial transcriptomics

techniques and computational tools would open new avenues to probe the evolutionary history and origins of LR stem cell specification. It would be interesting to include crop species (e.g. wheat, maize, rice, tomato) as model systems to understand LR stem cell specification. This will not only help to understand the evolutionary trajectory of this developmental process but also contribute to design crops with robust root systems that can cope with climate change.

Declaration of Interests

The authors declare no competing interests.

Further reading

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Figures

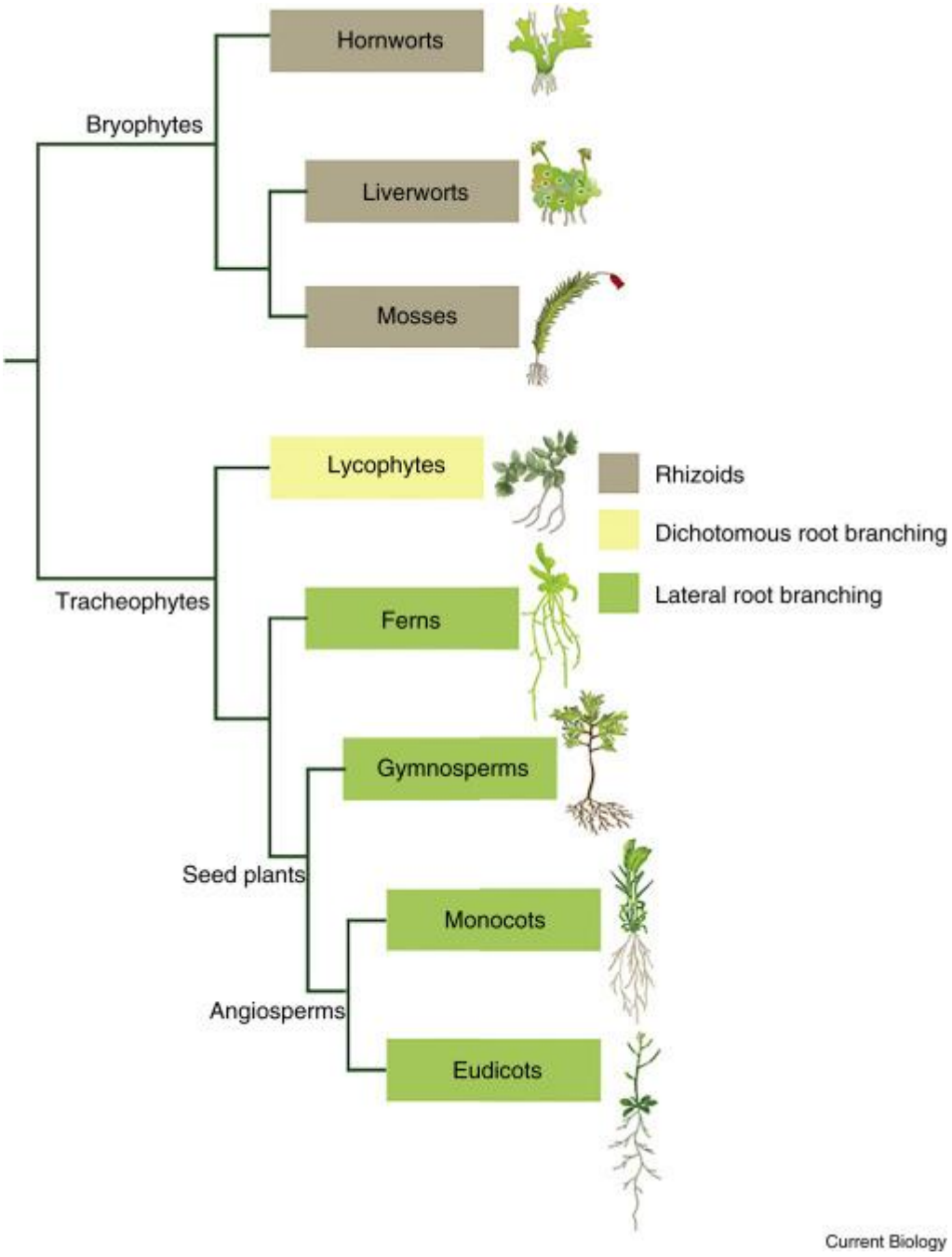

Figure 1: Evolution of root branching in land plants

A cartoon phylogenetic tree illustrating examples of root branching types in the extent embryophytes. Bryophytes, which comprise hornworts, liverworts and mosses, are rootless and develop rhizoids. Lycophytes, which comprise isoetales, lycopodiales and selaginellales, develop root axis that bifurcate dichotomously at their root tips.

Euphyllophytes, which comprise ferns, horsetails and seed plants, develop root axis that produce endogenously lateral roots.

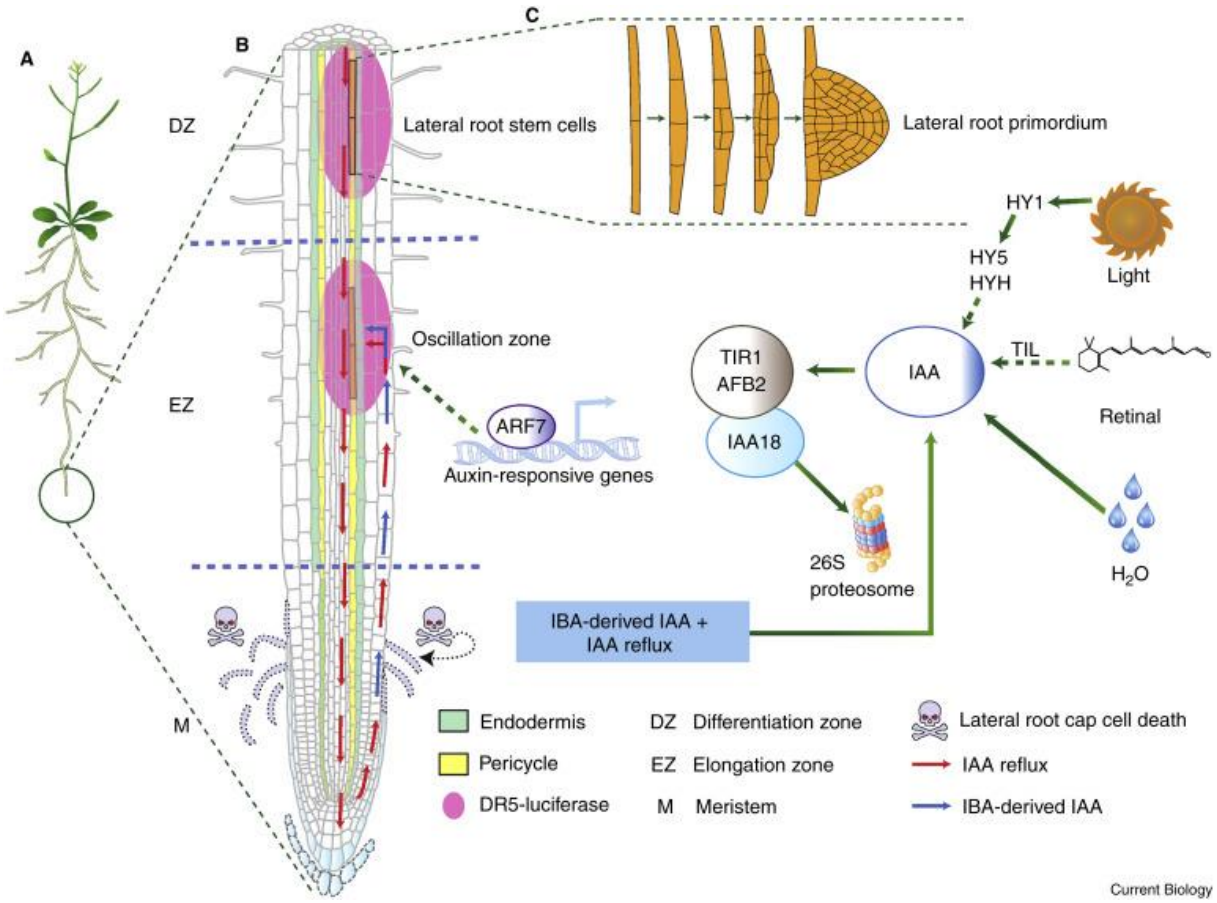

Figure 2: Role of positional cues in LR stem cell specification in Arabidopsis.

(A) Arabidopsis plant. **(B)** Schematic illustration of root tip showing different cell types and tissues (e.g. endodermis (green), pericycle (yellow) LR stem cells (orange)). Auxin controls LR stem cell specification in the elongation zone through gene expression oscillation as indicated by *DR5:LUC* reporter (pink). The persistence of *DR5:LUC* peak above the oscillation zone is an indication of a successful LR stem establishment. There are two sources of auxin that may control LR stem cell specification: shoot-derived auxin (red arrows) and IBA-derived IAA (blue arrows). Light and water promote IAA accumulation in the oscillation zone. Retinal also promotes this process *via* TIL. When IAA content increases in the protoxylem and/or in the abutting XPP cells, IAA18 forms co-receptor complex with TIR1 and/or AFB2 and is sent for degradation through the 26S proteasome. This releases transcriptional activity of ARF7 and thereby promotes LR stem cell specification. Continuous and dashed green lines indicate proven and possible interactions, respectively. **(C)** LR initiation and emergence. The specified XPP cells undergo a series of tightly coordinated cell divisions to form LR root primordium.

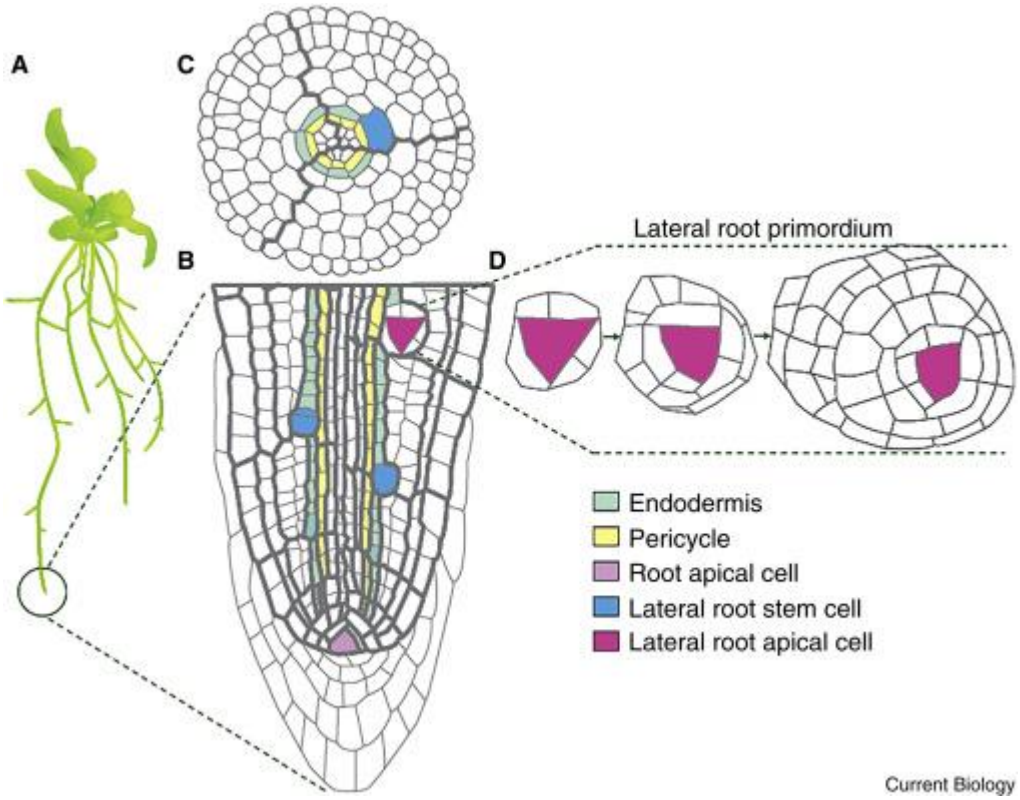

Figure 3: Lateral root development in C-fern

(A) C-fern plant illustration showing shoot-born roots from which LRs develop. **(B)** A longitudinal section of C-fern root showing different cell types and tissues (e.g. root apical cell (light pink), endodermis (green) and pericycle (yellow)). One cell within endodermis (colored in blue) expands before undergoing a series of formative cell divisions marking the earliest stages of LR stem cell specification. **(C)** A cross section of C-fern root showing the earliest stage of LR stem cell establishment (colored with blue). **(D)**The specified LR stem cell continues to divide to establish a LR apical cell in the middle (colored with magenta) and daughter merophytes, which in turn divide to form LR primordium.