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Lateral root formation in Arabidopsis: a well-ordered LRexit

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Abstract:	Lateral roots are crucial for increasing surface area of root systems to explore heterogeneous soil environments. Major advances have recently been made in the model plant Arabidopsis thaliana to elucidate the cellular basis of lateral root development and the underlying gene regulatory networks that control morphogenesis of the new root organ. This has provided the foundation on which to understand the sophisticated adaptive mechanisms that regulate how plants pattern their root branching to match the spatial availability of resources like water and nutrients in their external environment. We review new insights into the molecular, cellular and environmental regulation of LR development in Arabidopsis.

Cover Letter

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Dear Susanne,

Please find enclosed our invited review manuscript for Trends in Plant Sciences entitled “Lateral root formation in *Arabidopsis*: *a well-ordered LRexit.”* The manuscript focuses on reviewing key scientific and technological advances relating to Arabidopsis lateral root development over the past 5 years and the novel mechanistic insights that have resulted from these studies. Our manuscript is meant to synergise with the co-submitted review paper by Muller et al which reviews lateral root development in other species.

Our manuscript has been prepared with close attention to your instructions to authors. It contains 4 figures and 1 Table. Please note that one figure (number 4) requires artistic assistance from you journal.

Yours sincerely

Professor Malcolm J Bennett

Highlights (889/900 characters, including spaces)

- Major advances have recently been made in *Arabidopsis thaliana* to elucidate the cellular basis of lateral root development and the underlying gene regulatory networks.
- New 4D imaging approaches are revolutionising the field's perspective of lateral root morphogenesis.
- Recent studies reveal that biomechanical interactions between the new primordia and overlying tissues impact organ initiation and morphogenesis. We propose a new mechanism termed the *developmental traffic light* model to explain how mechanical signals influence LRP patterning.
- Lateral root research has progressed beyond studying individual genes to characterising gene regulatory networks by exploiting innovative systems and -omics based approaches.
- Arabidopsis roots employ regulatory mechanisms to sense the availability of water and nutrients and adapt their pattern of branching to optimise resource capture.

Lateral root formation in *Arabidopsis*: *a well-ordered LRexit*

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Keywords: Auxin, biomechanics, live microscopy, cell fate, environmental patterning

Lateral roots are crucial for increasing surface area of root systems to explore heterogeneous soil environments. Major advances have recently been made in the model plant *Arabidopsis thaliana* to elucidate the cellular basis of lateral root development and the underlying gene regulatory networks that control morphogenesis of the new root organ. This has provided the foundation on which to understand the sophisticated adaptive mechanisms that regulate how plants pattern their root branching to match the spatial availability of resources like water and nutrients in their external environment. We review new insights into the molecular, cellular and environmental regulation of LR development in *Arabidopsis*.

New dimensions to lateral root morphogenesis in *Arabidopsis*

Lateral roots originate primarily from pericycle tissue in angiosperm species [1,2]. The pericycle consists of a single cell layer surrounding the vascular tissues and which is overlain by endodermal, cortex and epidermal tissues (Fig. 1). In *Arabidopsis,* lateral roots derive from six pericycle cell files overlaying the xylem pole (Fig. 1) [3–5]. Phloem-pole pericycle (PPP) cells are reported to be mitotically dormant, while xylem-pole-pericycle (XPP) cells retain stem cell activity after leaving the primary root meristem and can therefore form LRs [6].

In *Arabidopsis*, lateral root development can be divided in five main steps (Figure 1A): (1) **pre-branch site formation** which takes place in the basal meristem/elongation zone [7,8]; (2) **LR initiation** which features pericycle nuclear migration to a common cell wall between pairs of founder cells (LRFCs) followed by an asymmetric cell division in the differentiation zone [9,10]; (3) **LR morphogenesis** in which the LRFCs divide further to form a lateral root primordium (LRP) that eventually acquires a root meristem organization [11,12]; (4) concomitant **LR**

emergence where the new organ grows through overlaying tissue layers to emerge from the parent root in the differentiation zone [13] and finally (5) **LR meristem activation** corresponding to the initiation of cell divisions in the newly emerged lateral root meristem [14].

Until recently, *Arabidopsis* lateral root development was studied in a 2D manner, primarily visualising primordia stages from a 'side on' viewpoint (Fig 1) and not considering the wider 3D morphogenic events taking place. Advanced microscopy approaches such as confocal imaging and Light Sheet Fluorescence Microscopy (LSFM, [15]) has helped revolutionise the field's perspective, providing static [16–19] or real-time [5,16,19,20] 3D visualization of lateral root development (Figure 1B). For example, LR formation features a stereotypical sequence of cell layer generation [5,11]. However, 4D time-lapse imaging studies of LR morphogenesis revealed that after the first anticlinal cell division the pattern of divisions does not follow a specific sequence [5,12,21]. There is a high level of plasticity in the spatio-temporal regulation of cell divisions with a higher order of rules to form the shape of a new root tip.

Patterning under pressure

LRP originate from pericycle cells located deep within the parental root and have therefore to pass through overlying endodermal, cortical and epidermal tissues before emerging [22] (Figure 1). This involves complex biomechanical interactions between the overlying tissues and the LRP that impact organ initiation and morphogenesis [21,23–25].

During LR initiation, root cells surrounding new primordia actively adapt and remodel their properties to accommodate organ emergence [21]. For instance, during organ initiation LRFCs swell prior to asymmetric division which would require adjacent xylem and/or endodermal

cells to adjust their volumes to allow the radial growth of pericycle cells (Figure 1) [25]. Intercellular connectivity between the LRFC and surrounding cells are likely to be involved in coordinating cell volume adjustments through control of callose deposition by plasmodesmata remodelling enzymes which regulate initiation of lateral roots [26]. Interestingly, endodermal cell ablation is sufficient to trigger mitosis in the underlying pericycle cell [27]. Nevertheless, auxin co-treatment is required to observe an anticlinal division of the induced pericycle cells and initiate the lateral root organogenesis program [27]. In addition the cell wall remodelling enzyme EXPANSIN A1 (EXPA1), which may modulate the mechanical properties of the pericycle cell wall, is also required for radial expansion of LRFCs and to ensure the correct positioning of the first anticlinal divisions (Table 1) [28]. Hence, sufficient pericycle width appears necessary to trigger asymmetric pericycle cell divisions during lateral root initiation [28].

During LR morphogenesis, primordia have to develop under mechanical pressure from surrounding tissues. These mechanical constraints, rather than a stereotypical pattern of cell division, are responsible for determining LRP shape [5,21]. Plants, like animals, regulate morphogenesis of new organs by employing a stem cell niche regulated by a mitotically inactive organising centre [29]. Interestingly, the organising centre appears once LRP form 4 cell layers (stage IV; Figure 2C) and just prior to growing through the endodermis [12]. The endodermis contains an impermeable barrier termed the Casparian Strip (Figure 2) that was originally thought to regulate elemental and nutrient movement between inner and outer root tissues. However, the mechanical properties of the Casparian Strip may also provide information that impacts development of LRP. During the time course of LRP development, the mechanical properties of the endodermis cells are remodelled through an auxin-dependent pathway and this is necessary for LRP development to progress from stage V

onwards in a wild-type fashion [21]. We suggest that the Casparian strip could behave like a *"developmental traffic light"* that holds back new LR (red light) until the organising centre and stem cell niche are set up (amber light) allowing the new organ to break through the endodermis and overlying tissues before emerging into the soil (green light) (Figure 2). This checkpoint system could also provide a mechanism for regulation of LR development by endogenous or environmental signals, allowing only LRP in optimal conditions to emerge [30].

Breakthroughs in Lateral Root Emergence

New LRP must reprogram overlying cells to aid organ emergence. The hormone auxin functions as a local signal released by new LRP to facilitate this progression [22,25]. Key processes targeted by auxin in overlying cells include modifying their hydraulic properties, cell walls and Casparian strip [21,23,25,31–33]. Regarding hydraulics, auxin represses the expression of almost every member of a family of water channels termed aquaporins [23]. Aquaporin genes encode plasma membrane (PIP class) or tonoplast (TIP class) localised water channels that regulate hydraulic properties of plant cells. Auxin appears to fine-tune the hydraulic properties of cells in the LRP and overlying tissues through its regulation of PIP and TIP genes spatial expression. Over-expressing and/or mutating PIP and TIP class aquaporins significantly delays LR emergence (Table 1) [23,33]. Hence, auxin-dependent spatio-temporal regulation of aquaporin expression appears critical for emergence of LRP. The importance of these water channels results from new LRP becoming symplastically isolated from surrounding tissues soon after initiation through closure of plasmodesmata [26].

Auxin originating from new LRP also causes overlying cells to modify their cell walls and undergo cell separation to enable new organs to emerge [22]. Auxin does this by triggering the sequential induction of the PIN3 auxin efflux carrier and then the LAX3 influx carrier in cells directly overlying new LRP [34]. A 3D root mathematical model indicated that, collectively, the distinct temporal patterns of induction of different classes of auxin carrier was necessary for preferential hormone accumulation in cells overlying LRP. This functions to focus the auxin-dependent induction of cell wall remodelling enzymes and, consequentially cell separation, in advance of the emerging organ [22,34].

Reactive oxygen species (ROS) have also been proposed to function as important signals during auxin-regulated LR formation. ROS treatment increases LR density and can restore LR formation in lines in which auxin-mediated cell wall accommodation and remodelling in LRP-overlying cells is disrupted. ROS are deposited in the apoplast of overlying cells during LR emergence following induction of RESPIRATORY BURST OXIDASE HOMOLOGS (RBOH) (Table 1) [35]. Disrupting (or enhancing) expression of RBOH in LRP or overlying root tissues decelerates (or accelerates) LR development and emergence. Hence, RBOH-mediated ROS production appears to facilitate LR outgrowth by promoting remodelling of overlying root cell walls. ROS levels generated within LRP are also important for organ emergence [35,36]. The MYB36 TF controls the expression of a subset of peroxidase genes (e.g. *PER9*) in boundary cells at the base of developing LRP. Mutating *MYB36* causes LRP to adopt a flattened shape compared to the dome-like appearance of WT LRP, resulting in slower organ emergence. Reducing the levels of hydrogen peroxide (H_2O_2) in *myb36-5* rescues the LR mutant phenotype (Table 1). This suggests that MYB36-dependent induction of peroxidases reduces H_2O_2 levels thus defining the outer boundary of the growing LRP [37].

Lateral Root Gene Regulatory Networks: ***learning lessons from primary root development***

A large number of genes that regulate meristem patterning and maintenance in primary roots are also expressed during equivalent processes in lateral root development. For example, AP2-/ERF PLETHORA (PLT) transcription factors are major regulators of the gene regulatory network controlling primary root meristem patterning and maintenance [38]. *PLT* genes are also expressed during LRP development [39]. Interestingly, *PLT3*, *PLT5*, and *PLT7* were shown to control the onset of *PLT1, PLT 2*, and *PLT4* gene expression in developing LRP (Figure 3). Accordingly, the triple *plt3plt5plt7* mutant exhibits a PLT-null phenotype in which the first round of periclinal cell divisions, which creates a two layered LRP, is disrupted, resulting into a highly disorganized LRP with no properly defined meristem (Table 1). Surprisingly, transgenic expression of any PLT member from stage I onwards is sufficient to restore the formative periclinal divisions and subsequent organization of root meristem-like identities. This suggests that rather than target specificity of PLTs, precise timing in *PLT* expression controls critical events during LRP formation [13].

Gene regulatory networks dependent on the GRAS-family SCARECROW (SCR)/SHORT-ROOT (SHR) transcription factors control the transition from stage II to stage III in LRP development (Figure 1) and thereby impact patterning of the quiescent centre (QC) at stage IV/V [12,40]. The observed onset of QC marker gene expression is concomitant of a major transition from an early morphogenesis phase to a late, meristem organization phase (Figure 1). Interestingly, the *scr* mutant is still able to establish a QC and a functional root meristem later in development, thus illustrating the robustness of the LR regulatory network [12]. In the primary root tip SCR and PLT proteins were shown to directly induce expression of QC marker

WOX5 by cooperative interaction with TCP to coordinate LR outgrowth in a time- and space-dependant manner (Figure 3) [41].

Lateral Root Gene Regulatory Networks: *emerging properties*

Lateral root research has recently moved beyond studying the role of individual or a few genes to characterising many genes that compose regulatory networks controlling lateral root development employing systems and -omics based approaches. LR-related transcriptomic datasets have recently been produced in different conditions, monitoring gene expression dynamics during root branching in an unbiased fashion [42–46]. These offer unprecedented resources to explore single gene expression dynamics and identify new candidate regulators of LR formation. More importantly understanding how these components interact to form regulatory networks is crucial if we want to understand the emerging properties of LR development [47,48].

LRP can be induced in a highly synchronised manner by gravistimulation [23,46,49]. Researchers have therefore exploited gravistimulation-based LR induction to sequentially sample root bending zones every 3 hours and to generate a high resolution time course transcriptomic dataset spanning LR induction to organ emergence and meristem activation [46]. This unique transcriptome resource offers unprecedented insights to explore expression dynamics during LR organogenesis, ranging from a single gene up to the network scale. More than 8,000 (out of 22,000) genes were differentially expressed during LRP formation. Clustering identified 77 distinct classes of gene expression profiles, highlighting the complex regulation of LR formation [46]. Interestingly a number of circadian-clock regulated genes were identified, revealing a surprising clock re-phasing mechanism during LR initiation, which

were proposed to ‘insulate’ the LRP from diurnal fluctuations in water fluxes which may interfere with organ emergence [46].

This LR transcriptomic dataset has been used to infer the topology of the gene regulatory network coordinating lateral root morphogenesis [50]. The predicted GRN controlling primordia patterning was organised in two main auxin-regulated modules, an early network dependent on ARF7 and ARF19 (Figure 3), and a later network, involving ARF5. In general, genes in the early ARF7-associated sub-network are expressed in all primordia cells during early stages of LRP development and then their expression decreases and is confined to the base of LRP. Concomitant with this transition, expression of genes associated with the ARF5 module rises. This module includes many transcription factors involved in primary root meristem organization whose expression is observed in central and tip LRP zones [13,50]. Interestingly, the inference approach indicated that these two sub-networks were linked by multiple mutual inhibition relationships [50]. This topology is predicted to confer a toggle switch behaviour to the GRN [51–53]. Altogether, this suggests that a switch between a module controlling early stages of LR development and a second module regulating meristem organisation occurs between 20h and 30h after gravistimulation i.e. at the transition from stage IV to V. Interestingly, this corresponds to the appearance of the organising centre identity in LRPs that marks a major transition in LRP cell divisions and expansion [12]. Thus, the predicted topology of the network suggests systems-scale mechanisms that contribute to the patterning of LRP. Network analysis further revealed an unexpected role for very long chain fatty acids (VLCFA) acting downstream of the transcription factor PUCHI to control cell proliferation during lateral root formation (Trinh et al, PNAS, in press).

LR transcriptome analysis and meta-analysis approaches also contributed to identify numerous small signalling-peptide encoding genes involved in intercellular communications during root branching [54,55]. Pairs of peptides and peptide-receptor complexes were shown to contribute to the spatial patterning of the lateral root initiation by mediating cell-to-cell signalling [24,56–59]. Recently, the secreted peptide TARGET OF LBD SIXTEEN 2/PAMP-INDUCED PEPTIDE-LIKE 3 (TOLS2/PIPL3) and its receptor RLK7 were shown to cooperate in inhibiting LR initiation near pre-existing LRFC in a non-cell autonomous manner, thus controlling minimal spacing between initiated LRs (Figure 3) [60]. Together with the intricate networks controlling hormones distribution and signalling across root tissues, peptide signalling pathways provide a new level of complexity in cell-to-cell communication that can contribute to the overall organisation, especially spatial patterning, in the root branching process [61,62].

In summary, network scale analyses revealed interconnectivity, redundancy and multiple feedback loops provide plasticity at a cellular level, confer robustness to LRP development at a multicellular scale, and allow for the integration of plant systemic cues. These network properties are likely to be crucial for the root developmental machinery to adapt to the myriad environment signals that they are exposed to in highly heterogeneous soil environments [17].

Divining Roots in search of moisture

Recent studies have revealed that plant roots employ sophisticated regulatory mechanisms to sense and respond by branching to the availability of water and nutrients. Soil consists of air pockets, stones, nutrient rich and poor patches, whilst the spatial and temporal

distribution of moisture also varies. Regulating where a lateral root will form is therefore a critical decision to maximise efficiency of foraging in soil. One of the most important soil resources for plant roots to obtain is water. It has long been known that osmotic stress can slow down lateral root emergence in *Arabidopsis* and that this response is ABA dependent [63]. In roots of barley (*Hordeum vulgare* cv.) and maize (*Zea mays*), water deficit caused lateral roots to no longer initiate [64]. A recent article revealed this is controlled by an abscisic acid (ABA)-dependent regulatory mechanism termed xerobranching [65]. The effect of water deficit could be mimicked by ABA treatment, but mutants defective for key PYR/PYL ABA receptors were resistant to both signals. Additional experiments revealed that ABA accumulated in root tip tissues when exposed to a water deficit, blocking LR initiation possibly by attenuating the oscillatory auxin response network in the basal meristem.

A related adaptive response termed lateral root hydropatterning describes the ability of roots to differentiate between contact with moist soil or air, then trigger preferential branching towards water. Xerobranching (the inhibition of LR initiation by drought stress) and hydropatterning (the preferential LR emergence towards the wettest side of the root) appear to be mechanistically distinct since the latter response is unaffected in ABA mutant backgrounds [65,66]. Instead, modelling studies led authors to propose a “sense-by-growth” mechanism for hydropatterning in which uptake of external water into the root elongation zone conveyed a means to perceive water availability [66]. Recent research has revealed that water availability controls LR initiation in the elongation zone via post-translational modification of AUXIN RESPONSE FACTOR 7 (ARF7). This key LR regulator encodes a transcription factor that contains four SUMO modification sites. Mutating all 4 ARF7 SUMOylation sites disrupts hydropatterning [67]. When roots are exposed to air, ARF7 SUMOylation rapidly increases. SUMOylation of ARF7 enables this transcription factor to

recruit the Aux/IAA repressor protein, IAA3. This led to a model where the post-translational modification of ARF7 by SUMOylation on the dry side of a root causes IAA3 recruitment, repressing expression of target genes like *LBD16* that are required for LR initiation and hydropatterning (Figure 4) [67].

Branching out in search of nitrogen

Absorption of nutrients represents another crucial function of a root system and lateral roots in particular [17]. Nutrients are often dispersed in soil and therefore the ability by roots to sense nutrients is vitally important for efficient resource acquisition. Nitrate is a key nutrients that has a profound effect on lateral root development, whose formation and elongation is induced under mild N stress, but this is impaired during exposure to high N or prolonged N stress (recently reviewed in [68–70]). A recent study reported the importance of C-TERMINALLY ENCODED PEPTIDES (CEPs) in roots experiencing low N, where root stele-expressed CEPs travel to the shoot and accumulate in the leaf phloem, where they bind to two leucine-rich receptor kinases (LRR-RK), CEPR1 and CEPR2 [71]. This triggers the expression of *CEP DOWNSTREAM 1* (*CEPD1*) and *CEPD2*, which induce the expression of *NITRATE TRANSPORTER2.1* (*NRT2.1*) in roots exposed to N-rich conditions [72]. This boosts N uptake, but inhibits lateral root growth, as *NRT2.1* KO mutants have increased lateral root initiation [73]. A close family member, NRT1.1, coordinates a multitude of genes involved in nitrogen branching response [74]. This dual-affinity transporter and transceptor is phosphorylated under low nitrate conditions by CBL-Interacting Protein Kinase 23 (CIPK 23), turning the protein into a high-affinity nitrate carrier [75]. Interestingly, the non-phosphorylable form of NRT1.1 no longer represses lateral root initiation [76]. Phosphorylation of NRT1.1 negatively effects local auxin build-up in LRP through its ability to

co-transport auxin, which is inhibited in high nitrate patches, accounting for the local build-up of auxin, triggering localised emergence of lateral roots (Figure 4) [76,77].

Shining light on lateral root development

Recent studies have also revealed a key role for light on LR development. When roots were illuminated but the shoot kept in the dark, root growth and lateral root emergence was reduced [78]. Interestingly the bZIP transcription factor ELONGATED HYPOCOTYL5 (HY5) KO allele is incapable of directing root growth in response to exposing the shoot to light. HY5 stability is regulated by the COP1 ubiquitin ligase, which degrades HY5 in the dark [79]. Accordingly, the *cop4-1* mutant showed no difference in root growth in different shoot and root light treatments. Stabilized HY5 in the shoot is transported down to the root via the phloem to induce LR formation [78]. However, adding Far-red (FR) light could increase HY5 levels to a state in which lateral root primordia development was arrested [80]. The authors hypothesize this is through the binding of HY5 to the *ARF19* promoter, reducing its expression which negatively effects *PIN3* and *LAX3* auxin transport proteins in the overlaying cortex (Figure 4) [80]. Hence, light perception in the shoot can very finely regulate the auxin machinery locally in cells overlying LR primordia, to alter organ emergence. Interestingly, HY5 in shoots promotes carbon assimilation and in roots can induce expression of *NRT2.1* [78] whilst negatively regulating *AMT1;2* [81], which increases nitrate and reduces ammonium uptake, respectively. Collectively, this reveals novel root functions for HY5 and flags it as a key node in an array of plant environmental responses.

Concluding Remarks and Future Directions

Major advances have been made elucidating the molecular and cellular basis of lateral root development using the model plant *Arabidopsis.* Nevertheless, lateral root morphogenesis in other plant species like maize and rice exhibits far greater anatomical variation than observed in *Arabidopsis*, reflecting the diversity of root classes [82]. However, very little is currently known about the underlying gene regulatory networks and mechanisms driving the morphological diversity of these different LR classes in crops. Despite this, genetic studies in crop models like maize and rice have identified common hormone signals and genes with Arabidopsis that regulate lateral root development [83,84].

Surprisingly, limited attention has been paid to the role of biomechanics in the regulation of lateral root development, to date. However, increasing evidence points to the central role for mechanical signals during development in both plants and animals [21,85]. Lateral root development is a fascinating system to address these questions given that, unlike leaf primordia formation that takes place on the flanks of the shoot apical meristem, new root organs originate deep within parental root tissues. Live cell imaging, e.g. light sheet microscopy, will further advance the field in observing the dynamics of lateral root morphogenesis in real-time. Such research could generate fundamentally new knowledge about mechanical patterning and signalling in plants. For example, uncovering how LRP QC specification is synchronised with the new organ disrupting the Casparian strip could reveal novel mechanical signalling components and mechanisms linking cell walls and cell cycle regulation.

The rich 'omic' resources available for *Arabidopsis* has helped initiate efforts to elucidate the underlying gene regulatory networks that control morphogenesis of the new root organs. GRN approaches have revealed system properties that confer important features in LRP development: ability to stabilize or, conversely, trigger changes in cell identity, capacity to organize those changes in time and space (patterning), capacity to reach a similar output from various transcriptomics scenarios (robustness) and at the same time, capacity to integrate external cues that may influence the GRN final state. This latter plasticity property plays a prominent role for root system adaptation to environmental signals. This will also provide the foundation on which to probe the sophisticated adaptive mechanisms that regulate how plants pattern root branching to match spatial availability of resources like water and nutrients in their external environment.

In parallel with -omics data collection and studies, advances in cell tracking, sampling and transcriptome analysis will generate a more detailed cartography of gene expression in developing LRP at various stages. When combined with time-course transcriptomic datasets, this will allow exploration of GRN at high temporal and spatial resolution. Moreover, cross-referencing with other omics datasets, such as epigenomics, or phosphoproteomics, will help decipher the complex regulation levels resulting in the observed transcriptomic profiles and properties in LRP development [86]. Further studies will be needed in *Arabidopsis* to explore and characterise the emerging properties of the lateral root GRN and how it integrates environmental signals, and how it compares to mechanisms in other plant species (see Outstanding Questions).

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Figure Legends

Figure 1. The birth of a new lateral root. *The five main steps of lateral root formation in the classic longitudinal view (A). Beginning with the formation of the pre-branch sites in the meristem and elongation zone. Followed by the initiation of lateral root founder cells (LRFC) by the nuclear migration of two pericycle cells and their first anticlinal division in the differentiation zone. During morphogenesis the LRFCs divide periclinally as well as radially to form a three/dimensional lateral root primordia (LRP). The LRP grows through the overlaying tissue layers to emerge from the primary root. After emergence the lateral root will acquire an active meristem. Colour coding was used according to the model described by Malamy and Benfey* [11]*. Six files of pericycle cell files contribute to the LRP seen in a radial perspective (B)* [5]*. Common colours (yellow or dark yellow) highlight daughter cells derived from individual pericycle founder cell files.*

Figure 2. The lateral root primordia (LRP) must break through the overlaying endodermis hardened with the Casparian strip before emergence. *Three time points of confocal time-lapse images highlight the thinning of the endodermis during LR morphogenesis and emergence (A). A 3D stack of images of an Arabidopsis root expressing the membrane marker (YFP-PIP1;4) and a Casparian strip marker (ESB1-mCherry) was captured every 10 min over a period of 20 h. Single slice images are displayed in the side view (A) and front view (B). The process of emerging through the endodermal barrier could function as a "developmental traffic light" in which the stage I to III LRP is initially stopped by the tough Casparian strip barrier in the overlaying endodermal tissue (C). When the lights hit orange the Casparian strip gets cues from the LRP to slowly and locally break down to let the LRP pass, which gives the LRP time to form the organising centre and stem cell niche. When the lights on green the LRP breaks through the endodermis and undergoes a drastic shape change from flat topped to dome shaped, while resuming its emergence through the cortex and epidermis.*

Figure 3. Feeding GRN with spatial and temporal information result in robust spacing and patterning of developing LRP. *Auxin synthesis, transport and response modules are sequentially triggered during lateral root initiation. The starting point for lateral root initiation is the nuclear migration in LRFC controlled by the module of SLR/IAA14–ARF7 (ARF19). ARF7 and FLP controlled the PIN3 transcription and ARF7-regulated FLP transcription factors form a coherent feed-forward loop controlling PIN3 transcription. The LBD16 and LBD18 transcription factors control cell cycle genes through E2FA transcription factor. LBD18 and ARFs form a double positive feedback loop, by binding directly to the ARF19 promoter and through the protein-protein interactions with ARF7 and ARF19* [87]*. ARF7, ARF19 module control PLT-dependant responses through SHR/SCR turnover to control LR patterning* [13]*.*

TOLS2 peptide interact with RLK7 to control PUCHI and LR spacing [60]*. A plant-specific B3 transcription factors FUS3 and LEC2 interact together to induce expression of the auxin biosynthetic gene YUC4 through binding to its promoter elements in LRFCs* [88]*. The phloem pole-expressed CEP5 and its proposed leucine-rich repeat (LRR) receptor XIP1 as well as another peptide GOLVEN 6 (GLV6), might also be involved in the LRI* [56]*. Abbreviations: A-type CYCLIN-Dependent KINASE A1.1 (CDKA1); AUXIN-RESPONSE FACTOR 7, 19 (ARF7, ARF19); C-TERMINALLY ENCODED PEPTIDE5 (CEP5); CYCLINB1.1 (CYCB1); FOURLIPS (FLP); FUSCA3 (FUS3); LATERAL ORGAN BOUNDARIES 16, 18 (LBD16, LBD18; LEAFY COTYLEDON2 (LEC2); RECEPTOR-Like KINASE 7 (RLK7); SOLITARY-ROOT (SLR); TEOSINTE-branched CYCLOIDEA PCNA (TCP); TARGET OF LBD SIXTEEN 2 (TOLS2); WUSCHEL-Related HOMEOBOX 5 (WOX5); XYLEM INTERMIXED WITH PHLOEM 1 (XIP1); YUCCA4 (YUC4).*

Figure 4. The soil and light environment plays a key role in lateral root (LR) positioning. *White light (WT) coming in from the top does converts PhyA into its inactive form, leaving COP1 to break down HY5* [79]*. However in WT+FR light, COP1 is broken down through stabilized PhyA mediated degradation and subsequently HY5 accumulates* [80]*. HY5 is transported through phloem to inactivate LRP primordia development through inducing its own local expression and indirectly reducing LAX3 and PIN3 in the overlaying tissue layers, possibly through ARF19 interaction. In patched of low nitrogen (N) soil, NTR1.1 becomes phosphorylated by CIPK23. Phosphorylation causes NRT1.1 to perform a dual function as nitrogen and auxin transporter, which blocks auxin accumulation in the lateral root primordia (LRP). In high N soil, NRT1.1 is unphosphorylated, blocking its auxin transport function and allowing auxin to build up in the LRP to promote emergence* [74–77]*. During periods of root growth through air gaps, xerobranching, abscisic acid (ABA) accumulates and binds to its*

receptor family PYR/PYL to indirectly reduce IAA levels and therefore lateral root founder cell (LRFC) initiation. However, when one side is in contact with water in the soil, ARF7 induces downstream targets such as LBD16 to initiate LRFC division on the side in contact with water, termed lateral root hydropatterning [67]*. Contrary, on the air side ARF7 is SUMOylated causing recruitment of IAA3 repressor proteins resulting in ARF7 protein degradation and inhibition of LR formation.*

Table 1. Overview of mutant and transgenic lines affected in lateral root development.

Category	Mutants	Paper	Lateral root phenotype	Stage affected
Auxin Signalling	*35S:TOLS2*	[60]	Reduced number of LRs	LR initiation
	puchi	[89]	Reduced number of LRs	LRP development/ emergence
	CASP::shy2-2	[25]	No LRs	LR initiation
Auxin transport	*aux1*	[90]	Reduced number of LRs	LR initiation
	lax3	[22]	Reduced number of LRs	Stage I development
Auxin conjugation/ degradation	*dao1*	[91]	Increased number of LRs	?
	gh3.1,2,3,4,5,6	[91]	Increased number of LRs	?
Auxin Biosynthesis	*yuc4*	[88]	Reduced number of LRs	?
Meristem establishment	*plt3plt5plt7*	[39]	Reduced emergence	Promotes initiation, reduces emergence
Transcription factors	*flp*	[92]	Reduced number of LRs	Delay in stage I
	fus3	[88]	Reduced number of LRs	Delayed emergence
	lec2	[88]	Reduced number of LRs	?
Peptides	*ralf34*	[57]	Increased number of LRs	initiation
	irAtRALF1	[93]	Increased number of LRs	?
	ida	[24]	Decreased LR density	Delayed emergence
	CEP5	[58]	Decreased LR density	stage I and II
Receptors	*xip1*	[58]	Decreased LR density	initiation and development
	the1	[59]	Increase number of stage I LRP	patterning and development of LRP
	GLV 1-11 OE	[94]	Reduced LR density	initiation and development
Kinases	*aur1-2 aur2-2*	[95]	Decreased LR density	Decreased initiation and emergence
	hae hsl2	[24]	Decreased LR density	Delayed emergence
	mkk4/mkk5	[96]	Reduced LR density	Delayed emergence
	MPK3SR and MPK6SR	[96]	Reduced LR density	Delayed emergence
Cell wall	*expa1*	[28]	Reduced number of LRs	Initiation
	gpat4 gpat8	[97]	Reduced number of LRs	Delayed emergence and deformation
	dcr	[97]	Reduced number of LRs	Delayed emergence and deformation
	bdg	[97]	Reduced number of LRs	Delayed emergence and deformation
	lrd5/xeg113	[31]	Increase rate of emergence	Emergence
Oxygen and ROS	*rap2.12,rap2.2,rap2.3,hre1,hre2*	[98]	Increased number of LRs	Delayed emergence
	erf VII	[98]	Increased number of LRs	Initiation
	robhc rbohd rbohe	[35]	Decreased LR density	Delayed emergence
	myb36	[37]	Decreased LR density	Delay in stage IV
	per7	[36]	Decreased LR density	Initiation
	per57	[36]	Decreased LR density	Initiation
Water transport	*pdbg1,2*	[26]	Increased LR density	Patterning and spacing
	pip2;1-1 and pip2;1-2	[23]	Reduced number of LRs	Delayed development
	tip1;1 tip2;1 tip2;1	[33]	Reduced number of LRs	Delayed emergence
Light sensing	*hyh/hy5*	[80]	Increased number of LRs	Initiation and emergence
SUMOylation	*ots1 ots2*	[67]	Reduced number of LRs	?
	siz1	[99]	Reduced number of LRs	?
Circadian clock	*toc1-1*	[46]	Decreased LR density	Delayed initiation and emergence

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Fig 1

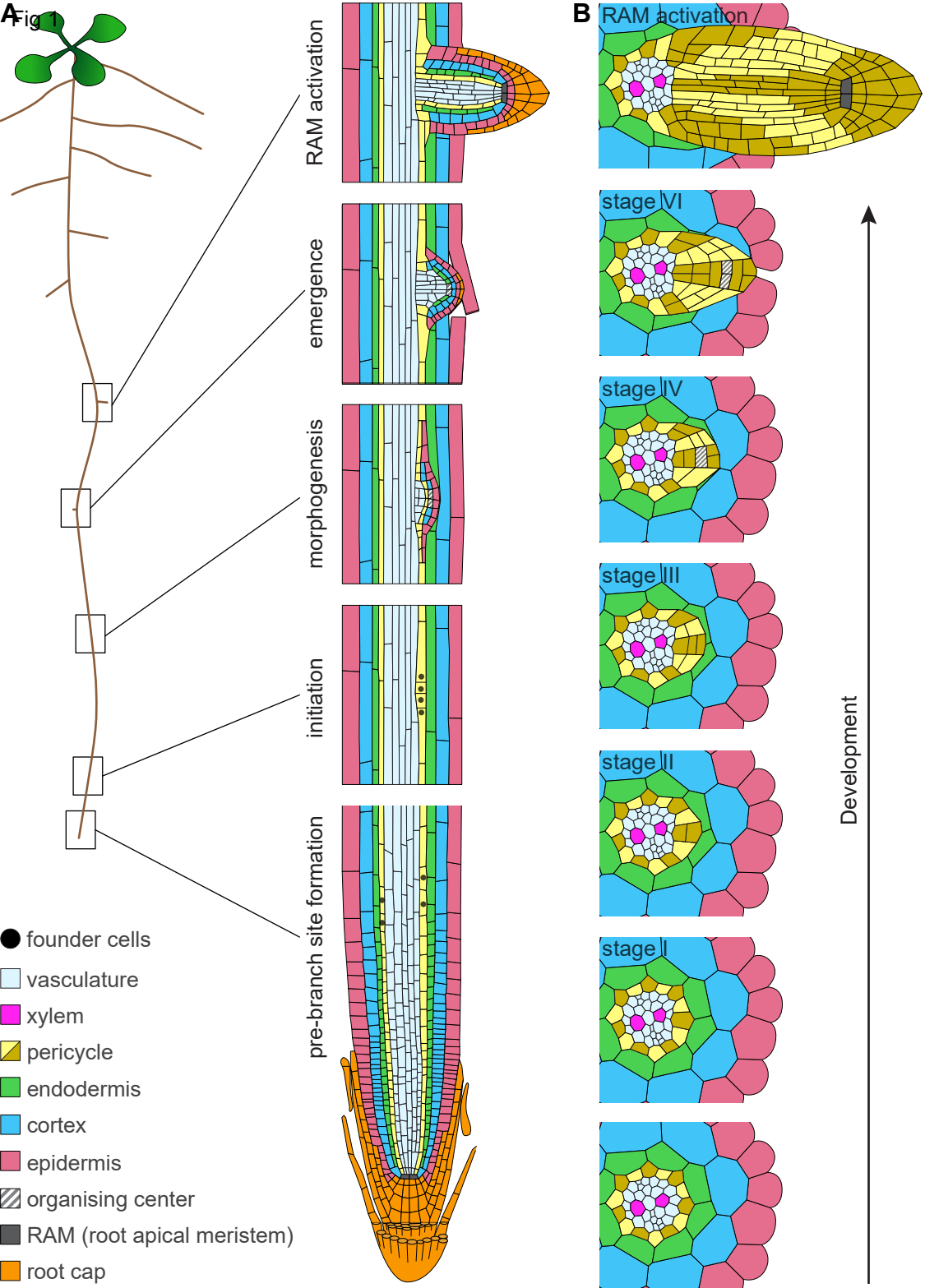

A
RAM activation
emergence
morphogenesis
initiation
pre-branch site formation
B
RAM activation
stage VI
stage IV
stage III
stage II
stage I
Development
founder cells
vasculature
xylem
pericycle
endodermis
cortex
epidermis
organising center
RAM (root apical meristem)
root cap

Fig 2

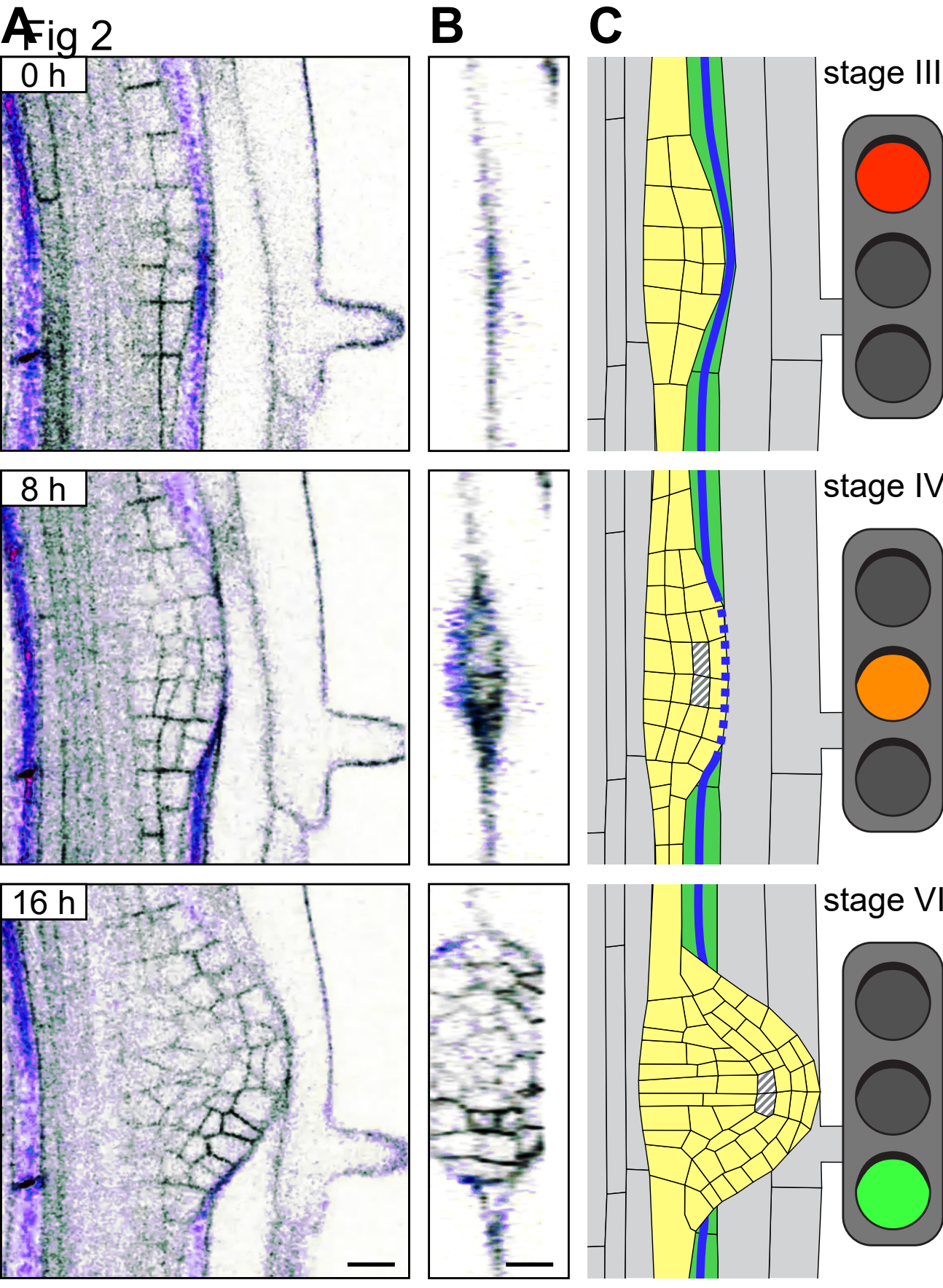

A
B
C
0 h
8 h
16 h
stage III
stage IV
stage VI
side view
front view
organising center
Casparian strip
endodermis
lateral root
ESB1::ESB1-mCherry
UBQ10::YFP-PIP1;4

Fig 3

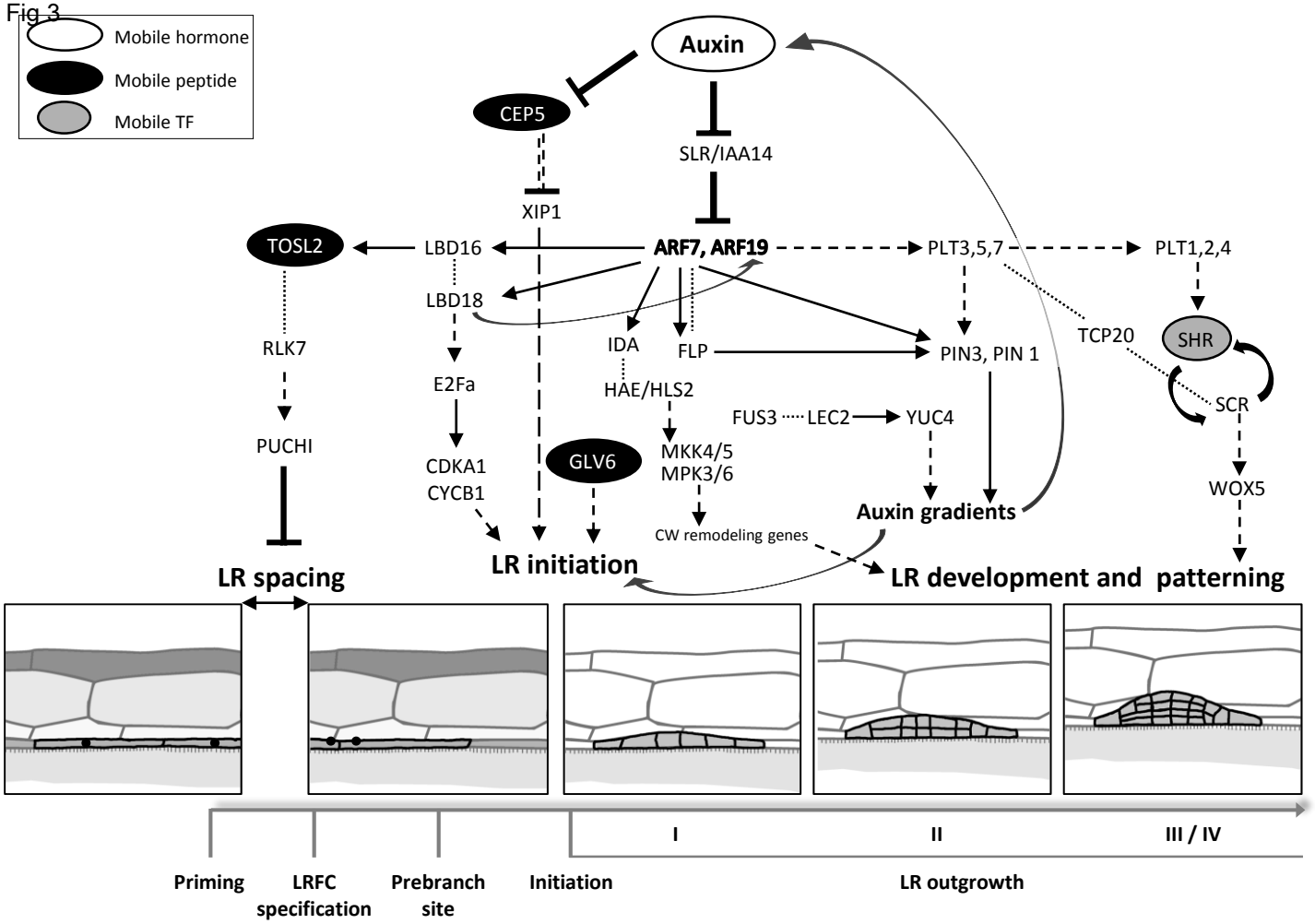
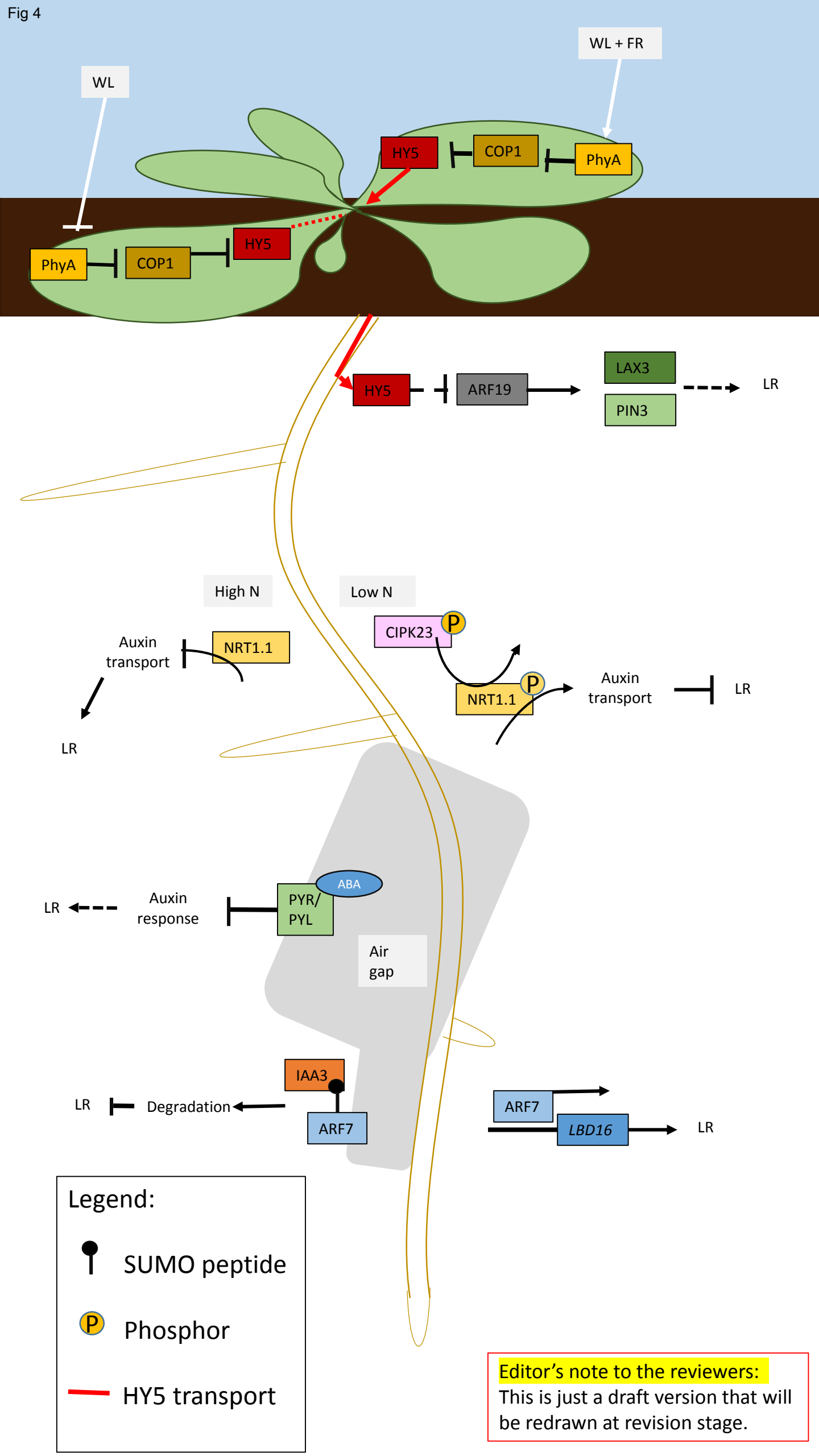


Figure 3: Feeding GRN with spatial and temporal information result in robust spacing and patterning of developing LRP. Auxin synthesis, transport and response modules are sequentially triggered during lateral root initiation. The starting point for lateral root initiation is the nuclear migration in LRFC controlled by the module of SLR/IAA14–ARF7(ARF19). ARF7 and FLP controlled the *PIN3* transcription and ARF7-regulated FLP transcription factors form a coherent feed-forward loop controlling *PIN3* transcription. The LBD16 and LBD18 transcription factors control cell cycle genes through E2FA transcription factor. LBD18 and ARFs form a double positive feedback loop, by binding directly to the ARF19 promoter and through the protein-protein interactions with ARF7 and ARF19. ARF7, ARF19 module control PLT-dependant responses through SHR/SCR turnover to control LR patterning. TOLS2 peptide interact with RLK7 to control PUCHI and LR spacing. A plant-specific B3 transcription factors FUS3 and LEC2 interact together to induce expression of the auxin biosynthetic gene YUC4 through binding to its promoter elements in LRFCs. The phloem pole-expressed CEP5 and its proposed leucine-rich repeat (LRR) receptor XIP1 as well as another peptide GOLVEN 6 (GLV6), might also be involved in the LRI. *Abbreviations: A-type CYCLIN-Dependent KINASE A1.1 (CDKA1); AUXIN-RESPONSE FACTOR 7, 19 (ARF7, ARF19); C-TERMINALLY ENCODED PEPTIDE5 (CEP5); CYCLINB1.1 (CYCB1); FOURLIPS (FLP); FUSCA3 (FUS3); LATERAL ORGAN BOUNDARIES 16, 18 (LBD16, LBD18; LEAFY COTYLEDON2 (LEC2); RECEPTOR-Like KINASE 7 (RLK7); SOLITARY-ROOT (SLR); TEOSINTE-branched CYCLOIDEA PCNA (TCP); TARGET OF LBD SIXTEEN 2 (TOLS2); WUSCHEL-Related HOMEOBOX 5 (WOX5); XYLEM INTERMIXED WITH PHLOEM 1 (XIP1); YUCCA4 (YUC4).*

Fig 4



Editor's note to the reviewers:
This is just a draft version that will be redrawn at revision stage.

Outstanding Questions Box (1000/2000 characters, including spaces)

- Lateral root (LR) development is a two-step process that eventually leads to the formation of a new root meristem. Which cues control the transition to lateral root primordium organisation?

- During LR morphogenesis, mechanical constraints from overlying endodermal, cortical and epidermal tissues, rather than a stereotypical pattern of cell division, determine primordia shape. Is this mechanical information contributing to organ patterning? How does the LRP signal to the endodermal Casparian strip to locally break down?

- How are environmental and systemic signals integrated within the LR gene regulatory network to regulate root architecture at the whole plant level?

- How does the *Arabidopsis* LR gene regulatory network differ compared to other plant species? Is it also comparable to the different lateral root classes described in other species?

- How can the use of single-cell analysis increase our understanding of transcriptomics, lineage tracing and epigenetics during LR morphogenesis?

Reviewer comments: Lateral root formation in Arabidopsis: a well-ordered LRexit

Reviewer #1: The current manuscript by Banda et al is well written and has a clear story line. It will serve as a nice update summarising all the latest advancements made towards a better understanding of LR formation. Also the illustrations, except the provisional one, are of a high standard. I only have a few comments that I would like to see addressed by the authors.

- page 3 line 1: it would suggest to replace pushing with growing. Pushing has a more passive tone, while there is a lot of data showing that there is an active signalling between the LRP and overlying cell layers and hence this is a rather active process. Similar goes for page 4 line 19, breaking is again passive and would suggest that the endodermis has no role during LR development and emergence.

We agree with the reviewers comment and replaced this term in the manuscript.

- I like the traffic light model, but why do the authors prefer to suggest that this is an CS-dependent mechanism. Environmental signals have a very strong effect on LR development and emergence. And how would they explain the phenotype of the myb36 mutant that has no CS for a large part of the root?

Whilst normal Casparian strips are indeed absent in plant roots lacking a functional MYB36, David Salt's group have shown that they are replaced by an ectopic lignin layer (located in the corners of endodermal cells; Kamiya et al, 2015, PNAS) which, like wildtype, forms close to the root apex (D. Salt, personnel communication). Like wildtype, myb36 roots block propidium Iodide staining of inner root tissues, suggesting that the mutant's modified CS retains a similar degree of chemical integrity (D. Salt, personnel communication). Nevertheless, the myb36 mutant exhibits a flat dome shaped LRP which may indicate differences in CS physical integrity.

- page 5, section regarding the PIPs and TIPs. Although I am aware of the work on PIPs and TIPs during LR formation and their relation to auxin, I think this interaction is displayed a little bit too simplistic. Don't PIPs and TIPs also need movement of other ions in order to drive the flow and transport of water? Why have the authors not discussed a bit more whether water movement depends on PIPs or rather the plasmodesmata?

We have clarified why PIP/TIPs are important (rather than PD) in controlling water fluxes, stating "The importance of these water channels results from new LRP becoming symplastically isolated from surrounding tissues soon after initiation through closure of plasmodesmata [26]."

- Have the authors considered that the expression of MYB36 at the boundary of the LRP (in endodermal cells not pericycle) is there just to setup the formation of the CS in the LRP?

That's an interesting question. We agree that the original CS (in endodermal cells surrounding the LRP) needs to link up with the new CS forming in LRP, to create a continuous barrier following LR emergence. Time lapse movies generated in our lab using an ESB1:VENUS reporter would concur with this.
Note: MYB36 is also expressed in pericycle cells at the boundaries of LRP where it controls expression of peroxidases that are proposed to stiffen cell walls/stop division (Fernández-Marcos 2017) and presumably aid creating the LRP dome structure.

- Table 1: Please change CASP1::IAA3/SHY2 to CASP1::shy2-2 as only the stabilised version of IAA3 expressed in the CASP1 domain blocks LR formation whereas the wildtype IAA3/SHY2 does not.
Done

Reviewer #2: Jason Banda et al reviewed new insights into the molecular, cellular and environmental regulation of LR development in Arabidopsis, proposed a new mechanism termed the " developmental traffic light" model to explain how mechanical signals influence LRP patterning, which gave a vivid visualization of mechanical induced LR patterning. Are there any reports about gene regulatory networks of Casparian strip formation correlated with LR development? I might miss the references.

That's an interesting question. The GRN's controlling LR development and Casparian strip (such as ARF7 and MYB36) are induced in (roughly) similar areas behind the root apical meristem (in basal meristem and elongation zones, respectively). However, while LRP form at discrete positions, CS formation is continuous. We reason that LR development and Casparian strip remodelling (rather than formation) networks are more likely to be coordinated to facilitate organ emergence.

IN this review, authors claimed that there are two main auxin-regulated modules, an early network dependent on ARF7 and ARF19 and a later network, involving ARF5. I don't fully agree with this scenario since ARF7-IDA-HAE/HSL2 module plays a critical role in LR emergence.

Please note that we are referring to the LR regulatory networks operating in the primordia to control morphogenesis and NOT in overlaying cells that regulate organ emergence (for which the reviewer is correct in stating the ARF7-IDA-HAE/HSL2 module operates). We have clarified this point in the text.

In figure 3, if the colored LR primodia which marked cells involving into LR formation will be more readable.
Now actioned.

Reviewer #3: Dear the authors,
This review is definitely a nice summary on the lateral root developmental mechanism and its regulation by water and nutrients mainly on nitrate. The only suggestion I have is about the "Future directions", I know the main focus of this manuscript is the classical model plant Arabidopsis and in the past decades most of the progresses has been acquired on it. I am wondering how much of this nice mechanism during lateral root initiation and elongation in Arabidopsis can be replicated in the other crop model plants like rice and maize, those monocot plants do perform very differently and more complex . So it would be great to pinpoint the ideas and linkage between dicot and monocot plants in the future directions.
We do mention this valid point at the start of the future directions section stating, *"Major advances have been made elucidating the molecular and cellular basis of lateral root development using the model plant Arabidopsis. Nevertheless, lateral root morphogenesis in other plant species exhibits far greater anatomical variation than observed in Arabidopsis, reflecting the diversity of root classes [82]."*

However, the reviewer is entirely correct that we do not comment on how conserved the molecular mechanisms underpinning LRP initiation, patterning and emergence are between Arabidopsis and crops like rice and maize. To address this valid point, we have added additional sentences and additional references as follows

"However, very little is currently known about the underlying gene regulatory networks and mechanisms driving the morphological diversity of these different LR classes in crops. Despite this, genetic studies in crop models like maize and rice have identified common hormone signals and genes with Arabidopsis that regulate lateral root development [83, 84]."

Fig 2

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Fig 1

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