Auxin-dependent control of a plasmodesmal regulator creates a negative feedback loop modulating lateral root emergence

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Lateral roots originate from initial cells deep within the main root and must emerge through several overlying layers. Lateral root emergence requires the outgrowth of the new primordium (LRP) to coincide with the timely separation of overlying root cells, a developmental program coordinated by the hormone auxin. Here, we report that in Arabidopsis thaliana roots, auxin controls the spatiotemporal expression of the plasmodesmal regulator PDLP5 in cells overlying LRP, creating a negative feedback loop. PDLP5, which functions to restrict the cell-to-cell movement of signals via plasmodesmata, is induced by auxin in cells overlying LRP in a progressive manner. PDLP5 localizes to plasmodesmata in these cells and negatively impacts organ emergence as well as overall root branching. We present a model, incorporating the spatiotemporal expression of PDLP5 in LRP-overlying cells into known auxin-regulated LRP-overlying cell separation pathways, and speculate how PDLP5 may function to negatively regulate the lateral root emergence process.
lateral root branching is critical for rapidly increasing the surface area of the root system architecture to aid nutrient and water uptake\(^1\). Lateral root primordium (LRP) originate from xylem pole pericycle derived “founder” cells which, through a series of formative cell divisions, creates a growing dome-shaped primordia that eventually passes through endodermal, cortical, and epidermal layers of the primary root\(^2,3\). This developmental program requires intercellular coordination between emerging LRP and overlying cells, facilitated by auxin released from the tip of growing LRP to trigger cell wall separation\(^4\), which prompted us to examine if PDLP5 expression is similarly regulated by shoot-supplied auxin. For this, shoots were removed from the three GUS reporter lines two days before their remaining roots were stained. The result revealed a substantial reduction of GUS staining in PDLP5pro:GUS roots (Fig. 1b) as well as in LAX3pro:GUS and DR5:GUS control roots (Supplementary Fig. 2). We concluded that shoot-derived auxin likely controls the highly-localized PDLP5 expression in LRP-overlying cells. Next, we tested auxin-dependent induction of PDLP5 expression using the GUS reporter in the presence and absence of auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) or auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA). Five micromolar NPA treatment abolished GUS expression in both DR5:GUS and PDLP5pro:GUS roots (Fig. 1c) while 1 µM NAA induced intense GUS staining (Supplementary Fig. 3a). A lower concentration of NAA (0.1 µM) allowed us to discern that NAA induced GUS staining specifically and distinctively in regions where LRPCs were formed (Fig. 1d; Supplementary Fig. 3b).

We also examined if other hormones such as SA, which induces PDLP5 expression in leaves\(^16\) has a similar effect inducing PDLP5 in roots. This experiment showed that exogenous SA treatment could induce a strong PDLP5 expression in roots as determined by GUS staining and RT-PCR (Supplementary Fig. 3a, c). However, in contrast to discrete GUS stains induced by auxin along the roots, SA-treated roots showed a uniform staining pattern. Other hormones such as cytokinin, jasmonic acid, and abscisic acid, had little to no effects on PDLP5 induction in roots, although cytokinin seems to reduce the area of PDLP5pro:GUS expression within the protoxylem (Supplementary Fig. 3a). Lastly, auxin treatment increased PDLP5 transcript levels in WT seedling roots (Supplementary Fig. 3c) and PDLP5-GFP accumulation at plasmodesmata between the epidermis and cortex cells in PDLP5pro:PDLP5-GFP/pdlp5-1 roots (Fig. 1e). Collectively, these data corroborate that PDLP5 expression is an auxin-dependent response.

Next, to gain insights into the regulatory mechanism controlling PDLP5 expression in roots, we examined the impact of the auxin response mutants, iaa28-1 and shy2-2 on PDLP5pro:GUS. IAA28 and SHY2 genes encode repressors that inhibit the transcription factors ARF7 and ARF19, respectively, during LR formation\(^18,19\). LR development is suppressed in iaa28-1\(^20\) while ectopic LRP initiation is increased in shy2-2\(^21\). GUS staining revealed PDLP5 expression was barely detectable in cells above a few early-stage LRP formed in iaa28-1 roots (Fig. 1f; Supplementary Fig. 4). In contrast, PDLP5 expression was strongly upregulated in En cells above the large number of unemerged LRP in shy2-2 roots (Fig. 1f; Supplementary Fig. 4). We examined shy2-2 roots under the microscope and could find few to no LR past stage V, as reported elsewhere\(^21\). The differential regulatory effect of iaa28-1 and shy2-2 may reflect the recent report\(^22\) that SHY2/IAA3 requires ARF targets to be SUMOylated before it can interact and repress their transcriptional activity. Next, we performed chromatin immunoprecipitation (ChIP) assays using anti-ARF19 which revealed binding to PDLP5 promoter fragments (Fig. 1g). Collectively, these results indicate that the PDLP5 expression in overlying cells during LR development requires auxin through the regulatory molecules IAA28 and ARF19, but not likely through SHY2.

Results

Auxin induces PDLP5 expression in LRP-overlying cells. To determine which root cell types express PDLP5, roots of a transgenic PDLP5pro:GUS reporter line were analyzed (Fig. 1a). We compared the PDLP5pro:GUS pattern to the auxin response reporters DR5:GUS and LAX3:proGUS under the same experimental conditions. While the GUS reporter was detected in lateral root forming zones and within LRPCs, DR5:GUS roots and LAX3:proGUS roots were excluded from LRPCs but induced in LRP-overlying cells (Fig. 1a; Supplementary Fig. 1). In PDLP5pro:GUS roots, expression was also detected in the protoxylem and groups of cells along the main root axis (Supplementary Fig. 1). Furthermore, PDLP5pro:GUS expression occurred in a distinct spatiotemporal manner during all eight stages of LRP development\(^2\), starting in endodermal (En) cells during organ initiation and early development, then subsequently in cortical (Co) and epidermal (Epi) cells as primordia emerged through these cell layers (Fig. 1a).

The PDLP5pro:GUS staining pattern in LRP-overlying cells was similar to several known auxin-regulated genes involved in LRP emergence such as LAX3 except that the latter gene is not expressed in En cells (Fig. 1a). It is known that LAX3pro:GUS expression in LRP-overlying cells is driven by shoot-supplied auxin\(^4\), which prompted us to examine if PDLP5 expression is similarly regulated by shoot-supplied auxin. For this, shoots were removed from the three GUS reporter lines two days before their remaining roots were stained. The result revealed a substantial reduction of GUS staining in PDLP5pro:GUS roots (Fig. 1b) as well as in LAX3pro:GUS and DR5:GUS control roots (Supplementary Fig. 2). We concluded that shoot-derived auxin likely controls the highly-localized PDLP5 expression in LRP-overlying cells. Next, we tested auxin-dependent induction of PDLP5 expression using the GUS reporter in the presence and absence of auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA) or auxin transport inhibitor 1-N-naphthylphthalamic acid (NPA). Five micromolar NPA treatment abolished GUS expression in both DR5:GUS and PDLP5pro:GUS roots (Fig. 1c) while 1 µM NAA induced intense GUS staining (Supplementary Fig. 3a). A lower concentration of NAA (0.1 µM) allowed us to discern that NAA induced GUS staining specifically and distinctively in regions where LRPCs were formed (Fig. 1d; Supplementary Fig. 3b).

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PDLP5 Localizes to plasmodesmata in LRP-overlying Cells. Following observing that PDLP5-GFP localizes to plasmodesmata in root cells treated with auxin (Fig. 1e), we investigated if the protein preferentially accumulates at plasmodesmata of LRP-overlying cells during LR emergence. To this end, we tracked PDLP5-GFP localization to plasmodesmata in LRP-overlying cells at different emergence stages (Fig. 2a).

PDLP5pro:PDLP5-GFP exhibited highly specific spatiotemporal expression patterns in LRP-overlying cells, appearing first in En cells during early LRP development followed by overlying Co and then Epi cells as new primordia grew outwards (Fig. 2a). In each stage, PDLP5-GFP fluorescence revealed a typical plasmodesmal localization pattern—punctate signals at the cell wall junctions: at stage II, PDLP5-GFP signals were associated with two En cells positioned directly above the newly-forming LRP and specifically plasmodesmata at En–En, En–Pe, and En–Co cell wall junctions. At stages III and V, plasmodesmal signals were detected primarily in the walls surrounding two Co cells located directly above the two initial En cells (Fig. 2a, darts). Strong plasmodesmal labelings were detected at the Co–En and Co–Epi junctions as well as at the
cell wall junctions between those two Co cells. Notably, intense but non-punctate fluorescent signals were sometimes detected at En–En and Co–Co cell junctions that would soon separate (Fig. 2a, carets). While PDL5-GFP signals disappeared in separated walls (Fig. 2a, double arrows), PDL5-GFP signals at the plasmodesmata of the other junctional walls persisted after LR emergence (Fig. 2a, b). Finally, PDL5-GFP expression and localization patterns described in LRP-overlying cells were identical in the pdlp5-1 background complemented with the functional PDL5pro:PDL5-GFP reporter (Supplementary Fig. 6a).

PDL5 restricts cell-to-cell movement in roots. Based on its demonstrated role as a plasmodesmal regulator in aerial tissues, we reasoned that PDL5 may function to restrict cell-to-cell movement in LR-overslapping cells during LR emergence. Since PDL5 restricts plasmodesmal permeability by stimulating callose deposition in leaf cells, we hypothesized that it might function similarly in root cells. We evaluated plasmodesmal callose levels in LR-overslapping cells using aniline blue staining. Aniline blue binds to callose to give a yellow fluorescence in ultraviolet light. Although staining was successful in detecting plasmodesmata (Supplementary Fig. 6b), it was not possible to stain plasmodesmal callose consistently to discern if there were measurable differences in plasmodesmal callose levels between WT and pdlp5-1 roots. Therefore, we utilized the Arabidopsis transgenic line pER8:PDL5 that we had described elsewhere, which expresses PDL5 under the control of an estradiol-inducible promoter, to assess if we could correlate ectopic PDL5 induction and plasmodesmal callose levels in root tip cells where callose staining was possible. This experiment revealed that estradiol treatment increased a statistically significant amount of callose deposition at cell-cell junctions in pER8:PDL5 roots compared to mock-treated roots (Fig. 2c; Supplementary Fig. 7). These results suggest PDL5 functions in root cells to restrict cell-to-cell movement via stimulating callose deposition.

Since directly assessing plasmodesmal permeability across a few internal root cells is not currently possible, we designed a new experimental set-up that would allow us to evaluate PDL5 function indirectly in restricting cell-to-cell movement across the En cell layer. To this end, we created a movement reporter line, in which expression of free GFP is driven by an En-specific promoter derived from the genomic DNA encoding Casparian membrane protein 1 (CASP1)4—CASP1pro:GFP. For a control reporting CASP1 expression domain, we also created a non-mobile reporter replacing free GFP using ER-targeted citrine YFP—CASP1pro:ER-YFP. These reporter lines were then introduced into the estradiol-inducible pER8:PDL5 plants to monitor alterations in GFP movement out of the En cell layer in the presence of ectopically induced PDL5 in roots. In CASP1pro:ER-YFP roots, fluorescent signals were confined to En layer as expected, and this pattern did not change by estradiol-treatment (Fig. 2d). In contrast, in CASP1pro:GFP/pER8:PDL5 F1 roots, GFP fluorescence was detected not only in the En layer but also in the neighboring cell layers, Co and Pe, indicating that plasmodesmata at the En–Co and En–Pe junctions are permeable to GFP under normal growth conditions. This movement pattern, however, was altered by PDL5 induction; GFP could still move and accumulate in the Pe layer, but it could not move into the Co layer of 64% of the seedlings examined (Fig. 2d). This result suggests that PDL5 is capable of restricting plasmodesmal permeability in root cells.

PDL5 is required for lateral root branching and emergence. Next, we investigated if the spatiotemporal expression of PDL5 in LR-overslapping cells has roles in LR development and root branching. To facilitate LR phenotyping using histochemical staining, we introduced DR5:GUS into PDL5OE and pdlp5-1 mutant backgrounds. Compared to DR5:GUS Col-0 controls 8-days-post germination (dpg), approximately 30 and 70% fewer secondary and tertiary roots were formed in PDL5OE;DR5:GUS seedlings, and by 11 dpg, 25 and 50% fewer secondary and tertiary roots formed, respectively (Fig. 3a, b; Supplementary Fig. 8, 9a, b). In contrast, 50 and 70% more tertiary roots were formed at 8 dpg and 11 dpg, respectively, in pdlp5-1;DR5:GUS seedlings (Fig. 3a, b). Nevertheless, in spite of increased tertiary root numbers, root density remained comparable to that in WT seedlings because the secondary root length in pdlp5-1 mutants was also increased by 30% (Supplementary Fig. 9c, d).

To gain better insight into how PDL5 may impact the LR number and growth, we next evaluated LRP emergence rates using an LR-induction assay to monitor and compare all stages of LRP development. Four-day-old WT, pdlp5-1, and PDL5OE seedlings grown vertically on agar plates were rotated 90° to induce the formation of new LRP at the root bend as each seedling turns towards the new gravitropic vector (Fig. 3c).

Developmental stages of the induced LRP in each seedling (y-axis) was recorded from 12 to 48 h post-induction (hpi) (x-axis), then the distribution of LRPs at each stage at each time point was calculated as a percentage (z-axis) (Fig. 3d). During LRP initiation and early developmental stages 0–IV observed at 12, 18, and 24 hpi, LRP stage distributions exhibited no significant differences among all three genotypes. However, during LRP emergence stages V–VIII observed at 36, 42, and 48 hpi, LRP emergence occurred faster in pdlp5-1 seedlings, while it was severely delayed in PDL5OE. Specifically, compared to WT, 32% more pdlp5-1 LRP were in stage VIII at 42 hpi, and 17% more had emerged by 48 hpi; meanwhile, no PDL5OE LRP had emerged even by 48 hpi (Fig. 3d). Normal LRP emergence rate was restored by crossing pdlp5-1 with PDL5pro:PDL5-GFP,
demonstrating that the LRP emergence defect in the mutant is due to loss of PDLP5 (Supplementary Fig. 9e). Collectively, our results suggest that PDLP5 negatively regulates the rate of LRP emergence.

**PDLP5 modulates auxin accumulation in LRP-overlying cells.** Compared to WT, pdlp5-1 often had expanded DR5:GUS staining in the LRP zone and root tip, while DR5:GUS expression was generally weaker throughout PDLP5OE roots (Supplementary

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**Fig. 2 PDLP5 localizes to plasmodesmata in LRP-overlying cells.**

- **a** Representative confocal images showing 2D maximum intensity projections of 10 μm-thick cross-sections of PDLP5pro:PDLP5-GFP. Plasmodesmata marked by punctate PDLP5-GFP signal (in cartoons, red dots) can be seen at the cell junctions. PDLP5-GFP signals vary in color ranging from blue to green, yellow, and white depending on fluorescence intensity as indicated in calibration bar of the fluorescence intensity included on the right. The dashed arcs are positions of LRP; open arrowheads, separated cell walls; carets, strong PDLP5-GFP signal in cell walls just prior to separation. Scale bars, 25 μm.

- **b** A representative 3-D rendering of a confocal image, showing a 2-D maximum intensity projection of a 45 μm-thick cross-section of cells overlying an emerged LRP (not rendered) in PDLP5pro:PDLP5-GFP root. Signals at other junctions (Co–Co, Ep–Ep) were rare at the emergence stage we used for modeling. Inset, a cross-sectional view of the image taken from the boxed area. PDLP5-GFP-labeled plasmodesmata in different cell junctions are color-coded.

- **c** pER8:PDLP5 root tips stained with aniline blue after 24 h of 10 μM estradiol or mock treatment. Estradiol- and mock-treated n = 25; scale bars, 25 μm.

- **d** PDLP5 induction can prevent GFP movement from En to Co root tissue in pER8:PDLP5 × CASP1:GFP F2 crosses. Mock n = 24, Treated n = 47; scale bars, 20 μm.
Fig. 10). This led us to consider whether PDLP5 may modulate LR progression during emergence by affecting auxin accumulation and/or distribution within the newly forming LRP zone. To test this hypothesis using live cell imaging, we crossed the auxin reporter DR5:3VENUS with pdlp5-1 and PDLP5OE plants. Fluorescence associated with 3VENUS in the nuclei allowed us to monitor auxin distribution from induced LRP into the overlying cells in real time (Supplementary Fig. 11a). Those emerging LRPs were observed between 22–36 hpi, and 27 hpi was found to be an optimal time point to quantify nuclei under our experimental conditions for two reasons: at this time point, the stage IV–V LRP would be approaching the Co cells, and the LRP and overlying Co nuclei were more distinguishable from each other. The number of fluorescent overlying Co cells increased in pdlp5-1 roots, while it decreased in PDLP5OE (Fig. 4a; Supplementary Fig. 11b). We have also examined the DR5:3VENUS signal intensities in LRP-overlying Co cells, but the variance in the fluorescence intensity of DR5:3VENUS was too high to show any statistical differences (Supplementary Fig. 13). The Box plot analysis of overlying Co cell numbers revealed that while 50% of WT seedlings had 3–5 DR5:3VENUS-positive Co cells, this range was skewed lower in PDLP5OE, with 50% of seedlings having only 2–4 fluorescent Co cells, and skewed higher in pdlp5-1 mutants, with 50% having 4–5 positive Co cells (Fig. 4b). These results suggest that PDLP5 negatively regulates the spread of auxin through overlying Co cells during LRP emergence.

To gain insight into the functional relationship between PDLP5 and downstream auxin-responsive genes required for

**Fig. 3 PDLP5 is required for normal LR emergence.**

a LR development in WT, pdlp5-1, and PDLP5OE expressing DR5:GUS. Arrowheads, tertiary roots.
b Quantification of total lateral root numbers (both emerged and un-emerged secondary [2°], tertiary [3°], and quaternary [4°] roots). n ≥ 30 per seedling set. Bars, standard deviation. Asterisks, significance determined by student T-test (P < 0.01). c A diagram depicting the gravitropic assay and position of the root bend LRP. d Time-course of LRP development and emergence at the root bend of 4-day-old seedlings following gravistimulation. x-axis, hrs post-induction (hpi); y-axis, LRP developmental stage; z-axis, distribution of LRP at different stages per time point; n ≥ 20 seedlings per set.
LR emergence, we investigated whether the expression of LAX3, a key late auxin response gene induced in LRP-overlying root cells, might be affected by pdlp5-1 or PDLP5OE. To this end, the expression of LAX3pro:LAX3-YFP was monitored in WT, pdlp5-1, and PDLP5OE LRPs over the time course of 14–36 hpg. LAX3-YFP fluorescence was detectable in LRP-overlying Co cells as early as 16 hpi in pdlp5-1, at which time no LAX3-YFP signals could be detected in WT Co cells (Fig. 4c). By 22 hpi, almost two-fold more pdlp5-1 mutant seedlings than WT expressed LAX3-YFP in Co cells, whereas PDLP5OE seedlings were substantially delayed expressing LAX3-YFP until 36 hpi (Fig. 4c, d; Supplementary Table 1, 2).

To determine the impact of PDLP5 loss on the location of LAX3 expression, we quantified how many LRP-overlying Co cells expressed LAX3pro:LAX3-YFP in WT and pdlp5-1 backgrounds between 26–36 hpi (stages IV–VI), (Fig. 4c). We found that LAX3-YFP was expressed in more LRP-overlying Co cells in pdlp5-1 during this time range; ~50% of

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**Diagram:**
- **LRP**
- **Auxin**
- **Mechanical pressure**
- **En**
- **PD closure**
- **ARF7/19**
- **Auxin Importer**
- **Turgor pressure**
- **PDLP5**
- **Stimulation of LRP emergence through overlying cells**
**Fig. 4** PDLPS affects LAX3pro:LAX3-YFP or DR5:3VENUS expression domain. a Representative confocal images of DR5:3VENUS in each genetic background. Overlying Co cells were imaged as confocal z-stacks (2D maximum intensity projections of 115.4 µm thick confocal volumes), and the number of overlying Co cells with auxin-induced DR5:3VENUS was quantified at 27 hpg. The nuclei within LRP and Co cells are pseudo-colored white and green, respectively, to aid their 3D positioning in 2D images. Scale bars, 20 µm. b A box plot showing quantification of the number of LRP-overlying Co cells with DR5:3VENUS signal at 27 hpg. Asterisks, statistical significance determined by student T-test (P < 0.05) on three biological repeats. c Representative confocal images of LAX3pro:LAX3-YFP in a time course following gravistimulation. Arrowheads, LAX3-YFP expression in overlying Co cells. Scale bars, 50 µm. d Quantification of relative occurrence of LAX3-YFP signal in Co cells at 22 hpg, based on the data presented in Table S1. Asterisks, statistical significance determined by student T-test (P < 0.01). Bars, standard deviation. n ≥ 30 per seedling line. e Representative images of LAX3pro:LAX3-YFP showing signal in LRP-overlying cortical cells in either the WT or pdlp5-1 background. Images are 2D maximum intensity projections of 3.69 µm thick confocal volumes. Scale bars, 25 µm. f, g Graphs showing the percentage of total seedlings with specific numbers of LRP-overlying Co cells (f) or cell files (g) expressing LAX3pro:LAX3-YFP in WT or pdlp5-1; f: g n = 32. h A model illustrating a negative feedback loop between auxin and PDLPS-dependent plasmodesmal closure integrated with positive and negative signaling pathways dependent on auxin and turgor pressure. Depicted is stage III-IV LRP, approaching endodermis. Orange ovals, closed plasmodesmata.

LAX3pro:LAX3-YFP/pdlp5-1 seedlings had 4–5 Co cells with LAX3-YFP, compared to only ~25% of LAX3pro:LAX3-YFP/WT (Fig. 4f). Furthermore, ~40% of LAX3pro:LAX3-YFP/pdlp5-1 seedlings had signal in 3 cell files, compared to ~25% of LAX3pro:LAX3-YFP/WT (Fig. 4g). Finally, LAX3-YFP appeared earlier in pdlp5-1 LRP-overlying Epi cells compared to WT (Supplementary Fig. 12). Since LAX3 expression is dependent on targeted auxin accumulation in LRP-overlying cells, our results suggest that loss of PDLPS could increase auxin diffusion from these cells, thereby expanding the LAX3 expression domain.

Collectively, our study reveals that auxin upregulates PDLPS progressively in root cells overlying newly forming LRP; PDLPS localizes to plasmodesmal and can regulate cell-to-cell movement in roots, as it does in leaves; and the absence of PDLPS allows expansion of auxin distribution in LRP-overlying cells, enhancing LR emergence.

**Discussion**

Our study reveals a negative feedback loop linking the plasmodesmal regulator PDLPS with auxin in LRP-overlying cells. This regulatory circuit may function to sympelasmically isolate LRP-overlying cells while ensuring organ emergence occurs at a normal rate. Starting soon after founder cell division begins, auxin induces spatiotemporal expression of PDLPS in the LRP-overlying cells, where PDLPS localizes to plasmodesmata. We have shown that inducible PDLPS expression in roots stimulates callose deposition and closes plasmodesmata, inhibiting cell-to-cell movement of En-expressed GFP into outer Co cells. PDLPS negatively feedback-regulates auxin in LRP-overlying cells, altering the timing and number of overlying cells expressing auxin marker DR5 and LAX3. Lastly, the lack of PDLPS increases higher-order LRP development and emergence rate.

Due to the highly spatiotemporal nature of PDLPS expression in the LRP-overlying cells, and our previous data proving that PDLPS closes plasmodesmata, we hypothesized that the overlying cells might become sympelasmically isolated during LRP emergence. However, it was reported by Benitez-Alonso et al. that GFP expressed under the phloem-specific SUC2 promoter was present in parental root cells including LRP-overlying endodermal cells during stages I–II. Since PDLPS is expressed in cells overlying nascent LRP, observing GFP within these cells would at first seem to contradict our hypothesis on PDLPS’s role for plasmodesmal regulation in those cells. However, GFP can remain quite stable within tissue for many hours after expression. Thus, it is possible that the GFP had already moved from the phloem into outer cell layers prior to PDLPS expression during early LRP formation, and the signal Benitez-Alonso et al. detected was actually residual fluorescence from GFP present in that tissue. Another technique for observing the potential effects of PDLPS on cell-to-cell connectivity in LRP-overlying cells would, therefore, be to observe it in real-time as shown in the report by Oparka et al. That report noted the unloading of phloem-loaded carboxy fluorescin (CF) dye into emerging LRP but no dye movement from cells within LRP to LRp-overlying cells. Our experiment showed that CF was unloaded into the cells of nascent (stage I–II) but no dye movement occurred from the cells of the LRP into cells overlying them, both in wild-type and the pdlp5-1 roots (Supplementary Fig. 13). Our results seem to indicate that LRP s are not sympelasmically connected with overlying cells, even from the earliest stages of development.

While these results meant we could not use CF phloem loading to directly test PDLPS-dependent isolation of LRP-overlying cells, it led to the unexpected insight that one of the earliest steps during LRP development is its isolation from outer root cells. This isolation may prevent the loss of important growth signals from the nascent LRP into the overlying tissues. Supporting this, it was recently discovered that a cuticle layer, functioning as a diffusion barrier, is deposited at the outermost cell wall of developing LRP at stage I–II; our results make it tempting to speculate that the sympelasmic isolation of LRP from outer cells may coincide with the cuticle deposition. It would be an interesting future investigation to detail when and how the plasmodesmal disconnection occurs between LRP and overlying cells. Meanwhile, direct examination of the PDLPS’s impact on cell-to-cell connectivity of LRP-overlying cells would have to wait until a new technique is developed, allowing real-time analysis of a movement tracer out of those cells.

We summarize the PDLPS-auxin functional relationship in our model (Fig. 4h) and integrate key factors, such as the ARF7/19 module and cell turgor, which are vital for normal LRP development and emergence. The ARF7/19 pathway upregulates auxin transporters such as PIN3 in En and LAX3 in Co and Epi cells, which in turn reinforce positive feedback to elevate auxin accumulation in LRP-overlying cells. At the same time, turgor pressure rises within the overlying cell as it is compressed by the growing LRP, slowing the LRP emergence rate. Together, these positive and negative signaling components contribute to ensuring a safe passage for the new organ, while creating internal space for the developing organ. Although our illustration focuses on these events in the En layer, based on the PDLPS expression pattern, this regulatory programming would likely repeat itself in overlying Co and Epi layers during later stages of LRP emergence.

In addition, auxin movement into LRP-overlying En cell induces the expression of PDLPS via an ARF7/19 pathway, which stimulates plasmodesmal closure. Alternatively, high levels of auxin accumulating in the En could be sufficient to induce PDLPS and thus restricting the number of cells undergoing a feed-forward increase in auxin response. In this scenario, auxin-dependent PDLPS expression in outer cell layers might occur cell-autonomously without needing auxin to flow from the LRP to the...
endodermis. As for the signal that feedbacks to auxin, we consider an indirect effect from blocking cell-to-cell movement by closing plasmodesmata or a direct effect exerted from PDLP5 or both. One of the indirect effects of plasmodesmal closure could be on maintaining the turgor of the LRP-overlying cell against the mechanical pressure imposed by the growing LRP. The maintenance of turgor in overlying cells has been shown to be necessary to prevent a rapid loss of the cell volume and to slow the emergence process, which is consistent with the emergence phenotype exhibited in pdlp5-1.

PDLP5 expression in LRP-overlying cells appears to be regulated by shoot-driven auxin and mediated by the regulatory module ARF7/19. PDLP5 appears to be an early auxin response gene based on its impact on the expression of the late auxin response gene LAX3, and our ChIP assay results revealing PDLP5 auxin-dependent induction is mediated via ARF7/19. This regulatory module is essential for LRP initiation and emergence processes and controls the spatiotemporal expression of key downstream effectors, such as SHY2 and LAX3. Although all auxin-responsive genes depend on the ARF7/19 module for induction, SHY2 is expressed in the overlying En and LAX3 in the overlying Co and Ep, whereas PDLP5 is expressed sequentially in all three layers. This differential expression pattern suggests additional determining factor(s) exist specific for SHY2 and LAX3, but not for PDLP5 expression.

In addition to providing insight into plasmodesmata–auxin interactions, our data raises an interesting question about how PDLP5-mediated plasmodesmal closure might help to elevate turgor pressure in LRP-overlying cells. Water can move between cells through plasmodesmata; hence, changes in plasmodesmal permeability would impact cellular turgor pressure. A developing LRP is thought to sense mechanical resistance in the overlying En cells and abort if turgor of these cells does not decrease as the growing LRP pushes against them. Therefore, it is an interesting possibility that plasmodesmal closure in LRP-overlying cells may be necessary for modifying the turgor pressure needed for normal progression of LRP emergence. Equally, it would also be possible that turgor pressure (increased in overlying cells as LRP pushes through) may augment plasmodesmal closure. Indeed, changes in turgor pressure are known to alter plasmodesmal permeability.

Another interesting question our data raises is how auxin-dependent induction of PDLP5 during LR emergence might regulate root architecture, altering LR branching patterns. Both primary and secondary root lengths, as well as lateral root numbers, are reduced in PDLP5-OE seedlings, while secondary root length and tertiary root numbers are increased without affecting the primary root length in pdlp5-1 mutants. It is known that plant root architecture changes in response to internal and external nutrient states, and certain nutrients are linked to specific root morphological and architectural modifications. For example, while severe nitrogen deficiency inhibits overall root growth, mild nitrogen deficiency stimulates lateral root emergence, and elongation. Notably, this emergence and elongation is driven by auxin accumulating in later stage LRPs, at a similar time to when the first differences between WT and pdlp5-1 root emergence rate can also be observed. Future investigation may uncover a yet unknown signaling mechanism that suppresses PDLP5 in the roots so that when such a nutrient foraging program is activated, greater lateral root emergence and elongation can occur.

Symplasmic isolation of overlying cells is likely facilitated by callose-dependent plasmodesmal closure, based on our data that PDLP5 ectopic expression in roots stimulates plasmodesmal callose deposition. We hypothesize a mechanism exists to fine-tune auxin accumulation controlling outward LRP growth and cell separation pathways. Symplasmic isolation in these two domains may be necessary for the optimal build-up of turgor pressure in LRP and overlying cell, respectively.

In conclusion, our findings reveal a role for plasmodesmata fine tuning the LR emergence program via a negative feedback mechanism modulating auxin response in LRP-overlying cells.

**Methods**

**Plant materials, growth conditions, and genetic crosses.** All Arabidopsis thaliana genotypes were in the Col-0 genetic background, except for shy2-2 in Ler, and nad28-1 in Ws. Seedlings were grown vertically in 0.5× MS agar under continuous light at 22 °C. Plants in soil were grown in 16 h light at 22 °C. All the genetic crosses were genotyped to identify homozygous mutations when necessary (Supplementary Table 3). Genomic DNA was isolated from segregating F2 plants followed by PCR analyses using gene-specific primers.

**GUS assay and LRP quantification.** GUS solution (100 mM sodium phosphate buffer, pH 7.0, 10 mM EDTA, 0.5 mM each potassium ferrocyanide and potassium ferricyanide, 1.24 mM X-Gluc, and 0.1% Triton X-100) was vacuum-infiltrated into plant tissue for 5 min, then removed from vacuum and incubated in 37 °C for 3–12 h, followed by a series of ethanol washes. Stained tissues were imaged using a Zeiss Axioskop 2 microscopy. LRP were quantified by counting both the emerging LR and unemerged LRP, as determined by DR5:GUS staining of the primordia, under a dissecting microscope (1.2× magnification). LRP stages were determined by examining ethanol-cleared, GUS-stained tissue using a 40× water lens.

**Chromatin immunoprecipitation, PCR, and qPCR analyses.** A ChIP assay was performed on Col-0 and a knock-out allele, arf19-223 using 2–3 g root tissue pre-treated with 1 µM NAA and fixed under vacuum with 1% formaldehyde for 15 min. Nuclei were extracted following the protocol described previously and ChIP was performed, using home-made anti-ARF19 antibody33 following the method basically as described previously. Briefly, 200 µl of sheared chromatin (average fragment size of 400 bp) was added to 1 ml Immunoprecipitation Buffer (50 mM Hepes, pH 7.5, 150 mM KCl, 5 mM MgCl2, 0.1% Triton X-100) and incubated along with 3 µg of anti-ARF19 at 4 °C. Protein G Dynabeads® (Invitrogen) were then added and further incubated at 4 °C overnight. Input and ARF19-immunoprecipitated DNA was used for qPCR with SYBR green master mix and primers (Supplementary Table 4). Oligos were designed to two regions of the PDLP5:HF111 (At1g70690) promoter (Supplementary Fig. 5). These regions contain putative ARFs likely corresponding to ARF19 binding sites. Anti-ARF19 immunoprecipitated DNA is normalized to input chromatin using an internal control (TUB3) not bound by ARF19. All qPCR reactions were performed as triplicate technical replicates using a Light Cycler 480 qPCR machine and are representative of three biological repeats. Genomic-PCR and RT-PCR were performed as previous described, using primers listed in Supplementary Table 4.

**Confocal microscopy and image processing.** For PDLP5pro:PDLP5-GFP localization, seedlings were stained for 10–15 min in 5 µg/ml propidium iodide at 7 dpq. Fluorescent imaging was performed on a Zeiss AxioObserver Z1 inverted light microscope using a LSM 710 scanhead. For DR5:VENUS imaging, a LD LCI Plan-Apochromat 25×/0.8 Imm Korr DIC objective was used, with a 514 nm excitation laser and 515–550 nm filter (for VENUS) and a 585–758 nm (for propidium iodide) emission filters. For PDLP5pro:PDLP5-GFP, a C Apochromat 40×/1.2 W Korr objective was used, with a 485 nm excitation filter and 500–550 (GFP) emission filter, and detected with a BiGaAsP (Bi Gallium Arsenide Phosphide) Detector. Image brightness, contrast and gamma were adjusted to enhance the images via ZEN 2011 software. The 3D model of PDLP5pro:PDLP5-GFP in overlying cells was created using Amira 5.6 software to render separate channels, highlighting GFP signal and interpolating root cell shape from the propidium iodide outline. For counting LAX3pro:LAX3-YFP cells, a 25×/0.8 Imm Korr DIC objective was used, with a 514 nm excitation laser and a 575–610 nm BP filter (YFP) and a 543–735 nm BP filter (propidium iodide), and YFP was detected with the BiGaAsP Detector. For monitoring the timing of LAX3pro:LAX3-YFP expression, the cortical cell fluorescence at the root bend was monitored at different time points using a Zeiss LSM 780 confocal upright light microscope using a W Plan-Apochromat 20×/0.1 DIC M27 75 nm objective and the 415–445 nm excitation line of an argon laser with 520–550 nm band pass emission filter. Images are presented as 3-D composites of 30 µm-thick z-stacks. Aniline-blue stained callose imaging was performed on a Zeiss LSM880 multiphoton confocal microscope, using an LD LCI Plan-Apochromat 25×/0.8 Imm Korr DIC M27 objective, with a 780 nm multiphoton laser and 410–552 nm emission filters.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Data supporting the findings of this study are available in the manuscript and its supplementary files or are available from the corresponding author upon request. The
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Author contributions

R.S. produced major experimental data, and R.S. and J.-Y.L. designed the experiments and wrote the manuscript. X.W. helped collecting LAX3 images in a time course and K.H. carried out ChiP assays. B.-C.Y. expressed design expression vector system and J.C. helped with image processing. During revision, J.-Y.L. performed CF loading with A.N.’s help collecting confocal images, and T.T. helped with supplementary RT-PCR. M.J.B. helped with data interpretation and manuscript editing.

Competing interests

The authors declare no competing interests.

Additional information

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