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The auxin influx carrier LAX3 promotes lateral root emergence

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Lateral roots originate deep within the parental root from a small number of founder cells at the periphery of vascular tissues and must emerge through intervening layers of tissues. We describe how the hormone auxin, which originates from the developing lateral root, acts as a local inductive signal which re-programmes adjacent cells. Auxin induces the expression of a previously uncharacterized auxin influx carrier LAX3 in cortical and epidermal cells directly overlaying new primordia. Increased LAX3 activity reinforces the auxin-dependent induction of a selection of cell-wall-remodelling enzymes, which are likely to promote cell separation in advance of developing lateral root primordia.

The efficient colonization of soil by plant roots is dependent on the initiation of new lateral roots. In the model plant *Arabidopsis thaliana*, lateral roots are derived from pericycle cells adjacent to each xylem pole[1–4]. These lateral root founder cells undergo a well defined programme of oriented cell divisions and expansion to form a primordium[1–4]. To emerge, primordia must pass through several intervening parental root tissues. In *Arabidopsis*, these comprise single layers of endodermal, cortical and epidermal cells[5] (Fig. 1a). However, in other plant species, such as rice, lateral root primordia must negotiate up to 15 intervening layers of cells[4,6]. Researchers have speculated for over a century that lateral root development and cell separation processes in adjacent root tissues must be tightly coordinated to minimize tissue damage and reduce the risk of infection[4]. Several observations suggest that parental root tissues actively participate in the lateral root emergence process. First, genes encoding cell-wall-remodelling enzymes have been reported to be expressed in cells next to lateral root primordia in *Arabidopsis* roots[7–9]. Second, in several plant species, cells from root tissues overlaying new primordia are recruited to form a temporary root cap that assists organ emergence[4,10–12]. Despite these observations, the signals exchanged between lateral root primordia and overlaying cells that coordinate organ emergence remain to be characterized.

The plant hormone auxin provides a crucial signal during lateral root development[12]. Auxin initially triggers the division of lateral root founder cells in the pericycle tissue[1,13]. Subsequent patterning of tissues within a lateral root primordium also requires the establishment of an auxin response gradient with its maximum at the tip[14]. Here we show that auxin derived from the lateral root apex regulates lateral root emergence by acting as a local inductive signal to adjacent cells in the parental root. Our study reveals that lateral root emergence is a highly regulated process using a common signal to synchronize lateral root development and emergence processes.

RESULTS

LAX3 facilitates lateral root emergence

Auxin provides a key inductive signal during lateral root development[12]. It enters plant cells either through carrier-mediated uptake or by membrane diffusion[15]. Mutations in the high-affinity auxin-uptake carrier AUX1 (ref. 16) result in reduced numbers of lateral roots[17] (Fig. 1d, f). We investigated whether any of three closely related *Arabidopsis* sequences termed *LAX* (*Like AUX1*) *1, 2* and *3* genes performed a lateral-root-related developmental function(s) by isolating knockout mutations[18] in each of their coding sequences (Supplementary Information, Fig. S1).

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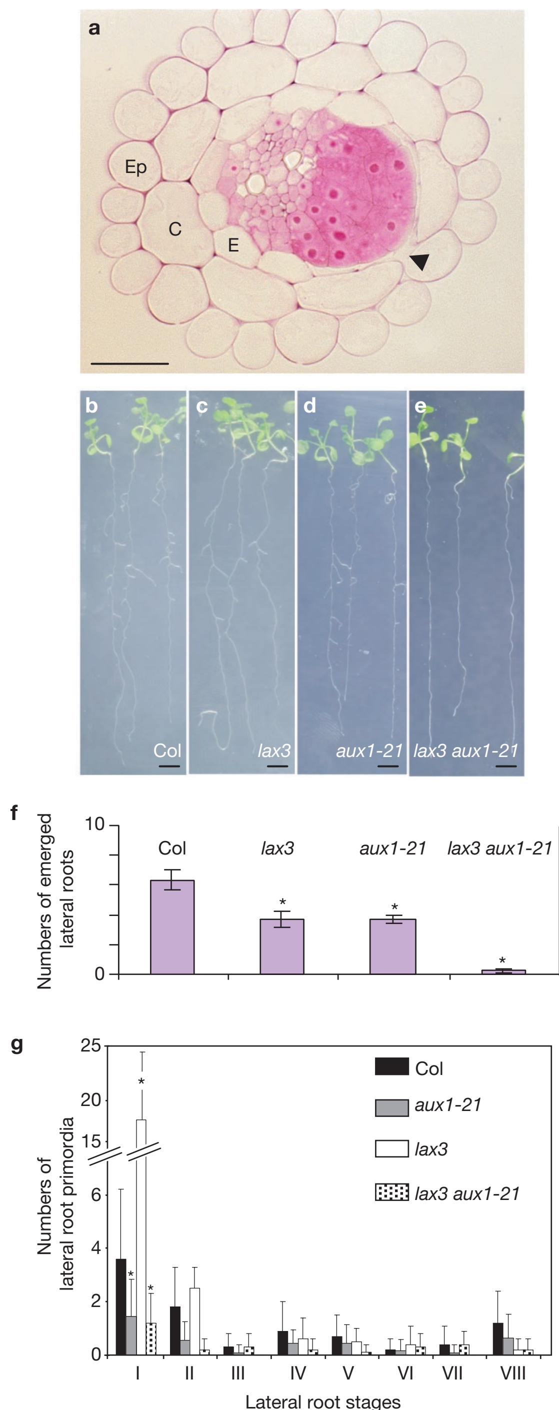

Figure 1 AUX1 and LAX3 are required for lateral root development. (**a**) Radial cross-section of an *Arabidopsis* root containing a lateral root primordium (LRP) emerging through endodermal (E), cortical (C) and epidermal (Ep) cell layers that are undergoing cell separation (denoted with an arrow). (**b**–**e**) Lateral root phenotype of 12-day-old wild-type (Col, **b**), *lax3* (**c**) *aux1-21* (**d**) and *aux1 lax3* (**e**) seedlings. (**f**) Numbers of emerged lateral roots (mean ± s.d.). Asterisks indicates a significant difference from the wild-type control ($P < 0.05$, Student's *t*-test; $n = 23$ (Col), 17 (*lax3*), 17 (*aux1-21*), 20 (*lax3 aux1-21*)). (**g**) Numbers of LRP at specific developmental stages in 8-day-old wild-type, *aux1*, *lax3* and *aux1 lax3* roots (expressed as stages I–VIII, according to ref. 3; mean ± s.d., $n = 10$ for each groups of seedlings). Asterisks indicates a significant difference from the wild-type control ($P < 0.05$, Student's *t*- test). Scale bars are 20 µm (**a**) or 3 mm (**b**–**e**).

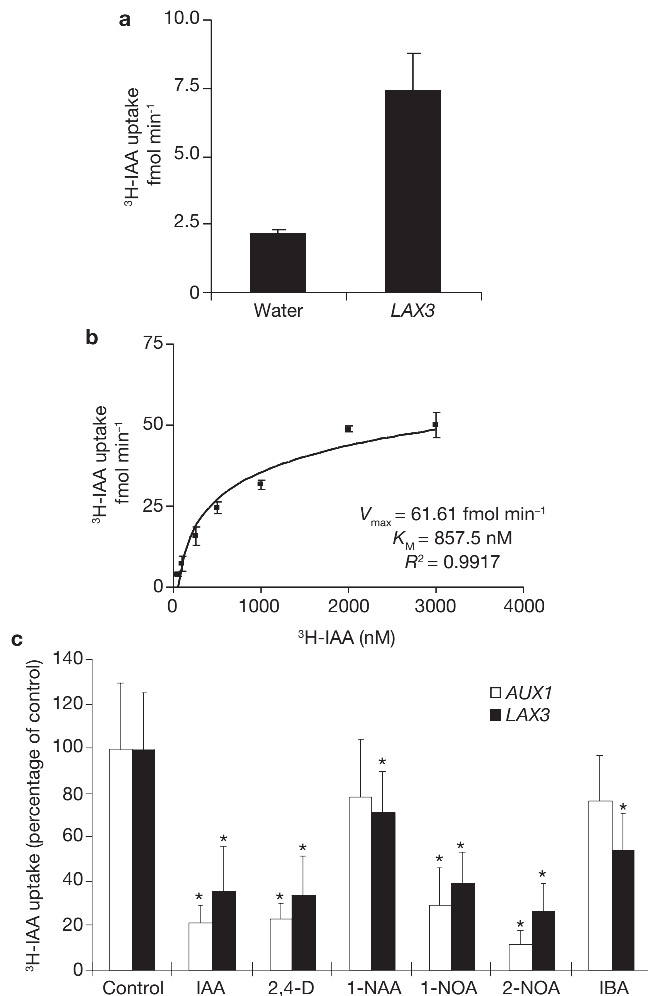

Figure 2 LAX3 encodes a high-affinity auxin influx carrier. (**a**) Uptake of ^{3}H-IAA into *Xenopus* oocytes injected with water or *LAX3*–cRNA at pH 6.4. Oocytes injected with *LAX3* cRNA showed increased ^{3}H-IAA (100 nM) uptake when compared with water-injected controls (data are mean ± s.d., $n = 8$ oocytes). (**b**). Kinetic analysis of ^{3}H-IAA uptake by LAX3. Mean ± s.d. of IAA uptake rates at the indicated concentration are shown ($n = 8$ for each concentration). Experiments were repeated on oocytes from two different frogs and showed similar results. (**c**). Auxin analogues affect both AUX1- and LAX3-mediated IAA uptake similarly. Uptake of ^{3}H-IAA (100 nM) into oocytes injected with either *LAX3*- or *AUX1*–cRNA were examined in the presence of excess unlabelled IAA (20 µM), the auxin analogues 2,4-D (20 µM) and 1-NAA (20 µM), and the naturally occurring auxin form IBA (20 µM). In addition, incubation with the specific auxin uptake inhibitors 1-NOA (naphthoxy-1-acetic acid, 20 µM) and 2-NOA (20 µM) reduced uptake. Data are mean ± s.d., $n = 8$ oocytes. Asterisks indicate a significant reduction ($P < 0.05$, Student's *t*-test) in transport into oocytes between AUX1 or LAX3 control oocytes and oocytes incubated with different auxins or auxin influx carrier inhibitors.

Characterization of the *lax1* and *lax2* seedling roots revealed that their lateral root phenotypes were comparable to wild-type (data not shown). In contrast, in *lax3* mutant seedlings the number of emerged lateral roots was reduced by about 40%, which was comparable to *aux1* (ref. 17; Fig. 1c, d, f; Supplementary Information, Fig. S1c). The *aux1 lax3* double mutant showed a much more severe lateral root phenotype (Fig. 1e, f; Supplementary Information, Fig. S1c), effectively blocking lateral root formation until at least 14 days after germination. To gain further insight into the developmental basis for the reduced number of

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emerged lateral roots in the *aux1* and *lax3* mutant backgrounds, the total number (Supplementary Information, Fig. S1e) and distribution of stages of lateral root primordia (Fig. 1g) were determined in wild-type versus mutant roots. Consistent with its known lateral root initiation defect[18], *aux1* mutants formed approximately half the number of primordia than wild-type, whereas *lax3* roots formed up to threefold more lateral root primordia than wild-type (Supplementary Information, Fig. S1e). The reduced number of emerged lateral roots in the *lax3* mutant therefore cannot be due to a lateral root initiation defect as in the case of *aux1*. Instead, the *lax3* mutation disrupts the development and emergence of lateral roots as the majority of primordia in either *lax3* or *aux1 lax3* roots accumulate at stage I (Fig. 1g).

LAX3 encodes a high affinity auxin influx carrier

The lateral root phenotypes of *aux1* and *lax3* single and double mutants are likely to result from reduced uptake of auxin in root cells. *AUX1* has recently been demonstrated to encode a high-affinity auxin-uptake carrier[16]. The close homology between *AUX1* and *LAX3* genes suggests that the latter sequence may also function as an auxin influx carrier. We directly tested protein function by heterologously expressing the *LAX3* sequence in *Xenopus* oocytes (Fig. 2) and human U20S cells (Supplementary Information, Fig. S2a). Transport assays using tritiated indole-3-acetic acid (^{3}H-IAA) revealed that LAX3-expressing cells in both heterologous systems showed increased levels of auxin accumulation, compared with the negative control (Fig. 2a; Supplementary Information, Fig. S2a). Transport of ^{3}H-IAA in *LAX3*-expressing oocytes was saturable, with a K_M value of about 860 nM (Fig. 2b), which was comparable to AUX1 (ref. 16). As with AUX1, addition of unlabelled IAA effectively competed with ^{3}H-IAA for uptake in *LAX3*-expressing oocytes (Fig. 2c). The addition of the auxin analogue 2,4-dichlorophenoxyacetic acid (2,4-D) or auxin influx carrier inhibitors 1-NOA or 2-NOA competitively inhibited uptake of ^{3}H-IAA in *LAX3*-expressing oocytes (Fig. 2c). In contrast, the lipophilic, membrane-permeable auxin analogue 1-naphthaleneacetic acid (1-NAA) caused a less significant decrease in ^{3}H-IAA uptake in *LAX3*-expressing oocytes (Fig. 2c). These results are consistent with whole-plant studies measuring auxin influx carrier substrate and inhibitor specificities[19,20] and demonstrate that *LAX3* encodes a high-affinity IAA uptake carrier.

Our heterologous expression studies (Fig. 2) suggest that *aux1* and *lax3* lateral root phenotypes result from reduced auxin uptake in mutant root cells. Indeed, the lateral root defects of *aux1, lax3* and *aux1 lax3* lines could be rescued when mutant roots were grown in the presence of 1-NAA (Supplementary Information, Fig. S2c–e). The ability of 1-NAA to rescue the lateral root phenotypes suggests that IAA levels in mutant root cells are suboptimal. Thus we directly measured IAA abundance in mutant and wild-type roots using GC-SRM-MS[21]. Although IAA levels in *lax3* and *aux1* root tissues were reduced, we did not observe a marked additive effect on root IAA abundance in the double mutant (Supplementary Information, Fig. S2f). Hence, the severity of the *aux1 lax3* lateral root phenotype is not caused by a major reduction in root IAA, but may result from subtle changes in the distribution of IAA between root tissues that regulate lateral root development.

LAX3 functions in cells overlaying new lateral root primordia

To determine which root tissues express *LAX3* and *AUX1*, promoter fragments for each gene were fused to the GUS reporter. These *LAX3* and *AUX1* promoter fragments have been demonstrated to be sufficient to drive their wild-type gene to rescue its mutant phenotype (ref. 17; Supplementary Information, Fig. S3). Reporter-based studies reveal that *AUX1* (ref. 17) and *LAX3* (Fig. 3) are expressed in distinct groups of cells during lateral root development. *AUX1* expression is limited to dividing cells at all stages of lateral root development until after organ emergence[17]. In contrast, *LAX3* is initially expressed in newly expanding and mature root stele tissues (Fig. 3a–c), but is later excluded from groups of dividing pericycle cells (Fig. 3e–h) and is absent from lateral root primordia until after emergence (Fig. 3d). *LAX3* expression was also induced in a small number of cortical cells directly adjacent to stage II primordia (Fig. 3e–g; Supplementary Information, Fig. S4), then later in the epidermis as stage V lateral roots are emerging (Fig. 3h; Supplementary Information, Fig. S4).

LAX3 appears to promote the development of newly initiated lateral root primordia indirectly from adjacent root epidermal/cortical and/or stele tissues. We investigated which of these root tissues requires LAX3 to facilitate lateral root development. Compared with wild-type, very few lateral roots were visible in the *aux1 lax3* mutant up to 14 days after germination (Fig. 1b

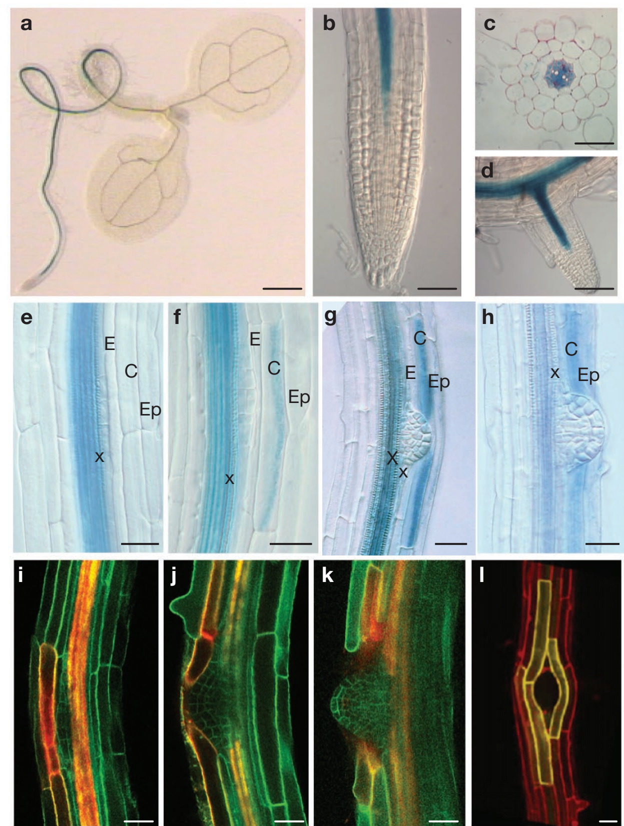

Figure 3 LAX3 is expressed in mature tissues adjacent to developing LRP. (**a**–**h**) *LAX3pro:GUS* reporter expression was detected in seedling root stele tissues (**a**), expanding root stele cells (**b**), stele tissues within a radial cross-section (**c**) of an *Arabidopsis* root; but later excluded from new lateral root primordia from stage I (**e**), stage II (**f**), stage V (**g**) and stage VII (**h**) until after lateral root emergence (**d**). Endodermal (E), cortical (C) and epidermal (Ep) cell layers are shown. (**i**–**l**) Root cells were detected (green) using the LTi6a–GFP plasma membrane marker. *LAX3pro:LAX3–YFP* expression (orange) was specifically detected in mature stele and cortical cells overlaying stage III (**i**), stage V (**j**) and newly emerged stage VII LRP (**k**). (**l**) Confocal scans of cells on the primary root surface stained with propidium iodide (red) revealed that the *LAX3pro:LAX3–YFP* reporter (yellow) demarked a subset of epidermal cells that were physically separating from one another. Scale bars are 500 µm (**a**), 50 µm (**b**), 40 µm (c), 100 µm (**d**) and 25 µm (**e**–**l**).

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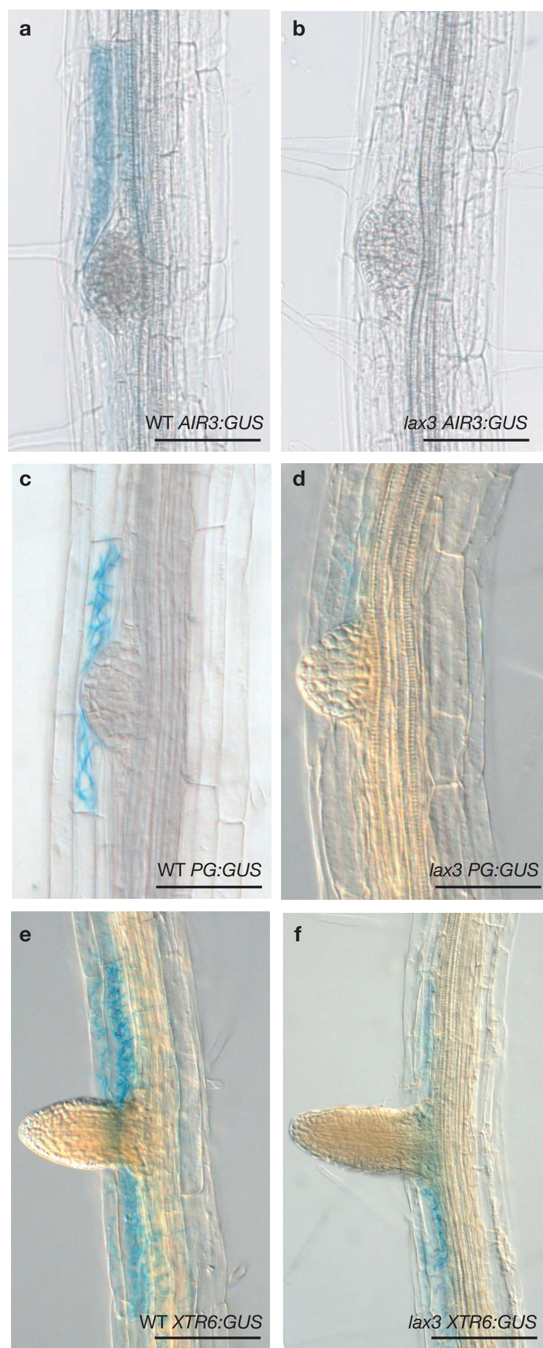

Figure 4 LAX3 regulates the expression of cell-wall-remodelling enzymes. (**a**–**f**) 7-day-old GUS-stained transgenic roots expressing *AIR3pro:GUS* (**a**, **b**), *PGpro:GUS* (**c**, **d**) and *XTR6pro:GUS* (**e**, **f**) reporters in either wild-type (**a**, **c**, **e**) or *lax3* (**b**, **d**, **f**) backgrounds. Scale bars are 50 µm (**a**–**f**).

versus e). However, expressing LAX3 in mutant stele plus epidermal/cortical tissues (using the native *LAX3* promoter) restored lateral root emergence (Supplementary Information, Fig. S3). In contrast, expressing LAX3 under the regulation of the stele-expressed *SHORTROOT* promoter[22] failed to rescue lateral root emergence (Supplementary Information, Fig. S3). Hence, the LAX3 epidermal/cortex (but not stele) expression domain seems essential. Indeed, confocal sections through transgenic roots expressing a functional LAX3–YFP fusion protein (in orange) revealed that the auxin influx carrier was not present at the surface of stele cells (Fig. 3i). In contrast, LAX3–YFP expressed in epidermal and cortical cells adjacent to developing lateral root primordia was clearly localized at the plasma membrane (Fig. 3i) where the fusion protein would facilitate auxin influx.

We next addressed how LAX3 could facilitate lateral root development when only expressed in small groups of cortical and epidermal cells directly overlaying developing lateral root primordia (Fig. 3i–l). Confocal scans of cells on the primary root surface (stained red with propidium iodide) revealed that the LAX3–YFP reporter (in yellow) was only expressed in a subset of these epidermal cells (Fig. 3l). Remarkably, only those epidermal cells marked with LAX3–YFP were physically separating from one another. The three epidermal cells expressing LAX3–YFP in Fig. 3l are from two adjacent cell files. Cell separation occurs exclusively between LAX3–YFP-expressing cells from these adjacent files. No cell separation was observed between LAX3–YFP-expressing cells in the same file, LAX3–YFP-expressing and non-expressing cell files, or any other non-expressing cells. Confocal images therefore suggest that LAX3 facilitates lateral root emergence by promoting the separation of epidermal and cortical cells overlaying primordia.

LAX3 regulates the spatial expression of several classes of cell-wall-remodelling enzymes

Scanning electron microscopy has revealed that in *Arabidopsis*, cells adjacent to emerging lateral root primordia remain intact but separate from one another along their middle lamella[9]. We analysed a variety of root transcript profiling datasets to identify candidate cell-wall-remodelling genes induced by auxin in the relevant tissues (see Supplementary information, Table S1 for further details). A number of mRNAs encoding cell-wall-related functions were identified, including a subtilisin-like protease[7] (AIR3), pectate lyase[23] (PLA2), polygalacturonase[24] (PG), a xyloglucan:xyloglucosyl transferase[25] (XTR6), expansin[26] (EXP17) and a glycosyl hydrolase[27] (GLH17).

We initially tested whether auxin-induction of these cell-wall-remodelling genes was dependent on LAX3 function. Given the substrate specificity of the auxin influx carrier (Fig. 2), LAX3-regulated genes should show reduced induction with IAA in the mutant, compared with wild-type backgrounds, whereas the membrane-permeable auxin 1-NAA should induce comparable levels of expression. Quantitative reverse transcriptase PCR (qRT–PCR) assays revealed that the majority of the cell-wall-remodelling genes studied (with the exception of *GLH17*) showed the predicted pattern (Supplementary Information, Fig. S5a). Promoter sequences from several of these cell-wall-related genes were fused to the GUS reporter to monitor their spatial patterns of expression in transgenic roots. Every cell-wall-related gene studied was expressed in cells directly overlaying new lateral root primordia (Fig. 4; Supplementary Information, Fig. 5c–d) similarly to *LAX3pro:GUS* (Fig. 3f; Supplementary Information, Fig. S4). IAA treatment confirmed that cell wall reporters were auxin-inducible (ref. 7; Supplementary Information, Fig. S5c–d). Every cell-wall-related promoter:GUS reporter expressed in cells overlaying new primordia was observed to be LAX3-dependent (Fig. 4). For example, expression of the *AIR3pro:GUS* reporter in wild-type root cells overlaying lateral root primordia (ref. 7, Fig. 4a) was significantly reduced in the *lax3* background (Fig. 4b). Similarly, *PGpro:GUS* and *XTR6pro:GUS* expression was reduced in root cells overlaying lateral root primordia in *lax3* (Fig. 4d, f), compared with wild-type (Fig. 4c, e). Hence, LAX3 seems to function by targeting the auxin-inducible expression of these cell-wall-related enzymes to cells overlaying lateral root primordia.

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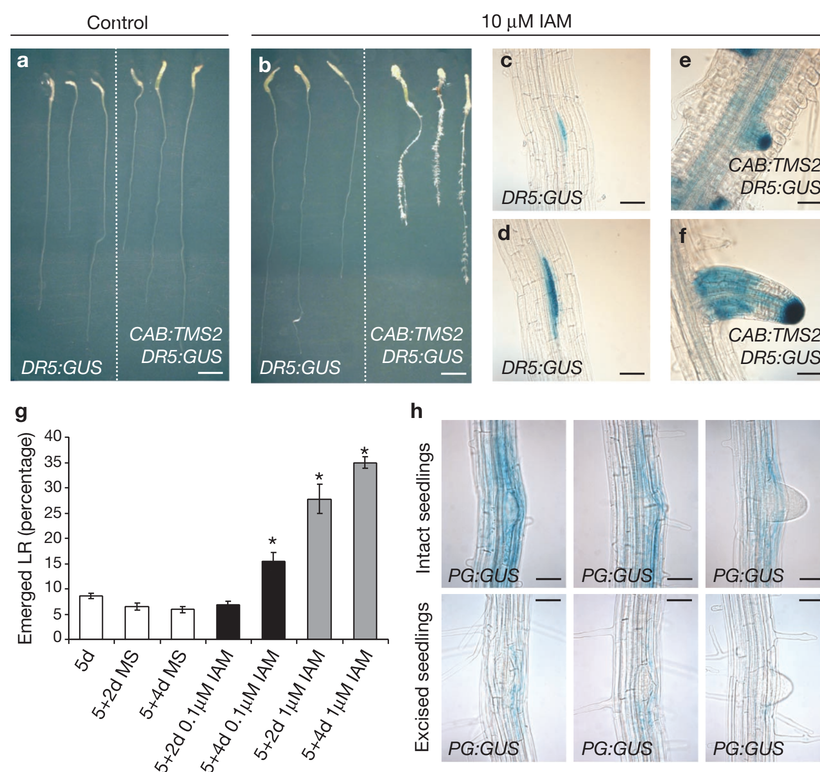

Figure 5 Lateral root emergence relies on an aerial source of auxin in a concentration-dependent manner. (**a**–**b**) *DR5:GUS* controls (left) or *CAB:TMS2 DR5:GUS* (right) seedlings were grown on Murashige and Skoog (MS) medium for 5 days after germination, then their cotyledons and leaves were excised and transferred to MS (**a**) or MS supplemented with 10 mM indole-3-acetamide (IAM, **b**) for 4 days. (**c**–**f**) Close-up view of lateral root primordium in *DR5:GUS* controls (**c**–**d**) or *CAB:TMS2 DR5:GUS* (**e**–**f**) seedlings treated with IAM as described above. Note that *CAB:TMS2* primordia developed because of the availability of an aerial auxin source, whereas *DR5:GUS* control LRP development was arrested. (**g**) Transgenic seedlings expressing *CAB:TMS2* were grown on MS medium for 5 days after germination, then their cotyledons and leaves were excised and transferred to MS or MS containing 0.1 or 1 mM IAM for a further 2 or 4 days. Roots were cleared and the number of emerged and non-emerged primordia was expressed as a percentage of the total number of lateral roots. Data are mean ± s.d., $*P < 0.05$; $n = 271$ (5d, primordial), 257 (5 + 2d, MS), 292 (5 + 4d, MS), 213 (5 + 2d, 0.1 μM IAM), 264 (5 + 4d, 0.1 μM IAM), 218 (5 + 2d, 1 μM IAM), 208 (5 + 4d, 1 μM IAM)). (**h**) *PG:GUS* seedlings were grown for 5 days on MS plates and either left intact (upper panels) or aerial tissues were excised (lower panels) then transferred to MS media for another two days. Roots were GUS-stained for 2 h. DIC imaging revealed that GUS staining was reduced at every stage of lateral root primordia monitored in *PG:GUS* seedlings. Scale bars are 2 mm (**a**, **b**), 40 μm (**c**–**f**) and 50 μm (**h**).

We examined the functional importance of individual cell-wall-remodelling enzymes by isolating knockout mutations in several of these genes. Phenotypic studies revealed that the *glh17* KO line showed a small but significant difference in the number of non-emerged LRP, compared with wild-type (Supplementary Information, Fig. S6a). Knockout mutations in other cell-wall-related genes did not significantly disrupt lateral root emergence (Supplementary Information, Fig. S6a). Many cell-wall-remodelling genes are members of large gene families, hence, because of genetic redundancy, knockout mutations in individual genes do not always have a mutant phenotype. We conclude that LAX3 coordinates the spatial expression of several classes of cell-wall-related enzymes, which are likely to act collectively to promote lateral root emergence.

Auxin derived from lateral root primordia induces cell wall-related enzymes

What is the source of auxin that promotes cell-wall-remodelling enzyme expression? This auxin is clearly not synthesized in root tissues as excision of aerial sources of auxin (cotyledons and leaves) blocks lateral root emergence (ref. 28; Fig. 5a). Consistent with the need for an aerial source of auxin to drive lateral root emergence, excised seedling roots contain only early-stage lateral root primordia, which express the auxin-responsive reporter *DR5:GUS* at a low level (Fig. 5c, d). Compellingly, lateral root emergence can be rescued in excised seedlings expressing the *CAB:TMS2* transgene[29] which can convert the inactive auxin precursor indole-3-acetamide (IAM) to IAA in the remaining green hypocotyl tissues (Fig. 5b, e, f). Increasing IAM levels caused a greater proportion of the lateral root primordia to emerge (Fig. 5g), demonstrating a clear relationship between the strength of the aerial auxin source and lateral root emergence.

The discrete pattern of expression of the auxin-inducible cell-wall-remodelling genes opposite new lateral roots (Figs 4, 5h; Supplementary Information, Fig. S5c–d) suggests that the aerial source of auxin necessary for organ emergence is channelled via primordia to overlaying cells. To test this model, we examined whether reducing the auxin source to lateral root primordia (by excising aerial tissues) results in a reduced auxin response in overlaying cells by monitoring the *PG:GUS* reporter (Fig. 5h). Cells overlaying lateral root primordia of intact seedlings stained strongly for *PG:GUS*, whereas reporter activity was significantly reduced in equivalent cells of excised seedlings (Fig. 5h). Hence, perturbing the auxin source in lateral root primordia clearly reduces auxin-responsive gene expression

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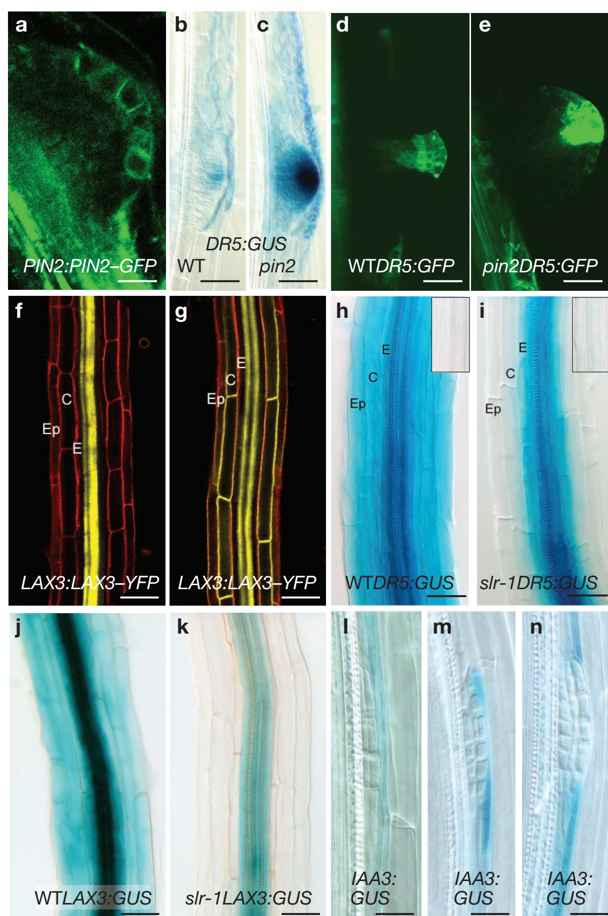

Figure 6 *LAX3* expression is positively regulated by auxin. (**a**) Confocal image of *PIN2pro:PIN2–GFP* expression (green) in primordia before emergence is restricted to the epidermis. (**b**, **c**) *DR5pro:GUS* (blue) and (**d**, **e**) *DR5pro:GFP* expression (green) is stronger in *pin2* (**c**, **e**) mutant primordia and adjacent cells, compared with wild-type (WT) (**b**, **d**). (**f**, **g**) Confocal images of root tissues expressing *LAX3pro:LAX3–YFP* expression (yellow) after treatment with 0.01 μM IAA (**f**) or 0.1 μM IAA (**g**). Root cells are stained with propidium iodide (red). (**h**, **i**) *DR5pro:GUS* expression (blue) in wild-type (**h**) and *slr-1* (**i**) root tissues after 6-h auxin treatment with 10 μM 1-NAA. Inset panels represent GUS staining in untreated controls. (**j**, **k**) *LAX3pro:GUS* expression (blue) in wild-type (**j**) and *slr-1* (**k**) root tissues after 6-h auxin treatment with 10 μM 1-NAA. (**l**–**n**) *IAA3pro:GUS* expression (blue) was localized to endodermal cells in direct contact with stage II (**l**), stage III (**m**) and stage IV (**n**) primordia. Scale bars are 10 μm (**a**), 20 μm (**b**–**e**, **l**–**n**), 40 μm (**f**–**g**) and 25 μm (**h**–**k**).

in overlaying cells. To further test this model, we examined the effect of altering the strength of the auxin signal at the lateral root primordia apex by investigating the emergence phenotype of the auxin transport mutant *pin2*. Lack of PIN2 function impairs basipetal transport in the main root[30]. Expression analysis of *PIN2pro:PIN2-GFP* in lateral root primordia suggests PIN2 performs a similar role during emergence of lateral root primordia (Fig. 6a). Consistent with this function, auxin accumulation, as monitored by DR5 reporters, was significantly higher in *pin2* primordia, compared with the wild-type control (Fig. 6b–e). Moreover, emergence of lateral root primordia was significantly enhanced in the *pin2* mutant (Supplementary Information, Fig. S6b). Our results suggest that auxin accumulation at the lateral root apex facilitates primordia emergence through the LAX3-dependent auxin induction of cell wall remodelling genes.

LAX3 expression is induced by auxin in epidermal and cortical tissues

Several lines of evidence demonstrate that *LAX3* expression is also induced by auxin in root cells overlaying lateral root primordia. First, qRT–PCR assays revealed that *LAX3* expression was upregulated by auxin (Fig. 7a, g). Second, auxin treatment of *LAX3*-promoter-driven YFP and GUS reporters induced expression in mature root cortical and epidermal tissues (Figs 6g, j, 7c–f). Third, the *LAX3* promoter sequence contains several copies of the auxin response element motif (AuxRE)[31]. Fourth, mutations in the ARF7 and ARF19 transcription factors[32] that bind the AuxRE motif block LAX3 auxin induction (Fig. 7g). Fifth, a gain-of-function mutant form of IAA14 termed solitary root (*slr-1*; ref. 33) can block auxin induction of *LAX3* mRNA (Fig. 7g) and completely abolished auxin-inducible *LAX3*-driven GUS reporter expression in root epidermal and cortical tissues (Fig. 6k). We conclude that LAX3 expression is auxin inducible and is mediated by the auxin signalling components ARF7, ARF19 and IAA14/SLR.

Lateral root emergence is also dependent on auxin responses in the endodermis

LAX3 expression is strictly limited to the cortex and epidermis (Figs 3, 6g, 7c–f), yet lateral root primordia must pass through endodermal, cortical and epidermal layers of root tissues to emerge (Fig. 1a). We investigated whether the endodermal auxin response was also important for lateral root emergence. The *slr-1* mutation does not block the expression of the auxin-inducible reporter *DR5pro:GUS* in the endodermis, in contrast to its strong inhibitory effect in cortex and epidermis (Fig. 6i), consistent with *IAA14pro:GUS* spatial expression (Fig. 7h). Instead, auxin-regulated endodermal gene expression is controlled by the distinct auxin signalling component *SHY2/IAA3* (ref. 34). An *IAA3pro::GUS* reporter revealed that *IAA3* expression was only detected in endodermal cells directly adjacent to lateral root primordia (Fig. 6l–n). Genetic evidence confirms that *IAA3* endodermal expression is functionally important for lateral root emergence. *shy2-24*, an *IAA3* loss-of-function allele that enhances endodermal auxin responsiveness, shows accelerated lateral root emergence, compared with wild-type (Supplementary Information, Fig. S6c). Conversely, *shy2-2,* an *IAA3* gain-of-function allele that reduces the auxin responsiveness of mature endodermal cells, delayed lateral root emergence (Supplementary Information, Fig. S6d). Quantitative RT–PCR assays for *GLH17* and *PLA2* revealed that *IAA3* is likely to influence the rate of lateral root emergence by regulating the auxin inducible expression of cell-wall-remodelling genes (Supplementary Information, Fig. S5b).

DISCUSSION

Auxin provides a key signal during lateral root development[12], triggering the initial mitotic division of lateral root founder cells in the pericycle tissue and patterning lateral root primordia[14]. The results of this study suggest that auxin has another equally important function, which is to promote organ emergence.

Auxin derived from lateral root primordia seems to re-programme overlaying cells in adjacent tissues. In endodermal cells, the auxin-dependent degradation of the IAA3 repressor triggers the expression of selected cell-wall-remodelling enzymes to initiate cell separation in this tissue (Fig. 8a). Auxin derived from the primordium also induces the expression of an auxin influx carrier, LAX3, in adjacent cortical

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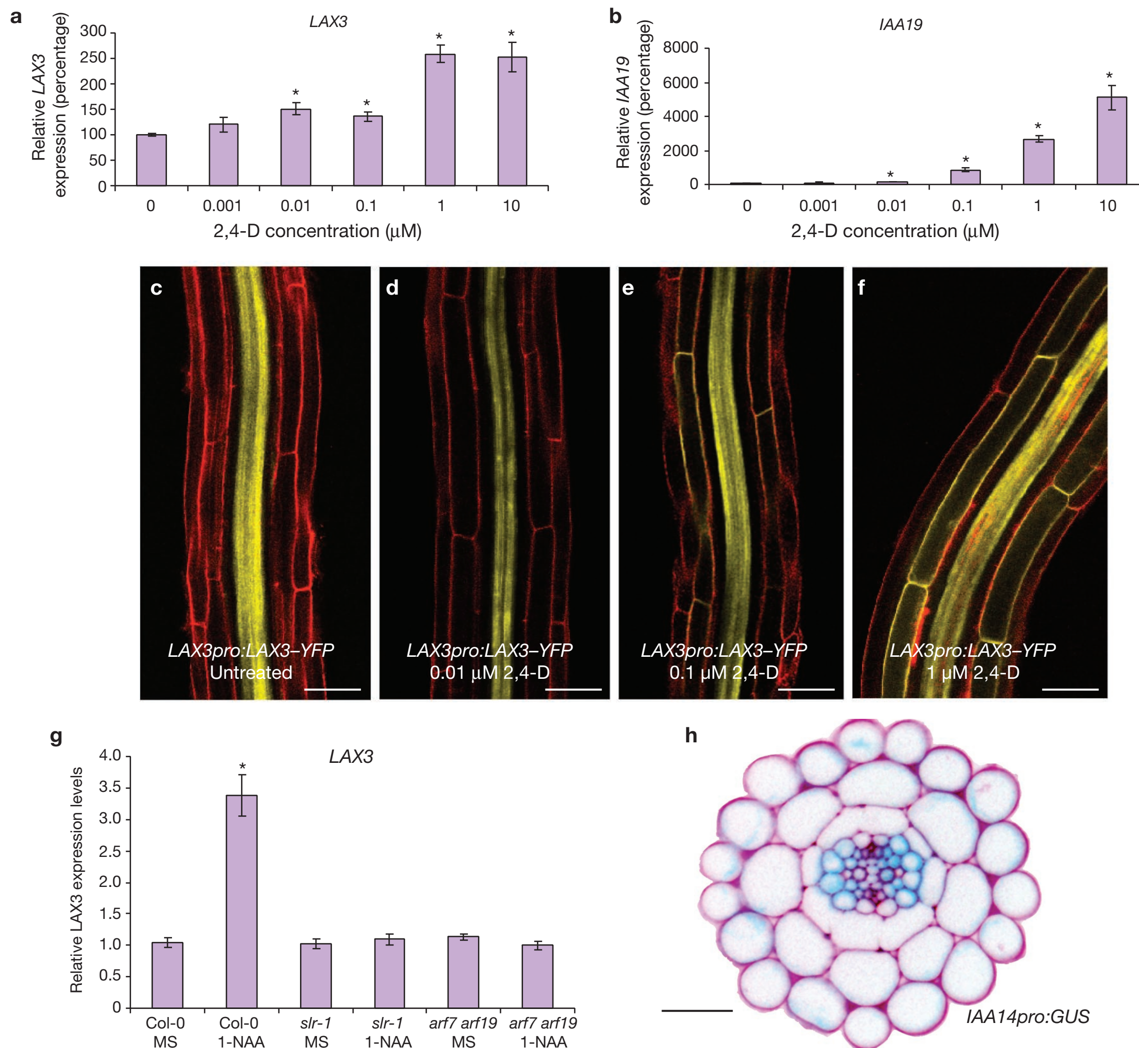

Figure 7 *LAX3* is upregulated by auxin in an ARF and IAA14-dependent manner. (**a**, **b**) qRT–PCR measurements of *LAX3* (**a**) and *IAA19* (**b**) mRNA abundance in *Arabidopsis* seedlings after treatment with increasing concentrations of the synthetic auxin 2.4-D. (**c**–**f**) Confocal images of root tissues expressing *LAX3pro:LAX3–YFP* (yellow) after treatment with 0 (**c**) 0.01 (**d**) 0.1(**e**) or 1.0 (**f**) μM 2,4-D. Root cells were stained with propidium iodide (red). (**g**) RNA abundance of *LAX3* in 14-day-old wild-type (Col-0), *slr-1* and *arf7 arf19* mutant seedling roots treated for 6 h with either 1 μM 1-NAA or no hormone (MS). RNA abundance was determined by qRT–PCR (data are means ± s.d., $^*P < 0.05$, Student's *t* test, $n = 3$). (**h**) Cross-section of GUS-stained mature root tissues expressing an *IAA14pro:GUS* reporter (blue) in epidermal, cortical and xylem-pole pericycle cells. Scale bars are 50 μm (**c**–**f**) and 20 μm (**h**).

cells following the degradation of the IAA14/SLR repressor (Fig. 8b). The resulting LAX3-dependent accumulation of auxin upregulates the expression of a distinct set of cell-wall-remodelling enzymes that initiate cortical cell separation (Fig. 8c). Auxin derived from later-stage primordia in closer proximity to overlaying epidermal cells induce expression of *LAX3*, and then cell-wall-remodelling genes (Fig. 8d), causing cell separation and ultimately resulting in lateral root emergence.

PIN and AUX/LAX classes of auxin transport proteins have key roles in transmitting or localizing the inductive IAA signal, respectively. Although PIN class of auxin efflux carrier expressed by the lateral root facilitates the transmission of the inductive IAA signal, the ability to localize the auxin response to cells directly overlaying lateral root primordia is dependent on the auxin influx carrier LAX3. Expression of *LAX3* is itself auxin inducible. This regulatory arrangement creates a positive-feedback loop that explains the striking 'all or nothing' pattern of LAX3 expression in cortical and epidermal cells adjacent to lateral root primordia (Fig. 3l). LAX3-expressing cells will become more efficient sinks for auxin. LAX3 therefore functions to amplify the signal emitted by the lateral root primordium tip while limiting its action to a few cells in close proximity with this auxin source.

The restricted pattern of *LAX3* expression in outer root tissues is important for the localized induction of cell-wall-remodelling genes such as *AIR3*, *PG* and *XTR6* (Fig. 4). High levels of auxin cause cell separation in *Arabidopsis* roots[35,36]. However, these studies showed that cells were sloughed off abnormally, whereas under normal conditions cell separation was highly localized (Figs 1a, 3l). Our results explain how the activities of auxin-regulated cell-wall-remodelling enzymes are precisely targeted during lateral root emergence without compromising the integrity of the surrounding root tissues.

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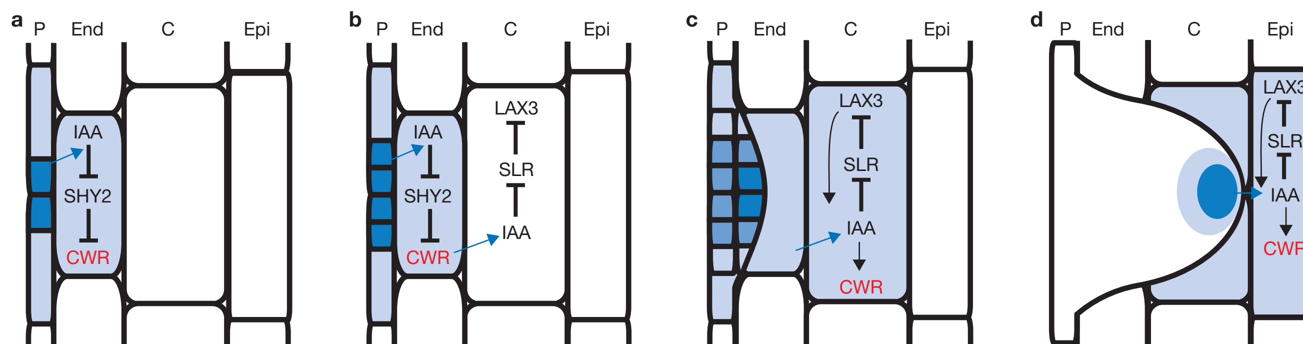

Figure 8 Model for auxin-dependent lateral root emergence. (**a**) Auxin (IAA) originating from dividing pericycle (P) cells induces cell-wall-remodelling (CWR) gene expression in adjacent endodermal (End) cells by targeting the degradation of the SHY2/IAA3 repressor protein. (**b**) Auxin derived from the lateral root primordium also induces expression of the auxin influx carrier LAX3 in adjacent cortical cells (C) by targeting the degradation of the SLR/IAA14 repressor protein. (**c**) LAX3 expression increases cell permeability to auxin, creating a positive-feedback loop. Increased auxin accumulation induces CWR expression. (**d**) At a later stage of primordium development, auxin induces LAX3 expression in adjacent epidermal (Epi) cells. The expression of CWR in a few cells of the different layers facilitates the emergence of lateral root primordium. Cellular auxin responses are represented as a blue colour gradient.

LAX3 regulates the expression of several cell-wall-remodelling enzymes, including a subtilisin-like protease (AIR3; ref. 7), PLA2 (ref. 23) and PG[24]. The PLA2 and PG enzymes are likely to be particularly important for cell separation during lateral root emergence as they cleave demethylated pectin, a substrate which is enriched in the middle lamella of root cells overlaying lateral root primordia[9]. Intriguingly, the endodermally expressed IAA3 protein seems to regulate an overlapping but distinct set of cell-wall-remodelling enzymes, compared with LAX3, most probably reflecting differences in the cell-wall composition of these root tissues.

Our study has revealed that the developmental progression and emergence of lateral root primordia are strictly coordinated. Mutants that disrupt the lateral root emergence process also affect the developmental progression of lateral roots, as illustrated by the increased proportion of stage I primordia in *lax3* mutant roots (Fig. 2g). The endodermis and cortex tissues form concentric circles of cells around the pericycle, in which lateral root primordia initiate. Targeting cell separation between endodermal and cortical cells overlaying the stage I primordia would help reduce the tissue tension created by these encircling tissues, enabling meristematic cells to divide periclinally and produce a new second layer that will protrude out into the parental root.

The *lax3* and *shy2-2* mutants that disrupt lateral root emergence also show significantly increased numbers of lateral root primordia, compared with wild-type (ref. 34; Fig. 1g). We hypothesize that the delay in developmental progression of lateral roots in these backgrounds causes leaf-derived auxin to accumulate to supra-optimal levels in the root pericycle, stimulating the ectopic initiation of new primordia. However, under normal circumstances in wild-type seedlings, shoot-derived auxin promotes lateral root emergence rather than initiation[28]. Consistent with this conclusion, excision of shoot tissues blocks lateral root emergence but not initiation (ref. 28; Fig. 5a–f). Similarly, the *mdr1* mutation reduces shoot to root auxin transport but does not affect the number of lateral roots initiated[37]. Hence, shoot-derived auxin is not normally a limiting factor for lateral root initiation.

In summary, our study has revealed that cells in the parent root overlaying new lateral root primordia actively participate in organ emergence. Roots use a transcellular auxin signalling network designed to synchronize lateral root development and emergence processes. □

METHODS

Generation of plant materials. Insertion lines for the *Arabidopsis LAX1* (At5g01240), *LAX2* (At2g21050) and *LAX3* (At1g77690) genes were identified as described previously[18]. To create the *LAX3pro:GUS* reporter, a 1.6 kbp *LAX3* promoter region was cloned upstream of the *uidA* gene in a BIN19-derived vector. The functional *LAX3pro:LAX3–YFP* construct was generated as described previously[38] for *AUX1pro:AUX1–YFP*. *SHRpro:LAX3–YFP* was created by replacing the promoter sequence of *LAX3pro:LAX3–YFP* with 2 kbp of the *SHR* promoter sequence[22]. *AIR3pro::GUS*[7] was provided by Bert van der Zaal (Institute of Biology, Leiden University, The Netherlands). *PGpro:GUS* and *XTR6pro:GUS* constructs were obtained by PCR amplification of a promoter region (approximately 1 kb) from each gene, then cloned upstream of the *uidA* sequence in pBI101.3. Transformation of *Agrobacterium* (C58) and *Arabidopsis* were performed as described previously[38].

Characterization of mutant and transgenic plant material. *Arabidopsis thaliana* seedlings were grown as described previously[1]. The total number and stages of lateral root primordia were counted according to methods used previously[3]. Auxin measurements in roots were performed as described previously[21]. Confocal analysis on lines expressing YFP and/or GFP reporters was performed as described previously[39]. GUS assays were performed as described previously[3,40]. GUS stained seedlings were viewed using differential interference contrast optics as described previously[3].

Heterologous expression of AUX1 and LAX3 in *Xenopus* oocytes. Capped RNA (cRNA) of *LAX3 or AUX1* was transcribed *in vitro* with SP6 RNA polymerase using the mMESSAGE mMACHINE kit (Ambion). Oocyte preparation and ^{3}H-IAA uptake assays were performed as described previously[16].

Quantitative RT–PCR. Total RNA was extracted using the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from total RNA using Superscript III (Invitrogen). Quantitative PCR was performed using SYBR Green QPCR Master Mix (Stratagene). Target quantifications were performed with specific primer pairs designed using Beacon Designer 4.0 (Premier Biosoft International). Expression levels were either normalized to *ACTIN2* (At3g18780) or *EF-1-alfa* (At1g07940). All qRT–PCR experiments were performed in triplicates and the values presented represent means ± s.d.

Note: Supplementary Information is available on the Nature Cell Biology website.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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