RESEARCH ARTICLE

Lateral root emergence in *Arabidopsis* is dependent on transcription factor LBD29 regulation of auxin influx carrier *LAX3*

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ABSTRACT

Lateral root primordia (LRP) originate from pericycle stem cells located deep within parental root tissues. LRP emerge through overlying root tissues by inducing auxin-dependent cell separation and hydraulic changes in adjacent cells. The auxin-inducible auxin influx carrier LAX3 plays a key role concentrating this signal in cells overlying LRP. Delimiting *LAX3* expression to two adjacent cell files overlying new LRP is crucial to ensure that auxin-regulated cell separation occurs solely along their shared walls. Multiscale modeling has predicted that this highly focused pattern of expression requires auxin to sequentially induce auxin efflux and influx carriers *PIN3* and *LAX3*, respectively. Consistent with model predictions, we report that auxin-inducible *LAX3* expression is regulated indirectly by AUXIN RESPONSE FACTOR 7 (ARF7). Yeast one-hybrid screens revealed that the *LAX3* promoter is bound by the transcription factor LBD29, which is a direct target for regulation by ARF7. Disrupting auxin-inducible *LBD29* expression or expressing an LBD29-SRDX transcriptional repressor phenocopied the *lax3* mutant, resulting in delayed lateral root emergence. We conclude that sequential *LBD29* and *LAX3* induction by auxin is required to coordinate cell separation and organ emergence.

KEY WORDS: *Arabidopsis*, Root development, Lateral root emergence, Auxin, LBD29

INTRODUCTION

Lateral root (LR) emergence represents a crucial developmental program enabling new primordia to breach the overlying endodermal, cortical and epidermal cell layers and enter the

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surrounding soil environment (Péret et al., 2009; Fig. 1A). This process is tightly controlled in order to limit damage to the parental root from which a new lateral root primordium (LRP) originates. The hormone signal auxin and several of its signaling and transport components have been demonstrated to play an essential role during LRP emergence (Swarup et al., 2008; reviewed in Lavenus et al., 2013). Auxin has a specialized transport machinery, and its signal triggers the degradation of AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins via the SCF^{TIR1} complex (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The degradation of AUX/IAA proteins (that function as transcriptional repressors) allows interacting transcriptional proteins termed AUXIN RESPONSE FACTORs (ARFs) to change auxin responsive gene expression in order to elicit developmental responses (Dharmasiri and Estelle, 2004).

In the model plant *Arabidopsis thaliana*, LAX3, an auxin influx carrier belonging to the AUX/LAX gene family, plays an important role during LR development, by facilitating the emergence process (Péret et al., 2009; Swarup et al., 2008). Swarup et al. (2008) proposed a model in which an increased level of auxin in the cortical cells overlying LR primordia, induces *LAX3* expression by targeting the degradation of the IAA14/SLR repressor protein, allowing ARF7 and ARF19 to activate auxin responsive genes. The *arf7 arf19* double mutant and the *iaa14/slr-1* gain-of-function mutant show impaired expression of *LAX3*, suggesting that *LAX3* expression is mediated by the auxin signaling pathway module IAA14/SLR-ARF7-ARF19 (Swarup et al., 2008). In the cortical cells, the increase of *LAX3* expression triggers a positive-feedback loop stimulating further auxin uptake from LRP. The consequence of auxin accumulation is the induction of expression of a set of cell wall remodeling genes, such as polygalacturonases and xyloglucan endotransglucosylase/hydrolase, which are involved in pectin polymer cleavage and cell wall loosening, respectively (Laskowski et al., 2006; Swarup et al., 2008). Consistent with the model that the LAX3 positive feedback loop is important for emergence, the expression of these cell wall remodeling enzymes at the emergence site is LAX3 dependent (Swarup et al., 2008). Hence, by inducing cell wall remodeling in overlying cells and triggering their separation, the growing primordium is able to pass through the outer root cell layers and emerge (Péret et al., 2009; Swarup et al., 2008).

LAX3 is expressed in just two cell files overlaying new LRP (Swarup et al., 2008; Fig. 1B). To understand how this striking pattern of *LAX3* expression is regulated, we developed a mathematical model that captures the network regulating its expression and auxin transport within realistic three-dimensional cell and tissue geometries (Péret et al., 2013). Our model revealed that, for the *LAX3* spatial expression to be robust to natural variations in root tissue geometry, the efflux carrier PIN3 is also
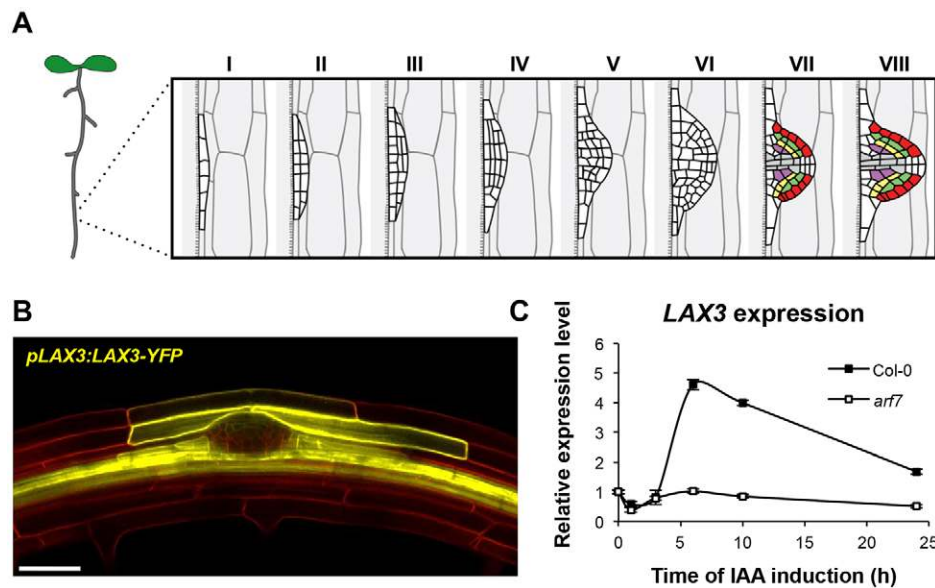
Fig. 1. *LAX3* induction in the outer tissue during lateral root emergence is ARF7 dependent. (A) Lateral root formation proceeds in eight developmental stages (I-VIII; Casimiro et al., 2003). (B) A functional LAX3-YFP fusion is used to monitor LAX3 accumulation in the overlaying tissues during LR emergence. Cell walls are stained red with propidium iodide. Scale bar: 50 µm. (C) *LAX3* induction by auxin (1 µM IAA) was monitored in wild-type (Col-0) compared with *arf7* mutant by qPCR. Data represent the mean±s.e. of four technical replicates; three biological replicates were performed.

required. To prevent *LAX3* from being transiently expressed in multiple cell files, the model predicted that *PIN3* and *LAX3* genes must be induced consecutively. Consistent with this prediction, the translational inhibitor cycloheximide was observed to block auxin upregulation of transcript abundance of *LAX3* (but not PIN3; Péret et al., 2013). Hence, *LAX3* appears to function as a secondary (rather than primary) auxin responsive gene. However, the underlying molecular basis of *LAX3* induction by auxin remained unresolved.

In this study, we initially describe that ARF7 is essential for auxin-dependent *LAX3* induction during LR emergence. However, we demonstrate using several independent lines of experimental evidence that ARF7 regulates LAX3 indirectly. Instead, we report that the LATERAL ORGAN BOUNDARIES-DOMAIN/ ASYMMETRIC LEAVES 2-LIKE (LBD/ASL) transcription factor (TF) family member LBD29 functions as a direct positive regulator of *LAX3* auxin-dependent expression. Our results position LBD29 at a key node downstream of auxin and ARF7 in the LR emergence regulatory network.

RESULTS
Transcription factor ARF7 regulates auxin-inducible *LAX3* expression indirectly

We initially addressed the ARF-dependent regulatory mechanisms controlling auxin-inducible *LAX3* expression. It was previously reported that the abundance of *LAX3* mRNA is reduced in an *arf7* mutant background, suggesting that *LAX3* expression is dependent on this ARF transcription factor (Okushima et al., 2007; Swarup et al., 2008). To test this, a RT-qPCR-based assay was designed to transcript profile *Arabidopsis* wild-type (WT) and *arf7* mutant roots exposed to external auxin (1 µM IAA) for varying lengths of time. Transcript profiling revealed that auxin induction of *LAX3* mRNA was abolished in the *arf7* mutant background (Fig. 1C). Similarly, when *pLAX3:GUS* and *pLAX3:LAX3-YFP* reporters (Swarup et al., 2008) were expressed in *arf7*, auxin induction of both reporters was abolished (Fig. S1AB). Hence, ARF7 appears to be crucial for auxin induction of *LAX3* expression.

We next addressed whether ARF7 regulated *LAX3* auxin induction directly or indirectly. Whilst the *LAX3* promoter sequence contains a canonical ARF binding motif (−939 bp), transcript profiling of auxin-treated WT roots revealed that

upregulation of *LAX3* mRNA was first detected 6 h after hormone induction (Fig. 1C). Induction of primary auxin-responsive transcripts is typically detected within minutes rather than hours, suggesting that *LAX3* may be a secondary auxin-responsive gene and hence not a direct target for ARF7 regulation (Péret et al., 2013; Mellor et al., 2015). This is consistent with bioinformatics analysis of transcriptomic data from the *arf7 arf19* mutant complemented by a dexamethasone (DEX)-inducible ARF7-GLUCOCORTICOID RECEPTOR (GR) fusion protein under its native *ARF7* promoter (as described in Lavenus et al., 2015), which categorized *LAX3* as a positive indirect target of ARF7 (Fig. S1C).

To directly test the functional importance of the AuxRE motif within the *LAX3* promoter for auxin induction and gene activity, we performed targeted *in vitro* mutagenesis studies. The *LAX3* (−939 bp) AuxRE motif and two mutant variants (termed IVM1 and IVM2; Fig. S2A) were re-introduced (as part of a functional *pLAX3:LAX3-YFP* transgene) into an *aux1 lax3* mutant background. Phenotypic analysis revealed that, like the wild-type *pLAX3:LAX3-YFP* reporter, both IVM1 and IVM2 promoter transgene variants retain their ability to rescue LR emergence in the *aux1 lax3* mutant background (Fig. S2B). Confocal imaging confirmed that both IVM1 and IVM2 variants of the *pLAX3:LAX3-YFP* reporter also retained their ability to be induced by auxin in root cortical cells (Fig. S2C).

To independently assess the role of the AuxRE versus other regulatory regions, a *LAX3* promoter deletion approach was also employed (Fig. 2). The 1.98 kb *LAX3* promoter was truncated at four different positions (Fig. 2A; termed Δ1, Δ2, Δ3 and Δ4), to create increasingly shorter promoter fragments, then fused to the *LAX3-YFP* reporter and transformed into the *aux1 lax3* double mutant. Multiple transgenic lines expressing each *pLAX3:LAX3-YFP* promoter deletion were initially scored for complementation of the *aux1 lax3* LR phenotype. All Δ1 and Δ2 promoter deletion lines fully complemented the *aux1 lax3* LR defect (Fig. 2B) and retained auxin-inducible expression (Fig. 2C,D). This result demonstrates that the 826 bp sequence upstream of the start of the *LAX3* coding sequence (that does not contain the *AuxRE*) is sufficient to drive auxin-inducible expression and promote LR emergence. In contrast, all Δ3 promoter deletion lines only partially complemented the *aux1 lax3* LR phenotype, whereas no complementation was observed for
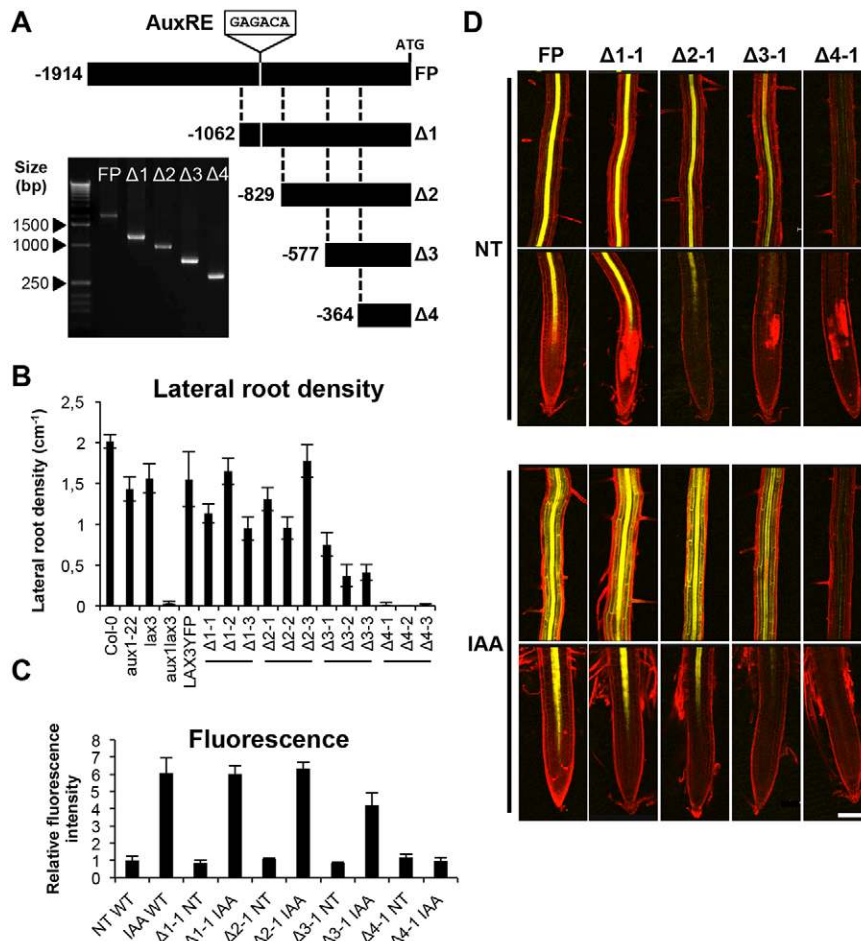
Fig. 2. *LAX3* induction by auxin is independent of the canonical auxin-response element (AuxRE). (A) Representation of *LAX3* full promoter (FP) from −1914 to start codon (ATG). Promoter deletions (named Δ1, Δ2, Δ3 and Δ4) were generated (see insert for fragment sizes verification) and cloned upstream of LAX3-YFP. The canonical AuxRE situated at −939 bp is indicated [complementary strand of the TGTCTC sequence described by Ulmasov et al. (1997)]. (B) Lateral root density measurements (number of LRs per length of primary root). Error bars are s.e.m. (*n*≥8). (C) Fluorescence intensity measurement of cortical cells is relative to the mock control of the corresponding transgenic line (*n*≥8 corresponding to at least four strips out of two independent roots). (D) Laser-scanning confocal images of LAX3-YFP fusion driven by the full promoter (FP) or promoter deletions in non-treated (NT) or auxin treated (1 μM IAA for 16 h) conditions. Cell walls are stained red with propidium iodide. Scale bar: 100 μm.

any of the Δ4 promoter deletion lines (Fig. 2B). Quantitative analysis of Δ3 and Δ4 reporter lines following auxin treatment gave similar results (Fig. 2C,D). Hence, multiple independent lines of evidence suggest that the *AuxRE* promoter element is not necessary for auxin-inducible *LAX3* expression. Instead, other regulatory motifs encoded closer to the start of the *LAX3* coding sequence appear to be functionally important. We conclude that ARF7 regulates auxin-inducible *LAX3* expression indirectly, raising the question about which transcription factor(s) acts between ARF7 and *LAX3* in the LR emergence gene regulatory network.

Identification of putative *LAX3* transcriptional regulators

In order to identify putative transcription factors that regulate *LAX3*, a yeast one-hybrid (Y1H) assay was performed. As bait, the *LAX3* promoter (1374 bp) was fused to *lacZ* and *HIS3* reporter genes. In the Y1H system, reporter gene expression is activated when a TF interacts with the DNA bait. A root TF collection (Gaudinier et al., 2011) containing >650 genes was fused to a transcription activation domain (AD) and used as a prey. A total of 17 root stele-expressed TFs were found to bind to the *LAX3* promoter sequence (Table S1). These transcription factors included five homeodomain leucine zipper (HD-Zip) proteins (HAT22, ATHB52, ATHB6, PHV/ ATHB9, ATHB40), two zinc finger-homeodomain (ZF-HD) proteins (HB21, HB30) and the LBD/ASL protein LBD29.

LBD29 represents the most promising candidate identified in the Y1H screen for an intermediary transcriptional regulator between ARF7 and *LAX3* based on several criteria. First, *LBD29* expression is induced minutes after auxin treatment (Okushima et al., 2007).

Second, characteristic of many primary auxin-response genes, *LBD29* expression is strongly induced following cycloheximide (CHX) treatment (Okushima et al., 2007). Third, *LBD29* expression can be induced in *pARF7:ARF7-GR/arf7 arf19* seedlings following treatment with DEX plus auxin (Okushima et al., 2007). Fourth, ARF7 has been shown to bind to *LBD29* promoter fragments containing *AuxRE* motifs using EMSA and ChIP-PCR techniques, respectively (Okushima et al., 2007; Lavenus et al., 2015). Finally, overexpression of *LBD29* can also partially restore LR formation in the *arf7 arf19* mutant background (Okushima et al., 2007).

To directly test the functional importance of LBD29 for *LAX3* auxin-inducible expression, we employed the *lbd29-1* T-DNA insertion line (SALK_071133). In this SALK line (the only insertion line currently available for this gene), the T-DNA sequence is inserted in the *LBD29* promoter region 3′ of the nearest *AuxRE* to the transcription start site (Fig. S3A). Whilst the position of the T-DNA insert does not disrupt the coding sequence, RT-qPCR analysis revealed that despite a higher basal level of expression, it significantly attenuated (>10-fold compared with WT) *LBD29* auxin-inducible expression (Fig. S3B,C). Interestingly, *LAX3* mRNA abundance was no longer auxin inducible in the *lbd29-1* insertion line compared with the WT (Fig. 3A), suggesting that this T-DNA allele attenuates the ability of LBD29 to function as an auxin-inducible positive regulator of *LAX3* expression. To verify this, the *lbd29-1* insertion line was also crossed with the *pLAX3:LAX3:YFP* reporter (Swarup et al., 2008). In the WT, LAX3-YFP is constitutively expressed in central root stele tissues, plus a small number of cortical cells (and later in
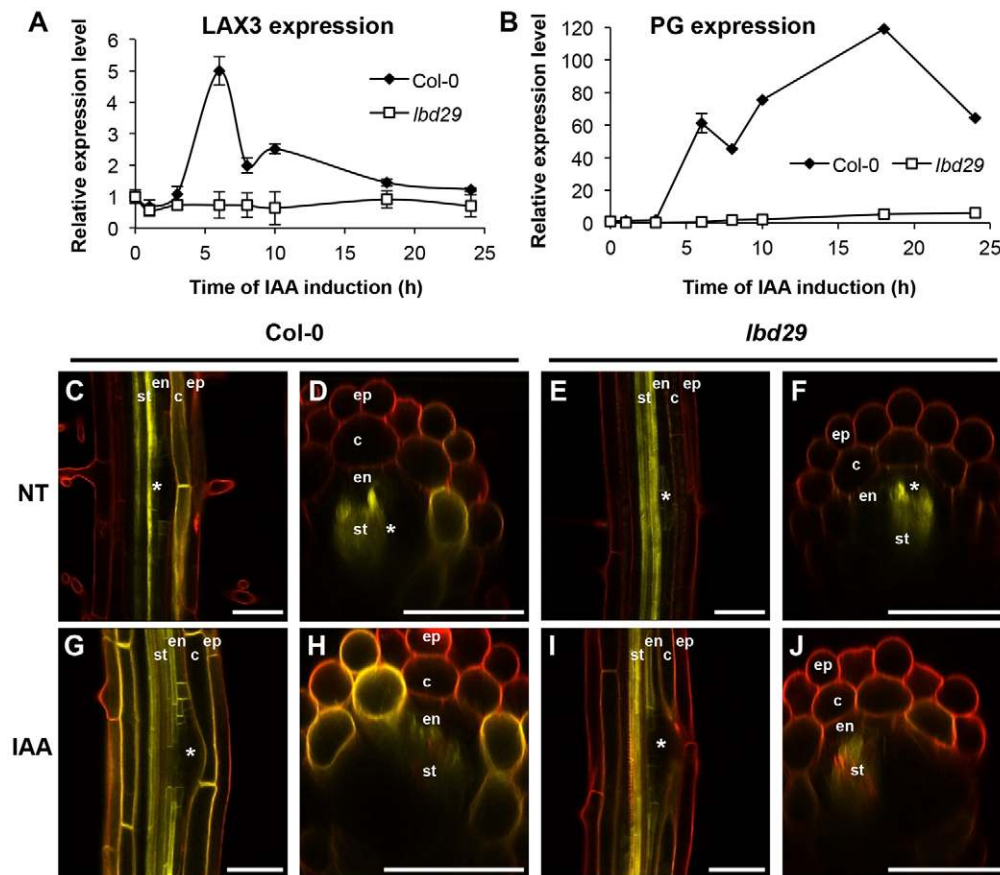
Fig. 3. LBD29 controls *LAX3* induction during lateral root emergence. 5-day-old seedlings of Col-0 and *lbd29* were treated with 1 µM IAA for 0, 1, 3, 6, 8, 10, 18 and 24 h. *LAX3* (A) and *PG* (B) mRNA levels were quantified by RT-qPCR. Data represent the mean value±s.e. of four technical replicates and the experiment was performed in triplicate. (C-J) Expression pattern of pLAX3: *LAX3-YFP* in non-treated (NT) control and after auxin treatment (1 µM IAA) in wild type (Col-0) and mutant (*lbd29-1*). Cell walls are stained red with propidium iodide. Scale bars: 50 µm. Asterisks indicate a LR primordium. ep, epidermis; c, cortex; en, endodermis; st, stele. Data represent the mean±s.e. of four technical replicates; three biological replicates were performed.

epidermal cells) overlying new LR primordia (Fig. 3C,D) (Swarup et al., 2008). However, in the *lbd29-1* mutant background the *pLAX3:LAX3-YFP* reporter was no longer expressed in cortical cells overlying new primordia (Fig. 3E-F). IAA treatment also could not induce ectopic expression of *LAX3* in the *lbd29-1* mutant (Fig. 3I,J) compared with the WT (Fig. 3G,H).

We next examined the effect of the *lbd29-1* allele on auxin-regulated genes controlled by LAX3. For example, the *POLYGALACTURONASE* (*PG*) gene is auxin and LAX3 dependent and is specifically expressed at sites of LR emergence (Swarup et al., 2008). Consistent with a role for LBD29 mediating upregulation of *LAX3*, *lbd29-1* roots also failed to show induced *PG* expression following auxin treatment in contrast to the WT (Fig. 3B). Hence, LBD29 appears to not only control upregulation of *LAX3* via auxin in outer root tissues but also (indirectly) impacts expression of LAX3-dependent downstream targets of the LR emergence gene regulatory pathway.

LBD29 facilitates lateral root emergence

Transcript profiling and reporter studies have demonstrated that LBD29 is essential for auxin-inducible expression of *LAX3* and other genes involved in cell separation in cells overlying new LRP (Fig. 3). Logically, as a regulator of *LAX3* expression (and its downstream targets), disrupting the induction of LBD29 (in the case of the *lbd29-1* allele) should cause a *lax3*-like mutant phenotype and delay LR emergence. To detect any change in the *lbd29-1* LR emergence rate, we employed a gravistimulation-based bioassay (Péret et al., 2012), which after subjecting seedlings to a 90° gravity stimulus, leads to the highly synchronized temporal development of a new primordium on the

outer side of the root bend (Lucas et al., 2008). For example, 18 h after a gravistimulation, WT root bends contain mainly stage II LRP, whereas by 42 h, many primordia were close to emergence (stage VI or VII) or already emerged (stage e; Fig. 4). In the case of *lax3*, 18 h after a gravity stimulus, mutant roots displayed proportionately more stage I primordia compared with the WT (Fig. 4A). However, the biggest phenotypic difference was observed 42 h after the gravistimulus, since no emerged LR were detected in *lax3* compared with the WT, and most primordia were still at earlier stage IV or V (Fig. 4A). Phenotypic analysis of the *lbd29-1* insertion line revealed an even greater delay in LR development compared with *lax3*, featuring a higher proportion of stage IV and V primordia (Fig. 4A), consistent with LBD29 acting upstream of LAX3 in the LR emergence regulatory pathway.

To provide additional independent genetic evidence to probe the function of LBD29 during LR emergence, we produced a transgenic line expressing the *LBD29* genomic sequence fused to the SRDX transcriptional repressor domain in the WT (Col-0) background. The resulting gLBD29-SRDX fusion protein is designed to repress LBD29 target genes by blocking their transcription, thereby mimicking an *lbd29* loss of function allele. Phenotypic analysis of two independent gLBD29-SRDX lines revealed that both exhibited delayed LR development and featured a higher proportion of stage V primordia versus the WT, as in *lbd29-1* (Fig. 4B), consistent with LBD29 acting as a positive regulator of the LR emergence regulatory pathway. Further independent confirmation of the role of LBD29 during LR emergence was generated by expressing a translational fusion of the LBD29 protein to the vYFP marker in the *lbd29* mutant background. The *lbd29-1* line transformed with the pLBD29:LBD29-vYFP construct
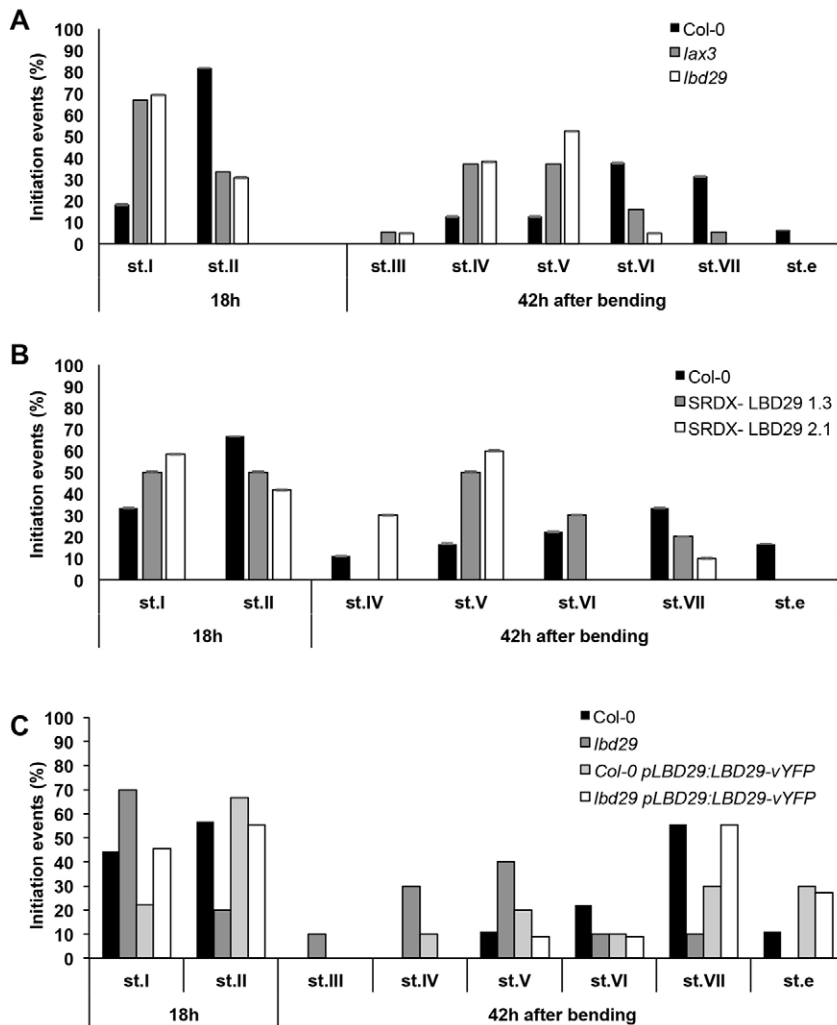
Fig. 4. Gravistimulation assays in 3-day-old-seedlings of WT, lax3, lbd29 and SRDX-LBD29 lines. Phenotypic analysis of LR emergence was achieved by synchronizing LR formation with a gravistimulus for 18 h and 42 h. Compared with WT (Col-0), LR emergence is delayed in *lax3* and *lbd29* mutants (A) and SRDX-LBD29 lines (B). Expression of the LBD29 protein fused to the vYFP reporter fully restores LR formation in the *ldb29* mutant (C). Data shown are percentage and the error bars represent s.e., *n*=20 for Col-0, lax3 and lbd29; *n*=18 and 16 for SRDX-LBD29 1.3 and 2.1, respectively and *n*=14 for pLBD29:LBD29-vYFP.

exhibited full restoration of the wild-type LR phenotype using the LR bending assay (Fig. 4C).

Further characterization of WT versus the *lbd29-1* T-DNA insertion line was performed to investigate any additional alterations of its LR phenotype in 10-day-old seedlings. This revealed that the number of emerged LRs was reduced in the *lbd29-1* insertion line (Fig. S4A-C) but that neither the stages of LR primordia distribution (Fig. S4D) nor the total number of LR primordia (Fig. S4E) was affected in *lbd29-1* compared with the WT. Taken together, these results suggest that the reduced number of emerged LR in the *lbd29-1* insertion line is not related to a defect in LR initiation or primordia development, but is due to slower organ emergence.
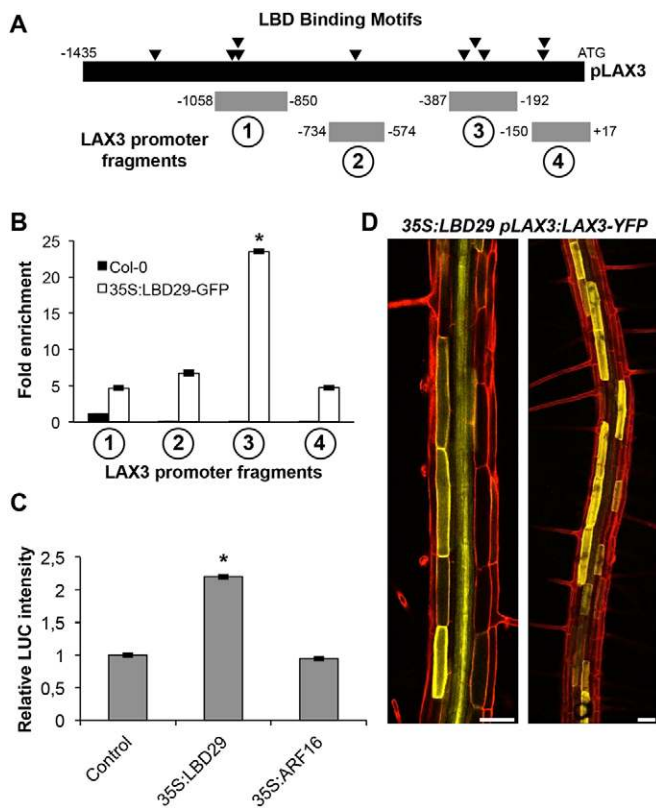
LBD29 directly controls *LAX3* expression

Several independent lines of evidence reported in this paper suggest that LBD29 regulates LR emergence by controlling *LAX3* expression. To test whether the LBD29 transcription factor binds directly to the *LAX3* promoter *in planta*, we performed chromatin immunoprecipitation (ChIP) qPCR analysis using an anti-GFP antibody on nuclear samples prepared from WT (Col-0) (as a negative control) and *35S:LBD29-GFP* lines. Four regions of the *LAX3* promoter were tested by ChIP-qPCR for LBD29-GFP enrichment (Fig. 5A). All four regions exhibited enrichment (∼5-fold) but in a fragment spanning −387 to −192 bp from the start codon of the *LAX3* promoter, LBD29-GFP enrichment was enriched

∼25-fold compared with the WT control (Fig. 5B). *In silico* sequence analysis identified 10 LBD/AS2 family binding motifs (Husbands et al., 2007) in the *LAX3* promoter (Table S2). Three of these motifs occurred within the fragment that exhibited the highest enrichment for LBD29-GFP using ChIP-qPCR (Fig. 5B). Our ChIP-based results suggest that LBD29 directly binds to the *LAX3* promoter *in planta*, presumably activating its expression.

To validate that LBD29 functions as a transcriptional activator for *LAX3* expression, we tested the ability of LBD29 to transactivate a LUC-Trap reporter (Lau et al., 2011) fused to the *LAX3* promoter. The *pLAX3:LUC* plasmid was co-expressed in protoplasts derived from tobacco suspension cells with other test plasmids and imaged using the dual luciferase transient expression assay. A two-fold increase in relative luciferase activity was detected when the *pLAX3: LUC* reporter was co-transfected with the *LBD29* effector plasmid (Fig. 5C) but not with the *ARF16* control (Fig. 5C). Our transient expression data suggest that LBD29 can operate as a positive transcriptional regulator of *LAX3* expression.

We reasoned that if LBD29 positively regulates *LAX3* expression, an LBD29 overexpression line is likely to cause overexpression of a LAX3 reporter even in the absence of exogenous auxin. To test this, we crossed the *pLAX3:LAX3-YFP* reporter (Swarup et al., 2008) with the overexpression line of LBD29 (*35S:LBD29-GFP*) (Okushima et al., 2007). Lines homozygous for both transgenes displayed ectopic expression of the LAX3-YFP reporter in both root

Fig. 5. **LBD29 directly regulates *LAX3* expression.** (A) Black triangles indicate LBD binding motif positions on *LAX3* promoter as predicted by AthaMap (Steffens et al., 2004). *LAX3* promoter fragments 1 to 4 are also displayed with their relative start and end nucleotides from the start codon (ATG). (B) ChIP was performed on the wild type (Col-0) and *LBD29* overexpressing line (35S:LBD29-GFP). Data shown are qPCR quantification of each DNA fragment. Relative enrichments of LBD29-GFP proteins were analyzed at four regions of the *LAX3* promoter. Transgenic roots of the *LBD29-O^{ex}* line were analyzed by ChIP using anti-GFP antibodies. Col-0 was used as negative controls (black rectangular). Values were normalized to internal controls (relative to input and to TUB2). Data represent the mean±s.e. of four technical replicates and two biological replicates were performed. (C) Relative luciferase (LUC) intensity is shown for each protoplast assay in control, 35S:LDB29 and 35S:ARF16. Transactivation with the reporter construct (pLAX3::fLUC), the effector constructs (35S::LBD29 and 35S::ARF16), the internal standard (35S::rLUC) and the negative control (35S::GUS) were used in this assay. Induction is expressed relative to the normalized luciferase activity of the GUS (negative control). The data represent the mean value±s.e. of six measurements and the experiment was performed in triplicate. (D) Overexpression of LBD29 triggers ectopic LAX3-YFP expression in all parts of the root (left, middle confocal section and right, surface view) compared with wild-type control in Fig. 1. Cell walls are stained red with propidium iodide. Scale bars: 50 µm. *P<0.05 compared with control (Student's *t*-test).

cortical and epidermal cells in the absence of exogenous auxin application (Fig. 5D). Taken together, these results suggest that LBD29 positively regulates *LAX3* transcription.

LBD29 is expressed in the LRP and cells directly overlying the new organ

An earlier study investigating the spatial and temporal expression patterns of selected members of the LOB/AS2 gene family during root development reported (using GUS-based reporters) that *LBD29* is expressed in LRP (but not overlying) cells (Okushima et al., 2007). Given the large body of evidence we have assembled showing that LBD29 is required to bind directly to the *LAX3* promoter to activate its expression, we generated new fluorescence-

based *LBD29* transcriptional and translational reporter lines to address its binding ability.

We initially fused an ER-localized CFP reporter to the *LBD29* promoter sequence. Transgenic lines expressing the *pLBD29:erCFP* transcriptional reporter clearly exhibited a CFP signal in new LRP plus a small number of cells directly overlying new primordia (Fig. 6A,B). Roots were clearly observed to express the *LBD29* driven erCFP reporter in cortical cells overlying Stage I/II LRP, coincident with *pLAX3:LAX3-YFP* expression first being detected (Fig. 1; Swarup et al., 2008). The *pLBD29:erCFP* transcriptional reporter was also clearly expressed in endodermal cells overlying LRP (Fig. 6A,B). However, no *LAX3* expression was detected in this tissue (Fig. 1; Swarup et al., 2008), suggesting that additional transcriptional repressor proteins may be required to impose the observed pattern of *LAX3* spatial expression.

A similar spatial expression pattern was observed after fusing the *LBD29* genomic sequence to a single copy of YFP (VENUS) and creating the *pLBD29:gLBD29:Venus* translational reporter line (Fig. 6C,D). The *pLBD29:gLBD29:Venus* reporter clearly exhibited a nuclear-localized YFP signal in a subset of cells directly overlying new LRP (Fig. 6C,D), consistent with *LBD29* encoding a transcription factor. In addition, temporal analysis of the *pLBD29:gLBD29:Venus* reporter line (Fig. 6) revealed that its induction preceded *LAX3* expression (Fig. 1B). Finally, RT-qPCR assays revealed that the *pLBD29:gLBD29:Venus* reporter line when used to complement the *lbd29-1* LR emergence defect (Fig. 4C) was also able to restore auxin-inducible LAX3 expression (Fig. S5). In
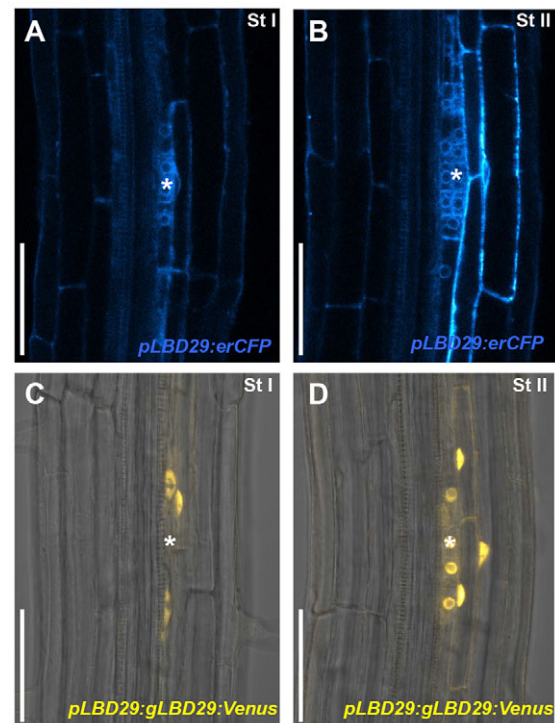
Fig. 6. ***LBD29* expression pattern during lateral root emergence.** (A,B) Laser-scanning confocal image of early stage lateral root primordia (LRP) in a transgenic line expressing a *LBD29* transcriptional reporter composed of its promoter fused to an endoplasmic reticulum-localised CFP reporter (*pLBD29:erCFP*). (C,D) Laser-scanning confocal image of early stage LRP in a transgenic line expressing a *LBD29* translational reporter composed of its promoter and genomic coding sequence fused to the Venus version of YFP (termed *pLBD29:gLBD29:Venus*). Scale bars: 50 µm. Asterisks indicate LRP at the stage denoted in each panel.

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summary, our results are consistent with the proposed role of LBD29 as a transcriptional regulator of *LAX3*.

DISCUSSION
LBD family members perform distinct regulatory roles during lateral root development
LBD/ASL genes encode a plant-specific family of transcription factors (Husbands et al., 2007) that have been implicated in a variety of developmental processes during leaf, flower and root morphogenesis (Iwakawa et al., 2002; Majer and Hochholdinger, 2011; Okushima et al., 2007, 2005; Soyano et al., 2008; Xu et al., 2008). In *Arabidopsis thaliana*, the LBD gene family contains 43 members, of which the *LOB* gene is the founding member (Shuai et al., 2002). *LOB* plays a role in organ separation and lateral organ development and encodes a transcription factor, expressed at boundaries within shoot tissues (Shuai et al., 2002). In roots, family members *LBD16*, *LBD18* and *LBD29* play important roles during LR development (Okushima et al., 2005, 2007; Lee et al., 2009; Lavenus et al., 2015; Fig. 7A). During LR initiation, LBD16 has been shown to play an important role promoting asymmetric cell division of LR founder cells, controlling polarized nuclear migration to the common cell pole between pairs of founder cells (Goh et al.,
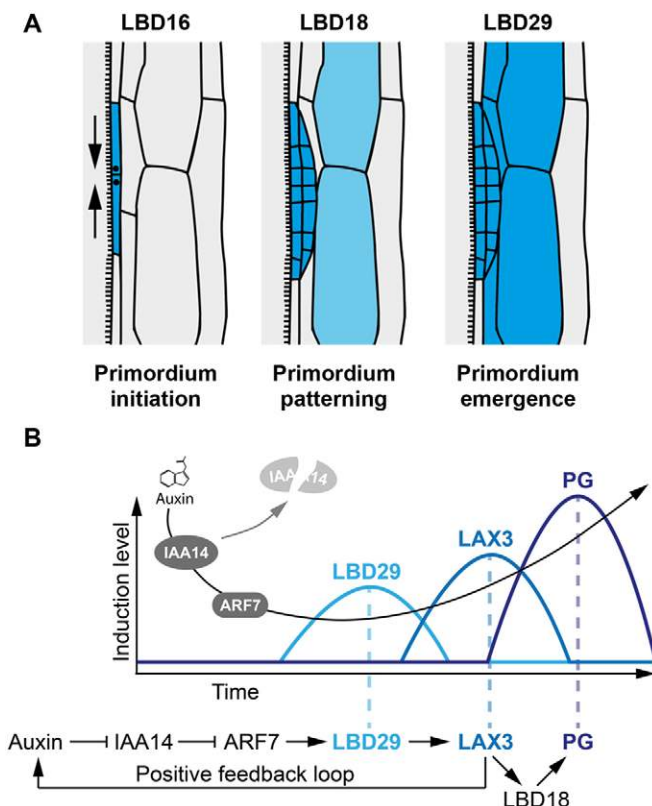

Fig. 7. LBD transcription factors control various stages of lateral root formation. (A) LBD16 is expressed early on during lateral root (LR) formation and controls LR initiation by acting on nuclear migration (arrows). LBD18 is expressed in the LR primordium and overlying tissues where it acts downstream of LAX3. We show here that LBD29 acts upstream of LAX3 to control LR emergence. (B) Temporal control of the LR emergence gene regulatory network. Auxin triggers the degradation of IAA14, which releases ARF7 so it can activate LDB29 (early gene). Consequently, LBD29 directly activates LAX3, which creates a positive feedback loop through its auxin influx activity. This allows for high auxin levels to induce cell wall-remodeling genes such as *PG* (late genes) and promotes cell separation that facilitates LR emergence.

2012). LBD18 and LBD33 positively regulate the cell cycle via the transcriptional regulation of *E2Fa* (Berckmans et al., 2011). LBD29 has also been reported to be involved in the regulation of the cell cycle progression during LR formation (Feng et al., 2012).

In this current study, we demonstrate that LBD29 plays a key role during LR emergence. Several other LBD genes have been linked with this developmental program. Like *lbd29-1*, the *lbd16-1* and *lbd18-1* single mutants also displayed a reduction in LR emergence, which was enhanced in the *lbd16-1 lbd18-1* double mutant (Lee et al., 2009; Okushima et al., 2007). *LBD18* has been demonstrated to function downstream of the auxin influx carrier *LAX3* during LR emergence (Lee et al., 2014). In addition, the triple mutant *lbd16-1 lbd18-1 lbd33-1* displayed a further reduction in the number of LRs emerged compared with any of the single or double mutants mentioned above (Goh et al., 2012). These observations may indicate the presence of functional redundancy between selected members of the LBD gene family. Alternately, it could reflect that these LBD genes play roles during distinct (but interacting) LR developmental processes such as organ initiation, patterning and emergence, which have additive phenotypic effects when disrupted in a multiple mutant background.

Any distinct regulatory functions that LBD family members play during LR development cannot be explained simply on the basis of each gene exhibiting contrasting spatio-temporal expression patterns (Fig. 7A). For example, whilst *LBD16* and *LBD29* are both expressed in pericycle cells prior to LR initiation (Goh et al., 2012; Fig. 6C), only transgenic lines expressing a LBD16-SRDX transcriptional repressor protein block the initial asymmetric cell division in LR founder cells (Goh et al., 2012), whereas *gLBD29-SRDX* lines are defective in organ emergence (Fig. 4). This suggests that LBD16 and LBD29 proteins target distinct sets of genes during LR development. Currently, the molecular basis of LBD16 and LBD29 target specificity is unclear. Several LBD proteins have been reported to interact with other family members or distinct classes of transcription factors that may help confer target specificity. For example, LBD18 and LBD33 dimerize to regulate cell cycle by activating *E2FA* transcription during LR initiation (Berckmans et al., 2011) and LBD6 (AtAS2) interacts with a MYB transcription factor AtAS1 during leaf development (Xu et al., 2003). In summary, LBD family members play key regulatory roles throughout plant development, including *LBD16*, *LBD18* and *LBD29*, which are critical for LR development.

Auxin-regulated lateral root emergence is dependent on *LBD29*
Auxin functions as a key regulatory signal during LR development (reviewed by Lavenus et al., 2013). In *Arabidopsis*, auxin alters the expression of large numbers (>2000) of genes during LR development via ARF transcription factors such as ARF7 and ARF19 (Okushima et al., 2005; Vanneste et al., 2005). ARF7 is particularly important as it functions to activate gene regulatory networks that control LR initiation, patterning and emergence (Lavenus et al., 2015; Swarup et al., 2008). ARF7 (and ARF19) regulates LR development (in part) by activating the expression of several auxin-inducible LBD family members (Okushima et al., 2005), as ectopic expression of *LBD16* and *LBD29* can partially rescue the *arf7 arf19* LR-less phenotype (Okushima et al., 2007). *LBD16* and *LBD29* represent key nodes within the LR gene regulatory network (GRN; Lavenus et al., 2015). ChIP-qPCR experiments have recently demonstrated that *LBD16* and *LBD29* represent direct targets for ARF7 binding and regulation (Lavenus et al., 2015).

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Whilst *LBD16* function was closely associated with the GRN controlling early stages of LR initiation (Goh et al., 2012; Lavenus et al., 2015), the role for *LBD29* had been less clear until our genetic studies employing *lbd29-1*, *pLBD29:gLBD29:Venus* rescue of *lbd29-1* and *gLBD29-SRDX* lines revealed that *LBD29* mediates auxin-regulated LR emergence (Fig. 4 and Fig. 7A). The current study has also revealed that LBD29 is essential for the auxin-inducible expression of the auxin influx carrier *LAX3* (Fig. 3 and Fig. 7B) by directly binding the *LAX3* promoter (Table S1; Fig. 5). Promoter deletion experiments show that a region between −570 and −363 is essential auxin induction by *LAX3* (Fig. 2, between Δ3 and Δ4). *In silico* analysis of the *LAX3* promoter sequence allowed the identification of a high number of LBD binding sites immediately downstream of this region (Fig. 5 and Table S2). Consistently, ChIP q-PCR experiments confirmed that LBD29 binds this region of the promoter, which triggers *LAX3* induction in response to auxin.

During LR emergence, cell separation in tissues overlying new organs requires auxin induction of cell wall-remodeling genes, such as PG that degrades pectin, leading to cell wall breakdown (Laskowski et al., 2006). Auxin-inducible PG expression in cells overlying LRP is dependent on the auxin influx carrier LAX3 (Swarup et al., 2008). The absence of *PG* expression following auxin treatment in the *lbd29-1* line (Fig. 3B) suggests that LBD29 controls *PG* expression via its regulation of *LAX3*. LBD18 has been reported to induce the expression of another cell wall-remodeling gene, *EXPANSIN 14* (*EXP14*), during LR emergence (Lee et al., 2012). Hence, both *LBD18* and *LBD29* regulate the induction of cell wall-remodeling enzymes that promote LR emergence. However, *LBD29* and *LBD18* function at distinct positions in the LR emergence GRN, upstream and downstream of *LAX3*, respectively (Fig. 7B; Lee et al., 2014).

Sequential induction of *LBD29* and *LAX3* by auxin is required for LR emergence

Delimiting *LAX3* expression to two adjacent cortical and epidermal cell files overlaying new LRP is crucial for ensuring auxin-regulated cell separation occurs solely along their shared walls (Swarup et al., 2008; Fig. 1B). To understand how *LAX3* spatio-temporal expression is regulated, Péret et al. (2013) developed a mathematical model that captures the network regulating its expression and auxin transport within realistic three-dimensional cell and tissue geometries. To prevent *LAX3* from being transiently expressed in multiple cell files overlying LRP, the model predicted that this auxin influx carrier must be induced by auxin after an auxin efflux carrier (later demonstrated to be encoded by *PIN3*; Péret et al., 2013). Hence, *PIN3* and *LAX3* expression would need to be sequentially induced by auxin. Given that *PIN3* is regulated as a primary auxin-responsive gene, *LAX3* would be required to be induced as a secondary auxin-responsive gene. ARF7 regulating *LAX3* auxin-inducible expression via an intermediary transcriptional factor (*LBD29*) would fulfill this temporal regulatory requirement. Hence, the sequential induction of *LBD29* and *LAX3* by auxin (Fig. 7B) represents an important regulatory motif within the LR GRN that is required to coordinate cell separation and organ emergence.

MATERIALS AND METHODS
Plant materials and growth conditions
The *Arabidopsis* ecotype Columbia (Col-0) was used as the wild type in all experiments. The *35S:LBD29-GFP* in the *arf7 arf19* mutant background, *arf7* (*nph4-1*), *arf19-1*, *lbd29* and *lax3* mutants have been previously described (Okushima et al., 2007, 2005; Feng et al., 2012; Swarup et al., 2008). The genomic LBD29-SRDX lines in Col-0 background (gLBD29-SRDX 1.3 and 2.1) were produced as described in Goh et al. (2012). Seeds were surface sterilized and plated on 0.5× MS medium (Sigma), 1% bacto-agar (Appleton Woods). Seeds were stratified at 4°C overnight and grown vertically (22°C, continuous light). Medium was supplemented either with 1 µM IAA (indole-3-acetic acid) (Sigma) or 10 µM cycloheximide (CHX) (VWR International).

Expression analysis using RT-qPCR
RNA extractions, reverse transcription and quantitative PCR were performed as previously described (Péret et al., 2013). Primer sequences for *LAX3* and *PG* have also been described (Péret et al., 2013).

Root phenotyping analysis
Three-day-old seedlings grown on vertical plates were subjected to 90° gravistimulations for 18 and 42 h (Voß et al., 2015). In addition, 10-day-old seedlings grown vertically were harvested to analyze the developmental stages of LR primordia. In this phenotypic study, the total number and stages of LR primordia were counted and determined as described by Malamy and Benfey (1997). Root length was measured using ImageJ (ImageJ 1.40 g).

Histochemical analysis and microscopy
GUS staining and clearing was done as previously described (Péret et al., 2013). Confocal microscopy was performed using a Leica SP5 confocal laser-scanning microscope (Leica Microsystems). Cell walls were stained using propidium iodide (Sigma) (10 µg/ml) for 2 min.

Yeast one-hybrid assays
The experiments were performed as described in Gaudinier et al. (2011). Interactions were called for TFs that activated at least one reporter assay.

Chromatin immunoprecipitation and quantitative PCR
Chromatin immunoprecipitation (ChIP) and subsequent quantitative PCR (input DNA dilution 1000×) were performed as previously described (Lavenus et al., 2015). Primers were designed to amplify 150-200 bp fragments and are listed in Table S3. Relative enrichment of the target region was normalized against TUB3 (TUBULIN BETA CHAIN 3, AT5g62700). Relative enrichments of LBD29-GFP proteins were analyzed at four regions of the *LAX3* promoter. Transgenic roots of the *LBD29-O^{ex}* line were analyzed by ChIP using anti-GFP antibodies. Values were normalized to internal controls (relative to input and to TUB2). Data represent the mean± s.e. of four technical replicates, and two biological replicates were performed.

Transient expression assays
Transient expression assay was performed on protoplasts as previously described (Bielach et al., 2012). Protoplasts were co-transfected with 1 µg reporter plasmid containing the luciferase (*LUC*) reporter gene, 1 µg plasmid effector and 2 µg normalization construct expressing *Renilla LUC* gene (De Sutter et al., 2005). Firefly luciferase (*fLUC*) activity values were normalized with the luciferase activity derived from the internal standard plasmid coding for the *Renilla* luciferase (*rLUC*) gene under the control of 35S CaMV promoter. Both luciferase activity were measured subsequently on a Synergy H1 with double injector (Biotek). The mean value (±s.e.) was calculated from six measurements on three independent experiments.

Cloning for luciferase and Y1H assays
For the luciferase assays, a genomic DNA sequence corresponding to 1374 bp upstream of the start codon of the *LAX3* gene was isolated and then amplified using the forward and reverse primers 5′-ATAAAT<u>CTGCAGA</u>-GTCATGATCCTTTT-3′ and 5′-TCTTTAAATAGA<u>CCATGG</u>AAAAGC-TTTTTC-3′ containing *Pst*I and *Nco*I sites, respectively, and ligated into a LucTrap vector (Lau et al., 2011) to generate luciferase fusion. The coding sequence of LBD29 was amplified to introduce *Hin*dIII and

*Bam*HI restriction sites at each extremity respectively (5′-ATCAAGCTT-ATGACTAGTTCCAGCT CTAGCTCT-3′ and 5′-GATGGATCCATATC-ACGAGAAGGAGATGTAGCC-3′) and subsequently cloned into pJIT60 vector to generate the plasmid effector (Schwechheimer et al., 1998). Full-length *ARF16* cDNA was introduced to the pJIT60 vector, using *Bam*HI and *Eco*RI restriction sites (5′-AAAACGGGATCCAAAAATATGATAAAT-GTGATGAATCCA-3′ and 5′-AAAGAATTCGCCAAGTTATACTACA-ACGCTCTCACT-3′). pJIT60 vector contained a double cauliflower mosaic virus 35S promoter.

Chimeric constructs were also created for the Y1H system. Genomic DNA from Col-0 was used to amplify 1422 bp of the *LAX3* promoter, just upstream of the translational start codon (5′-TTCTGCTTTTTGAATATT-ACACCATT-3′ and 5′-TTTTCTCTTCTTCTCTCAGTTTCTTTAGC-3′) and was cloned into pENTR 5′-TOPO TA vector (Invitrogen). The correct clone was recombined with pMW2 (HIS3 reporter vector) and pMW3 (*lacZ* reporter vector) (Brady et al., 2011) using LR clonase II. Because the stele-expressed transcription factor collection (Gaudinier et al., 2011) did not contain LBD29 transcription factor, a construct was generated to clone the *LBD29* coding sequence into the pDest-AD-2 µ plasmid. The cDNA sequence of *LBD29* was amplified (5′-CACCATGACTAGTTCCAGCTC-3′ and 5′-CGAGAAGGAGATGTAGCCAAAATT-3′) and cloned into the pENTR-D-Topo entry vector (Invitrogen). The entry vector was used in a gateway LR cloning reaction (LR clonase II; Invitrogen) with pDest-AD-2 µ (Gaudinier et al., 2011) to create a GAL4-activation domain fusion Y1H prey vector.

Cloning for promoter deletions and IVMs

The *LAX3* promoter was cloned from pENTR11-LAX3-YFP (Swarup et al., 2008) into pBluescript KM+ (Invitrogen) using unique *Kpn*I and *Spe*I restriction sites. The plasmid was PCR amplified using primers Lx3-25, 5′-TTTCTAAGAAATTAGTGGGTTAAATAAAGC-3′ and Lx3-26, 5′-AGTCTCCTTTTTAGCCCCATGCTTTTACAATGG-3′, which were designed to modify a single nucleotide within the auxin-response element (GAGACA to GAGACT). PCR amplification was carried out using *Pfx* proofreading DNA polymerase. Purified PCR products were digested with *Dpn*I, treated with T4 polynucleotide kinase (NEB) and ligated with T4 ligase (NEB). Point-mutated promoters were cloned back into pENTR11-LAX3-YFP and sequenced to check no other mutations were created during the PCR.

For PCR-generated promoter deletions, a combination of primers Lx3-R2, 5′-TTCTAAGTAATTCCCTGCGACC-3′ and (*Kpn*I)-Lx3-22, 5′-CCGGTACCTTTCTAAGAAATTAGTGGGTTA-3′ for Δ2 and (*Kpn*I)-Lx3-23, 5′-CCGGTACCAATATGTTTTATTCATTGTTTC-3′ for Δ4 were used. PCR amplification was carried out using *Pfx* proofreading DNA polymerase. Purified PCR products were digested with *Dpn*I and cloned into pENTR11-LAX3-YFP using *Kpn*I and *Spe*I restriction sites.

For deletions generated using restriction enzymes, *Kpn*I and *Mun*I for Δ1 or only *Bam*HI for Δ3 were used. The correct band was gel purified, if necessary 3′ overhang was filled using T4 DNA polymerase (NEB) and both fragments ligated using T4 ligase. Constructs were then cloned in the binary pGWB7 vector using the Gateway LR reaction (Invitrogen).

Author contributions

S.P., A.L., Y.D., A.G., T.G., R.S., A.B., J.L., I.C., K.H., K.S., E.B., H.F., S.M.B., B.S., B.P. and M.J.B. conceived, designed and performed experiments. S.P., A.L., B.P. and M.J.B. wrote the paper.

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