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

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SUMMARY STATEMENT

The transcription factor LBD29 regulates the induction of the auxin transporter LAX3 during emergence of lateral roots in order to fine-tune its temporal expression pattern and cell separation.

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 critical to ensure auxin-regulated cell separation occurs solely along their shared walls. Multiscale modeling has predicted 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 LAX3 auxin inducible expression is regulated indirectly by the AUXIN RESPONSE FACTOR ARF7. Yeast-1-hybrid screens revealed 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.
INTRODUCTION

Lateral root emergence represents a critical developmental program enabling new primordia to breach the overlying endodermal, cortical and epidermal cell layers and enter the 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 primordia (LRP) originates. The hormone signal auxin and several of its signaling and transport components have been demonstrated to play a critical 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 (ARF) 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 arf7arf19 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 a set of cell wall remodeling genes expression, 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 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 up-regulation 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 lateral root 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 LEAVES2-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 lateral root 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 LAX3 mRNA abundance 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 wildtype (WT) and arf7 mutant roots exposed to external auxin (1uM IAA) for varying lengths of time. Transcript profiling revealed that auxin induction of LAX3 mRNAs 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 critical 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 (-939bp), transcript profiling of auxin treated WT roots revealed that up-regulation of LAX3 mRNA was first detected six hours 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 (-939bp) AuxRE motif and 2 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 wildtype *pLAX3:LAX3-YFP* reporter, both IVM1 and IVM2 promoter transgene variants retain their ability to rescue lateral root 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 kbp *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 then 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* lateral root phenotype. All Δ1 and Δ2 promoter deletion lines fully complemented the *aux1 lax3* lateral root defect (Fig. 2B) and retained auxin inducible expression (Fig. 2 C and D). This result demonstrates that the 826 base pairs 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 lateral root emergence. In contrast, all Δ3 promoter deletion lines only partially complemented the *aux1 lax3* lateral root phenotype whereas no complementation was observed for 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 and 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 lateral root 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 transcription factor (TF) collection (Gaudinier et al., 2011) containing >650 genes were 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 transcriptional 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 LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES2-Like (LBD/ASL), 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 lateral root 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 to wildtype) LBD29 auxin inducible expression (Fig. S3B and C). Interestingly, LAX3 mRNA abundance was no longer auxin inducible in the lbd29-1 insertion line compared to 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 wildtype, LAX3-YFP is constitutively expressed in central root stele tissues, plus a small number of cortical cells (and later in epidermal cells) overlying new lateral root primordia (Fig. 3C and 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 and J) compared to WT (Fig. 3G and 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 specifically expressed at sites of LR emergence (Swarup et al., 2008). Consistent with a role for LBD29 mediating up-regulation of LAX3, lbd29-1 roots also failed to show induced PG expression following auxin treatment in contrast to WT (Fig. 3B). Hence, LBD29 appears to not only control auxin up-regulation of LAX3 in outer root tissues but also (indirectly) impacts expression of LAX3-dependent downstream targets of the lateral root emergence gene regulatory pathway.

**LBD29 facilitates lateral root emergence**

Transcript profiling and reporter studies have demonstrated that LBD29 is critical 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 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, 18h after a gravistimulation WT root bends contain mainly stage II LRP, whereas by 42h many primordia were close to emergence (stage VI or VII) or already emerged (stage e; Fig. 4). In the case of lax3, 18 hours after a gravity stimulus, mutant roots displayed proportionately more stage I primordia compared to WT (Fig. 4A). However, the biggest phenotypic difference was observed 42h after the gravistimulus, since no emerged LR were detected in lax3 compared to 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 to 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 lateral root emergence, we produced a transgenic line expressing the LBD29 genomic sequence fused to the SRDX transcriptional repressor domain in wildtype (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 2 independent gLBD29-SRDX lines both exhibited delayed LR development and featuring a higher proportion of stage V primordia versus wildtype comparable to 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 lateral root 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 exhibited full restoration of the wild-type lateral root 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 lateral root phenotype in 10-day-old seedlings. This revealed that the number of emerged LR was reduced in the *lbd29-1* insertion line (Fig. S4A, B and C) but that neither the stages of LR primordia distribution (Fig. S4D) nor the total number of LR primordia (Fig. S4E) were affected in *lbd29-1* compared to WT. Taken altogether, these results suggest that the reduced number of emerged LR in *lbd29-1* insertion line is not related to a defect in lateral root initiation nor primordia development, but is due to slower organ emergence.

**LBD29 positively regulates LAX3 expression directly**

Several independent lines of evidence reported in this paper suggest that LBD29 regulates lateral root 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) PCR analysis using an anti-GFP antibody on nuclear samples prepared from WT (Col-0) (as negative control) and *35S:LBD29-GFP* lines. Four regions of the LAX3 promoter were tested by ChIP-PCR for LBD29-GFP enrichment (Fig. 5A). All four regions exhibited enrichment (~5 fold) but in a fragment spanning -387 to -192bp from the start codon of the LAX3 promoter, LBD29-GFP enrichment was enriched ~25 fold compared to the WT control (Fig. 5B). *In silico* sequence analysis identified 10 LOB/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-PCR (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 suggests that LBD29 can operate as a positive transcriptional regulator of LAX3 expression.

We reasoned that if LBD29 positively regulates LAX3 expression, an LBD29 over expression line is likely to cause over-expression 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 over-expression 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 cortical and epidermal cells in the absence of exogenous auxin application (Fig. 5D). Taken together, these results are consistent with 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 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-localised 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 \textit{pLAX3:LAX3-YFP} expression first being detected (Fig. 1; Swarup et al., 2008). The \textit{pLBD29:erCFP} transcriptional reporter was also clearly expressed in endodermal cells overlying LRP (Fig. 6A and B). However, no \textit{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 \textit{LAX3} spatial expression.

A similar spatial expression pattern was observed after fusing the \textit{LBD29} genomic sequence to a single copy of YFP (VENUS) and creating the \textit{pLBD29:gLBD29:Venus} translational reporter line (Fig. 6C and D). The \textit{pLBD29:gLBD29:Venus} reporter clearly exhibited a nuclear localized YFP signal in a subset of cells directly overlying new LRP (Fig. 6C and D), consistent with \textit{LBD29} encoding a transcription factor. In addition, temporal analysis of the \textit{pLBD29:gLBD29:Venus} reporter line (Fig. 6) revealed that its induction preceded \textit{LAX3} expression (Fig. 1B). Finally, RT-qPCR assays revealed that the \textit{pLBD29:gLBD29:Venus} reporter line when used to complement the \textit{lbd29-1} LR emergence defect (Fig. 4C) was also able to restore auxin-inducible \textit{LAX3} expression (Fig. S5). In summary, our results are consistent with the proposed role of \textit{LBD29} as a transcriptional regulator of \textit{LAX3}.

**DISCUSSION**

\textit{LBD} family members perform distinct regulatory roles during lateral root development

\textit{Lateral Organ Boundaries-Domain/Asymmetric Leaves2-Like} (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; Okushima et al., 2005; Soyano et al., 2008; Xu et al., 2008). In \textit{Arabidopsis thaliana}, the LBD gene family contains 43 members, of which the \textit{LOB} gene is the founding member (Shuai et al., 2002). The \textit{LOB} gene 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 \textit{LBD16}, \textit{LBD18} and \textit{LBD29} play important roles during lateral root development (Okushima et al., 2005; Okushima et al., 2007; Lee et al., 2009; Lavenus et al., 2015; Fig. 7A). During lateral root 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., 2012). LBD18 and LBD33 positively regulate the cell cycle via the transcriptional regulation of \textit{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 lateral root emergence. Several other \textit{LBD} genes have been linked with this developmental program. Like \textit{lbd29-1}, the \textit{lbd16-1} and \textit{lbd18-1} single mutants also displayed a reduction in LR emergence, which was enhanced in the \textit{lbd16-1 lbd18-1} double mutant (Lee et al., 2009; Okushima et al., 2007). \textit{LBD18} has been demonstrated to function downstream of the auxin influx carrier \textit{LAX3} during lateral root emergence (Lee et al, 2014). In addition, the triple mutant \textit{lbd16-1 lbd18-1 lbd33-1} displayed a further reduction in the number of LR emerged compared to 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 \textit{LBD} gene family. Alternately, it could reflect that these \textit{LBD} genes play roles during distinct (but interacting) lateral root 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 \textit{LBD} family members play during lateral root development cannot be explained simply on the basis of each gene exhibiting contrasting spatio-temporal expression patterns (Fig. 7A). For example, whilst \textit{LBD16} and \textit{LBD29} are both expressed in pericycle cells prior to lateral root 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 lateral root 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 lateral root 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 that are critical for lateral root development.

**Auxin regulated lateral root emergence is dependent on LBD29**

Auxin functions as a key regulatory signal during lateral root development (reviewed by Lavenus et al., 2013). In *Arabidopsis* auxin alters the expression of large numbers (>2000) of genes during lateral root 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 lateral root initiation, patterning and emergence (Lavenus et al., 2015; Swarup et al, 2008). ARF7 (and ARF19) regulates lateral root 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 lateral root gene regulatory network (GRN; Lavenus et al., 2015). ChIP-PCR experiments have recently demonstrated that LBD16 and LBD29 represent direct targets for ARF7 binding and regulation (Lavenus et al., 2015).
Whilst *LBD16* function was closely associated with the GRN controlling early stages of lateral root initiation (Goh et al., 2012; Lavenus et al., 2015), the role for *LBD29* has 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 lateral root emergence (Fig. 4 and 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 7B) by directly binding the *LAX3* promoter (Table S1; Fig. 5). Promoter deletion experiments show that a region between -570 and -363 is essential for *LAX3* auxin induction (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 *Expansin14* during lateral root emergence (Lee et al., 2012). Hence, both *LBD18* and *LBD29* regulate the induction of cell wall-remodeling enzymes that promote lateral root emergence. However, *LBD29* and *LBD18* function at distinct positions in the lateral root 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 critical 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 the PIN3 gene; 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; Okushima et al., 2005; Feng et al., 2012; Swarup et al., 2008). The genomicLBD29-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 ½ 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 Ltd).

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 90º gravistimulations for 18 and 42 hours (Voß et al., 2015). In addition, 10-day-old seedlings grown vertically were harvested to analyze the developmental stages of lateral root primordia. In this phenotypic study, the total number and stages of lateral root primordia were counted and determined as described by (Malamy and Benfey, 1997). Root length was measured using ImageJ (ImageJ 1.40g).

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) (10µg/ml) for 2 minutes.

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 1000x) 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 CHAIN3, AT5g62700). Relative enrichments
of LBD29-GFP proteins were analyzed at four regions of the LAX3 promoter. Transgenic roots of the LBD29 Oex 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 ± standard error 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 of a reporter plasmid containing the Luciferase (LUC) reporter gene, 1 µg of plasmid effector and 2 µg of 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 (±se) 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′- ATAAATCTGCAGAGTCATGATCCTTTT -3′ and 5′- TCTTTAAATAGACCATGGAAAAGCTTTTTTC -3′ containing Pst1 and Nco1 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 HindIII and BamHI restriction sites at each extremity respectively (5′- ATCAAGCTTATGACTAGTTCCAGCTCTAGCTCT -3′ and 5′- GATGGATCCATATCACGAGAAGGAGATGTAGCC-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 BamHI and EcoR1 restriction sites. (5′- AAAACGGGATCCAAAAATATGATAAATGTGATGAATCCA-3′ and 5′-
AAAGAATTCGCCAAGTTATACTACAACGCTCTC-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 1422bp of the LAX3 promoter, just upstream of the translational start codon (5’-TTCTGCTTTTTGAATATTACACCATT-3’ and 5’-TTTTCTCTCTCTCTCAGTTTCTTTAGC-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-2µ 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-2µ (Gaudinier et al., 2011) to create a GAL4-activation domain fusion Y1H prey vector.

Cloning for promoter deletions and IVMs

LAX3 promoter was cloned from pENTR11-LAX3-YFP (Swarup et al., 2008) into pBluescript KM+ (Invitrogen) using unique KpnI and SpeI restriction sites. The plasmid was PCR amplified using primers Lx3-25 5’-TTTCTAAGAAATTAGGTTTTTTTTTTTAAATG-3’ and Lx3-26 5’-AGTCTCCTTTTTAGCCCCATGCTTTTTACAATGG-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 DpnI, 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’-TTCTAAGTAATTCCTGCGACC-3’ and (KpnI)-Lx3-22 5’-CCGGTACCTTTCTAAGAAATTAGTGGGT TA-3’ for ∆2 and (KpnI)-Lx3-23 5’-CCGGTACCAATATGTTTTATTCATTGTTTC-3’ for ∆4 were used. PCR amplification was carried out using Pfx proofreading DNA Polymerase. Purified PCR products were digested with DpnI and cloned into pENTR11-LAX3-YFP using KpnI and SpeI restrictions sites.

For deletions generated using restriction enzymes, KpnI and MunI for ∆1 or only BamH1 for ∆3 were used. The correct band was gel purified, if necessary 3’ overhang were 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).
Competing interests

No competing interests declared.

Author contributions


Funding

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References


Fig. 1. LAX3 induction in the outer tissue during lateral root emergence is ARF7 dependent.

(A) Lateral root formation proceeds in 8 developmental stages (St I to VIII, Casimiro et al., 2003).

(B) A functional LAX3-YFP fusion is used to monitor LAX3 accumulation in the overlaying tissues during lateral root emergence. Bar is 50µm. (C) LAX3 induction by auxin (1µM IAA) was monitored in wild-type (Col-0) compared to arf7 mutant by qPCR. Data represent the mean ± standard error of four technical replicates and three biological replicates were performed.
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 -939bp is indicated (complementary strand of the TGTCTC sequence described by Ulmasov et al., 1997).

(B) Lateral root density measurements (number of lateral roots per length of primary root). Error bars are SE of the mean (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 4 strips out of 2 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 (1µM IAA for 16h) conditions. Bar is 100µm.
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 during 0, 1, 3, 6, 8, 10, 18 and 24 hours (a-b). LAX3 (A) and PG (B) mRNA levels were quantified by RT-qPCR. Data represent the mean value ± standard errors 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 (1μM IAA) in wild-type (Col-0) and mutant (lbd29-1). Bars are 50μm. Asterisks indicate a lateral root primordium. ep: epidermis, c: cortex, en: endodermis, st: stele. Data represent the mean ± standard error of four technical replicates and three biological replicates were performed.
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 during 18h and 42h. Compared to 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 lbd29 mutant
(C). Data shown are percentage and the error bars represent standard error, 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.
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) Chromatin Immuno Precipitation 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 Ox 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 ± standard error 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 (Control = 35S::GUS) were used in this assay. Induction is expressed relative to the normalized luciferase activity of the GUS = negative control. Asterisk shows significant difference for LBD29 compared to the control (p<0.05, Student’s t-test). The data represent the mean value ± standard error of 6 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 to wild-type control in Fig. 1. Bars are 50µm. Asterisks show significant difference for LBD29 compared to the control (p<0.05, Student’s t-test).
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 (termed *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*). Bars are 50µm. Asterisks indicate LRP at the stage denoted in each panel.
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. LBD18 is expressed in the LR primordium and overalying 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 remodelling genes such as PG (late genes) and promotes cell separation that facilitates LR emergence.
Fig. S1. *LAX3* is an ARF7-dependent and secondary auxin responsive gene. (A-B) *LAX3* is expressed in the outer tissues in front of the LRP in the non-treated (NT) condition and induced in all outer tissues upon auxin (1µM IAA for 16h) treatment. (A) Transcriptional *LAX3-GUS* fusion in wild-type (Col-0) and mutant (*arf7*) backgrounds. (B) Translational *LAX3-YFP* fusion in wild-type (Col-0) and mutant (*arf7*) backgrounds. Bars are 100µm. Asterisks indicate a lateral root primordium. (C) Decomposition of the transcriptional response of *LAX3* to auxin into four independent components (shown in log₂ fold changes). This was obtained by mathematical analysis of the *LAX3* transcript levels monitored by genechip from *pARF7:ARF7-GR arf7 arf19* root tissues treated with 1 µM auxin (NAA) and any combination of 2 µM dexamethasone (DEX) and 10 µM cycloheximide (CHX) for 4 h. Domain A corresponds to DEX and CHX, domain B to DEX only, domain C to CHX only and domain D to mock treatment (0.1% DMSO). All domains were treated with auxin. DEX treatments activates ARF7-GR by allowing its targeting to the nucleus and CHX treatment blocks protein synthesis.
Fig. S2. *LAX3* induction by auxin does not depend on the canonical AuxRE in its promoter.

(A) *LAX3* promoter contains a single canonical (TGTCTC on reverse strand) Auxin Response Element (AuxRE) situated at -939bp from start codon (ATG). Two independent mutated versions of *LAX3* promoter were generated by In Vitro Mutagenesis (IVM1 and IVM2). (B) Expressing *LAX3*-YFP under the control of the full promoter (LAX3YFP) or the mutated version (IVM1 and IVM2, 2 independent transgenic lines each) fully restores the lateral root phenotype of the *aux1 lax3* mutant (n≥8). (C) The mutated version of the promoter still allows the induction of *LAX3*-YFP in response to auxin (1µM IAA for 16h) compared to non-treated (NT) condition. Bar is 100µm.
Fig. S3. *ldb29-1* SALK insertion line Salk_071133 displays a loss in *LBD29* auxin induction. (A) T-DNA is inserted in the 3’ region of the promoter. (B) This results in an increased basal level of *LBD29* expression compared to wild-type (WT) control. (C) *LBD29* induction by auxin (1µM IAA) is dramatically reduced in the *ldb29*-1 mutant compared to wild-type control.
Fig. S4. *ldb29-1* displays a reduced level of lateral root emergence but no initiation defect.

(A) Emerged lateral root number of the wild-type (Col-0) and *ldb29-1* mutant. (B) Lateral root primordium stages in the wild-type (Col-0) and *ldb29-1* mutant. (C) Total number of initiation events (emerged and non-emerged) in the wild-type (Col-0) and *ldb29-1* mutant. Values are mean (n=20 roots) and error bars are SE of the mean.
Fig. S5. RT-qPCR demonstrates that pLBD29::LBD29-Venus can rescue LBD29 and LAX3 auxin inducible expression in lbd29-1. 5 day old Arabidopsis seedlings were treated for 5 hours with 1µM IAA. RNA was extracted from root tissues (n>100) and cDNA prepared. Transcript abundance of vYFP (A), LBD29 (B) and LAX3 (C) genes was then checked using RT-qPCR in four technical replicates in either lbd29-1, lbd29-1 complemented with pLBD29::LBD29-vYFP and wild-type (Col-0) expressing the pLBD29::LBD29-vYFP reporter as the positive control. Primer details are provided in Table S4.
Table S1  List of putative candidate transcription factors regulating LAX3 expression identified by yeast one-hybrid.

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Table S2 *In silico* LAX3 promoter analysis reveals the existence of 10 putative LBD binding sites. The online tool AthaMap (http://www.athamap.de/) was used to analysis LAX3 promoter up to 2000 bp from ATG. Results of the analysis are given in the table, with additional sequence information for each motif.

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Table S3 Primer sequences used for the ChIP experiment. LAX3-4 corresponds to -150 to +17bp promoter fragment, LAX3-3 corresponds to -387 to -192bp, LAX3-2 is equivalent to -734 to -574bp, and LAX3-1 to -1058 to -850bp.

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Table S4 Primer sequences used for qRT-PCR experiments (Figure S5).

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