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

RUNNING TITLE: *LBD29* **and lateral root emergence**

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1   **SUMMARY STATEMENT**

2   The transcription factor LBD29 regulates the induction of the auxin transporter LAX3 during

3   emergence of lateral roots in order to fine-tune its temporal expression pattern and cell separation.

4

5   **ABSTRACT**

6   Lateral root primordia (LRP) originate from pericycle stem cells located deep within parental

7   root tissues. LRP emerge through overlying root tissues by inducing auxin-dependent cell

8   separation and hydraulic changes in adjacent cells. The auxin inducible auxin influx carrier

9   LAX3 plays a key role concentrating this signal in cells overlying LRP. Delimiting *LAX3*

10   expression to two adjacent cell files overlying new LRP is critical to ensure auxin-regulated

11   cell separation occurs solely along their shared walls. Multiscale modeling has predicted this

12   highly focused pattern of expression requires auxin to sequentially induce auxin efflux and

13   influx carriers *PIN3* and *LAX3*, respectively. Consistent with model predictions, we report that

14   *LAX3* auxin inducible expression is regulated *indirectly* by the AUXIN RESPONSE

15   FACTOR ARF7. Yeast-1-hybrid screens revealed the *LAX3* promoter is bound by the

16   transcription factor LBD29, which is a *direct* target for regulation by ARF7. Disrupting auxin

17   inducible *LBD29* expression or expressing an LBD29-SRDX transcriptional repressor

18   phenocopied the *lax3* mutant, resulting in delayed lateral root emergence. We conclude that

19   sequential *LBD29* and *LAX3* induction by auxin is required to coordinate cell separation and

20   organ emergence.

21   **INTRODUCTION**

22   Lateral root emergence represents a critical developmental program enabling new primordia

23   to breach the overlying endodermal, cortical and epidermal cell layers and enter the

24   surrounding soil environment (Péret et al., 2009; Fig. 1A). This process is tightly controlled in

25   order to limit damage to the parental root from which a new lateral root primordia (LRP)

26   originates. The hormone signal auxin and several of its signaling and transport components

27   have been demonstrated to play a critical role during LRP emergence (Swarup et al., 2008;

28   reviewed in Lavenus et al., 2013). Auxin has a specialized transport machinery, and its signal

29   triggers the degradation of AUXIN/INDOLE ACETIC ACID (AUX/IAA) proteins via the

30   SCF^{TIR1} complex (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). The degradation of

31   AUX/IAA proteins (that function as transcriptional repressors) allows interacting

32   transcriptional proteins termed AUXIN RESPONSE FACTORs (ARF) to change auxin

33   responsive gene expression in order to elicit developmental responses (Dharmasiri and

34   Estelle, 2004).

35   In the model plant *Arabidopsis thaliana*, LAX3, an auxin influx carrier belonging to the

36   *AUX/LAX* gene family, plays an important role during LR development, by facilitating the

37   emergence process (Péret et al., 2009; Swarup et al., 2008). Swarup et al. (2008) proposed a

38   model in which an increased level of auxin in the cortical cells overlying LR primordia,

39   induces *LAX3* expression by targeting the degradation of the IAA14/SLR repressor protein,

40   allowing ARF7 and ARF19 to activate auxin responsive genes. The *arf7arf19* double mutant

41   and the *iaa14/slr-1* gain of function mutant show impaired expression of *LAX3*, suggesting

42   that *LAX3* expression is mediated by the auxin signaling pathway module IAA14/SLR-ARF7-

43   ARF19 (Swarup et al., 2008). In the cortical cells, the increase of *LAX3* expression triggers a

44   positive-feedback loop stimulating further auxin uptake from LRP. The consequence of auxin

45   accumulation is the induction of a set of cell wall remodeling genes expression, such as

46   polygalacturonases and xyloglucan endotransglucosylase/hydrolase which are involved in

47   pectin polymer cleavage and cell wall loosening respectively (Laskowski et al., 2006; Swarup

48   et al., 2008). Consistent with the model that the LAX3 positive feedback loop is important for

49   emergence, the expression of these cell wall remodeling enzymes at the emergence site is

50   LAX3 dependent (Swarup et al., 2008). Hence, by inducing cell wall remodeling in overlying

51   cells and triggering their separation, the growing primordium is able to pass through the outer

52   root cell layers and emerge (Péret et al., 2009; Swarup et al., 2008).

53   *LAX3* is expressed in just two cell files overlaying new LRP (Swarup et al., 2008; Fig. 1B).

54   To understand how this striking pattern of *LAX3* expression is regulated, we developed a

55   mathematical model that captures the network regulating its expression and auxin transport

56   within realistic three-dimensional cell and tissue geometries (Péret et al., 2013). Our model

57   revealed that, for the *LAX3* spatial expression to be robust to natural variations in root tissue

58   geometry, the efflux carrier PIN3 is also required. To prevent *LAX3* from being transiently

59   expressed in multiple cell files, the model predicted that *PIN3* and *LAX3* genes must be

60   induced consecutively. Consistent with this prediction, the translational inhibitor

61   cycloheximide was observed to block auxin up-regulation of transcript abundance of *LAX3*

62   (but not PIN3; Péret et al., 2013). Hence, *LAX3* appears to function as a secondary (rather

63   than primary) auxin responsive gene. However, the underlying molecular basis of *LAX3*

64   induction by auxin remained unresolved.

65   In this study we initially describe that ARF7 is essential for auxin-dependent *LAX3* induction

66   during lateral root emergence. However, we demonstrate using several independent lines of

67   experimental evidence that ARF7 regulates LAX3 *indirectly*. Instead, we report that the

68   LATERAL   ORGAN   BOUNDARIES-DOMAIN/ASYMMETRIC   LEAVES2-Like

69   (LBD/ASL) transcription factor (TF) family member LBD29 functions as a *direct* positive

70   regulator of *LAX3* auxin-dependent expression. Our results position LBD29 at a key node

71   downstream of auxin and ARF7 in the lateral root emergence regulatory network.

72

73 **RESULTS**

74 **Transcription factor ARF7 regulates auxin inducible *LAX3* expression** *indirectly*

75 We initially addressed the ARF-dependent regulatory mechanisms controlling auxin inducible

76 *LAX3* expression. It was previously reported that *LAX3* mRNA abundance is reduced in an

77 *arf7* mutant background, suggesting that *LAX3* expression is dependent on this ARF

78 transcription factor (Okushima et al., 2007; Swarup et al., 2008). To test this, a RT-qPCR

79 based assay was designed to transcript profile *Arabidopsis* wildtype (WT) and *arf7* mutant

80 roots exposed to external auxin (1uM IAA) for varying lengths of time. Transcript profiling

81 revealed that auxin induction of *LAX3* mRNAs was abolished in the *arf7* mutant background

82 (Fig. 1C). Similarly, when *pLAX3:GUS* and *pLAX3:LAX3-YFP* reporters (Swarup et al., 2008)

83 were expressed in *arf7*, auxin induction of both reporters was abolished (Fig. S1AB). Hence,

84 ARF7 appears to be critical for auxin induction of *LAX3* expression.

85 We next addressed whether ARF7 regulated *LAX3* auxin induction *directly* or *indirectly.*

86 Whilst the *LAX3* promoter sequence contains a canonical ARF binding motif (-939bp),

87 transcript profiling of auxin treated WT roots revealed that up-regulation of *LAX3* mRNA was

88 first detected six hours after hormone induction (Fig. 1C). Induction of primary auxin

89 responsive transcripts is typically detected within minutes rather than hours, suggesting that

90 *LAX3* may be a secondary auxin responsive gene and hence *not* a direct target for ARF7

91 regulation (Péret et al, 2013; Mellor et al., 2015). This is consistent with bioinformatics

92 analysis of transcriptomic data from the *arf7 arf19* mutant complemented by a dexamethasone

93 (DEX) inducible ARF7-GLUCOCORTICOID RECEPTOR (GR) fusion protein under its

94 native *ARF7* promoter (as described in Lavenus et al., 2015) which categorized *LAX3* as a

95 positive indirect target of ARF7 (Fig. S1C).

96   To directly test the functional importance of the AuxRE motif within the *LAX3* promoter for

97   auxin induction and gene activity, we performed targeted *in vitro* mutagenesis studies. The

98   *LAX3* (-939bp) AuxRE motif and 2 mutant variants (termed IVM1 and IVM2; Fig. S2A) were

99   re-introduced (as part of a functional *pLAX3:LAX3-YFP* transgene) into an *aux1 lax3* mutant

100 background. Phenotypic analysis revealed that, like the wildtype *pLAX3:LAX3-YFP* reporter,

101 both IVM1 and IVM2 promoter transgene variants retain their ability to rescue lateral root

102 emergence in the *aux1 lax3* mutant background (Fig. S2B). Confocal imaging confirmed that

103 both IVM1 and IVM2 variants of the *pLAX3:LAX3-YFP* reporter also retained their ability to

104 be induced by auxin in root cortical cells (Fig. S2C).

105 To independently assess the role of the AuxRE versus other regulatory regions, a *LAX3*

106 promoter deletion approach was also employed (Fig. 2). The 1.98 kbp *LAX3* promoter was

107 truncated at four different positions (Fig. 2A; termed Δ1, Δ2, Δ3 and Δ4), to create

108 increasingly shorter promoter fragments, then fused to the *LAX3-YFP* reporter and then

109 transformed into the *aux1 lax3* double mutant. Multiple transgenic lines expressing each

110 *pLAX3:LAX3-YFP* promoter deletion were initially scored for complementation of the *aux1*

111 *lax3* lateral root phenotype. All Δ1 and Δ2 promoter deletion lines fully complemented the

112 *aux1 lax3* lateral root defect (Fig. 2B) and retained auxin inducible expression (Fig. 2C and

113 D). This result demonstrates that the 826 base pairs sequence upstream of the start of the

114 *LAX3* coding sequence (that does not contain the *AuxRE*) is sufficient to drive auxin inducible

115 expression and promote lateral root emergence. In contrast, all Δ3 promoter deletion lines

116 only   partially   complemented   the   *aux1   lax3*   lateral   root   phenotype   whereas   no

117 complementation   was   observed   for   any   of   the   Δ4   promoter   deletion   lines   (Fig.   2B).

118 Quantitative analysis of Δ3 and Δ4 reporter lines following auxin treatment gave similar

119   results (Fig. 2C and D). Hence, multiple independent lines of evidence suggest that the *AuxRE*

120   promoter element is not necessary for auxin inducible *LAX3* expression. Instead, other

121   regulatory motifs encoded closer to the start of the *LAX3* coding sequence appear to be

122   functionally important. We conclude that ARF7 regulates auxin inducible *LAX3* expression

123   *indirectly,* raising the question about which transcription factor(s) acts between ARF7 and

124   *LAX3* in the lateral root emergence gene regulatory network.

125

126   **Identification of putative *LAX3* transcriptional regulators**

127   In order to identify putative transcription factors that regulate *LAX3*, a yeast one-hybrid

128   (Y1H) assay was performed. As bait, the *LAX3* promoter (1374 bp) was fused to *LacZ* and

129   *HIS3* reporter genes. In the Y1H system, reporter gene expression is activated when a TF

130   interacts with the DNA bait. A root transcription factor (TF) collection (Gaudinier et al.,

131   2011) containing >650 genes were fused to a transcription activation domain (AD) and used

132   as a prey. A total of 17 root stele-expressed TFs were found to bind to the *LAX3* promoter

133   sequence (Table S1). These transcriptional factors included five homeodomain leucine zipper

134   (HD-Zip) proteins (HAT22, ATHB52, ATHB6, PHV/ATHB9, ATHB40), two zinc finger-

135   homeodomain (ZF-HD) proteins (HB21, HB30) and the LATERAL ORGAN

136   BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES2-Like (LBD/ASL), LBD29.

137   *LBD29* represents the most promising candidate identified in the Y1H screen for an

138   intermediary transcriptional regulator between ARF7 and *LAX3* based on several criteria.

139   First, *LBD29* expression is induced minutes after auxin treatment (Okushima et al., 2007).

140   Second, characteristic of many primary auxin response genes, *LBD29* expression is strongly

141   induced following cycloheximide (CHX) treatment (Okushima et al., 2007). Third, *LBD29*

142   expression can be induced in *pARF7:ARF7-GR/arf7 arf19* seedlings following treatment with

143   DEX plus auxin (Okushima et al., 2007). Fourth, ARF7 has been shown to bind to *LBD29*

144   promoter fragments containing *AuxRE* motifs using EMSA and ChIP-PCR techniques,

145   respectively (Okushima et al., 2007; Lavenus et al., 2015). Finally, overexpression of *LBD29*

146   can also partially restore lateral root formation in the *arf7 arf19* mutant background

147   (Okushima et al., 2007).

148   To directly test the functional importance of LBD29 for *LAX3* auxin inducible expression we

149   employed the *lbd29-1* T-DNA insertion line (SALK_071133). In this SALK line (the only

150   insertion line currently available for this gene) the T-DNA sequence is inserted in the *LBD29*

151   promoter region 3' of the nearest *AuxRE* to the transcription start site (Fig. S3A). Whilst the

152   position of the T-DNA insert does not disrupt the coding sequence, RT-qPCR analysis

153   revealed that despite a higher basal level of expression, it significantly attenuated (>10 fold

154   compared to wildtype) *LBD29* auxin inducible expression (Fig. S3B and C). Interestingly,

155   *LAX3* mRNA abundance was no longer auxin inducible in the *lbd29-1* insertion line compared

156   to WT (Fig. 3A), suggesting that this T-DNA allele attenuates the ability of LBD29 to

157   function as an auxin inducible positive regulator of *LAX3* expression. To verify this, the

158   *lbd29-1* insertion line was also crossed with the *pLAX3:LAX3:YFP* reporter (Swarup et al.,

159   2008). In wildtype, LAX3-YFP is constitutively expressed in central root stele tissues, plus a

160   small number of cortical cells (and later in epidermal cells) overlying new lateral root

161   primordia (Fig. 3C and D) (Swarup et al., 2008). However, in the *lbd29-1* mutant background

162   the *pLAX3:LAX3-YFP* reporter was no longer expressed in cortical cells overlying new

163   primordia (Fig. 3E-F). IAA treatment also could not induce ectopic expression of *LAX3* in the

164   *lbd29-1* mutant (Fig. 3I and J) compared to WT (Fig. 3G and H).

165   We next examined the effect of the *lbd29-1* allele on auxin-regulated genes controlled by

166   LAX3. For example, the *Polygalacturonase* (*PG*) gene is auxin and LAX3-dependent and

167   specifically expressed at sites of LR emergence (Swarup et al., 2008). Consistent with a role

168   for LBD29 mediating up-regulation of *LAX3*, *lbd29-1* roots also failed to show induced *PG*

169   expression following auxin treatment in contrast to WT (Fig. 3B). Hence, LBD29 appears to

170   not only control auxin up-regulation of *LAX3* in outer root tissues but also (indirectly) impacts

171   expression of LAX3-dependent downstream targets of the lateral root emergence gene

172   regulatory pathway.

173

174   **LBD29 facilitates lateral root emergence**

175   Transcript profiling and reporter studies have demonstrated that LBD29 is critical for auxin

176   inducible expression of *LAX3* and other genes involved in cell separation in cells overlying

177   new LRP (Fig. 3). Logically, as a regulator of *LAX3* expression (and its downstream targets),

178   disrupting the induction of LBD29 (in the case of the *lbd29-1* allele) should cause a *lax3-like*

179   mutant phenotype and delay LR emergence. To detect any change in *lbd29-1* LR emergence

180   rate, we employed a gravistimulation-based bioassay (Péret et al., 2012) which after

181   subjecting seedlings to a 90º gravity stimulus, leads to the highly synchronized temporal

182   development of a new primordium on the outer side of the root bend (Lucas et al., 2008). For

183   example, 18h after a gravistimulation WT root bends contain mainly stage II LRP, whereas by

184   42h many primordia were close to emergence (stage VI or VII) or already emerged (stage e;

185   Fig. 4). In the case of *lax3*, 18 hours after a gravity stimulus, mutant roots displayed

186   proportionately more stage I primordia compared to WT (Fig. 4A). However, the biggest

187   phenotypic difference was observed 42h after the gravistimulus, since no emerged LR were

188   detected in *lax3* compared to WT, and most primordia were still at earlier stage IV or V (Fig.

189   4A). Phenotypic analysis of the *lbd29-1* insertion line revealed an even greater delay in LR

190   development compared to *lax3*, featuring a higher proportion of stage IV and V primordia

191   (Fig. 4A), consistent with LBD29 acting upstream of LAX3 in the LR emergence regulatory

192   pathway.

193   To provide additional independent genetic evidence to probe the function of LBD29 during

194   lateral root emergence, we produced a transgenic line expressing the *LBD29* genomic

195   sequence fused to the SRDX transcriptional repressor domain in wildtype (Col-0)

196   background. The resulting gLBD29-SRDX fusion protein is designed to repress LBD29 target

197   genes by blocking their transcription, thereby mimicking an *lbd29* loss of function allele.

198   Phenotypic analysis of 2 independent gLBD29-SRDX lines both exhibited delayed LR

199   development and featuring a higher proportion of stage V primordia *versus* wildtype

200   comparable to *lbd29-1* (Fig. 4B), consistent with LBD29 acting as a positive regulator of the

201   LR emergence regulatory pathway. Further independent confirmation of the role of LBD29

202   during lateral root emergence was generated by expressing a translational fusion of the

203   LBD29 protein to the vYFP marker in the *lbd29* mutant background. The *lbd29-1* line

204   transformed with the pLBD29:LBD29-vYFP construct exhibited full restoration of the wild-

205   type lateral root phenotype using the LR bending assay (Fig. 4C).

206   Further characterization of WT versus the *lbd29-1* T-DNA insertion line was performed to

207   investigate any additional alterations of its lateral root phenotype in 10-day-old seedlings.

208   This revealed that the number of emerged LR was reduced in the *lbd29-1* insertion line (Fig.

209   S4A, B and C) but that neither the stages of LR primordia distribution (Fig. S4D) nor the total

210   number of LR primordia (Fig. S4E) were affected in *lbd29-1* compared to WT. Taken

211   altogether, these results suggest that the reduced number of emerged LR in *lbd29-1* insertion

212   line is not related to a defect in lateral root initiation nor primordia development, but is due to

213   slower organ emergence.

214

215   **LBD29 positively regulates *LAX3* expression *directly***

216   Several independent lines of evidence reported in this paper suggest that LBD29 regulates

217   lateral root emergence by controlling *LAX3* expression. To test whether the LBD29

218   transcription factor binds directly to the *LAX3* promoter *in planta*, we performed chromatin

219   immunoprecipitation (ChIP) PCR analysis using an anti-GFP antibody on nuclear samples

220   prepared from WT (Col-0) (as negative control) and *35S:LBD29-GFP* lines. Four regions of

221   the *LAX3* promoter were tested by ChIP-PCR for LBD29-GFP enrichment (Fig. 5A). All four

222   regions exhibited enrichment (~5 fold) but in a fragment spanning -387 to -192bp from the

223   start codon of the *LAX3* promoter, LBD29-GFP enrichment was enriched ~25 fold compared

224   to the WT control (Fig. 5B). *In silico* sequence analysis identified 10 *LOB/AS2* family binding

225   motifs (Husbands et al., 2007) in the *LAX3* promoter (Table S2). Three of these motifs

226   occurred within the fragment that exhibited the highest enrichment for LBD29-GFP using

227   ChIP-PCR (Fig. 5B). Our ChIP-based results suggest that LBD29 directly binds to the *LAX3*

228   promoter *in planta,* presumably activating its expression.

229   To validate that LBD29 functions as a transcriptional activator for *LAX3* expression, we tested

230   the ability of LBD29 to transactivate a LUC Trap reporter (Lau et al., 2011) fused to the *LAX3*

231   promoter. The *pLAX3:LUC* plasmid was co-expressed in protoplasts derived from tobacco

232   suspension cells with other test plasmids and imaged using the dual luciferase transient

233   expression assay. A two-fold increase in relative luciferase activity was detected when the

234   *pLAX3:LUC* reporter was co-transfected with the *LBD29* effector plasmid (Fig. 5C) but not

235   with the *ARF16* control (Fig. 5C). Our transient expression data suggests that LBD29 can

236   operate as a positive transcriptional regulator of *LAX3* expression.

237   We reasoned that if LBD29 positively regulates *LAX3* expression, an LBD29 over expression

238   line is likely to cause over-expression of a LAX3 reporter even in the absence of exogenous

239   auxin. To test this, we crossed the *pLAX3:LAX3-YFP* reporter (Swarup et al, 2008) with the

240   over-expression line of LBD29 (*35S:LBD29-GFP*) (Okushima et al., 2007). Lines

241   homozygous for both transgenes displayed ectopic expression of the LAX3-YFP reporter in

242   both root cortical and epidermal cells in the absence of exogenous auxin application (Fig.

243   5D). Taken together, these results are consistent with LBD29 positively regulates *LAX3*

244   transcription.

245

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

247   An earlier study investigating the spatial and temporal expression patterns of selected

248   members of the LOB/AS2 gene family during root development reported (using GUS based

249   reporters) that *LBD29* is expressed in LRP (but not overlying) cells (Okushima et al., 2007).

250   Given the large body of evidence we have assembled that LBD29 is required to bind directly

251   to the *LAX3* promoter to activate its expression, we generated new fluorescence-based *LBD29*

252   transcriptional and translational reporter lines to address its binding ability.

253   We initially fused an ER-localised CFP reporter to the *LBD29* promoter sequence. Transgenic

254   lines expressing the *pLBD29:erCFP* transcriptional reporter clearly exhibited a CFP signal in

255   new LRP plus a small number of cells directly overlying new primordia (Fig. 6A,B). Roots

256   were clearly observed to express the *LBD29* driven erCFP reporter in cortical cells overlying

257   Stage I/II LRP, coincident with *pLAX3:LAX3-YFP* expression first being detected (Fig. 1;

258   Swarup et al., 2008). The *pLBD29:erCFP* transcriptional reporter was also clearly expressed

259   in endodermal cells overlying LRP (Fig. 6A and B). However, no *LAX3* expression was

260   detected in this tissue (Fig. 1; Swarup et al., 2008), suggesting that additional transcriptional

261   repressor proteins may be required to impose the observed pattern of *LAX3* spatial expression.

262   A similar spatial expression pattern was observed after fusing the *LBD29* genomic sequence

263   to a single copy of YFP (VENUS) and creating the *pLBD29:gLBD29:Venus* translational

264   reporter line (Fig. 6C and D). The *pLBD29:gLBD29:Venus* reporter clearly exhibited a

265   nuclear localized YFP signal in a subset of cells directly overlying new LRP (Fig. 6C and D),

266   consistent with LBD29 encoding a transcription factor. In addition, temporal analysis of the

267   *pLBD29:gLBD29:Venus* reporter line (Fig. 6) revealed that its induction preceded *LAX3*

268   expression (Fig. 1B). Finally, RT-qPCR assays revealed that the *pLBD29:gLBD29:Venus*

269   reporter line when used to complement the *lbd29-1* LR emergence defect (Fig. 4C) was also

270   able to restore auxin-inducible LAX3 expression (Fig. S5). In summary, our results are

271   consistent with the proposed role of LBD29 as a transcriptional regulator of LAX3.

272

273   **DISCUSSION**

274   ***LBD* family members perform distinct regulatory roles during lateral root development**

275   LATERAL   ORGAN   BOUNDARIES-DOMAIN/ASYMMETRIC   LEAVES2-Like

276   (LBD/ASL) genes encode a plant-specific family of transcription factors (Husbands et al.,

277   2007) that have been implicated in a variety of developmental processes during leaf, flower

278   and root morphogenesis (Iwakawa et al., 2002; Majer and Hochholdinger, 2011; Okushima et

279   al., 2007; Okushima et al., 2005; Soyano et al., 2008; Xu et al., 2008). In *Arabidopsis*

280 *thaliana*, the LBD gene family contains 43 members, of which the *LOB* gene is the founding

281 member (Shuai et al., 2002). The *LOB* gene plays a role in organ separation and lateral organ

282 development and encodes a transcription factor, expressed at boundaries within shoot tissues

283 (Shuai et al., 2002). In roots, family members *LBD16*, *LBD18* and *LBD29* play important

284 roles during lateral root development (Okushima et al., 2005; Okushima et al., 2007; Lee et

285 al., 2009; Lavenus et al., 2015; Fig. 7A). During lateral root initiation, LBD16 has been

286 shown to play an important role promoting asymmetric cell division of LR founder cells,

287 controlling polarized nuclear migration to the common cell pole between pairs of founder

288 cells (Goh et al., 2012). LBD18 and LBD33 positively regulate the cell cycle via the

289 transcriptional regulation of *E2Fa* (Berckmans et al. 2011). LBD29 has also been reported to

290 be involved in the regulation of the cell cycle progression during LR formation (Feng et al.,

291 2012).

292 In this current study, we demonstrate that LBD29 plays a key role during lateral root

293 emergence. Several other *LBD* genes have been linked with this developmental program. Like

294 *lbd29-1*, the *lbd16-1* and *lbd18-1* single mutants also displayed a reduction in LR emergence,

295 which was enhanced in the *lbd16-1 lbd18-1* double mutant (Lee et al., 2009; Okushima et al.,

296 2007). *LBD18* has been demonstrated to function downstream of the auxin influx carrier

297 *LAX3* during lateral root emergence (Lee et al, 2014). In addition, the triple mutant *lbd16-1*

298 *lbd18-1 lbd33-1* displayed a further reduction in the number of LR emerged compared to any

299 of the single or double mutants mentioned above (Goh et al., 2012). These observations may

300 indicate the presence of functional redundancy between selected members of the *LBD* gene

301 family. Alternately, it could reflect that these *LBD* genes play roles during distinct (but

302 interacting) lateral root developmental processes such as organ initiation, patterning and

303 emergence, which have additive phenotypic effects when disrupted in a multiple mutant

304 background.

305 Any distinct regulatory functions that *LBD* family members play during lateral root

306 development cannot be explained simply on the basis of each gene exhibiting contrasting

307 spatio-temporal expression patterns (Fig. 7A). For example, whilst *LBD16* and *LBD29* are

308 both expressed in pericycle cells prior to lateral root initiation (Goh et al, 2012; Fig. 6C), only

309 transgenic lines expressing a LBD16-SRDX transcriptional repressor protein block the initial

310 asymmetric cell division in LR founder cells (Goh et al, 2012), whereas *gLBD29-SRDX* lines

311 are defective in organ emergence (Fig. 4). This suggests that LBD16 and LBD29 proteins

312 target distinct sets of genes during lateral root development. Currently, the molecular basis of

313 LBD16 and LBD29 target specificity is unclear. Several LBD proteins have been reported to

314 interact with other family members or distinct classes of transcription factors that may help

315 confer target specificity. For example, LBD18 and LBD33 dimerize to regulate cell cycle by

316 activating *E2FA* transcription during lateral root initiation (Berckmans et al., 2011) and LBD6

317 (AtAS2) interacts with a MYB transcription factor AtAS1 during leaf development (Xu et al.,

318 2003). In summary, *LBD* family members play key regulatory roles throughout plant

319 development including *LBD16, LBD18* and *LBD29* that are critical for lateral root

320 development.

321

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

323 Auxin functions as a key regulatory signal during lateral root development (reviewed by

324 Lavenus et al., 2013). In *Arabidopsis* auxin alters the expression of large numbers (>2000) of

325 genes during lateral root development via ARF transcription factors such as ARF7 and

326   ARF19 (Okushima et al., 2005; Vanneste et al, 2005). ARF7 is particularly important as it

327   functions to activate gene regulatory networks that control lateral root initiation, patterning

328   and emergence (Lavenus et al., 2015; Swarup et al, 2008). ARF7 (and ARF19) regulates

329   lateral root development (in part) by activating the expression of several auxin inducible *LBD*

330   family members (Okushima et al., 2005), as ectopic expression of *LBD16* and *LBD29* can

331   partially rescue the *arf7 arf19* LR-less phenotype (Okushima et al., 2007). *LBD16* and *LBD29*

332   represent key nodes within the lateral root gene regulatory network (GRN; Lavenus et al.,

333   2015). ChIP-PCR experiments have recently demonstrated that *LBD16* and *LBD29* represent

334   direct targets for ARF7 binding and regulation (Lavenus et al., 2015).

335   Whilst *LBD16* function was closely associated with the GRN controlling early stages of

336   lateral root initiation (Goh et al., 2012; Lavenus et al., 2015), the role for *LBD29* has been less

337   clear until our genetic studies employing *lbd29-1, pLBD29:gLBD29:Venus* rescue of *lbd29-1*

338   and *gLBD29-SRDX* lines revealed that *LBD29* mediates auxin regulated lateral root

339   emergence (Fig. 4 and 7A). The current study has also revealed that LBD29 is essential for

340   the auxin inducible expression of the auxin influx carrier *LAX3* (Fig. 3 and 7B) by directly

341   binding the *LAX3* promoter (Table S1; Fig. 5). Promoter deletion experiments show that a

342   region between -570 and -363 is essential for *LAX3* auxin induction (Fig. 2, between Δ3 and

343   Δ4). *In silico* analysis of the *LAX3* promoter sequence allowed the identification of a high

344   number of LBD binding sites immediately downstream of this region (Fig. 5 and Table S2).

345   Consistently, ChIP q-PCR experiments confirmed that LBD29 binds this region of the

346   promoter, which triggers *LAX3* induction in response to auxin.

347   During LR emergence, cell separation in tissues overlying new organs requires auxin

348   induction of cell-wall-remodeling genes, such as PG that degrades pectin, leading to cell wall

349   breakdown (Laskowski et al, 2006). Auxin inducible PG expression in cells overlying LRP is

350   dependent on the auxin influx carrier LAX3 (Swarup et al., 2008). The absence of *PG*

351   expression following auxin treatment in the *lbd29-1* line (Fig. 3B) suggests that LBD29

352   controls *PG* expression via its regulation of *LAX3*. LBD18 has been reported to induce the

353   expression of another cell wall remodeling gene *Expansin14* during lateral root emergence

354   (Lee et al., 2012). Hence, both *LBD18* and *LBD29* regulate the induction of cell wall-

355   remodeling enzymes that promote lateral root emergence. However, *LBD29* and *LBD18*

356   function at distinct positions in the lateral root emergence GRN, upstream and downstream of

357   *LAX3*, respectively (Fig. 7B; Lee et al., 2014).

358

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

360   Delimiting *LAX3* expression to two adjacent cortical and epidermal cell files overlaying new

361   LRP is critical for ensuring auxin-regulated cell separation occurs solely along their shared

362   walls (Swarup et al., 2008; Fig. 1B). To understand how *LAX3* spatio-temporal expression is

363   regulated, Péret et al. (2013) developed a mathematical model that captures the network

364   regulating its expression and auxin transport within realistic three-dimensional cell and tissue

365   geometries. To prevent *LAX3* from being transiently expressed in multiple cell files overlying

366   LRP, the model predicted that this auxin influx carrier must be induced by auxin *after* an

367   auxin efflux carrier (later demonstrated to be encoded by the *PIN3* gene; Péret et al., 2013).

368   Hence, *PIN3* and *LAX3* expression would need to be sequentially induced by auxin. Given

369   that *PIN3* is regulated as a primary auxin responsive gene, *LAX3* would be required to be

370   induced as a secondary auxin responsive gene. ARF7 regulating *LAX3* auxin inducible

371   expression via an intermediary transcriptional factor (*LBD29*) would fulfill this temporal

372   regulatory requirement. Hence, the sequential induction of *LBD29* and *LAX3* by auxin (Fig.

373   7B) represents an important regulatory motif within the LR GRN that is required to

374   coordinate cell separation and organ emergence.

375

376   **MATERIALS AND METHODS**

377   **Plant materials and growth conditions**

378   The *Arabidopsis* ecotype Columbia (Col-0) was used as the wild type in all experiments. The

379   *35S:LBD29-GFP* in the *arf7 arf19* mutant background, *arf7* (*nph4-1*), *arf19-1, lbd29,* and

380   *lax3* mutants have been previously described (Okushima *et al*., 2007; Okushima et al., 2005;

381   Feng *et al*., 2012; Swarup *et al*., 2008). The genomicLBD29-SRDX lines in Col-0

382   background (gLBD29-SRDX 1.3 and 2.1) were produced as described in Goh et al. (2012).

383   Seeds were surface sterilized and plated on ½ MS medium (Sigma) 1% bacto-agar (Appleton

384   Woods). Seeds were stratified at 4°C overnight and grown vertically (22°C, continuous light).

385   Medium was supplemented either with 1 µM IAA (indole-3-Acetic Acid) (Sigma) or 10 µM

386   cycloheximide (CHX) (VWR International Ltd)..

387   **Expression analysis using RT-qPCR**

388   RNA extractions, reverse transcription and quantitative PCR were performed as previously

389   described (Péret et al., 2013). Primer sequences for *LAX3* and *PG* have also been described

390   (Péret et al., 2013).

391   **Root phenotyping analysis**

392   Three-day-old seedlings grown on vertical plates were subjected to 90º gravistimulations for

393   18 and 42 hours (Voß et al., 2015). In addition, 10-day-old seedlings grown vertically were

394   harvested to analyze the developmental stages of lateral root primordia. In this phenotypic

395   study, the total number and stages of lateral root primordia were counted and determined as

396   described by (Malamy and Benfey, 1997). Root length was measured using ImageJ (ImageJ

397   1.40g).

398   **Histochemical analysis and microscopy**

399   GUS staining and clearing was done as previously described (Péret et al., 2013). Confocal

400   microscopy was performed using a Leica SP5 confocal laser-scanning microscope (Leica

401   Microsystems). Cell walls were stained using propidium iodide (Sigma) (10µg/ml) for 2

402   minutes.

403   **Yeast one hybrid assays**

404   The experiments were performed as described in (Gaudinier et al., 2011). Interactions were

405   called for TFs that activated at least one reporter assay.

406   **Chromatin Immunoprecipitation and Quantitative PCR**

407   Chromatin Immunoprecipitation (ChIP) and subsequent quantitative PCR (input DNA

408   dilution 1000x) were performed as previously described (Lavenus et al., 2015). Primers were

409   designed to amplify 150-200 bp fragments and are listed in Table S3. Relative enrichment of

410   the target region was normalized against TUB3 (TUBULIN BETA CHAIN3, AT5g62700).

411   Relative enrichments of LBD29-GFP proteins were analyzed at four regions of the *LAX3*

412   promoter. Transgenic roots of the LBD29 O^{ex} line were analyzed by ChIP using anti-GFP

413   antibodies. Values were normalized to internal controls (relative to input and to TUB2). Data

414   represent the mean ± standard error of four technical replicates, and two biological replicates

415   were performed.

416   **Transient Expression Assays**

417   Transient expression assay was performed on protoplasts as previously described (Bielach et

418   al., 2012). Protoplasts were co-transfected with 1 µg of a reporter plasmid containing the

419   Luciferase (LUC) reporter gene, 1 µg of plasmid effector and 2 µg of normalization construct

420   expressing Renilla LUC gene (De Sutter et al., 2005). Firefly luciferase (fLUC) activity

421   values were normalized with the luciferase activity derived from the internal standard plasmid

422   coding for the Renilla luciferase (rLUC) gene under the control of 35S CaMV promoter. Both

423   luciferase activity were measured subsequently on a Synergy H1 with double injector

424   (Biotek). The mean value (±se) was calculated from six measurements on three independent

425   experiments.

426   **Cloning for Luciferase and Y1H assays**

427   For the Luciferase assays, a genomic DNA sequence corresponding to 1374 bp upstream of

428   the start codon of the *LAX3* gene was isolated and then amplified using the forward and

429   reverse   primers   5′-   ATAAAT<u>CTGCAG</u>AGTCATGATCCTTTT   -3′   and   5′-

430   TCTTTAAATAGA<u>CCATGG</u>AAAAGCTTTTTC -3′ containing Pst1 and Nco1 sites,

431   respectively and ligated into a LucTrap vector (Lau et al., 2011) to generate Luciferase fusion.

432   The coding sequence of LBD29 was amplified to introduce HindIII and BamHI restriction

433   sites at each extremity respectively (5′ - ATC<u>AAGCTT</u>ATGACTAGTTCCAGCT

434   CTAGCTCT -3′ and 5′- GAT<u>GGATCC</u>ATATCACGAGAAGGAGATGTAGCC-3′) and

435   subsequently cloned into pJIT60 vector to generate the plasmid effector (Schwechheimer et

436   al., 1998). Full length ARF16 cDNA was introduced to the pJIT60 vector, using BamH1 and

437   EcoR1   restriction   sites.   (5'-

438   AAAACG<u>GGATCC</u>AAAAATATGATAAATGTGATGAATCCA-3'   and   5'-

22

439   AAA<u>GAATTC</u>GCCAAGTTATACTACAACGCTCTCACT-3'). pJIT60 vector contained a

440   double cauliflower mosaic virus 35S promoter.

441   Chimeric constructs were also created for the Y1H system. Genomic DNA from Col-0 was

442   used to amplify 1422bp of the LAX3 promoter, just upstream of the translational start codon

443   (5'-TTCTGCTTTTTGAATATTACACCATT-3'   and   5'-

444   TTTTCTCTTCTTCTCTCAGTTTCTTTAGC-3') and was cloned into pENTR^{TM} 5'-

445   TOPO®TA vector (Invitrogen). The correct clone was recombined with pMW2 (HIS3

446   reporter vector) and pMW3 (LacZ reporter vector) (Brady et al., 2011) using LR clonase II,...

447   Because the stele-expressed transcription factor collection (Gaudinier et al., 2011) did not

448   contain LBD29 transcription factor, a construct was generated to clone the LBD29 coding

449   sequence into the pDest-AD-2µ plasmid. The cDNA sequence of LBD29 was amplified (5'-

450   <u>CACC</u>ATGACTAGTTCCAGCTC-3' and 5'- CGAGAAGGAGA TGTAGCCAAAATT-3')

451   and cloned into the pENTR-D-Topo entry vector (Invitrogen). The entry vector was used in a

452   gateway LR cloning reaction (LR clonase II - Invitrogen) with pDest-AD-2µ (Gaudinier et al.,

453   2011) to create a GAL4-activation domain fusion Y1H prey vector..

454   **Cloning for promoter deletions and IVMs**

455   *LAX3* promoter was cloned from pENTR11-LAX3-YFP (Swarup et al., 2008) into

456   pBluescript KM+ (Invitrogen) using unique KpnI and SpeI restriction sites. The plasmid was

457   PCR amplified using primers Lx3-25 5'-TTTCTAAGAAATTAGTGGGTTAAATAAAGC-

458   3' and Lx3-26 5'-AGTCTCCTTTTTAGCCCCATGCTTTTACAATGG-3', which were

459   designed to modify a single nucleotide within the auxin response element (GAGACA to

460   GAGACT). PCR amplification was carried out using Pfx proofreading DNA Polymerase.

461   Purified PCR products were digested with DpnI, treated with T4 Polynucleotide Kinase

462 (NEB) and ligated with T4 Ligase (NEB). Point mutated promoters were cloned back into

463 pENTR11-LAX3-YFP and sequenced to check no other mutations were created during the

464 PCR.

465 For PCR generated promoter deletions, a combination of primers Lx3-R2 5'-

466 TTCTAAGTAATTCCCTGCGACC-3'   and   (KpnI)-Lx3-22   5'-

467 CCGGTACCTTTCTAAGAAATTAGTGGGTTA-3' for Δ2 and (KpnI)-Lx3-23 5'-

468 CCGGTACCAATATGTTTTATTCATTGTTTC-3' for Δ4 were used. PCR amplification was

469 carried out using Pfx proofreading DNA Polymerase. Purified PCR products were digested

470 with DpnI and cloned into pENTR11-LAX3-YFP using KpnI and SpeI restrictions sites.

471 For deletions generated using restriction enzymes, KpnI and MunI for Δ1 or only BamH1 for

472 Δ3 were used. The correct band was gel purified, if necessary 3' overhang were filled using

473 T4 DNA Polymerase (NEB) and both fragments ligated using T4 Ligase.

474 Constructs were then cloned in the binary pGWB7 vector using the Gateway LR reaction

475 (Invitrogen).

476

477 **Competing interests**

478 No competing interests declared.

479

480 **Author contributions**

481 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.

482 and M.J.B. conceived, designed and performed experiments. S.P., A.L., B.P. and M.J.B.

483 wrote the paper.

484

491

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614   **Figure legends**

615

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

617   **dependent.** (A) Lateral root formation proceeds in 8 developmental stages (St I to VIII,

618   Casimiro et al., 2003). (B) A functional LAX3-YFP fusion is used to monitor LAX3

619   accumulation in the overlaying tissues during lateral root emergence. Bar is 50µm. (C) *LAX3*

620   induction by auxin (1µM IAA) was monitored in wild-type (Col-0) compared to *arf7* mutant

621   by qPCR. Data represent the mean ± standard error of four technical replicates and three

622   biological replicates were performed.

623

624   **Fig. 2.** *LAX3* **induction by auxin is independent of the canonical Auxin Response**

625   **Element (AuxRE).** (A) Representation of LAX3 full promoter (FP) from -1914 to start codon

626   (ATG). Promoter deletions (named Δ1, Δ2, Δ3 and Δ4) were generated (see insert for

627   fragment sizes verification) and cloned upstream of LAX3-YFP. The canonical AuxRE

628   situated at -939bp is indicated (complementary strand of the TGTCTC sequence described by

629   Ulmasov et al., 1997). (B) Lateral root density measurements (number of lateral roots per

630   length of primary root). Error bars are SE of the mean (n≥8). (C) Fluorescence intensity

631   measurement of cortical cells is relative to the mock control of the corresponding transgenic

632   line (n≥8 corresponding to at least 4 strips out of 2 independent roots). (D) Laser scanning

633   confocal images of LAX3-YFP fusion driven by the full promoter (FP) or promoter deletions

634   in non-treated (NT) or auxin treated (1µM IAA for 16h) conditions. Bar is 100µm

635

636   **Fig. 3. LBD29 controls** *LAX3* **induction during lateral root emergence.** 5 day-old-

637   seedlings of Col-0 and *lbd29* were treated with 1 µM IAA during 0, 1, 3, 6, 8, 10, 18 and 24

638   hours (a-b). *LAX3* (A) and *PG* (B) mRNA levels were quantified by RT-qPCR. Data represent

639   the mean value ± standard errors of four technical replicates and the experiment was

640   performed in triplicate. (C-J) Expression pattern of pLAX3:*LAX3-YFP* in non-treated (NT)

641   control and after auxin treatment (1μM IAA) in wild-type (Col-0) and mutant (*lbd29-1*). Bars

642   are 50μm. Asterisks indicate a lateral root primordium. ep: epidermis, c: cortex, en:

643   endodermis, st: stele. Data represent the mean ± standard error of four technical replicates and

644   three biological replicates were performed.

645

646   **Fig. 4 Gravistimulation assays in 3-day-old-seedlings of WT, *lax3, lbd29* and SRDX-**

647   **LBD29 lines.** Phenotypic analysis of LR emergence was achieved by synchronizing LR

648   formation with a gravistimulus during 18h and 42h. Compared to WT (Col-0), LR

649   emergence is delayed in *lax3* and *lbd29* mutants (A) and SRDX-LBD29 lines (B).

650   Expression of the LBD29 protein fused to the vYFP reporter fully restores LR formation

651   in the *ldb29* mutant (C). Data shown are percentage and the error bars represent

652   standard error, n=20 for Col-0, lax3 and lbd29; n= 18 and 16 for SRDX-LBD29 1.3 and

653   2.1 respectively and n=14 for pLBD29:LBD29-vYFP.

654   **Fig. 5. LBD29 directly regulates *LAX3* expression.** (A) Black triangles indicate LBD

655   binding motif positions on *LAX3* promoter as predicted by AthaMap (Steffens et al., 2004).

656   *LAX3* promoter fragments 1 to 4 are also displayed with their relative start and end

657   nucleotides from the start codon (ATG). (B) Chromatin Immuno Precipitation was performed

658   on the wild-type (Col-0) and *LBD29* overexpressing line (35S:LBD29-GFP). Data shown are

659   qPCR quantification of each DNA fragment. Relative enrichments of LBD29-GFP proteins

660   were analyzed at four regions of the *LAX3* promoter. Transgenic roots of the LBD29 O^{ex} line

661   were analyzed by ChIP using anti-GFP antibodies. Col-0 was used as negative controls (black

662   rectangular). Values were normalized to internal controls (relative to input and to TUB2).

663   Data represent the mean ± standard error of four technical replicates, and two biological

664   replicates were performed. (C) Relative Luciferase (LUC) intensity is shown for each

665   protoplast assay in control, 35S:LDB29 and 35S:ARF16. Transactivation with the reporter

666   construct (pLAX3::fLUC), the effector constructs (35S::LBD29 and 35S::ARF16), the

667   internal standard (35S::rLUC), and the negative control (Control = 35S::GUS) were used in

668   this assay. Induction is expressed relative to the normalized luciferase activity of the GUS =

669   negative control. Asterisk shows significant difference for LBD29 compared to the control

670   ($p<0.05$, Student's t-test). The data represent the mean value ± standard error of 6

671   measurements and the experiment was performed in triplicate. (D) Overexpression of LBD29

672   triggers ectopic LAX3-YFP expression in all parts of the root (left, middle confocal section

673   and right, surface view) compared to wild-type control in Fig. 1. Bars are 50µm. Asterisks

674   show significant difference for LBD29 compared to the control ($p<0.05$, Student's t-test).

675

676   **Fig. 6. *LBD29* expression pattern during lateral root emergence. (A-B)** Laser scanning

677   confocal image of early stage lateral root primordia (LRP) in a transgenic line expressing a

678   *LBD29* transcriptional reporter composed of its promoter fused to an endoplasmic reticulum-

679   localised CFP reporter (termed *pLBD29:erCFP*). (C-D) Laser scanning confocal image of

680   early stage LRP in a transgenic line expressing a *LBD29* translational reporter composed of its

681   promoter and genomic coding sequence fused to the Venus version of YFP (termed

682   *pLBD29:gLBD29:Venus*). Bars are 50µm. Asterisks indicate LRP at the stage denoted in each

683   panel.

684

685   **Fig. 7.** *LBD* **transcription factors control various stages of lateral root formation.** (A)

686   LBD16 is expressed early on during lateral root (LR) formation and controls LR initiation by

687   acting on nuclear migration. LBD18 is expressed in the LR primordium and overalying

688   tissues where it acts downstream of LAX3. We show here that LBD29 acts upstream of

689   LAX3 to control LR emergence. (B) Temporal control of the LR emergence gene regulatory

690   network. Auxin triggers the degradation of IAA14 which releases ARF7 so it can activate

691   LDB29 (early gene). Consequently, LBD29 directly activates LAX3 which creates a positive

692   feedback loop through its auxin influx activity. This allows for high auxin levels to induce

693   cell wall remodelling genes such as PG (late genes) and promotes cell separation that

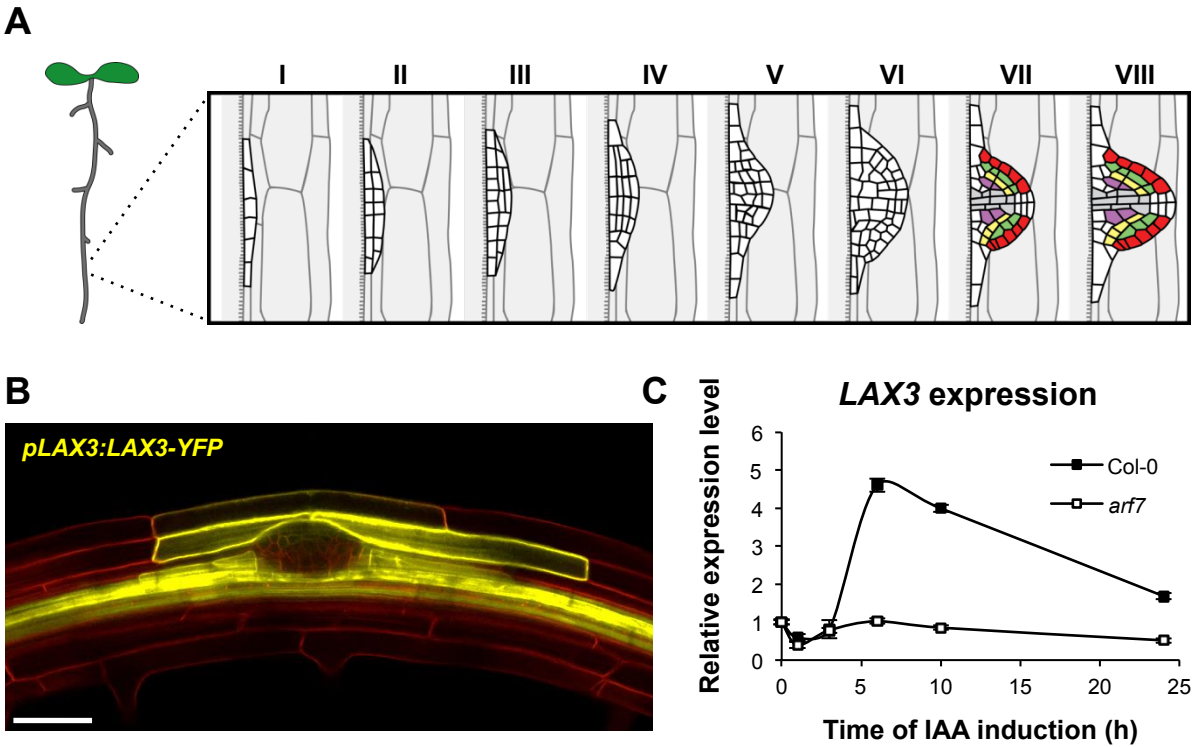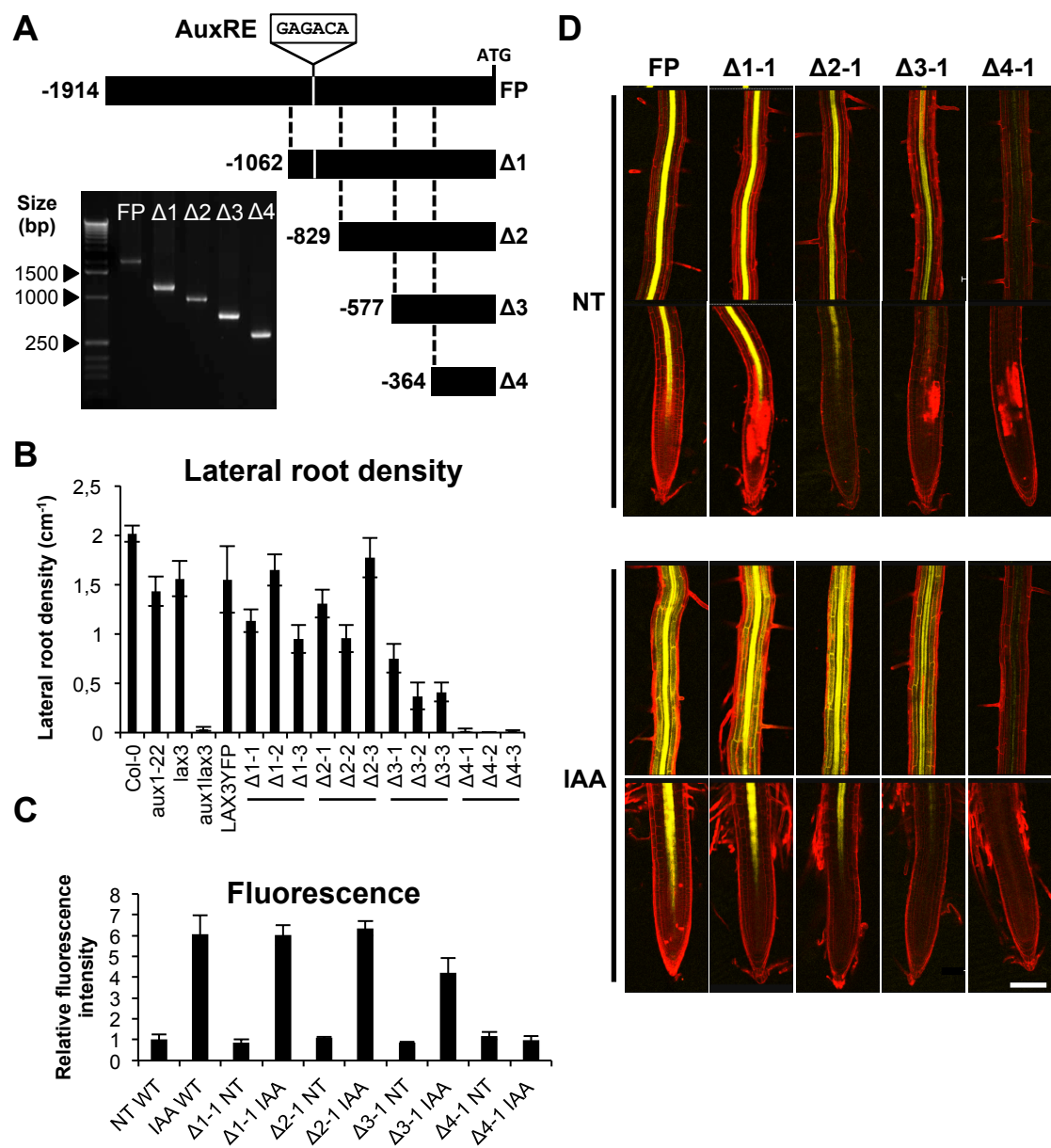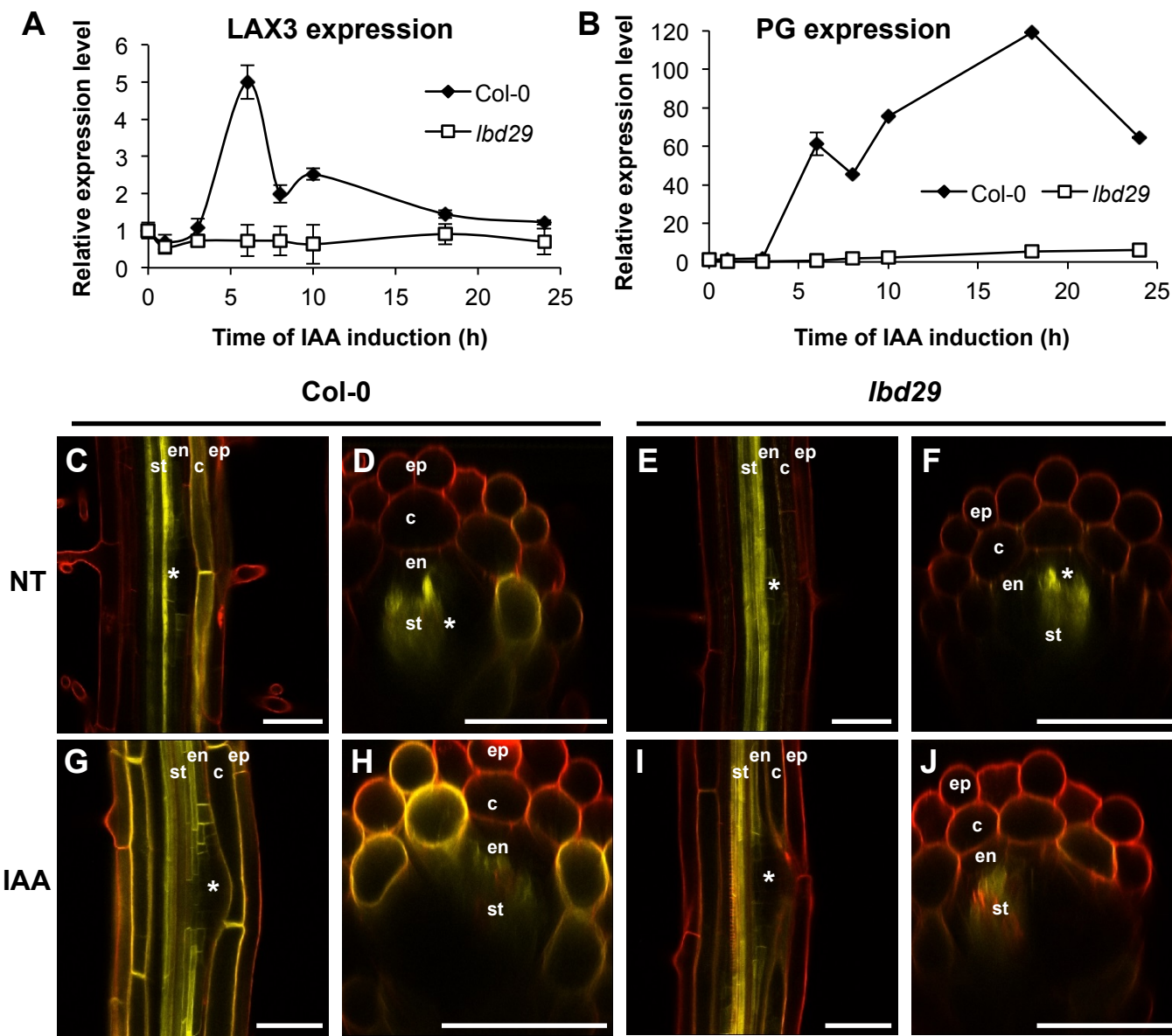694   facilitates LR emergence.

Figure 1

pLAX3:LAX3-YFP

C *LAX3* expression

Col-0
arf7

Relative expression level

Time of IAA induction (h)

Figure 2

Figure 3

Figure 4

Figure 5

A LBD Binding Motifs

-1435 ATG pLAX3

-1058 -850
-387 -192
LAX3 promoter fragments ①

-734 -574
②

③ -150 +17
④

B

Fold enrichment

■ Col-0
□ 35S:LBD29-GFP

*

① ② ③ ④
LAX3 promoter fragments

C

Relative LUC intensity

*

Control   35S:LBD29   35S:ARF16

D *35S:LBD29 pLAX3:LAX3-YFP*

Figure 6

Figure 7

A

LBD16   LBD18   LBD29

Primordium initiation   **Primordium patterning**   **Primordium emergence**

B

Auxin

IAA14

IAA14

ARF7

LBD29

LAX3

PG

Induction level

Time

Auxin ⊣ IAA14 ⊣ ARF7 → **LBD29** → **LAX3**   **PG**

Positive feedback loop

LBD18

Figure 1

A

B

pLAX3:LAX3-YFP

C

LAX3 expression

Col-0

arf7

Relative expression level

Time of IAA induction (h)

Figure 2

A

AuxRE [GAGACA]

-1914 ████████ FP (ATG)

-1062 ███████ Δ1

-829 ██████ Δ2

-577 ████ Δ3

-364 ██ Δ4

Size (bp): FP Δ1 Δ2 Δ3 Δ4
1500
1000
250

B

Lateral root density

Lateral root density (cm⁻¹)
Col-0, aux1-22, lax3, aux1lax3, LAX3YFP, Δ1-1, Δ1-2, Δ1-3, Δ2-1, Δ2-2, Δ2-3, Δ3-1, Δ3-2, Δ3-3, Δ4-1, Δ4-2, Δ4-3

C

Fluorescence

Relative fluorescence intensity
NT WT, IAA WT, Δ1-1 NT, Δ1-1 IAA, Δ2-1 NT, Δ2-1 IAA, Δ3-1 NT, Δ3-1 IAA, Δ4-1 NT, Δ4-1 IAA

D

FP Δ1-1 Δ2-1 Δ3-1 Δ4-1

NT

IAA

Figure 4

Figure 5

A

LBD Binding Motifs

-1435 ▼   ▼▼   ▼   ▼▼▼   ▼   ATG

pLAX3

-1058 ▢ -850
①

-734 ▢ -574
②

-387 ▢ -192
③

-150 ▢ +17
④

LAX3 promoter fragments

B

Fold enchriment

- ■ Col-0
- ▢ 35S:LBD29-GFP

*

① ② ③ ④

LAX3 promoter fragments

C

Relative LUC intensity

*

Control   35S:LBD29   35S:ARF16

D

35S:LBD29 pLAX3:LAX3-YFP

Figure 6

Figure 7

A

LBD16   LBD18   LBD29

Primordium initiation   Primordium patterning   Primordium emergence

B

Auxin
IAA14
IAA14
ARF7

Induction level

PG
LBD29
LAX3

Time

Auxin —| IAA14 —| ARF7 → **LBD29** → **LAX3**   **PG**

Positive feedback loop

LBD18