Lateral Organ Boundaries Domain16 and 18 Act Downstream of the AUXIN1 and LIKE-AUXIN3 Auxin Influx Carriers to Control Lateral Root Development in Arabidopsis

Han Woo Lee, Chuloh Cho, and Jungmook Kim*

Department of Bioenergy Science and Technology and Kumho Life Science Laboratory, Chonnam National University, Gwangju 500–757, Korea

ORCID IDs: 0000-0001-7997-1544 (C.C.); 0000-0003-1735-5564 (J.K.).

Several members of the Lateral Organ Boundaries Domain (LBD)/Asymmetric Leaves2-Like (ASL) gene family have been identified to play important roles in Arabidopsis (Arabidopsis thaliana) lateral root (LR) development during auxin response, but their functional relationship with auxin transporters has not been established yet. Here, we show that the AUXIN1 (AUX1) and LIKE-AUXIN3 (LAX3) auxin influx carriers are required for auxin signaling that activates LBD16/ASL18 and LBD18/ASL20 to control LR development. The lax3 mutant phenotype was not significantly enhanced when combined with lbd16 or lbd18. However, LBD18 overexpression could rescue the defects in LR emergence in lax3 with concomitant expression of the LBD18 target genes. Genetic and gene expression analyses indicated that LBD16 and LBD18 act with AUX1 to regulate LR initiation and LR primordium development, and that AUX1 and LAX3 are needed for auxin-responsive expression of LBD16 and LBD18. LBD18/SUPERMAN REPRESSIVE DOMAIN X in the lbd18 mutant inhibited LR initiation and LR primordium development in response to a gravitropic stimulus and suppressed promoter activities of the cell cycle genes Cyclin-Dependent Kinase A1;1 and CYCLINB1;1. Taken together, these results suggest that LBD16 and LBD18 are important regulators of LR initiation and development downstream of AUX1 and LAX3.

Root branching, such as lateral root (LR) formation, is critical for the absorption of water and nutrients, and for the anchorage of plants in soil (Hochholdinger and Zimmermann, 2008; Dastidar et al., 2012). In recent years, intensive research on the molecular mechanisms of LR formation has been conducted with Arabidopsis (Arabidopsis thaliana; Lavenus et al., 2013). The developmental events of LR formation include priming, initiation, primordium development, and the emergence of LRs, which are primarily regulated by auxin (Dubrovsky et al., 2008, 2011; De Smet, 2012; Lavenus et al., 2013). The pericycle cells associated with a xylem pole are primed by auxin in the basal meristem to become pericycle founder cells, and the founder cells divide asymmetrically for LR initiation, generating small daughter cells flanked by long daughter cells (Parizot et al., 2008; Péret et al., 2009a,b; Lavenus et al., 2013). These cells undergo a series of anticlinal divisions to produce a few initial cells. Further anticlinal, periclinal, and tangential divisions of lateral root primordium (LRP) cells generate a dome-shaped LRP that continues to grow and emerge through the cortex and epidermal layers of the primary root via cell separation.

In the Arabidopsis root, auxin is transported acropetally through the central cylinder toward the root tip and columella cells (Friml et al., 2003). Basipetal auxin transport from the root tip toward the basal region plays an important role in the control of auxin homeostasis in the root meristem (Band et al., 2014). The AUXIN1 (AUX1)/LIKE-AUXIN1 (LAX1) gene family encoding major auxin influx carriers comprise four highly conserved genes: AUX1, LAX1, LAX2, and LAX3 (Péret et al., 2012; Swarup and Péret, 2012). AUX1 regulates the initiation of LR by basipetal auxin transport (Swarup et al., 2001; Marchant et al., 2002; De Smet et al., 2007). LAX2 regulates vascular patterning in cotyledons (Péret et al., 2012). LAX3 promotes LR emergence by affecting auxin influx of outer endodermis and cortex cells (Swarup et al., 2008). The aux1 lax3 double mutations effectively blocked LR

1 This work was supported by the Next-Generation BioGreen 21 Program (grant no. PJ0104701), Rural Development Administration, Republic of Korea (grant to J.K.), Basic Science Research Programs through the National Research Foundation of Korea funded by the Ministry of Education (grant no. 2013R1A6A3A01062668), and Chonnam National University, 2013.

* Address correspondence to jungmkim@jnu.ac.kr.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jungmook Kim (jungmkim@jnu.ac.kr).

H.W.L. conducted most of the experiments, analyzed the data, and wrote the article; C.C. conducted specific experiments and analyzed the data; J.K. conceived the project, designed the experiments, analyzed the data, and wrote and edited the article.

www.plantphysiol.org/cgi/doi/10.1104/pp.15.00578
formation, indicating that these two auxin influx carriers are critical for LR formation (Swarup et al., 2008). Auxin efflux carriers, PIN-FORMED (PIN) proteins, have also been demonstrated to play a role in LR formation (Benková et al., 2003; Laskowski et al., 2008; Marhavý et al., 2013; Péret et al., 2013).

Auxin-responsive transcriptional regulatory modules for LR formation have been identified in Arabidopsis. The GATA transcription factor23 specifies LR founder cell identity in an INDOLE-3-ACETIC ACID INDUCIBLE28 (IAA28)-dependent auxin signaling for LR priming (De Rybel et al., 2010). SOLITARY-ROOT (SLR)/IAA14-Auxin Response Factor7 (ARF7)-ARF19 and others regulate nuclear polarization of LR founder cells (De Rybel et al., 2010; Goh et al., 2012a). Two Aux/IAA-ARF modules, SLR/IAA14-ARF7-ARF19 and BODENLOS/IAA12-ARF5, control the LR initiation and patterning process (Fukaki et al., 2002; Vanneste et al., 2005; De Smet et al., 2010). LR emergence is distinctively modulated in the endodermis and the cortex and epidermis by two different Aux/IAA-ARF modules. SHORT HYPOCOTYL2/IAA3-ARF7-ARF19 and BODENLOS/IAA12-ARF5, control the LR initiation and LRP development during the auxin response, and on the role of Aux/IAA-ARFs and LBD/Asymmetric Leaves2-Like (ASL) proteins, are regulated downstream of Aux/IAA-ARF modules. SHORT HYPOCOTYL2/IAA3-ARF signaling operates in the endodermis, whereas SLR/IAA14-ARF7-ARF19 signaling functions in the cortex and epidermis (Goh et al., 2012b; Lavenu et al., 2013).

Several transcription factors, in particular, Lateral Organ Boundaries Domain (LBD)/Asymmetric Leaves2-Like (ASL) proteins, are regulated downstream of Aux/IAA-ARF modules during LR development (Okushima et al., 2007; Lee et al., 2009a; Berckmans et al., 2011; Goh et al., 2012a; Lee et al., 2013a; Lee and Kim, 2013). ARF7 and ARF19 directly activate LBD16/ASL18 and LBD29/ASL16 (Okushima et al., 2007). LBD16 and related LBDs are involved in the symmetry breaking of LR founder cells for LR initiation (Goh et al., 2012b). LBD18/ASL20 regulates LR formation in conjunction with LBD16 downstream of ARF7 and ARF19 (Lee et al., 2009a,b) and contributes to both the initiation and emergence of LRs (Berckmans et al., 2011; Lee et al., 2013a; Lee and Kim, 2013). LBD18 transcriptionally activates the E2Fa transcription factor, which regulates the asymmetric cell division for LR initiation (Berckmans et al., 2011). LBD18 promotes LR emergence by acting as a specific DNA-binding transcriptional activator that directly up-regulates EXPANSIN14 (EXP14) encoding a cell wall-loosening protein (Lee et al., 2013a). LBD18 indirectly up-regulates EXP17, which promotes LR emergence during the auxin response (Lee and Kim, 2013). Although the auxin-responsive transcriptional cascade of the Aux/IAA-ARFs and LBD genes has been well characterized, no functional evidence has been provided yet on how auxin transport proteins are linked to LBD gene expression to control LR development during the auxin response, and on the role of LBD18 in LR initiation and LRP development.

In this study, we investigated the connection between auxin influx carriers, LAX3 and AUX1, and two auxin-responsive LBD genes, LBD16 and LBD18, during LR development by conducting genetic analysis with various multiple mutants derived from lbd16, lbd18, lax3, and aux1 and gene expression analysis. Our molecular genetic analysis results suggested that LBD16 and LBD18 are linked to auxin signaling via AUX1 for LR initiation and LRP development, in part via LAX3 for LR development, and that LBD18 acts downstream of LAX3 to control LR emergence in Arabidopsis. To confirm that LBD18 is involved in LR initiation and LRP development, we expressed LBD18: SUPERMAN REPRESSIVE DOMAIN X (SRDX), a dominant repressor of LBD18, under the control of its own promoter in wild-type or lbd18 mutant backgrounds and showed that LBD18:SRDX suppressed LR initiation events, periclinal divisions of primordium after LR initiation, and later stages of LRP development in response to a gravitropic stimulus. In addition, we identified a link between LBD18 and the regulation of cell cycle genes during LR initiation through the analyses of GUS expression under the cell cycle gene promoter Cyclin-Dependent Kinase A1;1 (CDKA1;1), CDKB1;1, or CYCLINB1;1 (CYCB1;1), a marker gene for LR initiation, in transgenic Arabidopsis expressing LBD18:SRDX, and of expression of these cell cycle genes by LBD18:GLUCOCORTICOID RECEPTOR (GR).

RESULTS

LR Phenotypes of Multiple Mutants Derived from lax3, lbd16, and lbd18 and Gene Expression Analysis

To investigate whether LBD16 and LBD18 are genetically linked to LAX3 in controlling LR development, we generated single, double, and triple mutants and analyzed LR phenotypes of these mutants. As shown in Figure 1, A and B, the mean ± se numbers of emerged LRs in lax3 lbd16 (1.83 ± 0.06) and lax3 lbd18 (1.86 ± 0.07) double mutants were slightly lower than in single mutants (lax3, 2.03 ± 0.08; lbd16, 2.1 ± 0.09; lbd18, 2.03 ± 0.08). The number of emerged LRs in lax3 lbd16 lbd18 triple mutants (0.65 ± 0.09) was considerably lower than that in lax3 lbd16 lbd18 (1.83 ± 0.06) and lax3 lbd18 (1.86 ± 0.07) double mutants, but to a lesser degree than that of lbd16 lbd18 double mutants (1.07 ± 0.09). The process of LRP development is divided by eight stages defined by specific anatomical characteristics and cell divisions (Malamy and Benfey, 1997). It has been previously shown that the lax3 mutation significantly increases the LRP number at stage I (Swarup et al., 2008), whereas the numbers at all LRP developmental stages in lbd16 or lbd18 single and double mutants were similar to those in the wild type (Lee et al., 2009b). The LRP numbers of lax3 at stage I (2.63 ± 0.12) were mitigated in lax3 lbd16 (1.80 ± 0.06) and lax3 lbd18 (1.77 ± 0.08) double mutants, and in lax3 lbd16 lbd18 (1.58 ± 0.06) triple mutants (Fig. 1C). These results indicated that LR development in lax3 mutants is dependent on LBD16 and LBD18 function.
Quantitative reverse transcription (qRT)-PCR analyses showed that the lax3 mutation significantly reduced expression of LBD16 and LBD18 in response to auxin after a 4- or 8-h treatment (Fig. 2A). Moreover, auxin-induced expression of the LBD18 target genes, EXP14 and EXP17 (Lee et al., 2013a; Lee and Kim, 2013), in the lax3 mutant decreased compared with that in the wild type (Fig. 2B). However, LAX3 expression with auxin was not affected by lbd16 or lbd18 single or double mutations (Fig. 2C). These results indicated that LAX3 acts upstream of LBD16 and LBD18 during the auxin response. GUS staining of ProLBD16:GUS, ProLBD18:GUS (Lee et al., 2009b), and ProLAX3:GUS (Swarup et al., 2008) transgenic Arabidopsis demonstrated that LBD18 and LAX3 were coexpressed in the cortex and epidermis from stages I to VII during LRP development and after the emergence of LRs (Supplemental Fig. S1). Although strong GUS staining was detected in the LRPs and emerged LRs of ProLBD16:GUS and ProLBD18:GUS Arabidopsis, we did not detect GUS staining in the same tissues of ProLAX3:GUS Arabidopsis (Supplemental Fig. S1). The overlapping expression pattern of LBD18 and LAX3 in the overlying tissues (Supplemental Fig. S1) and the decrease in the LBD18 transcript levels by the lax3 mutation (Fig. 2A) are consistent with the observation that GUS expression under the control of the LBD16 promoter was reduced in lax3 mutants compared with that in the wild type in the overlying tissues as well as in the LRP without or with auxin treatment (Fig. 2, D–G). In contrast, we only noted reduced GUS expression under the control of the LBD16 promoter in lax3 mutants compared with that in the wild type in the LRP with auxin treatment (Fig. 2, H–K). These results indicated that LAX3 promotes LBD18 expression in the overlying tissues.

**LBD18 Induces LR Formation and Expression of the Target Genes, EXP14 and EXP17, in lax3 Mutants**

To demonstrate that LBD18 functions downstream of LAX3 for LR emergence, we constructed dexamethasone (DEX)-regulated LBD18 transgenic Arabidopsis in the lax3 mutant background (Pro35S:LBD18:GR/lax3) by crossing these transgenic plants and the lax3 mutant. As shown in Figure 3A, LR densities in Pro35S:LBD18:GR/lax3 were rescued to the wild-type levels by DEX treatment for both 7 and 10 d, as compared with what was observed in the absence of DEX and in both the presence and absence of DEX in the lax3 mutants. EXP14 and EXP17 are up-regulated by LBD18 during the auxin response (Lee et al., 2013a; Lee and Kim, 2013). Using qRT-PCR analysis, we showed that EXP14 and EXP17 expression was greatly induced in the lax3 mutants by DEX treatment of Pro35S:LBD18:GR/lax3 plants for 2 and 8 h (Fig. 3B). These results demonstrated that LBD18 controls LR formation downstream of LAX3. However, LR formation by LBD18 in the lax3 mutants under the control of the LBD18 promoter requires exogenous auxin treatment (Fig. 3C, Supplemental Fig. S2), indicating that lax3 mutation caused defects in LR formation, which could
not be rescued by LBD18 normally expressed under its own promoter but could be complemented by ectopic overexpression of LBD18. We further showed that LBD18 expression under the control of the LBD18 promoter induced expression of EXP14 and EXP17 in lax3 mutants at levels similar to those that can be gained by LBD18 expression in the wild-type background (Fig. 3, D and E). These results together with genetic and gene expression analysis data (Figs. 1 and 2) demonstrated that LBD18 regulates expression of EXP14 and EXP17 downstream of LAX3 during the auxin response.

**LBD18 Up-Regulates POLYGALACTURONASE Expression as a Secondary Response**

POLYGALACTURONASE (PG) is induced by auxin, and this depends on proper auxin influx regulated by LAX3 (Swarup et al., 2008). PG enzymes cleave demethylated pectin, a substrate enriched in the middle lamella of root cells overlaying LRP, and are important for cell separation during LR emergence (Hadfield and Bennett, 1998; Laskowska et al., 2006; González-Carranza et al., 2007). We investigated whether PG is regulated by LBD16 and/or LBD18. We generated ProPG::GUS in the lbd16, lbd18, or lbd16 lbd18 mutant backgrounds and conducted GUS expression analyses (Fig. 4A). GUS staining detected in overlaying tissues of the LRP was significantly reduced in lbd18 compared with that in the wild type, but not in lbd16 (Fig. 4A). GUS staining in lbd16 lbd18 double mutants was similar to that in lbd16 single mutants. Consistent with these observations, qRT-PCR analysis of the GUS transcripts confirmed that the lbd18 mutation decreased GUS expression of ProPG::GUS, but the lbd16 mutation did not (Fig. 4B). DEX treatment of Pro35S:LBD18:GR plants resulted in a sustained increase in the PG transcript levels after 4 h of treatment (Fig. 4C), whereas the same treatment of Pro35S::LBD16:GR plants did not alter the PG transcript levels until 48 h of treatment. PG expression in Pro35S::LBD18:GR plants was not induced by DEX treatment in the presence of cycloheximide after 8 and 12 h of incubation (Fig. 4D), indicating that PG is a secondary response gene of LBD18. We further demonstrated that LBD18 up-regulates PG expression in tissues where LBD18 is normally expressed (Fig. 4E).
Analysis of LR Phenotypes of Multiple Mutants Derived from aux1, lax3, lbd16, and lbd18

AUX1 facilitates LR initiation by promoting auxin accumulation in the root apex, and later LR emergence through the uptake in the LRP (Marchant et al., 2002; Swarup et al., 2008). Strong GUS expression in the LRP and emerged LRs of ProLBD16::GUS and ProLBD18::GUS (Supplemental Fig. S1) overlaps with that of ProAux1::GUS (Marchant et al., 2002), indicating that a potential link may exist between LBD18 and AUX1 during LR development. To investigate a functional link between LBD16/LBD18 and AUX1 for LR development, we generated multiple mutants derived from lbd16, lbd18, and aux1-22 and analyzed LR initiation, LRP development, and LR emergence of these multiple mutants. As shown in Figure 5, A and B, the numbers of emerged LRs in aux1 lbd16 (1.08 ± 0.09), aux1 lbd18 (1.11 ± 0.07) double mutants, and aux1 lbd16 lbd18 (0.35 ± 0.06) triple mutants decreased additively compared with that of corresponding single mutants (aux1, 1.8 ± 0.06; lbd16, 1.61 ± 0.12; and lbd18, 1.86 ± 0.08) and double mutants (lbd16 lbd18, 0.65 ± 0.08), indicating that LBD16/LBD18 and AUX1 combinatorially contribute to LR emergence. In contrast, the numbers of primordia at the initiation stage in aux1 lbd16 (0.86 ± 0.04) and aux1 lbd18 (0.88 ± 0.03) double mutants increased compared with that in aux1 (0.57 ± 0.07), but decreased compared with that in lbd16 (1.17 ± 0.07) or lbd18 (1.11 ± 0.04; Fig. 5C), indicating

![Image](https://example.com/image.png)

Figure 3. Induction of LRs in lax3 mutants by LBD18:GR with DEX treatment. A, LR densities of Pro35S::LBD18:GR/lax3 plants. Plants were grown vertically for 7 or 10 d in the absence or presence of 10 μM DEX, and LR numbers per unit root length (#/cm) measured were plotted. Asterisks denote statistical significance (**, P < 0.01). DAG, Days after germination. B, Expression analysis of EXP14 and EXP17 in the wild type (WT), lax3, and Pro35S::LBD18:GR/lax3 plants. Seven-day-old seedlings were incubated with DEX for 2 or 8 h in the light and subjected to qRT-PCR. -8 h, Samples that were incubated with mock treatment for 8 h. Data are the mean ± SE of three independent biological replications. Asterisks denote statistical significance (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). C, The effects of auxin on LR densities of Pro35S::LBD18:GR/lax3 plants. Plants were grown vertically for 7 d in the absence or presence of 10 μM DEX and 0, 0.05, or 0.1 μM auxin 2,4-dichlorophenoxyacetic acid (2,4-D), and LR numbers per unit root length (#/cm) measured were plotted. Asterisks denote statistical significance (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). D, Expression analysis of EXP14 and EXP17 in the wild type, lax3, and Pro35S::LBD18:GR/lax3 plants. Plants were grown for 7 d in the absence or presence of 10 μM DEX and subjected to qRT-PCR. Data are the mean ± SE of three independent biological replications. Asterisks denote statistical significance (*, P < 0.05). E, Expression analysis of EXP14 and EXP17 in the wild type, aux1, lax3, aux1 lax3, and Pro35S::LBD18:GR/lax3 plants. Plants were grown for 7 d in the absence or presence of 10 μM DEX and subjected to qRT-PCR. Data are the mean ± SE of three independent biological replications. Asterisks denote statistical significance (*, P < 0.05).
that the aux1 phenotype requires LBD16 and LBD18 gene function during LR initiation and at early stages of the LRP development. The aux1 lbd16 lbd18 triple mutants (0.68 ± 0.04) exhibited a further decreased LRP number compared with that of aux1 lbd16 or aux1 lbd18 mutants. At stage II, lbd16 and lbd18 mutations in aux1 rescued the LRP number reduced by aux1 mutation to the wild-type levels. Similar patterns were observed at stages II to VI, albeit the aux1 impact on the LRP numbers was small. These results indicated that LBD16 and LBD18 function together with AUX1 to control LR initiation and early LRP development.

To define a combinatorial role of AUX1 and LAX3 in LBD16 and LBD18 function during LR development, we further generated lbd16 and lbd18 mutations in aux1 lax3 double mutants. Double mutations in AUX1 and LAX3 dramatically reduced numbers of emerged LRs (0.06 ± 0.11) compared with that of single mutants (aux1, 1.8 ± 0.06; and lax3, 2.03 ± 0.08; Figs. 1B and 5, B and D), consistent with a previous report (Swarup et al., 2008). The lbd16 (0.03 ± 0.02 for

Figure 4. LBD18 activates PG expression. A, GUS expression analysis of ProPG:GUS in the wild type (WT), lbd16, lbd18, or lbd16 lbd18 double mutant backgrounds. Seven-day-old light-grown seedlings were subjected to GUS staining. Scale bars = 50 μm. B, qRT-PCR analysis of ProPG:GUS in the wild type, lbd16, lbd18, or lbd16 lbd18. Seven-day-old seedlings were subjected to qRT-PCR for GUS expression. Relative transcript levels are plotted. Data are the mean ± se of three independent biological replications. Asterisks denote statistical significance (**, P < 0.01). C, Time course expression analysis of PG in Pro35S:LBD16:GR and Pro35S:LBD18:GR plants. Seven-day-old plants were incubated without or with DEX for the indicated time and subjected to qRT-PCR for PG expression. Relative transcript levels are plotted. Data are the mean ± se of three independent biological replications. D, The effect of cycloheximide (CHX) treatment on DEX-induced expression of PG. Bars represent se. Data are the mean ± se of three independent biological replications. Asterisks denote statistical significance (*, P < 0.05; and **, P < 0.01). E, Expression of PG following treatment with DEX in Pro35S:LBD18:GR plants. Pro35S:LBD18:GR seedlings were grown for 7 d in the absence (black squares) or presence (white squares) of DEX. Expression of PG was then analyzed by qRT-PCR and plotted as relative expression levels. Ubiquitin-Conjugating enzyme E2 (UBC) was used as a negative control. Asterisk denotes statistical significance (*, P < 0.05).
7 d; and 1.57 ± 0.13 for 14 d) or lbd18 (0.20 ± 0.09 for 7 d; and 2.03 ± 0.18 for 14 d) single mutation in aux1 lax3 double mutants further reduced the emerged LR numbers of aux1 lax3 mutants (0.60 ± 0.11 for 7 d; and 2.64 ± 0.18 for 14 d), and the lbd16 lbd18 (0.03 ± 0.02 for 7 d; and 0.38 ± 0.07 for 14 d) double mutations additively reduced the emerged LR number of aux1 lax3 mutants, which is due to the independent roles of AUX1, LBD16, and LBD18 during LR emergence (Fig. 5D; Supplemental Fig. S3). Numbers of primordia of aux1 lax3 double mutants at stage I were not significantly altered by lbd16 and lbd18 single or double mutations, whereas those numbers from stages III to VII were significantly reduced by the lbd16 mutation (Fig. 5E). The lbd18 or lbd16 lbd18 mutation greatly reduced the LR numbers from stages V to VII. These results indicated that both LBD16 and LBD18 control LR development in combination with LAX3 and AUX1 but at different stages after stage III and after stage V, respectively.

AUX1 and LAX3 Are Involved in Auxin-Responsive Expression of LBD16 and LBD18

qRT-PCR analysis of auxin-responsive expression of LBD16 or LBD18 in aux1 mutants showed that the aux1 mutation did not alter LBD16 expression, but decreased LBD16 expression slightly after 8 h of auxin treatment (Fig. 6A). However, aux1 lax3 double mutations reduced the expression of LBD16 and LBD18 by approximately 50% in response to auxin after a 4- or 8-h treatment compared with those of wild-type and aux1 or lax3 mutants (Figs. 2A and 6B). These results show that both LAX3 and AUX1 are involved in auxin-responsive expression of LBD16 and LBD18. These
qRT-PCR data are consistent with the observation that aux1 mutation reduced GUS expression in the primordium under the control of the LBD16 or LBD18 promoter in response to auxin compared with that in the wild type (Fig. 6, C–J).

Expression of LBD18:SRDX in lbd18 Mutant Caused Inhibition of LR Initiations, Periclinal Divisions in the Initiated LRP, and Progression of LRP Development

The present genetic analyses with multiple mutants consisting of aux1, lax3, and lbd18 indicated that LBD18 is involved in controlling LR initiation and LRP development. Our previous study showed that any alterations in the numbers of the LRPs from stages I to VII were not detected in lbd18 compared with those in the wild type (Lee et al., 2009b). To further investigate the role of LBD18 during LR initiation and LRP development, we constructed transgenic Arabidopsis expressing LBD18 fused to an SRDX repression domain under its own promoter and analyzed LR phenotypes. The SRDX chimeric repressors cause dominant repression of target genes, allowing the functional analysis of plant transcription factors with genetic redundancy (Hiratsu et al., 2003). This approach has been previously utilized to reveal a unique function of LBD16 in the polar nuclear migration prior to LR initiation (Goh et al., 2012a). A complete block of LR formation has been observed with ProLBD16:LBD18:SRDX transgenic plants in the wild-type background (Goh et al., 2012a). We also observed the same phenotype with ProLBD16:LBD16:SRDX transgenic plants in wild-type as well as in the lbd16 mutant backgrounds (Supplemental Fig. S4). In contrast, ProLBD16:LBD18:SRDX transgenic plants in the wild-type background displayed wild-type appearance with normal LR formation (Fig. 7A), whereas ProLBD16:LBD18:SRDX transgenic plants in the lbd18 mutant background exhibited more reduced LR formation compared with that of the lbd18 mutant (Fig. 7, B and C). Consistent with these observations, a significant dominant repression of EXP14, a direct target of LBD18 (Lee et al., 2013a), by LBD18:SRDX was found in both the absence and presence of auxin in the lbd18 mutant (Fig. 7D). None of eight ProLBD16:LBD18:SRDX lines analyzed showed a complete block of LR formation, unlike ProLBD16:LBD16:SRDX transgenic plants. Determination of LRP numbers at all developmental stages of ProLBD16:LBD18:SRDX lines showed wild-type distribution of LRPs (Supplemental Fig. S5). We then conducted an LR induction experiment, which allows the direct analysis of LR development kinetics as opposed to the analysis of the LRPs and LRs at a given plant developmental stage (Péret et al., 2012). We applied a gravitropic stimulus inducing initiation of LR to wild-type plants grown vertically for 30 h by rotating the agar plate through 90°. We then measured the number of newly developed LRPs on the convex side of the curves after 18 or 42 h of gravitropic induction and determined the relative distribution of the LRPs at stages I to VIII. We found that ProLBD16:LBD18:SRDX lines displayed 40% block of LR initiation events compared with those of the wild type.
and lbd18 mutant 18 h postgravitropic induction (pgi) in LR induction experiments with a gravitropic stimulus (Fig. 7E). Moreover, distribution of the LRP's at stages I to VIII showed that developmental transition from stage I to II was significantly inhibited 18 h pgi and LRP development was significantly delayed 42 h pgi, compared with that of the wild type (Fig. 7F). These results showed that LBD18:SRDX suppressed LR initiation events, periclinal divisions of primordium after LR initiation, and later stages of LRP development in response to a gravitropic stimulus. In contrast, LBD18: SRDX did not suppress auxin-induced LR initiations compared with the wild type and lbd18 mutant (Supplemental Fig. S6), indicating that exogenous auxin overrides dominant repression of LR initiation by LBD18:SRDX, activating LR initiation.
**LBD18 Regulates Expression of Cell Cycle Genes CDKA1;1 and CYCB1;1**

Several lines of evidence suggested that the regulated cell cycle is essential for asymmetric divisions of a subset of pericycle cells during LR initiation (De Smet, 2012). For example, progression of pericycles through the cell cycle is prevented by the treatment of auxin transport inhibitor in Arabidopsis seedlings or in a gain-of-function slr mutant blocking early auxin response (Fukaki et al., 2002; Himanen et al., 2002; Vanneste et al., 2005). Auxin activates many cell cycle-related genes, such as cyclins and CDKs, before pericycle division (Himanen et al., 2002). Various cell cycle genes have been identified to play a role in LR initiation (Himanen et al., 2002, 2004; Vanneste et al., 2005; Ren et al., 2008; Nieuwland et al., 2009; Sanz et al., 2011). To identify a link between LBD18 function and the regulation of cell cycle during LR initiation and LR development, we first made multiple mutants, lbd16 and/or lbd18, crossed to transgenic Arabidopsis harboring the promoter of cell cycle genes, CDKA1;1, CDKB1;1, or CYCB1;1, fused to the GUS reporter gene (Hemerly et al., 1993; Doerner et al., 1996; de Almeida Engler et al., 1999). However, none of the mutations in lbd16, lbd18, or lbd16 and lbd18 altered GUS expression in the LRPs due to the genetic redundancy with other LBD gene family members (Supplemental Fig. S7). We then constructed transgenic Arabidopsis expressing LBD18:SRDX under its own promoter and harboring CDKA1;1, CDKB1;1, or CYCB1;1-GUS in the lbd18 mutant background, and found that LBD18:SRDX greatly repressed GUS expression in the CDKA1;1 promoter but slightly suppressed GUS expression under the CDKB1;1 promoter (Fig. 8A and B). LBD18:SRDX completely removed GUS expression under the CYCB1;1 promoter in the primordium and greatly reduced GUS staining in the root tip (Fig. 8C). LBD16:SRDX greatly reduced GUS expression under the CDKB1;1 promoter in the root (Fig. 8B). However, LBD16:SRDX did not affect GUS expression under the CDKA1;1 promoter (Fig. 8A). These results showed that LBD18:SRDX repressed the promoter activities of cell cycle genes, CDKA1;1 and CYCB1;1. We further showed that LBD18:GR up-regulates expression of CDKA1;1 and CYCB1;1, but not that of CDKB1;1, under the control of the Cauliflower mosaic virus 35S promoter (Fig. 8D) and under the control of the LBD18 promoter (Fig. 8E). These results indicated that LBD18 plays a role in the regulation of cell cycle genes. Cycloheximide treatment of Ppro35S: LBD18:GR transgenic plants in the presence of DEX prevented DEX-inducible expression of CDKA1;1 and CYCB1;1 (Supplemental Fig. S8), demonstrating that induction of these cell cycle genes by LBD18 is a secondary response requiring new protein synthesis.

**DISCUSSION**

Emerged LR densities of lax3 lbd16 and lax3 lbd18 were similar to those of lax3, lbd16, or lbd18 single mutants (Fig. 1B). However, the lax3 mutation in lbd16 lbd18 double mutants significantly reduced the emerged LR density to some extent, but not in an additive manner (Fig. 1B). These results indicate that, although LAX3 and LBD16 or LBD18 function largely in a linear pathway to control LR emergence, LAX3 also acts in parallel with LBD16 and LBD18 to some extent during LR emergence. Auxin-inducible expression of LBD16 and LBD18 and LBD18-regulated target genes, EXP14 and EXP17, significantly decreased in lax3 mutants as compared with the wild type (Fig. 2, A and B). In contrast, double mutations in LBD16 and LBD18 did not affect the expression of LAX3 in response to auxin (Fig. 2C). Altogether, genetic and gene expression analysis results indicate that LAX3 acts in part upstream of LBD16 and LBD18 during the auxin response, whereas LBD16 and LBD18 are not critically involved in auxin-responsive LAX3 expression. Consistent with this, overexpression of LBD18 in lax3 fully restored the defect in LR emergence of lax3 to the wild-type levels with concomitant overexpression of LBD18 target genes, EXP14 and EXP17 (Fig. 3, A and B). However, in the case of using the LBD18 promoter to express LBD18 in lax3, the defect in LR formation in lax3 was rescued to some extent, although only in the presence of auxin. This result indicates that lax3 mutation produced some defects in LR formation that are caused by other components and/or transcription factors acting downstream of LAX3 as well as the defect in LBD18 function under the LBD18 promoter. Auxin activates the signaling pathway, inducing expression of certain transcription factor genes along with which LBD18 acts to promote LR formation to a certain degree in the absence of lax3 function. We further showed that LBD18 up-regulates expression of EXP14 and EXP17 in lax3 mutants under the control of the LBD18 promoter (Fig. 3, D and E), indicating that LBD18 can regulate its target gene expression in the absence of LAX3 function in tissues where LBD18 is normally expressed. Expression of PG, encoding a cell wall remodeling enzyme, whose expression is dependent upon LAX3 function (Swarup et al., 2008), was down-regulated in the overlaying tissues of the LRP in lbd18 and up-regulated by LBD18 expression under the control of the Cauliflower mosaic virus 35S promoter and under the control of the LBD18 promoter (Fig. 4). Taken together, these results demonstrated that LBD18 modulates its target gene expression downstream of LAX3 to promote LR emergence. Overlapping expression patterns of LAX3 and LBD18 in the overlaying tissues of the LRP (Supplemental Fig. S1) and reduced GUS expression under the control of the LBD18 promoter by lax3 mutation in the same tissue (Fig. 2, D-G) are consistent with the notion that LAX3 induces LBD18 expression by promoting auxin influx into the cortex and epidermis to facilitate LR emergence. We noted significant auxin-inducible expression of LBD16, LBD18, EXP14, and EXP17 in lax3, which may be due to involvement of other auxin transporters. Two additional LAX genes, LAX1 and LAX2.
Kerr and Bennett, 2007; Ugartechea-Chirino et al., 2010), could be involved redundantly in auxin-responsive expression of LBD16 and LBD18. Several studies have demonstrated that PINs are involved in LR development (Benková et al., 2003; Laskowski et al., 2008; Marhavý et al., 2013; Péret et al., 2013). However, it remains to be determined whether other LAXs and PINs are involved in auxin-responsive expression of LBD16 and LBD18 to control LR development.

It has been reported that the increased number of primordia at stage I in lax3 (Fig. 1C) was due to accumulation of leaf-derived auxin at supraoptimal levels.
in the root pericycle, thus stimulating the ectopic initiation of new primordia in lax3, but not due to the defects in LR initiation (Fig. 5C; Swarup et al., 2008). We found that lbd18 mutation reduced the primordium number at stage I, which was exaggerated by lax3 mutation (Fig. 1C). This result indicates involvement of LBD18 in LR initiation. A previous study showed that lbd18 mutation did not affect the distribution of the LRP s at all developmental stages, but significantly reduced the number of emerged LRs as compared with the wild type, indicating that the emergence of LRs in the lbd18 mutant is a rate-limiting step in LR formation compared with the wild type (Lee et al., 2009b). The present genetic analysis results with multiple mutants of aux1 or lax3 provided the evidence that LBD18 is involved in LR initiation and LRP development in addition to playing a role in LR emergence (Figs. 1 and 5). This notion was further confirmed by the analysis of LR development kinetics using transgenic Arabidopsis expressing LBD18:SRDX under its own promoter in the lbd18 mutant background (Fig. 7). We found that LBD18 target gene expression and LR formation by LBD18:SRDX were not affected in wild-type plants when compared with that in the lbd18 mutant background, indicating that wild-type LBD18 homo dimers (Lee et al., 2013b) might have higher DNA-binding affinity to the target promoter and/or higher transcriptional activity than LBD18:SRDX homodimers and thus can overcome dominant repression by LBD18:SRDX.

A model integrating the present data and previous reports (Marchant et al., 2002; Okushima et al., 2007; Swarup et al., 2008; Lee et al., 2009a,b, 2013a; Berckmans et al., 2011; Goh et al., 2012a; Lee and Kim, 2013) into a gene regulatory pathway for LR development is presented in Figure 9. LBD16 plays a role in the polar nuclear migration in the LR founder cell after LR prim ing (Goh et al., 2012a), whereas LBD18 is involved in asymmetric anticlinal division during LR initiation and periclinal divisions of the LRP initiated in the xylem pole pericycle cells and in the progression of LRP development through the regulation of cell cycle genes such as CDKA1;1 and CYCB1;1 (Figs. 7 and 8). LBD18 also activates E2Fa expression for LR initiation (Berckmans et al., 2011). LBD16 and LBD18 function together to regulate LRP development with AUX1 (Figs. 5 and 6). LAX3 activates the SLR/IAA14-ARF7-ARF19 signaling module (Swarup et al., 2008), thus inducing LBD18 expression (Figs. 1–3). LBD18 in the endodermis and cortex in turn directly activates EXP14 and indirectly activates EXP17 and other EXPs (Kim and Lee, 2013; Lee et al., 2013a; Lee and Kim, 2013) for cell wall loosening, and induces PG expression at a later time (Fig. 4) for cell wall remodeling, which facilitates local cell separation. PG enzymatically modifies the structures of the cell wall, rendering it more responsive to wall loosening mediated by expansions (Cosgrove, 2000; Péret et al., 2009a, 2009b). These molecular events help promote the emergence of LRP through the outer cell layers of the primary root. As ARF7 induces expression of ARF19 and LBD16 in

---

**Figure 9.** A model showing the molecular signaling network of LBD16 and LBD18 via the auxin influx carriers AUX1 and LAX3 during LR development. The column shaded with lines indicates the xylem. The thick arrow and dotted arrow indicate direct and indirect activation of target genes, respectively. P, Pericycle; En, endodermis; C, cortex; Ep, epidermis.
response to auxin signal, and subsequently ARF19 induces LBD18 expression (Okushima et al., 2007; Lee et al., 2009a), the ARF7/ARF19-LBD16/LBD18 transcriptional network via the AUX1/LAX3 auxin influx carriers plays a key role in controlling LR development from polar nuclear migration of LR founder cells to initiation, primordium development, and emergence of LR during the auxin response.

**MATERIALS AND METHODS**

**Plant Growth and Tissue Treatment**

Arabidopsis (Arabidopsis thaliana; Col-0) seedlings were grown and treated as described previously (Park et al., 2002). For the treatment of hormone and chemicals, plants were grown in a 16-h photoperiod on 3MM Whatman paper (GE Healthcare) on top of agar plates, and the filter paper with the seedlings was then transferred to a plate containing plant hormone or chemicals, plants were grown in a 16-h photoperiod on 3MM Whatman paper with the filter paper. Mutants and wild-type seeds were sown in small paper plates, and seedlings were grown and treated as described previously (Jeon et al., 2010). All real-time RT-PCR analyses were conducted in triplicate biological replications and subjected to statistical analysis. PCR conditions and primer sequences are shown in Supplemental Table S1.

**RNA Isolation, Reverse Transcription-PCR, and qRT-PCR Analysis**

Following treatment, Arabidopsis plants were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from frozen Arabidopsis using TRI Reagent (Molecular Research Center). For reverse transcription (RT)-PCR analysis, total RNA was isolated using an RNasey plant mini kit (Qiagen) and subjected to RT-PCR analysis with the Access RT-PCR System (Promega) according to the manufacturer’s instructions. Real-time RT-PCR was carried out using a Quantitect SYBR Green RT-PCR kit (Qiagen) in a CFX96 real-time system using a C1000 thermal cycler (Bio-Rad) as described previously (Jeon et al., 2010). All real-time RT-PCR analyses were conducted in triplicate biological replications and subjected to statistical analysis. RT-PCR conditions and primer sequences are shown in Supplemental Table S1.

**Microscopy and Histochimical GUS Assays**

For whole-mount visualization, the seedlings were then cleared in 80% (v/v) ethanol for 24 h and mounted in 90% (v/v) glycerol and observed under the Leica DM2500 microscope with differential interference contrast according to Malamy and Benfey (1997). Histochimical assays for GUS activity were performed with 5-bromo-4-chloro-3-indolyl glucuronide, as described previously (Jefferson and Wilson, 1991). Samples were observed using a DM2500 microscope (Leica) at 200- or 400-fold magnification with differential interference contrast.

**Statistical Analysis**

Quantitative data were subjected to statistical analysis for every pairwise comparison, using the software for Student’s t test (Predictive Analytics Software for Windows, version 20.0).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: LBD16 (AT2g45420), LBD18 (AT1g77690), CYCB1;1 (AT3g54180), EXP17 (At4g37490), GR (At5g56320), and GR/lax3 (At1g77690), PG (At5g14650), CDA11 (At3g48750), CDKB1;1 (At1g54180), CYCB1;1 (At4g37490), EXP14 (At5g56320), and EXP17 (At4g01630).

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** GUS expression of ProLBD16:GUS, ProLBD18:GUS, and ProGRGUS transgenic Arabidopsis during LR development.

**Supplemental Figure S2.** LR densities of wild-type, lax3, and ProLBD16:GR/lax3 plants without or with DEX.

**Supplemental Figure S3.** LR phenotypes of aux1 lax3, aux1 lax3 lbd16, aux1 lax3 lbd16, and aux1 lax1 lbd16 lbd18 plants.

**Supplemental Figure S4.** Analysis of LR formation of ProLBD16:SRDX transgenic plants in Col-0 and lbd16 mutant backgrounds.

**Supplemental Figure S5.** Analysis of the numbers of primordia at different stages of LR development in 7-d-old wild-type (Col-0), lbd18, ProLBD16:SRDX/Col-0, and Proaux1-LBD16:SRDX/lbd18 plants.

**Supplemental Figure S6.** Differential interference contrast images of LR initiations of wild-type (Col-0), lbd18, and Proaux1-LBD16:SRDX/lbd16 plants without or with auxin.

**Supplemental Figure S7.** Analysis of GUS expression of transgenic Arabidopsis harboring the promoter of cell-cycle genes fused to GUS in lbd16, lbd18, or lbd16 lbd18 mutant backgrounds.

Lee et al.
Supplemental Figure S8. The effect of cycloheximide treatment on DEK-induced expression of CDKA1;1 or CycB1;1 in Pro_AUX::LB16:GR transgenic Arabidopsis.

Supplemental Table S1. Oligonucleotides and PCR conditions.

ACKNOWLEDGMENTS

We thank Dr. Bennett for las3, aux1–21, LAX3;GUS, and PG;GUS seeds and Dr. De Veylder for the CDKA1;1::GUS, CDKB1;1::GUS, and CYCB1;1::GUS seeds, and Arabidopsis Biological Resource Center for transfer DNA insertion mutants.

Received April 20, 2015; accepted June 8, 2015; published June 9, 2015.

LITERATURE CITED


Networks of LB16 and LB18 with AUX1 and LAX3.

1805


Copyright © 2015 American Society of Plant Biologists. All rights reserved.

Downloaded from on January 30, 2018 - Published by www.plantphysiol.org


