Running head: *LBD18/ASL20 Regulates Arabidopsis Lateral Root Formation*

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LBD18/ASL20 Regulates Lateral Root Formation in Combination with LBD16/ASL18 Downstream of ARF7 and ARF19 in Arabidopsis

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ABSTRACT

The LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE (LBD/ASL) genes encode proteins harboring a conserved amino acid domain, referred to as the LOB domain. While recent studies have revealed developmental functions of some LBD genes in Arabidopsis and in crop plants, the biological functions of many other LBD genes remain to be determined. In this study, we have demonstrated that lbd18 mutant evidenced a reduced number of lateral roots and lbd16 lbd18 double mutants exhibited a dramatic reduction in the number of lateral roots compared to lbd16 or lbd18. Consistent with this observation, significant GUS expression in ProLBD18:GUS seedlings was detected in lateral root primordia as well as in the emerged lateral roots. Whereas the numbers of primordia of lbd16 or lbd18 or lbd16 lbd18 mutants were similar to those observed in the wild-type, the numbers of emerged lateral roots of lbd16 and lbd18 single mutants were reduced significantly. lbd16 lbd18 double mutants exhibited additively reduced numbers of emerged lateral roots compared to single mutants. This finding indicates that LBD16 and LBD18 may function in the initiation and the emergence of lateral root formation via a different pathway. LBD18 was shown to be localized into the nucleus. We determined whether LBD18 functions in the nucleus by using a steroid regulator inducible system in which the nuclear translocation of LBD18 can be regulated by dexamethasone (DEX) in wild-type, lbd18, and lbd16 lbd18 mutant backgrounds. Whereas LBD18 overexpression in wild-type backgrounds induced lateral root formation to some degree, other lines manifested the growth-inhibition phenotype. However, LBD18 overexpression rescued lateral root formation in lbd18 and lbd16 lbd18 mutants without inducing any other phenotypes. Further, we demonstrate that LBD18 overexpression can stimulate lateral root formation in arf7 arf19 mutants with blocked lateral root formation. Taken together, our results suggest that LBD18 functions in the initiation and
the emergence of lateral roots, in conjunction with \textit{LBD16}, downstream of \textit{ARF7} and \textit{ARF19}. 
INTRODUCTION

The *LBD/ASL* genes (hereafter referred to as *LBD*) encode proteins harboring an LOB (lateral organ boundaries) domain, which is a conserved amino acid domain that is detected only in plants, indicative of its function in plant-specific processes (Iwakawa et al., 2002; Shuai et al., 2002). There are 42 Arabidopsis *LBD* genes, which have been assigned to 2 classes. Class I comprises 36 genes and class II comprises 6 genes (Iwakawa et al., 2002; Shuai et al., 2002). The class I proteins harbor LOB domains similar to those observed in the LOB protein, whereas the class II proteins are less similar to the class I proteins which include the LOB domain, as well as regions outside of the LOB domain. The LOB domain is approximately 100 amino acids in length and harbors a conserved 4-Cys motif with a CX₂CX₆CX₃C spacing, a Gly-Ala-Ser (GAS) block, and a predicted coiled-coil motif with a LX₆LX₃LX₆L spacing, reminiscent of the Leu-zipper found in the majority of class I proteins (Shuai et al., 2002). None of the class II proteins were predicted to form coiled-coil structures.

Although we currently understand very little about the biological roles of the *LBD* genes, there have been some reports describing the developmental functions of *LBD* genes in Arabidopsis on the basis of gain-of-function studies. The gain-of-function mutants of *LBD36/ASL1*, designated as *downwards siliques1* (*dsl1-D*), showed shorter internodes and downward lateral organs such as flowers (Chalfun-Junior et al., 2005). Although the *lbd36* loss-of-function mutants did not show morphological phenotypes, the analysis of *lbd36 as2* double mutants showed that these 2 members act redundantly to control cell fate determination in the petals. Another Arabidopsis gain-of-function mutant, *jagged lateral organs-D* (*jlo-D*), generates strongly lobed leaves and the shoot apical meristem prematurely arrests organ initiation, terminating in a pin-like structure (Borghi et al., 2007). During embryogenesis, *JLO* (=*LBD30/ASL19*) is necessary for the initiation of cotyledons and development beyond...
the globular stage. The results of misexpression experiments indicate that during postembryonic development, JLO function is required for the initiation of plant lateral organs. Recent study showed that the LOB domain of AS2 cannot be functionally replaced by those of other members of the LOB family, indicating that dissimilar amino acid residues in the LOB domains are important for characteristic functions of the family members (Matsumura et al., 2009).

Thirty-five \textit{LBD} genes in rice have been identified from the genome sequences of the 2 rice subspecies-a \textit{japonica} rice (Nippobare) and an \textit{indica} rice (9311) (Yang et al., 2006). Analyses of rice mutants have provided evidence of the involvement of a variety of rice \textit{LBD} genes in lateral organ development. \textit{CROWN ROOTLESS1 (CRL1)}, encoding a LBD protein, is crucial for crown root formation in rice (Inukai et al., 2005). The \textit{crl1} mutant showed auxin-related phenotypes, such as decreased lateral root number, auxin insensitivity in lateral root formation, and impaired root gravitropism. A rice AUXIN RESPONSE FACTOR (ARF) appears to directly regulate \textit{CRL1} expression in the auxin signaling pathway (Inukai et al., 2005). \textit{ADVENTITIOUS ROOTLESS1 (ARL1)} encodes an auxin-responsive protein with an LOB domain that controls the initiation of adventitious root primordia in rice, and turned out to be the same gene as \textit{CRL1} (Liu et al., 2005).

Lateral roots of Arabidopsis are derived from a subset of the pericycle cells (pericycle founder cells), which are positioned at the xylem poles within the parent root tissues (Casimiro et al., 2003). The mature pericycle cells dedifferentiate to form lateral root primordium (LRP), which undergoes consistent anticlinal and periclinal cell divisions to generate a highly organized LRP (Malamy and Benfey, 1997). The LRP emerges from the parent root via cell expansion, and the activation of the lateral root meristem results in continued growth of the organized lateral root. A growing body of physiological and genetic evidence has been collected to suggest that auxin plays a profound role in lateral root
formation. For example, many auxin-related mutants have been shown to affect lateral root formation (Casimiro et al., 2003). It was demonstrated that lateral root formation in Arabidopsis was shown to be regulated by ARF7 and ARF19 via the direct activation of $LBD_{16}$ and $LBD_{29}$/ASL16 (Okushima et al., 2007). Overexpression of $LBD_{16}$ and $LBD_{29}$ induced lateral root formation in the absence of ARF7 and ARF19, and the dominant repression of $LBD_{16}$ inhibited lateral root formation, thus suggesting that these LBDs function downstream of ARF7- and ARF19-mediated auxin signaling during lateral root formation. The results of selection and binding assays demonstrated that a truncated LOB protein harboring only the conserved LOB domain can preferentially bind to unique DNA sequences, which is indicative of a DNA-binding protein (Husbands et al., 2007). Recently, $LBD_{18}$ was shown to regulate tracheary element differentiation (Soyano et al., 2008).

In the present study, we demonstrated that $LBD_{18}$ is involved in the regulation of lateral root formation, based on the analysis of loss-of-function mutants and the complementation of $lbd_{18}$ and $lbd_{16} lbd_{18}$ mutants by DEX-inducible $LBD_{18}$ expression. Double mutations in $LBD_{16}$ and $LBD_{18}$ resulted in a synergistic reduction in the number of lateral roots, particularly in the initiation and the emergence, compared to that of either $lbd_{16}$ or $lbd_{18}$ single mutants. This finding is suggestive of a combinatorial interaction of $LBD_{16}$ and $LBD_{18}$ in the process of lateral root formation. $LBD_{18}$ expression in $arf_{7}$ and $arf_{19}$ mutants by the DEX-inducible system increases the number of lateral roots, thus demonstrating that $LBD_{18}$ functions downstream of $ARF_{7}$ and $ARF_{19}$ in lateral root formation.
RESULTS

Phenotype Analysis of lbd16, lbd18, and ld16 lbd18 Mutants

In an earlier study, we constructed a transgenic Arabidopsis that expressed the GR-fused stabilized-iaa1 protein harboring an amino acid change in domain II (Park et al., 2002). DEX treatment of this transgenic Arabidopsis evoked dramatic auxin-related phenotypes and repressed the auxin induction of a variety of Aux/IAA genes (Park et al., 2002; Ku et al., 2009). We previously assessed the effects of DEX-inducible iaal on auxin-regulated gene expression, focusing on early genes, with the Affymetrix full genome array, and subsequently identified a transcriptome downstream of iaal during the auxin response (Lee et al., 2009). In order to characterize the biological functions of auxin response genes downstream of iaal, we analyzed a variety of Arabidopsis T-DNA insertion mutants, but none of them evidenced notable morphological phenotypes. Then, we constructed double mutants of similarly classified single mutants and examined auxin-related phenotypes (data not shown). We found that among them, lbd16-1 lbd18-1 double mutants exhibited significantly reduced numbers of lateral roots. Fig. 1, A and B show the schematic view and the results of the PCR analysis of the homozygous T-DNA insertion mutants of lbd16-1 and lbd18-1 single mutants. Fig. 1C shows the results of RT-PCR analysis of homozygous T-DNA insertion mutants of lbd16-1 and lbd18-1, as well as lbd16-1 lbd18-1. We examined the auxin-related phenotypes and morphological changes of these single mutants, but detected no significant phenotypes except for the change in lateral root formation. One example demonstrates the insignificant difference in root growth inhibition between these mutants and the wild-type plants, with varying concentrations of indole-3-acetic acid (IAA) (Supplemental Fig. S1). However, we did note a significant reduction in lateral root number for lbd18-1 as well as lbd16-1 when
compared to the wild-type plants at 5 and 8 d after germination (DAG) (Fig. 2, A-C). The
lbd16-1 lbd18-1 double mutants showed substantially reduced numbers of lateral roots
compared to lbd16-1 and lbd18-1 under a dissecting microscope. The addition of auxin 2,4-
dichlorophenoxyacetic acid (2,4-D) to the lbd16-1, lbd18-1, and lbd16-1 lbd18-1 mutants
rescued the reduced lateral root number to wild-type levels (Fig. 2D). These results indicate
that LBD18, in combination with LBD16, is involved in lateral root formation during the
auxin response.

**GUS Expression Patterns of ProLBD16:GUS and ProLBD18:GUS Transgenics**

It has been previously reported that in ProLBD16:GUS seedlings, strong GUS activity was
detected in the root stele and the lateral root primordia (Okushima et al., 2007). We
additionally assessed the expression of GUS at three different stages of lateral primordium
formation (Malamy and Benfey, 1997) and during lateral root emergence in transgenic
Arabidopsis harboring the 1.4 kbp LBD16 promoter-GUS fusion construct (ProLBD16:GUS) that
we generated (Fig. 3, A-D). We noted strong GUS expression not only in the root stele (A-D)
and the lateral root primordia (B), but also in the developing (B), emerged (C), and mature
(D) lateral roots in 7-d-old light-grown ProLBD16:GUS seedlings. Upon treatment with auxin,
IAA, strongly enhanced GUS expression was detected in the primary roots and lateral roots
(Fig. 3, A and D, lower panels). We noted intense GUS expression in developing primordia
(Fig. 3B, lower panels). In order to understand the role of LBD18 in lateral root formation, we
generated transgenic Arabidopsis harboring the 2.0 kbp LBD18 promoter-GUS fusion
construct (ProLBD18:GUS) and examined GUS expression during lateral primordium formation
and lateral root emergence (Fig. 3, E-H). In 7-d-old light-grown ProLBD18:GUS seedlings,
significant GUS activity was noted in the root stele (Fig. 3E), similar to what was noted in the
The GUS staining patterns of ProLBD16:GUS seedlings were also assessed for three different stages of lateral root development. GUS activity was observed in the lateral root primordia as well as the emerging lateral root (Fig. 3, F and G). However, no GUS activity was detected in the lateral root stele (Fig. 3H). Treatment with auxin, IAA, resulted in enhanced GUS expression in the primary and lateral roots (Fig. 3, E-H, lower panels). These GUS expression patterns are consistent with the role of LBD18 in lateral root formation during the auxin response. Overlapping GUS expression patterns of ProLBD16:GUS and ProLBD18:GUS during the formation of lateral root primordium and during the lateral root emergence suggest a common function in lateral root initiation and emergence.

Analysis of Primordia and Emerged Lateral Roots in lbd16, lbd18, and lbd16 lbd18 Mutants

Lateral root development can be divided into 4 steps: stimulation and dedifferentiation of pericycle cells, ordered cell division and re-differentiation to generate a highly organized lateral root primordium, emergence of the lateral root primordium via cell expansion, and the activation of the lateral root meristem to permit the continued growth of the organized lateral root (Malamy and Benfey, 1997; Casimiro et al., 2003). In order to determine the step of lateral root formation in which LBD16 and LBD18 act, we enumerated the lateral root primordia and emerged lateral roots of lbd16-1, lbd18-1, and lbd16-1 lbd18-1 mutants by photographing the roots with a light microscope equipped with a camera. As shown in Fig. 4, the numbers of primordia in the lbd16 and lbd18 single or double mutants were similar to the numbers observed in the wild-type. However, the numbers of emerged lateral roots of lbd16 and lbd18 single mutants were significantly reduced as compared to the wild-type. The lbd16 lbd18 double mutants exhibited additively reduced numbers of emerged lateral roots.
compared to the single mutants. We further analyzed the effects of lbd16 and lbd18 mutations on primordium development by counting the numbers of primordia from stage I to stage VIII on the basis of classification made by Malamy and Benfey (1997), showing that primordium development was not significantly affected at every stage examined (Fig. 4B). These results suggest that LBD16 and LBD18 may be involved in the initiation and the emergence of lateral root formation.

**Gain-of-Function Analysis of LBD16 and LBD18**

GFP fusion proteins of LBD16 and LBD18 are localized in the nucleus in protoplasts isolated from Arabidopsis mesophyll cells (Fig. 5). To evaluate the gain-of-function phenotypes of LBD16 and LBD18 in the nucleus, we utilized a steroid regulator inducible system via the fusion of the full-length coding region of LBD16 or LBD18 in-frame with the hormone-binding domain of the glucocorticoid receptor (GR) to generate transgenic Arabidopsis (Pro35S:LBD16:GR or Pro35S:LBD18:GR) in Col-0 wild-type background. As observed in the RNA-gel blot analysis of these transgenic lines, 2 bands corresponding to endogenous LBD16 or LBD18 and LBD16:GR or LBD18:GR transcripts were detected, and the transgenic lines exhibiting high expression ratios of the GR fusion transcripts over the endogenous LBD16 or LBD18 transcripts (indicated by filled triangle) were selected for phenotype analysis (Fig. 6A). DEX treatment significantly stimulated lateral root numbers in all 4 lines of Pro35S:LBD16:GR transgenic plants when compared to the wild-type plants, as well as in the absence of DEX (Fig. 6, B and C). This observation is similar to a previous report describing the stimulation of lateral root formation via LBD16 overexpression in arf7 arf19 mutants (Okushima et al., 2007). In contrast, 2 lines (#14-7 and #38-2) of Pro35S:LBD18:GR transgenic plants in the presence of DEX exhibited severely reduced root
length and lateral root numbers, and the 25-2 line showed some degree of reduction in both root length and lateral root number (Fig. 6, B and D, and Supplemental Fig. S2). When the DEX concentrations were varied from 0.25 to 5 μM with the #38-2 and #25-2 lines, similar results showing dose-response curves were generated (Supplemental Fig. S3). The 13-3 line, which expresses the lowest levels of the \textit{LBD18:GR} transcripts among the lines tested, showed insignificant changes in both root length and lateral root number upon treatment with DEX. These findings demonstrated that the expression levels of the \textit{LBD18:GR} transcripts are correlated with phenotype severity in the roots. However, when we examined the #28-9 line which expresses lower levels of the \textit{LBD18:GR} transcripts than those of both the 14-7 and 38-2 lines, but higher levels of the transcripts than those of the 13-3 line, we noted statistically significant stimulations of lateral root number by various concentrations of DEX (Fig. 6E). These results indicate that appropriate levels of \textit{LBD18} expression may be required for the stimulation of lateral root formation, but higher levels of \textit{LBD18} expression could result in the inhibition of primary and lateral roots. These results also showed that LBD18, as well as LBD16, function in the nucleus to regulate lateral root formation. In addition, consistent with the observed inhibition of root growth with high levels of \textit{LBD18} expression, the hypocotyl lengths of 4 different lines of \textit{Pro35S:LBD18:GR} transgenic plants were reduced significantly by DEX treatment (Fig. 7).

**Overexpression of LBD18 Rescues Lateral Root Formation of lbd18 Single Mutants and lbd16 lbd18 Double Mutants**

Although we have observed statistically significant increases in the numbers of lateral roots in \textit{Pro35S:LBD18:GR} transgenic plants (the #28-9 line) with various concentrations of DEX (Fig. 7E), other lines evidenced severe root growth inhibition phenotypes. To address
this mixed phenotype problem, we attempted to determine whether \textit{LBD18} could rescue the wild-type phenotype in \textit{lbd18} and \textit{lbd16 lbd18} mutants, using \textit{Pro\textsubscript{35S}:LBD18:GR}. Transgenic \textit{lbd18} or \textit{lbd16 lbd18} mutants expressing \textit{LBD18:GR} transcripts were generated by crossing the mutants with \textit{Pro\textsubscript{35S}:LBD18:GR}, as demonstrated by the T-DNA insertions and expression patterns of \textit{LBD18:GR} and \textit{LBD18} (Fig. 8A). Quantitative RT-PCR analysis of the \textit{LBD18} transcripts demonstrated that the transgenic mutant plants express the \textit{LBD18:GR} transcripts to a level comparable to the \textit{LBD18} transcript level of the wild-type plants (Fig. 8B). DEX treatment of \textit{Pro\textsubscript{35S}:LBD18:GR} in the \textit{lbd18} mutant backgrounds resulted in a significant increase in the number of lateral roots (Fig. 8, C and D). A similar level of induction of lateral root formation was observed in the \textit{lbd16 lbd18} double mutant backgrounds. No other significant phenotypes were detected with these transgenic mutants. These results show that \textit{LBD18} is responsible for its mutant phenotype, and also that \textit{LBD18:GR} is functional in lateral root formation. We found that while DEX treatment of \textit{Pro\textsubscript{35S}:LBD18:GR} in \textit{lbd18} mutant backgrounds can complement \textit{lbd18} at almost a wild-type level, that of \textit{Pro\textsubscript{35S}:LBD18:GR} in \textit{lbd16 lbd18} can induce lateral root formation at the same level as can be achieved by DEX treatment of \textit{Pro\textsubscript{35S}:LBD18:GR} in \textit{lbd18}, but cannot fully complement the \textit{lbd16 lbd18} double mutants. These findings suggest that while \textit{LBD16} and \textit{LBD18} play roles in lateral root formation, they may also perform a distinctive role in a different pathway.

**\textit{LBD18} Induces the Formation of Lateral Roots in \textit{arf7 arf19} Mutants**

The results of our earlier study demonstrated that the auxin-upregulated expression of \textit{LBD16}, \textit{LBD18}, and \textit{LBD29} genes was repressed dramatically by DEX treatment and were inhibited completely by double mutations of \textit{ARF7} and \textit{ARF19}, thereby indicating that these \textit{LBD} genes might be regulated by \textit{ARF7} and \textit{ARF19} in auxin signaling (Lee et al., 2009). It
has also been previously reported that LBD16 and LBD29 overexpression induces lateral root formation in the absence of ARF7 and ARF19, and that dominant repression of LBD16 inhibits lateral root formation (Okushima et al., 2007). To further demonstrate the positive role played by LBD18 in lateral root formation, we constructed DEX-regulated LBD18-transgenic plants in arf7-1 arf19-1 mutant backgrounds (Pro35S:LBD18:GR/arf7 arf19) by crossing these transgenic plants and mutants. The RT-PCR analysis of the LBD18:GR transcripts revealed fewer transcripts in Pro35S:LBD18:GR/arf7 arf19 compared to that of the Pro35S:LBD18:GR used for the construction of this transgenic mutant (Fig. 9A). As shown in Fig. 9B, we noted a significant induction of lateral root numbers in Pro35S:LBD18:GR/arf7 arf19 after 12 days of DEX treatment, as compared to what was observed in the absence of DEX and in both the presence and absence of DEX in the arf7 arf19 mutants. DEX treatment of wild-type plants did not affect lateral root number (data not shown). These results reveal that LBD18 positively regulates lateral root formation downstream of ARF7 and ARF19.
DISCUSSION

Recent studies have demonstrated the functions of some LBD genes in lateral organ development in Arabidopsis and in crop plants, such as rice and maize. For many Arabidopsis LBD genes, loss-of-function mutation phenotypes were not apparent with the exception of LBD6, which performs a role in leaf development (Semiarti et al., 2001; Xu et al., 2003). However, several loss-of-function mutants of rice and maize LBD genes have been reported to evidence clear phenotypes (Inukai et al., 2005; Liu et al., 2005; Bortiri et al., 2006; Taramino et al., 2007; Evans, 2007). However, the ectopic expression of several LBD genes, as wild-type or as proteins fused to a transcriptional repression domain, yielded morphological phenotypes, providing clues as to the biological functions of these LBD genes (Shuai et al., 2002; Chalfun-Junior et al., 2005; Borghi et al., 2007; Okishima et al., 2007). For example, the overexpression of LBD16 or LBD29 induced lateral root formation in the absence of ARF7 and ARF19 and the dominant repression of LBD16 activity inhibited lateral root formation; these findings suggest that these LBDs function downstream of ARF7- and ARF19-dependent auxin signaling in lateral root formation (Okishima et al., 2007). In this study, we demonstrated that LBD16 and LBD18 are involved in lateral root formation at the emergence step, based on the results of analysis of lbd16 and lbd18 single or double loss-of-function mutants. LBD18 overexpression using a DEX-inducible system was shown to complement lateral root formation in lbd18, as well as the lbd16 lbd18 mutants, without inducing any other phenotypes. LBD18 overexpression also induced lateral root formation in arf7 arf19 mutants with blocked lateral root formation. Collectively, these results suggest that LBD18 performs a function in lateral root formation, particularly during the initiation and emergence steps, in conjunction with LBD16 downstream of ARF7 and ARF19.

The lbd16 lbd18 double mutants exhibited substantially reduced numbers of lateral roots
compared to the lbd16 or lbd18 single mutants (Fig. 2), thereby suggesting that LBD16 and LBD18 might function in a combinatorial manner in lateral root formation. Because we failed to detect a protein-protein interaction between LBD16 and LBD18 in a yeast 2-hybrid assay (data not shown), LBD16 and LBD18 might function redundantly in different pathways or might require auxiliary factors for their functional interaction. Some degree of lateral root formation could still be detected in the lbd16 lbd18 double mutants, thereby indicating the existence of additional components for lateral root formation.

In the transgenic line expressing appropriate levels of the LBD18:GR transcripts in the wild-type background, a statistically significant increase in lateral root number was induced by treatment with various concentrations of DEX (Fig. 6E). In contrast, the transgenic lines expressing higher levels of the LBD18:GR transcripts exhibited significantly halted root growth, significantly decreased numbers of lateral roots, and reduced hypocotyl length (Figs. 6 and 7, and Supplemental Figs. S2 and S3). These strong phenotypes may be related to the effects of ectopic tracheary element-like cells in non-vascular cells, as previously reported (Soyano et al., 2008). These results also indicate that appropriate LBD18 expression levels might be necessary for the induction of lateral root formation in wild-type plants. In order to further demonstrate that increased LBD18 expression can stimulate lateral root formation and that LBD18 is responsible for the mutant phenotype, we generated the transgenic mutants, Pro35S:LBD18:GR in lbd18 mutant background, as well as in lbd16 lbd18 mutant background that express the LBD18:GR transcripts at wild-type level or slightly higher. We noted that DEX treatment can induce lateral root formation in both lbd18 and lbd16 lbd18 mutant backgrounds (Fig. 8, C and D), thus clearly demonstrating that LBD18 plays a role in lateral root formation. GUS expression detected in the developing lateral roots of ProLBD18:GUS seedlings (Fig. 3) is also consistent with the role of LBD18 in lateral root formation. In previous studies, it has been determined that in ProLBD16:GUS seedlings, GUS expression can
be detected in the lateral root primordium (Okushima et al., 2007). We determined that while the GUS expression of Pro\textsubscript{LBD16}:GUS seedlings is strong in the root stele and the developing and fully emerged lateral roots, that of Pro\textsubscript{LBD18}:GUS is restricted to the primordia and the emerging lateral roots, but is not detected in the lateral root stele. These GUS expression patterns suggest that LBD16 and LBD18 may have an overlapping function in the formation of lateral roots and that LBD16 may play an additional role in the continued growth of lateral roots compared to LBD18.

Lateral roots are formed from mature root pericycle cells located adjacent to the proxylem poles of the parent root (Casimiro et al., 2003). The developmental events of lateral root formation include priming, initiation, primordium development, and the emergence of lateral roots (Fukaki and Tasaka, 2009). Recent studies with Arabidopsis have shown that auxin transport and signaling are necessary for lateral root initiation and primordium development. The auxin influx carrier, AUX1-dependent basipetal auxin transport, regulates the initiation of the lateral root (De Smet et al., 2007). The AUX1-like auxin influx carrier, LAX (Like AUX1)3, promotes lateral root emergence (Swarup et al., 2008). Multiple pin mutations induced dramatic defects in root patterning, including the development of the lateral root primordium (Benkova et al., 2003), thereby suggesting that auxin efflux regulated by PIN proteins is also required for lateral root formation. Auxin signaling mediated by the Aux/IAA and ARF families of transcriptional regulators has been determined to be required for lateral root formation. Various gain-of-function mutants of Aux/IAA genes, slr-1, shy2/iaa3, msg2/iaa19, axr5/iaa1, iaa28, and crane/iaa18, yielded dramatically reduced numbers of lateral roots (Tian and Reed, 1999; Rogg et al., 2001; Fukaki et al., 2002; Tatematsu et al., 2004; Yang et al., 2004; Uehara et al., 2008). Whereas mutations in ARF19 had a little effect on their own, and ARF7 mutations resulted in impairments in hypocotyl phototropism, a variety of phenotypes that were not detected in these single mutants were observed in arf7
arf19 double mutants, including a reduction in lateral root formation (Harper et al., 2000; Okushima et al., 2005; Wilmoth et al., 2005), suggesting that ARF7 and ARF19 regulate lateral root formation in a redundant fashion. A novel regulator of lateral root primordium development, PUCHI, which encodes AP2/EREBP, has been identified, and has been shown to play a role in lateral root morphogenesis by altering the pattern of cell divisions during the early stages of primordium development (Hirota et al., 2007). It has been suggested that PUCHI functions downstream of auxin signaling.

LBD16 has been previously proposed to be involved in the initiation step of lateral root formation, based on the observation that the dominant repression of LBD16 profoundly inhibited lateral root formation (Okushima et al., 2007). Our present study involving loss-of-function mutant analysis demonstrated that, while the numbers of primordia of lbd16 and lbd18 single or double mutants were similar to those of the wild-type strain, the numbers of emerged lateral roots of the single mutants were reduced significantly compared to the wild-type (Fig. 4A). In the lbd16 lbd18 double mutants, additively reduced numbers of emerged lateral roots were noted as compared to the single mutants. Moreover, the numbers of primordia at stage I to stage VIII were not affected by lbd16 and lbd18 mutations (Fig. 4B). These findings reveal that LBD16 and LBD18 are likely to be involved in the initiation and the emergence of lateral root formation and function additively in a different pathway. Recent study showed that GUS of ProLBD18::GUS transgenics is expressed in immature tracheary elements and that LBD18 functions in differentiation of tracheary elements (Soyano et al., 2008). It is, therefore, possible that the phenotype of lateral root emergence in the lbd16 lbd18 mutant might be related to the vascular differentiation in the lateral root primordium.

The results of microarray analysis demonstrated that the loss of ARF7 and ARF19 function completely suppressed the expression of LBD16, LBD18, and LBD29 in response to auxin (Okushima et al., 2005). The ectopic expression of LBD16 or LBD29 in arf7 arf19
mutants induced an increase in lateral root numbers (Okushima et al., 2007). We have also determined that ectopic LBD18 expression in arf7 arf19 mutants as the result of DEX treatment induced a significant increase in lateral root numbers (Fig. 9). Thus, we propose that LBD16, LBD18, and LBD29 might act in a combinatorial fashion to regulate lateral root formation downstream of ARF7 and ARF19 during the auxin response. The results of our previous study demonstrated that the DEX-regulated expression of iaa1 with a domain-II mutation resulted in a severe inhibition of lateral root formation and the suppression of LBD16 and LBD18 expression in response to auxin (Park et al., 2002 and Lee et al., 2009). Reduced lateral root formation has also been noted in endogenous gain-of-function iaa1/axr5 mutants (Yang et al., 2004). The arf7 or arf19 mutations resulted in a significant suppression of auxin-mediated LBD16 expression (Lee et al., 2009). The expression of LBD18 and LBD29 in response to auxin in the arf7 or arf19 mutants was inhibited almost to the basal levels (Lee et al., 2009). In the arf7 arf19 double mutants, LBD16, LBD18, and LBD29 expression was inhibited completely in response to auxin. In P_{IAA1}:GUS 7-day-old light-grown seedlings, we have detected strong GUS expression in various tissues, including the lateral roots (Lee et al., 2009). In P_{ARF7}:GUS and P_{ARF19}:GUS seedlings, GUS expression was also detected in various tissues including the lateral roots, but the expression patterns of these 2 GUS fusion genes are distinct. Partial overlap in the GUS of P_{ARF7}:GUS seedlings was detected in the root stele and in the developing lateral roots, whereas the GUS of P_{ARF19}:GUS seedlings was shown to be expressed in the entire region of the primary and lateral roots (Okushima et al., 2005). Collectively, these results indicate that LBD16 and LBD18 might be regulated downstream of the IAA1-ARF7/ARF19 transcriptional regulator system in auxin signaling to modulate lateral root formation at the initiation and the emergence steps.
MATERIALS AND METHODS

Plant Growth and Tissue Treatment

*Arabidopsis thaliana* seedlings were grown under a 16-h photoperiod and treated, as described previously (Park et al., 2002).

Plasmid Construction and Arabidopsis Transformation

The promoter region of *LBD16* or *LBD18*, which encompasses 1416 bp from -1436 bp to -20 bp or 2034 bp from -2054 bp to -20 bp relative to the AUG initiation codon, respectively, was isolated with PCR primers harboring *Hind*III (N-terminus) and *Xma*I (C-terminus) sites for *LBD16* and *Xba*I (N-terminus) and *Xma*I (C-terminus) sites for *LBD18* using the *Pfu* DNA polymerase (Stratagene) from the genomic DNA of Arabidopsis Col-0. These PCR products were inserted into pGEM®-T Easy vector (Promega). The DNA fragments were cut with their corresponding restriction enzymes and subcloned into pBI101 (Clontech) in place of the CaMV 35S promoter. The full-length *LBD16* and *LBD18* coding regions were synthesized via PCR using primers harboring *Xba*I (N-terminus) and *Xho*I (C-terminus), then ligated into pBI-deltaGR (Lloyd et al., 1994) as translational fusions with the glucocorticoid receptor hormone binding domain (GR) to generate the constructs, Pro~35S:~LBD16:~GR and Pro~35S:~LBD18:~GR, respectively. Transgenic Arabidopsis plants harboring these constructs (Pro~35S:~LBD16:~GR or Pro~35S:~LBD18:~GR) were then generated via *Agrobacterium*-mediated transformation (Bechtold et al., 1993). T3 homozygous transformants were generated and amplified (Park et al., 2002). All constructs were confirmed via DNA sequencing prior to plant transformation. The oligonucleotides utilized for PCR and the PCR conditions are
Isolation and Phenotypic Analysis of Arabidopsis lbd16, lbd18 Single and lbd16 lbd18 Double T-DNA Insertion Mutants

Arabidopsis lbd16-1 (SALK_095791) and lbd18-1 (SALK_038125) T-DNA insertion mutants from ABRC were verified by PCR with primers designed with the T-DNA primer design program, which is available from the Salk Institute Genomic Analysis Laboratory (SIGNAL) (http://signal.salk.edu/). The homozygous T-DNA insertion mutant lines were isolated with the primers shown in Supplemental Table S1. Double lbd16 lbd18 mutants were generated by crossing of lbd16-1 (female) with lbd18-1 (male), and the resultant homozygous lines isolated were verified by PCR. Single copy T-DNA in lbd16 and lbd18 was verified by backcrossing these mutants into wild-type plants and assessing the segregation of T-DNA by PCR. The null mutations of lbd16, lbd18, and lbd16 lbd18 were further verified by RT-PCR analysis. Phenotypic analysis of these T-DNA insertion mutants was conducted for root length, hypocotyl length, number of lateral roots, gravitropic response, and morphological changes, as described previously (Park et al, 2002). For the analysis of the effects of 2,4-D on the lateral root number, we used 5-d-old seedlings grown vertically 4 d after transfer to the media containing the indicated concentrations of 2,4-D (Dharmasiri et al., 2005).

Generation of Pro35S:LBD18:GR Transgenic Arabidopsis in lbd18 or lbd16 lbd18 or arf7 arf19 Mutant Backgrounds

Transgenic mutants, Pro35S:LBD18:GR/lbd18 or Pro35S:LBD18:GR/lbd16 lbd18, were generated by crossing lbd18-1 or lbd16-1 lbd18-1 (female) with Pro35S:LBD18:GR (male).
Homozygous lines were isolated on the basis of genotyping and the PCR detection of genomic DNA for the \textit{LBD18:GR} transgene. Expression levels of the \textit{LBD18:GR} transcripts in Arabidopsis overexpressing \textit{Pro}_{35S}:\textit{LBD18:GR} in \textit{lbd18-1} or \textit{lbd16-1 lbd18-1} mutants were determined by RT-PCR analysis.

\textit{arf7-1 arf19-1} mutants were acquired from ABRC and confirmed by genotyping (http://signal.salk.edu) and lack of lateral-root phenotype (Okushima et al., 2005). Arabidopsis overexpressing \textit{Pro}_{35S}:\textit{LBD18:GR} in \textit{arf7-1 arf19-1} mutants was generated by crossing \textit{arf7-1 arf19-1} (female) with \textit{Pro}_{35S}:\textit{LBD18:GR} (male). Homozygous lines were isolated according to genotype and the lack of the lateral-root phenotype for \textit{arf7-1 arf19-1} and also the PCR detection of genomic DNA for the \textit{LBD18:GR} transgene. Expression levels of the \textit{LBD18:GR} transcripts in Arabidopsis overexpressing \textit{Pro}_{35S}:\textit{LBD18:GR} in \textit{arf7-1 arf19-1} mutants were determined by RT-PCR analysis. The oligonucleotides and PCR conditions utilized are provided in Supplemental Table S1.

\textbf{RNA Isolation, RNA-Gel Blot Analysis, RT-PCR, and Real-Time RT-PCR}

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, Inc.). Total RNA was separated on 1.2% agarose gel, transferred to nylon membranes, and hybridized for 3 h with $^{32}$P-labeled DNA probes at 68°C using 10 ml of QuickHyb solution (Stratagene), then washed. The blots were subsequently exposed to X-ray film. The DNA probes for RNA gel-blot analysis were RT-PCR amplified, subcloned into pGEM®-T Easy vector (Promega), then confirmed by DNA sequencing. For RT-PCR analysis, total RNA was isolated using an RNasy plant mini kit (Qiagen) and subjected to RT-PCR analysis with an Access RT-PCR System (Promega) according to the manufacturer’s
instructions. Real-time RT-PCR was conducted with a QuantiTect SYBR Green RT-PCR kit (Qiagen) in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research). In order to determine the copy number of the transcripts in the treated sample, real-time PCR was performed on each sample with a known quantity of the \textit{in vitro} transcribed RNA (Promega), yielding specific threshold values (Ct). A standard curve was generated to demonstrate the linear correlation between log [copy numbers of the RNA] and the Ct. The copy numbers of the transcripts of unknown samples were subsequently calculated from this standard curve. RT-PCR conditions, primer sequences, and DNA probes used in this study are provided in Supplemental Table S1.

\textbf{Nuclear Localization of EGFP:LBD18 Fusion Proteins in Arabidopsis Protoplasts}

\textit{LBD18} full-length DNA was PCR-amplified using \textit{Pfu} DNA polymerase (Stratagene) and inserted into the \textit{Pro\textsubscript{35S}:EGFP} vector (Lee et al., 2008) at the \textit{XhoI} sites as a translational fusion, thereby yielding the \textit{Pro\textsubscript{35S}:EGFP:LBD18} DNA construct. PCR-amplified DNA sequences were used for subcloning after verification by DNA sequencing. These plasmids were purified using a Qiagen Plasmid Midi kit prior to protoplast transformation. The protoplasts from Arabidopsis mesophyll cells were prepared as described previously (Lee et al., 2008). Protoplasts isolated from the rosette leaves of 2 to 3-week-old Arabidopsis plants grown under a 16-h photoperiod were transfected with plasmid DNA by polyethylene glycol (PEG)-mediated protoplast transformation and incubated for 18 h in the light at room temperature. The nuclear localization of the GFP-fusion proteins was monitored by capturing GFP images with a TCS SP5 AOBS\textsuperscript{®} spectral confocal and multiphoton microscope system (Leica Microsystems). Confocal images of the GFP-fusion proteins were acquired at the Korea Basic Science Institute, Gwangju Center. Ar (488 nm) and HeNe lasers (633 nm) were
utilized for GFP excitation and autofluorescence of chlorophyll excitation, respectively. Hoechst 33342 (Sigma) was utilized at 12 ng/ml to stain the nuclei of the protoplasts. The protoplasts were incubated with Hoechst 33342 for 15 min at room temperature in darkness, rinsed 3 times in culture medium, and the excess buffer was drained and replaced with fresh culture medium.

**Statistical Analysis**

Quantitative data on the phenotypes such as measurements of root length, number of lateral roots, and hypocotyls length were subjected to statistical analysis for every pair-wise comparison, using the software for Student’s t-Test (Predictive Analytics Software for Windows version 17.0).
SUPPLEMENTAL DATA

The following materials are available in the online version of this article.

Supplemental Figure S1. Dose-response curves for root elongation of lbd16, lbd18, and lbd16 lbd18 mutants on IAA.

Supplemental Figure S2. Lateral roots and primary root lengths of Pro35S:LBD16:GR and Pro35S:LBD18:GR transgenic plants.

Supplemental Figure S3. Root lengths of Pro35S:LBD18:GR transgenic plants in the presence of varying concentrations of DEX.

Supplemental Table S1. Oligonucleotides used for PCR and PCR conditions.

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FIGURE LEGENDS

Figure 1. PCR analysis of the lbd16, lbd18, and lbd16 lbd18 mutants. A, Location of T-DNA insertions of LBD16 and LBD18 in Arabidopsis. T-DNA insertions are indicated by triangles. LBD16 and LBD18 are composed of two exons indicated by E1 and E2 and one intron indicated by a grey line. L and R, left and right borders of T-DNA. Solid lines and dotted lines indicate the locations of the primers for the RT-PCR and the primers for the PCR of genomic DNA, respectively. Numbers indicate the position of the first nucleotide of the primer relative to the AUG initiation codon. B, PCR analysis of T-DNA insertion mutants. Genomic DNA isolated from each mutant was subjected to PCR with primers specific for LBD16 or LBD18 and the primers for T-DNA. S or T, PCR products amplified by specific primers (S) or specific primers and T-DNA primers (T). WT, wild-type Col-0. C, RT-PCR analysis of T-DNA insertion mutants. Total RNA isolated from 7-d-old light-grown seedlings was subjected to RT-PCR. The ACTIN7 mRNA was utilized as a loading control.

Figure 2. Lateral-root phenotype of lbd16, lbd18, and lbd16 lbd18 mutants. A and B, Lateral roots of T-DNA insertion mutants and wild type. Pictures were taken 5 d (A) or 8 d (B) after germination. Bars = 1 cm. C, Number of lateral roots at different days after germination. The open column and closed column indicate the lateral root numbers of the plants grown vertically 5 and 8 d after germination, respectively. n>29. Lateral roots were counted under a dissecting microscope. Lateral root numbers per unit root length (cm) measured were plotted. D, Number of lateral roots with varying concentrations of 2,4-D. Lateral root numbers of 5-d-old seedlings grown vertically were counted 4 d after transfer to media containing the indicated concentrations of 2,4-D. n>18. Lateral root numbers per unit root length (cm) measured were plotted. ** denotes statistical significance with p<0.01.

Figure 3. GUS expression in ProLBD16:GUS and ProLBD18:GUS transgenics. A-D, GUS expression in 7-d-old light-grown ProLBD16:GUS seedlings. Seedlings were incubated without or with 20 μM of IAA for 4 h. Pictures were obtained after 8 h of incubation with X-Glu. E-H, GUS expression in 7-d-old light-grown ProLBD18:GUS seedlings. Seedlings were incubated without or with 20 μM of IAA for 8 h. Pictures were obtained after 16 h of incubation with X-Glu. A and E, GUS expression of a primary root with or without IAA. Bar = 100 mm. B and F, GUS expression of primordia of the developing lateral root with or without IAA. C and G, GUS expression of an emerged lateral root with or without IAA. D and H, GUS expression of a lateral root with or without IAA. Stages of lateral roots were based on Malamy and Benfey (1997). Bar = 50 mm.

Figure 4. Numbers of lateral roots of lbd16, lbd18, and lbd16 lbd18 mutants. A, Numbers of
primordia of lateral roots or emerged lateral roots. Plants were grown vertically for 8 d after germination. Primordia or emerged lateral roots were photographed with a camera affixed to a Leica CME Trinocular light microscope at 100- or 400-fold magnification and counted on the basis of Malamy and Benfey (1997). n>10 per column. ** denotes statistical significance with p<0.01. B, Numbers of primordia at given stages before emergence of lateral roots. Stages I to VIII of primordia were based on the classification by Malamy and Benfey (1997).

Figure 5. Nuclear localization of EGFP fusion proteins of LBD18. Pictures represent epifluorescence (GFP), autofluorescence (chloroplast), merge, and black and white field images of mesophyll protoplasts transfected with plasmid DNAs. Bar = 10 μm.

Figure 6. Analysis of lateral root numbers and primary root lengths in Pro35S:LBD16:GR and Pro35S:LBD18:GR transgenic plants. A, RNA-gel blot analysis of LBD16, LBD18, LBD16:GR, and LBD18:GR expression in wild-type, Pro35S:LBD16:GR, and Pro35S:LBD18:GR transgenic plants. Numbers on the upper gel indicate the line number of homozygous transgenic Arabidopsis. Twenty μg of total RNA isolated from 10-d-old light-grown seedlings was subjected to RNA-gel blot analysis using LBD16 or LBD18 DNA probes. Transcripts of LBD16 and LBD18 are indicated by a thick arrow and the corresponding GR fusion transcripts are indicated by an open arrow. A closed triangle indicates the Arabidopsis transgenic lines used for phenotypic analysis. Total RNA from wild-type plants was isolated from the plants treated for 4 h with 20 μM of IAA. B, Representative seedlings of Pro35S:LBD16:GR and Pro35S:LBD18:GR treated with or without DEX. Number within the bracket indicates the line number of Arabidopsis transgenic plants. Plants were grown vertically for 8 d in the absence (-) or presence (+) of 10 μM of DEX. C, Lateral root number of various lines of Pro35S:LBD16:GR transgenic plants without or with DEX. Plants were grown vertically for 8 d in the absence or presence of 10 μM of DEX and lateral root numbers per unit root length (cm) measured were plotted. Bars indicate SE. n>20. ** denotes statistical significance with p<0.01. D, Lateral root number of various lines of Pro35S:LBD18:GR transgenic plants without or with DEX. Plants were treated as described in the legend of Fig. 7C. n>20. * denotes statistical significance with p<0.05. E, Lateral root number of #28-9 line of Pro35S:LBD18:GR transgenic plants without or with DEX. Plants were grown vertically for 8 d with varying concentrations of DEX and lateral root numbers per unit root length (cm) measured were plotted. n>21. Number below the column indicates the line number of Arabidopsis transgenic plants (C and D).

Figure 7. Hypocotyl lengths of Pro35S:LBD18:GR transgenic plants. A, Representative plants of 7-d-old dark-grown Pro35S:LBD18:GR treated with or without DEX. B, Hypocotyl lengths of 7-d-old light- or dark-grown Pro35S:LBD18:GR treated without or with DEX. n>20. Bars
indicate SE. * and ** denote statistical significance with $p<0.05$ and $p<0.01$, respectively.

**Figure 8.** Complementation of lbd18 or lbd16 lbd18 by LBD18:GR. A, PCR analysis of Pro$_{35S}$:LBD18:GR/lbd18-1 or iPro$_{35S}$:LBD18:GR/bd16-1 lbd18-1 transgenic mutants compared with lbd18-1 and lbd16-1 lbd18-1 mutants. Seven-d-old plants grown on MS plate were harvested for genomic DNA, followed by PCR analysis for T-DNA insertion and total RNA was also isolated, followed by RT-PCR analysis using the primers for the LBD18:GR fusion transcripts or the LBD18 transcripts. ACTIN7 mRNA was used as a loading control. B, Quantitative RT-PCR analysis of the LBD18 transcripts in Pro$_{35S}$:LBD18:GR/lbd18-1 or Pro$_{35S}$:LBD18:GR/bd16-1 lbd18-1 transgenic mutants compared with lbd18-1 and lbd16-1 lbd18-1 mutants. Relative abundance of the LBD18 transcripts are shown compared to wild type. C, Lateral roots of Pro$_{35S}$:LBD18:GR/lbd18-1 or Pro$_{35S}$:LBD18:GR/bd16-1 lbd18-1 transgenic mutants compared with lbd18-1 and lbd16-1 lbd18-1 mutants without or with DEX. Plants were incubated and treated as described in Fig. 7C, but for 12-d. Bars indicate SE. n>24. ** denotes statistical significance with $p<0.01$. D, Lateral roots of transgenic mutants and wild type. Pictures were taken 12 d after germination.

**Figure 9.** Induction of lateral roots in arf7 arf19 mutants by activation of LBD18:GR with DEX treatment. A, RT-PCR analysis of Pro$_{35S}$:LBD18:GR/arf7-1 arf19-1 transgenic mutants compared with arf7 arf19 and Pro$_{35S}$:LBD18:GR for the LBD18:GR transcripts. Seven-d-old plants grown on MS plate were harvested for total RNA, followed by RT-PCR analysis using the primers for the LBD18:GR fusion transcripts. ACTIN7 mRNA was used as a loading control. B, Lateral roots of Pro$_{35S}$:LBD18:GR/arf7-1 arf19-1 transgenic mutants compared with arf7 arf19 without or with DEX. Plants were incubated and treated as described in Fig. 7C, but for 12-d. The number in the picture indicates the average lateral root number ± SE. n>18.
A) Col-0 (IAA treated for 4 h) and Pro\textsubscript{2P}:LBD16:GR

B) WT, Pro\textsubscript{2P}:LBD16:GR (#5-2), Pro\textsubscript{2P}:LBD18:GR (#38-2) with DEX

C) Lateral root number per root at 4 days

D) Lateral root number per root at 4 days

E) WT and Pro\textsubscript{2P}:LBD18:GR #28-9 with DEX
