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The establishment of asymmetry in *Arabidopsis* lateral root founder cells is regulated by LBD16/ASL18 and related LBD/ASL proteins

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SUMMARY

In most dicot plants, lateral root (LR) formation, which is important for the construction of the plant root system, is initiated from coordinated asymmetric cell divisions (ACD) of the primed LR founder cells in the xylem pole pericycle (XPP) of the existing roots. In *Arabidopsis thaliana*, two AUXIN RESPONSE FACTORs (ARFs), ARF7 and ARF19, positively regulate LR formation through activation of the plant-specific transcriptional regulators *LATERAL ORGAN BOUNDARIES-DOMAIN 16/ASYMMETRIC LEAVES2-LIKE 18* (*LBD16/ASL18*) and the other related *LBD/ASL* genes. The exact biological role of these LBD/ASLs in LR formation is still unknown. Here, we demonstrate that LBD16/ASL18 is specifically expressed in the LR founder cells adjacent to the XPP before the first ACD and that it functions redundantly with the other auxin-inducible LBD/ASLs in LR initiation. The spatiotemporal expression of LBD16/ASL18 during LR initiation is dependent on the SOLITARY-ROOT (SLR)/IAA14-ARF7-ARF19 auxin signaling module. In addition, XPP-specific expression of LBD16/ASL18 in *arf7 arf19* induced cell divisions at XPP, thereby restoring the LR phenotype. We also demonstrate that expression of LBD16-SRDX, a dominant repressor of LBD16/ASL18 and its related LBD/ASLs, does not interfere in the specification of LR founder cells with local activation of the auxin response, but it blocks the polar nuclear migration in LR founder cells before ACD, thereby blocking the subsequent LR initiation. Taken together, these results indicate that the localized activity of LBD16/ASL18 and its related LBD/ASLs is involved in the symmetry breaking of LR founder cells for LR initiation, a key step for constructing the plant root system.

KEY WORDS: *Arabidopsis*, Lateral root initiation, Asymmetric cell division, Auxin, LBD/ASLs

INTRODUCTION

Asymmetric cell division (ACD) generates two daughter cells with different sizes, shapes, compositions, and fates. It is crucial for the generation and maintenance of cell diversity in multicellular organisms. In vascular plants, ACD is important for the establishment of the apical-basal and radial axes during early embryogenesis, for the generation of various types of tissues from the stem cells in the shoot and root apical meristems during postembryonic development, and for the creation of cell-type diversity in tissues, such as stomatal patterning and development (Abrash and Bergmann, 2009; Müller et al., 2009; Petricka et al., 2009; De Smet and Beeckman, 2011).

In vascular plants, the root system is formed through continuous growth of root tips and branching from the existing roots or shoot organs, which enables the plant to absorb water and nutrients efficiently and to sustain growth of its aerial parts (Péret et al., 2009). Root branching usually occurs postembryonically from a small number of differentiated cells in the existing roots or shoots, leading to the generation of lateral roots (LRs) and adventitious roots. In *Arabidopsis thaliana*, LR is initiated from a pair of differentiated pericycle cells of the three cell files adjacent to the protoxylem (Péret et al., 2009). These xylem pole pericycle (XPP) cells, which are the presumed LR founder cells in a zone close to the root meristem (basal meristem), exhibit firstly intense auxin response (auxin response maximum), and then undergo the first anticlinal and ACDs in a coordinated manner (De Smet et al., 2007; De Rybel et al., 2010; Moreno-Risueno et al., 2010). These ACDs produces two small daughter cells as well as larger flanking cells that will continue to divide to form the LR primordium (Benková and Bielach, 2010; De Smet and Beeckman, 2011).

These initial events of LR formation are mainly regulated by auxin. Particularly, auxin signaling through the AUXIN RESPONSE FACTOR transcriptional activators, ARF7 and ARF19, and auxin/indole-3-acetic acid (Aux/IAA) repressors, including SOLITARY-ROOT (SLR)/IAA14, is crucial for LR initiation (reviewed by Fukaki et al., 2007; Fukaki and Tasaka, 2009). The function of these genes was revealed by molecular genetic studies of two LR mutants – *slr-1*, which has no LRs owing to a gain-of-function mutation in the *SLR/IAA14* gene, and the *arf7 arf19* double mutant, which has few LRs (Fukaki et al., 2002; Fukaki et al., 2005; Okushima et al., 2005). Auxin triggers the degradation of SLR/IAA14, following the de-repression of ARF7 and ARF19, and then activates the expression of downstream genes for LR initiation. Okushima et al. (Okushima et al., 2007) have demonstrated that ARF7 and ARF19 regulate the transcriptional activation of *LATERAL ORGAN BOUNDARIES-DOMAIN16/ASYMMETRIC LEAVES2-LIKE18* (*LBD16/ASL18*) and *LBD29/ASL16* in roots via direct binding to the auxin response elements (AuxREs) in their promoter region. Overexpression of *LBD16* or *LBD29* partially restores the LR-less phenotype of the *arf7 arf19* double mutant whereas overexpression of LBD16-SRDX, a dominant repressor, decreases LR formation, strongly suggesting that these LBD/ASL proteins positively regulate LR formation

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downstream of SLR/IAA14-ARF7-ARF19 signaling (Okushima et al., 2007). However, the above experiments did not provide information on the specific site of LBD16 or LBD16-SRDX activity in LR formation because a constitutive *35S* promoter was used. The *Arabidopsis* genome has 42 genes encoding LBD/ASL proteins (AS2/LOB family), which are involved in various aspects of plant growth and development through transcriptional regulation (Iwakawa et al., 2002; Shuai et al., 2002; Matsumura et al., 2009; Majer and Hochholdinger, 2011). Although the auxin-inducible *LBD16*- and *LBD29*-related genes play important roles in the development of the root system not only in dicot but also in monocot plants (Inukai et al., 2005; Liu et al., 2005; Fukaki et al., 2007; Fukaki and Tasaka, 2009), the mechanisms by which these LBD/ASL proteins regulate root formation, and specifically how and where LBD16/ASL18 regulates LR initiation, remain unknown.

Here, we demonstrate that LBD16/ASL18 is expressed specifically in the LR founder cells adjacent to the XPP before the occurrence of ACD, and it is dependent on the SLR/IAA14-ARF7/19 signaling module. In addition, inhibition of LBD16/ASL18 and its related LBD/ASLs, specifically at LR founder cells, blocks nuclear migration, ACDs, and LR initiation. Our data indicate that local activation of LBD16/ASL18 and its related LBD/ASLs at XPP establishes the asymmetry of *Arabidopsis* LR founder cells, resulting in ACDs for LR initiation, which is a key step for constructing the plant root system.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana accession Columbia (Col) was used in this study. The *lbd16-1* (SALK_095791), *lbd18-1* (SALK_038125), *lbd33-1* (SAIL_95_H10) and *35S::LTI6b-GFP* lines were obtained from the Arabidopsis Biological Resource Center (Alonso et al., 2003). The *nph4-1*, *arf7-1* (SALK_040394), *arf19-1* (SALK_009879), *pLBD16::GUS*, *pLBD29::GUS* and *pLBD33::GUS* lines have been described previously (Okushima et al., 2005; Okushima et al., 2007). The *arf7-2* (CGA [R146] to TGA [stop]) and *arf19-5* (CAA [Q52] to TAA [stop]) were isolated in our lab. GAL4-GFP enhancer trap line, J0121 was obtained from the Nottingham *Arabidopsis* Stock Centre. *pCycB1;1::CycB1;1(NT)-GUS*, *DR5::GUS*, and *DR5rev::erGFP* lines were kindly provided by Peter Doerner (University of Edinburgh, UK), Tom Guilfoyle (University of Missouri, USA), and Jiří Friml (Ghent University, Belgium), respectively. Seeds were germinated under sterile conditions on 1× Murashige-Skoog medium with 1% sucrose. Plants were grown at 23°C under continuous light. For the root auxin sensitivity assays, seedlings were transferred 5 days after germination (DAG) to vertically oriented agar plates containing 0.1 or 1 μM α-naphthalene acetic acid (NAA). The number of LRs and root length were determined using a dissecting microscope and ImageJ software (NIH). LR density indicates the number of emerged LR per portion of the primary root where LRs are present. All data are the mean values for each plant considered. Experiments were repeated twice, and similar values were obtained in each experiment. Statistical analysis of data was performed using the Student's *t*-test.

Vector construction and plant transformation

Primers used for plasmid construction are listed in supplementary material Table S1. To generate the LBD16-GFP and LBDs-SRDX expressed under the control of their own regulatory region, genomic fragments of *LBD16*, *LBD17*, *LBD29* and *LBD33* genes, including the 2.5 kb promoter region (with the 5′-untranslated region) and coding region (with intron), were amplified from genomic DNA and cloned into pDONR221 or pENTR D-TOPO using Gateway technology (Invitrogen, Carlsbad, CA), and then transferred to pGWB-GFP (C) or pGWB-SRDX which contain the Gateway cassette in front of the GFP coding sequence or SRDX (LDLDLELRLGFA) linker sequence, respectively. The *pLBD17::GUS* construct was generated by fusing a promoter fragment (~2.5 kb) in front of the GUS coding sequence of pBI301 (Uehara et al., 2008). NLS-GFP and NLS-tagRFP were constructed by using the nuclear localization signal (NLS) of SV40. The fusion genes were cloned into pENTR D-TOPO and then transferred to the pGWB501:pLBD16, which contains *LBD16* promoter in front of the Gateway cassette of pGWB501 (Nakagawa et al., 2007). *LBD16* coding sequence was cloned into pGWB501:5xUAS, which contains 5xUAS in front of Gateway cassette. The transformation of *Arabidopsis* plants was performed via floral dipping using *Agrobacterium tumefaciens* (strain C58MP90) (Clough and Bent, 1998).

Microscopy

GUS staining, fixation, and whole-mount clearing preparation of roots were performed essentially as described previously (Malamy and Benfey, 1997), and samples were observed with a Leica DM6000 microscope equipped with Nomarski optics (Leica Microsystems, Wetzlar, Germany). For confocal microscopy, roots were counterstained with propidium iodide (10 μg/ml) and analyzed with an Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan). For in vivo time-lapse imaging, 4-day-old plants grown vertically were turned 90° degrees for 6 hours, and then placed with a block of solid medium horizontally under the confocal microscope equipped with Z focus drift compensating system (ZDC) (FV-1000, Olympus). Images were taken every 5 minutes and processed with ImageJ software.

RESULTS

LBD16/ASL18 is specifically activated in the pairs of XPP cells at the presumptive LR initiation site

A previous study has shown that *LBD16/ASL18* positively regulates LR formation as a direct target of ARF7 and ARF19 (Okushima et al., 2007). However, it was not determined which developmental steps of LR formation are regulated by LBD16/ASL18 and its related LBD/ASLs. In order to investigate when and where LBD16/ASL18 functions in LR formation, we examined its spatiotemporal expression pattern in *Arabidopsis* roots by expressing a translational fusion of LBD16 and the green fluorescent protein (GFP) under the control of its own endogenous regulatory region (*genomicLBD16-GFP*, *LBD16-GFP*) in *Arabidopsis*. To avoid possible artifacts due to ectopic expression or overexpression, we introduced *LBD16-GFP* into the *lbd16-1*, a knockout mutant of *LBD16* (Okushima et al., 2007). We confirmed that the LBD16-GFP was functional because the expression of *LBD16-GFP* restored the LR phenotype in both the *lbd16-1 arf7-1* and *lbd16-1 arf19-1* double mutants in which the LR density was decreased (supplementary material Fig. S1). The *arf7-1* and *lbd16-1* single mutants showed slightly decreased number of LRs, whereas the *arf19-1* single mutant showed a normal number of LRs, indicating the redundant functions of ARFs (ARF7 and ARF19) and those among the LBD/ASLs in LR formation (supplementary material Fig. S1) (Okushima et al., 2005; Okushima et al., 2007). LR formation in the *lbd16-1 arf7-1* and *lbd16-1 arf19-1* double mutants, however, was strongly reduced, suggesting that the potential to form LRs retained in the *arf7-1* and *arf19-1* single mutant is partially dependent on LBD16 (supplementary material Fig. S1). However, *LBD16-GFP* did not restore the LR phenotype in the *lbd16-1 arf7-1 arf19-1* triple mutant, which had no LRs, the same phenotype as the *arf7-1 arf19-1* (supplementary material Fig. S1) (Okushima et al., 2005; Okushima et al., 2007). These results indicate that *arf7-1 arf19-1* mutations are epistatic to *lbd16-1*, consistent with the previous model that ARF7 and ARF19 redundantly regulate LR formation via activity of LBD/ASLs (Okushima et al., 2007; reviewed by Fukaki and Tasaka, 2009).

In *LBD16-GFP* seedlings, LBD16-GFP was strongly expressed in the early steps of LR initiation at the pairs of specific XPP cells in three cell files and the expression was localized in the nuclei (Fig. 1A-C), supporting the idea that LBD16 is linked to LR initiation events and functions in the nuclei as a transcriptional regulator (Okushima et al., 2007).

In *Arabidopsis*, LR formation is initiated by the ACDs of the pair of XPP cells (Péret et al., 2009; Benková and Bielach, 2010; De Rybel et al., 2010). At first, two adjacent XPP cells are specified as LR founder cells, and they accumulate auxin. Then, their two nuclei migrate towards the common cell wall, establishing cell polarity. After that, both LR founder cells divide asymmetrically, thereby producing two small daughter cells and two larger flanking cells. To monitor how LBD16-GFP expression links to the ACDs of LR founder cells in detail, we performed time-lapse imaging on XPP cell files during LR formation induced by gravity-stimulated root bending (Laskowski et al., 2008). In this assay, root gravitropic bending induces auxin accumulation at the outer side of the curvature, which could in turn induce LR formation at the bending point (Laskowski et al., 2008). When the 4-day-old *LBD16-GFP* seedlings were gravistimulated by turning the agar plate 90° for 6 hours, and then placed horizontally under the confocal microscope, the expression of LBD16-GFP could be observed in the specific XPP on the outside of the curvature about 6 hours after gravistimulation (supplementary material Movie 1). After about 11 hour, two nuclei of the pair of XPP cells migrated towards the common cell walls, followed by the first anticlinal ACDs (Fig. 2A; supplementary material Movie 1). The expression level of LBD16-GFP gradually increases with time as it approaches the first ACDs (supplementary material Movie 1). These data strongly suggest that LBD16/ASL18 is involved in these early steps of LR initiation, including nuclear migration of LR founder cells and their subsequent ACDs.

A synthetic auxin response reporter, *DR5rev::erGFP*, known as one of the earliest markers for LR initiation (Benková et al., 2003; Dubrovsky et al., 2008), is expressed before the first ACD of the LR founder cells at XPP. To compare the timing of both *DR5* and *LBD16* promoter activity during LR initiation, we generated transgenic lines by crossing the *DR5rev::erGFP* line with a transgenic plant expressing NLS-tagRFP driven by the *LBD16* promoter (*pLBD16::NLS-tagRFP*, NLS: nuclear localization signal). As shown in Fig. 1D-I, *DR5rev::erGFP* and *pLBD16::NLS-tagRFP* are co-expressed in the same XPP cells before and after the first ACD of the LR founder cells during early stages of LR initiation. These observations indicate that specific activation of LBD16/ASL18 at the LR founder cells coincides with localized auxin response maximum in XPP cells.

Specific expression of LBD16/ASL18 in the LR founder cells is regulated by the SLR/IAA14-ARF7-ARF19-dependent auxin signaling module

ARF7 and ARF19 activate *LBD16/ASL18* expression via direct binding to its promoter region (Okushima et al., 2007). To investigate the effects of the SLR/IAA14-ARF7-ARF19-dependent auxin signaling on *LBD16/ASL18* expression in the LR founder cells, we examined the expression of LBD16-GFP in the *slr-1* and *arf7 arf19* mutant backgrounds, which were severely impaired in LR initiation (Fukaki et al., 2002; Fukaki et al., 2005; Okushima et al., 2005; Vanneste et al., 2005; Okushima et al., 2007). Compared with the localized activation of LBD16-GFP in the LR founder cells at wild-type XPP cells (Fig. 3A), both the *slr-1* and *arf7 arf19* mutants showed very low expression of LBD16-GFP without any localized activation in the XPP (Fig. 3B,C). Whereas the *arf7 arf19* mutant retained weak XPP-specific expression of LBD16-GFP (Fig. 3C), the *slr-1* mutant showed low expression of LBD16-GFP only in the endodermal cells but not in the pericycle cells (Fig. 3B, yellow asterisks). These observations indicate that normal auxin signaling regulated by the SLR/IAA14-ARF7-ARF19 module is necessary for the localized activation of *LBD16/ASL18* expression in the LR founder cells at XPP.

Targeted expression of LBD16/ASL18 in XPP induces LR formation in the absence of ARF7 and ARF19 activity

In order to investigate whether targeted expression of LBD16/ASL18 in XPP rescues the LR-less phenotype of the *arf7 arf19* double mutant, we produced transgenic *arf7 arf19* plants

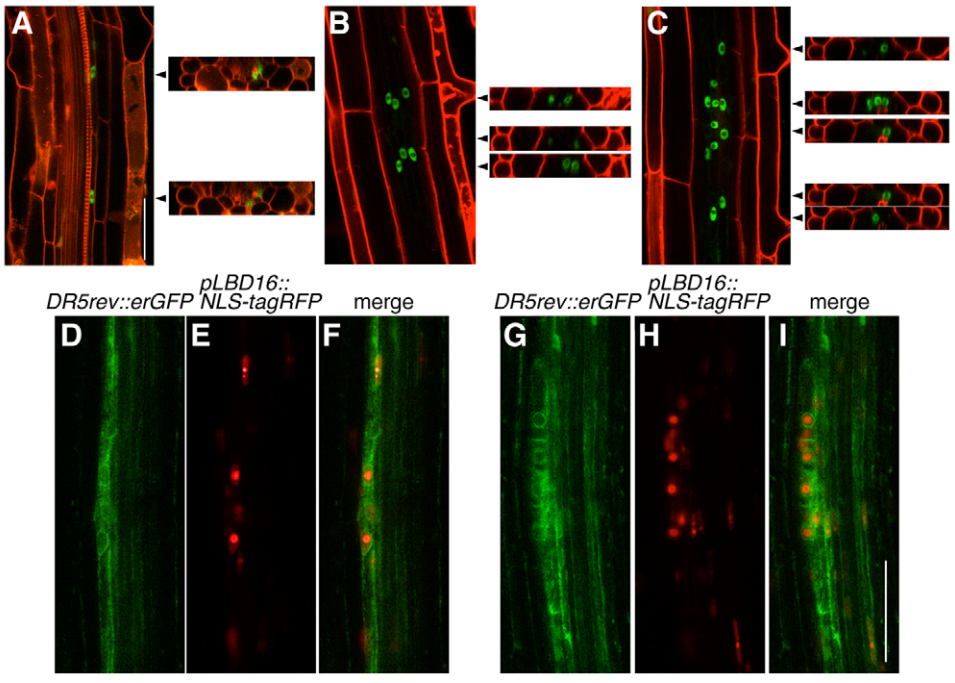

Fig. 1. Expression pattern of *LBD16-GFP* around the LR initiation sites. (**A-C**) LBD16-GFP is expressed in pairs of pericycle cells in three cell files adjacent to the xylem pole. Expression of LBD16-GFP, under its own regulatory region (*genomeLBD16-GFP*; *LBD16-GFP*), was observed using confocal laser scanning microscopy. The expression starts before nuclear migration toward the common cell wall (A) and the first ACD (B). After the first ACD, LBD16-GFP is specifically expressed in the LR initiation site (C). Green, LBD16-GFP; red, PI. (**D-I**) Expression pattern of *DR5rev::erGFP* and *pLBD16::NLS-tagRFP* during early stages of LR initiation. (D-F) Before the first ACD. (G-I) After the first ACD. Both reporter genes are co-expressed during early stages of LR initiation. Green, erGFP; red, NLS-tarp. Scale bars: 50 μm.

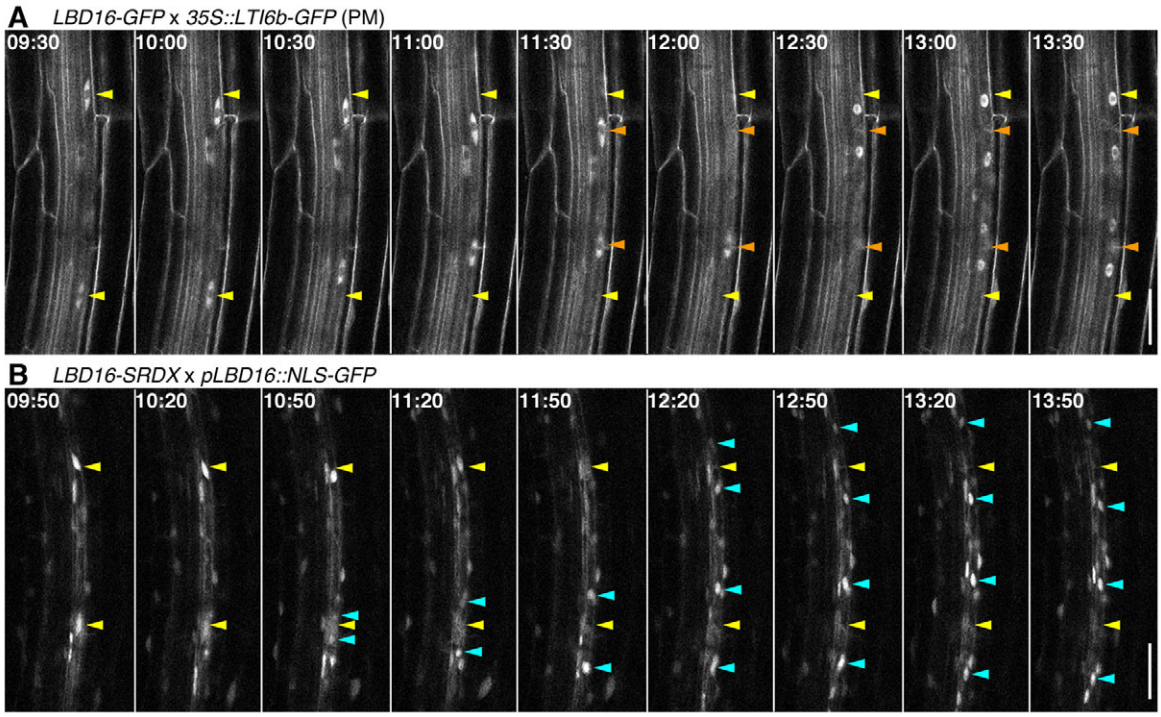

Fig. 2. Time-lapse imaging of *LBD16* expression during LR initiation events. (**A**) Time-lapse imaging of the nuclear migration and asymmetric cell division in the XPP of a wild-type root using *LBD16-GFP* x *35S::LTI6b-GFP* (PM) line. The yellow arrowheads indicate the initial position of the nucleus and the orange arrowheads show the position of the nucleus after migration. (**B**) Time-lapse imaging of symmetrical cell division in the XPP of a *genomeLBD16-SRDX* (LBD16-SRDX) root using a *pLBD16::NLS-GFP* line. The yellow arrowheads indicate the initial position of the nucleus and the aqua arrowheads show the divided nucleus. In A and B, the time (hour:minute) after the gravistimulation is indicated in each panel. Scale bars: 50 μm.

containing both the GAL4-VP16 driver construct in the enhancer trap line *J0121*, which is specifically active in XPP, and the *5xUAS::LBD16* effector construct (*J0121>>LBD16/arf7 arf19*) (Laplaze et al., 2005). Although neither the *J0121/arf7 arf19* nor *5xUAS::LBD16/arf7 arf19* seedlings are able to form LRs, the F_1 progeny from the genetic cross between *J0121/arf7 arf19* and *5xUAS::LBD16/arf7 arf19* seedlings (*J0121>>LBD16/arf7 arf19*) developed LRs (Fig. 4A,B), indicating that targeted expression of LBD16/ASL18 in XPP partially rescues the ability to form LRs in the *arf7 arf19* double mutant. We observed the *J0121>>LBD16/arf7 arf19* seedlings in detail by microscopy and found that targeted expression of LBD16/ASL18 in XPP results in strong activation of anticlinal cell divisions in XPP (Fig. 4C). Owing to highly activated cell division in XPP and close contacts of adjacent LR initiation sites, it was impossible to count the number of LR primordia in the *J0121>>LBD16/arf7 arf19* seedlings. These observations indicate that expression of LBD16/ASL18 in XPP is sufficient to promote cell divisions for LR initiation in the absence of ARF7 and ARF19.

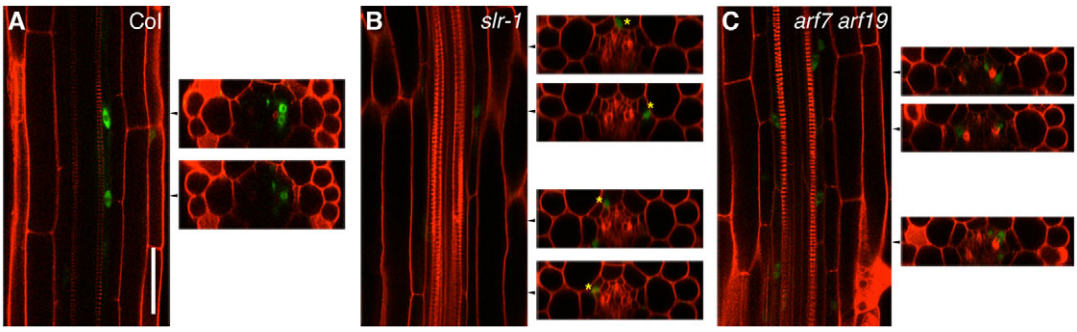

Fig. 3. LBD16-GFP expression in the LR founder cells is regulated by the SLR/IAA14-ARF7-ARF19-dependent auxin signaling module. (**A-C**) Expression pattern of *LBD16-GFP* in the wild-type Col (A), *slr-1* (B) and *arf7-1 arf19-1* (C) primary roots. Compared with the wild-type Col (A), the fluorescence intensity of LBD16-GFP was very weak in the *slr-1* (B) and *arf7-1 arf19-1* mutants (C). The yellow asterisks in B indicate the expression of LBD16-GFP in the endodermis of *slr-1* mutant. The pictures shown in the three panels were taken using the same camera settings. Green, LBD16-GFP; red, PI. Scale bar: 50 μm.

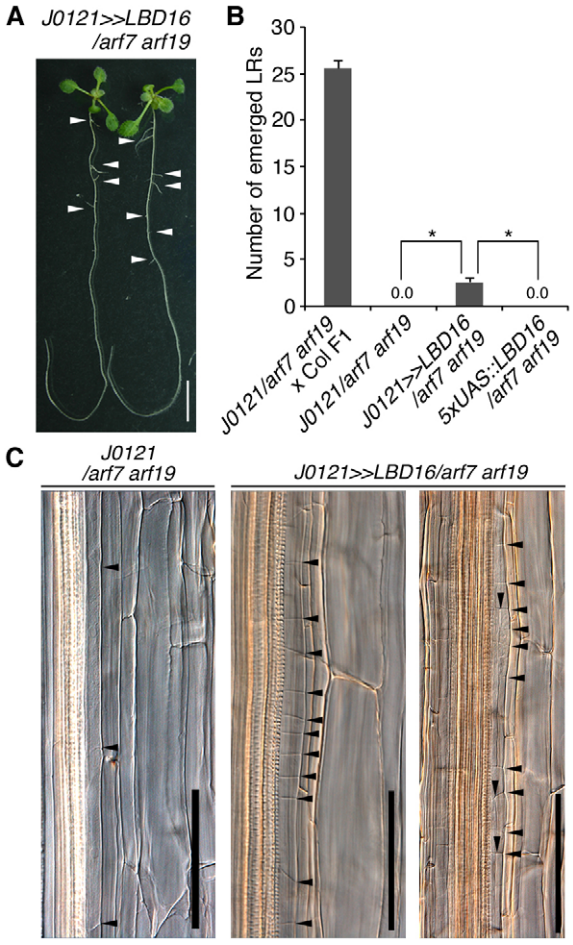

Fig. 4. XPP-specific expression of *LBD16* induces LR formation and anticlinal cell divisions in *arf7 arf19* double mutant. (**A**) Seedlings (12 days old) of *J0121>>LBD16* in *arf7 arf19* double mutant. The *arf7-2 arf19-5* was used as *arf7 arf19* in this experiment. The arrowheads indicate emerged LRs. (**B**) Number of LRs in the indicated lines at 12 DAG. The values of *J0121/arf7 arf19* or *5xUAS::LBD16/arf7 arf19* are 0.0±0.0 (mean ± s.e.m.). *n*>25. The error bars indicate s.e.m. The asterisks indicate a statistical difference (*P<0.01 by two-sided *t*-test). (**C**) *J0121>>LBD16/arf7 arf19* showed strong activation of cell division in XPP cells (middle and right) compared with *J0121/arf7 arf19* (left). The arrowheads indicate cell wall. Scale bars: 10 mm in A; 100 μm in C.

LBD16/ASL18 and related LBD/ASL proteins are required for LR formation

Compared with the *slr-1* and *arf7 arf19* mutants, the *lbd16-1* knockout mutant has a slightly reduced number of LRs, suggesting redundant functions between LBD16/ASL18 and its related LBD/ASL proteins (Fig. 5C; supplementary material Fig. S1) (Okushima et al., 2007). In *Arabidopsis*, there are 42 genes encoding LBD/ASL proteins (Iwakawa et al., 2002; Shuai et al., 2002; Matsumura et al., 2009; Majer and Hochholdinger, 2011). Five of these genes, namely *LBD16/ASL18*, *LBD17/ASL15*, *LBD18/ASL20*, *LBD29/ASL16* and *LBD33/ASL24*, are auxin-inducible and expressed in the LR primordia (supplementary material Fig. S2), and they belong to a subfamily in class I LBD/ASLs (Okushima et al., 2007; Lee et al., 2009; Majer and Hochholdinger, 2011). In addition, their auxin-inducible expression is positively regulated by ARF7 and ARF19 (Okushima et al., 2005; Okushima et al., 2007). T-DNA insertion knockout mutants are available for *LBD16/ASL18* (SALK_095791, *lbd16-1*), *LBD18/ASL20* (SALK_038125, *lbd18-1*) and *LBD33/ASL24* (SAIL_95_H10, *lbd33-1*) from the SALK collection, but no knockout lines of *LBD17/ASL15* and *LBD29/ASL16* are available in public collections. The *lbd16-1* and the *lbd18-1* single mutations slightly reduced the number of LRs without affecting the primary root growth, whereas the *lbd33-1* single mutation had no inhibitory effect on LR formation (Fig. 5A; supplementary material Fig. S3) (Okushima et al., 2007; Lee et al., 2009). The *lbd16-1 lbd18-1* double mutant had decreased LR density as previously reported (supplementary material Fig. S3) (Lee et al., 2009). We found that the *lbd16-1 lbd18-1 lbd33-1* triple mutant showed more reduction of LR number (Fig. 5A-C; supplementary material Fig. S3). We also found that, although the LR initiation density (number of non-emerged LR primordia and emerged LRs per primary root) in *lbd16-1 lbd18-1 lbd33-1* was lower than that of wild type, the non-emerged LR primordium density (number of non-emerged LR primordia per primary root) of *lbd16-1 lbd18-1 lbd33-1* was higher than that of wild type (supplementary material Fig. S4A,B), indicating that LBD16/18/33 are necessary not only for LR initiation but also for LR primordium development and emergence. In addition, we found that the *lbd16-1 lbd18-1 lbd33-1* mutant had more pre-LR initiation sites with DR5::GUS activity than wild-type Col, but the *lbd16-1 lbd18-1 lbd33-1* mutations did not affect the density of pre- and post-initiation sites with DR5::GUS activity (supplementary material Fig. S4C), suggesting that the *lbd16-1 lbd18-1 lbd33-1* mutations decrease LR initiation events without affecting the specification of LR founder cells. However, LR primordia at Stage I after ACDs were observed in the *lbd16-1 lbd18-1 lbd33-1* mutant, suggesting that the asymmetry of the LR founder cells was established in the *lbd16-1 lbd18-1 lbd33-1* mutant (supplementary material Fig. S4D). These observations are consistent with our hypothesis that LBD16/ASL18 and related LBD/ASL proteins, including at least LBD18/ASL20 and LBD33/ASL24, function redundantly in LR formation.

Expression of LBD16-SRDX under its own regulatory region blocks LR formation

In order to directly confirm the functional redundancy of LBD16/ASL18 and related LBD/ASL members in LR formation, we constructed *Arabidopsis* transgenic plants expressing LBD16-SRDX under its own regulatory region (*genomeLBD16-SRDX, LBD16-SRDX*), instead of CaMV *35S* promoter as used in a previous study (Okushima et al., 2007). SRDX is an artificial repression domain for the construction of chimeric repressors of transcriptional factors, which is originally identified from EAR-motif repression domain (Hiratsu et al., 2003). SRDX-fused transcriptional activator dominantly represses the transcription of its target genes, even in the presence of endogenous and functionally redundant transcription activators (Hiratsu et al., 2003). We found that about half of the independent transgenic lines of *LBD16-SRDX* have no or fewer LRs (*n*=20). The inhibitory effect of LBD16-SRDX was more obvious when transformed into the *lbd16-1* knockout mutant (11 out of 12 independent lines). In the severely affected *LBD16-SRDX* lines in wild-type background, LR formation was completely blocked even 12 DAG (Fig. 5A,C), but the primary root length was not affected (Fig. 5B). Expression of *LBD16-SRDX* also inhibited the auxin-induced LR formation but had no effect on auxin-inhibited primary root growth (Fig. 6A-C), whereas exogenous auxin promotes LR formation but inhibits

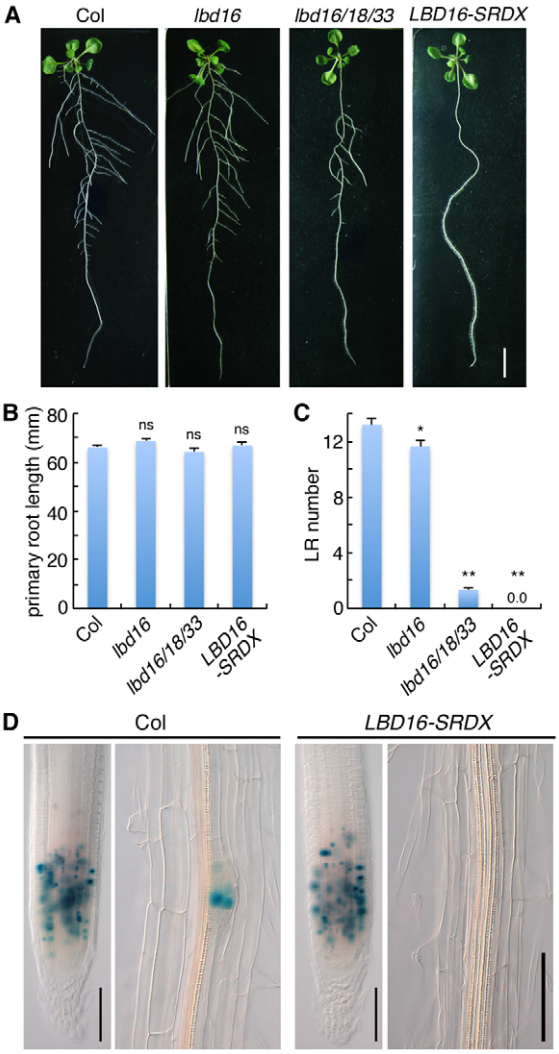

Fig. 5. LBD16/ASL18 and its related LBD/ASL proteins are required for LR formation. (**A**) Twelve-day-old seedlings of Col, *lbd16*, *lbd16 lbd18 lbd33* triple mutant and *LBD16-SRDX*. (**B**,**C**) Primary root length (B) and LR number (C) of Col, *lbd16*, *lbd16 lbd18 lbd33* triple mutant and *LBD16-SRDX* at 10 DAG. The value of *LBD16-SRDX* is 0.0±0.0 (mean ± s.e.m.). *n*=20. The error bars indicate s.e.m. The asterisks indicate a statistical difference from Col (*P<0.05, **P<0.01 by two-sided *t*-test). (**D**) Expression pattern of *pCyclinB1;1::CycB1;1(NT)-GUS* (CycB1;1-GUS) in Col and *LBD16-SRDX*. CycB1;1-GUS expression in root tip and mature root region is shown. Scale bars: 10 mm in A; 100 μm in D. ns, not significant.

primary root growth in the wild type. Furthermore, no anticlinal cell divisions related to LR initiation were observed in the *LBD16-SRDX* plants, even when treated with exogenous auxin (Fig. 6D). This was confirmed by CycB1;1-GUS expression: no GUS spots were observed in the mature root region of *LBD16-SRDX* plants (Fig. 5D). These observations strongly suggest that the *LBD16-SRDX* competitively inactivates the redundant function of LBD16/ASL18 and the other related LBD/ASLs where *LBD16/ASL18* is induced, thereby specifically inhibiting LR initiation.

We also examined the effects of the other related LBD/ASL members (LBD17/ASL15, LBD29/ASL16 and LBD33/ASL24) fused with SRDX under the control of their own regulatory region. We analyzed ten independent *genomeLBDs-SRDX* lines each, but there were no obvious phenotypes as observed in *LBD16-SRDX* lines, suggesting that LBD17-, LBD29- and LBD33-SRDX could not competitively inactivate the redundant function of LBD/ASLs in wild-type background. However, expression of *LBD29-SRDX* in *lbd16-1* resulted in no, or fewer, LRs (supplementary material Fig. S5), whereas both *LBD17-SRDX* and *LBD33-SRDX* had slight effects on LR formation in *lbd16-1*. These phenotypic differences might be due to the differences in their promoter activity, protein stability or effect of transcriptional repression among these LBDs-SRDX proteins. These experiments also indicate the importance of LBD16/ASL18 and related LBD/ASL members for LR initiation.

LBD16-SRDX does not affect local activation of auxin response in specific XPP cells

We examined whether expression of *LBD16-SRDX* affects the establishment of the auxin response maximum in the primary roots using the *DR5::GUS* reporter. In *LBD16-SRDX* seedlings, *DR5::GUS* was normally expressed in the root tip (Fig. 7A,B). However, strong *DR5::GUS* activity was observed in the two adjacent XPP cells even though anticlinal cell division is completely blocked (Fig. 7C-F). These observations indicate that specification of LR founder cells with localized auxin response maximum is not inhibited by LBD16-SRDX, suggesting that the upstream pathway of SLR/IAA14-ARF7-ARF19 signaling occurs in the *LBD16-SRDX* plants.

Interestingly, we noticed that the number of pericycle cells with intense auxin response was dramatically increased in the *LBD16-SRDX* plants (Fig. 7C,D,G). A twofold increase in number of *DR5::GUS* spots (including pre-initiation spots and LR primordia with auxin response) per centimeter of primary root were observed in 7-day-old *LBD16-SRDX* seedlings (Fig. 7G). *pLBD16::GUS* reporter also showed an expression pattern similar to that of *DR5::GUS* in the *LBD16-SRDX* plants in which the number of strong *pLBD16::GUS* sites was increased (Fig. 7H). These results suggest that inhibition of LR initiation by LBD16-SRDX increases the frequency of LR founder cells with auxin response maximum in the *LBD16-SRDX* seedlings.

LBD16-SRDX inhibits the polar nuclear migration and ACD in XPP cells with auxin response maximum

In wild type, the nuclei of two adjacent XPP cells migrate to the common cell walls to establish the asymmetry of these cells before the first ACD for LR initiation. To observe the position of the nucleus in XPP cells of *LBD16-SRDX* plants, NLS-GFP was expressed under the control of the *LBD16* promoter. In *LBD16-SRDX* plants, each nucleus was localized in the center of the XPP cells (Fig. 7I), suggesting that LBD16-SRDX inhibits nuclear migration in XPP cells. However, it was unclear whether LBD16-SRDX inhibits nuclear migration in the presumptive LR founder cells with auxin response maximum. To clarify this point, we performed time-lapse imaging during LR initiation events in *LBD16-SRDX* plants. Unlike wild-type plants, gravistimulated root bending could not induce LR formation in *LBD16-SRDX* plants (data not shown). However, relatively strong fluorescence of *pLBD16::NLS-GFP* was observed in pairs of XPP cells in the central bending region of *LBD16-SRDX* plants as in the wild-type plants, consistent with the assumption that LR founder cells were specified in the *LBD16-SRDX* plants as indicated above (Fig. 2B, Fig. 7D,F,H; supplementary material Movie 2). Surprisingly, although the nuclei did not migrate to the common cell walls in

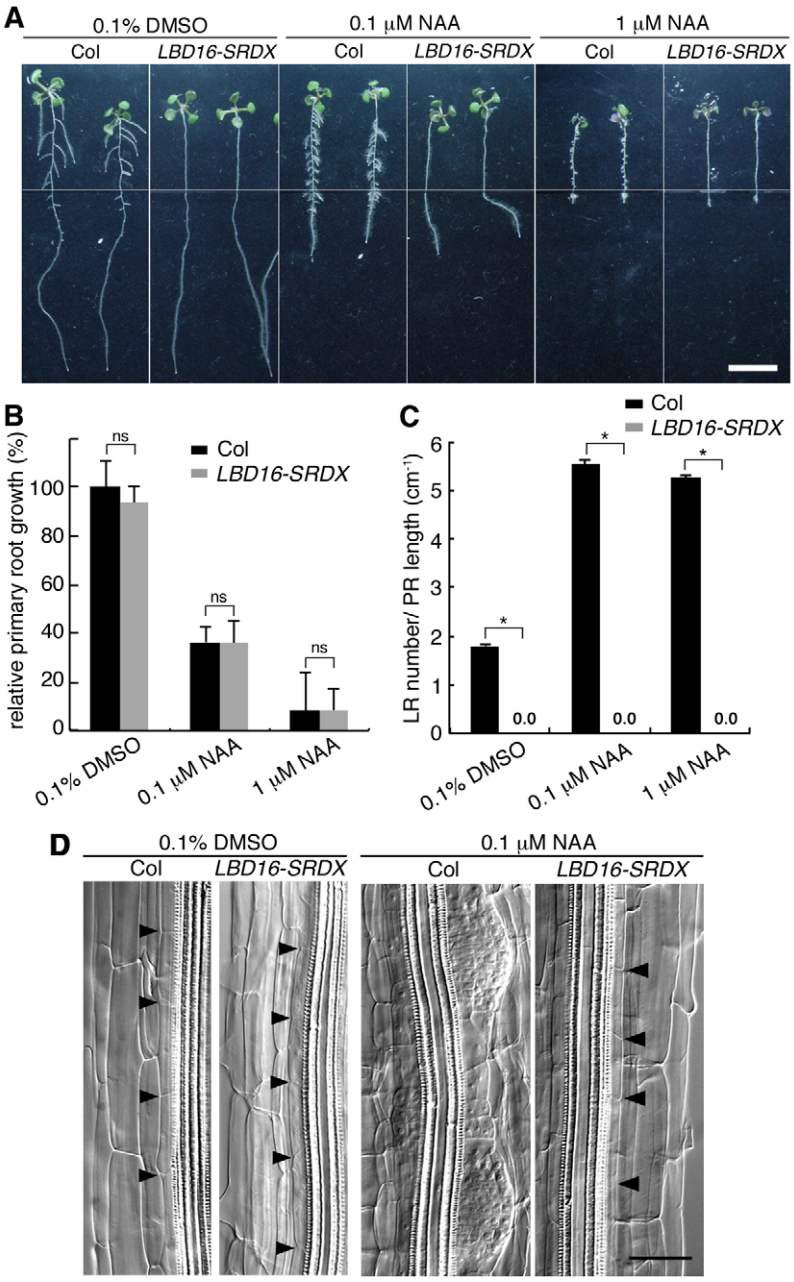

Fig. 6. Auxin-induced LR formation is completely inhibited by LBD16-SRDX. (**A**) Four-day-old *LBD16-SRDX* seedlings were transferred onto NAA-containing or NAA-free media, and incubated for an additional 72 hours. (**B**) Root elongation of seedlings on NAA-free, 0.1 μM and 1.0 μM NAA medium for 72 hours. The data are represented as growth of primary root relative to growth on NAA-free medium. The error bars represent s.e.m. *n*=20. ns, not significant. (**C**) LR number per primary root length after NAA treatment for 72 hours. The values of *LBD16-SRDX* are 0.0±0.0 (mean ± s.e.m.). *n*=20. The error bars represent s.e.m. The asterisks indicate a statistical difference between Col and *LBD16-SRDX* (**P*<0.001 by two-sided *t*-test). (**D**) LBD16-SRDX inhibits auxin-induced cell division in XPP. Nomarski images of Col and *LBD16-SRDX* grown on NAA-free or 0.1 μM NAA medium for 36 hours at 4 DAG. The arrowheads indicate cell wall positions. Scale bars: 10 mm in A; 50 μm in D.

LBD16-SRDX plants, symmetric cell divisions occurred in such XPP cells with auxin response monitored by *pLBD16::NLS-GFP* about 11 hours after gravistimulation (Fig. 2B; supplementary material Movie 2; *n*=5). However, these symmetrically divided XPP cells in *LBD16-SRDX* plants did not show additional divisions until 32 hours after gravistimulation, at which time a few rounds of divisions were usually observed in the wild-type plants. These observations suggest that LBD16/ASL18 and related LBD/ASLs establish the asymmetry of LR founder cells to the first ACDs for LR initiation.

DISCUSSION

In multicellular organisms, cell differentiation mediated by ACD is important for the development of new organs. In flowering plants, LRs are formed from the apparently differentiated pericycle cells adjacent to the XPP, which divide asymmetrically to produce central small and flanking large cells with different fates (De Smet et al., 2008). Local auxin response maximum of specific XPP cells is important for the polarization of LR founder cells before the first ACD. However, it remains unknown how auxins regulate this initial step of LR initiation. Here we showed that LBD16/ASL18 and related LBD/ASL members play important roles in the establishment of asymmetry of LR founder cells.

We demonstrated that LBD16/ASL18 is specifically expressed in pairs of XPP cells in three cell files before the first anticlinal ACD (Fig. 1A-C, Fig. 2A). The fluorescent intensity of LBD16-GFP was very weak in the *slr-1* and *arf7 arf19* mutants, indicating that the local activation of *LBD16* expression is regulated by the SLR/IAA14-ARF7-ARF19-dependent auxin signaling module (Fig. 1D-I, Fig. 3). In the roots, *ARF7* is expressed in the stele including XPP, whereas *ARF19* is broadly expressed (Okushima et al., 2005), strongly suggesting that the local ARF7-ARF19-dependent auxin signaling should be required to achieve the activation of *LBD16* expression in LR founder cells. Interestingly,

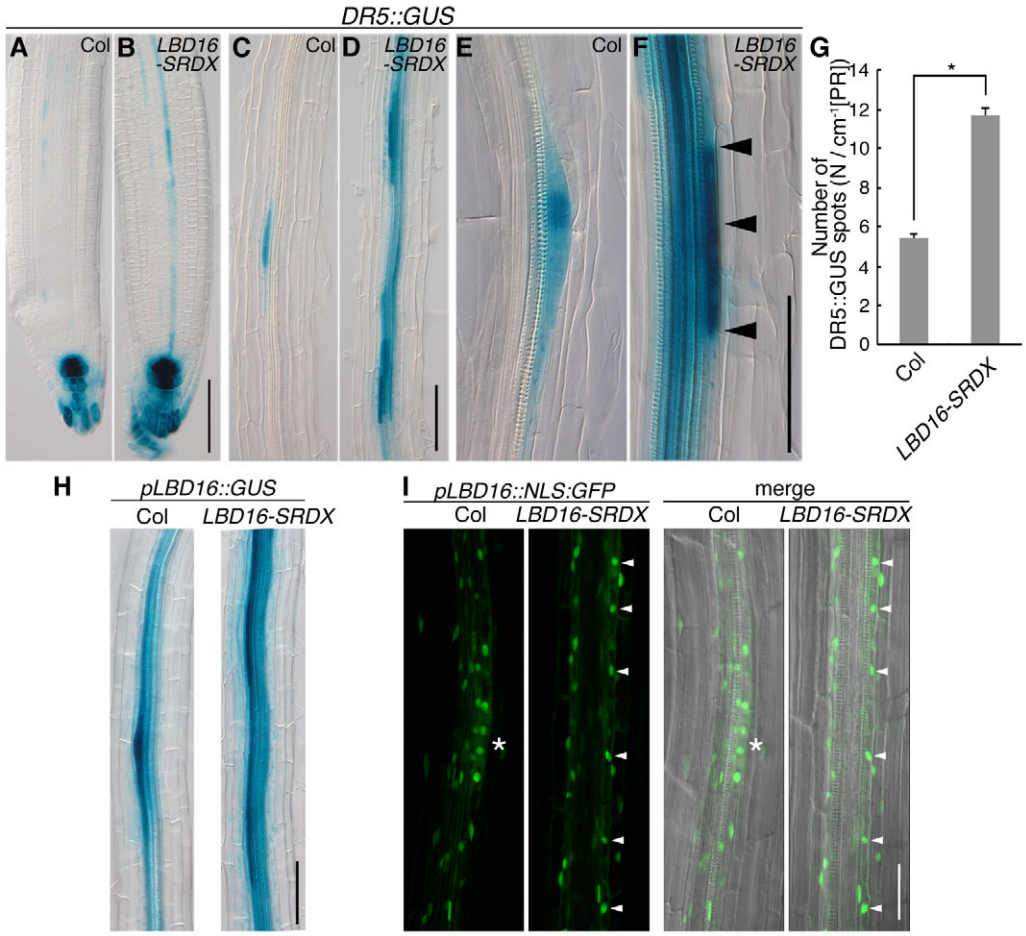

Fig. 7. LBD16-SRDX inhibits polar nuclear migration without affecting local activation of auxin response in LR founder cells. (**A**,**B**) Expression of *DR5::GUS* in the root tip of Col (A) and *LBD16-SRDX* (B) seedlings (5 DAG). (**C-F**) Expression of *DR5::GUS* in the mature root region of Col (C,E) and *LBD16-SRDX* (D,F) seedlings (5 DAG). Magnified views of the pericycle cells expressing *DR5::GUS* are shown (E,F). The arrowheads indicate the cell wall. (**G**) The number of *DR5::GUS* spots increases in the *LBD16-SRDX* roots. Pre-initiation sites (LR founder cells in the xylem pole pericycle), LR primordia and emerged LRs with DR5::GUS signal were counted in Col and *LBD16-SRDX* at 7 DAG. n=15. *P<0.001 by two-sided t-test. (**H**) Expression pattern of *pLBD16::GUS* in the *LBD16-SRDX* roots. *LBD16* promoter activity in mature root region of Col and *LBD16-SRDX* seedlings (5 DAG) is shown. (**I**) Nuclear position visualized by NLS-GFP driven by *LBD16* promoter in Col and *LBD16-SRDX* roots. The asterisks indicate LR initiation sites and the arrowheads show the XPP nuclei. Scale bars: 100 μm in B,D,F,H; 50 μm in I.

weak expression of *LBD16-GFP* was observed only in the endodermis but not in XPP in the *slr-1* mutant although the *slr-1* mutation does not affect XPP identity shown with *J0121* expression, a marker for XPP cell files (Fig. 3) (Vanneste et al., 2005). It is known that the *slr-1* mutant has higher auxin content in the root, which seems to be due to disturbed auxin homeostasis (Vanneste et al., 2005). The increased auxin content in *slr-1* mutant might alter the expression pattern of *LBD16-GFP* in the roots, independently from the SLR/IAA14-ARF7-ARF19 module. Taken together, these observations indicate the importance of normal SLR/IAA14-ARF7-ARF19-dependent auxin signaling for LBD16/ASL18 expression in LR founder cells.

The specific XPP cells expressing LBD16/ASL18 are supposed to be the LR founder cells, which are specified by priming events in the basal meristematic region dependent on oscillatory gene expression, including *GATA23* (De Rybel et al., 2010; Moreno-Risueno et al., 2010). Because LBD16/ASL18 was not expressed in the basal meristem (supplementary material Fig. S2) and LBD16-SRDX did not interfere with the establishment of local activation of the auxin response (Fig. 7C-F), our results suggest that auxin-inducible LR priming events are not directly regulated by LBD16/ASL18 and its related LBD/ASLs. This is also supported by the fact that the SLR/IAA14-ARF7-ARF19 signaling module is not involved in specification of LR founder cells (De Smet et al., 2007; De Rybel et al., 2010). The SLR/IAA14-ARF7-ARF19 signaling module is thought to be required for controlling the polar migration of the nuclei in LR founder cells, because excess application of auxin to the *slr-1* and *arf7 arf19* mutants causes abnormal nuclear migration and ACDs (De Rybel et al., 2010). Here, we could clarify more precisely the contribution of the SLR/IAA14-ARF7-ARF19 module to the control of the polarity of LR founder cells. We hypothesize that the SLR/IAA14-ARF7-ARF19 module activates the expression of auxin-inducible LBD/ASLs, including LBD16/ASL18, in response to local activation of auxin response in LR founder cells, thereby inducing the symmetry break for polarization of LR founder cells (Fig. 8).

The *Arabidopsis* genome has 42 genes encoding LBD/ASL proteins, which are separated into two groups characterized by the presence (class I) or absence (class II) of leonine-zipper-like domain (Matsumura et al., 2009; Majer and Hochholdinger, 2011).

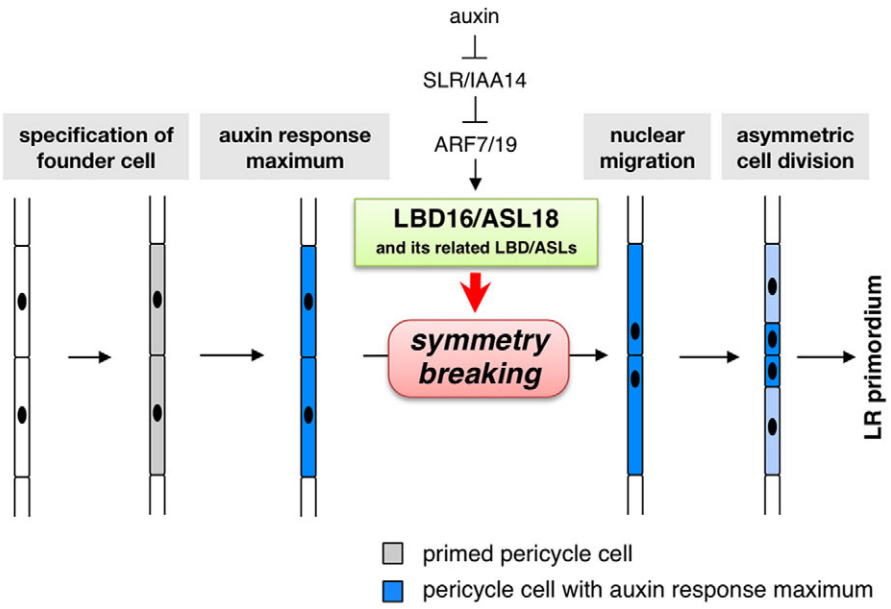

Fig. 8. Schematic model for the role of LBD16/ASL18 and related LBD/ASLs in LR initiation. LBD16/ASL18 is activated in the LR founder cells dependent on SLR/IAA14-ARF7-ARF19 auxin signaling module, and promotes the symmetry breaking for polarization of LR founder cells, probably through transcriptional regulation with its related LBD/ASLs, thereby leading the nuclear migration and ACD for LR initiation and subsequent LR primordium development.

The class I LBD/ASLs play an important role in defining organ boundaries and are involved in various plant developmental processes, including embryogenesis and development of root, leaf, and inflorescence. The five LBD/ASLs, LBD16/ASL18, LBD17/ASL15, LBD18/ASL20, LBD29/ASL16 and LBD33/ASL24, belong to the subtype C in the class I group, and they are all auxin-inducible and expressed at the LR initiation site and LR primordia (Okushima et al., 2007; Lee et al., 2009; Matsumura et al., 2009) (this study). In addition, only these five LBD/ASLs were identified as auxin-inducible LBD/ASLs in the XPP cells (De Smet et al., 2008). We demonstrated that multiple mutants of these LBD/ASLs and transgenic lines of *LBD16-SRDX* and *LBD29-SRDX* had no, or fewer, LRs, suggesting that LBD16/ASL18 and its related auxin-inducible LBD/ASLs have redundant functions in LR initiation. SRDX technology has been utilized in several studies of transcriptional factors to overcome functional redundancy (Hiratsu et al., 2003; Kubo et al., 2005; Ishida et al., 2007; Koyama et al., 2007; Mitsuda et al., 2007; Groszmann et al., 2008; Soyano et al., 2008). Although expression of SRDX-fused transcriptional activators might have unexpected effects, including competing with unrelated proteins for DNA binding or might form aberrant transcriptional complexes, the various transgenic plants expressing SRDX-fused transcriptional activators exhibit a phenotype similar to the loss-of function mutants (Hiratsu et al., 2003; reviewed by Mitsuda and Ohme-Takagi, 2009). LBD16-SRDX driven by constitutive CaMV *35S* promoter had various effects on plant development, such as inhibition of primary root growth, aberrant leaf morphology in addition to inhibition of LR formation (Okushima et al., 2007). However, such additional effects were not found in the *LBD16-SRDX* (*genomeLBD16-SRDX*) plants (Fig. 5A), suggesting that the *LBD16-SRDX* specifically inhibits the functions of endogenous LBD16/ASL18 and its related LBD/ASLs only where LBD16/ASL18 could be expressed. As the *Arabidopsis* LR primordia express several non-auxin-inducible *LBD/ASLs*, including *LBD37/ASL39*, *LBD38/ASL40* and *LBD41/ASL38*, which belong to the class II LBD/ASLs (supplementary material Fig. S6) (Brady et al., 2007), the possibility that non-auxin-inducible LBD/ASLs expressed in LR primordium might be also involved in LR formation with the auxin-inducible LBD/ASLs still remains. Among the *LBDs-SRDX* that we tested, *LBD16-SRDX* inhibits LR initiation most efficiently, strongly suggesting that LBD16/ASL18 is one of the key regulators for ACDs of the LR founder cells (Fig. 5; supplementary material Fig. S5).

Recently, Berckmans et al. (Berckmans et al., 2011) reported that LBD18/ASL20 and LBD33/ASL24 function as a heterodimer and regulate the cell cycle reactivation on LR initiation through transcriptional activation of cell cycle controller, E2Fa (Berckmans et al., 2011). LBD18/ASL20 is also involved in the LR primordium development and emergence (Lee et al., 2009), as well as in the xylem tissue differentiation (Soyano et al., 2008). These findings also suggest that LBD16/ASL18 and related LBD/ASL members might regulate a variety of developmental steps during LR formation. Furthermore, recent analysis of the *sidecar pollen* (*scp*)*/lbd27/asl29* mutant in *Arabidopsis* showed that LBD27/ASL29 is important for the correct timing and control of division orientation of microspore but not for nuclear migration (Oh et al., 2010). The general and specific functions of LBD/ASL proteins in the regulation of cell division in plants are worth investigating further.

We also found that expression of *LBD16-SRDX* increased the number of XPP cells with auxin response maximum without inducing the nuclear migration and ACDs (Fig. 2B, Fig. 7D,G,I). This result suggests that there might be a mechanism for lateral inhibition of LRs through LBD16/ASL18-dependent LR initiation, thereby controlling the frequency of LR formation. In fact, either the *bdl/iaa12* or *mp/arf5* mutation causes the clustered LRs under some conditions, suggesting that ARF-Aux/IAA auxin signaling modules play the role for the control of LR distribution (De Smet et al., 2010). In the *LBD16-SRDX* plants, it was not confirmed whether the new initiation events occurred between existing LR founder cells or whether the number of primed cells were increased in the basal meristem. However, auxin responses detected by both *DR5::GUS* and *pLBD16::GUS* were enhanced in the root stele of the *LBD16-SRDX* seedlings (Fig. 6D,H). As observed in the *slr-1* mutant, which is defective in LR initiation, the increased auxin response maximum in *LBD16-SRDX* might be due to the feedback regulation through the control of active auxin biosynthesis, auxin transport and/or signaling (Vanneste et al., 2005; Swarup et al.,

2008). In *LBD16-SRDX* seedlings, the enhanced auxin response might induce additional LR initiation or disturb priming events in the root basal meristem, thereby increasing the number of XPP cells with auxin response maximum. We hypothesize that there might be a feedback loop in which LBD16/ASL18 and its related LBD/ASLs positively regulate nuclear migration and ACDs of LR founder cells while these LBD/ASLs or LR initiation events negatively act upon LR initiation in the neighboring regions. Unfortunately, we could not detect the increased auxin response maximum in the *lbd16 lbd18 lbd33* triple mutant in which both LR initiation and LR primordium development and emergence were inhibited (supplementary material Fig. S4), suggesting that a mechanism for lateral inhibition of LRs still works in the *lbd16 lbd18 lbd33* triple mutant through the remaining LBD/ASL-dependent LR initiation.

In summary, our study has demonstrated that LBD16/ASL18 and related LBD/ASL proteins regulate the establishment of asymmetry in *Arabidopsis* LR founder cells. Identification and analysis of the downstream targets of LBD16/ASL18 and its related LBD/ASLs will further our understanding of the mechanisms regulating the polarization of LR founder cells, subsequent ACDs and LR primordium development, which are key steps for constructing the plant root system.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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