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ORIGINAL RESEARCH

Ectopic Expression of *LBD18*/*ASL20* Results in Arrest of Plant Growth and Development with Repression of *AINTEGUMENTA* and *PLETHORA* Genes

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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. Recent studies have revealed developmental functions of some *LBD* genes in *Arabidopsis* and crop plants, but the biological functions of many other *LBD* genes remain to be determined. Our recent study has shown that *LBD18* functions in lateral root formation in combination with *LBD16* downstream of AUXIN RESPONSE FACTOR7 (ARF7) and ARF19 in auxin signaling. In this study, we showed that *LBD18*-transgenic lines expressing high levels of the transcripts in steroid regulator inducible system exhibit growth inhibition of aerial parts of the plants and epinastic symptoms on the newly formed leaves upon treatment of dexamethasone (DEX). We found that *AINTEGUMENTA* (*ANT*) known as one of the main controllers of plant organ size and *PLETHORA* (*PLT*) homologues essential for root development were downregulated by DEX treatment. These results implicate that *LBD18* might be involved in the regulation of plant organ size and root development via the *ANT* and *PLT* genes. In addition, we showed that *ANT* can be downregulated by DEX in the presence of cycloheximide, a protein synthesis inhibitor, indicating that *ANT* might be a direct target gene for LBD18.

Keywords Auxin · *LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE* · *AINTEGUMENTA* · *PLETHORA* · *LBD16* · *LBD18*

Abbreviations

LBD	Lateral organ boundaries domain
ASL	*ASYMMETRIC LEAVES2-LIKE*
ARF	*AUXIN RESPONSE FACTOR*
DEX	Dexamethasone
GR	Hormone binding domain of glucocorticoid receptor
ANT	*AINTEGUMENTA*
PLT	*PLETHORA*
AS2	*ASYMMETRIC LEAVES2*
KNOX	*KNOTTED1-like homeobox*
BP	*BREVIPEDICELLUS*
KNAT	*KNOTTED1-like gene*
STM	*SHOOT MERISTEMLESS*
BOP	*BLADE-ON-PETIOLE*
BBM	*BABY BOOM*
AIL	*ANT-like*
AXR	*AUXIN-RESISTANT1*
ARGOS	*AUXIN-REGULATED GENE CONTROLLING ORGAN SIZE*

Introduction

The *LBD/ASL* genes (hereafter referred to as *LBD*) encode proteins harboring a LOB domain that is found only in

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plants (Iwakawa et al. 2002; Shuai et al. 2002). The LOB domain, approximately 100 amino acids in length, possesses a conserved four-Cys motif with a $CX_2CX_6CX_3C$ spacing, a Gly-Ala-Ser (GAS) block, and a predicted coiled-coil motif with a $LX_6LX_3LX_6L$ spacing reminiscent of a Leu-zipper (Shuai et al. 2002). Forty-two *Arabidopsis LBD* genes have been assigned to two classes. Class I and class II comprise 36 and six genes, respectively (Iwakawa et al. 2002; Shuai et al. 2002). Class I proteins contain an LOB domain similar to that of the LOB protein, whereas class II proteins are less similar to class I proteins including the LOB domain as well as regions outside the LOB domain.

Although we understand little about the biological roles of the *LBD* genes, there have been some reports describing the developmental functions of *LBD* genes in *Arabidopsis*. Among them, *ASYMMETRIC LEAVES2* (*AS2*, =*LBD6*) has been well characterized. The *AS2* gene of *Arabidopsis* regulates formation of a symmetric lamina and establishment of venation (Semiarti et al. 2001). In *as2* mutants, the transcripts of class I *KNOX* (*BP*, *KNAT2*, and *KNAT6*) were found to accumulate in the leaves relative to wild type, suggesting that AS2 represses the expression of these homeobox genes. The *AS2* gene also functions in the establishment of adaxial–abaxial polarity in conjunction with the *AS1* gene encoding a MYB domain-containing protein (Xu et al. 2003). Ectopic expression of *AS2* at high levels resulted in repression of the *KNOX* genes, *BP*, *KNAT2*, and *KNAT6*, but not of the related *SHOOT MERISTEMLESS* (*STM*) gene, and in the replacement of abaxial cell types with adaxial cell types (Lin et al. 2003; Iwakawa et al. 2007). This suggests that *AS2* is sufficient to induce the adaxial cell fate and repress *KNOX* gene expression. *BLADE-ON-PETIOLE(BOP)1* and *BOP2* control *Arabidopsis* lateral organ cell fate through upregulation of *AS2* and *LOB* as well as repressing class I *KNOX* gene expression (Ha et al. 2007). A 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). The gain-of-function mutant of *LBD36*/*ASL1* designated *downwards siliques1* (*dsl1-D*), exhibited shorter internodes and downward lateral organs such as flowers similar to *bp* mutants (Chalfun-Junior et al. 2005). Analysis of *lbd36 as2* double mutants indicated that these two members act redundantly to control cell fate determination in petals. Another *Arabidopsis* gain-of-function mutant, *jagged lateral organs-D* (*jlo-D*), develops strongly lobed leaves and the shoot apical meristem arrests organ initiation prematurely, terminating in a pin-like structure (Borghi et al. 2007). Misexpression experiments suggest that during postembryonic development, *JLO* function is required to initiate plant lateral organs. It was demonstrated that LBD16 and LBD29 regulate lateral root formation downstream of ARF7 and ARF19 (Okushima et al. 2007). LBD18 was shown to regulate differentiation of tracheary elements (Soyano et al. 2008).

Our recent studies showed that *LBD18* functions in lateral root formation, particularly in the emergence step, in combination with *LBD16*, downstream of *ARF7* and *ARF19* in auxin signaling, based on the loss-of-function and complementation studies (Lee et al. 2009a; 2009b). In the present study, we show that *Arabidopsis LBD18*-transgenic lines expressing high levels of the transcripts exhibit growth inhibition of aerial parts including leaves, as well as epinastic effects on the newly formed leaves along with downregulation of *ANT* and its homologue *PLT* genes upon DEX treatment. We also found that *ANT* can be directly downregulated by LBD18. This result indicates that *LBD18* might regulate *ANT* and *PLT* genes for the regulation of plant organ size and root development.

Materials and Methods

Plant Growth and Tissue Treatment

Arabidopsis thaliana seedlings were grown for a 16-h photoperiod on 3MM Whatman filter paper on top of agar plates containing 0.5× Murashige Skoog (MS) media salts with vitamins, 1.5% sucrose, 2.5 mM Mes (pH5.7), and 0.8% phytoagar at 23°C. The filter paper with the seedlings was then transferred to plates containing 0.5× MS with chemicals (10 μM DEX or 50 μM cycloheximide) without agar and incubated for a given period of time with gentle shaking in the light at 23°C. DEX at 10 μM concentration was added in the media or sprayed five times every day when grown in the soil (Park et al. 2002).

RNA Isolation, RT-PCR, Real-Time RT-PCR, and Northern Blot Analysis

Following treatment, *Arabidopsis* plants were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from frozen *Arabidopsis* using TRI Reagent® (Molecular Research Center, Inc.). For RT-PCR analysis, total RNA was isolated using an RNeasy plant mini kit (Qiagen) and subjected to RT-PCR analysis with the Access RT-PCR System (Promega) according to the manufacturer's instructions. Real-time RT-PCR was performed with QuantiTect SYBR Green RT-PCR kit (Qiagen) in a Rotor-Gene 2000 real-time thermal cycling system (Corbett Research). To determine the copy number of the transcripts in the treated sample, the real-time PCR was performed for each

sample with a known amount of the in vitro transcribed RNA (Promega), yielding specific threshold values (C_t). A standard curve was generated to show linear correlation between log [copy numbers of the RNA] and the C_t. Copy number of the transcripts of unknown samples was then calculated from this standard curve. RT-PCR conditions, primer sequences, and DNA probes are shown in Supplemental Table 1. For Northern blot analysis, total RNA was separated on 1.2% agarose gel, transferred to nylon membranes, hybridized with ^{32}P-labeled DNA probes at 68°C for 3 h using 10 ml of QuickHyb solution (Stratagene), and then washed. The blot was subsequently exposed to X-ray film.

Results and Discussion

Our previous study showed that in a transgenic line expressing appropriate levels of the *LBD18:GR* transcripts in wild-type background, a statistically significant increase in lateral root number was induced by DEX treatment at various concentrations, but the transgenic lines expressing higher levels of the *LBD18:GR* transcripts exhibited significantly halted root growth and thus lateral root number (Lee et al. 2009b). To further investigate the possible involvement of *LBD18* in other aspects of plant growth and development, we treated DEX solution onto the

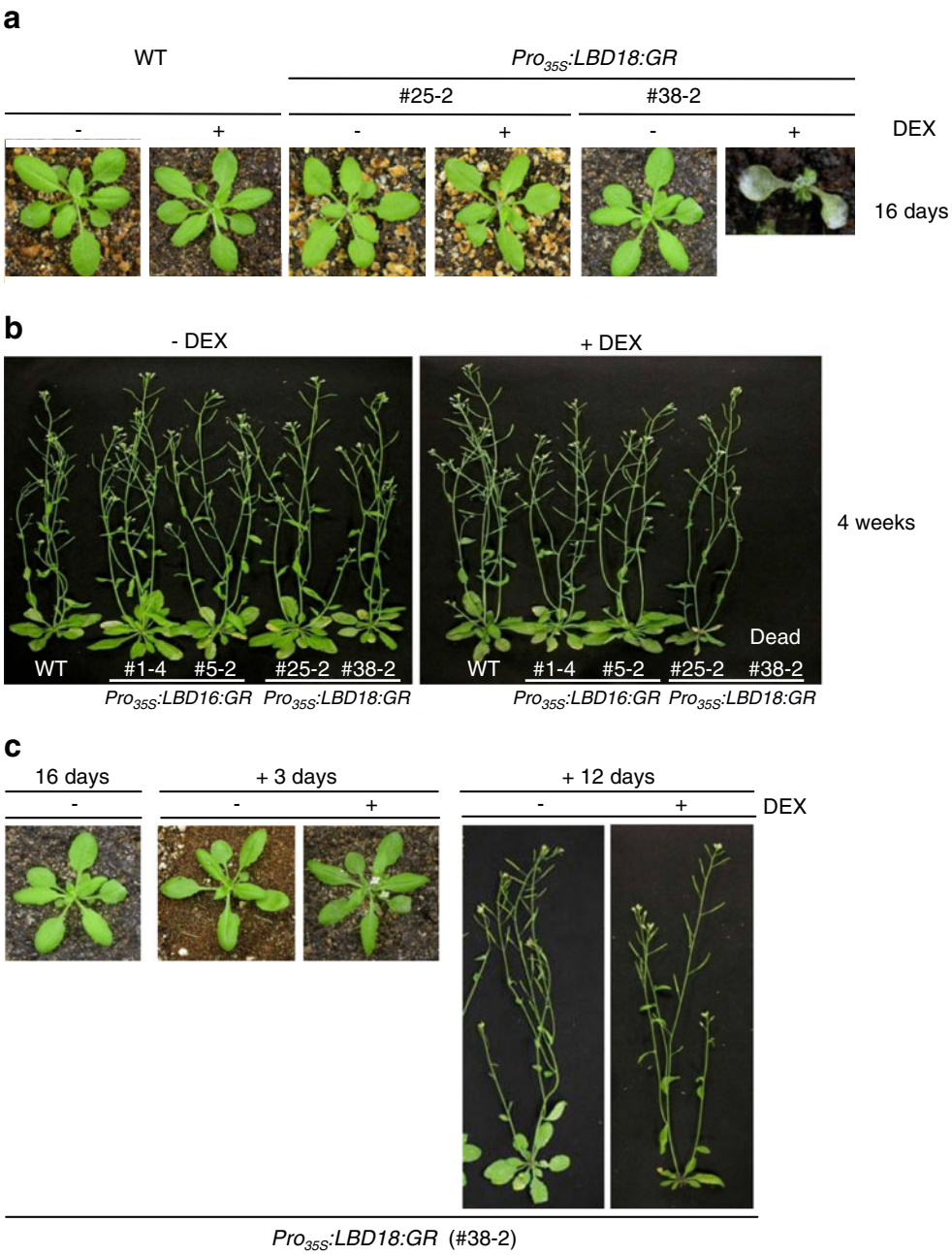

Fig. 1 Morphological phenotypes of *Pro$_{35S}$:LBD18:GR* transgenic *Arabidopsis*. **a** Morphological phenotypes of 16-day-old *Pro$_{35S}$:LBD18:GR* plants grown on the soil with DEX treatment. Solution with or without 10 μM of DEX was sprayed onto the plants five times every day after 10-day growth in the absence of DEX. The *numbers* on the pictures indicate transgenic line number. − or + indicates without or with DEX treatment. **b** Morphological phenotypes of 4-week-old *Pro$_{35S}$:LBD18:GR* or *Pro$_{35S}$:LBD16:GR* transgenic *Arabidopsis* on the soil with DEX treatment. **c** Phenotypes of *Pro$_{35S}$:LBD18:GR* plants with DEX treatment after 16-day growth in the absence of DEX. Plants grown for 16 days on the soil were sprayed with or without 10 μM of DEX for 3 and 12 days

aerial parts of *Pro$_{35S}$:LBD18:GR* as well as *Pro$_{35S}$:LBD16:GR* transgenic plants after germination on the soil (Fig. 1). While we did not find any morphological change in *Pro$_{35S}$:LBD16:GR* associated with DEX treatment, we observed significant DEX-induced inhibition of leaf formation and eventual death 16 days after germination of *Pro$_{35S}$:LBD18:GR* (#38-2) plants. The transgenic line (#25-2) expressing relatively lower levels of *LBD18:GR* transcripts or control plants (Fig. 1a and b) showed normal growth without DEX treatment. When DEX solution was applied to the transgenic line #38-2 16 days after germination, milder but significant levels of growth inhibition such as smaller number and reduced length of primary inflorescence were observed and this transgenic line could grow into mature plants (Fig. 1c). However, we observed that the leaves are epinastic, when DEX was applied to this transgenic line at an earlier time point (Fig. 2a and b). We further examined the effects of DEX treatment to *Pro$_{35S}$:LBD18:GR* (#38-2) at varying days after germination. Similar epinastic leaves and growth inhibition started to appear after DEX treatment, indicating that ectopic expression of *LBD18* affects differentially on the adaxial and abaxial surface of the leaf.

Change in the expression of *KNOX* genes encoding homeodomain transcription factors has been shown to be

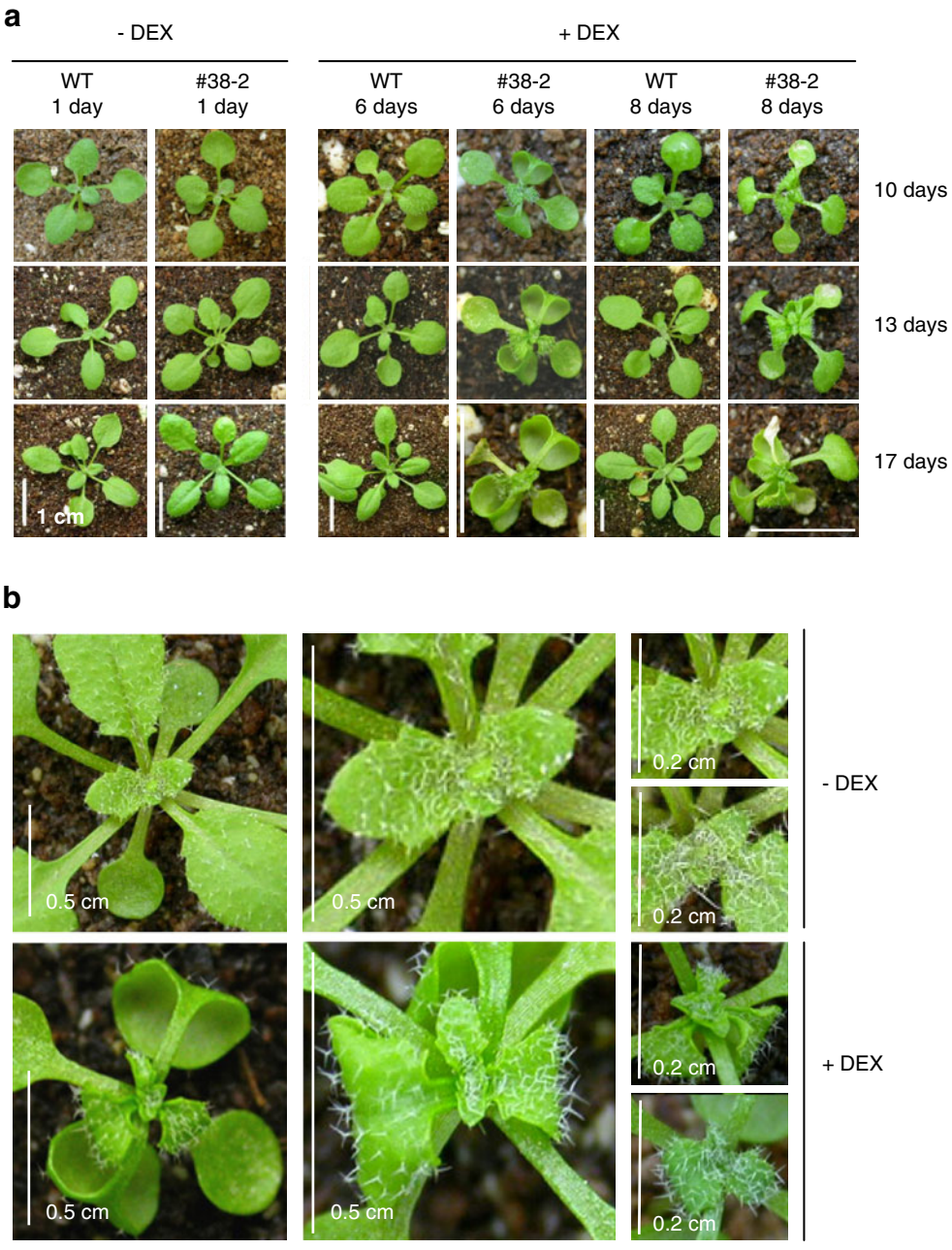

Fig. 2 DEX-inducible leaf phenotype of *Pro$_{35S}$:LBD18:GR* transgenic *Arabidopsis*. **a** Leaf phenotype of *Pro$_{35S}$:LBD18:GR* induced by DEX treatment. Plants grown for a given day on the soil were sprayed with or without 10 μM of DEX for the indicated growth period. Days on top of the picture denotes days after germination when DEX was started to be sprayed. Days on the right of the picture indicates total days for plant growth including days of DEX spray. The number *38-2* on the pictures indicates transgenic line number. *Bars* 1 cm. **b** Enlarged pictures of 13-day-old *Pro$_{35S}$:LBD18:GR* plants without or with DEX in which DEX solution was started to be sprayed at 6 days of growth

associated with lateral organ development and cell fate determination (Lin et al. 2003; Xu et al. 2003; Borghi et al. 2007). We thus examined the expression of *KNOX* genes, *BP*, *KNAT2*, *KNAT6*, and *STM*, in *Pro*$_{35S}$*:LBD18:GR* (#38-2) plants by RT-PCR to test whether regulation of *KNOX* gene expression might be related to *LBD18* overexpression effect on developmental phenotypes noted in the leaf. However, the expression of these genes was not changed with the increasing incubation time up to 8 days after DEX treatment of 7-day-old transgenic plants (Fig. 3), suggesting that the effect of *LBD18* overexpression on lateral organ development may not be related to the *KNOX* gene pathway.

AINTEGUMENTA (*ANT*) is known as one of the main controllers of plant organ size. Its homologous genes, *ANT*-like (*AIL*) genes including *PLT* genes, have also been identified (Boutilier et al. 2002; Aida et al. 2004; Nole-Wilson et al. 2005; Galinha et al. 2007). To test whether *LBD18* might modulate the expression of *ANT* and *AIL*s, we determined the transcript levels of *ANT* and seven *AIL*s by RT-PCR upon incubation of 7-day-old *Pro*$_{35S}$*:LBD18:GR* plants in the presence of DEX (Fig. 4a). We found that *ANT* expression started to decrease in 3-day incubation compared with the absence of DEX and very significantly decreased in 8-day incubation. We also observed decreased transcript levels of *BABY BOOM*(*BBM*)/*AIL2*, *PLT1*/*AIL3*, *PLT2*/*AIL4*, and *PLT3*/*AIL6* compared with the absence of DEX. The RT-PCR results were further supported by quantitative real-time RT-PCR assayed for the samples incubated for 5 and 8 days with DEX (Fig. 4b). These results indicate that *ANT* and *AIL* might be the potential genes regulated downstream of *LBD18* for controlling plant organ size and root development.

Relevance of phenotypes observed in leaf tissues in terms of potential developmental function of *LBD18* could be sought from the observation that the *LBD18* transcripts can be significantly detected by RT-PCR not only in roots but also in other tissues such as aerial parts of 2-week-old plants and rosette leaves of 6-week-old plants (Lee et al. 2009a). We attempted to measure the transcript levels of these genes in *lbd18* loss-of-function mutant, but could not detect any changes in the transcript levels compared with those of wild type (data not shown). This result could be due to the genetic redundancy of *LBD* gene family. Ectopically overexpressed *LBD18* might also affect the downstream genes regulated by the other LBD members, as it has been reported that overexpression of several *LBD* members affects shoot growth (Nakazawa et al. 2003).

We further examined expression of *ANT* in response to DEX in *Pro*$_{35S}$*:LBD18:GR* at early time points such as 15 min, 30 min, or 1, 2, 4, or 8 h (Fig. 5a). We found that the *ANT* transcript levels significantly decreased even in 15 min by DEX treatment, whereas without DEX treatment, the *ANT* transcript levels were not changed until 8-

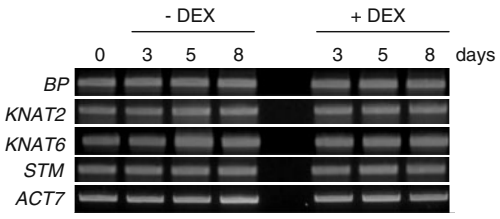

Fig. 3 RT-PCR analysis of *KNOX* genes in *Pro*$_{35S}$*:LBD18:GR* with or without DEX treatment. Seven-day-old light-grown *Pro*$_{35S}$*:LBD18:GR* (#38-2) seedlings after germination were incubated with 10 μM of DEX on MS plate for the indicated days. The total RNAs isolated from the whole seedlings were subjected to RT-PCR analysis with the primers for the indicated *KNOX* gene transcripts

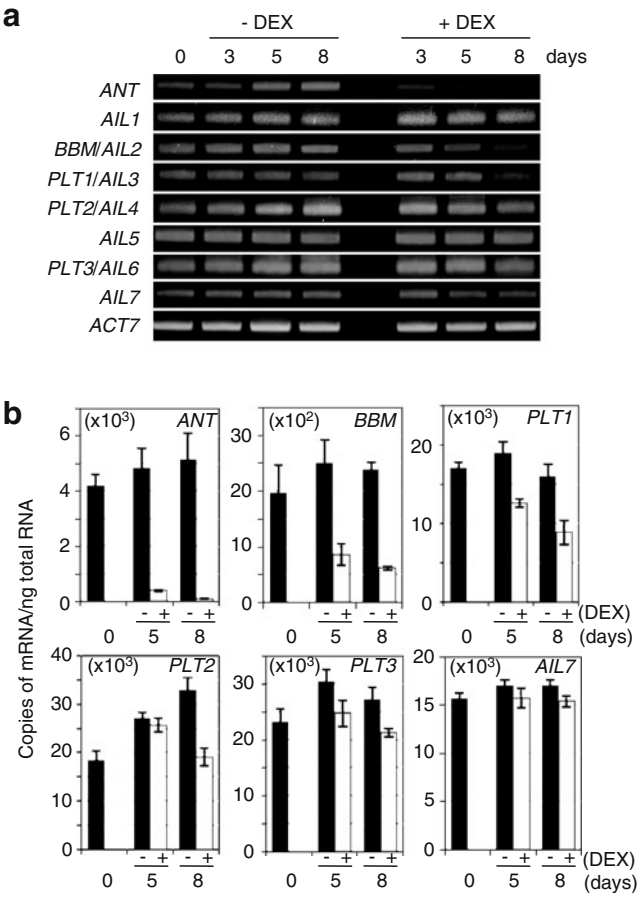

Fig. 4 Analysis of *ANT* and *AIL* expression in *Pro*$_{35S}$*:LBD18:GR* transgenic plants. **a** RT-PCR analysis of *ANT* and *AIL* genes in *Pro*$_{35S}$*:LBD18:GR* with or without DEX. Plants were treated and analyzed as described in the legend of Fig. 3 with the primers for *ANT* and *AIL* genes. **b** Real-time RT-PCR analysis of *ANT* and *AIL* genes in *Pro*$_{35S}$*:LBD18:GR* with or without DEX. Copies of the transcripts from plants treated with or without DEX were plotted per nanogram of total RNA after normalization to *ACTIN7* RNA. Multiplication of the *number in the brackets* inside the graph by the number in the *y*-axis generates copy numbers of the corresponding transcripts. The mean values and standard errors from biological triplicate experiments were plotted

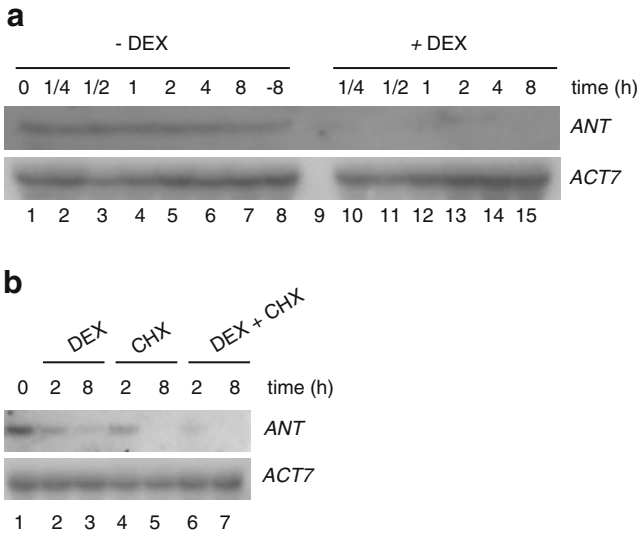

Fig. 5 Expression analysis of *ANT* in response to DEX and/or cycloheximide treatment. **a** Early time-course expression analysis of *ANT* without or with DEX. Seven-day-old light-grown *Pro*$_{35S}$*:LBD18:GR* (#38-2) seedlings were incubated without or with DEX for the indicated time in the light and subjected to Northern blot analysis with the *ANT* or *ACT7* DNA probe. *−8 h* indicates the samples incubated with mock for 8 h. *ACT7* was used a loading control. **b.** Expression analysis of *ANT* with DEX, cycloheximide, or DEX and cycloheximide. *Pro*$_{35S}$*:LBD18:GR* (#38-2) seedlings were incubated with DEX (*lanes 2* and *3*), cycloheximide (*lanes 4* and *5*), DEX and cycloheximide (*lanes 6* and *7*) for 2 (*lanes 2*, *4*, or *6*) or 8 h (*lanes 3*, *5*, or *7*) in the light, followed by Northern blot analysis with the *ANT* or *ACT7* DNA probe. *Lane 1* represents the sample before treatment

h incubation. We then tested whether *ANT* gene expression is directly regulated by LBD18 by analyzing the transcript levels in response to DEX in the presence of cycloheximide, a protein synthesis inhibitor (Fig. 5b). *Pro*$_{35S}$*:LBD18:GR* transgenic seedlings were incubated with DEX, cycloheximide, or DEX and cycloheximide for 2 or 8 h, followed by Northern blot analysis with the *ANT* DNA probe. Treatment of cycloheximide to *Pro*$_{35S}$*:LBD18:GR* seedlings decreased the *ANT* transcript levels to some extent at 2 h and to the undetectable levels at 8 h, indicating that continued protein synthesis might be required to maintain the basal levels of the *ANT* transcripts. When *Pro*$_{35S}$*:LBD18:GR* seedlings were incubated with both DEX and cycloheximide for 2 h, the *ANT* transcript levels were decreased at much higher levels compared with cycloheximide treatment alone. This result showed that *ANT* can be downregulated by LBD18 without new protein synthesis, suggesting that *ANT* might be a direct target gene for downregulation by LBD18.

ANT is a member of the AP2 subgroup of the AP2/ERF family of plant-specific transcription factors (Krizek 2003) and functions in the regulation of the size of all lateral shoot organs including floral organs through controlling cell number (Elliott et al. 1996; Klucher et al. 1996; Mizukami and Fischer 2000). Loss of *ANT* function reduces the size of all lateral shoot organs including floral organs by decreasing cell number. Conversely, ectopic expression of the *ANT* gene in *Arabidopsis* resulted in enlargement of embryonic and all shoot organs including floral organs by increasing cell number. *ANT* also contributes to organ polarity (Krizek 1999; Mizukami and Fischer 2000; Nole-Wilson and Krizek 2006).

ARGOS (auxin-regulated gene controlling organ size) has a similar enlargement effect on aerial organs as *ANT* when overexpressed in transgenic *Arabidopsis*. *ARGOS* appears to be upstream of *ANT* in the signaling cascade, as the loss of *ANT* function blocks the organ-enlargement effect of *ARGOS* (Hu et al. 2003). The induction of *ARGOS* by auxin is attenuated in *auxin-resistant1* (*axr1*), and overexpression of *ARGOS* partially restores *axr1* organ development, suggesting that the action of ARGOS is downstream of AXR1. We have observed insignificant changes in the *ARGOS* transcript levels in *Pro*$_{35S}$*:LBD18:GR* transgenic plants upon DEX treatment within the time frame in which the *ANT* transcript levels are significantly downregulated by DEX (data not shown). Therefore, *LBD18* might be independent of this *AXR1-ARGOS-ANT* auxin signaling cascade.

The genes homologous to *ANT*, *BBM*/*AIL2*, *PLT1*/*AIL3*, *PLT2*/*AIL4*, and *PLT3*/*AIL6*, also encode AP2 class of transcription factors (Nawy et al. 2008). *PLT1* and *PLT2* genes were first identified as developmental effectors for the establishment of the stem cell niche during embryonic pattern formation (Aida et al. 2004). Both *plt1* and *plt2* single mutants display a slight but significant reduction in the growth rate of the root and in the number of meristematic cells. In *plt1 plt2* double mutants, root growth is extremely reduced compared with the wild-type and each single mutant, and the number of meristematic cells is also highly reduced. The reduced root growth was due to reduction in the rate of cell production as well as the size of mature cells. Interestingly, the *plt1 plt2* double mutants produce numerous lateral roots from the pericycle of the primary root because of highly reduced size of root meristem. It was suggested that these *PLT* genes are required to root meristem maintenance in lateral roots. It was further shown that a clade of four *PLT* homologues, *BBM*, *PLT1*, *PLT2*, and *PLT3*, is necessary for root formation (Galinha et al. 2007). *plt1 plt2 plt3* triple mutants were rootless, showing linkage between rootless phenotype and the three *PLT* genes (Galinha et al. 2007). The progeny of plants segregating different *plt* and *bbm* allele combinations lack root and hypocotyl. Gain- and loss-of-function experiments suggest that *PLT* genes are master switches for root development. Thus downregulation of *PLT* genes by LBD18 might contribute to root phenotype of DEX-treated *Pro*$_{35S}$*:LBD18:GR* plants. However, it remains to be determined whether LBD18 downregulates *BBM*,

PLT1, *PLT2*, and *PLT3* genes in wild type, as a very weak GUS expression in the root tip was detected with transgenic *Arabidopsis* harboring the 2.0-kb *LBD18* promoter-GUS fusion construct compared with the expression of these *PLT* homologue genes in the root tip (Aida et al. 2004; Galinha et al. 2007; Lee et al. 2009b).

The induction of *PLT1* and *PLT2* transcription in lateral root primordia requires the Aux/IAA-ARF system, but occurs significantly later than that of primary auxin response genes, in between 5 and 24 h after auxin action (Aida et al. 2004). A recent report showed that the *PLT1* and *PLT2* genes are direct targets of the transcriptional co-repressor TOPLESS (TPL) of IAA12 (Smith and Long 2010). LBD18 might be an intriguing molecular link between auixn-responsive ARF action and *PLT* transcription for controlling root development.

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