EXPANSINA17 Up-Regulated by LBD18/ASL20 Promotes Lateral Root Formation During the Auxin Response

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Expansins are non-hydrolytic cell wall-loosening proteins involved in a variety of plant developmental processes during which cell wall modification occurs. Cell wall remodeling proteins including expansins have been suggested to be involved in cell separation to facilitate the emergence of lateral roots (LRs) through the overlaying tissues of the primary root. LBD18/ASL20 activates EXPANSINA14 (EXPA14) expression by directly binding to the EXPA14 promoter to enhance LR emergence in Arabidopsis thaliana. Here we show that EXPA17 is another target gene regulated by LBD18 to promote LR formation in Arabidopsis. We showed that nuclear translocation of the LBD18:GR fusion protein expressed under the Cauliflower mosaic virus (CaMV) 35S promoter or under the LBD18 promoter by dexamethasone treatment results in an increase in EXPA17 transcript levels. β-Glucuronidase (GUS) expression under the EXPA17 promoter, which is detected only in the roots of the wild type, was reduced in the LR primordium and overlaying tissues in an lbd18 mutant background. The number of emerged LRs of the EXPA17 RNAi (RNA interference) Arabidopsis lines was significantly lower than that of the wild type. Overexpression of EXPA17 in Arabidopsis increased the density of emerged LRs in the presence of auxin compared with the wild type. LR induction experiments with a gravitropic stimulus showed that LR emergence is delayed in the EXPA17 RNAi plants compared with the wild type. In addition, EXPA4 expression was also detected in overlaying tissues of the LR primordium and was inducible by LBD18. Taken together, these results support the notion that LBD18 up-regulates a subset of EXP genes to enhance cell separation to promote LR emergence in Arabidopsis.

**Keywords:** Arabidopsis thaliana • Auxin • Cell wall loosening factor • LBD18 • EXP17 • Lateral root formation.

**Abbreviations:** ARF, auxin response factor; Aux/IAA, auxin/indole acetic acid protein; CaMV, Cauliflower mosaic virus; DEX, dexamethasone; EXP, EXPANSIN; GUS, β-glucuronidase; LBD/ASL, LATERAL ORGAN BOUNDARIES DOMAIN/ASYMMETRIC LEAVES2-LIKE; LR, lateral root; LRP, lateral root primordium; pgi, post-gravistropic induction; qRT-PCR, quantitative reverse transcription–PCR; RNAi, RNA interference; RT–PCR, reverse transcription–PCR.

**Introduction**

Plant roots are important for uptake of water and nutrients and anchorage in soil, which is necessary for plant growth and development (Hochholdinger and Zimmermann 2008). The main part of the root system is initiated with the production of lateral roots (LRs) from the primary root (Péret et al. 2009a). Arabidopsis LRs initiate from pericycle founder cells after the priming of the xylem pole pericycle cells to divide by auxin signaling in the basal meristem and undergo a series of anticlinal divisions to produce a few initial cells. Further anticlinal and periclinal divisions create a lateral root primordium (LRP) that continues to grow and emerge through the cortex and epidermal layers of the primary root via cell separation. Activation of the LR meristem leads to continued growth of the organized LR (Parizot et al. 2008, Péret et al. 2009a, Péret et al. 2009b).

The developmental events of LR formation are mainly regulated by auxin. In particular, two auxin response modules involving AUXIN RESPONSE FACTORS (ARFs), ARF7 and ARF19, and an Aux/IAA (auxin/indole acetic acid protein), a negative regulator of ARFs, SOLITARY-ROOT/IAA14, and ARF5-BOUNDLOS/IAA12, have been identified to regulate the LR initiation and patterning process (Fukaki et al. 2002, Okushima et al. 2005). ARF7 and ARF19 regulate LR formation in Arabidopsis via activation of LATERAL ORGAN BOUNDARIES DOMAIN16/ASYMMETRIC LEAVES2-LIKE18 (LBD16/ASL18) and LBD29/ASL16 (Okushima et al. 2007). LBD18/ASL20 regulates LR formation in conjunction with LBD16 downstream of ARF7 and ARF19 (Lee et al. 2009a, Lee et al. 2009b). LBD18 along with LBD23/ASL24 regulate the initiation of LRs via transcriptional activation of the E2Fa transcription factor, which regulates the asymmetric cell division (Berckmans et al. 2011).

The LBD gene family encodes a unique class of transcription factors harboring a conserved plant-specific LOB domain,
and 43 LBD members exist in Arabidopsis (Iwakawa et al. 2002, Shuai et al. 2002). A variety of studies have revealed the developmental functions of certain LBD genes in establishment of leaf polarity (Lin et al. 2003, Xu et al. 2003), tracheary element development (Soyano et al. 2008), the auxin-dependent apical–basal polarity and patterning processes in the embryo (Borghi et al. 2007, Bureau et al. 2010), the correct timing and orientation of asymmetric cell division during male gametophyte development (Oh et al. 2010) and LR formation in Arabidopsis (Okushima et al. 2007, Lee et al. 2009b), as well as the developmental functions of their homolog in Oryza sativa (Inukai et al. 2005, Liu et al. 2005) and Zea mays (Bortiri et al. 2006, Evans 2007, Taramino et al. 2007). We have demonstrated that LBD18 acts as a specific DNA-binding transcriptional activator that directly up-regulates EXP14, promoting LR emergence (Lee et al. 2013). A previous microarray analysis of LBD18-up-regulated genes has revealed 27 candidate genes that may be involved in LR emergence (Lee et al. 2013). Some of them encode cell wall remodeling proteins such as SUBTILISIN-LIKE PROTEASE (AILR3) and EXP17, and have been suggested to be important for cell separation during LR emergence, and are regulated downstream of the auxin influx carrier Lkteux1 (LAX3) (Neuteboom et al. 1999, Cosgrove 2000a, Swarup et al. 2008, Lee et al. 2013).

Expansins are cell wall-loosening proteins that directly induce extension of the cell wall by disrupting non-covalent bonding between the cellulose microfibrils and associated matrix polysaccharides in the cell wall (McQueen-Mason et al. 1992, McQueen-Mason and Cosgrove 1995, McQueen-Mason and Cosgrove 2000). A large body of gene expression studies and gain-of-function and antisense expression approaches has shown that these proteins play roles in various processes involving cell wall-loosening during plant growth and development. Expansins are encoded by a multigene superfamilies in plants and divided into four families, α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB) (Sampedro and Cosgrove 2005). The Arabidopsis genome contains 36 EXP genes, comprising 26 α-EXP genes, six β-EXP genes, three EXLA genes and one EXLB gene (hereafter referred to as EXP) (Sampedro and Cosgrove 2005). EXP10 was shown to play a role in leaf growth and pedicel abscission using antisense expression of EXP10 (Cho and Cosgrove 2000). EXP7 and EXP18 are expressed specifically in the root hair cells and regulate the initiation of root hairs (Cho and Cosgrove 2002, Lin et al. 2011). EXP1 is specifically expressed in guard cells, and overexpression of EXP1 in Arabidopsis causes acceleration of light-induced stomatal opening by decreasing the volumetric elastic modulus, suggesting its role in stomatal opening (Zhang et al. 2011). Simultaneous suppression of four expansin genes, EXP1, EXP3, EXP5 and EXP10, using an artificial microRNA via inducible repression, leads to growth suppression during leaf development (Goh et al. 2012). Many functional roles of expansins in plant growth and development have been identified from the studies with transgenic crops such as leaf formation in the meristem and fruit ripening in tomato (Reinhardt et al. 1998, Brummel et al. 1999), enhancement of plant growth and root hair elongation in rice (Choi et al. 2003, ZhiMing et al. 2011), cell size, crystalline cellulose formation, cell wall polymer composition and the timing of axillary meristem development in Petunia hybrida (Zenoni et al. 2004, Zenoni et al. 2011), cell expansion in primary and secondary tissues in aspen (Gray-Mitsumune et al. 2008), root system architecture responses to abiotic stresses in soybean (Guo et al. 2011), plant growth and fruit production in cotton (Xu et al. 2012) and storage development in sweet potato (Noh et al. 2013).

In the present study, we showed that EXP17 is up-regulated by LBD18 in response to auxin. β-Glucuronidase (GUS) expression analysis showed that EXP17 is expressed in overlaying tissues of developing LRPCs and that its expression is diminished by lbd18 mutation. Loss-of-function studies using the RNA interference (RNAi) approach and gain-of-function studies demonstrated that EXP17 is involved in facilitating LR emergence. We found that EXP4, whose expression is detected in overlaying LRP tissues, is inducible by LBD18. These results and our previous report (Lee et al. 2013) support the notion that LBD18 up-regulates a subset of EXP genes as a part of transcriptional responses to promote LR emergence and that one distinct developmental function of EXP genes is to regulate LR development via cell wall remodeling in Arabidopsis.

### Results

**EXP17 is up-regulated by LBD18**

Our previous microarray analysis suggested that EXP17 might be up-regulated by LBD18 (Lee et al. 2013). If EXP17 is regulated downstream of LBD18, it should be auxin inducible at a later time point than the induction of LBD18 by auxin. Consistent with this prediction, EXP17 displayed delayed expression kinetics upon auxin treatment compared with LBD18 (Fig. 1A). Dexamethasone (DEX) treatment of Pro35S:LBD18-GR (Lee et al. 2009b) seedlings induced EXP17 expression, whereas mock treatment of Pro35S:LBD18-GR plants did not (Fig. 1B). Pro35S:LBD18-GR plants were treated with DEX in the presence or absence of cycloheximide, a protein synthesis inhibitor, to investigate whether EXP17 is a primary or secondary response gene of LBD18 (Fig. 1B). EXP17 was not induced by DEX treatment in the presence of cycloheximide after a 2 h incubation, but was induced after an 8 h incubation to some extent, much more than with DEX treatment alone, suggesting that EXP17 might be a secondary response gene of LBD18. We used ProLBD18:LBD18-GR plants (Lee et al. 2013) to show that LBD18 regulates EXP17 expression when LBD18 is expressed under its own promoter. DEX treatment induced EXP17 expression in ProLBD18:LBD18-GR plants compared with that in mock-treated plants, indicating that LBD18 regulates EXP17 expression under the LBD18 promoter (Fig. 1C). In addition, we also tested whether LBD18 could induce EXP4 expression, as EXP4 is up-regulated by auxin, and auxin-induced EXP4
expression is repressed by stabilized iaa1-GR in a microarray analysis (Lee et al. 2009a). Auxin induced EXP4 expression, and DEX treatment of Pro35S:LBD18-GR plants induced EXP4 expression, after a 2 h treatment (Supplementary Figs. S1A, B, S2A). Cycloheximide treatment of Pro35S:LBD18-GR plants enhanced DEX-induced EXP4 expression, indicating that a labile repressor might be involved in LBD18-regulated expression of EXP4.

**EXP17 is expressed during lateral root development**

We generated ProEXP17::GFP-GUS transgenic Arabidopsis to investigate the tissue-specific expression pattern of EXP17 during LR development. GUS staining was only detected in the roots, the LRP and overlaying tissues of new LRPs such as endodermis, cortex and epidermis (Fig. 2). However, the GUS staining in the tip of LRPs was reduced after stage IV of LR development, but remained unaltered at the flank region (Fig. 2D–H). These GUS expression patterns indicate that EXP17 might play a role in tissues where LR development and emergence occur. GUS expression analysis of ProEXP4::GFP-GUS transgenic Arabidopsis also showed that EXP4 was expressed in overlaying tissues of the LRP and root stele (Supplementary Fig. S3). In contrast to EXP17, GUS expression was detected in the leaf vein of ProEXP4::GFP-GUS Arabidopsis.

**Loss of function in LBD18 caused reduced expression of GUS in overlaying tissues of LRPs in ProEXP17::GFP-GUS**

To investigate whether EXP17 is an endogenous target of LBD18, we generated ProEXP17::GFP-GUS transgenic Arabidopsis in the lbd18 mutant background (ProEXP17::GFP-GUS/lbd18) and conducted GUS expression analysis. GUS staining was only detected in the root, and was decreased in the lbd18 mutant background compared with that in the wild type (Fig. 3A, B). Auxin treatment enhanced GUS staining in ProEXP17::GFP-GUS Arabidopsis (Fig. 3C). The lbd18 mutation diminished auxin-enhanced GUS expression of ProEXP17::GFP-GUS Arabidopsis (Fig. 3D). GUS staining detected in the endodermis, cortex and epidermis region of LRPs at stages II and IV in the wild type (Fig. 3E, F) decreased significantly in the lbd18 mutant background (Fig. 3G, H). Quantitative reverse transcription–PCR (qRT-PCR) analysis of the GUS transcripts confirmed that the lbd18 mutation decreased GUS expression of ProEXP17::GFP-GUS (Fig. 3K). These results showed that a loss-of-function mutation in LBD18 reduced the expression of EXP17 in the overlaying tissues such as endodermis, cortex and epidermis.

**EXP17 knock-down by RNAi results in reduced lateral root formation**

To evaluate the role of EXP17 in LR formation, we generated an RNAi construct to knock-down transcript levels of EXP17 in Arabidopsis (Pro35S:LhGR:EXP17RNAi), as EXP17 knock-out T-DNA insertion mutants are not available. This construct allows the formation of a hairpin RNA containing approximately 149 bp (from the start codon) of the EXP17 coding region under the control of the 3SSLhGR that encodes the DEX-responsive LhGR transcription factor (Wielopolska et al. 2005). Five Pro35S:LhGR:EXP17RNAi Arabidopsis transgenic lines were obtained, exhibiting significantly decreased levels of the EXP17 transcripts following DEX treatment (Fig. 4A). Three EXP17RNAi transgenic lines exhibiting the most reduced EXP17 transcript levels (#2-2, #4-4 and #5-3) by DEX were selected for phenotypic analysis. DEX treatment of these transgenic lines following DEX treatment (#2-2, #4-4 and #5-3) by DEX were selected for phenotypic analysis. DEX treatment of these transgenic lines significantly reduced the expression of EXP17 by 43% (#4-4) [1.80 ± 0.15 (mean ± SD)] and 32% (#5-3) (2.14 ± 0.15), respectively, compared with that without DEX treatment (#4-4,
3.15 ± 0.11; #5-3, 3.14 ± 0.13) (Fig. 4C, D). The processes of LR development can be divided into eight stages by specific anatomical characteristics and pericycle cell divisions (Malamy and Benfey 1997). LR emergence occurs after stage VII. We determined the LRP number at seven given developmental stages and found that DEX treatment of these three RNAi transgenic lines displayed a slight but statistically significant increase in LRP number at stages II (#4-4, 0.85 ± 0.03; #5-3, 0.86 ± 0.04) and III (#2-2, 0.51 ± 0.03; #4-4, 0.53 ± 0.04; #5-3, 0.49 ± 0.03) compared with the absence of DEX at stages II (#4-4, 0.75 ± 0.04; #5-3, 0.73 ± 0.04) and III (#2-2, 0.39 ± 0.02; #4-4, 0.38 ± 0.03; #5-3, 0.36 ± 0.02) (Fig. 4E). Total numbers of LRPs of DEX-treated RNAi lines were similar to those of mock-treated RNAi lines (Fig. 4E), indicating that the effect of reduced expression of EXP17 on LR development was relatively marginal compared with that on LR emergence. We did not observe any other notable phenotypes in the morphometric aspect of these EXP17RNAi lines. These results suggest that EXP17 is involved in LR emergence. We further examined if exogenous auxin treatment could have a differential effect on LR density in EXP17RNAi lines compared with that in the wild type. As shown in Fig. 4F, exogenous auxin treatment significantly decreased LR density in EXP17RNAi lines compared with that in the wild type.

**Overexpression of EXP17 in Arabidopsis enhanced auxin-stimulated lateral root formation**

To evaluate the gain-of-function phenotype of EXP17 during LR formation, we constructed transgenic Arabidopsis that overexpresses EXP17 under the control of the constitutive Cauliflower mosaic virus (CaMV) 35S promoter (Pro35S:EXP17). Three independent transgenic lines expressing high levels of EXP17 expression were chosen for phenotypic analysis (Fig. 5A). The densities of emerged LRs in these transgenic lines overexpressing EXP17 increased to some extent compared with that of the wild type (Fig. 5B, C). However, equal densities of LRPs were found between the transgenic lines and the wild type (Fig. 5D). We examined if exogenous auxin treatment could provide a differential effect on LR density in Pro35S:EXP17 transgenic lines compared with that in the wild type. As shown in Fig. 5E, exogenous auxin treatment significantly enhanced LR density in Pro35S:EXP17 transgenic lines...
compared with that in the wild type. These results show that EXP17 is involved in facilitating LR formation by enhancing outgrowth and emergence of the LRP during the auxin response.

**Lateral root emergence is delayed in the Pro35S:LhGR:EXP17RNAi Arabidopsis**

In order to analyze further the impact of EXP17 RNAi or overexpression on LR development, we conducted an LR induction experiment which allows a more direct and quantitative analysis of LR development kinetics (Péret et al. 2012) compared with the analysis of the numbers of LRPs and LRs at a given developmental stage of plants. LRs can be initiated by either mechanical (Ditengou et al. 2008, Richter et al. 2009) or gravitropic (Lucas et al. 2008) stimuli. A gravitropic stimulus was applied to EXP17 RNAi plants, EXP17-overexpressing plants and wild-type plants grown vertically for 3 d by rotating the agar plate through 90°, and the number of newly developed LRPs on the convex side of the curves was measured after 18 or 42 h gravitropic induction. LRPs were grouped according to developmental stages as defined previously (Malamy and Benfey 1997), but stage VIII was added in to indicate emerged LRPs (Péret et al. 2012). Wild-type plants showed accumulation of stage I and II LRPs 18 h post-gravitropic induction (pgi) and accumulation of stages VI, VII and VIII 42 h pgi in both the absence and presence of DEX (Fig. 6A). Both Pro35S:LhGR:EXP17RNAi treated with DEX and Pro35S:EXP17 transgenic plants showed similar stages of LR formation at 18 h pgi compared with the wild type or the EXP17 RNAi plants without DEX, indicating that the early stages of LR development were not affected by EXP17 RNAi or EXP17 overexpression (Fig. 6B, C). However, Pro35S:LhGR:EXP17RNAi plants treated with DEX showed accumulation of stages IV–VIII with a decreased profile at 42 h pgi compared with the absence of DEX, whereas Pro35S:EXP17 transgenic plants showed similar stages of LR formation compared with the wild type. This result indicates that
Fig. 4 Inhibition of LR formation in *Pro35S:LhGR:EXP17RNAi* Arabidopsis. (A) Quantitative RT–PCR analysis of the *EXP17* transcripts in wild-type and *Pro35S:LhGR:EXP17RNAi* transgenic plants in the absence (filled squares) or presence (open squares) of DEX. Total RNAs were isolated from 7-day-old seedlings, and each RNA sample was subject to qRT–PCR. # indicates the line number of *EXP17RNAi* plants. The numbers within the bracket indicate the relative expression levels of *EXP17* by DEX treatment compared with in the absence of DEX. Data are the mean ± SE of three independent biological experiments. Asterisks denote statistical significance at *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***)

(B) Quantitative RT–PCR analysis of *EXP4, EXP11* and *EXP14* transcripts in *Pro35S:LhGR:EXP17RNAi* transgenic plants in the absence (filled squares) or presence (open squares) of DEX. Total RNAs were isolated from 7-day-old seedlings, and each RNA sample was subject to qRT–PCR. Data are the mean ± SE of three independent biological experiments.

(C) LR phenotypes of *Pro35S:LhGR:EXP17RNAi* transgenic plants. Seven-day-old seedlings of wild-type (Col-0) and *Pro35S:LhGR:EXP17RNAi* transgenic plants (#2-2, #4-4, #5-3) in the absence or presence of DEX are shown.

(D) Number of emerged LRs (LR number per unit primary root length; #/cm). Error bars indicate the SE (n = 20). Asterisks denote statistical significance at *P* < 0.05 (*), *P* < 0.01 (**) and *P* < 0.001 (***)

Role of *EXPA17* in lateral root formation (continued)
Fig. 5 Enhanced LR formation in Arabidopsis plants overexpressing EXP17 compared with that in the wild type. (A) RT–PCR analysis of Pro35S:EXP17 transgenic plants compared with the wild type for the EXP17 transcripts. Total RNAs were isolated from 7-day-old seedlings, and each RNA sample was subject to RT–PCR. # indicates the line number of Pro35S:EXP17 plants. Black triangles indicate the Arabidopsis transgenic lines used for phenotypic analysis. (B) LR phenotypes of Pro35S:EXP17 transgenic plants. Seven-day-old seedlings of wild-type and Pro35S:EXP17 transgenic plants are shown. (C) The number of emerged LRs (LR numbers per unit primary root length; #/cm) of 7-day-old wild-type and Pro35S:EXP17 transgenic plants are shown. Asterisks denote statistical significance at $P < 0.05$ (*). (D) Analysis of the number of primordia at different stages of LRP development in 7-day-old wild-type and Pro35S:EXP17 transgenic plants. (E) The number of emerged LRs (LR number per unit primary root length; #/cm) of 7-day-old wild-type and Pro35S:EXP17 transgenic plants in the presence of the auxin 2,4-D. Asterisks denote statistical significance at $P < 0.05$ (*) and $P < 0.01$ (**).
LR emergence is delayed in the EXP17RNAi line, which is consistent with the decreased number of emerged LRs as shown in Fig. 4D.

Discussion

A variety of the components involved in LR initiation and emergence processes have been identified and the molecular mechanism for auxin-regulated LR initiation is well studied (Péret et al. 2009a, Péret et al. 2009b, De Smet et al. 2010). It has been suggested that cell wall remodeling enzymes and expansions might cause cell separation in overlying root tissues to promote LRP emergence (Laskowski et al. 2006, Swarup et al. 2008, Péret et al. 2009a). However, the biological functions of the genes involved in this process and the signaling pathway are largely uncharacterized. Here we showed that EXP17 encoding a cell wall-loosening factor is up-regulated by LBD18 and plays a role in LR emergence during the auxin response.

EXP17 expression is induced in response to auxin at a later time point than that of LBD18 (Fig. 1A). LBD18 activated the expression of endogenous EXP17 under the control of the LBD18 promoter (Fig. 1C). Loss-of-function in LBD18 caused reduced GUS expression in overlying tissues of LRP s in ProEXP17:GFP-GUS transgenic Arabidopsis (Fig. 3). However, DEX-induced expression of EXP17 in ProEXP17:GFP-GUS transgenic Arabidopsis was prevented by treatment with the protein synthesis inhibitor, cycloheximide (Fig. 1B). These results indicate that LBD18 regulates EXP17 expression in response to auxin as a secondary response requiring new protein synthesis. DEX treatment of ProLBD18-LBD18-GR plants caused a relatively small increase in EXP17 expression compared with that in ProEXP17:GFP-GUS plants (Fig. 1C), and the reduction of EXP17 expression in the lbd18 mutant background was also relatively small (Fig. 3), indicating that additional transcription factors might be necessary for the full activation of EXP17.

Overexpression of EXP17 in Arabidopsis caused an increase in the number of emerged LRs per primary root length compared with that in the wild type (Fig. 5B, C). Exogenous auxin treatment dramatically enhanced LR density in ProEXP17 transgenic plants compared with that of the wild type (Fig. 5E). We have recently shown that EXP14-overexpressing Arabidopsis also displayed an increased number of emerged LRs in response to auxin (Lee et al. 2013). These results together suggest that LBD18 up-regulates both EXP14 and EXP17 in response to auxin to cause cell wall-loosening to promote LR emergence. Enhanced LR formation by EXP17 overexpression is also consistent with the GUS expression detected in the overlying tissues of Arabidopsis (Figs. 2, 3). Interestingly, GUS staining of ProEXP17:GFP-GUS Arabidopsis was detected only in the roots (Fig. 3A, C), whereas ProEXP14:GFP-GUS and ProEXP4:GFP-GUS plants displayed GUS expression in leaf veins and roots (Lee et al. 2013; Supplementary Fig. 3). These results indicate that EXP17 may have a specialized role in root development. This hypothesis is consistent with the observation that EXP17RNAi lines exhibited a significant decrease in the emerged LR number (Fig. 4D), whereas the exp14 mutant showed wild-type levels of LRs (Lee et al. 2013). We also found that GUS expression was detected in the overlying tissues of ProEXP4:GFP-GUS Arabidopsis and that EXP4 expression was induced by LBD18 (Supplementary Figs. S1–S3). The microarray data showed that EXP9 is also inducible by LBD18 (Lee et al. 2013). These results indicate that one transcriptional response by LBD18 to auxin is the induction of a subset of EXP genes to facilitate the emergence of the LRs via cell wall-loosening.

Expansins are primary wall-loosening factors that directly induce turgor-driven wall extension (Cosgrove 2000a, Cosgrove 2000b). Secondary wall-loosening factors such as...
xyloglucan endoglycosylase/hydrolases, glucanases and polygalacturonase, which enzymatically modify the structures of the cell wall, render it more responsive to wall-loosening events primarily mediated by expansins (Cosgrove 2000b, Swarup et al. 2008, Péret et al. 2009). Thus the relatively small increase in the emerged LRs in Arabidopsis overexpressing EXPI7 or EXPI4 may have been due to the limited amounts of those secondary wall-loosening factors. Auxin treatment of these transgenic plants then activated various signaling events including induction of the secondary wall-loosening factors, causing synergistic effects on LR emergence with EXPI7 or EXPI4 overexpressed in the DEX-treated Arabidopsis. The number of emerged LRs decreased substantially in the LR- or LRP-related phenotypes in the DEX-treated EXPI7RNAi plants (Fig. 4D), and the number of LRPs at stages II and III increased to some extent in EXPI7RNAi lines compared with that in the wild type (Fig. 4H). LR induction experiments with the wild type, EXPI7-overexpressing transgenic plants and EXPI7 RNAi plants (Fig. 6) showed that early stages of LR development are not affected by EXPI7 overexpression or EXPI7 RNAi, and that LR emergence is delayed by EXPI7 RNAi. These results together suggest that EXPI7 plays a role in LR emergence.

As loss-of-function EXPI7 mutants are not available, we used an RNAi approach to investigate the role of EXPI7 in LR formation. Although the CaMV 35S promoter was used to express a hairpin RNA to knock-down the EXPI7 transcript levels, the transcript levels of other EXPI family members such as EXPI4, EXPI11 or EXPI14, which are highly homologous to EXPI7, were not altered in the EXPI7 RNAi lines with or without DEX treatment (Fig. 4B). Moreover, we did not detect any apparent phenotypes other than the LR- or LRP-related phenotypes in the DEX-treated EXPI7 RNAi lines. We observed the opposite phenotype in the LR density of Pro35S:EXPI7 plants compared with the DEX-treated EXPI7 RNAi lines (Figs. 4, 5). These results showed that the LR-related phenotypes of the EXPI7RNAi lines or Pro35S:EXPI7 plants correlated with the levels of the EXPI7 transcripts, demonstrating the role of EXPI7 in LR formation. However, the cell-specific role of EXPI7 during LRP development remains to be determined.

Materials and Methods

Plant growth and tissue treatment

Arabidopsis thaliana (Col-0) seedlings were grown and treated as described previously (Park et al. 2002). Plants were grown under a 16 h photoperiod on 3MM Whatman filter paper on top of agar plates, and the filter paper with the seedlings was then transferred to a plate containing plant hormone or chemicals (20 μM IAA, 10 μM DEX or 50 μM cycloheximide) and incubated for a given period of time with gentle shaking in the light at 23°C. The light intensity was approximately 120 μmol m−2 s−1 and was provided by three wavelength daylight color fluorescent bulbs (Kumho Electric Co.).

Plasmid construction and Arabidopsis transformation

To produce DEX-inducible RNAi transgenic plants, the EXPI7 coding region was subcloned into pDONR™/221 (Invitrogen) using the Gateway® BP recombination reaction, yielding pDONR-EXPI7RNAi. This construct was subcloned into destination vector pOpOff2 (hyg) (Wielopolska et al. 2005) using the Gateway® LR recombination reaction to yield Pro35S:GFP-EXPI7RNAi. Transgenic Arabidopsis plants were selected on Murashige and Skoog (MS) plates containing hygromycin (25 μg ml−1) and rifampicin (25 μg ml−1) after Agrobacterium transformation. To generate the promoter–GUS transgenic Arabidopsis, the promoter region of EXPI7 encompassing 1,584 bp from −1,585 to −1 bp relative to the AUG initiation codon was isolated by PCR from genomic DNA of Arabidopsis, and subcloned into pDONR™/221 (Invitrogen) using the Gateway® BP recombination reaction to yield pDONR-EXPI7. This construct was subcloned into the pBGWFS7 vector (destination vector, VIB, Belgium) using the Gateway® LR recombination reaction to yield Pro35S:GFP-GUS. Pro35S:GFP-GUS plasmid was then introduced into Arabidopsis by Agrobacterium-mediated transformation. Transgenic Pro35S:GFP-GUS Arabidopsis in a lbd18 mutant background (Pro35S:GFP-GUS/lbd18) was generated by crossing lbd18-1 (female) with Pro35S:GFP-GUS (male). Homozygous lines were isolated by genotyping for Pro35S:GFP-GUS and the lbd18-1 mutant. To generate transgenic Arabidopsis overexpressing EXPI7, the full-length EXPI7 coding region was isolated by PCR from Arabidopsis cDNA, and subcloned into pDONR™/221 (Invitrogen) using the Gateway® BP recombination reaction to yield pDONR-EXPI7. This construct was subcloned into the pB7WG2.0 vector (destination vector, VIB) using the Gateway® LR recombination reaction to yield Pro35S:EXPI7. The Pro35S:EXPI7 plasmid was introduced into Arabidopsis by Agrobacterium-mediated transformation. T3 homozygous transformatants were prepared and amplified. All constructs were confirmed by DNA sequencing prior to plant transformation. The primer sequences used in this study are shown in Supplementary Table S1.

RNA isolation, RT–PCR and qRT–PCR analysis

Following treatment, Arabidopsis plants were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated from frozen Arabidopsis using TRI Reagent® (Molecular Research Center, Inc.). 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 carried out using a QuantiTect SYBR Green RT–PCR kit (Qiagen) in a Rotor–Gene 2000 Real-Time Thermal Cycling system (Corbett Research) as described previously (Jeon et al. 2010). All quantitative real-time RT–PCRs were conducted in triplicate biological replications and subjected to statistical
an analysis. RT–PCR conditions and primer sequences are shown in Supplementary Table S1.

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

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

Supplementary material
Supplementary data are available at PCP online.

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Disclosures
The authors have no conflicts of interest to declare.

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