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ORIGINAL ARTICLE

ASYMMETRIC LEAVES2-LIKE38, one member of AS2/LOB gene family, involves in regulating ab-adaxial patterning in Arabidopsis lateral organs

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Abstract *ASYMMERTIC LEAVES1 (AS1)* and *AS2* in Arabidopsis are essential in determining leaf cell fates of adaxial axis. Here, we report *ASYMMERTIC LEAVES2-LIKE38* (*ASL38/LBD41*), is an important gene of the *LATERAL ORGAN BOUNDARY DOMAIN* family. *ASL38/LBD41* was detected in the adaxial and internal domain between the ab-adaxial domains of leaves. For explaining *ASL38/LBD41* role in Arabidopsis development, a construct of the sense-expressing *ASL38/LBD41* was transformed to Arabidopsis (Col-0); thus gained both overexpressing and cosuppressing *35S:ASL38/LBD41* plants. Rosette leaves of these overexpressing plants showed narrow (Class I) to radial symmetric needle-like patterns (Class II), compared with those of wild-type control, exhibiting adaxilized defect. However, rosette leaves of these cosuppressing plants showed narrow (Class I) to radial symmetric needle-like patterns (Class II), in contrast to those of wild-type (Col-0), exhibiting abaxilized defect. Furthermore, phenotypes observed in *asl38-1* mutants suggest a redundant function for *ASL38/LBD41* in boosting adaxial and/or inhibiting cell fate in abaxial axis. Together, our data suggest that *ASL38/LBD41* might play a role of specialization in adaxial cell fate in Arabidopsis lateral organs.

Keywords ASYMMERTIC LEAVES2-LIKE38 (ASL38/LBD41) gene · Arabidopsis · Adaxial/abaxial polarity · Overexpression · Cosuppression · asl38-1 mutant

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Introduction

The primordium of leaf blades is as a result of the peripheral section in shoot apical meristem (SAM). When the primordia of leaves form, it is necessary to build the proximodistal, mediolateral, and ab/adaxial axes (Hudson 2000). The ab/adaxial factor might be most crucial of all among these axes (Bowman et al. 2002).

Recently, a lot of researches have revealed a few genes that function in building ad-abaxial axes of leaf blades. In this homeodomain/Leu zipper (*HD-ZIP*) (class III) family, five members function in special adaxiality of lateral organs (Emery et al. 2003; Prigge et al. 2005; McConnell et al. 2001; McConnell and Barton 1998). With miR165 and miR166 targeting the mRNAs of this family genes; in abaxial cells, the down-regulation is determined (Williams et al. 2005; Kim et al. 2005; Bao et al. 2004; Tang et al. 2003). Under the L*er* (Landsberg *erecta*) background, *as2* mutant shows that the pattern of adaxial cells is transformed into the pattern of abaxial cells (Xu et al. 2003). However, its overexpressing lines (*35S:AS2*) exhibit obviously adaxial and radial leaf blades (Xu et al. 2003; Lin et al. 2003). In the AS2/LOB domains, the LOB domain is essential, and it cannot be shifted via that of other members in the family (Matsumura et al. 2009). *bop1 bop2* double mutants exhibit that adaxial cell pattern is transformed into abaxial cell pattern (Ha et al. 2007). On the other hand, in

KANADI (*KAN*) and *YABBY* (*YAB*) families, some members exhibit abaxial pattern (Eshed et al. 1999; Sawa et al. 1999; Siegfried et al. 1999; Kerstetter et al. 2001; Emery et al. 2003; Eshed et al. 2004; Candela et al. 2008).

Here, we report that *ASL38/LBD41* gene is one member of the Arabidopsis *LATERAL ORGAN BOUNDARY DOMAIN* family, and studies its mode in expression level. Our data indicate that during Arabidopsis leaf growth and development, such expression mode is essential. We constructed sense *35S:ASL38/LBD41* to transform Arabidopsis, and attained *35S:ASL38/LBD41* plants that were both overexpression and cosuppression mutants. By studying these mutants, our findings indicate that *ASL38* function is specification of adaxial cell fates. Furthermore, phenotypes observed in *asl38-1* mutant suggested that *ASL38/LBD41* might play a character of specialization of adaxial cell fate in lateral organs of Arabidopsis.

Materials and methods

Arabidopsis material, constructing vector and genetic transformation

Arabidopsis plants were sown in MS medium for 7 days, and then transferred to green room and grown under 21 ± 2 °C (Meng 2015; Meng et al. 2015).

The *ASL38/LBD41* (At3g02550) codes a zone, which is a DNA fragment (214–1005 in the full-length Arabidopsis *ASL38/LBD41*). This DNA fragment is enlarged derived from 20-day-old plant cDNA of Arabidopsis wild-type (Col-0). And then the DNA was built in the sense direction and seated in downstream of the 35S promoter (Fig. 2a). By sequencing, we confirm the *35S:ASL38/LBD41* construct.

The *35S:ASL38/LBD41* plants were produced via vacuum infiltration of Arabidopsis Col-0 by usualizing *GV3101* strain (Meng and Yao 2015). All *35S:ASL38/LBD41* transgenic lines (T2) were confirmed through PCR via usualizing an *nptII* primer (Meng et al. 2009a, b).

Molecular biological experiments

Total RNA was secluded via usualizing Trizol. To perform RT-PCR research, we synthesized cDNA by 1 μg of total RNA by usualizing an oligo dT 18 primers and MMuLV Reverse Transcriptase. To perform PCR experiments, in each cDNA, 1/4 volume was selected, and usualized them as a template.

To perform *ASL38/LBD41* and *ASL37/LBD40* PCR amplification, conditions below is used, that is, at 94 °C for 4 min (for denaturation), and then for 40 cycles (for cosupression of *ASL38/LBD41*) and 33 cycles (for overexpression of *ASL38/LBD41*) of 94 °C for 45 s, 60 °C for 45 s, 72 °C for 1 min, at last at 72 °C for 7 min. The PCR primers: *ASL38/LBD41-F* (5′-tcggaaagggtgtagtgag-3′) and *ASL38/LBD41-R* (5′-aggacgaaggtgattgggac-3′); *ASL37/LBD40-F* (5′-tacgaaaaggctgcagtgaa-3′) and *ASL38/LBD41-R* (5′-ggtaccaccacgtgatttcc-3′) (Shuai et al. 2002).

For assaying the expressing change of abaxial polarity genes in *35S:ASL38/LBD41* lines, RT-PCR was implemented, its conditions are below in details: at 94 °C for 4 min (for denaturation), for 35 (for *FIL, YAB* and *PHB*) cycles of 94 °C for 40 s, 58 °C for 40 s, and 72 °C for 1 min, and at last, a incubation at 72 °C for 7 min. Gene-specific primers used were described by Lin et al. (2003).

Morphological analyses

We performed morphological experiments, which refer to methods in Fuchs (1963) and Baum and Rost (1996). Also we completed experiments of scanning electron microscopy using Quanta 200 FEG SEM (Alvarez et al. 1992). *FIL* probe was produced via cDNA and synthesized by using DIG-labeled antisense RNA via usualizing T7 RNA polymerase. *FIL* probes were produced, which refer to methods in Eshed et al. (2001), and *ASL38/LBD41* probes were produced via usualizing *ASL38/LBD41 F* (5′-tcggaaagggtgtagtgag-3′) and *R* (5′-aggacgaaggtgattgggac-3′).

Results

Site accumulation of *ASL38/LBD41* transcripts during vegetative growth

In the wild-type Columbia ecotype, similar to previous report (Shuai et al. 2002), *ASL38/LBD41* gene transcripts can be detected, at variable levels, in all tissues examined, for example, cauline leaves, roots, rosette leaves, inflorescence stems and blossom flowers (Fig. 1a), suggesting *ASL38/LBD41* was expressed throughout Arabidopsis tissues. Furthermore, for better detecting *ASL38/LBD41* transcripts in Arabidopsis, in situ hybridization of *ASL38/LBD41* was constructed. In transverse sections of 10-day-old Arabidopsis (Col-0), relatively weak signals were detected in terms of *ASL38/LBD41* transcripts in this zone of the expected primordium on leaf blades (P0) and early primordia (P1 and P2) can be seen. With primordia developed, stronger signals in adaxial region were detected. In general, with leaf blades developed, the relative signals became weaker (Fig. 1b). These data suggest that *ASL38/LBD41* might regulate adaxial leaf blade of Arabidopsis.

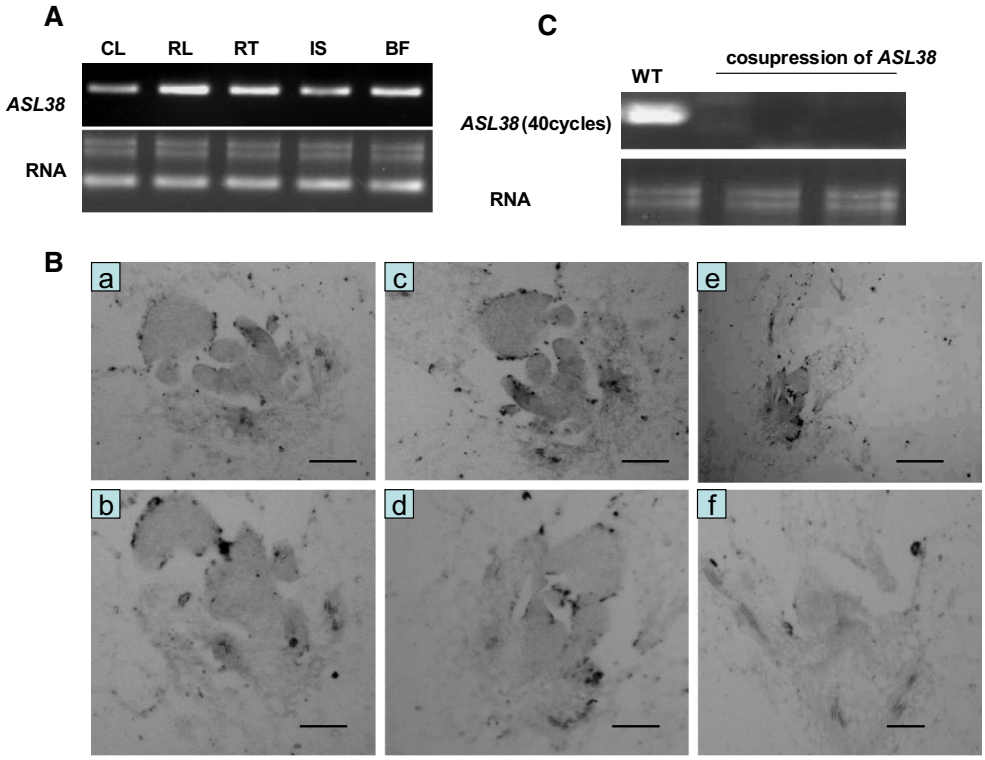

Fig. 1 Expression of some genes in wild-type and *35S: ASL38/LBD41* plants. **a** In rosette leaves, cauline leaves, roots, inflorescence stems and blossom flowers, *ASL38/LBD41* can all be detected, at variable levels. *RL* rosette leaves, *CL* cauline leaves, *RT* roots, *IS* inflorescence stems, *BF* blossom flowers. **b** in situ hybridization with an *ASL38/LBD41* probe in wild-type. **a**–**f** are serial sections of a vegetative meristem with 10 um interval. *Bars* 100 um. **c** *ASL38/LBD41* mRNA levels are completely inhibited in sense *35S:ASL38* plants using primers of coding regions, in contrast to those of wild-type (Col-0). RNA was used as a control

Identification of *35S:ASL38/LBD41* Arabidopsis seedlings

For explaining the function of *ASL38/LBD41*, we performed the sense- expressing status of the *ASL38/LBD41* in the 35S promoter, which is a strong promoter (Fig. 2b). The *35S:ASL38/LBD41* transgenic plants were made. Using PCR assay, seventy-two transgenic plants containing the *nptII* reporter gene were proved (Meng et al. 2009a, b). In these identified seedlings, 30 with narrow rosette leaves (Class I) and 22 with radially- symmetric needle-like leaves (Class II) were found. While residual 20 identified seedlings are proved via PCR, they were not obviously diffinct with Col-0 (data not shown). RT-PCR findings in these 52 (52/72) transgenic plants with aberrant phenotypes were performed. In 12 with narrow rosette leaves (Class I) and 9 with radially-symmetric needle-like leaves (Class II) (Fig. 3a–d, g, h; arrow), RT-PCR findings indicated that both transgenic *ASL38/LBD41* and endogenous were entirely inhibited, for example, not any expression of endogenous *ASL38/LBD41* mRNA compared with Col-0 was observed (Fig. 1c). These findings indicate that in the *ASL38/LBD41* plants, the cosuppression is observed. These identified plants (9 + 12/30) of RT-PCR analysis were selected for further analysis. Besides these, 18 with narrow rosette leaves and 13 with radially-symmetric needle-like leaves (Fig. 3l, m; arrow) showed overexpression of *ASL38/LBD41* by RT-PCR analysis (Fig. 2a). In this same construct, independent transgenic seedlings always fluctuate >100-fold in transgene expression level and cosupression frequently evokes gene silencing (Chen et al. 2007; Holtorf et al. 1995; Jones et al. 1985). Thus, it was not surprised that both cosupression and overexpression were triggered in *35S:ASL38/LBD41* seedlings.

Overexpression and cosuppression of *ASL38/LBD41* show Ab-adaxial polarity defects

The vasculature of rosette leaves displayed a reticulate pattern (Fig. 3j) in wild-type plants, whereas midvein often disappeared and secondary vein drastically simplified in the cosupressing *35S:ASL38/LBD41* rosette leaves (Fig. 3e). Since the establishment of a vascular system requires the existence of different ab/adaxial axes (Lin et al. 2003), these alternations of the vasculature of the cosupressing *35S:ASL38/LBD41* leaves may result from

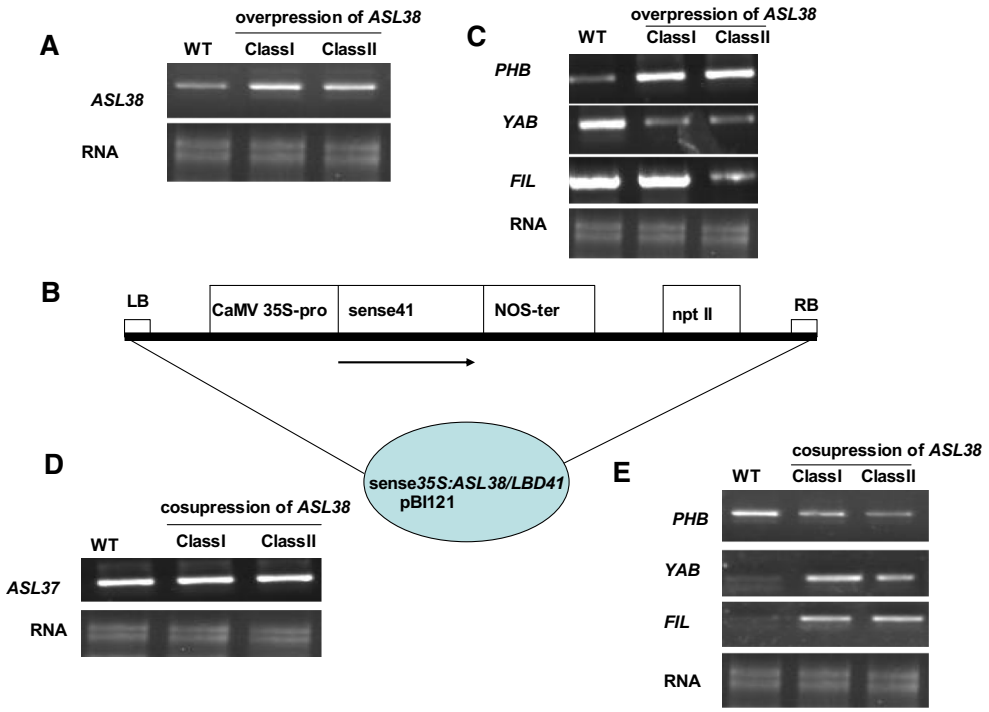

Fig. 2 Expression of some genes in *35S: ASL38/LBD41* plants. **a** *ASL38* expression levels are enhanced in overexpressing *35S:ASL38* plants, in contrast to those of wild-type. **b** Scheme of the sense *35S:ASL38/LBD* constructs. The vector used for the introduction of the 793-bp *Arabidopsis 35:ASL38/LBD41*cDNA sense orientation into *Arabidopsis*. The sense *ASL38/LBD41*cDNA insert is flanked by the cauliflower mosaic virus 35S promoter at the 5′ end and by the transcriptional terminator at the 3′ end. *LB* left border, *RB* right border, *nptII* neomycin phosphotransferase gene whose expression confers plant resistance to kanamycin. **c** In overexpression of *ASL38/LBD41,* expression of *FIL* and *YAB3* genes is decreased, whereas expression of *PHB* genes is enhanced, in contrast to that of wild-type (Col-0). **d** *ASL37* expression leaves of cosupressing *35S:ASL38/LBD41* plants were compared with those of wild-type. **e** *35S:ASL38/LBD41* cosupression enhances expression of *FIL* and *YAB3* genes, whereas it decreases expression of *PHB* genes, in contrast to those of wild-type (Col-0). Levels of RNA of genes above are determined by RT-PCR analysis using 1 ug of total RNA from each sample. RNA was used as a control

the loss of polar gene expression. For further explaining the function of *ASL38/LBD41*, we analyzed the dissecting characteristics of these *35S:ASL38/LBD41* rosette leaves. In rosette leaves of wild-type, an obvious ab-adaxial polarity is visible. Tightly packed, extended palisade mesophyll cells locate at the adaxial side, while irregular or round spongy mesophyll cells, loosely packed, at the abaxial side (Fig. 4a). However, in overexpressing and cosuppressing *35S:ASL38/LBD41* radially-symmetric needle-like leaves (Class II), the polarity was disoriented; as the palisade mesophyll cells were well developed on abaxial side as well as adaxial side in overespressing *35S:ASL38/LBD41* leaf blades (Fig. 4n), and the spongy mesophyll cells were well developed on adaxiality except as on abaxiality in cosuppressing *35S:ASL38/LBD41* leaves (Fig. 4d).

In the elementary vascular tracts of Arabidopsis Col-0 leaf blades, ab/adaxial antagonism was obvious via the positioning of distinct tissues. Xylem located adaxially, while phloem positioned abaxially (Fig. 4b, c). In a transverse section of overexpressing and cosuppressing *35S:ASL38/LBD41* rosette leaves, regardless of narrow leaf blades (Class I) or radially-symmetric needle-like leaves (Class II), the primary tracts showed alterable xylem/phloem array, for example, xylems twined by phloems of cosuppressing *35S:ASL38/LBD41* leaves (Fig. 4d, h, e, f, i, j), and phloems situated amid xylems of overexpressing *35S:ASL38/LBD41* leaves (Fig. 4m–p).

Like the internal tissues, polar deficiency was also obvious in the epidermal cells of *35S:ASL38/LBD41* leaf blades. By scanning electron microscopy analysis, the wild-type abaxial epidermis was arranged via a wavelike surface containing no uniform measured cells, which contains long cells (Fig. 5a; arrow), whereas the control (Col-0) adaxial surface of leaf blades was arranged through a flat epidermis containing consistently measured cells (Fig. 5b). In comparison, the abaxial epidermises of overexpressing *35S:ASL38/LBD41* narrow rosette leaves (Class I) reveal a compound of ab/adaxial leaf blades (Fig. 5l). Their adaxial surface was not distinct from that of control (Col-0) leaves (data not shown). Moreover, the epidermis of the radially-symmetric needle-like leaves (class II)

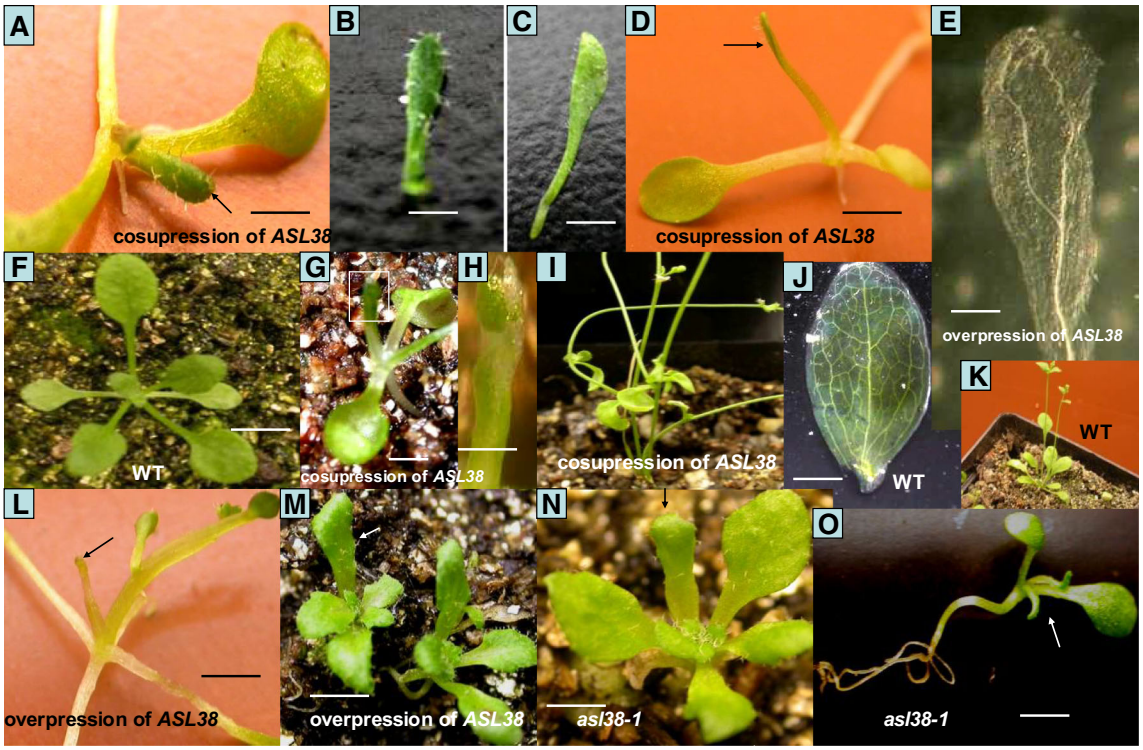

Fig. 3 Phenotypes of *35S:ASL38/LBD41* plants and *asl38-1* mutants. **a**, **d** A 14-day-old cosupressing *35S:ASL38/LBD41* plant with a narrow rosette leaf. *Arrow* indicates a narrow rosette leaf. **b**, **c** A cosupressing *35S:ASL38/LBD41* narrow rosette leaf grown for 14 and 30-day-old, respectively. Arrow indicates a narrow rosette leaf. **e** Vascular-pattern of *35S:ASL38/LBD41* leaf. **f** *Arabidopsis thaliana* ecotype Columbia wild-type. **g** Cosupressing *35S:ASL38/LBD41* plant with the radial symmetric needle-like leaf. **h** The radial symmetric needle-like leaf (*box*) of **g** shows a higher magnification. i Cauline leaves of cosupressing *35S:ASL38/LBD41* plants curled downward. **j** Vascular-pattern of wild-type leaf. **k** Wild-type grown in flowerpot. **l** Overexpressing *35S:ASL38* plant with the radial symmetric needle-like leaf. **m** Overexpressing *35S:ASL38* plant with a narrow rosette leaf. *Arrow* indicates a narrow rosette leaf. **n**, **o** *asl38-1* mutants with narrow rosette leaves. *Arrow* indicates a narrow rosette leaf. *Bars* 1.0 mm

reveal adaxial blade traits, as the epidermis containing consistently measured cells can be observed (Fig. 5h). On the contrary, the adaxial epidermises of cosuppressing *35S:ASL38/LBD41* narrow rosette leaves (Class I) reveal an undulating surface resembling those of wild-type abaxial blade; and on this side, long cells were well observed (Fig. 5c, g; arrow). Their abaxial epidermises were not distinct from that of control (Col-0) leaves (data not shown). Moreover, the distal, middle and proximal epidermis in the radially- symmetric needle-like leaves (class II) reveal abaxial blade traits in cosuppressing *35S:ASL38/LBD41* narrow rosette leaves, as a lot of long cells can be observed (Fig. 5i–k; arrow).

To obtain molecular evidence for the *ASL38/LBD41* function, by utilizing RNA in situ hybridization, we detected the expressing traits of the *FIL* polar gene. In Col-0 seedlings, transcripts of the *FIL* gene store up in younger leaves and their expressions decrease on the adaxial side with younger leaf growing (Sawa et al. 1999; Siegfried et al. 1999) (Fig. 6a; arrow). In cosuppressing *35S:ASL38/LBD41* plants with narrow (Class I) and needle-like rosette leaves (Class II), *FIL* expression was entirely detected in the ab/adaxial side of the developing leaf blades and the apex meristem (Fig. 6c, d; arrow), suggesting lateral organ of these *35S:ASL38/LBD41* plants was abaxialized patterning. However, in overexpressing *35S:ASL38/LBD41* plants with narrow (Class I) and needle-like rosette leaves (Class II), *FIL* was not detected in the ab-adaxial side of the developing leaf and the apex meristem (Fig. 5e, f; arrow), suggesting lateral organ of these *35S:ASL38/LBD41* plants was adaxialized patterning. In general, our data lead to the conclusion that the cosuppressing *35S:ASL38/LBD41* leaf blades closely link to the loss of adaxial polarity, but overexpressing *35S:ASL38/LBD41* leaf blades are closely relative to the loss of abaxial polarity.

Aberrant phenotype of the overexpressing *35S:ASL38/LBD41* trichomes indicate that *ASL38/LBD41* is multiple functions. There was a decrease in the divergent section of trichomes on the *35S:ASL38/LBD41* radial needle-like leaves (class II), to two (Fig. 5h) rather than the three to four branches of wild- type (Fig. 5d). Also, on *35S:ASL38/*

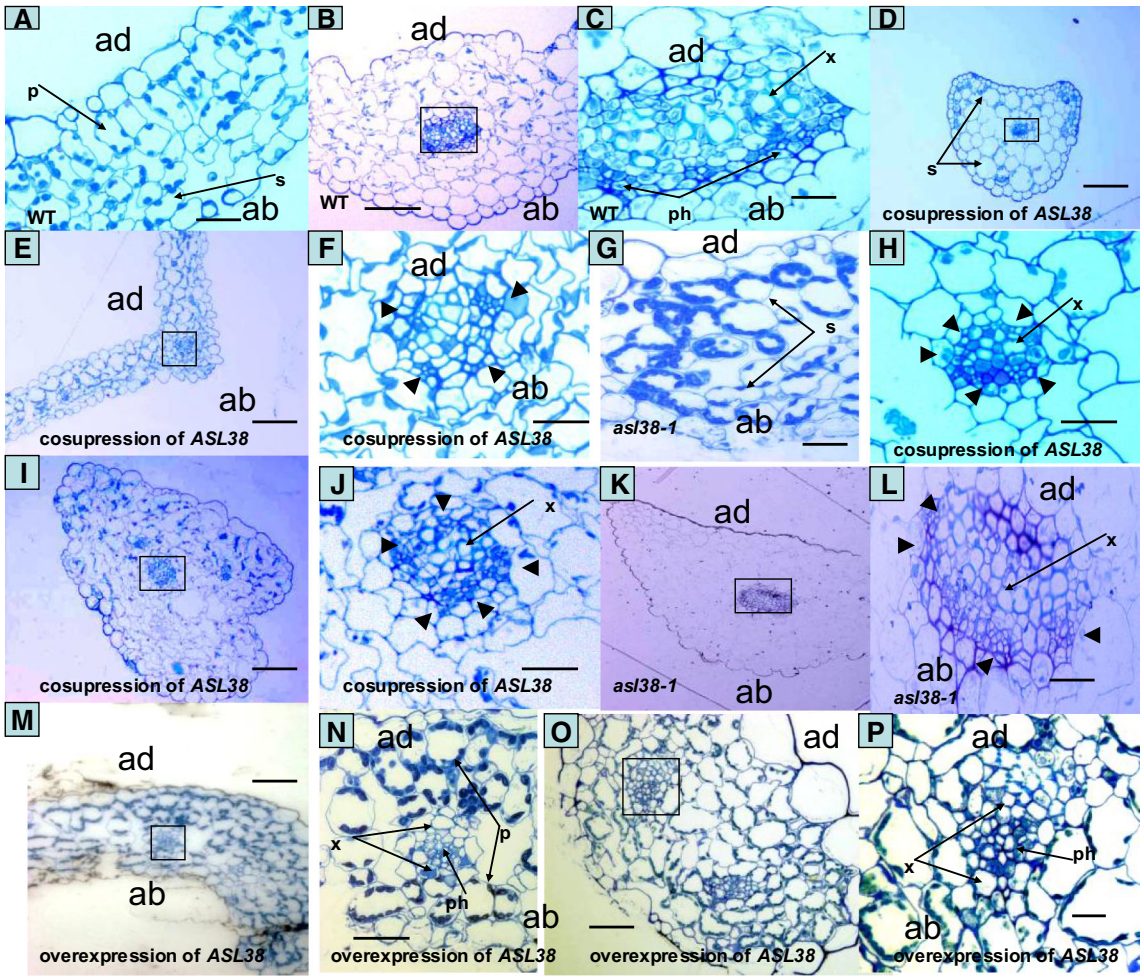

Fig. 4 Cross-section of *35S:ASL38/LBD41* plants and *asl38-1* mutants. **a** Cross-section through the rosette leaf blade of a wild-type. **b** Cross-section through the rosette leaf petiole of a wild- type. **c** is the magnified views of the regions boxed in **b**. **d**, **i** Cross-section through the radial symmetric needle-like rosette leaves of cosupressing *35S:ASL38/LBD41* plants. **h**, **j** are the magnified views of the regions *boxed* in **d** and **i**, respectively. **e** Cross-section through the narrow rosette leaves of cosupressing *35S:ASL38* plants. **f** the magnified views of the big box regions in **e**. **g** Cross-section through the *narrow* rosette leaf blade of *asl38-1* mutants. **k** Cross-section through the narrow rosette leaf petiole of *asl38-1* mutants. **l** is the magnified views of the regions *boxed* in **k**. **m**, **o** Cross-section through the narrow and radial symmetric needle-like rosette leaves of overexpressing *35S:ASL38/LBD41* plant, respectively. **n**, **p** The magnified views of the *box* regions in **m**, **o**, respectively; *Bars* 50 um in **a**, **b**, **d**, **e**, **g**, **i**, **k**, **m** and **o**; *Bars* 20 um in **c**, **f**, **h**, **j**, **l, n** and **p**. *ab* abaxial, *ad* adaxial, *p* palisade mesophyll, *s* spongy mesophyll, *ph* phloem, *x* xylem

LBD41 leaves, both the size and the number of the trichome support cells were increased, and protruded from the leaf blade surface (Fig. 5h); in contrast to those of wild-type Col-0 (Fig. 5d).

ASL37 expression analysis in cosuppressing *35S:ASL38/LBD41* seedlings

While our data indicated that *ASL38/LBD41* was probably involved in ab-adaxil polarity, it is reasonable that our findings may be produced via an artifact through the influence of the outcome of RNAs of *ASL38/LBD41*, influencing levels of undisclosed genes of the *AS2/LOB* family, which contains 42 members and unidentified members. In *LBDs* gene family, *ASL37/LBD40* is the most similar to *ASL38/LBD41*. Since *ASL38/LBD41* is highly homologous to *ASL37* both in the *AS2/LOB* domains (58 % identical amino acids) and the C-terminal halves (38 % identical amino acids), it is possible that *ASL38* and *ASL37* would have the overlapping functions. Thus, RT-PCR analysis was performed for examining *ASL37* expression levels in cosuppressing *35S:ASL38/LBD41* plants. Our data indicated that *ASL37* expression levels in cosuppressing *35S:ASL38/LBD41* plants were consistent with those in control plants (Fig. 2d), suggesting that these aberrant phenotypes of cosuppressing *35S:ASL38/LBD41* plants were triggered by endogenous RNAs of suppressing *ASL38/LBD41* other than those of suppressing *ASL37* or

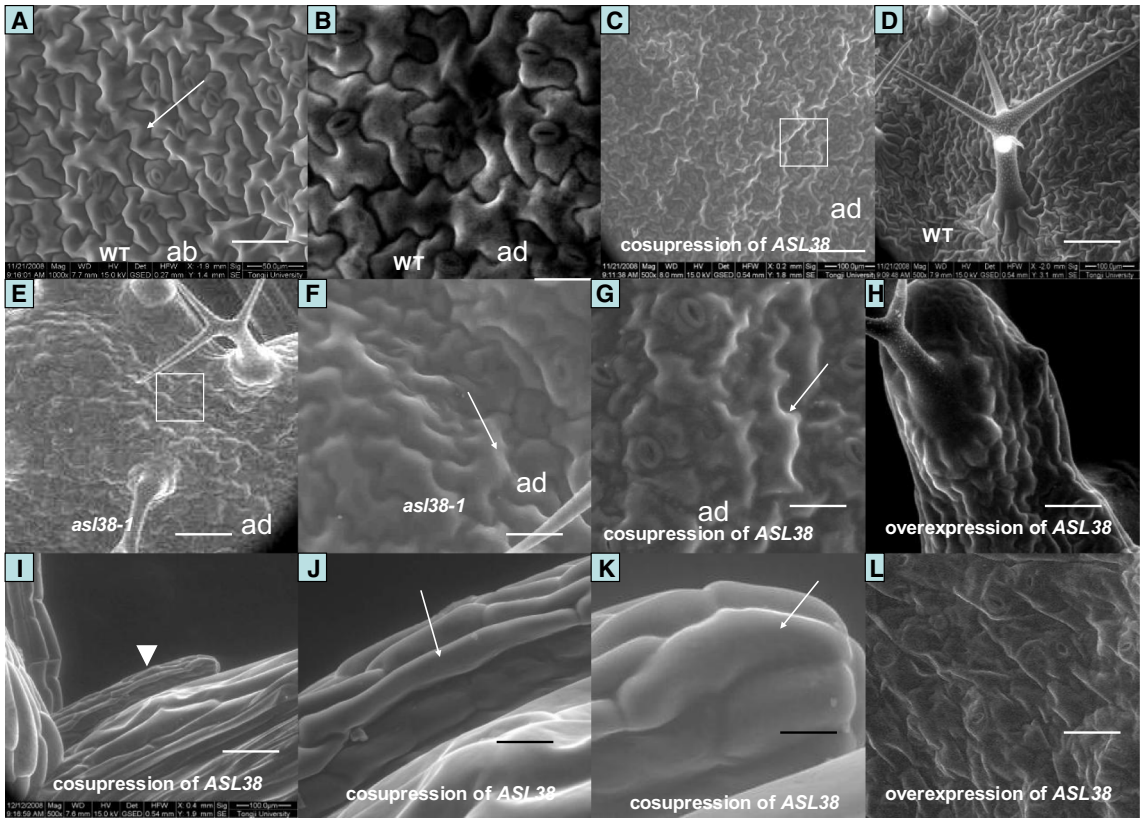

Fig. 5 Epidermal Surface of *35S:ASL38/LBD41* Plants and *asl38-1* mutants. **a** Abaxial epidermis of a wild-type (wt) rosette leaf showing an undulating surface composed of irregular cell size. *Arrow* indicates elongated cell. **b** Adaxial epidermal cells of a wild-type (wt) rosette leaf showing uniform cell size. **e**, **f** The adaxial epidermises of the *asl38-1* narrow rosette leaf blades resembling a wild-type abaxial epidermis. Arrow indicates elongated cell. **c**, **g** The adaxial epidermises of the cosupressing *35S:ASL38/LBD41* narrow rosette leaf blades resembling a wild-type abaxial epidermis. *Arrow* indicates elongated cell. **i–k** The adaxial tip, middle and proximal epidermises of the cosupressing *35S:ASL38* radially-symmetric, needle-like leaf (*arrowhead*) resembling a wild-type abaxial epidermis. *Arrow* indicates elongated cell. **d** Abaxial epidermis of a wild-type (wt) rosette leaf with three to four branches. **h** The epidermises of the overexpressing *35S:ASL38/LBD41* radially-symmetric, needle-like leaf resembling a wild-type adaxial epidermis. **l** The abaxial epidermises of the overexpressing *35S:ASL38/LBD41* narrow leaf resembling a wild-type adaxial epidermis. *Bars* 50 um in **a**, **b**, **f**, **g**, **h**, **j**, **k,** and **l**; *Bars* 100.0 um in **c–e** and **i**

ASL38 and *ASL37*. Previous reports (McHale and Koning 2004; Andersson et al. 2001; Oeller et al. 1991; Hamilton et al. 1990; Ecker and Davis 1986; Rothstein et al. 1987; Smith et al. 1988; Van der Krol et al. 1988) have proved that antisense mRNA and sense has been proved to be a helpful tool for suppressing the expression of particular genes; more importantly, the mRNA level of this requested protein in this gene family can be only altered, on the other hand, other genes in this family keep up no influence (Chen et al. 2007; Andersson et al. 2001; Ganeteg et al. 2001; Zhang et al. 1997).

T-DNA insertions of *ASL38/LBD41* expression

In order to further assure that these aberrant phenotypes of *35S:ASL38/LBD41* plants do result from *ASL38/LBD41* overexpression and cosuppression, we gained a *asl38* mutant of T-DNA insertion, which is from the Salk collection (SALK-090708, T4; *asl38-1*). In 22 *asl38-1* mutants, 5 with narrow rosette leaves were acquired; suggesting that *ASL38/LBD41* might be participated in leaf enlargement (Fig. 3n, o). Remaining 17 *asl38-1* mutants (17/22) displayed to be analogical leaf enlargement pattern to control (Col-0) plants at either younger or older stages (data not shown). Phenotypes analysis indicated that the polarity was disturbed in the subepidermis of the *asl38-1* narrow rosette leaves, which indicates on the both abaxial and adaxial side, there are spongy mesophyll cells (Fig. 4g). Moreover, in arrangement of the primary vascular bundles, partially abaxialized rosette leaves appeared in *asl38-1* plants, namely, leafstalks showed vasculature with half-moon-traits; where xylem was observed on the interior and phloem was observed on the external (Fig. 4k, l). This is interpreted as partially abaxialized vasculature (Ha et al. 2007). By scanning electron microscopy analysis, the adaxial epidermises of *asl38-1* narrow rosette leaf

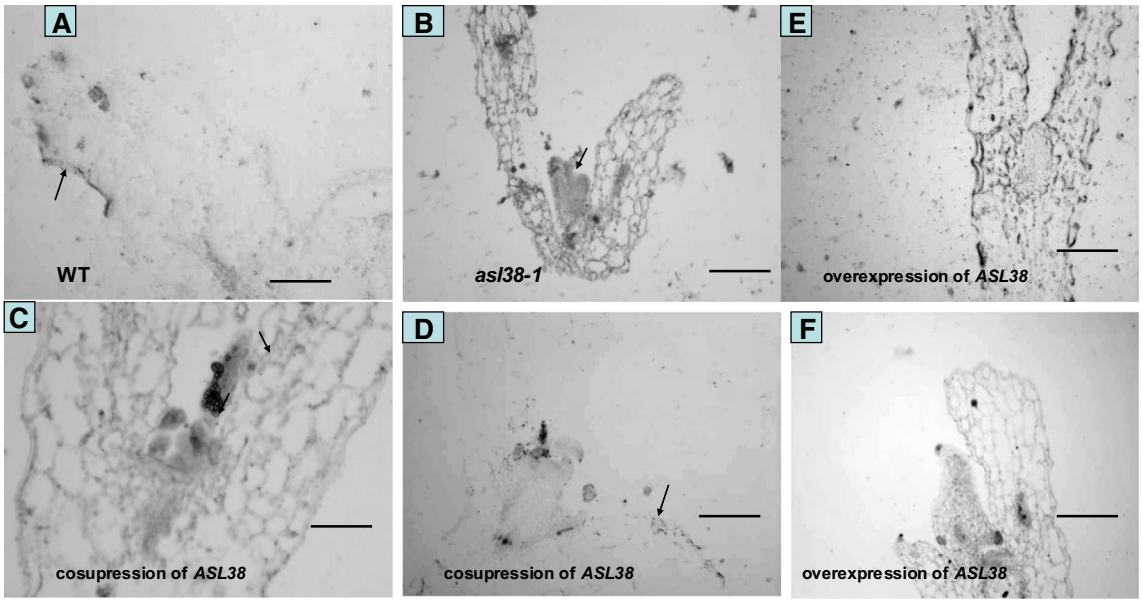

Fig. 6 RNA in situ hybridization analysis of *FIL* expression in *35S:ASL38/LBD41* Plants and *asl38-1* mutants. In 12-day-old Col (**a**); *asl38-1* mutants (**b**); cosupressing *35S:ASL38/LBD41* Class I seedlings (**c**); cosupressing *35S:ASL38* Class II seedlings (**d**); overexpressing *35S:ASL38* Class I seedlings (**e**); overexpressing *35S:ASL38* Class II seedlings (**f**). Arrows indicate expression of *FIL* in (**a**). *Arrows* indicate ectopic expression of *FIL* in **b**–**d**. *Bars* 100 um

blades showed an undulating surface resembling those of wild-type abaxial blade; and on this side, long cells were well observed (Fig. 5e, f; arrow). Their abaxial surface was not distinct from that of control (Col-0) leaves (data not shown). Furthermore, in *asl38-1* with narrow rosette leaf blades, by utilizing RNA in situ hybridization, we detected the status of the *FIL* expression. In control (Col-0) seedlings, the *FIL* transcripts were seen in younger leaves, and when younger leaf grows their expressions were decreased on the adaxial surface (Siegfried et al. 1999; Sawa et al. 1999) (Fig. 6a; arrow), whereas in *asl38-1* plants with narrow rosette leaves, *FIL* expression was entirely detected in the ab-adaxial side of the developing leaves and the apex meristems, which was never found in that of wild-type (Fig. 6b; arrow). At adult stages, *asl38-1* plants displayed to be analogical to control (Col-0) seedlings (data not shown). These partially abaxilized phenotypes seen in *asl38-1* mutant might be as a result of the redundant function in *LBD/ASL* family.

Discussion

LOB is considered as playing an important character in building boundary of plant lateral organ development. When the meristem and lateral organs initiate, *LOB* can establish boundary or communicate signaling (Shuai et al. 2002). LOB is known as a nuclear protein, which participates in regulation of transcriptional level (Husbands et al. 2007). Although we have well understood their some functions, such as *AS2* (Xu et al. 2003; Lin et al. 2003), in this family, many genes are not fully studied.

Aberrant phenotypes of some transgenic plants are triggered by *ASL38/LBD41* cosupression

Independent transformed plants containing the identical construct always fluctuate >100-fold in transgene expression level and cosupression frequently evokes gene silencing (Chen et al. 2007; Holtorf et al. 1995; Peach and Velten 1991; Jones et al. 1985). In our work, wild-type exhibits evident *ASL38/LBD41* transcripts by RT-PCR analysis, whereas 21 (9 + 12) *35S:ASL38/LBD41* transgenic plants with similar traits yield no signal in the code regions corresponding to endogenous *ASL38/LBD41* mRNA, strongly suggesting that these *35S:ASL38/LBD41* plants are co-suppressed (Chen et al. 2007; Dougherty and Parks 1995; van der Krol et al. 1990; Napoli et al. 1990). Interestingly, the 21 *35S:ASL38/LBD41* plants show similar traits that xylems are surrounded by phloems in xylem/phloem arrangements (traits of abaxially lateral organ), suggesting cosuppression, but not overexpression, of sense *35S:ASL38/LBD41*. Furthermore, had sense *35S:ASL38/LBD41* in transgenic plants overexpressed, xylem/phloem arrangements would have been different or even reversed (Lin et al. 2003; McConnell and Barton 1998; McConnell et al. 2001). Sense or antisense suppression is a helping instrument for building *Arabidopsis* plants which knocks

out specific protein, more significantly, specific in the sense transgenic plants in which the mRNA expression of the requested protein is influenced, whereas other genes in this family are not influenced (Andersson et al. 2001; Ganeteg et al. 2001; Zhang et al. 1997). Sense cosupression can be induced by divergent mechanism that is, at present, still poorly understood. Chen et al. (2007) thought that RNA silencing was triggered by sense cosupression evoked by siRNAs. The mechanism of *ASL38/LBD41* cosupression remains to be elucidated.

ASL38/LBD41 specifies adaxial cell fate

A lot of studies imply there is a link between cosuppressing *35S:ASL38/LBD41* and fate of abaxial cells or overexpressing *35S:ASL38/LBD41* and fate of adaxial cells. Cauline leaves of cosuppressing *35S:ASL38/LBD41* plants curled downward, toward the abaxial side, suggesting that these *35S:ASL38/LBD41* plants may be involved in abaxialized defect. More significantly, blade expansion of rosette leaves of *35S:ASL38/LBD41* overexpression and cosuppression were radically limited. The similar narrow leaf blades have been reported in *35S:AS2* overexpression, curling upward, hence are believed to be adaxialized organs (Lin et al. 2003). Mutations that related to ab-adaxial axes induce decreased blade elognation (Bowman et al. 2002), which agrees with the hypothesis that juxtaposition of ab/adaxial and abaxial regions is needed to leaf growth (Waites and Hudson 1995). In extreme cases, *35S:ASL38/LBD41* rosette leaves exhibit radially-symmetric, needle-like appearance. Similar to these extreme leaves of these *35S:ASL38/LBD41* plants, in the *Antirrhinum,* mutants of *PHAN* lead to the radial and symmetric needle-like leaf blades, which are thought to be the results of abaxialization (Waites and Hudson 1995), and loss-of-function mutant of *phb-phv-rev* triple exhibits a single radial cotyledon and hypocotyl, which are believed to be abaxialized organs (Emery et al. 2003). *ae3-1* leaves have a radially-symmetric, needle-like look, which is considered resulting from the defective adaxial identity (Huang et al. 2006). These radial cotyledon, hypocotyl, and rosette leaves all show very similar phenotypes, namely, the radially-symmetric, needle-like traits are always closely related to ab-adaxial polarity, suggesting that the needle-like leaves of *35S:ASL38/LBD41* plants may be the adaxial-abaxial defective organs. The development with needle-like leaves cannot further produce laminae (Xu et al. 2003). Together, added losses with asymmetric growth are kept company via decreased blade expansion (Eshed et al. 2004). This phenomenon accords the classic theory that the build of ab/adaxial axis is necessary to laminae development (Sussex 1954, 1955).

The examination of the internal tissues reveals that adaxial side of the rosette leaves of cosupressing *35S:ASL38/LBD41* plants are spongy mesophyll, which is different from that extended cells of the palisade mesophyll locate at the adaxial surface in wild-type; whereas abaxial side of overexpressing *35S:ASL38/LBD41* rosette leaves are palisade mesophyll. In a vascular-pattern of Arabidopsis leaf blades, ab-adaxial axis is organized via arranging distinct tissues. Xylem is positioned adaxially, whereas phloem abaxially. However, in a transverse section of cosuppressing *35S:ASL38/LBD41* rosette leaf blades, most primary bundles display distinct xylem/phloem arrangements, i.e., they typically show phloem-surrounding-xylem patterns; whereas overexpressing *35S:ASL38/LBD41* rosette leaves reveal xylem-surrounding-phloem patterns. In previous reports, a link has been found, for example, in *kan1-kan2* or *bop1-bop2* double mutant, *phb-phv-rev* triple mutant and *phan* mutant leaf blade growth (Ha et al. 2007; Huang et al. 2006; Eshed et al. 2004; Emery et al. 2003; Waites and Hudson 1995), vascular-pattern presents a pattern of xylem surrounded by phloem, and the vascular-shape deficiencies are always closely relative to the missing of adaxial axis. For another, in adaxial *phb-1d/+* leaf blades, amphivasal positioning is observed, in which the tissue of phloem is enclosed via xylem (McConnell and Barton 1998); and in leaf blades of adaxial *35S:AS2* lines, phloem is enclosed via xylem tissue (Lin et al. 2003). The above observations demonstrate that there is a close linkage between the vascular patterning and ab/adaxial axis, and that the cosuppressing *35S:ASL38/LBD41* leaf blades closely link to the loss of adaxial polarity and overexpressing *35S:ASL38/LBD41* leaf blades closely link to the loss of abaxial polarity. By scanning electron microscopy analysis, the abaxial epidermises of overexpressing *35S:ASL38/LBD41* reveal adaxial blade traits of wild-type, as a even face made of uniformly sized cells can be observed. On the contrary, the adaxial epidermises of cosuppressing *35S:ASL38/LBD41* narrow rosette leaves show an undulating surface resembling those of wild-type abaxial blade; and on this side, long cells were well observed.

Phenotypic analyses of overexpression and cosupression of *ASL38/LBD41* reveal that the *ASL38/LBD41* is necessary to specify the adaxial position in leaf blades. To better understand if overexpression and cosuppression of *ASL38/LBD41* is closely related to ab-adaxial later lateral organs of shoots, the molecular evidence of *ASL38/LBD41* function was attained by examining the expressing modes of the *FIL* via utilizing RNA in situ hybridization. Our data showed that *FIL* was entirely detected in the abaxial and adaxial surfaces of the developing leaf blades and apex meristems; whereas *FIL* was not detected in the abaxial and adaxial surfaces of the developing leaves and the apex

meristems in overexpressing *35S:ASL38/LBD41* plants, which was never found in that of wild-type.

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