The Over-Expression of Two Transcription Factors, ABS5/bHLH30 and ABS7/MYB101, Leads to Upwardly Curly Leaves

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Abstract
Proper leaf development is essential for plant growth and development, and leaf morphogenesis is under the control of intricate networks of genetic and environmental cues. We are interested in dissecting these regulatory circuits genetically and report here the isolation of two Arabidopsis dominant mutants, abnormal shoot5-1D (abs5-1D) and abs7-1D identified through activation tagging screens. Both abs5-1D and abs7-1D display an intriguing upwardly curly leaf phenotype. Molecular cloning showed that the elevated expression of a bHLH transcription factor ABS5/T5L1/bHLH30 or a MYB transcription factor ABS7/MYB101 is the cause for the abnormal leaf phenotypes found in abs5-1D or abs7-1D, respectively. Protoplast transient expression assays confirmed that both ABS5/T5L1 and ABS7/MYB101 are targeted to the nucleus. Interestingly, the expression domains of auxin response reporter DR5::GUS were abnormal in leaves of abs5-1D and ABS5/T5L1 over-expression lines. Moreover, cotyledon venation analysis showed that more areoles and free-ending veins are formed in abs5-1D. We found that the epidermis-specific expressions of ABS5/T5L1 or ABS7/MYB101 driven by the Arabidopsis Meristem Layer 1 promoter (PAML1) were sufficient to recapitulate the curly leaf phenotype of abs5-1D or abs7-1D. In addition, PAML1::ABS5 lines exhibited similar changes in DR5::GUS expression patterns as those found in 35S-driven ABS5/T5L1 over-expression lines. Our work demonstrated that enhanced expressions of two transcription factors, ABS5/T5L1 and ABS7/MYB101, are able to alter leaf lamina development and reinforce the notion that leaf epidermis plays critical roles in regulating plant organ morphogenesis.

Introduction
A major difference between plant and animal development is the de novo formation of plant organs such as leaves in post-embryonic development [1]. Advances in the past decade have uncovered elaborate regulatory pathways governing the morphing of pluripotent cells in plant apical meristems, both the shoot apical meristem (SAM) and the root apical meristem, into distinct organs [2–4]. For example, the proper establishment of a leaf is under the control of intricate networks of genetic pathways and environmental cues [5,6]. As leaf primordia are emerging from the SAM, these pathways and factors work in concert to ensure the coordinated development of leaf primordia along three dimensions: the proximo-distal, the medio-lateral, and the adaxial-abaxial axes, into leaves that show asymmetric features along these axes [6].

In most plants, one key aspect of leaf development is the proper coordination of adaxial and abaxial growth to maintain relatively flat leaves that are maximized for photosynthesis [6,7]. A growing list of genetic factors regulates the establishment of leaf adaxial and abaxial identities [6,7]. The class III homeodomain-leucine zipper (HD-ZIP) transcription factors genes PHABULOSA (PHB), PHAVOLUTA (PHV), and REVOLUTA (REV) are factors that promote the adaxial fate [7–10]. These genes were first identified through gain-of-function mutants in which leaves show adaxialization, and loss-of-function mutations of PHB, PHV and REV show reduced adaxial fate and concurrently an abaxialization of leaves [7–10]. On the other hand, at the abaxial side of the leaf, a group of factors antagonistic with the HD-ZIPs work to determine the abaxial fate [7,11]. These include KANADI (KAN) family transcription factors and epigenetic regulation through micro-RNA165/166 [12,13]. In addition, the YABBY (YAB) genes, ASYMMETRIC LEAVES1 (AS1) and AS2 genes have also been demonstrated to participate in leaf adaxial-abaxial polarity determination [14–16].

Curly leaf mutants are one group of mutants that show aberrant adaxial-abaxial growth coordination, giving rise to upward or downward leaf curvature. The classical Antirrhinum mutant cincinnata clearly demonstrated that leaf surface curvature is under genetic regulation [17]. Indeed, genetic works in Arabidopsis have identified a number of curly leaf mutants, such as the incursa (icu) series of mutants [18,19]. Many genes defined by these mutants are potential regulators of gene expression at
epigenetic, transcriptional or post-transcriptional levels [20]. The
usu1 mutant, also isolated as curly leaf, is defective in a polycomb-
group gene involved in chromatin remodeling while the ICL2
encodes the putative catalytic subunit of the eukaryotic type DNA
polymerase α [20,21]. Consistent with a role of microRNA in
regulating leaf development, microRNA related mutations can
also lead to leaf curling phenotypes. For instance, HASTY/ICU3
codes for a member of the importin-β family nucleocytoplasmic
transport receptors that might be involved in the nuclear export of
microRNAs [22]. Genetically dominant curly leaf mutant have
also been reported. Gain-of-function mutations in Class III HD-
ZIP transcription factor gene AhHB15, alternatively known as
ICU4 or CORONA, caused upwardly curly leaves due to
mutations in its microRNA processing site [23]. Phytohormone
auxin also participates in the regulation of abaxial-adaxial polarity.
The over-expression of Arabidopsis IAMT1, which encodes an
indole-3-acetic acid (IAA) carboxyl methyltransferase that
presumably converts active auxin IAA to inactive methyl-IAA ester,
cause dramatic hyponastic leaf phenotypes [24]. Moreover,mu-
tations that are impaired in the auxin induced degradation of
AUX/IAA proteins could also lead to curly leaves [25–27]. The
fact that numerous factors have been shown to be able to modulate
leaf curvature suggests that higher plants utilize complex
regulatory schemes to ensure the proper development of leaves.

Taking a genetic approach, we have identified and character-
ized factors that are involved in the regulation of plant leaf and
shoot development [28–29]. Here, we report the identification of
two upwardly curly leaf mutants in Arabidopsis, designated abs5-
1D (abnormal shoot-1-Dominant) and abs7-1D. We cloned ABS5
and ABS7 and demonstrated that ABS5 encodes a bHLH
transcription factor bHLH30 and ABS7 encodes a MYB
transcription factor MYB101, and both ABS5 and ABS7 were
targeted into the nucleus. Interestingly, auxin homeostasis and leaf
venation development were altered in abs5-1D. We assayed
potential transcriptional activation activities of ABS5 and ABS7
and found that ABS7 is capable of activating reporter gene
expression, while ABS5 alone is not. We further showed that the
expression of ABS5 or ABS7 specifically in the epidermis was
sufficient to cause leaf curvature similar to those of abs5-1D and
abs7-1D, reconfirming the importance of epidermis in regulating
leaf development. Although the phenotypes of abs5-1D and abs7-
1D were results of ectopically expressed genes, our work do
make the utilities of gain-of-function genetic approaches in
uncovering potential regulators of plant development and these
two genes may be exploited in the future for generating curly leaf
traits when desired.

Results

The isolation of a dominant curly leaf mutant, abs5-1D

We are interested in the regulatory schemes that ensure the
proper development of plant leaves and have carried out genetic
screens for mutants with altered leaf and shoot morphologies [28–
30]. Through screening activation-tagged Arabidopsis mutant
pools, we identified a dominant leaf development mutant and
designated it abnormal shoot-5-1D (abs5-1D; D for dominant)
(Figure 1A). The most prominent phenotype of abs5-1D is the
upward curling of leaf margins, in contrast to the slightly
downward curvature usually observed in wild type (Figure 1A–
B). Examination of the transverse sections of the eighth rosette
leaves from five-week-old wild type and abs5-1D plants confirmed
our visual observations (Figure 1B–C). Closer examination of leaf
anatomy revealed that although the general arrangements of the
palisade and spongy mesophyll cells are not grossly changed in
abs5-1D, the number of cells composing the vascular bundles were
increased in abs5-1D compared with that of wild type (Figure 1D–
E). Moreover, there were also defects associated with floral
development in abs5-1D, namely an increase in the number of
secondary inflorescences (Table 1). Taken together, these ob-
servations suggest that the mutation in abs5-1D leads to pleiotropic
developmental defects.

The up-regulation of At1g68810 causes abs5-1D
phenotypes

Since abs5-1D was isolated from activation-tagged T-DNA
mutant pools, we tested whether abs5-1D phenotypes co-
segregated with T-DNA insertion(s). Southern blot analysis of 16
F2 progenies from a cross between abs5-1D and wild type showed
that a single T-DNA insertion was detected in all the plants
showing an abs5-1D-like phenotype, indicating a close linkage
between the abs5-1D mutation and the T-DNA insertion
(Figure 2A). We next recovered the plant genomic sequences
flanking the T-DNA insertion site via plasmid rescue. Blast search
against the Arabidopsis whole genome sequences revealed that the
activation T-DNA was inserted in the intergenic region between
genes At1g68800 and At1g68810 (Figure 2B). The T-DNA right
border was 204 bp upstream of the At1g68810 start codon. In

Figure 1. Phenotypes of abs5-1D. A. Leaf rosettes of five-week-old
wild type and abs5-1D mutant. To have a clear view of the rosette
leaves, the inflorescence stems were removed prior to photographing.
B–C. Overview of the transverse sections of the eighth rosette leaf from
three-week-old wild type (B) and abs5-1D (C). Bars: 500 μm. D–E. 
Transverse sections of the mid-vein regions of wild type (D) and abs5-
1D (E) leaf. Bars: 50 μm.
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addition, northern blot analysis showed that the accumulation of At1g68810 transcripts was greatly increased in abs5-1D compared to that of wild type (Figure 2C). To confirm that the over-expression of At1g68810 led to the abs5-1D phenotypes, a vector harboring a full-length cDNA of At1g68810 under the control of the constitutive cauliflower mosaic virus 3SS promoter was constructed and transformed into wild type Arabidopsis. Independent transgenic lines recapitulated the leaf curling up phenotypes of abs5-1D in T1 and T2 generations (Figure 2D). The up-regulation of At1g68810 in these over-expression (OE) lines was confirmed by semi-quantitative RT-PCR (Figure 2E). Moreover, At1g68810 OE lines also showed increased secondary inflorescence numbers (Table 1). Taken together, these data established that the developmental phenotypes associated with abs5-1D mutation are due to the enhanced expression of At1g68810 and ABS5 gene is At1g68810. At1g68810 was previously identified as TM05-LIKE1 (T5L1) and was implicated in the regulation of vascular tissue development [31].

**ABS5/T5L1 encodes a putative bHLH transcription factor**

ABS5 is annotated to encode a protein of 368 amino acids and protein sequence analysis revealed that ABS5 is likely a putative transcription factor belonging to the basic helix-loop-helix (bHLH) family [32]. In Arabidopsis, there are at least 147 members in the bHLH family and ABS5/T5L1 was previously annotated as bHLH390 [32].

As an initial attempt to understand the function of **ABS5/T5L1**, we examined its tissue expression profile via semi-quantitative RT-PCR with cDNAs obtained from various wild type Arabidopsis tissues. Figure 3A shows that ABS5 transcripts accumulated in all tissues examined. **ABS5/T5L1** expression was relatively lower in aerial part of two-week-old seedlings and older rosette leaves but is highly expressed in roots and stems (Figure 3A). We next explored the sub-cellular localization of ABS5/T5L1 protein. Vectors expressing eGFP alone or the ABS5-GFP fusion protein under the control of 35S promoter were used to transform wild type leaf protoplasts. Nuclei of protoplasts were labeled via staining with the fluorescent dye Hoechst33342 used to transform wild type leaf protoplasts. Nuclei of protoplasts ABS5-GFP fusion protein under the control of 35S promoter were (Figure 3A). We next explored the sub-cellular localization of rosette leaves but is highly expressed in roots and stems relatively lower in aerial part of two-week-old seedlings and older protoplasts expressing signals in both the cytosol and the nucleus (Figure 3B). In contrast, Hoechst33342 staining (Figure 3B). These data show that ABS5/T5L1 resides in the nucleus, consistent with its potential function as a transcription factor.

**ABS5/T5L1 over-expression alters auxin homeostasis and cotyledon vein patterns**

Given that auxin plays a key role in leaf morphogenesis we next examined whether auxin homeostasis is altered in abs5-1D. The expression patterns of the synthetic DR5::GUS reporter gene were used to deduce the distributions of auxin maxima [34]. In wild type background, the strongest DR5::GUS signals coincided with the positions of the hydathodes in cotyledons and the first true leaves of two-week-old seedlings (Figure 4A–B). However, in abs5-1D/+ heterozygous background, DR5::GUS activities were less restricted but more evenly distributed along the entire leaf margin compared to that of wild type (Figure 4C–D). As illustrated in Figure 4E–G, stronger and more diffused GUS signals were also found in leaf marginal areas in transgenic lines over-expressing ABS5/T5L1 in DR5::GUS background. These observations led us to investigate whether other auxin related processes are also disturbed in abs5-1D. Previous studies have implicated that both the initiation and differentiation of vascular strands are regulated by auxin transport and signaling in leaves [35,36]. Since Arabidopsis cotyledons display simple and predictable patterns of vasculature development, we compared mature cotyledon vein patterns of wild type and abs5-1D. Under our growth conditions, wild type cotyledons predominantly displayed two, three or four areoles (45.1%, 40.9% and 13.8%, respectively) (Table 2; Figure 4I–J). The proportions of cotyledons with four or more areoles were increased while those with two or three areoles were decreased in abs5-1D (Table 2; Figure 4I–J). Notably, 6.8% of abs5-1D cotyledons developed veins with five areoles, which is usually not seen in wild type (Figure 4I–J). In addition, in abs5-1D cotyledons with three or four areoles, vein patterns were usually more complex than those of wild type, due to the presence of multiple free-ending tertiary veins (Figure 4J). Taken together, these data suggested that auxin homeostasis and vascular development are likely perturbed by the abs5-1D mutation.

### Table 1: Comparison of the average number of secondary inflorescences of wild type, abs5-1D and ABSS/T5L1 OE lines.

| Genotype | Average Number of Secondary Inflorescences | Number of Plants Scored |
|----------|------------------------------------------|--------------------------|
| WT       | 3.44±0.11                                | 25                       |
| abs5-1D  | 4.60±0.14**                              | 25                       |
| ABSS OE-5| 4.64±0.15**                              | 25                       |
| ABSS OE-10| 5.00±0.17**                             | 25                       |

Data were presented in the form of mean± standard error (SE). Differences between wild type and each of the mutant lines were evaluated by a p-value generated by one-sided t-test (**: p<0.01). doi:10.1371/journal.pone.0107637.t001

The identification of a second dominant curly leaf mutant, abs7-1D

During the course of our work, we isolated another curly leaf mutant, which was designated abs7-1D, also from our activation tagging T-DNA mutant pools (Figure 5A). Overall abs7-1D displayed upwardly curly leaf phenotypes that were reminiscent of abs5-1D (Figure 5A–B). However, there are several distinctions between the two mutants. First, the overall plant stature of homozygous abs7-1D was considerably smaller than that of wild type while the size of abs5-1D is comparable to that of wild type (Figure 5C). Second, the timing of leaf curling is different in abs5-1D and abs7-1D (Figure 5C–I). In abs5-1D, the upward leaf curling was more obvious in newly emerged young leaves while old leaves were only slightly curled up in marginal areas (Figure 5E,H). On the contrary, in abs7-1D young leaves at the center of the rosette were not curled up, the upwardly curling leaf phenotype was more conspicuous in mature leaves in abs7-1D.
To understand the cellular basis of the "curly leaf" phenotypes in abs5-1D and abs7-1D, we measured the average number and length of abaxial and adaxial epidermal cells in wild type and mutants at the developmental stages when their "curly leaf" phenotypes were most obvious (Figure S1). Statistical analysis of the measurements showed that the number of epidermal cells on either the adaxial or the abaxial side of the leaves was about the same in abs5-1D or abs7-1D compared to that of wild type, suggesting epidermal cell proliferation was not grossly altered in abs5-1D or abs7-1D (Figure S1A, C). On the other hand, although the average length of abaxial epidermal cells of abs5-1D or abs7-1D was comparable to that of wild type, the average length of adaxial epidermal cells of abs5-1D or abs7-1D was significantly reduced compared to that of wild type (Figure S1B, D). These observations suggest that the "upwardly curly leaf" phenotypes in abs5-1D or abs7-1D is probably due to more restricted expansion of leaf epidermal cells on the adaxial side.

The leaf phenotypes of abs7-1D co-segregated with T-DNA insertion(s) (Figure 5C,F,I). To understand the cellular basis of the "curly leaf" phenotypes in abs5-1D and abs7-1D, we measured the average number and length of abaxial and adaxial epidermal cells in wild type and mutants at the developmental stages when their "curly leaf" phenotypes were most obvious (Figure S1). Statistical analysis of the measurements showed that the number of epidermal cells on either the adaxial or the abaxial side of the leaves was about the same in abs5-1D or abs7-1D compared to that of wild type, suggesting epidermal cell proliferation was not grossly altered in abs5-1D or abs7-1D (Figure S1A, C). On the other hand, although the average length of abaxial epidermal cells of abs5-1D or abs7-1D was comparable to that of wild type, the average length of adaxial epidermal cells of abs5-1D or abs7-1D was significantly reduced compared to that of wild type (Figure S1B, D). These observations suggest that the "upwardly curly leaf" phenotypes in abs5-1D or abs7-1D is probably due to more restricted expansion of leaf epidermal cells on the adaxial side.

The leaf phenotypes of abs7-1D co-segregated with T-DNA insertion(s) (Figure 6A). Through plasmid rescue, we identified a T-DNA insertion 102 bp upstream of the start codon of At2g32460 (Figure 6B). Given the dominant nature of abs7-1D, we tested whether the over-expression of At2g32460 was the cause for curly leaves in abs7-1D. Figure 6C shows that independent At2g32460 OE lines phenocopied abs7-1D and the up-regulations of At2g32460 in these lines were confirmed via semi-quantitative RT-PCR (Figure 6D). These results indicate that enhanced expression of At2g32460 underlines the leaf curling up phenotypes of abs7-1D and ABS7 is At2g32460. ABS7 encodes a member of the Arabidopsis MYB family transcription factors and was designated MYB101 [37]. Phylogenetic studies have shown that ABS7/MYB101 and four other MYB transcription factors (MYB33, MYB65, MYB97 and MYB120) belong to a small family called the GAMYBs [37]. We next analyzed the accumulation of ABS7/MYB101 transcripts in different Arabidopsis tissues. As shown in Figure 6E, ABS7/MYB101 transcripts were only detected in flowers and siliques by semi-quantitative RT-PCR. This is consistent with previous finding that ABS7/MYB101 is highly expressed in seeds and floral tissues [38]. Consistent with its identity as a transcription factor, ABS7-GFP localized to the nucleus in protoplast transient expression assays (Figure 6F). These results suggested that although ABS7/
MYB101 is not normally expressed in leaves, it is able to change leaf morphology when artificially over-expressed. The isolation of loss-of-function mutations in ABS5/T5L1 and ABS7/MYB101

To further examine the roles that ABS5/T5L1 and ABS7/MYB101 play in plant development, we sought for loss-of-function alleles of ABS5/T5L1 and ABS7/MYB101. A transposon tagged line (SM_3_20727) and a T-DNA insertional line (SALK_149918) were obtained from ABRC for ABS5/T5L1 and ABS7/MYB101, respectively [39,40]. PCR and sequencing analysis confirmed that the transposon was inserted in the 5’ untranslated region (UTR) of ABS5/T5L1, 19 bp upstream of its start codon, and the homozygous line was named abs5-1 (Figure S2A–B). Semi-quantitative RT-PCR analysis showed that the accumulation of ABS5/T5L1 transcripts was reduced in abs5-1 (Figure S2C). Under our growth conditions, we did not observe major developmental abnormalities with abs5-1, suggesting that the partial loss of ABS5/T5L1 is not detrimental to plant growth (Figure S2D).

For the putative ABS7/MYB101 knockout line SALK_149918, T-DNA was confirmed to be inserted in the second exon of ABS7/MYB101, 1408 bp downstream of the start codon, and the homozygous line was named abs7-1 (Figure S3A–B). Full-length ABS7/MYB101 transcripts were not detected in abs7-1 (Figure S3C). However, abs7-1 plants were indistinguishable from wild type plants, suggesting ABS7/MYB101 is dispensable for normal plant growth and development, at least under lab conditions and there might be additional genes sharing redundant functions with ABS7/MYB101 (Figure S3D).

Since the ICU genes are known regulators of leaf curvature [19–23], we next tested whether the “upwardly curly leaf” phenotype in abs5-1D and abs7-1D is related to changes of the expression levels of ICU genes. We compared the accumulation of ICU1, ICU2, ICU3 and ICU4 transcripts in wild type, loss-of-function and activation-tagged lines of ABS5/T5L1 and ABS7/MYB101 using semi-quantitative RT-PCR. No significant changes in the expression levels of any ICU genes were observed in these plants (Figure S4A–B). These data suggested that the over-expression of ABS5/T5L1 or ABS7/MYB101 may influence leaf lamina developments via pathways that are not mediated by ICU1-4 genes.

Trans-activation activity assays of ABS5/T5L1 and ABS7/MYB101

To test whether ABS5/T5L1 and ABS7/MYB101 could function as transcription activators, we carried out trans-activation activity assays in yeast. The open reading frames of ABS5 and ABS7 were fused to the 3’ end of the GAL4 DNA binding domain (BD) to generate pBD-ABS5 and pBD-ABS7 vectors, respectively. The empty vector containing only the GAL4 DNA binding domain served as a negative control and Arabidopsis WRKY33 gene was used as a positive control [41]. Each construct was co-transformed with pGADT7 into yeast strain AH109. The expression of three reporter genes, HIS3, ADE and LucZ were assayed. As expected, all yeast transformants grew on SD/-Trp-Leu medium (Figure 7A). However, only yeast transformants...
AB5S and AB57 Regulate Leaf Curvature

Epidermal expression of AB5S/T5L1 or AB57/MYB101 is sufficient to cause leaf curvature

Epidermis is an integral part of plant leaf and has been shown to regulate many aspects of plant growth and development [42]. To investigate the potential impact of AB5S/T5L1 or AB57/MYB101 over-expression in the epidermis, fusion constructs with AB5S/T5L1 or AB57/MYB101 cDNA under the control of epidermal layer specific Arabidopsis Meristem Layer 1 promoter (PAtML1) were generated [43]. Transgenic lines harboring PAtML1::GFP were used to verify the epidermis-specific expression profile (Figure 8A). Interestingly, multiple lines that express PAtML1::AB55 showed the curly leaf phenotype, similar to that observed in abs5-1D (Figure 8B). Moreover, transgenic lines with epidermal-specific expressions of AB5S/MYB101 phenocopied abs5-1D (Figure 8C). Next, we tested whether the epidermal-specific expression of AB5S/T5L1 is sufficient to alter auxin homeostasis. We transformed DR5::GUS plants with PAtML1::ABS5 construct and assayed GUS activities in independent transgenic lines that exhibited the upward curling leaf phenotype. Figure 8D-F showed that auxin distributions as indicated by the expressions of DR5::GUS were increased in PAtML1::ABS5 lines in a way that is similar to what was found in abs5-1D or the AB5S/T5L1 OE lines. Taken together, these results indicate that specific over-expression AB5S/T5L1 or AB57/MYB101 in the epidermal layer alone was sufficient to alter leaf development, reinforcing the idea that the epidermis plays an important role in plant organ shape determination.

Discussion

Leaf development is one of the fundamental processes ensuring robust phototrophic growth for higher plants and mechanisms are in place to coordinate the establishment of leaf polarities [6]. In this study, we report the isolation of two dominant leaf polarity mutants, abs5-1D and abs5-1D, both displayed an “upwardly curly leaf” phenotype (Figures 1 and 5).

We established that the over-expression of a bHLH transcription factor AB55/T5L1 was responsible for the intriguing curly leaf phenotype in abs5-1D. Furthermore, we found that the homeostasis of phytohormone auxin, as indicated by the expression pattern of auxin reporter gene DR5::GUS, was also disturbed in abs5-1D mutants (Figure 4A-G). Auxin is a key regulator of leaf morphogenesis and vasculature development [44]. A number of Arabidopsis auxin signaling mutants display “curled up” leaf phenotype similar to that of abs5-1D. For example, bodenlos (bdl) mutant, the gain-of-function mutant allele of the IAA12 gene, showed a leaf curling up phenotype [45]. Mutation in bdl allele dampens the auxin induced degradation of IAA12 protein via the ubiquitin-proteasome pathway [45]. Interestingly, mutations in several other IAA genes that have comparable impact on IAA proteins, including in IAA3 (sby2-2), and IAA17 (axr3-3, axr3-1 and icu6) also give rise to similar defects in leaf morphology [46,25,26].
ABS5 and ABS7 Regulate Leaf Curvature

Consistent with disturbed auxin homeostasis and auxin’s involvement in leaf vasculature development, we also determined that abs5-1D has abnormal cotyledon venation patterns (Figures 1 and 4). We found that both the complexity and the number of free ending veins were increased in abs5-1D cotyledons compared with those of wild type (Figure 4H–J). During leaf vasculature development, the canalization hypothesis indicates that the convergence of auxin polar transport to the tip of the developing leaf primordia and the subsequent inward flow of auxin is critical for the establishment of leaf vasculature [35,36,44]. The flow of auxin defines the expression domains of auxin efflux carrier PIN1, and the polarized PIN1 localization further enhances the polar transport of auxin [36]. In developing young leaves, both the differentiation of procambial cells and the formation of new vascular strands depend on auxin polar transport via PIN1 [36,44]. On the other hand, genetic screens for mutants defective in vein patterns have also identified genes involved in auxin signaling [47,48]. It is possible that altered auxin distribution in abs5-1D affects the polar auxin transport process, which in turn leads to abnormalities in vasculature development.

Previous studies have shown that ABS5/T5L1 is the closest homolog of TMO5, a direct target of MONOPTEROS/AUXIN RESPONSE FACTOR5 (MP/ARF5) [31]. Both TMO5 and T5L1 are expressed in the vasculature of the embryo and in the xylem precursor cells in the root meristem [31]. The tmo5 t5l1 double mutants are impaired in periclinal vascular cell divisions and developed less vascular tissue in the roots [31]. Higher order mutants of genes in the TMO5 clade showed more severe vascular tissue defects [31]. Our observations that gain-of-function abs5-1D mutants developed more complex leaf vascular tissues are in line with this report, suggesting that ABS5/T5L1 may promote the formation of veins. TMO5 clade proteins form heterodimers with LONESOME HIGHWAY (LHW) clade bHLH transcription factors [31]. When ectopically expressed, the TMO5/LHW dimer is able to induce periclinal cell divisions in non-vascular cells [31]. We showed that the curly leaf phenotype in abs5-1D is probably due to mis-coordinated growth of the adaxial and abaxial sides of the leaf. Since ABS5/T5L1 alone is not able to activate reporter gene expression, its activity may depend on the availability of its partners, such as the LHW proteins. One possibility for the “curled up” leaf phenotype of abs5-1D might be that the expression domains of LHW proteins in leaves are not evenly distributed on the abaxial and adaxial sides of leaves. Alternatively, there might be additional pathways that regulate differential growth of the adaxial and abaxial sides of leaves.

In this study, we show that ABS7 encodes MYB101, which is a member of a small group of Arabidopsis MYB genes called the GAMYBs [38,49,50]. First identified in barley, GAMYB was named so for its involvements in phytohormone gibberellin (GA) mediated processes [49]. Studies in cereals and Arabidopsis have shown that GAMYBs are essential for GA-mediated programmed cell death in aleurone tissues during seed germination and in tapetum during anther maturation [50–53]. A recent report

### Table 2. Quantification of cotyledon vein patterns in wild type and abs5-1D.

| Genotype | Total* | One Areole | Two Areoles | Three Areoles | Four Areoles | Five Areoles |
|----------|--------|------------|-------------|---------------|--------------|--------------|
| WT       | 423    | 1 (0.2%)   | 191 (45.1%) | 173 (40.9%)   | 58 (13.8%)   | N.A.         |
| abs5-1D  | 412    | N.A.       | 22 (5.3%)   | 112 (27.2%)   | 250 (60.7%)  | 28 (6.8%)    |

Ten-day-old wild type or abs5-1D seedlings were de-colored with 70% ethanol and examined under a Nikon SMZ1500 stereoscope. Cotyledon vein patterns were scored based on the number of areoles formed.

*total numbers of cotyledons examined for genotype.

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Figure 5. Isolation of abs7-1D. A. Phenotypes of representative two-week-old wild type and abs7-1D seedlings. B. Comparison of individual leaves detached from plants shown in (A). From left to right are the two cotyledons and the first four rosette leaves (Upper panel: wild type; Lower panel: abs7-1D). C. Comparison of the overall plant statues of three-week-old wild type, abs5-1D and abs7-1D. D-F. Transverse sections of the ninth rosette leaves of three-week-old wild type (D), abs5-1D (E) and abs7-1D (F). G-I. Transverse sections of the first rosette leaves of three-week-old wild type (G), abs5-1D (H) and abs7-1D (I).

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showed that \textit{ABS7}/\textit{MYB101}, as well as two other Arabidopsis \textit{GAMYBs}, \textit{MYB97} and \textit{MYB120}, are highly expressed in mature pollen grains and pollen tubes and three genes share redundant functions in regulating proper pollen tube reception [54]. Several Arabidopsis \textit{GAMYBs}, particularly \textit{MYB33} and \textit{MYB65}, are direct targets of \textit{miR159} family microRNAs [50,55]. However, \textit{ABS7}/\textit{MYB101} is not likely to be regulated by \textit{miR159a/b}, because its expression pattern is not changed in any of the \textit{miR159} mutant combinations and the sequence of putative microRNA targeting site in \textit{ABS7}/\textit{MYB101} is slightly different from those of \textit{MYB33} and \textit{MYB65} [50,55]. Interestingly, loss of both \textit{miR159a} and \textit{miR159b} or the over-expression of a mutant form of \textit{MYB33} with an abolished \textit{miR159} targeting site results in a curled-up leaf phenotype that is similar to that of \textit{abs7-1D}, suggesting that
MYB33 and MYB65 might share similar functions with ABS7/MYB101 and these functions are normally suppressed by miR159s [50]. In line with previous findings, we showed that ABS7/MYB101 likely functions as a transcription activator via yeast trans-activation assay [54]. Although normally ABS7/MYB101 transcripts do not accumulate in leaves, our findings showed that mis-expressed ABS7/MYB101 is able to regulate leaf morphology, possibly through the activation of down-stream target genes and the lack of regulation of ABS7/MYB101 transcripts accumulation by miR159 in leaves.

Lastly, we found that epidermal-specific expression of ABS5/T5L1 or ABS7/MYB101 driven by the AtML1 promoter was sufficient to cause upwardly curly leaves and epidermal-specific ABS5/T5L1 expressions can alter leaf auxin homeostasis (Figure 8). Our findings are consistent with previous studies that the leaf epidermis plays important roles in organ shape determination and plant development [42,56,57]. For example, epidermal-specific expression of brassinosteroid biosynthesis, signaling or inactivating genes are sufficient to promote or restrict the growth of the whole plant [56]. Leaf margin development provides another example of the involvement of epidermis in regulating plant organ morphogenesis [57]. Recent evidence suggests that mesophyll cells are also involved in the epidermal control of leaf development. Arabidopsis ANGUSTIFOLIA3 (AN3) gene, encoding a transcription co-activator, has been identified as a critical mobile factor in coordinating leaf epidermal and mesophyll cell proliferation [58]. AN3 transcripts can only be detected in the mesophyll layer, yet AN3 protein is able to move between the epidermal layer and the mesophyll layer [58]. Retaining AN3 protein in the mesophyll layer failed to complement the leaf development defects in an3 mutant, indicating the inter cell layer movement of AN3 is essential to ensure proper leaf morphogenesis [58]. We did not determine the possibility of inter-cellular mobility for ABS5/T5L1 and ABS7/MYB101. However, our findings show leaf curvature can be manipulated through the epidermis alone and reinforce the notion that the epidermis plays important roles in leaf development.

Materials and Methods

Plant Materials and Growth Conditions

Wild type Arabidopsis and all mutants used in this study are in the Columbia-0 background. Arabidopsis seeds were sown on commercial soil mix (Pindstrup, Denmark) and stratified for two days at 4°C before placed in a growth room maintained at approximately 22°C under continuous illumination (~100 μmol·m⁻²·s⁻¹).

Transposon insertional line SM_3_20727 and T-DNA line SALK_146872C were obtained from the Arabidopsis Biological

Figure 7. Transcriptional activation analysis of ABS5/T5L1 and ABS7/MYB101 in yeast. Yeast strain AH109 was transformed with a negative control vector (pBD), a positive control pBD-WRKY33, pBD-AB5 or pBD-AB57, respectively. Each of the BD vectors was co-transformed with an empty AD vector, pGADT7. A–C. Growth of yeast transformants on the SD/-Trp-Leu medium (A), the SD/-Trp-Leu-His medium plus 5 mM 3-AT (B) or the SD/-Trp-Leu-His-Ade medium (C). D. Activation of the LacZ gene analyzed via filter lifting X-gal assays.
performed as described in [28]. Bright field images and fluorescent signals from Hoechst33342, GFP and chlorophyll autofluorescence were monitored using a Leica DM5000B fluorescent microscope (Leica, Germany).

Yeast Trans-activation Assays

For transcriptional activation activity assays, ORFs of ABS5/T5L1 and ABS7/MYB101 were cloned into the pGBK7 vector (pBD), which contains the GAL4 DNA binding domain, to generate pBD-ABS5 and pBD-ABS7, respectively. The empty pGBK7 vector was used as a negative control and the Arabidopsis WRKY33 gene was included as a positive control [41]. Yeast strain AH109 was used. Each of the BD vectors was co-transformed with an empty AD vector, pGAD17. Yeast transformation and reporter gene activities were assayed according to manufacturer’s instructions (Clontech, USA).

Supporting Information

Figure S1 Statistical analysis of the average number and length of epidermal cells of wild type, abs5-1D and abs7-1D. (TIF)

Figure S2 Identification of a loss-of-function mutant allele of ABS5/T5L1. (TIF)

Figure S3 Identification of a loss-of-function mutant allele of ABS7/MYB101. (TIF)

Figure S4 Accumulation of ICUI1-4 transcripts in wild type, loss-of-function and activation-tagged lines of ABS5/T5L1 and ABS7/MYB101. (TIF)

Table S1 Primers used in this study. (PDF)

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Author Contributions

Conceived and designed the experiments: XL FY. Performed the experiments: RA RW HW SL JS YQ. Analyzed the data: LA. Contributed reagents/materials/analysis tools: LA. Wrote the paper: XL.
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