STM/BP-Like KNOXI Is Uncoupled from ARP in the Regulation of Compound Leaf Development in Medicago truncatula

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INTRODUCTION

KNOTTED-like homeobox (KNOX) genes regulate both embryonic and postembryonic development in plants. KNOX genes fall into two subclasses, Class I KNOX (KNOXI) and Class II KNOX (KNOXII) based on sequence similarity, gene structure, and expression pattern (Hay and Tsiantis, 2010). KNOXI genes, which are evolutionarily close to maize (Zea mays) KN1 (Vollbrecht et al., 1991), are expressed in the shoot apical meristem (SAM) of both monocot and eudicot plants and play crucial roles in the maintenance of SAM and regulation of leaf complexity across vascular plants (Long et al., 1996; Hake et al., 2004; Barkoulas et al., 2008; Hay and Tsiantis, 2010). KNOXII genes display diverse expression patterns, and their function is not clear (Serikawa et al., 1997; Byrne et al., 2002).

The KNOXI gene family from Arabidopsis thaliana consists of SHOOTMERISTEMLESS (STM), BREVIPEDICELLUS (BP)/KNOTTED-like in A. thaliana 1 (KNAT1), KNAT2, and KNAT6 (Lincoln et al., 1984; Long et al., 1996). STM is expressed during early embryogenesis and marks the entire SAM. The loss-of-function stm mutant failed to establish the SAM during embryogenesis (Long et al., 1996; Belles-Boix et al., 2006). BP contributes redundantly with STM to SAM maintenance (Byrne et al., 2002; Venglat et al., 2002), and loss of BP plants showed a mildly dwarfed phenotype. KNAT2 and KNAT6 showed redundant and antagonistic roles with KNOXI genes (Belles-Boix et al., 2006; Ragni et al., 2008). In the SAM, KNOXI genes increase the cytokinin (CK) level by activating the expression of the CK biosynthesis gene ISOPENTENYLTRANSFERASE7 and decrease the gibberellic acid level by inhibiting the GA 2-oxidase1 gene (Sakamoto et al., 2001; Jasinski et al., 2005; Yanai et al., 2005; Bolduc and Hake, 2009). Such high CK–low gibberellic acid conditions are required for the SAM to maintain its activity. Ectopic expression of KNOXI genes significantly altered leaf development (Lincoln et al., 1994; Belles-Boix et al., 2006; Shani et al., 2009). In simple-leafed species, such as Arabidopsis, a lobed leaf margin was observed in transgenic plants overexpressing KNOXI. However, in compound-leafed species, overexpression of KNOXI genes dramatically increased the degree of leaflet reiteration (Hareven et al., 1996; Hay and Tsiantis, 2006).

A MYB transcription factor, ASYMMETRIC LEAVES1 (AS1) in Arabidopsis, ROUGH SHEATH2 (RS2) in maize, and PHANTASTICA (PHAN) in Antirrhinum majus (together known as ARP factors) is a negative regulator of KNOXI genes (Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Guo et al., 2008; Lodha et al., 2013). Leaf forms can be classified into two major types: simple leaves and compound leaves. A simple leaf has a single unit of undivided blade, and a compound leaf consists of multiple discontinuous blades. The ARP-KNOXI regulatory module is well established in simple-leafed species. In Arabidopsis, a mutually exclusive expression pattern is observed between AS1 and STM in the shoot apex. STM represses AS1
expression in presumptive stem cells to maintain the undifferentiated meristematic state (Byrne et al., 2000). Furthermore, ARP proteins act in the leaf to restrict KNOXI, such as BP, to the SAM. The mutually exclusive expression domains of ARP and KNOXI distinguishes leaf founder cell from meristem cell fate in the SAM (Long et al., 1996; Hay and Tsiantis, 2006). By contrast, some compound-leafed species, such as tomato (Solanum lycopersicum) and Cardamine hirsuta, show a reactivation of KNOXI expression during leaf development. An overlap of the expression domains of ARP and KNOXI was observed in these species (Hareven et al., 1996; Bharathan et al., 2002; Hay and Tsiantis, 2006; Champagne et al., 2007; Shani et al., 2009). However, expression analysis of ARP and KNOXI in pea (Pisum sativum), a compound-leafed legume belonging to the large inverted repeat-lacking clade (IRLC), shows they are expressed in complementary domains, like simple-leafed species and unlike tomato (Tattersall et al., 2005).

The lack of KNOXI expression in leaf primordia of the IRLC group indicates that KNOXI genes may not be involved in compound leaf development in these legumes (Hofer et al., 2001; Champagne et al., 2007). Further studies demonstrate that the FLORICAULA/LEAFY (LFY) putative orthologs, pea UNIFOLIATA (UNI) and Medicago truncatula SINGLE LEAFLET1 (SGL1) function in place of KNOXI to regulate compound leaf development (Hofer et al., 1997; Wojciechowski et al., 2004; Champagne et al., 2007; Wang et al., 2008). These findings raise questions on whether the ARP-KNOXI regulatory circuitry is still conserved in IRLC since the developmental process of the compound leaf has shifted away from the KNOXI-mediated module and how the LFY orthologs take over roles in compound leaf patterning in IRLC. However, the lack of KNOXI and ARP loss-of-function mutants in legumes hindered understanding of this distinct genetic regulation mechanism in compound leaf formation. Here, we address these questions by characterizing the Tnt1 retrotransposon-tagged ARP and STM/BP-like KNOXI mutants in the model legume M. truncatula. We show that M. truncatula ARP (PHAN) and STM/BP-like KNOXI genes exhibit conserved functions. However, no genetic interactions between PHAN and STM/BP-like KNOXI genes were observed, suggesting that STM/BP-like KNOXI genes are uncoupled from PHAN. Furthermore, different responses to ectopic expression of KNOXI and SGL1 reveal that KNOXI and SGL1 regulate parallel pathways in leaf development, and SGL1 probably functions in a stage-specific manner in regulating the indeterminate state of developing leaves in M. truncatula. Comparison of these developmental effects also sheds light on possible roles of other regulators in compound leaf patterning.

RESULTS

Identification of a M. truncatula Mutant with Defects in Leaf Development

To identify additional regulators that control leaf development, a M. truncatula mutant population (~13,000 independent lines) generated by tobacco (Nicotiana tabacum) Tnt1 retrotransposon insertional mutagenesis (Taddege et al., 2008) was screened. One mutant line, NF2810, with obvious changes in leaf morphology was identified. Compared with the wild type, the mutant plant displayed downward-curved leaves with pronounced serrations on the leaf margin and needle-like stipules (Figures 1A to 1D) and occasionally produced leaves with ectopic leaflets and asymmetric lateral leaflets (Supplemental Figures 1A and 1B). In addition, mutant leaves exhibited elongated serrated tips at the margin area (Supplemental Figures 1C and 1D). At the reproductive stage, the petiole length in the mutant was significantly decreased, suggesting a compression of the leaf proximal-distal axis (Figures 1C and 1E). However, the length of rachis in the mutant did not show significant change (Figure 1F). Scanning electron microscopy analysis showed that the length of petiole epidermal cells in the mutant was drastically reduced compared with that in the wild type, suggesting that the reduced cell length accounted for the shortened petiole in the mutant (Figures 1G and 1H). The length of rachis epidermal cells was indistinguishable between the mutant and the wild type, but the cells in the mutant appeared thinner (Figures 1I and 1J). In addition, leaf epidermal cells were examined, and no obvious difference was observed between the mutant and wild type (Supplemental Figures 1E to 1H). Anatomical analysis revealed that 30% of midveins of the mutant leaf displayed ectopic vascular bundles (n = 10). Moreover, the phloem was enlarged on the abaxial side of the leaf of the mutant compared with that in the wild type (Figures 1K and 1L). Expanded phloem was also observed in the petiole of the mutant, suggesting that mutant leaves were partially abaxialized (Supplemental Figures 1I and 1J).

YABBY and HD-ZIP III gene families are implicated in the establishment of abaxial and adaxial domains, respectively (Moon and Hake, 2011; Townsley and Sinha, 2012). To further evaluate the defects in leaf polarity of the mutant, the expression levels of YABBY and HD-ZIP III genes were analyzed (Supplemental Figure 2 and Supplemental Data Sets 1 and 2). Transcript levels of HD-ZIP III gene members varied between the wild type and mutant, but most YABBY genes were upregulated in the mutant, supporting the observation of abaxialized leaves in the mutant. In addition to the defects in leaf morphology, flower development in the mutant was also affected (Supplemental Figures 3A to 3J). The width of floral organs, such as the vexillum, was reduced in the mutant (Supplemental Figure 3K). The flowers were able to develop into seedpods, but the number of pods produced in the mutant was fewer than the wild type (Supplemental Figure 3L).

The Mutant Phenotype Is Associated With an ARP Ortholog in M. truncatula

To identify the gene associated with the mutant phenotype, thermal asymmetric interlaced-PCR was performed to recover the flanking sequences of Tnt1 retrotransposon from the mutant. Based on PCR genotyping results, one flanking sequence segregating with the mutant phenotype was identified. A full-length genomic sequence was obtained using this flanking sequence to search against the M. truncatula genomic sequences in the National Center for Biotechnology Information database. The full-length coding sequence of 1080 nucleotides was obtained by RT-PCR. Alignment between the coding sequence (CDS) and the genomic sequence revealed that one intron is located at 5' untranslated region of the gene (Figure 2A). Genomic PCR analysis was performed to detect the insertion site of the Tnt1 retrotransposon. While an ~1.1-kb PCR fragment was amplified in the wild type, an ~6.4-kb PCR fragment...
containing a single Tnt1 retrotransposon (~5.3 kb) was amplified in the mutant (Figure 2B). Sequence comparison revealed that the Tnt1 retrotransposon was inserted into the 3' end of the second exon of this gene (Figure 2A; Supplemental Table 1). RT-PCR analysis showed that the full-length transcripts were interrupted in the mutant (Figure 2C). Quantitative RT-PCR (qRT-PCR) analysis was performed to measure gene expression levels using primers designed to amplify fragments upstream and downstream of the Tnt1 insertion site. The results showed that transcript levels of this gene were very low (<9%) in the mutant, compared with that in the wild type (Supplemental Figure 4). Phylogenetic analysis revealed that this gene is a member of the MYB-domain protein family and evolutionarily closer to the pea ARP gene CRISPA (CR1), which is the ortholog of A. majus PHAN (Figure 2D; Supplemental Data Set 3) (Waites et al., 1998). BLAST analysis was performed against the M. truncatula genome sequence database (version Mt4.0V1,
Amino acid sequence comparison revealed high sequence similarity between this protein and ARP proteins from other plant species (Supplemental Figure 5). Based on these data, this gene is identified as the putative ARP ortholog in *M. truncatula* and named PHAN. A Tnt1-tagged mutant of PHAN was recently reported (Ge et al., 2014). Because the Tnt1 insertion is located in the same position in the PHAN protein sequences of the mutants, the PHAN allele (Ge et al., 2014) is identical to the one in this study.

To further confirm that the Tnt1 insertion in PHAN is responsible for the mutant phenotype, PCR reverse genetic screening of DNA pools from the Tnt1-tagged mutant population was performed (Tadege et al., 2008), but it failed to uncover additional alleles of *phan* from ~18,000 mutant lines. To rule out the possibility that the phenotype of *phan* is caused by other unknown mutations, PHAN knockdown plants were generated using RNA interference (PHANRNAi). The expression level of PHAN was dramatically reduced in the PHANRNAi transgenic plants (Figure 2C). The leaf phenotype of the transgenic plants resembled that of *phan* (Figure 2E). Furthermore, a genomic fragment including the promoter and coding sequencing of PHAN was stably introduced into *phan* plants. The *phan* mutant phenotype was fully complemented (Figures 2F and 2G). Collectively, these data confirm that loss of function of PHAN resulted in developmental defects in the mutant. Expression pattern analysis based on the *M. truncatula* Gene Expression Atlas revealed relatively high levels of PHAN in vegetative buds, seeds, and pods (Supplemental Figure 6A). To determine the expression pattern more comprehensively, a PHAN promoter-GUS (β-glucuronidase) reporter gene was constructed and introduced into wild-type plants. GUS expression was detected in almost all organs (Supplemental Figures 6B to 6J), indicating broad roles of PHAN in the development of *M. truncatula*.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Molecular Cloning of PHAN in *M. truncatula.  

(A) PHAN gene structure and Tnt1 insertion site. Boxes represent exons and lines represent intron. Vertical arrow marks the location of Tnt1 retrotransposon in the *phan* mutant. Horizontal arrows mark the gene fragment used for the construction of the PHANRNAi transgene.  
(B) PCR identification of the *phan* mutant. An ~6.4-kb PCR fragment containing a single Tnt1 retrotransposon (~5.3 kb) was amplified in the mutant. Wild-type and PHANRNAi transgenic plants were used as controls.  
(C) RT-PCR analysis of full-length transcripts of PHAN transcripts in vegetative buds of the *phan* mutant and PHANRNAi transgenic plants. ACTIN was used as control.  
(D) Phylogenetic analysis of PHAN and ARP genes from other species. Alignments used to generate the phylogeny are presented in Supplemental Data Set 3.  
(E) Leaves of PHANRNAi transgenic plants mimic the phenotype of *phan*.  
(F) and (G) Genetic complementation of *phan*. Representative leaves of *phan* and *phan* transformed with the PHANpro:PHAN construct are shown in (F) and (G), respectively. Bars = 1 cm in (E) to (G).  
[See online article for color version of this figure.]
It has been shown that overexpression of AS1 in Arabidopsis generated elongated and downwardly curling leaves (Theodoris et al., 2003), and overexpression of SI-PHAN in tomato and tobacco produced ectopic blade outgrowth (Zoulias et al., 2012). To investigate the effects of ectopic PHAN expression, a cauliflower mosaic virus (CaMV) 3SS promoter-driven PHAN transgene was introduced into the wild type. The transgenic plants did not display any obvious change in leaf morphology, suggesting that increased ARP activity results in different developmental responses among species (Supplemental Figures 6K and 6L).

**STM/BP-Like KNOXI Activity Is Sufficient for Increasing Leaf Complexity**

In a previous report, three class I (KNOX) and three class II (KNOXI) KNOX genes were isolated in *M. truncatula* (Di Giacomo et al., 2008). By BLAST searching the *M. truncatula* genome sequence database (version Mt4.0V1, www.jcvi.org/medicago/), two additional KNOXI genes and two additional KNOXII genes were found. Phylogenetic analysis revealed that KNOX1 and KNOX6 were STM-like class I genes; KNOX2 was a BP-like class I gene; and KNOX7 and KNOX8 were KNOTTIN2/6-like class I genes (Supplemental Figure 7). In addition, Expression Atlas analysis showed that KNOX1, 2, and 6 genes displayed similar expression patterns, and the patterns are different from that of KNOX7 and KNOX8 (Supplemental Figure 8). To start with, we focused on KNOX1, KNOX2, and KNOX6 because the conserved regulatory module was reported mainly between STM/BP-like KNOXI genes and ARP (Kim et al., 2003b; Luo et al., 2005; Tattersall et al., 2005; Hay and Tsiantis, 2006). Previous studies showed that ectopic expression of KNOXI genes in compound-leaved species results in a dramatic increase in leaf complexity through promoting CK biosynthesis (Hareven et al., 1996; Jasinski et al., 2005; Yanai et al., 2005; Champagne et al., 2007). To investigate whether KNOXI genes play such roles in *M. truncatula*, KNOX1, KNOX2, and KNOX6, under control of the constitutive CaMV 3SS promoter, were introduced into the wild type. One of the KNOXII genes, KNOX4, was also overexpressed as a control. Transgenic plants (OX-KNOXI) with increased expression levels of STM/BP-like KNOXI genes exhibited a similar reiteration of higher order leaflets along elongated petiolules, and variations in leaflet number and shape were also observed (Figures 3A to 3F). These observations demonstrate that the ectopic STM/BP-like KNOXI activity is sufficient for increasing leaf complexity in *M. truncatula*. The transgenic plants overexpressing KNOX4 did not show obvious changes in leaf morphology, suggesting distinct functional roles between KNOXI and KNOXII genes (Figure 3G).

**STM/BP-Like KNOXI Genes Are Not under Negative Control of PHAN**

To assess if PHAN has conserved function, the PHAN coding sequence under control of the CaMV 3SS promoter was introduced into the Arabidopsis as1 mutant. The mutant phenotype was fully complemented, and the ectopic expression of BP in as1 was repressed in the transgenic plants, indicating functional equivalence between *M. truncatula* PHAN and Arabidopsis AS1 (Figures 4A to 4D). Previous studies showed that ARP is a negative regulator of KNOXI genes (Tsiantis et al., 1999; Kim et al., 2003b; Tattersall et al., 2005; Hay and Tsiantis, 2006; Guo et al., 2008). To investigate whether PHAN plays a conserved role to repress the STM/BP-like KNOXI genes in leaves of *M. truncatula*, the expression levels and domains of KNOXI1, 2, and 6 genes were analyzed. qRT-PCR data showed that the expression levels of KNOXI1, 2, and 6 genes remained unchanged in both leaf and petiole in the *phan* mutant, compared with that in the wild type (Figures 4E and 4F). The spatial localization of KNOXI1, 2, and 6 in the wild type and the *phan* mutant was further compared by RNA in situ hybridization analysis. In the wild type, the expression of KNOXI1, 2, and 6 genes was detected in the SAM but excluded from incipient leaf primordia (P0) and developing leaf primordia (Figures 4G, 4I, and 4K). In the *phan* mutant, expression patterns of three KNOXI genes were essentially the same as those in the wild type (Figures 4H, 4J, and 4L), indicating that loss of PHAN did not lead to ectopic expression of the STM/BP-like KNOXI genes.

**Simultaneous Disruption of STM/BP-Like KNOXI Genes Cannot Rescue the phan Phenotype**

To better understand the functions of STM/BP-like KNOXI genes, a PCR reverse genetic screening of the Tnt1-tagged mutant population was performed to isolate relevant loss-of-function mutants. Two, two, and three independent mutant lines were identified for KNOXI1, KNOX2, and KNOX6, respectively. The Tnt1 retrotransposon was detected in the exons of these genes (Figures 5A to 5C; Supplemental Table 1). RT-PCR analysis showed that full-length transcripts of the three genes were abolished in respective homozygous mutant plants (Figure 5D). Transcript levels of KNOXI1, 2, and 6 genes in the mutant alleles were further measured by qRT-PCR using primer pairs designed to amplify fragments upstream and downstream of the Tnt1 insertion sites (Supplemental Figure 9). The results revealed that the expression levels of KNOXI1, 2, and 6 were extremely low (<5%) in the mutants. Loss of function in KNOXI1, KNOX2, or KNOX6 did not lead to obvious defects in SAM maintenance and leaf morphology (Figure 5E). To assess functional redundancy among STM/BP-like KNOXI genes, double mutants and triple mutants were generated. No obvious developmental changes were observed in double mutants derived from different cross combinations among knox1, knox2, and knox6. Simultaneous disruption of three KNOXI genes resulted in semidwarf plants (Supplemental Figures 10A and 10B), suggesting that STM/BP-like KNOXI genes are required for plant vegetative growth. The leaves in the triple mutant were normal, indicating that STM/BP-like KNOXI genes are not involved in compound leaf patterning in *M. truncatula* (Figure 5E).

To further investigate potential genetic interactions between PHAN and STM/BP-like KNOXI genes, double, triple, and quadruple mutants were generated among the relevant mutants. Knockout of any or all KNOXI1, 2, and 6 genes in the *phan* background did not rescue leaf defects, such as downward-curled leaves (Figure 5F) and short petioles (Supplemental Figures 10C and 10D). These genetic evidences in combination with the expression pattern analysis (Figures 4G to 4L) demonstrate that PHAN does not negatively regulate the expression of STM/BP-like KNOXI genes in *M. truncatula*. 
STM/BP-Like KNOXI Genes Do Not Repress PHAN

In Arabidopsis, STM represses AS1 expression in the meristem, and the as1 mutant can partially rescue the stm phenotype (Byrne et al., 2000; Hay et al., 2006). To investigate the possible suppression of STM/BP-like KNOXI genes on PHAN, the spatial and temporal localizations of PHAN were examined by RNA in situ hybridization in the wild type and knox1, 2, and 6 mutants. In the wild type, PHAN mRNA was detected throughout the whole SAM and in both adaxial and abaxial sides of developing leaf primordia (Figures 6A and 6B). Transverse sections through developing leaflets showed that PHAN expression was more localized in the leaflet in the P6 primordium (Figure 6C). In the older P7 primordium, PHAN transcripts were confined to the adaxial side of the medial portion of leaf lamina (Figure 6D). Such adaxial expression of PHAN was also detected in the rachis and petiole at this developmental stage (Figure 6E). The PHAN expression pattern was further assessed in the knox1, 2, and 6 mutant backgrounds. RNA in situ hybridization revealed that expression patterns of PHAN in SAM of the single/double/triple knox1, 2, and 6 mutants (Figures 6G to 6I and 6K) were similar to that of the wild type (Figures 6A and 6B). The expression domain of PHAN was also adaxialized in developing leaf lamina of the knox1 mutants (Figures 6J and 6L), similar to the wild type (Figure 6D). qRT-PCR data further confirmed that the expression levels of PHAN were unchanged in vegetative buds and other plant tissues between the wild type and the knox1, 2, and 6 mutants (Supplemental Figure 11).

These observations suggest that STM/BP-like KNOXI genes do not suppress the expression of PHAN in M. truncatula.

Compromising Auxin Transportation Mediated by SLM1 Does Not Affect STM/BP-Like KNOXI Gene Expression

It has been shown that proper auxin transport regulated by PINFORMED1 (PIN1) and AS1 converge to repress BP expression in Arabidopsis, and the defects of the pin1 mutant are partially rescued in the pin1 bp double mutant (Hay et al., 2006). A PIN1 ortholog, SMOOTH LEAF MARGIN1 (SLM1), has been identified in M. truncatula (Zhou et al., 2011). Auxin distribution is impaired in the slm1 mutant, indicating conserved roles of SLM1 in auxin transport. To determine whether the auxin/SLM1 module is a possible repressor of STM/BP-like KNOXI in M. truncatula, the expression levels of KNOXI genes were analyzed in the slm1 mutant (Figure 7A). The transcript levels of KNOX1, 2, and 6 genes remained essentially unchanged in slm1, indicating that SLM1 is not involved in the repression of KNOXI genes. To further test whether spatial and temporal expression of STM/BP-like KNOXI genes contributed to the defects of slm1, double, triple, and quadruple mutants among slm1 and knoxi mutants were generated (Figure 7B). The phenotype of the slm1-1 knox2-1 double mutant is similar to that of slm1-1. On the other hand, the slm1-1 knox1-1 knox6-1 triple mutant is semidwarf, displaying fewer clustered leaves and shortened stems. Leaflet initiation was severely reduced when all three KNOXI genes were
simultaneously disrupted in slm1-1. These observations suggest that SLM1 and STM/BP-like KNOXI genes probably function redundantly in leaf initiation. However, the defects in floral development and seed production in slm1 could not even be partially rescued by introducing various knoxi mutations (Figure 7C). This result suggests that the slm1 mutant phenotype could not be attributed to the STM/BP-like KNOXI genes in M. truncatula, which is distinctly different from that in Arabidopsis. To further test whether PHAN and SLM1 converge to repress KNOXI expression, the phan slm1 double mutant was generated. Leaves of the phan slm1 double mutant showed an additive phenotype (Figure 7D). The expression levels of KNOX1, 2, and 6 genes did not change in the phan slm1 double mutant (Figure 7E), further confirming that STM/BP-like KNOXI genes are not under the negative regulation of either SLM1 or PHAN.

Ectopic Expression of SGL1 Fails to Increase Leaf Complexity but Mimics phan Phenotype

As previously suggested, the LFY putative ortholog, SGL1, functions in place of KNOXI genes to regulate compound leaf development (Champagne et al., 2007; Wang et al., 2008). To determinate whether increased SGL1 activity is sufficient to increase leaf complexity, SGL1 was overexpressed under control of the CaMV 35S promoter. Overexpression of SGL1 (OX-SGL1) did not produce extra leaflets on rac- chis in transgenic plants (Figure 8A), indicating that increased SGL1 activity does not alter the indeterminacy of developing leaves. It was observed that the leaves of OX-SGL1 plants displayed a downward-curl leaf margin and elongated stipule (Figures 8A and 8B), which partially mimic the phenotype of phan (Figures 1A and 1D). This
observation suggests two possibilities: SGL1 represses PHAN expression in OX-SGL1 transgenic plants, or SGL1 expression is upregulated in the phan mutant. However, qRT-PCR results showed that transcript levels of PHAN (Figure 8C) and SGL1 (Figure 8D) were not altered in OX-SGL1 or phan, indicating no direct interaction between them at the transcriptional level. To further investigate the genetic interaction between PHAN and SGL1, the sgl1 phan double mutant was generated (Figures 8E to 8H; Supplemental Figure 12). The double mutant showed additive defects in leaf phenotype by displaying downward-curled simple leaves. Furthermore, sgl1 phan developed a significantly shorter petiole than the sgl1 or phan single mutant (Figure 8I), suggesting an additive interaction between SGL1 and PHAN in the development of leaf proximal-distal axis.

The KNAT2/6-Like Class I Gene KNOX7 Is Likely Repressed by PHAN

It has been shown that AS1 represses the expression of KNAT2 and KNAT6, in addition to BP in Arabidopsis (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Guo et al., 2008). To test if KNAT2/6-like Class I genes were negatively regulated by PHAN in M. truncatula, qRT-PCR was performed to analyze expression levels of KNOX7.
and KNOX8 in different tissues. A significant increase in the level of KNOX7 transcript was detected in the petiole of the phan mutant, while no change was found in the leaf blade (Figures 9A and 9B). No significant difference was detected in KNOX8 expression in either leaf blade or petiole in phan, compared with the wild type (Figures 9A and 9B). The spatial localizations of KNOX7 were examined by in situ hybridization analysis. The results showed that KNOX7 was expressed in the SAM but excluded from incipient leaf primordia (P0) and developing leaf primordia in the wild type, which is similar to that of KNOX1, 2, and 6 genes. In the phan mutant, however, ectopic expression of KNOX7 was detected in the P0 primordia, and a signal was also detectable in the P1 primordia (Figures 9C to 9F). These data indicate that KNOX7 may be under the negative regulation of PHAN. Future identification and characterization of loss-of-function KNOX7 and KNOX8 mutants are needed to provide more convincing evidence.

DISCUSSION

The Function of ARP Genes Is Species Specific

Loss of function of PHAN in A. majus resulted in abaxialized leaves (Waites et al., 1998). However, Arabidopsis as1 and
maize rs2 mutants did not show obvious defects in leaf polarity (Schneeberger et al., 1998; Serrano-Cartagena et al., 1999). Moreover, in compound-leaved species, various leaf patterning was observed when ARP orthologs were suppressed. Down-regulation of ARP in tomato resulted in altered leaf number and leaf shape (Kim et al., 2003a). A mutation in AS1 in C. hirsuta led to increased leaf number by developing extra leaflets (Hay and Tsiantis, 2006). The pea cri mutant developed abaxialized leaflets and ectopic stipules and had only minor effects on leaf complexity (Tattersall et al., 2005). In M. truncatula, the length of rachis in the phan mutant was similar to that of the wild type, although leaves with longer rachis were observed on some nodes in the phan mutant (Ge et al., 2014). The most obvious defects displayed in the phan mutant were narrow laminae and shortened petioles, which were similar to those in the cri mutant. It should be noted that the Tnt1 insertion is located at the 3' end of the PHAN coding sequence, and the 9% PHAN expression detected in the mutant could result in some residual function. Although both M. truncatula and pea are IRLC members, their leaf complexities are different. M. truncatula has the simplest compound leaf form consisting of only three leaflets with the same identity, while pea possesses a more complex leaf form including highly specialized tendrils. Even so, neither leaflet number nor leaf identity was altered in phan and cri mutants regardless of their leaf complexity. According to these observations, it appears that PHAN orthologs may play limited roles in the elaboration of compound leaves in IRLC species. Furthermore, increased ARP activities led to different phenotypic output among species. Overexpression of AS1 in Arabidopsis or PHAN in M. truncatula did not alter leaf complexity, such as reiteration of lobes or leaflets (Theodoris et al., 2003). However, ectopic expression of PHAN in tomato produced an

Figure 7. The Auxin/SLM1 Module Is Not a Repressor of STM/BP-Like KNOXI Genes.
(A) Expression levels of the KNOXI genes in vegetative buds of the wild type and slm1-1. Transcript levels were measured by qRT-PCR. Values are the means and so of three biological replicates.
(B) Plants of the wild type and mutants derived from different cross combinations among slm1-1 and knoxi mutants.
(C) Seedpod production in the wild type and mutants. The inset shows the flower phenotype of slm1-1 (left) and slm1-1 knox1-1 knox2-1knox6-1 quadruple mutant (right). Arrows point to the fused floral organs. Numbers are presented as means ± so (n = 5).
(D) Leaves of slm1-1 and phan slm1-1 double mutant.
(E) Expression levels of KNOXI genes in vegetative buds of the wild type and phan slm1-1 double mutant. Values are the means and so of three biological replicates.
[See online article for color version of this figure.]
ectopic adaxial domain in leaves, leading to defects in leaf patterning. Thus, these observations imply that ARPs function in a species-specific manner during leaf development. We performed a comparative analysis and summarized ARP expression patterns among species (Supplemental Figure 13).

Although pea is a member of IRLC and *Lotus japonicus* is not, the expression patterns of *CRI* and *Lj-PHANα/b* are similar. Their transcripts were detected at sites of leaf initiation (P0), but excluded from the SAM, forming a mutually exclusive pattern with *KNOXI* genes. To our surprise, *M. truncatula* PHAN has a distinct expression pattern, compared with those in *Arabidopsis*, *C. hirsute*, and other legume species (Byrne et al., 2000; Luo et al., 2005; Tattersall et al., 2005; Hay and Tsiantis, 2006) (Supplemental Figure 13). *M. truncatula PHAN* is diffusely expressed throughout the SAM, showing an overlapping expression domain with *KNOXI* genes. The diffused expression of *M. truncatula PHAN* in the SAM is similar to that in tomato and distinctly different from that in pea, even though *M. truncatula* and pea are evolutionarily close to each other. On the other hand, ARP expression domains are associated with the adaxial side of leaf primordia at early developmental stages in compound-leafed species. For example, *PHAN* displayed adaxial expression in the P3/P4 leaf primordia of tomato (Kim et al., 2003a, 2003b), P1 of *L. japonicus* (Luo et al., 2005), and P3 of pea (Tattersall et al., 2005) (Supplemental Figure 13). In *M. truncatula*, *PHAN* transcripts were detected in both the adaxial and abaxial sides of leaf primordia from P1 to P5. However, *PHAN* mRNA was confined to the adaxial side of the leaf at the late developmental stage (P6/P7). The adaxial expression of *PHAN* during leaf development implies that it may play a role in leaf polarity maintenance, as evidenced by the partially abaxialized leaf and upregulated *YABBY* expression in the *phan* mutant. Taken together, these results suggest that the roles of ARP orthologs vary with species, resulting in different developmental effects among species.

**STM/BP-Like KNOXI Genes Are Uncoupled from PHAN**

In *Arabidopsis*, STM represses *AS1* expression in the SAM, and the *as1* mutant can rescue the *stm* phenotype (Byrne et al., 2000). A similar regulatory relationship was also reported in tomato where Le-T6 is a negative regulator of *PHAN* (Kim et al., 2003b). Moreover, *PHAN* negatively regulates *STM* in *A. majus* (Tsiantis et al., 1999) and *BP* orthologs in *Arabidopsis*, *C. hirsuta*, tomato, and pea (Kim et al., 2003b; Tattersall et al., 2005; Hay and Tsiantis, 2006; Guo et al., 2008). In this study, we investigated the relationship between *PHAN* and *STM/BP-like KNOXI* genes in *M. truncatula*. Unsurprisingly, our results provide evidences that argue against the conserved relationship between *PHAN* and *STM/BP-like KNOXI* genes reported previously. First, the *PHAN* expression level and pattern did not change in single and multiple mutants of both *STM-like* and *BP-like KNOXI* genes. Similarly, the expression levels of *STM/BP-like KNOXI*...
genes did not show significant changes in the phan mutant either. In another report, STM/BP-like KNOXI genes were upregulated slightly (<2-fold) in shoot buds of the phan mutant (Ge et al., 2014). However, such an increase of KNOXI expression in phan is not comparable with the upregulation of BP in as1 (>100-fold) (Hay et al., 2006). Second, genetic evidence showed that knockout of STM/BP-like KNOXI genes failed to rescue the phan phenotype. These results together indicate that it is unlikely that the conserved regulatory circuitry between PHAN and STM/BP-like KNOXI genes exist in M. truncatula. Additionally, previous studies reported that BP expression is repressed by PIN1-mediated auxin transport in Arabidopsis. Loss of BP activity partially rescued the defects in the pin1 mutant (Hay and Tsiantis, 2006). In M. truncatula, however, compromising SLM1 activity did not lead to ectopic expression of the BP-like KNOXI gene. Furthermore, knockout of any or all STM/BP-like KNOXI genes failed to rescue the defects in flower development and seed production in the slm1 mutant. Overall, our data support that STM/BP-like KNOXI genes are uncoupled from PHAN.

The suppression of growth along the proximal-distal axis was observed in arp mutants in several species, and the trait has been associated with ectopic expression of KNOXI genes (Tattersall et al., 2005; Hay et al., 2006; Hay and Tsiantis, 2006). In Arabidopsis, KNAT2 and KNAT6 are under the negative regulation of AS1 (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Guo et al., 2008). Our data also showed that the KNAT2/6-like Class I gene KNOX7 is upregulated in the petiole of the phan mutant, suggesting that ectopic expression of KNOX7 may contribute to the phan phenotype. The expression of KNOX8 remains essentially unchanged between the wild type and phan, indicating that KNOX8 may not be associated with PHAN. The expression domain of KNOX7 overlapped with that of PHAN in the SAM, although KNOX7 was repressed in the incipient leaf primordia. Similar overlapping between PHAN and TKN1 was observed in the SAM of tomato (Kim et al., 2003b). This observation implies that other genes may be involved in the repression of KNOX7, and the regulation mechanism of KNOX7 is not universal in different developmental domains. It has been suggested that KNOXI genes play redundant roles in SAM maintenance in Arabidopsis (Byrne et al., 2002; Belles-Boix et al., 2006), rice (Tsuda et al., 2011), and maize (Bolduc et al., 2013). In this study, simultaneous disruption of three STM/BP-like KNOXI genes did not affect SAM function in M. truncatula. Therefore, future identification of loss-of-function mutants of KNAT2/6-like KNOXI genes will help clarify (1) whether STM/BP-like KNOXI genes and KNAT2/6-like KNOXI genes function redundantly in SAM maintenance; (2) whether KNAT2/6-like KNOXI genes are repressors of PHAN; and (3) how the developmental process of compound leaves in IRLC shifts away from the regulation mechanism mediated by the KNOXI gene family.

Figure 9. Expression of KNAT2/6-like Class I Gene KNOX7 in the Wild Type and the phan Mutant.

(A) and (B) Transcript levels of the KNOX7 and KNOX8 in leaf blade (A) and petiole (B) of the wild type and the phan mutant. Transcript levels were measured by qRT-PCR. Values are the means and SD of three biological replicates. **P < 0.01. (C) to (F) Expression patterns of KNOX7 in the wild type (C) and phan (D). Longitudinal sections of SAM are shown. No expression was detected using a control sense KNOX7 probe (E) and (F). P, plastochron. Bars = 50 μm.

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**KNOXI and LFY Orthologs May Regulate Parallel Pathways in IRLC**

Previous studies have found that LFY orthologs, instead of KNOXI genes, are essential for compound leaf development in IRLC species (Champagne et al., 2007). Loss of function of LFY orthologs in pea and *M. truncatula* resulted in the formation of simple leaves (Hofer et al., 2001; Wang et al., 2008). Moreover, leaf complexity was increased in pea *afila* mutants in which the LFY/UNI expression level was upregulated (Mishra et al., 2009). These observations imply that LFY activities are necessary and sufficient for regulating indeterminacy during leaf development. However, to date, there has been no direct evidence that increased activities of LFY orthologs could prolong the window of morphogenetic plasticity during leaf development in IRLC species. In this study, comparative analysis of OX-KNOXI and OX-SGL1 transgenic plants shed light on the possible roles of the LFY ortholog in leaf development in *M. truncatula*. At present, there are two hypotheses to interpret how the LFY orthologs replace KNOXI to promote indeterminacy in IRLC. One hypothesis is that LFY regulates the same target genes of KNOXI, and the other hypothesis is that LFY and KNOXI regulate parallel pathways (Champagne et al., 2007). Our data show that OX-KNOXI can increase leaf complexity probably through the conserved pathway. Should SGL1 have the same regulatory mechanism as KNOXI, overexpression of SGL1 would be able to promote indeterminacy in developing leaves. However, we were surprised to find that leaf complexity was not changed in OX-SGL1 plants. These observations support the hypothesis that KNOXI and LFY regulate parallel pathways instead of regulating the same targets in IRLC.

The unexpected phenotypic output in response to the ectopic expression of SGL1 raises a new question: Why does OX-SGL1 fail to induce an increase in the degree of leaflet reiteration in *M. truncatula*? In tomato, overexpression of KNOXI in specific domains of developing leaves resulted in different leaf complexity, suggesting that KNOXI functions in a spatial- and temporal-dependent manner (Shani et al., 2009). It is possible that distinct leaf developmental windows exist in IRLC species, thus allowing SGL1 to function in a specific stage or domain. On the other hand, LFY may function with its corregulator UNUSUAL FLORAL ORGANS (UFO) in leaf development of IRLC species. It has been shown that ectopic expression of UFO led to dissected leaves, similar to ectopic expression of KNOXI in Arabidopsis (Ingram et al., 1995; Lee et al., 1997). Moreover, the lobed leaf form requires LFY activity (Lee et al., 1997), indicating that UFO promotes indeterminacy in leaf development in a LFY-dependent manner. In addition, loss of function of STAMINA PISTILLOIDA, the ortholog of UFO in pea, led to a reduction of leaflet number (Taylor et al., 2001). Therefore, UFO orthologs may play an important role in recruiting LFY orthologs into compound leaf patterning in IRLC. Characterization of the loss-of-function UFO ortholog mutant in *M. truncatula* and a comparison of the phenotype and regulatory targets between OX-SGL1 and OX-UFO may help to provide insight into the roles of the UFO/LFY cascade in compound leaf patterning.

**METHODS**

**Plant Materials and Growth Conditions**

*Medicago truncatula* (ecotype R108) plants were grown in the greenhouse at 22°C day/20°C night temperature, 16-h-day/8-h-night photoperiod, and 70 to 80% relative humidity. *Arabidopsis thaliana* (Landsberg erecta) plants were grown in a growth chamber at 20°C and a daylength of 18 h. The *Arabidopsis as1* allele (CS16272, ecotype Landsberg erecta) was obtained from TAIR.

**Gene Constructs**

To make the PHANRNAi construct, a 390-bp fragment of PHAN was PCR amplified from wild-type *M. truncatula* and cloned into the pENTR/D-TOPO cloning vector (Invitrogen), then transferred into the pANDA35K vector by attL × attR recombination reactions (Invitrogen). To make the complementation construct, a 2611-bp PHAN promoter sequence plus 1080-bp PHAN coding sequence was PCR amplified and cloned into the pHGWF57 vector (Karimi et al., 2002). To generate the PHANpro:GUS construct, a 2611-bp promoter region of PHAN was amplified and transferred into the pHGWF57 vector (Karimi et al., 2002) for gene expression pattern analysis. For overexpression of PHAN, KNOX1, KNOX2, KNOX4, KNOX6, and SGL1, the CDS of these genes were amplified and cloned to the pEarley-Gate 100 vector (Earley et al., 2006), respectively. Primer sequences are listed in Supplemental Table 3.

**Stable Plant Transformation**

Gene constructs were introduced into disarmed Agrobacterium tumefaciens using the freezing/heat shock method. Agrobacterium strain EHA105, harboring various vectors, was used for *M. truncatula* transformation as described (Cosson et al., 2006). The numbers of transgenic lines are listed in Supplemental Table 2.

**GUS Staining and Scanning Electron Microscopy Analysis**

GUS activities were histochemically detected as described previously (Zhou et al., 2011). For scanning electron microscopy, leaf tissue samples were first fixed in fixative solution (3% glutaraldehyde in 25 mM phosphate buffer, pH 7.0) overnight, dehydrated in graded ethanol series, and then critical point dried. The Hitachi TM-3000 scanning electron microscope was used for observation of samples at an accelerating voltage of 15 kV.

**RNA Extraction, RT-PCR, Real-Time PCR Analysis, and Statistical Analysis**

Total RNA from different organs, such as leaf, petiole, and shoot apices, was extracted from 4-week-old plants. RT-PCR and real-time PCR analysis were performed as described previously (Zhou et al., 2012). The single-factor ANOVA method was used to estimate if the difference is significant in analysis of gene expression level and plant phenotype.

**RNA in Situ Hybridization**

The 752-, 587-, 632-, 881-, and 777-bp fragments were isolated from the CDS of PHAN, KNOX1, KNOX2, KNOX6, and KNOX7, respectively. The PCR products were labeled with digoxigenin. RNA in situ hybridization was performed on shoot apices of 6-week-old plants as previously described (Zhou et al., 2011).

**Phylogenetic Analysis**

Alignment of multiple sequences was performed using ClustalW2 with default parameters (alignment type, slow; protein weight matrix, gonnet; Gap open, 10; Gap extension, 0.1). The neighbor-joining phylogenetic tree was constructed using the MEGA 6 software suite (http://www.megasoftware.net). The most parsimonious trees with bootstrap values from 1000 trials were shown.
Accession Numbers

Sequence data from this article can be found in the National Center for Biotechnology Information GenBank under the following accession numbers: KNOX1, Medtr2g024390; KNOX2, Medtr1g077080; KNOX3, Medtr1g012860; KNOX4, Medtr5g011070; KNOX5, Medtr3g106400; KNOX6, Medtr5g085880; KNOX7, Medtr5g033720; KNOX8, Medtr1g084060; KNOX9, Medtr4g116545; KNOX10, Medtr2q461240; Mt-PHAN, Medtr7g061550; PHAN, CAA06612; CRI, AAG10600.1; Gm-PHANa, NP_001236839.1; Gm-PHANb, NP_001235251.1; Lj-PHANa, AAX21343.1; Lj-PHANb, AAX21344.1; AS1, NP_181299.1; and AAG10600.1; Gm-PHANa, NP_001236839.1; Gm-PHANb, NP_001235251.1; KNOX7, Medtr5g033720; KNOX8, Medtr1g084060; KNOX9, Medtr4g116545; KNOX10, Medtr2q461240; Mt-PHAN, Medtr7g061550; PHAN, CAA06612; CRI, AAG10600.1; Gm-PHANa, NP_001236839.1; Gm-PHANb, NP_001235251.1; Lj-PHANa, AAX21343.1; Lj-PHANb, AAX21344.1; AS1, NP_181299.1; and Ch-AS1, ABF59515.1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Developmental Defects in Leaves of the phan Mutant.

Supplemental Figure 2. Transcript Levels of YABBY and HD-ZIP III Gene Families in M. truncatula.

Supplemental Figure 3. Developmental Defects in Flower Organs of the phan Mutant.

Supplemental Figure 4. Transcript Levels of PHAN in Wild Type and the phan Mutant.

Supplemental Figure 5. Alignment of ARP Proteins in Different Species.

Supplemental Figure 6. Expression Patterns of PHAN and Over-expression of PHAN.

Supplemental Figure 7. Phylogenetic Analysis of Members of KNOX Gene Family in M. truncatula and Arabidopsis.

Supplemental Figure 8. Expression Patterns of KNOXI Genes.

Supplemental Figure 9. Transcript Levels of KNOX1, 2, and 6 in the Wild Type and knox1, 2, and 6 Mutants.

Supplemental Figure 10. Genetic Interactions among phan and knoxi Mutants.

Supplemental Figure 11. Transcript Levels of PHAN in the Wild Type and knox1, 2, and 6 Mutants.

Supplemental Figure 12. Flower Phenotype of the Wild Type, phan, sgf1, and phan sgf1.

Supplemental Figure 13. Summary of the Expression Patterns of ARP Genes among Species.

Supplemental Table 1. List of Mutant Alleles.

Supplemental Table 2. List of the Number of Transgenic Lines.

Supplemental Table 3. Primers Used in This Study.

Supplemental Data Set 1. Alignments Used to Generate the Phylogeny Presented in Supplemental Figure 2A.

Supplemental Data Set 2. Alignments Used to Generate the Phylogeny Presented in Supplemental Figure 2C.

Supplemental Data Set 3. Sequence Alignment Used to Generate the Phylogeny Presented in Figure 2D.

Supplemental Data Set 4. Sequence Alignment Used to Generate the Phylogeny Presented in Supplemental Figure 7.

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AUTHOR CONTRIBUTIONS

C.Z. and Z.-Y.W. designed the research. C.Z., L.H., G.L., M.C., C.F., and X.C. performed the experiments. C.Z. and Z.-Y.W. wrote the article.

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