PHANTASTICA in compound leaf regulation in legume

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Regulation of compound leaf development by PHANTASTICA in Medicago truncatula

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Plant leaves, simple or compound, initiate as peg-like structures from the peripheral zone of the shoot apical meristem (SAM), which requires KNOXI homeobox transcription factors to maintain its activity. The MYB domain protein encoded by the ASYMMETRIC LEAVES1/ROUGH SHEATH2/PHANTASTICA (ARP) gene, together with other factors, excludes KNOXI gene expression from incipient leaf primordia (P0) to initiate leaves and specify the leaf adaxial identity. However, the regulatory relationship between ARP and KNOXI is more complex in compound-leafed species. Here, we investigated the role of ARP and KNOXI genes in compound leaf development in Medicago truncatula. We show that Medicago phantastica mutant exhibited severe compound leaf defects, including curling and deep serration of leaf margins, shortened petioles, increased rachises, petioles acquiring motor organ characteristics and ectopic development of petiolules. On the other hand, Medicago brevipedicellus (bp) mutant did not exhibit visible compound leaf defects. Our analyses show that the altered petiole development requires ectopic expression of ELONGATED PETIOLULE1 (ELP1), which encodes a LOB domain protein and the distal margin serration requires the auxin efflux protein MtPIN10 in the mtphan mutant.

Key words: Medicago truncatula, PHANTASTICA (PHAN), BREVIPEDICELLUS (BP), ELONGATED PETIOLULE1 (ELP1), MtPIN10, SINGLE LEAFLET1 (SGL1)
Introduction

Plant leaves are the primary photosynthetic organs and play a key role in plant growth and biomass production. Leaves are derived from leaf founder cells developed at the periphery of the shoot apical meristem (SAM), a pluripotent structure capable of self-renewal. The meristematic activity of SAM is maintained by class I KNOTTED-LIKE HOMEOBOX genes (KNOXIs) (Long et al., 1996; Clark et al., 1996). Early events marking the recruitment of leaf founder cells to the incipient leaf primordia (P0; P for plastochron) at the peripheral zone of SAM involve downregulation of KNOXI gene expression, expression of the MYB domain transcription factor gene, ARP for ASYMMETRIC LEAVES1 (AS1) in Arabidopsis (Byrne et al., 2000), ROUGH SHEATH2 (RS2) in maize (Zea may) (Timmermans et al., 1999; Tsiantis et al., 1999) and PHANTASTICA (PHAN) in Antirrhinum (Waites and Hudson, 1995; Waites et al., 1998), and formation of auxin activity maxima (Reinhardt et al., 2000; Vernoux et al., 2000). It has been shown that AS1 acts together with the Lateral Organ Boundary (LOB) domain transcription factor, AS2, to exclude expression of KNOXI genes in incipient leaf primordia and SHOOT MERISTEMLESS (STM, a KNOXI protein) acts to exclude ASI expression in the SAM, and these regulatory relationships are not only important for the maintenance of the meristematic activity of SAM but also for development of leaf primordia in Arabidopsis (Byrne et al., 2000; Semiarti et al., 2001; Byrne et al., 2002; Guo et al., 2008). Leaf primordia initiate and expand along the proximodistal, mediolateral and adaxial-abaxial axes. ARP and the class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIPIII) genes are known to specify the leaf adaxial identity (Emery et al., 2003; McConnell et al., 2001; McConnell and Barton, 1998). On the other hand, YABBY (Golz et al., 2004; Siegfried et al., 1999) and KANADI (Kerstetter et al., 2001; Emery et al., 2003) specify the leaf abaxial identity.

Plant leaves can be categorized as either simple or compound. A simple leaf is composed of a single undivided blade and a compound leaf is composed of multiple blades known as leaflets. In compound-leafed species, specific regions of the leaf margin acquire a transient meristematic activity and initiate leaflet primordia in a species-specific pattern. The KNOXI genes are initially downregulated in incipient leaf primordia and subsequently reactivated to promote the leaf marginal meristematic activity to initiate leaflet primordia in compound-leafed species such as...
tomato (*Solanum lycopersicum*) and *Cardamine hirsuta* (Kim et al., 2003; Hay and Tsiantis, 2006; Barkoulas et al., 2008). In *C. hirsuta as1* mutants, *STM* is ectopically expressed in leaf primordia, leading to an increased compoundness of leaves (Hay and Tsiantis, 2006; Barkoulas et al., 2008). However, the regulatory relationship between *ARP* and *KNOXI* genes is more complex in tomato, (Zoulas et al., 2012). It has been shown that downregulation of the tomato *ARP* gene, *SlPHAN* results in radialized or peltately-palmate compound leaves and a loss of the typical wild-type pinnate compound leaves (Kim et al., 2003).

In *C. hirsuta* and tomato, downregulation of *KNOXI* genes results in compound leaves with reduced leaflets (Hay and Tsiantis, 2006; Shani et al., 2009; Burko et al., 2013). In tomato, overexpression of the *KNOXI* gene, *LeT6*, results in supercompound leaves (Hareven et al., 1996). Intriguingly, in the Inverted Repeat Lacking Clade (IRLC) of legumes, including garden pea (*Pisum sativum*) and Medicago species, the *KNOXI* genes are not expressed in leaf primordia and therefore not likely involved in compound leaf development, although the genetic evidence is lacking (Hofer et al., 2001; Champagne et al., 2007; Peng et al., 2011). Instead, the *FLO/LFY* orthologs, *UNIFOLIATA* (*UNI*) and *SINGLE LEAFLET1* (*SGL1*), play a key role in compound leaf development in pea and *M. truncatula*, respectively (Hofer et al., 1997; Wang et al., 2008). Pea *crispa* (*cri*) mutants, in which the pea *PHAN* gene is mutated, exhibit leaf adaxial-abaxial and proximodistal polarity defects and ectopic stipules on the adaxial lamina surface, but, the typical pinnate compound leaf pattern is not altered (Tattersall et al., 2005). RNA in situ hybridization shows patchy expression of the pea *KNOXI* gene, *BREVIPEDICELLUS* (*BP*), in leaves of *cri* mutants (Tattersall et al., 2005). This is thought to be associated with the development of ectopic stipules on the adaxial lamina surface in *cri* mutants (Tattersall et al., 2005).

Auxin convergent points or activity maxima mediated by auxin efflux transporter PIN proteins mark and precede the initiation of leaf primordia in both simple- and compound-leaved species (Barkoulas et al., 2008; Koenig et al., 2009). In compound-leaved species, auxin activity maxima also mark and precede leaflet and lobe initiation (Koenig et al., 2009; Ben-Gera et al., 2012). In
tomato entire (e) mutants that affect the auxin response inhibitor, \textit{SlIAA9} (Zhang et al., 2007; Koenig et al., 2009), the auxin signal monitored by the auxin response sensor, DR5, expands to include the complete leaf margin (Ben-Gera et al., 2012). Similar to e mutants, tomato goblet (gob) mutants affecting the \textit{CUC} transcription factor gene develop only primary leaflets (Berger et al., 2009). Inhibition of auxin transport or activity suppresses the \textit{GOB} overexpression phenotype (Ben-Gera et al., 2012), consistent with the notion that auxin mediates \textit{GOB}-regulated leaf patterning. Downregulation of both \textit{E} and \textit{GOB} results in complete loss of leaflet initiation and strong auxin signals throughout the leaf margin (Ben-Gera et al., 2012). These observations support the hypothesis that proper leaflet initiation and separation requires distinct boundaries between regions of lamina growth and adjacent regions of growth suppression (Ben-Gera et al., 2012).

In \textit{M. truncatula}, mutations in the \textit{PIN10} gene encoding an auxin efflux transporter result in complete loss of serrations at the distal leaf margin and a variable number of leaflets likely due to fusion of leaf primordia during leaf development (Peng and Chen, 2011; Zhou et al., 2011). Interestingly, in Arabidopsis, AS1 and auxin converge to suppress the expression of \textit{KNAT1/BP} and promote the leaf fate, and the interactions between auxin, AS1 and KNOX activities control both leaf initiation and leaf form (Hay et al., 2006).

To address the role of \textit{ARP} and \textit{KNOXI} genes in compound leaf development in \textit{M. truncatula}, we isolated and characterized \textit{Tnt1} retrotransposon insertion mutants of Medicago \textit{PHAN} and \textit{BP} genes. Our results show that \textit{mtphan} mutant exhibits multiple defects in compound leaf development, including curling and deep serration of leaf margins, shortened petioles, increased rachises, petioles acquiring motor organ characteristics and ectopic development of petiolules. On the other hand, \textit{mtbp} mutant did not exhibit visible defects in compound leaf development. We show that the altered petiole development requires ectopic expression of \textit{ELONGATED PETIOLULE1} (\textit{ELP1}) and the leaf margin serration requires the auxin efflux protein MtPIN10 in the \textit{mtphan} mutant. However, development of ectopic tissues on the leaf adaxial surface does not require the \textit{MtBP} activity.
Results

Isolation of *Medicago truncatula phantastica* mutant

Genome analysis identified a single *ARP* gene, *MtPHAN* (*Medtr7g061550.1*) in *M. truncatula* and four copies of *ARP* genes in soybean (*Glycine max*). In *Lotus japonicus*, a tandem repeat of *ARP* genes, *LjPHAN1* and *LjPHAN2*, has been reported (Luo et al., 2005). Amino acid sequence alignments of *ARP* genes from legume and non-legume species revealed extensive amino acid sequence similarities in the NH2-terminal MYB domain and the COOH-terminal domain amongst the sequences (Fig. S1). Phylogenetic analysis grouped the legume *ARP* genes into a single clade, in which the pea *PHAN* ortholog, *CRI*, was most closely related to *MtPHAN* (Fig. S2A). The *ARP* genes examined also exhibited a conserved intron-exon structure, one intron and two exons (Fig. S2B).

Using a reverse genetic screen (Cheng et al., 2011), we isolated an insertion mutant of the *MtPHAN* gene with the tobacco *Tnt1* retrotransposon inserted at the 3’ end of the coding region (Fig. 1A; Fig. S1). Reverse transcription (RT)-PCR analysis indicates that the isolated mutant lacked the full-length transcripts of the corresponding gene (Fig. 1B). Quantitative RT-PCR analysis using primers located upstream of the *Tnt1* insertion site revealed that the transcript level was extremely low compared with wild-type (w.t.; R108) plants (Fig. 1C), suggesting that *mtphan* is a loss-of-function or partially reduced function mutant.

*MtPHAN* regulates leaf margin development, lateral leaflet placement and leaf adaxial-abaxial polarity

In w.t. plants, adult leaves were trifoliate with one terminal leaflet and two lateral ones attached to a rachis and a petiole, respectively (Fig. 1D, E). Leaflets were folded upward along the central axis during early stages of development and subsequently unfolded to expose the adaxial surface (Fig. 1D; Fig. S3C). The distal margin of leaflets was slightly serrated (Fig. 1E, I).
In contrast to the flat w.t. leaflets (Fig. 1E, G), the proximal margin of leaflets of compound leaves and the juvenile leaf of the *mtphan* mutant curled downward (Fig. 1D, F, H). This phenotype was observed in young leaflets when they were still folded along the central axis (Fig. S3C, D), however, microscopic dissection of shoot buds revealed that curling of the proximal leaf margin did not occur when laminae were only a few millimeters in length. Leaf primordia development was not different between *mtphan* and w.t. plants (Fig. S3A, B). The distal margin of *mtphan* mutant leaflets did not curl. Instead, it exhibited deeper serrations than the w.t. counterpart (Fig. 1E, F, I, J). These results indicate that *MtPHAN* plays roles in distal and proximal leaf margin development.

In a w.t. compound leaf, a pair of lateral leaflets always developed symmetrically on the petiole (Fig. 1D, E, K). Inspection of a large number of plants revealed that 107 out of 131 (82%) *mtphan* mutant plants developed asymmetric lateral leaflets on petioles (Fig. 1K, L-1 to L-3), although the number of compound leaves with asymmetric lateral leaflets was variable among plants, ranging from one to all compound leaves in three weeks-old plants. This phenotype was, however, much less pronounced in 70 day-old plants.

Next, we compared leaf epidermal cell morphologies between w.t. and *mtphan* plants at different developmental stages, using scanning electron microscopy (SEM). In 21 day-old plants, epidermal cell size and shape of both terminal and lateral leaflets were not different between w.t. and *mtphan*. However, in 70 day-old plants, epidermal cells of the adaxial surface of fully-expanded leaves (leaves on the 4th node from the top) of the *mtphan* mutant were smooth and appeared to be less differentiated, in contrast to the jigsaw puzzle-like leaf pavement cells of corresponding w.t. plants, while the leaf abaxial epidermal cells were similar between *mtphan* and w.t. plants, suggesting that leaf adaxial differentiation or identity was affected in mature *mtphan* mutant plants (Fig. S4A-H). In addition, some ectopic tissues frequently formed on the adaxial surface of leaflets in 70 day-old *mtphan* plants, in contrast to w.t. plants (Fig. 1M, N; inset). SEM images show that these ectopic tissues appeared as ring-like extrusions with distinct
boundaries and consisted of rod-shaped smooth cells (Fig. 1O, P). Based on the morphological changes, we conclude that MtPHAN regulates lamina adaxial-abaxial polarity and this regulatory role appears to be strongly dependent on developmental stages.

MtPHAN regulates petiole, rachis and petiolule development

We next examined the role of MtPHAN in petiole and rachis development. In four week-old plants, rachises and petioles developed on the apical nodes 1 to 3 were not different between w.t. and the mtphan mutant (Fig. S5A-D). However, rachises and petioles on the 4th and 5th nodes from the top were longer and shorter, respectively, in the mtphan mutant than were in w.t. plants (Fig. S5A-D). Measurements further showed that epidermal cells of rachises and petioles on the apical 4th and 5th nodes were longer and shorter, respectively, in the mtphan mutant than were in w.t. (Fig. S5E), suggesting that MtPHAN regulates petiole and rachis development primarily through modulation of cell elongation.

Differences in rachis and petiole development between w.t. and mtphan were much pronounced in 70 day-old plants (Figs. 2A, B; S6). While petioles and rachises developed coordinately along the primary stem in w.t. plants, resulting in a narrow range of rachis/petiole ratios (0.3 to 0.5) (Fig. S6), rachises and petioles of leaves on the apical nodes 1-4 were much longer and shorter, respectively, in mtphan mutant than were in w.t. plants (Fig. S6C, D). The rachis length on the apical nodes 5 to 7 was not much different between w.t. and mtphan mutant; however, the petiole length was still largely reduced. As results, high rachis/petiole ratios were observed in mtphan plants compared with w.t. plants (Fig. S6C-E).

In a w.t. compound leaf, lateral leaflets were attached to the petiole through pulvini (Fig. 2A, C; asterisks). In an mtphan compound leaf, lateral leaflets were attached to the petiole through pulvini and petiolules (Fig. 2B, D). This phenotype was variable amongst plants but was much pronounced in 70 day-old plants. These results suggest that MtPHAN is also involved in the suppression of petiolule development in w.t. plants.
SEM analysis shows that petiole epidermal cells were greatly reduced in length and altered in morphology in 70 day-old *mtphan* mutant compared with w.t. counterparts (Fig. 2E-H). Petiole epidermal cells of the *mtphan* plants developed longitudinal and transverse folds on their surface (Fig. 2F-H). These morphological modifications resembled those of pulvini (Fig. 2A-H). Cross section images show that w.t. petioles exhibited the characteristic adaxial-abaxial polarity with one large vascular bundle and two small ones at the abaxial and adaxial side, respectively (Fig. 2I). By contrast, petioles of the *mtphan* plants contained an enlarged central vascular bundle (Fig. 2J), resembling that of pulvini. On the other hand, rachis epidermal cells were elongated and smooth in both w.t. and *mtphan* mutant plants (Fig. S7A, B). Taken together, these results reveal a novel role of *MtPHAN* in the maintenance of petiole identity through preventing ectopic acquisition of the motor organ characteristics in petioles.

*MtPHAN* regulates stipule development

In a w.t. leaf, a pair of stipules with 3-5 digitations developed at the base of the petiole (Couzigou et al., 2012) (Fig. S8A). Both the size of stipule lamina and number of digitations were significantly reduced in the *mtphan* mutant compared with w.t. plants (Fig. S8A, B). SEM analysis shows that epidermal cells of both stipule lamina and digitation were less curving in *mtphan* than were in w.t. plants, suggesting altered stipule epidermal cell differentiation or stipule identity in the *mtphan* mutant (Fig. S8C-F).

Functional rescue of *mtphan* mutant defects by *MtPHAN*

To confirm that the observed *mtphan* mutant phenotypes were indeed caused by the mutation of the *MtPHAN* gene, we introduced the *MtPHAN* genomic sequence including its promoter and the coding sequence fused to the green florescence protein (GFP) into the *mtphan* mutant by stable transformation. Fig. S9 shows that the *mtphan* mutant phenotypes were rescued in stable transgenic plants. Therefore, the mutation in the *MtPHAN* gene was responsible for the *mtphan* mutant phenotypes.
MtPHAN functionally rescued Arabidopsis as1 mutant phenotypes

To test whether Medicago and Arabidopsis PHAN genes are functional orthologs, we introduced the same MtPHAN genomic sequence-GFP fusion construct into the Arabidopsis as1-1 mutant by stable transformation. Fig. S10 (A, B) shows that both leaf and inflorescence defects of the as1-1 mutant were rescued in transgenic lines by MtPHAN. Laser confocal microscopic analysis reveals that high MtPHAN-GFP signals were localized in nuclei of leaf epidermal cells at both adaxial and abaxial surfaces in transgenic as1-1 plants (Fig. S11A-C).

Next, we tested whether the MtPHAN sequence can rescue the expression of AS1 downstream target genes. It has been shown that AS1 negatively regulates KNAT1/BP, but not STM in Arabidopsis (Byrne et al., 2000). In transgenic as1-1 plants expressing MtPHAN, the ectopic expression of KNAT1/BP was suppressed in leaves, similar to that of w.t. plants (Fig. S10C). The expression of STM was, however, not affected in leaves of transgenic plants as expected (Fig. S10C). These results indicate that (1) MtPHAN and AS1 are functional orthologs and (2) the MtPHAN promoter sequence is correctly recognized in Arabidopsis.

MtPHAN tissue-specific expression

RNA in situ hybridization shows that MtPHAN transcripts were strongly expressed in incipient and developing leaf primordia, and in lamina tissues (Fig. 3A). MtPHAN transcripts were also detectable in SAM, albeit at a lower level (Fig. 3A). As a negative control, an MtPHAN sense probe did not result in any detectable signals (Fig. 3B).

Interactions between MtPHAN and ELP1

ELONGATED PETIOLULE1 (ELP1) encoding a LOB domain protein is required to maintain the motor organ identity in M. truncatula (Chen et al., 2012; Zhou et al., 2012). Ectopic expression
of *ELP1* results in both petioles and rachises acquiring motor organ characteristics, i.e. reduced cell size and altered cell surface morphology (Chen et al., 2012). Since the altered petiole morphologies in mature *mtphan* plants (Fig. 2) resemble that of petioles of *ELP1* ectopic expression lines (Chen et al., 2012), we hypothesized that *ELP1* is possibly involved in this process. To test this hypothesis, we first examined *ELP1* gene expression in rachises and petioles of mature *mtphan* mutant plants. RT-PCR results show that *ELP1* was ectopically expressed in petioles but not in rachises of *mtphan* plants, in contrast to w.t. plants (Fig. 4A). On the other hand, *ELP1* was similarly expressed in pulvini of both *mtphan* and w.t. plants (Fig. 4A). Fig. 4A also shows ectopic expression of *ELP1* in petioles of an *ELP1* overexpression line as previously reported (Chen et al., 2012).

We next generated *mtphan elp1* double mutant (Fig. 4F). Similar to the *elp1* mutant, *mtphan elp1* double mutant developed petiolules in place of pulvini (Figs. 4E, F; S12, arrows and asterisks). Measurements show that the reduced petiole length in 70 day-old *mtphan* plants was restored in the *mtphan elp1* double mutant to the level of w.t. and *elp1* mutant plants, whereas the elongated rachis phenotype of the *mtphan* mutant plants remained unchanged in the double mutant (Fig. 4B-F). SEM analysis shows that the reduced cell size and altered cell morphology of petioles of mature *mtphan* plants were restored in the double mutant to that of w.t. and *elp1* mutant (Figs. 4G, H). These results indicate that the altered development of petioles but not that of rachises of mature *mtphan* plants is a result of ectopic expression of *ELP1* in petioles.

Distal leaf margin serration requires the auxin efflux protein, MtPIN10

The leaf distal margin serration was much deeper in the *mtphan* plants than was in w.t. plants (Fig. 1I, J). Since the auxin efflux protein MtPIN10 is required for the leaf distal margin development in *M. truncatula* (Peng and Chen, 2011; Zhou et al., 2011), we tested whether MtPIN10 is involved in this process in the *mtphan* mutant. For this, we generated *mtphan mtpin10* double mutants. Fig. 5 shows that *mtphan mtpin10* double mutants exhibited compound leaves with smooth leaf margins, resembling that of the *mtpin10* single mutant. This genetic interaction result suggests that the development of the deeply-serrated leaf margin of *mtphan*...
The mutant is dependent on auxin activity maxima mediated by the auxin efflux protein MtPIN10. The proximal leaf margin of the *mtphan mtpin10* double mutant remained curled as the *mtphan* single mutant, indicating that *MtPIN10* is not involved in the *MtPHAN*-dependent proximal leaf margin development (Fig. 5).

Genetic analysis of *MtBP* in compound leaf development

The role of the class I *KNOX* genes in compound leaf development in *M. truncatula* has not been tested genetically. To investigate involvements of *MtBP* in compound leaf development, we isolated a *M. truncatula* mutant with *Tnt1* retrotransposon inserted in the first exon of *MtBP* (Fig. S13A). RT-PCR analysis shows that *MtBP* transcripts were not detectable in vegetative shoot buds in the *mtbp* mutant, suggesting that *mtbp* is a null allele (Fig. S13B). As a control, we show that *FUSED COMPOUND LEAF1 (FCL1)* (Peng et al., 2011) was similarly expressed in *mtbp* and w.t. plants (Fig. S13B). Phenotypic analyses reveal that *mtbp* mutant did not show obvious defects in compound leaf development (Fig. S13C, D, H, I). We generated *mtbp mtphan* double mutants (Fig. S13C, D). In mature *mtbp mtphan* double mutant plants, extra tissues developed similarly as the *mtphan* single mutant on the adaxial leaf surface (Fig. S13C, D; inset), suggesting that *MtBP* is not responsible for the development of ectopic tissues in the *mtphan* mutant.

RNA in situ hybridization shows that *MtBP* transcripts were strongly detected in SAM, but they were excluded from incipient (P0) and subsequent leaf primordia (Fig. 3C). In the *mtphan* mutant, although *MtBP* transcripts were detected in SAM, they were similarly excluded from leaf primordia as in w.t. plants (Fig. 3D). Real-time RT-PCR results show that *MtBP* gene expression was upregulated in shoot buds in 21 and 70 day-old *mtphan* mutant compared with w.t. plants (Fig. S13E). However, its expression was extremely low in leaves of both *mtphan* mutant and w.t. plants at different developmental stages (Fig. S13E). Similarly, transcripts of two other *KNOXI* genes, *MtSTM* and *MtKNOX6* were also upregulated in shoot buds in the *mtphan* mutant but remained extremely low in leaves of both *mtphan* mutant and w.t. plants (Fig. S13F, G).
Genetic interactions between *MtPHAN* and *SGL1*

SINGLE LEAFLET1 (*SGL1*) has been shown to act as an indeterminate factor in the control of lateral leaflet initiation in *M. truncatula*, a similar role played by *KNOXI* in tomato and *C. hirsuta* (Wang et al., 2008). Given the known *KNOXI-ARP* interactions, we investigated genetic interactions between *MtPHAN* and *SGL1*. For that, we generated *mtphan sgl1* double mutants (Figs. 6; S14). *mtphan sgl1* double mutants exhibited simple leaves with downwardly curled and deeply serrated leaf margins and simplified stipules (Figs. 6A, B; S14A-D). These results suggest that *MtPHAN* and *SGL1* act independently in compound leaf patterning and polarity development. *sgl1* mutants produced cauliflower-like inflorescences due to defects in flower meristem development (Fig. 6C). *mtphan sgl1* double mutants produced small cauliflower-like inflorescences, resembling that of the *sgl1* single mutants (Fig. 6C). On the other hand, the petiole length was further reduced in the *mtphan sgl1* double mutant compared with single mutants, suggesting additive interactions between *MtPHAN* and *SGL1* in leaf petiole development (Fig. 6D).

Discussion

In this study, we demonstrate novel roles of Medicago *PHANTASTICA* in trifoliate leaf development, in addition to its conserved role in regulating leaf adaxial-abaxial polarity. In particular, our work shows that (1) *MtPHAN* is required to maintain the petiole identity by repressing ectopic expression of *ELONGATED PETIOLULE1* (*ELP1*) in petioles; (2) *MtPHAN* represses rachis and petiolule but promotes petiole development; (3) *MtPHAN* maintains proper leaf margin development and lateral leaflet placement and (4) *MtPHAN* promotes stipule development.
*MtPHAN* is required to maintain the petiole identity by repressing ectopic expression of *ELONGATED PETIOLULE1 (ELP1)*

The petiole identity was altered in mature *mtphan* mutant plants, as indicated by (1) petioles exhibited a nearly radial symmetry in contrast to wild-type petioles with an adaxial-abaxial polarity and (2) petiole epidermal cells were small and developed longitudinal and transverse folds on their surfaces. This structural feature, absent in wild-type petioles, resembles but is not identical to that of pulvini (Chen et al., 2012). It has been shown that the motor organ identity is controlled by the *ELP1* gene. *ELP1* is expressed in the motor organ precursor cells and its expression is restricted to the motor organ (Chen et al., 2012; Zhou et al., 2012). Ectopic expression of *ELP1* results in petioles and rachises acquiring motor organ characteristics (Chen et al., 2012). Our gene expression analysis shows that in mature *mtphan* mutant plants *ELP1* was ectopically expressed in petioles, but not in rachises, indicating a specific effect of the *mtphan* mutation on *ELP1* gene expression. Our *mtphan elp1* double mutant analysis shows that the altered petiole development in mature *mtphan* mutant plants was rescued when *ELP1* is mutated, confirming the involvement of ectopic *ELP1* gene expression in the acquisition of motor organ characteristics in petioles of mature *mtphan* plants.

In Arabidopsis, AS1 forms a complex with AS2, a LOB domain protein, and the AS1-AS2 nuclear complex interacts with promoter elements and represses transcription of *KNOX* genes and other downstream targets such as *ETTIN (ETT)/AUXIN RESPONSE FACTOR3 (ARF3)* (Guo et al., 2008; Iwasaki et al., 2013). It is not clear whether the same AS1-AS2 complex functions in compound leaf development in *M. truncatula*. Several AS2-like sequences are present in the Medicago genome. Future work is needed to examine the role of Medicago AS2 ortholog(s) in compound leaf development. It is intriguing that *ELP1* also encodes a LOB domain protein, although it is most closely related to LOB, the founding member of the LBD family, but not AS2.

*MtPHAN* represses rachis and petiollule but promotes petiole development
PHANTASTICA has been shown to play a role in leaf adaxial-abaxial polarity and proximodistal axis development in simple- and compound-leafed species. In compound-leafed species such as C. hirsuta, tomato and garden pea, PHAN plays a role in leaf patterning and development, albeit in species-specific manner. For example, in pea cri mutants, compound leaves remain pinnate, though multiple polarity defects are observed in mutant leaves. By contrast, downregulation of the tomato PHAN gene, SlPHAN in the leaf proximal region leads to absence of the adaxial domain in the proximal region, resulting in cup-shaped simple leaves or peltately-palmate compound leaves (Kim et al., 2003; Zoulias et al., 2012).

In three-week old mtphan mutant plants, leaves developed on the 4th and 5th nodes had slightly increased rachis length and reduced petiole length compared with wild-type leaves. In 70 day-old mtphan mutant plants, however, young leaves had extremely increased and reduced rachises and petioles, respectively, compared with those of w.t. plants. This results in significantly large rachis/petiole ratios. In older leaves, the petiole length was reduced but the rachis length was not increased, still resulting in large rachis/petiole ratios for mtphan mutant leaves. Cell size measurement results show that epidermal cells of rachises and petioles were increased and reduced, respectively, in the mtphan mutant plants compared with wild-type plants, suggesting that MtPHAN primarily regulates rachis and petiole cell differentiation.

In mature mtphan mutant plants, we observed a large number of compound leaves developed petiolules at the proximal ends of pulvini of lateral leaflets. By contrast, lateral leaflets were attached to petioles by pulvini in w.t. plants. This abnormality was present, but less prevalent in three week-old mtphan mutant plants. This phenotype, together with the effect of the mtphan mutation on rachis elongation, supports that MtPHAN normally represses the development of rachises and petiolules in wild-type compound leaves.

MtPHAN maintains proper leaf margin development and lateral leaflet placement.
Deep serration and curling of distal and proximal leaf margins in the *mtphan* mutant indicate that *MtPHAN* plays key roles in leaf margin development. Previous studies have shown that auxin convergent points mediated by auxin efflux transporter PIN proteins are prerequisites for the initiation of leaf primordia and leaf serrations in simple- and compound-leafed species and for the initiation of leaflet primordia in compound-leafed species. In Medicago, *MtPIN10* plays an essential role in distal leaf margin development indicated by the smooth leaf margin phenotype of *mtpin10* mutants (Peng and Chen, 2011; Zhou et al., 2011). Because the deeply serrated distal leaf margin of the *mtphan* mutant was changed to the smooth margin in *mtphan mtpin10* double mutants, similar as the *mtpin10* mutant, MtPIN10-mediated auxin maxima are required for the *MtPHAN*-regulated distal leaf margin development in Medicago.

In mature *mtphan* mutant plants, leaf epidermal cells on the adaxial surface appeared to be less differentiated, compared with the jigsaw puzzle-like epidermal cells of wild-type plants. In addition, ectopic tissues developed on the adaxial surface of mature *mtphan* mutant plants. These phenotypes suggest that the adaxial-abaxial polarity was altered in the *mtphan* mutant. *AS1/RS2/PHAN* is known to negatively regulate expression of *KNOX* genes in Arabidopsis and other species. This is shown by ectopic expression of *KNOX* genes in leaves and formation of ectopic tissues or meristems on the leaf adaxial surface or sinuses in *arp* mutants.

Depending on the timing and extent of spatiotemporal expression of *KNOX* genes and species studied, overexpression of *KNOX* genes may lead to different developmental consequences. Analysis of tomato mutants suggests that weak *LeT6* (a tomato *KNOX* gene) overexpression and *SIPHAN* downregulation lead to *LeT6* overexpression phenotypes, leaf lobing and compoundness; whereas strong *LeT6* overexpression and *SIPHAN* downregulation lead to *SIPHAN* downregulation phenotypes, such as cup-shaped and wire-like leaves (Janssen et al., 1998; Kim et al., 2003). In maize *rs2* mutants, *KNOX* genes may not be overexpressed to a high level to exhibit leaf lobing or *PHAN* downregulation phenotypes (Timmermans et al., 1999; Tsiantis et al., 1999). In pea *cri* mutants, some ectopic stipules are formed on the adaxial leaf surface and this is attributed to patches of *BP* ectopic expression in leaflets (Tattersall et al., 2005).
We isolated a knock-out mutant of the *Medicago* BP gene. Phenotypic analysis of *mtphan mtbp* double mutant plants shows that ectopic tissues developed on the adaxial leaf surface in mature *mtphan mtbp* double mutant similarly as in *mtphan* single mutant, suggesting that *MtBP* is not involved in this process. Consistent with this conclusion, RNA in situ hybridization shows that *MtBP* transcripts are detected in SAM but not in leaf primordia and leaflets in both wild-type and *mtphan* mutant plants. Interestingly, quantitative RT-PCR shows that the expression level of *MtBP*, and *MtSTM* and *MtKNOX6*, two other KNOXI genes in *M. truncatula*, was elevated in vegetative shoot buds in the *mtphan* mutant compared with wild-type plants, supporting a negative regulation of *MtKNOXI* genes by *MtPHAN* in the shoot apex. In Medicago, three KNOXI genes have been identified thus far, it is possible that redundant gene functions among the class I KNOX genes exist to mask phenotypes of single mutants. Future experiments are required to further test this hypothesis.

In tomato plants with *PHAN* downregulation, the characteristic pinnate compound leaves change to peltately-palmate compound leaves (Kim et al., 2003; Zoulias et al., 2012). Interestingly, we observed altered leaflet placement in young *mtphan* mutant plants. However, the number of compound leaves with asymmetric lateral leaflets and the degree of displacement were variable. It is not yet clear how *MtPHAN* regulates leaflet placement. In the *mtpin10* mutant, we also observed asymmetric leaflets in compound leaves (Peng and Chen, 2011). Since *mtpin10 mtphan* double mutants resemble *mtpin10* single mutant, we reasoned that *PHAN* may be involved in the synchronized lateral leaflet initiation through *MtPIN10*-mediated auxin activity maxima.

**MtPHAN** promotes stipule development

In the *mtphan* mutant, stipules were greatly reduced in size and digitations and altered in epidermal cell identity. Recently, it has been shown that *Medicago NODULE ROOT* (*NOOT*) and pea COCHLEATA (*COCH*) genes, orthologs of the Arabidopsis BLADE-ON-PETIOLE (*BOP*) gene, play a role in stipule and nodule development (Couzigou et al., 2012). The stipule
phenotypes of *noot* and *coch* mutants resemble that of the *mtpfan* mutant, raising the possibility that *PHAN* and *BOP* may interact to regulate stipule development in *Medicago*.

Our observation that *mtpfan* mutant phenotypes are strongly dependent on developmental stages appears to be consistent with previous studies, which show that AS1-AS2 has multiple downstream targets (Iwasaki et al., 2013) and multiple pathways including chromatin modification, cell proliferation, ribosomal proteins, and *trans*-acting siRNA biogenesis are involved in regulating leaf development in the *asl* or *as2* background (Horiguchi et al., 2011; Kojima et al., 2011; Ishibashi et al., 2012; Nakagawa et al., 2012; Xu et al., 2012). Alternatively, *mtpfan* represents a partially reduced function mutant and the residual level of gene expression in the mutant may be sufficient to mask some phenotypes. However, some *mtpfan* mutant phenotypes, such as serration and curling of distal and proximal leaf margins and asymmetric placement of lateral leaflets, appear early and thus may not be in support of the later hypothesis.

There are reports that leaf adaxial-abaxial polarity and expansion in some *phan* mutants are conditional. For example, in Arabidopsis *asl* mutants, a high rate of radialized leaves is only observed in the Landsberg *erecta* genetic background (Xu et al., 2003). The Antirrhinum *phan* mutants exhibit completely abaxialized, needle-like leaves at a late developmental stage or at a low temperature (17°C). The first leaves are usually broader and heart-shaped in Antirrhinum *phan* mutants than wild-type plants (Waites and Hudson, 1995; Waites et al., 1998). In maize *rs2* mutants, about 90% of the plants display narrow, bladeless leaves in the B73 genetic background, whereas this phenotype is rare in the Mo17 background (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). The conditional and stage-dependent phenotypes observed in *phan* mutants may be explained by existence of redundant genes or complex mechanisms (Tattersall et al., 2005).

Genetic interactions between *MtPHAN* and *SINGLE LEAFLET1 (SGL1)*
SGLI, the Medicago FLO/LFY/UNI ortholog, is required to maintain a transient indeterminacy for the initiation of lateral leaflets in Medicago, a role played by the class I KNOX genes in compound-leafed species outside the IRLC legumes (Wang et al., 2008). Consistent with this, sgl1 mutants have simple leaves with shortened petioles (Wang et al., 2008; Peng et al., 2011). Interestingly, phenotypes of the sgl1 mtphan double mutant suggest independent functions of MtPHAN and SGL1 in compound leaf patterning and leaf margin development. On the other hand, the further reduced petiole phenotype in sgl1 mtphan double mutants suggests additive interactions between SGL1 and MtPHAN in petiole development. MtPHAN and SGL1 functions appear to be consistent with their partially overlapping expression patterns. SGL1 is highly expressed in SAM and leaf primordia, but, its expression is rapidly decreased in expanding leaves (Wang et al., 2008); whereas, MtPHAN is weakly expressed in SAM but strongly expressed in leaf primordia and leaflets.

Materials and methods

Plant materials and growth conditions

Medicago phan (mtphan) and bp (mtbp) mutants were obtained from reverse screens of the Tnt1 retrotransposon insertion pool in the R108 background (Cheng et al., 2011). Mutants were backcrossed to R108. The BC1 mutant and its descendants were used for phenotypic characterization. sgl1, mtpin10 and elp1 mutants, all in R108 background, were as described previously (Wang et al., 2008; Peng and Chen, 2011; Chen et al., 2012). Arabidopsis as1-1 was from the Arabidopsis Stock Center. Arabidopsis and Medicago plants were grown in growth chambers and glasshouses (16h/8h day/night light cycle; 22°C/20°C day/night temperature), respectively.

Sequence alignment and phylogenetic analysis

ARP amino acid sequences were aligned using ClustalX 1.83. The aligned sequences were edited using BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The maximum parsimony
phylogenetic tree was reconstructed by PAUP 4.0 beta 10 in Geneious Pro 5.6.3, with 1,000 bootstrap replications.

Scanning electron microscopy

Scanning electron microscopy was performed as previously described (Wang et al., 2008). Briefly, plant tissues were fixed with 2.5% glutaraldehyde in phosphate saline solution (pH 7.0) overnight, followed by fixation with 1% OsO4 at 4°C for 1 hour. The fixed samples were dehydrated with an ethanol series, critical point dried and mounted for imaging.

Genetic complementation

Medicago PHAN genomic DNA, including promoter and coding sequences (without the stop codon) was cloned into a binary vector in frame with the green florescent protein (GFP). The complementation construct was introduced into the mtphan mutant plants by Agrobacterium tumefaciens EHA105-mediated stable transformation, as previously reported (Wang et al., 2008) and into the Arabidopsis as1-1 mutant by A. tumefaciens GV3101-mediated stable transformation.

RNA isolation, RT-PCR and quantitative PCR

Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen) and quantified with a Nanodrop Spectrophotometer. Reverse transcription was performed using Qiagen SuperScript II Kit (Qiagen). Quantitative PCR was conducted on 7900HT Fast Real-Time PCR system (Applied Biosystems). An Medicago ACTIN gene was used as the control. LinRegPCR (Ramakers et al., 2003) and SDS2.2.1 program (Applied Biosystems) were used for data analysis. Statistical analysis was carried out using Student t-tests. Primer sequences are listed in Supplemental Table 1.
RNA in situ hybridization

RNA in situ hybridization was performed as previously described (Ferrandiz et al., 2000) with minor modifications. The \textit{MtPHAN} probes correspond to a 445-bp sequence of the \textit{MtPHAN} coding sequence. The \textit{MtBP} probes correspond to a 613-bp sequence of the \textit{MtBP} coding sequence. Eight μm sections from shoot apices of 2- to 4-week-old seedlings were processed and hybridized with digoxigenin-labeled sense and antisense probes.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or NCBI GenBank under the following accession numbers: Mt PHAN (DQ468322), GmPHAN1 (NP_001236839.1), GmPHAN2 (NP_001235251), GmPHAN3 (KC737842), GmPHAN4 (KC737843), LjPHAN1 (AAX21343.1), LjPHAN2 (AAX21344.1), ELP1 (JQ653161.1), Mt STM (EF128056.1), Mt BP (EF128057.1), Mt KNOX6 (EF128061.1), SGL1/UNI (AY928184.1), FCL1 (HQ695002), AS1 (AT2G37630), STM (AT1G62360) and BP/KNAT1 (AT4G08150).

Supplemental Data

Supplemental Fig. S1. Amino acid sequence alignment of MtPHAN and its related sequences.

Supplemental Fig. S2. Phylogenetic analysis and gene structures of \textit{MtPHAN} and its related sequences.

Supplemental Fig. S3. Leaf primordia development of \textit{mtphan} mutant.

Supplemental Fig. S4. Leaf epidermal cell morphologies of \textit{mtphan} mutant.

Supplemental Fig. S5. Compound leaf phenotypes of \textit{mtphan} mutant seedlings.

Supplemental Fig. S6. Compound leaf phenotypes of 70 day-old \textit{mtphan} mutant plants.
Supplemental Fig. S7. SEM analysis of rachis epidermal cells of compound leaves of 70 day-old w.t. (A) and mtphan mutant (B) plants.

Supplemental Fig. S8. Altered stipule development of mtphan mutant.

Supplemental Fig. S9. Functional rescue of mtphan mutant by MtPHAN.

Supplemental Fig. S10. Functional rescue of Arabidopsis asl mutant by MtPHAN.

Supplemental Fig. S11. Nuclear localization of MtPHAN-GFP fusion proteins in transgenic Arabidopsis plants.

Supplemental Fig. S12. Compound leaf phenotypes of mtphan elp1 double mutant.

Supplemental Fig. S13. Characterization of mtbp and mtphan mtbp mutant plants.

Supplemental Fig. S14. Compound leaf phenotypes of sgl1 mtphan double mutant.

Supplemental Table 1. Primer sequences used in this study.

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Literature cited


reveals context-dependent effects on compound leaf development. Plant Cell 23: 2106-2124


Figure legends

**Fig. 1.** Compound leaf phenotypes of the mtphan mutant. (A) MtPHAN gene structure and Tnt1 insertion site. Closed boxes, exons; open boxes, 5’ and 3’ untranslated regions; horizontal lines, introns. (B) RT-PCR analysis of MtPHAN gene expression in 21 day-old shoot buds. Lane 1, wild-type (w.t.; R108); Lane 2, mtphan. A MtACTIN gene was used as a loading control. PCR cycles used were 32 and 28 for MtPHAN and MtACTIN, respectively. (C) Quantitative RT-PCR analysis of MtPHAN gene expression in 21 day-old plants. MtACTIN was used as the reference gene. (D) Images of 21 day-old w.t. (left) and mtphan mutant (right). (E, F) Close-up views of adaxial (left) and abaxial (right) side of w.t. (E) and mtphan (F) compound leaves. (G, H) Cross-sections of leaflets of w.t. (G) and mtphan mutant (H). (I, J) Close-up views of distal leaf margins of w.t. (I) and mtphan mutant (J). (K, L-1 to L-3) Close-up views of the typical symmetric lateral leaflet placement in w.t. (K) and various lateral leaflet placements in the mtphan mutant (L-1 to L-3). (M, N) Compound leaves of 70 day-old w.t. (M) and mtphan (N) plants. Inset shows ectopic tissues developed on the adaxial leaf surface of the mutant. (O, P) SEM images show ectopic tissues and epidermal cell morphologies on the adaxial leaf surface of the mtphan mutant. Scale bars, 1 cm (E, F, M, N); 2 mm (G, H, K, L); 5 mm (I, J).

**Fig. 2.** Development of ectopic petiolules and altered petioles in mtphan plants. Representative 70 day-old w.t. (A) and mtphan mutant (B) compound leaves. Bottom panels are close-up images. Horizontal lines indicate the regions where SEM (E-H) and cross sections (I, J) were made. Scale bars, 1 cm. SEM images of w.t. (C) and mtphan mutant (D) compound leaves. Asterisks, pulvini; arrows, ectopic petiolules (A-D). SEM images of petioles of 70 day-old w.t. (E) and mtphan mutant (F-H). Cross sections of petioles of 70 day-old w.t. (I) and mtphan mutant (J). Scale bar, 100 um.

**Fig. 3.** RNA in situ hybridization of MtPHAN and MtBP. (A) A longitudinal section of a vegetative shoot bud of w.t. plants showed MtPHAN transcripts in SAM (asterisk), leaf primordia at various stages and young leaves. (B) An adjacent section hybridized with a sense probe did not yield any signals, serving as a negative control. (C) MtBP transcripts were detected
in SAM (asterisk), but not in leaf primordia at various stages and young leaves of w.t. plants. (D) Similarly, MtBP transcripts were detected in SAM (asterisk), but not in leaf primordia at various stages and young leaves of mtphan mutant plants. Scale bars, 100 µm.

**Fig. 4.** Medicago ELP1 is required for the altered petiole identity in mature mtphan mutant plants. (A) RT-PCR analysis of ELP1 gene expression in rachises, petioles and pulvini of 70 day-old w.t., mtphan and 35S::ELP1 plants. MtACTIN was used as a loading control. PCR cycles were 32 and 28 for ELP1 and MtACTIN, respectively. (B) Measurements of the petiole length of compound leaves on the apical 3rd node of 70 day-old w.t., mtphan, elp1 and mtphan elp1 double mutant plants. Shown are means ± s.d.; n=12 and statistical significances were calculated by Tukey’s Honest Significance Test (p<0.05). (C-F) Images of compound leaves on the apical 3rd node of 70 day-old w.t. (C), mtphan (D), elp1 (E) and mtphan elp1 (F). Top and bottom panels, adaxial and abaxial views, respectively. (G, H) SEM images of petioles of leaves on the 3rd node of mature mtphan (G) and mtphan elp1 mutant (H). Scale bars, 1 cm (C-F) and 100 µm (G, H).

**Fig. 5.** Compound leaf phenotypes of mtphan mtpin10 double mutant. (A, B) Adaxial (A) and abaxial (B) views of compound leaves of 70 day-old w.t., mtphan, mtpin10 and mtphan mtpin10 double mutant (from left to right). Inset shows a close-up view of the distal leaflet margin of mtphan mtpin10 double mutant. Scale bars, 1 cm.

**Fig. 6.** Compound leaf phenotypes of sgl1 mtphan double mutant. (A) Compound leaves of 70 day-old w.t., mtphan, sgl1 and mtphan sgl1 double mutant (from left to right). (B) Close-up images of stipules. (C) Flowers and inflorescences of w.t., mtphan, sgl1, and mtphan sgl1 double mutant (from left to right). (D) Measurements of petiole lengths in compound leaves on apical nodes 1 to 7 of four week-old w.t., mtphan, sgl1 and mtphan sgl1 double mutant plants. Shown are means ± s.e.; n=11. Scale bars, 1 cm (A-C).
Fig. 1. Compound leaf phenotypes of the mtphan mutant. (A) MtPHAN gene structure and Tnt1 insertion site. Closed boxes, exons; open boxes, 5’ and 3’ untranslated regions; horizontal lines, introns. (B) RT-PCR analysis of MtPHAN gene expression in 21 day-old shoot buds. Lane 1, wild-type (w.t.; R108); Lane 2, mtphan. A MtACTIN gene was used as a loading control. PCR cycles used were 32 and 28 for MtPHAN and MtACTIN, respectively. (C) Quantitative RT-PCR analysis of MtPHAN gene expression in 21 day-old plants. MtACTIN was used as the reference gene. (D) Images of 21 day-old w.t. (left) and mtphan mutant (right). (E, F) Close-up views of adaxial (left) and abaxial (right) side of w.t. (E) and mtphan (F) compound leaves. (G, H) Cross-sections of leaflets of w.t. (G) and mtphan mutant (H). (I, J) Close-up views of distal leaf margins of w.t. (I) and mtphan mutant (J). (K, L-1 to L-3) Close-up views of the typical symmetric lateral leaflet placement in w.t. (K) and various lateral leaflet placements in the mtphan mutant (L-1 to L-3). (M, N) Compound leaves of 70 day-old w.t. (M) and mtphan (N) plants. Inset shows ectopic tissues developed on the adaxial leaf surface of the mutant. (O, P) SEM images show ectopic tissues and epidermal cell morphologies on the adaxial leaf surface of the mtphan mutant. Scale bars, 1 cm (E, F, M, N); 2 mm (G, H, K, L); 5 mm (I, J).
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Fig. 4. Medicago ELPI is required for the altered petiole identity in mature mtphan mutant plants. (A) RT-PCR analysis of ELPI gene expression in rachises, petioles and pulvinus of 70 day-old w.t., mtphan and 35S::ELPI plants. MaACTIN was used as a loading control. PCR cycles were 32 and 28 for ELPI and MaACTIN, respectively. (B) Bar graph showing the length of rachis and petiole of w.t., mtphan, elp1 and mtphan elp1. The bars with different letters are statistically different at p<0.05. (C-F) Images of compound leaves on the apical 3rd node of 70 day-old w.t. (C), mtphan (D), elp1 (E) and mtphan elp1 (F). Bottom panels, close-up views. (G, H) SEM images of petioles of leaves on the 3rd node of mature mtphan (G) and mtphan elp1 mutant (H). Scale bars, 1 cm (C-F) and 100 μm (G, H).
Fig. 5. Compound leaf phenotypes of *mtphan mtpin10* double mutant. (A, B) Adaxial (A) and abaxial (B) views of compound leaves of 70 day-old *w.t., mtphan, mtpin10* and *mtphan mtpin10* double mutant (from left to right). Inset shows a close-up view of the distal leaflet margin of *mtphan mtpin10* double mutant. Scale bars, 1 cm.
Fig. 6. Compound leaf phenotypes of sgl1 mtphan double mutant. (A) Compound leaves of 70 day-old w.t., mtphan, sgl1 and mtphan sgl1 double mutant (from left to right). (B) Close-up images of stipules. (C) Flowers and inflorescences of w.t., mtphan, sgl1, and mtphan sgl1 double mutant (from left to right). (D) Measurements of petiole length on compound leaves on apical nodes 1 to 7 of four week-old w.t., mtphan, sgl1 and mtphan sgl1 double mutant plants. Shown are means ± s.e.; n=11. Scale bars, 1 cm (A-C).