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Auxin efflux transporter MtPIN10 regulates compound leaf and flower development in *Medicago truncatula*

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Plant diversity in nature is to a large extent reflected by morphological diversity of their leaves. Both simple and dissected (with multiple blades or leaflets) leaves are initiated from shoot apical meristem (SAM) in a highly ordered fashion. Similarly, development of leaflets from leaf marginal meristem (marginal blastozone) is also highly ordered. How morphological diversity of plant leaves is regulated remains an important topic of studies on plant form evolution. Here, we describe isolation and characterization of loss-of-function mutants of auxin efflux transporter *MtPIN10* of a legume species, *Medicago truncatula*. *Mtpin10* mutants exhibit defects in diverse developmental processes including leaf and leaflet development. Cross species genetic complementation demonstrates that *MtPIN10* and Arabidopsis *PIN1* are functional orthologs. Double mutant analyses reveal complex genetic interactions between *MtPIN10* and Medicago *SINGLE LEAFLET1* (*SGL1*) and *CUP-SHAPED COTYLEDON2* (*MtCUC2*), three regulatory genes involved in developmental processes including dissected leaf and flower development.

Introduction

In nature, plant leaves exhibit remarkable morphological diversity and can be classified as either simple with a single blade or compound with multiple blades known as leaflets. How divergent leaf morphology is determined remains an important topic of studies on plant form evolution. Both simple and compound leaves are initiated from the periphery of shoot apical meristem (SAM) that consists of pluripotent stem cells capable of self renewal. Development of leaf primordia from the SAM requires downregulation of *KNOTTED1*-like homeobox transcription factors (*KNOXIs*) and convergence of activity maxima of the plant hormone auxin at incipient sites of organ initiation.[1-4] In simple leafed species such as *Arabidopsis thaliana*, the *KNOXI* genes are permanently downregulated in leaves. In tomato and several other species with compound leaves, the *KNOXI* genes are re-activated in developing leaves and are required for compound leaf development. In some compound leafed species of the legume family (Fabacaea), including pea (*Pisum sativum*) and *Medicago truncatula*, *FLORICAULA* (*FLO*)/*LEAFY*(*LFY*) transcription factor, *UNIFOLIATA*(*UNI*)/*SINGLE LEAFLET1*(*SGL1*), plays a key role in place of *KNOXI* in compound leaf development,[5-7] consistent with independent origins of compound leafed species during evolution. Recent studies have shown, however, that differential deployment of conserved molecular mechanisms contributes to diversification of leaf forms during evolution.[8-10]

In *Cardamine hirsuta* and tomato (*Solanum lycopersicum*), both with compound leaves, auxin activity maxima converge at incipient sites of leaf primordia in the SAM and of leaflet primordia in leaf margins (marginal blastozones) and condition the outgrowth of leaf and leaflet primordia.[11,12] In *C. hirsuta*, loss-of-function mutations in auxin efflux transporter *PIN-FORMED1* (*PIN1*) gene impair polarized outgrowth and result in simplified leaves, demonstrating involvement of a fundamental mechanism in compound leaf development.[12] In *A. thaliana*, auxin is required for initiation of lateral organs in the SAM, development of serrations in leaf margins and differentiation of vascular tissues.[13-17] Polarized outgrowth during organ initiation requires auxin efflux transporter PIN1-mediated auxin activity maxima.[13,14,17,18] Consistent with this, Arabidopsis *pin1* mutants exhibit pin-like inflorescence stems devoid of reproductive lateral organs, proliferation of vascular tissues and reduced serrations in leaf margins.[19-22]

Recent studies demonstrate that plant-specific NAC transcription factors play a regulatory role in compound leaf development in diverse species.[8,9] The NAC family of transcription factors, including *CUP-SHAPED COTYLEDON1, 2, 3* (*CUC1, 2, 3*) in *A. thaliana*, *NO APICAL MERISTEM* (*NAM*) in petunia, *GOBLET* (*GOB*) in tomato and *CUPULIFORMIS* (*CUP*) in *Antirrhinum majus*, are expressed in boundary cells and function to suppress boundary cell growth and promote boundary formation.[23-26] In addition, NAC transcription factors activate *KNOXI* gene expression and are involved in meristem maintenance.

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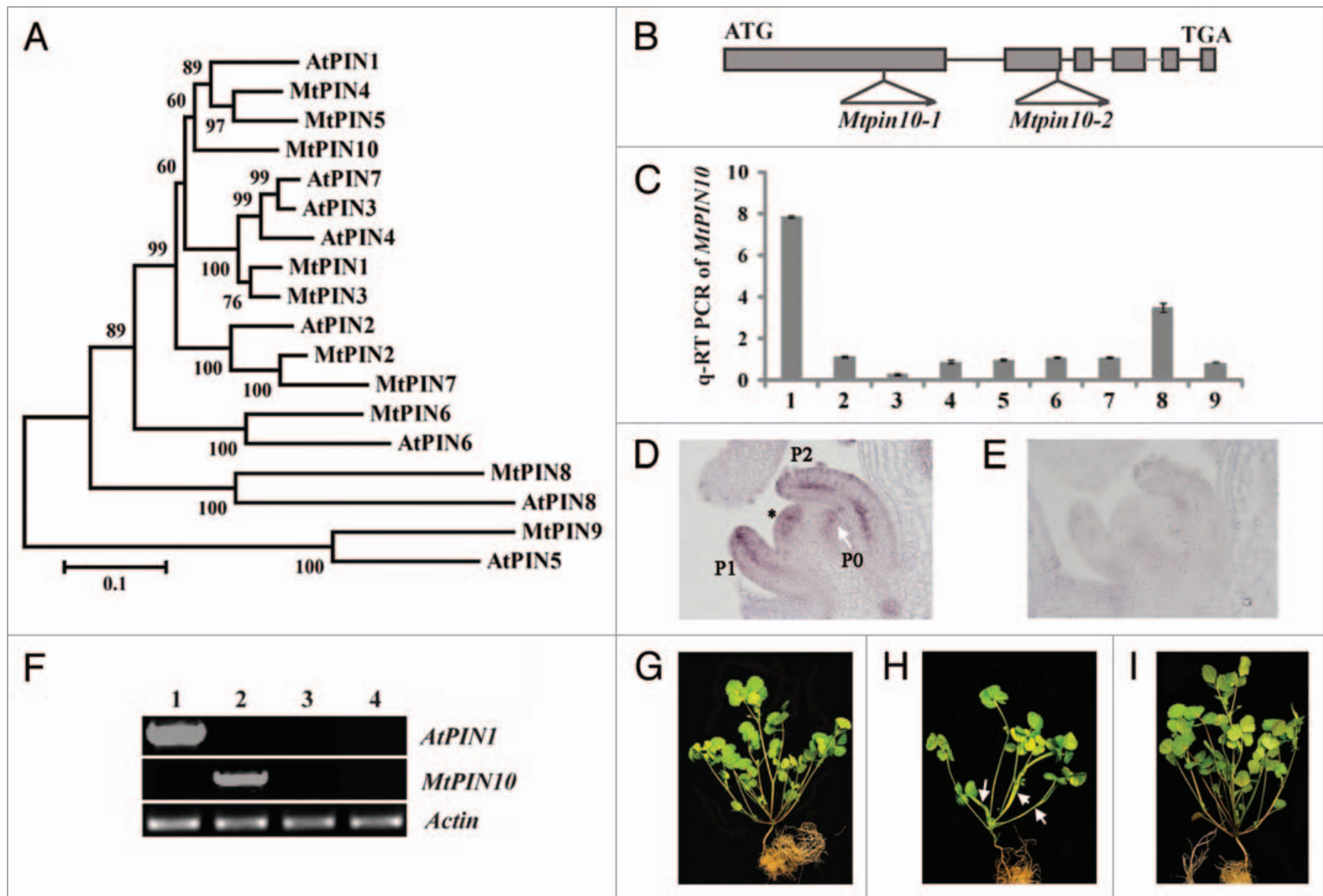

Figure 1. Phylogenetic relationships of Medicago and Arabidopsis PINs and genetic complementation of *Mtpin10*. (A) Phylogenetic relationships of Medicago and Arabidopsis PINs. MtPIN4, MtPIN5 and MtPIN10 are clustered with AtPIN1. (B) *MtPIN10* gene structure and *Tnt1* insertion sites in *Mtpin10-1* and *Mtpin10-2* mutants. (C) Quantitative RT-PCR analysis of *MtPIN10* expression pattern. *MtPIN10* expression was normalized with an internal control, *MtActin*. 1, shoot bud; 2, young leaf; 3, mature leaf; 4, rachis; 5, petiole; 6, stem; 7, root; 8, flower and 9, immature pod. (D) RNA in situ hybridization. *MtPIN10* transcripts were detected in the shoot apical meristem (asterisk), and P0, P1 and P2 compound leaf primordia. In P1 and P2 leaf primordia, *MtPIN10* transcripts were detected in both epidermal and vascular cells. (E) No signal was detected in an adjacent tissue section hybridized with a sense probe. (F) RT-PCR analysis of *AtPIN1* and *MtPIN10* gene expression. Lanes 1–4, *Mtpin10-1* mutant transformed with an Arabidopsis *PIN1::PIN1:GFP* construct, wild-type (R108), *Mtpin10-1* and *Mtpin10-2* mutants, respectively. *MtActin* was used as a loading control. (G-I) Growth defects and genetic complementation of *Mtpin10* mutants. Shown were six-week-old wild type (G), *Mtpin10-1* (H) and *Mtpin10-1* transformed with an Arabidopsis *PIN1::PIN1:GFP* construct (I). In *Mtpin10* mutants, leaves were often fused (H, arrows).

Loss-of-function mutants of NAC transcription factors are impaired in shoot meristem development and boundary formation, leading to fusion of leaflets and simplified leaves in several compound leafed species and smooth leaf margins in both compound and simple leafed species.[8,9,27]

It has been shown that the lineage in the legume family, the inverted repeat-lacking clade (IRLC), including pea and *M. truncatula*, utilizes the *FLO/LFY* pathway in place of *KNOXI* genes in compound leaf development.[5-7,28] To investigate the role of auxin efflux transporter PIN1-mediated polar auxin transport in compound leaf development and interactions with the *FLO/LFY* ortholog, *SGL1*, and the *NAM/CUC* ortholog, *MtCUC2*, we isolated two *pin10* mutant alleles in *M. truncatula* and characterized mutant phenotypes in detail. Double mutants were constructed between *Mtpin10* and *sgl1*, and *Mtcuc2* to examine their genetic interactions. Cross-species genetic complementation was performed to examine the degree of functional conservation of *MtPIN10* and the Arabidopsis *PIN1*.

Results and Discussions

Isolation of *Medicago truncatula pin10* mutants. Arabidopsis *pin1* mutants exhibit pronounced defects in shoot apical meristem, inflorescence stem, lateral organ and vascular tissue development. To study the role of *M. truncatula PIN1* homolog in compound leaf development, we compared sequences of Medicago PIN proteins (MtPIN1-MtPIN10) [29] with the Arabidopsis counterparts (PIN1-PIN8). Phylogenetic analysis indicated that MtPIN4, MtPIN5 and MtPIN10 are clustered with the Arabidopsis PIN1 (**Fig. 1A**). In addition, *MtPIN4*, *MtPIN5* and *MtPIN10* genes share a similar intron-exon structure as the Arabidopsis *PIN1* (**Fig. 1B**) and the encoded proteins share 71%, 65% and 65%

amino acid sequence identities with PIN1, respectively. In silico gene expression analysis indicated that *MtPIN4* and *MtPIN10* are expressed in similar types of tissues in *M. truncatula*, albeit *MtPIN4* being much highly expressed; whereas *MtPIN5* expression was not detectable in almost all tissues except that a low level of expression was detected in seed coat (**Fig. S1**).

Using a reverse genetics approach, we isolated two independent alleles of *Mtpin10* mutant, *Mtpin10-1* and *Mtpin10-2*, from a *M. truncatula Tnt1* retrotransposon insertion mutant population.[30] However, no mutants of *MtPIN4* were uncovered from the screen. Flanking sequence analysis showed that *Mtpin10-1* has the *Tnt1* inserted in the first exon and *Mtpin10-2* in the second exon of *MtPIN10* (**Fig. 1B**). RT-PCR using primers flanking the full-length coding sequence did not detect any *MtPIN10* transcripts in both alleles (**Fig. 1E**), indicating that both are knockout mutants. Both mutants were backcrossed with the wild-type parent. In F2 populations, one quarter of F2 plants (135/536 and 43/180, respectively) were homozygous for *Tnt1* insertion in *MtPIN10* and exhibited mutant phenotypes, including abnormal phyllotaxy, and leaf and flower development (**Fig. 1G and H; Fig. S2**). Because *Mtpin10* mutants are sterile, heterozygous plants are maintained. Functional conservation between *MtPIN10* and the Arabidopsis *PIN1* is confirmed by cross-species genetic complementation of *Mtpin10-1* with an Arabidopsis *PIN1::PIN1:GFP* contruct (**Fig. 1I**). *Mtpin10* mutant phenotypes are discussed below.

Abnormal cotyledon development in *Mtpin10* mutants. In contrast to wild-type plants that had two separate cotyledons, *Mtpin10* mutants frequently exhibited three or four cotyledons and fusion between them (**Fig. 2A and B**). This is similar to Arabidopsis *pin1* mutants that frequently exhibit three partially fused cotyledons. To investigate early developmental stages in which cotyledon abnormality occurred, embryos from different developmental stages were dissected. Microscopic analysis indicated that abnormal embryos with an increased number of cotyledons were frequently observed in heart-stage embryos in *Mtpin10* heterozygous plants (**Fig. 2F**). By contrast, all heart-stage embryos in wild-type plants had two distinct cotyledons (**Fig. 2E**). No apparent abnormalities were observed in embryos at earlier stages in *Mtpin10* mutants (data not shown). These results suggest that loss-of-function mutations in *MtPIN10* results in an early embryonic defect, leading to development of an increased number of partially fused cotyledons.

Compound juvenile leaf in *Mtpin10* mutants. In wild-type Medicago plants, the first leaf, or the so-called juvenile leaf, is always simple and leaves developed later are trifoliate, consisting of a pair of lateral leaflets, a terminal leaflet, a petiole and a pair of stipules. By contrast, the juvenile leaf in *Mtpin10* mutants was always compound (**Fig. 2C and D**). In fact, the first few leaves including the juvenile leaf frequently had four leaflets with an even pinnate configuration in *Mtpin10* mutants (**Fig. 2D**). Occasionally, the two distal leaflets fused, leaving two visible mid-veins.

***Mtpin10* mutants developed abnormal compound leaves with smooth margins.** *Mtpin10* mutants exhibited pronounced defects in compound leaf development, including an increase in leaflet number, fusion of leaves and leaflets and an altered placement of lateral leaflets (**Figs. 1G, H, 2G and H**). SEM analysis indicated that compound leaf primordia were initiated from the periphery of the SAM in a sequential order in wild-type plants (**Fig. 2J**). In a P2 stage leaf primordium, terminal and lateral leaflet, and stipule primordia were clearly visible and a boundary between stipule and lateral leaflet primordia was established (**Fig. 2J**). By contrast, compound leaf primordia appeared to be much broader with less recognizable leaflet primordia at the P2 stage in *Mtpin10* mutants (**Fig. 2K**). In some cases, almost the entire periphery of the SAM developed into compound leaf primordia (**Fig. 2L**), which may explain fusion of leaves and altered phyllotaxy seen in *Mtpin10* mutants (**Figs. 1H and 2H**).

Plant leaves, both simple and compound, exhibit characteristic leaf margin morphologies such as smooth, serrated or lobed margins due to secondary morphogenesis. Auxin activity maxima mediated by PIN1 are required not only for the initiation and differentiation of compound leaf primordia but also for the elaboration of margins of both simple and compound leaves.[14,27,31,32] Wild-type Medicago plants had serrated leaf margins (**Fig. 2G**). However, all leaves in *Mtpin10* mutants had smooth margins (**Fig. 2H** and inset). In *Mtpin10-1* plants stably transformed with an Arabidopsis *PIN1::PIN1:GFP* construct, which is sufficient to complement *A. thaliana pin1* mutants, all mutant phenotypes including smooth leaf margins were rescued (**Figs. 1I and 2I**). These results indicate that *MtPIN10* and the Arabidopsis *PIN1* are functional orthologs and *MtPIN10* plays a key role in diverse developmental processes ramified in *Mtpin10* mutants.

Abnormal floral development. In contrast to Arabidopsis *pin1* mutants, which exhibit a naked inflorescence meristem devoid of lateral organs, *Mtpin10* mutants developed flowers. However, inflorescence and flower were abnormal and plants were sterile. In wild-type Medicago plants, one to three flowers developed on a single stalk (**Fig. 3A**). In *Mtpin10* mutants, however, up to 10 flowers were formed and clustered on a stalk (**Fig. 3B and C**). And, unlike wild-type flowers that have a pea flower-like closed structure, all flowers were precociously opened in *Mtpin10* mutants (**Fig. 3A–C**). Medicago flowers have pentamerous organs in the outermost four whorls (sepals, petals, and outer and inner stamens) and a central carpel (**Fig. 3D**).[7,33] Floral organs were reduced in *Mtpin10* mutants, although the degree of reduction was variable among flowers (**Fig. 3E–G**). In the mutants, different floral organs were frequently fused. Unlike the central carpel enclosed by a stamen tube in wild-type plants, *Mtpin10* mutants had an exposed central carpel (**Fig. 3D–G**). In some flowers (5/60), two carpels were formed (**Fig. 3F**). It is noticeable that petals were much less serrated and petal teeth were fatter and shorter in *Mtpin10* mutants than wild-type counterparts (**Fig. 3H–K**).

SEM analysis indicated that early flower development appeared to be normal in *Mtpin10* mutants (**Fig. 3L and O**). In wild-type plants, development of floral organ primordia occurred along the abaxial-adaxial axis (**Fig. 3M**).[7,33] In *Mtpin10* mutants, the developmental polarity along the abaxial-adaxial axis was less pronounced (**Fig. 3P**). In wild-type plants, all floral organ primordia were clearly differentiated in a late S5 stage (**Fig. 3N**).

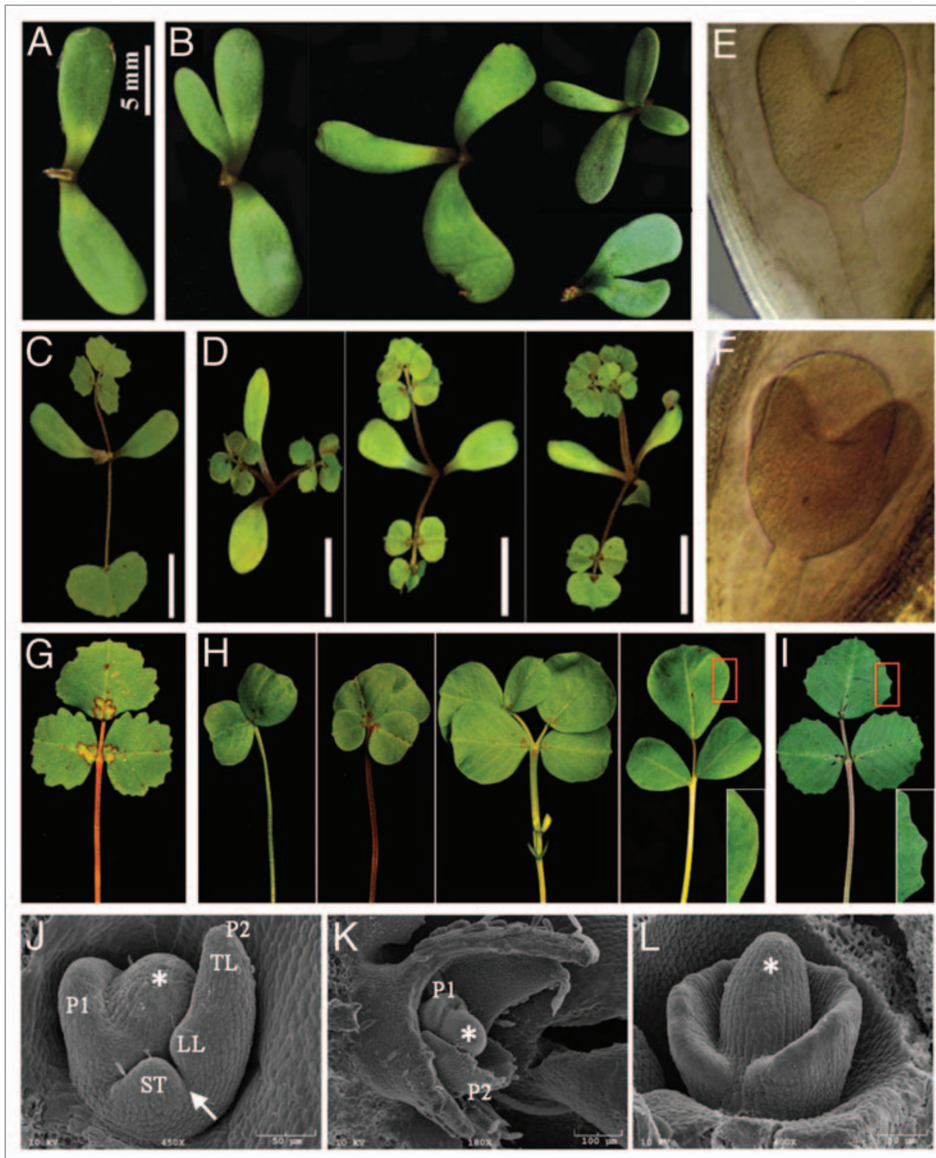

Figure 2. *Mtpin10* phenotypes. (A and B) *Mtpin10* mutants frequently exhibited three to four cotyledons and partial fusion of cotyledons (B). By contrast, all wild-type plants exhibited two separate cotyledons (A). (C and D) The juvenile leaf was always compound in *Mtpin10* mutants (D), but simple in wild-type plants (C). (E and F) Heart-stage embryos always had two cotyledons in wild-type plants (E). However, in *Mtpin10* (+/-) plants, three cotyledons were frequently observed (F). (G and H) Compound leaves of wild-type (G) and *Mtpin10-1* mutant (H). (G) A representative compound leaf of wild-type plants (R108). (H) Representative compound leaves of *Mtpin10-1* mutant. Observed were a wide range of abnormal leaves, including leaves lacking a terminal leaflet, leaves with multiple leaflets with fusion of terminal leaflets, leaves with multiple leaflets presumably derived from fusion of two leaves and trifoliate leaves with altered symmetry of lateral leaflets. In all cases, leaf margins were always smooth (H, inset), unlike leaf margin serrations in wild-type plants (G). (I) Genetic complementation of *Mtpin10-1* mutant with an Arabidopsis *PIN1::PIN1:GFP* construct. Shown was a representative compound leaf of *Mtpin10-1* transformed with the Arabidopsis *PIN1* gene. Wild-type compound leaves and leaf margin serrations (inset) were restored. (J–L) SEM analysis of leaf development. Compound leaf primordia were initiated from the periphery of SAM (asterisk). (J) In wild-type P2 leaf primordia, terminal (TL) and lateral leaflet (LL) and stipule (ST) primordia were clearly recognizable and a boundary between lateral leaflet and stipule primordia (arrow) was established. (K) In *Mtpin10* mutants, compound leaf primordia were initiated from the periphery of SAM but appeared to be much broader and without recognizable leaflet primordia at P2. (L) In some cases, almost the entire periphery of SAM gave rise to compound leaf primordia, which may explain fusion of leaves in mutants.

By contrast, floral organ primordia were misshaped and differentiation of common primordia in the second whorl, which would normally give rise to petals and stamens, was impaired in *Mtpin10* mutants (**Fig. 3P and Q**).

***MtPIN10* was expressed in the shoot apical meristem and leaf primordial.** Quantitative RT-PCR was first performed to investigate tissue specific expression pattern of *MtPIN10*. The results showed that *MtPIN10* was expressed in vegetative shoot apices and flowers (**Fig. 1C**). In mature leaves, however, *MtPIN10* expression was relatively low (**Fig. 1C**). These results are consistent with in silico expression analysis of *MtPIN10* (**Fig. S1**).

Next, RNA in situ hybridization was performed to identify cell types in which *MtPIN10* was expressed. Using an *MtPIN10*-specific probe, *MtPIN10* transcripts were detected in the SAM and P0 leaf primordia (**Fig. 1D**). In P2 and P3 leaf primordia, a high level of *MtPIN10* expression was confined to vascular and epidermal cells, as well as in tips of leaf and leaflet primordia (**Fig. 1D**; data not shown). As a negative control, a sense probe did not detect any signals (**Fig. 1E**). *MtPIN10* expression in the SAM suggests that it may play a role in SAM maintenance, whereas its expression in incipient sites of leaf initiation, and vascular and epidermal cells in developing leaf primordia is consistent with its role in mediating auxin transport in leaf and leaflet initiation and differentiation.

Cross-species genetic complementation of *Mtpin10*. To evaluate functional conservation of *MtPIN10* and the Arabidopsis *PIN1*, we introduced an Arabidopsis *PIN1::PIN1-GFP* construct that is sufficient to rescue *A. thaliana pin1* mutants into *Mtpin10-1* mutant via *Agrobacterium tumefaciense*-mediated transformation. Independent transgenic lines that were obtained exhibited wild-type morphologies, including two separate cotyledons, simple juvenile leaf, trifoliate adult leaves, serrated leaf margins, normal phyllotaxy and fertile flowers (**Figs. 1I and 2I**). These results

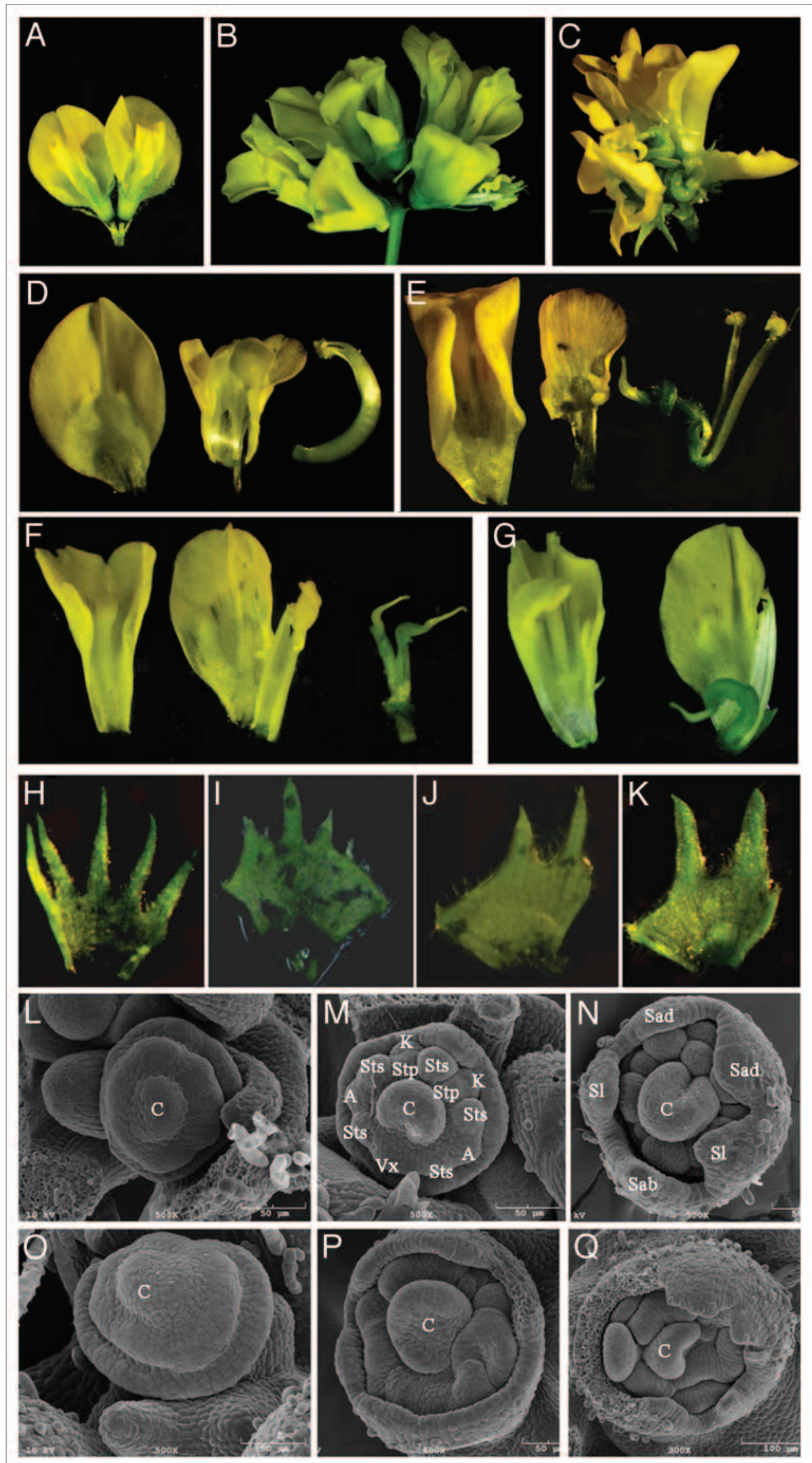

Figure 3. Floral defects in *Mtpin10* mutants. (A–C) Flowers developed on inflorescence stalks. Wild-type plants developed 1–3 flowers on each stalk (A), whereas *Mtpin10* mutants developed up to 10 flowers on a stalk (B). In contrast to a closed pea flower-like structure of wild-type flowers (A), flowers in *Mtpin10* mutants were always precociously opened (B and C). (D–K) Dissected floral organs. A wild-type flower had a standard petal (or vexillum), two keel petals, two fused alae petals, a central carpel and 10 fused stamens (D), and a circular sepal with five teeth (H). In *Mtpin10* flowers, floral organs were frequently missing and had irregular shapes (E–G); the central carpel was always exposed (E–G); stamens were not fused (E); two carpels were occasionally formed (5/60); and the circular sepal had a reduced number of teeth that were shorter and fatter than the wild-type counter parts (H–K). (L–Q) SEM analysis of flower development. Early stages of floral meristem development appeared to be similar in wild-type (L) and *Mtpin10-1* mutant (O). Between S4 and S5 stages, common primordia in the second whorl gave rise to petal and stamen primordia along the abaxial-adaxial direction in wild type (M). In *Mtpin10-1*, the developmental polarity was less pronounced and common primordia in the second whorl failed to properly differentiate, resulting in misshaped floral organ primordia (P). At a late S5 stage, all floral organ primordia were clearly developed in wild-type (N), but not in *Mtpin10-1* mutant (Q). C, carpel; Vx, vexillum; K, keel petal; A, alae petal; Sts, antesepal stamen; Stp, antepetal stamen; Sab, abaxial sepal; Sad, adaxial sepal; Sl, lateral sepal.

indicate that the Arabidopsis *PIN1* completely rescued *Mtpin10* mutant phenotypes and *MtPIN10* is the Medicago *PIN1* ortholog.

Genetic interactions between *sgl1* and *Mtpin10*. Previous studies have shown that *M. truncatula SGL1* is required for compound leaf and flower development.[7] To examine genetic interactions between *Mtpin10* and *sgl1*, we made crosses between *sgl1-1* and *Mtpin10-1* and generated *sgl1-1 Mtpin10-1* double mutants. Phenotypic analysis indicated that all leaves in the double mutants were simple, resembling

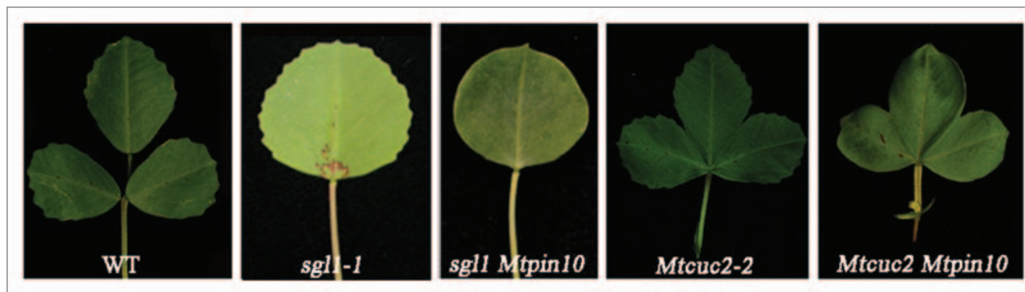

Figure 4. Genetic Interactions of *Mtpin10* and *single leaflet1* (*sgl1*), and *cup-shaped cotyledon2* (*Mtcuc2*). Shown were mature leaves of wild-type, *sgl1-1, sgl1-1 Mtpin10-1*, *Mtcuc2-2* and *Mtcuc2-1 Mtpin10-1* plants. Leaves of *sgl1 Mtpin10* double mutants are simple with smooth margins. Leaves of *Mtcuc2-2 Mtpin10-1* double mutants are partially fused with smooth margins.

the *sgl1* single mutant (**Fig.** 4). However, leaf margins of the double mutants were smooth, resembling the *Mtpin10* single mutant. On the other hand, flowers of the *sgl1 Mtpin10* double mutants were cauliflower-like, resembling *sgl1*. Taking together, these results indicate that *sgl1* is genetically epistatic to *Mtpin10* in leaflet development and inflorescence differentiation, and *Mtpin10* is epistatic to *sgl1* in control of leaf margin serration.

Genetic interactions between *Mtcuc2* and *Mtpin10*. It has been recently shown that *MtCUC2* encoding a NAC domain transcription factor is required for maintenance of the SAM and development of organ boundaries in *M. truncatula* (Cheng et al., unpublished results). *Mtcuc2-1*, a strong allele, does not form any shoot structures; whereas *Mtcuc2-2*, a weak allele, exhibits partial fusion of cotyledons and leaflets but with wild type-like leaf margin serrations (**Fig.** 4). To evaluate genetic interactions between *Mtcuc2* and *Mtpin10*, we constructed *Mtcuc2-2 Mtpin10-1* double mutants. The double mutants exhibited fusion of cotyledons and leaflets that resembles the *Mtcuc2-2* single mutant and smooth leaf margins that resembles the *Mtpin10-1* single mutant. These results indicate that *Mtcuc2* is genetically epistatic to *Mtpin10* in boundary separation and leaf patterning; whereas *Mtpin10* is epistatic to *Mtcuc2* in leaf margin serration. Similar to tomato *GOB*, *MtCUC2* is the only *CUC*-like gene identified in *M. truncatula* genome. Yet, *Mtcuc2* mutants exhibited pronounced defects in shoot apical meristem maintenance and boundary separation. Shoots can be developed in regenerated *Mtcuc2-1* mutant through tissue culture. However, both *Mtcuc2-1* and *Mtcuc2-2* mutant alleles had wild type-like serrated leaf margins (**Fig.** 4). These results suggest that *MtCUC2* may not play a prominent role in leaf margin serration, like *NAM*/*CUC* genes from other species do. Alternately, some unidentified *MtCUC2* homologs that may exist in the *M. truncatula* genome mask the role of *MtCUC2* in leaf margin formation.

Our mutant studies identified a key role for *MtPIN10* encoding an auxin efflux transporter in diverse developmental processes in *M. truncatula*. The role of *MtPIN10* in embryonic cotyledon development is similar to the Arabidopsis *PIN1*. However, the role of *MtPIN10* in compound leaf development appears to be different from the *PIN1* ortholog in *C. hirsuta*, a compound leafed close relative of *A. thaliana*. The role of *MtPIN10* in inflorescence and floral meristem development also appears to be different from the Arabidopsis *PIN1*. In addition, our double mutant analysis revealed complex genetic interactions of *MtPIN10* with *SGL1* and *MtCUC2* in both leaf and flower development. Our studies revealed that *MtPIN10* but not *MtCUC2* plays a prominent role in leaf margin serration. Cross-species genetic complementation studies not only support functional conservation of *MtPIN10* and the Arabidopsis *PIN1* but also raise some interesting questions how fundamental mechanisms are differentially deployed to regulate developmental processes in independent lineages of plants during evolution.

Materials and Methods

Plant growth. *M. truncatula* seeds were treated and germinated as previously described in reference 7.

Phylogenetic analysis. Cluster X was used for multiple sequence alignments as previously described in reference 34. Phylogenetic trees were constructed using neighbor-joining, maximum parsimony and UPGMA algorithms implemented in MEGA software suite (www.megasoftware.net)[35] with 1,000 bootstrap replicates.

Reverse genetics screening of *Mtpin10* mutants. To isolate *Mtpin10* mutants, PCR-based reverse genetics screening of the Medicago *Tnt1* mutant population was performed as previously described in reference 30. Briefly, nested PCRs using the following nested forward and reverse primers, MtPIN10-N1-F, MtPIN10-N2-F, MtPIN10-N1-R and MtPIN10-N2-R, Tnt1-F, Tnt1-F1, Tnt1-R and Tnt1-R1 were performed and a total of 18 super-pools of DNA samples prepared from 9,000 *Tnt1* insertion mutant lines were screened. Positive PCR products were subsequently confirmed in lower level DNA pools and individual lines. The PCR products were purified, cloned and sequenced using Tnt1-F2 or Tnt1-R2 primer. Resulting flanking sequences were used to determine *Tnt1* insertion sites in *MtPIN10*. MtPIN10-F and MtPIN10-R primers were used to amplify *MtPIN10* transcripts by reverse transcription (RT)-PCR, and MtPIN10-q-F and MtPIN10-q-R primers were used in quantitative RT-PCR analysis of *MtPIN10* gene expression.

RNA in situ hybridization. RNA in situ hybridization was performed as previously described in references 7, 36 and 37. Briefly, *MtPIN10* probes were generated from a non-conserved region of *MtPIN10*. Ten micrometer sections prepared from

Table 1. Primers used in this study

Name	Forward primer	Reverse primer
MtPIN10-RT	ATG ATA AGT GCT TTA GAC TTA TAC	TCA AAG TCC CAA TAA AAT GTA GTA AAC
MtPIN10-N1	AAA GTC CTC CCC TTT TTA CCA TAT CC	AAA TAT GCA AAA GTT GGG TTC ACA GT
MtPIN10-N2	ACC ATA TCC CTG TCT TCT TCC A	ACT ACC TAA CTA GCT ATT GCC CT
AtPIN1-RT	CTA TGA TCC TCG CTT ACG GCTC	GTT TAG CAG GAC CAC CGT CTT C
MtPIN10-q	CCA TGT CTT TAG AGG TGG TGG TG	CAC TGG TCC TTC TTT GTC CAC AC
MtActinB-RT	TCT TAC TCT CAA GTA CCC CAT TGA GC	GTG GGA GTG CAT AAC CTT CAT AGA TT
MtActinB-q	TCA ATG TGC CTG CCA TGT ATG T	ACT CAC ACC GTC ACC AGA ATC C

shoot apices of 2-week-old wild type plants were hybridized with digoxigenin-labeled sense or antisense probes. Primer sequences are listed in **Table 1**.

Tissue clearing. Flowers and seeds dissected from young pods 1-4 d after pollination were cleared in Hoyer's solution (7.5 g gum Arabic, 100 g chloral hydrate, 5 ml glycerol and 60 ml ddH_2O). Ovules and embryos were further dissected and observed under microscope (specify the type of microscope used).

Scanning electron microscopy. Shoot apices of 2- to 4-week-old seedlings were subjected to vacuum infiltration in a fixative solution (3% glutaraldehyde in 25 mM phosphate buffer, pH 7.0) for 1 h and then incubated at 4°C overnight. Plant tissues were further fixed with 1.0% osmium tetroxide in the same fixative buffer overnight and dehydrated in a graded ethanol series. Scanning electron microscopy (SEM) was performed as described previously in reference 7.

RNA Extraction, RT-PCR, qRT-PCR. Roots, stems, leaves, vegetative shoots, inflorescence shoots, flower buds, young pods and young seeds were collected from *M. truncatula* wild-type (R108) plants. Vegetative shoot buds were collected from wild-type and homozygous *Mtpin10-1* and *Mtpin10-2* mutants. Total RNA was extracted using Trizol (Invitrogen) and treated with Turbo DNase I (Ambion). Three micrograms of total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen) with olig$(dT)_{20}$ primer. Two microliters of 1:20 diluted cDNA were used as templates. Gene-specific primers used are listed in **Table 1**. Quantitative RT-PCR was performed as previously described in reference 38. The expression level was normalized with *M. truncatula ACTIN* (tentative consensus no. 107326).

Stable plant transformation. An Arabidopsis *PIN1::PIN1:GFP* construct was introduced into *Agrobacterium tumefaciens* EHA105 strain by electroporation. *Mtpin10* heterozygous plants were transformed following the protocol previously reported in reference 39.

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Note

Supplemental material can be found at
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