The Trans-Acting Short Interfering RNA3 Pathway and NO APICAL MERISTEM Antagonistically Regulate Leaf Margin Development and Lateral Organ Separation, as Revealed by Analysis of an argonaute7/lobed leaflet1 Mutant in Medicago truncatula

Chuanen Zhou, a Lu Han, a Chunxiang Fu, a Jiangqi Wen, b Xiaofei Cheng, b Jin Nakashima, b Junying Ma, b Yuhong Tang, b Yang Tan, a Million Tadege, c Kirankumar S. Mysore, b Guangmin Xia, d and Zeng-Yu Wang a, 1

a Forage Improvement Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401
b Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401
c Institute of Agricultural Biosciences, Oklahoma State University, Ardmore, Oklahoma 73401
d Key Laboratory of Plant Cell Engineering and Germplasm Innovation, Ministry of Education, School of Life Sciences, Shandong University, Jinan 250100, China

Leaf shape elaboration and organ separation are critical for plant morphogenesis. We characterized the developmental roles of LOBED LEAFLET1 by analyzing a recessive mutant in the model legume Medicago truncatula. An ortholog of Arabidopsis thaliana ARGONATE7 (AGO7), Mt-AGO7/LOBED LEAFLET1, is required for the biogenesis of a trans-acting short interfering RNA (ta-siRNA) to negatively regulate the expression of AUXIN RESPONSE FACTORS in M. truncatula. Loss of function in AGO7 results in pleiotropic phenotypes in different organs. The prominent phenotype of the ago7 mutant is lobed leaf margins and more widely spaced lateral organs, suggesting that the trans-acting siRNA3 (TAS3) pathway negatively regulates the formation of boundaries and the separation of lateral organs in M. truncatula. Genetic interaction analysis with the smooth leaf margin1 (slm1) mutant revealed that leaf margin formation is cooperatively regulated by the auxin/SLM1 (ortholog of Arabidopsis PIN-FORMED1) module, which influences the initiation of leaf margin teeth, and the TAS3 ta-siRNA pathway, which determines the degree of margin indentation. Further investigations showed that the TAS3 ta-siRNA pathway and NO APICAL MERISTEM (ortholog of Arabidopsis CUP-SHAPED COTYLEDON) antagonistically regulate both leaf margin development and lateral organ separation, and the regulation is partially dependent on the auxin/SLM1 module.

INTRODUCTION

New organs are formed from meristems throughout the life cycle of a plant. Well-organized and regulated cell division and expansion are crucial for the patterning and elaboration of organ primordia, requiring a mechanism for reiterative specification of new boundaries (Aida and Tasaka, 2006; Berger et al., 2009). Auxin is one of the major plant hormones that has vast effects on plant developmental processes. The local auxin activity gradient generated by the auxin efflux regulator PIN-FORMED1 (PIN1) is one such mechanism that facilitates initiation of organs at the flanks of the shoot apical meristem (SAM). The polar localization of PIN1 in auxin-conducting cells determines the direction of auxin flow. Loss of function of PIN1 leads to defects in the separation of lateral organs (Vernoux et al., 2000; Reinhardt et al., 2003). Although the auxin accumulation pattern regulated by PIN1 is a prerequisite for the identification of boundaries during organ initiation, the creation of such boundaries also requires coordinated actions with other genes such as NAC-domain transcription factor genes (Aida and Tasaka, 2006; Blein et al., 2008; Rast and Simon, 2008; Berger et al., 2009). Leaf shape depends on the pattern of serrations and the degree of indentation of leaf margins. Previous studies have shown that two key processes are required for the development of leaf margins in Arabidopsis thaliana. The first process is the local auxin activity gradient generated by PIN1 (Hay et al., 2006). The formation of marginal serration tips is tightly correlated with auxin activity maxima, and the formation of lateral veins is defined by internalizing auxin through the center of the serrations. Perturbation of auxin transport by 1-N-naphthylphthalamic acid results in smooth leaf margins (DeMason and Chawla, 2004; Hay et al., 2006; Barkoulas et al., 2008; Zhou et al., 2011a). The second process is the repression of growth at the sinus by CUP-SHAPED COTYLEDON2 (CUC2). Two feedback loops involving PIN1 and CUC2 have been conceptualized for the formation of leaf serrations in Arabidopsis (Blisborough et al., 2011).

Trans-acting short interfering RNAs (ta-siRNAs) are endogenous siRNAs that act in trans and direct the cleavage of complementary mRNA. The production of ta-siRNAs requires components from both microRNA and siRNA biogenesis pathways, including SUPPRESSOR OF GENE SILENCING3 (SGS3), RNA-DEPENDENT RNA
The roles of ta-siRNAs in leaf patterning and leaf polarity vary substantially among species. The Arabidopsis ago7 mutants display increased leaf length and downward-curled leaf margin; however, the mutants do not show obvious leaf polarity defects (Hunter et al., 2003, 2006; Fahlgren et al., 2006). Tomato (Solanum lycopersicum) WIRY genes are involved in ta-siRNA biogenesis, and the wiry mutants show shoestring leaves with defects in leaf blade expansion (Yifrach et al., 2012). In the legume species Lotus japonicus, mutations in REDUCED LEAFLET1 (REL1) and REDUCED LEAFLET3 (REL3), which respectively encode the orthologs of Arabidopsis SGS3 and AGO7, result in abaxialized leaflets with decreased leaflet numbers (Yan et al., 2010). In monocots, more obvious developmental defects have been observed in ta-siRNA pathway-related mutants. Blocking of the TAS3 ta-siRNA pathway in rice (Oryza sativa) leads to complete deletion or abnormal formation of the SAM (Liu et al., 2007; Nagasaki et al., 2007). In maize (Zea mays), the leafbladeless1 mutant, which carries a mutation in SGS3, develops radially symmetric, thread-like leaves with partial or complete loss of adaxial cell identity (Timmermans et al., 1998; Juarez et al., 2004; Noguera et al., 2007). However, the maize ago7 mutant, ragged seedling2, maintains dorsiventral polarity of the leaf (Douglas et al., 2010).

A well-defined adaxial-abaxial domain in leaves is crucial for subsequent leaf expansion and leaf shape (Braybrook and Kuhlemeier, 2010; Wang et al., 2011). Independent studies have shown that the ta-siRNA pathway is broadly involved in the determination of dorsiventral polarity of leaves among species (Timmermans et al., 1998; Juarez et al., 2004; Liu et al., 2007; Yan et al., 2010). Therefore, the ta-siRNA pathway probably plays a key role in leaf morphogenesis. In the model legume Medicago truncatula, the orthologs of Arabidopsis PIN1 and CUC have been identified and named SMOOTH LEAF MARGIN1 (SLM1) and NO APICAL MERISTEM (NAM), respectively (Zhou et al., 2011a; Cheng et al., 2012). The slm1 mutant exhibits a smooth leaf margin as a result of diffuse auxin distribution, suggesting the conserved roles of the auxin/SLM1 module in different plant species (DeMason and Chawla, 2004; Barkoulas et al., 2008; Koenig et al., 2009; Zhou et al., 2011a). In addition, the loss of function of SLM1 or NAM in M. truncatula leads to defects in the separation of lateral organs, indicating that both of them are positively correlated with the promotion of organ boundaries. However, thus far, it is not clear how plants prevent organs/tissues from overseparation during differential growth and morphogenesis. The regulator that plays negative roles in the formation of boundaries remains unclear. In this study, we analyzed the developmental roles of the TAS3 ta-siRNA pathway in M. truncatula by characterizing a mutant with lobed leaf margins, lobed leaflet1 (lol1). Molecular analyses show that LOL1 is an ortholog of Arabidopsis AGO7, which is specifically required for the biogenesis of TAS3 ta-siRNA. Loss of function of AGO7/LOL1 leads to pleiotropic defects in different plant organs as a result of the ectopic expression of target ARF genes. The prominent phenotype of the ago7/lol1 mutants is a lobed leaf margin and more pronounced lateral organ separation, implying that the TAS3 ta-siRNA pathway functions as a repressor during the formation of boundaries of organs/tissues in M. truncatula. Genetic interaction analyses with other mutants suggest that the TAS3 ta-siRNA pathway and NAM antagonistically regulate leaf margin elaboration and lateral organ separation, and that such regulation is partially dependent on the auxin/SLM1 module.

RESULTS

LOL1 Regulates Leaf and Flower Development

Four mutant lines showing obvious changes in leaf margins were identified from screening ~10,000 M. truncatula mutants tagged by the transposable element of tobacco (Nicotiana tabacum) cell type1 (Tnt1). In contrast with the serrated leaf margin in the wild type (Figure 1A), all of these mutants displayed lobed leaf margins (Figure 1B). The mutants were thus named lobed leaflet1 (lol1-1 to lol1-4). Although M. truncatula is a compound leaf species, its leaf development is heteroblastic. The first true leaf, also called the juvenile leaf, has simple leaf morphology (Figures 1C and 1D). All adult leaves that subsequently develop are in trifoliate form (Figures 1A and 1C). In the juvenile leaf, ~43% of the mutants showed one or two extra lateral leaflets (Figures 1E to 1G). In the adult phase, lol1-1 exhibited lobed and elongated adult leaves (Figure 1B; see Supplemental Table 1 online). These observations suggest that loss of function of LOL1 has different effects on leaf development at the juvenile and adult stages. Anatomical analysis revealed that the phloem was enlarged on the abaxial side of leaves and the palisade mesophyll cells became smaller in lol1-1 compared with those of the wild type (Figures 1H and 1I). Cross sections of petioles in the wild type showed that three vascular bundles were arranged in a polar fashion, and two of them were located on the adaxial side of the petiole (Figure 1J). By contrast, the number of vascular bundles on the adaxial side of the petiole was decreased in lol1-1 (Figure 1K). In addition, the shape of cross sections of petioles in lol1-1 was changed to nearly round. These observations indicate that the polarity of lateral organs is altered and that these organs are partially abaxialized in lol1-1.

Flowers developed in lol1-1 were abnormal and the plants were infertile. The M. truncatula flower contains three types of petals. The largest petal (vexillum) is located at the adaxial position of the flower. Two lateral petals (alae) and two fused short petals (keel) are fused at the basal region and are situated at the abaxial side of the flower (Figures 2A to 2D) (Benlloch et al., 2003). More widely...
spaced keel and alae were observed in lol1-1, suggesting that LOL1 affects the boundary formation of floral organs (Figures 2E to 2H). Furthermore, the lesion in LOL1 resulted in defects in the central carpel, anther dehiscence, and pollen viability (Figures 2I to 2P). As a result, mature plants of all four alleles of LOL1 failed to develop seedpods.

LOL1 Encodes an ARGONAUTE Protein

Flanking sequences of Tnt1 retrotransposon in lol1-1 were recovered by thermal asymmetric interlaced PCR, and one of the sequences was confirmed to be associated with the mutation. A genomic sequence of 4088 nucleotides was obtained by blasting this flanking sequence against the M. truncatula genomic sequences. The full-length coding sequence (CDS) of LOL1 contains 3057 nucleotides (Figure 3A). PCR amplification of the LOL1 genomic sequence from the four alleles and the wild type revealed that all mutants carried a single 5.3-kb insertion (Figure 3B). Further analysis showed that a Tnt1 retrotransposon was inserted in exons of LOL1 in lol1-1, lol1-2, and lol1-3. However, lol1-4 carried a native retrotransposon, Medicago RetroElement1 (Rakocevic et al., 2009) (Figure 3A). The expression of LOL1 was interrupted in all four lol1 alleles (Figure 3C). Furthermore, the defects in lol1-1 were fully rescued after transforming the LOL1 CDS driven by the native or the cauliflower mosaic virus 35S promoter into homozygous mutant plants (see Supplemental Figure 1 and Supplemental Table 1 online). No obvious phenotypic changes were observed in LOL1-overexpressing lines in the wild type (see Supplemental Figure 1 online). Bioinformatic analysis showed that LOL1 belongs to the ARGONAUTE protein family and is the putative ortholog of the Arabidopsis AGO7 in M. truncatula (see Supplemental Figure 2 and Supplemental Data Set 1 online). To allow consistent nomenclature, we renamed LOL1 as AGO7.

RT-PCR analysis revealed that AGO7 was expressed in almost all tissues. The expression level of AGO7 was relatively high in flowers, stems, and leaves, and relatively low in cotyledons, pods, and roots (Figure 3D). RNA in situ hybridization was conducted to further examine the spatial expression patterns of AGO7. At the vegetative stage, preferential expression of AGO7 was observed on the adaxial side of the emerging leaf primordia and developing leaflets but not in the SAM (Figures 3E to 3G). In the reproductive stage, higher levels of AGO7 expression were detected in the floral meristem and developing floral organs such as petals and ovules (Figures 3H to 3J). These data indicate that AGO7 plays a role in

![Figure 1](image-url)
plant development in both the vegetative and reproductive stages. In addition, the asymmetric expression pattern in leaf primordia implies that AGO7 is probably involved in the establishment of adaxial/abaxial leaf polarity, as also evidenced by the defects in dorsiventral polarity in the ago7-1 mutant (Figures 1H to 1K).

**AGO7 Functions in the TAS3 ta-siRNA Pathway in *M. truncatula***

It has been shown that AGO7 is specifically required for TAS3 ta-siRNA (Montgomery et al., 2008). To determine whether AGO7 plays a similar role in the TAS3 ta-siRNA pathway in *M. truncatula*, a putative TAS3 ta-siRNA gene (AC186679.3) was obtained by conducting a BLAST database search. Two 21-nucleotide–long TAS3 ta-siRNAs were identified based on the sequences of the TAS3 ta-siRNA precursor and miR390 in *M. truncatula* (Figure 4A). The TAS3 ta-siRNAs showed high similarity with those in other species (Figure 4B). Furthermore, the transcript levels of TAS3 5′D7 (+) and TAS3 5′D8 (+) were dramatically reduced, whereas miR390 was accumulated in ago7-1, indicating that the AGO7 gene is required for the biogenesis of TAS3 ta-siRNA in *M. truncatula* (Figure 4C).

Microarray analysis was performed to determine the target genes of TAS3 ta-siRNAs in *M. truncatula* (see Supplemental Data Sets 2 and 3 online). The expression levels of three *M. truncatula* ARF genes were upregulated dramatically in ago7-1, suggesting that they are the putative targets of TAS3 ta-siRNAs. Phylogenetic analysis revealed that one ARF (AC158497_40.1, designated ARF3) is the putative ortholog of *Arabidopsis* ARF3. The other two (AC152176_2.1 and AC150891_17.1, designated

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**Figure 2. lol1-1/ago7-1 Mutant of *M. truncatula* Shows Defects in Flower Development.**

(A) Flower phenotype in the wild type. Arrow points to the bottom of fused alae and keel.
(B) to (D) Dissected floral organs of the wild type. The top view of vexillum (B), the top view (left) and bottom view (right) of fused alae and keel (C), and the top view of a dissected sepal (D). Arrows point to the bottom of fused alae and keel.
(E) Flower phenotype in the lol1-1/ago7-1. Arrowhead points to the overseparated alae and keel.
(F) to (H) Dissected floral organs of the lol1-1/ago7-1. The top view of vexillum (F), the separated alae and keel (G), and the top view of a sepal (H). Arrowheads point to the basal regions of overseparated alae and keel.
(I) and (J) The side view of the central carpel in the wild type (I) and lol1-1/ago7-1 (J). The insets in (I) and (J) show the top view of central carpels.
(K) and (L) Scanning electron microscopy analysis of the central carpel in the wild type (K) and lol1-1/ago7-1 (L). Arrow in (K) points to the closed central carpel. Arrowhead in (L) points to the opened central carpel and exposed ovules.
(M) and (N) Scanning electron microscopy analysis of anthers in the wild type (M) and lol1-1/ago7-1 (N). Arrow in (M) points to the dehiscing anther. Arrowhead in (N) shows defect in anther dehiscence in lol1-1/ago7-1.
(O) and (P) Pollen staining in the wild type (O) and lol1-1/ago7-1 (P). The size of pollen and anther sacs was uneven and pollen was partially viable in lol1-1/ago7-1.

A, alae; AN, anther; C, carpel; FI, filament; K, keel; OV, ovule; WT, wild type.

Bar in (A) to (J) = 2 mm; bar in (K) to (P) = 200 μm.
ARF4a and ARF4b, respectively) are evolutionarily closer to Arabidopsis ARF4 and show high identity (71%) with each other. Therefore, they are probably the duplicated ARF4 orthologs in M. truncatula (see Supplemental Figure 3 and Supplemental Data Set 4 online). ARF3 and ARF4a/b are highly expressed in multiple organs, implying that they play broad roles during plant development (see Supplemental Figure 4 online). Further analysis showed that all three ARF genes contained two recognition sites (A and B) that are complementary to the TAS3 ta-siRNAs (Figure 4D). Quantitative RT-PCR (qRT-PCR) analysis showed

Figure 3. Molecular Cloning and Expression Pattern of AGO7 in M. truncatula.

(A) Schematic representation of the gene structure of AGO7. Three exons (block) and two introns (line) are shown. Numbers indicate nucleotide positions of the site of mutations.
(B) PCR amplification of AGO7 from the wild type and ago7 mutants. A single insertion (∼5.3 kb) was detected in each mutant line.
(C) RT-PCR analysis of AGO7 transcripts in the wild type and ago7 mutants. Actin was used as the loading control. Three technical replicates were performed.
(D) RT-PCR analysis of AGO7 expression in different plant organs. Actin was used as the loading control. Three technical replicates were performed.
(E) to (J) In situ hybridization analysis of AGO7 mRNA in vegetative and reproductive apices of the wild type. Bar = 50 μm in (E) to (J).
(E) to (G) Longitudinal sections of the SAM at stages 3 and 4 ([E] and [F], respectively). The sense probe was hybridized and used as the control (G).
(H) to (J) Longitudinal sections of the floral apical meristem at stages 5 and 7 ([H] and [I]). The sense probe was hybridized and used as the control (J).
AB, abaxial; AD, adaxial; AN, anther; C, carpel; P, petal; S, stage; WT, wild type.
Figure 4. Characterization of Putative TAS3 ta-siRNA and Target Genes in M. truncatula.

(A) A diagram represents the biogenesis of predicted TAS3 ta-siRNAs from TAS3 transcript directed by miR390. Putative ta-siRNAs are shown alternately in yellow and green.

(B) Alignment of TAS3 ta-siRNAs among Mt (M. truncatula), Lj (L. japonicus), At (Arabidopsis), and Os (rice).

(C) RT-PCR analyses of TAS3 5’D7(+), TAS3 5’D8(+), and miR390 in the wild type (WT) and ago7-1. EF1a was used as a control. Three technical replicates were performed.

(D) Diagram represents TAS3 ta-siRNA and the coding sequence of three putative target ARF genes.

(E) Relative expression level of three ARF genes in the juvenile leaf (first foliage leaf) and second foliage leaf of the wild type and ago7-1. Values are the mean and SD of three biological replicates. *P < 0.05; **P < 0.01.
Figure 5. Involvement of AGO7 in Leaf Margin Development in *M. truncatula*.

(A) and (B) Observation of margin cells in the wild type (WT) (A) and ago7-1 (B) by scanning electron microscopy. Arrows point to the margin cells. Arrowheads point to the epidermal cells.

(C) and (D) Transverse sections of leaf margin in the wild type (C) and ago7-1 (D). Arrow points to the ridge-like structure on the surface of margin cells in the wild type. Arrowhead points to the smooth surface of margin cells in ago7-1.

(E) to (H) Observation of margin cells at the teeth tips in the wild type (E and G) and ago7-1 (F and H). Margin cells harboring the auxin response marker DR5 (*DR5rev:GFP*) were observed by scanning electron microscopy (E and F) and confocal microscopy (G and H). Cells with the ridge-like structure on the surface are marked in green in (E) and (F).
that expression levels of the three ARF genes were significantly upregulated in ago7-1 leaves at different stages (Figure 4E; see Supplemental Figure 5 online). These results suggest that ARF3 and ARF4a/b are the putative targets of the TAS3 ta-siRNA pathway.

**The TAS3 ta-siRNA Pathway Regulates Leaf Margin Development in *M. truncatula***

One prominent phenotype of ago7-1 is the conversion of the serrated leaf margin to the lobed leaf margin. In the wild type, leaf margin tips started to initiate along the leaf margin after the formation of leaflet primordia (see Supplemental Figures 6A to 6H online). Compared with the wild type, ago7-1 exhibited more pronounced leaf serration and sinuses (see Supplemental Figures 6I to 6P online). In addition, a ridge-like structure was observed on the surface of leaf margin cells in the wild type. By contrast, ago7-1 exhibited a smooth surface of leaf margin cells that was similar to that of epidermal cells (Figures 5A to 5F). These observations indicate that leaf margin cells in ago7-1 probably lost the determination of cell fate.

To monitor auxin responsiveness in leaf margin cells, the *DR5rev:green fluorescent protein* (GFP) reporter was transformed into wild-type and ago7-1 plants. By examining the GFP expression signal, a higher level of auxin responsiveness was observed in the margin cells of ago7-1 compared with those in the wild type (Figures 5G and 5H). GFP signal also offers a convenient way of visualizing cell size. At the tooth tip, the size of margin cells in ago7-1 was similar to that of the wild type (Figures 5G and 5H). In the sinuses, the margin cells (purple color) of the wild type were morphologically elongated compared with epidermal cells (yellow color) (Figure 5I). However, the size of margin cells in the sinus of ago7-1 was similar to that of epidermal cells (Figure 5J). Furthermore, large numbers of small, nonelongated margin cells were observed in the sinus of ago7-1 compared with the wild type (Figures 5K and 5L). Taken together, these data demonstrate that the lobed leaf of ago7-1 is caused by increased cell proliferation activity and repressed elongation of margin cells in the sinus of the leaf margin (Figures 5M and 5N).

**Lobed Leaf Margin in ago7-1 Is Caused by the Ectopic Expression of ARF3**

In fully expanded leaves, the expression of ARF3 was increased by ~13-fold in ago7-1, whereas ARF4a and ARF4b were upregulated by approximately twofold compared with the wild type (see Supplemental Figure 5 online), implying that the ectopic expression of ARF3 is the primary cause of the ago7-1 phenotype. Three experiments were performed to verify this hypothesis. First, the expression pattern of ARF3 was compared between the wild type and ago7-1 by in situ hybridization. In the wild type, a weak expression of ARF3 was detected in SAM. In the leaf primordia at the plastochron 2 (P2) stage, abaxial expression of ARF3 was displayed (Figure 6A). By contrast, ARF3 was highly expressed over a much broader region throughout the leaf primordia in ago7-1, suggesting that ARF3 expression is repressed by the TAS3 ta-siRNA pathway during leaf primordia development (Figure 6A).

Second, ARF3 knockdown (*ARF3knockdown*) plants were generated using RNA interference in the ago7-1 background. The expression level of ARF3 was dramatically reduced in ago7-1 transgenic plants, and the lobed leaf margin was completely recovered (Figures 6B to 6G). The transcript levels of ARF4a and ARF4b in the *ARF3knockdown* ago7-1 plants were similar to that in ago7-1 (see Supplemental Figure 7 online). These data suggest that the lobed leaf margin in ago7-1 was specifically caused by the misexpression of ARF3. Third, to further confirm the above observations, the original ARF3 cDNA (OX-ARF3) and a mutated ARF3 cDNA carrying two altered ta-siARF target sites (OX-ARF3mut; Figure 6B) were introduced into wild-type plants under the regulation of the cauliflower mosaic virus 3SS promoter, respectively. The expression level of ARF3 was significantly upregulated in both the OX-ARF3 and the OX-ARF3mut transgenic plants (Figure 6C). OX-ARF3 plants exhibited downward-curled leaves and showed more serrations along the leaf margin (Figures 6H to 6J). By contrast, OX-ARF3mut transgenic plants displayed obvious lobed leaf margin, mimicking the ago7-1 phenotype (Figures 6K to 6M). Taken together, these observations demonstrate that the lobed leaf margin in ago7-1 is caused by the ectopic expression of ARF3.

**ARF3 Expression Pattern and Auxin Responsiveness Is Altered in the Leaf Margin of ago7-1**

To further evaluate the expression pattern of ARF3 in the leaf, the transcript level of ARF3 was examined in different regions of leaves in the wild type and ago7-1 by dissecting the leaves into three regions (Figure 6N). In wild-type leaves, the expression of ARF3 was similar in the middle and marginal (outer) regions, and the expression in both of these regions was lower than that of the inner region of the leaf. In ago7-1, a significantly higher level of ARF3 expression was detected in the marginal region than in the middle region (Figure 6N). Characterization of transgenic plants carrying *DR5:GUS and DR5rev:GFP* constructs revealed that β-glucuronidase (GUS) activity or GFP signal was slightly increased in lateral leaf primordia and highly increased in floral organs and leaf margins, especially in the leaf teeth of ago7-1 (Figure 6O; see Supplemental Figure 8 online). The results indicate the auxin responsiveness was changed. To test whether the transcription of ARF3 depends on auxin, the expression level of

*Figure 5. (continued).*

(i) to (L) Observation of margin cells in the leaf sinus in the wild type ([I] and [K]) and ago7-1 ([J] and [L]). The margin cells are marked in purple and epidermal cells are marked in yellow in (I) and (J). Arrows point to the elongated margin cells in the wild type. Arrowheads point to the small and unelongated margin cells in ago7-1. (M) and (N) A schematic illustration of the developmental difference of leaf margin cells in the wild type (M) and ago7-1 (N). Bar in (A) to (D) = 20 μm; bar in (E) to (L) = 100 μm.
ARF3 Expression Pattern and Auxin Distribution Is Altered in ago7-1.

Figure 6. ARF3 Expression Pattern and Auxin Distribution Is Altered in ago7-1.
ARF3 was measured in leaves treated with indole-3-acetic acid. No change in ARF3 expression was observed in response to exogenous application of auxin (see Supplemental Figure 9 online). Therefore, the alteration of the ARF3 expression pattern in leaf margins was not caused directly by altered auxin responsiveness in ago7-1.

Formation of Lobed Leaf Margin in ago7-1 Is Partially Dependent on the Auxin/SLM1 Module

A previous study showed that SLM1 regulates leaf development by generating local auxin activity gradients (Zhou et al., 2011a). Compared with the wild type, the expression levels of SLM1 were reduced in both leaf buds (Figure 7A) and fully expanded leaves in ago7-1 (Figures 7B and 7C). The results were further confirmed by assaying the expression of the SLM1 promoter–GUS reporter in wild-type and ago7-1 backgrounds (Figures 7D to 7G).

The role of SLM1 in the formation of lobed leaf margin in ago7-1 was further examined by generating a double mutant between slm1-1 and ago7-1 (Figures 7H to 7K). Both the slm1-1 single mutant and slm1-1 ago7-1 double mutant could produce a few leaf teeth at the early stage, and the numbers of teeth are similar between them (see Supplemental Figure 10 online). The leaf margin in slm1-1 eventually became smooth because of a disruption of auxin distribution (Figure 7J) (Zhou et al., 2011a). However, a few serrations were formed in the slm1-1 ago7-1 double mutant (Figure 7K). To further compare the leaf shape among the wild type and mutants, the terminal leaflets were outlined and overlapped with different colors (Figure 7L). The length and width of the terminal leaflet in the double mutant were intermediate between those of the two single mutants. Moreover, the contours of the terminal leaflet in the double mutant were similar to that of the wild type and distinct from those in single mutants. These observations suggest that the lobed leaf in ago7 is partially dependent on the auxin/SLM1 module.

The TAS3 ta-siRNA Pathway and NAM Antagonistically Regulate Leaf Margin Serration Development and Lateral Organ Separation

A recent study showed that NAM, a putative M. truncatula ortholog of NAM/CUC, is involved in the development of organ boundaries in M. truncatula (Cheng et al., 2012). The strong allele, nam-1, cannot maintain the SAM, resulting in a seedling-lethal phenotype. nam-2 is a weak allele that can produce leaves and shoots showing fused leaflets and wild-type-like leaf margins (Cheng et al., 2012). Previous studies showed that NAM/CUC plays conserved roles among species in the regulation of both leaf margin development and lateral organ separation among species (Blein et al., 2008). Therefore, we hypothesized that NAM is also involved in the elaboration of the leaf margin in M. truncatula. Compared with serrated leaf margins in the wild type (Figures 8A to 8C), the leaf teeth in nam-2 became relatively smooth, indicating that NAM is involved in the promotion of leaf margin serrations (Figures 8D to 8F). To investigate the roles of NAM in the formation of lobed leaf margin in ago7-1, an ago7-1 nam-2 double mutant was generated (see Supplemental Figure 11 online). Compared with ago7-1, the degree of indentation in the leaf margin was decreased in the double mutant, confirming that NAM indeed positively regulates the formation of leaf margin serration (Figures 8G to 8K). These data further suggest that the TAS3 ta-siRNA pathway and NAM antagonistically regulate the development of leaf margins in M. truncatula.

To further elucidate the genetic interaction between NAM and the TAS3 ta-siRNA pathway, the developmental pattern of leaflets was examined. The adult leaves of the wild type exhibit trifoliate form. In nam-2, 82% of leaves were simplified because of fused leaflets. In contrast with nam-2, the frequency of fused leaflets in the ago7-1 nam-2 was dramatically decreased to 5% (Figure 8L). Furthermore, the elongated rachis and petiole in ago7-1 were suppressed in the double mutant (Figures 8M and 8N). These observations indicate that the TAS3 ta-siRNA pathway and NAM also antagonistically regulate the separation of lateral leaflets.

Leaf Margin Development Regulated by NAM Is Dependent on SLM1

To determine whether NAM functions in concert with auxin polar transport to promote leaf margin serration development, a nam-2 slm1-1 double mutant was generated (see Supplemental Figure 11 online). The leaf margin of the double mutant resembled that of slm1-1, indicating that leaf margin development regulated by
NAM is dependent on SLM1 (Figures 9A to 9C). Moreover, the frequency of fused leaflets in the double mutant increased (Figure 9D), suggesting that NAM and SLM1 play redundant roles in the separation of leaflets. In addition, the transcript level of NAM was downregulated in slm1-1 compared with that in the wild type (Figure 9E). The spatial localization of NAM in slm1-1 was further examined by in situ hybridization (Figure 9F). Whereas NAM mRNA was easily detected in the developing leaf margins of the wild type, an obvious reduction in NAM expression in leaf margins of slm1-1 was observed. The qRT-PCR and in situ hybridization results showed that proper auxin distribution is required for NAM expression.

**DISCUSSION**

The Conserved TAS3 ta-siRNA Pathway Has Different Roles in Leaf Development

Arabidopsis AGO7 and its orthologs (REL3 in *L. japonicus*, WIRY2 in tomato, SHOOTLESS4 in rice, and RAGGED SEEDLING2 in *M. truncatula*)...
maize) have been shown to play key roles in the TAS3 ta-siRNA pathway (Hunter et al., 2003; Nagasaki et al., 2007; Douglas et al., 2010; Yan et al., 2010; Yifhar et al., 2012). In this study, **AGO7** (LOL1) is identified as the *M. truncatula* ortholog of Arabidopsis AGO7. Three ARF genes were upregulated in ago7. These data further support that the regulation mechanism of the TAS3 ta-siRNA pathway is well conserved in both dicots and monocots. Blocking of the TAS3 ta-siRNA pathway has different effects on leaf pattern among species. For example, tomato *wiry2/ago7* alleles exhibited leaflet number variations at different developmental stages. *L. japonicus rell* showed a reduced number of leaflets (Yan et al., 2010). By contrast, the leaflet number of adult leaves in both *ago7* and *OX-ARF3mut* plants did not change. Previous studies indicate that the leaf patterns of both tomato and *L. japonicus* are determined in response to the Class I KNOTTED-like homebox (*KNOX1*) expression domain (Hareven...
et al., 1996; Champagne et al., 2007). *M. truncatula* belongs to the inverted repeat–lacking clade of legumes (Fabaceae), and SINGLE LEAFLET1, the FLORICAULA/LEAFY ortholog, functions in place of KNOX1 to regulate compound leaf development (Wojciechowski et al., 2004; Champagne et al., 2007; Wang et al., 2008). It could be speculated that different determination mechanisms of compound leaf development result in diverse responses to the loss of ta-siRNA. The investigation and comparison of gene functions among species are critical for obtaining insight into the general or specialized gene functions (Efroni et al., 2010; Yifhar et al., 2012).

**ARF3 Regulates Leaf Shape by Maintaining Proper Leaf Polarity**

Mutants with defects on adaxial-abaxial polarity display filamentous organs, demonstrating the importance of boundary formation for morphogenesis (Townsley and Sinha, 2012; Nakata and

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**Figure 9. Leaf Margin Development Regulated by NAM Is Dependent on SLM1.**

(A) to (C) Adult leaves of *nam-2* (A), *slm1-1* (B), and *nam-2 slm1-1* (C). Arrow points to the clustered leaflets. Arrowheads indicate the fused leaflets.

(D) Percentage of fused leaflets in the 6-week-old wild type and mutants (*n* = 50).

(E) Transcript levels of NAM in the wild type (WT) and *slm1-1*. Transcript levels were measured by qRT-PCR using leaf buds from 6-week-old plants. Values are the means and SD of three biological replicates.

(F) In situ hybridization and expression patterns of NAM in leaf primordia of the wild type and *slm1-1*. Empty boxes mark the leaf margin. Bar in (A) to (C) = 5 mm; bar in (F) = 50 μm.
Leaf margin cells are a type of boundary cell that forms a specific structure to separate the adaxial and abaxial epidermis of the leaf blade. Such structures are required for proper leaf blade growth and are also regulated by leaf adaxial-abaxial polarity (Sarojam et al., 2010; Szakonyi et al., 2010; Wang et al., 2011; Yamaguchi et al., 2012; Nakata and Okada, 2013). In this study, the dorsiventral polarity of the leaf is altered in ago7 as a result of the ectopic expression of ARF3. In accordance with the polarity defects, the development of leaf margin cells is also abnormal as evidenced by the loss of their cell identity. Therefore, by regulating the establishment of leaf polarity, ARF3 is probably indirectly involved in the formation of leaf margin cells. On the other hand, the pattern of cell proliferation and expansion is a major factor affecting organ shape (Nakata and Okada, 2013). Accompanying the defects in margin cell identity in ago7, the density of margin cells was increased and the cell size was decreased in the sinus of the leaf margin, suggesting that both cell proliferation and expansion are changed. Such irregular cell development results in the overgrowth of leaf margin teeth, which finally promotes the formation of lobed leaf margins.

The TAS3 ta-siRNA Pathway and the Auxin/SLM1 Module Play Essential but Different Roles in Leaf Margin Formation in *M. truncatula*

Auxin is known as the key hormone for elaboration of leaf margins (Hay et al., 2006; Barkoulas et al., 2008; Koenig et al., 2009; Bilsborough et al., 2011; Zhou et al., 2011a). The differential distribution of auxin between cells of a tissue is crucial for auxin-mediated developmental processes (Vanneste and Friml, 2009; Byrne, 2012). Based on the expression of DR5 reporter constructs, auxin responsiveness was altered along the leaf margin of ago7. It has been suggested that auxin distribution is dependent on the dorsiventral pattern (Zgruski et al., 2005; Nakata and Okada, 2013). Therefore, the increased auxin response in ago7 is likely a result of the changed leaf polarity induced by ectopic expression of ARF3. In turn, ARF3 is implicated in leaf development by responding to auxin signaling (Pekker et al., 2005). Taken together, these findings imply that crosstalk between the TAS3 ta-siRNA pathway and auxin distribution probably exists during leaf margin formation (Figure 10).

In *M. truncatula*, loss of function of SLM1 results in the smooth leaf margin (Zhou et al., 2011a). In contrast with *slm1*, the *slm1 ago7* double mutant formed serrations (Figure 7). The TAS3 ta-siRNA pathway represses the degree of indentation by suppressing the expression of ARF3. Such regulation is independent of the SLM1-mediated mechanism but could be enhanced by the auxin/SLM1 module. This is evidenced by the observation that the degree of indentation in *slm1 ago7* is not as pronounced as ago7, although the SLM1 expression is partially repressed in ago7 (see Supplemental Figures 6J and 10B online). These data suggest that elaboration of leaf margin formation requires two different processes and corresponding regulators: (1) the auxin/SLM1 module regulates the initiation of leaf margin teeth; and (2) the TAS3 ta-siRNA pathway determines the degree of marginal indentation (Figure 10).

![Figure 10. A Proposed Model Illustrating the Functional Roles of the TAS3 ta-siRNA Pathway, NAM, and SLM1 in Leaf Margin Development and Lateral Organ Separation.](image-url)

**The TAS3 ta-siRNA Pathway and NAM Antagonistically Regulate the Degree of Indentation of Leaf Margin in a Context-Dependent Manner**

Leaf margin patterning is dependent on genetic networks involving transcription factors and hormone signaling (Byrne, 2012). A recent study proposes a fine model of the interdependent feedback loops among PIN1, CUC2, and auxin distribution. In this model, proper auxin activity gradients established by both CUC2 and PIN1 are required for producing regularly spaced marginal serrations (Bilsborough et al., 2011). As a result, the *pin1* and *cuc2* loss of function mutants fail to form teeth in *Arabidopsis*. In *M. truncatula*, auxin maxima established by SLM1 at the teeth tips is crucial for the formation of leaf serrations (Zhou et al., 2011a). Leaf margin development is also partially dependent on NAM. Unlike the smooth leaf margin of *cuc2* in *Arabidopsis*, *nam-2* still developed a less serrated leaf margin. This finding does not contradict the conserved roles of NAM in the initiation of leaf margin teeth because *nam-2* is a weak allele. The *slm1 nam* double mutant showed a smooth leaf margin, indicating that NAM is dependent on SLM1 to promote leaf serrations. In addition, the expression of NAM varied with auxin distribution as revealed by the repression of NAM in *slm1*. These data indicate that NAM and

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**Image References:**
- Figure 10: A Proposed Model Illustrating the Functional Roles of the TAS3 ta-siRNA Pathway, NAM, and SLM1 in Leaf Margin Development and Lateral Organ Separation.
- TAS3 ta-siRNA pathway
- AGO7
- ta-siARF
- ARF3,4a,b
- SLM1
- Auxin accumulation patterns
- Leaf margin cell fate/ lateral organ polarity
- Initiation of leaf marginal teeth
- Formation of marginal indentation & separation of lateral organs

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**Further Reading:**
- Hay et al., 2006
- Barkoulas et al., 2008
- Koenig et al., 2009
- Bilsborough et al., 2011
- Zhou et al., 2011a
- Vanneste and Friml, 2009
- Byrne, 2012
SLM1 play conserved roles in the regulation of leaf marginal structure in *M. truncatula*. It was proposed that the relative balance between growth promotion and growth repression results in the ultimate leaf shape (Byrne, 2012). In *M. truncatula*, a growth balance was observed in the elaboration of leaf margins. On one side, the TAS3 ta-siRNA pathway represses the degree of indentation at the leaf margin by suppressing ARF3. On the other side, NAM promotes the formation of leaf serrations in an auxin-independent manner. These observations suggest that the TAS3 ta-siRNA pathway and NAM antagonistically act on the elaboration of leaf margin serrations (Figure 10).

Different plant species exhibit large variations in leaf shape. For example, both Arabidopsis and *M. truncatula* display serrated leaves, whereas the entire leaf margin in *L. japonicus* is without teeth. Although the TAS3 ta-siRNA regulation mechanism is highly conserved, blocking the synthesis of TAS3 ta-siRNAs did not yield the same phenotypic changes in leaf margin among species. In Arabidopsis, ago7 shows elongated leaves with increased serrations in the leaf margin (Hunter et al., 2003). In *M. truncatula*, leaf serrations are strongly enhanced to form leaf lobing in ago7. However, in *L. japonicus*, the TAS3 ta-siRNA pathway–related mutants, rel1 and rel3, show smooth leaf margins similar to the wild type, although the leaf polarity changed dramatically (Yan et al., 2010). It seems that the role of the TAS3 ta-siRNA pathway in leaf margin morphogenesis depends on the prior state of competence. These observations are reminiscent of the different context-specific developmental events when KNOX1 is expressed ectopically in compound leaf species and simple leaf species (Efroni et al., 2010). For example, the ectopic activity of KNOX1 causes a substantial increase in leaflet number in tomato but leads to lobed leaves, rather than induction of leaflets, in Arabidopsis (Shani et al., 2009). Therefore, the distinct developmental identities of leaf margins among species imply that the regulation of leaf shape by the TAS3 ta-siRNA pathway is species specific. We propose a possible regulation mechanism in which the TAS3 ta-siRNA pathway influences leaf margin development in a species-specific manner by interacting with SLM1 and NAM through the auxin patterning and signaling pathway (Figure 10).

**METHODS**

**Plants Materials and Growth Conditions**

*Medicago truncatula* ecotype R108 was used in this study, NF3499 (ago7-1), NF6270 (ago7-2), NF6665 (ago7-3), and NF4633 (ago7-4) mutant lines were identified from a Tnt1 retrotransposon-tagged mutant collection of *M. truncatula* (Tadte et al., 2008). Plants were grown at 24°C (day) and 20°C (night), 16-h day and 8-h night photoperiods, 70 to 80% relative humidity, and 150 µmol/m²/s light intensity.

**Molecular Cloning of AGO7 and Identification of Insertion Sites in AGO7**

Thermal asymmetric interlaced PCR was performed as previously described (Tadte et al., 2008). PCR and RT-PCR were performed to amplify the AGO7 genomic DNA sequence and AGO7 CDS sequence. The Tnt1 or Mere1 retrotransposon flanking sequences from ago7-2, ago7-3, and ago7-4 were PCR amplified (MJ Research PTC-200 Thermal Cycler) using a combination of Tnt1- or Mere1-specific primer and AGO7 gene-specific primer (see Supplemental Data Set 5 online). The PCR products were purified and cloned into pGEM-T Easy vector (Promega) for sequencing, and the sequences were aligned (NCBI blastn: Align two or more sequences) with the AGO7 genomic DNA sequence to determine the retrotransposon insertion sites.

**Microscopy and Photography**

Leaves and flowers at different stages were collected. For scanning electron microscopy analysis, sample fixation was performed as previously described (Zhou et al., 2011a). The Hitachi TM-3000 Tabletop Scanning Electron Microscope was used for observation. For fluorescence imaging, samples were observed with a Leica TCS SP2 AOBS confocal laser scanning microscope using the 488-nm line of an argon laser for the GFP signal, and emission was detected at 510 nm (Leica Microsystems). The silhouettes in Figure 8 were produced by Adobe Photoshop CS5 software. The original photos were adjusted using the commands levels and brightness/contrast, and then changed to black/white using the command black and white.

**Histology, GUS Staining, and Pollen Staining**

The adult leaves and petioles were fixed in 3% glutaraldehyde in a phosphate buffer. Dehydrated samples were embedded in LR white resin (London Resin). Sample sectioning and staining were performed as previously described (Zhou et al., 2011a). Leaf buds and fully expanded leaves were collected for GUS staining. GUS activity was histochemically detected as previously described (Jefferson et al., 1987). To determine pollen viability, flowers of the wild type and ago7-1 were collected. Sample staining was performed as previously described (Zhou et al., 2011a).

**In Situ Hybridization Analysis**

The fragments of 525-bp AGO7, 621-bp ARF3, and 638-bp NAM CDS were amplified by PCR. The PCR product was labeled with digoxigenin-11-UTP (Roche Diagnostics). RNA in situ hybridization was performed on shoot apices or inflorescence apices of 4-wk-old wild-type or sim1-1 plants as previously described (Zhou et al., 2011a).

**Plasmids and Plant Transformation**

To obtain the SLM1 genomic clone for functional complementation of the ago7 mutant, the 2.5-kb promoter sequence plus 4.2-kb AGO7 genomic sequence was amplified using the primers AGO7-PRO-F and AGO7-PRO-R, and transferred into the Gateway plant transformation destination vector pEarleyGate 301 (Earley et al., 2006) using the Gateway LR reaction (Invitrogen). To generate the AGO7 overexpression construct, the CDS of AGO7 was amplified using the primers AGO7-CDS-F and AGO7-CDS-R (see Supplemental Data Set 5 online). The PCR product was purified and cloned into pEarleyGate 100 (Earley et al., 2006). To construct the ARF3RNAi vector, a 602-bp fragment of ARF3 CDS was PCR amplified from wild-type *M. truncatula* and transferred into the Gateway plant transformation destination vector pANDA35K. To construct the ARF3RNAi vector, silent mutations were introduced into two ta-siARF target sites of ARF3 CDS using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and then transferred into pEarleyGate 100 vector. The DSrev:GFP reporter construct was previously described (Zhou et al., 2011a). The leaves of *M. truncatula* ago7-1 and the wild type were transformed with the Agrobacterium tumefaciens EHA105 strain harboring the various destination vectors (Cosson et al., 2006; Crane et al., 2006).

**Phylogenetic Analysis**

The protein sequences of ARGONAUTE family members and ARF RESPONSE FACTOR family members were used for phylogenetic
analysis. Alignments were performed using ClustalW2 with default parameters (alignment type, slow; protein weight matrix, gonnet; gap open, 10; gap extension, 0.1). A phylogenetic tree was constructed by MEGA4 using the neighbor-joining method with 1000 bootstrap replications (http://www.megasoftware.net/).

RNA Extraction, RT-PCR, qRT-PCR, and Microarray Analysis

The shoot meristem tissues of 4-wk-old wild-type and mutant plants were collected for RNA isolation. Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and cleaned with the DNA-free Kit (Ambion). cDNA synthesis for RT-PCR and real-time RT-PCR was performed using SuperScript III reverse transcriptase (Invitrogen) as previously described (Zhou et al., 2011a). For qRT-PCR, triplicate biological samples were collected. SYBR Green (Sigma-Aldrich) was used as the reporter dye. qRT-PCR was performed with an ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Data were analyzed using the SDS 2.2.1 software (Applied Biosystems). The protocol for the amplification and detection of ta-siARF was adapted from a previous report (Varkonyi-Gasic et al., 2007). The stem-loop reverse transcription primers and ta-siARF-specific and miR390-specific PCR primers were designed and used for RT-PCR. Primer sequences used are listed in Supplemental Data Set 5 online.

For microarray analysis, ago7-1 mutants and wild-type-like plants in a segregating F2 population were used. Shoot apices of 4-wk-old plants were collected from triplicate biological replicates of the above samples for RNA extraction. RNA purification, probe labeling, hybridization, and scanning for microarray analysis were conducted as previously described (Zhou et al., 2011b). Data normalization between chips was conducted using the robust multichip average technique (Irizarry et al., 2003). Presence/absence calls for each probe set were obtained using dChip software (Li and Wong, 2001). Gene selections for pairwise comparison were made based on associative analysis (Dozmorov and Centola, 2003) in Matlab software (MathWorks). In this method, the background noise presented between replicates and technical noise during microarray experiments were measured by the residual pre-extracted F2 plants. The double phenotype was identified by PCR. Primer sequences used are listed in Supplemental Data Set 5 online.

Supplemental Data

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

Supplemental Figure 1. Complementation of ago7-1 by AGO7 and Overexpression of AGO7 in the Wild Type.

Supplemental Figure 2. Phylogenetic Tree and Alignment of Mt-AGO7 with Other Members of the ARGONAUTE Family.

Supplemental Figure 3. Phylogenetic Relationships of ARF Proteins in M. truncatula, Arabidopsis, and Rice.

Supplemental Figure 4. Expression Profiling of the ARF3, ARF4a, and ARF4b Transcripts.

Supplemental Figure 5. The Relative Expression Level of Three ARF Genes in the Wild Type and ago7-1.

Supplemental Figure 6. Leaf Margin Development in the Wild Type and ago7-1.

Supplemental Figure 7. Relative Expression Level of ARF4a and ARF4b in Leaves of the Wild Type, ago7-1, and ARF3RNAi ago7-1.

Supplemental Figure 8. Auxin Distribution in the Wild Type and ago7-1.

Supplemental Figure 9. Relative Expression Level of ARF3 in Leaves Treated with Auxin.

Supplemental Figure 10. Phenotype of Leaf Margin at the Early Developmental Stage.

Supplemental Figure 11. Phenotype of 40-d-Old Plants of the Wild Type and Mutants.

Supplemental Table 1. Analysis of Leaf Growth in ago7-1 Mutant, Wild-Type, and Transgenic Plants.

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

Supplemental Data Set 2. Genes Upregulated More Than Twofold in ago7-1 Mutants Compared with Those of the Wild Type.

Supplemental Data Set 3. Genes Downregulated More Than Twofold in ago7-1 Mutants Compared with Those of the Wild Type.

Supplemental Data Set 4. Alignment Used to Generate the Phylogeny Presented in Supplemental Figure 3.

Supplemental Data Set 5. Primers Used in This Study.

ACKNOWLEDGMENTS

The authors thank Amy Mason, Jackie Kelley, and Katie Wenzell for critical reading of the article, greenhouse staff for assistance with plant care, and the Samuel Roberts Noble Electron Microscopy Laboratory for the scanning electron microscopy work. This work was supported by the Samuel Roberts Noble Foundation, the National Science Foundation (Grant EPS-0814361), and the BioEnergy Science Center. The BioEnergy Science Center is a U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the Department of Energy Office of Science.

AUTHOR CONTRIBUTIONS

The Trans-Acting Short Interfering RNA3 Pathway and NO APICAL MERISTEM
Antagonistically Regulate Leaf Margin Development and Lateral Organ Separation, as Revealed by Analysis of an argonaute7/lobed leaflet1 Mutant in Medicago truncatula
Chuanen Zhou, Lu Han, Chunxiang Fu, Jiangqi Wen, Xiaofei Cheng, Jin Nakashima, Junying Ma, Yuhong Tang, Yang Tan, Million Tadege, Kirankumar S. Mysore, Guangmin Xia and Zeng-Yu Wang
Plant Cell; originally published online December 24, 2013;
DOI 10.1105/tpc.113.117788

This information is current as of February 6, 2021

Supplemental Data /content/suppl/2013/12/23/tpc.113.117788.DC1.html
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