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Genome-wide characterization of *AINTEGUMENTA-LIKE* family in *Medicago truncatula* reveals the significant roles of *AINTEGUMENTAs* in leaf growth

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AINTEGUMENTA-LIKE (AIL) transcription factors are widely studied and play crucial roles in plant growth and development. However, the functions of the *AIL* family in legume species are largely unknown. In this study, 11 *MtAIL* genes were identified in the model legume *Medicago truncatula*, of which four of them are *MtANTs*. *In situ* analysis showed that *MtANT1* was highly expressed in the shoot apical meristem (SAM) and leaf primordium. Characterization of *mtant1 mtant2 mtant3 mtant4* quadruple mutants and *MtANT1*-overexpressing plants revealed that *MtANTs* were not only necessary but also sufficient for the regulation of leaf size, and indicated that they mainly function in the regulation of cell proliferation during secondary morphogenesis of leaves in *M. truncatula*. This study systematically analyzed the *MtAIL* family at the genome-wide level and revealed the functions of *MtANTs* in leaf growth. Thus, these genes may provide a potential application for promoting the biomass of legume forages.

KEYWORDS

***Medicago truncatula*, *AILs*, *ANT*, leaf size, leaf morphogenesis**

Introduction

The AINTEGUMENTA-LIKE (AIL) transcription factors have been widely studied in plants (Klucher et al., 1996; Mizumoto et al., 2009; Karlberg et al., 2011; Li and Xue, 2011; Rigal et al., 2012; Horstman et al., 2014; Kuluev et al., 2015; Bui et al., 2017; Ding et al., 2018; Zhao et al., 2019b; Liu et al., 2020; Shen et al., 2020; Miao et al., 2021; Han et al., 2022). They belong to the APETALA 2 (AP2)-like subfamily, which is characterized by two putative DNA-binding AP2 domains and one conserved linker region (Kim et al., 2006). The AP2-like subfamily can be divided into three groups: euAP2, basalANT, and

euANT (Kim et al., 2006). Unlike *euAP2* genes, the *basalANT* and *euANT* genes can not be recognized by *miR172* and are differentiated by specific sequence signatures (Kim et al., 2006; Dipp-Álvarez and Cruz-Ramírez, 2019).

In *Arabidopsis thaliana*, the euANT proteins are also known as AILs and consist of *ANT*, *AIL1*, *AIL2*/*BBM*/*PLT4*, *AIL3*/*PLT1*, *AIL4*/*PLT2*, *AIL5*/*PLT5*, *AIL6*/*PLT3* and *AIL7*/*PLT7* (Nole-Wilson et al., 2005; Horstman et al., 2014). *ANT* mainly regulates cell division and cell differentiation in leaves and floral organs. For instance, *ANT* regulates the size of lateral organs by controlling cell proliferation during organogenesis (Mizukami and Fischer, 2000). *ANT* also participates in the establishment of adaxial-abaxial polarity. *ANT* acts with the abaxial-specifying gene *FILAMENTOUS FLOWER* (*FIL*) to up-regulate the expression of the adaxial gene *PHABULOSA* (Nole-Wilson and Krizek, 2006). Meanwhile, *ANT* and *AIL5*/*6*/*7* function partially redundantly in flower development, and the direct targets of *ANT* and *AIL6* include *LEAFY* and other genes involved in polarity establishment, meristem and flower development, as well as auxin signaling pathway (Krizek, 2009; Krizek, 2015; Yamaguchi et al., 2016; Krizek et al., 2020; Krizek et al., 2021). *ANT*, *AIL6*, and *AIL7* regulate SAM function; the SAM of the *ant ail6 ail7* triple mutant terminates after the production of only a few leaves (Mudunkothge and Krizek, 2012). Furthermore, *BBM*/*AIL2*, *PLT1*/*AIL3*, *PLT2*/*AIL4* and *PLT3*/*AIL6* regulate cell proliferation during embryogenesis and root apical meristem maintenance (Horstman et al., 2014). These genes function in stem cell identity in the root and promote cell division of the stem cell daughters (Galinha et al., 2007). *PLT1*/*AIL3* and *PLT2*/*AIL4* also play essential roles in the quiescent center specification and stem cells maintenance (Aida et al., 2004). *BBM*/*AIL2* is involved in the embryo and endosperm development, and ectopic expression of *BBM*/*AIL2* leads to the production of somatic embryos on seedlings (Boutilier et al., 2002; Chen et al., 2022). Moreover, *AIL5*, *AIL6*, and *AIL7* execute an extra function in phyllotaxy stability and lateral root emergence (Prasad et al., 2011; Hofhuis et al., 2013; Pinon et al., 2013).

The roles of *AIL* genes in panicle, root, seed, leaf, flower, and chloroplast development have been reported in other species. *OsAILs* are involved in panicle branching, panicle structure regulation, and crown root initiation in rice (Kitomi et al., 2011; Harrop et al., 2019; Luong et al., 2021). In poplar, *PtAIL1* plays a positive role in adventitious root formation (Rigal et al., 2012). In *Medicago truncatula*, ectopic expression of *AtANT* under the control of a seed-specific promoter generates larger seeds and improves the germination rate (Confalonieri et al., 2014). In maize, *ZmANT1* regulates leaf and vascular tissue development, chloroplast development, and photosynthesis (Liu et al., 2020). In *Nicotiana tabacum*, *Cucurbita moschata*, *Triticum aestivum*, and *Brassica rapa*, putative orthologs of *ANT* positively regulate organ size. For example, *NtANT* increases the size of leaf and corolla by promoting cell division and expansion (Kuluev et al., 2015), ectopic expression of *CmoANT* accelerates the growth of grafted plants and promotes the size of silique and leaf (Miao et al., 2021), ectopic expression of *TaANT* enlarges plant size by promoting cell proliferation (Zhao et al., 2019b), and ectopic expression of the *BrANT* increases stomatal density and organ size (Ding et al., 2018).

Alfalfa (*Medicago sativa*) has the characteristics of high biomass yield, good forage quality, high adaptability to growing conditions, and palatability for ruminants, and has been called the "Queen of Forage" (Radović et al., 2009). Alfalfa cultivars are allogamous, self-incompatibly, autotetraploid plants with a complex genome, which leads to difficulty in genomics research (Zhu et al., 2005). *Medicago truncatula* has been adopted as a model legume species for a range of genetics and genomics studies. The *AIL* genes have been well studied in many species, but the information and functions of *AIL* genes are largely unknown in legume species. In this study, the genome-wide identification and characterization of the *AIL* gene family was performed in *M. truncatula*. Eleven *MtAIL* genes were identified and their phylogenetic relationship, gene structure, and protein motifs were analyzed. Furthermore, the expression patterns of *MtANTs* showed that *MtANT1* was highly expressed in the SAM and leaf primordium. Loss-of-function mutants of *MtANTs* were isolated and the *mtant1 mtant2 mtant3 mtant4* quadruple mutant was generated. The quadruple mutant exhibited obvious defects in leaf size, while, transgenics overexpressing *MtANT1* produced enlarged leaves. Cellular level analysis indicates that *MtANTs* regulate leaf size mainly through cell proliferation. Our study provides detailed information on the *MtAILs* and demonstrates that *MtANT* genes play vital roles in leaf growth in *M. truncatula*.

Materials and methods

Plant material and growth conditions

Medicago truncatula ecotype R108 was used as the wild type in this study. *mtant1-1*, *mtant2-1*, *mtant3-1*, and *mtant4-1* were identified from a tobacco (*Nicotiana tabacum*) *Tnt1* retrotransposon-tagged mutant population of *M. truncatula* (Tadege et al., 2008). The seeds were scarified with sandpaper and treated at 4°C for 7 days. The germinated seeds were planted in a nursery seedling plate for 3 weeks. Then, the seedlings were transferred to soil and grown at 22°C ± 2°C under long-day conditions (16-h light and 8-h dark), with a relative humidity of 70%–80%.

Identification and phylogenetic analysis of AIL genes in *M. truncatula*

To identify the AIL proteins in *M. truncatula*, 8 AILs in *Arabidopsis thaliana* and 10 AILs in *Oryza sativa* were used to

execute BLASTP Search against the sequence database of the *Medicago truncatula* in Phytozome (https://phytozome-next.jgi.doe.gov/). We selected the fullest transcripts for the study, and other splice variants were excluded. To investigate the phylogenetic relationships of AILs in different species, 8 AIL proteins in *Arabidopsis thaliana*, 10 AIL proteins in *Oryza sativa*, 11 AIL proteins in *Pisum sativum*, 9 AIL proteins in *Lotus japonicus*, 18 AIL proteins in *Glycine max* and 11 identified AIL proteins in *Medicago truncatula* were used to construct the phylogenetic tree. The phylogenetic trees were generated with the Neighbor-Joining method and 1000 Bootstrap Replications using MEGA6.06 (Kumar et al., 2008). The amino acid sequences of the AtAILs were obtained from The Arabidopsis Information Resource (TAIR) database (http://www.Arabidopsis.org/). The OsAILs protein sequences were obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/). The GmAILs and LjAILs protein sequences were obtained in Phytozome, and the PsAILs protein sequences were obtained in the Pea Genome Database (https://www.peagdb.com/index/) (Yang et al., 2022).

Gene structure, conserved domains and motif analysis

Exon and intron structures analysis of MtAIL genes were determined by aligning the CDS sequences and their corresponding genomic DNA sequences using the Gene Structure Display Server (GSDS 2.0, http://gsds.gao-lab.org/) (Wang et al., 2019). Conserved motifs in MtAIL proteins were analyzed with the Multiple Em for Motif Elicitation (MEME, https://meme-suite.org/meme/tools/meme) with the following parameters: maximum of motif width, 80; minimum width of motif, 4; maximum motif number, 10 (Wang et al., 2018). The MtAIL proteins were aligned using Clustal X2 (Larkin et al., 2007), and GeneDoc software was used for homology shading (Brocard-Gifford et al., 2004).

RT-PCR and qRT-PCR analysis and statistical analysis

For gene expression pattern analysis, total RNA was extracted from the leaves, vegetative buds, flowers, stems, petioles, pods, and roots. To analyze the relative expression levels of *MtANT1* in the overexpressing plants, RNA was extracted from 30-d-old mature leaves of wild type and transgenic plants. For RT-PCR analysis, RNA was extracted from vegetative buds of wild type and mutant lines. RNA extraction, cDNA synthesis, qRT-PCR, and RT-PCR analyses were performed as described previously (Zhou et al., 2011). The primers used for qRT-PCR and RT-PCR are listed in Supplementary Table S1. *T*-test was used to compare the means of different populations.

In situ hybridization analysis

For RNA *in situ* hybridization, the 583-bp CDS of *MtANT1*, the 546-bp CDS of *MtANT2*, and the 502-bp CDS of *MtANT3* were amplified. The PCR products were cloned into the pGEM-T vector (Promega). The sense and anti-sense probes were made according to the previous report (Zhao et al., 2019a). 30-d-old wild type vegetative buds were used for RNA *in situ* hybridization as previously described (Zhou et al., 2011). The primers used for RNA *in situ* hybridization are listed in Supplementary Table S1

Plasmids and plant transformation

To obtain the *MtANT1* overexpression construction, the full-length CDS of *MtANT1* was obtained by PCR amplification and inserted into the pENTR/D-TOPO vector (Invitrogen), and then recombined with final vector pEarleyGate 100, using the Gateway LR reactions (Invitrogen) (Earley et al., 2006). The primers used are listed in Supplementary Table S1. The *35S:MtANT1* construct was introduced into *Agrobacterium* strain EHA105. For stable transformation, leaves of wild type were used for transformation (Cosson et al., 2006).

SEM analysis

SEM was performed as described previously (Zhang et al., 2022). Briefly, leaves were fixed, dehydrated, critical point dried, and observed for imaging.

Results

Identification and phylogenetic analysis of AILs in *M. truncatula*

Eleven putative MtAIL proteins were identified by BLAST Search in Phytozome. The length of 11 MtAILs proteins ranged from 402 to 688 amino acids. The gene locus, exon number, amino acid length, molecular weight (Mw), and chromosome location are listed in Table 1. Based on the gene locus, these *MtAIL* genes showed uneven distribution on the *M. truncatula* chromosomes. Chromosome 1, 2, 3, and 7 contained one *MtAIL* gene, respectively. Both chromosome 5 and 8 contained two *MtAIL* genes, chromosome 4 contained four *MtAIL* genes, and no *MtAIL* gene

was located on chromosome 6 (Table 1). To further investigate the evolutionary relationship between MtAIL proteins and homologs in other species, a phylogenetic tree was constructed, including 8 AtAILs, 10 OsAILs, 9 LjAILs, 18 GmAILs, 11 PsAILs and 11 MtAILs (Figure 1). Based on the phylogenetic analysis, all the *MtAIL* genes were named according to their closest *Arabidopsis* orthologs. Furthermore, 67 AIL proteins were classified into six clades: ANT, AIL1, AIL2, AIL3/AIL4, AIL5, and AIL6/7. MtAILs were close to PsAILs, and GmAILs were more closely related to LjAILs. Moreover, every GmAIL gene contained more than one copy. OsAILs were separated from others mainly because of the species differences. ANT clade contained four members of MtAILs which were named MtANT1 to MtANT4. Clades AIL1, AIL2, AIL3/AIL4, AIL5, and AIL6/7 contained seven MtAILs which were named MtAIL1 to MtAIL7 (Figure 1). Phylogenetic analysis also showed that MtAIL1 was more closely related to MtANTs, MtAIL2 was close to MtAIL3 and MtAIL4, MtAIL5 was clustered with MtAIL6 and MtAIL7 (Figure 1).

TABLE 1 *AIL* gene family in *M. truncatula* .

Name	Locus	CDS (nt)	Exons	Length (aa)	MW (kDa)	Chromosome location
MtANT2	Medtr4g097520	1977	9	658	73.8	chr4:40188318..40192060 forward
MtANT1	Medtr1g017400	1995	9	664	74.13	chr1:4844539..4848969 reverse
MtANT3	Medtr3g103460	1986	9	661	73.18	chr3:47751101..47755318 forward
MtAIL1	Medtr8g020510	1722	9	573	63.95	chr8:7209111..7212535 forward
MtANT4	Medtr5g015070	1635	9	544	61.52	chr5:5176272..5179958 reverse
MtAIL5	Medtr4g127930	1557	9	518	55.82	chr4:53232819..53237003 reverse
MtAIL2	Medtr7g080460	2067	9	688	76.57	chr7:30617122..30621534 reverse
MtAIL3	Medtr2g098180	1578	9	525	58.7	chr2:41962850..41966348 reverse
MtAIL4	Medtr4g065370	1644	9	547	61.25	chr4:24560916..24564307 reverse
MtAIL6	Medtr5g031880	1545	9	514	56.95	chr5:13680654..13684967 reverse
MtAIL7	Medtr8g068510	1209	9	402	44.79	chr8:28586113..28591359 reverse

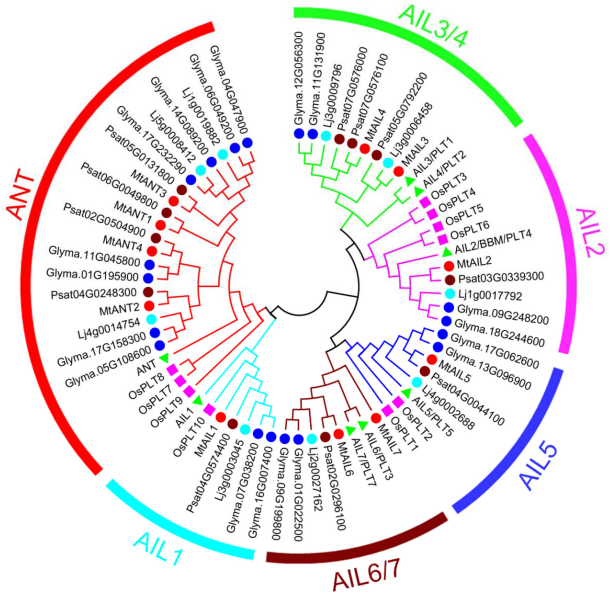

FIGURE 1
Phylogenetic tree analysis of AIL transcription factors from *Medicago truncatula*, *Arabidopsis thaliana*, *Oryza sativa*, *Pisum sativum*, *Lotus japonicus* and *Glycine max*. The Neighbor-Joining tree was constructed using full length protein sequences from *M. truncatula* (11), *Arabidopsis* (8), *Oryza sativa* (10), *Pisum sativum* (11), *Lotus japonicus* (9) and *Glycine max* (18) in MEGA6.06 with 1000 bootstrap replicates. The red, brown, light blue and blue circles indicate *M. truncatula*, *Pisum sativum*, *Lotus japonicus* and *Glycine max*, respectively. The pink boxes and green triangles indicate *Oryza sativa* and *Arabidopsis*.

Gene structures and conserved motifs analysis

To further study the diversification of *MtAILs*, the corresponding gene structures of *MtAILs* were analyzed. The exon/intron organization of *MtAILs* was investigated by aligning the coding sequences and corresponding genomic sequences. All the *MtAILs* displayed 9 exons and 8 introns (Figure 2), suggesting that the gene structures of the *MtAIL* family are conserved during evolution. Previous studies showed that AILs belong to the AP2-like transcription factor subfamily, which is characterized by two AP2 domains (Nole-Wilson et al., 2005; Licausi et al., 2013). According to this, the amino acid sequences of MtAILs were aligned, and two conserved AP2 domains were shown in MtAILs, including the N-terminal AP2-R1 and C-terminal AP2-R2 domains (Supplementary Figure S1). To get a better understanding of the protein sequence characteristics of MtAILs, the motifs of MtAILs were analyzed. Online MEME search was performed and 10 conserved motifs were identified in MtAILs (Figure 3). The motifs 1, 2, 3, 4 were found in each MtAIL. Among them, motifs 1, 2 and 3 were found to be similar to the AP2-R1-linker-AP2-R2 region of AP2-like proteins. Motif 4 was located downstream of the AP2-R2 domain and sequence analysis suggests that it may function as a nuclear localization signal (Aida et al., 2004; Dipp-Álvarez and Cruz-Ramírez, 2019). Motif 5 contained the euANT2 motif (WLGFSLF), and motif 6 was the euANT3 motif (PKLEDFLG). Motif 5 and 6 were conserved motifs of euANT clade protein and existed in almost all the MtAILs (Kim et al., 2006; Dipp-Álvarez and Cruz-Ramírez, 2019). Motif 7 was absent in MtAIL5, 6, 7 proteins, suggesting that motif 7 is lost before the MtAIL5, 6, 7 divergence. Motifs 8 and 9 were specific to MtANT1, 2, 3 and MtAIL1, while motif 10 was only presented in MtANTs, implying that the distribution of motifs among specific groups is related to their functional divergence (Figure 3).

Expression patterns of *MtANTs* in *M. truncatula*

In *Arabidopsis*, *ANT* plays an important role in the regulation of organ initiation, organ development, and cell proliferation (Mizukami and Fischer, 2000; Horstman et al., 2014). In order to investigate the function of *MtANTs*, the expression patterns of *MtANTs* were analyzed. The expression levels of *MtANT1*, *MtANT2*, *MtANT3*, and *MtANT4* were measured by quantitative real-time PCR (qRT-PCR) in different organs, including leaf, vegetative bud, flower, stem, petiole, pod, and root. qRT-PCR results showed that the relative expression levels and patterns of *MtANTs* varied in different organs, but all the *MtANTs* were expressed at the lowest levels in leaves (Figure 4A). Among the four *MtANTs*, the expression of *MtANT1* was much higher than the other three genes in vegetative buds. Additionally, the expression level of *MtANT1* in vegetative buds was higher than other tissues (Figure 4A). To gain spatial information about the expression patterns, *in situ* hybridization was performed for *MtANT1*, *MtANT2*, and *MtANT3*. *In situ* hybridization was not performed for *MtANT4* because it showed very low expression levels in vegetative buds. Strong *MtANT1* signals were detected in SAM, leaf primordia at stage 1 and stage 2, and leaves at stage 7 (Figure 4B). *MtANT2* and *MtANT3* transcripts were not detected in the SAM, and fewer transcripts were detected in leaf primordia and leaves (Figures 4C–D). The overall transcript level of *MtANT1* was much higher than those of *MtANT2* and *MtANT3* (Figures 4B–D). The sense probes were used as the negative controls and did not show any signal (Figures 4E–G).

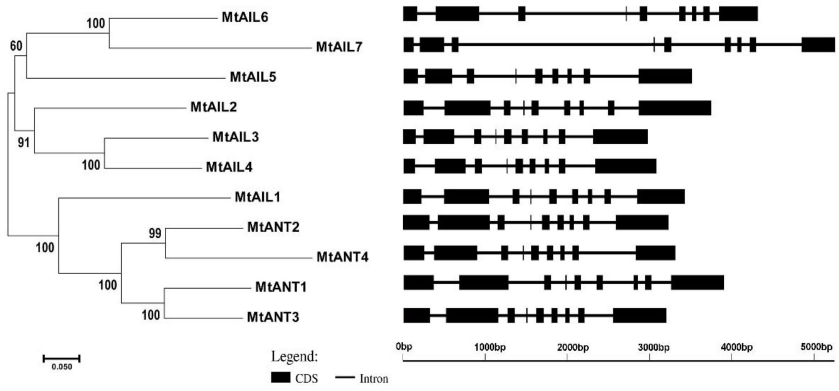

FIGURE 2
The phylogenetic relationship and exon/intron structural analyses of all *AILs* in *M. truncatula*. Introns are represented by black lines and exons are represented by black boxes. Phylogenetic tree was constructed using MEGA6.06 by the Neighbor-Joining method.

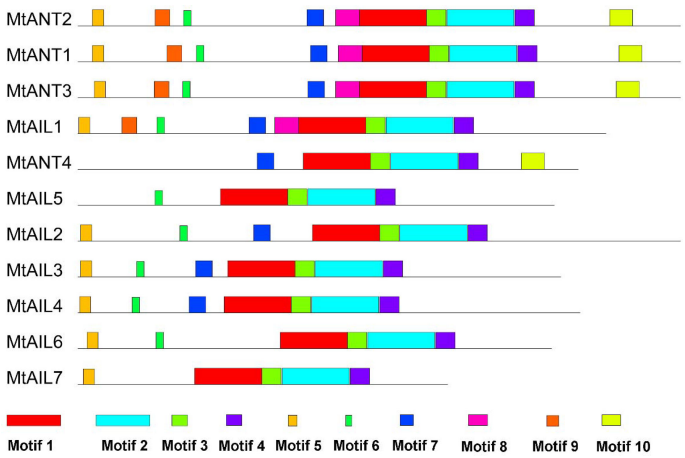

FIGURE 3
Motifs in MtAIL proteins. The motifs in MtAILs were indicated using MEME online tool. Motifs were represented by the boxes with different numbers and colors.

MtANTs are necessary for leaf size maintenance

To investigate the function of *MtANTs* in leaf morphogenesis, a reverse genetic screening was performed on a *Tnt1* retrotransposon-tagged mutant population of *M. truncatula* (Cheng et al., 2014). Insertional mutant alleles were identified in *MtANT1*, *MtANT2*, *MtANT3* and *MtANT4*. Sequence analysis showed that a single *Tnt1* was inserted in the sixth exon of *MtANT1* in *mtant1-1*, the first exon of *MtANT2* in *mtant2-1*, the first exon of *MtANT3* in *mtant3-1*, and the second exon of *MtANT4* in *mtant4-1* (Figures 5A–D). Reverse transcription PCR (RT-PCR) data showed that the transcripts of *MtANT1-4* were interrupted in the *mtant1-1*, *mtant2-1*, *mtant3-1*, and *mtant4-1* mutants, respectiveley (Figures 5E–H). Subsequently, the leaves of mutants were observed. Compared with the wild type, *mtant1-4* mutants did not show obvious defects in leaf morphology and compound leaf pattern (Figures 6A–E). To assess functional redundancy among *MtANTs*, *mtant1-1 mtant3-1* and *mtant2-1 mtant4-1* double mutants were generated. The *mtant1-1 mtant3-1* double mutant exhibited relatively smaller leaves (Figure 6F), but the *mtant2-1 mtant4-1* double mutant did not show obvious differences in leaf

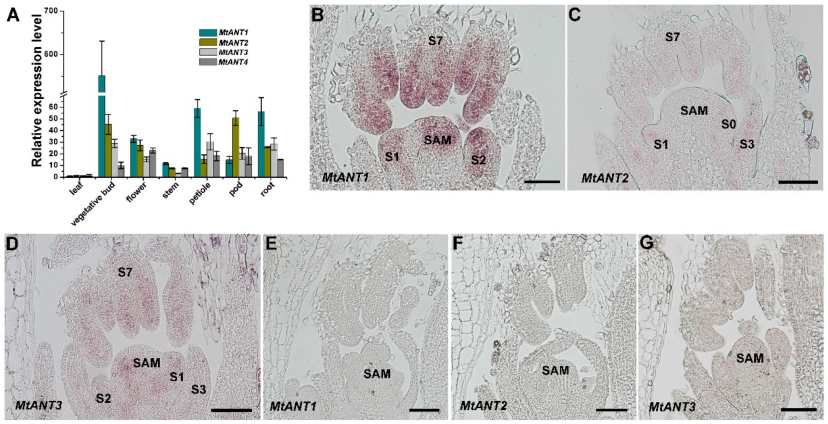

FIGURE 4
Expression patterns of *MtANTs* in *M. truncatula*. **(A)** qRT-PCR analysis of *MtANTs* expression in different organs. *MtUBIQUITIN* was used as the internal control. Bars represent means ± SD of three biological replicates. **(B-D)** *In situ* hybridization analysis of *MtANT1* **(B)**, *MtANT2* **(C)** and *MtANT3* **(D)** in the vegetative buds of wild type. Longitudinal sections of SAM and leaf primordia at the different developmental stages are shown. SAM, shoot apical meristem; S, stage. Bars = 100 μm. **(E–G)** The sense probes were used as the negative controls. SAM, shoot apical meristem; Bars = 100 μm.

pattern compared with wild type (Supplementary Figure S2). Then, high order mutants were generated in the *mtant1-1* background, since *MtANT1* showed the strongest expression in leaf primordia. Simultaneous disruption of *MtANT1*, *2*, *3* or *MtANT1*, *2*, *3*, *4* resulted in a significantly smaller leaf phenotype than that in the wild type (Figures 6G, H). It was worth noting that the leaf phenotypes of *mtant1-1 mtant2-1 mtant3-1* triple mutant and *mtant1-1 mtant2-1 mtant3-1 mtant4-1* quadruple mutant were similar (Figures 6G, H), indicating that *MtANT4* play a limited role in leaf development. Moreover, the length and width of leaves in *mtant1-1 mtant2-1 mtant3-1 mtant4-1* were reduced compared with those of wild type (Figures 6I–L). Compared with the wild type, the length/width ratio was increased, and the leaf area was significantly decreased in *mtant1-1 mtant2-1 mtant3-1 mtant4-1* (Figures 6M–P), demonstrating that simultaneous disruption of *MtANT1*, *2*, *3*, *4* resulted in smaller leaves in *M. truncatula*. To explore the cellular basis for the alteration in leaf dimensions of wild type and *mtant1-1 mtant2-1 mtant3-1 mtant4-1* mutant, we viewed the epidermal cells by scanning electron microscopy (SEM). Results showed that no differences were found for both epidermal cell size and cell number per unit area in the wild type and *mtant* quadruple mutant (Figures 6Q–S), indicating that the smaller leaf area of *mtant* quadruple mutant was resulted from cell proliferation.

MtANT1 is sufficient for increasing leaf size

The aforementioned findings indicated that *MtANTs* play positive roles in regulating leaf size. To determine whether increased expressions of *MtANTs* are sufficient to produce larger leaves, *MtANT1* was chosen to be overexpressed under the control of *CaMV 35S* promoter in the wild type because of its highest expression level in SAM and leaf primordia. Compared with wild type, the expression levels of *MtANT1* were increased by 93- to 669-fold in 35S:*MtANT1* transgenic plants (Supplementary Figure S3). Then, the leaf sizes in *35S:MtANT1* lines and wild type were compared. The *35S:MtANT1-1*, *-6*, and *-8* lines, with the highest expression levels, displayed larger leaves compared with those in wild type (Figures 7A–F). We measured the leaf areas of *35S:MtANT1* transgenic plants and wild type. The transgenic lines showed an increase in the area of leaves in comparison with those in wild type plants (Figures 7G–I). In addition, the length, width and length/width ratio of leaves of *35S:MtANT1-6* transgenic line were measured. The results showed that the length of leaves in *35S:MtANT1-6* was similar to that in the wild type, the leaves of transgenic plant were wider than wild type, and the length/width

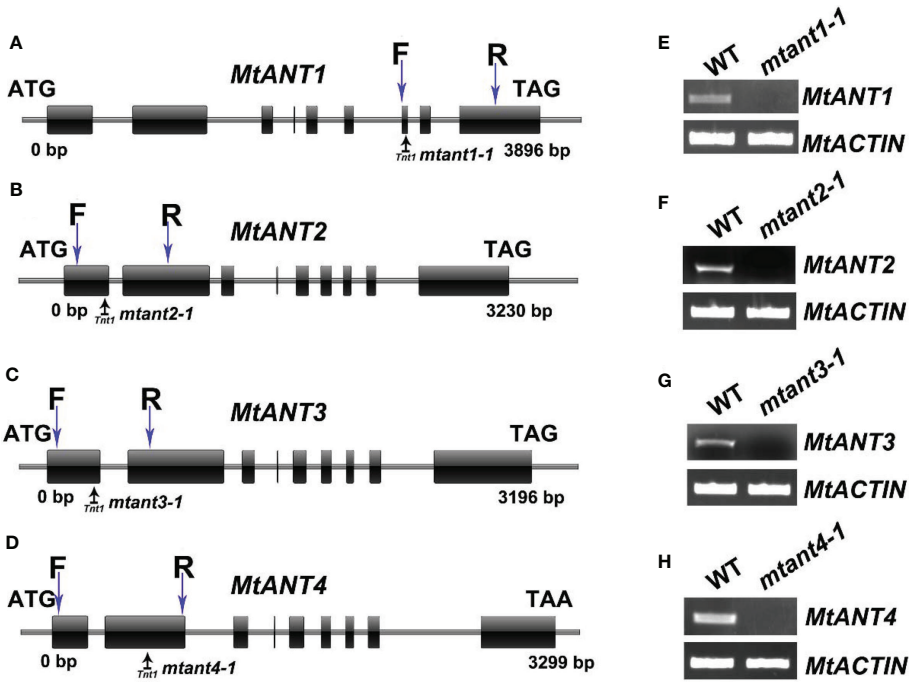

FIGURE 5
Mutant screening of *MtANTs* in *M. truncatula*. **(A–D)** Schematic diagram of the gene structures of *MtANT1-MtANT4*. The positions of the ATG start and TGA/TAA stop codons are shown. Black vertical arrows mark the location of *Tnt1* retrotransposons. Blue vertical arrows mark the location of primers used for RT-PCR. Exon is represented by a box, and intron is represented by a line. **(E–H)** RT-PCR shows the transcripts of *MtANTs* in wild type and *mtant* mutants. *MtACTIN* was used as the control.

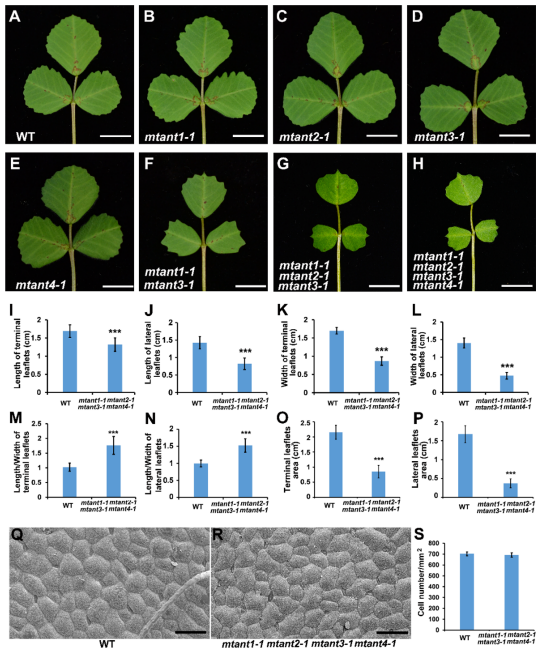

FIGURE 6
Phenotype analyses of leaves in mtant mutants. **(A–H)** Leaf phenotypes of wild type **(A)**, mtant1-1 **(B)**, mtant2-1 **(C)**, mtant3-1 **(D)**, mtant4-1 **(E)**, mtant1-1 mtant3-1 **(F)**, mtant1-1 mtant2-1 mtant3-1 **(G)** and mtant1-1 mtant2-1 mtant3-1 mtant4-1 **(H)**. Bars = 1 cm. **(I–L)** Leaf length **(I–J)** and width **(K, L)** in terminal leaflets and lateral leaflets of wild type and mtant1-1 mtant2-1 mtant3-1 mtant4-1. **(M–P)** Length/width ratio **(M, N)** and leaf area **(O, P)** of terminal leaflets and lateral leaflets in wild type and mtant1-1 mtant2-1 mtant3-1 mtant4-1. **(Q, R)** Scanning electron microscopy analyses of the adaxial surface of leaves in wild type **(Q)** and mtant1-1 mtant2-1 mtant3-1 mtant4-1 mutant **(R)**. Bars = 50 μm. **(S)** Cell number per unit area of the adaxial surface of leaves in the wild type and mtant1-1 mtant2-1 mtant3-1 mtant4-1 mutant. Bars represent means ± SD. ***P < 0.001.

ratio of *35S:MtANT1-6* was decreased (Figures 7J–L). These observations suggest that ectopic expression of *MtANT1* is able to promote the leaf width and leaf size in *M. truncatula*. The epidermal cells of wild type and *35S:MtANT1-6* plants were also analyzed by SEM. The data showed that the cells size and cell number per unit area were similar between wild type and *35S:MtANT1-6* plants (Figures 7M–O), further demonstrating that the larger leaf area of *35S:MtANT1* transgenic plants was resulted from increased cell proliferation. Overall, *MtANTs* control leaf growth by promoting cell proliferation rather than cell expansion.

Discussion

AINTEGUMENTA-LIKE (AIL) proteins belong to the AP2-like family, and play vital roles in plant developmental process and stress response (Horstman et al., 2014; Meng et al., 2015a; Meng et al., 2015b). AIL transcription factors have been extensively studied in different species, but their functions in the model legume, *M. truncatula*, are largely unknown. According to the phylogenetic analysis, four MtANT genes are identified. MtANT1 and MtANT3 are clustered in one clade, while MtANT2 and MtANT4 are clustered in another clade, indicating the tandem duplication followed by genomic reshuffling in *M. truncatula*. The MtAIL proteins are more closely related to *Pisum sativum* homologs, indicating that their *MtAIL* and *PsAIL* genes may diverge from a common ancestor. Compared with other AILs, most *GmAIL* genes are presented in multiple copies, suggesting that these genes are a product of whole-genome duplication events and relatively slow process of diploidization during the evolutionary process. *AIL2/BBM/PLT4*, *AIL3/PLT1*, *AIL4/PLT2* and *AIL6/PLT3* were reported to regulate the root stem cell niche patterning in *Arabidopsis*, and *OsPLT1-6* are specifically expressed in the primodium of crown root and lateral root in rice (Galinha et al., 2007; Li and Xue, 2011). Based on the phylogenetic tree analysis, we speculate that MtAIL2, 3 and 4 and their homologous proteins in the same clade among leguminous species may be involved in the regulation of root and root nodule development. Gene structure is an important indicator for gene function and classification, of which intron gain or loss is the consequence of selection pressures during evolution (Mattick, 1994). In our study, all the *MtAIL* genes evolve into the same exon-intron structures, further supporting their close evolutionary relationship. Motifs and domains are involved in various regulatory processes including interactions between proteins, transcriptional activity, and DNA binding (Liu et al., 1999). The numbers and distribution of motifs in MtAILs are different, implying that MtAIL members have some differences in function. However, two AP2 domains are highly conserved among the MtAIL proteins, suggesting that the AP2 domains are evolutionarily conserved and necessary for the correct structure of AIL proteins. Moreover, proteins sharing the unique motifs in one cluster are likely to exert similar functions (Du et al., 2012; Zhao et al., 2019b). A unique motif (motif 10) was displayed in four MtANT members. This finding raises a question on whether motif 10 is related to the leaf growth, and future characterization of the function of motif 10 will clarify this point.

AtANT participates in organ size control, floral organ initiation and development (Mizukami and Fischer, 2000; Horstman et al., 2014; Manchado-Rojo et al., 2014), the *ANT*-homologous genes are associated with panicle branching, panicle structure and inflorescence development in rice (Kitomi et al., 2011; Luong et al., 2021). But we observed that *MtANTs* only affect the leaf growth in *M. truncatula*. The *mtant* quadruple mutant and *MtANT1* overexpression plants didn't exhibit other phenotypic changes, such as plant height, floral organ size and inflorescence structure. In addition, MtAIL1 is closer to MtANTs, and the developmental defects of *mtant* quadruple mutant may be masked by MtAIL1. Thus, the multiple

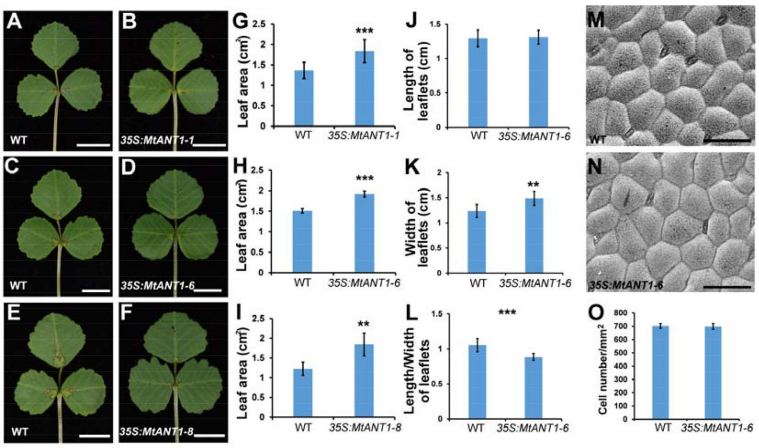

FIGURE 7
Phenotype analyses of leaves in *35S:MtANT1* plants. **(A–F)** Leaf phenotypes of wild type **(A, C, E)** and *35S:MtANT1-1, -6, -8* transgenic plants **(B, D, F)**. Bars = 1 cm. **(G–I)** Leaf area of wild type and *35S:MtANT1-1, -6, -8* plants. **(J–L)** Length **(J)**, width **(K)** and length/width ratio **(L)** of leaves in wild type and *35S:MtANT1-6* plants. **(M–N)** Scanning electron microscopy analyses of the adaxial surface of leaves in wild type **(M)** and *35S:MtANT1-6* plants **(N)** Bars = 50 μm. **(O)** Cell number per unit area of the adaxial surface of leaves in the wild type and *35S:MtANT1-6* plants. Bars represent means ± SD. ***P < 0.001, **P < 0.01.

mutations of *MtAIL1* and *MtANTs* may lead to severe developmental defects. In *Arabidopsis*, *N. tabacum*, *C. moschata*, *T. aestivum*, and *B. rapa*, ectopic expression of *ANT* enlarged the size of leaves (Mizukami and Fischer, 2000; Ding et al., 2018; Zhao et al., 2019b; Miao et al., 2021). In accordance with these reports, a similar phenotype of larger leaves was shown in *35S:MtANT1* plants. These results indicate that *ANT* genes also exert conserved function in increasing leaf area among different species. Biomass is critical for the evaluation of forage grass quality. Therefore, overexpression of *ANT* in legume forages, such as alfalfa, will be helpful to improve forage production.

Previous study showed that *ANGUSTIFOLIA3* (*AN3*) functions as a transcriptional activator of the GRF-INTERACTING FACTOR (GIF) family, and it is probably a potential target of *ANT* in promoting organ growth in *Arabidopsis* (Krizek et al., 2020). The loss-of-function mutant of *AN3* exhibits smaller leaf size, while ectopic expression of *AN3* results in larger leaves (Kim and Kende, 2004; Horiguchi et al., 2005; Lee et al., 2009). The potential regulatory relationship and phenotypes in *Arabidopsis* remind us that the paralogous gene of *AN3* in *M. truncatula* may play a similar role. In addition, *CYCD3* genes encode D-type cell cycle proteins which play key roles in the switch from cell proliferation to cell differentiation (Dewitte et al., 2003; Menges et al., 2006). The expression of *CYCD3;1* is prolonged in the leaves of *35S:ANT* plant to maintain the meristematic competence of cells during organogenesis in *Arabidopsis* (Mizukami and Fischer, 2000). Similarly, *CYCD3.2* is also the downstream target of the AIL1 transcription factor in poplar (Karlberg et al., 2011). According to these reports, it raises the possibility that *MtANTs* regulate the cell cycle genes to determine the leaf size.

The leaf development process includes three intertwined stages: leaf initiation in the SAM, primary morphogenesis, and secondary morphogenesis in which expansion and proliferation of cells occur (Poethig, 1997; Dengler and Tsukaya, 2001; Shani et al., 2009; Bar and Ori, 2014). Leaf complexity is determined during primary morphogenesis. *M. truncatula* is a compound-leafed species, whose adult leaves are trifoliate. In this study, we found the leaf complexities in both quadruple mutant and *MtANT1*-overexpressing plants were unchanged. Therefore, we propose that *MtANTs* mainly regulate the secondary morphogenesis of leaves in *M. truncatula*.

In addition, auxin plays an important role in leaf development by influencing cell proliferation and cell expansion (Tadege et al., 2011; Wang et al., 2021). In *Arabidopsis*, overexpression of the auxin-inducible gene *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*) generates larger leaves and prolongs the expression of *ANT*. *ARGOS* functions downstream of the auxin signaling pathway and upstream of *ANT* in the regulation of leaf size (Hu et al., 2003). So, the relationship among auxin related pathways, *MtANTs* and *MtARGOS* should be investigated in the future.

Conclusion

In this study, we performed genome-wide analyses and identified *AIL* genes in *M. truncatula*. We characterized *MtANT* genes expression profiles in different tissues,

suggesting that *MtANT* genes play important roles in the leaf morphogenesis of *M. truncatula*. Simultaneous disruption of *MtANTs* resulted in smaller leaves and overexpression of *MtANT1* led to larger leaves, demonstrating that *MtANTs* are vital for leaf size maintenance. However, they cann't influence the leaf complexity. Further study is needed to elucidate the molecular mechanism of *MtANTs* that are involved in leaf size development.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

XW, CZ and LH designed the research. XW, JJZ and JZ performed the research and analyzed the data. XW and LH wrote the paper. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1050462/full#supplementary-material

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