ChinaRxiv 中文

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-202107.00052

Cloning, Expression, and Promoter Analysis of the LtAGO1 Gene from *Liriodendron tulipifera* Postprint

Authors: Wei Lingmin, Wen Shaoying, Ma Jikai, Xia Hui, Li Jiayu, Wu Xujia, Li Huogen

Date: 2021-07-20T00:00:00+00:00

Abstract

Leaf primordia originate from the peripheral zone of the shoot apical meristem (SAM), and the AGO1 gene plays an important role in the differentiation process of leaf primordia. To deeply investigate the morphogenetic mechanism underlying leaf primordia differentiation into leaf organs, this study employed Liriodendron tulipifera as experimental material. The full-length cDNA and promoter sequence of LtAGO1 were obtained using RT-PCR and RACE cloning techniques, and its function was predicted; the tissue expression pattern of LtAGO1 in the genus Liriodendron was analyzed via RT-qPCR. Meanwhile, transgenic Arabidopsis thaliana lines harboring ProAGO1 :: GUS were obtained through resistance screening and DNA identification, and phenotypic analysis along with GUS histochemical staining were further performed on T2 generation positive plants. The results demonstrated: (1) The LtAGO1 gene contains a 3,300 bp open reading frame, encoding 1,100 amino acids, with a molecular weight of 122.14 kD and a theoretical isoelectric point (pI) of 9.36. (2) Amino acid sequence analysis indicated that LtAGO1 possesses two typical AGO gene domains, Gly-rich-AGO1 and Piwi; homology analysis revealed that the LtAGO1 protein is most closely related to the AGO1 protein of Cinnamomum micranthum (RWR84608.1). (3) Tissue expression specificity analysis showed that the relative expression level of LtAGO1 in different tissues of Liriodendron tulipifera followed the order: stamen > flower bud > petal > sepal > leaf > pistil > leaf bud > stem; the relative expression level of LtAGO1 at different leaf developmental stages in Liriodendron tulipifera was: leaf bud sprouting stage > young leaf stage > senescence stage > mature stage; AGO1 expression in the genus Liriodendron was higher in leaf margins than in other leaf regions, and expression in the sinus region of Liriodendron tulipifera leaves was higher than in the leaf tip region. (4) Transgenic lines exhibiting polarity defects in both mediolateral and proximal-distal axes, leaf margin serration, and double

flower phenotypes were obtained. GUS histochemical staining revealed that the LtAGO1 promoter drives stable GUS gene expression at the apex of leaf buds, with stronger expression in newly differentiated petioles, and specific expression in the vascular bundles of stems, leaves, flowers, and fruits at the mature stage. The GUS activity intensity driven by the LtAGO1 promoter was leaf apical bud > flower > vascular bundle, which is consistent with the real-time quantitative PCR results. In summary, these findings indicate that the LtAGO1 gene is specifically expressed in the apical meristem and participates in the developmental processes of leaf and floral organs through regulation by multiple pathways. This study provides a theoretical foundation for further understanding the basic function of the LtAGO1 gene in Liriodendron tulipifera and its mechanism in regulating leaf shape development.

Full Text

Cloning, Expression, and Promoter Analysis of the LtAGO1 Gene from *Liriodendron tulipifera*

WEI Lingmin, WEN Shaoying, MA Jikai, XIA Hui, LI Jiayu, WU Xujia, LI Huogen*
(Key Laboratory of Forest Genetics & Biotechnology of Ministry of Education, Co-Innovation Center for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing, 210037)

Abstract

Leaf primordia originate from the peripheral zone of the shoot apical meristem (SAM), and the AGO1 gene plays a crucial role in leaf primordium differentiation. To investigate the morphogenetic mechanisms underlying leaf primordium differentiation into leaf organs, we cloned the full-length cDNA and promoter sequence of LtAGO1 from *Liriodendron tulipifera* using RT-PCR and RACE technologies and predicted its function. RT-qPCR was employed to analyze the tissue expression patterns of LtAGO1 in *Liriodendron* species. Meanwhile, transgenic *Arabidopsis thaliana* lines carrying *ProAGO1::GUS* were obtained through resistance screening and DNA identification, and phenotypic and GUS histochemical staining analyses were performed on T2 generation positive plants.

The results showed: (1) The LtAGO1 gene contains a 3,300 bp open reading frame encoding 1,100 amino acids, with a molecular weight of 122.14 kD and a theoretical isoelectric point (pI) of 9.36. (2) Amino acid sequence analysis revealed that LtAGO1 possesses two typical AGO domains: Gly-rich-AGO1 and Piwi. Homology analysis indicated that LtAGO1 is most closely related to the AGO1 protein from *Cinnamomum micranthum* (RWR84608.1). (3) Tissue-specific expression analysis demonstrated that LtAGO1 expression levels varied across different tissues of *L. tulipifera*: stamen > floral bud > petal > calyx > leaf > pistil > leaf bud > stem. Expression across different leaf developmental

stages was: leaf bud sprouting stage > young leaf stage > senescence stage > mature stage. AGO1 expression was higher in leaf margins than in other leaf parts, with expression in leaf sinus regions exceeding that in leaf tips. (4) Transgenic lines exhibited polarity defects in both the mediolateral and basal-apical axes, serrated leaf margins, and double-petal flower types. GUS histochemical staining revealed that the LtAGO1 promoter drove stable GUS expression at leaf bud apices, with stronger expression in newly differentiated petioles, and specific expression in vascular bundles of mature stems, leaves, flowers, and fruits. GUS activity strength was: leaf apical bud > flower > vascular bundle, consistent with RT-qPCR results.

In summary, LtAGO1 is specifically expressed in apical meristems and participates in leaf and floral organ development through multiple regulatory pathways. This study provides a theoretical foundation for further understanding the basic functions of LtAGO1 and its regulatory mechanisms in leaf shape development in *L. tulipifera*.

Keywords: *Liriodendron tulipifera*, AGO1, leaf polarity, GUS, tissue expression

---

Introduction

Leaves are the primary organs for photosynthesis and transpiration in higher plants, with leaf primordia originating from the peripheral zone of the shoot apical meristem (SAM). During morphogenesis, when cells transition from division to growth, leaf primordia undergo polar differentiation (Bowman & Eshed, 2000). Previous studies have demonstrated that AGO1 mutations affect leaf primordium differentiation and organ polarity determination (Wu et al., 2009; Liu & Nonomura, 2016). Wang (2019) obtained *Arabidopsis ago1-27* mutants with needle-like leaves and *ago1-38* mutants with curled, bud-like compound leaves through point mutations. Li et al. (2014) overexpressed *AtAGO1* in *Arabidopsis*, resulting in serrated leaf margins. In model plants rice and maize, knockout, overexpression, and complementation studies have shown that AGO1 deficiency reduces seed set rate and pollen fertility, while AGO1 overexpression causes adaxial leaf curling and reduced plant height (Li et al., 2019; Xu, 2014). In tomato, SlmiR168 targets and regulates *SlAGO1a* expression, enhancing resistance to low potassium stress (Liu et al., 2020). *Arabidopsis ago1-27* mutants are more sensitive to flooding than wild-type plants and, together with AGO4, regulate hypoxia stress signal transduction under low oxygen conditions (Elena et al., 2020). Yao et al. (2021) found that the HvtAGO1-encoded protein plays an important role in the regulatory pathway for stripe disease resistance in hulless barley. Current research indicates that AGO1 responds to stress by inducing jasmonic acid (JA) signaling pathway genes and activating JA responses (Liu et al., 2018), demonstrating that AGO1 participates in plant stress response regulation through multiple pathways. Additionally, studies have found that AGO1d

is involved in wheat anther and pollen grain development (Feng, 2018), and *Arabidopsis* root meristem maintenance requires AGO1 activity for cell proliferation (Adrien et al., 2019). Thus, AGO1 plays crucial regulatory roles in organ polarity determination, meristem differentiation, floral organ development, and stress responses.

Both *Liriodendron tulipifera* and *Liriodendron chinense* are ornamental landscape trees valued for their distinctive leaf shapes and elegant stems. Their leaves exhibit morphological differences between three-lobed and five-lobed forms, making them ideal materials for studying leaf shape diversity and cultivar improvement in foliage plants. In recent years, Yang et al. (2014) confirmed that nine *LtNAC* genes in *L. tulipifera* participate in leaf senescence. Ma et al. (2018, 2019) elucidated the four-stage morphological development process from leaf primordium to leaf in *L. chinense*, identified and validated ten differentially expressed genes related to leaf development from transcriptome data, and found that *LcKNOX6* causes disordered leaf phyllotaxy, deeply lobed leaves, and sterility in *Arabidopsis*. However, the mechanisms underlying the distinctive leaf shape formation in *L. tulipifera* remain unclear, and whether AGO1 participates in leaf shape development regulation and its relationship with leaf and flower bud formation in *L. tulipifera* require further investigation.

Plant growth and development processes are controlled by gene expression levels, which are influenced by promoters containing cis-acting elements (Dey et al., 2015). Studies have found that *Arabidopsis AtADR* and *AtAIF* promoters regulate flower and anther development (Dai et al., 2019; Shih et al., 2014), indicating that promoters play important roles in regulating plant traits. This study employed RACE cloning technology to obtain the full-length cDNA of *LtAGO1* from *L. tulipifera*, performed bioinformatic predictions and tissue-specific expression analysis to preliminarily understand its function, and focused on analyzing the *LtAGO1* promoter sequence and tissue expression specificity to establish a foundation for investigating the regulatory mechanisms of the *LtAGO1* promoter on related genes.

1.1.1 Tissue Sampling

To clarify expression differences of this gene among different leaf parts in *Liriodendron* species, we selected *L. tulipifera* (South Carolina provenance) and *L. chinense* (Wuyishan provenance) with consistent phenological stages from the *Liriodendron* provenance test forest at Xiashu Teaching Forest Farm of Nanjing Forestry University. In April 2018, three healthy individuals were selected, and three parts of young leaves (leaf margin, leaf base, and leaf middle) were collected from each, following the method of Shen et al. (2003). To understand the tissue expression pattern of this gene in *L. tulipifera*, eight tissue samples (leaf, stem, stamen, pistil, floral bud, calyx, leaf bud, and petal) were collected in April. To investigate temporal expression differences during leaf development, leaves were collected from March to August 2018 at the leaf bud sprouting stage, young leaf stage, mature leaf stage, and senescence stage, following the method

of Xiao (2014).

To further clarify the location of leaf margin morphogenesis, the leaf margin was divided into seven parts: a, c, and e represent protruding parts; b and d represent sunken parts; f is the petiole; and g is the central leaf part. Previous phenological observations revealed that *L. tulipifera* leaves show maximum morphological variation in July. Therefore, different-sized leaves were collected in July 2018, and samples were separated to 1 g using sterilized scissors, following the method of Li (2015). All samples were collected in triplicate and stored at -80 °C for RNA extraction.

1.1.2 Biochemical Reagents

The plant total RNA extraction kit was purchased from TIANGEN Biotech. PrimeScript™ RT Master Mix (Perfect Real Time) reverse transcription kit, 3 -Full RACE Core Set with PrimeScript™ RTase kit, SMARTer® RACE 5 /3 Kit, PrimeSTAR® Max DNA Polymerase high-fidelity enzyme, DL 2000 DNA marker, and SYBR Premix Ex Taq enzyme were purchased from Takara. Gel-Stain nucleic acid dye, EasyPure Quick Gel Extraction Kit, and Blunt vector were purchased from TransGen Biotech. ClonExpress® Ultra One Step Cloning Kit recombinase was purchased from Vazyme Biotech. *Escherichia coli* T1 and *Agrobacterium* GV3101 were purchased from Weidi Bio. The overexpression vector PBI121-GUS was provided by our laboratory. Primers were synthesized by GenScript (Nanjing), with details listed in Table 1 .

1.2.1 Target Gene Cloning

Based on the *L. tulipifera* transcriptome database (http://ancangio.uga.edu/content/liriodendron-tulipifera), EST sequences annotated as AGO1 were screened. PCR primers were designed using Oligo7 software. Using cDNA, 3 RACE cDNA, and 5 RACE cDNA from *L. tulipifera* leaf buds as templates, three target fragments of *LtAGO1* were amplified. The PCR system was 50 L with the following program: 98 °C for 3 min; 35 cycles of 98 °C for 10 s, 58 °C for 30 s, 72 °C for 1 min; and final extension at 72 °C for 5 min. Target fragments were ligated into the Blunt vector and transformed into *E. coli* T1 competent cells for sequencing. The full-length cDNA sequence was assembled using DNAMAN software. The open reading frame was predicted using NCBI ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/), and primers were designed to verify cloning accuracy. Based on the validated *AGO1* CDS sequence, the upstream promoter sequence was identified from the *L. chinense* (Lushan provenance) NJFU-Lchi-2.0 genome sequencing results (Chen et al., 2019). Verification primers were designed using homology-based cloning (Table 1). DNA from sampled tissues was extracted using a modified CTAB method (Ma et al., 2007) and used as template to amplify approximately 2,000 bp of promoter sequence for sequencing confirmation.

1.2.2 Bioinformatic Analysis of LtAGO1

NCBI Conserved Domain (https://www.ncbi.nlm.nih.gov/cdd) was used to predict conserved domains of LtAGO1 protein. ExPASy Protparam (https://web.expasy.org/protparam/) was used to analyze physicochemical properties. SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_{automat}.pl?page=npsa_{sopma}.html) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) were used to predict secondary and tertiary structures, respectively. SignalP 5.0 Server (http://www.cbs.dtu.dk/services/SignalP/) predicted signal peptides, and PSORT (https://wolfpsort.hgc.jp/) predicted subcellular localization. Homologous sequences were identified using NCBI BLASTx. ClustalX was used for homology analysis, and MEGA 7 was used to construct a Neighbor-Joining phylogenetic tree. PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) was used to analyze cis-acting elements in the cloned promoter sequence.

1.2.3 Expression Analysis of LtAGO1

Total RNA was extracted from different developmental stages and tissues of *Liriodendron* species using the plant total RNA extraction kit and reverse-transcribed to synthesize first-strand cDNA. The cDNA was diluted 20-fold and used as template for RT-qPCR. Fluorescent quantitative PCR primers were designed using Oligo7 software. Real-time qPCR was performed using the *Liriodendron* reference gene *Actin97* (Tu et al., 2019), with primer sequences listed in Table 1. The 20 L reaction system contained: 10 L SYBR Premix Ex Taq II, 0.4 L each of forward and reverse primers (5 $mol \cdot L^{-1}$), 0.4 L ROX Reference Dye II, 2 L template (100 ng), and 6.8 L RNase-free ddH_2O. The amplification program was: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 60 °C for 34 s.

1.2.4 Vector Construction and Transformation

To determine whether the cloned 2,001 bp upstream sequence of LtAGO1 possessed promoter activity, the PBI121 overexpression vector was digested with Xba I and Hind III. After electrophoresis and gel confirmation, target fragments were recovered. The PCR product with restriction sites was cloned into the digested PBI121 vector using Vazyme homologous recombinase, transformed into *E. coli* for amplification, and the GUS expression vector PBI121-ProAGO1-GUS was obtained. The correctly identified plasmid was transformed into *Agrobacterium* GV3101 and introduced into wild-type *Arabidopsis* via floral dip (Clough & Bent, 1998). T0 generation seeds were screened on 1/2 MS medium containing 50 $mg \cdot L^{-1}$ kanamycin. T1 generation plants were identified by PCR using promoter sequencing primers, with wild-type plants as negative controls. Confirmed transgenic plants were screened to the T2 generation for GUS staining analysis.

1.2.5 Phenotypic Observation of Transgenic Arabidopsis

Seeds of transgenic and wild-type *Arabidopsis* were sterilized with 75% ethanol and 10% sodium hypochlorite, washed three times with pure water, and sown on 1/2 MS medium (pH 5.8). After stratification at 4 °C for 2 days, plants were grown in a growth chamber at 25 °C with a 16 h light/8 h dark photoperiod and light intensity of 5,000 lx. Root length of 10-day-old seedlings was measured and recorded, with five replicates.

1.2.6 GUS Histochemical Staining of Transgenic Arabidopsis

According to the Solarbio GUS kit instructions, T2 generation *ProAGO1::GUS* transgenic plants at different growth stages (4, 6, 9, 12, 16, 20, and 25 days) and tissues were stained following Jefferson (1987), incubated overnight at 37 °C, destained with 75% ethanol 5–6 times, and photographed under a stereomicroscope. Wild-type plants infiltrated with GV3101 empty strain served as negative controls, and 35S::GUS empty vector-transformed *Arabidopsis* served as positive controls.

2.1 Full-Length cDNA Acquisition of LtAGO1

A 3,592 bp intermediate fragment of AGO1 was obtained from *L. tulipifera* leaf buds. RACE cloning yielded 440 bp and 636 bp sequences for the 5 and 3 ends (Figure 2 [Figure 2: see original paper]A), which were assembled into a 4,258 bp full-length cDNA sequence. ORF Finder predicted a 78 bp 5 -UTR, 880 bp 3 -UTR containing 13 polyA signals, and a 3,300 bp ORF encoding 1,100 amino acids. Primers flanking the ORF were designed for amplification and sequencing verification, confirming the ORF length matched the assembled sequence without variation sites. Protein structure analysis revealed two conserved domains: Gly-rich-AGO1 and Piwi, with the Piwi domain located at the C-terminus containing RNA 5 -end binding sites and mRNA cleavage active sites (Figure 2B). These results verified the cDNA sequence, and the gene was designated *LtAGO1*.

2.2 Secondary and Tertiary Structure Prediction of LtAGO1 Protein

As shown in Figure 2C, the protein comprises extended strands, α-helices (h), β-turns (t), and random coils (c), with random coils being most abundant (52.14%). α-helices (28.57%) and extended strands (13.83%) were also prominent, while β-turns were least common (5.46%). To further understand protein structure, the tertiary structure was predicted and modeled (Figure 2D), showing highest similarity to AGO2 protein with 100% confidence. SignalP 5.0 Server prediction yielded a low D-value (0.0016), suggesting the encoded protein lacks a signal peptide and is non-secretory. Subcellular localization prediction indicated scores of 0.3 for microbodies, 0.3 for nucleus, 0.1 for cytoplasm, and 0 for plasma membrane, suggesting nuclear and microbody localization.

2.3 Homology Alignment and Phylogenetic Analysis of LtAGO1

BLASTx analysis against NCBI databases revealed homology with AGO1 proteins from *Cinnamomum micranthum*, *Phoenix dactylifera*, and *Musa acuminata* (72.97%-76.33% similarity). Multiple sequence alignment showed the C-terminus of LtAGO1 is relatively conserved compared to the N-terminus, with a conserved Piwi domain present in all homologs (Figure 3 [Figure 3: see original paper]). The high sequence identity with AGO1 proteins from various plant species indicates evolutionary conservation. Phylogenetic analysis clustered *L. tulipifera* AGO1 most closely with *C. micranthum* AGO1 (RWR84608.1), followed by *P. dactylifera* (XP_{008812792}.1) and *M. acuminata* (XP_{009386429}.1), while showing more distant relationships with *Jatropha curcas* (XP_{012079244}.1) and *Hevea brasiliensis* (XP_{021670505}.1) (Figure 4 [Figure 4: see original paper]).

2.4 Spatiotemporal Expression Analysis of LtAGO1

qRT-PCR analysis of LtAGO1 expression in eight *L. tulipifera* tissues showed expression in all tissues but with significant variation (Figure 5 [Figure 5: see original paper]A). Highest expression occurred in stamens and floral buds, significantly higher than other tissues, followed by petals. Lower expression was detected in calyx, floral buds, leaves, leaf buds, and pistils. The expression pattern was: stamen > floral bud > petal > calyx > leaf > pistil > leaf bud > stem, suggesting important roles in floral organ development.

To investigate interspecific expression differences among leaf positions, qRT-PCR was performed on leaf base, middle, and margin (Figure 5B). In *L. tulipifera*, expression was: margin > middle > base. In *L. chinense*, expression was: margin > base > middle. AGO1 was predominantly expressed in leaf margins of both species, with overall expression in *L. chinense* being 4.5-7.5 times higher than in *L. tulipifera*, particularly in middle and base regions, indicating interspecific spatial distribution differences.

Temporal expression analysis during leaf development (bud sprouting stage 1-2, young leaf stage 3, mature stages 4-6, and senescence stage 7) showed decreasing expression during bud swelling (Figure 5C). During growth, expression negatively correlated with leaf size, reaching minimum at maximum leaf area. In senescence, expression increased compared to late growth stage but remained lower than in bud sprouting stage. Expression was substantially higher during primordium formation than in mature leaves. The developmental expression pattern was: leaf bud sprouting stage > young leaf stage > senescence stage > mature stage, indicating high expression during active meristematic growth.

To further clarify expression sites during leaf margin indentation, qRT-PCR was performed on protruding (tips a, c, e) and sunken (b, d) margin parts (Figure 5D). Expression in petioles far exceeded other parts, with higher expression in

sunken regions (b) than protruding regions (c). The expression pattern was: petiole > leaf sinus > leaf tip, suggesting LtAGO1 plays an important role in leaf margin lobe formation.

2.5 LtAGO1 Promoter Cloning, Vector Construction, and Identification

PCR amplification using *L. tulipifera* genomic DNA yielded a 2,001 bp promoter sequence (Figure 6 [Figure 6: see original paper]A). The promoter fragment was ligated into plant expression vector PBI121 (Figure 6D). Colony PCR of *E. coli* showed amplification products around 2,000 bp, consistent with positive controls (Figure 6B). Sequencing results matched database sequences. Double digestion produced two bands: one at 12,758 bp and another matching the ProAGO1 size, confirming successful promoter insertion into PBI121.

2.6 Cis-Acting Element Analysis of LtAGO1 Promoter

PlantCARE analysis of the LtAGO1 promoter revealed essential elements for RNA polymerase II transcription initiation (CAAT-box and TATA-box), numerous light-responsive elements (Box 4, G-box, GT1-motif, MYB), defense-related elements (TC-rich repeats), MeJA-responsive elements (CGTCA-motif, TGACG-motif), salicylic acid-responsive element (TCA-element), ABA-responsive element (MYC), cold stress element (as-1), anaerobic induction element (ARE), and growth regulation elements (meristem expression element CCGTCC-box, zein metabolism element O2-site) (Table 2).

2.7 Detection and Phenotypic Observation of Transgenic Plants

PCR screening identified 11 positive *ProAGO1::GUS* transgenic lines (Figure 7 [Figure 7: see original paper]C). T2 generation plants showed root length of (0.5 ± 0.2) cm compared to (1.5 ± 0.2) cm in wild-type, with well-developed lateral roots (Figure 7D). Leaf area was smaller than wild-type, with notched apexes and albinism in the second true leaf pair. At 30 days, two phenotypic lines were distinguished: dwarf plants (Figure 7F) with delayed bolting, sterile double-petal flowers, and smaller rosette leaves (Figure 7E). Line 1 showed one leaf extending mediolaterally with narrow base and fan shape; other leaves were half the wild-type area with asymmetric development in mediolateral and basal-apical axes, oblique leaf bases, and curved petioles. Line 2 had two fewer rosette leaves than wild-type, deeper leaf notches, one leaf 5–10 times larger than others with wedge-shaped base and heart-shaped apex, oblique leaf bases, linear or strap-shaped leaf development from base to apex, and short petioles. These results suggest LtAGO1 promoter influences polarity differentiation in both mediolateral and basal-apical axes from primordium initiation, with increasing variation over time, ultimately maintaining serrated leaf margin phenotypes.

2.8 LtAGO1 Promoter Activity Analysis

GUS expression driven by the LtAGO1 promoter showed stage-specific patterns during plant development (Figure 8 [Figure 8: see original paper]). No GUS activity was detected at 4 and 6 days post-germination (Figure 8B1-B2). During leaf bud differentiation (days 9–25), the promoter drove stable GUS expression at leaf bud apices, with strongest activity in newly differentiated petioles (Figure 8B3-B7). During reproductive growth, expression occurred in main leaf veins, serrated leaf margin tips, pedicels, sepals, and pistils, indicating expression in vascular bundles of mature flowers, pods, leaves, and stems (Figure 8B8-B11). GUS activity strength was: leaf apical bud > floral organ > vascular bundle, characterizing it as a meristem-specific promoter.

2.9 LtAGO1 Protein Co-Expression Network

The LtAGO1 co-expression network (Figure 9 [Figure 9: see original paper]) showed interactions with stress-responsive genes (*DCLs*, *RDR6*, *SGS3*) and miRNA-mediated leaf polarity differentiation genes (*HEN1*, *HYL1*). Small RNAs (20–24 nt) respond to various stresses (Chen et al., 2002; Zhang et al., 2008), with biogenesis depending on DCL, AGO, and RDR family proteins (Saito & Siomi, 2010). SGS3 and RDR6 cooperatively convert single-stranded RNA to dsRNA, inducing post-transcriptional gene silencing (PTGS) to reduce pathogen damage (Yoshikawa et al., 2013). RDR6 also regulates trans-acting siRNA pathways with AGO7, SGS3, and DCL4, targeting auxin response factors involved in leaf abaxialization (Peragine et al., 2004). *NbDCL1* silencing in tobacco causes dwarfism and leaf malformation. HYL1 regulates *HD-ZIP III* genes via miRNA to maintain flat leaf development (Yu et al., 2005). HEN1 was first discovered related to floral organ development (Chen et al., 2002) and regulates most miRNAs in *Arabidopsis* (Yu et al., 2006), participating in ABA signaling regulation with DCL1-3 (Park et al., 2002). AGO1 interacts with these genes to coordinately regulate stress responses and development.

3 Discussion and Conclusion

This study cloned the *LtAGO1* gene from *L. tulipifera*. Bioinformatic analysis revealed high similarity with other species' AGO1 proteins and nuclear subcellular localization, consistent with results in *Populus euphratica* (Li et al., 2018). Tissue-specific analysis showed *LtAGO1* expression was significantly higher during leaf primordium differentiation than in mature and senescent stages, with high expression in young leaf margins but only petiole expression in mature leaves. *ZmAGO1a* showed highest expression in maize young leaves and silking-stage ears (Xu, 2014), with higher expression in new leaves than old leaves (Xu et al., 2014). During *L. tulipifera* reproductive growth, *LtAGO1* expression was highest in stamens, followed by floral buds, similar to studies in longan (Yang, 2015) and apple (Lu et al., 2013). These results indicate AGO1 participates in leaf primordium differentiation during seedling stages and is abundant in

rapidly dividing floral organs during reproductive stages.

Promoters are transcriptional regulation centers. This study predicted multiple light-responsive, hormone-inducible, meristem expression, and abiotic stress response elements in the *LtAGO1* promoter. Further research confirmed ProAGO1 successfully drives GUS expression with spatiotemporal specificity: expression in apical meristems during seedling stage, strongest in newly differentiated petioles as apices differentiate new leaves, and later in vascular bundles of mature flowers, pods, leaves, and stems. Vaucheret et al. (2006) analyzed *ProAGO1::GUS Arabidopsis* and found AGO1 expressed throughout development, most abundantly in meristematic and vascular tissues. This indicates AGO1 expression is not limited to apical meristems but also occurs in other lateral meristem regions, though the underlying mechanisms remain unreported.

Leaf polarity differentiation involves primordium cells sensing polarity signals and responding accordingly, with differentiation along three polarity axes (adaxial-abaxial, basal-apical, and mediolateral) determining leaf morphogenesis (Du et al., 2018). This study obtained *Arabidopsis* lines with curved petioles and lost polarity in mediolateral and basal-apical axes via *ProAGO1::GUS*. Downregulating *SlAGO1* in tomato caused abaxial trichomes similar to adaxial surfaces and defective leaflet petiole development (Wang et al., 2015). *LtAGO1* promoter expression also promoted ectopic meristematic activity, causing wedge-shaped leaf bases and heart-shaped notched apices. AGO1 loss-of-function mutants show leaf curling, dwarfism, and reduced fertility (Wu et al., 2009; Liu & Nonomura, 2016). Kidner et al. (2005) found AGO1 regulates leaf polarity through *STM* gene-mediated stem cell function. Thus, both AGO1 deficiency and overexpression affect leaf polarity loss, suggesting AGO1 may regulate leaf morphogenesis through two distinct pathways. Current understanding indicates leaf primordium and leaf development require active *STM* and silenced *KNOX I* genes. AGO1 determines adaxial leaf and petal development by positively regulating *STM* and inhibiting *KNOX I* expression (Yang et al., 2006). Leaf morphogenesis (e.g., hole formation, margin splitting) is associated with programmed cell death (PCD) in key regions (Arunika et al., 2004), and adaxially curled leaves result from PCD in abaxial mesophyll cells (Zhang et al., 2009). Whether AGO1 regulates leaf shape diversity by controlling cell differentiation direction and speed, and how it responds to primordium differentiation initiation signals, requires further investigation. In reproductive development, our expression lines showed delayed bolting, large double-petal flowers, and sterility, consistent with Li et al. (2019). This may occur because floral organs differentiate from apical meristems where AGO1 is continuously and stably expressed, enabling participation in leaf and floral organ morphogenesis. Whether related genes are co-regulated by AGO1 requires further study.

This study first analyzed the spatiotemporal expression profile of *LtAGO1* in different tissues of *L. tulipifera*, clarified its promoter expression pattern in *Arabidopsis* meristems, and preliminarily explored *LtAGO1* regulation of leaf

shape development. Future work will construct *LtAGO1* overexpression vectors for poplar transformation and investigate protein interactions between LtAGO1 and key leaf development *KNOX I* family genes. These results will provide theoretical references for analyzing AGO1 function in woody plant growth and development and practical significance for promoting genetic improvement of leaf morphology in ornamental foliage trees.

References

ADRIEN T, PATRICIA B, MARIE CC, et al., 2019. Cell cycle–dependent regulation and function of ARGONAUTE1 in Plants[J]. Plant Cell, 31(8): 1734-1750.

ARUNIKA HL, GUNAWARDENA AN, JONE S, et al., 2004. Programmed cell death pemodels lace plant leaf shape during development[J]. Plant Cell, 16(1): 60-73.

BOWMAN JL, ESHED Y, 2000. Formation and maintenance of the shoot apical meristem[J]. Trends Plant Sci, 5(3): 110-115.

CHEN J, HAO Z, GUANG X, et al., 2019. Liriodendron genome sheds light on angiosperm phylogeny and species-pair differentiation[J]. Nat Plants, 5(1): 18-25.

CHEN X, LIU J, CHENG Y, et al., 2002. HEN1 functions pleiotropically in Arabidopsis development and acts in C function in the flower[J]. Development, 129(5): 1085-1094.

CLOUGH SJ, BENT AF, 1998. Floraldip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana[J]. Plant J, 16(6): 735-743.

DAI SY, HSU WH, YANG CH, 2019. The gene ANTHER DEHISCENCE REPRESSOR(ADR) controls male fertility by suppressing the ROS accumulation and anther cell wall thickening in Arabidopsis[J]. Sci Rep, 9(5112):

DEY N, SARKAR S, ACHARYA S, et al., 2015. Synthetic promoters in planta[J]. Planta, 242(5): 1077-1094.

DU F, GUAN C, JIAO Y, 2018. Molecular mechanisms of leaf morphogenesis[J]. Mol Plant, 11(9): 1117-1134.

ELENA L, FEDERICO B, MARIA JL, et al., 2020. ARGONAUTE1 and ARGONAUTE4 regulate gene expression and hypoxia tolerance[J]. Plant Physiol, 182(1): 287-300.

FENG N, 2018. Transcriptome and miRNA analyses of young inflorescences in wheat(Triticum aestivum L.)[D]. Beijing: Chinese Academy of Agricultural Sciences: 57-63.

JEFFERSON RA, 1987. Assaying chimeric genes in plants: the GUS gene fusion system[J]. Plant Mol Biol Rep, 5(4): 387-405.

LI HY, YANG X, WANG SB, 2018. Cloning and subcellular localization of ARGONAUTE1 gene from Populus euphratica[J]. J Henan Univ Sci Technol, 39(5): 68-73.

LI SF, ZAO Y, JI LS, 2014. Influences of AGO1 to the development of the Arabidopsis leaves with the serration margin[J]. J Hebei For Univ, 35(1): 51-57.

LI SY, 2015. Gibberellin impact on lotus plant height and cloning and expression analysis of its related genes[D]. Wuhan: Huazhong Agricultural University: 25-34.

LI YH, YANG YQ, LIU Y, et al., 2019. Overexpression of OsAGO1b induces adaxially rolled leaves by affecting leaf abaxial sclerenchy matous cell development in Rice[J]. Rice, 12(1): 60.

LIU C, XIN Y, XU L, et al., 2018. Arabidopsis ARGONAUTE 1 binds chromatin to promote gene transcription in response to hormones and stresses[J]. Dev Cell, 44(3): 348-361.

LIU H, NONOMURA KI, 2016. A wide reprogramming of histone H3 modifications during male meiosis I in rice is dependent on the Argonaute protein MEL1[J]. J Cell Sci, 129(19): 3553-3561.

LIU X, TAN CC, CHENG X, et al., 2020. miR168 targets Argonaute1A mediated miRNAs regulation pathways in response to potassium deficiency stress in tomato[J]. BMC Plant Biol, 20(1): 477.

LIVAK KJ, SCHMITTGEN TD, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method[J]. Methods, 25(4): 402-408.

LU Y, MA C, MENG D, et al., 2013. Clone and expression of AGO1 gene and development-related miRNA in apple(Golden Delicious)[J]. J Chin Agric Univ, 18(5): 69–76.

MA JK, MEI GG, LIU HH, et al., 2019. Overexpression of a Novel LcKNOX transcription factor from Liriodendron chinense induces lobed leaves in Arabidopsis thaliana[J]. Forests, 11(1): 33.

MA JK, WEI LM, LI JY, et al., 2018. The analysis of genes and phytohormone metabolic pathways associated with leaf shape development in Liriodendron chinense via De Novo transcriptome sequencing[J]. Genes, 9(12):

MA M, GUO KQ, GUO QR, 2007. Studies on genomic DNA extraction of forest species with improved CTAB method[J]. Biotechnology, 17(3): 36-38.

PARK W, LI J, SONG R, et al., 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana[J]. Curr Biol, 12(17): 1484-1495.

PERAGINE A, YOSHIKAWA M, WU G, et al., 2004. SGS3 and SGS2/SDE1/RDR6 are required for juvenile development and the production of trans-acting siRNAs in Arabidopsis[J]. Genes Dev, 18(19): 2368-2379.

SAITO K, SIOMI MC, 2010. Small RNA-mediated quiescence of transposable elements in animals[J]. Dev Cell, 19(5): 687-697.

SHEN ZG, YU D, HU ZH, et al., 2003. Comparative and the correlative studies on the structure and the content of anthraquinones in 3 Aloe leaves[J]. Acta Bot Boreal-Occidental Sin, 2003, 23(7): 1148-1153.

SHIH CF, HSU WH, PENG YJ, et al., 2014. The NAC-like gene AIF acts as a repressor that controls anther dehiscence by regulating genes in the jasmonate biosynthesis pathway in Arabidopsis[J]. J Exp Bot, 65(2):

TU ZH, HAO ZY, ZHONG WP, et al., 2019. Identification of suitable reference genes for RT-qPCR assays in Liriodendron chinense (Hemsl.) sarg[J]. Forests, 10(5): 441-457.

VAUCHERET H, 2006. Post-transcriptional small RNA pathways in plants: mechanisms and regulations[J]. Genes Dev, 20(7): 759-771.

WANG T, LI R, WEN L, et al., 2015. Functional analysis and RNA sequencing indicate the regulatory role of Argonaute1 in tomato compound leaf development[J]. PLoS ONE, 10(10): e140756.

WANG YY, 2019. New function of AGO1 in arabidopsis small RNA pathway[D]. Shenzhen: Shenzhen University: 38-53.

WU L, ZHANG Q, ZHOU H, et al., 2009. Rice microRNA effector complexes and targets[J]. Plant Cell, 21(11):

XIAO HJ, 2014. Cloning and functional analysis of leaf senescence ce-related genes in Capsicum Annuum L.[D]. Yangling: Northwest A & F University: 43-76.

XU DD, 2014. Cloning and functional identification of maize AGO1a gene[D]. Beijing: Chinese Academy of Agricultural Sciences: 27-39.

XU X, LI DD, LI SJ, et al., 2014. Clone and expression analysis of AGO genes from Zea mays L[J]. Northwest Bull Bot, 34(3): 449-453.

YANG L, HUANG W, WANG H, et al., 2006. Characterizations of a hypomorphic argonautel mutant reveal novel AGO1 functions in Arabidopsis lateral organ development[J]. Plant Mol Biol, 61(1-2): 63-78.

YANG MM, 2015. The study of cloning and expression analysis of AGO gene from embryogenic callus in Dimocarpus longan Lour[D]. Fuzhou: Fujian Agriculture and Forestry University: 56-64.

YANG Y, XU M, LUO Q, et al., 2014. De novo transcriptome analysis of Liriodendron chinense petals and leaves by illumina sequencing[J]. Gene, 534(2): 155-162.

YAO XH, WANG Y, AN LK, et al., 2021. Identification and expression analysis of HvtAGO1 gene in response to barley leaf stipe in Tibetan Hulless Barley[J]. Acta Bot Boreal-Occidental Sin, 41(1): 20-28.

YOSHIKAWA M, IKI T, TSUTSUI Y, et al., 2013. 3' fragment of miR173-programmed RISC-cleaved RNA is protected from degradation in a complex with RISC and SGS3[J]. Pnas, 110(10): 4117-4122.

YU B J, CHEN X, EBRIGHT YW, et al., 2006. HEN1 recognizes 21-24 nt small RNA duplexes and deposis a methyl group onto the 2' OH of the 3' terminal nucleotide[J]. Nucl Acid Res, 34(2): 667-675.

YU B, YANG Z, LI J, et al., 2005. Methylation as a crucial step in plant microRNA biogenesis[J]. Science, 307(5711): 932-935.

ZHANG G, XU Q, ZHU X, et al., 2009. SHALLOT-LIKE1 is a KANAD I transcription factor that modulates rice leaf rolling by regulating leaf abxial cell development[J]. Plant Cell, 21(3): 719-735.

ZHANG JF, YUAN LJ, SHAO Y, et al., 2008. The disturbance of small RNA pathways enhanced abscisic acid response and multiple stress responses in Arabidopsis[J]. Plant Cell Environ, 31(4): 562-574.

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv —Machine translation. Verify with original.