



Overexpression of the *LcCUC2-like* gene in *Arabidopsis thaliana* alters the cotyledon morphology and increases rosette leaf number

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ABSTRACT

Background. The unique ‘mandarin jacket’ leaf shape is the most famous trait of *Liriodendron chinense* and this characteristic gives *L. chinense* aesthetic and landscaping value. However, the underlying regulatory mechanism of genes involved in the leaf development of *L. chinense* has remained unclear.

Methods. Based on transcriptome data of leaves at different developmental stages from *L. chinense*, we identified differentially expression genes (DEGs) functioning in leaf development. A candidate gene named *LcCUC2-like* (*LcCUC2L*) had high similarity in sequence with *Arabidopsis thaliana* *CUC2*, and used for further research. We isolated the full-length *LcCUC2L* gene and its promoter from *L. chinense*. Subsequently, we analyzed the function of the *LcCUC2L* gene and its promoter activity via transformation into *A. thaliana*.

Results. In this study, we found that the *LcCUC2L* and *AtCUC2* are homologous in sequence but not homologous in function. Unlike the role of *AtCUC2* in leaf serration and SAM formation, the *LcCUC2L* mainly regulates cotyledon development and rosette leaf number. Histochemical β -glucuronidase (GUS) staining revealed that *LcCUC2L* was expressed in the cotyledons of *A. thaliana* seedlings, indicating that the *LcCUC2L* may play a role in cotyledon development. Ectopic expression of *LcCUC2L* resulted in long, narrow cotyledons without petioles, abnormal lamina epidermis cells and defective vascular tissue in cotyledons, and these results were consistent with the *LcCUC2L* expression pattern. Further analysis showed that overexpression of *LcCUC2L* also induced numerous rosette leaves. Also, *LcCUC2L* and other related genes showed a severe response in *L. chinense* by introducing exogenous auxin stimulation, partly revealed that *LcCUC2L* affects the leaf development by regulating the auxin content.

Conclusions. These results suggest that *LcCUC2L* may play a critical role in leaf development and morphogenesis in *L. chinense*, and our findings provide insight into the molecular mechanisms of leaf development in *L. chinense*.

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INTRODUCTION

The leaf, as an indispensable plant organ, functions in photosynthesis, respiration, and photoperception (Li et al., 2007). Leaf shape is one of the most important characteristics affecting plant productivity and survival. Previous studies have shown that compared with unlobed leaves, lobed leaves have larger specific leaf areas, which can enhance the competitive ability of the plant for light resources and improve photosynthetic rates (Semchenko & Zobel, 2007). Moreover, dissected leaves allow adjustment of leaf surface temperature and allow strong adaptability to stress (Vogel, 2009). In recent years, researchers have paid much attention to the regulatory mechanisms of leaf shape development (Biltsborough et al., 2011). In addition to environmental factors, many genetic factors, such as complex gene interactions, gene expression patterns, and microRNAs, and active hormonal regulation influence leaf shape development (Dkhar & Pareek, 2014). To date, many studies of leaf shape development have focused on herbaceous plants such as *A. thaliana* (Kessler & Sinha, 2004), *Cardamine hirsuta* (Barkoulas et al., 2008), *Medicago truncatula* (Peng et al., 2011), and *Solanum lycopersicum* (Chitwood et al., 2014). However, in woody plants, the mechanisms of leaf shape development remain poorly understood.

Liriodendron chinense (Hemsl.) Sarg, a valuable endemic tree belonging to the magnolia family (Magnoliaceae), is distributed mainly in southern China and northern Vietnam (Fang, 1994; Wang, 1997). *L. chinense* is an attractive species for ornamental use, with a distinctive leaf shape similar to that of the traditional Chinese mandarin jacket. Normally, *L. chinense* has one deep lobe on each side of the leaf margin (Yang et al., 2014). Due to its unique leaf shape, some investigators have focused on leaf development in *L. chinense*. To date, some progress has been made in this field. For instance, a comparison of *L. chinense* leaf transcripts among various developmental stages has been performed (Ma et al., 2018). Ectopic expression of *KNOX6* from *L. chinense* in *A. thaliana* was found to result in numerous lobed leaves. It has been proposed that *LcKNOX6* might participate in leaf development in *L. chinense* (Ma et al., 2020). Also, some *LcAP2/ERFs* also seems involved in leaf morphogenesis through STC analysis in various tissues and the further anatomical assay of the leaf bud (Zong et al., 2021).

CUC, a member of the NAC transcription factor family with a highly conserved N-terminal domain and a variable C-terminal domain (Taoka et al., 2004), was first isolated from *A. thaliana* (Aida et al., 1997). Leaves usually originate from protuberant clusters of cells around the shoot apical meristem (SAM) (Kessler & Sinha, 2004). Experimental evidence suggests important roles of *CUC2* in SAM formation and organ boundary formation, with *cuc1 cuc2* double mutants exhibiting a single cup-shaped cotyledon and a deficiency in embryonic SAM (Aida et al., 1997). Moreover, *CUC* transcription factors are commonly called plant dissectors partially due to their functions in leaf margin development (Biltsborough et al., 2011). Early reports showed that the expression of the *CUC2* gene is modulated by auxin (Vernoux et al., 2000; Aida et al., 2002; Furutani et al., 2004). *PINFORMED 1* (*PIN1*), as the main auxin transporter (Galweiler et al., 1998), is regulated by *CUC2* during serration formation (Kawamura, Horiguchi & Tsukaya, 2010). Auxin, *PIN1*, and *CUC2* have been demonstrated to compose a regulatory network involved

in leaf serration formation (Bilsborough et al., 2011). Belonging to a class of plant-specific microRNAs, miR164 is a significant regulatory factor for normal plant development (Mallory et al., 2004). It has been confirmed that *CUC2* is the target gene of miR164 (Rhoades et al., 2002). miR164 negatively regulates *CUC2*, and their balance influences the depth of leaf lobation (Nikovics et al., 2006). In addition, *DEVELOPMENT-RELATED PcG TARGET IN THE APEX4* (*DPA4*) inhibits leaf serration formation by negatively regulating *CUC2* expression independent of miR164 (Engelhorn et al., 2012). Investigators have proven that *NGATHA-LIKE 1* (*NGAL1*) directly inhibits *CUC2* expression by binding to the *CUC2* promoter and thereby negatively regulates the formation of leaf margin serration (Shao et al., 2020). In addition, *cuc2 cuc3* double mutants exhibit a lack of an axillary meristem (Hibara et al., 2006), indicating that *CUC2* might be involved in axillary meristem initiation. Previous studies have suggested that *CUC2* plays important roles in adventitious shoot formation (Daimon, Takabe & Tasaka, 2003) and floral organ development (Aida et al., 1997; Baker et al., 2005; Kamiuchi et al., 2014; Gonzalez-Carranza et al., 2017). Moreover, *CUC2* plays a role in internode development, with *BpCUC2* overexpression leading to internode shortening in *Betula pendula* (Liu et al., 2019). Taken together, these observations suggest that *CUC2* participates in both growth and development in plants.

In this study, *LcCUC2L* and its promoter were isolated from *L. chinense* and characterized by conducting bioinformatics analysis. Assessments of the functions of *LcCUC2L* in transgenic *A. thaliana* plants were performed using overexpression assays. As a result, GUS staining revealed that *LcCUC2L* was expressed in the cotyledons of *A. thaliana* seedlings, which indicated that *LcCUC2L* may play a role in cotyledon development. Intriguingly, almost all the transgenic *A. thaliana* lines displaying abnormal cotyledons and an increased rosette leaf number. Further SEM observation and venation pattern analysis showed that overexpression of *LcCUC2L* induced abnormal lamina epidermis cells and defective vascular tissue of cotyledons. Hormone determination and RT-qPCR results indicated that *LcCUC2L* affects leaf development by regulating the auxin content and the expression of genes involved in auxin synthesis, transport, and leaf shape development in *A. thaliana*. Our results will help to reveal the molecular mechanisms of leaf development in *L. chinense*.

MATERIALS & METHODS

Plant materials, growth conditions, and treatments

Samples of leaves at different developmental stages (Tu et al., 2019) were harvested from an adult *L. chinense* tree in Xiashu, Jurong County, Jiangsu Province, China (119°13'20'E, 32°7'8'N) (Yang et al., 2014). Plant materials were maintained at −80 °C prior to analysis.

Wild-type (WT) *A. thaliana* (Columbia-0), transgenic *A. thaliana*, *Nicotiana benthamiana* (Ben) plants, and *L. chinense* seedlings were cultivated at 23 °C under a long-day photoperiod (16 h light, 8 h dark) in a growth chamber (70% relative humidity).

For the IAA treatment, *L. chinense* seedlings grown in the incubator for 5 months were sprayed with 200 μM IAA on leaves and then sampled at 48 h. Water was used as the control. The samples were placed in an ultra-low-temperature freezer for further analysis.

DNA, RNA extraction, and RT-qPCR

A DNasesecure Plant Kit (Tiangen) was used for DNA isolation from *L. chinense*. RNA was extracted from *L. chinense* and *A. thaliana* leaves using the RNeasy Pure Plant Kit (Tiangen). Subsequently, first-strand cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa) in accordance with the manufacturer's protocol. The *LcCUC2L* transcript levels in leaves of different developmental stages (Tu et al., 2019) were investigated by RT-qPCR. Moreover, we also detected *LcCUC2L* expression levels under IAA treatment. *L. chinense* Actin 97 was used as the internal control. In addition, the transcription levels of *LcCUC2L* and some other genes in WT and transgenic lines were examined using specific primers (Table S1). *A. thaliana* Actin 2 was used as the internal quantitative control. RT-qPCR was carried out using the SYBR Premix EX Taq kit (TaKaRa). We applied the $2^{-\Delta\Delta C_T}$ method to analyze the data from relative quantification (Livak & Schmittgen, 2001).

Cloning and bioinformatics analysis

Based on the known transcriptome database (Ma et al., 2018), the *CUC2* gene was identified in *L. chinense*. The full-length *LcCUC2L* gene was obtained using RT-PCR and RACE. The *LcCUC2L* gene was assembled with the middle region, 5' sequence and 3' sequence. ORF Finder was used for prediction of the open reading frame (ORF) and protein sequence. The structure of the *LcCUC2L* gene was analyzed by the online software GSDS (<http://gsds.gao-lab.org/>). Analysis of the physicochemical characteristics of the proteins was conducted online at the following website: (<https://web.expasy.org/protparam/>). PredictProtein was used to forecast protein secondary structure. Subcellular localization was predicted by applying WoLF PSORT. Protein conserved domain analysis of *LcCUC2L* was performed with NCBI (<https://www.ncbi.nlm.nih.gov/cdd/?term>). The amino acid sequences of the *CUC2* gene in other plant species were acquired from GenBank (<http://www.ncbi.nlm.nih.gov/genbank>). Multiple sequence alignment of *LcCUC2L* and its homologous genes was performed with ClustalW (<https://www.genome.jp/tools-bin/clustalw>). The results were uploaded to ESPript 3.0 (<http://esprict.ibcp.fr/ESPript/cgi-bin/ESPript.cgi>). A phylogenetic tree was constructed using MEGA 7.

After consulting the published *L. chinense* genome data (Chen et al., 2019), approximately 2 kb of genomic DNA upstream of the start codon of *LcCUC2L* was cloned. The PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) was used to predict the cis-acting elements of *ProLcCUC2L*.

Subcellular localization of *LcCUC2L*

The whole coding sequence (CDS) of *LcCUC2L* without a stop codon was ligated to a linearized *pBI121-eGFP* vector. The recombinant vector was transformed into *Agrobacterium tumefaciens* GV3101 (ShiFeng). And the 35S::eGFP was used as the control. Positive clones and the *P19* vector were cultured in Luria-Bertani (LB) medium at 28 °C. Next, the cells were collected and suspended in buffer containing 10 mM MgCl₂ and 150 μM acetosyringone (AS). Two bacterial suspensions were mixed and injected into tobacco leaves (Sparkes et al., 2006). After 48 h of injection, the tobacco leaves were stained

with the nuclear dye 4', 6-diamidino-2-phenylindole (DAPI) to visualize the nucleus. Subsequently, the fluorescent signals in the epidermis cells of tobacco leaves were examined with a confocal laser scanning microscope (LSM 710, Zeiss, Germany).

Plasmid construction and genetic transformation

The full-length ORF of *LcCUC2L* was cloned into the linearized overexpression vector *pBI121*. Similarly, the sequence of *ProLcCUC2L* was inserted at the *HindIII/NcoI* site of the *pCAMBIA1301* vector by substituting the CaMV35S promoter, yielding the construct *ProLcCUC2L::GUS*. The *Agrobacterium*-mediated floral dip method was used for the genetic transformation of *A. thaliana* (Clough & Bent, 1998). Transgenic *A. thaliana* seeds were screened on $\frac{1}{2}$ MS medium containing antibiotics. Using leaf genomic DNA as templates, T1-positive transgenic plants were identified by PCR using specific primers. We acquired T3 transgenic plants through continuous screening for three generations.

Venation pattern analysis and scanning electron microscopy

To compare cotyledon vein patterns of transgenic and WT *A. thaliana* plants, the cotyledons were bleached with 75% (v/v) ethanol and observed using stereomicroscopy. For cotyledon epidermal cell analysis, the cotyledons of transgenic and WT *A. thaliana* plants were fixed in FAA fixative solution (38% formalin, 50% ethanol, and glacial acetic acid). After critical point drying (CPD), the samples were coated with an Edwards E-1010 ion sputter golden coater and examined with a scanning electron microscope (FEI Quanta 200 FEG MKII).

Auxin content measurement

Basal rosette leaves samples of 0.6 g were harvested from 3-week-old WT and *LcCUC2L* transgenic *A. thaliana* plants and immediately frozen in liquid nitrogen. Determination of indole-3-acetic acid (IAA) in *A. thaliana* leaves was performed by ELISA (Huding, Shanghai, China). Three replicates were used for each sample.

Histochemical staining to detect GUS activity

The β -Galactosidase Reporter Gene Staining Kit (Leagene) was used for GUS histochemical staining in *ProLcCUC2L* transgenic and WT *A. thaliana* plants. In this study, seven-day-old seedlings and fourteen-day-old seedlings were processed. Moreover, leaves at different stages of development (10, 20 and 30 days of age) from plants planted in soil were subjected to GUS staining. All materials were treated with GUS staining solution and incubated overnight at 37 °C, followed by bleaching with 75% (v/v) ethanol.

Statistical analysis

Both the control and the experimental lines had three repetitions. The data are expressed as the mean $\pm$ standard deviation (SD). Statistical significance was determined with Student's *t*-test. $P < 0.05$ was regarded as significant (*), and $P \leq 0.01$ was considered highly significant (**).

RESULTS

Cloning and sequence analysis of LcCUC2L and ProLcCUC2L

The full length of the *LcCUC2L* gDNA sequence is 4,374 bp and consists of three exons and two introns (Fig. 1A). *LcCUC2L* (accession number: lcCUC2MW629054) contains a 1,017 bp ORF (Data S1), which encodes 338 amino acids and has a predicted molecular weight of 37.32 kDa and a theoretical PI of 8.45. The instability index was 44.46, indicating that the protein is unstable. The secondary structure of *LcCUC2L* was predicted to consist of helix (3.25%), loop (84.02%) and strand (12.72%) structures. Subcellular localization prediction showed that the *LcCUC2L* protein was mainly distributed in the cell nucleus. The amino acid sequences of the *LcCUC2L* protein in *L. chinense* and other plants were analyzed via multiple sequence alignment. Furthermore, the amino acid sequence of *LcCUC2L* was compared with the sequences of other CUC2 proteins in *Arabidopsis lyrata* subsp. *lyrata*, *A. thaliana*, *Ananas comosus*, *Cardamine hirsute*, *Glycine max*, and *Zea mays*. Similar to the N-terminus of other CUC2 proteins, the N-terminus of the *LcCUC2L* protein contains a conserved NAC domain, with five conserved subdomains (A, B, C, D, E), indicating that these proteins belong to the CUC subfamily of the NAC family. In addition, CUC genes harbor a variable CTD domain. The K motif (PSKKTKVPSTIS), V motif (EHVSCFS), L motif (SLPPL) and W-motif (WNY) can be observed in CUC2. In contrast, CUC1 does not have a K motif and there is only an L motif in CUC3 (Larsson et al., 2012). *LcCUC2L* harbors a variable CTD domain, and the K motif, L motif, V motif and W motif were observed in the *LcCUC2L* protein (Fig. 1B). The V motif of CUC1 and CUC2 has been identified as a miR164 recognition site (Rhoades et al., 2002). As shown in Fig. 1C, which indicated the *LcCUC2L* might be recognized by miR164. Moreover, phylogenetic tree analysis confirmed that CUC proteins could be classified into three clads: CUC1, CUC2, and CUC3. The *LcCUC2L* protein is most closely related to the *Ananas comosus* CUC2 protein (Fig. 1D). In conclusion, these results show that the sequence is highly homologous to CUC2, so we named it *LcCUC2L*.

To explore the function of *LcCUC2L*, a 2,028 bp (Data S2) sequence upstream of the translational initiation site (ATG) of the *LcCUC2L* gene, which contains the promoter sequence of *LcCUC2L*, was amplified using the DNA template of *L. chinense*. PlantCARE software was used to analyze the *cis*-acting elements of *ProLcCUC2L*. As shown in Table 1, some *cis*-acting elements are involved in light regulation, such as AE-box, ATCT-motif, Box4, G-Box, G-box, I-box, TCCC-motif, and chs-CMA1a. LTR and WUN motifs associated with low-temperature responsiveness and wound responsiveness, respectively, exist in the sequence. In addition, some hormone-related elements were identified, including the abscisic acid responsive regulatory motif (ABRE), auxin-responsive element (TGA-element) and MeJA-responsiveness elements (CGTCA-motif and TGACG-motif). The predicted results revealed that the core promoter elements of *ProLcCUC2L* contained TATA-box and CAAT-box.

Expression pattern analysis of LcCUC2L

The transcript levels of *LcCUC2L* were determined by RT-qPCR in leaves of different developmental stages (Fig. 2A). The highest expression levels of *LcCUC2L* were observed

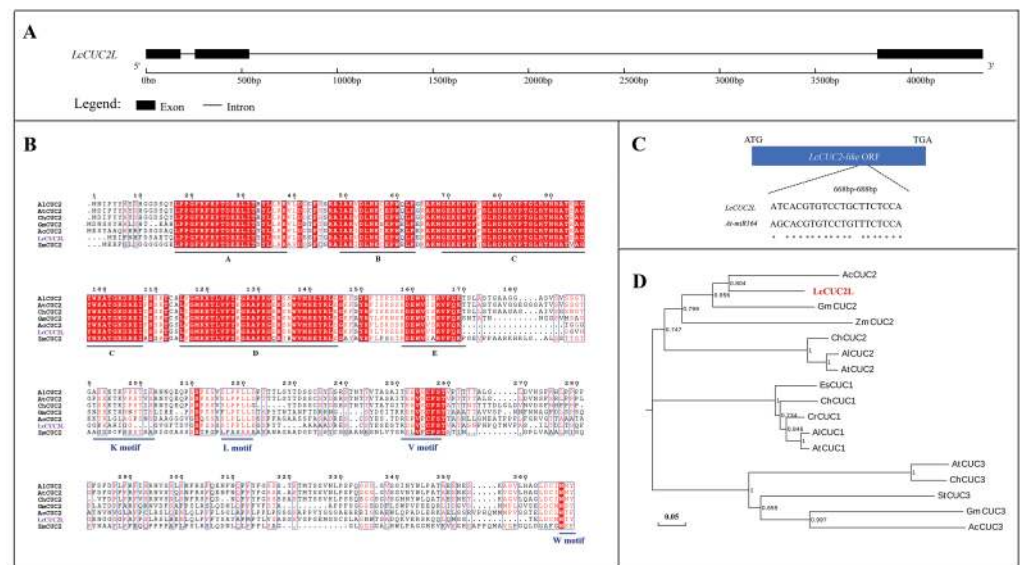


Figure 1 Sequence analysis of *LcCUC2L*. (A) *LcCUC2L* gene structure diagram. (B) Homology analysis of the *LcCUC2L* protein. Amino acid sequence alignment of *LcCUC2L* and CUC2 proteins in other plants. ALCUC2: *Arabidopsis lyrata* subsp. *lyrata* (XP_002866009.1), AtCUC2: *Arabidopsis thaliana* (OAO90779.1), ChCUC2: *Cardamine hirsuta* (ACL14370.1), GmCUC2: *Glycine max* (XP_003541838.1), AcCUC2: *Ananas comosus* (OAY67566.1), and ZmCUC2: *Zea mays* (PWZ16696.1). (C) Nucleotide sequence alignment of the *LcCUC2L* and *AtCUC2* that targeted by miR164. (D) Phylogenetic analysis of *LcCUC2L* and CUC proteins from other plants. EsCUC1: *Eutrema salsugineum* (XP_006407005.1), ChCUC1: *Cardamine hirsute* (ACL14369.1), CrCUC1: *Capsella rubella* (XP_006296616.1), ALCUC1: *Arabidopsis lyrata* subsp. *lyrata* (XP_002882916.1), AtCUC1: *Arabidopsis thaliana* (BAB20598.1), ALCUC2: *Arabidopsis lyrata* subsp. *lyrata* (XP_002866009.1), AtCUC2: *Arabidopsis thaliana* (OAO90779.1), ChCUC2: *Cardamine hirsuta* (ACL14370.1), GmCUC2: *Glycine max* (XP_003541838.1), AcCUC2: *Ananas comosus* (OAY67566.1), ZmCUC2: *Zea mays* (PWZ16696.1), AtCUC3: *Arabidopsis thaliana* (AAP82630.1), GmCUC3: *Glycine max* (XP_003523523.1), StCUC3: *Solanum tuberosum* (NP_001275002.1), ChCUC3: *Cardamine hirsuta* (ACL14365.1), and AcCUC3: *Aquilegia coerulea* (ACL14364.1).

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in the leaf bud, and the lowest abundance of *LcCUC2L* transcripts were in mature leaves (Fig. 2B, Table S2). The results indicated that *LcCUC2L* may play an important role in leaf bud development.

Based on cis-element sequence analysis of the *LcCUC2L* promoter, we predicted that *LcCUC2L* might be associated with auxin. To confirm the effect of auxin on *LcCUC2L* expression, we sprayed 200 μ M IAA on the leaves of *L. chinense*. The application of IAA significantly inhibited the expression of *LcCUC2L* (Fig. 2C, Table S3), which suggested that IAA represses *LcCUC2L* expression.

To further assess the expression pattern of *LcCUC2L*, *ProLcCUC2L* was fused to the GUS reporter and subsequently transferred into *A. thaliana* (hereafter *ProLcCUC2L*). After screening in media containing hygromycin and DNA detection, we obtained 8 transgenic lines (Fig. S1). Histochemical GUS staining was applied to detect the expression levels of the GUS gene in transgenic *A. thaliana* plants, which displayed inducible activity of *ProLcCUC2L*. In the 7-day-old and 14-day-old transgenic seedlings, GUS staining was

Table 1 Details of cis-acting elements of the *ProLcCUC2L* in *L. chinense*.

Name	Number	Sequence	Function
ABRE	3	CACGTG/ ACGTG	cis-acting element involved in the abscisic acid responsiveness
AE-box	1	AGAAACAA	part of a module for light response
ARE	2	AAACCA	cis-acting regulatory element essential for the anaerobic induction
ATCT-motif	1	AATCTAATCC	part of a conserved DNA module involved in light responsiveness
Box 4	1	ATTAAT	part of a conserved DNA module involved in light responsiveness
CAAT-box	27	CAAT/CAAAAT	common cis-acting element in promoter and enhancer regions
CGTCA-motif	3	CGTCA	cis-acting regulatory element involved in the MeJA-responsiveness
G-Box	2	CACGTGAAA/ CACGTG	cis-acting regulatory element involved in light responsiveness
G-box	2	CACGTG/CACGTC	cis-acting regulatory element involved in light responsiveness
I-box	1	atGATAAGGTC	part of a light responsive element
LTR	1	CCGAAA	cis-acting element involved in low-temperature responsiveness
TATA-box	8	TATAA/TATA/ ccTATAAAaa/TATACA	core promoter element around -30 of transcription start
TCCC-motif	1	TCTCCCT	part of a light responsive element
TGA-element	1	AACGAC	auxin-responsive element
TGACG-motif	3	TGACG	cis-acting regulatory element involved in the MeJA-responsiveness
chs-CMA1a	1	TTACTTAA	part of a light responsive element
WUN-motif	1	AAATTTCCCT	wound-responsive element

mainly detected in the cotyledon lamina and roots (Fig. 2D). In contrast, low GUS activity was observed in the cotyledon petiole and hypocotyl of the transgenic seedlings. GUS activity was not detected in the true leaves of 14-day-old transgenic seedlings. These results indicated that *LcCUC2L* may have participated in cotyledon development in the transgenic *A. thaliana* seedlings.

Confocal images with DAPI nuclear staining (blue) were taken 48 h after transfection, showing GFP (green) expression that indicates the subcellular localization of *LcCUC2L*. Figure 2E shows that the green fluorescence of the control 35S::GFP was distributed throughout the cells. In contrast, the green fluorescence of the 35S::*LcCUC2L-eGFP* fusion protein was observed in the nucleus, suggesting that *LcCUC2L* localized to the nucleus. This result was fully consistent with the subcellular localization result of the *AtCUC2* protein in *A. thaliana* (Taoka et al., 2004).

Overexpression of *LcCUC2L* regulates leaf development in *A. thaliana*

To investigate the functions of *LcCUC2L*, we obtained transgenic *A. thaliana* plants overexpressing *LcCUC2L* under the control of the *CaMV* 35S promoter. After screening

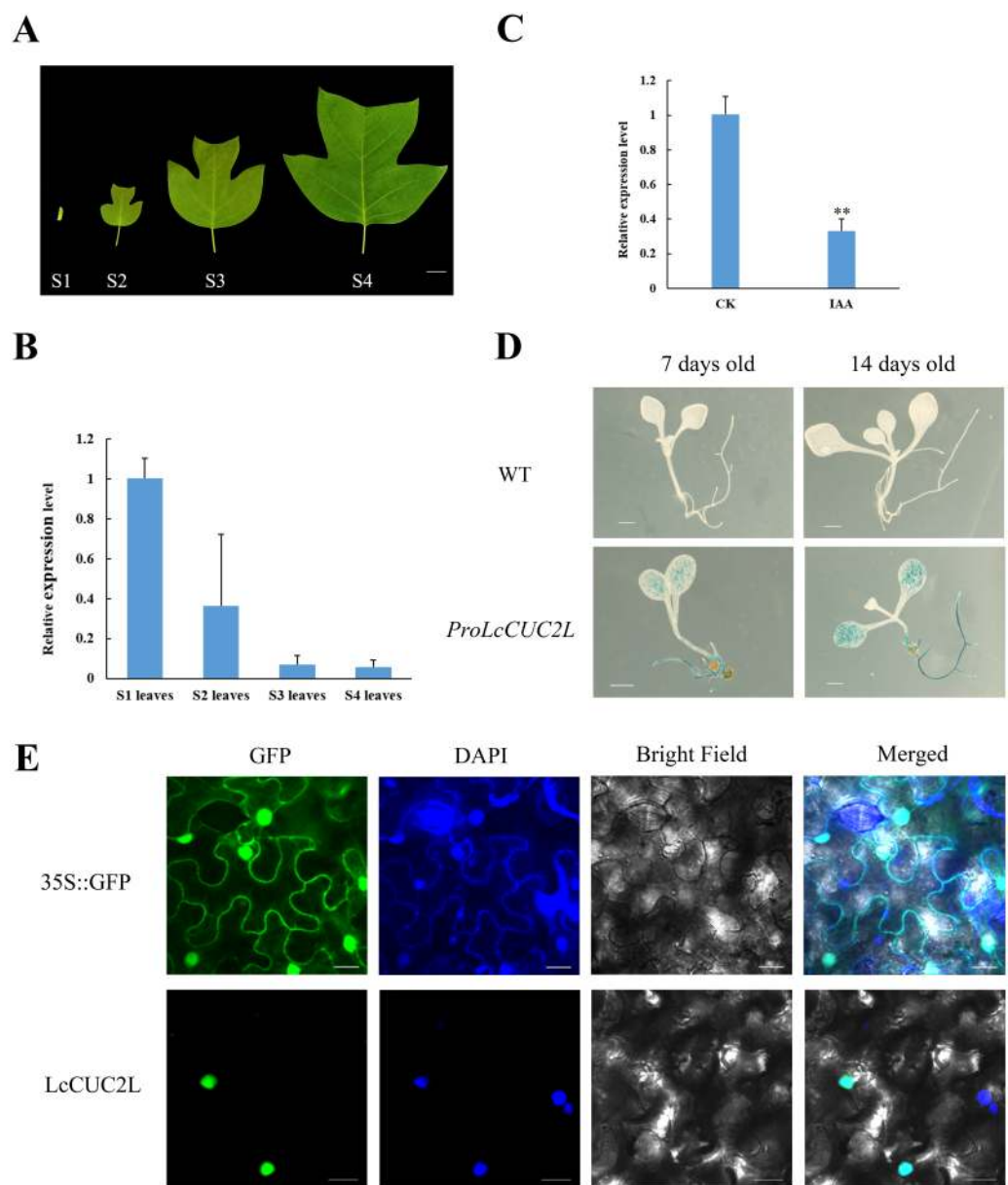


Figure 2 Expression pattern analysis of *LcCUC2L*. (A) Leaves of four different developmental stages in *L. chinense*. S1 leaves: leaf bud; S2 leaves: newly expanded leaves; S3 leaves: larger leaves; S4 leaves: mature leaves, bars = two cm. (B) Transcript levels of *LcCUC2L* in leaves of different developmental stages in *L. chinense*. (C) Expression level of *LcCUC2L* under 200 μ M IAA. (D) Histochemical analysis of GUS in transformed *A. thaliana* leaves, bars = 0.1 cm. (E) Subcellular localization of *LcCUC2L* protein, bars = 2,000 nm. Note: In B and C data are shown as the mean $\pm$ SD, B and C data are based on three repetitive experiments. $**P < 0.01$ and $*P < 0.05$, Student's *t*-test.

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on selective medium, seven positive transgenic plants were acquired. Subsequently, three high expression lines with the same phenotypes (OE 1, OE 3, and OE 5) were selected for phenotype analysis (hereafter 35S::*LcCUC2L*-OE1, 35S::*LcCUC2L*-OE3, and 35S::*LcCUC2L*-OE5) (Fig. 3F, Table S4). We found that true leaves began to emerge on or near the 7th day,

as shown in Fig. 3A. The results revealed significant variation in the cotyledon phenotypes between WT and 35S::LcCUC2L plants. In contrast to WT cotyledons, the cotyledons of the 35S::LcCUC2L plants appeared as long strips and without petioles, and they had narrower blades compared to the WT cotyledons. Moreover, we compared vascular development between the WT and 35S::LcCUC2L cotyledons. Two kinds of veins exist in WT cotyledons: primary veins and subprime veins. Several subprime veins branch from the midvein and then unite to form areoles separated by veins (Figs. 3B, 3C) (Sieburth, 1999). In contrast, 35S::LcCUC2L plant cotyledons displayed an incomplete vascular pattern and had only a single central strand (Figs. 3B, 3C). In conclusion, the 35S::LcCUC2L cotyledons exhibited significant deficiencies in vascular development. To examine the cotyledon cell changes in the transgenic plants, we imaged the epidermal cells of 35S::LcCUC2L and WT with scanning electron microscopy (SEM). Scanning electron microscopy observations revealed that in WT cotyledons, the epidermal cells were organized in a specific pavement-like pattern (Fig. 3D) (Koyama et al., 2007). Remarkably, the shape and arrangement of the epidermal cells in the 35S::LcCUC2L cotyledons were similar to those of the WT petiole, with both being arranged regularly and exhibiting a rectangular shape. Taken together, the findings revealed large variations in cotyledon morphology between the 35S::LcCUC2L and WT *A. thaliana* plants, demonstrating that *LcCUC2L* affects cotyledon development in *A. thaliana*.

Ten days later, we transplanted seedlings of the homozygous transgenic lines and WT *A. thaliana* plants into nutrient soil. We observed that the rosette leaves of the 30-day-old 35S::LcCUC2L plants grew in clusters and were small and numerous (Figs. 3E, 3G). The 35S::LcCUC2L-OE1 and 35S::LcCUC2L-OE3 had 20 rosette leaves on average and the 35S::LcCUC2L-OE5 had 18 rosette leaves on average (Table S5), which was considerably more than the number in WT (Fig. 3H). These results indicated that *LcCUC2L* influences leaf development in *A. thaliana*.

***LcCUC2L* regulates leaf development by upregulating the expression of some genes related to auxin and leaf shape development**

Many recent investigations have indicated that auxin plays a vital role in leaf development (Xiong & Jiao, 2019). The 35S::LcCUC2L plants amassed more IAA than the WT *A. thaliana* plants (Fig. 4A, Table S6). To explore whether *LcCUC2L* regulates leaf development by affecting auxin signaling, the expression levels of auxin biosynthesis genes and auxin transport genes were examined (Table S7). The transcript levels of the auxin biosynthetic genes *YUCCA* (*AtYUC2* and *AtYUC4*) (Fig. 4B), an auxin influx carrier (*AtAUX1*), and efflux carriers (*AtPIN1*, *AtPIN3*, and *AtPIN4*) (Fig. 4C) were increased in *LcCUC2L*-expressing plants compared to WT *A. thaliana* plants. However, *LcCUC2L* overexpression had little effect on the expression level of *AtYUC6*. The results imply that the effects of *LcCUC2L* in leaf development might be enhanced by changes in auxin biosynthesis and polar transport. Additionally, we analyzed some genes related to leaf shape development, i.e., *KNAT1*, *KNAT2*, *KNAT6* (for *KNOTTED*-like from *A. thaliana*), *SHOOTMERISTEMLESS* (*STM*), and *DPA4*. We found that 35S::LcCUC2L plants presented much higher transcript levels of *KNAT6*, *KNAT2*, *DPA4*, and *AtCUC2* than WT

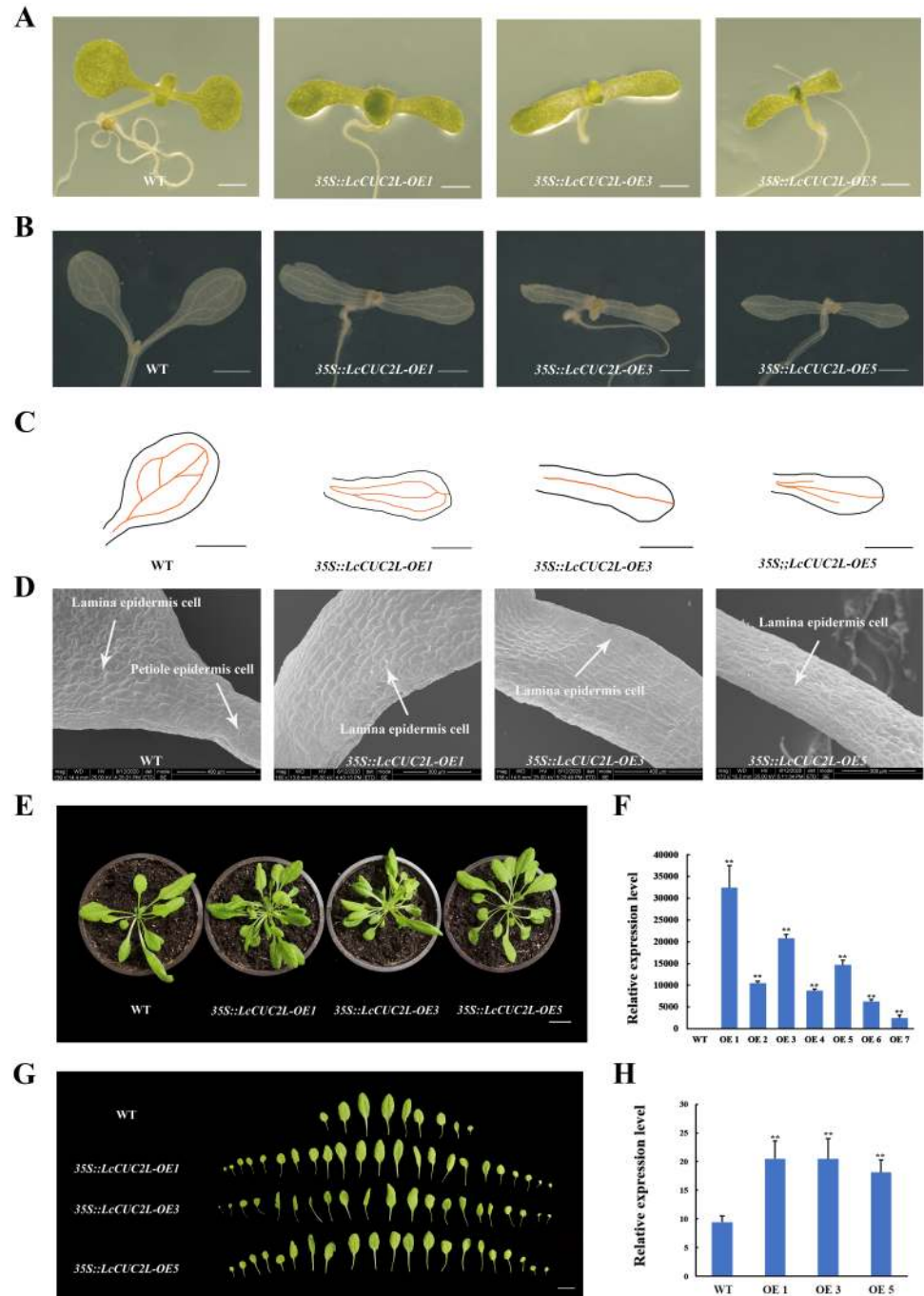


Figure 3 The phenotypes of the *LcCUC2L* overexpression transgenic lines and WT *A. thaliana* plants. (A) Seven-day-old cotyledons of WT and 35S::LcCUC2L plants, bars = 0.1 cm. (B) Venation patterns of WT and 35S::LcCUC2L plants, bars = 0.1 cm. (C) Illustrations of the vascular patterns of 7-day-old cotyledons of WT and 35S::LcCUC2L plants. (D) SEM images of cotyledons of WT and 35S::LcCUC2L plants. (E) 30-day-old WT and 35S::LcCUC2L *A. thaliana*, bars = 2 cm. (F) qRT-PCR analysis of *LcCUC2L* expression in WT and transgenic plants. Values are means $\pm$ SD ($n = 3$). Data are based on three repetitive experiments. ** $P < 0.01$ and * $P < 0.05$, Student's *t*-test. (G) The rosette leaves of WT and 35S::LcCUC2L *A. thaliana*, bars = 2 cm. (H) The number of rosette leaves in WT and 35S::LcCUC2L *A. thaliana*. Values are means $\pm$ SD ($n = 18$). ** $P < 0.01$ and * $P < 0.05$, Student's *t*-test.

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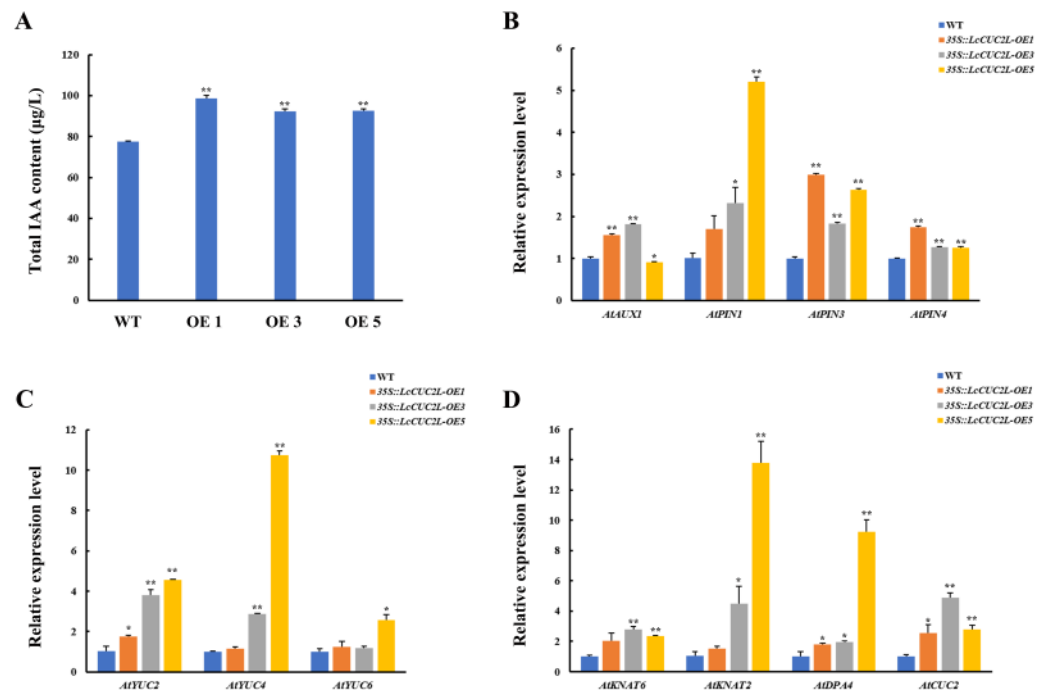


Figure 4 *LcCUC2L* promoted auxin accumulation in leaves and upregulated the expression of some genes in transgenic *A. thaliana* plants (35S::LcCUC2L). (A) Total IAA content in the rosette leaves of three-week-old seedlings of WT and 35S::LcCUC2L *A. thaliana* plants. (B) Transcript levels of auxin biosynthetic genes (*AtYUC2*, *AtYUC4*, and *AtYUC6*) in WT and 35S::LcCUC2L *A. thaliana* plants. (C) Transcript levels of auxin influx carriers (*AtAUX1*) and efflux carriers (*AtPIN1*, *AtPIN3*, and *AtPIN4*) in WT and 35S::LcCUC2L *A. thaliana* plants. (D) Transcript levels of some genes involved in the leaf shape development (*AtKNAT2*, *AtKNAT6*, *AtDPA4*, and *AtCUC2*) in WT and 35S::LcCUC2L *A. thaliana* plants. Note: In A, B, C, and D data are shown as the mean $\pm$ SD, based on three repetitive experiments. ** $P < 0.01$ and * $P < 0.05$, Student's *t*-test.

Full-size [DOI: 10.7717/peerj.12615/fig-4](https://doi.org/10.7717/peerj.12615/fig-4)

A. thaliana plants (Fig. 4D). The results indicate that the overexpression of *LcCUC2L* led to the upregulation of some genes related to leaf shape development and thus affected leaf development in *A. thaliana*.

DISCUSSION

Leaf shape is an important plants traits. Early studies of leaf shape development have focused on model plants, but there is still limited in woody plants. *L. chinense* has a distinctive leaf shape (Fig. 2A), which provides a wealth of reference information to uncover the molecular mechanisms of leaf shape development. Based on a comparison of *L. chinense* leaf transcripts among different developmental stages, the *CUC2* gene was identified as a candidate gene related to leaf development (Ma et al., 2018). Subsequently, we identified the full-length sequences of *LcCUC2L* and its promoter isolated from *L. chinense* and analyzed their functions via overexpression analysis.

In this study, we discovered that overexpression of *LcCUC2L* strongly affected cotyledon development in transgenic *A. thaliana*, resulting in long, narrow cotyledons without

petioles (Fig. 3A) but with abnormal epidermal cells (Fig. 3D). In the early stages of leaf organogenesis, the proximal–distal, medial–lateral and adaxial–abaxial axes are established (Tsukaya, 2006; Kidner & Timmermans, 2007). The proximal–distal axis domains of WT *A. thaliana* leaves are composed of the lamina and the petiole (Jover-Gil et al., 2012). In the present study, abnormal epidermal cells were present in the cotyledons of the 35S::LcCUC2L plants, with those in the lamina resembling the epidermal cells of the wild-type petiole (Fig. 3D). Previous studies have reported blade growth on the petiole and a diminished proximal domain in the *bop1 bop2* double mutant (Ha et al., 2007). In addition, the *as2* mutation causes the petiole to curl upward and to sometimes produce leaflets (Iwakawa et al., 2007). These phenotypes are associated with the ectopic expression of meristem class I homeobox KNOX genes, i.e., *KNAT2*, *KNAT6*, *STM* and *BP* (Ha et al., 2003; Iwakawa et al., 2007; Ikezaki et al., 2010; Jun, Ha & Fletcher, 2010). To our surprise, overexpression of *LcCUC2L* in *A. thaliana* promoted the expression of *KNAT2* and *KNAT6* (Fig. 4D). Further analyses suggested that the ectopic expression of *LcCUC2L* regulated the proximal–distal axis by enhancing the expression of *KNAT2* and *KNAT6*; thus, the cotyledons of 35S::LcCUC2L *A. thaliana* tended to develop as long, narrow cotyledons without petioles and exhibited abnormal epidermal cells.

Furthermore, cotyledon vein development in 35S::LcCUC2L plants was defective (Figs. 3B, 3C). It is widely known that auxin plays an indispensable role in vascular development (Aloni, 1995). Mutations in some genes related to the auxin signaling pathway (*AUXIN RESPONSE FACTOR 5* (*ARF5*), *AUXIN RESISTANT 6* (*AXR6*) and *IAA12*) contribute to incomplete vascular development (Przemeck et al., 1996; Hobbie et al., 2000; Hamann et al., 2002). Moreover, evidence suggests that polar auxin transport plays a significant role in determining leaf vascular patterns. Studies have shown that the expression of *CUC* genes is regulated by *PIN1* (Aida et al., 2002) and that blocking PIN-mediated polar auxin transport (PAT) results in cotyledon fusion (Liu, Xu & Chua, 1993). It has been proposed a feedback regulation network of leaf margin development among auxin, *PIN1*, and *CUC2* (Maugarny et al., 2016). The auxin content and *PIN1* transcript level of 35S::LcCUC2L plants were higher than those of WT *A. thaliana* plants in this study (Figs. 4A, 4C). Bioinformatic analysis revealed that the auxin-responsive element is present in *ProLcCUC2L* (Table 1). The authors identified three upstream transcription factors that bind to the auxin response element (CACATG) of the *BpCUC2* promoter (Liu et al., 2018). Our results revealed that the expression of *LcCUC2L* markedly decreased under IAA treatment (Fig. 2C). These findings imply that *LcCUC2L* may be related to the metabolism of auxin. Based on the above results, we speculate that *LcCUC2L* elevated auxin content by upregulating local auxin biosynthesis and polar transport, which affected cotyledon development. Moreover, GUS activity was detected in the cotyledons of transgenic *A. thaliana* seedlings (Fig. 2D). This finding is in line with the fact that *LcCUC2L* regulates cotyledon development in transgenic *A. thaliana* plants.

Ectopic expression of *LcCUC2L* produced numerous rosette leaves (Figs. 3G, 3H). Leaves are lateral organs that develop continuously at the flanks of the SAM in flowering plants (Kessler & Sinha, 2004). The cotyledons of *cuc1 cuc2* double mutant seedlings are fused into a single cup-shaped cotyledon without a SAM (Aida et al., 1997). In addition, the KNOX

gene is required to maintain the development and function of the SAM (Scofield, Dewitte & Murray, 2014). Overexpression of *LcCUC2L* led to upregulated expression of *KNAT2* and *KNAT6* (Fig. 4D), indicating that *LcCUC2L* may regulate SAM development by affecting the expression of *KNAT2* and *KNAT6*. Moreover, *LcCUC2L* was prominently expressed in the leaf buds (Fig. 2A), which is in accordance with *BpCUC2* having the highest expression level in buds (Liu et al., 2018), suggesting that *LcCUC2L* may function in the early stage of leaf development. SAM and leaf tissue can be observed in the leaf buds of *L. chinense* (Ma et al., 2018). We assumed that the development of the SAM was influenced by the overexpression of *LcCUC2L*, which increased the number of rosette leaf. These findings call for further research to verify this possibility.

Research has demonstrated that the *CUC1* and *CUC2* genes descended from a common ancestor that has undergone two duplications and sequence loss events in the evolutionary process (Bowers et al., 2003). Previous data have shown no obvious divergence between *CUC2* and the ancestral gene, while *CUC1* has diverged from the ancestral gene, resulting in a different function (Hasson et al., 2011). For example, both *CUC1* and *CUC2* participate in primordium development, while *CUC2* and *CUC3* were proven to be involved in leaf margin development (Nikovics et al., 2006; Hasson et al., 2011). Moreover, the phylogenetic tree showed that *LcCUC2L* and *AtCUC1* were not clustered in one branch (Fig. 1D). We found that *LcCUC2L* had the nearest evolutionary distance to *AcCUC2* (Fig. 1D) and the amino acid sequence similarity of *LcCUC2L* and *AtCUC2* was 52.36%. These results indicated that there may be functional differentiation between *LcCUC2L*, *AtCUC2* and *AtCUC1*.

Studies in *A. thaliana* have suggested that *cuc1 cuc2* double mutants showed cotyledon fusion and defective SAMs, revealing a key role for *CUC* genes in cotyledon development and SAM formation (Aida et al., 1997). However, our study showed that overexpression of *LcCUC2L* strongly conferred long, narrow cotyledons without petioles (Fig. 3A) and abnormal epidermal cell (Fig. 3D) phenotypes. Moreover, *LcCUC2L* showed strong expression in cotyledons (Fig. 2D), which further confirmed that *LcCUC2L* might play a role in regulating cotyledon development. In *A. thaliana* seedlings, *AtCUC2* was expressed in the boundaries between the cotyledons, the first true leaves, and the SAM. This finding is in line with *CUC2* being a boundary-specific gene (Larsson et al., 2012; Takada et al., 2001). During leaf development, GUS activity tended to become weaker in the sinus of the leaves, which indicated that local inhibition of the *CUC2* gene may cause serration (Nikovics et al., 2006). *A. thaliana* has serrated leaves, and a smooth leaf margin phenotype was observed in the *cuc2* mutant, suggesting that *CUC2* participates in leaf serration in *A. thaliana* (Hasson et al., 2011). Accordingly, it can be inferred that the expression pattern of the *AtCUC2* gene is closely related to its function. However, the *LcCUC2L* gene was highly expressed in the cotyledons and affected the cotyledon development. Moreover, the *ProLcCUC2-GUS* line showed strong expression in the roots as well as cotyledons (Fig. 2D). This phenomenon interested us, and the role of *LcCUC2L* in root development might our next research direction.

CUC1 was detected in the whole area of the cotyledons. 35S::*AtCUC1* transgenic plants showed small lobed cotyledons with defective vasculature (Hibara, Takada & Tasaka, 2003). The phenotype was similar to that of the *LcCUC2L* overexpressing plants. Both play role in cotyledon development. In the cotyledons of 35S::*AtCUC1*, adventitious SAMs were observed on the adaxial surface of this region (Hibara, Takada & Tasaka, 2003). To date, we have not found that *LcCUC2L* could induce adventitious SAMs in transgenic *A. thaliana* plants. The above results show that the *LcCUC2L* and *CUC* genes seem to be homologous in sequence, but non-homologous in function. Moreover, differences in regulatory mechanisms exist between herbaceous and woody plants, and the expression of *LcCUC2L* in *L. chinense* may yield different results than that in *A. thaliana*. Therefore, to test the above hypothesis, it is necessary to transfer the *LcCUC2L* gene into *L. chinense*.

In summary, these observations suggest that *LcCUC2L* may play a critical role in leaf shape development in *L. chinense*. Our results lay a foundation for future studies of the mechanisms of leaf shape development and provide initial insight into the functions of the *CUC2* gene in *L. chinense*.

CONCLUSIONS

In this study, histochemical GUS staining revealed that *LcCUC2L* was expressed in the cotyledons of *A. thaliana* seedlings, which indicated that *LcCUC2L* may play a role in cotyledon development. Ectopic expression of *LcCUC2L* resulted in long, narrow cotyledons without petioles and increased rosette leaf number. Further analysis showed that overexpression of *LcCUC2L* induced abnormal lamina epidermis cells and defective vascular tissue in cotyledons. Hormone determination and RT-qPCR results indicated that *LcCUC2L* affects leaf development by regulating the auxin content and the expression of genes related to auxin and leaf shape development. These findings indicate that *LcCUC2L* may influence leaf development in *L. chinense* and provide insights into the regulatory mechanisms of leaf development in *L. chinense*.

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Shaoying Wen conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Jiayu Li performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Ziyuan Hao performed the experiments, prepared figures and/or tables, and approved the final draft.
- Lingmin Wei performed the experiments, prepared figures and/or tables, took part in the seeds of transgenic *A. thaliana* collection, and approved the final draft.
- Jikai Ma and Yaxian Zong performed the experiments, prepared figures and/or tables, was involved in the plant materials collection, and approved the final draft.
- Huogen Li conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

DNA Deposition

The following information was supplied regarding the deposition of DNA sequences:

The sequences described here are available in the [Supplemental Files](#) and at GenBank: LcCUC2 [MW629054](#).

Data Availability

The following information was supplied regarding data availability:

The raw data are available in the [Supplementary Files](#).

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.12615#supplemental-information>.

REFERENCES

- Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M. 1997.** Genes involved in organ separation in Arabidopsis: an analysis of the cup-shaped cotyledon mutant. *The Plant Cell* **9**(6):841–857 DOI [10.1105/tpc.9.6.841](https://doi.org/10.1105/tpc.9.6.841).
- Aida M, Vernoux X, Furutani M, Traas M, Tasaka M. 2002.** Roles of PIN-FORMED1 and MONOPTEROS in pattern formation of the apical region of the Arabidopsis embryo. *Development* **129**(17):3965–3974 DOI [10.1101/gad.242202](https://doi.org/10.1101/gad.242202).

- Aloni R. 1995. *The induction of vascular tissues by auxin and cytokinin*. Dordrecht: Kluwer Academic.
- Baker CC, Sieber P, Wellmer F, Meyerowitz EM. 2005. The early extra petals1 mutant uncovers a role for MicroRNA miR164c in regulating petal number in Arabidopsis. *Current Biology* 15(4):303–315 DOI 10.1016/j.cub.2005.02.017.
- Barkoulas M, Hay A, Kougioumoutzi E, Tsiantis M. 2008. A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. *Nature Genetics* 40(9):1136–1141 DOI 10.1038/ng.189.
- Bilsborough GD, Runions A, Barkoulas M, Jenkins HW, Hasson A, Galinha C, Laufs P, Hay A, Prusinkiewicz P, Tsiantis M. 2011. Model for the regulation of Arabidopsis thaliana leaf margin development. *Proceedings of the National Academy of Sciences of the United States of America* 108(8):3424–3429 DOI 10.1073/pnas.1015162108.
- Bowers JE, Chapman BA, Rong JK, Paterson AH. 2003. Unravelling angiosperm genome evolution by phylogenetic analysis of chromosomal duplication events. *Nature* 422(6930):433–438 DOI 10.1038/nature01521.
- Chen J, Hao Z, Guang X, Zhao C, Wang P, Xue L, Zhu Q, Yang L, Sheng Y, Zhou Y, Xu H, Xie H, Long X, Zhang J, Wang Z, Shi M, Lu Y, Liu S, Guan L, Zhu Q, Yang L, Ge S, Cheng T, Laux T, Gao Q, Peng Y, Liu N, Yang S, Shi J. 2019. Liriodendron genome sheds light on angiosperm phylogeny and species-pair differentiation. *Nature Plants* 5(1):18–25 DOI 10.1038/s41477-018-0323-6.
- Chitwood DH, Ranjan A, Kumar R, Ichihashi Y, Zumstein K, Headland LR, Ostria-Gallardo E, Aguilar-Martinez JA, Bush S, Carriedo L, Fulop D, Martinez CC, Peng J, Maloof JN, Sinha NR. 2014. Resolving distinct genetic regulators of tomato leaf shape within a heteroblastic and ontogenetic context. *Plant Cell* 26(9):3616–3629 DOI 10.1105/tpc.114.130112.
- Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *The Plant Journal: for Cell and Molecular Biology* 16(6):735–743 DOI 10.1046/j.1365-3113x.1998.00343.x.
- Daimon Y, Takabe K, Tasaka M. 2003. The CUP-SHAPED COTYLEDON genes promote adventitious shoot formation on calli. *Plant and Cell Physiology* 44(2):113–121 DOI 10.1093/pcp/pcg038.
- Dkhar J, Pareek A. 2014. What determines a leaf's shape? *EvoDevo* 5(1):47–59 DOI 10.1186/2041-9139-5-47.
- Engelhorn J, Reimer JJ, Leuz I, Goebel U, Huettel B, Farrona S, Turck F. 2012. DEVELOPMENT-RELATED PcG TARGET IN THE APEX 4 controls leaf margin architecture in Arabidopsis thaliana. *Development* 139(14):2566–2575 DOI 10.1242/dev.078618.
- Fang Y. 1994. Geographical distribution and spatial pattern of Liriodendron chinense (Hemsl.) Sarg. *Journal of Nanjing Forestry University* 000(002):13–18 (in Chinese) DOI 10.3969/j.jssn.1000-2006.1994.02.003.
- Furutani M, Vernoux T, Traas J, Kato T, Tasaka M, Aida M. 2004. PIN-FORMED1 and PINOID regulate boundary formation and cotyledon development in Arabidopsis embryogenesis. *Development* 131(20):5021–5030 DOI 10.1242/dev.01388.

- Galweiler L, Guan C, Muller A, Wisman E, Mendgen K, Yephremov A, Palme K. 1998. Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. *Science* 282(5397):2226–2230 DOI 10.1126/science.282.5397.2226.
- Gonzalez-Carranza ZH, Zhang X, Peters JL, Boltz V, Szecsi J, Bendahmane M, Roberts JA. 2017. HAWAIIAN SKIRT controls size and floral organ number by modulating CUC1 and CUC2 expression. *PLOS ONE* 12(9):e0185106 DOI 10.1371/journal.pone.0185106.
- Ha CM, Jun JH, Nam HG, Fletcher JC. 2007. BLADE-ON-PETIOLE1 and 2 control Arabidopsis lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. *Plant Cell* 19(6):1809–1825 DOI 10.1105/tpc.107.051938.
- Ha CM, Kim GT, Kim BC, Jun JH, Soh MS, Ueno Y, Machida Y, Tsukaya H, Nam HG. 2003. The BLADE-ON-PETIOLE 1 gene controls leaf pattern formation through the modulation of meristematic activity in Arabidopsis. *Development* 130(1):161–172 DOI 10.1242/dev.00196.
- Hamann T, Benkova E, Baurle I, Kientz M, Jurgens G. 2002. The Arabidopsis BODENLOS gene encodes an auxin response protein inhibiting MONOPTEROS-mediated embryo patterning. *Genes & Development* 16(13):1610–1615 DOI 10.1101/gad.229402.
- Hasson A, Plessis A, Blein T, Adroher B, Grigg S, Tsiantis M, Boudaoud A, Damerval C, Laufs P. 2011. Evolution and diverse roles of the CUP-SHAPED COTYLEDON genes in arabidopsis leaf development. *Plant Cell* 23(1):54–68 DOI 10.1105/tpc.110.081448.
- Hibara K, Karim MR, Takada S, Taoka K, Furutani M, Aida M, Tasaka M. 2006. Arabidopsis CUP-SHAPED COTYLEDON3 regulates postembryonic shoot meristem and organ boundary formation. *Plant Cell* 18(11):2946–2957 DOI 10.1105/tpc.106.045716.
- Hibara K, Takada S, Tasaka M. 2003. CUC1 gene activates the expression of SAM-related genes to induce adventitious shoot formation. *Plant Journal* 36(5):687–696 DOI 10.1046/j.1365-313X.2003.01911.x.
- Hobbie L, McGovern M, Hurwitz LR, Pierro A, Liu NY, Bandyopadhyay A, Estelle M. 2000. The axr6 mutants of Arabidopsis thaliana define a gene involved in auxin response and early development. *Development* 127(1):23–32 DOI 10.1016/S0070-2153(00)50008-8.
- Ikezaki M, Kojima M, Sakakibara H, Kojima S, Ueno Y, Machida C, Machida Y. 2010. Genetic networks regulated by ASYMMETRIC LEAVES1 (AS1) and AS2 in leaf development in Arabidopsis thaliana: KNOX genes control five morphological events. *Plant Journal* 61(1):70–82 DOI 10.1111/j.1365-313X.2009.04033.x.
- Iwakawa H, Iwasaki M, Kojima S, Ueno Y, Soma T, Tanaka H, Semiarti E, Machida Y, Machida C. 2007. Expression of the ASYMMETRIC LEAVES2 gene in the adaxial domain of Arabidopsis leaves represses cell proliferation in this domain and is critical for the development of properly expanded leaves. *Plant Journal* 51(2):173–184 DOI 10.1111/j.1365-313X.2007.03132.x.

- Jover-Gil S, Candela H, Robles P, Aguilera V, Barrero JM, Micol JL, Ponce MR. 2012. The MicroRNA pathway genes AGO1, HEN1 and HYL1 participate in leaf proximal-distal, venation and stomatal patterning in arabidopsis. *Plant and Cell Physiology* 53(7):1322–1333 DOI 10.1093/pcp/pcs077.
- Jun JH, Ha CM, Fletcher JC. 2010. BLADE-ON-PETIOLE1 coordinates organ determinacy and axial polarity in arabidopsis by directly activating ASYMMETRIC LEAVES2. *Plant Cell* 22(1):62–76 DOI 10.1105/tpc.109.070763.
- Kamiuchi Y, Yamamoto K, Furutani M, Tasaka M, Aida M. 2014. The CUC1 and CUC2 genes promote carpel margin meristem formation during Arabidopsis gynoecium development. *Frontiers in Plant Science* 5:165–173 DOI 10.3389/fpls.2014.00165.
- Kawamura E, Horiguchi G, Tsukaya H. 2010. Mechanisms of leaf tooth formation in Arabidopsis. *Plant Journal* 62(3):429–441 DOI 10.1111/j.1365-3113X.2010.04156.x.
- Kessler S, Sinha N. 2004. Shaping up: the genetic control of leaf shape. *Current Opinion in Plant Biology* 7:65–72 DOI 10.1016/j.pbi.2003.11.002.
- Kidner CA, Timmermans MCP. 2007. Mixing and matching pathways in leaf polarity. *Current Opinion in Plant Biology* 10(1):13–20 DOI 10.1016/j.pbi.2006.11.013.
- Koyama T, Furutani M, Tasaka M, Ohme-Takagi M. 2007. TCP transcription factors control the morphology of shoot lateral organs via negative regulation of the expression of boundary-specific genes in Arabidopsis. *Plant Cell* 19(2):473–484 DOI 10.1105/tpc.106.044792.
- Larsson E, Sundstrom JF, Sitbon F, Von Arnold S. 2012. Expression of PaNAC01, a Picea abies CUP-SHAPED COTYLEDON orthologue, is regulated by polar auxin transport and associated with differentiation of the shoot apical meristem and formation of separated cotyledons. *Annals of Botany* 110(4):923–934 DOI 10.1093/aob/mcs151.
- Li L, Kang D, Chen Z, Qu L. 2007. Hormonal regulation of leaf morphogenesis in Arabidopsis. *Journal of Integrative Plant Biology* 49(001):75–80 DOI 10.1111/j.1744-7909.2006.00410.x.
- Liu C, Xu H, Han R, Wang S, Liu G, Chen S, Chen J, Bian X, Jiang J. 2019. Over-expression of BpCUC2 influences leaf shape and internode development in betula pendula. *International Journal of Molecular Sciences* 20(19):4722–4740 DOI 10.3390/ijms20194722.
- Liu C, Xu H, Jiang J, Wang S, Liu G. 2018. Analysis of the promoter features of BpCUC2 in Betula platyphylla x Betula pendula. *Plant Cell Tissue and Organ Culture* 132(1):191–199 DOI 10.1007/s11240-017-1324-2.
- Liu C, Xu Z, Chua NH. 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. *Plant Cell* 5(6):621–630 DOI 10.1105/tpc.5.6.621.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 25(4):402–408 DOI 10.1006/meth.2001.1262.

- Ma J, Mei G, Liu H, Li H. 2020. Overexpression of a novel LcKNOX transcription factor from *liriodendron chinense* induces lobed leaves in *Arabidopsis thaliana*. *Forests* 11(1):33–45 DOI 10.3390/f11010033.
- Ma J, Wei L, Li J, Li H. 2018. The analysis of genes and phytohormone metabolic pathways associated with leaf shape development in *liriodendron chinense* via de novo transcriptome sequencing. *Gene* 9(12):577–592 DOI 10.3390/genes9120577.
- Mallory AC, Dugas DV, Bartel DP, Bonnie B. 2004. MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. *Current Biology* 14(12):1035–1046 DOI 10.1016/j.cub.2004.06.022.
- Maugarny A, Gonçalves B, Arnaud N, Laufs P. 2016. CUC transcription factors: to the meristem and beyond. *Plant Transcription Factors* 229–247 DOI 10.1016/j.cub.2004.06.022.
- Nikovics K, Blein T, Peaucelle A, Ishida T, Morin H, Aida M, Laufs P. 2006. The balance between the MIR164A and CUC2 genes controls leaf margin serration in *Arabidopsis*. *Plant Cell* 18(11):2929–2945 DOI 10.1105/tpc.106.045617.
- Peng J, Yu J, Wang H, Guo Y, Li G, Bai G, Chen R. 2011. Regulation of compound leaf development in *medicago truncatula* by fused compound Leaf1, a class M KNOX gene. *Plant Cell* 23(11):3929–3943 DOI 10.1105/tpc.111.089128.
- Przemeck GK, Mattsson J, Hardtke CS, Sung ZR, Berleth T. 1996. Studies on the role of the *Arabidopsis* gene MONOPTEROS in vascular development and plant cell axialization. *Planta* 200(2):229–237 DOI 10.1080/14622200307243.
- Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bonnie B, Bartel DP. 2002. Prediction of plant microRNA targets. *Cell* 110(4):513–520 DOI 10.1016/S0092-8674(02)00863-2.
- Scofield S, Dewitte W, Murray JA. 2014. STM sustains stem cell function in the *Arabidopsis* shoot apical meristem and controls KNOX gene expression independently of the transcriptional repressor AS1. *Plant Signaling & Behavior* 9:e28934 DOI 10.4161/psb.28934.
- Semchenko M, Zobel K. 2007. The role of leaf lobation in elongation responses to shade in the rosette-forming forb *Serratula tinctoria* (Asteraceae). *Annals of Botany* 100(1):83–90 DOI 10.1093/aob/mcm074.
- Shao J, Meng J, Wang F, Shou B, Chen Y, Xue H, Zhao J, Qi Y, An L, Yu F, Liu X. 2020. NGATHA-LIKEs control leaf margin development by repressing CUP-SHAPED COTYLEDON2 transcription. *Plant Physiology* 184(1):345–358 DOI 10.1104/pp.19.01598.
- Sieburth LE. 1999. Auxin is required for leaf vein pattern in *Arabidopsis*. *Plant Physiology* 121(4):1179–1190 DOI 10.1104/pp.121.4.1179.
- Sparkes IA, Runions J, Kearns A, Hawes C. 2006. Rapid, transient expression of fluorescent fusion proteins in tobacco plants and generation of stably transformed plants. *Nature Protocols* 1(4):2019–2025 DOI 10.1038/nprot.2006.286.
- Takada S, Hibara K, Ishida T, Tasaka M. 2001. The CUP-SHAPED COTYLEDON1 gene of *Arabidopsis* regulates shoot apical meristem formation. *Development* 128(7):1127–1135 DOI 10.1007/s004290100164.

- Taoka K, Yanagimoto Y, Daimon Y, Hibara K, Aida M, Tasaka M. 2004.** The NAC domain mediates functional specificity of CUP-SHAPED COTYLEDON proteins. *The Plant Journal* **40**(4):462–473 DOI [10.1111/j.1365-313X.2004.02238.x](https://doi.org/10.1111/j.1365-313X.2004.02238.x).
- Tsukaya H. 2006.** Mechanism of leaf-shape determination. *Annual Review of Plant Biology* **57**(1):477–496 DOI [10.1146/annurev.arplant.57.032905.105320](https://doi.org/10.1146/annurev.arplant.57.032905.105320).
- Tu Z, Hao Z, Zhong W, Li H. 2019.** Identification of suitable reference genes for RT-qPCR assays in *Liriodendron chinense* (Hemsl.) Sarg. *Forests* **10**(5):441–456 DOI [10.3390/f10050441](https://doi.org/10.3390/f10050441).
- Vernoux T, Kronenberger J, Grandjean O, Laufs P, Traas J. 2000.** PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development* **127**(23):5157–5165 DOI [10.1007/s004290000122](https://doi.org/10.1007/s004290000122).
- Vogel S. 2009.** Leaves in the lowest and highest winds: temperature, force and shape. *New Phytologist* **183**(1):13–26 DOI [10.1111/j.1469-8137.2009.02854.x](https://doi.org/10.1111/j.1469-8137.2009.02854.x).
- Wang Z. 1997.** Conservation of genetic resources of *Liriodendron chinense* (Hemsl.) Sarg and Prospect of cross breeding. *Forestry Science and Technology* **9**(3):8–10 (in Chinese).
- Xiong Y, Jiao Y. 2019.** The diverse roles of auxin in regulating leaf development. *Plants* **8**(7):243–256 DOI [10.3390/plants8070243](https://doi.org/10.3390/plants8070243).
- Yang Y, Xu M, Luo Q, Wang J, Li H. 2014.** De novo transcriptome analysis of *Liriodendron chinense* petals and leaves by Illumina sequencing. *Gene* **534**(2):155–162 DOI [10.1016/j.gene.2013.10.073](https://doi.org/10.1016/j.gene.2013.10.073).
- Zong Y, Hao Z, Tu Z, Shen Y, Zhang C, Wen S, Yang L, Ma J, Li H. 2021.** Genome-wide survey and identification of AP2/ERF genes involved in shoot and leaf development in *Liriodendron chinense*. *BMC Genomics* **22**(1):807–823 DOI [10.1186/s12864-021-08119-7](https://doi.org/10.1186/s12864-021-08119-7).