Overexpression of a Novel *LcKNOX* Transcription Factor from *Liriodendron chinense* Induces Lobed Leaves in *Arabidopsis thaliana*

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Abstract: *Liriodendron chinense* is a common ornamental tree that has attractive leaves, which is a valuable trait for use in landscape architecture. In this work, we aimed to identify the potential genes that control and regulate the development of *L. chinense* leaf lobes. Based on the transcriptome data for the leaf developmental stages we previously generated, two candidate genes were identified in this study. *KNOTTED-LIKE HOMEOBOX(KNOX)*, encoding homeobox family proteins, play a large role in leaf lobe and leaf complexity regulation. Here, two full length *KNOX* genes from *L. chinense* were amplified and named *LcKNOX1* and *LcKNOX6* according to their sequence similarities with the respective *Arabidopsis thaliana KNOX* family genes. Overexpression vectors were constructed and subsequently transformed into wild type (WT) *A. thaliana*. Additionally, *LcKNOX6* was expressed in tobacco leaves to examine its subcellular localization, and the *35S::LcKNOX6* transgenic *A. thaliana* leaf cells were imaged with the use of SEM. The expression of several genes that participate in *KNOX* gene regulation were validated by quantitative real-time PCR. The results show that *LcKNOX1* produces almost the same phenotype as that found in WT *A. thaliana*. Notably, the *LcKNOX6-1* lines presented deep leaf lobes that were similar to *L. chinense* leaf lobes. Two *35S::LcKNOX6* lines induced an abnormal growth phenotype whose seeds were abortive. In short, these results indicate that the *LcKNOX6* gene might affect leaf development in *A. thaliana* and provide insights into the regulation of *L. chinense* leaf shaping.

Keywords: leaf shape; primordia initiation; axes growth; transcription factors; phytohormone

1. Introduction

Leaves present tremendous diversity due to their adaption to variable environments, and they play an essential role in plant photosynthesis and transpiration. Since photosynthesis in leaves is vital to plant growth and survival, variations in leaf shape may reflect natural selection according to function [1]. For instance, the decreased hydraulic resistance in deeply lobed leaves may serve as a mechanism for promoting water balance under drought conditions [2]. Leaf shape diversity is usually determined by alterations of the margin [3]. Recently, a report indicated that leaf shape might be an important but overlooked factor that affects the interactions between plants and leaf-processing herbivores [4]. Over the years, leaf shape has been examined in studies of the relationship between the environment and variation, as well as in studies of the evolution of species of angiosperms [3,5,6].

Liriodendron chinense is an ornamental tree that is used in the Chinese landscapes thanks to its straight stem, graceful crown, and attractive leaf shape, which prominently resembles an imperial

robe from the Qing Dynasty. Hence, it has the local name Robe Tree in the southeastern region of China. The *Liriodendron* genus is an old lineage that includes two distinct species from the Tertiary period: *L. chinense*, an endangered species in Southern China and Northern Vietnam, and *L. tulipifera*, a dominant species in Eastern North America [7]. Among the species in the genus, the leaves' morphologies exhibit slight variation in the depth of the lobes and the numbers of serrations below the two lobes. However, the most common leaf trait is the presence of two lobes that create the valuable feature that resembles a robe. Consequently, we examined the crucial genes that are involved in leaf lobe development in *L. chinense*.

The morphology of a leaf can be simple, such as that of *A. thaliana,* or be dissected, which is sometimes called compound found in species such as *Cardamine hirsute*, a relative of *A. thaliana* [5,8]. Regardless of their final shape, leaves initiate from a highly active dome called the shoot apical meristem (SAM), which is the central part involved in plant organogenesis. In the SAM, many regulators are involved in SAM maintenance and organ development [9,10]. Among these regulators, the *KNOX* family genes, which encode the three amino acid loop extension (TALE) homeodomain proteins, play vital roles in leaf developmental regulation [11,12]. The first *KNOX* gene was examined in maize in the foci of cells along the lateral veins that form knots and was characterized as part of the TALE homeobox superfamily [13–15]. *KNOX* genes can be divided into three subfamilies: class I, II and III. In *A. thaliana*, the *KNOX* class I family includes *SHOOT MERISTEMLESS(STM)*, *KNAT1*(*KNOTTED-LIKE HOMEOBOX* from *Arabidopsis thaliana1*)/*BREVIPEDICELLUS(BP)*, *KNAT2* and *KNAT6,* all of which are pivotal in the regulation of the SAM; the *KNOX* II family includes *KNAT3* and *KNAT7*; the *KNOX* class III family includes other *KNOXs* called *KNOXM* [16,17]. Class I *KNOX* genes can regulate leaf complexity in angiosperms, are expressed in the SAM, and are essential for SAM maintenance [18]. In plants with simple leaves, the class I *KNOX* genes are exclusively expressed in the meristem and stem, but some of them are shut down in leaf primordia [19]. In dissected leaves, they are expressed in leaf primordia [20]. KNOX proteins can maintain the key pluripotent cell population, which generates the entire aboveground body of vascular plants [19]. They generally regulate several target genes that mediate hormonal homeostasis in the meristem and interact with another subclass of homeodomain proteins. Generally, the KNOX proteins include three domains: The N-terminal MEINOX domain, the HD domain at the C-terminus and the ELK domain followed by the HD domain. The MEINOX region comprises two domains: KNOX1 and KNOX2. A study reported that the KNOX1 domain plays a role in its target gene suppression, and the KNOX2 domain is thought to be indispensable for homodimerization [21]. In fact, the KNOX proteins can cooperate with other regulators through the homeodomain, which is a conserved domain of the homeobox family and is typically composed of three α-helices that are connected by two loops that are approximately 60 amino acids long.

Even though the first plant *homeobox* gene was cloned from maize over 20 years ago, only recently have studies focused on *KNOX* gene function in diverse developmental processes, as well as *KNOX* functions have been found to contribute to developmental transitions during land plant evolution [19]. Though some studies have identified genes regulated by *KNOX*, further research in many species is needed. The transcriptome data for different stages leaves development in *L. chinense* have been examined [22]. In a previous work, a few candidate genes that participate in leaf shape development, including the *KNOX* genes, were selected from the differential expressed genes. In this work, the two most differentially expressed candidate genes were selected, and we examined these two key genes, which are members of the most important transcription factor family of genes that determine leaf morphology [23–27]. Though several studies have focused on the regulation by *KNOX* family genes in several species, they have not yet been examined in *L. chinense*. Ectopic *KNAT1* expression in *A. thaliana* was found to result in a transition from simple to lobed leaves [28]. Additionally, overexpression of *KNAT6* was found to induce the lobed leaves in *A. thaliana* [27]. Following to these studies, we aimed to examine whether *LcKNOX1* and *LcKNOX6* have the function to produce leaf lobe growth. Additionally, the *LcKNOX1* and *LcKNOX6* genes are the most likely candidate genes due to their significantly

differential expressions that were found during *L. chinense* leaf development in our previous work. Hence, the *LcKNOX1* and *LcKNOX6* genes were isolated from *L. chinense* as the putative key factors involved in leaf lobe development. Next, we overexpressed these two *KNOX* genes in wild type (WT) *A. thaliana* (Col-0). Detailed information on the conserved domain features, expression patterns and subcellular localizations of the two genes are reported here. Consequently, we obtained three different phenotypes from *35S::LcKNOX6* transgenic seedlings whose leaves presented several lobes. Notably, the *35S::LcKNOX6-1* line's leaf shape with two apparent lobes bore resemblance to the *L. chinense* leaf shape. These results indicated that *LcKNOX6* gave rise to leaf lobes and complexity in *A. thaliana*. This finding may provide insights into *L. chinense* leaf formation and contribute to a better understanding the critical regulators involved in leaf development.

2. Method

2.1. Materials and Plant Growth Conditions and Homozygote Screening

L. chinense trees were planted in a provenance trial plantation in the Xiashu forest nursery, which is located in Jurong County, Jiangsu Province, China (119°13′20″ E, 32°7′8″ N), in 1993. In June 2018, we sampled the tender leaves and buds of the sample trees whose provenances originated from Wuyi Mountain, Fujian Province, China.

The ecotype of *A. thaliana* that was used in transgenic assay was the wild type Col-0. Prior to sowing, seeds were sterilized with 70% ethanol for 30 s, flushed three times with distilled water for 20 s, soaked in bleach (10%) for 15 min, and washed five times with distilled water for 30 s. The putative transgenic seeds were dipped on solidified 1/2 MS (Murashige Skoog) medium with 0.8% (*w/v*) agar, 30 mM kanamycin and 1% (*w/v*) sucrose; the pH was adjusted to 5.7. Then, they were vernalized in a 4 °C refrigerator, left in darkness for 2 d, and nursed in a Sanyo MLR-350 chamber (Sanyo Electric Co., Osaka, Japan) under long day conditions (illumination at 23 °C for 16 h and darkness at 16 °C for 8 h). Once the seeds germinated two cotyledons, we transferred the seedlings into pots with a 1:1:1 mixture of soil, vermiculite and sand. Subsequent molecular detection was achieved via specific primer amplification, and several positive plants were generated. The seedlings of the T3 generation were subjected to transgene expression analysis.

2.2. RNA Extraction and cDNA Synthesis

The total RNA was isolated from *L. chinense* leaves and bud tissues and *A. thaliana* leaves tissues by using a DP441-RNAprep Pure Plant Kit (Tiangen, Beijing, China) and following the manufacturer's instructions. RNA was quantified by measuring absorbance at 260 nm, and its integrity was checked through denaturing agarose gel electrophoresis. Then, the cDNA (complementary DNA) was synthesized from 500 ng of the total RNA by using the TransScript All in One First-Strand cDNA Synthesis SuperMix for PCR (Transgen Biotech, Beijing, China) according to the manufacturer's protocol in 20 μL of total volume.

2.3. 5′ and 3′ Rapid Amplification of cDNA Ends and Sequence Analysis

To identify the candidate gene sequences that encode KNOX proteins in *L. chinense*, their unigenes were selected from the DEGs (differentially expressed genes) in *L. chinense* leaves at different stages in a transcriptome database [22]. Then, the 3′ and 5′ RACE (rapid-amplification of cDNA ends) primers were designed according to the transcriptomic annotated *KNOX1* and *KNOX6* sequences, respectively. The RACE reaction was conducted with the use of a SMARTer®RACE 5′/3′Kit (Takara Biomedical Technology, Dalian, China). The open reading frame (ORF) of the two *LcKNOX* genes was generated and ligated into pEASY-Blunt cloning vectors by using a pEASY-Blunt Zero Cloning Kit (Transgen biotech, Beijing, China). Next, the vectors were transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Transgen biotech, Beijing, China). The plasmid DNA was isolated from the positive *Escherichia coli* cells and sequenced by Genscript Biotech Corp (Genscript, Nanjing, China).

The ORFs were predicted by the NCBI (National Center for Biotechnology Information) ORF Finder(https://www.ncbi.nlm.nih.gov/orffinder/) and verified by sequencing (Genscript, Nanjing, China). The alignment was analyzed with the DNAMAN software (Lynnon Biosoft, QC, Canada) and compared to the *A. thaliana KNOX* genes online (https://www.arabidopsis.org/index.jsp). All the primers used for PCR are listed in Table S1.

2.4. Recombinant Plasmid Construction and Plant Transformation

To generate the plant transformation constructs, the *LcKNOX1* and *LcKNOX6* coding regions (without a stop codon) were amplified and ligated into a modified pBI121 vector downstream of the 35S CaMV (Cauliflower mosaic virus) promoter via the *Xba*I and *Bam*HI sites by using QuickCut enzymes (Takara Biomedical Technology, Dalian, China) and the ClonExpress®Ultra One Step Cloning Kit, respectively. We introduced the recombinant plasmids into *Agrobacterium tumefaciens* (GV3101) and used the floral dipping approach to transform the plasmids into *A. thaliana* (Col-0).

Since the lobed phenotype on which we focused occurs in *35S::LcKNOX6* seedlings, the sequence coding *LcKNOX6* was introduced into the binary pCAMBIA 1302 vector. The pCAMBIA1302 vector was digested by the *Xba*I and *Bam*HI sites by using QuickCut enzymes (Takara Biomedical Technology, Dalian, China). Then, the *LcKNOX6* ORF sequence was inserted into the digested pCAMBIA1302 vector. The *LcKNOX6::GFP* fusion construct was transformed into *A. tumefaciens* strain GV3101, which was injected into *Nicotiana benthamiana* leaves separately. A green fluorescent protein (GFP) fluorescence signal was observed 2 days after infiltration by using an LSM 710 confocal microscope (Zeiss, Jena, Germany) [29]. After multigeneration inbreeding and screening, the T3 generation of *35S::LcKNOX6*-2 transgenic *A. thaliana* was recruited for further assays.

2.5. SEM Observation and Subcellular Localization Analysis

We sampled the third leaves of *35S::LcKNOX6*-2 and WT *A. thaliana* (six week old) in an SEM assay. The *35S::LcKNOX6* transgenic seedlings and WT leaves were rinsed in a formalin acetic-acid alcohol (FAA) fixation solution composed of 38% formalin, glacial acetic acid, and 50% ethanol overnight at 4 °C. Next, all the samples were fixed in 1% OsO_4 for 1 h. Then, the samples were dried with an EMITECH K850 critical point dryer (Emitech, Ashford, UK). Next, they were coated with an Edwards E-1010 ion sputter golden coater (Hitachi, Tokyo, Japan). The leaves and siliqua epidermis of WT and *35S::LcKNOX6*-2 were observed using an FEI Quanta 200 FEG MKII scanning electron microscope (FEI, Eindhoven, Netherlands) under a suitable pressure (1.94×10^{-3} Pa) at 10–20 KV of high voltage (HV). To investigate the subcellular localization of *LcKNOX6*, the GFP fluorescence signal in tobacco leaves was observed 2 days after infiltration with the use of an LSM 710 confocal microscope (Zeiss, Jena, Germany).

2.6. Quantitative Real-Time PCR of Gene Expression

As previously reported, we selected a few genes related to leaf shape formation that are regulated by *KNOX* family genes in *A. thaliana*. We designed their primers to verify their expression in *35S::LcKNOX6* transgenic seedlings (Table S1). Since we barely harvested any *35S::LcKNOX6-1* seeds and few *35S::LcKNOX6-3* seeds were obtained due to the severely weak growth of these two lines, the third leaves of *35S::LcKNOX6*-2 and WT *A. thaliana* (six week old) were sampled to isolate the total RNA. Then, the total RNA was diluted to 500 ng and reverse transcribed by using a 5× PrimeScript RT Master Mix for Real Time (TaKaRa, Shiga, Japan) in a 20 μL of reaction protocol including 2 μL of cDNA (diluted five times), 10 μL of SYBR Premix Ex Taq (2×), 0.8 μL of each specific primer (10 mM), 0.4 μL of ROX Reference Dye II (50×), and 6.8 μL of double distilled H_2O (ddH_2O) (TaKaRa, Shiga, Japan). All designed primers were 18–25 bp and generated PCR produces 80–250 bp in size; the temperature of melting (Tm) ranged from 58 to 60 °C. The primers are listed in Table S1. The reference housekeeping gene *Actin97*(*ACT97*) of *L. chinense* was used for normalization [30]. The PCR conditions were as follows: 60 s at 95 °C for the initial denaturation, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C,

15 s at 95 °C, 30 s at 60 °C, and 15 s at 95 °C for annealing [31]. The standard deviations (SD) were calculated by triplicates.

3. Results

3.1. Cloning and Sequence Analysis of KNOX Genes

The genes were cloned from *L. chinense* buds and mixed leaves samples because previous reports claimed that some class I *KNOX* genes are only expressed in the SAM and that their expression might be non-existent in leaf primordia. Based on the transcriptome results of gene expression from different leaf development phases, the expression of *LcKNOX1* and *LcKNOX6* is significantly different during leaf development. Thus, we selected these two *KNOX* genes for further study in the relation to leaf lobes and development. Constructs corresponding to the two full-length *LcKNOX* genes were generated for overexpression under the 35S promoter in *A. thaliana* (Col-0). Consequently, the *LcKNOX1* and *LcKNOX6* protein sequences were aligned to that of the *A. thaliana* class I KNOX protein family, which has been shown to regulate *A. thaliana* leaf development. The results showed that the two *LcKNOX* genes were similar to *KNAT1* and *KNAT6*, so we named them *LcKNOX1* and *LcKNOX6*, respectively. *LcKNOX1* and *LcKNOX6* are comprised of three conserved domains: a MEINOX domain, including two domains at the N-terminus called KNOX1 and KNOX2; an ELK domain; and a homeodomain at the C-terminal, which is vital for combination with itself or other regulatory functional domains according to previous studies (Figure 1).
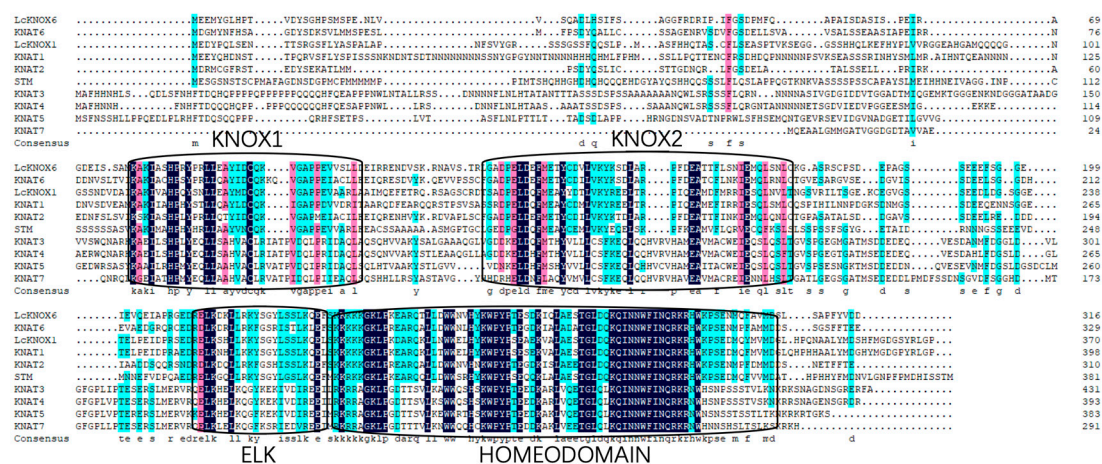

**Figure 1.** The sequences of the LcKNOX1 and LcKNOX6 proteins.

The full length of the *LcKNOX1* and *LcKNOX6* genes were found to 1394 and 1258 bp, respectively. The open reading frame of *LcKNOX1* was 1110 bp, and this frame was found to encode 370 amino acids with a molecular mass of 41.5 kDa. The hydrophilicity was calculated as −0.755, with an instability coefficient of 47.52, indicating that the LcKNOX1 protein was likely to be a stable and hydrophobic protein. The *LcKNOX6* ORF was 948 bp, it encoded 316 amino acids, and the molecular weight of the protein was found to be 50.15 kDa (Table 1).

Table 1. Analysis of the protein physical and chemical properties.

Gene	Amino Acids (aa)	Molecular Weight (kDa)	PI Value	Molecular Formula	Fat Coefficient	Instability Coefficient	Hydrophilicity
LcKNOX1	370	41564.43	5.88	$C_{1816}H_{2814}N_{512}O_{575}S_{17}$	62.54	47.52	−0.755
LcKNOX6	316	35783.25	5.19	$C_{1580}H_{2457}N_{429}O_{494}S_{13}$	70.41	50.15	−0.653

3.2. Phenotype of Transgenic A. thaliana

LcKNOX1 and *LcKNOX6*, both of which encode homeobox family protein sequences, were introduced into the binary pBI121 vector downstream of the 35S CaMV promoter. The overexpression of *LcKNOX1*, whose sequence is similar to that of the *KNAT1* gene in *A. thaliana*, did not result in any significant phenotypic changes compared to the WT *A. thaliana* (Figure 2a,b). The *35S::LcKNOX6* transgenic plants could be divided according to three observed phenotypes that corresponded to different lines. Notably, one of the *35S::LcKNOX6* phenotypes displayed by the *35S::LcKNOX6-1* line was characterized by lobed leaves (Figure 2c,f,i) that were apparently similar to *L. chinense* leaves (Figure 2g), but the leaves of the transgenic *35S::LcKNOX6-1* phenotype were considerably smaller than the WT leaves (Figure 2b,c,f,i). The growth of this line was extremely weak, and the line did not produce many seeds. Two other phenotypes of *35S::LcKNOX6* were displayed by the lines *35S::LcKNOX6-2* and *35S: 35S::LcKNOX6-3*. The *35S::LcKNOX6-2* leaves had a few extra lobes, and some leaves were observed to be dissected (Figure 2e). In the *35S::LcKNOX6-3* line, the individuals were abnormal, with weak and abortive growth (Figure 2d).
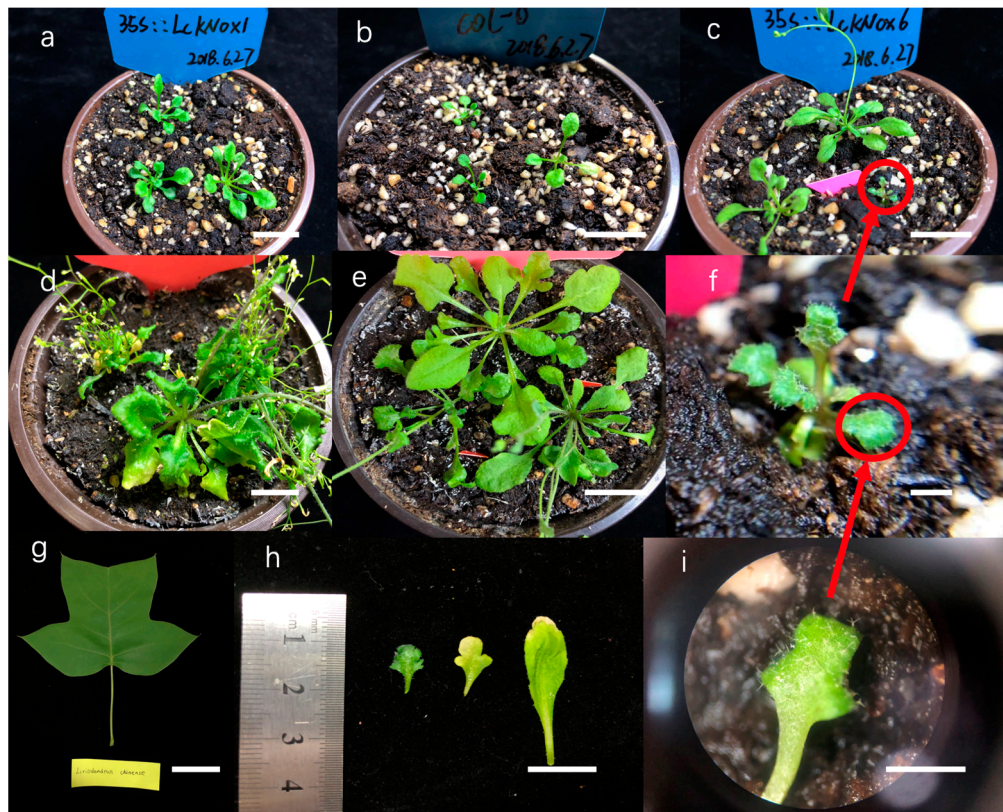

Figure 2. The wild type (WT) and transgenic *35S::LcKNOX Arabidopsis thaliana* and *Liriodendron chinense* phenotypes. (**a**) The first generation of the *LcKNOX1* transgenic seedlings; (**b**) WT *A. thaliana* (Col-0); (**c**) *35S::LcKNOX6-1* transgenic *A. thaliana* seedlings (the red circle is the positive transgenic plant of *35S::LcKNOX6-1*); (**d**) *35S::LcKNOX6-3* line exhibited dissected and abnormal leaves and abnormal growth, and their growth was weak; (**e**) *35S::LcKNOX6-2* line presented increased lobes; (**f**) one leaf from the positive transgenic plant of *35S::LcKNOX6-1*; (**g**) one leaf from *L. chinense*; (**h**) *35S::LcKNOX6-3*, *35S::LcKNOX6-2* and WT leaves of *A. thaliana*; and (**i**) one leaf of *35S::LcKNOX6-1*. Bar = 3 cm (**a**–**e**); bar = 5 mm (**f**,**i**); bar = 5 cm (**g**); and bar = 1 cm (**h**).

3.3. Transgenic Tissue SEM Imaging

To examine the leaf cell changes in the transgenic plants, we imaged the *35S::LcKNOX6* leaf margins, especially at the sinuses, which are known to contain ectopic stipules and meristems. At the sinus of

the *35S::LcKNOX6* plant leaf, we imaged many stomata along the lobe. Generally, twenty stomata were found on imaged part of the *35S::LcKNOX6* leaf margin, but only four stomata were found on the imaged part of the WT leaf margin. (Figure 3a,b). The cells at the *35S::LcKNOX6* leaf lobe were smaller than the cells at the WT leaf margin. Moreover, the siliqua epidermis was examined, and the result showed that the siliqua epidermis cells in the *35S::LcKNOX6* phenotype were shorter and smaller than siliqua epidermis cells of the WT phenotype. The cells of *35S::LcKNOX6* siliqua epidermis were almost 35 μm long, but the length of WT siliqua epidermis cell was more than 50 μm. (Figure 3c,d).
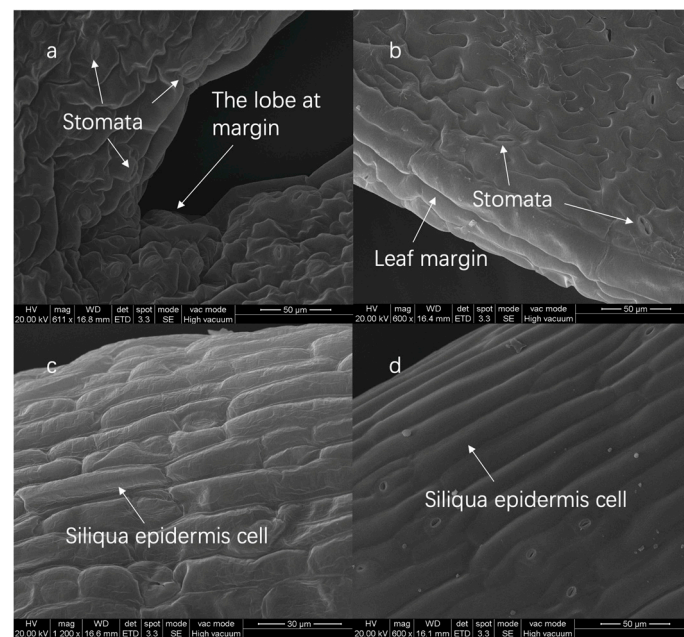

Figure 3. The SEM imaging leaf margin and siliqua epidermis. (**a**) *35S::LcKNOX6* leaf lobe at the margin; (**b**) WT *A. thaliana* leaf margin; (**c**) siliqua epidermis of *35S::LcKNOX6*; and (**d**) siliqua epidermis of WT *A. thaliana*. Some stomata on leaf margins and siliqua epidermis cells are pointed out by white arrows. Bar = 50 μm (**a**,**b**,**d**), and bar = 50 μm (**c**).

3.4. Subcellular Localization and Analysis with a Reporter Gene for β-glucuronidase (GUS) Staining in Transgenic Seedlings

Before the subcellular localization assay, we used LOCALIZER (http://localizer.csiro.au/) and Gneg-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Gneg/#), two online tools, for the prediction of subcellular localization [32]. The predictions both showed that *LcKNOX6* is a cytoplasmic protein. Then, a confocal microscope was used to analyze the subcellular localization of *LcKNOX6* with a GFP vector in *N. benthamiana* leaves. The result indicated that the *LcKNAT6::GFP* fusion protein was localized to the plasma membrane, nucleus and the cytoderm of cells, which is consistent with some transcription factor patterns (Figure 4).
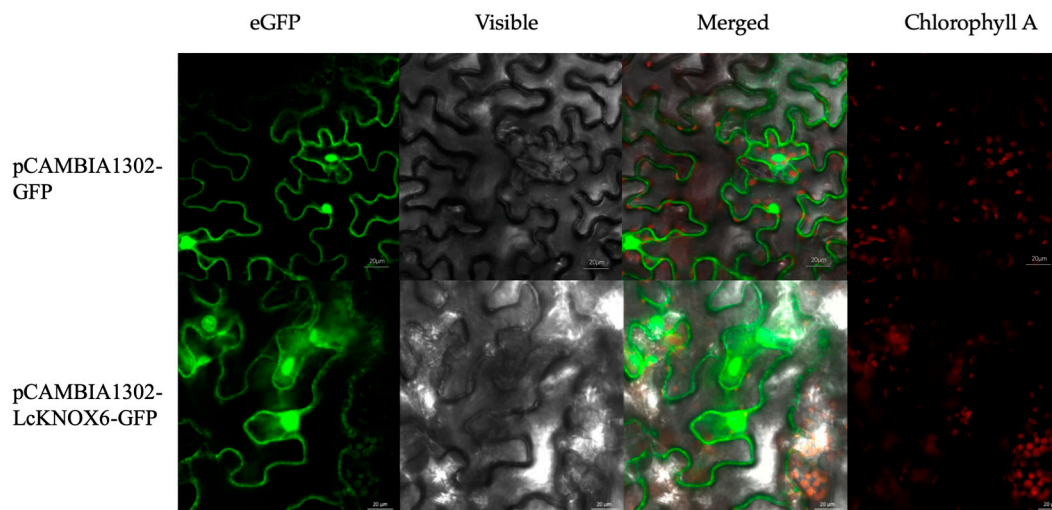
Figure 4. *35S::LcKNOX6* subcellular localization in *Nicotiana benthamiana* leaves.

3.5. The Expression of Genes Related to KNOX in Transgenic A. thaliana

Based on the abnormal leaf phenotype in *35S::LcKNOX6* seedlings, we examined the regulation of the other genes by *LcKNOX6* in *A. thaliana*. Thus, we selected *KNAT1*, *KNAT2*, *KNAT6*, *AS2*, *PIN1* and *GA20ox*, six genes in *A. thaliana*, on the basis of reports showing that they are involved in *KNOX* regulation [20,26,28,33–35]. Then, their expressions in the WT and transgenic *A. thaliana* leaves were generated. We validated that *KNAT1* expression was decreased, whereas the expression levels of *KNAT2* and *KNAT6* did not obviously change in transgenic *A. thaliana* compared to those in WT *A. thaliana*. We found that *AS2* can cooperate with *AS1* gene to repress *KNOX* gene expression. Likewise, the expression of *AS2*, an upstream regulator of *KNOX* genes, in transgenic seedlings presented a much lower expression level than the expression level in WT (Col-0). *PIN1*, which encodes an auxin efflux transporter protein, plays a critical role in leaf margin establishment. The decreased *PIN1* expression may have been due to *LcKNOX6* overexpression. *GA20ox* expression also decreased, which was consistent with the reports that KNOX proteins can bind the *GA20ox* gene promoter and repress its expression [36–38]. This result indicates that the *LcKNOX6* protein may have a function that is related to the binding of the promoter of the *GA20ox*. *KNAT1* was also found in *A. thaliana*, suggesting that *LcKNOX6* participates in the regulation of *KNAT1* (Figure 5).
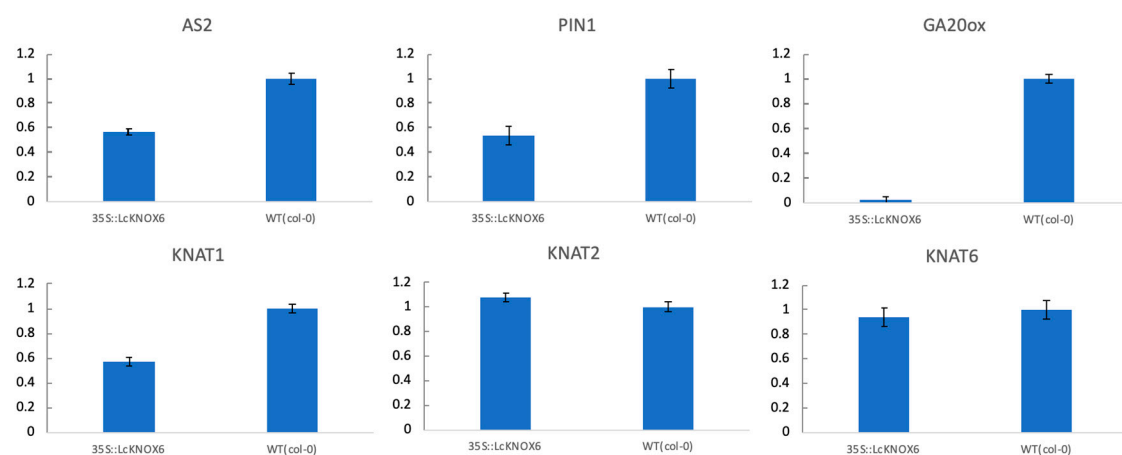
Figure 5. The expression of genes that may be regulated by the KNOX protein in *35S::LcKNOX6 A. thaliana* ($n = 3$ replications for each validation).

4. Discussion

4.1. Function of Domains in the LcKNOX Genes

Generally, *KNAT1*, *KNAT2* and *KNAT6* belong to class I *KNOX* family, which plays a crucial role in leaf morphology. The other classes *KNOX* families, e.g., the class II *KNOX* family, do not show a direct correlation with leaf shape, and they may have other functions in plant development. The sequences of the LcKNOX proteins were aligned to the *A. thaliana* KNOX family protein sequences. According to their similarity to the respective *A. thaliana KNOX* family genes, we named the two identified genes *LcKNOX1* and *LcKNOX6*. The two *LcKNOX* genes were both found to have the three typical domains of the *KNOX* family, including the MEINOX domain, the ELK domain, and the homeodomain. They correspond with the conserved structures of the class I *KNOX* genes found in other species [39,40]. Apparently, class I *KNOX* genes have a few different sequences that encode proteins from class II *KNOX* genes. Indeed, the N-terminal MEINOX domain is composed of KNOX1 and KNOX2 two subdomains, of which the KNOX1 subdomain participates in suppressing target gene expression, and the KNOX2 subdomain, which is essential for KNOX function, is thought to be necessary for homodimerization [15,21]. In addition, the C-terminal homeodomain (HD), which has three extra amino acids between helix1 and helix2, is a characteristic DNA-binding domain that is a conserved domain in the homeobox family and is typically composed of three α-helices that are connected by two loops that are approximately 60 amino acids long [11,41].

4.2. Roles of KNOX Genes During Lobed Leaf Formation

To investigate whether these two genes are involved in leaf development, we overexpressed *LcKNOX1* and *LcKNOX6* in *A. thaliana*. Overall, *35S::LcKNOX6* produced three phenotypes that were represented by three different lines. Firstly, the *35S::LcKNOX6-1* line exhibited two lobes on each side of the leaf that apparently resembled the leaf lobes of *L. chinense*. However, the growth the *35S::LcKNOX-1* line was extremely weak, and we obtained hardly any seeds. In a few leaves of the *35S::LcKNOX-2* line, which exhibited the most commonly observed phenotype for *35S::LcKNOX6*, the number of lobes was induced. The third phenotype was severely abnormal, and the seedlings tended to be abortive. These results indicate that the *LcKNOX6* gene might play a key role in leaf development and outgrowth in *L. chinense*. The overexpression of *KNAT1*, a *homeobox* gene of *A. thaliana*, and *knl*, a *homeobox* gene of maize, in *A. thaliana* resulted in a highly abnormal leaf morphology of obviously lobed leaves [20,28,42]. Before this work, *35S::LcKNOX6* plants were predicted to form lobed leaves and even leaflets. Therefore, the result is consistent with previous reports [28,43]. The overexpression of *KNAT6* induces the lobed leaves phenotype in *A. thaliana* [27]. The overexpression of five rice *homeobox* (*OSH*) genes under the control of the 35S promoter resulted in almost all of the transgenic seedlings showing abnormal phenotypes [44]. In *A. thaliana*, the *KNAT6* gene is expressed in the embryonic SAM and can contribute to *STM* maintenance, as well as SAM and organ separation [26]. However, the *35S::LcKNOX1* transgenic seedlings presented little phenotypic change in *A. thaliana*. The subcellular localization result indicates that the *LcKNOX6* gene is localized in the plasma membrane, nucleus and the cytoderm of cells in tobacco leaves. This result was also consistent with most of the transcription factor subcellular localization patterns.

4.3. KNOX Gene Regulations

The *KNOX* genes, which serve as pleiotropy factors in plant development, could maintain SAM function by regulating the expression of other key genes. Due to the abnormal phenotypes in *35S::LcKNOX6* seedlings, and according to previous studies, a few genes that may be regulated by the *KNOX* gene were examined in *35S::LcKNOX6* leaves. The *LcKNOX6-1* line was dead before the flowers bloomed, and the *LcKNOX6-3* plants were seriously abortive such that we were unable to obtain enough T3 generation materials for further assays. Thus, only the *35S::LcKNOX1-2* line was used in the assays. Generally, the KNOX family genes affect leaf development by regulating several genes, such as

AS1/AS2, *CUC*, *GA20ox* and *PIN1* [45–50]. Additionally, the *KNOX* family gene structure contained the HD conserved domain which can combine with other genes [11,41]. Thus, we detected the expression of *KNAT6* to exclude the interference of *KNAT6*. Therefore, we examined the expression of several genes by using the quantitative real-time PCR method. In fact, a few *35S::LcKNOX6* seedlings were prone to being abortive and dwarfed, which is similar to the behavior of the *as2* mutant [51]. This might mean the *LcKNOX6* gene plays a role in *AS2* gene suppression. The suppression of *AS2* in *35S::LcKNOX6* leaves has been validated. *AS2* has been identified to participate in the regulation of *KNOX* genes by complexing of the *AS2* gene and the *AS1* gene to repress the *KNOX* gene expression [33]. *PIN1* was also decreased in *35S::LcKNOX6* leaves. The ectopic expression of the *KNOX* gene in leaves was found to perturb local *PIN1*-dependent gradients of auxin activity, which leads to lobe or leaflet outgrowth in *A. thaliana* [52,53]. However, the decreased *PIN* expression in *35S::LcKNOX6* may have be due to the leaves that complete the regulation between *KNOX* and *PIN*. In our study, we generated a phenotype that was so abnormal that the resulting plant was dwarfed and abortive. Additionally, some *KNOX* genes have the ability to bind the promoter of the *GA20ox* gene, which is an indispensable biosynthesis enzyme that mediates gibberellin gradients. For instance, the NTH15 protein, which belongs to the KNOX protein family, binds to a 5 bp dyad symmetric sequence, GTGAC in the first intron of *Ntc12*, which encodes the GA20ox protein in tobacco in vitro [38]. *KNOX* can bind to a sequence, TGAC, to regulate the target gene. In potato, TALE proteins, *StBEL5* (*Solanum tuberosum BEL 5*) and *POTH1* (*potato homeobox 1*) form a bond, a *StBEL5-POTH1* heterodimer, that targets a composite 10 bp sequence composed of two TGAC cores and has greater binding affinity for the *ga20ox1* promoter [48]. The validation of *GA20ox* showed its apparent suppression in the *35S::LcKNOX6* transgenic line, and this result is consistent with the results of previous studies. However, this activity remains to be investigated in *L. chinense*. Additionally, the expression of *KNAT2* and *KNAT6* were found to have little change, whereas the expression of *KNAT1* was decreased. The *KNOX* family genes were recently examined in a study of the evolution and variation of plant morphology. For example, the relationship between *A. thaliana* and *C. hirsute* was examined to determine the differences between simple and dissected leaves, and the results showed that the *KNOX* homeobox genes are repeatedly manipulated to generate natural variation in leaf shape [54]. In most cases, *KNOX* plays an integral part in leaf complexity and lobe formation. The previous studies indicated that alterations in *KNOX* activity might contribute to the evolution of divergent leaf morphologies between *A. thaliana* and *C. hirsute,* two relatives [8,34]. In fact, the *Liriodendron* genus includes a sister species pair of *L. chinense* and *L. tulipifera,* which are relic lineage from the Tertiary period [55]. The depth of two lobes is an essentially different morphologic trait between these two species. Whether the *Liriodendron* genus morphology has the similar regulatory model of divergence is a question that needs further investigation. Furthermore, the regulation underlying the *KNOX* diversification of leaf morphology also needs further research.

5. Conclusions

Based on the transcriptome data, we aimed to identify the candidate genes that may be involved in leaf shape regulation in *L. chinense*. Then, two candidate *KNOX* genes of *L. chinense* were selected with the use of extensive reports on the regulation of the *KNOX* genes of leaf development in other species. In this work, the full-length sequences of two *LcKNOX* ORFs were obtained in *L. chinense*. Then, the ORFs of these two genes were overexpressed in WT *A. thaliana* (col-0) to preliminarily identify their function. Consequently, we generated three abnormal phenotypes in *35S::LcKNOX6* that presented lobed leaves. Among the lines, the *35S::LcKNOX6-1* leaf shape was very similar to that of *L. chinense*. The *35S::LcKNOX6-1* and *35S::LcKNOX6-3* lines were prone to being dwarfed and abortive. The *35S::LcKNOX6-2* line presented numerous lobed leaves. In addition to the phenotypes, gene expression with respect to *KNOX* regulation was also mediated in the *35S::LcKNOX6-2* seedlings. These results indicate that *LcKNOX6* might be involved in *L. chinense* leaf development and even in plant growth regulation.

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