The upregulated LsKN1 gene transforms pinnately to palmately lobed leaves through auxin, gibberellin, and leaf dorsiventrality pathways in lettuce

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Summary
Leaf shape represents a vital agronomic trait for leafy vegetables such as lettuce. Some lettuce cultivars produce lobed leaves, varying from pinnately to palmately lobed, but the genetic mechanisms remain unclear. In this study, we cloned one major quantitative trait locus (QTL) controlling palmately lobed leaves. The candidate gene, LsKN1, encodes a homeobox transcription factor, and has been shown previously to be critical for the development of leafy heads in lettuce. The LsKN1 allele that is upregulated by the insertion of a transposon promotes the development of palmately lobed leaves. We demonstrated that LsKN1 upregulated LsCUC2 and LsCUC3 through different mechanisms, and their upregulation was critical for the development of palmately lobed leaves. LsKN1 binds the promoter of LsPID to promote auxin biosynthesis, which positively contributes to the development of palmately lobed leaves. In contrast, LsKN1 suppresses GA biosynthesis to promote palmately lobed leaves. LsKN1 also binds to the promoter of LsAS1, a dorsiventrality gene, to downregulate its expression. Overexpression of the LsAS1 gene compromised the effects of the LsKN1 gene changing palmately to pinnately lobed leaves. Our study illustrated that the upregulated LsKN1 gene led to palmately lobed leaves in lettuce by integrating several downstream pathways, including auxin, gibberellin, and leaf dorsiventrality pathways.

Keywords: palmately lobed leaves, pinnately lobed leaves, KNOX1, transposon, phytohormones.

Introduction
Plant leaf is the primary organ to harvest light energy through photosynthesis (Tsukaya, 2013). Though the function of leaves is conserved, their shapes may vary dramatically among different plant species (Chitwood and Sinha, 2016; Tsukaya, 2018). The underpin mechanisms for the high divergence of leaf shapes are not well understood (Drost et al., 2015). Leaf shape may have evolved to adapt to natural habitats (Nicotra et al., 2011). However, closely related plant species with similar distribution regions or niches can vary considerably in leaf shapes (Byrne, 2012). Furthermore, leaf shape may vary among individuals within a natural population of the same wild species (Hickey, 1973). Leaf shapes of some cultivated species have experienced considerable modifications during domestication to increase yield or fit agricultural practices (Rowland et al., 2020). For leafy vegetables, leaf shape per se is the target of artificial selection (Sedivy et al., 2017).

The prominent polymorphisms of leaf shape are single leaf versus compound leaves, and non-lobed leaves versus lobed leaves. Fossil evidence suggested that the ancestors of angiosperm were unifoliate, and, therefore, compound leaves had evolved from single leaves and developed convergently in different plant lineages (Ledford, 2018). Compared with single leaves, compound leaves possess many benefits, such as better gas exchange and less tissue damage from herbivores (Higuchi and Kawakita, 2019). It was hypothesized that compound leaves were more developmentally elastic and flexible than single leaves, allowing a wide range of mutations to produce new phenotypic manifestations (Sisó et al., 2001). Though lobed leaves were anatomically and developmentally different from compound leaves, the regulation of the development of lobed leaves in some plant species resembled that of compound leaves (Chang et al., 2019). Lobed leaves contained higher plasticity of spatial extension and responded more quickly to compete for limited light sources than non-lobed leaves (Baker-Brosh and Peet, 1997). Plants with lobed leaves acquired better adaptions to low temperatures in high latitudes or cold regions than those with non-lobed leaves (Sedivy et al., 2017). The complexity of lobed leaves had a negative correlation with leaf hydraulic resistance, and lobed leaves were hypothesized to help plants achieve water balance under dry atmospheric conditions (Nicotra et al., 2011).

The molecular mechanisms for the development of lobed or compound leaves have been studied elusively in model species such as tomato (Lycopersicon esculentum), Cardamine hirsute, Medicago truncatula, and Lotus japonicus (Champagne et al., 2007). Lobed leaves have auxin maxima at the tip of leaf lobes, accompanied with high expression level of CUP-SHAPED COTYLEDON (CUC) genes in the sinuses. Overexpression of CUC1 and its homologs CUC2 and CUC3 promote leaflet separation and leaflet formation, leading to the development of leaf lobes (Aida et al., 1997; Blein et al., 2008; Takada et al., 2001; Vroemen et al., 2003). Reduced expression of NAM/CUC boundary
genes suppresses all marginal outgrowths and consequently reduces the number of leaf lobes (Vroemen et al., 2003).

The leaf lobe pattern was orchestrated by several pathways, and one of them involved the KNOXI homologs, a family of homeobox transcription factors (Vollbrecht et al., 1991). Members of the KNOX1 family were mainly expressed in shoot meristems and subtending stems to maintain meristematic activity. The KNOXI family participated in the initiation of lateral organs (Hake et al., 2004). KNOX1 mRNA transports between cells through ribosomal RNA-processing protein 44A, which was critical to regulate the stem cell-dependent processes in plants (Kitagawa et al., 2022). Up-regulation of the KNOXI genes after the formation of leaf primordia is unique for plants with lobed or compound leaves (Efroni et al., 2010; Hareven et al., 1996; Janssen et al., 1998; Piazza et al., 2010; Shani et al., 2010; Veit, 2009). Increased expression of a KNOXI homolog in tomato led to super-compound leaves with thousands of lobed leaflets (Janssen et al., 1998). The KNOXI gene in tomato is negatively regulated by a BEL-like homeodomain protein BIPINNATA (BIP), and the mutation of the bip gene boosted the complexity of leaf morphology in tomato (Nakayama et al., 2021). Arabidopsis contained four KNOXI homologs including SHOOTMERISTEMLESS (STM), KNAT1, KNAT2, and KNAT6 (Hake et al., 2004). Overexpression of KNAT1 in Arabidopsis transformed entire leaves into lobed leaves by repressing the AS1 and AS2 genes (Chuck et al., 1996; Ori et al., 2000). The expressions of the KNOXI genes are regulated by several other transcription factors besides AS1 and AS2. For example, BLADE ON PETIOLE1 (BOP1) and BOP2, members of the BTB ankyrin family repress the expression of the KNOXI family and play critical roles in maintaining a border between meristem organ compartments (Khan et al., 2014).

Besides the KNOXI family, several other families were also shown to regulate the development of lobed leaves or compound leaves. The polymorphism of lobed leaves and non-lobed leaves in cotton, rapeseed, and watermelon was genetically controlled by the polymorphic LMI1 homologs, which also encode homeobox transcription factors (Andres et al., 2017; Sicard et al., 2014; Vlad et al., 2014; Wei et al., 2017). The UNIFOLIATA (UNI) in pea and SINGLE LEAFLET1 (SGL1) in Medicago, orthologs of the Arabidopsis floral meristem identity protein LEAFY (LFY), played critical roles in leaf shapes (Hofer et al., 1997). Pea uni mutant and Medicago sgl mutant reduced the complexity of compound leaves (Gourlay et al., 2000; Wang et al., 2008). In addition, PALMATE-LIKE PENTAFOLIATA1 (PALM1), a zinc finger protein, controlled the development of the trifoliate leaves in Medicago through negatively regulating SGL1 in lateral leaflet regions (Chen et al., 2010). PINNATE-LIKE PENTAFOLIATA1 (PINN1), a BEL1-like homeodomain protein, attenuated the expression of SGL1 in the terminal leaflet regions (He et al., 2020). SILAM1, the ortholog of WOX1 in tomato, facilitated secondary leaflet initiation and maintained the morphology of compound leaves (Wang et al., 2021). Moreover, the SMOOTH LEAF MARGIN1 (SLM1) protein in M. truncatula, which is an auxin efflux carrier protein and is the ortholog of PIN-FORMED1 (PIN1) from A. thaliana, regulates the complexity of leaves (Zhou et al., 2011). Loss of function in MtG3Aox1, an enzyme for GA biosynthesis, promotes serration on the blade margin and increases leaf complexity. The above data suggested the antagonistic role of phytohormones GA and auxin in the regulation of lobed leaves and compound leaves, with auxin increasing, and GA decreasing leaf complexity (Bar and Ori, 2015).

Cultivated lettuce (Lactuca sativa), domesticated from prickly lettuce (L. serriola), is one of the most important green leafy vegetables worldwide (Zhang et al., 2017). Lettuce is also a model species for hydroponics, and an ideal plant to be engineered to produce oral vaccines or valuable pharmaceuticals (Daniell et al., 2001; Kanamoto et al., 2006; Lal et al., 2007; Power et al., 2021). Both cultivated and wild lettuce have the polymorphism of lobed and non-lobed leaves. The polymorphism of lobed leaves and non-lobed leaves in cultivated lettuce was inherited from its progenitor L. serriola, and the causal gene is located on Chromosome 3 (Wei et al., 2021). Previous studies focused on pinnately lobed leaves in lettuce, but it is a daunting challenge to uncover the genetic and molecular mechanisms underlying the development of palmately lobed leaves in lettuce. In this study, we conducted a bulked segregant analysis (BSA) combined with RNA-seq (BSR) to dissect the genetics underlying the complexity of leaf lobes in lettuce. We fine mapped and cloned a major QTL controlling lobe complexity of lettuce leaves. We validated the candidate gene with a complementation test and investigated its molecular mechanism in detail. Our results opened the door to the molecular regulation of lobed leaves, and are useful in breeding programs to develop lettuce cultivars with ideal leaf shapes.

Results

Genetic analysis of the complexity of leaf lobe in lettuce

To investigate the genetic mechanism underlying palmately lobed leaves in lettuce, we crossed an inbred line (FZ-118) of palmately lobed leaves with a wild lettuce accession (L. serriola, CGN04971) of pinnately lobed leaves (Figure 1a). The F1 population had palmately lobed leaves similar to those of the inbred line FZ-118, suggesting that the gene(s) controlling palmately lobed leaves is dominant. The F1 individuals were self-pollinated to generate an F2 segregating population. Individuals from the F2 population showed a continuous distribution of lobe complexity, ranging from pinnately lobed leaves to palmately lobed leaves, clarifying the lobe complexity as a quantitative trait in lettuce (Figure 1b).

We performed BSR to dissect the genetics underlying the lobe complexity in the F2 population. The differences of allele frequencies, Δ(SNP-index), between an extremely lobed pool and a pinnately lobed pool, were plotted along the nine chromosomes of lettuce. The plot figure demonstrated that three major loci contributed to the complexity of leaf lobes in the F2 segregating population, located on chromosomes 2, 3, and 7, respectively (Figure 1c).

To verify the potential loci controlling the lobe complexity, we designed Cleaved Amplified Polymorphic Sequence (CAPS) markers at the three potential loci, and screened the F2 population. Analysis of Variance (ANOVA) illustrated that the three loci were significant associated with lobe complexity (P < 0.001). We defined the loci on chromosomes 2, 3, and 7 as Palmately Lobed Leaf 1, 2, and 3, respectively. ANOVA of the F2 population suggested that the PLL1, PLL2, and PLL3 explained 16.5%, 9.7%, and 22.0% of the variance on lobe complexity in the F2 population. This study focused on the PLL3 locus on chromosome 7, which contributes the highest phenotypic variation explained (PVE) of 22.0%.

The candidate gene for PLL3 is the LsKN1 gene

An individual from the F2 population, which was heterozygous at the PLL3 locus but homozygous at the PLL1 and PLL2 loci, was...
self-pollinated to generate an F3 family. Individuals from the F3 family produced either palmately lobed or pinnately lobed leaves, with no intermediate phenotypes (Figure 2a). Of the 405 individuals from this F3 family, 302 had palmately lobed leaves, and 103 had pinnately lobed leaves, which fits the Mendelian ratio of 3:1 ($\chi^2 = 0.98$, $P > 0.05$). BSR method was used to investigate the genetics of lobe complexity in the F3 family, pointing to a single locus on chromosome 7 (PLL3) (Figure 2b).

We therefore used this F3 family to fine map and clone the PLL3 gene. We genotyped additional 599 individuals from the F3 family using a series of genetic markers in the candidate region, and consequently delimited the PLL3 gene between markers CAP18.5 and CAP18.9, in an interval of 400 kb on chromosome 7 (Figure 2c).

The candidate region contains eight genes, with LsKN1 as the only differentially expressed one between the pinnately lobed and the palmately lobed leaves (Table S1). The LsKN1 gene was reported to be critical for the development of leafy heads in crisphead lettuce (Yu et al., 2020). We investigated the polymorphism of the LsKN1 gene between the two parents. The parent CGN04971 with pinnately lobed leaves had the wild-type allele. The parent FZ-118 with the palmately lobed leaves had the mutated allele LsKN1▽, with an insertion of 3935 bp CACTA-like transposon at +99 bp. The above result was identical to the allele indispensable for the development of leafy heads in lettuce (Figure 2d) (Yu et al., 2020). The CACTA-like transposon is present in the LsKN1▽ allele from the parent with palmately lobed leaves. Black boxes represent the exons of the candidate gene. The bottom panel shows the predicted proteins encoded by the two alleles. The mutated protein lost the 79 amino acids at the N-terminal. (e) The expression of the LsKN1 gene in the F3 family. Five individuals with pinnately lobed leaves and five individuals with palmately lobed leaves were randomly chosen from the F3 family. Data represent mean ± SD (n = 3). ** denotes $P < 0.01$.

Figure 1 Complexity of leaf lobes. (a) Leaves of parents FZ-118, CGN04971, and their F1 hybrid. (b) Continuous distribution of lobe complexity in the F2 population. The bottom figure shows the distribution of lobe complexity (number of lobes per leaf). Bars = 2 cm. (c) Plot of $\Delta$ (SNP-index) between the extremely palmately lobed pool and pinnately lobed pool constructed from the F2 population. Three loci associated with the complexity of leaf lobe are detected in the F2 population.

Figure 2 Genetic cloning of the PLL3 gene. (a) An F3 family had individuals with pinnately lobed leaves (top) and palmately lobed leaves (bottom). Bars = 2 cm. (b) Plot of $\Delta$(SNP-index) between the pinnately lobed pool and palmately lobed pool from an F3 family. (c) Linkage map of PLL3. Number of recombs refers to the number of recombinants between a marker and the PLL3 gene among 1004 individuals from the F3 family. (d) Schematic representation of LsKN1, the candidate gene of PLL3. The CACTA-like transposon is present in the LsKN1▽ allele from the parent with palmately lobed leaves. Black boxes represent the exons of the candidate gene. The bottom panel shows the predicted proteins encoded by the two alleles. The mutated protein lost the 79 amino acids at the N-terminal. (e) The expression of the LsKN1 gene in the F3 family. Five individuals with pinnately lobed leaves and five individuals with palmately lobed leaves were randomly chosen from the F3 family. Data represent mean ± SD (n = 3). ** denotes $P < 0.01$. 
its expression. We hypothesized that the upregulated expression of the LsKN1 gene transformed pinnately lobed leaves to palmately lobed leaves.

Verification of the function of LsKN1 on the complexity of leaf lobe

We then used a complementation test to verify the function of LsKN1 on palmately lobed leaves. We chose an individual from the F3 family with a homozygous LsKN1 allele producing pinnately lobed leaves for further study. This individual was self-pollinated to generate a homozygous line (PINN) with pinnately lobed leaves. Similarly, an isogenic line named PALM, with homozygous LsKN1 and palmately lobed leaves, was generated for further study.

We transformed a fragment of 10 220 bps containing the LsKN1 allele, including the CACTA-like transposon, into the PINN line. We obtained three independent transformants, and all of them produced palmately lobed leaves (Figure 3a). All three T1 populations showed a 3:1 Mendelian segregation ratio (P > 0.05), and the palmately lobed leaves co-segregated with the insert, confirming LsKN1 as the PLL3 gene controlling palmately lobed leaves in lettuce. The expression of LsKN1 showed a deep increase in the transformants, supporting the correlation between its expression level and the leaf lobe complexity (Figure 3a). We further overexpressed the LsKN1 gene in the PINN line. The two overexpression lines had palmately lobed leaves, in contrast to the pinnately lobed leaves in the wild-type PINN (Figure 3b).

Figure 3 Verification of the function of LsKN1 on lobe complexity. (a) Complementation test. Transformation of LsKN1' changed pinnately lobed leaves (PINN) to palmately lobed leaves (COM#1-3) (left panel). qRT-PCR shows high expression of LsKN1 in positive complementation lines (right panel). Data represent mean ± SD (n = 3). ** denotes P < 0.01. (b) Overexpression of LsKN1. Overexpression of LsKN1 changed pinnately lobed leaves (PINN) to palmately lobed leaves (OE#1-2) (left panel). qRT-PCR shows high expression of LsKN1 in the two overexpression lines (right panel). Data represent mean ± SD (n = 3). ** denotes P < 0.01. (c) Knockout of LsKN1 using CRISPR/Cas9. Knockout of LsKN1 changed the palmately lobed leaves (PALM) to pinnately lobed leaves (CR#1-3) (left panel). Modification of the sequences in the LsKN1 gene in the knockout plants (right panel). The sgRNA sequences are indicated with a horizontal line. Dash lines refer to deletion, and inserted nucleotides are in blue. PAM sequences are in red. Bars = 2 cm.

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We then knocked out the LsKN1 gene in PALM to further confirm its function on lobe complexity. We constructed a recombinant CRISPR/Cas9 vector with sgRNA specific to the coding region of the LsKN1 gene and transformed it into the PALM line. Three knockout lines were obtained, and all of them had pinnately lobed leaves, in contrast to palmately lobed leaves in PALM (Figure 3c). Our knockout results further verified that the upregulated LsKN1 contributed to the palmately lobed leaves in lettuce.

A large number of LsKN1 target genes are differentially expressed between palmately and pinnately lobed leaves

We sequenced the RNA extracted from the young leaves of the LsKN1 knockout mutants with pinnately lobed leaves in the T1 generation and its recipients, which have the LsKN1 allele and palmately lobed leaves. There are 389 differentially expressed genes (DEGs) between the pinnately lobed and palmately lobed individuals (Table S2). Of them, 76 genes are the potential targets of LsKN1 according to the ChiP-seq results of LsKN1 (Table S3; Figure S2). Some of the 76 target genes were predicted to be of LsKN1 according to the ChIP-seq results of LsKN1 (Table S3; Figure S2). Of them, 76 genes were the potential targets of LsKN1 according to the ChiP-seq results of LsKN1 (Table S3; Figure S2). Some of the 76 target genes were predicted to be associated with leaf development, such as LsCUC3 (LG9882694), LsHB (LG8751215), LsGA20ox1 (LG9790044), LsYAB3 (LG6576535), LsAS1 (LG4386200), and LsCKX3 (Ls5469613).

Our previous study also identified 581 DEGs between LsKN1 mutant and its wild type (Table S4; Yu et al., 2020). Note that the 581 DEGs were detected between two genotypes with nonlobed leaves. A comparison between the 581 DEGs under nonlobed leaf background with the 389 DEGs under lobed leaf background revealed 303 DEGs specific to the latter (Figure S2). These lobed-leaf specific 303 DEGs included Genes critical for leaf development, such as LsCUC3 (LG9882694), LsGA20ox1 (LG9790044), LsYAB3 (LG6576535), LsCUC2 (LG7605596), LsPIN5 (LG1109276), LsTCP10 (LG1159713), LsTCP18 (LG4391812), and LsCKX3 (Ls5469613).

LsKN1 binds to the promoter of LsCUC3 and upregulates its expression

Previous studies suggested that the CUC gene family was associated with serrations or lobed leaves (Blein et al., 2008). The lettuce genome contains three CUC homologs. LsCUC3 represented an ortholog of Arabidopsis CUC3, while LsCUC2a and LsCUC2b were duplicated after divergence of Asteraceae and Brassicaceae as the ortholog of Arabidopsis CUC2 (Figure S3). ChiP-seq of LsKN1 suggested that LsCUC3 was a target of LsKN1, but LsCUC2a and LsCUC2b were not (Figure 4a; Table S3). LsKN1 directly bound the promoter region of LsCUC3 in the yeast one-hybrid (Y1H) assay (Figure 4b). Further electrophoretic mobility shift assays (EMASs) demonstrated that LsKN1 bound to the LsCUC3 promoter between the -2205 and -1790 bps (Figure 4c).

We carried out dual-luciferase assay (LUC) to investigate the effects of LsKN1 on the expression of LsCUC3. The LsKN1 gene bolstered the activity of LsCUC3 promoter significantly (Figure 4d). Surprisingly, LsKN1 and LsKN1\n contributed to the development of palmately lobed leaves. The overexpression of the LsCUC3 gene in PINN led to palmately lobed leaves (Figure 4f; Figure 4g). On the contrary, the knock-out of the LsCUC3 gene in PALM resulted in pinnately lobed leaves, in contrast to palmately lobed leaves in PALM (Figure 4h). Our results showed that LsKN1 controlled palmately lobed leaves through LsCUC3, which is a repressive epistatic to LsKN1 for its effects on the development of palmately lobed leaves.

Like the LsCUC3 gene, LsCUC2b was differentially expressed between palmately lobed leaves and pinnately lobed leaves (Figure 5a). However, ChiP-seq results suggested that LsCUC2b was not a target of LsKN1. In Arabidopsis, ATHB1, an HD Zip1 transcription factor, bound to the promoter region of CUC2 and miR164, directly and indirectly upregulating the expression of CUC2 (Miguez et al., 2020). Interestingly, ChiP-seq data suggested that LsHB was a target of LsKN1 in lettuce, although LsCUC2b was not (Figure 5b). Y1H assay showed that LsKN1 bound to the promoter region of LsHB (Figure 5c). LUC results showed that the LUC activity driven by the promoter of LsHB considerably increased when the LUC reporter vector co-expressed with the LsKN1 gene. Similarly, the LUC activity driven by the promoter of LsCUC2b considerably increased when the LUC reporter vector co-expressed with the LsKN1 gene. Furthermore, the upregulated LsKN1 gene contributed to the development of palmately lobed leaves through both LsCUC2 and LsCUC3 genes, which are mainly expressed in the sinuses of lobes (Figure 5a).

LsKN1\n promotes auxin biosynthesis to enhance palmately lobed leaves

Auxin modifications of leaf margins (Zhou et al., 2011). We found that the auxin content in the palmately lobed leaves of PALM is significantly higher than that in the pinnately lobed leaves of the LsKN1 knockout mutants (Figure 6a). The high concentration of auxin in palmately lobed leaves was also demonstrated in the LsDR5::GUS transformants (Figure 6b). GUS activity was detected in all leaf veins in palmately lobed leaves, in contrast to limited to the main veins in pinnately lobed leaves. The activity of GUS at the margin of palmately lobed leaves was much higher than that of pinnately lobed leaves (Figure 6b).

To investigate whether auxin affects the complexity of leaf lobes in lettuce, we treated the leaves of PINN with Naphthaleneacetic acid (NAA). The pinnately lobed leaves changed to pinnately lobed leaves after it was treated with N-1-naphthylphthalamic acid (NPA), an auxin-transport inhibitor (Figure 6c). We conclude that LsKN1 upregulates auxin biosynthesis, contributing to the development of palmately lobed leaves in lettuce.
The ChIP-seq results suggested that LsKN1 binds to the promoter region of the LsPID gene (Figure 6d), which controls PIN polarity and mediates changes in auxin flow. Y1H (Figure 6e) assay and EMSAs (Figure 6f) showed that LsKN1 bound to the promoter region of LsPID. LUC assay showed that the LUC activity driven by the promoter of LsPID considerably increased when the 

![Figure 4](image-url)

Figure 4  LsKN1 binds to the promoter of LsCUC3 and upregulates its expression. (a) ChIP-seq analysis of LsKN1 binding activity in the promoter region of LsCUC3. The y-axis represents the number of reads in the ChIP-seq. The x-axis shows the position of the reads in the gene. (b) Y1H assay for LsKN1 and the promoter sequences of LsCUC3. Transformants were grown on the SD-Leu medium with 500 ng/mL AbA. Positive control, transformants of p53-AbAi and pGADT7-p53; negative control, transformants of p53-AbAi and pGADT7. The region of promoter is shown in (a). (c) EMSA showing the binding of LsKN1 to the promoter of LsCUC3. (d) Dual-luciferase assay showing the effects of LsKN1 on LsCUC3. The left panel shows the diagram of reporter and effector vectors used in the dual luciferase assay. The right panel shows the LUC activity when the LUC gene driven by the promoter of LsCUC3 was co-expressed with an empty vector (EV) or LsKN1\textsuperscript{\textregistered}. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05. (e) qRT-PCR analysis of LsCUC3 expression in LsKN1 and LsKN1\textsuperscript{\textregistered} genotypes randomly chosen from the F\textsubscript{3} family. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05. (f) Overexpression of LsCUC3 changed pinnately lobed leaves to palmately lobed leaves. (g) Knockout of LsCUC3 using CRISPR/Cas9. Knockout of LsCUC3 changed palmately lobed leaves to pinnately lobed leaves (left panel). Sequence modification in the LsCUC3 gene in the knockout mutants (right panel). The sgRNA sequences are indicated with a horizontal line. Dash lines refer to deletion. PAM sequences are in red. Bars = 2 cm.

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LUC reporter vector co-expressed with the LsKN1 gene (Figure 6g). The LUC results indicated that LsKN1 upregulates the expression of LsPID. qRT-PCR showed that the expression of LsPID in LsKN1 genotypes with palmately lobed leaves was significantly higher than that in LsKN1 genotypes with pinnately lobed leaves from the F3 family, consistent with the upregulation of LsPID by LsKN1 (Figure 6h).

We further knocked out the LsPID gene in the PALM line to confirm its function on lobe complexity. We constructed a recombinant CRISPR/Cas9 vector with sgRNAs specific to the coding region of the LsPID gene and transformed it into the PALM line. Two knockout lines were obtained, and both of them changed from palmately lobed leaves to pinnately lobed leaves (Figure 6i). Our knockout results further verified that the LsPID gene contributes to palmately lobed leaves in lettuce.

LsKN1\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{V}}}}}} suppresses GA biosynthesis to promote palmately lobed leaves

GA was shown to promote the elongation of plant leaves and inhibit leaf shape complexity (Hay et al., 2002; Smith et al., 1996). To investigate whether GA affects the complexity of leaf lobes in lettuce, we treated the leaves of PALM with GA. The results showed that the leaves changed to pinnately lobed with the increase of GA concentration in treatments (Figure 6j).

Figure 5 LsKN1 upregulates the expression of the LsCUC2 gene through LsHB. (a) qRT-PCR analysis of LsCUC3 and LsCUC2b in PINN and PALM. Data represent mean ± SD (n = 3). ** denotes significance level of P < 0.01. The LsCUC genes are mainly expressed in the sinuses of lobes in PINN and PALM. (b) Chip-seq analysis of LsKN1 binding activity in the promoter region of LsHB. The y-axis represents the number of reads in the Chip-seq. The x-axis shows the position of the reads in the gene. (c) Y1H assay for LsKN1 and the promoter sequences of LsHB. Transformants were grown on the SD-Leu medium with 300 ng/mL AbA. See Figure 4b for details. The region of promoter is shown in (b). (d) Dual-luciferase assay. The left panel shows the LUC activity when the LUC gene driven by the promoter of LsHB was co-expressed with an empty vector (EV) or LsKN1\textsuperscript{\textsuperscript{\textsuperscript{\textsuperscript{V}}}}. The right panel shows the LUC activity when the LUC gene driven by the promoter of LsCUC2b was co-expressed with an empty vector (EV) or LsHB. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05. (e) qRT-PCR analysis of the expression of the LsHB gene in LsKN1 and LsKN1\textsuperscript{\textsuperscript{\textsuperscript{V}}} genotypes in the F3 family. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05. (f) Overexpressing LsCUC2b increased the complexity of leaf lobes (left). qRT-PCR analysis of the LsCUC2b gene in overexpression line (right). Bar = 2 cm. RNA was extracted from the sinuses of lobes. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05.

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The ChIP-seq results suggested that LsKN1 bound to the promoter regions of *LsGA3ox1* and *LsGA20ox1*, two critical genes in the GA biosynthesis pathway (Figure 6k). However, the LUC assay could not confirm the regulatory role of LsKN1 on the expression of *LsGA20ox1* (Figure 5c). We hypothesized that the low expression of the *LsGA3ox1* gene triggered by LsKN1 contributed to the development of palmately lobed leaves. Y1H assay indicated that LsKN1 bound to the promoter sequence of *LsGA3ox1* (Figure 6m). LUC assay showed that the proteins LsKN1 and LsGA3ox1 co-expressed significantly decreased the LUC activity, indicating a negative regulatory role of LsKN1 on the expression of *LsGA3ox1*. These results suggest that LsKN1 plays a dual role in the development of palmately lobed leaves by promoting auxin biosynthesis and suppressing GA biosynthesis.
LsKN1\(^{\triangledown}\) had the same binding ability to the promoter of LsGA3ox1 (Figure S4d). The LUC results indicated that LsKN1 suppresses the expression of LsGA3ox1. qRT-PCR showed that the expression of LsGA3ox1 in LsKN1\(^{\triangledown}\) genotypes with pinnately lobed leaves was significantly lower than that in LsKN1 genotypes with pinnately lobed leaves from the F\(_2\) family (Figure 6n), consistent with above conclusion that LsKN1 suppresses the expression of LsGA3ox1. We knocked out the LsGA3ox1 gene in a genotype with pinnately lobed leaves. The Lsga3ox1 knockout mutant changed to pinnately lobed leaves (Figure 6o). Our knockout results further verified that the downregulation of LsGA3ox1 by LsKN1\(^{\triangledown}\) contributes to pinnately lobed leaves in lettuce.

The effects of LsKN1 on lobe complexity depend on LsAS1 and Lettuce Lobed Leaf (LLL) genes

Our previous study showed that LsKN1 promotes leafy heads through dorsiventrality (Yu et al., 2020). LsAS1 was the direct target of LsKN1, and it was downregulated in pinnately lobed leaves compared to pinnately lobed leaves. To investigate whether LsAS1 was required to develop palmately lobed leaves, we overexpressed LsAS1 in cultivar PI595096, which had the LsKN1\(^{\triangledown}\) genotype and pinnately lobed leaves. The overexpression line of LsAS1 changed from pinnately lobed leaves to pinnately lobed leaves, which was similar to the pinnately lobed leaves in LsKN1 knockout mutants (Figure 7a).

It was shown recently that one single locus controls the polymorphism of lobed and non-lobed leaves in Lactuca, but the causal gene (Lettuce Lobed Leaves, LLL) has not been identified (Wei et al., 2021). We crossed the F2-118 of palmately lobed leaves with a cultivar of non-lobed leaves. In the F2 population, we observed segregation of LLL and III genotypes (Table S5). We compared the phenotypes of PLL1/PLL2/PLL3 under the genetic background of LLL and III. Interestingly, all III homozygotes had non-lobed leaves, while all LLL homozygotes and LLL/III heterozygotes had palmately lobed leaves. Therefore, the LLL gene was recessive epistatic to LsKN1 in regulation of palmately lobed leaves in lettuce (Figure 7b).

Discussion

The pleiotropism of LsKN1\(^{\triangledown}\) depends on genetic background

In our previous study, we crossed a romaine lettuce and a crisphead lettuce to construct a segregating population to investigate the genetics underlying leafy heads. We showed that the insertion of a CACTA-like transposon upregulated the expression of the LsKN1\(^{\triangledown}\) gene to contribute to the development of leafy heads in lettuce (Yu et al., 2020). In the current study, we crossed a lettuce cultivar of palmately lobed leaves with a wild lettuce of pinnately lobed leaves to investigate the genetics underlying palmately lobed leaves. Surprisingly, the LsKN1\(^{\triangledown}\) gene is also responsible for the polymorphism of pinnately and palmately lobed leaves in lettuce, and the upregulated LsKN1\(^{\triangledown}\) allele controls palmately lobed leaves. The LsKN1\(^{\triangledown}\) allele, when introduced into stem lettuce, did not cause any noticeable phenotypic changes (Yu et al., 2020). Therefore, the LsKN1\(^{\triangledown}\) allele showed pleiotropic effects on the development of lettuce leaves, but the phenotype depended on genetic background. We predict that the multiple phenotypes derived from LsKN1\(^{\triangledown}\), such as leafy heads and palmately lobed leaves, may occur in one plant if it has the corresponding genetic background, such as the LLL allele and other heading QTLs, respectively.

We showed that the LLL gene controlling lobed leaves was recessive epistatic to the LsKN1\(^{\triangledown}\) gene on palmately lobed leaves. Unfortunately, the LLL gene has not been cloned yet. With the identification of the LLL gene in the future, it will be interesting to investigate the molecular mechanism underlying the epistatic effects of LLL to LsKN1\(^{\triangledown}\).

LsKN1 controls palmately lobed leaves through multiple pathways

This study showed that LsKN1 controlled palmately lobed leaves through multiple pathways (Figure 7c). First, the auxin pathway plays important roles in the regulation of palmately lobed leaves triggered by LsKN1. ChIP-seq results showed that the target genes of LsKN1 included LsYUC4 (LG197724), LsYUC2 (LG8849068), LsYUC8 (LG8736671), LsAA3 (LG8735159), and LsYUC8 (LG8735159). The pleiotropism of LsKN1\(^{\triangledown}\) depends on genetic background.}

![Figure 7](image-url)

Figure 7 Effects of LsKN1\(^{\triangledown}\) on palmately lobed leaves depend on LsAS1 and LLL. (a) Overexpression of LsAS1 in LsKN1\(^{\triangledown}\) genotype changes palmately lobed leaves to pinnately lobed leaves. (b) The Lettuce Lobed Leaves (LLL) gene is recessive epistatic to LsKN1 in regulating palmately lobed leaves in lettuce. Bars = 2 cm. (c) The network of LsKN1 regulating palmately lobed leaves in lettuce.
LsAA9 (LG3303130), which are from the auxin biosynthesis and signaling pathways. The pinnately lobed leaves of individuals with the LsKN1 allele had significantly higher auxin concentration than the pinnae lobed leaves of individuals with the LsKN1 allele. In Arabidopsis, the overexpression of the YUC genes led to the overproduction of auxin. PIN1 and YUC genes synergistically control leaf development. The yuc1 pin1-S double mutants have pin-like leaves (Cheng et al., 2006; Wang et al., 2011). The mutation of a YUC homolog in Medicago suppresses lateral leaflet development (Zhao et al., 2020). The expression of YUCs in the leaves mediates the development of leaf margins and subsequently promotes blade outgrowth.

In contrast to auxin, GA reduces leaf complexity. The ChiP-seq of LsKN1 suggested that LsKN1 bound to the promoter regions of LSGA3ox1 and LSGA20ox1. Both LSGA3ox1 and LSGA20ox1 were differentially expressed between pinnately lobed leaves and pinnately lobed leaves. Knockout of the LSGA3ox1 gene in an individual with pinnately lobed leaves led to pinnately lobed leaves. GA treatment of the Mouse-ear mutant inhibited the super-compound leaf phenotype of tomato (Hay et al., 2002). In Arabidopsis, KNOXI directly inhibits the activities of GA biosynthesis genes GA3ox1 and GA20ox1 to reduce the concentration of GA in SAM (Chen et al., 2004; Sakamoto et al., 2001).

The ARP (AS1/RS2/PHAN) gene family plays essential roles in maintaining stem cells and the initiation of lateral organs (Li et al., 2005; Timmermans et al., 1999; Tsiantis et al., 1999; Waite et al., 1998). Mutation of the ARP gene family affects not only cell properties but also leaf pattern formation. The AS1 gene is expressed in leaf primordia, bract primordia, and flower organ primordia (Byrne et al., 2000; Tsiantis et al., 1999). In Arabidopsis, the down-regulation of STM leads to the expression of the AS1 gene at the leaf initiation site, while AS1 interacts with AS2 to inhibit the expression of other class I KNOX genes in the leaf primordium, resulting in leaf differentiation (Guo et al., 2008; Ori et al., 2000). Furthermore, AS1 and AS2 may form a complex to regulate the establishment of leaf polarity (Sun et al., 2002; XU et al., 2003). The rosette leaves of the as1 mutant are smaller and round, leaf margins are lobed, and leaf edges curl downward, resulting in asymmetric leaves (Byrne et al., 2000; Ori et al., 2000).

Our previous study has shown that LsKN1 promotes leafy heads by downregulating LsAS1 (Yu et al., 2020). The current study revealed the involvement of the LsKN1–LsAS1 module in the development of pinnately lobed leaves. Therefore, the LsKN1 gene may regulate different traits (phenotypes) through similar pathways, and the ultimate phenotypes caused by the LsKN1 gene are genetic-background dependent.

LsKN1 controls LsCUC homologs through different mechanisms

NAM/CUC is a vital transcription factor family regulating leaf lobes (Sourer et al., 1996; Weir et al., 2004). CUC2 gene and the other two homologs CUC1 and CUC3 are necessary for organ boundary specification (Aida et al., 1997; Takada et al., 2001). It is believed that the leaf margin development of Arabidopsis is controlled through two steps. The pattern of serration is determined first, independent of CUC2 and miR164. The balance between CUC2 and miR164 then determines the degree of serration (Nikovics et al., 2006). During the development of leaf margin, CUC2 promotes the establishment of the PIN1 convergence point, which produces the maximum auxin at the serrated end of leaf margin (Bilsborough et al., 2011; Cooke et al., 2002). Auxin maximally inhibits CUC2 at the tip of the sawtooth and promotes tooth growth. While CUC2 is required early in serration formation, CUC3 acts later to maintain serration (Hasson et al., 2011).

The LsKN1 gene had high expression of LsCUC2b and LsCUC3, both promoting leaf complexity. LsKN1 bound to the promoter region of the LsCUC3 gene to upregulate its expression. In comparison, LsKN1 upregulated the expression of LsHB, which in turn upregulated the expression of LsCUC2b. In Arabidopsis, ATHB indirectly promotes the expression of CUC2 by inhibiting miRNA164, and ATHB also directly activates the expression of CUC2 to a certain extent (Miguel et al., 2020). Our study showed that LsHB in lettuce directly and significantly upregulated LsCUC2b.

In both LsKN1 and LsKN1 genotypes, the LsCUC homologs were only expressed in the sinuses of leaf lobes. The CUC homologs in Arabidopsis are also expressed in the sinuses, where growth and proliferation are reduced (Bilsborough et al., 2011; Kierzkowski et al., 2019; Nikovics et al., 2006). Interestingly, the high expression of LsCUC homologs in overexpression lines was also limited to the sinuses of leaf lobes but not in lobe tips. The uneven spatial distribution of LsCUC genes in leaves is most likely caused by miRNA164, which is present in lobe tips to silence the CUC genes (Hasson et al., 2011; Nikovics et al., 2006).

Materials and methods

Construction of F2 segregating population

An inbred line F2118 with palmately lobed leaves was derived from the progeny of a looseleaf cultivar PI595096 and a crisphead cultivar PI64570A. The inbred line was crossed with L. serriola accession CN04971, which has pinnately lobed leaves. PI595096 and PI64570A were ordered from the USDA GRIN (http://www.ars-grin.gov), while CN04971 was ordered from CGN, Netherland (https://www.wur.nl/). The F2 hybrids were selfed to generate a segregating F2 population. The lettuce plants were grown on the campus of Huazhong Agricultural University in Wuhan, China.

Genetic analysis of the loci controlling palmately lobed leaves

BSR genetic analysis followed the method described previously (Su et al., 2020). From the segregating population, 25 individuals with the most complex palmately (or pinnately) lobed leaves were chosen to construct a “palmately (or pinnately) lobe pool”. Equal amount of leaf tissues from the chosen individuals were mixed for each pool, and RNA was extracted using TRIzol reagent (Invitrogen). RNA was quantified and assessed using a Qubit Fluorimeter and a Nanodrop spectrophotometer (Novogene, Beijing, China). The non-directional paired end RNA-Seq libraries were constructed using the Illumina TruSeq RNA sample preparation kit, version 2, and sequenced using the Illumina HiSeq 2500 platform to obtain 125-bp paired-end reads. The raw data was filtered to remove low-quality reads. Clean RNA-seq data were mapped to the lettuce genome assembly v8 (Reyes-Chin-Wo et al., 2017), and SNPs were called. Allele (nucleotide) frequency for each SNP was calculated. The difference of allele frequencies between the two pools, Δ(SNP-index), was plotted along the nine chromosomes of lettuce. A region with a high Δ value was considered to potentially harbor a gene controlling the complexity of lobed leaves.

ANOVA model was used to quantify the effects of the candidate loci on leaf lobes using ANOVA function in R package ‘car’ (Fox et al., 2012). We used the number of lobes per leaf to
represent leaf complexity. Leaf complexity for 237 individuals from the F2 population was determined using the number of lobes in the 8th leaf of the 45-days-old seedling. One CAPS marker was designed for each candidate locus and was genotyped for all individuals (Table S5). ANOVA was used to assess the association between candidate locus and leaf complexity, and then to calculate the variations of leaf complexity explained by the candidate loci.

**Quantitative reverse transcription PCR (qRT-PCR)**

Total RNA was extracted from the young leaves of 2.5-month-old plants using TriZol reagent (Invitrogen, Carlsbad, CA, USA), and treated with DNase I (ThermoFisher Scientific, https://www.thermofisher.com) to remove contaminated genomic DNA. First strand cDNAs were synthesized using a HiScript® II Q RT SuperMix for qPCR Reagent Kit (Vazyme Biotech, China). After confirming no genomic DNA contamination, qPCR was performed using the AceQ® qPCR SYBR Green Master Mix (Vazyme #Q111) in the LightCycler 480 System (Roche, Basel, Switzerland). qPCR was carried out using the 384-well-plate-based real-time PCR platform (Roche, Basel, Switzerland). Actin was used as a reference gene. Primers were designed using PREMIER 5.0 (Table S5).

Data represent mean ± SD (n = 3). The statistical significances for gene expression difference were determined using Student’s t test.

**Transformation and complementation tests**

For complementation test, the full-length of the LsKN1 gene was PCR amplified from lettuce with palmately lobed leaves (F2-118) and purified using gel extraction kit (Omega bio-tek, Norcross, USA), and then inserted into HindIII-linearized binary vector pRI011-GFP using ClonExpress II One Step Cloning Kit (Vazyme, China). The resulting construct was transformed into Agrobacterium GV3101.

To overexpress the LsKN1 gene, its full-length cdNA sequence was inserted into the pHei1sGate8 (Invitrogen, USA), pRI101-GFP vector driven by the CaMV 35S promoter. The construct was transformed into Agrobacterium strain GV3101 using the freeze-thaw method. Transgenic plants were generated cotyledon explants, using heat shock method. The plasmid was homologous recombined with the linearized vector, then transformed into E. coli using heat shock method. The plasmid was amplified, and transformed into Agrobacterium using freeze-thaw method.

**Dual-luciferase assay**

The dual-luciferase reporter assay was performed as described previously (Hellens et al., 2005). The coding region of LsKN1 was PCR amplified using the primers listed in Table S5. The PCR products were digested with SpeI and EcoRI and cloned into a pRTBD vector driven by a 35S constitutive promoter to generate an effector plasmid. The primers listed in Table S5 was used to PCR amplify the natural LsCUC3, LsHB, LsPID, LsCUC2b promoter sequences, which was digested using HindIII and BamHI, and inserted into a pGreen-LUC vector to drive the firefly luciferase reporter gene as reporter plasmids. The plasmid containing the Renilla luciferase gene, driven by the 35S promoter, was used as the control plasmid. The effector, reporter, and internal control plasmids were mixed at a ratio of 4:1: The mixtures were introduced into leaves of three-week-old Nicotiana tabacum using Agrobacterium infiltration. The activities of firefly and Renilla luciferase were quantified 2.5 d after infiltration with a Dual Luciferase Assay kit (Promega), and luminescence was recorded using a GloMax 96 Microplate Lumimeter (Promega). The firefly luciferase activity (Luc) was normalized to the Renilla luciferase activity (Ren). The assay was performed with three biological replicates, and the error bars represent the standard errors of the means from three independent experiments.

**Electrophoretic mobility shift assay (EMSA)**

The EMSA assay was performed using the chemiluminescent EMSA kit according to a previously described method (Thermo, No 20148). The coding sequences of LsKN1 were digested using EcoRI and cloned into a E. coli pMAL-c2x (NEB) vector to generate the expression vector. MBP-LsKN1 was purified by affinity purification with maltose. The expression of the MBP-LsKN1 was expressed by induction with 1 mM IPTG at 16 °C for 8–10 h in an orbital shaker. The bacteria were harvested by centrifugation and washed with prechilled PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4) and then resuspended in PBS. The LsKN1 protein was purified from the crude extract using affinity chromatography with an amylose resin (NEB). To prepare the probe for EMSAs, fragments of LsCUC3 promoter were PCR amplified and their 3’ ends were labeled with biotin according to the manufacturer’s recommendation (Thermo, No89818). The competitor was an unlabeled version of the same LsCUC3 promoter fragment. The cold competitor was used to test whether LsKN1 specifically binds this probe. For the binding reaction, the LsKN1 protein, probes (20 fmol each), and competitor DNA (896 fmol) were incubated in the binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% Glycerol, 5 mM MgCl2, 0.05% NP-40) with the presence of 3 μg/μl poly (dI-dC) at room temperature for 45 min. The complex was separated using 6% native SDS-PAGE in 0.5X TBE buffer. The signal was detected using horseradish peroxidase (HRP) conjugated to streptavidin and an ECL substrate (Thermo, No 89880).

**Yeast-one-hybrid assay**

Yeast-one-hybrid assay was performed according to the manufacturer’s instruction (Clontech). The coding region of LsKN1 was digested with EcoRI and BamHI and then cloned into a pGADT7 vector to generate a prey vector. After confirmation by DNA sequencing, the E. coli was incubated at 37 °C in dark for 24 h on LB media. Plasmid was extracted using Plasmid Mini Kit II D6945 (Omega). The natural LsCUC3, LsHB, LsPID promoter sequences were amplified using primers listed in Table S5, digested with Xhol and SacI, and inserted into the bait vector (pAbAi). The bait vectors were confirmed through DNA sequencing. The E. coli was incubated at 37 °C in dark for 24 h on LB media. Plasmid was extracted using Plasmid Mini Kit II D6945 (Omega). The recombinant constructs were linearized using BstBI.
and transformed into the Y1HGold yeast strain. After being cultured at 28 °C in dark for three days on Ura-dropout medium, the strains were screened using colony PCR. The second transformation recombinant constructs were the plasmid of prey vector and the bait-reporter yeast strain. After confirmation by DNA sequencing, the plates were incubated for 3 days at 28 °C in dark on the Leu-dropout plates. Matings were performed with the wild-type strain as a negative control. A positive control, and transformants of p53-AbAi and pGADT7-p53 as a positive control, and transformants of p53-AbAi and pGADT7 as a negative control.

Accession numbers
All data supporting the results of this study can be found within and in the supplementary table files. The raw data of ChiP-seq, RNA-seq of BSR pools, and RNA-seq of LsKN1 knockout mutants and its recipients have been deposited in the National Center for Biotechnology Information (NCBI) under the BioProject ID PRJNA576072, PRJNA797355, and PRJNA797273, respectively. The sequences of LsKN1, LsCUC3, LsHB, LsCUC2b, LsPID, LsGA3ox1 identified in this study are available in the NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession number LOC111890976, LOC111880143, LOC111865154, LOC111921537, LOC111880697, LOC11190 9350.

Author contributions
M.W. performed the experiments; C.Y. helped on vector construction; D.L. and R.W.M. helped in genome sequences. W.Z. and X.W. did bioinformatic analysis. H.K. conceived and designed the experiment; M.W. wrote the manuscript with the help from H.K., J.C., X.W., R.W.M., and D.L.

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Competing interests
The authors declare no competing interests.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Alignment of LsKN1 and LsKN1▽ amino acid sequences.

Figure S2 Venn diagram for DEGs for LsKN1. The blue circle is DEGs between LsKN1▽ and LsKN1 plants that have non-lobed leaves. The red circle is DEGs between LsKN1▽ and its knockout mutant, both with lobed leaves. The green circle represents potential targets of LsKN1 according to ChIP-seq results.

Figure S3 Neighbor-joining (NJ) phylogenetic tree for CUC homologs from lettuce and Arabidopsis. Amino acid sequences were used. Bar represents changes per site.

Figure S4 Dual-luciferase assay. (a) The left panel shows the diagram of reporter and effector vectors used in the dual luciferase assay. The LUC activity is similar when the LUC gene driven by the promoter of LsCUC3 was co-expressed with LsKN1▽ or LsKN1. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05. (b) The left panel shows the diagram of reporter and effector vectors used in the dual luciferase assay. The right panel shows that LsKN1▽ has no effects on the expression of LsCUC2b. Data represent mean ± SD (n = 3). P = 0.56 > 0.05. (c) The left panel shows the diagram of reporter and effector vectors used in the dual luciferase assay. The right panel shows that LsKN1▽ has no effects on the expression of LsGA20ox1. Data represent mean ± SD (n = 3). P = 0.33 > 0.05. (d) The left panel shows the diagram of reporter and effector vectors used in the dual luciferase assay. LsKN1 and LsKN1▽ have similar effects on the expression of LsGA3ox1. Data represent mean ± SD (n = 3). * denotes significance level of P < 0.05.

Table S1 Annotation of genes in the candidate region.

Table S2 389 DEGs between the LsKN1▽ knockout mutant with pinnately lobed leaves and its recipient with palmately lobed leaves.

Table S3 Candidate targets of LsKN1▽ obtained by ChIP-seq analysis.

Table S4 581 DEGs between LsKN1▽ mutant and its wild type.

Table S5 Primers used in this study.