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Upregulation of a *KN1* homolog by transposon insertion promotes leafy head development in lettuce

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Leafy head is a unique type of plant architecture found in some vegetable crops, with leaves bending inward to form a compact head. The genetic and molecular mechanisms underlying leafy head in vegetables remain poorly understood. We genetically fine-mapped and cloned a major quantitative trait locus controlling heading in lettuce. The candidate gene (*LsKN1*) is a homolog of *knotted 1* (*KN1*) from *Zea mays*. Complementation and CRISPR/Cas9 knockout experiments confirmed the role of *LsKN1* in heading. In heading lettuce, there is a CACTA-like transposon inserted into the first exon of *LsKN1* (*LsKN1*▽). The transposon sequences act as a promoter rather than an enhancer and drive high expression of *LsKN1*▽. The enhanced expression of *LsKN1*▽ is necessary but not sufficient for heading in lettuce. Data from ChIP-sequencing, electrophoretic mobility shift assays, and dual luciferase assays indicate that the LsKN1▽ protein binds the promoter of *LsAS1* and down-regulates its expression to alter leaf dorsoventrality. This study provides insight into plant leaf development and will be useful for studies on heading in other vegetable crops.

leafy head | transposon | leaf development | dorsoventrality

Lettuce (*Lactuca sativa*), a member of the Asteraceae family, is one of the most important green vegetables worldwide. It was domesticated from wild lettuce (*Lactuca serriola*) through a single domestication event (1). Its stems (stem lettuce) or leaves (leafy lettuce) may be harvested and consumed. Leafy lettuce can be further classified into several different horticultural types based on their architecture, such as crisphead (iceberg), butterhead, romaine, and loose leaf. Heading (forming a spherical tight leafy head) is a unique trait for the crisphead type. The spherical leafy head develops during three stages: seedling, rosette, and heading (2). The rosette leaves in crisphead lettuce are usually flat and expanded and function as photosynthetic organs. After the plant produces about 10 to 12 leaves, the emerging new leaves fold inward to form a leafy head.

In addition to lettuce, several other vegetables have leafy heads, such as endive (*Chicorium endivia*), cabbage (*Brassica oleracea* var. *capitata*), Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), and mustard (*Brassica juncea*). Heading in leafy vegetable crops improves leaf texture, facilitates harvesting, and increases shelf life. Although heading is an important horticultural trait, little is known about the genetic and molecular mechanisms responsible for heading. Analysis of recombinant inbred lines from cabbage yielded 18 quantitative trait loci (QTLs) associated with heading (3). microRNAs may also regulate the heading phenotype. The overexpression of *miR319a* down-regulated its target gene (*BrpTCP4-1*) and led to the altered development of the leafy head in Chinese cabbage (4). A comparison of the *B. rapa* and *B. oleracea* genomes provides evidence that genes controlling dorsoventrality were subjected to strong selection bias in the heading cultivars of these two vegetable crops (5).

Leaf size and shape are determined by cell division and expansion along adaxial–abaxial, proximal–distal, and medial–lateral axes. Heading in vegetable crops is considered to be a developmental disorder of leaf dorsoventrality (i.e., incongruous cell division/expansion along the adaxial–abaxial axis). Genes involved in leaf dorsoventrality include *AS1*, *AS2*, *miR390*, *TAS3*, *KAN*, *YABBY*, *ARF3*, *ARF4*, *PHB*, and *REV* (6–12). Genes associated with dorsoventrality are directly or indirectly regulated by key developmental regulators of leaves, such as members of the KNOXI (knotted-1 like homeobox gene) family (13–16). The KNOXI family is a group of transcription factors with a homeobox domain that plays a critical role in establishing and maintaining the shoot apical meristem and organ separation (16–19). Four members of KNOXI are present in the genome of *Arabidopsis thaliana*, including *STM* (*SHOOTMERISTEMLESS*), *KNAT1* (also named *BREVIPEDICELLUS*), *KNAT2*, and *KNAT6*. KNOXI proteins are required in the leaf to delay cellular differentiation and to induce the initiation of leaflets on the petiole (20–22). Transgenic overexpression of the *KNOX1* gene in *Arabidopsis* resulted in abnormal leaf morphology characterized by severely lobed leaves (23, 24). Similarly, elevated expression of the *LeT6* gene, a *KNOXI* homolog in tomato, led to highly ramified leaves (25). Immunohistochemistry and in situ hybridization experiments indicated that the differences in the leaf forms of *Cardamine hirsuta* and *Arabidopsis thaliana* are associated with the altered expression of *KNOXI* genes. Moreover, KNOXI proteins accumulated in the complex leaves of *C. hirsuta* but not in the simple leaves of *Arabidopsis* (20).

Leaf heading is a composite trait that includes formation of a head, head shape, head size, and head compactness, etc. In this study, we focused mainly on the genetic and molecular mechanisms of forming a leafy head in lettuce. A crisphead (heading)

Significance

Heading, which facilitates harvest and contributes to taste and shelf life, is an important trait in some vegetable crops. However, litter is known about its genetic and molecular mechanism. Here, we report the genetic cloning of a key regulator (*LsKN1*) of heading in lettuce, which is homologous to the *knotted 1* (*KN1*) gene in maize. The *LsKN1* gene is upregulated by the insertion of a CACTA-like transposon. Its enhanced expression suppressed the *LsAS1* gene and consequently altered leaf polarity and led to a heading phenotype.

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The authors declare no competing interest.

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cultivar was crossed with a romaine (nonheading) cultivar. In the F_2 population, the heading phenotype was analyzed using Bulked Segregant RNA-seq (BSR-seq). Based on the genetic results, we constructed an $F_{2:3}$ family in which the phenotypic segregation of heading and nonheading was controlled by a single major QTL. This major QTL was then cloned using map-based cloning procedures. The causal mutation was identified, and its function was verified using a complementation test and by knocking out the candidate gene using CRISPR/Cas9 technology. The evolution and impact of the causal mutation were studied in detail. This study provides insight into our understanding of the genetics underlying heading and sheds light on leaf development. Our results will facilitate studies on the genetic and molecular mechanisms of heading in other important leafy vegetable crops, such as cabbage.

Results

Structural Basis of Leafy Heads in Lettuce. To investigate the structural basis of leaf heading, we compared the leaf shapes from a heading cultivar (PI536839, a crisphead type) and a nonheading cultivar (PI344074, a romaine type) of lettuce (Fig. 1*A*). The leaves of the crisphead parent are much wider than those of the romaine parent and have serrated margins with an altered pattern of veins (Fig. 1 *B* and *C*). The most striking structure in crisphead is the main vein on the leaves, which bends adaxially to form a leafy head (Fig. 1*B*). To dissect the structural difference, we prepared paraffin sections using the heading and nonheading leaves of lettuce at the four-leaf stage. In the nonheading lettuce, cells in the adaxial domain were deeply stained by Toluidine blue and were compactly arranged, exhibiting a well-established adaxial–abaxial polarity (Fig. 1*D*, *Left*). In contrast, cells in the adaxial domain of heading lettuce were loosely arranged, and the adaxial and abaxial domains do not show obvious difference (Fig. 1*D*, *Right*).

Map-Based Cloning of a QTL Controlling Leafy Head in Lettuce. To understand the genetics underlying the heading phenotype in lettuce, we constructed an F_2 segregating population by crossing the heading lettuce (PI536839) with the nonheading lettuce (PI344074). Individuals from the F_2 population showed continuous distribution of phenotypes ranging from tight heading to nonheading, which provides evidence that heading is a quantitative trait (Fig. 1*E*). We used BSR-seq to identify genetic loci controlling heading in this population. The heading pool was prepared by mixing equal amounts of tissue from 20 individuals with tight heads while the nonheading pool was prepared by mixing equal amounts of tissue from 20 nonheading individuals. RNAs were extracted from the two pools and sequenced. Analysis of the data revealed prominent peaks on chromosomes 4 and 7, suggesting two major loci for heading in lettuce (Fig. 1*F*). We consequently named the loci on chromosomes 4 and 7 *Lettuce Heading Locus* (*LHL1*) and *LHL2*, respectively. The ordinal logistic regression model suggests a 8.4 times increase in odds of forming a head when an individual has the heading allele at the *LHL1* locus, and the ANOVA model suggests that this allele explains 13.0% of the variance in heading phenotype (Phenotypic Variation Explained [PVE]). On the other hand, the ordinal logistic regression model suggests a 7.3 times increase in odds of forming a head when an individual has the heading allele at the *LHL2* locus, and the ANOVA model suggests that this allele explains 14.5% of the variance in heading phenotype. ANOVA showed no interactions between the two loci ($P = 0.08$). The PVE by the *LHL2* locus was estimated to be 22.3% using QTL IciMapping (26).

In this study, we focused on the *LHL2* gene. First, we selected some F_2 individuals that were heterozygous at the *LHL2* locus but homozygous at the *LHL1* locus. We self-pollinated these individuals, which yielded $F_{2:3}$ families. Their heading phenotypes were investigated. Among the 24 $F_{2:3}$ families that were studied, a family that we named JQ1 was chosen to fine-map the *LHL2* gene because the heading and nonheading phenotypes were segregating and readily distinguishable in this family. To minimize the effects of errors caused by environmental effects and phenotyping errors, we chose 577 plants with the strongest heading phenotypes from a population of ∼5,000 individuals for further analysis. The *LHL2* gene was ultimately mapped to a region between 18.54 and 18.99 Mb on chromosome 7 (*SI Appendix*, Fig. S1). This region contains 11 predicted genes, including a gene encoding a homolog of the KN1 protein from maize and the STM protein from *Arabidopsis* (27, 28) (Dataset S1). Like the KN1 protein from maize, the predicted KN1 protein in lettuce (LsKN1) has conserved KNOX and homeobox domains. Moreover, their protein sequences have a similarity of 66.9% (*SI Appendix*, Fig. S2). The two alleles of the *LsKN1* gene were PCR-amplified and sequenced. The major difference between the *LsKN1* alleles from the nonheading parent (wild type, *LsKN1*) and the heading parent (*LsKN1*▽) is a 3,935-bp insertion in the first exon of *LsKN1*▽ (Fig. 1*G* and *SI Appendix*, Fig. S3). A BLASTN analysis provided evidence that the insertion is a typical CACTA-like transposon, which has 3-bp target site duplication and terminal inverted repeat sequences (CACTACTAGAAAAA/TTTTTCTAGTAGTG). *LsKN1* is the only gene in the candidate region known to be associated with leaf development.

***LsKN1* Plays An Essential Role in Heading in Lettuce.** To test whether the *LsKN1* gene contributes to heading, we conducted a complementation test. A nonheading individual from the $F_{2:3}$ population, which is homozygous for the nonheading allele at the *LHL2* locus and homozygous for the heading allele at the *LHL1* locus, was transformed with the *LsKN1*▽ allele. For convenience, this line is hereafter referred to as Non-Heading Genotype (NHG). The NHG was transformed with a 10,220-bp sequence from the heading parent that contained the promoter region, the CACTA-like transposon, introns, exons, and the terminator of the *LsKN1*▽ allele. We obtained three positive transformants. Each of these transgenic plants displayed a heading phenotype (Fig. 2*A*). The heading and nonheading phenotypes segregated in the progeny of these transformants and cosegregated with the insert, confirming that *LsKN1*▽ is the *LHL2* gene controlling leaf heading in lettuce (Fig. 2*B*).

To knock out the heading gene, we constructed a recombinant CRISPR/Cas9 vector with two single-guide RNAs (sgRNAs) specific to the coding region of the *LsKN1*▽ gene. The heading parent was transformed with this vector. Six transformants were obtained. The sequences of PCR products derived from these transformants showed that deletions occurred in the vicinity of the sgRNA target sites (Fig. 2*C*). As expected, homozygous knocking out of the *LsKN1*▽ gene in the crisphead parent changed the phenotype from heading to nonheading, further validating its essential role in the formation of leafy heads in lettuce (Fig. 2*A*).

Reshuffling of the *LsKN1* Gene by a CACTA-like Transposon. Based on our finding that knocking out the *LsKN1*▽ gene in a heading parent led to a nonheading phenotype, we concluded that heading is caused by a gain-of-function mutation. Strikingly, the *LsKN1*▽ gene in the heading parent had a 3,935-bp insertion of a CACTA-like transposon in its first exon (99 bp downstream of the translational start codon). This insertion appears to not have knocked out *LsKN1* but instead up-regulated its expression. To understand the mechanism underlying this phenomenon, we analyzed the transcripts of the *LsKN1*/*LsKN1*▽ alleles in the nonheading and heading parents. The RT-PCR results showed that the region downstream of the transposon insertion site was transcribed in both the nonheading and the heading parents (Fig. 3 *A* and *B*). However, the region upstream of the insertion site was transcribed only in *LsKN1* and not in *LsKN1*▽ (Fig. 3 *A*

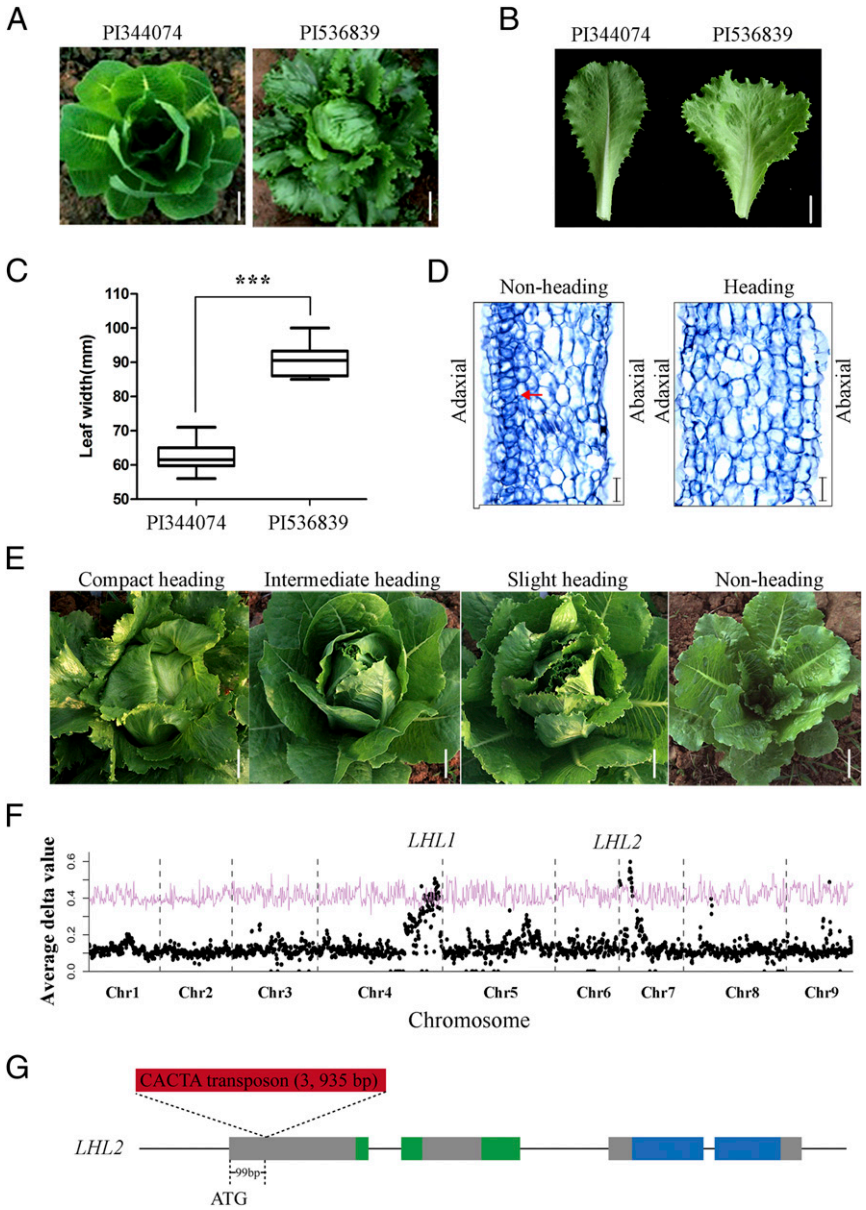

Fig. 1. Map-based cloning of a gene controlling heading in lettuce. (*A*) Parental lines. A romaine-type lettuce (nonheading, *Left*) and a crisphead-type lettuce (heading, *Right*) were chosen as parents to construct a segregating population. (Scale bar, 5 cm.) (*B*) Leaf shape of the nonheading and heading parents. (Scale bar, 2 cm.) (*C*) Leaf width of the nonheading and heading parents at the 10-leaf stage. Box plots display median (line), interquartile range (box), and whiskers (extending 1.5 times the interquartile range). $n = 30$. The statistical significances were determined using Student's *t* test. $^{***}P < 0.001$. (*D*) Longitudinal section of the nonheading and heading leaves. The red arrow indicates cells compactly arranged in the adaxial domain present in nonheading leaves but absent in heading leaves. (Scale bar, 25 μm.) (*E*) Heading phenotypes of the F_2 population. The heading phenotypes ranged from a compact head to no head. (Scale bar, 5 cm.) (*F*) BSR-seq analysis of heading. The *x* axis represents the nine chromosomes of lettuce. The *y* axis indicates the differences in allele frequencies between the two extreme pools. The confidence interval was obtained by simulating each SNP with a sequencing depth of 1,000 times assuming equal frequencies of the two nucleotides at each SNP in the two pools ($P < 0.05$). (*G*) Schematic representation of the candidate gene at the *LHL2* locus. The boxes represent exons and lines linking boxes are introns. Green region encodes a KNOX domain; blue region encodes a homeobox domain.

and *B*). This finding indicated that transcription of the *LsKN1*▽ allele was altered by the transposon insertion in its first exon. To identify the transcriptional start site of the *LsKN1*/*LsKN1*▽ alleles, we performed 5' rapid amplification of cDNA ends (5′-RACE) for each parent. In the nonheading parent, *LsKN1* produces two transcripts differing only in the size of the 5′ untranslated region (5′ UTR): 163 and 37 bases (Fig. 3 *A* and *C*). In the heading parent, two *LsKN1*▽ transcripts were also obtained, both missing the 5′ UTR of *LsKN1*; one transcript starts 43 bases downstream of the transposon insertion site within exon1, while the other starts 21 bases upstream of the 3′ end of the transposon (Fig. 3 *A* and *C*). Consequently, the first 99 bp of coding sequence from the *LsKN1* gene is missing in both of the *LsKN1*▽ transcripts, which is consistent with the results from our RT-PCR experiments. Nevertheless, the LsKN1▽ protein contains both the conserved KNOX and homeobox domains found in LsKN1 (*SI Appendix*, Fig. S2).

Overexpression of the *LsKN1* Gene Is Key to Its Heading Function. qRT-PCR assays showed that the expression of the *LsKN1*▽

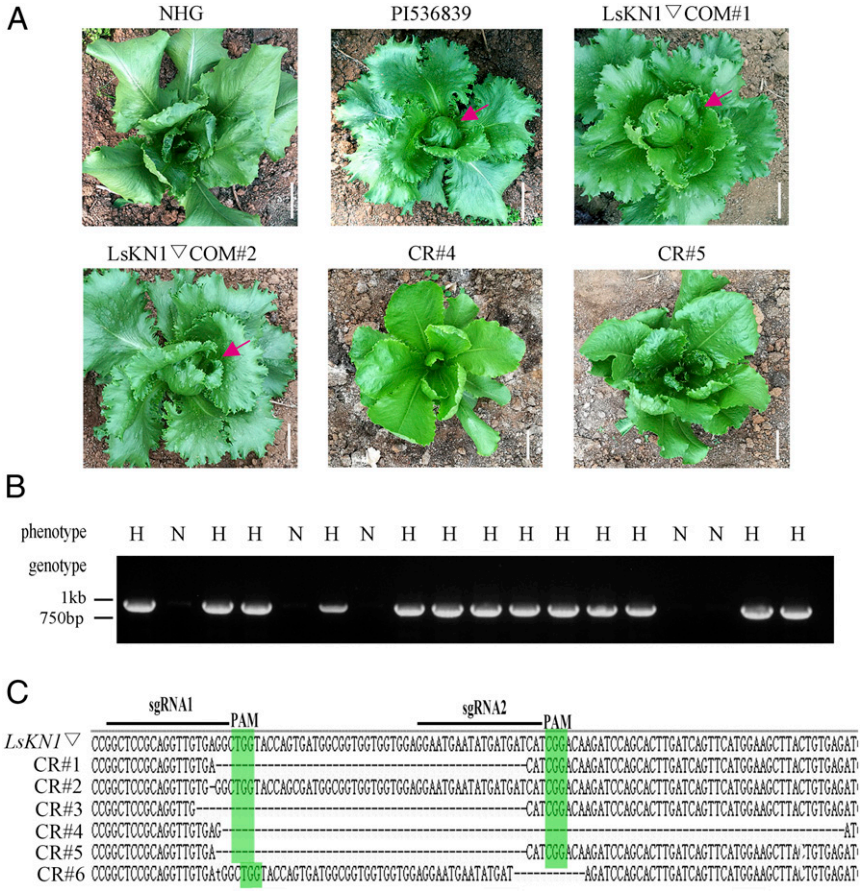

Fig. 2. Essential role of the *LsKN1* gene in heading. (*A*) Phenotypes of the *LsKN1*▽ complemented plants and CRISPR/Cas9 mutants. NHG, a nonheading line from the $F_{2:3}$ population, was used for complementation. PI536839, heading parent. LsKN1▽COM#1 and LsKN1▽COM#2 are positive transformants containing the full-length *LsKN1*▽ gene. CR#4 and CR#5 are the CRISPR/Cas9 mutants of the *LsKN1*▽ gene in the background of PI536839. The magenta arrows indicate the leafy heads. (Scale bar, 5 cm.) (*B*) Segregation of the heading phenotype in the T_1 generation of LsKN1▽COM#1. Phenotypes "H" and "N" refer to heading and nonheading, respectively. (*C*) Sequences around the target sites in the CRISPR/Cas9 mutants. Dashed lines indicate the region deleted during the CRISPR/Cas9 experiment. sgRNA sequences are indicated above the *LsKN1*▽ sequences by a thick line. PAM sequences are indicated by green shading.

allele was considerably higher than the expression of the *LsKN1* allele (Fig. 4*A*). We hypothesized that the heading function of the *LsKN1*▽ allele was due to its up-regulated expression. To test this hypothesis, the NHG was transformed with an overexpression vector containing a CaMV 35S promoter driving the expression of the *LsKN1*▽ gene. We obtained 19 transgenic plants, and only 3 of them developed normally and set seeds, while the others were dwarf, had abnormal leaves, and did not set seeds. qRT-PCR assays demonstrated that the expression of *LsKN1*▽ was enhanced in the transformants (Fig. 4*A*). As expected, we detected high-level accumulation of the LsKN1▽ protein and a firm head in the overexpression lines, which were undetectable in the nonheading genotype or knockouts of *LsKN1* (Fig. 4 *B–D* and *SI Appendix*, Fig. S4). Leaf heading in the overexpression lines occurred considerably earlier than in the heading parent. The third leaf of the transgenic line was obviously curved along the main vein. In contrast, the third leaf of the heading parent was straight (*SI Appendix*, Fig. S5). These findings indicate that enhanced expression of the *LsKN1*▽ gene promotes heading in lettuce.

The CACTA-like Transposon Acts as a De Novo Promoter. Since the major sequence variation between the *LsKN1*▽ and *LsKN1* alleles is the insertion of the CACTA-like transposon, the up-regulated expression of *LsKN1*▽ is most likely caused by the CACTA-like transposon. To test this hypothesis, we used the dual luciferase reporter system to evaluate the effects of the CACTA-like transposon on gene expression. The native promoter of the *LsKN1* gene, the native promoter of the *LsKN1* gene plus the CACTA-like transposon, and the CACTA-like transposon alone were used to drive the expression of the gene encoding firefly luciferase in the leaves of *Nicotiana benthamiana* (Fig. 5*A*). We found that the promoter plus the CACTA-like transposon and the transposon alone produced significantly higher firefly luciferase activity than the promoter of the *LsKN1* gene (Fig. 5*B*). Moreover, firefly luciferase activity produced by the *LsKN1* native promoter plus the CACTA-like transposon is similar to the firefly luciferase activity produced by the CACTA-like transposon alone. Thus, the CACTA-like transposon does not appear to drive elevated levels of *LsKN1*▽ gene expression by serving as an enhancer of the *LsKN1* promoter. Instead, the robust promoter activity of the CACTA-like transposon appears responsible for driving the elevated expression of the *LsKN1*▽ gene in heading lettuce.

Next, we investigated whether the CACTA-like transposon changed the expression pattern of the *LsKN1*▽ gene. In the nonheading parent, *LsKN1* is mainly expressed in inflorescences and weakly expressed in vegetative tissues, such as inner leaves, outer leaves and roots. However, in the heading lettuce, the expression of *LsKN1*▽ is greatly increased and abundantly expressed in the inner leaves, outer leaves, inflorescences, roots, and leaf primordia (Fig. 5*C* and *SI Appendix*, Fig. S6). Therefore,

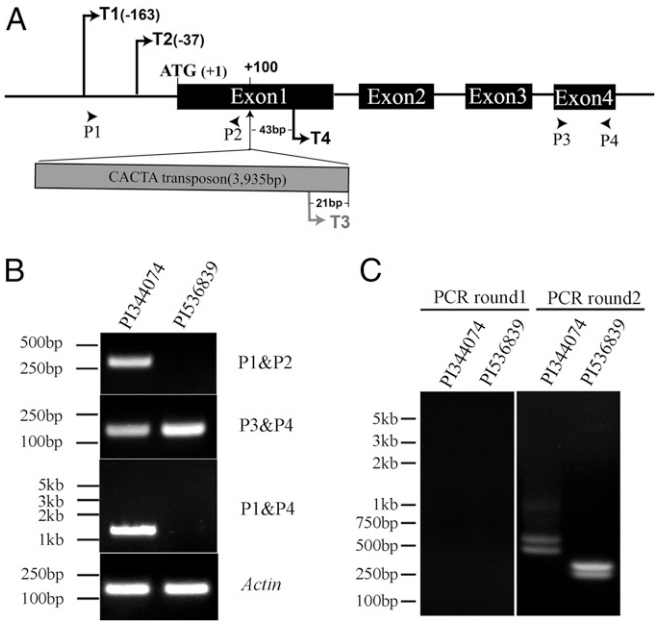

Fig. 3. Transcription of the *LsKN1* gene. (*A*) Schematic representation of the different transcription start sites of the *LsKN1* gene in the nonheading and heading parents. The different transcription start sites were determined using 5′ RACE (*C*). Transcripts vary only in their transcription start sites. T1 and T2 indicate the transcription start sites for the *LsKN1* gene from the nonheading lettuce (PI344074). T3 and T4 indicate the different transcription start sites for the *LsKN1*▽ gene in heading lettuce (PI536839). The relative positions of the primers (P1 through P4) used to measure gene expression in B are indicated by black arrowheads. (*B*) Expression of the *LsKN1* gene. *Actin* was used as a reference gene. (*C*) 5′ RACE analysis of the *LsKN1* gene in the nonheading and heading lettuce. The products from the first round of PCR were used as the templates for the second round of PCR.

the insertion of the CACTA-like transposon in the *LsKN1*▽ gene not only up-regulated its expression but also changed its expression pattern.

***LsKN1*▽ Is Necessary but Not Sufficient for Heading.** We previously sequenced the RNAs from a natural genome-wide association study (GWAS) population of lettuce (1). Of the 175 genotypes investigated, 51 genotypes had high *LsKN1* gene expression (*SI Appendix*, Fig. S7). A primer pair specific to the insertion of the CACTA-like transposon from the *LsKN1*▽ allele was used to screen the GWAS population. Strikingly, all of the 51 genotypes that had high *LsKN1* gene expression had the *LsKN1*▽ allele (with CACTA-like transposon insertion), while the other 86 genotypes with the *LsKN1* allele and 38 genotypes with the *LsKN1*▽ allele had low expression (Dataset S2). These 51 genotypes include all 22 crisphead cultivars (i.e., 22 heading cultivars) in the GWAS population. However, the other 29 genotypes do not form heads. Thus, we conclude that high-level expression of the *LsKN1* gene is necessary but not sufficient for heading in lettuce.

To further test this hypothesis, we overexpressed the *LsKN1* gene in a cultivar of stem lettuce using the same vector (Fig. 4*D*). Although high-level expression of the *LsKN1* gene was detected in overexpression lines of stem lettuce, no obvious phenotypic changes were observed between the transformants and nontransformed controls (*SI Appendix*, Fig. S8). Thus, we verified the hypothesis that up-regulated expression of the *LsKN1* gene is necessary but not sufficient to develop a leafy head in lettuce.

The Transposon in *LsKN1*▽ Was Inserted after Domestication. The 29 genotypes with the *LsKN1*▽ allele but no heading phenotype include cultivars from different horticultural types such as romaine, butterhead, loose-leaf, and stem lettuce (Dataset S2). To study the origin of the *LsKN1*▽ allele, we screened 451 accessions of wild relatives of lettuce (i.e., *L. serriola*, *Latuca saligna*, and *Latuca virosa*) collected from all over the world. The CACTA-like transposon insertion in *LsKN1* was not found in any of the wild relatives (Dataset S3). We conclude that the insertion of the CACTA-like transposon in *LsKN1* occurred after domestication and before the divergence of stem lettuce and leafy lettuce ~2,000 y ago (1).

Duplication and Diversification of *KN1* Homologs in Lettuce. The *KN1* gene in maize is expressed mainly in the meristem. This expression pattern is critical for maintaining meristem identity and normal plant development (29, 30). Unlike *KN1* in maize, *LsKN1* is also expressed in leaves (Fig. 5*C*). We searched the lettuce genome and found a second *KN1* homolog on chromosome 4, which is hereafter referred to as *LsKN2* (*SI Appendix*, Fig. S2). *KN1* homologs were also obtained from sunflower and representative species from other plant families (Fig. 6*A*). Lettuce and sunflower have two copies of *KN1*, while only one copy is found in other species, such as *Arabidopsis*, tomato, cabbage, and sorghum. The phylogenetic tree is consistent with the duplication of the *KN1* gene occurring during the early divergence of the Asteraceae.

Our analysis of the RNA-seq data from the panel of 240 lettuce accessions in our GWAS population indicated that the *LsKN2* gene either is not expressed or is expressed at extremely low levels in leaves—in striking contrast to the high-level expression of the *LsKN1*▽ paralog in some genotypes (*SI Appendix*, Fig. S7). RT-PCR assays demonstrated that the *LsKN2* gene was expressed mainly in the shoot apex and the inflorescence, but not in the leaf and the root. Thus, the expression pattern of *LsKN2* is similar to the expression pattern of the *KN1* gene in maize (Fig. 6*B*). Therefore, it is possible that the *LsKN2* gene is the functional counterpart of the *KN1* gene in maize and that the *LsKN1* gene is a paralog with a distinct expression pattern.

Although the expression patterns of the two *KN1* paralogs in lettuce are distinct, their sequences are highly conserved (*SI Appendix*, Fig. S2). Based on this, we hypothesized that these two *KN1* paralogs encode biochemically nearly identical proteins. To test this hypothesis, we overexpressed the *LsKN2* gene in the NHG. We obtained a total of 26 positive lines, and 1 of them showed a heading phenotype (Fig. 6 *C* and *D*). The remaining 25 lines were dwarf, had a leaf abnormality such as lack of differentiation of abaxial and adaxial faces, and did not set seeds. The phenotypes of overexpression lines of the *LsKN2* gene resembled those of the transformants that overexpressed the *LsKN1*▽ gene (see above). We conclude that the two *KN1* paralogs in the lettuce genome encode proteins with similar functions and that their distinct expression levels and patterns are responsible for their distinct effects on leaf development.

LsKN1 Binds the Promoter of *LsAS1* to Suppress Its Expression. To determine how LsKN1▽ promotes heading, we performed a ChIP-sequencing (ChIP-seq) experiment using leaves from the GFP-tagged LsKN1▽ overexpression lines and the GFP antibody. The ChIP-seq data indicate that LsKN1▽ may have thousands of targets in the lettuce genome (Dataset S4). The potential targets include genes controlling leaf dorsoventrality, such as *LsAS1* (*LG4386200*), *LsYABs* (*LG3311975*, *LG6549654*), *LsKAN2* (*LG6579663*), *LsAGO10* (*LG9772461*), and *LsARF4* (*LG2240867*). Since the heading leaves in lettuce exhibit disrupted adaxial identity (Fig. 1*D*), and the *AS1* gene has been shown to prompt adaxial cell identity in *Arabidopsis*, we chose *LsAS1* as our first candidate to validate its involvement in the heading trait. ChIP-seq showed that there is a prominent peak in the promoter region of the *LsAS1* gene in the LsKN1▽-immunoprecipitated group (Fig. 7*A*). Moreover, electrophoretic mobility shift assays (EMSAs) demonstrated that LsKN1▽ binds to the promoter of the lettuce homolog of the

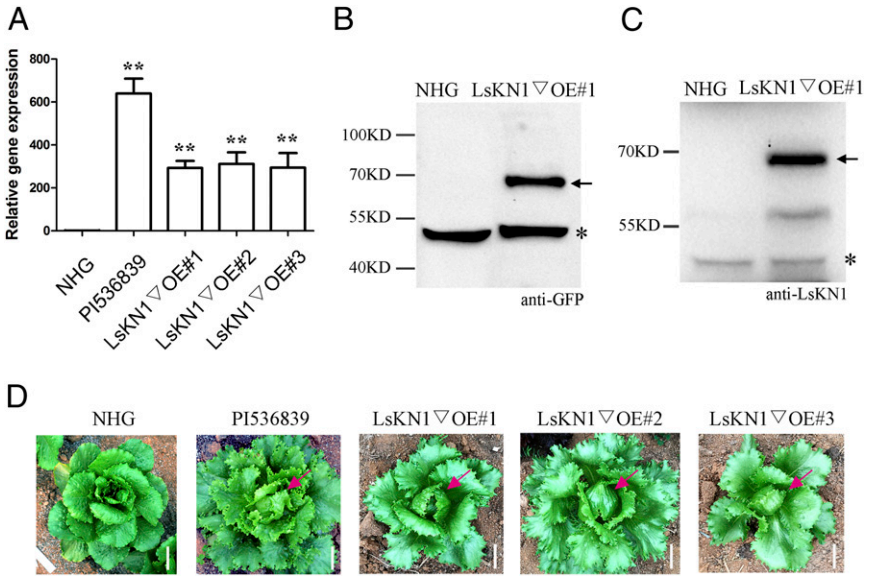

Fig. 4. Overexpression of the *LsKN1*▽ gene leads to heading. (*A*) qRT-PCR analysis of *LsKN1* expression in NHG, heading parent PI536839, and *LsKN1*▽ overexpression lines. LsKN1▽OE#1 to LsKN1▽OE#3, *LsKN1*▽ overexpression lines under NHG genetic background. Data represent mean ± SD ($n = 3$). The statistical significances for gene expression difference were determined using Student's *t* test between NHG and PI536839 or between NHG and *LsKN1*▽ overexpression lines. $^{**}P < 0.01$. (*B* and *C*) Immunoblot analysis of LsKN1▽ protein levels in overexpressing plants. Immunoreactive bands were detected using anti-GFP (*B*) and anti-LsKN1 antibodies (*C*). Arrows indicate the LsKN1▽-GFP fusion proteins, and single asterisks (*) indicate nonspecific bands used as loading controls. (*D*) Heading phenotype of the lines overexpressing *LsKN1*▽. Magenta arrows indicate head formation in the overexpression lines. (Scale bar, 5 cm.)

Arabidopsis gene *AS1* (*LsAS1*) between −401 and −8 bp (Fig. 7*B*). The shift of the LsKN1▽-DNA complex shifted further as the LsKN1 antibody was added in the EMSA and disappeared when nonlabeled competitor DNA containing the LsKN1▽-binding site in the *LsAS1* promoter was added to the EMSA. These data indicate that LsKN1▽ specifically binds the promoter of the *LsAS1* gene (Fig. 7*B*). The interaction between LsKN1▽ and the promoter of the *LsAS1* gene was also confirmed using a yeast one-

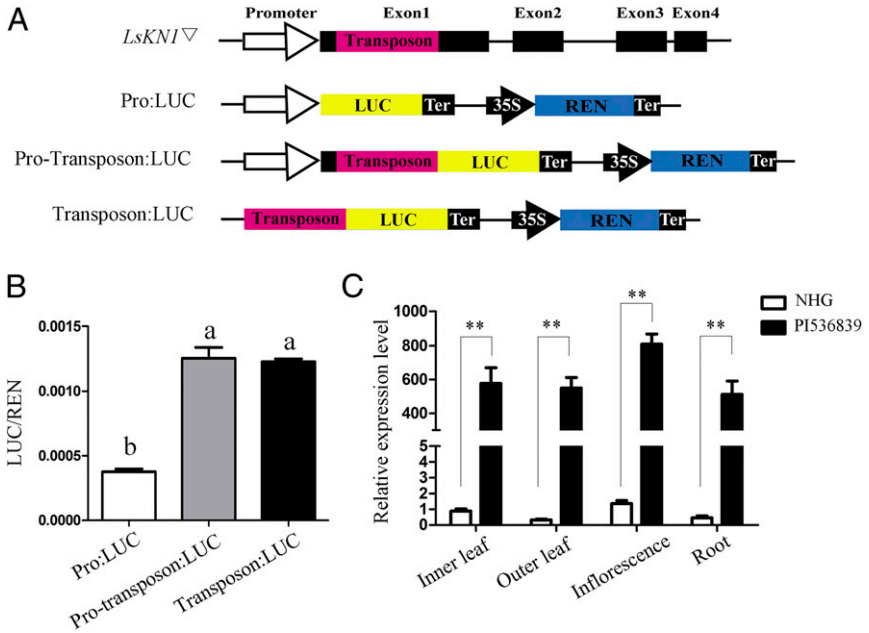

Fig. 5. Activation of *LsKN1* expression by the CACTA-like transposon. (*A*) Diagrams of the *LsKN1*▽ gene and reporter vectors used in the dual luciferase assay. Pro:LUC, firefly luciferase gene driven by the promoter of the *LsKN1* gene. Pro-Transposon, firefly luciferase gene driven by the promoter of the *LsKN1* gene fused to the CACTA-like transposon. Transposon:LUC, firefly luciferase gene driven by only the CACTA-like transposon. LUC, firefly luciferase; REN, renilla luciferase. (*B*) Dual luciferase assay. The LUC/REN ratio is indicated by the *y* axis. Different letters indicate statistically significant differences determined by one-way ANOVA test ($P < 0.05$). (*C*) Expression pattern of the *LsKN1* gene in the nonheading and heading lettuce. *Actin* was used as a reference gene. Data represent mean ± SD ($n = 3$). The statistical significances for gene expression difference were determined using Student's *t* test. $^{**}P < 0.01$.

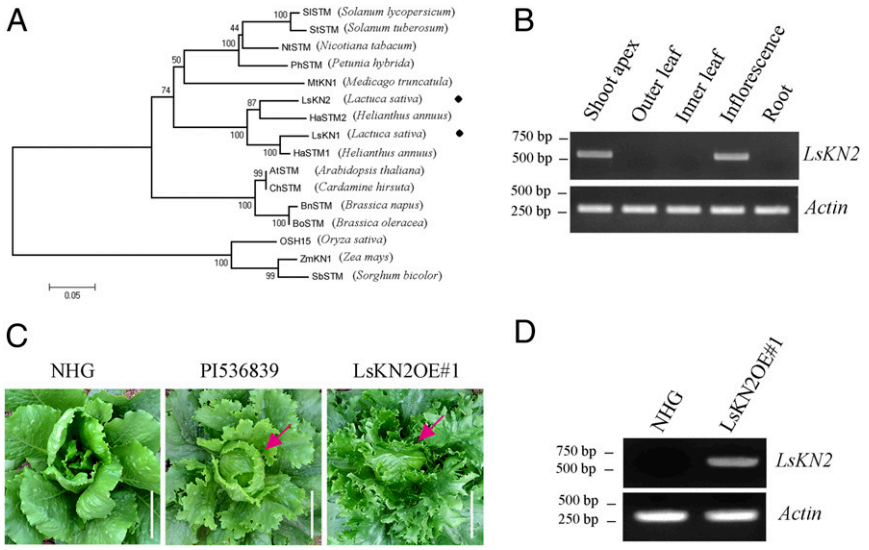

Fig. 6. Duplication and diversification of the *KN1* homologs in lettuce. (*A*) Phylogenetic tree constructed with *KN1* homologs from lettuce, sunflower, and representative plant families. (*B*) Expression of the native *LsKN2* gene in different tissues. (*C*) Heading phenotype of a *LsKN2* overexpression line. Magenta arrows indicate the leafy heads. (Scale bar, 5 cm.) (*D*) Expression of the *LsKN2* gene in the overexpression line.

hybrid experiment (Fig. 7*C*). Additionally, we performed a dual luciferase assay to test whether LsKN1▽ can affect the expression of the *LsAS1* gene. We found that, when the *LsAS1* promoter drove the expression of the LUC reporter gene, transiently coexpressing the *LsKN1*▽ gene with the LUC reporter plasmid reduced the accumulation of LUC activity. Thus, *LsKN1*▽ suppressed the expression of the *LsAS1* gene in this assay (Fig. 7 *D* and *E*). qRT-PCR analysis also showed that the expression of the *LsAS1* gene was significantly less in the heading parent PI536839 as well as in the *LsKN1*▽-overexpressing line compared with the NHG (Fig. 7*F*). To confirm the role of the *LsAS1* gene in the heading phenotype associated with the *LsKN1*▽ gene, we overexpressed the *LsAS1* gene in the heading parent. As is shown in Fig. 7 *G* and *H*, the plants overexpressing *LsAS1* showed hyponastic leaves at an early stage and fail to fold inward, consistent with the above hypothesis that the *LsKN1*▽ gene regulates heading through the *LsAS1* gene. Interestingly, the overexpression lines showed a weak heading phenotype at the late rosette leaf stage, suggesting that the *LsAS1* gene is not the only target of LsKN1▽. Based on these findings, we conclude that LsKN1▽ directly binds the promoter of the *LsAS1* gene and suppresses its expression, which consequently lead to the alternation of leaf dorsoventrality and to the heading phenotype in lettuce.

Discussion

Heading Is a Complex Trait. To study the genetics of heading in lettuce, we constructed a segregating population through crossing a romaine lettuce with a crisphead lettuce. The romaine type of lettuce has a weak heading phenotype in which its leaves grow to form tightly clustered leaves but do not curve and fold in upon themselves as leaves in a crisphead type. It was chosen as the nonheading parent in this study to minimize the complexity of the heading trait in the segregating population. In the segregating F_2 population, only two loci (*LHL1* and *LHL2*) that control the development of a leafy head were identified. The *LHL1* gene has not yet been cloned, but *LsAS1* and *LsKN2*, which are located at the positions of 302.9 and 94.0 Mb, both on chromosome 4, are excluded as the candidate gene for *LHL1*, as the confidence interval of the *LHL1* gene is from 310.4 to 366.4 Mb ($P = 0.05$). With the cloning of the *LHL1* gene in the future, it will be interesting to study the relationship (such as relative positions in a pathway) between the *LHL1* and *LsKN1* genes. The two loci could not explain all of the heading phenotypes in the segregating population, consistent with additional locus/loci contributing to heading in this population. The new locus/loci were overlooked in the original BSR-seq analysis, probably due to growing season differences and/or the overwhelming effects of *LHL1* and *LHL2* in the F_2 population.

The heading alleles for both *LHL1* and *LHL2* are from the crisphead parent. Considering that the romaine parent also has a weak heading phenotype, the romaine parent should harbor one or more loci controlling heading as well. A segregating population derived from a cross between romaine and loose-leaf cultivars would be required to dissect the loci controlling the weak heading phenotype in the romaine cultivar. It was hypothesized previously that heading in *B. rapa* depends on natural variation in many key regulators of plant development, such as *BrpGL1*, *BrpESR1*, *BrpSAW1*, *BrARF3.1*, *BrARF4.1*, *BrKAN2.1*, *BrKAN2.3*, and *BoATHB15.2*, most of them controlling abaxial identity (3, 5). The *LsKN1* gene characterized in this study functions through *LsAS1*, a gene controlling adaxial identity. In future studies, it will be interesting to study the effects of *KN1* homologs on heading in other vegetables and at the same time to investigate the effects of abaxial genes on heading in crisphead lettuce.

Unusual Mechanism for the Up-Regulation of the *LsKN1*▽ Gene. The insertion of a transposon into the exon of a gene usually abolishes its function because these insertions introduce premature stop codons and/or because of the silencing effects of transposons (31). It is intriguing that the insertion of a CACTA transposon in the first exon of the *LsKN1*▽ gene produced a gain-of-function allele (Fig. 2) with greatly enhanced expression (Fig. 4*A*). High expression of the *LsKN1* gene is strongly associated with the insertion of CACTA-like transposon. However, not all accessions with the insertion showed high expression, probably due to sequence divergence after insertion or genetic background. Indeed, our data indicate that this transposon serves as a de novo promoter rather than an enhancer of the native promoter of the *LsKN1* gene, similar to the transposon T10 (32). A BLAST search of the lettuce genome using this CACTA transposon as a query indicated that there are dozens of copies of this transposon in the lettuce genome. The element

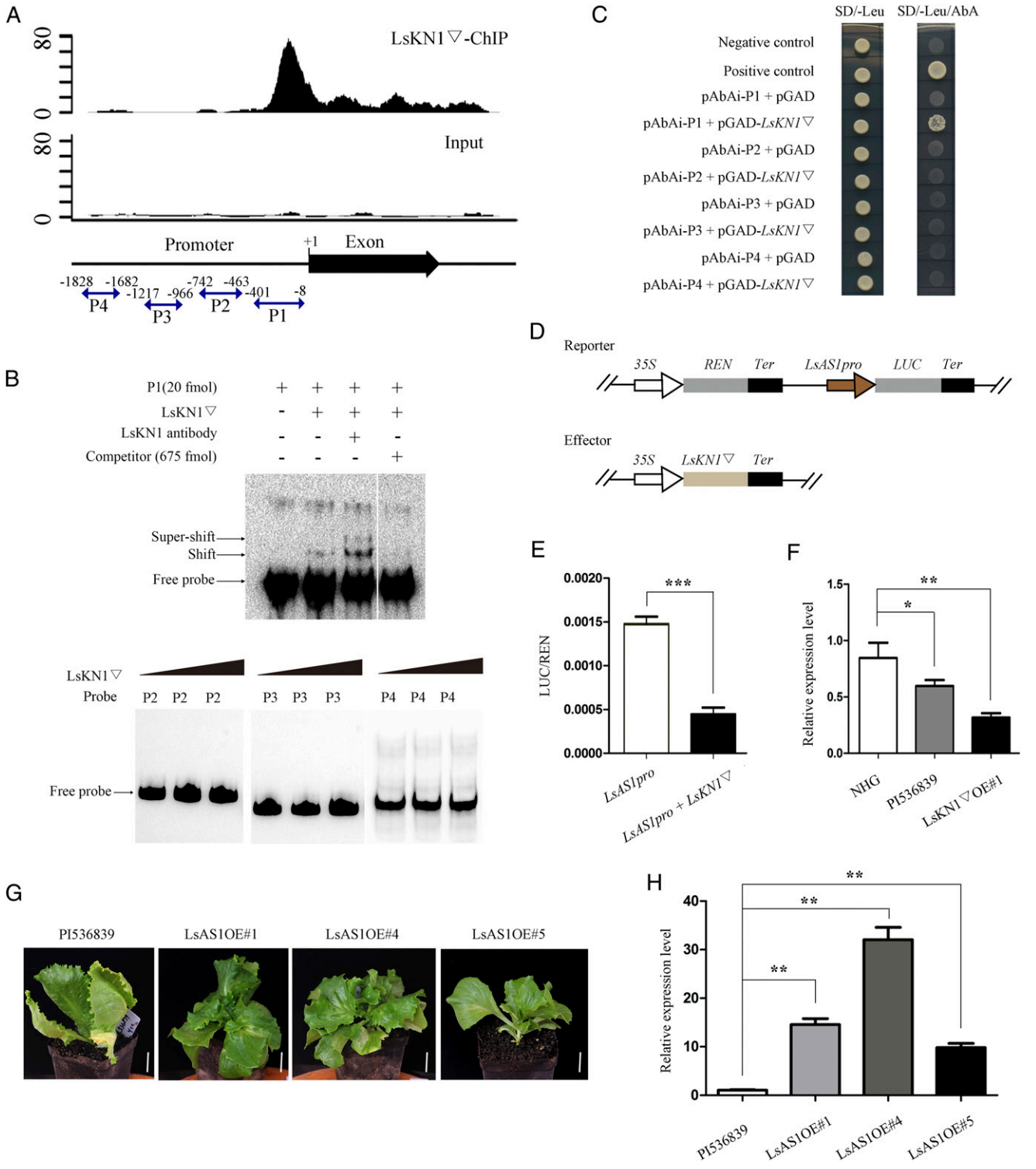

Fig. 7. *LsKN1▽* represses the expression of *LsAS1*. (*A*) ChIP-seq analysis of LsKN1▽-binding activity on the promoter region of *LsAS1*. The number of reads is indicated by the *y* axis. The position of the reads on the gene is indicated by the *x* axis. P1 through P4 indicate the DNA fragments used as probes in EMSA and as baits in the yeast one-hybrid assay. (*B*) *LsAS1* promoter-binding activity of LsKN1▽. EMSAs indicate that LsKN1▽ directly binds the P1 fragment of the *LsAS1* promoter (*Top*) and that LsKN1▽ does not bind P2, P3, and P4 (*Bottom*). (*C*) Yeast one-hybrid assay. Transformants were grown on the SD/–Leu medium and the medium with 200 ng/mL aureobasidin A. Negative control, transformants of p53-AbAi and pGADT7; positive control, transformants of p53-AbAi and pGADT7-p53. The regions of P1 to P4 fragments are shown in *A*. (*D*) Diagram of reporter and effector vectors used in the dual luciferase assay (*E*). (*E*) Dual luciferase assay. *LsAS1pro*, *LsAS1* promoter driving the expression of the LUC gene. *LsAS1pro* + *LsKN1▽*, coexpression of the LUC gene driven by the *LsAS1* promoter and *LsKN1▽*. Data represent mean ± SD ($n = 3$). ***$P < 0.001$. (*F*) *LsAS1* expression in NHG, PI536839, and a *LsKN1▽* overexpression line. Relative expression was quantified using qRT-PCR. Data represent mean ± SD ($n = 3$). **$P < 0.01$. *$P < 0.05$. (*G*) Phenotype of *LsAS1* overexpression plants. *LsAS1* was driven by CaMV *35S* promoter, and the heading parent PI536839 was used as the transgenic receptor. (Scale bar, 2 cm.) (*H*) qPCR analysis of *LsAS1* gene expression. LsAS1OE#1, LsAS1OE#4, and LsAS1OE#5 are the *LsAS1* overexpression lines. Data represent mean ± SD ($n = 3$). Student's *t* test was used. **$P < 0.01$.

inserted into the *LsKN1*▽ gene is a truncated one, with 1,900 bp deleted. It remains unknown whether this deletion is associated with the promoter activity of this particular transposon.

The Molecular Mechanism of Heading. *KN1* is a master regulator in plant meristem that contributes to stem-cell maintenance and the initiation of lateral organs (30). In this study, we showed that the *LsKN1*▽ gene makes a major contribution to leafy head traits in lettuce but has no obvious effect on the development of meristems since the knockout plants showed a normal vegetative and reproductive development. It is most likely that *LsKN2*, a homolog of *LsKN1* in lettuce, is functionally similar to the *KN1* gene in maize, since *LsKN2* is expressed mainly in the shoot apex. We speculate that the *LsKN1* gene in lettuce is a diversified homolog of *KN1* and expressed in different tissues. The LsKN1 transcription factor may have thousands of direct targets in the lettuce genome (Dataset S4). The LsKN1 target that we mainly focused on in this study is the gene encoding the *LsAS1* transcription factor, which controls dorsoventrality in *Arabidopsis*. In *Arabidopsis*, *STM* negatively regulates the expression of *AS1* gene. *AS1* also negatively regulates other *KNOXI* genes including *KNAT1* and *KNAT2*. The mutual repression between *STM* and *AS1* plays a pivotal role in maintenance of meristems and specification of lateral organs (33). Studies on *B. oleracea* and *B. rapa* provided evidence that some key genes involved in leaf adaxial–abaxial development were strongly selected during the development of the heading trait (5). In *B. rapa*, overexpression of *miR319a* down-regulated its target gene (*BrpTCP4-1*), leading to the altered development of the leafy head (4). Members of the TCP family may directly regulate dorsoventrality genes, such as *AS1* (34). Alternatively, TCP proteins may down-regulate the expression of the *STM* gene, which in turn regulates the expression of dorsoventrality genes (35). Other *LsKN1* targets in addition to *LsAs1* may also contribute to the heading phenotype. The function of the *LsKN2* gene on heading remains unknown, although we showed that overexpression of the *LsKN2* gene had a similar phenotype to that of the *LsKN1* overexpression lines. However, like *KN1* in maize, the *LsKN2* gene is expressed mainly in meristems. Knockout of *KN1* in maize caused dramatic plant-architecture change (29). Due to its expression pattern (in meristems), we predict that knockout of the *LsKN2* gene in lettuce may change the heading phenotype, mainly due to its effects on meristem development rather than dorsoventrality. We also noticed that some overexpression lines of *LsKN1*▽ and *LsKN2* genes developed abnormal plants and were often sterile, very likely caused by high expression levels.

Heading is a complicated trait that has been shown in this and previous studies to be controlled by many loci. Multiple pathways, including phytohormone pathways, synergistically control leaf development. Indeed, as more genes controlling heading are cloned, we anticipate that a large number of genes associated with many different pathways will be linked to heading. The genes and pathways involved in heading might be conserved among evolutionarily diverse vegetable crops such as cabbage and lettuce, although the particular mutations leading to the heading phenotype may be different. With the effects of the *LsKN1* gene and its target *LsAS1* on heading characterized in this study, their orthologs and other components in this pathway should be considered in future studies on heading in other vegetable crops such as cabbage.

Methods

Plant Materials and Growth Conditions. *Lactuca* materials were ordered from the collections of the US Department of Agriculture Germplasm Information Network (https://www.ars-grin.gov/) and the Netherlands Center for Genetics Research (https://www.wur.nl/en/Research-Results/Statutory-research-tasks/Centre-for-genetic-Resources-the-Netherlands-1.htm) or collected and maintained by our laboratory (Datasets S2 and S3). Plants were grown in a field on the campus of Huazhong Agriculture University, Wuhan, China.

Tissue Section and Microscopy. The main veins of heading leaves at the curled position and their counterparts in the nonheading leaves (derived from NHG plants) were excised and immersed in 50% FAA buffer (50% ethanol, 5% acetic acid, 3.7% formaldehyde). After application of a vacuum for 10 min, samples continued to be fixed in the solution at room temperature overnight. The samples were sequentially dehydrated by a series of ethanol solutions (50, 70, 85, 95, and 100% for two times), and then the ethanol was gradually replaced by xylene to make the samples clear. Then samples were further immersed in an increasing concentration of paraffin and finally embedded in the absolute paraffin. Sections were performed by using a Rotary Microtome (Leica), and the slides were stained by 0.5% Toluidine blue. Images were taken using a bright-field microscope (ZEISS).

Genetic Analysis of the Heading Trait. BSR-seq was used to identify regions controlling heading following the procedure of Su et al. (36). In this study, a heading pool was prepared by mixing equal amounts of tissue from 20 individuals with tight heads. A nonheading pool was prepared by mixing equal amounts of tissue from 20 nonheading individuals. Total RNA was extracted from the two pools using the TransZol reagent (TransGen). RNA-seq was performed with the Illumina Hiseq2500 platform. Approximately 5 Gb of clean data were obtained from each pool. Reads were aligned to the lettuce genome (37), and single-nucleotide polymorphisms (SNPs) were identified. For each SNP, the allele frequency difference was calculated and plotted at the relevant position along the nine chromosomes of lettuce. A peak of ΔSNP values on these plots is consistent with a region harboring a gene contributing to heading.

Two models were used to quantify the effects of loci identified using BSR-seq. The first model, the ordinal logistic regression model, was used to analyze the ordered categorical phenotypic variable in a statistically rigorous manner. A second model, an ANOVA model, was used, under the assumption of linear relationships between different classes of the phenotypic value, to generate outputs that are more intuitive and therefore easier to interpret. The genetic parameters were also calculated using the method of QTL IciMapping (26).

Plant Transformation. Transformation of lettuce followed the procedure of Curtis et al. (38). First, seeds were sterilized with 50% bleach for 10 min and then washed with a large amount of water. After sterilization, seeds were germinated on half-strength Murashige and Skoog (MS) media at 22 °C in a growth chamber. Cotyledons from 5-d-old seedlings were excised and immersed in a suspension of Agrobacterium for 10 min. The infected cotyledons were cocultivated in the dark on the half-strength MS media containing 1 mM acetosyringone. After 2 d of dark cultivation, the cotyledons were transferred to a selection and shoot-inducing media (1x MS, 0.1 mg/L 1-naphthlcetic acid, 0.1 mg/L 6-BA, 40 mg/L Kan, 300 mg/L Timentin) and grown in long-day conditions (16 h of light followed by 8 h of dark) at 22 °C. The emerging young buds were excised and transferred to half-strength MS media containing 300 mg/L Timentin to induce rooting. Finally, the transgenic plants were grown in soil in a growth chamber or protected field on the campus of Huazhong Agricultural University.

For complementation assays, the full-length *LsKN1*▽ gene was amplified using PCR from heading lettuce (PI536839) and purified using a gel extraction kit (Omega). This PCR product was inserted into a HindIII/PmlI-linearized binary vector pCAMBIA1301 using the ClonExpress II One Step Cloning Kit (Vazyme). The construct was transformed into Agrobacterium GV3101 using electro-transformation. A nonheading lettuce (NHG) that was homozygous for the *LsKN1* allele was obtained from the segregating population. This NHG was self-pollinated. The resulting seeds were used for the transformation experiments.

The coding sequence (CDS) of the *LsKN1*▽ or *LsAS1* gene was amplified and recombined with the BamHI linearized p06 vector, which uses the CaMV 35S promoter to drive the expression of the inserted sequence. The coding sequence of an enhanced GFP tag was fused to the 3′ end of each insert to yield a C-terminal fusion protein. After DNA sequence analysis, the construct was electro-transfected into GV3101, and then the strains were cultivated in the dark at 28 °C for 3 d. The cells that grew on the selective media were used for the transformation experiments.

Gene Expression Analysis and Immunoblotting. Total RNA was extracted from leaves using the TransZol reagent (TransGen). A total of 5 μg of the total RNA was treated with DNase I (Thermo) to remove the contaminating genomic DNA and then reverse-transcribed using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega). qRT-PCR was performed using the

QuantStudio 6 Real-Time PCR System (Thermo). *Actin* was used as a reference gene. To exclude genomic DNA contamination, no PCR products were amplified using RNA as template. For immunoblotting, total protein was extracted from young leaves using the radioimmunoprecipitation assay lysis buffer (25 mM Tris·HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). After denaturation, samples were resolved on 10% SDS gels and blotted to polyvinylidene difluoride membranes (Millipore). The membranes were blocked in phosphate-buffered saline (PBS) containing 5% skim milk and 0.1% Tween 20. The membranes were probed with anti-GFP (Abcam) or anti-LsKN1 antibodies at a 1:2,000 dilution, and, after washing, the membranes were probed with a horseradish peroxidase (HRP)-coupled goat anti-rabbit IgG secondary antibody (Abbkine). The immunoreactive bands were detected using the Clarity Western ECL substrate (Bio-Rad).

Dual Luciferase Assay. The dual luciferase assay was performed according to the manufacturer's instructions (Promega). A total of 2,000 bp from the *LsKN1* promoter, the CACTA-like transposon, and the *LsKN1* promoter followed by the CACTA transposon were amplified and separately cloned into the pGreenII 0800-LUC reporter vector to drive the expression of the firefly luciferase gene (LUC). The Renilla luciferase reporter gene (REN) driven by the CaMV 35S promoter in the same vector as the LUC reporter gene was used as an internal reference standard in each transformation. The construct was transiently expressed in tobacco leaves using Agrobacterium-mediated transformation. Two days after injection, the luciferase activity was measured using the Tecan Infinite 200 PRO luminometer.

EMSA. EMSAs were performed according to the manufacturer's instructions (Thermo). First, the coding sequence of the *LsKN1*▽ gene was cloned into the expression vector pMAL-c2x (NEB). The expression of the MBP-LsKN1▽ fusion protein was induced overnight in the C41 expression strain with 1 mM isopropyl β-D-1-thiogalactopyranoside at 20 °C in an orbital shaker. The bacteria were harvested by centrifugation and washed with prechilled PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) and then resuspended in PBS. The bacteria were lysed using an ultrasonic homogenizer. Then, the LsKN1▽ protein was purified from the crude extract using affinity chromatography with an amylose resin (NEB). For the EMSAs, the probes were prepared by amplifying DNA fragments from the *LsAS1* promoter and labeling their 3′ ends with biotin according to the manufacturer's recommendation (Thermo). An unlabeled version of this same *LsAS1* promoter fragment (competitor) and LsKN1 antibody were used to test whether LsKN1▽ specifically binds this probe. For the binding reaction, the LsKN1▽ protein, probes (20 fmol each), and competitor DNA (675 fmol) were incubated in the binding buffer (10 mM Tris·HCl, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 2.5% Glycerol, 5 mM $MgCl_2$, 0.05% Nonidet P-40) with the presence of 3 μg/μL poly (dI•dC) at room temperature for 20 min. The complex was resolved on 6% native gels and blotted to nylon membranes. The signal was detected using HRP conjugated to streptavidin and an ECL substrate (Thermo).

5′ RACE. To determine the transcript starting point, 5′ RACE was performed using the FirstChoice RLM-RACE Kit (Ambion). Briefly, total RNA was extracted from young leaves of the nonheading and heading parents. After removal of 5′ PO_4 from the degraded transcripts utilizing a calf intestinal alkaline phosphatase treatment, the messenger RNAs were further decapped using TAP enzyme to remove the 5′ guanosine end caps and ligated to an adapter (Ada). The ligation product was reverse-transcribed using MMLV (Promega) to yield first-strand complementary DNA. The first round of PCR was performed using primers specific for the adapter (Ada1_F) and the *LsKN1*-coding sequence (LHL446R). Nested primers (Ada_2 and LHL164R) were used to amplify the products. The PCR products were resolved on a 1% agarose gel. The bands were recovered and ligated into the T-A cloning vector (Takara). The clones containing inserts were sequenced.

Construction of a Phylogenetic Tree. The amino acid sequences of the KN1 homologs from different species were retrieved from the EnsemblPlants database (http://plants.ensembl.org/index.html). The protein sequences were aligned using Clustal X (39). A maximum-likelihood phylogenetic tree was constructed using the MEGA 7.0 (40). Bootstrapping was performed with 1,000 replications.

ChIP-seq Assay. The ChIP assay was carried out as described by Saleh et al. (41). For preparation of crude extracts of nuclei, leaves of 3-wk-old *LsKN1*▽ overexpression plants were cut into pieces and submerged in a solution containing 1% formaldehyde for 10 min for cross-linking. Samples were rinsed with prechilled water, frozen, and ground into a fine powder using prechilled mortars and pestles. The samples were further suspended in cold nuclei isolation buffer (0.25 M sucrose, 15 mM piperazine-1,4-bisethanesulfonic acid, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1 mM $CaCl_2$, 0.9% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mg/mL pepstatin A, and 2 mg/mL aprotinin). The extracts were filtered through four layers of Miracloth (Millipore) and centrifuged at 11,000 x *g* for 20 min at 4 °C. The pellet was resuspended in the cold nuclei lysis buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate, 1% Triton X-100, 1 mg/mL pepstatin A, and 1 mg/mL aprotinin). The chromatin was sheered to yield small fragments with lengths ranging from 200 to 1,000 bp with an average size of 500 bp. For the immunoprecipitation of chromatin, the sheered chromatin was diluted 10-fold with dilution buffer (50 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mg/mL pepstatin A, and 1 mg/mL aprotinin) and precleared by incubation with salmon sperm DNA pre-equilibrated Protein A agarose beads (Bio-Rad). The precleared chromatin was subsequently incubated with an anti-GFP antibody (Abcam, Ab290) overnight at 4 °C. The immunocomplexes were recovered by incubating them with salmon sperm DNA pre-equilibrated Protein A agarose beads for 1 h at 4 °C. After washing the immune complexes one time with low-salt wash buffer (150 mM NaCl, 20 mM Tris·HCl, pH 8, 0.2% SDS, 0.5% Triton X-100, and 2 mM EDTA), one time with high-salt wash buffer (500 mM NaCl, 20 mM Tris·HCl, pH 8, 0.2% SDS, 0.5% Triton X-100, and 2 mM EDTA), one time with LiCl wash buffer (0.25 M LiCl, 1% sodium deoxycholate, 10 mM Tris·HCl, pH 8, 1% Nonidet P-40, and 1 mM EDTA), and two times with TE buffer (1 mM EDTA and 10 mM Tris·HCl, pH 8), the immunocomplexes were eluted with elution buffer (0.5% SDS and 0.1 M $NaHCO_3$), and the cross-linking was reversed with an incubation at 65 °C overnight. The chromatin was purified with a Proteinase K digestion, phenol-chloroform extraction, and an ethanol precipitation. The purified DNA was subsequently sequenced using the HiSeq X Ten-PE150 platform. Finally, reads were aligned to the lettuce genome v8 using HISAT2 (42). Peak calling was performed using MACS (43).

Yeast One-Hybrid Assay. Yeast one-hybrid assay was performed according to the manufacturer's instructions (Clontech). DNA fragments from the *LsAS1* promoter were amplified and cloned into the bait vector (pAbAi) at the XhoI site. All of the bait vectors were confirmed by sequencing. The recombinant constructs were linearized using BbsI or BstBI and transformed into the Y1HGold yeast strain. After growth at 28 °C in the dark for ∼3 d on Ura-dropout media, the strains were screened using colony PCR. Strains with properly integrated bait plasmids were used as bait-reporter strains for the second transformation. The CDS of the *LsKN1*▽ gene was amplified and inserted into pGAD-T7 between EcoRI and BamHI to generate a prey vector. After confirmation by DNA sequencing, the bait-reporter yeast strain was transformed with the prey vectors and selected by aureobasidin A on the Leu-dropout media.

Preparation of LsKN1 Antibody. The CDS of the *LsKN1*▽ gene was amplified and cloned into the pET-28a vector between BamHI and SalI sites and transformed into BL21 cells for recombinant protein expression, followed by purification using Ni-NTA resins (GE). The purified recombinant proteins were injected into rabbits to produce the polyclonal antibodies. The antibodies specific to LsKN1 proteins were first enriched from the antiserum by Super-Beads protein A Magnetic beads (Bio-Rad) and were further purified by the LsKN1▽-coupled *N*-hydroxy-succinimide-activated beads (Smart Life Sciences).

Data Availability. The gene (*LsKN1*) identified in this study can be found in GenBank (LOC111890976). The raw data of ChIP-seq, RNA-seq of the BSR pools, and RNA-seq of the GWAS materials have been deposited in GenBank under accession numbers PRJNA576072, PRJNA602578, and PRJNA394784, respectively.

All study data are included in the article and supporting information.

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