LsaYAB7, A Homologous Gene of *FILAMENTOUS FLOWER*, Participating In The Regulation of Adaxial-Abaxial Polarity of Leaves In Lettuce

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Abstract

Background: The plant-specific YABBY transcription factor plays important roles in the development of lateral organs, the establishment of adaxial-abaxial polarity and abiotic stress response. However, the function of *YABBY* gene family in lettuce (*Lactuca sativa*), an important leaf vegetable is still unclear yet.

Results: In this study, we analyzed the chromosomal distribution, gene structure and cis-transcriptional elements of the *YABBY* gene family in lettuce. Phylogenetic analysis of *YABBY* genes in lettuce, *Arabidopsis*, maize and rice classified them into five groups. In addition, the expression profile of *YABBY* genes in lettuce indicated that they may play distinct functions in different tissues and developmental processes. The subcellular localization analysis and transactivation assay showed that *LsaYAB7* probably functions as a transcription factor in the nucleus. Furthermore, the ectopic expression of *LsaYAB7* exhibited abaxially curled leaves, not only in *Arabidopsis*, but also in lettuce.

Conclusion: Altogether, *LsaYAB7* is a key functional gene in determining the adaxial-abaxial polarity of lettuce leaves. Our study laid foundation for the molecular research of functional genes in lettuce.

Background

In the process of plant growth and development, diverse regulators participate in leaf morphology and development, such as GRF (GROWTH-REGULATING FACTOR), TCP (TEOSINTE BRANCHED1, CYCLOIDEA, and PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR) and YABBY. Among them, YABBY genes encode plant-specific transcription factors that are especially widely distributed in seed plants [1–3]. They play an important role in the development of plant lateral organs, the establishment of adaxial-abaxial polarity and abiotic stress response [4–6]. The YABBY transcriptional factor contains a C2–C2 zinc-finger domain in the N-terminal and a high mobility group box in the C-terminal region [7]. There are six members in *YABBY* gene family in *Arabidopsis thaliana*: *YABBY1/FIL* (*FILAMENTOUS FLOWER*), *YABBY2*, *YABBY3*, *YABBY4/INO* (*INNER NO OUTER*), *YABBY5* and *CRC* (*CRABS CLAW*) [8]. These six *AtYAB* genes have distinct and redundant functions in regulating morphogenesis of plant organs [9]. Accordingly, *CRC* and *INO* are usually called "flower-specific *YABBY* genes", while *FIL*, *YABBY2*, *YABBY3* and *YABBY5* are called "nutritional *YABBY* genes" [10]. *FIL*, *YABBY2* and *YABBY3* are expressed in polar manner and functionally redundant in all lateral organs including cotyledons, petals, sepals, carpels and stamens with different expression levels [9]. *FIL*, *YABBY2* and *YABBY3* express respectively at a high, moderate and low level and jointly determines the fate of abaxial cells [9–12]. *CRC* is specifically expressed in the carpels and nectaries, and *crc* mutant shows non-fusion of carpels and abnormal developed nectaries [7]. While *INO* is expressed in the abaxial region of outer integument, and *INO* mutation leads to loss of polarity differentiation in ectoovule [13]. The *FIL/YAB3* double mutant shows linear cotyledons and leaves, and abnormal vascular tissue development [2]. The triple mutant *fil/yab3/yab5* and quadruple mutant *fil/yab2/yab3/yab5* are short and dense, lack apical dominance and polarity of lateral organs [2, 9]. *YAB2* shows functional redundancy with *YAB3* and *CRC*, which regulate leaf development and participating in the synthesis of cell wall [14].

Leaf development, and in particular, the establishment of the adaxial-abaxial polarity, is regulated by a precise genetic network. *phantastica* (*phan*) in *Antirrhinum* is the first mutant affecting the leaf adaxial-abaxial polarity, with the leaves showing more and more radial symmetry, and the leaf at the top of the stem became needle-like [15, 16]. In *Arabidopsis thaliana*, the fate of the abaxial cells is comprehensively determined by the interaction between YABBY, KANADI and auxin response factor (ARF3/4) and miR165/166 [9, 11, 17–21]. Upon establishment of the adaxial-abaxial polarity YABBY is initially expressed in the leaf primordium, and then fixed in the abaxial region, acting downstream of KANADI, which functions antagonistically to the adaxially expressed HD-ZIPIII gene that also limited by miR165/166 [18, 22–27]. AS1 and AS2 promoted the expression of HD-ZIPIII in the adaxial side and inhibited the expression of miR165/166, Kanadi and YABBY genes in the adaxial side [28, 29]. TAS3 trans-acting short interference RNA (TAS3 ta-siRNAs) limits the expression of ARF3/4 genes to the abaxial side [30–32].

At present, many YABBY genes in different plants have been found to play an important role in leaf adaxial-abaxial polarity establishment, floral organ recognition, and SAM development. In rice, *OsYAB1* participates in the feedback regulation of GA synthesis by the specific binding to the cis-acting element of *GA3ox2* promoter [33]. *OsYAB2* controls shedding [34]. *OsYAB3* was closely related to maize *ZmYAB14* and *Arabidopsis FIL*. *OsYAB3*-RNAi showed twisted leaves without ligule and auricle [35]. *OsYAB4* overexpression in *Arabidopsis* exhibited retardation of development, loss of apical dominance at flowering stage, more stems, tufted flowers and less fruiting, similar to *FIL/YAB3* overexpression lines [36]. *DROOPING LEAF 1 (DL1)* plays a regulatory role in midvein formation and carpel development in rice [37]. The *CRC* homologous gene in maize, regulates plant structure by affecting leaf length and width, leaf angle, internode length and diameter [38]. In tomato, a YABBY-like FAS protein influences the formation of carpels [39]. In addition, the YABBY genes are also associated with abiotic tolerance such as drought, salt and ABA stress in soybean [6]. The negative regulative role of YABBY gene under drought and salt stress is also reported in upland cotton [40].

Altogether, YABBY genes are pivotal regulators in leaf development [41], however, in lettuce (*Lactuca sativa* L.), a popular leafy vegetable in the world, and corresponding studies on the YABBY gene function are rarely reported, due to the relatively slow progress in development biological research in lettuce. In this study, we carried out the studies on the YABBY genes in lettuce. Nine YABBY genes were identified in lettuce, and furthermore, their gene structure, protein conservative motif, cis-acting elements and expression profile in different tissues were studied. Moreover, the regulatory roles of some YABBY gene in leaf development, especially in adaxial-abaxial polarity establishment in lettuce were revealed by genetic study. This study laid the foundation for further exploration on the molecular regulatory network of lettuce leaf development.

Results

Identification of the YABBY gene family in lettuce

In order to identify YABBY gene family in lettuce, both BLASTP and Hidden Markov Model (HMM) searches were performed. Initially, the amino acid sequences of six YABBY transcription factors in *Arabidopsis* were used as query sequences for performing a BLASTP search in lettuce genome database. Additionally, HMM profiles of the YABBY (PF04690) domains were searched against the lettuce genome database (<https://phytozome.jgi.doe.gov>). Finally, a total of 9 candidate YABBY genes were identified in lettuce. Further lettuce database searching using these candidate YABBYs as queries against the draft genome and predicted mRNAs resulted in no further hits. Thus, in total, 9 proteins were identified as LsaYABs, and their coding genes were designated as *LsaYAB1* to *LsaYAB9*, according to their distribution in the lettuce genome (Table 1). Based on the location information of 9 *LsaYABs* genes on chromosomes, it was found that 9 *LsaYABs* genes were distributed on 6 chromosomes (chromosomes 3, 4, 5, 6, 7 and 8) (Fig. 1A). Among them, Chromosomes 3 carries three *LsaYABs*, chromosome 7 has two, whereas chromosomes 4, 5, 6, and 8 each contains one *LsaYAB* gene. By comparing the protein sequences of *LsaYABs*, it was found that all of them contained conserved YABBY and C2-C2 zinc-finger domains (Fig. 1B).

The proteins encoded by the *LsaYABs* were predicted to show significant differences in their sizes and physicochemical properties (Table 1). Their predicted protein lengths varied from 168 to 228 amino acids and molecular weights ranged from 18.61 kDa to 25.09 kDa (Table 1). Exon numbers of the *LsaYABs* genes ranged from 3 to 7, with *LsaYAB2*, *LsaYAB3*, *LsaYAB4*, *LsaYAB7* containing seven exons and *LsaYAB5* containing only three. Their lengths of nucleotides are relatively short, none of them was longer than 700bp. In addition, YABBY protein sequences showed large variations in predicted isoelectric point (pI) values (ranging from 6.29 to 9.43).

Table 1
Characteristics of the YABBY proteins in lettuce

Name	Accession No.	Chr	CDS (bp)	Exon No.	Length (aa)	MW (KDa)	PI
LsaYAB1	Lsat_1_v5_gn_0_27800.2	Chr04	507	5	168	18.61	6.29
LsaYAB2	Lsat_1_v5_gn_3_3040.1	Chr03	687	7	228	25.09	8.15
LsaYAB3	Lsat_1_v5_gn_3_53601.1	Chr03	552	7	183	19.97	9.28
LsaYAB4	Lsat_1_v5_gn_3_128981.1	Chr03	666	7	221	24.67	7.27
LsaYAB5	Lsat_1_v5_gn_5_94941.2	Chr05	561	3	186	20.62	9.28
LsaYAB6	Lsat_1_v5_gn_6_2400.1	Chr06	669	6	222	24.57	9.43
LsaYAB7	Lsat_1_v5_gn_7_9041.1	Chr07	672	7	223	24.60	7.21
LsaYAB8	Lsat_1_v5_gn_7_43360.1	Chr07	600	6	199	22.02	6.53
LsaYAB9	Lsat_1_v5_gn_8_58340.3	Chr09	648	5	215	24.02	8.66

Characterization and phylogenetic analysis of the *LsaYAB* genes

In order to study the phylogenetic relationship of the nine *LsaYABs* genes, a Neighbor-Joining phylogenetic tree containing *YABBY* genes from *Arabidopsis*, lettuce, rice and maize was constructed by using MAGE 7.0 software (Fig. 2). In this analysis, 9 *LsaYABs* genes were divided into 5 groups, named FIL/YAB3, YAB2, CRC, INO and YAB5, respectively (Fig. 2) [33, 42]. Among them, FIL/YAB3 contains the largest number of family members, with four *LsaYABs*, namely *LsaYAB2*, *LsaYAB4*, *LsaYAB6* and *LsaYAB7* belonging to this group (Fig. 2). YAB5 contains three members from lettuce, namely *LsaYAB5*, *LsaYAB8* and *LsaYAB9*; CRC and INO each contain only one member from lettuce (Fig. 2). However, there is no *LsaYABs* in YAB2 group, indicating that functional loss may have occurred in genome replication events. Monocots (rice and maize) and dicotyledous (*Arabidopsis* and lettuce) have low gene similarity and were separated into each group. Most of the *LsaYAB* genes are closer to those in *Arabidopsis*, except for *LsaYAB1* and *LsaYAB3* (Fig. 2). It implied that these two *YABBY* genes might be involved in the conserved function of monocots and dicotyledous.

Gene structures often diverges during the evolution of multi-gene families. Therefore, we analyzed the gene structures and conserved motif characteristics of the *LsaYABs*. The phylogenetic tree of *LsaYABs* was used in gene structures and conserved motif analysis (Fig. S1). As shown in Fig. S2A, the *LsaYABs* within each group had similar exon–intron organizations. Most *LsaYAB* genes harbored 5–7 exons, with the exception of *LsaYAB5*, which has 3 exons. Tools of the MEME web server (<http://meme.sdsc.edu/meme/meme.html>) were used to analyze motif distributions and to conduct domain predictions. A total of 8 motifs were identified in the *LsaYABs* (Fig. S2B). The predicted motif models are listed in Fig. S3. *LsaYABs* contain at least two domains, with Motif 2 representing the zinc finger domain and Motif 1 the *YABBY* domain. The *YABBY* gene family has a very close relationship in evolution and distribution. *LsaYABs* in the same group usually show similar protein conservative motif types and sequences within. For example, *LsaYAB4* and *LsaYAB7* have 7 identical motifs, with same sequence. *LsaYAB2* and *LsaYAB6* have six identical motifs in exactly the same order. Among them, Motif 5 and Motif 8 exist in *LsaYAB4* and *LsaYAB7*, Motif 6 exists in *LsaYAB5* and *LsaYAB8*. These motifs, which exist specifically in different subfamilies, may play important roles in the functional differentiation of *YABBY* gene family proteins.

The cis-elements in gene promoter play critical roles in the initiation of gene expression. Investigation of cis-regulatory elements in *LsaYAB* genes was performed within 2000 bp upstream from the putative translation start site using the PlantCARE database. Various cis-elements related to hormone and stress responses were identified in the *LsaYAB* promoters (Fig. S4). *LsaYABs* contain a large number of cis-acting elements related to light responsiveness (G-Box, MRE, GT1-motif, ACE, 3-AF1 binding site) and MeJA-responsiveness (CGTCA-motif, TGACG-motif). Additionally, it contains many cis-acting elements related to stress resistance (drought and low temperature response) and hormone response (abscisic acid and gibberellin induction). *LsaYAB3*, *LsaYAB6*, and *LsaYAB7* contain meristem expression element (CAT-box), indicating that these genes may play roles in the development of plant meristems. *LsaYAB3* also contains cis-regulatory element related to flavonoid biosynthetic genes regulation element (Fig. S4). The *LsaYAB* genes in the same phylogenetic cluster showed moderate consistency in their distributions of the cis-elements, indicating that the promoters of these *LsaYABs* diverged.

Phylogenetic tree was constructed for 9 *Lactuca sativa*, 6 *Arabidopsis thaliana*, 13 Maize and 8 *Oryza sativa* YABBY proteins. There were 5 phylogenetic clusters, highlighted with different colors, designated as CRC, INO, YAB2, YAB5, FIL/YAB3. Numbers at the nodes indicate bootstrap values; values lower than 30% are not shown.

Expression profiles of YABBY genes in different tissues

To characterize the function of the *LsaYAB* genes, we firstly evaluated their expression levels in the root, stem, cotyledon, leaf, bud, flower and seed of lettuce. It was revealed that most of the genes showed relatively high expression in the flower and seed, while low expression in the in root and stem (Fig. 3). *LsaYAB1*, the putative homologous gene of *Arabidopsis INO*, specifically expressed in the flower. And *LsaYAB3*, the putative homologous gene of *Arabidopsis CRC*, expressed highly in bud and flower. *LsaYAB7* is a putative homologous gene of *Arabidopsis FIL* and it has the highest expression in floral organs, which is consistent with the expression pattern of *AtFIL* gene [9]. *LsaYAB7* is also expressed in leaves and floral organs.

To investigate the expression pattern of *LsaYAB7*, we constructed a vector containing β -glucuronidase (GUS) reporter gene driven by the promoter of *LsaYAB7* (2 kb sequence upstream of the initiation codon of *LsaYAB7*), and genetically transformed into *Arabidopsis*. GUS activity was observed in the cotyledons and the root tip at cotyledon-stage (Fig. 3B). After two true leaves were full expanded, GUS signal was seen in petioles of cotyledons, but not in cotyledons and true leaves (Fig. 3B). The GUS activity in the initiation of lateral roots were also observed at two-leaf-stage (Fig. 3B). GUS staining was also found in the apical stem and the petioles, but not the cotyledons at the four-leaf-stage (Fig. 3B). These results indicated that *LsaYAB7* expressed in the tissues undergoing cell proliferation, which was consistent with that of *AtFIL*.

LsaYAB7 putatively functions as a transcription factor in the nucleus

LsaYAB7 in *FIL* group shows a similar expression pattern to *AtFIL*, an important regulator of leaf development. Therefore, we further investigate the function of *LsaYAB7*. As the *LsaYAB* proteins are putative TFs, subcellular location observation and transactivation assay of *LsaYAB7* were firstly investigated. We constructed the *35S::LsaYAB7-GFP* vector and transformed it into lettuce protoplast cells. The empty vector was used for control. As shown in Fig. 4A, green fluorescence of GFP was observed within the nucleus in the protoplast cell transformed by *35S::LsaYAB7-GFP*. While the protoplast cell transformed by empty vector exhibited ubiquitous green fluorescence. Agrobacterium with recombinant plasmid *35S::LsaYAB7-GFP* was transfected into tobacco leaves too. Also, the fluorescence signal was detected in the nucleus (Fig. 4B), indicating that the *LsaYAB7-GFP* localized to the nuclear. In the transcriptional activity, full-length *LsaYAB7* was constructed into yeast expression vector, pGBK-T7. The empty vector pGBK-T7 and pGBK-T7-OsMYB103L which was proved to have transcriptional activity were designed as the negative and positive control, respectively [47]. These recombinant plasmids were transformed into yeast strain Y2HGOLD. They showed similar growth states without Tryptophan (Trp) under different diluted concentration (Fig. 4C), indicating the transformation made few influences on

yeast growth. The yeast cells expressed full-length *LsaYAB7* could grow with AbA (Aureobasidin A) and turn blue with X- α -galactoside, similar to the positive control, indicating that *LsaYAB7* had transcriptional activity in yeast (Fig. 4C). These results implied that *LsaYAB7* probably functioned as a transcriptional factor in the nucleus.

***LsaYAB7* functioned in leaf adaxial-abaxial polarity establishment in *Arabidopsis* and lettuce**

To further investigate the function of the *LsaYAB7* gene, we constructed the over-expression plasmid of *LsaYAB7* gene driven by 35S promoter, and respectively transformed into *Arabidopsis* using Agrobacterium-mediated transformation method and lettuce using leaf disc method. A total of 12 transgenic lines were obtained and three of which with relatively higher expression (Fig. 5A) were further used for phenotype investigation. Compared with transgenic negative line (CK) plants, all homozygous transgenic lines (10-day-old) from T1 generation exhibited outward curled cotyledons (Fig. 5B); along with the growing process, the leaves curled abaxially became long-narrow, and this phenotype turned more obvious with the increase of the leaf ages. In addition, the *LsaYAB7*-OE plants were short, and the flowering stage was delayed (Fig. 5). Conclusively, we demonstrated that over-expression of *LsaYAB7* in *Arabidopsis* caused abaxialization of the leaf, which is consistent with the phenotype of *AtFIL* overexpression [43].

In the lettuce transformation, PCR identification showed that the *LsaYAB7* gene has been successfully transformed into the lettuce genome (Fig. 6). qRT-PCR detection of the relative expression of the *LsaYAB7* gene in the transgenic plants showed that the expression of *LsaYAB7* was significantly up-regulated compared with that in the transgenic negative line (CK) (Fig. 6A). Compared with non-transgenic plants, the leaves of transgenic lines curled abaxially (Fig. 6B). These results indicated that the overexpression of *LsaYAB7* affected the leaf adaxial-abaxial polarity and led to outward curling of leaves in lettuce, which was consistent with the phenotype of ectopic expression of *LsaYAB7* in *Arabidopsis*.

Discussion

The YABBY gene family plays an important role in abaxial–adaxial polarity establishment, carpel morphogenesis, shoot apical meristem restriction, floral differentiation, and development of lateral organs [22]. In this study, we firstly identified 9 YABBY genes in lettuce and cloned *LsaYAB7*, the homologous gene of *AtFIL*, which regulates leaf abaxial–adaxial polarity in *Arabidopsis* and lettuce. Overexpressed *LsaYAB7* in *Arabidopsis thaliana* and lettuce could result in obvious curled leaves, which is consistent with the phenotype of *AtFIL* overexpression line in *Arabidopsis*. According to the phylogenetic analysis, the YABBY genes in lettuce, *Arabidopsis thaliana*, rice and maize could be divided into five groups (Fig. 2), among which the group of FIL/YAB3 contains the most of *LsaYABs*, as well in rice and maize [33, 42]. There was no *LsaYAB* genes found in YAB2 group, which might be lost during the interspecific differentiation. In addition, it was also found that the gene structures and conserved motifs of *LsaYABs* in the same group were conserved to a certain extent (Fig. S2).

The functions of *AtYAB* genes were found to be involved in the development of abaxial tissue of lateral organs. In this study, the expression profiles of 9 *LsaYAB* genes in lettuce were analyzed and *LsaYAB* genes were identified to be widely expressed in various organs (Fig. 3). It showed that the expression of *LsaYAB* gene was significantly up-regulated in lateral organs such as leaves, flowers and seeds, which implied that *LsaYAB* gene, similar to *AtYAB* genes, plays a certain role in the development of lateral organs. Nine *LsaYABs* were all found to be highly expressed in flowers, of which *LsaYAB1* was detected to be expressed specifically in flowers (Fig. 3A). *LsaYAB1* is a homologous gene of *AtINO*, which has the highest expression in abaxial region of the outer integument [12]. It suggested that *LsaYAB1* might have the same functions with its homologous gene *AtINO*. *LsaYAB7* was mildly expressed in cotyledon and true leaf. However, its expression reached the highest level in the bud and gradually decreased at flowering stage (Fig. 3A). It might be similar with the expression pattern of *AtFIL* in Arabidopsis, that its expression was accumulated in the flower primordium at the beginning and gradually restricted to the abaxial region of the flower organs with the differentiation of floral organs [9].

The amino acid sequence alignment of *LsaYABs* and *AtYABs* showed that all of the YABBY genes contained two highly conserved protein domains, C2-C2 zinc finger and YABBY, which might be associated with similar molecular functions (Fig. 1B). However, the expression patterns of *LsaYABs* showed diversity in different organs and development stages, which suggested that they may function in different temporal and spatial ways (Fig. 3). The transcription elements analysis of *LsaYABs* showed that the putative promoters of *LsaYABs* contains a large number of light response-related cis-acting elements, indicating that the expression of *LsaYABs* may be greatly influenced by light. In addition, some *LsaYABs* have gibberellin, abscisic acid, methyl jasmonate, ethylene, low temperature and drought response elements in their putative promoter sequences. Abscisic acid, methyl jasmonate and salicylic acid play important roles in response to abiotic stress and disease resistance. It may indicate that some *LsaYABs* were not only related to the development of plant lateral organs, but also closely related to hormone signaling transduction and stress responses. Cis-acting elements related to regulate flavonoid synthesis were also found in the putative promoter of *LsaYAB3*, indicating that *LsaYAB3* might also be involved in the regulatory pathway of flavonoid synthesis.

Conclusions

Although there have been lots of researches on the functions of YABBY genes, the YABBY genes in lettuce have not been identified and characterized yet. In this study, the members of YABBY gene family in lettuce were firstly identified and conserved protein domains, gene structures, conserved motif and cis-acting elements of these *LsaYABs* were also analyzed. The overexpressed *LsaYAB7*, a homologous gene of *AtFIL*, both in Arabidopsis and lettuce resulted in the curled leaves and tufted flowers like the overexpression lines of *AtFIL* in Arabidopsis. These results in this research have laid a foundation for the functional investigation of the YABBY genes in lettuce. However, the phenotypes of *LsaYAB7* loss-of-function lines in lettuce, the downstream regulation genes of *LsaYAB7* and the functional analysis of other *LsaYAB* genes, would need a further study. This study is of great significance to analyze the

molecular mechanism of leaf shape variation in lettuce and laid a foundation for molecular breeding of different leaf shapes in lettuce.

Methods

Plant materials

'L3', a loose-leaf type cultivar of lettuce (*Lactuca sativa* L.), was used in this study. The seeds of 'L3' were provided by Beijing vegetable Technology Research Center. Spread lettuce seeds evenly in petri dishes covered with filter paper and add ddH₂O to keep the filter paper moist. After 1 day of treatment at 4 °C, the lettuce seeds were transferred to a light incubator, and the seeds could sprout after 2 days. The germinated seeds were transferred to a burrow tray to raise seedlings. When 5-6 true leaves of lettuce were grown, they were transplanted in a protected field (seedling spacing 6-8 cm) and watered every 5-7 days.

Genetic transformation

The lettuce transformation method were modified according to the previous description [44]. To obtain the lettuce explants used for genetic transformation, we firstly soaked the full lettuce seeds in 70% ethanol for 1 minute and washed them with aseptic water for 3 times, then added 10% bleaching disinfection water for 15 minutes and washed them with aseptic water for 5 times. Finally, the sterile seeds were sown on Murashige and Skoog (MS) solid medium and cultured at 25 °C for 16 hours to grow two cotyledons. The cotyledons of lettuce seedlings were taken using scissors and were co-cultured with *Agrobacterium tumefaciens* for 15 min. The infected leaves were placed on co-culture medium MS1 (MS with 0.5mg/L 6-BA and 0.1 mg/L NAA) at 25 °C dark condition for 48 h for 2 days. Then the leaves were transferred into differentiation medium MS2 (MS with 0.5 mg/L 6-BA, 0.1 mg/L NAA, 50mg/L Kan and 50 mg/L Tim) at 25 °C, 16-h-light/8-h-dark condition for about 2-4 weeks to obtain kana-resistant differentiation seedlings. The buds were cut off, transferred to MS3 medium (MS with 0.046 mg/L 6-BA, 0.026 mg/L NAA, 50 mg/L Kan and 50 mg/L Tim) for culture, and then transferred to the medium MS4 (MS with 20 mg/L Kan and 50 mg/L Tim) for roots generation. After the root system is developed, the seedlings were transferred to soil.

Identification of *YABBY* gene family in lettuce

In order to identify the *YABBY* genes in the genome of lettuce, 15 full-length amino acid sequences of AtYABBY members were obtained from Arabidopsis thaliana genome database TAIR (<https://www.arabidopsis.org>). Using them for inquiry sequences to blast the protein sequence of all members of YABBY gene family in lettuce was obtained by BLASTp comparison search in Phytozome v12 (<http://phytozome.jgi.doe.gov/pz/>). Then, we used SMART (<http://smart.embl-heidelberg.de>) and PFAM (<http://pfam.xfam.org>) to analyze the sequence of the candidate members, excluding the members that do not contain conservative domains, all members of the YABBY transcription factor family of lettuce were finally obtained. The physical and chemical properties of lettuce YABBY proteins, such as

molecular weight and isoelectric point, were analyzed on ExPASy website (<https://web.expasy.org/protparam/>). Multiple sequence alignment analysis of 9 *LsaYABs* protein sequences was carried out by DNAMAN software.

Prediction of promoter cis-elements in *LsaYABs*

The 2 000 bp regions upstream of the start codon of *LsaYABs* were downloaded from Phytozome V12. The cis-acting elements of these sequences were predicted on website PlantCare (<http://bioinformatics.psb.ugent.be/webtools/PlantCare/html/>), and were predicted.

Construction of phylogenetic tree

Multiple sequences alignment of YABBY proteins in lettuce were carried out using DNAMAN software, and the phylogenetic tree was constructed by Neighbor-Joining method using MEGA 7.0 software. The Bootstrap value was set to 1000 times, and P-distance and Complete Deletion models were selected for analysis.

RNA extraction and qRT-PCR

A certain amount of plant tissue was taken with RNase-free 's 2mL centrifuge tube and quickly placed in a liquid nitrogen tank. Total plant RNA was extracted by polysaccharide polyphenol plant total RNA extraction kit (TIANGEN). RNA was reverse transcribed into cDNA using a reverse transcription kit (*TransScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix). The synthesized first strand cDNA was diluted 5 times for real-time quantitative PCR amplification. The reaction was carried out by Roche480 real-time quantitative PCR system. The *YABBY* genes and reference Actin gene were amplified in each sample and three biological repeats were set in each sample. The preparation and reaction conditions of the system refer to the kit specification *TransStart*® TopGreen qPCR Super Mix Kit (TransGen, Beijing, China). The fluorescence signal of the first 15 cycles of the PCR reaction was used as the fluorescence background signal. The Ct value of the sample was used to calculate the relative expression of the gene by $2^{-\Delta\Delta CT}$. Sequences of primers used for qRT-PCR are listed in Supplementary Table S1.

Subcellular localization and transactivation assay of *LsaYAB7*

According to the CDS sequence of *LsaYAB7* downloaded from Phytozomev12 database, specific primers with *EcoR*Ⅰ and *Sal*Ⅰ restriction sites and vector homologous arm sequence were designed to amplify the cDNA fragment of target gene (excluding stop codon). After amplified fragment was ligated into the pSAT6-EGFP-N1 vector digested by *EcoR*Ⅰ and *Sal*Ⅰ by the method of homologous recombination. The *Agrobacterium* containing recombinant plasmid LC-*LsaYAB7*-GFP and empty vector were injected into tobacco leaves. After 12 h and 48 h, the tobacco leaves were observed under a Nikon A1R/A1 confocal microscope with wavelengths of 514 nm (Nikon, Tokyo, Japan).

In order to detect the transcriptional activation activity in yeast cells, its full-length coding sequence, N-terminal DNA binding domain and C-terminal activation domain of LsaYABBY7 were cloned into pGBKT7 vector at *EcoR* I and *Bam*H I sites by homologous recombination. The empty vectors pGBKT7 were used as negative control. All of these vectors were transformed into yeast Y2HGold containing AUR1C and MEL1 reporter genes respectively. After transformation, the yeast clones were cultured on SD/-Trp and SD/-Trp/ABA/X- α -GAL plates at 30 °C for 2-4 days. The primers used for vector construction were listed in Table S1.

Plasmid construction and plant transformation

The cloning primers of *LsaYAB7* was designed based on the genome sequences of lettuce (*Lactuca sativa* V8) from the Phytozome (<https://phytozome.jgi.doe.gov>). The coding sequences (CDS) of *LsaYAB7* was cloned and constructed into the overexpression vector pEZR(K)-LC driven by 35S promoter using 2X Seamless Cloning Mix (Biomed, CL117-01, Beijing). The primers used for vector construction were listed in Table S1.

The *LsaYAB7* CDS were PCR-amplified and introduced into the LC vector. The recombinant constructs were transformed into *Arabidopsis* and Lettuce using Agrobacterium-mediated transformation following the floral dip method and the leaf disk method respectively [44, 45]. Transgenic plants were then screened on solid MS medium containing 50 μ g/ml kanamycin (Kana). Resistant seedlings were transferred to soil and further verified by PCR and qRT-PCR.

Histochemical GUS staining

The *LsaYAB7* promoter containing 2000 bp upstream from ATG were cloned into modified pCambia vector to drive GUS gene expression. The recombinant plasmid was transformed into *Arabidopsis* and the tissues from different developmental stages of transgenic *Arabidopsis* plants were used for GUS staining. The tissues were stained in the solution (50 mM phosphate buffer, pH 7.0, containing 1 mM Glc and 10% methanol) at 37 °C for 16 h. Then the tissues were immersed in 70% ethanol for several hours to remove chloroplast. Finally, the tissues were subjected to microscopic observation using a light microscope (SMZ1500; Nikon), and digital images were taken (Fi1; Nikon).

Abbreviations

MW: Molecular weight

ARF: auxin response factor

DL1: DROOPING LEAF 1

TCP: TEOSINTE BRANCHED1, CYCLOIDEA, and PROLIFERATING CELL NUCLEAR ANTIGEN BINDING FACTOR

FIL: FILAMENTOUS FLOWER

INO: INNER NO OUTER

CRC: CRABS CLAW

TF: transcription factor

MS: Murashige and Skoog

pI: Isoelectric point

qRT-PCR: Quantitative real-time polymerase chain reaction

GRF: GROWTH-REGULATING FACTOR

AbA: Aureobasidin A

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The links to the databases used in this study were listed as follows.

1. Arabidopsis thaliana genome database TAIR (<https://www.arabidopsis.org>)
2. Phytozome v12 (<http://phytozome.jgi.doe.gov/pz/>)
3. SMART (<http://smart.embl-heidelberg.de>)
4. PFAM (<http://pfam.xfam.org>)
5. ExPASy website (<https://web.expasy.org/protparam/>)
6. PlantCare (<http://bioinformatics.psb.ugent.be/webtools/PlantCare/html/>)

The public access to all these databases is open.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

D. L., D. Z., J. Z., and B. Z. designed experiments. K. L., Z. Z. and B. Z. identified and characterized the YABBY genes family in lettuce. K. L., Z. Z., and X. L. carried out the experiment. K.L., B.Z., and D. L. wrote the paper. All authors discussed the results and commented on the manuscript.

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Figures

Figure 1

Chromosomal locations and sequence alignments of LsaYABs. (A) Chromosomal locations of LsaYAB genes. Chromosome 0 indicated the sequences that could not be assembled onto any other chromosomes. (B) Alignment of the conserved domains, C2C2 and YABBY, of different YABBY proteins in lettuce and *Arabidopsis thaliana*. The conserved C2C2 and YABBY domains were respectively marked in red rectangles.

Figure 2

Phylogenetic relationship between YABs from Arabidopsis, rice, maize and lettuce.

Figure 3

Functional characterization of *LsaYAB* genes. (A) qRT-PCR analysis of the *LsaYAB* genes transcript in different tissues of the lettuce 'L3'. Actin was used as a reference gene. (B) GUS staining of pYAB7::GUS transgenic Arabidopsis lines during cotyledon-stage, two-leaf-stage and four-leaf-stage.

Figure 4

Characterization of the LsaYAB7 protein. (A) Subcellular localization of the LsaYAB7-GFP protein in lettuce protoplasts. The fluorescence of LsaYAB7-GFP fusion protein concentrated in the nucleus. Scale Bar = 5 μ m. (B) Subcellular localization of the LsaYAB7-eGFP fusion protein in *Nicotiana benthamiana* leaves. Scale bar: 20 μ m. (C) Transactivation assay of LsaYAB7 protein in yeast. The full-length of the LsaYAB7 was fused to DNA sequences containing a GAL4 DNA-binding domain in pGBKT7 (BD). The construct was expressed in the yeast strain Y2HGold with PGBDT7-OsMYB103L as a positive control and the void vector as a negative control (pGBKT7). The transformants with different diluted concentrates were dropped on the SD/-trp and SD/-trp/AbA/ X- α -Gal plates. After 2–4 days at 30°C, possible transcriptional activation functions of LsaYAB7-full length were observed.

Figure 5

Expression of LsaYAB7 gene in the Arabidopsis LsaYAB7-OE/At plants and phenotyping (A) Expression assay of LsaYAB7 in the LsaYAB7-OE/At lines. The transgenic negative line (CK) was used as negative control. (B) The phenotypes of T1 progeny of LsaYAB7-OE/At lines and transgenic negative line (CK). The rosette stage of CK and LsaYAB7-OE/At line were showed in a and b, respectively. The abaxial and adaxial of leaves in CK and LsaYAB7-OE/At line were exhibited in c and d, respectively. The inflorescence of CK and LsaYAB7-OE/At line were in e and f, respectively. The whole seedlings of CK and LsaYAB7-OE/At line at flowering stage were showed in g.

Figure 6

Phenotypes of the over-expression lines of LsaYAB7 in lettuce. (A) Verification of positive overexpression lines of LsaYAB7-OE/lettuce by PCR. The primers for verification were designed from LsaYAB7 and vector, respectively. (B) The relative expression level of LsaYAB7 in LsaYAB7-OE/lettuce lines. The transgenic negative line (CK) was used as negative control. (C) The phenotypes of T1 progeny of LsaYAB7-OE/lettuce and transgenic negative line (CK) in lettuce. The rosette stage of CK and LsaYAB7-OE/lettuce line were showed in a, respectively. The abaxial and adaxial of leaves in CK and LsaYAB7-OE/lettuce line were exhibited in b and c.

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