Identification of GROWTH-REGULATING FACTOR transcription factors in lettuce (Lactuca sativa) genome and functional analysis of LsaGRF5 in leaf size regulation

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Abstract

Background: GROWTH-REGULATING FACTORs (GRFs), a type of plant-specific transcription factors, play important roles in regulating plant growth and development. Although GRF gene family has been identified in various plant species, a genome-wide analysis of this family in lettuce (Lactuca sativa L.) has not been reported yet.

Results: Here we identified 15 GRF genes in lettuce and performed comprehensive analysis of them, including chromosomal locations, gene structures, and conserved motifs. Through phylogenetic analysis, we divided LsaGRFs into six groups. Transactivation assays and subcellular localization of LsaGRF5 showed that this protein is likely to act as a transcriptional factor in the cell nucleus. Furthermore, transgenic lettuce lines overexpressing LsaGRF5 exhibited larger leaves, while smaller leaves were observed in LsaMIR396a overexpression lines, in which LsaGRF5 was down-regulated.

Conclusions: These results in lettuce provide insight into the molecular mechanism of GRF gene family in regulating leaf growth and development and foundational information for genetic improvement of the lettuce variations specialized in leaf character.

Keywords: Lettuce, GROWTH-REGULATING FACTOR, Leaf development, MicroRNA396, Genome-wide analysis

Background

As one of the plant-specific transcription factors, GROWTH-REGULATING FACTORs (GRFs) are important regulators in plant growth and development. The first identified GRF functions on gibberellic acid (GA)-induced stem elongation [1]. In various studies, the functions of GRF genes were found in development of leaf, stem, seed and root by regulating cell proliferation or cell expansion to form large organs [1–8], other new functions in flowering, stress response and plant longevity were uncovered recently [9–15]. Two conserved domains, the QLQ (Gln, Leu, Gln, InterPro: IPR014978, PFAM: PF08880) domain, which is considered to be a protein-protein interaction domain, and WRC (Trp, Arg, Cys, InterPro: IPR014977, PFAM: PF08879) domain, which is supposed to be involved in DNA binding, are found in the N-terminal of GRF family proteins [1, 2, 8, 16–18].

GRF family genes are found to be involved in plant growth, development and regeneration. The seedlings with overexpressed miR396-resistant AtGRF1 or AtGRF3
showed shorter roots [9]. BuGRF2 could increase seed weight and oil content by upregulating the expression of chloroplast-related genes in rapeseed (Brassica napus) [19]. In rice (Oryza sativa), OsGRF4 played an important role in grain weight [20, 21]. GRF proteins form functional transcriptional complex with the transcription cofactor GRF-Interacting Factors (GIF) [22], and GRF-GIF chimeras could dramatically boost regeneration in various species [23]. Furthermore, most Arabidopsis thaliana GRF genes play important roles in leaf size control [6–8]. Among the nine AtGRF genes, six GRF genes, AtGRF1, AtGRF2, AtGRF3, AtGRF4, AtGRF5 and AtGRF9, were proved to function in leaf development [3, 4, 12]. Overexpression of AtGRF1 and AtGRF2 respectively cause larger leaves with increased cell size, while atgrf1/2/3 triple mutant showed smaller and narrower leaves [2]. Overexpression of AtGRF5 exhibit bigger leaves due to increased cell number but not cell size [3]. The function of AtGRF5 could not be replaced by other AtGRFs, though some functions of them overlap [3, 12]. AtGRF9 contribute to determining final leaf size, although it has a minor role in cell proliferation [3, 24].

miRNAs, about 20 nucleotides (nt) in length, are single-strand, non-coding, small-molecular-weight RNAs, which could regulate gene expression through target mRNA cleavage or/and translational inhibition [25–27]. Genome-wide analyses reveal that GRFs and a few bZIP transcription factor genes are the major targets of microRNA396 (miR396) [28]. miR396 shares nearly perfect sequence complementarity with the transcript of WRC RNA396 (miR396) [28]. miR396 is expressed in various plant species, including sepal-petal identity, by regulating the expression of GRF genes in Arabidopsis [29, 30]. Correspondingly, miR396a and miR396b regulate leaf growth and development by repressing the expression of AtGRFs [29, 30]. MiR396a regulates flower formation, including sepal-petal identity, by regulating the expression of AtGRF gene [15]. Additionally, the miR396-AtGRF module could also regulate adaxial–abaxial (Ad-Ab) polarity formation during leaf morphogenesis [31].

The ever-developing whole-genome sequencing technology identifies GRF genes in various plant species, such as Arabidopsis thaliana [2], Oryza sativa [16], Zea mays [32], Brassica rapa [33], Solanum lycopersicum [34], Pyrus bretschneideri, Vitis vinifera [35], Brassica napus [36], Nicotiana tabacum [37] and Populus trichocarpa [38]. Lettuce (Lactuca sativa L.) is an important leafy vegetable, the leaf size of which has significant meaning for production. The whole genome sequence of lettuce cultivar ‘Salinas’ has been recently released [39]. However, the GRF gene family in lettuce has not been evaluated yet. In this study, we identified 15 GRF genes in lettuce and respectively named them based on their chromosomal location. They were divided into six groups according to phylogenetic tree of LsaGRFs and AtGRFs. To investigate the transcriptional factor character, LsaGRF5 was specifically studied for its subcellular location and transcriptional activity. In addition, LsaGRF5 was found to be cleaved as a target of miR396. Using transgenic plants, LsaGRF5 could enhance the leaf growth in lettuce and Arabidopsis thaliana when overexpressed, while smaller leaves were obtained through overexpression of Lsa-miR396 in lettuce. These results may provide a foundation for further elucidation of the function of LsaGRFs and Lsa-miR396 in leaf growth regulation in lettuce.

Results

Identification of the GRF genes and miR396s in lettuce

To identify the GRF gene family in lettuce, the GRF genes from Arabidopsis thaliana, rice and tomato were firstly used as the query sequences for BLASTN searching in lettuce genome database. Thus, thirteen candidate GRF genes were obtained in lettuce. GRF protein contains two conserved functional domains, QLQ and WRC. Therefore, QLQ and WRC were used for the Hidden Markov Model (HMM) search, and another two candidate LsaGRF genes were identified. Finally, the amino acids sequences of all fifteen candidate LsaGRF genes were applied for BLASTP searching, and no further hits were got. Thus, there are totally 15 LsaGRF genes identified in lettuce genome. Based on their respective location on the chromosomes, we designated them as LsaGRF1 to LsaGRF15 respectively (Fig. 1A). LsaGRFs were distributed on each chromosome of lettuce, except for chromosome 1 and 7, and the most (five LsaGRFs) occurred on chromosome 6 (Fig. 1A). The basic information of LsaGRF1–15, including gene ID, chromosomal location, length of gene and protein, PI value and exon numbers, was listed in Table 1. Most LsaGRFs had 3 or 4 exons, while LsaGRF4 and LsaGRF9 had 2 exons and LsaGRF7 had 6 exons (Table 1). In addition, the predicted isoelectric point (pl) values of LsaGRF preproteins were between 6 and 9, except that the pl values of LsaGRF4 and LsaGRF7 were higher than LsaGRF9 (Table 1). The amino acid sequence alignment of 15 LasGRF was showed in Fig. S1. All of the 15 LsaGRFs contained the conserved WRC domain, but only 13 LsaGRFs had complete QLQ domain. There is no QLQ domain in LsaGRF4, and LsaGRF7 has only an incomplete QLQ domain (Fig. 1B).

The GRF genes are known as the target of miR396s. To date, two MIR396s in Arabidopsis (Ath-MIR396s) and seven MIR396s in rice (Osa-MIR396s) are identified. There are five MIR396s, MIR396a to MIR396e, in lettuce genome [39]. The phylogenetic trees of five Lsa-MIR396s and MIR396s in lettuce, Arabidopsis and rice were shown in Fig. S2A and B, respectively, based
on their stem-loop sequences. The target sequence of miR396s locates at the end of the WRC domain (Fig. 1C). Although the stem-loop structures were totally distinct, five Lsa-miR396s were highly conserved in the mature region with only two nucleotides difference (Fig. 1C and D). Five Lsa-MIR396s were located on chromosome 1, 5, 7 and 8 respectively, among which chromosome 5 contained two Lsa-MIR396s, Lsa-MIR396a and Lsa-MIR396d (Fig. 1A). The identified GRF genes and miR396s in major species were listed and compared with those in lettuce (Table S1).
To explore the phylogenetic relationship of GRF gene family in different species, phylogenetic analysis, intron-exon and motif characteristics of LsaGRFs were performed. Phylogenetic analysis of GRF family in lettuce was firstly assessed and visualized using a Neighbor-Joining phylogenetic tree (Fig. 2A). All of the 15 LsaGRFs were divided into two groups (including 9 and 6 LsaGRFs respectively), each of which contained two small subgroups in the phylogenetic tree. Gene structure and motifs were considered to have a divergence during gene evolution. Therefore, the gene structures and motifs were listed in phylogenetic tree’s order (Fig. 2B). Most LsaGRF genes contain three or four exons. The LsaGRF genes containing the same number of exons were in the same group. For examples, LsaGRF4 and LsaGRF9 both have two exons. LsaGRF11, 1, 2 and 13 have four exons and the rests in another group have three exons expect LsaGRF7 (Fig. 2B). In conserved motifs analysis, motif 1 (yellow rectangle) and motif 2 (purple rectangle) were present to be the WRC and QLQ protein domain. As shown in Fig. 2B, the LsaGRF proteins in the same branch of phylogenetic tree have similar position and numbers of QLQ and WRC domains. All these analyses showed that the phylogenetic relationship of LsaGRF genes was highly consistent with the gene structures and motif divergence of GRF genes in lettuce.

The functions of GRF genes in Arabidopsis and rice were extensively studied. The phylogenetic relationship of GRF genes in lettuce, Arabidopsis and rice were helpful for putative function prediction of GRF genes in lettuce. There are totally 12 and 9 GRF genes in rice and Arabidopsis, respectively. The Neighbor-Joining phylogenetic tree of GRF genes from lettuce, rice and Arabidopsis showed that there were six groups according to the tree (Fig. 3). It was revealed that the phylogenetic relationship of GRF genes in lettuce, rice and Arabidopsis

| Name   | Accession No.       | Chr  | CDS (bp) | Exon No. | Length (aa) | MW (KDa) | pI   |
|--------|---------------------|------|----------|-----------|-------------|----------|------|
| LsaGRF1| Lsat_1_v5_gn_2_135541.1 | Chr02 | 990      | 3         | 329         | 36.06    | 8.76 |
| LsaGRF2| Lsat_1_v5_gn_3_14660.1  | Chr03 | 1227     | 4         | 408         | 44.51    | 6.24 |
| LsaGRF3| Lsat_1_v5_gn_3_85521.1  | Chr03 | 999      | 4         | 332         | 37.74    | 8.73 |
| LsaGRF4| Lsat_1_v5_gn_3_133500.1 | Chr03 | 702      | 2         | 233         | 27.17    | 9.63 |
| LsaGRF5| Lsat_1_v5_gn_4_92941.1  | Chr04 | 951      | 3         | 316         | 36.58    | 8.81 |
| LsaGRF6| Lsat_1_v5_gn_4_159961.1 | Chr04 | 1551     | 4         | 516         | 56.20    | 7.70 |
| LsaGRF7| Lsat_1_v5_gn_5_54781.3  | Chr05 | 603      | 6         | 200         | 22.70    | 9.27 |
| LsaGRF8| Lsat_1_v5_gn_5_141200.1 | Chr05 | 990      | 3         | 329         | 37.54    | 8.24 |
| LsaGRF9| Lsat_1_v5_gn_6_10680.1  | Chr06 | 459      | 2         | 152         | 16.89    | 8.66 |
| LsaGRF10| Lsat_1_v5_gn_6_17460.1 | Chr06 | 897      | 3         | 298         | 33.12    | 6.44 |
| LsaGRF11| Lsat_1_v5_gn_6_70601.1 | Chr06 | 1113     | 4         | 370         | 40.08    | 8.23 |
| LsaGRF12| Lsat_1_v5_gn_6_75441.1 | Chr06 | 1014     | 3         | 337         | 37.60    | 8.74 |
| LsaGRF13| Lsat_1_v5_gn_6_81681.1 | Chr06 | 1029     | 4         | 342         | 36.78    | 7.09 |
| LsaGRF14| Lsat_1_v5_gn_8_86361.1 | Chr08 | 1071     | 3         | 356         | 40.63    | 8.97 |
| LsaGRF15| Lsat_1_v5_gn_9_78540.1 | Chr09 | 1233     | 4         | 410         | 44.10    | 7.67 |

Fig. 2 Gene structures and motif composition of LsaGRFs. A The gene structures of LsaGRFs. B The motif composition of LsaGRF proteins with MEME. Twenty motifs are represented by different colored boxes. The same color rectangle in different proteins referred to the same motif. The sizes of motifs are proportional to their sequence lengths. The order of LsaGRF genes were based on the phylogenetic tree shown on the left of the figures. The size scales of gene and protein length were indicated at the bottom.
were divergence. There were two groups, group I and VII, harboring GRF genes from lettuce, rice and Arabidopsis, which indicated that these LsaGRFs had putative orthologous genes in both rice and Arabidopsis. In group II and IV, there were just LsaGRFs and AtGRFs, but no GRF genes from rice, while there was no GRF gene from Arabidopsis in group VI. Group III contained only two LsaGRFs, LsaGRF4 and LsaGRF9, but no AtGRFs or OsGRFs, while group V contained three OsGRFs and one AtGRF gene, but no LsaGRF gene. Interestingly, the role of AtGRF5 could not be taken over by other members of AtGRFs, though there were partly overlapping functions between AtGRFs [3, 12]. Therefore, we chose the putative homolog gene of AtGRF5, LsaGRF5, based on the phylogenetic relationship derived from phylogenetic tree for further functional analysis (Fig. 3).

Phylogenetic tree was constructed for 15 Lactuca sativa, 9 Arabidopsis thaliana and 12 Oryza sativa
GRF proteins. There were 7 phylogenetic clusters designated as I–VII. LsaGRFs were written with red fronts. The cluster III that contained GRFs only from lettuce was highlighted with green and the cluster V without LsaGRFs was shaded in red. The LsaGRF5, which was selected for further investigation, were highlighted in yellow. The scale bar represents 0.1 amino acid changes per site.

**Functional characterization of LsaGRF5**

To characterize the putative functions of LsaGRF5, the expression profile of LsaGRF5 was detected. From the quantitative real-time PCR (qRT-PCR) results, the expression levels of LsaGRF5 in roots and leaves were relatively low, while those in the bud were significantly high (Fig. 4A). However, in mature flowers with mature pollens and pistils, the expression level of LsaGRF5 was much lower comparing that in the bud (Fig. 4A), indicating that LsaGRF5 probably function in flower development. Meanwhile, we also detected the expressions of Lsa-miR396a, putatively regulating the expression of GRF genes, in these tissues. We found that Lsa-miR396a were relatively highly expressed in stem, cotyledon and mature flower, while significantly much lower in buds (Fig. 4A). The tissues with high Lsa-miR396a expression, e.g. mature flowers and stems, showed relatively low expression of Lsa-miR396a, indicating that LsaGRF5 might be regulated by Lsa-miR396a.

The LsaGRF proteins are putative TFs. We chose the LsaGRF5 and performed its subcellular location observation and transactivation assay. We isolated the protoplast cell of lettuce and transformed the vector containing 35S:LsaGRF5-GFP and 35S:Ghd7-mCherry, which was reported to locate in nuclear, into lettuce protoplast cells. The empty vector was used for control. As shown in Fig. 4B, green fluorescence of GFP and red fluorescence of mCherry were totally overlapped in the protoplast cell transformed by 35S:LsaGRF5-GFP, indicating that the LsaGRF5-GFP and Ghd7-mCherry have the same nuclear localization. While the protoplast cell transformed by empty vector exhibited ubiquitous green fluorescence, excepting the overlapped region with the red nuclear fluorescence of Ghd7-mCherry (Fig. 4B). Therefore, LsaGRF5 located in the nucleus.

To identify which part of the LsaGRF5 protein had the transcriptional activity, we divided the LsaGRF5 into two parts based on the conserved protein domains. One part is the N-terminal region (1–154 aa) containing QLQ and WRC domains and the other, 155–317 (Fig. 4C). Full-length and two partial LsaGRF5s were constructed into yeast expression vector, pGBD-T7-OsMYB103L, as a positive control and the negative control plastid (pGBKT7). The transformants with different diluted concentrates were dropped on the SD-/trp and SD-/trp/AbA/X-alpha-gal plates. After 2–4 days at 30°C, possible transcriptional activation functions of LsaGRF5-full length and LsaGRF5155–317 were observed.
These recombinant plasmids were transformed into yeast strain Y2HGold. They showed similar growth states without Tryptophan (Trp) under different diluted concentration (Fig. 4D), indicating that the recombinant plasmids were indeed transformed into the yeast cells and the transformation made few influences on the yeast growth. The yeast cells expressed full-length GRF5 and GRF5\textsubscript{155-317} could grow with AbA (Aureobasidin A) and turn blue with X-alpha-galactoside, which were the same as the positive control (Fig. 4D). These results suggested that the C-terminal contributed to the transcriptional activity of LsaGRF5, while the N-terminal containing QLQ and WRC domains did not.

**LsaGRF5 is a miR396a target gene in lettuce**

The GRF gene family is known as the target of miR396 [29, 30]. To verify this in lettuce, we firstly predicted the complementarity between Lsa-miR396 and Lsa-GRFs. Lsa-miR396a shared nearly perfect complementarity with 14 LsaGRFs except LsaGRF9 (Fig. S3). The free energies of duplex structures were all lower than −30 kcal/mol except for LsaGRF9 (−30.6 kcal/mol) (Fig. S3). It means that all LsaGRF genes except LsaGRF9 probably were the targets of Lsa-miR396a. We chose LsaGRF5 for further verification, and performed the 5’ RNA ligase-mediated (RLM) rapid amplification of cDNA ends (RACE) assay. The results showed that the 1～10 bp of the target sequence in LsaGRF5 did not exist in the sequencing results, which means the transcript of LsaGRF5 was cleaved at base 10 of the miR396 target site (Fig. 5). Therefore, LsaGRF5 was probably the target of Lsa-miR396a. Function analysis in vivo could further clarify the regulatory relationship between LsaGRF5 and Lsa-miR396.

**The phenotypes of LsaGRF5 and Lsa-miR396 overexpression lines**

To investigate the function of LsaGRF5, we constructed the overexpression lines in lettuce. LsaGRF5 driven by CaMV 35S promoter was transformed into the lettuce cultivar of Romaine type ‘YDALI’ (YDL). Eleven independent transgenic lines were obtained. Transformation verification was carried out through a pair of primers located on 35S promoter and LsaGRF5, respectively. The results showed that five out of eleven were positive transgenic lines with the same band as the positive control (Fig. 6A). qRT-PCR assay revealed that the expression of LsaGRF5 increased 5～15 folds in these five lines (Fig. 6B). Two lines, LsaGRF5-OE5 and LsaGRF5-OE11, with high LsaGRF5 expressions were used for further phenotypic analysis. The leaves of LsaGRF5-OE5 and LsaGRF5-OE11 were significantly bigger than these of YDL transformed by empty vector (OE-) (Fig. 6C). The bigger leaves also existed in LsaGRF5 overexpressed in Arabidopsis (Fig. S4). From these results, LsaGRF5 could enhance the leaf growth, and this function was conserved in Arabidopsis.

To figure out the functional relevance between Lsa-miR396a and LsaGRF5, we also overexpressed Lsa-miR396a in YDL. Three positive transgenic lines were obtained by PCR of genomic DNA (Fig. 6A). The transcriptional levels of Lsa-miR396a were about 8～15 times higher in overexpression lines compared with negative control (Fig. 6B). Two overexpression lines with high expression level of Lsa-miR396a showed smaller leaves, the opposite phenotype of LsaGRF5-OE lines, and the results revealed that they were suppressed in Lsa-miR396a-OE lines (Fig. 6D). To quantify the leaf growth changes in these transgenic lines, we measured the length and width of the outermost leaves at 10-leaf stage. The leaves in LsaGRF5 overexpression lines were significantly larger than these of YDL transformed by empty vector (OE-) both in length and width (Fig. 6E and Table S2). However, the leaf length and width of Lsa-grm396a overexpression lines were significantly smaller than these of wide type ‘YDL’ (Fig. 6E and Table S2). These results suggested that LsaGRF5 was identified as a regulatory factor in leaf size, while Lsa-miR396a was also found to function in leaf size by the regulation of expression of LsaGRF5.

**Discussion**

GROWTH-REGULATING FACTOR, a type of plant specific transcription factor, plays important roles in plant growth and development. Our studies focus on the genome organization, conservation and function of GRFs in lettuce. 15 GRF genes were identified based on the recently released genome data of lettuce cultivar ‘Salinas’ [39]. The regulatory genes are considered to be preferentially retained after genome duplications [41]. The GRF gene family has subjected to two expansions with one occurred through the whole-genome triplication in the common ancestor of eudicots and the other one occurred during independent whole-genome duplications in various plants [8]. Among the 15 identified LsaGRFs, not all LsaGRF genes contained both QLQ and WRC domains. LsaGRF7 contains only WRC domain, and LsaGRF4 contains two WRC domains but no QLQ domain, which might be the results from whole-genome duplication and recombination, and might play distinct roles in plant growth regulation (Fig. 2B). Interestingly, LsaGRF9, like rice and maize GRF10, had truncated C-terminal [14, 16, 42]. Overexpression of ZmGRF10 may break the homeostasis of GRF/GIF (GRF INTERACTION FACTOR) to affect leaf growth, whether or not
LsaGRF9 functioned in the same way needs to be further addressed [42]. Through subcellular location and transactivation assay, LsaGRF5 probably worked as transcriptional factor in cell nucleus. Notably, AtGRF5 was proved to be able to interact with GIF1 to regulate cell proliferation in leaf primordium [3]. In addition, GIF proteins have recently been reported to play a role in transcription regulation not only by the interactions with GRFs but also with various chromatin remodeling proteins [12, 43]. Whether there were functional GIF genes in lettuce, and if LsaGRF5 could interact with GIF to regulate leaf growth needs further investigation. It is already known that miR396 directly cleaves the GRF genes on their complementary sequence to suppress their expression [30]. In this study, we firstly predicted that there were 14 GRF genes, except for LsaGRF9, as the putative targets of Lsa-miR396 based on the free energies of duplex structures analysis. The cleavage sites of LsaGRF5 were confirmed by 5' RACE in vivo. At the same time, the expression of LsaGRF5 was significantly decreased in Lsa-miR396a-OE lines. Among nine GRF genes in Arabidopsis, AtGRF5 and AtGRF6 were found not to be the target of miR396 [29, 30]. AtGRF5, AtGRF6 and LsaGRF5 belonged to group VII, while LsaGRF9 was in group III (Fig. 3), suggesting that the regulation pattern of miR396-GRFs might be distinct in lettuce and Arabidopsis thaliana. Recently, AtGRF5 was found to function in chloroplast development, nitrogen signaling and senescence, besides leaf development [44]. Therefore, besides some conserved function characteristic of GRF genes, it is valuable to know whether LsaGRF5 has other functions.

Expression profile of genes would help us to predict their potential biological function. The expression patterns of GRFs have been previously investigated. They usually express in growing zones of roots and shoots where cell proliferation occurs [2, 3, 10, 11, 13, 15]. Here, we detected the expression of LsaGRF5 in root, stem, cotyledon, leaf, bud and mature flower, and revealed that LsaGRF5 was highly expressed in bud where cell proliferation occurs violently. Moreover, expression level of AtGRF is suppressed during plant aging [2, 30]. The expression of LsaGRF5 in bud was significantly higher than that in mature flower, which is consistent with previous results that GRFs functioned in the early stages of the growth and development in different tissues [2, 29, 30].
In previous researches, the strong expressions of all \textit{AtGRF} genes were found in the shoot apical region and flower buds, where no morphological changes were observed \cite{2}. Instead, the alterations in leaf growth and development were detected, though the expression levels of \textit{AtGRF} genes were very low in leaves \cite{2}. These results were consistent with those in this study. All \textit{AtGRF} genes have low expression level in leaf and high in root, bud and...
mature flower, which is consistent with our expression results of LsaGRF5 in our manuscript [2]. The expression patterns of other LsaGRF genes should be detected in future work. The overexpressed AtGRF5 could increase the leaf area by increasing the cell number, but not cell area [3]. The LsaGRF5 overexpression lines also exhibited the larger leaves, which caused by increased cell number or area should be deeply investigated.

Conclusion
In this study, we firstly identified all of the GRF gene family members in lettuce. The phylogenetic relationship of these GRF genes in lettuce with their counterparts in Arabidopsis thaliana and rice and conserved motif were evaluated. GRFs were well-known as target genes of miR396s. Therefore, thus is a daily use, we also characterized the chromosomal location of the stem-loop sequences features of Lsa-miR396s. Furthermore, LsaGRF5 could probably function as a transcriptional factor in cell nucleus through subcellular location observation and transactivation assay. Overexpression of LsaGRF5 could stimulate the leaf growth leading to bigger leaves, while overexpression of Lsa-miR396a exhibited smaller leaves with suppressed expression of LsaGRF5. In summary, the expression of LsaGRF5 was regulated by Lsa-miR396 through the cleavage of complementary sequences to control leaf growth. Our findings will facilitate further understanding of the functions of GRF genes and help elucidating the leaf development mechanism in lettuce.

Methods
Plant materials and growth conditions
The lettuce (Lactuca sativa L.) cultivar of Romaine type, cv. ‘YIDALI’ (YDL), was used for transformation in this study. ‘YIDALI’ (YDL) was a commercial variety cultivated by Beijing Vegetable Research Center and the seeds were also provided by Beijing Vegetable Research Center, Beijing Academy of Agriculture and Forestry Science, Beijing, China. The sterilized lettuce seeds were grown on Murashige and Skoog (MS) medium plus 3% sucrose and 0.6% agar (pH 5.8) at 25 °C in a 16-h-light/8-h-dark cycle. The full expanded cotyledons were used for transformation. The transgenic lettuce plants were grown in a growth chamber under a photoperiod of 16-h light (200 μmol m⁻² s⁻¹) and 8-h dark at 25 °C. When the fifth true leaf was fully expanded, the lettuce plants were transplanted into a greenhouse in Beijing Vegetable Research Center under standard greenhouse conditions.

For lettuce transgenic lines, we collected the seeds of T1 progeny from T0 seedlings, which were grown in a growth chamber described above. The T1 seeds of GRF5 overexpression lines were screened on the plate containing Kanamycin. The T1 generation of transgenic lines transformed with the empty vector (OE-) were used as the control and the seeds of it were screened simultaneously. And then the seedlings were transferred to the same size pots for genotyping. The T1 seeds of Lsa-miR396a overexpression lines and wide-type control ‘YDL’ were sown directly in soil in the greenhouse described above without antibiotic screening and then we detected the transformation positive lines using genomic PCR and qRT-PCR. The phenotypes of transgenic positive and wide-type lines were observed in greenhouse.

Identification of GRF genes in lettuce
The genome sequences of lettuce (Lactuca sativa V8) were downloaded from the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). The sequences of Lsa-miR396s were obtained from PmiREN (Plant miRNA Encyclopedia, http://www.pmiREN.com) [45]. The sequences of AtGRFs and OsGRFs were retrieved from the Arabidopsis thaliana Information Resource (http://www.arabidopsis.org/) and China Rice Data Centre (http://www.ricedata.cn/) respectively. The amino acid and nucleotide sequences of AtGRFs and OsGRFs were used for BLASTP and BLASTN (E < 0.01) searching in Phytozome (Lactuca sativa V8) to obtain a list of putative LsaGRF genes. Subsequently, the amino acid sequences of the obtained putative LsaGRFs were iteratively used for BLAST searching. The conserved protein domains in the GRF proteins, including QLQ (PF08880) and WRC (PF08879), were used for searching in the lettuce Protein Database in GRAMENE [46], and the protein domains of LsaGRFs were confirmed in the Pfam database (http://pfam.sanger.ac.uk/) (E-value < 1 × 10⁻⁴) [47]. Finally, the results were supplemented using the HMMER software.

Characterization of LsaGRFs and miR396s
The molecular masses of the putative GRF proteins were calculated using the Compute pi/Mw tool of ExPaSy (http://web.expasy.org/compute_pi/). Schematic LsaGRF gene structure diagrams were drawn using the Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/). Protein sequence motifs were predicted using the MEME program (http://meme.sdsc.edu/meme/). The physical position of each LsaGRF gene on the ten Chinese cabbage chromosomes was determined from the Chinese cabbage database (BRAD, http://Chinesecabbagegd.org/brad/) and marked on each chromosome using the MapInspect software (http://mapinspect.software.informer.com). The combination of phylogenetic tree, gene, and protein structures was generated using the iTOL tool (http://itol.embl.de) [48].
**Phylogenetic analysis**

To construct the phylogeny of the GRFs from various species, multiple sequence alignments for all GRF amino acid sequences were conducted using MEGA 7.0 with default settings [49]. Phylogenetic analyses were carried out with a Neighbor-Joining method using MEGA 7.0 [49].

**Lettuce protoplast isolation and subcellular location of LsaGRF5**

The coding sequence of LsaGRF5 was cloned into the pSAT6-EGFP-N1 vector. The 35S:LsaGRF5-GFP and 35S:MIR396a-GFP plasmids (1 μg/μL, 5 μL each) were transformed into protoplasts of the lettuce ‘YDL’ by means of polyethylene glycol treatment [50]. Transformed protoplasts were observed using a fluorescence microscope (Leica TCS SP5). Images were analyzed with Image LAS AF software. Ghd7-mCherry was used as controls for nuclear [51]. Primers used were listed in Table S3.

**Transactivation assay based on the yeast GAL4 system**

The transcriptional activity of LsaGRF5 was evaluated in yeast cells. The full-length coding sequence, N-terminal LsaGRF5 DNA-binding domain (1–154 aa) and the C-terminal putative activation domain (155–317 aa) were (respectively) cloned into pBD-GAL4 vector. The empty vectors pGBKT7 and GAL4 were used as negative and positive controls, respectively. All of these constructs were individually introduced into cells of yeast strain Y2HGold containing the AUR1-C and MEL1 reporter genes. Yeast cell transformation was carried out using the instructions in the Yeast maker™ Yeast Transformation System 2 User Manual. The yeast transformants were grown on SD/−Trp and SD/−Aba and X-alpha-gal plates for 2–4 d at 30 °C to identify transactivation activity (Yeast Protocols Handbook; Clontech, Mountain View, CA, USA). Primers used were shown in Table S3.

**RNA extraction and qRT-PCR**

Total RNA was extracted from the leaves using a plant RNAeasy kit (Tiangen, Beijing, China). RNA was reverse transcribed into cDNA with a PrimeScript™ RT reagent Kit (Takara, Osaka, Japan). Real-time PCR reactions were performed using the SYBR Green I Master Mix and were quantified in a Light Cycler 480 II instrument (Roche, Basel, Switzerland). The PCR program comprised an initial step at 94 °C for 30 s, followed by 40 cycles of 94 °C for 10 s and 58 °C for 30 s. Amplification was followed by heating for 1 min at 60–95 °C for melting curve analysis. Each reaction was performed with three replications using 5 μL of Master Mix, 0.25 μM of each primer, 1 μL of diluted cDNA, and DNase-free water to a final volume of 10 μL. Three biological replicates were collected for each sample. According to Yu et al. 2020 [52], the lettuce actin genes were used as internal controls to normalize the transcript levels of target genes. Relative gene quantification was calculated by the comparative ΔΔCt method [53]. The average 2−ΔΔCt values were used to determine differences in gene transcript levels. The lengths of PCR products were among 300 to 500 bp. The PCR products were sequenced in a commercial DNA sequencing company. The primers were designed using the Primer Premier 6.0 software and were shown in Table S3.

**Plasmid construction and plant transformation**

The cloning primers of LsaGRF5 and Lsa-MIR396a were designed based on the genome sequences of lettuce (Lactuca sativa V8) from the Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). The coding sequences of LsaGRF5 and Lsa-MIR396a were cloned from the lettuce ‘YDL’ and constructed into the overexpression vector pEZR(K)-LC [54] driven by 35S promoter using 2 X Seamless Cloning Mix (Biomed, CL117–01, Beijing). The primers used for vector construction were listed in Table S3. The recombinant constructs were transformed into Lettuce using Agrobacterium-mediated transformation following the leaf disk method [55]. The seeds sterilized with 10% bleach were sowed on Murashige and Skoog MS media at 22 °C, 16-h-light/8-h-dark, in a growth chamber. After 5 days, cotyledons were cut and immersed in a suspension of Agrobacterium (OD600 = 0.6) for 15 min and then were transferred to the co-cultivation media (MS with 1 mM acetosyringone) in the dark for 2 days. The co-cultivated cotyledons were subjected to the selection and shoot-inducing media (1x MS, 0.1 mg/L 1-naphthylacetic acid, 0.1 mg/L 6-BA, 50 mg/L Kan, 300 mg/L Timentin) at 22 °C for about 2 weeks. The young buds were transferred to the root-inducing media (MS, 300 mg/L Timentin). Finally, the resistant seedlings were transferred to soil and further verified by PCR and qRT-PCR.

**MiR396 cleavage site analysis**

A 5′ RLM-RACE was used to detect the miR396 cleavage site in miR396 target genes and was performed accordingly [56]. Total RNA of ‘YDL’ leaves was extracted using a plant RNAeasy kit (Tiangen, Beijing, China). Then 5′ adaptor ligation RNA was prepared according to the kit (NEB, M0204, MA, USA). The fist chain cDNA was synthesized based on PrimeScript™ RT reagent Kit (Takara, RR047, Osaka, Japan). Furtherly, the RLM-RACE reactions were amplified with the enzyme TKS (Takara, Cat# AI51320A, Osaka, Japan) and 2 X TransStart Fast-Pfu PCR SuperMix (TransGene, Cat# AS221–01, Beijing,
Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). The sequences of Lsa-miR396s were obtained from PmiREN (Plant miRNA Encyclopedia, http://www.pmiren.com). All of the datasets supporting the results of this article are included within the article and its additional files. The phylogeny data were uploaded to the TreeBASE repository (https://www.treebase.org). The accession number is 28704 and the data could be checked on the website: http://purl.org/phylo/treebase/phylovs/study/TB2:528704.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Plant ethics
Experimental materials were obtained from Beijing Vegetable Research Center and we have got the permission to use. Experimental research and field studies comply with Beijing Vegetable Research Center guidelines. The wide-type lettuce used in this study was a commercial variety cultivated by the Beijing Vegetable Research Center and was not deposited in a publicly available herbarium.

Authors’ contributions
D. L, D. Z, and B. Z. designed experiments. B. Z, Y. T, Z. S, and D. Z. identified and characterized the GRF genes family in lettuce. B. Z, Y. T, K. L, Z. Z, and X. L carried out the experiment. B. Z, and D. L. wrote the paper. All authors discussed the results and commented on the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The genome and protein sequences of lettuce (Lactuca sativa V8) were downloaded from the Phytozone (https://phytozone.jgi.doe.gov/pz/portal.html). The sequences of Lsa-miR396s were obtained from PmiREN (Plant miRNA Encyclopedia, http://www.pmiren.com). All of the datasets supporting the results of this article are included within the article and its additional files. The sequences of AtGRFs and OsGRFs were retrieved from the Arabidopsis thaliana Information Resource (http://www.arabidopsis.org/) and China Rice Data Centre (http://www.nicedata.cn/) respectively. The sequences, alignments and phylogeny data were uploaded to the TreeBASE repository (https://www.treebase.org). The accession number is 28704 and the data could be checked on the website: http://purl.org/phylo/treebase/phylovs/study/TB2:528704.

Supplementary Information
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Additional file 1: Figure S1. The amino acid sequences alignment of LsaGRF genes. Figure S2. Phylogenetic analysis of Lsa-miR396s. Figure S3. The degree of Lsa-miR396a complementarity to all LsaGRFs. Figure S4. The phenotypes of overexpression lines of LsaGRFs in Arabidopsis.

Additional file 2: Table S1. Number of GRF genes and miR396s have been identified in different species.

Additional file 3: Table S2. The length and width of LsaGRF5-OE and Lsa-miR396a-OE leaves.

Additional file 4: Table S3. The sequences of Primers used in this study.

Additional file 5. The original gel images used in this article.
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