

# Gene Characterization and Protein Expression Analysis of *LsARF2* in Lettuce (*Lactuca sativa* L.) Under High Temperature

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## Abstract

In this study, our objective is to investigate the gene characteristics of *LsARF2*, an auxin response factor in leaf lettuce, and analyze how its protein expression responds to high temperature. To achieve this, we cloned the *LsARF2* gene and utilized subcellular localization and yeast one-hybrid assays to determine its subcellular location and transcriptional activity. Subsequently, Western blot analysis was employed to track the protein expression pattern of *LsARF2*. The findings unveiled that the coding sequence of the *LsARF2* gene spans 2196 base pairs, encoding 731 amino acids. Although *LsARF2* was found localized in the cell nucleus, it demonstrated no transcriptional activation activity. Remarkably, the protein expression of *LsARF2* exhibited a noticeable downward trend when subjected to elevated temperatures. Our research underscores the substantial impact of elevated temperatures on the protein expression of *LsARF2* in leaf lettuce. The responsiveness of *LsARF2* protein expression to high-temperature stress suggests its pivotal role in bolting. Further investigation through transgenic methods will be essential to conclusively elucidate its precise function. Additionally, following exogenous hormone treatment, the shifting expression pattern of *LsARF2* implies its potential responsiveness to both GA<sub>3</sub> and IAA. Moreover, it exerts an influence on the floral development of lettuce.

**Keyword:** leaf lettuce, high temperature, *LsARF2*, subcellular localization, protein expression, exogenous hormone

## 1. Introduction

With the continuous growth of the global population, the quantity and quality of crops have become increasingly critical for ensuring stable and sustainable food supply. Lettuce (*Lactuca sativa* L.), as an essential vegetable, is widely utilized in human diets. However, during the cultivation of lettuce, the phenomenon of bolting often leads to reduced yields, significantly impacting agricultural productivity. Therefore, investigating the mechanisms behind high-temperature-induced bolting in leaf lettuce and developing cultivation management strategies to regulate bolting timing, preventing or delaying bolting, and ensuring year-round production, holds significant theoretical guidance and practical application prospects.

Early bolting in lettuce refers to the premature formation of flower stalks and inflorescences in lettuce plants under specific environmental conditions, thereby affecting normal leaf growth and yield accumulation. Bolting in lettuce is a complex physiological phenomenon influenced by various factors, including temperature, plant hormones, photoperiod, light intensity, plant age, and genetic factors. Temperature is one of the most significant factors affecting lettuce bolting. Higher temperatures often induce lettuce plants to enter the flowering stage prematurely, leading to bolting (Hurkman et al., 2009). Optimal growth temperatures help maintain leaf growth in lettuce, while excessively high temperatures may trigger flowering responses (Hao et al., 2022). Plant hormones, especially auxins (IAA), abscisic acid (ABA), gibberellins (GA), play crucial roles in regulating lettuce bolting (Aloni et al., 2009; Li et al., 2021; Wang et al., 2022). Photoperiod refers to the duration of alternating light and darkness and plays a significant role in plant growth and flowering (Imaizumi & Kay, 2006). Some lettuce varieties are prone to bolting under short day conditions, while they tend to prioritize leaf growth

under long day conditions. Light intensity also impacts lettuce flowering (Aloni et al., 2009). Excessive or insufficient light intensity can both lead to flowering initiation and subsequent bolting. The health of the plants also influences bolting tendencies. Plants stressed by factors such as pests and diseases may be more prone to entering the flowering stage (Hwang et al., 2019).

While temperature is a major factor influencing lettuce bolting, its molecular regulatory mechanisms remain incompletely understood. Past research indicates that plants adjust their growth and developmental patterns in response to environmental changes by initiating a series of signal transduction pathways. These pathways often involve the participation and coordination of multiple genes. The FT (FLOWERING LOCUS T) gene and SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1) gene are regulatory genes involved in plant flowering (Wickland & Hanzawa, 2015; Richter et al., 2019). Temperature can impact the expression levels of FT and SOC1 genes (Wendell et al., 2017). In some plants, higher temperatures may promote the expression of the FT gene, leading to the premature initiation of flowering.

Plant hormone auxin plays a crucial role in regulating plant growth and development, with its endogenous form primarily being indole-3-acetic acid (IAA) (Gomes & Scortecci, 2021). There are two pathways for artificial synthesis of plant auxin, relying on the synthesis of tryptophan (Trp). Under high temperatures, the hypocotyls and inflorescence stems of *Arabidopsis* seedlings significantly elongate, and the concentration of IAA increases upon seedling emergence, indicating a correlation between high temperature-induced elongation of hypocotyls and auxin. Recent research suggests that elevated temperatures upregulate the expression of the plant auxin synthetic gene *YuC8*, and *Arabidopsis atyuc8* mutants control the elongation of hypocotyls under high temperatures. This indicates that utilizing the plant auxin synthetic pathway can increase the plant's auxin concentration, thereby inducing hypocotyl elongation (Hu & Xu, 2016; Hu et al., 2003).

Apart from the *YuC8* gene, other genes are also involved in regulating hypocotyl and inflorescence stem length via auxin. For instance, auxin-induced genes ARGOS (Auxin-Regulated Gene Involved in Organ Size) and IAA-Ala Resistant3 (IAR3) play essential roles in *Arabidopsis*. ARGOS limits hypocotyl and inflorescence stem length by suppressing auxin signal transduction, while IAR3 participates in auxin biosynthesis to regulate endogenous auxin levels (Oh et al., 2014; Bowers et al., 2014). Furthermore, the ARF (Auxin Response Factors) family is a significant component of the auxin signal transduction pathway, capable of regulating hypocotyl and inflorescence development (Guilfoyle & Hagen, 2007; Xing et al., 2011). Beyond auxin response to high temperatures, auxin also responds to other environmental factors such as light, nutritional status, and plant hormones by modulating plant growth and development. For instance, under low-temperature conditions, auxin promotes hypocotyl elongation in *Arabidopsis* seedlings and achieves this process through gene expression regulation (Lee et al., 2019). Additionally, auxin also participates in plant reproductive development, regulating the growth and differentiation of floral organs. In flower development, auxin, through the regulation of ARF family genes, participates in the development of anther and ovary, influencing flower structure and morphology (Moubayidin, Ostergaard, & Voß, 2018; Goetz et al., 2006).

The signaling pathway of auxin is a complex network that involves the plant's response to various environmental signals. High temperature is a common stress factor in plant growth and development, and it has a significant impact on the regulation of the auxin signaling pathway. The auxin signaling pathway is primarily regulated through the degradation of the auxin-SCF E3 ubiquitin ligase complex mediated by TIR1/AFB proteins (Qi et al., 2022). Upon binding to TIR1/AFB, auxin promotes the degradation of Aux/IAA proteins, releasing ARF proteins for DNA binding and transcriptional activation. Under high-temperature stress, the auxin signaling pathway undergoes multifaceted regulation.

Firstly, high temperature affects auxin synthesis and distribution. Synthesis and transport of auxin are inhibited under high-temperature conditions, leading to a decrease in auxin levels and consequently impacting the normal functioning of the auxin signaling pathway (Sakata et al., 2010; Goetz et al., 2006). In wheat inflorescences exposed to high temperature, the expression of *TAA1* and *YUC* genes decreases significantly, inhibiting the auxin biosynthesis pathway. This results in an upregulation of *PIN* and *LAX* gene expression, possibly to increase auxin import and transport to compensate for the impaired auxin biosynthesis pathway (Higashitani, 2013). High temperature can also enhance the activity of other ubiquitin ligases in the auxin pathway, further regulating the auxin signaling pathway (Del Pozo & Manzano, 2014).

Lastly, high temperature can affect other components of the auxin signaling pathway. For instance, under high-temperature conditions, the degradation of Aux/IAA proteins and the transcriptional activity of ARF proteins can be inhibited, thereby affecting the normal functioning of the auxin signaling pathway (Zenser et al., 2001; Lv et al., 2020).

The ARF (AUXIN RESPONSE FACTOR) family comprises a considerable number of members, each exhibiting substantial functional variations. *Arabidopsis thaliana*, as a model plant, has witnessed in-depth investigations into *AtARFs*. Within *Arabidopsis*, a total of 23 *AtARF* genes have been identified, with 22 being full-length ARF genes; *AtARF2,3* contains a premature stop codon within its DNA-binding domain (DBD), rendering it a putative pseudogene (Lv et al., 2020; Remington et al., 2004; Okushima et al., 2005). Research has found that mutations in *AtARF2* lead to delays in processes such as flowering, senescence of cauline leaves, floral organ abscission, and fruit maturation (Ellis et al., 2005). Conversely, mutations in *AtARF1* enhance the phenotypes of *AtARF2*, suggesting partial functional redundancy between *AtARF1* and *AtARF2*. *AtARF1* mutations also elevate the expression of *Aux/IAA* genes, hinting at its role as a transcriptional repressor. Schruff (Schruff et al., 2006), through the study of *AtARF2* loss-of-function mutants, observed enlarged seeds and accelerated cell division and elongation across various organs, suggesting a role for *AtARF2* as an inhibitor of cell division and elongation. *AtARF3* significantly impacts the formation of floral organs in *Arabidopsis*; mutations in this gene result in increased sepal numbers, decreased stamen numbers, and defects in apical development (Ellis et al., 2005). Research indicates the involvement of *AtARF5* in embryo pattern formation and vascular tissue development in *Arabidopsis* (Cole et al., 2009). Both *AtARF6* and *AtARF8* play roles in regulating flower development (Tabata et al., 2010). Beyond *Arabidopsis*, the study of the ARF family extends to numerous other species. In maize, 31 ARF gene family members have been identified, with 14 containing auxin-responsive elements in their promoter regions, and 18 *ZmARF* genes are predicted as potential targets of small RNAs (Goetz et al., 2006). *OsARF1* represents the first full-length ARF family gene cloned from a monocot; it exhibits significantly higher expression in rice embryo tissues compared to nutritional tissues. Silencing *OsARF1* leads to phenotypes including weak growth, small and curled leaves, and sterility (Tian et al., 2005). De et al. found that overexpressing *SLARF9* transgenic plants produce smaller fruits due to reduced cell division activity, while silencing *SLARF9* plants exhibit contrasting phenotypes. This indicates that *SLARF9* negatively regulates cell division activity during the early stages of tomato fruit development. *SLARF2* is implicated in regulating tomato fruit ripening processes (Xing et al., 2011). In recent years, with the advancement of whole-genome studies in various species, ARF family members have been sequentially identified in plants such as *Arabidopsis*, tomato, cucumber, barley, pineapple, and peach, encompassing 23, 21, 15, 20, 20, and 18 family members respectively (Attia et al., 2009; Tiwari et al., 2003; Jong et al., 2015; Hao et al., 2015; Wu et al., 2011; Liu et al., 2013). These findings establish a solid foundation for investigating the functions and mechanisms of ARF transcription factor families in plant growth and development processes.

Previous studies have predominantly focused on the impact of ARF2 on plant morphogenesis, leaf shape, flower and fruit development, with limited reports on ARF2's response to high temperature. In this experiment, through gene cloning, subcellular localization, transcriptional activation analysis of the target gene *LsARF2*, and protein expression analysis after exposure to high temperature, we aim to uncover *LsARF2*'s response to high-temperature stress. As earlier research has already established the induction of bolting in leaf lettuce by high temperatures (Bing et al., 2019), the current study will provide a solid foundation for further delving into the relationship between *LsARF2* and high-temperature-induced bolting in leaf lettuce.

## 2. Materials and Methods

### 2.1 Plant Materials

Leaf lettuce variety GB-30 was selected for this study. Fully developed lettuce seeds were germinated by sowing pale seeds into a 50-cell tray. Germination occurred after approximately 3 days. The leaf lettuce seedlings, with 2 to 3 true leaves, were transplanted into 6 cm × 8 cm nutrient pots. When the seedlings reached the growth stage of 6 leaves and 1 heart, they were subjected to high-temperature treatment. The seedlings were divided into two groups: the control group was exposed to a day/night temperature of (20±2) °C/(13±2) °C, while the high-temperature group was exposed to a day/night temperature of (33±2) °C/(25±2) °C, with other conditions remaining the same. The seedlings were cultivated until reaching a certain size [19]. The entire plant was used as experimental material for cloning the gene. Tobacco (*Nicotiana tabacum*) plants were cultivated until they reached an appropriate size (4-6 true leaves).

### 2.2 Bacterial Strains and Plasmids

*Escherichia coli* strain DH5α in competent state was obtained from Baorui Doctor Biotechnology (Beijing) Co., Ltd. The yeast strain AH109 in competent state and plasmids PBI-121, pCambia1304, pFGC5941, and PGK7 (all of which are preserved in our laboratory) were used in this experiment.

### 2.3 Phylogenetic Relationships Analysis of *LsARF2*

The ARF2 of sesame, tomato, and other species were obtained in the corresponding plant database, and the species database was downloaded from Phytozome V13 (<https://phytozome-next.jgi.doe.gov/>). Next, all ABF protein sequences were aligned using MEGA7.0 software. The construction of phylogenetic tree was completed by MEGA 7.0 software using maximum likelihood method and 1000 boot-straps, and beautified on iTOL (<https://itol.embl.de/>).

### 2.4 Cloning of *LsARF2*

Based on the sequence information, primers for cloning the full-length coding sequence (CDS) of *LsARF2* were designed. The *LsARF2* forward primer was 5'-ATGACATCTTCAGAGGTTTC-3', and the reverse primer was 5'-CTAAACATCCTCAGGACTTG-3'. The *LsARF2* CDS was amplified using leaf lettuce cDNA as a template.

### 2.5 Subcellular Localization of *LsARF2*

Designing homologous arms based on the vector information of pBI121, selecting NdeI and SalI enzyme cutting sites, and designing homologous primers for cloning the full-length *LsARF2* CDS. *LsARF2* Forward primer: 5'-agaacacgggggactctagaATGACATCTTCAGAGGTTTC-3'; Reverse primer: 5'-gactgaccaccgggggatccCTAAACATCCTCAGGACTTG-3'. Amplifying the *LsARF2* gene CDS full length containing homologous arm sequences using cDNA from leaf lettuce stems as a template, isolating and recovering the target band after gel electrophoresis. The recovered product is stored at -20 °C for later use.

### 2.6 Construction of Subcellular Localization Vector for *LsARF2*

The plant expression vector pBI121-GFP was digested using the restriction enzymes NdeI (#R0111V) and SalI-HF (#R3138V) to perform double digestion, resulting in the linearization of the vector. This linearized pBI121-GFP vector was then mixed with *LsARF2* gene fragment in a 1:3 molar ratio in a 0.2 mL PCR tube for homologous recombination. The reaction mixture contained 2× Seamless cloning Mix (5 µL), approximately 80 ng of linearized vector, 50-100 ng of insert fragment, and was supplemented with ddH<sub>2</sub>O to a final volume of 10 µL. The recombination reaction was carried out at 50 °C for 45 minutes. The resulting ligation products were then transformed into competent *Escherichia coli* DH5α cells. The recombinant plasmid was named pBI121-*LsARF2*. Samples of the transformed bacteria were sent to the sequencing department at Beijing Genomics Institute for sequencing. Upon successful sequencing, the samples were used for plasmid extraction. The extracted plasmids were streaked onto new resistance plates, and the bacterial growth was preserved by mixing with 40% sterilized glycerol in a 1:1 ratio and storing at -80 °C for future use.

### 2.7 *Agrobacterium*-Mediated Transient Transformation of Tobacco

Inoculate *Agrobacterium* strains containing recombinant plasmids pBI121-*LsARF2*, pBI121-*LsARF5*, and pBI121-*LsARF8*, as well as *Agrobacterium* strains containing the empty vector pBI121-GFP, into LB liquid medium supplemented with rifampicin (25 mg·L<sup>-1</sup>) and kanamycin (50 mg·L<sup>-1</sup>). Incubate the cultures overnight at 28 °C with agitation at 200 rpm for activation. Repeat this activation step once.

When the OD<sub>600</sub> of the bacterial cultures reaches approximately 0.8, centrifuge at 2000 rpm for 10 minutes and remove the supernatant.

Resuspend the bacterial pellets in 10 mL of suspension buffer, then centrifuge again and discard the supernatant. Repeat this wash step.

Resuspend the bacterial pellets in infiltration solution and let them stand at room temperature for about 3 hours.

Dilute the infiltration solution to an OD<sub>600</sub> of approximately 0.5 just before infiltration.

Select healthy and mature leaves (upper leaves) and use a 2 mL syringe to inject the backside of tobacco leaves with the bacterial suspension, about 200-500 µL per injection, ensuring the entire leaf surface is covered with the suspension. The leaves should appear moist, resembling a water-soaked appearance.

Healthy and mature tobacco leaves (upper leaves) were selected. Using a 2 mL syringe, the backside of the tobacco leaves was injected with the bacterial suspension, approximately 200-500 µL, until the entire leaf was covered with the suspension, making the leaf moist and appearing water-soaked. After two to three days, when the expression of the fused protein was relatively high, the infiltrated leaves were cut into small squares of about 0.5 cm in size. Cell nuclei were stained using a 5 mg·L<sup>-1</sup> DAPI solution. The plant material was immersed in the DAPI solution for 20-30 minutes, and then removed to create slices. These slices were observed and photographed using a confocal laser scanning microscope.

### 2.8 Validation of Transcriptional Activation Activity of *LsARF2*

According to the sequence structure features and considering the positions of introns and exons, primers for homologous cloning of the full-length CDS and both C-terminal and N-terminal sequences of *LsARF2* were designed (refer to Supplementary Table 3). Using cDNA from leaf lettuce GB30 stems as a template, the aforementioned gene sequences were cloned. Employing homologous cloning technique, the target fragments were ligated into the pGBKT7 vector, resulting in the construction of yeast one-hybrid recombinant plasmids, namely pGBKT7-*LsARF2*, pGBKT7-*LsARF2N*, and pGBKT7-*LsARF2C*.

The constructed plasmids were then transformed into competent yeast strain AH109 using the following procedure:

- (1) Thaw 100  $\mu$ L of yeast strain AH109 in an ice bath. Sequentially add 2-4  $\mu$ g of pre-chilled target plasmid, 10  $\mu$ L of Carrier DNA (denatured at 95  $^{\circ}$ C for 5 min, rapidly cooled on ice, repeated once), and 400  $\mu$ L of liquid culture medium (PEG/LiAc) to a sterile 1.5 mL microcentrifuge tube. Gently mix by pipetting up and down, and place the tube in a 30  $^{\circ}$ C water bath for 30 minutes, gently swirling every 10 minutes.
- (2) Transfer each microcentrifuge tube to a 42  $^{\circ}$ C water bath for 15 minutes, gently swirling every 5 minutes.
- (3) Centrifuge at 5000 rpm for 1 minute, remove the supernatant, and resuspend the pellet in 500  $\mu$ L of sterile ddH<sub>2</sub>O. Centrifuge again for 1 minute, and discard the supernatant.
- (4) Resuspend the pellet in 100  $\mu$ L of sterile ddH<sub>2</sub>O. Take 50  $\mu$ L from each suspension and spread onto SD-T solid culture medium. Place the plates in a 30  $^{\circ}$ C constant-temperature incubator, and invert them for 2-3 days.

Refer to the instructions for AH109 competence transformation method for specific steps.

- (5) After bacterial colonies appear on solid SD-T medium, observe growth and capture images. Pick a single clone and culture it in 500  $\mu$ L of liquid SD-T medium at 30  $^{\circ}$ C and 200 rpm for 2-3 days. Perform PCR validation for each culture and confirm successful sequencing, indicating the presence of recombinant plasmids pGBKT7-*LsARF2*, pGBKT7-*LsARF2N*, pGBKT7-*LsARF2C*, and empty vector pGBKT7 in yeast.

- (6) Apply the turbid yeast cultures onto solid SD-THA medium in a streaking manner, and add X- $\alpha$ -gal for coloration. Place the plates in a 30  $^{\circ}$ C constant temperature incubator, invert them, and cultivate for 2-3 days. Observe the coloration reaction and capture images.

### 2.9 *LsARF2* Western Blot

Samples of leaf lettuce stems subjected to high-temperature treatment for 0, 8, 16, and 24 days were prepared. OsACT1 was selected as the internal reference protein, and Goat Anti-rabbit IgG/HRP was used as the secondary antibody. Total protein was extracted from the plant material, followed by SDS-PAGE gel electrophoresis, protein transfer to a membrane, immunoblotting (blocking with blocking solution, primary antibody incubation, secondary antibody incubation), and visualization using the NBT/BCIP staining method. The samples were collected at specific time points to assess the expression of *LsARF2* under high-temperature conditions. The Western blot protocol was executed following laboratory safety and procedure guidelines, while adhering to the instructions provided by reagents and equipment used.

### 2.10 Exogenous Hormone Spraying

The exogenous hormone spraying experiment on leaf lettuce included four treatments: spraying with 40 mg/kg indole-3-acetic acid (IAA); spraying with 25 mg/kg gibberellic acid (GA<sub>3</sub>); combined spraying with 40 mg/kg IAA and 25 mg/kg GA<sub>3</sub>; and control group sprayed with water (CK). Each treatment included 30 lettuce plants. For the treatment with IAA alone, samples were collected at 0, 2, 4, 6, 8, 12, 18, and 24 days after spraying. For the treatments with GA<sub>3</sub> alone and combined GA<sub>3</sub> and IAA spraying, samples were collected at 0, 2, 4, 6, 8, 12, and 16 days after spraying. The control group sprayed with water was sampled at 0, 2, 4, 6, 8, 12, 16, 18, and 24 days after spraying. Sampling was performed on stems and leaves, with stem samples taken from the upper part within 1 cm below the stem tip and leaf samples collected from the third to fourth leaves below the stem tip.

### 2.11 RNA Extraction and Quantitative Real-Time PCR Analysis (qRT-PCR)

The samples obtained from the exogenous hormone spraying experiment were used in this study. Total RNA was extracted from collected samples, using Fast Pure Universal Plant Total RNA Isolation Kit (Vazyme, Nan Jing). Synthesis Super Mix (Trans Gen Biotech, Beijing, China) was used to synthesize the first-stand cDNA of samples, which was then subjected to a 5-fold dilution series (1, 1/10, 1/100, 1/1000, 1/10000) for fluorescent quantitative PCR reactions. The reaction program was as follows: 95  $^{\circ}$ C denaturation for 3 min; 95  $^{\circ}$ C denaturation for 20 s, 53  $^{\circ}$ C annealing for 20 s, 72  $^{\circ}$ C extension for 20 s (40 cycles); finally, from 65  $^{\circ}$ C to 95  $^{\circ}$ C,

with an increase of 0.5 °C per step and a 30 s hold at each step to detect fluorescence values and generate melt curves. The reaction program for the reference gene was the same as that for the target gene, with each sample run in triplicate. Calculation and graphing were performed using the 2- $\Delta\Delta C_t$  method. The internal reference gene used was 18S ribosomal RNA of lettuce.

### 2.12 Data Analysis

The data obtained from quantitative real-time PCR were calculated using the 2- $\Delta\Delta C_t$  method [104]. Statistical analysis was performed using the SPSS 12.5 software (International Business Machine, Chicago, IL) with one-way analysis of variance (ANOVA) ( $\alpha = 0.05$ ). Graphs were generated using Origin9 software (Origin Lab, Northampton, MA, USA).

## 3. Results

### 3.1 Obtaining the Full-Length *LsARF2* Gene in Leaf Lettuce

PCR amplification yielded a specific band of *LsARF2* with a length of 2196 bp (Figure 1). The sequencing results showed 100% consistency with the sequence in the lettuce gene database, indicating the successful cloning of the CDS sequence of *LsARF2*. Further analysis using NCBI ORFfinder (<https://www.ncbi.nlm.nih.gov/orffinder/>) revealed that this gene encodes 731 amino acids.

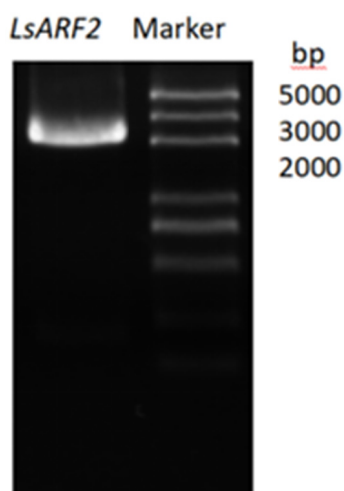


Figure 1. The amplification of *LsARF2* genes

### 3.2 Evolutionary Tree Analysis of *LsARF2* Protein

The amino acid sequence of obtained *LsARF2* was aligned against the NCBI protein database, revealing significant homology with ARF2 proteins from 10 plant species including sunflower and Arabidopsis. Using MEGA7.0 software, an evolutionary tree was constructed to analyze the amino acid sequence of *LsARF2*. The sequences in the tree can be classified into three categories: tomato and sesame form one group, *LsARF2* and sunflower are in a separate group due to their close relationship as members of the Asteraceae family, and Arabidopsis and rubber tree belong to another category (Figure 2)

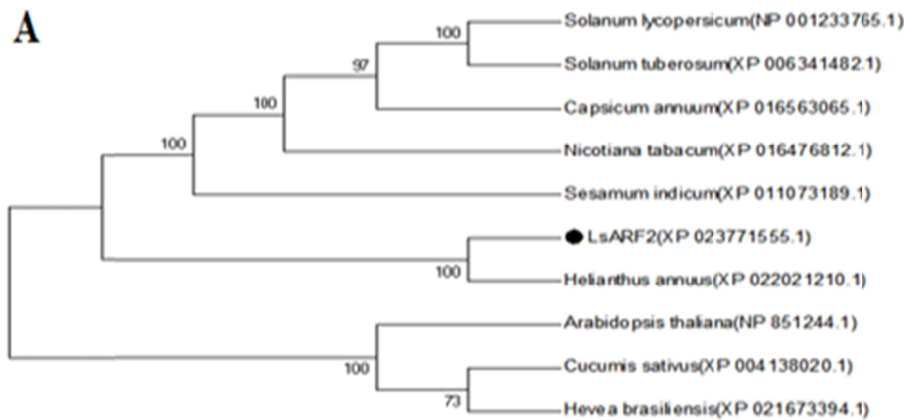


Figure 2. Phylogenetic Analysis of *LsARF2*

### 3.3 Bioinformatics Analysis of *LsARF2*

The amino acid sequence encoded by the *LsARF2* gene was subjected to analysis using the ProtParam software. The predicted molecular weight of the protein was 81.8 kDa, and its theoretical isoelectric point was 6.5. Classified as a hydrophilic protein, the subcellular localization of the *LsARF2* protein was determined using the Psort software, revealing a primary localization within the cell nucleus.

Conducting a conservation motif analysis on the amino acid sequence of *LsARF2* using the MEME software, a total of 8 conserved motifs were identified. Among these motifs, Motif 1 and Motif 5 were situated within the DBD domain of *LsARF2*, while Motif 2 and Motif 4 were positioned within the MR conserved domain of *LsARF2*. Furthermore, Motif 6 and Motif 8 were observed within the CTD domain. These findings indicate that *LsARF2* exhibits highly conserved motifs within the typical three conserved domains of the ARF protein family.



Figure 3. Conservative motif analysis of LsARF2

### 3.3 Subcellular Localization Analysis of LsARF2

The figure depicts the subcellular localization experiment of *LsARF2* conducted using the tobacco leaf back-injection method. DAPI staining is shown in blue, marking the position of cell nuclei, while the green color represents green fluorescent protein (GFP) as well as the fusion protein of *LsARF2* and GFP. According to the experimental results, the fluorescence signal of the empty vector pBI121-GFP is observed in both the cell nucleus and cell membrane, with a relatively high expression level. In contrast, the fusion protein of *LsARF2* with other members of the LsARF family and GFP shows fluorescence signal exclusively in the cell nucleus, indicating a higher expression level. This outcome confirms the subcellular localization of the *LsARF2* protein within the cell nucleus, and the substantial expression within this compartment suggests its characteristics as a nuclear protein. As a growth hormone response factor, *LsARF2* may interact with downstream genes within the cell nucleus to regulate its expression. This observation aligns with the predictions made by subcellular localization software. The discovery provides crucial insights for further exploring the functionality and regulatory mechanisms of *LsARF2*.



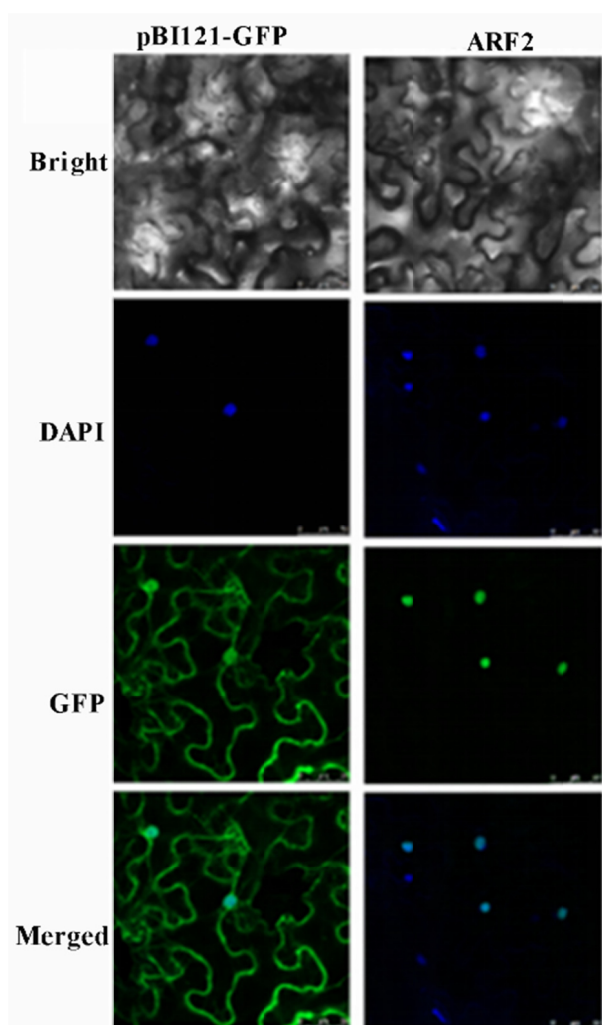


Figure 3. Subcellular localization of LsARF2

### 3.4 Validation Analysis of *LsARF2* Transcriptional Activation Activity

The yeast one-hybrid method was employed to validate the transcriptional activation activity of *LsARF2*. The results are shown in Figures 3-5. Yeast strains containing plasmids pGBKT7, pGBKT7-*LsARF2*, pGBKT7-*LsARF2N*, and pGBKT7-*LsARF2C* were able to grow normally on SD-Trp yeast selective medium, indicating successful transformation of the respective vectors into AH109 yeast cells. These yeast strains containing different plasmids were streaked onto SD-Trp-His-Ade + X- $\alpha$ -gal yeast triple-deficient medium. Notably, control plasmid pGBKT7 and recombinant plasmids pGBKT7-*LsARF2*, pGBKT7-*LsARF2N*, and pGBKT7-*LsARF2C* did not grow on the yeast triple-deficient medium and did not form colonies. These results suggest that *LsARF2* might possess transcriptional inhibitory activity.

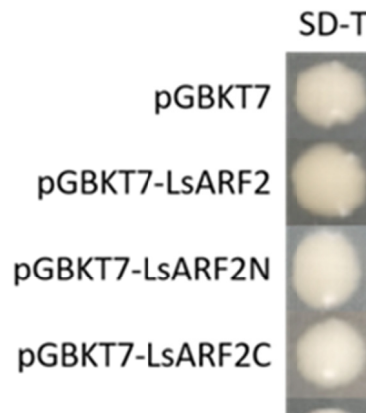


Figure 4. Validation of LsARF2 Transcription Activation Activity

### 3.5 Analysis of *LsARF2* Protein Expression

Immunoblot validation was performed on the LsARF2 protein in leaf lettuce. The results indicated that the LsARF2 protein exhibited specific bands in the control group on days 8, 16, and 24. Notably, the control group displayed higher protein expression on day 16. Meanwhile, a faint band corresponding to LsARF2 was observed on day 8 of high-temperature treatment, while no detectable bands were found on days 16 and 24, suggesting a significant suppression of protein expression under high-temperature conditions. This implies that the expression of LsARF2 protein in leaf lettuce is distinctly inhibited to varying degrees by high temperature, suggesting its potential role in the process of bolting in leaf lettuce (Figure 5).

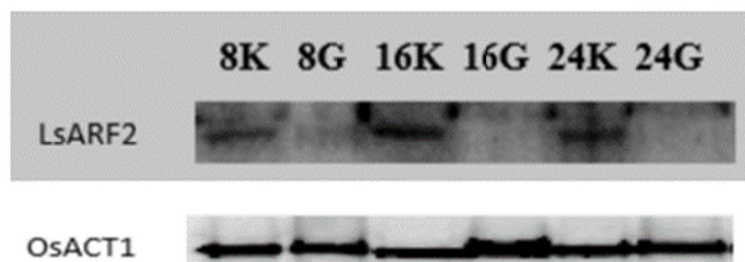


Figure 5. Western blot analysis of LsARF proteins

### 3.6 Effect of Exogenous Hormone Spraying on the Expression Level of *LsARF2*

The relative expression levels of treatments with GA3, IAA, and combined GA3 and IAA spraying were relatively low in the early stages and rapidly increased on the 12th day, showing a significant difference compared to the control group with fold changes of 6.2 and 24.2, respectively. The treatment with IAA spraying alone exhibited higher expression levels on the 4th and 18th days (Figure 6).

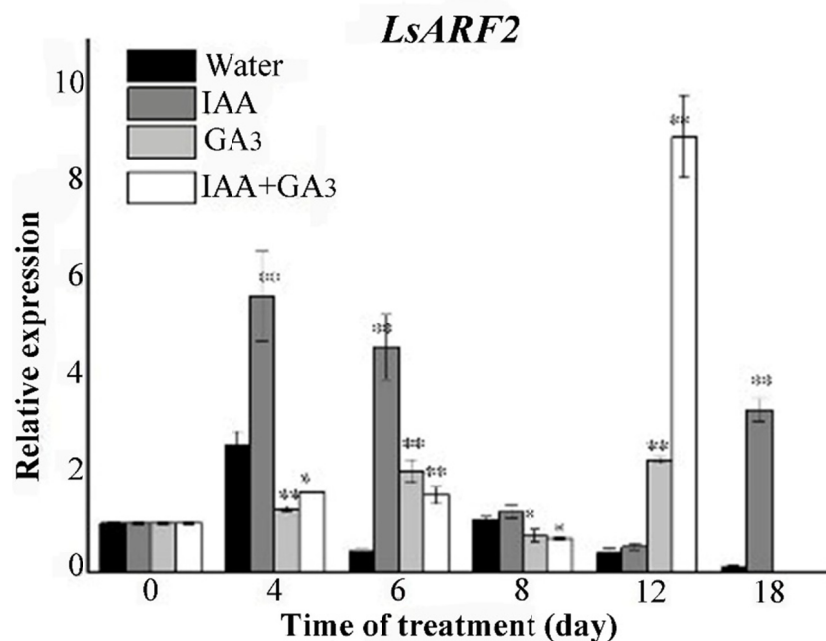


Figure 6. *LsARF2* relative expression after hormone spraying

Note. The standard error is shown, and \* indicates  $p < 0.05$  while \*\* represents  $p < 0.01$ .

#### 4. Discussion

Auxin, a crucial plant hormone, plays a significant role in regulating various aspects of plant growth and development. Recent research has yielded substantial insights into its involvement in processes such as cell growth, division, tissue differentiation, organ formation, phototropism, and apical dominance, among others (Souter & Lindsey, 2000; Bohn-Courseau, 2010; Benková et al., 2003). In lettuce, upon entering the bolting phase, a notable transformation occurs with rapid stem elongation. Studies have indicated that auxin is indispensable for stem elongation in plants (Ross et al., 2001). Additionally, Research has found that elevated temperatures significantly elevate auxin levels in lettuce stems, and exogenous auxin application stimulates stem elongation and bolting (Hao et al., 2018). Therefore, it is plausible to infer that auxin plays a pivotal role in the bolting process of lettuce.

Current research has demonstrated that many developmental processes regulated by auxin are governed by gene expression (Weijers & Wagner, 2016; Roosjen et al., 2018). As a critical transcription factor family in the auxin signaling pathway, AUXIN RESPONSE FACTORS (ARFs) hold a pivotal position. ARF proteins interact with auxin response elements via their DNA-binding domains (DBD) at the amino-terminal end. This interaction, coupled with the modulation of downstream target gene expression via activation or repression domains, enables ARFs to mediate auxin signaling and elicit appropriate responses (Tiwari, Hagen, & Guilfoyle, 2003). Earlier studies have indicated that the carboxy-terminal (C-terminal) CTD domain of ARF proteins shares structural similarities with domains III and IV of the Aux/IAA family proteins. These domains can form heterodimers, inhibiting the activity of ARF proteins and subsequently limiting their ability to regulate downstream gene expression. With increased auxin concentrations, the SCFTIR1 complex specifically recognizes Aux/IAA proteins and promotes their ubiquitination, leading to their degradation by the 26S proteasome. The intricacies of auxin signaling involve ARF family members competing for auxin-binding sites and mutual competition between ARF and Aux/IAA family proteins (Chapman & Estelle, 2009). Ploense et al.'s study uncovered interactions between ARF5 and IAA18 in Arabidopsis mutants, further emphasizing the complexity of the auxin signaling regulatory network (Ploense et al., 2009).

ARF2, as a responsive factor in the auxin signaling pathway, has been extensively studied primarily in model plants. Previous research on *AtARF2* in Arabidopsis revealed its multifaceted regulatory role in growth and development. Studies by Okushima Y found *AtARF2*'s involvement in flower organ formation in Arabidopsis (Okushima et al., 2005). *AtARF2*'s involvement in the regulation of aging pathways in Arabidopsis, alongside the research conducted by Guilfoyle and Hagen, which identified its role in transcriptional repression within

*Arabidopsis* (Guilfoyle & Hagen, 2003). *SlARF2* was found to negatively regulate tomato fruit ripening (Breitel et al., 2016). Meanwhile, research by Yu Xiwen in leafy vegetables highlighted *BnaARF2*'s significance in regulating seed size in *Brassica napus* (Yu, 2020). Within the ARF gene family, Li Yunfeng identified *LsARF3* as a crucial factor affecting bolting in leaf lettuce (Li et al., 2022). *LsARF2* contains typical conserved domains (DBD, MR, and CTD) of the ARF family, consistent with most ARF gene members identified in *Arabidopsis*, tomato, and canola (Guilfoyle & Hagen, 2003; Kumar et al., 2011; Zhang et al., 2016). This suggests its affiliation with the ARF transcription factor family in leaf lettuce, indicating its potential importance in bolting.

ARF2 is known to regulate senescence and floral organ abscission (Liu et al., 2018; Lim et al., 2010). Studies conducted on *Arabidopsis* have revealed that ARF2 acts as a suppressor of auxin signaling to regulate tissue and organ development. ARF2 also responds to other hormones and participates in various physiological processes in plants. For instance, in *Arabidopsis*, ARF2 functions as a negative regulator in response to ABA, playing a role in regulating cell division and differentiation in the root meristem (Promchuea et al., 2017). In this study, the relative expression levels of treatments with GA<sub>3</sub>, IAA, and a combination of GA<sub>3</sub> and IAA were initially low, and then rapidly increased on the 12th day. This phase corresponds to entering the differentiation of bract scales and the initiation of floral primordia differentiation. In contrast, the treatment with IAA alone showed higher expression levels on the 4th and 18th days. In the IAA exogenous application treatment, the expression of the ARF2 gene was upregulated on the 18th day, which coincided with the initiation of bract scale differentiation, possibly preparing for the differentiation of floral primordia.

Our study underscores the significant impact of high temperature on the expression of LsARF2 protein in leaf lettuce. The expression of LsARF2 protein in leaf lettuce responds to high-temperature stress, implying its crucial involvement in bolting. Further transgenic approaches will be necessary to definitively ascertain its function. Following exogenous hormone application, the expression pattern of LsARF2 changes, indicating its potential responsiveness to both GA<sub>3</sub> and IAA, thereby playing a role in lettuce floral development. The promoter of ARF2 contains cis-acting elements responsive to various hormones. In subsequent investigations, elucidating the interactions of ARF2 with other hormones will further unravel the intricacies of hormone-mediated processes.

## 5. Conclusion

In this study, the CDS sequence of *LsARF2* was successfully cloned. Subcellular localization experiments revealed that LsARF2 protein is localized in the nucleus, aligning with the nucleus-localized SlARF2 in tomato and *CaARF2* in chili pepper (Yuan-Yuan et al., 2012; Yu et al., 2017). The results harmonize with findings in other species. Through yeast one-hybrid assays, *LsARF2*'s transcriptional activation was investigated, suggesting it lacks activation but potentially exhibits transcriptional repression activity. This outcome is consistent with Guilfoyle et al.'s discovery that *AtARF2* functions as a transcriptional repressor in *Arabidopsis* (Guilfoyle & Hagen, 2003). Western blot results revealed that *LsARF2* protein expression was variably inhibited under high-temperature conditions. Additionally, GmARF2a's highest expression in flowers, followed by stem tips, roots, stems, and leaves, further emphasizes ARF2's vital role in flower development (Yin, 2021).

In conclusion, the expression of *LsARF2* protein in leaf lettuce responds to high-temperature stress, suggesting its crucial involvement in bolting. Subsequent transgenic approaches will be needed to definitively determine its function. After exogenous hormone application, the expression pattern of *LsARF2* changed, indicating its may response to both GA<sub>3</sub> and IAA. Furthermore, it played a role in the floral development of lettuce.

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## Appendix A

### Lettuce ARF Gene Information

Gene	Location	CDS/bp	Number of encoded amino acids per protein
LsARF1	XM_023874903.1	1968	655
LsARF2	XM_023915787.1	2196	731
LsARF2-like1	XM_023906629.1	1476	491
LsARF2-like2	XM_023902210.1	2370	789

## Appendix B

### Primer Sequences for LsARF2

Gene	primer(5'-3')	Length
LsARF2-F	agaggaggacctgcatatgATGACATCTTCAGAGGTTTCAAGCA	2193
LsARF2-R	cgacggatccccgggaattcAACATCCTCAGGACTTGAAGAAGA	
LsARF2N-F	agaggaggacctgcatatgATGACATCTTCAGAGGTTTCAAGCA	1104
LsARF2N-R	cgacggatccccgggaattcCTCAAGGATCTCCATTAGATTCTG	
LsARF2C-F	agaggaggacctgcatatgGTACGATGGGATGAAACATCTACTG	1089
LsARF2C-R	cgacggatccccgggaattcAACATCCTCAGGACTTGAAGAAGA	

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**Authors contributions**

Xiyi Yang: Investigation, Methodology, Writing (original draft). Kai Jia: Data curation, Visualization, Methodology, Writing (original draft). Junxuan Zhu: Formal analysis, Data curation, Writing (review & editing). Yijun Zhang: Methodology, Writing (review). Yufeng Tian: Visualization. Zhengyang Qi: Formal analysis. Yingyan Han: Project administration, Supervision. Chaojie Liu: Project administration. JingHong Hao: Conceptualization, Funding acquisition, Resources, Supervision, Writing (review & editing). All authors read and approved the final manuscript.

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**Data sharing statement**

No additional data are available.

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