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Full Length Article

Cloning of Auxin Response Factor 2 (*ARF2*) Gene and its Expression Analysis at High Temperature in Lettuce (*Lactuca sativa*) during Bolting

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Abstract

Auxin response factor as a type of transcription factor that regulates auxin responsive gene expression is an important part of auxin research. In order to study the mechanism of *LsARF2* gene in lettuce bolting, full-length CDS sequence of *LsARF2* gene was obtained by cloning technology and analyzed by biological software. Real-time quantitative PCR (qRT-PCR) was used to analyze *LsARF2* gene expression at different temperatures. Sequence analysis showed that the open reading frame of this gene was 2196 bp and encoded a protein of 731 amino acids with a conserved *ARF* family. qRT-PCR analysis showed that the expression of *LsARF2* in stems was significantly higher than that in the roots and leaves. The gene was down-regulated in easy-bolting varieties under high temperature treatment. The results showed that *LsARF2* gene may play an important role in lettuce bolting. © 2018 Friends Science Publishers

Keywords: Leaf lettuce; *LsARF2*; Cloning; Gene; Expression analysis

Introduction

Leaf lettuce (*Lactuca sative* L.) native to the Mediterranean coast (Han *et al*., 2013), is a vegetable from the genus *Lactuca* (Family Asteraceae). The leaves of lettuce are used for food. In recent years, the areas in China used for lettuce cultivation have gradually expanded, as this vegetable is well loved by consumers. The lettuce plant prefers a cool climate and grows best at 15–20°C. Temperatures that exceed 30°C will result in poor growth, a tendency to bolt, and a decrease in food quality, leading to substantial crop losses. This problem requires an urgent solution in the annual production of lettuce.

Auxins, a class of plant hormones secreted by active plant cells, regulate the direction of plant growth, which has an important role in various aspects of plant growth and development, including cell growth and division, flower development, tissue differentiation, root and stem growth, tropisms, and apical dominance (Souter and Lindsey, 2000; Benková *et al*., 2003; Woodward and Bartel, 2005a; Bohn-Courseau, 2010; Tromas and Perrot-Rechenmann, 2010). In recent years, great progress has been made in the understanding of auxin signal transduction pathways. Auxins function biologically by regulating the expression of genes associated with plant development (Dharmasiri and Estelle, 2004; Kepinski and Leyser, 2005). Some studies have demonstrated that auxins are plant hormones necessary for plant stem elongation (Ross *et al*., 2001). In our previous study, we found that the auxin concentration in stems and leaves of lettuce increased significantly during bolting. As such, it has been deduced that auxins play an important role in lettuce bolting.

In most plants, auxin response genes are regulated by two families of transcriptional factors, auxin response factors (*ARF*) and *Aux/IAA* transcriptional factors (Tiwari *et al*., 2003; Woodward and Bartel, 2005b; Audrandelalande *et al*., 2012; Zouine *et al*., 2014). A typical ARF has a B3 DNA-binding domain at its N-terminus, and domains III and IV (Ulmasov *et al*., 1997; Guilfoyle and Hagen, 2001), which are similar to that of *Aux/IAA* proteins at its C-terminus. ARF proteins can specifically bind to TGTCTC auxin response elements (*AuxREs*) in promoters of auxin response genes, and the amino acid composition in the central region of an ARF protein determines whether the protein acts as a transcriptional activator or repressor (Ulmasov *et al*., 1999). It is widely believed that some *ARF* proteins affect transcription when they bind with AuxREs but lose activity when they bind with *Aux/IAA* proteins. When auxin concentration reaches a certain level, *Aux/IAA* proteins are degraded, and the activity of *ARF* proteins are recovered, thus facilitating the regulation of downstream gene expression (Chapman and Estelle, 2009). There are

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many members in the *ARF* family, and significant differences in function exist among different *ARF* proteins. Sessions *et al.* (1997) found that *AtARF3* plays an important role in the formation of floral organs in *Arabidopsis*. Another study indicated that *AtARF5* participates in the formation of embryonic patterns and development of vascular tissues (Cole *et al.*, 2009). Goetz *et al.* (2006) elucidated that *AtARF8* is a negative regulator of fruit development in *Arabidopsis*, and that the suppression of its expression induces parthenocarpy in *Arabidopsis*. Sagar *et al.* (2013) suggested that *SlARF4* is involved in the regulation of sugar metabolism during tomato fruit development, and that *SlARF7* regulates auxin signal transduction and mediates auxin and gibberellin signal transduction during the tomato fruit setting and development processes (De *et al.*, 2009, 2011). In a study on transgenic tomatoes, De *et al.* (2015) found that *SlARF9* is a negative regulator of cell division during the early stages of tomato fruit development. Ren *et al.* (2017) found that the expression of the *SlARF2* gene in tomato might be positively regulated by auxins and gibberellins, and negatively regulated by ethylene. Based on the study of *SlARF2* overexpression in transgenic tomato, it was deduced that *SlARF2* could regulate lateral root formation and floral organ senescence in tomato via regulation of the expression of auxin and ethylene response genes. *ARF2* is an important transcription factor in plants. It is involved in the signal transduction pathways of many hormones and plays an important role in plant growth and development. In our group's previous analysis of differential proteins between the high-temperature bolting group and the control group, we found that the *ARF2* protein was a down-regulated protein, presumably related to bolting. However, the mechanism of action of the *ARF2* gene in lettuce and its association with lettuce bolting remain unclear. Therefore, in the present study, cloning of the *LsARF2* gene and bioinformatics analysis were performed, and real-time quantitative PCR technique was used to analyze the relative expression levels of *LsARF2* during various stages of treatments at different temperatures, with the purpose of facilitating further investigation of the relevant mechanisms of action during lettuce bolting.

Materials and Methods

Plant Material and Growth Conditions

Leaf lettuce easily-bolting varieties GB-30 is numbered and conserved in our laboratory. Select 150 full seeds and sown in a sand/soil/peat (1:1:1 v/v) mixture, and grown in the artificial climate chamber of Beijing University of Agriculture (14 h light; 10 h dark; 20 ± 2°C and 12000lx during the day; 13 ± 2°C at night; and 60%-70% relative humidity). Seedlings were planted in 10 cm pots with six leaves, and two days of acclimation. 15 plants were selected for their roots, stems and leaves, frozen in liquid nitrogen and stored at -80°C in a refrigerator. After that, the plants were divided into two groups. The high-temperature group was moved to another growth chamber and treated with high temperatures of 33 and 25°C during the day and night. The other environmental conditions were unchanged. The control group was kept under the standard greenhouse conditions as described above. On the 0, 8^{th}, 16^{th}, 24^{th} and 32^{th} days of treatment, the stems were taken as experimental materials and were repeated three times and stored at -80°C.

Methods

Total RNA extraction and cDNA first strand synthesis: Spin Column Plant Total RNA Purification Kit (Songon Biotech, Shanghai, China) was used to extract the total RNA of lettuce, and then TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) was used to reverse transcribe the RNA to cDNA. All cDNA strands obtained were stored at –20ºC for use as a template for cloning the *LsARF2* gene and for the qRT-PCR.

LsARF2 Gene Cloning and Real-time Fluorescent Quantitative PCR

The CDS sequence of *LsARF2* from transcriptome sequencing was used as a template, and Primer Premier 5 software was used to design the primers for the cloning (*LsARF2*-F and *LsARF2*-R) and qRT-PCR experiments (q*LsARF2*-F and q*LsARF2*-R). The 18S gene of lettuce was used as an internal reference gene (Table 1). The primers were synthesized by Sangon Biotech Co. Ltd.http://xueshu.baidu.com/s?wd=author:(Maha%20Sagar)%20&tn=SE_baiduxueshu_c1gjeupa&ie=utf-8&sc_f_para=sc_hilight=person

cDNA from lettuce stems was used as a template for cloning the *LsARF2* gene. The reaction conditions used were as follows: pre-denaturation at 98°C for 3 min; 35 cycles of denaturation at 98°C for 20 s, annealing at 53°C for 20 s, and extension at 72°C for 3 min; followed by a final extension of 72°C for 8 min. The PCR products were stored at 4°C. Agarose gel electrophoresis was carried out on the PCR products, and EasyPure Quick Gel Extraction Kit (TransGen Biotech, Beijing, China) was used to recover the target band, which was then ligated to the pTOPO-Blunt (Aidlab Biotechnologies Co., Ltd, Beijing, China). The ligated vector was then transformed into *Escherichia coli* DH5α competent cells (Bao Biological Engineering Co., Ltd, Dalian, China), and the bacterial culture was sent to Sangon Biotech Co. Ltd. for sequencing. The cDNAs of various lettuce tissues and stem cDNAs from the control and high-temperature groups at various time points were used as templates for the qRT-PCR. The reaction conditions were as follows: pre-denaturation at 95°C for 3 min; followed by 38 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s. The $2^{-\Delta\Delta Ct}$ relative quantitation method was used to

calculate the relative expression of the *LsARF2* gene.

Bioinformatics Analysis of the *LsARF2* Gene Sequence

The self-optimized prediction method with alignment (SOPMA) by NPS@ (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.plpagenpsa_sopma.html) was used to predict the two grade structure of amino acids. the NCBI CD-search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) was used to analyze the conserved structures of the protein. ProtParam (http://web.expasy.org/protparam) was used for online analysis of protein parameters. NCBI Protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for online search of homologous sequences for the *LsARF2* amino acid sequence. SignalP 4.1 (http://www.cbs.dtu.dk/services/ SignalP/) is used for signal peptide analysis. MEME software was used to analyze conserved motifs. ProtComp (http://linux1.softberry.com/berry.phtml) and PSORT (https://psort.hgc.jp/) were used to predict subcellular localization of proteins. SWISS-MODEL (https://www.swissmodel. expasy.org/) was used to predict the tertiary structure of proteins. Neighbor-joining methods in MEGA7 (Kumar *et al*., 2016) were used to construct phylogenetic trees.

Statistical Analysis

The experiment was performed in three replicates. The shown data represent the means ± SD of three replications, and were statistically analyzed using analysis of variance (ANOVA) by SPSS 10.0 (International Business Machine, Chicago, IL). Duncan's multiple analysis and Student's t-test were used to identify significant differences among groups ($P < 0.05$, $P < 0.01$). Figures were drawn using Origin Pro 8.0 SR4 (Origin Lab, Northampton, MA, USA).

Results

Leaf Lettuce *LsARF*2 Gene Cloned

A 2196 bp specific band was obtained from the PCR amplification (Fig. 1). The identity between the sequencing results and the transcriptome sequence was 100%. *LsARF2* gene encodes for 731 amino acids (Fig. 2).

Analysis of the Amino Acid Sequence and Domain of LsARF2

By using the self-optimized prediction method with alignment (SOPMA) by NPS@ to predict the amino acid secondary structure of *LsARF2*, it was found that the protein is composed of irregular coils, extended strands, α helices and β turns, with proportions being 48.15%, 22.02%, 21.61%, and 8.21%, respectively. Thereafter, DNAMAN 7.0 was used to compare *ARF2* gene sequences in lettuce, sunflower, tomato, and *Arabidopsis*. It was found that the *LsARF2* protein is similar to proteins of other species and possesses conserved structures, such as FCKTL, RGQPRRH, RFTGTIVGIEE, and VVYTDDEGDMMLVGDDPWQEFCGMVRKI (Fig. 3).

Subsequently, the NCBI CD-search was used to analyze the conserved structures of the protein, and it was found that the protein has four structural domains, namely B3, Auxin_resp, Herpes_TK superfamily, and *AUX_IAA* (Fig. 4). The B3 structural domain is the region in the DNA to which *ARF* proteins bind to, Auxin_resp is the conserved structural domain of the *ARF* family, and *AUX_IAA* is

Table 1: The primers used in the study

Name of primer	Sequence of primer
LsARF2-F	ATGACATCTTCAGAGGTTTC
LsARF2-R	CTAAACATCCTCAGGACTTG
LsARF2-qF	AATTGCTGAGCTGGACGAGT
LsARF2-qR	ACGGATCATCTCCAACAAGC
Ls18S-F	CCTGCGGCTTAATTTGACTC
Ls18S-R	AACTAAGAACGGCCATGCAC

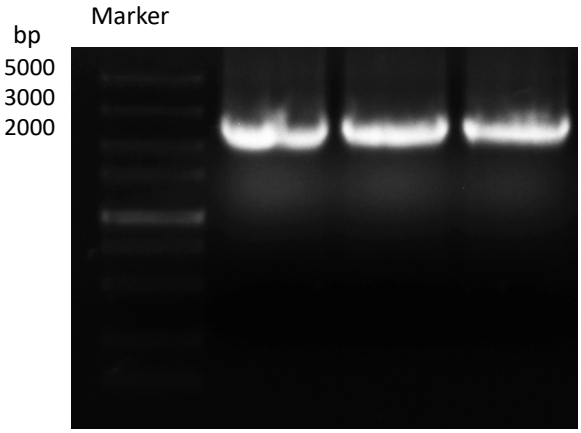

Fig. 1: The amplification of *LsARF2* genes

Fig. 2: Nucleotide and amino acid sequence of *LsARF2*

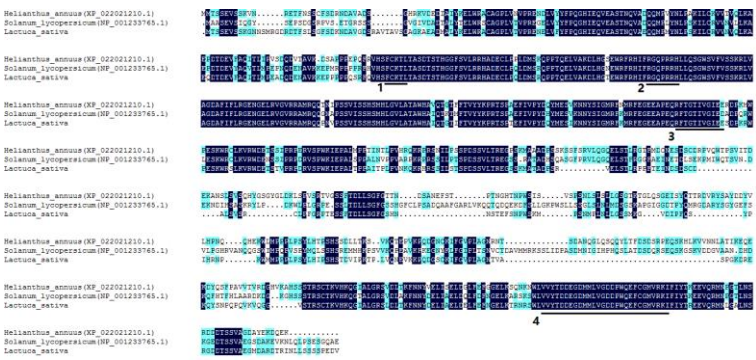

Fig. 3: Alignment and analysis of amino acid sequences of *LsARF2*

1 represents FCKTL domain，2 represents RGQPRRH domain，3 represents RFTGTIVGIEE domain，4 represents VVYTDDEGDMMLVGDDPWQEFCGMVRKI domain

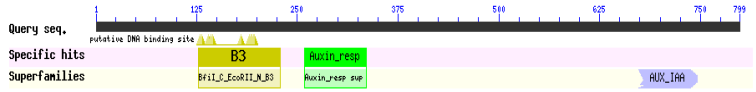

Fig. 4: Conserved domain of *LsARF2* protein

the binding site for the dimerization of ARF proteins. Because most ARF proteins have these three structural domains, and based on the amino acid sequences mentioned above, it appeared that the LsARF2 protein might have the same functions as the ARF family of proteins.

Bioinformatics Analysis of LsARF2 Protein

The ProtParam tool was used to analyze the amino acid sequence encoded by the *LsARF2* gene. The LsARF2 protein had a molecular weight of 81.8 kDa and a theoretical isoelectric point of 6.5. It had high levels of serine, proline, and leucine (10.5%, 7.8% and 7.3% respectively), low levels of tyrosine, tryptophan, and cysteine (1.9%, 1.6%, and 1.2%, respectively), a total of 89 negatively charged residues (Asp + Glu), a total of 85 positively charged residues (Arg + Lys), an instability index of 62.5 (unstable protein), and a mean hydrophilicity value of –0.56 (predicting it to be a hydrophilic protein). Signal P 4.1 analysis of the amino acids showed that the protein did not contain a signal peptide or transmembrane domains, and was not a secreted protein. ProtComp and PSORT were used to predict the sublocalization of the LsARF2 protein, and results showed that it was located mainly in the nucleus. The *LsARF2* protein may bind specifically to the TGTCTC auxin response elements on the promoters of primary auxin response genes in the nucleus in order to activate or inhibit gene expression and regulate auxin signal transduction.

Subsequently, the MEME software was used to analyze conserved motifs of *LsARF2*, and it was found that the protein has three highly conserved motifs in the conserved structural domains of B3, Auxin_resp, and *AUX_IAA* (Fig. 5). SWISS-MODEL was used to predict the tertiary protein structure of *LsARF2*. It was found that the structure matches that of several ARF protein models, and that it has the unique conserved structures of the ARF family. This result is consistent with the outcome of the analysis performed with the NCBI CD-search (Fig. 6).

Phylogenetic Analysis of LsARF2 Protein in Leaf Lettuce

The *LsARF2* amino acid sequence obtained was aligned with *ARF2* sequences in the NCBI protein database and was found to have a high degree of homology with the *ARF2* protein from 10 types of plants, such as *Helianthus annuus*, *Arabidopsis*, and sesame. The MEGA 7.0 software tool was used to construct a phylogenetic tree of the highly homologous protein sequences. These 11 amino acid sequences were clearly divided into three groups, with tomato and sesame being the first category; leaf lettuce and sunflower being the second category, Lettuce and *Helianthus annuus* are both crops from the family *Asteraceae*, with a closer degree of homology; *Arabidopsis* and cucumbers are the third category (Fig. 7).

Analysis of *LsARF2* Gene Expression

Analysis of *LsARF2* gene expression in various organs, the expression of *LsARF2* in stems was significantly higher than that in the roots and leaves (Fig. 8).

The expression levels of the *LsARF2* gene in the treatment group of GB-30 varieties showed an overall similar trend in both short-term and long-term treatments, and the relative expression of the *LsARF2* gene in the high-temperature group was lower than that in the control group. (Fig. 9). These differences were significant on h 3, 6, 12, 24,

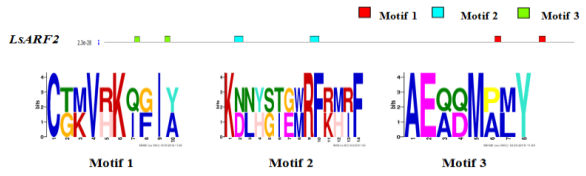

Fig. 5: Motif composition analysis of *LsARF2*

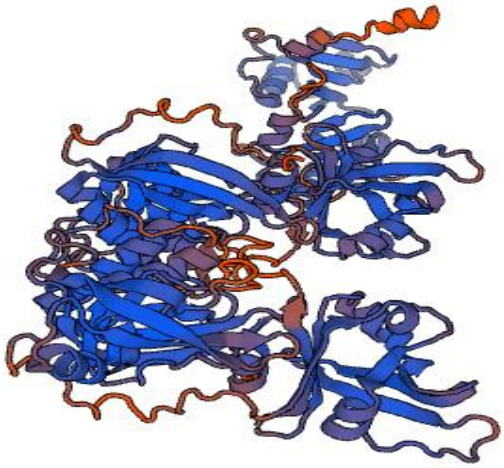

Fig. 6: The prediction of *LsARF2* protein structures

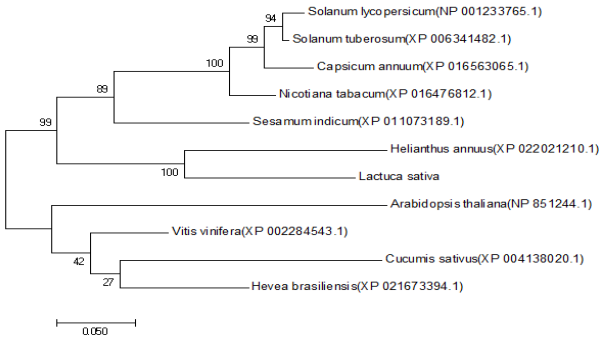

Fig. 7: Phylogenetic tree

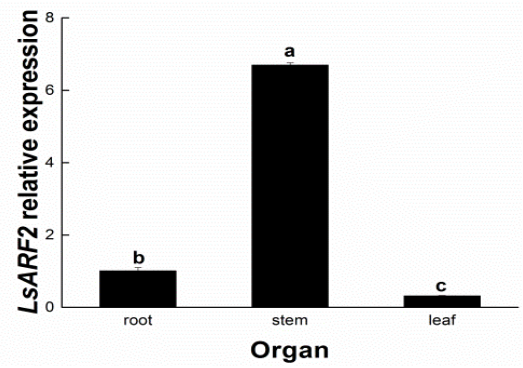

Fig. 8: Relative expression of *LsARF2* gene in different organs

(the same minuscule indicates that the difference is not significant, whereas different minuscules imply significant difference. $P < 0.05$)

48 and on Days 16, 24 and 32.

Discussion

Auxin is involved in most of the developmental processes regulated by gene expression (Weijers and Wagner, 2016; Roosjen *et al.*, 2017). As an important transcription factor in auxin signal transduction pathway, ARF family plays an important role in this pathway (Tiwari *et al.*, 2003). At present, the research on the regulation mechanism of ARF protein on downstream genes has made some progress. Guilfoyle (2007) postulated that under low auxin concentrations, the AUX/IAA protein would bind with the ARF protein and inhibit its activity, thereby preventing ARF from regulating its downstream genes. Under high auxin concentrations, auxin would bind to the transport inhibitor response 1 (TIRI) receptor in the SCF^{TIRI} complex so that it can specifically recognize the AUX/IAA protein and cause it to enter the ubiquitination pathway for degradation. This will activate ARF protein activity, thereby regulating the expression of downstream genes.

ARF2 is an important member of the *ARF* gene family. There are many related studies. Ellis *et al.* (2005) and Lim *et al.* (2010) studied *AtARF2* mutants in *Arabidopsis*, and found that its expression positively regulated leaf senescence. Okushima *et al.* (2005) suggested that *AtARF2* is a pleiotropic regulator of plant development. Hao *et al.* (2015) identified two paralogs of *SlARF2* in the tomato genome, *SlARF2A* and *SlARF2B*, and found that silencing of these two genes can severely inhibit tomato fruit ripening, thus concluding that *SlARF2* plays an important role during the tomato fruit ripening process. In addition, Breitel *et al.* (2016) believed that *SlARF2* can regulate tomato fruit ripening, and by studying *SlARF2* in transgenic tomato plants, they concluded that *SlARF2* initiates a complex ripening process by linking the signals of ethylene with those of other hormones. In recent years, with the development of whole-genome studies on various species, *ARF* family members in *Arabidopsis*, tomato, cucumber and other plants have been successively identified (Remington *et al.*, 2004; Wu *et al.*, 2011; Liu and Hu, 2013). These research developments provide a solid foundation for the investigation of the roles and mechanisms of action of the *ARF* transcriptional factor family during plant growth and development, and the involvement of these factors in signal transduction pathways of auxins and other plant hormones.http://xueshu.baidu.com/s?wd=author:(Okushima,%20Y)%20Ctr%20Plant%20Gene%20Express,%20Albany,%20CA%2094710%20USA&tn=SE_baiduxueshu_c1gjeupa&ie=utf-8&sc_f_para=sc_hilight=personhttp://xueshu.baidu.com/s?wd=author:(Yanwei%20Hao)%20&tn=SE_baiduxueshu_c1gjeupa&ie=utf-8&sc_f_para=sc_hilight=personhttp://xueshu.baidu.co

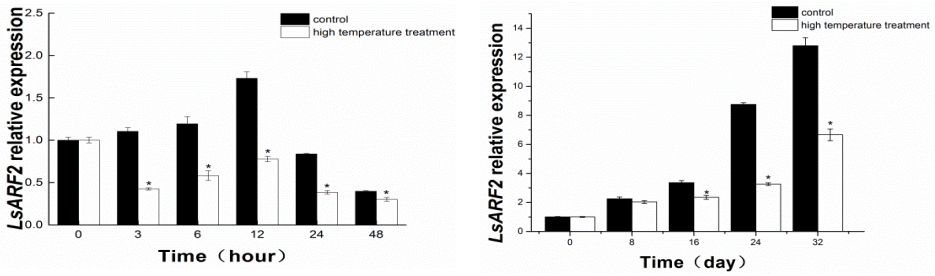

Fig. 9: Relative expression analysis of *LsARF2* under 20/13℃, 33/25℃ of GB-30
(* stands for P <0.05)

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In this study, we cloned and sequenced a *ARF* gene from lettuce, *LsARF2*. We have found that the *LsARF2* protein has a conserved structure typical of the *ARF* protein family, and its subcellular localization is predicted in the nucleus. And we used many analysis software to analyze *LsARF2* protein and laid a foundation for studying the function of *LsARF2* protein. Through expression analysis of this gene in various organs, we hypothesize that this gene may elicit its effects in the stems. The analysis of *LsARF2* gene expression level in GB-30, a species that bolts readily at different temperatures, showed that the difference between the high-temperature group and control group began to emerge on the eighth day of treatment. On the 16th day of treatment, the difference became significant, with the level of *LsARF2* gene expression decreasing significantly owing to significant repression at high temperatures, and the lettuce stems in the high-temperature group began to grow rapidly. Therefore, it was inferred that bolting is related to the repressed expression of the *LsARF2* gene. The study by Okushima *et al*. (2005) showed that *AtARF2* is involved in floral organ formation in *Arabidopsis*. The findings of Hao *et al.* (2015) and Breitel *et al.* (2016) indicate that *SlARF2* participates in the tomato fruit ripening process. Collectively, these research findings suggest that the *ARF2* gene may have an important role in plant reproduction and development.

Conclusion

In this study, the CDS sequence of *LsARF2* gene was cloned and its bioinformatics analysis was carried out, which laid the foundation for future work. *LsARF2* protein has a conserved structure of *ARF* protein family. qRT-PCR analysis showed that the expression of *LsARF2* in stems was significantly higher than that in the roots and leaves. And the expression of this gene in the stems under control group was significantly higher than that in the high temperature treatment. *LsARF2* may be intimately associated with high-temperature bolting in lettuces. In subsequent studies, we will construct an RNA interference vector for the *LsARF2* gene to obtain *LsARF2*-silenced plants for phenotype analysis and molecular level studies. This will further clarify the effector mechanisms of the *LsARF2* gene in lettuce bolting.

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