INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19F–064/2019/22–5–1211–1216
DOI: 10.17957/IJAB/15.1189
http://www.fspublishers.org

Full Length Article

Analysis of the Auxin Response Factor 5 (*ARF5*) Gene in Lettuce *(Lactuca sativa*) and its Expression Pattern During Bolting in Response to High Temperature

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Abstract

Bolting is currently a major problem in achieving true annual production of lettuce. The *ARF5* gene is associated with a high temperature bolting mechanism, and the full-length cDNA sequence of the *LsARF5* gene was obtained by cloning and was analyzed by biology-related software. Moreover, real-time quantitative PCR (qRT-PCR) was used to amplify the target gene, which further demonstrated that the gene is associated with bolting. The difference in expression levels of the *LsARF5* gene at different temperatures was analysed. Sequence analysis indicated that the open reading frame was 2517 bp and encoded 838 amino acids. In addition, qRT-PCR analysis indicated that the expression of the gene was downregulated in varieties susceptible to bolting under high temperature treatment. Together, the results indicate that the *LsARF5* gene may have an effect on lettuce bolting. © 2019 Friends Science Publishers

Keywords: Lettuce; *LsARF5*; Gene clone; Expression Analysis

Introduction

Lettuce (*Lactuca sativa* L.) is an annual or biennial herbaceous crop species native to the Mediterranean coast (Su *et al.*, 2016). People generally eat lettuce leaves as components of salads. Lettuce has a growth period of 90–100 days. This growth period is suitable to cold weather, and the species is most suitable for growth at 15–20°C. When the temperature is continuously higher than 25°C, the growth of lettuce will worsen, the quality of the leaves declines and the leaves taste slightly bitter, which affects the palatability. The consequences of a large reduction in production and income reduction are caused by bolting. This problem in urgent need to of resolution in terms of the annual production of leaf lettuce.

Auxins have great importance in leaf lettuce bolting. Flower bud differentiation is a landmark switch from vegetative growth to reproductive growth during plant growth and development. This differentiation is a process in which the shoot tip tissue of a plant no longer produces leaf primordia or axillary bud primordia and differentiates into flowers or inflorescences (Wu *et al.*, 2015). Bolting is a phenomenon in which two-year-old vegetables such as leafy vegetables, root vegetables, and bulbs grow from the leaf buds after flower bud differentiation (Zhou, 1996). Auxins are involved in the regulation of plant growth and development, especially the radicle formation (Rademacher *et al.*, 2012), floral organ primordium formation (Yamaguchi *et al.*, 2013), and shoot tip meristem development (Aida, 2002). This regulation is achieved by auxin response factors (ARFs) and auxin inhibitors (Aux/IAAs). ARFs and Aux/IAAs represent two important families of transcription factors whose members play a key role in plant physiological growth and regulate auxin-responsive gene expression. Most of the data on the biological function of ARF originated from the phenotypes of the ARF gene function-deletion mutants in Arabidopsis, and many useful phenotypes have been obtained thus far. It has been shown that ARFs are transcription factors that mediate the auxin response, regulate auxin response gene expression and play a functional role in auxin signalling (Hagen and Guilfoyle, 2002).

ARFs are targeted to TGTCTC sequences or related DNA-binding protein auxin response elements (AuxREs). When auxin concentrations are low, AUX/IAA proteins bind to ARF proteins, inhibit protein activity, and inhibit the ARF protein regulation of downstream genes. When auxin concentrations are high, auxin binds to TIR1 receptors in the SCF-TIR1 complex, enabling it to specifically recognize the AUX/IAA protein; this phenomenon allows the AUX/IAA protein to degrade via the ubiquitination pathway, activate ARF protein activity, and thereby regulate downstream gene

To cite this paper: Pan, G., Z. Qi, J. Hao, S. Fan, 2019. Analysis of the auxin response factor 5 (*arf5*) gene in lettuce *(lactuca sativa*) and its expression pattern during bolting in response to high temperature. *Intl. J. Agric. Biol*., 22: 1211–1216

expression (Tiwari, 2003). In auxin-responsive promoters, ARF is activated or blocked by a glutamine-rich activation domain (AD) or an inhibitory region (RD) lacking a glutamine-rich region (Guilfoyle, 2007). Therefore, ARFs can directly bind to auxin response elements and can activate or inhibit gene expression (Guilfoyle and Hagen, 2007), while Aux/IAAs and ARFs act directly to inhibit the auxin response process (Dreher *et al.*, 2006).

ARFs are inseparable from the relationship between plant growth and development, and participates in the process from plant embryo formation to ageing and death. *Arabidopsis thaliana* ARF gene promoter and reporter gene fusions and *in situ* hybridization result in the specificity and diversity of ARF gene expression, when studying the expression pattern of the ARF gene. For example, *ARF5* is likely to play a role in plant embryo development and vascular bundle differentiation (Przemeck *et al.*, 1996). *ARF5/MONOPTEROS* (*MP*), which is a mutant of Arabidopsis embryo development, is a member of the ARFs. Deletion of *MP* causes Arabidopsis embryos to lose polarity and fail to form root meristems and normal cotyledons (Berleth and Jurgens, 1993). In the *MP* mutants, the vascular bundle was significantly reduced, auxin transport was abnormal, and MP encoded *ARF5*.

The mechanism of action of the *ARF5* gene in leaf lettuce and its relationship to the convulsion of leaf lettuce are unclear. Therefore, in this study, we cloned the *LsARF5* gene for bioinformatics analysis, and analyzed the relative expression of *LsARF5* under different temperature treatments by qRT-PCR to further study its mechanism of action in lettuce.

Materials and Methods

Experimental Details and Treatments

Experimental details: The documented easily bolting leaf lettuce variety GB-30 was preserved in our laboratory. Seedings were grown in an artificial climate chamber at Beijing University of Agriculture. It is used widely and tends to replace the hole seeding, because the bowl seeding has no harm to the stock when transplanted. The lettuce seeds were directly sown in seedling trays (50 mm high with 50 mm diameter from the neck, 22 mm from bottom, and number of holes 5×10) containing a sand: soil: peat (1:1:1 v/v) mixture and kept in the net-house. After germination, 50 uniform seedlings were maintained in each tray. Water is regularly dosed to make the plants generally consistent throughout their growth periods.

Treatments: Seedlings with three leaves were transplanted in a 10 cm nutrient pots. When the leaf lettuce plants were grown to six true leaves, they were transferred to a smart artificial climate chamber. The culture conditions included a temperature of 20/13°C (day/night, the same convention is used below), the illumination was 12000 lx/0 lx, the photoperiod was 14h/10h, and the humidity was 60%. After 2–3 days of growth, the seedlings were divided into two groups for temperature treatment: 20/13°C (control) and 33/25°C (high temperature). Other environmental conditions were unchanged.

Plant Harvesting

Stems and mature leaves were taken as experimental materials on days 0, 8, 16 and 24 of the treatment. At each stage, immediately select the plants with good growth, cut off the roots, leaves, stems and shoots, and take the whole stem. After liquid nitrogen freezing, the sampled material was stored at-80°C until further analysis. qRT-PCR sample was sampled and subjected to three independent replicates. There were three repetitions in each treatment. On the one hand, three repeated sampling constitute one replication; on the other hand, three samples were mixed together as one replication.

Total RNA Extraction and Synthesis of First-strand cDNA

The total RNA in the leaf lettuce material was separately extracted using an RNA extraction kit, and then reverse-transcribed using a cDNA First Strand Synthesis Kit to synthesize first-strand cDNA. The resulting cDNA was stored at -20°C and used as a template for *LsARF5* gene cloning and real-time fluorescent quantitative PCR.

A Total RNA Rapid Extraction Kit was purchased from Shenggong Bioengineering Co., Ltd.; PrimeSTAR High-Fidelity enzyme, SYBR® Premix Ex Taq™ II; and E. coli DH5$^{\alpha}$ competent cells were purchased from Yubao Biotech Co., Ltd.; A plasmid extraction kit (EasyPure HiPure Plasmid MiniPrep Kit), EasyPure Quick Gel Extraction Kit and cDNA First Synthesis Kit (TransScript First-Strand cDNA Synthesis SuperMix) were purchased from Beijing Quanjin Biotechnology Co., Ltd.; pTOPO-blunt cloning vector were purchased from Beijing Aidelai Biotechnology Co., Ltd.; and other biochemical reagents were analytically pure reagents.

LsARF5 Gene Cloning and Real-time PCR

Using the *LsARF5* sequence as a template for transcriptome sequencing, Primer Premier 5 software was used to design cloning primers (*LsARF5-F* and *LsARF5-R*) and qRT-PCR primers (*LsARF5-qF* and *LsARF5-qR*), and leaf lettuce 18S was used as an internal reference gene (Table 1). The primers were synthesized by Shenggong Bioengineering Company.

The *LsARF5* gene was cloned from the cDNA of leaf lettuce stalks. The reaction procedure was as follows: pre-denaturation at 98°C for 3 min; 35 cycles of denaturation at 98°C for 15 s, annealing at 53°C for 15 s, extension at 72°C for 3 min; and a final extension at 72°C for 8 min, after which the products were stored at 4°C. The PCR products

were subjected to agarose gel electrophoresis, and the target band was recovered using a DNA gel recovery kit, ligated into a pTOPO-blunt cloning vector, and introduced into *E. coli* DH5α competent cells. The bacterial liquid was then sent to a bioengineering company for sequencing. The qRT-PCR was performed using stem cDNA from each control group and high temperature group as a template. Reaction procedure was as follows: pre-denaturation at 94°C for 3 min, followed by 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s.

Statistical Analysis

The experiment was performed in three replicates. The relative expression of the *LsARF5* gene was calculated via the 2-ΔΔCt relative quantitative analysis method, and the data were analysed via SPSS 20.0 software for variance and significance. Figures were constructed using OriginPro 9.0 software. The DNAMAN7.0 software was used to sequence the *ARF5* gene of leaf lettuce, sunflower, pepper and *Arabidopsis thaliana*. The conserved structure of the protein was analyzed using NCBI CD search and found to have three domains, B3, Auxin_resp, and AUX_IAA. The phylogenetic tree was constructed using the Maximum Likelihood methods using MEGA 7.0 software.

Results

LsARF5 Gene Cloning

A 2517 bp specific band was obtained by PCR amplification (Fig. 1), and the sequence was 100% identical to the sequence in the transcriptome. Analysis using NCBI ORFfinder revealed that the reading frame encodes 838 amino acids (Fig. 2).

Amino Acid Sequence and Domain Analysis of Lettuce *LsARF5*

The secondary structure of the *LsARF5* amino acid was predicted by NPS SOPMA (Fig. 3). The protein was found to consist of random coils, α-helices, extended chains and β-turns, with contents of 52.98, 25.42, 16.23 and 5.37%, respectively. It was found that *LsARF5* has high similarity to ARFs in other species.

The B3 domain is a region where ARF family proteins bind DNA, Auxin resp is a conserved domain of the ARF family, and AUX_IAA is a binding site for ARF protein dimerization. Most of the ARF proteins have these three domains (Fig. 4). Combined with the amino acid sequence analysis results, it can be inferred that the *LsARF5* protein may function the same as members of the ARF family of proteins.

Bioinformatics Analysis of the *LsARF5* Protein

The amino acid sequence encoded by the *LsARF5* gene was

Table 1: The primers used in the study

Name of primer	Sequence of primer
LsARF5-F	ATGGCTGCCATTGAAGAGAAGCTC
LsARF5-R	TCATGTGGGTCCCACCCAAGTAC
LsARF5-qF	CGCTCATGCCGCCGCTAATAG
LsARF5-qR	CGGTATCTCACCAACGGAATCACG
Ls18S-F	GTGAGTGAAGAAGGGCAATG
Ls18S-R	CACTTTCAACCCGATTCACC

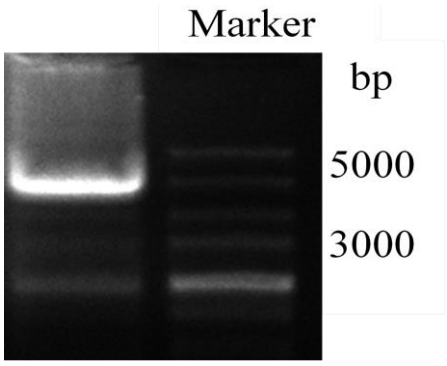

Fig. 1: The amplification of *LsARF5* genes

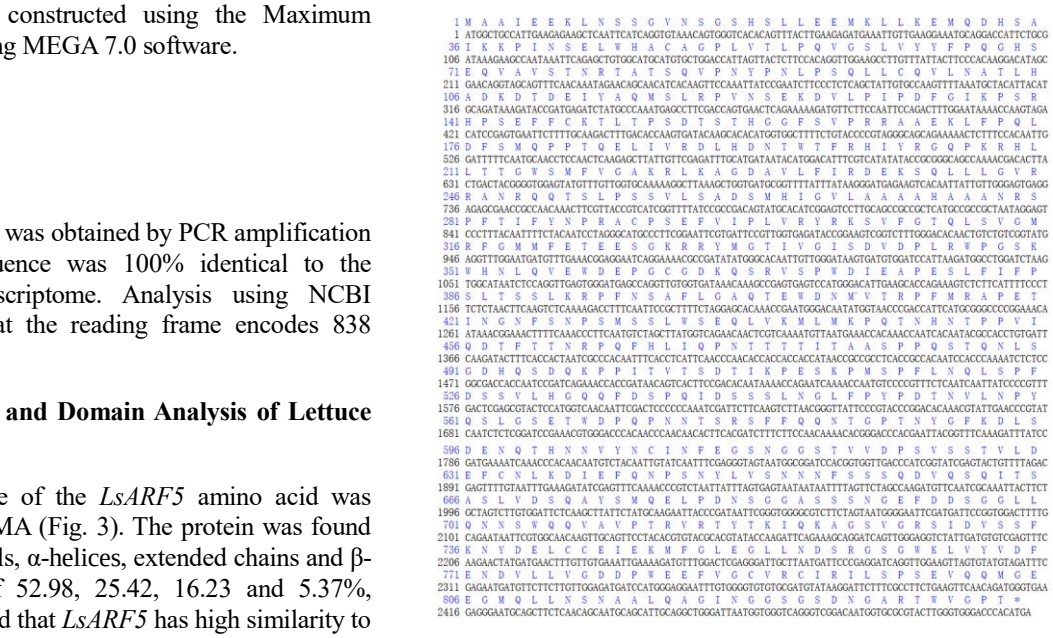

Fig. 2: Nucleotide and amino acid sequence of *LsARF5*

analysed by ProtParam software. The molecular weight of the *LsARF5* protein was 92.7 kD. The theoretical isoelectric point was 5.32, and the contents of serine, leucine and proline were 11.6, 8.4 and 7.2%, respectively. Furthermore, the tyrosine, tryptophan, and cysteine content were lower 2.1, 1.7 and 1.3%, respectively. The total number of negatively charged residues (Asp + Glu) was 88, whereas the total number of positively charged bases (Arg + Lys) was 66. The instability index was 50.46, which indicated an unstable protein, and the average hydrophobicity was -

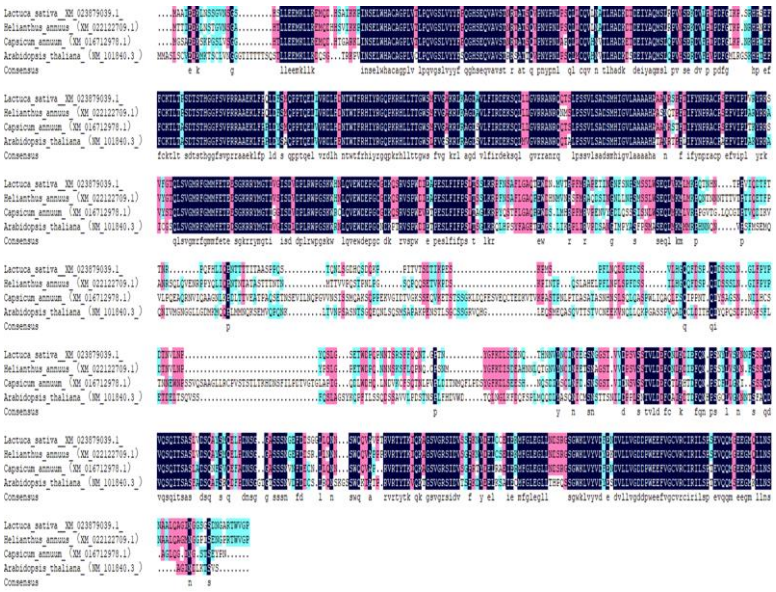

Fig. 3: Alignment and analysis of amino acid sequences of *LsARF5*

Fig. 4: Conserved domain of *LsARF5* protein

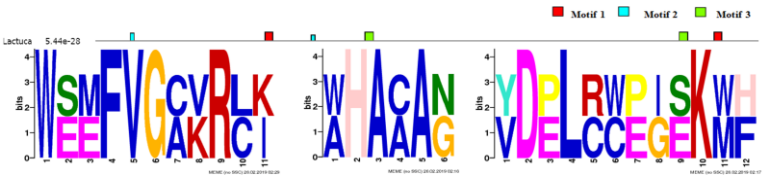

Fig. 5: Motif composition analysis of *LsARF5*

0.475, which suggested the prediction of a hydrophilic protein. As analysed by SignalP 4.1, the protein has no signal peptide, does not contain a transmembrane structure, and is not a secreted protein. PSORT software was used to predict the subcellular localization of the protein, and the results showed that the *LsARF5* protein was mainly localized to the nucleus.

The conserved motif of *LsARF5* was analysed by MEME software and found to have three highly conserved motifs located within the B3, Auxin_resp, and AUX_IAA conserved domains (Fig. 5). Further, SWISS-MODEL was used to infer the tertiary structure of the *LsARF5* protein; this protein matched the model of multiple ARF proteins and contained the conserved structure unique to the ARF family. These results are consistent with those of the NCBI CD-search analysis (Fig. 6).

Phylogenetic Analysis of the *LsARF5* Protein

The amino acid sequence of the obtained *LsARF5* gene was aligned in the NCBI protein database, and it was found to have high homology with the *ARF5* protein in 11 plants such as sunflower, *A thaliana* and pepper. It can be seen from Fig. 7 that the twelve amino acid sequences are clearly divided into two categories, among which lettuce, sunflower and artichoke are the first types, because all three are members of the Asteraceae. Arabidopsis and the Brazilian rubber tree are included in the second category (Fig. 7).

Analysis of *LsARF5* Gene Expression

It can be seen from Fig. 8 that an overall expression of the *LsARF5* gene in the treatment groups of GB-30 was the same, showing a first rising and then falling trend. The relative expression of the *LsARF5* gene in the control group was higher than that in the high temperature group at each period. These differences were clearly significant on the 8th, 16th and 24th days. Based on the above analysis, high temperature inhibited the expression of the *LsARF5* gene in GB-30, suggesting that the *LsARF5* gene may be involved in leaf lettuce bolting.

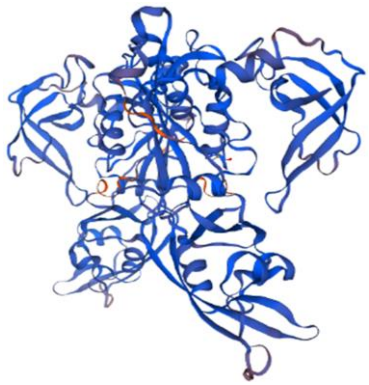

Fig. 6: The prediction of *LsARF*5 protein structures

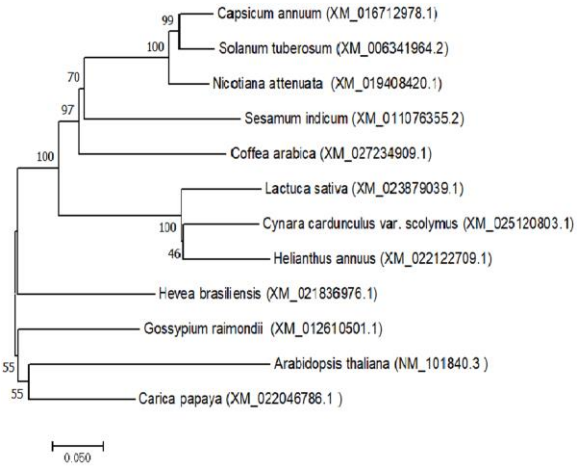

Fig. 7: Phylogenetic tree

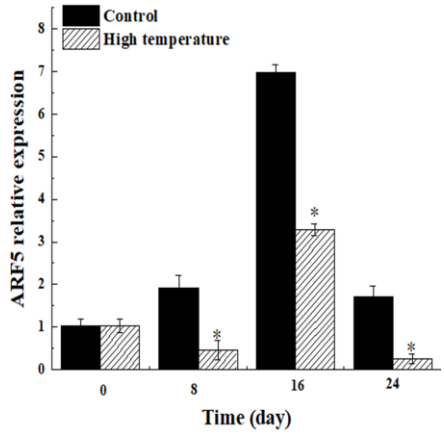

Fig. 8: Relative expression analysis of *LsARF5* under 20/13℃, 33/25℃ of GB-30 (* stands for P＜0.05)

Discussion

Plant growth and development are coordinated by interacting hormones, and the interaction between auxin signaling is critical for a wide range of processes during plant development (Wolters and Jürgens, 2009). In this study, it was found that ARF has robust expression differences and functions in different tissues and organs, but most of its functions are related to the development of vascular bundles, and indirectly affect the physiological metabolism of plants. A more comprehensive and in-depth study of the molecular mechanisms of the ARF gene structure, response to auxin signaling, and regulation of plant growth and development would help ultimately reveal the function of auxin in plant growth and development. Most developmental processes in which auxin is involved in the regulation are regulated by gene expression (Weijers and Wagner, 2016; Roosjen *et al.*, 2018). The ARF family of important transcription factors involved in the auxin signal transduction pathway plays an important role in this pathway. Different ARF genes are expressed differently in different tissues and organs, and the expression of these genes is affected by different signals. The regulation of the auxin response gene is an extremely complex process. ARF proteins bind to AuxREs and activate the expression of target genes under the action of the AD region of the ARFs, which respond to auxin signals (Ulmasov *et al.*, 1999). Thus, AuxRE elements are competitive binding sites that are competitive between not only the members of the large family of ARF proteins, but also the members of both the ARF protein family and the Aux/IAA protein family. Moreover, interactions between *ARF5* and *IAA12* and between *ARF5* and *IAA18* have been discovered (Weijers *et al.*, 2006; Chapman and Estelle, 2009; Ploense *et al.*, 2009).

In recent years, with the development of genome-wide studies of various species, members of the ARF family have been identified in plant species such as Arabidopsis, tomato, cucumber and apple (Hardtke and Berleth, 1998; Wenzel and Chuetz, 2007; Udaya *et al.*, 2007; Rahul *et al.*, 2011). This knowledge lays a good foundation for studying the functions and mechanisms of the ARF transcription factor family members in plant growth and development, and for future research on auxin and other plant hormone signal transduction pathways. *ARF5* is an important member of the ARF gene family. The first mutant of the *AtARF* gene identified in Arabidopsis is a monopteros mutant, which was later shown to be an allele of *AtARF5* (Hardtke and Berleth, 1998). In *A thaliana*, *AtARF5* was most abundantly expressed in embryos and vascular bundles (Remington *et al.*, 2004; Wu *et al.*, 2011). In tomato, *SlARF5* is expressed the most from the young fruit stage to the fruit ripening stage (Liu and Hu, 2013.). In poplar, the expression level of the *AtARF5* homologous gene *PoptrARF5.1* is slightly higher in roots than in stems and leaves, and *PoptrARF5.2* expression is highest in flower buds (Li *et al.*, 2015).

Conclusion

The expression level of the *LsARF5* gene was significantly downregulated in the easily bolting variety GB-30 and was

significantly inhibited by high temperature. This bolting may be related to the inhibition of *LsARF5* gene expression, and the stem began to grow rapidly when the leaves were treated with high temperature. The CDS of the *ARF5* gene of leaf lettuce was cloned and analyzed by bioinformatics, which laid a foundation for future experiments. qRT-PCR analysis showed that the relative expression of this gene at high temperature was significantly lower than that in the control group. Thus, the *LsARF5* gene may be associated with bolting. Subsequent work will further elucidate the mechanism of action of the *LsARF5* gene in leaf lettuce during bolting.

Acknowledgements

This work was financially supported by the 2018 Joint Funding Project of Beijing Natural Science Foundation-the Municipal Education Commission (KZ201810020027), the National Natural Science Foundation of China (Grant No. 31372057), and Earmarked Fund for Beijing Leaf Vegetables Innovation Team of Modern Agro-industry Technology Research System (BAIC08).

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[Received 19 Apr 2019; Accepted 07 Jun 2019; Published (online) 10 Nov 2019]