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# Cloning and expression analysis of heat-shock transcription factor gene *CaHsfA2* from pepper (*Capsicum annuum* L.)

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**ABSTRACT.** The heat-shock transcription factor (Hsf) gene *CaHsfA2* (GenBank accession No. JX402923) was cloned from the *Capsicum annuum* thermotolerant line R9 by combining the techniques electron cloning and rapid amplification of cDNA ends. The gene, which is 1436 bp in length, had an open reading frame of 1089 bp that encoded 362 amino acids. There was an 831-bp intron between positions 321 and 322 of the cDNA. The deduced amino acid sequence of *CaHsfA2* contained the conserved domains of Hsf, including DNA binding domain, adjacent domain with heptad hydrophobic repeats (A/B), activator motifs, nuclear localization signal, and nuclear export signal, and it had the highest *E* value of hypothesized annotation of HsfA2. *CaHsfA2* had the nearest phylogenetic relationship with *HsfA2* from *Lycopersicon peruvianum* and *Mimulus guttatus*, which was consistent with its botanical classification. After heat-shock treatment at 40°C for 2 h, the expression of *CaHsfA2* was observed in different tissues of thermotolerant cultivar R9 and thermosensitive line B6; however, the expression levels of the

 

*CaHsfA2* gene were significantly different as follows: expression in B6 leaf > stem > flower > root, and expression in R9 flower > leaf > stem ≈ root.

**Key words:** *Capsicum annuum* L.; Gene cloning; Gene expression; *HsfA2*

## INTRODUCTION

Temperature is an important factor that affects plant growth. However, with the development of economics, the amount of greenhouse gasses such as $CO_2$ and $CH_4$ is increasing greatly, which leads to global climate warming. Agriculture production is facing a challenge from the high temperature stress or heat stress. According to the present temperature rising trends, the crop yield would decrease 1.5% per decade (Lobell and Gourdji, 2012) if there are no suitable improvements.

Raising thermotolerant varieties is one important way to assure the production of the crops under heat stress; the efficiency of these varieties highly depends on the illustration of thermotolerant mechanisms at the morphological, cytological, and molecular levels. Plenty of research about thermotolerant physiological activities of the crops have been reported; however, such studies at the molecular level are scarce (Wahid et al., 2007). The accumulation of heat-shock proteins (Hsps) is very important for the development of plant thermotolerance under heat stress. The transcription of the Hsps is induced by combining heat-shock transcription factor (Hsf) with the heat-shock element (HSE), which makes the plant acquire thermotolerance (von Koskull-Döring et al., 2007). As the prevalent Hsf in plants, HsfA2 is intensely induced by heat stress and has strong activation ability on the transcription of the Hsp gene during prolonged heat stress. All of these are important for the formation of plant thermotolerance (Schramm et al., 2006). Until now, the function of the gene *HsfA2* in thermotolerance formation has been researched in *Solanum lycopersicum* (Scharf et al., 1993), *Arabidopsis thaliana* (Charng et al., 2007), *Oryza sativa* (Yokotani et al., 2008), and *Lilium longiflorum* (Xin et al., 2010).

Pepper (*Capsicum annuum* L.) is a typical non-thermophyte. Heat stress will appear when the temperature is above 32°C, which can cause serious pollination and fertilization problems and result in blossom and fruit dropping. The thermosensitivity problem has become a serious barrier during *C. annuum* production (Lu et al., 2009). Therefore, in order to gain additional information about the function of *CaHsfA2* and the molecular mechanism of pepper thermotolerance formation, the pepper thermotolerant line R9 was used as material to clone *CaHsfA2* by combining the techniques electron cloning and rapid amplification of cDNA ends (RACE). The bioinformatic analysis of the deduced protein sequence was performed. Furthermore, the expression of *CaHsfA2* in different tissues was analyzed in different thermotolerant pepper cultivars by real-time polymerase chain reaction (PCR).

## MATERIAL AND METHODS

### Plant materials

The pepper thermotolerant line R9 and thermosensitive line R6 were used as materials.

 

R9 was introduced from the Asia Vegetable Research and Development Center (AVRDC), and the original code was PP0042-51. B6 was selected by the pepper research group in the College of Horticulture, Northwest A&F University.

## Cloning the *CaHsfA2* gene in pepper

### *Pepper growth conditions and stress treatments*

The thermotolerant line R9 was planted in the regular manner, and the seedlings with 4-5 leaves were raised in an illumination incubator (GXZ-380C, Jiangnan Instrument Factory, China) under 25°/15°C and full-light conditions. Both the thermoperiod and the photoperiod are 12 h. After 3 days, the temperature was changed from 25° to 40°C for heat-shock treatment. Two hours later, the young leaves of 5 pepper seedlings were mix-collected and quick frozen with liquid nitrogen, and the total RNA was extracted immediately (Zhu et al., 2011).

### *Total RNA extraction and cDNA synthesis*

Total RNAs of pepper leaves were extracted with the Total RNA kit (TIANGEN, China) and cDNA was synthesized by the SMART kit (Clontech, USA) according to manufacturer instructions.

### *Cloning the CaHsfA2 gene*

The two *CaHsfA2* fragments, 387 and 348 bp, were assembled using ContigExpress with homologous sequences that were identified by the Basic Local Alignment Search Tool (BLAST) with the tomato *LpHsfA2* (X67601.1) sequence as the query in the pepper expressed sequence tag (EST) database of GenBank. According to the nucleotide sequence of these 2 fragments, forward primers HsfA2-F1 and HsfA2-F2 and reverse primers HsfA2-R1 and HsfA2-R2 were designed using Primer Premier 5.0. The 3'- and 5'-sequences of *CaHsfA2* were amplified using the nested primer constructed by the 4 primers and the general primer in the SMART kit as shown in Figure 1. The nucleotide sequence of the primers are given in Table 1. For 3'-RACE, HsfA2-F1/RA31 was used in the first amplification and HsfA2-F2/RA32 in the second amplication; for 5'-RACE, HsfA2-R1/UPM was used in the first amplification and HsfA2-R2/RA54 in the second amplification. The primers were synthesized by biological engineering (Shanghai).

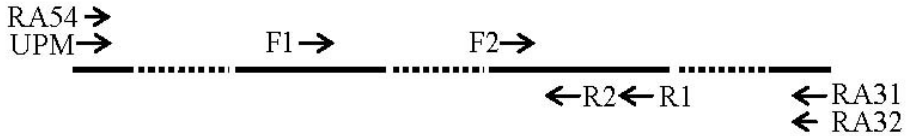


**Figure 1.** Relative position of the primers used in *CaHsfA2* amplification by the RACE technique. *Solid lines* in the middle represent the known *CaHsfA2* fragments obtained by electron cloning technology. *Dashed lines* represent the unknown fragments. *Solid lines* on both sides represent the added sequences when the first chain of cDNA was synthesized.

**Table 1.** Primer sequences used in this investigation.

| Primer name | Primer sequence (5'→3') | Application |
|---|---|---|
| HsfA2-F1 | GTTCTCTACCACATTGCTCCCT | 3'-RACE |
| HsfA2-F2 | TATTGAAATGTTATTCTCTGCTG | 3'-RACE |
| RA31 | CTGATCTAGAGGTACCGGATCCTTTTTTTTTTTTTTTTTV | 3'-RACE |
| RA32 | CTGATCTAGAGGTACCGGATCC | 3'-RACE |
| HsfA2-R1 | TTCTAATTCCTCACCCCATACAG | 5'-RACE |
| HsfA2-R2 | TCACCAAGCAACTCTTCCCAAAT | 5'-RACE |
| UPM1 | CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT | 5'-RACE |
| UPM2 | CTAATACGACTCACTATAGGGC | 5'-RACE |
| RA54 | AAGCAGTGGTATCAACGCAGAGT | 5'-RACE |
| HsfA2-F3 | ACATGGGGATCCATCTTAATTGTAT | Reverse PCR |
| HsfA2-R3 | CAAGGAAAATATTAATTCTGCACTT | Reverse PCR |
| HsfA2-F4 | CTTCATTGTTTGGGACTCTCAT | RT-PCR |
| HsfA2-R4 | CCTCCATTCCATAATAACCTACTT | RT-PCR |
| UBI3-F | TGTCCATCTGCTCTCTGTTG | RT-PCR |
| UBI3-R | CACCCCAAGCACAATAAGAC | RT-PCR |

The UPM primer used in RACE contains 0.4 M UPM1 and 2 M UPM2.

The amplified products of 3'- and 5'-RACE were ligated to the pMD19-T vector after being purified for sequencing. The full-cDNA sequence of *CaHsfA2* was assembled by ContigExpress with sequencing results. The primers HsfA2-F3 and HsfA2-R3 (Table 1) were designed to clone the full cDNA of the pepper heat-shock transcription factor gene *CaHsfA2* using *pfu* polymerase.

## *CaHsfA2* sequence analysis

The open reading frame (ORF) of *CaHsfA2* was confirmed using the ORF Finder of the National Center for Biotechnology Information (NCBI) and then translated into the amino acid sequence. The full-*CaHsfA2* cDNA was submitted to Heatster web site (http://www.cibiv.at/services/hsf/), which was established by Nover and Scharf, to infer whether *CaHsfA2* belonged to Hsf. According to the HsfA2 amino acid sequences from different plants that were collected by Heatster and NCBI, the neighbor joining phylogenetic tree of *CaHsfA2* was constructed by MEGA 5.0 with a multiple-sequence alignment that was constructed using ClustalW, a phylogenetic tree measurement of 1000 bootstraps, a Poisson model, and pairwise deletion.

## *CaHsfA2* gene intron analysis

The pepper thermotolerant line R9 was cultivated in the regular manner. The seedlings with 4-5 leaves were collected and quick-frozen with liquid nitrogen, and the DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Zhu et al., 2011). The full *CaHsfA2* was specifically amplified using primer HsfA2-F3 and HsfA2-R3 (Table 1). By comparing the lengths of the DNA- and cDNA-amplified products, whether gene *CaHsfA2* contained introns can be inferred. The position and the length of the intron were confirmed through homologous comparison of the *CaHsfA2* DNA sequence with its cDNA sequence after being purified and sequenced.

## *CaHsfA2* tissue-specific expression analysis

The pepper materials of the thermotolerant line R9 and the thermosensitive line B6

were cultured in the regular manner until the flower buds appeared, and then the heat-shock treatment was performed as described in the Section Pepper growth conditions and stress treatments. After a 2-h treatment, the young leaves, flower buds, stems, and roots from 5 plants of the 2 pepper lines were mix-collected and quickly frozen with liquid nitrogen for the total RNA extraction and cDNA synthesis. According to the position of the intron in *CaHsfA2*, the specific gene primers HsfA2-F4 and HsfA2-R4, which span the intron (Table 1), were designed for the real-time quantitative PCR (RT-PCR) using the SYBR Premix Ex Taq II kit (TaKaRa, Japan), the cDNA of different pepper tissues as the template, and the ubiquitin binding protein gene *UBI-3* as the reference gene with the primer pair UBI3-F and UBI3-R (Table 1) (Wan et al., 2011). All of the PCRs were carried out in triplicate, and the data analysis was done with Bio-Rad iQ5 (version 2.1) and Excel 2003.

## RESULTS

### Isolation of the *CaHsfA2* gene

The full-length cDNA of the pepper heat-shock transcription factor gene *CaHsfA2* was obtained by combining electron cloning and the RACE technique using the first strand of cDNA from the leaves of the pepper thermotolerant line R9 after a 2-h treatment at 40°C as the template. The amplified products of 3'- and 5'-RACE were about 580 and 1000 bp, respectively. The total length of the assembled cDNA was 1436 bp (Figure 2). The full-length *CaHsfA2* cDNA sequence was amplified using a primer pair that was designed according to the full-length cDNA sequence and high-fidelity *pfu* polymerase. The sequence analysis showed that the full-length cDNA was 1089 bp, extending from nucleotides 52-1140 and encoding 362 amino acids (Figure 3).

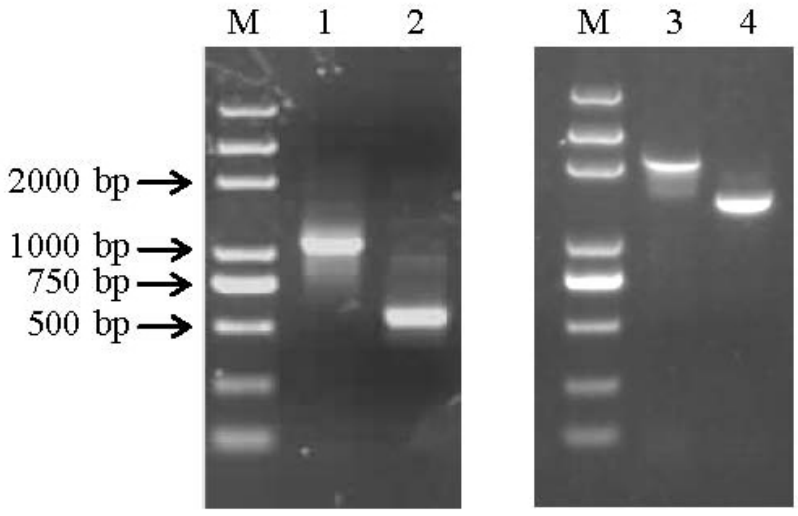


**Figure 2.** Cloning of the pepper heat-shock transcription factor gene *CaHsfA2*. *Lane M* = marker. *Lanes 1* and *2* = amplified product of 5'- and 3'-RACE, respectively. *Lanes 3* and *4* = all-length of DNA and cDNA.

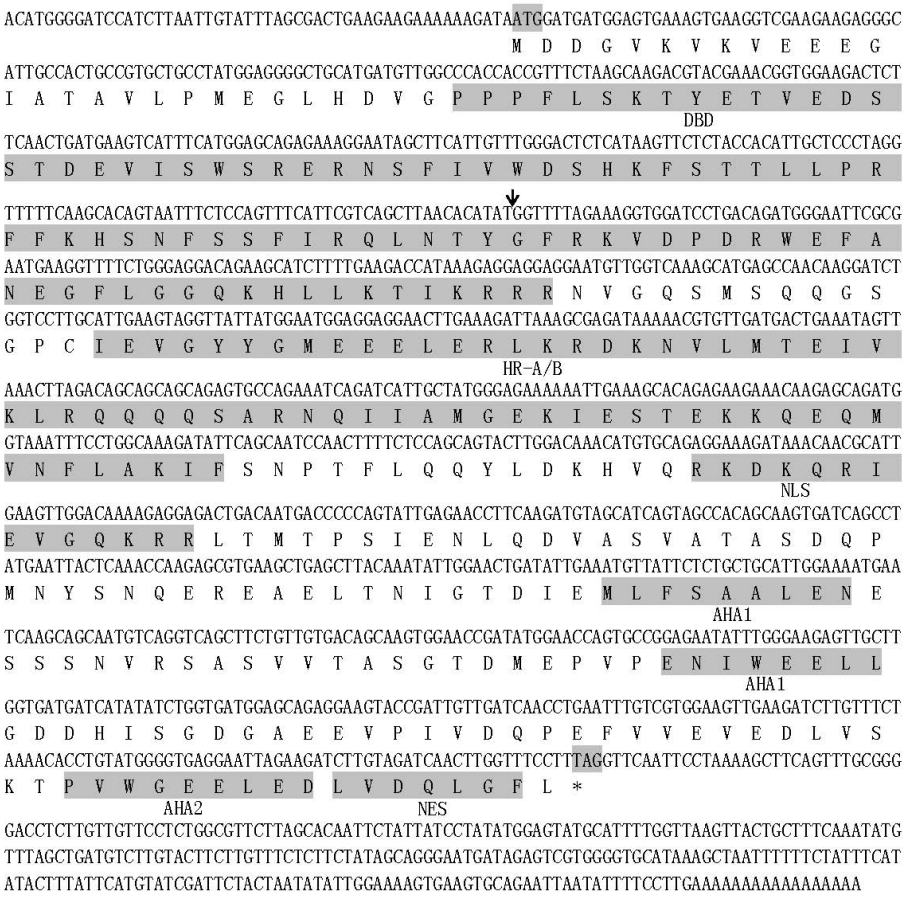


**Figure 3.** cDNA sequence and deduced amino acid sequence of *CaHsfA2*. The conserved domains are highlighted in gray. The letters and marks in the alignment represent: DBD = DNA binding domain; HR-A/B = hydrophobic repeat regions A/B; NLS = nuclear localization signal; AHA = activator motifs; NES = nuclear export signal. The position of inverted intron is indicated by an arrow. ATG is the start codon, and TAG is the stop codon.

## *CaHsfA2* sequence analysis

### *CaHsfA2 sequence characteristic analysis*

The deduced amino acid sequence of *CaHsfA2* was used for sequence analysis by the Heatster web site. The results showed that the deduced amino acid sequence of *CaHsfA2* contained the main conserved domains, including a DNA binding domain (DBD), oligomerization domain (OD), nuclear localization signal (NLS), activator motifs (AHA), and nuclear export signal (NES). The deduced *CaHsfA2* showed the highest *E* value of tentative annotation with HsfA2 (1e-201), and the *E* value with HsfA9, HsfA6/A7, HsfA6, and HsfA1 (2.3e-127, 1.1e-116, 1.3e-116, and 1.5e-96, respectively). Thus, we inferred that the cloned gene in this study belonged to the *HsfA2* family, and we named it *CaHsfA2* (JX402923).

## *CaHsfA2 phylogenetic tree analysis*

The neighbor joining phylogenetic tree of HsfA2 was constructed based on the multiple sequence alignment of the amino acid sequences of HsfA2 (or HsfA2a) from 31 plant species. As shown in Figure 4, HsfA2 of *Lycopersicon peruvianum* and *Mimulus guttatus* were the best matches of pepper CaHsfA2, which agreed with their botanical classification. Similar results were observed among other plant species, such as *Glycine max*, *Lotus japonicus*, *Arachis hypogaea*, and *Medicago truncatula* of family Leguminosae; *Malus domestica* and *Prunus persica* of family Rosaceae; *Arabidopsis thaliana*, *Thellungiella halophila* and *Brassica napus* of family Cruciferae; *Manihot esculenta* and *Ricinus communis* of family Euphorbiaceae, and *Oryza sativa*, *Hordeum vulgare*, *Sorghum bicolor*, *Zea mays*, *Setaria italica*, and *Brachypodium distachyon* of family Gramineae.

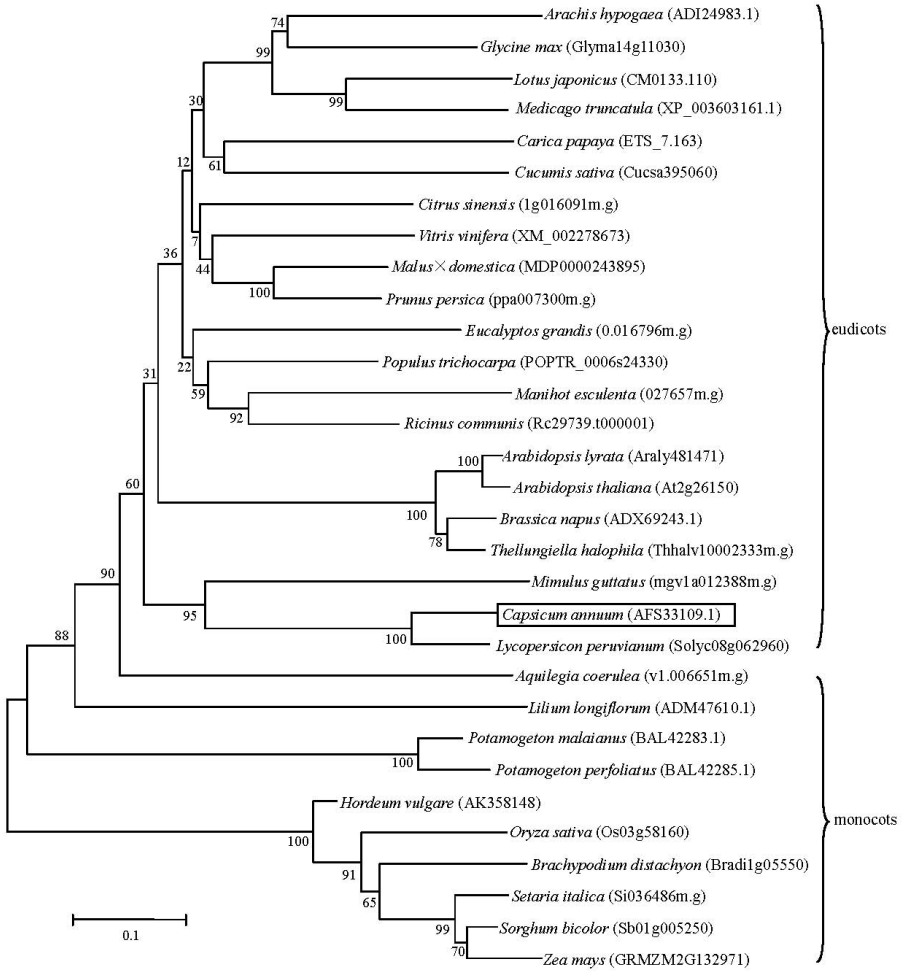


**Figure 4.** Neighbor-joining phylogenetic tree of HsfA2 from different species. The pepper CaHsfA2 is indicated by the open box. The numbers in the joint are the confidence levels. The genetic distance is indicated by bar line.

### *CaHsfA2 gene intron analysis*

The full-length DNA sequence of *CaHsfA2*, spanning 2200 bp, was cloned using the full-length primers of cDNA and the total DNA of the pepper thermotolerant line R9 as template. After sequencing and homologous comparison, an 831-bp intron was found between positions 321 and 322 (DBD region) of the *CaHsfA2* cDNA (Figure 3).

### *CaHsfA2* gene tissue-specific expression analysis

*CaHsfA2* was expressed in different tissues of the pepper thermotolerant line R9 and the thermosensitive line B6 after heat-shock treatment for 2 h; however, the expression levels were greatly different (Figure 5). In B6, the order of the *CaHsfA2* gene expression in different issues was leaf > stem > flower > root, while in R9, the order was flower > leaf > stem ≈ root, and the difference in different tissues was also obvious ($\alpha = 0.05$). In general, the *CaHsfA2* expression level in B6 was higher than that in R9.

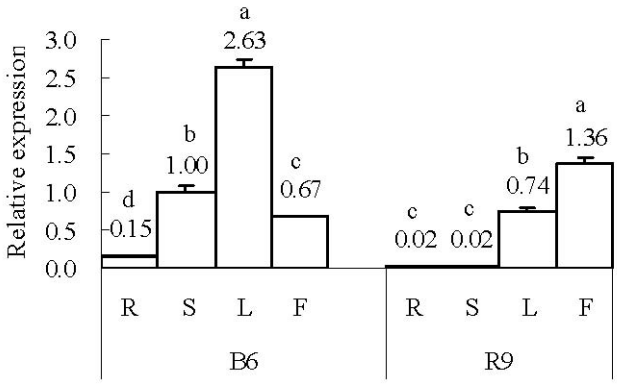


**Figure 5.** Expression of the *CaHsfA2* gene in different pepper tissues after heat-shock treatment. B6 = pepper thermosensitive line; R9 = pepper thermotolerant line; R = root; S = stem; L = leaf; F = flower. The numbers in the figure represent the relative expression in different pepper tissues. The letters represent the obvious difference.

## DISCUSSION

Heat-shock transcription factor A2 (HsfA2) does not only play an important role in the development of thermotolerance in plants but also regulates the plant's ability to resist other environment stresses such as highlight (Nishizawa et al., 2006), hypoxia (Banti et al., 2010), high salt, and osmotic stress (Ogawa et al., 2007; Yokotani et al., 2008). Thus, the cloning of *CaHsfA2* and the study of its biological function are very important to further understand the thermotolerance and other abiotic stress-tolerance mechanisms in pepper.

With the development of sequencing technology, plenty of plant ESTs are published, which provides great convenience in cloning the important genes in the plant that have not been sequenced. In this study, the combination of the electron cloning with the RACE technique to clone the pepper *CaHsfA2* gene (Figure 1) not only boosted the efficiency but also assured the accuracy. Two fragments of the *CaHsfA2* gene have been obtained through electron

cloning technology. Based on this, the nested PCR was performed for 3'- and 5'-RACE, and the full-length cDNA sequence was obtained. The amino acid sequences that were deduced from *CaHsfA2* contained all of the conserved domains (Figure 3). The position of the intron was also conserved as a heat-shock transcription factor (Scharf et al., 2012). What is more, the *E* value of the hypothetical gene annotation was the highest with HsfA2. This meant that the gene belonged to the heat-shock transcription factor gene family, which made a good foundation of our research.

Today, the molecular systematics based on the genetic relationships is one of the most active research areas in plant taxonomy (Whelan et al., 2001). In this study, after the amino acid sequence phylogenetic analysis of pepper CaHsfA2 and HsfA2 (or HsfA2a) from 31 other plant species, we found that CaHsfA2 had the closest genetic relationship with *Lycopersicon peruvianum* and *Mimulus guttatus* (Figure 4). This agreed with its botanical classification. Additionally, the genetic relationships of HsfA2 in the other species that had similar botanical classifications were also close. This implied that the HsfA2 evolved together with the evolution of the species, which was consistent with the report by Scharf et al. (2012). The chloroplast *rbcL* gene (encoding the large subunit of rubisco), *atpB* gene (encoding the chloroplast ATP synthase b subunit), and the ITS (internal transcribed spacer) region of 18S rDNA are used most commonly in molecular systematical analysis (Whelan et al., 2001). If the *HsfA2* gene can be identified in more plants, it may become a candidate marker gene for molecular systematic analysis.

The *HsfA2* gene expression was induced by heat shock, and it correlated positively with the plant's level of thermotolerance (Charng et al., 2007; Scharf et al., 2012). In this study, after being treated at 40°C for 2 h, the *CaHsfA2* expression was detected in the 2 pepper lines. The expression level in the heat-sensitive line B6 was higher than that in the heat-tolerant line R9 as a whole, which was not coherent with the heat tolerance (Figure 5). Xin et al. (2010) found that the *HsfA2* expression in heat-tolerant cultivar leaves of *Lilium longiflorum* reached its peak 1 h after the heat treatment, and then it decreased slightly. In contrast, in the heat-sensitive cultivar, the expression peaked 12 h after the heat shock and then decreased significantly. In this study, the samples were collected 2 h after the heat shock. We speculated that this might be the time when the *CaHsfA2* expression decreased in R9 but increased in B6. Busch et al. (2005) and Schramm et al. (2006) both thought that the inconsistency between the plant heat tolerance and *HsfA2* expression pattern was related to dynamic *HsfA2* expression levels during the heat shock. Therefore, it is necessary to further analyze the dynamic *HsfA2* expression levels during heat stress to clarify the relationship between *CaHsfA2* expression and pepper heat tolerance.

After heat shock, the *CaHsfA2* expression level in the leaves of the thermosensitive line B6 was much higher than that in the flower. This was coincident with the idea that the reproductive growth of plant is more sensitive to the heat stress than the vegetative growth (Snider and Oosterhuis, 2011). However, the *CaHsfA2* expression level in the flower of the thermotolerant line R9 was much higher than that in the leaves. Giorno et al. (2010) reported similar findings in *Solanum lycopersicum* cv Saladette. This was probably because HsfA2 was involved in the anther's normal development in addition to its key role in the development of thermotolerance (Scharf et al., 2012). In Frank's investigation (2009), under normal temperature conditions, the *HsfA2* expression level in microspores of a *Solanum lycopersicum* thermotolerant cultivar was higher than that in the thermosensitive cultivar, which resulted in a higher basal thermotolerance. This might be the reason why R9 was more thermotolerant than B6.

In summary, we have presented the isolation of a pepper heat-shock transcription factor gene, *CaHsfA2*. The phylogenetic relationship of the *CaHsfA2* deduced amino acid sequence with the HsfA2 amino acid sequence from other plant species was similar to the botanical classifications. We speculated that HsfA2 could be used as a candidate marker gene for molecular phylogenetic research. After heat stress, *CaHsfA2* was expressed in different tissues of various thermotolerant pepper lines; however, the expression patterns were different. The relationship between the gene and the formation of pepper thermotolerance require further research.

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