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Heat shock transcription factors expression during fruit development and under hot air stress in Ponkan (*Citrus reticulata* Blanco cv. Ponkan) fruit

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ABSTRACT

Heat shock transcription factors (Hsfs) play a role in plant responses to stress. Citrus is an economically important fruit whose genome has been fully sequenced. So far, no detailed characterization of the *Hsf* gene family is available for citrus. A genome-wide analysis was carried out in *Citrus clementina* to identify *Hsf* genes, named *CcHsf*s. Eighteen CcHsfs were identified and classified into three main clades (clades A, B and C) according to the structural characteristics and the phylogenetic comparison with *Arabidopsis* and tomato. MEME motif analysis highlighted the conserved DBD and HR-A/B domains, which were similar to Hsf protein structures in other species. Gene expression analysis in Ponkan (*Citrus reticulata* Blanco cv. Ponkan) fruit identified 14 *Hsf* genes, named *CrHsf*, as important candidates for a role in fruit development and ripening, and showed seven genes to be expressed in response to hot air stress. *CrHsfB2a* and *CrHsfB5* were considered to be important regulators of citrate content and showed variation in both developmentally-related and hot air-triggered citrate degradation processes. In summary, the data obtained from this investigation provides the basis for further study to dissect Hsf function during fruit development as well as in response to heat stress and also emphasizes the potential importance of *CrHsf*s in regulation of citrate metabolism in citrus fruit.

1. Introduction

Heat shock transcription factors (Hsfs) are important components in sensing and signaling different environmental stresses (von Koskull-Doring et al., 2007). *Arabidopsis*, which served as the prototype for the Hsf family, has a set of 21 Hsf encoding genes with 15 members belonging to class A, five members to class B and one to class C (Nover et al., 2001; Baniwal et al., 2004). Genome wide identification of Hsf family members has been fully described in rice, tomato, apple, Chinese cabbage and other species (Nover et al., 2001; Baniwal et al., 2004; Wang et al., 2009; Giorno et al., 2012; Ma et al., 2014). Commonly, all plant Hsfs have DNA binding domains (DBD) at the N-terminal and an oligomerization domain (HR-A/B) (Nover et al., 2001). Other Hsf functional modules include a nuclear localization signal (NLS) essential for nuclear import, leucine-rich export sequences important for nuclear export (NES), and a less conserved C-terminal activator domain, the so-called AHA motifs (Doring et al., 2000; Nover et al., 2001).

The functions of *Hsf* genes have been widely studied in model plants, such as *Arabidopsis* and tomato. For example, *HsfA1a* has been known as a master regulator of heat response in tomato, since it cannot be replaced by any other Hsf proteins (Mishra et al., 2002); *Arabidopsis* plants overexpressing *HsfA2* showed not only higher levels of thermotolerance but also increased resistance to salt or osmotic stress (Ogawa et al., 2007), oxidative stress (Zhang et al., 2009) and anoxia (Banti et al., 2010). Despite structural similarities, *HsfA4* acted as a potent activator of heat shock gene expression, whereas *HsfA5* was inactive and inhibited *HsfA4* activity (Baniwal et al., 2007). In contrast to class A *Hsfs*, a considerable number of *Hsfs* assigned to classes B and C have no evident function as transcription activators on their own (Czarnecka-Verner et al., 2000), but a highly conserved -LFGV- tetrapeptide forms the core of a repressor domain in class B *Hsfs* (Ikeda and Ohme-Takagi, 2009). However, under certain conditions of

Abbreviations: Hsf, heat shock transcription factor; DBD, DNA binding domains; TF, transcription factor; DAF, days after flowering; RH, relative humidity; GC–MS, gas chromatography–mass spectrometry; qRT-PCR, quantitative real-time PCR; H2-T-H3, helix-turn-helix motif; HSE, heat stress element

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Table 1
Primers used to analyze gene expression patterns by quantitative real-time PCR (qRT-PCR) in the present research.

Gene name	Forward primer	Reverse primer
CrHsfA1a	GAAAGCACTATTAGCATCCCTG	CCATCTGGCGAAACATCC
CrHsfA1b	GGCAATCCTCTTCACGTTCC	ACTTTCGGGCATTATTTCTGG
CrHsfA2	CACCTGTGGGTCTGGATTG	ACCAGCATTTGGGTTGTCG
CrHsfA3	GGGGAAACCTCACCATACCT	TACCTGATCCGATGCCTACC
CrHsfA4a	TGTTGATGCTCGCCCTAAAT	GCTTGCACAGGAATGGTGGT
CrHsfA4b	GTGGCGGTAGTTTCATTGTC	AGATGCCTCTGTCCTCGTATG
CrHsfA5a	ATGGAGCAGAGGCAGGAGAAT	CCACGATTGGCTTTGATTGAT
CrHsfA6	AATTAGCCCAGCAGAAGGAT	AAGTTCAGCCAGGTCACCAT
CrHsfA7	CTGGTAGCGAAGTGGGTGAT	AACTGATGCCGAATGCTGAT
CrHsfA8	CCAGCAGCAGATGTTGTCATT	AACTTCTTCGAGCATAGTGCC
CrHsfA9b	CAAAGCGTGCCTGAGTCTGT	AAGCCATCGTCATCCATAAGAG
CrHsfB1	TTATCGGACGAGAATGCG	GACCCACAGCTTCCTTGC
CrHsfB2a	GAACTTTCTGGAGGCCGAGT	CTGCCATTTCCATCCAAGC
CrHsfB2b	CGACGGATCGACCTTCATAG	TCTCACCTCTTCGGAAACAATC
CrHsfB3	CCTAAGGGATGAGAATAAGCGT	AGCCACTAAATCAAGCAACTCC
CrHsfB4	GCACCCACATTCACATTCTC	CAGCCAGCACCACTACTCAT
CrHsfB5	AGCCAGTCGTCTCCAAGAAC	AACTCAGCCGGTGACCATAC
CrHsfC1	TTTTGTCAGTCATCTCCGTCAC	CATCACTTGCCTGCTTTGC
Actin	CATCCCTCAGCACCTTCC	CCAACCTTAGCACTTCTCC

appropriate promoter architecture, the heat shock-induced tomato *HsfB1* can act as co-activator cooperating with *HsfA1a* (Kotak et al., 2004). With the exception of tomato, the *Hsf* gene function has rarely been reported in fruit.

Organic acids are also involved in many stress processes (Marschner and Marschner, 2012), especially in fruit storage under heat treatment, which has been widely studied in various fruits. For example, organic acids content was observed to decrease in apples under heat treatments (Klein and Lurie, 1990); lower organic acid content was detected in peach fruit exposed to hot air (Lara et al., 2009); lower titratable acid was observed in navel oranges treated with hot air (Shellie and Mangan, 1998); citrate content decreased under hot air treatment in citrus fruit (Chen et al., 2012; Yun et al., 2013). Also, expression of some genes related to citrate metabolism has been identified in response to stress. For example, increased levels of *citrate synthase* gene expression enhanced Al tolerance (delaFuente et al., 1997; Anoop et al., 2003); lack of *Aco1* enzymatic activity in mitochondria increased zinc tolerance in *Saccharomyces cerevisiae* (Guirola et al., 2014); the *CitAco3–CitIDH2/3–CitGAD4* cascade was enhanced under hot air treatment in Ponkan fruit (Chen et al., 2012). However, the regulation of organic acid responses to environmental stress and the role of related transcription factors have rarely been investigated and are not understood.

Citrus, which is one of the most important horticultural crops, is characterized by citrate accumulation and degradation during different fruit developmental stages. It has been shown previously that fruit citrate content can also be influenced by heat stress (Chen et al., 2012; Yun et al., 2013). However, the possible relationship between *Hsf* genes, the transcription factors (TFs) most closely related to heat shock, and citrate metabolism has not been reported. In the present study, sequences of citrus *Hsf* genes were isolated based on genomic information, and phylogenetic and structural analysis performed. A developmental series of Ponkan (*Citrus reticulata* Blanco cv. Ponkan) fruit was collected for temporal expression analysis of *Hsf* genes, and the expression of *Hsf* genes in response to hot air treatment were characterized in mature Ponkan fruit. The possible role of *Hsf* genes in citrate metabolism is discussed.

2. Materials and methods

2.1. Plant materials and treatments

Ponkan fruit (*C. reticulata* Blanco cv. Ponkan) of uniform size were collected for the developmental series from orchards in Quzhou, Zhejiang, China. Nine fruits were selected from three different trees at each sampling point at 60 (S1), 90 (S2), 120 (S3), 150 (S4), 180 (S5) and 200 (S6) days after flowering (DAF) in 2012. S1 and S2 were characterized by extensive cell division, while S3 and S4 were characterized by cell expansion. S5 was the fruit ripening stage when fruit growth slowed down and the pulp reached its final size. S6 was the harvest stage when fruit had reached commercial maturity. For heat treatment, mature Ponkan fruits picked in 2011 were subjected to the following treatments: (1) Hot air treatment: the fruits were kept in a chamber at 40 °C, >90% relative humidity (RH) for two days followed by storage at 10 °C, 85%–95% RH; and (2) control fruits were stored at 10 °C with 85%–95% RH. The fruits were sampled at 0, 2, 10, 20 days after storage. The initiation of the treatments is referred to as 0 day in storage. Each sample consisted of nine fruits for each treatment, separated into three replicates with three fruits in each. Fruit at each sampling point were transported to the laboratory as soon as possible, the flesh was taken and immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Identification and classification of Hsfs in the citrus genome

Hsf genes were isolated from the *Citrus clementina* genome (Wu et al., 2014) (http://www.citrusgenomedb.org) based on annotation and BLAST. Firstly, the sequences indicated as belonging to Hsfs were downloaded and assembled with the CAP3 Sequence Assembly

Table 2
List of *Hsf* genes in the *Citrus clementina* genome.

Gene name	Genome number	Scaffold	Start	Stop	Size (aa)	MW (kDa)	pI
CcHsfA1a	Ciclev10020928m	3	40240644	40244399	497	55.32	5.31
CcHsfA1b	Ciclev10015269m	2	1187218	1192976	516	56.29	4.91
CcHsfA2	Ciclev10008617m	1	4143948	4146608	384	43.09	4.8
CcHsfA3	Ciclev10011531m	6	21074422	21078686	505	56.20	4.96
CcHsfA4a	Ciclev10015472m	2	34516499	34518924	403	46.00	5.09
CcHsfA4b	Ciclev10015413m	2	26097343	26099320	412	46.69	5.17
CcHsfA5	Ciclev10005059m	9	17075598	17077856	358	40.31	5.92
CcHsfA6	Ciclev10006902m	9	4098428	4100155	360	41.85	5.48
CcHsfA7	Ciclev10020718m	3	44141518	44144453	367	41.71	4.94
CcHsfA8	Ciclev10015541m	2	26890335	26894409	390	44.96	4.65
CcHsfA9	Ciclev10008116m	1	10527419	10529892	478	53.53	4.68
CcHsfB1	Ciclev10026058m	7	3182426	3186292	329	36.55	6.95
CcHsfB2a	Ciclev10005403m	9	28757274	28759075	330	36.00	6.61
CcHsfB2b	Ciclev10021287m	3	7047024	7049430	309	34.08	4.94
CcHsfB3	Ciclev10005649m	9	23409097	23411093	264	30.39	5.81
CcHsfB4	Ciclev10001482m	5	40075099	40076671	383	42.77	8.09
CcHsfB5	Ciclev10016707m	2	33354368	33355106	208	24.08	9.56
CcHsfC1	Ciclev10008768m	1	6358030	6359568	355	39.67	6.02

Size: deduced protein size; MW: molecular weight; and pI: isoelectric point.