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Genome-wide analysis of the *Hsp70* family genes in pepper (*Capsicum annuum* L.) and functional identification of *CaHsp70-2* involvement in heat stress

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ABSTRACT

Hsp70s function as molecular chaperones and are encoded by a multi-gene family whose members play a crucial role in plant response to stress conditions, and in plant growth and development. Pepper (*Capsicum annuum* L.) is an important vegetable crop whose genome has been sequenced. Nonetheless, no overall analysis of the *Hsp70* gene family is reported in this crop plant to date. To assess the functionality of *Capsicum annuum Hsp70* (*CaHsp70*) genes, pepper genome database was analyzed in this research. A total of 21 *CaHsp70* genes were identified and their characteristics were also described. The promoter and transcript expression analysis revealed that *CaHsp70s* were involved in pepper growth and development, and heat stress response. Ectopic expression of a cytosolic gene, *CaHsp70-2*, regulated expression of stress-related genes and conferred increased thermotolerance in transgenic *Arabidopsis*. Taken together, our results provide the basis for further studied to dissect CaHsp70s' function in response to heat stress as well as other environmental stresses.

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1. Introduction

Heat stress (HS) is a highly significant environmental factor that has been known to represent seriously threat to plant growth and development and causes great economic loss of crop yield worldwide [1]. The sessile plants have evolved mechanisms to cope with HS and thus confer increased thermotolerance, which called heat shock response (HSR) [2]. In HSR, the induced expression and accumulation of a group of conserved HS proteins (Hsps) lead to the acquired thermotolerance by maintaining homeostasis of protein folding [3,4]. Based on their apparent molecular mass, plant Hsps are divided into five families: small Hsps (sHsps), Hsp60, Hsp70, Hsp90 and Hsp100 [5]. As the most abundant proteins induced by an elevated temperature, Hsp70s are ubiquitous and highly conserved across all domains of life and are crucial housekeeping proteins (70-kD heat shock cognate, Hsc70), indicating that the Hsp70 members are essential for plant during normal growth and under HS condition [6,7].

Identification and characterization of Hsp70 proteins was pursued as early as in the 1960s [8]. Hsp70s are considered to be the most highly conserved Hsps [5] and are ATP-dependent chaperones having two major functional domains, a conserved ∼44-kD N-terminal ATPase domain (nucleotide binding domain; NBD) and a ∼18-kD substrate binding domain (SBD) with a ∼10-kD variable C-terminal "lid" [9]. Hsp110 proteins are included in Hsp70 family due to their high sequence and structural homology to Hsp70s, while the inserted acidic region in SBD or C-terminal extension leads to Hsp110s' larger size [9,10]. NBD performs regulatory role and SBD binds transiently to hydrophobic amino acid residues or surfaces that are exposed in nonnative states [11,12]. Successive cycles of substrate proteins binding and release are coupled to the intrinsic ATPase activity of Hsp70s, which requires the activation by cohort system, Hsp70 co-chaperones such as the DnaJ-type molecular chaperones [5]. Based on subcellular localization, the plant *Hsp70* gene family is divided into four subfamilies: cytosol, endoplasmic reticulum (ER), mitochondria and plastids, which can be distinguished with the unique and highly conserved motifs at the C-terminus of each subfamily, EEVD, HDEL, PEAEYEEAKK and PEGDVIDADFTDSK, respectively [13]. The phylogenetic divergence and characteristic roles of individual Hsp70 proteins are likely to be closely related to their location in different subcellular compartments [7,12].

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In the past 50 years, major advances in Hsp70 molecular chaperones research have been made [5,14]. Various members of Hsp70 proteins are present in different cellular compartments. In contrast to *Escherichia coli*, that possesses three members, higher organisms contain large numbers of *Hsp70* members, 14 in *Saccharomyces cerevisiae* [15], 18 in *Arabidopsis* [12], at least 12 in spinach [13], 32 in *Oryza sativa* [9], 20 in *Populus trichocarpa* [16] and 61 in *Glycine max* [17]. The *Hsp70* genes are necessary for plant development and the responsible for HS or low temperature in *Arabidopsis* [18–20], as well as in other species, such as hybrid polar [16], rice [21] and *Sorghum bicolor* [22]. Heat induction of plant *Hsp70s* is mediated by heat shock transcription factors (Hsfs), nevertheless, Hsp70 proteins may function as negative feedback regulators of Hsf activity [20], indicating that the biological function of plant *Hsp70* gene family is diverse. However, as Hsp70 family is a multigenic family, functional redundancy may exist among the plant *Hsp70* members, which will disturb the investigation of biological function of each *Hsp70*.

Pepper (*Capsicum annuum* L.) as an economically important horticultural crop is threatened by various stresses, such as drought, salinity, HS and viruses [23,24]. HS can cause serious pollination and fertilization problems during the pepper reproductive stage, which will significantly reduce the yield and quality of pepper fruits. Although Hsp70s are required for plants to cope with HS, few members of this family were characterized in pepper [25,26], and very little is known about their functions in the mechanisms of tolerance to HS. Thus, a genome-wide analysis of *CaHsp70* genes will help to reveal the underlying complex molecular mechanisms of CaHsp70 proteins in response to HS.

In this study, a total of 21 *CaHsp70* genes were identified. A phylogenetic tree combining Hsp70 proteins from different species was constructed to test the evolutionary relationships. Promoter analysis revealed that various *cis*-acting elements involved in stress response were present in the promoter regions of *CaHsp70* genes. And we also determined the protein structures of CaHsp70s. The comprehensive gene expression analysis was investigated in various organs, in response to HS and exogenous substances ($CaCl_2$ and EGTA). The function of a cytosolic gene, *CaHsp70-2*, was characterized in the mechanism of tolerance to HS in transgenic *Arabidopsis*. Our study provides a foundation to better understand the role of *CaHsp70* genes in pepper HS defense response and valuable information for further investigation into the function of this significant gene family.

2. Materials and methods

2.1. Genome-wide identification of *CaHsp70* genes in pepper

The Hidden Maekov Model (HMM) profile of the Hsp70 domain (PF00012) was downloaded from Protein family (Pfam) database (http://pfam.xfam.org/) and was used to search the Pepper Genome Database (PGD, http://peppergenome.snu.ac.kr/ , CM334 and Zunla-1 genomes) [23,27]. The tomato Hsp70 protein sequences [28] were also used as query sequences to search against the PGD and the NCBI database. All output genes with E-value ≤ 0.001 were collected and examined for the presence of the Hsp70 domain by Pfam and SMART (http://smart.embl-heidelberg.de/), and the incorrectly putative genes were rejected. The non-redundant and identified genes were assigned as *Capsicum annuum Hsp70* (*CaHsp70*) genes.

2.2. Sequence analysis

The chromosomal location data, protein sequences, genomic sequences and intron numbers of identified *CaHsp70s* were derived from PGD. The protein sequences were analyzed with EXPASY PROTOPARAM (http://www.expasy.org/tools/protparam.html) to obtain the number of amino acids, molecular weight, theoretical isoelectric point (pI) and instability index (with a value >40 considered as unstable) [29]. The subcellular locations were predicted using WoLF PSORT program (http://wolfpsort.org/).

Exton/intron organization of *CaHsp70* genes was illustrated with Gene Structure Display Server program (GSDS, http://gsds.cbi.pku.edu.cn/index.php). The identification of CaHsp70 conserved motifs was performed using MEME program (http://meme-suite.org/tools/meme), with the following parameters: number of repetitions: any; maximum number of motifs: 15; and the optimum motif widths: 6–200 amino acid residues. The conserved domains annotation was performed using Pfam and SMART, and the conserved domains were aligned using the Promals3D structural alignment program (http://prodata.swmed.edu/promals3d/promals3d.php).

2.3. Phylogenetic analysis

The full amino acid sequences of Hsp70 members from different plant species were aligned using CLUSTALW program. The gene IDs of *Hsp70* members from above species were shown in Table S1. The parameters for alignment by CLUSTALW were: gap open penalty, 10; gap extension penalty, 0.2; protein weight matrix, gonnet; residue-specific gap penalties, on; hydrophilic penalties, on; gap separation distance, 0; end-gap separation penalty, on; use negative matrix, on; delay divergent cutoff (%): 30. An unrooted neighbor-joining (NJ) phylogenetic tree was constructed using MEGA 5.10 software [30] with the bootstrap test replicated 1000 times, pair wise deletion and a Poisson model.

2.4. Promoter analysis

The transcription start site was designated +1. The upstream regions (1500 bp, from −1500 to +1 bp) of *CaHsp70* genes were obtained from PGD, and the *cis*-acting regulatory elements were determined by the program PlantCARE online (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.5. Plant growth conditions and treatments

The pepper line R9 was used in this study. Seedlings were grown in a growth chamber with a condition of 26/20 °C day (16 h)/night (8 h) till the age of 6–8 true leaves. For the tissue specific expression analysis, the young leaves, stems and roots from seedling plants and the flower buds from adult plants treated with HS of 40 °C for 2 h were collected, the different tissues under normal condition were used as the control [31]. To study the effect of Ca^{2+} and HS on the expression of *CaHsp70* family genes, the seedlings were pretreated with an equal volume of distilled water, 20 mM ethylene glycol bis(2-aminoethylether)-*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA) or 100 mM calcium chloride ($CaCl_2$) dissolved in water for 1 h [32], followed by exposure to HS (40 °C) for 0, 1, 2, 4 h. This treatment was initiated during the light period and all the samples were harvested within the light period. The leaves were collected and frozen with liquid nitrogen for total RNA extraction and cDNA synthesis. Each treatment was conducted with three biological replicates, and samples from five plants were collected for each replicate.

2.6. Gene expression analysis

Total RNA from fresh tissues was extracted using Total RNA kit (BioTeke, Beijing, China) and reverse-transcribed using SuperScriptIII Reverse Transcriptase (Takara, Dalian, China), the operational procedure followed the manufacturer's procedure, and

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