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Comprehensive Genomic Analysis and Expression Profile of *Hsp70* Gene Family Related to Abiotic and Biotic Stress in Cucumber

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Abstract: Heat shock protein 70 (*Hsp70*) is a class of HSPs involved in plant growth and development, stress response and regulation. The *Hsp70* proteins exist widely in the plant world, but the detail information about *Hsp70s* is still unclear in cucumber. Based on the available cucumber genome, a total of 12 *Hsp70* genes (*CsHsp70-1* to *CsHsp70-12*) were identified in this study, and they were distributed among five out of seven chromosomes. The *CsHsp70s* were divided into four groups based on a phylogenetic analysis by using protein sequences from cucumber and other plants, and their conserved motifs were relatively conserved. Gene duplication analysis showed that segmental duplication is the main driving force of expansion in cucumber *CsHsp70* genes. Promoter analysis of *CsHsp70* genes showed that they contained many *cis*-acting elements involved in hormone and stress responses. Expression analysis by RNA-seq and qRT-PCR indicated that the expression of most *CsHsp70* genes was associated with multiple biotic and abiotic stresses in cucumber. This study introduces the characteristics of cucumber *CsHsp70* genes and the regulation of their expression levels in various abiotic and biotic stresses, which provided a basis for functional exploration and utilization of *CsHsp70* genes in the future.

Keywords: cucumber; heat shock protein 70 (*Hsp70*); stress response; expression pattern; gene family

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1. Introduction

Various abiotic and biotic stresses have huge impacts on the growth and development of crop plants, and thus reduce the yield and economic benefit. To deal with these adverse factors, plants produce quite a lot of induced proteins due to their ability to protect other proteins from denaturation or aggregation under stresses. Among these proteins, heat shock proteins (HSPs) are a class of molecular chaperones originally discovered according to their increased levels when cells are suffered by heat stress [1,2]. HSPs are highly conserved and widely distributed in both the prokaryotic and eukaryotic organisms, and they can be divided into five major sub-families, namely, HSP100s, HSP90s, HSP70s, HSP60s, and small HSPs (sHSPs), on the basis of their molecular weights (MWs) [3]. Among these families, HSP70s were considered to be the most evolutionarily conserved HSPs in every kingdom, from prokaryotes (DnaK) and eukaryotes (HSP70). The MW of HSP70s is mostly between 68–78 kDa, and all members of HSP70s comprise a highly conserved N-terminal nucleotide

binding domain (NBD) for ATP-binding and a substrate binding domain (SBD) for binding to the substrate located in the C-terminal region [4]. The two domains are connected by an intermediate domain (about 15 kDa). In plants, four types of *Hsp70s* were widely distributed in different subcellular compartments: mitochondria, cytoplasm, chloroplast, and endoplasmic reticulum (ER) [5]. And *Hsp70s* in other subcellular compartments were also observed. For example, a tobacco nuclear-localized HSP70, NtHSP70-1, is involved in nuclear DNA stability for preventing the fragmentation and degradation during heat stress [6].

Plant *Hsp70* genes were composed of a multi-gene family and have been extensively identified in a variety of plants, such as rice [7,8], pepper [9], potato [10], tobacco [11], cabbage [12], tomato [13,14], pumpkin [15], chrysanthemum [16], and radish [17]. In previous studies, many recognized *Hsp70* genes have been characterized to quickly express and accumulate under a number of abiotic stresses, such as heavy metal [14,18], salt [19], drought [20,21], heat [7,16], UV and high intensity light stress [22], as well as biotic stresses, such as pathogen and virus infection [23,24]. These reports revealed the possible roles of *Hsp70* genes in improving the tolerance of plants to various stresses. For example, overexpression of *GhHSP70-26* markedly enhances drought resistance and reduces cell membrane damage and reactive oxygen species (ROS) accumulation in transgenic tobacco plants, whereas *GhHSP70-26* silenced cotton plants displayed increased sensitivity to drought stress, indicating that *GhHSP70-26* positively regulated drought stress in cotton [25]. Overexpression and silencing of *RsHSP70-20* in radish cotyledons showed that *RsHSP70-20* paly a positive role in regulating of heat stress response in radish [17]. Many adverse abiotic and biotic stress treatments (such as heat, salt stress and pathogen infection) can induce ER stress by affecting the accumulation of unfolded and misfolded proteins in the ER [26]. BiP (binding protein) is a subfamily of HSP70 and one of the most abundant chaperones involve in ER stress. There are three *BiP* genes in pepper, where overexpression of *CaBiP1*/*CaHsp70-8* promoted tolerance to ER stress and a variety of environment stresses in transgenic *Arabidopsis* plants [27]. Additionally, the developmental role of the *Hsp70* genes were also experimentally characterized in some plant species. For instance, *Arabidopsis* plants have a deficiency in *AtHSP70-15* displayed severe growth retardation and accelerated wilting due to impaired stomatal closure [23]. *OsHsp70cp-2*, encoding a plastid-localized *Hsp70*, was involved in two Tic translocon components (Tic110 and Tic40) and important for amyloplast development in rice [28].

Cucumber (*Cucumis sativus* L.) is one of the most crucial vegetable crops, but its growth and development are frequently challenged by a number of unfavorable environmental conditions [29]. Since the *Hsp70* gene family in cucumber have been identified in a previous report [30], the detail information, especially abiotic and biotic stress responses of *CsHsp70* genes was still limited. In this study, we focus attention on the identification and characterization of *Hsp70* family genes in cucumber, and systematically analyzed their evolutionary relationship, conserved motif, and *cis*-acting regulatory elements in their promoter regions. In addition, the expression patterns of *CsHsp70* genes under multiple abiotic and biotic stresses were also determined by qRT-PCR and public RNA-seq data. The findings of our study provide valuable information for revealing the roles of *CsHsp70* genes, which maybe serve as molecular resources for developing new resistant varieties for cucumber breeding.

2. Results

2.1. Genome-Wide Identification of the Hsp70 Family Genes in Cucumber

In total, 12 *Hsp70* genes were identified in the cucumber genome, which were designated as *CsHsp70-1* to *CsHsp70-12* according to their location on the seven cucumber chromosomes (Table 1). The CDS lengths of *CsHsp70* genes varied from 1716 bp (*CsHsp70-3*) to 2697 bp (*CsHsp70-7*), encoded proteins varied from 571 to 898 amino acids (aa) in length with MW values ranging from 61.99 to 100.14 kDa. Other physicochemical parameters of all *CsHsp70* proteins, including pI and GRAVY values, were also examined. The pIs of the *CsHsp70* proteins varied from 5.09 (*CsHsp70-11*) to 5.69 (*CsHsp70-5*), suggesting that all of

them are acidic proteins. Besides, the GRAVYs of the *CsHsp70s* were varied from −0.498 (*CsHsp70-7*) to 0.040 (*CsHsp70-3*) (Table 1).

Table 1. Identification and characterization of *Hsp70* gene family in cucumber.

Gene	Accession No. (v2)	Accession No. (v3)	Chromosome: Location	CDS/bp	Physicochemical Parameters of Proteins			
					AA	pI	MW/kDa	GRAVY
CsHsp70-1	Csa2G070310.1	CsaV3_2G009170	Chr2: 5,427,208 .. 5,429,443 (+)	1953	650	5.18	71.27995	−0.422
CsHsp70-2	Csa2G122520.1	CsaV3_2G011210	Chr2: 8,176,614 .. 8,180,834 (+)	2124	707	5.18	75.39607	−0.295
CsHsp70-3	Csa3G147740.1	CsaV3_3G013380	Chr3: 9,928,649 .. 9,930,364 (−)	1716	571	5.52	61.98548	0.040
CsHsp70-4	Csa3G391900.1	CsaV3_3G022450	Chr3: 19,117,817 .. 19,123,998 (−)	2289	762	5.63	85.39136	−0.422
CsHsp70-5	Csa4G179170.1	CsaV3_4G014660	Chr4: 8,965,693 .. 8,969,625 (−)	2043	680	5.69	73.1238	−0.323
CsHsp70-6	Csa4G295440.1	CsaV3_4G024840	Chr4: 11,823,184 .. 11,826,273 (+)	1944	647	5.16	70.86926	−0.403
CsHsp70-7	Csa4G617390.1	CsaV3_4G032260	Chr4: 19,669,174 .. 19,679,176 (+)	2697	898	5.27	100.13606	−0.498
CsHsp70-8	Csa5G149330.1	CsaV3_5G001960	Chr5: 4,469,887 .. 4,473,757 (+)	1998	665	5.10	73.42913	−0.474
CsHsp70-9	Csa5G512930.1	CsaV3_5G026450	Chr5: 17,912,303 .. 17,915,006 (+)	1959	652	5.10	71.45378	−0.442
CsHsp70-10	Csa5G514500.1	CsaV3_5G026520	Chr5: 17,969,849 .. 17,972,430 (−)	1947	648	5.21	71.04657	−0.415
CsHsp70-11	Csa7G312930.1	CsaV3_7G024760	Chr7: 10,868,096 .. 10,872,494 (+)	2001	666	5.09	71.51079	−0.250
CsHsp70-12	Csa7G446710.1	CsaV3_7G033150	Chr7: 17,685,920 .. 17,691,540 (−)	2532	843	5.40	92.89913	−0.430

2.2. Evolutionary Relationships of CsHsp70 Proteins

To explore the evolutionary relationships of the *CsHsp70* proteins, a phylogenetic tree was generated using the protein sequences of the *Hsp70* family members from 12 *CsHsp70s* in cucumber, 18 *AtHsp70s* in *Arabidopsis* [31], 32 *OsHsp70s* in rice [8], 21 *CaHsp70s* in pepper [9], 22 *ZmHsp70s* in maize [32], and 21 *SlHsp70s* in tomato [13]. The resulting phylogenetic tree revealed that the above *Hsp70* proteins were clustered into four groups (A–D) based on the bootstrap support (Figure 1). Group A was the largest group and contained the most *Hsp70* proteins, while group B had the fewest. In addition, groups A and D covered the most numbers of *CsHsp70s* (four members each), while one and three *CsHsp70s* fell into groups B and C, respectively (Figure 1).

2.3. Conserved Motif Analysis of CsHsp70 Proteins

To study the evolutionary divergence of *Hsp70* proteins in cucumber, the number and arrangement of conserved motifs were predicted with the MEME tool. As a result, we identified ten motifs and their details are shown in Supplementary Table S3. In addition, these motifs were widely found in the *CsHsp70* proteins with several exceptions (Figure 2). For example, *CsHsp70-2* in group C had an additional motif 7 in the N-terminus, while *CsHsp70-10* in group A harbored an additional motif 9 in its C-terminus. Motifs 9, 4 and 6 were absent in *CsHsp70-3*, *CsHsp70-7* and *CsHsp70-11*, respectively. Additionally, *CsHsp70-4*, *CsHsp70-7* and *CsHsp70-12* in group D missed motif 2, and both of *CsHsp70-4* and *CsHsp70-7* lacked motif 3 (Figure 2). These findings reflect the diversity and conservation among *CsHsp70s*.

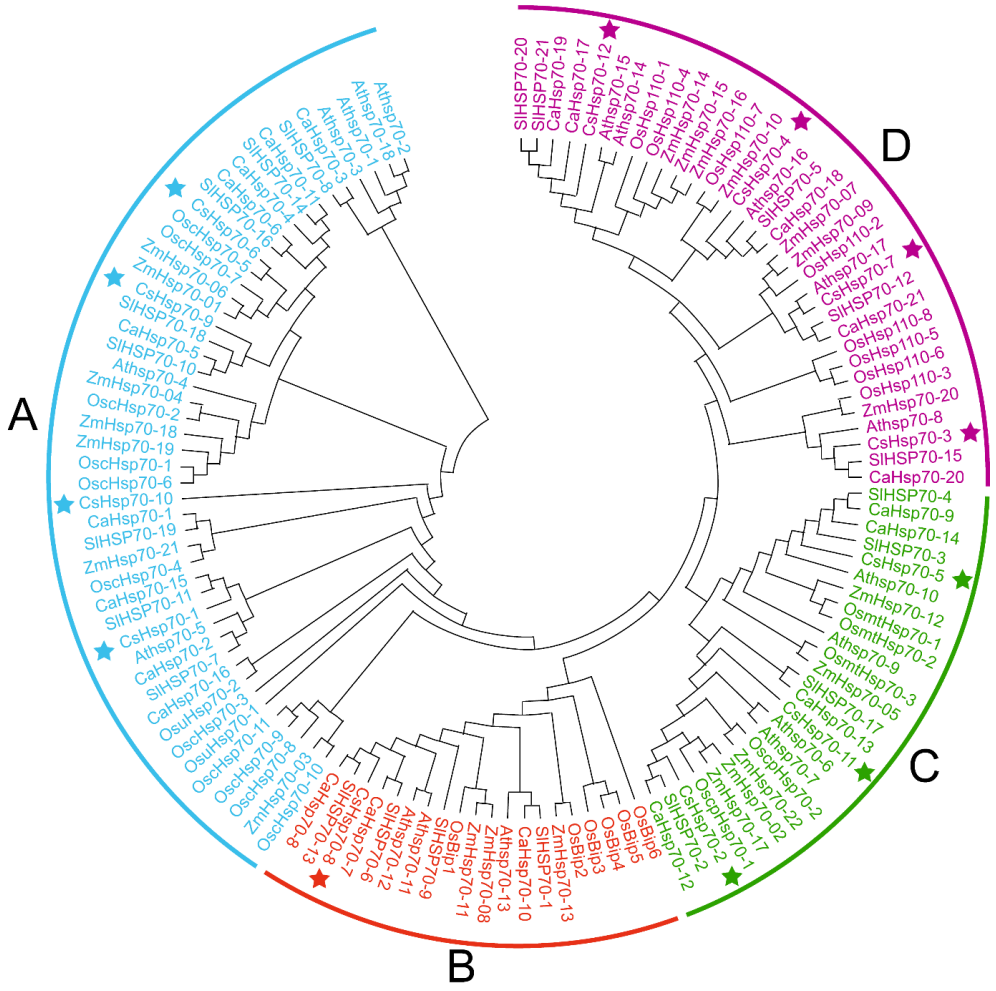

Figure 1. Phylogenetic tree of *Hsp70* proteins in cucumber (*Cucumis sativus*), *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), pepper (*Capsicum annuum*), maize (*Zea mays*), and tomato (*Solanum lycopersicum*). Four groups (**A**–**D**) are represented by different colors, and *CsHsp70s* are represented by stars. The accession numbers of *Hsp70* proteins are provided in Supplementary Table S2.

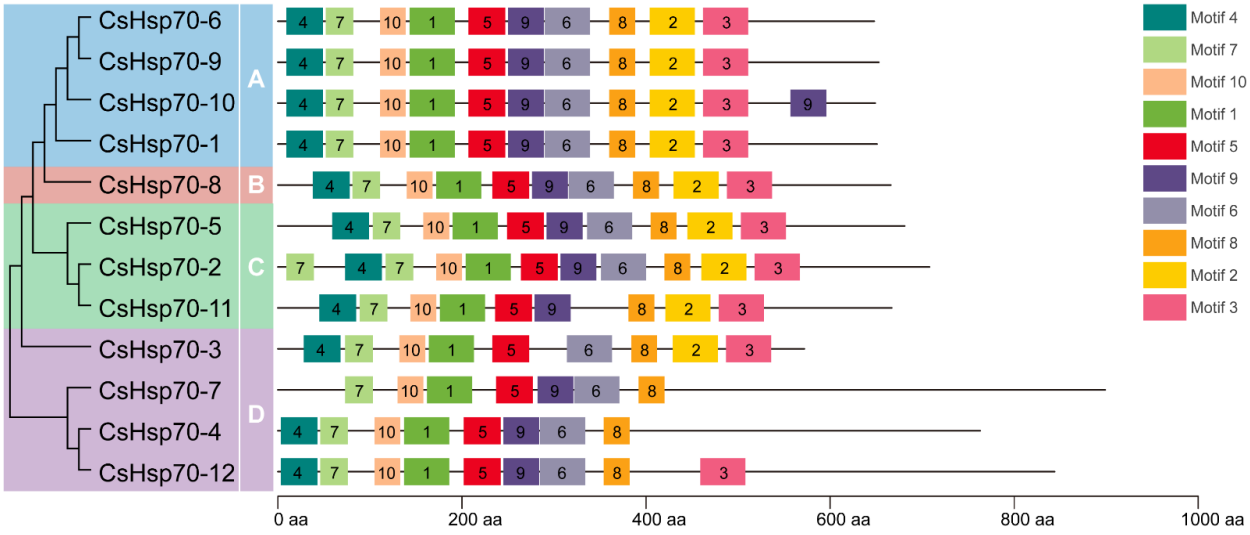

Figure 2. Conserved motif analysis of *CsHsp70* proteins on the basis of the phylogenetic relationship. Ten motifs were marked by different colors and length of box denotes motif length.

2.4. Chromosomal Location and Gene Duplication Analysis of the CsHsp70 Genes

Chromosomal location analysis indicated that 12 *CsHsp70* genes were distributed on five out of the seven chromosomes (Chrs) in cucumber genome (Figure 3). Chrs 4 and 5 had the highest number of *CsHsp70* genes (three genes each), while Chrs 2, 3 and 7 each contained two *CsHsp70* genes. Gene duplication event analysis displayed that two *CsHsp70* genes, *CsHsp70-6* and *CsHsp70-10*, were made up of one segmental duplication event (Figure 3).

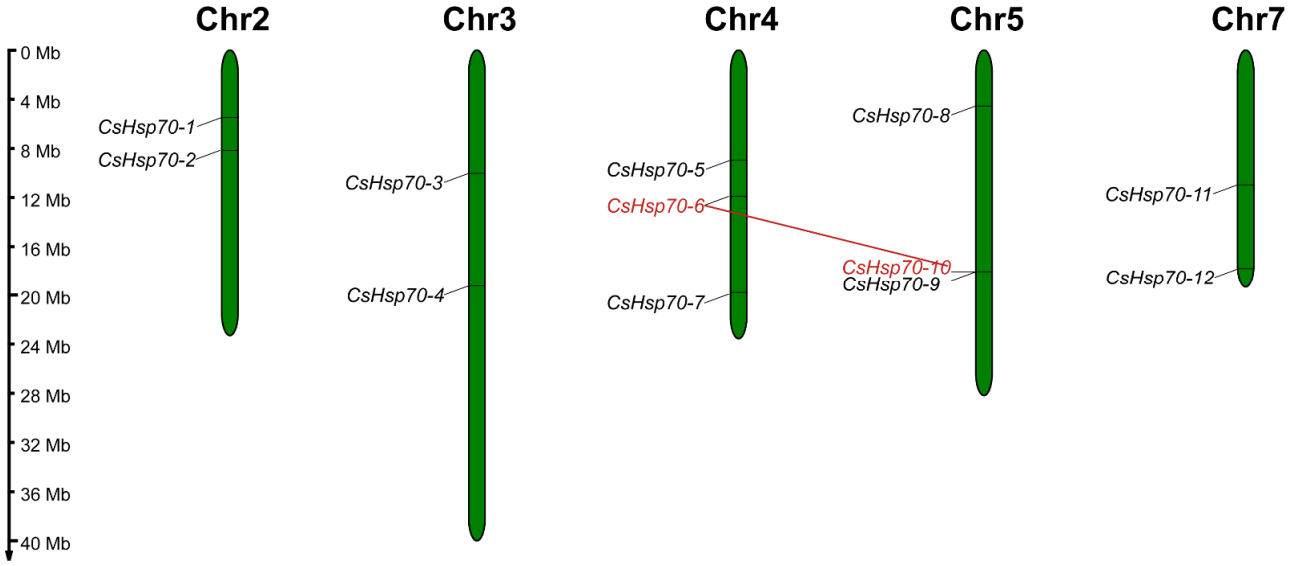

Figure 3. Distribution of the *CsHsp70* genes on the five chromosomes of cucumber. The bar located on the left side representing the chromosome length was showed in megabase (Mb), and segmental duplication genes are marked with red and connected with red lines.

2.5. Cis*-Acting Regulatory Element Analysis of the CsHsp70 Genes*

To investigate the potential functions of *CsHsp70* genes, *cis*-acting regulatory elements of the promoter regions of *CsHsp70* genes were examined. We divided the identified *cis*-elements into two categories: stress-responsive and phytohormone-responsive (Figure 4). Nine kinds of stress-responsive *cis*-elements were filtered out, including ARE involved in anaerobic induction, GC-motif involved in anoxia response, LTR involved in low-temperature response, MBS involved in drought-related response, STRE involved in stress-responsive, TC-rich repeats involved in defense and stress responses, W-box involved in fungal elicitor response, WRE3 and WUN-motif involved in wounding responses (Figure 4). Each *CsHsp70* gene contained at least two kinds of stress-related *cis*-elements in their promoter regions, among which *CsHsp70-4* had the largest number of stress-responsive elements. It should be noted that ARE involved in anaerobic induction were the most abundant *cis*-element and was present in all *CsHsp70* promoter regions, particularly in *CsHsp70-10*, suggesting that the *CsHsp70* genes are potentially regulated by anaerobic induction. In addition, many hormone-responsive *cis*-elements were identified in the *CsHsp70* genes in response to distinct phytohormones, such as abscisic acid (ABA, including ABRE), auxin (including TGA-element and AuxRR-core), methyl jasmonate (MeJA, including CGTCA-motif and TGACG-motif), ethylene (including ERE), salicylic acid (SA, including TCA-element), and gibberellin (GA, including P-box, TATC-box and GARE-motif) (Figure 4). It is worth noting that the distribution pattern of hormone-responsive *cis*-elements differed among *CsHsp70* genes. For example, ABRE was most abounding in the promoter regions of *CsHsp70-7* and *CsHsp70-8* (nine each), suggesting that they may mostly be associated with the ABA response, while *CsHsp70-1*, *CsHsp70-5*, *CsHsp70-9* and *CsHsp70-12* promoters contained none. P-box, TATC-box and GARE-motif were GA-

responsive *cis*-elements, and they were unequally located in the 2, 4 and 2 *CsHsp70* genes, respectively (Figure 4).

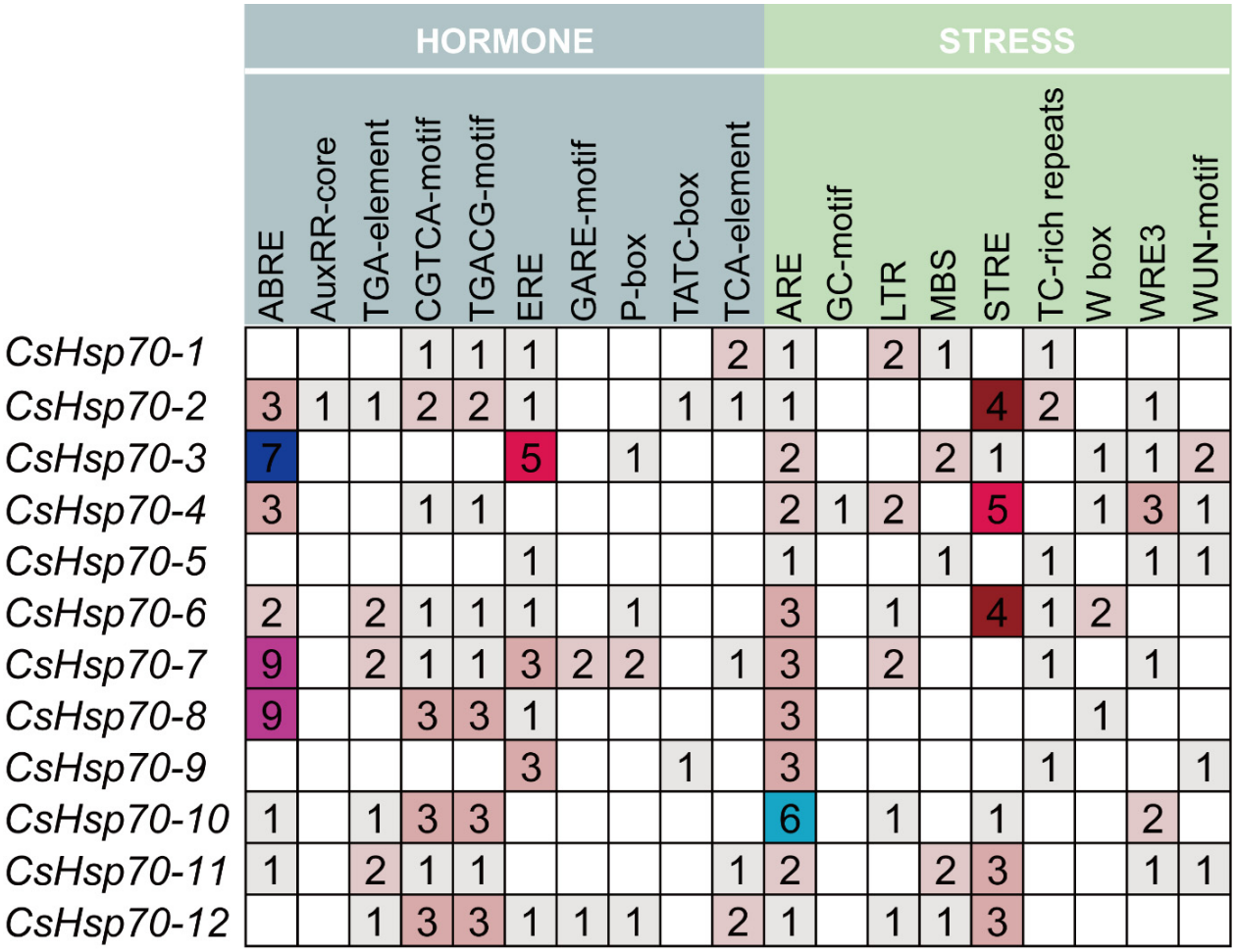

Figure 4. Phytohormone- and stress-responsive *cis*-elements in *CsHsp70s* promoter regions. The amounts of *cis*-elements in *CsHsp70s* promoter regions were displayed as different colors and numbers in the grid.

2.6. Expression Analysis of CsHsp70 Genes under Different Abiotic Stresses

To identify the roles of *CsHsp70* genes in response to abiotic stress, the TPM values of *CsHsp70* genes under salt, temperature, and photoperiod treatments were obtained by public RNA-seq data [33–35], and these transcription levels were assembled hierarchically in a heatmap. Under salt stress, the transcription levels of *CsHsp70-6* and *CsHsp70-9* were dramatically elevated in the leaf and root (Figure 5A). In addition, the transcription levels of *CsHsp70-1*, *CsHsp70-3*, *CsHsp70-10*, and *CsHsp70-11* were observably increased in the leaf, while *CsHsp70-5* and *CsHsp70-8* exhibited significantly increased expression levels in the root (Figure 5A). Under different temperature and photoperiod combined conditions, the expression levels of *CsHsp70-1*, *CsHsp70-10*, and *CsHsp70-11* were induced by high temperature under short days, while their transcription levels were repressed by high temperature under long days (Figure 5B), suggesting that they were involved in differential temperature-photoperiod environments.

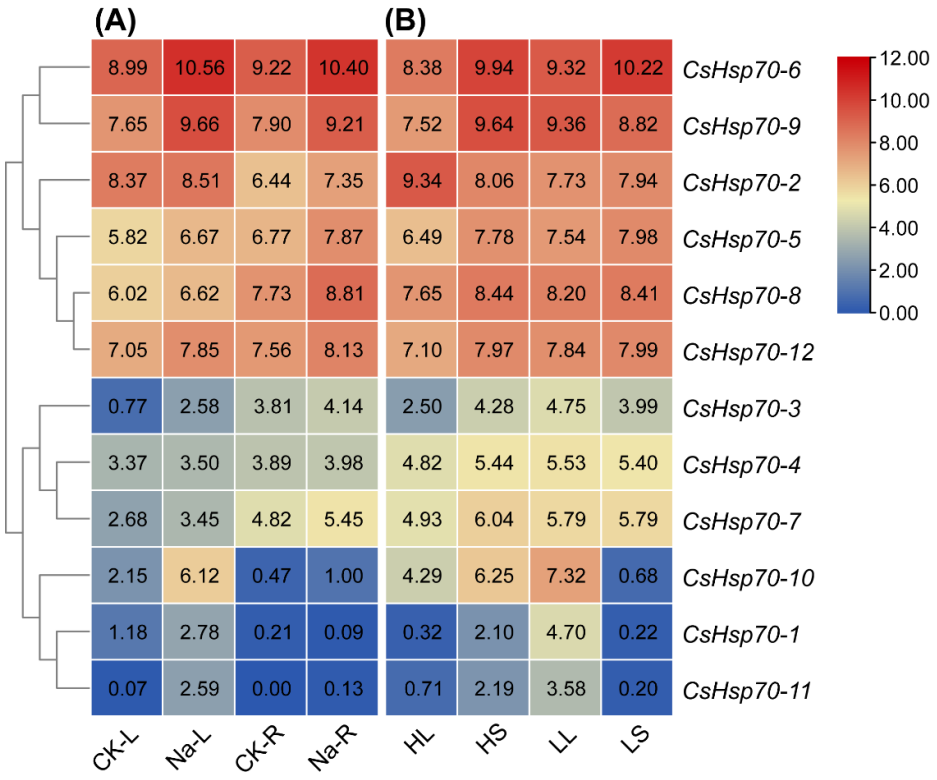

Figure 5. The transcription levels of *CsHsp70* genes under salt stress (**A**), different temperature and photoperiod combined conditions (**B**) based on RNA-seq data. The red to blue colors on the scale located on the right side representing high to low gene expression, which were calculated as the log2(TPM+1) values. CK-L and Na-L, leaf sample from control and salt-stressed plants; CK-R and Na-R, root sample from control and salt-stressed plants. HL and LL, high and low temperature under long day; HS and LS, high and low temperature under short day.

To understand if *CsHsp70* genes were regulated by drought and ER stress, the expression patterns of the seleted *CsHsp70* genes under PEG and DTT conditions were measured by qRT-PCR. Under drought stress, *CsHsp70-1* displayed a markedly up-regultaion expression from 2 h to 8 h, and its expression peaked at 8 h, while the other three *CsHsp70* genes (*CsHsp70-6*, *CsHsp70-8*, *CsHsp70-9* and *CsHsp70-10*) exhibited obviously decreased transcription levels at certain points under drought stress (Figure 6A). Under ER stress, the transcripts of *CsHsp70-6*, *CsHsp70-8* and *CsHsp70-9* were obviously increased at all test time points, while *CsHsp70-1* display distinctly down-regultaion expression. *CsHsp70-10* displayed an increased expression at the earlier time point (4 h), but its expression showed an obvious decline at the later time points (8 h and 12 h) (Figure 6B).

2.7. Expression Analysis of CsHsp70 Genes in Response to Different Biotic Stresses

A previous study showed that the expression levels of *CsHsp70* genes were altered by the *Pseudoperonospora cubensis* infection [30], implying their roles in regulating biotic stress. To further understand whether *CsHsp70* genes were responses to various biotic stresses, the expression profiles of *CsHsp70* genes under three different biotic stresses, including PM (*Sphaerotheca fuliginea*), RKN (*Meloidogyne incognita*), and DM (*Pseudoperonospora cubensis*) were examined using the available RNA-seq data [36–38]. Upon the PM infection, the transcriptional levels of *CsHsp70-6*, *CsHsp70-9* and *CsHsp70-10* were increased in both resistant and susceptible cucumber cultivars (Figure 7A). *CsHsp70-2*, *CsHsp70-5*, *CsHsp70-11*, and *CsHsp70-12* displayed increased transcriptional levels after PM treatment in the resistant cucumber line SSL508-28, while their expression was unaltered in the susceptible cucumber line D8 (Figure 7A). After the treatment with RKN inoculation, four *CsHsp70* genes, *CsHsp70-1*, *CsHsp70-3*, *CsHsp70-10* and *CsHsp70-11*, were found to be up-regulated

in both resistant and susceptible cucumber cultivars (Figure 7B). We also determined the transcriptional levels of *CsHsp70* genes from Vlaspik (DM susceptible line) and PI197088 (DM resistant line) after DM infection (Figure 7C). The results showed that *CsHsp70-6*, *CsHsp70-7*, *CsHsp70-8*, and *CsHsp70-9* showed a markedly up-regulation trend after DM inoculation in both resistant and susceptible cucumber lines, suggesting their possible roles in DM invasion. Notably, *CsHsp70-10* was down-regulated after the DM inoculation in Vlaspik (DM susceptible), while its expression was increased to a different extent in PI197088 (DM resistant) cucumber plants (Figure 7C). Therefore, some *CsHsp70* genes might be associated with biotic stress responses in cucumber.

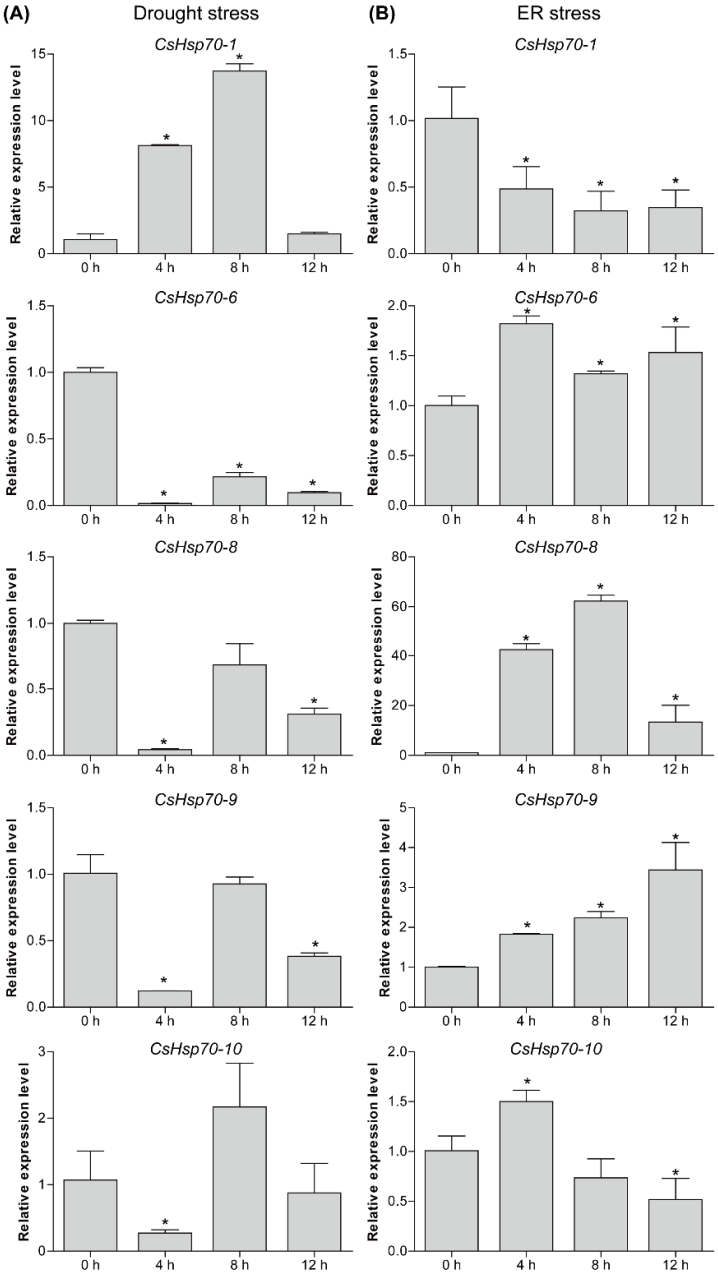

Figure 6. Transcription levels of five selected *CsHsp70* genes under drought (**A**) and ER stress (**B**) treatments, as examined using qRT-PCR. Asterisks indicate significant differences examined by the student's *t*-test ($p < 0.05$).

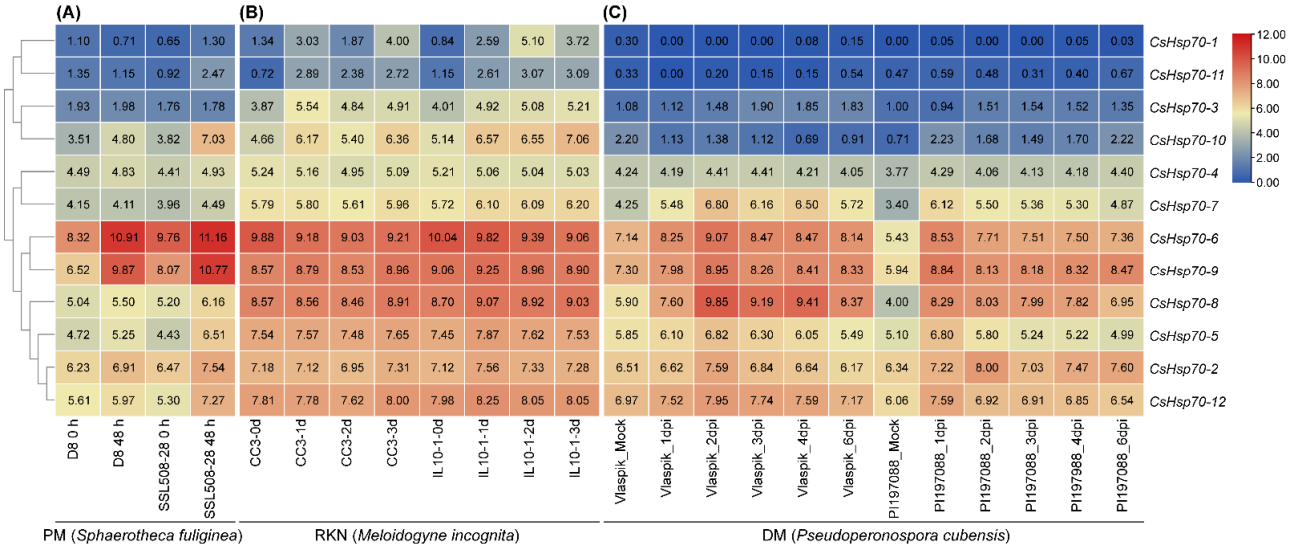

Figure 7. Heat map of differential transcription of *CsHsp70* genes under inoculation of PM (*Sphaerotheca fuliginea*) (**A**), RKN (*Meloidogyne incognita*) (**B**), and DM (*Pseudoperonospora cubensis*) (**C**). The transcriptional levels of *CsHsp70* genes are displayed as log2 transformed TPM values. Different colors on the scale located on the right side represent differential gene expression, which decrease from red to blue.

3. Discussion

Hsp70 proteins are widespread in every kingdom and mainly reported in heat shock responses. In this work, 12 *Hsp70* genes were identified and systematically analyzed in cucumber. The protein sequences of the *Hsp70s* were highly conserved, but the number of *Hsp70* family genes varied greatly in different plant species. For instance, the numbers of the *Hsp70* gene family were 18 in *Arabidopsis*, 20 in potato [10], 21 in pepper [9], 21 in pumpkin [15], 24 in common bean [19], 32 in rice [7,8], 34 in radish [17], 52 in cabbage [12], 61 in tobacco [11], and 83 in chrysanthemum [16]. The reason for the different gene numbers of the above plant species may be due to the size of the genome. However, there was a disproportionate number of *Hsp70* genes and a disproportionate genome size in several of the plant species, suggesting that the *Hsp70* gene family has also undergone different duplication events. For example, three tandem and two segmented duplicated gene couples were observed among the 10 *StHsp70* genes in potato [10]. Among the 52 *BoHSP70* genes in cabbage [12], one tandem and 25 segmental duplication events were identified. Collinearity analysis of pumpkin *Hsp70* genes showed that nine gene pairs were segmentally duplicated [15]. However, only two *CsHsp70* genes made up to one segmental duplication event in the cucumber genome (Figure 3). Therefore, species–specific duplication or deletion during evolution may have resulted in differences in the numbers of the *Hsp70* family genes across distinct plant species. In addition, the number of *Hsp70s* in different plants varied in each group (Figure 1), indicating that the roles of these *Hsp70s* belonging to different groups may undergo variations by environmental selection, resulting in the gain and/or loss of some functional genes.

According to the phylogenetic analysis, the *Hsp70* family members can be clustered into four groups (Figure 1), which is consistent with the previous reports [4,8]. High sequence similarity of members in a gene family often indicates similar roles of them in different organisms. The *CsHsp70s* show closer phylogenetic relationships with the *Hsp70s* in dicots (*Arabidopsis*, pepper and tomato) than those in monocots (rice and maize), suggesting that *Hsp70* genes might have similar functions in cucumber as their homologous genes in other plants. It is worth noting that each group comprised *Hsp70s* from the detected plants (Figure 1), implied that the *Hsp70* gene family has undergone species-specific expansion during evolution in these plants. In addition, most *CsHsp70s* exhibit

much similar conserved motif organization patterns (Figure 2), further supporting the relatively slow evolutionary process of the *CsHsp70* genes. Nevertheless, further research to reveal the specific evolution process is still needed.

The determination of gene expression patterns provide insights into studying molecular functions of genes involved in diverse processes. It had been reported that most *CsHsp70s* were highly expressed in various cucumber tissues and also have differential expression under heat, salt, and *P. cubensis* infection, implying that they may take part in developmental processes of cucumber [30]. In the present study, many hormone- and stress-responsive *cis*-elements identified in the promoters of *CsHsp70s* revealed the involvement of them in cucumber stress tolerance (Figure 4). We thus further analyzed the transcriptional levels of *CsHsp70* genes in response to distinct abiotic and biotic stresses. Our heatmap data showed that 8 out of 12 *CsHsp70* genes exhibited significantly increased expression levels in leaf or root under salt stress (Figure 5A), implying that they have positive functions in the responses to salt stress. A previous report showed that overexpression of *Erianthus arundinaceus EaHSP70* promoted tolerance of sugarcane plants to salt stress [39]. Expression of *EsHSP70* by both constitutive and stress inducible promoters conferred tolerance to salt stress in transgenic *Arabidopsis* plants [40]. DTT is a redox reagent and act as an ER stress inducer by affecting disulfide bond formation, and previous study revealed that the expression levels of rice *OsBiP4* and *OsBiP5* were highly and specifically activated under DTT-induced ER stress [41]. In this study, the expression of selected five *CsHsp70* genes responded to varying degrees under ER stress induced by DTT treatment, among which *CsHsp70-8* extremely up-regulated (Figure 6B). *CsHsp70-8* had HDEL (ER retention signal) in its C-terminus and it was clustered closely with CaBiP1/*CaHsp70-8* and other BiPs in group D (Figure 1). Therefore, it can be speculated that *CsHsp70-8* performed vital roles in ER stress and other environmental stresses. In addition, several *CsHsp70* genes displayed altered expression under drought and differential temperature-photoperiod environments. Similar results were also observed in previous reports, such as potato [10], tomato [13], pumpkin [15], and chrysanthemum [16], in which a large proportion of *Hsp70* genes display inducibility in response to at least one or more abiotic stresses.

Previous studies revealed that the expression levels of *HSP70s* can also respond to pathogen invasion, suggesting that they are an integral part of plant immunity [42,43]. In this study, PM, RKN and DM invasion observably altered the transcription levels of 7, 4 and 8 *CsHsp70* genes, respectively (Figure 7). Notably, *CsHsp70-6* and *CsHsp70-9* displayed up-regulated expression levels in both susceptible and resistant cucumber lines under PM and DM treatments (Figure 7A,C), indicating that the two *CsHsp70* genes might act as positive regulators in resistance to *S. fuliginea* and *P. cubensis* infection. In addition, the expression of *CsHsp70-10* displayed increased levels in both susceptible and resistant cucumber lines under PM and RKN treatments, while its expression levels were opposite between resistant and susceptible cucumber plants under DM treatment (Figure 7). The changes in *Hsp70* expression observed in this study suggested that some *CsHsp70* genes play important roles in response to pathogen invasion of cucumber.

4. Materials and Methods

4.1. Database Searches and Annotation of the Hsp70 Members in Cucumber

To identify *Hsp70* family members in cucumber, we used the Hidden Maekov Model (HMM) profile of the *Hsp70* domain (PF00012) acquired from the Pfam database (http://pfam.xfam.org/, accessed on 1 May 2023) for hmmsearch (HMMER version 3.3.2) against the Cucurbit Genomics Database (CuGenDB, http://cucurbitgenomics.org/, accessed on 1 May 2023). In addition, the full-length *Hsp70* amino acid sequences from rice [8], pepper [9], and tomato [13], were also retrieved and used as query sequences to perform BLAST (basic local alignment search tool) search against the CuGenDB database. The resulting candidate cucumber *Hsp70s* were confirmed by SMART (http://smart.embl-heidelberg.de/, accessed on 1 May 2023), for the presence of the *Hsp70* domain (PF00012). The physicochemical properties of *CsHsp70* proteins, including molecular weight (MW),

isoelectric point (pI), and GRAVY (grand average of hydropathicity), were predicted using ProtParam server on ExPASy (http://web.expasy.org/protparam/, accessed on 1 May 2023).

4.2. Phylogenetic Analysis and Conserved Motif Identification

Multiple sequence alignments were conducted with the MAFFT tool (https://www.ebi.ac.uk/Tools/msa/mafft/, accessed on 1 May 2023) by using full-length protein sequences of *Hsp70s* from different plant species, and a Neighbor-Joining (NJ) phylogenetic tree was generated with MEGA 7.0 software using a bootstrap option of 1000 replications. MEME (http://meme-suite.org/tools/meme/, accessed on 1 May 2023) was used to predict the conserved motifs of *CsHsp70s* with the default settings, with the exception of the maximum number of diverse motifs was set as 10.

4.3. Prediction of Chromosomal Locations, Gene Duplication, and Cis-Acting Regulatory Elements of CsHsp70 Genes

The chromosomal locations of *CsHsp70* genes were obtained from CuGenDB and drawn with the MG2C tool (http://mg2c.iask.in/mg2c_v2.1/, accessed on 1 May 2023) according to a previous report [29]. Gene duplication analysis was performed with the MCScanX software. The 2000 bp sequences in the 5′ flanking region from the promoters of *CsHsp70* genes were extracted from CuGenDB, and then the PlantCARE tool (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 1 May 2023) was used to determine the cis-acting regulatory elements present in the putative promoters of *CsHsp70* genes.

4.4. Expression Analysis of the CsHsp70 Genes via RNA-Seq Data

To analyze the expression patterns of *CsHsp70* genes under various stresses, the RNA-seq data were obtained from NCBI database with the BioProject IDs of PRJNA285071 (downy mildew, DM) [36], PRJNA321023 (powdery mildew, PM) [37], PRJNA419665 (root-knot nematode, RKN) [38], and PRJNA380322 (temperature and photoperiod) [33]. The RNA-seq data of cucumber under salt stress (CK-L and Na-L were represented as the leaf sample from control and salt-stressed plants, while CK-R and Na-R were represented as the root sample from control and salt-stressed plants) were also downloaded under the BioProject IDs of PRJNA477930 and PRJNA511946 [34,35]. The expression levels of *CsHsp70* genes were calculated as transcripts per million reads (TPM) on the basis of the methods in previous studies [29,44]. The expression heatmaps were visualized with TBtools [45].

4.5. Plant Materials and Stress Treatments

Chinese Long cucumber inbred line 9930 seedlings were treated with drought and ER stress. In brief, two-leaf stage cucumber seedlings were transferred to Hoagland nutrition solution containing PEG-6000 (10%) and 2 mm dithiothreitol (DTT, a reducing agent that blocks the formation of disulfide bridge), for drought stress and endoplasmic reticulum (ER) stress, respectively. The leaf samples were harvested for each stress at various time points (0, 4, 8 and 12 h) and frozen with liquid nitrogen. Each stress was completed with three biological replicates.

4.6. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis of the CsHsp70 Genes

Total RNA was isolated using the RNAsimple Total RNA Kit (Tiangen, Beijing, China), and cDNA was synthesized for qRT-PCR by the HiScript® III RT SuperMix for qPCR (Vazyme, Nanjing, China). The qRT-PCR was carried out on the Roche LightCycler 480 (LC480) system using TransStart Top Green qPCR SuperMix (+Dye II) (TransGen, Beijing, China) with three independent replicates. The amplification reaction included an initial 30 s denaturation at 95 °C, subsequently 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative transcriptional levels of the *CsHsp70* genes were calculated by using the cucumber β-*Actin* gene (Csa2G301530) as an internal control. The $2^{-\Delta\Delta Ct}$ method was implemented to

calculate the relative gene transcriptional level of *CsHsp70* genes, among which the samples at 0 h were set as 1.0. The qRT-PCR primers were shown in Supplementary Table S1.

5. Conclusions

In the present work, we identified 12 *CsHsp70* genes from the *C. sativus* genome. The proteins encoded by these genes contained different amounts of amino acids and ranged from 61.99 to 100.14 kDa in size. The analyses of evolutionary relationship, conserved motif and gene duplication provide insights into the evolutionary process for the *Hsp70* gene family in cucumber. The promoter analysis showed that the *CsHsp70* genes may be involved in the hormone and stress responses of cucumber. Expression analysis by RNA-seq and qRT-PCR suggested that the *CsHsp70* genes participate in the regulation of responses to distinct abiotic and biotic stresses. The findings provided by this work will not only offer new insights into the functions of *CsHsp70* genes but also help to develop new resistant varieties for cucumber breeding.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9091057/s1. Table S1: The gene-specific primers used for qRT-PCR. Table S2: *Hsp70* proteins from different plant species used in this study. Table S3: Sequences and lengths of motifs among cucumber *Hsp70* proteins.

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