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Research Article

Genome Wide Analysis *of Citrus sinensis* Heat Shock Proteins

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Background: Plant and animal cells possess a ubiquitous protein known as heat shock proteins (HSPs). Hsps were originally described in relation to heat shock and against abiotic and biotic stresses. Heat shock protein was classified in other crops on the bases of single classes or all classes but in *Citrus sinensis* Hsps groups, classes, subfamilies and members were not classified and characterized up to our knowledge.

Objectives: Present study was focused on the identification and grouping of *C. sinensis Hsps (CsHsps)* classes, members among classes, their phylogenetic relationship, gene structure, conserved motifs and identification of proteins by using bioinformatics tools and analyses.

Materials and Methods: Genomic, Peptide and CDS sequences of CsHsps were downloaded from phytozome. MEGA 7 used for the phylogenetic analysis, GSDS for gene structure, UGENE for the multiple sequence alignment and MEME suite for the conserved motif analysis.

Results: The genome size of *C. sinensis* was 367 Mb, Chromosome number (2n)18, having 151 Hsps with six groups CsHsp10, 20, 40, 60,70 and 90. CsHsp20 was the largest group having 54 members, followed by CsHsp60 and CsHsp70 both having 30 members respectively.

Conclusion: CsHsps members within a class shared more similar gene and protein structure. CsHsp 60, CsHsp 70 and CsHsp90 shared more conserved and similar amino acid pattern. Each class had some important proteins such as Cpn in CsHsp10, Hypothetical proteins in CsHsp20 and 40, Dnak in CsHsp60, Molecular chaperone in CsHsp70 and Hsp90 in CsHsp90. These proteins are produced by cells in response to stresses in citrus. Chaperonins and some hypothetical proteins identified in CsHsps, help in ATP synthesis and protein degradation. This is genome wide analysis and classification sets the groundwork for future investigations to fully characterize functionally the Citrus Hsps families and underscores the relevance of Hsps response to abiotic and biotic stresses in Citrus.

Keywords: Abiotic stress; Chaperones; *Citrus* spp.; Protein assembly; Protein expression

1. Background

Fluctuating temperature is regarded as stress that has serious effects on crop production worldwide; however, additional stress factors with temperature causing cell damage, functioning alteration and osmotic or oxidative stress (1). Opportunistic behavior of plants compromised their ability to avoid exposure to adverse conditions. Citrus plant physiology and morphology is also affected by heat and water deficit (2), and causes reduction in productivity (3,4). However, modification in structure, regeneration ability and production of secondary metabolites insured their survival and prosperity. The response of plants against infections caused by biotic stress is mediated by Heat shock proteins (Hsps). Hsps genes mechanism of genetic expression against stresses is still unidentified, but the similar stimulus could be produced by pathogen attack resulting in same metabolic changes as those observed under abiotic stress activation. Besides high temperature response, heat shock proteins are also responsive

Table 1. Functions and classes of Hsps in *C. sinensis*

Hsp Classes	Pfams#	CsHsp group	Function
Hsp10	PF00166	4 members	Hsp10 functions with Hsp60 as double-ring assemblies rotationally symmetrical subunits
Hsp20	PF00011	54 members	ATP-independent molecular chaperones, avoiding protein denaturation
Hsp40	PF00226	5 members	DnaJ proteins, constitute largest families, Regulate the ATPase activity of Hsp70 in binding to partially denatured protein
Hsp60	PF00118	25 members	maintain integrity of cellular proteins in response to environmental changes
Hsp70	PF00012	25 members	Fundamental role in plant developmental processes and functions during heat stress
Hsp90	PF00183	6 members	assist protein folding, signal transduction, cell-cycle control, protein degradation, genomic silencing, and protein trafficking
Hsp100	PF00004	0 members	unfoldase activity in the presence of ATP

* Hsp40 having no pfam number for identification mostly researcher used Hsp40 as a key word.

against cold stress, heavy metals and reactive oxygen species (ROS) (5).

Heat shock proteins against heat stress were first identified in *Drosophila melanogaster*. Heat shock proteins are grouped with HSP70, HSP90, HSP100 and HSP60/GroE families having high molecular weight protein, and Hsp20 or other 15-42kDa small heat shock proteins (sHSPS) having low molecular weight proteins (6). Small heat shock proteins such as HSP20 aggregates oligomeric protein complexes comprising 9 to 50 subunits (200–800kDa) and are ATP-independent molecular chaperones functioned by preventing protein denaturation in both eukaryotic and prokaryotic cells (6).

Hsp40/Dnaj proteins, function as a cofactor of Hsp70 in ATPase activity regulation, facilitate the reversible binding to partially denatured protein substrates to avoid the aggregation of themselves or with other molecules (7). The binding of ATP to ATPase domain and subsequent hydrolyzation to change the conformation of the binding domain is prerequisite for the association between Hsp70 proteins and substrates, in the Hsp70-Hsp40 co-chaperone system. Thus, various Hsp70's substrates could specifically bind to its least conserved C-terminal at a higher affinity. However, J-domain of Hsp40s is necessary for activating the ATPase domain of Hsp70s because the ATPase activity of Hsp70s is extremely weak (8).

Molecular chaperone is basic part of cellular network and protein folding which inheld Hsp70 proteins as central component (9), and was identified and characterized in early 1960. Two major functional domains exist in HSP70s, one is a conserved ~44-kD N-terminal ATPase domain (NBD), which is also called the nucleotide binding domain and the other is a ~18-kD substrate binding domain (SBD) with a ~10-kD variable C-terminal "lid" (10).

All eukaryotes and prokaryotes have highly conserved Hsp90s chaperone proteins. ATP is obligatory for the functioning of Hsp90s which is the major species of molecular chaperones (11). Although Hsp90s are expressed in most organisms, their expression increases regarding to response against stresses. Hsp90s are very specific for their client proteins and are distinct from many other well-characterized molecular chaperones. Steroid hormone receptors and signaling kinases are signal-transduction proteins and act as substrates for Hsp90s (12). Beside performing the major function of assistance in protein folding, Hsp90s also play key role in signal transduction, cell-cycle control, protein degradation, genomic silencing, and protein trafficking (12,13). Hsp's major groups, members within a group and reported functions in *C. sinensis* are presented in **Table 1**. Heat shock proteins responsive to heat stress was not characterized in the citrus upto our knowledge.

2. Objectives

The objective of present study was identification and grouping of *C. sinensis* CsHsps, total members among classes, their phylogenetic relationship, gene structure, conserved motifs and identification of proteins by using different bioinformatics tools and analyses.

3. Material and Methods

3.1. Identification of Hsp's

Heat shock proteins of *C. sinensis* and closely related member *Citrus clementine*, their classes and members within a class were identified using online genetic database tool named "Phytozome" (http://www.phytozome.org/citrus/) also taking *Arabidopsis thaliana* as a model plant (14). Classification of Hsp's in different classes were performed using the "Hidden Markov Model" profile of the Pfam 32.0 (https://pfam.

Table 2. Hsps classes, Pfams number and member within classes of *C. sinensis*, *C. celementina* and *A. thaliana*.

Hsp Classes	Pfams	*Citrus sinensis*	*Citrus celementina*	*Arabidopsis thaliana*
Hsp10	PF00166	4	4	5
Hsp20	PF00011	54	61	26
Hsp40	PF00226	5	5	21
Hsp60	PF00118	25	20	23
Hsp70	PF00012	25	29	19
Hsp90	PF00183	6	6	7
Hsp100	PF00004	0	0	0
Hsp(X)	Unidentified	32	32	57
Total	*hsp	151	157	158

*= 'hsp' used as keyword on phytozome against *C. sinensis* to identify total Hsp's.

xfam.org/) (15) through performing BLAST (basic local alignment search tool) of protein sequence of *C. sinensis, C. clementine* and *A. thaliana* by motif PF00166 (Hsp10), PF00011(Hsp20), PF00118(Hsp60), PF00012(Hsp70), PF00183(Hsp90) and Hsp40 used as a keyword to search the genome sequence database.

3.2. Phylogenetic Analysis

Peptide sequence of all the *C. sinensis* Hsps classes were downloaded from phytozome. Multiple sequence alignment and neighbor joining phylogenetic tree was constructed by MEGA 6.0 (16). Phylogenetic tree constructed by bootstrap method with 1000 replicate by passion method (17).

3.3. Genes Sequence Retrieval

Genomic and coding sequences of all the six CsHsp groups were retrieved taken from Phytozome. Gene structure of CsHsp10, CsHsp20, CsHsp40, CsHsp60, CsHsp70 and CsHsp90 were constructed (http://gsds.cbi.pku.edu.cn/) by using gene structure display server (GSDS) 2.0 online software (18).

3.4. Multiple Sequence Alignments

Peptide sequences of *C. sinensis* of Hsp's groups (CsHsp10, CsHsp20, CsHsp40, CsHsp60, CsHsp70 and CsHsp90) used for multiple sequence alignments of peptide sequences by MEGA 6.0 (16) genetic tool and Unipro UGENE (Integrated Bioinformatics Tools) (19) to study the relationship between groups and within a group.

3.5. Identification of Conserved Motifs

Conserved protein motifs of the deduced protein sequences were also detected and mapped using MEME version 4.12.0 (http://www.meme.sdscedu/meme/meme.html) (20). Maximum number of motifs found were a set of 25, the distribution of one single motif was "any number of repetitions" and the other parameter was "search given strand only". Conserved motifs were annotated by NCBI blast against protein to find specific proteins.

4. Results

4.1. Identification of CsHsps

Heat shock protein has protein 99% similarity among *C. sinensis* and *C. celementina*, hence both citrus species and *A. thaliana* were used to identify the classes of Hsp10, 20, 40, 60, 70, 90 and 100 from Phytozome. *C. clementina* has almost identical number of Hsps members within a class and total Hsps with *C. sinensis* (**Table 2**).

4.2. Phylogenetic Relationships of All the Hsp Groups of Citrus Sinensis

Peptide sequences of all the Hsp's from six major classes (Hsp 10, 20, 40, 60, 70 and 90) were downloaded and NJ-phylogenetic tree was constructed. The NJ phylogenetic tree showed that all the Hsps genes from *C. sinensis* are divided into five big clades (**Fig. 1**). Phylogenetic tree separated all the classes into separate clusters on the bases of NJ-bootstrap analysis. CsHsp10 (green color) and CsHsp70(red color) formed two separate but neighbor clusters which depicted that proteins of CsHsp10 and CsHsp70 having almost similar amino acid sequences and conserved motifs. CsHsp60 (light blue) having neighbor joining clusters with CsHsp70 (red color) and CsHsp10 (green color) showed more similarity. Fifty out of fifty-four members of CsHsp20 (yellow color) formed a separate cluster leaving the four members of CsHsp20 which were grouped with sHsp40 (purple) and CsHsp90 (blue color).

4.3. Gene Structure Analysis of CsHsps

Gene structure was analyzed along with the exon/intron

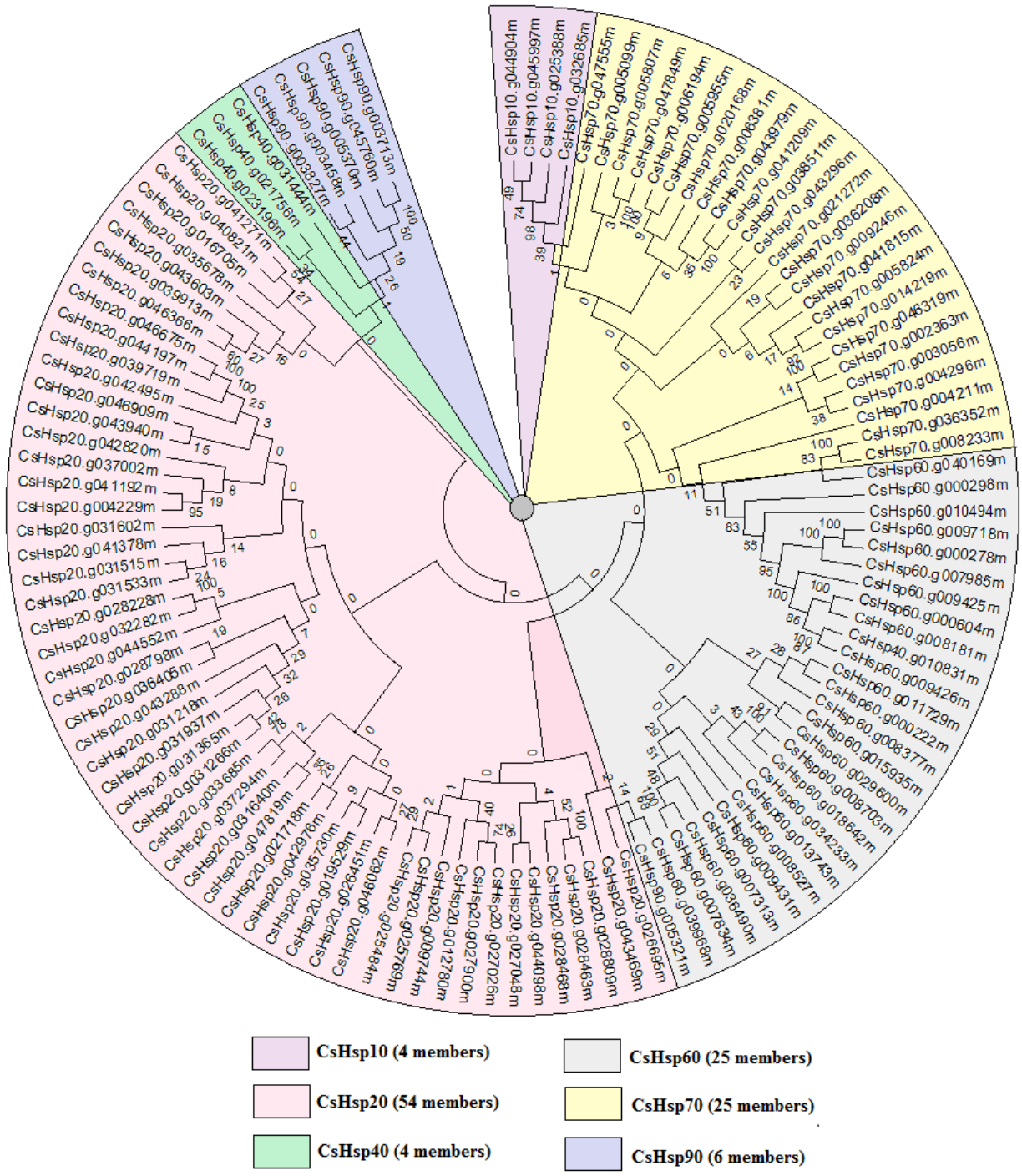

Figure 1. Phylogenetic tree constructed based on the amino acid sequences of 118 *C. sinensis* heat shock proteins, separated in six major classes CsHsp10, CsHsp20, CsHsp40, CsHsp60, CsHsp70 and CsHsp90 indicated with different colors. The phylogenetic tree was constructed using MEGA 7.0 with neighbor joining phylogenetic method. Bootstrap values in percentages (1000 replicates) are indicated on the nodes.

organization in the coding and genomic sequence of the entire CsHsp gene after removing redundant genes with in a class or group (**Fig. 2**). Gene size within the Hsps of *C. sinensis* ranges from 1kb to 9kb. Gene structure of all the classes of Hsp such as Hsp10, Hsp20, Hsp40, Hsp60, Hsp70 and Hsp90 shared similar structure in each class. The size of genes from 2-5kb with relatively a smaller number of exon/introns were observed in CsHsp10, 20 and 40 except one member CsHsp40 (CsHsp4g10831m) with 8kb gene size and 10 introns.

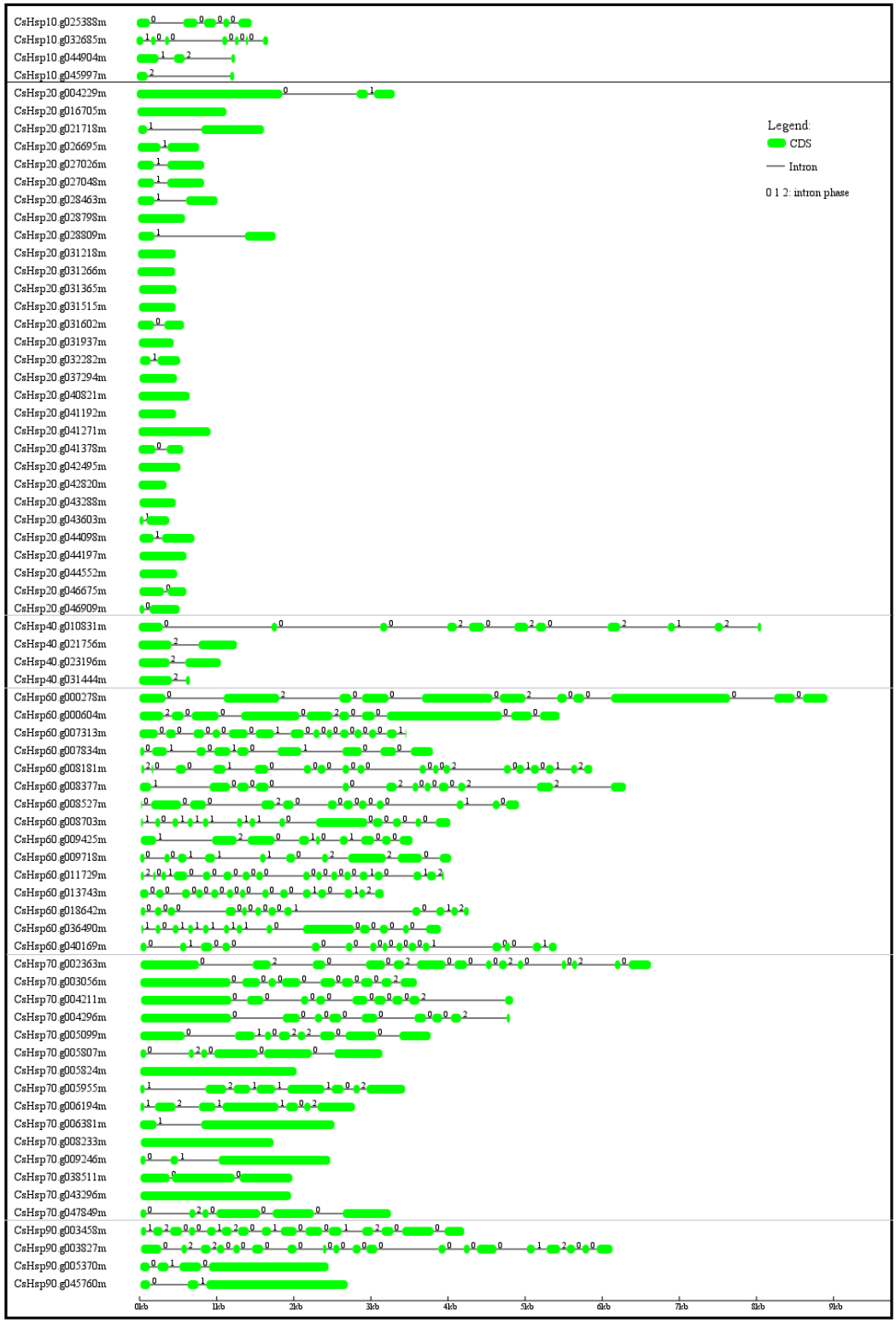

Figure 2. Annotated gene structure of *C. sinensis* Hsps. Exons and introns of CsHsp five classes genes are plotted using green boxes and black lines, respectively. Gene structure was constructed by online tool GSDS 2.0 (gene structure display server).

CsHsp60 fifteen members have 4-9kb gene size with maximum number of introns of each gene. CsHsp70 and 90 members indicated gene size ranges from 2-7kb with varied number of exon and intron.

Table 3. Hsps protein conservation in *C. sinensis* of six major classes CsHsp 10, 20, 40, 60, 70 and 90 constructed by Unipro UGENE.

Hsps groups	Graph of conservation of proteins	Members
CsHsp10		4
CsHsp20		31
CsHsp40		4
CsHsp60		25
CsHsp70		25
CsHsp90		4

4.4. Conservation of Proteins

Peptide sequence conservation of *C. sinensis* Hsp's after removing redundant or truncated genes is constructed by UGENE bioinformatics software and presented in graphical structure (**Table 3**). It is indicated that the amino acid sequences of CsHso70 (25 members) and CsHsp90 (4 members) have more than 5 conserved motifs in all their members within a class. CsHsp20 with 31 members, aligned by Clustal-W showed very little conservation. A smooth pattern of conserved motifs was also observed in CsHsp10, CsHsp40 and CsHsp90 (**Table 3**).

4.4.1. CsHsp10 Conserved Motifs

Identified Cpn10 and GroES protein motifs in the three members of CsHsp10 when BLAST is performed for conserved motifs. CsHsp10.g044904m, CsHsp10. g025388 and CsHsp10.g032685 having amino acid at positions 2nd, 29th, 32nd, 43rd and 49th in the conserved region of the identified cpn10 and GroES, are different (**Fig. 3A**).

4.4.2. CsHsp20 Conserved Motifs

Fourteen members of CsHsp20 have conserved motif of 40 amino acids. Amino acids observed at position 10th and 39th of conserved protein in these members are responsible to differentiate the members in different genes. The identified conserved motifs had shown more similarity to one of the Hypothetical protein COLO4 in the literature (**Fig. 3B**).

4.4.3. CsHsp40 Conserved Motifs

Four members of CsHsp40 have less similarity in amino acid of conserved motifs. Amino acid at positions 3rd, 9th, 10th, 11th, 17th, 21st, 32nd, 33rd, 34th, 36th and 40th of conserved motifs are responsible to differentiate the members in different genes. Conserved protein motifs are BLAST and identified Hypothetical protein reported in genome of allotetraploid *Gossypium barbadense* reveals genomic plasticity and fiber elongation in cotton evolution (**Fig. 3C**).

4.4.4. CsHsp60 Conserved Motifs

Nine members of CsHsp60 have conserved motifs chaperonin and T-complex. All the nine-members have more similar alignment of conserved amino acid in sequences. Identified chaperonins are involved in productive folding of proteins (**Fig. 4A**).

4.4.5. CsHsp70 Conserved Motifs

Ten members of CsHsp70 having more conserved proteins for two identified proteins were Hsp70/Dnak (**Fig. 4B**) and HSPA (**Fig. 4C**).

4.4.6. CsHsp90 Conserved Motifs

Three types of conserved motifs were observed in four members of CsHsp90. CsHsp 90 with different amino acid was observed at position 15th, 28th, 44th and 50th in first conserved motifs Hsp90 proteins (**Fig. 5A**). Conserved motifs of molecular chaperone with different amino acid was observed at positions 7th, 11th, 22nd and

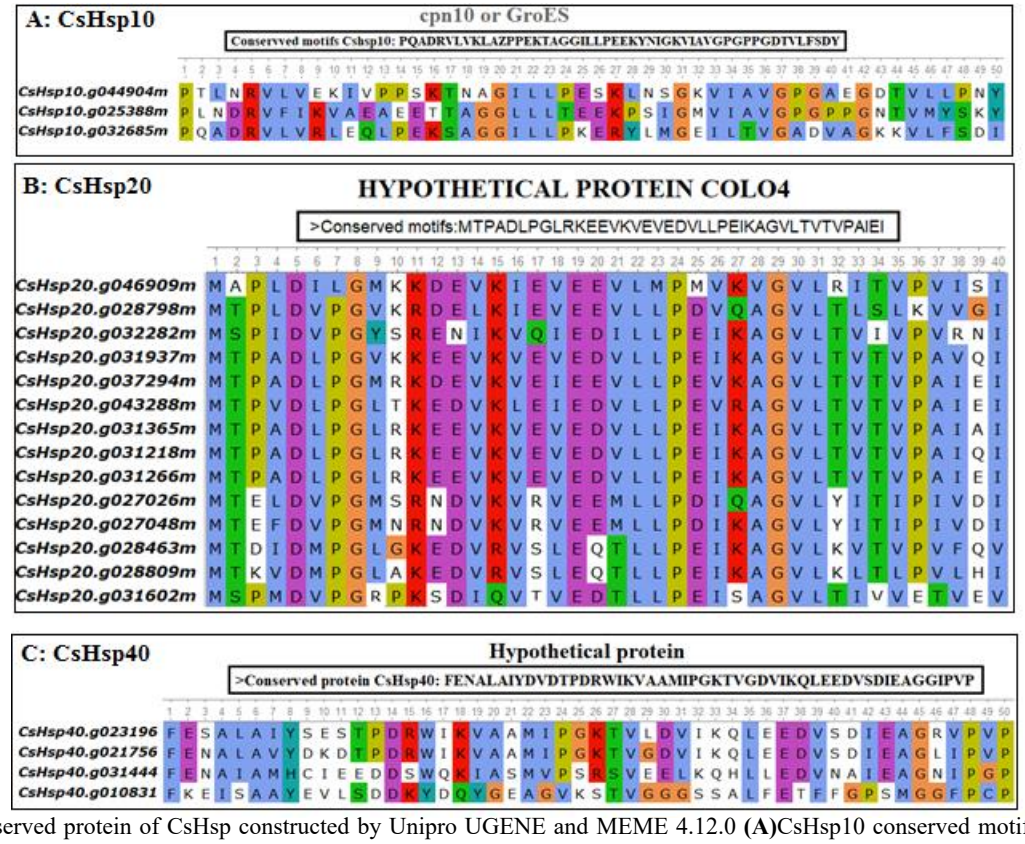

Figure 3. Conserved protein of CsHsp constructed by Unipro UGENE and MEME 4.12.0 **(A)**CsHsp10 conserved motifs of fifty amino acids. **(B)** CsHsp20 conserved motifs of forty amino acids. **(C)** CsHsp40 conserved proteins of fifty amino acids.

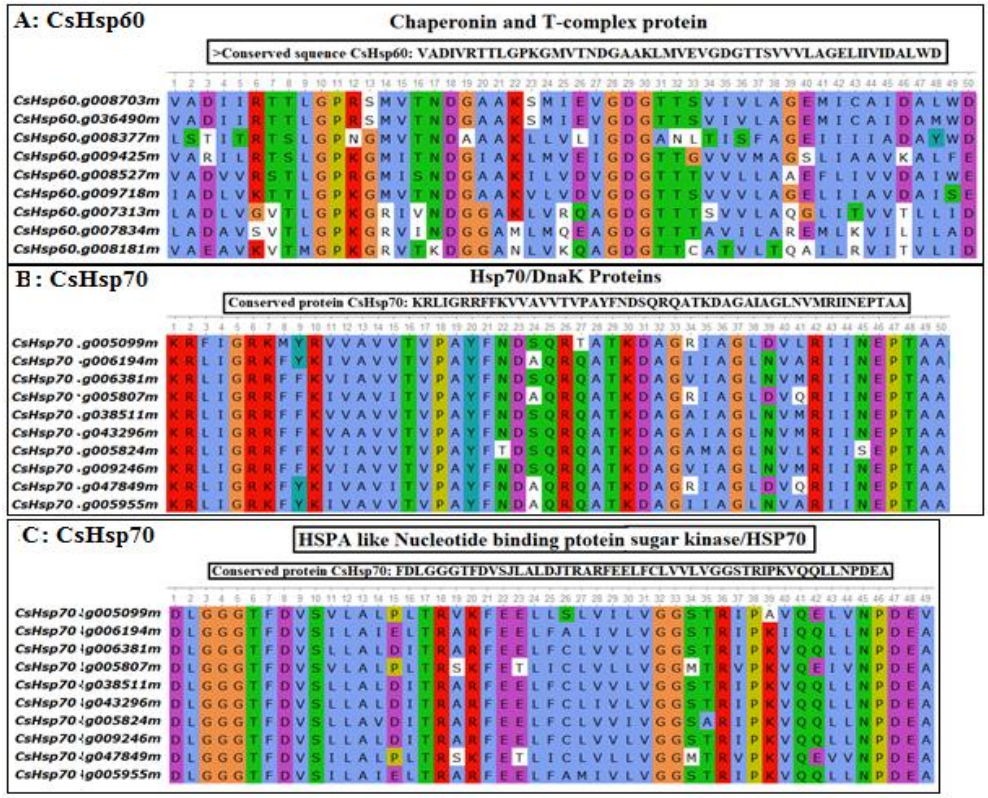

Figure 4. Conserved protein of CsHsp constructed by Unipro UGENE and MEME 4.12.0 **(A)** CsHsp60 conserved identified motifs of fifty amino acids. **(B)** CsHsp70 first conserved motifs of fifty amino acids. **(C)** CsHsp70 conserved motifs of forty-nine amino acids.

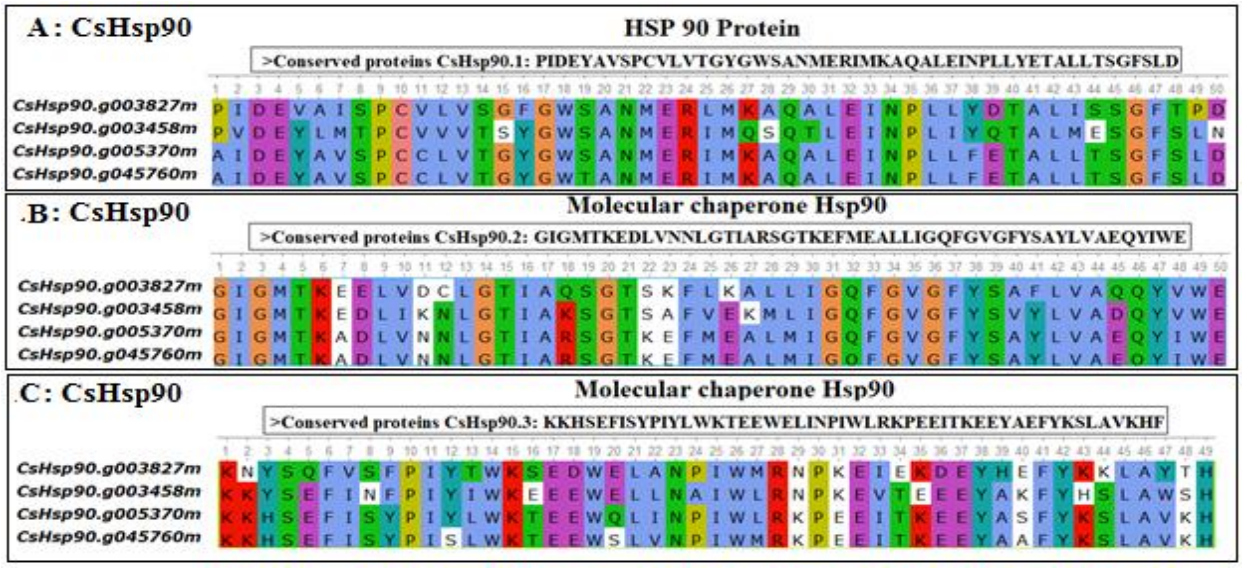

Figure 5. Conserved protein of CsHsp90 three conserved (**A, B** and **C**) motifs of fifty amino acid constructed by Unipro UGENE and MEME 4.12.0.

23rd amino acid (**Fig. 5B**). Third and last conserved motifs molecular chaperone has different amino acids at positions 29th, 31st, 41st and 48th of conserved region (**Fig. 5C**).

5. Discussion

Phylogenetic tree analysis identified 151 members of *C. sinensis* Hsps with six major classes Hsp10, Hsp20, Hsp40, Hsp60, Hsp70 and Hsp90. The tree distinguished different classes of CsHsp's in different cluster which showed that protein sequences within the members of the family were similar. It is reported in literature that Hsp10 helps in protein folding in mitochondria while Hsp70 in protein folding (assembly & refolding) in endoplasmic reticulum (21), and due to that reason both classes originate from the same branch of cluster and fall in neighbor cluster. Hetero-oligomeric chaperonins such as GroES acts as a detachable lid for the cavity of cylindrical cpn60 tetradecamer and creates a folding chamber that encloses polypeptide substrates. CsHsp60 was observed in close cluster to both CsHsp10 and CsHsp70 so considered closer relative. Hsp40 helps in chaperoning intermediate filament while Hsp90 in myosin folding and sarcomere formation, both classes observed in same cluster. CsHsp40 (g010831m) was in the cluster of CsHsp60 but when we blast Hsp40 in Phytozome they fall in the CsHsp40 class. Fifty members of CsHsp20 were observed in a separate cluster, while reported literature showed that Hsp20 helps in the synthesis of ATP-independent molecular chaperones, avoiding protein denaturation (21).

After removing redundant sequences, we got 72 members for gene structure analyses with six major classes CsHsp10 (4 member), CsHsp20 (30 members), CsHsp40 (4 members), CsHsp60 (15 members), CsHsp70 (15 members) and CsHsp90 (4 members). Gene size observed in all members ranged 500-9000bp. All the CsHsps gene family members within the same class shared almost similar gene structures in terms of intron number or exon length. CsHsp 10, CsHsp 20 and CsHsp 40 found in mitochondria, plasma membrane and cytosol respectively having 2-4 introns known as cytosolic members (22). CsHsp60 found in Mitochondria, CsHsp70 and CsHsp90 found in cytoplasm comprises organelle-type having more than 6-15 introns. Only one member of CsHsp40 (g010831m) also having 8 member and considered as an organelle type structure as compared to rest of three members (22).

Peptide sequence conservation of Hsps of *C. sinensis* showed conservation among groups such as all the members of CsHsp70 and CsHsp90 has more conserved and similar aligned amino acids followed by CsHsp60. CsHsp10, CsHsp 20 and CsHsp40 members indicate less conserved motifs.

The conserved protein of CsHsp10 has Cpn and GroES domains starting after 65 number amino acid in three members. Cpn10 co-operates with chaperonin 60 (cpn60 or GroEL), an ATPase, to assist the folding and assembly of proteins which are found in eubacterial cytosol, as well as in the matrix of mitochondria and chloroplasts (23). Identified CsHsp10 conserved motifs

can also assist in folding and assembly of proteins.

Alpha crystalline and hypothetical protein were identified in fourteen members of CsHsp20 which, start after 600 scale position when amino acids were aligned. Similar Alpha crystallin/Hsp20 domain has been found in *Macleaya cordata* (24) and hypothetical protein COLO4_18416 is identified in *Corchorus olitorius* (25).

Hypothetical protein was observed in four members of CsHsp40 which start from 118 to onward. Identified protein is also similar to hypothetical protein identified in *G. barbadense* genome sequence which provides insight into the evolution of extra-long staple fiber and specialized metabolites (26) reported in cotton.

Chaperonin and T-complex protein are the functional annotations of proteins (27) and CDD/SPARCLE: are functional classification of proteins via subfamily domain architectures were found in ten members of CsHsp 60. CsHsp60 conserved protein starts from 365 sites to onward, protein size ranges from 1-1774.

DnaK/Hsp70 is identified in ten members of CsHsp70 which play an essential role in the initiation of phage lambda DNA replication, where it acts as ATP-dependent and involved in chromosomal DNA replication, possibly through an analogous interaction with the DnaA protein (28). Nucleotide-Binding Domain of the sugar kinase/ HSP70 was also identified in the same ten members of CsHsp70 proteins. which regulate enzymes catalyze ATP phosphoryl transfer or hydrolysis coupled to a large conformational change, in which the two domains close around the nucleotide (29). Sequences of the *Arabidopsis* Hsp70 gene family was used to identify and annotate the Citrus Hsp70 genes represented in the CitEST database in a past study (30). Molecular chaperone and Hsp90 chaperone as signal transduction proteins, including steroid hormone receptors and a broad range of protein kinases, are identified in all four members of CsHsp90 (31).

6. Conclusion

We performed a comprehensive analysis of the *C. sinensis* heat shock proteins. *C. sinensis* Hsps gene family covering phylogeny, gene structure, motif conservation, and identified proteins. A total of 151 with 72 full-length CsHsps genes were identified in the *C. sinensis* genome, all of which are clustered into some big cluster and some small distinct groups. Exon/ intron structure and motif compositions are found to be relatively conserved in each classes and subgroup members. The citrus genome contains cytosolic gene structure in CsHsp10 and CsHsp20 members, and organelle type gene structure in CsHsp60, CsHsp70 and CsHsp90. Cpn and GroES identified in CsHsp10 helped in assist in folding and assembly of proteins. Chaperonin and some hypothetical protein identified in some classes of CsHsps. CsHsp70 and CsHsp90 helps in ATP synthesis and protein degradation. The classification will help to further dissect the rule and functional analysis of the identified genes.

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Data archiving statement

All the genome data related genomic, CDS and peptide sequence were available at https://phytozome.jgi.doe.gov/pz/portal.html#!search?show=KEYWORD&method=Org_Csinensis. Additional proteins are identified by NCBI protein blast tool to target a specific protein region https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome database.

Conflict of Interest

There is no conflict of interest for this manuscript.

Author contribution

Waqar shafqat performed the work and wrote the manuscript. Muhammad Jafar Jaskani and Rizwana Maqbool planned and supervised the work. Rizwana Maqbool, Ahmad Sattar Khan and Summar Abbas Naqvi helped in performing softwares and genetic tools. Zulfiqar Ali and Iqrar Ahmad Khan edited and improved the manuscript.

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