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ORIGINAL PAPER

Genome-wide analysis of *Hsp70* and *Hsp100* gene families in *Ziziphus jujuba*

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Abstract

The *Ziziphus* species are naturally tolerant to a range of abiotic stresses. Therefore, it is expected that they are an enriched source of genes conferring stress tolerance. Heat shock proteins (Hsps) play a significant role in plants in imparting tolerance against abiotic stress conditions. To get an insight into potential Hsp function in *Ziziphus*, we performed a genome-wide analysis and expression study of *Hsp70* and *Hsp100* gene families in *Ziziphus jujuba*. We identified 21 and 6 genes of the *ZjHsp70* and *ZjHsp100* families, respectively. Physiochemical properties, chromosomal location, gene structure, motifs, and protein domain organization were analysed for structural and functional characterization. We identified the contribution of tandem and segmental gene duplications in expansions of *ZjHsp70s* and *ZjHsp100s* in *Z. jujuba*. Promoter analysis suggested that *ZjHsp70s* and *ZjHsp100s* perform diverse functions related to abiotic stress. Furthermore, expression analyses revealed that most of the *Z. jujuba Hsp* genes are differentially expressed in response to heat, drought, and salinity stress. Our analyses suggested *ZjHsp70-3*, *ZjHsp70-5*, *ZjHsp70-6*, *ZjHsp70-16*, *ZjHsp70-17*, *ZjHsp70-20*, *ZjHsp100-1*, *ZjHsp100-2*, and *ZjHsp100-3* are potential candidates for further functional analysis and with regard to breeding new more resilient strains. The present analysis laid the foundation for understanding the molecular mechanism of *Hsps70* and *Hsp100* gene families regulating abiotic stress tolerance in *Z. jujuba*.

Keywords Genome-wide analysis · Heat shock protein (Hsp) · Gene family · Expression analysis · qRT-PCR · *Z. jujuba*

Introduction

In recent decades, abiotic stress has caused severe economic losses of crop production globally. Abiotic stress hampers the biochemical and physiological processes of crop plants, which negatively affects their growth and development, thus cumulatively affecting crop yield as well as food quality (Wahid 2007; Hasanuzzaman et al. 2013). The global climate model has proposed that temperatures will rise by 1.4 to 5.8 °C by the end of the twenty-first century due to the increase in atmospheric greenhouse gases (Planton et al. 2016). So far, the progressively changing climate has affected food security (Coumou and Rahmsdorf 2012), mainly in South Asia and sub-Saharan Africa, but as well on the global level (Battisti and Naylor 2009). As the sessile nature of crop plants means that they cannot relocate to avoid stress, they have therefore evolved molecular tolerance mechanisms to stressful conditions (Khan et al. 2019). Heat shock proteins (Hsps) are produced in response to stress as a molecular defence mechanism that is very important for the survival and growth of plants (Hasanuzzaman et al. 2013). Members of the *Hsp* gene family play significant roles in developmental processes in addition to abiotic stress conditions such as drought, heat, salinity, cold, and heavy metal toxicity (Khan et al. 2019). The Hsps have been associated with responses to the infection by

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pathogens such as nematodes, playing a role in programmed cell death (PCD) during the remodelling of leaves (Maimbo et al. 2007; Rowarth et al. 2020). The Hsps act as essential molecular chaperones in the transport and assembly of nascent proteins, refolding, and exclusion of denatured proteins in a cell under abiotic stress conditions and enhance tolerance (Hasanuzzaman et al. 2013). They are classified into five major families: Hsp100, Hsp90, Hsp70, Hsp60, and small Hsp (10–40 kDa) based on their molecular weight (Khan et al. 2019; Hasanuzzaman et al. 2013).

The Hsp70 protein is a central part of the cellular network of folding catalysts and chaperones (Mayer and Bukau 2005). It contains two important functional domains, NBD (N-terminal ATPase domain) of ~ 44-kDa, and a ~ 18-kDa SBD (substrate-binding domain) (Dragovic et al. 2006). The various roles of the *Hsp70* gene family have been characterised in several plants. For instance, in *Arabidopsis thaliana*, the *cpHsc70-1* mutant showed defective phenotypes when germinated seeds were subjected to heat stress (Su and Li 2008). The *M. sativa MsHsp70* was induced by heat stress and transgenic lines of *A. thaliana* overexpressing *MsHsp70* have enhanced tolerance to drought stress (Li et al. 2017). *Hsp70s* have also been demonstrated to play significant functions under stress condition in wheat (Duan et al. 2011), cucumber (Li et al. 2014), pepper (Guo et al. 2014), and rubber trees, etc. (Zhang et al. 2009). Similarly, the Hsp100/Clp (**C**aseinol**y**tic **P**rotease) proteins belong to the AAA+ ATPase superfamily and these chaperones are also crucial in modulating plant thermotolerance traits (Mishra and Grover 2016). Hsp100 proteins contain several domains (amino (N)–terminal, nucleotide-binding domain 1 (NBD1), middle domain, nucleotide-binding domain 2 (NBD2), and a carboxyl (C)–terminal domain). This gene family is divided into ClpATPases class I (ClpA, ClpB, ClpC, ClpD, ClpE) with two ATP binding domains and ClpATPases class II (ClpM, ClpN, ClpX, ClpY) with a single ATP binding domain (Mishra and Grover 2016). The *A. thaliana* genome contains 7 class-1 Clp/Hsp100 protein-encoding genes including 4 *ClpB* proteins 2 *ClpC*, 1 *ClpD* protein, but no members of the *ClpA* or the *ClpE* subclass (Lee et al. 2007). Mutants of *A. thaliana*, rice, and maize plants with defective *Hsp100* expression showed severe sensitivity to heat stress (Hong and Vierling 2000; Nieto-Sotelo et al. 2002; Lin et al. 2014). Additionally, overexpression of *Hsp100* in *A. thaliana* and rice increased plant thermotolerance (Queitsch et al. 2000; Katiyar-Agarwal et al. 2003).

Ziziphus is native to Asia and mainly found in central and Southwest Asia, central India, and China (Outlaw et al. 2002; Pandey et al. 2010; Sabir et al. 2020). The *Ziziphus* species including *Z. jujuba* (Chinese *jujuba*) and *Z. nummularia* are well adapted to the arid and semiarid region and tolerant to a range of abiotic stresses including heat, drought, salinity, chilling, and metal toxicity (Pandey et al. 2010; Sabir et al. 2020). Therefore, they are expected to house a repository of genes responsible for abiotic stress tolerance (Pandey et al. 2010; Padaria et al. 2016; Panzade et al. 2020). For example, transcriptomic profiling in *Z. nummularia* identified several drought-responsive genes (Yadav et al. 2018a; Yadav et al. 2018b). Furthermore, the heat stress-inducible isoform *ZnJClpB1-C* of *Hsp100* and the abscisic acid-stress-ripening gene (ASR) from *Z. nummularia* have been shown to enhance heat stress tolerance in *N. tabacum* and drought stress tolerance in *E.coli* cells (Padaria et al. 2016; Panzade et al. 2020). Similarly, *SbHsp70-1* from the stress-tolerant cereal crop *Sorghum bicolor* also confers thermal tolerance in *E. coli* (Mulaudzi-Masuku et al. 2015).

In this study, we surveyed the newly available genome sequence of *Z. jujuba* for *Hsp70* and *Hsp100* gene family members and conducted stress challenge experiments as the first step in identifying the role of these *Hsp* families in stress tolerance.

Materials and methods

Genome-wide identification of *Hsp70* and *Hsp100* gene family members in *Z. jujuba*

The *Hsp70* and *Hsp100* gene family members in *Z. jujuba* were identified by performing a BLASTP search against *Z. jujuba* genome database (https://www.genome.jp/kegg-bin/show_organism?org=zju) by using orthologous amino acid sequences of *Arabidopsis thaliana*, *Rhamnella rubrinervis*, and *Cannabis sativa*, as queries (*e*-value of 1×10^{-5} and identity of > 60% as the threshold). The Hidden Markov model (HMM) of the Hsp70 and Hsp100 protein family also used to search the candidate amino acid sequences. All of the retrieved amino acid sequences were analysed for the presence of the Hsp70 and Hsp100 conserved protein domains using SMART (http://smart.embl-heidelberg.de/) tool (Letunic et al. 2012). Protein sequences with redundancy and truncated domains were removed. Then, the coding sequences (CDS), genomic sequences, amino acid sequences, chromosomal location data, and nucleotide sequences of 2000-bp upstream from the translation initiation codon were retrieved from the *Z. jujuba* genome database (Table S1).

Predicted analysis of physico-chemical properties and sub-cellular localization

The full-length protein coding sequences of Hsp70 and Hsp100 gene family members were analysed in silico for various physico-chemical parameters (amino acid composition, theoretical isoelectric point (pI), molecular weight, grand average of hydropathicity (GRAVY), aliphatic index, etc.) using the Expasy ProtParam tool (http://us.expas y.org/tools/protp

aram.html) (Garg et al. 2016). Sub-cellular localization was predicted using CELLO v.2.5 (http://cello.life.nctu.edu.tw/cello2go/alignment.php) (Yu et al. 2014).

Analyses of gene structure, motifs, and domain architecture

The intron/exon structure of the *Z. jujuba Hsp70* and *Hsp100* gene family was analysed based on the alignment of genomic DNA sequences with corresponding CDS sequences, by using the Genes Structure Display Server tool (GSDS, http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2015). The conserved motifs of Hsp70 and Hsp100 protein families were identified using amino acid sequences in the MEME tool (Multiple Expectation Maximization for Motif Elicitation, http://meme-suite.org/tools /meme). The parameters were used as follows: Optimum motifs: 6 to 50 amino acid residues, the maximum number of motifs: 20, a number of repetitions: any (Bailey and Elkan 1995). Domains of Hsps protein were analysed by using the SMART tool (http://smart.embl-heidelberg.de/) (Letunic et al. 2012).

Microsatellite mining and identification

The SSR Locator (http://microsatellite.org/ssr.php?info) software was used to extract microsatellites (Da Maia et al. 2008) with mono-, di-, tri-, tetra-, penta, hepta-, octa-, nova-, or decanucleotide motifs. SSRs with a total length of ≥ 20 bp with the parameters described by Gao et al. (2011) were used to identify potential SSRs across the *ZjHsp70* and *ZjHsp100* genes.

Multiple sequence alignment and phylogenetic analysis

In the present study, two phylogenetic trees were constructed to classify and assess the phylogenetic relationships of the *Z. jujuba Hsp70* and *Hsp100* gene families. The first tree was limited solely to *Z. jujuba* Hsp70 and Hsp100 amino acid sequences and the second contained Hsp70 and Hsp100 amino acid sequences from *Ziziphus jujuba*, *Arabidopsis thaliana*, *Cannabis sativa*, and *Rhamnella rubrinervis* (retrieved from NCBI and TAIR database (Table S2)). Multiple sequence alignments of all Hsp70 and Hsp100 amino acid sequences were carried out using ClustalW with default parameters (Larkin et al. 2007). The phylogenetic trees were constructed using the Neighbour-Joining (NJ) method implemented in MEGA 7.0.26 software, with a bootstrap value 1000 (Tamura et al. 2013).

Chromosomal locations and duplication of *Hsp70* and *Hsp100* in *Z. jujuba*

The chromosomal localization of *Hsp70* and *Hsp100* genes family was analysed using MapChart software (Voorrips 2002), according to the chromosomal position data available at the *Ziziphus* database. Coding protein pairs with ≥ 50% identity and covering ≥ 90% protein length were considered as duplicated genes (Wang et al. 2017). If genes from the same species were placed in the same clade of the tree, these were further analysed to identify whether the potential duplication was of the tandem or segmental type. If the paralogous pair was separated by five or fewer genes within a 100-kb region, they were designated as tandemly duplicated genes. If the paralogs were separated by > 5 genes or they were mapped onto duplicated chromosomal blocks, they were designated as segmental duplications (Zhang and Li 2018; Liu et al. 2018; Zhang et al. 2015a, b).

Promoter analysis of *Hsp70* and *Hsp100* gene families

Potential cis-elements of the promoter regions were analysed in the 2000-bp sequences upstream of the TSS (transcriptional start site) of each of the *Hsp70* and *Hsp100* gene using the PLACE database (http://www.dna.affrc.go.jp/PLACE/).

Plant material, growth conditions, and stress treatments

Seeds of Indian *jujuba* variety "Maharwali" were sterilised for 10 min using a 10% hypochlorous acid and rinsed with distilled water three times. Upon scarification, seeds were grown in a growth chamber (at National Phytotron Facility, Indian Agricultural Research Institute, New Delhi, India) maintained at 22 °C, 16-h light/8-h dark photoperiod, RH 60–70%. One-month-old seedlings were exposed to various stresses. The heat stress at 42 ± 1 °C was produced by raising the temperature at 1 °C per 10 min until the temperature reaches 42 °C and the same temperature was continued for 2 h (Vishwakarma et al. 2018). The drought and salinity stress treatments were produced by seedlings being transferred to 400 mM mannitol or 300 mMNaCl for 5 h. The seedlings, which were grown under normal condition and kept in water for the same duration at 25 ± 2 °C, were used as controls (Singh et al. 2016; Liu et al. 2018). Total RNA isolation was carried out from leaves using the Spectrum™ Plant Total RNA Kit and RNase-free DNase I (Sigma-Aldrich, USA) was used to get eradicate genomic DNA as per the manufacturer's protocol. First-strand synthesis of cDNA was performed using the SuperScript III First-Strand Synthesis System (Sigma-Aldrich, USA). Specific oligonucleotide primers were designed using the IDT primer quest tool (Table S3). Real-time expression analysis was carried out using three technical replicates of each biological replicate using the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific, USA) according to the manufacturer's

recommendations. Quantifications were normalised using the actin housekeeping gene of *Z. nummularia* as an internal control (Sun et al. 2009). The steps used for amplification were 95 °C for 30s followed by 32 cycles at 95 °C for 3 s and 62 °C for 30s. Ct values were estimated using the 2−ΔΔCT method (Livak and Schmittgen 2001).

Statistical analysis

Student's *t* test was used to evaluate the data at a significance level of $p < 0.05$.

Results

Genome wide identification of the *Hsp70* and *Hsp100* gene family members in *Z. jujuba*

A total of 21 *Hsp70* and 6 *Hsp100* unique full-length genes were identified in *Z. jujuba* genome, which were designated as *ZjHsp70*-1 to *ZjHsp70*-21 and *ZjHsp100*-1 to *ZjHsp100*-6. The CDS sequences of *ZjHsp70* and *ZjHsp100* genes ranged from 1448 to 2754 base pairs and 2427 to 2901 base pairs, respectively, with predicted protein lengths ranging from 481 to 917 and 808 to 966 amino acids with varying sub-cellular locations predicted (Table 1 and Table S1). The majority of ZjHsp70s were predicted to be localised to the cytoplasm. The subcellular locations of ZjHsp100s were more varied with three localised to the cytoplasm, two in the chloroplasts, and one to the mitochondrion.

Physio-biochemical properties

Results indicated that there were significant variations among ZjHsp70s and ZjHsp100s proteins in physical and chemical properties (Table S1). As per the instability index, 4 out of 21 ZjHsp70s and 3 out of 6 ZjHSsp100s were considered as unstable proteins (ZjHsp70-1, ZjHsp70-2, ZjHsp70-14, ZjHsp70-17, ZjHsp100-3, ZjHsp100-5, and ZjHsp100-6), (Instability index > 40.4). The aliphatic index is a feature of

Table 1 Features of *Hsp70* and *Hsp100* in *Z. jujuba*

Name	Locus ID	Chr	CDS (bp)	Exon	No. of aa	MW (kDa)	Subcellular localization
Hsp70-1	zju:107432045	1	2310	11	769	85.92	Nuclear
Hsp70-2	zju:107405039	1	2571	10	856	94.34	Cytoplasmic
Hsp70-3	zju:107406481	1	1968	1	655	71.85	Cytoplasmic
Hsp70-4	zju:107405906	1	1956	1	651	71.21	Cytoplasmic
Hsp70-5	zju:107426626	1	1965	1	654	71.74	Chloroplast
Hsp70-6	zju:107412565	2	2139	8	712	75.88	Cytoplasmic
Hsp70-7	zju:107411081	2	2133	3	710	78.27	Cytoplasmic
Hsp70-8	zju:107411863	2	1965	6	654	72.53	ER
Hsp70-9	zju:107411934	2	1977	6	658	73.41	ER
Hsp70-10	zju:107411076	2	1953	2	650	71.18	Nuclear
Hsp70-11	zju:107413527	3	1947	2	648	70.80	Cytoplasmic
Hsp70-12	zju:107413482	3	2487	3	828	92.32	ER
Hsp70-13	zju:107413529	3	1965	2	654	71.51	Cytoplasmic
Hsp70-14	zju:107416864	4	2754	15	917	101.49	Cytoplasmic
Hsp70-15	zju:107423197	7	1719	3	572	61.85	Cytoplasmic
Hsp70-16	zju:107426507	9	1659	3	552	62.94	Cytoplasmic
Hsp70-17	zju:107426748	9	2049	6	682	73.38	Mitochondrial
Hsp70-18	zju:107429814	11	2001	9	666	73.36	ER
Hsp70-19	zju:107431116	11	1941	2	646	70.82	Cytoplasmic
Hsp70-20	zju:107407469	u	1770	6	589	65.05	Cytoplasmic
Hsp70-21	zju:107408891	u	1448	3	481	53.61	ER
Hsp100-1	zju:107422833	1	2793	10	930	104.81	Cytoplasmic
Hsp100-2	zju:107415872	4	2637	9	878	99.08	Cytoplasmic
Hsp100-3	zju:107419106	5	2733	7	910	101.39	Cytoplasmic
Hsp100-4	zju:107420312	6	2466	9	822	91.40	Mitochondrial
Hsp100-5	zju:107423408	7	2901	12	966	105.97	Chloroplast
Hsp100-6	zju:107403384	u	2427	8	808	89.49	Chloroplast

thermostability, and ZjHsp70-15 and ZjHsp100-3 had the highest aliphatic index of 102.6 and 96.24, respectively.

Analyses of phylogenetic relationship, gene structure, motifs, and domain architecture

Based on sequence divergence the *Z. jujuba*, Hsp70 and ZjHsp100 gene family members were grouped into seven and three sub-groups respectively (Fig. 1a). Group I and VII were clustered in the Hsp110/SSE subfamily, with the group I members located in the ER, and cytoplasm and group VII members located in cytoplasm and nucleus. Group II and VI were clustered in the DnaK subfamily and located in both the cytoplasm and nucleus. Members of group III were located in cytoplasm. Group IV members were also located in the cytoplasm and ER. To get detailed information on structural variations among the *Hsp70s* and *Hsp100s* genes in *Z. jujiba*, we analysed the intron/exon structure. The length and number of introns in all *ZjHsp70* and *ZjHsp100* genes were observed to differ from 0 to 13 and 5 to 11, respectively (Fig. 1b). The conserved motifs of *ZjHsp70* and *ZjHsp100* genes varied considerably between family members (Fig. 1c, Table S4). The type, order, and number of motifs were found to be similar in the proteins within a single sub-family and different in the proteins between sub-families..

The result of in silico domain identification revealed that all the *ZjHsp70* proteins possess the conserved Hsp70 (PF00012.17) and MreB_Mbl (MreB/Mbl protein) domains. However, the MreB_Mbl domain was absent in ZjHsp70-14 and ZjHsp70-15 proteins, and interestingly ZjHsp70-16 has two Hsp70 and MreB_Mbl domains (Table S5). Similarly, all ZjHsp100 class proteins possessed the conserved domains belonging to Clp_N (Clp amino-terminal domain, pathogenicity island component), AAA (ATPase family associated with various cellular activities), AAA_2 (Cdc48 subfamily), and ClpB_D2-small (C-terminal, D2-small domain, of ClpB protein) domain. However, ZjHsp100-2 and ZjHsp100-4 lack the ClpB_D2-small domain.

Identification and occurrence patterns of different SSRs

A total of 13 and 3 perfect SSR loci (ranging in size from 20 to 30 bp) were identified within *ZjHsp70* and *ZjHsp100* genes (Table 2) using SSR locator software. In case of *ZjHsp70*, SSRs were divided into five types composed of mono-, di-, tri-, tetra-, and hexa-motifs. However, in *ZjHsp100*, only mono- and di- types of SSR motifs were found. The highest number of SSR loci was located in introns (56%) followed by 5′ UTR (38%), with only one SSR loci identified in the 3′ UTR.

Comparative analysis of the ZjHsp70s and ZjHsp100s in *Z.jujuba*, *A. thaliana*, *C. sativa*, and *R. rubrinervis*

Comparative genomics allows the analysis of the same protein families between diverse plant species. In the present study, a

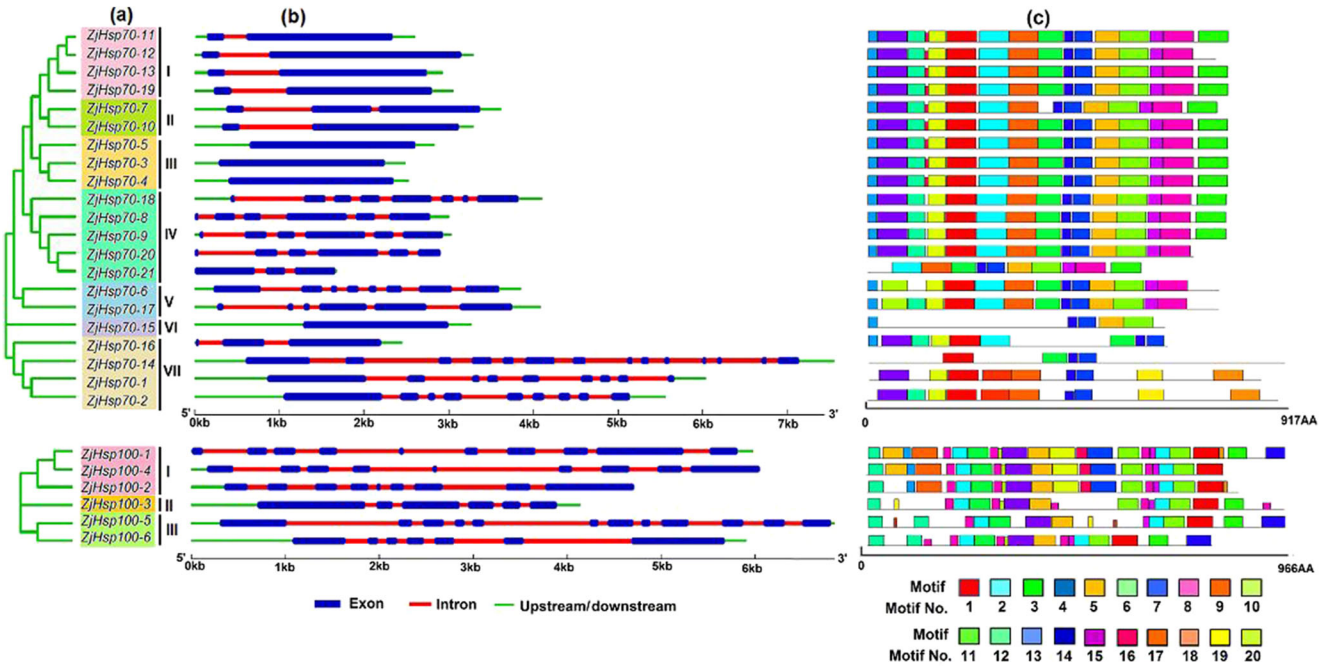

Fig. 1 Phylogenetic relationships, gene structure and conserved motifs analysis in the ZjHsp70 and ZjHsp100 family protein in *Z. jujuba.* **a** The unrooted phylogenetic tree shows sub-families of *ZjHsp70s* (I–VII) and *ZjHsp100s* (I–III) are highlighted with different coloured backgrounds. **b** Gene structure analysis of *ZjHsp70* and *ZjHsp100* gene family members. The sizes of the exons and introns can be estimated using the scale given at the bottom. **c** Conserved motif analysis of the ZjHsp70 and ZjHsp100 family protein in *Z. jujuba.* Each coloured box represents a motif in each of the ZjHsp70 and ZjHsp100 proteins

Table 2 SSRs loci identified in *Hsp70* and *Hsp100* genes in *Z. jujuba*

S no.	Name	SSR type	SSR start	SSR end	SSR length (bp)	SSR position
1	*ZjHsp70-1*	(T)22	354	376	22	5′ UTR
2	*ZjHsp70-1*	(T)22	2368	2390	22	Intron
3	*ZjHsp70-2*	(T)20	3893	3913	20	Intron
4	*ZjHsp70-6*	(A)20	161	181	20	5′ UTR
5	*ZjHsp70-8*	(T)20	201	221	20	Intron
6	*ZjHsp70-14*	(T)26	405	431	26	5′ UTR
7	*ZjHsp70-14*	(T)20	460	480	20	5′ UTR
8	*ZjHsp70-15*	(ATTTTT)4	3194	3218	24	3′ UTR
9	*ZjHsp70-17*	(TCT)8	569	593	24	Intron
10	*ZjHsp70-17*	(TTTA)5	694	714	20	Intron
11	*ZjHsp70-18*	(T)20	3845	3865	20	5′ UTR
12	*ZjHsp70-20*	(TA)14	239	267	28	Intron
13	*ZjHsp70-20*	(AT)12	1054	1078	24	Intron
14	*ZjHsp100-2*	(TG)15	612	642	30	Intron
15	*ZjHsp100-4*	(A)22	24	46	22	5′ UTR
16	*ZjHsp100-6*	(TC)13	1712	1738	26	Intron

phylogenetic relationship of ZjHsp70s and ZjHsp100s was studied with the orthologous protein sequences from *A. thaliana*, *C. sativa*, and *R. rubrinervis* (Figs. 2 and 3). All the Hsp70s were divided into four groups (I, II, III, IV), with group I members in the majority with 26 genes. Groups II, III, and IV contained 13, 11, and 16 members respectively. The Hsp100s were divided into 3 groups. Group I was the largest, containing 14 genes. Group II included 5 members and group III consisted of 4 members (Table S6). The combined phylogenetic tree further identified the paralogous and orthologous relationships between the *Hsp70* and *Hsp100* gene family members. Mainly the paralogous pairs of each species contained duplicated genes, indicating the events of species-specific *Hsp70* and *Hsp100* gene duplication. Ten pairs of

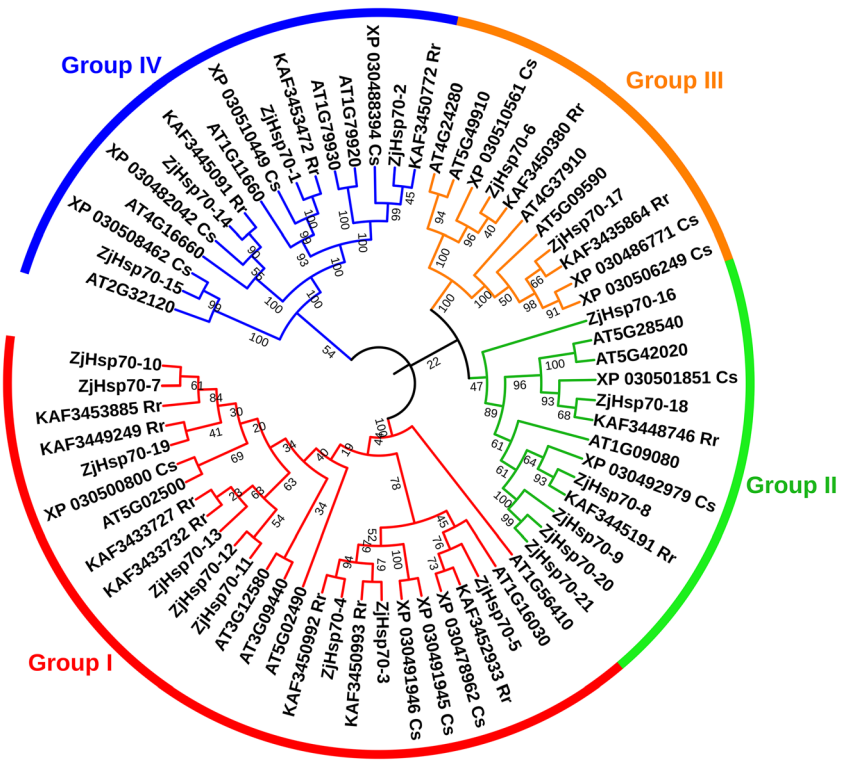

Fig. 2 Phylogenetic relationship analysis of ZjHsp70 with its orthologs. Phylogenetic tree constructed by using amino acid sequences of *Z. jujuba*, *A. thaliana*, *R. rubrinervis*, and *C. sativa*. The red, green, orange, and blue colour of each node represents group I, group III, group III, and group IV respectively

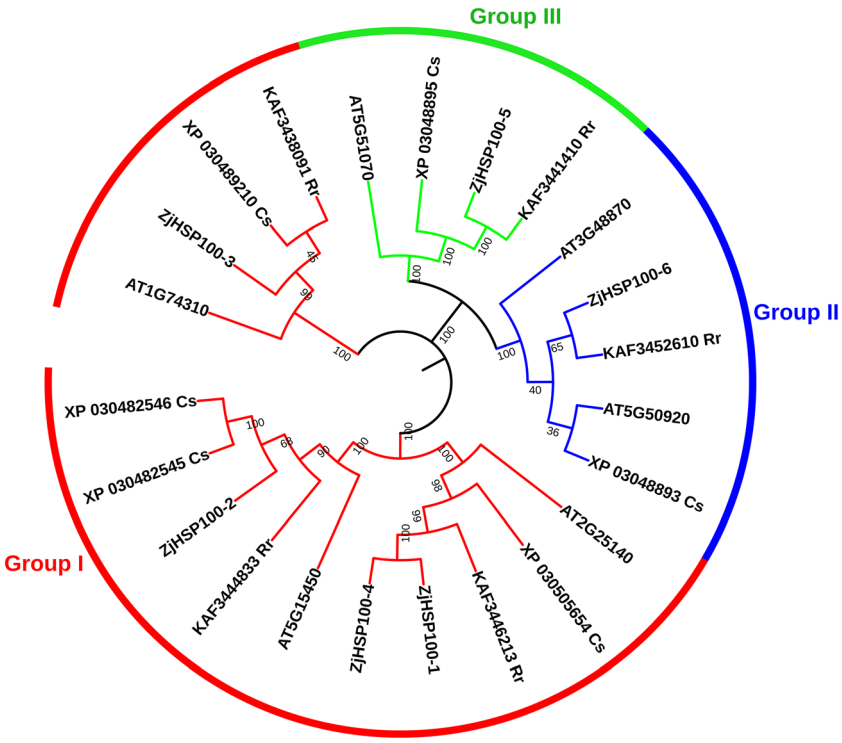

Fig. 3 Phylogenetic analysis of ZjHsp100 with its orthologs. Phylogenetic tree constructed by using amino acid sequences of *Z. jujuba*, *A. thaliana*, *R. rubrinervis*, and *C. sativa*. The red, blue, and green colour of each node represents group I, group III, and group III respectively

paralogous genes *Hsp70* were identified. A total of 13 pairs of orthologous genes derived from a common ancestral gene have been identified in different plant species, with the highest numbers (10) identified in *Z. jujuba* and *R. rubrinervis*. In the case of *ZjHsp100*, one pair of paralogous genes and a total of 5 pairs of orthologous genes were identified.

Chromosomal locations and gene duplication

Among the 12 chromosomes of *Z. jujuba* genome, the *Hsp70* and *Hsp100* genes were unevenly dispersed across a total of 7 and 5 chromosomes respectively (Fig. 4). The uneven distribution of *ZjHsp70* and *ZjHsp100* genes indicates a genetic divergence in the process of evolution. The results illustrated that there were three and one paralogous pairs observed in the *ZjHsp70* and *ZjHsp100* gene family respectively (Figs. 2 and 3). One of the three *ZjHsp70* paralog gene pairs, *ZjHsp70-7:ZjHsp70-10*, was identified as tandem duplication found on chromosome 2 in a head-to-tail orientation in 24.36 kb and no other genes were present in between them. In addition, a segmental duplication was observed in the paralogous pair *ZjHsp70-11:ZjHsp70-12* as they were separated by 381.19 kb and present in tail-to-head orientation on chromosome 3. Similarly, the second segmental duplication in the paralogs *ZjHsp100-1:ZjHsp100-4* was also separated by a large distance of 13,138 kb, with *ZjHsp100-1* on chromosome 1 segmentally linked to *ZjHsp100-4* on chromosome 6. These results revealed that segmental duplication has played an important role in the expansion of the *Hsp70* and *Hsp100* gene family in *Z. jujuba.* Due to the lack of chromosomal location data of the paralogous pair *ZjHsp70*-20:*ZjHsp70*-21, the exact type of duplication could not be determined.

Promoter analysis

In order to identify the upstream cis-regulatory elements of *Hsp70* and *Hsp100* genes in *Z. jujuba*, the upstream regulatory regions (URRs) were analysed using the Plant Place tool (Table S7). The analysis showed the presence of 35 and 33 types of different cis-regulatory elements was situated in *Hsp70* and *Hsp100* genes of *Z. jujuba* respectively. Among them, many cis-regulatory elements were gene-specific. The identified cis-regulatory elements were grouped into three categories based on their different roles, as putative abiotic stress-responsive, hormone-responsive, and light-responsive elements.

In the URRs of the *ZjHsp70* gene family, abiotic stress-responsive cis-regulatory elements including heat stress-responsive, dehydration-responsive, salt stress-responsive elements, plant growth and development, and stress response were prominent. The gibberellin responsive elements, cytokinin response regulator (RR) binding motif, abscisic acid-responsive element, salicylic acid (SA)-induced elements, were the major hormonal responsive cis-regulatory elements observed. The URRs of *Hsp70-1*, *Hsp70-3*, *Hsp70-5*, *Hsp70-6*, *Hsp70-11*, *Hsp70-13*, *Hsp70-15*, *Hsp70-16*, and *Hsp70-18* genes contained the highest numbers of nearly all identified cis-regulatory

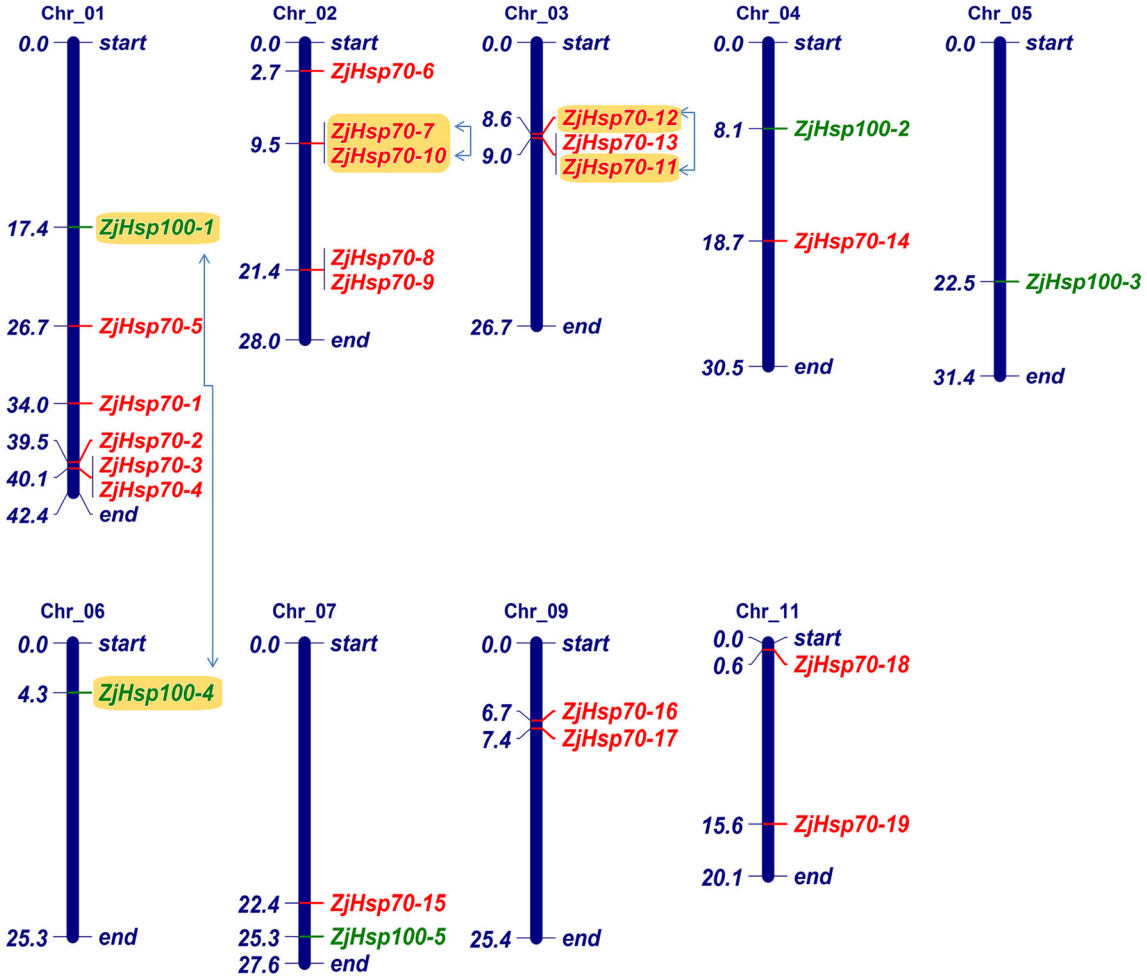

Fig. 4 Chromosomal locations and duplications of *ZjHsp70* and *ZjHsp100* gene family members on *Z jujuba* chromosomes. The red and green colouring indicates *ZjHsp70* and *ZjHsp100* genes, respectively. Segmentally duplicated genes are indicated by blue arrow; Tandemly duplicated *ZjHsp* genes are indicated by yellow boxes

elements. The URRs of the *Hsp100* gene family of *Z. jujuba* contained prominent abiotic stress-responsive, light-responsive, and hormonal responsive cis-regulatory elements as found in the *Hsp70* gene family. The URRs of *ZjHsp100-1*, *ZjHsp100-5*, and *ZjHsp100-6* genes contained the highest numbers of nearly all identified cis-regulatory elements. This in silico analysis of cis-regulatory elements indicated that these genes are likely involved in diverse transcriptional regulatory functions.

Expression analysis of *ZjHsp70* and *ZjHsp100* genes under abiotic stress conditions

In the present analysis, to get insight into which *ZjHsp70* and *ZjHsp100* genes are potentially involved in the response to abiotic stress conditions, the expression profiles of all *ZjHsp70* and *ZjHsp100* genes were analysed using acute stress experiments, as previously used in Liu et al. 2018, Singh et al. 2016, Mulaudzi-Masuku et al. 2015, to provide a comparative analysis. Expression levels of *ZjHsp70-3*, *ZjHsp70-5*, *ZjHsp70-6*, *ZjHsp70-16*, *ZjHsp70-17*, and *ZjHsp70-20* were upregulated in response to three of the abiotic stresses exposed (Fig. 5), with *ZjHsp70-1*, *ZjHsp70-8*, and *ZjHsp70-12* upregulated in response to the salinity and drought stress conditions. *jHsp70-4*, *ZjHsp70-10*, and *ZjHsp70-14* gene were observed to be upregulated in response to the drought stress conditions, whilst *ZjHsp70-19* was observed to be upregulated in heat and salinity and downregulated in drought stress. Among the *ZjHsp100* genes, *ZjHsp100-1*, *ZjHsp100-3*, *ZjHsp100-4*, and *ZjHsp100-6* were upregulated in response to heat stress (Fig. 5) and *ZjHsp100-2* and *ZjHsp100-3* upregulated in response to drought stress. Interestingly, *ZjHsp100-1* and *ZjHsp100-4* genes were upregulated but *ZjHsp100-2*, *ZjHsp100-3*, *ZjHsp100-5*, and *ZjHsp100-6* genes were downregulated in response to salinity stress condition. These results demonstrated that *ZjHsp70* and *Hsp100* may potentially play collective roles in abiotic stress tolerance.

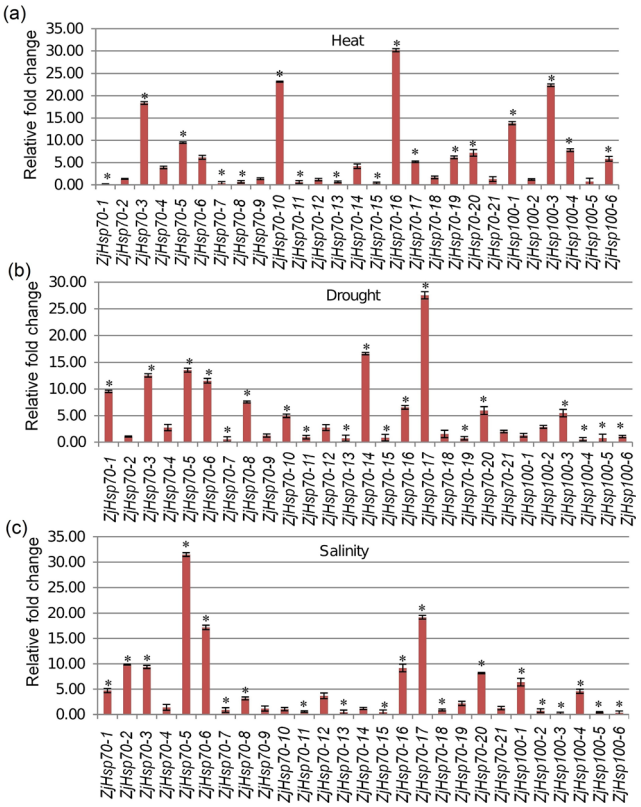

Fig. 5 Expression analyses of *ZjHsp70* and *ZjHsp100* gene family members under heat (**a**), drought (**b**), and salinity (**c**) stress conditions. Statistical significance within genes was indicated with an asterisk. Statistical significance was indicated with an asterisk ($P < 0.05$). Error bars represents ± SD from three technical replicates

Discussion

Ziziphus species have significant abiotic stress-tolerant abilities; suggesting they may be potential repositories of genes responsible for abiotic stress tolerance. Therefore, we performed in silico analysis to characterise *Hsp70* and *Hsp100* gene families in *Z. jujuba* and also analysed gene expression of candidate *Hsps* to identify potential stress-responsive genes, which could assist the bio-prospecting of genes via expression study and further functional analysis.

We identified 21 and 6 members of the *Hsp70* and *Hsp100* gene family in *Z. jujuba* respectively. The number of identified genes in *Z. jujuba* is comparatively less than rice (32 Hsp70, 9 Hsp100), cotton (30 Hsp70), and *A. thaliana* (18 Hsp70, 6 Hsp90) (Lin et al. 2001; Jung et al. 2013; Zhang et al. 2014). This might be because all of the genomes of these other species contain more predicted genes (i.e. cotton 41,330, 2287 Mb (Yang et al. 2020), *M. domestica* 15,345, 690 Mb (Scott et al. 2014), rice 49,066, 430 Mb (Mustafiz et al. 2016) as compared to *Z. jujuba* (32,808, 437.65 Mb) (Liu et al. 2014). The ZjHsp70 and ZjHsp100 family proteins have diverse biophysical properties. The Hsp70 and Hsp100 in *Z. jujuba* comprised a multi-genic family and different members were localised in different cellular organelles, as has been identified previously in other species such as *S. tuberosum*, *N. tobacum*, and *S. italica* (Liu et al. 2018; Song et al. 2019; Singh et al. 2016; Lee et al. 2007). This indicated the different patterns of organelle localization may be associated with particular molecular mechanisms in relation to abiotic stress tolerance.

The instability index indicates the in silico stability of protein, and 17 out of 21 *Z. jujuba* proteins were found to be stable proteins (Gonzalez-Diaz et al. 2007). The single ZjHsp70-6 protein localised in chloroplasts was the most stable among the 17 stable proteins, which suggested their potential role in maintaining homeostasis of photosystem II under stress condition (Su and Li 2010). The role of members situated in the chloroplast mostly includes contributions to the formation of thylakoid membrane, maintaining the normal process of photosystem II under heat stress, and assisting in protein translocation (Liu et al. 2007; Zhong et al. 2013). A similar result was observed in *N. tobacum* where members localised in the chloroplast (NtHsp70-46 to NtHsp70-49) were stable proteins (Song et al. 2019). The *ZjHsp70* genes were unevenly situated on chromosomes, generally near to both ends of the chromosomes (Fig. 4), similar to *Hsp70* genes mapped in *N. tabacum* and *A. thaliana* (Song et al. 2019). The *ZjHsp100* genes were randomly present associated across 5 different chromosomes across the genome. Gene duplication events of entire genomes, chromosomal segments, and individual genes play a role in the expansion of gene family members (Cannon et al. 2004; Maere et al. 2005). These events also contribute to the evolution of novel gene functions and potentially play an important role enhancing plant environmental adaptability (Huang et al. 2015). We identified 3 and 1 pairs of *ZjHsp70* and *ZjHsp100* respectively that have undergone tandem and segmental gene duplication, indicating the importance of these events in the amplification of the *ZjHsp* gene family members.

Furthermore, we analysed the gene structures of *Hsp70* and *Hsp100* gene families. Their intron-exon organization was highly similar within the sub-families, as defined by the phylogenetic analyses (Fig. 1a). For example, in sub-family I, all of the *ZjHsp70* members had a single intron, whilst in sub-family III, no introns were observed. These observations indicate that some introns were lost and gained during the structural evolution between the *ZjHsp70* genes, as previously identified in *S. tuberosum* (Liu et al. 2018; Zhang et al. 2015a, b). This variation in intron/exon structures between the different gene family groups provides the possibility for alternative splicing events, which could potentially increase the diversity of gene function (Barbazuk et al. 2008). In the motif study, the number, type, and order of motifs in the ZjHsp70 and ZjHsp100 proteins between the sub-families were also similar, but differed between the proteins from the other sub-families.

Large numbers of SSRs are distributed in eukaryotic genomes and may play an important role in gene regulation (Gao et al. 2011; Xue et al. 2018). SSR markers have been used in marker-assisted selection to enhance the efficiency of heat stress tolerance plant breeding in various crops including barley, potato, and rice (Jha et al. 2014; Shamsudin et al. 2016). In this study, a total of 16 perfect potential SSRs were mined from both *ZjHsp70* and *ZjHsp100* genes. Most SSRs were predicted in the intergenic region (5′ UTRs and 3′ UTRs). Similar observations were reported in the previous studies in *Brassica rapa* (Hong et al. 2007), *Rosaceae* species (Moe et al. 2011), rice, and *A. thaliana* (Fujimori et al. 2003; Lawson and Zhang 2006).

A comparative phylogenetic tree was constructed to obtain insight into the evolutionary relationships of Hsps and to identify paralogs within species and orthologs between species. We used the amino acid sequences of Hsp70 and Hsp100 genes from *C. sativa* and *R. rubrinervis* as a close relatives and sequences from *A. thaliana* as an out group. Based on the phylogenetic tree, ZjHsp70s and ZjHsp100 could be divided into four and three groups respectively based on their sequence relatedness (Liu et al. 2018; Chaudhary et al. 2019; Singh et al. 2016). A total of 10 pairs of paralogs in the Hsp70s were identified which included 3 pairs from *Z. jujuba*, 4 pairs from *A. thaliana*, 2 pairs from *C. sativa*, and 1 pair from *R. rubrinervis*. In the case of ZjHsp100s, 1 pair of paralogous genes from *Z. jujuba* and 1 pair from *C. sativa* were identified. This implies that the expansion of these gene families in each species was species-specific approach (Liu et al. 2018). The cis-regulatory elements in promoter regions play an important role in the functional divergence of the *Hsp* genes. Analysis of the cis-regulatory element showed three types of cis-regulatory elements, including hormonal responsive elements, stress-responsive elements, and light-responsive elements (Song et al. 2019). Among the majority of *Hsp70* and *Hsp100* promoter sequences, cis-regulatory elements related to abiotic stress were identified (Song et al. 2019). In the recent past, Song et al. (2019) also reported these three types of cis-regulatory elements in *N. tabacum*. For instance, the CCAATBOX1, LTRE1HVBLT49, and LTRECOREATCOR15 contribute to metabolic regulation of heat stress (Deng et al. 2018). The MYCCONSENSUSAT and MYBCORE contribute to metabolic regulation of drought stress and GT1GMSCAM4 contributes to metabolic regulation of salinity stress (Senavirathne et al. 2017). However, the DOFCOREZM motif is the most overrepresented cis-element in both *Hsp70* and *Hsp100* gene family involved in plant metabolism and drought condition (Corrales et al. 2014). Similarly the overrepresented DOFCOREZM cis-element was observed in the Hsp70 of *P. patens* a high dehydration tolerant plant, which can be recover even after dehydration to 92% loss of fresh weight (Tang et al. 2016). Due to the presence of diverse abiotic stress-responsive cis-elements, *ZjHsp70* and *ZjHsp100* could play significant roles in response to diverse abiotic stresses. Hence, the present investigation offers a theoretical foundation for further studies of the actual mechanism of action (Song et al. 2019). Nonetheless, no correlation between the cis-regulatory elements of the promoter sequences of *ZjHsp* and the expression pattern of genes was observed. This may be due to several reasons. Firstly, it may be due to the spatio-temporal pattern of gene expression during the growth and development of plants. Secondly, there may be unidentified cis-regulatory elements which contribute to the diversity of the expression patterns observed, or finally, it may be due to the particular nature (level and duration) of the stress challenge (Song et al. 2019).

Previous analysis has also reported that *Hsp70* and *Hsp100* genes play important roles under abiotic stress conditions including heat, drought, and salinity in other plants (Chaudhary et al. 2019; Liu et al. 2018; Singh et al. 2016; Mulaudzi-Masuku et al. 2015). In this study, the expression profiles of the *ZjHsp70* and *ZjHsp100* genes in response to heat, salinity, and drought stress were analysed (Fig. 5). The results showed that most of the *ZjHsp70* and *ZjHsp100* genes were induced by abiotic stresses. Not all *Hsp70s* have the ability to increase the stress tolerance of plants, some are intimately involved in the development of plants (Noel et al. 2007), which would not be detected in this study.

Conclusion

In the present study, a total of 21 and 6 *Hsp70* and *Hsp100* gene family members were identified in *Z. jujuba* using *an* in silico approach. Analysis of their gene distributions, genomic organization, gene structures, and protein structures suggested a complex evolutionary history for these two Hsp families. Segmental and tandem duplications were identified suggesting their role in the expansion of *ZjHsp70* and *ZjHsp100* gene families. Based on promoter analysis, the results indicated that *ZjHsp70s* and *ZjHsp100s* were involved in abiotic stress tolerance and development, which was validated in certain genes by expression analysis in response to heat, drought, and salinity. Thus, the present investigation suggested that *ZjHsp70-3*, *ZjHsp70-5*, *ZjHsp70-6*, *ZjHsp70-16*, *ZjHsp70-17*, *ZjHsp70-20*, *ZjHsp100-1*, *ZjHsp100-2*, and *ZjHsp100-3* are potential candidate genes for further functional analysis in crop improvement research.

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Authors' contributions KPP conceived, designed experiments, performed the most of analysis, experiments, wrote and revised the manuscript. SSK conducted the analysis and help in manuscript writing. NRC and BH also help in manuscript writing.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval N/A

Consent to participate N/A

Consent for publication N/A

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