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Expression analysis of the apple *HSP70* gene family in abiotic stress and phytohormones and expression validation of candidate *MdHSP70* genes

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Heat shock protein 70 (HSP70) is one kind of molecular chaperones which are widely found in organisms, and its members are highly conserved among each other, with important roles in plant growth and development. In this study, 56 *HSP70* genes were identified from the apple genome database. Analysis of gene duplication events showed that tandem and segmental duplication events play an important role in promoting the amplification of the *MdHSP70* gene family. Collinearity analysis showed that *HSP70* family members of apple were more closely related to *HSP70* family members of Arabidopsis, tomato and soybean. The promoter region of the apple *HSP70* genes contains a large number of *cis*-acting elements in response to hormones and stress. Tissue-specific expression analysis showed that some of the genes were associated with various stages of the apple growth process. Codon preference analysis showed small differences between codon bases 1 and 3 in the apple *HSP70* genome, and the codon base composition had a small effect on codon usage preference. The multiple expression patterns of the *MdHSP70* gene suggested that *MdHSP70* gene members play important roles in growth and development and in response to hormonal and abiotic stresses. The yeast two-hybrid (Y2H) demonstrated that MdHSP70-53 interacts with MdDVH24_032563. The qRT-PCR analysis showed that most *MdHSP70* members' hormonal and abiotic stresses (*MdHSP70-6*, *MdHSP70-26* and *MdHSP70-45*) appeared to be highly expressed. To further elucidate the function of MdHSP70 (6, 26, 45), we introduced them into tobacco to confirm subcellular locations and noted that these genes are located in the cytoplasm and cell membrane. This study serves as a theoretical basis for further studies of the *MdHSP70* gene and helps to further investigate the functional characterization of *MdHSP70* gene.

Keywords Apple, *HSP70*, Phytohormones, Abiotic stress, Bioinformatics, Subcellular proteins

Plants are often subjected to a variety of adverse environmental influences such as high temperature, drought, cold damage, heavy metals and pathogens during their growth and development. When subjected to these adverse stimuli, plants employ a wide range of defense mechanisms at the morphological, biochemical, physiological, cellular or molecular levels to adapt their organisms to the environmental influences to a certain extent in order to improve their survivability[1,2]. Heat shock proteins (HSPs), as the most common molecular components of stress-related genes, are triggered and up-regulated for expression when plants are exposed to almost all types of stresses thereby protecting the organisms from injury[3–5]. HSP plays an important role in assisting de novo protein modification[6], intracellular protein transport[7], immune complex formation and breakdown and redundant protein degradation[8,9]. According to the molecular weight size, HSP can be categorized into five major families: HSP100, HSP90, HSP70, HSP60 and small molecule HSP[10]. HSP70 and HSP90 proteins are responsible for the folding of other proteins in the cell, with HSP70 binding unnaturally occurring proteins, while HSP90 binds substrates that are usually naturally occurring proteins[11]. In eukaryotes, the interrelationship between HSP70 and HSP90 involves the transfer of folded proteins from HSP70 to HSP90 proteins[12]. The two

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molecular chaperones may functionally interact with each other through PfHop-mediated pathways, with HSP70 being one of the most widely studied heat shock proteins[13].

HSP70 and its homologs consist of a 45 kDa N-terminal ATPase structural domain and a 25 kDa C-terminal substrate-binding structural domain, which is further subdivided into a 15 kDa β-sandwich subdomain and a C-terminal α-helix subdomain[14]. The subcellular localization of HSP70 was complex and could be present in various organelles such as the cytosol, cell wall, cytoplasm, nucleus, chloroplasts, cell membrane and mitochondria[15–22]. Different subcellularly localized HSP70 proteins play different important functions during plant growth and development. For example, mitochondria in the Arabidopsis *mthsc70-1* mutant exhibit abnormalities and altered respiratory systems, which is achieved by inhibiting the cytochrome c oxidase (COX) pathway and activating an alternative respiratory pathway, thereby inhibiting normal growth in Arabidopsis[23]. The HSP70 chaperone protein, localized in the cytoplasm, helps to target misfolded cytoplasmic proteins to the ubiquitin ligase San1p and promote degradation[24]; ohta et al. found that *OsERdj7* is involved in translocation of some seed proteins (SSPs) in the endoplasmic reticulum as well as degradation of misfolded or unfolded proteins, which contributes to the maintenance of protein quality controlling in the endosperm endoplasmic reticulum of rice (*Oryza sativa*)[25]. Mitochondrial *HSP70* expression was up-regulated during salt-induced programmed cell death (PCD) in rice root tip cells, which may inhibit temperature and H_2O_2 ion-induced PCD in rice protoplasts by maintaining mitochondrial membrane potential ($\Delta\psi$) and inhibiting the amplification of reactive oxygen species (ROS)[26]. This is mainly in terms of increased cell viability, reduced DNA denaturation gradient and chromatin condensation[27].

Members of the *HSP70* gene family have now been characterized in many plants, and their response to abiotic and biotic stresses has been well studied in many plants. Date palm (*Ziziphus jujuba*) contains 21 *HSP70* genes, with *ZjHSP70-4*, *ZjHSP70-10*, and *ZjHSP70-14* expression up-regulated under drought conditions, and *ZjHSP70-19* expression up-regulated under high temperature and salt stresses[28]; in the 61 *Hsp70* gene family members of tobacco (*Nicotiana tabacum*), most of the tobacco *HSP70* gene promoter sequences (40/61 *NtHSP70* genes) contain *cis*-regulatory elements associated with abiotic stress. For example, ETH mediated wound signal transmission to prevent tissue necrosis, abscisic acid (ABA) is involved in the metabolic regulation of drought stress, and salicylic acid (SA) plays an important role in the invasion of pathogenic microorganisms and enhances the resistance of tobacco to Mosaic disease[29]. Among the 20 *StHsp70* genes identified by potato (*Solanum tuberosum*), *StHSP-7* is highly expressed under drought and stress conditions, *StHsp-11* is expressed under salt and drought stress, and *StHSP-16* is expressed under drought and low temperature stress, *StHSP-20* was significantly up-regulated and expressed under drought, high temperature and low-temperature stresses, *StHSP-7* was expressed at higher levels under ABA and Gibberellic acid (GA_3) treatments, and *StHSP-17* was up-regulated under ABA, GA_3 and SA treatments[30]; In addition to being associated with plant stress tolerance, Hsp70 is also an important protein indispensable for plant growth and development. *AtHSP70-15* might be a key factor in the correct folding of cytosolic proteins, and silencing of the *AtHSP70-15* gene resulted in the resultant plant mutants showing significant inhibition of growth and even severe wilting when compared to the control plants[31]; *Δcphsc70-1* mutant plants exhibit heterochromatic cotyledons, misshapen leaves, growth retardation, and impaired root growth, and *Δcphsc70-1* and *Δcphsc70-2* double-knockout mutants are lethal to female gametes and reduce the efficiency of male gamete transmission[32]. Besides, it has also been found that heat treatment promotes plant infection with different RNA viruses (TMV, PVX, CMV and WMV), heterologous expression of *AtHSC70-3* enhances viral infection in tobacco, and silencing of *NbHSP70c-1* inhibits viral proliferation and motility, suggesting that *HSP70* exerts a positive regulatory role in virus accumulation or motility[33].

The apple (*Malus domestica*) is a major fruit grown in temperate regions around the world[34]. It is a major source of flavonoids in the human diet and is one of the most nutritious and widely consumed fruits in the world[35]. Consumption of apples may reduce the risks of diabetes, heart disease, cancer and asthma[36]. Therefore apples have a very important role both as a cash crop and for medicinal purposes. As one of China's main economic fruits, Northwest China is an advantageous production area for apples due to its favorable climatic conditions. But drought and salt stress affected the normal physiological metabolism of apples, slowed down growth and development, and even caused plant death, limiting the development of China's apple industry. Enhancing the ability of apples to cope with abiotic stresses through genetic engineering techniques is important for improving apple traits as well as increasing yields. Although the gene family has been identified in multiple species by previous authors, there is no systematic analysis of the evolutionary relationships of the *HSP70* gene family in monocotyledonous and dicotyledonous plants, and there are fewer studies on the function of the *HSP70* gene family in apple. In this study, we identified the *HSP70* gene family in the apple genome, analyzed its expression pattern under methyl jasmonate (MeJA), GA_3, ABA, and salt, drought, and low-temperature stresses, and screened for genes responsive to the stresses. Meanwhile, phylogenetic analyses of four monocotyledonous (millet, maize, rice, sorghum) and five dicotyledonous (apple, soybean, tomato, Arabidopsis, grape) plant *HSP70* gene families, providing new insights into the evolution of the *HSP70* gene family.

Result
MdHSP70 gene family identification and sequence analysis
A total of 56 apple *HSP70* family genes were identified using Arabidopsis amino acid sequence alignment, and named *MdHSP70-1* to *MdHSP70-56* based on their chromosomal locations (Fig. 1). They were located on 14 chromosomes, and the maximum number of genes located on Chr1 was 22. At the same time, obvious gene clusters appeared on Chr1, Chr7, Chr9 and Chr12. Analysis of their physicochemical properties showed that the amino acid length of the *MdHSP70* gene family ranged from 58 to 891 aa, with the longest being *MdHSP70-50* and the shortest being *MdHSP70-32*. The pI values ranged from 4.49 to 10.19 and most of them were less than 7. It is presumed that this family is mainly acidic proteins. The relative molecular weights were *MdHSP70-32*
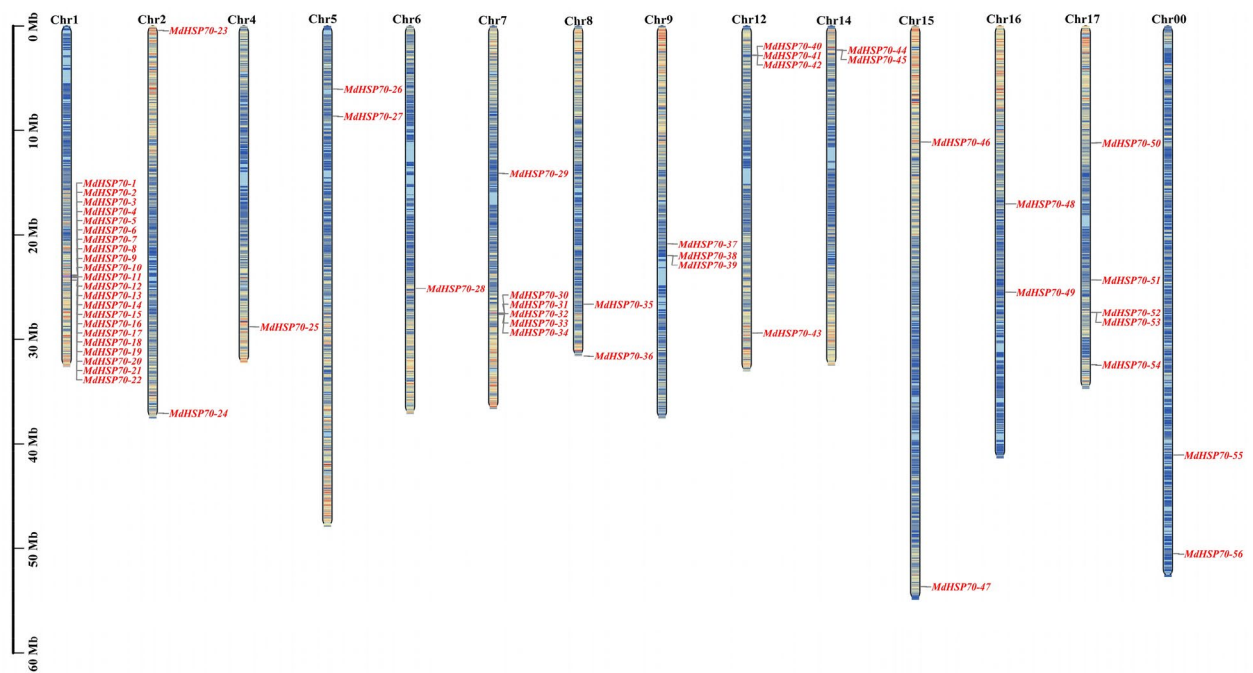
Fig. 1. Distribution of *MdHSP70* genes on different chromosomes. The scale on the left indicates the chromosome length (Mb), and each chromosome is labeled with the *MdHSP70* gene on the right side. Different colors indicate gene density, where red indicates high-density regions and blue indicates low-density regions.

(6691.45) to *MdHSP70-50* (99,483.34), with α-helices ranging from 9.26 to 100%, and β-turns ranging from 0 to 12.24% (Table 1).

Phylogenetic tree analysis of the *MdHSP70* gene family

The results showed (Fig. 2) that all *HSP70* family members could be categorized into seven subfamilies. According to the number of genes contained in each subfamily, The highest number of family V genes was 97, accounting for 35.80% of the total number of genes, and the lowest number of family I genes was 2, accounting for 0.74% of the total number of genes. Family I only contained the tomato *HSP70* gene, and family VI only contained the *MdHSP70* gene. Family III did not contain the Arabidopsis and rice *HSP70* genes, and family VII did not contain the grape, soybean, and tomato *HSP70* genes. Family II, VI, and V contained the nine species studied. Further analysis of their evolutionary relationships revealed that *MdHSP70* family members are more closely related to Arabidopsis *HSP70* family members, tomato *HSP70* family members, and soybean *HSP70* family members; the above results suggest that plant *HSP70s* are evolutionarily distinct, and that *MdHSP70* family members are more closely related to dicotyledonous plants than to monocotyledonous plants.

Covariance analysis of the *MdHSP70* gene family

Among *HSP70* family members in monocotyledon plants, sorghum (31), and maize (25) contained the most and least members, respectively. Among dicotyledon plants, apple (56) and Arabidopsis (16) contained the most and least members, respectively. Therefore, the study of the *HSP70* family in the above plants is helpful to understand the reasons for the expansion of gene numbers.

In the monocotyledon *HSP70* family, millet (Fig. 3A) has 3 pairs of collinear genes, which are distributed on Chr3, Chr5 and Chr9. Since the gene *Sela.5G376100.1* is collinear with the other three genes at the same time, it is speculated that the cross replication of chromosomes in the evolution process leads to the repetition of fragments. Maize (Fig. 3B) has 4 pairs of collinear genes, which are distributed on Chr2, Chr4, Chr5 and Chr6. In rice (Fig. 3C), 4 pairs of collinear genes were distributed on Chr1, Chr3, Chr5, Chr11 and Chr12. *LOC_Os05g38530* and other two genes also showed fragment repetition. Sorghum (Fig. 3D) has one pair of collinear genes, distributed on Chr1 and Chr3. In dicotyledonous *HSP70* family, apple (Fig. 3E) has 4 pairs of collinear genes, which are distributed on Chr1, Chr2, Chr4, Chr7, Chr9, Chr12, Chr15 and Chr17. There are 7 pairs of collinear genes in soybean (Fig. 3F), which are distributed on Chr2, Chr5, Chr7, Chr8, Chr12, Chr13, Chr14, Chr15, Chr16, Chr17 and Chr19. Tomato (Fig. 3G) had 5 pairs of collinear genes, distributed on Chr3, Chr4, Chr6, Chr8, Chr9 and Chr10, and the gene *Solyc06T00041* and *Solyco3T001718* showed fragment repetition. Arabidopsis (Fig. 3H) has 3 pairs of collinear genes, which are distributed on Chr1, Chr3, Chr4 and Chr5. In grape (Fig. 3I), 5 pairs of collinear genes were distributed on ChrUn, Chr3, Chr6, Chr8, Chr9 and Chr13, and fragment repetition occurred in genes *VIT_213s0019g01430*, *VIT_208s0007g00130* and *VIT_206s0004g04510*. It is noteworthy that all nine species studied showed clusters of genes at chromosomal locations. The above results

Gene ID	Gene name	Full length (bp)	Length of CDS (bp)	Length of amino acid (aa)	pI	Molecular weight (kD)	Alpha helix (%)	Beta turn (%)	Random coil (%)
MD01G1126500	MdHSP70-1	2841	1950	649	5.12	71.13568	42.99	7.09	31.74
MD01G1126900	MdHSP70-2	543	543	180	8.64	10.67124	37.22	9.44	34.44
MD01G1127100	MdHSP70-3	449	372	123	5.07	13.6977	12.2	9.76	46.34
MD01G1127200	MdHSP70-4	5388	2508	835	6.54	92.29032	35.93	6.35	36.05
MD01G1127400	MdHSP70-5	2221	1710	569	7.92	62.54622	31.81	7.03	36.91
MD01G1127500	MdHSP70-6	1926	1578	525	6.36	57.77998	34.29	8.00	35.05
MD01G1127600	MdHSP70-7	1357	735	244	6.33	26.5071	31.56	10.25	31.97
MD01G1127700	MdHSP70-8	662	639	212	4.97	22.79504	27.36	8.96	40.57
MD01G1128200	MdHSP70-9	8806	2409	802	6.47	89.82163	39.03	11.97	25.31
MD01G1128400	MdHSP70-10	381	381	126	9.32	14.72206	34.13	3.97	34.92
MD01G1128500	MdHSP70-11	525	525	174	6.09	19.11412	28.16	9.77	35.63
MD01G1128600	MdHSP70-12	956	546	181	5.95	20.44561	35.91	8.29	33.70
MD01G1128700	MdHSP70-13	399	399	132	8.42	14.7001	24.24	6.82	42.42
MD01G1128800	MdHSP70-14	368	327	108	5.1	12.17269	9.26	8.33	46.30
MD01G1129500	MdHSP70-15	1525	1254	417	5.53	45.8121	36.69	10.31	32.37
MD01G1129700	MdHSP70-16	1066	987	328	5.15	36.52553	32.93	8.84	34.76
MD01G1129800	MdHSP70-17	2448	1593	530	6.26	58.42889	34.15	7.92	34.91
MD01G1130000	MdHSP70-18	273	273	90	6.7	9.69345	27.78	11.11	40.00
MD01G1130100	MdHSP70-19	489	489	162	5.01	18.24513	32.72	5.56	39.51
MD01G1130300	MdHSP70-20	928	621	206	7.83	22.6528	30.58	7.77	36.41
MD01G1133300	MdHSP70-21	982	957	318	6.47	35.18613	35.22	8.18	34.28
MD01G1133400	MdHSP70-22	16,618	1557	518	5.52	56.57718	35.91	8.88	32.05
MD02G1005800	MdHSP70-23	1962	1962	653	5.21	71.44106	43.49	7.20	30.32
MD02G1315900	MdHSP70-24	10,382	2466	821	6.24	92.02934	41.66	6.09	37.39
MD04G1201500	MdHSP70-25	3573	942	313	8.49	34.97121	45.69	7.99	30.35
MD05G1038000	MdHSP70-26	591	591	196	7.77	21.34206	31.12	12.24	29.59
MD05G1049700	MdHSP70-27	3148	2031	676	5.79	72.78052	43.49	6.66	30.18
MD06G1113000	MdHSP70-28	4015	2004	667	5.17	73.57629	45.58	6.75	29.84
MD07G1116300	MdHSP70-29	288	288	95	9.35	10.39614	30.53	10.53	26.32
MD07G1196600	MdHSP70-30	3938	861	286	10.19	32.48961	21.33	6.99	46.85
MD07G1196700	MdHSP70-31	2161	1722	573	8.2	63.90818	46.07	7.33	29.84
MD07G1197000	MdHSP70-32	177	177	58	4.91	6.69145	100.00	0.00	0.00
MD07G1197200	MdHSP70-33	1896	1200	399	6.72	44.90254	34.59	8.27	36.34
MD07G1197700	MdHSP70-34	1927	1524	507	5.82	55.57735	31.36	7.30	37.67
MD08G1205600	MdHSP70-35	2854	1782	593	9.06	65.6409	34.23	7.25	36.09
MD08G1250700	MdHSP70-36	231	231	76	9.63	8.2356	44.74	9.21	32.89
MD09G1212600	MdHSP70-37	5301	2568	855	5.17	96.61861	43.51	2.81	39.88
MD09G1218100	MdHSP70-38	360	360	119	4.62	12.75891	77.31	2.52	20.17
MD09G1218200	MdHSP70-39	6860	1128	375	6.95	40.52351	32.53	6.67	37.33
MD12G1025700	MdHSP70-40	3654	1641	546	7.1	60.26688	32.78	8.79	34.43
MD12G1025800	MdHSP70-41	3797	1533	510	5.79	55.90361	30.00	7.84	37.65
MD12G1025900	MdHSP70-42	606	606	201	5.95	22.09036	24.38	7.46	41.79
MD12G1215200	MdHSP70-43	3317	1989	662	5.2	73.48552	44.26	6.80	30.66
MD14G1024500	MdHSP70-44	4803	1542	513	5.66	56.58745	34.11	7.80	33.92
MD14G1024700	MdHSP70-45	1259	948	315	6.45	34.22305	45.71	6.98	33.33
MD15G1150500	MdHSP70-46	1968	1869	622	4.94	68.7431	45.02	6.11	31.67
MD15G1436200	MdHSP70-47	3815	2088	695	5.11	74.07873	39.42	6.62	32.66
MD16G1192600	MdHSP70-48	2673	1749	582	5.42	62.46004	33.51	6.36	38.66
MD16G1239900	MdHSP70-49	792	792	263	8.6	29.51759	48.29	7.98	27.00
MD17G1127600	MdHSP70-50	7612	2676	891	5.47	99.48334	47.70	2.92	37.37
MD17G1201200	MdHSP70-51	3480	2040	679	5.78	72.66439	45.07	6.63	28.42
MD17G1225800	MdHSP70-52	246	222	73	4.49	7.54344	53.42	8.22	35.62
MD17G1226100	MdHSP70-53	1971	1971	656	5.26	72.10454	41.16	6.55	34.60
MD17G1263000	MdHSP70-54	462	462	153	5.12	16.50985	35.95	10.46	32.03
MD00G1176700	MdHSP70-55	876	876	291	6.46	31.71715	29.55	12.03	27.15
MD00G1207500	MdHSP70-56	654	654	217	5.06	23.64796	29.95	7.37	39.17

Table 1. Information and protein secondary structure analysis of *MdHSP70* gene family.

suggest that the *HSP70* gene family has a high degree of homology, in which segmental duplications may play an important role in the expansion of the family.

The collinearity analysis among monocotyledonous leaves of rice, millet, sorghum and maize showed (Fig. 4A) that there were 49 gene pairs between rice and millet, 30 gene pairs between rice and sorghum, 16 collinear pairs between rice and maize, 31 gene pairs between millet and sorghum, and 17 collinear pairs between millet and maize. There were 15 collinear pairs between sorghum and maize, indicating that the relationship between rice and millet was closer, followed by millet and sorghum, and the least collinear pairs between sorghum and maize, indicating that the relationship between sorghum and rice was estranged; Collinear analysis of dicotyledon apple, Arabidopsis, tomato, grape and soybean *HSP70* family members found (Fig. 4B) that there were 36 gene pairs between apple and Arabidopsis, 28 gene pairs between apple and tomato, 17 gene pairs between apple and grape, 16 gene pairs between apple and soybean, and 31 gene pairs between Arabidopsis and tomato. There are 16 gene pairs between Arabidopsis and grape, 14 gene pairs between Arabidopsis and soybean, 15 gene pairs between tomato and grape, 15 gene pairs between tomato and soybean, and 8 gene pairs between grape and
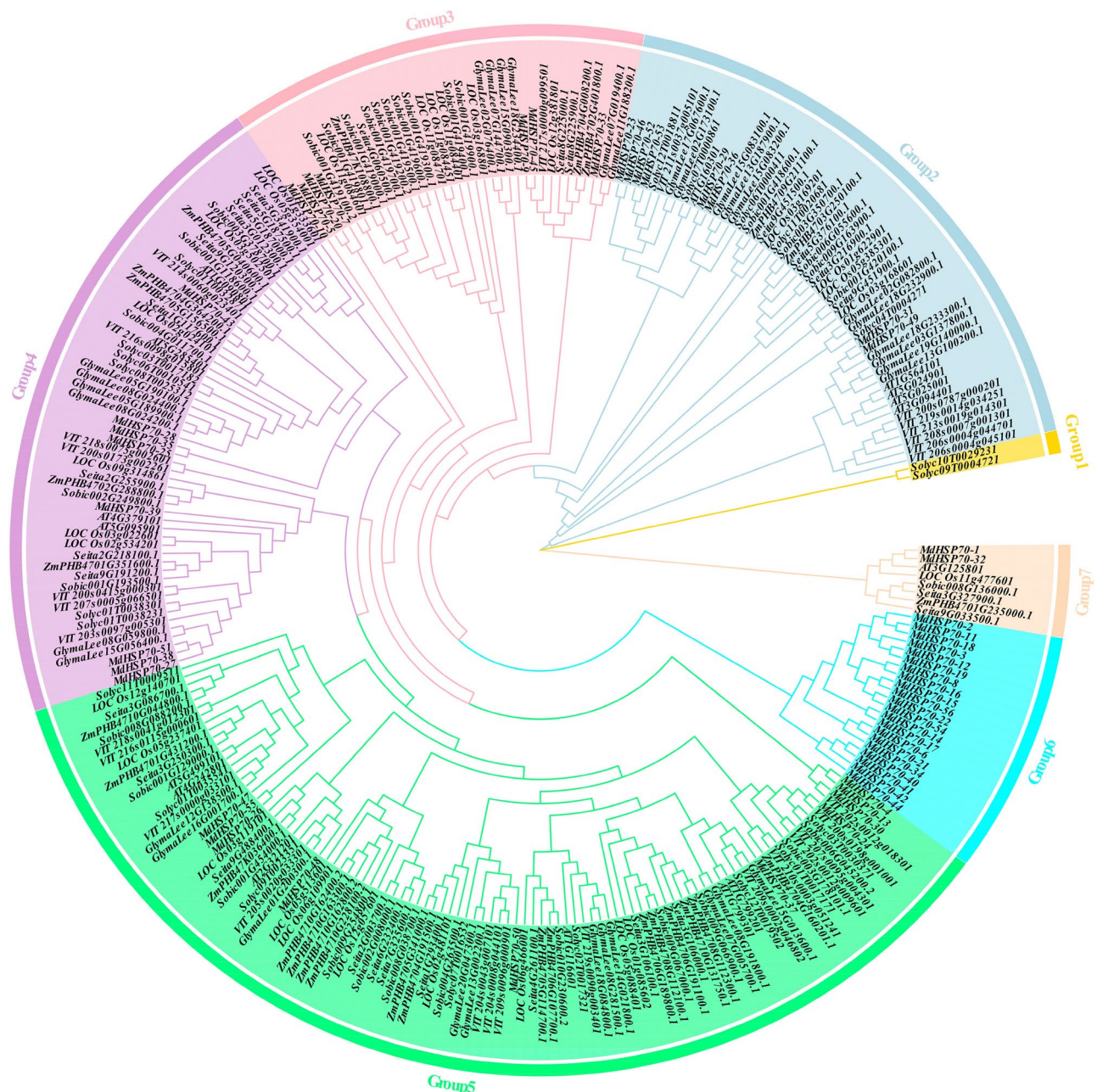

Fig. 2. Phylogenetic analysis of the *MdHSP70* gene family. Phylogenetic tree was constructed using HSP70 protein sequences. The NJ method was used and the bootstrap value was set equal to 1000.
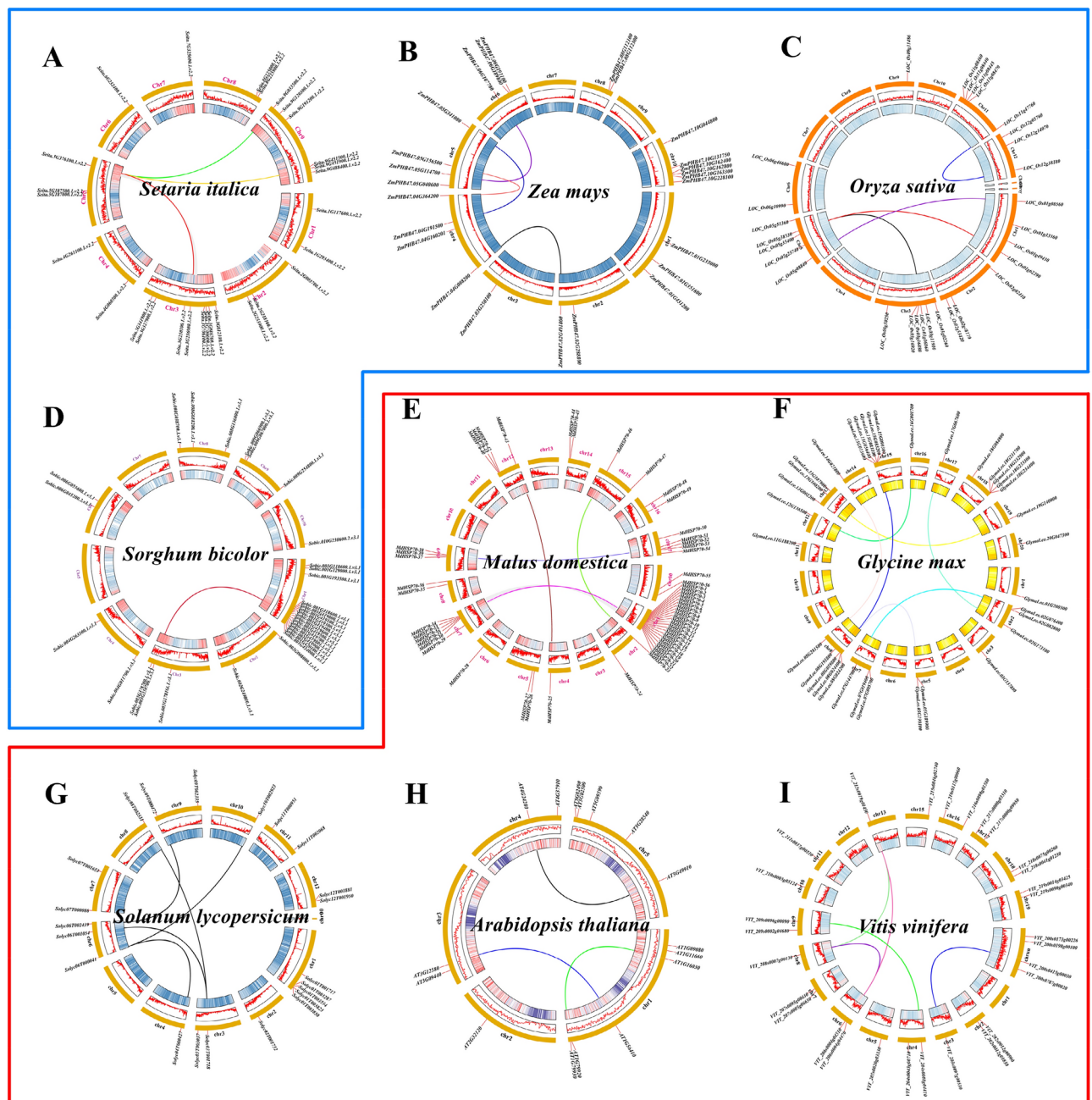
Fig. 3. Intraspecic collinearity analysis of *HSP70* gene family. (**A**)–(**I**) Represent the collinear heat maps of species millet, maize, rice, sorghum, apple, soybean, Arabidopsis, tomato and grape. The blue line shows monocotyledonous plants and the red line shows dicotyledonous plants.

soybean. The results indicated that apple and Arabidopsis were more closely related, while grape and soybean were more closely related.

MdHSP70 family protein interaction and screening and validation of proteins interacting with MdDVH24_032563

To determine the interactions between MdHSP70 and related proteins, we constructed an HSP interaction network using a STRING database with apple proteins. The results showed that there were interactions among the 20 MdHSP70 proteins, and the interactions among the 20 MdHSP70 proteins formed a protein interaction network (PPI network) (Supplementary Fig. S1). It is worth noting that DVH24_004410 and DVH24_032563 are proteins containing the OUT domain, which belong to the UDP-glycosyltransferase family (UGt). Since the UDP-glycosyltransferase family plays an important role in the regulation of plant growth and abiotic stress tolerance, and the thickness of the lines represents the intensity of the interaction. We can find that MdHSP70-43, MdHSP70-14, MdHSP70-35, MdHSP70-11, MdHSP70-10, MdHSP70-53, MdHSP70-50, MdHSP70-8, and
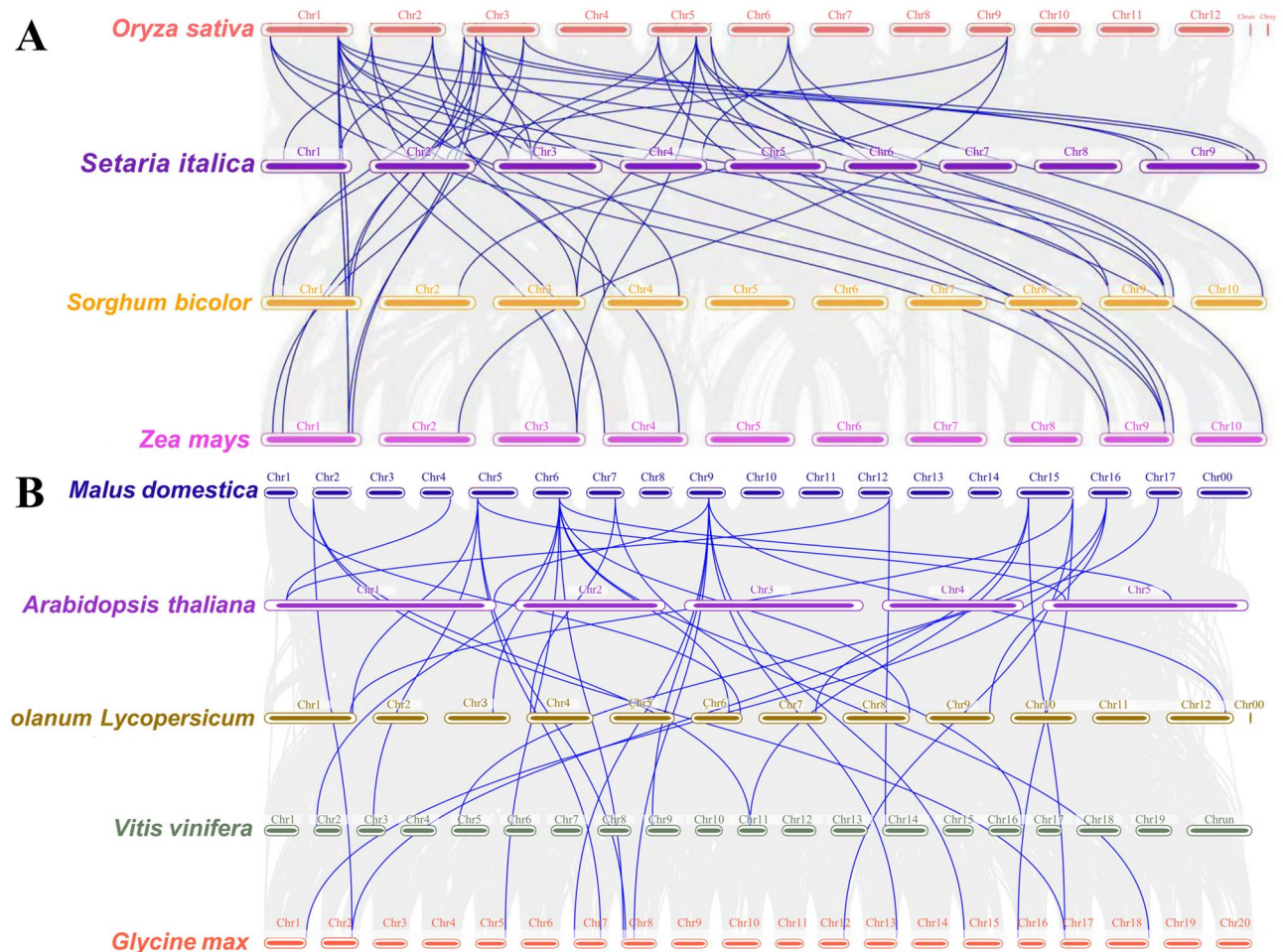
Fig. 4. Interspecific collinearity analysis of *HSP70* gene family. (**A**) The collinearity of the *HSP70* gene in monocotyledonous plants such as rice, millet, sorghum and maize. (**B**) The collinearity of HSP70 gene in dicotyledonous plants such as apple, Arabidopsis, tomato, grape and soybean. The blue lines represent collinear genes.

MdHSP70-13 are the major interacting proteins compatible with MdDVH24_004410, MdDVH24_032563, and it is speculated that these proteins play an indispensable role in improving the growth and development of apple plants and abiotic stress tolerance. In order to verify the interactions between the proteins even further, we constructed MdDVH24_032563-pGBADT7, MdHSP70-53-pGBKT7 vectors, cloned the above genes and transformed them into E. coli DH5α (Supplementary Fig. S2). Co-transformation of pGBADT7 with pGBKT7, pGBADT7 with MdHSP70-53-pGBKT7, MdDVH24_032563-pGBADT7 with pGBKT7, and MdDVH24_032563-pGBADT7 with MdHSP70-53-pGBKT7 into Y2H yeast-receptor cells was performed to Validation. AD/BD, AD/MdHSP70-53-BD, and MdDVH24_032563-AD/BD grew normally in DDO and DDO/X media and did not blotch or turn blue on QDO and QDO/X, indicating that MdHSP70-53-BD and MdDVH24_032563-AD are not self-activating. MdDVH24_032563-AD/MdHSP70-53-BD appeared as white spots on QDO and QDO/X as well as DDO and DDO/X media and turned blue, suggesting the presence of an interaction between MdDVH24_032563 and MdHSP70-53 (Fig. 5).

Analysis of *cis*-acting elements of the *MdHSP70* gene family

Cis-acting elements are non-coding DNA sequences that regulate transcriptional initiation of related genes. In this study, PlantCARE software was used to analyze the 2000 bp promoter sequence upstream of the *MdHSP70* coding region, and a total of 9 *cis*-acting elements in response to hormone signals and stress responses were identified (Fig. 6). Hormone response elements include MeJA, SA, indole-3-acetic acid (IAA), gibberellin (GA) and ABA, and light responsive *cis*-elements (LREs). The *cis*-regulatory factors related to abiotic stress include low-temperature response elements, drought-induced response elements and stress-related elements. The promoter region of *MdHSP70-15* contains only GA response elements, and the promoter region of *MdHSP70-56* contains only SA response elements. Most of the genes contain MeJA response elements, GA response elements, ABA response elements and drought induced response elements.
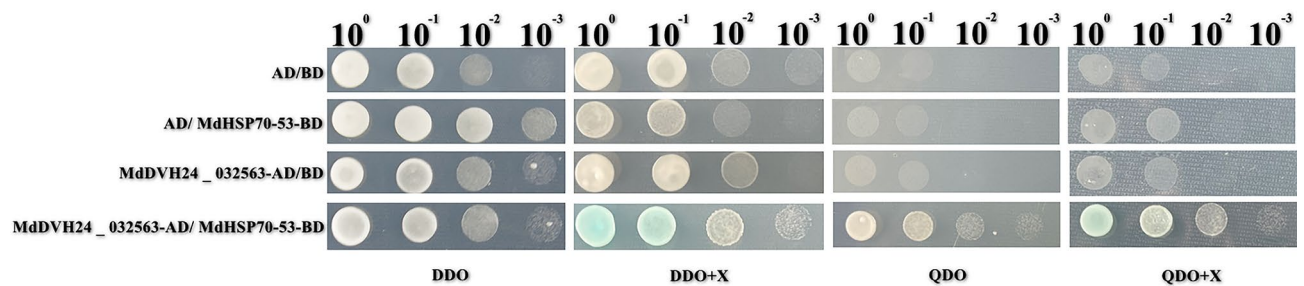
Fig. 5. Y2H analysis of MdHSP70-53 protein. AD and BD represent pGADT7 and pGBKT7, respectively. DDO indicates SD/-Leu/-Trp, and QDO indicates SD/-Ade/-His/-Leu/-Trp. X indicates X-α-gal.
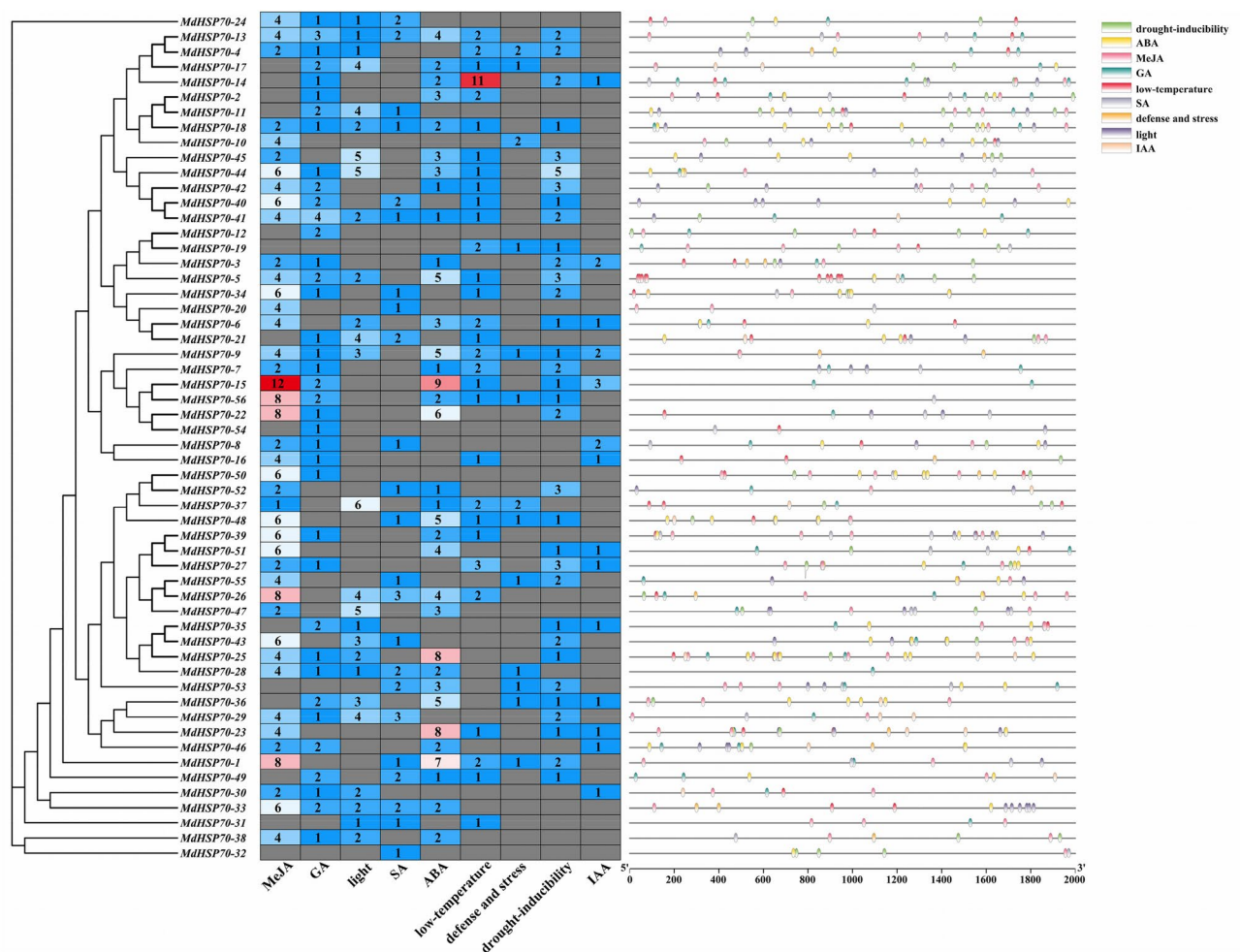

Fig. 6. *Cis*-acting element analysis of the first 2000 bp of the *MdHSP70* gene family. Different elements are marked with different colors. Numbers indicate the number of *cis*-acting elements.

Analysis of *MdHSP70* gene family structure and conserved motifs and conserved domains

The gene structure of 56 *MdHSP70* gene family genes was arranged according to the phylogenetic tree, and the analysis results showed (Fig. 7) that the *MdHSP70* gene family was divided into three groups according to the evolutionary tree relationship. The first group is composed of *MdHSP70-1*, *MdHSP70-32*, *MdHSP70-23*, *MdHSP70-46*, *MdHSP70-52*, *MdHSP70-53*, and *MdHSP70-33*, except that *MdHSP70-32* and *MdHSP70-52* contain only motif1. Other genes contained motif1, motif2, motif3, motif4 and motif6. The second group consists of 45 genes, almost all containing motif5 and motif9 except for a few genes. The third group of genes consists of *MdHSP70-29*, *MdHSP70-36*, *MdHSP70-31* and *MdHSP70-49*, and it contains motif3. The N-terminal starts with motif1 and motif7, and the C-terminal ends with motif8. It is worth noting that motif1, motif3 and motif7 appeared frequently in the entire *MdHSP70* gene family, suggesting that motif1, motif3 and motif7 are

important and highly conserved domains of this gene family. The number of introns and exons of *MdHSP70* gene in different subfamilies is different, and the exon number is between 1 and 16. *MdHSP70-24* has the highest number of exons, with 16. The exon length of *MdHSP70-22* is the longest, about 16500 bp, and the exon length of *MdHSP70-32* is the shortest. The number and distribution of introns and exons within the same group also are different, with only a few having highly conserved properties. The conserved structural domains of the MdHSP70 gene family show that except for the *MdHSP70-24* gene, the conserved structural domains of all genes are *HSP70*.

Tissue-specific expression analysis of *MdHSP70* gene family

The expression of 47 *MdHSP70* genes in different plant organs at different periods was analyzed by searching the Apple MDO database, expressed value is FPKM (Supplementary Fig. S3). Comparing the expression of these genes in apple seed, stem, bud, leaf, flower and fruit stages, these genes can be categorized into three classes. Class I contained only *MdHSP70-11* gene, which were highly expressed in almost all four periods except for low expression at flowering stage, and the highest expression was found at leaf and fruit stages, indicating that the genes in this class might be related to leaf growth and fruiting in apple, and their role in fruiting was more obvious. Class II contained 33 genes which were expressed at lower levels in five periods. Class III contained 9 (*MdHSP70-23*, *MdHSP70-27*, *MdHSP70-37*, *MdHSP70-44*, *MdHSP70-45*, *MdHSP70-47*, *MdHSP70-49*, *MdHSP70-50*, *MdHSP70-53*) genes, and these genes were more highly expressed in some periods. We found that 16 *MdHSP70* genes were expressed at the seed stage, accounting for about 34% of the total retrieved genes, and the expressions of *MDHSP70-1* and *MdHSP70-47* were the highest; twenty genes were expressed at rooting stage, accounting for 43% of the total retrieved genes, and the expression of *MdHSP70-1* was the highest. In the germination stage, the expression of *MdHSP70* increased gradually with the increase of time, *MdHSP70-1*, *MdHSP70-23*, *MdHSP70-37*, *MdHSP70-47*, *MdHSP70-50* were the most obvious. In the leaf growth period stage, *MdHSP70* increased first and then decreased with the increase of time. The expression of *MdHSP70* in flowering stage was lower than that in germination stage. The expression level of *MdHSP70* in flowering stage was lower than that in leaf growth period stage: the expression level of *MdHSP70* in fruit stage showed a trend from increasing to decreasing, then increasing and decreasing again. As a whole, *MdHSP70-1*, *MdHSP70-23*, *MdHSP70-27*, *MdHSP70-37*, *MdHSP70-47* and *MdHSP70-50* participated in all stages of apple growth and were highly expressed, suggesting that these genes played an important role in apple growth and development.

Codon preference analysis of *MdHSP70* gene family

The frequency of relative synonymous codons in *MdHSP70* genome was analyzed (Supplementary Table S1). It was found (Fig. 8A) that RSCU ≥ 1 for 30 codons: AAG, UCU, CCU, CUU, GGU, GUG, CCA, GUU, AUU, ACU, UGA, GCU, UUG, AGG, GCC, AAC, AUG, UUU, CAU, UCA, UAU, UGU, GAG, GAU, ACC, CAG, AGU, AGC, AUC, AGA, among the 30 codons, 14 of them have the third codon U, accounting for 26.4% of the total codons. Seven of the third codons are G, accounting for 15.2% of the total codons, five of the third codons are C, accounting for 7.1% of the total codons, and four of the third codons are A, accounting for 5.3% of the total codons, indicating that the *MdHSP70* protein genome codons prefer ending in U or G (Fig. 8B). The average values of CAI, CBI, Fop, and NC of *MdHSP70* family members are 0.209, − 0.030, 0.391, and 48.750, respectively. The content of GC of *MdHSP70* family members ranges from 39 to 56%, and the content of GC3s ranges from 30 to 78%. The average for GC and GC3s was 42.7% and 44.6% respectively (Supplementary Table S2). A total of 18 genes had NC values less than 50, which were: *MdHSP70-1*, *MdHSP70-8*, *MdHSP70-12*, *MdHSP70-16*, *MdHSP70-18*, *MdHSP70-19*, *MdHSP70-26*, *MdHSP70-28*, *MdHSP70-29*, *MdHSP70-32*, *MdHSP70-36*, *MdHSP70-38*, *MdHSP70-42*, *MdHSP70-46*, *MdHSP70-49*, *MdHSP70-52*, *MdHSP70-53*, *MdHSP70-54*. This indicates a strong codon preference for these 18 genes (Fig. 8C). The correlation analysis results of *MdHSP70*
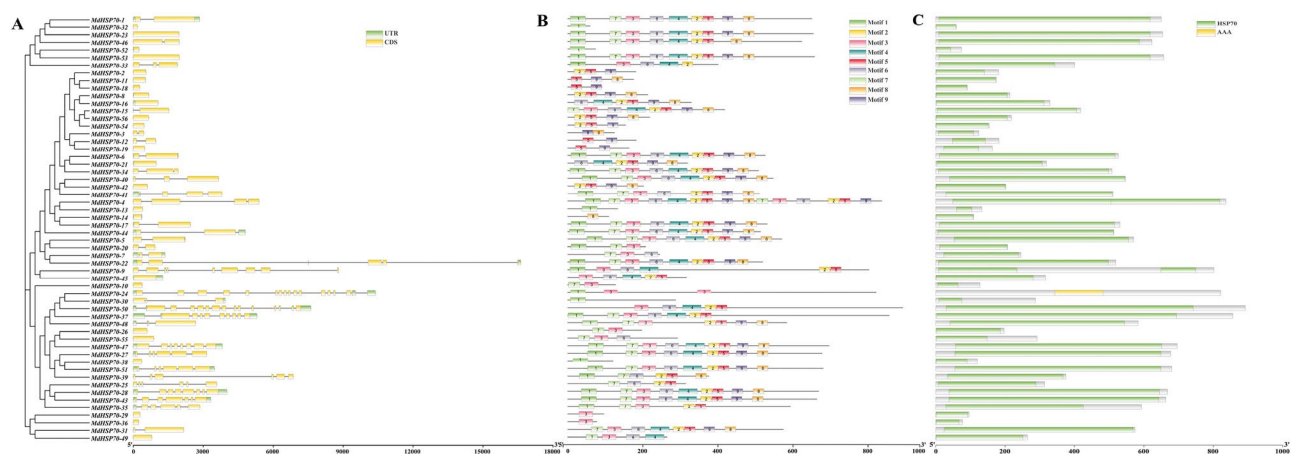

Fig. 7. *MdHSP70* gene family structure and conserved motif analysis. (**A**) Exon–intron structure of *MdHSP70* gene. (**B**) Conserved motifs; different motifs are marked with different colors and the numbers above represent different motifs. (**C**) Conserved structural domain of the *MdHSP70* genes.
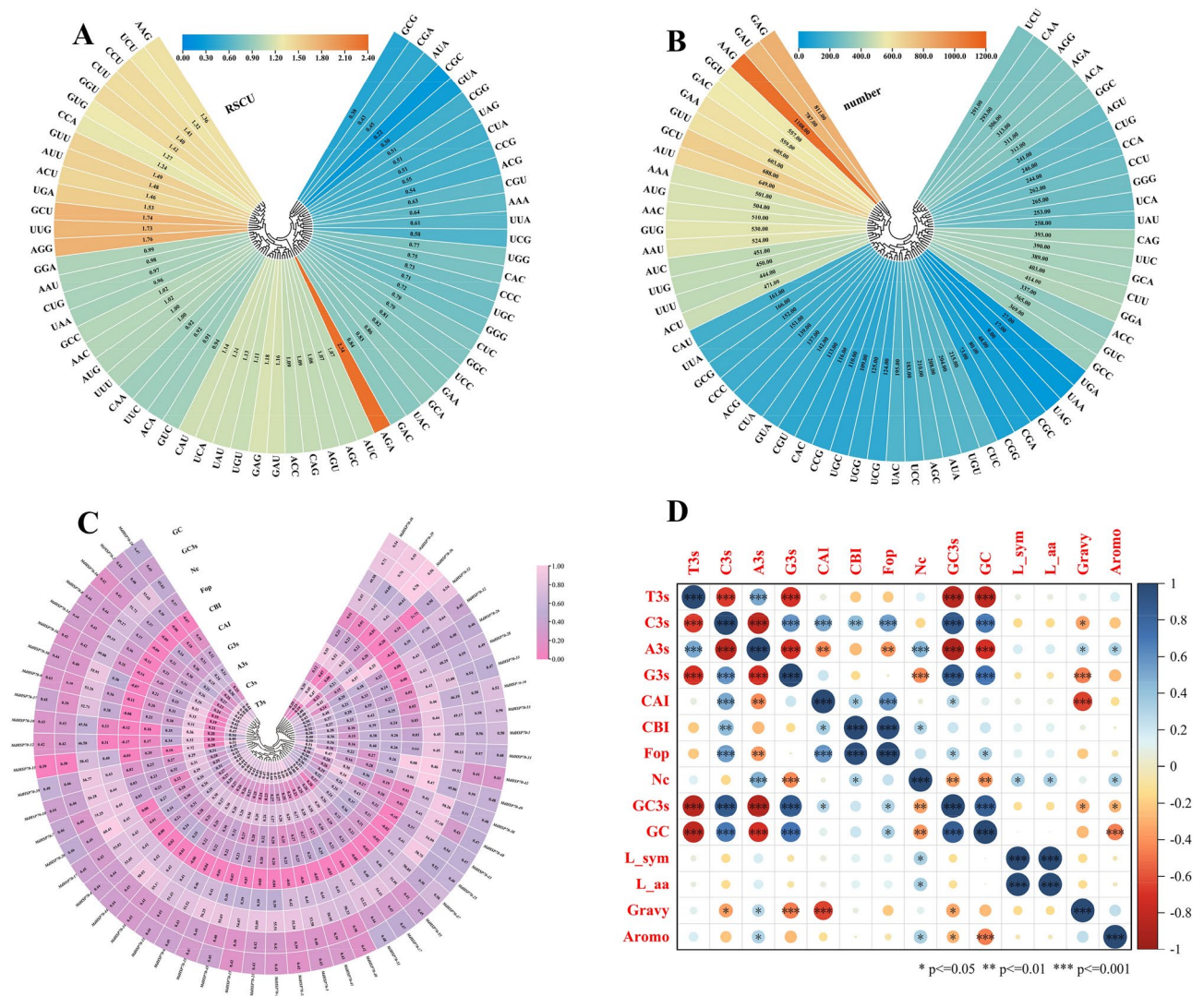
Fig. 8. Analysis of the use and number of synonymous codons associated with codons in the *MdHSP70* gene family. (**A**) Use of synonymous codons. (**B**) Preferred number of synonymous codons. (**C**) Analysis of *MdHSP70* gene family codon parameters. "A3s, G3s, C3s, T3s" refers to the frequency of the third base corresponding to the synonymous codon; "CAI" is the codon adaptation index; "CBI" refers to the codon bias index; "FOP" is the frequency of optimal codon occurrence; "ENc" is the number of effective codons; "GC3s" is the number of the third codon (G + C); and GC is the gene count (G + C). (**D**) Correlation analysis of *MdHSP70* gene family codons. Blue color indicates a positive correlation, red color indicates a negative correlation, and white color indicates no correlation. The darker the color and the larger the circle, the stronger the correlation, and vice versa. The number of observations (n) for the correlation coefficient is 141.

genome codon using bias correlation indicators (Fig. 8D) showed that there is a very significant ($P < 0.001$) positive correlation between GC value and GC3s value, indicating that there is little difference between the first and third base of *MdHSP70* genome codon. The GC values were not significantly ($P < 0.05$) correlated with the CBI and FOP values, and the GC3s values were not significantly ($P < 0.05$) correlated with the CBI and FOP values, suggesting that codon base composition has a small effect on codon usage bias.

qRT-PCR analysis of *MdHSP70* gene family

The results of quantitative fluorescence analysis of apple leaves treated with different conditions showed that (Fig. 9). 41 *MdHSP70* gene expressions were up-regulated under both hormone and abiotic stress treatments. Under hormone treatment conditions, *MdHSP70-23*, *MdHSP70-26*, and *MdHSP70-45* had the highest expression under MeJA, GA$_3$, and ABA treatments, respectively, which were 44.36-fold, 43.96-fold, and 51.73-fold higher than the control. Under abiotic stress treatment conditions, *MdHSP70-45* had the highest expression after NaCl treatment, which was about 91.70-fold of the control; *MdHSP70-6* had the highest expression after PEG treatment, which was about 95.30-fold of the control; and *MdHSP70-45* had the highest expression after 4 °C treatment, which was about 93.82-fold of the control.

The expression of the remaining members was down-regulated under individual treatments, for example, *MdHSP70-10*, *MdHSP70-14*, *MdHSP70-16*, *MdHSP70-18* and *MdHSP70-56* gene expressions were down-regulated after MeJA treatment; *MdHSP70-14*, *MdHSP70-34*, and *MdHSP70-39* were down-regulated in gene expressions after GA$_3$ treatment; and *MdHSP70-14*, *MdHSP70-16*, *MdHSP70-34*, and *MdHSP70-39* were down-regulated in gene expression after ABA treatment. Likewise, under abiotic stress treatments, the expression of *MdHSP70-3* and *MdHSP70-10* were down-regulated after PEG treatment, and *MdHSP70-2* and *MdHSP70-10* gene expressions were down-regulated after low temperature treatment at 4 °C. It is noteworthy that most *MdHSP70* genes were more sensitive to stress treatments relative to hormone treatments, suggesting that *MdHSP70* genes play an important role in abiotic stresses in plants. The relationship between *MdHSP70* and hormones and abiotic stress remains to be verified in our further experiments.

Prediction of transmembrane structural domains and subcellular localization analysis of the *MdHSP70* gene

Seven genes were found to have transmembrane structures on the Transmembrane Structure Domain Prediction website. They are MdHSP70-4, MdHSP70-13, MdHSP70-28, MdHSP70-30, MdHSP70-35, MdHSP70-43, and MdHSP70-50 (Supplementary Fig. S4). Whereas, MdHSP70-6, MdHSP70-26, and MdHSP70-45 do not have transmembrane structures, suggesting that they are localized only to a organelle. Subcellular localization can be used to detect the exact location in the cell where specific proteins are present and act. They are transiently expressed using recombinant plasmids ligated with fluorescently labeled vectors, and their location is determined by observing fluorescence in the cell through an inverted microscope. Subcellular localization is not only used to study the structure and function of cells, but can also be used to study cell signaling, the interactions that exist between proteins, and to track the location and activity of cell surface receptors. To further determine the subcellular location of *MdHSP70*, we cloned the genes of *MdHSP70* (6, 26 and 45) and constructed the overexpression vectors *35S::MdHSP70-6-GFP*, *35S::MdHSP70-26-GFP*, *35S::MdHSP70-45-GFP*, and the empty vector 35S::GFP was used as a positive control to observe green fluorescence. The identification results showed
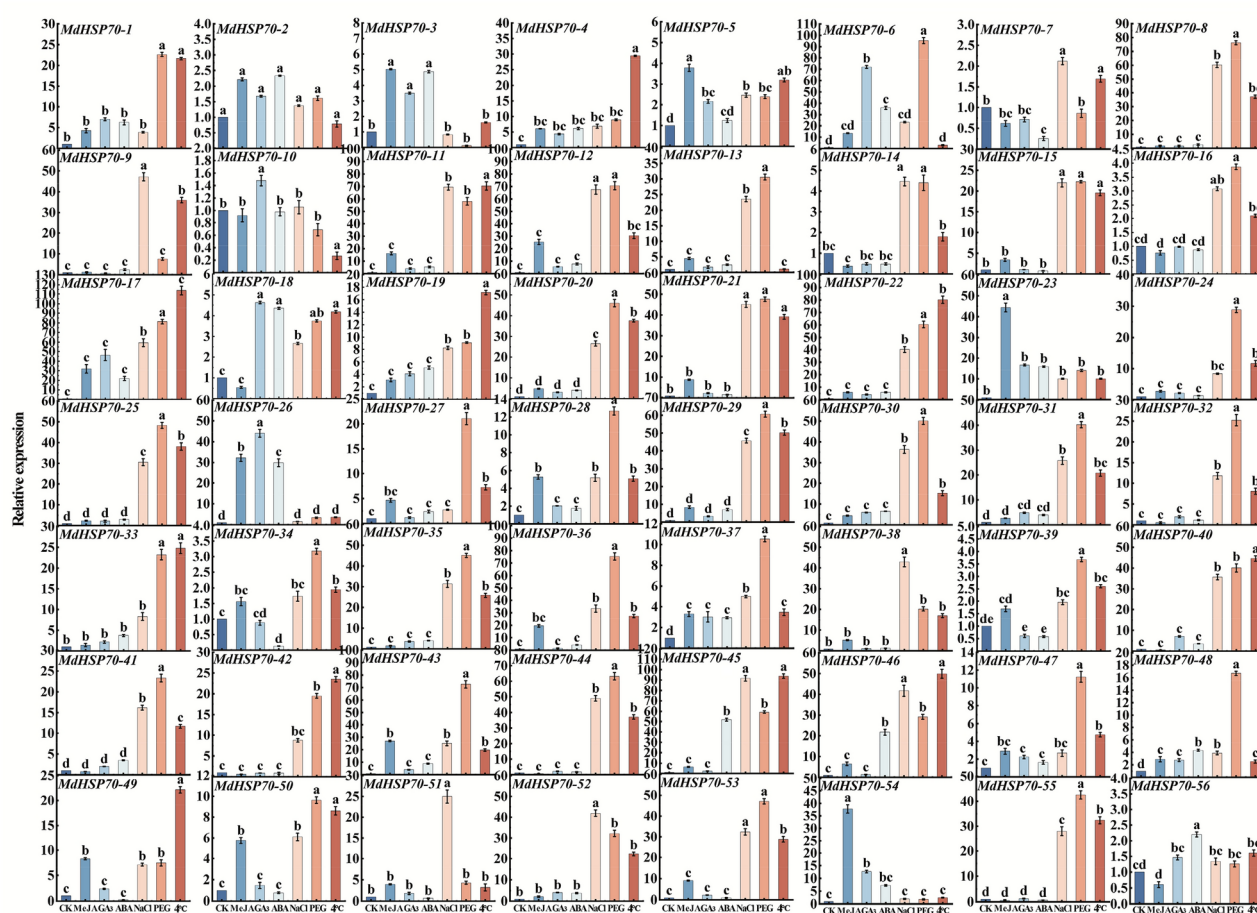

Fig. 9. Expression levels of *MdHSP70* gene family under abiotic stress and plant hormone treatment. Statistical analysis is performed by one-way ANOVA with Tukey's honestly significant difference (HSD) test. The control unstressed expression level, which was assigned with a value of 1. Error bars represent the mean ± SE from three biological repeats. Different letters denote signifcant differences, whereas the same lowercase letters indicate no statistical difference ($P < 0.05$).

that the overexpression vectors *35S::MdHSP70-6-GFP*, *35S::MdHSP70-26-GFP*, and *35S::MdHSP70-45-GFP* observed green fluorescence in the cell membrane (Fig. 10).

Discussion

As an important member of the heat shock protein family, HSP70 protein is widely distributed in eukaryotes and plays an important role in plant growth and development and adaptation to stress[37]. It has been intensively and meticulously studied in Arabidopsis (*Arabidopsis thaliana*), pepper (*Capsicum annuum*), sugarcane (*Saccharum officinarum*), and sorghum (*Sorghum bicolor*), among other plants, with abundant results[38–41]. At present, genome-wide identification of *HSP70* gene family in tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), rice (*Oryza sativa*) and soybean (*Glycine max*) has been reported[29,30,42,43], *HSP70* gene has also been identified in woody plants such as *Citrus reticulata* and *Populus trichocarpa*[44,45]. Identification and analysis of *HSP70* gene family in apples will provide a theoretical basis for the study of their and it is speculated that these proteins play an indispensable role in improving the growth and development of apple plants and abiotic stress tolerance. In this study, 56 *MdHSP70* genes were identified from the apple genome database, their numbers were similar to those of soybean (*Glycine max*) (61)[43], cabbage (*Brassica oleracea*) (52)[46] and tobacco (*Nicotiana tabacum*) (61)[29], More than pumpkin (21) (*Cucurbita moschata*)[47], potato (*Solanum tuberosum*) (25)[30] and maize (*Zea mays*) (25)[48]. This should be related to the paleopolyploidy event of the species genome or *HSP70* gene evolutionary diversity. The proteins encoded by the 56 *MdHSP70* genes differed in sequence length, isoelectric point, and molecular weight, suggesting diversity among members of the *MdHSP70* gene family.

Although the position of genes on chromosomes is determined by previous evolutionary events[49], gene replication is the fundamental mechanism of species evolution and the generation of new genes, mainly in three ways: fragment replication, tandem replication, and gene translocation, which are often the basis for the expansion of plant gene families[50]. Tandem replication is an important mechanism for gene family expansion, and genes in tandem replication usually appear in clusters[51]. We found that *HSP70* genes clusters appeared on multiple chromosomes, indicating that tandem replication was the main reason for the expansion of *HSP70* gene in apple. Phylogenetic trees help to analyze the evolutionary relationships between genes of species, and studies have shown that genes with close phylogenetic relationships usually have similar properties or functions[5]. In this study, a phylogenetic tree of HSP70 protein sequences in monocotyledonous and dicotyledonous plants was constructed, and the conclusion was drawn that *MdHSP70* was closely related to dicotyledonous plants but was estranged to monocotyledonous plants. Zhou et al. in their study on dicotyledonous plants cucumber found that the *HSP70* genes in dicotyledonous plants *Arabidopsis thaliana*, chili peppers, and tomato were more closely phylogenetically related to those in cucumber than those in monocotyledonous plants[52]. The present study is similar to the results of Zhou et al.[52]. It is hypothesized that the function of the *HSP70* gene in apple may be similar to its homologs in other plants, and that the *HSP70* gene family may have undergone species-specific amplification during the evolution of these plants. In addition, it was also found that *MdHSP70* and *Arabidopsis thaliana* had the most collinear genes in dicotyledonous plants. This also indicates that apple is more closely related to the Arabidopsis *HSP70* family to some extent. This is similar to the findings of Yin et al.[53] who found that *chrysanthemum lavender* and Arabidopsis *HSP70* are highly conserved.

Protein interaction network analysis and validation of protein interaction experiments showed that UGts interacted with MdHSP70 protein, and UGts are widely involved in the biosynthesis of secondary metabolites, thereby regulating plant growth and development and response to abiotic and biological stresses[54]. Overexpression of Arabidopsis glycosyltransferase genes UGT79B2 and UGT79B3 significantly improved plant abiotic stress tolerance to low temperature, drought and salt stress[55]. The interaction between MdHSP70 protein and UGts protein indicates that they have similar functions. Since most genes of *MdHSP70* are up-regulated in response to hormonal and abiotic stresses in qRT-PCR, our analysis of MdHSP70 protein also supports this view.

The expression patterns of genes are related to their corresponding functions, and the analysis of their expression patterns can be used to predict the molecular functions of genes involved in different processes to some extent[46]. Previous studies have found that the *HSP70* gene is expressed in different tissues and organs, and different members have different tissue specificities[43,46]. This study predicted that during the growth and development of apple, the expression of some genes has obvious tissue specificity. For example, *MdHSP70-16* is expressed in the root stage, germination stage and fruit-bearing stage, and *MdHSP70-43* is expressed in the seed stage, germination stage and highly in the fruit stage, some genes (*MdHSP70-1*, *MdHSP70-23*, *MdHSP70-27*, etc.) were also highly expressed at seed, root, germination, leaf, flower, and fruiting stages, suggesting that the *HSP70* family of genes is closely related to the growth of apple plants. This kind of situation also appeared in other species, and the sprouts of cabbage *BoHSP70-5*, *BoHSP70-19*, *BoHSP70-35* and *BoHSP70-52* showed high expression, *BoHSP70-13* and *BoHSP70-40* are highly expressed in roots, flowers and shoots[46]; For the 113 *HSP70* genes contained in the wheat (*Triticum aestivum*) genome, most of them (*TaHsp70-049*, *TaHsp70-040*, *TaHsp70-088*, etc.) are highly expressed in the root system during the nutritive growth period; A few genes (*TaHsp70-003*, *TaHsp70-008*, etc.) were highly expressed at germination and leaf stages (*TaHsp70-033*, *TaHsp70-133*)[56].

Eukaryotic genomes contain 64 codons that encode 20 different amino acids and three stop codons, and all amino acids except methionine (Met) and tryptophan (Trp) are encoded by more than one codon. Different codons encoding the same amino acid are synonymous with each other, and different synonymous codons are not used with the same frequency. The codons that are used frequently are "preferred codons", while others are "non-preferred codons", which is called "the phenomenon of codon preference". Components of codon preference include RSCU (relative synonymous codon usage), ENC (effective number of codons), CBI (codon adaptation index), Fop (frequency of optical codons), GC, GC3s (The GC content of the third position of all codons in the gene, except methionine, tryptophan, and stop codons, and the frequency of G and C appearing in the third position of the codon). Codon use bias is closely related to GC content. Mutations at the first and second codon bases are usually non-synonymous mutations with low mutation the rate, while mutations at the third
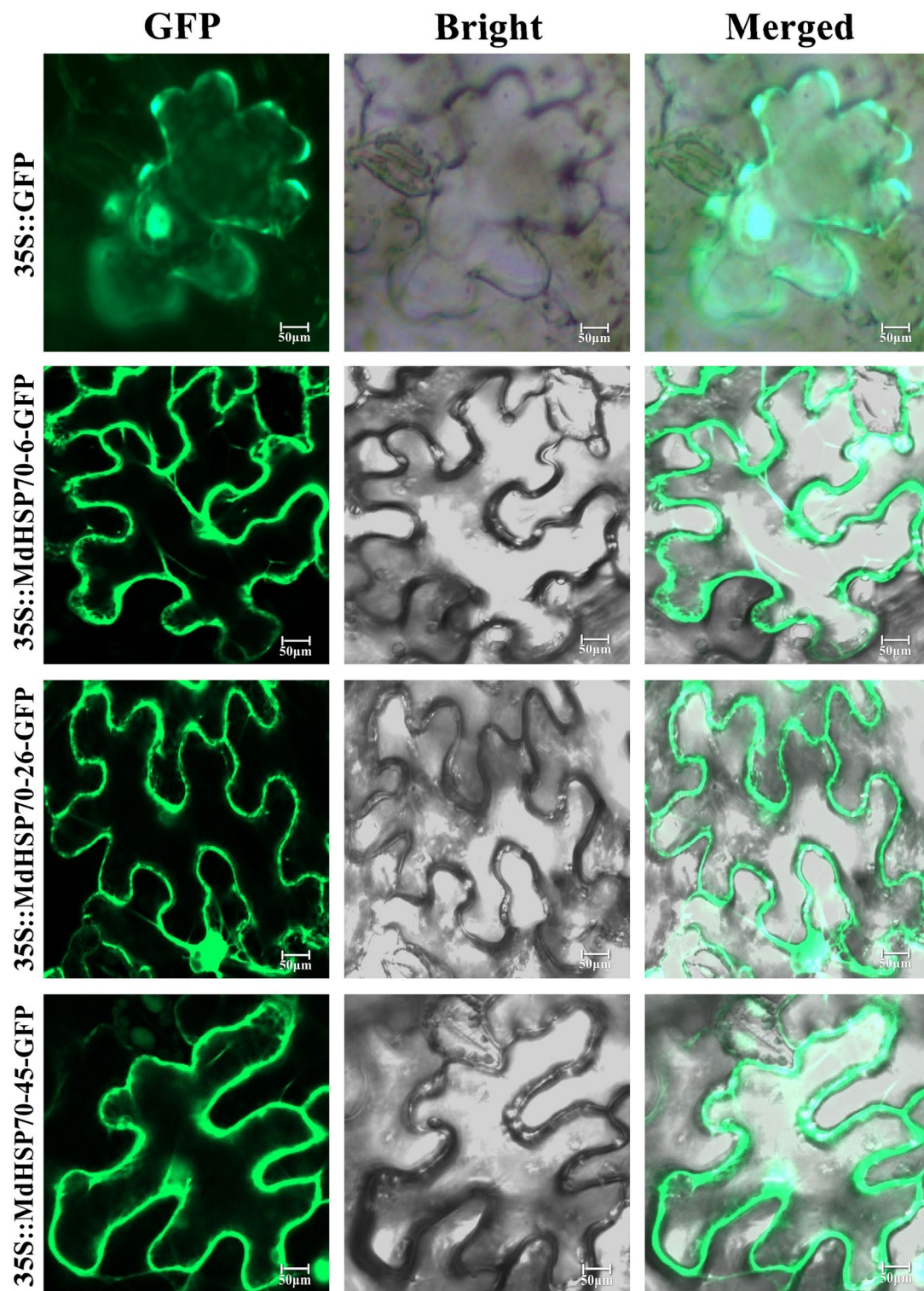
Fig. 10. Subcellular localizations of *MdHSP70-6*, *MdHSP70-26*, and *MdHSP70-45* in tobacco leaves. *35S::GFP* was used as the control.

codon base are usually synonymous mutations. Therefore, the composition of the third codon base can be used as the main basis for analyzing codon use bias. Codon preference is also an important tool for effective analysis of evolutionary traits in prokaryotes and eukaryotes[57]. While nucleotide composition has been reported to play an important role in the formation of codon preferences in nuclear genomes in plants, natural selection has been reported to play an important role in the formation of codon preferences in plant mitochondrial genomes and chloroplast genomes[58,59]. In this study, the main factors affecting the codon preference of *MdHSP70* were

analyzed. Neutral analysis showed that there was a significant correlation between GC and GC3s in the genome of *MdHSP70*. The mean NC value was 48.750, and the ENC value of 18 genes was less than 50, which suggested that the use of these gene codons was strongly biased. There are 30 codons with RSCU ≥ 1, indicating that these codons are high-frequency codons and the codon preference ends in U or G.

Studies have shown that *cis*-acting elements can regulate the expression of stress response genes under stress environments[60], ABA promotes plant abiotic stress tolerance to salt, low temperature and drought stress[61], and MeJA is a plant volatile, acting as an important cell regulator, mediating different developmental processes and defense responses[62]. MeJA regulates many processes of plant growth and development and mediates various plant defense responses[63]. MeJA is a volatile jasmonic acid compound that is commonly used to induce defense responses in seed plants[64]. GA promotes cell elongation[65], while controlling plant growth and modulating growth responses to environmental stress[66]. Zhou et al. found that the expression of *CsHsp70-1*, *CsHsp70-3*, *CsHsp70-10*, and *CsHsp70-11* was up-regulated in cucumber using RKN treatment, and hypothesized that the CsHsp70 genes might be related to the biotic stress response in cucumber[52]. He et al. found that *RsHSP70-20* may positively regulate heat tolerance in radish plants by scavenging reactive oxygen species and enhancing membrane permeability[67]. Guo et al. found that ectopic expression of the chili pepper gene *CaHsp70-2* regulated the expression of stress-related genes and resulted in enhanced heat tolerance in transgenic Arabidopsis[68]. In this study, the *cis*-acting elements of MeJA, ABA and GA_3 were detected in the promoter region of *MdHSP70* gene, and it was found that most of the genes contained MeJA response elements, GA_3 response elements, ABA response elements and drought induction response elements. It is suggested that the *MdHSP70* gene plays an important role in coping with high temperature, NaCl salt and drought stress. In order to further analyze the changes of gene expression under stress, we performed qRT-PCR analysis on 56 *MdHSP70* genes, and the results showed that a few of genes showed down-regulation in response to stress, which is due to the fact that some of the genes were up-regulated in response to external stress, and some of the genes were down-regulated in order to maintain the normal growth of the cells and stabilize the cellular state. Through the analysis of the action elements, we found that ABA, MeJA and GA_3 are the important action elements of the HSP70 family. At the same time, the effects of these hormones on plant abiotic stress have been carefully studied by predecessors. The genes *StHSP70-7* and *StHSP70-17* were highly expressed in potato under ABA and GA_3 treatments, and the *StHSP70-7* gene was up-regulated under drought stress conditions. the *StHSP70-11* gene was up-regulated under two abiotic stresses (salt and drought), and *StHSP70-16* was up-regulated under drought and cold stress conditions[30]. In soybean, 29 *GmHSP70* genes were up-regulated under drought stress[43]. qRT-PCR analysis showed that these hormones were positively expressed in *MdHSP70-23*, *MdHSP70-26*, *MdHSP70-45* and other genes, while *MdHSP70-6* was also found to be positively expressed under abiotic stress, which also provided a certain theoretical basis for further study of HSP70 family genes.

Subcellular localization provides insight into the specific site of a protein's action or its product, where only proteins in close to each other act and thus perform normal functions. Many HSP70 can bind to the cell membrane, which suggests that HSP70 plays a role in the functions performed by the membrane under stress conditions. HSP70 was localized in the cytoplasm and had the functions of protein refolding, movement of polypeptides between cell membranes, and degradation of misfolded proteins, but all of them need to juggle with the assisting participation of HSP40 in the process[22,69]. Because HSP70 binds to substrates when assisting protein folding, and requires ATPase to catalyze the conversion of HSP70 from the ATP-bound state to the ADP-bound state, in which the ATPase cycle is controlled by HSP40, which has the role of activating ATPase[14,70]. In this study, recombinant plasmids *35S::MdHSP70-6-GFP*, *35S::MdHSP70-26-GFP* and *35S::MdHSP70-45-GFP* were transferred into Bengal's tobacco by transient expression, and were found to be mainly localized in cell membranes, which was similar to the results of previous studies, therefore It was speculated that HSP70 plays an important role in the exercise of cell membrane functions and speculate that it is involved in protein folding and polypeptide movement between membranes in plants.

Conclusions

In the present study, we analyzed the role of the *MdHSP70* genes in the development of Malus domestica leaf by combining the physicochemical properties, phylogenetic relationships, and gene expression patterns of the *HSP70* gene family members in apple. We hypothesized that (1) A total of 56 *MdHSP70* family genes were identified using Arabidopsis amino acid sequence alignment. (2) *MdHSP70* family members are more closely related to dicotyledonous plants than to monocotyledonous plants, apple and Arabidopsis were more closely related. (3) Most of the *MdHSP70* genes contain MeJA response elements, GA response elements, ABA response elements and drought induced response elements. (4) Motif1, motif3 and motif7 are important and highly conserved domains of *MdHSP70* gene family. (5) *MdHSP70-1*, *MdHSP70-23*, *MdHSP70-27*, *MdHSP70-37*, *MdHSP70-47* and *MdHSP70-50* participated in all stages of apple growth and were highly expressed. (6) The yeast two-hybrid (Y2H) demonstrated that MdHSP70-53 interacts with MdDVH24_032563. (7) Under hormone treatment conditions, *MdHSP70-23*, *MdHSP70-26*, and *MdHSP70-45* had the highest expression under MeJA, GA_3, and ABA treatments, Under abiotic stress treatment conditions, *MdHSP70-45* had the highest expression after NaCl treatment, *MdHSP70-6* had the highest expression after PEG treatment, and *MdHSP70-45* had the highest expression after 4 °C treatment. (8) The overexpression vectors *35S::MdHSP70-6-GFP*, *35S::MdHSP70-26-GFP*, and *35S::MdHSP70-45-GFP* observed green fluorescence in the cell membrane. This research provides evidence for regulating *HSP70* gene expression in response to hormonal and abiotic stresses to improve abiotic stress tolerance in apples.

Materials and methods

Plant materials and stress treatment

The selected test material was the 'Gala' apple seedling stored in the Fruit Tree Physiology and Biotechnology Laboratory of Gansu Agricultural University. Stem segments of single buds were placed on 50 mL MS solid medium and exposed to LED white light (25 °C for 16 h; After 30 days of subculture at 20 °C dark for 8 h), the test-tube seedlings with good growth and no pollution were selected and completely removed from the medium on a super-clean workbench and the roots were washed with sterilized water. They were fixed using filter paper and then continued to be cultured in MS liquid medium containing 400 mM NaCl, 10% PEG, 0.2 mM ABA, 0.1 mM GA$_3$, and 0.1 mM MeJA, respectively, and were subjected to 4 °C low-temperature stress treatments in a low-temperature plant incubator, with normal-growing in vitro seedlings serving as controls. The treatment duration was 24 h, and three replicates were set for each treatment. The leaves were sampled at the end of the treatment and stored at –80 °C for subsequent experiments.

Identification of *MdHSP70* gene family

Download Arabidopsis (https://www.arabidopsis.org/, accessed on 23 May 2023) HSP70 protein sequence, and in the Rosaceae genome website (https://www.rosaceae.org/, accessed on 26 May 2023) using Arabidopsis protein sequence alignment sequences of apple, To compare the results using the Pfam (http://pfam.sanger.ac.uk/search, accessed on 28 May 2023) remove excluding conservative structure domain and repetitive sequences, 56 *MdHSP70* gene family, named after its position on the chromosome.

Prediction of physicochemical properties and chromosomal localization of the *MdHSP70* gene family

Theoretical isoelectric points, amino acid sizes, and molecular weights of proteins were analyzed using the ExPASy database (https://web.expasy.org/, accessed on 5 September 2023). Chromosome localization prediction was performed with the online software MG2C (http://mg2c.iask.in/mg2c_v2.1/index.html, accessed on 8 September 2023). Secondary structures (alpha-helix, beta-turn, irregular curl) were predicted using the online software PRABI- GERLAND (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?%20page=npsa_sopma.html, accessed on 10 September 2023); and secondary structures (alpha-helix, beta-turn, irregular curl) were predicted using the online software Protein.

Conserved motif, gene structure and conserved domain of *MdHSP70* gene family

Gene annotation files (gff.) for apple were downloaded from the Phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html, accessed on 15 September 2023). Conserved motifs in apple HSP70 protein were identified using the online software Multiple Em for Motif Elicitation (MEME) Version 5.0.5 (http://meme-suite.org/tools/meme, accessed on 18 September 2023) (9 motifs were set) and visualized using TBtools 1.6 software to visualize the conserved motifs; the online software GSDS2.0 (http://gsds.cbi.pku.edu.cn/, accessed on 5 November 2023) was used to visualize the gene structure analysis; using (https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, accessed on 1 July 2024) to search for *MdHSP70* conserved structural domains, and use Pfam (http://pfam.sanger.ac.uk/search, accessed on 1 July 2024) for authentication. Conserved structural domains were mapped using TBtools 1.6 software and modified using Adobe Illustrator CS5 software.

Analysis of *cis*-acting elements of *MdHSP70* gene family

The upstream 2000 promoter region of all apple *HSP70* family genes (fasta.gff.) was extracted using TBtools 1.6, and the results obtained were visualized and predicted on the online website PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 8 November 2023) and TBtools 1.6 for prediction and visualization, respectively.

Phylogenetic tree, collinearity and protein network analysis of *MdHSP70* gene family

Phylogenetic analysis of 271 *HSP70* amino acid sequences from nine species, including apple, Arabidopsis, and rice, was performed using MEGA7 software, and the neighbor-joining method was used to construct a phylogenetic tree. The Arabidopsis *HSP70* amino acid sequences were obtained from the Arabidopsis genome website (https://www.arabidopsis.org/, accessed on 10 November 2023), the rice *HSP70* amino acid sequences were obtained from the rice genome website (http://rice.uga.edu/, accessed on 15 November 2023), and the rice *HSP70* amino acid sequences were obtained from the Rosaceae Genome website (https://www.rosaceae.org/, accessed on 20 November 2023) to obtain the MdHSP70 protein sequence, and the *HSP70* family structural domain login number PF00012 via the HMMER (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan, accessed on 26 November 2023) website, and the HSP70 protein sequence via the online website Phytozome13 (https://phytozome-next.jgi.doe.gov/, accessed on 30 November 2023) to download the amino acid sequences of grape, soybean, tomato, millet, sorghum, and maize species using the login number PF00012, and the duplicates were removed using Pfam (http://pfam.sanger.ac.uk/search, accessed on 1 December 2023) to remove the duplicates using the ClustalX software for multiple comparison of amino acid sequences of apple, tomato, soybean, grape, *Arabidopsis thaliana*, rice, maize, millet, sorghum, constructed a phylogenetic tree and self-checked it using MEGA5.0 software, (bootstrap) duplicates were set with a default value of 1000, and used the online tool iTOL (https://itol.embl.de, accessed on 3 December 2023) for landscaping; covariance analysis between *MdHSP70* family members was performed using genome sequence (fasta.) and gene annotation files (gff.) in TBtools 1.6 software. Genome sequences (fasta.) and gene annotation files (gff.) of apple, tomato, soybean, grape, Arabidopsis, rice, millet, maize, and sorghum were downloaded through the online website Phytozome13 (https://phytozome-next.jgi.doe.gov/, accessed on 6 December 2023), and each species was analyzed by performing covariance analysis among members of the *HSP70* family of *MdHSP70* through the

TBtools1.6 software. The covariance analysis of the *HSP70* family members was analyzed with the covariance analysis between species of *HSP70* family members of dicotyledonous plants apple, grape, tomato, soybean, and Arabidopsis and monocotyledonous plant rice, millet, maize, and sorghum species. *MdHSP70* family interacting proteins were predicted by String (http://string-db.org/, accessed on 9 December 2023).

Yeast two-hybrid

Using MdHSP70-53-pGBKT7 and MdDVH24_032563-pGBADT7 qPCR primers designed and synthesized by Sangon Biotech (Shanghai) (Supplementary Table S3). The recombinant vectors MdHSP70-53-BD and MdDVH24_032563-AD vectors were obtained by ligation transformation using homologous recombination reagents. Y2H yeast competent cells were co-transformed with AD and BD, AD and MdHSP70-53-BD, MdDVH24-AD with BD and MdDVH24_032563-AD with MdHSP70-53-BD and cultured on SD/-Leu/-Trp, SD/-Leu/-Trp/-His/-Ade and SD/-Leu/X-α-Gal, SD/-Leu/-Trp/-His/-Ade/X-α-Gal nutrient-deficient medium, respectively. After incubation at 30 °C for 2–3 d, the yeast co-transformants were observed to grow on the different defective medium. The growth of yeast co-transformants on different defective media was observed at 30 °C for 3d, and the interactions between MdHSP70-53 and MdDVH24-032563 proteins were analyzed.

Tissue-specific expression analysis of *MdHSP70* gene family

Gene expression of apple growth stages was retrieved from the Apple MDO (http://bioinformatics.cau.edu.cn/AppleMDO/index.php, accessed on 12 December 2023)[71], database using the *MdHSP70* gene accession number, organized in Excel 2016 and visualized using TBtools 1.6 software.

Codon preference analysis of *MdHSP70* gene family

Codon use characteristics of CDS sequences of the *MdHSP70* gene were analyzed using CodonW 1.4.2 online software (http://codonw.sourceforge.net, accessed on 18 December 2023), including relative synonymous codon usage (RSCU), effective codon (ENC), and codon bias index (CBI), Codon Adaptation Index (CAI), Frequency of Optimal Codon Use (Fop), T3s, C3s, A3s, G3s, CAI, CBI, Nc, Fop, GC, GC3s, L_sym, L_aa, GRAVY, and Aromo parameter correlation analysis.

Fluorescence quantitative qRT-PCR analysis of *MdHSP70* gene family

RNA was extracted from leaves treated with 0.1 mM MeJA, 0.1 mM GA_3, 0.2 mM ABA, 400 mM NaCl, 10% PEG, 4 °C and control (normal-growing seedlings) using 56 gene qPCR primers designed and synthesized by Sangon Biotech (Shanghai) (Supplementary Table S3). The RNA was converted to cDNA by the kit (Prime Script RT reagent Kit, TaKaRa), and analyzed by PCR (LightCycler® 96 Real-Time PCR System, Roche, Switzerland), using 6 μL ddH_2O, 2 μL cDNA, 2 μL of upstream and downstream primers, and 10 μL of SYBR enzyme as the reaction system, and GAPDH as the internal reference gene. Roche, Switzerland). The relative gene expression was analyzed by the $2^{-\Delta\Delta CT}$ method.

Prediction of transmembrane structural domains and subcellular localization analysis of the *MdHSP70* genes

Use (https://services.healthtech.dtu.dk/services/TMHMM-2.0/, accessed on 1 July 2024) for MdHSP70 transmembrane structure prediction; *MdHSP70-6*, *MdHSP70-26*, *MdHSP70-45* were cloned using forward and reverse primers, and then ligated into the vector (pCAMBIA1300-GFP), and the untargeted GFP was used as an empty vector as a control. The recombinant plasmid and empty vector were introduced into *Agrobacterium tumefaciens* GV3101 and cultured, centrifuged at $5000 \times g$ for 10 min, and the organisms were collected. The bacteria were resuspended using buffer (50 mL sterilised distilled water + 500 μL MES + 200 μL $MgCl_2$ + 75 μL As) to an OD_{600} of 0.7–0.8. The resuspended bacterial solution was punched from the dorsal surface of the leaves of Benji's tobacco, which had been grown for about 25 d, using a 2 m L disposable syringe, and incubated in the dark at 25 °C for 48 h and then transferred to the normal light for 1 d. On the bacterial solution-injected A 0.2 mm × 0.2 mm microscopic sample was cut from the leaf injected with the bacterial solution with a scalpel and placed under an AX10 fluorescence inverted microscope (ZEISS, Germany) for observation and photographs.

Statistical analysis

The data were analyzed using IBM SPSS statistical 21, and figures were made using Origin 2018. The results were pre-sented as the mean values ± standard error (SE) of at least three independent experiments. Using Duncan's multiple interval test ($P < 0.05$) analyzed the statistical differences between treatments.

Data availability

All data generated or analysed during this study are included in this published article (and its supplementary information files). The datasets generated and/or analysed during the current study are available in the TAIR (The Arabidopsis Information Resource, https://www.arabidopsis.org/), GDR (GENOME DATABASE FOR ROSACEAE, https://www.rosaceae.org/), Phytozome13 (https://phytozome-next.jgi.doe.gov/) and String (http://string-db.org/) repositories.

Material availability

Our research material is 'Gala' apple test tube seedlings were provided by the Fruit Seedling Research Laboratory (36° 29′ N, 103° 21′ E) of Gansu Agricultural University. We have the right to collect plants, conduct experimental and field research on apples. At the same time, we comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of

Wild Fauna and Flora, and all methods were carried out in accordance with relevant guidelines.

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Author contributions

Conceptualization, J.M. and M.L; Methodology, M.L, and M.S; Software, Y.F and W.M; Validation, Z.B; Formal analysis, M.L; Writing—original draft preparation, M.L.; Writing—review and editing, G.L and J.M; All authors reviewed the manuscript.

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