The Hsp70 Gene family in *Solanum Lycopersicum*; Genome-wide Identification and Expression Analysis Under Heavy Metals Stresses

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Abstract

The Heat shock protein-70 (Hsp70) gene family is one of the protective mechanisms; however, it has not been widely studied in tomatoes. Therefore, the current study provides the first report genome-wide analysis of the Hsp70 gene family in tomato (*Solanum lycopersicum* L.) under five heavy metals (Cd^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and Fe^{2+}) stresses. We identified 23 candidate genes of the *Hsp70* gene family based on the PF00012 domain through bioinformatics studies, including gene structure, distribution, synteny, phylogenetic tree, protein-protein interactions, gene ontology, and previous RNA-seq data analysis followed by qRT-PCR analysis. Based on the phylogenetic analysis, the 23 candidate genes were classified into five subfamilies where the same subfamily contains similar SlHsp70 proteins. Many pairs of SlHsp70 gene duplications have appeared, consisting of tandem and segment duplication. In addition, analysis of previous RNA-seq besides the gene ontology gave us significant evidence about the vital roles of these genes during tomato development and growth. The SlHsp7s showed different responses, which were varied depend on different plant tissues and types of heavy metal. Some of the SlHsp70s were up-regulated after heavy metal exposure, such as Cd^{2+}/ *SlHsp70-23* and Mn^{2+}/ *SlHsp70-8*. Still, down-regulated others such as Fe^{2+}/ *SlHsp70-18*. Finally, our gene expression analysis revealed the significant roles of the Hsp70s, especially, *SlHsp70- 3, SlHsp70-8, SlHsp70-12, SlHsp70-19*, and *SlHsp70-23*, with the different heavy metals treatments.

1. Introduction

Tomato (*Solanum lycopersicum* L.) is a common and economically essential crop worldwide after potato with 177 million tons production (Cheng et al. 2020, Liu et al. 2020). Tomato among the plants is considered the most vulnerable to environmental stress (Abewoy 2018). The different kinds of abiotic stresses such as drought, salt, heavy metals, and extreme temperature negatively affect every tomato life stage, causing about 70% yield losses (Atkinson &Urwin 2012, El-Sappah et al. 2019). The response to heavy metal stress involves a complicated signal transduction network that is activated by sensing the heavy metals and is characterized by the synthesis of stress-related proteins and signaling molecules, and finally, the transcriptional activation of specific metal-responsive genes to counteract the stress (El-Sappah et al. 2012). The typical consequence of most abiotic stresses, including heavy metal (BERTAMINI 2001), is the increased production of reactive oxygen species (ROS). These ROS [i.e. superoxide radical (O^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-)] are extremely toxic to plants (El-Sappah 2013, Maksymiec 2007). They caused damage to DNA, proteins, lipids and chlorophyll (Schutzendubel &Polle 2002). However, plants are well equipped with an antioxidant system consisted of antioxidant enzymes (superoxide dismutase, peroxidase, catalase, glutathione reductase), low non-enzymatic molecule antioxidants (proline, tocopheroles, carotenoids, glutathione, ascorbic acid) to counter the oxidative stress to protect plants from oxidative injuries (Apel &Hirt 2004). Accordingly, it is important to understand the mechanisms that help tomatoes withstand different stress conditions. Plants, including tomatoes, have advanced a range of mechanisms to acclimatize to altering environmental conditions (Becklin et al. 2016).

The molecular chaperones heat shock proteins (HSPs) are one sort of plant response to environmental stresses (El-Sappah et al. 2017, Sung et al. 2003). Several classes of HSP have been identified in plants with molecular weights ranging from 10 to 200KD, where the *Hsp70* is one of them (El-Sappah et al. 2017). Hence, the induction of HSPs proteins is thought out as a critical protective, eco-physiologically adaptive, and genetically conserved response of organisms to environmental anxiety. Thus, they accomplish a key function in the hostility of stress by re-establishing normal protein conformation and cellular homeostasis (Rhee et al. 2009). Hsp70 families are highly conserved and encoded by a multi-gene family, which regulates the plant development as well as can be exist under different abiotic and biotic stresses (Liu et al. 2018, Song et al. 2019). In plants, *Hsp70* gene families can be divided into four major subgroups with different cellular localization depending on their motifs at the C-terminus; plastids (PEGDVIDADFTDSK motif), mitochondria (PEAEYEEAKK) motif, cytosol (EEVD motif), and the endoplasmic reticulum (HDEL motif) (Luo et al. 2015).

The Hsp70 family has been identified in many plants such as arabidopsis 18 genes (Lin et al. 2001); potato 20 genes (Liu et al. 2018); soybean 61 genes (Zhang et al. 2015); pepper 21 genes (Guo et al. 2016), maize 22 genes (Jiang et al. 2020), and tobacco 61 genes (Song et al. 2019). Many heat shock proteins, especially the Hsp70, were induced by heavy metal stress (Khan et al. 2019). For instance, the Hsp70 sub-family, DnaK (Bip), was up-regulated in rice seedlings under the Cd and Cu contamination (Ahsan et al. 2007). A similar finding was in *Populus trichocarpa*, *Glycine max*, *Arabidopsis thaliana* (Lomaglio et al. 2015, Neumann et al. 1994). Furthermore, the cultured flax on heavy metal treated media induced many heavy metal-binding proteins, including *Hsp70*, while *Hsp83* showed a down-regulation (Kosová et al. 2011).

Given the importance of resisting environmental hazards in tomatoes, and the whole tomato genome is now available (Wang et al. 2018), the identification and characterization of stress tolerance genes have become very urgent to tomato plant breeders and scientists. Therefore, the current study provides the first report genome-wide analysis of the *Hsp70* gene family in tomato under the heavy metals stresses. We identified 23 *Hsp70* genes using bioinformatics methods. The phylogenic and the interaction within this gene family was also declared, as well as their expression profile of different tomato tissues under some stress was mentioned revealed to the previous RNA sequencing data. Furthermore, the expression of all 23 *Hsp70* genes under different heavy metals stresses was measured. Our data will be essential to understand the functional structure and the genomic organization of the *Hsp70* gene members in the whole tomato genome that will be advantageous in further functional genomics characterization.

2. Materials And Methods

2.1. The identification of Hsp70 genes in tomato

The tomato sequences were obtained from the Solanaceae Genomics Network (https://solgenomics.net/) and then BioEide 7.0 software was used for the local database construction. The candidate tomato *MTP* genes were utilized used the HMM profile of the Hsp70 domain (Pfam: PF00012) (http://www.sanger.ac.uk/Software/Pfam). The blast of putative Hsp70 protein sequences has been done compared to tomato whole genome, at the NCBI (http://blast.ncbi.nlm.nih.gov/blast.cgi), SPud DB tomato Solanaceae Genomics Network (https://solgenomics.net) and phytozome (https://phytozome.jgi.doe.gov/).

The examination of all obtained protein sequences has been done at E-value < 10^{-5} for detecting the Hsp70 domain, using SMART (http://smart.embl-heidelberg.de/)tools (Letunic et al. 2004). All genomic information about the selected *Hsp70* gene family, such as chromosomal location and CDS, was obtained from the phytozome website database (https://phytozome.jgi.doe.gov/). The Hsp70 family's proteins were analyzed to obtain their characterization, such as the molecular weight, the number of atoms, the amino acid number, isoelectric point, and instability index using EXPASY PROTOPARAM (http://www.expasy.org/tools/protparam.html) (Gasteiger et al. 2003). Furthermore, the theoretical PI and molecular weight were obtained using ProtParam Tool (http://web.expasy.org/portparam).

2.2. Phylogenetic analysis

In addition to tomato, the potato and arabidopsis Hsp70 protein sequences have been downloaded from the Spud DB Potato Genomics Resources (http://solanaceae.plantbiology.msu.edu/) and the Arabidopsis Information Resources (TAIR) (http://arabidopsis.org). The CLUSTALX 2.0 software with default parameters has been used after that for Hsp70 proteins sequences multiple alignments. The previous alignment has been inserted into MEGA 6.0 software with a Neighbor-Joining method for the phylogenetic tree construction, and then, at last, the bootstrap analysis has been done at 1,000 iterations with a pair-wise gap deletion mode (Tamura et al. 2011).

2.3. Chromosomal locations, synteny analysis, and protein-protein interactions

The tomato gene database (https://phytozome.jgi.doe.gov/) supports us by the chromosomal position information of *Hsp70* genes, which have been used to generate the genetics map by MapChart software. After that, two genes in the same species, located in the same clade of the phylogenetic tree, were defined as being coparalogs to identify whether tandem and segmental duplication events had occurred. On the other hand, the tomato gene database (https://phytozome.jgi.doe.gov/) has been further used with target genes for detecting the coordinates of the segmental duplications. The paralogs were regarded to be the results of tandem duplicated when two genes separated by five or fewer genes in a 100 kb region (Tang et al. 2008). Additionally, the coparalogs were considered segmental duplications if they were located on duplicated chromosomal blocks (Wei et al. 2007). Smith-Waterman algorithm (http://www.ebi.ac.uk/Tools/psa/) had been used for the calculation of the local alignments of two protein sequences. The synteny relationship with the chromosomal distribution for each *SlHsp70* was introduced using circos (http://circos.ca/) (Krzywinski et al. 2009). Furthermore, for more knowledge about the cellular function of the Hsp70 protein family, the functional interactions between all expressed studied proteins are essential. The amino acid sequences of all the family members used for protein-protein interaction studies used the STRING database (https://string-db.org/).

2.4. Gene structures and motif analyses

The structure of all *Hsp70* gene family members was analyzed to detect the intron/exon and their organization, and this has been done using both genomic and CDS sequences with the online tools of the Genes Structure Display Server program (GSDS, http://gsds.cbi.pku.edu.cn/index.php) (Hu et al. 2015). The conserved motif has been also detected for the gene family members using a Multiple EM for motif elicitation (MEME) (http://meme.nbcr.net/meme3/meme.html) depends on the amino acid sequences (Bailey et al. 2006). The motif identification processes were adapted as a total maximum of 10 motifs in number and optimum motifs: 6 to 200 amino acid residues.

2.5. Protein modeling, prediction and the gene ontology annotation (GO).

The Phyre2 web has been used for protein modeling, prediction, and analysis of the SlHsp70 and this at intensive mode (sbg.bio.ic.ac.uk/phyre2/)(Kelley et al. 2015). Blast2GO v3.0.11 (https://www.blast2go.com) and OmicsBox sofware have been used with the identified Hsp70 protein sequences for GO annotation (Conesa &Götz 2008).

2.6. Gene expression analysis based on the RNA-seq data

In our research, we checked the previous RNA-based data analyses for obtaining the real Hsp70 family genes expression during normal conditions. The expression data were downloaded from Tomato functional genomics databases (http://ted.bti.cornell.edu/pgsc_download.shtml) for some of the tomato organs such as leaves, roots, and flowers. Then the gene expression has been analyzed using the cufflinks (version: 2.2.1). Finally, after dividing absolute FPKM values by their mean and transformed the ratio by log2, the MeV 4.5 was used to cluster the expression data as a heat map (http://heatmapper.ca/) (Babicki et al. 2016, Saeed et al. 2006).

2.7. Growth Conditions and Heavy Metal Treatments

In this study, the tomato M82 line was cultivated during the Autom of 2020 at the experimental greenhouse of Yibin University (China). First, the seeds were washed with 10% hypochlorous acid and distilled water. The seeds have been germinated using water-saturated filter paper, and then transferred to fertilized pittmoss soil with germination conditions of 16 hours light (27ºC) and 8.0 dark (18ºC) with a relative humidity of 70%.Thirty-day-old tomato were placed in 1/2 Hoagland solutions (pH 6.0) with different heavy metal treatments 0.1 mM $CdCl_2$, 0.1 mM $CoCl_2$, 0.5 mM $FeSO_4$,1 mM $MnSO_4$ and 0.5 mM $ZnSO_4$ respectively, while normal 1/2 Hoagland solutions as the control (CK) (Desoky et al. 2019, Gao et al. 2020). Then, 24 h later, the leaves and roots of tube plantlets were collected and used as RNA extraction materials.

2.8. qRT-PCR analysis

The Trizol reagent (Invitrogen, USA) has been used to isolate the RNA from all plant samples (leaf and root), and the cDNA synthesis SuperMix Kit (Transgen, Beijing) used for the construction of the cDNA. The specific primers of all selected genes have been designed used Primer 5.0 (Table S1) with B-actin as a housekeeping gene. The real-time PCR was performed with the following reagents volumes:10 μL SYBR premix Taq (2×) mixture, 1 μL of cDNA,0.5μL of each primer, and 8 μL of ddH_2O for a total volume of 20. The cycles have been adapted as follows: 95°C for 10 minutes; 40 cycles at 95°C for 15 seconds, and 60°C for 60 seconds. The relative expression has been calculated based on the Livak equation of $2^{-\Delta\Delta CT}$ values for three replicates of every sample (Livak &Schmittgen 2001).

Statistical analysis

The Student's t-test has been used to calculate the data at a significance level of 0.05 in Excel software. Three biological replicates of expression analyses have been performed with ± standard deviation (SD) at $p < 0.05$. The significant variations between means were compared at $p < 0.05$ (Duncan's Multiple Range Test). Furthermore, COSTAT computer software (CoHort Software version 6.303, Berkeley, CA, USA) was used for the statistical analysis.

3. Results

3.1. The identification of *Hsp70* genes in tomato

A total set of 31 genes were identified at the beginning of the research, and after excluding the incompleted domain genes, we finally selected 23 candidate genes for further evaluation and study. The genes have been given new names from *SlHsp70-1* to *SlHsp70-23*, as shown in Table 1. The whole set of tomato 12 chromosomes contributed to harboring the *Hsp70* genes, except chromosome number five, which does not carry any of these genes. Furthermore, the molecular weight varied between all genes, ranging from 21273.47 to 98787.40 kDa. The total numbers of negative L-alpha-aspartyl and L-glutamic acid residues (Asp + Glu) were the dominant in all studied genes except *SlHsp70-12.* Moreover the total number of gene intron was varied between the studied family members except for the two genes *SlHsp70-9* and *SlHsp70-13*, which have no intron. The content of every studied gene from their aminoacids were varied (ranging from 186 and 890 amino acids). Besides, all of the studied 23 genes have an acidic isoelectric point (PI), which indictor for *Hsp70* genes rich content in acidic amino acids.

Table 1
The characteristics of Hsp70 genes in tomato.

Hsp70 gene	Sequance ID	Location	(-)	(+)	MW	aa	Total no. of atoms	Instability	Aliphatic index	Intron	PI
SlHsp70-1	Solyc01g106210	SL2.50ch01:94157485..94161397	89	84	72969.84	681	10334	38.21	87.27	5	5.75
SlHsp70-2	Solyc06g076020	SL2.50ch06:47192489..47195586	102	82	71008.48	648	9984	32.79	82.02	1	5.04
SlHsp70-3	Solyc03g082920	SL2.50ch03:52794869..52798836	114	92	73457.21	667	10382	29.90	85.95	6	5.07
SlHsp70-4	Solyc10g086410	SL2.50ch10:65236863..65240232	100	81	70779.21	644	9949	35.11	82.39	1	5.07
SlHsp70-5	Solyc01g106260	SL2.50ch01:94215968..94220340	86	81	71876.57	670	10151	38.91	85.04	5	5.95
SlHsp70-6	Solyc07g043560	SL2.50ch07:57457649..57465996	134	121	98787.40	890	13932	39.17	80.89	12	5.91
SlHsp70-7	Solyc02g080470	SL2.50ch02:44673835..44685301	110	98	84108.25	753	11771	42.99	78.62	8	6.02
SlHsp70-8	Solyc06g052050	SL2.50ch06:35713191..35716219	102	82	67513.37	619	9543	29.09	87.74	8	5.04
SlHsp70-9	Solyc03g117630	SL2.50ch03:66724560..66726524	99	83	71849.40	654	10088	31.01	87.72	0	5.21
SlHsp70-10	Solyc01g099660	SL2.50ch01:89839013..89842124	112	98	74641.87	669	10568	34.03	87.16	6	5.36
SlHsp70-11	Solyc07g005820	SL2.50ch07:655717..659235	103	86	71953.43	654	10115	33.75	81.10	1	5.15
SlHsp70-12	Solyc03g117620	SL2.50ch03:66722304..66723457	18	30	21273.47	186	2997	46.72	73.33	1	9.37
SlHsp70-13	Solyc09g075950	SL2.50ch09:67581791..67583521	69	53	62723.17	576	8852	42.31	98.49	0	5.56
SlHsp70-14	Solyc11g020040	SL2.50ch11:10015582..10019521	101	90	74493.21	692	10544	27.93	84.36	7	5.36
SlHsp70-15	Solyc11g066100	SL2.50ch11:51773141..51775439	100	82	71458.91	654	10036	33.10	80.69	1	5.10
SlHsp70-16	Solyc04g011440	SL2.50ch04:3894918..3898067	100	82	71389.83	651	10030	32.91	80.78	1	5.13
SlHsp70-17	Solyc12g043110	SL2.50ch12:39110693..39115806	130	106	93882.82	852	13211	42.35	80.06	8	5.23
SlHsp70-18	Solyc12g043120	SL2.50ch12:39096307..39100382	130	105	92996.38	846	13051	42.45	77.74	8	5.22
SlHsp70-19	Solyc08g082820	SL2.50ch08:65489311..65493585	113	93	73200.96	666	10357	30.84	87.85	7	5.10
SlHsp70-20	Solyc08g079170	SL2.50ch08:62804339..62810456	98	91	65165.56	579	9124	36.18	67.03	6	5.99
SlHsp70-21	Solyc01g103450	SL2.50ch01:92060728..92065237	98	85	74896.54	703	10598	25.83	86.13	7	5.20
SlHsp70-22	Solyc11g066060	SL2.50ch11:51740558..51743431	101	90	77141.77	698	10869	33.98	84.14	2	5.51
SlHsp70-23	Solyc09g010630	SL2.50ch09:3965253..3968837	100	82	71224.69	649	10008	35.04	82.53	1	5.13

(-), (+), MW, aa and PI refer to, the total number of negatively charged residues (Asp + Glu), the total number of positively charged residues (Arg + Lys), molecular weight, amino acid number and isoelectric points, respectively.

3.2. Phylogenetic analysis of *Hsp70* gene families

The phylogenetic tree was done using 19 *Hsp70* gene members from potato and 20 genes from arabidopsis besides the 23 *Hsp70s* of tomato using their amino acid sequences with Neighbor-Joining methods at MEGA5.0 software and 100 replicants bootstrap value. The *Hsp70* families, according to this tree, lie in four groups which were Group I, II, III, and IV (Fig. 1). Group, I contained large numbers of *Hsp70* compared to other groups. It contained eight, nine, and six genes from tomato, potato, and arabidopsis, respectively, while group IV contained the second largest number of studied 23 genes of tomato, which were six genes.

3.3. Chromosomal locations and synteny analysis of *Hsp70* gene family

Further studies have been done on all members of the *SlHsp70* gene family, which aimed to further identify their location on the tomato chromosomes and their effect on the gene duplication. As mentioned before, all sets of the *Hsp70* genes are distributed on all tomato chromosomes except chromosome 5, which does not harbor any genes of the studied *Hsp70* family. Chromosomes 1,3, and 11 harbors the largest number of the *Hsp70* gene family, where they carry ten whole of this family genes. Furthermore, for the evolution of novel functions and gene family expansion, we investigated gene duplication and divergence. Our results revealed segmental and tandem gene pair duplication from PGDD (Plant Genome Duplication Database) with circos for examining the duplications behavior on *Hsp70* gene family members. One hundred twenty pairs of collinearity gene pairs with identity ranging from 50–100% were detected and these due to the exist of the two types of duplication, segment and tandem (Table S2). The segment duplication resulted in many homologies of *Hsp70* genes between the chromosomes within the tomato genome, such as what occurs with the genes, *SlHsp70-3*, *SlHsp70-10*, and *SlHsp70-13* (Fig. 2). On the other hand, there are five obvious tandem replicated gene clusters on chromosomes 1, 3, 8, 11, and 12.

3.4. Phylogenetic analysis, gene structures, and motif analyses of the tomato *Hsp70* gene family

The *Hsp70* gene members were divided into five subfamilies (A, B, C, D and E). Subfamily A was the largest among all the subfamilies with eight genes, followed by subfamily E with six genes, while subfamily D contains only one gene (Fig. 3a). There was variation in exon-intron between different genes where they showed a high degree of similarity in the same subfamily that supports the close evolutionary relationships of tomato *Hsp70* gene family members (Fig. 3c). Our analysis showed that all of the *Hsp70* family members contained a greatly varied number from intron except in the two genes *SlHsp70-9* and *SlHsp70-13* which have no intron. The largest intron is often indicated the complexity of gene structure. We analyzed all of the conserved motifs of *SlHsp70* using MEME depends on the amino acid sequences with ten motifs (Fig. 3b, and Table 2). All of the studied motifs contain 50 amino acids, except motif 4, 6, and 8 with 29 amino acids, motif 41, 33, and 29, respectively. The largest number of common motifs were 1,3 and 8, which have been noticed within all the subfamilies except D, followed by the motifs 4, 7, and 6, which disappear in only two of the studied Hsp70 genes. The number, type, or order of motifs pretty much was observed to similar within the same subfamily moreover between different families.

Table 2
Analysis of the 10 conserved motifs of SlHsp70 genes in tomato.

Motif	Logo	Best possible match	Width
1		VKBAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAJAYGLDKK	50
2		LLDVTPLSLGJETAGGVMTKLIPRNTTIPTKKEQVFSTYSDNQPGVLIQV	50
3		EKNVLVFDLGGGTFDVSJLTIEEGIFEVKATAGDTHLGGEDFDNRLVNHF	50
4		TRARFEELNMDLFRKCMEPVEKCLRDAKLDKSDIHEVVLVGGSTRIPKVQ	41
5		EGERARTKDNNLLGKFELSGIPPAPRGVPQIEVCFDIDANGILNVSAEDK	50
6		FNGKEPCKSINPDEAVAYGAAVQAAILSG	33
7		ERLIGDAAKNQAAMNPENTVFDAKRLIGRRFSDP	50
8		FKRKHKKDISGBPRALRRLRTACERAKRTLSSTAQTTIEIDSLYEGIDFY	29
9		FKRKHKKDISGBPRALRRLRTACERAKRTLSSTAQTTIEIDSLYEGIDFY	21
10		YKGEEKQFSPEEISAMVLTKMKEIAEAFL	29

3.5. Protein modeling, prediction, protein-protein interactions and GO enrichment analysis

Phyre 2 web portal for protein modeling (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) has been processed using amino acid sequences of SlHsp70s (Fig. 4 and tables. S 3). The eleven predicted models of the SlHsp70s were based on c5tkyA_, c5e84B_, c3d2fC, c2khoA, c3c7nB_and c5obuA_ templates with 100%. On other hand, the STRING investigation of protein-protein interactions showed the physical (direct) and the functional (indirect) associations (Fig. 5 and Table S4). The result showed different interactions within the studied proteins where the total number of nodes was 22 with an average of 8. 82. The STRING database analysis showed 97 edges with 14 expected one and showed 13 expected local network clusters, which were CL:4682, 4141, 4138, 4661, 4411, 4655, 4020, 4084, 4021, 4025, 3997, 4023 and 4339. Cluster 3997 was the biggest, containing 12 of the *Hsp70* proteins (Table S5). Moreover, our protein analysis showed the appearance of two common domains within the checked members of the Hsp70 family, which were PF00012 (23 proteins) and PF06723 (19 proteins), (Table S6). Besides, our checked proteins family showed five pathways according to the Kyoto Encyclopedia of Genes and Genomes (KEGG), which were sly04141 (14 proteins), sly03060 (3 proteins), sly04144(10 proteins), sly03040 (10 proteins), and finally sly03018 (two proteins) (Table S6). Similarly, sub-cellular localization, molecular function, and biological process were predicted by GO enrichment analysis (Fig. 6 and Table S5). In sub-cellular localization analysis, the predicted distribution scores of Hsp70 proteins were as following; 2/4% in vaculoar membranes, 3.5/8% in the chloroplast, and 9/42% in endoplasmic reticulum lumen. Noticeably, the *SlHsp8* gene was localized in 21 sub-cellular compartments out of all 28, which underlined the significant role of *SlHsp8* in metal stress resistance. Collective scores of Hsp70 protein molecules during biological processes were as following; response to heat and cadmium was 3/4%, while cellular response to stress was 17/23% and cellular response to chemical stimulus was 15/23%. More precisely, *SlHsp8*, *SlHsp12*, and *SlHsp19* play a key role in response to different stresses. Molecular function analysis revealed significant roles of *SlHsp12 and SlHsp23* in heavy metal processes.

3.6. Gene expression analysis based on the RNA-seq data

Previous RNA- RNA-seq data for the expression profile of the *Hsp70* genes in different tomato tissues have been used for screening the expression level of these genes family in flower buds, opened flowers, 1 cm fruits, 2 cm fruits, 3 cm fruits, mature green fruits, breaker fruits, breaker + 10 fruits, roots, and leaves (Fig. 7, and Table S8). Depending on previous RNA seq the highest expression levels were detected with *SlHsp70-23* in all selected tissues except in breaker + 10 fruits, where the *SlHsp70-15* recorded the highest level. Moreover, the lowest expression level was detected with the *SlHsp70-10 and SlHsp70-11*.

3.7. qRT-PCR analysis of the SlHsp70 genes under different environmental stresses

As shown in Fig. 8, different expression patterns of different SlHsp70s in tomato leaves and roots were observed under the five heavy metals stresses. The 23 *Hsp70* genes showed differential vaulues with different types of heavy metals in root and leaf : In root, Cd^{2+} enhanced the expression of *SlHsp70-1, SlHsp70-2, SlHsp70-3, SlHsp70-4, SlHsp70-5, SlHsp70-8, SlHsp70-10, SlHsp70-11, SlHsp70-12, SlHsp70-13, SlHsp70-19* and *SlHsp70-23* but decreased the expression of *SlHsp70-7, SlHsp70-17* and *SlHsp70-20* ; Co^{2+} decreased the expression levels of *SlHsp70-7, SlHsp70-18, SlHsp70-20* and *SlHsp70-21* but increased the expression levels of *SlHsp70-1, SlHsp70-3, SlHsp70-5, SlHsp70-8, SlHsp70-10, SlHsp70-11, SlHsp70-12, SlHsp70-13, SlHsp70-15, SlHsp70-19*, and *SlHsp70-23*; the *SlHsp70-3, SlHsp70-8, SlHsp70-17, SlHsp70-19* and *SlHsp70-23*, recorded the highest expression but the expression of other two genes *SlHsp70-14* and *SlHsp70-21* it decresd under Mn^{2+} contamination ;Zn^{2+} increased the expression of *SlHsp70-3, SlHsp70-8, SlHsp70-10, SlHsp70-11, SlHsp70-12, SlHsp70-19* and *SlHsp70-23* while it decresed the expression of *SlHsp70-14, SlHsp70-18* and *SlHsp70-21* ; Fe^{2+} increased the expression levels of *SlHsp70-3, SlHsp70-8, SlHsp70-19* and *SlHsp70-23* but decresed the expreseeion of *SlHsp70-18, SlHsp70-20* and *SlHsp70-21.* In leaf, Cd^{2+} enhanced the expression of *SlHsp70-1, SlHsp70-2, SlHsp70-3, SlHsp70-4, SlHsp70-5, SlHsp70-8, SlHsp70-10, SlHsp70-12, SlHsp70-13, SlHsp70-15* and *SlHsp70-23* while decrese the expression of *SlHsp70-7*, *SlHsp70-17* and *SlHsp70-20*; Co^{2+} increased the expression of *SlHsp70-1, SlHsp70-3, SlHsp70-4, SlHsp70-5, SlHsp70-8, SlHsp70-10, SlHsp70-12, SlHsp70-13, SlHsp70-15 SlHsp70-19* and *SlHsp70-23* but decreased the expression of *SlHsp70-7, SlHsp70-14, SlHsp70-17* and *SlHsp70-18*; Fe^{2+} increased the expression of *SlHsp70-17* and *SlHsp70-23* but reduce the expression of *SlHsp70-18*, *SlHsp70-20* and *SlHsp70-21* ; Mn^{2+} decrease the expression of *SlHsp70-14* and *SlHsp70-21* but enhanced the expreesion of *SlHsp70-3, SlHsp70-8, SlHsp70-12* and *SlHsp70-23*; Zn^{2+}, decrese the expression of *SlHsp70-7, SlHsp70-14, SlHsp70-18* and *SlHsp70-21* but enhanced the expression of *SlHsp70-1, SlHsp70-3, SlHsp70-8, SlHsp70-10, SlHsp70-12* and *SlHsp70-23*.

4. Discussion

The Hsp70 considers one of the conserved protein family, which is essential for plants as it participates in multiple roles such as protection of other proteins during stress, assistance in protein translocation, and protein biogenesis (Ray et al. 2016). The Hsp70 has previously been studied in several plants, such as Arabidopsis (Lin et al. 2001), potato (Liu et al. 2018), pepper (Guo et al. 2016), and cotton (Rehman et al. 2020), while this is the first study of *Hsp70* in tomato. Therefore, our study implements a genome-wide identification and expression analysis of the tomato *Hsp70* gene family based on *Hsp70* gene family selection, phylogeny, chromosomal locations, gene structure, motif sequences, protein-protein interactions, synteny analysis, and the expression profile based on previous RNA sequencing data or current environmental stresses treatments. The tomato *Hsp70* gene family was 23 genes distributed in four groups based on the phylogenetic analysis of *Hsp70* gene families in tomato, Arabidopsis, and potato (Fig. 1) as what mentioned in the other plants, such as the studies of Liu et al. (2018), who after phylogenetic analysis confirmed the exist of the four Hsp70 groups in potato. Furthermore, based on the phylogenetic tree and motif analysis of the tomato Hsp70 proteins, the studied family was consist of five subfamilies (Figs. 3), which are A, B, C, D, and E, where within the same sub-families,the most closely related and similar Hsp70 were found. This information related to gene structure from exons, introns, and motif sequences were identical to the previous studies in which a similar gene structure was found within the same subfamilies (Liu et al. 2018). For an instant, most of the gene members of subfamily A contain one intron. Howerver, most of subfamily E members contain eight introns,where as most of the subfamily C contains seven introns. These outcomes referred that during the structural evolution of tomato Hsp70, some intron gain and loss events may have occurred. Besides, the exons are lower than the introns in the gain/loss rate due to higher selection pressure in the exons sequences (Harrow et al. 2006). Besides, with all these observations, we have got to make sure that the placement divergences in intron number consider shared events that are more related to the evolution (Babicki et al. 2016, Jeffares et al. 2006, Rogozin et al. 2012).

Furthermore, the gene in which the introns exist and the host organism are two main determining factors of the intron evolutionary fate (Jeffares et al. 2006). In our study, to more knowledge about the gene annotation and the expansion mechanism of the Hsp70 gene family in tomatoes, we investigate the gene synteny and duplication analysis (Figs. 2 and Table S2). The exist of two or more genes on the same chromosome is often related to tandem duplication, while segmental duplication often occurs on different chromosomes (Schlueter et al. 2007). Our studies showed five tandem duplication pairs while there are more than 100 segmental duplications, such as the pairs *SlHsp70-7/ SlHsp70-17*, *SlHsp70-7/ SlHsp70-18*, and *SlHsp70-10/ SlHsp70-15.* In-plant gene families, the gene duplications events in its types followed by divergence, consider standard features and more related to secondary plant metabolism genes (Ober 2005). Hence, our results about the gene duplications confirmed their essential roles in the *Hsp70* genes family expansion.

On the other hand, we used amino acid sequences of the SlHsp70s for predicting the protein 3D structure. Moreover some additional relevant information such as the structures of related proteins where its three-dimensional structure usually specifies the protein's function (Fig. 4 and tables S3) (Büyükköroğlu et al. 2018). The c3d2fC template (100% confidence) has been used for modeling most of Hsp70 members except with (*SlHsp70-1* / *SlHsp70-5*), *SlHsp70-12*, *SlHsp70-13* and *SlHsp70-20* which used the c2khoA, c5gjjA, c5tkyA and C5nnrD templates respectively.

On the other hand, the protein-protein interaction considers an essential side of plant systems biology, which provides us more knowledge about the regulation of the plant developmental processes with plant and environmental interactions (Struk et al. 2019). Our protein-protein interactions analysis showed different interactions between the 23 proteins, which formed five pathways (Fig. 5).

On the other hand, gene ontology is a fundamental analysis to study each gene's different contributions across the living organisms (Consortium 2010). Moreover, gene ontology classes and concepts have been used to define the relationships and a gene function existing between these concepts (Purwantini et al. 2014). our gene ontology analysis showed the vital role of the tomato's SlHsp70 genes with heavy metals and other stresses (Fig. 6 and Table S7).

Furthermore, the GO showed the Hsp70 genes' molecular function where more most of them participate in metal-related processes such as unfolded protein binding,protein folding chaperone ion binding misfolded protein binding, and the oxidoreductase activity. HSP70 like DnaJ make a set of prominent cellular machines as aresult of the compination between the their chaperones with co-chaperones, to prevent the accumulation of newly synthesized proteins as aggregates and ensure the proper folding of protein during their transfer to the destination (Al-Whaibi 2011).

Thus, we investigated the expression profile of all members of the *Hsp70* gene family from previously published RNA-sequencing data, which showed the expression of all gene members in all selected tomato tissues (Fig. 7 and Table S8). Worth evidence has been obtained about the essential roles of tomato Hsp70 genes after tissue expression evaluation. For instance, the exclusive increased expression as stage progress of the five genes *SlHsp70-8, SlHsp70-13, SlHsp70-14, SlHsp70-20*, and *SlHsp70-23* in plant fruit, while 14 of the studied Hsp70 showed a significant increase in their expression at the open flower than the closed one indicating that they might participate in early fruit and flower development, respectively. Besides, the vital expected roles of *SlHsp70-23* in all plant tissues and developmental stages. However, only *SlHsp70-10* and *SlHsp70-11* were rarely expressed in all examined tissues from all *SlHsp70* genes. The documented reduction in some gene expression is an essential factor for maintaining the gene duplicates and ancestral functions (Qian et al. 2010). Hence in our study, the reduction of *SlHsp70-10* and *SlHsp70-11* expression is expected to be vital for keeping their biological functions and maintain them from losing during the cell evaluation. Our finding agreed with Rehman et al. (2020), who investigated the differential expression analysis profiles of the Hsp70 family in cotton, which revealed their essential roles in plant development and tolerance. Generally, many Hsp70 genes have low expression while very few Hsp70 genes have high expression in developmental processes or specific organs (Chauhan et al. 2012, Neta-Sharir et al. 2005). There were 61, 32, 30, 18, 22, and 20 Hsp70 genes identified in *Nicotiana tabacum*, *Oryza sativa, Gossypium raimondii*, *Arabidopsis thaliana*, *Zea mays* and *Solanum tuberosum*, respectively (Jiang et al. 2020, Liu et al. 2018, Song et al. 2019). Most of the Hsp70 families in different plants exhibited different expression profiles under different environmental stresses (Zhang et al. 2015). The qRT-PCR has been processed for more authenticity after the transcriptome data analysis; however, the minor asymmetry between both analyses due to different growth conditions and tomato varieties, which finally affect the SlHsp70s expression. The transcription of Hsp70s in response to various heavy metals varied and complicated, although the gene expression response to different stresses is usually reflected in corresponding gene roles. In our study, the expression of Hsp70 members was different by a different type of heavy metals. For instant, the Cd^{2+} enhanced the expression of some genes such as *SlHsp70-1, SlHsp70-2, SlHsp70-3*, and *SlHsp70-4*, while it causes inhibition to others such as *SlHsp70-7* and *SlHsp70-14.* The Co^{2+} enhanced the expression of some genes such as *SlHsp70-15, SlHsp70-19*, and *SlHsp70-23* while it causes inhibition of others such as *SlHsp70-7* and *SlHsp70-14.* Zn^{2+} increased some genes, such as *SlHsp70-12* and *SlHsp70-23*, while it causes decreases in others. As the same as the Mn, which enhanced the expression of some genes such as *SlHsp70-8* and *SlHsp70-19* while it decreases the expression of *SlHsp70-18 and SlHsp70-21*. Finally, some genes showed high expression after Fe^{2+} exposure, such as *SlHsp70-3* and *SlHsp70-8*, while negatively affecting others. Generally, the five genes *SlHsp70- 3, SlHsp70-8, SlHsp70-12, SlHsp70-19*, and *SlHsp70-23* showed the highest expression under heavy metal tratments. Our finding agreed with many of transcript analysis in many plant species in *Populus trichocarpa (*Lomaglio et al. 2015), *Lycopersicon peruvianum* L.(Neumann et al. 1994), *Glycine max* (Hossain et al. 2012), *Arabidopsis thaliana* (Sarry et al. 2006), *Populus tremula* (Kieffer et al. 2008), and *Populus nigra* (Lomaglio et al. 2015) which showed the high levels of their *Hsp70* under the Cd stresses. Also, Spijkerman et al. (2007) studied the essential roles of HSPs 70A in *Chlamydomonas acidophila* under both Fe^{2+}, and Zn^{2+} stresses. Another study by Basile et al. (2015) used the *Hsp70* s as specific markers of heavy metals pollution in *Lemna minor.* Also, our finding agreed with Kosová et al. (2011), who recorded a high expression level of *Hsp70* in flax after treated the cultured plant with the heavy metals while the *Hsp83* showed down-regulation. Moreover, many other studies showed the essential roles of the Hsp70 with different heavy metal stresses in Arabidopsis (Lee &Ahn 2013), Poplar (Augustine et al. 2015), and rice (Rodríguez-Celma et al. 2010). As more knowledge about the *Hsp70* family is absent, our studies will be essential for investigating Hsp70s molecular roles in tomatoes under various heavy metals stresses.

5. Conclusions

Tomato plants, which are grown widely, are more sensitive to environmental stress than other plants due to their exposure to many environmental stresses such as heavy metals. Therefore, we performed bioinformatics and expression studies on the *SlHsp70* gene family, where we selected 23 genes in tomato as family members. The analysis of their organizations, distributions interactions, and structures supplied us with great evidence for the complicated evolutionary history of this studied family in tomato, as same as the protein-protein interaction and the 3D modeling showed. Moreover, the *SlHsp70* gene family members showed many duplications distributed between tandem and segment duplications that are the major cause of the expansion of the genes. The RNA-seq data from previous studies besides the gene ontology indicated the multiple roles of these gene family members during the normal and abnormal environmental conditions. Finally, our expression analysis of *SlHsp70* genes at different tomato tissues showed different responses to heavy metals (Cd^{2+}, Co^{2+}, Mn^{2+}, $Zn^{2+,}$ and Fe^{2+}). Our expression analysis revealed the significant roles of the Hsp70s, especially, *SlHsp70- 3, SlHsp70-8, SlHsp70-12, SlHsp70-19*, and *SlHsp70-23*, under heavy metals treatments. The comprehensive studies of this gene family will help facilitate additional research about the molecular functions and evolutionary history of the tomato *Hsp70* gene family.

Declarations

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Figures

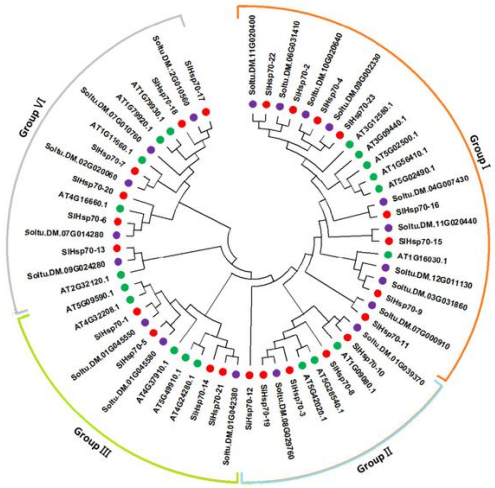

Figure 1

Figure 1

Phylogenetic tree of the 61 Hsp70 proteins: 23 Tomato (marked by red circle) ,19 Arabidopsis (green circle) and potato 19 (move circle). ClustalX1.83 was used for protein alignments and the phylogenetic tree's construction Neighbor-Joining (NJ) level with MEGA5.0 software at 1,000 replications bootstrap.

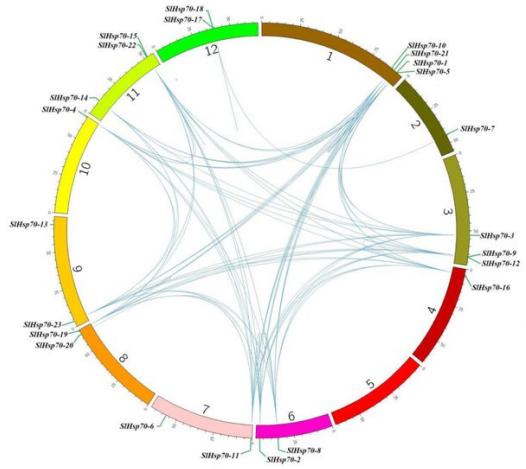

Figure 2

Figure 2

Genome-wide synteny analysis for Hsp70 gene family at 12 tomato chromosomes. The blue lines represented the syntenic orthologs and paralogs and showed the segment duplication while the he tandem duplicated genes were referred by red boxes..

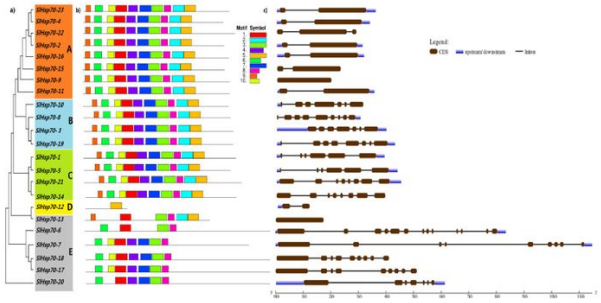

Figure 3

Figure 3

Phylogenetic relationship, gene structure and conserved motif analysis of SlHsp70 genes; a) The neighbor-joining phylogenetic tree was constructed with MEGA7 using SlHsp70 amino acid sequences with 1000 times replicate. b) The motif composition of SlHsp70 proteins using ten conserved motifs is represented by the unique colour mentioned in the box on the top lift. c) Exon-intron structure of the Hsp70 tomato proteins where dark green boxes presented the exons, and the black lines represent the introns. The blue boxes represented the untranslated regions (UTRs), with size scales detailed at the bottom.

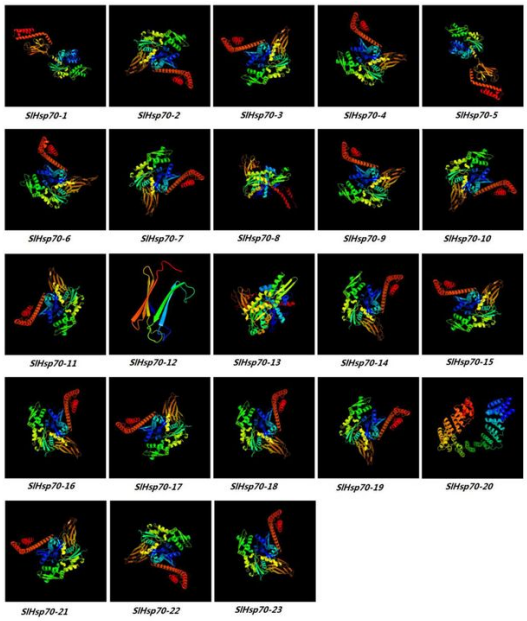

Figure 4

Figure 4

Predicted 3D models of tomato SlHsp70 proteins . Models have been generated by using the Phyre 2 server in intensive mode. Models were visualized by rainbow colour from N to C terminus and organized in SlHsp70-1, SlHsp70-2,…to SlHsp70-23,

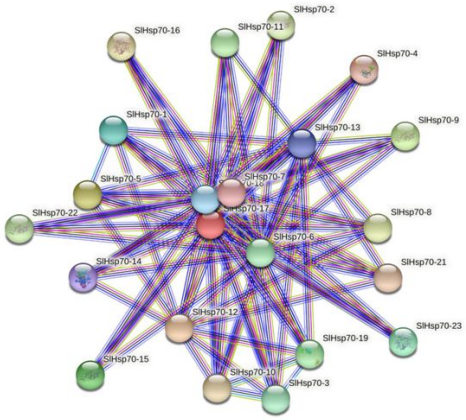

Figure 5

Figure 5

protein-protein interaction among the Hsp70 family members in tomato.

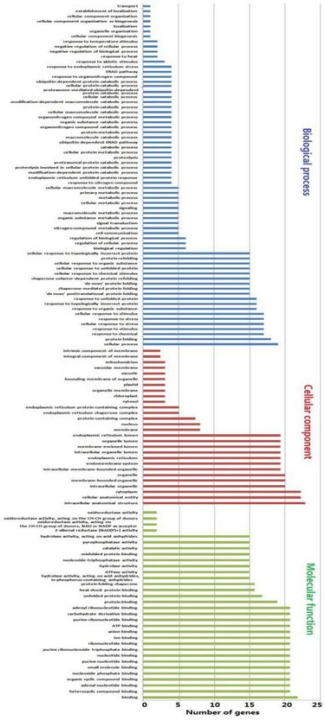

Figure 6

Figure 6

Gene Ontology analysis of tomato Hsp70 genes. Gene ontology showed the distribution of every SlHsp70 genes in the plant, where a red colour column mentioned the cellular component. In contrast, the biological processes in which the Hsp70 family participate were mentioned by the blue colour column, and the molecular function was mentioned by green colour.

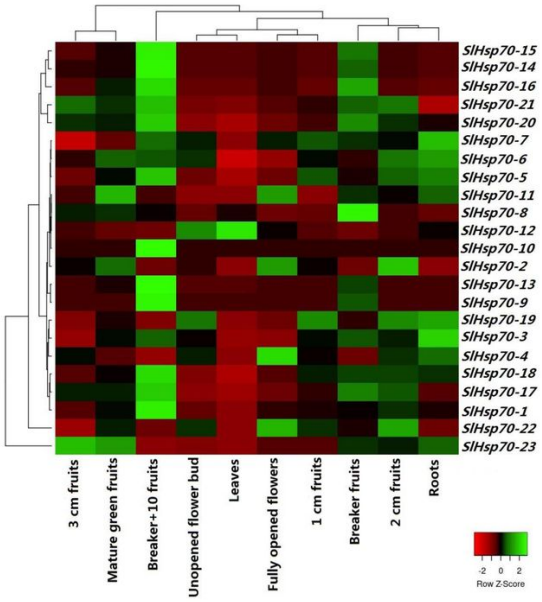

Figure 7

Figure 7

The heat map of the 23 SlHsp70 genes expression profiles in different tomato tissues based on the RNA-seq (http://ted.bti.cornell.edu/).

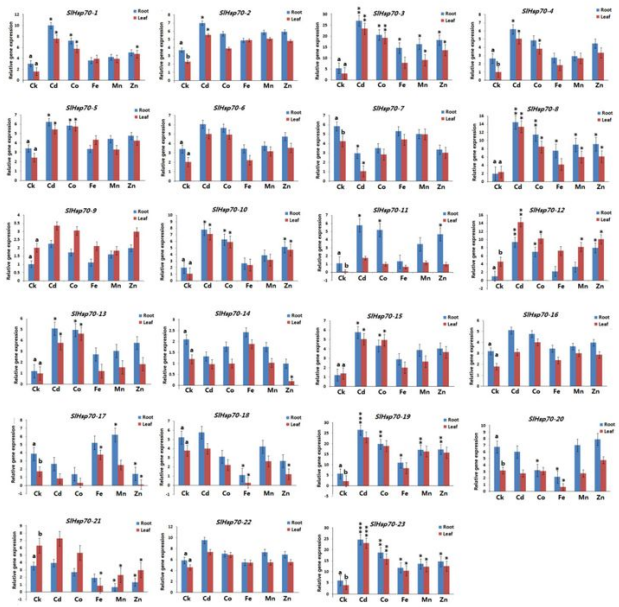

Figure 8

Figure 8

The qRT-PCR expression of the tomato SlHsp70 genes from root and leaf samples under various metal ion stresses. The reactions were normalized using the β-actin reference gene. The standard deviations have been represented by the error bars from three independent technical replicates. The mean expression levels of three replicates were analyzed with the five heavy metals treatments (Cd2+, Co2+, Fe2+, Mn2+ and Zn2+) using t-tests ($p < 0.05$) while the CK represent control samples . Asterisks indicate significant differences between the treatment samples and the corresponding control samples in roots and leaves. ($n = 9$, $p < 0.05$, Student's t-test).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Geneontology.xlsx
- S5.clusters.xlsx
- TableS1.primersforHsp70familyexpression.docx
- TableS2Sl.MTP.synteny.xlsx
- TableS6.domain.xlsx
- previousRNAseq.xlsx
- renamedc7acf.xlsx
- stringinteractions.xlsx