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Original Research

Genome-Wide Analysis and Expression Profiling of *SlHsp70* Gene Family in *Solanum lycopersicum* Revealed Higher Expression of *SlHsp70-11* in Roots under Cd^{2+} Stress

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Abstract

Background: Tomato is an important part of daily food, rich source of multitude nutrients, suitable candidate for bio-pharmaceutical production due to berry size and has numerous health benefits. Transcriptional regulation of metalloregulatory heat shock protein-70 family plays pivotal role in plants tolerance against abiotic stress factors including salinity, heat, cold, drought and trace metal elements such as cadmium (Cd^{2+}). **Methods**: Here, we provide comprehensive report on *in-silico* identification of *SlHsp70* family genes in tomato (*Solanum lycopersicum*) and their expression in tomato *via* qPCR analysis under broad range of trace metal elements. **Results**: *In-silico* analysis revealed 23 *SlHsp70* family genes in tomato, phylogenetically divided into four groups I–IV and displayed expression in all tissues. Gene Ontology (GO) analysis revealed that SlHSP70 proteins were membrane localized which were involved in metal ions translocation and oxidoreductase activity to counter hyper-accumulation of reactive oxygen species (ROS). **Conclusions**: Cd^{2+} is a widespread heavy metal soil contaminent which is continously polluting fertile soils, a knotty issue which has serious implications over photosynthesis, nitrogen assimilation, minerals and water absorption by plants. Plants exposure to Cd^{2+} and subsequent qRT-PCR analysis revealed increased expression of *SlHsp70-11* in tomato roots, which can be employed in breeding low Cd^{2+} enriched tomato varieties.

Keywords: *SlHsp70*; *Solanum lycopersicum*; RNA-seq; qRT-PCR; trace metal elements stresses

1. Introduction

Latest genetic engineering tools have increased commercial importacne of tomato (*Solanum lycopersicum* L.) by increasing nutritional value *via* bio-fortification, improving shelf life, developing berry size and bio-pharmaceuticals [1–3]. Tomato is being cultivated on a large scale in different soil types and under different biotic and abiotic stress conditions [4,5]. Tomato crop is highly vulnerable to harsh environmental conditions [6]. Abiotic stresses including salinity, drought, trace metal elements and high temperature cause severe yield loss upto 70% [7,8]. Trace metal elements stresses induce a complex signaling pathway which subsequently activates transcription of metal-responsive genes [9,10]. Trace metal elements stresses also induce over-accumulation of

reactive oxygen species (ROS) such as hydroxyl (OH^-), superoxide radical (O^-) and hydrogen peroxide (H_2O_2), which result in damaging essential biomolecules such as lipids, chlorophyll, DNA and proteins [11–13]. In response, metal chelating molecules, variation in gene expression and antioxidants such as peroxidase (POD), glutathione reductase (GTR), superoxide dismutase (SOD), catalase (CAT) and ascorbic acid (ABA) protect plants from ROS [14,15].

Hsp70 genes are induced under sudden and rapid increase in temperature. Initiation of transcription of *Hsp70* genes in plants is a protective eco-physiological adaptation and a conserved genetic response against abiotic stress factors. Initiation of Hsp70 family gene transcription in response to abiotic stressors aids acclimatisation by restoring normal confirmations of damaged metabolites and maintaining cellular homeostasis [16]. The *Hsp70* gene family is very conserved and comprised of multiple genes which play key role in regulating plant developmental processes to endure abiotic stress conditions [17,18]. Polypeptides of HSP70 family in plants differ in their molecular weights (MW) from 10 kDa to 200 kDa [19,20]. HSP70 proteins bind with heat-denatured metabolic proteins to protect them from aggregation and refold them to their original quaternary conformation to perform normal functions. These proteins are predominantly involved in translation, translocation and metal homeostasis as metal contents exceed normal limit [21,22]. On the basis of C-terminal, HSP70 family protiens are divided into four subgroups, which are differnetially localized and function in almost all cell oraganells. For example, HSP70 of cytosol, plastids and mitochondria harbor EEVD, PEGDVIDADFTDSK and PEAEYEEAKK motifs, respectively [23].

Plants absrob soil-born trace metal elements such as Cadmium (Cd^{2+}) by roots which has serious implications over metabolic processes including photosynthesis, nitrogen fixation, oxidative stress, carbohyderate metabolism, water and nutrient absorption which, consequently, result in stunted roots and loss in biomass and yield [24]. Cd^{2+} stress induces numerous gene families including HMA, P-ytpe ATPases, Hsp70, Hsp83 and Hsf. DnaK (Bip), a member of HSP70 sub-family displayed overexpression in Aarabidopsis, rice and soybean under Cd^{2+} stress [25–27]. Similarly, expression of BnHMA gene family in *Brassica napus* was upregulated under Cd^{2+} stress [28]. Flax cultivated in growth medium supplemented with trace metal elements exhibited irregular expression of number of heavy metal-binding proteins, i.e., members of *Hsp70* family were over-expressed while members of *Hsp83* were down-regulated [29]. Cd^{2+} stress indirectly induces oxidative pathways under different metals including *ZnSOD*, *FeSOD*, *MnSOD* and *POD* [30].

To develop Cd^{2+} stress tolerant tomato varities, it is prerequisite to retrieve and characterize genes in roots responsible for trace metals tolerance. Availability of whole sequenced genome has enabled to retrieve and characterize abiotic stress tolerance gene family in tomato [31]. In this study, we performed widely used *in-silico* analyses for genome-wide identificaiton of *Hsp70* family genes in tomato. Further, we performed *in-vitro* expression analyses of *Hsp70* family genes under trace metal elements stress with the help of robust and reproducible qRT-PCR technique. Speceific names were assigned to gene sequences based on homology and evolutionary relationship with Arabidopsis and potato *Hsp70s* by constructing an outgroup rooted phylogenetic tree. This study will provide pave the way to precisely engineer tomato plants for enhanced tolerance for Cd^{2+} stress.

2. Materials and Methods

2.1 Retrival of Hsp70 Genes Sequences

Whole genome of tomato (*S. lycopercicum*) was downloaded from Solanaceae Genomics Network (https://solgenomics.net/) database to construct a local database by employing BioEide7.0 (bioedit.software.informer.com). In order to retrieve tomato *SlHsp70* gene sequences, *Arabidopsis thaliana AtHsp70* gene sequences were BLAST-analyzed against newly constrctued local tomato database. The Hidden Markov Model (HMM) was employed to validate *SlHsp70* gene sequences and their functional domains particularly by running against each *AtHsp70* gene (Pfam: PF00012). Incomplete raw reads were excluded and assigned specific name to each gene according to Mendel database for plant gene families listed in Commission on Plant Gene Nomenclature (CPGN) (mbclserver.rutgers.edu/CPGN/), International Society of Plant Molecular Biology (ISPMB) [32]. In order to further validate retrieved *SlHsp70* genes, BLAST analyses were also performed in NCBI genome database by selecting tomato whole genome (blast.ncbi.nlm.nih.gov/blast.cgi), SPud DB tomato Solanaceae Genomics Network (solgenomics.net) and phytozome (phytozome.jgi.doe.gov/).

All retrieved SlHSP70 polypeptide sequences were further validated at E-value $<10^{-5}$ for detection of functional domains by deploying SMART (smart.embl-heidelberg.de/) tools. All *SlHsp70* gene sequences were analysed for chromosomal localization. SlHSP70 family proteins were *in-silico* characterized for their molecular weights (MW), total number of atoms, total number of amino acids, Aliphatic index, isoelectric point (pI) and instability index by employing EXPASY PROTOPARAM (expasy.org/tools/protparam.html) [33,34]. Theoretical pI and total MW of each polypeptide was analyzed by employing ProtParam (web.expasy.org/portparam).

2.2 Phylogenetic Tree Construction of SlHsp70 Family Genes in S. lycopercicum

Arabidopsis AtHSP70 family proteins were downloaded from Arabidopsis Information Resource (TAIR) (arabidopsis.org) and potato StHSP70 family pro-

teins from the Spud DB Potato Genomics Resources (solanaceae.plantbiology.msu.edu/). Multiple sequence allignments of HSP70 polypeptide sequences of Arabidopsis, potato and tomato were performed in ClustalX 2.0 with default parameters [35]. Phylogenetic analysis were performed to construct an outgroup rooted tree by using 20 *A. thaliana* AtHSP70, 19 *S. tuberosum* StHSP70 and 23 *S. lycopercicum* SlHSP70 protein sequences by performing alignment using Neighbor-Joining (NJ) method in MEGA7.0 with following parameters: 2000 bootstrap replication values, pair-wise gap deletion mode and Poisson model [36,37].

2.3 Chromosomal Localization, Synteny and Protein-Protein Interaction Analysis

The phytozome plant genome database (phytozome.jgi.doe.gov/) was explored by selecting tomato to collect information obout localization of *SlHsp70* genes and a genetic map was constructed by employing MapChart tool [38]. Genes of similar species were placed in the same group of an outgroup rooted tree, defined as co-paralogs, which were further analyzed to identify tandem duplicatons, segmental duplications and their coordinates. To investigate evolutionary events such as gene duplicaiton and divergence for vigourous gene functions as well as genetic expansion, we explored PGDD (Plant Genome Duplication Database) by deploying circos. Co-paralogs were considered tandemly duplicated when both of them were non-homologous and distance between their loci was $<$100 kb [39] and segmentally duplicated if located on different chromosomes [40]. Smith-Waterman algorithm (ebi.ac.uk/Tools/psa/) was employed to calculate local alignment of two proteins and synteny analyses were performed by employing circos with default parameters (circos.ca/). Finally, amino acid sequences of all SlHSP70s were used to construct protein-protein interaction network with the help of STRING (version: 11.5) proteome database (string-db.org/) by selecting *Solanum lycopercicum* as an organism.

2.4 Structure and Motif Analyses of SlHsp70s in S. lycopercicum

Genomic and CDS sequences of each *SlHsp70* gene were analyzed to examine number and order of introns and exons with the help of Genes Structure Display Server 2.0 (GSDS 2.0) program (gsds.cbi.pku.edu.cn/index.php) by adjusting following parameters: (a) Intron: color-black, shape-line and line width-3, (b) CDS: color-dark maroon, shape-round corner rectangle and height-12 and (c) UTR: color-blue, shape-rectangle and height-10. Protein sequence of each SlHSP70 was also analyzed to identify conserved motifs with the help of Multiple EM for motif elicitation (MEME) tool (meme.nbcr.net/meme3/meme.html) with following parameters: number of motifs-10 and optimum amino acid residues per motif were 6 to 200 [41].

2.5 Protein Modeling and GO Enrichment Analysis

Phyre2 web (sbg.bio.ic.ac.uk/phyre2/) was utilised in intensive mode to undertake protein modelling of SlHSP70 polypeptides [42]. All predicted models of SlHSP70 proteins were based on c5tkyA, c5e84B, c3d2fC, c2khoA, c3c7nB and c5obuA templates with 100% identification. *In-silico* direct physical and indirect functional interactions among proteins were predicted by using STRING (version 11.5) database. GO enrichment analyses were performed to predict localization of SlHSP70 proteins within cellular components (red bars), expected molecular function (green bars) and participation in possbile biological processes (blue bars) (Fig. 1 & **Supplementary Table 1**). To perform GO enrichment analysis for each SlHSP70 protein, OmicsBox and Blast2GO v3.0.11 (www.blast2go.com) were employed [43].

2.6 RNA-Seq Data Analysis

We analyzed RNA-seq data under normal conditions and constructed a heatmap to explore expression profiles of all *SlHsp70* family genes in different tissues of *S. lycopercium* including flower bud, unopened flowers, fully opened flowers, fruits (1 cm, 2 cm and 3 cm), mature green fruits, breaker fruits, breaker + 10 fruits, leaves and roots (Fig. 2 and **Supplementary Table 2**). RNA-seq data and PFKM values were downloaded from tomato functional genomics database (ted.bti.cornell.edu/pgsc_download.shtml) and analyzed using cufflinks v2.2.1. FPKM values were divided by their mean, transformed into log2 ratio and clustered into expression data in the form of heat map (heatmapper.ca/) with the help of MeV4.5 with default parameters [44,45].

2.7 SlHsp70 Genes Expression Analysis via qRT-PCR under Trace Metal Elements Stresses

The seeds of tomato (line M82) were cultured in greenhouse of Yibin University in mid Autumn 2020. In order to perform surface sterilization, seeds were soaked in 10% hypochlorous acid for 5 mins and then washed thrice with ddH_2O. After sterilization, seeds were placed on wet filter paper for germination, germinated seedlings were detached from paper and anchored in pots filled with Miracle-Gro® and Metro-Mix® 200 soil mixture and placed in greenhouse under following conditions 16 hours light at 27 ºC, 8 hours dark at 18 ºC and 70% humidity. Thirty days old tomato seedlings were uprooted, roots were washed with ddH_2O to remove soil particles and dipped in 1/2 Hoagland solution (pH 6.0) supplemented with different concentrations of five trace metal elements such as 1 mM $MnSO_4$, 0.1 mM $CdCl_2$, 0.5 mM $FeSO_4$, 0.1 mM $CoCl_2$, 0.5 mM $ZnSO_4$ and normal 1/2 Hoagland solution as control (CK) [46,47]. To extract RNA for qRT-PCR anslysis, leaves and roots of tomato seedlings treated with Hoagland solution supplemented with different concentrations of trace metal elements and normal Hoagland solution was removed after

Table 1. Characterization of *SlHsp70* genes in *S. lycopercicum*.

Gene name	Sequence 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

(-), negatively charged amino acid residues (Asp + Glu); (+), positively charged amino acid residues (Arg + Lys); MW, Molecular Weight; aa, Total amino acid residues; pI, isoelectric points.

24 h and stored in liquid nitrogen.

We used TRIzol™ Reagent (Thermo Fisher Scientific, USA) for total RNA extraction and SuperMix Kit (TransGen, Beijing) was used for cDNA synthesis. Gene specific primers were manually designed (**Supplementary Table 3**) and β-actin was selected as an internal control during qRT-PCR [48]. qRT-PCR was performed with following recation mixture 10 μL of SYBR premix Taq 2X, 1 μL of cDNA as a template, 0.5 μL of each forward primer and reverse primer and 8 μL of ddH_2O. PCR conditions were as follow: 95 °C for 10 min, 95 °C for 15 s, total 40 cycles, 60 °C for 60 s and three scientific replications. Relative expression level of each *SlHsp70* gene was measured with the help of Livak equation ($2^{-\Delta\Delta CT}$) [49].

3. Results

3.1 Identification of SlHsp70 Family Genes in S. lycopercicum

Hsp70 genes sequences were retrieved by downloading and subsequently analyzing *S. lycopercicum* genome database, incomplete raw reads were excluded and finally 23 complete candidate *Hsp70* gene sequences were selected. All genes were assigned scientific name given as *SlHsp70-1* to *SlHsp70-23* (Table 1). Gene synteny analysis revealed that all 12 tomoto chromosomes harbored *Hsp70* genes except chromosome 5. Total number of amino acids in each SlHSP70 polypeptide were 186 to 890, and molecular weight (MW) was 21273.47 Da to 98787.40 Da (Table 1). Comparatively, high contents of negative and acidic amino acids such as L-glutamic acid and L-alpha-aspartyl residues (Asp + Glu) were observed in almost all SlHSP70 proteins except SlHSP70-12 (Table 1). Isoelectric point (pI) of all 23 genes was also acidic (Table 1).

3.2 Phylogenetic Analysis of SlHsp70 Family Genes

Phylogenetic analysis revealed distribution of all *SlHsp70* genes in four groups I-IV (Fig. 3). Highest number of *Hsp70* genes were placed in Group I of phylogenetic tree which comprised of 6, 9 and 8 *Hsp70* genes of Arabidopsis, potato and tomato, respectively. Group II was smallest which comprised of five tomato *SlHsp70* genes, two potato *Hsp70* genes and three Arabidopsis *AtHsp70* genes, respecitvely. Group III comprised of four tomato *SlHsp70* genes, three potato *Hsp70* genes and five Arabidopsis *AtHsp70* genes. Group IV was second largest group which compried of six tomato *SlHsp70* genes, five potato *Hsp70* genes and five Arabidopsis *AtHsp70* genes (Fig. 3).

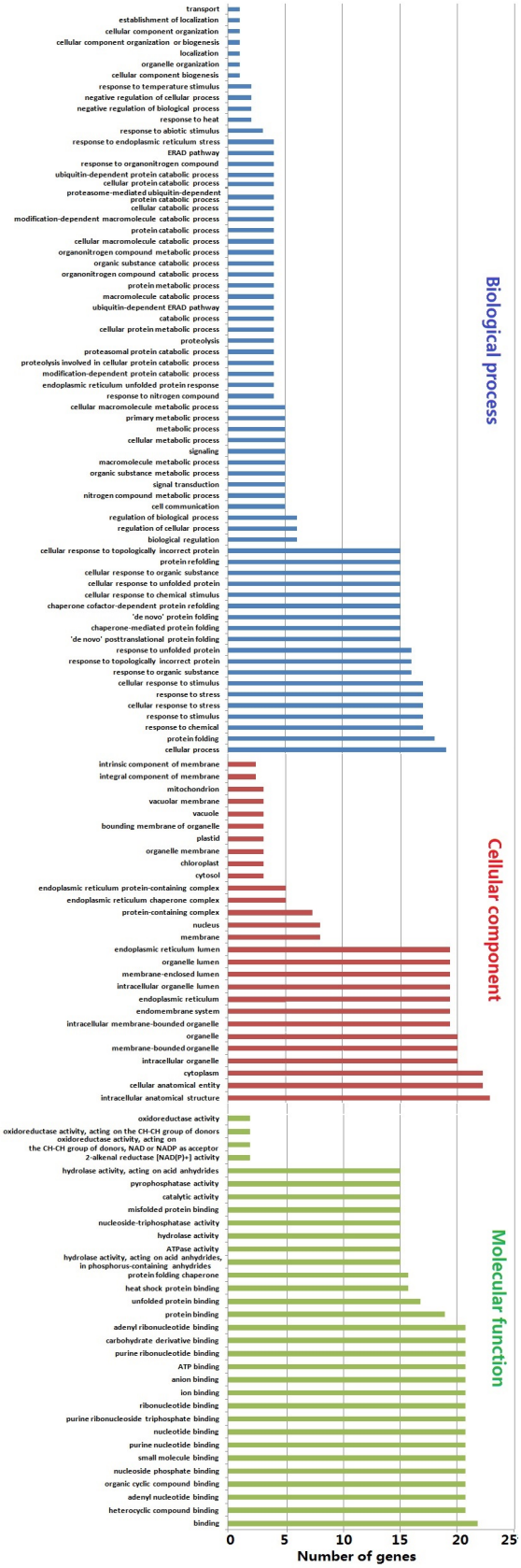

Fig. 1. GO enrichment analysis of *SlHsp70* genes in *S. lycopercicum*. Red columns represent cellular components in which *SlHsp70* genes displayed expression, blue columns represent biological processes in which *SlHsp70* genes played their specific roles and green columns represent expected molecular function of *SlHsp70* genes.

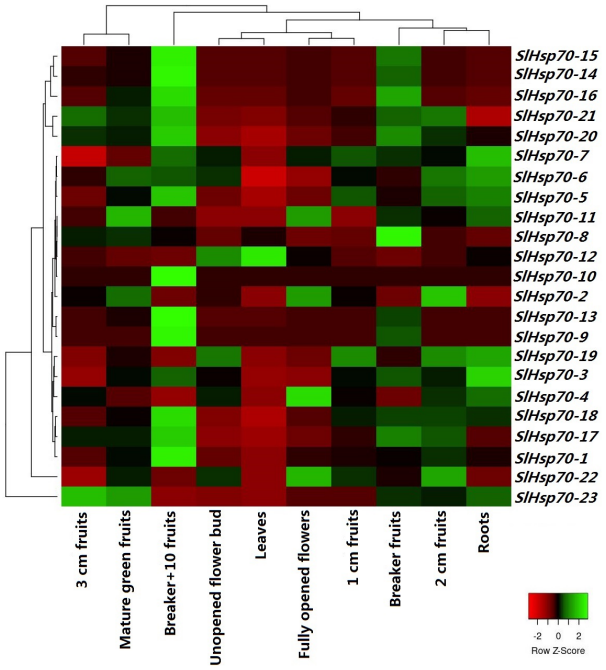

Fig. 2. Expression level of of all 23 *SlHsp70* in different tissues including roots, leaves, buds, flowers and fruits of different stages of *S. lycopercicum* based on RNA-seq (http://ted.bti.cornell.edu/).

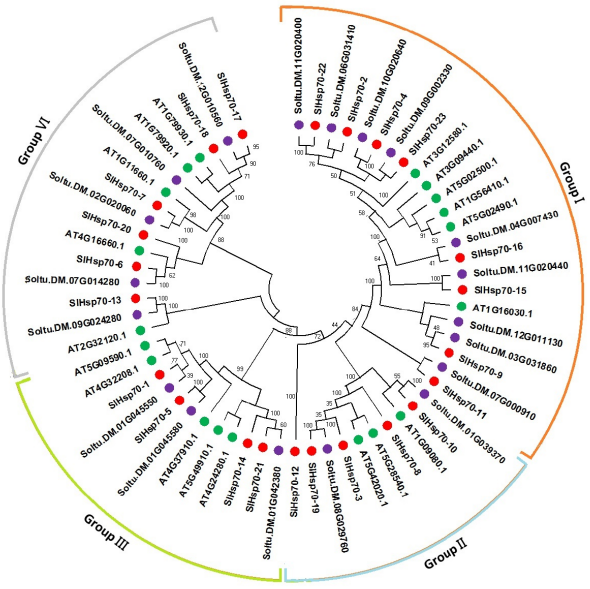

Fig. 3. Phylogenetic tree comprised of 23 *S. lycopercicum* (red circle), 19 *Arabidopsis thaliana* (green circle) and 19 *S. tuberosum* (purple circle) HSP70 protein sequences. ClustalX 2.0 was employed for protein alignment. Neighbor-Joining (NJ) method was used at 2000 bootstrap value to construct a phylogenetic tree in MEGA 7.0.

3.3 Chromosomal Localization and Gene Synteny Analysis

An uneven distribution of all *SlHsp70* genes on different chromosomes was detected except chromosome number 5 which did not harbor any *SlHsp70* gene. The highest number of *SlHsp70* family genes localized on any chromosome were following four: *SlHsp70-1*, *SlHsp70-5*, *SlHsp70-10* and *SlHsp70-21*, which were present on chromosome 1 (Fig. 4). Both segment and tandem duplication revealed 120 collinearity gene pairs with 50–100% duplication events (**Supplementary Table 4**). We noted 4 sister pairs among all 23 *SlHsp70* genes. Multiple segment duplication events of *SlHsp70-3*, *SlHsp70-10* and *SlHsp70-13* genes were also observed. On the contrary, four parlog pairs were observed having distance less then 5 kb which were tandemly duplicated gene clusters and localized on chromosome 1, 3, 8, 11, and 12 (Fig. 4 and **Supplementary Table 5**).

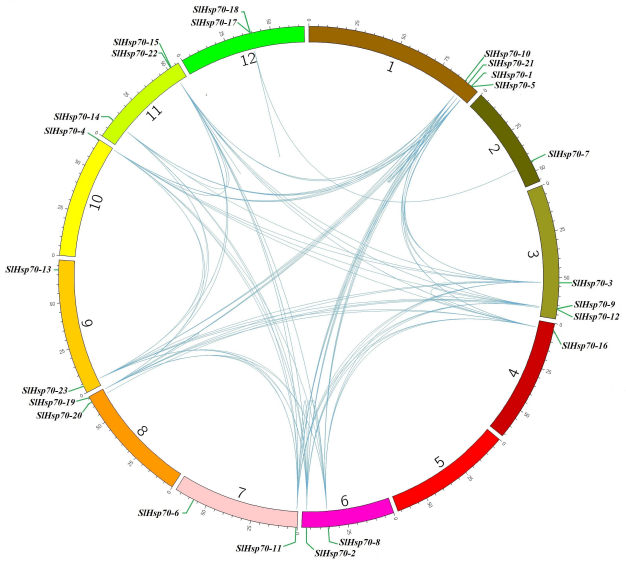

Fig. 4. Gene synteny analysis of *SlHsp70* genes in *S. lycopercicum*. Blue lines represent orthologs and paralogs to express segmental duplication while red boxes represent tandem duplication.

3.4 Gene Structure and Motif Analysis of SlHsp70 Genes

On the base of structure, all 23 *SlHsp70* gene family members were sub-divided into A, B, C, D and E subfamilies (Fig. 5a). Largest subfamily was A containing 8 genes, subfamily E was second largest with 6 genes and subfamily D was smallest with only 1 gene (Fig. 5a). Except for SlHsp70-9 and -13, which had no introns, the number of exons and introns varied across all sub-families. Highest degree of similarity in exon and intron numbers was observed with in sub-families (Fig. 5c). Each motif of SlHPS70 proteins comprised of 50 amino acids except motif 4, 6, and 8 which contained only 41, 33 and 29 amino acids, respectively. The motifs 1, 3 and 8 were found in

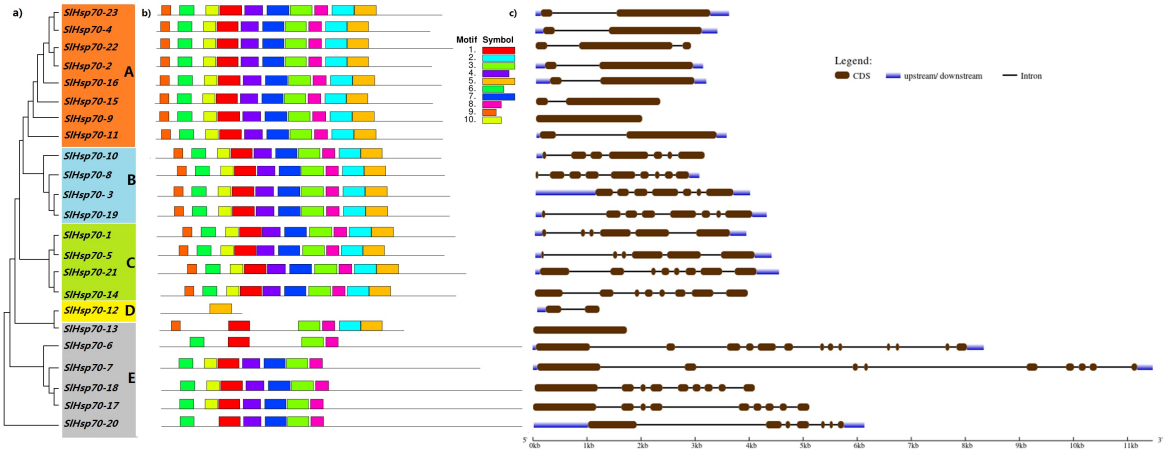

Fig. 5. Phylogenetic analysis, gene structure analysis and conserved motifs of *SlHsp70* genes in *S. lycopercicum*. (a) The neighbor-joining (NJ) method was used at 2000 bootstrap value to construct a phylogenetic tree of SlHSP70 amino acid sequences in MEGA 7.0. (b) Ten conserved motifs of all SlHSP70 proteins were presented in unique colour symbol shown in box. (c) Dark lines represent exons, black lines represent introns annd blue lines represent UTRs. Specific size scale is given below.

all subfamilies except D, and were followed by motifs 4, 6 and 7, which were likewise found in all subfamilies but two members of E. Noticeably, number, order and types of motifs were similar within a subfamily but different among subfamilies (Fig. 5b and Table 2).

3.5 Protein-Protein Interaction, 3D Modeling and GO Analysis

Amino acid sequences of SlHSP70 were analyzed *in-silico* to predict 3D protein structures as 3D conformation guarantees specific function of any protein (Fig. 6 & **Supplementary Table 6**). Except for SlHSP70-1 and -5, the rest of the SlHSP70 proteins were modelled using the c3d2fC template at a confidence level of 100 percent., while modeling of SlHSP70-12, -13 and -20 proteins was performed using c2khoA, c5gjjA, c5tkyA and C5nnrD templates. STRING database analysis revealed 22 nodes, 97 edges and following 13 local network clusters: 4141, 4411, 4655, 4661, 4020, 4021, 4023, 4025, 4084, 3997, 4339, 4682 and 4138. Among all, the biggest cluster was 3997 which comprised of 12 *SlHsp70* proteins (**Supplementary Table 7**). Protein analysis also revealed existence of two common protien domains (PF00012 in all 23 SlHSP70 proteins and PF06723 in 19 SlHSP70 proteins) and five KEGG pathways (such as sly04141 in 14 SlHSP70 proteins, sly03060 in 3 SlHSP70 proteins, sly04144 in 10 SlHSP70 proteins, sly03040 in 10 SlHSP70 proteins and sly03018 in 2 proteins) (**Supplementary Table 8**). Sub-cellular distribution percentages of SlHSP70 proteins were 2/4% in vaculoar membranes, 3.5/8% in chloroplast and 9/42% in endoplasmic reticulum (ER). *SlHsp70-8* was localized in 21/28 sub-cellular compartments. Overall percentages of SlHSP70 proteins in different biological processes were as follow: heat and cadmium resistance response was 3/4%, cellular stress response was 17/23% and chemical stress response was 15/23%.

3.6 Tissue Specific Expression Pattern of SlHsp70 Family Genes

Tissue specific expression of the gene *SlHsp70-1*, *-5*, *-9*, *-10*, *-13*, *-14*, *-15*, *-16*, *-17*, *-18*, *-20* and *-21* diplayed upregulated expression level in breaker + 10 fruits while *SlHsp70-8* exhibited highest expression level in breaker fruits. *SlHsp70-11* displayed upregulated expression level in fully opened flowers and mature green fruits while *SlHsp70-22* showed mild expression level in 2 cm fruit and upregulated expression level in fully opened flowers. Only single gene with upregulated expression level in leaves was *SlHsp70-12* but it also displayed very mild expression in unopened flower bud. *SlHsp70-6* exhibited mild expression level in roots, 2 cm fruit, breaker + 10 fruits and mature green fruits. *SlHsp70-19* displayed mild expression in roots, 1 cm fruit, 2 cm fruit and unopened flower bud. Finally, SlHsp70-23 showed mild expression in mature green fruit and 3 cm fruit (Fig. 2).

3.7 SlHsp70s Expression Profiling under Trace Metal Elements Stress

Each *SlHsp70* gene exhibited differential expression level on treatment of different trace metal elements in both leaves and roots. The roots of plants treated with Cd^{2+} and Co^{2+} solutions displayed upregulated expression of all *SlHsp70* genes except *SlHsp70-7*, *-17* and *-20*, while *SlHsp70-11* specifically showed upregulated expression in roots (Fig. 7). Similarly, Cd^{2+} and Co^{2+} solutions treatment resulted in upregulation of expression of all *SlHsp70* genes in leaves except *SlHsp70-7*, *-11*, *-17* and *-20*. Fe^{2+} treatment resulted in increased expression of *SlHsp70-17* and *-23* but decreased expression of *SlHsp70-18*, *-20* and *-21*. Mn^{2+} treatment resulted in decreased expression of

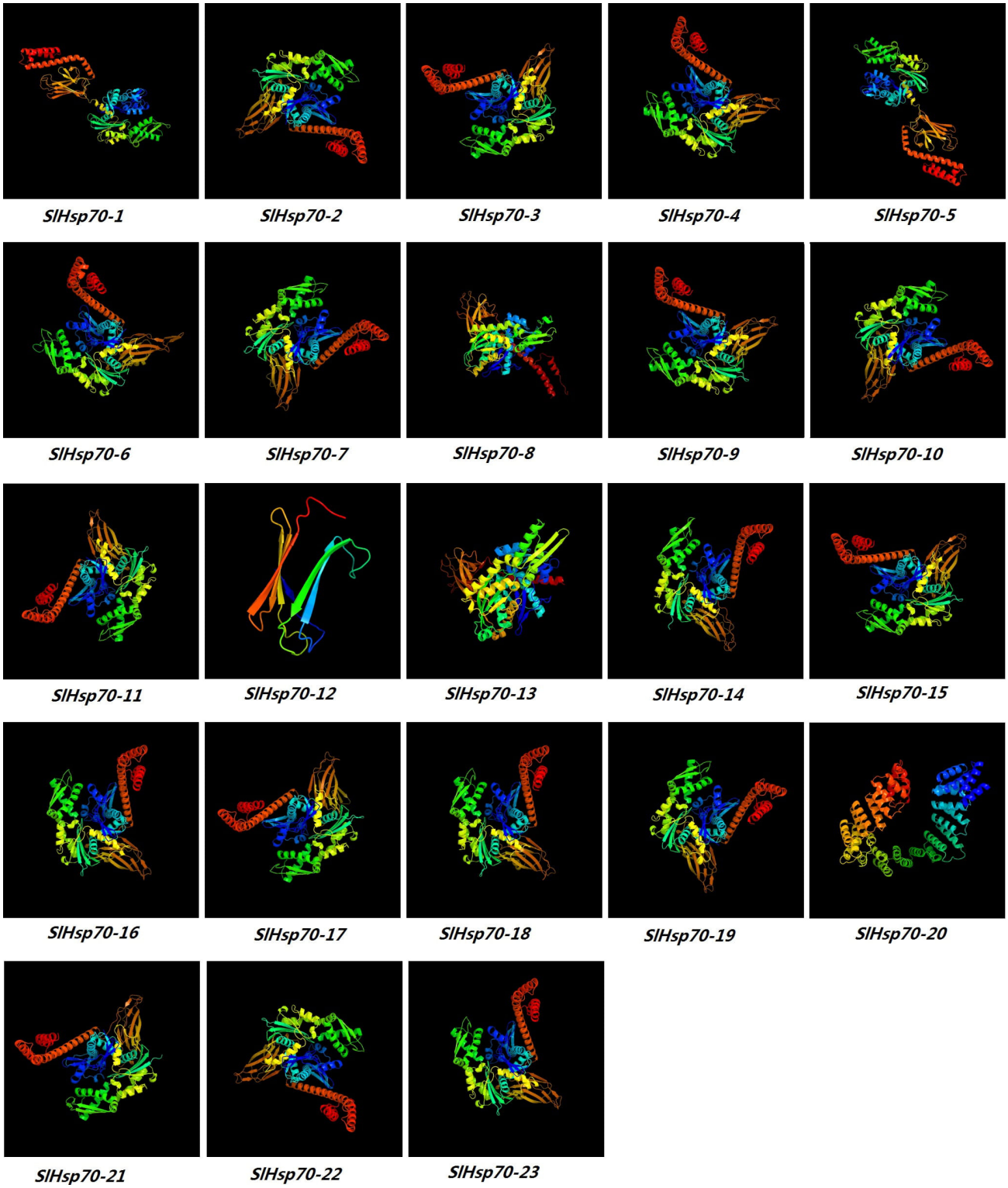

Fig. 6. Prediction of 3D structures of SlHSP70 proteins in *S. lycopercicum*. Phyre 2 server was used in an intensive mode to generat protein models, visualized by rainbow colours in direction from N to C terminus.

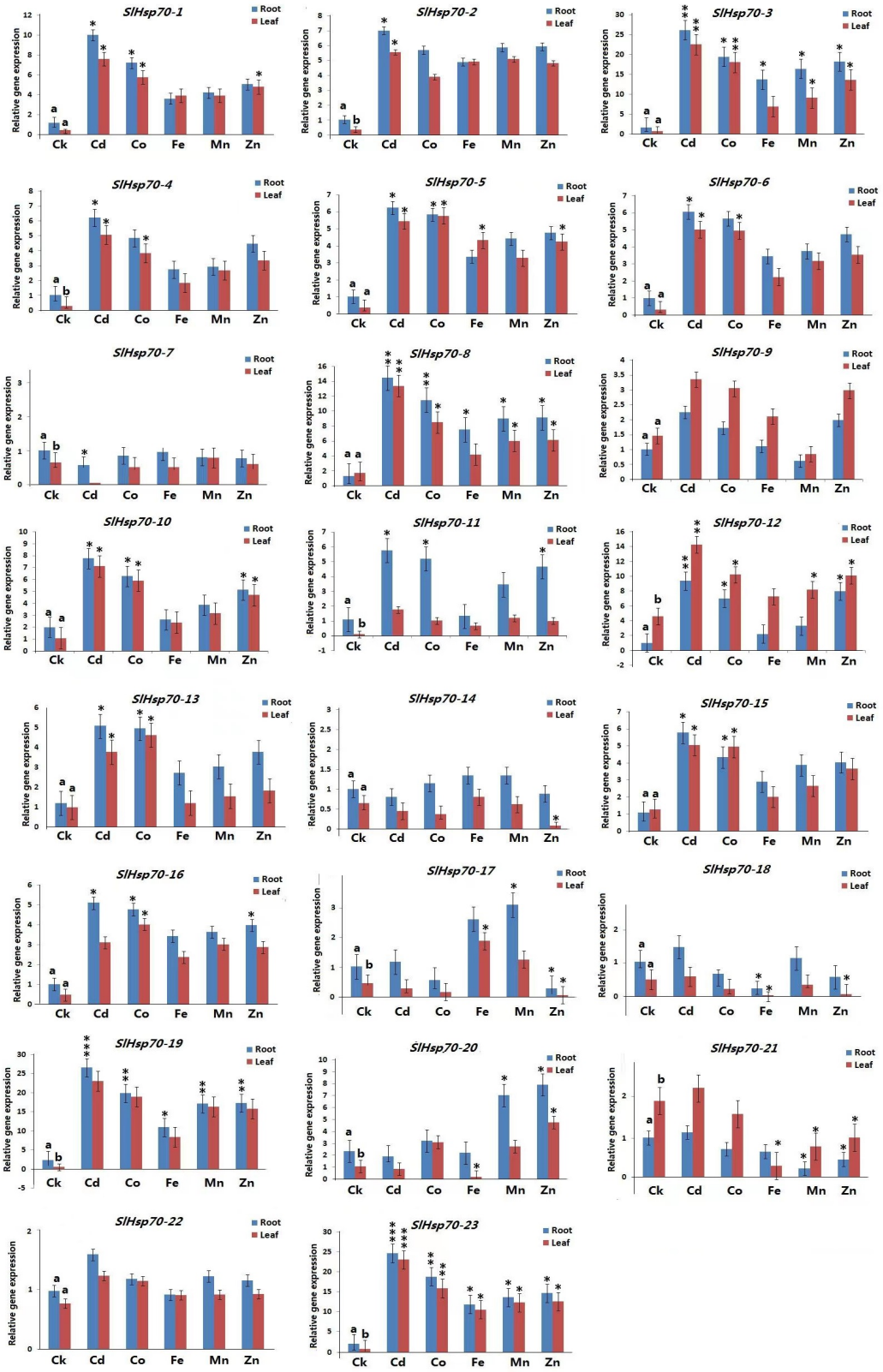

Fig. 7. qRT-PCR analysis of all 23 *SlHsp70* genes in root and leaf tissues under different metal stresses. β-actin gene was employed as an internal control. Standard deviation among three scientific repeats was shown by drawing error bars. Mean expression of three biological replicates of each *SlHsp70* under Cd, Co, Fe, Mn and Zn stress was taken by deploying *t*-test at $p < 0.05$ significance (n = 9). CK means control and asterisk (*) represents significant differential expression of *SlHsp70* genes in root and leaf under stress of specific heavy metal.

Table 2. Conserved motifs of 10 selected SlHSP70 proteins in *S. lycopercicum*.

Motif	Logo	Most probable matches	Width
1		VKBAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKK	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

SlHsp70-14 and *SlHsp70-21*, but increased expression of *SlHsp70-3*, *-8*, *-12* and *-23*. Zn^{2+} treatment resluted in decresed expression of *SlHsp70-7*, *-14*, *-18* and -21 but increased expression of *SlHsp70-1*, *-3*, *-8*, *-10*, *-12* and *-23* (Fig. 7).

4. Discussion

Trace metal element stress, such as Cd^{2+}, disrupts cell molecular mechanisms and homeostasis through enzyme denaturation, and plants respond by activating stress responsive genes [50]. HSP70s are stress induced proteins like aconitases, cation diffusion facilitator (CDF), ferroportin (FPN), iron regulatory proteins (ZIP) and copper importer (CTR), which are conserved among multiple plant species and play pivotal role in metal homeostasis, stability and translocation of mRNA and catalysis [51,52]. Metal efflux is precisely regulated by membranous transporters, ensuring homeostasis. Chaperones and co-chaperones of HSP70 like DnaJ proteins play key role in maintaining integrity of proteins and proper folding during translocation [53]. The overexpression of 22 *Hsp70* genes in switchgrass under Cd^{2+} stress shows their role in repair system and specific bio-indicators of Cd^{2+} stress [54,55]. GO analysis also endorsed their role in heat and trace metal elements stress, ion binding, unfolded protein binding, misfolded protein binding and membrane proteins (Fig. 6, **Supplementary Tables 7,8**). These findings demonstrated that HSP proteins were in charge of restoring normal cell homeostasis in tomato under Cd^{2+} stress.

Commercial cultivation of tomato is being predominantly performed in greenhouse, where it is irrigated with recycled water containing very high Cd^{2+} contents. Roots are primary tissues exposed to Cd^{2+} stress and their 2-DE proteome profiling revealed upregulated TCA cycle, glycolysis, detoxification proteins and respiration while severe halt in carbon metabolism [56], corroborated by our qRT-PCR analysis of tomato plants exposed to Cd^{2+} stress. Gene duplication followed by divergence is a common phenomenon in plant gene families and often occurs in gene families responsible for modulating secondary metabolism such as terpene synthases, methyltransferases, 2-oxoglutarate-dependent dioxygenase, cytochrome-P450-dependent monooxygenase and α/β-hydrolases [57]. Duplication of multiple *SlHsp70* family genes indicated their key role during secondary metabolism to regulate growth of tomato plants (Fig. 4). All SlHSP70 proteins interact with each other which indicates their specific role in Cd^{2+} transportation in form of polymer (Fig. 8).

Cd^{2+} stress induces hyper-accumulation of ROS in root hairs of Arabidopsis which scavenges expression of genes involved in negative regulation of root elongation such as root hair-specific 10 (RHS10) and proline-rich extension-like receptor kinase (PERK5 & 8). Meanwhile, Cd^{2+} stress upregulates expression of root hair elongation and cell wall biosynthesis genes such as expansin (EXPA7

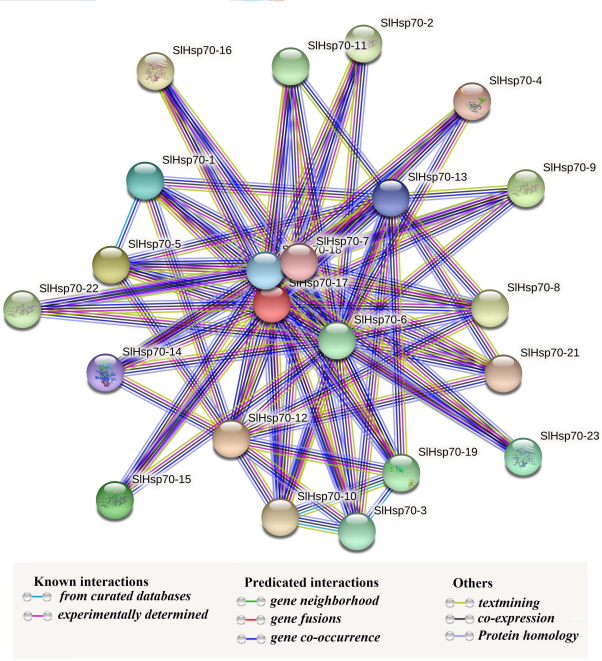

Fig. 8. Protein-protein interaction network building among SlHSP70 proteins in *S. lycopercicum*. Empty nodes: unknown 3D structure, fillled nodes: known or predicted 3D structure, colored node: query proteins and first shell of interactors and white nodes: second shell of interactors.

& EXPA18) and COBRA-like9 (COBL9), which commulatively results in long root hairs [58]. For example, overexpression of TaEXPA2 in wheat resulted in lower accumulate of Cd^{2+} ions and ROS [59]. Contrastingly, GO analysis revealed oxidoreductase activity of *Hsp70* genes in tomato (Fig. 6), which helps plants in countering the effect of reactive oxygen species (ROS), that might cause stunted root hairs. On the other hand, application of CuCd and ZnCd solutions resulted in stunted pea roots [15]. As a result, additional research is needed to explore the impact of Cd^{2+} stress on root length, root hair length, and ROS in tomato. Application of plant growth promoting bacteria (PGPB) in soils rich in heavy metals circumvents accumulation of Cd^{2+} ions in plant tissues and higher biomass accumulation [55].

In order to evade deleterious effects of Cd^{2+} stress, HSP70 proteins sequester it in vacuole. HSP70 family proteins are highly sensitive to Cd^{2+} stress and their expression could increse 2 to 10 fold on exposure [60]. For example, *KvHsp70* is induced under salt stress [61], *NtHsp70-1* under heat and drought stress [62], *StHsp70* under abiotic stress factors and hormones stress [17] to avoid their deletirious effects. In tomato, expression of all *SlHsp70* genes was upregulated in roots under trace metal elements stress which demonstrated their crucial role in heavy metals stress (Fig. 7). *SlHsp70-11* in tomato is an ortholog of *StHsp70-11* in potato which was overexpressed in roots under Cd^{2+} stress same as in potato which plays key role in detering

trace metal elements stress [17]. Similarly, *SlHsp70-12* in tomato is an ortholog of *StHsp70-12* in potato which displayed higher expression in leaves under Cd^{2+} stress which takes part in detering heat stress, similar results as observed in previous studies [63].

5. Conclusions

Cd^{2+} is a non-essential metalic pollutant widely distributed in agri-lands and easy to accumulate in plant tissues which, on ingestion, pose serious threats to plant health. Tomato is widely grown and commonly used food item grown on soils contaminated with trace metal elements. *SlHsp70* family genes play pivotal role in sequestration of these trace metal elements into vacuole to maintain homeostasis. *In-silico* analysis revealed 23 *SlHsp70*s in tomato which were phylogenetically divided into four groups I–IV along with orthologs of Arabidopsis and potato. RNA-seq based heat map exhibited upregulated expression of 18 out of 23 *SlHsp70* genes in breaker + 10 fruits, 2 in roots, 2 in flowers and 1 in leaves, which shows their key role in flowering and fruiting. qRT-PCR analysis under five trace metal elements stress specially Cd^{2+} revealed upregulaed expression of *SlHsp70-11* in roots and *SlHsp70-9* and *SlHsp70-12* in leaves. GO enrichment analysis revealed that *SlHsp70* proteins were integral parts of membranes to regulate cell transport, ATP binding, nucleotide binding, ion binding, oxidoreductase activity to deter ROS, responsive to abiotic stresses including heat and trace metal elements. In conclusion, *SlHsp70-11* can be employed in breeding of tomato varities with improved tolerance against Cd^{2+} stress.

Author Contributions

Conceptualization—MA (Manzar Abbas), YL, RGE and AHEl-S; Data acquision—AHEl-S, MA (Manzar Abbas), RGE and JL; Formal analysis—MA (Manzar Abbas), AHEl-S, SAS, MMI, VY, SZ, MA (Mubashir Abbas), NS, SSH, SA and ZN; Methodology—AHEl-S, RGE, MA (Mubashir Abbas), SZ, MMI, VY, JL, and AJR; Writing-original draft—MA (Manzar Abbas), and AHEl-S; Editing and proof-reading—MA (Mubashir Abbas), AHEl-S, AJR, KY and YL; Corresponding—AHEl-S, YL, and JL.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2706186.

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