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Research Article

Genome-Wide Analysis of HSP70 Family Protein in *Vigna radiata* and Coexpression Analysis Under Abiotic and Biotic Stress

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ABSTRACT

Heat shock protein 70 (Hsp70), a 70-kDa protein, also known as a molecular chaperone, is highly conserved. It plays a major role in cellular functions such as protein folding, regulation of protein degradation, translocation of proteins across membranes, receptor signaling, and protein assembly or disassembly. *Vigna radiata* is an important legume crop with available whole-genome sequence, but no such study on the HSP70 family is reported. A total of 32 *V. radiate* HSP70s (Vr-HSP70s) were identified and described. They are phylogenetically clustered into four subgroups. Vr-HSP70s show variations in intron/exon organization. This indicates that introns may play an essential role in gene regulating. The coexpression analysis of Vr-HSP70s revealed that these genes were involved in both abiotic and biotic stresses. Three cytoplasmic hub genes namely Vr-HSP70-C-14, Vr-HSP70-C-29, and Vr-HSP70-C-30 were found common in both stresses. Our findings provide directions for future studies to dissect functional analysis of Vr-HSP70s in response to abiotic and biotic stresses.

Keywords: gene expression, genome-wide analysis, heat shock proteins, HSP70s, *Vigna radiata*.

1. INTRODUCTION

HEAT SHOCK PROTEINS (HSPs) are ubiquitously abundant proteins of plant and animal cells. Since they were first reported in response to heat shock but are now found to be associated with both biotic and abiotic stresses, they are misnomers of stress proteins. They play a role in plant immunity (Park and Seo, 2015). They are also known as molecular chaperones. These highly conserved HSPs are classified into five families on the basis of molecular weight, namely, small HSP, HSP60, HSP70, HSP90, and HSP100 (Wang et al., 2004). Of all these, the HSP70 family is widely studied due to its role in plant development and its functions during abiotic and biotic stress. It was identified and characterized in the 1960s (Ritossa, 1996; Sung et al., 2001a).

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Hsp70s has the proofreading functions used to repair the misfold conformers of proteins and is also involved in supporting the folding process and proper folding of newly synthesized proteins, controlling the activity of regulatory proteins, refolding of misfolded and aggregated proteins, and membrane translocations of organelles (Bukau et al., 2000; Hartl and Hayer-Hartl, 2002; Pratt and Toft, 2003; Mayer and Bukau, 2005). Moreover, the major property of HSP70 is to bind unfolded polypeptides and release an ATP-dependent reaction (Schroda et al., 1999).

Hsp70s has two major domains, that is, a 45 kDa nucleotide-binding domain (NBD; N-terminal ATPase domain) and 15 kDa substrate-binding domain (SBD) with an $\sim$10-kDa C-terminal lid region. Both NBD and SBD are highly conserved (Yu et al., 2015; Guo et al., 2016). It is localized in chloroplast (CP), mitochondrion (MT), endoplasmic reticulum (ER), and cytoplasm (Yu et al., 2015). HSPs are mainly found in the cytoplasm and respond to various biotic and abiotic stresses. Moreover, HSPs found in other organelles play a role in protein homeostasis (Vierling, 1991; Boston et al., 1996). In prokaryotes, it is also called as Dank and plays a critical role in many cellular functions such as protein folding, regulation of protein degradation, translocation of proteins across membranes, receptor signaling and assembly, or disassembly of protein complex (Jiang et al., 2014; Yu et al., 2015).

Vigna radiata (also known as mungbean, green gram) belongs to the Fabaceae family crop and is a warm season legume species. It is a self-pollinated crop with a diploid chromosome number ($2n = 2x = 22$), having an estimated genome size of 579 Mb. *V. radiata* is mainly cultivated in East, South, and Southeast Asia (Kang et al., 2014). Archeological evidences and genetic diversity data suggested that the *V. radiata* was domesticated and cultivated in India (Fuller, 2007). Globally, India is the largest producer and consumer of *V. radiata*, which is around 50% of the total annual production, followed by China and Myanmar.

Sprouts of *V. radiata* are a good source of minerals having higher levels of folate, vitamin, dietary protein, fiber, and iron compared with other legume crops (Chen et al., 2015). It can be processed into porridge, soups, ice cream, and flour, which makes this crop versatile for human diet and forage useful to sheep (Tian et al., 2016). It is a short-duration legume crop (65–90 days) having low input requirements and wide adaptability (Nair et al., 2012; Kang et al., 2014). All these nutritional and adaptability attributes in an era of climate change have made this a much more promising crop.

In addition, like other legume crops it is also involved in atmospheric nitrogen fixation by root rhizobial symbiosis, which leads to improved soil texture and fertility (Graham and Vance, 2003). Productivity difference has been observed in the intercropping system in rice-wheat or rice-rice with a higher yield of cereal crops (Yaqub et al., 2010; Liu et al., 2016). In *V. radiata*, losses of productivity (80%–100%) due to various biotic and abiotic stresses are reported (HanumanthaRao et al., 2016; Rana et al., 2016; Alderfasi et al., 2017).

Genome-wide studies of HSP70s in abiotic and biotic stresses have been in various crops such as *Capsicum annuum* L. (Guo et al., 2016), *Populus* (Yer et al., 2016), soybean (Zhang et al., 2015), *Physcomitrella patens* (Tang et al., 2016), rice (Sarkar et al., 2013), and spinach (Guy and Li, 1998). There is no such study in this important crop.

Present work aims at mapping the available putative Hsp70 genomic data over the genome of *V. radiata* to get chromosomal maps of these protein families. It further aims at the prediction of motifs, intron/exon boundaries, and subcellular localization of these proteins. It also aims at a comparative analysis in monocot, dicot, and lower organisms along with the construction of gene regulatory network in biotic and abiotic stress in *V. radiata* to reveal its role as a putative candidate stress responsive gene.

2. MATERIALS AND METHODS

2.1. *Genome-wide identification of putative* V. radiate *HSP70 in* V. radiata

The hidden Markov model (HMM) profile search of the HSP70 domain (PF00012) was retrieved from the Pfam (protein family) database and used against *V. radiata* genome (National Center for Biotechnology Information [NCBI] accession: GCA_000741045.2) with an expected threshold *e*-value of 0.05 by using HMMER (http://hmmer.org). SMART (http://smart.embl-heidelberg.de), Pfam (https://pfam.xfam.org), and Conserved Domain Database (CDD; www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) were used to validate the presence of functional domain of HSP70s in the protein sequence. Finally, the nonredundant selected proteins were assigned as *V. radiate* HSP70 (Vr-HSP70). The ProtParam tool was used for the calculation of instability index (proteins with <40 score were considered stable), molecular weights, and theoretical isoelectric points (pI; https://web.expasy.org/protparam).

2.2. Sequence analysis, chromosomal location, and gene duplication

The tblastn function of NCBI blast was used for the retrieval of nucleotide sequence of Vr-HSP70 (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The chromosomal location and exon/intron number were identified from NCBI. Blast search of each gene was performed against the Vr-HSP70 gene for the identification of gene duplication with a threshold *e*-value of 0.05. A gene with a similarity above 80% was considered duplicate. Graphical presentation of *HSP70s* on *V. radiata* genome was carried out using the MapChart tool (Voorrips, 2002).

2.3. Subcellular localization prediction, intron/exon organization, and motif identification

Subcellular localization of Vr-HSP70 was predicted using the CELLO v.2.5: subcellular LOcalization predictor tool (http://cello.life.nctu.edu.tw). Identification of conserved motifs in Vr-HSP70s with parameters such as maximum number of motifs (10), number of repetitions (any), and motif width (6–50 amino residues) was predicted using MEME suit (Multiple Em for Motif Elicitation) 5.0.4 (http://meme-suite.org/tools/meme).

2.4. Multiple sequence alignment and phylogenetic and comparative analysis

Multiple sequence alignment of Vr-HSP70 was performed using ClustalW program (www.genome.jp/tools-bin/clustalw) and gene ID number, shown in Table 1. In the alignment of Vr-HSP70s, several parameters have been used such as protein weight matrix (Gonnet), gap open penalty (10), residue-specific gap penalty (on), gap extension penalty (0.2), gap separation distance (0), use negative matrix (on), hydrophilic penalties (on), delay divergent cutoff (%): 30, and end-gap separation penalty (on). Neighbor-joining (NJ) method was used for the construction of phylogenetic tree of Vr-HSP70s in MEGA 7 software (Kumar et al., 2016) with bootstrap replication of 1000 times, Poisson model, uniform rates, and pairwise deletion. Furthermore, the iTOL tool was used for visualization of modeled tree (https://itol.embl.de/). The evolutionary distances of Vr-HSP70s were computed by using the Poisson correction method.

For the phylogenetic analysis, monocot (rice), dicot (*V. radiata* and *Arabidopsis thaliana*), and lower organisms (*Saccharomyces cerevisiae*) were taken. All genomic data of the HSP70 family of these species were retrieved from Guo et al. (2016), Sarkar et al. (2013), and InterProScan database (www.ebi.ac.uk/interpro/entry/IPR013126/taxonomy).

2.5. Gene ontology annotation

The functional characterization of Vr-HSP70 sequences was performed using the Blast2GO server (Conesa et al., 2005). First it executes BLASTp to search homology sequences, followed by mapping and annotation to find the function of proteins. Furthermore, these proteins are classified into three subcategories of gene ontology (GO) term, namely, molecular functions, cellular components, and biological processes.

2.6. Coexpression analysis of Vr-HSP70 genes

Coexpression analysis of 32 Vr-HSP70 genes was performed using RNA-seq data available in public domain, related to biotic and abiotic stress in *V. radiata*. All the data were retrieved from Sequence Read Archive (SRA) of NCBI with BioProject accessions, PRJNA327304 (abiotic) and PRJNA312984 (biotic). In data of abiotic stress, desiccation tolerance experiment was performed in *V. radiata* under various time lines, namely, SRR3735179 (CK3h), SRR3735193 (CK6h), SRR3735547 (CK18h), SRR3735572 (CK24h), SRR3735589 (SY3h), SRR3735674 (SY6h), SRR3735739 (SY18h), and SRR3735764 (SY24h), where ''CK'' and ''SY'' represent the control and experimental group, respectively (Tian et al., 2016). In data of biotic stress, associated with bruchid resistance (VC6089A, SRR3184678, SRR3184680, SRR3184681 and SRR3184682) and bruchid susceptible (VC1973A, SRR3184693, SRR3184694, SRR3184702 and SRR3184703) were used (Lin et al., 2016; War et al., 2017). Quality control and preprocessing of reads were performed using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc) and Trimmomatic tool (Bolger et al., 2014). All cleaned reads were mapped onto gene sequences of 32 Vr-HSP70s to calculate the read count using RSEM (RNA-Seq by Expectation-Maximization) tool (Li and Dewey, 2011). Furthermore, read count values were used for hierarchal clustering using ClustVis web tool (https://biit.cs.ut.ee/clustvis).

Table 1. Details of Vr-HSP70 Identified in *Vigna radiata*

Name	*Accession No.*	*Chr*	*ORF (bp)*	*Introns*	*AA*	*Hsp70 domain (length aa)*	*Mut (kDa)*	*Isoelectric points (pI)*	*Instability index*	*Localization predicted*
Vr-HSP70-C-1	XP_014517324.1	10	681	2	226	9–176 (168)	25.21	6.23	36.32	Cyto
Vr-HSP70-C-2	XP_014496914.1	4	777	0	258	1–256 (256)	29.11	8.74	22.38	Cyto
Vr-HSP70-C-3	XP_014517323.1	10	1317	—	297	9–296 (288)	33.08	5.02	33.67	Cyto
Vr-HSP70-C-4	XP_014511307.1	8	1317	0	439	1–439 (439)	48.83	8.9	35.55	Cyto
Vr-HSP70-C-5	XP_014517322.1	10	1317	—	462	4–461 (458)	51.27	6.65	39.5	Cyto
Vr-HSP70-ER-6	XP_014523717.1	*	1428	5	476	1–476 (476)	52.73	4.96	29.7	ER
Vr-HSP70-C-7	XP_014496911.1	4	1497	3	498	9–497 (489)	55.67	8.46	32.34	Cyto
Vr-HSP70-Cl-8	XP_014520151.1	11	1722	1	573	28–538 (511)	62.12	5.72	39.42	C
Vr-HSP70-C-9	XP_014518968.1	10	1809	0	602	8–602 (595)	67.12	6.09	31.89	Cyto
Vr-HSP70-C-10	XP_014496910.1	4	1836	1	611	9–611 (603)	68.46	7.01	30.8	Cyto
Vr-HSP70-N-11	XP_014523180.1	*	1860	1	619	9–617 (609)	69.34	7.45	34.25	N
Vr-HSP70-C-12	XP_014496954.1	4	1944	1	647	9–618 (610)	70.77	5.12	34.49	Cyto
Vr-HSP70-C-13	XP_014510496.1	7	1944	0	647	8–617 (610)	70.93	5.3	32.28	Cyto
Vr-HSP70-C-14	XP_014493887.1	2	1950	1	649	9–618 (610)	71.01	5.1	34.66	Cyto
Vr-HSP70-C-15	XP_014497624.1	4	1950	1	649	9–618 (610)	71.12	5.16	34.34	Cyto
Vr-HSP70-C-16	XP_014513551.1	8	1947	1	648	9–618 (610)	71.22	5.13	33.87	Cyto
Vr-HSP70-C-17	XP_014495815.1	3	1953	1	651	9–618 (610)	71.31	5.07	36.23	Cyto
Vr-HSP70-C-18	XP_014496915.1	4	1881	—	642	9–616 (608)	71.65	9.05	31.81	Cyto
Vr-HSP70-C-19	XP_014515808.1	9	1968	1	655	10–619 (610)	71.94	5.3	33.42	Cyto
Vr-HSP70-M-20	XP_014518723.1	10	2025	5	674	53–647 (595)	72.45	5.75	41.56	M
Vr-HSP70-M-21	XP_014509742.1	7	2037	5	678	56–650 (595)	72.69	5.89	39.08	M
Vr-HSP70-Cl-22	XP_014518378.1	10	2070	6	689	53–648 (596)	73.66	5.28	28.41	C
Vr-HSP70-C-23	XP_014502537.1	6	2316	8	771	3–670 (668)	85.72	5.39	42.6	Cyto
Vr-HSP70-N-24	XP_014522749.1	*	2316	10	771	3–669 (667)	86.02	5.4	48.99	N
Vr-HSP70-C-25	XP_014518846.1	10	2382	0	793	8–601 (594)	87.73	8.01	31.8	Cyto
Vr-HSP70-N-26	XP_014515835.1	9	2544	9	790	1–639 (639)	88.4	5.7	41.06	N
Vr-HSP70-C-27	XP_014496831.1	4	1881	—	832	9–618 (610)	92.38	5.19	36.2	Cyto
Vr-HSP70-C-28	XP_014515834.1	9	2544	9	847	3–699 (697)	94.43	5.66	41.14	Cyto
Vr-HSP70-C-29	XP_014501310.1	5	2592	10	863	3–700 (698)	95.29	5.14	44.3	Cyto
Vr-HSP70-C-30	XP_014501311.1	5	2592	10	863	3–700 (698)	95.29	5.14	44.3	Cyto
Vr-HSP70-C-31	XP_014497815.1	4	2679	12	892	24–742 (719)	99.04	5.45	39.48	Cyto
Vr-HSP70-C-32	XP_014496918.1	4	1830	—	1012	412–1005 (594)	112.27	6.07	36.44	Cyto

*, Unplaced scaffold; AA, amino acids; C, chloroplast; Cyto, cytoplasmic/cytosolic; ER, endoplasmic reticulum; M, mitochondrial; N, nuclear; Vr-HSP70, *V. radiate* HSP70; ORF, open reading frame; Mut, molecular weight.

Gene regulatory network of normal, biotic, and abiotic samples was generated on the basis of expression values using ARACNe (Algorithm for the Reconstruction of Accurate Cellular Networks) plug-in, which is integrated in the Cyni Toolbox panel of Cytoscape software (Shannon et al., 2003). Hub genes were identified on the basis of degree and betweenness centrality.

3. RESULTS AND DISCUSSION

3.1. Identification and characterization of Vr-Hsp70 in V. radiata

HSP70 belongs to the class of molecular chaperones and is involved in the process of protein folding, refolding of misfolded proteins, and membrane translocation of secretory proteins and organelles; it is highly conserved in various organisms ranging from microorganisms to animals to plants (Mayer and Bukau, 2005; Yu et al., 2015). In plants, HSP70s play a major role in the developmental processes as well as in the response to various biotic and abiotic stresses (Guo et al., 2016; Yer et al., 2016).

In this study, a total of 47 proteins were identified, which contained PF00012 domains, and 37 HSP70s remained after removal of duplicate HSP70s. Five domains were discarded on the basis of SMART, Pfam, CDD, instability index, molecular weights, and theoretical isoelectric point scan. Finally, a total of 32 putative *Vigna* Vr-HSP70s were obtained for further downstream analysis. For convenience, all the HSP70s were designated as Vr-HSP70-C-1 to Vr-HSP70-C-32 based on their molecular weight in KDa, from lower to higher, along with cellular localization of protein.

Identified Vr-HSP70s and their accession numbers, amino acid (AA) length, molecular weight, isoelectric point, and subcellular localization are given in Table 1. The predicted molecular weights were found between 25.21 (Vr-HSP70-C-1) and 112.27 (Vr-HSP70-C-32) kDa and protein sequence lengths between 226 (Vr-HSP70-C-1) and 1012 (Vr-HSP70-C-32) amino acids. Of 32 proteins, 7 Vr-HSP70s were found to be unstable, having an instability index >40. Moreover, theoretical isoelectric points (pI) of Vr-HSP70s ranged from 4.96 (Vr-HSP70-ER-6) to 9.05 (Vr-HSP70-C-18) (Table 1 and Supplementary Data). Preliminary studies of such HSP70 families have been done in several other plants such as *Arabidopsis* (Sung et al., 2001a), rice (Sarkar et al., 2013), spinach (Guy and Li, 1998), soybean (Zhang et al., 2015), pepper (Guo et al., 2016), Populus (Yer et al., 2016), and moss (Tang et al., 2016).

This is the first report of HSP70 gene families in the genome of *V. radiate*. Study reveals the variation in HSP70 domains in *V. radiata*-like other plant species. For example, spinach genome contains 12 domains, 18 in *Arabidopsis* genome, 34 in poplar genome, 21 in moss genome and 32 domains in rice genome. Maximum 61 HSP70 domains have been reported in soybean which is due to twice gene duplication event in its evolution (Jaiswal et al., 2019). All this variation may be due to the genome size variation and predicted coding genes.

3.2. Sequence analysis, chromosomal location, and gene duplication

All the Vr-HSP70 genes were mapped onto *V. radiata* genome to detect the chromosomal position and found that all the genes were distributed throughout the genome except chromosome 1. Chromosomal variations of *HSP70* density were observed. The maximum (9 genes) number of genes was mapped over chromosome 4, followed by chromosome 10 (7 genes) and chromosome 9 (3 genes). Two genes were mapped on chromosome numbers 5, 7, and 8, while a single gene was mapped on chromosomes 2, 3, 6, and 11 (Fig. 1). Three genes without chromosomal localization were mapped on scaffolds (Vr-HSP70-ER-6, Vr-HSP70-N-11, and Vr-HSP70-N-24) (Table 1).

In this study, we found nine pairs of gene duplication events, while six genes were uniquely present, namely, Vr-HSP70-C-1, Vr-HSP70-ER-6, Vr-HSP70-Cl-8, Vr-HSP70-C-15, Vr-HSP70-Cl-22, and Vr-HSP70-C-31, as shown in Figure 1. A total of nine pairs of gene duplication of Vr-HSP70s were found, of which, two pairs were observed in the same chromosome such as Vr-HSP70-C-9 and Vr-HSP70-C-25, present in chromosome 10, whereas the second pair consisting of Vr-HSP70-C-2, Vr-HSP70-C-7, Vr-HSP70-C-10, Vr-HSP70-C-18, Vr-HSP70-C-27, and Vr-HSP70-C-32 was found in chromosome 4 (Table 2). In addition, the Vr-HSP70-N-11 gene was also present in the second pair, but it was found in an unplaced scaffold region of the genome. We found seven more pairs of gene duplication between chromosomes (Table 2). It was observed that mostly genes involved in duplication were cytoplasmic *HSP70s*.

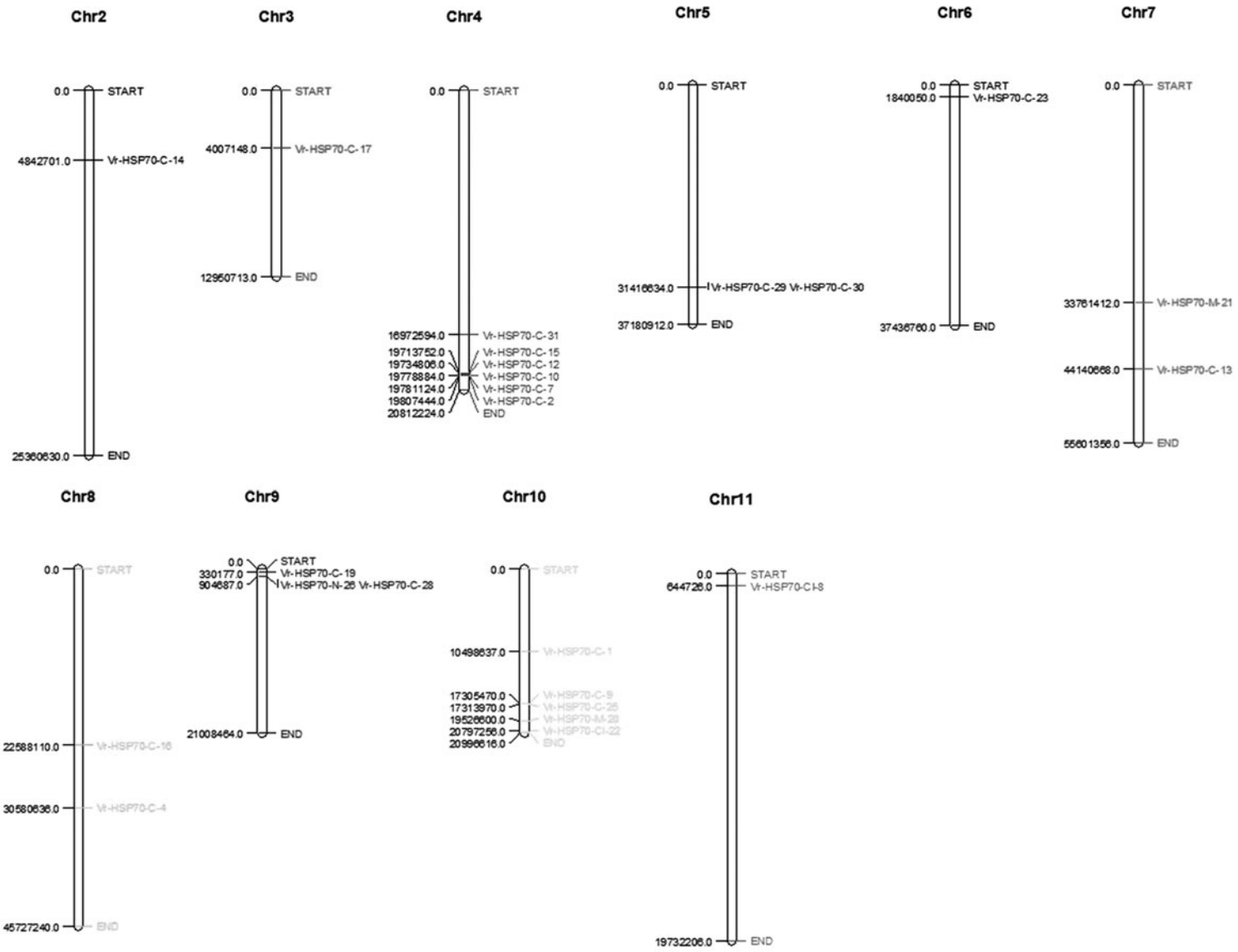

FIG. 1. Chromosome-wise distribution of Vr-HSP70s in *Vigna radiata* genome. Vr-HSP70, *V. radiate* HSP70.

Table 2. List of Gene Duplications Found in Vr-HSP70

Pairs	*Gene ID (chromosome No.)*
1	Vr-HSP70-C-3 (10), Vr-HSP70-C-4 (8), Vr-HSP70-C-5 (10)
2	Vr-HSP70-C-23 (6), Vr-HSP70-N-24 (unplaced scaffold)
3	Vr-HSP70-C-12 (4), Vr-HSP70-C-14 (2)
4	Vr-HSP70-C-13 (7), Vr-HSP70-C-19 (9)
5	Vr-HSP70-M-20 (10), Vr-HSP70-M-21 (7)
6	Vr-HSP70-C-16 (8), Vr-HSP70-C-17 (3)
7	Vr-HSP70-N-26 (9), Vr-HSP70-C-28 (9), Vr-HSP70-C-29 (5), Vr-HSP70-C-30 (5)
8	Vr-HSP70-C-9 (10), Vr-HSP70-C-25 (10)
9	Vr-HSP70-C-2 (4), Vr-HSP70-C-7 (4), Vr-HSP70-C-10 (4), Vr-HSP70-C-18 (4), Vr-HSP70-C-27 (4), Vr-HSP70-C-32 (4), Vr-HSP70-N-11 (unplaced scaffold)

Cannon et al. (2004) reported that tandem and segmental gene duplication has played a crucial role in the expansion and evolution of gene families in plant species. These gene families are made by insertion, deletion, and base substitution (Wen et al., 2017). This observation revealed that the Vr-HSP70 family members might be the result of such genomic expansions and rearrangement during the course of evolution. These observed gene duplication events of *HSP70* gene families reflect the diversification of function against various biotic and abiotic stresses of *V. radiata.*

3.3. Subcellular localization prediction, intron/exon organization, motif identification, and phylogenetic analysis

Subcellular localization analysis of Vr-HSP70s revealed that these proteins were distributed into five regions such as cytoplasmic (Cyto), endoplasmic reticulum (ER), chloroplast (C), nuclear (N), and mitochondrial (M). Maximum proteins were found in the cytoplasmic/cytosolic region, that is, 24, followed by 3 in nuclear. Chloroplast and mitochondrial shared two genes each, while one was found in ER (Table 1). In *V. radiata*, a total 24 cytosolic HSP70s were found, which is comparatively higher than rice (11) and Arabidopsis (5) and lower compared with soybean, that is, 34 (Sung et al., 2001a; Sarkar et al., 2013; Zhang et al., 2015).

Exon/intron organization of 32 Vr-HSP70s was analyzed to collect the information of gene structure. The maximum numbers of introns were found in Vr-HSP70-C-31, that is, 12, followed by 10 introns in Vr-HSP70-N-24, Vr-HSP70-C-29, and Vr-HSP70-C-30 each. While no introns were found in five Vr-HSP70s (Vr-HSP70-C-2, Vr-HSP70-C-4, Vr-HSP70-C-9, Vr-HSP70-C-13, and Vr-HSP70-C-25), no information of exon/intron was found for five genes in *V. radiata* data available in NCBI (Fig. 2).

Structural analysis of the exon/intron revealed that 15.62% of Vr-HSP70 genes were intron-less. In Vr-HSP70, the phylogenetic group 1b shares a similar exon/intron organization, that is, 0 or 1 intron. These findings are similar to the reported subcellular localization of *Arabidopsis* and Poplar *Hsp70* genes (Sung et al., 2001a; Yer et al., 2016). A huge variation was observed in the gene encoding cytoplasmic Vr-HSP70s; maximum genes have intron 0 or 1, but 5 cytoplasmic genes have an intron count above 8 (Table 1). These exon/intron organizations vary in different plant species. Higher introns were observed in *Vigna* HSP70 compared with *C. annuum* L. (Guo et al., 2016), spinach (Guy et al., 1998), and *Arabidopsis* (Sung et al., 2001a), and less compared with *P. patens* (Tang et al., 2016), soybean (Zhang et al., 2015), and rice (Sarkar et al., 2013). The gene structure diversity of HSP70 in *Vigna* species reflects not only an evolutionary process but also plays a major role in the regulatory mechanisms of complex environment of *Vigna* cells and tissues (Sung et al., 2001b).

Phylogenetic tree of 32 Vr-HSP70s was constructed using the NJ method to understand the evolutionary divergence of this gene family in *Vigna*. Four major clusters were found based on the analysis. Group 1 was the major largest cluster having 26 proteins with 4 subclusters, namely, 1a (12), 1b (9), 1c (1), and 1d (4). Cluster 3 was found with four proteins, whereas Cluster 2 and Cluster 4 were with only one (Fig. 3).

The phylogenetic tree of 96 HSP70s of *V. radiata* (32), rice (32), *Arabidopsis* (18), and *S. cerevisiae* (14) was constructed using the NJ method to understand the evolutionary relationship between them. Therefore, phylogenetic relationships of *V. radiata*, rice, *Arabidopsis*, and *S. cerevisiae* were divided into seven subgroups (group I to VII) (Fig. 4). Of all these groups, group I was the largest class with 25 HSP70s which

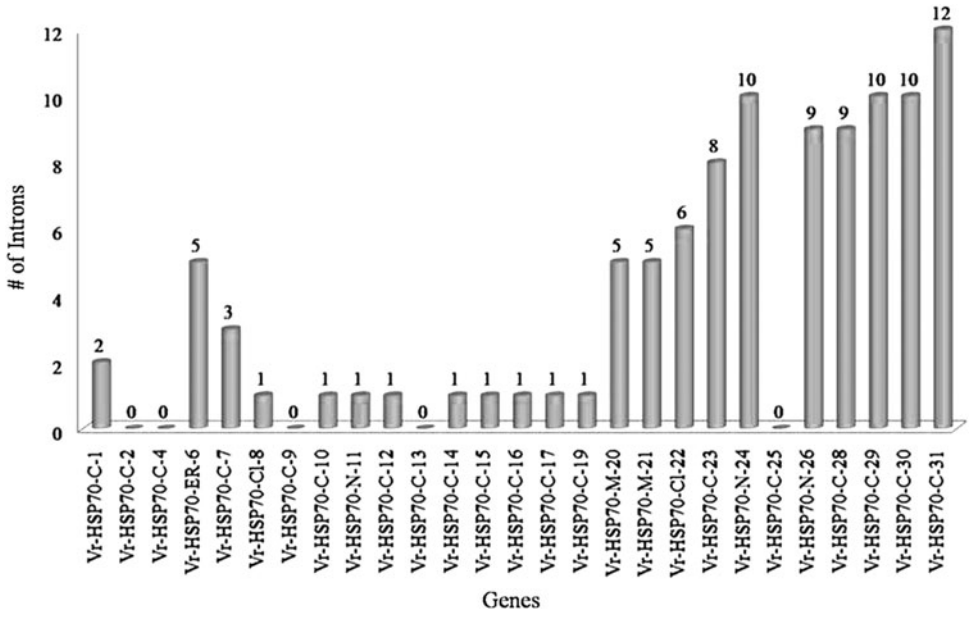

FIG. 2. Intron organization in Vr-HSP70 genes.

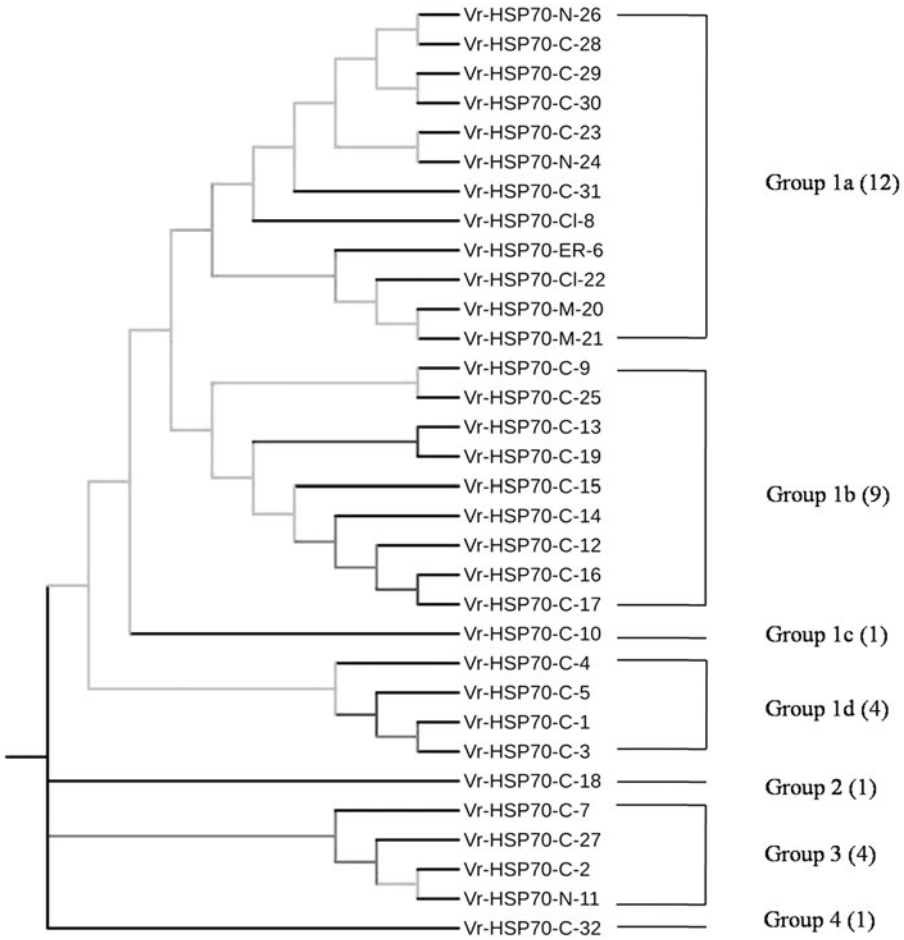

FIG. 3. Phylogenetic tree of 32 Vr-HSP70s using NJ method. NJ, neighbor-joining.

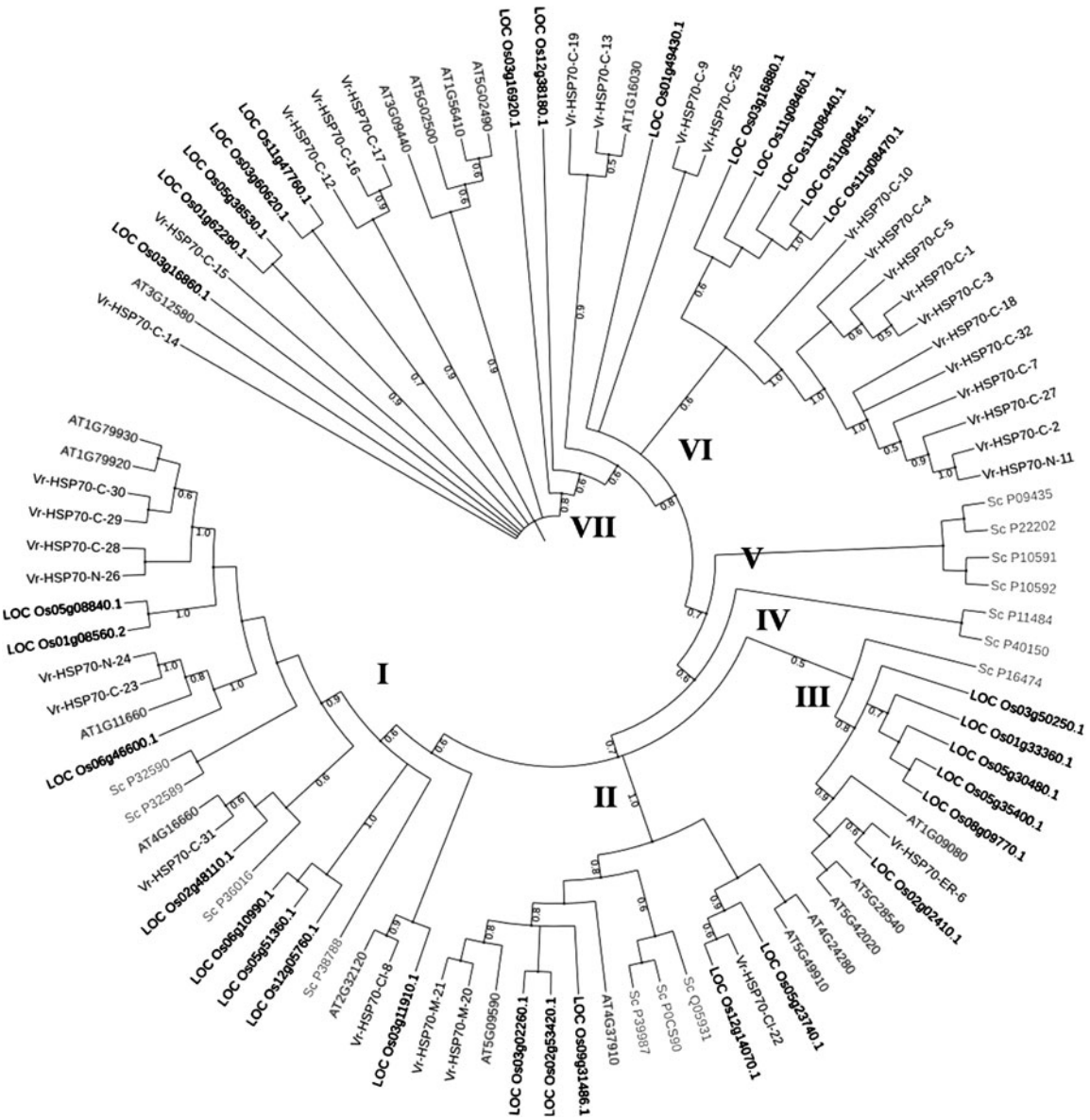

FIG. 4. Phylogenetic tress of HSP70 of *V. radiata*, rice, *Arabidopsis*, and *Saccharomyces cerevisiae*.

contains 8 each of *V. radiata* and rice, followed by 5 and 4 of *Arabidopsis* and *S. cerevisiae*, respectively. Whereas group VI was the second largest group with 16 HSP70, which contains 11 from *V. radiata* and 5 from rice. Moreover, Group IV and V only contain HSP70s of *S. cerevisiae*. These findings suggest the main features of the HSP70 families of plants, which were generated before the division of monocot and dicot. This evolutionary relationship of HSP70 revealed the orthologous and paralogous differences among species (Zhang et al., 2015).

Results of motif analysis of 32 Vr-HSP70s predicted 10 consensus motifs. High motif similarity was found in the N-terminal (ATP binding domain) region. The size of predicted motifs ranged from 21 to 50 amino acids, motifs 1, 2, and 5 have the maximum length, that is, 50 amino acids. While motifs 3 and 9 share the minimum length of 21 amino acids (Supplementary Fig. S1). Only four proteins, namely, Vr-HSP70-C-1, Vr-HSP70-C-2, Vr-HSP70-C-3, and Vr-HSP70-C-31, with ≤5 motifs. The highest number (>10) was found in 12 Vr-HSP70s (from 9 to 19 and 32). All the proteins were containing multiple repeats of shorter motifs (3). Only Vr-HSP70-C-32 was having motifs in both N and C terminals. Cytoplasmic Vr-HSP70s, being smaller sequences, were observed with high variability.

A phylogenetic analysis was carried out to understand the evolutionary relationship between domain structure and Vr-HSP70s. Using the NJ method, the phylogenetic tree of 32 Vr-HSP70 domains was constructed. The phylogenetic tree of 32 Vr-HSP70s was classified into 4 groups (clusters 1–4), which comprise 26, 1, 4, and 1 protein, respectively (Fig. 3). Group 1 was the biggest cluster and further split into four subgroups (subgroup 1a, 1b, 1c, and 1d). We found that a good number of internal branches had high bootstrap values that demonstrate the statistical reliability of potential homologous pairs. Phylogenetic tree analysis for functional prediction of HSP70s was also studied in various other crops such as rice, *Arabidopsis*, and poplar. Members of HSP70 in rice were separated into four clades (Sarkar et al., 2013), whereas in *Arabidopsis*, HSP70s were divided into two large clusters with seven subclusters (Lin et al., 2001) and poplar HSP70s were clustered in three subgroups (Yer et al., 2016). Cytoplasmic HSP70s in *Vigna* were found in all the four groups of phylogenetic tree, but 1b and 1d were the groups containing only cytoplasmic HSP70. In addition, group 1a contains mitochondrial, cytoplasmic, ER, chloroplast, and nuclear Vr-HSP70s. However, two mitochondrial and two chloroplast HSP70s were found in the same group, that is, 1a. Of the three nuclear HSP70s, two were found in group 1a and one in group 3. These results concluded that few members of groups were separated from their groups and these finding were also found in poplar, rice, and *Arabidopsis*.

Motif composition analysis was also carried out to check the reliability of phylogeny. A total of 10 different motifs were identified on the basis of domain composition of Vr-HSP70s and are found conserved among these sequences. We found that most of the closely related Vr-HSP70s have a common motif composition (Supplementary Fig. S1). We found conserved motif structures in phylogeny group 1a and 1b. This kind of motif variations between sequences specifies a potentially diverse function in regard to various biological functions (Puranik et al., 2012). Different subfamilies were having variable motif numbers, orders, and types, as reported in soybean (Zhang et al., 2015). Maximum conserved motifs were observed in the ATPase domain region of Vr-HSP70s as reported in soybean (Zhang et al., 2015) and rice (Sarkar et al., 2013).

3.4. Gene ontology annotation

GO and functional categorization of Vr-HSP70 revealed that maximum proteins were involved in ''binding'' and ''catalytic activity,'' GO terms of molecular function, that is, 30 and 7, respectively. In biological processes, only three proteins were found to be involved in ''cellular process,'' whereas one protein in each, namely, ''cell part,'' ''organelle,'' and ''cell'' under cellular component. Of all these proteins, only Vr-HSP70-M was found in all the GO terms (Supplementary Fig. S2).

3.5. Gene regulatory network analysis of Vr-HSP70 genes

HSP70 as a housekeeping gene plays an important role in protein regulation, cellular development, maintaining cell stability such as protein folding, and protecting organism from biotic and abiotic stress (Yu et al., 2015). The main objective of construction of gene regulatory network (GRN) based on coexpressional data is to delineate the putative candidate genes involved in stress regulation. The GRN model constructed on the basis of limited sample size shows the qualitative aspect of genes involved rather than the quantitative relationship of genes. Previously, it has been reported that single nucleotide polymorphisms (SNPs) of genes involved in GRN lead to perturbation in phenotype/trait resulting in eQTL discovery (Iquebal et al., 2017).

In abiotic stress, Vr-HSP70-C-31, Vr-HSP70-C-29, Vr-HSP70-C-30, Vr-HSP70-M-21, Vr-HSP70-Cl-22, Vr-HSP70-C-12, Vr-HSP70-M-20, Vr-HSP70-C-14, Vr-HSP70-C-13, Vr-HSP70-ER-6, Vr-HSP70-C-15, Vr-HSP70-C-17, and Vr-HSP70-C-16 were found highly expressed with very high effective read count throughout the samples. Three genes were found without any expression values such as Vr-HSP70-C-1, Vr-HSP70-C-2, and Vr-HSP70-C-7. In heat map (Fig. 5a), cytoplasmic *HSP70s* were highly expressed compared with other organelles. Furthermore, 29 genes were used for the coexpression network analysis (Supplementary Table S1).

Hub genes were selected on the basis of degree of connected genes. Five genes were considered hub with highest degree of 14 (Supplementary Table S2). Of five, four belong to cytoplasmic/cytosolic (Fig. 5b). Vr-HSP70-C-14, Vr-HSP70-N-26, and Vr-HSP70-C-28 were highly expressed in 18h and 24h, while Vr-HSP70-C-29 and Vr-HSP70-C-30 show low expression in experimental stages compared with control hour. Cytosolic HSP70 is highly expressed and acquired thermo tolerance due to reactivation of protein aggregates under stress condition (Sarkar et al., 2013). It plays a major role in response to various abiotic

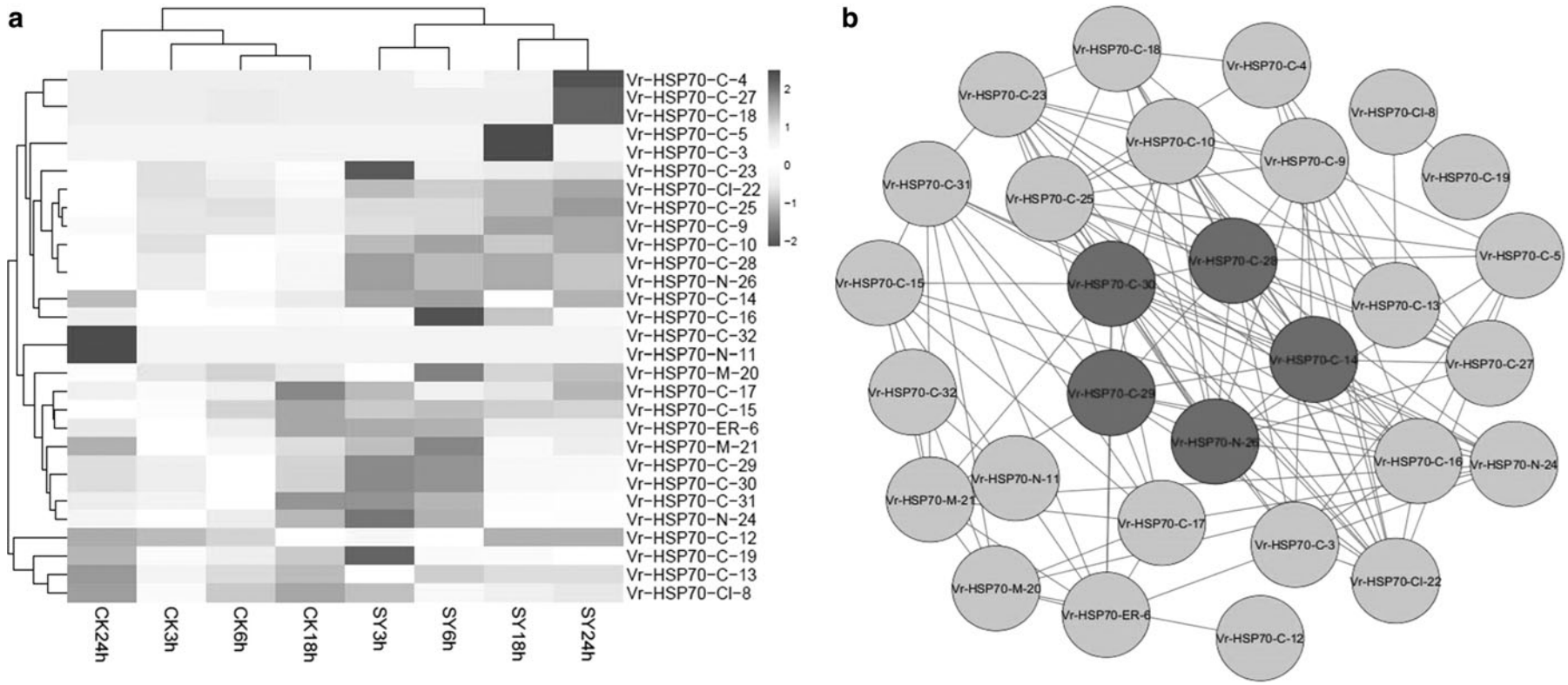

FIG. 5. **(a)** Coexpression analysis of Vr-HSP70 genes in abiotic stress and represented in the form of heat map. **(b)** Coexpression gene network of expressed genes, and inner dark nodes show the hub genes.

stresses such as heat stress in rice (Jung et al., 2013), heat and drought stress in *C. annuum* L. and *Populus* (Guo et al., 2016; Yer et al., 2016), highly expressed in salt, drought, heat, and cold stress responses in *Solanum tuberosum* (Liu et al., 2018), heat and drought stress in soybean (Zhang et al., 2015) and high expression enhances drought and salinity tolerance in sugarcane (Augustine et al., 2015b), and ABA, salt, and drought stresses in *P. patens* (Tang et al., 2016), cucumber (Li et al., 2014), and *Arabidopsis*, respectively (Swindell et al., 2007). It also plays a role in response to high and low temperatures in *Hevea brasiliensis* and *Castanea sativa* (Lopez-Matas et al., 2004; Zhang et al., 2009).

In biotic stress, expression values of Vr-HSP70-C-13 were found maximum in all the biotic samples, followed by Vr-HSP70-Cl-8, Vr-HSP70-M-20, and Vr-HSP70-C-16. Ten genes were found without any or very less expression values: Vr-HSP70-C-2, Vr-HSP70-C-3, Vr-HSP70-C-4, Vr-HSP70-C-5, Vr-HSP70-C-7, Vr-HSP70-C-10, Vr-HSP70-N-11, Vr-HSP70-C-18, Vr-HSP70-C-27, and Vr-HSP70-C-32 (Fig. 6a) (Supplementary Table S1). After coexpression analysis, a total of nine genes were found in hub genes with the highest degree, that is, 15 (Supplementary Table S2). Hub genes Vr-HSP70-C-13, Vr-HSP70-C-14, Vr-HSP70-C-15, Vr-HSP70-C-16, Vr-HSP70-C-19, Vr-HSP70-M-20, Vr-HSP70-C-29, Vr-HSP70-C-30, and Vr-HSP70-C-31 were found highly expressed in resistant samples compared with susceptible cultivar (Fig. 6b). *HSP70* plays a crucial role in plant immunity in microbial pathogenesis mainly during viral infections (Park and Seo, 2015) and is involved in replication of viruses and movement such as virion assembly, viral protein folding, and protein expression, which ultimately lead to viral infection (Aparicio et al., 2005; Nagy et al., 2011; Jiang et al., 2014).

HSP70 regulates the viral movement, replication, and reproduction, which eventually support viral infection. It is also reported that interaction between *HSP70* of *Nicotiana tabacum* and coat protein of *Potato virus* plays a vital role in viral infection (Boevink and Oparka, 2005; Hafrén et al., 2010). A similar observation is also reported in case of rice stripe virus (Jiang et al., 2014). Chen et al. (2008) reported that cytosolic *HSP70s* increase the infection of *Nicotiana benthamiana* by *Potato virus X*, *Watermelon mosaic virus*, *and Tobacco mosaic virus*. Interaction between cytosolic HSP70 and SGT1 involved regulating the *Arabidopsis* immune responses (Noël et al., 2007). Recent studies show that the silencing of *HSP70* gene in pepper increases the susceptibility to *Xanthomonas campestris pv. vesicatoria* (Xcv) infection (Kim and Hwang, 2015). *HSP70* has been reported to interact with *pepino mosaic virus capsid* in tomato (Mathioudakis et al., 2012). All these findings indicate that *HSP70s* are multifunctional in nature at different stages of the plant life cycle.

Vr-HSP70-C-14, Vr-HSP70-C-29, and Vr-HSP70-C-30 hub genes were found to be common in both the networks (Figs. 5b and 6b). Highly expressed cytosolic HSP70s are involved in maturation, seed development, and germination (DeRocher and Vierling, 1995; Sung et al., 2001a, 2001b). The effective count of all the highly expressed genes was above 1000. Moreover, these highly expressed genes were found in group 1 (1a and 1b) of phylogenetic tree.

These discovered candidate genes can be used in a variety of developments for improvement of biotic and abiotic stress resistance. Several such candidate genes, having attributes of resistance, have been reported in various crops, for example, AHAS1 gene in sunflower (Bulos et al., 2013), opaque2 gene in maize (Danson et al., 2006), and dehydrins involved in freezing stress in *Arabidopsis* (Puhakainen et al., 2004). AtHsp70–15 plays the key role in normal growth in response to heat response in *Arabidopsis* and its deficiency has been associated with turnip mosaic virus resistance (Jungkunz et al., 2011). Earlier it has been reported that a higher expression of cytosolic Hsc70–1 of *Arabidopsis* was involved in tolerance to heat at certain stress environments (Sung and Guy, 2003). Also, overexpression of ER *Hsp70* of tobacco lowers the ER stress and enhanced the tolerance level against drought. Overexpression of ER *Hsp70* has been found with better drought tolerance in tobacco (Usman et al., 2017).

Our findings may be useful in the development of HSP70 induced transgenic as it is already reported in *A. thaliana* and alfalfa (Lee and Schöffl, 1996; Ferradini et al., 2015). Augustine et al. (2015a) reported that *Erianthus arundinaceus HSP70* plays a key role in the regulation of formation of anisotropic interdigitation in plant drought stress in Erianthus and *HSP70* transgenic sugarcane. Also, expressions of *Trichoderma harzianum HSP70* transgenic enhance the heat and other abiotic stress resistances in *Arabidopsis* (Montero-Barrientos et al., 2010; Al-Whaibi, 2011).

CRISPR/Cas9-mediated gene editing can be used in *V. radiata HSP70* gene to increase tolerance to external stresses as well as quality management. Previously, Nitika and Truman (2018) developed a cell line of CRISPR/Cas9-mediated gene editing in genomic locus of *HSC70* by integrating a tandem-affinity (TAP) epitope tag.

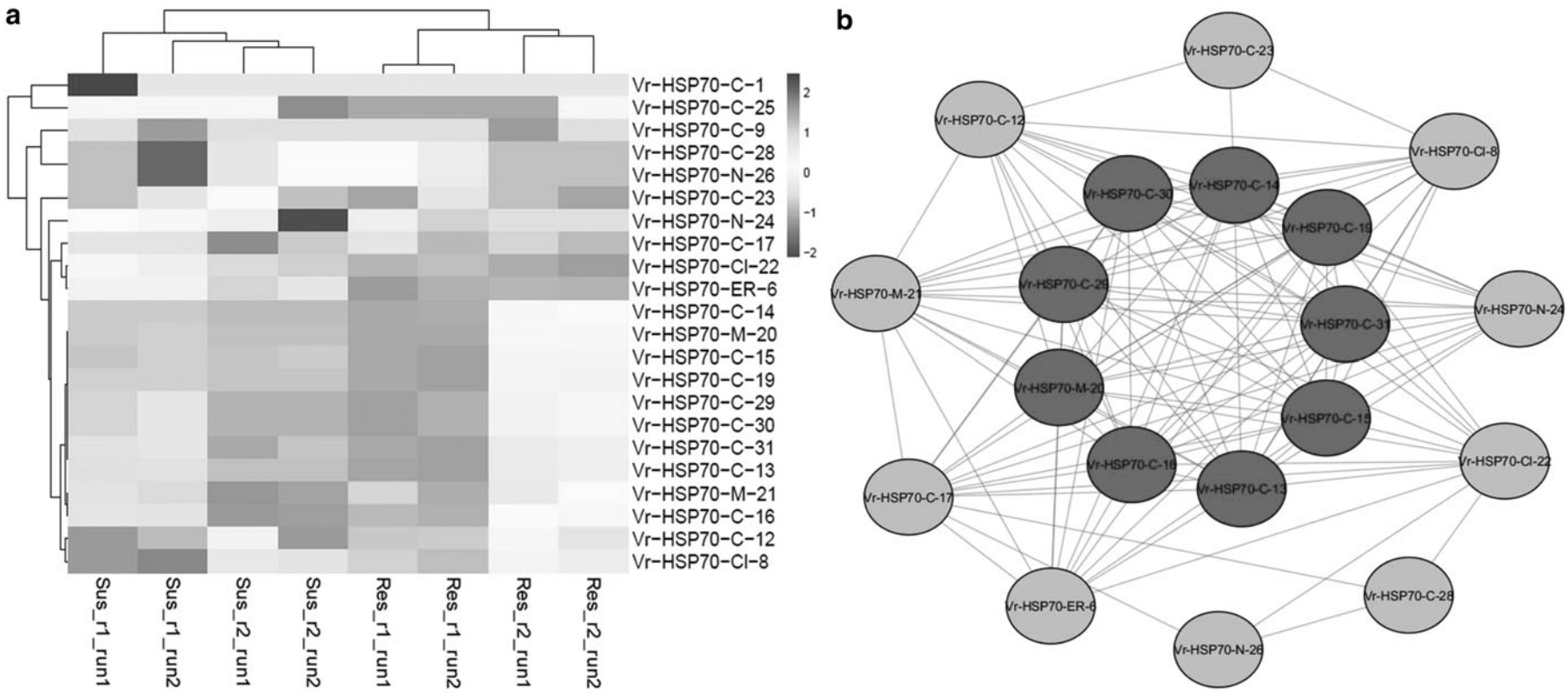

FIG. 6. **(a)** Coexpression analysis of Vr-HSP70 genes in biotic stress and represented in the form of heat map. **(b)** Coexpression gene network of expressed genes, and inner dark nodes show the hub genes.

4. CONCLUSIONS

In this study, a genome-wide study of the Vr-HSP70 family in *V. radiata* was performed and it investigated the expression profile in abiotic and biotic stresses and construction of gene regulatory network. A total of 32 Vr-HSP70s were identified from *V. radiata* genome. A comprehensive analysis of Vr-HSP70s was performed, including structural organization of protein, gene duplication, phylogeny, motif identification, expression profile, gene regulatory network, and variant identification. Results of overexpression analysis showed that most of the cytoplasmic/cytosolic Vr-HSP70 genes were involved in abiotic and biotic stress. This information lays the foundation for further functional and structural studies of Vr-HSP70 genes in *V. radiata* under various stresses. This work laid the foundation of exploration and characterization of the *HSP70* gene family and other agricultural applications and provides a fundamental clue for investigation of the HSP gene family.

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AUTHORS' CONTRIBUTIONS

D.K., S.J., P.K.Y., M.A.I., and R.S.J. conceived the theme of the study. R.S.J. did the computational analysis. R.S.J. and M.R. did expression and gene regulatory network analysis. R.S.J., S.J., and M.A.I. drafted the article. D.K., P.K.Y., and A.R. edited the article. All authors read and approved the final article.

AUTHOR DISCLOSURE STATEMENT

The authors declare that they have no conflicting financial interests.

SUPPLEMENTARY MATERIAL

Supplementary Data
Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2

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