Indian J. Genet., 78(1): 95-103 (2018)
DOI: 10.5958/0975-6906.2018.00011.1

Isolation and allelic characterization of finger millet (*Eleusine coracana* L.) small heat shock protein *EcHSP17.8* for stress tolerance

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(Received: August 2017; Revised: December 2017; Accepted: January 2018)

Abstract

Finger millet (*Eleusine coracana* L.) commonly known as ragi is an orphan cereal crop having significant climate-smart stress adaptation system. Apart from various abiotic stresses worldwide, heat stress is the major limiting constraint for crop yield and the loss of crop yield due to heat stress is increasing every year. Hence, to generate better heat tolerant crop plants there is a need to mine superior alleles. In this regard, efforts were made to isolate and characterize role of small heat shock protein gene *HSP17.8* from finger millet. Here, we obtained an EST of having Hsp20 domain based on the expression analysis under heat stress and identified complete gene using homology-based analysis. The obtained gene was named as *EcHSP17.8* based on its molecular weight. The complete Open Reading Frame (ORF) of *EcHSP17.8* is of 489 bp with peptide length of 162 aa. Expression analysis of this gene in finger millet revealed its maximum expression in roots tissue. This gene was found to be induced by heat (42^{o}C), desiccation, NaCl (250 mM), mannitol (200 mM) and oxidative stress; however maximum upregulation was observed under heat stress up to 40 folds. Expression analysis of this gene in 16 finger millet genotypes under heat stress revealed its maximum expression after 2h which remained upregulated up to 4h showing it as early responsive gene under heat stress. As this gene is well characterized for stress tolerance in other crops, the alleles of *HSP17.8* from crop like finger millet can be used as better candidate for generating heat tolerant crop plants.

Key words: Heat stress, chaperon, finger millet, open reading frame, ramachandran plot.

Introduction

Abiotic stresses are major constraints to the crop plants. These stresses are contributing significant yield losses by causing morphological, physiological, biochemical and molecular changes in crop plants (Bita and Gerats 2013). But plants evolved a variety of mechanisms to rapidly sense the changes and protect themselves from the environmental stress conditions (Sun et al. 2012). To cope with environmental changes, organisms up regulate a group of proteins known as heat shock proteins (HSPs) as a response to the stress conditions (Bakthisaran et al. 2015). Heat shock proteins (HSPs) are families of proteins and many members of this family shows chaperone activity. A minimum of 150 million years ago, small heat shock protein gene families indeed arise before the divergence of the dicots and monocots (Doyle and Donoghue 1993). Small and large heat shock protein families had diverse evolutionary lineages and patterns, and that gene replacement could be another evolutionary mechanism in the large heat shock proteins (i.e. pre-existing nuclear counterparts was replaced by eubacterial genes) (Huang et al. 2008). Plant small heat shock proteins did not group with any homologs from prokaryotes. Therefore, during the early evolution, plant small heat shock proteins would not have originated from the prokaryotes by gene transfer (Huang et al. 2008). On the whole, based on the pattern of sequence closeness, and evolutionary analysis revealed that the gene families of small heat shock proteins (smHSPs), arose by comparatively ancient duplication of genes followed by divergence in the sequence preceding to the radiation of

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angiosperms (Waters et al. 1996). HSPs are responsible for achieving protein folding, protein assembly, translocation and degradation under normal cellular condition. However, under cellular stress conditions they stabilize proteins, membranes, bind to partially denatured proteins and prevent irreversible aggregation (Wang et al. 2004). By this process, they maintain cellular homeostasis and protect plants under normal and stress conditions. Based on their molecular mass, heat shock proteins (HSPs) are divided into five different families: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, which are high molecular mass HSPs and low molecular mass HSPs also called small heat shock proteins (sHSPs) ranging from 12-40 kDa (Wang et al. 2004; Zhou et al. 2012). Among the five different families of HSPs, small heat shock proteins (sHSPs) are the most common in plants and share a common C-terminal α-crystalline domain (ACD) of having 90-amino acids (Vierling 1991; Waters et al. 1996; Mu et al. 2013). Because of their abundance and heterogeneity, they play significant role in stress tolerance (Sun et al. 2012). The sHSPs are further divided into six sub classes (class CI to class CVI), out of these CI, CII, CIII are localized in the nucleus or cytosol and the CIV, CV, CVI are localized in the plastids, endoplasmic reticulum (ER), and mitochondria, respectively (Low et al. 2000; Ma et al. 2006; Siddique et al. 2008; Jiang et al. 2009). Previous studies have shown that sHSPs are induced and accumulated by major abiotic stresses such as high temperature, salinity, drought and cold. Apart from these stresses sHSPs are regulated by different developmental stages of the plant i.e., germination, embryogenesis, pollen development and fruit maturation (Sun et al. 2001; Sun et al. 2002; Chauhan et al. 2012). Over expression of sHSP showed enhanced thermotolerance, osmotolerance, chilling and seed longevity. (Sun et al. 2001; Sanmiya et al. 2004; Perez et al. 2009; Mu et al. 2013).

The prime objective of this study was to identify and isolate small Hsp17.8 protein and its allelic variants from heat tolerant and heat sensitive accessions of finger millet, which is an excellent climate adaptation system and able to grow under non-optimal environmental conditions. Another objective was to develop marker for Hsp17.8 to differentiate heat tolerant and sensitive finger millet genotypes. Here, we identified *HSP17.8* gene based on expressed sequence tags (ESTs) from heat stressed finger millet, and characterized it under various abiotic stresses and in particular under heat stress in finger millet genotypes and also developed expression-based marker for crop improvement.

Materials and methods

Plant material and stress imposition

Finger millet genotype MR1 is used for gene isolation and expression analysis in different abiotic stresses. Seeds were sterilized with 70% ethanol and 2% sodium hypochlorite (NaClO) with intermittent double distilled water washings and placed on half strength MS (Murashige and Skoog) medium. Seedlings were allowed to grow for fifteen days under 16h light/8h dark cycle at 25°C in a culture room. These seedlings attaining three leaf stages after fifteen days were imposed for stress treatments. For NaCl (250mM), ABA (100µM) Mannitol (300mM), H_2O_2 (25mM) and DTT (2.5mM) stress imposition, respective chemicals were added to hydroponics solutions and seedlings were placed in to it. For heat treatment, seedlings were subjected to 42°C. For desiccation stress, seedlings were placed on a filter paper (90 × 100 mm) and allowed for drying. For tissue samples, matured leaf, shoot, root and panicle were collected along with complete seedlings. In all the stress treatments, the samples were collected after 2, 4, 6, 8 and 24 h. Tissue samples were immediately freezed in liquid nitrogen and stored at -80^0 C for further analysis.

RNA isolation and qRT-PCR analysis

RNA was isolated using Spectrum plant total RNA kit (Sigma, USA) from collected finger millet samples. DNA contamination during RNA isolation was eliminated using On-Column DNase I digestion set (Sigma, USA). The quality and quantity of RNA was assessed using gel electrophoresis and Nanodrop Spectrophotometer (Thermo scientific, USA). 1µg of RNA from every sample was used for reverse transcription using Prime script 1st strand cDNA synthesis kit (Takara, Japan) based on the manufacturer instructions. Each cDNA was diluted five times and 1µl was used as a template for qRT-PCR reaction which was carried out in a LightCycler 480 system (Roche, USA). The reaction conditions for qRT-PCR were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 5s, 60°C for 10s and 72°C for 25s. β-tubulin was used as internal reference gene (βTubulin-F 5'- ACTGGGGTATCAATCCATCATCT CCCCTAT-3', βTubulin-R 5'-GTT TCCATCTCCAGGAGCCAAA GCCA-3') for normalization (Ramakrishna et al. 2017). Delta CT method was used to calculate fold change in expression (Livak and Schmittgrn 2001; Kanakachari et al. 2016).

In silico analysis

NCBI BLAST program was used for identification of homologous sequences from different plant species (https://blast.ncbi.nlm.nih.gov/Blast.cgi.). SMART online tool (http://smart.embl-heidelberg.de/) was used for prediction of major domain in EcHSP17.8 protein. The CELLO Sub-Cellular Localisation predictor online tool was used for sub cellular localisation prediction (http://cello.life.nctu.edu.tw/). Multiple sequence alignment of EcHSP17.8 protein and other homologs were performed with CLUSTAL W program of Bioedit software (Hall 1999). The phylogenetic tree was constructed using the MEGA6 software (Hall 2013). MEME online tool was used to predict the motifs present in EcHSP17.8 and other proteins (http://meme-suite.org/). Various physiochemical properties of the protein were predicted through ExPASy online tool (http://us.expasy.org/tools/ protparam.html).

Results

Identification of EcHSP17.8 from heat stressed finger millet SSH library

We have generated heat stressed finger millet EST library using MR1 genotype and identified up and down regulated genes under heat stress condition. From these up regulated ESTs, we obtained an EST of Class II small heat shock protein having 459 bp length. Which was further submitted to National Center for Biotechnology Information (NCBI; Accession Number KY053292) as Hsp20 due to presence of HSP20 domain. We aligned this EST with other sequences of finger millet genotypes which were retrieved from RNA-seq data present in the public domain. Homology analysis predicted a complete open reading frame of 489 bp which was further amplified (Fig. 1) with forward primer EcHSP17.8-F 5'-ATGGAGGCGAGGATGTT CGGGCTG-3' and reverse primer EcHSP17.8-R 5'-TCACGCAACCTTGACCTCAATGGT-3' and confirmed with sanger sequencing.

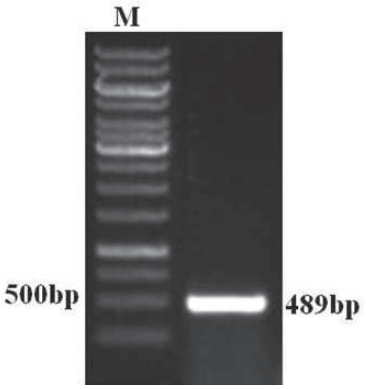

Fig. 1. PCR amplification of EcHSP17.8 using gene specific primers

Expression analysis of EcHSP17.8 under different stresses in finger millet

The expression level of *EcHSP17.8* was identified in different tissues of the plant including leaf, shoot, root, panicle, and seed using qRT-PCR. The highest expression of *EcHSP17.8* was noted in the root tissue. In panicle and seed, the transcript expression was almost similar (Fig. 2a). Further expression of *EcHSP17.8* was carried out under different abiotic, hormonal and oxidative stressors. The highest expression of this gene was observed up to 39 folds under heat stress at 2 h (Fig. 2b), and the lowest expression was observed under mannitol stress up to 2 folds (Fig. 2d). Under desiccation and H_2O_2 stresses its expression was limited up to 6 folds (Fig. 2e, f) and the highest expression was observed after 24h. Under NaCl stress, the expression was limited up to 15 folds with highest expression at 8h (Fig. 2c).

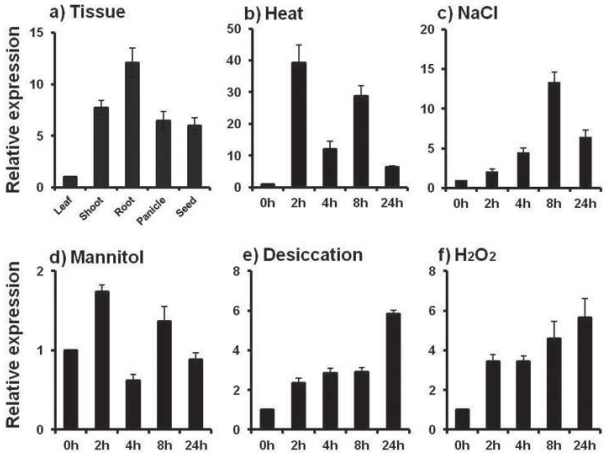

Fig. 2. Expression pattern of *EcHSP17.8* transcript in finger millet: a) Expression pattern of *EcHSP17.8* transcript in different vegetative organs of finger millet. b-f) Expression pattern of *EcHSP17.8* transcript in three leaf stage seedlings of finger millet seedlings treated with Heat (42^{o}C), NaCl (250mM), Mannitol (300mM), Desiccation, and H2O2 (25mM).

Structural analysis of EcHSP17.8

Analysis of EcHSP17.8 protein for major domains in SMART software showed presence of HSP20 domain ranging from 57 to 160 aa (Fig. S1a). The secondary structure analysis showed that 62 amino acid residues

(38.27%) are involved in alpha helices, 33 residues (20.37%) in extended strands, 11 residues (6.79%) in beta turns, and 56 residues (34.57%) in random coil formation (Fig. S1b). Since EcHSP17.8 is a chaperon, its major protein-protein interaction region was identified between 27 to 45 amino acids (Fig S1c). There are 27 negatively charged (Asp + Glu) and 26 positively charged (Arg + Lys) amino acids present in this protein. The atomic composition revealed presence of 2511 atoms ($C_{773}H_{1270}N_{222}O_{236}S_{10}$) in the protein. Aliphatic index of this protein is 77.65, whereas grand average of hydropathicity (GRAVY) was –0.412 confirming the hydrophobic nature of the protein (Fig S1d). CELLO sub-cellular localisation prediction tool predicted EcHSP17.8 as a cytoplasmic protein (Fig. S2). The extinction coefficient of this protein is 4470 M-1cm-1. The calculated exact mass of the protein is 17770.53 Da with an Isoelectric point (pI) of 5.96 (Table S1). The estimated half-life of this protein is 30 h, whereas the computed instability index was 37.03, which classifies the stable nature of the protein. There were four major motifs predicted in EcHSP17.8 when subjected to MEME motif prediction tool. Among the proteins analysed, proteins from *Brassica napus*, *Gossypium raimondii* and *Arabidopsis thaliana* were lack in Motif1 (Fig S3a). Each motif defines a pattern of defined width; Motif1 and Motif2 are having width of 50 amino acids whereas Motif2 and Motif4 are having width of 29 and 15 amino acids, respectively (Fig. S3b).

Molecular modelling of EcHSP17.8 protein

The 3D model for EcHSP17.8 protein was generated by I-TASSER. This software generated a total of five structural models based on the threading approach. The confidence of each generated model measured quantitatively by C-score. Out of the five models generated, model 1 had the best confidence score (C-sore) of –1.09 (Fig. 3a). C-score is calculated based on the convergence parameters of the structure assembly simulations and significance of threading template alignments. The typical range of C-score is -5 to 2. C-score of a higher value signifies a model with a higher confidence and vice-versa. The PROCHECK analysis of model 1 predicted Ramachandran plot (Fig. 3b). Statistical analysis of Ramachandran plot showed that 86 amino acid residues were in most favoured regions (64.2%), 34 residues in additionally allowed regions (25.4%), whereas 8 residues in generously allowed regions and 6 residues in disallowed regions (4.5%). An important aspect of the predicted Ramachandran plot is G factor. G factor value of predicted model 1 is -0.73, which falls into the usual category. G factor value is the overall average of parameters such as Dihedral angles (Phi-psi distribution, Chi1-chi2 distribution, Chi1 only, Chi3 and chi4, Omega) and Main-chain covalent forces (Main-chain bond lengths, main-chain bond angles).

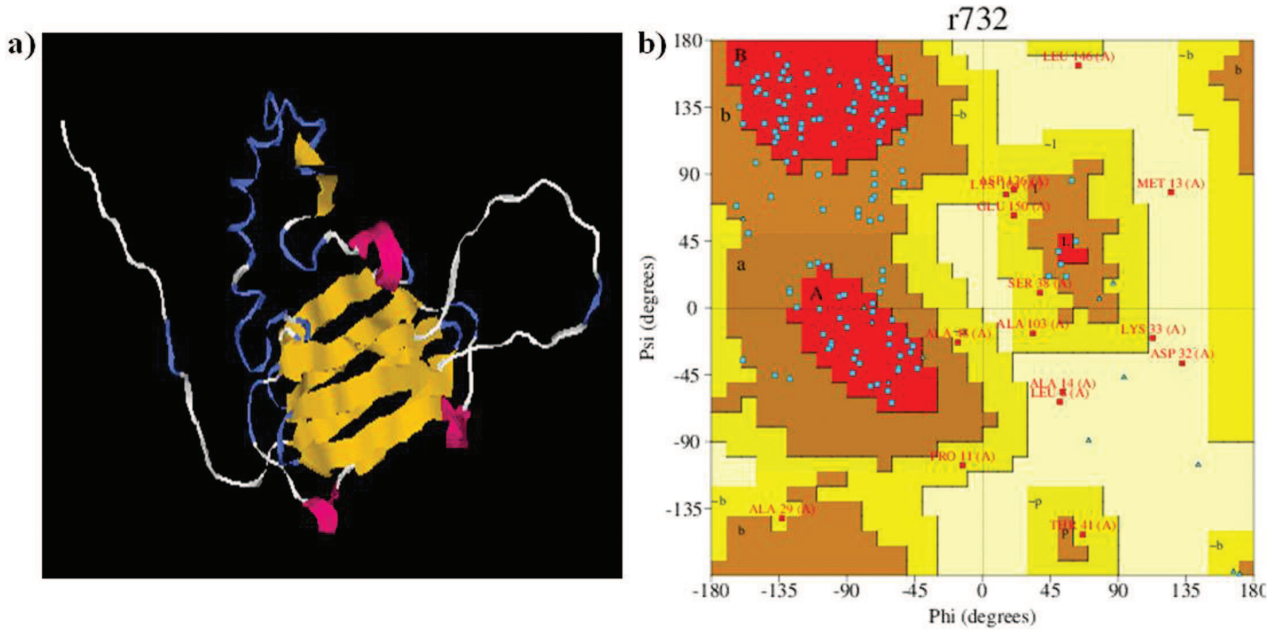

Fig. 3. 3D structure and Ramachandran plot prediction for EcHSP17.8 protein. (a) Predicted 3D model of EcHSP17.8 protein through I-TASSER server, representing model 1 from the server. (b) Ramachandran plot for predicted model 1 through PROCHECK server

Phylogenetic analysis of EcHSP17.8 among plant species

EcHSP17.8 has an open reading frame of 489 bp encoding a protein of 162aa. Multiple sequence alignment of EcHSP17.8 with small heat shock proteins from other species revealed that HSP20 domain was conserved among all peptides. Phylogenetic analysis showed that EcHSP17.8 protein was 96% similar to (*Zea mays*), 95% to (*Sorghum bicolour*), 92% to (*Brachipodium*), 89% to (*Triticum aestivum*), 76% to *Vitis vinifera* and *Brassica napus*, 71% to *Oryza sativa japonica*, 70% to *Gossypium raimondii*, and 64% to *Arabidopsis thaliana* (Fig. 4a). Evolutionary relationship of EcHSP17.8 with other proteins through MEGA analysis revealed that it is very close to *Zea mays* (Fig. 4b).

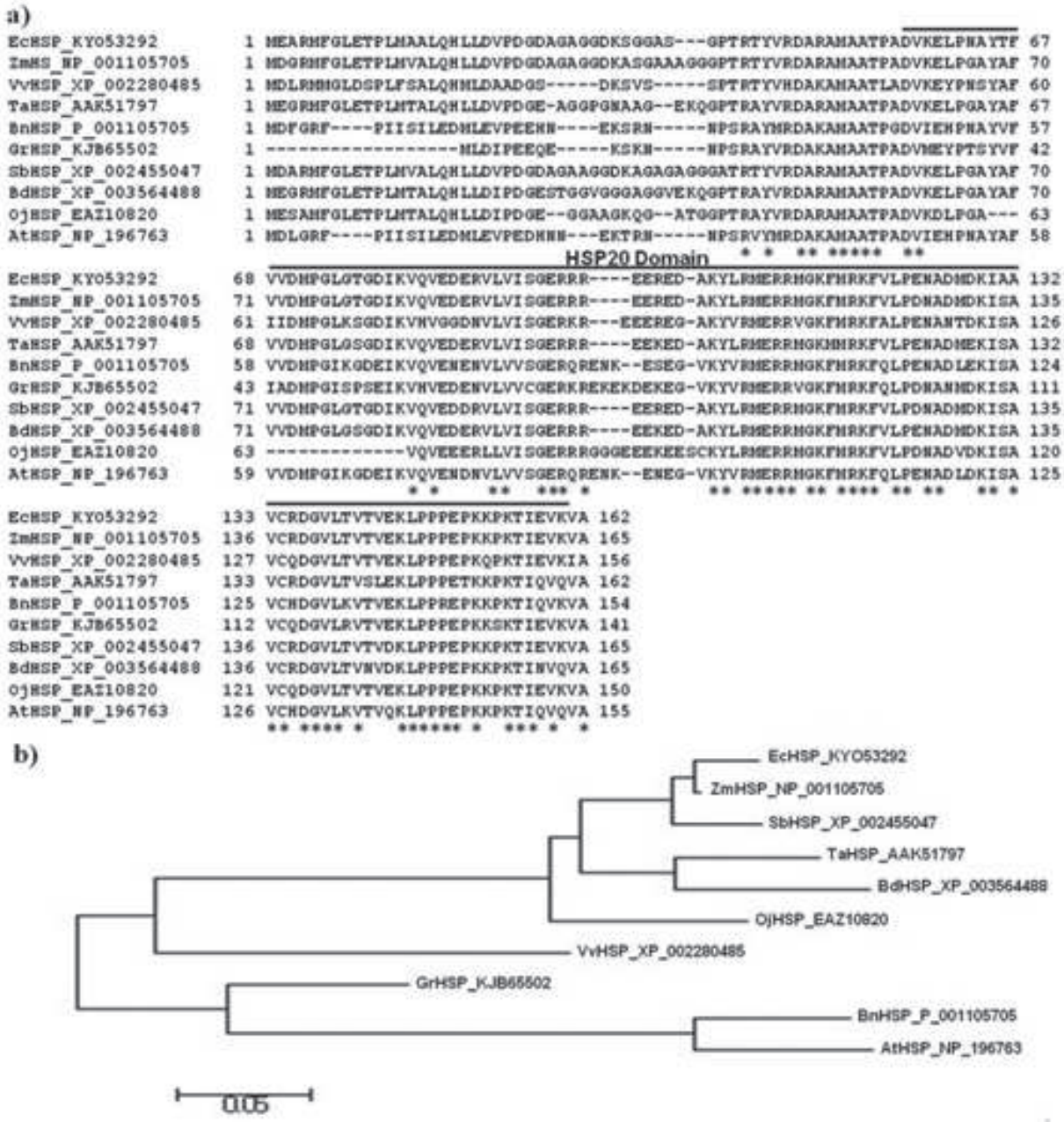

Fig. 4. Multiple sequence alignment and Phylogenetic analysis of EcHSP17.8. and other proteins. a) Multiple sequence alignment of EcHSP17.8 from *Finger millet*, *Zea mays*, *Vitis vinifera*, *Triticum aestivum*, *Brassica napus*, *Gossypium raimondii*, *Sorghum bicolor*, *Brachypodium distachyon*, *Oryza sativa japonica*, and *Arabidopsis thaliana*. b) Predicted Phylogenetic tree from multiple sequence alignment sequences

Sequence similarity analysis of EcHSP17.8 in different genotypes of finger millet

We have downloaded transcriptome data of finger millet genotypes namely IE6537, IE7079, GP1, and GP45 from NCBI and generated an assembly. Sequences of *EcHSP17.8* from these data and isolated sequence from MR1 genotype aligned together. The resultant ORFs showed nucleotide variations. When these sequences were aligned together, two nucleotides variation was observed in genotype GP1, whereas nine nucleotides variation and three nucleotide deletions was observed in genotype GP45 (Fig. 5a). When these ORFs from five genotypes were subjected to nucleotide translation, the resultant protein sequences were almost similar except one amino acid deletion in genotype GP-45. The amino acid glycine at 36th position is absent in HSP17.8 of genotype GP-45 (Fig. 5b).

Expression analysis of EcHSP17.8 under heat stress in different genotypes of finger millet

A total of 16 different finger millet genotypes were used to check expression of EcHSP17.8 under heat stress. Results of qRT-PCR expression analysis showed that *EcHSP17.8* is expressing in all the genotypes with a maximum expression at 2h heat stress in almost all the genotypes except INDAF8, RAU8, KOPN330 and GE1160 where it was maximum at 4 h. In almost all genotypes, expression reached its lowest level after 24 h. The upregulation of this gene in heat tolerant lines (1-8 genotypes) is found to be less than 40-fold, whereas it is more than 40-fold in heat sensitive lines (9-16 genotypes) (Fig. 6).

Discussion

Because of the sessile nature, plants cannot escape from unfavourable environmental conditions, therefore they developed more effective stress responsive

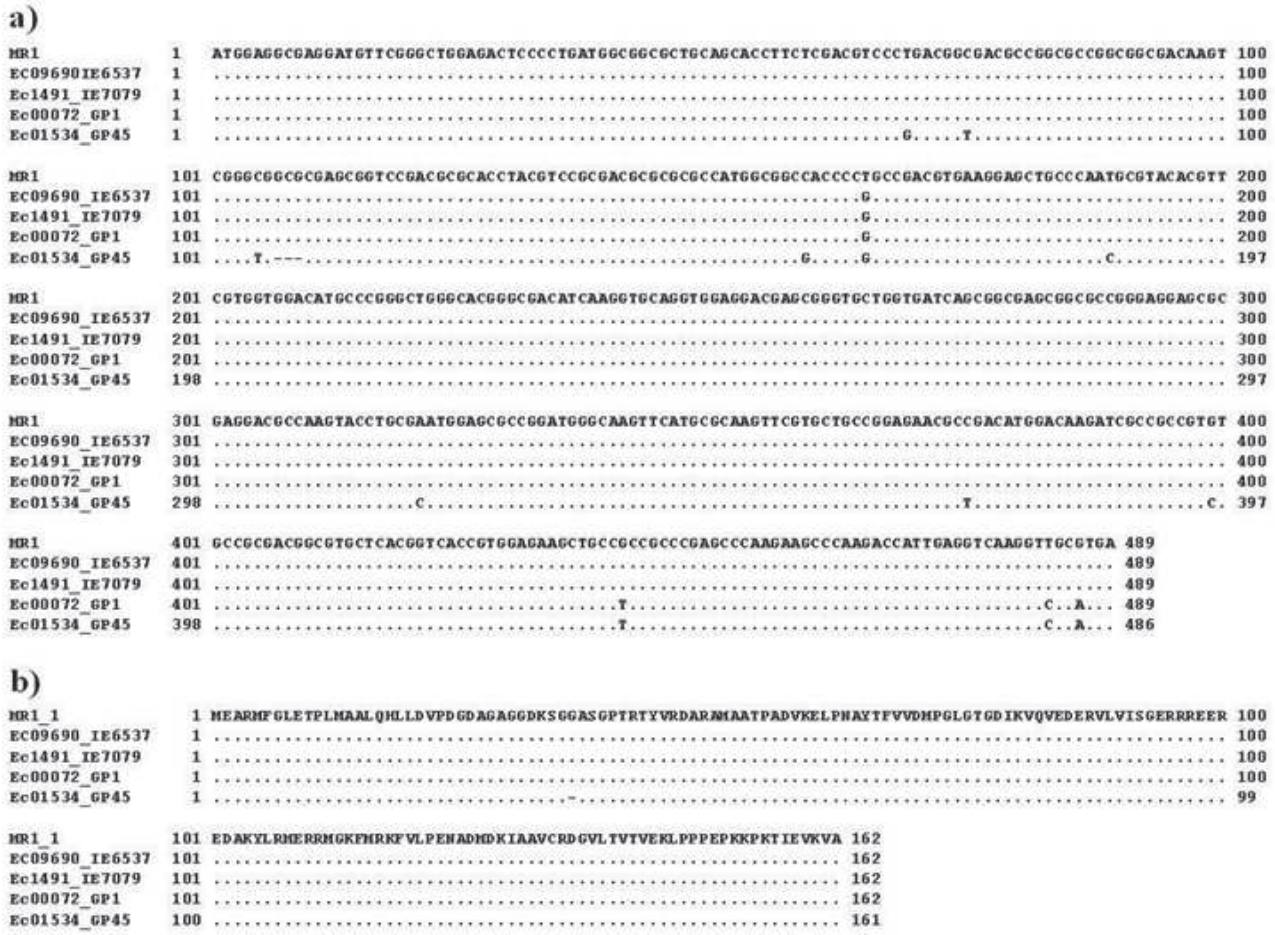

Fig. 5. Multiple sequence alignment of EcHSP17.8 nucleotide and protein sequences from different finger millet genotypes. Dots represent the similarity of sequence with MR1 genotype and difference in the nucleotide or amino acid is visible at respective position

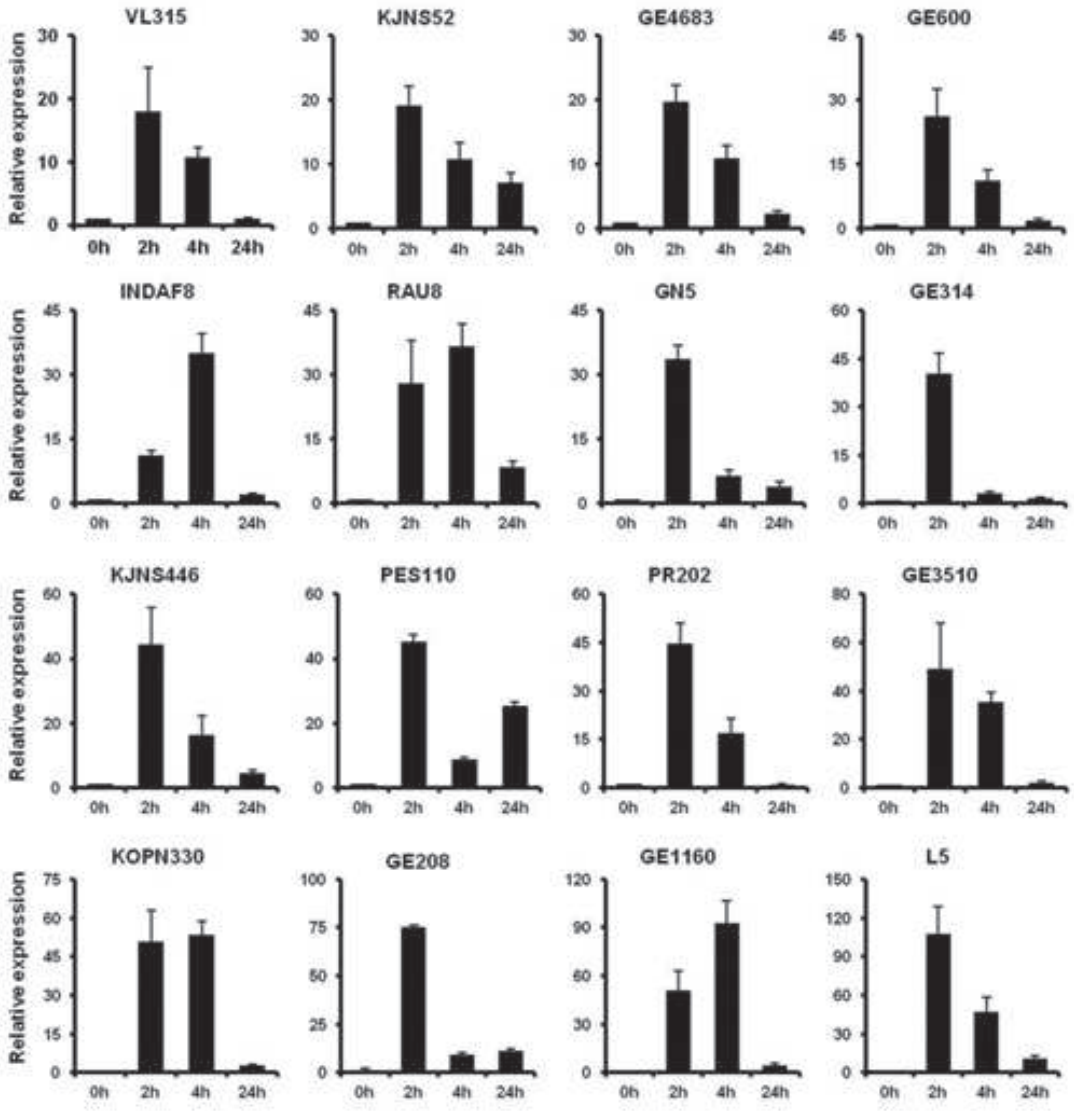

Fig. 6. Expression analysis of EcHSP17.8 in sixteen different genotypes of finger millet genotypes subjected to heat stress at indicated time points

systems than animals. In view of this and along with other stress tolerant proteins, the massive accumulation of HSPs in plants may indicate a special role for survival and recovery after stress conditions (Low et al. 2000). Many studies revealed that sHSP production under stresses is in correlation with stress tolerance. For example, small heat shock protein *OsHSP18.6* from rice, found expressed in different vegetative organs and also induced by diverse abiotic stresses (Wang et al. 2015). In the past, several studies demonstrated overexpression of sHSPs leading to stress tolerance in overexpressed plants. Overexpression of *AtHSP17*.8 in transgenic lettuce plants, showed dehydration and salt stress tolerance through modulation of ABA mediated signalling (Kim et al. 2013). Overexpression of cytosolic class I small heat shock protein ZmHSP16.9 from maize confers thermotolerance in transgenic tobacco plants (Sun et al. 2012). Transgenic *Arabidopsis* plants over-expressing *RcHSP17*.8 showed improved tolerance to heat, salt, osmotic and drought stresses (Jiang et al. 2009). Constitutive expression of *DcHSP17.7* from carrot into potato (*Solanum tuberosum* L.) also enhanced the membrane stability and *in vitro* tuberization (Ahn and Zimmerman 2006).

In the present study, we have isolated and characterized *EcHSP17.8* gene from finger millet which we obtained from subtractive cDNA library of heat

Table 1. Primary structural properties of EcHSP17.8 protein

S.No.	Primary structural properties	Theoretical prediction
1	Molecular weight (kDa)	17.5
2	Isoelectric point	5.96
3	Total no. of negatively charged residues (Asp + Glu)	27
4	Total no. of positively charged residues (Arg + Lys)	26
5	Extinction coefûcient (M-1 cm-1, at 280 nm)	4470
6	Aliphatic index	77.65
7	Grand average of hydropathicity (GRAVY)	-0.412

stressed finger millet seedlings. *In silico* analysis of *EcHsp17.8* showed stable protein formation with a usual G factor value of Ramachandran plot which signifies the protein functional stability. We checked expression pattern of *EcHSP17.8* in different tissues of finger millet and also showed that it was induced under different abiotic stresses. Its expression was upregulated up to 40 folds under heat stress condition. Its overall expression under other abiotic stresses was maximum up to 13 folds. Apart from heat stress condition, the basal expression of *EcHsp17.8* in different stresses signifies its role not only under heat stress but also in different abiotic stress conditions. Since it was highly expressed under heat stress, we checked its expression in 16 finger millet genotypes under heat stress condition. In all 16 genotypes, its expression was found to be maximum after 2 or 4h of stress. In heat tolerant genotypes, expression of this gene is not upregulated more than 40-fold however it is upregulated more than 40-fold in sensitive genotypes. This can help to develop expression-based marker using *Hsp17.8* to use for breeding purpose. Since in this study, we have not identified its promoter region, this will be future plan of work. The present findings suggest that this is an early stress responsive gene showing basic role as chaperon to protect other proteins from misfolding. Conclusively, *EcHSP17.8* is a promising candidate from finger millet for heat stress tolerance with a potential key role not only in heat stress but also in different abiotic stresses. As knowledge of genetic and molecular basis of thermotolerance in cereals is highly required to identify the beneficial alleles and genes (Yildiz & Terzi, 2008), this gene and it's alleles form tolerant genotypes are very valuable resource for molecular breeding programs to develop superior cultivars.

Authors' contribution

Conceptualization of research (AVS); Designing of the experiments (AVS, JCP); Contribution of experimental materials (NR); Execution of field/lab experiments and data collection (RC, SS); Analysis of data and interpretation (RC, SS, RST, AVS); Preparation of manuscript (RC, AVS, SM, SK, JCP).

Declaration

The authors declare no conflict of interest.

Acknowledgements

We acknowledge All India Coordinated Research Project on Small Millets, ICAR, GKVK, Bangalore-560065 for providing seed material and 'National Innovations in Climate Resilient Agriculture' (NICRA), a flagship project of Indian Council of Agricultural Research (ICAR), New Delhi, India for providing financial support.

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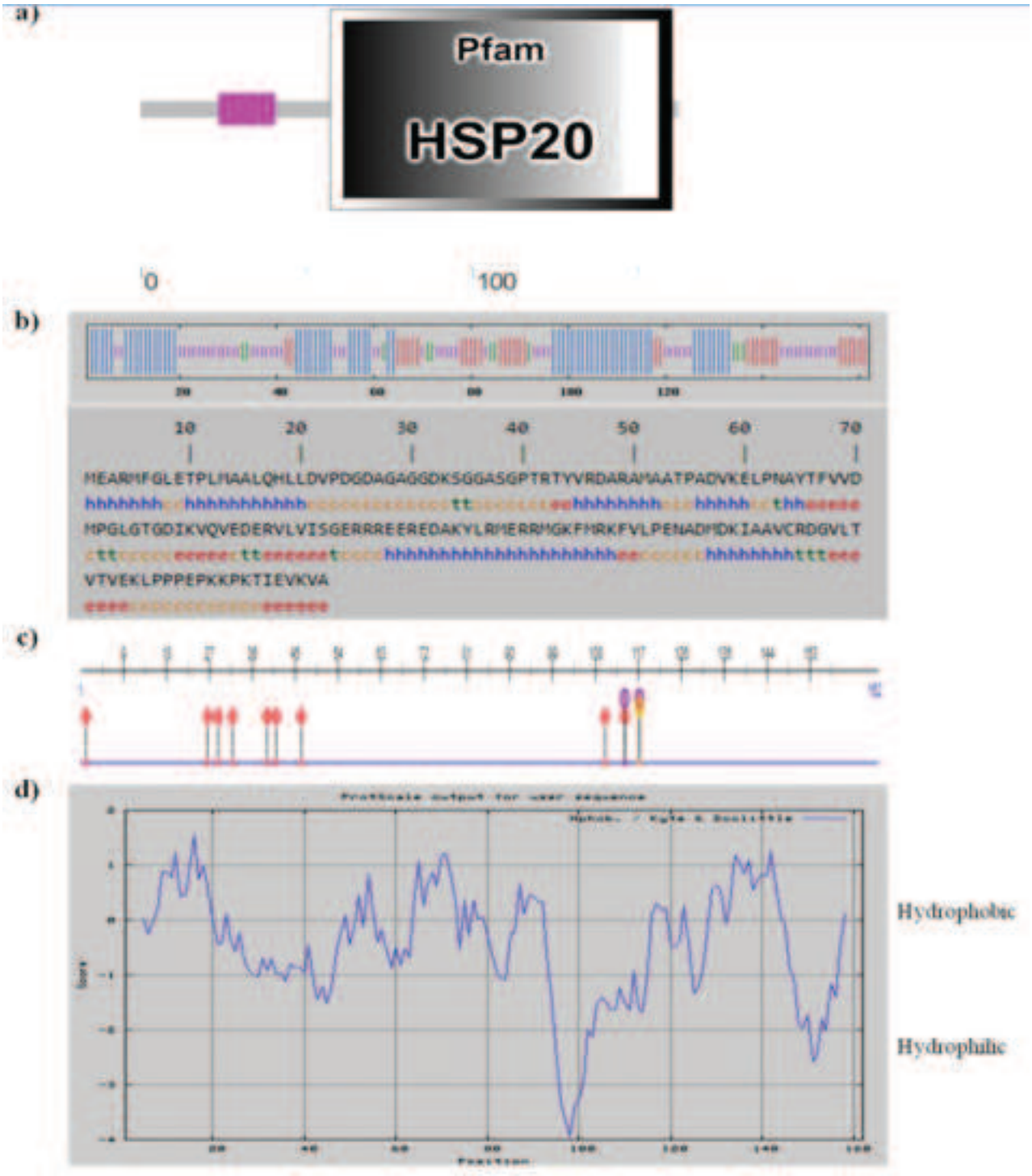
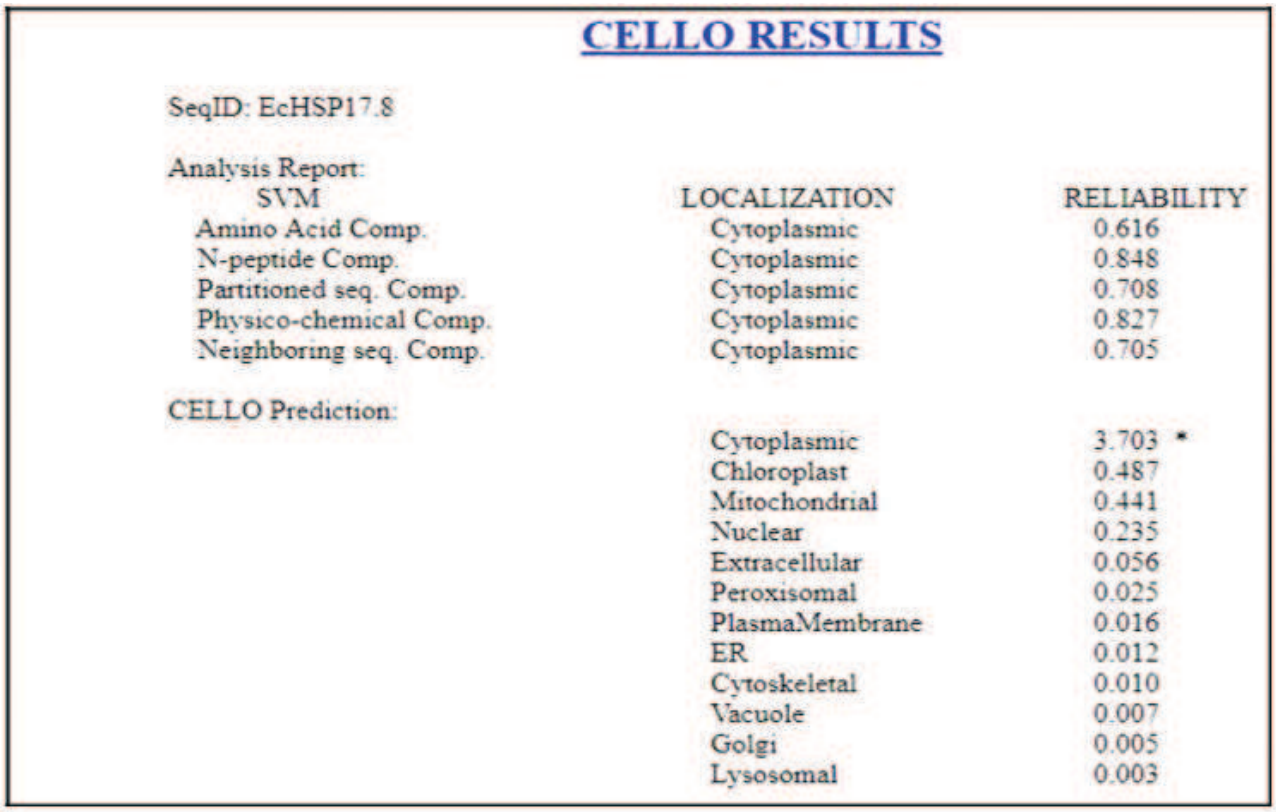


Supplementary Fig. S1: Bioinformatics analysis of EcHSP17.8 a) Functional domain prediction of EcHSP17.8 protein using SMART online tool. b) Predicted secondary structure of EcHSP20.c) Secondary structural characteristics of EcHSP17.8 protein by different case letters in different colours. d) Predicted protein protein interaction regions in EcHSP17.8. e) Letter (c) represents the random coils, letter (e) represents the extended strands, letter (h) represents the helices, and letter (t) represents the turns. e) Hydrophobic index prediction of EcHSP17.8 through ProtScale package of the ExPASy server



CELLO RESULTS

SeqID: EcHSP17.8

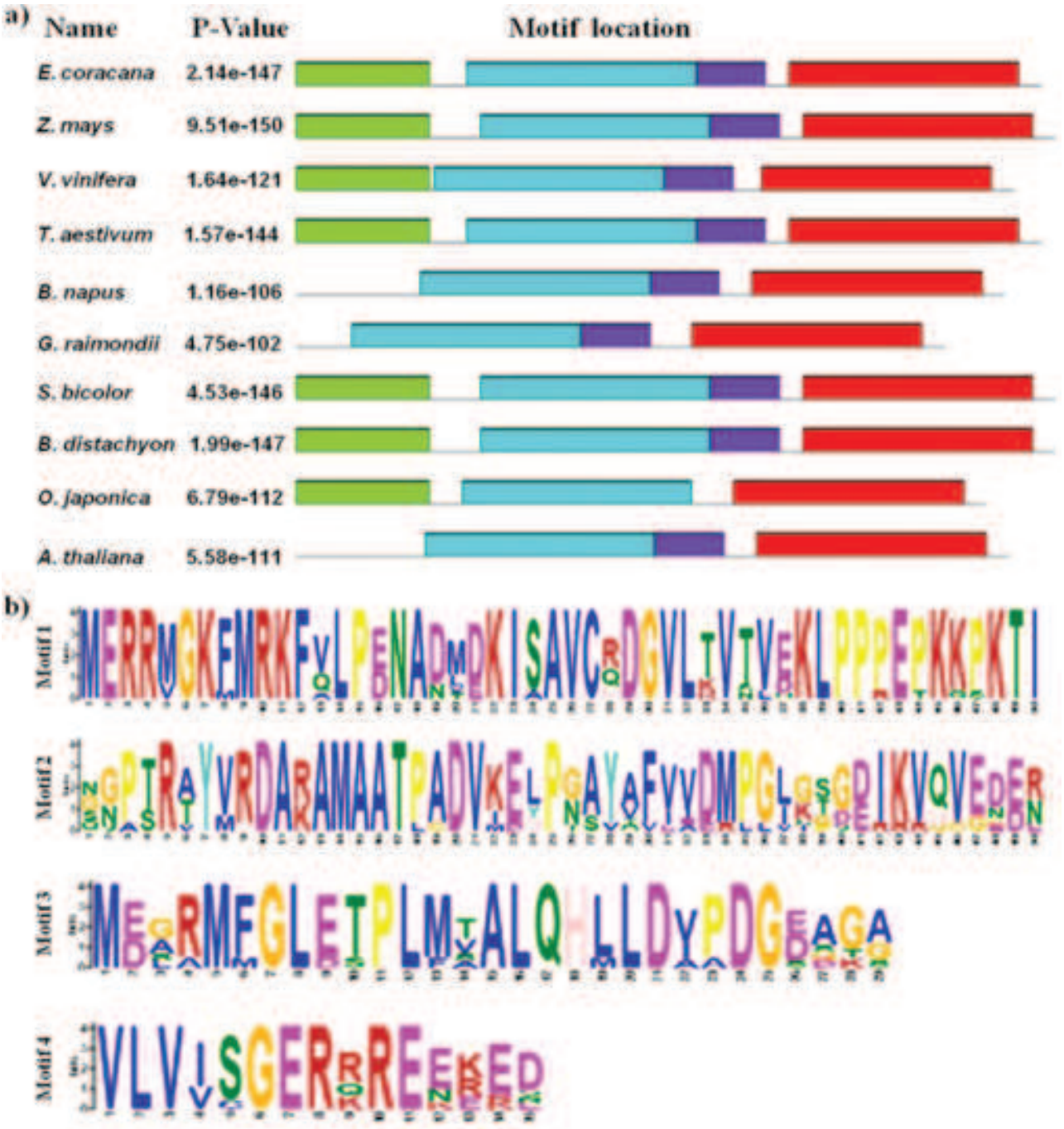
Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Cytoplasmic	0.616
N-peptide Comp.	Cytoplasmic	0.848
Partitioned seq. Comp.	Cytoplasmic	0.708
Physico-chemical Comp.	Cytoplasmic	0.827
Neighboring seq. Comp.	Cytoplasmic	0.705

CELLO Prediction:

Cytoplasmic	3.703 *
Chloroplast	0.487
Mitochondrial	0.441
Nuclear	0.235
Extracellular	0.056
Peroxisomal	0.025
PlasmaMembrane	0.016
ER	0.012
Cytoskeletal	0.010
Vacuole	0.007
Golgi	0.005
Lysosomal	0.003

Supplementary Fig. S2: Predicted sub cellular localization for EcHSP17.8 protein



Supplementary Fig. S3: Prediction of Motifs in EcHSP17.8 and other proteins. a) Predicted Motif locations and respective sequences of EcHSP17.8 from *Elucine coracana* and indicated other plant species through MEME online tool

Supplementary Table S1: Primary structural properties of EcHSP17.8 protein

S.No	Primary structural properties	Theoretical prediction
1	Molecular weight (kDa)	17.5
2	Isoelectric point	5.96
3	Total no. of negatively charged residues (Asp + Glu)	27.00
4	Total no. of positively charged residues (Arg + Lys)	26.00
5	Extinction coefficient (M-1 cm-1, at 280 nm)	4470.00
6	Aliphatic index	77.65
7	Grand average of hydropathicity (GRAVY)	-0.412