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ORIGINAL ARTICLE

Identification and characterization of a small heat shock protein 17.9-CII gene from faba bean (*Vicia faba* L.)

Ritesh Kumar[1] · Dhruv Lavania[1] · Amit Kumar Singh[1] · Manisha Negi[1] · Manzer H. Siddiqui[2] · Mohamed H. Al-Whaibi[2] · Anil Grover[1]

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Abstract We cloned and characterized the full-length coding sequence of a small heat shock protein 17.9 gene from faba bean encoding 160 amino acids and containing the conserved α-crystallin domain at the C-terminus. Homology and phylogenetic analysis suggested its proximity with the class II sHsp members of fabaceae family. Therefore, we name this gene as VfHsp17.9-CII. The VfHsp17.9-CII transcript showed a clear heat stress induction pattern in leaves of young seedlings and flowering plants. Transient expression of VfHsp17.9-CII fused with green fluorescent protein reporter indicated its nuclear localization. Overexpression of recombinant VfHsp17.9-CII protein in *Escherichia coli* cells increased tolerance of the bacterial cells to heat and arsenic stresses. The reduction of faba bean pollen viability in response to heat stress correlated with the accumulation pattern of VfHsp17.9-CII transcript in heat stressed pollen. It is suggested that VfHsp17.9-CII protein plays a key role in heat and heavy metal stress tolerance.

Keywords Faba bean · Heat stress · Nuclear localization · Pollen · Small heat shock protein · Transcript induction

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R. Kumar and D. Lavania contributed equally to this work.

✉ Anil Grover
anil.anilgrover@gmail.com

[1] Department of Plant Molecular Biology, University of Delhi, South Campus, New Delhi 110021, India

[2] Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia

Introduction

Faba bean (*Vicia faba* L.) is an important leguminous food crop (Al-Suhaibani 2009; Rubiales 2010). These plants experience heat stress (HS) indirectly during drought stress conditions and directly when the sowing dates of the crop are delayed (Stoddard et al. 2006). The rise of ambient temperatures due to the phenomenon of global warming is a major cause of concern for HS to all crops (Grover et al. 2013; Lavania et al. 2015). The onset of HS affects the physiology of faba bean plants in varied ways including major reduction of net photosynthetic assimilation rate and changes in membrane characteristics (Avola et al. 2008; Hamada et al. 2001). High sensitivity of pollen to HS has been noted in diverse legume and non-legume crops (Frank et al. 2009; Jagadish et al. 2010). Kitano et al. (2006) showed that pollen viability of soybean is HS sensitive. Notably, the reproductive stage of faba bean is also sensitive to HS (Patrick and Stoddard 2010).

In several recent studies, the molecular basis of the plant HS response has been exhaustively analyzed using model plant species like Arabidopsis, rice, and tomato. Sarkar et al. (2014) showed that the maintenance of homeostasis of cellular proteins is a critical component of the heat stress response (HSR) in rice seedlings and that heat shock proteins (Hsps) play a decisive role in protection of rice seedlings against HS. The elevated synthesis of Hsps under HS conditions has been noted to be a ubiquitous response across the spectrum of living organisms. It has further emerged that the accumulation of Hsps and the acquisition of heat tolerance are correlated processes (Lavania et al. 2015; Sarkar et al. 2014). Hsps function mostly as molecular chaperones, protecting cellular proteins from degradation and misfolding. Hsps comprise Hsp20, Hsp40, Hsp60, Hsp70, Hsp90, and Hsp100 classes (Sarkar et al.

2013a, b; Singh and Grover 2010). Hsp members representing these classes are located in endoplasmic reticulum, peroxisome, nucleus, chloroplast, and mitochondria. Apart from stress, Hsps have been implicated in plant development as these proteins are expressed at specific growth stages under non-stress, control conditions. It is indicated that Hsps play significant role in pollen development and in response of pollen to HS (Burke and Chen 2015; Frank et al. 2009; Ischebeck et al. 2014; Wei et al. 2010; Zinn et al. 2010).

Among the various plant Hsps, Hsp20, or sHsp class members (in the range of 16–40 kDa) are expressed in maximal amounts under HS conditions (Chen et al. 2014; Sarkar et al. 2009; Wang et al. 2014). These proteins form large oligomeric structures of 200–800 kDa range. The characteristic feature of sHsps is the presence of a conserved 80–100 amino acid sequence called α-crystallin domain (ACD). This domain is located toward the C-terminal end of these proteins and is important for oligomer formation and chaperoning activities. On the other hand, N-terminal end of these proteins has a variable amino acid sequence. sHsps are ATP-independent molecular chaperones which make complexes with denatured proteins to prevent their aggregation. The target proteins are retrieved from these complexes by Hsp100/Hsp70 and co-chaperones. Apart from stress-induced expression, sHsps are also developmentally regulated. Studies in maize indicate that during microsporogenesis, cytoplasmic class II sHsps are expressed before meiosis and during the meiotic prophase, while, cytoplasmic class I sHsps are expressed during pollen maturation (Atkinson et al. 1993; Dietrich et al. 1991; Hopf et al. 1992). As compared to model plant species, little work has been carried out on cloning and expression analysis of Hsps in legumes barring limited analysis of Hsps in common bean, lima bean, pea, and soybean (Ahsan et al. 2010; Derocher et al. 1991; Keeler et al. 2000; Lee et al. 1995; Lopes-Caitar et al. 2013; Simões-Araújo et al. 2003). This study aimed at the analysis of sHsps of faba bean. We cloned and sequenced the full-length sHsp17.9-CII coding sequence of faba bean (VfHsp17.9-CII). We further show the accumulation profile of VfHsp17.9-CII transcript in seedlings, leaves of flowering stage plants, and in pollen under HS conditions in faba bean. The heterologous overexpression of the recombinant VfHsp17.9-CII protein in *Escherichia coli* cells resulted in enhanced tolerance of bacterial cells to heat and heavy metal (arsenic) stresses.

Materials and methods

Plant materials and stress treatments

Faba bean (*V. faba* L.) seeds were sown in sterilized Soil-Rite Mix (KEL, India) in plastic pots with regular watering. Plants were grown under controlled conditions inside a plant growth chamber (Conviron, Canada) maintained at 23 °C, 16 h light/8 h dark cycle. HS treatments were given to faba bean plants at two stages of growth, namely, at 10-day-old seedling stage and at 10-week-old flowering stage. Details of stress regimes are shown in figures (see Results). All HS treatments were given in sets of three independent plants representing biological replicates inside the plant growth chamber. After each treatment, top-most leaf from each plant was harvested, frozen in liquid nitrogen, and stored at −80 °C until isolation of RNA.

Cloning and sequencing of VfHsp17.9-CII

Ten-day-old faba bean seedlings were subjected to 38 °C HS for 2 h. Total RNA was isolated from the top-most leaves with TRI Reagent (Sigma-Aldrich, USA) according to manufacturer's instructions. DNase treatment was given to the RNA after which it was column purified using RNeasy MinElute Cleanup Kit (Qiagen, USA). RNA purity, yield, and integrity were analyzed spectrophotometrically as well as by gel analysis as per the standard protocols. First-strand cDNA was synthesized using 2 µg total RNA and an oligo-(dT) 18 primer with RevertAid H Minus reverse transcriptase (Thermo Scientific, USA) according to manufacturer's instructions. The HS cDNA was used for PCR amplification using Phusion High-Fidelity DNA polymerase (New England Biolabs, USA) and primers complementary to the full-length coding sequence of *Glycine max* sHsp gene Glyma14g11430, downloaded from Phytozome database (see primer list in Table 1; http://www.phytozome.net). PCR conditions were as follows: denaturation at 98 °C for 20 s, annealing at 56 °C for 30 s, and extension at 72 °C for 20 s. After 35 cycles, reaction mixture was cooled to 4 °C. Cloning was carried out in pJET1.2/blunt cloning vector supplied with CloneJET PCR Cloning Kit (Thermo Scientific, USA). DNA sequencing was carried out using primers supplied in the kit.

Analysis of VfHsp17.-9-CII expression in vegetative and reproductive tissues

For quantitative real-time PCR (Q-PCR) analysis, VfHsp17.9-CII coding sequence-specific primers were designed using NCBI/Primer blast with default parameters and amplicon length of 80–120 bp (see primer list in Table 1). To normalize the variance among samples, eukaryotic elongation factor 1-alpha-specific primers (Table 1) were used as endogenous control, as described elsewhere (Gutierrez et al. 2011). High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) was used to synthesize cDNA from 2 µg of DNase-treated

Table 1 List of primers used in this study

Application of primers	Primer sequence (5′–3′)	Restriction site	Product length (bp)
Cloning in pJET1.2/blunt and sqRT-PCR			
VfHsp17.9-CII	F: ATGGATTTCAGAGTGATGGG	NA	483
	R: TCAGAAAACCTTAACCTCAATG	NA	
Actin	F: GAATTGCCTGATGGACAGGT	NA	481
	R: GCTTTGGGGATCCACATCTA	NA	
qRT-PCR			
VfHsp17.9-CII	F: CACGCTCTGCACCAAATGAC	NA	90
	R: GCCTTCGCATCTCTGACGTA	NA	
Eukaryotic elongation factor 1-alpha (ELF1A)	F: GTGAAGCCCGGTATGCTTGT	NA	150
	R: CTTGAGATCCTTGACTGCAACATT	NA	
Subcellular localization	F: CATGCCATGGATTTCAGAGTGATG	NcoI	493
	R: GACTAGTGAAAACCTTAACCTCAATGGT	SpeI	
Protein expression	F: GGAATTCATGGATTTCAGAGTG	EcoRI	498
	R: GCGTCGACTCAGAAAACCTTAACCTC	SalI	

Restriction enzyme sites in the primer sequence are underlined

F forward primer, *R* reverse primer, *NA* not applicable

RNA. Two hundred nanomolar of each primer mixed with Power SYBR Green PCR Master Mix (Applied Biosystems, USA) was used in Q-PCR, as per manufacturer's instructions. The reaction was carried out in 96-well optical reaction plates (Applied Biosystems, USA) in Mx3005P Q-PCR System (Agilent Technologies, USA). In total, three biological replicates were analyzed for each treatment. The average of three technical replicates for each sample was calculated to obtain the Ct value, and standard deviation and standard error were calculated. Relative expression values (fold changes) were calculated using the ΔΔCT method.

Faba bean actin transcript amplified using primers complimentary to *G. max* β-actin gene (GenBank: U60500.1; Table 1) was employed as internal control in semi-quantitative RT-PCR (sqRT-PCR). Primers complementary to the full-length coding sequence of VfHsp17.9-CII gene (Table 1) were used for expression analysis by sqRT-PCR. All sqRT-PCRs were repeated thrice.

Sequence analyses

ProtParam tool on ExPASy server (http://web.expasy.org/protparam/) was used for determination of physico-chemical properties of the amino acid sequence. Homology search was performed with BLASTp tool of NCBI database with the non-redundant protein sequences of fabaceae family (taxid: 3803). All non-redundant protein sequences which exhibited 64–99 % query coverage and 48–80 % identity were cataloged. Protein fold recognition server Phyre2 (Protein Homology/analogY Recognition Engine version 2.0; Kelly and Sternberg 2009) was used to generate the three-dimensional (3D) protein structure. Geneious R8 software suite (http://www.geneious.com) was used for multiple amino acid sequence alignment and phylogenetic analysis of the cataloged sequences. Global alignment with free ends and cost matrix Blosum 32 was used for multiple sequence alignment with a gap open penalty as 12 and a gap extension penalty as 3. A consensus phylogenetic tree was constructed using neighbor-joining (tree build method) and Jukes-Cantor (Genetics Distance model) with 1000 bootstrap replicates and consensus threshold percentage of 50.

Subcellular localization

cNLS Mapper software (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) was used for prediction of nuclear localization signals. WoLF PSORT (http://www.genscript.com/psort/wolf_psort.html) and PlantLoc (http://cal.tongji.edu.cn/PlantLoc/index.jsp) tools were used for prediction of subcellular localization. For transient expression in onion epidermal cells, the full-length coding sequence of VfHsp17.9-CII cloned in pJET1.2/blunt was PCR amplified without stop codon using Phusion High-Fidelity DNA polymerase (New England Biolabs, USA). The forward primer had NcoI site and reverse primer had SpeI site (Table 1). The PCR product was purified, digested with NcoI and SpeI, and ligated into binary vector pCAMBIA1302. The resultant recombinant plasmid contained VfHsp17.9-CII driven by CaMV35S promoter and in frame with a C-terminal fusion of green fluorescent protein (GFP). This fusion construct was introduced into onion epidermal peel cells by particle bombardment using

Biolistic PDS-1000/He particle delivery system (Bio-Rad, USA) as described previously (Lee et al. 2008) with some modifications. Empty pCAMBIA1302 vector expressing free GFP under CaMV35S promoter was used as control. Five micrograms of each plasmid were used to coat 0.6 mg of 1 micron gold particles per shot. The biolistic parameters used were 27 mmHg vacuum, accelerating pressure of 1100 psi, and 6 cm distance between the projectile source and the target onion epidermal peel. After incubation for 16 h at 28 °C in the dark, GFP fluorescence was detected under a confocal laser scanning microscope (Leica TCS SP5) with an argon laser at excitation wavelength of 488 nm. Nuclei were identified by DAPI (4′,6′-diamidino-2-phenylindole) staining.

Recombinant expression and purification of VfHsp17.9-CII protein

The full-length coding sequence of VfHsp17.9-CII cloned in pJET1.2/blunt was PCR amplified with EcoRI site in the forward primer and SalI site in the reverse primer (Table 1) using Phusion High-Fidelity DNA polymerase (New England Biolabs, USA). The PCR product was purified, digested with EcoRI and SalI, and ligated into pET-28a(+) expression vector (Novagen, USA). The resulting recombinant plasmid, pET-28a(+)-VfHsp17.9-CII, was transformed into *E. coli* strain BL21-CodonPlus-RIL. A single colony was inoculated in 5 ml Luria–Bertani (LB) broth containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol, overnight at 37 °C. Fresh LB medium containing antibiotics was inoculated with 100 μl of the overnight starter culture until OD_{600} reached 0.6. Isopropyl-β-D-thiogalactoside (IPTG) was then added to the medium at a final concentration of 0.5 mM to induce recombinant protein expression at 30 °C. After 4 h of induction at 30 °C, the cells were harvested by centrifugation at 4 °C. The pellet was resuspended in sodium dodecyl sulfate (SDS) containing sample loading buffer and boiled for 5 min. After boiling, samples were centrifuged and supernatants were separated on 12.5 % (w/v) SDS-gel and analyzed by Commassie brilliant blue staining. Purification of recombinant VfHsp17.9-CII protein from cell-free extracts was carried out using Ni–NTA agarose resin (Qiagen, USA) as per the manufacturer's instructions. Protein purity and molecular weight were assessed by 12.5 % (w/v) SDS-gel and Coomassie brilliant blue staining. Two hundred nanograms of purified protein were electroblotted onto 45 μm Hybond-C super nitrocellulose membrane (Amersham-Pharmacia, USA) as described by Towbin et al. (1979). Primary monoclonal antisera against 6 X Histidine tag (Sigma-Aldrich, USA) and secondary antisera of anti-mice horseradish peroxidase conjugate (Sigma-Aldrich, USA) were used as 1:10,000 dilution. Blots were developed using enhanced chemiluminescence (ECL) peroxidase system.

Bacterial stress tolerance and pollen viability experiments

E. coli BL21-CodonPlus-RIL cells harboring pET-28a(+)-VfHsp17.9-CII or pET-28a(+) were cultured overnight at 37 °C in LB medium containing antibiotics. Hundred microliters of these seed cultures were used to inoculate secondary cultures until mid-log phase (OD_{600} equal to 0.5), followed by the addition of IPTG to 0.5 mM concentration, and allowed to grow for 4 h at 30 °C. The cultures were diluted to OD_{600} equal to 0.1, with fresh LB medium containing antibiotics. Sodium arsenate and NaCl were used to administer heavy metal stress and salt stress, respectively. The cultures were serially diluted three times, and 5 μl from each dilution was spotted onto LB kanamycin–chloramphenicol basal plates supplemented with 0.5 mM IPTG or containing 0.5 mM sodium arsenate or 300 mM NaCl. HS was administered by incubating the diluted cultures ($OD_{600} = 0.1$) at 50 °C in a water bath, while unstressed control samples were incubated at 30 °C. After 0 h, 0.5 h, 1 h, and 2 h of HS, 100 μl of samples was removed and serially diluted five times, and 5 μl of each serial dilution was spotted onto LB kanamycin–chloramphenicol plates. After spotting, the plates were incubated for 16 h at 37 °C and photographed.

Pollen viability was measured by staining with fluorescein di-acetate (Sigma-Aldrich, USA), and fluorescing pollen grains were scored under Leica TCS SP5 confocal laser scanning microscope.

Results

Cloning and analysis of VfHsp17.9-CII coding sequence

The full-length coding sequence of VfHsp17.9-CII was PCR amplified from 2 h HS cDNA of faba bean using *G. max* sHsp-specific primers. The amplified product was cloned in pJET1.2/blunt cloning vector and sequenced using vector-specific sequencing primers. The full-length nucleotide sequence was submitted to GenBank under accession number KC249973.2. The nucleotide sequence and its translated sequence are shown in Fig. 1a. The translated sequence was composed of 160 amino acids (molecular weight 17864.3 Da; pI 5.95). It lacked Trp residue. The total numbers of negatively (Asp + Glu) and positively (Arg + Lys) charged residues were 26 and 24, respectively. Homology search within non-redundant protein sequences of fabaceae revealed highest identity of 80 % and query coverage of 99 % with cytoplasmic CII sHsp17.9 of *G. max*, XP_003550253.1. An ACD of 106 amino acids was noted at the C-terminus from position

53-158 as seen in NCBI conserved domain database. Predicted protein structure with Phyre 2 suggested the presence of Hsp20-like chaperone fold. In this analysis, 143 residues (89 % of the sequence) were modeled with 99.9 % confidence by the single highest scoring template. Based on Phyre 2 data, 3D structure comprising of 5 α-helices and 7 β-sheets was predicted (Fig. 1b). Multiple alignment with fabaceae protein sequences indicated maximum percentage identity with *G. max* Hsp17.9-CII (Fig. 2a). Phylogenetic analysis showed that the cloned faba bean sequence was grouped in sHsp CII-specific clade (Fig. 2b).

VfHsp17.9-CII expression in response to heat stress

Healthy, intact, and uniform-sized faba bean seedlings (10-day old) grown at 23 °C were subjected to increasing durations of HS treatments. Primary leaf from treated seedlings was harvested immediately after the 38 °C HS treatments for 1 h, 2 h, 3 h, and 4 h for RNA isolation, and expression analysis was carried out by Q-PCR. Expression of VfHsp17.9-CII transcript was not induced under unstressed control condition (Fig. 3). Upon exposure to HS, the expression was upregulated to ~350-fold relative to control after 1 h and reached its maxima of ~620-fold change after 2 h. Thereafter, the expression decreased to ~240-fold under 3 h HS and ~96-fold under 4 h HS (Fig. 3).

Subcellular localization of VfHsp17.9-CII

A monopartite NLS containing a single cluster of basic amino acids, PPQPKKPRTIEVKVF, was predicted in the cloned sequence near the C-terminus at position 146-160 by cNLS Mapper (Table 2). According to cNLS Mapper, CII and CIII sHsps of fabaceae family are predicted to contain a monopartite NLS at positions 139–146 and

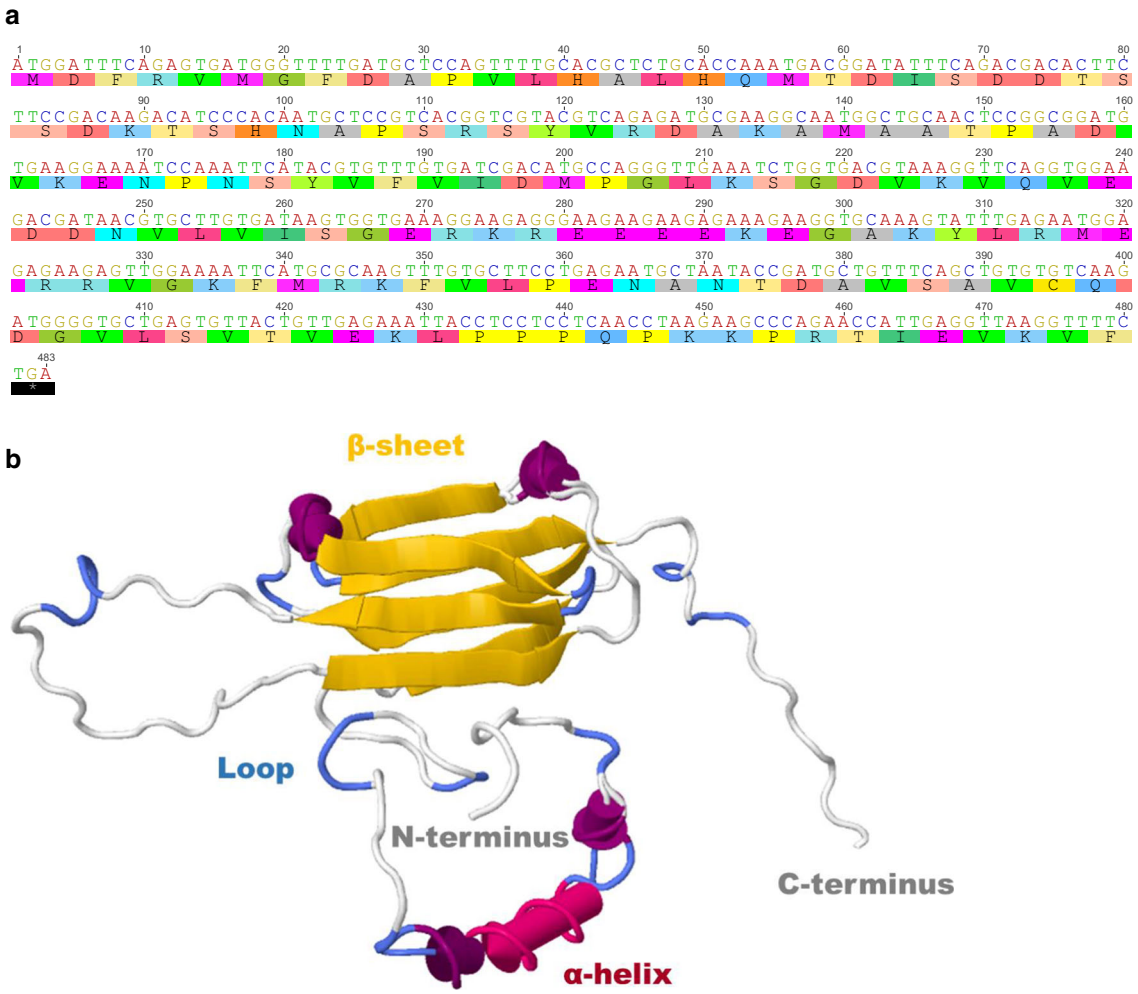
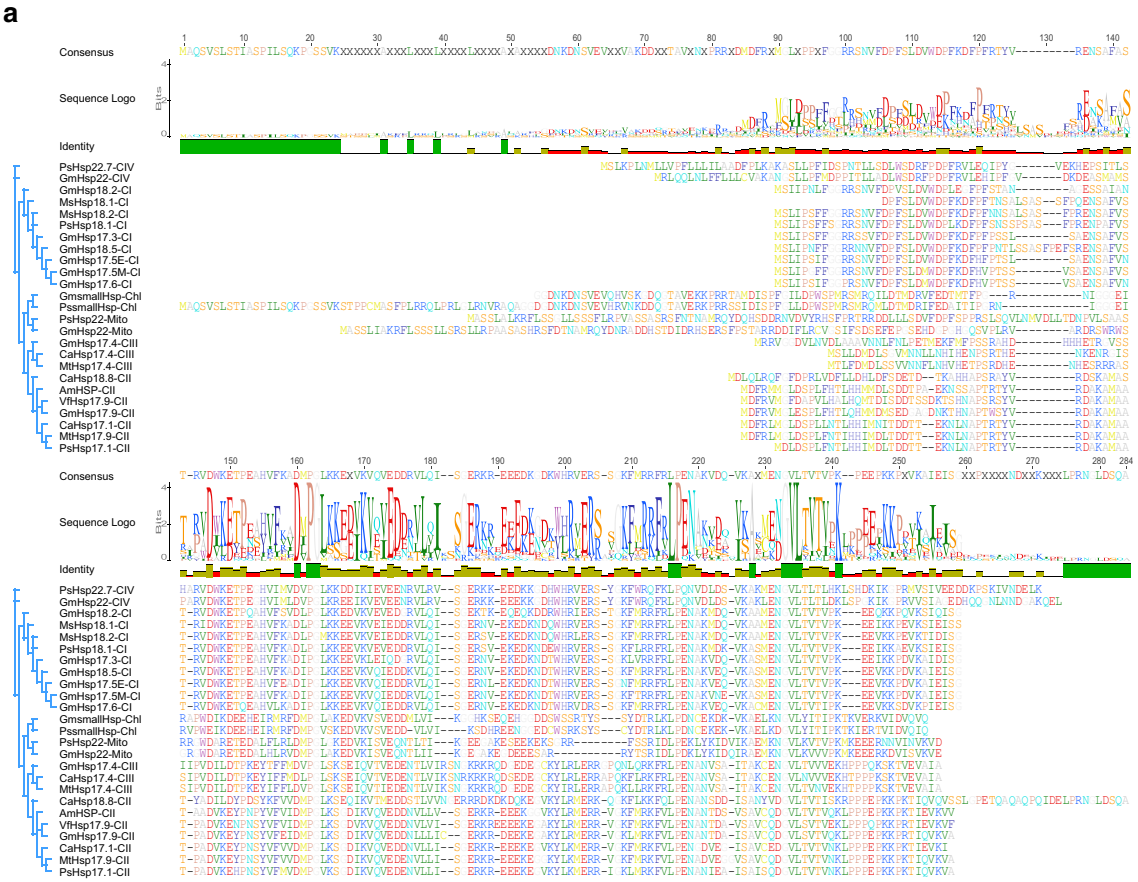
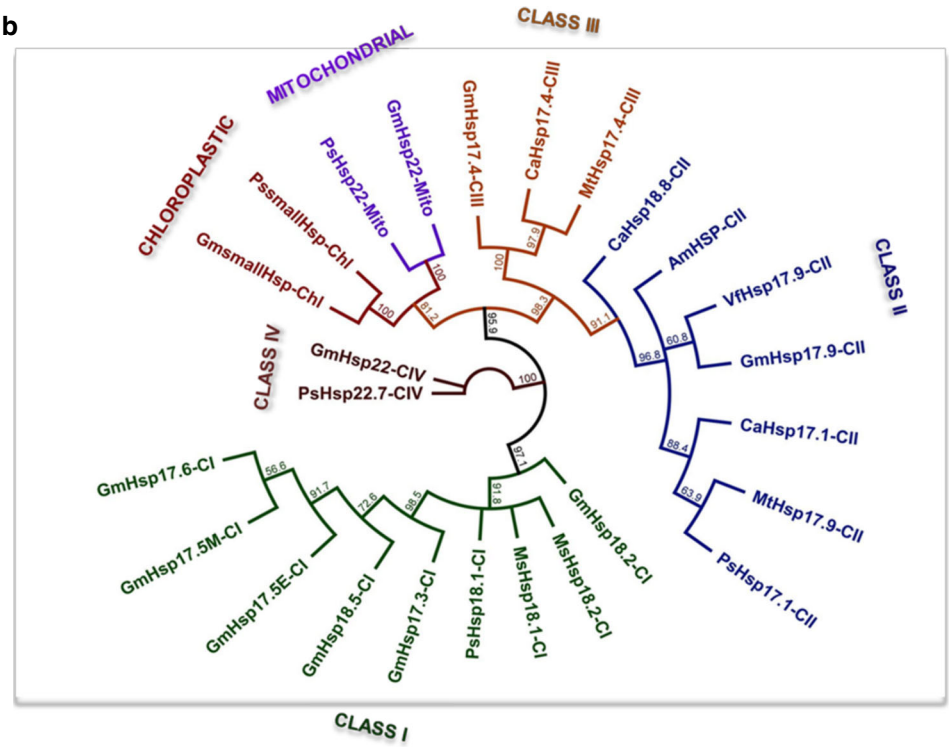


Fig. 1 Sequence and structural modeling of VfHsp17.9-CII. **a** Nucleotide and deduced amino acid sequence corresponding to the VfHsp17.9-CII coding sequence. Translation stop codon is indicated by *asterisk*. *Numbers on top* denote intervals of 10 nucleotides. **b** Three-dimensional structure prediction of VfHsp17.9-CII protein using Phyre 2 server. α-helices are indicated by *red color* rockets. β-Sheets are indicated by *yellow color planks* and turns are indicated as *colored threads*


a
Consensus
Sequence Logo
Identity
PsHsp22.7-ClV
GmHsp22-ClV
GmHsp18.2-CI
MsHsp18.1-CI
MsHsp18.2-CI
PsHsp18.1-CI
GmHsp17.3-CI
GmHsp18.5-CI
GmHsp17.5E-CI
GmHsp17.5M-CI
GmHsp17.6-CI
GmsmallHsp-ChI
PssmallHsp-ChI
PsHsp22-Mito
GmHsp22-Mito
GmHsp17.4-CIII
CaHsp17.4-CIII
MtHsp17.4-CIII
CaHsp18.8-CII
AmHSP-CII
VfHsp17.9-CII
GmHsp17.9-CII
CaHsp17.1-CII
MtHsp17.9-CII
PsHsp17.1-CII


b
CLASS III
MITOCHONDRIAL
CHLOROPLASTIC
GmHsp22-Mito
PsHsp22-Mito
PssmallHsp-ChI
GmsmallHsp-ChI
GmHsp17.4-CIII
CaHsp17.4-CIII
MtHsp17.4-CIII
CaHsp18.8-CII
AmHSP-CII
VfHsp17.9-CII
GmHsp17.9-CII
CaHsp17.1-CII
MtHsp17.9-CII
PsHsp17.1-CII
CLASS II
CLASS IV
GmHsp22-ClV
PsHsp22.7-ClV
GmHsp17.6-CI
GmHsp17.5M-CI
GmHsp17.5E-CI
GmHsp18.5-CI
GmHsp17.3-CI
PsHsp18.1-CI
MsHsp18.1-CI
MsHsp18.2-CI
GmHsp18.2-CI
CLASS I

◀**Fig. 2** Sequence comparison and phylogenetic analysis of VfHsp17.9-CII. **a** Multiple alignment of the deduced VfHsp17.9-CII amino acid sequence with diverse sHsps classes of fabaceae family members. Maximum coverage and percentage identity of amino acids are indicated. A consensus tree is shown at the *left* corner of sequence name. **b** Phylogenetic relationship of VfHsp17.9-CII with sHsps from different members of fabaceae family. The phylogenetic tree was constructed using neighbor-joining method with 1000 bootstrap replicates in Geneious R8 software suit. Consensus support threshold value of more than 50 % is indicated at each node. All the sequences used are obtained from GenBank on NCBI. Am, *Ammopiptanthus mongolicus*; Ca, *Cicer arietinum*; Gm, *Glycine max*; Mt, *Medicago truncatula*; Ps, *Pisum sativum*; Vf, *Vica faba*; CI-Class I, CII-Class II, CIII-Class III, CIV-Class IV, Chl-chloroplastic, Mito-mitochondrial. Accession number details are AmHSP-CII (AGS48404.1), CaHsp17.1-CII (XP_004501443.1), CaHsp17.4-CIII (XP_004510552.1), CaHsp18.8-CII (XP_004501442.1), GmHsp17.3-CI (NP_001235293.1), GmHsp17.4-CIII (XP_003528707.1), GmHsp17.5E-CI (P04794.1), GmHsp17.5 M-CI (XP_003529343.1), GmHsp17.6-CI (P04795.1), GmHsp17.9-CII (XP_003550253.1), GmHsp18.2-CI (XP_003519372.1), GmHsp18.5-CI (NP_001235177.1), GmHsp22-CIV (P30236.1), GmHsp22-Mito (NP_001235130.1), GmsmallHsp-Chl (P09887.1), MsHsp18.1-CI (P27879.1), MsHsp18.2-CI (P27880.1), MtHsp17.4-CIII (XP_003627410.1), MtHsp17.6-CII (XP_003603183.1), PsHsp17.1-CII (P19242.1), PsHsp18.1-CI (P19243.1), PsHsp22.7-CIV (P19244.1), PsHsp22-Mito (P46254.1), PssmallHsp-Chl (P09886.1), VfHsp17.9 (AGC51113.2)]

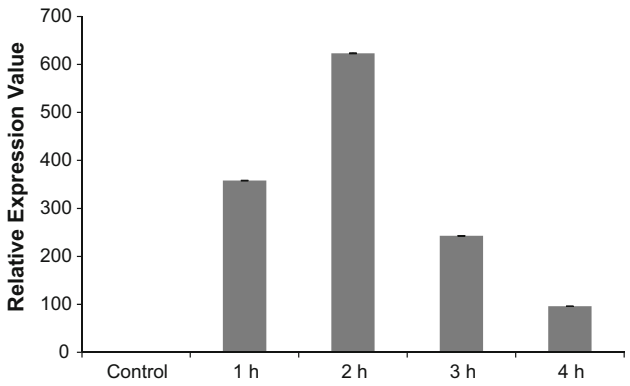


Fig. 3 Expression analysis of VfHsp17.9-CII under HS by Q-PCR. Expression level of faba bean elongation factor 1-alpha was used as internal control. *Y*-axis represents normalized relative expression value or fold change and *X*-axis represents different treatments namely, control and 38 °C HS for 1, 2, 3, and 4 h. The results are represented as means of three replicates ($n = 3$) ± SEM

76–89, respectively, depending on protein length (Table 2). An additional bipartite NLS was predicted in the CIII sHsps of fabaceae (Table 2). Cytoplasmic localization of VfHsp17.9-CII was predicted by WoLF PSORT and PlantLoc. We analyzed the subcellular localization of VfHsp17.9-CII by transient transformation of onion epidermal peel cells with a fusion construct of VfHsp17.9-CII and GFP driven by CaMV35S promoter. Empty vector expressing free GFP driven by CaMV35S promoter served as a control. VfHsp17.9-CII-GFP fusion protein was found to be localized in the nuclei of onion cells while the empty vector-transformed cells expressed GFP throughout the cytoplasm (Fig. 4).

Abiotic stress tolerance of *E. coli* cells overexpressing VfHsp17.9-CII protein

Recombinant VfHsp17.9-CII protein was expressed in *E. coli* BL21-CodonPlus-RIL cells. According to the construction of the recombinant plasmid, size of the induced protein is ~21.7 kDa containing 3.8 kDa of the C-terminal 6 X histidine tag/thrombin/T7 tag and 17.9 kDa of VfHsp17.9-CII. After induction with 0.5 mM IPTG for 4 h, SDS-gel analysis showed high level overexpression of 21.7 kDa recombinant VfHsp17.9-CII in crude extracts from bacterial cells transformed with pET-28a(+)-VfHsp17.9-CII but not in crude extracts from cells harboring empty pET-28a(+) vector (Fig. 5). The N-terminal 6 X histidine tag in the recombinant protein allowed its purification from cell-free extracts by affinity chromatography using Ni–NTA agarose which was confirmed by Commassie blue staining and further by Western blot analysis (Fig. 5).

To determine the effect of VfHsp17.9-CII overexpression on abiotic stress tolerance of *E. coli* cells, we compared the survival of bacterial cells harboring empty pET-28a(+) vector with that of the cells overexpressing VfHsp17.9-CII, by spot assay. The recombinant and empty vector cells showed similar growth on basal LB media, whereas the above cell types showed differential growth in the presence of stress treatments (Fig. 6a–e). While the bacterial cells transformed with pET-28a(+) empty vector did not grow on LB containing 0.5 mM arsenic, the cells transformed with pET-28a(+)-VfHsp17.9-CII grew at 0.5 mM arsenic (Fig. 6b). Under NaCl stress, the empty vector-transformed bacterial cells showed better survival with higher number of colonies than the cells transformed with recombinant VfHsp17.9-CII (Fig. 6c). Upon exposure to 50 °C HS, *E. coli* cells expressing recombinant VfHSp17.9-CII protein showed greater viability than cells containing the empty vector (Fig. 6e). The number of colonies was higher in recombinant *E. coli* cells compared to empty vector at all dilutions. The difference in growth was clearly visible after 1 h of HS where the recombinant cells showed growth up to 10^{-4} dilution, whereas vector-transformed cells showed lesser growth up to 10^{-2} dilution (Fig. 6e).

Analysis of the induction of VfHsp17.9-CII in flowering faba bean plants

The effects of HS were studied at flowering stage of faba bean plants. Flowering stage plants were subjected to HS

Table 2 Prediction of NLS and subcellular localization of various CII and CIII sHsps of fabaceae family

Gene	Sequence type	Position of NLS	NLS Score	Predicted subcellular localization		
				cNLS Mapper	WolfPSORT	PlantLoc
MtHsp17.6-CII	**PPEPKKPKTI**	**144**	**5.5**	Both cytoplasm and nucleus	Cytoplasm	Cytoplasm
CaHsp17.1-CII	**PPEPKKPKTI**	**143**	**5.5**	Both cytoplasm and nucleus	Cytoplasm	Cytoplasm
PsHsp17.1-CII	**PPEPKKPKTI**	**138**	**5.5**	Both cytoplasm and nucleus	Cytoplasm	Cytoplasm
AmHsp-CII	**PPEPKKPKTI**	**144**	**5.5**	Both cytoplasm and nucleus	Cytoplasm	Cytoplasm
VfHsp17.9-CII	**PPQPKKPRTIEVKVF**	**146**	**8.0**	Partially nucleus	Cytoplasm	Cytoplasm
GmHsp17.9-CII	**PPEPKKPRTI**	**145**	**6.5**	Partially nucleus	Cytoplasm	Cytoplasm
CaHsp18.8-CII	**PPEPKKPKTI**	**148**	**5.5**	Both cytoplasm and nucleus	Nucleus	Cytoplasm
CaHsp17.4-CIII	**IKSNRKRKRQDSE,SNRKRKRQDS/** *KSNRKRKRQDSEDEGCKYLRLE*	**75, 77**/*76*	**10.5, 12**/*9.5*	Nucleus	Cytoplasm	Cytoplasm
MtHsp17.4-CIII	**SNGKRKRQDG/** *KSNGKRKRQDGEDEGCKYIRLE*	**78**/*77*	**6**/*7*	Partially nucleus	Cytoplasm	Nucleus and Endoplasmic reticulum
GmHsp17.4-CIII	**IRSNGKRKRQDGE,RSNGKRKRQDG/** *RSNGKRKRQDGEDEGCKYLRLE*	**88, 89**/*89*	**6, 7**/*6.5*	Partially nucleus	Cytoplasm	Cytoplasm

Sequence, position, and score of monopartite (bold) and bipartite (italicized) NLS are shown

Each NLS sequence was scored based on which subcellular localization was predicted by cNLS Mapper

The subcellular localization analyzed with WolfPSORT and PlantLoc is also indicated

Species names are shown in Fig. 3

[38 °C (2 h) and 38 °C (4 h)] in growth chamber. The unstressed, control plants were maintained at 28 °C. The stress treatments showed no visible damage to plants or the flowers *per se* (Fig. 7a). Pollen viability was found to be significantly reduced in response to HS. A greater decline of pollen viability was noted after 38 °C (2 h) HS treatment as compared to 38 °C (4 h) HS treatment (Fig. 7b, c). HS treatments induced VfHsp17.9-CII transcripts in the top-most leaf and in pollen. In pollens, VfHsp17.9-CII expression was induced after 38 °C (2 h) HS treatment (Fig. 7d). Higher induction of VfHsp17.9-CII expression was noted after 38 °C (4 h) HS treatment of pollen (Fig. 7d).

Discussion

In order to improve heat tolerance of faba bean crop, it is imperative that the diversity of Hsps of this species is unveiled. There is only one report so far on Hsp profiling in faba bean in published literature. In this report, Nieden et al. (1995) analyzed Hsp17 expression in faba bean and reported that Hsp17 was mainly localized in protein bodies in mature seeds. There is little information on faba bean sHsp genes in public-domain database. To expand the work on Hsp biology of faba bean, we aimed at the analysis of faba bean sHsp transcripts using primers specific to *G. max* sHsp gene. Hsps are highly conserved proteins across plant species. For instance, Agarwal et al. (2002) showed that amino acid sequence of Hsp100 genes from soybean, Arabidopsis, and rice is significantly identical. The basis of using soybean sHsp gene primers for the analysis of faba bean sHsps was the fact that the soybean genome has been completely sequenced. Further, the expression profiles of soybean sHsps have been analyzed under abiotic and biotic stresses (Lopes-Caitar et al. 2013). The HS-regulated faba bean transcript noted in this study corresponds to a CII sHsp gene as evidenced by presence of an ACD in the C-terminus and high sequence homology and phylogenetic proximity with other CII sHsps of fabaceae family. The transcript expression of VfHsp17.9-CII was increased to the extent of ~620 fold change after 2 h of HS, indicating that VfHsp17.9-CII gene is highly heat responsive (Fig. 3). We thus report a novel CII sHsp of faba bean and name it VfHsp17.9-CII. Position of NLS in sHsps is a factor for deciding their classification under CII or CIII. The CII nuclear/cytoplasmic sHsps of Arabidopsis and rice contain distinct NLS near the C-terminus (Sarkar et al. 2009; Scharf et al. 2001). The prediction of a monopartite NLS near the C-terminus in addition to the predicted cytoplasmic localization further supported the classification of

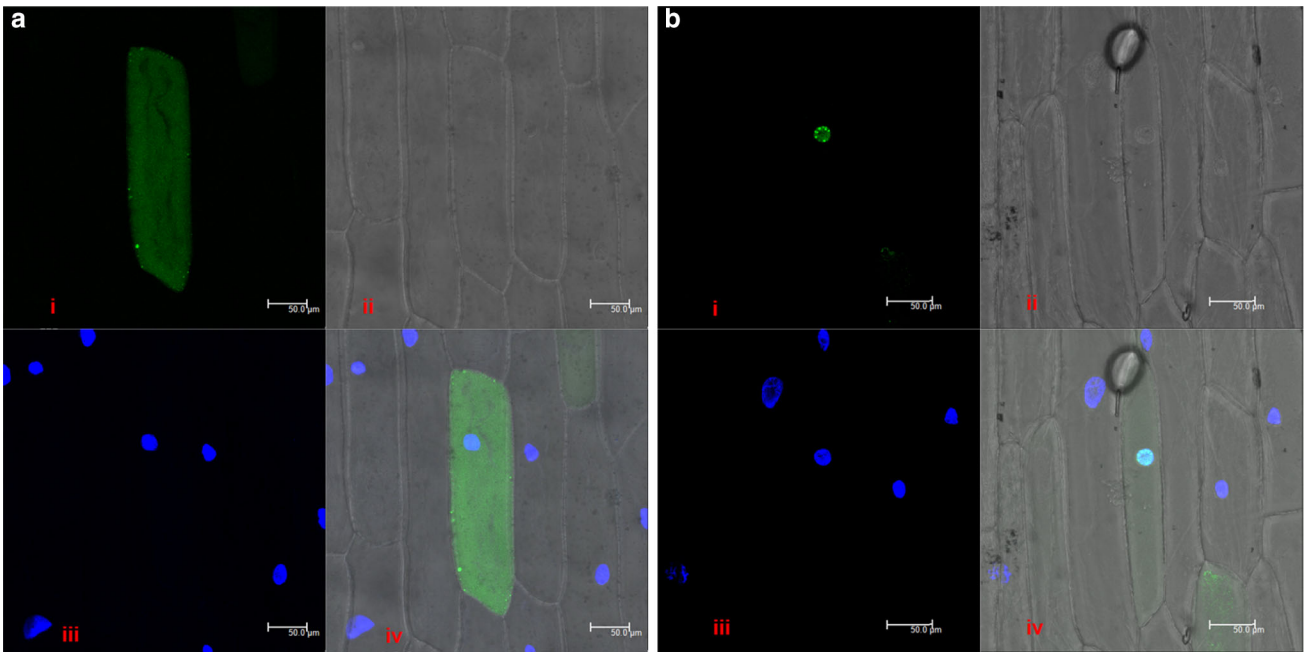


Fig. 4 Subcellular localization of VfHsp17.9-CII in onion epidermal peel cells. **a** Onion epidermal cells transformed with empty vector pCAMBIA1302. **b** Onion epidermal transformed with VfHsp17.9-CII-GFP fusion construct in pCAMBIA1302 vector. *i* represents GFP signal, *ii* represents bright field images, *iii* represents cells stained with DAPI (4′,6′-diamidino-2-phenylindole) to identify the nucleus, and *iv* represents merged image of *i*, *ii* and *iii*

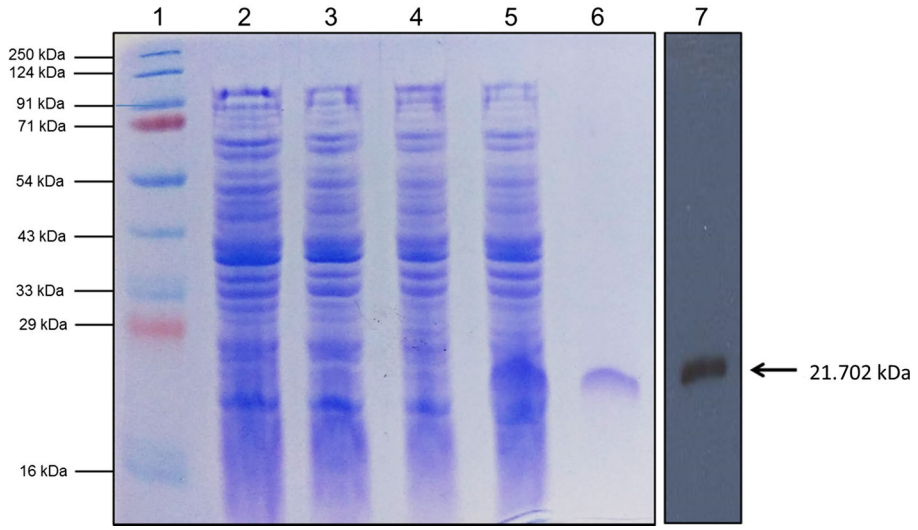


Fig. 5 Expression and purification of recombinant VfHsp17.9-CII expressed in *E. coli* BL21-CodonPlus-RIL cells. Protein samples were separated by 12.5 % SDS-gel and stained with either Coomassie brilliant blue or detected by Western blotting. Lane numbers are indicated on *top* of the picture. Lanes 1–6 were loaded with 10 µl of cell-free extract. *Lane 1* protein molecular mass marker with sizes shown on the left in kDa, *lane 2* cellular extract of uninduced empty vector pET-28a(+) cells, *lane 3* cellular extract of empty vector cells induced with 0.5 mM IPTG, *lane 4* cellular extract of uninduced recombinant pET-28a(+)-VfHsp17.9-CII cells, *lane 5* cellular extract of recombinant pET-28a(+)-VfHsp17.9-CII cells induced with 0.5 mM IPTG, *lane 6* affinity-purified recombinant VfHsp17.9-CII using 200 mM imidazole, *lane 7* 200 ng of purified recombinant VfHsp17.9-CII was electroblotted to nitrocellulose membrane and probed with monoclonal antibody to the $(His)_6$ epitope tag encoded by pET-28a(+) by the ECL procedure (*arrow marked*)

VfHsp17.9 as a CII sHsp gene. We noted that the GFP fusion of VfHsp17.9-CII was localized in the nuclei of transiently transformed onion epidermal peel cells.

In this study, we noted that overexpression of VfHsp17.9-CII in bacterial cells provides distinct advantage to the bacterial cells to combat HS and arsenic stress.

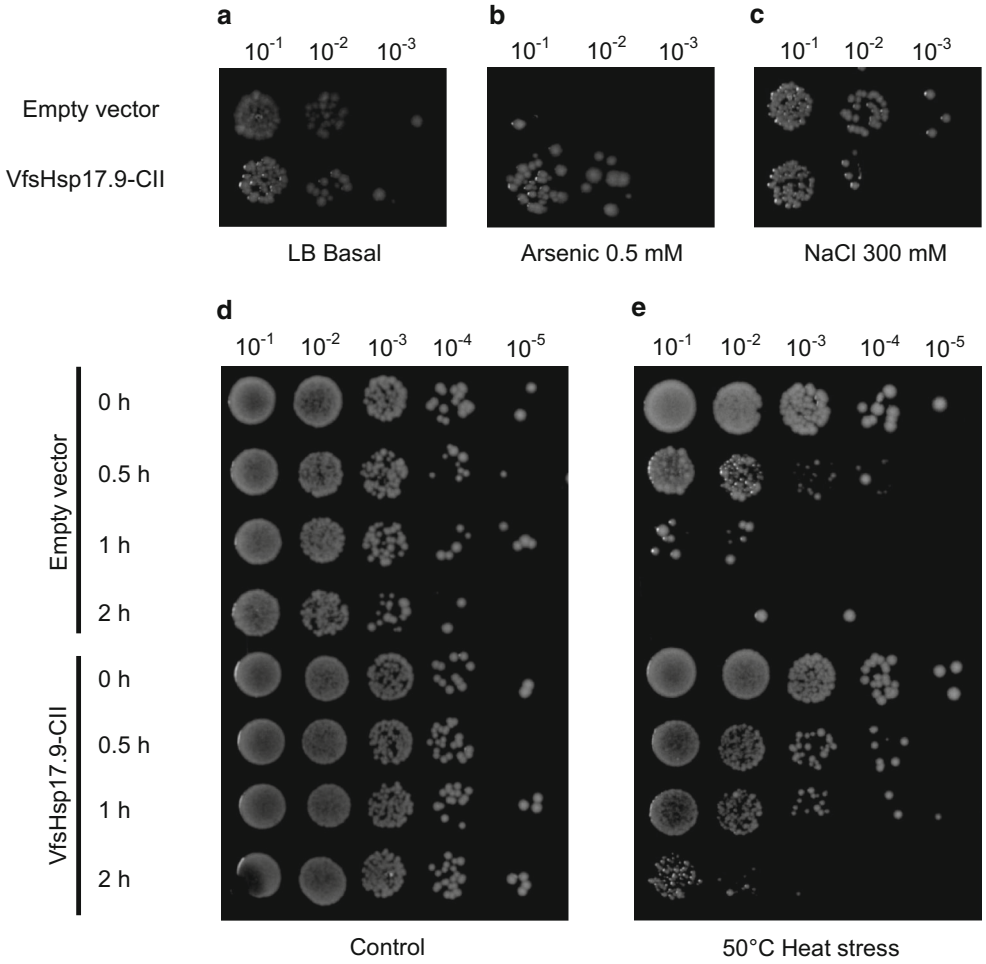


Fig. 6 Abiotic stress tolerance of *E. coli* cells expressing recombinant VfHsp17.9-CII. **a–c** Spot assay of BL21-CodonPlus-RIL cells harboring empty vector pET-28a(+) and pET-28a(+)-VfHsp17.9-CII on LB kanamycin–chloramphenicol basal plates supplemented with 0.5 mM IPTG or containing 0.5 mM sodium arsenate or 300 mM NaCl. Five microliters from 10^{-1} to 10^{-3} dilutions were spotted on **a** LB kanamycin–chloramphenicol/IPTG basal plates, **b** containing 0.5 mM sodium arsenate, and **c** 300 mM NaCl. **d–e** Spot assay for thermotolerance of BL21-CodonPlus-RIL cells harboring empty vector pET-28a(+) and pET-28a(+)-VfHsp17.9-CII on LB kanamycin–chloramphenicol plates. Five microliters from 10^{-1} to 10^{-5} dilutions were spotted after 0, 0.5, 1, and 2 h time points of **d** unstressed control cells grown at 30 °C and **e** cells heat stressed at 50 °C

The promoters of Hsps have been shown to harbor metal stress responsive elements or STREs and their expression is governed by both heat stress and heavy metal stress (Singh et al. 2012). Our observation is in consonance with several past studies where overexpression of different sHsps has been shown to be beneficial for induction of high heat tolerance and heavy metal stress tolerance (Lee et al. 2014; Soto et al. 1999; Wan et al. 2012; Yeh et al. 1997). The possible significance of induction of VfHsp17.9-CII gene under HS for faba bean plants remains unexplored. We noted that pollen viability of faba bean plants was drastically affected in response to HS. It was striking that the loss of pollen viability was higher after 38 °C (2 h) treatment as compared to 38 °C (4 h) treatment. Concurrently, we noted that the transcript expression of VfHsp17.9-CII in pollen was higher after HS of 38 °C for 4 h as compared to 38 °C for 2 h. In David Lily (*Lilium davidii* var. Willmottiae), LimHSP16.45 was found to be highly expressed during late zygotene to pachytene stages of meiotic prophase I in the pollen mother cells and its expression in the anthers was induced by HS (Mu et al. 2011). Expression of LimHSP16.45 was found to peak specifically at 4 h of 42 °C HS and 4 h of 4 °C cold stress exposure. We speculate that the sudden rise in temperature may have affected the faba bean pollens more severely and the pollens were more heat shocked in the 2 h HS treatment. With longer HS regime of 4 h, pollen may have developed mechanism(s) to reduce the loss of viability. This needs to be substantiated in future work. Higher damage to pollen viability occurred under conditions when pollen did not

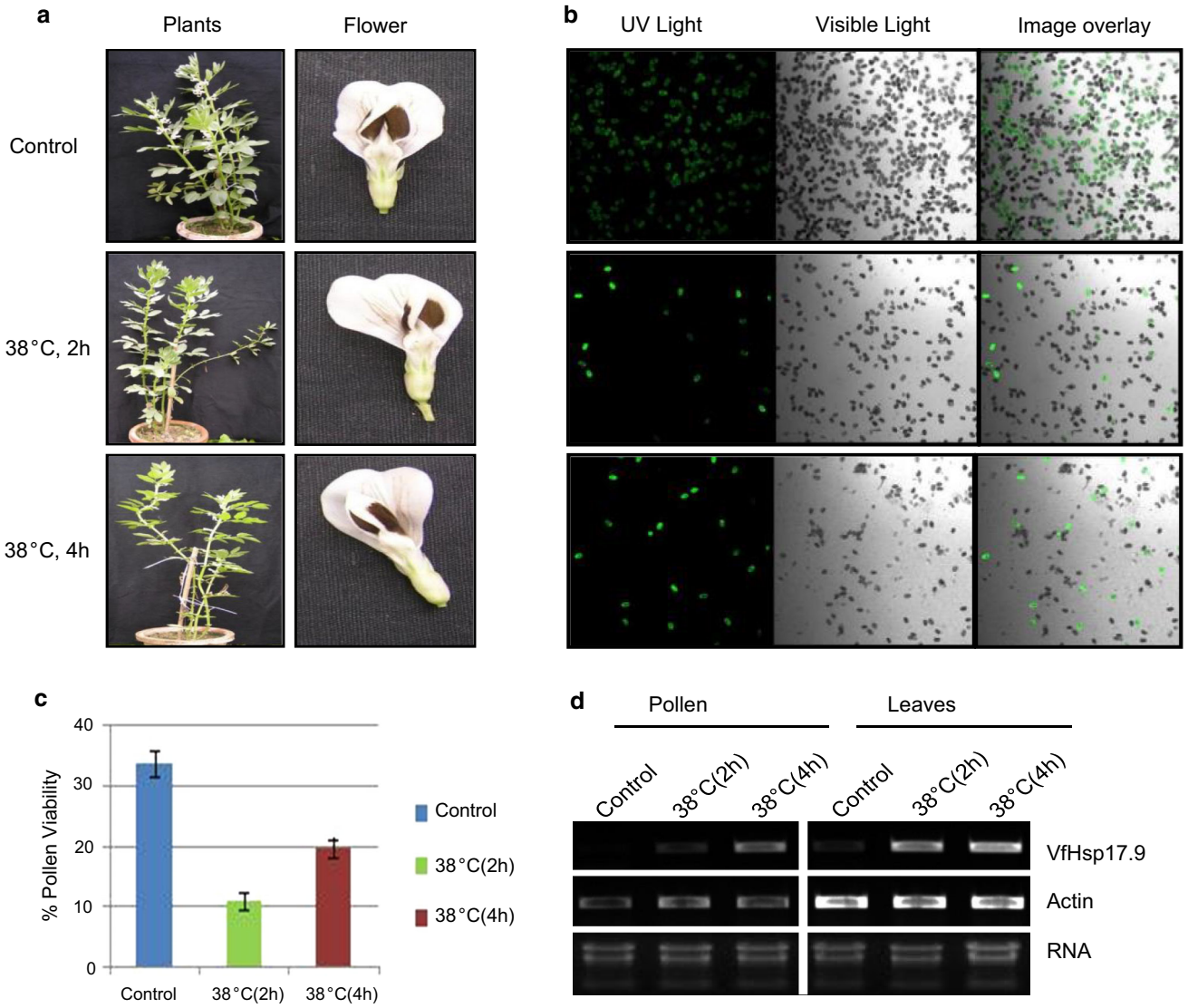


Fig. 7 **a** Analysis of intact faba bean plants and flowers in response to HS treatments. **b** Confocal laser scanning micrographs of FDA-stained faba bean pollen after HS treatments. Micrographs under ultraviolet, visible light, and after overlay of the two are shown. Control and HS treatments in **a** and **b** are indicated on the *left*. **c** Percentage pollen viability after HS as determined by FDA staining. **d** Transcript expression profile of VfHsp17.9-CII in pollen and topmost leaf of flowering faba bean plants. Actin is used as internal control

synthesize high levels of VfHsp17.9-CII transcript. Probably VfHsp17.9-CII has a role at later stages of HS response in the pollen or under prolonged HS. In rice, it has been suggested that the number of differentially regulated genes common to the late time point of HS (60 min) and the recovery period is higher than the number of differentially regulated genes common to the early time point of HS (10 min) and the recovery period (Sarkar et al. 2014). This indicates that recovery-specific gene expression changes start to occur under prolonged HS which might lead to specific adaptive changes to withstand the stress. We infer that VfHsp17.9-CII transcript levels and pollen viability are positively correlated under longer duration of HS. The detailed role of VfHsp17.9-CII in thermoprotection of faba bean plants under field-level HS conditions needs to be further analyzed through forward and reverse genetic approaches in future studies. While this study is a step forward, it must be appreciated that plant sHsps are encoded by multigene families. Arabidopsis genome contains 13 genes for sHsps and 25 genes for ACD proteins (Scharf et al. 2001). Likewise, rice genome contains 23 genes for sHsps and 17 genes for ACD proteins (Sarkar et al. 2009). Clearly, there is a need to unveil the entire family of faba bean sHsps in future research.

Author contribution statement RK, AKS, and MN coordinated with experiments on transcript expression analysis and sequencing. RK and DL coordinated with the experiments on subcellular localization, protein expression, protein purification, and abiotic stress tolerance in *E. coli*. RK, DL, and AG performed data analysis and drafted the manuscript. MHS and MHA provided the details on problems posed by heat stress in faba bean cultivation. AG coordinated this study.

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