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Saudi Journal of Biological Sciences

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## ORIGINAL ARTICLE

# Expression analysis of ClpB/Hsp100 gene in faba bean (*Vicia faba* L.) plants in response to heat stress



Ritesh Kumar <sup>a</sup>, Amit Kumar Singh <sup>a</sup>, Dhruv Lavania <sup>a</sup>, Manzer H. Siddiqui <sup>b</sup>,  
Mohamed H. Al-Whaibi <sup>b</sup>, Anil Grover <sup>a,\*</sup>

<sup>a</sup> Department of Plant Molecular Biology, University of Delhi, South Campus, New Delhi 110021, India

<sup>b</sup> Department of Botany and Microbiology, King Saud University, Riyadh, Saudi Arabia

Received 6 January 2015; revised 13 March 2015; accepted 18 March 2015

Available online 24 March 2015

## KEYWORDS

ClpB;  
Faba bean;  
Heat stress;  
Hsp100;  
Pollen;  
Protein expression;  
Thermotolerance;  
Transcript induction

**Abstract** Heat stress adversely affects the growth and yield of faba bean crop. Accumulation of ClpB/Hsp100 class of proteins is a critical parameter in induction of acquired heat stress tolerance in plants. Heat-induced expression of ClpB/Hsp100 genes has been noted in diverse plant species. Using primers complementary to soybean ClpB/Hsp100 gene, we analyzed the transcript expression profile of faba bean ClpB/Hsp100 gene in leaves of seedlings and flowering plants and in pollen grains. ClpB/Hsp100 protein accumulation profile was analyzed in leaves of faba bean seedlings using *Arabidopsis thaliana* cytoplasmic Hsp101 antibodies. The transcript and protein levels of faba bean ClpB/Hsp100 were significantly induced in response to heat stress.

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## 1. Introduction

Faba bean (*Vicia faba* L.) plants are extensively cultivated for food and fodder. They are grown in medial and western regions of Saudi Arabia (Al-Suhaibani, 2009). High atmospheric temperatures or terminal drought conditions cause heat stress (HS) on faba bean plants (Stoddard et al., 2006) leading to detrimental effects on growth and yield-related processes. The delay in sowing date also leads to HS on faba bean

plants (Adisarwanto and Knight, 1997). In desert ecosystems of Saudi Arabia, unstable weather conditions leading to drought and HS affect the cultivation of faba bean to a significant extent (Al-ghamdi and Al-Tahir, 2001; Abdelmula and Abuanja, 2007; Alghamdi, 2007; Al-Suhaibani, 2009).

HS profoundly affects cellular, biochemical and physiological activities and processes of plant cells. Recent molecular studies show that the expression levels of thousands of genes are simultaneously altered in response to HS (Sarkar et al., 2014). Proteins inside cells misfold upon exposure to HS leading to accumulation of non-functional toxic aggregates. Cells respond by activating mechanisms that reduce the aggregation of proteins and promote the disaggregation of large protein aggregates formed under stressful conditions. This is primarily achieved by increased expression of heat shock proteins (Hsps) in cells (Lavania et al., 2015a). Hsps, through their molecular chaperoning activities, aid in protein homeostasis. Hsps play

\* Corresponding author. Tel.: +91 11 24117693; fax: +91 11 24115270.

E-mail address: [anil.anilgrover@gmail.com](mailto:anil.anilgrover@gmail.com) (A. Grover).

Peer review under responsibility of King Saud University.



Production and hosting by Elsevier

<http://dx.doi.org/10.1016/j.sjbs.2015.03.006>

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critical roles in the development of heat tolerance (Grover et al., 2013; Lavania et al., 2015b). The chaperones represented by Hsp10/Hsp20/Hsp40/Hsp60/Hsp70 classes help to reduce protein aggregation. Hsp100 class of chaperones have a major role in disaggregation of aggregated proteins (Singh and Grover, 2010). Hsp100 is the only class of chaperones which functions in retrieving active proteins from stress-induced protein aggregates. Hsp100 proteins belong to the family of caseinolytic protease (Clp). More specifically, they are clubbed with ClpB proteins and are therefore referred as ClpB/Hsp100 proteins.

Most of the published work on plant Hsps is on model species such as Arabidopsis and rice (Lavania et al., 2015a). Legume crops are a rich source of dietary protein. They play an important role in maintaining soil fertility through symbiotic associations with nitrogen fixing bacteria. The molecular work on legumes is relatively less optimized. Hsps of faba bean are poorly analyzed (Lavania et al., 2015b). Previous studies indicate that ClpB/Hsp100 proteins play a critical role in acquisition of heat tolerance in several plant species (Queitsch et al., 2000; Nieto-Sotelo et al., 2002; Lin et al., 2014). This fact prompted us to examine the expression profile of ClpB/Hsp100 genes in faba bean. However, a major obstacle is the scarce information on nucleotide sequences of faba bean Hsp genes in public-domain databases. To overcome this obstacle, we recently proposed that genomic information available on model legume crops such as soybean can be exploited (Lavania et al., 2015b). The soybean genome has been completely sequenced (Schmutz et al., 2010). By making primers corresponding to soybean sHsp gene, we recently cloned the full-length coding sequence of sHsp17.9-CII gene of faba bean (GenBank accession number KC249973.1). In this study, we report transcript and protein accumulation profiles of faba bean ClpB/Hsp100 gene.

## 2. Materials and methods

### 2.1. Plant growth and stress treatments

*V. faba* L. seeds were obtained from the National Bureau of Plant Genetic Resources, New Delhi. The seeds were sown onto sterilized Soilrite Mix (obtained from KEL, Maharashtra, India) in plastic pots with regular watering. Plants were grown at 23 °C, 16 h light/8 h dark cycle in a growth chamber (Adaptis, Conviron). HS was given inside the chamber to intact plants at two growth stages namely, seedling stage (10-day-old) and reproductive or flowering stage. For recovery treatments following stress, plants were transferred to the control, growth conditions. Temperature and durations of stress and recovery treatments are shown in Figures (see Section 3). Treatments were given in sets of three independent plants representing biological replicates. Top-most leaves from each set were harvested separately, immediately frozen in liquid nitrogen and stored at -80 °C until isolation of RNA or protein.

### 2.2. cDNA synthesis, cloning and sequence analysis

Total RNA was isolated using TRI Reagent (Sigma-Aldrich) as per manufacturer's instructions. 2- $\mu$ g RNA was used for

first-strand cDNA synthesis using oligo (dT) primers and RevertAid H Minus reverse transcriptase (Thermo Scientific). Phusion High-Fidelity DNA polymerase (New England Biolabs) was used for semi-quantitative reverse transcriptase-PCR (sqRT-PCR). Stolf-Moreira et al. (2011) suggested the use of soybean  $\beta$ -actin (GenBank accession number GMU06500) as a reference gene for expression analysis. The primers complementary to soybean  $\beta$ -actin (forward primer-5'GAATTGCCTGATGGACGGT3' and reverse primer-5'GCTTTGGGGATCCACATCTA3') were used for amplifying *V. faba* actin transcript as an endogenous control in sqRT-PCR. Soybean ClpB/Hsp101 mRNA sequence (Glyma05g00540; GenBank accession number NM\_001251193.1; Lee et al., 1994) was used as query to search for the soybean genomes for homologs using BLASTN function of the Phytozome database (<http://www.phytozome.net>). ClpB/Hsp100 conserved domains were identified in NCBI conserved domain database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and SMART database (<http://smart.embl-heidelberg.de/>). WoLF PSORT ([http://www.gen-script.com/psort/wolf\\_psort.html](http://www.gen-script.com/psort/wolf_psort.html)) software was used for prediction of sub-cellular localization. Primers complementary to full-length coding sequence of soybean ClpB/Hsp100 gene (Glyma05g00540; forward primer - 5'ATGAATCCTGAG AAGTTTACTCA3' and reverse primer - 5'TCACTCTTC CATTTCATCAT CAT3') were used in sqRT-PCR. PCR conditions were as follows: initial denaturation at 98 °C for 30 s followed by 30 cycles of denaturation at 98 °C for 20 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 90 s. After 30 cycles, final extension at 72 °C for 5 min was given and the reaction mixture was then cooled to 4 °C. All sqRT-PCRs were repeated thrice with three biological replicates. Cloning was performed with CloneJET PCR Cloning Kit (Thermo Scientific) as per manufacturer's instructions. Nucleotide sequencing was carried out using sequencing primers supplied with the cloning kit.

### 2.3. Protein isolation and western blot analysis

Frozen top-most leaf tissue of different treatments was homogenized in 1.0 mL lysis buffer (HEPES buffer 25 mM pH 7.5, NaCl 500 mM, MgCl<sub>2</sub> 5 mM, EDTA 1 mM, Nonidet P-40 0.2% v/v, PMSF 1 mM). Cell debris was pelleted by centrifugation at 12,000 rpm for 10 min. 40  $\mu$ g of each protein sample was loaded onto a 10% (w/v) SDS-gel (with 3.9% w/v acrylamide stacking gel) and electrophoresed at 30 V. Proteins were electro-blotted onto 45  $\mu$ m Hybond-C super nitrocellulose membrane (Amersham-Pharmacia) as described by Towbin et al. (1979). Primary polyclonal antibodies against N-terminal region of *Arabidopsis thaliana* Hsp101 (Agrisera) and secondary antibodies of a goat anti-rabbit horseradish peroxidase conjugate (Sigma-Aldrich, Product number A6154) were used with dilutions of 1:5000 and 1:10,000, respectively. Western blots were developed using the enhanced chemiluminescence (ECL) peroxidase system.

### 2.4. Measurement of pollen viability

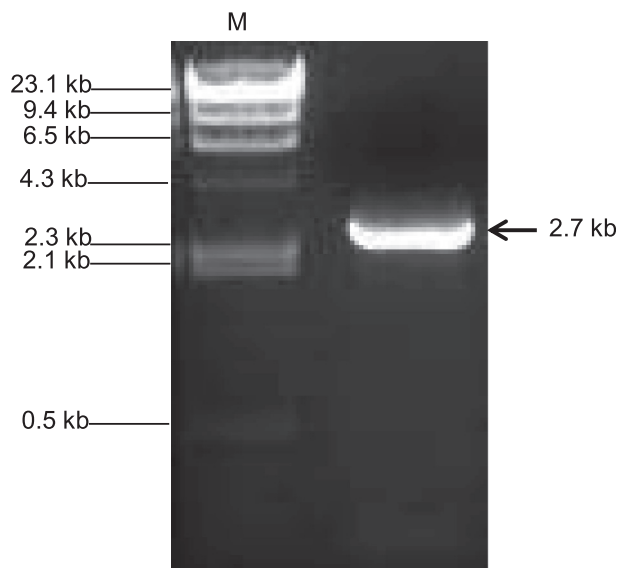
Fluorescein di-acetate (Sigma-Aldrich) staining was used to measure pollen viability and Leica TCS SP5 confocal laser

scanning microscope was used to score fluorescing pollen grains.

### 3. Results

Six ClpB/Hsp100 genes namely, Glyma04g06610, Glyma05g00540, Glyma08g26410, Glyma13g05920, Glyma18g49930 and Glyma19g03440 were identified in the soybean genome by sequence homology search in Phytozome database. These six genes represented one cytoplasmic (Glyma05g00540), four plastidial (Glyma08g26410, Glyma13g05920, Glyma18g49930 and Glyma19g03440) and one mitochondrial (Glyma04g06610) isoform of ClpB/Hsp100. The number of amino acids in the six genes ranged from 912 to 979 and all the ClpB/Hsp100 conserved domains namely, ClpN1, ClpN2, WalkerA1, WalkerB1, WalkerA2, WalkerB2 and ClpB2 small domain were noted in the six isoforms. Detailed characteristics of these genes are shown in Fig. 1. Using primers complementary to Glyma05g00540 gene, reverse transcriptase PCR was performed on cDNA from the top-most leaf of 10-day-old heat stressed seedlings. This reaction yielded an amplicon of nearly 2.7 kb (Fig. 2). It was cloned in pJET1.2/blunt cloning vector. We confirmed that the amplified product indeed represents faba bean ClpB/Hsp100 gene by partial end sequencing of the clone (data now shown). However, we are yet to determine the full-length nucleotide sequence of the clone.

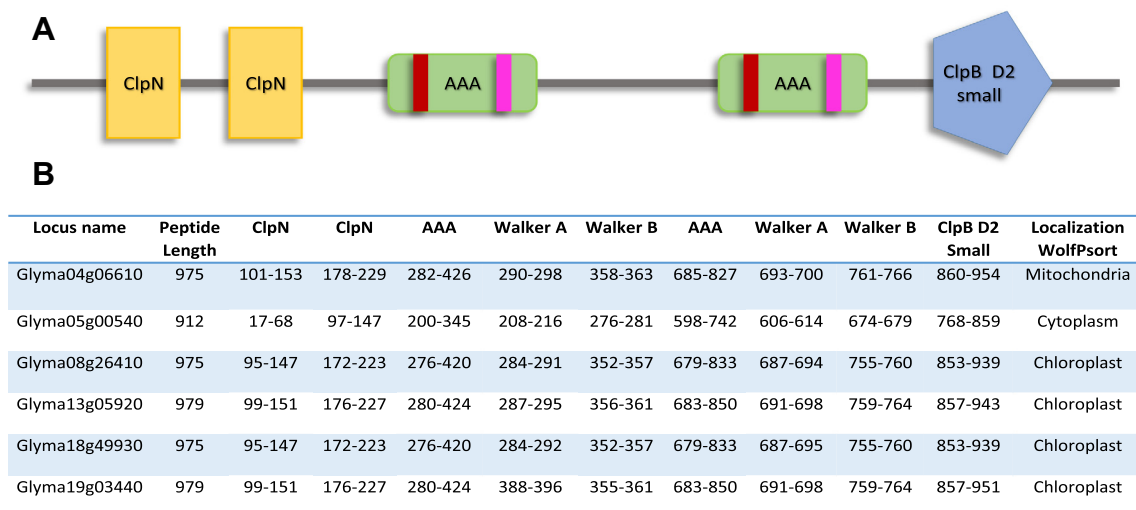
Semi-quantitative RT-PCR analysis was performed on the top-most leaf of seedlings after HS treatments at 38 °C for 2 h and 4 h. Under unstressed control conditions, faba bean ClpB/Hsp100 transcript was not expressed. Increase in expression was noted after the HS treatments (Fig. 3). Higher expression was seen after 2 h as compared to 4 h HS. Next, transcript levels were analyzed at recovery treatments of 1 h and 2 h after both HS regimes. Transcript levels decreased significantly after 1 h recovery treatments. Transcript levels declined to beyond detection limit after 2 h recovery treatments. Levels of the faba bean ClpB/Hsp100 transcripts were next analyzed in pollen and the top-most leaf of flowering stage plants (Fig. 4). In case of pollen, transcript was induced after 38 °C HS for 4 h and



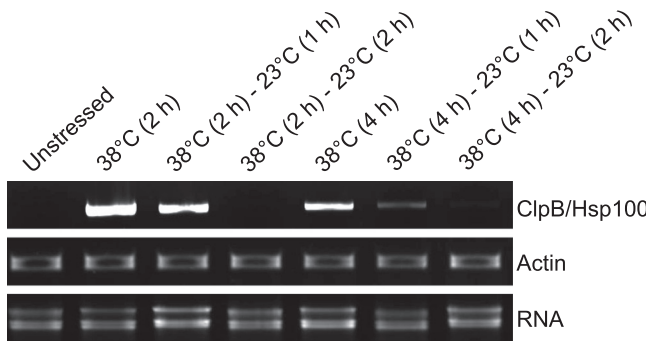
**Figure 2** PCR amplification of an approximately 2.7 kb product corresponding to faba bean ClpB/Hsp100 gene using primers complementary to Glyma05g00540 gene. The amplified product was loaded onto 1% agarose gel. M:  $\lambda$  DNA digested with HindIII as molecular size marker, the fragment sizes are given on the left of the photograph. An arrow indicates the position of PCR product.

not after HS for 2 h. In the case of leaf, the induced transcript levels were comparable after 2 h and 4 h HS treatments. The effect of HS (42 °C; 2 h and 4 h) was next measured on pollen viability. The pollen viability was decreased as a result of HS treatment. Importantly, the decrease in pollen viability was higher after 2 h HS treatment as compared to 4 h HS treatment.

To analyze the ClpB/Hsp100 protein levels in faba bean under unstressed control, HS and recovery treatments, western blot analysis was performed with polyclonal antibodies against *A. thaliana* Hsp101 (Fig. 5). ClpB/Hsp100 protein levels were below detection limits under unstressed control conditions.



**Figure 1** Selected details of soybean ClpB/Hsp100 genes. (A) General domain structure of ClpB/Hsp100 proteins. (B) Table showing six genes of soybean belonging to ClpB/Hsp100 family. Position of conserved domains is denoted. Predicted localization by WoLF PSORT is shown.



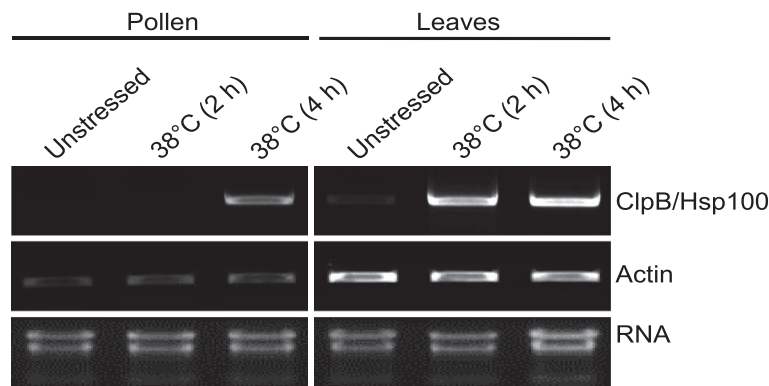
**Figure 3** Semi-quantitative RT-PCR analysis of faba bean ClpB/Hsp100 gene in top-most leaf of 10-day-old seedlings (top panel). Details of HS and recovery treatments are indicated on top. Actin was used as internal control (middle panel). Lower-most panel shows loading of RNA samples.

ClpB/Hsp100 accumulation was observed after 38 °C HS for 2 h; importantly, protein accumulation after 4 h HS was higher than that after 2 h HS. A one hour recovery post 4 h HS resulted in a significant increase in ClpB/Hsp100 accumulation while a 2 h recovery caused a severe decline in protein levels. The noted increase in protein level after a 3 h recovery may be due to biological variation in protein expression among different tissue samples.

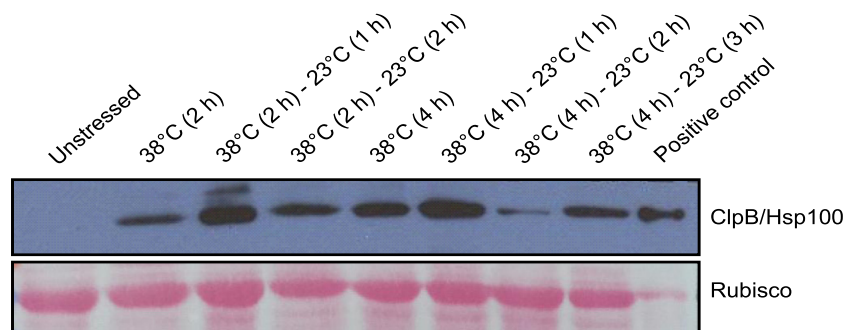
#### 4. Discussion

In plants, ClpB/Hsp100 proteins are strongly implicated in heat stress tolerance. All plants analyzed till date contain multiple species of these proteins. Arabidopsis contains three ClpB/Hsp100 genes representing cytoplasmic (AtClpB-C; At1g74310), plastidial (AtClpB-P; At5g15450) and mitochondrial (AtClpB-M; At2g25140) isoforms (Lee et al., 2006). Similarly, rice contains one cytoplasmic (OsClpB-C; Os05g44340), one plastidial (OsClpB-P; Os03g31300) and one mitochondrial (OsClpB-M; Os02g08490) isoform of ClpB/Hsp100 (Singh et al., 2010). In soybean, Lee et al. (1994) cloned and characterized a ClpB/Hsp100 gene. We noted that the soybean genome contains six ClpB/Hsp100 genes representing one cytoplasmic, one mitochondrial and four chloroplastic isoforms. Subsequently, we analyzed faba bean ClpB/Hsp100 form corresponding to cytoplasmic ClpB/Hsp100 isoform of soybean (Glyma05g00540) in this study. We could successfully amplify faba bean ClpB/Hsp100 cDNA corresponding to Glyma05g00540 primers. This amplicon was confirmed by nucleotide sequencing. However, we are left to obtain full-length sequence of the faba bean ClpB/Hsp100 gene. The complexity of ClpB/Hsp100 gene forms in faba bean genome can only be answered after the complete genome is sequenced.

Under stressful conditions, transcription and translation processes are globally repressed. Studies have shown that Hsp



**Figure 4** Semi-quantitative RT-PCR analysis of faba bean ClpB/Hsp100 gene in pollen and top-most leaf of flowering plants, as indicated. Details of HS treatments are indicated above the panels. Actin was used as internal control (middle panel). Lower-most panels show loading of RNA samples.



**Figure 5** Western blot analysis of faba bean ClpB/Hsp100 protein in top-most leaf of 10 day old seedlings. Details of HS and recovery treatments are indicated on top. Ponceau staining of ribulose biphosphate carboxylase oxygenase (Rubisco) is shown in lower panel to denote equal loading of proteins. 10 µg of total protein isolated from heat stressed *Arabidopsis thaliana* seedlings was used as positive control.



mRNAs including those of ClpB/Hsp100 genes are preferentially translated under HS conditions (Yángüez et al., 2013). Semi-quantitative RT-PCR analysis clearly indicates that transcript levels of the faba bean ClpB/Hsp100 gene are induced by HS. We further noted that polyclonal antibodies against *A. thaliana* Hsp101 cross-react with faba bean ClpB/Hsp100 protein indicating that ClpB/Hsp100 proteins are highly-conserved. Higher protein accumulation of faba bean ClpB/Hsp100 after 4 h HS as compared to 2 h HS indicates higher protein stability or preferential translation of its mRNAs. In western blot, the maximum levels of ClpB/Hsp100 protein were noted after recovery treatments indicating that transcript and protein levels follow different temporal kinetics.

The flowering stage plants of faba bean are highly sensitive to HS. Imposition of HS at this stage significantly affects pollen viability, thereby affecting yield (Patrick and Stoddard, 2010). We have previously observed that HS of 38 °C drastically affects the pollen viability in faba bean and that pollen viability at 38 °C is correlated with transcript expression profile of VfHsp17.9-CII gene (unpublished data). We noted that 42 °C HS similarly has a detrimental effect on pollen viability. Notably, in pollen tissues, the faba bean ClpB/Hsp100 transcript was undetected after 2 h HS of 38 °C and was induced strongly after 4 h HS at 38 °C. We speculate that higher levels of ClpB/Hsp100 transcript and protein might be present in pollen and play a role in the enhanced HS tolerance and pollen viability.

This study is the first report on ClpB/Hsp100 expression in leaves of seedlings and mature plants as well as in pollen of faba bean. We aim to obtain full-length cDNA sequences of various faba bean ClpB/Hsp100 genes in further course. We also wish to obtain comprehensive data on functionality of different ClpB/Hsp100 genes of faba bean plants in future experiments.

### Conflict of interest

The authors confirm that there is no conflict of interest for the information presented in the manuscript.

### Acknowledgements

RK is thankful to the University Grants Commission, India for fellowship. DL is thankful to the Council of Scientific and Industrial Research, Government of India for the research fellowship award. MHS and MHA-W thank project funding from the National Plan for Science and Technology Program, Saudi Arabia. AG gratefully acknowledges Visiting Professorship of the King Saud University, Saudi Arabia.

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