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ORIGINAL ARTICLE

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

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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.

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

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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. Topmost 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-μg 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 β-actin (GenBank accession number GMU06500) as a reference gene for expression analysis. The primers complementary to soybean β-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 (Glyma05g00 540; 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.genscript.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, $MgCl_2$ 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 μg 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 μm 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