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Expression analysis of genes encoding sHSP and Rab1 in *Hevea brasiliensis*

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Abstract

Drought induced low molecular weight (LMW) protein synthesis and changes in the level of translatable mRNA of two LMW proteins induced during water stress were studied in leaves of four clones of *Hevea* with varying level of drought tolerance. When young plants of four *Hevea* clones exposed to drought and high light stresses the chloroplasts accumulated a LMW membrane protein of 23.8 kDa. The amino acid sequence of the stress protein was elucidated by combining SDS PAGE followed by in-gel digestion and shotgun analysis (LC-MS/MS) and found to be a small heat shock protein (*HbsHSP23.8*). Another protein of similar LMW was found co-eluted with *HbsHSP23.8* upon purification by electro-elution from SDS PAGE and MALDI-TOF-MS analyses. This protein was identified as *Hevea brasiliensis* Rab1 (*HbRab1*) protein. The bioinformatics analysis indicated that *HbRab1* protein is a key regulator in vesicle trafficking in cells. On the other hand *HbsHSP23.8* is a chaperone that plays significant role in drought tolerance in young plants of *Hevea.* Gene expression analysis of *HbRab1* and *HbsHSP23.8* was carried out by real-time RT-PCR. Under drought conditions both genes were found over expressed in leaves however, the level of expression was different. The expression of *HbsHSP23.8* was significantly up-regulated by drought and high light conditions whereas in the case of *HbRab1* gene it was not obvious. This study compares the possible role of both these LMW proteins in young rubber plants for drought responses.

Keywords: *Hevea brasiliensis*, Drought stress, Stress protein, MALDI-TOF-MS, qPCR expression analysis.

INTRODUCTION

Natural rubber plant (*Hevea brasiliensis*) is the major source of rubber accounting around 99% of the world's total natural rubber production. To meet the increasing demand for natural rubber it has become necessary to produce more rubber by extending its cultivation to non-traditional areas. Drought, high solar radiation, low atmospheric humidity, high and low temperatures, high vapour pressure deficit (VPD), poor soils etc. are the adverse environmental conditions which limit the expansion of rubber cultivation to newer areas in many rubber producing countries including India [1,2,3]. Stressful environment caused by the above conditions is a limiting factor even in traditional rubber growing belt. One of the major abiotic constraints that can considerably reduce plant growth and crop yield is drought [4].

Plants require an adequate supply of water which is vital for cell expansion and all other growth and development. A deeper understanding of drought tolerance is very much essential in the increasing oscillations of climatic conditions. Multiple mechanisms at the morphological, physiological and molecular levels that contribute for drought tolerance in plants have been evolved. Proteins which play a key role in stress signaling, transcription regulation, protection of macromolecules, cellular detoxification, and an array of other cellular processes are the primary factors associated with drought tolerance [5]. Proteomic analyses of leaves from various plants have identified numerous drought responsive proteins involved in various physiological activities.

Small heat shock proteins (sHSPs) are ubiquitous stress proteins that act as chaperones with molecular mass ranging from 12 to 40 kDa [6] and get induced upon exposure to abiotic stresses including drought, cold, oxidized species and salinity [7]. Previous investigations have identified the expression of sHSP in *Hevea* clones under soil moisture deficit and high light intensity [8, 9]. Several proteins are found to be expressed in plants in response to stresses at pre- and post- transcriptional and translational levels [10]. Rab1 protein is a small GTPase and belongs to Ras superfamily that contains monomeric GTP-binding proteins with molecular masses of 20-40 kDa [11]. In plants RabGTPases are key regulators of vesicle trafficking and in *Hevea* Rab1 (*HbRab1*) protein have found to be involved in tapping-activated latex regeneration [12]. Role of such proteins in intracellular vesicle trafficking and abiotic stress responses in *Hevea* has not been studied.

The present study was initiated to identify drought induced low molecular weight proteins in *Hevea brasiliensis*. A 23.8 kDa stress protein that showed consistent over expression in drought induced clones identified as *HbsHSP23.8* and a protein that co-eluted with sHSP upon purification was identified as *HbRab1* protein. Further the expression analysis of *HbsHSP23.8* and *HbRab1*genes was quantified in four clones of *Hevea* with varying level of drought tolerance to identify the specific gene among

them for drought responsiveness.

MATERIALS AND METHODS

Plant material and Stress Induction

Budded stumps of four clones of *Hevea,* namely, RRII 105, RRIM 600, RRII 414, and RRII 430 were planted in large (35 x 65 cm) size polythene bags filled with around 30kg laterite rich garden soil per bag. The plants were grown under normal field conditions (twenty plants per treatment) with open sunlight. One set of plants of each clone was drought stressed by withholding irrigation for 10 days during the rain free summer season. Mid-day sun light load was around 1800µmol/m^2/s. A second set of plants was kept as irrigated controls. The impact of stress in these one year old plants after 10 days of withholding irrigation was assessed by measuring the net photosynthetic rate (P_N) using an infra-red gas analyzer (IRGA), LI-6400 (LI-COR U.S.A), chlorophyll fluorescence was measured by using PAM 2100 (Waltz, Germany) and PS II quantum yield (φ PSII) was calculated. Leaf samples from these plants were collected in liquid nitrogen and stored at -80ºC for further chloroplast protein and qPCR analysis.

Isolation of Chloroplast proteins

Type II (broken) chloroplast were isolated by the method of Reeves and Hall [13]. The leaf bits were homogenized with liquid nitrogen in a mortar and pestle into powder. The powdery samples (3g) were extracted with 5 ml of ice cold grinding buffer consisting of 20mM TrisHCl (pH 7.8), 10mM NaCl, 5mM $MgCl_2$, 350mM Mannitol, 2mM Ascorbate and filtered through four layered cheese cloth and centrifuged at 800g for 2 min at 4ºC. The pellet represented unbroken cells, tissue was removed and the supernatant was spun at 3500g for 5 min at 4ºC and the resulting pellet was suspended in 1ml of grinding buffer (pH 7.8) as chloroplast suspension. The chloroplast proteins were pelleted out by trichloroacetic acid (TCA)-acetone precipitation.

Purification of Low Molecular Weight protein

The air-dried chloroplast protein was solubilised in a small amount of 10% SDS and quantified by the method of Lowry *et al.,* [14]. Equal amount of chloroplast protein isolated from control and drought exposed plants was loaded onto a 10 % preparative SDS polyacrylamide gel with a reference well holding the standard molecular weight protein marker according to the method of Laemmli [15]. The protein profile was visualized by coomassie staining and destaining and a 23.8 kDa protein that showed

consistent expression in the profile from drought induced clones were excised from the gel and purified by electro-elution using the Bio-Rad Model 422 Electro-Eluter apparatus following the manufacturer's protocol.

Identification of co-eluted protein by MALDI-TOF-MS

For mass spectrometry analysis approximately 100 µg of 23.8 kDa stress protein was purified by the process of electro-elution from the chloroplast protein of clone RRIM 600 (drought tolerant, 10 days drought induction) resolved on 10% SDS gel. The purified protein was subjected to 2-DE. Gel pieces of 1.5 mm in diameter were excised manually from the 1mm thick gels and washed for 30min at room temperature under vigorous shaking with 400µl of 10mM ammonium bicarbonate solution containing 50% (v/v) acetonitrile. Gel pieces were dried for 15min in a vacuum concentrator after removing the supernatant. The rehydrated gel pieces were incubated in 150µl reduction solution (10mm DTT, 100mm ammonium bicarbonate) for 30min at 56 ºC. The reduction solution was then discarded and 100µl alkylation solutions (50mm iodoacetamide, 100mm ammonium bicarbonate) were added for 30 min in the dark at room temperature. For digestion, 5µl trypsin solutions (Sequencing grade modified trypsin, Promega, Madison and 10ng/µl in 5mM ammonium bicarbonate/5 % acetonitrile) were added to each sample. After incubation for 5h at 37 ºC, the reaction was stopped by adding 1µl of 1% TFA. For better extraction of peptides, the samples were stored overnight at 5 ºC. Without further purification, 1µl of supernatant was mixed with 2µl of matrix solution (5mg α-cyano-4-hydroxycinnamic acid in 40 % [v/v] acetone, 50 % [v/v] acetonitrile, 9.9 % [v/v] water and 0.1 % [w/v] TFA in water). 1µl from this mix was deposited onto the MALDI target. Tryptic peptides were analysed with a MALDI-TOF mass spectrometer (Bruker-Daltonics, Germany) in positive mode. Contamination by keratins and background ions from trypsin autolysis were removed from mass lists. Protein identification was performed by searching for proteins in the latest version of the NCBI nr database using the Mascot search engine. The following parameters were applied: Monoisotopic mass accuracy, peptide mass tolerance (0.1 Da); peptide charge state (1+); missed cleavages, 1; allowed variable modifications, oxidation (Met) and fixed modification, carbamidomethyl (C). Fragmentation of selected peptides was measured using the PSD mode.

Total RNA isolation and qPCR analysis

Total RNA was extracted from the leaf samples using Spectrum Plant Total RNA Kit (Sigma-Aldrich) and reverse transcribed using Superscript III first strand synthesis system (Invitrogen) following the manufacturer's instructions. Suitable primers were designed with Primer Express Software and got synthesized by Eurofins and are given

in **Table 1**. Real time PCR was performed using Light Cycler 480II, Roche Real Time PCR System. The reaction consisted of 1µl of 1:10time's diluted cDNA, 125nM of each forward and reverse primers and 10µl of SYBR Green I Master (Roche Diagnostics Gmbh, Germany) in a 20µl reaction volume. The reaction conditions included 95ºC for 7min, followed by 40 cycles of 95ºC for 20 s and 60ºC for 30 s. This was followed by a melt curve analysis (95ºC for 20 s, 60ºC for 1 min and 95ºC for 5 min). Reaction efficiency of both target genes and the endogenous control was calculated based on the formula, $E=10^{(-1/slope)}-1$ and the slope values of the primers were between -3.2 and -3.5. Three biological replications for each treatment were included in the qPCR analysis. No template controls (NTC) were run to assay for false positive signals and GAPDH was used as the endogenous control. Relative Quantification (RQ) values were used to study the fold change in the expression rate of these genes using Light Cycler 480 Software; release 1.5.0. The relative changes in gene expression from qPCR experiments were analyzed by $2^{-\Delta\Delta Ct}$ method [16]. Statistical analysis was performed with the relative quantification data using ANOVA. The difference between groups was assessed by means of the 2-tailed Student *t* test. P-value <0.05 was considered to be statistically significant.

Table 1: List of genes and the corresponding primers used for qPCR analysis

Sl no	Gene	Forward Primer (5′-3′)	Reverse Primer (5′-3′)
1	*HbsHSP23.8*	GATGTGGTCGACTCATTTTCTCCA	CTTTGACGTCCTGTTTGCTTAGCC
2	*HbRab1*	GGCTCTTGCAGATAAATATGGCATC	GCTGGTTAATTTTGATCGTCTGTGG
3	GAPDH	GCCTGTGATAGTCTTCGGTGTTAG	GCAGCCTTATCCTTGTCAGTGAAC

RESULTS AND DISCUSSION

To understand the role of drought induced 23.8 kDa protein, 4 clones of *Hevea brasiliensis* with varying level of drought tolerance were exposed to water deficit stress by withholding irrigation. The effective quantum yield of PS II (φ PS II) was found declining significantly in all four clones after exposure to drought stress by withholding irrigation for 10days (**Fig 1. A**). Among the clones RRIM 600 and RRII 430 showed better stability in PSII activity while the magnitude of inhibition was severe in clones RRII 105 and RRII 414. The efficiency of PS II photochemistry was analyzed by measuring the effective quantum yield of PSII (φ PS II) during steady state photosynthesis under light illuminated conditions therefore it is an indication of overall photosynthesis [17]. Similarly, the net photosynthetic CO_2 assimilation rate (P_N) under drought condition was significantly inhibited compared to

their respective irrigated control plants in clones RRII 105 and RRII 414 but was less inhibited in RRIM 600 and RRII 430 (**Fig 1.B**). The drastic reduction in P_N in clones RRII 105 and RRII 414 indicated their relative susceptibility to desiccation stress. Although clones RRIM 600 and RRII 430 recorded a significantly lesser rate of P_N than their respective irrigated controls, they maintained comparatively stable level of photosynthesis than the other two clones after 10 days of drought. These results confirmed that significant level of drought stress was set in these plants.

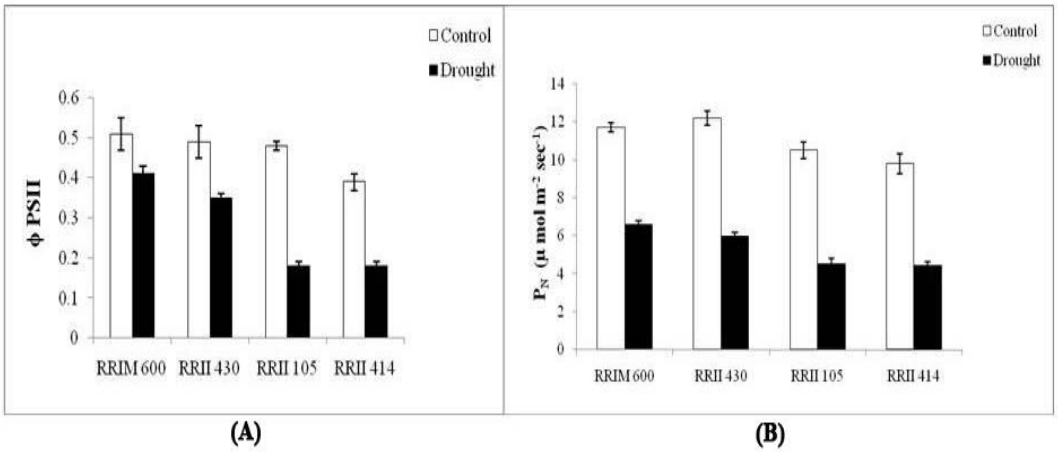

Fig 1: The effective quantum yield of Photosystem II (φ PS II) (A) and net photosynthetic rate (P_N) (B) in irrigated and drought imposed *Hevea* plants.

The chloroplast protein profile in all the clones studied from drought induced and irrigated control in field was obtained by coomassie staining of SDS polyacrylamide gels. A low molecular weight (LMW) protein of 23.8 kDa was observed to be over expressing consistently in drought imposed young plants of *Hevea.* The water deficit stress imposed plants accumulated significantly higher level of this stress protein in comparison to the control irrigated plants. The relative abundance of this stress protein was very prominent in relatively drought tolerant clones RRIM 600 and RRII 430 when compared to their respective irrigated counterparts. However the expression level of stress protein was not unique in clones RRII 414 and RRII 105 though susceptible to water deficit stress in terms of physiological parameters. RRII 414 showed expression within medium to optimum range but clone RRII 105 showed a relatively fair degree of expression of the stress protein (**Fig. 2**). Though RRII 105 is considered as drought susceptible the degree of susceptibility of this popular clone is debatable. This clone was graded as relatively drought tolerant in traditional rubber growing areas in earlier studies [17]. Recent reports indicated that this clone is comparatively drought and high light susceptible as observed from many studies

conducted in traditional as well as non-traditional drought prone areas of India [19,9,20]. Recent molecular studies on drought responsive genes have even graded this clone as moderately drought susceptible [21].

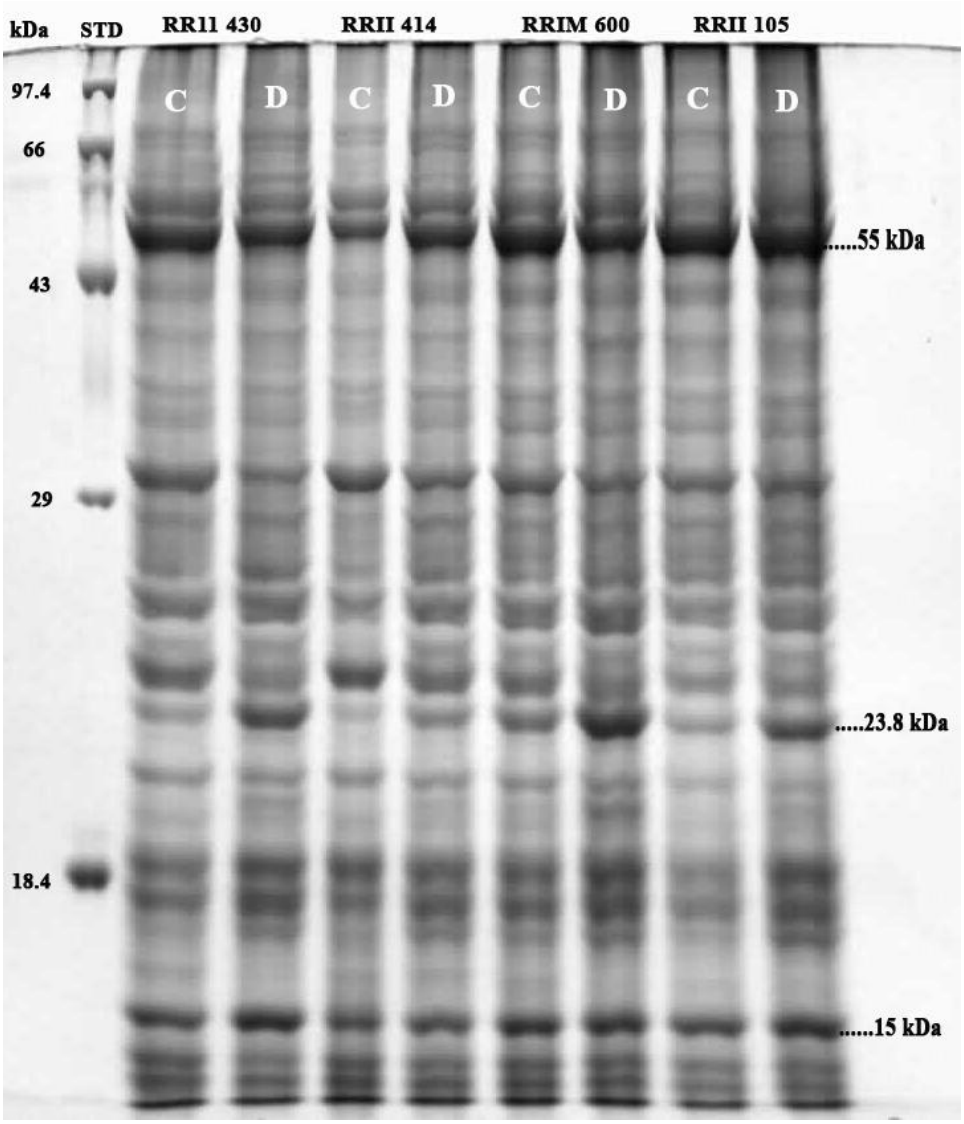

Fig 2: Chloroplast protein profile of 04 elite *Hevea* clones (RRII 430, RRII 414, RRIM 600, RRII 105). The plants were grown with (C) or without (D) irrigation for 10 days during summer season. The stress protein 23.8 kDa is indicated on the right side along with RuBisCO larger subunit (55kDa) and smaller subunit (15 kDa).

The amino acid sequence of the stress protein was already elucidated by combining SDS PAGE followed by in-gel digestion and shotgun analysis LC-MS/MS and mass spectrometry and was reported as a HSP type protein associated with chloroplast thylakoid membrane in *Hevea* [8]. In the present study a protein of similar molecular

weight that co-eluted with *HbsHSP23.8* upon purification by electro-elution was further subjected to 2-D gel electrophoresis and MALDI-TOF-MS (**Fig 3**).

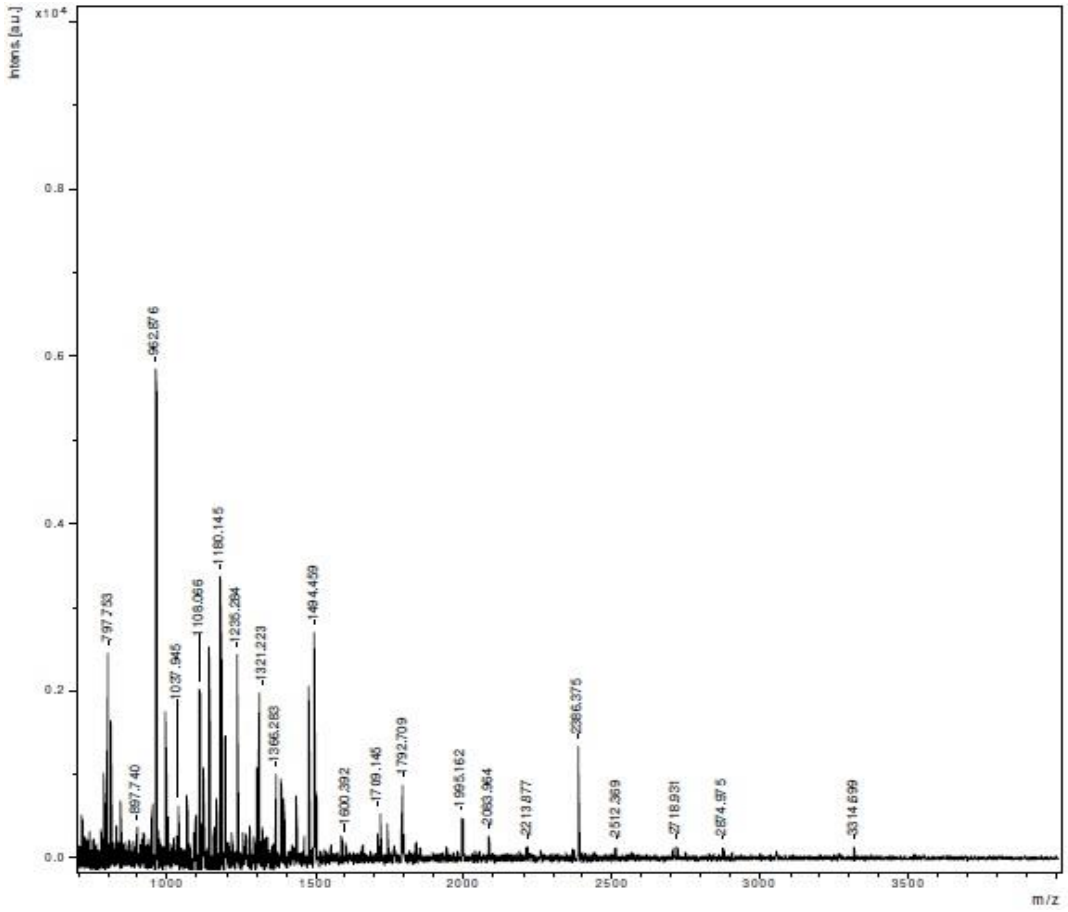

Fig 3: MALDI-TOF–MS mass spectrum of the tryptic digest of spot obtained in the linear TOF–MS for highest resolution and mass accuracy

Data obtained by mass spectrometry were used to identify this protein in the latest version of NCBI nr database using the Mascot search engine. The protein that showed maximum coverage of 58% with a stress protein was Ras related protein Rab1 from species *Beta vulgaris* with accession no Q39433 and predicted nominal mass (M_r) of 23943. The protein was further identified in swiss-prot database using the accession number and the nucleotide sequence of this protein was extracted from RefSeq (Reference Sequence). The RefSeq nucleotide sequence was subjected to BLAST-X search and *Hevea* (Rab1) hit obtained from BLAST result and the protein was identified as small GTP binding protein *HbRab1*. Small GTP binding proteins (G proteins) are monomeric G proteins that function as molecular switches which cycle between a GTP-bound active state and a GDP- bound inactive state found to be involved in the regulation of diverse and essential cellular processes, including

intracellular membrane trafficking, cytoskeleton organization, signal transduction, cell proliferation and gene expression [22,23].

In order to determine the association of *HbsHSP23.8* and *HbRab1* genes to drought tolerance/susceptibility quantitative expression analyses were made by qPCR and the results are depicted in **Fig 4**. Among the two genes investigated in this study, *HbsHSP23.8* was found significantly upregulated in the relatively drought tolerant clones RRIM 600 and RRII 430 than other two drought susceptible clones. In the case of RRII 105 the expression level was slightly higher than RRII 414 which showed optimum range of expression. Reports from earlier studies indicated that sHSPs play significant role in tolerances to a variety of abiotic and biotic stresses as well as key developmental processes [24,25]. In *Hevea* previous investigation has confirmed the existence of sHSP in chloroplast of thylakoid membrane of rubber plants experiencing drought and high solar light [8]. Recent studies through RT-PCR analysis have established the influence of *HbsHSP23.8* genes in enhancing rubber plants tolerance to environmental stresses including drought [26]. Significant level of up-regulation of *HbsHSP23.8* in tolerant clones of *Hevea* in this study indicates its strong association with drought tolerance.

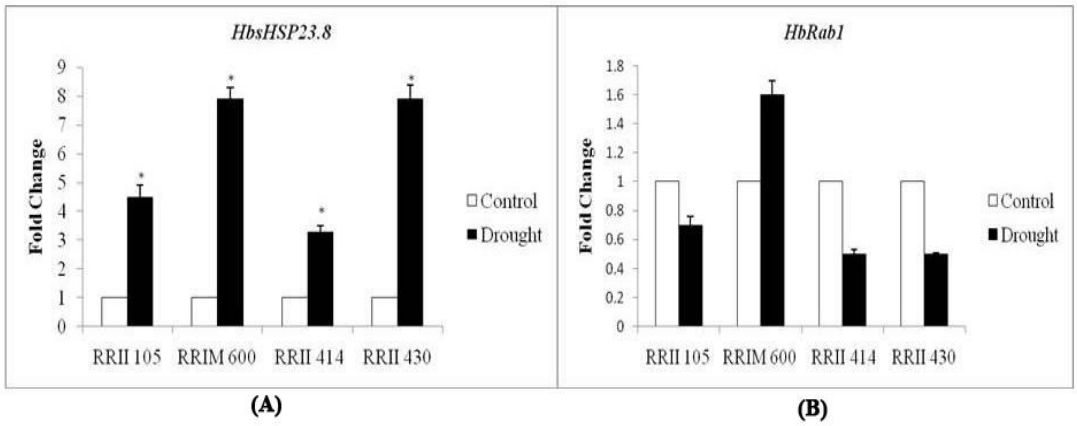

Fig 4: Expression analysis of *HbsHSP23.8* and *HbRab1* genes in four clones of *Hevea* under drought condition. Y axis indicated fold change in the expression of *HbsHSP23.8* (A) and *HbRab1* (B) mRNA in drought samples over their respective irrigated plants (Control). The mRNA were quantified by means of real-time polymerase chain reaction and normalized with the use of GAPDH mRNA. Values are expressed as mean ± SD (n=3/group). *P<0.05 Drought vs Control.

On the other hand the gene expression analysis of *HbRab1* indicated that its level did not show any significant change in all the four clones analyzed. In *Hevea* these small GTPases genes were reported to be expressed ubiquitously in all tissues suggesting

the functional requirement of these genes in diverse tissues and *HbRab1* were significantly induced in response to tapping and wounding through a jasmonic acid mediated signalling pathway. *HbRab1* expression increased strikingly with successive tapping of virgin trees suggesting the role of it in tapping-activated latex regeneration [12]. Vesicle trafficking though traditionally viewed as a constitutive housekeeping process, recent studies hence described the involvement of intracellular vesicle trafficking in plant stress adaptation in resistance to various environmental stresses [27]. Recent reports have indicated that transformation of a vesicle trafficking protein gene Rab7 from a stress tolerant plant *Pennisetum glaucum* to a stress-sensitive rice variety *Pusa Basmati-I (PB-I)* confers tolerance to drought and salinity stresses [28]. However, the expression pattern of *HbRab1* observed in this study is inconclusive hence needs to be further investigated.

In the present study transcripts of two LMW proteins viz. *HbsHSP23.8* and *HbRab1* from *Hevea brasiliensis* were validated for their association with drought stress tolerance. This was made possible by quantifying its expression in four different clones of which two were graded as drought tolerant (RRIM 600 and RRII 430) and remaining two as drought susceptible (RRII 105 and RRII 414). The data obtained from physiological and proteomics studies also supported the fact in the case of tolerant clones. RRIM 600 and RRII 430 were graded as abiotic stress tolerant clones in previous studies conducted on *Hevea* and hence suitable for drought and cold prone regions [29,17,30]. From the gene expression analysis data, *HbsHSP23.8* was identified to be strongly associated with drought responses in clones RRIM 600 and RRII 430 (upregulation of 7-8 fold) whereas in RRII 105 and RRII 414 the upregulation was 4 fold and 3.3 fold, respectively under drought. However in case of *HbRab1* no definite trend could be observed and hence further investigations are required by incorporating more number of clones with varying levels of drought tolerance/susceptibility.

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