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Research Article

Differential gene expression studies on *HsfA4d*, *HsfB2a* and *ClpD1/HSP100* during heat stress in the rice varieties N22 and Co51

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Abstract
In the present investigation, differential expression of two transcription factor genes (*HsfA4d and HsfB2a*) and a heat shock protein (*ClpD1/HSP100*) was evaluated in the rice varieties N22 and Co 51 using qRT-PCR. It was observed that gene expression levels were varying during ontogeny. Among the two transcription factor genes, *HsfB2a* which is under the influence of the master regulator *HsfA1s* was found to have enhanced expression levels than *HsfA4d*, which acts in a *HsfA1s* independent pathway. Gene expression level of the heat shock protein *ClpD1/HSP100* in the heat tolerant variety N22 was higher than that of the Co 51, implying that Co 51 is relatively less heat tolerant. N22 was observed to have higher heat-shock memory retention than that of Co51.

Keywords
Rice, Thermal Stress, qRT-PCR, Heat shock factors, HSP100, Heat stress memory

INTRODUCTION

Rice (*Oryza sativa* L.) is a model system for genetic and genomic studies in grasses and one of the major staple cereals in the world. Heat stress is one of the major abiotic stressors limiting plant biomass production and productivity, primarily in tropical and subtropical countries. The productivity of rice is threatened by high-temperature stress which is exemplified by climate change. When the ambient temperature is higher than the suitable growing temperature, enzymes and structural proteins lose their activity and quaternary structure, respectively, resulting in altered metabolism as well as structural damage to the plant cells. This may lead to abnormal growth, flowering, and seed yield reduction(Miller and Mittler 2006; Song *et al.,* 2010) upon prolonged exposure to high temperatures / heat stress. Key genes involved in heat stress response (HSR) pathways have been identified and evaluated for their role in tolerance / resistance mechanisms. Analysis of the rice genome reveals that approximately 2.6 *per cent* of the identified genes encode transcriptional factors (TFs) (Goff *et al.,* 2002) playing important regulatory roles in growth, development, and responses to adverse environmental conditions. Heat stress response is a universal phenomenon, displayed by the perception of signals by the HSFs which enhance or repress gene expression through their active binding to pentameric heat shock elements (HSE)(Nover *et al.,* 2001) present in the promoter region of heat shock protein (HSP) genes or HSF modifying enzymes. The rice HSF family consists of 25 genes (Wang *et al.,* 2009) and are divided into three conserved classes *viz.,* A, B, and C, based on sequence homology and domain architecture (Guo *et al.,* 2008). HSFs are present in a latent state under normal conditions and are activated upon heat stress by induction of trimerization and high-affinity binding to DNA

and by exposure of domains for transcriptional activity (Pérez-Rodríguez *et al.,* 2010).

The *HEAT SHOCK TRANSCRIPTION FACTOR A1s (HsfA1s),* popularly regarded as the ‘‘master regulator’’, directly regulates or fine-tunes the expression of heat stress-inducible genes (Ohama *et al.,* 2017). Under non-stress conditions, the heat shock proteins, namely, HSP70 and HSP90 repress *HsfA1* through repression of transactivation activity and nuclear localization, respectively. Following heat stress encounter, HsfA1s become active, undergo various post-translational modifications, and consecutively triggers a transcriptional cascade of other major heat-responsive TFs, including *DEHYDRATION-RESPONSIVE ELEMENT BINDING PROTEIN2A (DREB2A), HsfA2, HsfA7a, HsfBs*, and *MULTIPROTEIN BRIDGING FACTOR 1C (MBF1C).* It has been reported that among the members of the HSF family, *HsfAs* and *HsfBs* sub-families are significantly responsive to heat stress (Zhang *et al.,* 2012).

One of the downstream target gene family of HsfA1s is *HsfB*s (*HsfB1s, HsfB2s*). *HsfB*s suppresses the leaky expression of TFs under normal conditions in a negative feedback loop (Ohama *et al.,* 2016). They repress the activity of HsfA1s either by forming a HsfA1-HsfB2s complex or by masking the HSEs in the promoter of HsfA1s responsive genes under non-heat stress conditions and during attenuating periods (Ikeda *et al.,* 2011). Also, they act as coactivators of *HsfA1s* under heat stress conditions resulting in enhanced expression of HS inducible downstream Hsfs and HSPs, which are necessary for acquired thermotolerance. Few of the Hsfs are identified to function in a HsfA1independent pathway. The *HsfA4s* are induced by oxidative stress during HS and regulates the expression levels of ascorbate peroxidase1 (*APX1*), one of the H_2O_2 scavengers during HS (Ohama *et al.,* 2017).

HSPs are molecular chaperones, which function in protein folding and assembly, protein intracellular localization and secretion, and degradation of misfolded and truncated proteins. High-temperature stress disrupts cellular proteostasis, resulting in the accumulation of insoluble protein aggregates. For survival under stressful conditions, cells need to maintain a pool of native soluble proteins by preventing the formation of- and/or dissociation of- these aggregates. Five major families of HSPs / chaperones include HSP70 (DnaK) family, chaperonins family (GroEL and HSP60), HSP90 family, HSP100 (Caseinolytic Protease/Clp) family, and the small HSP (sHSP) family (Wang *et al.,* 2004). Plants possess multiple forms of HSPs that are localized to different cellular compartments (*i.e.,*cytoplasm / nucleus, chloroplast, or mitochondria). The HSP100 transcript / protein expression is induced during heat stress in rice (Agarwal *et al.,* 2003; Pareek *et al.,* 1995; Shen *et al.,* 2003). The HSP100 proteins, along with a plethora of other molecular chaperones, play crucial roles in the removal of stress-related protein aggregates induced by physiological, chemical, and pathological stresses. They show a unique capability to resolubilize and reactivate aggregated proteins, rather than the regular chaperone function of preventing protein aggregation and misfolding. The expression of these proteins are regulated by heat stress (HS) and developmental cues.

High-temperature stress results in various biological responses in rice plants. It is important to uncover the heat response mechanism in order to identify the underlying genes and then, breed truly heat tolerant varieties. Towards this goal, preliminary evaluation of three important heat responsive genes that comprises of HsfA4d, HsfB2a, and HSP100 was carried out using two rice varieties differing in heat tolerance viz., Nagina 22 (N22) (tolerant) (Bahuguna et al., 2017; Jagadish et al., 2010) and Co51. The findings of the study are reported herewith.

MATERIALS AND METHODS

The research was conducted during 2019 - 2020 at Tamil Nadu Agricultural University, Coimbatore. Co 51 (assumed to be thermo-sensitive) (Robin *et al.* 2019), and N22 (Nagina 22) (Waghmare *et al.* 2018), a thermo-tolerant variety, both belonging to *Oryza sativa* ssp. indica were used in this study. Pre-germinated seeds from both the varieties were sown in pro-trays and were transplanted to individual pots, 20 days after sowing (DAS). The plants representing both the varieties were raised in pairs (one for use as a control and the other for high-temperature stress experiment) in the greenhouse. 30 DAS, they were moved to plant growth chambers (Manufactured by M/s. Genesis Technologies, Thane, Maharashtra, India) wherein they were maintained at a temperature of 25 °C and 19 °C during the day (16h) and night (8h), respectively. Light intensity was maintained at approximately 35 Klux using white, fluorescent lamps, while relative humidity was maintained ~80 %. High-temperature stress treatment was carried out during the 50th, 60th, and 70th DAS.

During experimentation, the temperature of the first chamber (control chamber) was maintained at 25 °C, whereas, in the second chamber (heat stress chamber), the temperature was gradually raised from 25 °C at 6:00 a.m., to 37 °C at 12:00 noon (~2 °C rise for every 1 h) and back to 25 °C by 06:00 p.m. Immediately after heat stress, leaf tissues were collected from both the untreated (25 °C) and treated (37 °C) plants. The leaves were immediately flash-frozen in liquid nitrogen before being stored at -80 °C. The plants were always retained in their original chambers until the completion of the heat stress experiment.

Samples were obtained from only one tissue (Leaf) that were collected on different time periods (50, 60, and 70 DAS) and two different experimental conditions [Control (25 °C) and Treatment (37 °C)]. Leaf samples

obtained were used for RNA isolation and cDNA synthesis, in quick succession. Total RNA was extracted using the Nucleo-pore™RNASure Plant Kit (Genetix Biotech Asia Pvt. Ltd., Delhi, India) (Cat. #NP-84905) according to the manufacturer's instructions along with an on-column RNase free DNase I treatment (Genetix Biotech Asia Pvt. Ltd., Delhi, India) for removing DNA contamination. RNA concentration was quantified using a Nanodrop spectrophotometer. The integrity of RNA was assessed by 1% (w/v) agarose gel electrophoresis and ethidium bromide staining. 1 µg of total RNA was used to synthesize cDNA immediately using the iScript™ cDNA synthesis kit (cat #1708891) of Bio-Rad laboratories. The cDNA samples were subjected to qRT-PCR experiment.

Generally, in qRT-PCR, transcripts of stably expressed genes (reference genes) are employed for data normalization. Using the GeNorm algorithm, six reference genes, *viz.*, *Actin*, *Ubiquitin5, eEF1α, GAPDH,18S rRNA,* and *25SrRNA* were used to find the most stable candidate reference gene for data normalization. In GeNorm analysis, the most stable gene in a pool of candidate reference genes is the one with the lowest M value(Vandesompele *et al.,* 2002). With an assumption that the chosen genes are not co-regulated, a step-wise elimination of gene with the highest gene stability measure value M (0.5 or 1.5), the pair of genes *eEF1α* and *Ubiquitin5* were identified as promising 'stable candidate reference genes' for further downstream differential gene analysis experiments (data not shown). Hence, *Ubiquitin* was used as the reference gene for relative quantification of target genes namely *HsfA4d*, *HsfB2a*, and *Hsp100*. The NCBI GenBank reference sequence identity of the target and internal control genes from *Oryza sativa* spp. japonica is shown in **Table 1**.

Primers were designed using Primer 3 plus software (http://www-genome.wi.mit.edu). The design of primers followed a set of stringent criteria, as generally suggested in qRT-PCR protocols. Full length conserved domain sequence (CDS) for the selected genes was obtained from the GenBank of NCBI database. The following parameters were taken into consideration: optimal length between 20-22 bases; T_m between 55-60°C; length of amplification product around 200bp. The specificity and further validation of each primer were confirmed by comparing its sequence with all predicted rice coding sequences using the NCBI Primer BLASTN tool to ensure that at least one primer of each pair targets a unique site within the set of predicted rice CDS.

For each candidate gene primer pair, two technical replicate PCR reactions were conducted in a CFX Connect TM Real-Time PCR detection system (Bio-Rad Laboratories, Inc.). In each well of optical reaction plates (Bio-Rad Laboratories, Inc.), a PCR mix containing 1 µl of cDNA (100 ng/µl), 0.5 µl of 10 mM of each gene-specific primer, and 5 µl of SSO Advanced TM Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.) was taken with the final volume adjusted to 10 µl with DNase / RNase free water. The following thermal profile was used: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 45 seconds at recommended annealing temperature (T_a) **(Table 1)**, and extension for 30 seconds at 72°C. The melting curve profile is set from 65°C to 95°C with a ramp speed of 0.5°C min-1 for each fluorescence measurement. The samples were then brought down to 4°C for final storage. The baseline correction and Quantification cycle (Cq) was auto calculated by the CFX Connect TM Real-Time PCR detection software (Bio-Rad Laboratories, Inc.).

Table 1. Primers for qRT-PCR amplification

Gene	Gene ID (NCBI Accession Number)	Primer Sequence (5'-3')	Orientation	Product Length (bp)	T_a
UBI-5	LOC4332169	CCAGGACAAGATGATCTGCC	Forward	245	58
		AAGAAGCTGAAGCATCCAGC	Reverse		
HSP100	LOC4329520	CTCAATGATCCTGACAGACC	Forward	155	58
		CTCAATGATCCTGACAGACC	Reverse		
HsfA4d	LOC4339409	CCAGATAAACGGACCACTC	Forward	133	55.3
		CCAGTTGATCACGTACTGC	Reverse		
HsfB2a	LOC4336701	GTACCAAGCAAACTGCAGCC	Forward	145	57.4
		TCTGAGAGCTACAACAAACCACT	Reverse		

Comparative ΔCq method (Schmittgen and Livak 2008; Silver *et al.,* 2006) was used to evaluate the raw Cq values using ubiquitin as an internal control. Cq values for both, target and internal reference gene, were used for quantification of transcripts. The mRNA expression of target genes was quantified using the formula $2^{-\triangle\triangle Cq}$, where △△Cq is equal to $\triangle Cq_{treatment} - \triangle Cq_{control}$(Livak and Schmittgen 2001) and △△Cq values reflect the relative expression of the target genes. The data were graphically represented as fold changes normalized to internal control (**Fig. 1,2 and 3**).

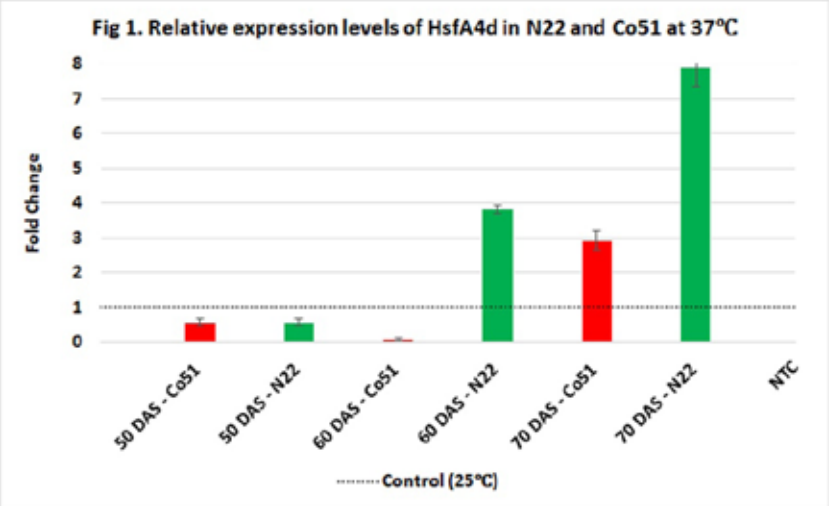
Fig 1. Relative expression levels of HsfA4d in N22 and Co51 at 37°C
Fold Change
8
7
6
5
4
3
2
1
0
50 DAS - Co51
50 DAS - N22
60 DAS - Co51
60 DAS - N22
70 DAS - Co51
70 DAS - N22
NTC
Control (25°C)

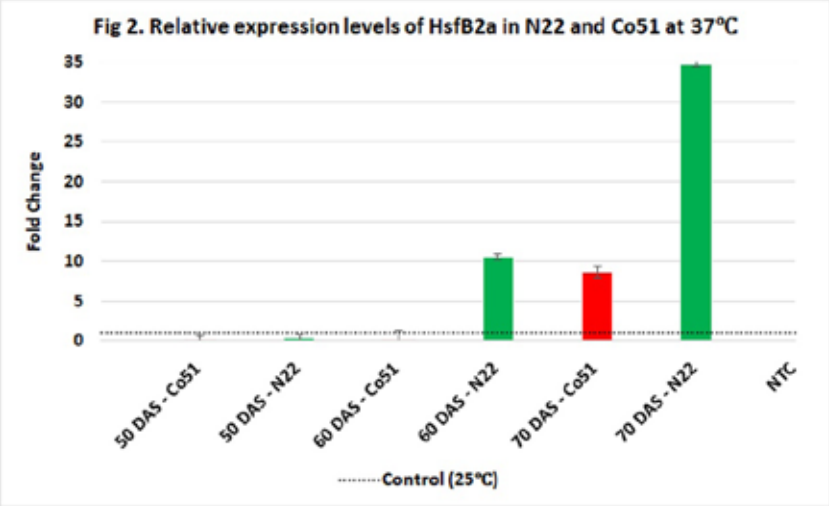
Fig 2. Relative expression levels of HsfB2a in N22 and Co51 at 37°C
Fold Change
35
30
25
20
15
10
5
0
50 DAS - Co51
50 DAS - N22
60 DAS - Co51
60 DAS - N22
70 DAS - Co51
70 DAS - N22
NTC
Control (25°C)

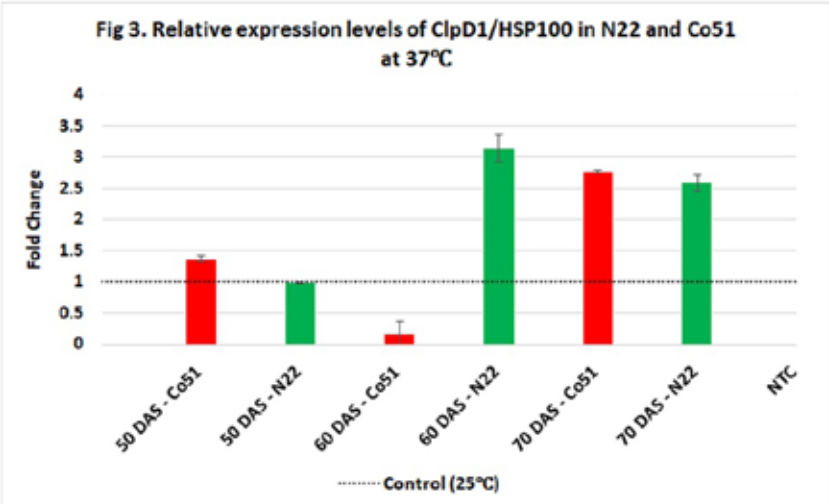
Fig 3. Relative expression levels of ClpD1/HSP100 in N22 and Co51 at 37°C
Fold Change
4
3.5
3
2.5
2
1.5
1
0.5
0
50 DAS - Co51
50 DAS - N22
60 DAS - Co51
60 DAS - N22
70 DAS - Co51
70 DAS - N22
NTC
Control (25°C)

RESULTS AND DISCUSSION

qRT-PCR analysis was used to investigate the expression levels of three genes in response to heat stress treatment during 50th, 60th and 70th DAS in rice (vegetative phase and pre-reproductive phase). Among the genes induced by heat stress, there is an extensive overlapping response of HSPs- and HSFs- genes to heat and other abiotic stresses(Ohama *et al.* 2017), which indicates that they are important elements in the crosstalk of different response pathways.

Relative expression levels of three genes *viz., HsfA4d, HsfB2a*, and *HSP100* were analysed in control and heat-stressed plants of the rice varieties Co51 and N22 based on the comparative △Cq method. Gene expression levels were quantified relatively by analysing normalized Cq values of the samples collected from 50th, 60th, and 70th DAS. Comparison of the temporal expression levels of *HsfA4d* in the rice variety Co51 revealed that the *HsfA4d* are highly induced only around 70th DAS, whereas levels of expression were observed to be down-regulated more around 60th DAS than 50th DAS. In N22, relative expression level of HsfA*4d* was found to be approximately 3 folds higher around 70 DAS when compared with Co51. Also, *HsfA4d* was observed to be down-regulated around 50 DAS whereas, 4-folds up-regulation was observed around 60 DAS.

The expression pattern of *HsfB2a* followed an identical pattern as that of *HsfA4d* in both the rice varieties, N22 and Co 51. But, the expression level of *HsfB2a* was found to be 4-folds higher than that of *HsfA4d* around 70 DAS. Among the two transcription factor genes, *HsfB2a* appears to be the major gene, regulating heat shock response in both Co 51 and N22 rice varieties. This is because, *HsfB2a* is directly under the control of the master regulator *HsfA1s*(Ohama *et al.,* 2017). Also, it is observed that the TF genes are down-regulated when the plants are exposed to heat stress during their first encounter, because they are already under the negative regulation of HSP70, HSP90 and HsfBs(Ohama *et al.* 2017). But their levels gradually enhance in subsequent encounters because of the activation of master regulator *HsfA1s*. This could also be due to the fact that the heat shock memory acquired in the first encounter as a result of epigenetic regulations or changes in small RNA species and transposon activities (Ding *et al.,* 2012; Zhang *et al.,* 2009) lasts for a longer timer period resulting in enhanced levels of protective heat shock factors / heat shock proteins. Hence, during the subsequent heat stress encounter, induction of the heat shock fact ors leads to their enhanced level of expression.

HSP100 expression level also follows a similar trend as that of the transcription factors *viz., HsfA4d* and *HsfB2a* in the variety Co51. In N22, expression of *HSP100* exhibits no change around 50 DAS, whereas it is increased by 3 folds around 60 DAS. In Co51, its expression is highly reduced from 50th to 60th DAS, and around 70th DAS, they show an enhanced expression. A possible explanation for the differences observed during the period 60 DAS in both N22 and Co 51 is that the variety N22 has a good heat shock memory than that of Co 51.

Higher levels of expression of *HsfA4d* and *HsfB2a* around 70 DAS could possibly imply that these genes might be active towards the reproductive phase in all the rice varieties. This could be due to the acquired heat stress memory. Also, they might upregulate the expression of downstream heat shock proteins and impart heat tolerance in both susceptible and tolerant rice varieties since reproductive phase is a highly sensitive but an important phase essential for the continuity of life. Higher expression level of *HsfA4d* and *HsfB2a* around 50th and 60th DAS in N22 indicates that the heat shock proteins are constantly being synthesized because of the acquired heat shock memory and are involved in imparting heat tolerance to N22 during its ontogeny. This could be the reason why N22 is regarded as one among the popular heat-tolerant varieties (Bahuguna *et al.,* 2017; Jagadish *et al.,* 2010).

In this study, we attempted to verify the levels of gene expression of *HsfA4d, HsfB2a* and *HSP100/ClpD* upon heat stress in the rice varieties N22 and Co 51. Out of two rice genotypes, N22 (heat tolerant) showed a higher gene expression levels for the HSPs and HSF genes that contributed to its greater heat tolerance. Based on the differential gene expression levels observed, we hypothesize that N22 acquires its heat tolerance because of the retention of greater heat stress memory for a longer time than that of Co 51. Hence, we conclude that the heat susceptible lines have poor retention of heat stress memory than heat tolerant lines which requires further investigation.

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