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MITIGATING HEAT INDUCED DAMAGES IN RICE (*ORYZA SATIVA* L.) BY HSP70 MODULATION

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Abstract

Stress induced cell damages leading necrosis is considered as major challenges in sustainable agriculture production in times of climate change and can be prevented by various factors particularly remediate effect of HSP70. Rice (*Oryza sativa* L.) thermo-tolerance at molecular level are still enigmatic hence expression pattern of heat shock protein (HSP70) was determined in eight rice cultivars subjected to heat stress (42±2ºC) followed by recovery (28±2ºC) treatments at early growth stage. Growth characteristics along with free radicals (H_2O_2) accumulation and level of lipid peroxidation (MDA) were determined as stress damage indicators while expression pattern of HSP70 protein was used as stress tolerance scoring. Results revealed that expression level of HSP70 was increased with duration of thermal stress and declined upon recovery. Among all the cultivars relatively "Sada" Hayat had maximum malondialdehyde (MDA) and H_2O_2 accumulation and late induction and fast decay of HSP70 showed susceptibility towards heat stress while such a damage was the least observed in K-95, having consistent and strong expression of HSP70 during heat stress and recovery treatments. This study concludes that prompt induction in expression of HSP70 during heat stress condition and slow decay during recovery for long-term memory is desirable trait for acquisition of thermo-tolerance in rice.

Key words: Oxidative stress, Reactive oxygen species, HSP70, Thermotolerance.

Introduction

Plant cells and tissues get high risk for injury whenever they face unfavorable environmental conditions, which culminate in stunted growth and yield loss due to alteration in metabolic functions (Wahid *et al.,* 2007). Heat stress among other abiotic stresses, showed multifarious consequences on growth and development of plants. All parts of the plants are affected by heat stress however, variable response is documented during phonological stages development (Halford, 2009). However, overproduction of reactive oxygen species (ROS) causing deterioration of membrane lipids (peroxidation) is a major and common heat stress response observed at all growth stages of plants including rice. In addition, misfolding and accumulation of misfolded proteins also found in cells and tissues subjected to thermal stress (Sarkar *et al.,* 2013).

Plants have developed numerous adaptive mechanisms to survive under unfavorable conditions. Under different stress conditions, plants express different types of stress proteins to assist native proteins and protect cellular metabolism (Allagulova *et al.,* 2003; Ingram *et al.,* 1996; Wahid *et al.,* 2007). In well-known stress responsive proteins, heat shock proteins (HSPs) are well known to mostly express under heat stress condition (Kang *et al.,* 2005). Over-expression of HSPs is an important adaptive mechanism manifested by plants to cope with unfavorable conditions, which enhances plant stress tolerance through hydration of cellular organelles and components due to their hydrophilic properties (Wahid & Close, 2007).

Various functional molecular biology approach for abiotic stress tolerance studies manifested that crop adaptation with changing environmental condition is highly correlated with expression level of heat shock protein HSP70 (Abreu *et al.,* 2013). Similarly, acclimation of many plant tissues and cells subjected to thermal stress manifested induction and accumulation of HSP70 (Gurley, 2000). It is because, many cellular activities including expression, post-translation modification, folding and targeting of newly expressed proteins and refolding of damaged of misfolded proteins, redox homeostasis, energy, biosynthesis and carbohydrate metabolisms are assisted by heat responsive polypeptide (Gorantla *et al.,* 2007; Zhang *et al.,* 2010). Therefore, thermotolerance in many cereal crops including *Oryza sativa* is a multifarious process, in which expression and control of many genes and proteins are involved, which are associated to key cellular activities (Zhang *et al.,* 2010). In this manner identification and subsequent characterization of various genes and proteins potentially linked to abiotic stress tolerance, is an important and practical step. It helps in elucidation of thermo-tolerance mechanism at molecular level followed by engineering rice plant having enhanced tolerance to a heat stress (Zou *et al.,* 2011). To cope with this issue unfortunately rice response to heat stress at molecular level is still enigmatic, despite its consideration as a model crop (Shah *et al.,* 2011; Kang *et al.,* 2010). Plant cell survival under unfavorable environmental condition require maintenance of proteins in their functional conformation and prevention from damaging during abiotic stresses. Under such circumstances heat-shock proteins (Hsps)/ chaperones stabilize membranes and proteins to help in refolding of misfolded proteins because HSPs are involved in protein folding, assembly and translocation (Lin *et al.,* 2014; Gorantla *et al.,* 2007). In this paper, we presented induction and accumulation of HSP70 in rice seedlings subjected to heat stress and discussed its significance in recovery potential of heat stressed rice.

In re-establishing cellular homeostasis and protein conformation HSPs may play important role in plant survival under stress conditions. Therefore, this study was designed to investigate the effect of heat stress on protein expression pattern especially identification of HSP70 and its role in thermo-tolerance at early growth stage of rice.

Materials and Methods

Growth and stress conditions: Eight rice cultivars were used in this study and allowed to grow under natural condition as well as in controlled growth chambers. From both growth conditions, 20 days old seedlings were subjected to heat stress by gradually increasing the temperature at 42±2°C and 35±2°C during day (16 h) and night (8 h), respectively. Relative humidity of the stress room and controlled growth chamber was adjusted approximately around 50%. After 24, 48 and 72 h of heat treatment leaf samples were collected at each time and similar time interval (24, 48 and 72 h) and sample collection was also done during recovery treatment (28±2°C). Before collection of leaf tissue and their subsequent storage (-80°C) for proteomic analysis, morphological and physiological attributes were recorded.

MDA and H_2O_2 quantification: Production and accumulation of hydrogen peroxide (H_2O_2) under normal and stressed conditions content was determined as described by Jessup *et al.,* (1994). For this purpose, 0.5 g leaf was homogenized with TCA (0.1%) followed by centrifugation for at 12,000 rpm for 15 min. Supernatant mixed with potassium iodide (KI) in potassium phosphate buffer (pH 7.0) and absorbance was observed at 390 nm.

Lipid peroxidation extent was determined by assaying melondialdehyde (MDA) production according to Heath & Packer, (1968). Tetracholoroacetic acid (TCA, 5%) was used to homogenize leaf tissue, followed by centrifugation at 12,000 rpm and 4°C for 15 minutes. Pellet was discarded, supernatant was added with thiobarbituric acid (TBA, 0.5%) and allowed to boil at 95°C for 30 minutes. The mixture then centrifuged at 7,500 rpm? after cooling and absorbance was taken at 532 nm and at later at 600 nm.

HSP70 expression analysis: Total soluble protein was extracted from leaf samples by pulverizing it with QB extraction buffer (pH 7.2). The pulverized tissue was centrifuged in a refrigerated benchtop micro-centrifuge at 13,000 rpm for 15 minutes. Using supernatant concentration of total soluble protein was quantified through Bradford, (1976) assay.

Initially total soluble proteins were subjected to proliferate on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmeli, 1970). Two electrophoresis were performed consecutively. One of the SDS-PAGE gel was used for transferring peptide bands to a membrane (nitrocellulose, 0.45 µm) while second gel was subjected to staining purpose using Coomassie Brilliant Blue (CBB) dye. About 100 µg of total soluble proteins from each sample were used for protein blotting. For western blotting, Tris-HCl (0.025 mmol/L) and glycine (125 mmol. L^{-1}), were used as transfer buffer (pH 8.3) at 4°C and 100 V current was supplied continuously for an hour.

Expression level of heat induced poly peptide (~70 kDa) were detected in stressed samples based on immunological reaction. For this antigen-antibody detection system, blotted membrane was incubated overnight in mouse monoclonal antibody (1º Ab) to HSP70 (Abcam, ab69561) using Tris-buffered saline (TBS, 10%). After incubation with monoclonal antibody membrane was washed with TBS buffer (1X) three times. This membrane was then allowed to incubate approximately 2 h with anti-rabbit goat secondary antibody (2º Ab, IgG) linked with alkaline phosphatase (AP) enzyme. Alkaline phosphatase (ALPH) buffer having NBT and BCIP as substrate was used according to Towbin *et al.,* (1979).

Statistical analysis

For statistical analysis LSD was applied to test the normality of data and for correlation Pearson's method was applied. Using SPSS17 software, ANOVA test was carried out to test the significance of physiological and biochemical attributes (Tables 2 and 3).

Results

The effect of short episodes of heat waves on plant cell survival fate governed under differential expression pattern of heat shock protein (HSP70), monitoring oxidative burst (H_2O_2) and deteriorated membrane lipids (MDA) was investigated. This study was accomplished at early growth stage of eight local rice cultivars. Data were carefully recorded, and findings are summarized in following paragraph, after statistical analysis.

Twenty days old seedlings of used rice varieties had average size except K-95, which showed slightly talle height, however significant heterosis exhibited by these cultivars, holding minus end by Sada Hayat and plus end by K-95. Rest of the cultivars fell in between. Total soluble protein content was slightly decreased in stressed plants and increased upon recovery. Over-production of H_2O_2 and MDA was also stress dependent and these two attributes were found cultivar-specific. Among the cultivars, K-95 had minimum H_2O_2 and MDA, which showed less protein degradation, whilst Sada Hayat behaved inverse (Table 1). There was a strong positive correlation between MDA and H_2O_2, but protein showed negative correlation with these attributes (Table 1).

Protein profiling revealed that there was differential expression of protein in heat stressed and non-stressed rice. Near 70 kDa marker protein, a newly induced protein band was observed in heat treated samples while this was absent in unstressed rice leaves (Figs. 1 & 2). The band intensity of this newly heat induced protein showed increment with the increment in duration of thermal stress. During recovery, band thickness was reduced and gradually disappeared after 72 h (R72). This new protein band was present in all varieties but with difference in band thickness and promptness was obviously due to difference in protein expression level and proper signal transduction, respectively. Some new protein bands were also observed near 90 kDa protein standard, in some cultivars only under stress condition, while they were missing in control and recovery treatments. Total protein fraction from the leaf samples of eight rice cultivars at seedling stage indicated quite a few numbers of heat responsive bands under heat stress condition as well as during recovery, which were not discernible in control samples (Figs. 1 & 2).

Table 1. Quantitative analysis of hydrogen peroxide (H2O2), melondialdehyde (MDA) and total protein of eight rice cultivars subjected to control (C), heat stress for 24h (T24), 48h (T48) and 72h (T72) followed by recovery treatment for 24h (R24), 48h (R48) and 72h (R72), at seedling stage (Mean ± S.E).

Attributes	Rice Cultivar	Control	Heat Shock			Recovery		
		C	T24	T48	T72	R24	R48	R72
Hydrogen peroxide (µmole/gFW)	IRI-6	3.45±0.21	10.98±0.87	26.38±3.20	34.90±0.54	31.45±1.21	23.31±0.22	18.23±0.31
	IRI-8	3.25±1.21	18.04±3.21	31.52±0.21	40.47±0.22	35.70±1.21	24.81±0.23	20.24±0.33
	DR-82	2.99±1.42	16.20±3.21	32.51±1.12	42.21±0.32	37.26±0.21	32.05±0.22	27.58±0.87
	DR-83	3.78±0.34	18.04±1.23	34.44±2.12	48.02±0.23	39.36±2.22	32.31±1.22	27.48±0.89
	DR-92	4.14±4.21	16.69±2.13	28.02±0.11	46.12±0.76	31.48±1.32	30.25±1.43	24.21±0.78
	K.95	2.88±2.12	15.75±2.13	19.54±0.15	33.58±0.33	24.23±0.54	17.23±1.22	12.42±0.86
	SDHT	4.06±2.12	19.39±3.21	37.21±0.21	50.21±0.85	35.46±0.33	30.75±0.26	28.01±0.12
	SHKR	3.68±2.31	14.10±3.21	25.20±3.12	31.15±0.54	25.83±0.31	19.23±1.24	14.23±0.65
Melondialdehyde (nmole/gFW)	IR-6	0.219±0.9	0.435±2.2	0.674±2.4	0.834±0.64	0.672±1.21	0.571±2.22	0.435±0.34
	IR-8	0.187±0.2	0.546±0.2	0.782±3.3	0.968±0.55	0.789±0.32	0.672±2.13	0.532±0.32
	DR-82	0.193±0.4	0.543±0.7	0.792±2.2	1.023±0.32	0.978±1.21	0.723±3.21	0.534±2.13
	DR-83	0.180±0.7	0.459±1.0	0.756±3.1	1.123±0.42	0.934±2.21	0.765±0.23	0.541±0.97
	DR-92	0.190±1.2	0.543±2.1	0.834±3.2	1.023±0.31	0.942±3.21	0.782±3.21	0.654±0.45
	K.95	0.160±3.2	0.367±3.2	0.532±2.3	0.683±0.41	0.567±0.23	0.456±3.22	0.345±0.31
	SDHT	0.191±0.2	0.491±2.1	0.872±2.1	1.232±0.22	0.989±1.78	0.823±0.21	0.678±2.13
	SHKR	0.193±1.2	0.467±0.8	0.671±0.2	0.824±0.76	0.678±2.12	0.547±0.23	0.435±3.23
Total protein (mg/gFW)	IRI-6	2.74±0.33	3.02±2.22	2.34±2.21	2.510±2.12	2.20±0.04	2.43±2.21	2.53±2.12
	IRI-8	2.60±0.97	2.95±2.32	2.50±2.76	2.44±1.22	2.35±2.26	2.33±0.23	2.43±0.02
	DR-82	2.21±1.21	2.93±2.11	2.65±0.56	2.47±0.11	2.25±3.01	2.65±1.12	2.29±3.23
	DR-83	2.64±0.66	2.62±0.88	2.81±1.23	2.14±0.03	1.94±0.98	2.23±0.81	2.34±0.21
	DR-92	2.59±0.43	2.72±0.22	2.44±3.23	2.36±2.67	2.70±0.21	2.57±0.22	2.37±0.33
	K.95	2.84±0.33	2.89±0.21	2.93±0.92	2.73±1.22	2.91±3.21	2.96±1.22	3.01±2.01
	SDHT	2.41±0.22	2.68±0.23	2.40±1.11	2.13±1.21	2.26±2.22	2.26±0.52	2.21±3.01
	SHKR	2.39±2.21	2.63±0.54	2.84±3.01	2.59±3.22	2.63±0.04	2.48±2.12	2.32±2.11

Table 2. ANOVA table of H_2O_2, MDA and total protein (TP) of rice, subjected to different temperature treatments and effect of Cultivars (C) and Treatments (T) on these attributes.

Factors	H_2O_2	MDA	TP
Cultivars (C)	0.000	0.000	0.002
Treatments (T)	0.000	0.000	0.421
Cultivars x Treatments (C x T)	0.002	0.001	0.213

Significance was considered at $p \leq 0.05$

Table 3. Correlation analysis using SPSS v. 17 among hydrogen peroxide (H_2O_2), melondialdehyde (MDA) and total protein of eight rice cultivars subjected to control (C), heat stress for 24h (T24), 48h (T48) and 72h (T72) followed by recovery treatment for 24h (R24), 48h (R48) and 72h (R72), at seedling stage.

	Correlations	H_2O_2	MDA	Protein
H_2O_2	Pearson correlation	1	0.969**	-0.443**
	Sig. (2-tailed)		0.000	0.001
	N	56		56
MDA	Pearson correlation	0.969**	1	-0.420**
	Sig. (2-tailed)	0.000		0.001
	N	56	56	56
Protein	Pearson correlation	-0.443**	-0.420**	1
	Sig. (2-tailed)	0.001	0.001	
	N	56	56	56

**. Correlation is significant at the 0.01 level (2-tailed)

However, the immunoblotting of these proteins revealed the differential expression of HSPs in all rice accessions at 24, 48 and 72 h time points under thermal stress. Western blotting analysis revealed that rice seedlings indicated the expression of polypeptides with an apparent molecular mass of 70 kDa during heat stress (Fig. 3). This heat induced polypeptide showed increment in band intensity with increase in the duration of applied heat stress, which was reduced during recovery periods. As the recovery time extended, the expression of HSP70 diminished in many varieties.

Maximum band thickness of HSP70 was observed in K-95 followed by IR-6 while minimum was evident in Sada Hayat, as compared to other cultivars. Band intensity of HSP70 was increased after 24 h of heat stress but some cultivars like DR-83 showed no differential expression even after 48 h of thermal stress. Maximum expression of HSP70 was evident after 72 h of heat treatment in all cultivars. During recovery, the level of HSP70 expression gradually declined and finally disappeared after 72 h of recovery (R72). This pattern of protein expression and accumulation was similar in all accessions, but thickness of protein band was significantly different (Fig. 3). Level of HSP70 expression presented in terms of percentage (%), comparing the cultivars under three different growth conditions. Rice cultivars manifested three distinct response for this attribute. The induction of HSP70 expression was rapid (K-95), Mild (IR-6) and late (Sada Hayat). Similarly, decline of HSP70 during recovery condition also had rapid (Sada Hayat) and late (K-95). These results suggest cultivarial variation of rice in expression of stress proteins to cope with adverse environmental conditions.

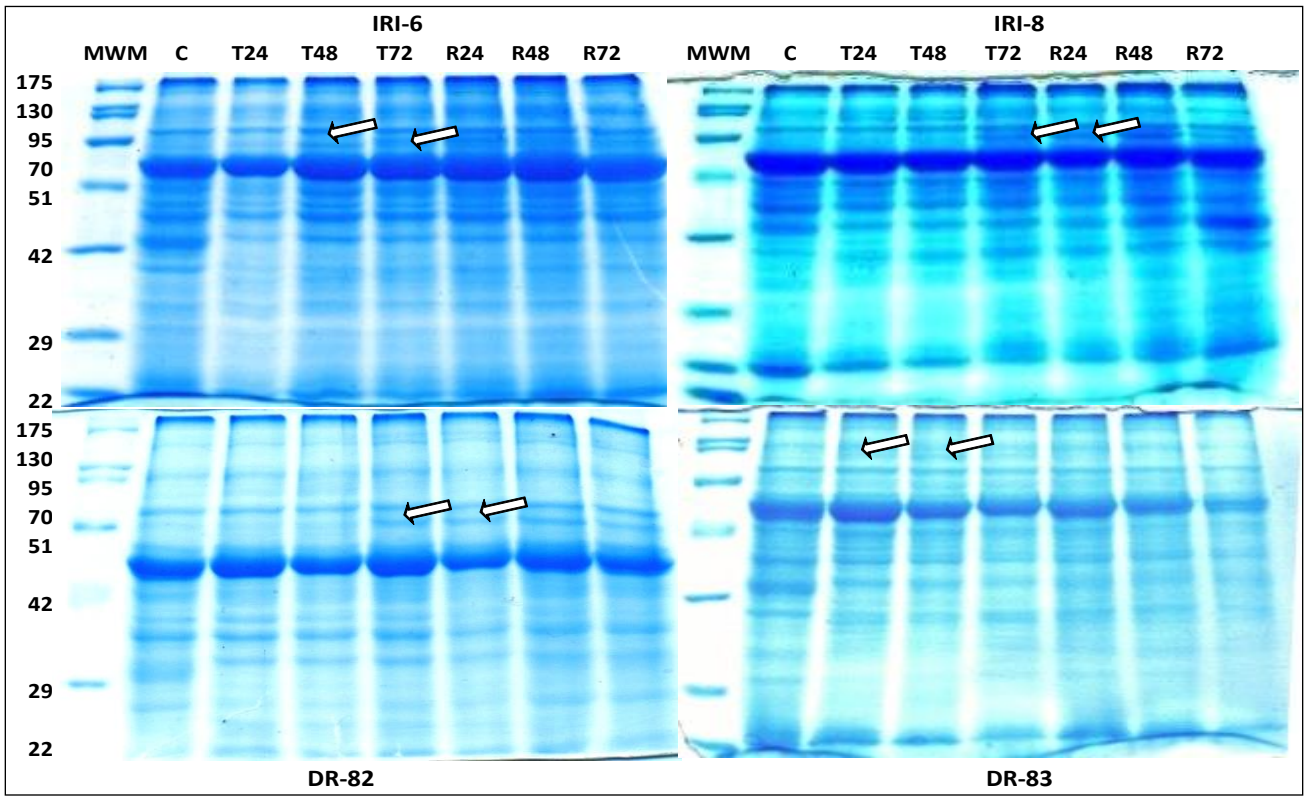

Fig. 1. SDS-PAGE profiling of leaf proteins of rice cultivars IR-6, IR-8, DR-82 and DR-83 at control (C), heat shock for 24h (T24), 48h (T48) and 72h (T72) and recovery treatments for 24h (R24), 48h (R48) and 72h (R72). Standard molecular weight markers were used to determine relative size of leaf proteins. Differential expression of proteins are indicated by arrow. In each well 50-60 µg protein were loaded, and the experiment were performed for 3 times.

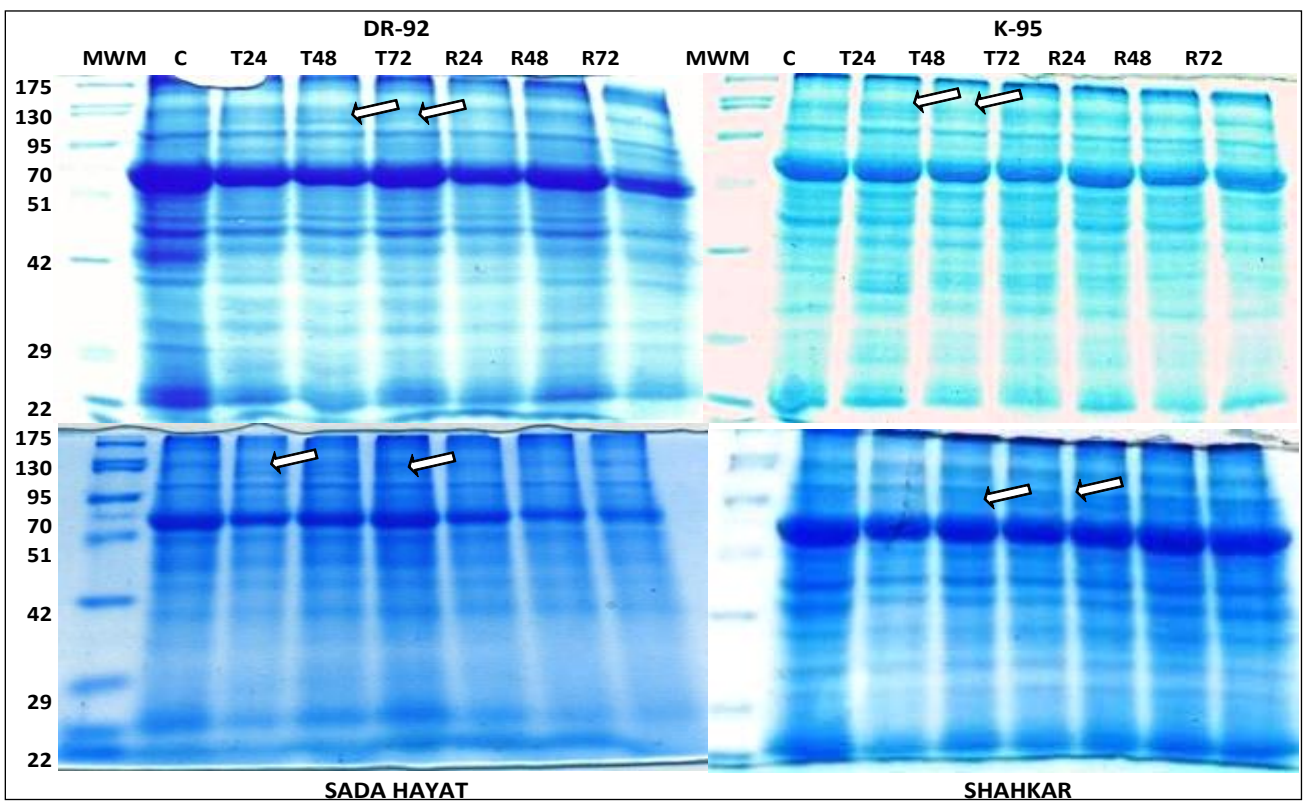

Fig. 2. SDS-PAGE profiling of leaf proteins of 20 days old seedlings of rice cultivars DR-92, K-95, Sada Hayat and Shahkar at control (C), heat shock for 24h (T24), 48h (T48) and 72h (T72) and recovery treatments for 24h (R24), 48h (R48) and 72h (R72). Standard molecular weight markers were used to determine relative size of leaf proteins. Differential expression of proteins are indicated by arrow. In each well 50-60 µg protein were loaded and the experiment were performed for 3 times.

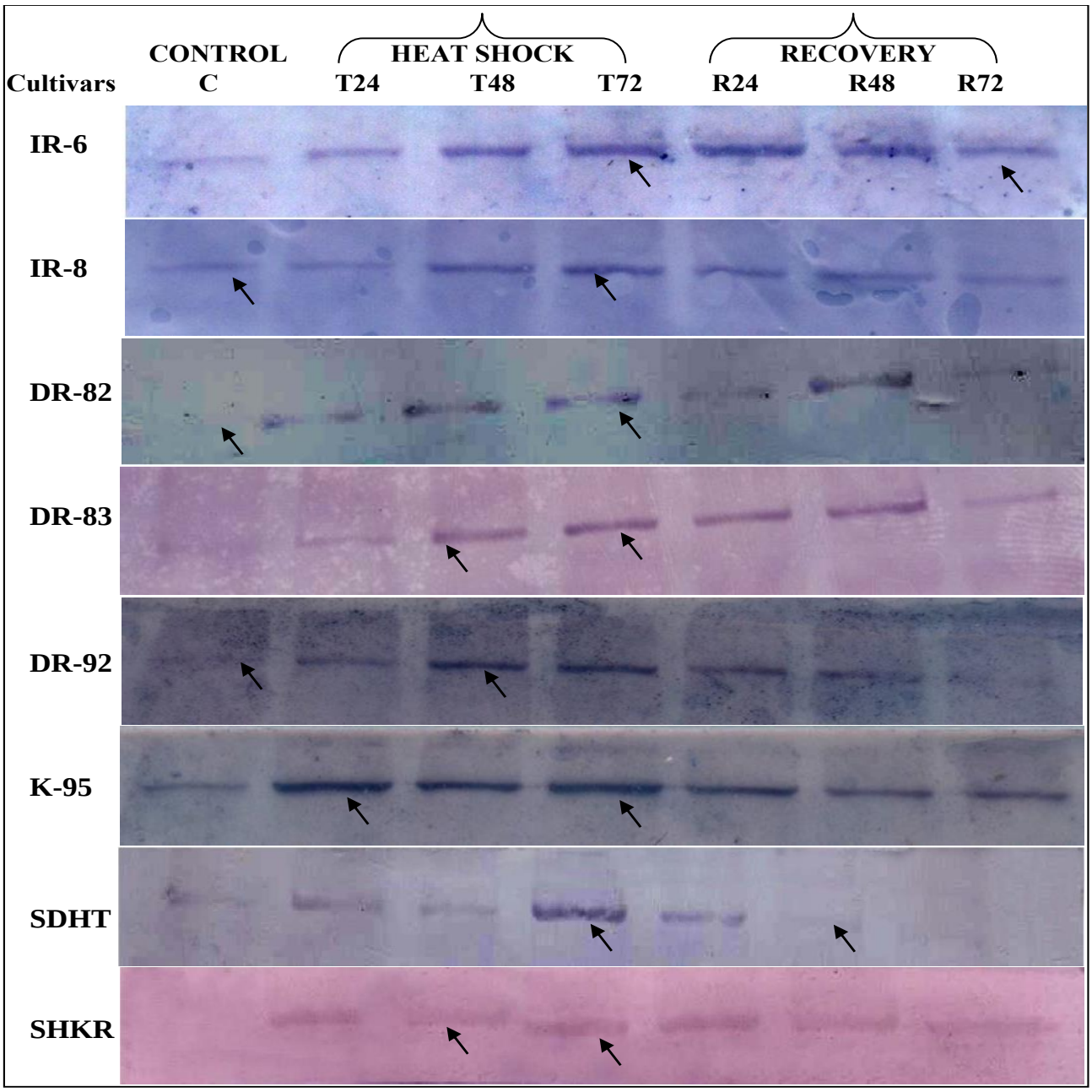

Fig. 3. Immuno-detection of HSP70 expression pattern in leaf tissue of 20 days old seedlings of rice cultivars IR-6, IR-8, DR-82, DR-83, DR-92, K-95, Sada Hayat and Shahkar, subjected to control (C), heat shock (42±1ºC) for 24h (T24), 48h (T48) and 72h (T72) followed by recovery treatments (28±1ºC) for again 24h (R24), 48h (R48) and 72h (R72). For this 50-60µg protein electrophoresed through SDS-PAGE and polypeptides were transferred to nitrocellulose membrane followed by antigen-antibody reaction and enzyme based detection. For detection NBT/BCIP was used in ALPH buffer. This experiment was performed atleast two times.

Table 4. Comparative analysis of expression pattern of HSP70 in terms of percentage (%), identified through immunological detection system, in eight rice cultivars, grown under control (C), heat shocked (T24, T48 and T72) and recovery (R24, R48 and R72) conditions.

Cultivars	Control	Heat shock			Recovery		
	C	T24	T48	T72	R24	R48	R72
IR-6	10	20	40	50	50	40	30
IR-8	10	10	20	30	20	20	10
DR-82	0	10	20	20	10	20	10
DR-83	5	10	20	30	20	20	10
DR-92	5	10	20	20	10	10	5
K-95	10	60	50	60	40	40	30
SDHT	2	10	5	40	20	5	0
SHKR	0	10	10	20	20	20	20

Discussion

In this study, induction and regulation of a heat sensitive polypeptides were elucidated in heat stressed rice leaf samples at early growth stage and subjected to western blotting for identification of HSP70, a molecular chaperone. In addition, it was correlated with cell homeostasis by monitoring oxidative stress damages in terms of cell membrane thermo-stability and lipid peroxidation. Excessive hydrogen peroxide and higher MDA level, manifested that heat stress application generated oxidative stress and membrane lipids were deteriorated, hence considered as stress damage indicators. While induction of HSP70 expression under

heat stress condition shows that cultivars have successfully generated signal transduction for stress tolerance mechanism because expression of HSPs generally increases under high temperature stress and subsequently organisms become able to tolerate lethal heat waves (Chang *et al*., 2007).

The pattern of HSP70 expression was found stress and cultivar dependant. Some cultivars, like Sada Hayat and DR-83 manifested delayed and non-specific response towards expression of HSP70 (Fig. 3) hence had maximum MDA and H_2O_2 as compared to K-95 which showed early and consistent expression of HSP70 (Fig. 3) during stress and this ability appeared to help this cultivar to tolerate high temperature, as evident from least oxidative stress and membrane damages in terms of H_2O_2 and MDA contents. This well controlled cell homeostasis might be due to proper folding of key cellular proteins and enzymes assisted by heat shock proteins (HSP70) for their targeted action which enable plant cells and tissues to cope with heat induced damages (Sarkar *et al.,* 2013) because plants are subjected to thermal stress and survive, intimately linked expression and accumulation level of molecular chaperone HSP70 and find that they have ability to reassemble misfolded ribosomal proteins and prevent deterioration of protein translation factory (Daugaard *et al.,* 2007). It is because expression of heat shock proteins (HSPs)/chaperones are involved in protein folding, assembly, translocation and degradation in many normal cellular processes, stabilizes proteins and membranes and can assist in refolding of miss-folded proteins under stress conditions and thus important to protect plants from necrosis (Sarkar *et al.,* 2013; Chang *et al.,* 2007). Some other studies also supported that HSPs plays essential role in deaggregation of damaged proteins and assist in reforming of non-native proteins structure under harsh environmental conditions as well as under normal condition (Hartl, 1996). It means that in susceptible cultivars viz., Sada Hayat and DR-83, accumulation of damaged and misfolded proteins was increased and similarly, protein translation and their proper folding was retarded due to unavailability of molecular chaperone for remedy because newly protein import and translocation processes are also linked with chaperonin activity of HSP70 (Frank *et al.,* 2009; Frydman, 2001; Bita *et al.,* 2011). In heat tolerant genotypes of grapes, favorable internal environment of plant tissues and better thermo-stability of biological membranes, under elevated temperature was strongly correlated with maximum number of genes of HSP70 and their expression level (Zhang *et al.,* 2005; Wang *et al.,* 2004). Similarly, in our study, cultivar K-95, exhibited early activation and higher expression of HSP70 during stress time and consistent accumulation and late disappearance during recovery time. That's why it showed minimum accumulation of H_2O_2 and MDA as compared to other crops. This attribute might have provided additional ability for K-95 to withstand in oxidative stress condition and give successful seedling establishment. On the other hand, efficient scavenging of reactive oxygen species like H_2O_2 is highly dependent on the activities of antioxidant enzymes like catalase (CAT) and ascorbate peroxidase (APX) and being protein in nature these key elements also need assistance of HSP70 not only in maintenance of their proper structure but for their proper functioning as well (Ali *et al.,* 2017).

Western blotting results revealed differential expression of HSP70 and by comparing with control cultivars, can be grouped in three categories. Some cultivars like DR-82 and Shahkar showed no protein band in control condition hence considered as zero percent (0%) expression while IR-6 and K-95, showed maximum expression hence considered 10%, however remaining cultivars viz. DR-83 and DR-92 had 5% and Sada Hayat had 2% and placed in between (2-5%). If we look the expression pattern of HSP70 at T24, then only K-95 had highest (50%), followed by IR-6 (20%) while remaining had approximately 10%. At T48, most of the cultivars just increased 10% while IR-6 showed 20% increment. Maximum duration of heat stress (T72), manifested that maximum expression level were 50 and 60 %, in IR-6 and K-9, respectively. Remaining showed less than 30% except Sada Hayat (40%).

After application of heat stress, the stressed plants allowed to recover the heat induced damages at normal growth temperature, are called recovery condition. Interesting pattern of HSP70 expresion observed during recovery condition, where cultivars showed significant differential response in terms of sustainable accumulation and resistant to decline the protein. Sada Hayat showed rapid decay of HSP70 protein and completely disapeared after 72 h of recovery time (R72) while IR-6 and K-95 showed consistent expression and accumulation, approximately 30% of the highest value even after 72 h of recovery (R72) while the rest of the cultivars had mild response (Table 4).

At this level it can be inferred that rice heat stress tolerance are highly dependent on early signal transduction for expression of HSP70 and and late decay of HSP70 in post stress timings (Fig. 3). Similar results also observed in other grains like Kausar *et al.,* (2013, reported that thermotolerant barlay manifested high level of HSP70 while heat susceptible had very low HSP70. In this way, it can be concluded, that cultivar, IR-6 and K-95 are thermotolerant, DR-82, DR-92 and Shahkar are moderately heat tolerant while IR-8, DR-83 and Sada Hayat are heat susceptible. Secondly, development of thermo-tolerant rice varieties under the threat of global warming and food security issues, expression pattern and level of HSP70 should be considered as potential biomarkers in future rice breeding programs.

Conflict of interest

The authors declare to have no conflict of interest.

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