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Characterization of Physiological Responses and Deciphering Differential Expression of Heat stress Responsive Candidate Genes in Rice under High Temperature

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Abstract

With increasing global temperature (by 1.5-4.8 °C by 2100) and its negative impact on crop productivity of major food crops including rice (by 41% by 21st century), a study was undertaken to assess the genes involved in maintaining crop metabolism and sustenance under high temperature. Rice, most important staple food crop feeds around 3 billion people, hence producing improved heat tolerant varieties is necessary. High temperatures produce new group proteins called 'Heat Shock Proteins (HSPs), whose transcription is guarded by heat shock transcription factors (Hsfs). HSPs refold proteins maintaining functional conformation, aiding in host-defence mechanisms. The aim of this research was to assess physiological and biochemical changes and to analyze key genes expressed due to high temperature exposure. Expression profiling of five heat responsive genes (OsHSP26.7, OsHSP16.9, OsHSP-DnaJ, OsHSP18 and 60Kda-chaperon), in rice showed up-regulation. Pollen fertility, spikelet fertility, photosynthetic pigments like chlorophyll a, chlorophyll b and total chlorophyll content decreased under heat stress while membrane stability index, proline and Malondialdehyde was increased. This study suggested OsHSP26.7 as most responsive gene under stress and rice genotypes RRF-127, Annada with heat tolerant adaptive mechanisms and better performance under high temperatures. These findings were observed to be in correlation with the phenological, biochemical and expression analysis studies carried out with five different heat responsive genes. This can be taken as a base for heat tolerance response of rice crop, which may be useful for further validation studies of the candidate genes responsive for heat stress in rice.

Keywords: Heat shock proteins, chlorophyll, malondialdehyde, proline, spikelet fertility

1. Introduction

Rice is the most important staple food crop in the world, directly feeding more than 3 billion people across Asia, Africa, and Latin America. It is cultivated in more than 159 mha every year by households more than 100 millions in at least 114 countries across Asia and Africa. In the developing world, rice is the source of 27% dietary energy and 20% of dietary protein. It's been reported that the productivity of rice crop is to be reduced by 41% by the end of 21st century (Ceccarelli et al., 2010), due to increase in earth's surface temperatures. In the past three decades, surface temperatures of the earth have become warmer than it was during any preceding decade since 1850, due to rapid increase in green house gas concentrations. Global mean surface temperatures

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increase by 1.5 to 4.8 °C by 2100 (IPCC., 2014). Plant growth and performance was damaged irreversibly due to increasing temperatures, majorly affecting the yield of crop and also quality of the yield (Wahid et al., 2007). With increasing concerns about global warming, the effect of temperatures stress on rice production has become a major focus in many countries in tropical, subtropical and temperate regions that produce rice. Heat stress affects the growth of rice plants at all stages like vegetative, reproductive and/or ripening phasesby causing reduction in the photosynthesis rate and stomatal conductance. In particular, spikelet sterility induced by high temperatures during flowering is a serious problem, because it directly reduces yield (Mohammed et al., 2011).

In response to heat stress, plants adjust themselves to the metabolism and morphology suitable to sustain in the stress conditions. Generally high temperature induces expression of heat shock proteins (HSPs) and normal cellular protein production, at least in part, is suppressed. The heat stress not only affects the morphological and phenological parameters but also the physiological and biochemical parameters like plant water relationship, photosynthesis, assimilate partitioning, hormonal changes, cell membrane stability, production of secondary plant metabolites, antioxidative metabolism, synthesis of heat shock proteins, cell signalling etc (Mondal et al., 2014). The sheath blight incidence and severity were negatively correlated with maximum temperature, minimum temperature, evening relative humidity and rainfall and positively correlated with morning relative humidity and sunshine hours during both the crop growing seasons (Kaur et al., 2015). The ten wheat genotypes by gradual increase in temperature from control (25 °C) to 30 °C 1 h, 35 °C 1 h, 40 °C 2 h and 46 °C 3 h in order to investigate its effect on RWC, chlorophyll, osmolytes accumulation, lipid peroxidation and activity of antioxidants SOD, CAT, GPOX, APX and GR. Heat stress induce the activities of all five ROS and decline in RWC and chlorophyll b in NIAW-34, AKAW-4627 and NIAW-917 wheat genotype which found to be stable under heat stress (Satbhai et al., 2015). The male sterile plants were identified in M2 generation under high temperature condition (Coimbatore) and the reverted lines (with more than 60% spikelet fertility) in the low temperature region (Gudalur) were planted again in the high temperature condition to confirm their Temperature sensitive Genic male sterilirty (TGMS) nature. Seven plants (comprising five plants from ADT 39 and two plants from CR 1009) isolated from M3 generation recorded to have 100 % pollen and spikelet sterility (Anitha devi et al., 2010). Terminal heat stress in wheat, High Phenotypic Coefficient of Variation (PCV), Genotypic Coefficient of Variation (GCV), heritability, Genetic advance (GA), GA (%) of mean was observed in the characters viz., proline content, no. of tillers per plant, no. of spikes per plant, no. of grains per spike and yield per plant under late heat stress condition. The genotypes viz. GW 2011-403, WSM 135, GW 2008-153, RAJ 4358, RAJ 4362, J-07-47 & UP 2783 were found to be high yielders may exploit towards heat tolerance (Mohanty et al., 2016). Increasing severity of high temperature worldwide presents an alarming threat to wheat in India as late planting of wheat is very common due to the wide spread intensive rice-wheat cropping system particularly in north-west India. As a result, wheat crop has to face the problem of terminal heat stress. It causes a series of morpho-anatomical, physiological and biochemical changes, which affect plant growth and development and results in reduced yield (Suryavansh et al., 2016). The low temperature also affects the rice cultivation mainly in two stages of development i.e. seedling and booting. In both of them, cold temperature has harmful effects on crop productivity. Low temperature during the reproductive stage in rice causes degeneration of spikelets, incomplete panicle exsertion and increases spikelet sterility thus reducing grain yield (Loitongbam et al., 2017). HSPs can improve or stabilise photosynthesis, partitioning of assimilates, nutrient and water use efficiency and the thermal stability of cellular membranes. Damaged protein restoration is also aided by some of the HSPs and molecular chaperones. Heat tolerance genetics was poorly understood as it is complex and controlled by multiple genes (Farooq et al 2009). Many HSPs have been reported and their genetics (controlling genes, location of genes, dominance/recessive) are known. Besides this, plants also develop different physiological and biochemical mechanisms in response to various environmental stresses. Osmoregulation is one of the important biochemical phenomena in plants to cope up adverse environmental conditions. Proline accumulates in many plant species under a broad range of stress conditions such as water shortage, salinity, extreme temperatures, and high light intensity. Proline is considered to be a compatible solute. Photosynthetic pigments are important to plants mainly for harvesting light and production of reducing power such as ATP and NADPH. Both the chlorophyll a (CHLa) and (CHLb) are prone to high temperatures. Heat stress induced changes in the ratio of CHLa and CHLb and carotenoids (Farooq et al., 2009). The chlorophyll content decreased to a significant level at high temperatures in plants. During high temperature stress, ROS (reactive oxygen species) are produced as secondary stress which reacts with unsaturated fatty acids in membranes and results in lipid peroxidation, which leads to MDA accumulation. As the purity and functions of biological membranes are sensitive to heat stress, tertiary and quaternary structures of membrane proteins gets transformed boosting up the permeability of membranes, which is obvious from enhanced electrolyte leakage. This increased the electrical conductivity under heat stress, resulting the decreased membrane thermal stability index (Hemantaranjan et al., 2014). With these considerations, the current study was undertaken to characterize heat tolerance mechanism in rice at phenological, biochemical and molecular levels.

2. Materials and Methods

2.1. Plant material

The experimental material of present investigation comprised of fourteen rice genotypes belonging to landraces, established varieties and susceptible varieties (GP-145-103, SL-62, Dagaddeshi, Nagina-22, Swarna, GP-145-55, CGZR-1, Annada, Poornima, Karma mashuri, ARB-6-11, GP-145-40, MTU-1010, RRF-127) planted in trays separately with proper spacing and maintained in green house at 28±2 °C in summer 2018-19. Summer field conditions were mimicked by flooding the trays with water and increasing the green house temperature. Heat stress is given at the end of vegetative stage, before the panicle initiation of plant by gradually increasing the green house temperature from 30 °C at 6:00 am to 42 °C at 11:00 am. Constant temperature of 42 °C was maintained for 6 hrs continuously. The rice plants were treated with high temperature stress until 17:00 pm, with gradual adjustments to the greenhouse's temperature down to 28 °C–30 °C at night (18:00 to 6:00). This stress is given continuously for 6 days from the beginning of the respective stage. The morphology of plant after stress treatment is shown in Figure 1. Leaf samples were collected after stress treatment from control and stress plants for further studies. For RNA isolation leaf samples are collected in liquid nitrogen and stored at -80 °C.

2.2. Membrane stability index (MSI)

Membrane stability index is measured by electrolyte leakage. Leaf samples from all fourteen rice genotypes were collected from control and stress conditions. Weigh 1 g of leaf samples and wash it with de-ionized water to remove the residues present on them. Cut them into small pieces of 1cm and place in test tubes containing 15 ml de-ionized water and incubate at 24 ˚C for 12 h. Electrical conductivity (EC1) of the solution was measured using conductivity meter. Subsequently, the samples were autoclaved at 120 ˚C for 20min and then cooled to room temperature. Now the final Electrical conductivity (EC2) of the solution was measured.

Electrolyte leakage from the leaf samples is calculated by the formula:

Electrolyte leakage (EL %)=(EC1÷EC2)×100

2.3. Pollen fertility

Pollen fertility is calculated using staining methods with the help of stains like aectocarmine (2%). Anthers are collected from all the genotypes under control and stress conditions, and they are stored in ethanol (70%) to arrest the stage. Then the anthers are punched using needle and pollen is distributed on the microscope slide and place a drop of acetocarmine (2%) dye. Cover the slide with cover slip and observe under microscope at 40x just after preparation of slide. The deeply stained/normal looking pollen grains are counted as the viable pollen and the colourless/shrivelled pollen are counted as the non-viable pollen. The pollen fertility is calculated using the formula.

Pollen fertility (%)= (no.of viable pollen ÷ total no. of pollen in the microscopic field)×100

2.4. Spikelet fertility

Spikelet fertility at maturity was used to screen heat tolerance. It is calculated by counting the empty and filled grains. Three randomly selected matured panicles were harvested from all the fourteen genotypes in stress and control conditions. The harvested panicles were manually threshed and the numbers of filled, unfilled and total number of grains per panicle are recorded. These readings were taken by pressing each floret between forefinger and thumb to determine if the grain is filled or not. Both partially and fully filled spikelets were categorized as filled spikelets. The negative effect (% decrease from control) was determined in every genotype. Spikelet fertility is calculated as shown below

Spikelet fertility (%) = (no. of filled grains)÷(total no. of grains formed (florets)×100

2.5. Chlorophyll estimation

Total chlorophyll CHLa and CHLb contents from the leaves were estimated as per Arnon method (Arnon., 1949). The values were expressed in milligram per gram fresh weight.

2.6. Measurement of proline

Proline content was estimated following the method described by Bates et al. (1973). Express the proline content on fresh weight basis (µ mol g^{-1} fresh weight).

2.7. Estimation of MDA content

MDA content (an indicator of lipid peroxidation) was calculated using the method given by Heath and Packer (1986). MDA content is expressed as nmoles MDAg-1 fresh weight.

2.8. Statistical analysis

The OPSTAT software developed at BHU was applied for statistical analysis.

2.9. RNA extraction

Total RNA was isolated using TRIzol (Invitrogen, USA) using a manufacturer's protocol. cDNA was synthesized using BIORAD iScript TM cDNA Synthesis Kit as per manufacturer's instructions.

2.9.1. Semi-quantitative RT-PCR and Gel Electrophoretic Analysis

Semi-quantitative RT-PCR reactions were carried out with 20 µl of the reaction (APS Taq polymerase) solutions using gene specific primers (given in Table 1) and Actin gene primers as internal control. The resultant PCR product was then resolved on 1.5% Agarose gel followed by digitalization of fluorescence data to numerical values using GelQuant. NET Analyzer.

Table 1: Details of primers used for semi quantitative RT-PCR analysis[7]

Sl. No.	Gene	Locus ID	Forward primer (5'-3')	Reverse primer (5'- 3')	Tm (˚C)	Product size (bp)
1.	OsHsp 26.7	LOC_Os03g14180	CGTGAGGGTTTAAGCAGTGT	AGCTCAGTGTCTCAGCCTTG	62	250
2.	OsHsp 16.9	LOC_Os01g04270	GTGATGGCCAGTCAAGTAGA	CTGCATCTCTGTTGGATCAC	61	219
3.	OsHspDnaJ	LOC_Os01g01160	GAAGACAAGTCTGGCTGGAG	CACAGCACACCCTTCTAACC	62	246
4.	OsHsp 18	LOC_Os01g08860	AAGAAGAAGAGGCGATCGAG	CTTGATGTCGGAGGACTTGA	61	259
5.	60 kDa chaperon	LOC_Os10g32550	AGTTCGAGCCTCAGATGTTG	ACCAACTTCAGCTTCACTGG	62	239

3. Results and Discussion

3.1. Effect of high temperature on Membrane stability index (MSI)

Uninterrupted function of cellular membranes is very important under stress conditions, for photosynthesis and respiration processes to occur precisely. Hence,cell membrane stability was reported to play critical role under conditions of high temperature, as a major component of heat tolerance. Membrane stability index is calculated by measuring the relative electrolyte leakage from cells due to injury occurred under stress. Electrolyte leakage is the measure of electrical conductivity of the tissues. Wide variation in the fold increase of membrane stability among the tested genotypes was recorded which ranged from 19.8% fold increase in RRF-127 to 292.9% in MTU-1010. Previous studies reported that heat tolerant genotypes show low levels of electrolyte leakage, indicating less injury to the membrane (Hemantaranjan et al., 2014). In the present study, the lowest levels of electrolyte leakage was observed in RRF-127 with 19.8% increase under stress when compared with control and reported as heat tolerant genotype followed by Nagina-22 (21.1%), Annada(27.5%), CGZR-1 (51.7%) and Karma mashuri (69.8%) as shown in Figure 1.

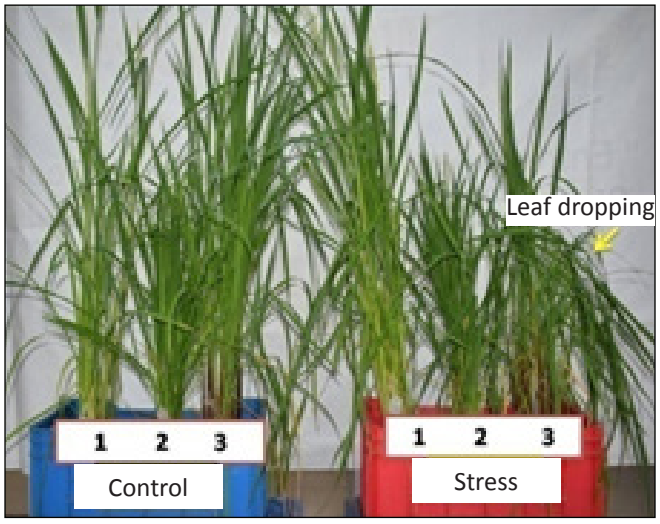

Figure 1: Morphological changes in tolerant and susceptible rice genotypes under high temperature; 1: Nagina-22 (Check) 2: RRF-127 (Tolerant) 3: MTU-1010 (Susceptible)

3.2. Effect of high temperature on pollen fertility

Wide variation is observed in the pollen fertility of the tested genotypes under stress. The fold decrease in pollen fertility ranged from 14.4% in RRF-127 to 66.5% in MTU-1010. Many studies reported that pollen fertility decreases under stress conditions and the genotypes showing lowest decrease in pollen fertility are considered as the tolerant ones for heat stress (Fahad et al., 2018). In the present study lowest decrease in the pollen fertility, under stress conditions was observed in RRF-127 (14.4%) followed by Nagina-22 (18.1%), GP-145-103 (24.9%), Annada (28.3%) and CGZR-1 (37.9%) as shown in Figure 2.

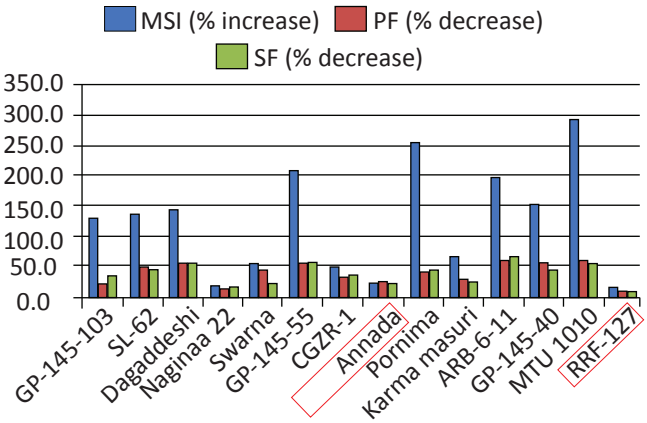

Figure 2: Effect of high temperature on phenological traits in diverse rice genotypes

3.3. Effect of high temperature on spikelet fertility

Heat stress affects spikelet fertility by decreasing pollen germination and increasing respiration rates, which subsequently reduces yield. High temperatures reduce seed size and number, which ultimately affects '100 seed weight'. This occurs because high temperatures decrease translocation of photo assimilates, seed reserves biosynthesis that takes place in leaves during seed filling. Wide variation is observed in the spikelet fertility of the tested genotypes under stress. The fold decrease in spikelet fertility ranged from 14% in RRF-127 to 71% in ARB-6-11. Many studies reported the decreased spikelet fertility under heat stress conditions and the genotype having the lowest change in spikelet fertility under stress conditions when compared to

control is considered as the tolerant genotype (Mohammed et al., 2010). In the present study, lowest decrease in spikelet fertility under stress conditions was observed in RRF-127 (14%) followed by Nagina-22 (19%), Annada (25%), Karmamashuri (31%) and CGZR-1 (42%) as shown in Figure 2.

3.4. Effect of high temperature on Chlorophyll content

A significant decrease in chlorophyll content (CHLa, CHL band total chlorophyll) was observed in all rice genotypes under temperature stress. The mean CHLa content under control conditions was recorded as 2.529 mg g-1 leaf tissue and it ranged from 1.610 mg g^{-1} in MTU-1010 to 3.410 mg g^{-1} in CGZR-1 (Table 2). While mean CHLa content under stress conditions was recorded as 1.874 mg g^{-1} leaf tissue and it ranged from 0.523 mg g^{-1} in swarna to 2.743 mg g^{-1} in CGZR-1 (Table 3). Lowest fold decrease in CHLa content was recorded in the genotype Nagina-22 (1.0 fold) followed by Karma mashuri (1.0 fold), Annada (1.0 fold), GP-145-103 (1.2 fold) and CGZR-1 (1.2 fold).The mean CHLb content under control conditions was recorded as 1.303 mg g^{-1} leaf tissue and it ranged from 0.370 mg g^{-1} in GP-145-40 to 2.820 mg g^{-1} in GP-145-55 (Table 2). While mean CHLb content under stress condition was recorded as 0.873 mg g^{-1} leaf tissue and it ranged from 0.203 mg g^{-1} in RRF-127 to 2.300 mg g^{-1} in GP-145-103 (Table 3). Minimum fold decrease in CHLb content was recorded in the genotype Annada (1.0 fold) followed by ARB-6-11 (1.0 fold), GP-145-103 (1.0 fold), Karma mashuri (1.1 fold) and GP-145-40 (1.1 fold). The mean total chlorophyll content under control conditions was recorded as 3.831mg g^{-1} leaf tissue and it ranged from 2.023 mg g^{-1} in MTU-1010 to 5.820 mg g^{-1} in GP-145-55 (Table 2). While mean total chlorophyll content under stress conditions was recorded as 2.747 mg g^{-1} leaf tissue and it ranged from 0.853 mg g^{-1} in Swarna to 4.970 mg g^{-1} in GP-145-103 (Table 3). Lowest fold decrease in total chlorophyll content was recorded in genotype Annada (1.0 fold) followed by GP-145-103 (1.0 fold), Karma mashuri (1.0 fold), GP-145-40 (1.1 fold) and CGZR-1 (1.2 fold). Heat stress imposed at the vegetative stage, undoubtedly decreased all CHLa, CHL band total chlorophyll content. It is reported that CHLa content is more sensitive to abiotic stress as compared to CHLb (Mafakheri et al., 2010). The ability to synthesize more chlorophyll under high temperature stress is a good criterion for the species

Table 2: Mean and Range for biochemical traits of all rice genotypes under control conditions

Genotype	Chlorophyll a control (mg g^{-1})	Chlorophyll b control (mg g^{-1})	Total chlorophyll Control (mg g^{-1})	Proline control (µ mol g^{-1} f.wt)	MDA control (nmols g^{-1} f.wt)
	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
GP-145-103	2.710±0.000	2.310±0.006	5.023±0.004	0.064±0.000	0.293±0.008
SL-62	2.993±0.003	2.150±0.000	4.833±0.003	0.064±0.000	0.785±0.008
Dagaddeshi	2.400±0.000	1.290±0.000	3.690±0.001	0.093±0.000	0.237±0.015
Naginaa 22	2.547±0.003	1.817±0.003	4.360±0.000	0.068±0.001	0.419±0.016
Swarna	1.720±0.000	1.047±0.003	2.763±0.003	0.265±0.004	0.366±0.006
GP-145-55	3.000±0.000	2.820±0.000	5.820±0.000	1.001±0.000	0.284±0.000
CGZR-1	3.410±0.000	1.440±0.010	4.847±0.009	0.173±0.004	0.097±0.008
Annada	2.693±0.003	0.620±0.000	3.317±0.003	0.182±0.002	0.243±0.009
Pornima	3.090±0.000	1.060±0.000	4.147±0.003	0.374±0.000	0.590±0.004
Karma masuri	2.540±0.000	1.930±0.000	4.470±0.000	0.067±0.001	0.301±0.012
ARB-6-11	2.700±0.000	0.493±0.003	3.190±0.001	0.977±0.001	0.411±0.006
GP-145-40	1.900±0.000	0.370±0.000	2.070±0.000	1.650±0.006	0.639±0.006
MTU 1010	1.610±0.000	0.413±0.003	2.023±0.003	0.519±0.001	0.150±0.008
RRF-127	2.600±0.000	0.480±0.000	3.077±0.003	0.111±0.000	0.219±0.004
Mean	2.529	1.303	3.831	0.407	0.372
Minimum	1.610	0.370	2.023	0.064	0.097
Maximum	3.410	2.820	5.820	1.65	0.785
CD (*p*=0.05)	0.004	0.01	0.01	0.007	0.026
SEm±	0.002	0.004	0.003	0.002	0.009
SEd±	0.002	0.005	0.005	0.003	0.013
C.V.	0.106	0.473	0.151	0.995	4.134

tolerant to stress (Poljakoffand Gale, 1975).

3.5. Effect of high temperature on Proline content

Accumulation of proline as an osmolyte under high temperature stress was observed in all rice genotypes under study. Proline acts as a compatible solute, i.e. it can accumulate to high concentrations in the cell cytoplasm without interrupting cellular structure and metabolism [33]. The mean proline content under control conditions was recorded as 0.407 µ mol g^{-1} fresh weight and it ranged from 0.064 µ mol g^{-1} in GP-145-103 to 1.65 µ mol g^{-1} in GP-145-40 (Table 2), while the mean proline content under stress conditions increased to 1.213 µ mol g^{-1} fresh weight and ranged from 0.095 µ $molg^{-1}$ in SL-62 to 2.044 µ $molg^{-1}$ in GP-145-55 (Table 3). The highest Fold increase in proline content was recorded in the genotype Nagina-22 (20.6 Fold) followed by CGZR-1 (11.4 Fold), RRF-127 (11.2 Fold), Annada (10.5 Fold), GP-145-103 (7.0 Fold) and Karma mashuri (6.0 Fold). Proline is one of the most studied solutes and high proline content in plants under abiotic stress is frequently observed in several species (Bajji et al., 2001) and may act as a regulatory or signalling molecule to activate multiple responses that are part of the adaptation process (Claussen 2005). An elevated level of proline during stress conditions reduce the osmotic potential and help in diffusion of water into the cells by maintains high turgor potential in the cell (Basu et al., 2002). Other functions of proline accumulation were also proposed, including stabilization of macromolecules (Schobertand Tschesche, 1978), sink for carbon and nitrogen after stress recovery (Farooq et al., 2009), and as a scavenger against reactive oxygen species. The concentrations of increased proline under stress conditions are directly proportional to the intensity of heat stress.

3.6. Effect of high temperature on MDA (Malondialdehyde) content

Lipid peroxidation in the cell membranes is the destructive effect of oxidative damage caused due to heat stress. MDA content is the ultimate result of lipid peroxidation, which changes cells membrane stability. MDA content has been widely used as a criterion for assessing abiotic stress in various plants (Jain et al., 2001). The mean MDA content under control conditions was recorded as 0.372 $nmolg^{-1}$

Table 3: Mean and Range for biochemical traits of all rice genotypes under stress conditions

Genotype	Chlorophyll a Stress (mg/g)	Chlorophyll b Stress (mg/g)	Total Chlorophyll Stress (mg/g)	Proline Stress (µ mol/g f.wt)	MDA stress (nmols / g f.wt)
	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.	Mean±S.E.
GP-145-103	2.173±0.003	2.300±0.000	4.970±0.000	0.247±0.008	0.945±0.004
SL-62	2.340±0.000	1.290±0.000	3.630±0.000	0.095±0.003	0.901±0.027
Dagaddeshi	1.720±0.000	0.837±0.015	2.560±0.012	0.380±0.003	1.563±0.006
Naginaa 22	2.550±0.000	1.240±0.000	3.790±0.000	1.402±0.000	1.617±0.009
Swarna	0.523±0.003	0.323±0.003	0.853±0.003	1.767±0.000	0.512±0.002
GP-145-55	1.140±0.000	0.620±0.000	1.760±0.000	2.044±0.001	0.432±0.000
CGZR-1	2.743±0.003	1.107±0.003	3.890±0.000	1.975±0.001	0.326±0.004
Annada	2.680±0.000	0.930±0.000	3.210±0.000	1.903±0.000	1.430±0.002
Pornima	1.760±0.000	0.410±0.000	2.163±0.003	1.906±0.001	1.228±0.006
Karma masuri	2.547±0.003	1.800±0.000	4.343±0.003	0.404±0.000	0.748±0.004
ARB-6-11	2.203±0.003	0.480±0.000	2.687±0.003	1.941±0.001	1.271±0.006
GP-145-40	1.290±0.000	0.340±0.000	1.930±0.000	1.667±0.006	1.684±0.004
MTU 1010	1.540±0.000	0.340±0.000	1.623±0.003	0.921±0.000	0.226±0.007
RRF-127	0.933±0.003	0.203±0.003	1.133±0.003	0.146±0.001	0.385±0.004
Mean	1.874	0.873	2.747	1.213	0.922
Minimum	0.523	0.203	0.853	0.095	0.226
Maximum	2.743	2.300	4.970	2.044	1.684
CD (*p*=0.05)	0.006	0.012	0.011	0.009	0.025
SEm±	0.002	0.004	0.004	0.003	0.009
SEd±	0.003	0.006	0.005	0.004	0.012
C.V.	0.202	0.83	0.238	0.435	1.634

fresh weight and it ranged from 0.097 nmolg^{-1} in GP-145-55 to 0.785 nmol g^{-1} in RRF-127 (Table 2), while the mean MDA content under stress conditions increased to 0.922 nmolg^{-1} fresh weight and ranged from 0.265 nmolg^{-1} in Annada to 1.684 nmolg^{-1} in GP-145-40 (Table 3). The highest Fold increase in MDA content was recorded in the genotype Dagaddeshi (6.6 Fold) followed by Annada (5.9 Fold), Nagina-22 (3.9 Fold), CGZR-1 (3.4 Fold) and GP-145-103 (3.2 Fold). Studies reported that MDA content is relatively more in heat susceptible varieties under stress conditions due to increased lipid peroxidation (Kazim et al., 2013). The heat tolerant genotypes recorded relatively low MDA content under stress than susceptible ones. In this present study the lowest amount MDA content under stress was recorded in RRF-127 with 1.1 Fold increase followed by Nagina-22 (1.4 Fold), CGZR-1 (1.5 Fold), Annada (1.8 Fold), GP-145-103 (2.1 Fold) and Karma mashuri (2.5 Fold).

3.7. Expression pattern of high temperature responsive genes in rice genotypes under high temperature stress

Semi quantitative RT-PCR was performed to analyze the expression pattern of five differentially expressed (OsHSP26.7, OsHSP16.9, OsHSP-DnaJ, OsHSP18 and 60Kda-chaperon) transcripts in rice under high temperatures and control conditions. Figure 3 represents the differential expression of genes under high temperature. Semi quantitative RT-PCR of OsHSP26.7 gene has shown up-regulation under heat stress conditions in all the rice genotypes. The rice genotype RRF-127 showed the highest up-regulation of 14.3 Fold increase followed by Annada (13.9 Fold), Karma mashuri (11.5 Fold), GP-145-103 (8.6 Fold) and CGZR-1 (3.7 Fold). Previous studies reported that

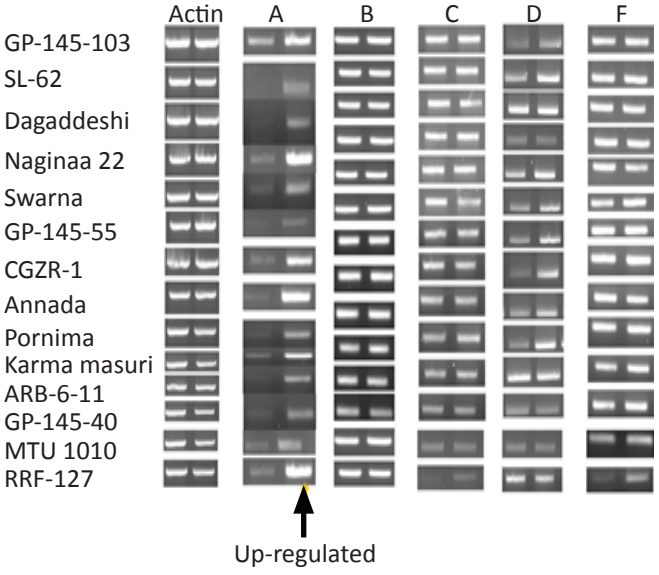

Figure 3: Semi quantitative RT-PCR analysis of heat stress responsive genes in different rice genotypes under high temperature. (A) OsHSP26.7, (B) OsHSPDnaJ, (C) OsHSP18, (D) OsHSP16.9, (E) 60kDa Chaperon

OsHSP 26.7 gene belongs to sHSP (small heat shock proteins) class of genes and showed strong up-regulation under heat stress conditions in comparison to control (Chandel et al., 2013). OsHSP16.9 gene, in the present investigation has shown up-regulation in almost all rice genotypes under heat stress conditions. RRF-127 showed the highest up-regulation of 10.0 Fold increase followed by Annada (3.7 Fold), CGZR-1 (3.4 Fold), GP-145-103 (3.2 Fold) and Karma mashuri (3.1 Fold). It's been reported that OsHSP16.9 gene belongs to sHSP (small heat shock proteins) class of genes and showed slight up-regulation under heat stress conditions when compared with control. Semi quantitative RT-PCR of OsHSP-DnaJ gene, has shown almost the same expression under control and stress conditions and no convincing difference was observed in heat stress. Previous studies also reported that gene OsHSP-DnaJ also showed no significant change in the expression under control and stress. OsHSP18 gene showed up-regulation in almost all genotypes of rice under heat stress. GP-145-103 has shown highest up-regulation of 17.7 Fold increase followed by CGZR-1 (14.1 Fold), Annada (13.8 Fold), GP-145-55 (11.9 Fold) and poornima (8.9 Fold). This gene also belongs to the family sHSP(small heat shock proteins) class of genes and showed up-regulation under heat stress conditions. 60Kda chaperon gene has shown up-regulation in almost all the genotypes under heat stress, but the up-regulation was by minimal levels. Annada has shown highest up-regulation of 1.9 Fold increase followed by CGZR-1 (1.5 Fold), Nagina -22 (1.4 Fold), GP-145-103 (1.3 Fold) and Karma mashuri (1.3 Fold). 60Kda-chaperon was also reported to be up-regulated by minimal levels under heat stress but it is lately induced under stress(Chandel et al., 2013).Small heat shock proteins (sHSPs) are the largest ubiquitous HSP subgroup whose molecular weight ranges from 12 t0 42 KDa.Small HSPs sequence analysis indicates that members belonging to this family include evolutionarily divergent N-terminal part, which is followed by a conserved α-crystallin domain and a short C-terminal tail (De Jong et al., 1993). It's been recognized that ten separate sHSPs families have been conserved in both monocots and dicot plants, which indicates the diversity for sHSPs mechanisms (Scharf et al., 2001). Among the ten, sHSPs encoded by 4 families are localized to cytoplasm and other 6 families are localized to different cellular organelles like nucleus, chloroplasts, endoplasmic reticulum, mitochondria and peroxisomes (Basha et al., 2010). In vivo studies reported that sHSPs function as molecular chaperons. Plants synthesize large amounts of sHSPs when they are exposed to high temperature suggesting that they play a major role for enduring thermo-tolerance in plants (Charng et al., 2006). Some regulatory proteins called heat stress transcription factors (Hsfs) control the transcription of heat shock protein genes. Plants have atleast 21 Hsfs, each one having its own specific role in regulation. They also cooperate in all phases of responses due to periodical stress like triggering, maintanence and

recovery. The smHsps cannot refold non-native proteins, but they can bind to partially folded or denatured substrates proteins, preventing irreversible unfolding or wrong protein aggregation (Mogk et al., 2003). Recent findings showed that the smHSPs 18.1 isolated from *Pisumsativum*, as well as the sHsps 16.6 from *Synechocystis* sp. PCC6803 under *in vitro* conditions, binds to unfolded proteins and allows further refolding by HSP70 and HSP100 complexes (Guan et al., 2004). A very strong positive qualitative relation was noticed between the thermo-tolerance and accumulation of sHSPs in plastids.The current model for sHSP chaperone activity was defined based on studies of a cytosolic sHSP family named as Class I sHSPs (sHSP-CI), which represent the most abundant sHSP in plants. According to this model, a large homo-oligomer is formed by the assembly of sHSPs, which binds to denatured proteins by ATP-independent fashion, and keeps them in folding competent state. Then this complex cooperates with molecular chaperons like HSP70 and HSP90, which are ATP-dependent to refold those denatured proteins. Notably, sHSP has a much larger binding stoichiometry than other molecular chaperones, which has led to the speculation that sHSP functions as a reservoir to stabilize the flood of denatured proteins in response to stress (Lee et al., 2000). Based on this mechanism it is observed that heat-induced sHSPs oligomers dissociation may expose the hydrophobic patches that were buried in the oligomeric interface, which leads to binding and stabilization of denatured proteins under heat stress and imparts thermo-tolerance.

3.8. Correlation between phenological, biochemical traits and gene expression under high temperature stress

Characterization of rice genotypes contrasting in their response to high temperature stress was performed at the phenological and biochemical level by determination of MSI, Pollen fertility, Spikelet fertility, chlorophyll content, free proline content and MDA content under temperature stress condition. Pollen fertility and Spikelet fertility were positively correlated and MSI was negatively correlated with the yield per plant which was significant (Table 4). The remaining traits showed non-significant correlation with yield per plant. Chlorophyll-a degradation was positively correlated with chlorophyll-b and total chlorophyll content and it was negatively correlated with MDA levels and was significant, which correctly indicated the increase in MDA levels under high temperatures with decreased chlorophyll content. Chlorophyll-b degradation was positively correlated with chlorophyll-a, total chlorophyll content and it was negatively correlated with proline content and was significant, which indicated the increase in proline accumulation under high temperature with decreased chlorophyll content.

Table 4: Correlation of Phenological and biochemical traits

	MSI	Pollen fertility	Spikelet fertility	CHL a	CHL b	CHL total	Proline	MDA	Yield plant^{-1}
MSI	1								
Pollen fertility	-0.503^{NS}	1							
spikelet fertility	-0.499^{NS}	0.999**	1						
chl a	0.054^{NS}	0.076^{NS}	0.048^{NS}	1					
chl b	-0.142^{NS}	0.173^{NS}	0.157^{NS}	0.653*	1				
chl total	-0.079^{NS}	0.130^{NS}	0.108^{NS}	0.867**	0.931**	1			
proline	0.095^{NS}	0.104^{NS}	0.099^{NS}	-0.147^{NS}	-0.552*	-0.394^{NS}	1		
MDA	-0.054^{NS}	-0.344^{NS}	-0.340^{NS}	-0.543*	-0.305^{NS}	-0.397^{NS}	-0.215^{NS}	1	
Yield plant^{-1}	-0.640*	0.642*	0.643*	0.202^{NS}	0.016^{NS}	0.101^{NS}	0.072^{NS}	-0.396^{NS}	1

4. Conclusion

The rice genotypes RRF-127 (a drought tolerant advanced rice breeding line) and Annada (popular rice variety), were identified as heat tolerant rice varieties based on the component traits analysis. Further out of five genes under study, the gene OsHSP26.7 involved in wrong protein aggregation and irreversible unfolding of protein have shown high expression.

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