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Expression Dynamics of Genes and microRNAs at Different Growth Stages and Heat Treatments in Contrasting High Temperature Responsive Rice Genotypes

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Abstract

The global warming-driven climate change is becoming a major challenge for rice cultivation in Asia and Africa. High-temperature stress impairs the physiology and growth of rice plant, and ultimately results in reduced grain yield. This study was aimed to decipher the physiological and molecular changes occurring during different growth stages of heat-tolerant (N22) and -susceptible (Vandana) rice cultivars under three different heat treatments. Chlorophyll content, membrane integrity, gas exchange parameters and expression of genes and miRNAs were analyzed in N22 and Vandana at seedling, vegetative, and reproductive growth stages after exposing to short and long duration of high temperature stress, and recovery. A number of genes and miRNAs showed dynamic changes in their expression patterns at different growth stages and heat treatments, highlighting the necessity to understand gene regulation before employing the genes for modification through transgenic or gene editing approaches. Predominantly N22 showed distinct and unique capability to reprogram its physiological and molecular machinery during prolonged heat stress at reproductive stage, suggesting that the dynamics in gene regulation is crucial to determine its heat tolerant ability. The study has larger implications in deploying genes for the development of heat tolerant rice cultivars through breeding, transgenic, and genome editing approaches.

Keywords Heat stress · *Oryza sativa* · N22 · Vandana · MDA · Photosynthesis

Abbreviations

ARF	Auxin response factor
Chl	Chlorophyll
Ci	Internal CO_2 concentration
CWIP	Cell wall integrity protein
E	Transpiration rate
FRH	Fertility restorer homologue
g_s	Stomatal conductance
Hsf	Heat shock transcription factor
HSP	Heat shock protein
MDA	Malondialdehyde
miRNA	MicroRNA
N22	Nagina22
NF-Y	Nuclear transcription factor-Y
P_N	Net photosynthetic rate
SPL	Squamosa promoter binding protein
SPS	Sucrose phosphate synthase

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Introduction

Rice is the primary staple food crop ensuring food security of more than half of the world's population. Increasing population and shrinking farm resources (land, water, labor, and farm inputs) demands the increased productivity of rice to meet the challenges of global food requirements. Climate change is one of the major impediments in achieving the goals of rice production enhancement. Extreme temperatures and unpredicted precipitation are the major factors of climate change which adversely affect rice growth, development,

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and productivity (Krishnan et al. 2011). The rise in global average surface temperature by 0.85 °C in the last century (IPCC 2013), and reports of regional-scale warming over 1.5 °C in at least one season is an alarming situation for rice cultivation in various parts of the world (Allen et al. 2018). During the growing season, an increase of 1 °C in the minimum temperature could decrease rice grain yield by 10% (Peng et al. 2004). Another study reported that an increase of 1 °C in global mean temperature is estimated to reduce the global production of rice by 3.2 ± 3.7% (Zhao et al. 2017) which varies at regional scales (Zhang et al. 2017; Li et al. 2018). For India, however, estimates from four independent methods predict more severe temperature impacts, with average yield loss 6.6 ± 3.8% per degree Celsius increase (Zhao et al. 2017). Heat stress impairs all the growth stages of rice, especially the seedling and reproductive stages (Sailaja et al. 2014a, 2015; Shi et al. 2017).

High temperature affects the physiological, biochemical, and molecular pathways of plants primarily by influencing membrane fluidity, ROS accumulation, cellular concentration of ions, gene regulation, and protein denaturation (Hasanuzzaman et al. 2013). The key biological processes such as photosynthesis and chlorophyll biosynthesis are also affected severely due to heat stress (Hermann and Gabriel 2013; Sailaja et al. 2014a, 2015). A change in the malondialdehyde (MDA) content is a major indicator of high-temperature stress (Wahid et al. 2007). These changes in physiological and biochemical processes in turn, activate different stress regulated protein-coding genes and non-coding microRNAs (miRNA) through different signal transduction pathways (Sailaja et al. 2014a; Su et al. 2017; Mangrauthia et al. 2017, 2018; Jalmi et al. 2018). Regulation of genes encoding heat-shock transcription factors, heat-shock proteins, and other stress-associated genes have been very well studied during high temperature stress (Fragkostefanakis et al. 2015; Guo et al. 2016). In addition, miRNAs are also known to play regulatory roles during heat stress response in rice (Sailaja et al. 2014a, 2015; Li et al. 2015; Mangrauthia et al. 2017).

In our previous reports, we analyzed the expression of protein-coding genes and miRNAs, and established relationship with the physiological and biochemical changes during high temperature stress. During these studies, we noticed that physiological/biochemical response as well as genes/miRNAs expression during high temperature stress is highly dynamic in rice cultivars, and it is very much influenced by growth stages, stress treatments, and genotypes (Sailaja et al. 2014a, b; 2015; Mangrauthia et al. 2017). Therefore, experiments were aimed to further probe the differential response of heat susceptible (Vandana) and tolerant (N22) rice cultivars during three different heat treatments at different growth stages by analyzing a set of protein-coding genes and miRNAs expression, along with physiological and biochemical processes. Although the information on protein-coding genes and miRNAs expression at seedling stage (Sailaja et al. 2014a) and partial information on gene expression at reproductive stage (Sailaja et al. 2015) of both cultivars has already been reported in a different context to study the differential response of tolerant genotype in comparison to the susceptible one, but different growth stages/heat treatments were not compared together earlier. Here, in this study we included the data of protein-coding genes and miRNAs expression from these previous reports to compare the expression dynamics at different growth stages. It has been done for the benefit of readers as understanding the dynamics of protein-coding genes and miRNAs expression at different growth stages and heat treatments would be easy.

In this study, we deciphered the dynamics of protein-coding genes and miRNAs expression under three different heat stress treatments, i.e. short duration stress (SDS), long duration stress (LDS), recovery (REC), and three different growth stages (seedling, vegetative and reproductive) in heat tolerant (N22) and susceptible (Vandana) rice cultivars. Thirteen protein-coding genes and nine miRNAs were selected based on their critical role during high temperature and other abiotic stresses (Table S1). In addition, physiological attributes such as gas exchange parameters, chlorophyll, and MDA content were also analyzed to support the gene expression results.

Material and Methods

Plant Material and Stress Treatments

Seeds of N22 and Vandana cultivars were germinated in Petri plates on a wet filter paper at 28 °C. After 15 days, seedlings were transferred into 12'' earthen pots. Two seedlings per pot were maintained for further experiments. For heat treatment at vegetative stage, high-temperature (42 °C) stress was imposed after 45 days of transplantation. At reproductive stage, heat stress was imposed during the flowering initiation stage. Heat stress treatments were given as described in our previous reports (Sailaja et al. 2014a, 2015). For the short duration stress (SDS), heat treatment (42 °C/36 °C, day/night) was given for 24 h and for long duration stress (LDS), heat treatment was given for 5 days. For the recovery (REC) treatment, plants were transferred to ambient temperature (30 °C/24 °C, day/night) for 24 h after being exposed to 4 days' stress treatment at 42 °C/36 °C (day/night). Plants maintained at ambient temperature (30 °C/24 °C, day/night) were treated as control. For all the experiments, a minimum of three biological replicates were used.

Chlorophyll Pigment Measurement

Chlorophyll pigments were measured in fourth leaf (vegetative stage) and flag leaf (reproductive stage) using the method described by Lichtenthaler and Wellburn (1983). 100 mg of leaf sample was extracted in 25 ml of 80% acetone. The absorbance of Chl a, Chl b, and carotenoids was measured using UV-Spectrophotometer at 663.2, 646.8, and 470 nm wavelengths, respectively.

Lipid Peroxidation Assay

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in the leaves of both control and heat treated samples by following the method described by Heath and Packer (1968). About 200 mg of fresh leaf tissue was homogenized and extracted in 10 ml of 0.25% TBA made in 10% trichloroacetic acid (TCA). The extract was heated at 95 °C for 30 min in a water bath and then quickly cooled on an ice bath. After centrifugation at 11,200 × g for 10 min, the absorbance of the colored supernatant was measured at 532 nm. Correction of non-specific turbidity was done by subtracting the absorbance value taken at 600 nm. MDA concentration was determined using the extinction coefficient of 155 mM^{-1} cm^{-1}.

Leaf Photosynthesis Parameters

The photosynthesis of fully expanded mature leaves was measured in triplicates using LI6400XT portable photosynthesis measuring system (LI-COR Environmental, USA) connected to Leaf Chamber Fluorometer (6400–40, LI-COR, USA), which was used as a light source. Leaf temperature was maintained at 35 °C, and PAR (photosynthetically active radiation) was maintained at 1,000 µmol m^{-2} s^{-1}. Measurements were made at ambient CO_2 levels. The mean CO_2 concentration during measurements was 387 µmol mol^{-1}. Consequently, net photosynthetic rate (P_N), stomatal conductance (g_s), transpiration rate (*E*), and intercellular CO_2 concentration (C_i) were measured.

Statistical Analysis

The data were analyzed by ANOVA (analysis of variance) using a statistical computer package Statistix Ver.8.1. It was analyzed as per CRD (Completely Randomized Design). The differences between treatments and cultivars were estimated using HSD (Honest Significant Difference) test (Table 1).

Protein-Coding Genes and miRNAs Expression

Gene sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) and miRNA sequences were downloaded from miRBase (http://microrna.sanger.ac.uk). Nine miRNAs and thirteen protein-coding genes having established roles during high-temperature stress and other abiotic stresses (Table S1) were used here for expression analysis. Nine miRNAs were miR156, 160, 162, 167, 168, 169, 397, 398, and miR1884. Thirteen protein-coding genes used for expression analysis were heat shock transcription factors (*OsHsfA2a, OsHsfA2e, OsHsfA7*), heat shock proteins (*HSP70, HSP81.1*), superoxide dismutase (*SOD*), sucrose-phosphate synthase 1 (*SPS*), cytochrome c oxidase assembly protein (*CYT-C-Oxi*), squamosa promoter-binding-like protein 10 (*SPL*), cell wall integrity protein (*CWIP*), auxin response factor (*ARF*), nuclear transcription factor-Y (*NF-Y*) subunit A-3, and unknown protein similar to ferredoxin (*Osfd*). In addition, the expression of a fertility restorer homologue gene (*FRH*, AK101861; forward primer 5′-TTACGCCAC GCTGATTGAGG 3′ and reverse primer 3′- CCGCTCCGC ATTACACAACC 5′) was also analyzed in this study. Details of primers and methods of RNA extraction and quantitative

Table 1 Statistical analysis of the physiological parameters analyzed in this study

S.No	Prbf							
	Parameters	Treatments (T)	Genotypes (G)	Stage(S)	G×T	T×S	G×S	G×T×S
1	Photosynthesis (P_N)	0.0000(***)	0.0000(***)	0.0000(***)	0.0000(***)	0.0000(***)	0.2205 (ns)	0.0082(**)
2	Stomatal conductance (g_s)	0.0000(***)	0.0000(***)	0.0000(***)	0.0006(***)	0.0161(*)	0.7085(ns)	0.0118(*)
3	Intercellular CO_2 (Ci)	0.0000(***)	0.9267(ns)	0.0269(*)	0.3186(ns)	0.0089(**)	0.6068(ns)	0.0001(***)
4	Transpiration (E)	0.0000(***)	0.0000(***)	0.0000(***)	0.0000(***)	0.0002(***)	0.8369(ns)	0.0228(*)
9	Chl a	0.0000(***)	0.1015(ns)	0.0147(*)	0.0001(***)	0.1011(ns)	0.3984(ns)	0.0003(***)
10	Chl b	0.0000(***)	0.7391(ns)	0.7322(ns)	0.0010(**)	0.8281(ns)	0.9007(ns)	0.0019(**)
11	Total Chl	0.0000(***)	0.1729(ns)	0.0446(*)	0.0002(***)	0.2064(ns)	0.5314(ns)	0.0004(***)
12	Carotenoids	0.0000(***)	0.0091(**)	0.2938(ns)	0.0001(***)	0.0205(*)	0.2481(ns)	0.0007(***)
13	Chl a/b	0.0000(***)	0.0002(***)	0.0000(***)	0.0001(***)	0.0011(**)	0.1815(ns)	0.1183(ns)
15	MDA	0.0000(***)	0.0000(***)	0.0000(***)	0.0000(***)	0.0000(***)	0.0063(**)	0.0018(**)

*Statistical significance at level of 0.05 (*); 0.01 (**); 0.001 (***) and ns denotes not significant

real time PCR (qRT-PCR) were same as published in a previous study (Sailaja et al. 2014a).

RNA Isolation and qRT-PCR

Total RNA and small RNA were isolated using mirVana miRNA isolation Kit (Ambion, Austin, TX, USA) from fourth leaf at vegetative stage and flag leaf at reproductive stage. RNA was isolated from control as well as heat stress samples. cDNA synthesis from mRNAs was done using Improm-II reverse transcription system (Promega) and qRT-PCR was performed using SYBR Premix Ex-Taq (Takara). Actin was chosen as an internal control. For miRNAs quantification, cDNA synthesis was done using miscript II reverse transcription kit (Qiagen). Quantitative PCR (qRT-PCR) was performed using miscript SYBR green PCR kit (Qiagen). U6 SnRNA was chosen as an internal control. Reactions of qRT-PCR and analysis using a comparative threshold cycle (C_T) were performed as described earlier (Livak and Schmittgen 2001; Sailaja et al. 2014a; Mangrauthia et al. 2017). Three biological replicates and three technical replicates were used for the experiment.

Results

N22 and Vandana Showed Different Expression Patterns of Protein-Coding Genes after Stress Treatments

Heat stress showed distinct phenotypic responses in N22 and Vandana at seedling and reproductive growth stages. N22 showed lesser necrosis, chlorosis, and rolling of leaves than Vandana (Fig. 1). Both the genotypes showed drastically different expression patterns of genes at three growth stages after the heat stress treatments (Fig. 2). At seedling stage, N22 showed up-regulation of 3 genes while Vandana showed up-regulation of 8 genes after LDS. In contrast, N22 showed up-regulation of 13 genes while Vandana showed up-regulation of 10 genes after REC. Both the genotypes showed similar response in number of upregulated genes after SDS. At vegetative stage, N22 showed up-regulation of 10 genes while Vandana showed up-regulation of 3 genes after SDS. In contrast, Vandana showed up-regulation of 12 genes while N22 showed up-regulation of 7 genes after LDS. Both the genotypes showed similar response in number of upregulated genes after REC. At reproductive stage, N22 showed up-regulation of 10 genes while Vandana showed up-regulation of 4 genes after LDS. In contrast, Vandana showed up-regulation of 13 genes while N22 showed up-regulation of 9 genes after REC. Both the genotypes showed

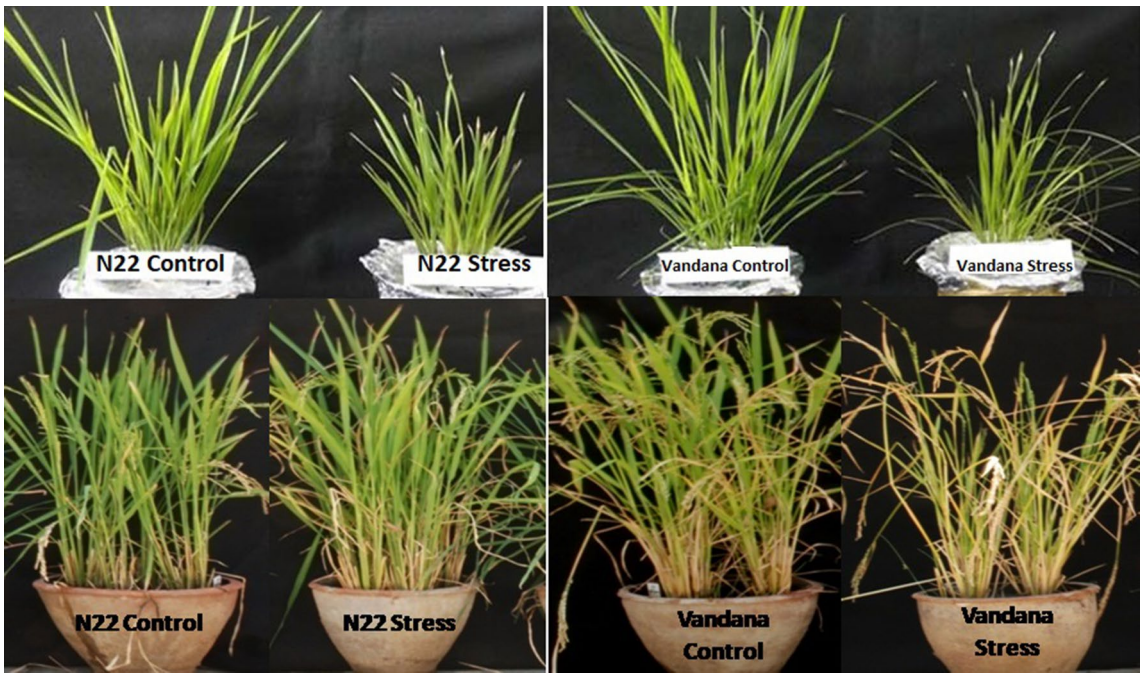

Fig.1 Effect of long duration heat stress (42 °C/36 °C, day/night for 5 days) on N22 and Vandana at seedling (upper panel) and reproductive (lower panel) growth stages. Vandana showed more leaf necrosis and rolling than N22

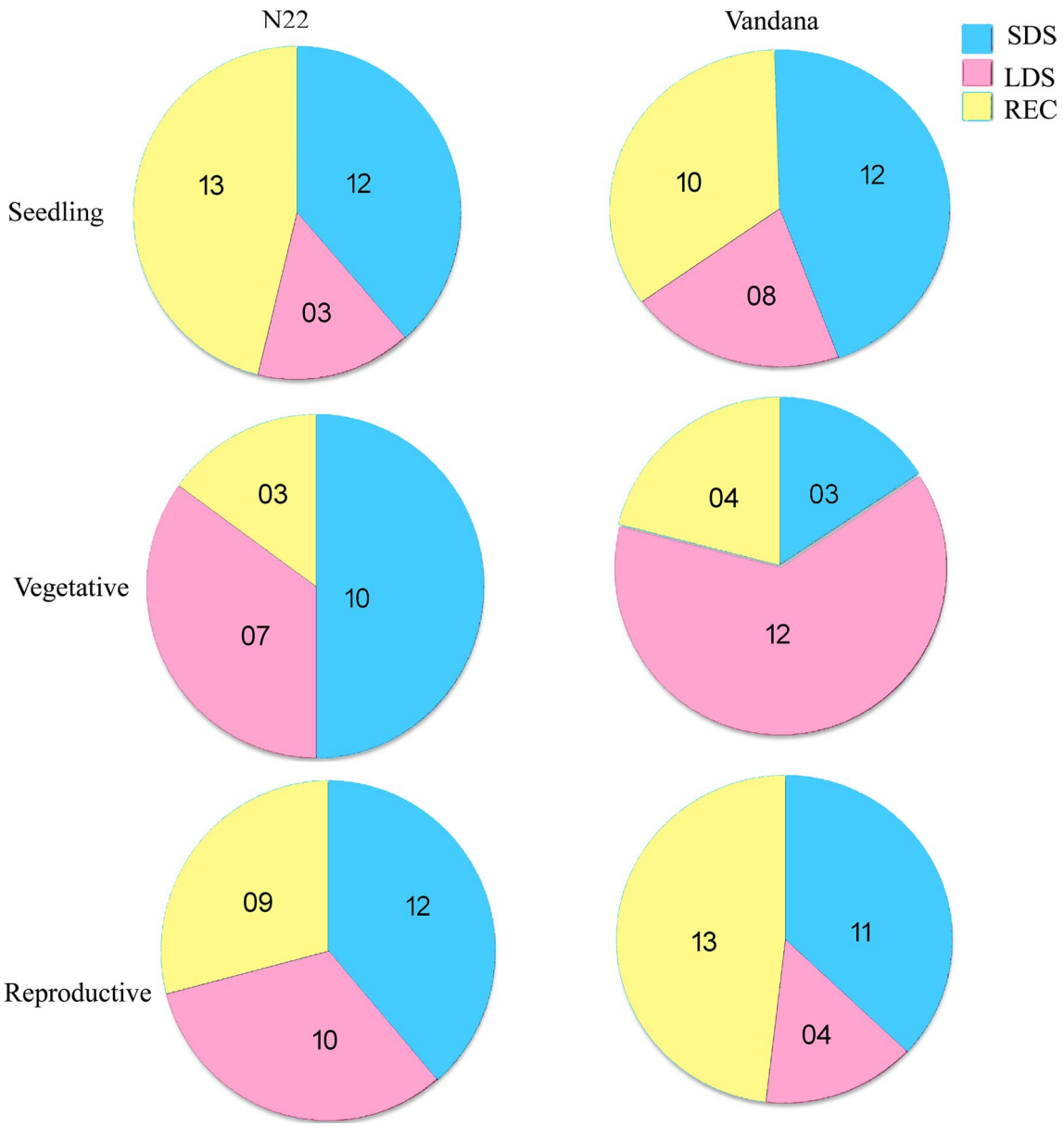

Fig. 2 An overview of number of genes showing up-regulation after heat stress treatments (SDS-short duration stress, LDS-long duration stress, and REC-recovery) in N22 and Vandana at seedling, vegetative, and reproductive growth stages

similar response in number of upregulated genes after SDS. Overall, the tolerant genotype N22 showed more number of up-regulated genes after REC at seedling stage, after SDS at vegetative stage, and after LDS at reproductive stage.

Heat Shock Transcription Factors

Among the three heat shock transcription factors (Hsfs), *OshsfA2a* showed similar expression pattern at seedling and vegetative stages in N22. Its expression was upregulated after SDS and REC but downregulated after LDS. Notably, it showed very high expression at reproductive stage after each of the three heat stress treatments, and particularly after SDS and LDS, i.e. 49.0 and 8.80 fold up-regulation, respectively. In contrast, the susceptible cultivar Vandana showed downregulation of *OshsfA2a* after SDS and LDS at reproductive stage (Fig. 3a). *OshsfA2e* showed similar expression pattern at vegetative and reproductive stages of N22 after heat stress treatments. It was upregulated after SDS and LDS but downregulated after REC. Its expression was increased to 6.9 fold at reproductive stage after SDS. Expression pattern of this gene at seedling stage was opposite to the expression pattern

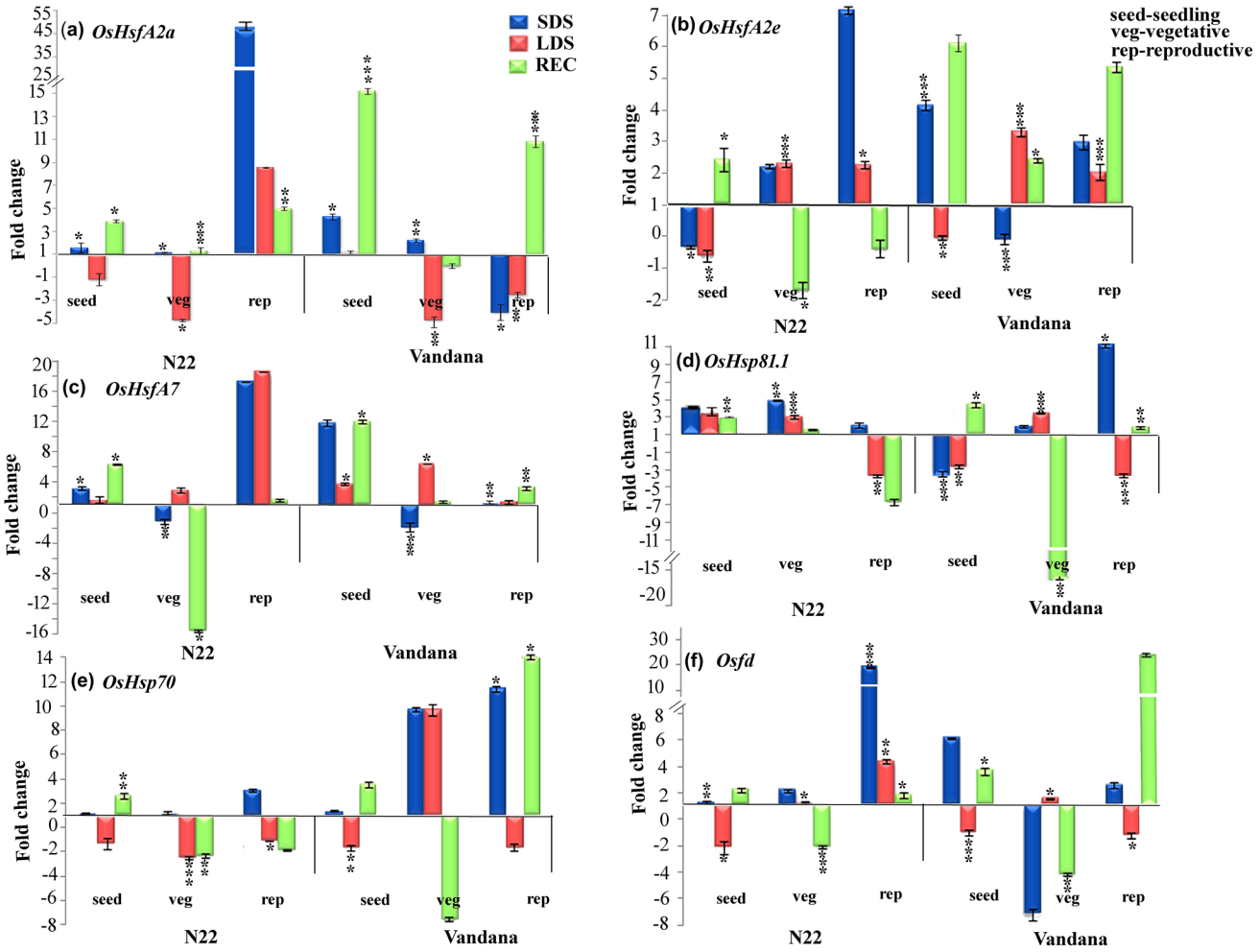

Fig. 3 Expression analysis of genes encoding heat-shock transcription factors *(OsHsfA2a, OsHsfA2e,* and *OsHsfA7)*, heat shock proteins *(OsHsp81.1* and *OsHsP70)* and an iron sulfur cluster-binding protein (*Osfd*) in different growth stages of N22 and Vandana after heat stress treatments. X axis: samples; Y axis: fold change in gene expression with respect to control sample. Error bars indicate the mean ± S.E. of three biological replicates. Statistical significance was determined by performing one-way ANOVA analyses (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

obtained in other two growth stages after heat stress treatments. In Vandana, its expression was increased at reproductive stage after all the three heat treatments, however, the level of up-regulation was higher in tolerant cultivar N22 after SDS and LDS treatments (Fig. 3b). Another heat shock transcription factor, *OshsfA7* showed increased expression at seedling and reproductive stages of N22 after all the heat treatments. However, its expression was more at reproductive stage, i.e.17.0 and 18.0 fold up-regulation after SDS and LDS treatments, respectively. In contrast, the level of up-regulation of this gene was relatively very less in susceptible cultivar Vandana after heat treatments at reproductive stage (Fig. 3c). The heat shock transcription factors showed higher expression in N22 than Vandana after heat treatments at reproductive stage.

Heat-Shock Proteins

In N22, *OsHsp81.1* transcript showed increased expression at seedling and vegetative stages after heat treatments. Whereas at reproductive stage, it showed decreased expression after LDS and REC. Vandana showed increased expression after SDS treatment at vegetative and reproductive stages, and maximum expression was noticed at reproductive stage (11.0 fold up-regulation). After LDS, expression decreased at seedling and reproductive stages while at vegetative stage it increased by 3.4 fold (Fig. 3d). *OsHsp70* transcript showed increased expression after SDS in all the three growth stages of N22. In Vandana, increased expression of *OsHsp70* after SDS was more pronounced to the extent of 9.0 fold (vegetative) and 11.0 fold (reproductive). Its expression was reduced in both the genotypes after LDS

at all the growth stages except for the vegetative stage of Vandana. Increased expression after REC was observed at seedling stage of both the genotypes and reproductive stage of Vandana (Fig. 3e). After heat treatments, the fold change expression of heat shock proteins was more in susceptible cultivar Vandana than N22 at reproductive stage.

Iron Sulfur Cluster Binding Protein (Osfd)

Osfd, a gene encoding a putative iron-sulfur cluster-binding protein showed increased expression in N22 after heat stress treatments at reproductive stage. In Vandana, higher expression of *Osfd* transcript was observed after SDS at seedling and reproductive stages by 6.0 and 2.0 fold, respectively. N22 showed up-regulation while Vandana showed downregulation after LDS at reproductive stage (Fig. 3f). Further, N22 showed ~ 19.5-fold up-regulation in comparison to 2.5-fold up-regulation in Vandana after SDS at reproductive stage. Therefore, the fold change expression of *Osfd* was more in N22 than Vandana at reproductive stage after SDS and LDS treatments.

Sucrose Phosphate Synthase (SPS)

SPS showed increased expression after three heat stress treatments at reproductive stage of N22. Specifically, its expression was 6.0 (SDS) and 13.6 (LDS) fold more in comparison to control. Further, up-regulation of this gene was observed in all the three growth stages after SDS. Vandana also showed increased expression after all the heat stress treatments at reproductive stage, however, the degree of up-regulation was less compared to N22. Notably, N22 showed 13.6 fold up-regulation in comparison to 1.8 fold up-regulation observed in Vandana after LDS at reproductive stage (Fig. 4a).

Superoxide Dismutase (SOD)

In N22*, SOD* was up-regulated at seedling stage but downregulated at reproductive stage after heat-stress treatments. At vegetative stage, it was upregulated after SDS and LDS but downregulated after REC. Similar to N22, Vandana showed decreased expression of *SOD* at reproductive stage after heat-stress treatments (Fig. 4b).

Cytochrome C Oxidase (Cyt-C-Oxi)

Cyt-C-Oxi showed increased expression at reproductive stage of N22 after heat-stress treatments. At seedling and vegetative stages, increased expression was noticed after SDS treatment. In Vandana, the gene showed enhanced expression at seedling stage after the heat treatments. At reproductive stage, N22 showed 14.2 and 7.2 fold up-regulation while Vandana showed 1.3 fold up-regulation and 5.0-fold downregulation after SDS and LDS, respectively (Fig. 4c).

Transcription Factors

Three transcription factors- SPL, NF-Y, and ARF showed similar expression patterns in N22 at seedling stage, i.e. up-regulation after SDS and REC and downregulation after LDS. Similarly, in vegetative stage, *SPL* and *NF-Y* genes showed similar expression pattern i.e. up-regulation after SDS and downregulation after LDS and REC. *SPL* and *ARF* showed up-regulation and *NF-Y* showed downregulation after all the heat stress treatments in reproductive stage. Similar to N22, Vandana showed up-regulation of *ARF* after heat treatments at reproductive stage. Notably, *SPL* was 10.8 fold upregulated in N22 but 2.0 fold downregulated in Vandana after LDS at reproductive stage. (Fig. 4d, e,f).

Cell Wall Integrity Protein (CWIP) and Fertility Restorer Homologue (FRH)

CWIP showed up-regulation in N22 after all the heat treatments while Vandana showed downregulation after LDS at reproductive stage. Maximum up-regulation of 12.5 fold was noticed at reproductive stage of N22 after SDS. Similar expression pattern of this gene was observed in both the genotypes at seedling sage. Vandana showed significant downregulation (33.0 fold) of *CWIP* at vegetative stage after SDS (Fig. 5a). *FRH* showed very high expression at reproductive stage of N22 i.e. 80.0 and 24.0-fold up-regulation after SDS and LDS, respectively. On the contrary, Vandana showed downregulation of this gene after SDS and LDS. The extent of up-regulation of *FRH* was more in N22 than Vandana after REC (Fig. 5b). Overall, the heat tolerant N22 showed higher expression of these genes than the susceptible cultivar Vandana after heat treatments at reproductive stage.

N22 and Vandana Showed Different Expression Patterns of microRNAs After Heat Stress Treatments

At seedling stage, N22 showed up-regulation of 7 miRNAs while Vandana showed up-regulation of only one miRNA after REC. In contrast, at vegetative stage, N22 showed up-regulation of only one miRNA while Vandana showed up-regulation of 6 miRNAs after REC. At reproductive stage, N22 showed up-regulation of 7 miRNAs while in Vandana, none of the miRNAs were upregulated after REC. Significant difference in the number of upregulated miRNAs of two genotypes was observed after LDS treatment at seedling and reproductive stages (Fig. 6). Taken together, Vandana showed more number of up-regulated miRNAs after SDS

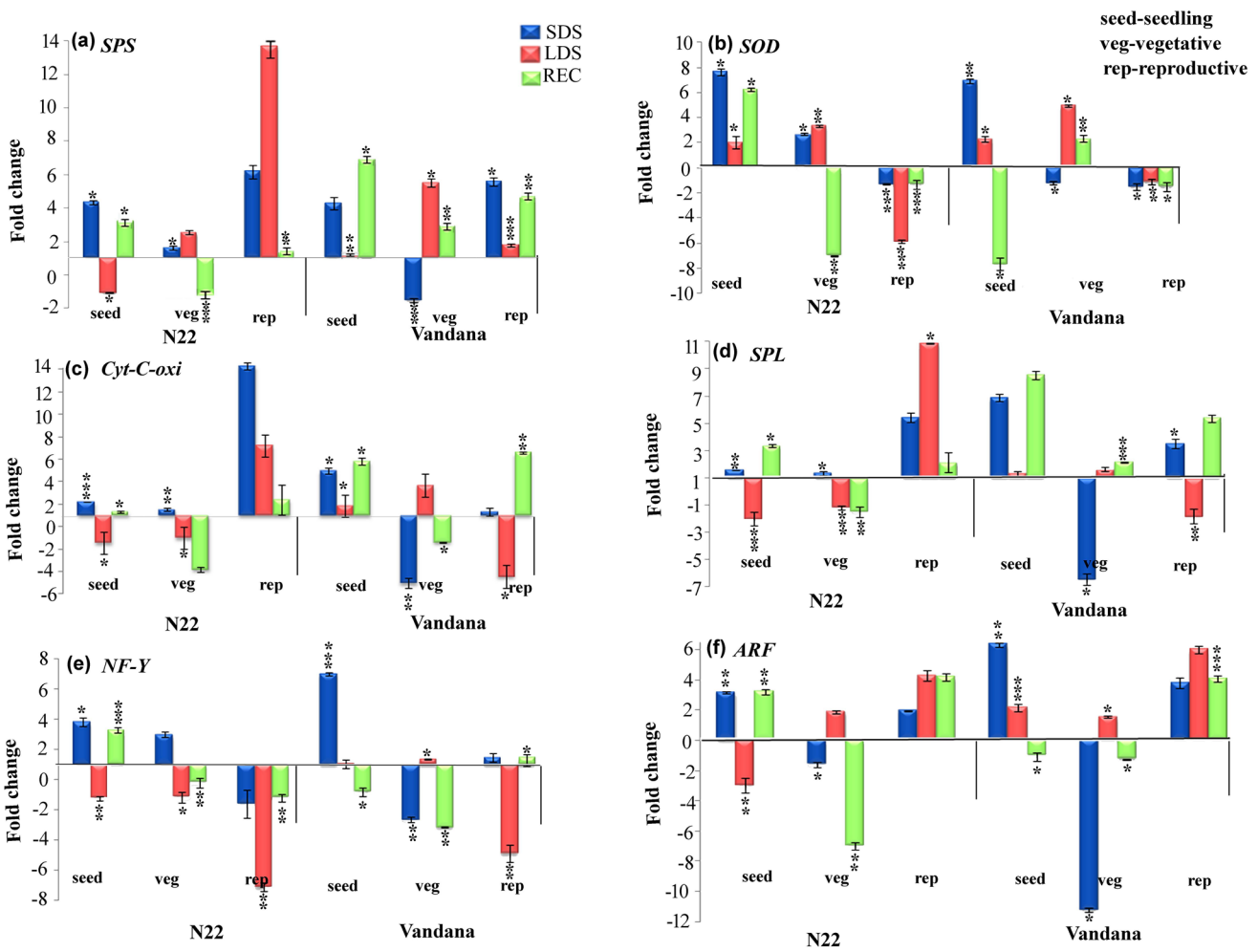

Fig. 4 Expression analysis of gene-encoding sucrose phosphate synthase *(SPS)*, superoxide dismutase *(SOD)*, cytochrome-C oxidase (*Cyt-C-oxi*), and transcription factors squamosa promoter-binding protein (*SPL*), nuclear transcription factor-Y (*NF-Y*) and auxin response factor (*ARF*) in different growth stages of N22 and Vandana after heat stress treatments. X-axis: samples; Y-axis: fold change in gene expression with respect to control sample. Error bars indicate the mean ± S.E. of three biological replicates. Statistical significance was determined by performing one-way ANOVA analyses (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

and LDS treatments while N22 showed more number of upregulated miRNAs after REC.

miR156

N22 showed downregulation while Vandana showed up-regulation after SDS and LDS treatments at seedling and reproductive stages. N22 showed 9.0 and 8.3 fold downregulation while Vandana showed 1.8 and 1.13 fold up-regulation of miR156 at reproductive stage after SDS and LDS treatments, respectively. In contrast, N22 showed up-regulation while Vandana showed downregulation after REC at seedling and reproductive stages (Fig. 7a). At reproductive stage, N22 showed 6.0 fold up-regulation but Vandana showed 66.6 fold downregulation of miR156 after REC.

miR160

miR160 showed up-regulation after SDS and REC treatments and downregulation after LDS at seedling and reproductive stages of N22. In comparison to N22, Vandana showed opposite expression of this miRNA at reproductive stage (Fig. 7b). N22 showed 3.0 and 2.8 fold up-regulation while Vandana showed 2.7 and 12.5 fold downregulation of miR160 at reproductive stage after SDS and REC, respectively. Further, N22 showed 1.16 fold downregulation but Vandana showed 4.6 fold up-regulation of miR160 at reproductive stage after LDS.

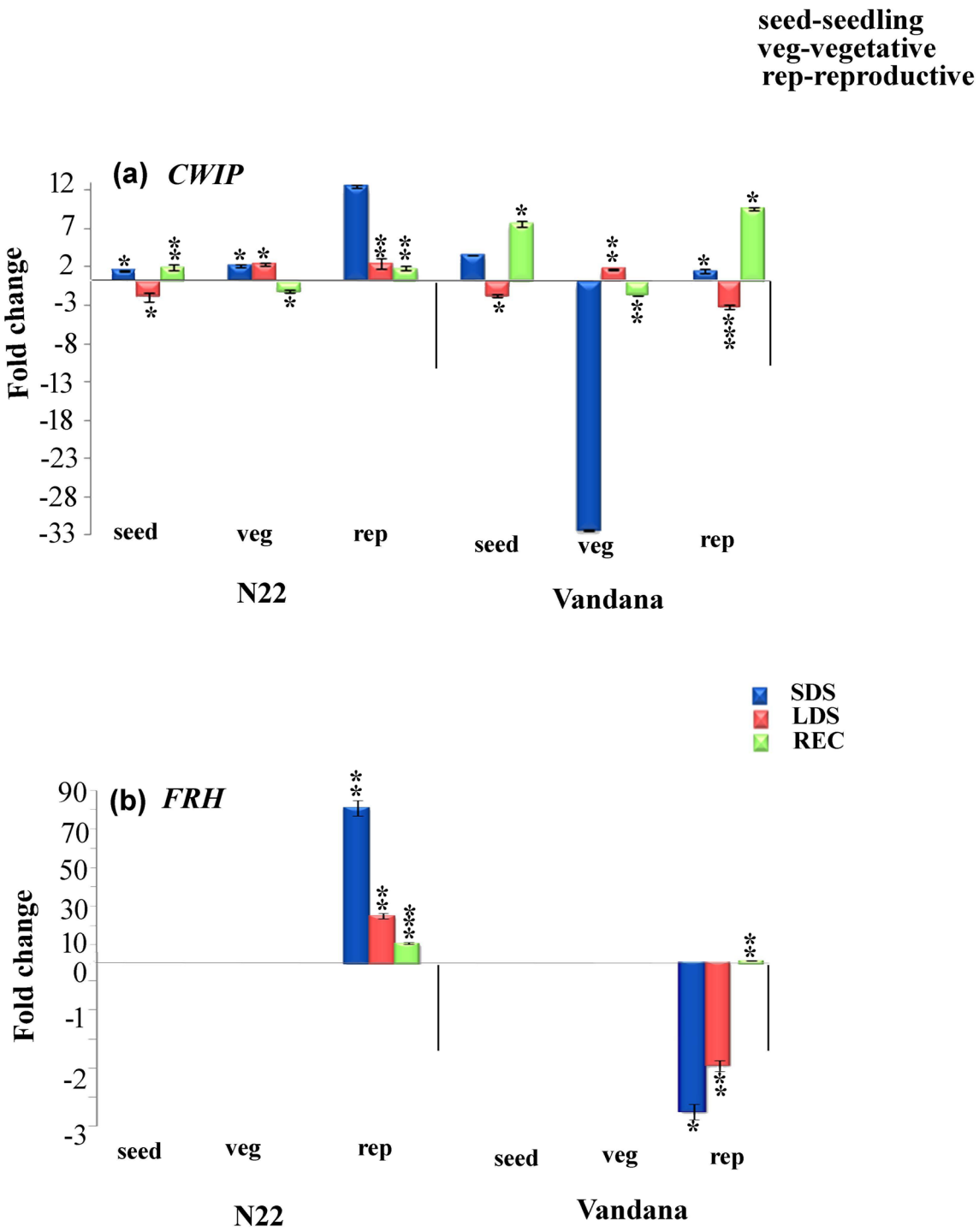

Fig. 5 Expression analysis of genes encoding cell wall integrity protein *(CWIP)*, and fertility restorer homologue (*FRH*) in different growth stages of N22 and Vandana after heat stress treatments. X-axis: samples; Y-axis: fold change in gene expression with respect to control sample. Error bars indicate the mean ± S.E. of three biological replicates. Statistical significance was determined by performing one-way ANOVA analyses (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

miR162

miR162 showed downregulation after SDS and LDS during three growth stages of N22. Vandana showed increased expression by 18.0 fold after SDS at reproductive stage. Both the genotypes showed opposite expression patterns in three growth stages after REC and it was more pronounced at reproductive and seedling stages (Fig. 7c). N22 showed 1.3

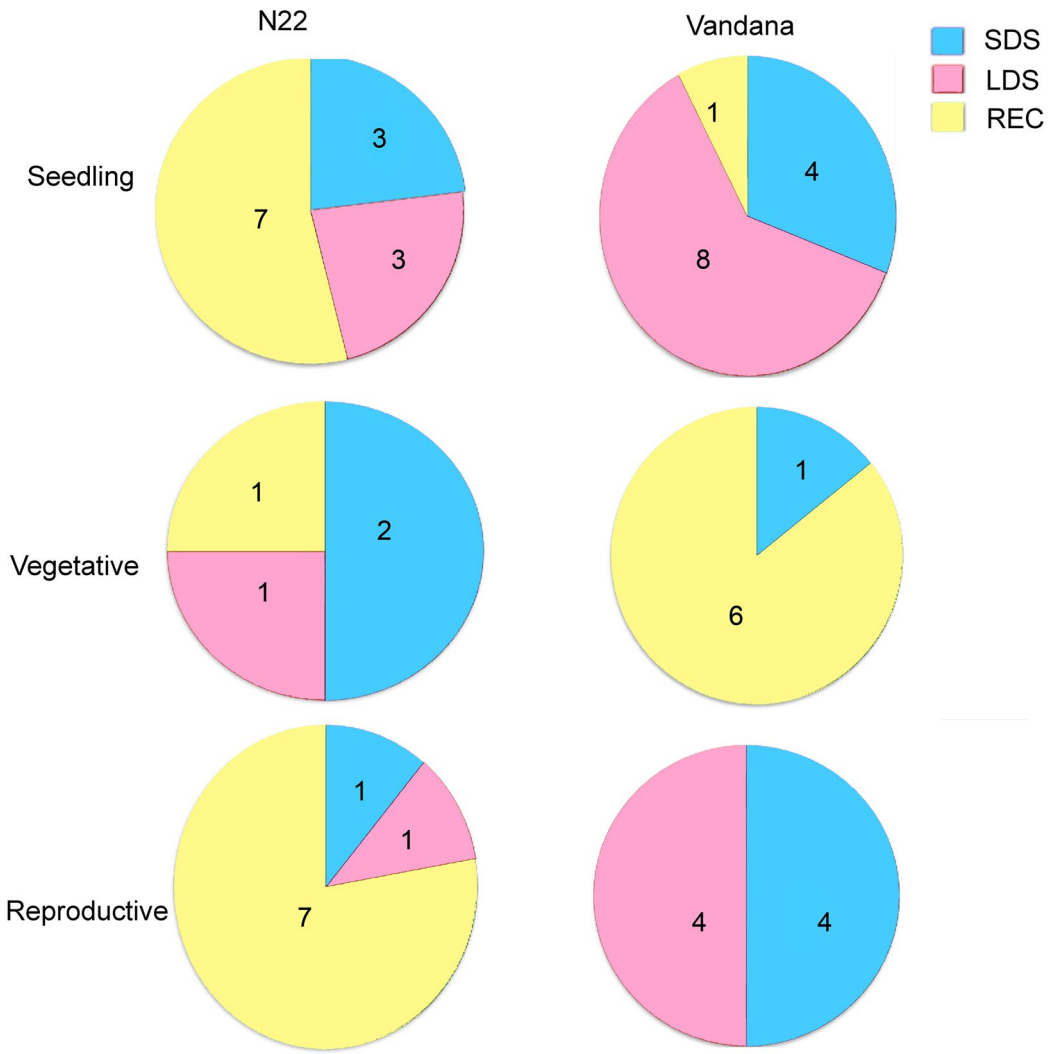

Fig. 6 An overview of number of microRNAs showing increased expression after heat stress treatments in N22 and Vandana at different growth stages

and 8.3 fold up-regulation while Vandana showed 35.0 and 9.0 fold downregulation after REC at seedling and reproductive stages, respectively. Further, N22 showed 17.5 fold downregulation while Vandana showed 18.6 fold up-regulation of miR162 at reproductive stage after SDS. After LDS, N22 showed 33.3 fold downregulation in comparison to 5.5 fold down-regulation in Vandana at reproductive stage.

miR167

miR167 showed downregulation (1.6 fold) in N22 and up-regulation (2.2 fold) in Vandana after SDS at seedling stage. Conversely, at vegetative stage, up-regulation in N22 (2.0 fold) and downregulation (8.8 fold) in Vandana was observed after SDS. At reproductive stage, contrasting expression pattern of miR167 was noticed after REC, i.e. up-regulation (4.0 fold) in N22 and downregulation (15.0 fold) in Vandana (Fig. 7d).

miR168

N22 showed similar expression patterns at seedling and reproductive stages, i.e. downregulation after SDS and LDS and up-regulation after REC. However, its expression pattern at vegetative stage was opposite to the expression pattern obtained at seedling and reproductive stages. Expression pattern of miR168 in Vandana was opposite to N22 at vegetative and reproductive stages. Specifically, at reproductive stage, N22 showed significant downregulation after SDS (6.0 fold) and LDS (87 fold) while Vandana showed up-regulation after SDS (42.0 fold) and LDS (5.0 fold). Further, N22 showed up-regulation (29.7 fold) while Vandana showed downregulation (1.5 fold) after REC (Fig. 7e).

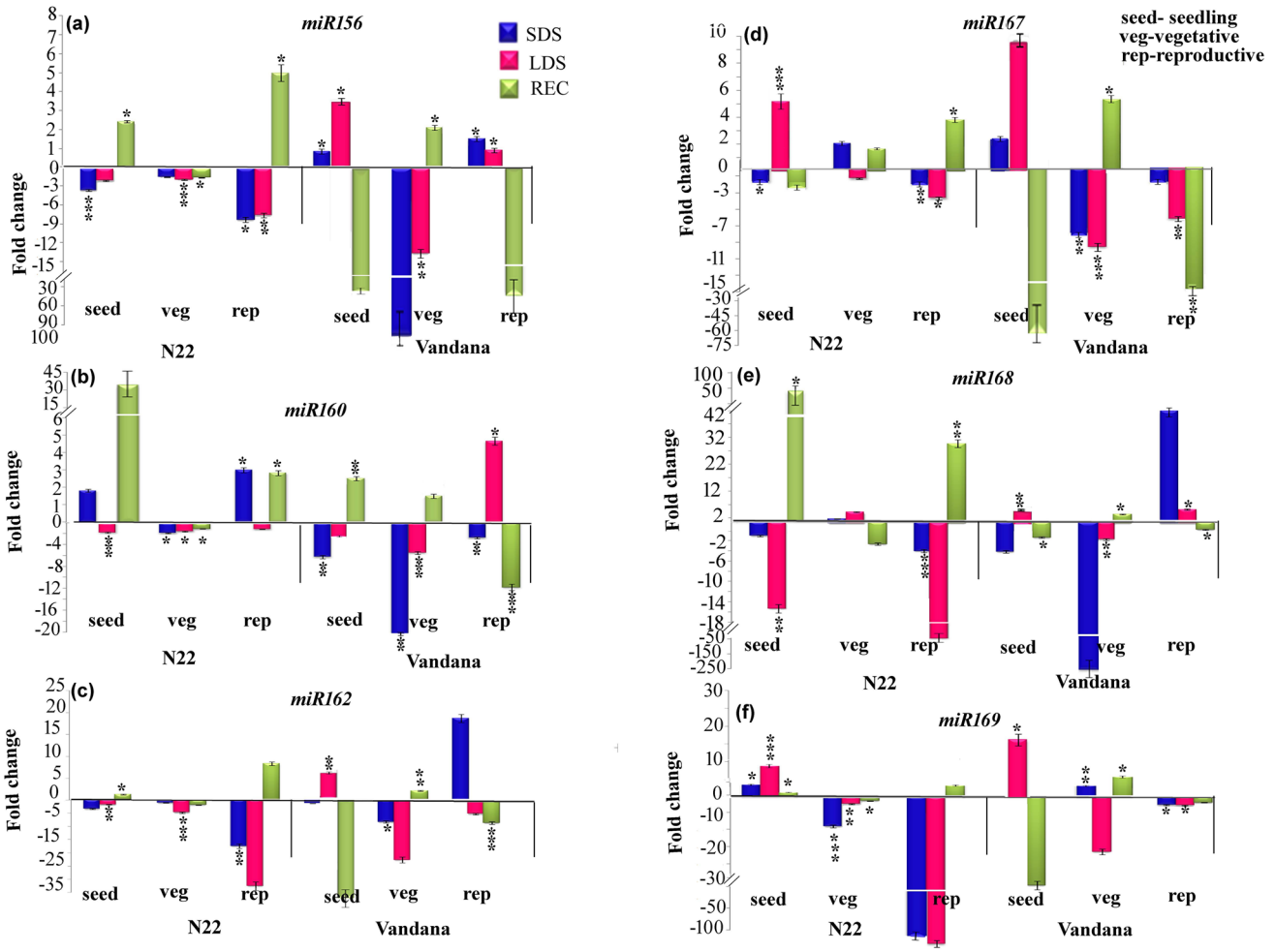

Fig. 7 Expression analysis of microRNAs (miR156, miR160, miR162, miR16, miR168, and miR169) in different growth stages of N22 and Vandana after heat stress treatments. X-axis: samples; Y-axis: fold change in miRNA expression with respect to control sample. Error bars indicate the mean ± S.E. of three biological replicates. Statistical significance was determined by performing one-way ANOVA analyses (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

miR169

miR169 showed contrasting expression pattern after heat treatments at seedling and vegetative stages of N22. It was upregulated at seedling stage but downregulated at vegetative stage after SDS, LDS, and REC. Significant downregulation of this miRNA in N22 was noticed after SDS (101 fold) and LDS (112 fold) at reproductive stage. Similar to N22, Vandana also showed downregulation after SDS (5.5 fold) and LDS (6.0 fold) at reproductive stage, however, the degree of downregulation was very high in N22 as compared to Vandana. Expression pattern was opposite in N22 and Vandana after REC at reproductive stage, where N22 showed up-regulation (2.0 fold) while Vandana showed downregulation (1.80 fold) (Fig. 7f).

miR397

In N22, miR397 showed downregulation at three growth stages after SDS and LDS treatments. There was no expression at reproductive stage after LDS. N22 showed 130 fold downregulation while Vandana, showed 32.0 fold up-regulation after SDS at reproductive stage. Similarly, N22 did not show expression of miR397 while Vandana showed 5.40 fold up-regulation after LDS at reproductive stage. After REC, N22 showed up-regulation by 16.0 fold while Vandana showed downregulation by 190 fold at seedling stage (Fig. 8a).

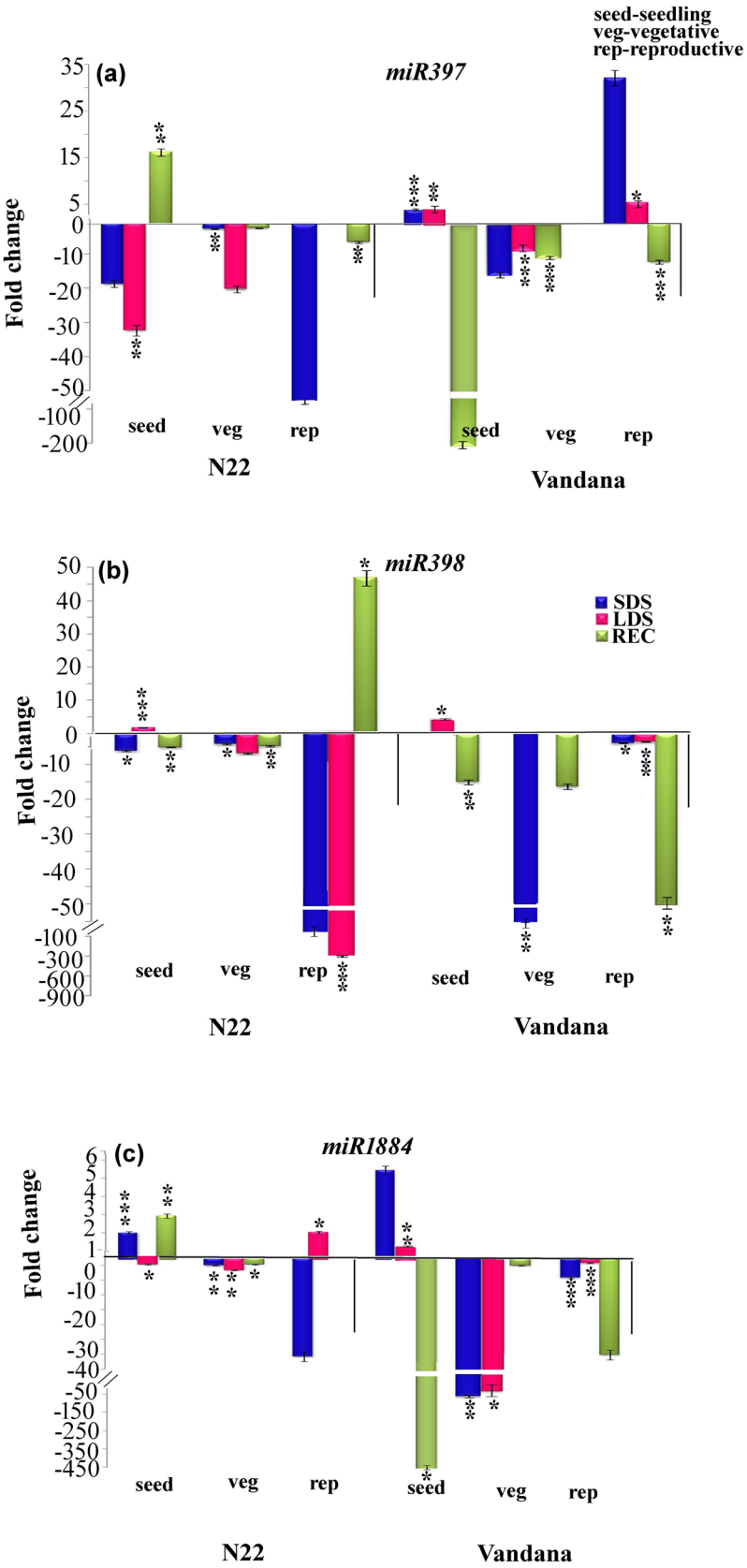

Fig. 8 Expression analysis of microRNAs (miR397, miR398, miR1884) in different growth stages of N22 and Vandana after heat stress treatments. X axis: samples; Y axis: fold change in miRNA expression with respect to control sample. Error bars indicate the mean ± S.E. of three biological replicates. Statistical significance was determined by performing one-way ANOVA analyses (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$)

miR398

N22 and Vandana showed similar expression pattern of miR398 at three growth stages after heat treatments except for REC at reproductive stage. Notably, the degree of downregulation of miR398 was very high in N22 as compared to Vandana after SDS and LDS at reproductive stage. N22 showed 193.4 and 312 fold downregulation in comparison to 2.5- and 2.1-fold downregulation obtained in Vandana at reproductive stage after SDS and LDS, respectively. Further, N22 showed 47 fold up-regulation while Vandana showed 49 fold downregulation after REC at reproductive stage (Fig. 8b).

miR1884

In N22, miR1884 showed up-regulation only after SDS and REC at seedling stage and LDS at reproductive stage. For all other treatments, it was downregulated at three growth stages of N22. Similarly, in Vandana, it was downregulated at three growth stages and heat treatments except at seedling stage SDS and LDS. Contrasting expression pattern between N22 and Vandana was observed at reproductive stage-LDS and seedling stage-REC (Fig. 8c). N22 showed 33.3 fold downregulation in comparison to 6.36 fold downregulation obtained in Vandana at reproductive stage after SDS. Further, N22 showed 2.0 fold up-regulation while Vandana showed 1.44 fold downregulation of miR1884 at reproductive stage after LDS. After REC, N22 did not show any expression while Vandana showed 33 fold down-regulation of this miRNA at reproductive stage.

Chlorophyll Pigments

Chl a, Chl b, and total chlorophyll content were measured in control and heat stress-treated plants of N22 and Vandana ($P < 0.001$) at vegetative and reproductive growth stages. Statistical analysis showed that chlorophyll pigments were significantly affected after heat treatments (Table 1). At vegetative stage, N22 showed 17%, 32%, and 50% reduction of Chl a after SDS, LDS, and REC, respectively. Chl b was less affected after the heat treatments as only 13% reduction after LDS and 34% reduction after REC was recorded. Total chlorophyll content was reduced to the extent of 14%, 28.2%, and 47% after SDS, LDS, and REC, respectively. The carotenoids content was reduced by 21%, 35%, and 46% after SDS, LDS, and REC, respectively. At reproductive stage of N22, Chl a was not affected after SDS (0.97 mg/g FW) when compared with control (0.96 mg/g FW), but it was reduced by 8% and 24% after LDS and REC, respectively. Notably, the content of Chl b was increased by 28% and 16% after SDS and LDS, respectively. After REC, the content of Chl b was restored to the level of control. Total chlorophyll content was not much affected after SDS (1.29 mg/g FW) and LDS (1.17 mg/g FW) as compared to control (1.21 mg/g FW), however, a reduction of 19% was recorded after REC. Similarly, carotenoids content was not significantly affected after SDS (0.56 mg/g FW), LDS (0.52 mg/g FW), and REC (0.50 mg/g FW) treatments when compared with control (0.52 mg/g FW) at reproductive stage (Fig. 9).

The susceptible genotype Vandana showed 51% and 52% reduction of Chl a after LDS and REC treatments at vegetative stage. Similarly, 27% and 17% reduction of Chl b, and 46% and 44% reduction of total chlorophyll was noticed after LDS and REC treatments, respectively. Chl a, b, and total chlorophyll were not affected after SDS treatment. Carotenoids content was reduced by 10%, 54%, and 57% after SDS, LDS, and REC, respectively. At reproductive stage, Vandana showed 41%, 61%, and 62% reduction of Chl a after SDS, LDS, and REC, respectively. Similarly, 32%, 48%, and 49% reduction of Chl b, and 39%, 59%, and 60% reduction of total chlorophyll was recorded after SDS, LDS, and REC, respectively. Carotenoids content was reduced by 43%, 57%, and 55% after SDS, LDS, and REC, respectively at reproductive stage (Fig. 9).

Malondialdehyde (MDA) Content

Significant differences were observed in MDA content after heat stress treatments at vegetative and reproductive stages of N22 and Vandana ($P < 0.001$). Both the genotypes showed similar trend of lipid peroxidation at vegetative stage, as indicated by MDA content after heat stress treatments. The MDA content in N22 was increased from 0.65 µM mg-^{1}FW (control) to 3.34 µM mg-^{1}FW after SDS, 5.21 µM mg-^{1}FW after LDS, and restored to 0.20 µM mg-^{1}FW after REC. Similarly, the MDA content in Vandana was increased from 1.94 µM mg-^{1}FW (control) to 3.98 µM mg-^{1}FW after SDS, 8.79 µM mg-^{1}FW after LDS, and restored to 1.13 µM mg-^{1}FW after REC. At reproductive stage, the MDA content in N22 was increased from 0.62 µM mg-^{1}FW (control) to 1.89 µM mg-^{1}FW after LDS and 1.72 µM mg-^{1}FW after REC. Notably, it was decreased to 0.48 µM mg-^{1}FW after SDS. In Vandana, LDS treatment showed an increase in MDA content (3.05 µM mg-^{1}FW) while it was decreased after SDS (1.60 µM mg-^{1}FW) and REC (0.48 µM mg-^{1}FW), in comparison to control (1.86 µM mg-^{1}FW) (Fig. 10).

Gas Exchange Parameters

The net photosynthesis rate (P_N), conductance (g_s), transpiration rate (E), and intercellular CO_2 concentration (Ci) were measured in control and heat-treated samples of both the genotypes at vegetative and reproductive growth stages (Table 2). Significant differences were observed between treatments ($P < 0.001$) for all the gas exchange parameters analyzed here.

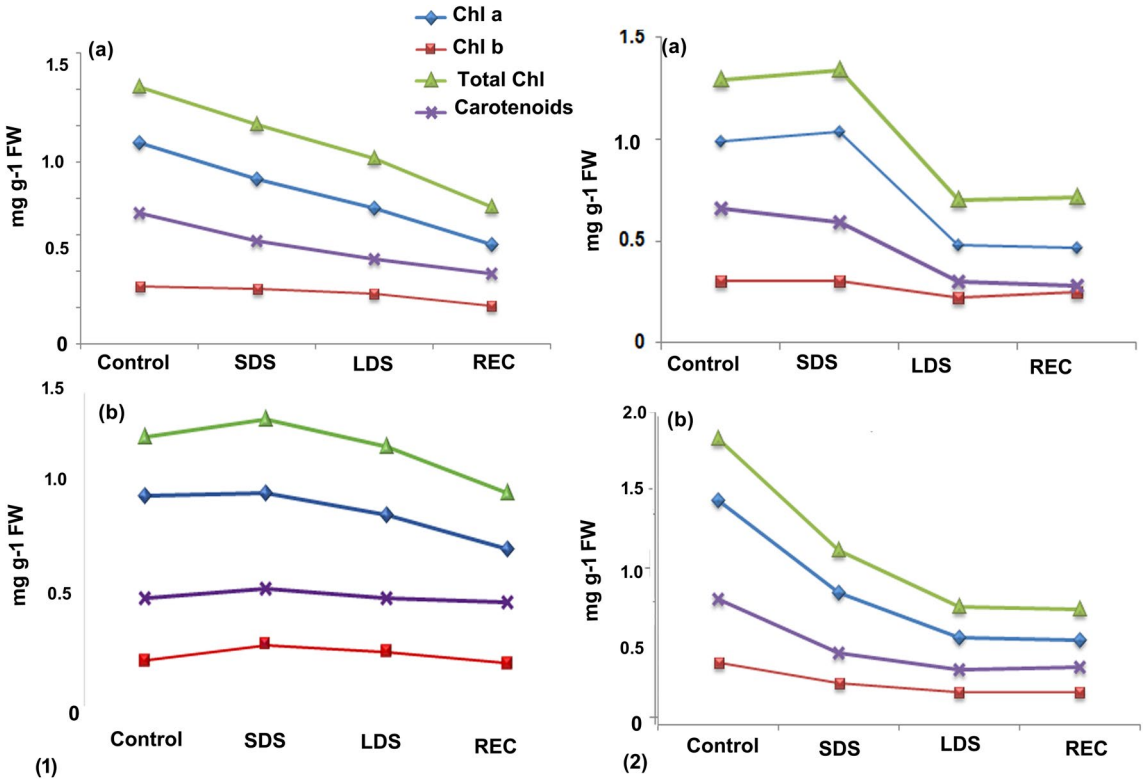

Fig. 9 Effect of high temperature stress on chlorophyll and carotenoids content of N22 (1) and Vandana (2) at vegetative (**a**) and reproductive (**b**) growth stages. The chlorophyll and carotenoids content were measured in control as well as heat-treated samples of N22 and Vandana. The values shown in graphs represent the mean of three biological replicates

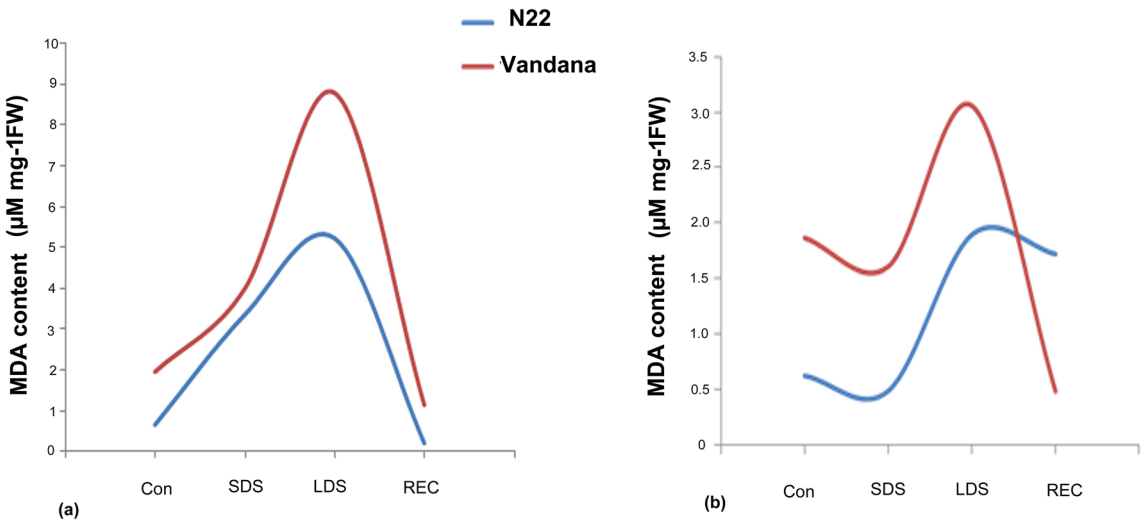

Fig. 10 Effect of high temperature stress on malondialdehyde (MDA) content of N22 and Vandana at vegetative (**a**) and reproductive (**b**) growth stages. The MDA content was measured in control as well as heat-treated samples of N22 and Vandana. The values shown in graphs represent the mean of three biological replicates

Significant differences were observed ($P < 0.001$) between the genotypes also. In comparison to control, LDS and REC samples showed a reduction of P_N by 54% and 42% in N22, and 90% and 83% in Vandana, respectively at vegetative stage. Similarly, at reproductive stage, P_N was reduced by 25% and 49% in N22, and 77% and 86% in Vandana after LDS and REC

Table 2 Gas exchange parameters measured in N22 and Vandana after heat stress treatments at vegetative and reproductive growth stages

Stage	Cultivar	Parameter	Control	SDS	LDS	REC
Vegetative stage	N22	Photosynthesis rate (P_N) umol m^{-2} s^{-1}	13.22^{abcd}	9.00^{efgh}	6.08^{hi}	7.62^{gh}
		Stomatal conductance(g_s) mol m^{-2} s^{-1}	0.088^{cdef}	0.176^{abcd}	0.153^{bcd}	0.112^{cdef}
		Intercellular CO_2 concentration (C_i)	153.7^{de}	293.3^{ab}	310.3^{ab}	244.0^{abcde}
		Transpiration rate mmol[H_2O]m^{-2} s^{-1}	5.1^{d}	7.8^{bcd}	7.2^{bcd}	5.9^{d}
	Vandana	Photosynthesis rate (P_N) umol m^{-2} s^{-1}	12.7^{bcd}	7.5^{gh}	1.2^{j}	2.1^{j}
		Stomatal conductance(g_s) (mol m^{-2} s^{-1})	0.118^{bcdef}	0.151^{bcde}	0.015^{f}	0.021^{ef}
		Intercellular CO_2 concentration (C_i)	211.0^{bcde}	300.1^{ab}	254.3^{abcd}	212.6^{bcde}
		Transpiration rate (mmol[H_2O]m^{-2} s^{-1})	5.53^{d}	6.93^{cd}	0.74^{f}	1.23^{f}
Reproductive stage	N22	Photosynthesis rate (P_N) umol m^{-2} s^{-1}	16.4^{a}	13.9^{bcde}	12.3^{fgh}	8.4^{abc}
		Stomatal conductance(g_s) mol m^{-2} s^{-1}	0.209^{abc}	0.208^{a}	0.288^{cdef}	0.110^{abc}
		Intercellular CO_2 concentration (C_i)	244.5^{abcde}	261.7^{ab}	308.3^{abcd}	256.2^{abc}
		Transpiration rate mmol[H_2O]m^{-2} s^{-1}	9.1^{abc}	10.2^{a}	11.5^{de}	5.0^{ab}
	Vandana	Photosynthesis rate (P_N) umol m^{-2} s^{-1}	14.50^{abc}	15.32^{ij}	3.36^{j}	2.04^{ab}
		Stomatal conductance(g_s) mol m^{-2} s^{-1}	0.100^{cdef}	0.247^{bcdef}	0.140^{def}	0.071^{ab}
		Intercellular CO_2 concentration (C_i)	146.41^{e}	272.39^{a}	331.83^{a}	336.41^{ab}
		Transpiration rate mmol[H_2O]m^{-2} s^{-1}	5.74^{d}	10.32^{d}	5.73^{ef}	1.93^{ab}

Means followed by different letters in the same column are significantly different at $P < 0.05$ according to Fisher's LSD test

treatments, respectively. Stomatal conductance was increased by 100%, 74%, and 27% in N22 after SDS, LDS, and REC treatments, respectively at vegetative stage, whereas Vandana showed 27% increase of g_s after SDS, and 87% and 82% decrease after LDS and REC, respectively. At reproductive stage, g_s was increased by 37% after LDS and decreased by 47% after REC in N22, while Vandana showed 147% and 40% increase after SDS and LDS, respectively, and 29% decrease after REC. Intercellular CO_2 concentration was increased by 90%, 102%, and 58% after SDS, LDS, and REC, respectively, at vegetative stage in N22, while Vandana showed 42% and 20% increase after SDS and LDS respectively. At reproductive stage, Ci was increased by 7%, 26%, and 4.7% in N22, and 86%, 126%, and 129% in Vandana after SDS, LDS, and REC treatments, respectively. Transpiration rate was increased by 53%, 41%, and 15% at vegetative stage of N22 after SDS, LDS, and REC treatments, respectively, while Vandana showed 25% increase after SDS but 86% and 78% decrease after LDS and REC, respectively. At reproductive stage, N22 showed an increase of *E* by 12% and 26% after SDS and LDS respectively and 45% decrease after REC, while Vandana showed 80% increase after SDS and 66% decrease after REC (Table 2).

Discussion

This study was aimed to analyze the physiological and molecular changes in heat-tolerant (N22) and susceptible (Vandana) rice cultivars at different growth stages and heat treatments. N22 has been used for mapping QTLs/genes associated with high-temperature tolerance in rice (Jagadish et al. 2008; Poli et al. 2013; Prasanth et al. 2016; Shanmugavadivel et al. 2017; Kilasi et al. 2018). Similarly, Vandana has been used as susceptible check cultivar for heat-tolerance studies along with N22 because of the similarity in their maturity duration and growth stages (Jagadish et al. 2008; Sailaja et al. 2014a; Mangrauthia et al. 2017). In previous studies, we have demonstrated the contrasting response of N22 and Vandana during high-temperature stress (Sailaja et al. 2014a, b, 2015; Mangrauthia et al. 2017, 2018).

Gene expression analysis and physiological observations of two contrasting rice cultivars at three growth stages suggested that heat tolerant N22 uses its molecular and physiological machinery more efficiently at reproductive stage after heat treatments. Among the three growth stages, reproductive stage showed the most distinctly different response between N22 and Vandana. *OshsfA2a, OsHsfA7, Osfd, SPS, Cyt-C-oxi, SPL, ARF, CWIP*, and *FRH* showed up-regulation in N22 at reproductive stage after SDS, LDS, and REC. Conversely, the susceptible cultivar Vandana did not show much efficient gene regulation after heat treatments, and differences between two cultivars were more obvious at the crucial reproductive stage. Among the 14 genes analyzed here, N22 showed up-regulation of 10 genes in comparison to 4 up-regulated genes of Vandana after LDS at reproductive stage. Most of the genes were downregulated in reproductive stage of Vandana after LDS treatment, suggesting that long exposure of high temperature is detrimental to susceptible genotype. *OshsfA2a, Cyt-C-Oxi, SPL, CWIP,* and *FRH* genes showed 8.8, 14.2, 10.8, 2.5, and 25 fold up-regulation in N22 while in Vandana the same set of genes showed downregulation by 2.5, 5.0, 2.0, 3.5, and 1.8 fold after LDS at reproductive stage, respectively. The heat shock transcription factor *OshsfA7* was 18.0 fold up-regulated in N22 in comparison to 1.4 fold up-regulation in Vandana after LDS at reproductive stage. Similarly, sucrose phosphate synthase coding gene showed 13.6 fold up-regulation in N22 in comparison to 1.8 fold up-regulation in Vandana after LDS at reproductive stage. Taken together, the gene expression analysis clearly suggests that N22 regulates its genes more efficiently during prolonged heat stress at reproductive stage which ensures higher grain yield in N22 than in Vandana under high temperature stress (Sailaja et al. 2015; Prasanth et al. 2017). Similar to protein-coding genes, expression pattern of miRNAs was distinct between both the genotypes at reproductive stage. miR156, miR160, and miR168 showed opposite expression patterns between N22 and Vandana at reproductive stage after each of the three heat treatments. Members of miR156 family showed induced expression during heat in *Arabidopsis* (Stief et al. 2014), *Brassica rapa* (Yu et al. 2012), and wheat (Xin et al. 2010; Kumar et al. 2014) but repressed in cassava (Ballen-Taborda et al. 2013). Other miRNAs also showed opposite expression patterns in either one or two heat treatments. Most of the miRNAs showed a significant degree of downregulation in N22 suggesting the up-regulation of their target genes. Similar to protein-coding genes, miRNAs also showed very distinct expression patterns between N22 and Vandana after LDS treatment at reproductive stage.

The expression analysis of protein-coding genes and miRNAs was further supported by the physiological observations. N22 showed 8% reduction while Vandana showed 61% reduction of Chl a after LDS at reproductive stage. Further, N22 showed 16% increase while Vandana showed 48% reduction of Chl b after LDS at reproductive stage. In an interesting study, photo-protective response was affected and CO_2 assimilation was decreased in Chl b-deficient mutant lines of wheat under high temperature, suggesting a close connection between chl b content and efficiency of CO_2 assimilation capacity under heat stress (Brestic et al. 2016). The carotenoids content was unaffected in N22 while it was reduced by 57% in Vandana after LDS at reproductive stage. Carotenoids are known to shield chlorophylls and help in photosynthesis and stress acclimation in leaves (Dhami and Cozzaneli 2020). The net photosynthetic rate was reduced by 25% in N22 and 77% in Vandana after LDS at reproductive

stage. The lesser reduction in P_N may contribute to heat tolerance ability of N22 (Krishnan et al. 2011; Shi et al. 2015). These physiological observations provide clues to how heat tolerant N22 performs better than Vandana during prolonged high temperature stress at reproductive stage.

Majority of genes showed differential expression pattern at different growth stages of a genotype. For example, expression pattern of *OshsfA2e* at seedling stage of N22 was contrary to the expression pattern obtained in its vegetative and reproductive growth stages. Likewise, Vandana showed opposite expression pattern of *OshsfA2e* at seedling and vegetative stages after SDS and LDS. *Osfd* was up-regulated after REC at seedling and reproductive growth stages but downregulated at vegetative growth stage of both the genotypes. Similar to protein-coding genes, expression of miRNAs was also highly dynamic at different growth stages of rice cultivars. To mention few, miR156 was up-regulated after REC at seedling and reproductive stages but downregulated at vegetative stage of N22 while Vandana showed upregulation at vegetative stage and downregulation at seedling and reproductive stages. Similarly, miR167 was up-regulated after LDS at seedling stage but down-regulated at vegetative and reproductive stages of both the genotypes. Briefly, the expression pattern of protein-coding genes and miRNAs was highly dynamic at different growth stages and heat treatments in both the genotypes. This suggests that expression of these genes is very much tightly regulated in plants which are controlled by the nature of stress and growth stage. Tissue and treatment specific induced expression of heat shock transcription factors *OsHsfA2a* and *OsHsfA7* has been reported previously (Chauhan et al. 2011).

Overall, the molecular and physiological studies suggested that both N22 and Vandana have clear differences in regulation of gene expression and physiological processes, and the differences are more pronounced during reproductive stage and long duration heat stress treatment. This study brings valuable information about protein-coding genes and miRNAs regulation at different growth stages and heat treatments in susceptible and tolerant rice cultivars. The information on differential regulation of protein-coding genes and miRNAs at different growth stages and heat treatments will be highly useful for deploying the protein-coding genes or miRNAs for incorporating heat stress tolerance trait in commercial rice cultivars through breeding/transgenic/gene editing approaches.

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Author Contributions SKM, NS, SRV- conceived and designed the experiments; SB, MSB, KNS, TV- performed the experiments; SB, DS, SKM- analyzed the data; DS and PRR- assisted in physiology experiments; SKM, SB, NS—drafted the manuscript.

Compliance with Ethical Standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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