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Comparative Study of Susceptible and Tolerant Genotype Reveals Efficient Recovery and Root System Contributes to Heat Stress Tolerance in Rice

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Abstract In changing climate scenario, heat stress caused by increased atmospheric CO_2 is a major concern for rice productivity. There is a need to decipher the mechanisms of heat stress susceptibility and tolerance response of rice cultivars considering that high temperature is detrimental to growth and development of rice crop. The present study was designed to understand the heat stress response in heat-susceptible (Vandana) and heat-tolerant (N22) cultivars of rice. Rice seedlings were subjected to short-duration (24 h, SDS) and long-duration (5 days, LDS) heat stress (42 °C/36 °C, day/night). Besides the heat stress, recovery response (REC) of both the cultivars was also studied. Physiological parameters (chlorophyll content and membrane thermostability) and root/shoot length analysis revealed that N22 has better efficiency in recovering from heat stress. In particular, root tissue of N22 showed increased thermotolerance during SDS and LDS when compared with Vandana. In addition to physiological studies, gene expression pattern of 13 genes including heat shock transcription factors and heat shock proteins and 9 microRNAs (miRNAs) was analyzed in root and shoot of both the genotypes during various treatments. Gene and miRNA expression studies showed that root tissue of N22 was more responsive during SDS and LDS, suggesting important function of roots in heat stress tolerance. Further, during recovery, root tissue of both the genotypes showed more significant change in gene expression than shoot which signifies the vital role of plant root system in heat stress recovery response. Very high expression of an unknown iron-sulfur cluster-binding protein *OsFd* involved in electron transport activity was observed in root tissue of N22 during all the stress treatments. This study shows that better recovery and efficient root system play an important role in heat tolerance trait of N22.

Keywords N22 · Vandana · *HSP* · *OsHsf* · miRNAs · *Oryza sativa*

Abbreviations

CWIP	Cell wall integrity protein
SOD	Superoxide dismutase
SPS	Sucrose phosphate synthase
SPL	Squamosa promoter binding protein
NF-Y	Nuclear transcription factor-Y
ARF	Auxin response factor
HSP	Heat shock protein
Hsf	Heat shock transcription factor
miRNA	microRNA

Introduction

Abiotic stresses such as drought, salinity, and extreme temperature lead to an average yield loss of >50 % in most of the crop plants (Boyer 1982; Bray et al. 2000). Heat stress due to increased temperature is a growing agricultural problem limiting plant growth and productivity in many parts of the world. Transitory or constantly high temperatures cause an array of morpho-anatomical, physiological, biochemical, and molecular changes in plants, which affect plant growth and

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development and may lead to a drastic reduction in economic yield (Wahid et al. 2007). Plants have evolved a variety of responses and mechanisms to elevated temperatures that minimize damage and ensure protection of cellular homeostasis. Understanding the plant adaptation and tolerance mechanism to heat stress is crucial for the development of heat-tolerant cultivars for improving productivity in warm climatic regions.

The projected rise in global mean temperature is expected to have a serious impact on rice production and productivity (Matsui et al. 2007), which is cultivated in diverse ecosystems facing threat of extreme temperatures (Mohammed and Tarpley 2009). Heat stress is one of the most detrimental abiotic stresses for shoot and root growth of rice, and it is expected to become a more significant problem in the future. Root growth is more sensitive than shoot growth to elevated temperatures because of their lower optimal growth temperature (Huang et al. 2012). However, most of the studies performed in rice heat stress have been confined to shoot tissue, and the emphasis is generally on stress response rather than recovery. Recovery from heat stress is equally important for high-temperature tolerance in plants. Recovery from high-temperature stress can be evaluated by comparing the growth and development of rice plants grown in heat stress and followed by optimum temperature conditions after a specified period of heat stress. The ability to recover from stress, speed of regrowth, and vigor upon recovery of rice vary with the developmental stage at which heat stress is given, the duration of stress, extent of stress, and other ambient conditions such as water status in soil, vapor pressure deficit, light conditions, and also the cultivars. Currently, very less information is available on heat stress recovery, which would be an important criterion in the selection of cultivars for heat stress tolerance.

Changes in the expression profile of some genes encoding important proteins may play an important role in plant adaptation to heat stress (Zou et al. 2009; Jung and An 2012; Sarkar et al. 2013). In rice, a large number of heat stress-responsive genes have been reported. These genes include heat shock proteins (*HSPs*), heat shock transcription factors (*Hsf*s), antioxidant enzymes, and various transcription factors (TFs) which are induced during stress conditions (Wahid et al. 2007; Jung et al. 2013; Sarkar et al. 2013). They function not only in the protection of cells from stress by production of important metabolic proteins but also in gene regulation and signaling. The accumulation of HSPs under the control of Hsfs is assumed to play a central role in heat stress response and in acquired thermotolerance in plants (Kotak et al. 2007; Vierling 1991; Gupta et al. 2010). Heat stress also alters the activity of antioxidant enzymes due to the increased production of reactive oxygen species (ROS) (Kochhar and Kochhar 2005). Most of these gene expression studies in rice have been performed under 1–2 h of heat stress. However, response of genes under prolonged heat stress is still not very clear, which is important in understanding the adaptive mechanism of rice genotypes during high temperature.

In addition to protein-coding genes, reports have shown that noncoding microRNAs (miRNAs) also play a crucial role during biotic and abiotic stresses in plants (Sunkar and Zhu 2004; Zhou et al. 2010; Jeong and Green 2013). MicroRNAs are small RNAs, 22–24 nt in size, known to play important regulatory roles in plants by cleavage of target mRNAs or by translational repression (Bartel 2004). There have been fewer studies on heat stress-associated miRNAs compared to other abiotic stress-responsive miRNAs (Jeong and Green 2013). In wheat, 12 miRNAs were identified in response to heat stress (Xin et al. 2010). Similarly, five conserved and four novel miRNAs were reported in *Brassica rapa* (Yu et al. 2011). In *Populus*, 52 miRNAs from 15 families were reported as heat stress-responsive (Chen et al. 2012). In rice, several miRNAs were reported under various abiotic stresses such as drought (Zhou et al. 2010), salinity (Zhao et al. 2009), and cold (Lv et al. 2010); however, very few (Jeong et al. 2011; Jeong and Green 2013; Sailaja et al. 2014) have reported miRNA expression during high temperature.

The present study was aimed at analyzing the effect of high-temperature treatment for 24 h and 5 days in tolerant and susceptible cultivars of rice. Two contrasting rice genotypes, Nagina22 (N22) as tolerant and Vandana as susceptible, were selected based on an earlier report (Jagadish et al. 2008). We studied the physiological traits and expression pattern of stress-associated genes in root and shoot tissues of both the cultivars after stress as well as after recovery from stress. Thirteen genes including *Hsfs*, *HSPs*, antioxidants, and transcription factors were selected for expression analysis. Further, expression analysis of nine miRNAs was performed in root and shoot tissues to understand the role of miRNAs in high-temperature response in heat-tolerant rice cultivar N22 and heat-susceptible Vandana.

Materials and Methods

Plant Materials

Rice (*Oryza sativa*) seeds of cultivars N22 and Vandana were surface-sterilized in 0.1 % $HgCl_2$ aqueous solution for 3–4 min followed by three to four washes with distilled water. Seeds were then germinated in petri dishes at 28 °C for 3 days on moist blotting paper in dark. After germination, sprouted seeds were transferred to plastic cups containing Yoshida medium (Yoshida et al. 1976). Seedlings were grown in a growth chamber under 13 h of light and 11 h dark by maintaining diurnal temperatures 30 °C day/24 °C night. Four batches of seedlings with 40 plants in each batch were maintained for both the cultivars. Another four batches of seedlings with similar conditions were kept as biological replicates.

Heat Stress Treatments

Eight-day-old seedlings were subjected to high temperature for heat stress treatment. Treatment of heat stress was given for short duration (24 h) and long duration (5 days). Two batches of seedlings were maintained in ambient temperature (30 °C/24 °C, day/night), while the other two batches were exposed to high temperature (42 °C/36 °C, day/night). Out of the two batches exposed to 42 °C/36 °C, one batch was maintained at this temperature for 5 days (termed as long-duration stress, LDS), while another batch was transferred back to 30 °C/24 °C on the 4th day after heat stress treatment. Therefore, this batch was subjected to 24 h of recovery (30 °C/24 °C) after a heat stress treatment of 4 days. Out of the remaining two batches maintained in ambient temperature, one batch was maintained in the same conditions for 5 days (termed as control), while the other batch was subjected to high-temperature treatment on the 4th day for 24 h (termed as short-duration stress, SDS). On the 13th day, a total of 16 samples were harvested from four batches (control, SDS, LDS, and recovery response (REC)) including root and shoot tissues of N22 and Vandana. These samples were used further for physiological and molecular studies.

Root and Shoot Length

The root and shoot length of 13-days-old N22 and Vandana seedlings was measured in 16 samples immediately after harvesting. Measurement was taken from control as well as heat stress samples. Ten seedlings of each set and biological replication were taken for this study, and a mean value was used to interpret the results.

Membrane Thermal Stability

The membrane thermal stability (MTS) of leaf samples was measured following the procedure described by Haque et al. (2009). The leaf samples of control and stress treatments were washed three times and collected in 15 ml sterile conical tubes with 10 ml deionized water. Two sets of each sample were prepared, one set designated as control was maintained at 28 °C, while the other set was treated in water bath at 52 °C for 1 h. Here, three replications were maintained for both sets. After the treatment, control and treated tubes were kept at room temperature for 24 h. The initial conductance was measured using conductivity meter. Thereafter, all the tubes were autoclaved at 121 °C at 15 lbs for 20 min, and the next day, the final conductance was measured. This ensures complete electrolyte leakage from the plant tissue. The relative injury (RI) was calculated using the following formula:

$$\text{Injury}(\%) = E{-}I/F \times 100$$

Where, I = initial conductance, E = elevated temperature conductance, and F = final conductance after autoclaving.

Chlorophyll Content

The chlorophyll content was quantified using the acetone method as described by Zhang et al. 2009. Total chlorophyll was extracted from 100 mg leaf sample in 25 ml of 80 % acetone. Using UV-Spectrophotometer, *Chla* and *Chlb* were measured at 663.2 and 646.8 nm wavelengths, respectively.

Statistical Analysis

Physiological parameters were analyzed statistically using two-way analysis of variance (ANOVA, Steel and Torrie 1997), and treatment means were compared using least significant difference (LSD) ($T < 0.05$) implemented in software Statistics 8.1.

Genes, miRNA Sources, and Primer Designing

Gene sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov), and miRNA sequences of *O. sativa* were downloaded from miRBase (http://microrna.sanger.ac.uk). Primers for gene quantitative polymerase chain reaction (qPCR) (Table 1) were designed using Primer 3 software (http://frodo.wi.mit.edu/). Mature miRNA-specific PCR forward primers were designed based on miRNA sequences (Table 2). Nine miRNAs, miR156, 160, 162, 167, 168, 169, 397, 398, and miR1884, were selected for real-time PCR quantification. These miRNAs were chosen based on the reports on the role of these miRNAs in abiotic stress response in various crops.

RNA Isolation

Total RNA and small RNA were isolated by using mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). RNA was isolated separately from root and shoot tissues subjected to control and stress treatments. Samples of both the cultivars were harvested from two biological replicates.

Quantitative Real-Time PCR of Gene Transcripts and miRNAs

Complementary DNA (cDNA) synthesis of mRNAs was done using Improm-II Reverse Transcription System (Promega), and quantitative real-time PCR (qRT-PCR) was performed using SYBR Premix Ex-Taq (Takara). Actin was chosen as an internal control, and all the reactions were run in triplicate. Amplification conditions maintained were 50 °C for

Table 1 List of genes and primer sequences used for gene expression analysis

S. No.	Gene name	Accession no.	Forward primer	Reverse primer
1	Sucrose-phosphate synthase 1 (*SPS*)	AK065273	GCCATATCGTGCGGATGTCA	CCTGCACTGGCATAGTGTCCA
2	*OsHsfA2a*	AK069579.1	TTCAAGCACAGCAACTTCTCCA	GCATCGCATTCCGCCATTG
3	*OsHsfA2e*	AK068660.1	TCCGCTGCTGCTGTTAGGTG	GAATCAGGCGAGCGCAATCT
4	*OsHsfA7*	AK064271.1	GGCTCCTGGTGAGGTTGTGA	TCCGCTGTTGCCTTCTCTCG
5	Cytochrome c oxidase assembly protein (*Cyt-C-Oxi*)	AK103187.1	GCTTACACGCTCAGGATTGTCG	CCAGCAGCCACTCTTCATCTG
6	*HSP70*	AK100676.1	TGAGGAGAAGATGAGCGTGGTG	CGCCGAAGCAGACAATAGCC
7	*HSP81.1*	AK102426.1	CTGGCTGCTGGTGCTGATGT	ATCGTTGTGCTTGGTGGTCA
8	Unknown protein similar to ferredoxin (*OsFd*)	AK100064	TGTGGTGGTCTGCCTAGTGA	GAGCCATAAGCATGGAGGAA
9	*CWIP*	AK242237	AAGAGAAGCTCGTGGATGCAGA	AGCAGCCGCCTCATGGCAAT
10	*SOD*	AK110251.1	CCAGTGAACACAATGCTGGA	CTTGAGCCAGACGAACAACA
11	Squamosa promoter-binding-like protein 10 (*SPL*)	AK068702.1	TGTGAATTGGATGACGCGAGA	TTGATGGAGCACCGGATCTTC
12	*NF-Y* subunit A-3	AK069348	TTCGTTCTATGGTGGTGCTGT	TTCGGTGGCTGGTTCTATTGG
13	*ARF*	AK243230	CCGAGATGTTCTGCATCGACA	ATGGTAAGCCTCCTCGCCAAC
14	ACTIN	AK100267	CAGCCACACTGTCCCCATCTA	AGCAAGGTCGAGACGAAGGA

10 min for preholding stage, 95 °C for 10 min for holding stage, 40 cycles of denaturing at 95 °C for 15 s and annealing/extension at 60 °C for 30 s, followed by a disassociation stage (melting curve analysis). Reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

For miRNA quantification, cDNA synthesis was done by using miScript II Reverse Transcription Kit (Qiagen). Quantitative PCR (qPCR) was performed using miScript SYBR Green PCR Kit (Qiagen). U6sn RNA was chosen as an internal control (Ding et al. 2011). Reactions were run in triplicate, and the amplification conditions maintained were 95 °C for 15 min, 40 cycles of denaturing at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s, followed by a disassociation stage (melting curve analysis).

Table 2 List of primer sequences utilized for miRNA expression study

S. No.	miRNA	Primer sequence
1	miR156	TGACAGAAGAGAGTGAGC
2	miR160	TGCCTGGCTCCCTGTATG
3	miR162	TCGATAAACCTCTGCATC
4	miR167	TGAAGCTGCCAGCATGAT
5	miR168	TCGCTTGGTGCAGATCGG
6	miR169	CAGCCAAGGATGACTTGC
7	miR397	TCATTGAGTGCAGCGTTG
8	miR398	TGTGTTCTCAGGTCACCC
9	miR1884	AATGTATGACGCTGTTGA
10	U6 snRNA	CGATAAAATTGGAACGATACAGA

Analysis

The comparative threshold cycle (C_T) method was used to quantify the relative expression levels in real-time PCR. ΔC_T was calculated by the difference between C_T target and C_T reference. Further $\Delta\Delta C_T$ values are calculated using the formulae $\Delta\Delta C_T = \Delta C_T$ of stress sample $- \Delta C_T$ control sample, and then fold difference was calculated from $2^{-\Delta\Delta Ct}$. Similarly, ΔC_T standard deviation was calculated as given at www3.appliedbiosystems.com/.../general documents/cms_042380.pdf. In case of downregulation of genes/miRs, $\Delta\Delta C_T$ calculation was positive. Here, if the test sample has a value of 0.20, it suggests 1/5 the amount of target RNA as the calibrator.

Results

Shoot/Root Length

Both shoot and root lengths showed significant difference in varieties, treatments, and variety × treatment interaction (Fig. 1a, b). After SDS, N22 showed 21 % reduction in shoot length, whereas Vandana showed 14 % reduction. Likewise, in LDS, N22 showed 36 % reduction, whereas Vandana showed only 25 % reduction. After REC, N22 shoot length was reduced by 26 %, while Vandana showed 31 % reduction as compared to control. Interestingly, there was a 10 % recovery in N22 and further reduction in Vandana shoot length when compared to LDS. After SDS, the root length increased by 16 % in N22 but decreased by 12 % in Vandana. After LDS, N22 root length decreased only 5.7 % while 24 % in

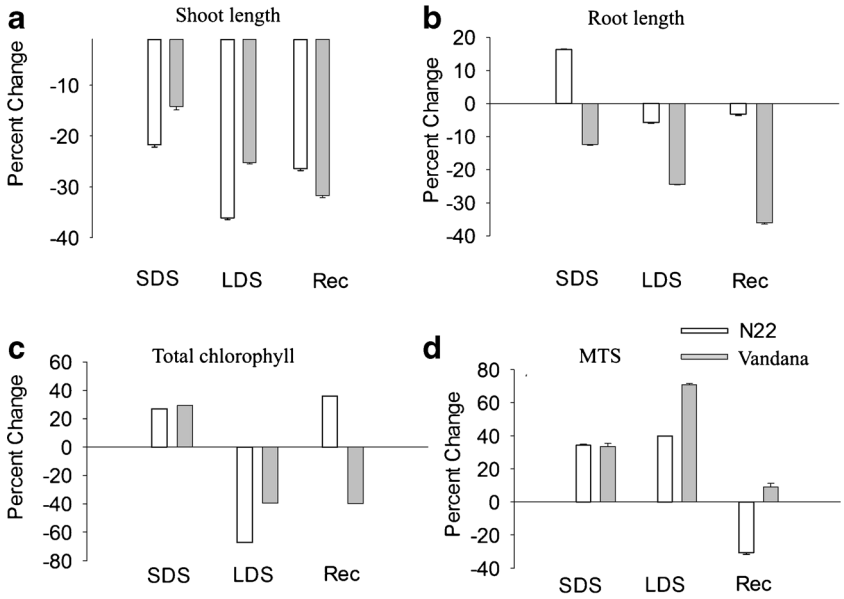


Fig. 1 Physiological studies of shoot and root of rice cultivars during short-duration stress (*SDS*), long-duration stress (*LDS*), and recovery (*REC*), **a** shoot length, **b** root length, **c** total chlorophyll content, and **d** membrane thermostability (*MTS*)

Vandana. Significantly, at REC, there was a reduction of only 3 % in N22 but 36 % in Vandana as compared to control. When recovery treatment was compared with LDS, there was an increase of 2.7 % in N22, while Vandana showed no recovery.

Chlorophyll Content and Relative Injury Score

Total chlorophyll content in shoot tissue of control and treated samples is shown in Fig. 1c. After SDS, total chlorophyll content increased to 26 % in N22 and 29 % in Vandana. After LDS, the reduction in chlorophyll content was 67 % in N22 and 39 % in Vandana. When REC was compared with LDS, 31.3 % increase of chlorophyll content was observed in N22, whereas Vandana showed no difference. In control conditions, the relative injury (RI) was 5.5 in N22 and 13.6 in Vandana. After SDS and LDS, the RI was increased by 34.2 and 39.9 % in N22 and 33.6 and 70.8 %, respectively in Vandana. After REC, N22 showed 30 % decrease in RI, whereas Vandana showed 8.9 % increase compared to control (Fig. 1d).

Expression of Selected Genes

The expression of 13 selected genes was compared in shoots and roots of N22 and Vandana each after SDS, LDS, and REC. In the case of N22, *OsHsfA2a* and *OsHsfA7* were upregulated in shoot, while *OsHsfA2e* and *OsHsfA7* were upregulated in root after SDS (Fig. 2a). The other transcripts *OsFd*, *SPS*, *SOD*, *Cyt-C-Oxi*, *SPL*, *NF-Y*, *ARF,* and *CWIP* also showed increased expression in both shoot and root, but expression of *Cyt-C-Oxi*, *SPL*, *ARF*, and *CWIP* was higher in root. Interestingly, *OsFd* transcript was significantly upregulated in root with 21-fold change. Thus, root showed higher expression of transcripts after SDS. After LDS, *OsHsfA2a*, *OsHsfA2e, OsFd*, *SPS*, *Cyt-C-Oxi*, *SPL*, *ARF,* and *CWIP* were downregulated in shoot but upregulated in root (Fig. 2b). *OsFd* transcript was highly upregulated (16.4-fold) in root as in SDS. Transcript expression after LDS was compared with the expression observed after SDS (Fig. 2c). Here, *OsHsfA2a*, *HSP81.1,* and *HSP70* displayed organ-specific expression being downregulated in shoot but upregulated in root. The other genes viz, *OsHsfA2e* and *OsHsfA7*, *OsFd*, *SPS*, *SOD*, *Cyt-C-Oxi*, *SPL*, *NF-Y*, *ARF,* and *CWIP* showed decreased expression in both the organs. After REC, all the transcripts were upregulated in both organs (Fig. 3a). *OsFd* (46-fold) and CWIP (15.2-fold) showed a remarkably high expression in root. Similarly, *Cyt-C-Oxi, SPL*, *ARF, OsHsfA7, OsHsfA2e,* and *OsHsfA2a* showed higher expression in root than shoot. When expression after REC was compared with LDS, all the genes showed upregulation except *OsHsfA7*, which was downregulated in root (Fig. 3b).

The susceptible cultivar Vandana was also analyzed for gene expression pattern in root and shoot during heat stress treatments. After SDS, all the transcripts except *HSP81.1* showed upregulation in both the organs (Fig. 4a). Further, all the genes except *OsHsfA7* and *SPS* showed higher expression in shoot than in root. Among the three Hsfs, *OsHsfA7* was significantly upregulated in shoot (11.8-fold) and root (13.3-fold) after SDS. After LDS, upregulation of *OsHsfA2a*, *OsHsfA7, SPS*, *SOD*, *Cyt-C-oxi*, SPL, *NF-Y*, and *ARF* in both the organs was recorded (Fig. 4b). Further, *OsHsfA2e, HSP81.1*, *OsFd,* and *CWIP* were downregulated in shoot and upregulated in root. Comparing LDS with SDS, *OsHsfA2a*, *OsFd*, *SOD,* and *Cyt-C-Oxi* showed downregulation in shoot and upregulation in root (Fig. 4c), and most of the

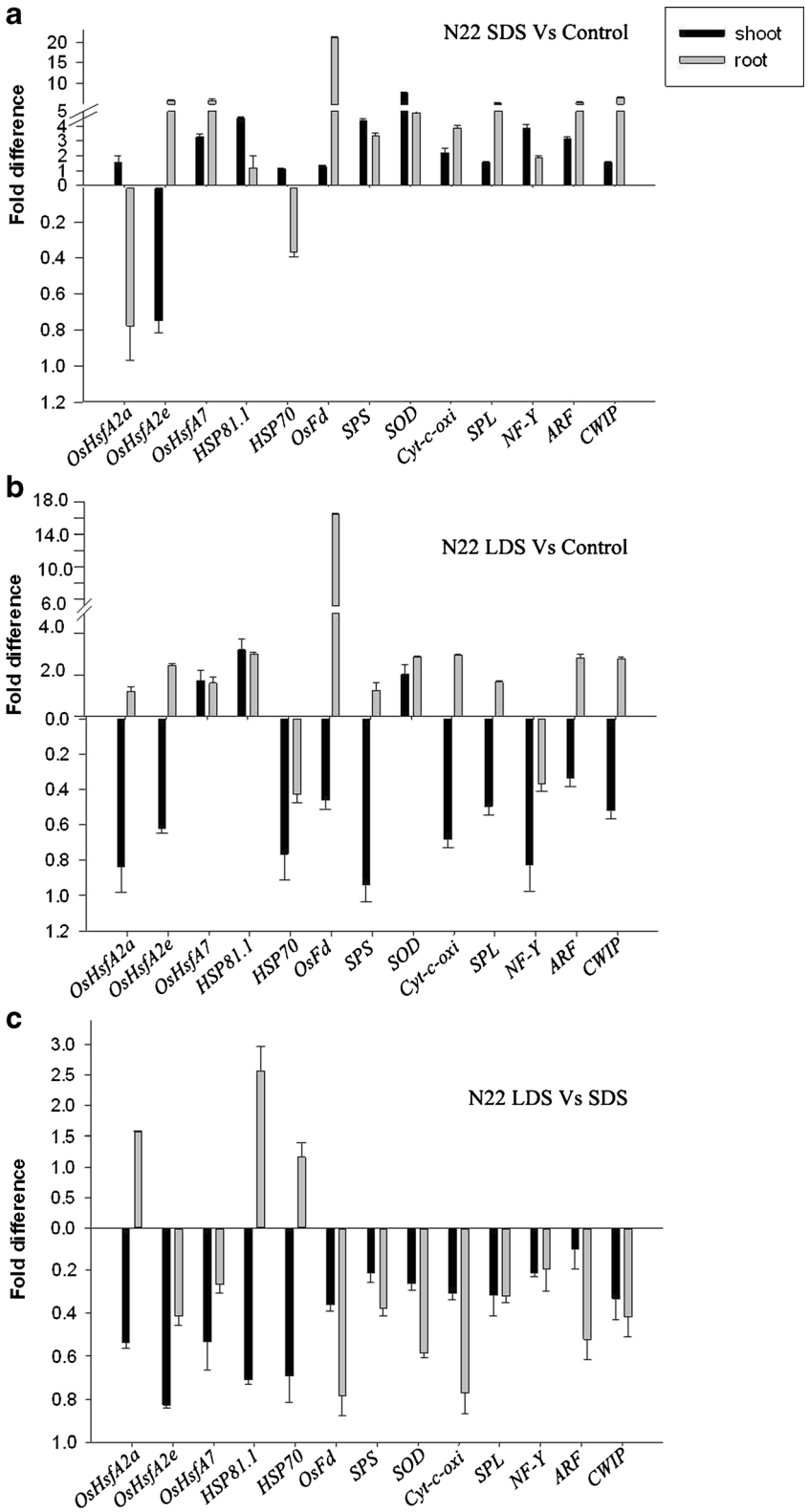


Fig. 2 Expression of 13 genes in shoot and root of heat-tolerant N22; **a** gene expression during SDS in comparison to control, **b** gene expression during LDS in comparison to control, and **c** gene expression during LDS in comparison to SDS

other genes were downregulated in both organs. After REC, most of the genes were upregulated in both organs compared to control (Fig. 5a). The increased expression of *OsHsfA2a* and *OsHsfA7* was 15.2- and 12-fold in shoot and 80.3- and 74.8-fold in root, respectively. Three genes *OsHsfA2e, NF-Y,* and *ARF* showed downregulation in shoot and upregulation in root. *OsFd*, *SPS*, *SPL,* and *CWIP* showed 16.7-, 22.9-, 17-, and 13.8-fold upregulation in roots, respectively. When gene

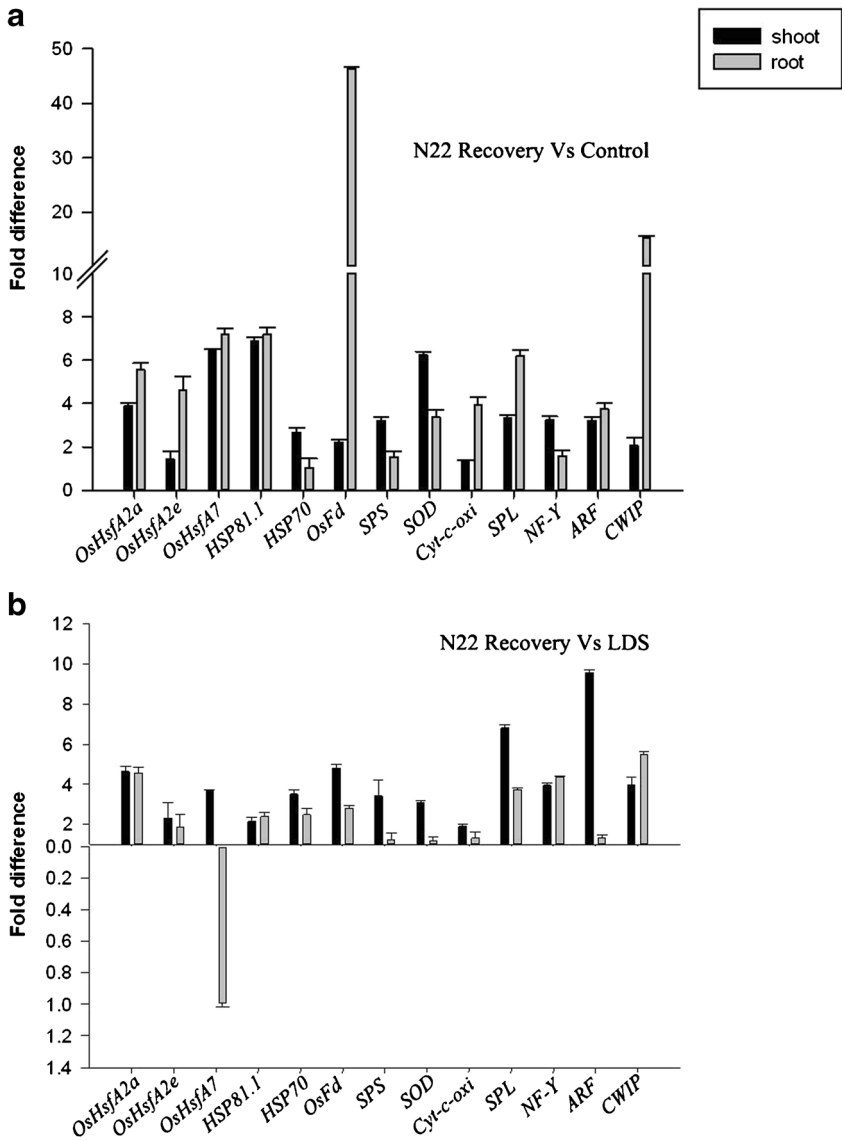


Fig. 3 Expression of 13 genes in shoot and root of heat-tolerant N22; **a** gene expression during recovery in comparison to control and **b** gene expression during recovery in comparison to LDS

expression after REC was compared with LDS, most of the transcripts showed increased expression in root and shoot (Fig. 5b). In particular, *OsHsfA2a* transcript showed 14.3-fold upregulation in shoot and 58-fold in root. The other two Hsfs, *OsHsfA2e* and *OsHsfA7*, showed 8.2- and 3.10-fold increased expression in shoots and 13.7- and 18.7-fold in roots, respectively. *OsFd*, *Cyt-C-Oxi,* and *SPL* showed 10.2-, 4.6-, and 13.8-fold upregulation in root, respectively, which was higher in comparison to shoot. The highest fold change expression in REC was in roots rather than shoots.

Expression of miRNAs

Expression analysis of miRNAs after SDS was carried out in N22 in our previous study (Sailaja et al. 2014). In this study, expression of the same nine miRNAs in N22 was analyzed after LDS and REC also. Out of nine miRNAs, six miRs were downregulated in both shoot and root after LDS (Fig. 6a), and three miRNAs showed tissue differential expression. It is important to note that all the miRNAs showed downregulation in root after SDS and LDS. Most of the miRNAs were upregulated in shoot and downregulated in root after REC (Fig. 6b). In Vandana, miR156 and miR1884 showed upregulation in both shoot and root after SDS. On the other hand, miR162 was downregulated in both (Fig. 7a). miR167 and miR397 showed upregulation in shoot and downregulation in root. The reverse was true of miR160 and miR168. After LDS, miRs 156, 168, 169, 398, and 1884 were upregulated in both shoot and root (Fig. 7b). Four miRNAs—miR160, 162, 167, and 397 showed differential expression in shoot and root. miRs 162,167 and 397 were upregulated in shoot and downregulated in root. When REC was compared with control, all miRNAs except miR160 showed downregulation in shoot and

root (Fig. 7c). miR160 was upregulated in shoot and downregulated in root.

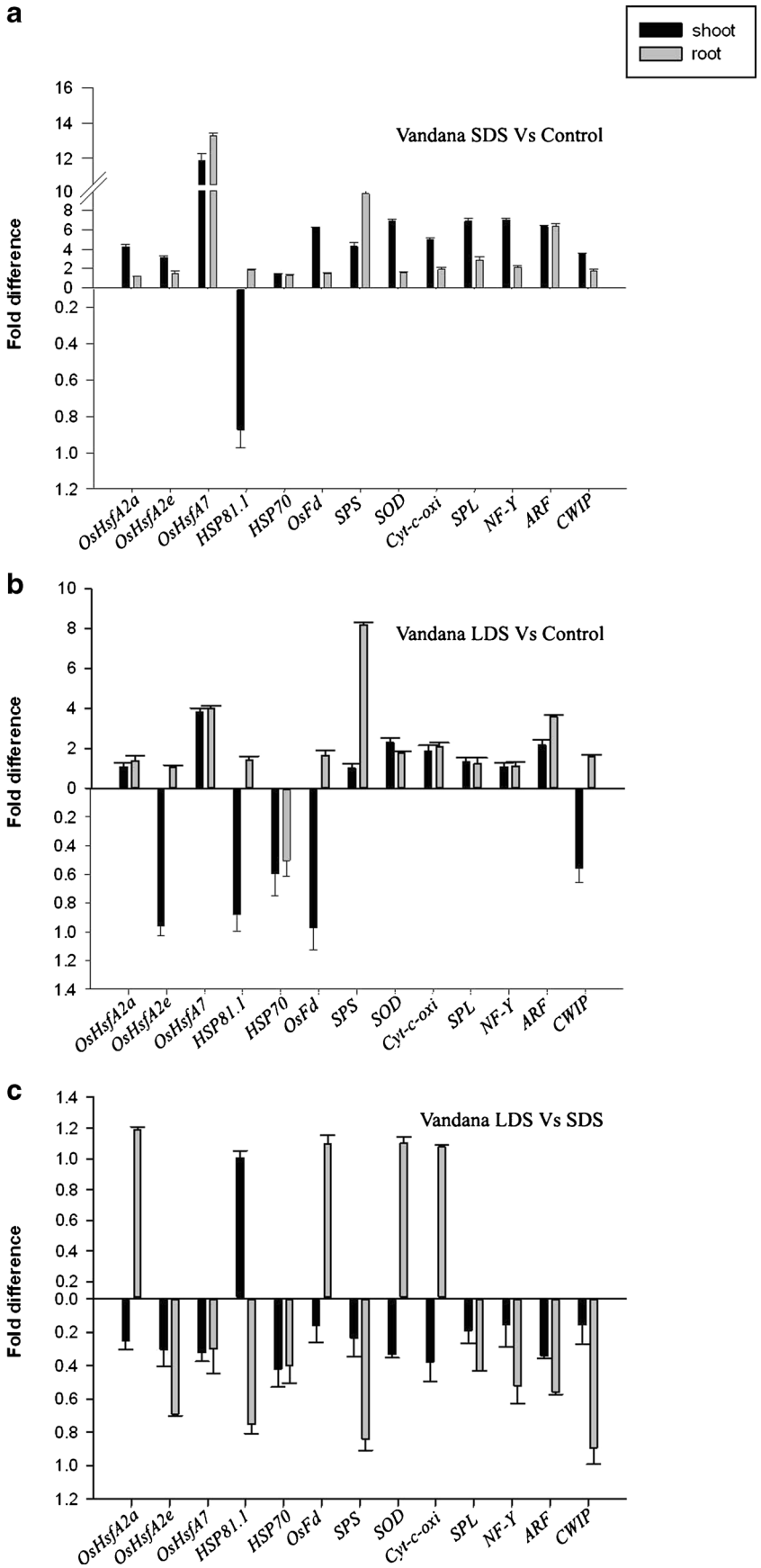
Discussion

The present study was carried out to understand the differential response of shoots and roots of two contrasting genotypes for physiological traits and expression of selected genes after short stress, long stress, and recovery after heat stress. Physiological studies have shown that heat-tolerant and heat-susceptible cultivars of rice behave differently at seedling stage during heat stress treatments. Our study showed that shoot length was reduced in both the genotypes after SDS and LDS, and N22 showed more significant reduction than Vandana. However, root length was reduced in Vandana after SDS (12 %) and LDS (24 %). High temperature causes decrease in root biomass, root number, total length, and metabolic activities (Nielsen 1974; McMichael and Burke 2002; Xu and Huang 2000a, b; Huang and Liu 2003). Interestingly, N22 showed increased root length (16 %) after SDS and a slight reduction (5.7 %) after LDS, suggesting root growth as an important feature of plant helping in faster response to stress. Root systems play critical roles in whole plant adaptation to heat stress (Huang et al. 2012). Heat-tolerant cultivars of *Agrostis stolonifera* had increased production of new roots and lower root mortality compared to heat-sensitive cultivars under heat stress (Huang and Liu 2003). However, LDS resulted in reduction of root length of N22 probably due to increased protein degradation and cell death in prolonged heat stress. A study in *Agrostis scabra* and *A. stolonifera* roots during prolonged period of heat stress showed increased protein degradation and cell death (Huang et al. 2012). Given the fact that heat stress limits rice production through its adverse effects on shoot and root growth (Hansen et al. 2006), the tolerant N22 showed more plasticity and quick response to a favorable change in temperature profile. The differences between N22 and Vandana were more pronounced at recovery stage. N22 showed significant gain in shoot and root length during recovery, while Vandana did not show any rescue, rather it deteriorated. Vandana showed reduced root length during REC when compared with LDS, indicating the inability of this heat-susceptible cultivar to recover after LDS.

Both N22 and Vandana showed increased chlorophyll content after SDS, while it was reduced after LDS. The increase in total chlorophyll content during SDS could be due to chloroplast development (increased thylakoid number per chloroplast) which in turn may be the result of osmotic shock rather than heat stress, as reported in a previous study (García-Valenzuela et al. 2005). Increased total chlorophyll content is a typical response of plants to stress conditions (Romero-Aranda et al. 2001). On the other hand, the decrease in total chlorophyll content during LDS may be due to induction of leaf senescence which causes decline in chlorophyll content and in photochemical efficiency (Veerasamy et al. 2007). The other possibility is increased protein degradation during LDS in rice cultivars. It has been reported that chlorophyll loss is linked to protein degradation (Hashimoto et al. 1989). During recovery, N22 showed a significant increase in chlorophyll content (31.3 %) over LDS, while Vandana did not show any change, indicating the resilience of N22 by increasing its chlorophyll content. Further, Vandana (70.8 %) showed much higher relative injury (RI) than N22 (39.9 %) after LDS, suggesting that prolonged heat stress affects membrane thermostability of Vandana more adversely than N22. The increase in RI during heat stress indicates occurrence of cell membrane damage (Blum and Ebercon 1981). In addition, recovery shows decrease in RI of N22 as compared to control sample, suggesting better response of N22 after the temperature shift. An introgression line derived from indica and wild rice showed that susceptible introgression line seedlings exhibited more RI than tolerant seedlings during heat stress (Lei et al. 2013).

Gene expression study showed upregulation of almost all the 13 transcripts in root and shoot of both the genotypes after SDS. Interestingly, N22 showed higher expression of *CWIP*, *ARF*, *SPL*, *Cyt-C-Oxi*, *OsFD,* and *OsHsfA7* in root as compared to shoot. Particularly, *OsHsfA2e* showed 5.9-fold upregulation in root of N22 which generally shows more expression in shoot than root under normal conditions (Liu et al. 2010). Vandana showed higher expression of majority of the transcripts in shoot. Expression of *OsHsfA2a*, *OsHsfA2e*, *OsFd*, *SPS*, *Cyt-C-Oxi*, *SPL*, *ARF*, and *CWIP* was increased in root but decreased in shoot of N22 during LDS. Unlike N22, Vandana showed upregulation of most of the transcripts in both shoot and root. The study suggested that heat-tolerant cultivar N22 shows more upregulation in root than in shoot atleast for the selected genes. Further, expression of majority of transcripts in N22 was reduced in both the tissues during LDS when compared with SDS, suggesting that prolonged stress did affect the gene expression and molecular functions of cells adversely. Here, roots of N22 after LDS showed downregulation of all transcripts except *OsHsfA2a*, *HSP70,* and *HSP81.1*. Microarray analysis suggested predominant expression of *HSP70* with its co-chaperones Hsp40 and STI1 in rice seedlings (Sarkar et al. 2013). *HSP81.1*, a member of Hsp90 family, is a heat-inducible gene that shows more abundance in roots, suggesting a specific role of *OsHSP81.1* in root growth or function (Yabe et al. 1994; Zou et al. 2009). Our study provides evidence that *OsHSP81.1* gets upregulated in root of N22 after LDS.

Fig. 4 Expression of 13 genes in shoot and root of heat-susceptible Vandana; **a** gene expression during SDS in comparison to control, **b** gene expression during LDS in comparison to control, and **c** gene expression during LDS in comparison to SDS


a
shoot
root
Vandana SDS Vs Control
Fold difference
16
14
12
10
8
6
4
2
0
0.2
0.4
0.6
0.8
1.0
1.2
OsHsfA2a
OsHsfA2e
OsHsfA7
HSP81.1
HSP70
OsFd
SPS
SOD
Cyt-c-oxi
SPL
NF-Y
ARF
CWIP
b
Vandana LDS Vs Control
Fold difference
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OsHsfA2a
OsHsfA2e
OsHsfA7
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Vandana LDS Vs SDS
Fold difference
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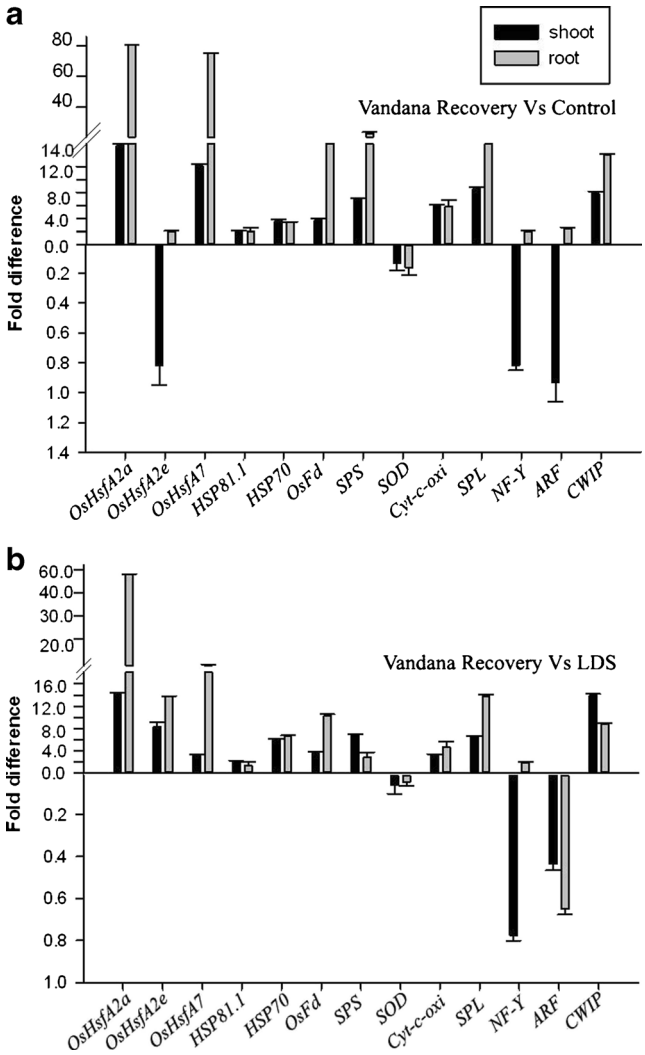

Fig. 5 Expression of 13 genes in shoot and root of heat-susceptible Vandana; **a** gene expression during recovery in comparison to control and **b** gene expression during recovery in comparison to LDS

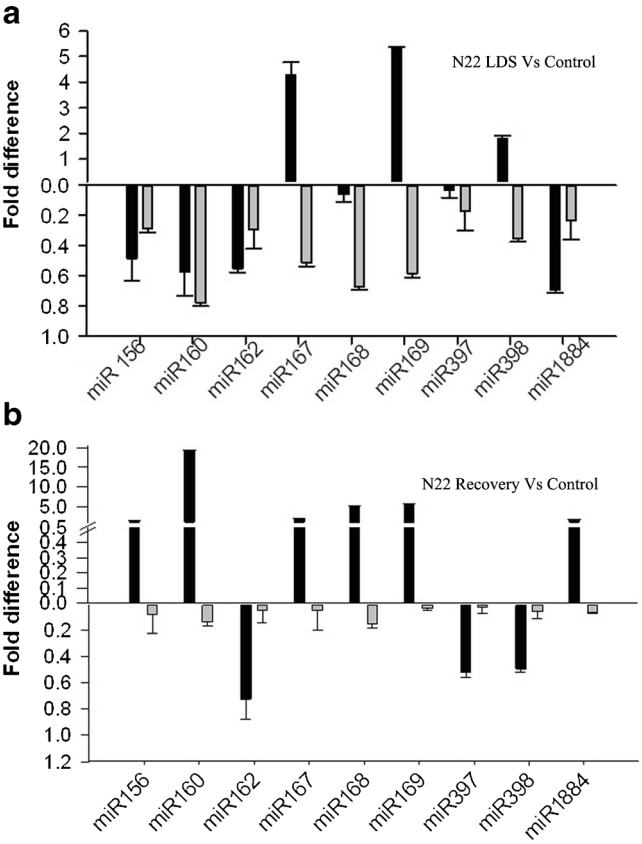

Fig. 6 MicroRNA expression in shoot and root of heat-tolerant N22; **a** miRs expression during LDS in comparison to control and **b** miRs expression during recovery in comparison to control

Recovery of N22 showed upregulation of all the 13 transcripts when compared with control. In particular, *OsFd* and *CWIP* showed 46- and 15-fold upregulation in REC root of N22. REC root of Vandana showed very high expression of *OsHsfA2a* and *OsHsfA7* to the extent of 80- and 74-fold, respectively. Further, expression of *OsFd*, *SPS*, *SPL,* and *CWIP* was quite high in Vandana root after REC. While comparing REC with LDS, N22 and Vandana showed upregulation of almost all the transcripts in root and shoot. Expression of *OsHsfA2a* (58.0-fold), *OsHsfA2e* (13.0-fold), *OsHsfA7* (18.0-fold), *OsFd* (10.2-fold), and *SPL* (13.8-fold) was very high in REC root of Vandana. These results clearly show that it is the root and not shoot which is more actively involved in the recovery response phase atleast for the sample of genes chosen for study.

In recent years, knowledge of the mechanisms underlying plant responses to heat stress has widened (Wahid et al. 2007; Sarkar et al. 2013). However, the biological mechanisms linked with root tolerance to high temperatures are far less explored, despite the significance of functional root systems in plant adaptation to heat stress. Roots play vital functions in plant survival in extreme temperatures and show higher sensitivity to adverse environmental circumstances (Nielsen 1974). The present study performed in contrasting genotypes of rice suggests that roots play a critical role in heat stress response. Physiological and gene expression studies after SDS and LDS suggest that roots of heat-tolerant N22 show more response during high-temperature stress. Our results are consistent with studies which demonstrated that high soil temperature is more detrimental than high air temperature for whole plant growth (Xu and Huang 2001; Liu and Huang 2005). During the recovery phase, root tissue of both the cultivars showed higher expression of most of the genes, suggesting that molecular response of heat stress recovery is more significant in root than shoot.

MicroRNAs are small noncoding regulatory RNA molecules that regulate gene expression posttranscriptionally and play a critical role in regulating the intrinsic normal growth of cells and development of organisms as well as in maintaining the integrity of genomes (Bartel 2004). There is a strong evidence that miRNAs are hypersensitive to abiotic stresses

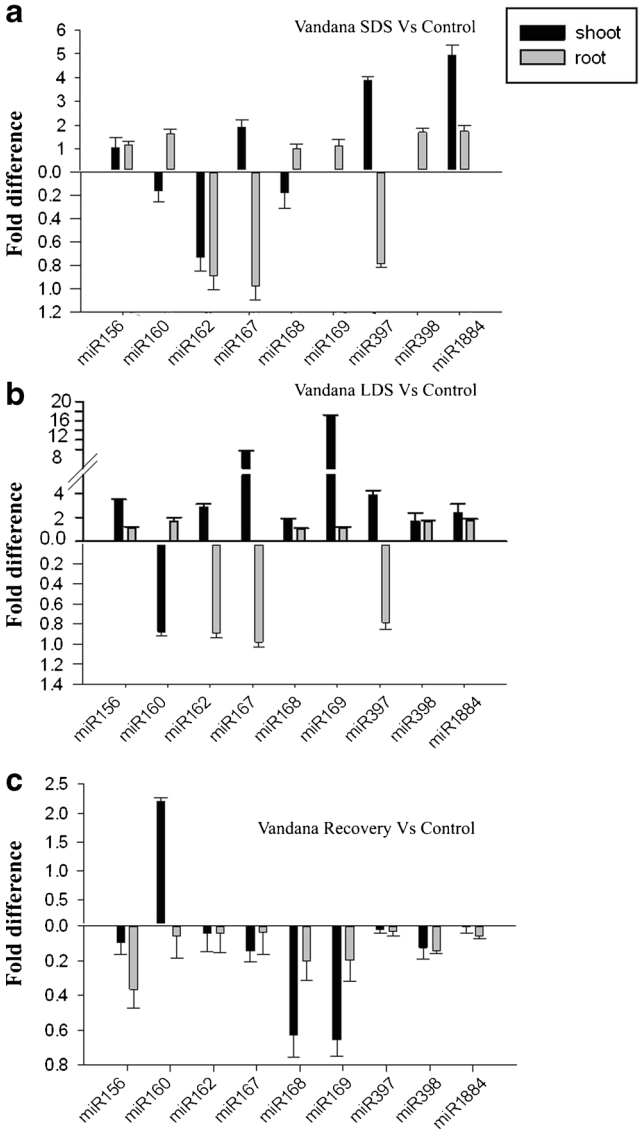

Fig. 7 MicroRNA expression in shoot and root of heat-susceptible Vandana; **a** miRs expression during SDS in comparison to control, **b** miRs expression during LDS in comparison to control, **c** miRs expression during recovery in comparison to control

(Barrera-Figueroa et al. 2013; Sunkar and Zhu 2004; Jeong and Green 2013; Mangrauthia et al. 2013). In *Arabidopsis*, miR398 was found to target two closely related Cu/Zn superoxide dismutase coding genes, cytosolic CSD1 and chloroplastic CSD2, and a reduced level of miR398 led to improved tolerance of transgenic lines under oxidative stress conditions (Sunkar et al. 2006). Likewise, in rice, miR169g was induced by drought stress (Zhao et al. 2007). In a previous study, we analyzed the expression of nine miRNAs after SDS in N22 (Sailaja et al. 2014). Here, the expression of those nine miRNAs was analyzed during LDS and recovery also. Most of the miRNAs were downregulated in both root and shoot after SDS and LDS, suggesting the increased expression of their target genes during heat stress conditions. Further, root of N22 was more responsive than shoot as it showed downregulation of all the miRNAs. This is more evident from the fact that after REC, most of the miRNAs showed upregulation in shoot while all the miRNAs showed downregulation in root. This is significant as it indicates increased expression of target genes and the necessity to regulate them. In contrast, susceptible rice cultivar Vandana showed increased expression of most miRNAs during SDS and LDS. This suggests that key regulators of gene expression such as miRNAs are more precisely induced in tolerant rice cultivar N22 during heat stress. In addition, roots of Vandana showed downregulation of the miRNAs during recovery, strengthening our premise that roots play an important role in heat stress response in rice genotypes. There were distinct differences between N22 and Vandana in the shoots also. In contrast to shoots of N22, Vandana shoots showed downregulation of all miRNAs except miR160 during recovery.

Taken together, the present study suggests that N22 has a better ability in recovering from heat stress, providing it the resilience that contributes to its heat tolerance. This trait of N22 would be highly useful in breeding for the changing climate scenario where temperature often rises and falls in an unpredicted manner. It is interesting to note that after SDS and LDS, most of the genes showed higher expression in roots of N22 than its shoot. Further, gene expression after recovery showed that roots of both the cultivars were more responsive than shoots. This finding lays emphasis on the important function of root system in heat stress response of plants and supports previous results (Huang et al. 2012). Interestingly, *OsFd* showed very high expression in roots of N22 during SDS, LDS, and also recovery. It would be interesting to further investigate the function of this gene in heat stress tolerance. The annotation shows it as iron-sulfur cluster binding-protein involved in electron transport activity and is located in chromosome1 (locus Os01g0730500). This gene is adjacent to a major QTL reported for heat tolerance on chromosome1 (Cao et al. 2003). In addition, roots of N22 showed downregulation of all the miRNA during heat stress treatments, thereby increasing the expression of target genes. Demonstrating the role of miRNAs in N22 and Vandana is an important addition towards understanding the heat stress tolerance and susceptibility in rice genotypes. Thus, we have attempted to answer why N22 is more heat-tolerant than Vandana at seedling stage and shown the important role of roots and recovery phenomenon in heat stress response using a set of genes and miRNAs. It would be interesting to enlarge this study to a genome-wide scale and further investigate how N22 roots maintain growth and activity during SDS and LDS and which metabolic processes of N22 may be involved in better recovery and root tolerance to heat stress. Genetic mapping of heat tolerance-related traits is in progress using a mapping population derived from the cross between N22 and Vandana and also using N22 mutants for heat tolerance (Poli et al. 2013).

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