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Heat-responsive microRNAs regulate the transcription factors and heat shock proteins in modulating thermo-stability of starch biosynthesis enzymes in wheat (*Triticum aestivum* L.) under the heat stress

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Abstract

Heat-responsive miRNAs regulate the expression of the transcription factors (HSFs) and majority of the heat stress-associated genes (HSPs). Here we report identification of few heats - responsive miRNAs in wheat (*Triticum aestivum* L.)through *de novo* sequencing on Illumina Hiseq 2000. Validation of identified miRNAs in endospermic tissues of thermotolerant (HD2985) and thermosusceptible (NIAW-34-34) wheat cultivars using real time PCR showed up-regulation of 4 micro RNAs (tae-miR156, tae-miR167, tae-miR395b and tae-miR398) and down-regulation of 6 micro RNAs (tae-miR159a, tae-miR159b, tae-miR160, tae- miR171a, tae-miR319, and tae-miR1117) in response to the heat stress. Target analysis of identified miRNAs showed HSF3, HSFA4a, HSP17, HSP70 and superoxide dismutase (SOD) as most probable target genes. Expression profiling of identified target genes under heat stress (42°C, 2 h) showed 2.34, 1.33 fold (HSF3), 2.45, 1.44 fold (HSFA4a), 3.9, 1.9 fold (HSP17), 5.6, 2.4 fold (HSP70), 1.9, 1.2 fold (SOD) and 2.7, 1.6 fold (catalase) increase in the expression in HD2985 and NIAW-34-34 cultivars of wheat compared to control. A defragmented, small and pleated starch granule structure was observed in sample with low expression of target genes (NIAW-34-34) compared to intact, robust and globular starch granules in samples with high expression of target genes (HD2985). Transcript profiling and activity assay of soluble starch synthase (SSS) showed less transcript accumulation and activity in heat shock treated samples compared to control sample in both the cultivars.

Keywords: Heat responsive transcription factors; Heat shock proteins; Heat stress; Milky- dough; miRNA; Quantitative real-time PCR; Soluble starch synthase; Starch granule; (*Triticum aestivum* L.); Wheat.
Abbreviations: CAT_Catalase; HSFs_Heat shock transcription factors; HSP_Heat shock protein; HS_Heat stress; miRNA_Micro RNA; qRT-PCR_Quantitative real-time PCR; ROS_Reactive oxygen species; SOD_Superoxide dismutase.

Introduction

Plants interact with various climatic factors (such as irradiation, temperature, drought etc.) during their growth and development. These climatic factors influence the various metabolic and developmental processes inside the plant system. To ameliorate such stresses, plants have evolved different ways and morphological adaptations. Abiotic stresses trigger some mechanism of defense inside the plant system such as change in the gene expression profile of stress associated proteins (SAPs), which are not expressed under normal conditions (Feder, 2006). Heat immediately changes the cellular state, alters membrane fluidity and lipid composition, and initiates the signaling cascades that ultimately lead to transcript accumulation for genes encoding protective and chaperone activities. Heat stress causes change in the level of enzymes, cellular membrane structure, photosynthetic activity and protein metabolism (Singla et al., 1997). It has been reported that high temperature changes the properties of nucleus, endoplasmic reticulum, mitochondria, and chloroplasts of rice plant (Pareek et al., 1998). Heat stress also induces oxidative stress in plants caused by the generation and accumulation of superoxides (O^{-}_{2}), hydrogen peroxides (H_2O_2) and hydroxyl radicals (OH^{-}), which are commonly known as reactive oxygen species (ROS; Breusegem et al., 2001). miRNAs are highly conserved class of small 21-24 nucleotides RNA molecules which are the key regulators of plant gene expression (Carrington and Ambros, 2003; Bartel, 2004). Recent studies have confirmed that miRNAs are involved in the regulation of a variety of plant abiotic stress-responses by regulating transcription factors and genes. Sunkar and Zhu (2004) have reported that the expression of miR393 is strongly up-regulated by cold, dehydration, and NaCl treatments. miR397b and miR402 are slightly up-regulated by all the stress treatments, whereas miR319c appears to be up-regulated by cold. miR389a appears to be down regulated by all the stress treatments. Thus, miRNAs may be used as a promising tool to improve plant yields, quality or resistance to various environmental stresses. Heat stress causes sudden changes in the genotypic expression because of an increase in the expression of heat shock transcription factors, heat-shock proteins (HSPs), antioxidant enzymes and alterations in cell-signaling (Gupta et al., 2010, Kumar et al., 2013). The induction and synthesis of HSPs due to high temperature exposure are common phenomena in all living organisms from bacteria to human beings (Gupta et al., 2010). They play important role in solving the problem of mis-folding and aggregation of

nascent proteins under the heat stress (Iba, 2002). Transcription of HSP genes is controlled by regulatory proteins called as heat shock transcription factors (HSFs). HSFs and HSPs are regulated by miRNAs which acts as driver for the expression of these stress associated genes (SAGs). Wheat (*Triticum aestivum* L.) is the second most important staple food crop of the world providing the maximum carbohydrate required in the diet (Kumar et al., 2013). Wheat is very sensitive to high temperature especially at grain-filling and seed hardening stages (Kumar et al., 2012a). The terminal heat stress is one of the major problems in wheat growing areas mainly because of the elevation in temperature being increasingly observed, the problem is likely to exacerbate with the climate change (Mitra and Bhatia, 2008) adding to the exasperation of all concerned with food production during the grain- filling stage. Exposure to high temperatures at grain-filling causes significant reductions in grain yield (Macas et al., 2000; Mullarkey and Jones, 2000; Tewolde et al., 2006, Kumar et al., 2013). Both grain numbers as well as grain weight are sensitive to the elevated temperature (Ferris et al., 1998). About 70 percent of dry matter in cereal grains is contributed by starch, thus the synthesis and deposition of starch may be an important determinant of the size of the grain and ultimately yield. Control of starch synthesis in wheat endosperm is vested largely in the activity of soluble starch synthase (SSS; Jenner et al., 1993, Kumar et al., 2013) which is extremely sensitive to high temperature (Keeling et al., 1994). In present investigation, we have identified heat-responsive miRNAs from wheat at grain-filling stage and were validated in tolerant and susceptible cultivars under differential heat stress. Target genes of identified miRNAs were predicted and their expression pattern was studied in endospermic tissue of thermotolerant and thermosusceptible wheat cultivars grown under the elevated temperature. Comparative expression analysis of miRNAs and their target genes and its effect on the quality and quantity of starch granule biosynthesis has also been studied.

Results and Discussion

Identification of conserved heat responsive miRNA in wheat

The cDNA sequencing library for small RNA was prepared from HD2985 (control and HS treated) cultivar of wheat by using illumina HiSeq RNA Library Preparation Kit. Illumina HiSeq 2000 was used to perform high throughput sequencing of the library after cluster generation. The raw data has been uploaded on NCBI BioProject data base with accession number PRJNA172054. A total of 44 mature conserved miRNAs were found in *Triticum aestivum* from miRBase sequence database release 18. We could observe change in the expressions of 10 miRNAs (tae-miR156, tae-miR167, tae-miR395b, tae-miR398, tae-miR159a, tae-miR159b, tae-miR160, tae-miR171a, tae-miR319 and tae-miR1117) in response to heat stress (HS) of 42°C for 2 h (fold-change from -0.026 to 3.99; Table 1). Out of 10 miRNAs, four miRNAs (tae-miR156, tae-miR167, tae-miR395b and tae-miR398) were significantly up-regulated and six miRNAs (tae-miR159a, tae-miR159b, tae-miR160, tae-miR171a, tae-miR319 and tae-miR1117) were significantly down-regulated. Under HS, the significant variation in the expression of these identified miRNA suggests that miRNAs play crucial roles in thermotolerance of wheat.

Validation of identified heat-responsive miRNA

To validate the identified conserved miRNAs, all the 10 miRNAs sequences (4 up-regulated and 6 down-regulated) were subjected to quantitative real time- PCR under the differential heat stress and the expressional variation was studied in thermotolerant (HD2985) and thermosusceptible (NIAW-34-34) wheat cultivars. The expression patterns of 4 up-regulated (tae-miR156, tae-miR167, tae-miR395b and tae-miR398) and 6 down-regulated (tae-miR159a, tae-miR159b, tae-miR160, tae-miR171a, tae-miR319 and tae-miR1117) heat-responsive miRNAs conformed to the digital expression pattern revealed by NGS (Illumina HiSeq 2000) (Fig. 1). The change in the relative fold expression of miR156, miR167, miR395b, miR398, miR159a, miR159b, miR160, miR171a, miR319 and miR1117 was 4.1, 2.7, 2.5, 2.1, 0.031, 0.032, 0.8, 0.41, 0.82, 0.7 (HD2985) and 1.69, 1.6, 1.73, 1.31, 0.018, 0.019, 0.32, 0.21, 0.41 and 0.45 (NIAW-34-34) fold (Table 1). We could observe significant variations in the expression of identified miRNAs in both the cultivars under the heat stress. miR160 was more responsive to heat stress in wheat, which is in conformity with the observation in *Populus tomentosa* (Chen et al., 2012). Yu et al., (2011) identified five conserved and four novel miRNA families that were responsive to heat stress in *Brassica rapa*. Since wheat genome is partially sequenced, only a few heat-responsive miRNAs has been discovered. Understanding the mechanism of miRNAs in regulating the tolerance of wheat grown under elevated temperature through their specific target genes will be of immense importance in developing climate smart crop.

Identification of miRNA specific target genes

We used psRNATarget, a plant small RNA target analysis server http://plantgrn.noble.org/psRNATarget/. (Dai and Zhao, 2011) to predict the number of mRNA target genes of these miRNAs (Table 1). The targets for the up-regulated miRNAs showed the presence of heat shock protein 90, squamosal promoter binding protein (tae-miR156), small heat shock proteins (tae-miR167), heat shock protein 90 (tae-miR195b), heat shock 70 kda mitochondrial-like, Cu/Zn-SODs gene (tae-miR398). In case of down-regulated miRNA, we could observe the targets such as heat shock protein 70, MYB3, mob1 like protein, alkaline phosphatase family protein, HSF3, cytochrome P450, cob alkaline adenosyl transferase, NB-ARC domain containing protein, histone protein associated genes, HSFA4a etc. miRNA regulate the gene or family of genes (targets) in manipulating the activity of enzymes and in turn the tolerance level of different metabolic and biological pathways inside the plant system.

Validation of target genes using quantitative real time PCR

Few identified target genes were selected for the validation using the tool of real time PCR. Primers were designed using the gene fisher primer designing software. The transcript profiling of HSFA4a showed 2.45 fold (HD2985) and 1.44 fold (NIAW-34) increase in the expression compared to control. Similarly 2.34 fold (HD2985) and 1.33 fold (NIAW-34) increase in the expression was observed in case of HSF3. With the increase in the expression of HSF, an increase in the expression of HSPs was observed as evident from the figure 2. The relative change in the fold expression was 5.6, 2.4 fold (HSP70) and 3.9, 1.9 fold (HSP17) in HD2985 and NIAW-34 cultivars of wheat. Our results are in consistent with the previous observation done in case of *Arabidopsis* (Sung et al., 2001; Sun et al., 2001).

Table 1. Relative fold change in miRNAs expression in heat stress treated wheat wheat cultivar as compared to control.

Up-regulated				
Mature miRNA	Log fold Change in HD2985	Log fold Change in NIAW-34	Number of mRNAs targeted	Annotation of the target genes
tae-miR156	4.1	1.69	30	Heat shock protein 90, Squamosal promoter binding protein
tae-miR167	2.7	1.6	3	Small heat shock proteins, ARF
tae-miR395b	2.5	1.73	16	Heat shock protein 90
tae-miR398	2.1	1.31	23	Heat shock 70 kda mitochondrial-like, Cu/Zn-SODs gene
Down regulated				
tae-miR159a	0.031	0.018	36	Heat shock protein 70, MYB3, Mob1 like protein, Alkaline phosphatase family protein, HSF3, Cytochrome P450, Cob alkaline adenosyl transferase
tae-miR159b	0.032	0.019	21	Heat shock protein 70, MYB3, HSF3, Ubiquitin, carboxyl terminal hydrolase, NB-ARC domain containing protein
tae-miR160	0.8	0.32	5	Heat shock protein 70, ARF, TPR domain protein
tae-miR171a	0.41	0.21	5	Heat shock protein 70
tae-miR319	0.82	0.41	16	TCP, Histone protein associated genes, HSFA4a
tae-miR1117	0.7	0.45	-	-

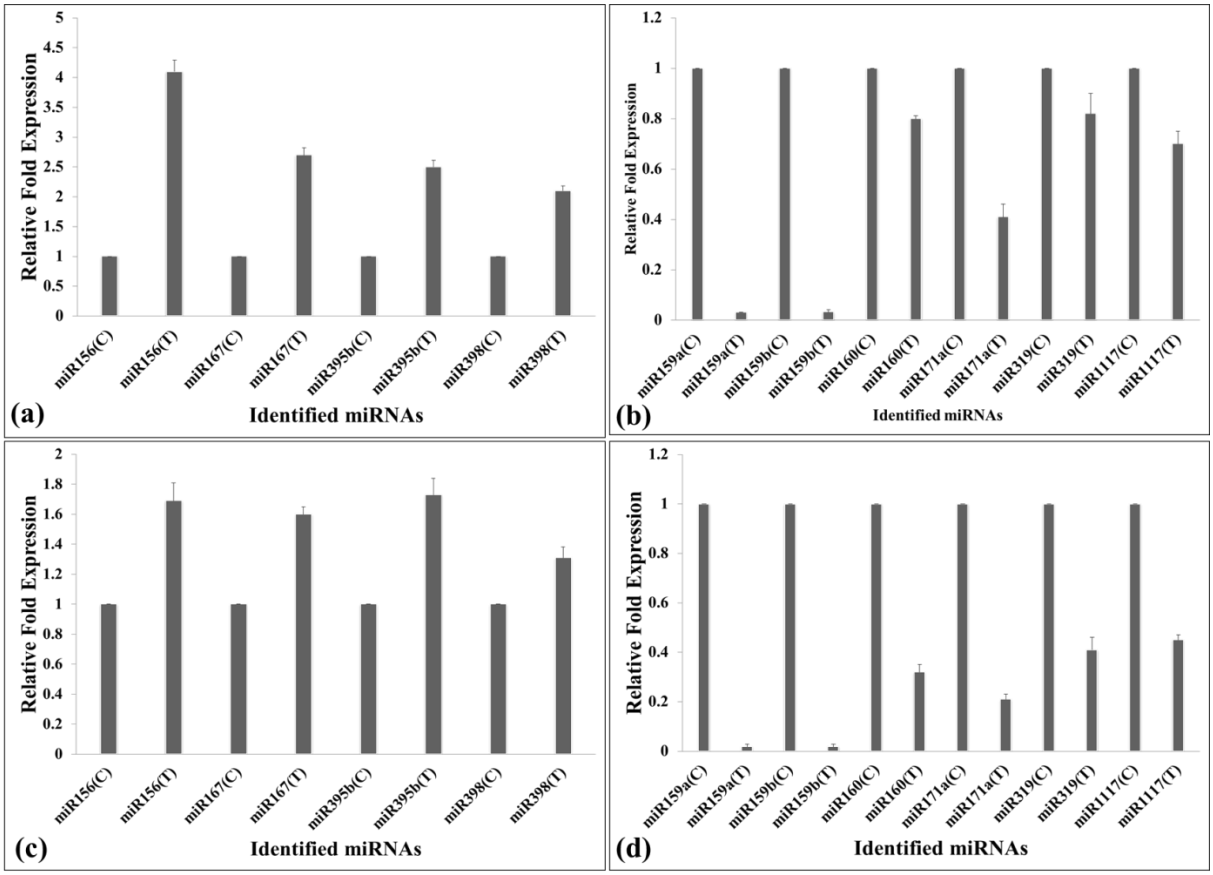

Fig 1. Transcript profiling of identified heat-responsive microRNA in HD2985 (thermotolerant) and NIAW-34 (thermosusceptible) cultivars of wheat under heat stress; (a & b) Up-regulated and down-regulated miRNA in HD2985, (c & d) Up-regulated and down-regulated miRNA in NIAW-34; C- control (22°C), T- treated (42°C, 2 h).

Heat stress causes an abrupt increase in the expression of stress-associated proteins (SAPs) and accumulation of such SAPs like HSPs has been directly implicated in the induction of thermotolerance in plants (Kumar et al., 2012b). Scarpeci *et al*., (2008) have reported that HSFs (*HSFA4a* and *HSFA2*) regulate the genes involved in stress response in *Arabidopsis.* HSFA2 and HSFA4a proteins play an important role in the induction of defense system in response to the oxidative stress and HSFA4a is the principal candidate to function as H_2O_2 sensor in *Arabidopsis* (Miller and Mittler, 2006). HSF3 has been reported to induce many heat-inducible genes and are involved in enhancing the thermotolerance of *Arabidopsis* (Yoshida et al., 2008). The stress inducible expression of HSPs is regulated mainly at the transcriptional level by HSFs that bind to conserved regulatory elements located in the promoters of HSP genes, referred to as heat shock elements (HSEs) (Krishna, 2004). HSFs perceive the elevation in temperature through different signaling molecules like H_2O_2, kinases and increase the expression of HSPs and other stress associated proteins inside the cell in order to protect the nascent protein from denaturation or aggregation under HS.

Expression profiling of antioxidant enzymes

In the present investigation, a comparative study was carried out with respect to superoxide dismutase (SOD) and catalase (CAT), since both the enzymes have been implicated in controlling the peroxide levels inside the cell under the heat stress. Transcript profiling at milky-dough stage showed 1.9 fold (HD2985) and 1.2 fold (NIAW-34) increases in the expression of SOD in response to HS of 42°C for 2 h (Fig. 3a). Similarly CAT expression profiling showed 2.7 (HD2985) and 1.6 (NIAW-34)-fold increase in the expression under HS (Fig. 3b). Expression of both the antioxidant enzymes was significantly high under HS, but the percentage increase in the expression was observed more in HD2985 (thermotolerant) as compared to NIAW-34 (thermosusceptible). SOD converts free O_2^- radicals to H_2O_2 and O_2 (Breusegem et al., 2001) and CAT scavenges the accumulated H_2O_2 to non-toxic levels or form water and oxygen (Mittler, 2002). Thus the abundance of transcript of both the antioxidant enzymes at milky-dough stage suggests its role in modulating the defense system under HS. Heat stress causes impairment in mitochondrial functions that results in the induction of oxidative damage (Davidson and Schiestl, 2001; Larkindale and Knight, 2002; Vacca et al., 2004). Various reports have shown that in plants the transcript and protein levels of many reactive oxygen species (ROS) scavenging enzymes are elevated by HS (Miller and Mittler, 2006, Secenji et al., 2010, Kumar et al., 2012a).

Change in total antioxidant capacity under differential heat shock

The total antioxidant capacity (AOC) was assayed using CUPRAC method. Plants were exposed to differential HS (T_0- 22°C, T_1- 30°C, T_2- 35°C and T_3- 40°C) for 2 h. During grain-filling stage, the total AOC estimated was 165.53 (T_0), 190 (T_1), 201.7 (T_2) and 227.6 (T_3) µM Fe/ 100 µl in HD2985 compared to 128.3 (T_0), 152.3 (T_1), 169.6 (T_2) and 185.5 3 (T_3) µM Fe/ 100 µl in NIAW-34 (Fig. 4). The maximum total AOC was observed in response to HS of 40°C for 2 h in both the cultivars. Total AOC was observed very low in susceptible cultivar when compared to tolerant under differential heat stress. An increase in the total antioxidant capacity was observed under different abiotic stress conditions in plants (Chatzissavvidis et al., 2008). In this study, we could observe proportional increase in the expression of heat-responsive miRNAs with that of total antioxidant capacity in thermotolerant and thermosusceptible wheat cultivars under the heat stress which makes us to infer that miRNAs regulate the activity of different stress-associated proteins like antioxidant enzymes and HSPs inside the cells and in turn enhances the total antioxidant capacity and thermotolerance level of the plant.

Structural analysis of starch granule under heat stress

The structure of starch granules was studied in HD2985 and NIAW-34 wheat cultivars under heat stress (42°C, 2 h) using scanning electron microscope (SEM). We could observe small granule with sparse distribution in case of HD2985 exposed to HS compare to control. Similarly disintegrated granules with pleated structure and fissure on the surface were observed in case of NIAW-34 exposed to HS compared to control (Fig. 5). HS influences the activity of key enzymes involved in granule biosynthesis as it is evident from the SEM of starch granules. It disrupts the synthesis and filling of starch granules inside endosperm (Kumar et al., 2013). HS during maize seed development interferes with endosperm starch biosynthesis and reduces seed size, an important component of yield (Singletary et al., 1994). Terminal HS speeds up the development of spike reducing spikelet number and thus, the number of grains per spike (Porter and Gawith, 1999). The results were correlated with that of the expression profile of identified heat-responsive miRNAs. We could observe decrease in the expression of miRNAs as well as different stress-associated genes in samples having defragmented and distorted starch granule synthesis under heat stress (NIAW-34).

Soluble Starch synthase activity assay under heat stress

The effect of temperature on soluble starch synthase (SSS) activity was studied in the endosperm of HD2985 and NIAW-34 cultivars of wheat at 20 days after anthesis (DAA). The soluble starch synthase activity was observed very low in HS (42°C, 2 h) treated samples as compared to control. The SSS activity estimated in case of HD2985 (control and HS treated) was 0.89 and 0.68 U/ h compared to 0.39 and 0.16 U/ h in case of NIAW-34 (Fig. 6). A significant decrease in the SSS activity was observed in both the cultivar under HS. High temperature reduces the SSS activity which is required for the amylopectin synthesis (Delvalle et al., 2005), an important constituent of starch in the endosperm. Heat stress during during endosperm development is an important yield limiting factor in wheat (Fokar et al., 1998). Endosperm development is affected by heat stress because assimilate translocation, grain-filling duration and rate are influenced directly by changes in ambient temperature (Liu et al., 2011). Increase in the expression of stress associated genes protects the SSS enzymes from denaturation under HS as it is evident from the present investigation.

miRNA as a master regulator

miRNA has emerged as master regulators of plant growth and development. Evidence suggests that miRNAs play an important role in plant stress responses. The expression profiles of most of the miRNAs that are implicated in plant growth and development are significantly altered during the stress. When a miRNA is down-regulated, it is reported that its target genes shows up-regulation post-transcriptionally. In this study, tae-miR159a, tae-miR159b, tae-miR160 and tae-

Table 2. List of miRNA specific primers used for the validation using quantitative real - time PCR (qRT-PCR).

Mature miRNA	Primer Sequences (5'-3')	Tm (°C)
tae-miR156	UGACAGAAGAGAGUGAGCACA	60
tae-miR167	UGAAGCUGCCAGCAUGAUCUA	60
tae-miR395b	UGAAGUGUUUGGGGGAACUC	60
tae-miR398	UGUGUUCUCAGGUCGCCCCCG	60
tae-miR159a	UUUGGAUUGAAGGGAGCUCUG	60
tae-miR159b	UUUGGAUUGAAGGGAGCUCUG	60
tae-miR160	UGCCUGGCUCCCUGUAUGCCA	60
tae-miR171a	UGAUUGAGCCGUGCCAAUAUC	60
tae-miR319	UUGGACUGAAGGGAGCUCCCU	60
tae-miR1117	UAGUACCGGUUCGUGGCACGAACC	60

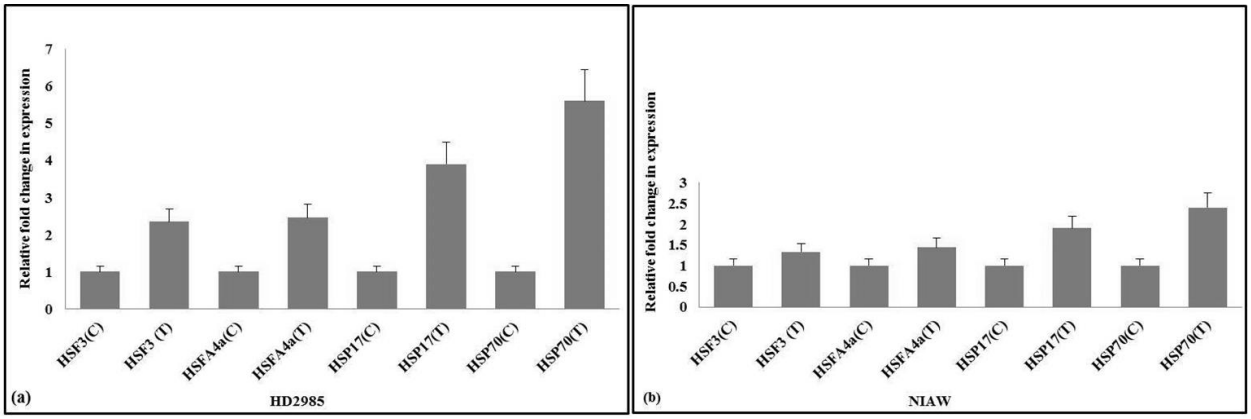

Fig 2. Transcript profiling of heat shock transcription factors (HSFs) and heat shock proteins (HSPs) in thermotolerant and susceptible cultivars of wheat under the heat stress; HSFs (HSF3 and HSFA4a) and HSPs (HSP17 and HSP70); (a) HD2985 (thermotolerant) and (b) NIAW-34 (thermosusceptible) cultivars of wheat at grain-filling stage; C- control (22°C), T- treated (42°C, 2 h).

miR171a were differentially expressed and all were down-regulated under the heat stress. Target analysis showed, HSP70 to be the most probable target of these miRNAs. The low expression of above miRNAs causes up-regulation of HSP70 under the heat stress, suggesting that miRNA and HSP70 are part of defense cascade operating in wheat grown under elevated temperature (Kumar et al., 2013). miR398 has Cu/Zn-SODs gene as its target (Jones-Rhoades et al., 2004) and it indirectly activates a series of defense responses through the reactive oxygen species (ROS) pathway (Kotak et al., 2007). In the present study, ARF (auxin-responsive factor), was predicted to be the putative targets of miR160 and miR167, which maintains the suitable IAA level in developing grains for regulating the progress of grain-filling. MiR159 has been predicted to regulate the expression of MYB family transcription factors which are involved in the regulation of ABA. During grain-filling, the content of ABA was positively correlated with cell division and grain-filling rate (Yang et al., 2006). Recently, it has been reported that approximately half of the detected miRNAs were up-regulated, whereas the rest were down-regulated with the development of rice grains in Indica rice filling phase (6–20 days after fertilization) (Lan et al., 2012). Thus, the expression of all these heat-responsive transcription factors, heat stress-associated genes etc. are ultimately regulated by miRNAs (a master regulator). Based on the results obtained in the present investigation, a model has been proposed showing the regulation path of miRNAs and its correlation with ROS scavenging enzymes, HSFs and HSPs under the terminal heat in plants (Fig. 7). Heat stress causes abrupt increase in the expression of signaling molecules (kinases) and heat-responsive miRNAs. Heat stress activated miRNAs and cascade of signaling molecules involved in modulating the tolerance by enhancing the expression of stress associated genes. Heat-responsive transcription factors (HSFs) act as sensors for H_2O_2 and O_2^-, activate antioxidant enzymes and regulate the expression of HSPs. Heat stress also causes oxidative burst inside the cells especially in organelles like mitochondria and chloroplast. ROS such as H_2O_2 and O_2^- are thought to function as early signals for HS in plants. ROS is scavenged by antioxidant enzymes like SOD and catalase. The nascent proteins and enzymes are protected from the elevated temperatures by HSPs and other SAPs and ultimately the thermotolerance level of the plant is increased.

Materials and Methods

Plant material and stress treatment

Seeds of HD2985 and NIAW-34 cultivars of wheat were procured from the Division of Genetics, Indian Agricultural Research Institute, New Delhi. Pre-treated seeds (0.5% bavistin for 30 min) of both the cultivars were sown in 32 pots (16 pots for each cultivar) inside the regulated chambers (optimum temperature regime of 26/22°C, humidity 60%, photoperiod 16 h and light intensity 350 µmol/m^2/s) at the National Phytotron Facility. Plants were divided into four groups (3 groups for HS treatments and 1 group for growth stage specific analysis). Both the cultivars (3 groups) were exposed to HS at grain-filling stage (Feeke scale 11.2) inside chamber in a sinusoidal mode, regulation from 25°C to 42°C by microprocessor. The temperature was increased with an increment of 1°C per 10 min from 8 am onwards till it reaches 42°C. The plants were kept at 42°C for 2hr and further the temperature was decreases in the same fashion. The pots were uniformly irrigated before the treatment. After

Table 3. List of target genes specific primers used for the quantitative real-time PCR (qRT-PCR)

Gene	Primer Sequences (5'-3')	Tm (°C)
HSP70(F)	CTTCGTCCAGGAGTTCAAGC	60
HSP70(R)	GTCGATCTCGATGGTGGTTT	60
HSF3(F)	CGTTCCTGAACAAGACGTATCA	60
HSF3(R)	TGAAGTACTTGGGGAGGAGGT	60
HSFA4a(F)	TATCGAGGGTCAGCAGAAAAAT	60
HSFA4a(R)	TCTTTCCGTGATGATCAGATTG	60
HSP17(F)	AGTGGGTAGCGAGTTTCCTGTGAT	60
HSP17(R)	CAAACAACCACCAGTACGCACGAA	60
SOD(F)	TCCTTTGACTGGCCCTAATG	60
SOD(R)	CTTCCACCAGCATTTCCAGT	60
CAT(F)	CAAGAGCGATTCATCAACAGAT	60
CAT(R)	AGACCAGTAGGAGAGCCAGATG	60
β-Act(F)	GCGGTCGAACAACTGGTATT	60
β-Act(R)	GGTCCAAACGAAGGATAGCA	60

Note: F- forward primer, R- reverse primer, Act- actin, SOD- superoxide dismutase, CAT- catalase

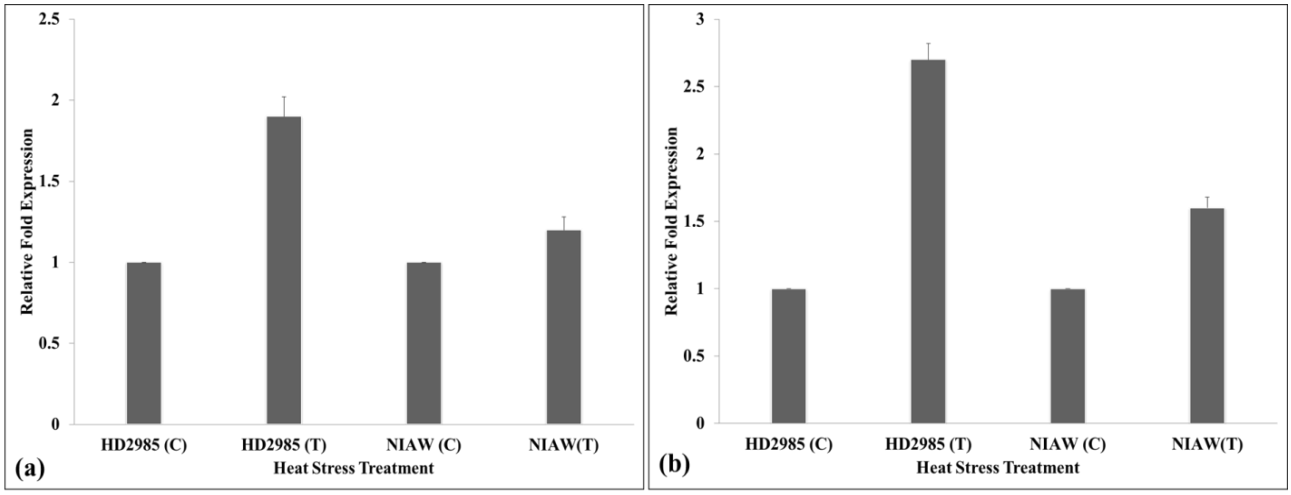

Fig 3. Expression profiling of antioxidant enzymes in thermotolerant (HD2985) and thermosusceptible (NIAW-34) cultivars of wheat under heat stress; (a) Relative change in fold expression of superoxide dismutase (SOD) (b) Relative change in fold expression of catalase (CAT); C- control (22°C), T- treated (42°C, 2 h).

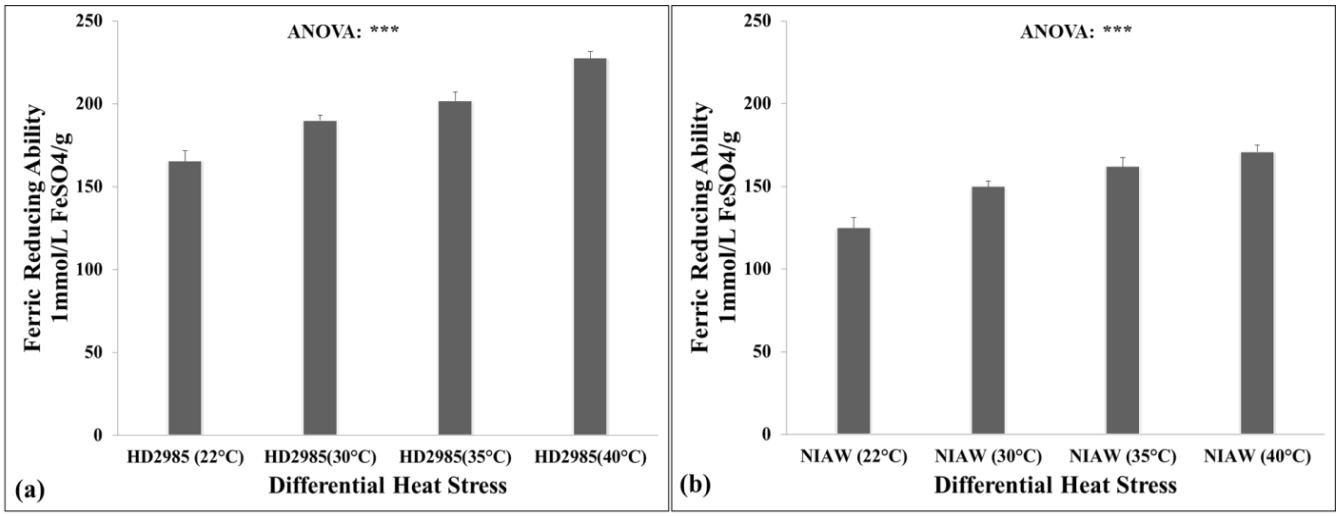

Fig 4. Total antioxidant capacity (AOC) profiling in thermotolerant (HD2985) and thermosusceptible (NIAW-34) cultivars of wheat exposed to differential heat stress at grain-filling stage; **(a)** HD2985 exposed to differential HS and **(b)** NIAW-34 exposed to differential HS; ANOVA significance levels: *, $P < 0.05$;**, $P < 0.001$;***, vertical bars indicate SE. (n = 3).

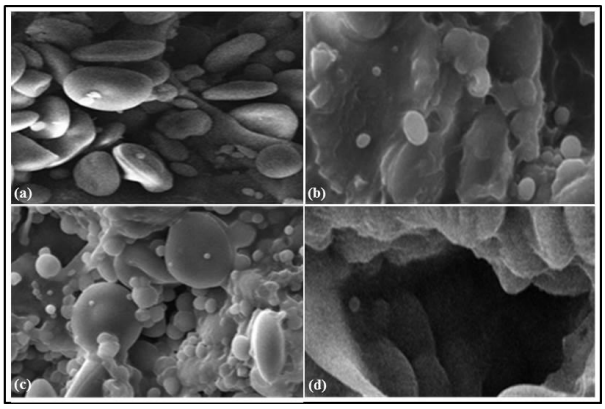

Fig 5. Scanning Electron Microscopy (SEM) of starch granules in thermotolerant (HD2985) and thermosusceptible (NIAW-34) cultivars of wheat; **(a)** HD2985 (control at 22°C), **(b)** HD2985 (heat stress treated at 42°C, 2 h), **(c)** NIAW-34 (control at 22°C), **(d)** NIAW-34 **(**heat stress treated at 42°C, 2 h); HS was given at grain-filling stage.

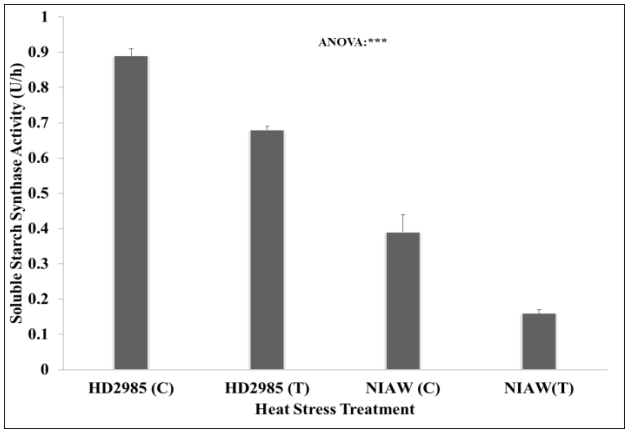

Fig 6. Soluble starch synthase activity assay in HD2985 and NIAW-34 cultivars of wheat at grain-filling stage; C- control, T- treated; ANOVA significance levels: *, P < 0.05;**, P < 0.001;***, vertical bars indicate SE. (n = 3).

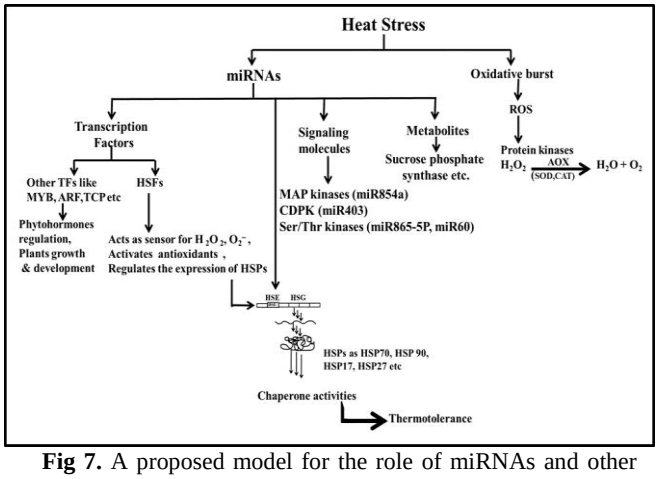

Fig 7. A proposed model for the role of miRNAs and other gene network (target genes) involved in modulating the thermotolerance of wheat under elevated temperature based on present investigation; HSE- Heat stress elements, SAG- Stress associated genes, TFs- Transcription factors, ROS- Reactive oxygen species, PDC- Programmed cell death, AOX- Antioxidant enzymes.

the stress treatment, endosperm samples were harvested in triplicates and immediately suspended in liquid nitrogen for subsequent analyses.

miRNA specific cDNA synthesis and quantitative real- time PCR (qRT-PCR)

Total RNA was extracted from the collected samples using the Millipore RNA isolation kit (Genetix, UK) and quantification was done using QubitTM 2.0 fluorometer (Invitrogen, UK). RNA integrity was verified on 1.2% agarose gel. First strand cDNA synthesis was performed by using Ncode Vilo miRNA cDNA synthesis kit (Invitrogen, UK) according to the manufacturer's instructions. For the expression study of miRNA, qRT-PCR was performed using Express SYBR GreenER qRT- PCR super mix universal (Invitrogen, UK). Reactions were performed on the CFX96 Real- Time PCR platform (Biorad, UK). Final concentration of cDNA used for qRT-PCR was 20 ng/µl. Forward primers sequences of miRNAs for qRT-PCR were designed from NCode miRNA Database at http://escience.invitrogen.com/ncode (Table 2). The Universal qPCR Primer provided in the Ncode Vilo kit was used as the reverse primer in the qPCR reaction. For each treatment, expression was performed using duplicate biological replicates and triplicate technical replicates. Quantitative PCR was performed in 20 µl reactions using miRNA gene specific primers. The thermal profile for qPCR was: 50°C for 2 min, 95°C for 2 min, followed by 39 cycles each consisting of 95°C for 15 sec and 60°C for 1 min, followed by melting at temperature between 65-95°C with an increment of 0.5 °C for 10 sec. Wheat β-actin gene was used for normalization. Data analysis was performed using software provided by BioRad, UK. The Comparative C_t ($2^{-\Delta\Delta Ct}$) method was used to calculate the changes in gene transcript as a relative fold difference between an experiment and calibrator sample (Pffafl, 2001).

Prediction of miRNA specific target genes

A web based plant small RNA target analysis server ie. psRNATarget (http://plantgrn.noble.org/psRNATarget/.) (Dai and Zhao, 2011) was used to predict the number of mRNA target genes of heat responsive miRNAs.

Validation of identified miRNAs specific target genes

Total RNA isolated from collected samples was used for the first strand cDNA synthesis. It uses oligo dT primers and the Superscript II reverse transcriptase (Invitrogen, UK) according to the manufacturer's instructions. First-strand cDNA was diluted to a final concentration of 1.0 ng/µl. Primers for qRT- PCR reactions were designed from the deduced sequence corresponding to the wheat *HSFA4a*, *HSF3*, *HSP17*, *HSP70*, *SOD* and *CAT* gene using Prime 3 primers designing software (Premier Biosoft, USA) (Table 3). For each stress condition as well as for controls, expression measurements were performed using duplicate biological replicates and triplicate technical replicates. Quantitative PCR was performed in 20 µl reactions using gene specific primers, 1µl (20 ng/µl) of cDNA as template and the SYBRGreenER qPCR SuperMix Universal (Invitrogen, UK). Reactions were performed on the CFX96 Real- Time PCR platform (Bio Rad, UK). The thermal profile for qPCR was: 3 min at 95°C, followed by 39 cycles each consisting of 95°C for 15 sec, 60°C for 30 sec and 72°C for 15 sec followed by plate reads. The expression levels of

wheat β-actin gene were used as internal standards for normalization of cDNA template using β-actin specific primers (Gen Bank accession no. AB181991.1) as shown in the Table 3. Data analysis was performed using software provided by Bio Rad (UK). The Comparative C_t ($2^{-\Delta\Delta Ct}$) method was used to calculate the changes in gene transcript as a relative fold difference between an experiment and calibrator sample (Pffafl, 2001). The primer specificity and the formation of primer-dimers were monitored by dissociation curve analysis and agarose gel electrophoresis on a 3% agarose gel.

Estimation of total antioxidant activity

The samples collected at grain-filling stage were used for the total antioxidant activity assay using the method of Benzie and Strain (1999) with slight modification. Aliquots of 100 µl sample were mixed with 3 ml FRAP (Ferric reducing antioxidant power) reagent and the absorbance of the reaction mixture at 593 nm was measured spectrophotometrically after incubation at 37°C for 10 min. For the calibration curve, five different concentration of $FeSO_4 7H_2O$ (100, 750, 500, 250 and 125 µmol/L) were used and the absorbance were measured at 593 nm. The values were expressed as the concentration of antioxidants having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4$.

Soluble starch synthase activity assay

Soluble starch synthase (SSS) activity was assayed by coupling reaction with pyruvate kinase. The ADP released by starch synthase is utilized by pyruvate kinase in the presence of phosphoenolpyruvate. The product of pyruvate kinase reaction *i.e*. pyruvate is then made to react with dinitrophenol hydrazine to give a brown colour, absorbance of which was read at 520 nm. In this procedure, the reaction mixture (final volume 56 µl) comprised 1.6 mM ADPG (adenosine diphosphate glucose), 0.7 mg amylopectin, and 15 mM DTT (dithiothreitol) in 50 mM Hepes NaOH (pH 7.5) with the 20 µl of crude enzyme used to start the reaction. After 20 min, the reaction was stopped by boiling for 1 min. After cooling, the mixture was added to 20 µl of a solution comprising 50 mM Hepes-NaOH (pH 7.5), 4 mM PEP (phosphoenolpyruvate), 200 mM KCl, 10 mM $MgCl_2$, and 1.2 IU pyruvate kinase, and then further incubated for 20 min at 30°C. The resulting solution was heated in a boiling-water bath for 1 min, and the homogenate was centrifuged at 10,000 × *g* for 10 min then 60 µl of the supernatant was mixed with 43 µl of a solution of 50 mM Hepes-NaOH (pH 7.5), 10 mM glucose, 20 mM $MgCl_2$ and 2 mM NADP. The enzymatic activity was measured as the increase in absorbance at 520 nm after the addition of 1.4 IU hexokinase and 0.35 IU glucose-6-phosphatedehydrogenase (Nakamura et al., 1989).

Statistical analysis

The experiment was conducted in a completely randomized design (CRD). The endosperm tissue from the control and HS treated wheat cultivars (HD2985 and NIAW-34) were collected randomly as three replicates. Data were analyzed using one-way analysis of variance (one-way ANOVA) and standard errors were depicted in histograms.

Conclusion

Here we report the identification of 10 heat-responsive miRNA in wheat using Illumina HiSeq 2000. We could observe significant variation in the expression of identified miRNA under HS. Significant changes in miRNA expression revealed key role of miRNAs in the heat stress-response. tae-miR159a, tae-miR159b, tae-miR160 and tae-miR171a were down-regulated under heat stress and HSP70 was predicted to be the possible target of these miRNAs. An increase in the expression of HSPs (HSP70 and HSP17) was observed with the increase in the expression of HSFs (HSF3 and HSFA4a) under the heat stress. Abundance of HSP17, HSP70 and antioxidants (SOD and CAT) in the endosperm of wheat indicate their roles in the defense system under the terminal heat. We could observe decrease in the SSS activity under HS which was reflecting in the form of disintegrate and distorted starch granule synthesis. To conclude, miRNA regulate the expression of SAGs and metabolic pathway associated genes which in turn modulate the tolerance of wheat under HS by manipulating the activity of key enzymes. The redox regulation of HSPs and HSFs functions as ROS sensors in plants is a matter of future investigation which needs to be addressed along with miRNAs and heat-stable proteins.

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