



Characterization of heat-responsive novel microRNAs in wheat identified based on miRNome analysis

Ranjeet R. Kumar², Mahesh Kumar^{1*}, Jyoti P. Singh³ and Kajal Arora¹

¹Division of Livestock Production and Range Management, ICAR-CAZRI, Jodhpur 342 003, India

²Division of Biochemistry, ICAR-IARI, New Delhi 110 012, India

³ICAR-National Bureau of Agriculturally Important Microorganism, Mau 275103, India

Received: January 25, 2024 Accepted: September 26, 2024

OPEN ACCESS

Editor-in-Chief
Praveen Kumar

Associate Editor
V.S. Rathore
P. Santra
R.K. Solanki

Managing Editor
N.R. Panwar

Editors
R.S. Tripathi
S. Soondarmurthy
U.R. Ahuja
R. Sharma
P.P. Rohilla
Raj Singh

Guest Editors
Mahesh Kumar
M.L. Dotaniya
Archana Verma

***Correspondence**
Mahesh Kumar
nirjal.mahesh@gmail.com

Citation

Kumar, M., Kumar, R.R., Singh, J.P. and Arora, K. 2025. Characterisation of heat-responsive novel microRNAs in wheat identified based on miRNome analysis. *Annals of Arid Zone* 64(1): 45-56
doi.org/10.56093/aaz.v64i1.147781
<https://epubs.icar.org.in/index.php/AAZ/article/view/147781>

<https://epubs.icar.org.in/index.php/AAZ>

Abstract: MicroRNAs function as primary regulators of plant growth and development. RNA-RNA interaction plays a crucial role in gene regulation. The miRNA regulates the expression of functional genes related to plant development and other physiological processes. Under stressful conditions, the expression of most miRNAs that play a role in plant growth and development undergoes significant changes. Here, we identified nineteen heat-responsive novel miRNAs using the reference genome of *Sorghum bicolor* and *Zea mays*. The novel miRNAs genes were validated in four contrasting wheat cultivars - BT Schomburgk, PBW 343 (thermosusceptible) and HD 3086, Raj 3765 (thermotolerant) under heat stress (HS) treated conditions during the seedling stage by quantitative real-time PCR. Almost all the novel miRNAs showed significant downregulation in all the wheat cultivars under heat stress. We have cloned and characterised candidate 2233 miRNA from HD 3086. Secondary structure prediction and energy dot plot analysis showed very high stability of the identified novel miRNAs. Most of the identified targets of miRNAs were associated with stress-responsive genes and heat shock proteins. A negative correlation was seen between microRNAs (miRNAs) and their target genes. These identified novel miRNAs can be used for the development of 'climate-smart' wheat crops.

Key words: Heat stress, heat responsive transcription factor, gene regulation, miRNA, qRT-PCR, stress-associated genes.

Human-caused global warming surpassed pre-industrial levels by an estimated 1°C, with a rate of increase of 0.2°C every decade. Heat stress is often defined as the rise in temperature beyond a threshold level for a period of time sufficient to cause irreversible damage to the plant growth and development. Increasing global temperature is negatively affecting global food production and crop productivity (Lesk *et al.*, 2016). Heat stress affects plants in various ways; it disrupts membrane proteins by unfolding or misfolding, disrupting membrane components by forming reacting oxygen

species (ROS), alters intermediary metabolism, and other physiological and developmental processes (Kaushal *et al.*, 2016). Plants sense changing temperatures via multiple regulatory networks of molecular sensors located in the various cellular compartments.

Transcription plays a pivotal role in the response to biotic and abiotic stresses. The discovery of small RNAs, such as microRNAs (miRNAs) and small-interfering RNAs, has brought to light the significance of post-transcriptional gene regulation. Noncoding RNAs transcribed from intergenic, intron, or exon regions are the building blocks of microRNAs that have a structure similar to a hairpin and are 21 nucleotides long. Following splicing and processing of pre-miRNA with m7 Gcap and 30 polyadenylations, microRNAs are synthesised by RNA polymerase II (Xie *et al.*, 2005). According to Borges and Martienssen (2015), this pri-miRNA is converted into pre-miRNA in the nucleus through cleavage by Dicer-like (DCL1) endonucleases and related proteins at least twice. Following this, HASTY protein exports pre-miRNAs to cytoplasmatic P-bodies. The cytoplasmic pre-miRNAs are converted into miRNA duplexes of 21-24 nucleotides in length through the action of DCL1-4 nucleases (Borges and Martienssen, 2015). Forming miRNA-induced silencing complexes (miRISCs), which direct target-specific mRNA cleavage or translational repression, mature miRNAs are loaded onto Argonaute 1 (AGO1) (Cui *et al.*, 2017).

Plants have developed several strategies to adapt to a challenging environment during the course of their existence. These strategies include the formation of specialized structures, the production of reactive oxygen species, and alterations in the expression of numerous genes. Post-transcriptional gene silencing (PTGS), which microRNA activates following transcription, is important for both developmental and stress tolerance processes. MicroRNA controls the process of chlorophyll biosynthesis (Ma *et al.*, 2014), blooming (Wu *et al.*, 2009), stress response (Guan *et al.*, 2013), and nucleic acid binding (Kruszka *et al.*, 2014). The utilization of genome-wide analysis and the development of high-throughput sequencing technologies have facilitated the discovery of novel miRNA variants and their corresponding targets (Chavez-montes *et al.*, 2014; You *et al.*,

2017). Heat-responsive miRNAs have been discovered in many crops such as rice, maize, and wheat, as discussed by Liu *et al.* in 2016. The validation of miRNA targets is insufficient, particularly in polyploid crops such as wheat. However, certain crops, such as rice and Arabidopsis, have been successfully validated for thermotolerance.

Heat shock proteins (HSPs) play a crucial role in plants' defence against different stresses by aiding in the process of protein refolding (Kotak *et al.*, 2007). There is mounting evidence that indicates the involvement of miRNA in the induction of HSP/HSF. The presence of miR156 in Arabidopsis led to the over-expression of HSP17.6A, HSFA2, and HSP22.0 transcripts (Yu *et al.*, 2012). Liu *et al.* (2017) utilised small RNA deep sequencing technologies to extract 102 miRNAs that are responsive to heat from the panicles of both heat-tolerant and heat-sensitive rice types. The heat stress response leads to the generation of reactive oxygen species (ROS) (Mittler, 2002). The miR398 serves as a stimulator for heat stress in Arabidopsis and wheat and is rapidly induced upon exposure to heat stress (Kumar *et al.*, 2015; Lu *et al.*, 2017). Heat stress triggers the upregulation of miR398 and downregulates the expression of its target genes CSD1, CSD2, and CCS (copper chaperone for superoxide dismutase). The miR398 transgenic line, which expresses variants of CSD1, CSD2, and CCS that are resistant to miR398, exhibited increased sensitivity to heat stress, as reported by Guan *et al.* in 2013. The study conducted by Yan *et al.* (2012) revealed that the Arabidopsis transgenic line overexpressing miR400 exhibited a higher susceptibility to heat stress compared to the wild-type plants.

A total of 32 miRNA families were discovered using High-throughput sequencing. Among these families, miR172, miR160, and miR156 are potentially associated with heat stress tolerance in wheat, as shown by Xin *et al.* (2010) and Khraiwesh *et al.* (2012). The miR160 molecule stimulates the activity of auxin response transcription factors (ARFs), which in turn regulate the expression of early auxin-responsive genes (Liu *et al.*, 2010). The study conducted by Stief *et al.* (2014) demonstrates that miRNA156 in Arabidopsis controls the ability to adapt to repeated episodes of heat stress by regulating the expression of SQUAMOSA

promoter binding protein-like (SPL) genes. In this study, a new candidate miRNA was isolated, cloned, and studied in wheat cv. HD2985 using heat stress (HS). Quantitative real-time PCR was used to confirm that the candidate miRNA was heat-responsive. We found that the expression of potential miRNAs correlates with their target. The data collected will be helpful in controlling wheat's tolerance mechanism by adjusting SAG expression in response to HS intensity.

Materials and Methods

Wheat seed varieties HD 3086, Raj 3765 (thermotolerance), PBW 343, and BT Schomburgk (thermosusceptible) were procured from IARI, Division of Genetics in India. Before sowing in containers, the seeds were treated with bavistin (0.5%). Twelve containers, six for each variety, were seed-sown in a regulated chamber ($22 \pm 3^\circ\text{C}$, 75% relative humidity, and 8 hours of light with an intensity of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) at the National Phytotron Facility, IARI, New Delhi. The mixture contained equal amounts of perlite and FYM. Weeding and irrigation were performed manually at consistent intervals. All four wheat cultivars were subjected to thermal stress treatment at the National Phytotron Facility, IARI. During the pollination phase, heat stress was applied to three containers from each variety (38°C for two hours), while the remaining three pots were maintained at a control temperature of 22°C . The heat stress was induced in a sinusoidal fashion via a microprocessor-regulated controller, with the temperature increasing by $1^\circ\text{C}/10 \text{ min}$ until it reached 38°C , at which point it was maintained for 2 hours; it subsequently decreased to $22^\circ\text{C} \pm 3$ in the identical manner.

Novel miRNA identification

Using the Miranalyzer pipeline (Kumar *et al.*, 2015) and a homology-based approach, potential precursor molecules for *Triticum aestivum* miRNAs were identified. These precursor sequences were extracted from the genome, and their secondary structures were predicted using the mfold web server (<http://mfold.rna.albany.edu/?q=mfold>).

Total microRNA isolation

MicroRNAs were isolated from various tissues of wheat cultivars, including root, stem,

and flag leaf, from both control and heat stress (HS)-treated samples, using the miPremier microRNA isolation reagent (Sigma-Aldrich, UK) following the manufacturer's instructions. Briefly, 100 mg of tissue was ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle. The powder was mixed with 750 μL of lysis buffer and incubated at 55°C for 5 minutes. The mixture was then centrifuged at maximum speed for 5 minutes, and the clear lysate was transferred to a filtration column. After centrifugation, the lysate was washed with 100% ethanol. The column underwent two to three washing steps before being air-dried. Finally, the miRNA was eluted into a fresh RNase-free Eppendorf tube using elution buffer. The quality of the isolated miRNA was assessed with a Bioanalyzer (Agilent, UK) before storage at -80°C for further use.

Polyacrylamide gel electrophoresis for miRNA

The integrity of the extracted total miRNA was evaluated using a 12% denaturing polyacrylamide urea gel, as described by Kumar *et al.* (2017). The gel was prepared by mixing 5.04 g of urea, 1.2 mL of 10X TBE, 4.48 g of 40% acrylamide, and DEPC-treated water to a final volume of 15 mL. Once the urea had completely dissolved, 10% ammonium persulfate (APS) and 60 μL of TEMED were added to initiate polymerization. The gel mixture was immediately poured into a preassembled mould. A preliminary electrophoresis run was carried out in 1X TBE buffer to remove residual ammonium persulfate, running at 90 V for 2 hours.

For sample preparation, total miRNA was mixed with an equal volume of loading buffer and heated to 95°C for 5 minutes to ensure denaturation. The samples were then electrophoresed on the gel for approximately 2 hours at 90 V in 1X TBE buffer, until the bromophenol blue dye reached the gel's edge. Following electrophoresis, the gel was stained in DEPC-treated water containing ethidium bromide for 10 minutes and visualized under UV light.

Cloning of novel miRNA and RT-PCR amplification

The Genefisher2 software (<http://bibiserv.techfak.uni-bielefeld.de/genefisher2/>) was utilized to design the miRNA-specific forward

Table 1. Sequence of heat-responsive novel mature miRNAs forward primers

Novel miRNA	Primer sequence (5'-3')	Tm
Candidate_7501	GCT CTT GGA TGG CCT GAC GA	59.7
Candidate_7521	GGT CCG GTA TTA CCG GGG ATT GA	60.0
Candidate_8160	GCT GAC GTG GCG GAC AAA	58.7
Candidate_8295	CGG CCC AAT TCC CTT CAT TAC TCA	59.2
Candidate_901	GCG CCC CTT CGA GAT TGA AGA A	59.7
Candidate_3691	GGC CTC ATC CAG ACC GAC AAA	59.5
Candidate_4449	GCT GTC CGA CG A TCC ACT GAT	59.1
Candidate_5631	CGC GAT CGA GCA CTC GAC AA	60.0
Candidate_8696	CCT CCG ATC CCC TCC GGT AA	60.0
Candidate_69	GCT CCC ATG GAA GTC CAC CAA	59.7
Candidate_4424	CCG GTCCGACG ATCCACTGA AAA	60.4
Candidate_6979	GTC CGG AAT TTC CGG GGA TTG AA	59.5
Candidate_1124	GGA CCG AAC CCG AAC CGA A	60.0
Candidate_2233	GCC TGG CAC TCG GGA AAT ATG A	59.5
Candidate_905	GCGCCCCTT CGA GAT TGA AGA AAA	59.8
Candidate_143	AAC GCC CCG CAC ACG CCG	59.4
Candidate_3415	GCG CAT ACT CGC TCT GTT TCG TAA	59.8
Candidate_4032	GGC CGG CGA TTG TCA TAG AGA AA	59.4
Candidate_2549	GCG ACT CCC TCC GTT CCT TTT TAA	59.8

primer, while the quality of the primer was assessed using the Oligo Analyzer tool (Integrated DNA Technologies, USA). For miRNA sequences with a melting temperature (T_m) below 60°C, G or C nucleotides were added to the 5' end and/or A nucleotides to the 3' end to increase the T_m. Conversely, if the T_m exceeded 60°C, the primer was modified by trimming its 5' end. The NCode Vilo miRNA cDNA Synthesis Kit (Invitrogen, UK) was employed to synthesize cDNA. The RT-PCR reaction mixture consisted of 1 µL of cDNA, 5 µL of 10X buffer, 1 µL each of forward (miRNA-specific) and reverse (universal) primers (Table 1), 2.5 µL of dNTP mix (2.5 mM), and 1 µL of hot-start Taq polymerase (5 U/µL). The amplification process involved an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 45 seconds, and 72°C for 30 seconds. A final elongation was performed at 72°C for 10 minutes, with a holding step at 4°C for 10 minutes. The amplified product was analyzed on a 12% PAGE gel alongside a suitable marker to confirm its size and structure. The product was purified using a silica-based column and subsequently cloned into a pGEM-T Easy vector. The recombinant plasmid was transformed into the E. coli DH5α strain, and the cloned

sequence was verified using the Sanger dideoxy sequencing method.

In silico characterization of novel miRNA

T. aestivum's reference draft genome sequence was obtained from the assembly ftp://ftp.ensemblgenomes.org/pub/release-20/plants/fasta/triticum_aestivum/dna/, version IWGSP1, July 2013. For distribution analysis, the identified miRNAs were mapped to the available reference draft genome sequence of *T. aestivum*. MFOLD software was used to anticipate secondary structures and analyse fold energy. Using the psRNATarget (<http://plantgrn.noble.org/psRNATarget/>), the probable target sites of miRNA candidates were found (Dai *et al.*, 2011).

Validation of miRNAs and their targets using quantitative real-time PCR

The quantitative real-time PCR expression analysis was used in this study to validate the putative miRNAs and their targets. Using a miPremier microRNA isolation kit (Sigma-Aldrich), miRNAs were extracted from the control and heat stress-treated samples of both cultivars. The purity of the isolated miRNAs was assessed using an Agilent Bioanalyzer. Reverse transcription of poly (A)-tailed total

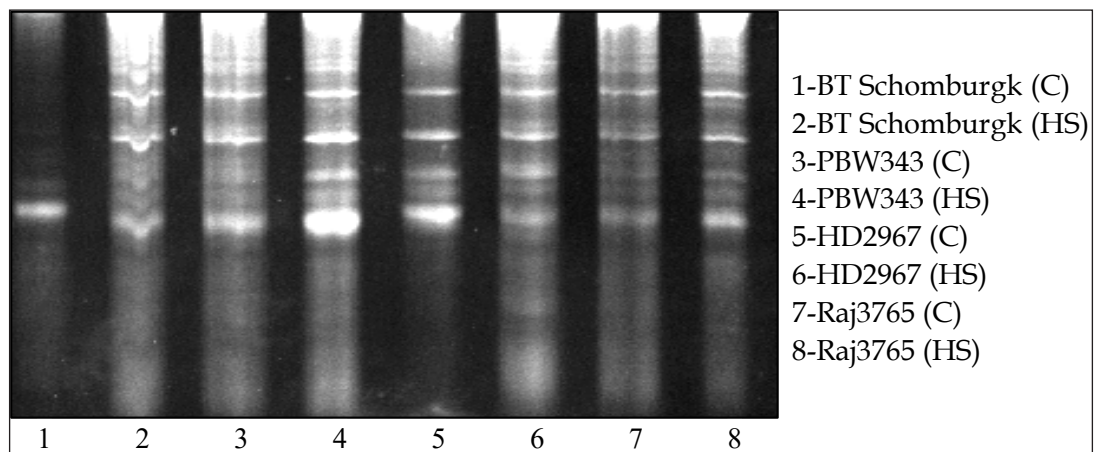


Fig. 1. Total small RNA isolation from wheat cultivars under control and heat stress treated leaf sample.

RNA was carried out with SuperScript III RT. Quantitative RT-PCR analysis was performed using SYBR® Premix Ex Taq™ II (Perfect Real Time) (TaKaRa, Japan) on a Bio-Rad CFX96 system. Reactions for each sample were conducted in triplicate, with U6 SnRNA (Clontech, USA) serving as the internal control gene. The relative expression levels of miRNAs were determined using the $2^{-\Delta\Delta C_t}$ method. For target gene analysis, first-strand cDNA was synthesized with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Quantitative real-time PCR was conducted on the Bio-Rad CFX96 platform using the EXPRESS SYBR GreenER miRNA qRT-PCR Kit (Invitrogen, UK). The β -actin gene (accession no. AF282624) was used as an

endogenous control to normalize C_t values, with expression levels calculated using the Pfaffl method.

Results

Wheat is prone to heat stress, which reduces grain yield and quality significantly. The miRNAs are one of the key molecular units that can increase a plant's overall tolerance level under stress.

De novo sequencing for novel miRNA identification

Novel miRNAs were identified in both control and heat-stress (HS)-treated samples of the wheat cultivar HD 2985 using input sequencing reads. Reference genomes of *Zea*

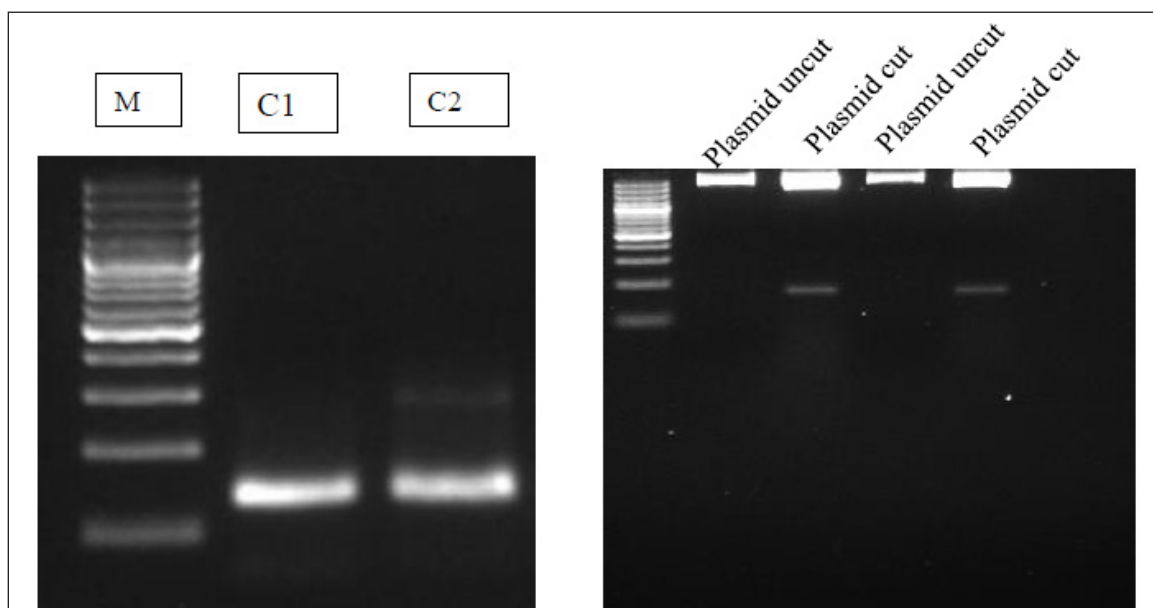


Fig. 2. PCR amplification of novel miRNA gene. M- 100 bp marker, C1- candidate_2233, C2-3415.

mays and *Sorghum bicolor* (build MSU6.1) were obtained from the PlantGDB database for miRNA identification in wheat (Kumar *et al.*, 2015). The MiRanalyzer tool, incorporating the miRNA pipeline and miRcheck, detected a total of 9 precursor miRNAs in the control samples and 10 precursor miRNAs in the HS-treated samples.

Visualization of novel microRNA on polyacrylamide (PAGE) gel

To investigate miRNA-mediated plant stress tolerance, leaf tissues from both control and HS-treated wheat cultivars were used for miRNA isolation (Fig. 1). Distinct miRNA bands were observed in all the cultivars.

Cloning of novel candidate miRNA

Out of the 19 heat-responsive candidate miRNAs identified, four miRNAs were randomly chosen for cloning. PCR amplification was performed using miRNA-specific forward primers along with a universal primer. A ~150 bp amplicon was observed on a 3% agarose gel (Fig. 2). The amplified product was then purified and ligated into the pGEM-T Easy vector. The ligated vector was transformed

into competent cells, and desirable colonies were selected using blue-white screening for plasmid isolation. The plasmid was digested with EcoRI, resulting in the release of a ~150 bp insert, which was visualized on an agarose gel. The amplified product was sequenced using Sanger's di-deoxy method.

In silico study of miRNA

The nucleotide sequence of miRNA was used to predict the secondary structure utilising the MFold website (<http://mfold.rna.albany.edu/?q=mfold>). Within the secondary structure of miRNA (Fig.3a), we identified numerous prominent and subtle grooves. The fold energy study was also conducted with the identical programme. The dots symbolise the combination of all potential folding patterns within a certain percentage, p , of ΔG_{mfe} , which is the minimum free energy. Here, p represents the maximum allowable deviation from ΔG_{mfe} . The varying levels of sub-optimality are indicated by different colors, ranging from two to eight (Fig. 3b). The base pairs with the most favourable characteristics have been highlighted using red and black colors, and

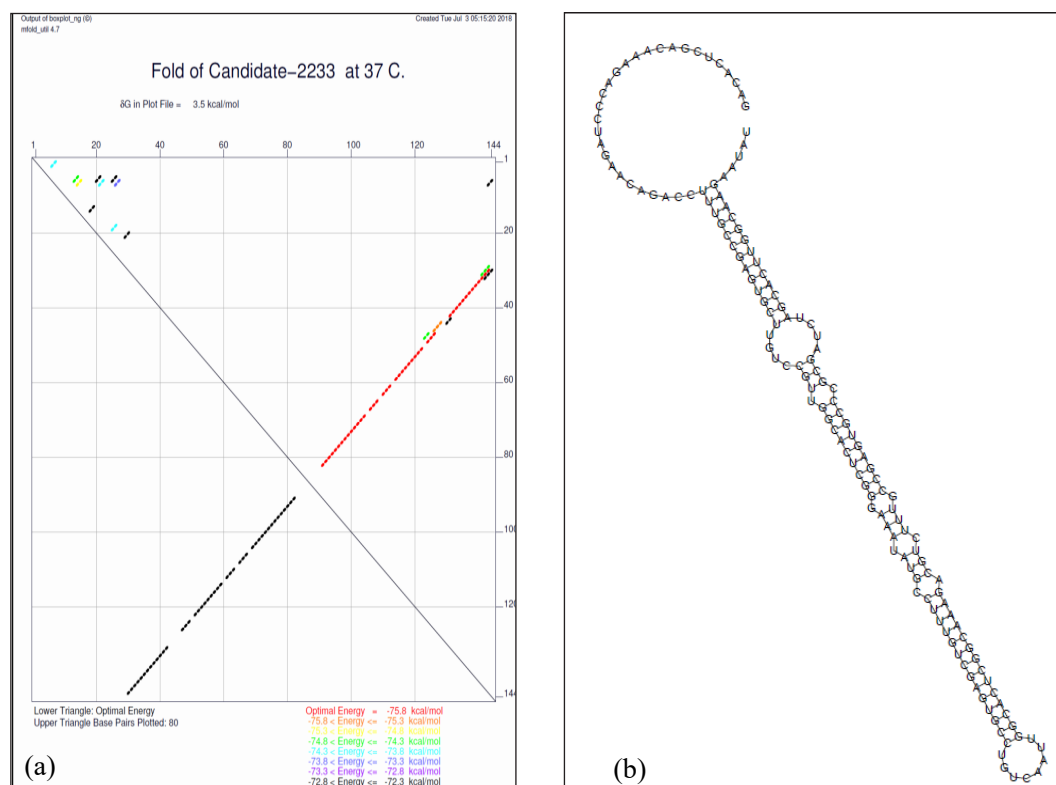


Fig. 3. Secondary structure and energy dot plot prediction of novel miRNA.

the observed energy value was determined to be -100.3 Kcal/mol.

Quantitative real-time PCR validation of miRNA

During stress, most miRNAs that are involved in plant growth and development changed their expression profiles. Nineteen novel miRNA genes Identified by the reference genome of *Zea mays* and *Sorghum bicolor* were validated in contrasting wheat cultivars BT Schomburgk, PBW 343 (thermos-susceptible) and HD 2967, Raj 3765 (thermotolerant) at the seedling stage. Most of the novel miRNAs showed

significant downregulation in all the cultivars under heat stress (Fig. 4a). Candidate_905 and candidate_1124 showed upregulation under heat stress (Fig. 4b). Significant differences in the expression of miRNAs in various tissues after heat shock demonstrated their heat-responsiveness (Fig. 4c). The selected candidate miRNAs' heat-responsiveness is confirmed by the variation in their expression under high temperature.

Expression analysis of target gene

The sequences of the novel miRNAs were analyzed using the psRNA target tool to identify

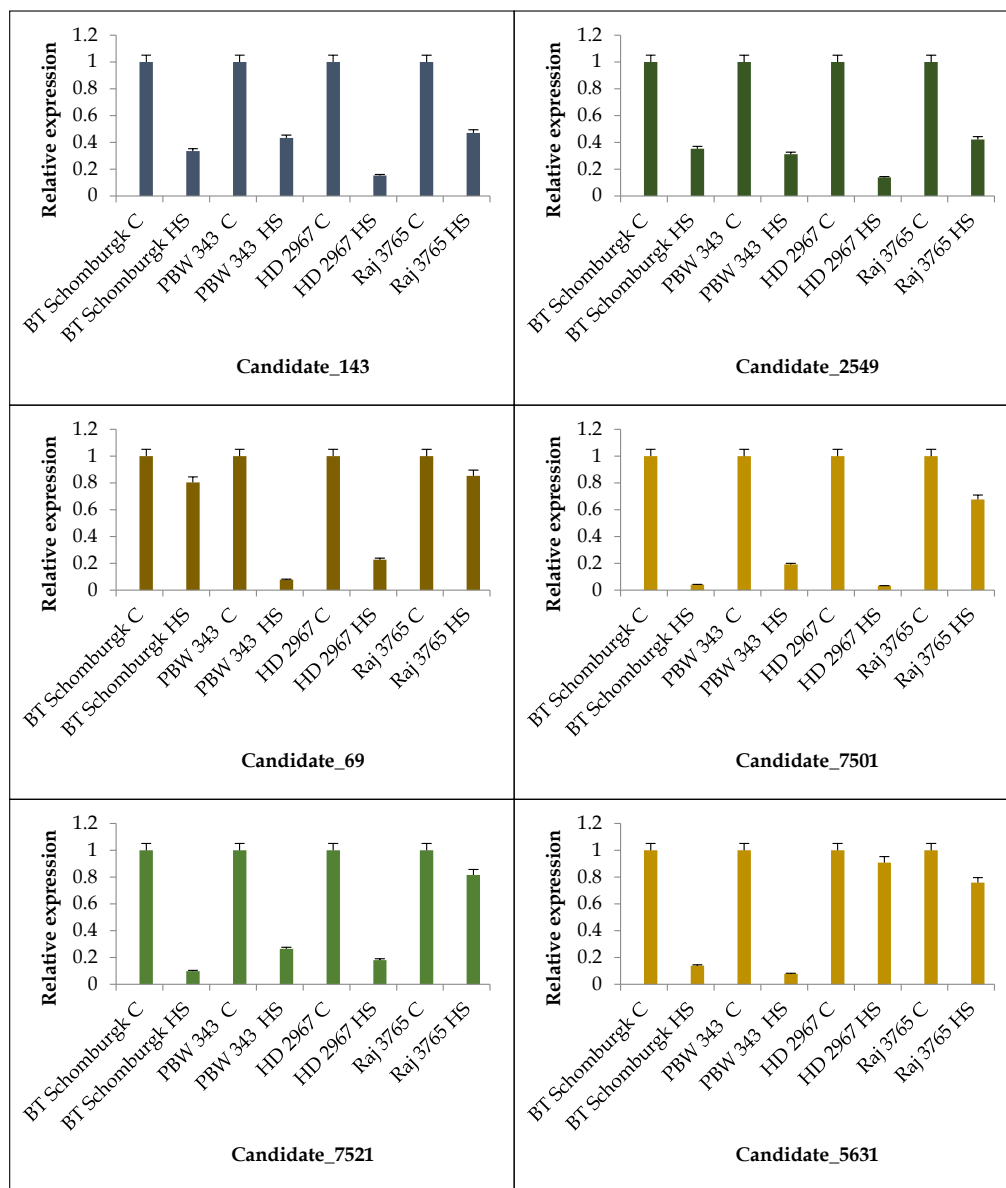


Fig. 4a. Relative expression of novel miRNAs in contrasting wheat cultivars under heat stress. Heat stress of 38° C was given for 2 h. U6 snRNA gene was used as endogenous control.

their corresponding targets. The target genes identified for the miRNAs were primarily heat shock proteins (sHSP) and antioxidant genes. All target genes showed upregulation under heat stress conditions (Fig. 5). An inverse correlation was observed between the expression levels of the miRNAs and their targets under heat stress in both thermotolerant and thermosusceptible wheat cultivars.

Discussion

A homology-based approach was employed to identify 19 novel miRNAs in *Triticum aestivum* using the reference genomes of *Zea*

mays and *Sorghum bicolor*, along with 132 miRNAs from miRBase v19. According to the miRBase repository (Dryanova *et al.*, 2008), deep sequencing technology has been extensively utilized to study both conserved and novel miRNAs in species such as *Arabidopsis*, *Oryza sativa* (rice), *Populus* (poplar), and *Physcomitrella*. When total miRNA was extracted and gel characterised, all cultivars under control and HS-treated conditions revealed distinct bands. According to Zhang (2015), the way that different types of miRNA, stress, tissues, and genotypes influence how miRNAs react to environmental challenges

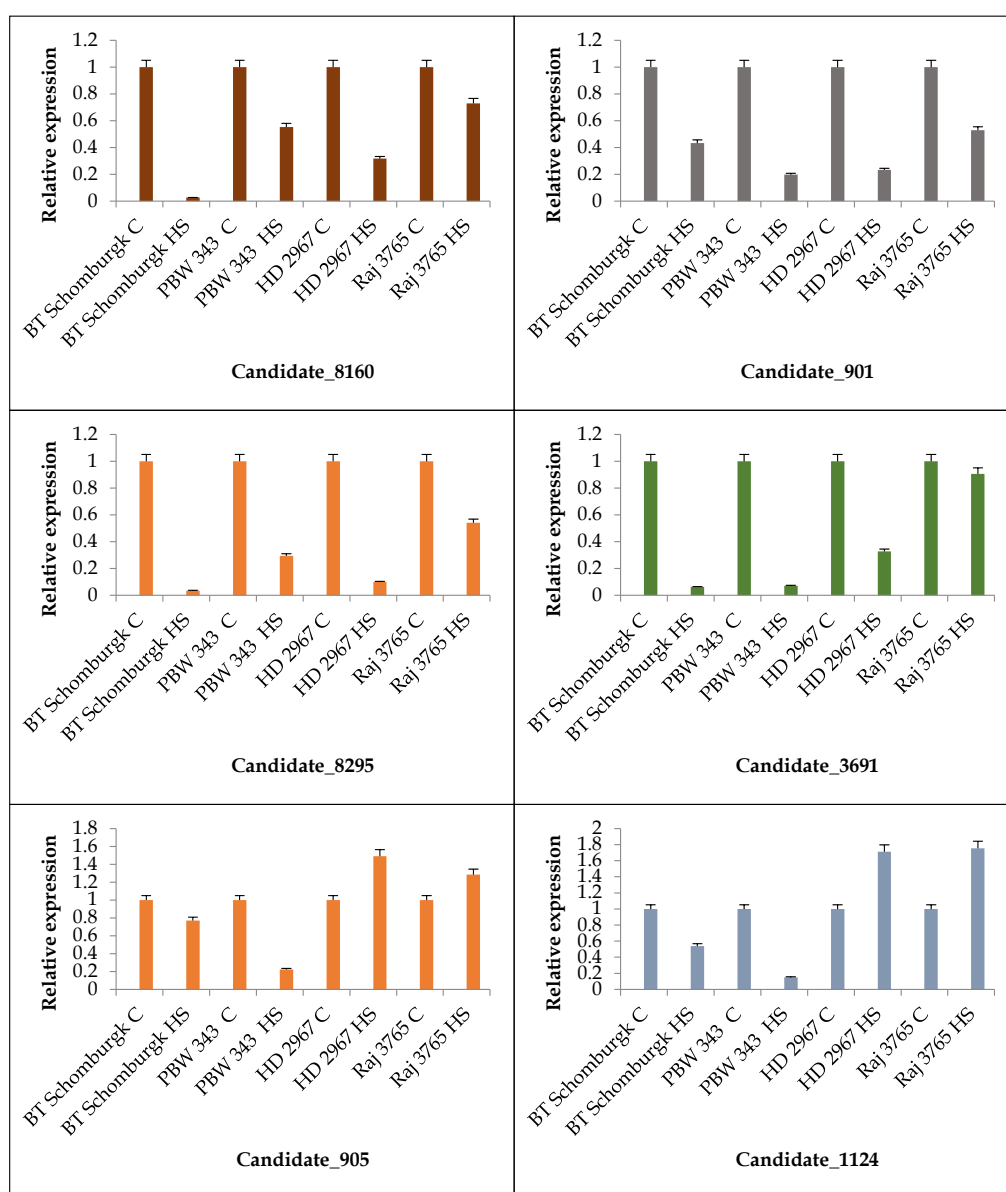


Fig. 4b. Relative expression of novel miRNAs in contrasting wheat cultivars under heat stress. Heat stress of 38° C was given for 2 h. U6 snRNA gene was used as endogenous control.

varies. We successfully cloned wheat miRNA candidates 2233 and 3415. According to Wu *et al.* (2010), miRNA cloning is an effective technique for profiling miRNA expression and exposing end heterogeneity, which may be significant for miRNA function. Rice miRNA was discovered by Sunkar *et al.* (2005) using computational and cloning methods. Yao *et al.* (2007) large-scale cloning and characterization of 23 new wheat miRNAs and their anticipated targets represents a first for the field. Under the HS, we observed a rise in the target genes' expression that the corresponding miRNAs had

triggered. Although making up just 1% of an organism's total protein-coding genes, miRNAs have been described as a master regulator of plant growth and development (Sunkar *et al.*, 2012; Lukasik *et al.*, 2013). It was discovered that different miRNAs control how heat stress causes the production of genes that respond to heat stress. For instance, it has been shown that Arabidopsis overexpresses miR156, which produces memory in response to heat stress (Stief *et al.*, 2014); conversely, heat stress causes miR159 to be downregulated, which controls the MYB transcription factors. Most miRNAs

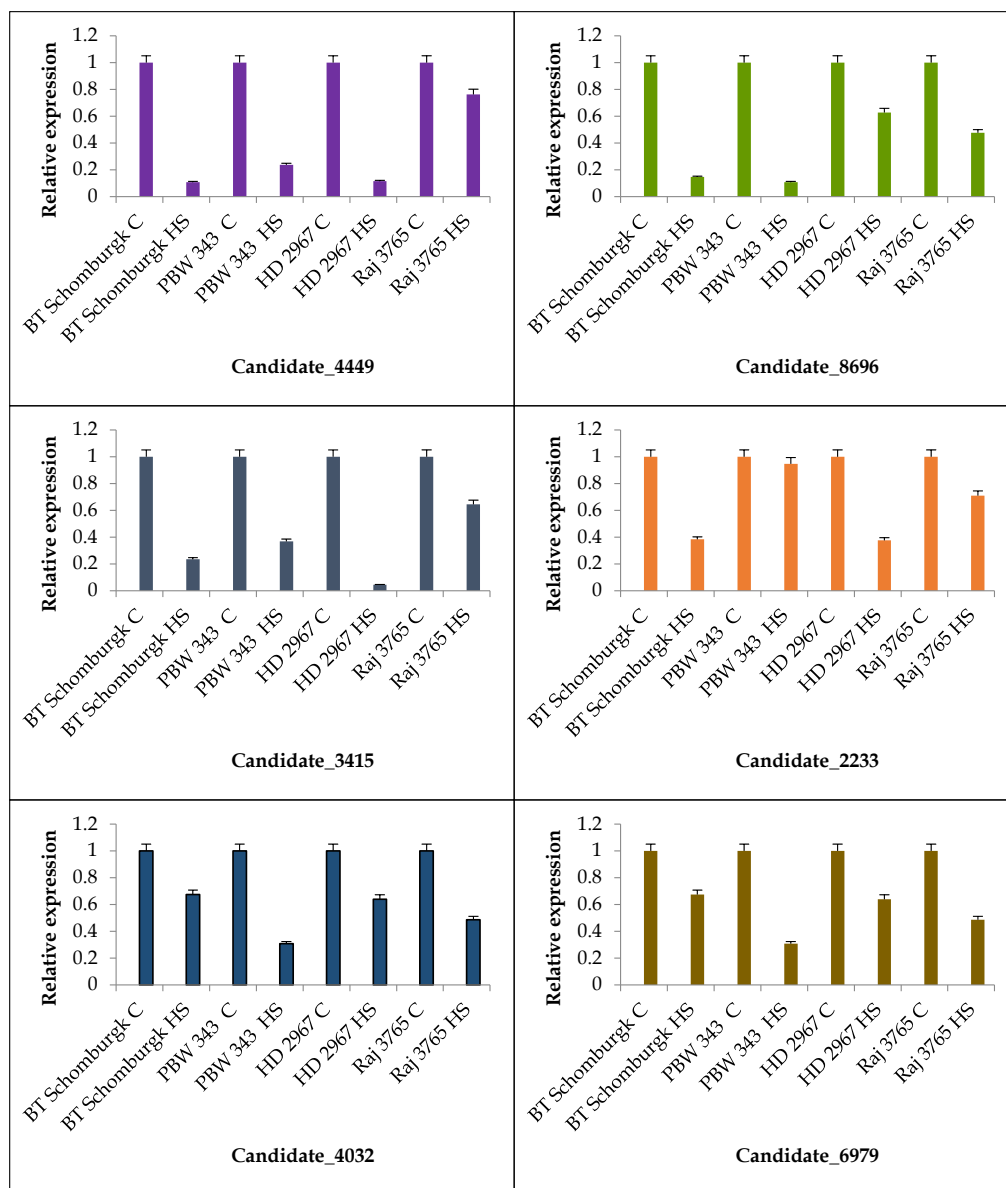


Fig. 4c. Relative expression of novel miRNAs in contrasting wheat cultivars under heat stress. Heat stress of 38°C was given for 2 h. U6 snRNA gene was used as endogenous control for normalizing the Ct values, Pfaffl method was used for the calculation.

* symbol indicates a significant difference ($p < 0.05$) between treatments (one-way ANOVA); vertical bars indicate s.e ($n = 3$).

involved in plant growth and development exhibited varying expression patterns in response to heat stress (HS). Stress-suppressed miRNAs allow the accumulation and activity of positive regulators, while stress-induced miRNAs lead to the downregulation of negative regulators of stress tolerance (Zhang, 2015). Consistent with Zhang *et al.* (2012), we also observed a concurrent decrease in miRNA transcripts and an increase in their target gene expression under HS. In thermotolerant and thermosusceptible wheat cultivars, an inverse relationship was evident between miRNA expression and their target gene levels during HS. Furthermore, a reduction in the differential expression of miRNAs was noted in response to HS. Multiple studies have demonstrated that abiotic stresses trigger the abnormal production of various miRNAs in a stress- and dose-dependent manner. Within the same plant species, different miRNAs show distinct responses to varying stressors, with some exhibiting expression patterns specific

to particular stress treatments. MicroRNAs are not directly involved in how plants react to abiotic stressors. Rather, stress-responsive genes, transcription factors, and other important elements of intricate gene networks that plants use to respond to various abiotic stressors are regulated by miRNAs. A thorough analysis of the relationships between the target genes and miRNA expression patterns showed both positive and negative correlations (Zhang *et al.*, 2012). The expression patterns of miRNAs and their targets can vary even among mRNAs within the same gene family or for the same target mRNA at different developmental stages, as noted by Lopez-Gomollon *et al.* (2012). Their study also highlighted a negative correlation between miRNA expression and their targets. Guan *et al.* (2013) reported that transgenic plants generally exhibited greater heat tolerance, while loss-of-function mutants of *csd1*, *csd2*, and *ccs* (a copper chaperone for copper/zinc superoxide dismutase: CSD1 and CSD2)

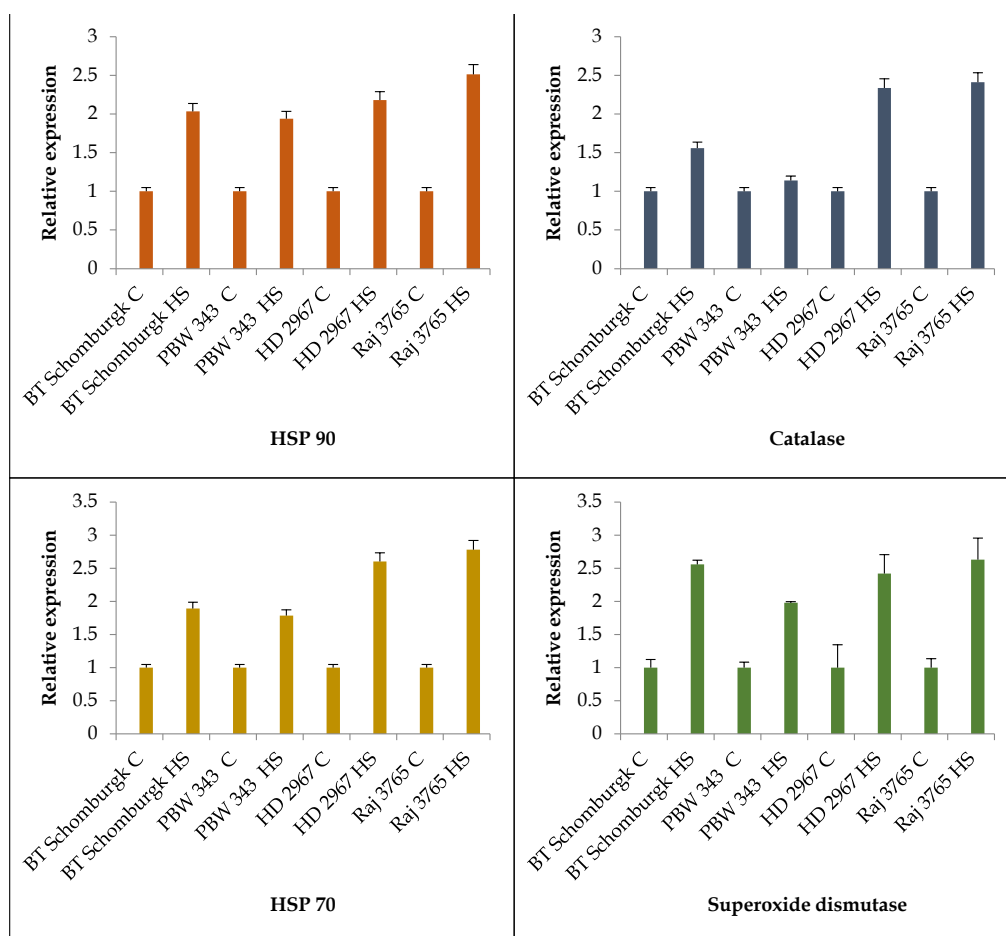


Fig. 5. Comparative expression analysis of identified novel miRNAs target gene under heat stress in contrasting wheat cultivars using quantitative real-time PCR. Vertical bars indicate s.e (n = 3).

showed increased expression of heat-responsive genes in *Arabidopsis*. Additionally, studies by Guan *et al.* (2013) and Naya *et al.* (2014) revealed that miR398 is overexpressed under heat stress, contributing to heat stress tolerance by facilitating the cleavage of *csd* mRNAs. Raised temperature stimulates the production of heat-responsive miRNAs, which in turn stimulates the expression of target genes and transcription factors. The plant's potential for thermo-tolerance under the HS is determined by the presence of these stress-associated proteins (SAPs). It is well recognised that a plant species' genotypes may vary in how well-adapted they are to abiotic stress. The miRNA-mediated control of genes might be a factor in this variation. In the current study, there were significant differences found between the transcripts of miRNAs and their corresponding targets under the heat stress (HS).

Conclusion

In conclusion, miRNA plays an important role in defence and metabolic pathways as a master gene regulator. In this study, we identified and characterized 19 novel miRNAs in wheat subjected to heat stress (HS) using a *de novo* assembly approach. Validation of the selected miRNAs confirmed their heat-responsive nature. Our findings offer valuable insights into the role of miRNAs during HS and present these newly identified gene resources as potential miRNA-based biomarkers for understanding and enhancing thermotolerance.

Acknowledgement

Financial assistance provided by the Indian Council of Agriculture Research (ICAR- IARI) is highly acknowledged. We also thank Director, ICAR- IARI for providing all the logistic support required for executing the research work.

Author contributions

MK, RRK-conceived and designed the experiments. MK, JPS - involved in the isolation and cloning of miRNA. RRK, MK, JPS- wrote the paper and edited the manuscript.

Conflict of interest

The authors declare that they have no competing interest.

References

- Borges, F. and Martienssen, R. A. 2015. The expanding world of small RNAs in plants. *Nature reviews Molecular Cell Biology* 16(12): 727-741.
- Chavez-montes, R.A., Rosas-Cardenas, D.F.F., De Paoli, E., Accerbi, M., Rymarquis, L.A., Mahalingam, G. and De Folter, S. 2014. Sample sequencing of vascular plants demonstrates widespread conservation and divergence of microRNAs. *Nature communications* 5(1): 3722.
- Cui, J., You, C. and Chen, X. 2017. The evolution of microRNAs in plants. *Current Opinion in Plant Biology* 35: 61-67.
- Dai, X., Zhuang, Z. and Zhao, P.X. 2011. Computational analysis of miRNA targets in plants: current status and challenges. *Briefings in Bioinformatics*, 12(2): 115-121.
- Dryanova, A., Zakharov, A. and Gulick, P. J. 2008. Data mining for miRNAs and their targets in the Triticeae. *Genome* 51(6): 433-443.
- Guan, Q., Lu, X., Zeng, H., Zhang, Y. and Zhu, J. 2013. Heat stress induction of mi R 398 triggers a regulatory loop that is critical for thermotolerance in *Arabidopsis*. *The Plant Journal* 74(5): 840-851.
- Kaushal, N., Bhandari, K., Siddique, K. H. and Nayyar, H. 2016. Food crops face rising temperatures: an overview of responses, adaptive mechanisms, and approaches to improve heat tolerance. *Cogent Food & Agriculture* 2(1): 1134380.
- Khraiwesh, B., Zhu, J.K., and Zhu, J. 2012. Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et Biophysica Acta* 1819(2): 137-148.
- Kotak, S., Larkindale, J., Lee, U., von Koskull-Döring, P., Vierling, E. and Scharf, K.D. 2007. Complexity of the heat stress response in plants. *Current opinion in plant biology* 10(3): 310-316.
- Kruszka, K., Pacak, A., Swida-Barteczka, A., Nuc, P., Alaba, S., Wroblewska, Z. and Szweykowska-Kulinska, Z. 2014. Transcriptionally and post-transcriptionally regulated microRNAs in heat stress response in barley. *Journal of Experimental Botany* 65(20): 6123-6135.
- Kumar, M., Kumar, R.R., Goswami, S., Verma, P., Rai, R.D., Chinnusamy, V. and Praveen, S., 2017. miR430: the novel heat-responsive microRNA identified from miRNome analysis in wheat (*Triticum aestivum* L.). *Indian Journal of Plant Physiology* 22:566-576.
- Kumar, R.R., Pathak, H., Sharma, S.K., Kala, Y.K., Nirjal, M.K., Singh, G.P., Goswami, S. and Rai, R.D., 2015. Novel and conserved heat-responsive microRNAs in wheat (*Triticum aestivum* L.). *Functional and Integrative Genomics* 15: 323-348.
- Lesk, C., Rowhani, P. and Ramankutty, N. 2016. Influence of extreme weather disasters on global crop production. *Nature* 529(7584): 84-87.

- Liu, H., Able, A.J., and Able, J.A. 2016. SMARTER de-stressed cereal breeding. *Trends in Plant Science* 21(11): 909-925.
- Liu, T., Zhang, L., Chen, G. and Shi, T. 2017. Identifying and characterizing the circular RNAs during the lifespan of Arabidopsis leaves. *Frontiers in Plant Science* 8: 1278.
- Liu, X., Huang, J., Wang, Y., Khanna, K., Xie, Z. and Owen, H.A. 2010. The role of miR160 in regulating plant development. *Plant Signaling & Behavior* 5(7): 842-845.
- Lopez-Gomollon, S., Mohorianu, I., Szittya, G., Moulton, V. and Dalmay, T. 2012. Diverse correlation patterns between microRNAs and their targets during tomato fruit development indicates different modes of microRNA actions. *Planta* 236: 1875-1887.
- Lu, Z., Xia, X., Jiang, B., Ma, K., Zhu, L., Wang, L. and Jin, B. 2017. Identification and characterization of novel lncRNAs in Arabidopsis thaliana. *Biochemical and Biophysical Research Communications* 488(2): 348-354.
- Lukasik, A., Pietrykowska, H., Paczek, L., Szweykowska-Kulinska, Z. and Zielenkiewicz, P. 2013. High-throughput sequencing identification of novel and conserved miRNAs in the Brassica oleracea leaves. *BMC Genomics* 14: 1-15.
- Ma, Z., Hu, X., Cai, W., Huang, W., Zhou, X., Luo, Q. and Huang, J. 2014. Arabidopsis miR171-targeted scarecrow-like proteins bind to GT cis-elements and mediate gibberellin-regulated chlorophyll biosynthesis under light conditions. *PLoS Genetics* 10(8): e1004519.
- Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* 7(9): 405-410.
- Stief, A., Altmann, S., Hoffmann, K., Pant, B. D., Scheible, W. R. and Bäurle, I. 2014. Arabidopsis miR156 regulates tolerance to recurring environmental stress through SPL transcription factors. *The Plant Cell* 26(4): 1792-1807.
- Sunkar, R., Girke, T. and Zhu, J.K. 2005. Identification and characterization of endogenous small interfering RNAs from rice. *Nucleic Acids Research* 33(14): 4443-4454.
- Sunkar, R., Li, Y. F. and Jagadeeswaran, G. 2012. Functions of microRNAs in plant stress responses. *Trends in Plant Science* 17(4): 196-203.
- Wu, G., Park, M.Y., Conway, S.R., Wang, J. W., Weigel, D. and Poethig, R.S. 2009. The sequential action of miR156 and miR172 regulates developmental timing in Arabidopsis. *Cell* 138(4): 750-759.
- Wu, L., Zhou, H., Zhang, Q., Zhang, J., Ni, F., Liu, C. and Qi, Y. 2010. DNA methylation mediated by a microRNA pathway. *Molecular cell* 38(3): 465-475.
- Xie, Z., Allen, E., Fahlgren, N., Calamar, A., Givan, S.A. and Carrington, J.C. 2005. Expression of Arabidopsis MIRNA genes. *Plant physiology* 138(4): 2145-2154.
- Xin, M., Wang, Y., Yao, Y., Xie, C., Peng, H., Ni, Z. and Sun, Q. 2010. Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum* L.). *BMC Plant Biology* 10: 1-11.
- Yan, K., Liu, P., Wu, C.A., Yang, G.D., Xu, R., Guo, Q.H. and Zheng, C.C. 2012. Stress-induced alternative splicing provides a mechanism for the regulation of microRNA processing in Arabidopsis thaliana. *Molecular cell* 48(4): 521-531.
- Yao, Y., Guo, G., Ni, Z., Sunkar, R., Du, J. and Zhu, J.K. 2007. Cloning and characterization of microRNAs from wheat (*Triticum aestivum* L.). *Genome Biology* 8(6): R96.
- You, C., Cui, J., Wang, H., Qi, X., Kuo, L.Y., Ma, H. and Chen, X. 2017. Conservation and divergence of small RNA pathways and microRNAs in land plants. *Genome Biology* 18: 1-19.
- Yu, S., Cao, L., Zhou, C.M., Zhang, T.Q., Lian, H., Sun, Y. and Wang, J.W. 2013. Sugar is an endogenous cue for juvenile-to-adult phase transition in plants. *Elife* 2: e00269.
- Zhang, B. 2015. MicroRNA: A new target for improving plant tolerance to abiotic stress. *Journal of Experimental Botany* 66: 1749-1761.
- Zhang, L., Hou, D., Chen, X., Li, D., Zhu, L., Zhang, Y., Li, J. *et al.* 2012. Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA. *Cell Research* 22: 107-126.