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ORIGINAL ARTICLE

MicroRNA profiles in *Sorghum* exposed to individual drought or heat or their combination

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Abstract

Sorghum is largely grown for food, fodder and for biofuel production in semi-arid regions where the drought or high temperature or their combination co-occur. Plant microRNAs (miRNAs) are integral to the gene regulatory networks that control almost all biological processes including adaptation to stress conditions. Thus far, plant miRNA profiles under separate drought or heat stresses have been reported but not under combined drought and heat. In this study, we report miRNA profiles in leaves of sorghum exposed to individual drought or heat or their combination. Approximately 29 conserved miRNA families represented by 80 individual miRNAs, 26 families represented by 47 members of less conserved or sorghum-specific miRNA families as well as 8 novel miRNA families have been identified. Of these, 25 miRNAs were found to be differentially regulated in response to stress treatments. The comparative profiling revealed that the miRNA regulation was stronger under heat or combination of heat and drought compared to the drought alone. Furthermore, using degradome sequencing, 48 genes were confirmed as targets for the miRNAs in sorghum. Overall, this study provides a framework for understanding of the miRNA-guided gene regulations under combined stresses.

Keywords Sorghum · Combined stress · Drought · miRNAs · Heat · Degradome

Abbreviations

ARFs	Auxin response factors
CDS	Coding sequences
FT	Flowering locus T
FUL	Fruitful
miRNAs	MicroRNAs
NAC transcription factors	NAM, ATAF1, CUC2 family of transcription factors
NFY TFs	Nuclear factor Y transcription factors
nt	Nucleotides; PCF6: Proliferating Cell Factor 6
RPTM	Reads Per Ten Million
SPLs	Squamosa promoter binding-like transcription factors
t-plots	Target plots
TAS3 siRNA	Trans-acting short-interfering RNA 3

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Introduction

Abiotic stresses such as drought and heat are major limiting factors for plant growth and development, consequently affecting crop production worldwide (Bartels and Sunkar 2005; Mittler and Blumwald 2010). Often both of these stresses co-occur in the field especially in the semi-arid regions of the world (Lawas 2018; Mittler 2006). The physiological responses of plants were affected to a greater magnitude under combined stresses compared to the individual stresses (Zandalinas 2018). Correspondingly, the effect of combined stresses (drought and heat) on crop yields are far greater (Mahalingam and Bregitzer 2019; Obata et al. 2015; Prasad et al. 2011). The plant responses to individual or combination of stresses are largely a reflection of altered gene expression programs (Guerra 2015). Previous studies have shown that the transcriptional regulation under individual drought or heat or their combination exhibit both shared and unique responses (Lawas 2018; Johnson et al. 2014; Rampino et al. 2012; Rizhsky et al. 2002; Shaar-Moshe et al. 2017). The commonly regulated transcripts include enzymes associated with reactive oxygen species detoxification system and sugar metabolism (photosynthesis and glycolysis) as well as heat shock proteins although the extent of regulation of these transcript levels differed greatly between individual or combined stresses (Lawas 2018; Johnson et al. 2014; Rampino et al. 2012; Obata et al. 2015; Rizhsky et al. 2002; Shaar-Moshe et al. 2017).

Sorghum (*Sorghum bicolor* (L.) Moench) is grown largely for its food grain production especially in the semi-arid tropical and sub-tropical regions of the world (FAO 2009) where drought and heat often co-occur in the field. Besides, it is also grown to use as fodder for livestock and feedstock for biofuel production (Mullet 2017). Sorghum has several stress-adaptive traits such as stay-green, high water-use efficiency and C4 photosynthesis that enable the plant species to endure stress conditions (Kapanigowda et al. 2012; Kedebe et al. 2001; Xu et al. 2000). It is ranked as one of the best crop plants that can tolerate drought and heat, therefore, emerged as a model to study molecular mechanisms underlying resistance to drought or heat and even their combination (Johnson et al. 2014). Indeed, transcriptional responses were assessed in sorghum subjected to individual drought or heat or their combination (Johnson et al. 2014). These analyses demonstrated that 1,190, 5,171 and 5,779 transcripts were differentially regulated under drought, heat, and their combination, respectively, suggesting that the overall transcriptional regulation differed greatly not only between individual drought and heat but also between individual and combined stresses (Johnson et al. 2014). The study identified the unique regulation of transcription factors (MYB and NAC transcription factors), chaperones, and genes associated with polyamine biosynthesis in sorghum under combined drought and heat (Johnson et al. 2014).

Although the transcriptional regulation is the most important process, miRNA-controlled post transcriptional gene regulation is also an important contributor to the overall regulation of gene expression in higher eukaryotes. The plant miRNAs have been shown to govern almost all biological processes ranging from maintaining stem cell status to cell and tissue differentiation to organismal growth and development (D'Ario et al. 2017; Rodriguez et al. 2016; Sunkar and Zhu, 2007). Additionally, miRNAs have been demonstrated to be important for gene regulation critical for adaptation to stress conditions as well as stress memory in plants (Saini et al. 2012; Song et al. 2019; Stief et al. 2014; Sunkar et al. 2012).

Previous studies have highlighted the roles of miRNAs in plants exposed to drought or heat. Interestingly, some of them have been shown to be responding differently to drought or heat. For instance, miR398 has been shown to be upregulated under heat while it was downregulated during drought (Guan et al. 2013; Lu et al. 2013). By contrast, miR159 was repressed by heat but upregulated by drought (Reyes et al. 2007; Wang et al. 2012). Attempts have been made to identify miRNAs that could be important for drought responses in sorghum (Katiyar et al. 2015). In the present study, we report miRNAs that are responding not only to individual drought or heat but importantly to the combined heat and drought stresses by sequencing small RNA libraries. The analysis enabled the identification 25 miRNAs, which are differentially regulated under these stresses. Interestingly, the miRNA profiles largely similar between heat and combined heat and drought treatments but differed under drought treatment. Additionally, the degradome analysis has validated 48 genes as targets for miRNAs including stress-regulated miRNAs in sorghum.

Material and methods

Plant growth conditions and stress treatments

High biomass Sorghum (*Sorghum bicolor* (L.) Moench) hybrid ES 5200 genotype seeds were sown in pots (0.15 m diameter × 0.75 m tall) filled with pure, fine sand and grown in growth chambers (Conviron Ltd., Winnipeg, Canada) at 28°/20 °C (day/night) with a photoperiod of 14 h/10 h (day/night) and a light intensity of 700 µmole m^{-1} s^{-1} and 50% humidity. After germination, sorghum seedlings were thinned to one plant per pot. Plants were drip-irrigated with half-strength Hoagland's nutrient solution three times a day to maintain pots at field capacity. To impose heat stress, sorghum seedlings grown at 28°/20 °C (day/night) for 30 days were transferred to a chamber maintained at 38°/30 °C (day/night) and continued until the plants were 80 days-old (treated for 50 days). Drought stress was imposed on plants maintained at 28°/20 °C (day/night) by withholding irrigation on day 73 and continued until day 80 of plant growth and development (8 days of progressive drought). To impose combined heat and drought stress, the above indicated heat and drought treatments were combined (30-day-old sorghum seedlings were transferred to heat stress while the drought stress was imposed on day 73 until day 80 (together, 50 days heat treatment and 8 days of progressive drought). At the end of the stress treatments (day 80), the topmost fully expended leaf (3rd leaf) from plants treated with drought, heat, and combined heat and drought along with control was harvested and snap frozen in liquid nitrogen.

Small RNA library construction and sequencing

Total RNA was isolated from the samples using the Trizol reagent according to the manufacturer's protocol. The RNA quality was verified on 1% agarose gel followed by quantification using Nanodrop spectrophotometer. Small RNA libraries were constructed using Illumina TruSeq small RNA protocol (Illumina Inc) and the sequences were bioinformatically processed as described previously (Hivrale et al. 2016). Briefly, raw data was processed to trim adapter sequences by using cutadapt, and then the short reads (< 19 nt) were removed, and a count was created for each unique sequence after removing redundant reads. Unique sequences were mapped to the sorghum genome (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor), mRNAs of sorghum, non-coding RNAs (rRNAs, tRNAs, snRNAs, snoRNAs) and repeats were removed by aligning to databases Rfam (http://www.sanger.ac.uk/Software/Rfam) and Repbase (http://www.girinst.org), and the Plant Repeats database (http://plantrepeats.plantbiology.msu.edu/downloads.html) using SOAP2. In brief, after trimming the adaptor using cutadapt, and extracting small RNAs, reads shorter than 20 nt and longer than 31 nt were removed. From these small RNAs, reads corresponding to rRNA, tRNA, snRNA and snoRNA were removed and the remaining sequences were processed for identification of conserved and novel miRNAs. First, a unique count was generated for each sequence after removing redundant reads. These unique small RNAs were used to search against the miRBase (release 22.1) for identification of known miRNA homologues. Novel miRNA identification and annotation were strictly based on miRNA* retrieval coupled with predictable fold back structures for the miRNA precursor sequences (Meyers et al. 2008).

Degradome library construction and identification of miRNA targets

For identifying miRNA targets, two degradome libraries were constructed using control seedlings or seedlings exposed to drought stress as described previously (Li et al. 2010, 2013). Briefly, the seeds were germinated and grown on the half-strength *MS* liquid medium for 15 days, which were maintained in a growth chamber with day/night temperatures of 31/22 °C and 14 h of day length. For drought treatment, 15 days-old seedlings were transferred to osmotic stress imposed by adding 25% PEG-8000 (Sigma, St. Louis, MO) to the medium for 48 h. Both control and PEG-stressed seedlings were harvested, ground to a fine powder using liquid nitrogen and total RNA was extracted using the Trizol reagent. Subsequently, poly-A + RNA was ligated to a 5'adapter containing a MmeI site at its 3'-end. The ligated products were reverse transcribed and amplified using 5 PCR cycles for obtaining the PCR product. The PCR products were digested with MmeI and the resulting fragments ligated to a double-stranded DNA oligonucleotide, purified and amplified for another ten PCR cycles and sequenced using Illumina platform. Degradome reads were filtered to remove low quality reads as well as adapter sequence, and the raw reads were used to derive unique sequences after removing redundant reads. The unique sequences were mapped to the Sorghum genome, sorghum cDNAs, as well as other non-coding RNA databases, including Rfam, repeat databases including Plant Repeat Databases and Repbase using SOAP2. The remained degradome reads were used for identifying miRNA targets among the annotated CDS from sorghum (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sbicolor) using the SeqTar algorithm (Zheng et al. 2012). Conserved miRNA target transcripts with fewer than 4 mismatches and with at least one valid read in the 9th to 11th positions of a miRNA binding site were considered as valid targets (Zheng et al. 2012). Sequences having up to 3.5 mismatches and more than 1% valid reads were considered as novel targets of miRNAs.

Results

Deep sequencing of small RNA libraries and their analysis

To identify miRNAs that are responsive to individual or combined stresses in the leaves of 80-day-old sorghum, four small RNA libraries were generated from control, drought, heat or drought plus heat-stressed plants and sequenced. A total of 28,338,681 reads from control, 27,427,142 from drought-treated, 31,541,978 from heat-exposed and 30,238,233 reads from combined drought plus heat-stressed leaves were obtained (Table1). The raw reads were further processed to obtain distinct reads from each library, which yielded 8,078,241, 7,289,746, 5,833,870, and 5,364,997 reads from control, drought, heat, and combined stress (drought plus heat) samples, respectively (Table 1). The unique small RNAs ranging between 20 and 30 nucleotides were analyzed for their size distribution and abundances, which revealed two peaks, one each at 21 and 24 nt sizes (Fig. 1) and is consistent with the characteristic feature of small RNA profiles in plants (Jagadeeswaran et al. 2012; Sattar et al. 2012; Yaish et al. 2015).

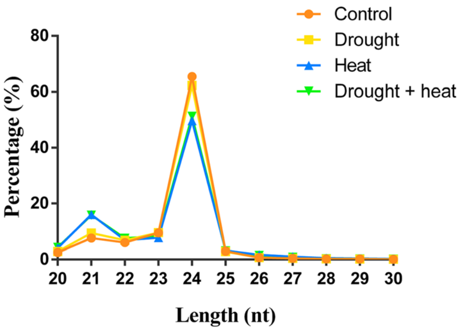

Fig. 1 Size distribution and their abundances of small RNAs in the control, drought stress, heat stress, and combined drought and heat stress, respectively

Conserved miRNAs, new homologs of known miRNA families and novel miRNAs in leaves of sorghum

Approximately 21–22 miRNA families are highly conserved among angiosperms (Sunkar and Jagadeeswaran 2008). Additionally, several other miRNA families are known to have restricted distribution, i.e., present only in dicots or monocots or only in specific lineages such as legumes, grasses etc., while some others appear to be species-specific (miRBase). Using in silico prediction or small RNA sequencing strategies, previous studies reported the occurrence of most conserved miRNAs including several novel miRNAs in young seedlings of Sorghum (Katiyar et al. 2012, 2015; Zhang et al. 2011). In this study, we have identified 127 unique small RNA sequences belonging to 55 miRNA families including 29 conserved families (miR156, miR159, miR160, miR162, miR164, miR166, miR167, miR168, miR169, miR171, miR172, miR319, miR390, miR393, miR394, miR395, miR396, miR397, miR398, miR399, miR408, miR437, miR444, miR528, miR530, miR827, miR894, miR1432 and miR2118) in the top most fully expanded (3rd leaf) leaf samples of 80 day-old sorghum (Table 2). Interestingly, only a small number of these families were abundantly expressed in leaves of mature plants. For instance, miR168 family was most abundantly expressed followed by miR827, miR156 and miR164 families. A notable observation was that the abundances of miR5564 (a non-conserved miRNA family) were even greater than the conserved miRNA families (Table 2). This was also the case in young seedlings of sorghum (Katiyar et al. 2012, 2015; Zhang et al. 2011). By contrast, several conserved families (miR319, miR390, miR393, miR394, miR395, miR397, miR398, miR399 and miR408,) were expressed at relatively low levels, i.e., below 1,000 RPTM (Table 2). Our analysis also revealed 59 new members (including 43 and 6 members belonging to miR5565 and miR5568 families, respectively) belonging to 10 miRNA families that are already reported from Sorghum (Table S1). TAS3 siRNAs are highly conserved across angiosperms and three TAS3 siRNAs were identified in this study (Table 2). Additionally, we identified 8 novel miRNAs based on miRNA* sequences present in the libraries (Table 3, Figure S1 and Table S3). Most of the identified novel miRNAs are represented by single locus in the genome whereas miRn3 has two loci (Table 3, Table S2).

Table 1 Sequenced reads and the categories to which the small RNAs were mapped

Category	Control		Drought		Heat		Drought plus heat	
	Reads	Unique sequence	Reads	Unique sequence	Reads	Unique sequence	Reads	Unique sequence
Total	28,338,681	8,078,241	27,427,142	7,289,746	31,541,978	5,833,870	30,238,233	5,364,997
Genome	18,481,782	5,531,728	16,941,272	4,960,806	13,962,795	3,038,546	13,974,582	3,195,822
mRNAs	5,847,203	474,903	4,949,216	471,260	5,057,809	348,510	5,419,653	377,605
ncRNAs	5,017,782	147,296	5,325,522	153,754	5,163,817	160,202	4,783,937	147,256
MIRNAs	3,828,011	4,486	3,238,446	4,233	3,633,812	5,060	4,138,183	5,056
Repeats	2,517,257	292,945	2,777,883	288,915	2,333,578	221,525	2,276,014	225,398

Table 2 Normalized expression levels (Reads per ten million) of known miRNAs under control and stress conditions

miRNA family	miRNA	miRNA sequence 5' → 3'	Control (RPTM)	Drought (RPTM)	Heat (RPTM)	Drought plus heat (RPTM)
miR156	miR156a,b,c,f,g,h,i	UGACAGAAGAGAGUGAGCAC	29,724	22,188	45,777	47,534
	miR156d	UGACAGAAGAGAGAGAGCACA	130	100	125	126
	miR156e	UGACAGAAGAGAGCGAGCAC	1,106	1,275	2,740	2,582
miR159	miR159a	UUUGGAUUGAAGGGAGCUCUG	2,210	2,050	3,142	3,072
	miR159b	CUUGGAUUGAAGGGAGCUCCU	8	5	8	14
miR160	miR160a,b,c,d,e	UGCCUGGCUCCCUGUAUGCCA	987	948	1,521	2,229
	miR160f	UGCCUGGCUCCCUGAAUGCCA	5	3	4	8
miR162	miR162	UCGAUAAACCUCUGCAUCCAG	1	0	1	3
miR164	miR164a,d,e	UGGAGAAGCAGGGCACGUGCA	8,058	5,665	7,008	5,669
	miR164b	UGGAGAAGCAGGGCACGUGCU	887	687	1,519	1,181
	miR164c	UGGAGAAGCAGGACACGUGAG	5	3	16	13
miR166	miR166a,b,c,d,h,i,j	UCGGACCAGGCUUCAUUCCC	1,077	1,472	1,033	1,207
	miR166e,g	UCGGACCAGGCUUCAAUCCCU	198	327	305	421
	miR166f	UCGGACCAGGCUUCAUUCCUC	1,124	1,802	1,592	2,142
	miR166k	UCGGACCAGGCUUCAUUCCU	257	536	383	419
miR167	miR167a,b,i	UGAAGCUGCCAGCAUGAUCUA	1,324	840	1,130	1,046
	miR167c,d,e,f,g,h	UGAAGCUGCCAGCAUGAUCUG	2,874	2,685	4,117	3,546
miR168	miR168	UCGCUUGGUGCAGAUCGGGAC	148,074	146,720	120,421	106,702
miR169	miR169a	CAGCCAAGGAUGACUUGCCGA	807	685	1,284	1,448
	miR169b,k	CAGCCAAGGAUGACUUGCCGG	66	61	374	328
	miR169c,h,m,n	UAGCCAAGGAUGACUUGCCUA	504	793	1,198	1,208
	miR169d-3p	GGGCGGUCACCUUGGCUAGC	28	15	114	113
	miR169d-5p	UAGCCAAGGAUGACUUGCCU	20	24	31	31
	miR169e,j	UAGCCAAGGAUGACUUGCCGG	6	11	33	30
	miR169f,g,l	UAGCCAAGGAUGACUUGCCUG	178	185	575	554
	miR169i	UAGCCAAGAAUGACUUGCCUA	0	0	2	1
	miR169o	UAGCCAAGGAUGAUUUGCCUG	1	0	1	1
	miR169p,q	UAGCCAAGAAUGGCUUGCCUA	7	11	19	21
miR171	miR171a,b,d,i,k	UGAUUGAGCCGUGCCAAUAUC	1,601	2,341	2,072	2,641
	miR171e,f	GUGAGCCGAACCAAUAUCACU	4	2	9	5
	miR171g,j	UGAUUGAGCCGCGCCAAUAUC	0	1	1	0
	miR171h	GGAUUGAGCCGCGUCAAUAUC	0	0	1	1
miR172	miR172a,c,d	AGAAUCUUGAUGAUGCUGCA	1,347	1,080	1,147	1,123
	miR172b	GGAAUCUUGAUGAUGCUGCA	13	5	12	7
	miR172e	UGAAUCUUGAUGAUGCUGCAC	34	25	33	37
	miR172f	AGAAUCCUGAUGAUGCUGCAC	0	0	1	0
miR319	miR319a,b	UUGGACUGAAGGGUGCUCCC	0	1	2	1
miR390	miR390	AAGCUCAGGAGGGAUAGCGCC	44	35	178	152
miR393	miR393a,b	UCCAAAGGGAUCGCAUUGAUC	184	189	166	232
miR394	miR394a,b	UUGGCAUUCUGUCCACCUCC	290	283	540	536
miR395	miR395a-j	GUGAAGUGUUUGGGGGAACUC	130	117	153	144
	miR395k	GUGAAGUGUUUGGAGGAACUC	1	1	0	0
	miR395l	GUGAAGUGCUUGGGGGAACUC	1	3	5	2
miR396	miR396a,b	UUCCACAGCUUUCUUGAACUG	343	195	449	444
	miR396c	UUCCACAGCUUUCUUGAACUU	372	241	598	548
	miR396d,e	CUCCACAGGCUUUCUUGAACUG	2,500	1,909	3,885	4,498
miR397	miR397-3p	UCACCGGCGCUGCACUCAAUU	27	40	26	26
	miR397-5p	UCAUUGAGUGCAGCGUUGAUG	16	12	7	8

Table 2 (continued)

miRNA family	miRNA	miRNA sequence 5' → 3'	Control (RPTM)	Drought (RPTM)	Heat (RPTM)	Drought plus heat (RPTM)
miR398	miR398	UGUGUUCUCAGGUCGCCCCCG	12	9	9	13
miR399	miR399a,c,h,j	UGCCAAAGGAGAAUUGCCCUG	3	3	3	5
	miR399b	UGCCAAAGGAGAGCUGCCCUG	2	1	3	4
	miR399d,i	UGCCAAAGGAGAGUUGCCCUG	54	28	36	34
	miR399e,f	UGCCAAAGGAGAUUUGCCCAG	0	0	1	2
miR408	miR408	CUGCACUGCCUCUUCCCUGGC	33	42	41	50
miR437	miR437a-w	AAAGUUAGAGAAGUUUGACUU	49	84	182	218
	miR437x-3p	AUUUGACUGACACGGAUUCUAGGA	7	3	3	3
	miR437x-5p	UAGAGUUGUCCUAAGUCAAACUUU	1	0	0	0
miR444	miR444a	UGCAGUUGCUGCCUCAAGCUU	173	213	350	463
	miR444b	UGUUGUCUCAAGCUUGUUGCC	193	109	91	86
	miR444c_1	UGCAGUUGUUGUCUCAAGCUU	855	871	1,461	1,526
	miR444c_2	UGCAGUUGUUGUCUCACGCUU	0	0	1	0
	miR444c_3	UGCAGUUGUUGUCUCCAGCUU	0	0	0	1
	miR444c_4	UGCAGUUGUUGUCUCGAGCUU	2	1	2	3
	miR444c_5	UGCAGUUGUUGUCUCUAGCUU	0	0	1	0
	miR444c_6	UGCAGUUGUUGUCUUAAGCUU	1	0	2	1
	miR444c_7	UGCAGUUGUUGUUUCAAGCUU	0	1	3	1
	miR444d_2	UGCAGUUGUUGCCUCAAGCUU	276	382	367	363
	miR444d_3	UUGUGGCUUUCUUGCAAGUUG	131	117	95	111
miR528	miR528	UGGAAGGGGCAUGCAGAGGAG	1251	875	515	547
miR530	miR530	UGCAUUUGCACCUGCACCUCC	25	50	17	32
miR827	miR827a	UUAGAUGACCAUCAGCAAACA	19,200	16,563	12,223	16,111
	miR827b	UUAGAUGACCAUCAGUAAACA	34	31	22	32
	miR827c	UUAGAUGACUAUCAGCAAACA	26	24	16	25
	miR827d	UUAGAUGAUCAUCAGCAAACA	30	27	18	22
	miR827e	UUAGAUGACCAUCAACAAACA	9	8	5	7
	miR827f	UUAGAUAACCAUCAGCAAACA	14	11	11	16
miR894	miR894	CGUUUCACGUCGGGUUCACCA	738	808	412	622
miR1432	miR1432	CUCAGGAGAGAUGACACCGAC	6,484	2,638	3,294	2,663
miR2118	miR2118-3p	UUCCUGAUGCCUCCCAUGCCUA	0	0	3	1
	miR2118-5p	GGCAUGGGAACAUGUAGGAAGG	1	0	1	1
miR5381	miR5381	AAGAUCUGUGGCGCCGAGC	0	0	0	0
miR5383	miR5383	AUGACAGAGCUCCGGCAGAGAUAU	1	1	0	0
miR5387	miR5387a	UAACACGAACCGGUGCUAAAGGAUC	3	1	3	3
	miR5387b	CGUGGCUCUGACCGGUGCUAAAGG	1	0	0	0
miR5564	miR5564a	UGGGGAAGCAAUUCGUCGAACA	268,946	289,404	278,348	378,408
	miR5564b	GCAAUUCGUCGAACAGCUUGA	12	6	14	12
	miR5564c-5p	AAUUCGUCGAACAGCUGCAGC	1	1	2	0
miR5565	miR5565a,b	AACACAUGUGGAUUGAGGCGAAUC	34	31	48	35
	miR5565c	UACACAUGUGGAUUGAGGUGA	64	51	81	79
	miR5565e	UUGUUUGGAUGUUGUCGGA	2	2	1	2
	miR5565f	UAGUCGGAUUUAUAUCAAUC	0	1	0	0
	miR5565g-3p	ACACAUGUGGAUUGAGAUGAAUAC	16	18	8	7
miR5566	miR5566	GAACAGCGGGGAGGUGCUGCC	5	0	1	1
miR5567	miR5567	CAUCGAAUCUUUAGACGUAUGC	0	0	0	1

Table 2 (continued)

miRNA family	miRNA	miRNA sequence 5’ → 3’	Control (RPTM)	Drought (RPTM)	Heat (RPTM)	Drought plus heat (RPTM)
miR5568	miR5568a	CAGAGCGACUUACAAUUUGGA	2	2	5	6
	miR5568b-3p	ACUAUGUAUCUAGAAAAGCUA	2	0	3	5
	miR5568b-5p	UUUCUAGGUACAUAGCUUUUG	0	0	0	1
	miR5568c-3p	ACUUACAGUUUGGAACGGAGG	2	0	2	2
	miR5568c-5p	UCUGUUCCAAAUUGUAAGUCG	2	0	0	0
	miR5568d-3p	AAAGUUGUGUAUCUAGAAAAG	2	0	1	2
	miR5568d-5p	UGGCUUUUCUAGAUACAUAGC	0	0	0	1
	miR5568e-3p	UAUCUAGAAAAGCUAAAACGU	0	1	1	0
	miR5568f-3p	GUCUUAUAAUUUGGAAUGGAG	1	0	1	1
	miR5568f-5p	UCCAUUCCAAAUUGUAAGAUG	1	0	0	1
miR5569	miR5569	UAUUGCAUGCUUGAACUAUGGUAA	2	3	3	3
miR5570	miR5570	AAAAGACAAAUCAGCAUGUCA	25	22	53	60
miR6217	miR6217a,b-3p	AAAAUUAUCGUAAAUAGAGGUGGC	1	0	0	0
	miR6217a,b-5p	UAGCCACUUUGAGUUACGAUAAUU	0	0	1	0
miR6219	miR6219-5p	GAACCGGGACUAAAGGUGGGACAU	2	1	2	1
miR6220	miR6220-3p	AUGCCUUAUAAUUUGGGAUGGAGA	0	0	0	0
miR6221	miR6221-5p	UUCUGACUUCUGGCCCCUGCU	0	0	1	0
miR6223	miR6223-3p	CUAGCAUGUUCCUCCUAAGAG	3	3	4	4
	miR6223-5p	UUCUUGGGAGGAGCAUGCUAG	1	4	3	5
miR6224	miR6224a,b,c-3p	CUUAUAUACUAGGACGGAGGG	1	0	0	0
miR6225	miR6225-5p	AACUAGACUCAAAAGAUUCAUCUC	5	8	4	5
miR6226	miR6226-3p	GAUUAGUCACGAUUAGUCGUCCGA	6	6	3	2
miR6227	miR6227-3p	CUCACAACACUUGCUAUUUGGG	1	0	0	0
	miR6227-5p	GGGCCCAAAUAGCAAGUGUUGUGA	0	1	0	0
miR6228	miR6228-3p	GUGGCAGUAGAAUUAAUGAAGGGA	2	2	1	1
miR6229	miR6229-5p	AUUCUCACUUGGGCGACGGAAAGG	3	1	0	1
miR6230	miR6230-3p	UAACAAGUUUAGGGAUCUAGA	1	0	0	1
	miR6230-5p	UUUUGGGUCCCUAAACUUGUU	0	0	0	1
miR6232	miR6232a-3p	UGGAUGUACCAAAAAAGUCAAAGC	0	0	0	1
	miR6232a-5p	GUCGCUUUGACUUUUUUGGUACAU	1	0	0	1
miR6233	miR6233-5p	UGUUGAGGCUGGAGCGAAACUCGG	1	1	1	1
miR6234	miR6234a,b-3p	UUAGCGUCAAGAGACGAACACACU	2	1	0	2
miR6235	miR6235-5p	UUGUGAGAGAAAAAUACUGUUGGC	0	0	0	1
tas3siRNA	TAS3	UUCUUGACCUUGCAAGACUUU	10	11	16	14
	TAS3	UUCUUGACCUUGUAAGGCCCU	0	0	0	1
	TAS3	UUCUUGACCUUGUAAGACCCA	1	1	2	1

Expression profiles of miRNAs under drought, heat or combined drought and heat

Using at least two-fold change as cut off, 21 conserved miRNAs belonging to 14 families (fifteen miRNAs belonging to nine families were upregulated whereas six miRNAs belonging to six families were downregulated) and four novel miRNAs were found to be differentially regulated under at least one or more of the stress conditions (Fig. 2, Table S3). Overall, there are 18 miRNAs that are found to be upregulated under heat or combined drought and heat treatment. Of these, only miR166k was found to be significantly upregulated under drought although eight other miRNAs were also upregulated but only mildly. Interestingly, similar miRNA profiles were observed under heat stress or combined drought and heat stresses (Fig. 2, Table S3). For instance, miR156e, miR164c, six members of miR169 family (miR169bk, miR169chmn, miR169d-3p, miR169e, miR169fgl and miR169pq), miR390, miR437a-w, miR444a, miR5570, miRn3a-b, miRn4 and miRn8)

Table 3 Expression of novel miRNAs

Novel miRNA	Control (RPTM)	Drought (RPTM)	Heat (RPTM)	Drought plus heat (RPTM)	miRNA sequence 5' → 3'	miRNA length (nt)
miRn1	253	167	100	118	CAUCGAAUCUUGCGGCACAUGCAU	24
miRn2	86	75	91	96	AGAGUGACAACGAGUAUGAAG	21
miRn3a-b	47	67	106	118	UCAUUAAGUUAGACUAGAGAA	21
miRn4	13	8	33	30	AUUACAUGAGCAUGACAGUGU	21
miRn5	86	90	99	132	UUGGAACUAACAAGUGGCAGU	21
miRn6	30	27	36	45	ACAAAGUUGUAGUUUCAUGAG	21
miRn7	26	22	43	45	UACACUAGAGAAGAACUGCGG	21
miRn8	91	169	205	220	UGUGAUCAUGCCAUACAUGAG	21

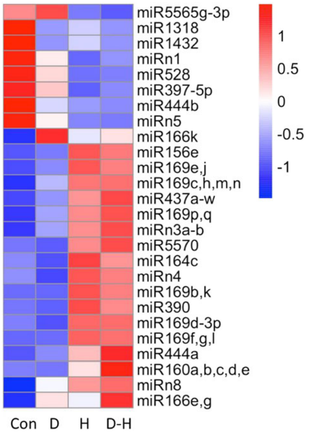

Fig. 2 Heatmap of differentially regulated miRNAs under different stress treatments (Con-Control, D-Drought, H-Heat and D-H—Combined Drought and Heat stress). The expression level of miRNA was normalized by Z-score. The bar represents the scale of relative expression levels of miRNAs

were commonly upregulated by heat as well as combined drought and heat stresses. miR160a-e, and miR166eg levels were also upregulated by combined drought and heat, while these were only mildly upregulated under heat stress alone. Interestingly, the extent of regulation for specific members differed under different stress conditions, i.e., miR166eg and miR166k levels were upregulated under all stress treatments, but the degree of upregulation of miR166eg was greater under combined drought and heat. Likewise, miR166k was strongly upregulated under drought stress (Fig. 2, Table S3).

Our analysis has identified 7 miRNAs (six known miRNAs and a novel miRNA) as downregulated under stress conditions (Table S3). Of these, only miR1432 displayed downregulation under all stress treatments (heat, drought, and combined drought and heat). The other six miRNAs that include three conserved miRNAs (miR444b, miR528, miR397-5p), and two sorghum-specific miRNA miR5565g-3p and miR5568h-3p, as well as a novel miRNA (miRn1) were downregulated by more than two-fold under heat, or combined drought and heat, while these were either slightly downregulated or unaltered under drought (Fig. 2, Table S3). Taken together, under heat alone or combined drought and heat, the levels of 24 miRNAs were differentially regulated, whereas under drought alone, only two miRNAs (miR166k and miR1432) were differentially regulated.

A few miRNAs exhibited opposite regulations under different stresses (Fig. 2, Table S3). For instance, miR164c, miR169d-3p, miR5570 and miRn4 were upregulated under heat and combined drought plus heat, but these were largely downregulated although only mildly under drought (Table 2). Contrastingly, miR5565g-3p was unaltered under drought but downregulated under heat or combined heat and drought. Furthermore, contrasting regulation was observed for members belonging to the same miRNA family under different treatments. For example, miR444a levels were significantly upregulated by heat stress and heat plus drought, whereas miR444b levels were downregulated under these conditions (Fig. 2, Table S3).

Degradome analysis for identifying miRNA targets in sorghum

In sorghum, several targets for miRNAs have been predicted using computational strategies (Du et al. 2010; Katiyar et al. 2012, 2015; Zhang et al. 2011), however, only few of them have been validated so far (Zhang et al. 2011). The degradome analysis is an effective approach for identifying miRNA targets through detection of cleavage on the target transcripts at the miRNA complementary region (Li et al. 2010, 2013). To confirm targets for the miRNAs in sorghum, two degradome libraries (one each from control and drought samples) were sequenced. The bioinformatic analysis of the reads has identified a total of 39 targets for the conserved miRNAs (28 targets from the control, and 32 targets from the drought-treated samples) (Table 4). The majority of the validated targets include transcription factors targeted by conserved miRNAs; six squamosa promoter binding like proteins targeted by miR156 family, six auxin response factors of which five are targeted by miR160 family and another auxin response factor was targeted by miR167 family, five growth regulating factors targeted by miR396 family, four scarecrow-like proteins targeted by miR171 family, three NAC-domain containing proteins targeted by miR164, three Homeodomain containing proteins targeted by miR166 family, two nuclear factor Y (NFY) transcription factors targeted by miR169 family, two Apetala-2 like transcription factors targeted by miR172, a MYB transcription factor targeted by miR159 and a transcription factor PCF6 targeted by miR319. Besides, three F-box containing proteins (2 targeted by miR393 and another targeted by miR394) and two Cu/Zn superoxide dismutases targeted by miR398 and a blue-copper containing protein targeted by miR408 have been identified. The validated targets of sorghum conserved miRNAs are shown in Table 4. The presented t-plots (target plots) depicts the abundances of the degradome signatures relative to their positions on the mRNA transcripts (Fig. 3).

We also searched for potential targets for other non-conserved miRNAs, which led to identifying nine targets for six miRNAs in sorghum. These include miR529 targeting putative protein phosphatase 2c, miR821 targeting ubiquitin-40S ribosomal protein S27a, miR5382 targeting uridine kinase-like protein 2, miR5565 has been found to target a calreticulin-like protein, an elongation factor and ribulose-bisphosphate carboxylase small chain protein, miR5568-5p appears to target uncharacterized protein (LOC8073821) while miR5568-3p appears to target NLP3 protein and miR5570 is targeting tropionin reductase homolog (Table 4; Figure S2). The presented t-plots depicts the abundances of the degradome signatures relative to their positions on the mRNA transcripts (Figure S2). Overall, our sorghum degradome analysis has identified 48 targets (39 targets for the conserved miRNA families, and nine targets for six less-conserved miRNAs) for the miRNAs in sorghum.

Discussion

Knowledge of miRNA guided stress-responsive posttranscriptional gene regulation can offer new insights into overall stress tolerance mechanisms in plants. So far, only drought-responsive miRNA profiles have been reported from sorghum seedlings (Katiyar et al. 2015). In this report, in addition to the drought, we report miRNA profiles under heat but also under the combined drought and heat treatment on mature plants.

The miR156 has been shown to be upregulated by heat stress in several plant species (Zhao et al. 2016). Heat-induced miR156 downregulates *SPL* transcription factors, which in turn activates *FLOWERING LOCUS T* (*FT*) and *FRUITFULL* (*FUL*) expression in *Arabidopsis* (Kim et al. 2015; Stief et al. 2014). More importantly, miR156 isoforms were shown to be important for heat stress memory in *Arabidopsis* (Cui et al. 2014; Stief et al. 2014), and this could be the case even in other plant species. In fact, miR156 levels were upregulated under heat or combined drought and heat in sorghum (Fig. 2, Table S3).

Auxin signaling is known to regulate variety of developmental processes in plants by activating or repressing auxin-responsive genes through transcription factors called Auxin Response Factors (ARFs) (Chandler, 2016). miR390 regulates the TAS3 precursor transcripts and the upregulated miR390 is likely to enhance the accumulation of TAS3-siRNAs that are targeting ARFs. NAC factors are known to transduce auxin signals required for a variety of organ initiation (lateral roots) and developmental activities (Xie et al. 2000). miR160 levels were upregulated in barley and sunflower *under abiotic stresses including heat (*Khaksefid et al. 2015; Kruszka et al. 2014). In sorghum, miR160a was shown to be upregulated under drought (Katiyar et al. 2015) in the present study, miR160a levels were almost unaltered under drought but was upregulated both under heat or drought combined with heat (Fig. 2, Table S3). miR160 is targeting five ARF genes (*ARF8*, *ARF13*, *ARF18*, *ARF22* and another *ARF*) in Sorghum (Table 4). miR390 levels were also upregulated under heat or combined drought and heat (Fig. 2). miR164 levels were found to be upregulated under heat and combined drought and heat stresses (Fig. 2). miR164 is targeting three NAC factors (NAC21/22/79 and 92) in Sorghum (Table 4). Taken together, the observed differential regulation of miR160, miR164 and miR390 in sorghum affecting their target genes, *ARFs* and *NACs*, differently under different stress conditions and possibly coordinating growth and developmental aspects under stress conditions.

Six different members of miR169 family were found to be upregulated under heat or drought combined with heat, while under drought, three of them were mildly upregulated and two of them were mildly downregulated (Fig. 2). Strong upregulation of several members of miR169 family both under heat and combined drought and heat suggested an important role for miR169 specifically in heat and combined stress conditions in sorghum. miR169 expression was shown to be increased (Du et al. 2017) or decreased (Du et al. 2017; Li et al. 2008) under drought in Arabidopsis. It appears that the slow soil drying decreases while a dehydration shock increases the miR169 levels in

Table 4 Target genes identified by degradome sequencing in sorghum

miRNA	Target gene ID	Alignment score	Control	Drought	Target site	Cleavage site	Target description
miR156e	Sb04g003175.1	1	+	+	1133–1152	1143	Squamosa promoter-binding-like protein 3
miR156f	Sb04g004940.1	1	+	+	823–842	833	Squamosa promoter-binding-like protein 4
miR156d	Sb07g027740.1	0	−	+	924–944	935	Squamosa promoter-binding-like protein 14
miR156d	Sb02g028420.1	0	−	+	775–795	786	Squamosa promoter-binding-like protein 17
miR156d	Sb03g044160.1	0	−	+	856–876	867	Squamosa promoter-binding-like protein 2
miR156d	Sb02g029300.1	0	−	+	1139–1159	1150	Squamosa promoter-binding-like protein 18
miR159b	Sb03g037680.1	2	+	+	972–991	982	Transcription factor GAMYB
miR160d	Sb06g022810.1	1	+	+	1387–1406	1397	Auxin response factor
miR160c	Sb06g033970.1	0.5	+	+	1239–1259	1250	Auxin response factor 13
miR160a	Sb04g026610.1	2	+	+	1454–1473	1464	Auxin response factor 8
miR160b	Sb10g027790.1	1	+	+	1750–1769	1760	Auxin response factor 18
miR160c	Sb01g019130.1	1	+	+	1333–1352	1343	Auxin response factor 22
miR164c	Sb04g023990.1	2	+	+	917–937	928	NAC domain-containing protein 79
miR164c	Sb06g019010.1	2.5	−	+	665–685	676	NAC domain-containing protein 92
miR164b	Sb08g021080.1	1	−	+	879–899	890	NAC domain-containing protein 21/22-like
miR166a	Sb01g019120.1	2	+	−	978–996	987	Homeobox-leucine zipper protein HOX9
miR166b	Sb03g002660.1	2	+	+	562–580	571	Homeobox-leucine zipper protein HOX29
miR166c	Sb01g013710.1	2	+	+	598–616	607	Homeobox-leucine zipper protein HOX32
miR167b	Sb06g032500.1	3	+	−	2520–2538	2531	Auxin response factor 12
miR169d	Sb01g011220.3	2.5	+	+	1013–1031	1023	nuclear transcription factor Y subunit A-1-like
miR169k	Sb01g045500.1	2.5	+	+	934–953	945	Nuclear transcription factor Y subunit A-7
miR171j	Sb04g032590.1	0	+	+	1128–1148	1139	Scarecrow-like protein 27
miR171j	Sb04g032570.1	0	+	+	1131–1151	1142	Scarecrow-like protein
miR171j	Sb06g024820.1	0	+	+	1143–1163	1154	Scarecrow-like protein 6
miR171j	Sb10g000520.1	1	−	+	751–771	762	Scarecrow-like protein
miR172d	Sb02g007000.1	0.5	−	+	1470–1489	1480	Floral homeotic protein apetala 2
miR172c	Sb01g003400.1	0.5	+	−	1467–1486	1477	Floral homeotic protein apetala 2
miR319b	Sb02g003070.1	1.5	+	−	1160–1178	1169	Transcription factor pcf6
miR393a	Sb09g003870.1	1	+	−	1554–1573	1564	Transport inhibitor response 1-like protein
miR393a	Sb06g014420.1	1	+	+	1503–1522	1514	Transport inhibitor response 1-like protein
miR394a	Sb03g044270.1	0	−	+	1377–1396	1387	F-box only protein 6
miR396e	Sb06g027465.1	1	+	−	447–468	458	Growth-regulating factor 3
miR396e	Sb04g034800.1	1	+	+	583–604	594	Growth-regulating factor 1
miR396e	Sb01g012170.1	1	+	+	683–704	694	Growth-regulating factor 9
miR396c	Sb01g009330.1	3	+	+	341–360	350	Growth-regulating factor 6
miR396e	Sb10g001350.1	1	−	+	783–804	794	Growth-regulating factor 5
miR398	Sb01g035350.2	2.5	−	+	165–185	176	Superoxide dismutase [cu–zn]
miR398	Sb07g023950.1	3.5	+	−	483–502	494	Superoxide dismutase [cu–zn]
miR408	Sb10g007520.1	3.5	+	+	735–754	744	Blue copper protein
sbi-miR529	Sb09g030150.1	3.5	−	+	666–682	674	Probable protein phosphatase 2c 52
sbi-miR5382	Sb02g029240.1	3.5	+	+	1158–1175	1167	Uridine kinase-like protein 2, chloroplastic

Table 4 (continued)

miRNA	Target gene ID	Alignment score	Control	Drought	Target site	Cleavage site	Target description
sbi-miR5565e	Sb05g003480.1	2.5	+	+	544–561	553	Ribulose bisphosphate carboxylase small chain
sbi-miR5565e	Sb08g002610.1	3	+	+	420–438	429	Elongation factor p
sbi-miR5565e	Sb01g002430.3	3.5	+	+	1120–1138	1129	Calreticulin-like
sbi-miR5568b-5p	Sb04g008450.1	3.5	+	+	955–975	965	Uncharacterized loc8073821
sbi-miR5568f-3p	Sb03g000490.1	3.5	+	+	3226–3245	3237	Protein nlp3
sbi-miR5570	Sb01g039880.1	3.5	–	+	932–951	943	Tropinone reductase homolog at5g06060
sbi-miR821e	Sb03g013260.1	3	+	+	709–729	719	Ubiquitin-40s ribosomal protein s27a

“ + ” indicates that cleavage on the target gene detected; “- ” indicates that cleavage on the target gene not detected

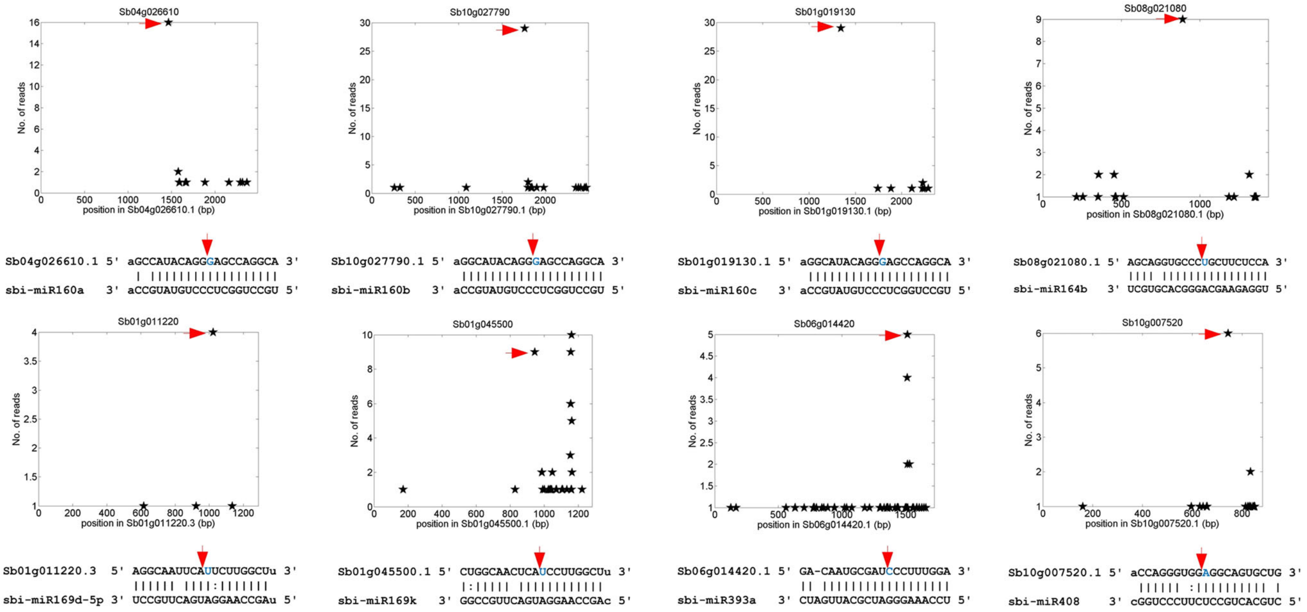

Fig. 3 Representative target-plots (t-plots) depicting cleavage on the miRNA-targeted transcripts. Arrows indicate signatures consistent with miRNA-directed cleavage. miRNA:mRNA alignments along with the detected cleavages are shown below the t-plots

Arabidopsis (Du et al. 2017). miR169 is shown to be downregulated under heat in Arabidopsis (Pegler et al. 2019) while upregulated in Arabidopsis and wheat (Zhao et al. 2016). The conflicting observations could be due to stress duration and intensity, tissue analyzed as well as other biases or species-specific responses of different plant species.

Interestingly, two miR444 members behaved differently under stresses, i.e., miR444a and miR444b showed upregulation and down regulation, respectively, under heat or combined heat and drought (Fig. 2; Table S3). A plausible explanation for this observation could be that the miR444a and miR444b are differentially regulated in the leaf tissue or miR444a is only upregulated in certain tissue while miR444b is downregulated in some other tissue within the leaves. The differential regulation of miR444a vs miR444b under stress conditions especially under heat or combined drought and heat is intriguing (Fig. 2). Interestingly, the module of miR444 and its target MADS-box factors appear to control biosynthesis of brassinosteroids, a plant hormone that could function in adaptation to drought and heat (Jiao et al, 2020; Nolan et al. 2020).

miR528 levels were downregulated by heat and combined drought and heat in sorghum (Fig. 2, Table S3). miR528 is targeting superoxide dismutase in wheat (Ravichandran et al. 2019*)*. It was previously shown that the stress-induced upregulation of Cu/Zn superoxide dismutases was dependent on downregulation of miR398, which was targeting them (Jagadeeswaran et al. 2009; Sunkar et al. 2006). The downregulated miR528 could contribute to the upregulation of superoxide dismutase in sorghum also, but further studies are required to determine this possibility.

Previous study that analyzed miRNA profiles in sorghum exposed to drought reported the upregulation of miR396bc, miR396de, miR160a and miR5385 levels and downregulation of miR169d-l, miR529, miR2118e, and miR2275 levels (Katiyar et al. 2015). In their study, miRNAs were analyzed from the leaves of 30-day-old seedlings. Our study utilized leaf samples from approximately 80-day-old sorghum plants that are subjected to 7 days of progressive drought stress or prolonged 50 days heat stress or their combination. A minimal overlap of similar regulation (miR160 and miR169 regulation) between the present study and Katiyar et al (2015) could be attributed to the differences in tissues, developmental stages, the severity and duration of the drought treatment.

miRNA-guided slicing takes place precisely between the 10th and 11th nt from the 5’ end of mRNA in the complementary region of the target transcript. Several of the target transcripts identified here for known miRNAs corresponded to previously defined targets found in other plant species (Li et al. 2010; Jones-Rhoades et al. 2006). Many of the identified targets for stress-responsive miRNAs are the transcription factors such as SPLs, NACs, ARFs, Homeobox leucine zipper proteins, NFY TFs, and MADS-box factors (Table 4) that are known to function in regulating various developmental processes. How all these differentially regulated miRNAs participate in adaptive processes associated with stress tolerance is an intriguing question?

In conclusion, the observed miRNA regulations in sorghum were varied between drought and heat; least number of regulations were observed under drought while this number was much higher under heat (Fig. 2). On the other hand, maximum overlap of similarly regulated miRNAs was observed between heat alone and combined heat with drought. Thus, the differential regulations under the combined drought and heat treatment were very similar to the miRNA profiles under heat stress. Notably, heat stress alone not only altered the expression of maximum number of miRNAs but also affected miRNA levels strongly in sorghum, compared to the combined drought and heat (Table 2). Consistent with our observations on miRNA regulations, previously it was shown that a large proportion of gene expression profiles under heat were shared (60%) with the combined drought and heat treatment, whereas only 13% of heat-responsive gene expression profiles were shared with the response to drought in sorghum (Johnson et al. 2014). These observations suggest that the heat has a dominant effect both on gene expression profiles (Johnson et al. 2014) and miRNA expression profiles (this study) under combined drought and heat. Overall, the presented results advance our understanding of miRNA-guided gene regulations, specifically under combination of stresses in sorghum.

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Declarations

Conflict of interests None of the authors have any competing interests.

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