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Evolutionary conservation of salinity responsive miRNAs from Indian wheat (*Triticum aestivum*) landrace, Kharchia Local

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ABSTRACT

Being temporal, majority of abiotic stresses induces responses in plants that needs to be regulated in energy efficient manner. These regulations are carried out by many regulatory molecules including microRNAs and transcription factors. Like protein coding genes, miRNAs are also conserved across the plant species to exhibit their conserved function during growth, development and response to biotic or abiotic stresses. The present study was carried out during 2020 and 2021 at National phytotron facility, ICAR-Indian Agricultural Research Institute, New Delhi. Control and salinity treated plants of Kharchia Local, a highly salt tolerant landrace of wheat (*Triticum aestivum* L.) from India, were grown in hydroponics and after sequencing and analysis of small RNA data, salinity responsive mature miRNA sequences from Kharchia Local were analyzed for their evolutionary relationship with sequences from the public databases. The phylogenetic study, sequence similarity (identity scores) and multiple sequence comparison was used for evolutionary conservation analysis. The study revealed that, miRNA sequences from Kharchia Local are diverse and did not group with the salinity responsive miRNAs from the database except miR1551. Interestingly, a total of 25 known or conserved miRNA families were identified as salinity responsive across the plant species. The miRNAs from Kharchia Local appears to play regulatory role in novel mechanisms of salinity tolerance.

Keywords: Abiotic stress, MicroRNA, mRNA target, Phylogenetic analysis, Seed sequence

The miRNAs are small non-coding RNAs that plays an important role in post-transcriptional gene regulation. The discovery of salinity-responsive miRNAs with their evolutionary conservation across plant species has opened new avenues for comprehending the fundamental adaptive strategies employed to thrive in saline environments (Shukla *et al.* 2008). Many plant miRNAs have been found to be up or down regulated following exposure to salt. These includes miR156, miR157, miR159, miR160, miR162, miR164, miR165, miR166, miR167, miR168, miR169, miR171 and miR172 (Lotfi *et al.* 2017). In plants, miRNAs are evolutionarily conserved indicating their conserved functions across the species during growth, development and stress responses. Across the plants, mature miRNAs are conserved evolutionarily and shows high degree of sequence similarity, which indicates their conserved functions across the species (Barik *et al.* 2015). Within miRNA family, there are many miRNAs whose sequences are highly similar, but differs by only one nucleotide base. When compared to precursor miRNAs, the mature miRNAs are highly conserved within their miRNA families (Pitchard *et al.* 2012)

Recent advancement in next generation sequencing (NGS) technology has enabled miRNA profiling easier and cost effective. Under different environmental conditions, profiling and validation of differentially expressed miRNAs have been studied in diverse plants species and now made available in curated public databases. Since the seed sequence of mature miRNA largely determines the specificity and binding ability to its target mRNAs (He *et al.* 2012), the phylogenetic analysis with mature sequences is likely to reflect true coevolutionary pattern under salt stress condition. In present study, the miRNA mediated coevolutionary regulation of salinity response in Kharchia Local, a highly salt tolerant landrace of wheat (*Triticum aestivum* L.) from India, was analyzed by sequence comparison and phylogenetic analysis with mature sequences of miRNA from different plant species. To understand functional conservation or divergence, 304 mature miRNA sequences were analyzed. The datasets consisted of top 15 upregulated and 15 downregulated miRNAs from Kharchia Local generated in the present study and 274 sequences retrieved from databases. From the 304 sequence datasets, we reconstructed phylogeny of mature

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miRNAs and identified different conserved and known miRNAs involved in salinity response in Kharchia Local.

MATERIALS AND METHODS

The present study was carried out during 2020 and 2021 at National Phytotron Facility, ICAR-Indian Agricultural Research Institute, New Delhi. Seeds of Kharchia Local were procured from ICAR-Central Soil Salinity Research Institute, Karnal, Haryana. Healthy seeds of Kharchia were germinated in a petri dish and uniform sized seedlings were selected for transplantation into a hydroponic system supplemented with Hoagland solution. These plants were raised in a glasshouse with a maintained temperature of 22/18°C (day/night), photoperiod 16/8 h (light/dark) and relative humidity of 60% at National Phytotron Facility, ICAR-Indian Agricultural Research Institute, New Delhi. After preconditioning, 10-days old seedlings were subjected to salt stress of EC 20 dS/m with a combination of salts NaCl:$CaCl_2$:Na_2So_4 (2:1:1) in a Hoagland solution.

After 1, 3 and 5 days of salinity treatment, tissue samples were collected from both control and treated plants. Total RNA was isolated using TRIZol method with two biological replicates. The quality of RNA was analyzed by using 1.2% denaturing agarose gel electrophoresis and NanoDrop spectrophotometer. For small RNA sequencing, pooled RNA samples from different time points (1, 3 and 5 days after treatment) and tissues (root and shoot) were used. After sequencing by Illumina HiSeq 2500, the generated raw data was deposited in SRA database under BioProject ID PRJNA1089600. Based on analysis, the $\log_2$ fold change values were used to select top 15 up and down regulated miRNA sequences for the analysis (Table 1). A total of 274 salinity responsive mature miRNA sequences available in open resource plant non coding RNA databases including

Table 1 Top 15 up and down regulated miRNAs from Kharchia Local under salt stress

Known name	Mature sequence	log2 Fold change	Expression
tae-miR9662a-3p	TTGAACATCCCAGAGCCACCG	5.950095094	Up
ata-miR9674b-3p	TGAATTTGTCCATAGCATCAG	5.750794064	Up
ppt-miR1053-5p	GATGGGTTATCTCAAAGTGGACTT	5.66831772	Up
tae-miR9672a-3p	CCACGACTGTCATTAAGCATC	4.580869063	Up
tae-miR9776	TTGGACGAGGATGTGCAACTG	3.995936704	Up
ata-miR169f-3p	GGCAAGTCCGTCCTTGGCTACA	3.803227036	Up
ghr-miR2949a-5p	ACTTTTGAACTGGATTTGCCGA	3.317882883	Up
aly-miR4224	CAACTGAGCTTCCTCATCTCT	3.317882883	Up
tae-miR5048-5p	TTTGCAGGTTTTAGGTCTAAGT	2.907275263	Up
ptc-miR6478	CCGACCTTAGCTCAGTTGGTG	2.880489627	Up
pla-miR11601	TGCTCTAAAAGATCGTAGTTC	2.803227036	Up
gma-miR4370	AGTAGACTCGTCCGATTTTGCGTA	2.732920382	Up
ath-miR837-3p	AAACGAACAAAAAACTGATGG	2.410883777	Up
tae-miR1123	TCCGTGAGACCTGGTCTCATAGA	1.64800518	Up
tae-miR9653a-3p	TTTGAGACTTTGGCCATGGCC	1.514501036	Up
sbi-miR6219-5p	GAACCGGGACTAAAGGTGGGACAT	-2.358453971	Down
hvu-miR5049d	TACAATTATTTAGGAACGGAG	-2.465938398	Down
tae-miR1120c-5p	TAATATAAGAACGTTTTTGAC	-2.506352666	Down
bdi-miR5166	TGCCCACCAGGGTTCGATCCA	-2.590744853	Down
ath-miR5654-3p	TGGAAGATGCTTTGGGATTTATT	-2.708396442	Down
tae-miR1125	AACCAACGAGACCAACTGCGGCGG	-2.736965594	Down
pab-miR11503	TCAAATAGAAAACAATGGACC	-2.816037165	Down
tae-miR1136	TTGTCGCAGGTATGGATGTATCTA	-2.965784285	Down
tae-miR1127b-3p	ACAAGTATTTCTGGACGGAGG	-3.035046947	Down
lja-miR11097c-3p	AAAAAACTTTACACCGTCGGT	-3.171368418	Down
ppe-miR1511-3p	ACCTGGCTCTGATACCATAAC	-3.321928095	Down
tae-miR1122b-3p	AGACTTATATGTAGGAACGGA	-3.321928095	Down
bdi-miR1878-3p	ATTTGTAGTGTTCAGATTGAGTTT	-3.395928676	Down
gma-miR9735	TACGGCTTAAGTTCAACTTTGGAG	-4.506352666	Down
mdm-miR10984a-3p	AGTCAATTACCTCATAAACTC	-5.717856771	Down

PncStress (Wu *et al.* 2020) and AsmiR database (Pradhan *et al.* 2023) were retrieved and used in the present study.

The dataset of total 304 miRNAs consisted of 274 downloaded sequences databases and 30 differentially expressed miRNAs from Kharchia. Phylogenetic tree was constructed using maximum likelihood method implemented in MEGAv11.0.8 software (Kumar *et al.* 2018), with bootstrap values calculated from 1000 trees using mature miRNA sequences. The pairwise sequence identities were calculated using SDTv1.2 (Muhire *et al.* 2014). miRbase and psRNATarget was used to check the identity of miRNA and its targets from Kharchia Local (Dia *et al.* 2018, Kozomara *et al.* 2019).

RESULTS AND DISCUSSION

Small RNA sequencing: Kharchia Local is a local landrace of bread wheat from India with high salinity tolerance. To identify miRNAs involved in salinity stress response in Kharchia Local, two small RNA libraries were constructed and subjected to high-throughput sequencing. A total of 31.5 and 30.2 million reads were generated for control and treated sample respectively (Table 2). After data processing and analysis, differentially expressed miRNAs in Kharchia Local under salt stress were identified. miRNAs having positive fold changes values are considered up-regulated whereas, miRNAs with negative fold change values are down regulated (Table 1).

Table 2 miRNA sequencing summary

Summary	Control	Treated
SRA	SRX23993959	SRX23995757
No. of Spots	31,520,133	30,257,145
Bases (bp)	1,576,412,250	1,512,857,250
GC Content (%)	49.3	22.7
No. of Reads	3,152,014	3,025,715
Size (GB)	1.0	0.776

Phylogenetic study: A total of 304 salinity responsive mature miRNA sequences were analysed to identify miRNA families involved in salinity response in plants. Based on the nucleotide similarity between miRNA sequences, the phylogenetic tree revealed the presence of different clustering patterns among miRNA sequences (Fig. 1). Within the tree, majority of miRNAs were diverged due to variation in nucleotide bases in the mature miRNA sequences that are novel or known. Whereas, 24 miRNA families were grouped together on the basis of similarity within mature miRNA sequences (Supplementary Table 1).

The main targets of these conserved miRNA families are transcription factors, hormonal receptors and enzymes involved in stress responses (Song *et al.* 2019). The family miR156, miR160, miR164, miR166 and miR167 targets SPL, NAC, HD-ZIP III and ARF family transcription factors respectively. Whereas, NF-YA, GRAS, APETALA2 and TCP family transcription factors are regulated by miR169, miR171, miR172 and miR319 respectively. In addition, the genes TIR1/AFBs, leaf curling responsiveness (LCR), ATP sulfurylase and GRF are post transcriptionally controlled by miR393, miR394, miR395 and miR396, respectively (Kumar *et al.* 2018, Yuan *et al.* 2024). Interestingly, miRNA sequences from Kharchia Local phylogenetically didn't cluster with the above conserved 24 miRNA families. The phylogenetic divergence is due to nucleotide variation in mature miRNA sequences of Kharchia Local. Among 30 sequences from Kharchia Local, miR1511 grouped with gma-miR1511 cluster due to high sequence similarity between sequences. To confirm, the sequences from Kharchia were searched for their identity in miRBase and found that all miRNAs are known (Kozomara *et al.* 2019).

Percent sequence identity: Conserved nature among the 24 miRNA families was evaluated based on identity scores between miRNA sequences (Fig. 2). Based on the sequence similarity the average identity scores for miRNA families ranged from 85–100%. Compared to other miRNAs; miR408, miR528, miR530 and miR2111 had 100% similarity within their sequences (Supplementary Table 1). This indicates, co-evolutionary conservation of above mentioned 24 miRNA families under salinity condition in plants.

In comparison, all miRNAs from Kharchia Local had less percent sequence identity scores with the 24 conserved miRNA families except KL-miR1511. The identity score for KL-miR1511 with gma-miR1511 was 85.00%, when compared with other miRNAs from Kharchia. Collectively, the miRNAs from Kharchia are conserved or known and possibly targeting mRNAs involved in salinity tolerance. One of the possible reasons for the variation in miRNA could be the ploidy level, that might have greater effect on expansion of miRNA families and evolution of new miRNA-target interactions for better adaptation under salinity stress condition in plants (Liu and Sun 2017).

Multiple sequence comparison: Most of the miRNAs in plants pairs with the target mRNAs with nearly perfect complementarity and induces its degradation. The pattern of cleavage is endo-nucleolytic in the middle of miRNA-mRNA duplex. In general, mature miRNA sequences has 5' seed region (2–8 bp), central region (9–12 bp) and 3' complementarity region (14–1 bp) (Filipowicz *et al.* 2008). In present study, the multiple sequence alignment of mature miRNA sequence was used to analyze nucleotide sequence variation among the 24 conserved miRNA families (Fig. 3). In majority of miRNA families, the sequence variation was observed at the 3' end of mature miRNA sequence. Interestingly, the seed region of most miRNA families are conserved at the 5' end (Fig. 3). The reason for the perfect and contiguous base pairing in between 2–8th position of seed region in the miRNA is to have great relationship in miRNA-target recognition (Filipowicz *et al.* 2008). On other hand, the 3' end of miRNA sequence has reasonable variations. This change in sequence might have possible role in either targeting multiple copies of same gene or multiple genes (Hashimoto *et al.* 2013).

miRNA targets of Kharchia Local: Targets of

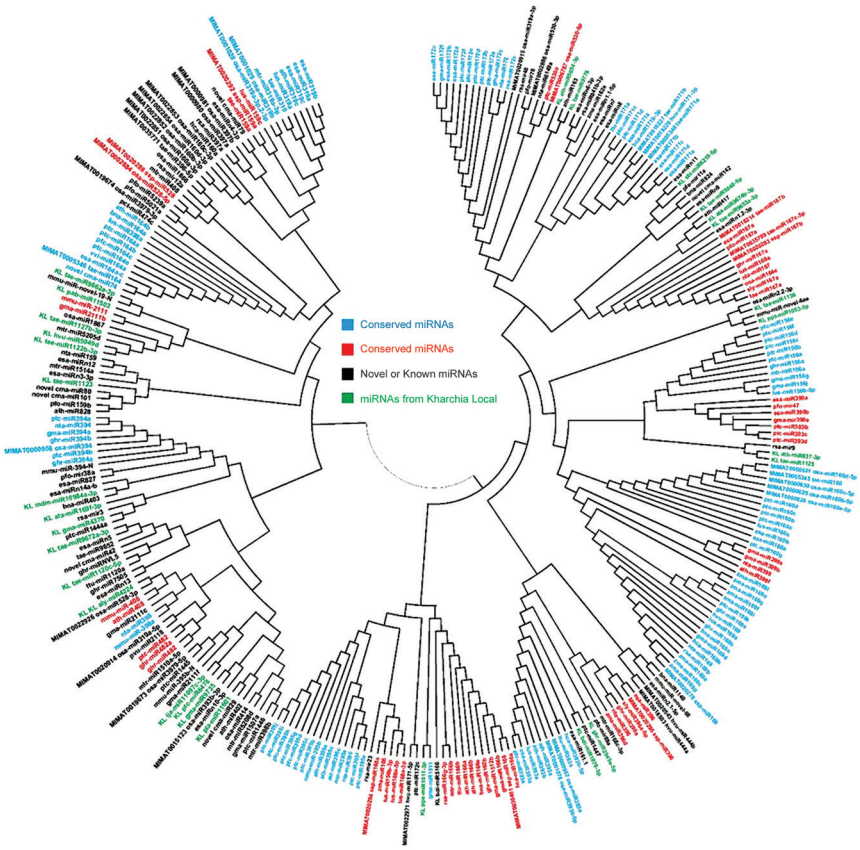

Fig. 1 Phylogenetic analysis of salinity responsive miRNAs from plants.

differentially expressed salinity responsive miRNAs from Kharchia Local were identified using psRNATarget server (Table 3). Tae-miR9662b-3p exhibited its expression only in tolerant wheat genotype (RAJ3765), under heat stress condition (Saroha *et al.* 2024). The potential involvement of miR9674 in responding to salt stress was identified. The expression of miR9674, in conjunction with tae-R9672a-3p, exhibited differential patterns between the salt-tolerant cv. Suntop and salt-sensitive cv. Sunmate, suggesting a potential role for these microRNAs in salt stress response in wheat (Zeeshan *et al.* 2021). Additionally, miRNA9776 has been documented to regulate a gene responsible for lipoxygenase, a key player in lipid metabolism (Zhao *et al.* 2020).

The overexpression of miR169d in *Arabidopsis* induces drought and heat tolerance by inhibiting NF-YA2 transcription factors (Gupta *et al.* 2024). In rice, miR169a promotes nitrogen utilization during nitrogen deficiency by targeting OsNF-YA_5 mRNA (Seo *et al.* 2024). The miR169 knockdown and overexpressed transgenic creeping bentgrass lines showed that, miR169 act as both positive and negative regulator under abiotic stress response and plant growth and development, respectively (Chen *et al.* 2024). Barley mesophyll cells experiencing drought stress showed increased expression of miR169b, suggesting a potential regulatory role for miR169b in response to drought conditions (Ferdous *et al.* 2017). Co-expression of SOS1 gene and the target gene of tdi-miR5048-p3_1ss15TG was reported in the ST genotype in wild emmer wheat (Yang *et al.* 2022). Similar co-expression pattern was observed in auxin signaling gene ARF8 and the tdi-miR393a_R+1/AFB2 module. miR5048 was found to target cytokinin dehydrogenase (CKX5), a gene known for its role in regulating cytokinin levels in plant development (Hyoung *et al.* 2020).

miR6478 is recognized for its crucial involvement in diverse biological processes, such as the mitogen-activated protein kinase (MAPK) signaling pathway, responses to plant hormones, and interactions with plant pathogens (Su *et al.* 2017). miR6478 has been implicated in the intricated

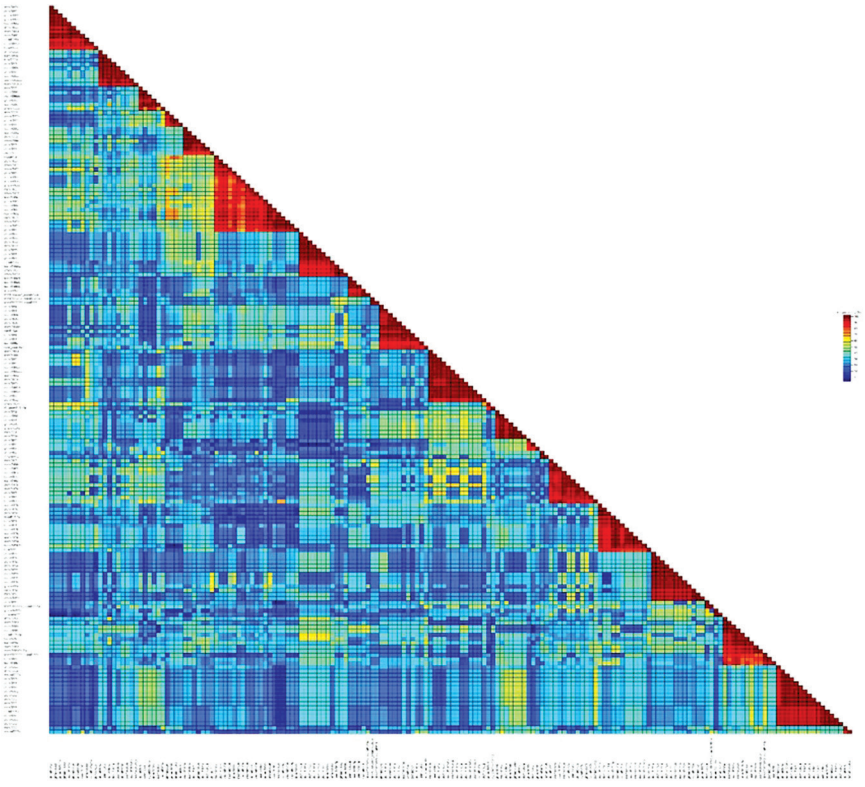

Fig. 2 Pairwise sequence identities of the conserved miRNAs.

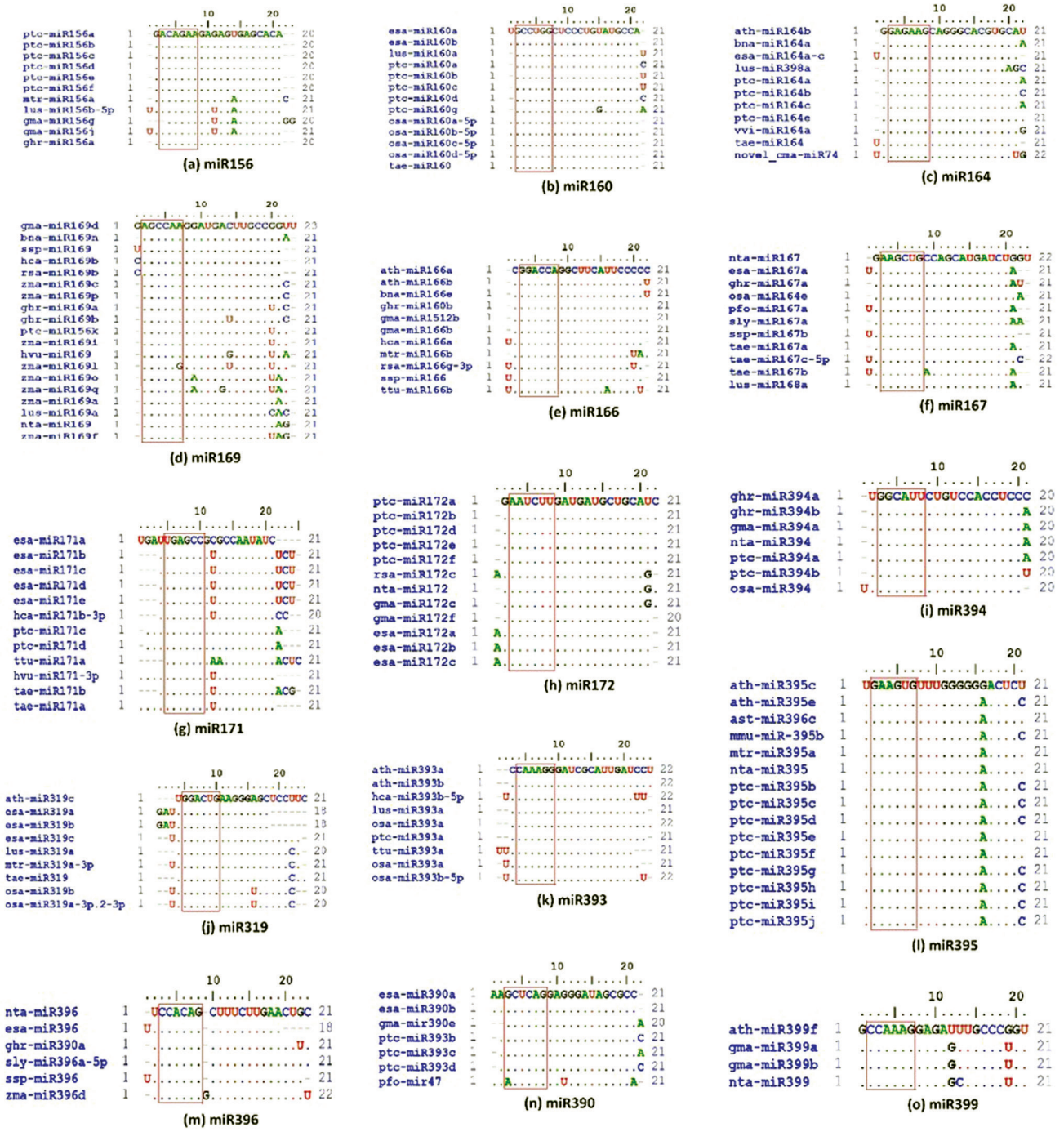

Fig. 3 Sequence comparison of conserved salinity responsive miRNA.

Seed region of mature miRNA sequence is highly conserved and highlighted in red box for miRNAs (a), miR156; (b), miR160; (c), miR164; (d), miR169; (e), miR166; (f), miR167; (g), miR171; (h), miR172; (i), miR394; (j), miR319; (k), miR393; (l), miR395; (m), miR396; (n), miR390; (o), miR399.

regulation of oxidation-reduction processes within biological systems (Sanz *et al.* 2019). Higher expression of miR6478 was observed following exposure to salinity stress in melon (Su *et al.* 2017). *Pseudomonas putida* RA-responsive microRNAs, particularly miR837-3p, were overexpressed in *Arabidopsis* (Jatan *et al.* 2020).

tae-miR1123 was established to be involved in resistance interaction between wheat and stripe rust suggesting a role play in the regulatory processes associated with the resistance of wheat plants to stripe rust (Feng *et al.* 2015). In *Phaseolus vulgaris* miR1511 exhibit responsiveness to metal toxicity. The gene sequence analysis of miR1511 in two *P. vulgaris* models, BAT93 (Mesoamerican) and G19833 (Andean), revealed a 58-bp deletion in G19833 encompassing the mature and star miR1511 sequence. The study experimentally confirmed the *P. vulgaris* models ALS3 gene as the target of miR1511, a gene crucial for aluminum detoxication in plants (Martin-Rodriguez *et al.* 2021).

Table 3 mRNA targets of salinity responsive miRNAs of Kharchia Local identified using psRNATarget server

miRNA	Target sequence	Target Acc.	Target location	Expect
KL_ata-miR169f-3p	GGUGGUGAAGGACGGACUUGUC	TraesCS7B02G041900.1	7B:41324016:41326616:1	2.5
KL_ata-miR9674b-3p	CCGAUGCUAUGGACAAGUUCA	TraesCS1D02G058000.1	1D:38257977:38259345:-1	0.5
KL_ath-miR5654-3p	ACAGAAACCCAAAGUAUCUUUCA	TraesCS1D02G376900.1	1D:453274017:453278271:1	2
KL_ath-miR837-3p	GUAUCAGUUUUUUGUCCGUUU	TraesCS6D02G367800.1	6D:455425056:455426440:1	1.5
KL_bdi-miR1878-3p	ACACUUGAUUUGAACAUUACAGAU	TraesCS5B02G369200.1	5B:548194676:548198598:1	2.5
KL_bdi-miR5166	GCGGUCGUGCCCUGGUGGGUA	TraesCS7D02G318500.1	7D:406900408:406901358:-1	2.5
KL_ghr-miR2949a-5p	AAUGGAGAUCCAGUUCAAAGGU	TraesCS3B02G222400.1	3B:283586705:283605449:1	2
KL_gma-miR4370	GCUGCAACAUCGGACGAGUCAACU	TraesCS7B02G167900.1	7B:234590456:234590876:1	2.5
KL_gma-miR9735	CUUGGAAGUUGAACUUGGGCUGGA	TraesCS2D02G249100.4	2D:294965872:294977343:1	3
KL_hvu-miR5049d	CUCCGUUCCUAAAUAAUUGUC	TraesCS6B02G434200.1	6B:702815241:702817606:1	1
KL_KL_aly-miR4224	CAAGAUAAGGAAGCUCAGUUG	TraesCS5B02G272100.2	5B:457389444:457390779:-1	1
KL_KL_aly-miR4224	AGGGUUGGGGAAGCUCAGUUG	TraesCS7A02G295400.3	7A:385427040:385430702:1	2
KL_lja-miR11097c-3p	UAUGGCGGUGUACAGUUUUUU	TraesCS4B02G151900.2	4B:247558199:247566991:1	2.5
KL_mdm-miR10984a-3p	CAGGUUAUGAGGUGGUUGACU	TraesCS6B02G073300.1	6B:49756445:49760981:-1	2
KL_pab-miR11503	UCUUCAUUGUUUUUUUUUUGA	TraesCS5D02G090000.1	5D:97261273:97274169:-1	2.5
KL_pla-miR11601	GGACCUCGAUCUUUUGGAGCA	TraesCS4D02G034800.1	4D:15795308:15797831:1	2.5
KL_ppe-miR1511-3p	ACAGUGGUAUCAGAGCCAGGU	TraesCS6A02G012300.1	6A:5671555:5676470:1	1.5
KL_ppt-miR1053-5p	UCGGCCCCUUUGAGAUAACCCUUC	TraesCS5B02G056000.1	5B:62027233:62029396:-1	2.5
KL_ptc-miR6478	UACCAACUGAGCUAAGGUCGG	TraesCS4A02G406400.1	4A:679256462:679260502:-1	0
KL_sbi-miR6219-5p	GAAGUUCACCUUUAGUCCCGGUUC	TraesCSU02G218500.1	322405960:322407604:-1	0.5
KL_tae-miR1120c-5p	GUCAAAAACGUUCUUAUAUUA	TraesCS3D02G241500.1	3D:335627686:335630660:1	0
KL_tae-miR1122b-3p	UCCGUUCCUAAAUAUAAGUCU	TraesCS7D02G277200.1	7D:265695957:265698602:1	1.5
KL_tae-miR1123	UCUAUGAGACCAGGUCUCACGGG	TraesCS2B02G338500.1	2B:483544376:483548719:1	0.5
KL_tae-miR1125	UCGUCGCAGUUGGUCUCGUUGGUU	TraesCS2A02G129800.1	2A:77744237:77745785:1	0
KL_tae-miR1127b-3p	CCUCCGUCCAGAAAUACUUGU	TraesCS4B02G356700.1	4B:647029967:647035594:-1	0
KL_tae-miR1136	UAGAUACAUCCAUACCUGCGACAA	TraesCS1D02G160700.1	1D:227651152:227655545:1	0
KL_tae-miR5048-5p	AUUUGGACCUAAAACCUGCGAA	TraesCS7B02G494500.1	7B:746046286:746055154:1	1
KL_tae-miR9653a-3p	UUCCAUGGCCGAAGCCUCAAA	TraesCS1B02G374100.2	1B:604548650:604553666:1	2
KL_tae-miR9662a-3p	CCGUGGCUCUGGGAUGUUCAG	TraesCS6A02G032200.1	6A:15933325:15935266:-1	0.5
KL_tae-miR9672a-3p	AUUGCUUGAUGAUAGUUGUGU	TraesCS5A02G281500.1	5A:490592865:490596129:-1	2.5
KL_tae-miR9776	CAGUCGUACGUCCUCGUCCAG	TraesCS3A02G108000.1	3A:72896734:72898021:-1	2.5

miR1136 exhibit higher expression in response to heat stress in HD2985 wheat plants. Heat responsiveness miRNA profiling of heat tolerant cultivar HD2985 was carried out in 12-days old seedlings and 44 miRNAs were found to be highly expressed (Raghupathy *et al.* 2016). tae-miR1136 regulates a diverse array of target genes, including OBSCN, DHX37, MRC1, STAB1, LOC105379283, SNORA14B, ZNF174, CALN1, UQCC2. This microRNA has been found to have regulatory role targeting 30S ribosomal protein S3 and exhibits significant interactions with 1725 pre-miRNA coding loci across various sub-genome (Ravichandran *et al.* 2019).

In recent years, area of plant salinity research has grown rapidly and accumulated large number of miRNA sequence datasets. The role of miRNA is well understood in some tolerant crops, but not fully explored in Kharchia Local under salt stress. In present study, the differentially expressed mature miRNA sequences from Kharchia Local were compared for conservation or divergence with available sequences in the public databases. Phylogenetic analysis revealed that, out of 304 salinity responsive miRNA sequences, 180 sequences were grouped into 24 miRNA

families based on their sequence similarity. Due to variation in sequence of Kharchia Local, the miRNAs diverged from the conserved miRNA families, indicating possible novel functional role in salinity tolerance. Under salinity condition both evolutionarily conserved and species-specific miRNAs are expressed and believed to have both conserved and specific functions in plants. Most importantly, majority of conserved miRNA family targets enzymes, transcription factors and hormonal receptors involved in the process of plant lifecycle. The novel or non-conserved miRNAs possess unknown specific functions and as a future direction, advanced molecular biology tools can be used as validation strategy to understand their roles. Genome editing techniques to alter base sequences in seed region of miRNA could be an effective approach to know their target partners. As a future study, our findings can be validated experimentally to elucidate regulatory mechanism underlying salinity tolerance and can used as a valuable genetic resource to develop salinity tolerant wheat varieties.

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