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Deep sequencing of small RNAs reveals ribosomal origin of microRNAs in *Oryza sativa* and their regulatory role in high temperature

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ABSTRACT

MicroRNAs are small noncoding regulatory RNAs which control gene expression by mRNA degradation or translational repression. They are significant molecular players regulating important biological processes such as developmental timing and stress response. We report here the discovery of miRNAs derived from ribosomal DNA using the small RNA datasets of 16 deep sequencing libraries of rice. Twelve putative miRNAs were identified based on highly stringent criteria of novel miRNA prediction. Surprisingly, 10 putative miRNAs (mi_7403, mi_8435, mi_12675, mi_4266, mi_4758, mi_4218, mi_8200, mi_4644, mi_14291, mi_16235) originated from rDNA of rice chromosome 9. Expression analysis of putative miRNAs and their target genes in heat tolerant and susceptible rice cultivars in control and high temperature treated seedlings revealed differential regulation of rDNA derived miRNAs. This is the first report of rDNA derived miRNAs in rice which indicates their role in gene regulation during high temperature stress in plants. Further studies in this area will open new research challenges and opportunities to broaden our knowledge on gene regulation mechanisms.

1. Introduction

MicroRNAs (miRNAs) are small (19–25 nucleotides) non-coding regulatory RNAs present in plants and animals (Bartel, 2004). They regulate gene expression and play an important role in plant growth, development and stress responses (Agarwal et al., 2015; Chen, 2004; Mangrauthia et al., 2017; Sunkar and Zhu, 2004). Next generation sequencing technology and comparative genomics have accelerated the discovery of conserved and novel miRNAs in different plants and animal species (Pritchard et al., 2012). The novel miRNAs have been annotated in rice by several researchers (Guo et al., 2012; Mutum et al., 2016; Paul et al., 2016). Earlier reports of deep sequencing of miRNAs showed significantly higher expression of conserved miRNA families than novel or non-conserved miRNAs (Chen et al., 2013; Mangrauthia et al., 2017; Sunkar et al., 2008). In model crop rice, it is assumed that most of the biologically important miRNAs have been discovered. Hence discovery rate of novel miRNAs in rice has reduced substantially with new stringent criteria of miRNAs prediction. In order to improve the novel miRNAs prediction, different criteria and bioinformatics tools have been suggested which evolved with time and experimental validations (Friedlander et al., 2008; Jeong et al., 2013; Meyers et al., 2008).

While annotating the novel miRNAs, it is a normal practice to filter the sequence reads derived from repetitive DNA, ribosomal RNA (rRNA) and transfer RNA (tRNA) (Jeong et al., 2013; Motameny et al., 2010; Mutum et al., 2016; Paul et al., 2016). However, one should wonder whether miRNAs may exist in such genetic loci also? In very few recent studies on animals, biologically important miRNAs have been discovered which originated from rDNA. Murine miR-712 and human miR-663 were identified as the most mechanosensitive miRNAs derived from pre-ribosomal RNA (Son et al., 2013). Another study identified novel miRNA in the rDNA region of *Drosophila* (Chak et al., 2015). In yet another interesting finding, re-evaluation of the miRBase list of miRNAs suggested that significant number of annotated mouse

Abbreviation list: miRNA, microRNA; siRNA, small interfering RNA; rDNA, Ribosomal DNA; rRNA, Ribosomal RNA; N22, Nagina22; qRT-PCR, Quantitative real time PCR; *OsFBX-OsFBX193*, F-box domain containing protein; *OsPE*, pectin esterase; *OsRPK*, Receptor-like protein kinase precursor; *OsBGP*, beta-galactosidase precursor; *OsGDCP*, glutaredoxin domain containing protein; *OsRRR*, Response regulator receiver domain containing protein; *OsGT*, glycosyltransferase

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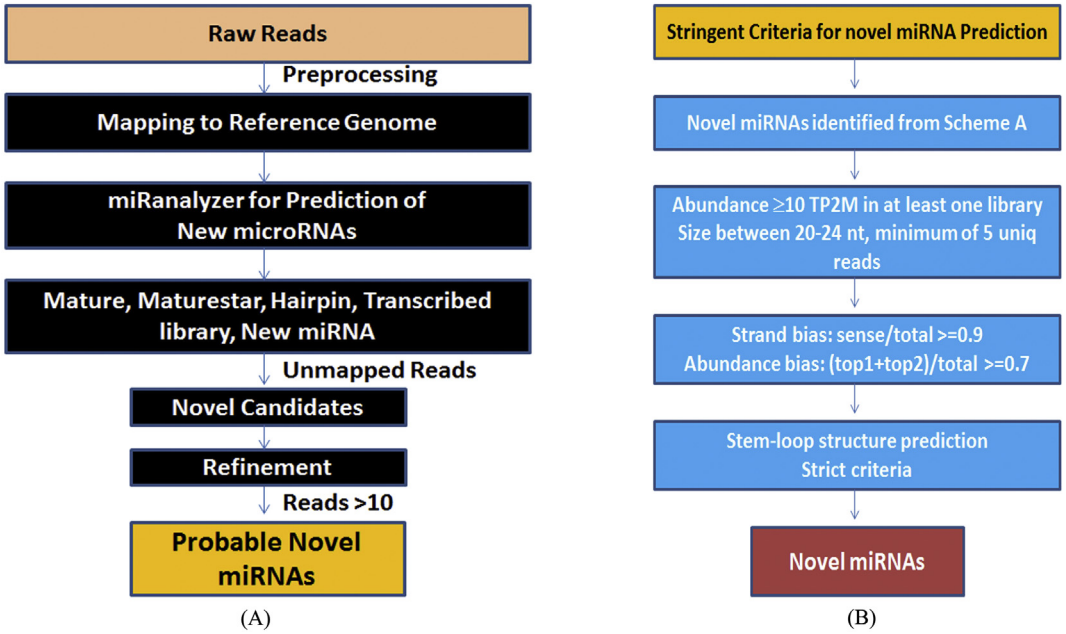

Fig. 1. (**A**) The outline of bioinformatics analysis work flow followed for prediction of novel miRNAs in rice. (**B**) The bioinformatics work flow for novel miRNA prediction by following stringent criteria. Novel miRNA identified from scheme 'A' were passed through different filters such as abundance, size, strand and abundance bias, and strict criteria of stem loop structure described in material and methods. TP2M, transcripts per 2 million.

Table 1
Summary of small RNA sequence assembly and analysis for prediction of novel miRNAs.

Samples	Total number of reads after quality filter and adapter removal	Number of unique reads after removal of known miRNAs	Number of unique reads matched genome	Number of reads filtered	Predicted miRNAs (5 model concordant) + reads count > 10
N22-CS	1,52,84,870	21,99,438	12,77,656	7,77,666	1020
N22-CR	1,42,51,351	20,52,166	7,43,593	5,14,983	344
N22-LS	1,83,97,168	7,44,611	4,22,838	8,26,222	311
N22-LR	1,82,47,384	24,83,255	4,11,450	13,31,302	289
N22-SS	94,18,764	9,85,091	5,60,269	21,86,359	433
N22-SR	3,04,36,715	21,01,806	8,33,410	1,05,53,929	666
N22-RS	2,08,04,542	14,85,977	8,07,125	14,95,041	605
N22-RR	1,55,57,190	21,52,059	4,14,445	3,73,193	213
Vandana-CS	1,94,85,806	24,76,314	15,94,700	3,49,281	1355
Vandana-CR	1,64,77,372	27,99,356	7,25,053	3,01,239	307
Vandana-LS	1,88,02,901	14,45,816	6,84,896	4,92,381	405
Vandana-LR	1,26,72,421	5,55,454	3,92,051	2,43,916	51
Vandana-SS	1,45,03,031	16,18,074	9,71,972	17,54,372	705
Vandana-SR	1,47,28,017	20,65,208	3,62,306	27,54,881	272
Vandana-RS	1,46,43,846	16,21,919	7,99,203	31,04,503	685
Vandana-RR	1,26,72,491	12,86,173	1,72,408	20,60,865	132

CS-control shoot, CR-control root, SS-short duration heat stress shoot, SR- short duration heat stress root, LS-long duration heat stress shoot, LR- long duration heat stress root, RS- recovery shoot, RR- recovery root.

miRNAs were derived from rRNAs. MicroRNAs which were mapped to rRNA sequences were miR-2182, miR-5102, miR-5105, miR-5109 and miR-5115 (Castellano and Stebbing, 2013). Among the plants, the ribosomal derived miRNAs were recently predicted in wheat, where the precursor sequences of three non-canonical putative miRNAs- ttu-42, ttu-48, and ttu-53 matched to ribosomal RNA (De Paola et al., 2016). There are no such reports from other plants.

With a hypothesis that there could be some more widely expressed novel miRNAs in rice which were not identified by earlier researchers because of bioinformatics filters removing miRNAs emanating from ribosomal DNA, the present study was designed to identify the novel miRNAs from rice originating from rRNA and repetitive DNA. Recently, we identified high temperature responsive miRNAs from root and shoot tissues of heat susceptible and tolerant rice cultivars (Mangrauthia et al., 2017), and analyzed the expression of high temperature responsive miRNAs from 16 small RNA libraries. The deep sequencing of miRNAs in two contrasting rice genotypes helped in identification of 162 miRNA families some of which showed specific expression with respect to genotype, treatment and tissue. The most abundant miRNAs were miR166, miR168, miR1425, miR529, mR162, miR1876, and miR1862. The high temperature tolerant rice genotype showed expression of osa-miR1439, osa-miR1848, osa-miR2096, osa-miR2106, osa-miR2875, osa-miR3981, osa-miR5079, osa-miR5151, osa-miR5484, osa-miR5792, and osa-miR5812 during high temperature, which were not observed in susceptible genotype. In this study, using the sequencing dataset of high temperature induced small RNAs, sequencing reads were utilized for annotation of novel miRNAs without filtering them for rRNA or repeat sequences. Highly stringent criteria were followed to annotate the novel miRNAs and their gene targets in rice. This is the first report showing the presence of putative novel miRNAs emanating from rRNA in rice and their possible role in high temperature response in plants.

Table 2
Newly identified microRNAs of *Oryza sativa* and their stem loop sequences.

S.No	MicroRNA	Stem loop sequence and length	Mature microRNA sequence	Length	Abundance bias	Strand bias	Origin and chromosome loci
1	mi_7403	TCGGCTCGGGGCGTGGACTGTTGT CGGCCGCGCCGGCGGCCAAAGCCCGGGGG CTCCGCGCCCCCGGCAGCCGTCGTCGGCGCAGCC GGTCACCGCGCGCCTCTGGCGCGCCCCT (115 bps)	GUCGUCGGCGCAGCCGGUCACC	22	0.816	1.0	rDNA, Chr 9
2	mi_8435	CCCTCGTGCCGGCGACGCATCATTCAAA TTTCTGCCCTATCAACTTTCGATGGTAGGATA GGGGCCTACCATGGTGGTGACGGGTGACGGAGAATTAGGG TTCGATTCCGGAGAGGGAGCCTG (123 bps)	CCUAUCAACUUUCGAUGGUAGG	22	0.706	1.0	rDNA, Chr 9
3	mi_12675	CGGGCGTCCCGCGGCGGCTCGACG GCGCGAGCGGCGTGGCCTCGCGGCGC CCGGCACCCAAGCGTGCCGGCGCTGCCAAGGCCACCTCGC GCGTGCCATTGGTCCCGGATGCCGC (115 bps)	ACGGCGCGAGCGGCGUGGCCU	21	0.956	1.0	rDNA, Chr 9
4	mi_1778	CATGGATACCCCTATCATATAGTGGT TCAGGACATCTCTCTTTCAAGGAAGCAG CTGGGATTCAACTTCCTTGAGGGTAGGAG TATTATGAAAGTATGTTAATTGTAGGTTATCA (115 bps)	ACAUCUCUCUUUCAAGGAAG	20	0.777	1.0	Chr 2
5	mi_4266	GGGCTGGGCTCGGGGGTCCGGCCCCG AACCCGTCGGCTGCCGGCGGACTGCTCG AGCTGCTCGCGCGGCGAGAGCGGGCCG CCGCGTGCCGGCCGGGGGACGGACCGG GAACGGCCCCCTCG (123 bps)	GUCGGCUGCCGGCGGACUGCUC	22	0.747	1.0	rDNA, Chr 9
6	mi_4758	TGGCCGTTTAGGCCACGGAAGTTTGA GGCAATAACAGGTCTGTGATGCCC TTAGATGTTCTGGGCCG CACGCGCGCTACACTGATGTATCC (91 bps)	UGCCCUUAGAUGUUCUGGGCC	21	0.849	1.0	rDNA, Chr 9
7	mi_4218	GGGCGTATCGCTGTGTTCCTTGAC GCCGTCGGCGCCGTGGGTTCTGTTGC GGCCCGGGGGCCTCGGTTGCCTCGC GCGCGAGCGCTCGG (89 bps)	UCCUUGACGCCGUCGGCGCCG	21	0.888	1.0	rDNA, Chr 9
8	mi_8200	TCGGTGCAGATCTTGGTGGTAG TAGCAAATATTCAAATGAGAACTTT GAAGGCCGAAGAGGAGAAAGGT TCCATGTGAACGGCACTTGCACA TGGGTAAGCCGATCCTAAGGG (113 bps)	AGCAAAUAUUCAAAUGAGAACUUU	24	0.863	1.0	rDNA, Chr 9
9	mi_2046	AGTTCTATAAAATGCCATCG TACAAGCGATTTTGTCCTAGAA ATACCATTGTCGTTAGGGTTCCAT CCATCCCACGCCGTTAACGGAACCTT AACGGCGATGGTATTTCTGGGACAAA ATCGTTTGTACGATGATATTTCATGGA ACTAGTAGACATTTCTTGAAATTG (169 bps)	AACGGCGAUGGUAUUUCUGGGA	22	0.755	1.0	Chr2&6
10	mi_4644	AGTTATCTTTTCTGCTTAACGGCCCGCC AACCCTGGAAACGGTTCAGCCGGAGG TAGGGTCCAGCGGCCGGAAGAGCACCGCACG (85 bps)	CUGCUUAACGGCCCGCCAACCCUG	24	0.861	1.0	rDNA, Chr 9
11	mi_14291	CTACCCATAAGCGAGATGCTCTCGGAAG ACGACAGCCCGCCCGGCCGCCGCGTGTCCG CCGCTCCCGACCCGGGGGCGGCGGCGACGCGCG TCGGACGGCGCGGGCTCGTCGCGGAGGACGTGCTA (127 bps)	ACGACAGCCCGCCCGGCCGCCG	22	0.764	1.0	rDNA, Chr 9
12	mi_16235	TTCACCTACGGAAACCTTGTTACGACTTCTCCT TCCTCTAAATGATAAGGTTCAATGGACTTCTC GCGACGTCGGGGGCGGCGAACCGCCCCCGTC GCCGCGATCCGAACACTTCACCGGACCATTCAATC (131 bps)	UUCUCGCGACGUCGGGGGCGG	21	0.938	1.0	rDNA, Chr 9

2. Materials and methods

2.1. Data sets, preprocessing and mapping

Small RNA sequencing reads used in this study were earlier used for identification of high temperature responsive known miRNAs in rice (BioProject ID: PRJNA322758) (Mangrauthia et al., 2017). 31,92,60,462 raw reads consisting 27,13,53,814, high-quality raw sequence were generated from small RNA isolated from root and shoot tissues of heat tolerant (Nagina 22) and susceptible (Vandana) rice genotypes. Small RNA was extracted from control and heat treated (short and prolonged heat stress and recovery) tissues. The *O. sativa* Nipponbare was used as a reference genome for mapping which was retrieved from the MSU Rice Genome Annotation Project Database and Resource (Kawahara et al., 2013). The HQ score small RNA reads were mapped to the reference genome of Nipponbare using bowtie (Langmead et al., 2009). Sequencing data is available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA322758/.

2.2. Discovery of microRNAs and prediction of target genes

Bioinformatics work flow followed for prediction of novel miRNAs is provided in Fig. 1. To predict the novel miRNAs candidates, miRanalyzer standalone version (Hackenberg et al., 2011) pipeline was used (Fig. 1A). The novel miRNA candidates which were predicted 5 times consistently with reads count > 10 were selected (Meng et al., 2012) (Table S1). Since the number of predicted novel miRNA was high, more stringent criteria for annotation of novel miRNAs were followed (Fig. 1B). For the stringent selection of novel miRNA, small RNAs of 20 to 24 nucleotides in length, with an abundance of ≥10 TP2M (transcripts per 2 million) in minimum one small RNA library, and a minimum of 5 unique reads, were selected. Strand bias was calculated by: Total abundance of sequences matching the sense strand/ Total abundance of all sequences matching both the strands. Further, abundance bias was calculated by: The sum of the abundances of the top two sequences for the precursor/Total abundance of all matching sequences. Small RNA sequences showing strand bias of ≥0.9 and abundance bias of ≥0.7 were selected for further analysis. These criteria were followed based on a previous study (Jeong et al., 2013). The stem-loop structure of selected small RNAs was predicted using mfold web server (http://www.bioinfo.rpi.edu/applications/mfold) (Zuker, 2003). Strict criteria for stem loop structure prediction were: 1-mature miRNA sequence should be in either of the arm of hairpin structure, 2-Number of mismatches 4 or less, 3- No loop or break in miRNA sequences, 4- asymmetric bulges should be minimal in size (one or two bases) and frequency (typically one or less), especially within the miRNA/miRNA* duplex. These criteria of stem loop structure prediction were followed as reported previously (Meyers et al., 2008). To further verify the putative novel miRNAs, the stem loop sequences were utilized to search for similarity with miRBase miRNA sequences using default parameters (http://www.mirbase.org/search.shtml). The origin of putative novel miRNAs was ascertained by mapping the sequence data in reference rice genome and NCBI-BLAST search tool. The target genes of identified miRNAs were predicted using plant miRNA target prediction program psRNATarget, 2011 release (Dai and Zhao, 2011).

2.3. Expression profiles

In order to analyze the expression of putative novel miRNAs and their target genes, quantification of transcripts was done using qRT-PCR. For miRNAs expression, small RNA was extracted from control (30 °C) and heat treated (42 °C for 6 h) 15 days old seedlings of rice cultivars N22 and Vandana using mirVana™ miRNA Isolation Kit (Ambion). cDNA for miRNAs was synthesized using miScript II RT Kit (Qiagen) which was used as template for miRNA quantification using miScript SYBR Green PCR Kit (Qiagen). For miRNAs expression analysis miScript Universal Primer (reverse primer provided in kit) and miRNA specific forward primer (Table S2) were used. Internal control for miRNAs quantification was U6 (Ding et al., 2011).

Similarly, expression analysis of predicted targets genes of novel miRNAs was done using qRT-PCR. RNA was extracted using RNeasy Plant Mini Kit (Qiagen). For isolation of RNA, the same plant samples (used for miRNA expression analysis) were used. Total RNA was converted to cDNA using Improm-II reverse transcription system (Promega) and Oligo dT primers. SYBR Premix Ex-Taq (Takara) kit was used for real time PCR reaction. It was run in an ABI GeneAmp 7500 Sequence Detection System. Internal control was *OsActin1* (Lee et al., 2011). The primer sequences of target genes are listed in Table S2. All the real time PCR reactions were run in three biological replications. Also, each reaction was performed in duplicate. The Dissociation Curves Software (Applied Biosystems) was used for construction of melting curves to ensure detection of a single specific product. We used $\Delta\Delta C_T$ method for calculation of expression change. The details of protocols and analysis followed for real time PCR of genes and miRNAs can be referred from our earlier study (Mangrauthia et al., 2017).

3. Results

In our previous study, we identified and analyzed the expression profile of known miRNAs and their gene targets using the small RNA sequence data set (Mangrauthia et al., 2017). Here, remaining sequences of sixteen small RNA libraries, classified as “unannotated” (excluding known miRNAs and reads matching to coding transcripts), were used to discover novel and potential miRNA candidates from rice. Novel miRNAs were searched irrespective of their origin from genome including repetitive DNA and rDNA. To accomplish this, these small RNA sequences were aligned with the *O. sativa* genome to identify genomic regions potentially harboring candidate pre-miRNA sequences whose hairpin-like structures were used for distinguishing miRNAs from other small noncoding RNAs. Using miRanalyzer standalone version, the candidate novel miRNAs predicted 5 times consistently and reads count > 10 were selected (Table 1 & Table S1). Since the number of predicted novel miRNAs was quite high, more stringent criteria were set to predict potential novel miRNAs. To follow the strict criteria, novel miRNAs were passed through several filters. The first filtering was done based on cut-offs for abundance and size. In the next filter, strand bias and abundance bias cut-offs were applied to differentiate the miRNA precursors from siRNA loci (Table S1). Finally, stem-loop structures of miRNA precursors were visually evaluated to confirm the accuracy of miRNA prediction. The predicted novel miRNAs were referred as putative miRNAs.

3.1. Identification of rDNA derived novel miRNAs in rice

Sixteen small RNA libraries yielded ~271 million high-quality raw sequences derived from small RNAs isolated from control and high temperature treated seedlings. These high quality reads were mapped to the reference genome of *O. sativa* Nipponbare (Langmead et al., 2009) (Table 1). A highly stringent criterion was followed for the prediction of novel miRNAs, and as a result 12 putative novel miRNAs were identified (Table 2). Stem loop sequences and target genes of putative novel miRNAs are given in Tables 2 and 3, respectively. The stem-loop secondary structures of ten putative miRNAs are shown in Fig. 2. Sequence similarity search of these stem loop sequences with miRBase miRNA sequences showed significant similarity with existing microRNA stem loop sequences submitted in miRBase (Fig. S1) thus confirming the robustness of prediction of these novel miRNAs. We performed BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to ascertain the origin of the newly identified putative miRNAs. The hairpin and mature miRNA sequences were used for searching the homologous sequences in rice genome. Interestingly, out of 12 novel miRNAs, 10 miRNAs were derived from rDNA (Table 2). After mapping

Table 3
Predicted target genes of novel miRNAs.

miRNA ID	Target acc.	Expect (E)	Target accessibility (UPE)	Target descriptions	Inhibition
mi_7403	LOC_Os06g13850.1	2.5	22.981	OsFBX193 - F-box domain containing protein	Translation
	LOC_Os11g43830.1	2.5	24.132	Pectin esterase	Cleavage
	LOC_Os06g13960.1	2.5	23.168	OsFBX194 - F-box domain containing protein	Translation
	LOC_Os02g20380.1	2.5	19.51	Expressed protein	Cleavage
mi_8435	LOC_Os11g47000.1	2	16.492	Receptor-like protein kinase precursor, putative, expressed	Translation
	LOC_Os01g65460.1	3	18.395	Beta-galactosidase precursor, putative, expressed	Cleavage
	LOC_Os02g07920.1	3	22.7	Expressed protein	Cleavage
mi_12675	LOC_Os10g34170.1	1.5	23.964	Glutaredoxin domain containing protein	Cleavage
	LOC_Os04g43650.1	2	24.498	L-allo-threonine aldolase	Cleavage
	LOC_Os07g23390.1	2	20.717	Expressed protein	Cleavage
	LOC_Os07g16130.1	2.5	21.498	Acetyltransferase GNAT family	Translation
	LOC_Os03g05640.1	3	20.461	Inorganic phosphate transporter	Cleavage
	LOC_Os01g56050.1	3	21.458	MATE efflux family protein	Cleavage
	LOC_Os02g10280.1	3	23.21	Transposon protein putative unclassified	Translation
	LOC_Os02g03010.1	3	22.897	Expressed protein	Cleavage
	LOC_Os04g56750.1	3	17.607	Zinc finger C-x8-C-x5-C-x3-H type family protein	Cleavage
	LOC_Os01g62490.1	3	17.775	Laccase precursor protein	Cleavage
	LOC_Os01g18170.1	3	20.787	Cupin domain containing protein	Translation
	LOC_Os01g04250.1	3	18.695	Expressed protein	Translation
	LOC_Os03g10940.1	3	19.874	Casein kinase II subunit alpha-2	Translation
mi_1778	LOC_Os10g25600.1	3	21.757	Expressed protein	Cleavage
	LOC_Os02g40510.1	3	16.464	Response regulator receiver domain containing protein	Cleavage
	LOC_Os07g49480.1	3	11.831	KIP1	Cleavage
	LOC_Os04g20270.1	3	23.512	Transcriptional regulator Sir2 family protein	Translation
mi_4266	LOC_Os06g07600.1	3.0	22.775	Uncharacterized glycosyltransferase, putative, expressed	Cleavage
mi_4758	LOC_Os06g31250.1	3	22.838	Retrotransposon protein, putative, unclassified, expressed	Cleavage
	LOC_Os07g17790.1	3	18.725	Retrotransposon protein, putative, Ty3-gypsy subclass	Translation
mi_4218	LOC_Os02g09720.1	2	24.349	Multidrug resistance protein, putative, expressed	Translation
	LOC_Os07g36820.2	2.5	24.582	Uncharacterized Cys-rich domain containing protein, putative, expressed	Cleavage
	LOC_Os01g65530.1	2.5	23.965	RNA recognition motif containing protein, putative, expressed	Cleavage
	LOC_Os08g33420.1	3	22.2	Agenet domain containing protein, expressed	Cleavage
mi_8200	LOC_Os09g16260.1	2.5	12.313	Expressed protein	Cleavage
	LOC_Os12g36730.1	3	15.626	Stripe rust resistance protein Yr10, putative, expressed	Cleavage
	LOC_Os12g36690.1	3	12.973	mla1, putative, expressed	Cleavage
	LOC_Os12g36720.1	3	15.11	RGH1A, putative, expressed	Cleavage
mi_2046	LOC_Os03g47050.1	1.5	19.2	Expressed protein	Cleavage
	LOC_Os02g03810.1	2.5	10.65	OsFBX34 - F-box domain containing protein	Cleavage
	LOC_Os01g31360.2	3	15.014	Expressed protein	Translation
mi_4644	LOC_Os03g41640.1	3	24.304	GRF zinc finger family protein, expressed	Cleavage
mi_16235	ChrSy.fgenesh.mRNA.20	2.5	24.206	Hypothetical protein	Cleavage
	LOC_Os01g05560.1	2.5	22.678	Expressed protein	Translation

the full cluster sequences to rice genome, we observed that all rDNA derived novel miRNAs were located on chromosome 9 of rice genome (Table S1).

3.2. Expression analysis

Expression analysis of putative miRNAs and their target genes was verified using qRT-PCR (Fig. 3). All the 12 putative miRNAs except mi8200 showed down-regulation during high temperature stress in heat tolerant rice cultivar N22. On the other hand, Vandana, the heat susceptible rice cultivar, showed up-regulation of 7 putative miRNAs during high temperature stress (Fig. 3A). This confirmed that novel miRNAs originating from rDNA are differentially expressed in heat susceptible and tolerant rice genotypes during high temperature stress. The expression of some of the selected target genes was also analyzed under heat stress condition in both the genotypes and their expression was correlated with the expression of corresponding miRNAs (Fig. 3B). Two gene targets of mi_7403 i.e., *OsFBX193* - F-box domain containing protein (*OsFBX*) and pectin esterase (*OsPE*) showed expected negative expression correlation in N22 while Vandana showed positive correlation. Receptor-like protein kinase precursor (*OsRPK*) and beta-galactosidase precursor (*OsBGP*) are predicted gene targets of mi_8435. Among these two targets, *OsRPK* showed negative correlation of expression in N22 and Vandana. The predicted target of mi_12675 is glutaredoxin domain containing protein (*OsGDCP*) which did not show negative expression correlation in both the genotypes. Response regulator receiver domain containing protein (*OsRRR*), the gene target of mi_1778 was negatively correlated in N22 while Vandana showed positive correlation of expression. The glycosyltransferase (*OsGT*) gene target of mi_4266 showed negative expression correlation in both the genotypes. Overall, among the 7 predicted gene targets, 5 showed expected negative correlation with corresponding miRNAs in heat tolerant N22 while susceptible cultivar Vandana showed expected expression correlation in case of only 2 genes (Fig. 3B).

3.3. Re-sequencing of rDNA regions harboring putative miRNA sequences from N22 and Vandana

Flanking primers were designed such that to cover the stem loop and mature miRNA sequences of all the rDNA derived putative miRNAs. These primers were used for the amplification and sequencing of putative miRNA genetic regions from both N22 and Vandana (Table S2). Sequences of both the rice genotypes were aligned with the reference genome sequence to observe the nucleotide changes in the stem loop and mature miRNA regions. Most of the putative miRNA genetic regions were highly conserved showing 100% similarity. Sequence variations in the stem loop and mature miRNA sequences were detected in the mi_7403, mi_12675, mi_4266 and mi_4758. In particular, mi_7403 showed significant variations in both stem loop and mature miRNA regions when compared with reference sequence (Fig. 4).

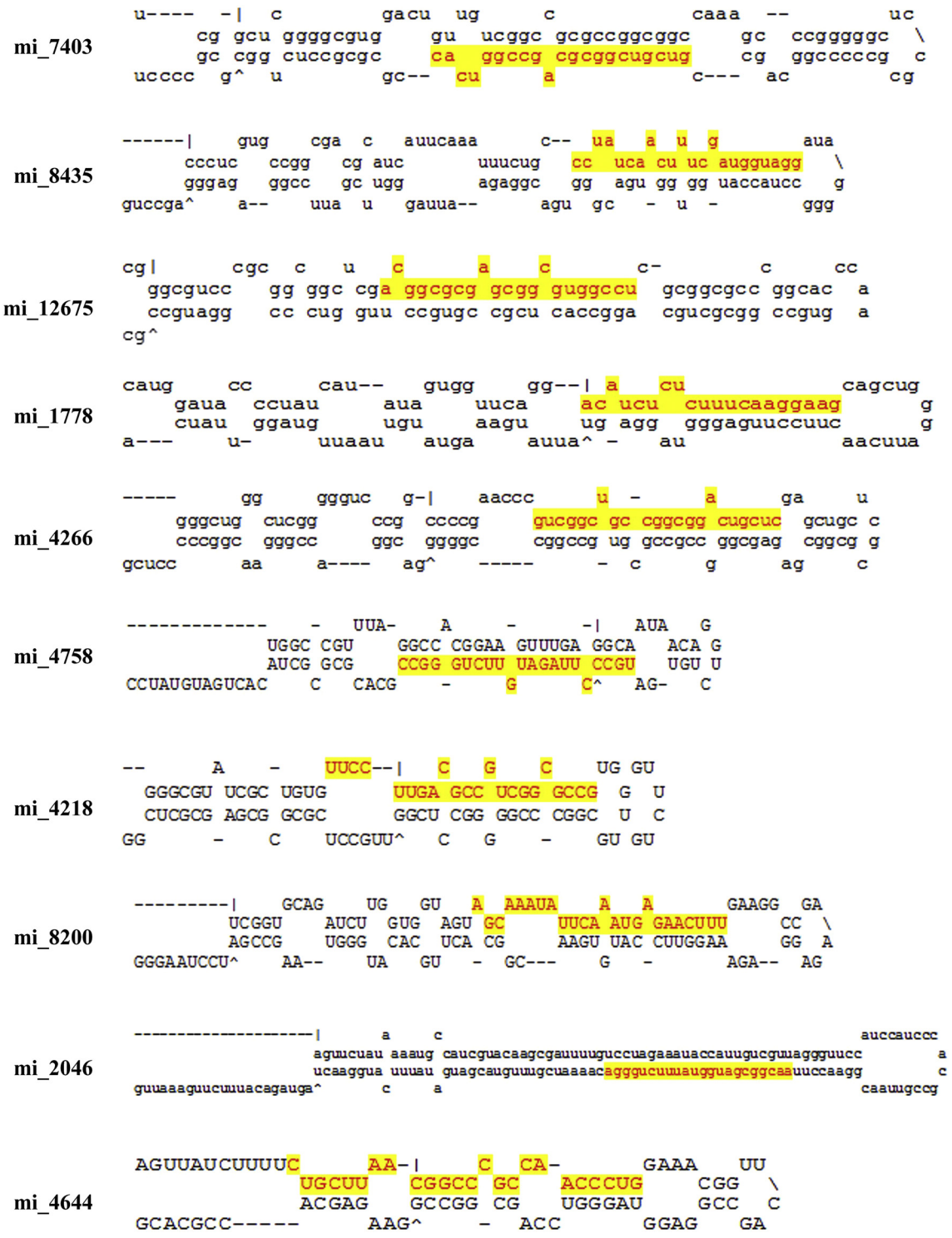

Fig. 2. The stem-loop secondary structures of predicted novel miRNAs. Mature miRNA sequences have been highlighted with yellow colour.

4. Discussion

Although many miRNAs have been discovered from a well-studied crop *O. sativa*, the large data set of small RNAs used in this study gave an opportunity for identification of new miRNAs in rice which originates from rDNA. Interestingly, all the rDNA derived novel miRNAs were mapped on Chromosome 9 in this study. It should be noted that nucleolar organizer, consisting of 17S-5.8S-25S rDNA units was reported at the end of telomere of the short arm of rice chromosome 9 (Matsumoto et al., 2005; Shishido et al., 2000). So far, very few studies have indicated the miRNAs originating from rDNA. miRNA-712 (miR-712) a mechanosensitive miRNA upregulated by disturbed flow in endothelial cells, was reported to be derived from preribosomal RNA (Pecinka et al., 2010). While, re-examining the miRBase list of miRNAs, Castellano and Stebbing, 2013 reported that 1% of annotated miRNAs of mammalian tissue could be mapped onto rRNAs. Some of the miRNAs derived from rRNA sequences were miR-2182, miR-5102, miR-5105, miR-5109 and miR-5115. miRNAs originating from rDNA in plants have not been reported previously except one study, where the predicted novel miRNAs were putatively derived from ribosomal RNA

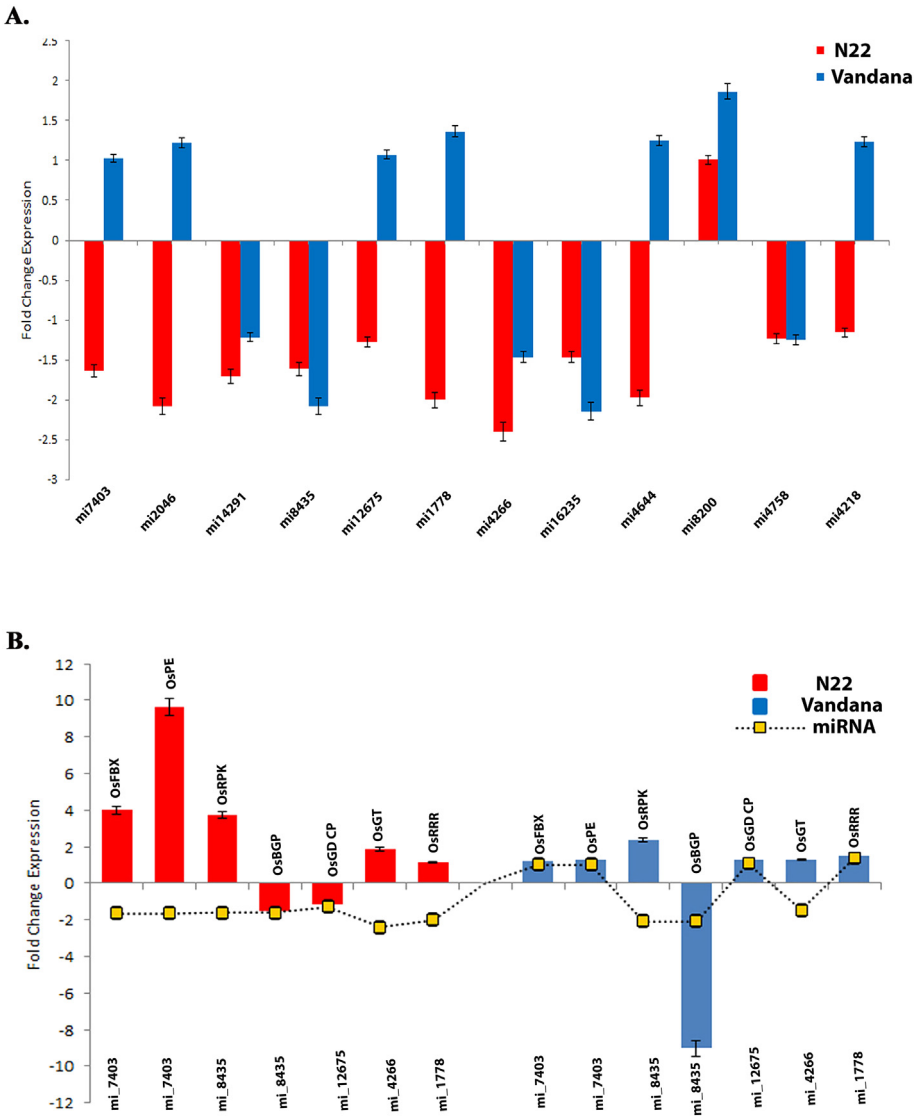

Fig. 3. (**A**) Expression verification of predicted novel miRNAs in N22 and Vandana. The fold change expression values of miRNAs in heat stress (in comparison to control) were plotted in Y axis. (**B**) Expression analysis of predicted target genes and their correlation with expression of corresponding miRNAs in N22 and Vandana. The fold change expression values of genes and miRNAs in heat stress (in comparison to control) were plotted in Y axis. Bars represent the mean ± S.E. of three biological replicates.

in durum wheat (De Paola et al., 2016). Chak et al. (2015) discovered a deeply conserved, noncanonical miRNA hosted by ribosomal DNA which was widely expressed and could not be identified by previous studies due to bioinformatics filters removing repetitive sequences. In another study, transcripts of subtelomeric repetitive sequences of rye (*Secale cereale* L.) showed high homology with rice and sorghum microRNAs and the possible role of miRNAs in the chromatin architecture was indicated (Tomas et al., 2013). Using deep sequencing approach, this is first report indicating origin of miRNAs from rDNA in rice. The most stringent criteria for prediction of miRNAs as suggested by Meyers et al. (2008) and Jeong et al. (2013) were followed.

In a recent study, Son et al. (2013) reported that miR-712 was derived from an unexpected source, pre-ribosomal RNA, in a XRN1-dependent, but DGCR8-independent and DICER1-dependent manner which suggests that pre-rRNA regions could be important site for processing of dicer mediated miRNAs. Further experiments which can demonstrate the processing of these identified putative miRNAs of rice by using rice *dcl-1* or other *dcl* mutants will strengthen this innovative area of research. Due to unavailability of *dcl1* mutant in rice, it would be important to develop miRNA pathway mutants in rice using genome editing technology which will facilitate the discovery of such non-traditional miRNAs. It will open new avenues to better understand the complexities of gene regulation mechanisms. Interestingly, miRNAs are processed by some noncanonical pathways also (Kim et al., 2009). For example, dme-mir-1003 was the first discovered mirtron that exists as an intron of pre-mRNA and is processed into pre-miRNA without the Drosha canonical processor (Ruby et al., 2007). This means that all protein-coding, noncoding, intergenic, and intragenic regions can become miRNA hosts (Yoshikawa and Fujii, 2016).

Identification of substantial number of putative novel miRNAs originating from rDNA in rice in this study could also be due to conducting of these experiments in high temperature stress. High temperature induced the activity of the silenced repetitive sequences in Arabidopsis and decondensation of centromeric and 5S rDNA sequences were observed (Tittel-Elmer et al., 2010). Decondensation of ribosomal 45S chromatin after heat stress was reported in *O. sativa* (Santos et al., 2011). Significant increase of rDNA transcription was induced by heat stress (Tomas et al., 2013). It should be noted that the comprehensive study of miRNAs regulated at three high temperature treatments {short 42 °C/36 °C (day/night) for 24 h, long 42 °C/36 °C for 5 days, and

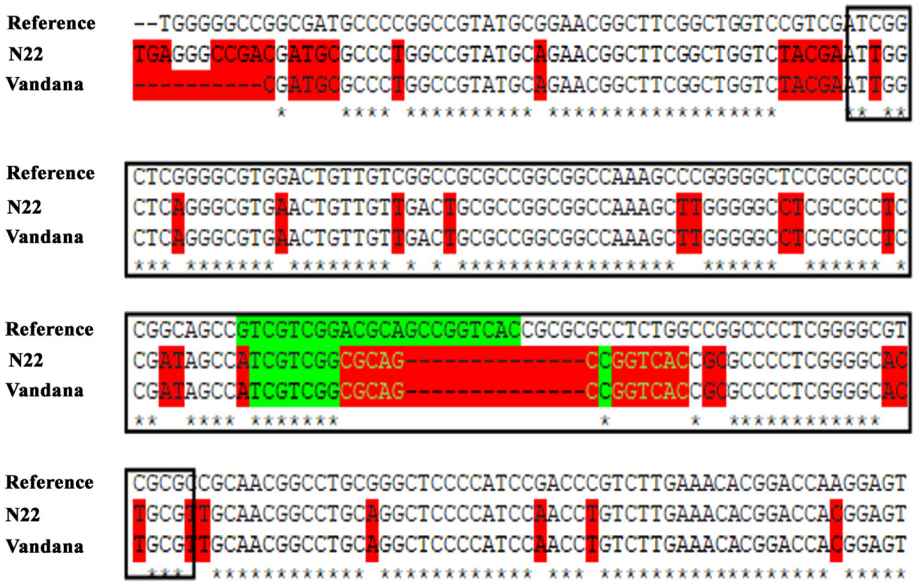

Fig. 4. Sequence alignment of mi_7403 genetic region. The black box indicates the stem loop sequence; green colour signifies mature miRNA sequences while red colour denotes the variable sequences. *O. sativa* Nipponbare was used as reference sequence.

recovery-42 °C/36 °C for 4 days followed by 30 °C/24° for 24 h} in shoot and root of susceptible and tolerant rice cultivars was first of its kind (Mangrauthia et al., 2017). Considering the important role of rRNA during high temperature stress, it would be interesting to further characterize the function of these rDNA derived miRNAs in gene regulation at high temperature.

Expression analysis suggested that rDNA derived putative miRNAs and their target genes are differentially regulated in heat tolerant N22 and susceptible Vandana cultivars. Most of the putative miRNAs showed down-regulation in N22 while majority were upregulated in Vandana during high temperature stress. Further, predicted target genes showed expected negative expression correlation in N22 while in Vandana, majority of target genes did not show negative correlation. In our previous study, we had shown that N22 regulates its genes more efficiently than Vandana during high temperature stress (Mangrauthia et al., 2017; Sailaja et al., 2014). Two predicted targets genes of mi_7403 were identified i.e. *OsFBX193* (F-box domain containing protein) and pectin esterase which showed desired negative expression correlation in N22 while Vandana showed positive correlation of expression with this putative miRNA. The regulation of these predicted target genes by mi_7403 during heat stress need to be functionally characterized. Interestingly, re-sequencing of putative miRNA genetic locus showed significant variations in stem loop sequence and mature miRNA of mi_7403. It would be important to further investigate the biological function of such miRNA candidates. Novel microRNA mi_14291 did not show any target genes against MSU Rice Genome Annotation Project Release 7 (*O. sativa* spp. japonica cv Nipponbare) embedded in psRNATarget tool which could be due to uniqueness of this putative miRNA to indica rice species.

5. Conclusion

In conclusion, identification of novel miRNAs from rDNA in rice supports the earlier observation of rDNA derived miRNAs. Sixteen small RNA libraries were used in this study for novel miRNAs identification which captured substantial proportion of expressed transcripts of rice. This study also indicates that due to enhanced transcription of rDNA during heat stress, rDNA derived miRNA discovery can be accelerated in samples collected from high temperature treatments. This is the first report pointing to the possible role of rDNA derived miRNAs in gene regulation during high temperature stress in plants. In plants, telomeric Nucleolar Organizing Regions are fragile sites and important for DNA repair and chromosomal stability. Our study shows a possible connection between temperature stress response and fragile sites, and indicates another layer of a regulatory player – rRNA derived miRNA in the complex activities carried out in this region. This could be a new area of research to further probe on rDNA derived miRNAs in plants and their role in regulating high temperature response.

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Author contributions

SKM, BS, NS, SRV, VRB, DS, PS designed research; SKM, BS, MP, BJ, SA, performed the research; SKM, BS, VVP analyzed the data; SKM, NS, BS, SRV wrote the paper.

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