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Prediction and Expression Analysis of miRNAs Associated with Heat Stress in *Oryza sativa*

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Abstract: Computational prediction of potential miRNAs and their target genes was performed to identify the miRNAs and genes associated with temperature response in rice. The data of temperature-responsive miRNAs of *Arabidopsis*, and miRNAs and whole genome data of rice were used to predict potential miRNAs in *O. sativa* involved in temperature response. A total of 55 miRNAs were common in both the species. A total of 27 miRNAs were predicted at the first time in rice. Target genes were searched for these 27 miRNAs in rice genome following stringent criteria. Real time PCR based on expression analysis of nine miRNAs showed that majority of the miRNAs were down regulated under heat stress for rice cultivar Nagina 22. Furthermore, miR169, miR1884 and miR160 showed differential expression in root and shoot tissues of rice. Identification and expression studies of miRNAs during heat stress will advance the understanding of gene regulation under stress in rice.
Key words: miRNA; *Oryza sativa*; *Arabidopsis*; target mRNAs; regulation

Temperature is one of the critical factors in rice yield that influence the grain filling by inhibiting the deposition of storage materials such as starch and proteins. Plants adopt diverse strategies to survive high or low temperature (Kondamudi et al, 2012). Temperature response in plants involves perception mechanisms, signal transduction networks, activation of stress-regulatory genes and - synthesis of diverse functional proteins (Mittal et al, 2009). Several genes and proteins that respond to temperature stress have been revealed. In addition to protein coding genes, recent studies showed that micro RNAs (miRNAs) also play a major role in stress response of plants by regulating number of transcription factors and metabolic genes (Sunkar et al, 2007). miRNAs are endogenous single-stranded small RNAs of 20-22 nt long regulating gene expression in plants and animals (Tang et al, 2003). These small RNAs get into the post-transcriptional gene silencing (PTGS) pathway leading either degradation of the target mRNA or translational repression. In plants, miRNAs were shown to be involved in plant development, biotic and abiotic stress responses (Zhao et al, 2007; Mishra et al, 2009; Jian et al, 2010; Li et al, 2010).

To date numbers of databases are available for computational prediction of miRNAs using cross species data. mirBase (http://microrna.sanger.ac.uk) is a comprehensive miRNA database which provides a set of precursor and mature miRNAs along with their sequence data. Currently, a total of 21264 mature miRNAs have been deposited in miRBase (as on August 2012). Similarly, the data pertinent to plant miRNA is available in the plant Micro RNA database (PMRD, http://bioinformatics.cau.edu.cn/PMRD/). Furthermore, the expression profile of miRNAs of various plant species during different stresses, such as rice oxidative stress microarray data and microarray data generated from *Populus*, *Arabidopsis*, tomato and maize, is also available. Twenty families of rice miRNAs were identified *in silico* based on conservation of sequences with *Arabidopsis* miRNAs (Bonnet et al, 2004). The computational identification of miRNAs and the prediction of target sites can be achieved using a Plant Small RNA Target (psRNA Target) Analysis Server (http://plantgrn.noble.org/psRNATarget). It reports all potential sequences complimentary to the given miRNA sequence with specified number of mismatches. Computational programs have been successfully applied to predict target genes in *Arabidopsis* (Adai et al, 2005), rice (Bonnet et al, 2004) and maize (Zhang et al, 2006) etc.

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In rice, computational analysis was carried out to generate a comprehensive list of putative miRNA targets which suggested half of the targets were conserved between *O. sativa indica* and *O.sativa japonica* (Archak and Nagaraju, 2007). The function of target genes can be predicted using different databases like UniProt (www.uniprot.org), a universal protein resource base which is catalogue of information on proteins.

For global climate change, heat stress is one of the major bottlenecks in rice production and productivity. Many efforts have been made to understand the regulation of genes under heat stress in rice (Mittal et al, 2009; Zou et al, 2009; Mittal et al, 2012). However, there are very limited researches on understanding the miRNAs expression profile during high temperature. The present study was aimed at prediction of temperature-responsive miRNAs in rice through computational approach using the expression data of temperature-responsive miRNAs of *A. thaliana*. The target genes of these predicted miRNAs were identified in the rice genome. Further, nine microRNAs were selected based on predicted data and available literatures for validation. Real time PCR expression analysis of miRNA was performed to understand the regulation of these miRNAs during heat stress in rice.

MATERIALS AND METHODS

Plant materials

Seeds of rice (*O. sativa*) cultivar Nagina 22 (N22) were surface sterilized in 0.1% $Hgcl_2$ soln for 3-4 mins followed by rinsing 3-4 times with distilled water. The seeds were then germinated in petri dishes at 28 °C for three days on moist blotting paper in dark. After germination, the sprouted seeds were transferred to Yoshida medium (Yoshida et al, 1976) under 13hrs of light and 11hrs dark by maintaining temperature of 30 °C day/24 °C night. Two sets were maintained in control conditions (30 °C/24 °C) with 8 plants in each set. One set of 12 days old seedlings was subjected to heat stress by exposing the seedlings to 42 °C/36 °C (day/night) for 24 hrs while another set was maintained in control conditions.

miRNAs and rice genome sources

There were 154 temperature-responsive mature miRNA sequences of *Arabidopsis* downloaded from PMRD database (bioinformatics.cau.edu.cn/PMRD/). The miRNA sequences of *O. sativa* were downloaded from miRBase. Rice genome sequence was retrieved from International Rice Genome Sequencing Project (IRGSP) website. In order to get rice sequences homologous with *Arabidopsis* miRNA sequences, BLAST search was performed using Rice Annotation Project Database (RAP-DB, http://rapdb.dna.affrc.go.jp/tools/blast). Also, small RNA and degradome data of rice miRNAs regulated by low and high temperature (http://mpss.udel.edu/rice_sRNA2/) were utilized to further refine the prediction of rice miRNAs regulated by temperature response (Jeong et al, 2011).

Prediction of miRNA secondary structures

Secondary structure prediction of putative rice miRNA backbone sequences and minimal free energy (MFE) of those secondary structures were performed by using RNA lifold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAalifold.cgi) and PMRD database. AMFE (adjusted MFE) was calculated by a formula (MFE/length of RNA sequence x 100). Based on AMFE value, minimal free energy index (MFEI) was calculated by the formula MFEI = AMFE/(G+C)%.

Prediction of miRNA targets

The target genes of predicted temperature-responsive miRNAs were searched in rice genome by using psRNATarget server. Here, miRNA sequences were aligned with rice genes available in TIGR (rice genome annotation project established by the institute for genome research). psRNATarget provides the options of minimum alignment score, maximum number of G–U wobble pairs, maximum number of mismatches and length of miRNA. All the parameters were maintained at the highest stringency levels to get minimum and best possible hits and later they were refined.

Refining of miRNA target genes

Using the psRNATarget database, the predicted miRNA targets were further refined by the criteria reported by Wang et al (2011).

Targets containing G: U base pairs were considered as mismatches. Each mismatch nucleotide in the miRNA: mRNA duplex was given the value 1.0. G: U base pairs and bulge nucleotides were given the value 0.5 and 2.0, respectively and with the maximum value of 3.5. The perfectly matched base pair in the position 8 to 12 of the duplex counting from the 5' end was considered. The hits possessing more than four

mismatches in the miRNA–mRNA pair were not considered.

Targets with two adjacent mismatches in positions 2-7 of the miRNA-mRNA target duplex were not taken into consideration.

Targets with mismatch score more than 2.5 within positions 1-7of the miRNA-mRNA duplex (5' of miRNA) were excluded. Furthermore, targets showing mismatches at the putative cleavage site (positions 10 and 11) in the seed region of the duplex target sequence were not taken into account.

Biological functions of target mRNAs

The biological functions of proteins encoded by predicted target mRNAs were analyzed using the UniProt database.

Quantitative Real Time PCR (qRT-PCR) validation of predicted miRNAs

Small RNAs were isolated from root and shoot tissues of 13 days N22 seedlings under control (30 °C/24 °C) and heat stress (42 °C/36 °C) by using mirVana miRNA isolation kit (Ambion, Austin, TX,USA). Each set (control and stress) of rice seedling was taken with three duplications for root and shoot samples. Mature miRNA-specific PCR forward primers were designed based on miRNA sequences downloaded from miRBase (Table 1). U6Sn RNA was chosen as an internal control (Ding et al, 2011). Addition of poly(A) tail to sRNAs by Poly(A) polymerase and cDNA synthesis were done by miscript reverse transcription kit (Qiagen). Quantitative PCR was performed using miscript SYBR green PCR kit (Qiagen). The 25 µl reaction volume consisting of 2.5 µl of normalized cDNA, 10X miscript universal primer, 10x miRNA specific primer and 12.5 microlitre 2X quantitative SYBR green PCR master mix was prepared. Reactions were performed in 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) with three duplications. Conditions for qRT-PCR were: 95 °C for 15 min, 40 cycles of denaturing at 94 °C for 15 s, annealing at 55 °C for 30 s, and extension at 70 °C for 30 s, followed by a disassociation stage (melting curve analysis). The comparative threshold cycle (Ct) method was used to quantify the relative expression levels. ΔCt was calculated by the difference between mean Ct target and mean Ct reference. Further, ΔΔCt values were calculated by the formula ΔΔCt =ΔCt of stressed sample-ΔCt of control sample and then fold difference was calculated by $2^{-\Delta\Delta Ct}$. Similarly, ΔCt standard deviation was calculated based on the information given at www3.appliedbiosystems.com/.../general documents/cms_042380.pdf.

Table 1. Primer sequences of miRNAs used for real time expression analysis.

miRNA	Primer sequennce
miR 156	TGACAGAAGAGAGTGAGC
miR160	TGCCTGGCTCCCTGTATG
miR 162	TCGATAAACCTCTGCATC
miR167	TGAAGCTGCCAGCATGAT
miR168	TCGCTTGGTGCAGATCGG
miR169	CAGCCAAGGATGACTTGC
miR397	TCATTGAGTGCAGCGTTG
miR398	TGTGTTCTCAGGTCACCC
miR1884	AATGTATGACGCTGTTGA
U6 snRNA	CGATAAAATTGGAACGATACAGA

RESULTS

In the present study, a total of 154 temperature-responsive miRNA sequences of *Arabidopsis* were retrieved from PMRD database. These miRNAs of *Arabidopsis* were searched against *O. sativa* miRNAs available in miRbase. Fifty five miRNAs were common in both *O. sativa* and *Arabidopsis* (Supplementary Table 1), however, roles of these miRNAs have not been predicted or established for temperature response in rice. Though, the target genes of these 55 common miRNAs are known in rice but their role has not been discussed in relation with temperature response. The remaining 99 miRNAs were unique to *Arabidopsis* and not found in *O. sativa* (Supplementary Table 2). However, when they were analyzed for homologous sequences in rice genome and fold back structures, 27 potential miRNAs in rice could be predicted. Further, these sequences were analyzed using rice small RNA and degradome data available at http://mpss.udel.edu/rice_sRNA2. The target gene sequences of these miRNAs were predicted using psRNATarget server and validated by the degradome data.

Prediction of fifty five conserved (common) miRNAs and biological significance

The conserved nature of miRNA sequences in both *O. sativa* and *Arabidopsis* species can be useful for prediction of the temperature-responsive miRNAs in rice. The target mRNAs were predicted in rice genome for the 55 miRNAs conserved in both the plant species. Among the 55 miRNAs, few of them showed the targets functionally related to temperature stress in rice genome. Targets of miR167c and 167d

are genes for heat repeat family proteins and targets of miR396a and 414 are coding for heat shock 70 kDa protein. In addition, miR414 targets heat shock protein 81-1 and calmodulin binding protein. Further, miR399b and 399e target for heat shock protein binding proteins. miRNA 413 targets like WRKY proteins that belonged to a large family of transcription factors. The other conserved miRNAs in rice, such as miR156, miR157, miR159 and miR160a, regulate the targets, which mainly include transcription factors like Squamosa promoter-binding protein-like (SPL-10 11), GAMYB transcription factors (GA-inducible MYB-transcription factor), MYB (myeloblastosis family) transcription factors and auxin response factors (ARFs), respectively. miR164a/c targets as NAC (Nascent polypeptide-associated complex)-domain containing gene. Further, these 55 conserved miRNAs were searched for sequencing signatures using rice degradome data available for rice miRNAs regulated by low and high temperature (Jeong et al, 2011). Interestingly, there were 43 miRNAs shown matched signatures in MPSS library and 25 of them showed signatures in the heat library (SHtD1I seedling, heat #1) (Table 2).

Table 2. The conserved 55 miRNAs showing signatures in MPSS library of small RNA and degradome data available at http://mpss.udel.edu/rice_sRNA2/.

Signatures found in MPSS library	Signatures found in heat library (SHtD1I seedling, heat #1)
miR156a, miR156g, miR156h	miR156a, miR156g, miR156h
miR159a, miR159b	miR159a, miR159b
miR160a	miR160a
miR162a	miR162a
miR164a, miR164c	miR164a, miR164c
miR166a	miR166a
miR167a, miR167c, miR167d	miR167a, miR167c, miR167d
miR168a	miR168a
miR169a, miR169b, miR169d, miR169g, miR169h	miR169a, miR169b, miR169g, miR169h
miR170a	miR170a
miR171a, miR171b	miR171b
miR172a, miR172b, miR172c	miR172a, miR172b
miR319a	
miR390	miR390
miR393	miR393
miR394	miR394
miR395b	
miR396a, miR396b	
miR397a, miR397b	
miR398a, miR398b	
miR399a, miR399b, miR399d, miR399e, miR399f	
miR408	

Prediction of new miRNAs and biological significance

Out of 154 temperature-responsive miRNAs of *Arabidopsis*, 99 miRNAs (supplementary Table 2) have not been reported in rice yet. Interestingly, when these miRNAs were searched in rice genome sequence for the existence of homologous miRNA sequences, 27 of them could be identified (supplementary Table 3). Further, these homologous sequences of rice were used for prediction of fold back structures (Fig. 1). MFE values of the predicted secondary structures ranged from -27.2 to -56.0 Kcal/mole and the MFEI values in range of 0.40 to 0.76 (Table 3). These putative miRNAs of rice were used to predict their targets in rice genome by following the stringent criteria as the G–U score at 0.5 and the total number of mismatches not more than three. The target sequences include many important stress-associated protein genes such as retrotransposon proteins, RNA-binding proteins, ATP binding proteins, mitochondrial 60S ribosomal protein L6, serine/threonine-protein kinase receptor precursor, zinc finger C3HC4 type family protein, ubiquitin-protein ligase 1, chloride channel protein, translation initiation factor IF-2, CDPK-related protein kinase, electron transporter, phosphatidylinositol 3- and 4-kinase family protein, leucine-rich repeat receptor protein kinase and several other transcription factors and proteins involved in signaling pathways, abiotic stress response and plant metabolism (supplementary Table 4). Further analysis was conducted by searching for signatures in MPSS library of rice small RNA and degradome data (Jeong et al, 2011) for these 99 miRNAs. The results showed that 10 miRNAs were signature mateched in MPSS libraries and two of them (miR165a and miR172e) showed signatures in heat library (Table 4). Interestingly, six miRNAs (miR172e, miR165a, miR400, miR829.1, miR857 and miR157a) showing signatures in MPSS library were also detected as novel rice miRNAs based on homology search and secondary structure prediction.

miRNAs target frequency and distribution

Most of the predicted target genes in rice are retrotransposons and transposons. In addition to this, few other targets of predicted miRNAs such as kinases, transcription factors, transporter proteins, nucleic acid binding proteins and ATP binding proteins were found in this study (Fig. 2a). The distribution and analysis of the targets of miRNAs among various chromosomes in rice were also done. The result indicated that the predicted targets of

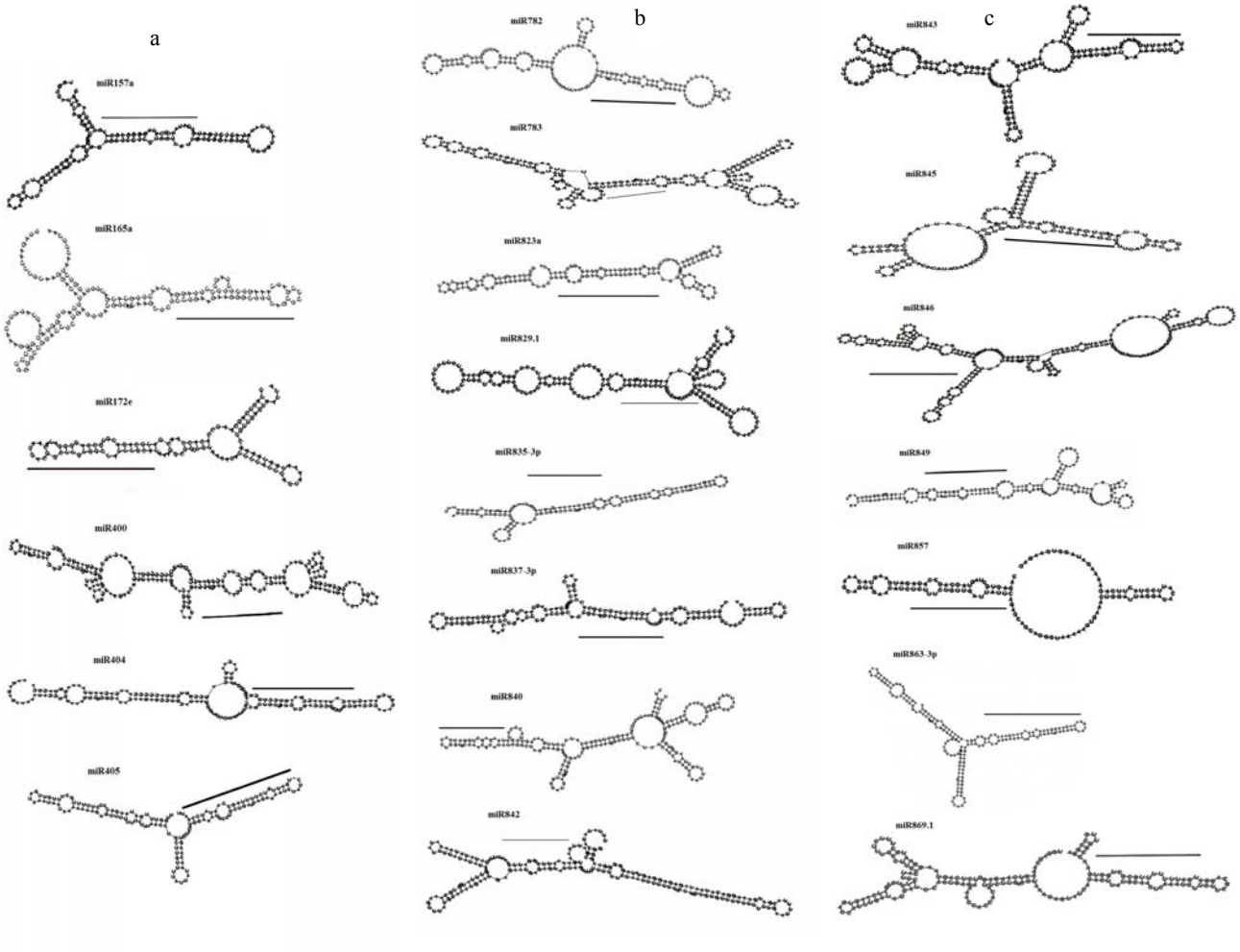

Fig. 1. Secondary structures of prediction of novel 21 rice miRNAs (a) miRNAs157a, 165a, 172e, 400, 404 and 405 (b) miRNAs 782, 783, 823, 829.1, 835-3p, 837-3p, 840, and 842(c) miRNAs 843, 845, 846, 849, 857, 863-3p and 869.1.
Secondary structure of other 6 novel miRNAs is not provided as they belong to same family and showed similar structure.

miRNAs were distributed among 12 chromosomes of rice. However, the chr 2, chr7, chr 3 and chr 8 of rice had the higher frequencies of predicted targets (Fig. 2b).

Expression analysis of microRNAs during heat stress

In order to understand the expression pattern of miRNAs, nice miRNAs were selected for real time expression analysis. These miRNAs include miR156, miR160, miR162, miR167, miR168, miR169, miR397, miR398 and miR1884. The selection of these miRNAs is based on present computational prediction and previous reports in different plant species. Real Time PCR analysis showed the differential expression pattern of these miRNAs in both root and shoot of rice seedlings under heat stress (Fig. 3). Among the 9 microRNAs, the majority of them were down regulated in both root and shoot during heat stress. In roots of rice seedlings, 7 miRNAs were down regulated while 2 miRNAs (miR168 and miR397) did not show expression during heat stress. The mean Ct value of these two miRNAs was reported with the value over 35.0 under heat stress. Out of 9 miRNAs, 3 miRNAs were up regulated and six were down regulated in shootunder heat stress. The up-regulated miRNAs in shoot included miR160, miR169 and miR1884. Among these 3 up-regulated miRNAs, miR 169 was up-regulated with the highest expression change of 2.3 fold. For miR160 and miR1884, the increased fold change was 1.8 and 1.5, respectively. Down-regulated miRNAs in shoot included miR156, miR162, miR168, miR167, miR397 and miR398. Notably, four miRNAs including miR156, miR162, miR167 and miR398 were down regulated in both root and shoot, while 3 miRNAs showed contrasting

Table 3. MFE and MFEI values of novel predicted miRNAs.

miRNA	Length of Precursor miRNA(bp)	Mature sequence length	Mismatches	A+U%	G+C%	MFE (-Kcal/mole)	AMFE	MFEI
miR157a	144	24	4	45.83	54.16	48.6	33.75	0.62
miR172e	125	21	1	59.2	40	30.0	24.0	0.60
miR165a	166	21	2	54	46	31.7	19.09	0.41
miR400	233	24	3	66.6	33.4	31.6	13.56	0.40
miR404	181	24	1	65.8	34.2	33.9	18.72	0.54
miR405a	151	24	2	70.9	29.1	31.5	20.86	0.71
miR782	177	17	1	59	41	31.1	17.51	0.42
miR783	201	21	4	54.73	45.27	52.6	26.16	0.58
miR823	154	24	0	55.9	44.1	30.1	19.54	0.44
miR829.1	201	17	1	50.8	49.2	40.1	19.95	0.40
miR835-3p	151	24	1	66.89	33.11	27.2	18.01	0.54
miR837-3p	176	21	5	65.4	34.6	33.0	18.75	0.55
miR840	219	17	1	50.7	49.3	56.0	25.57	0.52
miR842	208	17	1	75	25	38.40	18.46	0.74
miR843	204	22	3	65.7	34.3	33.4	16.37	0.48
miR845a	140	17	2	54.3	45.7	29.50	21.07	0.46
miR846	291	17	1	72.6	27.4	36.3	12.47	0.46
miR849	149	22	2	69.3	30.7	34.92	23.43	0.76
miR857	134	22	2	59	41	30.7	22.91	0.56
miR863-3p	181	24	1	68	32	33.9	18.72	0.58
miR869.1	191	24	1	57.6	42.4	40.9	21.41	0.50

expression for root and shoot tissue of rice seedlings during heat stress.

DISCUSSION

MicroRNAs play an important role in post-transcriptional gene regulation by targeting mRNAs for cleavage or repressing translation. These small RNAs are involved in modulating a variety of abiotic stress responses (Sunkar and Zhu, 2004). The information on miRNAs associated with temperature response in rice is very limited. Here, prediction of temperature associated miRNAs in *O. sativa* was done using the temperature regulated miRNAs expression data of *Arabidopsis* (PMRD, Zhang et al, 2009). Further study on expression of some of the selected miRNAs was analyzed using real time PCR analysis.

Out of 154 known temperature regulated miRNAs of *A. thaliana* (miRNAs retrieved from PMRD), fifty-five of them were common for both the species, *O.sativa* (miRNAs retrieved from miRbase) and *Arabidopsis*. A substantial number of miRNAs were found to be conserved among both species. The strict conservation of miRNAs suggests that the interactions between these miRNAs and their targets constitute essential processes and play evolutionary conserved roles. Based on the distinctiveness of the predicted targets and their functions, the temperature-responsive miRNAs had a strong propensity towards transcription factors and stress related proteins. The predicted targets of the most miRNAs included transcription factors like squamosa promoter binding protein (SPL) family, auxin-response factor, MYB family TF, homeobox-leucine zipper TF HB-14, homeodomain-leucine zipper protein, CCAAT-binding TF, scarecrow-like TF, floral homeotic protein

Table 4. Novel miRNAs showing signatures in rice small RNA and degradome data available at MPSS library (http://mpss.udel.edu/rice_sRNA2/).

miRNA	*Arabidopsis* sequence	Signature sequences in MPSS library
miR161	UGAAAGUGACUACAUCGGGGU	TGAAAGTGACTACATCGGGGT
miR172e*	GGAAUCUUGAUGAUGCUGCAU	GGAATCTTGATGATGCTGCAT
miR165a*	UCGGACCAGGCUUCAUCCCCC	TCGGACCAGGCTTCATCCCCC
miR173	UUCGCUUGCAGAGAGAAAUCAC	TTCGCTTGCAGAGAGAAATCAC
miR400	UAUGAGAGUAUUAUAAGUCAC	TATGAGAGTATTATAAGTCAC
miR829.1	AGCUCUGAUACCAAAUGAUGGAAU	AGCTCTGATACCAAATGATGGAAT
miR857	UUUCGUUGUCUGUUCGACCUU	TTTTGTATGTTGAAGGTGTAT
miR157a	UUGACAGAAGAUAGAGAGCAC	TTGACAGAAGATAGAGAGCAC
miR157d	UGACAGAAGAUAGAGAGCAC	TGACAGAAGATAGAGAGCAC
miR158a	UCCCAAAUGUAGACAAAGCA	TCCCAAATGTAGACAAAGCA

*, miRNAs showing signatures in the heat library

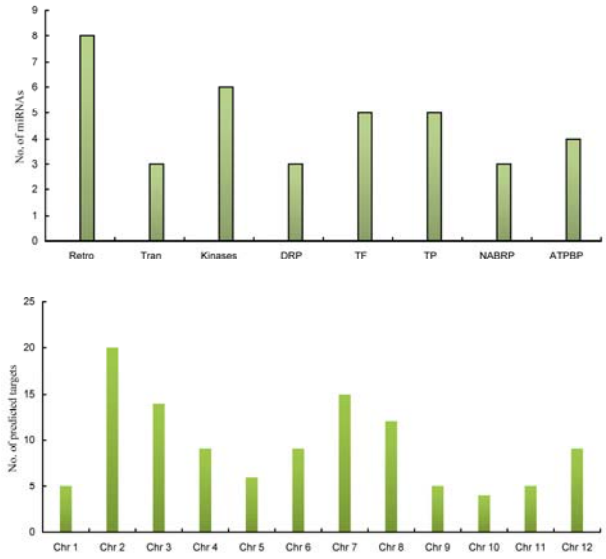

Fig. 2. Frequency of miRNAs targeting a specific class of protein in *O. sativa* (a) and distribution of new miRNAs targets on different chromosomes of rice (b).

Retro, Retrotransposons; Tran, Transposons; DRP, Disease resistance protein; TF, Transcription factors; TP, Transporter protein; NABRP, Nuleic acid binding related proteins; ATPBP, ATP binding proteins; Chr, Chromosome.

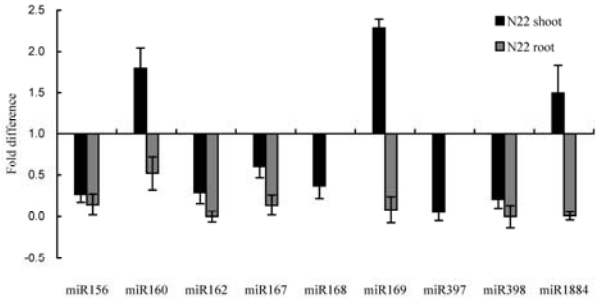

Fig. 3. Expression analysis of miRNAs during heat stress in rice using qRT-PCR.

APETALA2, AUX/IAA family protein (auxin response/Indole-3-acetic acid induced proteins), C3HC4-type zinc finger family proteins etc. These proteins are related to various abiotic stresses in different plant species (Liu et al, 2008).

In order to confirm the prediction data, those 55 common miRNAs were also tested for the presence of signatures in MPSS library and degradome data available at http://mpss.udel.edu/rice_sRNA2/. (Jeong et al, 2011) containing 62 small RNA libraries constructed from rice plants for deep sequencing with Illumina technology. These libraries represent several tissues from control plants and plants subjected to different environmental stress. All those information could be utilized in prediction analysis for further validation of our results. There were 43 miRNAs shown signatures in MPSS library. Notably, the presence of signatures of 25 miRNAs in heat library of rice small RNAs indicated their role in heat stress regulation in rice and *Arabidopsis*. Recent study in wheat showed that miR156, miR159, miR160, miR166, miR168, miR169 and miR827 were up-regulated under heat stress (Xin et al, 2010). These miRNAs were all covered in our study (Table 2). Moreover, a recent study in *Brassica rapa* also has shown that members of miR156 family were induced by heat stress (Yu et al, 2012). Among the conserved miRNAs, a few of their target genes were associated with heat stress directly. For example, miR414 targets two heat shock proteins, viz. HSP70 kDa protein, HSP 81-1 and a calmodulin binding protein. And, miR399b and 399e target heat shock protein binding proteins. Recent study also reported that HSPs played an important role in plant heat tolerance (Huang and Xu, 2008). Study in *B. rapa* showed that miR398-regulated genes BracCSD1 and BracCSD2 played an important role in thermotolerance through protection from oxidative damage (Yu et al, 2012). In this study, miR398 was predicted as temperature-responsive miRNA in *O.sativa.* miR398 targets Cu/Zn-SODs in rice, which are critical proteins functioning in mitigating oxidative stress (Jagadeeswaran et al, 2009).

In the present study, a total of 27 predicted novel miRNAs were obtained in rice. All these 27 miRNAs of *Arabidopsis* showed homologous sequences in rice genome. To validate the novel miRNAs, sequences of homologous rice miRNAs were used for secondary structure prediction and target genes prediction analysis (Fig. 1, Table 4). The MFEI gives best prediction of miRNAs. The average MFEI of miRNA precursors was higher significantly than those of tRNAs (0.64), rRNAs (0.59), and mRNAs (0.62–0.66) (Zhang et al, 2006). Among the predicted novel mRNAs, miR405a, 842 and 849 had higher MFEI than these values (Table 3). However, it would be difficult to calculate the real values of MFEI in this study since it relied mainly on precursor miRNA length and G+C content. We have selected precursor rice miRNA sequences based on homology with *Arabidopsis*, but both the plant species were significantly different in genome sequence and structure. To further confirm the role of these

predicted novel miRNAs in temperature response, expression analysis was performed. In addition, Jeong et al (2011) reported that 10 out of 99 miRNAs which were unique to *Arabidopsis* showed signatures in MPSS library of rice small RNA and degradome data (Jeong et al, 2011). Interestingly, six of them (miR172e, miR165a, miR400, miR829.1, miR857 and miR157a) showing signatures in MPSS library were detected in our study as novel predicted miRNAs in rice based on homology search and secondary structure prediction. This suggested that prediction of miRNA based on homology search was reliable and could be used to predict temperature-responsive miRNAs of rice based on the sequence data of *Arabidopsis*. Majority of miRNAs targets were retrotransposons and transposons, and kinases, transcription factors and transporter proteins as well (Fig. 2). Based on the highest frequency, it suggested that retroposons played an important role in temperature response. The retroelements would be activated particularly during certain stresses (Grandbastien, 1998). Higher frequency of miRNA targets to retrotransposon might be due to their higher abundancy in the rice genome or involvement in stress response. Roles of transporter proteins, transcription factors and kinases in abiotic stress response were well characterized (Nakashima et al, 2009; Yamada et al, 2010; Qin et al, 2011). The other interesting target proteins included RNA binding proteins (RBPs), ATP binding proteins, zinc finger C3HC4 type family protein, ubiquitin-protein ligase 1, DPK-related protein kinase, electron transporter, phosphatidylinositol 3- and 4-kinase family protein, leucine-rich repeat receptor protein kinase. Increasingly, diverse RBPs have been determined to perform crucial roles in post-transcriptional regulation of RNA metabolism during response to abiotic stresses (Jung et al, 2013). ATP binding proteins also play a crucial role in abiotic stress response of plants (Macovei et al, 2012).

Extreme heat can damage rice yield, grain quality, and plant growth irreversibly (http://www.irri.org). The high temperature (42 °C) is considered a maximum temperature, to exceeding 42 °C, rice yield can be reduced dramatically. In order to validate the expression of predicted miRNAs in heat stress response, rice tissues (root and shoot) were harvested from seedling with control (30 °C/24 °C) and heat stress (42 °C/36 °C) treatments. The qRT-PCR based expression study of miRNAs involved heat stress in rice demonstrated that the majority of the miRNAs were down regulated in response to heat stress. Three microRNAs miR169, miR1884 and miR160 showed differential expression in root and shoot of rice under heat stress. These miRNAs were up-regulated in shoot and down regulated in root. It was revealed that the miR169 family members were associated with drought response and high salt stress (Li et al, 2008; Zhao et al, 2009). Up-regulation of miR169 was observed during the drought stress induced by PEG (Zhao et al, 2007) as well as water-withholding conditions in rice leaves (Zhou et al, 2010). The present study implicates that miR169 might play an important role during heat stress since it showed 2.3 fold increased expression in shoot tissue of stressed seedlings as compare to control. Promoter sequence of miR169g contained two putative DRE (dehydration-responsive) cis-elements, causing the upregulation in response to drought and cold (Zhao et al, 2007). The down-regulation of miR169 in root tissue under heat stress condition had high concordance with the results reported, where miR169 showed reduced expression in root tissue under drought stress (Eldem et al, 2012). Down-regulation of miR1884 in rice shoots under cold stress was reported (Lv et al, 2010). Interestingly, it was upregulated in shoots of rice seedlings, while it was down regulated in roots when exposed to heat stress. Previous studies revealed that miR160 was involved in auxin signaling pathway through transcription factors (Rhoades et al, 2002). Studies on heat stress in wheat showed that miR160 was upregulated in shoot (Xin et al, 2010), similar results were observed in this study also. Three miRNAs with contrasting pattern of expression in root and shoot under heat stress suggested that they were playing key role in co-ordination of up and down regulation of set of target genes in two different tissues of rice.

Down-regulations of miR398, miR156, miR162, and miR167 were observed in both root and shoot, suggesting that the down regulated miRNAs might play an important role in rice seedling responses to heat stress. Especially, miR398 down-regulated in both tissues under heat stress implicated that the expression level of its target Cu/Zn superoxide dismutase would be increased. In *Brassica* and *Populous*, similar expression of miR398 was observed during heat stress. Similarly, down-regulation of miR156 was reported in rice during drought stress (Zhou et al, 2010). miR162 is known to target the gene encoding the RNA slicer enzyme DCL1 (dicer

like proteins) that plays a key role in the miRNA processing (Mallory and Vaucheret, 2006). And miR162 was down regulated under cadmium stress conditions in rice (Ding et al, 2011). In this study, the down-regulated miR168 played an important role in the production of the miRNAs through the target protein AGO I (Mallory and vaucheret, 2006). In addition, recent study in *Populous* has showed that miR168 was down regulated under heat stress (Chen et al, 2012). Therefore, down-regulation of miR167 both in rice root and shoot tissues suggests the important role of this miRNA in regulating the gene expression under heat stress.

CONCLUSION

In conclusion, the predicted miRNAs from our study were involved in temperature response in *O. sativa*. Based on miRNA expression data of *Arabidopsis* available at PMRD database, there were 55 miRNAs of rice (available at miRbase) which were related to temperature response. Furthermore, a total of 27 miRNAs were identified in *O.sativa* for the first time, and they might have a putative role in temperature response in rice. Target prediction using a very stringent criterion was conducted by searching for these miRNA sequences in *O. sativa* genome database, and many abiotic stresses related target genes were identified. The analysis suggests that the conserved nature of miRNAs across the species associates with a specific pathway regulation or trait. The expression analysis of selected miRNAs under heat stress could advance our understanding of the gene regulation and molecular pathways involved in heat stress response in rice.

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SUPPLEMENTAL DATA

The following materials are available in the online version of this article at http://www.sciencedirect.com/science/journal/16726308; http://www.ricescience.org.

Supplemental Table 1. Temperature responsive common miRNAs in *Arabidopsis thaliana* and *Oryza sativa*.

Supplemental Table 2. microRNA sequences unique to *Arabidopsis*.

Supplemental Table 3. List of temperature responsive miRNAs of Arabidopsis thaliana showing homology with the genome sequence of *Oryza sativa*.

Supplemental Table 4. Predicted target genes of 27 novel miRNAs in *Oryza sativa* genome.

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