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Research article

Identification and characterization of drought responsive miRNAs in a drought tolerant upland rice cultivar KMJ 1-12-3

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ABSTRACT

Shortfall of rain that creates drought like situation in non-irrigated agriculture system often limits rice production, necessitating introduction of drought tolerance trait into the cultivar of interest. The mechanism governing drought tolerance is, however, largely unknown, particularly the involvement of miRNAs, the master regulators of biochemical events. In this regard, response study on a drought tolerant rice variety KMJ 1-12-3 to 20% PEG (osmolality- 315 mOsm/kg) as drought stress revealed significant changes in abundance of several conserved miRNAs targeting transcription factors like homeodomain-leucine zipper, *MADS* box family protein, C2H2 zinc finger protein and *Myb*, well known for their importance in drought tolerance in plants. The response study also revealed significant PEG-induced decrease in abundance of the miRNAs targeting cyclin A, cyclin-dependent kinase, guanine nucleotide exchange factor, GTPase-activating protein, 1-aminocyclopropane-1-carboxylic acid oxidase and indole-3-acetic beta-glucosyl transferase indicating miRNA-regulated role of the cell cycle regulators, G-protein signalling and the plant hormones ethylene and IAA in drought tolerance in plants. The study confirmed the existence of four novel miRNAs, including osa-miR12470, osa-miR12471, osa-miR12472 and osa-miR12473, and the targets of three of them could be successfully validated. The PEG-induced decrease in abundance of the novel miRNAs osa-miR12470 and osa-miR12473 targeting RNA dependent RNA polymerase and equilibrative nucleoside transporter, respectively suggested an overall increase in both degradation and synthesis of nucleic acids in plants challenged with drought stress. The drought-responsive miRNAs identified in the study may be proved useful in introducing the trait in the rice cultivars of choice by manipulation of their cellular abundance.

1. Introduction

Plants are exposed to diverse environmental conditions, which induce multigenic response to restore the cellular homeostasis. Drought is one among the environmental factors or stresses that is highly prevalent affecting the agriculture world-wide, particularly the crop cultivation in the non-irrigated agriculture system because of reduction in precipitation and unpredictable rainfall (Pandey and Shukla, 2015; Dixit et al., 2014), which could be a result of changes in the global climate. A recent analysis of published data of yield of wheat and maize from 1980 to 2015 the world over has shown 21% and 40% reduction in their yield, respectively due to drought (Daryanto et al., 2016). A study also reported that even the advanced country like USA suffered 12% decrease in corn production in 2012 due to prolonged drought, indicating seriousness of challenge of the environmental factor to agriculture, and the food security as such (Fahad et al., 2017). The challenge of drought to rice is still greater because of being a paddy field crop, particularly susceptible to water shortage (Dixit et al., 2014). Any improvement in rice yield under drought stress shall go a long way in securing food for the ever-increasing world population. In this regard screening for

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drought resistant varieties of rice, irrespective of yield, is becoming increasingly important so as to understand the biochemical and molecular phenomena making the variety resistant to the stress.

Plants respond to dehydration stress through a myriad of developmental and physiological changes (Mishra et al., 2016). Pandey and Shukla (2015) have described in detail the morphological, physiological and biochemical changes induced by drought, especially in rice, including reduction in germination, inhibition in elongation and expansion, decrease in number of tillers and leaves, reduction in chlorophyll content, decrease in photosystem II activity and photosynthesis, reduction in water use efficiency and relative water content, accumulation of osmolytes, alteration in transcription of genes encoding defence related proteins and transcription factors, impairment of assimilate translocation, and increased spikelet sterility and reduced yield. The alteration in transcription of the genes is, however, the most important among the above drought induced changes. It has been reported that in rice transcription of more than 5000 genes are upregulated and more than 6000 genes are downregulated in response to drought stress (Maruyama et al., 2014). Several other researchers have also conducted similar studies, which led to the identification of a plethora of genes responsive to drought stress in rice, including those related to modulation of osmolytes, antioxidants, abscisic acid (ABA) and auxin signalling (Todaka et al., 2017; Wang et al., 2011). The genes of known relevance to drought stress can be broadly put into two categories (Pandey and Shukla, 2015). One category constitutes the genes that encode effector proteins, i.e. the proteins that are directly involved in biochemical and physiological processes, including the proteins involved in synthesis and accumulation of osmolytes, like proline (Verbruggen and Hermans, 2008), polyamines (Yang et al., 2007) and soluble sugars (Todaka et al., 2017), the enzymes that serve as antioxidants, like superoxide dismutase, catalase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase (Mishra et al., 2016), and the enzymes that facilitate synthesis of the plant hormones, like indole-3-acetic acid (IAA), cytokinin (CK) and abscisic acid (ABA), having crucial role in drought stress response and tolerance (Wang et al., 2007; Todaka et al., 2017). The second category of the genes encode regulatory proteins, mainly transcription factors that regulate the transcription of the effector genes. The drought-induced transcription factors belong to the families like AP2/ERF, NAC, MYB, bZIP, MYC, and ZnF (Mishra et al., 2016), and overexpression of the genes encoding several of them, including homoeodomain-leucine zipper (Zhao et al., 2014), C2H2-type zinc finger protein (Zhang et al., 2016), DREB (Sakuma et al., 2006) and ERF (Quan et al., 2010) have been shown to improve drought tolerance in plants. In addition to the transcription factors, several other proteins that form important components of environmental signal response and transduction, including the EF-hand family protein MtCaMP1, a calcium binding protein (Wang et al., 2013), the small GTP-binding protein Rab7 involved in intracellular vesicular trafficking (Agarwal et al., 2008), SIMAPK1, the mitogen-activated protein kinase involved in MAPK signalling (Wang et al., 2018), the DEAD-Box RNA helicase SIDEAD31 involved in several aspects of RNA metabolism (Zhu et al., 2015) and leucine-rich receptor-like kinase LRK2, a plasma membrane bound protein involved in a number of developmental processes and defence responses (Kang et al., 2017) have been demonstrated to increase drought tolerance in plants overexpressing them.

Apart from transcription factors, a robust class of ~21–24 nt (nucleotide) long small RNAs (sRNAs), called microRNAs (miRNAs), have emerged as the master regulators of gene expressions in past two decades, which in contrast to the transcription factors operate at the post transcriptional level. Recent research indicates that plants assign miRNAs as critical post-transcriptional gene-expression regulators to attenuate plant growth and development under stress conditions (Ding et al., 2013). MicroRNAs are derived from the secondary stem-loop structures formed by introns or the non-coding mRNAs, and ubiquitously serve as repressor of gene-expression by degradation of the target mRNAs or inhibition of their translation in higher eukaryotes (Akdogan et al., 2016). MicroRNAs in plants were first identified and functionally characterized in *Arabidopsis* by Reinhart et al. (2002), although sRNAs resembling miRNAs from animals were also reported in plant in the same year by Llave et al. (2002). Subsequently post-transcriptional regulation of gene expression by miRNAs has been well established influencing plant life process, including abiotic stress response and acclimation (Jones-Rhodes et al., 2006; Jones-Rhoades and Bartel, 2004; Chen and Rajewsky, 2007; Bartel, 2004; Lu and Huang, 2008). The cellular abundance of many miRNAs have been reported to be altered in response to drought stress, including that of miR398a/b in *Medicago truncatula* (Wang et al., 2011), miR169a/c in *Arabidopsis* (Li et al., 2008), miR166 and miR171 in barley and wheat (Kantar et al., 2010), and miRNAs of the family miR319 in rice (Zhou et al., 2010). Several miRNAs have also been identified in rice under drought stress at different developmental stages through genome wide profiling, *viz.* miR169g, miR393, miR397, miR408, miR167, miR395 and miR474, and these are shown to have huge impact on rice drought stress physiology (Zhao et al., 2007; Sunkar et al., 2008). Drought responsive miRNAs are reported to be involved in modulating ABA response, antioxidant defence, osmotic adjustment, photosynthesis and respiration, cell growth, and auxin signalling in plants (Ding et al., 2013). Despite the progress made so far in understanding the role of miRNAs in drought stress response/tolerance in plants, miRNA profiling and characterization have not been done so far in any drought tolerant rice variety. The present paper, therefore, reports identification and characterization of miRNAs in a rice cultivar KMJ 1-12-3 that shows high tolerance to drought stress.

2. Methods

2.1. Plant growth conditions and treatment

Seeds of a cultivated rice variety, *Oryza sativa* cv. KMJ 1-12-3, tolerant to drought were collected from the Regional Agricultural Research Station, Assam, India. The seeds were surface sterilized and kept in a beaker filled with water overnight. Next day the seeds were spread onto a large petri-dish containing moist tissue paper to aid germination. The germinated seeds containing visible outgrowths were then transferred on plastic net fastened around ~7 cm diameter glass bangle and placed at the top of a ~200 mL capacity tapering plastic containers filled with a known volume of 1/10th Hoagland's solution. The Hoagland's solution just touched the net. The seedlings were grown for 6 days with continuous care in a plant growth chamber maintained at 25 ± 2 °C and ~70% relative humidity. Light was provided through fluorescent bulbs maintaining an intensity of ~200 μmol m^{-2} s^{-1} with diurnal cycle of 14/10 h light/dark. Hoagland's solution (1/10th) was added in sufficient amount to just touch the net every day in the morning to make-up the loss due to evaporation. Drought treatment was given on the 7th day in the morning as 20% PEG-6000 (PEG). PEG-6000 was chosen for the treatment based on its use for application of drought stress in earlier studies (Kaufmann and Eckard, 1971; Yang et al., 2017). The treatment concentration was determined beforehand through pilot experiments of shoot growth inhibition where it was observed that 20% PEG (osmolality- 315 mOsm kg^{-1}) inhibited the growth of shoot by approximately 50% in 72 h (Supplementary file 1). IC_{50} of PEG for shoot growth inhibition in 72 h was 23.81% (Supplementary file 1). The treatment duration for the response study was restricted to 9 h so as to get information on initial response on miRNAs biosynthesis or formation because it is the initial response that determines survival of the organism subjected to an abiotic stress. The treatment for the study of the miRNA abundance level in the test plant began with an initial addition of a known volume of PEG stock solution (prepared in 1/10th Hoagland's solution) to the individual beakers (3 numbers) to raise the PEG concentration to approximately 1%. The seedlings were kept in dark for 30 min. After the initial treatment more of PEG stock solution (volume calculated beforehand considering the volume of the space in the plastic container up to the net) to raise the final PEG concentration to 20%. The remaining space up to the net was filled with 1/10th Hoagland's solution. Light was switched on after the application of PEG. After exposure of the seedlings to 20% PEG for 9 h under illumination the shoot and root tissues of both control and PEG-treated seedlings were harvested, made packets of ∼1 g in aluminium foil and immediately frozen in liquid nitrogen and stored at −80 °C for future use.

2.2. Small RNA library construction and sequencing

Quality of the RNA was confirmed by bioanalyzer, and only those samples showing RNA integrity number (RIN) greater than 7 were processed. The RNA concentration was estimated with Qubit 2.0 instrument applying the Qubit RNA HS Assay kit (Thermo Fisher Scientific). Small RNA library was prepared according to the manufacture's instruction using Ion Total RNA-Seq Kit v2.0 (Thermo Fisher Scientific). The Magnetic Bead Cleanup Module included in the kit was used to enrich the small RNAs (sRNA). The quality tested RNA was incubated with the magnetic beads and the binding solution supplied along with the kit maintaining a low ethanol concentration, which facilitated capture of the larger RNA species, such as mRNAs and rRNAs, on the magnetic beads leaving the sRNAs in solution. Incubation of the aspirated solution containing sRNAs further with the magnetic beads maintaining a high ethanol concentration facilitated the magnetic beads to bind the sRNAs, which were eluted with pre-heated (80 °C) nuclease-free water. The sRNA preparations were converted into representative cDNA libraries. Barcode and adapters were ligated to all the libraries. Barcoded library preparation enabled sequencing of multiple samples in a single, multiplexed sequencing run. The respective size distribution of the library was verified with Agilent 2100 Bioanalyzer using high-sensitivity DNA kit (Agilent, Santa Clara, CA). Libraries were diluted to 100 pM and an equimolar pool was prepared for clonal amplification. The template for sequencing was prepared from the pool of libraries on Ion Spheres using OneTouch 2 protocols and reagents that utilized Ion PI Hi-Q OT2 200 kit (Thermo Fisher Scientific). Briefly, the library was clonally amplified onto ion sphere particles (ISPs) through emulsion PCR and then enriched for the template-positive ISPs. The clonally amplified products were recovered from the system and the ISPs positive templates were enriched by binding to the streptavidin coated magnetic beads using Thermo Fisher Scientific's ES module of the Ion OneTouch 2, removing empty ISPs through washing steps. The positive templates were denatured and loaded on Ion PI chip kit using the manufacturer's protocol (Thermo Fisher Scientific). Sequencing was performed with the Ion PI Hi-Q sequencing 200 kit (Thermo Fisher Scientific) using the 200 bp chemistry with 160 flow (40 cycle) for small RNA sequencing format.

2.3. Small RNA bioinformatics and miRNA identification

The sequencing data was processed using a comprehensive analysis pipeline for sRNAs. First, pre-processing of the reads was performed using Fastx Toolkit where the reads were trimmed with quality below 17 and length below 17. The filtered in reads were aligned to rice genome (*Oryza sativa7*) using SHRiMP package and standard mapping parameters. mirDeep2 software was used for the quantification of the known miRNAs; the software utilizes the information on the mapped data and known miRNA precursor for the purpose and generates normalized counts (Supplementary file 2). The novel miRNAs were predicted based on the mapped reads and structural conformation with criteria of minimum 5 mapped reads. Structural stability and minimum free energy were calculated, which subsequently provided miRDeep scores where threshold of 0 and above were used as screening parameter. Differential analysis of miRNA abundance was conducted using DESeqR-package considering p-value < 0.05 as threshold (Love et al., 2014). DESeq takes raw counts of miRNA as input and performs normalization through geometrical mean. The basic criteria for qualifying a sRNA as miRNA were as described in Meyers et al. (2008), Wan et al. (2012), Zhang et al. (2006a) and Xie et al. (2012). Briefly: 1) the sRNA has at least 3 read counts, 2) there is perfect sequence match of sRNA mapped to the precursor, 3) the precursor is not less than 60 nt in length, 4) the precursor forms a hairpin structure when subjected to folding using mfold software (http://mfold.rna.albany.edu/?q = mfold/rna-folding-form), 5) the sRNA sequence occupies only one of the arms of the hairpin structure, 6) there is no more than six mismatches of the sRNA sequence on the opposite strand, 7) there is no loop or break between the miRNA/miRNA* duplex, 8) the minimum free energy (MFE) is less than or equal to −15 kcal/mol, 9) the minimum free energy index (MFEI) is not less than 0.66, and 10) the A + U content is within 30–70%. 

2.4. Study of the miRNAs abundance level by Northern blotting

The abundance level of representatives from both conserved and novel miRNAs identified through bioinformatics were validated by Northern blot analysis. Only the shoot of the control and 20% PEG treated plants was considered for validation of the NGS results. Total RNA was isolated from shoot of the control and PEG treated seedlings using Trizol. The quality of RNA was checked on 1.2% agarose gel and also on a nanodrop spectrophotometer (260/280∼2.0). For Northern blotting 10 μL of the individual RNA preparations containing 10 μg of good quality RNA was mixed with 10 μL of 2X loading buffer containing 0.025% bromophenol blue, denatured at 95 °C for 5 min and loaded into different lanes of 15% denaturing polyacrylamide gel (7 × 10 cm). The gel was run at 200 V until the dye reached the bottom. After the run was complete, the gel was removed and stained with EtBr to check the separation. The fully run PAGE gel was imposed on a nylon membrane for the transfer of the RNA separated using Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). The membrane was then air-dried, UV-cross linked at 150 mJ using a UV cross-linker (HoeferTM UVC 500 Cross linker) and stored at 4 °C until further use. The membrane was incubated or pre-hybridized in the hybridization buffer (Sigma) at 37 °C for 1 h. Probes for the individual miRNAs were designed beforehand, which included end-labelling of the DNA oligos complementary to the miRNA with [γ-^{32}P]dATP. End-labelling was done by incubating 2 μL of the individual oligos (10 μM) with 1 μL of radio-labelled ATP (5 mCi [γ-^{32}P]dATP mL^{-1}), 10 U polynucleotide kinase (PNK, Thermo Fischer Scientific), 2 μL of PNK buffer in a final volume of 20 μL at 37 °C for 1 h. The labelled probes were added individually to separate hybridization bottles each containing a pre-hybridized blot bathed in 5 mL of hybridization buffer. The hybridization bottles, each containing a blot, the labelled complementary probe and hybridization buffer were incubated for 16 h in a hybridization oven at 37 °C. The membrane from each bottle was then washed with 2X SSC +0.1% SDS (non-stringent) for 15 min at 32 °C and 1X SSC + 0.1% SDS (stringent) at 32 °C for another 15 min. The membrane was air dried and exposed to X-ray film for appropriate time. The exposed X-ray film was then developed, fixed and imaged on a gel-doc. The membrane was stripped-off the probe by washing it again with stringent and non-stringent wash buffers at 80 °C for 10 min each, and was then used to probe U6 RNA with the [γ-^{32}P]dATP probe prepared for a small stretch of its sequence and exposed to X-ray film for detection of the signal, which served as internal control. The images of miRNA signal and the corresponding U6 signal were subjected to densitometric analysis on a Gel-doc to show the abundance level of the individual miRNAs between the control and treated samples as relative values.

2.5. Study of the miRNA abundance level by miR-X qPCR

Total RNA was isolated from the root and shoot tissues as above. qPCR for the individual miRNAs was performed using miR-X qPCR kit

(Clontech) following the steps outlined in the instruction manual. The individual RNA preparations were polyadenylated and reverse transcribed using poly(A) polymerase and SMART™ M-MLV reverse transcriptase, respectively, included in the mRQ enzyme mix. SYBR® Advantage® qPCR Premix and mRQ 3′ primer along with miRNA-specific and U6-specific 5′ primers were then used for qPCR on LightCycler® 480II (Roche). Delta-delta Ct method (Pfaffl, 2001) was used for quantification of the cellular abundance of a miRNA, and the result was expressed as fold change in abundance of the miRNA in the samples from the PEG-treated plants over that from the control plants. Significance of differences ($p \leq 0.05$) in cellular abundance of a miRNA in the samples from the control and treated plants was tested by Paired 't' test.

2.6. Target prediction and validation

Targets were predicted for the conserved miRNAs and experimentally validated novel miRNAs in rice database using psRNAtarget software (http://plantgrn.noble.org/psRNATarget/) with default parameters of Schema V2 release. All miRNAs were bioinformatically found to have a number of targets, but the targets of expectancy 3.0 or less were only considered for annotation and validation. Complete CDS of the individual target genes whose transcription was to be studied was blast searched against the *Oryza sativa* L. sub sp. Indica using *EnsemblPlants* resource database (http://plants.ensembl.org/index.html) in order to verify the presence of the target sequence in the Indica sub-species as well. Real-time PCR (RT-qPCR) of the gene/target of interest was carried out to see the changes in their transcript abundance in response to the PEG treatment and possible relationship between their transcription vis-à-vis the cellular abundance of the concerned miRNAs. Total RNA was isolated from shoot tissue of the control and PEG treated plants using TRIZOL (Invitrogen, Life Technology). The RNA pellets of the individual plant samples were suspended in DEPC-water and the quality of preparation was checked on a nano-drop spectrophotometer. QuantiTect Reverse Transcription Kit (Qiagen) was used to convert RNA to cDNA. A total volume of 20 μL was used for all reverse transcription reactions using 1 μg gDNA free total RNA, as outlined in the kit manual. The first strand cDNA prepared was used as a template for RT-qPCR, which was carried out using QuantiTect SYBR Green PCR Kit (Qiagen) following the kit manual. Primers specific to the individual genes were designed using Primer Blast software at NCBI site (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primers designed were 20-mer or more nucleotide (nt) in length with Tm not less than 59 °C, and amplified fragments varying from 90 to 200 bp (Supplementary file 3). The PCR was carried out on LightCycler® 480II (Roche). The reaction cycle set was an initial denaturation at 95 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The PCR was set in triplicate for each gene for a cDNA preparation, and cDNA preparations from two biological samples were considered for the study of transcription of the individual gene ($n = 6$). The transcript abundance of actin gene in each sample was used for normalization. The transcription of a gene was shown as its transcript abundance in the sample from the treated plant relative to that from the control plant following Pfaffl (2001). Significance of differences ($p \leq 0.05$) in the transcript abundance of gene in the samples from control and treated plants was tested by Paired 't' test.

2.7. Validation of miRNA targets by 5′-RACE

5′RACE was performed using the Clonetech RLM-RACE kit. The 5′ adapter (1 μL) supplied along with the kit was incubated individually with 10 μg of total RNA extracted from shoot of the control and PEG treated plants together with other reagents for 1 h at 37 °C in a total volume of 10 μL for its ligation at the 5′ cleaved end of RNA that contains a free phosphate group. The 5′ adapter ligated RNA (2 μL) was then reverse transcribed by incubating with required amount of random decamers, M-MLV reverse transcriptase and other reagents at 42 °C for 1 h in a total volume of 20 μL to make the RACE library. For the identification of the target, 1 μL of the RACE library was PCR amplified using the gene-specific outer primer and 5′RACE outer primer for 30 cycles in a total volume of 50 μL. The PCR product so obtained was subjected to PCR again for 30 cycles taking 1 μL of it and using 5′RACE inner primer and gene-specific inner primer in a total volume of 50 μL. The entire PCR product was run on 1.5% agarose gel and the band of the predicted size was excised and the product was eluted using Qiagen gel extraction kit. The eluted amplicons were cloned into pGEMT easy vector by incubating them in 3:1 ratio at 4 °C overnight following the kit instruction manual. The cloned products (5 μL) were then transformed into DH5α competent cells, which were allowed to multiply in 1 mL of LB medium at 37 °C with shaking at 200 RPM, pelleted out by centrifugation and spread on LB plate containing ampicillin. The transformed colonies were subjected to colony PCR individually using SP6 T7 primers and the bands of expected size from different PCR reactions visualized on agarose gel were excised and sequenced. The number of colonies sequenced and that contained the sequence of the gene of interest were recorded. The primers used in PCR reactions are given in Supplementary file 3.

2.8. Validation of miRNA targets by dual-luciferase assay

Validation of the targets of two miRNAs, osa-miR12472 and osa-miR12473, cleaving C3HC4 zinc finger (*ZF*) and equilibrative nucleoside transporter (*ENT*), respectively was done using dual luciferase transient expression system. Dual luciferase assay was performed by transiently synthesizing a miRNA alongside transcription of its target gene in *Nicotiana benthamiana* (Liu and Axtell, 2015). Wild type *N. benthamiana* was grown in a greenhouse maintained at 24 ± 2 °C, 65–75% relative humidity and natural day/night illumination. The leaves of two months old plants were used for the experiment. The target oligos for the individual miRNAs were synthesized with the sense strand having AvrII restriction site extension at the 5′ end and the antisense strand with AgeI restriction site extension at the 3′ end. The sense and anti-sense strands were annealed on a thermocycler taking 5 μL each of 0.5 nM sense and antisense oligos and 1.67 μL 0.3 M NaCl to a final volume of 60 μL made up with PCR grade water. The initial temperature for annealing was set at 97 °C for 5 min with ramped-down to 20 °C at 0.1 °C/s. The annealed product was cloned separately into pGreen derived plasmid at 3′UTR of luciferase (Addgene ID 55206) and called 3′UTR sensor (sensor). For insertion, 1 μg of the plasmid was double digested with AverII and AgeI and the desired digested product was excised after separation and visualization on 1.2% agarose gel. The double digested product of ID55206 (sensor) was then ligated with the double stranded target oligo at 16 °C for 16 h using T4 DNA ligase and taking the vector and insert in 1:3 ratio. Similarly, miRNA overexpressor construct was made for the individual miRNA separately. The precursors of the individual miRNAs (hairpin region flanked with ~100 bp upstream and downstream sequence) were PCR amplified using forward primers having XhoI site and reverse primer having EcoRI site, and were cloned separately into pGreen II based vector (Addgene ID 55208). All the cloned constructs were transformed into DH5α, which was grown and plated for development of colonies following standard protocol. Cloning of the inserts was confirmed by colony PCR, followed by sequencing. The plasmids with right inserts were transformed into *A. tumefaciens* strain LBA4404 individually. Equal volumes of miRNA overexpressor and sensor were mixed and infiltrated in nine leaves considering three leaves per plant. A set of leaves were also infiltrated with *A. tumefaciens* carrying only the sensor construct. The leaf discs were harvested after 48 h and frozen immediately in liquid N_2. The preserved leaf tissues infiltrated with *A. tumefaciens* carrying both miRNA overexpressor and sensor constructs were used for the estimation of relative level of the target by dual luciferase assay, i.e. Renilla luciferase (R-Luciferase) and Fire-Fly luciferase (F-luciferase). Dual luciferase assay was performed using Dual-luciferase Reporter Assay System (Promega) following manufacturer's instructions using Glomax 20-20 luminometre (Promega). For the study of influence of a miRNA on transcription of F-luciferase, RNA was isolated from the leaves infiltrated with both sensor and miRNA overexpressor together and that infiltrated only with sensor, and reverse transcribed following standard protocol. RT-qPCR was performed using primers for F-Luciferase and R-Luciferase transcripts to validate that the relative transcription of F-luciferase corresponds to the results of F-Luciferase/R-Luciferase activity.

3. Results

3.1. Small RNA sequencing

The total raw sRNA reads obtained from sequencing of root and shoot tissues of the seedlings of the control and that treated with 20% PEG (~IC_{50}) for 9 h were 14802698 for control root (CR), 16726841 for treated root (TR), 12783171 for control shoot (CS) and 8438037 for treated shoot (TS), and the total filtered reads of nucleotide (nt) length 17 or more for these samples were 6791191, 6464494, 10348946 and 5427169, respectively (Table 1). The filtered reads that mapped to the rice genome were 84%, 76%, 96% and 98% in CR, TR, CS and TS, respectively (Table 1). Based on the number of reads mapped to the genome, the number of the likely miRNAs present varied; their numbers decreased with increase in the filter for the mapped reads. The total novel miRNAs predicted by miRDeep2 programme were 89, 103, 195 and 124, respectively in CR, CT, CS and TS. The number of predicted miRNAs decreased to 13, 19, 48 and 22, in CR, CT, CS and TS, respectively when the miRDeep2 score was raised to ≥5 (Table 1).

3.2. Family distribution of miRNAs

The conserved miRNAs identified belonged to 45 families in root (Fig. 1a) and to 50 families in shoot (Fig. 1b). Nearly 1/3rd of the miRNA families represented most of the miRNA members in both root and shoot with each family representing 5 or more of them. Among these the members of at least two families, including miR812 and miR818 were present exclusively in root (Fig. 1a), while the members of the family miR2118 were present only in shoot (Fig. 1b). Among the miRNA families that represented less than 5 miRNA members, at least five of them were present in root but not in shoot, while at least twelve of them were present in shoot but not in root (Fig. 1a and b). It was observed that the greater the number of miRNA members in a miRNA family, the higher was the abundance (reads) of miRNAs, both in root and shoot. However, in several cases, both in root and shoot, like that in the families miR535 and miR168, the abundance of the miRNAs was more compared to the numbers of the miRNA members they represented. The PEG treatment influenced the abundances of the miRNA members in several of the families, both in root (Fig. 1a) and shoot (Fig. 1b), but the number of miRNA families affected and the decrease in miRNA abundances in number of miRNA families in response to the PEG treatment was more in shoot than in root.

Root and shoot also differed greatly in response of their individual miRNAs to the 20% PEG treatment administered for 9 h. As many as 40 miRNAs showed two $\log_2$-fold or more decrease and 13 miRNAs showed two $\log_2$-fold or more increase in response to the PEG treatment (Fig. 2). In addition, the cellular abundance of 70 miRNAs showed at least 2-fold change ($\log_2$-fold change of one or more, but less than two) in response to the PEG treatment with the abundance level of 24 getting increased and 46 getting decreased (Supplemental file 4). Compared to shoot, much lesser number of miRNAs in root showed $\log_2$-fold change of two or more in response to the PEG treatment (Fig. 3). Among these seven miRNAs showed decrease and two miRNAs showed increase in response (Fig. 3). Among the remaining miRNAs showing at least 2-fold change in response to the PEG treatment, the cellular abundance of 24 increased and that of 26 decreased (Supplemental file 4). There occurred vast difference in the cellular abundance of miRNAs getting influenced by the PEG treatment in root and shoot, as only 48 miRNAs showing at least 2-fold or more change in response to the PEG treatment were synthesised both in root and shoot in common out of 82 and 184 miRNAs that were synthesized in root and shoot, respectively (Supplemental file 4).

3.3. Northern blot of the conserved and novel miRNAs

The presence of three of the conserved miRNAs, including miR1864e, miR1429-5p and miR156b-3p, could be successfully validated by Northern in shoot (Fig. 4). The cellular abundance of all these decreased in response to the 20% PEG treatment given for 9 h with miR156b-3p showing the maximum decrease and miR1429-5p showing the minimum decrease. The existence of four novel miRNAs, including osa-miR12470, miR12471, miR12472 and miR12473, could also be validated by Northern in shoot (Fig. 5). Among these, the cellular abundance of miR12470, miR12471 and miR12473 decreased, while that of miR12472 increased in response to the PEG treatment. The decrease in cellular abundance of miR12471 and increase in that of miR12472 in response to the PEG treatment was very high compared to the changes observed in the cellular abundance of the other novel miRNAs. The sequencing data revealed osa-miR12473 and osa-miR12471 to be of 21-nt in length, and osa-miR12472 and osa-miR12470 to be of 24-nt in length (Table 2). The miRNA* sequences of these miRNAs were also captured in sequencing (Table 2). The length of their precursors varied from 72 to 89 nt. Bioinformatics analysis of

Table 1

Small RNA bioinformatics. Roots and shoots (leaves) of the drought tolerant rice cultivar KMJ 1-12-3 were harvested after 20% PEG treatment to 6 days old seedlings for 9 h and were subjected to high throughput sequencing on Ion Proton system. The raw reads were processed by SHRiMP package and mirDeep2 software for identification and quantification of the known and novel miRNAs.

Read Distribution	Root control	Root treated	Leaf control	Leaf treated
Total no. of raw reads	14,802,698	16,726,841	12,783,171	8,438,037
Filtered reads (Q17, Length > =17)	6,791,191	6,464,494	10,348,946	5,427,169
% Filtered reads	46%	39%	81%	64%
Reads mapped on genome	5,725,376	4,934,123	9,931,918	5,313,388
Total no. of reads after t-RNA/ r-RNA filter	2345005	3359957	2542319	3640343
% reads mapped	84%	76%	96%	98%
miRNA with at least 1 mapped reads	450	397	489	442
miRNA with at least 3 mapped reads	361	334	416	387
miRNA with at least 5 mapped reads	332	307	387	350
Total Novel miRNA predicted	89	103	195	124
Total Novel miRNA predicted (mirdeep2 score > =1)	69	81	161	104
Total Novel miRNA predicted (mirdeep2 score > =5)	13	19	48	22

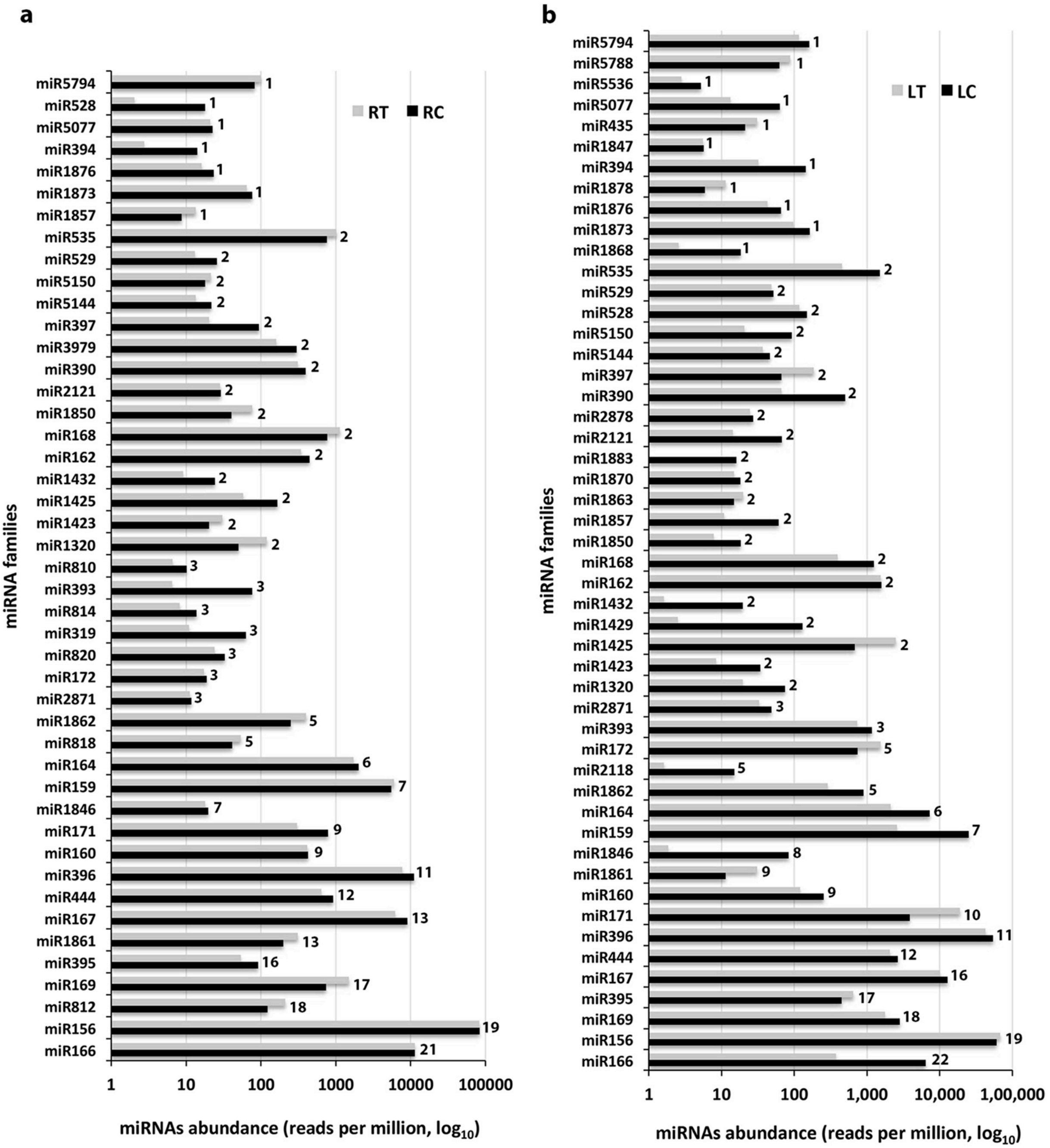

Fig. 1. Abundance of the conserved miRNAs in reads per million (RPM) in the individual miRNA families in root (a) and shoot (b) of control and 20% PEG treated (9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3. The numerical values against each miRNA family bar represent the number of individual conserved miRNAs represented by the family in root (a) and shoot (b) of the control and PEG treated seedlings out of the total identified through sequencing.

physical properties of the precursors showed that their MFE values varied from −30.50 to −51.54 kcal/mol and the MEFI values ranged from 1.10 to 1.66 (Table 2). The precursors formed perfect stem-loop structure (Supplemental file 2) necessary for dicing of the miRNA/miRNA* pair with mismatches of zero to 3 (Table 2). The reads per million (RPM) of each novel miRNAs was quite high, ranging from 15.13 to 391.63 (Table 2).

3.4. Micro-RNA abundance level study by Mir-X qPCR

The response of the miRNAs to the PEG treatment whose presence was validated in shoot by Northern was studied in both root and shoot by qPCR (Fig. 6). The results of qPCR for the conserved miRNAs in shoot were quite in agreement with that of the Northern, at least for miR1429-5p and miR1846e showing significant decrease in the abundance level in response to the 20% PEG treatment given for 9 h. However, miR156b-3p did not show any significant change in cellular abundance in shoot in response to the PEG treatment (Fig. 6), unlike the result of the Northern where a decrease in cellular abundance of the miRNA was observed (Fig. 4). In contrast, in root miR156b-3p showed highly significant decrease in cellular abundance in response to the PEG treatment. PEG-induced decrease in the cellular abundance was also observed for miR1864e, but unlike in shoot miR1429-5p did not show any significant change in cellular abundance in root. Similar to the conserved miRNAs, the results of qPCR for response of the novel miRNAs to the PEG treatment in shoot was quite comparable to that of

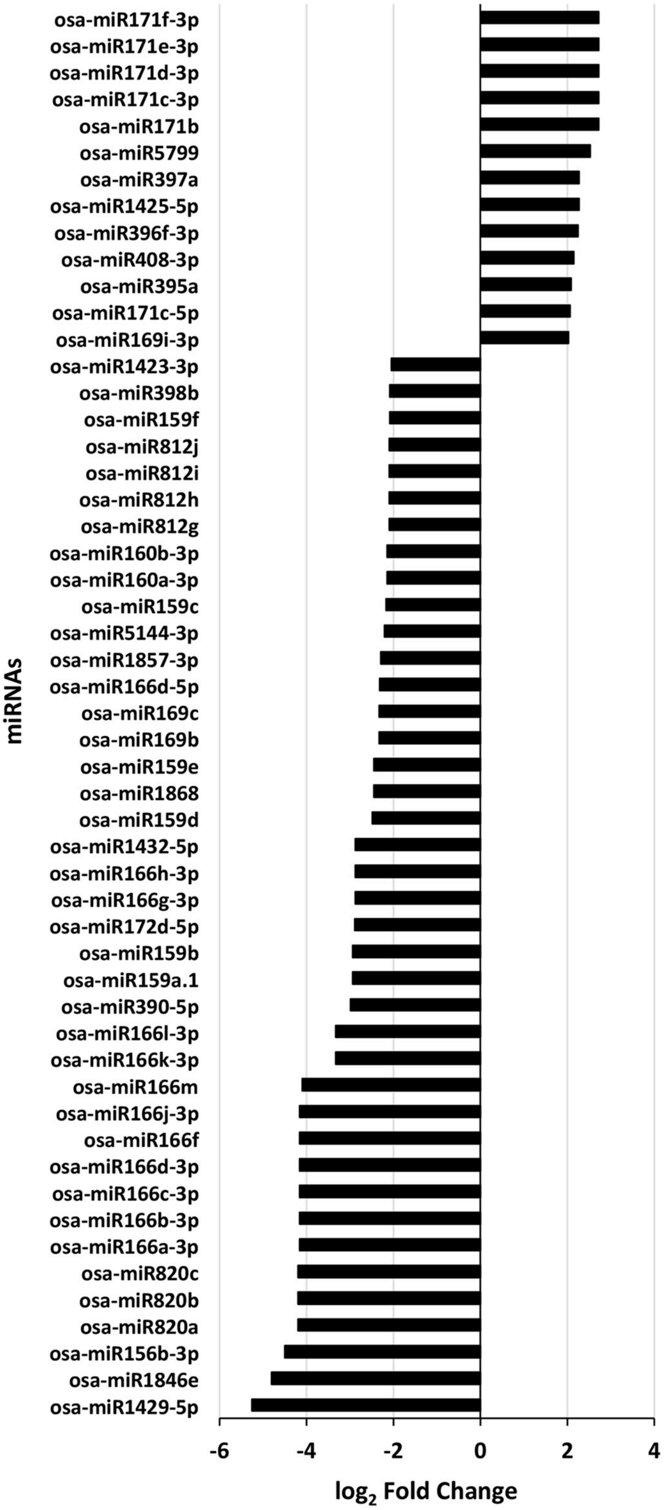

Fig. 2. Changes in cellular abundance ($\log_2$-fold) of the individual conserved miRNAs in shoot of the 20% PEG treated (9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3 over the control values. Only those miRNAs showing changes in abundance of two $\log_2$-fold or more have been listed. The fold change in abundance was calculated using the high throughput sequencing data.

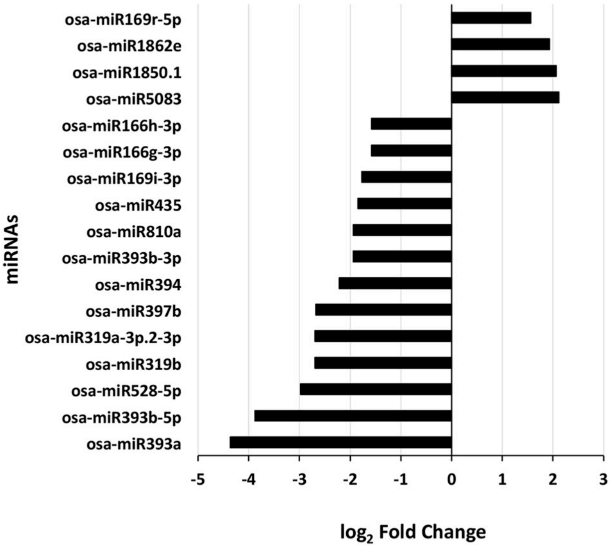

Fig. 3. Changes in cellular abundance of the individual conserved miRNAs in root of the 20% PEG treated (9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3 over the control values. Only those miRNAs showing changes in abundance of 1.5 $\log_2$-fold or more have been listed. The fold change in abundance was calculated using the high throughput sequencing data.

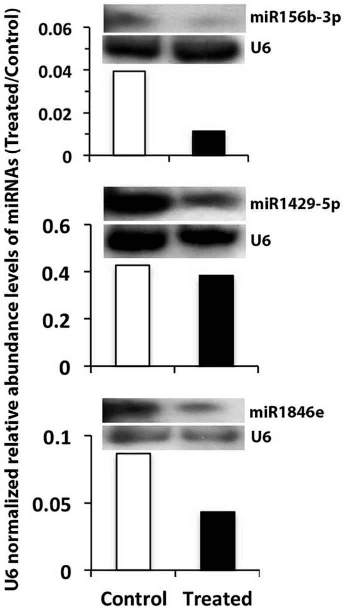

Fig. 4. Northern blots showing presence of a few conserved miRNAs in shoot of the control (C) and 20% PEG treated (T, 9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3. The empty and solid bars show relative abundance levels of the individual miRNAs in the shoot tissue of the control and treated seedlings, respectively with respect to U6 abundance level in the tissue. The relative abundance level values were obtained by densitometric analysis of the individual miRNA bands in the control and treated samples with respect to the U6 bands.

the results of the Northern (Fig. 5) with three miRNAs, including miR12470, miR12471 and miR12473, showing significant decrease in cellular abundance and miR12472 showing significant increase in cellular abundance. In the case of root also the cellular abundance of miR12470, miR12471 and miR12473 decreased in response to the PEG application, but the decrease was not statistically significant for miR12473. The cellular abundance of miR12472 increased highly significantly in root in response to the PEG application, similar to that observed in shoot.

3.5. Transcription analysis of the targets by RT-qPCR

The targets of all the conserved and novel miRNAs considered for

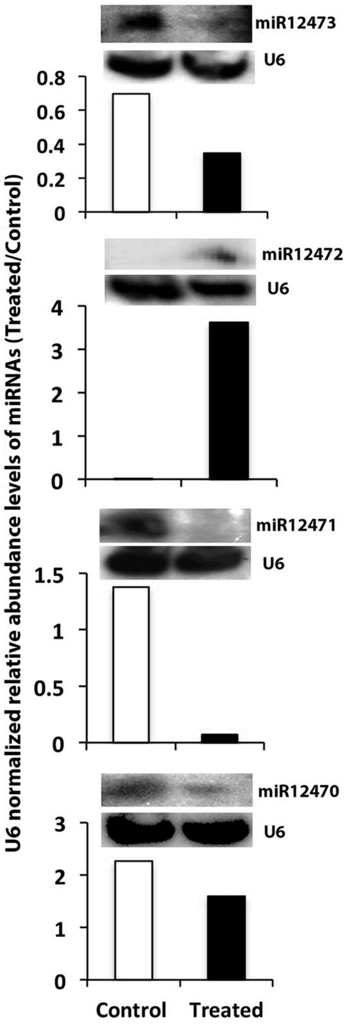

Fig. 5. Northern blots showing presence of a few novel miRNAs in shoot of the control (C) and 20% PEG treated (T, 9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3. Other details as in Fig. 4.

the study were predicted up to the expectancy (E) value of 3.0. The relative transcript abundance (treated/control) of the target genes (Fig. 7) and the relative cellular abundance (treated/control) of the miRNAs (Fig. 6) mostly showed a negative relationship. Among the targets of the conserved miRNAs, the transcription of *OsMPK1* and *R-SAM* (Radical SAM Enzyme), the targets of miR1429-5p and miR156b-3p, respectively upregulated highly significantly in both root and shoot in response to the short term (9 h) 20% PEG treatment of the seedlings. However, the transcription of *LRR* showed insignificant change in response to the PEG treatment in both root and shoot (Fig. 7) despite the miRNA targeting it, i.e. miR1846e, showing significant decrease in cellular abundance in response to the PEG treatment in both root and shoot (Fig. 6). Among the targets of the novel miRNAs, the targets of miR12470, miR12471 and miR12473, i.e. *RNAh* (RNA helicase SDE3), *IAGT* (Indol-3-acetic beta-glucosyl transferase) and *ENT* (equilibrative nucleoside transporter), respectively showed significant increase in transcription in both root and shoot in response to the PEG application (Fig. 7), in agreement with the PEG-induced decrease in cellular abundance of the miRNAs (Fig. 6). In contrast, the transcription of *ZF* (C3HC4 zinc finger) significantly decreased in both root and shoot tissues in response to the PEG application, which agreed with the PEG-induced significant increase in the cellular abundance of miR12472 targeting *ZF*.

Table 2

The novel miRNAs predicted by alignment of the sRNA sequences with the rice genome database and confirmed for their presence by Northern blot in shoot tissues of the control and 20% PEG treated (9 h) 6 days old seedlings along with the relevant information on the basic bioinformatics criteria qualifying them as miRNAs. MFE- Minimum Free Energy, MFEI- MEF index.

Novel miRNAs	Sequence	Maximum abundance among tissues in reads per million (RPM)	Length (nt)	miRNA*	miRNA/miRNA* mis-matches (nt)	Pre-cursor Length (nt)	A + U (%)	MFE (Kcal/mol)	MFEI
osa-miR12473	UGACAGAUAGAGAGUGAGCAC	15.13	21	GCUCACUCUCUUCUGUCAGA	1	88	47.72	−51.54	1.12
osa-miR12472	AUGAAUGUGGGAAAUGCUAGAAUG	58.09	24	UUCUAGCAUUUUCCACAUUAAUAU	1	72	68.05	−30.50	1.32
osa-miR12471	UCAAUAUGAAUGUGGGAAAUG	391.63	21	AUUUUCACAUUCAUAUUGAUG	0	89	76.40	−34.90	1.66
osa-miR12470	GUGGAACCUUGUUAAGGUCGGAAC	72.51	24	UCCGACCCAUACAAGGUUCCGU	3	87	48.27	−49.60	1.10

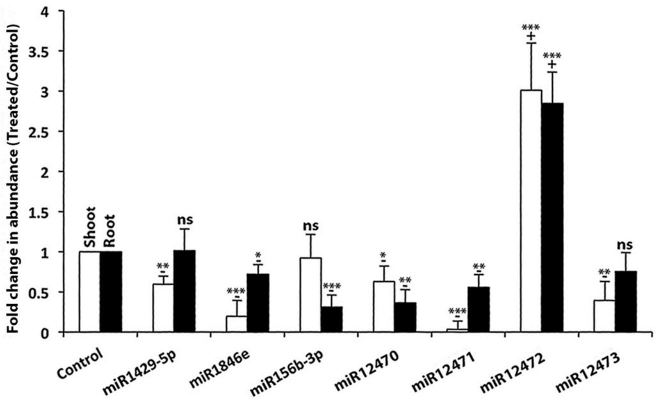

Fig. 6. Changes in the cellular abundance of select conserved and novel miRNAs in the root and shoot tissues of 20% PEG treated (T, 9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3 over the control level, as determined by miR-X qPCR. The empty and solid bars show relative level of the individual miRNAs in the shoot and root tissues, respectively, of the treated seedlings over the control level taken as unity. U6 level was taken as internal control for normalization of the abundance level of different miRNAs. The fold change value less than one and greater than one represented decrease and increase, respectively in the cellular abundance of a miRNA. The data are the mean ± SD of six independent estimations using two biological replicate samples. Asterisks against the individual columns indicate that PEG-induced changes in the cellular abundance of the miRNA differed significantly from the respective control cellular abundance level at $p \le 0.05$ (*), $p \le 0.01$ (**), or $p \le 0.001$ (***). ns = not significant.

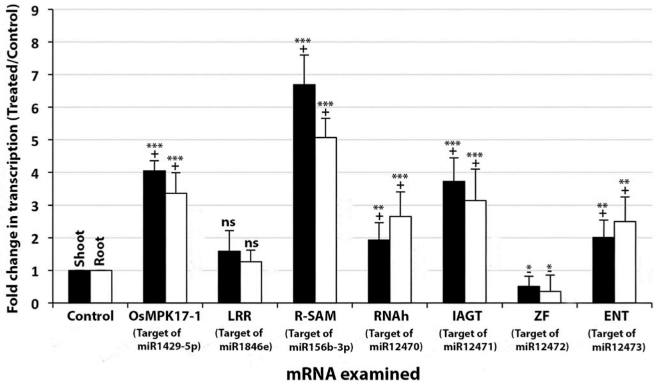

Fig. 7. Changes in the transcription or transcript abundance of the target genes of select conserved and novel miRNAs in the root and shoot tissues of 20% PEG treated (T, 9 h) 6 days old seedlings of *O. sativa* cv. KMJ 1-12-3 over the control level, as determined by RT-qPCR. The solid and empty bars against the individual miRNAs on the X-axis show relative transcript abundance of the target genes in the shoot and root tissues, respectively, of treated seedlings over the control level taken as unity. Transcription of *Actin* was taken as internal control for normalization of the transcription of various target genes. The fold change value less than one and greater than one represented decrease and increase, respectively in the transcript abundance of a target gene. The data are mean ± SD of six independent estimations using two biological replicate samples. Asterisks against the individual columns indicate that PEG-induced changes in transcription of the gene differed significantly from the respective control transcription level at $p \le 0.05$ (*), $p \le 0.01$ (**), or $p \le 0.001$ (***). ns = not significant.

3.6. Validation of target cleavage by 5′RACE and dual-luciferase assay

5′RACE successfully identified cleaved fragments of three novel miRNAs, including miR12473, miR12472 and miR12470 (Fig. 8). Sequencing of the 5′RACE cloned PCR products showed 5 out of 6, 3 out of 6 and 6 out 6 cloned products to contain cleaved fragment of *ENT* (equilibrative nucleoside transporter), *ZF* (C3H4 type zinc finger protein) and *RNAh* (RNA helicase SDE3), respectively. In order to validate the target of the three miRNAs further, dual luciferase assay was performed for them. It was possible to generate constructs for validation of the two targets, one each for miR12472 and miR12473; *ZF* for miR12472 and *ENT* for miR12473 (Fig. 9). Significant decrease in F-luciferase to R-luciferase activities ratio was obtained in the *N. benthamiana* leaves transfected with the sensor and miRNA overexpressor constructs for the individual miRNA and its target (Fig. 9a), suggesting that *ZF* and *ENT* were targeted by miR12472 and miR12473, respectively. The result was also supported from the decrease in transcription of F-luciferase in the above *N. benthamiana* leaves (Fig. 9b).

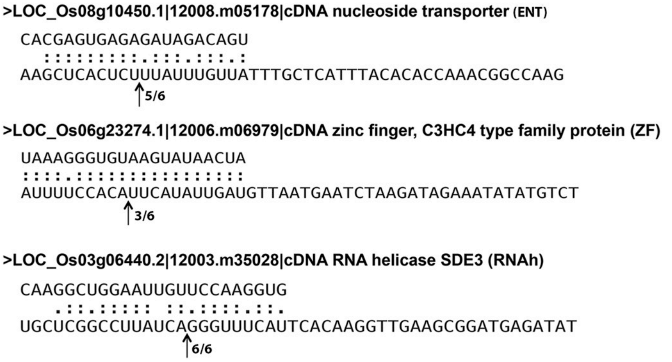

Fig. 8. The cleavage points, indicated by arrows, of equilibrative nucleoside transporter (*ENT*), C3HC4 zinc finger (*ZF*) and RNA helicase SDE3 (*RNAh*) targeted by the novel miRNAs osa-miR12473, osa-miR12472 and osa-miR12470, respectively, as obtained by 5′RACE PCR and cloning and sequencing of the PCR products. The figures besides the arrows indicate the number of clones of a gene product (mRNA) found to be having cleavage point ligated to the 5′ adaptor out of the total number of the PCR products cloned and sequenced.

4. Discussion

Last two decades of active research in understanding the role of miRNAs in regulation of cellular levels of mRNAs and determination of phenotype has increased the database of miRNAs considerably, as is reflected from the presence of ~1500 miRNAs in miRBase 21.0 database. Contribution to this database from rice itself is ~600 miRNAs. However, the discovery of new miRNAs is far from over, including that in rice, as is reflected from the fact that miRDeep2 in the present study with KMJ 1-12-3 rice cultivar predicted several of sRNAs caught in the sequencing in root and leaf samples to be possible miRNAs (Table 1), both in the control plant and that receiving 20% PEG (~IC_{50}) treatment for 9 h, fulfilling the criteria like the required number of read counts, hairpin formation by the precursors and the length of the precursors (Table 2., Supplemental file 2) (Meyers et al., 2008; Wan et al., 2012; Zhang et al., 2006a; Xie et al., 2012). The sequencing data, nevertheless, suggested that the biosynthesis of miRNAs could vary greatly depending upon the cultivars/varieties, the growth stage and the need of regulation of the molecular events at a particular stage of the plant's life, as is reflected from the presence of only 188 miRNAs in root (Figs. 1a) and 179 miRNA shoot (Fig. 1b) against as many as 600 miRNAs reported in rice.

The presence of the members of a miRNA family in shoot but not in root, and vice-versa (Fig. 1a and b) pointed towards their tissue-specific role. Possible specificity of miRNAs in regulating the biochemical and molecular events specific to root and shoot is also reflected from differential response of the miRNA members in miRNA families in the two tissues to the PEG-6000 application mimicking drought stress (Fig. 1a and b). Although the use of low molecular weight PEG, like PEG-6000, has limitation in drought stress application because of its chances of getting absorbed by root, such uptake has been found to be negligible, particularly of PEG of molecular weight 4000 or more on short time

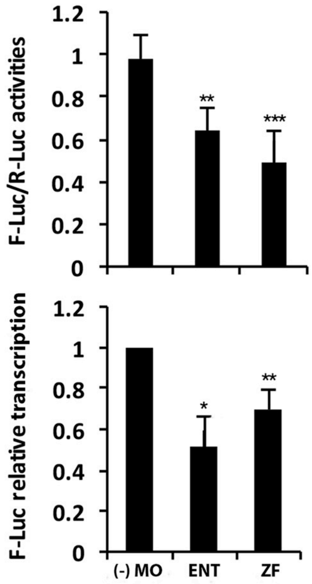

Fig. 9. Validation of the target cleavage by dual-luciferase assay. The miRNA target sequence of equilibrative nucleoside transporter (*ENT*) and C3HC4 zinc finger (*ZF*) were individually introduced in the 3′UTR of luciferase (Addgene ID 55206), which served as sensor. Similarly, the hairpin region flanked with 100 bp upstream and downstream sequences of the individual miRNAs was introduced separately into Addgene ID 55208 vector, which functioned as miRNA overexpressor. The recombinant plasmids were then individually transformed into *A. tumefaciens* strain LBA4404. Equal volumes of miRNA overexpressor and sensor were mixed and infiltrated in nine leaves considering three leaves per plant. A set of leaves were also infiltrated with *A. tumefaciens* carrying only the sensor. After 48 h the leaves were collected and dual-luciferase assay, Renilla and Fire Fly luciferase, was performed using Dual-luciferase Reporter Assay System (Promega). The result was expressed as F-Luc/R-Luc activities for the individual genes (a). RNA was isolated from the leaves infiltrated only with sensor and that infiltrated with both miRNA overexpressor and sensor, and RT-qPCR was performed for the F-Luciferase and R-Luciferase transcripts to see the relative transcript abundance of F-luciferase in the leaves infiltrated with sensor along with or without miRNA overexpressor as confirmation to the results of Renilla/Luciferase activity. F-Luciferase transcription in MO(-) was taken as unity (b). The results are mean ± sd of nine assays (biological replicates). ** and *** marked against the individual genes indicates that their transcription in the leaves infiltrated with miRNA overexpressor (MO) plasmid is significantly less from that in the leaves without infiltration of MO (-) plasmid at $p \leq 0.01$ and $p \leq 0.001$, respectively.

period of application (Lawlor, 1970), as uptake and accumulation of PEG by plant is inversely related to its molecular size and directly proportional to the exposure time, respectively (Janes, 1974). A very clear distinction in response of the two tissues to the PEG treatment is by the members of miR166 where root did not show any response, but shoot responded by decreasing the abundances of the miRNA together with synthesis of an additional miRNA. The decrease in abundance of the members of miR166 in response to the short period 20% PEG treatment could be very important keeping in view the fact that the changes induced by a stress at the initial stage of a sub-lethal dose are crucial in determining survival of the plant under long term exposure to a given level of stress. In fact, it is the initial response of plants that determines their survival to an abiotic stress in general. From the point of view of drought stress tolerance, drastic decrease in abundance of a few of the members of the miRNA family miR166, like of miR166a-3p, miR166b-3p, miR166g-3p, miR166c-3p, miR166d-5p, miR166i-3p, miR166g-5p, miR166k-5p, miR166l-3p and miR166l-5p targeting gene products, such as homeodomain leucine zipper (HD-Zip), MADS-box family protein, cyclin A3, cyclin dependent kinase (CDK), C2H2 zinc finger protein (ZnF), guanine nucleotide exchange factor (GEF), EF hand protein, GTPase activating protein (GAP) and expressed proteins suggested important role of these miRNAs in the drought tolerance process, as plants overexpressing several of their targets, including HD-Zip, MADS-box protein, ZnF and EF hand protein, have been reported to be better tolerant to drought compared to the wild type (Wang et al., 2013; Dezar et al., 2005; Zhao et al., 2014; Zhang et al., 2016; Yin et al., 2017). Moreover, the decrease in abundance of miR166 family members targeting *HD-Zip* has been reported in tea plant and maize subjected to drought stress with concomitant increase in transcription of the target gene (Guo et al., 2017; Aravind et al., 2017), emphasizing further the importance of the members of miR166 in enabling plant to survive under the challenge of drought stress. G protein signalling appears to play important role in drought tolerance in plants, as is reflected from the PEG-induced decrease in abundance of miR166 members targeting *GEF* and *GAP*. The importance of *GEF* in drought tolerance is indicated from the fact that GEF stimulates exchange of GDP for GTP to generate active form of GTPase capable of recognizing downstream targets, and overexpression of at least one such GTPase, *PgRab7*, confers drought tolerance in tobacco (Agarwal et al., 2008). GAP on the other hand facilitates functioning of G protein signalling by increasing the catalytic activity of GTPase. The importance of G protein signalling in drought response and tolerance in plant is also reflected from the PEG-induced decrease in abundance of the members of miR1425 in root that targets GDP dissociation inhibitor (Fig. 1a). Besides, the involvement of cyclin and CDK in drought tolerance, or abiotic stress tolerance in general, cannot be ruled out, as cyclin-CDK complex is believed to initiate cell cycle by initiating DNA replication (Yam et al., 2002).

miR159, miR1846, miR390, miR535 and miR5077 were other miRNA families whose members were synthesized in both root and shoot, but showed decrease in cellular abundance only in shoot as an initial response to the 20% PEG treatment. Among them only the target of miR1846e, the leucine rich repeat (LRR) protein, and the target of most of the members of miR159 (miR159a through 159f), the *Myb* family transcription factors, have some relevance to drought tolerance, as overexpression of LRR domain protein and *Myb* has been reported to confer drought tolerance in rice (Kang et al., 2017; Jung et al., 2008). Myb transcription factors are also among the most widely studied protein related to their involvement in abiotic stress response, including tolerance to drought stress in plants (Li et al., 2015). It may be important to note that in all these cases the changes in the cellular abundance of miRNA was seen only in shoot, not in root, suggesting that PEG treatment might be creating physiological dryness condition greater in shoot than in root. In fact, cellular abundances of several miRNAs, including that of osa-miR390-5p, osa-miR1846e, osa-miR535-5p, osa-miR5077 in this study (Fig. 2), have been found to decrease in the flag leaf of the drought tolerant rice cultivar Nagina 22 upon exposure of the plant to drought stress by withdrawing water (Balyan et al., 2017). The response of miRNAs to PEG treatment might also be highly tissue specific, as the reverse was noted for several members of the miRNA families, including that of miR528, miR397, miR1425 and miR393 (Fig. 1a).

One common effect of the short term 20% PEG treatment in root and shoot was the decrease in abundance of osa-miR394 in both the tissues, indicating an increase in turnover of proteins in both of them as a result of the PEG treatment because osa-miR394 targets F-box protein, and F-box proteins together with ubiquitin-conjugating enzyme (E2) are directly linked to degradation of cellular proteins (Gharat and Shaw, 2015; Glickman and Cienchanover, 2002). The cellular levels of a protein or protein family, nevertheless, could be regulated by different miRNAs in two tissues targeting the mRNAs encoding the proteins of same function, as is reflected from decrease in the cellular abundance of miRNA159a through miRNA159f in shoot (Figs. 1b and 2) and that of miR319a-3p.2-3p and miR319b in root (Figs. 1a and 3), all targeting *Myb* transcription factors.

The decrease in cellular abundance of miRNAs in most of the miRNA families in response to the short term 20% PEG treatment in both root and shoot (Fig. 1) suggested important positive role of the targeted effector/regulator proteins in drought tolerance. A feature common to both root and shoot was the decrease in abundance of miRNAs targeting *Myb* transcription factor family reportedly important for drought tolerance (Li et al., 2015). The decrease in cellular abundance of miR319 targeting *Myb* has also been reported in roots of the drought tolerant wheat cultivar cv. Sivas 111/33 in response to drought stress with concomitant increase in *Myb* transcript level compared with the control (Akdogan et al., 2016). Unlike this study, however, the grapevine (cv. Cabernet Sauvignon) exposed to drought showed significant decrease in cellular abundance of miR159c even in root with concomitant increase in transcription of *Myb*, the target gene (Pagliarani et al., 2017). The contribution to drought tolerance, however, may not be restricted to only these miRNAs, as shoot showed two log$_2$-fold or more decrease in abundance of several other miRNAs targeting leucine rich repeat (LRR), protein kinase (miR390-5p), EF hand protein (miR1432-5p), nuclear transcription factor Y (NF-Y, osa-miR169b,c), GATA zinc finger domain (osa-miR5144-3p) and superoxide dismutase (miR398b) well reported for their role in tolerance to the stress (Wang et al., 2013; Kang et al., 2017; Ni et al., 2013; Gupta et al., 2017). Drought tolerance might also be linked to the plant hormone ethylene, as at least 4 miRNAs, including miR812g, miR812h, miR812i and miR812j targeting 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) involved in ethylene biosynthesis showed more than two log$_2$-fold decrease in abundance, and ethylene is known to induce transcription of transcription factors like ethylene responsive factors (ERFs) and dehydration responsive element binding protein (DREB), which in turn regulate the transcription of a number of genes involved in abiotic stress tolerance, including drought stress tolerance (Muller and Munne-Bosch, 2015; Mizoi et al., 2012; Panda et al., 2014); it is well established that constitutive overexpression of *DREB* leads to enhanced transcription of drought-inducible genes that improves drought tolerance in *Arabidopsis* (Sakuma et al., 2006). In addition to the regulatory proteins, the enzyme like PM-H$^+$ATPase also appears to have some role in drought tolerance, as the miRNA miR1432 targeting the enzyme exhibited decrease in cellular abundance both in root and shoot upon the PEG treatment; althought PM-H$^+$ATPase is well known to generate protomotive force for ion movements under salt stress (Sahu and Shaw, 2009), its initial activation under drought stress is believed to trigger biosynthesis of osmolytes to facilitate drought stress tolerance (Gong et al., 2010).

The results of the Northern showing changes in the cellular abundance of both conserved (Fig. 4) and novel (Fig. 5) miRNAs in response to the short term 20% PEG treatment were mostly in agreement with the log$_2$fold changes in abundance of the miRNAs found in response to the PEG treatment by NGS (Figs. 2 and 3) indicating authenticity of the sequencing results. The results of the Northern (Fig. 5) also confirmed existence of the novel miRNAs miR12473, miR722, miR12471 and miR12470, which were predicted by bioinformatics (Table 2) fulfilling all the criteria, such as 1) the presence of mRNA*, 2) MFE of the precursor miRNAs was negative and much lower than the values of the tRNA and ribosomal RNA (Bonnet et al., 2004), 3) the  mean MFEI value of the precursors was higher than 0.64, which is MFEI of tRNAs and maximum among the known RNAs (Zhang et al., 2006a), 4) the length of the precursors was greater than the desired length of 60 nt with the miRNAs occupying only one of the arm of the concerned secondary structure and having less than five mismatches with miRNA* (Table 2, Supplemental file 2) necessary for getting diced to mature miRNAs (Zhang et al., 2005), 5) A + U contents of the precursors varied from 47.72% to 76.40% (Table 2) that is in agreement with the reports available (Zhang et al., 2006b; Panda et al., 2014), and finally 6) the miRNAs were 10 or more RPM in abundance, an important criteria for qualifying a sRNA as miRNA (Kozomara and Griffiths Jones, 2011). The discovery of these novel miRNAs indicated that although the current database of plant miRNAs for rice is quite rich, it is not yet saturated.

Mir-X qPCR further validated the presence of both conserved and novel miRNAs, not only in shoot, but also in root (Fig. 6). Significant differences in cellular abundance of most of the miRNAs between root and shoot also confirmed substantial difference in metabolism in the two tissues, similar to the result of NGS. Significant decrease in abundance of all the miRNAs, except of miR12472, in response to the 20% PEG application (Fig. 6) indicated an increase in overall metabolic activities in the plant promoting drought tolerance, as cellular abundance of miRNAs and the levels of their target mRNAs are mostly inversely related, although the relationship may not be statistically proportional (Akdogan et al., 2016; Pagliarani et al., 2017; Aravind et al., 2017). The increase in overall metabolic activities in the plant in response to the PEG application is also evident from the increase in the transcript abundance of the mRNAs targeted by most of the miRNAs showing PEG-induced decrease in cellular abundance (Fig. 7).

Although the targets of the conserved miRNAs, like miR1429-5p and miR156b-3p, showed much greater transcript abundance than others, functional significance of none of them in drought tolerance has been reported so far. *MAPK*, the target of miR1429-5p, however, could be contributing to the chemistry leading to drought tolerance in plants, as many enzymes and proteins become functionally active only after phosphorylation mediated by MAPK relay; it has been shown that overexpression of *SlMAPK1* in tomato increases drought tolerance of the plant (Wang et al., 2018). The biological role of none of the targets of the novel miRNAs identified are clearly defined, particularly with regard to drought tolerance, although these could be of great importance in tolerance to the stress. Among the targets of the novel miRNAs, indole-3-acetate beta-glucosyl transferase (IAGT) has been reported to manage the intracellular level of free IAA in the tissue depending upon the environmental condition (Ciarkowska et al., 2016). The level of IAA decreases significantly in leaves of maize seedlings under PEG stress applied through root medium (Wang et al., 2008). The observed decrease in the level of IAA could be mediated by increase in transcription of *IAGT*, which was found in the present case (Fig. 7) because of significant decrease in abundance of miR12471 in response to the PEG treatment (Figs. 5 and 6). With regard to RNA-helicase SDE3, the protein has been reported to have RNA-dependent RNA polymerase (RdRP) activity, similar to SDE1, which converts foreign genetic element (RNA) to dsRNA that is diced to produce siRNA for silencing the RNA concerned by RNAi (Dalmay et al., 2001). The phenomena is, however, not restricted only to the foreign genetic element. Endogenous mRNAs are also silenced by trans-acting-siRNAs (ta-siRNAs) and nat-siRNAs for which dsRNAs are to be produced by RdRP (William et al., 2011; Fei et al., 2013). Silencing of the coding genes, like of *PPR*, *NB-LRR* and *MYB* families, is resulted in by the formation of phased secondary siRNAs after initial action of miRNAs (Fei et al., 2013). The increase in transcript abundance of *RNAh-SDE3* as a result of decrease in abundance of the novel miRNA osa-miR12470 could be to silence the undesired gene products through the RNAi mechanism facilitated by ta-siRNAa and nat-siRNAs. The degradation of nucleic acids in turn must be necessitating their fresh synthesis. Nucleosides are the major components of nucleic acids, but their synthesis is restricted to selected cell types, and being hydrophilic, their efficient transport across the plasma membrane is mediated by two membrane proteins, concentrative nucleoside transporter and equilibrative nucleoside transporter, ENT (Molina-Arcas et al., 2009). The increase in transcription of *ENT* in response to the PEG treatment in this study (Fig. 7), mediated by decrease in abundance of the novel miRNA osa-miR12473 indicated the need of enhanced nucleic acid synthesis under drought stress, or abiotic stress in general. The confirmation of *RNAh-SDE3* and *ENT* as the targets of miR12470 and miR12473, respectively by 5′RACE PCR (Fig. 8), and that of *ENT* additionally by luciferase assay (Fig. 9) supports the involvement of miRNAs in synthesis and degradation of endogenous genetic material. Significant increase in abundance of the novel miRNA osa-miR12472 (Fig. 6) concomitant with significant decrease in transcript abundance of C3HC4 zinc finger (ZF) type RING finger (Fig. 7) supports negative relationship between the miRNA cellular abundance and the target mRNA level, and because most RING finger proteins are E3 ubiquitin ligase (Wu et al., 2014), a decrease in degradation of proteins under PEG treatment could be expected. PEG-induced decrease in cellular abundance of many conserved miRNAs targeting F-box protein of 26S proteosome system (Figs. 2 and 3), however, indicated considerable increase in protein degradation activity rather than decrease, which in fact might be necessary to cope with the changing metabolism in response to the stress.

5. Conclusion

The study thus led to identification of several drought (PEG)-responsive novel and conserved miRNAs, and presence of several of them could be validated successfully by Northern blot and Mir-X PCR. Family-wise cellular abundance study of the miRNA members revealed substantial difference in their response to the short term (9 h) 20% PEG treatment ($\sim IC_{50}$) in root and shoot, indicating tissue-specific response. Decrease in abundance of the miRNAs targeting homeodomain leucine zipper (HD-Zip), MADS-box family protein, cyclin A3, C2H2 zinc finger protein (ZnF), guanine nucleotide exchange factor (GEF), GTPase activating protein (GAP), EF hand protein, LRR protein, and Myb transcription factor even in response to short term exposure to 20% PEG treatment suggested that these could be important for drought stress tolerance in the plant. The evidence for their involvement in drought tolerance also comes from the fact that plants overexpressing genes like *HD-Zip*, *MADS*-box protein, *ZnF*, *EF* hand protein, *LRR* proteins and *Myb* TF are reportedly better tolerant to drought compared with the wild type (Wang et al., 2013; Dezar et al., 2005; Zhao et al., 2014; Zhang et al., 2016; Yin et al., 2017; Kang et al., 2017; Jung et al., 2008). The involvement of G protein signalling in drought tolerance was also visualized because of decrease in abundance of the miRNAs targeting GEF and GAP in response to the PEG treatment. Furthermore, the study showed possibility of increase in dynamics of protein degradation and synthesis under drought stress, as the cellular abundance of many miRNAs targeting F-box protein, a component of 26S proteasome system, decreased. The increase in protein degradation probably also accompanied an increase in degradation of nucleic acids, as revealed by decrease in abundance of osa-miR12470, a novel miRNA (Fig. 6), targeting *RNAh-SDE3* (Fig. 7), a RdRP enzyme initiating RNA silencing by RNAi. Together with these, an increase in transcription of *ENT* in response to the PEG treatment (Fig. 7) also suggested an increase in synthesis of nucleic acid. The picture that emerges from this short term PEG exposure study is that the plant initially tries to get rid of unwanted proteins and nucleic acids under the changing water stress conditions induced by the PEG treatment to give space to the proteins and nucleic acids necessary under the initial condition of the stress. The study also suggested that the drought tolerance may have another angle, i.e. may be mediated by the plant hormone ethylene, as is reflected from the decrease in abundance of the miRNAs (Fig. 2) targeting ethylene biosynthesizing enzyme ACO. The view stems from the fact that the hormone is well known to induce transcription of the transcription factors like *ERFs* and *DREBs*, which regulate the transcription of a number of genes contributing to abiotic stress tolerance in plants (Muller and Munne-Bosch, 2015; Mizoi et al., 2012; Panda et al., 2016). The regulation of tissue level of IAA might also be equally important in the drought tolerance process, as is reflected from the increase in transcript abundance of Indol-3-acetate beta-glucosyl transferase and decrease in cellular abundance of osa-miR12471 targeting the enzyme under drought stress. Together, the study suggested that a complex lot of biochemical machineries/processes participate to enable plants to overcome the challenge of drought stress, and miRNAs plays important role in it. A clear understanding of regulatory role of miRNAs, the master regulator of the biochemical processes, in drought tolerance in plant may allow introduction of the trait in the species of interest by manipulation of biosynthesis of the key miRNAs.

Author contributions

BPS conceived the work. BPS and TC designed the experiment. JPA and TC carried out most of the wet lab work. SM and TC carried out the luciferase assay. JPA and SS did screening of the rice cultivar for drought tolerance and finalized the PEG treatment concentration. PDN and NKC did sRNA sequencing and bioinformatics. BPS wrote the manuscript. BPS and SKP jointly revised the manuscript.

Conflicts of interest

The authors declare that they have no conflict of interests and non of the organizations supporting the work by the way of salary, laboratory facilities and research grant would loose or gain financially by publication of this manuscript.

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Appendix A. Supplementary data

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