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Identification of Tissue-Preferential Expression Patterns of Rice miRNAs

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ABSTRACT

It is imperative to understand the mechanisms of growth and development in higher plants for improving plant adaptation during different developmental stages. Plant microRNAs (miRs) play crucial regulatory roles in various developmental processes. As many as 15 miR families having multiple members are known to regulate plant development, yet the spatio-temporal expression patterns of individual members are not fully characterized. It is likely that different members of miR families can make specific contributions to the spatio-temporal control of targets. To understand the functional complexity of miRs and the amount of degeneracy existing in miR-mediated regulation of differentiated but developing tissues, we have identified the Osa-miR-sequences that are expressed in specific tissues. We adopted the approach of comparative miR profiling using next-generation sequencing technology followed by experimental validation. It was observed that 59 Osa-miR-sequences show tissue-preferential expression in local basmati rice variety; while 126 miRs belonging to 81 families are differentially regulated in these tissues. The 21 nt miRs were predominant in all tissues, but the 24 nt miRs were the most abundantly expressed. This indicates that target cleavage and chromatin state regulation are involved in organ development. This study also identified the expression patterns of individual members of Osa-miR families that were common and divergent between the indica and japonica rice varieties. The expression patterns of the predicted targets were also analyzed. The possible implications of the miR distribution patterns with respect to the regulation of their respective targets are discussed. J. Cell. Biochem. 114: 2071–2081, 2013.

KEY WORDS: microRNA; DEVELOPMENT; ILLUMINA SEQUENCING; *Oryza sativa*; TARGET GENES; EXPRESSION PATTERNS

The discovery of 19–24 nt long RNAs has defined a new regulation module within living cells that operates at both transcriptional and post-transcriptional levels. The microRNAs (miRs) represent an important class of these small regulatory molecules, which are involved in regulating the normal growth of cells, development of organisms and maintaining the integrity of genomes.

The miRs are transcribed as long primary transcripts (pri-miRs) from endogenous gene loci. The pri-miRs are processed, by a defined pathway mediated by DICER-like proteins, into the hairpin intermediates called precursor-miRNAs (pre-miRs) that are subsequently cleaved into 20–24 nt long duplexes. The mature miR is loaded into the ARGONAUTE protein-containing complex to form RNA induced silencing complex (RISC) [Baumberger and Baulcombe, 2005; Kim, 2005; Henderson et al., 2006; Voinnet, 2009] and targets the complementary transcripts to negatively regulate gene expression by repressing translation or degrading the targeted mRNAs [Dugas and Bartel, 2004; Bentwich, 2005].

As a class, miRs control various fundamental biological processes in plants like growth, organogenesis, pattern formation, organ polarity, hormone homeostasis, signal transduction, and hormone responses [Palatnik et al., 2003; Dugas and Bartel, 2004; Jones-Rhoades et al., 2006; Mallory and Vaucheret, 2006; Sanan-Mishra and Mukherjee, 2007; Voinnet, 2009]. Recently a role for miRs has been indicated in response to diseases and different environmental stresses [Sunkar and Zhu, 2004; Fujii et al., 2005; Ruiz-Ferrer and Voinnet, 2009; Sanan-Mishra et al., 2009; Pérez-Quintero et al., 2010].

The defective phenotypes or mutants of the miR biogenesis pathways and the identification of miR-targets have highlighted the role of miRs in regulating various aspects of morphogenesis [Laufs

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et al., 2004; Zhong and Ye, 2004; Millar and Gubler, 2005; Ori et al., 2007; Chen et al., 2010; Rubio-Somoza and Weigel, 2011]. At-miR165/miR166 is involved in organ axis specification, vascular development, and meristem function [Mallory et al., 2004; Li et al., 2005] while At-miR159/JAW controls cell division during leaf development. Juarez et al. [2004] reported Zm-miR166 as a mobile signal in leaf development in maize and Sly-miR319 was found to control leaf complexity in tomato [Ori et al., 2007]. These miRs regulate pattern and development of organs by targeting the transcription factor genes like HD-ZIP and TCP. At-miR164 controls the expression of the NAM/ATAF/CUC (NAC)-domain transcription factors; CUP-SHAPED COTYLEDONS (CUC-1, CUC-2) and SHOOT MERISTEMLESS1 (STM1) to regulate embryonic and floral meristem development, aerial organ boundaries, and leaf senescence [Aida et al., 1999; Laufs et al., 2004; Wang et al., 2004; Guo et al., 2005]. At-miR156 is required for the down-regulation of the SQUAMOSA PROMOTER BINDING LIKE (SPL) family members, which suppress flowering in Arabidopsis [Xie et al., 2012]. At-miR172 is involved in the regulation of AP2 and AP2-like proteins (TARGET OF EAT 1, TOE1), which are required for flowering and floral organ identity [Zhao et al., 2007; Wollmann and Weigel, 2010].

In recent years, an increasing number of miRs have been identified and functionally characterized from various plant species. Presently, 4,677 plant miRs from 52 species are listed in miRBase (release 18.0) of which 667 sequences have been reported from rice. Around 30 Osa-miR families are conserved across plant species and as many as 15 conserved Osa-miR families are known to regulate plant development by restricting accumulation of transcripts encoding crucial transcription factors [Jones-Rhoades et al., 2002; Llave et al., 2002; Mallory and Vaucheret, 2006; Axtell and Bowman, 2008].

The abundance of plant miR genes and their intriguing expression patterns in different physiologies or in different stages of development has been captured by microarray [Sato et al., 2011] and high throughput sequencing. This has allowed massive analysis of rice small RNAs [Sunkar et al., 2008; Zhu et al., 2008; Xue et al., 2009; Jeong et al., 2011] to study their dynamic expression patterns [Jeong et al., 2011; Wei et al., 2011]. It has been observed that members of the same miR family are differentially regulated in different organs indicating that these can make specific contributions in target regulation. It will therefore be interesting to check if the miR family members are operative in a defined spatio-temporal zone and whether variation in sequences of distinct miRs influence target regulation differentially. It will be important to check if expression patterns of members of the conserved families are also conserved across species.

In this work, global expression patterns of established *Oryza sativa* miRs (Osa-miRs) in the seedling leaves, seedling roots, and pre-pollinated flowers from early panicle stages, of indica rice variety Pusa basmati, were surveyed in an attempt to identify specific members of the miR families that have the preferential expression patterns in the young tissues. To follow the networks of these miRs in fine-tuning the growth and establishment of rice plants, different cDNA libraries were sequenced using the Illumina platform to compare the global expression status of the known rice miRs. The digital comparisons based on the sequencing data were experimentally validated using miR-arrays, northern analysis and RT-PCR. Our results revealed that members of 21, 7, and 31 Osa-miR families are specifically expressed across leaf, root and flower tissues, respectively while 126 Osa-miRs belonging to 81 families are differentially regulated in these tissues. The expression profiles of selected molecules were studied in detail across different development stages of the tissues. Enrichment analysis of these miRs and their targets helped identify the regulatory nodes operative in specific tissues under normal growing conditions, thus paving the way for further functional characterization of these miRs for understanding the tissue development in that region.

MATERIALS AND METHODS

PLANT MATERIALS AND GROWTH CONDITIONS

Seeds of Rice *var. Pusa Basmati* were surface sterilized with a solution of 10% commercial bleach (0.525% sodium hypochlorite) for 5 min, washed thoroughly with sterile water and placed on germinating sheets. The seeds were grown under controlled conditions, temperature (28 ± 2°C), relative air humidity (70%), and 16/8-h light/dark cycle. For further analysis, leaf and root tissue samples were harvested from 15 days old seedlings while pre-pollinated flower tissues from emerging panicles were collected from rice plants grown in a controlled green-house at 28 ± 2°C with 16 h of light.

RNA ISOLATION

Total RNA was extracted using guanidine iso-thiocyanate (GITC) protocol as described earlier [Sanan-Mishra et al., 2009] with modifications. For small RNA enrichment, high-molecular-weight RNA was precipitated out with 50% PEG 8000 in 5 M NaCl unless specified. The small RNA pool in the resultant supernatant was precipitated using ethanol, quantified and used as required.

PREPARATION OF CDNA LIBRARIES FOR DEEP SEQUENCING

The small RNA libraries were constructed as described. Briefly, low molecular weight RNA was enriched from 200 µg of total RNA by LiCl precipitation. Small RNAs in the size range of 18–30 nucleotides were fractionated using 15% denaturing PAGE. Samples from three different biological replicates were pooled and ligated first with the 5′ RNA adaptor (Illumina) and then with the 3′-RNA adaptor (Illumina). In each step, the ligated products were purified on 15% and 10% PAGE, respectively. After first strand synthesis, the cDNAs were amplified using adaptor specific primers in a PCR amplification of 15 cycles, each consisting of three steps: 10 s at 98°C, 30 s at 60°C, and 15 s at 72°C. The amplified products were purified using 6% PAGE and their quality check was performed on bioanalyzer (Agilent). The libraries thus made were used for deep sequencing on GAII sequencer (Illumina).

NORTHERN BLOT ANALYSIS

Total RNA (15 µg) or enriched RNA (25 µg) was resolved on a 15% denaturing PAGE and subsequently transferred to Hybond-N^+ membranes (GE Healthcare) using trans-blot semidry transfer cell (Bio-Rad). To generate specific miR detection probe, DNA oligonucleotides with perfect complementarity to the miR sequences were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. The

membranes were hybridized with a ^{32}P-labeled probe in a solution of 5× SSC, 0.01% heat-denatured salmon sperm DNA, 5× Denhart's solution, 5% Dextran sulfate, 0.05 M Sodium phosphate, 2.5 mM EDTA, and 0.4% SDS at 37°C for 24 h. The membranes were finally washed twice in 2× SSC, 0.5% SDS at 37°C, then exposed to phosphor screens for 24 h and visualized with a Typhoon Phospho-Imager (GE Healthcare). Biological replicates were probed and each probing was repeated thrice.

ANALYSIS OF THE SEQUENCING DATA

Deep sequencing on Illumina GA II resulted in approx. 11M tags per library. The data was delivered as sequences of 33 or 35 bases in length along with the base quality scores and read counts. Each sequence included variable amount (complete or in small parts) of the 3′-sequencing adaptor depending upon the length of the small RNA. The obtained raw sequence reads were processed computationally to remove the 3′-adapter sequences before further analysis. To identify the Osa-miRs from the sequenced small RNA pool, the rice datasets were compared against the rice sequences available from miRBase sequence database (Release 18.0) using local BLAST. Only those sequences showing 100% match to Osa-miRs were selected. The remaining sequences were processed and formatted for further analysis. The digital expression status of Osa-miRs within each data-set was generated by calculating the transcript per million (TPM) values for each selected sequence and comparing them across data sets.

TARGET PREDICTION AND REGULATORY NETWORK MODELING

To obtain potential gene targets for osa-miRs, the online tool psRNATarget [http://bioinfo3.noble.org/psRNATarget/; Dai and Zhao, 2011] was used to search for mRNA sequences complementary to miR sequences in TIGR Rice Genome mRNA (OSA release 5.0) data. Default parameters were used for prediction but mRNA sequences with a score of $\leq$3 were considered as potential targets. Transcripts targeted by tissue-preferential miRs were also obtained from PMRD [www.bioinformatics.cau.edu.cn/PMRD/] database. Enrichment analysis of tissue-preferential miR and its target was done by calculating the number of target transcripts for miR and number of targeting miR for transcripts. Cytoscape v 2.8.3 was used to model and visualize the miR–gene interaction and enrichment output.

SEMI QUANTITATIVE REVERSE TRANSCRIPTION-PCR

Total RNA from seedling leaf, seedling root, and pre-pollinated flower of Pusa Basmati was prepared using miRNeasy mini kit (Qiagen). Two micrograms of total RNA was used for first-strand cDNA synthesis using Superscript II (Invitrogen). PCR amplification (26–32 cycles) of cDNA was performed using specific primers to selectively amplify genes. Actin gene was also amplified and used as an internal control. PCR reaction conditions were standardized and the RT conditions for amplification of actin were as follows: 94°C for 2 min, (94°C for 30 s, 55–65°C for 30 s, 72°C for 30 s) × 25 cycles, and 72°C for 5 min.

MICROARRAY ASSAY

Total RNA isolated from the leaf, root and flowers obtained from rice seedlings were custom analyzed for global miR expression on miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which contains capture probes targeting miRs deposited on Sanger's database (Release 12.0). The hybridization was performed according to the miRCURY™ LNA array manual using a Tecan HS4800 hybridization station (Tecan, Austria).

RESULTS AND DISCUSSION

ANALYSIS OF RICE SMALL RNA SEQUENCES

To analyze the global expression patterns of rice miRs, small RNA libraries were prepared from the seedling leaves, seedling roots, and pre-pollinated flowers of local basmati (indica) rice, representing a comprehensive range of different young developmental stages. The libraries were deep sequenced on GAII sequencer (Illumina). Individual sequence reads along with their read counts were produced and further processed (see Materials and Methods Section). A total of 17458888, 5490031, and 6973783 small RNA raw reads were generated, consisting of 3632582, 1974196, and 3195276 unique sequences for the leaf, root, and pre-pollinated flower samples, respectively (Fig. 1). Our small RNA sequencing results showed that pre-pollinated flower library had the highest number of unique sequence tags as compared to leaf and root library (Fig. 1). The higher number of small RNA sequences in the floral tissues indicates a crucial role for small RNA guided regulons in the developing reproductive tissues [Wollmann and Weigel, 2010].

The preliminary processing of sequencing data sets revealed sequences of length ranging from 18 to 26 nt, with a predominance of 21–24 nt species (Fig. 2A). The 24 nt small RNAs emerged as the largest and the most diverse class across the three tissues followed by the 21 nt and 22 nt small RNAs. This pattern was consistent with earlier observations of the deep-sequencing data obtained from Japonica rice panicles [Jeong et al., 2011] and arabidopsis inflorescences [Kasschau et al., 2007]. It is proposed that the 24-nt RNA population consists mainly of siRNAs that may be required for the initiation and maintenance of DNA methylation at the repeat elements, including transposons and retro-elements [Groszmann et al., 2011; Hollister et al., 2011].

In our deep sequencing data sets, 242 known rice "miR-sequences" (as listed in the miRBase Sequence Database, Release 18.0) were identified (Supplementary Tables S1 and S2). The term "miR-sequence" is being used to refer to all the mature miRs that share exactly same sequence, for instance, the sequence UGCCAAAGGA-GAATTGCCCTG that is shared by miRs 399a, 399b, and 399c is regarded as a single miR-sequence. The database of rice available at miRBase (release 18.0) contains 467 miR-sequences ranging from 19 to 24 nt in length, representing 667 miRs. Among these sequences, the major category is that of the canonical 21 nt miRs that are known to regulate post-transcriptional gene expression. This is followed by the 22 nt and 24 nt miRs that have been implicated in siRNA production from target RNAs [Cuperus et al., 2010] and DNA methylation [Wu et al., 2010], respectively. We studied the size distribution of known Osa-miR-sequences present in our sequencing datasets, irrespective of their read frequencies (Fig. 2B). The 21-nt species represented a substantial number with more than 50% sequences belonging to this class. The 24 nt species emerged as the second dominant class with 35.9% sequences, while only 9.3% sequences belonged to the 22 nt species. This size distribution pattern was uniform across the three

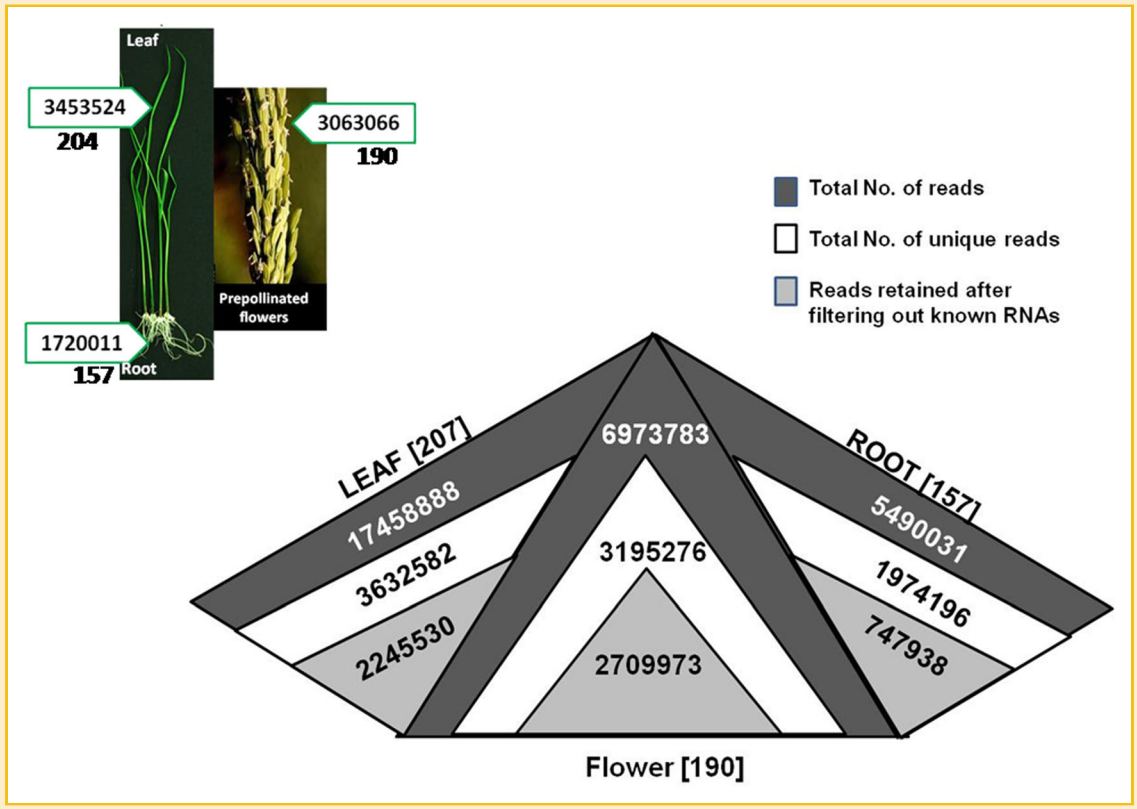

Fig. 1. Schematic representation of small RNA data analysis. Each section represents the seedling leaf, seedling root, and pre-pollinated flower tissues. It depicts the total number of reads obtained, the number of unique reads and reads retained after filtering out the known Osa-miR-sequences. The total number of unique Osa-miR-sequences identified in each tissue is provided within the brackets next to tissue name. Inset shows the total number of reads representing the foldable-sequences obtained from the seedling leaf, seedling root and pre-pollinated flower tissues. Leaf and root were harvested at the 14 days old stage and flowers were harvested at the mature stage when the panicles were ~15 cm in length.

tissues and is similar to earlier observations on the rice miRs [Zhu et al., 2008; Jeong et al., 2011].

However, on analyzing the cumulative expression status of the different size-category of miRs, by considering the total read frequencies, it emerged that the 20 nt miR-sequences are abundantly expressed in the leaf and the root while 21 nt miR-sequences have higher expression levels in the flower libraries (Fig. 2C). The observation that 20 nt miRs are expressed as the most abundant form suggests an important role being played by these miRs in plant development. This raises the possibility that miRs may be processed as different size variants. Although, larger derivatives (21 or 22 nt) of some of the 20 nt miRs were present in the deep sequencing data sets (Supplementary Fig. S1) it is not clear whether both the forms are functional during tissue development. It is established that most plant pre-miRs are processed by DCL1 activity to produce 21 nt miR:miR* duplexes, while DCL4, is known to produce 22 nt miRs [Wu et al., 2010]. Till date there is no report on the biogenesis and function of the 20 nt miRs. In rice, at least eight different DCLs have been reported [Kapoor et al., 2008] and it is likely that the other DCL isoforms may be involved in the processing of different size miRs.

To use the digital data obtained from deep sequencing as indicative miR expression signatures across tissues, it was required to confirm the consistency of the sequences and read frequencies obtained. So, three leaf small RNA libraries were prepared and sequenced, independently. From these datasets 178, 169, and 171 known Osa-miR-sequences could be identified (Supplementary Table S1). On comparing the digital expression status, 171 Osa-miR-sequences were found to be present in at least two datasets, with read frequencies ranging from approx. 1100000 to 1. However, the frequency of each of the Osa-miR-sequence was similar across libraries (Supplementary Table S1).

DIGITAL COMPARISON OF OSA-MIR-SEQUENCES ACROSS TISSUES

The digital data obtained from seedling leaf, seedling root, and pre-pollinated flowers of Pusa basmati rice was compared and analyzed to identify the expression patterns of Osa-miR-sequences available at miRBase (Release 18.0). For this comparison, the read values were normalized by obtaining the TPM values for each sequence within the library (Supplementary Table S2).

About 126 Osa-miR-sequences belonging to 81 different families could be identified across all the three tissues (Fig. 3) although with a different digital expression profile. It was observed that 38 Osa-miR-sequences belonging to 32 different families were absent from the leaf library, 52 Osa-miR-sequences belonging to 47 different families were absent from the floral library and 85 Osa-miR-sequences belonging to 72 different families were absent from the root library (Fig. 3; Supplementary Table S2).

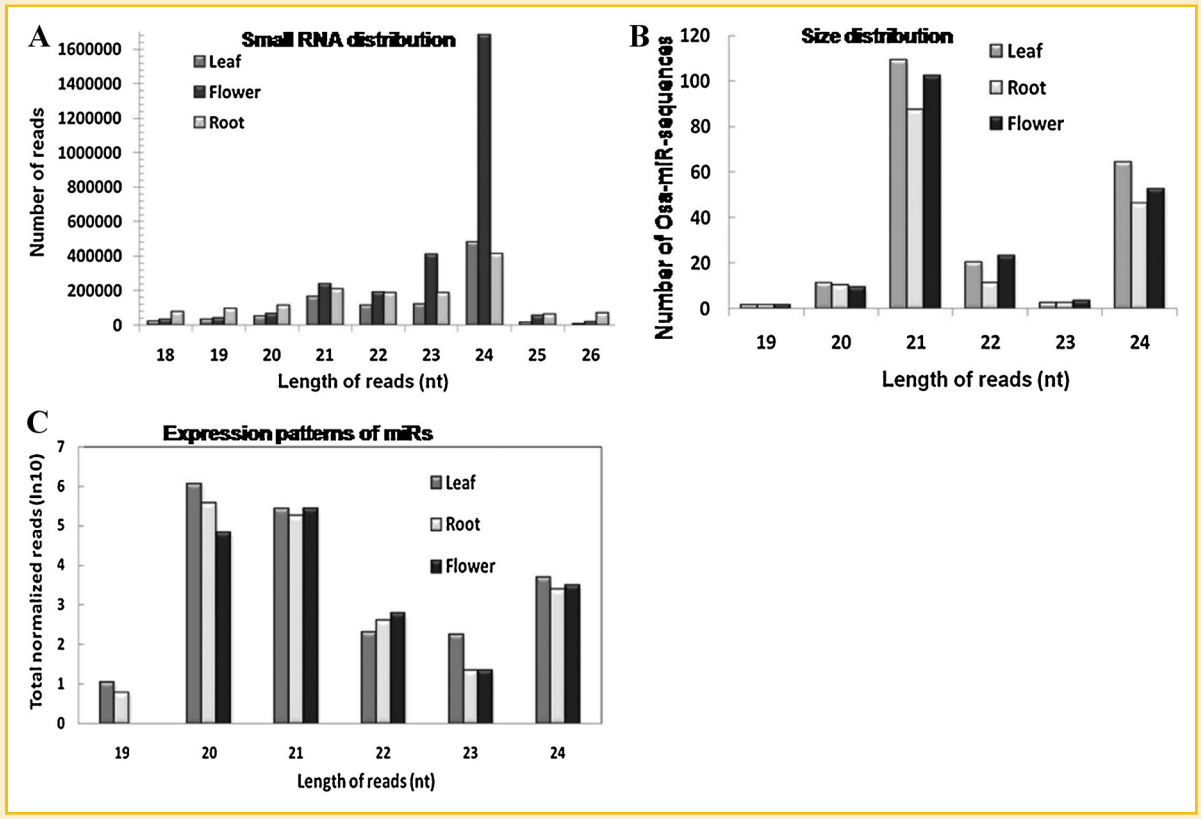

Fig. 2. Size distribution and abundance of small RNA and Osa-miR-sequences across different tissues. Small RNA libraries from seedling leaf, seedling root and pre-pollinated flowers were sequenced on Illumina platform. A: The size distribution of individual sequence tags across tissues. B: The size distribution of unique Osa-miR-sequences distributed across the three libraries irrespective of their read frequencies. C: The total normalized read frequencies of each size class of Osa-miR-sequence across the three libraries of small RNAs is plotted. The Osa-miR-sequences were filtered from the normalized data and the cumulative read frequencies of each size class was plotted.

Literature survey has shown that the expression of miRs varies from ubiquitous to temporal or tissue-specific [Llave et al., 2002; Aukerman and Sakai, 2003; Sunkar and Zhu, 2004; Jones-Rhoades et al., 2006]. A detailed analysis of our data sets revealed that majority of the known Osa-miRs were ubiquitously expressed but specific members of the Osa-miR families were expressed in a tissue-preferential manner (Table I). It was observed that 21 Osa-miR-sequences that represent 42 individual Osa-miRs were present specifically in the leaf libraries. Similarly, 7 Osa-miR-sequences (representing 15 Osa-miRs) were specific to the root libraries while 31 Osa-miR-sequences (representing 36 Osa-miRs) were specific to the pre-pollinated flower library (Fig. 3, Table I). Additionally, 26 leaf Osa-miR-sequences (representing 41 Osa-miRs) were over-expressed in flowers, 15 leaf Osa-miR-sequences (representing 21 Osa-miRs) were over-expressed in roots. It was observed that among the Osa-miR-sequences that exhibit a tissue-preferential expression, the 21 nt Osa-miR-sequences were predominant (Supplementary Fig. S2). This was followed by the 24 nt Osa-miR-sequences in the seedling leaf and root tissues and the 22 nt Osa-miR-sequences in the flowers (Supplementary Fig. S2). This might indicate a specific role for 24 nt miR mediated DNA methylation in leaf and root development and that of 22 nt miR mediated tasiRNA generation in the flower development.

It was intriguing to observe that the expression profiles of the Osa-miR-sequences behaved differently between the indica and japonica species [Jeong et al., 2011]. Osa-miR-444a, Osa-miR-1318, Osa-miR-1850 which have been shown to have a root-preferential expression in japonica rice variety [Jeong et al., 2011] were present in all three indica rice data sets (Supplementary Table S2) while the

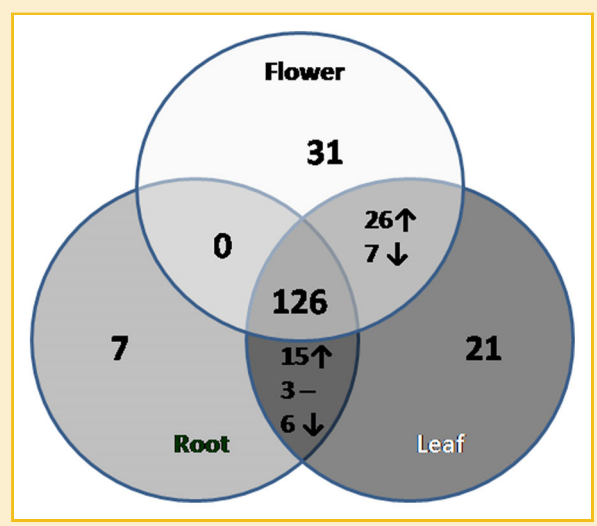

Fig. 3. Venn diagram to represent the expression domains of Osa-miR-sequences in different tissues. The number of miRs in each expression zone is indicated.

TABLE I. List of Osa-miR-Sequences that are Expressed in a Tissue-Preferential Manner

Leaf	Root	Flower
miR1428e_3p	miR1882a-h (miR1317_5p.2)*	miR169d
miR169f.1, g	miR3979_5p	miR1852
miR1848	**miR397b**	miR1857_5p
miR1877	miR3981_3p	miR1859
miR2123a-c	miR5082	miR2094_3p
miR2125	miR5152_3p	miR2118c,q
miR2872	**miR810a**	miR2118d
miR2875		miR2118f,j,m
miR2879		miR2118h-k
miR393b		miR2118p
miR395b,d,e,g-n,p-s,y		miR2275a,b
miR398b		miR2275c
miR399a-c		miR399h
miR5147		miR399j
miR5151		miR5162
miR5157a_3p,b_3p		**miR529a**
miR530_5p		miR5486
miR5500		miR5490
miR5501		miR5492
miR5504		miR5495
miR812o_5p		miR5496
		miR5499
		miR5509
		miR5517
		miR5518
		miR5521
		miR5522
		miR5525
		miR5530
		miR5534a
		miR5539

* miR1317_5p.2 is grouped together with miR1882 as they have similar sequences.

shoot-preferential Osa-miR-812p and Osa-miR-5804 were not present in any of the indica data sets. Similarly, all the flower-preferential miRs, namely Osa-miR-5485, Osa-miR-5789, Osa-miR-5793, Osa-miR-818f, that are reported in Japonica rice [Jeong et al., 2011], were absent from the indica rice data sets. However, specific molecules exhibited conserved expression zones between the indica and japonica varieties, like the expression of Osa-miR-3979 was specific to roots, expression of Osa-miR-529a was specific to flowers and expression of Osa-miR-395(b-e,g-n,p-s) was specific to leaves. This suggests a divergence in the regulation of individual miRs between the rice varieties. Most of the Osa-miR-sequences exhibiting a tissue-preferential expression pattern are not conserved in Arabidopsis and may represent a class of newly evolved molecules specific to rice.

GLOBAL EXPRESSION PROFILING

To validate the changes in the global miR expression profiles obtained from the digital data, rice miR-microarray was used with the total RNA isolated from leaf, root, and flower tissues. 98 Osa-miRs belonging to 54 families were differentially expressed between the leaf and root tissues the on the array (Fig. 4A, Supplementary Table S3). It was observed that 41 Osa-miRs were up-regulated in leaf tissues while 50 Osa-miRs were up-regulated in root. Osa-miRs belonging to 71 families were differentially expressed between the leaf and flower tissues the on the array (Fig. 4B, Supplementary Table S3). It was observed that 36 Osa-miRs were up-regulated in leaf

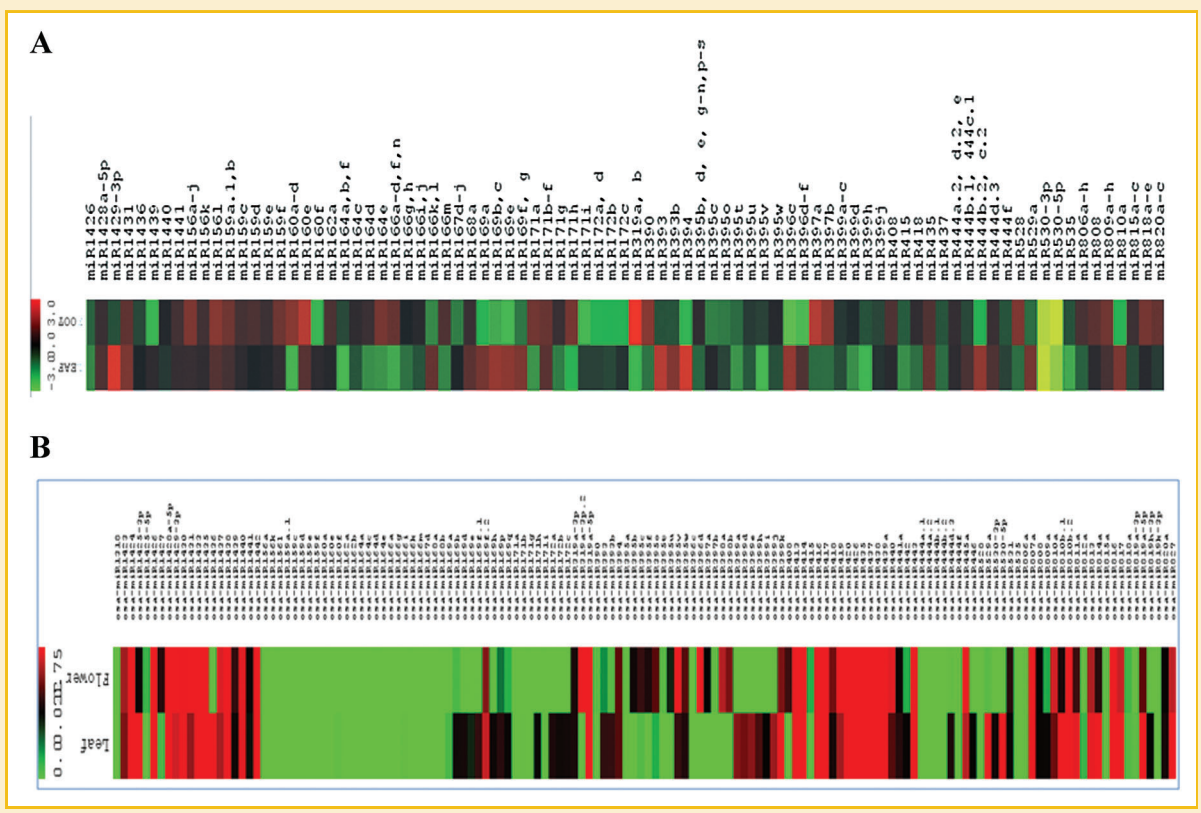

Fig. 4. Heat map obtained from the microarray data to show the differentially expression of Osa-miRs. A: leaf and root samples and (B) leaf and flower samples. The color scale shown at the left part illustrates the relative expression level of miRNA: shades of red represents an expression level above mean, shades of green represents expression lower than the mean. The clustering is performed on log2 [Hy3/Hy5] ratio, which passed the filtering criteria on variation across samples.

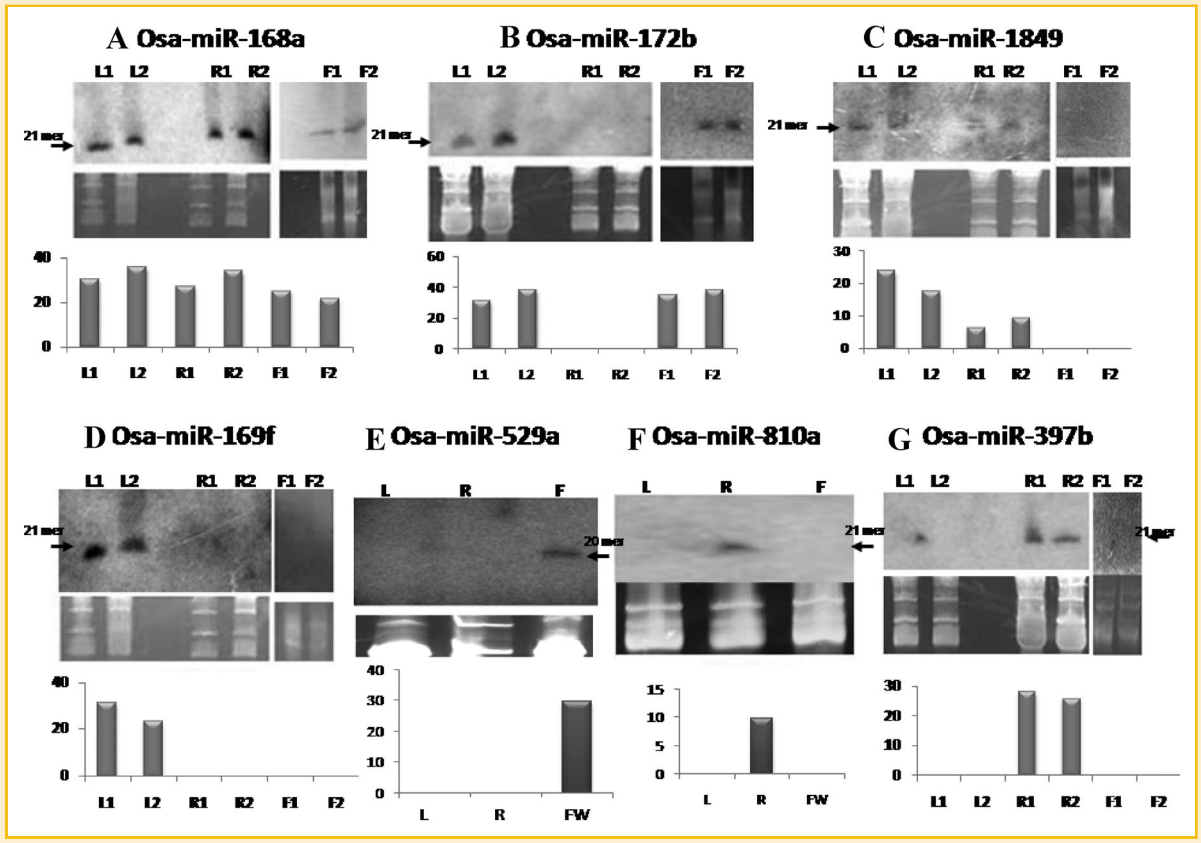

Fig. 5. Northern analysis of selected Osa-miRs showing low expression reads in the deep sequencing data. The position and intensity of the hybridized bands obtained with Osa-miR-168a; Osa-miR-172b; Osa-miR-1849; Osa-miR-169f; Osa-miR-529a; Osa-miR-810a; and Osa-miR-397b are shown. The top panel shows the miR bands on blots prepared with (A) total RNA blots and (B–E) enriched RNA blots obtained from leaves (L) and roots [R] of 15-day-old seedlings and pre-pollinated flowers [F]. Each sample was loaded at two different concentrations. 1 and 2 indicates loading of 15 μg and 25 μg RNA, respectively. Middle panel shows the 5S rRNA bands stained by ethidium bromide. Bottom panel shows the plot of the normalized radioactive band intensities for each lane.

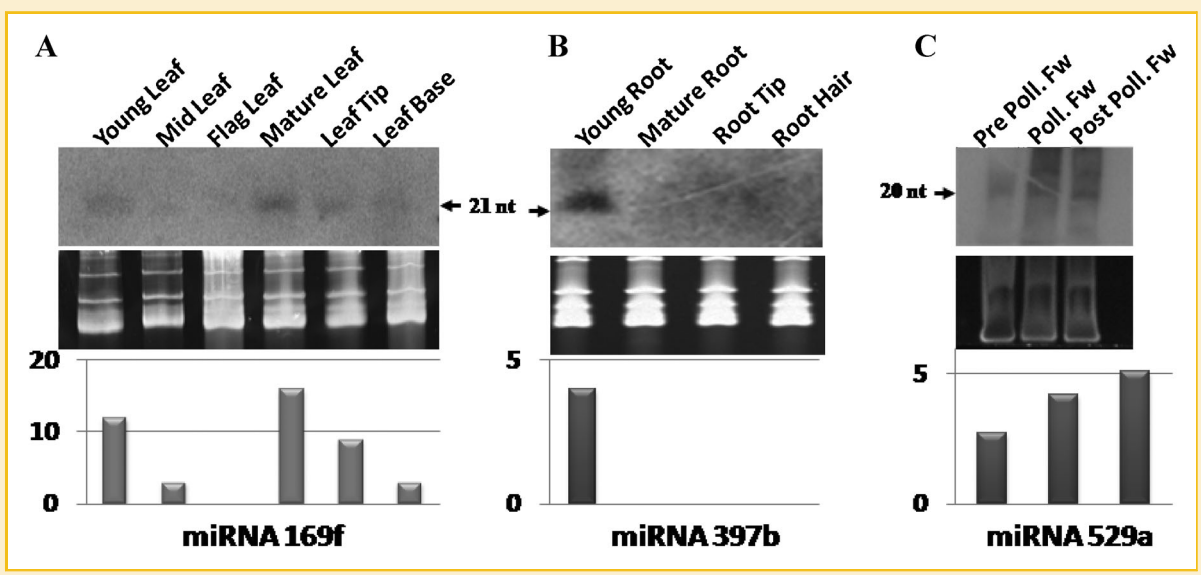

Fig. 6. Spatial expression patterns of tissue-preferential Osa-miRs. Enriched RNA (15 μg) isolated from different parts of the leaf, root and flower (as mentioned) were northern blotted and probed with end-labeled oligonucleotides complimentary to the respective miR sequences. The tRNA and 5S rRNA bands were visualized by ethidium bromide staining of polyacrylamide gels and served as loading controls. Bottom panel shows the plot of the normalized radioactive band intensities for each lane.

tissues while 50 Osa-miRs were up-regulated in flowers. The expression profiles of at least 26 Osa-miRs, that showed variation across leaf and root tissues and 43 Osa-miRs that showed variation across leaf and flower, on the array correlated with the digital expression profiles captured on the deep sequencing data. These included a large number of individual sequences that had relatively low read frequencies in the sequencing (Illumina) data.

It was observed that the individual miRs that were identified in specific libraries, on the basis of digital data analysis, also showed a tissue-preferential expression pattern on the miR-microarray. The expression patterns for a few of these Osa-miR-sequences were confirmed by northern blot analysis. Thus, we were able to identify 59 Osa-miR sequences that are expressed in a tissue-preferential manner (Table I).

TISSUE-PREFERENTIAL EXPRESSION PATTERNS: TRUE MIRS OR NOISE?

We observed that most of the miRs that were identified as having tissue-preferential expressions had TPM values ≤ 0.5. It was therefore required to verify whether the low frequency read miR-sequences were picked up as a result of system noise or they represented the true expression states. The expression profiles of a few sequences representing the less abundant reads (TPM < 0.3) were confirmed by northern analysis using specific probes. These included the tissue-preferential Osa-miR-169f, Osa-miR-172b, Osa-miR-397b, and Osa-miR-529a representing the conserved miRs and Osa-miR-1849 and Osa-miR-810a representing the rice-specific miRs. These miRs could be detected on small RNA enriched blots following a long exposure. For this analysis Osa-miR-168a (TPM 27815.74992) was used as experimental positive control and this could be easily detected on the total RNA blots within 2 h after exposure (Fig. 5A). The northern results thus corroborated that the low read frequency transcripts represent the low copy miRs (Fig. 5B–G, Supplementary Table S2).

ASSOCIATING MIR EXPRESSION PATTERNS TO SPECIFIC TISSUES

The expression domains of tissue-preferential Osa-miR-169f, Osa-miR-397b, and Osa-miR-529a were checked within the respective leaf, root, and flower tissues (Fig. 6). It was observed that within the seedling leaf expression of Osa-miR-169f was more in leaf tip as compared to the middle and basal region (Fig. 6A). Moreover, higher amounts Osa-miR-169f were present in the mature leaf as compared to seedling leaf while it could not be detected in the flag leaf tissues (Fig. 6A). The Osa-miR-397b could be detected only in the young roots and not in the mature roots, root tips and root hairs (Fig. 6B). Expression of flower-preferential Osa-miR-529a increased as the flowers matured (Fig. 6C).

CORRELATING THE TARGET AND MIR EXPRESSION PATTERNS

Plant miRs show high sequence complementarity to their target transcripts that leads to the cleavage of the targets. Thus, miRs and their targets are expected to show antagonistic expression levels. To check whether this correlation exists between the tissue-preferential Osa-miRs and their predicted targets, semi-quantitative reverse transcription-PCR analysis of selected molecules was performed (Fig. 7). The miR-target expression antagonism was observed for flower-preferential Osa-miR-399h and Osa-miR-2094-3p and their predicted targets (Fig. 7A). The root-preferential Osa-miR-397b and Osa-miR-810a also showed antagonistic expression levels with its predicted targets Laccase precursor and AT-Hook Protein 1,

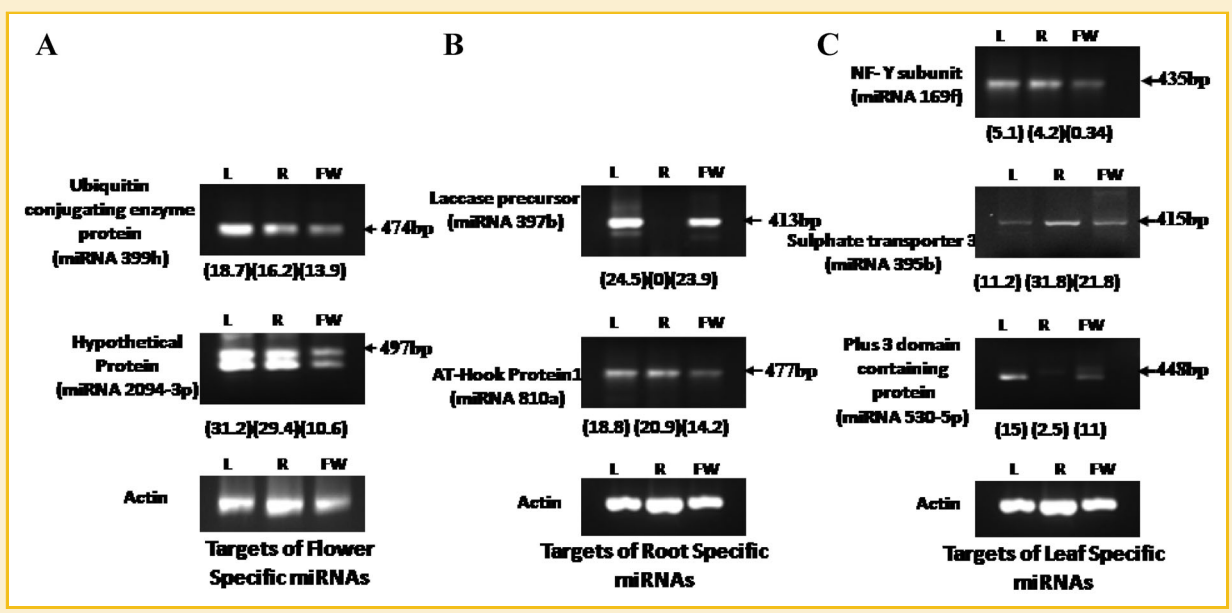

Fig. 7. Semi-quantitative reverse transcription PCR of various Osa-miR-targeted genes in L (seedling leaf), R (seedling root), and Fw (pre-pollinated flower). Total RNA from seedling leaf, seedling root and pre-pollinated flower of Pusa Basmati was prepared using miRNeasy mini kit [Qiagen]. Two micrograms of total RNA was used for first-strand cDNA synthesis using Superscript II (Invitrogen). PCR amplification (26–32 cycles) of cDNA was performed using primers to selectively amplify genes. Actin gene was also amplified and used as an internal control. A: targets of flower-preferential Osa-miRs. B: targets of root-preferential Osa-miRs. C: targets of leaf-preferential Osa-miRs. The figures below each lane are the values for normalized radioactive band intensities.

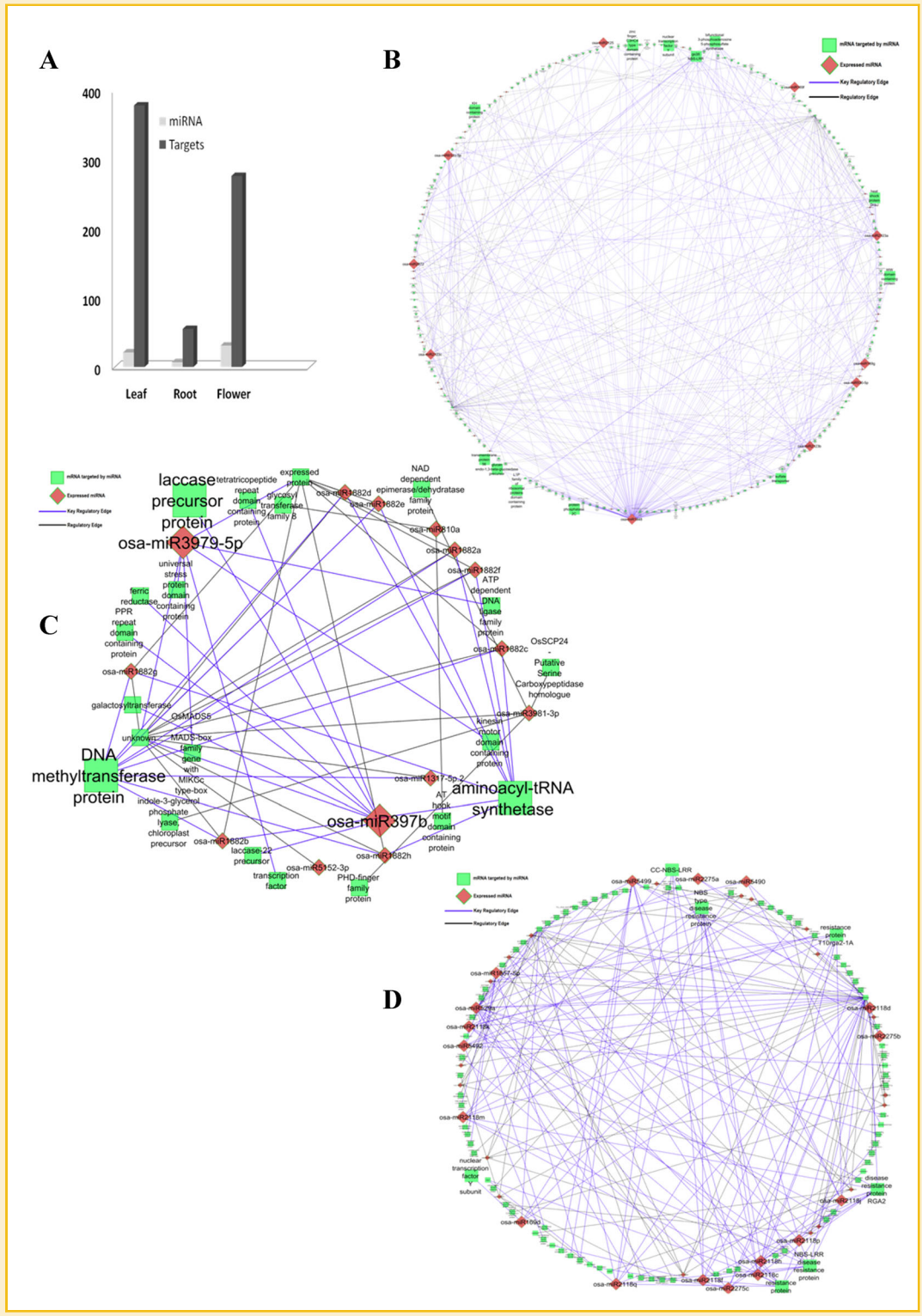

Fig. 8. miRNA-target regulatory model based on their interactions. A: Graphical representation of the distribution of the number of miRs and their predicted targets across tissues The miRNA-mRNA regulatory models for (B) Leaf (C) Root and (D) Flower tissues. Enrichment analysis of tissue-preferential miR and its target was done by calculating the number of target transcripts for miR and number of targeting miR for transcripts. Cytoscape v 2.8.3 was used to model and visualize the miR–gene interaction and enrichment output.

respectively (Fig. 7B). However, a correlative antagonistic expression pattern could not be observed for a few selected targets of leaf-preferential miRs (Fig. 7C). This could be attributed to the presence of other family members with redundant or overlapping expression domains. It is also possible that the correct targets for the specific family members have been missed due to the high stringency criteria of target selection.

To understand the functions of the tissue-preferential miRs, the targets of all the 59 putative tissue-preferential Osa-miR-sequences (Table I) were predicted using the computational algorithm psRNATarget [Dai and Zhao, 2011] and also checked against the PMRD database. For 59 Osa-miR-sequences, atleast one target genes with a stringent score <2.5, could be predicted. No target could be predicted for Osa-miR-1848 and Osa-miR-2879 using these criteria (Supplementary Table S4). A total of 686 transcripts were identified as targets for the tissue-preferential miRs including 378 for the leaf-preferential miRs, 276 for the flower-preferential miRs, and 55 for the root-preferential miRs (Fig. 8A). Osa-miR1848 was identified to be the key leaf-preferential miR as it was found to target 182 transcripts. Similarly, nuclear transcription factor Y subunit gene was identified to be the key target in the leaf as 53 different miRs were predicted to target this gene.

Tissue-preferential miR and its target enrichment analysis showed 51 nodes (miR/Gene) were significantly enriched apart from genes with unknown function or expression potential (Fig. 8B–D). Nuclear transcription factor Y subunit was the only gene to be enriched significantly in at least two out of three tissues analyzed. Enrichment modelling done using Cytoscape v 2.8.3 showed distinct post-transcriptional regulatory edges that connect enriched miR and its target genes.

CONCLUSIONS

In this work, we surveyed the global expression of known Osa-miRs in the roots, leaves, and flowers of rice seedlings in an attempt to follow their regulatory networks involved in fine-tuning the development of rice tissues. Our results revealed that members of 59 Osa-miR families are specifically expressed across leaf, root and flower tissues while 126 Osa-miRs belonging to 81 families are differentially regulated in these tissues. We predicted the targets for the tissue-preferential miRs and identified 51 potential interactive nodes that were significantly enriched in these tissues. The target miR correlation for selected miRs from each tissue was validated. The analysis revealed that expression of some miRs can be associated to specific organs under normal growing conditions. This study has paved the way for further functional characterization of these miR signatures for understanding tissue/organ formation.

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REFERENCES

Aida M, Ishida T, Tasaka M. 1999. Shoot apical meristem and cotyledon formation during arabidopsis embryogenesis: Interaction among the CUP-SHAPED COTYLEDON and SHOOT MERISTEMLESS genes. Development 126:1563–1570.

Aukerman MJ, Sakai H. 2003. Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15:2730–2741.

Axtell MJ, Bowman JL. 2008. Evolution of plant microRNAs and their targets. Trends Plant Sci 13:343–349.

Baumberger N, Baulcombe DC. 2005. Arabidopsis ARGONAUTE1 is an RNA slicer that selectively recruits microRNAs and short interfering RNAs. Proc Natl Acad Sci USA 102:11928–11933.

Bentwich I. 2005. Prediction and validation of microRNAs and their targets. FEBS Letts 579:5904–5910.

Chen HM, Chen LT, Patel K, Li YH, Baulcombe DC, Wu SH. 2010. 22-Nucleotide RNAs trigger secondary siRNA biogenesis in plants. Proc Natl Acad Sci USA 107:15269–15274.

Cuperus JT, Carbonell A, Fahlgren N, Garcia-Ruiz H, Burke RT, Takeda A, Sullivan CM, Gilbert SD, Montgomery TA, Carrington JC. 2010. Unique functionality of 22-nt miRNAs in triggering RDR6-dependent siRNA biogenesis from target transcripts in arabidopsis. Nat Struct Mol Biol 17:997–1003.

Dai X, Zhao PX. 2011. psRNATarget: A plant small RNA target analysis server. Nuc Acids Res 39:W155–159.

Dugas DV, Bartel B. 2004. MicroRNA regulation of gene expression in plants. Curr Opin Plant Biol 7:512–520.

Fujii H, Chiou TJ, Lin SI, Aung K, Zhu JK. 2005. A miRNA involved in phosphate-starvation response in arabidopsis. Curr Biol 15:2038–2043.

Groszmann M, Greaves IK, Albertyn ZI, Scofield GN, Peacock WJ, Dennis ES. 2011. Changes in 24-nt siRNA levels in arabidopsis hybrids suggest an epigenetic contribution to hybrid vigor. Proc Natl Acad Sci USA 108: 2617–2622.

Guo HS, Feia JF, Chua NH. 2005. MicroRNA directs mRNA cleavage of the transcription factor NAC1 to downregulate auxin signals for arabidopsis lateral root development. Plant Cell 17:1376–1386.

Henderson IR, Zhang X, Lu C, Johnson L, Meyers BC, Green PJ, Jacobsen SE. 2006. Dissecting *Arabidopsis thaliana* DICER function in small RNA processing, gene silencing and DNA methylation patterning. Nat Genet 38:721–725.

Hollister JD, Smith LM, Guo YL, Ott F, Weigel D, Gaut BS. 2011. Transposable elements and small RNAs contribute to gene expression divergence between *Arabidopsis thaliana* and *Arabidopsis lyrata*. Proc Natl Acad Sci USA 108:2322–2327.

Jeong DH, Park S, Zhai J, Ranjan Gurazada SN, Paoli ED, Meyers BC, Green PJ. 2011. Massive analysis of rice small RNAs: Mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage. Plant Cell 23:4185–4207.

Jones-Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. 2002. Prediction of plant microRNA targets. Cell 110:513–520.

Jones-Rhoades MW, Bartel DP, Bartel B. 2006. MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57:19–53.

Juarez MT, Kui JS, Thomas J, Heller BA, Timmermans MC. 2004. MicroRNA-mediated repression of rolled leaf1 specifies maize leaf polarity. Nature 428:84–88.

Kapoor M, Arora R, Lama T, Nijhawan A, Khurana JP, Tyagi AK, Kapoor S. 2008. Genome-wide identification, organization and phylogenetic analysis of Dicer-like, Argonaute and RNAdependent RNA Polymerase gene families and their expression analysis during reproductive development and stress in rice. BMC Genomics 9:451.

Kasschau KD, Fahlgren N, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Carrington JC. 2007. Genome-wide profiling and analysis of arabidopsis siRNAs. PLoS Biol 5:E57.

Kim VN. 2005. MicroRNA biogenesis: Coordinated cropping and dicing. Nat Rev Mol Cell Biol 6:376–385.

Laufs P, Peaucelle A, Morin H, Traas J. 2004. MicroRNA regulation of the CUC genes is required for boundary size control in arabidopsis meristems. Development 131:4311–4322.

Li H, Xu L, Wang H, Yuan Z, Cao X, Yang Z, Zhang D, Xu Y, Huang H. 2005. The putative RNA-dependent RNA polymerase RDR6 acts synergistically with ASYMMETRIC LEAVES1 and 2 to repress BREVIPEDICELLUS and microRNA165/166 in arabidopsis leaf development. Plant Cell 17:2157–2171.

Llave C, Xie Z, Kasschau KD, Carrington JC. 2002. Cleavage of scarecrow-like mRNA targets directed by a class of arabidopsis miRNA. Science 297: 2053–2056.

Mallory AC, Vaucheret H. 2006. Functions of microRNAs and related small RNAs in plants. Nat Genet 38:S31–S36.

Mallory AC, Reinhart BJ, Jones-Rhoades MW, Tang G, Zamore PD, Barton MK, Bartel DP. 2004. MicroRNA control of PHABULOSA in leaf development: Importance of pairing to the microRNA 5′ region. EMBO J 23:3356–3364.

Millar AA, Gubler F. 2005. The arabidopsis GAMYB-like genes, MYB33 and MYB65 are microRNA-regulated genes that redundantly facilitate anther development. Plant Cell 17:705–721.

Ori N, Cohen AR, Etzioni A, Brand A, Yanai O, Shleizer S, Menda N, Amsellem Z, Efroni I, Pekker I, Alvarez JP, Blum E, Zamir D, Eshed Y. 2007. Regulation of LANCEOLATE by miR319 is required for compound-leaf development in tomato. Nat Genet 39:787–791.

Palatnik J, Allen E, Wu X, Schommer C, Schwab R, Carrington J, Weigel D. 2003. Control of leaf morphogenesis by microRNAs. Nature 425:257–263.

Pérez-Quintero AL, Neme R, Zapata A, López C. 2010. Plant microRNAs and their role in defense against viruses: A bioinformatics approach. BMC Plant Biol 10:138.

Rubio-Somoza I, Weigel D. 2011. MicroRNA networks and developmental plasticity in plants. Trends Plant Sci 16:258–264.

Ruiz-Ferrer V, Voinnet O. 2009. Roles of plant small RNAs in biotic stress responses. Annu Rev Plant Biol 60:485–510.

Sanan-Mishra N, Mukherjee SK. 2007. A peep into the plant miRNA world. Open Plant Sci J 1:1–9.

Sanan-Mishra N, Kumar V, Sopory SK, Mukherjee SK. 2009. Cloning and validation of novel miRNA from basmati rice indicates cross talk between abiotic and biotic stresses. Mol Genet Genomics 5:463–474.

Sato Y, Antonio B, Namiki N, Motoyama R, Sugimoto K, Takehisa H, Minami H, Kamatsuki K, Kusaba M, Hirochika H, Nagamura Y. 2011. Field transcriptome revealed critical developmental and physiological transitions involved in the expression of growth potential in japonica rice. BMC Plant Biol 11:101.

Sunkar R, Zhu JK. 2004. Novel and stress-regulated micro-RNAs and other small RNAs from arabidopsis. Plant Cell 16:2001–2019.

Sunkar R, Zhou X, Zheng Y, Zhang W, Zhu JK. 2008. Identification of novel and candidate miRNAs in rice by high throughput sequencing. BMC Plant Biol 8:25.

Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. Cell 136:669–687.

Wang XJ, Reyes JL, Chua NH, Gaasterland T. 2004. Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. Genome Biol 5:R65.

Wei LQ, Yan LF, Wang T. 2011. Deep sequencing on genome-wide scale reveals the unique composition and expression patterns of microRNAs in developing pollen of *Oryza sativa*. Genome Biol 12:R53.

Wollmann H, Weigel D. 2010. Small RNAs in flower development. Eur J Cell Biol 89:250–257.

Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, Qi Y. 2010. DNA methylation mediated by a microRNA pathway. Mol Cell 38:465–475.

Xie K, Shen J, Hou X, Yao J, Li X, Xiao J, Xiong L. 2012. Gradual increase of miR156 regulates temporal expression changes of numerous genes during leaf development in rice. Plant Physiol 158:1382–1394.

Xue LJ, Zhang JJ, Xue HW. 2009. Characterization and expression profiles of miRNAs in rice seeds. Nucleic Acids Res 37:916–930.

Zhao K, Aranzana M, Kim S, Lister C, Shindo C, Tang C, Toomajian C, Zheng H, Dean C, Marjoram P, Nordborg M. 2007. An arabidopsis example of association mapping in structured samples. PLoS Genet 3:e4.

Zhong R, Ye ZH. 2004. amphivasal vascular bundle 1, a gain-of function mutation of the IFL1/REV gene, is associated with alterations in the polarity of leaves, stems and carpels. Plant Cell Physiol 45:369–385.

Zhu QH, Spriggs A, Matthew L, Fan L, Kennedy G, Gubler F, Helliwell C. 2008. A diverse set of microRNAs and microRNAlike small RNAs in developing rice grains. Genome Res 18:1456–1465.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's website.

Supplementary Table S1. List of all Osa-miR-sequences identified in the Illumina small RNA sequencing data obtained from the three leaf [NL] libraries.

Supplementary Table S2. List of all Osa-miR-sequences identified in the Illumina small RNA sequencing data obtained from leaf [NL], root [NR] and flower [FW] libraries.

Supplementary Table S3. The expression status of Osa-miRs in leaf root and flower libraries as observed by microarray hybridizations.

Supplementary Table S4. Predicted targets of Osa-miR-sequences

Supplementary Figure S1. Search results of the larger homologs of the 20 nt Osa-miRs in the deep sequencing data sets. The search results for individual sequences are separated by dotted lines. In each section the topmost sequence indicates the annotated sequence in miRBase. The sequences below this indicate the larger homolog's identified from the NGS data base

Supplementary Figure S2. Size distribution of Osa-miR-sequences which show tissue-preferential expression.