American Journal of Plant Sciences, 2022, 13, 1261-1281
https://www.scirp.org/journal/ajps
ISSN Online: 2158-2750
ISSN Print: 2158-2742

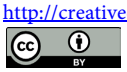
Mapping the Expression Profiles of Grain Yield QTL Associated miRNAs in Rice Varieties with Different Grain Size

Sudhir Kumar, Neeti Sanan-Mishra*

Plant RNAi Biology Group, International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi, India
Email: sudheerbgs20@gmail.com, *neeti@icgeb.res.in

How to cite this paper: Kumar, S. and Sanan-Mishra, N. (2022) Mapping the Expression Profiles of Grain Yield QTL Associated miRNAs in Rice Varieties with Different Grain Size. *American Journal of Plant Sciences*, **13**, 1261-1281.
https://doi.org/10.4236/ajps.2022.139086

Received: June 14, 2022
Accepted: September 20, 2022
Published: September 23, 2022



Abstract

microRNAs (miRNAs) are key regulatory molecules that fine tune gene expression at the transcriptional and post-transcriptional levels through the RNA silencing pathways. They play an important role in regulating plant growth, development and response to a/biotic stress conditions. Grain yield is a complex trait that is governed by the coordinated action of several genetic and environmental factors. A number of genes and miRNAs have been identified to affect the grain productivity and yield. In this study, we identified the miRNAs that map to grain yield QTLs in rice. The expression variations of these miRNAs and their target transcript were studied across different tissues of three *indica* rice varieties with different grain morphology. The varieties used include the extra-long and slender grained Pusa Basmati 1 (PB1), medium grain sized IR64 and the short grained Pokkali (PK). The windows for miRNA target correlation were captured and their putative role in regulating rice grain yield is discussed.

Keywords

miRNA, Grain Yield, Expression Analysis, Rice, IR64, Basmati, Pokkali

1. Introduction

Agriculture is a priority sector for ensuring incessant supply of food for the rapidly increasing world population [1]. Rice (*Oryza sativa* L.) is a common diet for over 3.5 billion people all over the world [2] and its consumption is increasing at a rate of 1.8 per cent annually, making it difficult to match the increase in its output to the exponentially increasing population [3]. So, rapid increase in

rice production over a short period of time and space will be critical to addressing the food demands.

Rice is a member of the family *Poaceae* and genus *Oryza.* It can be classified into three important subspecies: *japonica*, *indica and javanica*. The *japonica* varieties are sticky and short-grained, while *indica* varieties are long grained and non-sticky [3]. The rice plant needs a hot (22˚C to 37˚C) humid climate, prolonged sunshine and an assured supply of water. India is an important center for *indica* rice cultivation and the crop is grown in a diverse array of climatic conditions. In this study, three popular *indica* rice varieties were used namely, Pusa Basmati 1 (PB1), IR64 and Pokkali (PK), which are known for their extra-long, medium and short types of grains, respectively. PB1 is a semi-dwarf and short duration variety of rice with a maturing time of 145 - 155 days and average yield of 4.64 t/ha. It is relished all over the world as its grains are aromatic, extra-long and slender and have many other palatable qualities, such as white kernels, fluffy and long cooked kernels and moderate amylose content [4] [5]. IR64 represents another semi-dwarf, early maturing and disease resistance *indica* rice variety whose average growth duration is about 117 days. It has medium grain size as compared to the Basmati variety [6] [7] but produces more number of tillers and has average yield of 8.76 t/ha. The grains have excellent cooking quality, intermediate amylose content and intermediate gelatinization temperature [8]. PK rice is a salt-tolerant landrace that has tall plants with long duration of growth and low yield in grains of about yields about 2 - 5 t/ha. Its grain size is short, thick and bold [9] [10] [11].

Grain yield is a complex and multigenic agronomic trait that depends on several features such as, root architecture, plant height, shape and arrangement of leaves, panicle morphology and seed number, shape and size. The agronomic traits are controlled by specific regions of the rice genome known as Quantitative trait Loci (QTL), which contain several important genes and miRNAs. miRNAs are small genetic regulators of key genes and transcription factors that are indispensable for the proper growth and development of plants. They are involved in regulating various processes such as organ formation, primordial development, apical dominance, lateral root development, shoot branching, panicle formation etc. [12]. A large number of grain-yield related traits are also controlled by the miRNAs through their corresponding targets [13] [14]. The miRNAs such as miR159, miR160, miR165/166, miR395, miR417 and miR402, are important for the development, maturation and germination of seeds [15]. The miR397a and miR397b were identified to be associated with increased panicle branching, grain number and grain size by down regulating its target gene *Laccase13* (*OsLAC13*). Transgenic rice plants overexpressing miR397a/b exhibited increased primary and secondary branches and more grain number as compared to wild type [16]. Rice plants overexpressing osa-miR408 showed increased panicle branches and grain number that resulted in high grain yield [17]. Recent studies have shown miR396 affects grain size and shape in addition to panicle architecture and yield. Transgenic rice plants having knockout of miR396e and miR396f

showed improved grain size and panicle branching [18] [19].

Many rice QTLs associated with the grain yield related traits have been identified and cloned over the past two decades [17]. In this study, we identified the miRNAs related to grain yield QTLs and compared their expression profiles across various tissues of three indica rice varieties (PB1, IR64 and PK) with different grain morphology to capture the window for miRNA and target correlations.

2. Methods

2.1. Plant Materials

The mature and healthy rice seeds from Pusa-Basmati, IR64, and Pokkali were chosen for this study. Seeds were surface sterilized with 0.1% $HgCl_2$ and Teepol, rinsed with water and then soaked in water overnight. Seeds were grown on germination sheets for two weeks in growth room at ICGEB, New Delhi under controlled conditions of temperature (28˚C ± 2˚C), relative air humidity (70%), and 16/8-h light/dark cycle. The leaf tissue samples were harvested from 15-days old seedlings. Healthy 15-day old seedlings were also transferred to soil pots and grown till maturity under control conditions in the greenhouse to obtain reproductive tissues for RNA isolation. All tissue samples were harvested in two biological replicates and immediately frozen in liquid N_2.

2.2. Isolation of Total RNA

Total RNA was extracted from various rice tissues using guanidine isothiocyanate (GITC) based protocol as described previously [20]. Briefly, leaf tissue was homogenised in liquid nitrogen and GITC buffer was added. The mixture was allowed to thaw slowly in presence of phenol and chloroform and centrifuged at 13,000 rpm for 15 min. The upper aqueous phase was extracted twice with phenol and chloroform solution and precipitated with chilled ethanol. The pellet was obtained by centrifuging at 13,000 rpm for 15 min and it was washed twice using 75% ethanol each. The pellet of RNA was air dried at room temperature and dissolved in required amount of DEPC-treated water and stored at −20˚C.

2.3. cDNA Preparation

Around 500 µg of total RNA was used for reverse transcription to synthesize cDNA using Superscript reverse transcriptase III/IV (Invitrogen) as per manufacturer's specification. The synthesized cDNA was used as a template for stem-loop PCR, in which a gene-specific forward primer and universal reverse primer were used to get the amplification product.

2.4. Stem-Loop RT-PCR

Semi-quantitative stem-loop RT-PCR was performed by using cDNA prepared with 500 ng of total RNA. The primer sequences are provided in (**Table 1**). The amplified bands were analysed on agarose gel and band intensity/spot density

Table 1. List of primers used in the study.

Name	Sequence (5'-3')	Target gene
18S F.P.	CTACGTCCCTGCCCTTTGTACA	18S rRNA
18S R.P.	ACACTTCACCGGACCATTCAA	
miR408 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACG ACGCCAGG	mature miR408-3p
miR408 F.P.	GCTACTGCACTGCCTCTTC	
miR399e SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGGC	mature miR399e
miR399e F.P.	TCGTCGTGCCAAAGGAGATTT	
miR1427 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCGCCA	mature miR1427
miR1427 F.P.	ATAATTGCGGAACCGTGCGG	
miR2924 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTGGCG	mature miR2924
miR2924 F.P.	ATATTCTCGCTTGCTCCGGC	
miR5802 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCGCT	mature miR5802
miR5802 F.P.	CGCGCATGGACTGTACTTTGTAA	
miR5792 SL	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATGTC	mature miR5792
miR5792 F.P.	ATATTGATGACAGCGGTGGTTCG	
Plastocy F.P.	GAACGAACACACAGGGTAG	*Plastocyanin*
Plastocy R.P.	AAAACCTGCACGATGAC	
Ubiq F.P.	CTTGTGTCCTCTAATCATC	*Ubiquitin*
Ubiq R.P.	AAGGAGAATTACCACGA	
F-box F.P.	AATGGCGATGAACAAGCTA	*F-box*
F-box R.P.	AAGAACCTCGCCTCCAAGAA	
TMM F.P.	AATGCCGTTCGCTGCTCAA	*Too many mouth*
TMM R.P.	TGCCAACTCCCGACACAAA	
Sac F.P.	AAGGAGCCTTTCTTTTGCT	*SAC9*
Sac R.P.	ACATCAGATAGACCACCAA	
Exp F.P.	TACAGTGGTGATGTGCCAG	*Expressed protein*
Exp R.P.	AGCCTCTTTCTTCAGCCTTCT	
Universal R.P.	GTGCAGGGTCCGAGGT	Mature miRNA

was calculated with Alpha Imager software. The 18S rRNA transcripts were amplified as internal controls and used for normalizing the expression values to calculate relative abundance.

2.5. Analysis of Expression

The expression analysis was performed using two biological replicates and three technical replicates, for each set. The relative abundance was determined and a

graph was plotted using the average values. To check the significance of the expression difference, the normalized expression value was subjected to ANOVA (one-way analysis of variance) using Microsoft Excel. The means were compared with their respective controls at a significance level of $p < 0.05$.

2.6. QTL Analysis

The coordinates of the known grain yield related QTLs in rice genome were obtained from the Gramene database and their corresponding sequences were downloaded. The sequences corresponding to the known rice miRNA precursors were searched within the genomic sequences using high matching criteria of zero mismatch.

2.7. Target Prediction

To identify the transcripts that contain complementary sequences to allow miRNA binding, "psRNA Target finder", a miRNA target analysis server created mainly for plants [21], was employed using standard prediction parameters. The cut off expectation value was set to below 2.5 to filter the results. In all cases, where more than two significant targets for a miRNA were identified, only one target was selected for the validation experiments. The target selection was based on its role known or predicted role in determining grain yield.

3. Results and Discussion

3.1. Identification of miRNAs Mapping to Yield Related QTLs

The precursors of known rice miRNAs were mapped to genomic sequences of the grain yield-related QTLs and their chromosomal positions were plotted (**Figure 1**). This analysis identified 6 miRNAs (**Table 2**), indicating that these miRNAs may be involved in the regulation of agronomic traits in rice. Among these, four miRNAs, miR408-3p, miR399e, miR2924 and miR5802, were present on chromosome 1, while miRNA1427 was present on chromosome 8 and miR5792 was present on chromosome 10. Only miR399e was present on the minus strand, while all the others were found on the plus strand of chromosomes. Within

Table 2. List of miRNAs mapping to grain yield related QTLs.

miRNA	Sequence	Size	Chr. No.	Strand	Location
Osa-miR408-3p	CUGCACUGCCUCUUCCCUGGC	21nt	01	plus	12301661..12301873
Osa-miR399e	UGCCAAAGGAGAUUUGCCCAG	21nt	01	Minus	30477612..30477729
Osa-miR1427	UGCGGAACCGUGCGGUGGCGC	21nt	08	plus	9192581..91925837
Osa-miR2924	CUCGCUUGCUCCGGCCGCCAC	21nt	01	plus	11894321..11894440
Osa-miR5792	GAUGACAGCGGUGGUUCGGACAUC	24nt	10	plus	11842884..11842960
Osa-miR5802	AUGGACUGUACUUUGUAAAGCGGA	24nt	01	plus	13088727..13088790

Chr. No.: Chromosome number.

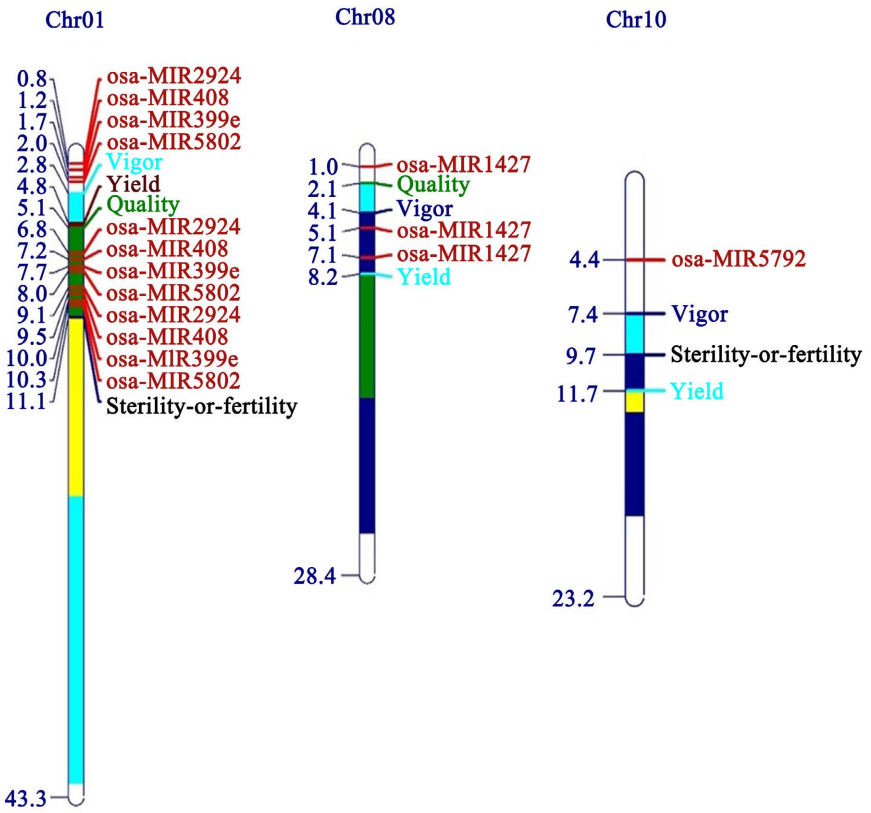


Figure 1. Karyoview of miRNAs mapping to grain yield related QTLs. The numbers on the left indicate the chromosomal location (in Mbps), while the miRNA IDs and QTL traits are written on the right.

this set of miRNAs, miR408-3p, miR399e, miR2924 and miR1427 are 21-nt in length, while miR5802 and 5792 are 24-nt in length. It has been reported that 21-nt miRNAs regulate their target genes by silencing the transcripts post-transcriptionally [22] [23] [24], whereas 24-nt miRNAs regulate their target genes at transcriptional levels [25] [26].

3.2. Prediction of miRNA Targets

miRNAs control the biological pathways by interacting with their target transcripts in a sequence-specific manner. The targets of miRNA associated with grain related QTLs were predicted (**Table 3**) and one transcript each was selected for expression profiling.

The miR408-3p targets the transcripts coding for plastocyanin-like domain containing protein, multicopper oxidase domain containing protein, helix-loop-helix DNA-binding domain containing protein and putative origin recognition complex subunit 6 (ORC6). *Plastocyanin* was selected for expression analysis because of its role in regulating rice grain yield.

The targets of miR399e were transcripts coding of DnaJ domain-containing protein and ubiquitin conjugating enzyme (UBC24). Among these *UBC24* (*PHO2*) was selected as it plays an important role in protein degradation and may

Table 3. Predicted targets of the grain yield related miRNAs.

miRNA	Target accession	Score	Chr. No.	Target transcript description
Osa-miR408-3p	LOC_Os03g15340.1	1.5	03	*Plastocyanin-like domain containing*
	LOC_Os01g02110.1	2	01	*Helix-loop-helix DNA binding domain containing*
	LOC_Os07g43540.1	2	07	*ORC6* (*origin recognition complex subunit 6*)
Osa-miR399e	LOC_Os05g48390.1	1.5	05	*Ubiquitin conjugating enzyme*
	LOC_Os05g45350.2	2.5	05	*Dnaj domain containing*
Osa-miR1427	LOC_Os06g49340.1	1.5	06	*Osfbduf35-F-box and DUF domain containing*
	LOC_Os04g32560.2	2.0	04	*ATP-dependent Clp protease, ATP-binding subunit clpa homolog CD4B*, chloroplast precursor
	LOC_Os03g04660.1	2.0	03	*Cytochrome P450 86A1*
Osa-miR2924	LOC_Os01g43440.1	2.0	01	*Too Many Mouths precursor*,
	LOC_Os11g32720.1	2.0	01	*zinc knuckle domain containing*
	LOC_Os07g47300.1	2.0	07	*spo0B-associated GTP-binding*
Osa-miR5792	LOC_Os01g25330.2	2.0	01	*Sac9*
Osa-miR5802	LOC_Os02g21770.1	2.0	02	*Putative expressed protein*
	LOC_Os08g40930.1	2.5	08	*Alpha amylase, catalytic domain containing*
	LOC_Os02g35010.1	2.5	02	*STE_MEKK_ste11_MAP3K.9-STE kinases* (homologous to yeast *sterile 7*, *sterile 11* and *sterile 20*)

influence grain yield [27].

The miR1427 was predicted to have stringent interactions with three transcripts, coding for protein containing F-box and DUF domains (*OsFBDUF35*), cytochrome P450-86A1 and chloroplastic ATP-dependent Clp protease (*CD4B*), respectively. The *OsFBDUF35* (*F-DUF*) transcript was chosen for profiling as it is implicated in auxin mediated floral meristem formation, floral organ identity determination and self-incompatibility [28] [29].

The miR2924 putatively targets the transcripts coding for precursor of *Too Many Mouths* (*TMM*), zinc knuckle domain containing protein and spo0B-associated GTP-binding protein, respectively. *TMM* is a receptor-like protein with a leucine-rich repeat sequence and is a regulator of stomatal development [30], so it was shortlisted for expression profiling.

The miR5792 was predicted to target the transcript coding for suppressor of actin mutation (SAC9). SAC9 is a PI phosphatase-like protein that terminates the Phosphatidylinositol (PtdIns 4,5 P2) signalling pathway to regulate physiological events such as vesicle targeting and membrane-cytoskeleton interactions [31].

The miR5802 was predicted to target three transcripts coding for Alpha amylase, a putative catalytic domain containing protein (LOC_Os02g21770.1) and

STE11/MEKK9 (ste11_MAP3K.9) kinase. The transcript for putative expressed protein correlated with grain length QTL, CQAL23, so it was selected for further analysis.

3.3. Expression Analysis of Selected miRNA-Target Modules

The expression profiles of miRNA and their corresponding target transcripts were generated using different tissues of three indica rice varieties, PB1, IR64 and PK, showing contrasting grain morphology. The profiles were checked at the vegetative stages by using tissues of immature root (IR) and immature leaf (IL) from seedlings and mature root (MR), mature leaf (ML) and flag leaf (FL) from the field grown plants. At the reproductive stage, the tissues used included complete enclosed total panicles (PAN), superior spikelets before anthesis (SSB), superior spikelets after anthesis (SSA), inferior spikelets before anthesis (ISB) and inferior spikelets after anthesis (ISA).

3.3.1. Osa-miR408-3p and *Plastocyanin* Transcript

It was observed that in PB1, expression of miR408-3p was lower in the vegetative tissues as compared to the reproductive tissues. MR and ML had relatively lower expression of miR408-3p than IR and IL, while FL displayed higher expression (**Figure 2(a)**). The expression levels were low in PAN tissue but increased in the spikelets, to levels observed in FL. Significant variations in expression levels were not observed in ISB, ISA, SSB and SSA tissues. The expression of target *plastocyanin* negatively correlated with miR408-3p expression in all the PB1 tissues, though larger differences were observed in MR, SSB, ISB and SSA (**Figure 2(a)**).

Analysis of IR64 tissues showed that the expression patterns of miR408-3p were similar to that observed in PB1 in most tissues but relatively higher in FL and lower in IR, ISA, ISB, SSA and SSB (**Figure 2(b)**). The expression levels of *plastocyanin* transcripts were high and inversely correlated with miR408-3p in IR and MR.

On analysing PK tissues, it was observed that the expression levels of miR408-3p were relatively higher in IR, IL, ML, FL and PAN (**Figure 2(c)**). The levels of *plastocyanin* transcripts were inversely correlated in all the tissues except MR. When compared to PB1 and IR64, very high expression of miR408-3p was seen in the FL and PAN and *plastocyanin* was high in the spikelet tissues of PK.

The results show that expression profiles of the miR408-3p were higher in the vegetative tissues of the short and bold grained PK varieties as compared to the long and slender grained PB1 and medium grained IR64. While expression profiles of its target, *plastocyanin* were relatively lower in the spikelets of the PB1 and IR64, but higher in the spikelets of PK. This indicates that miR408: *plastocyanin* node may play a crucial role in plant height and spikelet development, which in turn influence grain yield. miR408-3p is highly conserved across *Arabidopsis*, rice and other plants [32]. It is a copper homeostasis-related miRNA that controls panicle branching, grain weight and grain yield [17] [33]. It was

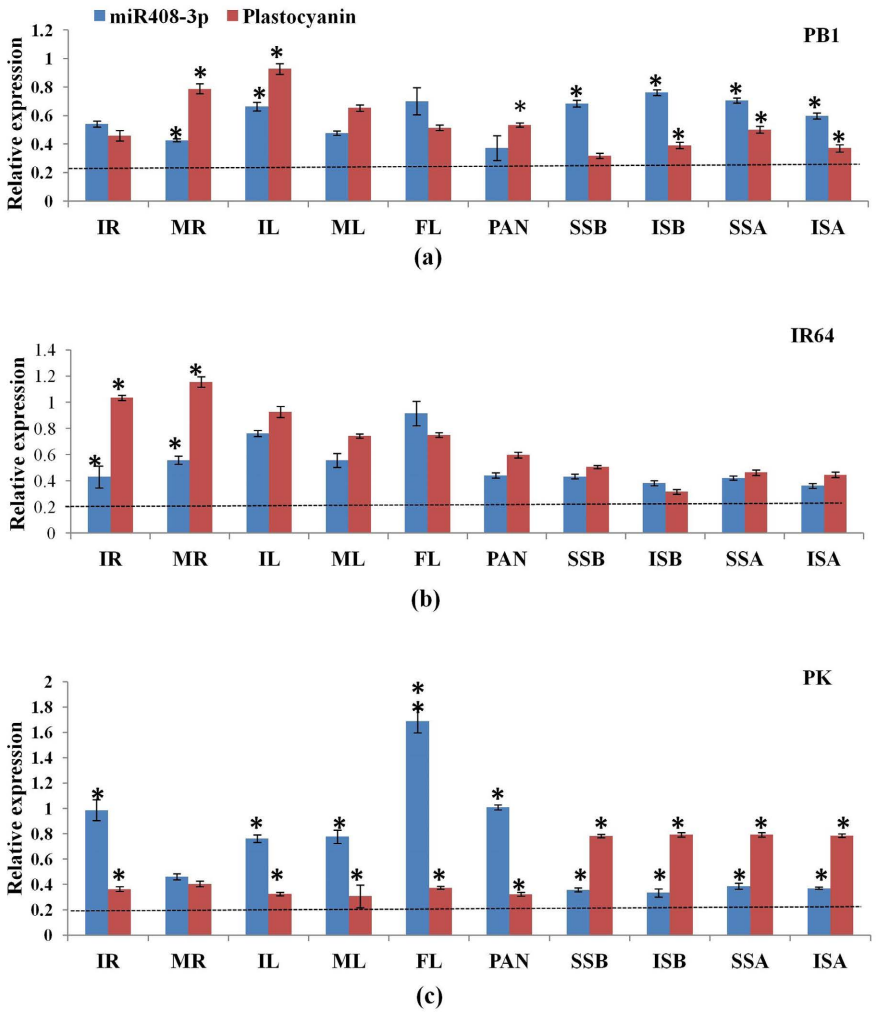


Figure 2. Expression profiles of osa-miR408-3p and its target, *Plastocyanin* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

shown that osa-miR408 targets *uclacyanin-like protein 8* (Os-UCL8) transcripts to regulate plastocyanin protein accumulation, copper homeostasis and rate of photosynthesis in rice [17] [33]. When osa-miR408 was overexpressed or *Os-ucl8* was knocked down or deleted, the number of panicle branches increased and plants produced more robust pollen, which led to an increase in grain yield [17]. Overexpression of *Os-UCL8* caused obvious abnormalities in pollen tube growth and pollination, affecting rice seed setting rate [34]. It was also reported that overexpression of *plantacyanin* (target of miR408) resulted in lower biomass and fewer seeds in plants [35].

3.3.2. Osa-miR399e and Ubiquitin Transcript

In PB1, expression levels of miR399e were high in IR but lower in MR. In leaf tissues, expression levels increased from IL to ML (Figure 3(a)). Very high expression was seen in FL and PAN, but these dropped to low levels in the spikelet tissues (Figure 3(a)). The expression levels of *ubiquitin* transcript negatively correlated with miR399e in the IR, MR, ML, FL and PAN tissues.

On comparing the expression patterns of miR399e in IR64 with PB1, it was clear that the levels were high in FL and PAN. However, a notable difference emerged as the miRNA levels, were lower in IR but higher in MR, IL and ML of IR64. The expression levels of miR399e were also relatively higher in ISB, ISA

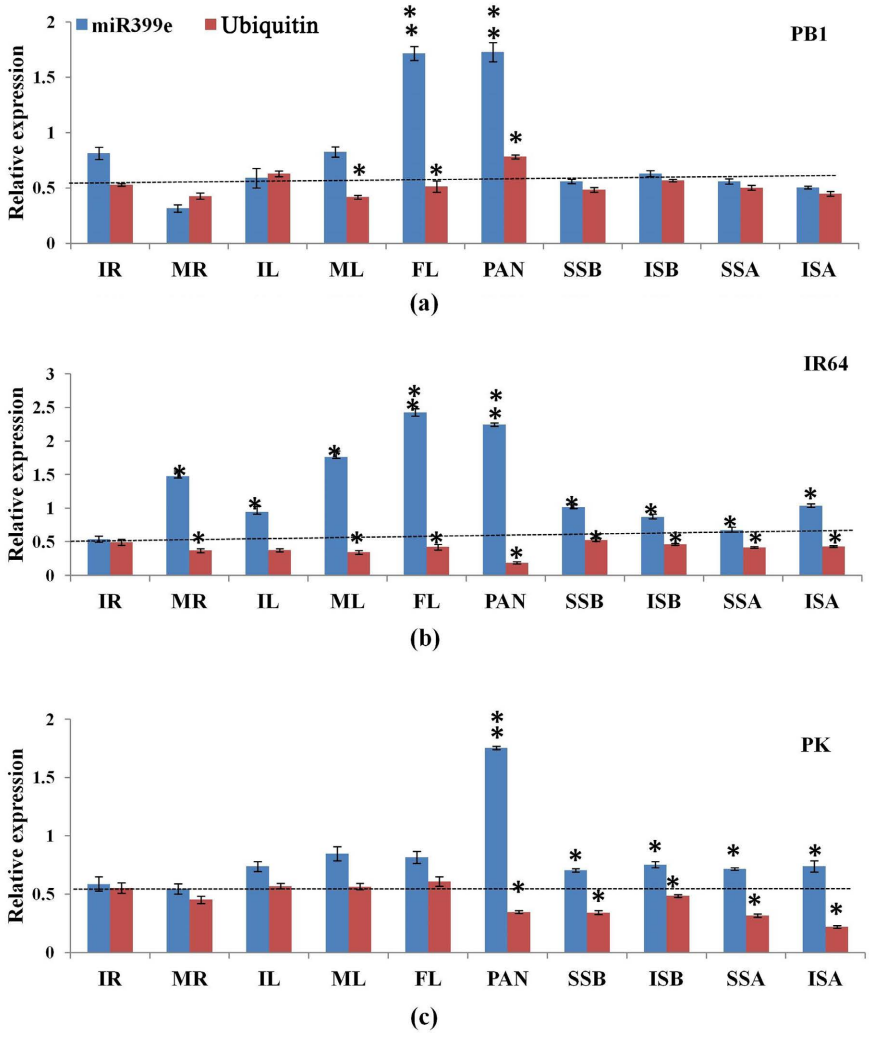


Figure 3. Expression profiles of osa-miR399e and its target, *Ubiquitin* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

and SSB (**Figure 3(b)**). Inverse correlation in expression levels of its target, *ubiquitin* was prominent in all the vegetative and reproductive tissue of IR64 except IR (**Figure 3(b)**).

The expression patterns of miR399e in the vegetative tissues of PK were similar to those in PB1 except for lower levels seen in FL tissues. At reproductive stages, the expression patterns of miR399e were relatively higher in PK and were largely similar to those observed in IR64 (**Figure 3(c)**). The level of *ubiquitin* transcript was relatively higher in the vegetative tissues of PK as compared to PB1 and IR64. Reverse correlation in expression of *ubiquitin* was visible in PAN tissues (**Figure 3(c)**).

The results show that expression of the miR399e was higher in the PAN tissues of all three varieties while expression of its target, *ubiquitin* were maintained at low levels. Differences in the expression patterns were seen in the FL tissues as the miRNA accumulated to higher levels in the long and slender grained PB1 and medium grained IR64 as compared to the short and bold grained PK varieties. In the spikelets miR399e levels were higher in PK compared to PB1 and IR64. The results indicate that miR399e: *ubiquitin* node may play an important role in influencing grain yield by enhancing nutrient accumulation and rate of grain filling. miR399e was identified and examined in *Arabidopsis* in response to phosphorous limitation [36] [37]. Overexpression of miR399e, enhanced accumulation of Pi and other nutrients in transgenic rice plants [38] [39]. The increase in nutrient content promotes plant growth, resulting in increased grain yields. *PH1* and *PT2* are two essential Pi transporters that regulate Pi homeostasis and their levels are controlled by miR399e [27] [40] [41]. Increase in miR399e expression under P deficiency also inhibited the expression of *PHO2* (*UBC24*), which encodes a *ubiquitin-conjugating enzyme* (E2). The ubiquitin enzymes modulate hormone sensitivity by regulating hormone biosynthesis, signalling pathways and transcription factors [42]. Loss of function of *gw2*, an E3 ubiquitin ligase, has been demonstrated to increase grain width and weight by increasing the rate of grain filling [43].

3.3.3. Osa-miR1427 and *F-DUF* Transcript

The expression profiles of miR1427 were higher in the vegetative tissues (IR, MR, IL, ML and FL) and PAN as compared to other reproductive tissues, in all three varieties. In the different spikelet tissues, there was no major difference in the expression levels of miR1427. The expression levels of its target, *F-DUF* transcript were low in all tissues, indicating an inverse correlation (**Figure 4**). In PK, levels of miR1427 were relatively higher in the spikelet tissues; though the *F-DUF* transcript was maintained at a low level in all the tissues (**Figure 4**).

The results indicate that miR1427: *F-DUF* node may not be playing a direct role in regulating grain yield or may be required in response to specific hormone signals to regulate flowering time and/or numbers. F-box proteins belong to one of the most widespread gene families in plants. They have a conserved domain of 40 - 50 amino acid at their N terminus and variable protein-protein interaction

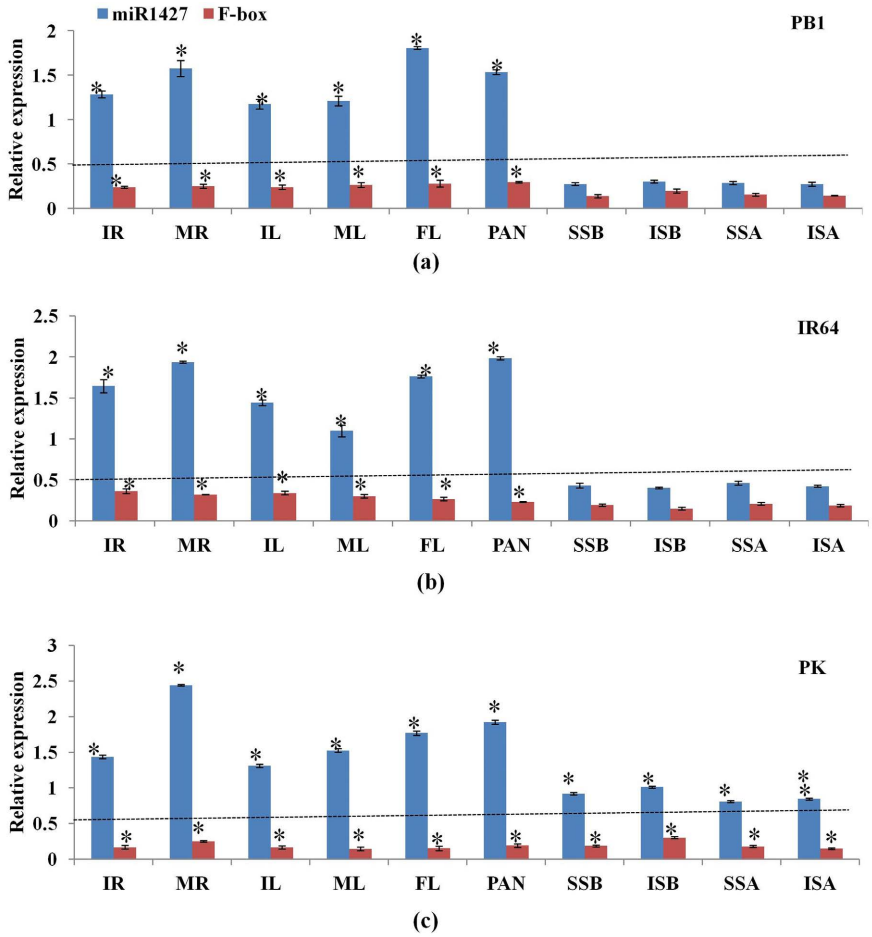


Figure 4. Expression profiles of osa-miR1427 and its target, *F-DUF* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

domains at the C-terminal. The variable domain can recognize numerous substrates to regulate many developmental processes such as photomorphogenesis, circadian clock regulation, self-incompatibility, hormonal responses, floral meristem and floral organ identity, senescence and a/biotic stress response [28] [29] [44] [45] [46] [47].

3.3.4. Osa-miR2924 and *Too Many Mouth* Transcript

The miR2924 and its target, *TMM*, were expressed in all tissues, but their expression patterns showed higher levels in vegetative tissues (IR, MR, IL and ML) as compared to the spikelets of PB1. The miR2924 expression levels were nearly similar in IR, MR, IL and FL but relatively lower in ML and PAN. PAN tissues exhibited higher levels of miR2924 expression as compared to ISB, ISA, SSB and SSA (**Figure 5(a)**). The levels of the target transcript were high and maximum

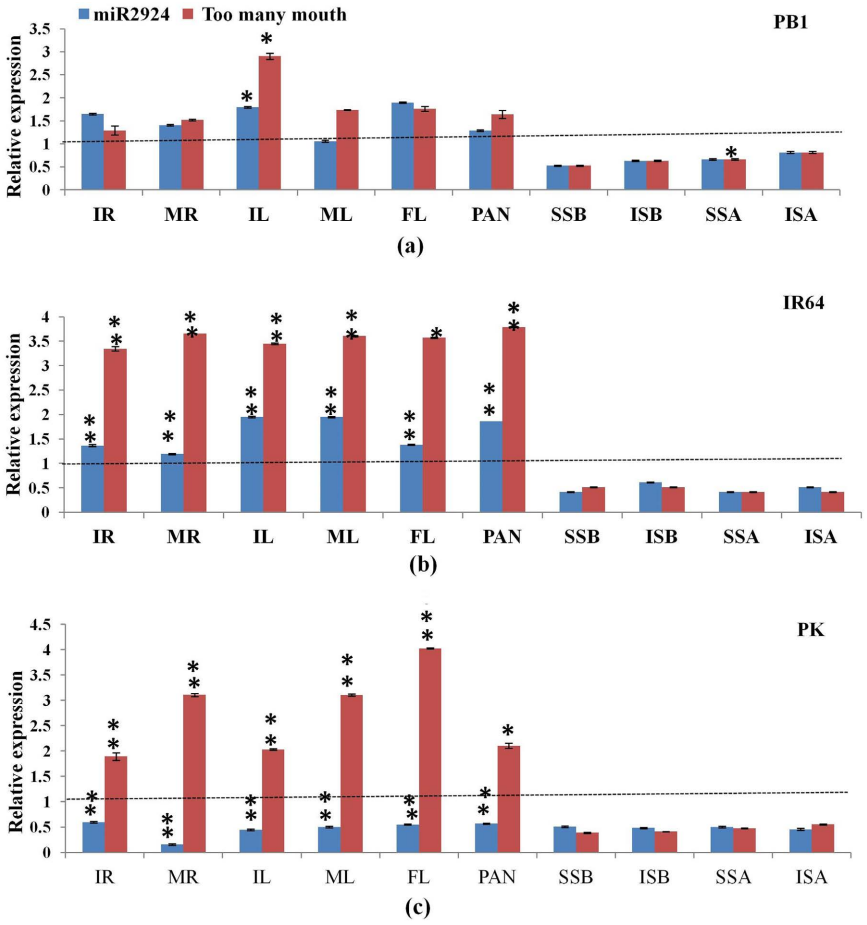


Figure 5. Expression profiles of osa-miR2924 and its target, *Too many mouth* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

expression was seen in IL. Substantial negative correlation could not be observed between the miR2924 and its target in any of the tissues in PB1. This may be likely if the miRNA does not cleave the transcript but repress its translation, hence more experiments are required to understand their interaction.

It was observed that in IR64, expression patterns of miR2924 were similar to that obtained in PB1. However, the *TMM* transcript, accumulated to higher levels in all the vegetative tissues and PAN. Low levels were maintained in all the spikelet tissues and no significant changes in levels were observed between them (**Figure 5(b)**). In contrast to the expression profiles seen in PB1 and IR64, the expression levels of miR2924 were down regulated in all the vegetative tissues and PAN in PK, while *TMM* transcript accumulated to high levels, similar to that seen in IR64 (**Figure 5(c)**).

The results show that expression profiles of the miR2924 were higher in the vegetative tissues of IR64 and PB1. The expression of its target, *TMM* showed negative correlation in IR64 and PK. The miR2924: TMM expression was low in the spikelets in all three varieties indicating that it may have an indirect role in grain formation. Mutation in *TMM* resulted in increase in the number of stomata on the leaf, but no stomata on the stem. The stomata regulate gaseous exchange and as a result affect photosynthesis which may impact the grain yield [48] [49]. TMM also enhanced meristematic activity in leaves by acting as a positive regulator of cell division [50] [51].

3.3.5. Osa-miR5792 and *SAC9* Transcript

The miR5792 expression was higher in vegetative tissues of PB1 as compared to the reproductive tissues. The expression levels were lower in MR than in the IR. The levels increased from IL to ML to FL and were even high in PAN (**Figure 6(a)**), while expression levels were very low in the spikelets (SSB, ISA, SSA and ISA). The expression of *SAC9* transcript was low in all tissues but levels were relatively very low in the spikelet tissues (**Figure 6(a)**).

The expression profiles of miR5792 and *SAC9* in IR64 were similar to those of PB1 tissues (**Figure 6(b)**). In PK vegetative tissues, the miR5792 expression levels were lower to that seen in PB1 and IR64, however, it accumulated to high levels in the PAN. Low levels of miR5792 were seen in the spikelets as in case of PB1 and IR64. The *SAC9* transcript accumulated to higher levels in IR, ML, FL, PAN and spikelet tissues when compared to PB1 and IR64 (**Figure 6(c)**).

The results show that high levels of miR5792 and low levels of *SAC9* correlated with the long grained PB1 and medium grained IR64 varieties while low levels of miR5792 and high levels of *SAC9* were seen in the short and bold grained PK variety. SAC proteins can be classified into three categories based on sequence homology [31]. Two of the categories; SAC 1 - 5 and SAC 6 - 8 show substantial sequence homology within themselves, but *SAC9* is very different from other SAC domain proteins and is categorized separately. Mutations in the *SAC9* gene cause elevated levels of PtdIns(4,5)P2 and Ins(1,4,5)P3 in the plants as compared to wild-type. The mutants also display a constitutive stress response, including overexpression of stress-induced genes, dwarfism, closed stomata, anthocyanin accumulation and over-accumulation of reactive-oxygen species [52].

3.3.6. Osa-miR5802 and *Expressed* Transcript

The miR5802 and its target are also present in all the tissues of the three varieties. In PB1, the expression of miR5802 was higher in IR than in MR. There was not much variation in the expression levels in IL, ML and FL. In PAN, SSB and SSA miR5802 expression levels were relatively higher (**Figure 7(a)**). The expression patterns of its target transcript showed a negative correlation in the vegetative and PAN tissues, but the transcript levels were high in all spikelet tissues (**Figure 7(a)**).

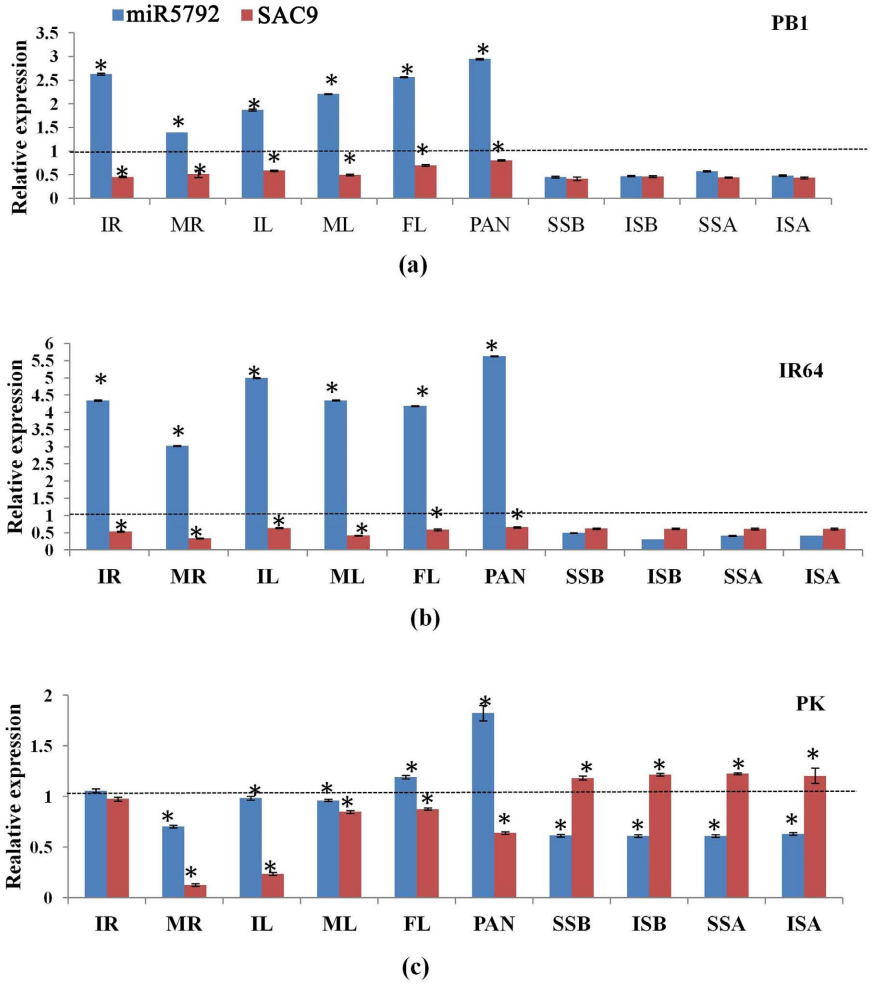


Figure 6. Expression profiles of osa-miR5792 and its target, *SAC9* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

In IR64, the expression levels of miR5802 were relatively low in IR, MR, ML, FL, PAN and spikelet tissues as compared to PB1 (Figure 7(b)). The target expression was higher in IR and MR and negatively related in most IR64 tissues except PAN, SSA and ISA (Figure 7(b)). The expression profile of miR5802 and its target in PK was similar to that seen in PB1, but the levels of its target transcript were higher in IR and ML (Figure 7(c)). Interestingly, the target transcript correlated with grain length QTL, CQAL23, and its expression levels were high in all reproductive tissues of the three varieties. Negative correlation with the miR5802 was not evident in the reproductive tissues even though miRNA levels were high, so more experiments are required to understand their interaction.

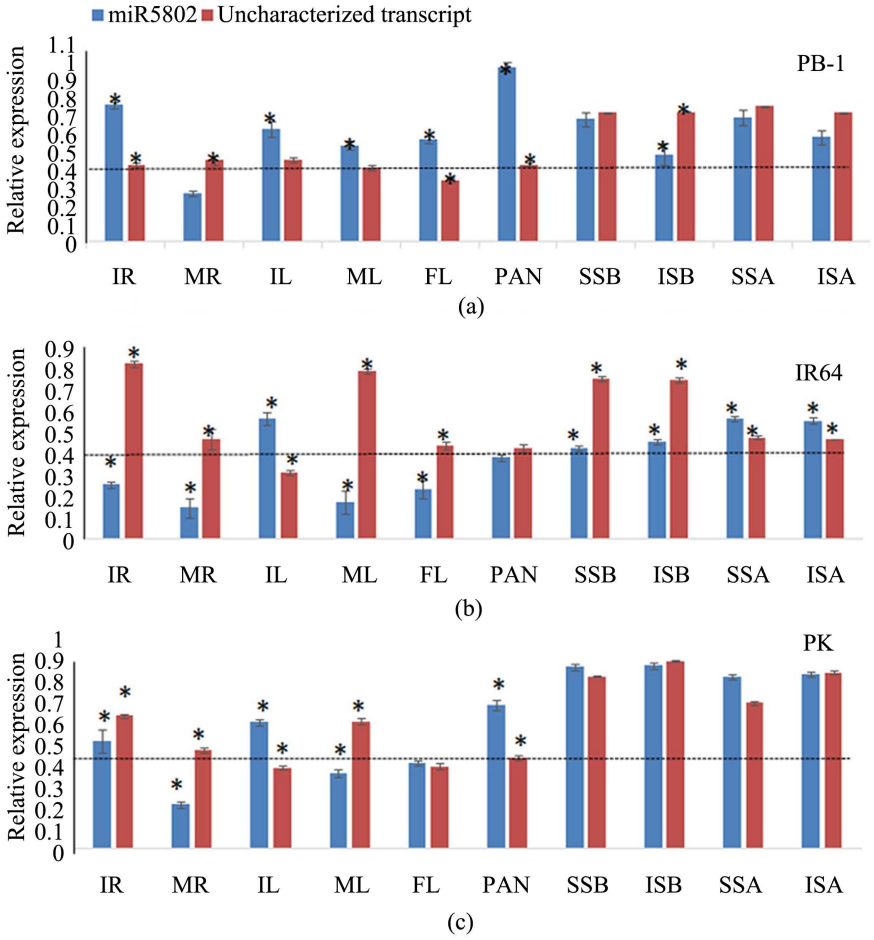


Figure 7. Expression profiles of osa-miR5802 and its target, *uncharacterised transcript* in different tissues of rice varieties PB1, IR64 and PK. IR—immature root, IL—immature leaf, MR—mature root, ML—mature leaf, FL—flag leaf, PAN—Panicle, SSA—superior spikelets after anthesis, ISA—inferior spikelets after anthesis, SSB—superior spikelets before anthesis, ISB—inferior spikelets before anthesis. The integrated density values (IDV) were calculated and normalized with respect to 18S rRNA transcripts and plotted as relative abundance. Significant differences are shown by asterisks (*p = 0.05, **p = 0.001, ***p = 0.001), as determined by ANOVA in Microsoft Excel. The error bars indicate the standard deviation.

4. Conclusions

In the present work, we report the expression profiles of rice miRNAs that map to yield-related QTLs using three rice varieties with varying seed morphology. The results show that these miRNAs are ubiquitously expressed but differentially regulated in the various tissues of the three rice varieties. The expression profiles of all target transcripts were negatively correlated in most cases. It was observed that expression profiles of miR408-3p were higher in the vegetative tissues, especially FL, of the short and bold grained PK varieties as compared to the long and slender grained PB1 and medium grained IR64. A clear window of negative correlation was observed for miR408-3p and *plastocyanin* in the tissues of short and bold grained PK varieties. The expression of the miR399e was high in the PAN

tissues in all three varieties and expression of *ubiquitin* was negatively correlated. However, stark difference was seen in the FL tissues as miR399e accumulated to higher levels in the long and slender grained, PB1 and medium grained IR64 as compared to the bold grained PK varieties, but expression of *ubiquitin* was low in all. The miR1427 and miR5792 expression profiles were high in the vegetative and PAN tissues, The expression of miR1427 targeted, *F-DUF* transcript and miR5792 targeted, *SAC9* transcript were maintained at low levels in the reproductive tissues of all three varieties. The miR5802 profiles were relatively higher in the reproductive tissues of PB1 and PK, while miR2924 profiles were higher in the vegetative tissues of IR64 and PB1. The miR5802 targeted *expressed* transcript showed negative correlation in the vegetative tissues of all varieties but spikelets of IR64. The expressions of miR2924 target, *TMM* were maintained at high levels in the reproductive tissues of all three varieties. *TMM* profiles were higher in IR64 and PK as compared to PB1.

Overall the expression profiles of miR408-3p: *plastocyanin*, miR399e: *Ubiquitin* and miR5802: *expressed transcript* showed the most difference in the three varieties whereas miR2924: *TMM*, miR5792: *SAC9* and miR1427: *F-DUF* were similar between IR64 and PB1 but different in PK. The genetic variations in the expression profiles seen in PB1, PK and IR64 may reflect on the differences in grain size, quality and yield of the three varieties. Therefore, a comprehensive functional analysis of miRNAs is required to acquire better knowledge of their role in regulating crop yields.

Acknowledgements

The authors are thankful for the funds received from SERB, Government of India and International Centre for Genetic Engineering and Biotechnology, New Delhi. SK is thankful to CSIR, India for Research Fellowship.

Author Contributions Statement

SK performed the experiments. NSM conceptualized and designed the experiments. Both authors wrote, revised and approved the manuscript

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] Abbasi, R., Martinez, P. and Ahmad, R. (2022) The Digitization of Agricultural Industry—A Systematic Literature Review on Agriculture 4.0. *Smart Agricultural Technology*, **2**, Article ID: 100042. https://doi.org/10.1016/j.atech.2022.100042

[2] Chen, T., Shabala, S., Niu, Y., Chen, Z.-H., Shabala, L., Meinke, H., Venkataraman, G., Pareek, A., Xu, J. and Zhou, M. (2021) Molecular Mechanisms of Salinity Tolerance in Rice. *The Crop Journal*, **9**, 506-520. https://doi.org/10.1016/j.cj.2021.03.005

[3] Khush, G.S. (1997) Origin, Dispersal, Cultivation and Variation of Rice. *Plant Mo-*

lecular Biology, **35**, 25-34. https://doi.org/10.1023/A:1005810616885

[4] Babu, N.N., Krishnan, S.G., Vinod, K.K., Krishnamurthy, S.L., Singh, V.K., Singh, M.P., Singh, R., Ellur, R.K., Rai, V., Bollinedi, H. and Bhowmick, P.K. (2017) Marker Aided Incorporation of Saltol, a Major QTL Associated with Seedling Stage Salt Tolerance, into *Oryza sativa* "Pusa Basmati 1121". *Frontiers in Plant Science*, **8**, Article No. 41. https://doi.org/10.3389/fpls.2017.00041

[5] Singh, V.K., Singh, B.D., Kumar, A., Maurya, S., Krishnan, S.G., Vinod, K.K., Singh, M.P., Ellur, R.K., Bhowmick, P.K. and Singh, A.K. (2018) Marker-Assisted Introgression of Saltol QTL Enhances Seedling Stage Salt Tolerance in the Rice Variety "Pusa Basmati 1". *International Journal of Genomics*, **2018**, Article ID: 8319879. https://doi.org/10.1155/2018/8319879

[6] Khush, G.S. and Virk, P.S. (2005) IR Varieties and Their Impact. International Rice Research Institute, Los Baños, 163 p.

[7] Okami, M., Kato, Y., Kobayashi, N. and Yamagishi, J. (2015) Morphological Traits Associated with Vegetative Growth of Rice (*Oryza sativa* L.) during the Recovery Phase after Early-Season Drought. *European Journal of Agronomy*, **64**, 58-66. https://doi.org/10.1016/j.eja.2014.12.006

[8] Mackill, D.J. and Khush, G.S. (2018) IR64: A High-Quality and High-Yielding Mega Variety. *Rice*, **11**, 1-11. https://doi.org/10.1186/s12284-018-0208-3

[9] Rana, P., Nainawatee, H.S., Sindhu, A., Jain, R.K. and Chowdhury, J.B. (1999) RAPD-Based Assessment of Diversity in Indica Rice Genotypes. *Indian Journal of Experimental Biology*, **37**, 1209-1212.

[10] Xie, J.H., Zapata-Arias, F.J., Shen, M. and Afza, R. (2000) Salinity Tolerant Performance and Genetic Diversity of Four Rice Varieties. *Euphytica*, **116**, 105-110. https://doi.org/10.1023/A:1004041900101

[11] Rajani, R., Das, S., Choudhury, P.R. and Mandal, A.B. (2010) Genotyping of a Mapping Population Derived from Salt Susceptible and Salt Tolerant Varieties (IR 28 × Pokkali) of Rice. *Indian Journal of Biotechnology*, **9**, 147-152.

[12] Rubio-Somoza, I. and Weigel, D. (2011) MicroRNA Networks and Developmental Plasticity in Plants. *Trends in Plant Science*, **16**, 258-264. https://doi.org/10.1016/j.tplants.2011.03.001

[13] Zheng, L.L. and Qu, L.H. (2015) Application of MicroRNA Gene Resources in the Improvement of Agronomic Traits in Rice. *Plant Biotechnology Journal*, **13**, 329-336. https://doi.org/10.1111/pbi.12321

[14] Peng, T., Teotia, S., Tang, G. and Zhao, Q. (2019) MicroRNAs Meet with Quantitative Trait Loci: Small Powerful Players in Regulating Quantitative Yield Traits in Rice. *Wiley Interdisciplinary Reviews RNA*, **10**, e1556. https://doi.org/10.1002/wrna.1556

[15] Das, S.S., Karmakar, P., Nandi, A.K. and Sanan-Mishra, N. (2015) Small RNA Mediated Regulation of Seed Germination. *Frontiers in Plant Science*, **6**, Article No. 828. https://doi.org/10.3389/fpls.2015.00828

[16] Zhang, Y.C., Yu, Y., Wang, C.Y., Li, Z.Y., Liu, Q., Xu, J., Liao, J.Y., Wang, X.J., Qu, L.H., Chen, F. and Xin, P. (2013) Overexpression of microRNA OsmiR397 Improves Rice Yield by Increasing Grain Size and Promoting Panicle Branching. *Nature Biotechnology*, **31**, 848. https://doi.org/10.1038/nbt.2646

[17] Zhang, J.P., Yu, Y., Feng, Y.Z., Zhou, Y.F., Zhang, F., Yang, Y.W., Lei, M.Q., Zhang, Y.C. and Chen, Y.Q. (2017) MiR408 Regulates Grain Yield and Photosynthesis via a Phytocyanin Protein. *Plant Physiology*, **175**, 1175-1185. https://doi.org/10.1104/pp.17.01169

[18] Duan, P., Ni, S., Wang, J., Zhang, B., Xu, R., Wang, Y., Chen, H., Zhu, X. and Li, Y. (2015) Regulation of OsGRF4 by OsmiR396 Controls Grain Size and Yield in Rice. *Nature Plants*, **2**, 1-5. https://doi.org/10.1038/nplants.2015.203

[19] Li, S., Gao, F., Xie, K., Zeng, X., Cao, Y., Zeng, J., He, Z., Ren, Y., Li, W. and Deng, Q. (2016) The OsmiR396c-OsGRF4-OsGIF1 Regulatory Module Determines Grain Size and Yield in Rice. *Plant Biotechnology Journal*, **14**, 2134-2146. https://doi.org/10.1111/pbi.12569

[20] Mittal, D., Mukherjee, S.K., Vasudevan, M. and Sanan-Mishra, N. (2013) Identification of Tissue-Preferential Expression Patterns of Rice miRNAs. *Journal of Cellular Biochemistry*, **114**, 2071-2081. https://doi.org/10.1002/jcb.24552

[21] Dai, X. and Zhao, P.X. (2011) psRNATarget: A Plant Small RNA Target Analysis Server. *Nucleic Acids Research*, **39**, W155-W159. https://doi.org/10.1093/nar/gkr319

[22] Vaucheret, H. (2006) Post-Transcriptional Small RNA Pathways in Plants: Mechanisms and Regulations. *Genes and Development*, **20**, 759-771. https://doi.org/10.1101/gad.1410506

[23] Zhu, Q.-H., Spriggs, A., Matthew, L., Fan, L., Kennedy, G., Gubler, F. and Helliwell, C. (2008) A Diverse Set of microRNAs and microRNA-Like Small RNAs in Developing Rice Grains. *Genome Research*, **18**, 1456-1465. https://doi.org/10.1101/gr.075572.107

[24] Wu, L., Zhou, H., Zhang, Q., Zhang, J., Ni, F., Liu, C. and Qi, Y. (2010) DNA Methylation Mediated by a microRNA Pathway. *Molecular Cell*, **38**, 465-475. https://doi.org/10.1016/j.molcel.2010.03.008

[25] Li, S., Castillo-González, C., Yu, B. and Zhang, X. (2017) The Functions of Plant Small RNAs in Development and in Stress Responses. *The Plant Journal*, **90**, 654-670. https://doi.org/10.1111/tpj.13444

[26] Ozata, D.M., Gainetdinov, I., Zoch, A., O'Carroll, D. and Zamore, P.D. (2019) PIWI-Interacting RNAs: Small RNAs with Big Functions. *Nature Reviews Genetics*, **20**, 89-108. https://doi.org/10.1038/s41576-018-0073-3

[27] Liu, T.-Y., Huang, T.-K., Tseng, C.-Y., Lai, Y.-S., Lin, S.-I., Lin, W.-Y., Chen, J.-W. and Chiou, T.-J. (2012) PHO_2-Dependent Degradation of PHO1 Modulates Phosphate Homeostasis in Arabidopsis. *The Plant Cell*, **24**, 2168-2183. https://doi.org/10.1105/tpc.112.096636

[28] Sullivan, J.A., Shirasu, K. and Deng, X.W. (2003) The Diverse Roles of Ubiquitin and the 26S Proteasome in the Life of Plants. *Nature Reviews Genetics*, **4**, 948-958. https://doi.org/10.1038/nrg1228

[29] Moon, J., Parry, G. and Estelle, M. (2004) The Ubiquitin-Proteasome Pathway and Plant Development. *The Plant Cell*, **16**, 3181-3195. https://doi.org/10.1105/tpc.104.161220

[30] Nadeau, J.A. and Sack, F.D. (2002) Control of Stomatal Distribution on the Arabidopsis Leaf Surface. *Science*, **296**, 1697-1700. https://doi.org/10.1126/science.1069596

[31] Zhong, R. and Ye, Z.-H. (2003) The SAC Domain-Containing Protein Gene Family in Arabidopsis. *Plant Physiology*, **132**, 544-555. https://doi.org/10.1104/pp.103.021444

[32] Kozomara, A., Birgaoanu, M. and Griffiths-Jones, S. (2019) miRBase: From microRNA Sequences to Function. *Nucleic Acids Research*, **47**, D155-D162. https://doi.org/10.1093/nar/gky1141

[33] Pan, J., Huang, D., Guo, Z., Kuang, Z., Zhang, H., Xie, X., Ma, Z., Gao, S., Lerdau,

M.T. and Chu, C. (2018) Overexpression of microRNA408 Enhances Photosynthesis, Growth, and Seed Yield in Diverse Plants. *Journal of Integrative Plant Biology*, **60**, 323-340. https://doi.org/10.1111/jipb.12634

[34] Zhang, F., Zhang, Y.-C., Zhang, J.-P., Yu, Y., Zhou, Y.-F., Feng, Y.-Z., Yang, Y.-W., Lei, M.-Q., He, H. and Lian, J.-P. (2018) Rice *UCL8*, a Plantacyanin Gene Targeted by mir408, Regulates Fertility by Controlling Pollen Tube Germination and Growth. *Rice*, **11**, 1-6. https://doi.org/10.1186/s12284-018-0253-y

[35] Song, Z., Zhang, L., Wang, Y., Li, H., Li, S., Zhao, H. and Zhang, H. (2018) Constitutive Expression of miR408 Improves Biomass and Seed Yield in Arabidopsis. *Frontiers in Plant Science*, **8**, Article No. 2114. https://doi.org/10.3389/fpls.2017.02114

[36] Fujii, H., Chiou, T.-J., Lin, S.-I., Aung, K. and Zhu, J.-K. (2005) A miRNA Involved in Phosphate-Starvation Response in *Arabidopsis*. *Current Biology*, **15**, 2038-2043. https://doi.org/10.1016/j.cub.2005.10.016

[37] Chiou, T.-J., Aung, K., Lin, S.-I., Wu, C.-C., Chiang, S.-F. and Su, C.-L. (2006) Regulation of Phosphate Homeostasis by microRNA in Arabidopsis. *The Plant Cell*, **18**, 412-421. https://doi.org/10.1105/tpc.105.038943

[38] Hu, B., Zhu, C., Li, F., Tang, J., Wang, Y., Lin, A., Liu, L., Che, R. and Chu, C. (2011) Leaf Tip Necrosis1 Plays a Pivotal Role in the Regulation of Multiple Phosphate Starvation Responses in Rice. *Plant Physiology*, **156**, 1101-1115. https://doi.org/10.1104/pp.110.170209

[39] Hu, B., Wang, W., Deng, K., Li, H., Zhang, Z., Zhang, L. and Chu, C. (2015) MicroRNA399 Is Involved in Multiple Nutrient Starvation Responses in Rice. *Frontiers in Plant Science*, 6, Article No. 188. https://doi.org/10.3389/fpls.2015.00188

[40] Lin, W.-Y., Huang, T.-K. and Chiou, T.-J. (2013) Nitrogen Limitation Adaptation, a Target of microRNA827, Mediates Degradation of Plasma Membrane-Localized Phosphate Transporters to Maintain Phosphate Homeostasis in Arabidopsis. *The Plant Cell*, **25**, 4061-4074. https://doi.org/10.1105/tpc.113.116012

[41] Park, B.S., Seo, J.S. and Chua, N.-H. (2014) Nitrogen Limitation Adaptation Recruits Phosphate2 to Target the Phosphate Transporter PT2 for Degradation during the Regulation of Arabidopsis Phosphate Homeostasis. *The Plant Cell*, **26**, 454-464. https://doi.org/10.1105/tpc.113.120311

[42] Santner, A. and Estelle, M. (2010) The Ubiquitin-Proteasome System Regulates Plant Hormone Signaling. *The Plant Journal*, **61**, 1029-1040. https://doi.org/10.1111/j.1365-313X.2010.04112.x

[43] Song, X.-J., Huang, W., Shi, M., Zhu, M.-Z. and Lin, H.-X. (2007) A QTL for Rice Grain Width and Weight Encodes a Previously Unknown RING-Type E3 Ubiquitin Ligase. *Nature Genetics*, **39**, 623-630. https://doi.org/10.1038/ng2014

[44] Clifford, R., Lee, M.-H., Nayak, S., Ohmachi, M., Giorgini, F. and Schedl, T. (2000) FOG-2, a Novel F-Box Containing Protein, Associates with the GLD-1 RNA Binding Protein and Directs Male Sex Determination in the *C. elegans* Hermaphrodite Germline. *Development*, **127**, 5265-5276. https://doi.org/10.1242/dev.127.24.5265

[45] Galan, J.-M., Wiederkehr, A., Seol, J.H., Haguenauer-Tsapis, R., Deshaies, R.J., Riezman, H. and Peter, M. (2001) Skp1p and the F-box Protein Rcy1p form a Non-SCF Complex Involved in Recycling of the SNARE Snc1p in Yeast. *Molecular and Cellular Biology*, **21**, 3105-3117. https://doi.org/10.1128/MCB.21.9.3105-3117.2001

[46] Kim, H.S. and Delaney, T.P. (2002) Arabidopsis SON1 Is an F-Box Protein That Regulates a Novel Induced Defense Response Independent of Both Salicylic Acid

and Systemic Acquired Resistance. *The Plant Cell*, **14**, 1469-1482. https://doi.org/10.1105/tpc.001867

[47] Smalle, J. and Vierstra, R.D. (2004) The Ubiquitin 26S Proteasome Proteolytic Pathway. *Annual Review of Plant Biology*, **55**, 555-590. https://doi.org/10.1146/annurev.arplant.55.031903.141801

[48] Yang, M. and Sack, F.D. (1995) The Too Many Mouths and Four Lips Mutations Affect Stomatal Production in Arabidopsis. *The Plant Cell*, **7**, 122227-2239. https://doi.org/10.2307/3870164

[49] Geisler, M., Yang, M. and Sack, F. (1998) Divergent Regulation of Stomatal Initiation and Patterning in Organ and Suborgan Regions of the Arabidopsis Mutants Too Many Mouths and Four Lips. *Planta*, **205**, 522-530. https://doi.org/10.1007/s004250050351

[50] Bhave, N.S., Veley, K.M., Nadeau, J.A., Lucas, J.R., Bhave, S.L. and Sack, F.D. (2009) Too Many Mouths Promotes Cell Fate Progression in Stomatal Development of Arabidopsis Stems. *Planta*, **229**, 357-367. https://doi.org/10.1007/s00425-008-0835-9

[51] Geisler, M., Nadeau, J. and Sack, F.D. (2000) Oriented Asymmetric Divisions that Generate the Stomatal Spacing Pattern in Arabidopsis Are Disrupted by the Too Many Mouths Mutation. *The Plant Cell*, **12**, 2075-2086. https://doi.org/10.1105/tpc.12.11.2075

[52] Williams, M.E., Torabinejad, J., Cohick, E., Parker, K., Drake, E.J., Thompson, J.E., Hortter, M. and DeWald, D.B. (2005) Mutations in the Arabidopsis Phosphoinositide Phosphatase Gene SAC9 Lead to Overaccumulation of PtdIns (4, 5) P2 and Constitutive Expression of the Stress-Response Pathway. *Plant Physiology*, **138**, 686-700. https://doi.org/10.1104/pp.105.061317