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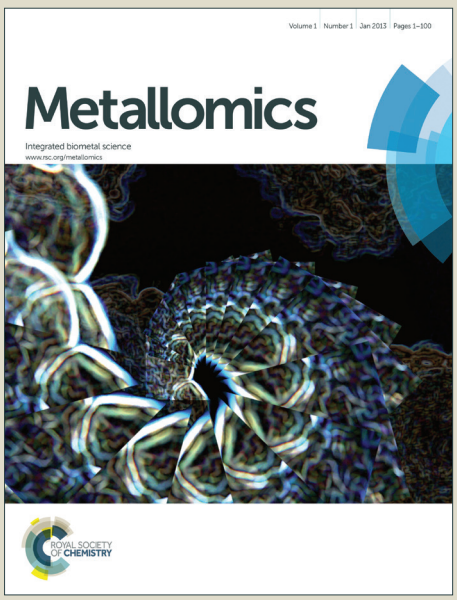

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Differential expression of microRNAs by arsenate and arsenite stress in natural accessions of rice

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ABSTRACT

Arsenic (As) contamination of rice (*Oryza sativa*) imposes serious threat to human health worldwide. Understanding the molecular mechanisms of As transport and accumulation in rice may provide promising solutions to the problem. MicroRNAs (miRNAs) are novel class of short, endogenous, non-coding small RNAs involved in wide variety of biological processes such as organ polarity, morphogenesis, floral transition, hormone signalling and adaptation to environment. In past, few studies led to the identification of differentially expressed miRNAs in rice in response to arsenite (AsIII) stress. However, studies related to differential miRNA expression involving rice natural accessions exposed to different species of As have not been carried out. Such studies are required to identify As-species responsive miRNAs in different rice accessions. In this study, we have carried out miRNAs profiling in contrasting As accumulating rice accessions using miRNA Array. We report identification of differentially expressed miRNAs in contrasting As accumulating rice cultivars in response to AsIII (25 μM) and AsV (50 μM) stress. A significant up-regulation in expression was observed among members of miR396, miR399, miR408, miR528, miR1861, miR2102 and miR2907 families in response to AsIII and AsV stress in both cultivars. In addition, members of miR164, miR171, miR395, miR529, miR820, miR1432 and miR1846 families were down-regulated. The differentially expressed miRNAs were subjected to validation of expression and bioinformatic analyses to predict and categorise the key miRNAs and their target genes involved in As stress. Analysis suggests As-species and rice accession specific miRNA might be responsible for the differential response of contrasting rice accessions towards AsIII and AsV stress. Study of proximal promoter sequences of the As-responsive miRNAs suggests that these identified miRNAs contain metal-responsive *cis*-acting motif and other elicitor and hormonal related motifs. Our study suggests miRNA-dependent regulatory mechanism during As species-specific stress in different rice accessions. Further analysis based on results obtained will be helpful in dissecting the molecular mechanism behind As responses in different rice accessions.

Keywords: Arsenic, Gene expression, Microarray, MicroRNA, Natural accessions, Rice.

INTRODUCTION

Arsenic (As), a toxic metalloid, found naturally in the earth crust is a global challenge to human being and other life forms. It is a major concern in many South-east Asian developing countries, where the amount of As in ground water is present more than the permissible level. Arsenic level in ground water of Bangladesh and West Bengal region of India exceeds upto 50 μgL^{-1} to World Health Organization guidelines[1] recommendations that is 10 μgL^{-1}. The presence of As in drinking water and food is a serious concern to human health.[2, 3] Arsenic contaminated water used for irrigation of paddy fields causes high As level in rice grains. Since rice is the staple crop growing in these areas, population living in these areas as well as around the world is currently under dietary threat to As toxicity.[4] Based on plethora of reports, it is established that As containing crops mainly rice are the primary avenue of As exposure to people. Studies also revealed that As is equally harmful to plants and it can limits uptake and accumulation of essential micro-nutrients and amino acids.[5, 6]

Owing to the huge negative effect on human health, studies related to As uptake, accumulation, transport and detoxification in rice has gained momentum in past few years with major objective to develop less As accumulating rice cultivars.[7] With the application of high throughput techniques like transcriptome and proteome profiling, knowledge about the underlying molecular mechanisms of As metabolism in rice has enhanced.[8-12] Arsenite (AsIII) and arsenate (AsV) are predominant inorganic species of As in soil and depending upon the changing redox potential and pH, these two inorganic As species are readily interconvertible.[13] AsV is the most prevalent form of As in aerobic soils and analogue of inorganic phosphate. Due to this chemical similarity, uptake of AsV is mediated through phosphate transporters into the plant cells.[14-17] AsIII, on the other hand, mainly predominates in anaerobic conditions such as flooded paddy soil and moves into the roots via nodulin 26-like intrinsic protein (NIP) aquaporin channels. NIP2 (Lsi1), which is known for its permeability to silicon, mediates the bidirectional transport of AsIII in rice.[17] One of the established mechanisms of As detoxification in plants depends on complexation with cellular thiols followed by sequestration into vacuoles. [19, 26, 27] Within the plant system, AsV is reduced to AsIII which is ultimately sequestered into vacuoles after conjugating with glutathione (GSH) or phytochelatin (PC).[10] The transcriptome modulation suggests that number of genes involved in diverse physiological processes might also be playing important role during AsIII and AsV stress.[9, 6, 12] Recently, rice NRAMP (Natural Resistance-

Associated Macrophage Protein) transporter, OsNRAMP1, has been reported to be involved in xylem loading and enhanced accumulation of As in *Arabidopsis*.[18]

Both the inorganic forms of As are highly toxic, of which AsIII interacts and binds with sulfydryl groups of proteins and therefore inhibits their functions. On the other hand, AsV interferes with phosphate metabolism by inhibiting phosphorylation and ATP synthesis.[3, 19, 20] Recent studies regarding a bacterial strain (GFAJ-1) showed that organisms can rely on As instead of phosphorus. These studies suggested that AsV can replace phosphate in biomolecules that are essential to sustain cell life.[21, 22] It was suggested that, within a bacterium, AsV interacts and replaces phosphate to form arsenylated analogs with small molecular weight metabolites such as NADH, ATP, glucose, and acetyl-CoA. It also substitutes phosphate at serine, tyrosine, and threonine residues to form arsenylated proteins.[21, 22] Apart from this, a series of recent studies report the substantial virtue of anti-cancerous activity of AsIII for curing acute promyelocytic leukemia patient.[23-25] These studies also described and successfully dissected the molecular mechanism of anti-cancerous property of AsIII.

miRNAs are established as critical determinant of growth and development of organism.[28-30] miRNAs comprise a major class of non-coding small RNAs which mediate silencing of their targets by either translation inhibition or mRNA cleavage in plants.[31, 28, 32-34] In addition to regulating plant growth and development, miRNAs are known to regulate gene expressions in response to nutritional deprivation as well as biotic and abiotic stresses.[35-39] Recently, role of miRNAs has been investigated during metal stress.[40-42] Using miRNA microarray, cadmium (Cd) responsive miRNAs have been identified in rice[36] and Brassica.[43] Through transcriptome sequencing Yu *et al.* (2012)[44] have identified AsIII responsive miRNAs in rice. In addition, miRNAs have been identified later through deep sequencing of small RNA library in the root of rice seedling under AsIII stress.[45, 46] However, despite having huge impact on gene expressions, a little is known about the proportion of miRNAs mediated regulation of regulatory network of As assimilation in rice. Most of these studies have been carried out on AsIII stress on specific rice germplasm. Studies suggests that transcriptomes of different rice germplasms are modulated differentially in response to AsIII and AsV stress.[9, 12] Therefore, it seems that differential miRNA expression pattern might be playing important role in modulating transcriptomes of rice germplasms in response to AsIII and AsV stress.

The study of natural variations is often considered as indispensable system now a day in biology.[47] Natural accessions of *Arabidopsis* are frequently used for understanding the mechanisms and pinpoint the causal genes underlying a particular allele.[48] Similar to *Arabidopsis*, a large genetic variation of rice germplasm has been identified from Indian subcontinent,[49] and these accessions can serve as a model to identify mechanisms that enable them to tolerate various stresses. Screening of rice accessions commonly growing in Indian subcontinent for As level in grains established a range of cultivar with varying potential of As accumulation, including High As accumulating Rice Germplasm (HARG) and Low As accumulating Rice Germplasm (LARG).[50, 51] However, limited information about the role of miRNAs in different species of As stress in different rice germplasm is available. This study has been carried out to investigate the role of miRNA mediated gene regulation in different species of As (AsIII and AsV) using contrasting As accumulating rice accessions (HARG and LARG). We have identified set of miRNA with differential expression and can have possible role in As stress. Some of these miRNAs show germplasm as well as As-species specific expression and might be playing important role in deciding contrasting response of these germplasms. Our data provide more insights about role of miRNA for understanding molecular basis of AsIII and AsV stress in HARG and LARG.

EXPERIMENTAL

Plant growth conditions and treatments

Seeds of HARG (IC-115730) and LARG (IC-340072) rice (*Oryza sativa* L. *indica*) genotypes[50] were utilized for the study. Seeds were surface sterilized in water containing 0.1% $HgCl_2$ for 30 s, washed with sterile water and kept for overnight in milli-Q water. Soaked seeds were transferred into Petri plates and incubated at 28º C for 3-4 days for germination. Germinated rice seedlings were transferred in modified Hewitt medium[52] and grown under controlled conditions, temperature (28±2° C), relative humidity (70%) and 16/8-h light/dark cycle for 10 days. Plants were subjected to AsIII [10 µM and 25 µM (stock solution 10 mM; $NaAsO_2$, ICN, USA)] and AsV [50 µM and 100 µM (stock solution 50 mM; Na_2HAsO_4, ICN, USA] stress for seven days. All nutrient solutions were changed twice per week, and pH was adjusted to 5.5 using 0.1 KOH or HCl. After that, seedlings were harvested, washed and stored immediately at -80° C for further RNA extraction.

Microarray experiment and data analysis

Total RNA was isolated from root samples using mirVanaTM miRNA isolation kit (Ambion, USA) according to manufacturer's instructions. Amount of RNA was measured with a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, USA). Presence of small RNAs was determined by denaturing polyacrylamide (15%) gel electrophoresis. RNA was labelled using FlashTag Biotin HSR RNA Labelling Kits (Genesphere, USA). Labelled RNA was hybridised at Affymetrix GeneChip miRNA-2.0 for 16 hrs. During hybridisation, washing and staining, Affymetrix GeneChip protocols (Affymetrix, USA) were strictly followed. Percentage of hybridization was analysed with Affymetrix miRNA QC Tool. Normalization of CEL files have been performed by using R-bioconductor setting $P \leq 0.05$.[53]

For construction of heat map related to expression of targets, the CEL file of our previous study that described the effect of AsV on rice seedlings was taken into consideration.[9] Affymetrix rice genome array probe IDs of targets were identified through searching at Rice Multi-platform Microarray Search tool (http://www.ricearray.org/element/search_single.shtml).[54] Expression map was generated using Dchip software.[55]

miRNA expression analysis

To validate the differential expression of mature miRNAs, TaqMan MicroRNA assay kit (Applied Biosystems, Foster City, CA) was used for selected miRNAs identified through microarray analysis. TaqMan assays specifically detect mature and biologically active miRNA. Total RNA (0.2 µg) was used for reverse transcription reaction using miRNA specific primers included in the TaqMan MicroRNA assay kit. Real-time PCR was performed using the ABI 7500 instrument (Applied Biosystems, Foster City, CA, USA). In a total volume of 20 µl of reaction mixture, 1.33 µl of complementary DNA templates were mixed with 10 µl of TaqMan Universal Master Mix No AmpEras UNG (Applied Biosystems, Foster City, CA) along with 1 µl of TaqMan small RNA assays (20X) were used. A standard TaqMan protocol with the reaction condition for RT-PCR were 50° C for 2 min and 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60° C for 60 s was followed. Ubiquitin (U6) was used as endogenous control for data normalization. The relative fold change of the miRNAs in the treated samples to the controls was calculated using the $2^{-\Delta\Delta CT}$ method. Expression of each miRNA was analysed in triplicate in three independent experiments.

Target prediction of miRNAs and expression analysis

To predict the targets, online miRNA target prediction tool psRNATarget (http://plantgrn.noble.org/psRNATarget/) was used by searching miRNAs in cDNA OSA1 release 5 as reference genome.[56] The default parameters were set and minimal weighed score <3.0 have applied to sort the potential targets. Gene ontology annotation of targets, especially the molecular function has been assigned by using AgriGO (http://bioinfo.cau.edu.cn/agriGO/) web-based analysis tool.[57]

To study, expression of target genes, quantitative RT-PCR (qRT-PCR) analysis was carried out. Approximately, 2 µg RNase free DNase treated total RNA isolated from different rice samples was reverse-transcribed using SuperScriptII (Fermentas, USA), following the manufacturer's recommendation. The synthesized cDNA was diluted 1:20 in DEPC water and subjected to qRT-PCR analysis using SYBR Green Supermix (ABI Biosystems, USA) in an ABI 7500 instrument (ABI Biosystems, USA). Rice actin gene was used as an internal control to estimate the relative transcript level of the gene tested. The list of different genes and oligonucleotides used is provided in Table S1.

Motif analysis in promoters of miRNAs

The 2-kbp promoter sequences of all differentially expressed miRNAs were retrieved from the plant miRNA database (http://bioinformatics.cau.edu.cn/PMRD/).[58] Sequences were individually searched for presence of *cis*-elements by using PlantCARE tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/).[59] The distribution of individual motifs among all the promoters was manually investigated.

Statistical analysis

Each experiment was carried out under completely randomized design with three replicates repeated at least thrice. The data were analyzed by student's unpaired t-test, and the treatment mean values were compared at $P \leq 0.05–0.001$.

RESULTS AND DISCUSSION

Inorganic arsenic species inhibit growth of HARG and LARG

Similar to the natural variation of *Arabidopsis*, rice accessions are also becoming potentially valuable in terms of studying various agronomically important traits and in revealing the biology of adaptive responses towards abiotic stresses. Due to the impact of As on nutritional values of rice and associated toxicity in human being, an extensive screening of native accessions of rice grown in Indian subcontinent for their inherent characteristics to accumulate varying levels of As in grains was performed.[6, 50, 51] The investigation revealed variation in As accumulation patterns existed in a diverse set of rice accessions. These accessions were categorised as High As accumulating Rice Germplasm (HARG) and Low As accumulating Rice Germplasm (LARG).[6] In addition, AsIII tolerant and sensitive accessions were screened from 303 rice genotypes on exposure to AsIII (10 μM and 25 μM) in hydroponic media. As accumulation analysis suggested contrast (13-fold difference) in As accumulation between HARG (IC-115730) and LARG (IC-340072) accessions. Studies also reported that a higher antioxidant potential and stress responsive amino acids level was seen in case of HARG compared to the LARG.[50] The differences between HARG and LARG are might be due to presence of evolutionary genetic variations. In this study, we studies growth of HARG (IC-115730) and LARG (IC-340072) accessions after exposure to two inorganic species of As (AsIII and AsV). Ten-day-old rice seedlings were grown in the presence of AsIII (10 and 25 μM) and AsV (50 and 100 μM) for 7 days. A substantial reduction in overall growth of both the cultivars was noted in presence of AsIII and AsV (Fig. 1A-B). However, the observed phenotypic difference was not significant in both the cultivars upon AsV treatment (Fig. 1B). Also, it is observed that As exposure stimulates more lateral root formation in both the cultivars. This suggests contrasting response of HARG and LARG accession towards different species of As. Studies have already reported that out of both inorganic As-species, AsIII is more toxic in comparison to AsV due to its effect on enzymes and proteins containing cysteine residue which leads to conformational distortion as well as prevents the disulphide bridge formation.[60]

Modulated expression of miRNAs in HARG and LARG in response to arsenic

To study possible involvement of miRNAs for the contrasting response of HARG and LARG towards AsIII and AsV stress, global miRNAs expression analysis was carried out. The high throughput techniques like microarray and small RNA library sequencing are frequently used

for studying the involvement of miRNAs in growth, development and towards stress response in organisms. Many stress related crucial miRNAs in *Arabidopsis* and Cd responsive miRNAs in rice were previously identified through microarray platform.[36, 40] Here, we have utilized microarray for studying the role of candidate miRNAs responsible for differential As response of HARG and LARG. We performed miRNA microarray using Gene Chip miRNA 2.0 arrays (Affymetrix) with RNA isolated from AsIII (25 μM) and AsV (50 μM) treated rice roots. This array contains complete set of miRNAs documented in miRBase release 15 that covers miRNA of 131 organisms including *Arabidopsis thaliana*, *Brassica napus*, *Glycine max*, *Populus trichocarpa*, *Sorghum bicolor*, *Triticum aestivum* and many others. miRNAome of rice is represented by the presence of 496 individual miRNAs on array.

Our analysis showed hybridization of a large number of miRNAs from rice in addition to other plants present on array. This may be due to high degree of sequence resemblance between miRNAs present on the array used in the analysis.[61] For detailed analysis, we have considered only those miRNAs which hybridized with probe sets of rice miRNAs to avoid any discrepancy. The analysis revealed that a set of miRNA from different families express differentially in both the cultivars in response to AsIII and AsV stress. Analysis revealed that 114 members of 30 miRNA families were differentially expressed upon AsIII exposure and among these 24 miRNAs were up- and 90 were down-regulated respectively in HARG (Fig. 2A). Similarly, 166 members of 62 miRNA families were differentially expressed and out of which 5 and 161 members were up- and down-regulated respectively in LARG (Fig. 2A). It is interesting to note that majority of miRNAs were down-regulated in LARG as compared to HARG. Of the identified set, only 5 miRNAs showed AsIII induced expression in LARG whereas 24 miRNAs were up-regulated in HARG. This suggests that modulated expression of miRNAs, in terms of number of miRNA between HARG and LARG, could be responsible for their variable responses towards AsIII stress in these cultivars.

Our phenotypic studies suggest that in contrast to AsIII, there is no significant change in growth of HARG and LARG under AsV stress (Fig. 1). This fact is also supported by many earlier studies which described that AsV is less toxic as compared to AsIII.[9, 62] Our analyses clearly suggest that AsIII modulate expression of significant number of miRNAs in comparison to AsV (Fig. 2). Of the total 81 miRNAs, 51 members are up- and 30 are down-regulated respectively in HARG (Fig. 2B). Likewise, 37 and 40 members are up- and down-regulated respectively in LARG (Fig. 2B). The analysis suggests that the expression of a

large number of miRNAs is significantly modulated in either HARG or LARG under AsIII and AsV stress. This might be one of the major reasons for the contrasting nature of cultivars towards AsIII and AsV stress. The details of miRNAs with significantly modulated expression under AsIII and AsV stress as well as in HARG and LARG are provided in Supplementary Fig. S1.

Quantitative RT-PCR analysis of miRNAs in HARG and LARG

Our analysis identified a set of miRNAs with modulated expression in response to AsIII and AsV stress in rice. Some of these miRNAs showed rice genotype-specific expression in response to specific As species. To further identify miRNAs with significant change in the expression, threshold value for the fold change expression was increased to 1.5 fold ($P \leq 0.05$). Interestingly, 14 miRNAs distributed in various families were identified through this analysis are listed in Table 1.

To validate the microarray data, quantitative real time PCR analysis of selected miRNAs from different gene families (miR164e, miR171g, miR395b, miR399h, miR528, miR529b, miR820a and miR1432) was performed. The analysis suggests that expressions of most of miRNAs are in accordance to that of microarray results. Similar to microarray data, a contrast expression between HARG and LARG was observed for some of the miRNAs in qRT-PCR results. A set of miRNA including miR171g, miR529b, miR820a and miR1432 was repressed in HARG whereas their expressions were significantly enhanced in LARG in response to AsIII stress (Fig. 3A). Surprisingly, microarray expression pattern of few miRNAs was not correlated with RT-PCR results. The example of such miRNAs are miR820a and miR1432 whose expressions were down-regulated in microarray in LARG, while in the qRT-PCR enhanced expression was observed under AsIII and AsV stresses (Fig. 3). One possible explanation of such inconsistency may be due to degenerate probe sets for the miRNAs, which may have cross hybridised with same miRNA of other species. However, Taqman probes used in qRT-PCR would have measured miRNA level accurately.

A differential expression pattern was observed among members within each miRNA family. In miR2907 family, miR2907a, miR2907d were significantly up-regulated whereas, miR2907b and miR2907c were down-regulated in LARG variety in response to AsIII stress. Similarly, miR162, miR166, miR396, miR529, miR1846 and miR1862 family members showed differential expression patterns (Table S2). In previous study, numerous AsIII responsive miRNAs including miR156, miR159, miR171, miR396, miR444, miR535,

miR820, miR827 and miR1432 were identified in AsIII using the Nipponbare rice cultivar.[18, 45, 46, 44] In our study, we have identified set of additional As-stress responsive miRNAs using different rice germplasm. These results suggested possible involvement of these miRNAs for controlling As responses and accumulation in different rice cultivars.

Target prediction of rice miRNA

Various studies have suggested that miRNAs are highly specific for their targets and inhibit target expression post-transcriptionally either through target cleavage or via translation inhibition.[63, 18, 64] To identify the potential targets of As-responsive miRNAs identified in this study, we have employed psRNATarget algorithm, a web-based program using default parameters.[56] miRNAs which showed significantly modulated expression profiles under AsIII and AsV stress in LARG and HARG were chosen for targets identification. Interestingly, most of the predicted targets identified belong to DNA binding proteins, transcription factors such as NAC domain-containing protein, nuclear transcription factor Y, growth-regulating factor 1, AP2, MADS-box, F-box, MYB and SPB family. Most of these transcription factors have been shown to play important role in plant development and during abiotic stresses.[26, 65, 66, 35, 67] Modulated expression of these transcription factors has been shown in several earlier studies,[9, 12] and it was speculated that these transcription factors might play prominent role in determining the physiological response like As tolerance/sensitivity in rice.[9] Numerous *loci* that encoded the metal transport protein, ATP-binding protein, protein kinases and methyltransferases were also predicted among as putative targets of identified miRNAs in this study (Table 2). Importantly, genes involved in the sulphur metabolism such as low affinity sulphate transporter, bifunctional 3-phosphoadenosine 5-phosphosulphate synthetase and ATP sulfurylases are identified as the targets of miRNAs with modulated expression. These genes act as sensor for sulphur limitation and constituent of integral part of the regulatory network of sulphate assimilation in plants.[68-70] Set of genes that maintains redox homeostasis of the cell constitutes is another promising category in the target list (Table 2). These include L-ascorbate oxidase precursor, copper ion binding protein, superoxide dismutase, ZIP zinc/iron transport family protein and blue copper protein precursor.

Gene ontology classification of targets of significantly differentially regulated miRNA targets (≥ 1.5-fold) in both the varieties under AsIII and AsV stress was also performed by using AgriGO online available tool.[57] The molecular characterizations of target

loci indicated that major proportion of target genes belongs to transcription factors followed by DNA binding proteins and genes involved in catalytic activity (Fig. 4).

Targets are negatively related with miRNA expressions

In our analysis, a large number of target *loci* of each miRNA were predicted by psRNATarget. In order to investigate the actual targets among putative targets identified by *in silico* analysis, we further analysed our results. First, we examined the transcript abundance of putative targets in the microarray analysis carried out in our previous study related to the transcriptome modulation during AsV stress in rice seedling.[9] It was observed that majority of targets of up-regulated miRNAs such as miR399, miR408, miR528, miR1861 and miR2907 was down-regulated in the analysis (Fig 5A). Similarly, the expression of targets of down-regulated miRNAs such as miR164, miR171, miR395, miR529, miR820, miR1432 and miR1846 were induced during AsV stress in rice (Fig 5C). This suggests a direct correlation between expression of identified miRNAs in this study and expression of targets during AsV stress.

Second, to validate the expression, qRT-PCR analyses of the putative targets of As-responsive miRNAs in root of HARG and LARG was carried out. It has been demonstrated that miR164 targets no apical meristem (NAM) protein, NAC-like transcription factors and Cup-shaped Cotyledon (CUC) of which CUC1 and CUC2 are necessary for the formation of boundaries between meristems, flower development and proper control of organ number in *Arabidopsis.*[65-67] Some NAC proteins, which are putative target of miR164 for instance NAC1 participate in transducing auxin signals for the development lateral roots.[71] Emergence of more lateral roots under As stressed condition (Fig. 1) might be due to modulated transcripts of NAC. miR171 is known to target scarecrow-like regulators which participated in signal transductions, root development and plant development.[72, 73] Our data clearly revealed an inverse relation between expression of miR171 and its predicted target Os06g01620 which encodes scarecrow-like 6 protein (Fig. 5C). miR395 regulates the expression of low-affinity sulphate transporter (SULTR2;1) and ATP sulfurylases involved in sulphate metabolism.[69] miR395 is strongly induced upon sulphate starved conditions and controls sulphur uptake required during plant growth and development as well as in stress conditions.[68, 74] It has been demonstrated that the demand of sulphur generally increases during As stress due to enhanced biosynthesis of sulphur containing amino acids and peptides required for As detoxification.[75-78] The down-regulation of miR395 in HARG and LARG and

thereby up-regulation of sulphate transporter (Os03g09940) supports involvement of regulated sulphur homeostasis during As stress. Importantly, significantly higher expression of Os03g09940 in HARG is in accordance with its higher As accumulation phenotype. This higher As uptake may require high cellular thiols and sulphur for detoxification regulated by miR395.

The expression of miR399 was induced in both cultivars as evident from microarray and RT-PCR results (Fig. 3 and 5A). The inorganic phosphate starvation induces miR399 that represses E2 conjugase and regulate phosphate assimilation in plants.[79] Due to structural analogy, AsV shares phosphate transporters for the uptake and translocation.[17, 80] In this study, a substantial high expression (nearly 15 fold) of miR399 in LARG was observed in AsIII and upto 3 fold in AsV stress compared to HARG. However, expression of one of the putative targets, ubiquitin conjugating enzyme, was not much repressed in HARG compared to LARG (Fig. 5B). The reason of miR399 induction upon As stress might be due to phosphate limitation because of competition with AsV. Another interesting observation, in this study, is the difference in expression of miR528 under AsIII and AsV exposure. The miR528 was induced upon AsIII stress whereas down-regulated upon AsV stress and simultaneously their target expression was also modulated (Table 1, Fig. 5A-B). In general, microarray expression of miR408, miR529, miR820, miR1432, miR1846, miR1861 and miR2907, and their corresponding targets showed inverse relation. Nonetheless, some inconsistency between expression patterns of target through microarray dataset and qRT-PCR was observed which might be due to differential As accumulating rice cultivars used in this study. These observations suggested that modulated expression of these miRNAs could be one of the prime reasons for less As accumulation phenotype of LARG.

Metal responsive *cis*-elements in promoters of miRNAs

Binding of *trans*-acting factors on *cis*-elements of promoter region of any gene and resulted changes in RNA polymerase complex are thought to be one of the critical determinants of gene expression. In past, the interaction of *cis*-elements with *trans*-factors such as stresses induced DNA binding proteins has been identified through extensive research.[81, 82] In this study, expression of several miRNAs from diverse families was modulated during As stress. It is important to study whether specific *cis*-elements and interacting *trans*-factors control their As-responsive expression. To study this, 2-kbp up-stream region of promoters of As-responsive miRNAs was analysed through PlantCare database.[59] Analysis revealed presence

of numerous conserved motifs in these promoters. Some of the conserved motifs include ARE elements (anaerobic induction), abscisic acid responsive elements such as ABRE, motif IIb and CE3, BOX-W1 (response to fungal elicitors), GC-motif (anoxic inducebility), TC-rich repeat for defence and stress response, LTR elements for low temperature, MYB and CCAAT-box elements for MYB binding site involved in drought inducibility, heat stress-responsive elements (HSE) amongst many promoters. Besides these, potential *cis*-acting elements responsive to TCA (Salicylic acid responsive), ERE (ethylene responsive) GARE, TATC and P-box (gibberellins responsive) and methyl jasmonate (MeJA) were also identified in the analysed promoters (Table S3).

The presence of metal recognition elements (MREs; 5'-TGCGCNC-3') in almost every promoter of miRNA genes with modulated expression in response to As stress is an interesting observation in this analysis. MRE-like sequence is a highly conserved motif commonly present in the promoters of metallotioneins in animals.[83] Various *trans*-acting regulatory factors interact with this motif and modulate expression in response to metal stress. MRE motif was evenly distributed in promoters of 14 studied miRNA families (Table S4). The presence of MREs indicates the existence of fine tuned mechanism by which expressions of miRNAs are under the control of *trans*-acting stress induced factors. Curiously, Skn-1_motif was present in almost every promoter of miRNAs with modulated expression during As stress. This motif is required for endosperm specific expression, and function in cooperation with other motifs. Again, the presence of methyl jasmonate responsive element (CGTCA and TGACG motif), abscisic acid responsive ABRE element, salicylic acid responsive TCA- element, gibberellin-responsive GARE-element and defence responsive TC-rich repeats clearly indicate stress induced *trans*-factors/proteins triggers transcriptional activation of these miRNAs. Taken together *cis*-element analysis of promoters suggested that most of the miRNAs identified in this study are stress responsive.

miRNAs expression and HARG and LARG phenotype

The presence of AsIII and AsV led to the significant modulation in miRNAs expression profile in HARG and LARG as listed in Table S2. Most of the identified miRNAs are conserved across plant species and are known to regulate growth and development as well as stress responses in plants. This suggests that AsIII or AsV are not solely responsible for induction of these miRNAs and As response may be linked to various other biotic, abiotic and environmental cues. Studies suggest that miR171, miR396, miR395, miR408 and

miR529 are responsive to salt, cold, mannitol and drought stress in rice.[40, 42, 84] However, a contrast expression profile of a set of miRNAs and their targets between HARG and LARG suggests that their contrasting As accumulating potentials may be regulated by miRNAs. miRNAs are known to work downstream to *trans*-acting proteins such as SA, JA, stress responsive and signal cascading factors. The *cis*-elements, present in the proximal promoters of various miRNAs with modulated expression in response to As stress suggests that regulations of these miRNAs are not only controlled with the SA and JA pathway, but also by GA, ethylene, ABA and other factors. Hence, an assessment of such factors including epigenetic changes which are known to regulate expression of miRNAs in LARG and HARG rice accessions will be required to dissect the molecular basis of adaptive trait.

CONCLUSIONS

Arsenic, highly toxic metalloid, is present in low amount in the environment and is a dreadful health hazard to millions of people across the globe. Inorganic forms of As, AsIII and AsV, present in the soil accumulate in the plant parts and contaminate food chain. There is an urgent need to develop strategies to restrict As accumulation in plants, however, this requires understanding of molecular mechanisms involved in As uptake, accumulation and detoxification. Previous studies based on microarray and deep sequencing has identified numerous differentially expressed genes in response to AsIII and AsV in rice. In addition, several AsIII-responsive miRNAs have been identified in rice. As uptake and transport mechanisms of these As species differ, it is important to investigate the involvement of AsIII- and AsV-responsive miRNAs and their targets. In this study, microarray hybridization was carried out to identify differentially expressed miRNAs in response to AsIII and AsV using two rice cultivars contrasting in As response and accumulation. Though, a number of miRNAs were differentially expressed in As species- and cultivar-specific manner, expression of 14 miRNAs was modulated in response to AsIII and AsV in both the cultivars. Among these miRNAs, members of miR396, miR399, miR408, miR528, miR1861, miR2102 and miR2907 families were significantly up-regulated whereas members of miR164, miR171, miR395, miR529, miR820, miR1432 and miR1846 families were down-regulated. Predicted targets for identified As-responsive miRNAs and study of *cis*-regulatory elements supported our finding regarding involvements of these miRNAs in As-stress. Altogether, our data indicate that differentially expressed miRNAs in HARG and LARG may encompass a critical sector of molecular responses for the As-stress adaptation in rice cultivars.

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FIGURE LEGENDS

Fig. 1: Representative picture showing effect of As-stress on morphology/phenotype of HARG and LARG rice cultivars. The rice cultivars, HARG and LARG, were germinated and allowed to grow for 5 days at 37 °C and then transferred to Hewitt solution. After 10 days of growth, the seedlings were treated with different concentrations of AsIII (A) and AsV (B) for 7 days under standard growth conditions. Photographs of representative seedlings of control and two cultivars were taken 7 d post treatment. White line in each panel indicates the scale bar (1 cm).

Fig. 2: Venn diagram representing numbers of differentially expressed miRNAs induced by As-stress in LARG and HARG. (A) Up/down-regulated miRNAs identified with a 1-fold change in expression under AsIII stress in rice cultivars HARG and LARG. (B) Up/down-regulated miRNAs identified with a 1-fold change in expression under AsV stress.

Fig. 3: qRT-PCR expression analysis of selected differentially expressing miRNAs identified through microarray analysis. (A) Expression levels of eight miRNAs between HARG and LARG rice cultivars exposed to AsIII stress. (B) Expression levels of eight miRNAs between HARG and LARG rice cultivars exposed to AsV stress. Data are reported as mean ± SE for three independent experiments.

Fig. 4: GO analyses of the targets of the 14 As-stress responsive miRNAs in rice. The blue bars indicate the enrichment of the GO terms in the miRNA targets in GO. The red bars indicate the percentage of reference genes present in different GO in rice.

Fig. 5: Expression profiles of As-induced miRNAs and their putative targets in HARG and LARG rice cultivars. (A) and (C) represents expression patterns of up- and down-regulated As-responsive miRNAs and their targets respectively. Arrows represent enhanced (A) and reduced (C) expression of miRNAs in As exposed rice seedlings. The heat map shown represent the expression profiles of putative target genes in AsV stress. Expression data for AsV stress treatment were used for the analysis.[9] The color scale for fold change values is shown at the bottom. (B) and (D) represents qRT-PCR analysis of target genes of up- and down-regulated miRNAs in HARG and LARG in response to AsIII and AsV stress respectively.

SUPPLEMENTARY INFORMATION

Fig. S1: Venn diagram showing the numbers of common and unique differential miRNAs induced by As-stress. (A) Up/down-regulated miRNAs identified with a 1-fold change in expression level, respectively, in rice cultivars HARG under AsIII and AsV stress. (B) Up/ down-regulated miRNAs identified with a 1-fold change in expression level, respectively, in rice cultivars LARG under AsIII and AsV stress.

Table S1: List of oligonucleotides used for the expression analysis of target genes.

Table S2: List of differentially expressed miRNAs in HARG and LARG rice cultivars in AsIII and AsV stress.

Table S3: Identified common motifs in the upstream regions of As-stress induced miRNAs.

Table S4: Metal-responsive elements in As-responsive miRNAs promoters.

Table 1: Expression patterns of AsIII and AsV stress induced miRNAs in HARG and LARG rice cultivars

S.No.	miRNAs	As III		As V	
		HARG	LARG	HARG	LARG
1	miR164	Down	Down	Down	Down
2	miR171	Down	Down	Down	Down
3	miR395	Down	Down	Down	Down
4	miR399	Down	Up	Up	Up
5	miR396	Down	Down	Up	Up
6	miR408	Up	Up	Up	Up
7	miR528	Up	Up	Down	Down
8	miR529	Down	Down	Down	Down
9	miR820	Down	Down	Down	Down
10	miR1432	Down	Down	Down	Down
11	miR1846	Up	Down	Down	Down
12	miR1861	Up	Up	Up	Up
13	miR2102	Up	Up	Up	Up
14	miR2907	Up	Up	Up	Up

miRNAs with fold change >1.5 and P-value <0.05.

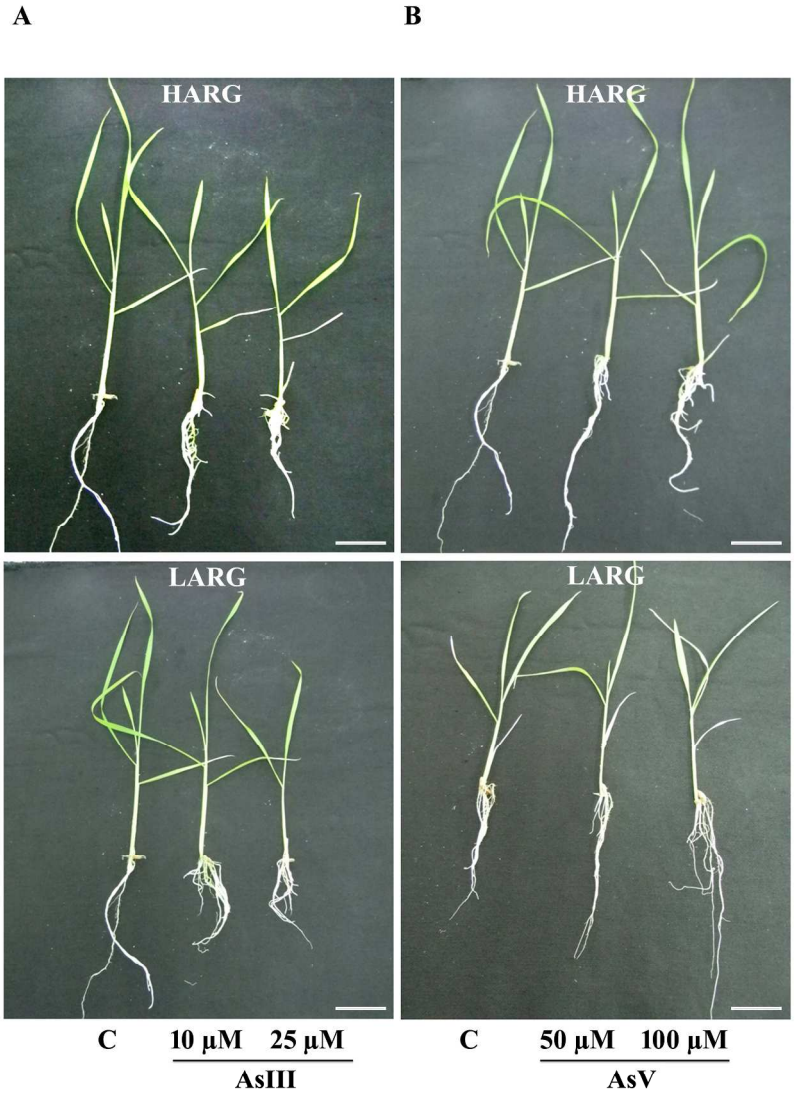
Table 2: Putative targets of As-responsive miRNAs

miRNA Accession	Target Accession	Target Start	Target End	Annotation
osa-miR164	Os06g23650	794	814	CUC2, No apical meristem (NAM) protein
	Os06g46270	954	974	NAC domain-containing protein
	Os12g41680	911	931	NAC domain-containing protein
	Os03g47310	43	63	transposon protein
	Os05g25960	368	388	no apical meristem protein
	Os04g41540	1060	1080	calmodulin
osa-miR171	Os02g44360	1351	1371	SCARECROW gene regulator
	Os06g01620	456	476	scarecrow-like 6
	Os02g19990	1598	1617	reticulon family protein
	Os07g01020	1190	1209	pyridoxin biosynthesis protein ER1
	Os01g65720	1069	1089	glycosyl hydrolase
	Os03g19070	1043	1063	long cell-linked locus protein
	Os08g31560	200	220	WD-repeat protein-like
	Os05g21120	3128	3147	retrotransposon protein
	Os07g33630	2566	2586	microtubule-associated protein TORTIFOLIA1
osa-miR395	Os03g09940	318	338	low affinity sulphate transporter 3
	Os03g53230	595	615	bifunctional 3-phosphoadenosine 5-phosphosulfate synthetase
	Os03g09930	111	131	sulfate transporter 2.1
	Os03g19750	832	851	transposon protein
	Os06g46480	1105	1124	expressed protein
	Os11g44580	1171	1190	disease resistance RPP13-like protein 1
osa-miR396	Os06g10310	282	302	growth-regulating factor 1
	Os06g02560	605	625	growth-regulating factor
	Os04g51190	535	555	growth-regulating factor
	Os02g47280	570	590	growth-regulating factor
	Os06g02560	613	633	growth-regulating factor
	Os02g47280	667	687	growth-regulating factor

	Os02g53690	570	590	atGRF5
	Os03g47140	947	967	atGRF2
	Os04g24190	828	847	growth-regulating factor 1
	Os04g48510	858	878	transcription activator
	Os03g51970	419	439	growth-regulating factor 1
	Os12g29980	732	752	atGRF2
	Os01g25330	1038	1058	SAC9
	Os11g35030	870	889	expressed protein
	Os11g35030	1557	1576	expressed protein
	Os12g29980	727	747	atGRF2
	Os02g58214	69	92	expressed protein
	Os01g27430	1352	1372	retrotransposon protein
	Os09g26000	1623	1645	glutamate receptor 2.8 precursor
	Os09g25960	1731	1753	glutamate receptor 2.7 precursor
	Os09g25990	1725	1747	glutamate receptor 2.6 precursor
	Os11g43410	2850	2870	NB-ARC domain containing protein
osa-miR399	Os05g48390	904	924	ubiquitin conjugating enzyme
	Os05g45350	1110	1130	electron transporter/ heat shock protein binding protein
	Os09g33830	1271	1291	solute carrier family 35, member F1
	Os03g13800	820	839	NHP2-like protein 1
	Os09g38330	2144	2163	expressed protein
	Os06g19660	1785	1804	nucleotide binding protein
	Os04g33860	495	514	expressed protein
osa-miR408	Os11g01470	1333	1353	retrotransposon protein
	Os03g05650	644	664	expressed protein
	Os02g43660	679	699	blue copper protein precursor
	Os10g41040	362	382	expressed protein
	Os08g37670	657	677	blue copper protein precursor
	Os07g43540	184	204	origin recognition complex subunit 6
	Os01g53880	1357	1376	OsIAA6 - Auxin-responsive Aux/IAA gene family member
	Os05g25120	79	98	hypothetical protein
	Os07g29660	66	86	hypothetical protein

	Os02g06250	98	118	phytosulfokine receptor precursor
	Os06g19130	66	86	conserved hypothetical protein
	Os09g21520	1514	1534	ATP binding protein
	Os09g08030	231	250	seven-transmembrane-domain protein 1
	Os09g08440	405	424	mtN3/saliva family protein
osa-miR528	Os08g04310	247	267	uclacyanin-2 precursor
	Os06g06050	2648	2668	F-box/LRR-repeat MAX2
	Os01g40150	2510	2530	translation initiation factor IF-2
	Os10g24090	577	597	expressed protein
	Os06g37150	1998	2018	L-ascorbate oxidase precursor
	Os07g38290	528	547	copper ion binding protein
	Os01g44330	41	61	L-ascorbate oxidase precursor
	Os09g33800	371	391	arabinogalactan protein
	Os08g44770	299	319	superoxide dismutase, chloroplast precursor
	Os01g03620	59	79	copper ion binding protein
	Os01g03640	45	65	copper ion binding protein
osa-miR529	Os09g31438	805	824	squamosa promoter-binding-like protein 9
	Os01g69830	1149	1168	teosinte glume architecture 1
	Os04g51830	1646	1666	cation transporter HKT7
	Os09g32944	1030	1049	teosinte glume architecture 1
	Os08g41940	1050	1069	teosinte glume architecture 1
	Os08g34820	482	501	F-box domain containing protein
	Os09g07300	11114	11134	BIG
	Os05g04150	149	169	expressed protein
	Os05g14010	80	99	expressed protein
	Os12g08760	82	102	carboxyvinyl-carboxyphosphonate phosphorylmutase
	Os01g65780	66	86	secondary cell wall-related glycosyltransferase family 8
	Os02g07720	1136	1155	choline-phosphate cytidylyltransferase B
	Os05g07090	1587	1606	glutaryl-CoA dehydrogenase, mitochondrial precursor
	Os03g57900	576	596	zinc finger A20 and AN1 domains-

				containing protein
	Os02g03620	40	59	ubiquitin ligase SINAT2
	Os04g28420	1846	1865	peptidyl-prolyl isomerase
osa-miR820	Os03g02010	304	324	DNA cytosine methyltransferase Zmet3
	Os11g13650	93	113	ATCSLC12
	Os11g03310	129	149	NAC domain-containing protein
	Os10g42196	4014	4033	expressed protein
osa-miR1432	Os03g59790	45	65	calcium-binding protein
	Os03g59770	117	137	calcium-binding allergen Ole e 8
	Os04g35590	485	505	thioesterase superfamily member
	Os05g07210	1166	1186	ZIP zinc/iron transport family protein
	Os08g36910	587	606	alpha-amylase isozyme 3D precursor
osa-miR1846	Os08g10350	477	497	expressed protein
	Os02g18870	76	95	anther-specific proline-rich protein APG
	Os01g70100	668	687	palmitoyltransferase ZDHHC9
osa-miR1861	Os01g63810	610	629	starch binding domain containing protein
	Os05g51790	578	599	ATP binding protein
	Os09g09500	1805	1824	lectin-like receptor kinase 7
	Os11g08950	1213	1232	ATP binding protein
	Os01g03090	740	759	SLL2
osa-miR2102	Os08g22780	110	129	retrotransposon protein
	Os01g12280	145	164	protein dimerization
	Os03g32610	763	782	hypothetical protein
	Os07g42834	1539	1558	retrotransposon protein
osa-miR2907	Os05g18660	460	481	F-box domain containing protein
	Os10g12130	987	1008	retrotransposon protein



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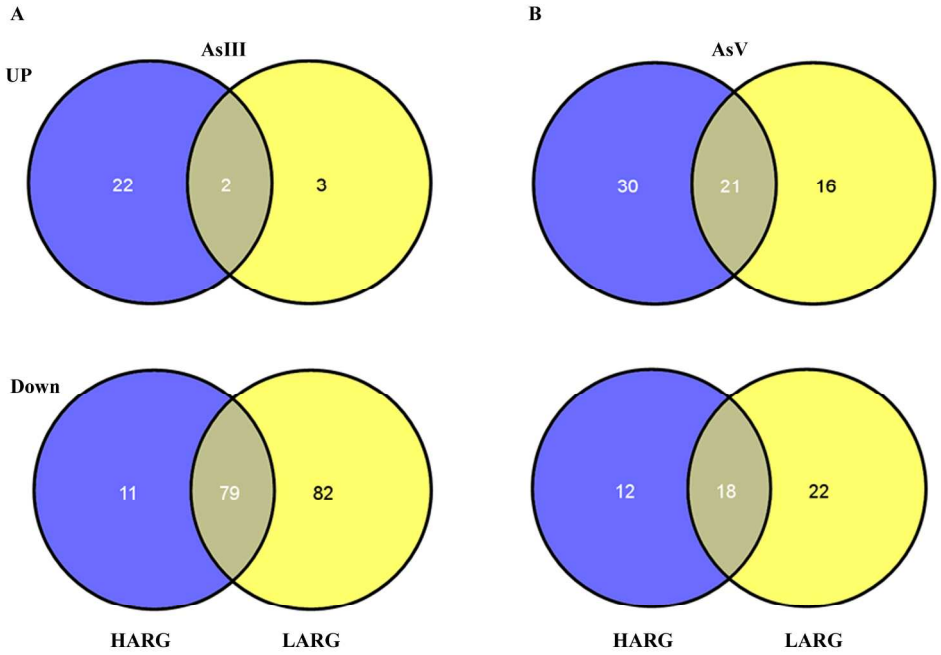
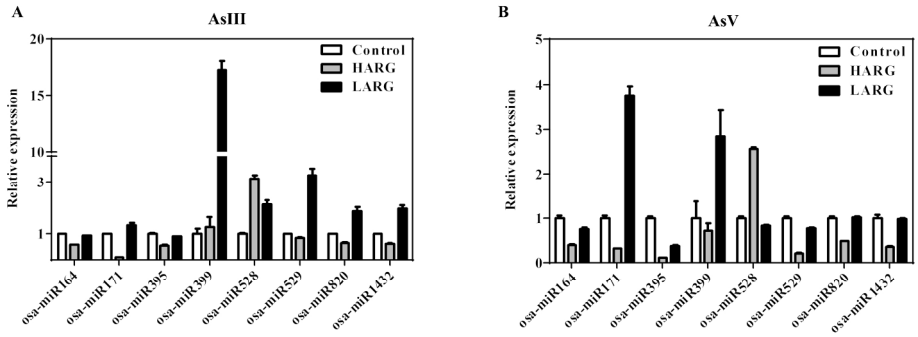


The Venn diagram showing the numbers of common and unique differentially expressed miRNAs induced by As-stress in LARG and HARG. (A) Up/down-regulated miRNAs identified with a 1-fold change in expression level under AsIII stress in rice cultivars HARG and LARG. (B) Up/ down-regulated miRNAs identified with a 1-fold change in expression level under AsV stress.



qRT-PCR expression analysis of selected miRNAs identified through microarray analysis. (A) Expression levels of eight miRNAs between HARG and LARG rice cultivars subjected to AsIII stress. (B) Expression levels of eight miRNAs between HARG and LARG rice cultivars subjected to AsV stress. Data are reported as mean ± SE for three independent experiments.
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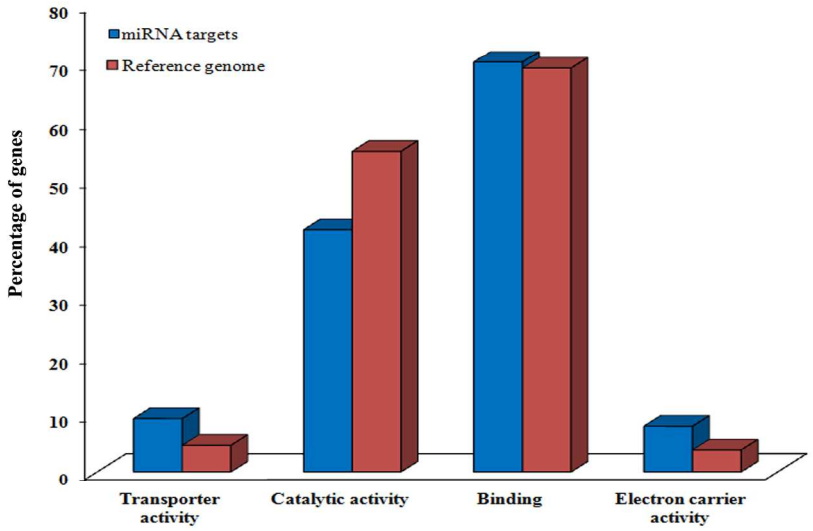

GO analyses of the targets of the 14 As-stress responsive miRNAs in rice. The yellow bars indicate the enrichment of the GO terms in the miRNA targets in GO. The purple bars indicate the percentage of reference genes present in different GO in rice.
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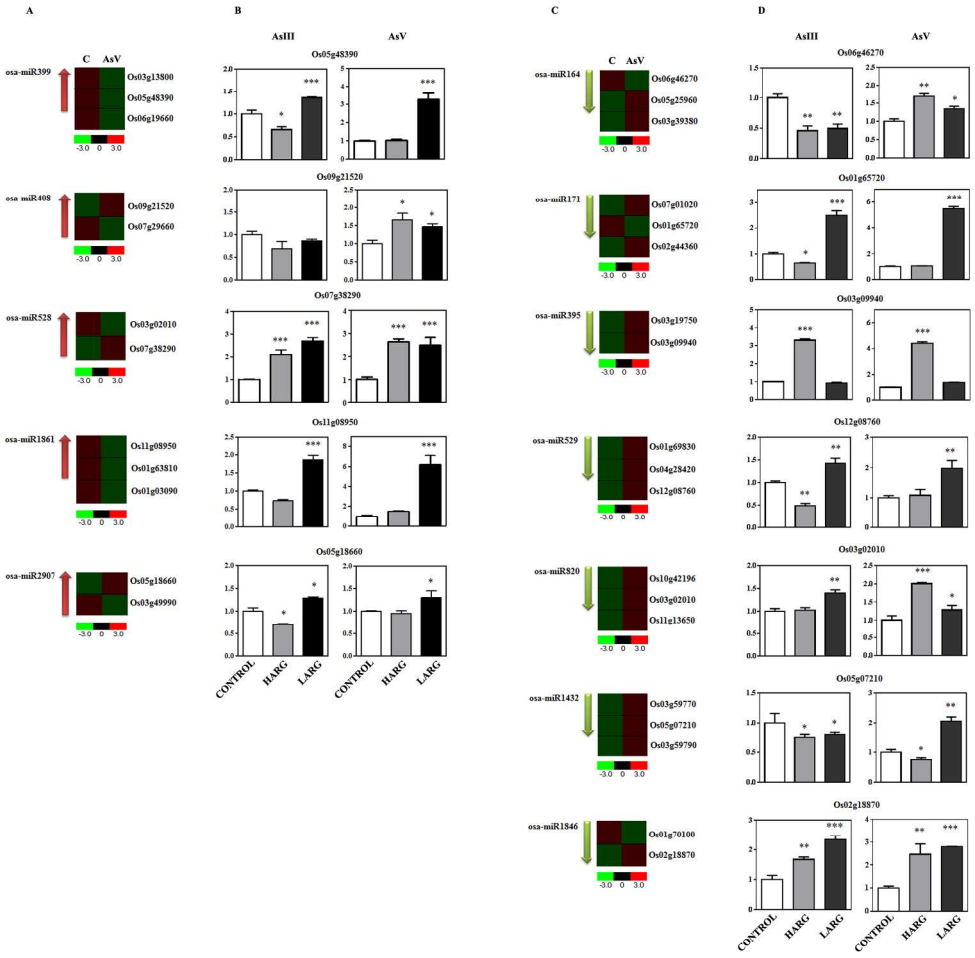

Expression profiles of As-induced miRNAs and their putative targets in HARG and LARG rice cultivars. (A) and (C) represents expression patterns of up- and down-regulated As-responsive miRNAs and their targets respectively. The heat map shown represent the expression profiles of putative target genes in AsV stress. Expression data for AsV stress treatment were used for expression analysis.9 The color scale for fold change values is shown at the bottom. (B) and (D) represents qRT-PCR analysis s of target genes of up- and down-regulated miRNAs in HARG and LARG in AsIII and AsV stress respectively.
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