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Molecular Identification and Analysis of Arsenite Stress-Responsive miRNAs in Rice

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Supporting Information

ABSTRACT: Arsenic is highly toxic to living organisms including humans and plants. To investigate the responsive functions of miRNAs under arsenite stress, an *indica* rice, Minghui 86, has been deeply sequenced, and a total of 67 arsenite-responsive miRNAs were identified in rice seedling roots, of which 5 were further validated experimentally. The potential targets of those differential miRNAs include some transcription factors, protein kinases, and DNA- or metal ion-binding proteins that are associated with cellular and metabolic processes, pigmentation, and stress responses. The regulatory roles of four miRNAs on their targets in response to arsenite were further confirmed by real time RT-PCR. Interestingly, osa-miR6256 was originally characterized as a putative exonic miRNA, supporting the notion that miRNAs may also originate from some exons in plants. The first identification of arsenite-responsive miRNAs at the whole genome-wide level will broaden the current understanding of the molecular mechanisms of arsenite responses in rice.

KEYWORDS: *rice, arsenite, miRNA, exon origination, deep sequencing*

INTRODUCTION

Arsenic (As) that is extremely toxic to living organisms is ubiquitously and abundantly present in the Earth's crust. In South and Southeast Asia, millions of people have suffered from As poisoning as a result of drinking As-contaminated water and the food chain,[1−3] which can be ascribed to release of As into the environment by natural processes, such as weathering of rocks, and various anthropogenic activities, such as mining and smelting. In addition, the wide use of As-containing herbicides and insecticides in weed and pest control, as well as wood preservatives and feed additives, has readily increased the extent of As contamination.[4]

It is well-known that arsenic is a nonessential metalloid but very toxic to plants. Compared to other cereals such as wheat and barley, rice is highly efficient in As uptake and accumulation,[5] particularly in As-contaminated soils. Xu et al.[6] revealed that As accumulation in rice shoots and grain significantly increased by 10−15-fold in flooded as compared with aerobic soil, because of the greatly increased bioavailability of As under flooded conditions. Because rice is a major food for populations in Southeast Asia, the elevated accumulation of As in grain may serve as one of the major sources of As exposure for those populations that rely mostly on rice for their diet.[2,7] In China, four As species, including arsenate [As(V)], arsenite [As(III)], monomethylarsonic acid [MMA(V)], and dimethylarsinic acid [DMA(V)], are found to be present in most rice samples, with the more toxic inorganic species [As(III) + As(V)] being predominant and accounting for 72% of total As in rice.[8] Thus, a reduction of As accumulation in rice is of great concern.

Plants vary considerably in their ability to accumulate As, with the range from excluders to hyperaccumulators.[4] In rice, a great variation in the ability of As accumulation also exists among different cultivars.[7] Rai et al.[9] found that three rice cultivars, Triguna, IR-36, and PNR-519, are relatively tolerant to As stress and accumulate greater arsenic, whereas IET-4786 is a sensitive rice cultivar that accumulates less arsenic. To further understand the genetic mechanisms behind phenotypic complexity, Dasgupta et al.[10] and Zhang et al.[11] identified and mapped one and four As tolerance and accumulation associated quantitative trait loci (QTLs) onto three chromosomes (2, 6, and 8) using a rice RIL and DH population, respectively. Fine-mapping and identification of genes controlling As tolerance and concentration that are located in the robust QTLs will provide deep insight into the genetic basis of As uptake, accumulation, and response.

To date, the molecular mechanisms of As uptake, translocation, and accumulation in higher plants have not been fully elucidated. It was shown that plant arsenic toxicity mainly derives from exposure to inorganic As(V) and As(III).[12] As(V) is the main As species in aerobic soils and taken up by plant roots through the phosphate transporters that have higher affinity for phosphate than for arsenate.[13] In contrast, As(III) is predominant in anaerobic environments such as submerged paddy soils. A number of plant aquaporins belonging to the nodulin26-like intrinsic protein (NIP) subfamily were demonstrated to be permeable to As(III).[14−17] In rice, a group of NIP III subgroup genes including *OsNIP2;1*, *OsNIP2;2*, and *OsNIP3;2* can mediate the bidirectional transport of As(III) across membranes.[4,14,16] However, OsNIP2;1 (Lsi1), which was first identified to function in permeability to silicic acid,[18] would be the major entry route for As(III) in rice.[16] Interestingly, OsNIP2;1 also contributes to the uptake of

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methylated As species, MMA and DMA.[19] Whereas As(III) is taken up by roots via OsNIP2;1, another silicon transporter, Lsi2,[20] mediates As(III) efflux in the direction of xylem and ultimately to the rice grain.[16] However, Lsi2 is not involved in the process of translocation of methylated As,[19] irrespective of its crucial role in the distribution of As(III) from roots to shoots. Whether in root, shoot, or grain, normally most arsenic will ultimately be sequestrated into vacuolars, a process that mainly depends on glutathione (GSH) and phytochelatins (PCs).[4,12] In *Arabidopsis*, two ABCC-type PC transporters (AtABCC1 and AtABCC2) were found to participate in the process of sequestration of arsenic into vacuolars; overexpression of AtABCC1 and AtABCC2 markedly enhanced the arsenic tolerance for *Arabidopsis* plants.[21]

Identification and characterization of critical protein-coding genes and regulatory miRNAs associated with As tolerance and concentration is a prerequisite for breeding plant cultivars with low As accumulation. This is a reasonable and feasible way to reduce the nutritional arsenic intake through the consumption of contaminated plants. However, only a small number of genes function in mediating the influx, efflux, compartmentation, and long-distance transport of As have been identified in plants.[4,12,13] To our knowledge, there are presently no papers regarding the identification of As stress-related regulatory miRNAs in rice.

MiRNAs, a class of endogenous noncoding small RNAs ~21 nt in length, can efficiently regulate the expression of their target genes at the post-transcriptional level and, hence, play crucial roles in diverse aspects of development, metabolism, apoptosis, and viral defense and in response to environmental stresses.[22−26] Therefore, it is intriguing to identify environmental stress-responsive miRNAs and further uncover their roles in regulation of plant adaptation. However, the functional significance of miRNAs in adaptive responses to environmental factors remains to be explored. By using a miRNA microarray platform, Zhou et al.[27] identified 30 drought-stress responsive miRNAs in rice. In *Arabidopsis*, the expression of miR169a was strongly inhibited in roots and shoots by nitrogen deprivation.[26] Transgenic experiments demonstrated the involvement of miR169a in the process of nitrogen bioavailability in soil.[26] In addition, miRNAs can also be induced by pathogen invasion, adding their roles in the involvement of plant−microorganism interactions.[28,29] Thus, extensive exploration of miRNA expression patterns under different stresses and identification of targets they act against are important steps in further understanding the function of miRNA and miRNA-dependent gene regulation.

In the present study, the rice miRNA expression pattern under arsenite stress was first investigated at the whole genome-wide level using the high-throughput, next-generation sequencing strategy. Totally, 67 miRNAs with relative expression change ≥2-fold were identified, of which 5 were further validated by real time RT-PCR. The potential targets of those differential miRNAs were predicted, and some of them were validated experimentally. In addition, a putative exonic miRNA that was significantly down-regulated after exposure to arsenite was identified. These results will add further evidence in the understanding of the molecular mechanisms of rice responses to As stress.

MATERIALS AND METHODS

Plant Culture and Treatments. The seeds of two rice cultivars, Minghui 86 and 93-11 (*Oryza sativa* L. *indica*), were first surface-sterilized with 0.5% NaOCl for 15 min, rinsed thoroughly, and soaked in deionized water overnight. Then, seeds were placed on a filter paper floating on a 0.5 mM $CaCl_2$ solution. After 5 days, all of the seeds were germinated, and seedlings were transferred to a 20 L container filled with half-strength Kimura solution[30] and directly precultured in a greenhouse for 21 days. Nutrient solution was renewed every 3 days. The 21-day-old seedlings were then transferred to 2 L pots (four plants per pot) containing 1 L of half-strength Kimura nutrient solution.[30] Totally, six pots were prepared, of which three pots were used as CK, and arsenite, in the form of $NaAsO_2$, was added to the other three pots at 25 μM. After different times (0, 2, 4, and 6 days) of arsenite treatment, rice roots were washed three times with an ice-cold desorption solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, and 5 mM MES (pH 5.5) to remove apoplastic As.[16,30] Then roots were harvested as a pool from the three replicates for each treatment (CK and As stress). After collection, all of the samples were immediately frozen in liquid nitrogen and stored at −80 °C until used.

Small RNA Library Construction and Deep Sequencing. Total RNA of each sample was isolated using a TRIzol kit and quantified by an Agilent 2100. Briefly, the samples were first processed by 15% denaturing polyacrylamide gel electrophoresis (PAGE), and then small RNAs of 18−30 nt in length were isolated from the gel and purified. Small RNAs were ligated to a 5′- and a 3′-adaptor sequentially, and RT-PCR was performed to convert RNA to DNA. The amplified PCR products were purified and subjected directly to the Solexa 1G Genome Analyzer for SBS sequencing (BGI-Shenzhen, China).

Analysis of Sequencing Data. The raw sequences were preprocessed by the BGI small RNA pipeline to remove low-quality reads. Furthermore, the reads with 5′-adaptor contaminants or having no 3′ adaptor and/or insertion fragment were removed, as were those containing the polyA tail. In high-quality reads, the vector sequences were trimmed off, and sequences of ≤18 and ≥30 nt were discarded as well. Finally, using the BLAST and OVERLAP programs, the clean small RNA sequences having perfect matches with known rRNA, scRNA, snoRNA, snRNA, and tRNA deposited in GenBank and Pfam databases, or overlapping with exons and introns of protein-coding genes, were excluded further. After that, other unique small RNA sequences were used as query to search against the miRBase database to identify known rice miRNAs.

Identification of Differentially Expressed miRNAs. To determine the differentially expressed miRNAs, the expression of miRNAs in the CK and As treatment samples were first normalized to obtain the expression of transcripts per million. If the normalized expression of a given miRNA is 0, its expression value is modified to 0.01. If the normalized expression of a given miRNA is <1 in both samples, this miRNA is removed from further analysis. Then, the fold change and *P* value were calculated, respectively. The calculation of fold change is based on the following formula:

$$\text{fold change} = \log_2(\text{As treatment}/\text{CK})$$

The Bayesian method, developed for analysis of digital gene expression profiles,[31] was employed to infer the statistical significance value (*P* value).[32] After Bayesian test, if the *P* value was ≤0.01 and the fold-change value was ≥2, a specific miRNA was considered to be differentially expressed.

Real Time Quantitative RT-PCR (qPCR). Real time qPCR was performed to validate the expression of five differentially expressed osa-miRNAs. The same RNA samples used for whole transcriptome sequencing were also employed, and total RNA of these tissues was extracted using a TRIzol kit. A stem loop primer was used to synthesize the miRNA cDNAs (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACNNNNNN-3′), where the letter N represents the sequence of the six nucleotides that hybridize to the 3′ end of the mature miRNA.[33] Mature miRNA-specific PCR forward primers were designed according to the relative miRNA sequences. The primers involved in real time qPCR are listed in the Supporting Information. Mature miRNAs cDNA was synthesized by adding 100 ng of total RNA, 0.5 μL of M-MLV reverse transcriptase (200 U/μL, Takara), 0.5 μL of stem loop primer

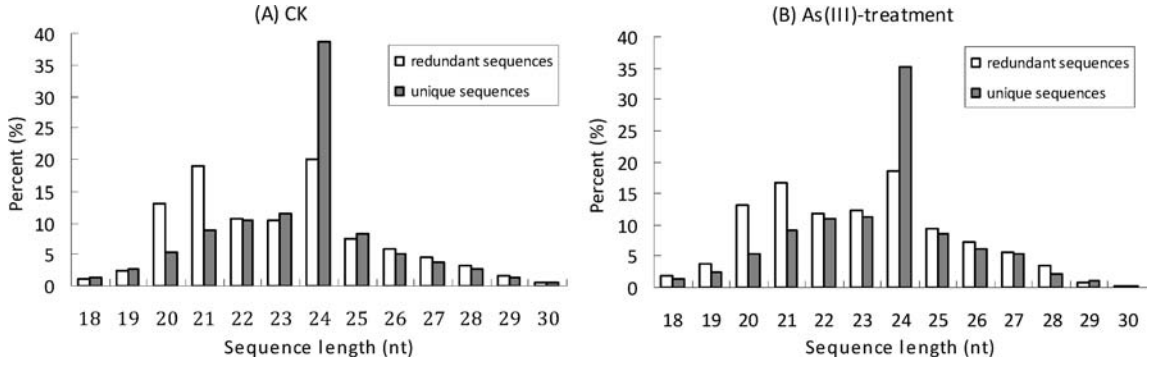

Figure 1. Length size distribution and abundance of small RNA sequences derived from CK (A) and As(III) treatment (B) samples in rice.

(2 μM), 1.0 μL of 5× enzyme buffer, 0.25 μL of dNTP (10 mM each, Takara), and 0.25 μL of RNase inhibitor (40 U/μL, Takara) mix in a final volume of 5.0 μL. The cDNA synthesis conditions were 42 °C for 60 min, 70 °C for 15 min, and a hold at 4 °C. The real time qPCR mix was 0.5 μL of cDNA, 0.8 μL of 10 μM primer mix, 0.4 μL of ROX, 10.0 μL of 2× SYBR Green Mix (Invitrogen), and 8.3 μL of ddH_2O to a final volume of 20 μL. A forward primer and a universal reverse primer (see the Supporting Information) were used in the quantitative PCR assays that were performed using an ABI7900 system (Applied Biosystems) and a standardized protocol. The real time qPCR conditions were 50 °C for 2 min and 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Then, the samples were heated from 60 to 95 °C to acquire the denaturing curve of the amplified products. In each reaction, U6 was used as positive PCR control. In addition, negative PCR controls (no cDNA template) were prepared to detect possible contamination for each miRNA. All reactions were run in triplicate.

The expression analysis of several target genes was also examined by real time qPCR. The RNA was first treated with DNase I and then reverse transcribed using an oligo(dT) primer and a PrimeScrip RT reagent kit (Takara) to generate cDNA, with a condition of 50 °C for 30−60 min. The target gene primers (see the Supporting Information) were then added to amplify the PCR products. Real time qPCR was carried out in an ABI7900 system (Applied Biosystems) using the SYBR Premix Ex Taq kit (Takara). The real time qPCR conditions for target gene amplification were 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 20 s, and 72 °C for 30 s. In each reaction, β-actin was used as the internal reference. Each experiment was replicated three times. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method.[34] The values of threshold cycle (CT, the fractional cycle number at which the fluorescence passes the fixed threshold),[33] were calculated by Rotor-Gene 6 software (Corbett Robotics, Australia). Then, the $2^{-\Delta\Delta CT}$ method was used to assess the relative changes in gene expression from real-time qPCR experiments.[34]

Prediction of miRNA Targets. The CleaveLand pipeline was utilized to identify the targets of differential miRNAs by searching rice degradome sequencing data integrated in the starBase database,[35,36] with a strict filter of Penalty score $\leq$4 and Cleavage tags $\geq$1. In addition, the psRNATarget, a plant miRNA target prediction server,[37] was also employed to predict putative miRNA target genes, with the default parameters. To reduce false positives, only those genes that were predicted by both CleaveLand and psRNATarget, and reported in the PmiRKB database as well,[38] were considered as the real miRNA targets. To analyze the molecular functions of targets, the WEGO server was employed to perform a homologous search against the GO database and to classify the targets into different functional categories on the basis of their GO term enrichments.[39]

RESULTS

Solexa Sequencing of Small RNA Transcriptomes. To uncover arsenite-responsive miRNAs, the small RNA (sRNA) transcriptomes of CK and arsenite-treated rice were sequenced, yielding totals of 17,182,731 and 18,695,511 raw sRNA reads, respectively. After low-quality reads were filtered out and adaptor sequences clipped, 16,243,833 and 17,681,819 clean reads with lengths ranging from 18 to 30 nt were obtained. These sequences represent 3,422,844 and 2,688,232 unique sRNAs (the sequence of a particular type with nonredundancy) in the CK and As treatment samples, respectively (see the Supporting Information). Comparison of unique and total sRNA reads of the two samples showed that the common sequences of total sRNA reads accounted for 83.17%, whereas the common sequences of unique sRNA reads occupied only 12.64% (see the Supporting Information). This observation indicated that the regulation of sRNAs in rice seedling roots with and without arsenite treatment shared many similarities. However, most of the unique sRNAs were library-specific, which might be produced for the respective physiological or environmental response activity regulation.

On the basis of the clean reads, the size distribution pattern of sRNAs was analyzed. As seen in Figure 1, the majority of unique reads were distributed between 21 and 24 nt in size, with the highest abundance in 24 nt, which accounts for 38.6 and 35.2% of total sRNAs for the CK and As treatment samples, respectively. In the redundant size distribution pattern, the numbers of 24 and 21 nt sequences were found to be significantly greater than other size sequences in the CK library, which account for 20.1 and 18.9% of total sRNA reads, respectively (Figure 1). This result is consistent with that of maize,[40] peanut,[41] safflower,[32] *Citrus trifoliate*,[42] and *japonica* rice.[43]

The reads matching noncoding RNAs including rRNA, scRNA, snoRNA, snRNA, and tRNA were removed from the data sets subsequently. To avoid the contamination of mRNA degradation, a small proportion of sRNAs overlapping with exons and introns of protein-coding genes was eliminated (Table 1). The remaining sRNAs were used to identify known miRNAs by searching the rice miRNA sequences deposited in miRBase. Totally, 2,592,873 and 2,100,094 reads representing of 4621 and 3162 unique pre-miRNA fragments were found to match well with 291 and 265 known pre-miRNAs in the CK and As treatment libraries, which account for 15.96 and 11.88% of total clean reads, respectively (Table 1).

Identification of Arsenite-Stress Responsive Osa-miRNAs. Totals of 54 and 13 osa-miRNAs that belong to 19 and 7 miRNA families were found to be significantly down- and up-regulated after the treatment of arsenite, respectively. Their relative expression changes were >2-fold (Table 2). If the criteria of fold change was modified to 1 (>1.0 or < −1.0), 105 and 19 osa-miRNAs were found to have markedly decreased or

Table 1. Distribution and Read Abundance of Small RNAs among Different Categories in Rice Seedling Roots

	CK		As(III) treatment	
category	total sRNAs	%	total sRNAs	%
clean reads	16243833	100	17681819	100
exon_antisense	42333	0.26	26163	0.15
exon_sense	100496	0.62	93696	0.53
intron_antisense	53821	0.33	21457	0.12
intron_sense	105566	0.65	51626	0.29
miRNA	2592873	15.96	2100094	11.88
rRNA	2503866	15.41	3658233	20.69
repeat	1099309	6.77	368898	2.09
siRNA	105227	0.65	136162	0.77
snRNA	21752	0.13	43673	0.25
snoRNA	13380	0.08	12921	0.07
tRNA	513649	3.16	486710	2.75
unannotated	9091561	55.97	10682186	60.41

increased their expression level under the arsenite stress condition, respectively (Figure 2; see the Supporting Information), of which 7 osa-miRNAs (osa-miR172c, osa-miR1432, osa-miR1318, osa-miR169a, osa-miR408, osa-miR397b, and osa-miR528) with ≥4-fold change in expression were identified (see the Supporting Information). Particularly, the expression of osa-miR172c, a member of the miR172 family, was changed extensively (−7.83-fold), which was weakly expressed in CK (sequencing reads 37), but thoroughly inhibited under arsenite stress. Conversely, osa-miR408 was not expressed in CK, but strongly induced after treatment with arsenite (8.33-fold; sequencing reads 57).

A differential expression pattern existed among members within each miRNA family under As stress. For example, in the miR444 family, osa-miR444a.1, osa-miR444a.2, osa-miR444d.1, osa-miR444d.2, and osa-miR444e were significantly up-regulated, whereas other miR444 family members were almost equally expressed in CK and As stress libraries (see the Supporting Information). A similar pattern was also observed in the miR166, miR167, miR169, miR171, miR396, miR812, and miR819 families. In each of these families, some members were extensively down-regulated, whereas the expression of other family members remained almost unchanged in rice seedlings with or without exposure to arsenite.

By BLASTN search of known miRNAs deposited in miRBase, we found that in the down-regulated osa-miRNA group, osa-miRNAs belonging to seven families (miR1318, miR1861, miR2121, miR810, miR812, miR815, and miR818) were rice species-specific, whereas the miR164, miR166, miR167, miR169, miR171, miR390, and miR396 families were conserved in the plant kingdom from moss to higher plants. By contrast, in the up-regulated osa-miRNA group, 8 osa-miRNAs belonging to 3 miRNA (miR444, miR528, miR827) families were monocot lineage-specific. However, another 3 miRNA (miR393, miR397, and miR408) families were widespread in monocot and dicot species.

Expression Validation of Differentially Expressed miRNAs by Real Time RT-PCR. Stem loop real time qPCR has been widely used to assess the expression level of miRNAs under different conditions and/or in different tissues. This method has been proven to be quantitative and sensitive.[33] Herein, five miRNAs including miR408, miR528, miR397b, miR1318, and miR390 were chosen to validate their expression experimentally. As expected, the three up-regulated miRNAs

Table 2. Significantly Differentially Expressed miRNAs with Fold Change > ±2 under Arsenite Stress by Deep Sequencing

miRNA	fold change	miRNAs	fold change	miRNAs	fold change
		Down-regulated miRNAs			
osa-miR172c	−7.830	osa-miR396g	−2.417	osa-miR812d	−2.314
osa-miR1432	−4.176	osa-miR396h	−2.417	osa-miR812h	−2.265
osa-miR1318	−4.174	osa-miR396i	−2.417	osa-miR812i	−2.260
osa-miR169a	−4.067	osa-miR815a	−2.404	osa-miR812c	−2.233
osa-miR164e	−3.607	osa-miR396f	−2.362	osa-miR812g	−2.218
osa-miR1861a	−3.292	osa-miR396d	−2.360	osa-miR166k	−2.209
osa-miR390	−3.092	osa-miR396e	−2.360	osa-miR812j	−2.125
osa-miR810b.2	−2.876	osa-miR2121a	−2.350	osa-miR169h	−2.121
osa-miR171g	−2.838	osa-miR819a	−2.344	osa-miR169i	−2.121
osa-miR167i	−2.811	osa-miR819c	−2.344	osa-miR169m	−2.121
osa-miR167e	−2.810	osa-miR819d	−2.344	osa-miR1427	−2.103
osa-miR818d	−2.749	osa-miR819e	−2.344	osa-miR169j	−2.093
osa-miR171h	−2.706	osa-miR819f	−2.344	osa-miR166l	−2.081
osa-miR819j	−2.599	osa-miR819g	−2.344	osa-miR169k	−2.065
osa-miR166i	−2.549	osa-miR819h	−2.344	osa-miR169l	−2.065
osa-miR166j	−2.549	osa-miR819k	−2.344	osa-miR815c	−2.065
osa-miR812b	−2.506	osa-miR812e	−2.336		
osa-miR3979−3p	−2.451	osa-miR2121b	−2.331		
osa-miR819i	−2.443	osa-miR812a	−2.331		
		Up-regulated miRNAs			
osa-miR408	8.332	osa-miR444a.1	3.151	osa-miR444a.2	2.627
osa-miR397b	5.363	osa-miR444d.2	3.151	osa-miR444e	2.574
osa-miR528	4.683	osa-miR827b	3.121	osa-miR319b	2.142
osa-miR827a	3.213	osa-miR397a	2.971		
osa-miR444d.1	3.173	osa-miR393	2.879		

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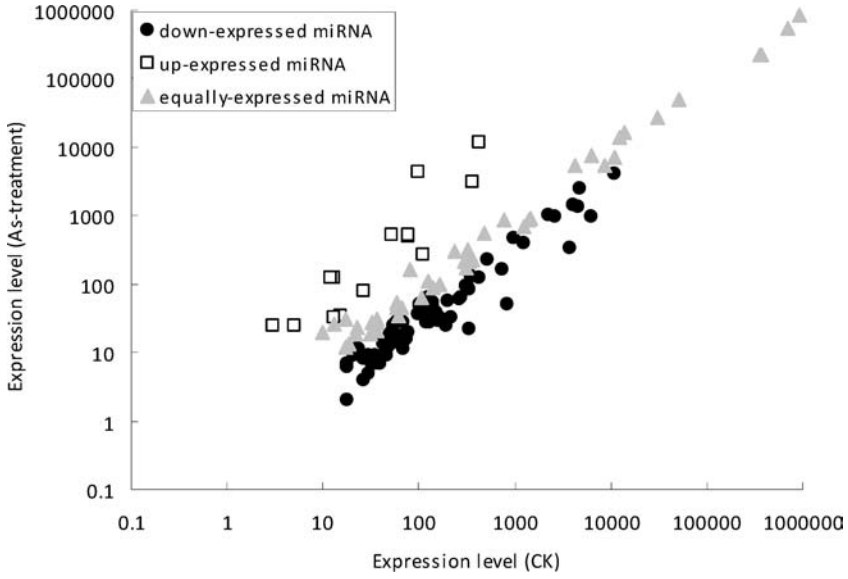

Figure 2. Comparison of expression pattern of miRNAs identified between the CK and As(III) treatment samples.

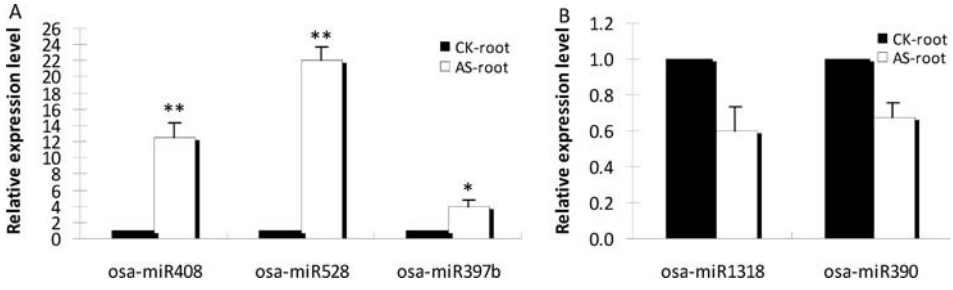

Figure 3. Expression validation of three significantly up-regulated (A) and two down-regulated (B) miRNAs in rice. Expression levels of osa-miRNAs in rice seedling roots under arsenite treatment and CK were examined using stem loop RT-PCR. The expression levels of miRNAs were normalized to the level of U6 snRNA. Fold changes in the expression level of miRNAs were estimated by the $2^{-\Delta\Delta CT}$ method relative to levels in CK samples. Data are reported as the mean ± SE for three independent experiments. The significance of difference between CK and arsenite treatment samples was calculated using the *t* test. ∗ and ∗∗ indicate significant difference from the CK sample at the 0.05 and 0.01 levels, respectively.

(miR408, miR528, and miR397b) still showed a strong tendency of increased expression level when challenged with arsenite ($2^{-\Delta\Delta CT}$ values = 12.31, 22.01, and 3.89, respectively; Figure 3). The extent of fold change of miR408 and miR528 was remarkably greater than the data obtained by Solexa sequencing, indicating that the two miRNAs were indeed directly involved in the process of arsenite response. By contrast, miR1318 still exhibited a suppressive expression under arsenite stress, although the extent of fold change was smaller but nearly statistically significant compared to the deep sequencing data (p value = 0.057). A similar case was also observed for miR390, which was down-regulated up to 1.49-fold under arsenite stress. Therefore, although the specific expression value of miRNAs assessed by real time qPCR and deep sequencing was not exactly identical, the differential expression trend or direction was similar for both methods.

Target Prediction and Their Functional Classification. To better understand the biological function of miRNAs with ≥2-fold changes in expression (MSD-miRNA), their target genes were predicted either by searching rice degradom sequences or full-length cDNAs. It was found that the number of potential targets varied from 1 to 25 per MSD-miRNA. These targets might participate in several biological processes, including biological regulation, cellular process, metabolic process, localization, pigmentation, etc. However, >78% of targets were enriched in the cellular process and metabolic process categories (Figure 4), indicative of their importance for rice growth and development. On the basis of the gene annotation information, the predicted targets of down-regulated miRNAs (DR-miRNAs) could be mainly classified into four functional categories, including transcriptional regulation, catalytic activity, binding, and transporter activity (Figure 4). Furthermore, it was observed that the targets of DR-miRNAs included nuclear transcription factor Y, AP2, and START domain containing proteins, some protein kinases, and DNA-binding domain containing proteins, etc. Compared to DR-miRNAs, genes belonging to five categories including binding, catalytic activity, electron carrier activity, transcription regulation, and transporter activity were predicted to be the potential targets of up-regulated miRNAs (UR-miRNAs), where electron carrier, metal ion transporter, and ion-binding proteins were especially enriched in the UR-miRNA group. Interestingly, in the UR-miRNA group MADS-box transcription factors were significantly overpresented in the transcription regulator activity category.

Time Course Analysis of miRNAs and Their Target Genes in Response to Arsenite. Temporal expression patterns of four miRNAs and their targets (Os06g03970.1, the target of miR390; Os05g38420.1, the target of miR397b; Os07g38290.1, the target of miR528; and Os04g51610.1, the

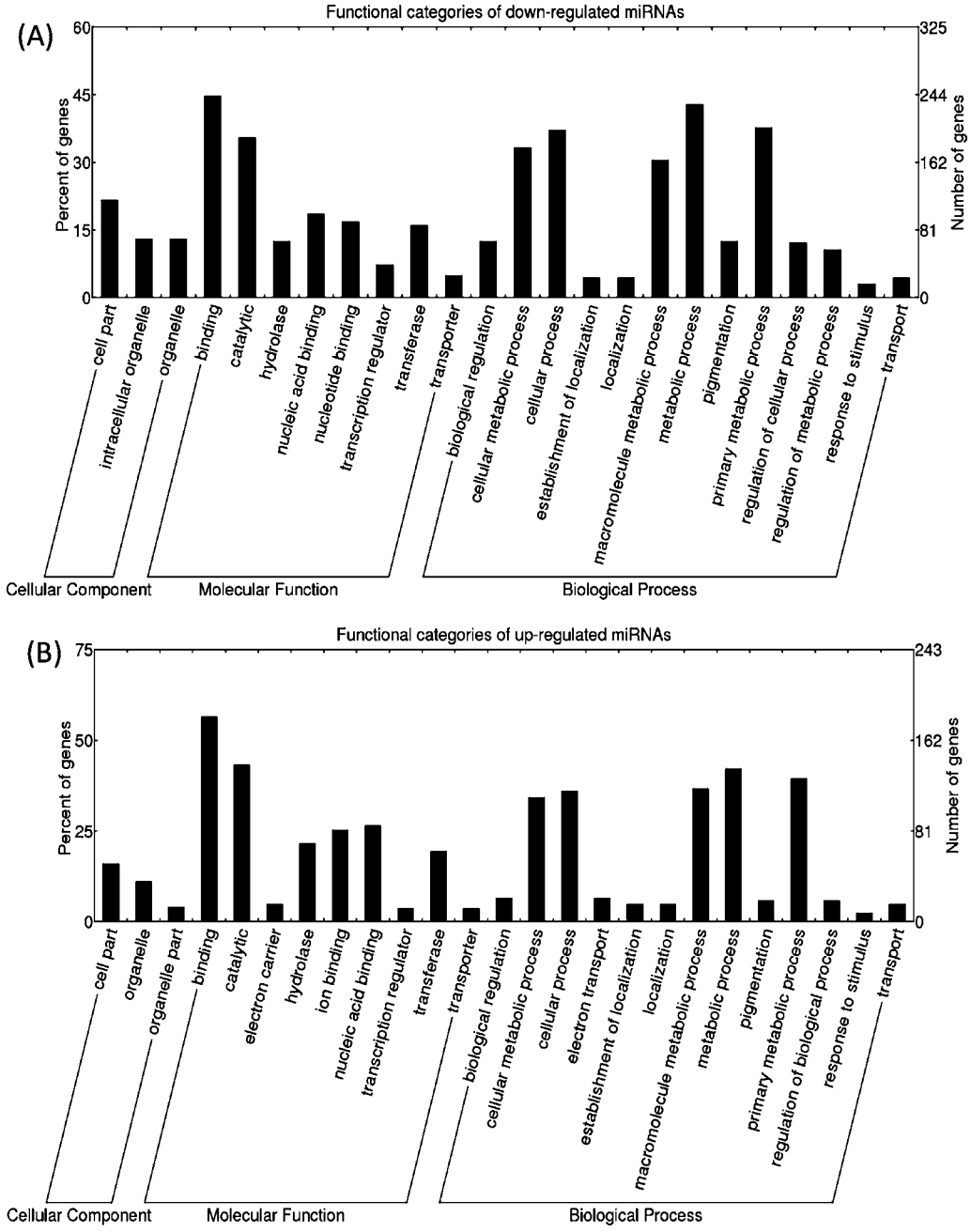

Figure 4. Functional classifications of the predicted target genes of significantly down- (A) and up-regulated (B) miRNAs.

target of miR1318) were assayed in both Minghui 86 and 93-11 rice seedling roots at different time points of arsenite treatment by real time RT-PCR (Figure 5). It was evident that miR390 and miR528 showed distinct expression pattern in Minghui 86 (Figure 5a,d) and 93-11 (Figure 5c,f), two rice cultivars that are sensitive and relatively tolerant to arsenite, respectively. In Minghui 86, the expression of miR390 was down-regulated from 2 to 4 days and subsequently recovered to nearly normal level at 6 days after exposure to arsenite (Figure 5a), whereas the expression of miR528 increased continuously from 2 to 6 days and reached its maximum expression level at 6 days after arsenite treatment (Figure 5c). However, in the tolerant variety 93-11, the expression of miR390 remained nearly constant from 0 to 2 days and then was inhibited from 4 to 6 days with the treatment of arsenite (Figure 5d), whereas miR528 quickly reached its maximum expression level at 2 days and then declined during the later stages (Figure 5f). The expression patterns of miR397b were similar in both Minghui 86 (Figure 5b) and 93-11 (Figure 5e), where it was strongly induced by arsenite, and reached its highest level at 4 days after exposure to arsenite. In addition, it was observed that the expression of miR1318 was down-regulated during the whole period of arsenite treatment in 93-11 (Figure 5g).

Contrary to the expression pattern of miRNAs, the transcript abundance of their targets increased or decreased along with the expression changes of the corresponding miRNAs at different time points after exposure to arsenite, only with the exception of miR390 in Minghui 86, for which the expression of Os06g03970.1 was up-regulated at 2 days, but markedly down-regulated at 4 days and then slightly recovered at 6 days after arsenite treatment (Figure 5a). However, the transcript abundance of Os06g03970.1 was almost exactly complementary

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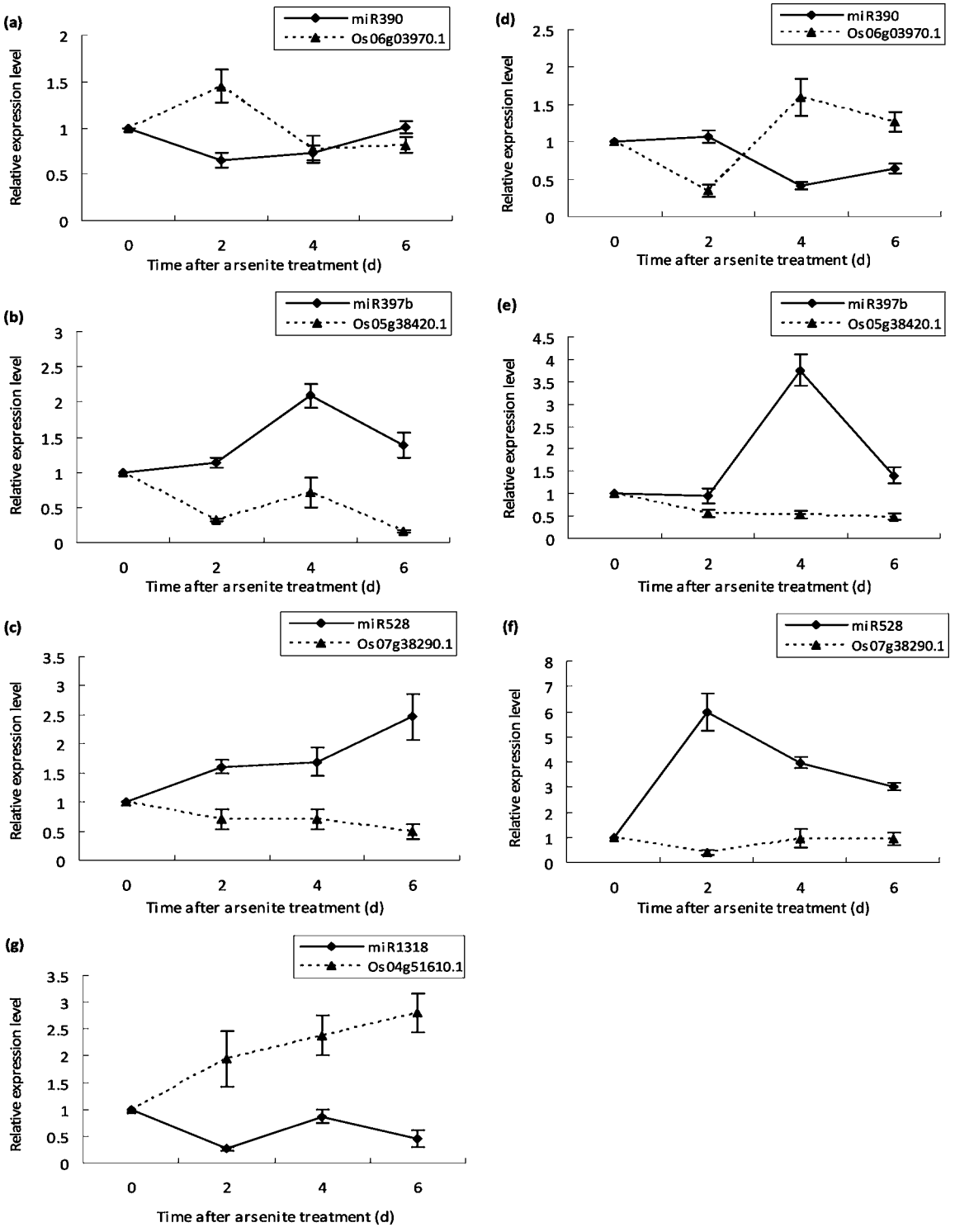

Figure 5. Expression profiling analysis of several miRNAs and their corresponding target genes in Minghui 86 (a–c) and 93-11 (d–g) rice seedling roots at different time points after exposure to arsenite. Experiments were performed in triplicates, and the results are reported as the mean ± SE for three replicates.

to the miR390 abundance in 93-11 (Figure 5d). In addition, the transcripts of Os05g38420.1 and Os07g38290.1 decreased during the whole stages of arsenite treatment in both Minghui 86 and 93-11, because of the negative effect of high miR397b and miR528 abundance (Figure 5c). The differences in expression pattern of miRNAs and their targets in Minghui 86 and 93-11 might indicate that the two rice cultivars should possess different mechanisms in response to arsenite stress. In general, the expression profiles of miRNAs were complementary, but not exactly opposite to their targets. Notwithstanding, the negative correlation in expression pattern between miRNAs and their targets further validated the regulatory role of miRNAs on their targets, and these results further indicated the role of miRNAs involved in the processes of arsenite response or tolerance in rice.

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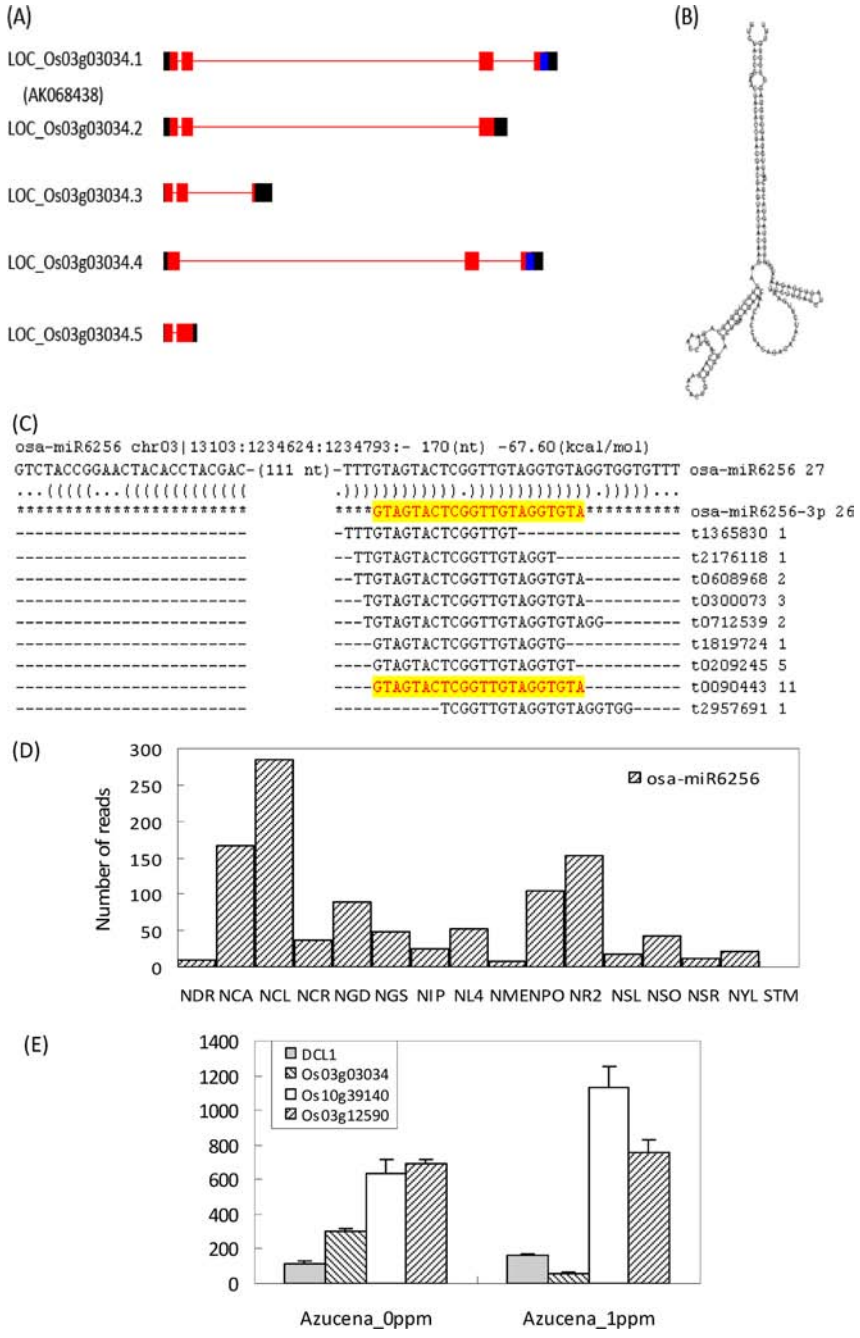

Figure 6. (A) Illustration of the Os03g03034 (AK068438) mRNA structure. Osa-miR6256 is located in the last exon of Os03g03034.1 and Os03g03034.4. The black, red, and blue boxes designate the UTR, CDS, and pre-miRNA of osa-miR6256, respectively. The red line indicates the introns. (B) Predicted hairpin structure of osa-miR6256. (C) Alignment of the osa-miR6256 stem-loop precursor and small RNAs generated from this *MIRNA* locus. Osa-miR6256-3p is shown in red. The numbers after the small RNA IDs are the numbers of sequencing reads. (D) Expression pattern of osa-miR6256 in different rice tissues with and/or without stress treatment. NDR, 14 day rice young root stressed in drought for 5days; NCA, 35-day-old rice callus; NCL, 14 day rice young leaf stressed in 4 °C cold for 24 h; NCR, 14 day rice young root stressed in 4 °C cold for 24 h; NGD, 10 day germinating rice seedlings grown in the dark; NGS, 3 day rice germinating seed; NIP, 90 day immature panicle; NL4, mature leaf; NME, 60 day crown vegetative meristematic tissue; NPO, mature pollen; NR2, 60 day mature root; NSL, 14 day young leaves stressed in 250 mM NaCl for 24 h; NSO, ovary and mature stigma; NSR, 14 day young roots stressed in 250 mM NaCl for 24 h; NYL, 14 day young leaves; STM, stem. (E) Expression profiles of *DCL1*, *Os03g03034*, *Os10g39140*, and *Os03g12590* in response to arsenate stress. The expression data were derived from the published microarray data GSE4471. Data are reported as the mean ± SE for three biological replicates.

Putative Exonic miRNA Found in Rice. After careful screening of the sRNA sequences perfectly mapped to the rice genome, 18 potential novel rice miRNAs were predicted in the CK sample (data not shown), which were further used to search against the noncoding regions consisting of intergenic and intronic sequences to localize the genomic positions where they originated. However, no hits were returned for osa-miR6256, which was subsequently used to search against the full-length cDNA sequences in rice. Interestingly, it was found that osa-miR6256 was located in the last exon of the AK068438 mRNA that encodes a flavonol synthase/flavanone 3-hydroxylase (FLS/F3H) Os03g03034.1. As annotated, five

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Table 3. Putative Exonic miRNA Osa-miR6256 and Its Potential Target Genes

miRNA ID	len (nt)	sequence 5′−3′	no. of reads[a] CK	no. of reads[a] As(III) treatment	fold change	predicted targets	target site	putative functions of targets
Osa-miR6256	22	GUAGUACUCGGUUGUAGGUGUA	26	0	−7.32	Os03g03034.1	1089...1109	flavonol synthase/flavanone 3-hydroxylase
			(1.601)	(0.01)		Os10g39140.1	1119...1139	flavonol synthase/flavanone 3-hydroxylase
						Os03g12590.1	1732...1753	coatomer subunit γ-1

[a]Numbers in parentheses indicate normalized sequencing reads in the CK and As treatment samples, respectively.

potential alternative splicing mRNAs could be transcribed from this locus. The gene *Os03g03034.1* should be actively expressed on the basis of the presence of AK068438. This gene was composed of four exons and three introns, with the osa-miR6256 miRNA present in its 3′-UTR region (Figure 6A).

Expression of osa-miR6256 in the CK and As treatment samples was examined by normalizing its sequencing reads to the total number of miRNA reads in each sample and multiplied by a million. We found that the expression of osa-miR6256 was strongly down-regulated after arsenite treatment (Table 3). Moreover, the tissue expression pattern of osa-miR6256 was examined by searching against the small RNA sequences of 16 untreated and 6 abiotic-treated diverse tissue libraries (GSE7107), using the miRDeep-P program.[44] It was found that except for stem, osa-miR6256 was constitutively expressed in all tissues examined, including mature roots, leafs, germinated seeds, mature pollen, ovary, and mature stigma (Figure 6D). Additionally, most of the sequenced small RNAs in this *MIRNA* locus were mapped to the strand producing the mature miRNA (Figure 6C). All of this evidence strongly supports the reality of this putative exonic miRNA.

To better understand its regulatory functions, the potential target genes of osa-miR6256 were predicted using the psRNATarget server and the BGI small RNA target prediction pipeline. Interestingly, osa-miR6256 was predicted to target its host gene *Os03g03034* (Table 3), a result that is similar to the first exonic miRNA identified in plants, osa-miR3981, that is located in the second exon of AK106348 and is demonstrated to target its host gene.[45] Besides, osa-miR6256 might also regulate the expression of a coatomer subunit γ-1 encoding gene *Os03g12590* and another FLS/F3H gene *Os10g39140* (Table 3). At present, only a few studies have described the localization of miRNAs in the exon of protein-coding transcripts.[45,46] The identification and characterization of osa-miR3981 and osa-miR6256 as potential exonic miRNAs may indicate that plants also use some exons as a miRNA source.

DISCUSSION

The mechanisms of arsenic toxicity to plants are quite different for As(III) and As(V). As(III) can react with sulfhydryl groups found in the amino acid cysteine, which results in loss of function of enzymes and proteins, thereby affecting many key metabolic processes in the cell.[47] As(V) can substitute inorganic phosphate in many biochemical processes, including the synthesis of ATP.[48] It is well-known that under anaerobic conditions, As(III) is the predominant form of arsenic, whereas As(V) dominants in an aerobic environment.[6,49] However, after being taken up by plant roots, As(V) is rapidly reduced to As(III). Accordingly, the majority of the toxic effects of As(V) to plants may be due to its reduction product, As(III).[50] Moreover, numerous studies have shown that As(III) is more toxic to plants in comparison to As(V),[51−53] although under hydroponic cultures, a proportion of As(III) is observed to be released to the external medium, where the efflux of As is mediated by OsNIP2;1 (Lsi1) in rice.[30] Accordingly, in this study, arsenite in the form of $NaAsO_2$ was adopted to stress challenge the rice cultivars Minghui 86 and 93-11, using a hydroponic cultivation strategy.

MiRNAs are ubiquitous in animals,[54] viruses,[55] and plants including unicellular green and red algae.[40,56−58] In each species, the miRNA repertoire is basically composed of a set of conserved miRNAs as well as many newly evolved lineage- or species-specific miRNAs.[59,60] Traditionally, two approaches, forward genetics and bioinformatics prediction, have been widely used for miRNA discovery in plants,[61] but they lack power in discovery of low abundant and tissue/condition-specific miRNAs. With its advantages in sequencing depth and sensitivity, the Solexa sequencing technology has revolutionized small RNA discovery,[62] and numerous miRNAs have been identified.[40,45] Here, >30 million sRNA sequences were produced after deep sequencing the cultivar Minghui 86, an *indica* rice that is sensitive to As.[63] It was observed that the members of three miRNA families (miR156a-j, miR166a-d, miR166f, miR166n, and miR168a) were more abundant both in CK and in As treatment samples, with the highest abundance found in miR156a-j, indicating that they played crucial roles in the regulation of plant seedling development.[64] In contrast, 386 miRNAs with low expression in the two samples (sequencing reads <100) were found (see the Supporting Information). Among those lowly expressed miRNAs, >50 were widely present in the plant kingdom, such as the miR159, miR160, miR164, miR166, miR172 family miRNAs, whereas in the remaining miRNAs, some were monocot-lineage-specific, for example, miRNA families 437, 821, and 2275, and others including the miR438, miR1423, miR806, and miR807 families were rice-species-specific. These observations support the notion that most of the nonconserved miRNAs are prone to low expression. Moreover, it was found that 56 of the 386 lowly expressed miRNAs were strongly induced or repressed by arsenite, thereby showing an environmentally inducible fashion. However, the expression of 185 miRNAs was not detectable either in CK or in As treatment samples (see the Supporting Information), indicating that these miRNAs might be expressed in a tissue- or developmental-stage-specific manner.

Heavy metal and other poisonous element pollution is an increasing and severe environmental problem, and aluminum (Al), cadmium (Cd), and arsenic (As), in particular, are extremely toxic to plants. To overcome such toxicity, plants have evolved various strategies to adapt to environmental stresses. Previous research revealed that the regulatory mechanisms of plants in response to stresses are complex, and many miRNA genes function in the processes of stress

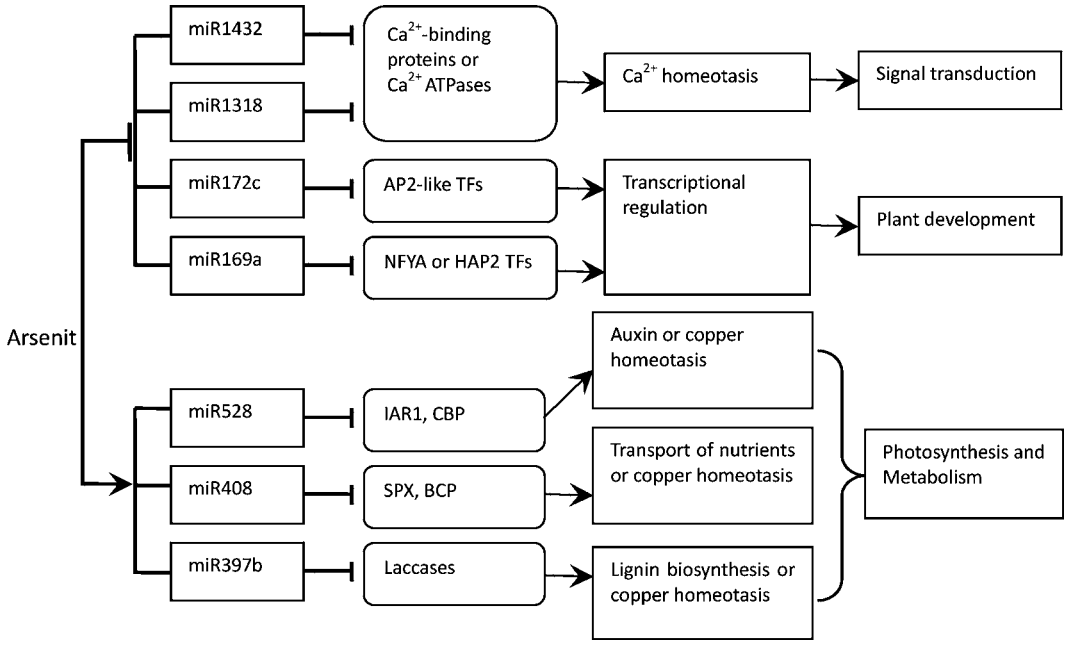

Figure 7. Proposed functional network of arsenite-responsive miRNAs in rice seedling roots. The arrows indicate positive regulation, and the nail shapes designate negative regulation. NFYA, nuclear factor Y, subunit A; IAR1, IAA-alanine resistance protein 1; CBP, Cu^{2+}-binding protein; SPX, SPX domain protein; BCP, blue copper protein.

response and/or stress tolerance. It was reported that there were 19 miRNAs, each being strongly induced and/or repressed in rice seedling roots under Al or Cd stress,[65,66] whereas >60 miRNAs were found to be involved in the process of arsenite response in this study (see the Supporting Information). Seven family members including miR1318, miR1861, miR2121, miR810, miR812, miR815, and miR818 were significantly down-regulated after the treatment of arsenite. However, no significant change in expression of these miRNAs was found after exposure to Al or Cd stress,[65,66] indicating that they might be arsenite-conditional-responsive miRNAs.

Presently, no reports on the identification of arsenite responsive miRNAs at the whole genome-wide level exist. Sixty-seven arsenite-responsive miRNAs were first identified herein. Previous studies showed that 4 of the 67 miRNAs (miR528, miR408, miR169a and miR1432) were directly involved in the process of stress response or stress tolerance. miR528, which targets copper proteins, cupredoxin, multicopper oxidase, and laccase genes,[65] was found to be down-regulated in response to shock drought stress in *Triticum dicoccoides*,[25] but highly induced under the Al,[65] Cd,[66] and As stresses. The opposite case was observed for miR1432, which was up-regulated under drought stress in *T. dicoccoides*,[25] but down-regulated remarkably in response to Cd[66] and arsenite. The expression pattern of miR408 and miR169 was intriguing. miR408 was strongly up-regulated both by water deprivation in *Medicago truncatula*[67] and arsenite, but was inhibited significantly under drought and Al stress in *O. sativa*.[27,65] In tomatoes, overexpression of miR169c enhanced their tolerance to drought stress.[68] Besides, miR169a was demonstrated to function in helping plants to cope with fluctuations in nitrogen availability in soil.[26] These results add further evidence that miRNAs play important roles in the regulation of plant adaptive responses to environmental stresses and nutrient deprivation. In addition, it was found that miR172 acts sequentially after miR156 to regulate plant development.[64,69] We found that the expression of miR172c was completely inhibited after treatment with arsenite. Thus, it is of interest to adopt the overexpression strategy to further explore the functional significance of miR172c in response to arsenite.

After exposure to arsenate or arsenite, a group of protein-coding genes including defense and stress-responsive transporters, heat-shock proteins, metallothioneins, sulfate-metabolizing proteins, and regulatory genes were found to be differentially expressed in rice (IR64) seedlings.[53] Using four rice cultivars that are tolerant and/or sensitive to arsenic, Rai et al.[9] uncovered that the strong activation of several of genes involved in the sulfate assimilation pathway and antioxidant defense system would be at least partially responsible for the high tolerance of Triguna and IR-36 to As. The arsenite stress would be transduced through the MAPK signaling cascade in rice.[70] At the whole small RNA transcriptome level, we first revealed that a group of transcription factors, protein kinases, and DNA-binding proteins were strongly activated, whereas a set of genes involved in electron transmission, ion binding and transport, and transcriptional regulation were extensively repressed in seedling roots of Minghui 86 under arsenite stress (Figure 4). These findings enhance our understanding of the mechanisms of rice responses to arsenic.

To our knowledge, few miRNAs have been located to the exon of protein-coding transcripts,[45,46] where osa-miR3981 is an exonic miRNA first identified in plants.[45] As discussed by Li et al.,[45] the presence of exonic miRNAs may present a new mechanism for posttranscriptional regulation of gene expression in plants. They speculated that protein-coding genes with hairpin structures might be negatively regulated by *DCL1*. To test this hypothesis, the expression profiles of *DCL1*, the host gene *Os03g03034*, and the target genes *Os10g39140* and *Os03g12590* of osa-miR6256 were analyzed using the published microarray data.[71] It is obvious that *DCL1* and *Os03g03034* exhibit completely adverse expression patterns under arsenate stress (Figure 6E), indicating that *DCL1* may negatively regulate the expression of *Os03g03034*. The *Os03g03034*

mRNA encodes a flavonol synthase/flavanone 3-hydroxylase (FLS/F3H), which is a key enzyme in the flavonoid biosynthetic pathway.[72] Flavonoids play important roles in diverse aspects of signaling transduction, UV protection, defense against pathogens and pests, and auxin transport regulation in plants.[73] Under arsenate stress, *Os03g03034* was strongly down-regulated, whereas the abundance of *Os10g39140* transcripts that encodes another FLS/F3H enzyme increased significantly (Figure 6E). Thus, osa-miR6256 might be involved in the regulation of flavonoid homeostasis by controlling flavonoid synthesis under arsenite stress. In addition, the putative target *Os03g12590*, a coatomer protein encoding gene that belongs to the Adaptin_N family and functions in mediating vesicles-mediated protein transport in cells, was also up-regulated (Figure 6E), thereby accelerating the speed of protein transport and update under arsenate stress.

On the basis of the results presented herein, a putative functional network of arsenite-responsive miRNAs in rice seedlings can be inferred. As illustrated in Figure 7, two groups of miRNAs with significant changes in expression were involved in the process of arsenite response. The first group of seven arsenite-responsive miRNAs consists of four As-down-regulated miRNAs including miR172c, miR1432, miR1318, and miR169a. In plants, miR172 targets a small group of AP2-like transcription factors (TFs) and plays a crucial role in plant development.[74] It was reported that miR172c was expressed in rice seedlings but not in developing grains.[43] Under As stress, the miR172c-mediated up-regulation of AP2-like mRNAs would possibly result in the phase transition from juvenile to adult stage. miR169, which targets several NFYA TFs, also participates in diverse processes including cell differentiation, respiration, and abiotic stress responses.[45] Zhao et al.[26] demonstrated that the *Arabidopsis* miR169a was strongly down-regulated, whereas its targets, NFYA family members, were strongly induced by nitrogen N starvation. Thus, down-regulation of miR169a would accelerate the absorption of nitrogen, thereby enhancing some processes of development and metabolism in rice seedlings in response to As stress. The additional two miRNAs, miR1432 and miR1318, both target calcium-binding proteins or Ca^{2+}-ATPase, which are involved in the processes of signal transduction. Accordingly, down-regulation of miR1432 and miR1318 would increase the activity of these proteins, hence triggering a series of cell biological actions by promoting influx or efflux of Ca^{2+} across biomembranes.

In the second group, three significantly up-regulated miRNAs including miR408, miR397b, and miR528 were included. miR408 was predicted to target mRNAs encoding major facilitator superfamily (MFS) proteins, including an SPX domain protein (AK066805). As previously reported, MFS proteins play a vital role in mediating the transport of a variety of substrates across membranes.[75] Accordingly, the up-regulation of miR408 may reduce the transport of nutrients such as monosaccharide to limit the nutrient consumption of plants. The targets of miR397b are some laccases, which may be involved in lignin biosynthesis and copper homeostasis.[76,77] In addition, miR408 and miR528 were also predicted to target blue copper proteins and Cu^{2+}-binding proteins (CBP), respectively. In plants, copper plays critical roles in diverse aspects, including photosynthetic and respiratory electron transport, oxidative stress protection, cell wall metabolism, and ethylene perception.[77] Thus, down-regulation of copper proteins might limit some nonessential biological processes to save copper for the most essential functions under stress challenges. Another target of miR528 was mRNAs encoding putative IAR1 proteins that function in control of cellular-free auxin levels,[45] possibly by transporting zinc, copper, or another inhibitory metal out of endoplasmic reticulum. Hence, up-regulation of miR528 would decrease the *IAR1* mRNA abundance, thereby keeping most IAA in conjugated forms, and protect rice seedlings from the damage of arsenite stress.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Information for RT-PCR primers, statistics of short RNA reads, and differentially expressed miRNAs described herein. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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