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Molecular Identification and Analysis of Cd-Responsive MicroRNAs in Rice

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Supporting Information

ABSTRACT: Cadmium (Cd) is a non-essential heavy metal with high toxicity to plants. MicroRNAs (miRNAs) are a class of small non-coding RNAs that play important roles in plant abiotic stress responses. To investigate whether miRNAs function in Cd stress response, miRNA expression profiles in rice (*Oryza sativa*) under Cd stress were monitored using microarray assays. A total of 12 Cd-responsive novel miRNAs predicted previously were identified, of which 4 were further validated experimentally. A total of 44 target genes were predicted for the Cd-responsive miRNAs, many of which appeared to regulate gene networks mediating environmental stresses. Several target genes were validated to show a reciprocal regulation by miRNAs. A transgenic approach was also used to determine the role of miRNAs in rice response to Cd stress. Overexpression of miR192 retarded seed germination and seedling growth under Cd stress. These results implied the role of novel miRNAs in the involvement of Cd tolerance of rice.

KEYWORDS: *Cadmium, gene regulation, microarray, microRNA, rice*

INTRODUCTION

Heavy metal pollution is an increasing environmental problem worldwide. Cadmium (Cd) is a non-essential trace element, which is considered the most toxic heavy metal. Cd has been shown to interfere with the uptake, transport, and use of essential nutrients and water, decrease photosynthesis, change enzyme activities, and also cause various symptoms, such as chlorosis, wilting, and root browning in plants.[1−3] Plants use a range of mechanisms to counteract Cd toxicity, including metal transport, chelation, and sequestration.[4] The production of phytochelatins and metallothioneins is a widespread mechanism of Cd detoxification in higher plants.[5] The expression of several metal transporters is essential for tolerance to Cd toxicity. The natural resistance-associated macrophage protein (*Nramp*) transporter was reported to function in Cd uptake in *Arabidopsis thaliana*.[6] The ATP-binding cassette (*ABC*) transporters also mediated the transport of Cd.[7] However, only a small number of genes functioning in Cd tolerance have been identified in plants, and the regulatory pathway involved is still largely unknown.

Small RNAs may be involved in the regulation/signaling of metal toxicity response.[8,9] MicroRNAs (miRNAs) are a class of endogenous non-protein-coding small RNAs of 21 nucleotides. The miRNA genes are processed from hairpin precursors by DICER-LIKE1 (DCL1) in plants or Drosha and Dicer in animals.[8,10] Mature miRNAs function within RNA-induced silencing complex (RISC) to negatively regulate the expression of specific mRNA targets by directing mRNA degradation or translational repression.[11,12] Most of miRNAs are highly conserved in evolution,[13] making it possible to identify putative miRNAs in other species using comparative genomics. To date, a number of computational programs have been successfully developed for predicting miRNAs.[14,15] With regard to the species-specific miRNAs, they can be predicted relying on the intragenomic matching between miRNA candidates and their targets coupled with support vector machine classification of miRNA precursors. Using such a strategy, Lindow et al.[16] computationally predicted 2100 novel miRNA candidates in rice (*Oryza sativa* L.) for all of the known rice mRNAs, a majority of which were not conserved across species (see Supporting Information 1). Because matching between miRNA candidates and targets is integral to the method, it is possible to predict new miRNAs that are specific to rice.

Recently, miRNAs have been reported to be crucial regulators of multiple physiological processes, including plant development, signal transduction, and adaption to abiotic stresses.[17−19] For example, miR395 and miR399 were induced by sulfate and phosphate starvation, respectively,[20,21] whereas miR398 was downregulated in response to copper (Cu) and iron (Fe) in *Arabidopsis*.[22] Recent studies also point to the role of miRNAs in the plant response to heavy metal stress.[23−26] Using microarray, 19 Cd-responsive miRNAs were identified and their target genes were predicted in rice.[27] Using high-throughput sequencing, 52 new miRNAs were profiled from *Medicago truncatula* seedlings exposed to HgII; most of these miRNAs were differentially regulated by the heavy metal.[26] In addition to being one of the most important world food crops, rice serves as a useful research model. In the present study, a total of 12 novel miRNA candidates predicted by Lindow et al.[16] were identified to be Cd-responsive using microarray assays in rice. Moreover, the prediction and transcription analysis of their target genes and the search for stress-related *cis* elements in the promoter regions of the miRNA genes

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provided molecular evidence for the possible involvement of miRNAs in the Cd tolerance of rice. Among the Cd-responsive miRNAs, miR192 functioned as a negative regulator of seed germination in rice under Cd stress. The identification of miRNAs and their target genes may provide new insights into the mechanism of heavy metal tolerance in plants.

MATERIALS AND METHODS

Plant Materials and Stress Treatments. The seeds of wild-type rice Zhonghua 11 (*Oryza sativa* L. subsp. *japonica*) were first surface-sterilized with 8% NaClO for 20 min, rinsed thoroughly, and soaked in deionized water for 2 days. Then, seeds were placed on a filter paper. After germination, seedlings were grown in plates under a 13 h light (29 °C)/11 h dark (22 °C) photoperiod. For Cd stress experiments, 60 μM $CdCl_2$, a moderate but not lethal Cd concentration, was chosen. The 1-week-old seedlings were treated with 60 μM $CdCl_2$ for 0−24 h. Untreated seedlings served as controls. After treatment, roots were separately harvested and pooled together for RNA extraction.

Microarray Assay Analysis. Microarray assays were performed by a service provider, LC Sciences (http://www.lcsciences.com). The 2100 miRNA sequences predicted by Lindow et al.[16] were used to identify Cd-responsive miRNAs on microarray chips. Microarray experiments were performed twice using distinct biological samples. The 1-week-old rice seedlings were treated with or without 60 μM $CdCl_2$ for 6 h. Approximately 20 plants were harvested, and root tissue was pooled to make one sample for RNA extraction. Total RNA samples were isolated with Trizol reagent (Invitrogen) from treated and control roots and size-fractionated using a YM-100 Microcon centrifugal filter (Millipore). The small RNAs (<300 nucleotides) were 3′-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. The purified small RNAs were labeled with Cy3 (control) and Cy5 (treated) fluorescent dyes. Hybridization was performed overnight on a μParaFlo microfluidic chip using a micro-circulation pump (Atactic Technologies). The custom μParaFlo microfluidic chip contained 992 probes after overlapping miRNAs, and other redundant high homology sequences were removed. Each probe was repeated 3 times on the chip to ensure microarray reproducibility. The hybridization buffer was 100 μL of 6× SSPE buffer [0.90 M NaCl, 60 mM Na_2HPO_4, and 6 mM ethylenediaminetetraacetic acid (EDTA) at pH 6.8] containing 25% formamide at 34 °C. After hybridization, signals were detected using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). For two color experiments, the ratio of the two sets of detected signals ($\log_2$ transformed and balanced) and p values of the t test were calculated; differentially detected signals were those with fold change > 1.5 and p values < 0.01.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Assay. Expression analysis of several miRNAs and their target genes were performed using quantitative real-time reverse transcription PCR (RT-PCR). Total RNA was isolated from 7-day-old seedlings at 0, 3, 6, 12, and 24 h after Cd exposure and treated with 5 units of RNase-free DNase I (TaKara) to remove DNA contamination. After inactivation of DNase I, RNA was reverse-transcribed using an oligo(dT) primer and a PrimeScript RT reagent kit (TaKara, Japan) to generate cDNA. Real-time PCR was carried out using SYBR Premix Ex Taq (TaKara, Japan) for detection of PCR products. Real-time PCRs were carried out on a Rotor Gene Q machine (parameters: 95 °C for 1 min, followed by 45 cycles of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s). All reactions were performed in triplicate. Quantification of gene expression was performed using the comparative CT method. Experiments were performed in triplicate, and the results were represented by means ± standard error (SE) of three replicates. The rice housekeeping gene *OsActin1* (Os03g0718100) was chosen as the internal loading control.[28,29] The primer pairs for the amplification of

Table 1. Cd-Responsive Novel miRNAs by Microarray Analysis

		chip 1				chip 2			
probe ID	sequence (5′ → 3′)	CK sample signal	Cd sample signal	log 2 (sample Cd/sample CK)	sample Cd/sample CK	CK sample signal	Cd sample signal	log 2 (sample Cd/sample CK)	sample Cd/sample CK
miR192	AUGAAUGUGGGCAAUGCUAGAA	842.89	523.41	−0.69	0.62	1126.98	611.01	−0.88	0.54
miR118	AGGGUCAUCGGUGACGGCUA	482.80	848.96	0.81	1.76	425.66	1611.73	1.92	3.79
miR59	ACGCGGAGGAGGUGGUGUUCU	66.64	295.87	2.22	4.44	162.91	335.53	1.04	2.06
miR1004	UCUUCUCUUCUUCAGUGAGGGAGGC	96.52	433.23	2.21	4.49	161.92	470.48	1.54	2.91
miR361	CCUAGAUCUGGCCGGAGGAGGA	355.50	947.55	1.34	2.67	663.49	1400.04	1.08	2.11
miR1060	UGGGCGGCAAGCUGACGUGGCA	517.36	1199.56	1.21	2.32	607.61	1178.44	0.96	1.94
miR441	CGUCCGACUGGUUUCCAGUCGGGGGCUC	13259.18	41904.20	1.64	3.16	15788.02	36734.14	1.22	2.33
miR248	CAUCCGACUGGUUUCCAGUCGGGGGCUC	13170.38	41472.37	1.64	3.15	16879.53	37620.00	1.16	2.23
miR848	GUUGCAGCAGCAGGGACAGU	1700.45	14788.06	3.06	8.70	2189.45	14422.92	2.72	6.59
miR588	GAUGACUCAUAUGUGACGGGC	279.16	520.49	1.01	1.86	215.18	488.37	1.18	2.27
miR1073	UGUCCUUGCUGGUGGCGGAC	1676.03	2238.45	0.42	1.34	1181.48	1998.63	0.76	1.69
miR191	AUCUUCGUAGGCGGGCGGUC	470.86	1994.61	2.15	4.24	306.35	960.66	1.65	3.14

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Table 2. Predicted Targets of Cd-Responsive miRNAs and Their Function Annotations

MiR ID	sequence (5′ → 3′)	target genes	target function
miR118	AGGGUCAUCGGUGACGGGCUA	Os02g32590	heat-shock factor protein 2
miR59	ACGCGGAGGAGGUGGUGUUCU	Os04g08640	cadmium tolerance factor
		Os04g30010	OsWAK45-OsWAK receptor-like protein kinase
		Os11g42280	F-box domain-containing protein
		Os01g09100	OsWRKY10 superfamily of rice TFs having WRKY and zinc finger domains
		Os03g05740	ras-related protein RHN1
		Os09g31454	myb-like DNA-binding domain-containing protein
miR1004	UCUUCUCUUCUUCAGUGAGGGAGGC	Os06g19980	myb-like DNA-binding domain-containing protein
		Os04g46940	copper-transporting ATPase 3
		Os02g48990	phosphatidylinositol transporter/transporter
		Os10g38580	glutathione *S*-transferase GSTU6
		Os03g63360	integral membrane protein
		Os08g09320	vacuolar protein sorting protein 72
		Os01g66110	ankyrin-like protein
		Os07g27460	serine/threonine-protein kinase 19
		Os05g07090	glutaryl-CoA dehydrogenase, mitochondrial precursor
miR361	CCUAGAUCUGGCCGGAGGAGGA	Os12g17900	ubiquitin-protein ligase
		Os10g22300	resistance protein
miR1060	UGGGCGGCAAGCUGACGUGGCA	Os10g30790	inorganic phosphate transporter 1-4
		Os10g30770	inorganic phosphate transporter 1-1
		Os02g12690	cytochrome P450 74A4
miR192	AUGAAUGUGGGCAAUGCUAGAA	Os09g39910	ATP-binding cassette subfamily F member 2
		Os12g40920	light-inducible protein CPRF-2
		Os06g19990	GPI-anchored protein
		Os07g03110	F-box domain-containing protein
		Os02g44990	
		Os10g33700	ATP-binding protein
		Os02g07960	
		Os04g35260	
		Os01g34620	OsGrx_S15.1 glutaredoxin subgroup II
		Os03g09210	NADH-ubiquinone oxidoreductase B16.6 subunit
		Os03g54130	cysteine protease 1 precursor
		Os06g08080	pyrophosphate-energized vacuolar membrane proton pump
		Os03g50070	membrane protein
		Os09g12230	ubiquitin-conjugating enzyme E2 17 kDa
		Os11g41860	ubiquitin-protein ligase
		Os02g45650	CAAX prenylprotease 1
		Os06g17390	auxin-independent growth promoter
		Os03g62070	IAA-amino acid hydrolase ILR1 precursor
		Os06g23274	zinc finger, C3HC4-type family protein
miR441	CGUCCGACUGGUUUCCAGUCGGGG	transponon	
	GCUC	retrotransposon	
miR248	CAUCCGACUGGUUUCCAGUCGGGG	transponon	
	GCUC	retrotransposon	

Actin1, pre-miRNAs (miRNA precursors), and target genes were listed in Supporting Information 2.

miRNA Target Prediction and *cis*-Acting Element Analysis. On the basis of sequence similarity search, a web-based computing system, miRU (Plant microRNA Potential Target Finder, http://bioinfo3.noble.org/miRNA/miRU_old.htm),[14] was used to predict target genes for the miRNA candidates provided by Lindow et al.[16] The miRU program reports all potential sequences, with mismatches no more than specified for each mismatch type. The minimal score among all 20-mers cannot exceed 3.0 with default parameters. The conservation of target complementarity in other plant species was also used for identifying miRNA targets and further reducing false positives. For *cis*-acting element analysis, about a 1500 bp sequence upstream of pre-miRNAs was extracted from TIGR Rice Genome Annotation (http://www.tigr.org/tdb/e2k1/osa1/data_download.shtml) and then scanned by PLACE (http://www.dna.affrc.go.jp/PLACE/), a database of nucleotide sequence motifs found in plant *cis*-acting regulatory DNA elements.[30]

Generation of Transgenic Rice Overexpressing miR192. The pre-miR192 sequence was amplified and subcloned into pCAMBIA 1301 between 35S promoter and NOS terminator with *Kpn*I and *Pst*I enzymes. The hygromycin (hyg) phosphotransferase gene (*hpt*) was inserted in T-DNA as a selectable marker (Figure 4). The rice variety Zhonghua 11 (*japonica*) was transformed by *Agrobacterium*-mediated transformation[31] and selected with hyg. All regenerated T0 transgenic plants were genotyped using the primer of *hpt* (forward, 5′-GTTTATCGGCACTTTGCATCG-3′; reverse, 5′-GGAGCATATACGCCCGGAGT-3′). GUS staining was also used to confirm the transgenic plants. The histological assay for GUS activity was performed at 37 °C using a reaction mixture: 50 mM phosphate buffer (pH 7.0) containing 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), 5% methanol, 10 μg/mL cycloheximide, and 1 mM dithiothreitol.[32] Expression levels of miR192 in transgenic rice plants were examined using real-time quantitative PCR (qPCR). Total RNA was extracted from roots of wild-type and transgenic seedlings using

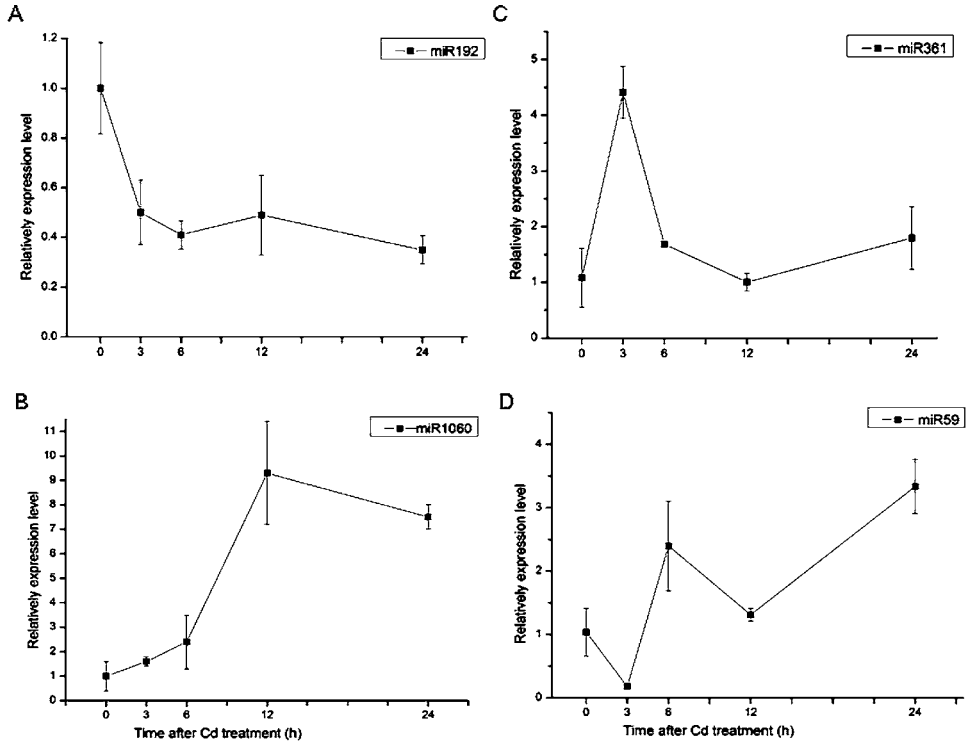

Figure 1. Temporal expression profiles of the four Cd-responsive miRNAs. Cd treatment time was 0, 3, 6, 12, and 24 h. *OsActin1* was used as a loading control.

Table 3. Stress-Related *cis*-Element Analysis of the Four Cd-Responsive miRNAs[a]

miRNA	*cis*-element analysis
miR192	ARE, HSE, DRE, and ERE
miR1060	ARE, ABRE, MYBCORE, and DRE
miR361	ARE, ABRE, GARE, HSE, and DRE
miR59	ARE, ABRE, GARE, and HSE

[a]ARE, anaerobic responsive element; ABRE, ABA responsive element; ERE, ethylene responsive element; GARE, gibberellin responsive element; HSE, heat stress responsive element; and DRE, dehydration responsive element.

Trizol reagent (Invitrogen) and then reverse-transcribed using oligo (dT) primer and a PrimeScript RT reagent kit (TaKara, Japan) to generate cDNA. Primers used for miR192 were as follows: forward, 5′-AGAGACCGTGTCGTTGT-3′; reverse, 5′-GGTTTGGATCTTTG-GAT-3′. Real-time PCRs were carried out on a Rotor Gene Q machine (parameters: 95 °C for 1 min, followed by 45 cycles of 94 °C for 10 s, 58 °C for 15 s, and 72 °C for 15 s). All reactions were performed in triplicate. Quantification of gene expression was performed using the comparative CT method. *OsActin1* (Os03g0718100) was chosen as a reference gene.

Germination and Seedling Growth Assays under Cd Stress. Sterile transgenic and wild-type seeds were sown on solid medium (0.8% agar) with 0 and 100 μM $CdCl_2$ and cultured vertically. The seedlings were grown in a growth chamber under cycles of a 13 h light (28 °C)/11 h dark (22 °C) photoperiod with 80% relative humidity (RH). After 7 days, plant growth was observed. Shoot length, root length, and root number was quantified.

RESULTS

Identification of Cd-Responsive Novel Candidate miRNAs. The 1-week-old rice seedlings were exposed to Cd-free (control) and 60 μM $CdCl_2$ for 6 h. Small RNAs from the two samples were isolated and analyzed using miRNA microarrays. Among the 992 putative miRNAs predicted by Lindow et al.,[16] a total of 12 putative miRNAs were detected to be Cd-responsive by microarray assays (Table 1). They were considered to be new miRNAs and named according to the probe ID on the microarray chips as follows: miR192, miR118, miR59, miR361, miR1060, miR1004, miR441, miR248, miR848, miR1073, miR191, and miR588 (Tables 1 and 2). miR192 was downregulated under Cd stress, whereas the other miRNAs were all upregulated. A sequence similarity search against the miRBase revealed that miR192 was highly similar to the known rice miR819 in sequence. The other upregulated

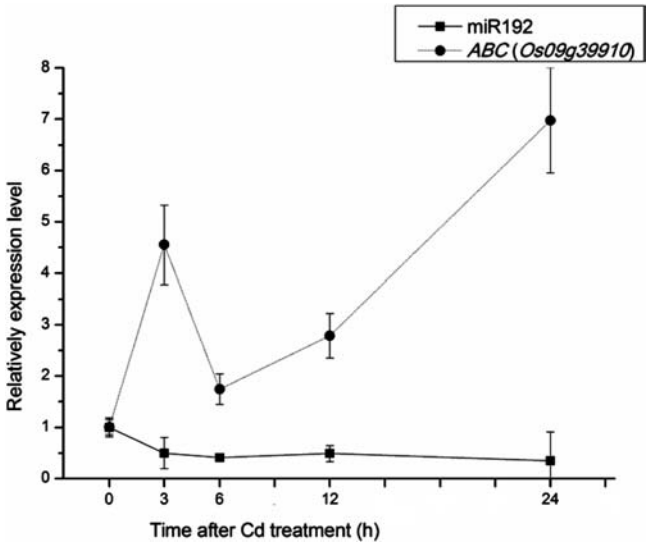

Figure 2. Expression analysis of miR192 and its target gene during Cd stress. Cd treatment time was 0, 3, 6, 12, and 24 h. *OsActin1* was used as a loading control.

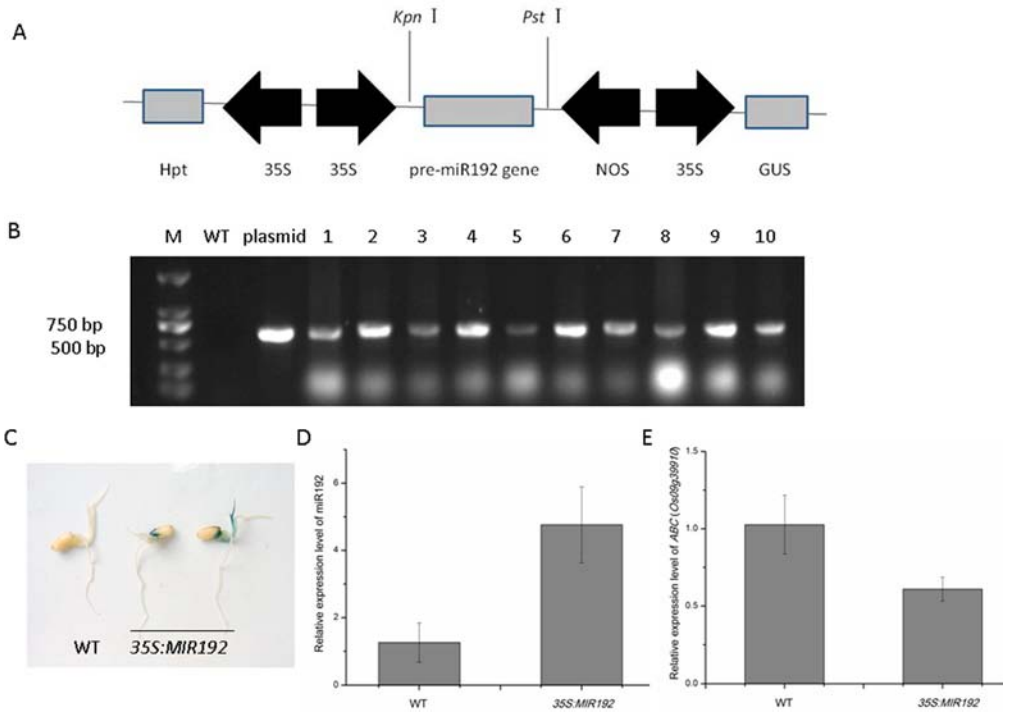

Figure 3. Overexpression of miR192 in transgenic rice plants. (A) T-DNA regions of plant expression vectors used to transform rice. The *Hpt* gene was used as a selection marker. (B) Genomic PCR analysis of transgenic rice plants. Genomic DNA was used as a template for PCR. The expected size of the product was 621 bp. M, DNA marker; WT, wild-type plant; and 1−10, transgenic plants. (C) GUS activity analysis of transgenic rice. (D) Expression levels of miR192 in wild-type and transgenic lines by qPCR. cDNAs were normalized using *OsActin1* gene. (E) Expression levels of *ABC* in wild-type and transgenic lines by qPCR.

miRNAs were found to be non-conserved between rice and *Arabidopsis*.

Validation and Time Course Analysis of the Identified miRNAs. To validate the identified rice miRNAs by microarray assays and further understand their temporal expression patterns, qPCR analysis was performed to detect the expression levels of four pre-miRNAs (precursor miRNAs): miR192, miR1060, miR361, and miR59 (Figure 1). These miRNAs showed different expression patterns at five time points during Cd stress. Expression levels of miR192 were downregulated, while miR1060 and miR361 levels were increased from 3 to 24 h after exposure to Cd treatment. miR59 levels decreased after 3 h of Cd stress and were increased after 6 h of Cd treatment. The qPCR results at 6 h after Cd exposure were consistent with microarray data for the miRNAs tested.

Prediction of Stress-Related *cis*-Acting Elements and Target Genes of miRNAs. In this study, the potential stress-responsive *cis* elements in the promoters of the four validated miRNAs were investigated. About a 1500 bp sequence upstream of the pre-miRNAs was extracted and analyzed using the PLACE database. As shown in Table 3, the stress-relevant *cis* elements included anaerobic responsive elements (AREs), ABA responsive elements (ABREs), gibberellin responsive elements (GAREs), ethylene responsive elements (EREs), and heat stress responsive elements (HSEs), suggesting that these miRNAs were probably involved in responses to various abiotic stimuli. All four miRNAs had AREs in their promoters, which respond to hypoxic, low-temperature, dehydration, and submergence conditions.[33,34]

To identify the potential target genes of the 12 newly identified miRNAs, the web-based prediction program miRU (Plant microRNA Potential Target Finder, http://bioinfo3.noble.org/miRNA/miRU_old.htm) was used.[14] With the miRU score of 3 or lower, a total of 44 target genes associated with Cd response were obtained from the rice genome, most of which were included in the list of miRNA targets provided by Lindow et al.[16] The 44 target genes predicted for the novel rice miRNA candidates expanded beyond transcription factor genes to include classes involved in stress responses and other metabolic and cellular processes. Information regarding these target genes was listed in Table 2, and they could be divided into several categories based on different characteristics. The first group contained target mRNAs involved in signal transduction, including OsWAK45-OsWAK receptor-like protein kinase and ras-related protein RHN1. The second group included ubiquitin system proteins and other proteins. The last group contained target mRNAs related to direct stress resistance, including ATP-binding cassette subfamily F member (*ABC*), cadmium tolerance factor, zinc finger, C3HC4-type family protein, dehydration-responsive element-binding protein 2A, glutaredoxin subgroup II, and heat-shock factor protein.

Real-Time PCR Analysis of Expression Profiling of the Corresponding Targets of Cd-Responsive miRNAs. To further validate the regulation role of miRNAs in Cd response, we also analyzed the expression profiling of corresponding targets of Cd-responsive miRNAs in rice seedlings exposed to 60 μM $CdCl_2$ for 24 h. As shown in Figure 2, miR192 expression was inhibited after exposure to Cd, whereas the abundance of *ABC* transcript increased. Real-time PCR results showed that the expression levels of target genes, *ABC* (the target of miR192), was just contrary to the expression levels of miR192. The negative expression patterns between miRNAs and their targets further validated the involvement of miRNAs in Cd response.

miR192 Had Negative Effects on the Seed Germination of Rice under Cd Stress. To determine whether miR192

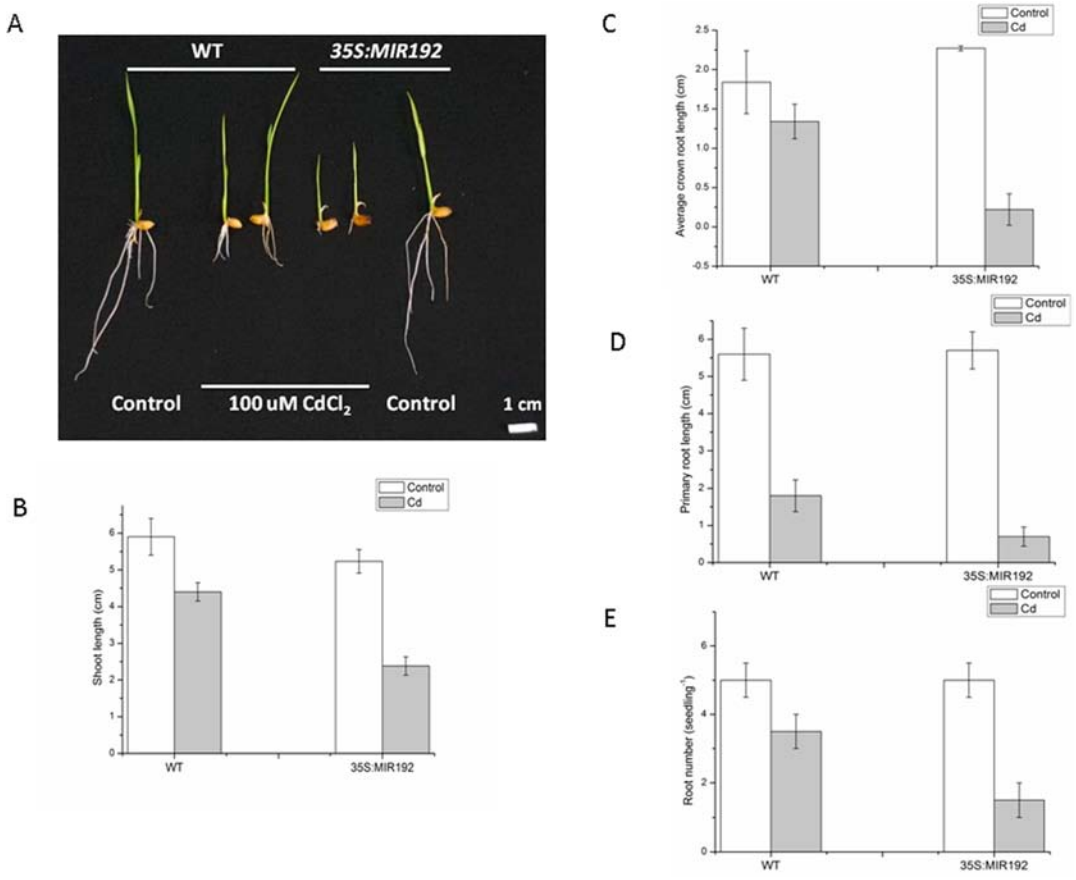

Figure 4. Germination and growth of wild-type and miR192 overexpressing seedlings on control media or media containing 100 μM $CdCl_2$. Pictures were taken after 7 days of growth. (A) Phenotypic comparison of wild-type and *35S:MIR192* transgenic rice plants under Cd stress. (B–E) Shoot length, root length, and root number of wild-type and *35S:MIR192* transgenic plants under Cd stress for 7 days. Each column represents the mean ± standard deviation (SD) of three independent experiments each with four replicates.

influences rice growth under heavy metal conditions, transgenic rice plants overexpressing miR192 under control of the cauliflower mosaic virus (CaMV) 35S promoter (*35S:MIR192* plants) were generated. Transgenic rice plants were verified by qPCR and GUS activity assay (Figure 3). The transcript levels of miR192 were substantially increased in transgenic plants, indicating that *35S:MIR192* plants overexpress miR192. *ABC* transcript levels were lower in the transgenic plants than in wild-type controls. These results further validated the negative regulation of miR192 in *ABC* transcripts.

We also examined the growth of miR192-overexpressing rice seedlings under Cd-stressed conditions. When the plants were grown under normal conditions, no noticeable differences in seed germination and seedling growth between wild-type and transgenic plants were observed. However, when the seeds were germinated on the media supplemented with 100 μM $CdCl_2$, the growth of *35S:MIR192* seedlings was significantly retarded in comparison to wild-type plants. Quantitative analyses confirmed that the shoot weight, root weight, and root length of *35S:MIR192* plants were significantly lower than those of wild-type plants when grown on Cd (panels B–E of Figure 4). These results demonstrated that miR192 had negative effects on the seed germination and seedling growth under Cd stress.

■ DISCUSSION

To date, more than thousands of rice miRNAs have been predicted, and nearly half of them have been identified by experiments.[35] On the basis of the perfect base pairing between miRNAs and their target mRNAs, Lindow et al.[16] predicted novel miRNA candidates for all known mRNAs in rice. Interestingly, this could predict new miRNAs that were specific to an organism, helping explain the difference between species that have very similar protein-coding genes but highly different phenotypes. For these predicted rice miRNAs, miRNA arrays were used to briefly identify the real/genuine miRNAs among candidate miRNAs. Candidate miRNAs whose hybridization signal was under the threshold value were discarded, and the remaining candidate miRNAs were identified. For Cd treatment, 60 μM $CdCl_2$ was chosen because of the fact that it caused serious damage to rice without resulting in death. miRNA microarray data demonstrated an altered expression profile of miRNAs in rice roots after exposure to 60 μM $CdCl_2$ for 6 h (Table 1). Among the 992 putative miRNAs predicted by Lindow et al.,[16] a total of 12 putative miRNAs were detected to be Cd-responsive by microarray assays. Moreover, 11 of the 12 putative new miRNAs were unique to the rice genome, suggesting the specific biological functions unique to one species.

To further validate the regulatory role of miRNAs in Cd response, the corresponding target mRNAs of Cd-regulated miRNAs were predicted and their expression profiles were analyzed (Table 2 and Figure 2). The 44 predicted target genes for Cd-responsive miRNAs were reported to be involved in several physiological processes, including defense and detoxification, antioxidant, and signal transduction. They could be

divided into several categories based on different characteristics. The first group contained target genes involved in signal transduction, including OsWAK45-OsWAK receptor-like protein kinase and ras-related protein RHN1. The second group included ubiquitin system proteins and other proteins. The ubiquitin-protein ligase and ubiquitin-conjugating enzyme, functioning in the degradation of abnormal proteins, were reported to be important for Cd resistance in yeast[36] and tomato (*Lycopersicon esculentum*).[37] These results suggest the role of the ubiquitin system in plant response to Cd stress.

The last group of target genes contained target mRNAs related to direct stress resistance. For example, miR192 was predicted to target *ABC* transporter, which was involved in heavy metal transport in yeast, mammals, and plants.[6,38] ABC transporters have been isolated from many plant species and reported to pump Cd conjugated to glutathione into vacuoles, resulting in less accumulation of Cd in cells.[39] In our study, the miR192 expression level was downregulated transcriptionally, whereas *ABC* transcript abundance increased under Cd treatment (Figure 2). In an earlier study, Moons reported that *Ospdr9*, a PDR-type ABC transporter, was rapidly and markedly induced by Cd and functioned in Cd sequestration in roots of rice seedlings.[40] The increased expression of *ABC* transporter under Cd stress was consistent with our results. To further validate the regulatory role of miR192 and *ABC* in the Cd response of rice, transgenic rice lines constitutively overexpressing miR192 were successfully obtained and confirmed. *ABC* transcript levels were lower in the transgenic plants than in wild-type controls, further validating the negative regulation of miR192 in *ABC* transcripts. Overexpression of miR192 significantly retarded seed germination and seedling growth under Cd stress compared to wild-type plants. These results suggested that miR192 overexpression increased Cd sensitivity in rice. To assess whether the increase in Cd sensitivity was because of higher metal accumulation, the Cd content in roots and shoots of transgenic rice lines needs to be compared to that of the same organs of wild-type plants in the future study. We believe that Cd accumulation and translocation within the plants would be important for investigating the real function of miR192 and *ABC* transporter in the rice Cd response.

■ ASSOCIATED CONTENT

Supporting Information

miRNAs predicted by Lindow et al. (Supporting Information 1) and primer pairs for the amplification of *OsActin1*, pre-miRNAs, and target genes (Supporting Information 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ABC, ATP-binding cassette; ABRE, ABA responsive element; ARE, anaerobic responsive element; DCL, DICER-LIKE; ERE, ethylene responsive element; GARE, gibberellin responsive element; HSE, heat stress responsive element; miRNA, microRNA; Nramp, natural resistance-associated macrophage protein; RISC, RNA-induced silencing complex; RT-PCR, reverse transcription polymerase chain reaction

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