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RESEARCH ARTICLE

Identification and expression analysis of conserved microRNAs during short and prolonged chromium stress in rice (*Oryza sativa*)

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Abstract

MicroRNAs (miRNAs) are one of the most critical epigenetic regulators of gene expression which modulate a spectrum of development and defence response processes in plants. Chromium (Cr) contamination in rice imposes a serious concern to human health as rice is used as staple food throughout the world. Although several studies have established the differential response of miRNAs in rice during heavy metal (arsenic, cadmium) and heat or cold stress, no report is available about the response of miRNAs during Cr stress. In the present study, we identified 512 and 568 known miRNAs from Cr treated and untreated samples, respectively. Expression analysis revealed that 13 conserved miRNAs (miR156, miR159, miR160, miR166, miR169, miR171, miR396, miR397, miR408, miR444, miR1883, miR2877, miR5072) depicted preferential up- or down-regulation (> 4-fold change; P value < 0.05). Target gene prediction of differentially expressed miRNAs and their functional annotation suggested the important role of miRNAs in defence and detoxification of Cr though ATP-binding cassette transporters (ABC transporters), transcription factors, heat shock proteins, auxin response, and metal ion transport. Real-time PCR analysis validated the differential expression of selected miRNAs and their putative target genes. In conclusion, our study identifies and predicts miRNA-mediated regulation of signalling pathway in rice during Cr stress.

Keywords *Oryza sativa* · MicroRNA · Cr stress · NGS · Transcriptome · Heavy metal toxicity

Introduction

Chromium (Cr) is one of the most abundant and harmful heavy metals reported in ground as well as surface water from all over the world (Vasilatos et al. 2010; Accoto et al. 2017; Castro-Rodríguez et al. 2014). It is a major concern in South Asian developing countries, where the level of chromium in ground water has extended way beyond the permissible level (0.5–50 μg/L) recommended by the World Health Organization (WHO) (Sharma et al. 2012). Naturally, Cr exists in a series of oxidation states; however, among the most stable states, III (trivalent) is an essential dietary nutrient, whereas VI (hexavalent) is highly toxic and carcinogenic. Cr (VI) is highly water-soluble and mobile that it mainly binds with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) oxyanion (Hayat et al. 2012). Due to its anti-corrosive and tanning properties, it is widely used in leather, paint, stainless steel and fertiliser industries. Unnao-Kanpur belt of Uttar Pradesh is one of the most industrialised regions in India and home to the most polluted stretch of river Ganga. The extent of Cr (VI) from anthropogenic activity is reported to be 3984

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μg/L resulting in > 250 times higher values (50–600 μg/L) of Cr in surface and ground water than the WHO permissible limit (Sharma et al. 2012). Therefore, ground water in industrialised cities in India and around the globe imposes serious concern to the human health as it is a major source of drinking water as well as used for irrigation of paddy fields (Tchounwou et al. 2012). Since rice is the major staple food in Asian countries, it serves as indirect source of Cr (VI) to the population residing in these geographical locations (Hassan et al. 2017). Previous studies have revealed that Cr (VI) equally harms rice plant by inhibiting seed germination and seedling growth and limiting the uptake and accumulation of nutrients (Choudhury and Panda 2005; Pandey et al. 2009). Therefore, understanding the underlying molecular mechanisms involved in Cr (VI) accumulation and its detoxification will help in the development of less Cr (VI)-accumulating rice plants.

For plants, Cr is a non-essential element; therefore, the uptake mechanism involves both passive and majorly active transport using carriers of essential elements localised in the parenchyma cells of xylem and companion cells of phloem (Ahmad et al. 2013). Plants possess several chelating molecules, such as phytochelatins (PCs) and metallothionines, to sequestrate Cr or other heavy metal into the vacuole. However, once the heavy metal accumulation crosses a threshold, it stimulates the expression of reactive oxygen species (ROS)-producing enzymes like NADPH oxidases and SOD, thus deteriorating the functioning of cell (Pandey et al. 2005). In addition, heavy metal stress also stimulates several defences signalling cascade, such as hormonal (mainly auxin, cytokinins and ethylene), calcium and MAPK signalling (Sun et al. 2010; Potters et al. 2007). Apart from stimulation of signalling pathways and other defence mechanisms, heavy metal stress also stimulates the expression of small non-coding RNA, such as microRNA (miRNA) (Tang et al. 2014). MiRNAs are ~ 22-nucleotide, endogenously expressed RNAs that bind with 3′UTR of mRNA and regulate gene expression at post-transcriptional level by either degradation or translational repression of mRNA (Saxena et al. 2017). MiRNAs play critical role in the overall growth and development of plant and have been investigated for their role during biotic and abiotic stresses (Noman and Aqeel 2017). Under stress, miRNAs exhibit time-dependent response and regulate key molecular pathways, such as ATP-binding cassette (ABC) transporters, ROS signalling and hormonal signalling (Pandey et al. 2015). In rice plant, several miRNAs, such as miR-144, miR-159, miR-166, miR-172, miR-199, miR-393, miR-397 and miR-408, have been reported as responsive miRNAs under heavy metal (cadmium and arsenic) and heat or cold stress (Sharma et al. 2015; Tang et al. 2014; Mangrauthia et al. 2017). In tobacco (*Nicotiana tabacum*), Cr-responsive miRNAs, such as miR-156, miR-166, miR-167, miR-169 and miR-171, have also been reported; however, their response in rice plant is still unknown (He et al. 2016). The negative impact of heavy metals depends on several factors, including metal type, its concentration, its oxidation state and its duration. The objective of the present study is to identify Cr-responsive miRNAs in the rice plant exposed to different durations (short and prolonged). This study will provide a better understanding of the regulatory mechanisms adaptive by rice plant in response to Cr stress.

Materials and methods

Plant culture and treatment

The seedling of rice (*Oryza sativa*) variety IR-64 was surface sterilized using sterile-containing 0.1% $HgCl_2$ followed by soaking in sterile Milli-Q water for 24 h. Seeds were distributed in four groups, namely (A) control 24 h, (B) treatment 24 h, (C) control 7 days and (D) treatment 7 days. Seeds were placed in autoclaved Petri plates and incubated at 25 ± 2 °C until germination. After 72 h, germinated seedlings of groups (A) and (C) were grown in Hewitt medium, while those of groups (B) and (D) were grown in modified Hewitt medium containing additional 100 μM of Cr (VI) prepared from stock solution of $K_2Cr_2O_7$. For groups (B) and (D), the media was replaced and pH was adjusted after every 72 h. The physiological conditions provided were 16 h light and 8 h dark photoperiod at 25 ± 2 °C temperature and 70% relative humidity (Dubey et al. 2010). After 24 h and 7 days, roots of seedlings grown in media supplemented with and without Cr (VI) were isolated, treated with liquid N_2 and stored at − 80 °C until further processing.

RNA extraction and quantification

Total RNA was isolated from the rice root of both the control and Cr (VI) treated using TRIzol reagent (Invitrogen, USA). Total RNA from all the samples was checked on 1× MOPS-formaldehyde agarose gel, and quality was verified by Agilent 2000 bioanalyser. Small RNAs were extracted using mirVana small RNA isolation kit (Ambion, Life Technologies, USA) as per manufacturer's instructions. RNA concentrations and purities were determined spectrophotometrically by measuring A260/A280 ratio using the NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). The samples were stored at − 80 °C until small RNA library preparation.

Small RNA library construction and miRNA sequencing

Four small RNA libraries were generated through reverse transcription (Illumina Inc., USA) of adapter-ligated RNA samples from both control and Cr-treated groups. The purified

cDNA libraries were sequenced on Illumina NextSeq500 sequencer. Srna-workbenchV3.0_ALPHA1 was used to trim the 3′ Trueseq adapter sequences. The raw sequence datasets were filtered based on the length criteria (minimum length 16 bp and maximum 35 bp) to remove redundant sequences. Sequences between 16 and 35 bp were checked for non-coding RNA (rRNA, tRNA, snRNA and snoRNA) contamination. Clean reads were mapped to the *Oryza sativa indica* genome sequence, and the mapper reads were used for the actual miRNA identification carried out by using miRDeep2 with the use of other short read aligner software called Bowtie 1.1.1. Further, homology search of these miRNA reads was carried out against matured miRNA sequences from miRbase using BLASTn analysis, allowing for two mismatches and three gaps. Precursors of reads annotated with rice miRNA sequences were predicted as known miRNAs, whereas unannotated miRNAs were considered as putative novel miRNAs. Stem–loop structures of each miRNA and their chromosomal location were determined using Mireap_0.22. Expression analysis of known miRNAs was performed using RPM (reads per million). The expression was calculated as RPM = R_{miRNA} / $R_{\mathrm{Total}} * 10^6$ and later normalized as $\log_2$ (fold change) = $\log_2$ RPM (treatment) / RPM (control). *P* value was calculated using edgeR software, and the miRNAs fulfilling the criteria ($\log_2$ fold change − 1 to + 1 and *P* value ≤ 0.05) were considered.

MiRNA expression and statistical analysis

Expression of selected differentially expressed miRNAs was also validated using quantitative stem–loop real-time PCR. The primers used in the study are listed in Table S1. The stem–loop reverse transcription was carried out using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per the manufacturer's instructions. The reaction mixture of 10 μL containing 1 μL RNA (200 ng), 1 μL stem–loop primer (1 μM), 1 μL U6 RT primer (1 μM), 1 μL dNTP mix (10 mM), 0.5 μL reverse transcriptase enzyme (200 U/μL), 0.5 μL RiboLock (20 U/μL), 2 μL buffer and 3 μL nuclease-free water was prepared. The reaction mixture was incubated at 25 °C for 5 min followed by 42 °C for 60 min. The reaction was terminated by heating at 70 °C for 5 min. Real-time PCR was performed using SYBR green fluorescence quantitative PCR reagent kit (Thermo Fisher Scientific, USA) and Piko Real-Time 96 (Thermo Fisher Scientific, USA). The 10 μL reaction mixture constituted 5 μL SYBR green real-time PCR Master Mix, 0.5 μL forward and reverse primers each (1 μM), 1 μL stem–loop cDNA product and 3 μL nuclease-free water. The PCR reaction conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 15 s and 55 °C for 30 s. All the reactions were performed in triplicates. MiRNAs crossing the threshold in < 35 cycles were considered to be expressed (Lagana et al. 2015). The relative expression of miRNA was measured using $2^{-\Delta\Delta Cq}$ after normalization to U6 snRNA used as internal control. Three independent experiments were performed to analyse the expression of each miRNA in triplicate.

Potential target identification and their functional annotation

MiRNAs with copy number >= 10 were considered for target prediction. The miRNA sequences were fed as input along with reference target miRNA sequences to miRanda-3.3 tool. Target genes were also predicted using psRNATarget (TIGR genome cDNA OSA1R5) with default parameters and a minimal weighed score of < 3.0. Common miRNA hits having minimum free energy <= − 25 were assumed to be the targets for reported miRNA. Gene ontology (GO) enrichment analysis was performed to classify the targets according to their biological, molecular and cellular functions. KEGG analysis was performed using KAAS (KEGG Automatic Annotation Server) (https://www.genome.jp/kaas-bin/kaas_main) to predict pathways in which the target genes are involved. Expression analysis of selected target genes was carried out by qRT-PCR analysis. Total RNA was isolated from root samples as mentioned for sequencing analysis till concentration determination. cDNA was prepared using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per the manufacturer's instructions. RT-PCR was performed as discussed previously using rice actin gene as internal control.

Statistical analysis

The relative differential expression of miRNAs as well as target genes between control and treatment group was compared using Student's unpaired *t* test. All the experiments were conducted in triplicate and repeated thrice, and the mean values were considered statistically significant at $P \leq 0.05$ to 0.001.

Results and discussion

High-throughput sequencing and identification of miRNA in *Oryza sativa*

To determine immediate and delayed roles of miRNA in response to Cr (VI) contamination, four small RNA libraries were generated from treated and untreated samples at different time points of exposure. After high-throughput sequencing, a total of 73,63,7470 raw reads, including of 47,03,9035 high-quality reads, were obtained (Table 1). The decrease in the number of reads in the treated samples suggests that Cr stress treatments affect small RNA

Table 1. Summary of four small RNA libraries prepared from control and Cr-stressed root of rice plant

Sequencing statistics	Treatment 24 h	Treatment 7 days	Control 24 h	Control 7 days
Total raw reads	14 57 0019	17 10 7027	18 85 6527	23 10 3897
Total number of reads after quality filtering	14 24 9037	16 79 4701	18 56 0241	22 77 1629
Total number of reads after adapter removal	98 17 326	12 70 5257	13 23 9350	11 27 7102
Percentage of reads mapped to mature miRNAs	90.55	85.71	88.67	88.76
Percentage of GC content	52	53	53	53
Known miRNA detected	298	214	281	287
Known miRNA detected with abundance >= 10	108	63	95	106
Putative novel miRNA detected	545	789	1014	790
Putative novel miRNA detected with abundance >= 10	154	277	333	226

metabolism extensively. Further, the small RNA sequences were mapped and searched against other RNA families, such as snRNA, snoRNA, tRNA and rRNA. We observed that nearly 90% of the total small RNA population belonged to miRNAs suggesting that all the libraries were enriched with miRNAs (Fig. 1). In order to identify conserved, species-specific and novel miRNAs from treated and untreated rice plant roots, miRNA reads obtained from different samples were aligned with mature miRNA sequences of *Oryza sativa* deposited in miRBase. The number of known miRNAs (512) detected from treated samples was low as compared to control samples (568), indicating the negative impact of Cr stress on biogenesis of miRNAs. Similar pattern was observed for putative novel miRNAs of treated (1334) and untreated samples (1804). Since novel miRNAs were not detected in miRBase database, they were not considered as true miRNAs in our study until experimental validation. Length distribution of miRNAs was evaluated, and it was observed that the length of miRNAs across all the samples lied between 21 and 25 nucleotides and majority of them were of 24 nucleotides (Fig. 2). Although the length distributions showed variation between treated and untreated samples, overall distribution was consistent with the common sizes of miRNAs. Another parameter to analyse the nature of miRNAs

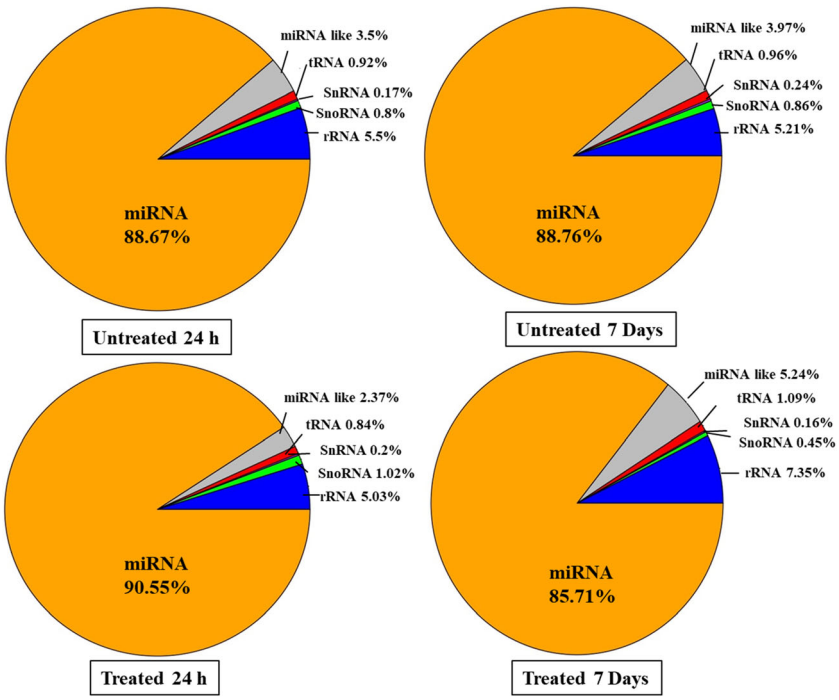

Fig. 1. Small RNA distribution across four libraries. All the libraries were found to be enriched in miRNAs

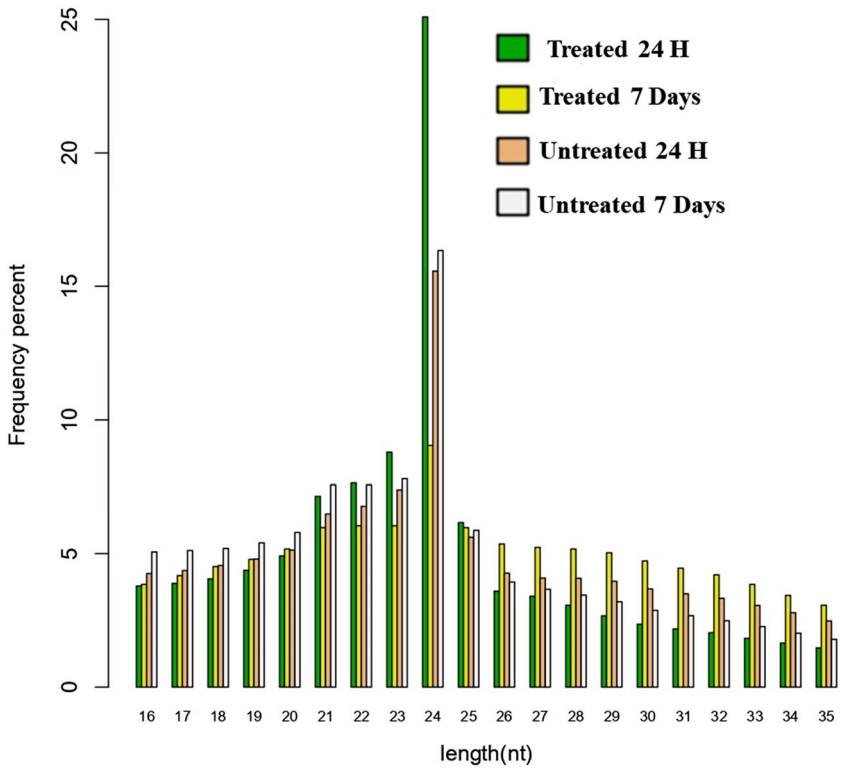

Fig. 2. Length distribution of RNA-seq reads. Maximum miRNAs were found to be of 24 nucleotide length

identified is to predict their chromosomal distribution as it indicates their differential expression (Saxena et al. 2018). We generated chromosomal distribution map using sequence alignment of miRNAs with *Oryza sativa* genome and observed that chromosome 1 harboured maximum number of miRNAs followed by chromosomes 2

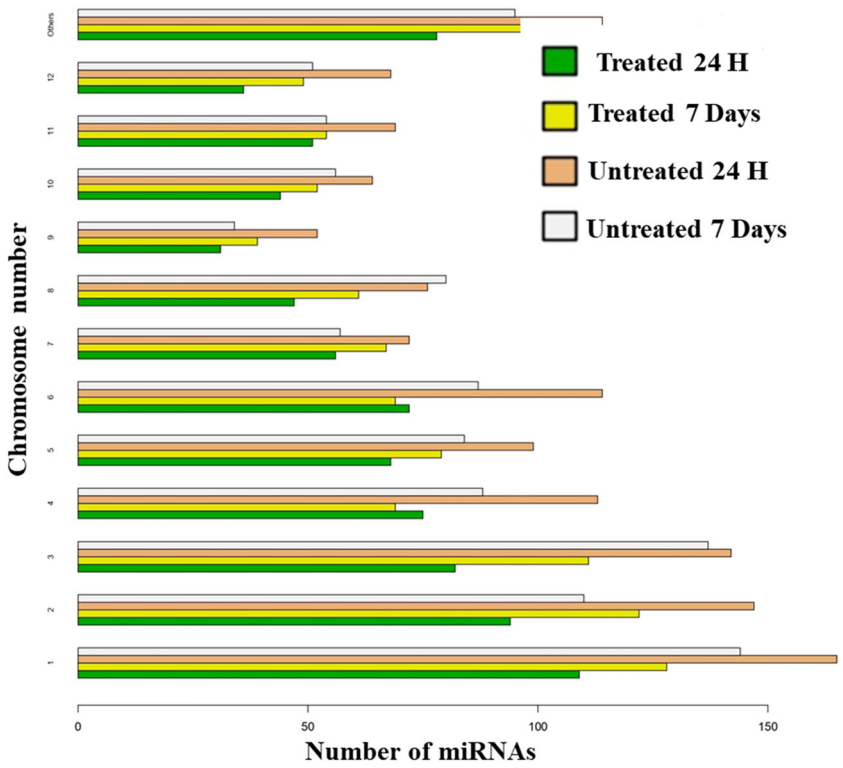

Fig. 3. Chromosomal distribution of RNA-seq reads. The histogram depicts variation in the distribution of miRNAs on different chromosomes of *Oryza sativa*

and 3. The scattered distribution of miRNAs between different chromosomes suggested that miRNAs might be depicting differential expression in response to Cr stress (Fig. 3).

MiRNAs depicted time-dependent differential expression during Cr stress

As discussed previously, a total of 512 and 568 known miRNAs were identified in treated and control samples, respectively. Out of these, 263 miRNAs were common or shared miRNAs between treated and untreated samples incubated for 24 h. Similarly, 207 were common or shared by treated and untreated samples incubated for 7 days. The unique miRNA count for control samples incubated for 24 h and 7 days was 18 and 80, respectively. However, the unique miRNA count for treated samples incubated for 24 h and 7 days was 35 and 7, respectively. To evaluate the differentially expressed miRNAs in treated samples as compared to control samples, the total read count for each miRNA was normalised and miRNAs with fold change > 1.5 and P value < 0.05 were analysed for their expression. Hierarchical clustering heat map was generated comparing control and treatment samples. A contrasting pattern of expression was observed between control (24 h and 7 days) and treated (24 h and 7 days) samples suggesting the expression of miRNAs alters in response to Cr stress (Fig. S1). Even the duration of stress changed the expression of miRNAs or miRNAs of the same family drastically (Fig. 4). Based on the fold change, we sorted several well-conserved preferentially up- or down-regulated miRNAs (> 4 fold change) (Table 2). We observed that upon short Cr stress (24 h), osa-miR156, osa-miR159, osa-miR396, osa-miR397, osa-miR2877 and osa-miR5072 were highly upregulated; however, osa-miR160, osa-miR169, osa-miR171, osa-miR408, osa-miR444 and osa-miR1883 were highly downregulated. On the other hand, prolonged stress provoked the upregulation of only two miRNAs (osa-miR166 and osa-miR171) and supressed the expression of only three miRNAs (osa-miR396, osa-miR444, and osa-miR5072). Interestingly, osa-miR171 was the only downregulated miRNA from 24 h that turns highly abundant after prolonged stress. Likewise, osa-miR396 and osa-miR5072 were the only upregulated miRNAs that got drastically downregulated after prolonged stress. Lastly, osa-miR444 was the only miRNA that was found to be down-regulated at both the time points. Validation of expression profile obtained from sequencing was performed by stem–loop RT-PCR for five miRNAs. We observed the drastic alteration in expression of osa-miR171, osa-miR396 and osa-miR5072. However, no significant changes was observed in expression of osa-miR444 and osa-miR1883. We observed that the relative expression of osa-miR171, osa-miR396, osa-miR5072 and osa-miR1883 was consistent with that of sequencing data. Only osa-miR444 showed inconsistent expression; however, the change was non-significant (Fig. 5).

Now, several highly up- or down-regulated miRNAs identified in our study have been previously reported as biotic or abiotic stress-responsive miRNAs. For instance, up-regulation of osa-miR156 during Cr stress was reported in tobacco (*Nicotiana tabacum*); however, under aluminium (Al) stress in rice and maize, miR156 was reported to be down-regulated (He et al. 2016; Lima et al. 2011). In contrast, osa-miR169 and osa-miR171 that were down-regulated in our study showed elevated expression in tobacco under Cr stress (Bukhari et al. 2015). Similarly, osa-miR160 which was found to be down-regulated in our study has been reported as upregulated under heat stress in *Arabidopsis* (Wang 2005). Besides, there were few miRNAs, namely osa-miR397 and osa-miR408, that have been reported to show similar expression under arsenic (As) stress in rice plant (Ma et al. 2015). Likewise, osa-miR2877 and osa-miR5072 have also been reported to depict similar expression under heat stress (Mangrauthia et al. 2017). These studies suggest that miRNAs were differentially expressed under different stress conditions and different time points. Interestingly, the response of osa-miR1883, a well-conserved miRNA identified in different rice plant species under biotic or abiotic stresses, is still unknown (Wu et al. 2017). For the first time, we report that osa-miR1883 is a heavy metal-responsive miRNA that gets down-regulated upon Cr stress.

Potential target genes regulate critical defence mechanisms during Cr stress

Previous studies have established that unlike in animals, miRNAs bind with highest possible specificity to its targets in plants and can regulate the expression of multiple targets simultaneously (Brousse et al. 2014). Therefore, to understand the biological functions of differentially expressed miRNAs under Cr stress, target genes were predicted using miRanda and psRNATarget. Initially, conserved or species-specific known miRNAs with copy number >= 10 were considered for target identification. Nearly, 900 target genes were predicted for miRNAs from 24 h treated and untreated samples, whereas, nearly 700 target genes were predicted for miRNAs from 7 days treated and untreated samples. Target genes of miRNAs depicting differential expression and having minimum free energy <= − 25 were considered for functional annotation. Gene ontology (GO) analysis and KEGG annotation were performed to predict the potential role of differentially expressed miRNAs. The potential target genes were classified into three categories based on their molecular functions, biological processes and cellular components. Among the molecular functions, the most represented GO terms were DNA binding, ATP binding and protein binding. Among the different biological processes, regulation of transcription,

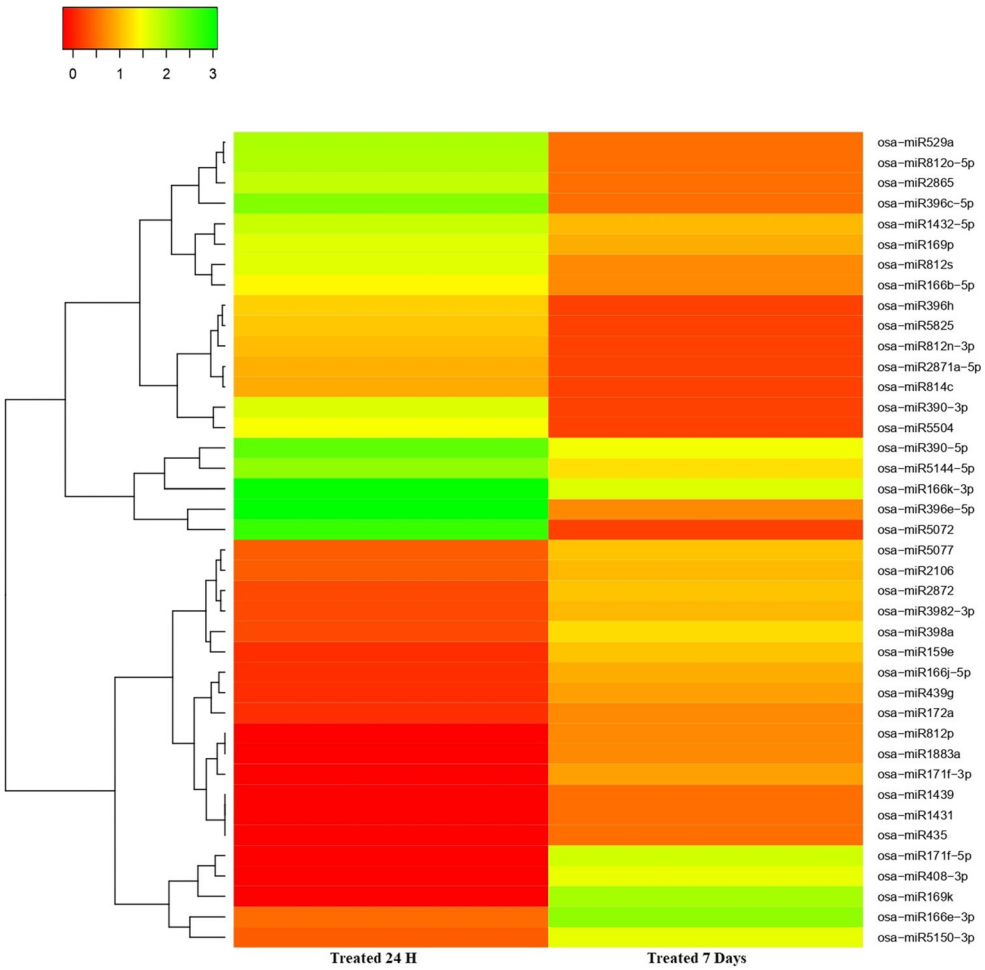

Fig. 4. Heat map depicting time dependent differential expression of miRNAs under Cr stress. Green colour denotes upregulation whereas red colour denotes downregulation. Potential target genes of miRNAs with fold change ≥ 4; *P* value ≤ 0.05 was considered for functional annotation

oxidation–reduction process and transmembrane transport were the most represented GO terms. Lastly, among the cellular component, nucleus and membrane represented the majority portion. KEGG pathway analysis is another method to analyse target gene products and their potential functions during metabolic processes (Kanehisa 2016). Maximum numbers of target genes were mapped to metabolic pathways, glycolysis/gluconeogenesis, starch and sucrose metabolism, biosynthesis of secondary metabolites and hormone signal transduction.

It is suggested that transcriptome as well as proteome of different plants depicts differential response during abiotic or biotic stress (Chakrabarty et al. 2009). Therefore, differentially expressed miRNAs might be one of the most critical regulators of transcriptome during stress. In our study, MADS-box, MYB, GRF1 and HOX10 were some of the critical transcriptional factors predicted to be the target of well-conserved differentially expressed miRNAs (Table 2). Previous studies have reported that most of these transcription factors play an important role in plant development and are involved in stress response (Shim et al. 2009; Wang et al. 2016a, b, 2017a, b). For instance, MADS-box proteins that regulate the gene expression are critical for coping with salinity and cold stress conditions (Kuo et al. 1997; Lozano et al. 1998). Osa-miR444, one of the most responsive miRNAs (down-regulated) in cadmium (Cd)-induced stress was predicted to target MADS-box (Ding et al. 2011). These observations are in parallel with our sequencing and qRT-PCR findings where osa-miR444 was found to be down-regulated after 24 h as well as 7 days Cr treatment. It is interesting to highlight that bacterial, fungal or viral infection in rice leads to up-regulation of osa-miR444 and suppression of MADS-box proteins, which in turn activates the antiviral RNA-silencing pathway (Wang et al. 2016a, b). Thus, it can be inferred that although supressed osa-miR444 during heavy metal stress might be protecting the plant, it may also be exposing the plant towards microbial infection. As previously mentioned, osa-miR396 was one of the two miRNAs whose expression was significantly increased immediately after Cr exposure (24 h) and significantly reduced after prolonged Cr exposure (7 days). Osa-miR396 was found to be targeting ATP-binding cassette (ABC) transporters and growth-regulating factor (GRF) which are predominantly involved in heavy metal detoxification and overall plant development (Wang et al. 2017a, b; Debernardi

Table 2. Summary of putative targets predicted for differentially expressed miRNAs in response to Cr stress

MiRNA accession	Target accession	Target start	Target end	Target annotation
osa-miR156	Os08g08220	2754	2774	ATP-binding protein
	Os05g13940	1467	1488	Early nodulin 75 protein
	Os03g01850	253	274	Cell division control protein 2
	Os01g20190	1198	1219	Transposon protein
osa-miR159	Os01g12700	804	823	Myb-like DNA-binding
	Os05g41166	1069	1088	Transcription factor GAMYB
	Os10g05230	336	355	Protein-binding protein
osa-miR160	Os06g47150	1844	1864	Auxin response factor 16
	Os09g29160	1451	1471	Expressed protein
	Os04g59430	1334	1354	DNA-binding protein
	Os07g31400	890	910	Transposon protein
	Os10g37770	644	664	Methyltransferase protein
	Os03g02440	2831	2851	Signal transducer
	Os12g41890	1433	1453	Cationic amino acid transporter 4
osa-miR166	Os04g49300	333	353	Retrotransposon protein
	Os03g56920	1533	1553	Conserved hypothetical protein
	Os10g33960	924	944	HB1 putative expressed protein
osa-miR169	Os07g06470	837	857	Nuclear transcription factor Y
	Os02g19970	1573	1593	Tyrosine aminotransferase
	Os07g06740	546	566	Calcium-dependent protein kinase
	Os03g46770	591	611	Glycine-rich RNA-binding protein 2
	Os04g51370	1535	1555	ATP-binding protein
osa-miR171	Os02g44370	1526	1546	Nodulation signalling pathway 2
	Os03g04300	2079	2099	Ankyrin repeat protein
	Os02g10510	892	912	DNA damage-inducible protein
	Os02g56550	169	189	ATP-dependent transporter
	Os05g37170	2001	2021	Transcription factor TGA6
osa-miR396	Os01g32750	579	599	TBP-associated subunit protein
	Os04g57050	853	873	Jasmonate O-methyltransferase
	Os01g52640	2356	2376	Ubiquitin-protein ligase
	Os01g08560	1991	2011	Heat shock 70
	Os06g10310	282	303	Growth-regulating factor 1
	Os12g29980	727	748	atGRF2
osa-miR397	Os01g62490	761	779	L-ascorbate oxidase precursor
	Os05g35770	356	374	Serine/threonine-protein kinase
	Os12g15530	794	812	Retrotransposon protein
osa-miR408	Os03g15340	85	105	Chemocyanin precursor
	Os01g03530	1551	1571	Copper ion-binding protein
	Os01g02110	730	750	Helix-loop-helix DNA-binding
	Os04g55940	292	312	Vacuolar cation/proton exchanger 3
	Os01g53880	1356	1376	OsIAA6-auxin-responsive
	Os03g11110	2052	2072	Metal ion-binding protein
osa-miR444	Os02g49840	613	633	MADS-box transcription factor 57
	Os08g33488	311	331	MADS-box transcription factor 23
	Os08g06510	1371	1391	Zinc finger
	Os08g33479	154	174	Expressed protein
osa-miR1883	Os01g73900	795	813	ATP-binding protein
	Os06g29320	1871	1889	Retrotransposon protein
	Os12g15260	300	318	Hypothetical protein
osa-miR2877	Os12g05040	12	35	Metal ion-binding protein
	Os03g55250	518	541	Cytochrome P450
	Os04g38780	911	934	MADS-box transcription factor 27
	Os04g47320	1747	1770	Mechanosensitive ion channel protein
osa-miR5072	Os03g50490	944	962	Glutamine synthetase
	Os07g09530	50	68	Protein-binding protein
	Os07g24100	425	443	Retrotransposon protein
	Os03g15250	1913	1931	Lectin-like receptor kinase

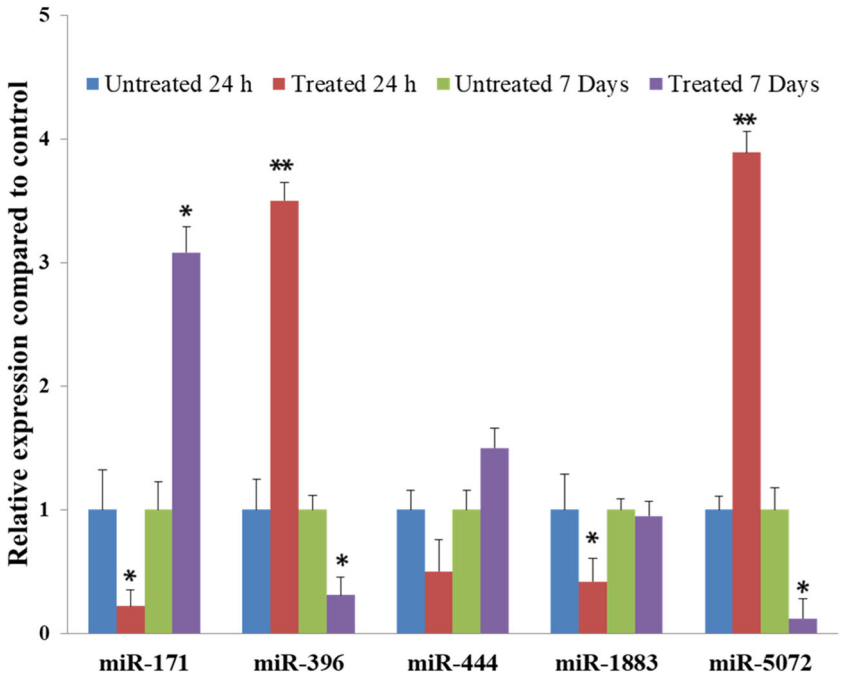

Fig. 5. qRT-PCR expression analysis of selected differentially expressing miRNAs. miRNAs identified through next-generation sequencing depicted similar expression pattern. Asterisk symbol refers to the significant difference between treatment and control

et al. 2012). This indicates that the plant possesses acute as well as prolonged defence mechanisms and osa-miR396 might be one of the critical agents of plant defence whose suppression elevates ATP-binding proteins and GRF expression.

Heat shock proteins (HSP) are another critical set of proteins that is preferentially expressed under stress to maintain functional and healthy proteomes (Shim et al. 2009). We detected osa-miR1883 to be targeting heat shock protein binding, suggesting decrease in the abundance of osa-miR1883 leads to the elevated expression of HSP to maintain cell homeostasis. Several genes that encode metal transport protein, such as protein kinases, ATP-binding protein, redox homeostasis of the cell, such as copper ion-binding protein, zinc ion-binding, iron ion homeostasis, and glutamate receptor were also found to be critical targets (Table 2). To prevent a plant

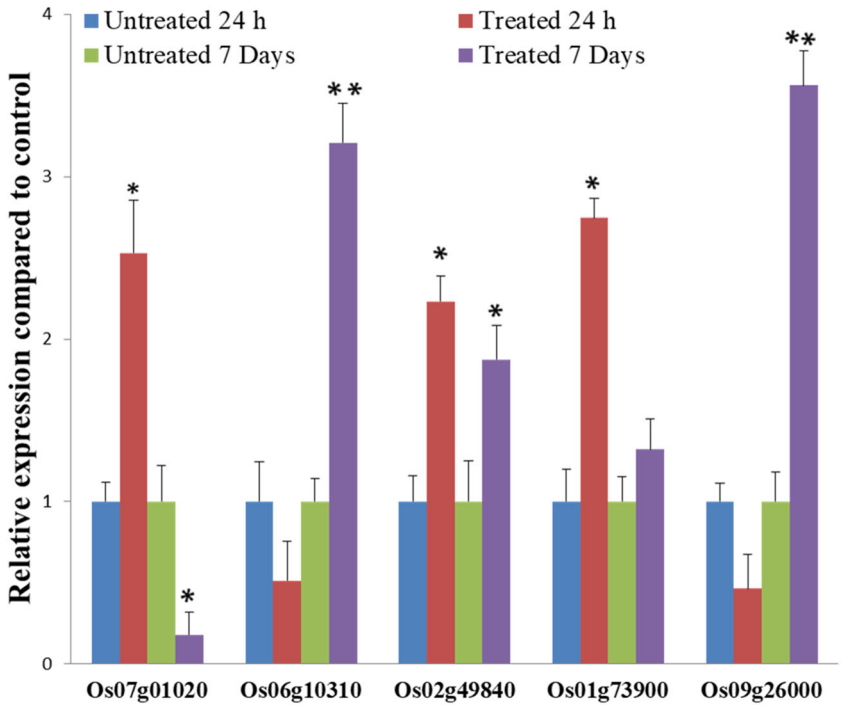

Fig. 6. qRT-PCR expression analysis of selected putative targets. Target loci were found to be negatively regulated by miRNAs. Asterisk symbol refers to the significant difference between treatment and control.

from heavy metal exposure, root remodelling can be used as one of the strategies. Plant growth hormones mainly auxin and cytokinin that regulate root formation modulate root system architecture in response to heavy metal stress (Krishnamurthy and Rathinasabapathi 2013). We observed that osa-miR160 targets ARF (auxin response factor); hence, downregulation of osa-miR160 under Cr stress might be causing elevated expression of auxin required to combat Cr stress. Similar ARF-mediated response of osa-miR160 in regulating root cap formation has been previously reported in Arabidopsis (Wang 2005). The relationship between auxin signalling and MAPK signalling in metal stress is still vaguely known. It has been reported that auxin signalling genes, namely *IAA*, *ARF* and *PIN*, are negatively regulated by MAPK signalling (Zhao et al. 2014). Interestingly, we observed that MAPK cascade was targeted by osa-miR159, suggesting Cr stress suppresses MAPK and induces auxin signalling. Hence, our study validates the observation that there exists an inverse correlation between auxin and MAPK signalling and osa-miR159 might be one of the epigenetic regulators of auxin response under heavy metal stress. qRT-PCR analysis of putative target genes of few differentially expressed Cr-responsive miRNAs revealed that targets are negatively regulated by miRNAs (Fig. 6). Overall findings of our study suggest that Cr induces differential expression in a set of miRNAs that are critical for regulating homeostasis in rice plant. The miRNAs clearly respond in a time-dependent manner; therefore, kinetic parameters of miRNA expression must be considered to link miRNA response during heavy metal stress. Our study also provides baseline knowledge for developing transgenic strategies through manipulating Cr stress-responsive miRNAs and/or their target mRNAs using target-mimics and artificial miRNAs.

Conclusion

We exposed the rice plants to Cr (VI) for different durations and prepared small RNA libraries from root samples. The libraries were sequenced and compared between treated and untreated groups. For the first time, a set of time-dependent differentially expressed Cr stress-responsive miRNAs were detected in rice plant highlighting their potential as detoxification agents. Potential target prediction and functional annotation further established that these miRNAs are among the critical molecular entities involved in rice plant defence against Cr stress. These results provide a novel understanding of miRNA-mediated regulation of rice response under Cr stress. Further validation and functional characterisation of these miRNAs may help in developing new strategies to combat heavy metal stress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Accoto V, Bullo P, Faccio R et al (2017) Hexavalent chromium: analysis of the mechanism of groundwater contamination in a former industrial site in the province of Vicenza (Northern Italy). Acque Sotter - Ital J Groundw 5

Ahmad A, Khan I, Diwan H (2013) Chromium toxicity and tolerance in crop plants. In: Crop improvement under adverse conditions 309–332

Brousse C, Liu Q, Beauclair L, Deremetz A, Axtell MJ, Bouché N (2014) A non-canonical plant microRNA target site. Nucleic Acids Res 42: 5270–5279

Bukhari SAH, Shang S, Zhang M, Zheng W, Zhang G, Wang TZ, Shamsi IH, Wu F (2015) Genome-wide identification of chromium stress-responsive micro RNAs and their target genes in tobacco (*Nicotiana tabacum*) roots. Environ Toxicol Chem 34:2573–2582

Castro-Rodríguez A, Carro-Pérez ME, Iturbe-Argüelles R, González-Chávez JL (2014) Adsorption of hexavalent chromium in an industrial site contaminated with chromium in Mexico. Environ Earth Sci 73:175–183

Chakrabarty D, Trivedi PK, Misra P, Tiwari M, Shri M, Shukla D, Kumar S, Rai A, Pandey A, Nigam D, Tripathi RD, Tuli R (2009) Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. Chemosphere 74:688–702

Choudhury S, Panda SK (2005) Toxic effects, oxidative stress and ultrastructural changes in moss Taxithelium nepalense (Schwaegr.) broth under chromium and lead phytotoxicity. Water Air Soil Pollut 167: 73–90

Debernardi JM, Rodriguez RE, Mecchia MA, Palatnik JF (2012) Functional specialization of the plant miR396 regulatory network through distinct microRNA-target interactions. PLoS Genet 8: e1002419

Ding Y, Chen Z, Zhu C (2011) Microarray-based analysis of cadmium-responsive microRNAs in rice (*Oryza sativa*). J Exp Bot 62:3563–3573

Dubey S, Misra P, Dwivedi S et al (2010) Transcriptomic and metabolomic shifts in rice roots in response to Cr (VI) stress. BMC Genomics 11:648

Hassan FI, Niaz K, Khan F, Maqbool F, Abdollahi M (2017) The relation between rice consumption, arsenic contamination, and prevalence of diabetes in South Asia. EXCLI J 16:1132–1143

Hayat S, Khalique G, Irfan M, Wani AS, Tripathi BN, Ahmad A (2012) Physiological changes induced by chromium stress in plants: an overview. Protoplasma 249:599–611

He X, Zheng W, Cao F, Wu F (2016) Identification and comparative analysis of the microRNA transcriptome in roots of two contrasting tobacco genotypes in response to cadmium stress. Sci Rep 6:32805

Kanehisa M (2016) Kegg bioinformatics resource for plant genomics and metabolomics. In: Methods in molecular biology 55–70

Krishnamurthy A, Rathinasabapathi B (2013) Auxin and its transport play a role in plant tolerance to arsenite-induced oxidative stress in *Arabidopsis thaliana*. Plant Cell Environ 36:1838–1849

Kuo M H, Nadeau E T, Grayhack E J (1997) Multiple phosphorylated forms of the Saccharomyces cerevisiae Mcm1 protein include an isoforminduced in response to high salt concentrations. Mol Cell Biol 17:819-832

Lagana A, Veneziano D, Spata T et al (2015) Identification of general and heart-specific miRNAs in sheep (Ovis aries). PLoS One 10: e0143313

Lima JC, Arenhart RA, Margis-Pinheiro M, Margis R (2011) Aluminum triggers broad changes in microRNA expression in rice roots. Genet Mol Res 10:2817–2832

Lozano R, Angosto T, Gómez P, Payán C, Capel J, Huijser P, et al (1998) Tomato flower abnormalities induced by low temperatures are associated with changes of expression of MADS-Box genes. Plant Physiol 117: 91–100

Ma C, Burd S, Lers A (2015) MiR408 is involved in abiotic stress responses in Arabidopsis. Plant J 84:169–187

Mangrauthia SK, Bhogireddy S, Agarwal S, Prasanth VV, Voleti SR, Neelamraju S, Subrahmanyam D (2017) Genome-wide changes in microRNA expression during short and prolonged heat stress and recovery in contrasting rice cultivars. J Exp Bot 68:2399–2412

Noman A, Aqeel M (2017) miRNA-based heavy metal homeostasis and plant growth. Environ Sci Pollut Res 24:10068–10082

Pandey V, Dixit V, Shyam R (2005) Antioxidative responses in relation to growth of mustard (Brassica juncea cv. Pusa Jaikisan) plants exposed to hexavalent chromium. Chemosphere 61:40–47

Pandey V, Dixit V, Shyam R (2009) Chromium (VI) induced changes in growth and root plasma membrane redox activities in pea plants. Protoplasma 235:49–55

Pandey C, Raghuram B, Sinha AK, Gupta M (2015) MiRNA plays a role in the antagonistic effect of selenium on arsenic stress in rice seedlings. Metallomics 7:857–866

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MA (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12:98–105

Saxena S, Jain A, Rani V (2017) MicroRNA-mediated MMP regulation: current diagnostic and therapeutic strategies for metabolic syndrome. Curr Gene Ther 17:214–227

Saxena S, Gupta A, Shukla V, Rani V (2018) Functional annotation of differentially expressed fetal cardiac microRNA targets: implication for microRNA-based cardiovascular therapeutics. 3. Biotech 8:494

Sharma P, Bihari V, Agarwal SK et al (2012) Groundwater contaminated with hexavalent chromium [Cr (VI)]: a health survey and clinical examination of community inhabitants (Kanpur, India). PLoS One 7:e47877

Sharma D, Tiwari M, Lakhwani D, Tripathi RD, Trivedi PK (2015) Differential expression of microRNAs by arsenate and arsenite stress in natural accessions of rice. Metallomics 7:174–187

Shim D, Hwang J-U, Lee J, Lee S, Choi Y, An G, Martinoia E, Lee Y (2009) Orthologs of the class A4 heat shock transcription factor HsfA4a confer cadmium tolerance in wheat and rice. Plant Cell 21:4031–4043

Sun P, Tian QY, Chen J, Zhang WH (2010) Aluminium-induced inhibition of root elongation in Arabidopsis is mediated by ethylene and auxin. J Exp Bot 61:347–356

Tang M, Mao D, Xu L et al (2014) Integrated analysis of miRNA and mRNA expression profiles in response to Cd exposure in rice seedlings. BMC Genomics 15:835

Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ (2012) Heavy metal toxicity and the environment. EXS 101:133–164

Vasilatos C, Megremi I, Economou-Eliopoulos M (2010) Geochemical characteristics of natural waters contaminated by hexavalent chromium, in Eastern Sterea Hellas, Greece. Sci Ann Sch Geol Aristotle Univ Thessaloniki Proc XIX CBGA Congr Thessaloniki, Greece 99:347–353

Wang J-W (2005) Control of root cap formation by microRNA-targeted auxin response factors in Arabidopsis. Plant Cell Online 17:2204–2216

Wang H, Jiao X, Kong X, Hamera S, Wu Y, Chen X, Fang R, Yan Y (2016a) A signaling cascade from miR444 to RDR1 in rice antiviral RNA silencing pathway. Plant Physiol 170:2365–2377

Wang S, Ren X, Huang B et al (2016b) Aluminium-induced reduction of plant growth in alfalfa (*Medicago sativa*) is mediated by interrupting auxin transport and accumulation in roots. Sci Rep 6:30079

Wang F-Z, Chen M-X, Yu L-J et al (2017a) OsARM1, an R2R3 MYB transcription factor, is involved in regulation of the response to arsenic stress in rice. Front Plant Sci 8:1868

Wang X, Wang C, Sheng H et al (2017b) Transcriptome-wide identification and expression analyses of ABC transporters in dwarf polish wheat under metal stresses. Biol Plant 61:293–304

Wu Y, Lv W, Hu L et al (2017) Identification and analysis of brown planthopper-responsive microRNAs in resistant and susceptible rice plants. Sci Rep 7:8712

Zhao FY, Wang K, Zhang SY et al (2014) Crosstalk between ABA, auxin, MAPK signaling, and the cell cycle in cadmium-stressed rice seedlings. Acta Physiol Plant 36:1879–1892