1 Characterization of wheat miRNAs and their target genes responsive
2 to cadmium stress

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Abstract

A increasing number of microRNAs have been shown to play important regulatory roles in plant responses to various metal stresses. However, little information about miRNAs especially miRNAs responsive to cadmium (Cd) stress is available in wheat. To investigate the role of miRNAs in responses to Cd stress, wheat seedlings were subjected to 250 μ M Cd solution for 6, 12, 24 and 48 h, and analyses of morphological and physiological changes as well as the expression of five miRNAs and their corresponding targets were carried out. Our results demonstrated that miRNAs and their targets were differentially expressed in leaves and roots of wheat seedlings exposed to Cd stress. Furthermore, miR398 may involve in oxidative stress tolerance by regulating its target CSD to participate in Cd stress. Among ten miRNA-target pairs studied, nine pairs showed complex regulation relationship in leaves and roots of wheat seedlings exposed to Cd stress. These findings suggested that miRNAs are involved in the mediation of Cd stress signaling responses in wheat. The characterization of the miRNAs and the associated targets in responses to Cd exposure provides a framework for understanding the molecular mechanism of heavy metal tolerance in plants.

Keywords: Wheat, microRNA, Target gene, Cadmium stress, Gene regulation

1. Introduction

Heavy metal contamination is an increasing environmental problem worldwide. Cadmium (Cd) is one of the most toxic heavy metals in the environment, posing serious threat to food security and crop production (Qiu et al., 2013) . Plants exposed to excess heavy metals can result in reduced biomass, retarded root growth, inhibition of cell proliferation, alterations in the photosynthesis rate (Fang et al., 2013; Jozefczak et al., 2014; Pérez-Chaca et al., 2014). To survive, plants respond and adapt to heavy metal stress by changes at morphological, physiological, genetic, and molecular levels (Tang et al., 2014). Great progress has been made in elucidating the complex response mechanisms involved in metal stress tolerance and in identifying a lot of metal stress responsive genes. To date, a number of heavy metal-responsive genes have been identified in various plant species, such as *Oryza sativa* (Shimo et al., 2011), *Solanum nigrum* (Xu et al., 2012), and *Triticum aestivum* (Qiu et al., 2013). However, the regulation of gene expression is a complex process, especially at the transcriptional level. MicroRNAs (miRNAs), recently recognized as important regulators of gene expression at the post-transcriptional level, have been found to be involved in plants response to a wide range of stresses (Sunkar et al., 2012; Budak et al., 2015b).

miRNAs are a class of small non-coding RNA molecules with 20-24 nucleotides in length that regulate gene expression at the transcriptional and post-transcriptional levels through mRNAs cleavage or repression of translation, depending on the complementarity between miRNA and their target genes (Akpinar et al., 2015). In

plants, miRNAs have been shown to play essential roles in plant growth and development and environmental stress responses (Zhou et al., 2012; Srivastava et al., 2013; Qiu et al., 2015). Increasing evidence has revealed that miRNA-mediated gene regulation plays a significant role in heavy metal regulatory networks. Using a direct cloning strategy, 13 conserved miRNAs representing 9 families have been isolated from *Brassica napus* and showed different responses to Cd stress (Huang et al., 2010). Ding et al. (2013) identified 12 Cd-responsive miRNAs in rice using microarray-based assay and most of these miRNAs were differentially regulated by Cd stress. A recent study using high-throughput sequencing found 15 known and 8 novel Cd-stress regulated miRNA families in radish roots exposed to CdCl₂ stress for 12 h. Furthermore, several Cd-responsive miRNAs have been found to target transcripts associated with metal transport and signaling (Xu et al., 2013). All of these findings demonstrate that a number of miRNAs play crucial roles in the regulation of plant responses to heavy metal stress.

As one of the world's most important crop species, some progress has been made in characterizing and analyzing miRNAs in the wheat genome (Budak et al., 2014, 2015a). Furthermore, several miRNAs have been found to play important roles in wheat response to a wide range of stresses such as salt (Wang et al., 2014), nutrient deficiency (Zhao et al., 2013), drought (Zhao et al., 2015). However, until now, little is known about miRNA expression patterns of wheat seedlings response to Cd stress, and there is even less understanding on miRNA targets for this regulation. In this study, physiological changes and the expression patterns of five miRNAs, which have

currently been released in the miRBase database, as well as their corresponding targets in wheat were investigated under Cd stress. The knowledge obtained from this study could be helpful in elucidating the potential roles of miRNAs in plant response to heavy metal stress and further understanding miRNA regulation in response to abiotic stress.

2. Materials and methods

2.1. Plant materials and treatment

The uniform seeds of wheat (*Triticum aestivum* L. cv. Zhengmai No. 004, obtained from Henan Academy of Agricultural Sciences) were surface sterilized in 0.1% HgCl₂ and germinated at 25 °C for 2 d in the dark. Germinated seeds were grown in a Petri dishes (diameter 18 cm, each containing 80 seeds) floating on a half-strength Hoagland's solution, in a growth chamber under a 12 h photoperiod at 800 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 70% relative humidity and 25°C/18°C (day/night). The plants were grown hydroponically for 7 d (with one fully expanded leaves) and then transferred to the same nutrient solution containing 250 μM CdCl₂ · 2.5H₂O for 6, 12, 24 and 48 h, respectively. Seedlings grown in Cd-free nutrient solution were treated as the control.

At each time point, roots and leaves of wheat seedlings from Cd-treated and control groups were separately harvested, immediately frozen in liquid nitrogen and stored at -80°C until use. For all physiological experiments, fresh leaf and root tissues harvested at five time points viz., 0, 6, 12, 24 and 48 h, were used. Then, plant height and root length of wheat seedlings were also measured using a scale.

2.2. Estimation of relative water content

Relative water content (RWC) in leaves was determined for each control and Cd treatment and it was calculated according to formula (Barrs and Weatherley, 1962):

$$\text{RWC (\%)} = [(\text{fresh weight} - \text{dry weight}) / (\text{turgid weight} - \text{dry weight})] \times 100$$

Leaves of the control and Cd stressed seedlings were collected and immediately weighed (fresh weight, FW). They were rehydrated in water for 24 h until fully turgid, surface dried, and re-weighed (turgid weight, TW). The tissues were then oven dried at 105°C for 24 h and re-weighed (dry weight, DW).

2.3. Determination of malondialdehyde (MDA) and photosynthetic pigments concentration

The concentration of MDA which is a product of lipid peroxidation was estimated by the thiobarbituric acid (TBA) as described by Predieri et al. (1995). Samples of leaves (0.2 g fresh weight, FW) were ground in 50 mM phosphate buffer (pH 7.8), and then centrifuged at 8,000×g for 15 min. One mL supernatant was combined with 2.5 mL thiobarbituric acid (TBA) incubated in boiling water for 30 min and then quickly cooled in an ice-bath. The mixture was centrifuged at 10,000×g for 5 min and the absorbance of supernatant was monitored at 532 and 600 nm. The value for non-specific absorbance at 600 nm was subtracted. MDA concentration was calculated by its molar extinction coefficient (155 mM⁻¹cm⁻¹) and the results expressed as μmol MDA g⁻¹ FW.

Measurement of chlorophyll *a* and *b* content of the control and Cd exposed plants was done as described by Lichtenthaler (1987). The photosynthetic pigments were extracted from seedling leaves with 10 mL of 80% acetone for 24 h in dark at room

temperature. The absorbance of supernatant was recorded at 663 nm and 645 nm using a spectrophotometer (*Lambda35*, *Perkin Elmer*, USA). The experiment was repeated three times and the concentration of chlorophyll *a* and *b* expressed as mg g⁻¹ FW.

2.4. Prediction of miRNA targets

To identify putative targets of miR156, miR159, miR164, miR398 and miR408, we used mature miR156, miR159, miR164, miR398 and miR408 as custom miRNAs and the wheat-expressed sequence tag (EST) database (TIGR Wheat Gene Index 9) as custom mRNAs to search for complementary hits by using psRNATarget (<http://bioinfo3.noble.org/psRNATarget/index.php?function=function3>) with default parameters settings (Zhang, 2005). Sequences with a penalizing score ≤ 3 were chosen as putative targets. Functional groups of predicted target genes were determined based on the annotations of the wheat EST database and BLAST search analysis.

2.5. Total RNA isolation and cDNA synthesis

Total RNA was isolated from each sample using RNAsimple total RNA kit (Tiangen, China) according to the manufacturer's instructions. Total RNA was treated with DNase I to remove contaminating genomic DNA. The integrity of the total RNA was checked by 2% agarose gel electrophoresis with ethidium bromide (EB) staining, and the concentration was determined with a NanoDrop^{TM} 1000 spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNA synthesis was performed using

Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, using 2 µg of total RNA and oligo (dT) primers.

2.6. Expression analysis of miRNAs and their target mRNAs by RT-qPCR

Quantitative real-time PCR (RT-qPCR) was performed using a Rotor-Gene 3000 real-time PCR detection system (Qiagen) according to the manufacturer's protocol. Gene-specific primers were designed using the Primer 5.0 (File S2). RT-qPCR reaction mix included 2 µL diluted cDNA, 0.3 µM of corresponding forward and reverse primers, and 10 µL of the Thunderbird SYBR Green PCR Master Mix in a final reaction volume of 20 µL with the following cycling conditions: 95°C for 2 min, 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. After amplification, a thermal denaturing cycle at 95°C for 15 s, 60°C for 15 s, and 95°C for 15 s was carried out to determine the dissociation curves and verify the specificity of the amplifications. All reactions were performed in biological triplicates, and the results were expressed relative to the expression levels of an internal reference gene, elongation factor 1 alpha-subunit (TEF1, GenBank accession No. M90077.1) in each sample using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

2.7. Statistical analysis

The experiment was performed in a completely random design with three replications. The data of morphological and physiological characteristics were subjected to a two-way analysis of variance (ANOVA) followed by the Duncan's multiple range tests at the 5% probability level using SPSS 16.0.

3. Results

3.1. Effect of CdCl₂ stress on plant height, root length and relative water content in wheat seedlings

Table 1 Changes of plant height, root length and relative water content in wheat seedlings subjected to 250 µM Cd solution for 0, 6, 12, 24 and 48 h.

Duration	Treatment	Plant height (cm)	Root length (cm)	Relative water content (%)
0h	CK	9.37±1.03bc	6.58±0.21cd	95.02±1.02a
6h	CK	9.47±1.51bc	6.82±0.32cd	94.21±2.04a
	Cd stress	9.05±0.53c	6.47±0.62d	78.32±1.25b
12h	CK	9.53±0.54bc	7.03±0.52bc	94.35±1.54a
	Cd stress	9.23±0.84c	6.56±0.24cd	66.24±0.98d
24h	CK	10.08±0.62ab	7.32±0.38b	95.67±1.54a
	Cd stress	9.47±0.32bc	6.66±0.34cd	71.42±0.84c
48h	CK	10.52±0.84a	8.58±0.52a	96.02±0.68a
	Cd stress	9.85±0.25b	6.72±0.62cd	67.28±0.95d

Data are means ± standard error of 20 replicates, and each mean in a column followed by the same letter indicates that there are no significant differences at 0.05 level according to Duncan's multiple range test.

Table 1 illustrates changes in plant height and root length in wheat seedlings under Cd stress in a time-dependent manner. Cd stress markedly hindered root and shoot elongation in the growth of wheat seedlings (Table 1). Compared with the control, Cd stress induced a marked decrease ($p < 0.05$) in the plant height and root length with prolonged Cd stress treatment. To further evaluate the responses to the Cd

stress, the relative water content of wheat seedlings grown at 250 μ M Cd solutions for 0, 6, 12, 24 and 48 h was estimated. The results indicated that the relative water content was strongly reduced in leaves of wheat seedlings with prolonged Cd exposure. Under the control conditions, it was maintained at 94-96%, whereas it gradually decreased to about 67% after 48 h under Cd stress.

3.2. Effect of CdCl₂ stress on MDA, Chl *a* and Chl *b* concentration in wheat seedlings

Fig.1. Changes of MDA, Chl *a* and Chl *b* concentration in wheat seedlings subjected to 250 μ M Cd solution for 0, 6, 12, 24 and 48 h. Data are means $\pm$ standard error of 6 replicates.

Changes of MDA, Chl *a* and Chl *b* concentration in wheat seedlings under continuous CdCl₂ stress were presented in Fig.1. As shows in Fig.1, with the elongation of Cd stress, MDA concentration gradually increased in wheat seedlings compared with the control. However, CdCl₂ stress resulted in a significant decrease in

the concentration of Chl *a* and Chl *b* in wheat seedlings compared with the control.

3.3. Target prediction of miRNAs in wheat seedlings

Table 2 Predicted targets of microRNA in wheat seedlings under Cd stress

miRNA	Predicted targets	Function	Accession number
miR156	Squamosa promoter-binding protein (SBP)	Development, flowering time	TC447879
miR159	MYB3 transcription factor	Signaling pathway and development	TC368630
miR164	NAC domain transcription factor	Signaling pathway, root development, response to oxidative stress	TC410195
miR398	Cu-Zn superoxide dismutase (CSD)	Copper homeostasis, response to oxidative stress	TC388772
miR408	Chemocyanin-like protein (CLP)	Stress-related proteins	TC395266

The identification of miRNA targets is an essential step towards the understanding of their regulatory function. Target prediction of miR156, miR159, miR164, miR398 and miR408 was carried out using the Web-based program psRNATarget. As expected, most of the predicted targets for *T. aestivum* have homologues of known miRNA targets in other plant species, such as squamosa promoter-binding protein (SBP) for miR156, MYB3 transcription factor for miR159, NAC domain transcription factor for miR164, Cu-Zn superoxide dismutases for miR398 or chemocyanin-like protein (CLP) for miR408 (Table 2). Putative target prediction for the miRNAs suggested that they may regulate various developmental processes and involve in oxidative stress tolerance.

3.4. Effect of CdCl₂ stress on miRNA expression in wheat seedlings

Fig.2. Expression level of five miRNAs in leaves and roots of wheat seedlings subjected to 250 μM Cd solution for 0, 6, 12, 24 and 48 h. The expression level of each miRNA in the control was arbitrarily set at 1. Error bars represent the standard deviations of three biological replicates.

In order to study the dynamic expression pattern of miRNAs under Cd stress, real-time PCR was performed on the leaves and roots of wheat seedlings at different time points (6, 12, 24 and 48 h) after Cd stress for five miRNAs (miR156, miR159, miR164, miR398 and miR408). Under Cd stress, the expression patterns of miR156, miR159, miR164, miR398 and miR408 in leaves were different from those in roots

(Fig.2). The expression of miR159, miR164, miR398 and miR408 were down-regulated in leaves, reaching their lowest expression level at 12 h, and then reached a relatively high expression level at 24 h. miR156 was down-regulated and remained at an extremely low expression level at all time points (6, 12, 24 and 48 h) in leaves. In the roots, the expression of miR156, miR159, miR164 and miR398 were rapidly up-regulated from 12 to 24 h, and then steadily decreased at 48 h. miR408 was up-regulated and remained at an extremely high expression level at all time points (6, 12, 24 and 48 h) in the roots. Thus, expression of miRNAs changed with time and also varied in different organs

3.5. Effect of CdCl₂ stress on target mRNAs expression in wheat seedlings

To further understand the mechanism of the interaction between miRNAs and their target genes, the expression levels of miRNA target genes (SBP, MYB, NAC, CSD and CLP) at different time points (6, 12, 24 and 48 h) after Cd stress were examined by RT-qPCR in leaves and roots of wheat seedlings. In the leaves, the transcript levels of MYB, NAC, CSD and CLP, as targets of miR159, miR164, miR398 and miR408 were down-regulated, gradually declined at 6 h, and then steadily increased at 12 and 24 h with the exception of slightly decreased at 24 h in CLP for miR408. The expression levels of MYB and NAC in the roots were sharply up-regulated at 6 h, and then gradually declined. CSD was significantly increased at 6 h, then sharply decreased at 12 h and again sharply increased at 24 and 48 h in roots (Fig. 3). The expression of CLP were up-regulated after 24 and 48 h in roots, reaching their highest expression at 48 h. SBP, as targets of miR156 remained at an extremely

low expression level at all time points (6, 12, 24 and 48 h) in roots compared in leaves.

Fig.3. Expression level of five target mRNAs in leaves and roots of wheat seedlings subjected to 250 μM Cd solution for 0, 6, 12, 24 and 48 h. Corresponding miRNAs are indicated in brackets. The expression level of each target mRNA in the control was arbitrarily set at 1. Error bars represent the standard deviations of three biological replicates.

3.6. Correlation between miRNAs and their target mRNAs

Fig.4. Negatively and positively correlated miRNA-target pairs in leaves (a, c, e) and roots (b, d, f) of wheat seedlings grown at 250 μ M Cd solutions for 0, 6, 12, 24 and 48 h. The expression level of miRNA and corresponding target mRNA in the control was arbitrarily set at 1. Error bars represent the standard deviations of three biological replicates.

In plants, miRNAs regulate gene expression majorly through targeting mRNAs for cleavage; thus, there might be an inverse correlation between a given miRNA and its targets. As expected, there are three miRNA/target pairs which show negative correlation in expression pattern under Cd treatment, including miR398-CSD in root

tissue, and miR159-MYB3 and miR408-CLP in both leaf and root tissues (Fig.4 c-f). Similarly, *miR164* showed an inverse relationship with its target NAC in the leaves of wheat seedlings under Cd stress except on the 24 h. However, the expression of SBP was positively correlated to *miR156* in both leaf and root tissues of wheat seedlings under Cd stress (Fig.4b). Interestingly, miR164-NAC also showed positive correlation in root tissue of wheat seedlings under Cd stress except on the 24 h. These results suggest that gene regulation may be more complicated under Cd stress condition.

4. Discussion

Cadmium (Cd) is a non-redox toxic heavy metal present in the environment. In plants, Cd could disturb various biochemical and physiological processes, leading to cell death and inhibition of growth (Fang et al., 2013; Qiu et al., 2013; Jozefczak et al., 2014). In accordance with previous work, our studies indicated that plant height, root length and chlorophyll content in wheat seedlings were markedly inhibited by Cd stress. Furthermore, Cd stress led to oxidative damage, measured by lipid peroxidation. The increase in MDA content and the decrease in chlorophyll content were observed in wheat seedlings exposure to Cd stress, suggesting that the plant growth inhibition must be, directly or indirectly, attributable to the Cd induced oxidative damage. Much progress has been made in unraveling the complex mechanisms behind Cd stress. The discovery of the role of miRNAs as gene regulators has led to a paradigm shift in the understanding of post-transcriptional gene regulation in plants. Recent findings illustrate that miRNAs might play a crucial role in regulation of plant genes at the posttranscriptional level in responding to metal

stress (Zhou et al., 2012; Srivastava et al., 2013; Budak et al., 2015b). Ding et al. (2011) found that most of the Cd-responsive miRNAs were down-regulated along with miR156, miR162 and miR390. Tang et al. (2014) also identified expression of miR156 to be suppressed upon exposure to Cd. In the present investigation, we found that miR156 was down-regulated and remained at an extremely low expression level in the leaves of wheat seedlings in response to the elongation of Cd stress, whereas the expression of miR398 were rapidly up-regulated from 12 to 24 h and miR408 was up-regulated and remained at an extremely high expression level at all time points in the roots of wheat seedlings under Cd stress. This result was in agreement with that of Ding et al. (2011), who found that miR398 and miR408 were reported to be up-regulated in response to Cd exposure in the root of rice. Using RT-qPCR, Zhou et al. (2012) also found that expression of miR398 was up-regulated in response to Cd exposure in the root of *Brassica napus*. These results indicated that miRNAs were responsive to Cd stress differentially in leaves and roots of wheat seedlings, suggesting that miRNAs might play different functions in the regulation of plant tolerance to Cd.

Notably, several key responsive enzymes for heavy metal detoxification were identified as target transcripts for miR398. miR398 was identified to detoxify superoxide radicals by directing the cleavage of its two targets, CSD1 and CSD2 (coding cytosolic and chloroplastic Cu/Zn superoxide dismutases, respectively) in *Arabidopsis thaliana* seedlings exposed to several abiotic stresses (Sunkar et al., 2006). CSD which is one target gene of miR398 as ROS (reactive oxygen species)

scavenger, is important for stress tolerance and survival in plant. Using RT-qPCR, Naya et al. (2014) found that miR398b was decreased and its target gene CSD1 were up-regulated in bean plants under Cu toxicity. The similar results were also detected in mulberry leaf treated with salt stress (Wu et al., 2015). Here, we found that miR398 was down-expression in wheat leaves, reaching their lowest expression level at 12 h of Cd exposure, whereas the expression of its target, a CSD gene, was up-expression. The decreased miR398 level in wheat seedlings resulted in accumulation of CSD mRNAs, which were important for catalyzing the dismutation of superoxide radicals into H₂O₂. Hence, oxidative stress induced miR398 down-regulation was considered a major machinery of detoxifying ROS by regulated its target CSD gene in wheat seedlings exposed to Cd stress. So we inferred that miR398 may involve in oxidative stress tolerance by regulating its target genes to participate in Cd stress.

In plants, miRNAs regulate gene expression majorly through targeting mRNAs for cleavage. Thus, miRNAs usually negatively regulate the accumulation of mRNAs and show an inverse correlation expression pattern in the same plant cells. Kantar et al. (2010) found that the expression profile of miR156 was negatively correlated with its target AV910992 in barley leaves under 8 h of dehydration stress. Our results are consistent with previous findings, indicating that three miRNA/target pairs (miR398-CSD in root tissue, and miR159-MYB3 and miR408-CLP in both leaf and root tissues) showed negative correlation in expression level under Cd stress condition. Interestingly, we observed that the transcript level of NAC was positively correlated with the accumulation of miR164 in the root of wheat seedlings upon the Cd stress.

This result was in agree with that of Phookaew et al. (2014), who found that the expression of miR164 and its target MesNAC was not inversely regulated in the roots of cassava during water deficit. Furthermore, the expression of miR156 and its target SBP in both leaf and root tissues of wheat seedlings in our study also showed positive correlation under Cd stress. There are several reasons why miRNA/target pairs do not show perfect negative correlation. One potential reason is that abiotic stress is a complicated progress, and it is also possible that the target is regulated by multiple miRNAs, the final expression level of the targets should be combined results for all the miRNAs targeting this gene (Khaksefidi et al., 2015). Another reason is that miRNAs have been demonstrated to establish temporal and spatial expression patterns of target genes (Zhou et al., 2012; Qiu et al., 2015). Therefore, expression patterns in a sample comprising multiple tissue types may conceal a reverse expression level for the miRNA and its targets. Thus, further investigation of the regulatory functions of miRNAs will deepen our understanding on plant response to heavy metal-induced toxicity.

Taken together, the results generated from this study suggested that Cd-responsive miRNAs showed time- and organ dependent expression patterns. Furthermore, a complex regulation relationship between miRNA and target expression profiles was validated in wheat seedlings under Cd stress using RT-qPCR. Comprehensive characterisation of the miRNAs and their target genes will contribute to our understanding of gene regulatory frameworks in plants, and may provide insights into the role of miRNAs and their targets in regulating plant tolerance to Cd

stress.

Acknowledgments

This research was supported by the National Science Foundation of China (U1404304 and No. 31500499), Program for Science & Technology Innovation Talents in Universities of Henan Province (16HASTIT019) and Innovative Research Team (in Science and Technology) in University of Henan Province (No. 15IRTSTHN020).

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