



# Genotypic difference of cadmium tolerance and the associated microRNAs in wild and cultivated barley

Jiahua Yu<sup>1</sup> · Liyuan Wu<sup>1</sup> · Liangbo Fu<sup>1</sup> · Qiufang Shen<sup>1</sup> · Liuhui Kuang<sup>1</sup> · Dezhi Wu<sup>1</sup>  · Guoping Zhang<sup>1</sup>

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## Abstract

Little study was performed to know how microRNAs (miRNAs) are responsive to cadmium (Cd) stress in barley (*Hordeum vulgare*). In this study, 16 small RNA libraries of shoot and root tissues from a wild barley accession (WB-1) and cultivated barley (Golden Promise) with contrasting Cd tolerance were constructed and sequenced. Moreover, a degradome library was constructed and analyzed to identify target genes of the miRNAs. Based on high-throughput sequencing, 216 conserved miRNAs (in 59 miRNA families) and 87 novel miRNAs were identified. A total of 238 target genes for 149 miRNAs (113 conserved and 36 novel miRNAs) were detected by the degradome analysis. Among these miRNAs, 45 miRNAs (40 conserved and 5 novel miRNAs) and 43 miRNAs (40 conserved and 3 novel miRNAs) showed differential expression in roots and shoots of two genotypes under Cd conditions. Compared with cultivar Golden Promise, the wild genotype WB-1 had genotype-dependent responses of miR156, miR159, miR166, miR167, miR171 and miR393, which regulate target genes including *SPL*, *MYB*, *HD-Zip*, *ARF*, *GRAS* and *TIR*. Correspondingly, WB-1 had lower Cd concentration and stronger Cd tolerance than Golden Promise. It indicates that miRNAs may play critical roles underlying genotypic difference of Cd tolerance in barley.

**Keywords** RNA sequencing · Degradome analysis · MiRNA · Target gene · Cd stress

## Introduction

Cadmium (Cd) is a toxic element, recently becoming a global agricultural contaminant in the form of Cd<sup>2+</sup> (ATSDR 2008). Soil Cd pollution is the consequences of metal mining, phosphate fertilizer overusing and other factors (Clemens et al. 2013; Khan et al. 2017). Unfortunately, Cd causes serious harm to human health including nephrotoxicity, osteoporosis and even cancer, mainly through the food chain (Jarup and Akesson 2009; Liu et al. 2009). Therefore, it is important to reduce Cd accumulation in crops. Cd can be readily taken up by plant roots, causing damages to plant growth, photosynthesis and nutrient uptakes (Khan et al.

2015; Uraguchi et al. 2009). In plants, many genes involved in Cd uptake, translocation and distribution have been identified, which are members of ABC (ATP-binding cassette), HMA (heavy metal ATPase), NRAMP (natural resistance-associated macrophage proteins) and ZIP (ZRT, IRT-like proteins) family genes (Kim et al. 2007; Siemianowski et al. 2014; Wu et al. 2016; Guerinot 2000). However, little study was performed to understand regulatory networks of microRNAs (miRNAs) involved in Cd tolerance in plants.

miRNAs are a special kind of endogenous non-coding small RNAs with the length of approximately 21 nucleotides (Voinnet 2009). Currently, there are 7,057 miRNAs from 73 plant species stored in the database miRBase (version 21; <http://www.mirbase.org>). It is well-known that miRNAs are involved in many biological processes such as plant growth, tissue development and various stress responses (Khraiwesh et al. 2012; Tang and Chu 2017). Furthermore, miRNAs were also reported in post-transcriptional regulatory networks under heavy metal stresses, such as arsenic (Ghosh et al. 2017; Yu et al. 2012), chromium (Bukhari et al. 2015), lead (He et al. 2014; Wang et al. 2015) and cadmium (Ding et al. 2011; He et al. 2016; Zhou et al. 2012). For instance,

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✉ Dezhi Wu  
wudezhi@zju.edu.cn

<sup>1</sup> Department of Agronomy, Key Laboratory of Crop Germplasm Resource of Zhejiang Province, Zhejiang University, Hangzhou 310058, China

miRNA166 was confirmed to regulate Cd accumulation and tolerance in rice, and overexpression of miR166 could reduce Cd accumulation in grains and Cd translocation from roots to shoots (Ding et al. 2018). However, miRNAs involved in Cd tolerance have not been reported in barley (*Hordeum vulgare*).

Barley is the fourth important cereal crop in the world, which is widely used in processing malts and livestock feed due to its high nutrition value. Therefore, Cd accumulation in barley grains poses a great threat to human health. In our previous study, the Cd concentration in barley grains was analyzed in 100 barley core accessions planted in the natural fields for 2 years. The results showed a wide genotypic difference in Cd accumulation (Wu et al. 2015). However, the molecular mechanism of Cd accumulation and tolerance in barley is still unknown. In this study, we identified miRNAs in response to Cd exposure in wild and cultivated barley, and performed a degradome library to reveal target genes of these miRNAs. Furthermore, we compared the differential expression profiles of miRNAs between Golden Promise and WB-1 to identify their distinct miRNA regulation of Cd tolerance. These Cd-responsive miRNAs and their target genes could provide useful information for molecular mechanisms underlying Cd tolerance in barley.

## Materials and methods

### Plant materials and hydroponics

In this study, a barley cultivar Golden Promise and a wild barley genotype WB-1 were used. All materials were preserved by the Provincial Key Laboratory of Crop Gene Resources of Zhejiang University, China. Seeds of the two genotypes were disinfected with 2% H<sub>2</sub>O<sub>2</sub> for 30 min, washed and soaked in deionized water for 2 h. Then, the seeds were germinated on moist filter papers in a growth chamber at 22 °C/18 °C (day/night) under dark conditions (Shen et al. 2017a, b). Light was supplied 5 days later. Seven-day-old seedlings were transferred to 6-L plastic pots filled with aerated one-fifth Hoagland (pH 6.0) solution, containing 1 mM KNO<sub>3</sub>, 1 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.4 mM MgSO<sub>4</sub>, 0.2 mM NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 20 μM Fe-EDTA, 3 μM H<sub>3</sub>BO<sub>3</sub> and 1.0 μM CuSO<sub>4</sub>. The nutrient solution was renewed every 3 days.

Twelve-day-old seedlings were treated with 5 μM CdCl<sub>2</sub>. The solution without CdCl<sub>2</sub> addition was used as the control. After 10 days treatments, root and shoot tissues of the two genotypes under treated and control conditions were sampled. Roots were washed 3 times with 5 mM CaCl<sub>2</sub> solution, and then were separated from the shoots. Three biological replicates were sampled for physiological analysis. Meanwhile, two biological replicates were set for small

RNA library construction, which were frozen immediately in liquid nitrogen and stored at –80 °C for use.

### Microelement and cadmium concentration determination

The root and shoot samples prepared as described above were dried in an oven at 70 °C for 2 days, and then digested completely in concentrated nitric acid solution using a microwave digestion instrument (Multiwave 3000, Anton Paar GmbH, Australia). There are interaction effects between Cd and other mineral elements. Therefore, the concentration of Cd and other mineral elements including Mn, Cu, Fe, and Zn was determined by ELAN® DRC-e ICP-MS (PerkinElmer SCIEX™, Concord, ON, Canada) following the manufacturer's procedure. Cd uptake by roots was calculated by the formula: Cd uptake = Cd content per plant/root dry weight. While Cd translocation to shoots was calculated by the formula: Cd translocation (%) = shoot Cd content/Cd content per plant × 100%. Three biological replicates were performed for the analysis.

### RNA extraction

Total RNA was extracted by Trizol reagents (Invitrogen, CA, USA) according to the manufacturer's instructions. Sixteen samples from two genotypes Golden Promise and WB-1 were used for small RNA library construction. The concentration and quality of total RNA were tested by Bioanalyzer 2100 (Agilent, CA, USA).

### Small RNA and degradome libraries construction and sequencing

Small RNA library construction was performed using TruSeq Small RNA Sample Prep Kit (Illumina, San Diego, USA) following the vendor's guidelines. In brief, 1 μg of total RNA was ligated to RNA 3' and 5' adapters. Reverse transcript reactions were carried out to create cDNA constructs, and the cDNAs were amplified with two primers that anneal to the ends of the adapters. Then, 140–160 bp (total length of RNA fragment and adapters) products were purified by 6% TBE PAGE Gel (Novex, USA). Finally, the constructed libraries were single-end sequenced (50 bp) by the Illumina HiSeq2500 platform (LC-BIO, Hangzhou, China).

To figure out the target genes for miRNAs, a degradome library was constructed by RNA pools used in the small RNA sequencing based on the methods as described by Ma et al. (2010). Briefly, poly (A)-enriched mRNA was isolated, and annealed with biotinylated random primers. RNAs containing 5'-monophosphates were ligated to a 5' RNA adapter. Then, the ligated products were used to generate first-strand cDNA by the reverse transcription and the PCR reactions.

Finally, we performed single-end sequencing (50 bp) by the Illumina Hiseq2500 as mentioned above.

The raw reads were deposited in the SRA database (<http://www.ncbi.nlm.nih.gov/sra/>) at NCBI with BioProject accession number PRJNA485436 and SRA accession number SRP157400 (Small RNA sequencing: SRR7687211-SRR7687226; Degradome sequencing: SRR7687210).

## Bioinformatics analysis

Raw reads of high-throughput small RNA sequencing were processed by the software ACGT101-miR in the LC-Sciences (Hangzhou, China) to remove the adapter sequences and junk reads through mapping against barley mRNA database ([ftp://ftp.ensemblgenomes.org/pub/plant/release-36/fasta/hordeum\\_vulgare/cds/Hordeum\\_vulgare.Hv\\_IBSC\\_PGSA\\_v2.cds.all.fa.gz](ftp://ftp.ensemblgenomes.org/pub/plant/release-36/fasta/hordeum_vulgare/cds/Hordeum_vulgare.Hv_IBSC_PGSA_v2.cds.all.fa.gz)), Rfam (<http://rfam.janelia.org>) and Repbase (<http://www.girinst.org/repbase>). The clean sequences with the length of 18–25 nt were blasted against miRBase (version: 21; <http://www.mirbase.org>). The matched reads mapped to the barley reference genome database ([http://plants.ensembl.org/Hordeum\\_vulgare/Info/Index](http://plants.ensembl.org/Hordeum_vulgare/Info/Index)), which were considered as conserved miRNAs. Meanwhile, the unmatched reads and their flanking 120 nt sequences were used to predict the hairpin structures by RNAfold software (<http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi>) to identify novel miRNAs.

For degradome analysis, raw reads were obtained by the Illumina Pipeline software (version 1.5), and then filtered out adaptor sequences and low quality reads. The proprietary program ACGT101-DEG v3.1 (LC-BIO, Hangzhou, China) and the public software Cleaveland v3.0 (Addo-Quaye et al. 2009) were used to analyze sequencing data. The degradome reads were mapped to barley mRNA database ([ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum\\_vulgare/cds/Hordeum\\_vulgare.Hv\\_IBSC\\_PGSA\\_v2.cds.all.fa.gz](ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/cds/Hordeum_vulgare.Hv_IBSC_PGSA_v2.cds.all.fa.gz)), and the annotations for target genes was obtained from the barley CDS database in IPK ([http://webblast.ipk-gatersleben.de/barley\\_ibsc/downloads/160517\\_Hv\\_IBSC\\_PGSA\\_r1\\_CDS\\_HighConf\\_REPR\\_annotation.fasta.gz](http://webblast.ipk-gatersleben.de/barley_ibsc/downloads/160517_Hv_IBSC_PGSA_r1_CDS_HighConf_REPR_annotation.fasta.gz)). All target genes were classified as category 0 to 4 in accordance with their abundance relative to the overall profiles of degradome reads (Addo-Quaye et al. 2008, 2009).

## Identification of Cd-responsive miRNAs

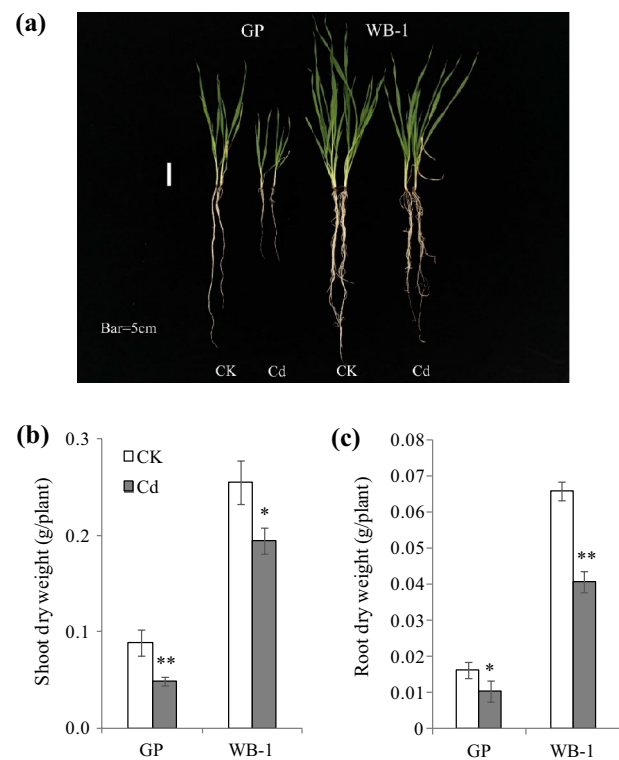
Cd-responsive miRNAs were identified based on the criteria as described by Wu et al. (2018). The abundance of miRNAs was normalized according to Li et al. (2016). The fold change of miRNAs between the Cd-treated and the control samples was calculated as the following formula: fold change =  $\log_2 N$ ,  $N = \text{Cd reads/control reads}$ . The miRNAs

were up-regulated with  $\log_2 N \geq 0.5$ , down-regulated with  $\log_2 N \leq -0.5$ .

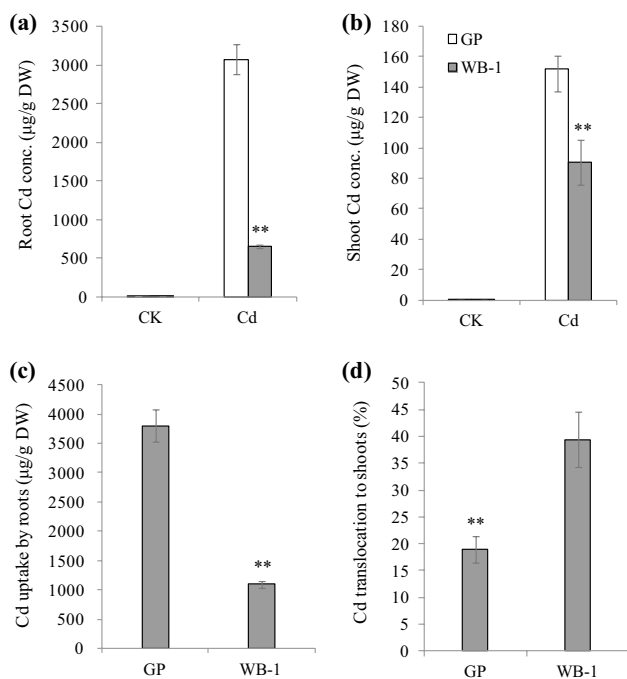
## Results

### The difference of Cd tolerance between Golden Promise and WB-1

After 10 days of 5  $\mu\text{M}$  Cd treatment, wild barley genotype WB-1 showed much stronger Cd tolerance than the cultivar Golden Promise (Fig. 1a). The dry weights of shoots and roots were reduced much more in Golden Promise than that in WB-1 after Cd treatment, especially 46.5% and 23.7% of the loss for shoot dry weights in Golden Promise and WB-1, respectively (Fig. 1b, c). Correspondingly, WB-1 accumulated much lower Cd concentration in the roots and shoots than Golden Promise, being 21.5% and 59.6% of Cd concentration in Golden Promise, respectively (Fig. 2a, b). In spite of much small biomass in Golden Promise, the Cd uptake by roots was significant larger in Golden Promise than that in WB-1, but it was opposite for Cd translocation from roots



**Fig. 1** Growth performance of two barley genotypes after Cd treatments. **a** Plant pictures of Golden Promise and WB-1 under 5  $\mu\text{M}$  Cd and control (CK) conditions for 10 days. Dry weights of shoots (**b**) and roots (**c**) of Golden Promise and WB-1 after Cd treatments. Data are means  $\pm$  SD of three biological replicates; \* indicates significant difference at  $P < 0.05$  by Tukey's test and \*\*  $P < 0.01$



**Fig. 2** The difference of Cd concentration, uptake and translocation between two barley genotypes. Cd concentration in roots (a) and shoots (b) of Golden Promise and WB-1 under control (CK) and 5  $\mu\text{M}$  Cd conditions for 10 d was determined by the ICP-MS. (c) Cd uptake by roots and Cd translocation to shoots (d) in Golden Promise and WB-1 under Cd stress were calculated as described in the methods. Data are means  $\pm$  SD of three biological replicates; \* indicates significant difference at  $P < 0.05$  by Tukey's test and \*\*  $P < 0.01$

to shoots (Fig. 2c, d). It indicates that higher Cd concentration in Golden Promise is due to its stronger Cd uptake in comparison with WB-1. Furthermore, other micrometals also showed lower concentration in WB-1 than in Golden Promise including Mn, Cu, Fe and Zn (Supplemental Fig. S1). These results indicated that WB-1 was more Cd tolerant than Golden Promise, mainly due to its low Cd uptake.

### Identification of conserved and novel miRNAs

To reveal molecular difference underlying contrasting Cd tolerance between Golden Promise and WB-1, 16 small RNA libraries from root and shoot tissues of two genotypes under Cd and control conditions were constructed. For small RNA libraries in shoots, 2,144,420, 2,178,580, 1,870,916 and 1,869,353 unique reads in the average were generated in Golden Promise (CK), Golden Promise (Cd), WB-1(CK) and WB-1(Cd), respectively (Supplemental Table 1). After raw reads filtering, 1,072,894, 1,091,675, 1,064,744 and 1,043,284 clean reads were obtained for the corresponding four groups, which were averagely accounted for 53.4% of the raw reads. Meanwhile, for small RNA libraries in roots, 2,325,952, 2,329,086, 3,452,555 and 2,864,046 unique

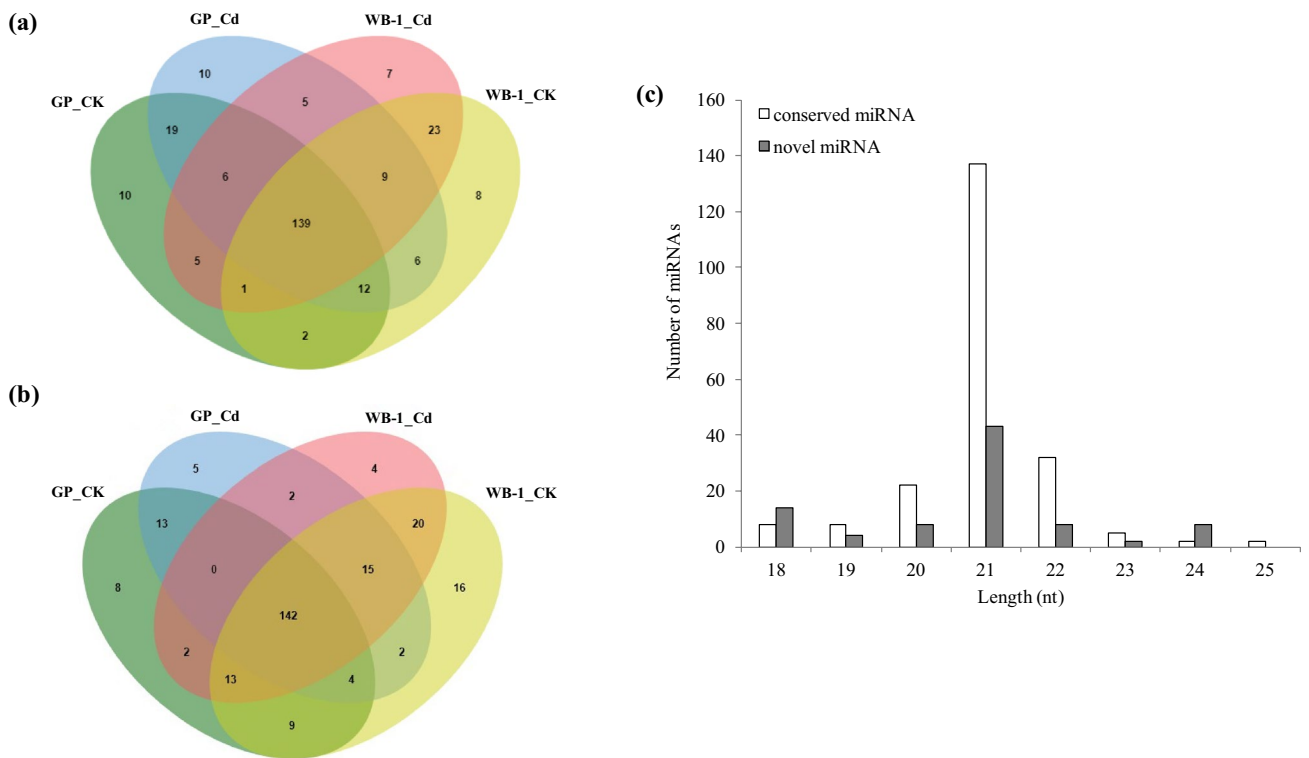
reads in the average, corresponding to 1,353,683, 1,378,257, 2,349,012 and 1,730,848 clean reads were obtained in Golden Promise (CK), Golden Promise (Cd), WB-1(CK) and WB-1(Cd), respectively, averagely accounting for 61.5% of the raw reads (Supplemental Table 1). The length of the clean reads distributed from 18 to 25 nt and 24 nt was the most dominant in all libraries (Supplemental Fig. S2).

A total of 303 miRNAs including 216 conserved miRNAs belonging to 59 families and 87 novel miRNAs were identified (Fig. 3 and Supplemental Table 2). For the length of these miRNAs, 21 nt miRNAs was the most dominant, which accounted for 63.4% and 49.4% of conserved and novel miRNAs, respectively (Fig. 3c). There were 262 and 255 miRNAs identified in shoot and root libraries, respectively (Fig. 3a, b). Among them, 235 miRNAs were found in both genotypes (Supplemental Table 2). The miR169 family was the largest miRNA families with 21 members identified in this study. Moreover, 6 families (miR156, miR166, miR167, miR171, miR396 and miR1122) were identified with more than 10 miRNAs (Supplemental Table 2).

### Genotypic difference of Cd responsive miRNAs in roots

In roots, 45 miRNAs were identified with differentially expressed levels after Cd treatment including 40 conserved miRNAs mainly belonging to miR156, miR166, miR167, miR169, miR396 families and 5 novel miRNAs (Table 1). For two genotypes, 17 up-regulated, 17 down-regulated and 10 slightly changed miRNAs were identified in Golden Promise, while 17 up-regulated, 11 down-regulated, and 17 slightly changed miRNAs were found in WB-1, respectively (Table 1). Some miRNAs were identified in both genotypes including 8 up-regulated miRNAs (5 families: miR167, miR319, miR394, miR396 and miR397) and 6 down-regulated miRNAs (3 families: miR169, miR172, miR396 and a novel miRNA). Based on degradome analysis and bioinformatics prediction, the target genes of these miRNAs mainly included TCP family transcription factor, growth-regulating factor, elongation factor, cytochrome P450 superfamily protein, laccase, calnexin, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein and disease resistance (Table 1).

On the other hand, there was genotypic difference of Cd responsive miRNAs between two genotypes. There were 7 miRNAs (5 families: miR159, miR166, miR168, miR390 and miR396) up-regulated in WB-1, which were slightly changed in Golden Promise, and these miRNAs target genes mainly included MYB domain protein, homeobox-leucine zipper protein family, receptor-like kinase, etc. Three miRNAs (2 members from miR156 and hvu-miR169c-3p\_2) were down-regulated in WB-1, but were not in Golden Promise. The target genes of these miRNAs encoded P-loop



**Fig. 3** Overview of miRNAs identified in two barley genotypes. **a** Venn diagrams of shoots and roots (**b**) indicate the number of miRNAs identified under control (CK), Cd treatment (5  $\mu$ M Cd) and over-

lap conditions in Golden Promise and WB-1. **c** Length distribution of the known and novel miRNAs

containing nucleoside triphosphate hydrolases superfamily protein, structure-specific endonuclease subunit and receptor-like protein kinase (Table 1).

### Genotypic difference of Cd responsive miRNAs in shoots

In shoots, 43 miRNAs were differentially expressed in response to Cd stress, including 40 conserved miRNAs mainly belonging to miR156, miR164, miR167, miR169, miR396, miR397 families and 3 novel miRNAs (Table 2). For two genotypes, 15 up-regulated, 16 down-regulated and 11 slightly changed miRNAs were identified in Golden Promise, while 8 up-regulated, 18 down-regulated and 17 slightly changed miRNAs were identified in WB-1, respectively (Table 2). For both genotypes, 3 miRNAs (hvu-miR167c-5p, hvu-MIR397a-3p and hvu-MIR397a-3p) up-regulated and 6 miRNAs (hvu-miR156a-3p\_1, hvu-miR164\_1 and four miR169 members) down-regulated were found for both genotypes (Table 2). The target genes of these miRNAs mainly included ubiquitin carboxyl-terminal hydrolase, cytochrome P450 superfamily protein, laccase, P-loop containing nucleoside triphosphate hydrolases superfamily protein, NAC domain containing

protein, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein, zinc finger protein and elongation factor.

For genotypic difference, hvu-miR390-5p, hvu-miR394-5p, hvu-miR396a-5p\_2 and hvu-miR444b\_1 were up-regulated in WB-1, but slightly changed in Golden Promise, and the target genes of these miRNAs mainly included receptor-like protein kinase, calnexin, growth-regulating factor and MADS-box transcription factor. Moreover, 7 miRNAs (hvu-miR156a-5p, hvu-miR168-3p, hvu-miR1122b-3p, hvu-miR5168-3p and 3 miRNAs from miR169) were down-regulated in WB-1, but were not in Golden Promise. These miRNAs target genes mainly encoded squamosa promoter-binding-like protein, receptor-like protein kinase and homeobox-leucine zipper protein family. Moreover, hvu-miR167a-5p\_1, hvu-miR167c-3p, hvu-miR530-5p, hvu-miR5048a, and PC-miR62 showed oppositely changed pattern between Golden Promise and WB-1, and the target genes of these miRNAs included auxin response factor, receptor kinase, UDP-glucose 4-epimerase and disease resistance protein (Table 2). In addition, hvu-MIR169g-3p targeted to non-specific phospholipase C2 was detected only in WB-1, which was down-regulated after Cd treatment.

**Table 1** Differentially expressed miRNAs and their target genes in roots of Gold Promise and WB-1 after Cd treatments

miRNAs name	Sequence	GP <sup>a</sup>	WB-1 <sup>b</sup>	Target gene	Transcript annotation	Degrade detection
hvu-miR156a-3p_1	GCTCACCTCTCTCTGTC AGC	0.05	-0.88	HORVU1Hr1G078160.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	Y
hvu-miR156b-3p	GCTCACTTCTCTCTCTGT CAGC	0.71	-0.70	HORVU6Hr1G028980	Cinnamoyl coa reductase 1	N
hvu-miR156d-3p	GCTCACTCCTCTTTCTGT CAGC	0.35	-0.50	HORVU4Hr1G025850.7	Structure-specific endonuclease subunit slx1	N
hvu-miR159a	TTTGGATTGAAGGGAGCT CTG	-0.29	1.17	HORVU3Hr1G079490.4	MYB domain protein 33	Y
hvu-miR160a-5p	TGCCTGGCTCCCTGTATG CCA	-0.65	-0.07	HORVU1Hr1G041770.6 HORVU2Hr1G089660.7 HORVU2Hr1G089670.2 HORVU6Hr1G026750.1	Auxin response factor 22 Auxin response factor 10 Auxin response factor 10 Auxin response factor 18	Y Y Y Y
hvu-miR164_2	GGAGAAGCAGGGCACGTG CA	0.64	-0.33	HORVU2Hr1G080460.8 HORVU2Hr1G044620.1 HORVU5Hr1G041400.1	NAC domain protein Undescribed protein Phytosulfokines 2	Y Y Y
hvu-miR164a-5p	TGGAGAAGCAGGGCACGT GCT	-1.32	-0.07	HORVU5Hr1G011650.2 HORVU7Hr1G072670	NAC domain containing protein 1 NAC domain containing protein 1	Y Y
hvu-miR166a	TCGGACCAGGCTTCATTC CCC	0.30	0.70	HORVU1Hr1G041790.2 HORVU5Hr1G010650.1 HORVU5Hr1G061410.29	Homeobox-leucine zipper protein family Homeobox-leucine zipper protein family Homeobox-leucine zipper protein HOX10	Y Y Y
hvu-miR166a-3p_1	CTCGGACCAGGCTTCATT CCCC	0.06	0.56	HORVU5Hr1G010650.1	Homeobox-leucine zipper protein family	Y
hvu-miR166a-3p_2	CTTCGGACCAGGCTTCAT TCCC	0.46	2.17	HORVU5Hr1G010650.1	Homeobox-leucine zipper protein family	N
hvu-miR166a-5p	GGAATGTTGTCTGGTCA AGG	1.06	0.41	HORVU1Hr1G076940.1 HORVU6Hr1G005350.2	Nucleotide-diphospho-sugar transferase family protein GPI mannosyltransferase 3	N N
hvu-miR167a-5p_1	TGAAGCTGCCAGCATGAT CTA	-0.84	-0.19	HORVU2Hr1G121110.32	Auxin response factor 6	Y
hvu-miR167b-3p	AGGTCATGCTGGAGTTTC ATC	1.17	-0.48	HORVU1Hr1G075520.2	Jacalin-related lectin 3	N
hvu-miR167c-5p	TGAAGCTGCCAGCATGAT CTGC	1.2	0.68	HORVU1Hr1G077630.2	Ubiquitin carboxyl-terminal hydrolase 25	N
hvu-miR167f-3p	AGATCATGCTGCAGCTTC ATT	1.69	0.09	HORVU4Hr1G016990.3	Cysteine desulfurase	N
hvu-miR168-5p	TCGCTTGGTGCAGATCGG GAC	-0.16	0.67	HORVU2Hr1G105050.1	Protein of unknown function (DUF581)	Y
hvu-miR169a-3p	TGGGCAAGTCACCCTGGC TACC	-2.9	-1.01	HORVU4Hr1G087430	rRNA N-glycosidase	N
hvu-miR169c-3p_2	GGCAAGCTGTCCTGGCT ACA	-0.44	-1.14	HORVU2Hr1G013460.2 HORVU3Hr1G068000.2 HORVU3Hr1G068010.1 HORVU3Hr1G079010.5	Strictosidine synthase-like 2 Receptor-like protein kinase 2 Leucine-rich repeat receptor-like protein kinase family protein Transcription factor-related	N N N N

**Table 1** (continued)

miRNAs name	Sequence	GP <sup>a</sup>	WB-1 <sup>b</sup>	Target gene	Transcript annotation	Degrade detection
hvu-miR169c-5p	TAGCCAAGGATGACTTGCC	-2.27	0.53	HORVU2Hr1G032130	Nuclear transcription factor Y subunit A-5	Y
hvu-MIR169d-3p_2	GGTGGGTCTTCTTGGCTA AC	-1.89	-1.23	HORVU4Hr1G081500.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
				HORVU4Hr1G081480.3	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
				HORVU4Hr1G081490.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
hvu-miR169h-3p	GCAAGTTGTTCTTGGCTA GC	-0.98	0.15	HORVU1Hr1G075540.3	Mitochondrial processing peptidase alpha subunit	Y
				HORVU5Hr1G108630.3	Cell division cycle 5	Y
hvu-miR169i-3p	AGGCAGTCTCCTTGGCTA GC	-2.43	-0.95	HORVU2Hr1G013680.1	Elongation factor 1-alpha	Y
hvu-miR169i-5p	TAGCCAAGGATGACTTGC CTG	-2.04	0.09	HORVU2Hr1G032130	Nuclear transcription factor Y subunit A-5	Y
hvu-miR171-3p	TGATTGAGCCGTGCCAAT ATC	0.72	0.13	HORVU1Hr1G053510.1	GRAS family transcription factor	Y
				HORVU6Hr1G063650.1	GRAS family transcription factor	Y
hvu-miR172b-5p	GCAGCACCACCAAGATTC ACA	-1.45	-0.78	HORVU6Hr1G088570.2	Clathrin interactor EPSIN 2	Y
hvu-miR319a-3p_2	TTGGACTGAAGGGTGCTC CCT	1.79	1.32	HORVU2Hr1G060120.1	TCP family transcription factor 4	Y
				HORVU5Hr1G103400.1	TCP family transcription factor 4	Y
hvu-miR390-5p	AAGCTCAGGAGGGATAGC GCC	0.10	0.93	HORVU1Hr1G043790.1	Leucine-rich repeat receptor-like protein kinase family protein	N
				HORVU2Hr1G091840.16	Receptor-like protein kinase 2	N
				HORVU2Hr1G124010.6	Receptor-like kinase	N
				HORVU7Hr1G007520.1	Leucine-rich repeat receptor-like protein kinase family protein	N
hvu-miR393-5p	TTCCAAAGGGATCGCATT GAT	1.02	0.05	HORVU1Hr1G021550.4	Transport inhibitor response 1-like protein	Y
				HORVU2Hr1G070800.3	Transport inhibitor response 1-like protein	Y
hvu-miR393b-3p	ATCATGCGATCCTTTTGG AAG	1.59	0.2	HORVU1Hr1G048580.3	Protein kinase superfamily protein	N
hvu-miR394-5p	TTGGCATTCTGTCCACCT CC	0.83	0.91	HORVU1Hr1G043940.3	Protein TIC110, chloroplastic	Y
				HORVU6Hr1G018370.1	Calnexin 1	Y
hvu-miR396a-5p_1	TCCAAAGGCTTTCTTGAA CTG	0.57	1.59	HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-miR396a-5p_2	TCCACAGGCTTTCTTGAA CTT	0.75	0.89	HORVU4Hr1G010080.6	Growth-regulating factor 6	Y
				HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-miR396c-5p	TCCACAGCTTTCTTGAA CTT	-0.78	-0.67	HORVU4Hr1G010080.6	Growth-regulating factor 6	Y
				HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-miR396e-3p	GTTCAATAAAGCTGTGGG AAA	0.46	1.11	HORVU2Hr1G087460.1	Nuclear transcription factor Y subunit B-3	N

**Table 1** (continued)

miRNAs name	Sequence	GP <sup>a</sup>	WB-1 <sup>b</sup>	Target gene	Transcript annotation	Degrado- dome detection
hvu-miR396e-5p	TTCCACAGCTTTCTTGAA CTG	0.62	1.26	HORVU4Hr1G010080.6	Growth-regulating factor 6	Y
				HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-miR396d-3p_1	GTTCAAGAAAGCCCATGG AAA	-0.73	0.40	HORVU3Hr1G070620.18	Auxin-responsive protein IAA6	N
				HORVU5Hr1G008980	2-methyl-6-phytyl-1	N
hvu-MIR397a-3p	TCACCGGCGCTGCACGCA ATG	inf	4.34	HORVU2Hr1G032890.2	Cytochrome P450 superfamily protein	N
hvu-MIR397a-5p	TTGAGTGCAGCGTTGATG AAC	5.61	3.76	HORVU3Hr1G086160.1	Laccase 17	Y
hvu-miR444b_2	TGCAGTTGCTGCCTCAAG CTTT	-1.23	-0.48	HORVU5Hr1G000370.3	MADS-box transcription factor 25	Y
				HORVU7Hr1G066380.4	Zinc finger (C3HC4-type RING finger) family protein	Y
hvu-MIR9662a-5p	GCGGCTCTGTGGTGTTC AGC	1.16	-0.23	HORVU2Hr1G100080.7	Protein strawberry notch homolog 1	N
				HORVU5Hr1G123930.2	Beta-fructofuranosidase, insoluble isoenzyme 3	N
PC-miR3	CTTGGTGATGGAACGTGT GAT	-3.11	-0.28	HORVU6Hr1G087330.4	ENTH/ANTH/VHS superfam- ily protein	Y
PC-miR4	TCATACGTTCCAACATCA ATA	0.00	-0.50	HORVU3Hr1G089450.15	-	N
PC-miR40	CCGACGATCTCCCGTGG	-1.29	2.02	HORVU5Hr1G004340.2	ADP-ribosylation factor family protein	Y
				HORVU4Hr1G090800.2	Charged multivesicular body protein 3	Y
PC-miR51	AATGCCCTTCCGTACATT TTC	-0.80	-0.79	HORVU1Hr1G061270.2	Disease resistance RPP13-like protein 4	N
PC-miR59	AACAATTTCTGACTTCATT	-2.44	-0.25	HORVU3Hr1G098660.3	Spermatogenesis-associated protein 20 isoform 2	Y

GP<sup>a</sup> and WB-1<sup>b</sup> represent the fold change between Cd treatment and control normalized reads in Golden Promise and WB-1. Fold change =  $\log_2 N$ ,  $N = \log_2$  (Cd reads/control reads). miRNAs were significantly up-regulated with fold change  $\geq 0.5$ , down-regulated with fold change  $\leq -0.5$ . <sup>c</sup>Degradome detection shows the target genes of miRNAs. Y and N indicate target gene in or not in the degradome sequencing library

## Discussion

In this study, we attempted to identify miRNAs involved in Cd tolerance, to reveal their post-transcriptional mechanisms underlying Cd tolerance in barley. Two barley genotypes (*cv.* Golden Promise and a wild accession WB-1) were grown in hydroponic culture and treated with 5  $\mu\text{M}$  Cd for 10 days. The growth performance of the wild accession WB-1 was much better than that of the cultivar Golden Promise after Cd treatment. Moreover, it was found that Cd concentration was much lower in shoots and roots of WB-1 than that in Golden Promise. These physiological parameters suggest that WB-1 had stronger Cd tolerance than Golden Promise due to its low Cd uptake (Fig. 2). On the other hand, 303 miRNAs were identified by sequencing small RNA libraries from root and shoot tissues of WB-1 and Golden Promise. Then, the miRNA expression profiles were compared between the two genotypes to figure out Cd-responsive

miRNAs in barley. Furthermore, to explore the molecular mechanism of these Cd-responsive miRNAs, target genes for these miRNAs were validated by the degradome sequencing.

Previous studies on miRNAs in response to Cd stress were studied in many crops including soybean (*Glycine max*), tobacco (*Nicotiana tabacum* L.), Chinese flowering cabbage (*Brassica parachinensis* L.) and so on (Fang et al. 2013; He et al. 2016; Zhou et al. 2017). In the present study, 40 miRNAs and 5 novel miRNAs were identified in response to Cd stress in barley roots, and 43 Cd responsive miRNAs (40 conserved and 3 novel miRNAs) were detected in barley shoots (Table 2). In addition, several miRNAs were responded differently in Golden Promise or WB-1 after Cd treatment. For instance, there were 11 miRNAs with differentially expressed levels in roots of WB-1, but they were slightly changed in roots of Golden Promise. It indicates that different genotypes may have distinct regulatory mechanisms in response to Cd stress. Similar studies



**Table 2** Differentially expressed miRNAs and their target genes in shoots of Gold Promise and WB-1 after Cd treatments

miRNAs name	Sequence	GPa	WB-1b	Target gene	Transcript annotation	Degrade detection
hvu-miR156a-3p_1	GCTCACCTCTCTCTGTC AGC	-0.54	-1.3	HORVU1Hr1G078160.1	P-loop containing nucleoside triphosphate hydrolases superfamily protein	Y
hvu-miR156a-5p	TGACAGAAGAGAGTGAGC AC	0.47	-1.04	HORVU3Hr1G094730.2	Squamosa promoter-binding-like protein 2	Y
				HORVU6Hr1G019700.2	Squamosa promoter-binding-like protein 3	Y
hvu-miR164_1	TGGAGAAGCAGGGCACGT GCG	-0.85	-0.61	HORVU5Hr1G011650.2	NAC domain containing protein 1	Y
				HORVU7Hr1G072670	NAC domain containing protein 1	Y
				HORVU5Hr1G041400.1	Phytosulfokines 2	Y
hvu-miR164a-5p	TGGAGAAGCAGGGCACGT GCT	-0.52	-0.21	HORVU5Hr1G011650.2	NAC domain containing protein 1	Y
				HORVU7Hr1G072670	NAC domain containing protein 1	Y
hvu-miR167a-5p_1	TGAAGCTGCCAGCATGAT CTA	1.87	-0.76	HORVU2Hr1G121110.32	Auxin response factor 6	Y
hvu-miR167b-3p	AGGTCATGCTGGAGTTTC ATC	-1.73	-0.2	HORVU1Hr1G075520.2	Jacalin-related lectin 3	N
hvu-miR167c-3p	ATCATGACTGACAGCCTC ATT	2.75	-1.22	HORVU5Hr1G077110.3	UDP-glucose 4-epimerase 4	N
hvu-miR167c-5p	TGAAGCTGCCAGCATGAT CTGC	1.19	0.8	HORVU1Hr1G077630.2	Ubiquitin carboxyl-terminal hydrolase 25	N
hvu-miR167f-3p	AGATCATGCTGCAGCTTC ATT	1.14	0.37	HORVU4Hr1G016990.3	Cysteine desulfurase	N
hvu-miR168-3p	CCCGCCTTGACCAAGTG AAT	-0.43	-0.56	HORVU4Hr1G031620.1	14-3-3 protein beta/alpha-A	N
				HORVU5Hr1G037570.4	Receptor-like protein kinase	N
hvu-miR169a-3p	TGGGCAAGTCACCCTGGC TACC	-3.05	-0.43	HORVU4Hr1G087430	rRNA N-glycosidase	N
hvu-miR169c-3p_1	AGGCGGTACCTTGGCTA GC	-2.81	0.43	HORVU3Hr1G108540.2	Two-component response regulator ARR8	Y
hvu-miR169c-3p_2	GGCAAGCTGTCCTTGGCT ACA	-0.1	-1.67	HORVU2Hr1G013460.2	Strictosidine synthase-like 2	N
				HORVU3Hr1G068000.2	Receptor-like protein kinase 2	N
				HORVU3Hr1G068010.1	Leucine-rich repeat receptor-like protein kinase family protein	N
				HORVU3Hr1G079010.5	Transcription factor-related	N
hvu-miR169c-5p	TAGCCAAGGATGACTTGC C	-2.06	-0.16	HORVU2Hr1G032130	Nuclear transcription factor Y subunit A-5	Y
hvu-miR169d-5p	CAGCCAAGGATGACTTGC CGG	-0.67	-1.31	HORVU2Hr1G032130	Nuclear transcription factor Y subunit A-5	Y
hvu-MIR169d-3p_1	TCCTTGGCTACACCTAGT TCT	0.24	-1.77	HORVU5Hr1G089950.4	Chromodomain-helicase-DNA-binding protein Mi-2 homolog	Y

**Table 2** (continued)

miRNAs name	Sequence	GPa	WB-1b	Target gene	Transcript annotation	Degrade detection
hvu-MIR169d-3p_2	GGTGGGTCTTCTGGCTA AC	−0.77	−1.76	HORVU4Hr1G081500.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
				HORVU4Hr1G081480.3	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
				HORVU4Hr1G081490.1	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	Y
hvu-miR169e-5p	TGCAGCCAAGGATGACTT GCC	−0.52	−0.89	HORVU6Hr1G071950.13	Zinc finger protein CON-STANS-LIKE 10	N
hvu-MIR169g-3p	TATCGGCGAGTTGTCTT GGC	0	−1.11	HORVU3Hr1G109160.2	Non-specific phospholipase C2	Y
hvu-miR169h-3p	GCAAGTTGTTCTGGCTA GC	−2.51	0.33	HORVU1Hr1G075540.3	Mitochondrial processing peptidase alpha subunit	Y
				HORVU5Hr1G108630.3	Cell division cycle 5	Y
hvu-miR169i-3p	AGGCAGTCTCCTTGGCTA GC	−0.85	−2.03	HORVU2Hr1G013680.1	Elongation factor 1-alpha	Y
hvu-miR169i-5p	TAGCCAAGGATGACTTGC CTG	−0.46	−1.01	HORVU2Hr1G032130	Nuclear transcription factor Y subunit A-5	Y
hvu-miR171-3p	TGATTGAGCCGTGCCAAT ATC	0.70	0.30	HORVU1Hr1G053510.1	GRAS family transcription factor	Y
				HORVU6Hr1G063650.1	GRAS family transcription factor	Y
hvu-miR172b-5p	GCAGCACCACCAAGATTC ACA	−1.31	0.33	HORVU6Hr1G088570.2	Clathrin interactor EPSIN 2	Y
hvu-miR319a-3p_2	TTGGACTGAAGGGTGCTC CCT	−2.35	−0.35	HORVU2Hr1G060120.1	TCP family transcription factor 4	Y
				HORVU5Hr1G103400.1	TCP family transcription factor 4	Y
hvu-miR390-5p	AAGCTCAGGAGGGATAGC GCC	−0.12	1.46	HORVU1Hr1G043790.1	Leucine-rich repeat receptor-like protein kinase family protein	N
				HORVU2Hr1G091840.16	Receptor-like protein kinase 2	N
				HORVU2Hr1G124010.6	Receptor-like kinase	N
				HORVU7Hr1G007520.1	Leucine-rich repeat receptor-like protein kinase family protein	N
hvu-miR393-5p	TTCCAAAGGGATCGCATT GAT	0.87	−0.13	HORVU1Hr1G021550.4	Transport inhibitor response 1-like protein	Y
				HORVU2Hr1G070800.3	Transport inhibitor response 1-like protein	Y
hvu-miR394-5p	TTGGCATTCTGTCCACCT CC	0.36	0.84	HORVU1Hr1G043940.3	Protein TIC110, chloroplastic	Y
				HORVU6Hr1G018370.1	Calnexin 1	Y
hvu-miR396a-5p_1	TCCAAAGGCTTTCTTGAA CTG	0.57	0.24	HORVU7Hr1G008680.14	growth-regulating factor 5	Y
hvu-miR396a-5p_2	TCCACAGGCTTTCTTGAA CTT	−0.13	0.64	HORVU4Hr1G010080.6	Growth-regulating factor 6	Y
				HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-miR396e-5p	TTCCACAGCTTTCTTGAA CTG	−0.60	−0.49	HORVU4Hr1G010080.6	Growth-regulating factor 6	Y
				HORVU7Hr1G008680.14	Growth-regulating factor 5	Y
hvu-MIR397a-3p	TCACCGGCGCTGCACGCA ATG	1.78	2.19	HORVU2Hr1G032890.2	Cytochrome P450 superfamily protein	N

**Table 2** (continued)

miRNAs name	Sequence	GPa	WB-1b	Target gene	Transcript annotation	Degrade detection
hvu-MIR397a-5p	TTGAGTGCAGCGTTGATG AAC	3.53	1.34	HORVU3Hr1G086160.1	Laccase 17	Y
hvu-miR444b_1	TGCAGTTGCTGTCTCAAG CTT	−0.3	1.88	HORVU5Hr1G000370.3	MADS-box transcription factor 25	Y
				HORVU7Hr1G066380.4	Zinc finger (C3HC4-type RING finger) family protein	Y
				HORVU2Hr1G080490.1	MADS-box transcription factor 27	Y
hvu-miR530-5p	TGCATTTGCACCTGCACC TAC	2.16	−0.82	HORVU3Hr1G075830.1	Chromosome 3B, genomic scaffold, cultivar Chinese Spring	Y
				HORVU3Hr1G075820.2	Chromosome 3B, genomic scaffold, cultivar Chinese Spring	Y
hvu-miR1122b-3p	AGACTTATATTTAGGAAC GGA	0.36	−0.68	HORVU1Hr1G093430	BnaC06g06240D protein	Y
hvu-miR5048a	TATATTTGCAGGTTTTAG GTCT	1.23	−0.78	HORVU7Hr1G065130.1	Receptor kinase 2	Y
				HORVU7Hr1G043150.1	Protein kinase superfamily protein	Y
hvu-miR5168-3p	TCGGACCAGGCTTCAATC CCT	0.50	−0.70	HORVU1Hr1G041790.2	Homeobox-leucine zipper protein family	Y
				HORVU5Hr1G061410.29	Homeobox-leucine zipper protein HOX10	Y
hvu-miR5168-5p	GGGTTGTTGTCTGGTTCA AGG	1.45	−0.46	HORVU5Hr1G123340.1	RING/U-box superfamily protein	N
				HORVU5Hr1G122660.4	Disease resistance protein	N
hvu-miR9774	CAAGATATTGGGTATTTT	1.02	0.02	HORVU5Hr1G088920.4	NADPH-cytochrome P450 reductase 2	Y
PC-miR14	TCAGTGCATCCCTCTGG AAT	0.55	−0.19	HORVU2Hr1G064140.13	Calcineurin-like metallo-phosphoesterase superfamily protein	N
				HORVU3Hr1G078110.7	E3 ubiquitin-protein ligase RING1	N
PC-miR62	TATTGGCTCGGCTCACTC AGG	−1.03	0.9	HORVU4Hr1G067060.7	Hsp70 nucleotide exchange factor fes1	N
				HORVU7Hr1G023770.19	Disease resistance protein	N
PC-miR82	TCAACAACAAGTATTATG GTAC	1.1	−0.32	HORVU4Hr1G033300.1	AAA-type ATPase family protein	N
				HORVU5Hr1G093770.4	Linoleate 9S-lipoxygenase 2-like	N

See Table 1 for more details

were reported in water spinach (*Ipomoea aquatic* Forsk.) and pakchoi (*Brassica chinensis* L.) (Shen et al. 2017a, b; Xue et al. 2014). Therefore, we hypothesized networks of Cd-responsive miRNAs in response to Cd stress in roots and shoots of barley (Supplemental Fig. S3 and S4).

Cd-responsive miRNAs could regulate transcriptional factors (TFs) and gene networks involved in ion homeostasis, plant development and metabolic processes, to enhance Cd tolerance in barley (Supplemental Fig. S3 and S4). GRAS (GAI, RGA and SCR) TFs are important for root

development, radial patterning and vascular tissue differentiation (DiLaurenzio et al. 1996; Lim et al. 2000; Pysh et al. 1999). Previously, miR171/GRAS module regulating Cd tolerance in rice and *B. napus* was reported (Ding et al. 2011; Zhou et al. 2012); while miR393/TIR 1 (transport inhibitor response 1-like protein) module was identified in auxin signaling pathway for mediating root growth and stress responses (Bai et al. 2017; Chen et al. 2011). In this study, hvu-miR171-3p and hvu-miR393-5p were up-regulated in roots of Golden Promise after Cd treatment, but

were not in WB-1, which probably caused the worse root growth in Golden Promise under Cd condition. However, hvu-miR159a targeting *HvMYB33* was up-regulated in roots of WB-1, but not for Golden Promise. MYB TFs have been reported to be associated with metal tolerance (Shen et al. 2008; Hu et al. 2017). Some miRNAs targeting important genes were involved in metal uptake and accumulation in roots. Three members of the miR166 family targeting HD-Zip (homeodomain-leucine zipper) TFs were up-regulated only in roots of WB-1. In rice, overexpression of miR166 could enhance Cd tolerance and regulate the expression of metal transporters OsHMA2 and OsHMA3 genes (Ding et al. 2018). Thus, we hypothesized that miR159a and miR166 might partially explain the mechanism of Cd tolerance in the roots of WB-1.

In shoots, hvu-miR156a-5p targeting SPL (squamosa promoter-binding-like proteins) was down-regulated in WB-1, but slightly changed in Golden Promise after Cd treatment. SPL TFs are involved in plant development (Cardon et al. 1999). Meanwhile, hvu-miR167a-5p\_1 was up-regulated in Golden Promise, but was down-regulated in WB-1 under Cd stress. The target gene of hvu-miR167a-5p\_1 encoded ARF6 (auxin response factor 6) protein, which played crucial roles in plant growth and development (Guilfoyle and Hagen 2007). Hence, these two miRNAs may be associated with the difference of shoot performance between two genotypes under Cd stress condition.

Additionally, some Cd-responsive miRNAs were identified with the same changed pattern in Golden Promise and WB-1 after Cd treatment, indicating that these miRNAs might be responsible for common adaptive responses to Cd stress in barley. For instance, miR397 was dramatically increased in roots and shoots of both genotypes after Cd treatment, as well as similar studies in (A) *thaliana* and (B) *parachinensis* (Gielen et al. 2016; Zhou et al. 2017). *LAC17* (laccase 17), as the target gene of hvu-MIR397a-5p, encodes a kind of copper-containing polyphenol oxidase, which regulates internal Cu homeostasis in plants (Bao et al. 1993; Solomon et al. 1996). Thus, decreasing gene expression of *LAC17* may enhance synthesis of essential copper-containing proteins such as SOD (Cu–Zn superoxide dismutase) and AO (ascorbate oxidase), in order to improve plant tolerance to oxidative stresses caused by Cd stress (Dixit et al. 2001; Hegedus et al. 2001; Abdel-Ghany and Pilon 2008). Hvu-miR319a-3p. 2 targeting *TCP4* (Teosinte branched1/Cycloidea/proliferating cell factor 4) was up-regulated in roots of both genotypes. Previous studies revealed that TCP was related to cell proliferation and hormone pathways in plants (Danisman et al. 2012; Sarvepalli and Nath 2011a, b; Schommer et al. 2014). Overexpression of *osa-miR319a* in creeping bentgrass (*Agrostis stolonifera*) showed more tolerance to abiotic stresses than the wild-type plants (Zhou and

Luo 2014). It was suggested that hvu-MIR397a-5p and hvu-miR319a-3p.2 probably played important roles in Cd tolerance for barley.

In conclusion, we compared the miRNAs profiles between wild and cultivated barley with contrasting Cd tolerance based on small RNA sequencing to identify 45 and 43 Cd-responsive miRNAs in roots and shoots. In comparison with cultivar Golden Promise, the wild genotype WB-1 had different responses of Cd-responsive miRNAs including miR156, miR159, miR166, miR167, miR171 and miR393, which might be related to Cd tolerance. The results could provide useful information for revealing molecular regulation mechanism of Cd tolerance in barley.

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**Authors' contributions** JH Yu and DZ Wu designed the research. JH Yu, LY Wu, LB Fu, QF Shen and LH Kuang performed the research. JH Yu, GP Zhang and DZ Wu analyzed the data. JH Yu and DZ Wu wrote the article.

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