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RESEARCH ARTICLE

# Identification of Zinc Deficiency-Responsive MicroRNAs in *Brassica juncea* Roots by Small RNA Sequencing

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## Abstract

The importance of zinc (Zn) as a micronutrient essential for plant growth and development is becoming increasingly apparent. Much of the world's soil is Zn-deficient, and soil-based Zn deficiency is often accompanied by Zn deficiency in human populations. MicroRNAs (miRNAs) play important roles in the regulation of plant gene expression at the level of translation. Many miRNAs involved in the modulation of heavy metal toxicity responses in plants have been identified; however, the role of miRNAs in the plant Zn deficiency response is almost completely unknown. Using high-throughput Solexa sequencing, we identified several miRNAs that respond to Zn deficiency in *Brassica juncea* roots. At least 21 conserved candidate miRNA families, and 101 individual members within those families, were identified in both the control and the Zn-deficient *B. juncea* roots. Among this, 15 miRNAs from 9 miRNA families were differentially expressed in the control and Zn-deficient plants. Of the 15 differentially expressed miRNAs, 13 were up-regulated in the Zn-deficient *B. juncea* roots, and only two, miR399b and miR845a, were down-regulated. Bioinformatics analysis indicated that these miRNAs were involved in modulating phytohormone response, plant growth and development, and abiotic stress responses in *B. juncea* roots. These data help to lay the foundation for further understanding of miRNA function in the regulation of the plant Zn deficiency response and its impact on plant growth and development.

Key words: Zn deficiency, *Brassica juncea*, microRNA, deep sequencing

## INTRODUCTION

Zinc (Zn) is an essential micronutrient for all organisms and serves as a cofactor for more than 300 enzymes, including Cu/Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase, alkaline phosphatase, proteases, and carbon anhydrase (Gonzalez-Guerrero *et al*. 2005). Zn is thus involved in a wide range of cellular processes, such as free radical defense, electron transport, cell proliferation, and reproduction in plants. Zn binding sites are key structural motifs in many transcriptional regulatory proteins (Kobae *et al*. 2004; Ishimaru *et al*. 2005). Therefore, Zn deficiency in plants leads to severe growth inhibition and the retardation of normal

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development. However, in recent years, Zn is becoming more apparent as a "trouble nutrient"; much of the world's soil exhibits moderate to intense Zn deficiency due to farming and natural occurrence, and this is often accompanied by Zn deficiency in humans (Jain and Brar 2010). Zn deficiency is also one of the most widespread factors limiting crop production, affecting 30% of the world's soils, including many agricultural lands in Australia, South-east Asia, Turkey, and the USA (Hacisalihoglu and Kochian 2003). Zn deficiency is known to induce the formation of longer roots, and less adventitious roots have been found in barley and wheat (Genc *et al*. 2007; Widodo *et al*. 2010). However, the detailed molecular mechanisms by which Zn deficiency modulates root system architecture (RSA) are poorly understood. Studies on the physiological and molecular mechanisms of plant responses to the stress of Zn deficiency are helpful for future research utilizing genetic approaches to improve plant Zn deficiency.

MicroRNAs (miRNAs) are 19-23-nucleotide-long noncoding, endogenous RNAs that play a regulatory role in the cell by negatively affecting gene expression at the post-transcriptional level, either by endonucleolytic cleavage or by translational inhibition (Song *et al*. 2010). Numerous studies have indicated that miRNAs play important roles in various aspects of plant development and in plant responses to abiotic stresses (Zhao *et al*. 2010). Many studies have indicated that plant miRNAs are evolutionarily conserved, suggesting that a homology-based approach using miRNAs from other species might be a powerful strategy for identifying or predicting novel plant miRNAs (Zhang *et al*. 2006; Ruan *et al*. 2009). Currently, there are several major methods used for identifying miRNAs. These include direct cloning using miRNA enriched libraries, microarray analysis and computational methods using comparative genomics. The computational method is effective in the identification of miRNAs, but is limited by the number of nucleotide sequences available in the database (Zhao *et al*. 2010). Recently developed high-throughput sequencing technologies provide a powerful approach for the identification and quantification of miRNAs (Morin *et al*. 2008; Kwak *et al*. 2009). The small RNA digitalization analysis based on Hi Seq high-throughput sequencing uses SBS-sequencing by synthesis, which can decrease the loss of nucleotides caused by the secondary structure. Benefits of this strategy include its small sample quantity requirements, its high-throughput nature, its high accuracy, and its use of a simply operated automatic platform. High-throughput sequencing technology makes it feasible to distinguish individual miRNA species that differ by only a few nucleotides (Kwak *et al*. 2009). Such analyses can obtain millions of small RNA sequence tags at one time, comprehensively identify small RNAs of certain species in certain conditions and construct the small RNA differential expression profile between samples, which could be used as a powerful tool for small RNA function research. Several studies have demonstrated the efficacy of deep sequencing in the identification of miRNAs. Deep sequencing strategies can identify miRNAs with both high sensitivity and specificity, and make it feasible to explore or annotate miRNAs in plants whose genomes are not yet sequenced (Kwak *et al*. 2009; Ruan *et al*. 2009; Song *et al*. 2010; Zhao *et al*. 2010).

Many miRNAs involved in modulating responses to heavy metal toxicity have been reported; however, miRNAs involved in the plant Zn deficiency response are almost completely unknown (Jain and Brar 2010). The identification and characterization of Zn deficiency-responsive miRNAs in plants would provide insight into the impact of Zn deficiency stresses on plant productivity. In this study, we provide the first large-scale cloning and characterization of Zn deficiency-responsive *Brassica juncea* miRNAs and their potential targets using next generation high-throughput sequencing technology and bioinformatics analysis. We identified 15 miRNAs that were differentially expressed in *B. juncea* roots under Zn deficiency. Bioinformatics analysis indicated that these miRNAs were involved in modulating auxin response, plant growth and development, and abiotic stress responses in *B. juncea* roots. Possible mechanisms for the role of these miRNAs in *B. juncea* exposed to Zn deficiency conditions are discussed.

## RESULTS AND DISCUSSION

### miRNAs detected by small RNA sequencing

Despite the important agronomy of *B. juncea*, information on the regulatory role of miRNAs in *B. juncea* is scant. Zn is a vital trace element that is known to affect the growth and stress tolerance of *B. juncea*. Several recent studies have addressed the physiological and molecular mechanisms of plant responses to Zn deficiency (Hacisalihoglu and Kochian 2003; Widodo *et al*. 2010). However, information on the role of miRNAs in the Zn deficiency response in plants is lacking. This study aimed to identify miRNA species present in *B. juncea* roots and to elucidate the miRNA response to Zn deficiency.

In this study, we identified miRNAs of *B. juncea* roots using small RNA sequencing (SRS) with Illumina-Solexa technology. A total of 18 881 933 and 21 396 825 reads in the control and the Zn deficiency-responsive *B. juncea* roots were obtained from the sequencing machine, respectively. After removing the low quality sequences and cleaning up the contaminated tags, 18 083 538 (97.96%) and 19 869 174 (94.86%) clean reads, representing 4 860 781 and 5 867 732 unique sequences, were obtained from the control and the Zn deficiency-responsive *B. juncea* roots (Fig. 1; Appendix A). The size of the small RNAs was not evenly distributed (Fig. 1). The normal length of a small RNA is between 18 and 30 nt. The length distribution analysis shows the size compositions of the small RNA samples. For example, miRNA is normally 21 or 22 nt, siRNA is normally 24 nt, and piRNA is normally 30 nt. Among these small RNAs, 28 237 479 total small RNAs (74.4%), representing 1 400 461 (15.1%) unique small RNAs, were common between the control and the treated *B. juncea* roots. Interestingly, 5 433 182 total small RNAs (14.3%), representing 4 467 271 unique small RNAs (47.8%), were specific to the Zn deficiency sample (Fig. 2).

To identify known miRNAs in *B. juncea*, all small

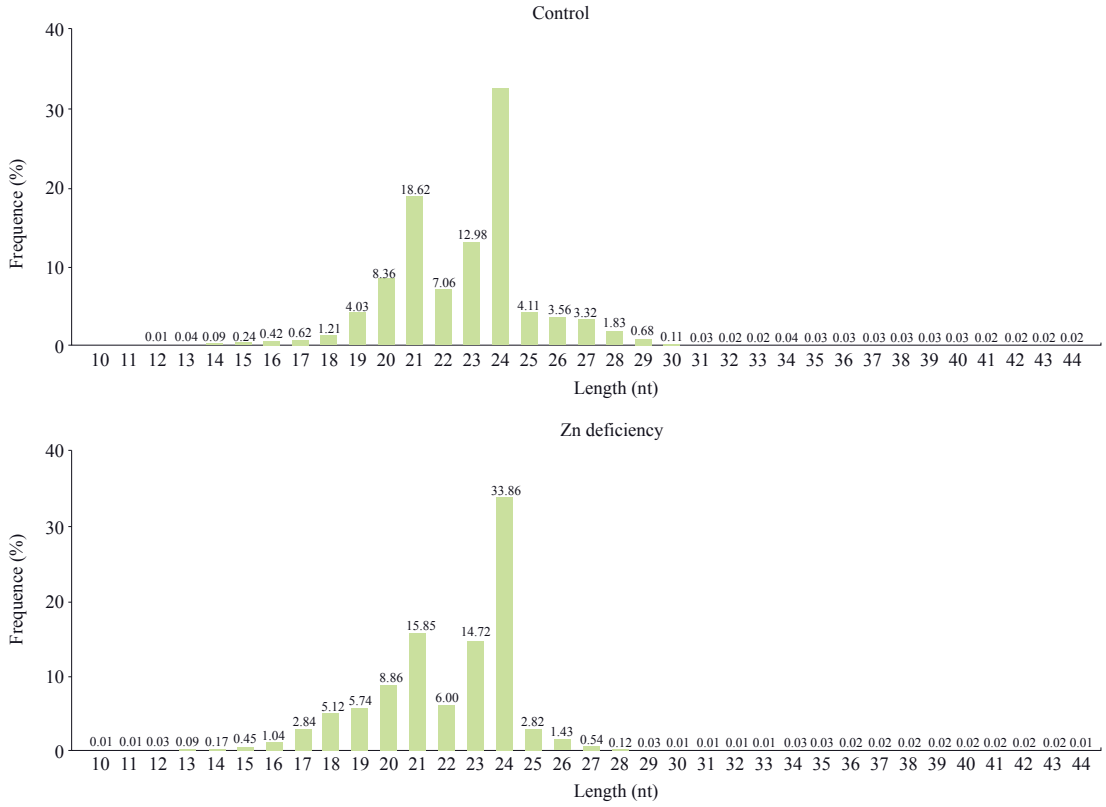
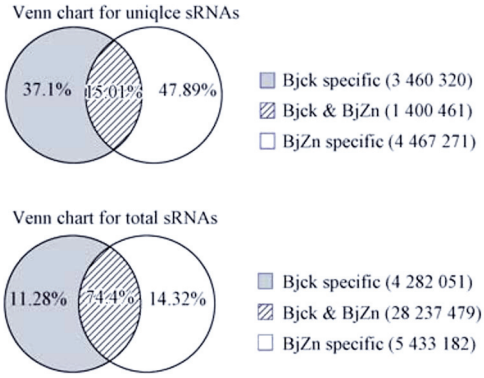



**Fig. 1** Length distribution of all small RNA tags in the two samples.




| Class | Unique sRNAs | Percent (%) | Total sRNAs | Percent (%) |
|---|---|---|---|---|
| Total_sRNAs | 9 328 052 | 1 | 37 952 712 | 1 |
| Ck & Zn | 1 400 461 | 0.1501 | 28 237 479 | 0.744 |
| Ck specific | 3 460 320 | 0.371 | 4282 051 | 0.1128 |
| Zn specific | 4 467 271 | 0.4789 | 5433 182 | 0.1432 |

**Fig. 2** Summary of common and specific small RNA sequences between control and Zn deficiency-treated *B. juncea* roots.

RNA sequences were blastn searched against the currently known miRNAs in the miRNA database (http://www.mirbase.org/). The known miRNA count, as well as base bias on the first position of identified miRNAs with certain length and on each position of all identified miRNAs, were also obtained by aligning small RNA to the mature miRNA (Appendices B and C). After Blastn searches and further sequence analysis, a total of 98 conserved miRNAs in control plants and 96 conserved miRNAs in Zn-deficient plants, belonging to at least 21 miRNA families, were identified in *B. juncea* roots (Appendix D). MiR156/157 dominated in both libraries and accounted for 72.2% (control) and 76.6% (treated) of all conserved miRNA reads. Several miRNA families such as miR159, 164, 165/166, 167, 168, and 172 had a moderate abundance of expression. In contrast, some miRNA families showed very low abundance of expression in roots and were identified with only several read counts. Families of *B. juncea* miRNAs are also diverse with respect to the number of members they contain. The largest miRNA family sizes identified were miR169 (15 members), miR156/157 (12 members), and miR165/166 (9 members). Several miRNA families (miR403, 408, 824, 845, and 858) had only one member detected in *B. juncea* roots. Most of the identified miRNA families have been shown to be conserved in a variety of plant species using a comparative genomics-based strategy.

Next-generation high-throughput sequencing also provides an alternative way to estimate the expression profiles of miRNA genes (Yao *et al*. 2007; Zhao *et al*. 2010). The number of reads reflects the level of miRNA enrichment. Fifteen miRNAs from 9 miRNA families were differentially expressed in the control and the Zn-deficient plants (Table 1, Fig. 3 and Appendix E). Among these 15 differentially expressed miRNAs, 13 miRNAs (miR158b, 160a, 160b, 160c, 169g*, 319a, 319b, 319c, 393a, 393b, 394a, 394b, and 398a) were upregulated in the Zn-deficient state; only 2 miRNAs (miR399b and miR845a) were downregulated in Zn deficiency (Table 1 and Appendix F, Fig. 3). Interestingly, the highly enriched miR156/157 and miR165/166 families were found at similar levels in both samples. We also found that different family members had vastly different expression levels. For example, the abundance of miR156/157 family members varied from 595 sequencing reads (miR156h) to 785 365 sequencing reads (miR157b). The existence of one or several dominant members in a miRNA family may suggest that this family's regulatory role may be primarily performed by the dominant members.

To validate the existence of the known and novel miRNAs, we tested the expression of 5 selected miRNAs by qRT-PCR. These miRNAs were significantly differentially expressed in the control and Zn-deficient samples. The results of the qRT-PCR analysis are shown in Fig. 4. This pattern of expression was consistent with our Solexa sequencing results.

## Target predictions for differentially expressed miRNAs

To better understand the functions of the identified *B. juncea* miRNAs and their potential roles in the Zn deficiency response, putative targets of these miRNAs were predicted using the described criteria and methods. The target genes of 15 miRNAs were predicted (Table 2). Bioinformatics analysis indicated that these miRNAs were involved in modulating phytohormone response, plant growth and development, and abiotic

**Table 1** The miRNA identified in control and Zn deficiency-treated *B. juncea* roots

| miR-name | Ck-std | Zn(-)-std | Fold-change (log2 Zn(-)/ck) | *P*-value | Sig-lable | Sequence (5′→3′) |
|---|---|---|---|---|---|---|
| Bj-miR156a | 30 099.586 | 27 705.883 | -0.1195513 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156b | 30 102.516 | 27 709.104 | -0.11952405 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156c | 30 099.586 | 27 705.832 | -0.11955392 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156d | 30 953.788 | 28 624.491 | -0.11286591 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156e | 30 050.204 | 27 659.882 | -0.11957979 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156f | 30 048.987 | 27 657.818 | -0.11962901 | 0 | | TGACAGAAGAGAGTGAGCAC |
| Bj-miR156g | 1 213.2582 | 814.1254 | -0.57556368 | 0 | | CGACAGAAGAGAGTGAGCAC |
| Bj-miR156h | 37.1609 | 29.9459 | -0.31142695 | 0.0001233 | | TTGACAGAAGAAAGAGAGCAC |
| Bj-miR157a | 40 348.576 | 39 526.152 | -0.02971022 | 9.41E-37 | | TTGACAGAAGATAGAGAGCAC |
| Bj-miR157b | 40 349.073 | 39 526.807 | -0.02970413 | 9.72E-37 | | TTGACAGAAGATAGAGAGCAC |
| Bj-miR157c | 40 299.802 | 39 580.508 | -0.02598262 | 1.66E-28 | | TTGACAGAAGATAGAGAGCAC |
| Bj-miR157d | 461.1376 | 429.6102 | -0.10216906 | 4.25E-06 | | TGACAGAAGATAGAGAGCAC |
| Bj-miR158a | 347.3325 | 474.0006 | 0.44857147 | 1.52E-82 | | TCCCAAATGTAGACAAAGCA |
| Bj-miR158b | 0.9954 | 3.1204 | 1.64838269 | 4.11E-06 | ** | CCAAATGTAGACAAAGCA |
| Bj-miR159a | 346.7242 | 521.612 | 0.58918853 | 1.40E-147 | | TTTGGATTGAAGGGAGCTCTA |
| Bj-miR159b | 20.6818 | 34.2239 | 0.72664242 | 1.57E-15 | | TTTGGATTGAAGGGAGCTCT |
| Bj-miR159c | 4.3686 | 7.4991 | 0.77954645 | 7.66E-05 | | TTTGGATTGAAGGGAGCTC |
| Bj-miR160a | 25.4928 | 93.7633 | 1.87893351 | 1.94E-171 | ** | ATGCCTGGCTCCCTGTATGCC |
| Bj-miR160b | 6.5806 | 74.9905 | 3.51041681 | 7.70E-275 | ** | TGCCTGGCTCCCTGTATGCCA |
| Bj-miR160c | 25.4928 | 93.2097 | 1.87039026 | 2.09E-169 | ** | ATGCCTGGCTCCCTGTATGCC |
| Bj-miR162a | 105.455 | 94.7196 | -0.15489261 | 0.0009517 | | TCGATAAACCTCTGCATCCAG |
| Bj-miR162b | 105.3997 | 94.7196 | -0.15413587 | 0.0010096 | | TCGATAAACCTCTGCATCCAG |
| Bj-miR164a | 644.5088 | 496.85 | -0.37538969 | 4.99E-81 | | TGGAGAAGCAGGGCACGTGCA |
| Bj-miR164b | 649.7069 | 500.1718 | -0.37736531 | 1.87E-82 | | TGGAGAAGCAGGGCACGTGCA |
| Bj-miR164c | 756.2679 | 445.3633 | -0.76391471 | 0 | | TGGAGAAGCAGGGCACGTGCG |
| Bj-miR165a | 4 552.9254 | 3 109.2385 | -0.55023254 | 0 | | TCGGACCAGGCTTCATCCCCC |
| Bj-miR165b | 4 543.3034 | 3 100.4308 | -0.55127296 | 0 | | TCGGACCAGGCTTCATCCCCC |
| Bj-miR166a | 7 534.5875 | 5 897.9301 | -0.35331981 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166b | 7 379.9165 | 5 778.5996 | -0.35288458 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166c | 7 374.1101 | 5 775.2275 | -0.35259118 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166d | 7 374.3866 | 5 775.2778 | -0.35263271 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166e | 7 380.0824 | 5 781.0154 | -0.35231401 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166f | 7 379.6953 | 5 780.8644 | -0.35227602 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR166g | 7 374.9949 | 5 777.9453 | -0.35208551 | 0 | | TCGGACCAGGCTTCATTCCCC |
| Bj-miR167a | 12 007.606 | 9 413.7783 | -0.35110276 | 0 | | TGAAGCTGCCAGCATGATCTA |
| Bj-miR167b | 12 006.555 | 9 411.3625 | -0.35134681 | 0 | | TGAAGCTGCCAGCATGATCTA |
| Bj-miR167c | 286.8355 | 200.26 | -0.5183493 | 7.69E-66 | | TAAGCTGCCAGCATGATCTTGT |
| Bj-miR167d | 158.7079 | 119.6829 | -0.4071569 | 1.88E-24 | | TGAAGCTGCCAGCATGATCT |
| Bj-miR168a | 11 352.701 | 7 566.847 | -0.5852714 | 0 | | TCGCTTGGTGCAGGTCGGGAA |
| Bj-miR168b | 11 413.309 | 7 603.7383 | -0.58593629 | 0 | | TCGCTTGGTGCAGGTCGGGAA |
| Bj-miR169a | 9.069 | 14.2432 | 0.65125793 | 3.08E-06 | | CAGCCAAGGATGACTTGCCGA |
| Bj-miR169b | 26.3776 | 42.0752 | 0.67365682 | 1.31E-16 | | CAGCCAAGGATGACTTGCCGG |
| Bj-miR169c | 26.267 | 40.2634 | 0.61621757 | 7.60E-14 | | CAGCCAAGGATGACTTGCCGG |
| Bj-miR169d | 17.198 | 13.2869 | -0.37223625 | 0.0020072 | | TGAGCCAAGGATGACTTGCCG |
| Bj-miR169e | 17.198 | 13.2869 | -0.37223625 | 0.0020072 | | TGAGCCAAGGATGACTTGCCG |
| Bj-miR169f | 18.9675 | 15.4007 | -0.30053361 | 0.0080004 | | TGAGCCAAGGATGACTTGCCG |
| Bj-miR169g | 18.8569 | 15.3001 | -0.30155144 | 0.0079817 | | TGAGCCAAGGATGACTTGCCG |
| Bj-miR169g* | 0.8848 | 2.9694 | 1.74674816 | 2.90E-06 | ** | GCAAGTTGACCTTGGCTCTGT |
| Bj-miR169h | 0.7742 | 1.1072 | 0.51613764 | 0.30284 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169i | 0.7742 | 1.1576 | 0.58035861 | 0.2407497 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169j | 0.7189 | 1.0569 | 0.55597587 | 0.282008 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169k | 0.7742 | 1.1072 | 0.51613764 | 0.30284 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169l | 0.7189 | 1.0569 | 0.55597587 | 0.282008 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169m | 0.8295 | 1.1072 | 0.41660196 | 0.3980452 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR169n | 0.7189 | 1.0569 | 0.55597587 | 0.282008 | | TAGCCAAGGATGACTTGCCTG |
| Bj-miR171c | 1.659 | 2.1138 | 0.34952499 | 0.3155621 | | TGATTGAGCCGTGCCAATATC |
| Bj-miR172a | 1 072.4118 | 566.405 | -0.92095309 | 0 | | AGAATCTTGATGATGCTGCAT |
| Bj-miR172b | 1 072.4118 | 566.4554 | -0.92082472 | 0 | | AGAATCTTGATGATGCTGCAT |
| Bj-miR172b* | 28.0918 | 18.6721 | -0.58926488 | 1.72E-09 | | AGAATCTTGATGATGCTGCAT |
| Bj-miR172c | 25.0504 | 15.6021 | -0.68309342 | 8.76E-11 | | AGAATCTTGATGATGCTGCAG |
| Bj-miR172d | 24.4974 | 15.4511 | -0.66491909 | 3.76E-10 | | AGAATCTTGATGATGCTGCAG |
| Bj-miR172e | 52.4787 | 29.2413 | -0.84372453 | 1.54E-29 | | GGAATCTTGATGATGCTGCAT |
| Bj-miR2111a | 0.9401 | 1.2079 | 0.36161489 | 0.4386833 | | TAATCTGCATCCTGAGGTTTA |
| Bj-miR2111b | 0.9401 | 1.2079 | 0.36161489 | 0.4386833 | | TAATCTGCATCCTGAGGTTTA |
| Bj-miR319a | 0.7742 | 5.3349 | 2.78468302 | 4.62E-17 | ** | TTGGACTGAAGGGAGCTCCCT |

(Continued on next page)

**Table 1** (Continued from preceding page)

| miR-name | Ck-std | Zn(-)-std | Fold-change (log2 Zn(-)/ck) | *P*-value | Sig-lable | Sequence (5′→3′) |
|---|---|---|---|---|---|---|
| Bj-miR319b | 0.8295 | 5.1336 | 2.629657 | 1.43E-15 | ** | TTGGACTGAAGGGAGCTCCCT |
| Bj-miR319c | 0.7189 | 1.7615 | 1.29294147 | 0.0041137 | ** | TTGGACTGAAGGGAGCTCCTT |
| Bj-miR390a | 31.8522 | 30.8518 | -0.04603836 | 0.5813986 | | AAGCTCAGGAGGGATAGCGCC |
| Bj-miR390b | 31.4098 | 29.5433 | -0.08838377 | 2.97E-01 | | AAGCTCAGGAGGGATAGCGCC |
| Bj-miR391 | 1.2166 | 1.5099 | 0.31159809 | 4.50E-01 | | TCGCAGGAGAGATAGCGCCAT |
| Bj-miR393a | 0.1106 | 1.4595 | 3.72205092 | 8.942E-07 | ** | TCCAAAGGGATCGCATTGATCC |
| Bj-miR393b | 0.1106 | 1.4595 | 3.72205092 | 8.942E-07 | ** | TCCAAAGGGATCGCATTGATCC |
| Bj-miR394a | 0.0553 | 2.2648 | 5.35596036 | 5.40E-12 | ** | TTTGGCATTCTGTCCACCTCC |
| Bj-miR394b | 0.0553 | 2.2648 | 5.35596036 | 5.40E-12 | ** | TTTGGCATTCTGTCCACCTCC |
| Bj-miR395a | 2.0461 | 2.3655 | 0.2092685 | 0.5155399 | | CTGAAGTGTTTGGGGGAACTC |
| Bj-miR395b | 1.0507 | 0.5536 | -0.92443496 | 0.0883947 | | CTGAAGTGTTTGGGGGGACTC |
| Bj-miR395c | 1.0507 | 0.5536 | -0.92443496 | 8.84E-02 | | CTGAAGTGTTTGGGGGGACTC |
| Bj-miR395d | 2.0461 | 2.3655 | 0.2092685 | 5.16E-01 | | CTGAAGTGTTTGGGGGAACTC |
| Bj-miR395e | 1.9908 | 2.3655 | 0.24879687 | 0.442006 | | CTGAAGTGTTTGGGGGAACTC |
| Bj-miR395f | 1.0507 | 0.5536 | -0.92443496 | 0.0883947 | | CTGAAGTGTTTGGGGGGACTC |
| Bj-miR396a | 34.9489 | 26.1209 | -0.42004244 | 8.26E-07 | | TTCCACAGCTTTCTTGAACTG |
| Bj-miR396b | 2.4885 | 1.3086 | -0.92725221 | 8.08E-03 | | TTCCACAGCTTTCTTGAACTT |
| Bj-miR397a | 11.7787 | 10.2672 | -0.19813753 | 0.1605558 | | TCATTGAGTGCAGCGTTGATGT |
| Bj-miR398a | 0.2212 | 1.6105 | 2.86408537 | 4.128E-06 | ** | TGTGTTCTCAGGTCACCCCTT |
| Bj-miR399b | 3.4838 | 1.7112 | -1.02565342 | 6.42E-04 | ** | TGCCAAAGGAGAGTTGCCCTG |
| Bj-miR399c | 3.5391 | 1.8119 | -0.96587919 | 1.06E-03 | | TGCCAAAGGAGAGTTGCCCTG |
| Bj-miR399f | 1.2166 | 1.4092 | 0.21202147 | 0.6153641 | | TGCCAAAGGAGATTTGCCCGG |
| Bj-miR400 | 2.5438 | 3.523 | 0.46981923 | 0.0859768 | | TATGAGAGTATTATAAGTCAC |
| Bj-miR403 | 152.8462 | 123.7092 | -0.30512789 | 2.22E-14 | | TTAGATTCACGCACAAACTCG |
| Bj-miR408 | 14.1565 | 21.8429 | 0.62569979 | 2.41E-08 | | ATGCACTGCCTCTTCCCTGGC |
| Bj-miR824 | 277.2688 | 250.2872 | -0.14770077 | 3.136E-07 | | TAGACCATTTGTGAGAAGGGA |
| Bj-miR845a | 2.5438 | 1.1576 | -1.13584842 | 0.0015589 | ** | CGGCTCTGATACCAATTGATG |
| Bj-miR858 | 3.5944 | 2.6171 | -0.45778192 | 8.74E-02 | | TTTCGTTGTCTGTTCGACCTT |

-std, normalized expression level of miRNA in a sample; sig-lable **, fold change (log2)>1 or fold change (log2)<-1, and *P*-value<0.01.

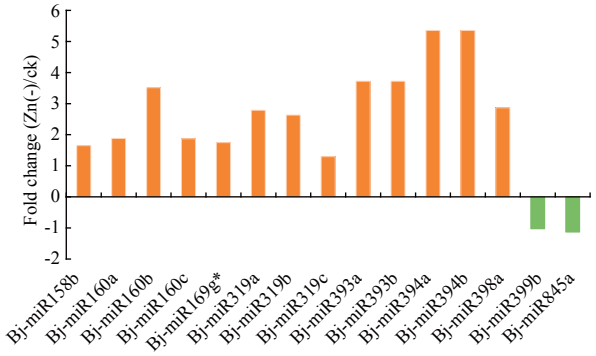



**Fig. 3** Differentially expressed miRNAs in *B. juncea* roots.

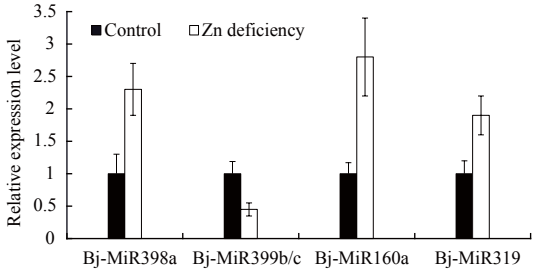



**Fig. 4** qRT-PCR analysis of five miRNAs in *B. juncea* roots.

**Table 2** Potential targets of the differentially expressed miRNAs in *B. juncea* roots

| miR-ID | Targets |
|---|---|
| Bj-miR158b | FUT1 |
| Bj-miR160a | Auxin response factors |
| Bj-miR160b | Auxin response factors |
| Bj-miR160c | Auxin response factors |
| Bj-miR169g* | CCAAT-binding transcription factors |
| Bj-miR319a | TCP transcription factors |
| Bj-miR319b | TCP transcription factors |
| Bj-miR319c | TCP transcription factors |
| Bj-miR393a | Basic helix-loop-helix (bHLH) transcription factor auxin receptors TIR1, AFB1, AFB2, and AFB3 |
| Bj-miR393b | Basic helix-loop-helix (bHLH) transcription factor auxin receptors TIR1, AFB1, AFB2, and AFB3 |
| Bj-miR394a | F-box transcriptional factor |
| Bj-miR394b | F-box transcriptional factor |
| Bj-miR398a | Cu/Zn SOD |
| Bj-miR399b | PHO2 |
| Bj-miR845a | - |

stress responses in *B. juncea* roots.

Among the 15 differentially expressed known miRNAs, only miR399b and miR845a were downregulated in the Zn-deficient *B. juncea* roots. The miR399 family is involved in systemic Pi-starvation signaling that is important for maintenance of Pi homeostasis in plants. Aung *et al*. (2006)

demonstrated that miR399 repressed the expression of target gene *PHO2*, an ubiquitin-conjugating E2 enzyme 24 which inhibited Pi uptake and accumulation. Therefore, expression of miR399 is advantageous for P uptake in plants. However, although the expression of miR399 was shown to be downregulated, the accumulation of P was increased in Zn-deficient roots. Huang *et al*. (2000) demonstrated that Zn deficiency induced the expression of *HVPT1/2* high-affinity P transporters in barley roots. Further study is needed to elucidate the role that these P transporters and P assimilation regulators have on P accumulation in Zn-deficient plants and their interaction with miR399.

The predicted target gene of miR319 was a subset of *TCP* transcription factors. *TCPs* control biosynthesis of the hormone jasmonic acid and thereby affect plant growth and development processes, especially in leaf (Nath *et al*. 2003). Three miR319 genes, miR319a, 319b, and 319c, were also upregulated in the Zn-deficient roots, consistent with the observed retarded growth phenotype. Predicted target genes of miR160 included *auxin response factors* (*ARFs*). miR393 targets transcripts that code for a *basic helix-loop-helix* (*bHLH*) transcription factor and for the auxin receptors *TIR1*, *AFB1*, *AFB2*, and *AFB3*. The target gene of miR394 was an F-box transcriptional factor. Three miR160 genes (miR160a, 160b, and 160c), two miR394 genes (miR394a and 394b), and two miR393 genes (miR393a and 393b), were significantly upregulated in the Zn-deficient roots. Auxin is a key phytohormone, mediating growth and developmental responses in plants. Zn deficiency affects primary root growth and lateral root formation in plants. These results suggest that Zn deficiency remodeled the auxin reception and signal transduction pathway by upregulating the expression of certain miRNA families including miR393, miR394 and miR160.

miR398 was confirmed to target copper/zinc superoxide dismutase 1 (CSD1) and copper/zinc superoxide dismutase 2 (CSD2), and has been shown to be downregulated under conditions of oxidative stress (Bonnet *et al*. 2004; Sunkar *et al*. 2005; Jones-Rhoades and Bartel 2006). Downregulation of miR398 is accompanied by an accumulation of *CSD1* and *CSD2* transcripts (Sunkar *et al*. 2005). These studies demonstrated that the downregulation of *CSDs* genes under stress conditions did not result from stress-related transcriptional induction of the *CSD* genes, but rather from the relaxation of miR398-directed cleavage. Yamasaki *et al*. (2007) demonstrated that copper (Cu) deficiency induces miR398, which then negatively regulates the translation of Cu/Zn SOD proteins. The Cu/Zn SOD was replaced by Fe SOD in plants (Lewis *et al*. 2010). This result indicates that miR398 mediates the level of free Cu in plants (Lewis *et al*. 2010). Like Cu, Zn is a vital element involved in Cu/Zn SOD biosynthesis. Consistent with the results for Cu deficiency, we found that Zn deficiency induces the upregulation of miR398a in *B. juncea* roots, implying that a similar pathway might exist in Zn-deficient plants. This putative pathway merits further study.

miR169 targets the CCAAT-binding transcription factors in plants. Zhao *et al*. (2007) found that two ABA-independent dehydration-responsive elements up-stream of the promoter region of the gene encode miR169g, suggesting that miR169g is involved in the ABA-independent stress response process. Li *et al*. (2008) showed that the *NFYA5* transcription factor is targeted by miR169. Overexpression of miR169 leads to excessive water loss through leaves and hypersensitivity to drought stress in *A. thaliana*. In this study, we found that the expression of miR169g* was upregulated in *B. juncea* roots subjected to Zn deficiency stress. The results indicate that miR169g* is involved in Zn deficiency-induced dehydration in plants. Further study of the function of these miRNAs could add new insights into the impact of Zn deficiency and other abiotic stresses on plant growth and development.

## MATERIALS AND METHODS

### Plant materials and growth conditions

To obtain seedlings, the seeds of *B. juncea* were sown under sterile conditions in Petri dishes containing 1/2 MS medium (Murashige and Skoog 1962) and solidified with 0.8% (w/v) agar (Sigma, St. Louis, MO, USA). 7-d-old seedlings were transferred into Hoagland solution (Hoagland and Arnon 1950) for plant growth in a sterilized green house. Cultures were maintained at 22-25°C under a 16-h photoperiod. 3-wk-old *B. juncea* seedlings were transferred to Hoagland

solution with or without $ZnCl_2$ for 7 d.

## Isolation of small RNAs, library preparationand deep sequencing

Total RNAs were extracted from control and Zn deficiency-treated *B. juncea* roots using Trizol (Invitrogen). Each RNA sample from three independent biological replicates was mixed with 2× loading buffer and then subjected to 15% denaturing polyacrylamide gel electrophoresis, after which the small RNA fragments of 18-28 nt were isolated from the gel and purified. Then the small RNAs were ligated sequentially to a 5′ adaptor and a 3′ adaptor and converted to DNA by RT-PCR (Liang *et al*. 2010). Approximately 20 μg of RT-PCR products were sequenced directly using the Solexa 1G genome analyzer according to the manufacturer's protocols (Shenzhen BGI, China). The experiment experimental processing of small RNA sequencing is shown in Appendix G.

The 50 nt sequence tags from Hi Seq sequencing first went through data cleaning, where low quality tags and several kinds of contaminants were excluded from the 50 nt tags. Length distributions of the cleaned tags were then summarized. The whole process is shown in Appendix H.

## miRNA prediction

The prediction of potential miRNAs and their precursor sequences was accomplished using criteria that were previously developed for plant miRNA prediction (Allen *et al*. 2005). This prediction strategy was implemented in the Mireap program developed by the BGI (Beijing Genome Institute, China). Mireap can be accessed from the following link: http://sourceforge.net/projects/mireap/. The known miRNA expression between two samples were compared in order to identify any differentially expressed miRNAs.

## Procedures for comparing the miRNA expression between two samples

Normalize the expression of miRNA in two samples (control and treatment) to get the expression of transcript per million (TPM). Normalization formula: Normalized expression= Actual miRNA count/Total count of clean reads×1 000 000.

Calculate fold-change and *P*-value from the normalized expression. Then generate the log2 ratio plot and scatter plot. Fold-change formula: Fold change=log2(treatment/control). *P*-value formula:

$$P(x|y)=\left(\frac{N_2}{N_1}\right)^{y}\frac{(x+y)!}{x!y!\left(1+\frac{N_2}{N_1}\right)^{(x+y+1)}}$$

$$C(y\leqslant y_{\min}|x)=\sum_{y=0}^{y\leqslant y_{\min}}p(y|x)$$

$$D(y\geqslant y_{\max}|x)=\sum_{y\geqslant y_{\max}}^{\infty}p(y|x)$$

## Mature miRNA stem-loop qRT-PCR

Total RNAs were extracted using TRIzol reagent (Invitrogen, USA) as described above. However, ethanol washes were avoided and nucleic acid precipitation steps were performed using 1:1 (v:v) isopropanol and 1:10 (v:v) 3 mol $L^{-1}$ sodium acetate (pH 5.2) to optimize small RNA molecule retrieval (Ravet *et al*. 2011). The stem-loop qRT-PCR was performed as previously described (Chen *et al*. 2005). Specific primers for each gene are listed in Appendix D. All RT-qPCRs were replicated three times using templates prepared from three independent samples.

## Target gene predictions

The rules used for target prediction are based on those suggested by Allen *et al*. (2005) and Schwab *et al*. (2005): (1) no more than four mismatches between sRNA & target (G-U bases count as 0.5 mismatches); (2) no more than two adjacent mismatches in the miRNA/target duplex; (3) no adjacent mismatches in positions 2-12 of the miRNA/target duplex (5′ of miRNA); (4) no mismatches in positions 10-11 of miRNA/target duplex; (5) no more than 2.5 mismatches in positions 1-12 of the of the miRNA/target duplex (5′ of miRNA); (6) minimum free energy (MFE) of the miRNA/target duplex should be ⩾75% of the MFE of the miRNA bound to it's perfect complement.

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**Appendix** associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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