Boron-deficiency-responsive microRNAs and their targets in *Citrus sinensis* leaves

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**Abstract**

**Background:** MicroRNAs play important roles in the adaptive responses of plants to nutrient deficiencies. Most research, however, has focused on nitrogen (N), phosphorus (P), sulfur (S), copper (Cu) and iron (Fe) deficiencies, limited data are available on the differential expression of miRNAs and their target genes in response to deficiencies of other nutrient elements. In this study, we identified the known and novel miRNAs as well as the boron (B)-deficiency-responsive miRNAs from citrus leaves in order to obtain the potential miRNAs related to the tolerance of citrus to B-deficiency.

**Methods:** Seedlings of 'Xuegan' [*Citrus sinensis* (L.) Osbeck] were supplied every other day with B-deficient (0 μM H₃BO₃) or -sufficient (10 μM H₃BO₃) nutrient solution for 15 weeks. Thereafter, we sequenced two small RNA libraries from B-deficient and -sufficient (control) citrus leaves, respectively, using Illumina sequencing.

**Results:** Ninety one (83 known and 8 novel) up- and 81 (75 known and 6 novel) down-regulated miRNAs were isolated from B-deficient leaves. The great alteration of miRNA expression might contribute to the tolerance of citrus to B-deficiency. The adaptive responses of miRNAs to B-deficiency might related to several aspects: (a) attenuation of plant growth and development by repressing auxin signaling due to decreased TIR1 level and ARF-mediated gene expression by altering the expression of miR393, miR160 and miR3946; (b) maintaining leaf phenotype and enhancing the stress tolerance by up-regulating NACs targeted by miR159, miR782, miR3946 and miR7539; (c) activation of the stress responses and antioxidant system through down-regulating the expression of miR164, miR6260, miR5929, miR6214, miR3946 and miR3446; (d) decreasing the expression of major facilitator superfamily protein genes targeted by miR5037, thus lowering B export from plants. Also, B-deficiency-induced down-regulation of miR408 might play a role in plant tolerance to B-deficiency by regulating Cu homeostasis and enhancing superoxide dismutase activity.

**Conclusions:** Our study reveals some novel responses of citrus to B-deficiency, which increase our understanding of the adaptive mechanisms of citrus to B-deficiency at the miRNA (post-transcriptional) level.

**Keywords:** Boron-deficiency, *Citrus sinensis*, Illumina sequencing, Leaves, MicroRNA

**Background**

Boron (B), an essential micronutrient for normal growth and development of plants, is involved in a series of important physiological functions, including the structure of cell walls, membrane integrity, cell division, phenol metabolism, protein metabolism and nucleic acid metabolism during growth and development of higher plants [1–5]. B-deficiency widely exists in many agricultural crops, including citrus. In China, B-deficiency is frequently observed in citrus orchards, and often contributes to the loss of productivity and poor fruit quality [3]. Li et al. reported that up to 9.0 % and 43.5 % of ‘Guanximiyou’ pummelo (*Citrus grandis*) orchards in Pinghe, Zhangzhou, China were deficient in leaf B and soil water-soluble B, respectively [6].

In plants, approx. 21-nucleotide-long microRNAs (miRNAs), one of the most abundant classes of non-coding small RNAs (sRNAs), are crucial post-transcriptional regulators of gene expression by repressing translation or directly degrading mRNAs in plants [7]. Evidence shows that miRNAs play key roles in plant response to nutrient deficiencies, including boron deficiencies.
deficiencies [8–13]. Identification of nutrient-deficiency-responsive miRNAs and their target genes has become one of the hottest topics in plant nutrition.

Plants have developed diverse strategies to maintain phosphorus (P) homeostasis, including miRNA regulations [11, 12]. MiR399, which is specifically induced by P-deficiency in Arabidopsis and rice, can regulate P homeostasis by negatively regulating its target gene UBC24 [13, 14]. Like miR399, miR827 is also highly and specifically induced by P-deficiency and is involved in the regulation of plant P homeostasis by down-regulating its target gene nitrogen limitation adaptation (NLA) in Arabidopsis [13]. In addition, many other P-deficiency-responsive miRNAs (i.e., miR1510, miR156, miR159, miR166, miR169, miR2109, miR395, miR397, miR398, miR408, miR447 and miR482) have been isolated from various plant species [15–21].

MiR397, miR398, miR408, and miR857, which are induced by copper (Cu)-deficiency, have been shown to play a role in the regulation of Cu homeostasis by down-regulating genes encoding nonessential Cu proteins such as Cu/Zn superoxide dismutase (SOD), laccases and plantacyanin, hence saving Cu for other essential Cu proteins such as plastocyanin, which is essential for photosynthesis [10, 22, 23].

In Arabidopsis, leaf miR395 was induced by sulfur (S)-deficiency. MiR395 targets ATP sulfurylases (APS) and sulfate transporter 2;1 (SULTR2;1), both of which are involved in the S metabolism. Their transcripts are greatly down-regulated in miR395-over-expressing transgenic Arabidopsis accompanied by increased accumulation of S in the shoot but not in the root. They concluded that miR395 play a role in the regulation of plant S accumulation and allocation by targeting APS and SULTR2;1 [24].

MiRNAs have been shown to play a role in the adaptation of plants to Fe-deficiency. Eight Fe-deficiency-responsive conserved miRNAs from five families had been identified in Arabidopsis roots and shoots and their expression profiles differed between the two organs [25]. Valdés-López et al. isolated ten up- and four down-regulated miRNAs, five up- and six down-regulated miRNAs, and seven up- and four down-regulated miRNAs from the leaves, roots and nodules of Fe-deficient common bean [17]. Waters et al. obtained eight differentially expressed miRNAs from seven conserved families in the rosettes of Fe-deficient Arabidopsis. Interestingly, Fe-deficiency led to increased accumulation of Cu in rosettes and decreased expression levels of miR397a, miR398a and miR398b/c, which regulate the mRNA levels of genes encoding Cu-containing proteins, implying a links between Fe-deficiency with Cu homeostasis [26].

Many N-deficiency-responsive miRNAs have been identified from Arabidopsis, soybean, maize and common bean. These miRNAs belong to at least 27 conserved families [10, 17, 27, 28]. In Arabidopsis, the expression of miR169 was inhibited by N-deficiency, while the expression levels of its target genes [i.e., NFYA2 (Nuclear Factor Y, subunit A2), NFYA3, NFYA5 and NFYA8] were increased [10, 13, 27, 29]. Transgenic Arabidopsis plants over-expressing miR169a had less accumulation of N and NFYA family members, and were more sensitive to N stress than the wild type, demonstrating a role for miR169 in the adaptation of plants to N-deficiency [29]. It is worth noting that some N-deficiency-responsive miRNAs (e.g., miR169, miR172, miR394, miR395, miR397, miR398, miR399, miR827, miR408 and miR857) are also responsive to other nutrient stresses (i.e., B, P, Fe, S and Cu deficiencies) in plants [8, 10], indicating the involvement of miRNA-mediated crosstalk among N, B, P, Fe, S and Cu under N-deficiency.

An increasing number of nutrient-deficiency-responsive miRNAs have been identified with different techniques [8–14]. Most research, however, has focused on N, P, S, Cu and Fe deficiencies, limited data are available on the differential expression of miRNAs and their target genes in response to deficiencies of other nutrient elements. Recently, we investigated miRNA expression profiles in response to B-deficiency in Citrus sinensis roots by Illumina sequencing and identified 134 (112 known and 22 novel) B-deficiency-responsive miRNAs, suggesting the possible roles of miRNAs in the tolerance of citrus plants to B-deficiency [8]. Previous studies showed that the responses of miRNAs to nutrient deficiencies differed between plant roots and shoots (leaves) [12, 17, 25]. In addition, there were great differences in B-deficiency-induced changes in major metabolites, activities of key enzymes involved in organic acid and amino acid metabolism, gas exchange and gene expression profiles between roots and leaves of C. sinensis [4, 30]. Therefore, B-deficiency-induced changes in miRNA expression profiles should be different between citrus roots and leaves.

In this study, we sequenced two small RNA libraries from B-deficient and -sufficient (control) citrus leaves, respectively, using Illumina sequencing, then identified the known and novel miRNAs as well as the B-deficiency-responsive miRNAs. Also, we predicted the target genes of these known and novel B-deficiency-responsive miRNAs and discussed their possible roles in the response to B-deficiency in citrus. The objective of this study is to identify the potential miRNAs related to the tolerance of citrus to B-deficiency.

Results

B and Cu concentrations in leaves

B concentration in 10 μM B-treated leaves was in the sufficient range of 30 to 100 μg g⁻¹ DW, while the value in 0 μM B-treated leaves was much less than 30 μg g⁻¹ DW (Fig. 1a) [31]. Visible B-deficient symptoms were
observed only in 0 μM B-treated leaves (data not shown). Therefore, seedlings treated with 0 μM B are considered as B-deficient, and those treated with 10 μM B are considered as B-sufficient. B-deficiency decreased leaf concentration of Cu (Fig. 1b).

Sequencing and analysis of two small RNA libraries from B-sufficient and -deficient citrus leaves

As shown in Table 1, 17,996,827 and 18,223,948 raw reads were generated from the libraries of B-sufficient and -deficient leaves, respectively. After removal of the contaminant reads like adaptors and low quality tags, 17,597,008 and 17,829,966 unique clear reads were obtained from the libraries of B-sufficient and -deficient leaves, comprising 3,673,054 and 4,654,829 unique clear reads, respectively. Among these reads, 11,726,078 clean reads (1,961,407 unique reads) from B-sufficient leaves and 11,372,875 clean reads (2,484,833 unique reads) from B-deficient leaves were mapped to C. sinensis genome (JGIversion 1.1, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Csinensis) using SOAP [32]. Exon, intron, miRNA, rRNA, repeat regions, snRNA, snoRNA and tRNA reads were annotated, respectively. After removal of these annotated reads, the remained unique reads that were used to predict novel miRNAs for B-sufficient and -deficient leaves were 3,237,407 and 4,179,224 reads, respectively.

Most of the clear sequences were within the range of 19–26 nt, which accounted for 89 % of the total clear reads. Reads with the length of 24 nt were at the most abundant, followed by the reads with the length of 21, 22, 23 and 20 nt (Additional file 1). Overall, the size distribution of sRNAs agrees with the results obtained on roots of

![Fig. 1](image-url) Effects of B-deficiency on B and Cu concentration in leaves. Bars represent mean ± SE (n = 3). Different letters above the bars indicate a significant difference at P < 0.05

| Treatments | Control | B-deficiency |
|------------|---------|--------------|
| Leaf Cu content (μg g⁻¹ DW) | 20 | 10 | 0 |
| Leaf B content (μg g⁻¹ DW) | 50 | 40 | 30 |

| Table 1 | Statistical analysis of sRNA sequencing data from B-sufficient and -deficient leaves of Citrus sinensis |
| B-sufficiency | Total sRNAs | B-deficiency | Total sRNAs |
|----------------|-------------|--------------|-------------|
| Raw reads | 17,996,827 | 18,223,948 |
| Clear reads | 3,673,054 (100 %) | 4,654,829 (100 %) |
| Mapped to genomic | 1,961,407 (67.64 %) | 11,372,875 (63.79 %) |
| Exon antisense | 28,626 (0.76 %) | 42,754 (0.92 %) |
| Exon sense | 77,868 (2.12 %) | 81,887 (1.76 %) |
| Intron antisense | 36,541 (0.99 %) | 46,940 (1.01 %) |
| Intron sense | 56,020 (1.53 %) | 67,594 (1.45 %) |
| miRNA | 44,496 (1.21 %) | 46,800 (1.01 %) |
| rRNA | 164,311 (4.47 %) | 263,999 (4.18 %) |
| repeat | 2,009 (0.01 %) | 2,978 (0.02 %) |
| snRNA | 8040 (0.05 %) | 3547 (0.08 %) |
| snoRNA | 3628 (0.02 %) | 4748 (0.03 %) |
| tRNA | 3,777 (0.04 %) | 2718 (0.02 %) |
| Unannotated sRNAs | 3,237,407 (88.14 %) | 4,179,224 (89.78 %) |
Citrus sinensis [8], fruits of C. sinensis [33] and Citrus trifoliata, and flowers of C. trifoliata [34]. This indicates that the data of sRNA libraries obtained by the Illumina sequencing are reliable.

Identification of known and novel miRNAs in citrus leaves
Here, a total of 734 known miRNAs were isolated from the two libraries (Additional file 2). The count of reads was normalized to transcript per million (TPM) in order to compare the abundance of miRNAs in the two libraries. The most abundant miRNA isolated from B-sufficient and -deficient libraries was miR157 (86,829.4201 and 48,091.4546 TPM, respectively), followed by miR166 (36,979.7525 and 26148.2271 TPM, respectively) and miR167 (24,944.5815 and 16,269.745, respectively). In this study, only these known miRNAs with normalized read-count more than ten TPM in B-sufficient and/or -deficient leaf libraries were used for further analysis in order to avoid false results caused by the use of low expressed miRNAs [8, 35]. After removal of these low expressed miRNAs, the remained 321 known miRNAs were used for further analysis (Additional file 3).

After removal of these annotated reads (i.e., exon, intron, miRNA, rRNA, repeat regions, snRNA, snoRNA and tRNA), the remained 3,237,407 and 4,179,224 reads from B-sufficient and -deficient libraries, respectively were used to predict novel miRNAs using the Mireap (http://sourceforge.net/projects/mireap/). Based on the criteria for annotation of plant miRNAs [7, 36], a total of 71 novel miRNAs were isolated from the two libraries (Additional file 4). Like the known miRNAs, novel miRNAs with normalized read-count less than ten TPM were not included in the expression analysis [7, 35]. After excluding these low expressed novel miRNAs, the remained 28 miRNAs were used for further analysis (Additional file 5).

Identification of B-deficiency-responsive miRNAs in citrus leaves
We identified 91 (83 known and 8 novel) up- and 81 (75 known and 6 novel) down-regulated miRNAs from B-deficient leaves. The most pronounced up- and down-regulated known (novel) miRNAs were miR5266 with a fold-change of 16.22 (novel_miR_95 with a fold-change of 17.61) and miR401 with a fold-change of -15.87 (novel_miR_236 with a fold-change of -18.48), respectively (Additional files 3 and 5).

Validation of high-throughput sequencing results by qRT-PCR
We analyzed the expression of 27 known miRNAs using stem-loop qRT-PCR in order to validate the miRNA expression patterns revealed by Illumina sequencing. The expression levels of all these miRNAs except for miR6214, miR5262 and miR7841 were comparable in magnitude to the expression patterns obtained by Illumina sequencing (Fig. 2). Obviously, the high-throughput sequencing allowed us to identify the differentially expressed miRNAs under B-deficiency.

Identification of targets for differentially expressed miRNAs and GO analysis
In this study, we predicted 489 and 17 target genes from the 70 known and 6 novel differentially expressed miRNAs, respectively (Additional files 6 and 7). GO categories were assigned to all these target genes based on the cellular component, molecular function and biological process. These target genes for the known and novel miRNAs were related to 12 and 3 components, respectively based on the cellular component. The most three GO terms for known miRNAs were membrane, chloroplast and plastid, while more than 42 % of the target genes for novel miRNAs belonged to membrane (Fig. 3a). Based on the molecular function, the target genes for the known and novel miRNAs genes were grouped into 11 and 9 categories, respectively, the highest percentage of three categories were nucleic acid binding, metal ion binding and transcription factor activity (Fig. 3b). In the biological process, the target genes were mainly focused on response to stress and developmental process for known miRNAs, and nucleic acid metabolic process, developmental process, response to stress and regulation of transcription for novel miRNAs, respectively (Fig. 3c).

qRT-PCR validation of target genes
To verify the expression of the target genes and how the miRNAs regulate their target genes, 77 genes targeted by 14 down- and 13 up-regulated miRNAs were assayed by qRT-PCR (Table 2). Among the 77 genes, the expression changes of 58 target genes showed a negative correlation with their corresponding miRNAs, implying that miRNAs might play a role in regulating gene expression under B-deficiency by cleaving mRNAs. However, the expression changes of the remained 19 target genes had a positive correlation with their corresponding miRNAs, which might be the results of the interaction of different target genes.

Discussion
Evidence shows that miRNAs are involved in the adaptive regulation of higher plants to nutrient deficiencies [8, 13, 17, 19, 24, 27, 37]. Here, we isolated 91 (83 known and 8 novel) up- and 81 (75 known and 6 novel) down-regulated miRNAs from B-deficient leaves (Additional files 3 and 5), indicating that B-deficiency greatly affected the expression profiles of miRNAs in leaves. The differentially expressed miRNAs isolated from leaves were more than from roots [i.e., 52 (40 known and 12 novel) up- and 82 (72
The majority of the differentially expressed miRNAs were isolated only from B-deficient roots or leaves, only 22 miRNAs were isolated from both. Moreover, among the 22 miRNAs, 11 miRNAs in roots and leaves displayed different responses to B-deficiency (Table 3). In conclusion, many differences existed in B-deficiency-induced changes in miRNA expression profiles between roots and leaves.

We found that miR159 was down-regulated in B-deficient leaves (Table 2), as previously obtained on salt-stressed sugarcane leaves [38]. Patade and Suprasanna showed that the up-regulation of MYB at 1 h of salt-stressed sugarcane leaves was accompanied by the down-regulation of miR159 [38]. However, the expression of miR159 was up-regulated in P-deficient soybean (Glycine max) roots and leaves [39]. MiR159 plays important roles in maintaining leaf phenotype by negatively regulating MYB transcription factors [40]. Dai et al. reported that the expression of OsMYB3R-2 was induced by various abiotic stresses, and that over-expression of OsMYB3R-2 enhanced tolerance to freezing, drought, and salt stress in transgenic Arabidopsis [41]. B-deficiency affects water uptake into the root, transport through the shoot, and loss of water from the leaves [42]. Thus, B-deficiency-induced down-regulation of miR159 might increase the expression of MYBs (Table 2), thus improving the tolerance of plants to B-deficiency.

qRT-PCR showed that all the four MYBs target genes (i.e., MYB domain protein 33, MYB domain protein 97, MYB-like HTH transcriptional regulator family protein and MYB domain protein 65) were induced by B-deficiency except for the last one. Similarly, the expression levels of MYB transcription factor (MYBML2) targeted by miR782, MYB-like HTH transcriptional regulator family protein and MYB domain protein 65 targeted by miR3946, and MYB-like HTH transcriptional regulator family protein and MYB transcription factor (MYBML2) targeted by miR7539 increased in response to B-deficiency except for MYB domain protein 65 (Table 2). B-deficiency-induced up-regulation of MYBs in citrus leaves agrees with the previous report that the expression of MYB85, MYB63 and MYB42 were up-regulated at the slight corking veins and the seriously corky split veins caused by B-deficiency in ‘Newhall’ navel orange (Citrus sinensis) leaves [43].

TIR1/AFB2 (TRANSPORT INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX PROTEIN2) Auxin Receptor (TAAR) family F-box proteins are involved in auxin perception and signaling. The expression of TAAR is regulated by miR393 [44]. MiR393 plays a key role in maintaining proper homeostasis of auxin signaling [45].
Fig. 3 GO of the predicted target genes for 70 (6) differentially expressed known (novel) miRNAs. Categorization of miRNAs target genes was performed according to cellular component (a), molecular function (b) and biological process (c).
| miRNA  | Fold change of miRNA | Accession  | Homology                      | Target genes                              | Relative change of target genes |
|--------|----------------------|------------|-------------------------------|-------------------------------------------|---------------------------------|
| miR158 | ~3.35603222**        | orange1.1g022993m | AT1G69840.1          | SPFH/Band 7/PHB domain-containing membrane-associated protein family | 1.9490**                         |
|        |                      | AT2G03210    | Lipase class 3 family protein |                                            | 1.6482**                         |
|        |                      | orange1.1g001709m | AT3G07400            |                                            | 0.7819*                          |
| miR159 | ~2.04145817**        | orange1.1g039708m | AT5G06100.2          | MYB domain protein 33                      | 1.1319*                          |
|        |                      | AT4G27330.1  | Sporocytes (SPL)          |                                            | 2.2016**                         |
|        |                      | orange1.1g046419m | AT4G26930.1          | MYB domain protein 97                      | 1.9078**                         |
|        |                      | AT3G11440.1  | MYB domain protein 65      |                                            | 0.8778**                         |
| miR160 | 1.81653886**         | orange1.1g004896m | AT2G28350.1          | ARF10                                      | 0.7870**                         |
|        |                      | AT4G30080.1  | TIR1              |                                            | 0.7150**                         |
|        |                      | AT1G77850.1  | TIR1              |                                            | 0.9153**                         |
| miR164 | ~2.28320824**        | orange1.1g030909m | AT1G6010.2           | NAC domain containing protein 1           | 0.5939**                         |
|        |                      | AT5G61430.1  | NAC domain containing protein 100 |                                      | 1.3247**                         |
|        |                      | orange1.1g017827m | AT5G08030.1          | Protein of unknown function, DUF642      | 0.5400**                         |
| miR158 | ~3.35603222**        | orange1.1g022993m | AT1G69840.1          | SPFH/Band 7/PHB domain-containing membrane-associated protein family | 1.9490**                         |
|        |                      | AT2G03210    | Lipase class 3 family protein |                                            | 1.6482**                         |
| miR393 | 1.66802767**         | orange1.1g010049m | AT3G18080.1          | B-S glucosidase 44                        | 0.8384**                         |
|        |                      | AT3g62980   | TIR1              |                                            | 0.7489**                         |
|        |                      | At4g03190   | AFB1              |                                            | 0.8195**                         |
|        |                      | At3g26810   | AFB2              |                                            | 0.7895**                         |
| miR408 | ~2.55840249**        | orange1.1g013075m | AT2g30210           | Laccase 3                                  | 1.5874**                         |
|        |                      | At5g07130   | Laccase 13         |                                            | 1.1251**                         |
| miR477 | 3.82198862**         | orange1.1g018483m | AT3G11340.1          | UDP-Glycosyltransferase superfamily protein | 0.6543**                         |
| miR782 | ~10.08402439**       | orange1.1g039969m | NM_001112290         | MYB transcription factor (MYBML2)         | 1.5782**                         |
|        |                      | Protein disulfide isomerase (PDILS-1) | |                                            | 0.908**                          |
| miR1446| 5.01671689**         | orange1.1g037028m | AT1G14920.1          | GRAS family transcription factor family protein | 0.7887**                         |
| miR1535| 1.58529156**         | orange1.1g001616m | AT3G63380.1          | ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein | 0.6757**                         |
|        |                      | AT1G16830.1  | ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein | | |
| miR2099| 10.31417531**        | orange1.1g017694m | AT3G22830.1          | Heat shock transcription factor A68       | 0.6459**                         |
| miR2643| ~2.52218131**        | orange1.1g013858m | AT5G05390            | Laccase 12                                 | 0.8814**                         |
| miR393 | 1.66802767**         | orange1.1g013075m | AT2g30210           | Laccase 3                                  | 1.5874**                         |
|        |                      | AT5g07130   | Laccase 13         |                                            | 1.1251**                         |
| miR477 | 3.82198862**         | orange1.1g018483m | AT3G11340.1          | UDP-Glycosyltransferase superfamily protein | 0.6543**                         |
| miR782 | ~10.08402439**       | orange1.1g039969m | NM_001112290         | MYB transcription factor (MYBML2)         | 1.5782**                         |
|        |                      | Protein disulfide isomerase (PDILS-1) | |                                            | 0.908**                          |
| miR1446| 5.01671689**         | orange1.1g037028m | AT1G14920.1          | GRAS family transcription factor family protein | 0.7887**                         |
| miR1535| 1.58529156**         | orange1.1g001616m | AT3G63380.1          | ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein | 0.6757**                         |
|        |                      | AT1G16830.1  | ATPase E1-E2 type family protein/haloacid dehalogenase-like hydrolase family protein | | |
| miR2099| 10.31417531**        | orange1.1g017694m | AT3G22830.1          | Heat shock transcription factor A68       | 0.6459**                         |
| miR2643| ~2.52218131**        | orange1.1g013858m | AT5G05390            | Laccase 12                                 | 0.8814**                         |
Table 2 qRT-PCR relative expression of experimentally determined or predicted target genes of selected miRNAs (Continued)

| miRNA  | Target Gene                                                                 | Fold Change |
|--------|------------------------------------------------------------------------------|-------------|
| miR3446− | orange1.1g004633m AT5G66850.1 Mitogen-activated protein kinase kinase kinase 5 | 1.6310**    |
|        | orange1.1g004928m AT2G25930.1 Hydroxyproline-rich glycoprotein family protein | 1.3981**    |
|        | orange1.1g036074m AT4G22200.1 Potassium transport 2/3 | 1.2999**    |
| miR3946− | orange1.1g023957m AT5G47370.1 Homeobox-leucine zipper protein 4 (HB-4)/HD-ZIP protein | 0.7342*     |
|        | orange1.1g041705m AT4G25980.1 Peroxidase superfamily protein                  | 1.5621**    |
|        | orange1.1g031837m AT1G08830.1 Copper/zinc superoxide dismutase 1             | 1.6638**    |
|        | orange1.1g016997m AT1G13310.1 Endosomal targeting BRO1-like domain-containing protein | 0.5406**   |
|        | orange1.1g014089m AT1G73390.1 Endosomal targeting BRO1-like domain-containing protein | 1.3404**   |
|        | orange1.1g027084m AT3G20560.1 PDI-like 5-3                                   | 1.0827*     |
|        | orange1.1g017665m AT3G04070.1 NAC domain containing protein 47               | 1.6886**    |
|        | orange1.1g010076m AT3G54700.1 Phosphate transporter 1/7                      | 1.7862**    |
|        | orange1.1g034408m AT1G33110.1 MATE efflux family protein                     | 1.5697**    |
|        | orange1.1g027612m AT1G04760.1 Vesicle-associated membrane protein 726       | 1.2270**    |
|        | orange1.1g027026m AT4G27670.1 Heat shock protein 21                          | 1.3134**    |
|        | orange1.1g020124m AT2G01060.1 MYB-like HTH transcriptional regulator family protein | 1.7116**  |
|        | orange1.1g011938m AT4G00050.1 Basic helix-loop-helix (bHLH) DNA-binding superfamily | 0.844**   |
|        | orange1.1g012387m AT4G00050.1 Basic helix-loop-helix (bHLH) DNA-binding superfamily | 1.6480**  |
|        | orange1.1g005832m AT1G06820.1 Carotenoid isomerase                          | 1.5524**    |
| miR39533  | orange1.1g016435m AT5G46590.1 NAC domain containing protein 96              | 0.7783**    |
|        | orange1.1g013411m AT2G16980.2 Major facilitator superfamily protein         | 0.5828**    |
|        | orange1.1g016066m AT2G16990.2 Major facilitator superfamily protein         | 0.4849**    |
| miR52271  | orange1.1g031467m AT2G42860.1 DnaJ/Hsp40 cysteine-rich domain superfamily protein | 0.4641**  |
|        | orange1.1g003885m AT5G49900.1 Chloride channel C                             | 0.844**     |
| miR5266  | orange1.1g033760m AT2G45290.1 Transketolase                                | 1.1778*     |
|          | orange1.1g017142m AT5G22290.1 NAC domain containing protein 89              | 1.1842*     |
| miR503710.12893993** | orange1.1g013645m AT2G16980.2 Major facilitator superfamily protein         | 0.5828**    |
| miR52621.64808069** | orange1.1g016066m AT2G16990.2 Major facilitator superfamily protein         | 0.4849**    |
| miR52271.8059848** | orange1.1g016435m AT2G16980.2 Major facilitator superfamily protein         | 0.4641**    |
| miR52670.52221.622392231** | orange1.1g003760m AT2G45290.1 Transketolase                                | 1.1778*     |
| miR52661.622392231** | orange1.1g013411m AT2G45290.1 Transketolase                                | 1.1778*     |
| miR5929−5.83479907** | orange1.1g009510m AT5G42480.1 Chaperone DnaJ-domain superfamily protein     | 1.3663**    |
| miR60253.39080972** | orange1.1g005832m AT1G06820.1 Carotenoid isomerase                          | 0.6716      |
|        | orange1.1g023118m AT2G21940.4 Shikimate kinase 1                            | 0.7012**    |
| miR6214−3.978202** | orange1.1g037660m AT5G37380.4 Chaperone DnaJ-domain superfamily protein     | 1.2352**    |
| miR6260−6.8442483** | orange1.1g010903m AT5G15130.1 WRKY DNA-binding protein 72                  | 3.2313**    |
| miR7539−4.033976** | orange1.1g003752m AT5G42480.1 Chaperone DnaJ-domain superfamily protein     | 1.3327**    |
| miR7841−10.61512382** | orange1.1g041450m AT3G42640.1 H+-ATPase 8                                  | 0.8903**    |

Both fold change of miRNAs and relative change of target genes are the ratio of B-deficient to –sufficient leaves. The value is an average of at least three biological replicates with three technical replicates; Target genes that had the expected changes in mRNA levels were marked in bold. * and ** indicate a significant difference at $P < 0.05$ and $P < 0.01$, respectively.
Table 3 List of differentially expressed miRNAs present in both roots and leaves

| miRNA   | Fold change | Roots                       | Leaves                      |
|---------|-------------|-----------------------------|-----------------------------|
| miR418  | 1.87710209* | 2.01596507*                 |                             |
| miR4413 | 3.76410603* | −5.94405631*                |                             |
| miR5037 | 4.79286276* | 10.12893993*                |                             |
| miR3946 | 5.08067725* | −1.66677822*                |                             |
| miR5259 | 6.34492626* | −5.83479070*                |                             |
| miR2099 | 13.49283335*| 10.3147531*                 |                             |
| miR2622 | 13.96750819*| 10.13868134*                |                             |
| miR2664 | 14.36084091*| −13.05830635*               |                             |
| miR5266 | −1.56149395*| 16.22392231*                |                             |
| miR394  | −5.15694535*| −1.66677822*                |                             |
| miR3513 | −5.84396568*| −7.04650639*                |                             |
| miR5492 | −6.7798681* | −5.48570888*                |                             |
| miR5534 | −7.1665574* | −2.89672418*                |                             |
| miR5029 | −7.36425252*| 6.19590225*                 |                             |
| miR5211 | −8.31439018*| 14.53849221*                |                             |
| miR1847 | −9.0000212* | 10.94295432*                |                             |
| miR158  | −10.05080647*| −3.35630222*                |                             |
| miR2921 | −10.13114959*| −11.0611889*                |                             |
| miR782  | −10.76475548*| −10.08402439*               |                             |
| miR1446 | −10.94721705*| 5.01671685*                 |                             |
| miR5074 | −10.94721705*| 10.74971862*                |                             |
| miR3443 | −11.47199392*| 9.96792062*                 |                             |

Data from Additional file 3 and Lu et al. [8]; ** indicates a significant difference at P < 0.01

Si-Ammour et al. showed that miR393 down-regulated all four TAAR genes by guiding the cleavage of their mRNAs, leading to the changes in auxin perception and some auxin-related leaf development [44]. Stress-induced increase in miR393 level may decrease the level of TIR1, a positive regulator of growth and development, thereby resulting in attenuation in growth and development during stress conditions [14]. Auxin response factors (ARFs) play a role in relaying auxin signaling at the transcriptional level by inducing mainly three groups of genes [i.e., Aux/IAA (Auxin/indole-3-acetic acid), GH3 and small auxin-up RNA (SAUR)] [46, 47]. MiR160 is predicted to target ARF10, ARF16 and ARF17. MiR160-directed regulation of Arabidopsis ARF17 is necessary for the normal growth and development of many organs, proper GH3-like gene expression and perhaps auxin distribution, while the ARF10 and ARF16 knockout mutants do not display obvious developmental anomalies [48]. Weakened plant growth and reduced metabolic rate are common survival strategies employed to divert energy and other resources to deal with stress conditions. It has been suggested that the stress-induced up-regulation of miR393 and miR160 might lead to the attenuation of plant growth and development under stress by repressing auxin signaling due to decreased TIR1 level and by suppressing the ARF-mediated gene expression, respectively, thus promoting plant stress tolerance [47]. Therefore, B-deficiency-induced up-regulation of leaf miR393 and miR160 might be an adaptive response of plants to B-deficiency, because the expression of the three genes targeted by miR160 and TIR1, AFB1, AFB2 and AFB3 targeted by miR393 was down-regulated by B-deficiency except for AFB3 (Table 2). Similarly, the expression of SAUR-like auxin-responsive protein family targeted by miR3946 was down-regulated in B-deficient leaves despite decreased expression of miR3946 (Table 2). By contrast, root miR3946 was up-regulated by B-deficiency [8].

Leaf miR164 was down-regulated by B-deficiency (Table 2), as previously observed on transient low nitrate-stressed maize leaves [28]. Water stress led to decreased expression of miR164 in cassava (Manihot esculenta) leaves, while its target gene MesNAC (No Apical Meristem) was strongly induced [49]. As expected, the expression of NAC domain transcriptional regulator superfamily protein and NAC domain containing protein 100 was induced in B-deficient leaves, while the expression of NAC domain containing protein 1 was depressed (Table 2). Over-expression of SNAC1 and OsNAC6 conferred drought and salt tolerance in rice [50, 51]. SINAC4-RNAi tomato plants became less tolerant to salt and drought stress [52]. Therefore, the down-regulation of miR164 in B-deficient leaves might be involved in the B-deficiency tolerance of plants by improving the expression of NAC. However, Xu et al. found that miR164 was up-regulated in maize leaves under chronic N limitation, and suggested that miR164 might function in remobilizing the N from old to new leaves to cope with the N-limiting condition via accelerating senescence due to decreased expression of NAC [28].

Leaf miR408 was down-regulated by B-deficiency (Table 2), as previously reported on N-deficient seedlings of Arabidopsis [27]. MiR408 targets genes encoding Cu containing proteins such as Cu/Zn SODs (CSDs), plantacyanin and several laccases [23]. Abdel-Ghany and Pilon observed that miR408 was induced under Cu starvation to down-regulate target gene expression and to save Cu for the most essential functional protein, concluding that might play a role in the regulation of Cu homeostasis [22]. Although B-deficiency decreased leaf concentration of Cu, its level was not lower than the sufficiency range of Cu in citrus leaves [53]. Thus, B-deficiency-induced decrease in miR408 might be advantageous to plant survival under B-deficiency by regulating Cu homeostasis and improving antioxidant (SOD) activity, because the expression of its four target genes was induced by B-deficiency.
except for *laccase 12* (Table 2). Indeed, SOD activity was higher in B-deficient *C. sinensis* leaves than in B-sufficient ones [54]. Also, SOD expression was up-regulated in B-deficient *Medicago truncatula* root nodules [55].

Leaf *miR477* was up-regulated by B-deficiency (Table 2), as previously reported on salt-stressed *Populus cathayana* plantlets [56]. NAC and GRAS transcription factors are target genes of *miR477*. NAC is involved in developmental process and stress responses [56], while GRAS proteins play a role in signal transduction and the maintenance and development of meristems [57]. Also, GRAS is the target gene of *miR1446* (Table 2), miR170 and miR171 [58], and NAC is the target gene of miR164, miR3953 and miR3946 (Table 2). This indicates the complex regulation in plant development and stress response.

WRKY proteins play important roles in plant responses to (a)biotic stresses, allowing plants to adapt to unfavorable environmental conditions including B-deficiency [59, 60]. Our results showed that leaf transcript of *miR6260* decreased in response to B-deficiency accompanied by increased expression of its target gene: *WRKY DNA-binding protein 72* (Table 2), which agrees with the previous reports that *WRKY3 DNA binding protein* expression was induced in B-deficient *M. truncatula* root nodules [55] and that *WRKY6* was up-regulated in B-deficient *Arabidopsis* roots [60]. Over-expression of various WRKY conferred tolerance to different abiotic stresses in different plant species, possible through the regulation of the reactive oxygen species system [61, 62]. Transgenic *Nicotiana benthamiana* plants over-expressing *GhWRKY139* had enhanced tolerance to salt and oxidative stress and increased expression of genes encoding antioxidant enzymes such as SOD, ascorbate peroxidase (APX), catalase (CAT) and glutathione-S-transferase (GST) [62]. Thus, leaf expression levels of antioxidant enzyme genes might be increased in response to B-deficiency. This agrees with our report that B-deficient citrus leaves had higher activities of SOD, APX, MDAR and GR [54]. Heat shock proteins (HSPs)/chaperones function in protecting plants against various stresses. As expected, the expression of *miR6260* was down-regulated in B-deficient leaves accompanied by decreased expression of its one target gene: *chaperone DnaJ-domain superfamily protein* (Table 2). Similarly, leaf expression levels of *miR5929* and *miR6214* were decreased by B-deficiency accompanied by increased expression levels of their corresponding target genes: *DnaJ-domain superfamily protein (AT5G42480.1 and AT5G37380.4)* (Table 2). However, the expression of *heat shock transcription factor A6B* targeted by *miR2099* were inhibited in B-deficient leaves despite down-regulated expression of *miR2099* (Table 2). Hydroxyproline-rich glycoproteins (HRGPs) are the most abundant cell wall structural proteins in dicotyledonous plants [63]. Hall and Cannon demonstrated that the cell wall HRGP RSH was required for normal embryo development in *Arabidopsis* [64]. Bonilla et al. observed that B-deficiency-induced aberrant cell walls of bean root nodules lacked covalently bound HRGPs [65]. Here, the expression of *HRGP family protein* (AT2G25930.1), a target gene of *miR3446*, was up-regulated in B-deficient leaves (Table 2), thus enhancing plant tolerance to B-deficiency. However, *miR3446* was down-regulated in B-deficient leaves, but its target gene (*HRGP family protein; AT1G49330.1*) was also depressed (Table 2).

B-deficiency lowered leaf expression level of *miR158* (Table 2), as previously obtained on N-deficient *Arabidopsis* seedlings [27] and B-deficient citrus roots [8]. The down-regulation of *miR158* means that its target genes: SPFH/Band 7/PHB domain-containing membrane-associated protein family, fucosyltransferase 2 and lipase class 3 family protein might be up-regulated in B-deficient leaves. However, qRT-PCR showed that the expression of the former two target genes was induced by B-deficiency, while the last one was down-regulated (Table 2). Lu et al. reported that *fucosyltransferase 2* and lipase class 3 family protein were down-regulated in B-deficient citrus roots accompanied by decreased expression of *miR158* [8].

The major facilitator superfamily (MFS) is the largest group of transport carriers, which are often coupled to the movement of another ion [66]. Kaya et al. reported that *ATRI*, which encodes a multidrug resistance transport protein of the MFS, was responsible for most of the tolerance of high B in *Saccharomyces cerevisiae*, concluding that *ATRI* was a B exporter [67]. In this study, leaf *miR5037* was induced by B-deficiency accompanied by decreased expression of its target gene: *MFS protein* (Table 2), thus decreasing B export from plants and improving plant tolerance to B-deficiency.

We found that leaf *miR5266* was induced by B-deficiency accompanied by increased expression of its target gene: *ammonium transporter 1:1* (Table 2), which disagrees with our report that the abundance of *miR5266* was lower in B-deficient citrus roots than in controls, while the expression level of *ammonium transporter 1:1* was higher in the former [8].

We observed that *miR3946* was inhibited in B-deficient leaves (Table 2), which disagrees with the previous report that *miR3946* was induced in B-deficient *C. sinensis* roots [8]. All the 17 target genes targeted by *miR3946* were induced by B-deficiency except for *homeobox-leucine zipper protein 4* (HB-4)/HD-ZIP protein, endosomal targeting BRO1-like domain-containing protein (*AT1G13310.1*), *MYB domain protein 65* and *SAUR-like auxin-responsive protein family* (Table 2). Previous studies showed that B-deficiency increased the expression levels of some transport-related genes and the abundances of some transport-related proteins in citrus roots [5, 8], thus improving the tolerance of plants to B-deficiency. *BOR1*, an efflux-type B transporter for xylem loading,
play a key role in the tolerance of plants to low B. *Arabidopsis bort-1* mutant was more sensitive to B-deficiency than the wild type [68]. *Oryza sativa* BOR1 has been demonstrated to be required for B acquisition by roots and translocation of B into shoots [69]. Thus, B-deficiency-induced up-regulation of leaf endosomal targeting BRO1-like domain-containing protein (AT1G73390.1), phosphate transporter 1:7, MATE efflux family protein, vesicle-associated membrane protein 726 (targeted by miR3946), potassium transport 2:3 (targeted by miR3446), ammonium transporter 1:1 (targeted by miR5266), Zn transporter 10 precursor (targeted by miR2648) involved in cell transport (Table 2) might contribute to the tolerance of citrus to B-deficiency. HD-ZIP transcription factors are found only in plants. The expression of *Hahb-4*, a member of *Helianthus annuus* (sunflower) subfamily I, strongly increased in water-stressed sunflower [70]. Subsequent study showed transgenic *Arabidopsis* plants over-expressing *Hahb-4* were more tolerant to drought by delaying the onset of senescence [71]. Huang et al. demonstrated that *PtrbHLH*, a basic helix-loop-helix transcription factor of *Poncirus trifoliata* might play a crucial role in cold tolerance via positively regulating peroxidase (POD)-mediated ROS scavenging [72]. Transketolase is a key enzyme of the pentose phosphate pathway (PPP) in plant cells. Our finding that *transketolase* was up-regulated in B-deficient leaves agrees with the report that transketolase activity in maize moderately increased in response to salt or oxidative stress [73]. In citrus, PPP has been suggested to play a role in the tolerance of plants to B-deficiency by providing reducing power (NADPH) and enhancing the antioxidant capacity [4]. Protein disulfide isomerases (PDIs), which act as molecular chaperones, play a role in the formation of proper disulfide bonds during protein folding [74]. Over-expression of a protein disulfide isomerase-like protein (PDIL) gene conferred Hg tolerance in transgenic plants, which had higher antioxidant capacity and lower levels of superoxide anion radicals, H$_2$O$_2$ and malondialdehyde (MDA) [75]. As shown in Table 2, the expression level of *PDILS-3* targeted by miR3946 was increased in B-deficient leaves. To conclude, down-regulation of miR3946 in B-deficient leaves might be an adaptive response of plants to B-deficiency. Carotenoid (Car) isomerase (CRTISO), which catalyzes the isomerization of poly-cis-carotenoids to all trans-carotenoids in higher plants, is a regulatory step for Car biosynthesis. *Arabidopsis* mutants of *crts*o had increased accumulation of poly-cis-carotenoids and reduced lutein concentration [76, 77]. Here, the expression of miR6025 was increased and its one target gene: CRTISO was decreased in B-deficient leaves (Table 2), thus impairing Car biosynthesis. This agrees with our report that B-deficient citrus leaves had lower Car concentration [54]. Plant phenolic secondary metabolites and their precursors are synthesized via the pathway of shikimate biosynthesis [78]. Shikimate kinase, a key enzyme for the biosynthesis of polyphenols, catalyzes the fifth reaction of the shikimate pathway. As shown in Table 2, the expression level of *shikimate kinase* 1 was down-regulated in B-deficient leaves and the expression of miR6025, which targets the gene, was up-regulated. This disagrees with our report that B-deficient citrus leaves displayed increased accumulation of phenolics [4]. Mitogen-activated protein kinase (MAPK) cascades play important roles in plant response to various stresses. Each MAPK cascade consists of MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). In plants, MAPKKKs have been shown to be involved in various stresses. Ning et al. showed that transgenic rice plants over-expressing DSM1 (a putative MAPKKK gene in rice) displayed higher tolerance to dehydration at the seedling stage by regulating ROS scavenging [79]. In this study, leaf transcript of miR3446 was decreased by B-deficiency and its target gene (MAPKKKS) was up-regulated under B-deficiency. This agrees with the report that MAPKKK genes were induced by drought, heat, salt, cold, IAA and jasmonic acid (JA) in *Arabidopsis* [80]. Our finding that leaf expression level of miR7539 decreased in response to B-deficiency, and its target gene (*phosphoenolpyruvate carboxylase, PEPC*) was induced by B-deficiency (Table 2). This agrees with our report that B-deficient citrus leaves had increased activity of PEPC and dark respiration [4].

Conclusion

We identified 734 known and 71 novel miRNAs from B-sufficient and -deficient citrus leaves using Illumina sequencing, and obtained 91 (83 known and 8 novel) up- and 81 (75 known and 6 novel) down-regulated miRNAs from B-deficient citrus leaves. Obviously, the expression of miRNAs was greatly altered in B-deficient leaves, which might play a role in the tolerance of plants to B-deficiency. In this study, we proposed a model for the responses of leaf miRNAs to B-deficiency by integrating the present results with the data available in the previous literatures (Fig. 4). The adaptive responses of leaf miRNAs to B-deficiency might be associated with several aspects: (a) attenuation of plant growth and development by down-regulating TIR1, ARF and AFB due to up-regulated miR393 and miR160, and by lowering the expression of SALIR-like auxin-responsive protein family targeted by miR3946, thus enhancing plant stress tolerance; (b) improving the expression of NACs due to decreased expression miR159, miR782, miR3946 and miR7539, hence maintaining leaf phenotype and enhancing the
stress tolerance; (c) activation of the stress responses and antioxidant system due to decreased expression of miR164, miR6260, miR5929, miR6214, miR3946 and miR3446; (d) decreased expression of MFS resulting from increased expression of miR5037, thus lowering B export from plants. In addition, B-deficiency-induced down-regulation of miR408 might be involved in the tolerance of plants to B-deficiency by regulating Cu homeostasis and enhancing SOD activity. In conclusion, our study reveals some adaptive mechanisms of citrus to B-deficiency.

Methods

Plant culture and B treatments

Both plant culture and B treatments were performed according to Yang et al. [5] and Lu et al. [8]. Briefly, 15-week-old seedlings of ‘Xuegan’ [Citrus sinensis (L.) Osbeck] grown in 6 L pots (two seedlings per pot) containing fine river sand were supplied every other day until dripping with B-deficient (0 μM H3BO3) or -sufficient (10 μM H3BO3) nutrient solution for 15 weeks. There were 10 replications per B treatment with 2 pots in a completely randomized design. At the end of the experiment, fully-expanded leaves from different replicates and treatments were collected at noon under full sun and frozen immediately in liquid N2. Leaf samples were stored at −80 °C until extraction. It’s worth mentioning that C. sinensis is polyembryonic seed development, an apomictic process in which many embryos are initiated directly from the maternal nucellar cells surrounding the embryo sac containing a developing zygotic embryo [81].

Isolation of leaf sRNAs, library construction and Illumina sequencing

About 0.1 g mixed frozen B-sufficient and -deficient leaves from five replications were used to extract RNA. Total RNA was extracted from frozen leaves using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. Two sRNA libraries were constructed according to Lu et al. [8]. High throughput sequencing was performed on a Solexa sequencer (Illumina) at the Beijing Genomics Institute (BGI), Shenzhen, China.

sRNA annotation and miRNA identification

Both sRNA annotation and miRNA identification were performed according to Lu et al. [8]. Briefly, software developed by the BGI was used to deal with the raw data from the Solexa sequencing. Clean reads were then used to analyze length distribution and common/specific sequences. Thereafter, the clear reads were mapped to C. sinensis genome (JGI version 1.1, http://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Csinensis) using SOAP, only perfectly mapped sequences were retained and analyzed further. rRNAs, tRNAs, snRNAs and snoRNAs were removed from the sRNAs sequences through BLASTn search using NCBI Genebank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi/) and Rfam (12.0) database (http://www.sanger.ac.uk/resources/databases/rfam.html) (e = 0.01). The remaining sequences were aligned with known plant miRNAs from miRBase 21 (http://www.mirbase.org/). Only the perfectly matched sequences were considered to be conserved miRNAs. Reads that were not annotated were used to predict novel miRNAs using a prediction software Mireap (http://
sourceforge.net/projects/mireap/), which was developed by the BGI, by exploring the secondary structure, the Dicer cleavage site and the minimum free energy of the unannotated small RNA tags which could be mapped to genome. In addition, we used MiTide: an integrated tool for the identification of miRNA-target interaction in plants (http://bis.zju.edu.cn/MiTide) [82] and DNAMAN 8 (http://www.lynnon.com/pc/framepc.html) to predict novel miRNA. Only these miRNA candidates that were simultaneously predicted by the three softwares were considered to be real novel miRNAs.

**Differential expression analysis of miRNAs**

Both the fold change between B-deficiency and -sufficiency and the P-value were calculated from the normalized expression of TPM [83]. A 1.5 log2-fold cut-off was set to determine up- and down-regulated miRNAs in addition to a P-value of less than 0.01 [8].

**Target prediction of miRNAs**

This was performed by RNAhybrid based on rules suggested by Allen et al. [84] and Schwab et al. [85].

**Functions of the potential targets of the differentially expressed miRNAs**

All targets of the differentially expressed miRNAs were mapped to GO terms in the database (http://www.geneontology.org/), and calculated gene numbers for each term. The GO results were expressed as three categories: cellular component, molecular function, biological process [8].

**Validation of miRNA expression by stem-loop qRT-PCR**

The detection of miRNA expression was performed using stem-loop qRT-PCR method, stem-loop primers for reverse transcription and primers for qRT-PCR were listed in Additional file 8. Total RNA was reversetranscribed using Taqman® MicroRNA Reverse Transcription Kit (USA), and SYBR® Premix Ex Taq™ II (Takara, Japan) kit was used for qRT-PCR. MiRNA special (forward) primers were designed according to the miRNA sequence but excluded the last six nucleotides at 3' end of the miRNA. A 5' extension of several nucleotides, which was chosen randomly and relatively GC-rich, was added to each forward primer to increase the melting temperature [86]. All the primers were assigned to Primer Software Version 5.0 (PREMIER Biosoft International, USA) to assess their quality. For qRT-PCR, 20 μL reaction solution contained 10 μL ready-to-use SYBR® Premix Ex TaqTM II (Takara, Japan), 0.8 μL 10 μM miRNA forward primer, 0.8 μL 10 μM Uni-miR qPCR primer, 2 μL cDNA template and 6.4 μL dH2O. The cycling conditions were 60 s at 95 °C, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s. qRT-PCR was performed on the ABI 7500 Real Time System. Samples for qRT-PCR were run in at least three biological replicates with two technical replicates. Relative miRNA expression was calculated using ddCt algorithm. For the normalization of miRNA expression, actin (AEK97331.1) was used as an internal standard and the leaves from control plants were used as reference sample, which was set to 1.

**qRT-PCR analysis of miRNA target gene expression**

Total RNA was extracted from frozen B-sufficient and -deficient leaves using TRIzol reagent (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. The sequences of the F and R primers used were given in Additional file 9. qRT-PCR analysis of miRNA target gene expression was performed using a ABI 7500 Real Time System according to Lu et al. [8].

**Experimental design and statistical analysis**

There were 20 pot seedlings per treatment in a completely randomized design. Experiments were performed with 3 replicates. Differences among treatments were separated by the least significant difference (LSD) test at P < 0.05 level.

**Availability of data and materials**

“The data set supporting the results of this article are available in the Gene Expression Omnibus repository under accession no GSE72108 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72108)”. The mature miRNA and precursor sequences will be submitted to miRBase registry and assigned final names after final acceptance of the manuscript.

**Additional files**

- Additional file 1: Length distribution of small RNAs from control and B-deficient leaves of Citrus sinensis seedlings. (DOC 81 kb)
- Additional file 2: List of known miRNAs in Citrus sinensis leaves. (DOC 1525 kb)
- Additional file 3: List of known miRNAs in Citrus sinensis leaves after removing these miRNAs with normalized read-count less than 10 TPM in the two miRNA libraries constructed from control and B-deficient leaves. (DOC 452 kb)
- Additional file 4: List of novel miRNAs in Citrus sinensis leaves. (DOC 158 kb)
- Additional file 5: List of novel miRNAs in Citrus sinensis leaves after removing these miRNAs with normalized read-count less than 10 TPM in two miRNA libraries constructed from control and B-deficient leaves. (DOC 198 kb)
- Additional file 6: List of target genes for parts of known miRNAs in Citrus sinensis leaves. (DOC 33 kb)
- Additional file 7: List of target genes for parts of novel miRNAs in Citrus sinensis leaves. (DOC 61 kb)
- Additional file 8: List of stem loop qRT-PCR primers. (DOC 160 kb)
Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
YBL carried out most of the experiments and drafted the manuscript; YPQ participated in the design of the study. LTY participated in the design of the study and coordination; FG participated in data analysis; YL directed the study; LSC designed and directed the study and revised the manuscript.

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