Boron Stress Responsive MicroRNAs and Their Targets in Barley

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Abstract

Boron stress is an environmental factor affecting plant development and production. Recently, microRNAs (miRNAs) have been found to be involved in several plant processes such as growth regulation and stress responses. In this study, miRNAs associated with boron stress were identified and characterized in barley. miRNA profiles were also comparatively analyzed between root and leave samples. A total of 31 known and 3 new miRNAs were identified in barley; 25 of them were found to respond to boron treatment. Several miRNAs were expressed in a tissue specific manner; for example, miR156d, miR171a, miR397, and miR444a were only detected in leaves. Additionally, a total of 934 barley transcripts were found to be specifically targeted and degraded by miRNAs. In silico analysis of miRNA target genes demonstrated that many miRNA targets are conserved transcription factors such as Squamosa promoter-binding protein, Auxin response factor (ARF), and the MYB transcription factor family. A majority of these targets were responsible for plant growth and response to environmental changes. We also propose that some of the miRNAs in barley such as miRNA408 might play critical roles against boron exposure. In conclusion, barley may use several pathways and cellular processes targeted by miRNAs to cope with boron stress.

Introduction

MicroRNAs (miRNAs) are a class of single strand, endogenous, non-coding small RNA molecules, which post-transcriptionally regulate gene expression in many organisms by targeting miRNAs for cleavage or translation suppression [1,2,3]. Increasing evidence demonstrates that miRNAs play an important role in many biological and metabolic processes including regulation of plant growth, development and response to biotic and abiotic stresses via interactions with their specific target miRNAs [4,5,6,7,8]. Boron (B) is an essential element for plants, and its deficiency generally causes growth defects mainly in young and growing parts of the plants, while excessive levels of B are toxic to plants [9,10]. A number of physiological processes are shown to be altered by B exposure. Deterioration of cell wall biosynthesis, metabolic deterioration by binding to the ribose moieties of ATP, NADH and NADPH, and inhibition of cell division and elongation are the most distinct symptoms of B toxicity [11,12,13]. However, plants also evolve mechanisms to cope with the presence of excessive amounts of metal ion. Although several studies have been performed on small RNAs and metal stressors such as mercury, cadmium, and aluminum [14,15,16], no studies have been reported on boron stress.

Barley (Hordeum vulgare L.) is one of the most important grain crops grown and cultivated worldwide [17]. Additionally, it is a well-studied model plant for triticacea research in terms of genetics, genomics, and breeding [18,19]. Although miRNAs in barley were identified in previous studies [19,20,21,22], compared with the number of identified miRNAs in other grain crops such as rice and maize, the number of known miRNAs in barley is still very insufficient. Initially, conventional approaches were extensively used for miRNA identification and contributed considerably to the miRNA exploration [8,23].

The purpose of this study is to identify tissue specific expression of miRNAs and their potential targets in barley exposed to high levels of boron. To achieve this goal, we identified miRNAs from the entire transcriptome RNA-seq data, which included more than 208 million reads generated from control and B-exposed roots and leaves of B-tolerant barley seedlings. Some of the identified barley miRNAs were validated in leaf and root tissues by quantitative RT-PCR. Additionally, ‘degradome sequencing’ approach was also employed for miRNA target identification in barley.
1) hvu-miR159

2) hvu-miR171

3) hvu-miR319a

4) hvu-miR1121

5) hvu-miR2024a

6) hvu-miR5049

Figure 1. The secondary stem-loop structures of several identified miRNAs in barley. Mature miRNA sequences are marked in red color. doi:10.1371/journal.pone.0059543.g001
Materials and Methods

Plant Materials and Boron Treatment

Barley (*Hordeum vulgare* L. cultivar Sahara) seeds were sterilized and placed into Petri dishes for germination at room temperature. Then, four-day-old seedlings were transferred into liquid culture flasks including nutrient solutions. The treatments were repeated at least three times with triple biological replicates. For toxicity experiments, toxic (1000 mM) and nontoxic (50 mM) concentrations of B were added to different flasks. Germinated seedlings were exposed to B-toxic or B-nontoxic conditions for approximately 24 hours.

RNA Isolation, cDNA Library Construction and Sequencing for Transcriptome Analysis

Total RNAs were extracted from barley root and leaf tissues using the TRIZOL Reagent (Invitrogen) according to the manufacturer’s instructions. The extractions were performed separately for each sample with three independent biological replicates and same amount of total RNA was subsequently pooled based on their concentration. The quality and quantity of purified RNAs were assessed with a Nanodrop 2000c spectrophotometer (Nanodrop Technologies, USA) and the presence of ribosomal RNA bands was determined by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). All RNA samples were stored at −80°C until further processing. The cDNA library construction and Illumina (Solexa) based-transcriptome sequencing experi-

| miRNA name   | Sequence (5’–3’)                        | LM  | LP  | MFEI | GC% | ΔG  |
|--------------|-----------------------------------------|-----|-----|------|-----|-----|
| hvu-mir-156  | UGACAGAAGAGAGAGAGCAC                    | 20  | 178 | 0.71 | 65.0| −83.20|
| hvu-mir-157  | UUGACAGAAGAGAGAGAGCAC                   | 21  | 178 | 0.93 | 52.0| −86.30|
| hvu-mir-159  | UUUGAUGUAAGGACGACU                     | 21  | 178 | 0.95 | 60.0| −56.00|
| hvu-mir-160  | UGGCGUGCUCCGUAGUAGACCA                  | 21  | 178 | 0.71 | 65.0| −83.20|
| hvu-mir-164  | UGGAGAAGAGGACGACU                      | 21  | 75  | 0.74 | 61.0| −34.10|
| hvu-mir-165  | CCGGACUCUCCCAUAGUAGCA                  | 20  | 100 | 0.51 | 62.0| −31.90|
| hvu-mir-166  | CCGGACUCUCCCAUAGUAGCA                  | 21  | 61  | 0.34 | 59.0| −12.50|
| hvu-mir-168  | GAUCUGCUCUAGGACUAGAAU                  | 24  | 106 | 0.81 | 75.0| −64.40|
| hvu-mir-169  | AAGCCAGAUGAGAUGAGAUG                  | 21  | 83  | 0.80 | 45.0| −30.10|
| hvu-mir-171  | UGAUGGAGGCGUAGGACUAAUC                | 21  | 137 | 0.97 | 55.0| −73.20|
| hvu-mir-172c | AGGAUGUAGGAGGAGUAG               | 21  | 54  | 0.60 | 41.0| −13.40|
| hvu-mir-319a | UUGGACUGAAGGAGGAGC                  | 20  | 186 | 0.90 | 52.0| −87.70|
| hvu-mir-319c | UUGGAAUGAGGAGGAGCA                | 20  | 78  | 0.55 | 45.0| −19.60|
| hvu-mir-397  | CCGUGUGAUGAGGAGGAGU                  | 21  | 133 | 0.98 | 67.0| −74.90|
| hvu-mir-399  | UGCAAGGAGAUGAGUAGGAGCC              | 21  | 113 | 0.65 | 46.0| −34.20|
| hvu-mir-408  | CUGUCUGCCUCUCCGUGG                   | 21  | 149 | 0.80 | 56.0| −67.50|
| hvu-mir-444b | UGCAGUUGCUAGCAGCUAGCU                 | 21  | 121 | 1.01 | 45.0| −55.20|
| hvu-mir-1120 | ACAUUUCAUAUUUAAUAGGAGGAGG           | 24  | 84  | 1.36 | 36.0| −41.30|
| hvu-mir-1121 | AGUAGUAGCACUACGCUU                      | 22  | 83  | 1.53 | 36.0| −45.90|
| hvu-mir-1122 | UUUGCAUCUAGCUAGCUAGU                 | 20  | 120 | 1.28 | 33.0| −50.70|
| hvu-mir-1126 | UCCACUAUGCAGCAGACAGGAGG              | 23  | 120 | 1.28 | 33.0| −50.70|
| hvu-mir-2004 | UUGUUGUUUAAGUGUUUUGAGAA            | 24  | 78  | 0.74 | 29.0| −16.90|
| hvu-mir-2007 | CAAGAUAUGGAGGUAUUUUGC              | 22  | 54  | 1.59 | 30.0| −25.90|
| hvu-mir-2014 | AGCAAAACAUUCAGAGCAGCA                  | 22  | 109 | 0.60 | 49.0| −32.20|
| hvu-mir-2019 | CCGUGGGCGUGCGUGCGGCG                       | 21  | 71  | 0.53 | 65.0| −24.70|
| hvu-mir-2023a | UUUGCCGUGAGCACGACUCA              | 22  | 113 | 0.74 | 55.0| −46.00|
| hvu-mir-2024a | GCAGUUGCAGUCUCAAGCUGU            | 20  | 118 | 1.02 | 44.0| −53.40|
| hvu-mir-2906 | AACCGGCCGCGUCGACACUUGG             | 21  | 254 | 0.77 | 63.0| −123.9|
| hvu-mir-2911 | UAGUGUGUAGGAGGUAUUUUGC           | 21  | 71  | 0.56 | 49.0| −19.6|
| hvu-mir-2914 | CAUGUGGUGUGACGUGGAGACG           | 23  | 63  | 0.61 | 56.0| −21.8|
| hvu-mir-5048 | UAUUGGAGGUGGUGUGUGACUAA              | 22  | 354 | 0.88 | 31.0| −96.90|
| hvu-mir-5049 | UCCUAAUAUCUGUGUUGUUGG             | 21  | 81  | 1.37 | 43.0| −47.80|
| hvu-mir-5051 | UUUGGCCACCUUGAAGACUGGGA       | 21  | 105 | 1.20 | 49.0| −61.90|
| hvu-mir-5052 | ACCCGGCCGACGUGGACGACUAGA           | 21  | 175 | 0.89 | 54.0| −85.00|
| hvu-mir-5066 | AAGUGUAGUAGGAGGUGGCU        | 21  | 80  | 0.33 | 44.0| −11.70|

LM: length of the mature miRNA; LP: length of the miRNA precursor sequence; MFEI: Minimal folding free energy index.
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Table 1. Barley miRNAs and features identified by high-throughput sequencing.
Table 2. The normalized read counts of the pre-miRNAs in each sample.

| miRNA name               | 50 µM B root reads | 1000 µM B root reads | 50 µM B leaf reads | 1000 µM B leaf reads |
|--------------------------|---------------------|-----------------------|--------------------|----------------------|
| hvu-miR156               | 146                 | 232                   | 28                 | 81                   |
| hvu-miR156a/miR156b/miR156c | 874                 | 848                   | 199                | 139                  |
| hvu-miR156c              | 22                  | 21                    | 0                  | 0                    |
| hvu-miR157               | 46                  | 25                    | 87                 | 43                   |
| hvu-miR159               | 746                 | 886                   | 196                | 339                  |
| hvu-miR160               | 211                 | 236                   | 4                  | 4                    |
| hvu-miR160o              | 525                 | 473                   | 190                | 283                  |
| hvu-miR164a              | 120                 | 177                   | 28                 | 20                   |
| hvu-miR165               | 104                 | 221                   | 58                 | 78                   |
| hvu-miR166c              | 80                  | 146                   | 44                 | 76                   |
| hvu-miR168               | 634                 | 890                   | 125                | 166                  |
| hvu-miR169               | 1711                | 937                   | 192                | 263                  |
| hvu-miR169c              | 3                   | 3                     | 3                  | 19                   |
| hvu-miR171               | 1450                | 1289                  | 477                | 1090                 |
| hvu-miR171a              | 264                 | 205                   | 26                 | 71                   |
| hvu-miR172               | 1473                | 699                   | 149                | 651                  |
| hvu-miR319c              | 31                  | 26                    | 5                 | 4                    |
| hvu-miR319/319a          | 171                 | 211                   | 0                 | 0                    |
| hvu-miR397               | 58                  | 51                    | 15                 | 2                    |
| hvu-miR399               | 124                 | 35                    | 31                 | 94                   |
| hvu-miR408               | 0                   | 0                     | 130                | 8                    |
| hvu-miR444a              | 562                 | 926                   | 4                  | 21                   |
| hvu-miR444b              | 83                  | 26                    | 14                 | 21                   |
| hvu-miR444c              | 236                 | 151                   | 36                 | 91                   |
| hvu-miR1120              | 869                 | 1261                  | 468                | 797                  |
| hvu-miR1121              | 2237                | 2115                  | 31                 | 11                   |
| hvu-miR1122              | 170                 | 277                   | 156                | 113                  |
| hvu-miR2004              | 4                   | 9                     | 118                | 77                   |
| hvu-miR2014              | 26                  | 22                    | 5                 | 2                    |
| hvu-miR2021              | 9                   | 22                    | 18                 | 6                    |
| hvu-miR2023a             | 38                  | 70                    | 34                 | 69                   |
| hvu-miR2024a             | 83                  | 26                    | 14                 | 21                   |
| hvu-miR2906              | 80                  | 85                    | 120                | 106                  |
| hvu-miR5048              | 1019                | 1169                  | 217                | 218                  |
| hvu-miR5049              | 37                  | 43                    | 26                 | 7                    |
| hvu-miR5051              | 35                  | 73                    | 27                 | 35                   |
| hvu-miR5053              | 277                 | 733                   | 126                | 20                   |
| hvu-miR5064              | 248                 | 250                   | 116                | 159                  |
| hvu-miR5066              | 8                   | 16                    | 30                 | 4                    |
| hvu-miR5141              | 993                 | 1247                  | 1199               | 175                  |
| hvu-miR5052              | 0                   | 0                     | 8                  | 0                    |
| hvu-miR5180a/miR5180b    | 278                 | 240                   | 25                 | 2                    |

The mapped read counts of each pre-miRNAs were normalized in terms of the length of pre-miRNA and total read numbers according to RPKM method (Reads Per kb per Million reads) [61]. doi:10.1371/journal.pone.0059543.t002

De novo Assembly of Transcriptome and Data Processing

Firstly, the image data obtained from sequencing platform were converted by base calling into sequence data which is commonly known as raw reads and typically stored in the fastq file format. In order to acquire high-quality clear reads, all raw sequence reads were filtered to remove adapters, the reads containing unknown nucleotides larger than 5% and low quality reads. Then, remaining clear reads were subjected to de novo transcriptome assembly using Trinity assembler with default settings [24]. Briefly, Inchworm, one of software module of Trinity, assembles reads with definite length of overlap in order to generate longer fragments which are termed contigs. Then, the reads are realigned to the newly formed contig so as to construct scaffolds which are basically derived from the contigs from the same transcript. By using paired-end information, these reads (pair-end clean reads) are mapped back to the resultant scaffolds to fill the intra-scaffolds gaps. Subsequently, the sequences generated by assembly of scaffolds are defined as unigenes which cannot be extended on either end by further assembly process. After obtaining non-redundant unigenes as long as possible using sequence clustering software, some of those unigenes were termed by singletons which are not part of any contigs.

Identification of miRNAs and Prediction of miRNA Precursors (Pre-miRNAs)

The RNA-seq generated more than 208 million clean reads (Kekec et al. Unpublished data) that were used to identify miRNAs and their targets. To identify potential miRNA precursors (pre-miRNAs), Blastn search was performed with an e-value of 1e-10 between unigene reads constructed from a total of four libraries for whole transcriptome of H. vulgare (Kekec et al. Unpublished data) that were used to identify with those short fragments. After size selection and purification through agarose gel electrophoresis, the selected fragments were enriched by PCR amplification with appropriate primers and eventually, the library sequencing experiments were performed using Illumina HiSeq™ 2000 instrument.
structure generation via the web-based computational software MFOLD 3.2 [25]. The default parameters of the software were adjusted for predicting secondary structure of the selected sequences and the minimal folding free energy index (MFEI) was calculated for each pre-miRNA sequence as described previously [26]. After identification of putative pre-miRNAs, we determined the localization of predicted mature miRNAs on the their own pre-miRNAs by mapping these mature miRNAs to the pre-miRNAs using BLAST search algorithm with default parameters. To consider whether these sequences are potential miRNAs with fold change over 2).

### Table 3. The expression level of boron-responsive miRNAs from highly B treated and control B applied barley leaf and root tissues.

| miRNA name | L–B expressed | L+B expressed | Fold change (Up/Down) | R–B expressed | R+B expressed | Fold change (Up/Down) |
|------------|---------------|---------------|-----------------------|---------------|---------------|-----------------------|
| miR156     | 28            | 81            | ↑ 2-fold (up-regulated)| 146           | 232           | Not significantly changed |
| miR156d    | 87            | 43            | ↓ 2-fold (down-regulated)| 46            | 25            | Not significantly changed |
| miR165     | 58            | 78            | Not significantly changed| 104           | 221           | ↑ 2-fold (up-regulated) |
| miR169c    | 3             | 19            | ↑ 6-fold (up-regulated)| 3             | 3             | Not changed |
| miR171     | 477           | 1090          | ↑ 2-fold (up-regulated)| 1450          | 1289          | Not significantly changed |
| miR171a    | 26            | 71            | ↑ 2-fold (up-regulated)| 264           | 205           | Not significantly changed |
| miR172     | 149           | 651           | ↑ 4-fold (up-regulated)| 1473          | 699           | ↓ 2-fold (down-regulated) |
| miR397     | 15            | 2             | ↓ 7-fold (down-regulated)| 58            | 51            | Not significantly changed |
| miR399     | 31            | 94            | ↑ 3-fold (up-regulated)| 124           | 35            | ↓ 3-fold (down-regulated) |
| miR408     | 130           | 8             | ↓ 16-fold (down-regulated)| Not detected in root library |
| miR444a    | 4             | 21            | ↑ 5-fold (up-regulated)| 562           | 926           | Not significantly changed |
| miR444b    | 14            | 21            | Not significantly changed| 83            | 26            | ↓ 3-fold (down-regulated) |
| miR444c    | 36            | 91            | ↑ 2-fold (up-regulated)| 236           | 151           | Not significantly changed |
| miR1121    | 31            | 11            | ↑ 2-fold (up-regulated)| 2237          | 2115          | Not significantly changed |
| miR2004    | 118           | 77            | Not significantly changed| 4             | 9             | ↑ 2-fold (up-regulated) |
| miR2014    | 5             | 2             | ↓ 2-fold (down-regulated)| 26            | 22            | Not significantly changed |
| miR2021    | 18            | 6             | ↓ 3-fold (down-regulated)| 9             | 22            | ↑ 2-fold (up-regulated) |
| miR2023a   | 34            | 69            | ↑ 2-fold (up-regulated)| 38            | 70            | Not significantly changed |
| miR2024a   | 14            | 21            | Not significantly changed| 83            | 26            | ↓ 3-fold (up-regulated) |
| miR5049    | 26            | 7             | ↓ 3-fold (down-regulated)| 37            | 43            | Not significantly changed |
| miR5051    | 27            | 35            | Not significantly changed| 35            | 73            | ↑ 2-fold (up-regulated) |
| miR5053    | 126           | 20            | ↓ 6-fold (down-regulated)| 277           | 733           | ↑ 2-fold (up-regulated) |
| miR5066    | 30            | 4             | ↓ 7-fold (down-regulated)| 8             | 16            | ↑ 2-fold (up-regulated) |
| miR5141    | 1199          | 175           | ↓ 6-fold (down-regulated)| 993           | 1247          | Not significantly changed |
| miR5180a/  | 25            | 2             | ↓ 12-fold (down-regulated)| 278           | 240           | Not significantly changed |
| miR5180b   |               |               |                        |               |               |                       |

L–B, B-free leaf; L+B, B-treated leaf; R–B, B-free root; R+B, B-treated root (\(p\) miRNAs with fold change over 2).

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from other types of RNAs, e.g. pre-miRNA with approximately >0.67 has been identified as more likely to be a miRNA [27], (vi) there is no large loop or break in the miRNA:miRNA*, and (vii) the miRNA has less than six mismatches with the opposite miRNA sequence (miRNA*) on the opposite arm [28,29,30].

### Computational Identification of miRNA Target Genes

Identification of miRNA-regulated gene targets is crucial for understanding miRNA functions. Therefore, the putative targets of *H. vulgare* miRNAs were identified by aligning the miRNAs with the high-quality unigene set obtained from the assembled transcripts and the singleton transcripts of barley *de novo* transcriptome libraries using the web-based psRNA Target Server (http://plantgrn.noble.org/psRNA target/) with default parameters of user-submitted option. Alignment between Hva-miRNA and its potential target(s) was evaluated by the parameters described by Zhang [31]. These computationally identified miRNA targets were further analyzed using BlastX searches with an e-value of 1e-10 against *Hordeum* EST sequences at NCBI database to identify putative gene homologs for confirmation.

### Gene Ontology (GO) Analysis of Potential miRNA Targets

In order to better understand the functional roles of miRNAs in barley, Blast2Go (B2G) software v2.3.1 was used to assign gene
ontology (GO) annotations of predicted target genes [32]. First, all miRNA target transcripts were arranged in a text file (Fasta format) as an input data and uploaded to the program for BlastX searches with an e-value of 1e-06. The BLAST results included: sequence length, gene name, e-value, similarity, Hit-length, Align-length, GenBank and Uniprot accession number as well as Gene Ontology IDs belonging to each target sequences. Next, the output file (.dat file) obtained from BlastX analysis was used to retrieve GO terms associated with each blast hit and Gene Ontology annotations. Finally, all miRNA targets representing genes with known function were categorized by biological process, cellular component and molecular function according to the ontological definitions of the GO terms.

**miRNA Target Cleavage Product (Degradome) Analysis**

In order to characterize the miRNA cleaved target library (degradome) of *H. vulgare*, we evaluated a dataset derived from the output generated by CleaveLand (v2.0) software (a pipeline for using degradome data to find cleaved small RNAs). The miRNA-directed cleavage site in the miRNA:mRNA alignment is represented by red arrow (Table S1). Stem-loop Reverse-transcription

Stem-loop RT primers for Hvu-MIR 156, Hvu-MIR 159, Hvu-MIR 164, Hvu-MIR 166, Hvu-MIR 168, Hvu-MIR 171, Hvu-MIR 395, Hvu-MIR 396, Hvu-MIR 414, Hvu-MIR 1120 and Hvu-MIR 5048 were designed according to Varkonyi-Gasic et al. [33] (Table S2). The miRNA stem-loop reverse transcription was carried out using 500 ng of total RNA samples of B-nontoxic (50 μM) and B-toxic (1000 μM) leaf and root samples (1 μL), 0.5 μL 10 mM dNTP mix, 1 μL stem-loop RT primer (1 μM) and 10.5 μL nuclease free water. Those components were also mixed separately for the different dilutions of total RNA stem-loop RT primer for cDNA synthesis and incubated for 5 min at 65°C, and then put into ice for 2 min. Thereafter, 4 μL first-strand buffer (5×), 2 μL 1 M DTT, 0.1 μL RNAseOUT (40 units/μL), and 0.25 μL SuperScript III (200 units/μL) were added to each tube. The RT reactions were fulfilled as 30 min at 16°C followed by 60 cycles of 30°C for 30 s, 42°C for 30 s and 72°C for 1 s. The control tubes included all components without RT primer (no RT or - RT) and RNA template (no RNA or - RNA).

**Quantitative Real-time PCR**

To verify some of the predicted *H. vulgare* miRNAs experimentally, and to measure and compare the expression levels of the miRNAs in root and leaf tissues treated with boron, qRT-PCR was conducted using SYBR Green I Master Kit (Roche, Germany) on a LightCycler® 480 Real-Time PCR System (Roche, Germany). For qRT-PCR analysis, 10 μL 2X Master mix, 0.1 μL (100 pmol) forward and 0.1 μL (100 pmol) reverse primers, 7.8 μL nuclease-free water and 2 μL RT stem-loop cDNA products were used. Forward primers were specifically designed for each individual miRNA, but 5’-GTGCAGGGTCCGAGGT-3’ was used as the universal reverse primer [33] (Table S3). The qRT-PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 41 cycles at 95°C for 10 s, 55°C for 20 s, and 72°C for 10 s. The melting curves were adjusted as 95°C for...
Table 4. Barley miRNA targets identified by degradome sequencing.

| miRNA name                      | Target gene name                                      | Target gene accession | Target gene number | Cleavage site |
|---------------------------------|-------------------------------------------------------|-----------------------|--------------------|--------------|
| hvu-miR156/                    | Squamosa promoter-binding-like protein (SLP)          | CL11026.Contig1_All   | 12                 | 789          |
| hvu-miR157                      |                                                        | CL11193.Contig1_All   | 11                 | 489          |
|                                 |                                                        | CL13226.Contig1_All   | 3                  | 613          |
|                                 |                                                        | CL38155.Contig1_All   | 12                 | 248          |
| hvu-miR159/                    | MYB transcription factor family                       | CL32877.Contig1_All   | 7                  | 161          |
| hvu-miR159a/                   |                                                        |                       |                    |              |
| hvu-miR159b                     |                                                        |                       |                    |              |
| hvu-miR160                      | Auxin response factor (ARF)                           | CL7269.Contig1_All    | 13                 | 232          |
| hvu-miR164a/                   | NAC transcription factor (NAC)                        | CL1686.Contig1_All    | 15                 | 800          |
| hvu-miR164b                     |                                                        | CL3897.Contig1_All    | 15                 | 801          |
|                                 |                                                        | CL6305.Contig2_All    | 13                 | 967          |
|                                 |                                                        | CL8731.Contig1_All    | 13                 | 1013         |
|                                 |                                                        | CL19527.Contig1_All   | 10                 | 311          |
|                                 |                                                        | Unigene5170_All       | 15                 | 868          |
|                                 |                                                        | Unigene29351_All      | 15                 | 953          |
| hvu-miR165/                    | Class III Homeodomain-leucine zipper (HD-ZIP III)     | CL153.Contig8         | 16                 | 452          |
| hvu-miR166c                     |                                                        | CL153.Contig11        | 17                 | 764          |
| hvu-miR168a(3p)/               | Argonaute protein (AGO1)                             | CL3360.Contig1        | 16                 | 720          |
| hvu-miR168b(3p)                 |                                                        |                       |                    |              |
| hvu-miR169                      | Nuclear transcription factor Y subunit (NF-Y)         | CL5590.Contig1        | 17                 | 943          |
|                                 |                                                        | CL3849.Contig1        | 15                 | 1123         |
|                                 |                                                        | CL2801.Contig1        | 13                 | 913          |
| hvu-miR172c/                   | AP-2 Transcription Factors                           | CL27047.Contig1       | 10                 | 906          |
| hvu-miR172d                     |                                                        |                       |                    |              |
| hvu-miR319a/                   | MYB transcription factor family                       | CL32877.Contig1       | 7                  | 201          |
| hvu-miR319c                     |                                                        |                       |                    |              |
| hvu-miR397                      | Laccase mRNA                                          | CL2226.Contig1        | 9                  | 527          |
| hvu-miR399                      | Phosphate transporter 2 (PHO2) and Putative ubiquitin conjugating enzyme (UBC) | CL876.Contig1 | 18 | 1629 |
|                                 |                                                        | CL876.Contig4         | 18                 | 813          |
| hvu-miR408                      | Heterotrimeric G protein alpha subunit or ATPase family gene 1 (AFG1) | CL30341.Contig1_All | 14 | undetermined |
|                                 |                                                        | Unigene31703_All      | 11                 | undetermined |
| hvu-miR444/                    | MADS-box transcription factor                         | CL1260.Contig1        | 19                 | 633          |
| hvu-miR444a/                   |                                                        | CL3271.Contig2        | 20                 | 344          |
| hvu-miR444b/                   |                                                        |                       |                    |              |
| hvu-miR444c                     |                                                        |                       |                    |              |
| hvu-miR1120                     | COV1-like protein                                     | CL58.Contig8_All      | 16                 | undetermined |
| hvu-miR1121                     | Serine/threonine protein kinase                       | CL3697.Contig1_All    | 13                 | undetermined |
|                                 |                                                        | Unigene28145_All      | 14                 | undetermined |
| hvu-miR1122                     | Phospholipase A2 and Universal stress protein (USP) and WIR1 | CL1.Contig23_All | 14 | undetermined |
|                                 |                                                        | CL2147.Contig2_All    | 13                 | undetermined |
|                                 |                                                        | CL2301.Contig1_All    | 3                  | undetermined |
| hvu-miR1126                     | Zinc finger ccc domain-containing protein             | CL6067.Contig1_All    | 12                 | undetermined |
|                                 |                                                        | CL6067.Contig2_All    | 10                 | undetermined |
|                                 |                                                        | CL6067.Contig3_All    | 10                 | undetermined |
| miRNA name     | Target gene name                                                                 | Target gene accession | Target gene number | Cleavage site |
|---------------|----------------------------------------------------------------------------------|-----------------------|--------------------|---------------|
| hvu-miR2004   | PHD finger family protein, AP-1 complex subunit, Subtilase family protein, Tetra/tricopeptide repeat-containing protein and Transcription elongation factor (TFIIS) family protein | CL1242.Contig3_All    | 18                 | undetermined  |
|               |                                                                                  | CL6239.Contig1_All    | 11                 | undetermined  |
|               |                                                                                  | CL904.Contig1_All     | 14                 | undetermined  |
|               |                                                                                  | CL162.Contig5_All     | 8                  | undetermined  |
|               |                                                                                  | CL17869.Contig1_All   | 11                 | undetermined  |
| hvu-miR2007   | Protein phosphatase and Serine/arginine repetitive matrix protein                 | CL2929.Contig1_All    | 12                 | undetermined  |
|               |                                                                                  | CL6012.Contig1_All    | 11                 | undetermined  |
| hvu-miR2014   | Phospholipid-translocating ATPase, GTP-binding protein, Ethylene responsive factor and Transcription factor jumonji | CL283.Contig1_All     | 12                 | undetermined  |
|               |                                                                                  | CL7041.Contig1_All    | 17                 | undetermined  |
|               |                                                                                  | CL2423.Contig1_All    | 8                  | undetermined  |
|               |                                                                                  | CL3225.Contig1_All    | 13                 | undetermined  |
| hvu-miR2019   | Tubulin-tyrosine ligase family                                                   | CL326.Contig1_All     | 14                 | undetermined  |
| hvu-miR2021   | Rough sheath 2-interacting KH domain protein (RIK), Lysophosphatidylcholine Acyltransferase, Respiratory burst oxidase-like protein F2 and Cytochrome P450 | CL527.Contig3_All     | 5                  | undetermined  |
|               |                                                                                  | CL318.Contig4_All     | 15                 | undetermined  |
|               |                                                                                  | CL2680.Contig1_All    | 12                 | undetermined  |
|               |                                                                                  | Unigene27511_All      | 8                  | undetermined  |
| hvu-miR2024a  | MADS box protein-like protein and Zinc finger family protein                    | CL3271.Contig2_All    | 20                 | undetermined  |
|               |                                                                                  | CL9100.Contig1_All    | 12                 | undetermined  |
| hvu-miR2906   | (E)-beta-caryophyllene/beta-elemene synthase                                     | CL40097.Contig1_All   | 7                  | undetermined  |
|               |                                                                                  | Unigene30593_All      | 7                  | undetermined  |
| hvu-miR2910   | glycine rich protein 3, glyceraldehyde-3-phosphate dehydrogenase, cytosolic, phosphatidylinositol-4-phosphate 5-kinase 9 and ubiquitin-associated protein | CL40314.Contig1_All   | 15                 | undetermined  |
|               |                                                                                  | CL386.Contig2_All     | 10                 | undetermined  |
|               |                                                                                  | CL5067.Contig2_All    | 12                 | undetermined  |
|               |                                                                                  | Unigene11586_All      | 16                 | undetermined  |
| hvu-miR2914   | Senescence-associated protein, CBL-interacting protein kinase 21                 | CL8337.Contig1_All    | 11                 | undetermined  |
|               |                                                                                  | CL660.Contig7_All     | 11                 | undetermined  |
| hvu-miR2916   | Senescence-associated protein                                                     | CL8337.Contig1_All    | 10                 | undetermined  |
| hvu-miR5048   | RPG1, Serine/threonine protein kinase NAC domain-containing protein 18 and Serine/threonine kinase-like protein | CL26250.Contig1_All   | 12                 | undetermined  |
|               |                                                                                  | CL2067.Contig1_All    | 16                 | undetermined  |
|               |                                                                                  | CL5978.Contig2_All    | 14                 | undetermined  |
|               |                                                                                  | CL421.Contig2_All     | 14                 | undetermined  |
| hvu-miR5049   | Tubby protein-like                                                               | CL9685.Contig1_All    | 9                  | undetermined  |
| hvu-miR5052   | Cyclophilin                                                                      | CL27515.Contig1_All   | 9                  | undetermined  |
| hvu-miR5053   | Chlorophyll a/b-binding protein and Predicted protein                            | CL40448.Contig1_All   | 13                 | undetermined  |
|               |                                                                                  | CL33769.Contig1_All   | 1                  | undetermined  |
5 s and 55°C for 1 min and then cooled to 40°C for 30 s. All reactions were repeated three times [30]. For each condition, the qRT-PCR experiments were run as biological triplicates and expression levels were normalized according to previous studies [8,19,29,33,34]. The relative fold change for each comparison was calculated by 2−ΔΔCt after normalization [33,34]. Error bars were derived from the three experiments in triplicate and error bars represent standard deviation.

Validation of Barley miRNA Target mRNAs by qRT-PCR

To verify the expression levels of identified 11 barley miRNAs, the mature miRNAs were measured via qRT-PCR. Relative expression levels of predicted barley miRNAs were compared in root and leaf tissues treated with excess boron. The expression profile of these miRNA targets was also measured using qRT-PCR and their specific PCR primers were listed in the Table S3. The reverse transcription reaction was performed with Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Germany) according to the manufacturer’s protocol. The qRT-PCR experiment was carried out as reported previously [34,35]. Briefly, 2 μL cDNA was amplified with 0.1 μL specific primers in a total volume of 18 μL using SYBR Green I Master (Roche) with LightCycler® 480 Real-Time PCR System. 18s rRNA (GenBank ID: AF147501) amplified with forward: GTGACGGGTGACGGAGAATT and reverse: GACACTAATGCGCCCGGTAT primers were used as a reference gene with triple replicates [36,37].

Results

Identification of Boron Responsive miRNAs in Barley

According to sequence similarity to known plant miRNAs, 31 known and 3 new miRNAs were identified. Previously, miR157, miR165, and miR319 have been identified in other plant species, but so far they have been undetermined in barley. Identified miRNAs in barley were located on either arm of the predicted pre-miRNA sequences. Of the 34 identified *H. vulgare* miRNAs, 47% of mature sequences were located in the 5’ arm of pre-miRNAs, while 53% were situated in the 3’ arm (Fig. 1; Fig. S1). The majority of these miRNAs were 21 nt long, followed by 22 nt, 20 nt and 23 nt, respectively (Table 1), which is consistent with miRNAs from other plant species [23,36]. In addition, our study showed that the average of MFEI was 0.66, which is higher than that of other types of RNA molecules such as tRNAs (0.64), rRNAs (0.39) and miRNAs (0.62–0.66) (Table 1) [29,37,38,39].

miR171) and non-conserved miR5141 were found abundantly in both libraries, but many others were detected with only a few in both libraries or could not be found in either library. We also found that some miRNAs are only expressed in either root or leaf tissues. The miR156c and miR319a were highly expressed in root, whereas miR408 was only detected in leaf. In addition, some miRNAs such as miR156, miR169, miR172, and miR1121 were highly expressed in root but miR2004 was highly expressed in leaf. Expression of most miRNAs was significantly changed in a tissue-specific manner under boron stress whereas the remaining miRNAs were found to be responsive in both tissues. In root tissue responding to boron stress, miR165, miR2004, and miR5051 were up-regulated whereas miR444b and miR2024a were down-regulated. miR156, miR169c, miR171, miR171a, miR444a, miR444c, miR2023a were up-regulated while miR156d, miR397, miR408, miR1121, miR2014, miR5049, miR3141, miR5180, and miR5180a were down-regulated in leaf tissue upon boron stress. In addition, some miRNAs, such as miR172, miR399, miR2021, miR5053 and miR5066 were expressed in both root and leaf (Table 3).

Target Identification of miRNAs in Barley Using Degradome Analysis

A total of 934 genes targeted by miRNAs were identified in barley by CleaveLand (v2.0) (Table 4). However, we could not identify the cleavage signature for some of the known miRNAs. The miRNA guided cleavage sites by degradome analysis are shown in Fig. 2 and Table S1. According to the results of blastn analysis of the identified miRNA targets, many of the targets were homologous to conserved target genes existing in other plants species; these targets included squamosa promoter-binding protein, auxin response factor (ARF), MYB transcription factor family, AP2 Transcription Factors, NAC transcription factor (NAC), AGO1, and class III homeodomain-leucine zipper (HD-ZIP III) proteins. Most of these targets were found to be responsible for plant growth and response to environmental changes. For example, the target transcript of miR160 was ARGOAUT1 protein (AGO1) family protein, which functions in plant development and in response to stress stimulus, such as NaCl and mannitol stress in rice. [40].

qRT-PCR Validation and Expression of *H. vulgare* miRNA Levels and their Targets

Eleven identified barley miRNAs and their targets were further investigated using qRT-PCR. Both conserved barley miRNAs (miR156, miR159, miR164, miR166, miR168, miR171, miR395 and miR396) and non-conserved barley miRNAs (miR1120 and miR5048) were detected. The expression levels of barley miRNAs and their targets were comparatively shown in Fig. 2. The miR159, miR164, miR166, miR171, and miR414 were induced in leaf, but were inhibited in root tissues exposed to boron stress.
Table 5. Gene Ontology analyses indicate that miRNAs and target in related to biological process, cellular component, molecular function process.

| miRNAs          | GO Biological Process | GO Cellular Component                          | GO Molecular Function                  | Target Gene | Target Description                                      |
|-----------------|-----------------------|------------------------------------------------|----------------------------------------|-------------|--------------------------------------------------------|
| hvu-miR156      | –                     | Organelle (plastid) and cellular part (nucleus) | DNA binding                            | CL13226.Contig1_All CL11026.Contig1_All CL11193.Contig1_All CL38155.Contig1_All | Squamosa promoter-binding protein |
| hvu-miR157      | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR159      | –                     | Intracellular organelle Nucleus                | Nucleic acid (DNA) binding             | CL32877.Contig1_All | MYB family transcription factor (GAMYb transcription factor family) |
| hvu-miR159a     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR159b     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR160      | Response to stimulus  | Organelle and nucleus                          | Nucleic acid (DNA) binding             | CL7269.Contig1_All | Auxin response factor (ARF) |
| hvu-miR164a     | –                     | –                                              | Nucleic acid (DNA) binding             | CL6305.Contig2_All CL1696.Contig1_All Unigene29395_All | NAC transcription factor (NAC) |
| hvu-miR164b     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR165      | Biological regulation | Intracellular organelle Nucleus                | Nucleic acid binding Transcription factor activity | CL153.Contig8_All CL153.Contig11_All | Class III Homeodomain-leucine zipper (HD-ZIP III) proteins |
| hvu-miR166c     | Cellular process      | –                                              | –                                      | –           | –                                                      |
| hvu-miR168a (3p)| Multicellular organisal process Reproduction Biological regulation Immune system process Response to stimulus Metabolic process Cellular process Developmental process | Nucleus Cytosol | Nucleic acid binding Catalytic activity | CL3360.Contig1_All | AGO1 (ARGONAUTE 1) |
| hvu-miR168b (3p)| –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR169      | Biological regulation Metabolic process Cellular process | Macromolecular complex Membrane-enclosed lumen Membrane-bounded organelle Nucleoplasm part | Nucleic acid binding | CL5590.Contig1_All CL3849.Contig1_All CL2801.Contig1_All | Nuclear transcription factor Y subunit (NF-Y) |
| hvu-miR172c     | Biological regulation Metabolic process Cellular process | Intracellular organelle Nucleus | Nucleic acid binding Catalytic activity | CL27047.Contig1_All Unigene3420_All | AP-2 Transcription Factors |
| hvu-miR172d     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR319a     | –                     | Nucleus Intra-cellular membrane-bounded organelle | Nucleic acid binding | CL32877.Contig1_All CL2226.Contig1_All | MYB transcription factor family |
| hvu-miR319c     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR397      | Metabolic process     | Extracellular region Organelle Cytoplasmic vesicle | Nucleic acid binding Catalytic activity | CL1278.Contig5_All | Laccase mRNA |
| hvu-miR399      | Biological regulation | –                                              | Catalytic activity                     | CL876.Contig1_All CL876.Contig4_All | Phosphate transporter 2 (PHO2) or Putative ubiquitin conjugating enzyme (UBC) |
| hvu-miR408      | Response to pheromone | Organelle (mitochondrion) Cytoplasmic part     | Binding Catalytic activity              | CL30341.Contig1_All Unigene31703_All | Heterotrimeric G protein alpha subunit or ATPase family gene 1 (AFG1) |
| hvu-miR444      | Biological regulation Metabolic process Cellular process | Organelle and nucleus | Binding Catalytic activity | CL1260.Contig1_All CL3271.Contig2_All | MADS-box transcription factor |
| hvu-miR444a     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR444b     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR444c     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR1120     | –                     | –                                              | –                                      | –           | –                                                      |
| hvu-miR1121     | Cellular process      | Membrane                                      | Binding Catalytic activity              | CL3697.Contig1_All Unigene28145_All | Serine/threonine protein kinase |
| hvu-miR1122     | Response to stimulus  | Organelle (mitochondrion) Membrane             | –                                      | CL1.Contig23_All CL2147.Contig2_All CL2301.Contig1_All | Phospholipase A2 or Universal stress protein (USP) or WR1 |
| miRNAs   | GO Biological Process | GO Cellular Component                  | GO Molecular Function                   | Target Gene                          | Target Description                                                                 |
|----------|-----------------------|----------------------------------------|-----------------------------------------|--------------------------------------|-------------------------------------------------------------------------------------|
| hvu-miR126 | Biological regulation | Membrane                               | Binding                                 | CL6067.Contig1_All                   | Zinc finger ccch domain-containing protein                                          |
|          |                       |                                        | Catalytic activity                      | CL6067.Contig2_All                   |                                                                                     |
|          |                       |                                        |                                         | CL6067.Contig3_All                   |                                                                                     |
| hvu-miR2004 | Cellular process     | Extracellular region                   | Binding                                 | CL1242.Contig3_All                   | PHD finger family protein or AP-1 complex subunit or Subtilase family protein or Tetratricopeptide repeat-containing protein or Transcription elongation factor (TFII) family protein |
|          | Localization         | Macromolecular complex                 | Catalytic activity                      | CL6239.Contig1_All                   |                                                                                     |
|          | Metabolic process    | Organelle                              | Transcription regulatory activity       | CL9044.Contig1_All                   |                                                                                     |
|          |                      |                                        |                                         | CL1622.Contig5_All                   |                                                                                     |
|          |                      |                                        |                                         | CL17869.Contig1_All                  |                                                                                     |
| hvu-miR2007 | Cellular process     | Membrane                               | Binding                                 | CL2929.Contig1_All                   | Protein phosphatase or Serine/arginine repetitive matrix protein                      |
|          | Metabolic process    |                                        | Catalytic activity                      | CL6012.Contig1_All                   |                                                                                     |
| hvu-miR2014 | Biological regulation | Membrane                               | Binding                                 | CL2833.Contig1_All                   | Phospholipid-translocating ATPase or GTP-binding protein or Ethylene responsive factor or Transcription factor jumonji |
|          | Cellular process     |                                        | Catalytic activity                      | CL7041.Contig1_All                   |                                                                                     |
|          | Localization         |                                        | Molecular transducer activity           | CL2423.Contig1_All                   |                                                                                     |
|          | Metabolic process    |                                        | Transporter activity                    | CL3225.Contig1_All                   |                                                                                     |
| hvu-miR2019 | Cellular process     | Membrane                               | Catalytic activity                      | CL326.Contig1_All                    | Tubulin-tyrosine ligase family                                                      |
|          | Metabolic process    |                                        |                                         | CL6012.Contig1_All                   |                                                                                     |
| hvu-miR2021 | Cellular process     | Nucleus                                | Antioxidant activity                    | CL5273.Contig3_All                   | Rough sheath 2-interacting KH domain protein (RIK) or Lysophosphatidylcholine Acyltransferase or Respiratory burst oxidase-like protein F2 or Cytochrome P450 |
|          | Metabolic process    | Intracellular membrane-bound organelle | Binding                                 | CL6280.Contig1_All                   |                                                                                     |
|          |                      |                                        | Catalytic activity                      | CL2680.Contig1_All                   |                                                                                     |
| hvu-miR2024a | Biological regulation | Membrane-bounded organelle            | Binding                                 | CL3271.Contig2_All                   | MADS box protein-like protein or Zinc finger protein                                 |
|          | Cellular process     |                                        |                                      | CL9100.Contig1_All                   |                                                                                     |
|          | Metabolic process    |                                        |                                      |                                        |                                                                                     |
| hvu-miR2906 | –                    | –                                      | –                                      | CL40097.Contig1_All                  | (E)-beta-caryophyllene/beta-elemene synthase                                        |
|          | –                    | –                                      | –                                      | Unigene30593_All                    |                                                                                     |
| hvu-miR2910 | Biological regulation | Membrane                               | Antioxidant activity                    | CL40314.Contig1_All                  | Glycine rich protein 3 or Glyceraldehyde-3-phosphate dehydrogenase, cytosoli or Phosphatidylcholine 4-phosphate-5-kinase 9 or Ubiquitin-associated protein |
|          | Cellular process     |                                        | Binding                                 | CL3863.Contig2_All                   |                                                                                     |
|          | Developmental process|                                        | Catalytic activity                      | Unigene11586_All                    |                                                                                     |
|          | Metabolic process    |                                        | Electron carrier activity               | CL5067.Contig2_All                   |                                                                                     |
|          | Multicellular organism process | - | - | CL1622.Contig5_All |                                                                                     |
|          | Reproduction         |                                        |                                         | CL6067.Contig1_All                   |                                                                                     |
| hvu-miR2911 | –                    | –                                      | –                                      | CL17424.Contig1_All                  | ASF/SF2-like pre-mRNA splicing factor SRP32 or Hydroxylproline-rich glycoprotein family protein |
|          | –                    | –                                      | –                                      | CL23524.Contig1_All                  |                                                                                     |
| hvu-miR2914 | Biological regulation | Membrane-bounded organelle            | Binding                                 | CL8337.Contig1_All                   | Senescence-associated protein or CBL-interacting protein kinase 21                    |
| hvu-miR2916 | –                    | Mitochondrion                          | Catalytic activity                      | CL6600.Contig7_All                   |                                                                                     |
|          | –                    | –                                      |                                        | CL6012.Contig1_All                   |                                                                                     |
| hvu-miR5048 | Cellular process     | Plastid                                | Binding                                 | CL6250.Contig1_All                   | RP1G or Serine/threonine protein kinase or NAC domain-containing protein 18 or Serine/threonine kinase-like protein |
|          | Metabolic process    |                                        | Catalytic activity                      | CL2067.Contig1_All                   |                                                                                     |
|          |                      |                                        |                                         | CL5978.Contig2_All                   |                                                                                     |
|          |                      |                                        |                                         | CL4212.Contig2_All                   |                                                                                     |
| hvu-miR5049 | Biological regulation | –                                      | Sequence-specific DNA binding transcription factor activity | CL9685.Contig1_All                  | Tubby protein-like                                                                  |
|          | Cellular process     | –                                      | –                                      | CL6012.Contig1_All                   |                                                                                     |
|          | Metabolic process    | –                                      | –                                      | CL27515.Contig1_All                  | Cyclophilin                                                                         |
| hvu-miR5052 | Cellular process     | Membrane                              | Binding                                 | CL40448.Contig1_All                  | Chlorophyll a/b-binding protein or Predicted protein                                 |
|          | Metabolic process    |                                        | Catalytic activity                      | CL7041.Contig1_All                   |                                                                                     |
| hvu-miR5053 | Cellular process     | Membrane                              | Electrorn carrier activity              | CL1791.Contig1_All                   | RNA polymerase beta subunit                                                          |
|          | Metabolic process    |                                        |                                         | CL1791.Contig1_All                   |                                                                                     |
| hvu-miR5056 | –                    | –                                      | –                                      | CL1791.Contig1_All                   |                                                                                     |
| hvu-miR5066 | Cellular process     | Membrane                              | Binding                                 | CL21592.Contig1_All                  | Carbohydrate transporter/sugar transporter or Serine/threonine protein kinase         |
|          | Metabolic process    |                                        | Catalytic activity                      | CL6.6.Contig12_All                   |                                                                                     |

**Table 5. Cont.**

Boron Responsive Barley miRNAs

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Although miR168 was induced, miR159, miR396, miR1120 and miR3048 were inhibited in both root and leaf upon excess boron exposure. The targets of miR159 and miR1120 were found to be up-regulated in both root and leaf upon boron stress, but miR395 and miR5048 target genes were down-regulated in root but remained at the same levels in leaf tissue upon boron stress. Additionally, miR171 target gene was down-regulated in leaf but up-regulated in root upon boron stress (Fig. 2).

Gene Ontology (GO) Analysis

According to the gene ontology analysis, the predicted targets were classified into three main categories: biological processes, cellular components, and molecular functions (Table 5). Of these, cellular and metabolic process in biological process, cell and organell part in cellular component, and binding and catalytic activity in molecular function were the most established categories.

Discussion

High-throughput sequencing technology has currently been successfully applied to identify miRNAs at whole genome scale in several plant species, including: soybean [41,42], peanut [43,44], barley [21,45], poplar [46], olive [47], Medicago [48], grapevine [49], rice [50], and cucumber [23]. However, almost all of the previous studies have been performed under normal growth conditions, few are associated with stress conditions. Li et al. [41] reported soybean miRNAs under three stress treatments (drought, salinity, and alkalinity) via high-throughput sequencing. Drought stress responsive miRNAs shows differential expression in response to heat stress in *Populus euphratica* and wheat [46,51]. In this study, we constructed RNA libraries from barley leaves and roots treated with boron stress compared to control conditions to identify boron stress-responsive miRNAs in barley using high-throughput sequencing.

Boron treatment affected the expression profiles of miRNAs in barley leaf and root tissues. The most striking ones with 16-fold and 12-fold changes were miR408 and miR5180, respectively. The remaining changes in the expression of miRNAs ranged between 2- to 7-fold (Table 3).

Recently, miR408 was identified in barley, which targets Cu-binding domain containing chemocyanin and blue copper protein [19]. In this study, we found that miR408 also potentially targets heterotrimeric G protein alpha (α) subunit and ATPase family gene 1 (AFG1). Heterotrimeric G proteins and ATPase gene family plays significant roles in signal transduction pathways in plants [52,53,54]. Fujisawa et al. [55] reported that suppression of α subunit gene expression causes abnormal morphology in rice. In response to water deficit, miR398 and miR408 were induced in *Medicago truncatula* [56]. In addition, expression of miR408 upon drought stress in barley was found to be induced in leaves, but unchanged in roots [19]. However, in *Oryza sativa*, miR408 expression was reported as 2.76-fold down-regulated 12 days after water withholding at tillering stage upon drought stress using microarray analysis [40]. In our study, expression of miR408 was down-regulated significantly (16-fold) upon excess boron treatment in barley leaves.

Previous studies reported the miRNA expression in a species-specific or tissue-specific manner [57,58]. miR168, miR319, miR396, and miR397 were induced by drought in *Arabidopsis thaliana* but were suppressed in *Oryza sativa* [59]. Additionally, the expression of miR399 was induced in shoots upon phosphate deficiency treatment, but it was accumulated in both shoots and roots [57]. In barley, miR166 was up-regulated in leaves, but down-regulated in roots; miR171 level was induced in leaves, but it was not affected in roots [19]. In our study, miR169c, miR171, and miR399 were up-regulated in leaves whereas miR397, miR444b were down-regulated in roots after exposure to high B concentration. The miR172 was down-regulated 2-fold in roots but up-regulated 4-fold in leaves in response to boron stress. The miR168c and miR171 was determined to be 6-fold up-regulated and 2-fold up-regulated in leaves under boron stress, respectively. In *Medicago truncatula*, miR169 and miR172 were up-regulated but miR171 and miR390 were down-regulated upon mercury exposure [16]. Similarly, miR171 was down-regulated but miR172 was up-regulated by cadmium exposure in *Brassica napus* [15]. However, in response to Al treatment, miR171 was up-regulated in *Medicago truncatula* [14].

Our study demonstrated that boron stress inhibited miR156a expression in barley leaves. However, we did not detect its expression in roots. In addition, the target of miR156a, SBP protein gene, was down-regulated in stressed leaves, but was unaltered in roots in response to boron stress (Fig. 2). This result is similar to the previous report [19], whereas not affected in roots upon drought stress. Expression of miR156 has been investigated in many studies as down-regulated in *Oryza sativa*, *Zea mays*, *Populus tremula*, *Populous trichocarpa* in response to drought stress, salt stress, cold stress, mechanical stress, while up-regulated in *Arabidopsis thaliana*, *Triticum aestivum*, *Nicotiana tabacum* upon salt stress, heat stress, viral infection, respectively [59]. Our study indicates that miR156 was also boron stress responsive in leaves upon excess boron treatment.

For better understanding of the functions of miRNAs, gene ontology analysis for miRNA target transcripts was performed. Sixty genes targeted by 34 miRNAs were found to be involved in 77 biological processes. These major processes are as follows: biological regulation, metabolic process, response to stimulus, cellular process, signal transduction, multicellular organismal process, reproduction, immune system process, developmental process, and localization. The most (24 out of 34) miRNAs participated in the cellular and metabolic processes, and the rest 12 miRNA families may be involved in other processes. For example, miR168 and miR2910 may have a role in plant reproduction, whereas miR160, miR2014 and miR2916 might be associated with signal transduction. Using gene ontology analysis, Mao et al. [23] reported that abscisic acid and salicylic acid stimulus might be regulated by miR159 and miR858 in cucumber. Furthermore, according to gene ontology analysis, 3 miRNAs (miR399, miR1122 and miR2014) were determined to be regulated in response to boron stress.

In conclusion, we identified 32 known and 4 new barley miRNAs, as well as 934 target genes using recently developed degradome analysis. The majority of the identified miRNAs were significantly responsive to boron stress in barley. In particular, the signal transduction mechanism in leaves regulated by miR408 plays an important role in boron tolerance in barley consistent with previous reports [40,60].

Supporting Information

**Figure S1** The sequences, additional properties, and stem-loop secondary structure of pre-miRNAs of *Hordeum vulgare* (DOC)

**Table S1** MicroRNA guided cleavage sites by degradome analysis. (DOCX)

**Table S2** Primers used for miRNA validation and measurement detected in this study. (DOCX)
| Table S3 | Primers used for target mRNA validation and measurement detected in this study. |
| --- | --- |

Author Contributions
Conceived and designed the experiments: TU. Performed the experiments: EO VE. Analyzed the data: TU VE SS AI SO HB. Contributed reagents/materials/analysis tools: TU SO BZ. Wrote the paper: TU EO SO BZ.

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