Identification and Profiling of microRNAs and Their Target Genes in Tibetan Hulless Barley in Response to Barley Leaf Stripe (BLS) Infection

Xiaohua Yao

Qinghai University

Yue Wang

Qinghai University

Youhua Yao

Qinghai University

Yixiong Bai

Qinghai University

Kunlun Wu

Qinghai University

Youming Qiao (✉ qhyoumingqiao@126.com)

Qinghai University

Research

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Abstract

Background

Tibetan hulless barley is widely grown on the Qinghai-Tibet Plateau, where it has served as a staple food for Tibetan people since the 5th century CE. Barley leaf stripe (BLS) is one of the most severe fungal diseases affecting the yield and quality of Tibetan hulless barley.

Results

Here, we compared the miRNA profiles before and after BLS in two Tibetan hulless barley genotypes: Z1141, a BLS-sensitive wild variety, and Kunlun14, a BLS-tolerant hybrid variety. A total of 36 conserved and 56 novel miRNAs were identified. Of these, 10 conserved and 10 novel miRNAs exhibited significantly changed expression between the normal and infected leaves of Kunlun14, respectively, while 3 conserved and 5 novel miRNAs exhibited significantly changed expression between the normal and infected leaves of Z1141, respectively. A total of 24 miRNAs were found in Z1141 and Kunlun14, and a further 546 putative target genes were predicted. Transcriptome sequencing analysis showed that among the 546 candidate genes, 131 had significant differences in expression between the normal and infected leaves of Kunlun14 and Z1141. Gene ontology, pathway, and Blast analyses indicated 10 candidate target genes that were involved in the barley disease resistance. These 10 candidate target genes may be regulated by 7 miRNAs. According to quantitative real-time PCR results, the 10 targets were negatively correlated with their corresponding miRNAs after infection with BLS.

Conclusions

The miRNAs and their target genes expressed in Tibetan hulless barley were identified and found to be associated with BLS resistance. Thus, these miRNAs and their target genes may be exploited via breeding programs or genetic engineering to improve BLS resistance in Tibetan hulless barley.

Background

Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) is a self-reproducing annual species that produces naked grains. This plant is widely cultivated on the Qinghai-Tibet Plateau and has served as a staple food for the Tibetan people since the 5th century CE [1, 2]. Grains of Tibetan hulless barley not only contain abundant nutritional components and physiologically-active compounds, but also are rich in edible pigments necessary the food manufacturing industry [3, 4].

Barley leaf stripe (BLS) is a serious fungal disease affecting the yield and quality of Tibetan hulless barley in the Qinghai-Tibet plateau [5]. BLS is generally carried by grains and infects the leaves, sheaths, and spikes of host plants[6]. BLS is common in the main cultivation areas of Tibetan hulless barley in

China, including Tibet, Qinghai, Gansu, Sichuan, and Yunnan [7]. Plant resistance of BLS is considered a complex trait, involving many disease resistance genes [8, 9, 10]. Quantitative trait locus (QTL) analysis is an efficient way to analyze some key traits when breeding for barley disease resistance [11, 12]. For example, applying QTL analysis to progeny crossed by BLS-resistant and -susceptible varieties identified two disease resistant genes, *Rdg1a* and *Rdg2a*, which are located on the 2HL and 7HS chromosomes, respectively [8, 9, 13, 14]. Overexpression of these two genes markedly enhanced the resistance of barley to BLS [15, 16, 17]. However, only a few genes have been discovered via QTL analysis. This suggests that we may have reached a bottleneck in the study of BLS through traditional gene mapping analysis, and thus new methods and perspectives are needed to explore novel plant genes directly or indirectly related to BLS resistance and resistance mechanisms.

MicroRNA (miRNA) is a type of non-coding small RNA consisting of 21–24 nt that plays various critical roles in the growth and development of plants, including how plants respond to abiotic and biotic stressors, such as pests and diseases [18, 19, 20]. High-throughput sequencing of microRNA has become a fast, efficient, informative, and cost-effective strategy for gene discovery [21, 22, 23]. Some miRNAs and their target genes contribute critically to regulating plant disease. For example, miR393 responds to bacterial disease by negatively regulating the auxin receptor genes TIR1, AFB2, and AFB4 [24]. Some artificial miRNAs (amiRNAs) in barley participate in regulating yellow dwarf disease under low temperature stress conditions [25], while in tomato plants, miR403 can regulate leaf-shortening disease by targeting the *AGO2 gene* [26]. Researchers analyzed miRNA the changes in cotton roots infected by verticillium wilt and found that the expression levels of at least 65 miRNAs changed, most of which were non-conservative miRNAs [27]. It was discovered that viral-inducible *OsAGO18* sequesters miR168 to ease the repression of rice *OsAGO1* by miR168 to enable antiviral defense in infected rice [28] .

With the development of high-throughput sequencing, increasing numbers of plant miRNAs are being discovered. We are currently aware of 47,618 mature miRNAs covering 271 species, of which 10,414 mature miRNAs from 82 plant species have been identified in the miRNA database (<http://www.mirbase.org/V21.0>). Among the latter, 812 miRNAs originate from *Arabidopsis*, 325 from maize, 738 from rice, 241 from sorghum, and 126 from wheat. By contrast, only 71 miRNAs have been found in barley, and not a single miRNA has yet been reported from Tibetan hulless barley. Here, to fill this knowledge gap and explore disease-resistant miRNAs, high-throughput sequencing was used to construct a miRNA database of disease resistance and susceptible Tibetan hulless barley varieties. From our results, eight miRNAs and 10 candidate target genes related to BLS resistance were found and their expression patterns were analyzed in detail. Our study results not only provide a data foundation for the functional verification of BLS resistance genes, but also provide information for the future selection and breeding of new disease-resistant Tibetan hulless barley varieties.

Materials And Methods

Plant materials and BLS treatment.

Wild Tibetan hulless barley accession 'Z1141' and the hybrid variety 'Kunlun14' were used to investigate BLS resistance. The proportion of field area (per square meter) of Kunlun14 and Z1141 was calculated from 2015 to 2017. Kunlun14 is a cultivar that can tolerate BLS, while Z1141 is susceptible to BLS (Fig. 1). In this study, we used ZS, ZN, KL14S, and KL14N, as experimental materials. First, the infected leaves of Z1141 were collected to construct the infection system of BLS in the laboratory. To complete this process, five pieces (5 mm × 5 mm) of one typical infected leaf from Z1141 were isolated, rinsed with 75% alcohol for 30 s and 2% sodium hypochlorite for 30 s, and then washed with distilled water three times. Finally, they were cultivated in a sealed Petri dish filled with potato dextrose agar (PDA) culture medium (200 g of dissolved peeled potato, 17 g of glucose, 16 g of agar to 1000 mL with distilled water) after drying off. The dish was placed in a biochemical incubator at 22–25 °C for 3 d. Non-contaminated mycelia were transferred to a new PDA culture dish four times until the colony was purified. First, three ensuing fungal cakes were removed with a perforator and cultivated in another new PDA culture medium, which was then placed in a biochemical incubator at 25 °C for 5–7 d until the whole Petri dish was filled with the BLS colony. Second, 300 grains of each Tibetan hulless barley were disinfected with 75% alcohol for 1 min, and then 150 grains were equally placed into three Petri dishes covered with the BLS colony, and another 150 grains were equally placed in three Petri dishes covered with the PDA culture medium without BLS colony. The above six dishes were then placed into a low-temperature incubator for two weeks at 6 °C. Finally, 300 grains of each variety were respectively planted in six flowerpots with vegetative soil and cultured in an artificial climate incubator (daytime: 14 h, at 20 °C; nighttime: 10 h, at 10 °C) for approximately 10 d. The second infected or normal leaf from the bottom of the seedling was removed, and leaves with at least three biological repeats were refrigerated at – 80 °C after freezing in liquid nitrogen.

Small RNA library construction and sequencing.

The total RNA of each leaf was extracted using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and the RNA purity was assessed using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). The RNA concentrations were then measured using the Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, MD, USA). The RNA integrity of each sample was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA integrity was further checked through a 1% agarose gel analysis. All RNA samples were then sent to Novogene (Beijing, China) to construct libraries for small RNA sequencing, where single-end reads (SE50) were sequenced on an Illumina HiSeq 2500 platform (Illumina Inc., CA, USA).

A total amount of 3 µg total RNA per sample was used as the input material for the small RNA library. Sequencing libraries were generated using the NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA), following the manufacturer's recommendations, with index codes added to label each sample with its corresponding sequences. PCR amplifications were performed using the Long Amp Taq 2X Master Mix, SR Primer for Illumina, and index (X) primer. The ensuing PCR products were then purified by 8% polyacrylamide gel analysis. DNA fragments corresponding to 140–160 bp—the length of

small non-coding RNA plus the 3' and 5' adaptors—were recovered and dissolved in 8 µL elution buffer. Lastly, library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. The clustering of index-coded samples was performed on a cBot Cluster Generation System that used the TruSeq SR Cluster Kit v3-cBot-HS (Illumina), following the manufacturer's instructions. After completing the cluster generation, the library preparations were sequenced on an Illumina HiSeq 2500/2000 platform, and 50-bp single-end reads were generated.

Prediction of miRNA.

The small RNA tags were mapped to reference sequences by Bowtie [29], with mismatching not allowed, in order to analyze their expression and distribution *vis-à-vis* the barley reference genome (ftp://ftp.ensemblgenomes.org/pub/plants/release-36/fasta/hordeum_vulgare/cds/Hordeum_vulgare.Hv_IBSC_PGSB_v2.cds.all.fa.gz) [30]. These reference-mapped small RNA tags were then used to search for known miRNA. The miRBase 22 served as the reference, with modified mirdeep2 [31] and srna-tools-cli software [32] used to obtain the potential miRNA and to draw the secondary structures. Custom scripts were used to obtain the miRNA counts as well as the base bias on the first position of an identified miRNA with a certain length and on each position of all identified miRNA, respectively.

Hairpin structural characteristics of the miRNA precursor may be used to predict novel miRNA. The available software, miREvo [33] and mirdeep2 [31], were integrated to predict novel miRNAs by exploring their secondary structure, dicer cleavage site, and minimum free energy of the small RNA tags non-annotated in the prior steps above. The occurrence of miRNA families identified from the samples in other species was then explored. In this analysis pipeline, for a known miRNA, miFam.dat 22 (<http://www.mirbase.org/ftp.shtml>) was used to search for families; likewise, a novel miRNA precursor was submitted to Rfam 32.0 (<http://pfam.xfam.org/>) to identify Rfam families.

Identification of differentially expressed miRNAs.

The miRNA expression levels were quantified as transcripts per million (TPM) using these criteria [34, 35]: normalization formula: $\text{normalized expression} = \text{mapped readcount} / \text{total reads} * 1000000$. Differential expression analysis of the four treatment groups was performed in the R software environment using the DESeq package (v1.8.3), for which the *P*-values were adjusted using the Benjamini & Hochberg method [36]. A corrected *P*-value of 0.05 was set, by default, as the threshold for a statistically significant level of differential gene expression.

Prediction of miRNA target genes, GO and KEGG pathway analysis.

Predicting the target gene of a given miRNA was carried out using psRNATarget for plants (<http://plantgrn.noble.org/psRNATarget/>) [37]. GO enrichment was conducted to analyze the likely functions of genes targeted by their miRNAs using the GO enrichment tool in PlantRegMap (<http://plantregmap.cbi.pku.edu.cn/>) [38]. Pathway analysis was performed using the Kyoto

Encyclopedia of Genes and Genomes (KEGG) database (<https://www.genome.jp/kegg/>) [38], for which KOBAS (KEGG Orthology Based Annotation System) (<http://kobas.cbi.pku.edu.cn>) was used to test the statistical enrichment of the target gene candidates identified in the KEGG pathways [39].

Validating the expression of BLS-responsive miRNAs and their target genes by qRT-PCR.

The expression of 10 target genes was validated by qRT-PCR. Total RNA per sample was extracted with the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara, Tokyo, Japan) and this RNA was then reverse-transcribed to cDNA using the PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan). The qRT-PCR was run using the TB Green premix Ex Taq II Kit in a Roche LC480 in order to detect the expression of the target genes. Total small RNA per sample was extracted using RNAiso for Small RNA (Takara, Tokyo, Japan), and this RNA was then reverse-transcribed into cDNA and validated using the Mir-X™ miRNA First-Strand Synthesis and TB Green™ qRT-PCR User Manual (Takara, Japan) in a Roche LC480. The primers used for this are listed in Table 4; here, *TC139057* and *5SrRNA* served as internal controls. The cycling parameters were 95 °C for 30 s (1 cycle); 95 °C for 5 s, 60 °C for 30 s (40 cycles); 95 °C for 5 s, 60 °C for 1 min (1 cycle); 40 °C for 5 s (1 cycle). Gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method [40] for which the C_t value is the average of three biological replicates with three technical replicates.

Results

Difference in BLS resistance between Kunlun14 and Z1141 barley.

The proportion of total leaf area that was diseased by BLS in KL14 was 12.80%, while it was nearly four times that in Z1141 (47.43%) (Fig. 1A and B). The proportion of field area (per square meter) diseased by BLS of Kunlun14 was 2.67%, while it was 92.67% for Z1141, based on field experiments conducted from 2015 to 2017 (Fig. 1C and D). The percentages differed significantly between the two barley cultivars ($P < 0.01$), confirming that Kunlun14 is a BLS-resistant cultivar, whereas Z1141 is BLS-susceptible.

Basic statistics of the small RNA data from Tibetan hulless barley libraries.

To identify BLS-related miRNAs in Tibetan hulless barley, small RNA libraries were constructed for Z1141 with (ZS) and without (ZN) BLS, and Kunlun14 with (KL14S) and without (KL14N) BLS. Totals of 15,141,660, 14,167,060, 13,591,121, and 12,992,954 raw reads were respectively generated from ZS, ZN, KL14S, and KL14N by high-throughput sequencing (Table 1). After removing the adaptors and junk reads, this left corresponding totals of 14,608,407, 13,573,623, 13,113,086, and 12,567,992 clean reads. Sequences within the range of 18–30 nt were then further analyzed. The length distributions from the libraries tested were similar overall, with most of the small RNAs being 19 to 24 nt in length. Among them, small RNAs with 20 nt of ZN were the most abundant, comprising 16.14% of the total reads (Fig. 2).

Identification of conserved and novel miRNAs in Tibetan hulless barley.

To identify conserved miRNAs from the small RNA libraries tested, reads with lengths of 18–30 nt were mapped onto *H. vulgare*-conserved miRNAs in the miRBase 22. This detected 36 conserved miRNAs in all the libraries tested, which belonged to 29 miRNA families (Table 2). Different families thus had different numbers of miRNAs. The family of hvu-miR5049 had the most members, with five miRNAs. The expression levels of different miRNAs differed significantly, with read numbers ranging from zero to several thousand. The 10 most highly-expressed miRNAs were hvu-miR159b, hvu-miR156a, hvu-miR5048a, hvu-miR5051, hvu-miR168, hvu-miR171, hvu-miR397a, hvu-miR171, hvu-miR5049e, and hvu-miR6198; their accumulated reads accounted for 99.50% of all total conserved reads. Among them, hvu-miR159b had the highest number of reads, with an average of 4476 per library, amounting to 68.81% of the total conserved reads (Fig. 3A).

After prediction, a total of 56 novel miRNAs were obtained (Table 3). The 10 most highly expressed novel miRNAs were hvu-novel-9, novel-1, hvu-novel-2, hvu-novel-16, hvu-novel-11, hvu-novel-46, hvu-novel-52, hvu-novel-7, hvu-novel-91, and hvu-novel-18, and their accumulative reads accounted for 96.21% of the total conserved reads. Among them, hvu-novel-2 had the highest number of reads, with an average of 68.81 reads in each library, which accounted for 34.31% of the total conserved reads (Fig. 3B).

Identification of miRNAs expressed in two Tibetan hulless barley genotypes infected with BLS.

Expression profiling analysis revealed that the expression of many miRNAs was modulated by BLS. To identify the differentially expressed miRNAs, Venn diagram analysis was used to predict both conserved and novel miRNAs. There were 28, 27, 19, and 22 conserved miRNAs detected in the libraries of KL14N, KL14S, ZN, and ZS, respectively; 12 of these miRNAs were common to the four groups (Fig. 4A; Additional file 1). There were 54, 28, 50, and 45 novel miRNAs detected in the libraries of KL14N, KL14S, ZN, and ZS, respectively. Of these, 25 were common to the four groups (Fig. 4B; Additional file 2). A total of 24 miRNAs differed significantly between KL14N vs. KL14S and ZN vs. ZS ($P < 0.05$) (Fig. 4C; Additional file 3), of which there were 11 conserved miRNAs and 13 novel miRNAs. The expression levels of 24 miRNAs are displayed in a heatmap (Fig. 5; Additional file 4). Based on their fold-change values, 20 miRNAs (including 10 up-regulated miRNAs and 10 down-regulated miRNAs) were found between KL14S and KL14N (Additional file 5), and eight miRNAs were detected between ZS and ZN (six up-regulated miRNAs and two down-regulated miRNAs; Additional file 5). Ultimately, 24 differentially expressed miRNAs were found, of which 11 were conserved miRNAs (hvu-miR6199, hvu-miR168-5p, hvu-miR6198, hvu-miR5049c, hvu-miR171-5p, hvu-miR156a, hvu-miR6181, hvu-miR397a, hvu-miR6195, hvu-miR168-3p, and hvu-miR159b) and 13 were novel miRNAs (hvu-novel-43, hvu-novel-119, hvu-novel-1, hvu-novel-45, hvu-novel-20, hvu-novel-111, hvu-novel-52, hvu-novel-46, hvu-novel-79, hvu-novel-94, hvu-novel-9, hvu-novel-11, and hvu-novel-91) (Fig. 4C). The differing miRNA expression patterns emphasized the uniqueness of the various miRNA responses to BLS; hence, this suggests that BLS differentially affects miRNA expression in Tibetan hulless barley cultivars with differing disease resistance.

Target gene prediction and analysis of 24 BLS-responsive miRNAs.

The psRNATarget server predicted 546 target genes of BLS-responsive miRNAs in Tibetan hulless barley that were identifiable for the 24 miRNAs (Additional file 6). Predicted target genes were classified and enriched via gene ontology (GO) and KEGG analyses. GO was used to describe the functional categories of the annotated unigenes and classify the transcripts with known annotated proteins. $P \leq 0.05$ was used as the threshold to determine significantly enriched GO terms. GO classification based on sequence homology revealed that 427 out of all the assembled genes were categorized (Additional file 7). The top 30 enriched GO terms are indicated in Fig. 6, which shows that the target genes of the BLS-responsive miRNAs were included in all three categories: biological process, cellular component, and molecular function. Specifically, the two most enriched categories were heterocyclic compound binding (207 genes) and organic cyclic compound binding (207 genes). Also, a term of copper ion binding (GO:0005507) was significantly enriched in 14 genes, most of which were *laccase* gene family members (Additional file 8). This indicated that *laccase* genes may play an important role in the response of Tibetan hulless barley to BLS infection.

A pathway-based analysis was performed using the KEGG pathway database to further elucidate the biological functions and interactions of the gene. We analyzed 546 target genes predicted by the KEGG database. A total of 75 genes were assigned to 64 pathways (Additional file 9). The three main pathways with the highest number of genes were glycerophospholipid metabolism, amino sugar and nucleotide sugar metabolism, and starch and sucrose metabolism. Top 20 enriched KEGG pathways are indicated in Fig. 7, one pathway of which was plant-pathogen interaction, which included four genes (Gene ID: HORVU4Hr1G051540, HORVU3Hr1G079210, HORVU7Hr1G008170, and HORVU7Hr1G055790). These annotations provide valuable information for investigating the disease-resistant pathway involved in BLS.

Transcriptome analysis showed that a total of 131 genes were significantly changed in Kunlun14 and Z1141 under BLS infection (Additional file 10). Among these genes, we concentrated on the functional genes and transcription factors possibly related to disease-resistant traits according to conserved domain analysis (<http://pfam.xfam.org/search>) and gene annotation at Swiss-Prot (<http://www.gpmaw.com/html/swiss-prot.html>), Uniprot (<https://www.uniprot.org/>), and NCBI (<https://www.uniprot.org/>). Additionally, considering the negative regulatory relationship between miRNA and target genes and that the relative transcript levels were greater than 20 in one library of KL14N, KL14S, ZN, or ZS (according to the results of Additional file 10), we found seven functional genes and three transcription factors associated with disease resistance. The reference genome sequence information of the candidate genes is shown in Additional file 11. Two members of the *P450* genes (Gene ID: HORVU5Hr1G096940 and HORVU5Hr1G096950) were found. Three members of the *WRKY* transcription factor genes (Gene ID: HORVU1Hr1G090190, HORVU4Hr1G011590, and HORVU7Hr1G083270) were found. *RGA* gene (Gene ID: HORVU4Hr1G027270), *LIN* (Gene ID: HORVU2Hr1G104640), *SAM* (Gene ID: HORVU3Hr1G067760), *PSD* (Gene ID: HORVU4Hr1G088470), and *NDB* (Gene ID: HORVU7Hr1G073050) were found. The miRNAs of these target genes were hvu-miR168-3p, hvu-miR156a, hvu-miR171-5p, hvu-miR159b, hvu-novel-91, hvu-novel-46, hvu-novel-52, and hvu-novel-11.

Expression levels of candidate miRNAs and their target genes in the barley response to BLS.

To validate the differential expression patterns of the BLS-responsive miRNAs, eight miRNAs and 10 target genes were randomly selected and their expression patterns were analyzed by quantitative real-time PCR (qRT-PCR) (Fig. 8). It was observed that the expression patterns of eight miRNAs were correlated with results obtained in the small RNA sequencing (RNA-Seq) study (Additional file 4). We also validated the expression of 10 target genes identified as BLS-responsive in this study. It was observed that the expression patterns of 10 target genes were correlated with results obtained in the RNA-Seq study (Additional file 4). Comparison of the expression patterns of miRNAs with their respective target genes showed that the steady-state levels of the miRNA transcripts were inversely related to the expression patterns of their target genes. For instance, the expression of the target gene (Gene ID: HORVU4Hr1G027270) was observed to be downregulated in contrast to the expression of the target miR168-3p, which was upregulated in the leaves after infection with BLS (Fig. 8A). On the contrary, the expression of the target genes (Gene ID: HORVU3Hr1G067760, HORVU4Hr1G088470, HORVU1Hr1G090190, HORVU7Hr1G083270, HORVU4Hr1G011590, HORVU5Hr1G096940, HORVU5Hr1G096950, and HORVU7Hr1G073050) was observed to be upregulated in contrast to the expression of their target miRNAs (hvu-miR171-5p, hvu-miR159b, hvu-novel-91, hvu-novel-46, hvu-novel-52, and hvu-novel-11) that were downregulated in the leaves after infection with BLS (Fig. 8C–J).

Discussion

Increasing numbers of miRNAs are being annotated in various plant species [41, 42]; however, few have been reported for barley [43, 44]. miRNAs played important roles in the process of plant resistance to disease [18]. In order to better understand the roles of miRNAs in the process of disease resistance, we aimed both to computationally identify different Tibetan hulless barley miRNAs and measure the expression of miRNAs before and after infection with BLS. After infection with BLS, a total of 24 differentially expressed miRNAs were identified, and many of their target genes, such as transcription factor WRKY, NBS-LRR resistance protein, and Cytochrome P450 were found to play important roles in plant growth, development and disease-resistant. Regarding the identification of Tibetan hulless barley miRNAs, 10 known miRNA families (hvu-miR156, hvu-miR168, hvu-miR6198, hvu-miR159, hvu-miR397, hvu-miR171, hvu-miR6195, hvu-miR5049, hvu-miR6199, and hvu-miR6181) and 13 novel miRNAs (hvu-novel-1, hvu-novel-11, hvu-novel-43, hvu-novel-45, hvu-novel-46, hvu-novel-52, hvu-novel-79, hvu-novel-91, hvu-novel-111, hvu-novel-119, hvu-novel-9, hvu-novel-20, and hvu-novel-94) differed significantly before and after infection with BLS, which was inferred to be related to the resistance of Tibetan hulless barley to BLS. Many of the 10 identified known miRNAs were known for their reported roles in responses to plant disease and other biotic and abiotic stresses [26].

The miR168 of barley was first isolated in 2010 [45], and it was later isolated from rice [46], barley [47], and cucumber [48] under drought, cadmium, aluminum, and salt stress. A promoter analysis using transgenic *A. thaliana* plants revealed the transcriptional activation of miR168 by fungal elicitors [49], which was upregulated to modulate leaf morphology and plant growth, signified its positive role during leaf curl virus in tomato [26]. In this study, the predicted target gene of hvu-miR168-3p was *IBR3* (Gene ID: HORVU4Hr1G027270), which was located on chromosome 4H and annotated to encode an acyl-CoA

dehydrogenase of *A. thaliana* in Swiss-Prot. In NCBI, HORVU4Hr1G027270 was annotated to encode a clone HV_Mba184-F11 of *H. vulgare* (GenBank accession no. AC256280.1) and LZ-NBS-LRR class *RGA* of *Aegilops tauschii* (GenBank accession no. AF446141.1). Nucleotide sequence consistency of them was 90.61% and 78.98%, respectively. Plants evolved a complex innate immune system that recognized specific pathogens via a specific group of gene products called resistance (R) proteins [50]. *RGAs* were involved in plant disease resistance [51, 52]. Thus, *RGA* appears to be crucial to the response of plant disease resistance. In this study, the expression of miR168-3p and its target gene HORVU4Hr1G027270 had opposite trends in the leaves after infection with BLS, which suggested that HORVU4Hr1G027270 might be regulated by hvu-miR168-3p during BLS-resistant of Tibetan hulless barley.

Kantar et al. suggested that miRNA156a in barley leaves responded to dehydration stress and played an important role in stress adaptation [45] (Kantar et al. 2010). miR156a targeted the squamosa promoter-binding protein-like genes, which played an important role in plant growth, development and abiotic stress [53]. In citrus, the overexpression of miR156a or knockout of its target genes *SPL3* and *SPL14* significantly increased the activity of wild kumquat callus [54]. In this study, the predicted target gene of hvu-miR156a was *LIN* (Gene ID: HORVU2Hr1G104640), which was located on chromosome 2H and annotated to encode an E3 ubiquitin-protein ligase of *Medicago truncatula* in Swiss-Prot. In NCBI, HORVU2Hr1G104640 was annotated to encode a predicted protein (NIAShv2044K07) of *H. vulgare* (GenBank accession no. AK366598.1), an E3 ubiquitin-protein ligase LIN-2 of *Zea mays*, and an E3 ubiquitin-protein ligase LIN-1 of *Brachypodium distachyon* (GenBank accession no. XM_010242010.3). Nucleotide sequence consistency of them was 99.95%, 84.88%, and 78.67%, respectively. In legumes, *LIN* controlled early infection by rhizobia and worked exclusively during rhizobial colonization [55]. In this study, the expression of hvu-miR156a and its target gene HORVU2Hr1G104640 had opposite trends, which suggested that HORVU2Hr1G104640 might be regulated by hvu-miR156a during BLS-resistant of Tibetan hulless barley.

miR171 was reported in pear and tomato and proved to vital for plant growth and development [56, 57]. In barley, overexpressing miR171 would lead to phase transitions and floral meristem determinacy [58]. In Arabidopsis, the expression of miR171 increased but the target gene AtSCL6-II decreased when both miR171 and AtSCL6-II were co-express transiently [59]. In addition, another study showed that miR171 overexpression altered the transition from vegetative to reproductive stage by activating the miR156 pathway [59]. Thus, the relationship between miR156 and miR171 in the regulation of BLS remains to be further studied. In this study, the predicted target gene of hvu-miR171-5p was *DDB_G0268948* (Gene ID: HORVU3Hr1G067760), which was located on chromosome 3H and annotated to encode a methyltransferase of *Dictyostelium discoideum* in Swiss-Prot. In NCBI, HORVU3Hr1G067760 was annotated to encode a predicted protein (NIAShv2113006) of *H. vulgare* (GenBank accession no. AK370649.1) and an S-adenosylmethionine-dependent methyltransferase of *Aegilops speltoides* (GenBank accession no. FJ236274.1) and *Triticum urartu* (GenBank accession no. FJ236273.1). Nucleotide sequence consistency of them was 99.73%, 93.76%, and 94.11%, respectively. S-adenosyl methionine (SAM), a widespread biological cofactor which was found in all aspects of life where it played very important roles in the transfer of methyl groups to DNA, proteins and small-molecule secondary

metabolites [60]. The methylation process played important roles in various disease processes and industrial chemical processing [60]. SAM-dependent methylation affected mating through altering the pheromone signaling pathway of the maize smut pathogens (*Ustilago maydis*) [61]. The second target gene of miR171 was HORVU4Hr1G088470, which was located on chromosome 4H and annotated to encode a phosphatidylserine decarboxylase proenzyme 1 of *Oryza sativa* L. *subsp. japonica* in Swiss-Prot. In NCBI, HORVU4Hr1G088470 was annotated to encode a BAC 615K1 of *H. vulgare* (GenBank accession no. AY485643.1) and a phosphatidylserine decarboxylase (PSD) of *Triticum monococcum* (GenBank accession no. AY485644.1). Nucleotide sequence consistency of them was 100% and 94.44%, respectively. PSD activity in oat (*Avena sativa*) root cells was involved in the changes of phospholipid composition of cell membrane during domestication under drought stress [62]. In this study, the expression of hvu-miR171 and its target genes HORVU3Hr1G067760 and HORVU4Hr1G088470 had opposite trends in the leaves after infection with BLS, which suggested that HORVU3Hr1G067760 and HORVU4Hr1G088470 might be regulated by hvu-miR171-5p during BLS-resistant of Tibetan hulless barley.

In this study, the targets of three miRNAs (hvu-novel-91, hvu-novel-46 and hvu-miR159b) were WRKYs. WRKY proteins was a large family of transcription factors in plants, which was involved in the regulation of plant development, aging and stress resistance [63, 64]. In rice, WRKY genes were involved in the resistance of the rice blast fungus *Pyricularia oryzae* [65]. Yokotani et al. showed that an *OsWRKY24* gene was upregulated in response to rice blast [66]. In Arabidopsis, the overexpression of *WRKY45* or *WRKY1* enhanced disease resistance and drought tolerance [44, 67]. In wheat, the overexpression of *WRKY45* enhanced disease resistance to multiple fungi [68]. In Swiss-Prot, the target of hvu-novel-91 was a WRKY (Gene ID: HORVU7Hr1G083270), which was located on chromosome 7H and annotated to encode a WRKY46 transcription factor of *A. thaliana*. The target of hvu-novel-46 was a WRKY (Gene ID: HORVU4Hr1G011590), which was located on chromosome 4H and annotated to encode a WRKY1 transcription factor of *Zea mays*. Furthermore, the target of hvu-miR159b was also a WRKY (Gene ID: HORVU1Hr1G090190), which was located on chromosome 1H and annotated to encode a WRKY53 transcription factor of *A. thaliana*. In NCBI, HORVU7Hr1G083270 was annotated to encode WRKY transcription factor 146 in *Triticum* (GenBank accession no. MF770640.1), WRKY transcription factor 70 in *Aegilops* (GenBank accession no. XM_020292626.1), and WRKY transcription factor 11 in *Aegilops* (GenBank accession no. EU665440.1), but a predicted protein in *H. vulgare* (AK360029.1). The nucleotide sequence consistency of these was 94.83%, 94.83%, 94.83%, and 87.96%, respectively. HORVU4Hr1G011590 was annotated to encode four predicted proteins (NIASHv2089N03, NIASHv1011A24, NIASHv3105H03, and FLbaf169m16) in *H. vulgare* (GenBank accession no. AK369358.1, AK354776.1, AK375802.1, and AK248640.1) and WRKY transcription factor 1 in *Aegilops* (GenBank accession no. XM_020293740.1), the nucleotide sequence consistency of which was 99.09%, 98.47%, 98.77%, 98.77%, and 89.94%, respectively. HORVU1Hr1G090190 was annotated to encode WRKY transcription factor 1 in *H. vulgare* (GenBank accession no. DQ863110.1) and WRKY transcription factor 55 in *Aegilops* (GenBank accession no. XM_020341128.1). Nucleotide sequence consistency of them was 100.00% and 87.077%, respectively. Research showed that miR159 played an important role in

disease symptom induction by a severe strain of *Cucumber mosaic virus* [69]. The inhibition of Fny-CMV-induced symptoms in *Arabidopsis* containing mutant alleles for the targets *MYB33* and *MYB65* of miR159ab confirmed the importance of miR159 in pathogenesis [69]. Phukan et al. showed *WRKY* included the WRKYGQK motif followed by a Cx4 5Cx22 23HxH or Cx7Cx23HxC zinc-finger motif [70]. In this study, the conserved domain results revealed that HORVU7Hr1G083270, HORVU4Hr1G011590, and HORVU1Hr1G090190 possess WRKY DNA-binding domains that belong to *WRKY* transcription factors. Most of the *WRKY* genes studied were found to be involved in the disease-resistant of plants. In apple, for example, *MdWRKY26* can induce eight pathogenesis-related (PR) genes to enhance host resistance to leaf spot disease [64]; *MdWRKY31* regulates plant resistance to *Botryosphaeria dothidea* through the salicylic acid signaling pathway by interacting with *MdHIR4* [71]. In *Arabidopsis*, the overexpression of *AtWRKY33* accelerated and intensified resistance to necrotrophic fungi [72]. These findings strongly suggested that *WRKY* functions critically in plant resistance to fungal disease. In our study, the expression of hvu-novel-91 was downregulated, while the expression of *WRKYs* was upregulated in the leaves after infection with BLS. The expression of miRNAs and their target genes had opposite trends in the leaves after infection with BLS, which suggested that HORVU7Hr1G083270, HORVU4Hr1G011590, and HORVU1Hr1G090190 might be regulated by hvu-novel-91, hvu-novel-46m, and hvu-miR159b during BLS-resistant of Tibetan hulless barley.

In Swiss-Prot, the target genes of hvu-novel-52 included *CYP81D11a* (Gene ID: HORVU5Hr1G096940) and *CYP81D11b* (Gene ID: HORVU5Hr1G096950), which were located on chromosome 5H and annotated to encode Cytochrome P450 (CYP450) in *A. thaliana*. In NCBI, HORVU5Hr1G096940 was annotated to encode a CYP450 in *Triticum* (GenBank accession no. KJ541960.1), a *CYP81B1v3* in *Lolium rigidum* (GenBank accession no. AB159039.1), a *CYP81A22* in *Echinochloa phyllopogon* (GenBank accession no. AB872310.1), and two predicted proteins in *H. vulgare* (AK363181.1 and AK375492.1). Nucleotide sequence consistency of them was 89.44%, 89.74%, 83.92%, 99.90%, and 95.65%, respectively. HORVU5Hr1G096950 was annotated to encode two predicted proteins (NIASHv3095M21 and NIASHv2013E24) in *H. vulgare* (GenBank accession no. AK375492.1 and AK363181.1), a CYP450 in *Triticum* (GenBank accession no. KJ541960.1), a *CYP81B1v3* in *L. rigidum* (GenBank accession no. AB159039.1), and a *CYP81A22* gene in *Echinochloa phyllopogon* (GenBank accession no. AB872310.1). Nucleotide sequence consistency of them was 99.39%, 95.34%, 93.75%, 89.21%, and 82.38%, respectively. Additionally, the conserved domain results showed that HORVU5Hr1G096940 and HORVU5Hr1G096950, possessing cytochrome P450 domains, belong to the CYP450 family. In rice, a CYP-like protein encoded by Os08g01480 helps plants to resist abiotic stresses [73]. In *Arabidopsis*, the enzymes encoded by *CYP94B3* and *CYP94C1* could promote the decomposition and inactivation of the plant hormone jasmonyl isoleucine complex, which affects the jasmonic acid metabolism pathway and thus affects the expression of stress-resistant genes [74]. In this study, the expression of hvu-novel-52 was downregulated, while the expression of HORVU5Hr1G096940 and HORVU5Hr1G096950 was upregulated in the leaves after infection with BLS, which suggested that HORVU5Hr1G096940 and HORVU5Hr1G096950 might be involved in the BLS disease process via the activity of hvu-novel-52 during BLS-resistant of Tibetan hulless barley.

Hvu-novel-11 targeted HORVU7Hr1G073050, which was located on chromosome 7H and annotated to encode the external alternative NAD(P)H-ubiquinone oxidoreductase B3 (NDB3) protein of in *A. thaliana*. In NCBI, HORVU7Hr1G073050 was annotated to a predicted protein (NIASHv2009M23) of *H. vulgare* (GenBank accession no. AK362739.1) and an external alternative NAD(P)H-ubiquinone oxidoreductase B2 (NDB2) of *Aegilops* (GenBank accession no. XM_020292911.1). Nucleotide sequence consistency of them was 99.80% and 93.42%, respectively. A series of treatment groups representing specific AOX and NDH genes were identified in response to mitochondrial inhibition, plastid inhibition and other abiotic stresses [75]. In this study, the expression of hvu-novel-11 was downregulated, while that of HORVU7Hr1G073050 was upregulated in the leaves after infection with BLS, and the expression of KL14S was significantly higher than that of ZS. This result suggested that HORVU7Hr1G073050 might be involved in the BLS disease process via the activity of hvu-novel-11 during BLS-resistant of Tibetan hulless barley.

Conclusion

In summary, 4 known miRNAs and 4 novel miRNAs were revealed in this empirical study, the potential roles of which in BLS resistance might involve the regulation of downstream target genes. The expression levels of 8 miRNAs and 10 target genes were tested, suggesting that these miRNAs and their target genes jointly played a vital role in the defense response of Tibetan hulless barley to BLS, although their relationship in the process of resistance to BLS requires further investigation and elucidation. Fourteen *laccase* genes from GO analysis and four plant-pathogen interaction genes from the KEGG analysis were deleted because the expression of these genes did not change significantly in the result of RNA-Seq; their function requires further study in the future. This study may improve our understanding of miRNA regulation in the response of Tibetan hulless barley to BLS infection in crop fields.

Abbreviations

QTL: quantitative trait locus; BLS: purple seed coat color; miRNA: microRNA; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PDA: potato dextrose agar; TPM: transcripts per million; KOBAS: KEGG Orthology Based Annotation System; KL14N: 'Kunlun14' without barley leaf stripe; KL14S: 'Kunlun14' with barley leaf stripe; ZN: 'Z1141' without barley leaf stripe; ZS: 'Z1141' with barley leaf stripe; RNA-Seq: RNA sequencing; SD: standard deviation; qRT-PCR: quantitative real-time PCR.

Declarations

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Availability of data and material

The data supporting the findings of this study are available within the article.

Authors' contributions

XHY and WY designed the research protocol, analyzed the data, carried out the experiment, and drafted the manuscript. YHY, and YXB assisted in the data analysis, supervised the protocol and prepared the manuscript. KLW and QYM finally approved the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Summary of small RNA sequencing data in four libraries from Tibetan hullless barley

Type	KL14N		KL14S		ZN		ZS	
	Count	Percentage (%)	Count	Percentage (%)	Count	Percentage (%)	Count	Percentage (%)
Raw reads	12992954	100	13591121	100	14167060	100	15141660	100
Clean reads	12567992	96.73	13113086	96.48	13573623	95.81	14608407	96.48
Total sRNA	8483600	65.29	7094377	52.20	7377096	52.07	8930242	58.98
Mapped sRNA	7698518	59.25	6055386	44.55	6643849	46.90	6554917	43.29
rRNA	3806241	29.29	3892847	28.64	3808842	26.89	4402208	29.07
tRNA	263883	2.03	151163	1.11	147841	1.04	230688	1.52
snRNA	2060	0.02	5209	0.04	1863	0.01	5210	0.03
snoRNA	6195	0.05	4168	0.03	3739	0.03	4789	0.03
miRNA	22408	0.17	2057	0.02	14273	0.10	5600	0.04
Exon	113098	0.87	69152	0.51	72516	0.51	54421	0.36
Intron	46160	0.36	5025	0.04	25199	0.18	9020	0.06
TAS	26	0.00	8	0.00	8	0.00	2	0.00
Repeat	293923	2.26	76478	0.56	195502	1.38	71723	0.47
Other ncRNA	1826849	14.06	1020473	7.51	1534211	10.83	1315953	8.69

KL14N = 'Kunlun14' without barley leaf stripe; KL14S = 'Kunlun14' with barley leaf stripe; miRNA = microRNA; TAS = ta-siRNA; ZN = 'Z1141' without barley leaf stripe; ZS = 'Z1141' with barley leaf stripe.

Table 2. The conserved miRNAs in Tibetan hulless barley

miRNA family	miRNA	Sequence	Length	Accession number
miR1120	hvu-miR1120	ACAUUCUUAUAUUAUGGGACGGAG	24	MIMAT0018499
miR1436	hvu-miR1436	ACAUUAUGGGACGGAGGGAGU	21	MIMAT0018501
miR156	hvu-miR156a	UGACAGAAGAGAGUGAGCACA	21	MIMAT0018207
miR159	hvu-miR159b	UUUGGAUUGAAGGGAGCUCUG	21	MIMAT0018209
miR168	hvu-miR168-3p	GAUCCCGCCUUGCACCAAGUGAAU	24	MIMAT0018216
	hvu-miR168-5p	UCGCUUGGUGCAGAUCCGGGAC	21	MIMAT0018215
miR171	hvu-miR171-5p	UGUUGGCUCGACUCACUCAGA	21	MIMAT0022971
	hvu-miR171-3p	UGAUUGAGCCGUGCCAAUAUC	21	MIMAT0018220
miR397	hvu-miR397a	CCGUUGAGUGCAGCGUUGAUG	21	MIMAT0018224
	hvu-miR397b-3p	AUCAACGCUGCACUCAACGGC	21	MIMAT0035814
miR444	hvu-miR444b	UGCAGUUGCUGUCUCAAGCUU	21	MIMAT0020543
miR5048	hvu-miR5048a	UAUUUGCAGGUUUUAGGUCUAA	22	MIMAT0020544
miR5049	hvu-miR5049a	UCCUAAAUACUUGUUGUUGGG	21	MIMAT0020545
	hvu-miR5049c	AGACAAUUAUUUUGGGACGGAGG	23	MIMAT0024803
	hvu-miR5049d	UACAAUUAUUUAGGAACGGAG	21	MIMAT0024813
	hvu-miR5049e	AAUUAUUUAGGUACAGAGGGA	21	MIMAT0024826
	hvu-miR5049f	AAUUAUAUGGAUCGGAGGGA	21	MIMAT0024829
miR5051	hvu-miR5051	UUUGGCACCUUGAAACUGGGA	21	MIMAT0020547
miR5052	hvu-miR5052	ACCGGCUGGACGGUAGGCAUA	21	MIMAT0020548
miR6177	hvu-miR6177	UACCAUGGACAGAAGGCACUUA	22	MIMAT0024800
miR6180	hvu-miR6180	AGGGUGGAAGAAAGAGGGCG	20	MIMAT0024804
miR6181	hvu-miR6181	UGCUCUUCAUGGACUGCGGCGCC	23	MIMAT0024805
miR6182	hvu-miR6182	UGAGUGUGUGAUGGAUGGCUUU	22	MIMAT0024806
miR6183	hvu-miR6183	UGAGCGAGUUGGCUGCAAGUUC	22	MIMAT0024807
miR6186	hvu-miR6186	CGAGGAAGGCGCUGAGAGAGA	21	MIMAT0024810
miR6191	hvu-miR6191	UAGAUUUGUCUAGAUUAUGAA	20	MIMAT0024816
miR6194	hvu-miR6194	UAUGGGGAUCUGACAGACGAG	21	MIMAT0024820
miR6195	hvu-miR6195	UGAGUACGUAGUAGGGAUGAG	21	MIMAT0024821

miR6198	hvu-miR6198	GCUCUGUCUUGGAUGGUCAUUC	22	MIMAT0024824
miR6199	hvu-miR6199	CCACAGAAUUCUCACAGUGAUGG	23	MIMAT0024825
miR6200	hvu-miR6200	UUUGGCCAACUAGAUCUAUGA	21	MIMAT0024827
miR6201	hvu-miR6201	UGACCCUGAGGCACUCAUACCG	22	MIMAT0024828
miR6202	hvu-miR6203	AGACGAUUAGAAGACCUGCAA	22	MIMAT0024831
miR6207	hvu-miR6207	UGGACGACCUGGGCGCCGACG	21	MIMAT0024835
miR6213	hvu-miR6213	ACAGAUUGCUCACAGACUGGUC	21	MIMAT0024841
miR6214	hvu-miR6214	CGACGACGACGAGCACGACA	20	MIMAT0024842

miRNA = microRNA.

Table 3. The novel miRNAs identified in this study of Tibetan hulless barley

Novel miRNAs	Sequence	Length
hvu-novel-1	UGAAGCUGCCAGCAUGAUCUGC	22
hvu-novel-100	UUCACGGGUCUGGACAAUA	19
hvu-novel-102	GCGUAUCUAGAUCGUUGAUGAUGU	24
hvu-novel-107	AGACGUCUCUCGUGUAGAAUAGG	24
hvu-novel-11	UCAGUGCGAUCCCUCUGGAAU	21
hvu-novel-111	AUAAUGUAGGACGUGUUUUGA	21
hvu-novel-119	AAAUCGGUGAUCUAGAACUACGCU	24
hvu-novel-12	UGACAGAAGAGAGUGAGCAC	20
hvu-novel-16	UUCAGGAGAGAUGACACCGACA	22
hvu-novel-18	UGCACUGCCUCUUCCCUGGC	20
hvu-novel-2	UCCACAGGCUUUCUUGAACUG	21
hvu-novel-20	UUCCACAGCUUUCUUGAACUG	21
hvu-novel-22	UGGAGAAGCAGGGCACGUGCA	21
hvu-novel-27	ACGACUGCGCGUACAUCGGACGGA	24
hvu-novel-28	GGAGUGUCUCUGAGAAAACAAG	22
hvu-novel-30	GUGUGCUGUAUCGGCUGUUGUUCU	24
hvu-novel-31	CCGCGGUAGAACAGAACCCACAUC	24
hvu-novel-33	CGUGGCCUACUGACCUGACGCGUC	24
hvu-novel-34	ACGAUCUAGAACUACGCGCAUGCC	24
hvu-novel-37	GAAUGCAUGCAGUCUACUAGUAGU	24
hvu-novel-38	UAGCCAAGGAUGAUUUGCCUGUG	23
hvu-novel-39	CGCAUGAAUGGAUCAAUGA	19
hvu-novel-42	AUGACGUUGGUGCUUGCAUUUUCC	24
hvu-novel-43	AUAAUGUAGGACGUGUUUUGAC	22
hvu-novel-45	UUUAGGACAAGUAUUUCCAGACGG	24
hvu-novel-46	AAACUGUGGCUGUCAAUUUUGGC	24
hvu-novel-48	AGGAUAUGAUGGCUCGCGGACGCG	24
hvu-novel-52	UUCCACAGCUUUCUUGAACUU	21

hvu-novel-55	GAAGACGUGCCUGGCUGUGUUACC	24
hvu-novel-56	AGUGCAACGACUGAGGCUUAGGCG	24
hvu-novel-57	UUUCCUGAUUUUGUUUUCACCGAGC	24
hvu-novel-58	GUUCUUUGCCGUCUGUAGUACGAA	24
hvu-novel-59	AGCGACUGUCCAUAGAUGAAAGC	24
hvu-novel-60	UCUGGUAGGCCUGUGUGUACGACC	24
hvu-novel-61	AGAAUAUGAGGGCUCGCGGACGCG	24
hvu-novel-65	AGCUCGUUUGAUCCUGAUG	19
hvu-novel-68	UGAAGCUGCCAGCAUGAUCUAAAC	23
hvu-novel-69	AGGAUAUGAGGGUUCAUGGACGUG	24
hvu-novel-7	UGAAGCUGCCAGCAUGAUCUGA	22
hvu-novel-70	GUUCUAUUGGACUGUCUGACCAGU	24
hvu-novel-72	AAUUGACGAUCUAGAUUAUGUGC	24
hvu-novel-73	GUGUAGCUCGCCGGUGAUUUGAUC	24
hvu-novel-74	AUGAGAACUUUGAAGACU	18
hvu-novel-76	AUCAGGAGAGAUGACACCGGC	21
hvu-novel-79	AAGGUCUCUGACGUCUGGUACGGG	24
hvu-novel-80	AUUAGACUGUGUGACGCCUUAGA	23
hvu-novel-81	CAACUAUUUCCGGACUGAGGGAGU	24
hvu-novel-83	UGACUGAAUUUUGCACUA	18
hvu-novel-86	CAAUUAACGACCUAGAACUACGCG	24
hvu-novel-87	AAGCUACAGUCGGGCCAGACCGGU	24
hvu-novel-88	AGGGACGAUCCGGUUACUAAAC	22
hvu-novel-89	AGCGAAGCCGACGGAUGCAUGACC	24
hvu-novel-9	UUGAACAUCCCAGAGCCACCG	21
hvu-novel-91	AAUUAGACGGUCUAGAUUGCGAGU	24
hvu-novel-94	CCGUCGUCGACAUGGCUG	18
hvu-novel-98	AAAUGUGAUCUGUACAAAAAAGAC	24

Table 4. Primer sequences used in the present study

MicroRNAs/Genes	Primer name	Primer sequences
hvu-miR168-3p	Forward-Primer	5'-GATCCCGCCTTGAC-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-miR156a	Forward-Primer	5'-GCAGTGACAGAAGAGAGTGA-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-miR171-5p	Forward-Primer	5'-GTGTTGGCTCGACTCAC-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-miR159b	Forward-Primer	5'-TTTGGATTGAAGGGA-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-novel-91	Forward-Primer	5'-AATTAGACGGTCTAGATT-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-novel-46	Forward-Primer	5'-AAACTGTGGCTGTCAAAT-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
hvu-novel-52	Forward-Primer	5'-GCAGTTCCACAGCTTTCTTG-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
Hvu-novel-11	Forward-Primer	5'-TCAGTGCGATCCCTC-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
5SrRNA (internal)	Forward-Primer	5'-GTCTGCCTGGGTGTCACGCAA-3'
	Universal Reverse	5'-GTGCAGGGTCCGAGGT-3'
HORVU4Hr1G027270	Forward-Primer	5'-TCTACCTGCCGTTCTAAGT-3'
	Reverse-Primer	5'-CCGTCCATACCAACATCAA-3'
HORVU2Hr1G104640	Forward-Primer	5'-CTCTGCGTGGAAGATTCA-3'
	Reverse-Primer	5'-TGATGAGCTGGACTGACT-3'
HORVU3Hr1G067760	Forward-Primer	5'-GGTCTACATACGGAGCAA-3'
	Reverse-Primer	5'-ACGGATGGAGTAATAAGCA-3'
HORVU4Hr1G088470	Forward-Primer	5'-GCTACTAATGTTGGCTCTATC-3'
	Reverse-Primer	5'-TGAATCCTGCTACCTCTTG-3'
HORVU7Hr1G083270	Forward-Primer	5'-TACACAACCTCCAAGCACTC-3'
	Reverse-Primer	5'-TACTCAGCACCTCCTCCT-3'

HORVU4Hr1G011590	Forward-Primer	5'-CGATGCCTTAATTGCTACG-3'
	Reverse-Primer	5'-TGTGATTGCCATTGCTGTA-3'
HORVU1Hr1G090190	Forward-Primer	5'-TCATCAGCATCACTACACA-3'
	Reverse-Primer	5'-GACACATCACCATCCTCTT-3'
HORVU5Hr1G096940	Forward-Primer	5'-GAAGGTGGTGGACGAGAT-3'
	Reverse-Primer	5'-TGAGACGATGGAGGAAGG-3'
HORVU5Hr1G096950	Forward-Primer	5'-CGAAGCCGACAAGAAGAG-3'
	Reverse-Primer	5'-TTCAGAAGCAGCGACATAG-3'
HORVU7Hr1G073050	Forward-Primer	5'-TTACGGAATGGCTGTCTG-3'
	Reverse-Primer	5'-TTACTTGCGGATACCTCAC-3'
<i>TC139057</i> (internal)	Forward-Primer	5'-GAAGGATGAGCAAAAGGCCCT-3'
	Reverse-Primer	5'-GGCAGGCAGACTCATTCTTCC-3'

Figures

Figure 1

Visible differences in leaves infected by BLS in two genotypes of Tibetan hulless barley plants. a, Phenotype of KL14N, KL14NS, ZN, and ZS. b, The ratio of diseased leaf area to total leaf area of KL14N, KL14NS, ZN, and ZS. c, Phenotype of the epidemic area of Kunlun14 and Z1141. d, the epidemic area of Kunlun14 and Z1141. Bars in B and D are means $\pm$ SD of three biological replicates; means labeled with different letters are significantly different at $P < 0.01$ (Tukey's test). BLS = barley leaf stripe; KL14N = 'Kunlun14' without barley leaf stripe; KL14S = 'Kunlun14' with barley leaf stripe; SD = standard deviation; ZN = 'Z1141' without barley leaf stripe; ZS = 'Z1141' with barley leaf stripe.

Figure 2

Length distribution of small RNAs in the control and BLS-infected leaves of Z1141 and Kunlun14. BLS = barley leaf stripe; KL14N = 'Kunlun14' without barley leaf stripe; KL14S = 'Kunlun14' with barley leaf stripe; ZN = 'Z1141' without barley leaf stripe; ZS = 'Z1141' with barley leaf stripe.

Figure 3

Abundance of the 10 most highly expressed conserved and novel miRNAs in the KL14N, KL14NS, ZN, and ZS Tibetan hulless barley libraries. a, Conserved miRNAs. b, Novel miRNAs. KL14N = ‘Kunlun14’ without barley leaf stripe; KL14S = ‘Kunlun14’ with barley leaf stripe; miRNA = microRNA; ZN = ‘Z1141’ without barley leaf stripe; ZS = ‘Z1141’ with barley leaf stripe.

Figure 4

Venn diagrams of Tibetan hulless barley miRNAs identified in the four libraries. A, Conserved miRNAs. B, Novel miRNAs. C, Venn diagrams of miRNAs before and after BLS infection of the Kunlun14 and Z1141 plants’ leaves. BLS = barley leaf stripe; KL14N = ‘Kunlun14’ without barley leaf stripe; KL14S = ‘Kunlun14’ with barley leaf stripe; miRNA = MicroRNA; ZN = ‘Z1141’ without barley leaf stripe; ZS = ‘Z1141’ with barley leaf stripe.

Figure 5

Heatmap showing the differentially expressed miRNAs of KL14N, KL14S, ZN, and ZS. The color scale indicates the log2-transformed values of the measured fold changes. KL14N = 'Kunlun14' without barley leaf stripe; KL14S = 'Kunlun14' with barley leaf stripe; miRNA = microRNA; ZN = 'Z1141' without barley leaf stripe; ZS = 'Z1141' with barley leaf stripe.

Figure 6

GO enrichment analysis of the target genes of four miRNAs responsive to BLS in Tibetan hulless barley. BLS = barley leaf stripe; GO = gene ontology; miRNA = microRNA.

Figure 7

KEGG analysis of target genes of four miRNAs responsive to BLS in Tibetan hulless barley. BLS = barley leaf stripe; KEGG = Kyoto Encyclopedia of Genes and Genomes; miRNA = microRNA.

Figure 8

Comparison of expression levels of eight miRNAs and ten target genes in leaves of KL14N, KL14S, ZN and ZS by qRT-PCR. The red histogram represented the relative expression values of miRNA by qRT-PCR, the black line represented the relative expression values of target genes by qRT-PCR. a–j, qRT-PCR analysis of miRNAs and their target genes responded to BLS. The 5SrRNA and TC139057 were used as internal gene of miRNAs and target genes. The data displayed in the histograms are expressed as the

means $\pm$ SD. BLS = barley leaf stripe; KL14N = 'Kunlun14' without barley leaf stripe; KL14S = 'Kunlun14' with barley leaf stripe; miRNA = microRNA; qRT-PCR = quantitative real-time PCR; SD = standard deviation; ZN = 'Z1141' without barley leaf stripe; ZS = 'Z1141' with barley leaf stripe.

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