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Identification of microRNAs and their corresponding targets involved in the susceptibility interaction of wheat response to *Puccinia striiformis* f. sp. *tritici*

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MicroRNAs (miRNAs) play very important roles in plant defense responses. However, little is known about their roles in the susceptibility interaction between wheat and *Puccinia striiformis* f. sp. *tritici* (*Pst*). In this study, two miRNA libraries were constructed from the leaves of the cultivar Xingzi 9104 inoculated with the virulent *Pst* race CYR32 and sterile water, respectively. A total of 1316 miRNA candidates, including 173 known miRNAs that were generated from 98 pre-miRNAs, were obtained. The remaining 1143 miRNA candidates included 145 conserved and 998 wheat-specific miRNAs that were generated from 87 and 1088 pre-miRNAs, respectively. The 173 known and 145 conserved miRNAs were sub-classified into 63 miRNA families. The target genes of wheat miRNAs were also confirmed using degradome sequencing technology. Most of the annotated target genes were related to signal transduction or energy metabolism. Additionally, we found that miRNAs and their target genes form complicated regulation networks. The expression profiles of miRNAs and their corresponding target genes were further analyzed by quantitative real-time polymerase chain reaction (qRT-PCR), and the results indicate that some miRNAs are involved in the compatible wheat-*Pst* susceptibility interaction. Importantly, tae-miR1432 was highly expressed when wheat was challenged with CYR32, and the corresponding target gene, predicted to be a calcium ion-binding protein, also exhibited upregulated expression but a divergent expression trend. PC-3P-7484, a specific wheat miRNA, was highly expressed in the wheat response to *Pst* infection, while the expression of the corresponding target gene ubiquillin was dramatically downregulated. These data provide the foundation for evaluating the important regulatory roles of miRNAs in wheat-*Pst* susceptibility interaction.

Introduction

MicroRNAs (miRNAs) are endogenous, non-coding small RNAs (sRNA) with typical length of 21–24 nucleotides (nt), which repress protein translation or degrade target mRNAs in eukaryotic organisms at the post-transcription level (Bartel 2004). Since the first discovery of plant miRNAs in Arabidopsis, miRNAs are reported to be involved in diverse plant processes including organ development, phase change, signal

Abbreviations – GO, gene ontology; hpi, hours post-inoculation; KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA; nt, nucleotides; PTI, pattern-triggered immunity; qRT-PCR, quantitative real-time polymerase chain reaction; sRNA, small RNA; XZ, Xingzi9104.

transduction, biogenesis regulation and stress responses etc. (Reinhart et al. 2002, Jones-Rhoades et al. 2006). Arabidopsis miR393, induced by the infection of plant pathogen *Pseudomonas syringae*, was the first reported miRNA involved in pathogen-associated molecular pattern-triggered immunity (PTI) by repressing auxin signaling (Navarro et al. 2006). Meanwhile, miR160 and miR167 were found to be induced by *P. syringae* infection and contributed to PTI by regulating auxin response factors (ARF) transcription factors (Fahlgren et al. 2007). miRNAs were also demonstrated to have divergent expression profiles when plants were infected with different viruses (Lang et al. 2011). Gupta et al. (2012) also found that eight wheat miRNAs showed diverse transcript profiles when resistant and susceptible wheat cultivars were challenged with stem rust. Interestingly, when poplar was infected with *Botryosphaeria dothidea*, none of its miRNAs were downregulated (Zhao et al. 2012). In 2013, Campo et al. (2013) identified a novel miRNA, osa-miR7695, which affected the plant resistance by regulating the innate immunity related macrophage protein 6 (OsNramp6). In the next year, Li et al. (2014) identified the miRNAs of resistance and susceptible cultivars from *Magnaporthe oryzae*, and they observed that the over-expression of miR160a and miR398b could improve the rice resistance with the inhibition of hypha growth. All these reports indicate that miRNAs play important roles in the interactions between plant and pathogens.

During the interactions between plants and pathogens, resistance and susceptibility have opposite functions. However, most studies have focused on the plant resistance mechanism, including resistance genes, defense genes and protein non-coding RNAs. Until 2002, Vogel et al. (2002) found that when the *PMR6* gene was knocked out, the resistance of *Arabidopsis* to powdery mildew was increased. This led to a question of whether a specific gene was essential for the successful infection of pathogens (Eckardt 2002, Pavan et al. 2010). The *MLO* gene was previously identified as a resistance gene. However, in subsequent studies, *MLO* was demonstrated to be an essential factor for the successful infection of powdery mildew to barley. *MLO* improved the plant susceptibility to powdery mildew through the down-regulated *PEN* gene (Hardham et al. 2007). In tobacco, the over-expression of barley RAC3 and RACB could improve the susceptibility to powdery mildew (Pathuri et al. 2009). When the *ROP6* gene was knocked out, the resistance of *Arabidopsis* to powdery mildew was improved (Poraty-Gavra et al. 2013). Meanwhile, the FERONIA receptor-like kinase (FER), which was confirmed to regulate the expression of *ROP6*, was also demonstrated to promote pathogen infection of plants (Kessler et al. 2010). All of these results indicate that there is a complex interaction among the susceptible genes, and this complex interaction can regulate the plant susceptibility. In recent years, miRNAs were also found that composed a miRNA-regulatory network to regulate the growth and stress responses (Galimov et al. 2015, Liu et al. 2015, Yao et al. 2015). However, there is still no report on the exploration of miRNAs and the corresponding complex regulatory network contributing to plants susceptibility to pathogens.

Wheat stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive wheat diseases worldwide. To control it, a breeding program with rational utilization of resistance varieties is still the most efficient way (Chen 2005). Therefore, understanding the mechanism of interactions between wheat and *Pst* will greatly facilitate the developing strategies of improving wheat cultivar resistance. Questions such as whether miRNAs also function in the compatible interaction between plant and virulent pathogen remains to be answered. In previous studies, several wheat miRNAs involved in various stress responses, such as heat stress, powdery mildew infection, drought stress and wheat rust infection, have been reported (Xin et al. 2010, Kantar et al. 2011, Gupta et al. 2012). Among these reports, miRNAs showed considerable specificity. The function of wheat miRNAs requires further analysis.

Seedlings of wheat cultivar Xingzi9104 (XZ) showed a typical compatible phenotype when challenged with *Pst* race CYR32 (Zhang et al. 2012). In this study, we shed light on the differentially accumulated miRNAs during this biological process by high-throughput sequencing. Corresponding target genes for these miRNAs were further detected by degradome sequencing. Identification of miRNAs and their targets provide a foundation of determining the miRNA-mediated regulatory networks during compatible interaction between wheat cultivar XZ and virulent *Pst* race CYR32.

Materials and methods

Plant materials and treatments

Wheat cultivar XZ, and *Pst* pathotype CYR32 were used in this study. XZ is an elite wheat germplasm possessing susceptibility to CYR32 at seedling stage (Zhang et al. 2012). Seeds were sown in pots and placed in a growth chamber under the following conditions: 16 ± 2°C with supplemental light for 16 h day^{-1} and water as needed. At the two-leaf stage, the first leaves were inoculated with fresh urediospores of *Pst* CYR32 (ST-I) or sterile water (ST-M) using a paintbrush. After inoculation, the plants were maintained in dark for 24 h with 100% relative humidity, and they were subsequently transferred

to a growth chamber under the same settings as above. Leaf tissues were sampled at 0, 24, 48 and 120 h post-inoculation (hpi) for RNA extraction.

Construction of RNA libraries and degradome sequencing

The total RNA for each sample was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the instructions. Samples were treated with DNAse I to remove contaminating genomic DNA for 30 min at 37°C. The RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

As wheat and *Pst* showed an intensive interaction at the initial infection from 24 to 48 hpi in our previous histological observation (Wang et al. 2007, Zhang et al. 2012), two miRNA libraries (ST-M and ST-I) were constructed using RNAs from 24 hpi, respectively. sRNAs (14–28 nt) were isolated from total RNA on a 15% denaturing polyacryamide gel electrophoresis (PAGE) gel and made ready for high-throughput sequencing. Sequencing was performed using an Illumina Genome Analyzer following the Illumina protocol. The 24 hpi RNA was also used for miRNA target identification by degradome sequencing. The purified cDNA library was used for cluster generation on Illumina's Cluster Station followed by sequencing on an Illumina GSIIx following the vendor's instructions. For each library, two biological replicates were included.

Small RNA sequencing data and miRNA abundance profile analysis

Sequencing data was processed using ACGT101-miR software (LC Sciences, Houston, TX) for hairpin prediction. Briefly, the raw reads were subjected to the Illumina pipeline filter (Solexa 0.3), and then the dataset was further processed with an in-house program, ACGT101-miR (LC Sciences, Hangzhou, China) to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA and snoRNA) and repeats. Subsequently, unique sequences 18–25 nt in length were mapped to wheat species precursors in miRBase 21.0 by BLAST search to identify known miRNAs and novel 3p- and 5p-derived miRNAs. Length variation at the 3′ and 5′ ends and one mismatch inside of the sequence were allowed in the alignment. The unique sequences mapping to wheat mature miRNAs in hairpin arms were identified as known miRNAs. The unique sequences mapping to the other arm of known wheat species precursor hairpin opposite to the annotated mature miRNA-containing arm were considered to be novel 5p- or 3p-derived miRNA candidates. The remaining sequences were mapped to other selected species precursors (*Triticum turgidum*, *Hordeum vulgare*, *Oryza sativa*, *Brachypodium distachyon*, *Sorghum bicolor*, *Zea mays*, *Aegilops tauschii*, *Saccharum* sp., *Saccharum officinarum*, *Festuca arundinacea*, *Elaeis guineensis*, *Brassica napus*, *Brassica rapa*, *Medicago truncatula*, *Glycine max*, *Populus trichocarpa*, *Arabidopsis thaliana*, *Gossypium raimondii*, *Solanum tuberosum*, *Malus domestica*, *Arabidopsis lyrata*, *Prunus persica*, *Vitis vinifera*, *Nicotiana tabacum*, *Manihot esculenta*, *Linum usitatissimum*, *Cucumis melo*, *Theobroma cacao*, *Carica papaya*, *Gossypium hirsutum*, *Solanum lycopersicum*, *Ricinus communis*, *Lotus japonicus*, *Citrus sinensis*, *Cynara cardunculus*, *Aquilegia caerulea*, *Rehmannia glutinosa*, *Hevea brasiliensis*, *Panax ginseng*, *Arachis hypogaea*, *Vigna unguiculata*, *Salvia sclarea*, *Helianthus tuberosus*, *Glycine soja*, *Digitalis purpurea*, *Brassica oleracea*, *Phaseolus vulgaris*, *Helianthus annuus*, *Citrus trifoliata*, *Citrus clementina*, *Bruguiera cylindrica*, *Bruguiera gymnorhiza*, *Citrus reticulata*, *Populus euphratica*, *Helianthus argophyllus*, *Helianthus ciliaris*, *Helianthus paradoxus*, *Helianthus petiolaris*, *Helianthus exilis*, *Avicennia marina*, *Gossypium arboreum*, *Gossypium herbaceum*, *Selaginella moellendorffii*, *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Picea abies*, *Pinus taeda*, *Pinus densata*, *Cunninghamia lanceolata*, *Acacia auriculiformis*, *Acacia mangium*) in miRBase 21.0 by BLAST search, and the mapped pre-miRNAs were further BLASTed against the wheat genomes to determine their genomic locations. The unmapped sequences were BLASTed against the wheat genome database (ftp://ftp.plantgdb.org/download/Genomes/TaGDB/TAbac175.bz2) and the mRNA database (ftp://ftp.plantgdb.org/download/Genomes/TaGDB/TAest175.bz2), and the hairpin RNA structures containing sequences were predicated from the flank 120-nt sequences using RNAFOLD software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

High-throughput sequencing abundance profile analysis was based on the sequence read number of each library. The first step was to normalize the miRNA sequence reads in the *Pst*-inoculated wheat plants and control plants to tags per million. The calculation of the *P*-value to compare miRNA expression between the different groups (ST-M and ST-I) was based on previously established methods (Audic and Claverie 1997). All calculations were performed on the BGI Bio-Cloud Computing platform (http://cloud.genomics.org.cn). miRNA tags per million of <1 were filtered out of both libraries.

Wheat degradome sequence analysis

Raw sequencing reads were obtained using Illumina's PIPELINE v1.5 software following sequencing image

analysis by Pipeline Firecrest Module and base-calling by Pipeline Bustard Module. The extracted sequencing reads were analyzed with the public software package CleaveLand 3.0 and BLASTed with XZ wheat transcripts generated from the same sample batch. Annotation of candidate target genes was performed using the Blast2GO Gene Ontology Functional Annotation Suite (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG). To predict the genes that were targeted by the most abundant miRNAs, computational target prediction algorithms (TargetFinder) were also used to identify miRNA binding sites. The GO terms and KEGG pathway of the most abundant miRNA targets were also annotated.

Primer design, cDNA synthesis and qRT-PCR

The procedures for the reverse transcription of miRNAs were as previously described (Feng et al. 2012). First-strand cDNA synthesis of target genes was carried out according to the manufacturers' instructions using the Promega RT-PCR system (Promega, Madison, WI) with the Oligo $(dT)_{18}$ primer. The translation elongation factor 1 alpha-subunit (EF) gene (GenBank accession no. M90077) was used as a control. Quantitative real-time polymerase chain reaction (qRT-PCR) assays were carried out using a CFX96 Real-Time System (Bio-Rad) with SYBR Green I (Invitrogen) as described before (Feng et al. 2011). Relative transcript levels of miRNAs and corresponding target genes in the *Pst*-inoculated plants at each time point were calculated as the fold-change vs mock-inoculated plants using the comparative $2^{-\Delta\Delta CT}$ method. Experiments were conducted in triplicate using newly extracted RNA for each replicate. All primers are listed in Table S5.

Results

Overview of the sRNA sequencing results

Using Illumina high-throughput sequencing, two sRNA libraries of XZ (ST-I and ST-M) were constructed. The distinct libraries (ST-I and ST-M) generated 11 897 494 and 10 848 949 raw reads, respectively (Fig. S1A, Supporting information). After removing low-quality and junk sequences (<15 nt), a total of 11 702 020 and 10 647 345 mapped reads were used for further analysis. Additionally, these mapped reads were compared with wheat mRNAs using Rfam and Repbase. After discarding 4 667 796 and 4 334 148 reads, excluding 4 141 327 and 3 279 374 no-hit sequences, the remaining sequences were analyzed for miRNA prediction (Fig. S1B). Among these sRNAs, most sRNAs were 21-nt and 24-nt in length. Meanwhile, the nucleotide bias position analysis showed that the first nucleotide of the miRNA candidates generally tended to be uracil (U) (Fig. 1).

Identification and bioinformatic analysis of miRNAs in wheat response to *Pst*

To explore and identify miRNAs that were specifically expressed when wheat was challenged with *Pst*, we compared the miRNAs in the mock (ST-M) and the test (ST-I) libraries. A total of 1533 and 1525 miRNAs were predicted in ST-I and ST-M, respectively (Fig. 2). Most miRNAs were co-expressed in ST-I and ST-M with only 37 and 29 miRNAs that were specifically expressed in ST-I and ST-M, respectively. We then mapped unique sRNAs to known wheat miRNAs (both conserved and non-conserved) in miRBase database with less than four mismatches. In total, we have identified 24 conserved miRNAs from 12 families and 149 non-conserved ones from 64 families (Table S1).

In addition to detecting known wheat miRNAs, the high-throughput sequencing data also enabled the discovery of novel miRNAs. We mapped unique sequences to the miRNAs of selected plants, such as rice, barley and maize, using miRBase; 145 sRNA could be mapped to the selected plants. The extended sequences could potentially form hairpins and could also be mapped to the *Triticum aestivum* genome. One hundred-six conserved miRNAs from 18 miRNA families were identified in wheat. An additional 39 non-conserved miRNAs from 21 miRNA families were also detected (Table S2).

Other than the data described above, a large number of sequences remained unmapped to selected miRNAs in miRBase. Clean reads, excluding reads mapped to mRNA, Rfam and Repbase, were further mapped to wheat genome database with no mismatches. We have annotated a total of 998 novel wheat miRNAs with their extended sequences for hairpins formation (Table S3).

We also compared relative expression of miRNAs in *Pst*-infected wheat to mock-inoculated controls using the reads' abundance. Identified wheat miRNAs showed various expression profiles in our comparison (Fig. 3).

Overview of the degradome sequencing results and target genes annotation

In higher plants, most miRNAs function by cleaving their corresponding targets, and the cleavage commonly occurs at the 10^{th} nucleotide of the complementary region between the miRNA and the mRNA (Jones-Rhoades et al. 2006). In this study, a high-throughput degradome sequencing library (DS-I) was constructed to predict the target genes of miRNA

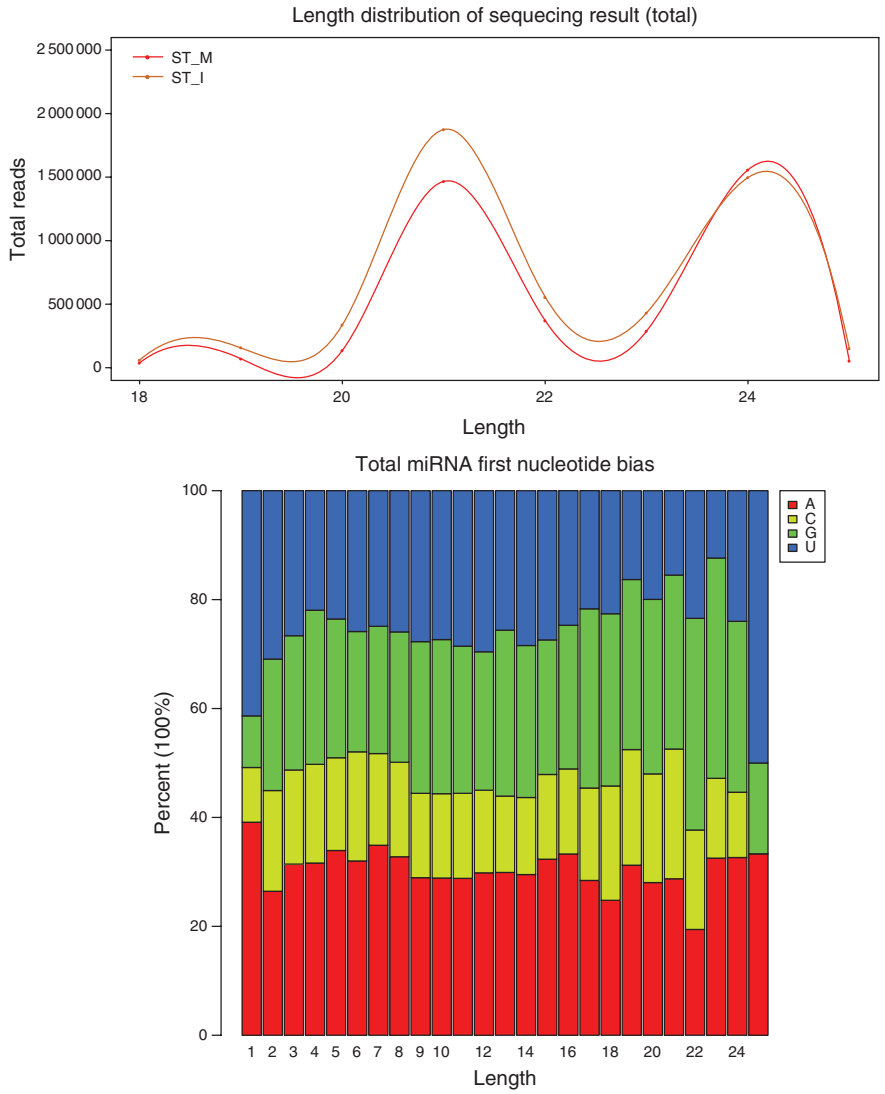


Fig. 1. Length distribution and nucleotide bias in each position of miRNAs in wheat.

during compatible interaction between wheat cultivar Xingzi9104 and *Pst* race CYR32. All of the miRNA candidates were mapped as described before (Addo-Quaye et al. 2009), 1394 unigenes with 3949 cleavage sites were predicted as the target genes of 570 miRNAs (Table S4). From the results of the degradome sequencing, we found that large numbers of miRNA targets remained undetected. Among them, the target genes of 16 known wheat miRNAs were identified, such as tae-miR398, which plays a role in regulating the expression of cytosolic Cu/Zn superoxide dismutase. However, the one-to-one regulation model was not prevalent, many miRNAs and targets constructed a complex regulation network (Fig. 4). This finding indicates that the regulatory mechanisms of miRNAs were very complex.

To further explore the detailed molecular mechanism of miRNAs in the compatible interaction between XZ and *Pst*, the transcripts, which were predicted as the targets of miRNAs, were aligned by BLASTX to the NCBI non-redundant protein database (nr) and the Swiss-Prot protein database using a cut-off E-value of 10^{-5} for the functional annotation (Fig. S2A). We found that few of the selections were predicted as known plant genes, and BLASTX results for Swiss-Prot protein database and nr database showed mutual hits. Gene functions of unigenes were classified by Gene ontology (GO) enrichment analysis. High confidence (similarity >80%) gene annotations for DS-I library from *H. vulgare*, *B. distachyon* and *O. sativa* were listed (Fig. S2B, C). These results indicated that the annotation was reliable. The

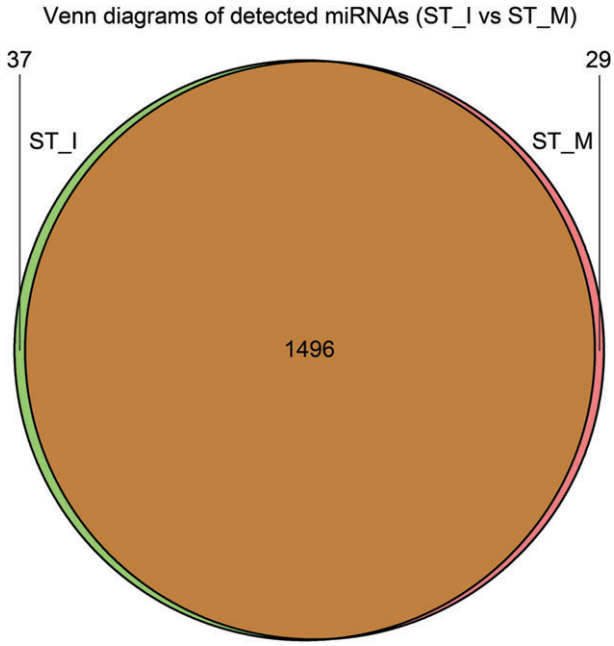


Fig. 2. Venn diagrams of detected miRNAs according to sequencing read number.

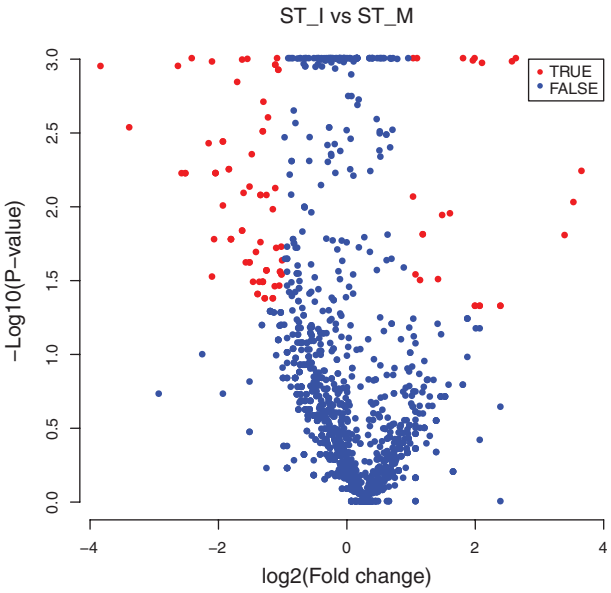


Fig. 3. Volcano plot of differentially expressed miRNAs in wheat cultivar Xingzi 9104 challenged with *Pst*. For each miRNA, sequence reads were divided by the total sequence number then multiplied by 1 000 000 (reads per million).

Argonaute protein (AGO) annotation indicated that the target genes were involved in various cellular processes, such as protein phosphorylation, transcription regulation, oxidation-reduction, ATP-binding, transferase activity, protein binding, protein kinase activity, DNA binding and catalytic activity (Fig. 5A). The KEGG pathway classification indicated that the target genes could be categorized into six functional groups. The candidate targets involved many important physiological and biochemical metabolisms, and our targets were mainly focused on carbohydrate metabolism, amino acid metabolism and energy metabolism (Fig. 5B). It is worth noting that the targets of miRNAs in wheat belonged to the mitogen-activated protein kinase (MAPK) signaling pathway, fatty acid degradation, oxidative phosphorylation, cell cycle, ubiquitin-mediated proteolysis and galactose metabolism (Table 1; Fig. S3). These pathways are known to be involved in the stress response, and these annotations provided a valuable resource for investigating specific processes, especially in plant–pathogen interactions. Further validation experiments for miRNAs and predicted corresponding target genes should be carried out in the future.

Expression profiles of wheat miRNAs and corresponding target genes during *Pst* infection

According to the results of bioinformatic analysis, we found that several miRNAs showed diverse expression trends in the wheat response to *Pst*. To explore the expression of miRNAs in wheat during *Pst* infection, 12 miRNAs were selected for qRT-PCR analysis (Fig. 6). Ata-miR1432-5p, tae-miR1138-p5, ata-miR1432-3p, PC-3p-7484, tae-miR1847-p3 and ata-miR5084-p5 were upregulated during the *Pst* infection process, and ata-miR1432-5p and PC-3p-7484 showed noticeable induction. Compared with the control, ata-miR1432-5p showed a stable upregulated trend with 7.28, 15.12 and 7.05-fold upregulation at 24, 48 and 120 hpi, respectively. The expression of PC-3p-7484 also showed similar trend, with 33.30, 52.69 and 41.78-fold upregulation. Meanwhile, tae-miR9670-p5, tae-miR159a-p5, tae-miR9779, PC-3p-28137, PC-3p-92379 and PC-5p-112398 showed a stable expression during the entire infection progress of *Pst*.

According to the results of degradome sequencing, we detected the targets of ata-miR1432-5p and PC-3p-7484. The targets of ata-miR1432-5p were calcium ion-binding protein family members, and the target genes of PC-3p-7484 were ubiquillin, mitochondrial-processing peptidase, tubby-like F-box protein and a hypothetical protein.

To confirm whether the targets of ata-miR1432-5p and PC-3p-7484 were involved in the compatible interaction between XZ and CYR32, the expression trends of all miRNA targets were analyzed using qRT-PCR (Fig. 7). The expression of

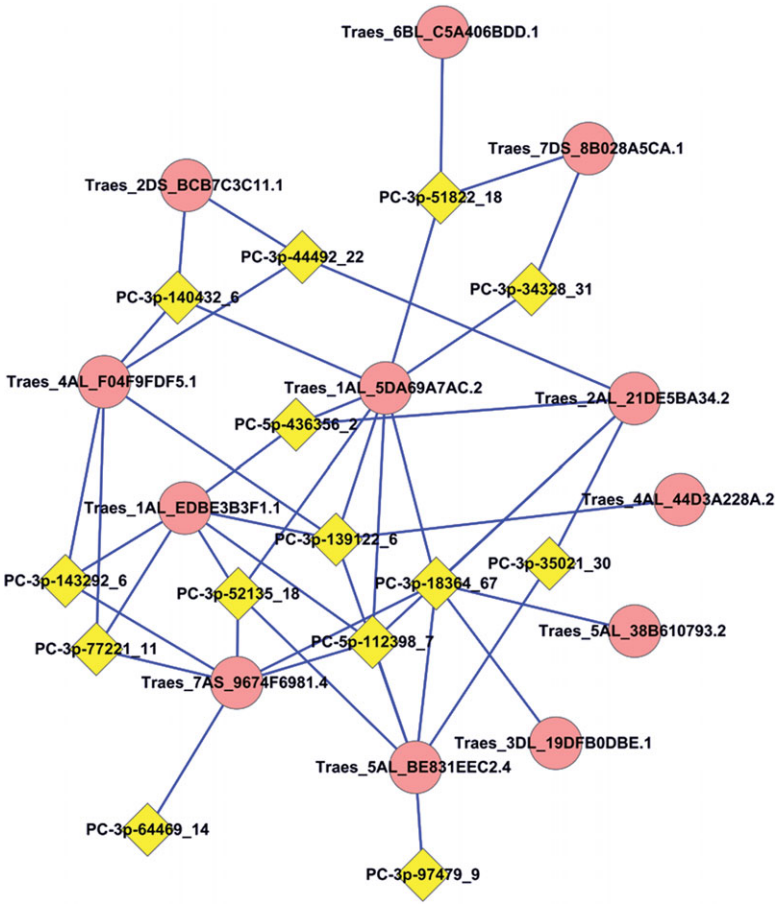
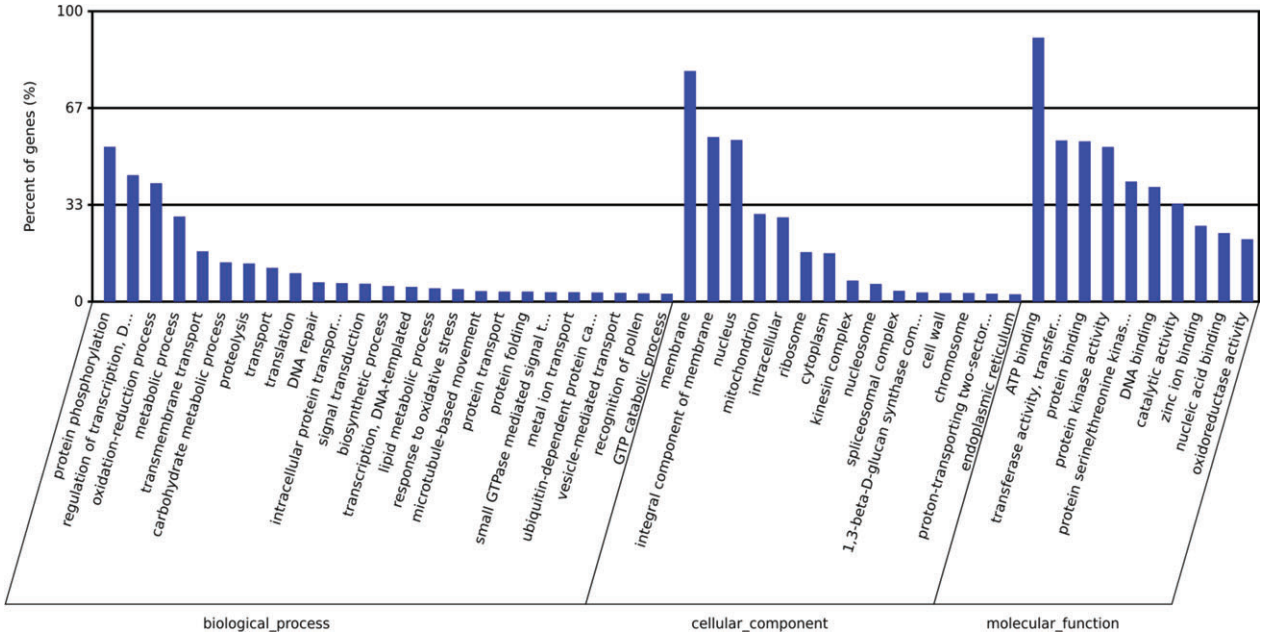


Fig. 4. Regulation network constructed with miRNAs and corresponding targets. Several miRNAs and their corresponding targets were selected to show the complex regulation network. One miRNA regulating several targets and one target being regulated by various miRNAs was the general phenomenon in this study.

two target genes was not detected, and the other seven genes showed diverse expression trends. Importantly, the expression of four target genes of ata-miR1432-5p was consistent. Although they were all upregulated, the expression peaks of miRNAs and target genes were divergent, which indicated that the regulation of ata-miR1432-5p and calcium ion-binding protein were true, and they play important roles in the wheat-*Pst* susceptibility interaction. As to PC-3p-7484, we found C81B58D98.1 and F8C1990BA.1 were first upregulated at 24 hpi, and they returned to the control level at 48 and 120 hpi, whereas 0B9EA6007.1 exhibited a stable downregulation during the entire *Pst* infection. Because PC-3p-7484 was highly induced in wheat challenged with *Pst* and 0B9EA6007.1 was highly deduced, we hypothesize that 0B9EA6007.1 was the real target gene of PC-3p-7484 in the wheat-*Pst* susceptibility interaction, and it also contributed to the susceptibility interaction. However, the molecular mechanism requires further study.

Discussion

miRNAs function in various plant biological progresses including organ development, phase change and defense responses (Bartel 2004). By constructing two miRNA libraries from wheat cultivar Xingzi9104 (ST-M and ST-I), we managed to reveal miRNAs and their corresponding target genes during wheat-*Pst* compatible interactions. All the mapped reads were analyzed by the length distribution of sRNAs, and the length distribution of miRNAs from our database showed consistence with typical size range for Dicer-derived products (Sunkar and Zhu 2004). miRNA sequences with a uracil nucleotide bias in the first position were cloned by loading into the RNA-induced silencing complex (RISC) with AGO proteins as described (Mi et al. 2008). We have also



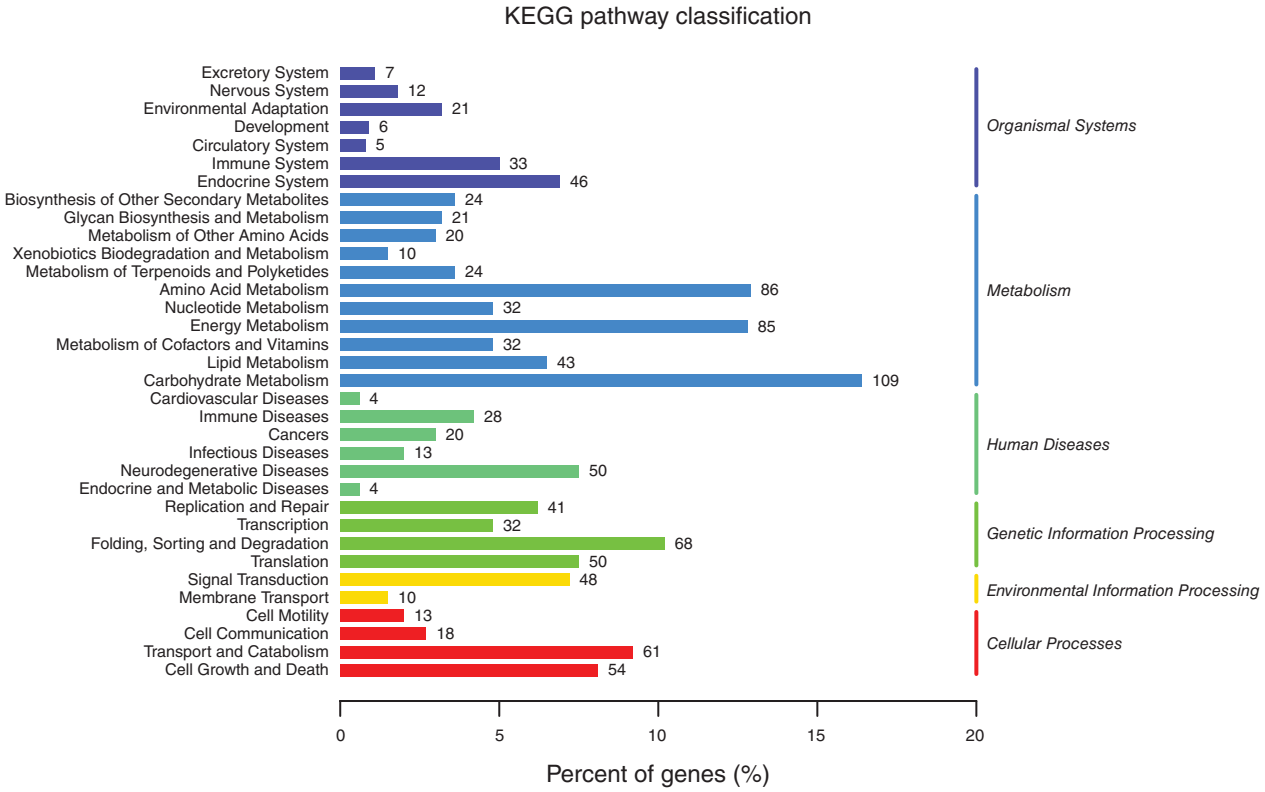


Fig. 5. GO annotation and KEGG pathway analysis for corresponding targets.

Table 1. Typical targets of miRNA identified by degradome sequencing.

miRNA names	Target annotation	Alignment score	Category	*P*-value
sbi-miR160a	Auxin response factor 8 (*Triticum urartu*)	2	1	1.95E-03
ata-miR169c-5p	CCAAT-binding transcription factor B (*Triticum aestivum*)	3.5	0	3.10E-03
bdi-miR156a_R-1	Squamosa promoter-binding-like protein 13 (*T. urartu*)	2	0	4.26E-03
tae-miR9676-5p	Acyl-protein thioesterase 2-like (*Oryza brachyantha*)	2	0	4.26E-03
PC-3p-39861_26	Unnamed protein product (*T. aestivum*)	3	1	4.79E-03
tae-miR5175-5p	Putative E3 ubiquitin-protein ligase HERC1 (*Aegilops tauschii*)	0.5	1	4.96E-03
PC-3p-207396_4	Probable protein ABIL1 (*Brachypodium distachyon*)	4	1	6.20E-03
PC-5p-119764_7	Transcriptional corepressor SEUSS-like (*B. distachyon*)	2.5	0	7.74E-03
PC-5p-10137_133	Cysteine-rich receptor-like protein kinase 27 (*A. tauschii*)	2.5	0	7.74E-03
PC-5p-46690_21	Protein FAR1-RELATED SEQUENCE 4-like isoform X1 (*Zea mays*)	1.5	0	9.28E-03
hvu-miR6197	Fructose-bisphosphate aldolase (*A. tauschii*)	2	0	1.27E-02
PC-3p-213573_4	Lipase (*T. urartu*)	2	0	1.27E-02
ata-miR167f-3p_L-1R+1	Unnamed protein product (*T. aestivum*)	2	1	1.48E-02
PC-5p-56871_16	Threonine dehydratase biosynthetic, chloroplastic-like (*B. distachyon*)	4	0	1.62E-02
tae-miR1120b-3p	Putative U3 small nucleolar RNA-associated protein 11 (*A. tauschii*)	1.5	1	1.69E-02
PC-5p-207189_4	Pyruvate kinase, cytosolic isozyme (*T. urartu*)	3.5	3	2.06E-02

found that the first nucleotide of novel wheat miRNA candidates tended to be 'U' base, which approved the quality of our sequencing results.

In total, 173 common miRNAs and 1143 wheat-specific miRNAs were identified from our database, which greatly increased the amount of known wheat miRNAs. It has been reported that plants express more species-specific miRNAs than conserved miRNAs (Li et al. 2012). We also found that the expression abundance of miRNAs showed a significant difference; most species-specific miRNAs exhibited a much lower expression level. Highly expressed miRNAs might be mainly involved in the basic biological pathways, whereas lower expressed miRNAs are responsible for regulating specific pathways (Glazov et al. 2008).

Several studies have identified target genes for miRNAs using degradome sequencing technology (Addo-Quaye et al. 2009, Li et al. 2010, Yang et al. 2013, Yao et al. 2015); we also detected the target genes for miRNAs by this technology. In this study, the transcriptome information of XZ was used to BLAST the sequence fragment; thus, the gene mapping and annotation were more accurate. The discovery of the target genes provided a valuable resource for investigating the regulatory function of miRNAs in the compatible interaction between XZ and CYR32. GO analysis for biological function indicated that these genes are mainly involved in metabolic processes and stress responses. In this study, the target genes of several conserved miRNAs were identified. For example, the targets of tae-miRNA160 in XZ were identified to be auxin response factor 8. In Arabidopsis, miR160 is involved in auxin signaling via the regulation of *ARF* genes (Jones-Rhoades et al. 2006). In rice, Zhou discovered five ARF encoding genes that are regulated by osa-miR160 (Zhou et al. 2010). In addition, many wheat-specific miRNA target genes were also detected, such as PC-5p-10137_133, the target of which was predicted to be a cysteine-rich receptor-like protein kinase 27. However, the target genes of several miRNAs target genes were not detected in this study, which could be because of the low expression level and specific regulation pathway in the compatible interaction between XZ and CYR32. In previous studies, not all miRNAs' target genes were identified using this method (Addo-Quaye et al. 2009, Li et al. 2010, Zhou et al. 2010). We know that some proteins are non-functional, even though the corresponding genes are expressed (Andrews and Hegeman 1976, Lovell 2003). However, it is unknown whether there were also certain types of miRNAs that expressed but were non-functional. This possibility requires further study.

Additionally, we found that miRNAs and target genes comprise a complex interaction network, not a one-to-one model. miRNAs may target multiple genes from the same gene family or genes involved in the same biochemical pathway. For instance, miRNA398 targets the whole family of Cu/Zn Superoxide Dismutase (SOD) genes (Bouche 2010). Interestingly, miRNA398 could also target gene families that are unrelated to Cu/Zn metabolism in a solo targeting way (German et al. 2009, Li et al. 2010). Our initial discoveries of miRNAs and their corresponding targets during wheat-*Pst* compatible interactions will greatly facilitate our knowledge about the molecular mechanism of wheat susceptibility to *Pst*.

During the last decade, several wheat miRNAs were reported to be involved in wheat response to biotic and abiotic stresses (Xin et al. 2010, Gupta et al. 2012, Akdogan et al. 2015, Eren et al. 2015). In this study, miRNAs

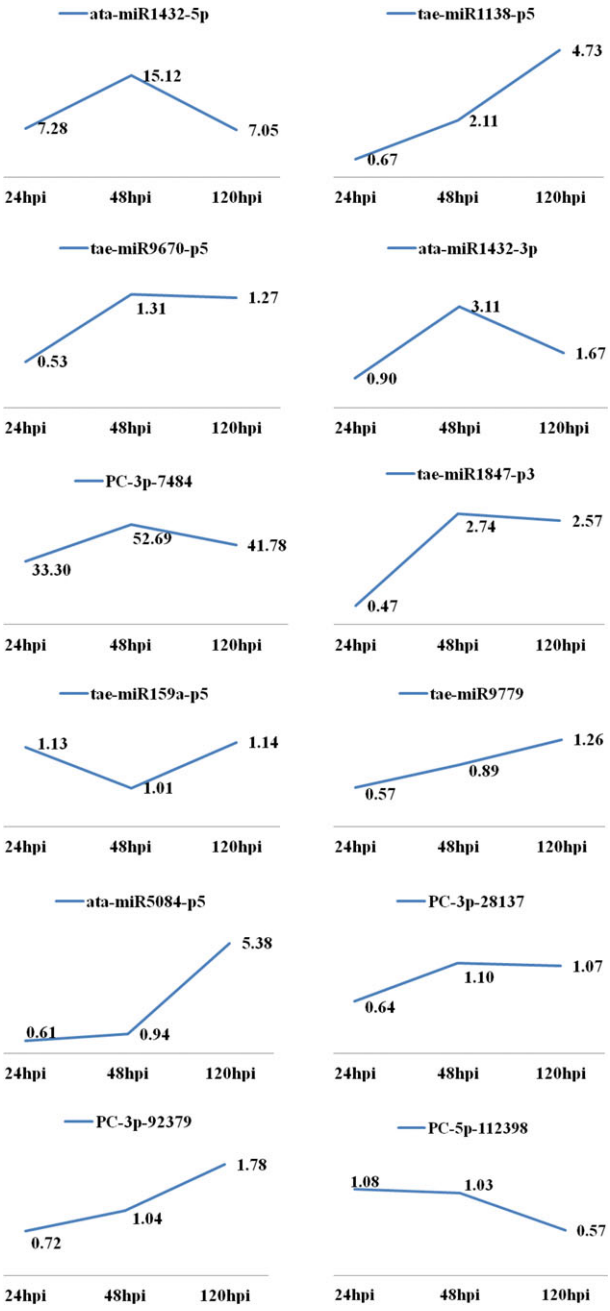


Fig. 6. Relative transcription levels of miRNAs in wheat response to *Pst*. According to the sequencing read number, 12 miRNAs were selected for transcript accumulation analysis in wheat after challenge with CYR32 (SI24, SI48 and SI120). The data were normalized to the expression level of wheat translation elongation factor 1 alpha-subunit (EF). The relative expression level of the target genes in the *Pst*-inoculated plants at each time point was calculated as the fold-change of the mock-inoculated plants at that time point using the comparative $2^{-\Delta\Delta CT}$ method. The experiments were repeated in triplicates of independent biological replicates using newly extracted RNA and synthesized cDNA samples.

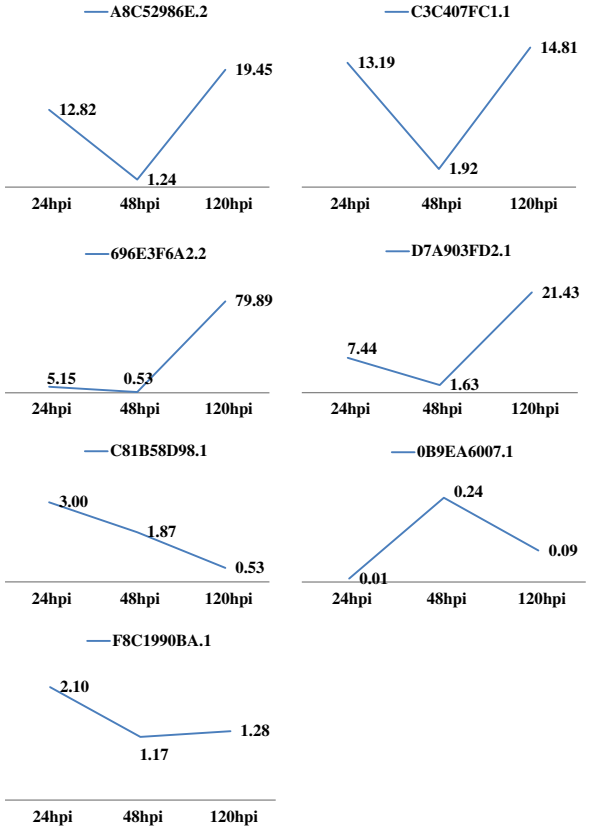


Fig. 7. Relative transcription levels of target genes in wheat response to *Pst*. Seven identified targets of miRNAs were selected for transcript accumulation analysis in wheat after challenge with CYR32 (SI24, SI48 and SI120). The data were normalized to the expression level of wheat translation elongation factor 1 alpha-subunit (EF). The relative expression level of the target genes in the *Pst*-inoculated plants at each time point was calculated as the fold-change of the mock-inoculated plants at that time point using the comparative $2^{-\Delta\Delta CT}$ method. The experiments were repeated in triplicates of independent biological replicates using newly extracted RNA and synthesized cDNA samples.

were also predicted to be responsive to *Pst* infection through bioinformatic analysis. We also used qRT-PCR to determine the expression level of some miRNAs at different time points, and we found that they showed diverse expression trends. Gupta et al. (2012) also found that the expression of miRNAs showed diverse trends when wheat was infected by the rust pathogen. It is interesting to note that the members of the same miRNA family were differentially regulated in the response to powdery mildew (Xin et al. 2010). The expression of detected target genes also showed a diverse trend. Combining the qRT-PCR results of miRNAs and target genes, we speculate that calcium ion-binding protein and ubiquillin can contribute to the compatible interaction positively and negatively, respectively. In another wheat-*Pst* interaction, calcium ion-binding protein was

also demonstrated to be a negative regulator of wheat resistance (Feng et al. 2011). Biquilins could function as ubiquitin receptors (Di Fiore et al. 2003), and they would contact with ubiquitinated proteins either to deliver them to the proteasome for degradation or to force them enter other destruction pathways (e.g. autophagy) (Marín 2014). The post-translational modification of proteins through the ubiquitin-proteasome system serves a critical regulatory role in most cellular processes. It was demonstrated that *AtUBP12*, *AtUBP13* and the tobacco homologue *NtUBP12* are negative regulators of plant immunity (Ewan et al. 2011). The detailed molecular mechanism will be explored in further studies.

In conclusion, we discovered a large amount of miRNAs that are involved in wheat-*Pst* compatible interaction by Solexa sequencing technology. We further analyzed the expression of some miRNAs, and the results indicated the significance of miRNAs in the wheat response to *Pst*. We also identified the target genes for the miRNAs, and several genes were related to stress responses. In addition, we found that the expression profiles of several miRNAs and the corresponding targets genes were induced/reduced. Considering their expression level, we speculate that calcium ion-binding protein and ubiquillin can positively contribute to the compatible interaction. The detailed function of miRNAs in plant susceptibility requires further detailed study.

Author contributions

H. F. conducted the majority of the experiments and wrote the main manuscript; T. W., C. F. and X. Z. analyzed the data and prepared the figures and tables; Q. Z. prepared the materials; and L. H., X. W. and Z. K. revised the manuscript. All authors reviewed the manuscript.

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References

Addo-Quaye C, Miller W, Axtell MJ (2009) CleaveLand: a pipeline for using degradome data to find cleaved small RNA targets. Bioinformatics 25: 130–131

Akdogan G, Tufekci ED, Uranbey S, Unver T (2015) miRNA-based drought regulation in wheat. Funct Integr Genomics. DOI: 10.1007/s10142-015-0452-1

Andrews KJ, Hegeman GD (1976) Selective disadvantage of non-functional protein synthesis in *Escherichia coli*. J Mol Evol 8: 317–328

Audic S, Claverie JM (1997) The significance of digital gene expression profiles. Genome Res 7: 986–995

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297

Bouche N (2010) New insights into miR398 functions in *Arabidopsis*. Plant Signal Behav 5: 684–686

Campo S, Peris-Peris C, Siré C, Moreno AB, Donaire L, Zytnicki M, Notredame C, Llave C, San Segundo B (2013) Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance. New Phytol 199: 212–227

Chen XM (2005) Epidemiology and control of stripe rust [*Puccinia striiformis* f. sp. *tritici*] on wheat. Can J Plant Pathol 27: 314–337

Di Fiore PP, Polo S, Hofmann K (2003) When ubiquitin meets ubiquitin receptors: a signalling connection. Nat Rev Mol Cell Biol 4: 491–497

Eckardt NA (2002) Plant disease susceptibility genes? Plant Cell 14: 1983–1986

Eren H, Pekmezci MY, Okay S, Turktas M, Inal B, Ilhan E, Atak M, Erayman M, Unver T (2015) Hexaploid wheat (*Triticum aestivum*) root miRNome analysis in response to salt stress. Ann Appl Biol 167: 208–216

Ewan R, Pangestuti R, Thornber S, Craig A, Carr C, O'Donnell L, Zhang C, Sadanandom A (2011) Deubiquitinating enzymes *AtUBP12* and *AtUBP13* and their tobacco homologue *NtUBP12* are negative regulators of plant immunity. New Phytol 191: 92–106

Fahlgren N, Howell MD, Kasschau KD, Chapman EJ, Sullivan CM, Cumbie JS, Givan SA, Law TF, Grant SR, Dangl JL, Carrington JC (2007) High-throughput sequencing of *Arabidopsis* microRNAs: evidence for frequent birth and death of MIRNA genes. PLoS One 2: e219

Feng H, Wang X, Sun Y, Wang X, Chen X, Guo J, Duan Y, Huang L, Kang Z (2011) Cloning and characterization of a calcium binding EF-hand protein gene *TaCab1* from wheat and its expression in response to *Puccinia striiformis* f. sp. *tritici* and abiotic stresses. Mol Biol Rep 38: 3857–3866

Feng H, Huang X, Zhang Q, Wei G, Wang X, Kang Z (2012) Selection of suitable inner reference genes for relative quantification expression of microRNA in wheat. Plant Physiol Biochem 51: 116–122

Galimov A, Hartung A, Trepp R, Mader A, Flück M, Linke A, Blüher M, Christ E, Krützfeldt J (2015) Growth hormone replacement therapy regulates microRNA-29a and targets involved in insulin resistance. J Mol Med 93(12):1369-79

German MA, Luo S, Schroth G, Meyers BC, Green PJ (2009) Construction of parallel analysis of RNA ends (PARE) libraries for the study of cleaved miRNA targets and the RNA degradome. Nat Protoc 4: 356–362
Glazov EA, Cottee PA, Barris WC, Moore RJ, Dalrymple BP, Tizard ML (2008) A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Res 18: 957–964
Gupta OP, Permar V, Koundal V, Singh UD, Praveen S (2012) MicroRNA regulated defense responses in *Triticum aestivum* L. during *Puccinia graminis* f.sp. *tritici* infection. Mol Biol Rep 39: 817–824
Hardham AR, Jones DA, Takemoto D (2007) Cytoskeleton and cell wall function in penetration resistance. Curr Opin Plant Biol 10: 342–348
Jones-Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57: 19–53
Kantar M, Lucas S, Budak H (2011) miRNA expression patterns of *Triticum dicoccoides* in response to shock drought stress. Planta 233: 471–484
Kessler SA, Shimosato-Asano H, Keinath NF, Wuest SE, Ingram G, Panstruga R, Grossniklaus U (2010) Conserved molecular components for pollen tube reception and fungal invasion. Science 330: 968–971
Lang Q, Jin C, Lai L, Feng J, Chen S, Chen J (2011) Tobacco microRNAs prediction and their expression infected with Cucumber mosaic virus and Potato virus X. Mol Biol Rep 38: 1523–1531
Li YF, Zheng Y, Addo-Quaye C, Zhang L, Saini A, Jagadeeswaran G, Axtell MJ, Zhang W, Sunkar R (2010) Transcriptome-wide identification of microRNA targets in rice. Plant J 62: 742–759
Li T, Chen J, Qiu S, Zhang Y, Wang P, Yang L, Lu Y, Shi J (2012) Deep sequencing and microarray hybridization identify conserved and species-Specific microRNAs during somatic embryogenesis in hybrid yellow poplar. PLoS One 7: e43451
Li Y, Lu YG, Shi Y, Wu L, Xu YJ, Huang F, Guo XY, Zhang Y, Fan J, Zhao JQ, Zhang HY, Xu PZ, Zhou JM, Wu XJ, Wang PR, Wang WM (2014) Multiple rice miRNAs are involved in immunity against the blast fungus *Magnaporthe oryzae*. Plant Physiol 164: 1077–1092
Liu XJ, Fu B, Chen DP, Hong Q, Cui J, Li J, Bai XY, Chen XM (2015) miR-184 and miR-150 promote renal glomerular mesangial cell aging by targeting Rab1a and Rab31. Exp Cell Res 336: 192–203
Lovell SC (2003) Are non-functional, unfolded proteins ('junk proteins') common in the genome? FEBS Lett 554: 237–239
Marín I (2014) The ubiquilin gene family: evolutionary patterns and functional insights. BMC Evol Biol 14: 63
Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, Li S, Zhou H, Long C, Chen S, Hannon GJ, Qi Y (2008) Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. Cell 133: 116–127
Navarro L, Dunoyer P, Jay F, Arnold B, Dharmasiri N, Estelle M, Voinnet O, Jones JD (2006) A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science 312: 436–439
Pathuri IP, Eichmann R, Hückelhoven R (2009) Plant small monomeric G-proteins (RAC/ROPs) of barley are common elements of susceptibility to fungal leaf pathogens, cell expansion and stomata development. Plant Signal Behav 4: 109–110
Pavan S, Jacobsen E, Visser RG, Bai Y (2010) Loss of susceptibility as a novel breeding strategy for durable and broad-spectrum resistance. Mol Breed 25: 1–12
Poraty-Gavra L, Zimmermann P, Haigis S, Bednarek P, Hazak O, Stelmakh OR, Sadot E, Schulze-Lefert P, Gruissem W, Yalovsky S (2013) The *Arabidopsis* Rho of plants GTPase *AtROP6* functions in developmental and pathogen response pathways. Plant Physiol 161: 1172–1188
Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16: 1616–1626
Sunkar R, Zhu JK (2004) Novel and stress-regulated microRNAs and other small RNAs from *Arabidopsis*. Plant Cell 16: 2001–2019
Vogel JP, Raab TK, Schiff C, Somerville SC (2002) PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. Plant Cell 14: 2095–2106
Wang CF, Huang LL, Buchenauer H, Han QM, Zhang HC, Kang ZS (2007) Histochemical studies of the accumulation of reactive oxygen species (O_2^- and H_2O_2) in the incompatible and compatible interaction of wheat – *Puccinia striiformis* f. sp. *tritici*. Physiol Mol Plant Pathol 71: 230–239
Xin M, Wang Y, Yao Y, Xie C, Peng H, Ni Z, Sun Q (2010) Diverse set of microRNAs are responsive to powdery mildew infection and heat stress in wheat (*Triticum aestivum* L.). BMC Plant Biol 10: 1471–2229
Yang JH, Liu XY, Xu BC, Zhao N, Yang XD, Zhang MF (2013) Identification of miRNAs and their targets using high-throughput sequencing and degradome analysis in cytoplasmic male-sterile and its maintainer fertile lines of *Brassica juncea*. BMC Genomics 14: 9
Yao FR, Zhu H, Yi C, Qu HX, Jiang YM (2015) MicroRNAs and targets in senescent litchi fruit during ambient storage and post-cold storage shelf life. BMC Plant Biol 15: 181
Zhang HC, Wang CF, Cheng YL, Chen XM, Han QM, Huang LL, Wei GR, Kang ZS (2012) Histological and cytological characterization of adult plant resistance to wheat stripe rust. Plant Cell Rep 31: 2121–2137
Zhao JP, Jiang XL, Zhang BY, Su XH (2012) Involvement of microRNA-mediated gene expression regulation in the

pathological development of stem canker disease in *Populus trichocarpa*. PLoS One 7: e44968
Zhou M, Gu LF, Li PC, Song XW, Wei LY, Chen ZY, Cao XF (2010) Degradome sequencing reveals endogenous small RNA targets in rice (*Oryza sativa* L. ssp. *indica*). Front Biol 5: 67–90

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Overview of wheat high-throughput sequencing.

Fig. S2. Overview of unigenes classification using Gene ontology (GO) enrichment analysis.

Fig. S3. Target genes of miRNAs participating in the signaling pathway found via KEGG analysis.

Table S1. Profile of the known *Triticum aestivum* microRNAs.

Table S2. Profile of novel microRNAs originating from other plant pre-miRNAs (PN-type) that can be mapped to *Triticum aestivum* Genome.

Table S3. Profile of potential candidate microRNAs originating from wheat.

Table S4. Identification and annotation of miRNA targets using degradome sequencing in adult XZ plants responding to *Pst*.

Table S5. Primers used in this study.

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