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Full Length Article

Identification of *Plasmopara viticola* Responsive microRNAs in Grapevine by Deep-Sequencing

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Abstract

Grapevine downy mildew caused by *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni is one of the most devastating grapevine diseases in the world. In plants, microRNAs (miRNAs) play important roles in biotic stress responses. Nevertheless, presently, the roles of *P. viticola*-responsive miRNAs in grapevine are not well-understood. In this study, we identified miRNAs that were regulated following the inoculation of grapevine leaves with *P. viticola* through high-throughput sequencing. In total, 123 vvi-miRNAs (including 100 known miRNAs and 23 novel ones) were identified from grapevine leaves. After inoculation, 92 miRNAs were downregulated, 30 were upregulated, and 1 did not change. Among those miRNAs that exhibited a change in regulation, the difference in post-inoculation expression was significant for 15 of them. Furthermore, the expression patterns of these 15 miRNAs were further shown, using RT-PCR, to be involved in the grapevine downy mildew response, and three were detected for the first time. A total of 152 target genes corresponded with the 15 miRNAs were predicted and categorized by Gene Ontology analysis. The miRNA targets were mainly involved in cellular processes and metabolism, and the expression patterns of the targets displayed a negative correlation with the corresponding eight vvi-miRNAs providing further evidence that these eight *P. viticola*-responsive miRNAs were reliable. These results will enhance our understanding of miRNAs regulatory mechanisms in response to *P. viticola* stress in grape. © 2019 Friends Science Publishers

Keywords: Grapevine; microRNAp; High-throughput sequencing; Downy mildew resistance; Target genes

Introduction

Grapevine is one of the most important and extensively cultivated fruits worldwide, and grape downy mildew (GDM) is a very destructive pathogen of this fruit (Figueiredo *et al.,* 2008). This disease is caused by the oomycete *Plasmopara viticola* (Berk. and Curt.) Berl. and de Toni (Gessler *et al.,* 2011). It can reduce yield or even cause complete crop failure. In China, the main strategies for controlling the disease have been fungicide applications and the cultivation of disease-resistant grape varieties (Liu *et al.,* 2001; Hu *et al.,* 2013). Nevertheless, these prevention strategies have had limited effectiveness. Long-term fungicide use leads to environmental contamination, and the cultivation periods of the disease-resistant varieties are often longer. Meanwhile, an inadequate understanding of grapevine *P. viticola* interactions have impeded the development of new disease prevention strategies.

MicroRNAs (miRNAs) are 22-nt, non-coding, single-stranded RNAs (Reinhart *et al.,* 2002). They are derived from a stem-loop precursor cleaved by a Dicer-like (DCL) enzyme at a specific site (Bartal, 2004), and they suppress target gene expression by inhibiting transcription and cleavage. In plants, miRNAs were first discovered in *Arabidopsis* (Reinhart *et al.,* 2002), and have since been identified in other plants as well. Information on 72 plant miRNA sequences is stored in the online database miRBase (http://www.mirbase.org/) (Jones *et al.,* 2008), and the dedicated plant miRNA database (PMRD) houses 8,433 miRNAs from 121 plants (http://bioinformatics.cau.edu.cn/PMRD) (Cui *et al.,* 2012). Previous studies have demonstrated that miRNAs maintain genetic stability, regulate plant growth, control signal transduction, and govern RNA metabolism (Chen, 2005; Wang and Li, 2007). Recent research indicated that miRNAs are also critical in plant stress responses (Shukla *et al.,* 2008; Leung and Sharp, 2010; Zhou *et al.,* 2010; Sunkar *et al.,* 2012).

In grapevine, many known and novel grapevine miRNAs responsive to abiotic and biotic stress have been studied by high throughput sequencing techniques (Sun *et al.,* 2015; Han *et al.,* 2016). To date, however, very little is

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known about the molecular processes that allow *P. viticola*-responsive miRNAs to regulate resistance to downy mildew. Thus, the present study aimed to investigate the expression profile of downy mildew-responsive miRNAs in grapevine. With Beta (a downy mildew-resistant variety) leaves as material, two sRNA libraries were constructed, and known and novel vvi-miRNAs were identified using high throughput sequencing techniques. The downy mildew-responsive miRNAs were identified *via* analysis of differentially expressed microRNAs (DEMs), and their expression levels were validated by qRT-PCR (quantitative real-time polymerase chain reaction). Next, DEMs target genes were predicted and assigned to a category by Go Ontology analysis. These findings could help to reveal the defense mechanisms of grapevine against *P. viticola* stress.

Materials and Methods

Plant Materials and Downy Mildew Inoculation

Three-year-old Beta grape (BG), with resistance to downy mildew inherited from a cross '*Vitis labrusea*' (Concord) × '*Vitis ripria*' (Carver), were provided by the Liaoning Academy of Agricultural Sciences. Following the method of Figueiredo *et al.* (2012), the grapevines were cultivated in a greenhouse for 10 weeks under natural diurnal variation and a temperature range of 5–28°C. *P. viticola* sporangia were collected from infected grape leaves showing disease symptoms in the vineyard and reproduced on the surface of disinfected grape leaves kept in the growth chamber. Sporangia were reclaimed by brushing, stored at -25°C. The third to fifth healthy and young leaves beneath the shoot apex were inoculated with a 1×10^5 sporangia/mL suspension (Wu *et al.,* 2010). After inoculation, the plants were kept with a 16h light/8h dark photoperiod at 25°C in a growth chamber. Sample leaves were collected at 0, 12, 24, 48, 72, and 96 h post-inoculation and immediately stored at -80°C. Five plants at each time points were pool together. Three replicates and two time points (0 and 12 h) were considered for high-throughput sequencing analysis, while 3 biological replicates and 6 time points were sample for real time PCR analysis.

Construction and Sequencing of a Small-RNA Library

Total RNA was extracted from each frozen sample with a Trizol reagent kit (Invitrogen, Carlsbad, C.A., U.S.A.). Total RNA was treated with DNase I for 30 min at 37°C and then purified by anhydrous ethanol precipitation. Total RNA purity and concentration were determined with a model ND5000 spectrophotometer (BioTeke Corp., Beijing, China). Libraries were established for the extracted small RNAs following the methods of Hafner *et al.* (2008). Small RNA sequencing was performed by Personalbio (Shanghai, China) using an Illumina Miseq system (Illumina, Inc., San Diego, CA, USA).

Identification on known and Putative Novel miRNAs

Illumina Pipeline (Illumina, Inc., San Diego, C.A., U.S.A.) was employed for image analysis and base pairing. Small RNAs were mapped to the grapevine *a* genome using SOAP2 (version 2.21). Matched sequences were subjected to a Blastn search in the NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, May 2018) with the blastall-p blastn-F F -e 0.01 parameters and Rfam (http://www.sanger.ac.uk/Software/Rfam, May 2018) databases. The matched sequences classified as follows: miRNAs, rRNAs, tRNAs, snRNAs, exons, introns, repeat-associate small RNAs, and snoRNAs. MicroRNA precursors were downloaded from the miRBase v. 21 (http://www.mirbase.org/, May 2018). The miRNA secondary structures were verified by mfold (http://www.bioinfo.rpi.edu/applications/mfold/, May 2018). Novel miRNAs were predicted by analyzing the remaining unknown small ribonucleic acids with MIREAP server (https://sourceforge.net/projects/mireap/) and further identified using the reported criteria (Meyers *et al.,* 2008; Sunkar *et al.,* 2008).

Analysis of differentially Expressed microRNAs (DEMs)

The relative expression level on each miRNA from the two small-RNA libraries was normalized using transcripts per million (TPM) reads. The following equation was employed to calculate the fold-change between the BG-C libraries and the BG-T: [Fold-change = $\log_2$(BG-T / BG-C)]. The miRNAs with fold-changes either > 1 or < -1 and with a $P \leq 0.001$ were deemed to be downregulated or upregulated, respectively, in response to *P. viticola* infection.

Analysis of the Expression Patterns of miRNAs

Each sample provided ~200 ng of total RNA. The DEMs were examined by real-time PCR. Primer v. 5.0 was used to design the miRNA-specific reverse transcription stem-loop primer as described by Varkonyi-Gasic *et al.* (2007). Reverse transcription was performed with Superscript II (Invitrogen, Carlsbad, C.A., U.S.A.). The reverse transcription products were used as real-time PCR templates, and all reactions were subjected to three parallel tests. The reactions were performed with an AB 7500 RT-PCR (Applied Biosystems Corp., Foster City, C.A., U.S.A.) and a SYBR Premix ExTaq kit (Takara Biomedical Technology Co. Ltd., Beijing, China). The reactions were run for 5 min at 95°C, followed by 40 cycles at 95°C for 10 s then 60°C for 34 s. Data were collected at 60°C. The 5s rRNA served as the reference gene. The miRNA expression levels were computed based on the 5s rRNA expression using the $2^{-\Delta\Delta CT}$ method.

Prediction of the miRNA Targets

Using mature miRNA sequences as queries, the target genes

were predicted with psRNATarget (http://plantgrn.noble.org/psRNATarget/), a plant miRNA target analysis database. The predicted miRNA targets should meet the following criteria reported by Dai and Zhao (2011). The target genes were subjected to Gene Ontology (GO) analysis with AgriGO (http://bioinfo.cau.edu.cn/agriGO/).

Analysis of the Expression Patterns of the miRNA Targets

Leaves from the Bate grapevine were inoculated with *P. viticola* as described above. Total RNAs were obtained at 0, 12, 24, 48, 72, and 96 hpi. The expression patterns of certain miRNA targets were analyzed by qRT-PCR. Total RNAs were processed with DNase I (New England Biolabs, Ipswich, MA, USA). After DNA digestion, first-strand cDNA was synthesized. Reverse transcription was performed using Superscript III (Invitrogen, Carlsbad, CA, USA). Quantitative real-time PCR was performed according to previously described using an AB 7500 RT-PCR (Applied Biosystems Corp., Foster City, C.A., U.S.A.) with a SYBR Premix ExTaq Reagent kit (Takara Biomedical Technology Co. Ltd., Beijing, China). The relative expression levels of these miRNAs relative to that of 5S rRNA were calculated using the $2^{-\Delta\Delta CT}$ method.

Results

Constructing and Sequencing Small-RNA Libraries

Two small-RNA libraries, namely, non-inoculated-treated (BG-C) and inoculated-treated 12 h (BG-T), were constructed to identify *P. viticola*-responsive microRNAs in grape. After sequencing, 14,834,643 raw total reads were generated from BG-C and 15,526,395 from BG-T (Table 1). After eliminating low-quality reads, 8,867,464 and 9,830,925 clean reads were obtained. Of these, 1,829,390 unique reads were determined for BG-C and 1,691,601 for BG-T. Ultimately, 1,079,340 reads in the BG-C library and 977,745 in BG-T were mapped onto the grapevine reference genome.

Further analysis categorized the matched small RNA sequences as miRNAs, tRNAs, rRNAs, snRNA, exons, introns, repeat-associate small RNAs, snoRNAs, or unannotated RNAs. Known miRNAs accounted for 1.11% of the total sequence reads in the BG-C library and 0.7% of those in BG-T. The unique small RNA sequences derived from known miRNAs accounted for only 0.18% and 0.14% of the total unique reads, respectively. Unannotated sRNA sequences constituted the highest proportion of unique sequences. Some novel miRNA candidates and other types of RNAs were probably included among these unannotated sRNA sequences.

Identification of Known miRNAs

To identify known miRNAs in grapevine, the clean reads were compared with a Blastn search against the miRBase v. 21.0 (May 2018), which had 186 known grapevine miRNAs. Blastn returned 100 known miRNAs for Beta grape. Among these, 88 were detected in both libraries and 12 in either one library or the other. The known miRNAs were classified into 47 families, including 25 non-conserved and 22 conserved. In this study, all 22 conserved miRNA families were detected. The MIR169 family had 13 members and was the largest of all those identified. The most abundant miRNA in both libraries was vvi-miR159c, followed by vvi-miR166 h.

Table 1: Reads generated by small RNA sequencing for Beta grape

Sequence reads	BG-C		BG-T	
Total Reads	14 834 643	100%	15 526 395	100%
Clean Reads	8 867 464	59.78%	9 830 925	63.32%
Unique Reads	1 829 390	12.33%	1 691 601	10.9%
Reads, mapping to genome	1 079 340	7.28%	977 745	6.30%

Identification of Putative Novel miRNAs

Based on the criteria of Ambros *et al.* (2003) and Meyers *et al.* (2008), putative novel miRNAs were identified. All unannotated small RNAs were mapped to the grapevine transcriptome database. Only 23 novel RNA precursors were perfectly matched the transcriptome sequences and were able to form proper secondary structures with an MFE of -18 kcal mol^{-1}. Novel miRNAs varied from 21 nt to 24 nt in length, and most were 21 nt long.

According to a MIREAP analysis (http://sourceforge.net/projects/mireap/), the range on the MFE of novel miRNA precursors was from -18.2 to -44.4 kcal mol^{-1}. The expression levels of most putative novel miRNAs were lower than those of the known miRNAs. In addition, 13 novel miRNAs were found, each with ≥ 10 TPM in the BG-C and BG-T libraries. The highest abundance was 298 TPM for vvi-new02. To identify *P. viticola*-responsive microRNAs, the novel miRNA expression levels in both BG-C and BG-T were compared. Three differentially expressed novel miRNAs (vvi-new01, vvi-new04, and vvi-new06) were significantly downregulated by > 2× ($P < 0.05$) in *P. viticola*-inoculated leaves.

Identification of differentially Expressed *Plasmopara viticola*-responsive microRNAs

The expression levels of miRNAs fluctuate in plants under biotic and/or abiotic stress (Ding *et al.,* 2009; Jin *et al.,* 2012; Wu *et al.,* 2014). We found that 92 miRNAs (of which 21 were novel) were downregulated, 30 (including two novel ones) were upregulated, and one did not change after inoculation with *P. viticola*. Thirteen of the downregulated miRNAs (of which 3 were novel) and 2 of the upregulated ones displayed significant differential expression (Tables S2 and S3). The most upregulated

miRNA was vvi-miR398c, and the most downregulated was vvi-miR319g.

Validating differentially Expressed microRNAs

Fifteen differentially expressed miRNAs were subjected to qRT-PCR to determine their expression patterns and conditions. The expression profiles of miRNAs at 0, 12, 24, 48, 72 and 96 hpi are shown in Fig. 1. Expression patterns of the 15 DEMs were classified into three groups. Group I consisted of nine vvi-miRNAs (black line, Fig. 1). Their relative expression levels were gradually downregulated and reached their minima at 72 h and then increased. Group II (red line, Fig. 1) expression levels first increased peaking at 12 h or 24 h and then declined until they reached their lowest points at 48 h or 72 h. The expression levels of Groups I and II were downregulated after *P. viticola* inoculation. Only vvi-miR398c and vvi-miR408 (blue line graph Fig. 1) were categorized in Group III, and their expression levels initially increased and reached a maximum at 72 h. The expression pattern of Group III was upregulated after *P. viticola* inoculation.

Predicted Target Gene of *P. viticola*-responsive miRNAs

To determine the functions of the miRNAs, it is necessary to identify their target genes. The psRNATarget database was employed to predict the target genes of the 15 DEMs (12 known and 3 novel miRNA candidates) associated with *P. viticola* infection. One hundred and fifty-two targets corresponding to 15 *P. viticola*-responsive vvi-miRNAs were predicted. According to previous studies, many target genes of conserved miRNAs encode transcription factors associated with plant growth and stress response (Llave *et al.,* 2002; Jones-Rhoades and Bartel 2004). For example, the main target genes of vvi-miR396 and vvi-miR171 are GRF and SCL, respectively. The target genes of non-conserved miRNAs were also detected (such as the ten predicted from vvi-miR3632-3p), but they were not as concentrated as those associated with conserved miRNAs. Among the 15 *P. viticola*-responsive vvi-miRNAs, vvi-miR398c corresponded to only one target gene, whereas the other miRNAs corresponded to multiple targets. The target genes of vvi-miR319efg, vvi-miR394c, vvi-miR3632-3p and vvi-new 06 coded for disease resistance. Certain target genes corresponding to the other miRNAs encoded structural proteins and those of unknown function.

Gene Ontology (GO) was applied to the identified targets to understand miRNA function more accurately. Fig. 2 shows that the putative target genes regulated cellular and metabolic processes as well as responses to stimuli. On the basis of GO and data from previous studies, transcription factors were identified in these target genes (Aukerman and Skai, 2003; Wang *et al.,* 2010, 2013; Han *et al.,* 2016; Kaur *et al.,* 2017). These included SCL (the putative target of vvi-miR171j), TCP (vvi-miR319efg), GAMYB (vvi-miR319efg), APETALA2 (AP2, vvi-miR172d), and bZIP (vvi-miR396a). Other putative target genes coded for the synthesis of proteins such as serine/threonine-protein kinase for vvi-miR396a and vvi-miR172d; E3 ubiquitin-protein ligase for vvi-miR408 and vvi-miR160b; NADPH-dependent diflavin oxidoreductase for vvi-miR319efg; disease resistance protein for vvi-miR3632-3p and vvi-new06; galacturonosyltransferase for vvi-new01; and LRR receptor-like serine/threonine-protein kinase for vvi-new04 and vvi-new06.

The Predicted Target Gene Expression Pattern Analysis

The expression patterns on the target genes of five known miRNAs (vvi-miR160b, vvi-miR171j, vvi-miR319e, vvi-miR172d and vvi-miR396a) and three novel miRNAs (vvi-new01, vvi-new04, and vvi-new06) during *P. viticola* infection were determined using qRT-PCR. The eight target genes were *ARF*17 (VIT_218s0001g04180), *SCL*15 (VIT_204s0023g01380), *TF APETALA*2 (VIT_207s0031g00220), *TF GAMYB* (VIT_213s0067g01630), *GRF*1 (VIT_200s0494g00010), galacturonosyltransferase 8 (VIT_200s0216g00020), LRR receptor-like serine/threonine-protein kinase (VIT_219s0014g00470), and disease-resistance protein At4g27220 (VIT_211s0052g00200). Transcription levels of the eight target genes were upregulated and negatively correlated with their respective miRNAs during *P. viticola* inoculation (Fig. 3).

Discussion

In the present study, high-throughput sequencing was effective in the investigation of small RNAs. This technique provided additional evidence that miRNAs play a vital role in plant responses to biotic and abiotic stress (Fahlgren *et al.,* 2007; Zhao *et al.,* 2007; Lu *et al.,* 2008; Zhang *et al.,* 2011;). Until now, however, comprehensive and systematic research on *P. viticola*-responsive grapevine miRNAs has not been reported. Through high-throughput sequencing and the sequenced grapevine genome, we identified *P. viticola*-responsive miRNAs from grapevine. We constructed two small-RNA libraries and obtained 1 829 390 and 1 691 601 unique reads, respectively. These small RNAs sequence can be classified as miRNAs, tRNAs, rRNAs, snRNA, exons, introns, repeat-associate small RNAs, snoRNAs, or unannotated RNAs.

The 186 known grapevine miRNAs are annotated and registered in miRBase v. 21.0. In this study, a total of 123 miRNAs (including 100 known and 23 novel miRNAs) were detected in the leaves of grapevine. Ninety-two of them were downregulated, 30 were upregulated, and 1 did not change. The analysis of differentially expressed miRNAs revealed that *P. viticola* stress significantly affected the expression profiles of certain vvi-miRNAs such as miR160, miR171, miR172, miR319, miR396, miR398,

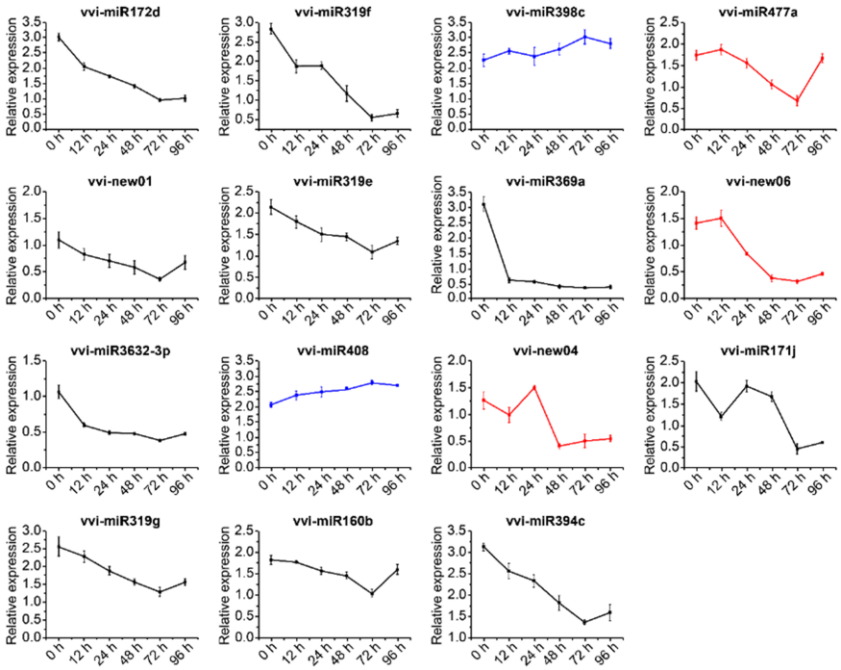

Fig. 1: Validation of downy mildew-responsive miRNAs by qRT-PCR
Quantitative analyses of 15 expression miRNAs with stem-loop real time RT-PCR at 0, 12, 24, 48, 72 and 96 h by $2^{-\Delta\Delta CT}$ method. 5S rRNA was used as the inner control. Y-axis indicated the fold changes of miRNA expression in Plasmopara viticola stress samples compared with the control samples. Data were averages of three PCR replicates of a single reverse transcription reaction. Error bar represent the standard deviation (SD) (n = 3)

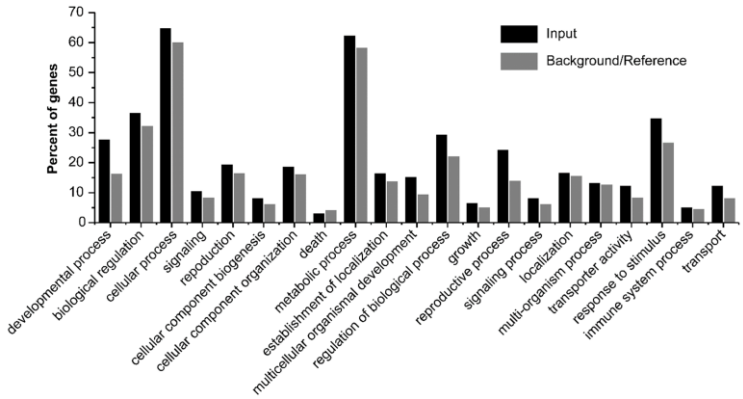

Fig. 2: Functional analysis of gene ontology for vvi-miRNA targets

miR408, miR477, and miR3632-3p. Most of the DEMs were conserved miRNAs. It is believed that these miRNAs were involved in plant growth and development. The fluctuating expression levels of these miRNAs may account for the fact that plant growth can change under biotic stress. It has been reported that miR319 is downregulated in *Oryza sativ*a (Lv *et al.,* 2010) and two grapevine varieties, 'Muscat Hamburg' (Sun *et al.,* 2015) and 'Chinese wild *V. pseudoreticulata*' (Han *et al.,* 2016). On the other hand, miR319 is upregulated in tomato (Naqvi *et al.,* 2010), sugarcane (Thiebaut *et al.,* 2011), and soybean (Li *et al.,* 2012). Li *et al.* (2012) suggested that the reversal of miR319 expression patterns under stress is the result of mitotic control by target gene TCP. Relative to non-conserved miRNAs, conserved miRNAs such as miR171, miR172, and miR319 show more pronounced differences. Twenty-three novel miRNAs were expressed at low levels, which coincide with previous findings (Kulcheski *et al.,* 2011; Wang *et al.,* 2011). The discovery of novel miRNAs could help to elucidate the interactions between pathogen and host.

miRNAs may regulate their target gene expression levels and patterns *via* cleavage and transcription inhibition, and target gene prediction contributes to understanding the regulatory mechanism of *P. viticola*-responsive miRNA. In this study, the target genes of 15 differentially expressed *P. viticola*-responsive vvi-miRNAs were predicted. The target genes of conserved miRNAs, including miR171 and

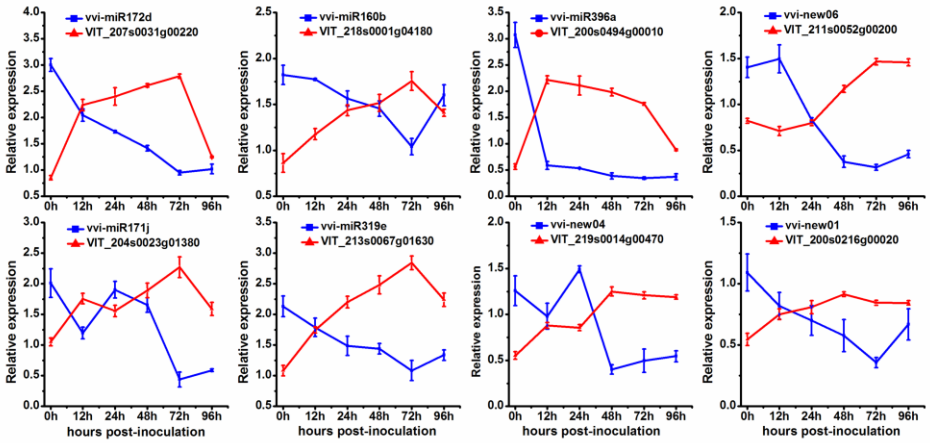

Fig. 3 qRT-PCR of expression patterns between vvi-miRNAs and their target genes

miR172, are mainly transcription factors controlling plant growth and development. miR171 and miR172 are two miRNAs that have been identified in other plant species as well (Gupta *et al.,* 2014; Khaksefidi *et al.,* 2015). Nevertheless, certain conserved miRNA targets code for stress responses and proteins associated with disease. For instance, vvi-miR319efg had a disease-resistance target RPP13 (VIT_212s0121g00050). These conserved miRNAs, then, could participate in the stress response. Members of the same miRNA family may have similar targets with similar functions.

These include vvi-miR319e, vvi-miR319f, and vvi-miR319g, which all have the same targets, VIT_213s0067g01630 and VIT_206s0009g02480, encoding the transcription factor GAMYB. Understanding the functions of these candidate target genes will elucidate the roles of *P. viticola*-responsive miRNAs in grapevine.

The application of qRT-PCR disclosed the expression modulations of eight predicted target genes associated with five known and three novel vvi-miRNAs from grapevine. The transcription levels of all eight target genes were upregulated after the grapevine leaves were inoculated with *P. viticola* and were negatively correlated with the corresponding vvi-miRNA during *P. viticola* infection. Therefore, *ARF*17, *SCL*15, *TF APETALA*2, *TF GAMYB*, *GRF*1, galacturonosyltransferase 8, LRR receptor-like serine/threonine-protein kinase, and disease resistance proteins might all be induced by *P. viticola* inoculation and play important roles in the *P. viticola* infection response. Previous experiments have shown that some members of the *GRF* family, such as *GRF*1, *GRF*2, and *GRF*3, participate in abiotic and biotic stress responses (Hewezi *et al.,* 2012; Casadeval *et al.,* 2013). Nevertheless, the functions of *ARF*17, *SCL*15, *TF APETALA*2, *TF GAMYB*, galacturonosyltransferase 8, and LRR receptor-like serine/threonine-protein kinase under *P. viticola* stress are still unknown and remain to be determined through future research.

Conclusion

A total of 123 vvi-miRNAs including 100 known miRNAs and 23 novel miRNAs were identified. Among them, 15 were DEMs during *P. viticola* stress in grapevine and their expression patterns were verified using real-time RT-PCR. The predicted results on the target genes of DEMs indicate that miRNAs may regulate some transcription factors, such as *ARF*17, *SCL*15, *TF APETALA*2, *TF GAMYB*, and *GRF*1 during *P. viticola* stress. The expression pattern of eight predicted targets were upregulated after the grapevine leaves were inoculated with *P. viticola*, and were negatively correlated with the corresponding vvi-miRNA during *P. viticola* infection. These findings contribute valuable information for further functional research of microRNAs in *P. viticola* infection response for grapevine and other plant species.

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