MicroRNA-like RNAs in plant pathogenic fungus *Fusarium oxysporum* f. sp. *niveum* are involved in toxin gene expression fine tuning

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Abstract MicroRNA-like RNAs (miRNAs) are short non-coding regulatory sRNAs which play an important role in regulating gene expression at the post-transcriptional level by targeting mRNAs for degradation or inhibiting protein translation. To explore the presence of miRNAs in *Fusarium oxysporum* f. sp. *niveum* (*Fon*) and analyze their expression at different propagules, two categories of sRNAs were identified from *Fon* hyphae and microconidia using illumina sequencing. A total of 650,960 and 561,114 unique sRNAs were obtained from the hyphae and microconidia samples. With a previously constructed pipeline to search for microRNAs, 74 and 56 miRNA candidates were identified in hyphae and microconidia, respectively, based on the short hairpin structure analysis. Global expression analysis showed an extensively differential expression of sRNAs between the two propagules. Altogether, 78 significantly differently expressed miRNAs were identified in two libraries. Target prediction revealed two interesting genes involved in

trichothecene production, necrosis and ethylene-inducing peptide 1 (NEP1) biosynthesis and in silico analysis indicated that they were down-regulated by *Fon*-miR7696a-3p and *Fon*-miR6108a. The expression levels of these two miRNAs were further validated by qRT-PCR and the results were consistent. The negative correlation of the expression levels between these two miRNAs and their potential target genes imply that they play a role in trichothecene and NEP1 biosynthesis. And this negative regulation for toxin-related gene expression is more specific in microconidia. The present study provides the first large-scale characterization of miRNAs in *Fon* and the comparison between hyphae and microconidia propagules gives an insight into how miRNAs are involved in toxin biosynthesis.

Keywords miRNA · *Fusarium oxysporum* f. sp. *Niveum* · Propagule · Toxin gene expression

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Introduction

Fungal pathogen *Fusarium oxysporum* is notorious for causing great losses in many crops. The *forma specialis* *Fusarium oxysporum* f. sp. *niveum* (*Fon*) affects watermelon, which was first identified and investigated in the 1890s after a devastating outbreak in the southern United States (Smith 1894). The soil-borne pathogen can lead both parasitism or saprophytism growth with four types of propagules including hyphae, microconidia, macroconidia and chlamydospores. During the parasitism growth, the mycelia or germinating spores penetrate into the roots. Once the hypha reach the vessel elements, microconidia are produced and transported by sap stream unless they are hindered by vessel end walls, in which case another round

of germination and penetration is repeated (Beckman and Roberts 1995; Martyn 2014).

Expression of genes related to development and virulence in plant pathogens is subtly manipulated (Dangl and Jones 2001), among which post-transcriptional regulation mechanism exert in many physiological process, such as immunoreaction (Xiao and Rajewsky 2009), development and differentiation (Bartel 2004). Nowadays, microRNAs, a non-coding RNAs of 18–25 nt, were found to be widespread in animals, plants, viruses and algae (Griffiths-Jones et al. 2006). They can negatively fine-tune gene expression by near perfect base pairing to cleave target genes in plants (Rhoades et al. 2002) or by imprecise complementary binding to inhibit target genes translation both in plants and animals (Lewis et al. 2003; Iwakawa and Tomari 2013; Wilczynska and Bushell 2015).

Though the first microRNA was discovered in early 1990s (Lee et al. 1993), the absence in fungal kingdom kept till the counterpart (microRNA-like small RNAs, milRNAs) was identified recently (Lee et al. 2010). Analysis of milRNAs from fungi *Neurospora crassa* and *Cryptococcus neoformans* showed that milRNAs negatively regulate protein expressions of reporter genes, which contain perfect complementary target sites (Lee et al. 2010; Jiang et al. 2012). Nevertheless, the mechanisms involved in targeted gene silencing by fungal milRNAs are mostly unknown, and so far little information about target recognition is available (Dahlmann and Kück 2015). Combined with the high-throughput sequence and bioinformatics approaches, more and more milRNAs were explored in fungus (Zhou et al. 2010; Kang et al. 2013; Chen et al. 2014); however, still relatively little is known about milRNAs and their expression profiles which should have important roles in virulence in pathogenic fungus *F. oxysporum*.

China is the largest producer of watermelon who contributes up to 67% share of the yield worldwide (<http://faostat3.fao.org/browse/Q/QC/E>). And the soil-borne disease caused by *Fon* is a major constraint in watermelon production. In this study, the milRNAs in pathogen *Fon* isolated from the main off-season watermelon producing area in China were investigated. Their expression profiles and potential target genes in two kinds of propagules were examined.

Materials and methods

Strain and sample preparation

Fusarium oxysporum f. sp. *niveum* (*Fon*) was isolated from infected watermelon grown in Hainan province, China and is deposited in the college of environment and plant protection, Hainan University. Hyphae and microconidia are

collected according to the literatures (Esposito and Fletcher 1961; Martyn 2014). Briefly, *Fon* were grown at 25 °C in 50 ml potato dextrose medium at stationary or on orbital shaker at 150 rpm in 150 ml Erlenmeyer flasks separately for 5 days in darkness. *Fon* hyphae were isolated from stationary culture by filtration through a sieve (Φ100 μm mesh) and microconidia were collected from shaking culture by filtration and centrifugation (5000 rpm, 10 min) followed by washing with sterilized distilled water and immediately frozen in liquid nitrogen and then stored at −80 °C. The purity of microconidia was confirmed by microscopic observation; samples with proportion of microconidia >95% were used for further analysis.

RNA extraction and sRNA sequencing

Total RNA was extracted using the AxyPrep Multisource Total RNA Miniprep Kit (Axygen Scientific, Inc., Hangzhou, China) according to the manufacturer's instructions. The quality and integrity of total RNA were analyzed using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). sRNAs fraction of *Fon* between 18 and 30 nucleotides (nt) was extracted using 15% polyacrylamide/8 M urea denaturing gel electrophoresis (PAGE) with radioactively labeled RNA as size markers. After ligated to 5' and 3' adapters sequentially using truncated rhythm T4 RNA ligase (Invitrogen, Carlsbad, CA), the sRNAs were transcribed into cDNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). PCR amplification was performed to enrich the adapter-modified cDNA libraries, which were sequenced with an Illumina Hiseq 2000 platform at Beijing Genomic Institute (BGI, Shenzhen, China). The sequenced short reads data were deposited to Gene Expression Omnibus database (GEO) at NCBI (<http://www.ncbi.nlm.nih.gov/geo/>) under the Accession No. GSE84384.

sRNA data analysis

After removing contaminant reads (reads shorter than 18 nt, reads with 5' primer contaminants or poly A, or without a 3' primer or insert tag) from the raw reads, the remaining sRNA tags were blasted against GenBank and Rfam database version 10.1 (Griffiths-Jones et al. 2005; Gardner et al. 2009) to annotate and then remove ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNA (snRNA), and small nucleolar RNA (snoRNA) using Basic Local Alignment Search Tool (BLAST).

Subsequently, all clean reads were mapped to *F. oxysporum* f. sp. *melonis* (*Fom*) genome, which was downloaded from the 'Ensembl Genomes' (http://fungi.ensembl.org/Fusarium_oxysporum_f_sp_melonis_26406/Info/Index)

(Ma et al. 2014), using Short Oligo Alignment Program (SOAP) (<http://soap.genomics.org.cn>) (Li et al. 2008). Furthermore, with the available annotation of repeat sequences in the *Fom* genome and use of a parallel overlap finding algorithm, small RNAs positioned at repeat loci were identified and annotated as repeat-associated small RNAs with software Repeat Masker (<http://www.repeatmasker.org/>).

Meanwhile, all small RNA tags were aligned to exons and introns of mRNA from *Fom* genome data with the software OVERLPAP developed by BGI (link or publication). In the previous alignment and annotation, some small RNA tags may be mapped to more than one category. To make every unique small RNA map to only one annotation, we followed the following priority rule: rRNA, etc. (in which Genbank > Rfam) > known miRNA > repeat > exon > intron. The software used for this work is BGI software tag2 annotation.

Identification of miRNAs

To identify potential miRNAs, all the unique reads were blasted against the miRBase 21.0 to identify the conserved miRNAs (Kozomara and Griffiths-Jones 2014). Meanwhile, the prediction of novel miRNAs was carried out using Mireap program (<https://sourceforge.net/projects/mireap>) which could identify known miRNAs and novel candidates with canonical hairpin structure. The following criteria were used to identify miRNA loci (Zuker 2003): (1) small RNAs form a stem loop structure (hairpin) with flanking sequences, and the most abundant species of the small RNA resides in the stem region. (2) The mature miRNA strand and its complementary strand (miRNA*) possesses 2-nucleotide 3' overhangs. (3) The secondary structures of the hairpins are stable, with minimum free energy of hybridization no greater than -18 kcal/mol. (4) The number of mature miRNA with predicted hairpin must be no fewer than five in the alignment result. Secondary structures of miRNA precursor (up to 340 nt) and their flanking sequences (20 nt) were examined by M fold.

Potential miRNA target prediction and data analysis

Potential targets of the miRNAs were predicted using the Target Finder (<https://github.com/carringtonlab/TargetFinder>) (Fahlgren et al. 2007; Fahlgren and Carrington 2010) and the psRobot program (<http://omicslab.genetics.ac.cn/psRobot>) (Wu et al. 2012). Criteria used by Allen et al. (2005) and Schwab et al. (2005) for target prediction were applied. Functional annotation of target genes was performed using various bioinformatics procedures, including annotation of BLAST, GO and KEGG. For specific methods, please referred to Gong et al. (2015).

Differential expression analysis of miRNAs

Differential expression analysis of two samples was performed using the DEGseq R package (Wang et al. 2010). MiRNAs expression levels were calculated according to the value of reads per million reads (RPM). Differentially expressed miRNAs were defined based on strict criteria (p value ≤ 0.05 and differential expression fold > 2). The procedure is as follows: (1) normalize the expression of sRNAs in hyphae and microconidia samples to get the expression of RPM. Normalization formula: normalized expression = actual miRNA count/total count of clean reads $\times 1,000,000$; (2) calculate the $\log_2$ fold change (hyphae/microconidia); (3) evaluate the p value using Fisher's exact test; and (4) graph a volcano plot that shows statistical significance versus fold change of expression on the y - and x -axes, respectively. p values < 0.01 were considered statistically significant.

sRNAs were divided into three groups according to their $\log_2$ fold change: (1) up-expressed sRNAs in hyphae: $\log_2$ fold change ≥ 1.0 ; (2) equally expressed sRNAs between hyphae and microconidia: $-1.0 < \log_2$ fold change < 1.0 ; (3) down-expressed sRNAs in hyphae: $\log_2$ fold change ≤ -1.0 . False discovery rates (FDR) were estimated, which were carried out by R statistical package version 3.0.1 (<http://www.r-project.org/>). MiRNAs with FDR < 0.05 for their expression in regression models were defined as differentially expressed miRNAs.

Stem-loop PCR detection of toxin-related miRNAs

Total RNA was extracted from *Fon* hyphae and microconidia, respectively, using AxyPrep Multisource Total RNA Miniprep Kit (Axygen) and treated with RNase-free DNase I (TaKaRa) according to the manufacturer's protocols. The quantity and quality of the isolated total RNA were verified using NanoVue spectrophotometry (GE HealthCare, USA) and gel electrophoresis. Mature miRNAs were detected using an end-point stem-loop RT-PCR method (Chen et al. 2005; Tang et al. 2010). A stem-loop-containing Reverse Transcription (RT) primer with its 5' end complementary to target the miRNA's last six nucleotides at the 3' end was designed. RT was performed at 16°C for 30 min, followed by 60 cycles of pulsed RT at 30°C for 30 s, 42°C for 30 s, and 50°C for 1 s using $5\ \mu\text{g}$ of total RNA, the stem-loop RT primer, and PrimeScript RT-PCR Kit (TaKaRa). With synthesized RT reaction products as DNA templates, Mature miRNAs RT-PCR was performed using an miRNA-specific forward primer and a universal reverse primer complementing the stem-loop part of the RT primer at 94°C for 2 min, followed by 35 cycles of 94°C for 15 s and 60°C for 1 min. The PCR products (about 60 bp) were analyzed by electrophoresis

on a 4% agarose gel in 1× tris–acetate EDTA buffer. All the primers used in this study are listed in Table 1. Meanwhile, all these PCR product fragments were inserted into the pMD-18T vector (TaKaRa) and then transformed into *Escherichia coli* strain DH5α to facilitate sequencing. The sequencing results were aligned with the primary predicted miRNAs to verify the accuracy.

Quantitative real-time PCR of toxin-related miRNAs and their targets

Based on the results of sequences alignment and gel electrophoresis, the expression level of two interesting toxin-related miRNAs and their target genes between the hyphae and microconidia samples were further analyzed using qRT-PCR. One-step quantitative real-time PCR was performed and analyzed using SYBR Premix Ex Taq II (Takara) in a PikoReal Real-Time PCR System (Thermo Scientific) according to the manufacturer's instructions. All the PCR primers listed in Table 1 were also used in this study; *Fom* actin gene (FOMG_05575) was selected as an internal reference.

Results

Sequence analysis of small RNAs

A total of 5,736,832 and 5,855,670 raw reads were generated from hyphae and microconidia of *Fon*, respectively. After removing the low quality and adaptor sequences,

5,193,149 and 5,744,648 clean reads were obtained, which represented 650,960 and 561,114 unique sRNAs sequences from hyphae and microconidia (Table 2). All the total sRNAs and unique sRNAs sequences were then compared with the *Fom* genome, and the sequences that perfectly matched to the genomes along their entire length were used for subsequent analysis. In view of limitation of genome data integrity and the sequence difference between *Fon* and *Fom*, we observed that only a part of sRNA sequences could mapped to *Fom* genome (Table 2).

The comparative analysis showed that the common “unique sRNAs” between the two samples were 9.89% (109,111), which constituted 87.95% (9,619,552) of the clean reads/total sRNAs (Fig. 1). The large number of sRNA sequences corresponding to a minority of the reads in only hyphae or microconidia samples suggested that *Fon* has a high degree of sRNA sequence complexity.

Moreover, the length distribution of sRNA varies between the two libraries (Fig. 2). The majority of sRNAs in hyphae samples are between 20 and 22 nt in length (about 30%), but the most frequent sRNAs in microconidia samples were found between 25 and 27 nt (>35%), indicating a different predominance of sRNAs in length between the two samples.

The composition of a small RNA dataset produced by deep sequencing is very complex; there are a lot of different categories of non-coding RNAs (ncRNA). With the alignment to different database mentioned above, the composition of small RNAs was annotated as rRNA, tRNA, miRNA, snRNA, snoRNA, etc. (Fig. 3). The largest fractions of total sRNA reads were the unannotated small

Table 1 Specific oligonucleotide primers for toxin-related miRNAs and their targets

Name	Brief description	Sequences (5′–3′)	Tm (°C)	Fragment size (bp)
Fon-miR7696a-3p	RT primer	GTTGGCTCTGGTGCAGGGTCCGAGGT/ ATTCGACCAGAGCCAACCTTCAA	–	59
	Forward primer	GCGGCGGTTCAATGAGAAC	60.0	
Fon-miR6108a	RT primer	GTTGGCTCTGGTGCAGGTCCGAGGTATT/ CGCACCAGAGCCAACGGTTCT	–	61
	Forward primer	CGGCGGTGAGAAGCGTAGAGA	60.0	
FOMG_13322	Forward primer	GGGCGGGGCTGGTGTATA	61.2	189
	Reverse primer	ATGGCGAAGGAGATGGGAA	60.0	
FOMG_19538	Forward primer	AGAAGACCAAGAACCCCCAAA	60.2	130
	Reverse primer	GCAGCCCAGTCGTAAATCCA	60.9	
Actin	Forward primer	GCCTTCTGTCCTGGGTCTTG	59.3	103
	Reverse primer	AATGTTGCCGTAGAGATCCTTTC	59.6	
URP	Universal reward primer for all miRNA end-point stem-loop RT-PCR	GTGCAGGGTCCGAGGT	53.0	–

The specificity of stem-loop RT primers to individual miRNA is conferred by a 6 nucleotide extension (lowercase letters) at the 3′ end; this extension is a reverse complement of the last six nucleotides at the 3′ end of the miRNA. Forward primers of miRNA are specific to their sequence but exclude the last six nucleotides at the 3′ end of the miRNA. A 5′ extension of 6–7 nucleotides (capital letters) is added to each forward primer to increase the melting temperature; these sequences were chosen randomly and are relatively GC rich

Table 2 Statistical summary of sRNAs from *Fon* hyphae and microconidia samples

	Hyphae Reads number (%)	Microconidia Reads number (%)
Total reads	5,736,832 (–)	5,855,670 (–)
High quality ^a	5,704,532 (100)	5,827,211 (100)
3' Adapter null ^a	2532 (0.04)	1588 (0.03)
Insert null ^a	29,555 (0.52)	2374 (0.04)
5' Adapter contaminants ^a	427,115 (7.49)	2,0469 (0.35)
Smaller than 18 nt ^a	51,404 (0.90)	58,061 (1.00)
polyA ^a	777 (0.01)	71 (0.00)
Clean reads/total sRNAs ^a	5,193,149 (91.04)	5,744,648 (98.58)
Unique sRNAs ^a	650,960 (11.41)	561,114 (9.63)
Total sRNAs matched to the genome ^b	1,181,001 (22.74)	855,001 (14.88)
Unique sRNAs matched to the genome ^c	166,027 (25.5)	216,243 (38.54)

Total reads: total sequenced reads; high quality: high quality reads with no N (N represents for ambiguous nucleotide A, T, G or C), no more than four bases whose quality score is lower than ten and no more than six bases whose quality is lower than 13; 3' adapter null: reads with no 3' adaptor; insert null: reads with no insertion; 5' adapter contaminants: 5' contaminants; smaller than 18 nt: reads less than 18 nt; polyA: reads with polyA; clean reads/total sRNAs: number of clean reads after adaptors and contaminants are removed which are used in the following analysis, also the number of total small RNAs sequences. Unique sRNAs: the kinds of total clean reads or small RNAs sequences

^aThe percentage to the high quality reads

^bThe percentage to the total sRNAs

^cThe percentage to the unique sRNAs

Fig. 1 Common and specific sequences between *Fon* hyphae and microconidia samples. Venn charts show the summary of unique (a) and total (b) sRNAs sequences between *Fon* hyphae and microconidia

RNA in both two libraries. Approximately, 31.9% (1,659,377) and 26.6% (1,530,958) of the reads were mapped to the known non-coding RNAs (ncRNAs) in hyphae and microconidia libraries, respectively, including

miRNAs, rRNA, tRNAs, snoRNAs, and snRNAs. Among all sRNAs, the reads of the miRNA were 44,246 (0.85%) and 29,923 (0.52%) in hyphae and microconidia libraries, respectively.

Fig. 2 Length distribution of sRNAs in both hyphae and microconidia libraries

Identification of miRNAs in *Fon*

All mappable sRNA sequences were compared with all the known miRNAs in miRBase to identify conserved miRNAs. A total of 57 and 34 conserved miRNA variants were identified in hyphae and microconidia, respectively (Tables S1 and S2). These conserved miRNAs belong to

A Pie chart for *Fon*_hyphae total sRNAs

B Pie chart for *Fon*_microconidia total sRNAs

Fig. 3 Distribution of small RNA among different categories. Charts **a** and **b** show the numbers of total sRNA tags matched to all categories of RNA

very different families and their read counts were markedly different. After searching for potential pre-miRNAs and predicting their hairpin-like structures with Mireap program, 17 and 22 unique sequences were identified as novel miRNAs in hyphae and microconidia (Tables S3 and S4). Thus, to a certain extent, our sequencing depth was sufficient to reflect the expression profiles of miRNAs in these two samples, and lots of conserved and unknown miRNAs were also presented. These results showed that plant pathogenic fungus *Fon* also encodes miRNAs. The identification of miRNAs in this fungus strongly supported the concept that the small RNAs are a conserved regulator of gene expression in eukaryotes (Bartel 2004).

The biogenesis of miRNAs has three steps: pri-miRNA, pre-miRNA, and mature miRNA. Lee et al. demonstrated that *N. crassa* generated miRNA by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE2, the exonuclease QIP and an RNase III domain-containing protein MRPL3 (Lee et al. 2010). A Blast homology search of the components in miRNA biogenesis showed that there were 3 Argonaute-like, 1 Dicer-like and 1 MRPL3-like proteins in *Fom* genome (Table 3), providing evidences for the miRNA existence in this fungus. We thus termed the miRNAs in *Fon* as *Fon*-miRNAs concordant with the nomenclature used in *N. crassa* (Lee et al. 2010).

Expression profiles of *Fon* hyphae and microconidia miRNAs

To compare miRNAs expression level in *Fon* hyphae and microconidia samples, the abundance of all candidate miRNAs in both hyphae and microconidia libraries was examined. Overall, approximately 60 known miRNAs and 18 novel miRNAs showed significant changes in expression (fold changes >2). Among these miRNAs with altered expression, 36 known miRNAs were specifically expressed in hyphae and 11 known miRNAs were specifically expressed in microconidia. In all 12 co-expressed miRNAs, 7 were up-regulated in hyphae and 6 were up-regulated in microconidia (Table 4). Meanwhile, ten novel miRNAs were specifically expressed in hyphae and 7 novel miRNAs were specifically expressed in microconidia. The only co-expressed novel miRNAs designated as miRNA novel_mir_2 were down-regulated in hyphae (Table 5).

Potential target gene prediction and screen of miRNAs involved in toxin biosynthesis

Hyphae and microconidia are two kinds of important propagules in *Fon*; transition of lifestyle is very important for successful invasion and completing its life cycle. Our

Table 3 Blast homology result of miRNA biogenesis factors in *Fon*

Name	Gene code	Homologs gene	Accession No.	E-value
QDE-like	FOMG_16409	<i>Penicillium marneffei</i> ATCC 18224 RNA interference and gene silencing protein (Qde2)	XM_002153707.1	0
	FOMG_18287			0
	FOMG_18792	<i>Metarhizium acridum</i> CQMa 102 QDE3 protein	XM_007811168.1	0
DCL	FOMG_07888	<i>Colletotrichum lindemuthianum</i> dicer-like-protein 1 gene	KR611018.1	3e−54
MRPL3-like	FOMG_03032	<i>Nectria haematococca</i> mpVI 77-13-4 mitochondrial 54S ribosomal protein YmL3	XM_003039827.1	0

objective was to better understand the roles of miRNAs and target genes identified in these two propagules. The miRNAs which showed differential expressions (fold changes >2) between two samples were screened for target predictions. In hyphae, 1127 target genes were predicted for 53 known miRNAs and 146 for 14 novel miRNAs. In microconidia, the numbers of target genes predicted for 31 known miRNAs and 19 novel miRNAs are 443 and 143.

We then analyzed the correlation between the expression of miRNAs and their target genes. By referring to BLAST annotation of all target genes, the miRNAs which potentially related to toxin synthesis could be identified. Consequently, we selected two miRNAs (named as Fon-miR7696a-3p and Fon-miR6108a) and two of their target genes likely involved in toxin synthesized in *Fon* (Table 6) for expression analysis. FOMG_13322, the predicted target gene of Fon-miR7696a-3p, is highly homologous with an unknown gene (Accession No. AAO34673.1) from trichothecene gene cluster of *Gibberella zeae* which is located in Supercontig_1.12 in the *Fom* genome. Taking into account that gene AAO34673.1 is only one among several genes related to trichothecene biosynthesis in *G. zeae*, we then searched and found more than two trichothecene biosynthesis-related genes within 50 kb up- and downstream of FOMG_13322 in Supercontig_1.12. These findings suggest that the target genes are likely trichothecene toxin genes and Fon-miR7696a-3p negatively regulate trichothecene toxin biosynthesis. Moreover, Fon-miR6108a can target to a gene encoding a necrosis and ethylene-inducing peptide 1 (NEP1)-like protein (NLP), whose product is a secreted toxin which enhances the virulence in several species (Gijzen and Nürnberger 2006; Ottmann et al. 2009).

miRNAs verification and qRT-PCR result

The end-point stem-loop reverse transcription (RT)-PCR is a highly sensitive and specific method to detect miRNA and has been widely used for the detection and quantification of miRNAs (Chen et al. 2005; Tang et al. 2006; Varkonyi-Gasic et al. 2007). To confirm the presence of Fon-miR6108a and Fon-miR7696a-3p in *Fon*, end-point

stem-loop RT-PCR was employed. Indeed, two corresponding mature miRNAs were detected in *Fon* (Fig. 4).

To verify the results of the digital gene expression analysis, expression levels of Fon-miR6108a and Fon-miR7696a-3p and target gene FOMG_13322 was evaluated by qRT-PCR (Fig. 5). As shown in Fig. 5, the qRT-PCR showed that Fon-miR6108a and Fon-miR7696a-3p were up-regulated in microconidia sample (Fig. 5a, b), when compared with that in hyphae sample. Meanwhile, the target gene FOMG_13322 in *Fon* microconidia and hyphae showed opposite expression pattern (Fig. 5c). These results are in agreement with the expression profiles obtained through in silico analysis. Although the mechanisms of target gene silencing by fungal miRNAs are unclear, both results showed a good coincidence with the idea that the expression of FOMG_13322 was suppressed by Fon-miR7696a-3p indicating that Fon-miR7696a-3p is probably involved in the post-translational regulation of FOMG_13322 in *Fon*.

Discussion

Since the discovery of the first miRNA lin-4 in *C. elegans* (Lee et al. 1993), an increasing amount of miRNAs has been discovered in animals, plants, and algae (Lagos-Quintana et al. 2001; Lee and Ambros 2001; Zhao et al. 2007; Grimson et al. 2008). But the existence of miRNAs in fungi is not extensively explored, and their roles in fungi remained largely unknown. The fungal microRNA-like RNAs (miRNAs) were firstly discovered in 2010 in the model filamentous fungus *N. crassa* (Lee et al. 2010). Since then, a few miRNAs have been identified in other fungi, such as *F. oxysporum* (Chen et al. 2014), *T. reesei* (Kang et al. 2013), *P. marneffei* (Lau et al. 2013) and *M. anisopliae* (Zhou et al. 2010). By both in silico and in vitro analysis, we demonstrated not only the existence of miRNAs for the first time in *Fon*, but also gained insights into their expression profiles and corroborated the regulatory function of miRNAs in toxin biosynthesis by targeting specific genes.

Table 4 List of known miRNAs which differentially expressed more than twofolds in *Fon*

Known miRNA name	Microconidia read number	Hyphae read number	log ₂ ratio (microconidia/hyphae) normalized	Up or down (microconidia to hyphae)
Fon-miR7696a-3p	0	5048	-12.7372	Down
Fon-miR166a	0	2406	-11.6682	Down
Fon-miR1886.1	0	484	-9.35461	Down
Fon-miR869.2	0	435	-9.20062	Down
Fon-miR156c	0	348	-8.87869	Down
Fon-miR7502e	0	333	-8.81512	Down
Fon-miR4414b	0	304	-8.68367	Down
Fon-miR2661	0	294	-8.63542	Down
Fon-miR7700-5p	0	284	-8.58549	Down
Fon-miR9722	0	247	-8.38411	Down
Fon-miR9897-5p	0	237	-8.32449	Down
Fon-miR167 h	0	236	-8.31839	Down
Fon-miR6032	0	231	-8.28749	Down
Fon-miR1144b	0	194	-8.03566	Down
Fon-miR7787-3p	0	157	-7.73037	Down
Fon-miR396e-3p	0	156	-7.72115	Down
Fon-miR8646	0	142	-7.58549	Down
Fon-miR5070-3p	0	136	-7.52321	Down
Fon-miR9386	0	103	-7.12225	Down
Fon-miR5226	0	101	-7.09396	Down
Fon-miR5161	0	100	-7.0796	Down
Fon-miR2641	0	93	-6.9749	Down
Fon-miR169r-3p	0	81	-6.7756	Down
Fon-miR395v	0	77	-6.70253	Down
Fon-miR5766	0	77	-6.70253	Down
Fon-miR6108a	0	74	-6.6452	Down
Fon-miR482b-5p	0	73	-6.62557	Down
Fon-miR1861a	0	67	-6.50183	Down
Fon-miR164	0	66	-6.48014	Down
Fon-miR408b	0	59	-6.31839	Down
Fon-miR898b	0	56	-6.2431	Down
Fon-miR535d	0	55	-6.2171	Down
Fon-miR157a-5p	35	3349	-6.01598	Down
Fon-miR1157-3p	0	47	-5.99033	Down
Fon-miR838-3p	0	34	-5.52321	Down
Fon-miR8175	0	28	-5.2431	Down
Fon-miR159b	0	14	-4.2431	Down
Fon-miR166a-3p	81	1683	-3.81271	Down
Fon-miR4348a	292	1690	-1.96873	Down
Fon-miR4340	47	250	-1.84694	Down
Fon-miR5072	215	917	-1.52833	Down
Fon-miR1112-5p	1687	7064	-1.50177	Down
Fon-miR3438-3p	67	247	-1.31802	Down
Fon-miR2916	2949	1873	1.21913	Up
Fon-miR1438	4695	2815	1.302245	Up
Fon-miR6194	2461	1386	1.392572	Up
Fon-miR3630-5p	12034	4240	2.069235	Up
Fon-miR172a	82	16	2.921807	Up

Table 4 continued

Known miRNA name	Microconidia read number	Hyphae read number	log ₂ ratio (microconidia/hyphae) normalized	Up or down (microconidia to hyphae)
Fon-miR168a-5p	1227	45	5.333321	Up
Fon-miR393-5p	14	0	5.37161	Up
Fon-miR408-5p	15	0	5.471146	Up
Fon-miR9774	17	0	5.651718	Up
Fon-miR1432	26	0	6.264695	Up
Fon-miR5078	35	0	6.693538	Up
Fon-miR394b-3p	47	0	7.118844	Up
Fon-miR528-5p	48	0	7.149217	Up
Fon-miR3440b-3p	112	0	8.37161	Up
Fon-miR167a	149	0	8.783423	Up
Fon-miR5564a	152	0	8.812182	Up
Fon-miR156 k-5p	210	0	9.2785	Up

Table 5 List of novel miRNAs which differentially expressed more than twofolds in *Fon*

Novel miRNA name	Microconidia read number	Hyphae read number	log ₂ ratio (microconidia/hyphae) normalized	Up or down (microconidia to hyphae)
Fon-novel_mir_1	0	688	-10.1001	Down
Fon-novel_mir_12	0	368	-9.1974	Down
Fon-novel_mir_5	0	54	-6.42873	Down
Fon-novel_mir_11	0	39	-5.95924	Down
Fon-novel_mir_10	0	36	-5.84376	Down
Fon-novel_mir_14	0	30	-5.58073	Down
Fon-novel_mir_3	0	22	-5.13327	Down
Fon-novel_mir_13	0	16	-4.67384	Down
Fon-novel_mir_6	0	15	-4.58073	Down
Fon-novel_mir_7	0	15	-4.58073	Down
Fon-novel_mir_2	38	15	1.667197	Up
Fon-novel_mir_18	10	0	4.648088	Up
Fon-novel_mir_17	20	0	5.648088	Up
Fon-novel_mir_15	21	0	5.718478	Up
Fon-novel_mir_30	25	0	5.970017	Up
Fon-novel_mir_21	30	0	6.233051	Up
Fon-novel_mir_25	65	0	7.348528	Up
Fon-novel_mir_22	5582	0	13.77273	Up

In our experiment, a large number of small RNAs from two propagules (hyphae and microconidia) in *Fon* were sequenced using a high-throughput sequencing technology. As expected, the miRNAs cloning frequency was very low (only approximately 0.68% of total sequences) in *Fon*, which supported the hypothesis that the accumulation level of miRNAs in these fungi is very low and thus difficult to detect by traditional sequencing approaches (Bartel 2004). In total, 53 miRNAs and 14 novel miRNA candidates were identified in *Fon* using Mireap program. The identification of miRNAs in this fungus supported the concept

that the small RNAs are a conserved regulator of gene expression in eukaryotes (Bartel 2004). Fungal miRNAs share many similarities with animal and plant miRNAs. They are all similarly processed from stem-loop RNA precursors and the majority of those require a Dicer-like enzyme for their biogenesis (Liu 2010). In addition, Argonaute-like proteins, the core components of RNA-induced silencing complex (RISC) are highly conserved in diverse eukaryotes including fungi. With the exception of Saccharomycotina, the majority of ascomycetes analyzed so far contain at least one pair of Dicer-like and Argonaute-

Table 6 Potential toxin-related miRNA and their potential targets

Homologs miRNA	Sequences	Targets name	Targets length (nt)	Homologs gene	Accession No.	E-value
Fon-miR7696a-3p	TTCAATGAGAACTTGAAG	FOMG_13322	2910	<i>Gibberella zeae</i> strain GZ3639 trichothecene gene cluster	AF359361.3	0
Fon-miR6108a	TGAGAAGCGTAGAGAAGAACC	FOMG_19538	762	<i>Fusarium oxysporum</i> f. sp. <i>erythroxili</i> necrosis and ethylene-inducing peptide (NEP1) gene	AF036580.1	4e-169

Fig. 4 Electrophoretogram of stem-loop PCR products from Fon-miR6108a and Fon-miR7696a-3p

like protein coding genes (Nakayashiki 2005; Drinnenberg et al. 2011).

Furthermore, target gene expression can be specifically knocked down by an RNAi-based method in many fungi (Catalanotto et al. 2002; Kadotani et al. 2004; Gong et al. 2007; Segers et al. 2007). In *Fom* genome, three Argonaute-like, one Dicer-like and one MRPL3-like protein were founded by Blast homology search in this study. All these results showed that plant pathogenic fungus *Fon* could also encode miRNAs and use them as a way of post-transcriptional regulation.

Pathogens secrete a wide range of cytolytic toxins into the apoplast and cytoplasm of plants to facilitate infection and to suppress host defenses (Ottmann et al. 2009). Especially in facultative parasites during necrotrophic lifestyle, various toxins are produced to enhance microbial

virulence (Gijzen and Nürnberger 2006). The most occurring *Fusarium* mycotoxins are trichothecene; they are potent inhibitors of protein translation in eukaryotes; moreover, toxins such as aflatoxins, fumonisin, zearalenone, and fusaric acid often appear synchronically and with overlapped function (Shi et al. 2017). Genes involved in trichothecene biosynthesis are usually clustered and closely linked (Hohn and Beremand 1989). Therefore, the spatiotemporal expression of these toxin genes in different types of propagules keeps intriguing.

Former researches have shown that miRNAs play regulatory roles in cellular differentiation and development (Bartel 2004), and knowledge about the expression patterns of miRNAs contributes to understanding of their functions. A recent study showed in *A. flavus*, Afl-miR-33 was predicted to target gene USTA which is responsible for ustiloxin B biosynthesis (Bai et al. 2015). Here, we found that two miRNAs are involved in regulating gene involved in toxin biosynthesis. Their expression level is evidently different in hyphae and microconidia which indicate that toxin biosynthesis is submitted to certain development stage. Our qRT-PCR results (Fig. 5) show that the expression level of miRNA is negative related with the expression level of target genes. However, whether miRNA negative tuning the target genes expression by cleaving target genes or inhibiting their translation is still needed to be proved by further investigation.

Conclusion

In the present work, two predicted miRNAs were identified in *Fon* with high-throughput sequencing. The differential expression profile of miRNAs and their target gene from hyphae and microconidia suggests that miRNAs may play a role in regulation of toxin-related genes expression. Taken together, our results provided new knowledge

Fig. 5 qRT-PCR validations of Fon-miR6108a, Fon-miR7696a-3p and the target gene FOMG_13322 expression levels

regarding the small RNA transcriptome and new insights into the function of miRNAs in toxin biosynthesis in fungi *Fon*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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