Exploring MicroRNA-Like Small RNAs in the Filamentous Fungus Fusarium oxysporum

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Abstract

RNA silencing such as quelling and meiotic silencing by unpaired DNA (MSUD) and several other classes of special small RNAs have been discovered in filamentous fungi recently. More than four different mechanisms of microRNA-like RNAs (miRNAs) production have been illustrated in the model fungus Neurospora crassa including a dicer-independent pathway. To date, very little work focusing on small RNAs in fungi has been reported and no universal or particular characteristic of miRNAs were defined clearly. In this study, small RNA and degradome libraries were constructed and subsequently deep sequenced for investigating miRNAs and their potential cleavage targets on the genome level in the filamentous fungus F. oxysporum f. sp. lycopersici. As a result, there is no intersection of conserved miRNAs found by BLASTing against the miRBase. Further analysis showed that the small RNA population of F. oxysporum shared many common features with the small RNAs from N. crassa and other fungi. According to the known standards of miRNA prediction in plants and animals, miRNA candidates from 8 families (comprising 19 members) were screened out and identified. However, none of them could trigger target cleavage based on the degradome data. Moreover, most major signals of cleavage in transcripts could not match appropriate complementary small RNAs, suggesting that other predominant modes for miRNA-mediated gene regulation could exist in F. oxysporum. In addition, the PAREsnip program was utilized for comprehensive analysis and 3 families of small RNAs leading to transcript cleavage were experimentally validated. Altogether, our findings provided valuable information and important hints for better understanding the functions of the small RNAs and miRNAs in the fungal kingdom.


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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. Sequencing raw data of small RNA and degradome libraries was deposited at the NCBI Sequence Read Archive (SRA) under accession no. SRP034883.

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Introduction

Small RNAs and their regulatory roles in plants and animals have aroused great interest in molecular biology during the last decade. Various small non-coding RNAs (ncRNAs), about 20–30 nucleotides (nt) long, deriving from endogenous or extrinsic pathways could regulate genes and genomes at different levels, which is known as post-transcriptional gene silencing (PTGS) [1], quelling [2] or RNA interference (RNAi) [3]. Current research has demonstrated that similar regulatory mechanisms exist in most eukaryotes, even in some eubacteria, archea [4] and unicellular organisms [5–7], which play fundamental roles in growth, development and stress responses.

MicroRNAs (miRNAs) [8–10], short interfering RNAs (siRNAs) [11] and piwi-interacting RNAs (piRNAs) [12] represent three major categories of functional small RNAs. In recent years, many other types of small RNAs with certain features have been constantly described and classified according to their origins, binding proteins or secondary structures, such as trans-acting siRNAs (tasiRNAs) [13], repeat associated siRNAs (rasiRNAs) [14], natural antisense-siRNAs (natsiRNAs) [15], tiny non-coding RNAs (tncRNAs) [16], heterochromatic small RNAs (hcRNAs) [17] and small scan RNAs (scnRNAs) [18], etc., which sufficiently exhibited a more complex and diverse roles of small RNAs.

Quelling, the first fungal RNA silencing phenomenon, was found in Neurospora crassa through the fact that the expression of an endogenous gene was attenuated by transformation with homologous sequences [2,19,20]. The following reports on meiotic silencing by unpaired DNA (MSUD) and repeat-induced point mutation (RIP) [21,22], fungal-specific RNA silencing mechanisms, further indicated that fungi possessed sophisticated RNA regulatory networks. However, the key components of RNA silencing, like RNA-dependent RNA polymerase (RdRP), AGO and Dicer proteins, are not universally present in fungi and lost in a considerable amount of fungal species including the model budding yeast Saccharomyces cerevisiae [23]. In some cases, the absent hall-markers could be found in its close relative [24], leading to the hypothesis that the inherent mechanisms of fungal
small RNAs are undergoing different stages and levels due to their diverse directions and rate of evolution.

In perspective of miRNAs, at least four kinds of miRNA-like RNAs (milRNAs) have been identified in the model fungus N. crassa, including a dicer-independent pathway. The production of these milRNAs didn't require QDE-1 (QUELLING DEFICIENT-1, an RdRP) and QDE-3 (a RecQ DNA helicase) but depended on Dicer, QDE-2 (an AGO protein), the exonuclease QIP and an RNase III domain-containing protein (MRPL3) [25]. Characteristics of these known milRNAs cannot meet the criteria for miRNA in plants or animals exactly. So, to some extent, study of fungal milRNAs would offer a new angle to better understand miRNAs and their regulatory roles in eukaryotic organisms. In recent years, the deep sequencing technology is being applied for fungal milRNAs investigation. Although many research works about small RNAs and/or their roles in PTGS have been reported recently. Herein, we combined research works about small RNAs and/or their roles in PTGS and only perfectly matched reads were maintained. As a result, 8,435,010 (84.48%) mapped reads, representing 493,304 (55.04%) unique, could be found at least one locus on genome. Annotation of these loci indicated that intergenic region was the major source for small RNA production, accounting for 95.0%, while only 3.3% and 1.7% were associated with exons and introns, respectively (Fig. 1B). However, a considerable fraction of unique reads with low abundance were matched on exons (38.5%), which could be degraded products of message RNAs or specific exon-derived small RNAs. Chromosomal distribution analysis showed that small RNAs were intensively generated from Chr02a, Chr02b, Chr04, scaffolds and mitochondrion with preference on sense or antisense strand (Fig. 1C).

Before miRNA prediction, mapped reads were screened against miRNAs, including rRNA, tRNA and snoRNA. Notably, 5,867,889 (58.77%) mapped reads were perfectly matched to ribosomal RNA, whereas tiny components come from rRNA and snoRNA (Table 1). The remaining 2,563,874 (25.68%) reads, designated as unknown reads, were used for miRNA prediction pipelines. Chromosomal distribution of unknown reads indicated that, Chr04, Chr08 and mitochondrion were major origins while Chr04, Chr07, Chr08 and Chr13 showed serious strand preference for small RNA generation (Fig. S2A). In the rRNA-derived small RNAs, 19 nt, 24 nt and 25 nt were abundant in total and unique reads showed relatively even distribution throughout 18 nt−30 nt (Fig. S2B). No obvious nucleotide bias was observed at the first nucleotide at the 5′ or 3′ end. Also, clean reads, mapped reads and unknown reads shared similar nucleotide bias to that of rRNA-derived small RNAs (Fig. S2C, D, E, F).

Conserved miRNAs screening
To identify miRNA homologs, unknown reads were searched against miRBase (Release 19.0) using BLAST and Perl scripts. Two mismatches or length difference were allowed for homolog determination. Although 21,264 hairpins and 25,141 mature miRNAs derived from 193 species were registered in miRBase, only three homologs with low abundance were finally confirmed, which were failed to be defined as miRNA candidates in F. oxysporum (fox-miRNAs) during subsequent analysis (Table S1). Because of the evolutionary divergence of miRNA genes, there is no common miRNA between plants and animals despite similar mechanisms for miRNA generation co-existed [39]. So, it makes sense that no miRNA orthologous was found between filamentous fungi and other eukaryotic species.
Prediction and identification of miRNAs

Due to the fact there is no commonly accepted standards for fungal miRNA prediction, both criteria of plants and animals were employed to comprehensively investigate miRNAs in *F. oxysporum*. miRDeep2 program was carried out with no reference option to predict miRNAs according to animal standards. As a result, 15 miRNA candidates were acquired. 13 of them passed significant randfold p-value test, and 6 of them were not classified as known ncRNAs (Table S2). Based on the features of plant miRNA precursors, a stringent pipeline integrating miRCheck was developed and performed for miRNA analysis. After contexts extraction, miRCheck judgment, single-strand evaluation and ortholog clustering, 35 families comprising 66 members were designated as fox-miRNAs, in which 6 families contained multiple members and 9 families were characterized by star strands (miRNA*) (Table S3). Subsequently, outputs of miRDeep2 and miRCheck were combined and manually confirmed according to their hairpin structures while fox-miRNA-4 was the only miRNA candidate co-existed in the two groups of results. At last, 15 miRNA candidates were subjected to experimental verification by RT-PCR, in which 10 of them showed positive results (Fig. 2C & Table 2). 11 putative targets of 7 fox-miRNAs were also...

Figure 1. Characterization of small RNAs in *F. oxysporum*. A: Size distribution of small RNAs. White and black columns represent unique and total reads of the small RNAs, respectively. B: Annotation of small RNA loci. Pie graphs show the proportions of small RNAs located in intergenic, exonic and intronic regions, respectively. C: Small RNA distribution on both strands of chromosomes.

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predicted using an online miRNA target search program, psRNATarget [40] (Table S4).

The sequence alignment (Fig. 2A), secondary structures (Fig. 2B) and coverage histograms of fox-miRNA precursors (Fig. S2G) were figured out with mature miRNAs indicated. A considerable conservation of the fox-miRNA precursors’ sequences was detected when blasted against another 13 genomes of Fusarium species, including 11 F. oxysporum strains, one F. graminearum, and one F. verticillioides (Table S5). Further alignments of fox-miRNA-4 precursors confirmed the conservation of the hairpin loop structure in F. oxysporum (Fig. S3).

Degradome library analysis

A degradome library deriving from RNA fragments with 5’ monophosphate was constructed and deep sequenced using polyA-enriched RNAs. Illumina/Solexa sequencing produced a total of 13,428,134 single-end reads. Quality screening was performed and 12,468,839 reads were selected for further analysis. Considering the relative low quality in the tail of reads, only front 26 nt were cut out and used for mapping analysis to ensure the specificity and minimize the false positive. Exactly, 454,679 (3.66%), 297,873 (2.40%) and 112,310 (0.90%) of the trimmed tags perfectly matched one or more positions in the genome, genes and transcripts, respectively (Table 3). When mapping transcripts, tag abundance, 5’ end alignment positions and cleavage site numbers on each single transcript were recorded and categorized as previously reports [41]. Generally, higher abundance and fewer numbers of cleavage sites are more likely to be the result of endonucleolytic cleavage rather than random degradation products. 16 transcripts with highest peak-to-total ratio were illustrated in Fig. 3, in which most signals were located in the 3’ UTR. However, these predominant cleavage sites do not have pairing small RNAs among unknown reads under conditions that no mismatch occurred at positions 10 and 11 of small RNAs. Other top 24 transcripts with strong signals also cannot match appropriate small RNAs either (Fig. S4).

Cleavage regulation of small RNAs in F. oxysporum

PAREsnip program was utilized to make an in-depth survey of cleavage sites in transcripts and corresponding small RNAs in F. oxysporum [42]. For each signal found, the alignment between small RNA and target was scored and the credibility was classified into five categories as defined in CleaveLand [43]. In total, 5,344 transcripts were found matching various amounts of degradome tags. Under strict parameters, 147 transcripts were finally identified as cleaved targets in which 39, 20, 77 and 11 transcripts were belonged to categories 0, I, II and III, respectively (Fig. S5). Among category 0, 3 families of small RNAs comprising 6 members were experimentally validated (Fig. 2C). The corresponding transcripts, FOXG00035T0, FOXG04985T0 and FOXG05275T0, encoded an esterase, a golgi integral membrane protein (Cln3) and a hypothetical protein, respectively (Fig. 4). These results reduced the likelihood of extensive small RNA-guided target cleavage as previously expected.

Discussion

Different biogenesis pathways of milRNAs identified in N. crassa and diverse sets of core proteins of RNA silencing uncovered in different fungi gave a hint that the regulatory functions of fungal miRNAs are ubiquitous but complicated [25,44]. Recently, deep sequencing technology has been proven to be an effective strategy for discovering novel miRNAs by generating more than ten millions of reads which was believed to cover every single RNA molecular expressed in the studied sample. Besides, the available genome data and the redundant Ago, Dicer and RdRP genes provided prerequisites and foundations for global discovery of miRNAs in F. oxysporum. In this work, small RNAs and tags of truncated transcripts were deep sequenced for exploring milRNAs and their potential targets in the filamentous fungus F. oxysporum, which would provide valuable evidence for better understanding miRNAs in fungi.

Small RNA populations of F. oxysporum was obviously different from plants and animals but shared many similarities with fungal species. First, the type of length distribution of small RNAs was similar with other fungi reported, including Metarhizium anisolae [20], Triehoderma reesei [29], Penicillium marneffei [30], and Sclerotinia sclerotiorum [31], but obviously distinguished from higher eukaryotes in which 21 nt and 24 nt classes were dominant [25,44]. Recently, deep sequencing technology has been proven to be an effective strategy for discovering novel miRNAs by generating more than ten millions of reads which was believed to cover every single RNA molecular expressed in the studied sample. Besides, the available genome data and the redundant Ago, Dicer and RdRP genes provided prerequisites and foundations for global discovery of miRNAs in F. oxysporum. In this work, small RNAs and tags of truncated transcripts were deep sequenced for exploring milRNAs and their potential targets in the filamentous fungus F. oxysporum, which would provide valuable evidence for better understanding miRNAs in fungi.
no defined length and strong nucleotide preference at both ends were different from qRNAs in N. crassa which have a strong preference for uridine at the 5' end (Fig. S2F). Next, 14.9% of total reads and 41.1% of unknown reads were located in mitochondrion, sharing similar percentage in N. crassa and indicating that the mitochondrion might be a major source for small RNA production in filamentous fungi. Forth, a class of abundant small RNAs equally matching two strands of particular regions was observed during miRCheck prediction, which was similar with the observation in fungus S. sclerotiorum [31]. Also like disiRNA (Dicer-independent small interfering RNA) in N. crassa, these small RNAs appeared to originate from loci producing overlapping sense and antisense transcripts (data not shown). Last, a large fraction of unique reads matching to exonic regions, which seemed to be ex-siRNA (exonic-siRNAs) reported in Mucor circinelloides [48, 49], were much more like degraded products of message RNAs for their low abundance and even distribution pattern. Additionally, the Fusarium oxysporum f. sp. lycopersici (Fol) lineage-specific regions, including total chromosomes 3, 6, 14, 15 and parts of chromosome 1 and 2, were calculated for small RNA mapping and it’s no obvious that they were major origins for small RNA generation. In short, these results confirmed the hypothesis that various types of small RNAs exist in fungi and mechanisms for their production might be universal or alternative among filamentous fungi.

No homologs of conserved miRNAs found in F. oxysporum by BLASTing against miRNA database suggested that fungal miRNAs have evolved independently from plants and animals. Given that there is no available standards for distinguishing miRNAs, criteria for miRNA identification in plants and animals were both employed in this study. After multiple rounds of data filtering, 15 miRNA candidates were screened out and 10 of them were finally identified as fox-miRNAs. Comparative analyses of the fox-miRNA precursors among 14 Fusarium species, indicated a considerable conservation of the sequence and secondary structure.
structure between different *F. oxysporum* strains and also, to a low extent, between different *Fusarium* species.

Unfortunately, all expected cleavage sites triggered by these fox-miRNAs were not validated by degradome data, suggesting that gene silencing mediated by fox-miRNAs might be independent, at least not completely dependent, on guiding transcripts cleavage [50]. Further analysis of degradome data showed that most strong signals were located in the 3'-UTR of target transcripts and no complementary small RNAs could match these coordinates appropriately. In category 0 of PAREsnip results, the majority of signals were located in the 3'-end positions of small RNAs loci were indicated, respectively. 8 families of small RNAs leading to target cleavage based on the degradome data. Moreover, most miRNA candidates from 8 families (comprising 19 members) were known standards of miRNA prediction in plants and animals, while some other fungi. According to the

<table>
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*The precursor sequences of miRNAs and 5'-end positions of small RNAs loci were indicated, respectively.

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Table 2. miRNAs of *F. oxysporum* identified in this study.

**Conclusions**

In this study, small RNA and degradome libraries were constructed and subsequently deep sequenced for investigating miRNAs and their potential cleavage targets on the genome level in the filamentous fungus *Fusarium oxysporum* f. sp. *lycopersici*. As a result, no conserved miRNAs was found by a cross check against miRBase. Further analysis showed that the small RNA population of *F. oxysporum* shared some common features with the small RNAs from *N. crassa* and some other fungi. According to the known standards of miRNA prediction in plants and animals, miRNA candidates from 8 families (comprising 19 members) were screened out and identified. However, none of them could trigger target cleavage based on the degradome data. Moreover, most major signals of cleavage in transcripts could not match appropriate complementary small RNAs, suggesting that other modes for miRNA-mediated gene regulation could exist in *F. oxysporum*. In addition, the PAREsnip program was utilized for comprehensive analysis and 3 families of small RNAs leading to in-depth analysis of small RNA and degradome libraries demonstrated many particularities involved in fungal small RNAs which were quite different from that of plants and animals. Our discovery provided significant amount of data and offered a new perspective for better understanding of the biogenesis and functions of small RNAs and miRNAs in eukaryotic kingdoms.
Figure 3. Scatter plot diagrams of degradome data on transcripts. The 16 most abundant signals with high peak-to-total ratio (>0.8) were selected and illustrated. The x and y axis of each diagram represent position of transcripts and the frequency of tags, respectively. Each tag perfectly matching the transcripts was plotted. However, none of these peak signals correspond to predicted cleavage sites of any small RNAs of *F. oxysporum*. These truncated transcripts might be particularly stable decay intermediates or products of special endonuclease.

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transcript cleavage were experimentally validated. Altogether, our findings provided direct evidence for milRNAs research and implied a more sophisticated system for small RNA generation and regulatory pathways in filamentous fungi.

Materials and Methods

**Fusarium oxysporum** strain and RNA isolation

The *Fusarium oxysporum* f. sp. *lycopersici* strain used in this study was kindly offered by Dr. Zhenchuan Mao (Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences). The virulence of this strain was confirmed by pathogenicity tests. The strain was taken to be activated and cultivated on PDA plates for 7 days. Mycelium was collected for RNA extraction using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The quality and quantity of RNA were determined by formaldehyde denaturing agarose gel electrophoresis and NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Total RNA was used for small RNA and degradome libraries construction as well as RT-PCR experiments (see below).

Small RNA deep sequencing

Small RNA library was constructed using Small RNA Sample Prep Kit v1.5 (Illumina) according to manufacturer’s instructions. Briefly, low molecular weight RNAs (15–30 nt) were isolated from 100 μg total RNA by a 15% TBE-urea denaturing polyacrylamide gel electrophoresis and NanoDrop 1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE). Total RNA was used for small RNA and degradome libraries construction as well as RT-PCR experiments (see below).

Degradome library construction

The degradome library was constructed following the previously published work as described by Ma *et al.* [53]. Different from the conventional protocol for PARE [54], a method similar to that used for transcriptome sequencing was utilized instead of the restriction endonuclease strategy. Approximately 200 ng of poly(A) RNA containing the 5′-monophosphates was used for adaptor ligation directly skipping the initial RNaseIII fragmentation step. Single-end deep sequencing read the first 35 nt that represented the 5′ends of the original truncate RNA fragments. Sequencing raw data of small RNA and degradome libraries was deposited at the NCBI Sequence Read Archive (SRA) under accession no. SRP034883.

Data analysis of small RNA and miRNA prediction

Perl programs were designed and written for data analysis in this section. For data preprocessing, the raw reads were firstly screened according to sequencing quality. The reads with more than four Phred scores below 15 (q15 = 4) were removed. High quality reads were subsequently processed by trimming adaptors, collapsing repeats and recording abundance. Only reads with reliable 3′ adaptor tail but no ‘N’ or adaptor contaminants ahead were cut out to generate clean reads. Clean reads ranging from 18–30 nt were mapped to *F. oxysporum* genome using SOAP [55] and only perfectly matched reads were subjected to further analysis. To perform chromosomal distribution analysis and small RNA loci annotation, average abundance was defined and calculated for reads with multiple loci. After removing rRNA, tRNA and snoRNA based on Rfam [56], SILVA [57] and GtRNAdb [58] databases, the remaining reads were designated as unknown reads and used for miRNA prediction. For conserved miRNA analysis, unknown reads were searched against the mature miRNAs in miRBase (Release 19.0) [59] using BLAST (-m 8). Customized Perl programs were used to find homologs under two mismatches or difference in length. For novel miRNA prediction, miRDeep2 [60] and miRCheck [61] were both used according to the criteria of animals and plants, respectively. To reduce the computation, unknown reads with abundance less than 3 times or loci number larger than 20 were excluded. miRDeep2 software was implemented with default and no reference miRNA options. In miRCheck approach, 100 bp flanks around each locus were extracted where adjacent loci less than 50 bp were combined and regarded as one. Then, each context was predicted by RNAfold [62] for secondary structure and examined by miRCheck with default parameters. Given that miRNAs were derived from single strand precursors relative to siRNAs, an
additional step was performed for single-strand verification. Only hairpins with coverage rate more than 10-fold between sense and antisense were retained (Fig. S6). Finally, outputted hairpins were clustered and mature miRNAs were manually checked for identification of \( F. \) oxysporum miRNA candidates. psRNA\_Target online was used for target prediction [40]. Detailed procedures for small RNA analysis and miRNA prediction can be seen in Fig. S1.

Bioinformatic analysis of degradome

Quality screening step was performed initially and low-quality nucleotides were trimmed at the tails of raw reads. To improve the mapping accuracy and keep the specificity, only first 26 nt of reads were extracted and collapsed as unique tags. PARE\_snip program was used with stringent parameters to comprehensively analyze regulatory interactions leading to mRNA cleavage [42]. Draft genome assembly and annotated file of \( F. \) oxysporum were produced by the Broad Institute (http://www.broad.mit.edu/).

RT-PCR assay for miRNAs

The experimental validation of small RNAs was performed using miR\_Cute miRNA First-Strand cDNA synthesis kit (Tiangen, KR201) and miR\_Cute miRNA qPCR detection kit (Tiangen, FP401). All reactions were repeated twice with two biological samples and detected by 2% (w/v) agarose gel electrophoresis. Primers used in this section were listed in Table S6.

Supporting Information

Figure S1 Flow chart of small RNA analysis and miRNA prediction.
(TIF)

Figure S2 Chromosomal distribution and nucleotide bias of small RNAs. A: Chromosomal distribution of unknown reads. B: Length distribution of rRNA-derived small RNAs. Nucleotide biases at both ends of clean reads (C), mapped reads (D), unknown reads (E) and rRNA-derived small RNAs (F). G: Histograms of small RNA coverage on fox-miRNA precursors. Base coverage was counted and recorded when mapping small RNAs onto genome. Short bars indicate the location of mature miRNAs. The asterisk represents the miRNA* sequence.
(TIF)

Figure S3 Alignment of the fox-miRNA-4 precursors in 11 Fusarium species.
(TIF)

References