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Review

Characterization and application of small RNAs and RNA silencing mechanisms in fungi

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ABSTRACT

Although extensively cataloged and functionally diverse in plants and animals, the role and targets of small RNAs remain mostly uncharacterized in filamentous fungi. To date, much of the knowledge of small RNAs in filamentous fungi has been derived from studies of a limited group of fungi, most notably in *Neurospora crassa*. While most of the recently discovered classes of small RNAs appear to be unique to fungi some are commonly found in eukaryotes. It is noteworthy that the RNA silencing protein machinery involved in small RNA biogenesis has also diverged greatly, particularly within filamentous fungi, and may explain the diversity of small RNA classes. In this review, we summarize important classes of eukaryotic small RNAs and provide a current analysis of the RNA silencing machinery based on available fungal genome sequences. Finally, we discuss opportunities for exploiting knowledge of small RNAs and RNA silencing for practical application such as engineering plants resistant to fungal pathogens.

1. Introduction

Small RNAs are the primary actors in RNA silencing. They selectively guide the RNA-induced silencing complex (RISC) to specific transcripts resulting in degradation of numerous genes involved in physiological and developmental processes, defense against selfish repetitive elements and invading parasites (Malone and Hannon, 2009; Ruiz-Ferrer and Voinnet, 2009; Katiyar-Agarwal and Jin, 2010). Novel sequencing technologies coupled with knowledge of the functional relevance of small RNAs as riboregulators has greatly accelerated interest in small transcriptome characterization (Le Roux *et al.*, 2011; Zhao *et al.*, 2007; Heisel *et al.*, 2008; Kawaji *et al.*, 2008; Lee *et al.*, 2010). In filamentous fungi, recent cataloging of small RNAs has revealed several unique classes of small RNAs, particularly in *Neurospora crassa*; although their targets and roles remain to be fully elucidated (Lee *et al.*, 2009, 2010). In other fungal species including plant pathogenic fungi, small RNA-mediated gene silencing is still in its initial exploration phase.

Small RNA molecules are mobile and can be taken up by cells. The treatment of fungal protoplasts with synthetic siRNAs directed toward *Aspergillus flavus* and *Aspergillus parasiticus aflD* (nor-1) has been shown to effectively silence gene expression (Khatri and Rajam, 2007; Abdel-Hadi *et al.*, 2011). In *Aspergillus nidulans*, siRNAs can also be up-taken during spore germination resulting in RNA silencing (Khatri and Rajam, 2007). Although such studies remain limited, these findings strongly suggest use of synthetic molecules as an attractive means to study RNA silencing *in vivo* as well as to

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elucidate possible role and targets of fungal small RNAs in growth, development and pathogenesis. Recently, host-induced gene silencing (HIGS) has been shown to be a promising strategy to control fungal disease (Nowara *et al.*, 2010; Yin *et al.*, 2010). In these studies, expression of dsRNA molecules in plants, specifically targeting fungal transcripts, resulted in RNA silencing of fungal targets and limited fungal infection. Thus, further characterization and analysis of small RNAs and RNA silencing machinery combined with the uptake and movement of synthetic siRNA in and between cells may result in valuable practical applications.

Here, we summarize (i) different classes of fungal small RNAs involved in posttranscriptional gene silencing (PTGS); (ii) describe current methodologies toward application and characterization of small RNA and RNA silencing in fungi; (iii) and discuss possible outcomes for the elucidation of the small RNA catalog with regards plant–fungal pathogen interactions.

2. Small RNAs

Here, small RNAs are defined as non-coding molecules of size 20–40 nucleotides (nt) with potential roles in RNA degradation or repression of translation. To date, many classes of small RNAs have been described including in fungi, and are classified based on their RNA precursor molecule and biogenesis mechanisms. Although small RNAs have been extensively studied in plants and animals, recent work in filamentous fungi has expanded the repertoire of eukaryotic small RNAs with the discovery of QDE-2-interacting small RNAs (qiRNAs), microRNA-like RNAs (milRNAs), Dicer-independent small interfering RNAs (disiRNAs) in *N. crassa*, endogenous short RNAs (esRNAs) in *Mucor circinelloides*, and LTR retrotransposon-siRNAs (LTR-siRNAs) and tRNA-derived RNA fragments (tRFs) in *Magnaporthe oryzae* and *Aspergillus fumigatus* (Lee *et al.*, 2009, 2010; Nunes *et al.*, 2011; Nicolas *et al.*, 2010; Jöchl *et al.*, 2008). Here, we briefly highlight the main features of these fungal small RNA classes. In addition, we describe microRNAs (miRNAs) because they are the best understood class of small RNAs in eukaryotes; although attempts to date have failed to identify canonical miRNAs in fungi (Lee *et al.*, 2010; Nunes *et al.*, 2011).

QDE-2-interacting small RNAs (qiRNAs)

At present, qiRNAs have only been described in *N. crassa*. They are approximately 21 nt long molecules and have a bias for uracil (U) at the 5′ end and for adenine (A) at the 3′ end. This class of small RNAs corresponds to both sense and antisense strands of mainly the ribosomal DNA repeat. Interestingly, aberrant RNAs (aRNAs) are induced by DNA-damage and also serve as precursors for qiRNA biogenesis. Although qiRNAs associate with QDE-2, their biogenesis requires only QDE-1, QDE-3 and Dicer (Lee *et al.*, 2009; Li *et al.*, 2010; Dang *et al.*, 2011).

tRNA-derived RNA fragments (tRFs)

tRFs have been identified in a number of deep sequencing studies (Kawaji *et al.*, 2008; Nunes *et al.*, 2011; Jöchl *et al.*, 2008). Usually, tRFs originate from either 5′ or 3′ of tRNA genes with lengths between 27 and 40 nt, although in some organisms 22 nt members have been also reported (Kawaji *et al.*, 2008; Nunes *et al.*, 2011). Biogenesis of tRFs primarily results from ribonuclease activity within or close to the anticodon loop, which generates tRNA-derived small RNAs corresponding to either tRNA halves. Additional smaller fragments may be generated through misnuclease activity on nucleotide sites exposed at the tips of arms of tRNA cloverleaf structure.

Recently, Rny1p and Angiogenin from *Saccharomyces cerevisiae* and human, respectively, have been identified as the requisite ribonucleases (Thompson and Parker, 2009; Yamasaki *et al.*, 2009). In addition, in HeLa cells Dicer has been shown to be responsible for the processing $tRNA^{Gln}$ into tRFs of 20 nt in length (Cole *et al.*, 2009). A growing body of evidence suggests that tRFs play a role in growth and development. First, tRNA cleavage results in tRNA depletion. This has been suggested to result in down regulation of protein synthesis in the human fungal pathogen *A. fumigatus* (Jöchl *et al.*, 2008). In *M. oryzae*, we have shown recently that tRFs were proportionally more abundant in the appressoria (a specialized host attachment structure) and spores compared to mycelia (Nunes *et al.*, 2011). This is consistent with tRFs playing a role in regulating protein synthesis. Mycelial tissues are actively growing requiring a full complement of tRNAs, which is in contrast to appressoria and spores.

Other recent data from humans suggest that tRFs have a sophisticated biogenesis mechanism that gives rise to distinct tRFs types having dynamic roles in RNA silencing. These tRFs have a 5′-phosphate and a 3′-hydroxyl group and were classified in two distinct types. tRFs type I are processed by Dicer from a mature tRNA molecule in the cytoplasm and are involved in down regulation of target transcripts in *trans*. Biogenesis of tRFs type II involves a single RNaseZ cleavage leaves a 5′-phosphate group and takes place close to the 3′ end of the tRNA cloverleaf molecule and occur in the nucleus at the 5′ end of tRFs type II. Also, the 3′ end of tRFs type II is derived from RNA polymerase III transcription termination. tRFs type II were not observed to have gene silencing activity, presumably because they show preferential association with the Argonautes 3 and 4. Interestingly, strong gene silencing (>80 %) was observed after the introduction of an oligonucleotide that was antisense to the overexpressed candidate tRF type II, but sense to the reporter gene. This scenario favored dsRNA formation and a biased association with Argonaute 2. These recent findings expand possible roles of tRFs in fungi and humans (Haussecker *et al.*, 2010).

miRNA-like small RNAs (milRNAs)

milRNAs were first discovered in *N. crassa* and named milRNAs due to similarities with miRNAs. This class has sizes of 19 and 25 nt as a consequence of four distinct biogenesis mechanisms involving different combinations of small RNA biogenesis components including dicers, QDE-2, the exonuclease QIP and an RNase III domain-containing protein MRPL3. Although all four RNA precursors vary in length and are transcribed from intergenic regions, milRNAs originate from hairpin RNA molecules, have U at the 5′ end and associate with Argonaute protein. To our knowledge, the roles of

milRNAs have not been reported to date (Lee et al., 2010; Li et al., 2010; Dang et al., 2011).

Dicer-independent siRNAs (disiRNAs)

Like milRNAs, disiRNAs have been reported only in *N. crassa* and their biogenesis is independent of Dicer. Current research suggests that dsRNA, originating from overlapping sense and antisense transcripts, are likely to be the precursor molecules for disiRNAs. DisiRNAs have bias for 5′ U and an average size of 22nt (Lee et al., 2010; Li et al., 2010; Dang et al., 2011).

Endogenous short RNAs (esRNAs)

esRNAs were discovered in *M. circinelloides* mycelia. The most predominant class originated from exons (exonic-siRNAs) and are produced by RNA-dependent RNA polymerase 1 (RdRp1) and Dicer-like 2 (DCL2). They are responsible for eliminating mRNA transcripts of the protein coding genes from which they are produced. PTGS is likely to be the silencing mechanism since methylation is not associated with silencing in *M. circinelloides*. In addition, biogenesis of esRNAs generated from transposons and intergenic regions also appear to require RdRp1 and DCL2 (Nicolas et al., 2010).

Small interfering RNAs (siRNAs)

siRNAs, generally defined as 21–24 nt long molecules derived from dsRNA precursor molecules, have been described in many eukaryotes including fungi. Within one strand complementary transcripts pairs and are further processed giving rise to endogenous-siRNAs (endo-siRNA). On the other hand, dsRNA containing exogenous RNA for instance trangene or viral RNA) give rise to exogenous-siRNAs (exo-siRNAs). Regardless of origin, Dicer associates and cleaves long dsRNA into short dsRNAs (21–24 nt) and AGO uses an ssRNA to target mRNA transcripts for degradation. In budding yeasts *Saccharomyces castellii* and *Candida albicans*, endo-siRNAs map to several genomic features including rRNA, tRNA, repetitive elements, ORF and others (Drinnenberg et al., 2009). Similar observations were recently made during characterization of small RNAs from mycelia and appressoria of *M. oryzae* (Nunes et al., 2011).

MicroRNA (miRNAs)

miRNAs have been extensively characterized in plants and animals. These small RNAs of 21–25 nt originate from endogenous miRNA genes. They are known to regulate a variety of processes including growth, development and response to abiotic and biotic stresses including during host–microbe interactions (Katiyar-Agarwal and Jin, 2010). However, to the best of our knowledge, canonical miRNAs have not been described in fungi. After transcription by RNA Pol II (some instances Pol III), single strand RNA molecules that may be as long as 1 kb in length folds back to form a hairpin structure referred to as the primary miRNA (pri-miRNA). In some cases, polycistronic pri-miRNAs give rise to different miRNA families. miRNAs originating from intronic regions are named Mirtrons and are produced from shorter pri-miRNAs (Kim et al., 2009; Kim, 2005; Winter et al., 2009). Interaction of pri-miRNA with DCL1 (plants) or Drosha (animals), and other proteins, triggers cleavage events around the base of the hairpin to create the precursor-miRNA (pre-miRNA) and, subsequently, to duplex miRNA (60–100 nt) that is transported to the cytoplasm by exportin-5 protein (Yi et al., 2003). In the cytoplasm, Dicer chops pre-miRNA into miRNA duplexes (dsRNA molecule having miRNA and miRNA*). The miRNA* strand is unwound from the duplex and eliminated, while the miRNA strand associates with AGO and is guided into RNA-induced silencing complex (RISC) to the target transcript for mRNA degradation or repression of translation (Kim, 2005; Ghildiyal and Zamore, 2009; Bartel, 2004; Esquela-Kerscher and Slack, 2006). Comprehensive reviews on miRNAs biogenesis and roles can be found elsewhere (Kim et al., 2009; Ghildiyal and Zamore, 2009; Bartel, 2004). Since the discovery of *lin-4*, the first reported miRNA in *Caenorhabditis elegans* (Bartel, 2004; Lee et al., 1993), an extensive list of miRNAs has been identified in algae, animals and plants. According to the last release (17 April 2011) of the miRBase (http://www.mirbase.org/index.shtml; accessed on July 05, 2011) the total number of miRNA entries is 16,772 (Griffiths-Jones et al., 2006, 2008; Griffiths-Jones, 2004; Kozomara and Griffiths-Jones, 2011). miRBase contains 207 entries for *C. elegans*, 106 annotated as being able to form a hairpin-structure molecule (Warf et al., 2011). Interestingly, Dicer-independent biogenesis of miRNAs has been reported in vertebrates (Yang and Lai, 2010). These additional pathways for canonical miRNA biogenesis likely increase miRNA complexity.

3. Current methodologies for further characterization of small RNA and RNA silencing in fungi

The ability to grow fungi in culture and availability or lack thereof a reference genome, and/or transcriptome, has largely determined strategies used to identify RNA silencing proteins and characterization of small RNAs. For plant pathogenic fungi, most studies regarding RNA silencing have emerged primarily from hemibiotroph and necrotroph organisms (Nunes & Dean, unpublished data, 2011).

The ability to perform homologous gene replacement is pivotal for functional characterization of the RNA silencing machinery. For instance, the functions of two dicer coding genes identified in *N. crassa*, *M. oryzae* and *M. circinelloides* genomes were characterized by gene knockout (Nicolás et al., 2007; de Haro et al., 2009; Catalanotto et al., 2004; Kadotani et al., 2004). In *N. crassa*, only the double Dicer mutant was defective to process dsRNAs into 25 nt siRNAs suggesting functional redundancy (Catalanotto et al., 2004). In contrast, Dicer 2 was shown to be essential for processing EGFP hairpin RNAs into small RNAs in *M. oryzae* (Kadotani et al., 2004). Furthermore, the Dicer 2 mutant was impaired to silence the LTR-retrotransposable element MAGGY (Murata et al., 2007). Thus, in *M. oryzae* only Dicer 2 is required to process hairpin-structured RNAs into siRNAs. In *M. circinelloides*, Dicer 1 mutants were able to silence *carB*, whereas Dicer 2 mutants did not (de Haro et al., 2009). For non-tractable fungi including obligate biotrophic plant pathogens, pioneering transformation strategies are being developed such as recently reported for the rust flax fungus, *Melampsora lini*

Table 1 – Number of fungal small RNA biogenesis/processing proteins.

Fungal Taxonomy	Dicer	Argonaute	RdRp	Exportin-5	RNase T2
Ascomycota					
- Pezizomycotina					
Ajellomyces capsulata (strain NAm1 / WU24)	2	3	3	1	1
Ajellomyces dermatitidis (strain SLH14081)	2	3	3	2	1
Arthroderma otae (strain CBS 113480)	2	3	3	0	0
Arthroderma benhamiae (strain CBS 112371)	2	3	3	1	1
Aspergillus clavatus (strain ATCC 1007 / CBS 513.65 / DSM 816 / NCTC 3887 / NRRL 1)	2	2	3	2	2
Aspergillus fumigatus (strain CEA10 / CBS 144.89 / FGSC A1163)	2	2	3 (2)	2	2
Aspergillus flavus (strain ATCC 200026 / FGSC A1120 / NRRL 3357 / JCM 12722 / SRRC 167) [a]	2 (3)	3	3	2	2
Aspergillus nidulans FGSC A4	2 (1)	2 (1)	2	2	3
Aspergillus niger (strain CBS 513.88 / FGSC A1513)	3	3	2	2	2
Aspergillus oryzae (strain ATCC 42149 / RIB 40)	3	3	3	2	2
Aspergillus terreus (strain NIH 2624 / FGSC A1156)	2	2	3	2	2
Botryotinia fuckeliana (strain B05.10) [a]	2	3	2	2	4
Chaetomium globosum	2	2	4	3	0
Coccidioides posadasii (strain C735)	2	3	3	3	1
Magnaporthe oryzae (strain 70-15 / FGSC 8958) [a]	2	4 (3)	4 (3)	3	2
Nectria haematococca (strain 77-13-4 / FGSC 9596 / MPVI)	2	3	4	4	2
Neosartorya fumigata (strain ATCC MYA-4609 / Af293 / CBS 101355 / FGSC A1100) [b]	2	2	2	2	2
Neosartorya fischeri (strain ATCC 1020 / DSM 3700 / FGSC A1164 / NRRL 181)	2	3	2	2	1
Neurospora crassa	2	3	4 (3)	4	1
Paracoccidioides brasiliensis (strain ATCC MYA-826 / Pb01)	2	2	0	2	1
Penicillium chrysogenum (strain ATCC 28089 / DSM 1075 / Wisconsin 54-1255)	2	3	2	2	2
Penicillium marneffei (strain ATCC 18224 / CBS 334.59 / QM 7333)	2	3	3	2	2
Phaeosphaeria nodorum [a]	2 (4)	7 (6)	4	3	2
Podospora anserina [a]	2	2	4	3	1
Pyrenophora tritici-repentis (strain Pt-1C-BFP) [a]	2	4	3	2	2
Sclerotinia sclerotiorum (strain ATCC 18683 / 1980 / Ss-1) [a]	2	3	3	1	3
Sporothrix schenckii	1	0	0	0	0
Talaromyces stipitatus (strain ATCC 10500 / CBS 375.48 / QM 6759 / NRRL 1006)	2	2	3	2	2
Trichophyton verrucosum (strain HKI 0517)	2	3	3	1	1
Uncinocarpus reesii (strain UAMH 1704)	2	3	0	3	1
- Saccharomycotina					
Ashbya gossypii (strain ATCC 10895 / CBS 109.51 / FGSC 9923 / NRRL Y-1056)	0	0	0	2	1
Candida albicans	0	1	0	1	4
Candida dubliniensis (strain CD36 / CBS 7987 / NCPF 3949 / NRRL Y-17841)	0	1	0	1	3
Candida glabrata	0	0	0	3	1
Candida tropicalis (strain ATCC MYA-3404 / T1)	0	1	0	2	3
Clavispora lusitaniae (strain ATCC 42720) [c]	0	0	0	2	2
Debaryomyces hansenii	0	1	0	2	1
Kluyveromyces lactis	0	1	3	1	1
Lachancea thermotolerans (strain CBS 6340)	0	0	0	2	1
Lodderomyces elongisporus	0	0	0	1	3
Pichia guilliermondii	0	0	0	2	1
Pichia pastoris (strain GS115)	0	0	0	3	1
Saccharomyces cerevisiae (strain YJM789)	0	0	0	1	1
Vanderwaltozyma polyspora (strain ATCC 22028 / DSM 70294)	0	1	3	3	1
Yarrowia lipolytica	0	0	0	2	2
Zygosaccharomyces rouxii (strain ATCC 2623 / CBS 732 / IFO 1130 / NBRC 1623 / NCYC 568)	0	0	0	2	2
- Taphrinomycotina					
Schizosaccharomyces japonicus (strain yFS275 / FY16936)	1	1	1	3	0
Schizosaccharomyces pombe	1	1	1	3	0

(continued on next page)

Table 1 (continued)

Basidiomycota					
- Agaricomycotina					
Coprinopsis cinerea (strain Okayama-7 / 130 / FGSC 9003)	3	8	8 (7)	2	0
Cryptococcus neoformans	4 (1)	2 (1)	2 (1)	4	1
Laccaria bicolor (strain S238N-H82)	0	0	0	2	3
Postia placenta (strain ATCC 44394 / Madison 698-R)	1	3	8	0	0
- Ustilaginomycota					
Malassezia globosa (strain ATCC 96807 / CBS 7966)	0	0	0	2	1
Ustilago maydis [a]	0	0	0	2	2

a Plant pathogenic fungi.
b Teleomorph of *Aspergillus fumigatus*.
c *Candida lusitaniae*.
Boxed lines = RNA silencing proteins are absent.
Green highlighted boxes = gene count agrees with Nakayashiki and Nguyen (2008).
Orange highlighted boxes = gene count disagrees with Nakayashiki and Nguyen (2008).
Numbers between parentheses correspond to the gene count reported in Nakayashiki and Nguyen (2008).

(Lawrence *et al.*, 2010). Only once these methodologies are fully developed, will it be feasible to expand knowledge and function of small RNAs in these important groups of fungi.

Identification and functional characterization of RNA silencing machinery

Since the publication of the *S. cerevisiae* genome sequence, over 400 fungal genome projects have been initiated. More than 100 are currently at the draft stages and over 50 are completed and publically available (Goffeau *et al.*, 1996; Ma and Fedorovab, 2010). The availability of fungal genomes has greatly facilitated the identification of coding protein genes in many fungal species. For instance, the sequence of well conserved Dicer, Argonaute and RNA-dependent RNA polymerase (RdRp) genes can be used to query and identify homologs in any given fungal species.

We took advantage of public genome sequences and mined the data for all fungal specific RNA silencing annotated domains for Argonaute (IPR003100), Dicer (IPR005034) and RdRp (IPR007855) using InterPro (Table 1). Here, we included 54 genomes, greatly expanding previous analyses (Nakayashiki and Nguyen, 2008; Nakayashiki *et al.*, 2006). Overall, we discovered that the number of RNA silencing gene homologs is quite variable among fungal species. Many fungi contain 2–3 copies of each gene, although some species such as *Schizosaccharomyces japonicus* and *Schizosaccharomyces pombe* contain only a single copy of each gene. Furthermore, we observed a total lack of the RNA silencing machinery in 13 species including *Clavispora lusitaniae*, *S. cerevisiae*, *Ustilago maydis*, *Ashbya gossypii*, *Candida glabrata*, *Laccaria bicolor*, *Lachancea thermotolerans*, *Lodderomyces elongisporus*, *Malassezia globosa*, *Pichia guilliermondii*, *Pichia pastoris*, *Yarrowia lipolytica* and *Zygosaccharomyces rouxii*. In a number of instances, our data was in disagreement with earlier reports. For instance, we found fewer number of Dicer genes in *A. flavus* and *Phaeosphaeria nodorum*. In other cases, we found more genes, such as all members of the RNA silencing machinery in *Cryptococcus neoformans* and Argonaute and RdRp in *M. oryzae*. It is likely that updated versions of the fungal genomes used here explain the observed differences. Alternatively, such as *A. nidulans*, manual examination revealed that one Dicer and one Argonaute protein were truncated and rendered non-functional (Hammond *et al.*, 2008b).

In addition, two other proteins were included in our analysis. First, exportin-5 (IPR013598) was included because it has been shown to transport numerous small RNAs from nucleus to cytoplasm and may also be considered as a RNA silencing machinery component. Although most of the fungal species have 1 or 2 genes encoding this transporter protein, *Nectria haematococca*, *N. crassa* and *C. neoformans* have four copies while *Arthroderma otae*, *Sporothrix schenckii* and *Postia placenta* have none (Table 1). RNase T2 (IPR001568) likely plays a role in tRFs biogenesis and was incorporated in our analysis. *Botryotinia fuckeliana* and *C. albicans* have 4 copies whereas most fungal species have 1 or 2 copies. Surprisingly, no candidate RNase T2 genes were identified in *A. otae*, *Chaetomium globosum*, *S. schenckii*, *S. japonicus*, *S. pombe*, *Coprinopsis cinerea*, and *P. placenta* (Table 1). These observations suggest that exportin-5- and RNase T2-like genes have diversified considerably within fungi. Is it formally possible that lack of identification is a function of extreme diversification or simply that the genome sequence encompassing these is incomplete. Exploration of transcriptome data may in some instances be a valuable tool to assist the identification of members of the RNA silencing machinery.

In situations where genomic and transcriptome resources are limited, the use of degenerative PCR primers may allow identification of Dicers-, Argonaute- or RdRp-like genes. Known Dicer, Argonaute and RdRp sequences from phylogenetically closely related species may be sufficiently conserved to identify such proteins in fungi lacking genome resources. Degenerate primers were successfully used to clone and characterize a Dicer coding gene in *M. circinelloides* (de Haro *et al.*, 2009). Alternatively, oligonucleotide probes corresponding to the conserved RNase III and dsRNA-binding domain of Dicer proteins may be used to screen DNA libraries of fungi of interest, particularly if libraries such as BACs are available.

Functional characterization of RNA silencing machinery using surrogate fungi

For non-tractable fungi, such as obligate biotrophs, the lack of reliable molecular genetic transformation methods greatly restricts efforts to functionally characterize the RNA silencing machinery. Work around strategies are possible, for example use of a surrogate fungus for functional complementation of genetic mutations. As an illustration, *Ustilago hordei* may be a promising surrogate to study the RNA silencing protein machinery in other Basidiomycota. It is able to process GUS hairpin RNAs into 25 nt long siRNAs and silence GUS transcripts (Laurie *et al.*, 2008). *U. hordei* deletion mutants can be used to functionally characterize genes of interest from related species based on functional complementation. This approach has been used successfully for the characterization of protein kinase genes from biotrophic Ascomycota and Basidiomycota fungi in tractable fungal substitutes. For instance, *Blumeria graminis* f. sp. *hordei Bka1* ORF effectively restored pathogenicity and appressorium maturation in a *M. oryzae ΔcpkA* mutant (Bindslev *et al.*, 2001). Similarly, the *Puccinia triticina* mitogen-activated protein kinase (MAPK), *PtMAPK1*, complemented the corresponding mutant in *U. maydis* (Hu *et al.*, 2007).

A second approach is based on synthetic reconstruction of RNA silencing. As reported here (Table 1) and elsewhere, several fungal species have lost the entire RNA silencing machinery (Nguyen *et al.*, 2008). *S. cerevisiae* and *U. maydis* lack RNA silencing machinery and represent excellent surrogates for reconstruction of RNA silencing mechanisms from non-tractable fungi. For example, introduction of Dicer and Argonaute from *S. castellii* restored RNA silencing in *S. cerevisiae* and resulted in silencing of endogenous retrotransposons (Drinnenberg *et al.*, 2009). RNA silencing reconstruction has also been shown to work by introduction of animal silencing machinery in *S. cerevisiae*. Suk *et al.* (2011) introduced human Argonaute 2, Dicer and HIV-1 transactivating response (TAR) RNA-binding protein (TRBP) into *S. cerevisiae* resulting in silencing of GFP. It is noteworthy that introduction of *S. castellii* Dicer and Argonaute were sufficient for RNA silencing in *S. cerevisiae*, while using human counterparts also required TRBP. The additional requirement of TRPB in budding yeast suggests subtle but significant differences in RNA silencing functionality across kingdoms. Although it remains to be demonstrated, the RNA silencing machinery from filamentous fungi can restore RNA silencing in yeasts, and these findings have great potential to test putative RNA silencing proteins from non-tractable Ascomycota and Basidiomycota fungi in *S. cerevisiae* and, possibly, in *U. maydis*, respectively.

A number of novel approaches are being developed to assess whether fungi, including obligate biotrophs, possess functional RNA silencing machinery. For example, Lawrence *et al.* (2010) took advantage of the gene-for-gene interaction, specifically between the *AvrL567* effector and its cognate receptor *L6*, to develop an elegant *Agrobacterium*-mediated transformation methodology for *M. lini*. In this work, flax carrying the *L6* receptor infected with a rust isolate expressing *Avr567* was transformed with *Agrobacterium* carrying a silencing cassette able to produce *AvrL567* siRNAs. *M. lini* silenced cells, exhibiting knocked down *AvrL567* effector protein levels, escaped host recognition and resulted in disease development and eventually urediospore production (Lawrence *et al.*, 2010).

Isolation of small RNAs from fungal infected plant tissues

To help explore the emergent role of RNA silencing and small RNAs in determining plant–pathogen interactions, elegant techniques developed to dissect pathogen infected plant tissues could be employed (Mosquera *et al.*, 2009; Portillo *et al.*, 2009; Hacquard *et al.*, 2010). Laser microdissection (LM) has been used in several instances to identify the transcriptome specifically associated with tissues infected with fungi such as *Colletotrichum graminicola* and *Melampsora larici-populina* (Portillo *et al.*, 2009; Hacquard *et al.*, 2010; Takahashi *et al.*, 2010; Nelson *et al.*, 2006; Tang *et al.*, 2006). Other methods have been designated to capture specific fungal cells such as haustoria (specialized feeding structures) from infected plants and used to prepare cDNA libraries such as haustoria specific library from *P. triticina* (Catanzariti *et al.*, 2011; Song *et al.*, 2011). Although promising, these methods have yet to be applied to study small RNA populations. An alternative approach is to first isolate small RNAs specifically bound to RNA silencing proteins (Lee *et al.*, 2009, 2010). This strategy was used to isolate small RNAs physically associated to Argonaute (QDE-2) protein in *N. crassa* using immunopurification (Lee *et al.*, 2009). A similar approach could be used to recover fungal small RNAs from infected plant tissue.

4. Challenges to using RNA silencing to explore plant–fungi interactions

A better understanding of the RNA silencing and small RNAs from both plants and fungi is likely to provide novel insights into plant–fungi interactions and present opportunities such as HIGS to develop disease resistant plants (Nowara *et al.*, 2010; Yin *et al.*, 2010). However, much more needs to be learned regarding small RNA classes and RNA silencing machinery in fungi. This basic knowledge will be essential for future manipulation of fungal pathogens transcriptome regulated by transgenic plants. We conclude with a number of questions that remain to be investigated.

Can RNA silencing be manipulated to interfere with the genetic diversity of plant pathogenic fungi?

Plant pathogenic fungi have evolved a variety of strategies to escape host defenses including maintenance of high genetic diversity at the population level. Many fungi contain substantial amounts of repetitive DNA, which may facilitate genome rearrangements possibly through active movement to retrotransposable elements. For instance, roughly 10 % of the *M. oryzae* genome is composed to repetitive elements. We speculate that this fungus utilizes LTR-siRNAs to regulate integration events of LTR-retrotransposable elements (Nunes *et al.*, 2011). However, it remains to be experimentally addressed. If this is a commonly employed mechanism, can HIGS be engineered to effectively silence fungal repetitive elements and restrict plant pathogen genetic diversity?

Can uptake of synthetic siRNAs or dsRNA molecules be used to manipulate important obligate biotroph fungi?

Recent findings have shown that *Aspergillus* spp. can uptake siRNAs and uptake of dsRNAs can trigger RNA silencing in *Phytophthora infestans* and *Moniliophthora perniciosa* (Khatri and Rajam, 2007; Abdel-Hadi *et al.*, 2011; Caribé dos Santos *et al.*, 2009; Whisson *et al.*, 2005). Furthermore, HIGS involving dsRNA movement from wheat into the *Puccinia striiformis* f. sp. *tritici* suggests that other plant pathogens may also uptake exogenous RNA molecules. Pathogens such as *Puccinia graminis* f. sp. *tritici* Ug99 (PgtUg99) has been reported to rapidly overcome plant genetic resistance (Singh *et al.*, 2011). siRNA/RNA silencing may be a promising tool for further functional characterization of genes in PgtUg99 particularly considering that use of synthetic siRNAs does not require time consuming cloning steps to develop silencing cassettes for fungal transformation (Abdel-Hadi *et al.*, 2011). Alternatively, the *Agrobacterium*-mediated transformation methodology developed by Lawrence *et al.* (2010), may be worthy of further investigation. With continued work, it is feasible that siRNA/RNA silencing approaches will be useful for controlling obligate biotroph fungal pathogens.

Can small RNAs from plant pathogenic fungi posttranscriptionally interfere with host defense genes?

Although evidence now suggests RNA molecules are transported from plant into fungal cells resulting in silencing of fungal transcripts, does the reverse occur? Can host defense gene transcripts induced upon fungal infection potentially be targeted by fungal small RNAs? Based on small RNAs produced by plant pathogenic fungi, can we predict likely host targets? Although highly speculative, addressing these questions, maybe rewarding.

Do plant pathogenic fungi produce specific populations of small RNAs in pathogenesis related structures such as appressoria or haustoria and are small RNAs acting during pre- or post-penetration events?

As previously described, our characterization of the small transcriptome of *M. oryzae* mycelia and appressoria revealed that tRFs were enriched in appressoria. However, more detailed studies are needed to more thoroughly examine changes occurring during pre- and post-appressorial development. In other plant pathogenic fungi, isolation of total RNA from haustoria tissue revealed important fungal transcripts playing essential roles in disease development. These same tissues need to be examined for small RNAs. Only once we understand the biogenesis of these molecules and how they are regulated, will we be able to fully exploit HIGS for development of durable and environmentally safe disease control.

Can RNA silencing invoked by mycovirus infection of plant pathogenic fungi be used to reduce virulence?

Similar to the role of RNA silencing mediated virus defense mechanisms in plants, recent findings suggest that fungal RNA silencing defends against mycoviruses. Hammond *et al.* (2008a) have shown that *Aspergillus* mycoviruses are both targeted and can suppress *A. nidulans* RNA silencing. In plant pathogenic fungi, it is known that mycoviruses can lead to a hypovirulent phenotype and growth impairments as observed in *Cryphonectria parasitica*, *Diaporthe perjuncta* and *M. oryzae* (Nuss, 2005; Moleleki *et al.*, 2003; Urayama *et al.*, 2010). Details of the role and mechanisms of RNA silencing/small RNA during the interaction of mycoviruses and plant pathogenic fungi remain limited. However, further examination may provide another opportunity to manage fungal plant pathogens through hypovirulence and reduced pathogenicity.

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REFERENCES

Abdel-Hadi, A.M., Caley, D.P., Carter, D.R.F., Magan, N., 2011. Control of aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus* using RNA silencing technology by targeting *aflD* (nor-1) gene. Toxins 3, 647–659.

Bartel, D.P., 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.

Bindslev, L., Kershaw, M.J., Talbot, N.J., Oliver, R.P., 2001. Complementation of the *Magnaporthe grisea ΔcpkA* mutation by the *Blumeria graminis* PKA-c gene: functional genetic analysis of an obligate plant pathogen. Mol. Plant Microbe Interact. 14, 1368–1375.

Caribé dos Santos, A.C., Sena, J.A.L., Santos, S.C., Dias, C.V., Pirovani, C.P., Pungartnik, C., Valle, R.R., Cascardo, J.C.M., Vincentz, M., 2009. dsRNA-induced gene silencing in *Moniliophthora perniciosa*, the causal agent of witches' broom disease of cacao. Fungal Genet. Biol. 46, 825–836.

Catanzariti, A.M., Mago, R., Ellis, J., Dodds, P., 2011. Constructing haustorium-specific cDNA libraries from rust fungi. Methods Mol. Biol. 712, 79–87.

Catalanotto, C., Pallotta, M., ReFalo, P., Sachs, M.S., Vayssie, L., Macino, G., Cogoni, C., 2004. Redundancy of the two dicer genes in transgene-induced posttranscriptional gene silencing in *Neurospora crassa*. Mol. Cell. Biol. 24, 2536–2545.

Cole, C., Sobala, A., Lu, C., Thatcher, S.R., Bowman, A., Brown, J.W., Green, P.J., Barton, G.J., Hutvagner, G., 2009. Filtering of deep sequencing data reveals the existence of abundant dicer-dependent small RNAs derived from tRNAs. RNA 15, 2147–2160.

Dang, Y., Yang, Q., Xue, Z., Liu, Y., 2011. RNA interference in fungi: pathways, functions and applications. Eukaryot. Cell 10, 1148–1155.

de Haro, J.P., Calo, S., Cervantes, M., Nicolas, F.E., Torres-Martinez, S., Ruiz-Vazquez, R.M., 2009. A single dicer gene is required for efficient gene silencing associated with two classes of small antisense RNAs in *Mucor circinelloides*. Eukaryot. Cell 8, 1486–1497.

Drinnenberg, I.A., Weinberg, D.E., Xie, K.T., Mower, J.P., Wolfe, K.H., Fink, G.R., Bartel, D.P., 2009. RNAi in budding yeast. Science 326, 544–550.

Esquela-Kerscher, A., Slack, F.J., 2006. Oncomirs – microRNAs with a role in cancer. Nat. Rev. Cancer 6, 259–269.

Ghildiyal, M., Zamore, P.D., 2009. Small silencing RNAs: an expanding universe. Nat. Rev. Genet. 10, 94–108.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., Oliver, S.G., 1996. Life with 6000 genes. Science 274, 546. 563–567.

Griffiths-Jones, S., Saini, H.K., van Dongen, S., Enright, A.J., 2008. miRBase: tools for microRNA genomics. Nucleic Acids Res. 36, D154–D158.

Griffiths-Jones, S., Grocock, R.J., van Dongen, S., Bateman, A., Enright, A.J., 2006. miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 34, D140–D144.

Griffiths-Jones, S., 2004. The microRNA registry. Nucleic Acids Res. 32, D109–D111.

Hacquard, S., Delaruelle, C., Legué, V., Tisserant, E., Kohler, A., Frey, P., Martin, F., Duplessis, S., 2010. Laser capture microdissection of uredinia formed by *Melampsora larici-populina* revealed a transcriptional switch between biotrophy and sporulation. Mol. Plant Microbe Interact. 23, 1275–1286.

Hammond, T.M., Andrewski, M.D., Roossinck, M.J., Keller, N.P., 2008a. *Aspergillus* mycoviruses are targets and suppressors of RNA silencing. Eukaryot. Cell 7, 350–357.

Hammond, T.M., Bok, J.W., Andrewski, M.D., Reyes-Domínguez, Y., Scazzocchio, C., Keller, N.P., 2008b. RNA silencing gene truncation in the filamentous fungus *Aspergillus nidulans*. Eukaryot. Cell 7, 339–349.

Haussecker, D., Huang, Y., Lau, A., Parameswaran, P., Fire, A.Z., Kay, M.A., 2010. Human tRNA-derived small RNAs in the global regulation of RNA silencing. RNA 16, 673–695.

Heisel, S.E., Zhang, Y., Allen, E., Guo, L., Reynolds, T.L., Yang, X., Kovalic, D., Roberts, J.K., 2008. Characterization of unique small RNA populations from rice grain. PLoS One 3, e2871.

Hu, G., Kamp, A., Linning, R., Naik, S., Bakkeren, G., 2007. Complementation of *Ustilago maydis* MAPK mutants by a wheat leaf rust, *Puccinia triticina* homolog: potential for functional analyses of rust genes. Mol. Plant Microbe Interact. 20, 637–647.

Jöchl, C., Rederstorff, M., Hertel, J., Stadler, P.F., Hofacker, I.L., Schrettl, M., Haas, H., Huttenhofer, A., 2008. Small ncRNA transcriptome analysis from *Aspergillus fumigatus* suggests a novel mechanism for regulation of protein synthesis. Nucleic Acids Res. 36, 2677–2689.

Kadotani, N., Nakayashiki, H., Tosa, Y., Mayama, S., 2004. One of the two dicer-like proteins in the filamentous fungi *Magnaporthe oryzae* genome is responsible for hairpin RNA-triggered RNA silencing and related small interfering RNA accumulation. J. Biol. Chem. 279, 44467–44474.

Katiyar-Agarwal, S., Jin, H., 2010. Role of small RNAs in host–microbe interactions. Annu. Rev. Phytopathol. 48, 225–246.

Kawaji, H., Nakamura, M., Takahashi, Y., Sandelin, A., Katayama, S., Fukuda, S., Daub, C.O., Kai, C., Kawai, J., Yasuda, J., Carninci, P., Hayashizaki, Y., 2008. Hidden layers of human small RNAs. BMC Genomics 9, 157.

Khatri, M., Rajam, M.V., 2007. Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene. Med. Mycol. 45, 211–220.

Kim, V.N., Han, J., Siomi, M.C., 2009. Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. 10, 126–139.

Kim, V.N., 2005. MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell Biol. 6, 376–385.

Kozomara, A., Griffiths-Jones, S., 2011. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 39, D152–D157.

Laurie, J.D., Linning, R., Bakkeren, G., 2008. Hallmarks of RNA silencing are found in the smut fungus *Ustilago hordei* but not in its close relative *Ustilago maydis*. Curr. Genet. 53, 49–58.

Lawrence, G.J., Dodds, P.N., Ellis, J.G., 2010. Transformation of the flax rust fungus, *Melampsora lini*: selection via silencing of an avirulence gene. Plant J. 61, 364–369.

Lee, H.C., Li, L., Gu, W., Xue, Z., Crosthwaite, S.K., Pertsemlidis, A., Lewis, Z.A., Freitag, M., Selker, E.U., Mello, C.C., Liu, Y., 2010. Diverse pathways generate microRNA-like RNAs and dicer-independent small interfering RNAs in fungi. Mol. Cell 38, 803–814.

Lee, H.C., Chang, S.S., Choudhary, S., Aalto, A.P., Maiti, M., Bamford, D.H., Liu, Y., 2009. qiRNA is a new type of small interfering RNA induced by DNA damage. Nature 459, 274–277.

Lee, R.C., Feinbaum, R.L., Ambros, V., 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. Cell 75, 843–854.

Le Roux, F., Davis, B.M., Waldor, M.K., 2011. Conserved small RNAs govern replication and incompatibility of a diverse new plasmid family from marine bacteria. Nucleic Acids Res. 39, 1004–1013.

Li, L., Chang, S.S., Liu, Y., 2010. RNA interference pathways in filamentous fungi. Cell Mol. Life Sci. 67, 3849–3863.

Malone, C.D., Hannon, G.J., 2009. Small RNAs as guardians of the genome. Cell 136, 656–668.

Ma, L., Fedorovab, N.D., 2010. A practical guide to fungal genome projects: strategy, technology, cost and completion. Mycology 1, 9–24.

Mosquera, G., Giraldo, M.C., Khang, C.H., Coughlan, S., Valent, B., 2009. Interaction transcriptome analysis identifies *Magnaporthe oryzae* BAS1-4 as biotrophy-associated secreted proteins in rice blast disease. Plant Cell 21, 1273–1290.

Moleleki, N., van Heerden, S.W., Wingfield, M.J., Wingfield, D., Preisig, O., 2003. Transfection of *Diaporthe perjuncta* with *Diaporthe RNA Virus*. Appl. Environ. Microbiol. 69, 3952–3956.

Murata, T., Kadotani, N., Yamaguchi, M., Tosa, Y., Mayama, S., Nakayashiki, H., 2007. siRNA-dependent and -independent post-transcriptional cosuppression of the LTR-retrotransposon MAGGY in the phytopathogenic fungus *Magnaporthe oryzae*. Nucleic Acids Res. 35, 5987–5994.

Nakayashiki, H., Nguyen, Q.B., 2008. RNA interference: roles in fungal biology. Curr. Opin. Microbiol. 11, 494–502.

Nakayashiki, H., Kadotani, N., Mayama, S., 2006. Evolution and diversification of RNA silencing proteins in fungi. J. Mol. Evol. 63, 127–135.

Nelson, T., Tausta, S.L., Gandotra, N., Liu, T., 2006. Laser microdissection of plant tissue: what you see is what you get. Annu. Rev. Plant Biol. 57, 181–201.

Nguyen, Q.B., Kadotani, N., Kasahara, S., Tosa, Y., Mayama, S., Nakayashiki, H., 2008. Systematic functional analysis of calcium-signalling proteins in the genome of the rice-blast fungus, *Magnaporthe oryzae*, using a high-throughput RNA-silencing system. Mol. Microbiol. 68, 1348–1365.

Nicolás, F.E., de Haro, J.P., Torres-Martínez, S., Ruiz-Vázquez, R.M., 2007. Mutants defective in a *Mucor circinelloides* dicer-like gene are not compromised in siRNA silencing but display developmental defects. Fungal Genet. Biol. 44, 504–516.

Nicolas, F.E., Moxon, S., de Haro, J.P., Calo, S., Grigoriev, I.V., Torres-Martinez, S., Moulton, V., Ruiz-Vazquez, R.M., Dalmay, T., 2010. Endogenous short RNAs generated by dicer 2 and RNA-dependent RNA polymerase 1 regulate mRNAs in the basal fungus *Mucor circinelloides*. Nucleic Acids Res. 38, 5535–5541.

Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J., Schweizer, P., 2010. HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. Plant Cell 22, 3130–3141.

Nunes, C.C., Gowda, M., Sailsbery, J., Xue, M., Chen, F., Brown, D., Oh, Y., Mitchell, T.M., Dean, R.A., 2011. Diverse and tissue-enriched small RNAs in the plant pathogenic fungus, *Magnaporthe oryzae*. BMC Genomics 12, 288.

Nuss, D.L., 2005. Hypovirulence: mycoviruses at the fungal–plant interface. Nat. Rev. Microbiol. 3, 632–642.

Portillo, M., Lindsey, K., Casson, S., Garcia-Casado, G., Solano, R., Fenoll, C., Escobar, C., 2009. Isolation of RNA from laser-capture-microdissected giant cells at early differentiation stages suitable for differential transcriptome analysis. Mol. Plant Pathol. 10, 523–535.

Ruiz-Ferrer, V., Voinnet, O., 2009. Roles of plant small RNAs in biotic stress responses. Annu. Rev. Plant Biol. 60, 485–510.

Singh, R.P., Hodson, D.P., Huerta-Espino, J., Jin, Y., Bhavani, S., Njau, P., Herrera-Foessel, S., Singh, P.K., Singh, S., Govindan, V., 2011. The emergence of Ug99 races of the stem rust fungus is a threat to world wheat production. Annu. Rev. Phytopathol. 49, 465–481.

Song, X., Rampitsch, C., Soltani, B., Mauthe, W., Linning, R., Banks, T., McCallum, B., Bakkeren, G., 2011. Proteome analysis of wheat leaf rust fungus, *Puccinia triticina*, infection structures enriched for haustoria. Proteomics 11, 944–963.

Suk, K., Choi, J., Suzuki, Y., Ozturk, S.B., Mellor, J.C., Wong, K.H., MacKay, J.L., Gregory, R.I., Roth, F.P., 2011. Reconstitution of human RNA interference in budding yeast. Nucleic Acids Res. 39, e43.

Takahashi, H., Kamakura, H., Sato, Y., Shiono, K., Abiko, T., Tsutsumi, N., Nagamura, Y., Nishizawa, N.K., Nakazono, M., 2010. A method for obtaining high quality RNA from paraffin sections of plant tissues by laser microdissection. J. Plant Res. 123, 807–813.

Tang, W., Coughlan, S., Crane, E., Beatty, M., Duvick, J., 2006. The application of laser microdissection to in planta gene expression profiling of the maize anthracnose stalk rot fungus *Colletotrichum graminicola*. Mol. Plant Microbe Interact. 19, 1240–1250.

Thompson, D.M., Parker, R., 2009. The RNase Rny1p cleaves tRNAs and promotes cell death during oxidative stress in *Saccharomyces cerevisiae*. J. Cell Biol. 185, 43–50.

Urayama, S., Kato, S., Suzuki, Y., Aoki, N., Le, M.T., Arie, T., Teraoka, T., Fukuhara, T., Moriyama, H., 2010. Mycoviruses related to chrysovirus affect vegetative growth in the rice blast fungus *Magnaporthe oryzae*. J. Gen. Virol. 91, 3085–3094.

Winter, J., Jung, S., Keller, S., Gregory, R.I., Diederichs, S., 2009. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat. Cell Biol. 11, 228–234.

Warf, M.B., Johnson, W.E., Bass, B.L., 2011. Improved annotation of *C. elegans* microRNAs by deep sequencing reveals structures associated with processing by Drosha and Dicer. RNA 17, 563–577.

Whisson, S.C., Avrova, A.O., Van West, P., Jones, J.T., 2005. A method for double-stranded RNA-mediated transient gene silencing in *Phytophthora infestans*. Mol. Plant Pathol. 6, 153–163.

Yang, J.S., Lai, E.C., 2010. Dicer-independent, Ago2-mediated microRNA biogenesis in vertebrates. Cell Cycle 9, 4455–4460.

Yamasaki, S., Ivanov, P., Hu, G.F., Anderson, P., 2009. Angiogenin cleaves tRNA and promotes stress-induced translational repression. J. Cell Biol. 185, 35–42.

Yi, R., Qin, Y., Macara, I.G., Cullen, B.R., 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 17, 3011–3016.

Yin, C., Jurgenson, J., Hulbert, S., 2010. Development of a host-induced RNAi system in the wheat stripe rust fungus *Puccinia striiformis* f. sp. *tritici*. Mol. Plant Microbe Interact. 24, 554–561.

Zhao, T., Li, G., Mi, S., Li, S., Hannon, G.J., Wang, X.J., Qi, Y., 2007. A complex system of small RNAs in the unicellular green alga *Chlamydomonas reinhardtii*. Genes Dev. 21, 1190–1203.