5 Small RNAs in Fungi

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I. Introduction

Fungi are one of the oldest groups of organisms on the Earth, where they play pivotal roles in maintaining life by participating in nutrient recycling and symbiosis with plants. Despite their benefits to the planet, they also put in risk the survival of other species, including humans, when they are genuine or opportunistic pathogens. Besides, we use several fungi for our own benefit in different aspects from food to the treatment of diseases. Thereby, understanding the biology of fungi at different levels ranging from ecology to molecular details is essential, although this kingdom received little attention in the past apart from some classical models such as yeasts, *Neurospora crassa*, and *Aspergillus nidulans*. Fortunately, the study of fungi is living now an exciting period with spectacular advances in our knowledge about the molecular mechanisms underlying the fascinating responses of these organisms to their faced environmental challenges. Several examples are showing how fungi can adapt rapidly to stressful situations that can put their lives at risk, such as the presence of antifungal drugs and high temperature (Calo et al. 2014; Chang et al. 2019; Kronholm et al. 2016; Noble et al. 2016; Slepecky and Starmer 2009). In this context, non-coding small RNAs (sRNAs) play essential roles in genome integrity preservation, gene expression, phenotypic plasticity, and the ability to interact with other organisms via the conserved eukaryotic RNA interference (RNAi) pathway, known also as the RNA-mediated gene silencing mechanism, which mainly represses the expression of target genes at the transcriptional or post-transcriptional level (Torres-Martínez and Ruiz-Vázquez 2017).

The phenomenon of RNAi was initially discovered in plants when the introduction of the chalcone synthase gene in petunia suppressed the expression of both the transgene and the endogenous gene, a phenomenon called co-suppression (Napoli et al. 1990). **Several RNAi pathways have been described in fungi** (Chang et al. 2012; Torres-Martínez and Ruiz-Vázquez 2017; Villalobos-Escobedo et al. 2016) and other species, but the **basic conserved machinery** (Fig. 5.1) comprises an RNase III protein, called Dicer, which produces the sRNA molecules from a double-stranded RNA (dsRNA) precursor. These sRNAs, which receive differ-

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Genetics and Biotechnology, 3rd Edition
The Mycota II
J.P. Benz, K. Schipper (Eds.)

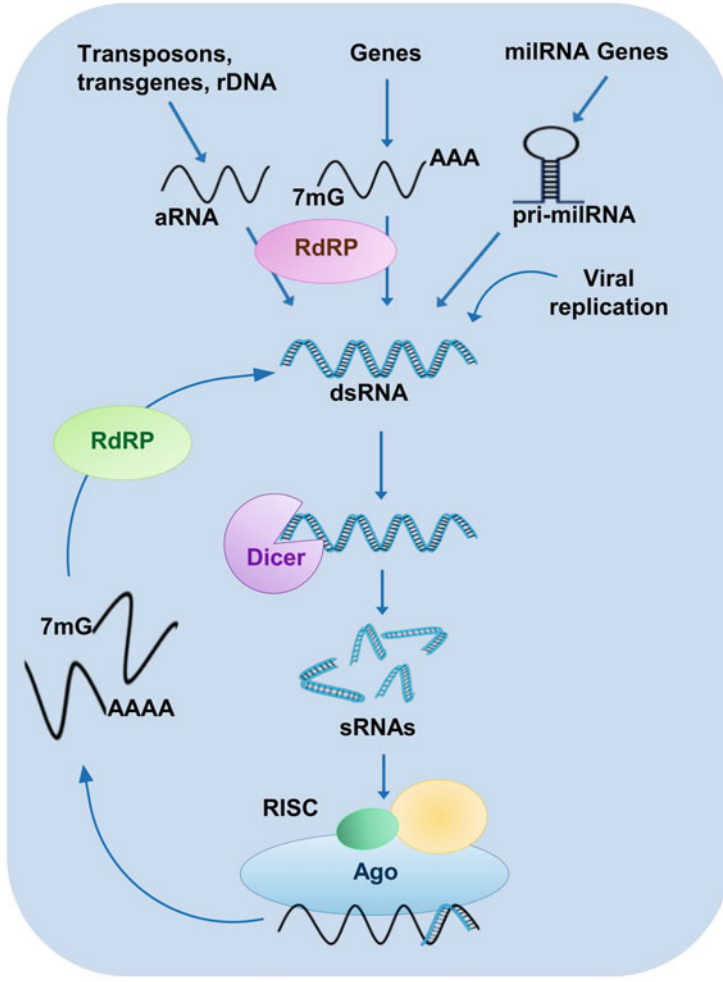

Fig. 5.1 A simplified model for RNAi pathways in fungi. Core components of the RNAi pathways are depicted (Table 5.2). Fungi show a wide diversity of specific RNAi-related pathways that carry out particular functions, which have been previously outlined in great detail (Calo et al. 2017; Chang et al. 2012; Torres-Martínez and Ruiz-Vázquez 2017; Villalobos-Escobedo et al. 2016)

ent names depending on their function and features (Table 5.1), are bound to an Argonaute (Ago) protein that uses one strand of these sRNAs to guide selective destruction, translational repression, or transcriptional suppression of target RNAs. Fungi, as plants and nematodes, present an additional RNA-dependent RNA polymerase (RdRP) protein that generates dsRNA from single-strand RNA (ssRNA) acting as inducers or amplifiers of sRNA signals (Torres-Martínez and Ruiz-Vázquez 2017). Genes coding for the key RNAi components are present in the genomes of most fungi, except for some relevant examples, such as *Cryptococcus deuterogattii* (Feretzaki et al. 2016), some species of the phylum Microsporidia and subphylum Ustilaginomycotina, and those of the subphylum Saccharomycotina and the class Wallemiomycetes (Choi et al. 2014; Nakayashiki et al. 2006; Laurie et al. 2008). Besides the core RNAi components, several accessories proteins with secondary functions have been described (Table 5.2). The **high number of fungi that may have a functional**

Table 5.1 sRNAs in fungi

Acronym	Name	Function	Features	References
siRNAs	Small-interfering RNAs	Genome integrity protection against integrative transgenes, non-integrative transgenes, transposons, and viruses	21–25 nt long with uracil at the 5′ end	Catalanotto et al. (2002) and Nicolás et al. (2003)
masiRNAs	MSUD-associated small-interfering RNAs	Genome integrity protection by silencing of unpaired DNA during meiosis	~25 nt long with uracil at the 5′ end	Hammond et al. (2013b)
qiRNAs	QDE-2-interacting small RNAs	Genome integrity protection in response to DNA damage	21–22 nt long with a strong preference for uracil at the 5′ end	Lee et al. (2009)
rasiRNAs	Repeat-associated small interfering RNA	Epigenetic silencing of transposons	21 nt long	Borgognone et al. (2018)
nat-siRNAs	Natural antisense transcript-derived siRNAs	Regulation of gene expression by targeting specific mRNAs	22–23 nt long	Drinnenberg et al. (2009)
disiRNAs	Dicer-independent esRNAs	Regulation of gene expression by targeting specific mRNAs	22 nt long with uracil at the 5′ end	Lee et al. (2010)
Ex-siRNAs	Exon- derived siRNAs	Regulation of gene expression by targeting specific mRNAs	Classes I and II, 23–24 nt long with a preference for uracil at the 5′ end Classes III and IV, different sizes with a strong preference for uracil in the penultimate position	Nicolas et al. (2010)
milRNA	miRNA-like small RNAs	Regulation of gene expression by targeting specific mRNAs	25 and 19 nt long with a strong preference for uracil at the 5'position	Lee et al. (2010)
rdRNAs	Rdrp-dependent degraded RNAs	Regulation of gene expression by a non-canonical dicer-independent mechanism?	Different sizes with uracil at the penultimate position	Trieu et al. (2015)
vsRNAs	Virus-derived siRNAs	Control of virus	20–22 nt with a strong preference for A at the 3′ position	Zhang et al. (2008)
priRNAs	Primal RNAs	Initiation of heterochromatin formation	22–23 nt long with uracil at the 5′ end	Halic and Moazed (2010)

Table 5.2 RNAi proteins in fungi

Core silencing proteins		
Protein family	Protein name	Function
Argonaute	AgoAGLQDE-2 (*N. crassa*)SMS2 (specific for MSUD in *N. crassa*)	They use one strand of sRNAs as a guide to bind target RNAs. They have endonuclease activity
RNase III protein	DicerDCRDCL	Riboendonuclease that produce sRNA from a dsRNA
RNA-dependent RNA polymerase	RdRPQDE-1 (*N. crassa*)SAD-1 (specific for MSUD in *N. crassa*)	They generate dsRNA from ssRNA. A DNA-dependent RNA polymerase activity has described for some of them
Accessory silencing proteins		
Protein family	**Protein name**	**Function**
RecQ DNA helicase	QDE-3 (*N. crassa*)	They promote aRNA production
RNA/DNA helicase	SAD-3 (specific for MSUD in *N. crassa*)Hrr1 (specific for heterochromatin formation in *S. pombe*)RnhA (*M. circinelloides*)	They act in unwinding nucleic-acid strands
SNF2 helicase-related proteins	SAD-6 (specific for MSUD in *N. crassa*)	Unpaired DNA detection during MSUD
Atypical RNase III protein	R3B2 (*M. circinelloides*)	Ribonuclease involved in the non-canonical RNAi pathway
Protein with RNase III domain	MRPL3 (*N. crassa*)	Dicer-independent biogenesis of some milRNAs
Exonuclease	QIP	They remove the passenger strand from the double-stranded siRNAs bound by ago proteins
Exoribonuclease	Dhp (*S. pombe*)	Dicer-independent biogenesis of sRNAs
Exonuclease	ERI-1 (*N. crassa*)	Biogenesis of disiRNAs
SAD-2	SAD-2 (specific for MSUD in *N. crassa*)	Constitutes a scaffold protein in MSUD
SAD-4	SAD-4 (specific for MSUD in *N. crassa*)	Production of masiRNAs
SAD-5	SAD-5 (specific for MSUD in *N. crassa*)	Production of masiRNAs

RNAi mechanism suggests that it plays an essential role in their biology, although in some particular cases its absence might provide some advantage (Drinnenberg et al. 2011; Nicolás et al. 2013; Wang et al. 2010). Despite the **original function of RNAi was to protect the genome** from deleterious movements of transposable elements (TEs) and viruses, the basic machinery has evolved in all groups of eukaryotes to generate specific pathways with **endogenous regulatory functions**. These pathways produce particular types of sRNAs that are involved in the regulation of gene expression (Table 5.1) (Cerutti and Casas-Mollano 2006). The most representative example of these regulatory sRNAs is the animal and plant microRNA (miRNA) (Ghildiyal and Zamore 2009). In fungi, various endogenous regulatory RNAi pathways have also been described (Calo et al. 2017; Lee et al. 2010; Nicolas et al. 2010; Yu et al. 2018). These pathways interact with each other at several levels, competing for and sharing substrates, components, and cross-regulating each other (Fig. 5.1) (Calo et al. 2017).

Several excellent reviews have examined the RNAi-related pathways, types of sRNAs and their associated functions in fungi (Chang et al. 2012; Torres-Martínez and Ruiz-Vázquez 2017; Villalobos-Escobedo et al. 2016). In this Chapter of The Mycota, we have updated the most recent discoveries and contributions to the field with particular emphasis on functional aspects. Readers interested in mechanistic details specific for each RNAi-related pathway are advised to go through the indicated reviews or the references in this Chapter.

II. Protective Small RNAs

Soon after the discovery of co-suppression in plants (Napoli et al. 1990), a similar RNAi phenomenon in response to transgenes, named **quelling**, was described in *Neurospora crassa*. In this mechanism, the expression of genes harbored in the genome was suppressed when copies of the same gene were introduced (Romano and Macino 1992). Similar responses to transgenes were observed in other fungi (Nicolás et al. 2003; Wang et al. 2010). The RNAi mechanism was well-characterized in *N. crassa* (Chang et al. 2012), which belongs to the Ascomycota phylum, and subsequently this characterization was extended to other significant fungal phyla such as Mucoromycota and Basidiomycota, represented by *Mucor circinelloides* (Torres-Martínez and Ruiz-Vázquez 2017) and *Cryptococcus neoformans* (Feretzaki et al. 2016; Janbon et al. 2010; Wang et al. 2012), respectively. These analyses and others described below revealed that RNAi pathways have a critical role in the maintenance of the genome integrity in response to TEs and other invading nucleic acids. Moreover, the RNAi mechanism as a guard of the genome soon acquired a secondary role in the function and evolution of centromeres, probably due to the high abundance of TEs in these specialized regions of the chromosomes (Friedman and Freitag 2017; Yadav et al. 2018).

A. Small RNAs in Genome Defense

RNAi-meditated protection mechanisms that participate in the maintenance of genome integrity have been identified in different stages of fungal life cycles, reflecting their importance.

1. Defense Responses During Vegetative Growth

The RNAi mechanism that acts in the defense against exogenous nucleic acids during vegetative growth received different names such as quelling in *N. crassa* (Romano and Macino 1992), mitotic-induced silencing (MIS) in *C. neoformans* (Wang et al. 2012) or gene silencing in *M. circinelloides* (Nicolás et al. 2003). However, they constitute basically the same mechanism with particular details in each fungus that affect the accessory RNAi proteins (Table 5.2) and sRNAs features (Table 5.1) (Chang et al. 2012; Torres-Martínez and Ruiz-Vázquez 2017). In these pathways, the long dsRNA is cleaved into double-stranded small-interfering RNAs (siRNAs) of 21–25 nucleotides, which bind to an Ago protein in the RNA-induced silencing complex (RISC) (Fig. 5.1). In most studied fungal models so far, the silencing is associated with a dramatic decrease of mature mRNA levels from target genes, whereas those of primary transcripts are not affected, indicating that it is mainly a **post-transcriptional gene silencing (PTGS)** phenomenon (Nicolás et al. 2003; Pickford and Cogoni 2003).

Full understanding of the function of the silencing mechanism in genome defense requires the **identification of the triggering signal**. Quelling and MIS are initiated when multiple transgene copies are integrated in tandem repeats in the genome (Cogoni et al. 1996; Romano and Macino 1992; Wang et al. 2010). This may also occur in *M. circinelloides* although transgenes are maintained in self-replicative plasmids, since in Mucorales these plasmids are prone to form concatemers and rearrange (Meussen et al. 2012; Michielse et al. 2004; Papp et al. 2013), which might favor the formation of tandem repeats. How the siRNA production is initiated in fungi is best characterized for quelling in *N. crassa*. Tandem repeats alone are not sufficient to induce siRNA production since it also requires double-stranded breaks and homologous recombination (HR) (Yang et al. 2015). Repetitive sequences are regions of genome instability due to hyperrecombination events caused by replication stress (Bzymek and Lovett 2001; Vader et al. 2011). Thus, the proposed model suggests that recombination intermediates produced during HR can be recognized by QDE-3, a putative RecQ DNA helicase involved in RNAi. This helicase might resolve the recombination intermediates into single-stranded DNA (ssDNA), after recruiting the ssDNA-binding complex (RPA) and the dual RdRP enzyme

QDE-1 with RNA/DNA-dependent RNA polymerase activities, which produces an aberrant single-stranded RNA (aRNA) and subsequently the dsRNA that activates the RNAi pathway (Fig. 5.1) (Liu et al. 2010).

In addition to transgenes, fungal genomes naturally contain two main types of tandem repetitive sequences, TE arrays, and ribosomal genes (rDNA), which are known to be a major source of genome instability (Butler 1992; Bzymek and Lovett 2001; Vader et al. 2011). In fact, several studies have revealed large amounts of sRNAs derived from both types of loci in different fungi (Dumesic et al. 2013; Janbon et al. 2010; Nicolas et al. 2010), including qiRNA (QDE-2-interacting small RNAs) and rasiRNAs (repeat-associated small-interfering RNA) originated from rDNA in *N. crassa* (Lee et al. 2009) and transposons in the basidiomycete *Pleurotus ostreatus* (Borgognone et al. 2018), respectively. Even though qiRNA and the transgenes-induced siRNAs have different origins, they require the same RNAi components and are the result of DNA damage (Chang et al. 2012; Zhang et al. 2013). In addition, the RNAi pathway suppresses transposon proliferation in several fungi (Borgognone et al. 2018; Janbon et al. 2010; Nolan et al. 2005; Wang et al. 2010), and it is required to maintain transgene tandem repeats (Yang et al. 2015) and retrotransposon arrays found in centromeres (Yadav et al. 2018).

An alternative mechanism to control TEs during vegetative growth has been described in *C. neoformans*. This mechanism identifies transposon transcripts due to the presence of suboptimal introns, which provokes that they stall on spliceosomes. A spliceosome-coupled and nuclear RNAi (SCANR) complex is able to recognize these transposons transcripts stuck on spliceosomes promoting siRNA production that leads to control of transposons (Dumesic et al. 2013).

Together, these observations suggest that the **principal function of the siRNA produced from repeat regions is to maintain genome stability**, which is supported by the identification of DNA damage-induced sRNAs and the involvement of Dicer enzymes in the maintenance of genome stability in plants and animals (Bonath et al. 2018; Francia et al. 2013; Lu et al. 2018; Michalik et al. 2012; Wei et al. 2012).

2. Defense Responses in the Sexual Cycle

In addition to the RNAi mechanisms that operate in the vegetative growth to protect the genome integrity, other RNAi pathways carry out this function during sexual reproduction. One of such RNAi mechanisms, called **sex-induced silencing (SIS)**, was described in *C. neoformans*. This is a PTGS mechanism that is triggered by tandem integration of a transgene array both in opposite-sex mating and unisexual reproduction. It shares the basic RNAi machinery required for MIS, including Ago, Dicer, and RdRP proteins (Wang et al. 2010, 2013). Beyond silencing exogenous transgenes, SIS plays a critical role in transposon control because RNAi mutants show an increase in transposition/mutation rate and elevated levels of siRNAs derived from repetitive TEs (Wang et al. 2010, 2013). Interestingly, the higher robustness of SIS compared to MIS might be related to the fact that transposons in *C. neoformans* are more active during mating than during vegetative growth (Wang et al. 2010, 2013).

An additional RNAi mechanism involved in defense of the genome during the sexual cycle has been described in species of the Ascomycota phylum, such as *N. crassa, Neurospora tetrasperma*, and *Gibberella zeae* (anamorph *Fusarium graminearum*) (Ramakrishnan et al. 2011; Shiu et al. 2001; Son et al. 2011), suggesting that it has evolved recently (Hammond 2017). This RNAi mechanism protects genome integrity through silencing of all those DNA sequences that remain unpaired during the meiotic prophase I. Consequently, it was named **Meiotic Silencing by Unpaired DNA (MSUD)** (Shiu et al. 2001). This mechanism seems to be mechanistically distinct from the SIS of *C. neoformans* because SIS is not triggered by unpaired DNA structures (Wang et al. 2010). The molecular mechanism of MSUD has been studied in detail in *N. crassa*, where it can be divided into a detection stage of unpaired DNA between homologous chromosomes and a silencing stage of unpaired DNA and any

related sequences found at other locations in the genome, regardless of their pairing state. Nine proteins are involved in MSUD, three of which correspond to the core components of the canonical RNAi pathway and the rest perform specific functions of this mechanism (Hammond 2017). In the latter group are included SAD-5 and SAD-6, which are the only proteins known to participate in the identification of unpaired sequences (Samarajeewa et al. 2014). Despite the poor characterization of the mechanism of unpaired DNA detection, this process is thought to trigger the production of an aRNA, which is recognized in the perinuclear region by the proteins involved in the silencing stage. Thus, a MSUD-specific RdRP SAD-1 and the helicase SAD-3 (Hammond et al. 2011, 2013a; Shiu and Metzenberg 2002; Shiu et al. 2001) generate a dsRNA molecule that is processed by Dicer DCL-1 into MSUD-associated small-interfering RNAs (masiRNAs) (Alexander et al. 2008). These are then bound by AGO SMS-2, and the passenger is removed by the exonuclease QIP (Lee et al. 2003; Xiao et al. 2010). The identification of masiRNAs derived from an unpaired transposon in a sexual cross suggests that MSUD protects the genome from transposons (Wang et al. 2015), although another hypothesis points to the protection of the genome from meiotic drive elements, called spore killers, which rearrange large genomic segments as part of the driving mechanism (Hammond 2017).

Spore killers, described in Ascomycota phylum, are single genes or complexes of genes that favor their own propagation through meiosis and/or gametogenesis by killing the meiotic products not containing them. The best characterized spore killer is *sk-2* of *N. crassa* (Turner and Perkins 1979). The *sk-2* drive mechanism requires that at least two distantly located genes, a resistance gene called *rsk* and a killer gene called *rfk*, inherit together during meiosis (Campbell and Turner 1987). Tight linkage between these two genes appears to be derived from chromosome rearrangements within the *sk-2* element (Harvey et al. 2014). The *sk-2*, and also *sk-3*, suppresses MSUD (Raju et al. 2007), suggesting that this blockage allows the evolution of chromosome rearrangements, which would be MSUD targets (Hammond 2017).

3. Antiviral Small-Interfering RNAs

The use of the RNAi pathway as an innate immune system against viruses was one of the first roles associated with this mechanism. Triggering of siRNA production by dsRNA or ssRNA viruses has been found in plants, flies, worms, mammals, and fungi (Harvey et al. 2011; Jeang 2012; Segers et al. 2007; Wilkins et al. 2005; Zambon et al. 2006). Among fungi, this response has been intensively studied in the ascomycete *Cryphonectria parasitica*, a filamentous fungus that is the causal agent of chestnut blight (Segers et al. 2007), and also described in *Colletotrichum higginsianum* (Campo et al. 2016), *Aspergillus nidulans* (Hammond et al. 2008), *Fusarium graminearum* (Yu et al. 2018), and *Sclerotinia sclerotiorum* (Mochama et al. 2018). *C. parasitica* produces an RNAi-mediated antiviral response by the production of virus-derived siRNAs (vsRNAs) (Table 5.1) that target and destroy viral sequences. Although this fungus has two *dicer*-like genes, four *ago*-like genes, and four *rdrp*-like genes, only genes *dcl2* and *agl2* are involved in the RNAi-mediated antiviral response. Thus, single deletion mutants in any of these two genes were defective in the production of vsRNAs and highly susceptible to mycovirus infections, resulting in a severely debilitated growth (Segers et al. 2007; Sun et al. 2009). Interestingly, the *C. parasitica* **RNAi mechanism also promotes recombination of viral genomic RNA**, a central component of virus evolution that contributes to the emergence of new viruses (Sun et al. 2009; Zhang and Nuss 2008).

The importance of the RNAi-mediated antiviral defense is reinforced by the fact that some **viruses present mechanisms of RNAi suppression**. Thus, the hypovirus *Cryphonectria* hypovirus 1 (CHV1-EP173), a mycovirus that infects *C. parasitica*, expresses the protein p29, a papain-like protease, that inhibits the RNAi pathway by repressing the transcriptional induction of *agl2* in response to virus infections (Sun et al. 2009). Likewise, the existence of a viral suppressor has also been demonstrated in *A. nidulans* (Hammond et al. 2008).

B. Small-Interfering RNAs in Chromosome Function

In previous sections, we have described several mechanisms that use siRNAs to protect the genome from TEs. These protective mechanisms seem to be adopted by eukaryotic cells during evolution to play other relevant functions in the cells. This might be the case for the **formation of heterochromatin** in different regions of the chromosomes, particularly in regional centromeres, where siRNAs play an essential role (Pidoux and Allshire 2005; Volpe and Martienssen 2011). Regional centromeres, contrary to point centromeres, are long ranging from few kilobases to megabases and made of repetitive DNA that consists of either arrays of satellite DNA or TEs or both (Roy and Sanyal 2011). TEs are proposed to play a more significant role in the evolution of this type of centromeres, and their domestication may have given rise to the *dh*/*dg* and α-satellite repeats present in the centromeres of fission yeast (*Schizosaccharomyces pombe*) and humans, respectively (Gao et al. 2015; Wong and Choo 2004).

In fission yeast, RNAi plays a critical role in heterochromatin formation, which it is required for normal centromere function leading to chromosome segregation (Pidoux and Allshire 2005). Dicer-independent sRNAs, named primal RNAs or priRNAs (Table 5.1), are the main effectors in the initiation of heterochromatin formation (Halic and Moazed 2010). The priRNAs are generated from ssRNAs to later form a complex with Ago1 that targets long non-coding centromeric transcripts. This complex recruits the RdRP to synthesize dsRNA, which is subsequently processed by Dicer into secondary siRNAs. These secondary siRNAs are loaded onto the RNA-induced transcriptional silencing complex (RITS), which includes Ago1. The binding of siRNA-containing RITS to the nascent transcripts in the centromeric regions recruits the Clr4 methyltransferase complex, which deposits the H3K9me mark and HP1 family proteins leading to heterochromatin formation (Martienssen and Moazed 2015; Ugolini and Halic 2018).

In addition to the establishment of heterochromatin formation in the centromeres, RNAi also seems to play an essential function in **centromere evolution** in species belonging to the phylum Basidiomycota. Here, RNAi and cytosine methylation are proposed to maintain repetitive transposon-rich centromeres, since RNAi-deficient species or mutants from RNAi-proficient species have shorter centromeres (Yadav et al. 2018). This agrees with the fact that fungi with RNAi systems have more total DNA corresponding to TEs than RNAi-deficient species, although fewer elements are functional, reflecting stringent control over transposition (Muszewska et al. 2017).

III. Small RNAs in the Regulation of Gene Expression

Soon after the discovery of RNAi, the focus on its defensive role gave way to a whole new research field based on the endogenous regulatory functions of sRNAs. It was initiated when miRNAs, previously identified and studied in *Caenorhabditis elegans* (Lee et al. 1993), were found to be conserved in most plants and animals. These discoveries opened an extensive period in which the regulatory roles of RNAi and **endogenous sRNAs (esRNAs)** were the hot topic in molecular biology for several years. Hundreds of miRNAs were found creating a regulatory network that might involve more than 60% of total genes in humans (Friedman et al. 2009) and regulate most of the complex biological processes in living cells such as development, differentiation, maintenance, cell death, and diseases associated with the misregulation of these molecules (Esteller 2011; López-Camarillo and Marchat 2013; Stefani and Slack 2008). During this time, miRNAs and other regulatory esRNAs were considered to be absent in fungi, until molecules similar to miRNAs and regulatory esRNAs were discovered in *Neurospora crassa* and *Mucor circinelloides*, respectively (Lee et al. 2010; Nicolas et al. 2010). However, the regulatory role of esRNAs has been scarcely studied in fungi because few

RNAi-deficient mutants show altered phenotypes in development or physiology. The most remarkable exceptions to this general rule are *M. circinelloides* and *Trichoderma atroviride*, since mutants in core components of the RNAi pathway show clear phenotypes associated with deregulation of large numbers of genes (Carreras-Villaseñor et al. 2013; Cervantes et al. 2013; de Haro et al. 2009; Nicolás et al. 2007, 2015; Trieu et al. 2015).

A. MicroRNA-Like Small RNAs

miRNAs are the most important small regulatory RNAs found in animals and plants as they negatively regulate the expression of hundreds of genes by either inducing degradation or repressing translation of the target mRNAs (Carthew and Sontheimer 2009; Ghildiyal and Zamore 2009). In fungi, an in-depth sequencing study of sRNAs interacting with Ago proteins in *N. crassa* revealed a complex system containing four different types of sRNAs that shared several similarities with miRNA, thereby named as miRNA-like small RNAs (milRNA) (Lee et al. 2010). These four classes of milRNAs were defined according to the RNAi components that were required for their biogenesis.

The synthesis of milRNAs type 1 (milR-1) depends on the activity of Dicer, QDE-2, QIP, and MRPL3 (Table 5.2), whereas the synthesis of milRNAs type 2 (milR-2) only relies on the catalytic activity of QDE-2, being totally independent of Dicer enzymes. \It is a conserved protein harboring a predicted RNase III domain similar to the well-known domains found in Dicer enzymes. The role of MRPL3 in other organisms is unknown, although it might be related to the fate of mRNAs during the synthesis of proteins, considering its association to the large subunit of the mitochondrial ribosomes (Smits et al. 2007). Biogenesis of milRNAs belonging to the type 3(milR-3) is quite similar to a pathway found in plants that only depends on the activity of Dicer enzymes (Carthew and Sontheimer 2009). The fourth type of milRNAs (milR-4) is the most unusual class as it requires MRPL3 and is partially dependent on Dicer enzymes (Lee et al. 2010).

Regardless of specific differences in the biogenesis of each type of milRNAs, all of them share three main features with the synthesis of miRNAs. First, they are produced from RNA precursors of a single strand that forms a typical hairpin structure. Second, the dsRNA region of the typical hairpin structure is processed to generate two complementary sRNAs, although only one of them is predominantly preserved while the other is degraded. Finally, a strong bias for uracil bases at the 5′ termini of the milRNA is commonly found in these sRNAs and shared with canonical miRNAs (Lee et al. 2010). Despite these similarities, the processing and the final features of milRNAs in *N. crassa* also present several fundamental differences compared with miRNAs in animals and plants. Thus, milRNA genes of *N. crassa* are not highly conserved in other species of fungi, indicating an early evolution of milRNAs independent of canonical miRNAs, which usually are highly conserved and share sequence, function and even tissue-specific expression in several species (Bartel 2004; Carthew and Sontheimer 2009). Additional divergences between milRNAs and miRNAs can be found at the beginning of their biogenesis. Thus, cleavage of the milRNA precursors (pri-milRNAs or pre-milRNAs) might be processed by the putative ribonuclease MRPL3, a conserved protein harboring a predicted RNase III domain similar to the well-known domains found in Dicer enzymes that has not been involved in miRNA biogenesis in plant and animals (Bartel 2004; Carthew and Sontheimer 2009). Also, pri-milRNAs are transcribed by RNA polymerase III, representing an additional significant difference compared to the well-established role of RNA polymerase II in the transcription of pri-miRNAs in plants and animals (Yang et al. 2013).

The function of **milRNAs has been linked to the regulation of the expression of target mRNAs** similarly to miRNAs (Lee et al. 2010). The first result supporting the functionality of milRNAs was the overexpression of target mRNAs in Dicer mutants of *N. crassa*, suggesting that target mRNAs are repressed by milRNAs (Lee et al. 2010). Correspondingly, mutants devoid in the Ago protein QDE2 (Table 5.2) also displayed increased levels of predicted target mRNAs and QDE2 co-precipitates with these target mRNAs. More

direct evidence showed that mutants lacking specific milRNA genes exhibited elevated levels of the mRNAs harboring predicted target sequences, indicating that these target sequences are regulated by milRNAs (Lee et al. 2010).

The discovery of milRNAs in *N. crassa*, a fungus of the Ascomycota phylum, proved that regulatory sRNAs are also produced in the lower branches of the Eukarya domain. Subsequently, other studies revealed the presence of similar regulatory sRNAs in fungi belonging to both the Ascomycota (*Fusarium oxysporum* (Chen et al. 2014), *Penicillium chrysogenum* (Dahlmann and Kück 2015), and *Trichoderma reesei* (Kang et al. 2013)) and Basidiomycota (*Coprinopsis cinerea* (Lau et al. 2013) and *Antrodia cinnamomea* (Lin et al. 2015)) phyla, suggesting that milRNAs have functional roles in these fungal groups. However, the thoroughly accomplished studies in *N. crassa* and other fungi could not identify phenotypes or specific functions associated with mutants enable to produce all milRNAs, such as deletion mutants lacking *dicer* or *ago* genes, or particular milRNA. Despite the absence of phenotypes, these studies uncovered several cases of expression profiles associated with specific developmental stages and groups of milRNAs targeting predicted mRNAs involved in the same functional roles (Jiang et al. 2017; Lau et al. 2013, 2018; Lin et al. 2015). Other studies have also identified **milRNAs that could control the production of compounds with industrial applications**, such as hydrolases and antibiotics (Dahlmann and Kück 2015; Kang et al. 2013).

The diversity of milRNAs across fungi, including sequence, number, expression pattern, and especially the lack of particular phenotypes associated with mutations in these regulators, represents a major obstacle that is preventing the unveiling of the different roles of milRNAs in fungal physiology. Nevertheless, all the studies describing fungal milRNAs have contributed to enlighten a heterogeneous world of endogenous regulatory sRNAs in Ascomycota and Basidiomycota phyla. The only common feature among all these fungi is the **lack of a unique or universal pathway for the production of milRNAs,** showing a scenario where the diversification of regulatory silencing mechanisms is the chosen strategy rather than the conserved pathways found in animals and plants (Bartel 2004; Carthew and Sontheimer 2009).

B. Exon-Derived Regulatory Endogenous Small RNAs

M. circinelloides accumulates several types of regulatory esRNAs generated by both Dicer-dependent and Dicer-independent RNAi pathways (Cervantes et al. 2013; Nicolas et al. 2010; Trieu et al. 2015). The Dicer-dependent esRNAs, named ex-siRNAs, are derived from exons of protein-coding genes and regulate the expression of the producing genes by degradation of the corresponding mRNAs (Nicolas et al. 2010). In addition to Dicer, the rest of components of the RNAi pathway involved in siRNA-mediated genome defense are also implicated in the biogenesis of ex-siRNAs, although they are combined to originate four different classes of ex-siRNAs (Nicolas et al. 2010). The four ex-siRNA classes not only differ in the biogenesis pathway but also in their binding to the main Ago proteins (Ago1) and structural characteristics, such as length, strand bias and 5′ nucleotide (Cervantes et al. 2013; Nicolas et al. 2010). The ex-siRNA-mediated regulation extends beyond the genes that produce them since hundreds of likely second targets genes are differentially expressed in deletion mutants for genes coding for RNAi proteins involved in ex-siRNA biogenesis (Nicolás et al. 2015). The massive alteration in gene expression in these mutants should affect genes that participate in the developmental and physiological processes affected in these mutants, which include vegetative growth, response to nutrient starvation and oxidative stress, asexual sporulation, mating, and development (Cervantes et al. 2013; de Haro et al. 2009; Nicolás et al. 2007, 2015; Trieu et al. 2015).

Analysis of esRNA accumulating in Dicer mutants in *M. circinelloides* revealed the existence of a new type of esRNAs, named rdRNAs (*rdrp*-dependent degraded RNAs). This led to the discovery of a **non-canonical RNAi mecha-**

nism in which participate the three RdRP enzymes of *M. circinelloides* and the Sad-3-like helicase *rnhA* (Calo et al. 2017; Trieu et al. 2015). The Dicer function is carried out by an atypical RNase III-like enzyme, named R3B2 (Table 5.2), which has been found only in Mucorales. The rdRNAs have characteristics of degradation products (Table 5.1), suggesting that **this mechanism is likely a degradation pathway** that controls the levels of specific mRNAs (Trieu et al. 2015). This pathway regulates the expression of hundreds of highly expressed genes involved in metabolism, regular cellular processes, and signaling; consequently, the deletion mutant lacking the key gene *r3b2* shows resistance to oxidative stress and defects in asexual sporulation, response to nutrient, and mating (Trieu et al. 2017). These kinds of Dicer-independent mechanisms are not restricted to *M. circinelloides,* but have been described in other fungi. In *Schizosaccharomyces pombe*, Dhp exoribonuclease drives a novel RNAi and exosome-independent pathway of epigenetic silencing and also plays a role in PTGS (Tucker et al. 2016). Similarly, R3B2 of *M. circinelloides* plays a relevant role in the canonical silencing mechanism in parallel to its function in the Dicer-independent degradation pathway (Trieu et al. 2015). Besides, Dicer-independent small non-coding RNAs (disiRNAs), described in *N. crassa,* are also produced by a Dicer-independent RNAi pathway from loci that generate overlapping sense and antisense transcripts as a result of convergent transcription (Lee et al. 2010). Although these sRNAs show structural characteristics corresponding to siRNAs, none of the components of the RNAi machinery is involved in their biogenesis (Lee et al. 2010).

The presence of esRNAs derived from protein-coding genes has also been detected in several fungi belonging to Ascomycota phylum, but only RNAi mutants of some species show a phenotype that suggests the involvement of esRNAs in regulation. Different components of the RNAi machinery are involved in light-dependent asexual reproduction and light-independent hyphal growth in *Trichoderma atroviride* (Carreras-Villaseñor et al. 2013), in vegetative growth in *Magnaporthe oryzae* (Kadotani et al. 2004), and formation of sexual spores after sexual interaction in *Fusarium graminearum* (Son et al. 2017). Despite these remarkable examples, the number of phenotypes observed in *M. circinelloides* and their strengths suggest that the **RNAi pathway plays a more relevant role in early-divergent fungi** than in more evolved fungi (Ascomycota and Basidiomycota), where milRNAs and other regulatory sRNAs may fine-tune gene expression (Villalobos-Escobedo et al. 2016).

C. Phenotypic Plasticity

In the previous sections, we described general RNAi-mediated regulatory mechanisms that affect the expression of hundreds of genes, but a specific regulatory RNAi mechanism has been revealed in *M. circinelloides* that target the mRNA of a particular gene. This mechanism was initially discovered in the screening for spontaneous **resistants to the antifungal drug** FK506 (tacrolimus). In addition to mutants in the gene *fkbA*, which encodes the FK506 target protein FKBP12, epimutant strains were isolated that transiently silenced the expression of *fkbA* (Calo et al. 2014). Interestingly, *M. circinelloides* isolated from humans or other animal hosts are able to produce a high number of FK506-resistant epimutants (Calo et al. 2014, 2017), suggesting that this mechanism may enable this opportunistic pathogen to readily adapt to different environments. Silencing of *fkbA* was accompanied by elevated levels of sRNAs and required the core components of the RNAi machinery (Calo et al. 2014, 2017). The analysis of RNAi proteins involved in this regulatory mechanism revealed the existence of an epimutational RNAi pathway in *M. circinelloides*, which is very similar to the ex-siRNA-producing pathways (Calo et al. 2017), but the set of RNAi proteins and their relevancy is different, suggesting that a distinct sRNA class is involved in this pathway (Calo et al. 2014, 2017). The generation of sRNAs depends on the production of a dsRNA generated by the action of one of the RdRPs (RdRP2) present in *M. circinelloides*, and not from antisense transcrip-

tion in *fkbA* locus (Calo et al. 2014). An unanswered question is why the accumulation of sRNAs from the *fkbA* locus is induced in response to the drug. One possible explanation is based on the high level of expression of *fkbA* that might lead to the formation of aRNA, triggering the silencing as it has been observed in plants (Gazzani et al. 2008).

The isolation of *M. circinelloides* epimutants exhibiting resistance to 5-fluoroorotic acid (5-FOA) has revealed that **RNAi-dependent epimutation plays a broad role** enabling rapid and reversible responses of this fungus (Chang et al. 2019). The distribution of RNAi-related mechanisms involved in the phenotypic plasticity in other fungi is unknown and requires further studies, but it is very promising that one of such mechanism could operate in response to multiple environments in *N. crassa* (Kronholm et al. 2016).

D. Small RNAs Associated with Antisense Transcripts

Noncoding RNAs that overlap with protein-coding regions as a consequence of convergent transcription, named natural antisense transcripts (NATs), have been identified in a high number in animals and plants (Faghihi and Wahlestedt 2009) and in a lesser extent in phylogenetically diverse fungi (Donaldson and Saville 2012). These NATs use different mechanisms to control gene expression, including transcriptional interference, chromatin remodeling, and dsRNA formation. The dsRNA can be a substrate of the RNAi machinery to produce nat-siRNAs, which has been observed only in *S. pombe* within the fungal kingdom (Donaldson and Saville 2012). In several fungi, the presence of NATs is not associated with the formation of nat-siRNAs, although NATs regulates gene expression and cellular processes (Nevers et al. 2018; Shao et al. 2017), including pathogenesis (Donaldson and Saville 2013).

The biogenesis of disiRNAs in *N. crassa* is related to nat-siRNAs because they derive from genes in which convergent transcription causes stalling of RNA polymerase II (Dang et al. 2016). The slow progression of polymerase II allows binding of the exonuclease ERI-1 (Table 5.2) to nascent mRNA provoking the generation of disiRNAs, although the precise role ERI-1 in disiRNA biogenesis is unknown (Dang et al. 2016). Like siRNAs, disiRNAs map to both strands of the genome and are bound to Argonaute protein QDE-2 (Lee et al. 2010). Interestingly, disiRNA loci are associated with DNA methylation and K3K9me3 (Dang et al. 2013), which are dependent on ERI-1 binding on mRNA and antisense transcription, whereas disiRNAs only contribute to these epigenetic modifications (Dang et al. 2016). The role of disiRNA in QDE-2-mediated DNA methylation is not clear, although a mechanism similar to siRNA-mediated heterochromatin formation in *S. pombe* by recognizing the nascent RNA and recruiting histone-modifying enzymes has been suggested (Dang et al. 2016).

E. Cross-Kingdom Regulation

Recent pieces of evidence have shown that sRNAs can move between interacting organisms to silence the gene expression *in trans* in the non-related species, a phenomenon called cross-kingdom or trans-kingdom RNAi (Kuan et al. 2016; Weiberg and Jin 2015; Weiberg et al. 2015). In fungi, it was observed for the first time in the aggressive plant pathogen *Botrytis cinerea*, which can transfer siRNAs during the infection to the plant host that hijack components of the plant RNAi pathway, **suppressing the expression of host immunity genes** (Weiberg et al. 2013). These sRNAs are derived from long-terminal repeat (LTR) retrotransposons and require the two Dicer-like enzymes of *B. cinerea* for their production. Consequently, deletion of both *dicer* genes leads to reduced virulence in plants (Weiberg et al. 2013). *B. cinerea* sRNAs are translocated by an unknown mechanism to the plant cell where they bind to one of the plant Ago proteins (AGO1 in *Arabidopsis thaliana*) to silence genes involved in plant immunity (Weiberg et al. 2013). Interestingly, the *A. thaliana* mutant in AGO1 is also resistant to another plant fungal pathogen, *Verticillium dahlia* (Ellendorff et al. 2009), and *V.*

dahlia sRNAs with predicted plant targets have been associated with plant AGO1, suggesting that this fungus also employs sRNAs and cross-kingdom RNAi for successful infection (Wang et al. 2016). Moreover, **sRNAs have been found in extracellular vesicles** secreted by *Malassezia sympodialis*, *Saccharomyces cerevisiae*, *Candida albicans*, *Paracoccidioides brasiliensis*, and *Cryptococcus neoformans* (Peres da Silva et al. 2015; Rayner et al. 2017). These proofs suggest that an unknown cell-to-cell transport mechanism must exist to translocate sRNAs to the plant that could be used by other pathogens to suppress host immune systems (Weiberg et al. 2015).

In addition to sRNA translocation from fungal pathogens into plants, **plants can also transfer sRNAs into fungi**. This was initially revealed when dsRNA targeting virulence genes of fungal pathogens produced by engineered plants were able to trigger silencing in the fungi and confer resistance to the infection (Koch et al. 2013; Nowara et al. 2010; Panwar et al. 2013; Tinoco et al. 2010). The use of this strategy, name host-induced gene silencing (HIGS), to control invading fungi has the potential to become an important disease-protection method (Cai et al. 2018a; Nunes and Dean 2012). Interestingly, the delivery of own plant sRNAs was later confirmed by the identification of specific plant sRNAs and miRNAs in fungal cells infecting plants (Cai et al. 2018b; Zhang et al. 2016). The delivery mechanism of sRNAs by the plant and taking up by fungi is basically unknown, but exosome-like vesicles have proven to play a critical role in the secretion of sRNAs by *A. thaliana* infected with *B. cinerea*. The fungus takes up these vesicles resulting in the silencing of fungal virulence-related genes, including genes involved in vesicle-trafficking (Cai et al. 2018b). Together these results suggest that cross-kingdom RNAi might play an essential role in the arms race between pathogens and host with bidirectional translocation of sRNAs. Moreover, the ability of the fungi to incorporate extracellular RNAs has been devised as a strategy **to control pathogens by direct application of dsRNAs or sRNAs** onto host plants or post-harvest products to silence target fungal genes and confers efficient disease control (Cai et al. 2018a).

IV. Conclusions

The study of RNAi in the fungal kingdom has revealed that it is a conserved mechanism present in most fungi with a primary function of genome protection against foreign invaders and TEs during vegetative and sexual cycles. More impressively, recent studies have additionally unveiled a whole regulatory layer composed of different classes of regulatory esRNAs that can control several target genes and biological processes. In some fungi, illustrated by *Mucor circinelloides*, the relevancy of the RNAi-related regulation is huge controlling physiological and developmental processes. In contrast, milRNAs seems to fine-tune gene expression, and more in-depth studies are required to validate their functional roles in particular biological processes experimentally. The regulation of these processes by RNAi-related mechanisms is transient and reversible, conferring substantial phenotypic plasticity for rapid adaptation, as it has been demonstrated for antifungal drug response. In addition to the regulation of endogenous processes, plant fungal pathogens produce sRNAs to regulate the expression of plant genes involved in plant immunity. On the whole, all the different studies describing esRNAs in fungi have contributed to enlighten a heterogeneous world of new regulatory mechanisms in Ascomycota, Basidiomycota, and Mucoromycota. The only common feature among all these fungi is the lack of a unique or universal pathway for the production of esRNAs, showing a scenario where the diversification of regulatory silencing mechanisms is the chosen strategy rather than the conserved pathways found in animals and plants.

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