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REVIEW

RNA silencing in fungi

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Abstract RNA silencing is an evolutionarily conserved mechanism in eukaryotic organisms induced by double-stranded RNA (dsRNA) and plays an essential role in regulating gene expression and maintaining genome stability. RNA silencing occurs at both posttranscriptional levels through sequence-specific RNA degradation or translational repression and at transcriptional levels through RNA-directed DNA methylation and/or heterochromatin formation. RNA silencing pathways have been relatively well characterized in plants and animals, and are now also being widely investigated in diverse fungi, some of which are important plant pathogens. This review focuses primarily on the current understanding of the dsRNA-mediated posttranscriptional gene silencing processes in fungi, but also discusses briefly the known gene silencing pathways that appear to be independent of the RNA silencing machineries. We review RNA silencing studies for a variety of fungi and highlight some of the mechanistic differences observed in different fungal organisms. As RNA silencing is being exploited as a technology in gene function studies in fungi as well as in engineering anti-fungal resistance in plants and animals, we also discuss the recent progress towards understanding dsRNA uptake in fungi.

Keywords RNA silencing, gene silencing, heterochromatin formation, fungi, small interfering RNA, micro-RNA

1 The RNA silencing mechanism in eukaryotes

Termed RNA interference (RNAi) in animals, co-suppression in plants and quelling in fungi, the RNA silencing mechanism has been widely investigated across eukaryotic organisms. Small non-coding RNA species play a crucial role in RNA silencing. These small RNA species act at the post-transcriptional level (posttranscriptional gene silencing or PTGS) and trigger the degradation of homologous target messenger RNAs (mRNA). Plants contain an extensive variety of small RNA species including small interfering RNA (siRNA), micro-RNA (miRNA), natural antisense RNA (natsiRNA), *trans*-acting small interfering RNA (tasiRNA), and repeat-associated or heterochromatic small interfering RNA (rasiRNA) (reviewed by Eamens et al., 2008). However, piRNAs (Piwi-interacting RNAs) have been identified in germline cells in mammals and *Drosophila melanogaster* and qiRNAs (Qde-2 associated RNAs) in the fungus *Neurospora crassa*, but they are not found in plants. Small RNAs (sRNAs) are also able to regulate gene expression at the transcriptional level (transcriptional gene silencing or TGS) through the RNA silencing pathway known as RNA-directed DNA methylation (RdDM) unique to plants. This is an epigenetic mechanism that plays an important role in controlling development and maintaining genome stability.

All these types of sRNAs are produced by a common mechanism, the processing of longer, double-stranded (ds) or hairpin (hp) precursor RNAs into 20–25 nucleotide (nt) small RNAs by an RNase III-like endonuclease known as Dicer (DCR) and/or Dicer-like (DCL). Although the processing is similar, the longer dsRNA or hpRNA templates have different origins. In plants, the replication of viruses gives rise to long dsRNA that is processed into 21–24 nt viral siRNAs (vsRNAs). The dsRNA can also be produced by transgenes expressing single-stranded RNAs, but how these single-stranded transgene transcripts are recognized and converted into dsRNA remains unclear. In contrast, precursor RNAs that give rise to miRNA, natsiRNA, tasiRNAs and rasiRNA are encoded within the genome (Fig. 1). Transcripts derived from miRNA genes are self-complementary and have the ability to form hairpin structures with the double-stranded region giving rise to a single 20–22 nt long mature miRNA. For natsiRNA and tasiRNA species, the production of precursor dsRNA is either due to partial complementarity of two independent natsiRNA transcripts or due to

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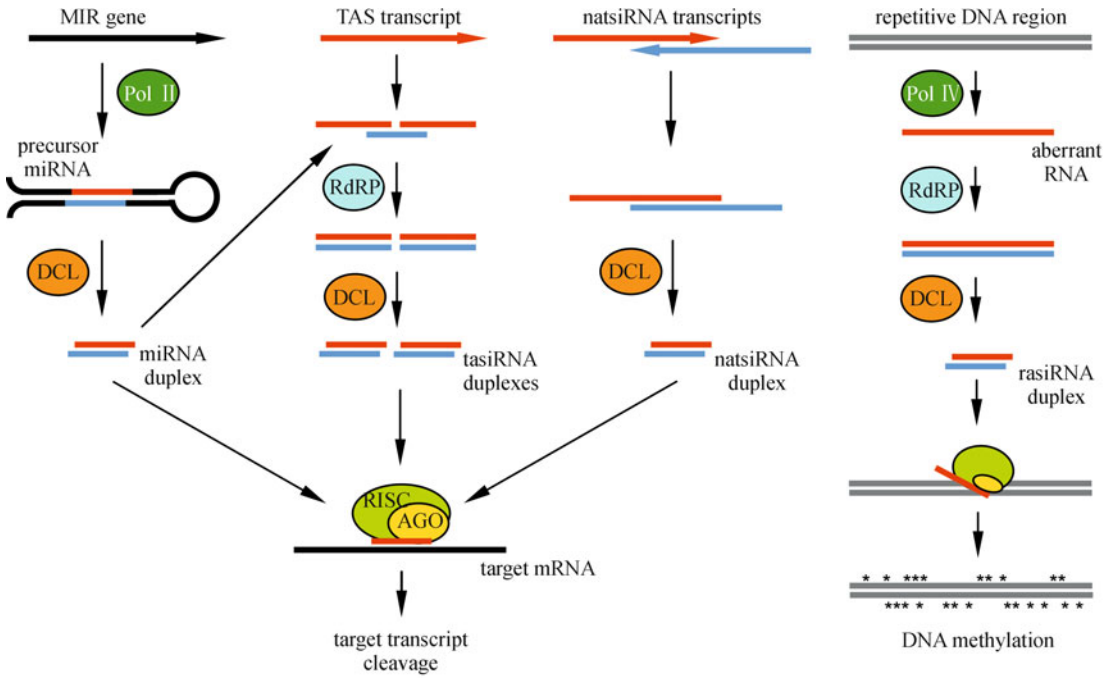

Fig. 1 The RNA silencing pathways in *Arabidopsis*. Within the plant genome RNA polymerase II (Pol II) transcribes miRNA genes giving rise to primary transcripts that have the ability to fold back to form hairpin structures. Natural sense and antisense transcripts produced by RNA Pol II have partial sequence overlap which can form double-stranded regions. TAS transcripts are produced by RNA Pol II and/or NRPD1a and serve as substrate to RdRP to produce dsRNA. All dsRNA molecules are processed by a specific DCL protein into sRNA species. These sRNAs are incorporated into RISC containing a specific AGO protein that mediates target transcript cleavage. rasiRNAs are generated by RNA Pol IV from repetitive DNA loci and direct sequence specific DNA methylation. The figure is adapted from Eamens et al., 2008 and simplified.

amplification of a single-stranded tasiRNA into dsRNA by an RNA-dependent RNA polymerase (RdRP), respectively. The dsRNA precursor for the 24 nt rasiRNAs is synthesized by an RdRP from aberrant single-stranded RNA (aRNA) transcribed from repetitive DNA. The miRNAs, tasiRNAs and natsiRNAs play an important role in plant development and stress response, while rasiRNAs are important in the RdDM process to maintain genome stability.

In animals biogenesis of sRNAs is largely similar to that in plants (reviewed by Ghildiyal and Zamore, 2009). Long dsRNAs are derived either from replicating viruses or from endogenous transcripts, such as those from transposons, produced by RdRP and cleaved by DCL into 20–21 nt long siRNAs. Transfection of animal cells with exogenous transcripts can also lead to the production of dsRNA and subsequent siRNA biogenesis. As in plants, primary miRNA transcripts (pri-miRNAs) in animals are able to form stem-loop structures and are encoded within the genome. These transcripts are recognized by the double-stranded RNA-binding protein DGCR8, which recruits the RNase III-family endonuclease Drosha, which in turn cleaves the pri-miRNA transcripts in the nucleus into precursor miRNAs (pre-miRNAs). The pre-miRNAs are exported from the nucleus into the cytoplasm by Exportin-5 and processed into mature miRNAs by DCL in conjunction with the double-stranded RNA-binding protein TRBP. Interestingly, *Drosophila* possesses two Dicers that act specifically in either siRNA or miRNA biogenesis, whereas mammals and *Caenorhabditis elegans* contain only a single Dicer that mediates both.

Animals also contain piRNAs that are 25–30 nt long and mainly found in germline cells where they function to silence transposons (reviewed by Malone and Hannon, 2009; Klattenhoff and Theurkauf, 2008). Unlike siRNAs and miRNAs, piRNAs are produced by Argonaute (AGO) cleavage which is a Dicer independent process. piRNAs arise from very long single-stranded RNA transcripts derived from large genomic clusters, but the detailed process of piRNA biogenesis is not fully understood. The best studied piRNAs derive from the *flamenco* cluster in *Drosophila*. *Flamenco* was first identified as a repressor of the transposons *gypsy*, *Idefix* and *ZAM*. *Flamenco* piRNAs appear to derive from a very long single-stranded RNA transcript of the *flamenco* locus. They are usually antisense to transposon mRNA and preferentially associate with the AGO proteins Piwi and Aubergine. Interestingly, piRNAs that associate with the AGO protein Ago3 correspond to transposon mRNA. Additionally to their preference for AGO proteins, sense and antisense piRNAs also have distinct biochemical properties. These observations led to the current ‘ping-pong’ model of piRNA biogenesis,

activated upon transposon transcription: Ago3 bound to piRNA, derived from transposon mRNA, catalyses the cleavage of antisense transposon transcript. The 5′ end of the cleaved transcript then associates with Piwi or Aubergine leading to the production of mature antisense piRNAs, which in turn mediate homology-dependent cleavage of target transposon mRNA. The 5′ end of the transposon mRNA cleavage product is thought to associate again with Ago3, creating a feed-forward loop repeating the process.

Mature siRNAs and miRNAs are loaded into the RNA-induced silencing complex (RISC) where they associate with an AGO protein, which has ribonuclease activity. The sRNAs then direct the RISC to homologous single-stranded mRNA, resulting in repression of gene expression. In plants, miRNAs generally mediate transcript cleavage, whereas in animals, translational repression occurs. This is thought to be due to the high complementarity of plant miRNAs to their target, whereas in animals most miRNAs only show a small region of complementarity to the 3′ untranslated regions of target mRNA, usually within the 5′ half of the miRNA. The 24 nt rasiRNAs mediate RdDM in plants, causing sequence-specific DNA methylation and transcriptional silencing of transposons and repetitive sequences. While it is known that piRNAs are involved in transposon silencing in germline cells, the underlying mechanisms and the proteins involved in this process remain largely unknown.

2 Evolution of RNA silencing machinery proteins in fungi

The Ascomycete fungus *N. crassa* possesses a number of different gene silencing mechanisms, two of which function at the post-transcriptional level. The presence of at least two distinct PTGS pathways led to the hypothesis that both pathways might have evolved from a single ancestral mechanism. Comprehensive phylogenetics analysis of fungal AGO, DCL and RdRP proteins showed clustering of these into two distinct clades, supporting this hypothesis (Nakayashiki, 2005; Nakayashiki et al., 2006). However, recent advances in fungal genome sequencing now allow for mining of so far unidentified RNA silencing proteins, and this revealed that not all fungal species contain two sets of these proteins. In fact, the genomes of several *Candida* species as well as *Ustilago maydis* and *Saccharomyces cerevisiae* do not seem to contain either *DCL* or *RdRP* genes, although *AGO*-like genes are sometimes present in the genomes. Initially it was assumed that these organisms had lost the genes and therefore do not possess a functional RNA silencing pathway. However, RNA silencing has been shown to be functional in *Ustilago hordei* (Laurie et al., 2008). The phylogenetic relationships of the majority of fungal organisms discussed in this paper are shown in Fig. 2.

Also, it was recently shown that a number of budding yeasts, namely *Candida albicans*, *Saccharomyces castellii* and *Kluyveromyces polysporus* produce sRNA species with modifications and size distribution that are typical of DCL processing (Drinnenberg et al., 2009). *In vitro* experiments using cellular protein extracts from these fungi showed cleavage of dsRNA into sRNAs and subsequently DCL proteins were identified. Interestingly, these proteins exhibit a domain organization distinct from other known DCL proteins, lacking the helicase and PAZ domains as well as one of the two RNase III domains. Additionally, these DCL proteins contain two (instead of one) dsRNA-binding domains (Drinnenberg et al., 2009). Deletion of the *DCL* and *AGO* genes in *S. castellii* resulted in loss of sRNAs whereas introduction of these genes into the RNA silencing-deficient yeast *S. cerevisiae* enabled silencing of a *gfp* transgene as well as the endogenous *URA3* gene. Furthermore, the introduction of *DCL* and *AGO* genes dramatically reduced endogenous transposon-derived mRNA levels (Drinnenberg et al., 2009). These findings suggest that some of the budding yeasts possess a different RNA silencing mechanism.

Most fungal species examined contain two or more *DCL*, *AGO* or *RdRP* genes; however, there are exemptions to this. *Schizosaccharomyces pombe* and *Cryptococcus cinereus*, for instance, contain one gene for each of the three RNA silencing proteins (Nakayashiki, 2005). Notably, *Aspergillus nidulans* also contains just a single *DCL* and *AGO*, whereas other *Aspergillus* species have at least two. This suggests a partial or sporadic loss of the silencing machinery genes in some fungi. However, some fungal species contain a large number of RNA silencing genes, presumably due to gene or genome duplication. For instance, the Basidiomycetes *Coprinopsis cinerea* and *Phanerochaete chrysosporium* have seven *RdRP* genes each and eight and five *DCL* genes respectively (Nakayashiki, 2005).

It remains unclear whether the loss of RNA silencing genes in some fungi suggests that their lifestyle no longer requires these proteins. *N. crassa* has evolved two sRNA-independent pathways (MSUD and RIP) that mediate the silencing of repetitive, highly similar sequences or unpaired genes. For *N. crassa* these two silencing mechanisms seem to entail an evolutionary cost and it remains unclear whether and how these sRNA-independent silencing systems would provide any advantage to the organism (reviewed by Galagan and Selker, 2004).

3 sRNA-independent gene silencing in fungi

The most comprehensive research on fungal gene silencing pathways has been conducted in the two model fungi *N. crassa* and *S. pombe*, but some knowledge has also been obtained for *Magnaporthe oryzae* and *A. nidulans*. *N.*

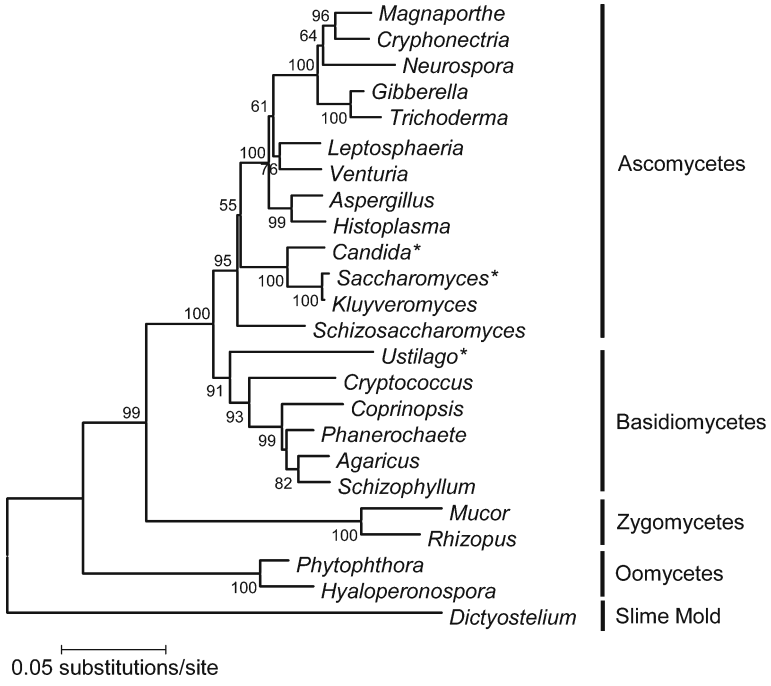

Fig. 2 Phylogenetic tree of selected fungal species based on the 18S ribosomal RNA sequences. Sequences were aligned using MAFFT and a rooted, neighbour joining tree was created in Mega4. 500 bootstrap trials were carried out and bootstrap values above 60% are shown. The asterisk (*) denotes genus in which some species have lost the RNA silencing machinery.

crassa possesses four pathways that mediate silencing of unpaired or repetitive genes (reviewed by Irelan and Selker, 1996; Catalanotto et al., 2006; Fig.3). Some of these pathways appear to be independent of sRNAs (Fig. 3). While this review focuses on the sRNA-dependent silencing, a short overview of the current understanding on the sRNA-independent pathways is included below (Fig. 3).

N. crassa possesses two PTGS mechanisms, quelling and meiotic silencing of unpaired DNA (MSUD). MSUD appears to be unique to *N. crassa* and functions to silence unpaired alleles during the diploid zygote stage. Unpaired sequences form looped regions that give rise to aberrant transcripts, which in turn mediate silencing of all homologous sequences within the genome, whether they are paired or not. How paired alleles recognize their partners and the mechanism through which silencing occurs is still poorly understood. Furthermore, whether sRNAs are involved in this process remains unclear, as no sRNAs have been identified in association with MSUD. The two TGS mechanisms present in *N. crassa*, repeat induced point mutation (RIP) and heterochromatin formation, are sRNA-independent mechanisms. RIP targets duplicated sequences longer than ~400 nt with a sequence identity of ~80%. RIP results from cytosine methylation, deamination and subsequent irreversible G:C to A:T transitions. Evidence for RIPed sequences has also been found in other fungi, and *M. oryzae*, *A. oryzae*, *Penicillium chrysogenum*, *Leptosphaeria maculans* and *Podospora anserina* are thought to have an active RIP pathway (Idnurm and Howlett, 2003; Braumann et al., 2008). The RIP mechanism is very similar to MIP (methylation induced premeiotically) in *Ascobolus immersus* and *C. cinerea*, which, in contrast to RIP, does not result in irreversible mutations. The second TGS pathway present in *N. crassa*, heterochromatin formation, has also been described in *S. pombe*, *S. cerevisiae* and *A. nidulans*. Heterochromatin formation occurs at tandem inverted arrays at the centromere region and the silent mating type locus in *S. pombe* and *S. cerevisiae* and, unlike other fungi, at the telomere region in *N. crassa*. Although in *A. nidulans* and *N. crassa* the mechanism is still poorly understood, it has been shown that in these fungi heterochromatin formation requires H3K9 methylation, with the exception of *S. cerevisiae* where loss of methyl marks at H3K4 and H3K74 seems to establish silent chromatin (Osborne et al., 2009). Loss of H3K9 methylation leads to reduced growth, but also to disruption of developmental processes regulating conidia formation in *A. nidulans*. Unlike *N. crassa*, where H3K9 methylation does not appear to require the RNA silencing machinery (Freitag et al., 2004; Cecere and Cogoni, 2009), heterochromatin formation in *S. pombe* is mediated by sRNAs and strictly requires the RNA silencing machinery, but it is unclear whether this is also the case in *A. nidulans*.

Another silencing process has recently been uncovered in *S. cerevisiae*, where transcriptional gene silencing can be mediated by short antisense transcripts. This is particularly interesting, as *S. cerevisiae* does not contain

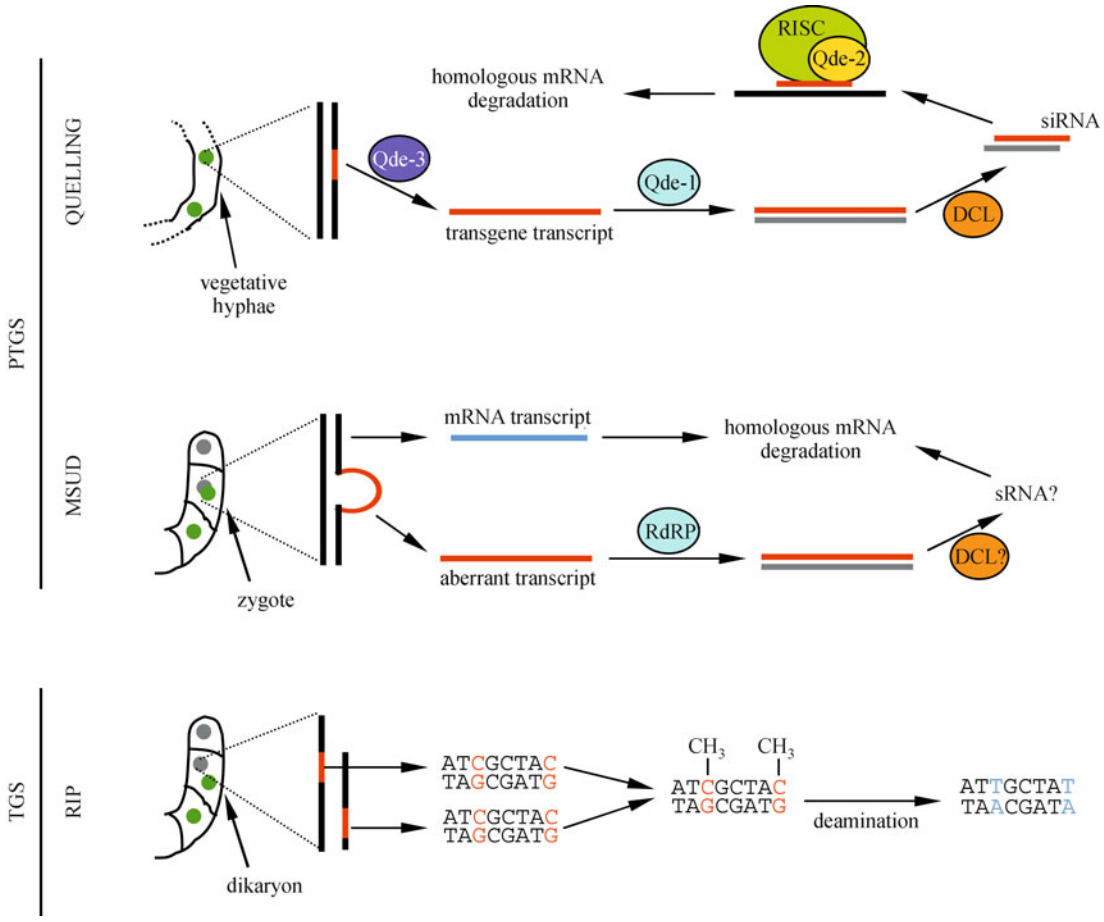

Fig. 3 The gene silencing pathways of *Neurospora crassa*. Quelling occurs during vegetative growth of the fungus and can be induced by the introduction of transgenes. Transcripts are converted into dsRNA by the RdRP Qde-1 and subsequent DCL processing results in siRNA production. These siRNAs are incorporated into the RNA-induced silencing complex (RISC) that contains the AGO protein Qde-2 and mediates degradation of homologous mRNA. Meiotic silencing of unpaired DNA (MSUD) occurs only during the zygote stage. Unpaired alleles give rise to aberrant transcripts that mediate degradation of all homologous transcripts. Whether this process is mediated by the RNA silencing machinery is still unclear. Repeat induced point mutation (RIP) is functional only during the dikaryotic stage. RIP recognizes repetitive sequences and results in cytosine methylation. Subsequent deamination leads to irreversible C:G to T:A transitions. This figure is adapted from Catalanotto et al., 2006 and simplified.

the RNA silencing machinery. This silencing pathway was discovered fortuitously during expression studies of the *PHO84* gene, which produces a short antisense transcript (Camblong et al., 2007). Normally, short non-coding transcripts are subject to degradation by the nuclear exosome complex (Davis and Ares, 2006). How the short antisense transcript induces transcriptional silencing of *PHO84* is not understood. However, inhibition of degradation and subsequent stabilization of the *PHO84* antisense transcript leads to recruitment of histone deacetylases resulting in H3K18 deacetylation and transcription repression of *PHO84* (Camblong et al., 2007). Introduction of a *PHO84* transgene, and therefore also its short antisense transcript, led to H3K18 deacetylation and transcriptional inhibition of both the transgene and the endogenous *PHO84* gene, suggesting that the antisense transcript can act *in trans* as well as *in cis* (Camblong et al., 2009).

Generally, heterochromatin formation in fungi results in H3K9 methylation and is independent of the RNA silencing machinery, with the exception of at least *S. pombe* where components of the RNA silencing machinery are required. Fungi generally possess TGS and PTGS mechanisms, but the intricate pathways through which silencing is achieved have only just started to emerge.

4 sRNA-dependent RNA silencing mechanism in fungi

RNA silencing is much less understood in fungi belonging to the Basidiomycete phylum. So much so that so far not a single gene that is essential for RNA silencing in Basidiomycete fungi has been identified. In contrast to

Ascomycetes, where gene silencing has already been extensively used as a tool to investigate gene function, in Basidiomycetes research is still focused on assessing whether RNA silencing is functional. The current understanding of RNA silencing pathways in fungi has come from studies in the Ascomycetes *N. crassa* and *S. pombe*, and to a lesser extent *M. oryzae*. As outlined below, extensive transgenic and mutational studies have resulted in the identification of proteins essential for effective gene silencing as well as the uncovering of distinct differences in gene silencing pathways among fungal organisms.

The discovery of quelling in *N. crassa* has marked the beginning of RNA silencing research in fungi. Macino and co-workers have carried out pioneering work, discovering and understanding this mechanism. Introduction of a truncated *al-1* or *al-3* gene resulted in impaired expression of endogenous *al-1* or *al-3* and reduced levels of spliced mRNA (Romano and Macino, 1992; Cogoni et al., 1994, 1996). The effect was shown to be independent of cytosine methylation (Cogoni et al., 1996) and subsequent work identified three genes that were essential in establishing and sustaining the quelling phenomenon: *Qde-1*, an RNA-dependent RNA polymerase; *qde-3*, a RecQ DNA helicase; and *qde-2*, identified as a homologue of the *C. elegans AGO* gene *rde-1* (Cogoni and Macino, 1997, 1999a, 1999b; Catalanotto et al., 2000). Subsequently, *al-1* sense and antisense sRNAs were identified. These sRNAs were 25 nt long and derived not only from the introduced truncated transgene but also from the endogenous target locus (Catalanotto et al., 2002). This study also provided the first insight into the mechanism of quelling, as it showed that Qde-1 and Qde-3 were essential for successful silencing and required for the production of siRNAs. However, in a *qde-2* mutant siRNAs were still detected, suggesting that Qde-2 acts downstream in the silencing pathway (Catalanotto et al., 2002). Although three proteins essential for establishment of silencing in *N. crassa* were identified, these did not include a DCL protein. Incubation of dsRNA with *N. crassa* protein extracts resulted in the production of sRNAs independently of an active quelling mechanism (Catalanotto et al., 2004). Through homology analysis two DCL genes were identified in *N. crassa*. Although the majority of dicer activity was conferred by Dcl-2, only a *dcl-1*/*dcl-2* double mutant was fully deficient in quelling, suggesting partial redundancy of the two dicer proteins (Catalanotto et al., 2004).

In all of these studies, quelling was achieved by introduction of truncated versions of the gene of interest, which yielded only a moderate level of silencing with low stability during vegetative growth (Romano and Macino, 1992). In fact, quelling seemed to be directly linked to the number of loci containing high transgene copy number with the transgenes arrayed in tandem repeats (Cogoni et al., 1996). Although the tandem repeat arrangement seemed to be essential for the establishment and maintenance of quelling, silencing associated with such repeats was usually unstable and released during vegetative growth (Romano and Macino, 1992). Goldoni et al. (2004) therefore followed a new approach by directly expressing dsRNA within the cell. Hairpin RNA (hpRNA) constructs containing a spliceable intron within the loop region had previously been shown to give highly effective silencing in plants (Smith et al., 2000) and the same approach was followed. A much higher quelling efficiency in *N. crassa* could be obtained using hpRNA constructs, and transformants showed stable silencing. However, stem length appeared to be critical for the efficiency of silencing (Goldoni et al., 2004). It was found that Qde-1 and Qde-3 are not required for efficient dsRNA-induced silencing within the cell (Goldoni et al., 2004).

Taken together, these results provided a first molecular model for quelling in *N. crassa*: Qde-3 is involved in the production of aberrant RNA transcripts, which serve as template for Qde-1 (an RdRP) to produce dsRNA (Catalanotto et al., 2006). Subsequent processing of the dsRNA by either of the two DCL proteins, and incorporation of siRNAs into the RISC complex containing Qde-2 leads to silencing of homologous transcripts (Catalanotto et al., 2006). Direct expression of dsRNA would bypass the first step, feeding the dsRNA molecule directly into dicer processing, thus explaining the Qde-1 and Qde-3 independent manner of dsRNA-induced silencing. This is in accordance with features of RNA silencing in animals and plants. The intricate regulatory processes in *N. crassa* are now subject to further investigation.

Studies in mammalian cells had shown that dsRNA or siRNA can trigger an interferon (IFN) response, which results in activation of IFN-stimulated genes. A large number of these genes encode proteins with antiviral properties, thus aiding viral immunity (Der et al., 1998; Goodbourn et al., 2000). A recent microarray study of *N. crassa* identified a number of genes that showed increased expression in response to treatment with dsRNA (Choudhary et al., 2007). Targeted analysis showed that dsRNA (not siRNA) has the ability to directly induce *qde-2* and *dcl-2* expression. This is a novel observation for fungi, although how this increased expression is achieved remains unclear. Additional evidence was provided suggesting that DCL proteins are required to maintain Qde-2 levels. Induction of *qde-2* transcription by dsRNA was maintained in a *DCL* mutant; however the Qde-2 protein levels did not increase significantly, suggesting that the DCL proteins are acting at a post-transcriptional level (Choudhary et al., 2007).

In *S. pombe*, RNA silencing is much less understood. This might be due to, as recent data begin to show, a much more complicated interaction between TGS and PTGS. Contrasting to *N. crassa*, in *S. pombe* both silencing processes require the RNA silencing proteins, which are single copy genes in *S. pombe* (Nakayashiki, 2005). *S. pombe* is distinct from other organisms in that it contains multiple complexes that have been implicated in

heterochromatin formation. The components of the two main complexes, RITS (RNA-induced transcriptional silencing complex) and RDRC (RNA-directed RNA polymerase complex), are well known and their function has been elucidated (reviewed by Verdel et al., 2009). The RITS complex contains three proteins, Chp1, Tas3 and Ago1 and also siRNAs, which bind to Ago1 and presumably guide the interaction of RITS with homologous DNA (Verdel et al., 2004). Deletion of any of these leads to loss of heterochromatin formation. Dcr1 is required for siRNA biogenesis, and RITS was shown to bind to chromatin in a Dcr1-dependent manner (Verdel et al., 2004). The RDRC complex also contains three proteins, Rdp1, Hrr1 and Cid12, which are essential for complex function (Motamedi et al., 2004). Rdp1 has been shown to be required for *in vitro* RdRP activity of RDRC, and for *in vitro* heterochromatin assembly (Sugiyama et al., 2005). The two complexes physically interact with each other, and this interaction strictly requires Clr4 and Dcr1 (Motamedi et al., 2004). Interestingly, deletion of any of the RDRC components leads to loss of siRNA incorporation into RITS. Furthermore, both complexes were found to interact with centromeric DNA and this interaction has led to the current model that nascent RNA recruits RDRC and RITS to the DNA locus via nascent RNA-siRNA interactions (Motamedi et al., 2004; Sugiyama et al., 2005). This nascent RNA is likely to be transcribed by RNA polymerase II (Pol II), as Pol II has been shown to be essential for the establishment of heterochromatin (Kato et al., 2005).

Because in *S. pombe* both TGS and PTGS are mediated by the same proteins, dissecting these pathways may pose difficulties. A recent study suggested that hpRNA-mediated silencing is independent of RITS and does not induce heterochromatin formation at the target locus (Sigova et al., 2004). This study was conducted using an *adh1*:*gfp* fusion as a target gene and hpRNA-mediated silencing was only observed for *gfp*, not the adjacent *adh1* gene. The transcription rate of the *gfp*:*adh1* target gene was not altered (Sigova et al., 2004), indicating that hpRNA-induced silencing occurs at the post-transcriptional level. Furthermore, deletion of any of the RITS (Chp1, Tas3, Ago1) or RDRC (Rdp1, Hrr1, Cid12) components as well as the chromatin remodelling proteins Swi6 or Clr4 resulted in loss of heterochromatin; however hpRNA-induced silencing was abolished only in a *rdp1*, *dcl1* or *ago1* strain but unaffected by deletion of the other components. Consistent with observations in other systems, siRNAs were still detected in an *ago1* mutant, suggesting that loss of *gfp* silencing is due to disruption of RISC formation and subsequent targeting of the homologous transcript. Curiously, hpRNA-mediated silencing and siRNA production were lost in an *rdp1* mutant, suggesting that in *S. pombe* efficient silencing requires an RdRP, but why RdRP is essential for dsRNA-mediated silencing remains unclear (Sigova et al., 2004).

Similar results were obtained using an hpRNA construct against the endogenous *ura4* locus: silencing was independent of RDRC and required Rdp1 (Iida et al., 2008). However, unlike the report by Sigova et al. (2004), in this latter study changes in heterochromatin formation were observed at the hpRNA-targeted endogenous locus. In a more recent study, Simmer et al. (2010) could detect secondary siRNAs from the *ura4* or *ade6* regions of *ura4*:*gfp* or *ade6:gfp* fusion genes targeted by a *gfp*-hpRNA construct. This suggests that silencing spread from the targeted *gfp* sequence to the entire fusion gene. However, the silencing of the *ura4* or *ade6* genes was unstable, and the authors suggested that PTGS might not be the prevalent mechanism in *S. pombe*. Interestingly, Simmer et al. (2010) could detect H3K9 methylation at the *ura4:gfp* target locus subsequently to *gfp* silencing. In accordance, silencing was lost in chromatin remodelling mutants *Swi6* or *Clr4*. Taken together, these studies indicate that hpRNA-mediated heterochromatin formation is possible *in trans*, but depends strongly on the genomic locus of the target gene. It has been proposed that *in trans* heterochromatin formation can only be efficiently initiated if the target gene is located in close proximity to heterochromatic regions, e.g. close to centromeric regions (Iida et al., 2008; Simmer et al., 2010). Interestingly, in both the Sigova et al. (2004) and Iida et al. (2008) studies, 23 nt and 25 nt long siRNAs were detected respectively, subsequently to the silencing event. It is conceivable that the same hpRNA approach could activate two different pathways characterized by distinct siRNA species; classic PTGS and silencing associated with heterochromatin formation. The two pathways may reinforce each other in a positive feedback loop.

There are several experimental approaches that can be used to study RNA silencing in fungi. One can either introduce a sense, antisense or an hpRNA construct targeting an endogenous gene or alternatively, first introduce a reporter transgene such as *gfp* or *gus* followed by a silencing construct. The use of reporter transgenes as a target has a significant advantage over the use of endogenous genes as silencing can be easily scored and does not lead to severe developmental impairment. Many of the gene silencing studies therefore made use of a *gfp* reporter system. Mayama and colleagues tested a range of constructs targeting *gfp* to identify the best silencing inducer for *M. oryzae*. As one might expect, the highest frequency of silencing was obtained using an hpRNA construct and the degree of silencing correlated with the abundance of hpRNA-derived siRNAs (Kadotani et al., 2003; Nakayashiki et al., 2005).

In contrast to *N. crassa*, where the sRNA species associated with quelling and DNA damage are 25 nt and 21 nt long (see section 7), respectively (Catalanotto et al., 2002; Lee et al., 2009), in *M. oryzae* sRNAs showed a size range of 19–30 nt (Kadotani et al., 2003; Kadotani et al., 2004). Since DNA methylation has been shown to be

associated with RNA silencing in *N. crassa* and *S. pombe*, it was expected that *M. oryzae* possesses a similar process. However, no changes in the methylation state of the target gene could be detected (Kadotani et al., 2003). A subsequent study showed that one of the two DCL proteins, MoDcl-2, is responsible for siRNA production and hpRNA-mediated transgene silencing (Kadotani et al., 2004). However, it was also shown that *MoDcl-1* was expressed at lower levels than *MoDcl-2* in WT cells, and over expression of *MoDcl-1* did result in silencing and siRNA production, suggesting that MoDcl-1 can function in the RNA silencing pathway (Kadotani et al., 2008). Dicer proteins in many eukaryotes have been shown to have exclusive function in distinct RNA silencing pathways. *MoDcl-2* was found to be upregulated during the sexual stage of *M. oryzae*, suggesting that the two dicers might have evolved distinct roles (Kadotani et al., 2008).

The most interesting of all fungal organisms investigated is probably the Basidiomycete *S. commune*. Schuurs et al. (1997) used a sense genomic clone to initiate silencing and achieved a high silencing frequency of around 90%. Cytosine methylation was detected at the endogenous target gene region, and addition of the methylation inhibitor, 5-azacytidine, resulted in loss of silencing. However, de Jong et al. (2006) showed that silencing induced by an hpRNA construct was not associated with cytosine methylation. It was also found that sense transgene-induced silencing and cytosine methylation was not transferred to the second nucleus in a dicaryon, whereas hpRNA-mediated silencing acted *in trans* to silence homologous genes in dicaryotic cells (Schuurs et al., 1997; de Jong et al., 2006). These observations suggest that at least two independent RNA silencing mechanisms might exist in this organism, depending on the nature of the silencing initiator. Unlike *S. commune*, cytosine methylation occurred in *C. cinereus* and *L. bicolor* following the introduction of an hpRNA construct (Wälti et al., 2006; Kemppainen et al., 2009). These findings are reminiscent of the observations in *S. pombe*.

Only few studies have focused on identifying the RNA silencing machinery in fungi other than Ascomycota or Basidiomycota. Recent studies carried out in the Ruiz-Vázquez lab on the Zygomycete *Mucor circinelloides* have provided significant insights into a gene silencing mechanism that is different from the ones found in other fungi. The first study investigated the possibility to induce silencing using a self-replicating plasmid containing a cDNA or genomic clone of the endogenous *carB* gene (Nicolás et al., 2003). The efficiency of silencing using this type of plasmid was very low with only 3% of transformants showing silencing. This was dramatically improved when an intron containing hpRNA construct was used (de Haro et al., 2009). The silencing phenotype correlated with reduced levels of spliced mature mRNA of the endogenous gene, but interestingly unspliced mRNA levels remained unchanged (Nicolás et al., 2003). This suggested a post-transcriptional gene silencing process, which is consistent with the observed unchanged cytosine methylation levels of the target gene. The most remarkable discovery in this study was the size distribution of the siRNAs. Antisense siRNAs were found to be 21 nt in size, whereas sense siRNAs were 25 nt long. Although the 21 nt antisense siRNAs were the predominant species, both were consistently detected in subsequent experiments (Nicolás et al., 2003, 2007; de Haro et al., 2009). These siRNA species were preferentially produced from the 3′ region of the silenced gene and derived not just from the target region, but from the entire open reading frame (ORF) (Nicolás et al., 2003). *M. circinelloides* contains two *DCL* genes, *dcl-1* and *dcl-2*, and deletion of *dcl-1* does not result in impaired silencing or siRNA accumulation (Nicolás et al., 2007). However, *dcl-1* mutants showed a reduced growth rate and abnormal hyphal morphology (Nicolás et al., 2007), a rare observation for fungi, as RNA silencing mutants generally do not show an observable phenotype. The second dicer, Dcl-2, is essential for hpRNA-induced silencing and siRNA production, although Dcl-1 appeared to contribute a residual level of dicer activity. A double mutant was difficult to obtain and was found to be completely impaired in silencing (de Haro et al., 2009). Interestingly, *dcl-2* showed a reduced spore production phenotype compared to WT, suggesting that in *M. circinelloides* both DCL proteins might play a role in developmental processes, also a novel observation in fungi.

5 miRNAs in fungi

In higher eukaryotes miRNAs play an important role in developmental processes. As described earlier, miRNAs derive from genome-encoded transcripts that have the ability to form imperfect hairpin structures. To date, no miRNA has been identified in fungi. There are two possibilities for this: first, fungal genomes do not encode for miRNAs or miRNA-like sRNAs; and second, miRNAs do exist in fungi but have not yet been detected. Several possible scenarios could prevent detection of miRNAs in fungi. The most widely used method to identify miRNAs is to sequence the endogenous small RNA population. Previous sequencing projects have shown that a large amount of endogenous fungal small RNAs are derived from the rDNA locus, as in *N. crassa*, or the centromere region, as in *S. pombe* (Cam et al., 2005; Cecere and Cogoni, 2009; Djupedal et al., 2009). The higher the proportion of these sRNAs, the smaller the chance of identifying possible miRNAs that might be lowly expressed. In addition, miRNAs might be involved in developmental or adaptive processes that have not yet been investigated. To date, fungal siRNA sequencing projects have used vegetative tissue rather than different developmental stages. Furthermore, fungal miRNAs, if present,

might have features distinct from known plant and animal miRNAs and thus have so far evaded detection.

It was initially thought that miRNAs might have evolved in parallel with multicellular organisms. However, this notion was challenged by the recent finding that the single-celled algae *Chlamydomonas reinhardtii* contains miRNAs (Molnár et al., 2007; Zhao et al., 2007). Sequencing of sRNA population from *Chlamydomonas* indentified 68 candidate miRNA precursor genes, and at least 80% of the matching sRNAs are derived from the putative stem region. These potential precursor miRNAs were classed into three groups: (1) miRNAs that resembled plant precursor miRNAs and were less than 150 nt in size, (2) longer precursor miRNAs that form almost perfect hairpin structures with the potential to produce multiple sRNAs, and (3) miRNAs that exhibited quite different characteristics from any known plant or animal miRNAs (Molnár et al., 2007). These characteristics included less frequent appearance of the miRNA* (the opposite strand or passenger strand of the miRNA) compared to the miRNA and, consistently, close proximity of the predicted mature miRNA to the loop region (18–24 nt). Small RNAs resembling plant tasiRNAs were also identified in *Chlamydomonas* (Molnár et al., 2007; Zhao et al., 2007).

In an independent study, 19 putative miRNA precursor sequences were identified in *Chlamydomonas* (Zhao et al., 2007). Notably, no plant or animal homologues for the putative *Chlamydomonas* miRNAs could be identified and interestingly, no potential homologues in other green algae were found. Experiments using cell extracts showed that miRNAs are likely to associate with proteins such as AGO. Incubation of an *in vitro* transcribed mRNA, a predicted target for one of the putative miRNAs, with *Chlamydomonas* cell extracts, resulted in mRNA cleavage. That miRNAs in *Chlamydomonas* act *in vivo* through target cleavage was confirmed by 5′ RACE (Zhao et al., 2007). This was further proven by effectively silencing endogenous *Chlamydomonas* genes using artificial miRNA constructs designed using endogenous miRNA backbones (Molnar et al., 2009; Zhao et al., 2009).

Another single-celled organism shown to contain miRNAs is the amoeba *Dictyostelium discoideum*. Sequence analysis of sRNAs isolated from two developmental stages yielded the discovery of 5 putative precursor miRNA sequences (Hinas et al., 2007). As had been observed for *Chlamydomonas*, a search for homologues of these putative miRNAs in the miRBase database was not successful. Two of the predicted miRNAs, mica1190 and mica1198, were 22 nt in size and showed increased expression during development. To identify a possible biogenesis pathway, several RNA silencing mutants were generated and miRNA expression assessed. Expression was unchanged in most mutants, except in the *DCL* mutant *DrnB*, where mica1198 expression was lost, and in a strain lacking the *RdRP* homologue *RrpC*, where mica1190 expression increased (Hinas et al., 2007).

6 RNA silencing in Oomycetes

Although Oomycetes are not part of the fungal kingdom and belong to the Stramenopiles, they exhibit fungal-like features and are included here. The first Oomycete for which gene silencing following the introduction of a transgene was shown is *Phytophthora infestans*. Introduction of sense or antisense copies of *inf1* resulted in loss of *inf1* expression from both, the transgene and endogenous gene (van West et al., 1999). The efficiency of silencing was very low, but dramatically improved when an hpRNA construct was employed (Ah-Fong et al., 2008). Silencing did not correlate to changes in cytosine methylation, but was stably inherited in non-transgenic progeny derived from transgenic parents. This suggested that silencing in *P. infestans* is stable once established, irrespective of whether the inducer is still present. This phenomenon was termed internuclear gene silencing (van West et al., 1999). Further investigation showed that *inf1* silencing was released in the presence of the methylation inhibitor 5-azacytidine and the histone deacetylase inhibitor trichostatin-A (van West et al., 2008). Silencing was restored when the inhibitors were removed, but not in the non-transgenic nuclei that were initially silenced by internuclear gene silencing. Cytosine methylation could not be detected, suggesting that it has no direct role in the silencing. Taken together, these observations suggested another mechanism that mediates gene silencing between nuclei in heterokaryotic cells, likely through changes in heterochromatin formation (van West et al., 2008).

Although gene silencing is functional in *P. infestans* (van West et al., 1999; Latijnhouwers and Govers, 2003; Blanco and Judelson, 2005; Judelson and Tani, 2007) and has been shown to also work in *Phytophthora parasitica* (Gaulin et al., 2002), the mechanism is poorly understood and to obtain stably silenced strains has been difficult. To facilitate gene function analysis, a different method has been developed based on the delivery of *in vitro* synthesized dsRNA into protoplasts allowing transient silencing of a gene of interest (Whisson et al., 2005). This consistently resulted in reduced mRNA levels of the target gene, although with wide variation in silencing efficiency. However, one disadvantage is that the greatest effect of silencing seems to occur at different time points depending on the target gene, thus requiring a large amount of transformants and analysis subsequently to dsRNA introduction. Nevertheless, this technique is now widely used in *P. infestans* particularly for high throughput gene function studies.

7 The role of RNA silencing in genome stability

To this date the physiological role(s) of the identified gene silencing pathways in fungal organisms remains largely

unclear, as only a few fungi have been shown to exhibit visible phenotypic changes when gene silencing is disrupted. The reasons could be two-fold. Firstly, RNA silencing is involved only in some of the specialized developmental processes that have not yet been well characterized, so the phenotypes are not easily recognizable. Alternatively, RNA silencing simply does not play an important role in fungal development. It is worth noting that in the model plant Arabidopsis strong developmental defects are associated with the disruption of the miRNA pathway but not with the deletion of the RdDM pathway involved in transcriptional silencing of transposons and repetitive DNA. Interestingly, miRNAs have not been identified in fungi, which could suggest that in fungi RNA silencing functions primarily on the transcriptional level.

This possibility is supported by the findings of Cecere and Gogoni (2009), who showed that the rDNA locus in *N. crassa* gives rise to sense and antisense transcripts and is associated with siRNAs. Furthermore, the copy number of rDNA repeats within the locus is reduced in quelling-deficient mutants, suggesting that RNA silencing is involved in stabilizing this region. Although quelling mutants showed no significant reduction in H3K9 methylation of the rDNA region, wild-type cells showed increased H3K9 methylation in the rDNA locus, suggesting that this region is transcriptionally silent. Thus, the RNA silencing machinery might be involved in maintaining the stability of the rDNA locus in *N. crassa* (Cecere and Cogoni, 2009).

Recent studies have suggested that RNA silencing may also play an important role in *N. crassa* DNA repair. It was shown that *qde-2* levels were induced by DNA damage (Lee et al., 2009). Since earlier experiments suggested that dsRNA directly induces *qde-2* (Choudhary et al., 2007), it was hypothesized that DNA damage somehow results in the production of dsRNA (Lee et al., 2009). Consistent with this hypothesis, Lee et al. (2009) identified a new class of 21 nt sRNAs, termed qiRNAs, for their association with Qde-2 under DNA damaging conditions, which mapped primarily to the rDNA locus. They also showed that DNA damage resulted in an induction of RNA transcripts from the rDNA locus that was independent of RNA polymerase I, normally responsible for the transcription of the rDNA locus, and suggested that these aberrant RNA (aRNA) transcripts serve as template for dsRNA production leading to induction of *qde-2* (Lee et al., 2009). In fact, their results indicated that production of both aRNA and dsRNA depended on Qde-1 (Lee et al., 2009). Thus, in addition to its usual role in synthesizing dsRNA, Qde-1 could also be responsible for aRNA production, possibly by interaction with the Replication Protein A that might recruit Qde-1 to ssDNA (Nolan et al., 2008). These findings have led to the hypothesis that quelling, via qiRNAs, is involved in blocking rRNA biogenesis to slow down protein synthesis subsequent to DNA damage (Nolan et al., 2008).

In *S. pombe* heterochromatin formation occurs at the silent mating type locus, the telomere regions and the tandem inverted repeats of the centromere region. Similarly to the rDNA locus of *N. crassa*, the centromere region is thought to give rise to non-coding transcripts, which might overlap to form dsRNA that is then processed into siRNAs (Volpe et al., 2002). In fact, secondary structure prediction of centromere-derived transcripts showed that these transcripts contain partially double-stranded regions that could be processed by DCL *in vitro* (Djupedal et al., 2009). These non-coding transcripts have been shown to accumulate in *ago1*, *dcr1* and *rdp1* strains (Volpe et al., 2002). Also, siRNAs were detected that overlap with the centromere region, indicating that siRNAs are derived from these non-coding transcripts and are involved in heterochromatin formation (Cam et al., 2005; Djupedal et al., 2009). In fact, Ago1 and Rdp1 have been found to be associated with all major heterochromatic loci in *S. pombe*, providing further evidence that RNA silencing is important in heterochromatin formation (Cam et al., 2005).

8 RNA silencing as defense against invasive nucleic acids

RNA silencing in plants and animals is known to play a key role in defense against transposons and viruses (Waterhouse et al., 2001; Buchon and Vaury, 2006). It has therefore been hypothesized that RNA silencing also functions in fungi to protect from viral infections. Nuss and co-workers were the first to show that, at least in a *Cryphonectria parasitica*-hypovirus interaction, this appears to be the case (Segers et al., 2006; Segers et al., 2007). The hypovirus CHV1-EP7123 encoded papain-like protease p29 was shown to inhibit hpRNA-mediated *gfp* silencing as well as to reverse established *gfp* silencing in *C. parasitica* (Segers et al., 2006). The relative levels of fluorescence in *gfp*-silenced cells dramatically increased in the presence of a functional p29 gene but not a non-functional p29 gene containing a premature stop codon. The p29 suppressor was also shown to inhibit PVX/*gfp* vector-induced *gfp* silencing in the 16C *Nicotiana benthamiana* line (Segers et al., 2006). Plants infected with PVX/*gfp* showed stable silencing of the *gfp* transgene, as did plants carrying the mutated p29 gene. In contrast, plants infected with PVX/*gfp*-p29 showed strong fluorescence in young developing leaves (Segers et al., 2006).

That RNA silencing is involved in viral defense was also suggested by the finding that *DCL* mutants showed increased susceptibility to viral infections. *C. parasitica dcl-2* and *dcl-1*/*dcl-2* mutants showed increased susceptibility to hypovirus CHV1-EP713 and reovirus My-RV1-Cp9B21 in comparison to the wild-type and the *dcl-1* single mutant (Segers et al., 2007). Accordingly, vsRNAs could be detected in the wild-type and the *dcl-1* mutant

strains but not in the *dcl-2* or *dcl-1/dcl-2* mutants (Zhang et al., 2008). Interestingly, Zhang et al. (2008) also reported that viral infection resulted in up to 15-fold increase of *dcl-2* expression, whereas *dcl-1* expression only increased by 2-fold. Notably, infection with a virus lacking p29 resulted in a further increase in *dcl-2* expression, suggesting that p29 represses the induction of *dcl-2*. This induction seems to be reliant on *agl-2*, one of four *AGO* genes in *C. parasitica*, as no *dcl-2* induction was observed in an infected *agl-2* mutant (Sun et al., 2009). In fact, *agl-2* expression also increased when p29 was not present. However, this induction of gene expression was not dependent on virus infection as hpRNA-induced *gfp* silencing also resulted in considerably increased *dcl-2* and *agl-2* transcript levels. Similar to the observation with the *DCL* mutants, *agl-1*/*3*/*4* strains showed a phenotype indistinguishable from infected wild-type strain, whereas an *agl-2* mutant showed increased susceptibility (Sun et al., 2009). These observations have led to the current model suggesting that *dcl-2* is required for sRNA production and *agl-2* for sRNA incorporation into RISC and subsequent target transcript cleavage.

The possible involvement of RNA silencing in viral defense in fungi was also investigated in mycovirus MoV2-infected *M. oryzae* cultures (Himeno et al., 2010). As transcripts of MAGGY, a long terminal repeat (LTR) retrotransposon, were shown to be increased in a *MoDcl-2* mutant strain (Murata et al., 2007; see discussions below), any inhibition of RNA silencing by MoV2-encoded proteins should result in increased accumulation of MAGGY mRNA. However, this was not the case, suggesting that MoV2 does not encode a silencing suppressor (Himeno et al., 2010). In fact, none of the mycovirus-encoded proteins showed the ability to suppress *gfp* silencing in *N. benthamiana*. Nevertheless, sRNAs matching the MoV2 genome were identified, although the proportion of vsRNAs was very low in comparison to those of host *M. oryzae*-derived siRNAs. The presence of non-viral sRNAs, with a peak in size distribution at 21 nt, suggested that *M. oryzae* could also possess a TGS pathway involving siRNAs that is similar to the process controlling the rDNA region in *N. crassa* (Lee et al., 2009) and the centromere repeat region in *S. pombe* (Verdel et al., 2004).

There is also evidence that RNA silencing could protect from other invading elements such as transposons. Introduction of the LTR-retrotransposon MAGGY into a *dcl-2* mutant of *M. oryzae* resulted in increased accumulation of MAGGY transcripts and loss of siRNA accumulation compared to the wild-type and *MoDcl-1* mutant strains (Murata et al., 2007). However, it was shown that *MoDcl-1*/*2*-independent suppression of MAGGY transposition occurred during progression of the cells, suggesting the presence of another silencing mechanism. In fact, although the transposition rate was reduced in progressed populations, transcript levels did not change, suggesting a post-transcriptional rather than transcriptional regulation of MAGGY (Murata et al., 2007). Interestingly, other endogenous transposable elements did not seem to be targets of the RNA silencing machinery, since transcript levels remained unchanged in a *MoDcl-2* mutant background where siRNAs could not be detected (Murata et al., 2007).

9 The use of RNA silencing as a functional genomics tool

Molecular tools available to study gene function in fungi are limited, particularly because homology-dependent recombination in gene knockout studies is technically difficult to achieve. Thus, RNA silencing has become an important alternative, especially in cases where a complete gene knockout causes a lethal phenotype. Therefore, most of the available data on RNA silencing in fungi comes from studies investigating if the introduction of a transgene induces homology-dependent gene silencing. In many of these studies, the main objective was to understand fungal pathogenicity or fungal-host interactions, rather than studying the RNA silencing mechanism itself. Only very few of these studies characterized sRNA species in association with the silencing phenomenon (Table 1) or identified the optimal silencing inducer. Ascomycete species where RNA silencing has been used as a functional genomics tool include *Aspergillus oryzae* (Yamada et al., 2007), *A. parasiticus*, *A. flavus* and *Fusarium graminearum* (McDonald et al., 2005), *A. nidulans* (Hammond and Keller, 2005; Hammond et al., 2008), *Bipolaris oryzae* (Moriwaki et al., 2007), *Cladosporium fulvum* (Hamada and Spanu, 1998; Lacroix and Spanu, 2009), *Histoplasma capsulatum* (Rappleye et al., 2004; Bohse and Woods, 2007), *Trichoderma harzianum* (Cardoza et al., 2006; Cardoza et al., 2007) and *Venturia inaequalis* (Fitzgerald et al., 2004). Early investigations into gene silencing in these fungi involved the introduction of a sense or antisense construct targeted against the gene of interest, usually with very low silencing rates. However, since the demonstration that hpRNA constructs were most effective at inducing silencing in plants (Waterhouse et al., 1998; Smith et al., 2000), this hpRNA transgene-based strategy has been widely adopted and has become the method of choice for many fungi (reviewed by Nakayashiki and Nguyen, 2008).

Using hpRNA constructs worked efficiently in the Basidiomycetes *Cryptococcus neoformans* (Liu et al., 2002), *Agaricus bisporus* (Eastwood et al., 2008; Costa et al., 2009), *Schizophyllum commune* (Schuurs et al., 1997; de Jong et al., 2006), *Laccaria bicolor* (Kemppainen et al., 2009), *C. cinnera* (Namekawa et al., 2005; Wälti et al., 2006; Heneghan et al., 2007) and *U. hordei* (Laurie et al., 2008). Attempts to silence genes in *U. maydis* have been unsuccessful and *U. maydis* has now been shown not to possess the RNA silencing genes (Keon et al., 1999).

Table 1 Isolated sRNA species associated with RNA silencing events in fungi

organism	silencing initiator	small RNAs	modifications	reference
N. crassa[A]	sense transgene	25 nt	–	Catalanotto et al., 2003
	DNA damage (qiRNAs)	21 nt	U at 5′ end	Lee et al., 2009
S. pombe[A]	centromeric siRNAs	22/25 nt	U and monophosphates at 5′ end	Djupedal et al., 2009
	RITS associated	22–22 nt	–	Cam et al., 2005
	Ago1 associated	22–23 nt	U at 5′ end	Bühler et al., 2008
	hp-intron construct	23 nt	–	Sigova et al., 2004
	hp-intron construct	25 nt	–	Iida et al., 2008
M. oryzae[A]	MoV2 infection	21 nt	A or U at 5′ end	Himeno et al., 2010
	sense, antisense or hairpin constructs	19–23 nt	–	Kadotani et al., 2003
	hp-loop construct	20–30 nt	–	Kadotani et al., 2004
A. nidulans[A]	hp-loop construct	~25 nt	–	Hammond and Keller, 2005
C. parasitica[A]	hp-intron construct	~25 nt	–	Segers et al., 2005
	viral infection	21 nt	–	Zhang et al., 2008
C. albicans[A]	cloned endogenous RNAs	21/22 nt	A or U at 5′ end	Drinnenberg et al., 2009
S. castellii[A]	cloned endogenous RNAs	22/23 nt	U at 5′ end	Drinnenberg et al., 2009
K. polysporus[A]	cloned endogenous RNAs	22/23 nt	U at 5′ end	Drinnenberg et al., 2009
U. hordei[B]	hp-intron construct	25 nt	–	Laurie et al., 2008
M. circinelloides[Z]	sense or hp-intron construct	21 nt sense 25 nt antisense	–	Nicolás et al., 2003
P. infestans[O]	sense, antisense or hp-loop constructs	21 nt	–	Ah-Fong et al., 2008

[A]: Ascomycete; [B]: Basidiomycete; [Z]: Zygomycete; [O]: Oomycete.

Simultaneous silencing of multiple genes has also been investigated in a number of studies by utilizing chimeric hpRNA constructs containing sequential arrangements of sense and antisense sequences of the genes of interest separated by a spacer. This strategy was shown to work efficiently in the Ascomycetes *A. fumigatus* and *V. inaequalis* (Fitzgerald et al., 2004; Mouyna et al., 2004). Silencing of a gene family containing six genes in *C. fulvum* proved more difficult, yielding a range of silencing levels as well as increased expression of the targeted genes, with some off-target effects (Lacroix and Spanu, 2009). A chimeric hpRNA construct has also been successfully used in the Basidiomycete *C. neoformans* (Liu et al., 2002).

10 Direct dsRNA or siRNA delivery as a tool to induce silencing

The approach to induce silencing based on physical delivery or "feeding" of dsRNA into fungi has now been tested in a number of studies because of its therapeutic potential for human and plant diseases. Early research showed that direct injection of dsRNA into *C. elegans*, or feeding *C. elegans* with bacteria expressing dsRNA, efficiently induces RNAi (Fire et al., 1998; Timmons and Fire, 1998). In *C. elegans* this dsRNA can spread to other cells, whereas some of the *Drosophila* and mammalian cell types seem unable to transmit the signal (Winston et al., 2002; Roignant et al., 2003; Duxbury et al., 2005). Evidence for active nucleic acid uptake was found in *Drosophila* S2 cell cultures. Uptake of dsRNA targeting the *luciferase* gene, measured by *luciferase* activity, was strongly length dependent, with longer nucleic acids entering the cells more efficiently (Saleh et al., 2006). A kinetic study showed that dsRNA of 200 nt and longer resulted in *luciferase* silencing one hour after incubation, whereas 21 nt dsRNA had no effect, even after 30 h incubation. Fluorescent labeling showed fast internalization with a distinct subcellular localization pattern. Strikingly, siRNAs did not accumulate and DNA only accumulated to low levels, suggesting that the mechanism is specific for long dsRNA (Saleh et al., 2006).

To assess if such active nucleic acid uptake also occurs in fungi, Disney et al. (2003) investigated *C. albicans* cells by adding nucleic acids directly to the fungal culture. Linear double-stranded or hairpin structures of both, DNA and RNA, were tested and all found to accumulate to an at least 10-fold higher intracellular than extracellular concentration. Interestingly, linear structures were taken up more readily than hairpin structures and all nucleic acids were found to be stable within the cell. The addition of NaN_3 to the culture, a metabolic inhibitor, reduced the

accumulation by 10-fold (Disney et al., 2003). These results suggested the presence of an active dsRNA uptake mechanism at least in *C. albicans*.

Another, almost accidental discovery provided further supporting evidence. When studying small non-coding RNAs in *A. fumigatus*, Hüttenhofer and co-workers found that cloned RNA species also included sequences mapping to the ribosomal region of *S. cerevisiae* (Jöchl et al., 2008). As culturing of *A. fumigatus* cells was carried out in yeast extracts containing medium, it was suspected that *A. fumigatus* can utilize nucleic acids from the environment via an uptake system. In a subsequent study, active uptake of bacterial 5S rRNA into *A. fumigatus* conidia was tested (Jöchl et al., 2009). The kinetics of this process was slow, and uptake did not occur until 4–5 h after inoculation. However, addition of 5S rRNA to mycelial cultures resulted in almost immediate internalization. As had been seen with *Drosophila* cells, DNA molecules were not internalized into *A. fumigatus*, although DNA was found to bind to the cell wall (Jöchl et al., 2009).

To investigate whether this RNA uptake mechanism can be exploited to induce silencing, 21 nt siRNAs directed against endogenous genes were added to germinating *A. fumigatus* conidia and a target transcript reduction of 30%–60% was observed (Jöchl et al., 2009). Independently, Khatri and Rajam (2007) showed that addition of synthetic 23 nt siRNAs to germinating *A. nidulans* spores resulted in sequence-specific silencing of the target gene. siRNA concentrations added to the medium did not seem to influence the onset or efficiency of silencing, and target transcript silencing was slowly released over time. The authors speculated that the incomplete silencing might be due to the presence of only a single siRNA species, rather than a range of siRNA molecules that would be produced by long hpRNAs (Khatri and Rajam, 2007). Barnes et al. (2008) found that addition of a commercially available smartpool of four 19 nt *gus*-siRNAs (Dharmacon, UK) to *A. niger* protoplasts induces *gus* silencing in a concentration-dependent manner. Using only a single siRNA also resulted in concentration-dependent silencing, but increased the silencing efficiency by about 20%. In both studies, silencing was released after 72 h (Barnes et al., 2008).

In a recent study, *in vitro* synthesized long dsRNA was used to transfect the Basidiomycete *Moniliophthora perniciosa* (Caribé dos Santos et al., 2009). Twenty-eight days after either $CaCl_2$/PEG transformation or electroporation of protoplasts with dsRNA, target transcript levels were efficiently reduced, in some cases by 95% in comparison to untransformed wild-type cells. This physical delivery approach was also sufficient to reduce the endogenous *Prx1* transcript levels by 23%–87% (Caribé dos Santos et al., 2009). In addition, simultaneous silencing of members of the hydrophobin gene family was attempted. Electroporation proved more efficient and target transcript levels were reduced by about 90%. Delivery by $CaCl_2$/PEG resulted in a wider range of silencing efficiency for the different targeted genes, presumably because dsRNA delivery is not as efficient. Nevertheless, these data clearly demonstrated that RNA silencing can be efficiently induced by direct physical delivery of dsRNA into the cell (Caribé dos Santos et al., 2009). Similarly, in *Coprinopsis cinerea* the commercially available pSUPER RNAi System™ (OligoEngine™, USA) was tested (Costa et al., 2008). This system is based on a short hpRNA construct comprising a user defined sense and antisense target sequence separated by a 9 nt spacer. The efficacy of a 19 nt stem length construct at inducing *gfp* silencing was investigated. Not unexpectedly, the efficiency of silencing was found to vary, with one strain showing an almost complete loss of *gfp* expression (Costa et al., 2008).

11 Concluding remarks

The understanding of RNA silencing mechanisms present throughout the fungal kingdom has increased over the last decade. Many genes essential for RNA silencing have been identified. However, with the exception of *N. crassa* and *S. pombe*, the intricate details of the post-transcriptional gene silencing pathways remain poorly understood for the majority of fungi. Although DNA methylation has been observed as a result of transgene-mediated silencing in *S. pombe* and *S. commune*, in other fungi, this was not the case. It has been suggested that the observed DNA methylation is dependent on the genomic context of the investigated transgene. The underlying mechanism responsible for transgene-mediated silencing and DNA methylation in *S. pombe* and *S. commune* remains unknown.

Interestingly, the size of sRNAs isolated in conjunction with RNA silencing in fungi is much more variable between species (Table 1). This could be due to stronger diversification of the RNA silencing pathway to adjust to the lifestyle of the particular fungus. Since the detailed mechanisms that underlie RNA silencing is much less understood in many fungi, different sRNA species could simply represent different pathways, as in plants, that have so far not been sufficiently dissected. It is also possible that in fungi different pathways interplay, leading to the observation of different sized sRNAs for a seemingly similar experiment (see hpRNA-induced silencing in *S. pombe*). However, plants with great evolutionary distance also show strong divergence of sRNA sizes. Gymnosperms predominantly accumulate 21 nt sRNAs, as they lack DCL3, whereas in angiosperms 24 nt sRNAs are the predominant species, with 21, 22 and 23 nt RNAs also being present (Morin et al., 2008).

C. parasitica is so far the only fungal organism where the size of the isolated sRNA species is clearly dependent on the type of silencing inducer, be it an hpRNA transgene or an infecting virus (Table 1). This suggests that PTGS and virus-induced gene silencing may activate two

different pathways that produce distinct sRNAs. Thus, understanding RNA silencing in the Ascomycete *C. parasitica* could help dissect a potentially more complicated and interlinked pathways in *S. pombe,* another Ascomycete.

Deletion of parts of the RNA silencing machinery in plants or animals often incurs developmental defects. Interestingly, only in very few fungi phenotypic changes have been observed upon deletion of DCL, AGO or RdRP proteins. Thus, it remains unknown whether RNA silencing also plays an essential role in fungal development. In *M. circinelloides* RNA silencing mutants showed impaired growth and reduced spore production, phenotypic changes that are easily observed during growth of the fungus. No such changes have been observed in other fungi, but deletion of these genes often resulted in loss or impaired heterochromatin formation, affecting genome stability in many fungi. Loss of heterochromatin silencing has not been reported to result in an observable phenotype in fungi. However, loss of chromatin silencing could affect specialized developmental stages, stress responses and adaptive processes, resulting in a phenotype that becomes obvious during such conditions. Thus, future research could investigate the role of RNA silencing in fungi during these conditions and, for pathogenic fungi, fungal-host interaction.

Most fungal organisms contain two sets of RNA silencing machinery proteins. Phylogenetic analyses showed that these cluster into two clades, suggesting that the two pathways, quelling and transcriptional silencing, might require distinct sets of proteins. Contrasting this assumption is the finding that in most fungi, which possess two DCL proteins, only one DCL is essential for mediating TGS and PTGS. This suggests that fungi might possess another, so far undetected RNA silencing pathway, possibly similar to the miRNA pathway in plants, which could play a role in developmental processes or be involved in the interaction with host species. Interestingly and contrasting to plants, in some fungi RdRP is essential for hpRNA-mediated gene silencing. The mechanistic behind that is still unknown. RNA silencing has been used successfully as a tool in gene function studies in a number of fungal systems. As has been reported for plants, using hairpin constructs as silencing inducer results in efficient and stable silencing in fungi. Therefore, hpRNA-mediated silencing is a useful approach for studying gene function, particularly for fungi where targeted gene knockout is challenging or lethal. However, hpRNA-induced PTGS could be complicated by the possible DNA methylation of the target gene that appears to occur in some fungi in a locus-dependent manner. As evidence emerges that some fungi possess a mechanism for active uptake of dsRNA or even siRNA, the potential problem with hpRNA transgenes could be alleviated by developing an alternative gene silencing technology based on transient delivery of dsRNA or siRNA. Such an approach could potentially allow for high throughput gene knockdown to facilitate gene function studies. However, the success of such an approach would depend on whether the transiently induced silencing can persist in the absence of the initial dsRNA or siRNA inducer. Further understanding of the RNA silencing pathways in fungi, and the process through which fungi are able to internalize dsRNAs and siRNAs and induce silencing of homologous target sequences would help to answer this question. This understanding would also have implications in the current efforts by many to develop RNAi-mediated anti-fungal strategies in both plants and animals.

References

Ah-Fong A M V, Bormann-Chung C A, Judelson H S (2008). Optimization of transgene-mediated silencing in *Phytophthora infestans* and its association with small-interfering RNAs. Fungal Genet Biol, 45(8): 1197–1205

Barnes S E, Alcocer M J C, Archer D B (2008). siRNA as a molecular tool for use in *Aspergillus niger*. Biotechnol Lett, 30(5): 885–890

Blanco F A, Judelson H S (2005). A bZIP transcription factor from *Phytophthora* interacts with a protein kinase and is required for zoospore motility and plant infection. Mol Microbiol, 56(3): 638–648

Bohse M L, Woods J P (2007). RNA interference-mediated silencing of the YPS3 gene of *Histoplasma capsulatum* reveals virulence defects. Infect Immun, 75(6): 2811–2817

Braumann I, van den Berg M, Kempken F (2008). Repeat induced point mutation in two asexual fungi, *Aspergillus niger* and *Penicillium chrysogenum*. Curr Genet, 53(5): 287–297

Buchon N, Vaury C (2006). RNAi: a defensive RNA-silencing against viruses and transposable elements. Heredity, 96(2): 195–202

Cam H P, Sugiyama T, Chen E S, Chen X, FitzGerald P C, Grewal S I S (2005). Comprehensive analysis of heterochromatin- and RNAi-mediated epigenetic control of the fission yeast genome. Nat Genet, 37(8): 809–819

Camblong J, Beyrouthy N, Guffanti E, Schlaepfer G, Steinmetz L M, Stutz F (2009). Trans-acting antisense RNAs mediate transcriptional gene cosuppression in *S. cerevisiae*. Genes Dev, 23(13): 1534–1545

Camblong J, Iglesias N, Fickentscher C, Dieppois G, Stutz F (2007). Antisense RNA stabilization induces transcriptional gene silencing via histone deacetylation in *S. cerevisiae*. Cell, 131(4): 706–717

Cardoza R E, Hermosa M R, Vizcaíno J A, González F J, Llobell A, Monte E, Gutiérrez S (2007). Partial silencing of a hydroxymethylglutaryl-CoA reductase-encoding gene in *Trichoderma harzianum* CECT 2413 results in a lower level of resistance to lovastatin and lower antifungal activity. Fungal Genet Biol, 44(4): 269–283

Cardoza R E, Vizcaíno J A, Hermosa M R, Sousa S, González F J, Llobell A, Monte E, Gutiérrez S (2006). Cloning and characterization of the erg1 gene of *Trichoderma harzianum*: effect of the erg1 silencing on ergosterol biosynthesis and resistance to terbinafine. Fungal Genet Biol, 43(3): 164–178

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(11): 825–836

Catalanotto C, Azzalin G, Macino G, Cogoni C (2000). Gene silencing in worms and fungi. Nature, 404(6775): 245

Catalanotto C, Azzalin G, Macino G, Cogoni C (2002). Involvement of small RNAs and role of the qde genes in the gene silencing pathway in *Neurospora*. Genes Dev, 16(7): 790–795

Catalanotto C, Nolan T, Cogoni C (2006). Homology effects in *Neurospora crassa*. FEMS Microbiol Lett, 254(2): 182–189

Catalanotto C, Pallotta M, ReFalo P, Sachs M S, Vayssie L, Macino G, Cogoni C (2004). Redundancy of the two dicer genes in transgeneinduced posttranscriptional gene silencing in *Neurospora crassa*. Mol Cell Biol, 24(6): 2536–2545

Cecere G, Cogoni C (2009). Quelling targets the rDNA locus and functions in rDNA copy number control. BMC Microbiol, 9(1): 44–54

Choudhary S, Lee H C, Maiti M, He Q, Cheng P, Liu Q, Liu Y (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol, 27(11): 3995–4005

Cogoni C, Irelan J T, Schumacher M, Schmidhauser T J, Selker E U, Macino G (1996). Transgene silencing of the *al-1* gene in vegetative cells of Neurospora is mediated by a cytoplasmic effector and does not depend on DNA-DNA interactions or DNA methylation. EMBO J, 15(12): 3153–3163

Cogoni C, Macino G (1997). Isolation of quelling-defective (qde) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*. Proc Natl Acad Sci USA, 94(19): 10233–10238

Cogoni C, Macino G (1999a). Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature, 399(6732): 166–169

Cogoni C, Macino G (1999b). Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. Science, 286(5448): 2342–2344

Cogoni C, Romano N, Macino G (1994). Suppression of gene expression by homologous transgenes. Antonie van Leeuwenhoek, 65(3): 205–209

Costa A M S B, Mills P R, Bailey A M, Foster G D, Challen M P (2008). Oligonucleotide sequences forming short self-complimentary hairpins can expedite the down-regulation of *Coprinopsis cinerea* genes. J Microbiol Methods, 75(2): 205–208

Costa A S M B, Thomas D J I, Eastwood D, Cutler S B, Bailey A M, Foster G D, Mills P R, Challen M P (2009). Quantifiable downregulation of endogenous genes in *Agaricus bisporus* mediated by expression of RNA hairpins. J Microbiol Biotechnol, 19(3): 271–276

Davis C A, Ares M Jr (2006). Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA, 103 (9): 3262–3267

de Haro J P, Calo S, Cervantes M, Nicolás F E, Torres-Martínez S, Ruiz-Vázquez 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(10): 1486–1497

de Jong J F, Deelstra H J, Wösten H A B, Lugones L G (2006). RNA-mediated gene silencing in monokaryons and dikaryons of *Schizophyllum commune*. Appl Environ Microbiol, 72(2): 1267–1269

Der S D, Zhou A, Williams B R G, Silverman R H (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci USA, 95(26): 15623–15628

Disney M D, Haidaris C G, Turner D H (2003). Uptake and antifungal activity of oligonucleotides in *Candida albicans*. Proc Natl Acad Sci USA, 100(4): 1530–1534

Djupedal I, Kos-Braun I C, Mosher R A, Söderholm N, Simmer F, Hardcastle T J, Fender A, Heidrich N, Kagansky A, Bayne E, Wagner E G H, Baulcombe D C, Allshire R C, Ekwall K (2009). Analysis of small RNA in fission yeast; centromeric siRNAs are potentially generated through a structured RNA. EMBO J, 28(24): 3832–3844

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(5952): 544–550

Duxbury M S, Ashley S W, Whang E E (2005). RNA interference: a mammalian SID-1 homologue enhances siRNA uptake and gene silencing efficacy in human cells. Biochem Biophys Res Commun, 331(2): 459–463

Eamens A, Wang M B, Smith N A, Waterhouse P M (2008). RNA silencing in plants: yesterday, today, and tomorrow. Plant Physiol, 147(2): 456–468

Eastwood D C, Challen M P, Zhang C, Jenkins H, Henderson J, Burton K S (2008). Hairpin-mediated down-regulation of the urea cycle enzyme argininosuccinate lyase in *Agaricus bisporus*. Mycol Res, 112: 708–716

Fire A, Xu S, Montgomery M K, Kostas S A, Driver S E, Mello C C (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature, 391(6669): 806–811

Fitzgerald A, Van Kan J A L, Plummer K M (2004). Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats. Fungal Genet Biol, 41(10): 963–971

Freitag M, Lee D W, Kothe G O, Pratt R J, Aramayo R, Selker E U (2004). DNA methylation is independent of RNA interference in *Neurospora*. Science, 304(5679): 1939

Galagan J E, Selker E U (2004). RIP: the evolutionary cost of genome defense. Trends Genet, 20(9): 417–423

Gaulin E, Jauneau A, Villalba F, Rickauer M, Esquerré-Tugayé M T, Bottin A (2002). The CBEL glycoprotein of Phytophthora parasitica var. nicotianae is involved in cell wall deposition and adhesion to cellulosic substrates. J Cell Sci, 115(23): 4565–4575

Ghildiyal M, Zamore P D (2009). Small silencing RNAs: an expanding universe. Nat Rev Genet, 10(2): 94–108

Goldoni M, Azzalin G, Macino G, Cogoni C (2004). Efficient gene silencing by expression of double stranded RNA in *Neurospora crassa*. Fungal Genet Biol, 41(11): 1016–1024

Goodbourn S, Didcock L, Randall R E (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol, 81(Pt 10): 2341–2364

Hamada W, Spanu P D (1998). Co-suppression of the hydrophobin gene *HCf-1* is correlated with antisense RNA biosynthesis in *Cladosporium fulvum*. Mol Gen Genet, 259(6): 630–638

Hammond T M, Bok J W, Andrewski M D, Reyes-Domínguez Y,

Scazzocchio C, Keller N P (2008). RNA silencing gene truncation in the filamentous fungus *Aspergillus nidulans*. Eukaryot Cell, 7(2): 339–349

Hammond T M, Keller N P (2005). RNA silencing in *Aspergillus nidulans* is independent of RNA-dependent RNA polymerases. Genetics, 169(2): 607–617

Heneghan M N, Costa A M S B, Challen M P, Mills P R, Bailey A, Foster G D (2007). A comparison of methods for successful triggering of gene silencing in *Coprinus cinereus*. Mol Biotechnol, 35(3): 283–296

Himeno M, Maejima K, Komatsu K, Ozeki J, Hashimoto M, Kagiwada S, Yamaji Y, Namba S (2010). Significantly low level of small RNA accumulation derived from an encapsidated mycovirus with dsRNA genome. Virology, 396(1): 69–75

Hinas A, Reimegård J, Wagner E G, Nellen W, Ambros V R, Söderbom F (2007). The small RNA repertoire of *Dictyostelium discoideum* and its regulation by components of the RNAi pathway. Nucleic Acids Res, 35(20): 6714–6726

Idnurm A, Howlett B J (2003). Analysis of loss of pathogenicity mutants reveals that repeat-induced point mutations can occur in the Dothideomycete *Leptosphaeria maculans*. Fungal Genet Biol, 39(1): 31–37

Iida T, Nakayama J I, Moazed D (2008). siRNA-mediated heterochromatin establishment requires HP1 and is associated with antisense transcription. Mol Cell, 31: 178–189

Irelan J T, Selker E U (1996). Gene silencing in filamentous fungi: RIP, MIP and quelling. J Genet, 75(3): 313–324

Jöchl C, Loh E, Ploner A, Haas H, Hüttenhofer A (2009). Development-dependent scavenging of nucleic acids in the filamentous fungus *Aspergillus fumigatus*. RNA Biol, 6(2): 178–186

Jöchl C, Rederstorff M, Hertel J, Stadler P F, Hofacker I L, Schrettl M, Haas H, Hüttenhofer A (2008). Small ncRNA transcriptome analysis from *Aspergillus fumigatus* suggests a novel mechanism for regulation of protein synthesis. Nucleic Acids Res, 36(8): 2677–2689

Judelson H S, Tani S (2007). Transgene-induced silencing of the zoosporogenesis-specific *NIFC* gene cluster of *Phytophthora infestans* involves chromatin alterations. Eukaryot Cell, 6(7): 1200–1209

Kadotani N, Murata T, Quoc N B, Adachi Y, Nakayashiki H (2008). Transcriptional control and protein specialization have roles in the functional diversification of two dicer-like proteins in *Magnaporthe oryzae*. Genetics, 180(2): 1245–1249

Kadotani N, Nakayashiki H, Tosa Y, Mayama S (2003). RNA silencing in the phytopathogenic fungus *Magnaporthe oryzae*. Mol Plant Microbe Interact, 16(9): 769–776

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(43): 44467–44474

Kato H, Goto D B, Martienssen R A, Urano T, Furukawa K, Murakami Y (2005). RNA polymerase II is required for RNAi-dependent heterochromatin assembly. Science, 309(5733): 467–469

Kemppainen M, Duplessis S, Martin F, Pardo A G (2009). RNA silencing in the model mycorrhizal fungus *Laccaria bicolor*: gene knock-down of nitrate reductase results in inhibition of symbiosis with Populus. Environ Microbiol, 11(7): 1878–1896

Keon J P R, Owen J W, Hargreaves J A (1999). Lack of evidence for antisense suppression in the fungal plant pathogen *Ustilago maydis*. Antisense Nucleic Acid Drug Dev, 9(1): 101–104

Khatri M, Rajam M V (2007). Targeting polyamines of *Aspergillus nidulans* by siRNA specific to fungal ornithine decarboxylase gene. Med Mycol, 45(3): 211–220

Klattenhoff C, Theurkauf W (2008). Biogenesis and germline functions of piRNAs. Development, 135(1): 3–9

Lacroix H, Spanu P D (2009). Silencing of six hydrophobins in *Cladosporium fulvum*: complexities of simultaneously targeting multiple genes. Appl Environ Microbiol, 75(2): 542–546

Latijnhouwers M, Govers F (2003). A *Phytophthora infestans* G-protein β subunit is involved in sporangium formation. Eukaryot Cell, 2(5): 971–977

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(1): 49–58

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(7244): 274–277

Liu H, Cottrell T R, Pierini L M, Goldman W E, Doering T L (2002). RNA interference in the pathogenic fungus *Cryptococcus neoformans*. Genetics, 160(2): 463–470

Malone C D, Hannon G J (2009). Small RNAs as guardians of the genome. Cell, 136(4): 656–668

McDonald T, Brown D, Keller N P, Hammond T M (2005). RNA silencing of mycotoxin production in *Aspergillus* and *Fusarium* species. Mol Plant Microbe Interact, 18(6): 539–545

Molnar A, Bassett A, Thueneman E, Schwach F, Karkare S, Ossowski S, Weigel D, Baulcombe D (2009). Highly specific gene silencing by artificial microRNAs in the unicellular alga *Chlamydomonas reinhardtii*. Plant J, 58(1): 165–174

Molnár A, Schwach F, Studholme D J, Thuenemann E C, Baulcombe D C (2007). miRNAs control gene expression in the single-cell alga *Chlamydomonas reinhardtii*. Nature, 447(7148): 1126–1129

Morin R D, Aksay G, Dolgosheina E, Ebhardt H A, Magrini V, Mardis E R, Sahinalp S C, Unrau P J (2008). Comparative analysis of the small RNA transcriptomes of *Pinus contorta* and *Oryza sativa*. Genome Res, 18(4): 571–584

Moriwaki A, Ueno M, Arase S, Kihara J (2007). RNA-mediated gene silencing in the phytopathogenic fungus *Bipolaris oryzae*. FEMS Microbiol Lett, 269(1): 85–89

Motamedi M R, Verdel A, Colmenares S U, Gerber S A, Gygi S P, Moazed D (2004). Two RNAi complexes, RITS and RDRC, physically interact and localize to noncoding centromeric RNAs. Cell, 119(6): 789–802

Mouyna I, Henry C, Doering T L, Latgé J P (2004). Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. FEMS Microbiol Lett, 237(2): 317–324

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(18): 5987–5994

Nakayashiki H (2005). RNA silencing in fungi: mechanisms and applications. FEBS Lett, 579(26): 5950–5957

Nakayashiki H, Hanada S, Nguyen B Q, Kadotani N, Tosa Y, Mayama S (2005). RNA silencing as a tool for exploring gene function in ascomycete fungi. Fungal Genet Biol, 42(4): 275–283

Nakayashiki H, Kadotani N, Mayama S (2006). Evolution and diversification of RNA silencing proteins in fungi. J Mol Evol, 63 (1): 127–135

Nakayashiki H, Nguyen Q B (2008). RNA interference: roles in fungal biology. Curr Opin Microbiol, 11(6): 494–502

Namekawa S H, Iwabata K, Sugawara H, Hamada F N, Koshiyama A, Chiku H, Kamada T, Sakaguchi K (2005). Knockdown of LIM15/DMC1 in the mushroom *Coprinus cinereus* by double-stranded RNA-mediated gene silencing. Microbiology, 151(11): 3669–3678

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(6): 504–516

Nicolás F E, Torres-Martínez S, Ruiz-Vázquez R M (2003). Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. EMBO J, 22(15): 3983–3991

Nolan T, Cecere G, Mancone C, Alonzi T, Tripodi M, Catalanotto C, Cogoni C (2008). The RNA-dependent RNA polymerase essential for post-transcriptional gene silencing in *Neurospora crassa* interacts with replication protein A. Nucleic Acids Res, 36(2): 532–538

Osborne E A, Dudoit S, Rine J (2009). The establishment of gene silencing at single-cell resolution. Nat Genet, 41(7): 800–806

Rappleye C A, Engle J T, Goldman W E (2004). RNA interference in *Histoplasma capsulatum* demonstrates a role for -(1,3)-glucan in virulence. Mol Microbiol, 53(1): 153–165

Roignant J Y, Carré C, Mugat B, Szymczak D, Lepesant J A, Antoniewski C (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*. RNA, 9(3): 299–308

Romano N, Macino G (1992). Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol Microbiol, 6(22): 3343–3353

Saleh M C, van Rij R P, Hekele A, Gillis A, Foley E, O'Farrell P H, Andino R (2006). The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nat Cell Biol, 8(8): 793–802

Schuurs T A, Schaeffer E A M, Wessels J G H (1997). Homology-dependent silencing of the SC3 gene in *Schizophyllum commune*. Genetics, 147(2): 589–596

Segers G C, van Wezel R, Zhang X, Hong Y, Nuss D L (2006). Hypovirus papain-like protease p29 suppresses RNA silencing in the natural fungal host and in a heterologous plant system. Eukaryot Cell, 5(6): 896–904

Segers G C, Zhang X, Deng F, Sun Q, Nuss D L (2007). Evidence that RNA silencing functions as an antiviral defense mechanism in fungi. Proc Natl Acad Sci USA, 104(31): 12902–12906

Sigova A, Rhind N, Zamore P D (2004). A single Argonaute protein mediates both transcriptional and posttranscriptional silencing in *Schizosaccharomyces pombe*. Genes Dev, 18(19): 2359–2367

Simmer F, Buscaino A, Kos-Braun I C, Kagansky A, Boukaba A, Urano T, Kerr A R W, Allshire R C (2010). Hairpin RNA induces secondary small interfering RNA synthesis and silencing *in trans* in fission yeast. EMBO Rep, 11(2): 112–118

Smith N A, Singh S P, Wang M B, Stoutjesdijk P A, Green A G, Waterhouse P M (2000). Total silencing by intron-spliced hairpin RNAs. Nature, 407(6802): 319–320

Sugiyama T, Cam H, Verdel A, Moazed D, Grewal S I S (2005). RNA-dependent RNA polymerase is an essential component of a self-enforcing loop coupling heterochromatin assembly to siRNA production. Proc Natl Acad Sci USA, 102(1): 152–157

Sun Q, Choi G H, Nuss D L (2009). A single argonaute gene is required for induction of RNA silencing antiviral defense and promotes viral RNA recombination. Proc Natl Acad Sci USA, 106(42): 17927–17932

Timmons L, Fire A (1998). Specific interference by ingested dsRNA. Nature, 395(6705): 854

van West P, Kamoun S, van't Klooster J W, Govers F (1999). Internuclear gene silencing in *Phytophthora infestans*. Mol Cell, 3 (3): 339–348

van West P, Shepherd S J, Walker C A, Li S, Appiah A A, Grenville-Briggs L J, Govers F, Gow N A R (2008). Internuclear gene silencing in *Phytophthora infestans* is established through chromatin remodelling. Microbiology, 154(5): 1482–1490

Verdel A, Jia S, Gerber S, Sugiyama T, Gygi S, Grewal S I S, Moazed D (2004). RNAi-mediated targeting of heterochromatin by the RITS complex. Science, 303(5658): 672–676

Verdel A, Vavasseur A, Le Gorrec M, Touat-Todeschini L (2009). Common themes in siRNA-mediated epigenetic silencing pathways. Int J Dev Biol, 53(2-3): 245–257

Volpe T A, Kidner C, Hall I M, Teng G, Grewal S I S, Martienssen R A (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science, 297(5588): 1833–1837

Wälti M A, Villalba C, Buser R M, Grünler A, Aebi M, Künzler M (2006). Targeted gene silencing in the model mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by expression of homologous hairpin RNAs. Eukaryot Cell, 5(4): 732–744

Waterhouse P M, Graham M W, Wang M B (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc Natl Acad Sci USA, 95(23): 13959–13964

Waterhouse P M, Wang M B, Lough T (2001). Gene silencing as an adaptive defence against viruses. Nature, 411(6839): 834–842

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(2): 153–163

Winston W M, Molodowitch C, Hunter C P (2002). Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science, 295(5564): 2456–2459

Yamada O, Ikeda R, Ohkita Y, Hayashi R, Sakamoto K, Akita O (2007). Gene silencing by RNA interference in the koji mold *Aspergillus oryzae*. Biosci Biotechnol Biochem, 71(1): 138–144

Zhang X, Segers G C, Sun Q, Deng F, Nuss D L (2008). Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. J Virol, 82(6): 2613–2619

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(10): 1190–1203

Zhao T, Wang W, Bai X, Qi Y (2009). Gene silencing by artificial miRNAs in *Chlamydomonas*. Plant J, 58: 157–164