Title: Evolution and diversification of RNA Silencing Proteins in Fungi

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Abstract

Comprehensive phylogenetic analyses of fungal Argonaute, Dicer, and RNA-dependent RNA polymerase-like proteins have been performed to gain insights into the diversification of RNA silencing pathways during the evolution of fungi. A wide range of fungi including ascomycetes, basidiomycetyes and zygomycetes possesses multiple RNA silencing components in the genome whereas a portion of ascomycete and basidiomycete fungi apparently lacks the whole or most of the components. The number of paralogous silencing proteins in the genome differs considerably among fungal species, suggesting that RNA silencing pathways have significantly diversified during evolution in parallel with developing the complexity of life cycle or in response to environmental conditions. Interestingly, orthologous silencing proteins from different fungal clades are often clustered more closely than paralogous proteins in a fungus, indicating that duplication events occurred before speciation events. Therefore, the origin of multiple RNA silencing pathways seems to be very ancient, likely having occurred prior to the divergence of the major fungal lineages.

Introduction

Since the breakthrough discovery of RNA interference (RNAi) in 1998 (Fire et al. 1998), accumulating evidence has indicated that double stranded RNA (dsRNA) is an important regulator in various types of gene silencing, such as mRNA degradation, translation inhibition, chromatin remodeling and DNA elimination (reviewed by Matzke and Birchler （2005)), which are here collectively referred to as RNA silencing. RNA silencing has been reported to occur in all four eukaryotic kingdoms; Animalia (Fire et al. 1998), Plantae (Van der Krol et al. 1990), Fungi (Romano and Macino 1992), and Protista (Ngo et al. 1998), but not in the prokaryotic kingdom Monera. Therefore, the RNA silencing machinery may have emerged before the divergence of the major eukaryotic lineages.

Genetic and biochemical studies have led to the identification of several fundamental components of the RNA silencing machinery such as the Argonaute and Dicer proteins (reviewed by Tomari and Zamore (2005)). Dicer is a member of the RNase III family of nucleases that processes long dsRNA into 21- to 26-nt-long RNA duplexes that serve as specificity determinants in the RNA silencing pathways. Argonaute proteins are highly basic proteins that contain two conserved domains, namely PAZ and Piwi domains. Argonaute proteins are essential components of multi-subunit effector complexes such as the RNA induced silencing complex (RISC). Recent structural studies have suggested that one of the four mouse Argonaute proteins, AGO2, functions as the slicer element of RISC that cleaves target messenger RNAs in the RNAi pathway (Liu et al. 2004). In organisms such as nematodes, plants, and fungi, RNA-dependent RNA polymerase (RdRP) also plays important roles in RNA silencing pathways. *Neurospora crassa* and *Arabidopsis* mutants deficient in the RdRP genes

have no co-suppression, a form of RNA silencing induced by homologous transgenes (Cogoni and Macino 1999; Dalmay et al. 2000; Mourrain et al. 2000). RdRP may be involved in the initiation step of RNA silencing by converting "aberrant" RNA into dsRNA. RdRP has also been proposed to account for signal amplification for the systemic spread of silencing in plants and *C. elegans* (Mlotshwa et al. 2002).

Two major RNA silencing pathways have been identified in animals and plants; namely, small interfering RNA (siRNA)-directed pathway and microRNA (miRNA)-directed pathway (Tomari and Zamore 2005). In the *Drosophila* genome, two Dicer-like proteins, Dcr-1 and Dcr-2, and at least four Argonaute-like proteins, AGO1, AGO2, Piwi, and Aubergine have been identified. Lee *et al*. (2004) showed that *Drosophila Dcr-1* mutants were defective in processing miRNA precursors, whereas *Dcr-2* mutants had reduced siRNA levels and a complete RNAi defect in the eye. Similarly, Okamura *et al*. (2004) showed that *Drosophila* AGO1 is essential for mature miRNA production, whereas AGO2 is responsible for siRNA-directed RNAi. Therefore, it seems that in *Drosophila* the miRNA and siRNA silencing pathways are mediated by different sets of silencing proteins, Dcr-1 and AGO1, and Dcr-2 and AGO2, respectively.

In the model filamentous fungus *N. crassa*, distinct sets of silencing protein components may also be responsible for two different forms of RNA silencing; quelling and meiotic silencing by unpaired DNA (MSUD) (Galagan et al. 2003). A series of studies has indicated that quelling in *Neurospora* corresponds, at least partly, to the siRNA silencing pathway in higher eukaryotes (Cogoni and Macino 2000). This process seems to be mainly mediated by a set of RNA silencing proteins, Qde-1 (RdRP) (Cogoni and Macino 1999), Qde-2 (Argonaute) (Cogoni and Macino 2000), and Dcl-2 (Dicer) (Catalanotto et al. 2004). Another *N. crassa* dicer Dcl-1/Sms-3 was also

reported to be redundantly involved in the quelling pathway (Catalanotto et al. 2004). However, Dcl-2 appeared to have stronger activity to produce siRNAs *in vitro* (Catalanotto et al. 2004). In addition, the Dcl-2 ortholog was solely responsible for siRNA biogenesis (Kadotani et al. 2004) in *Magnaporthe oryzae* (formerly *M. grisea*) (Couch and Kohn 2002; Kato et al. 2000), a fungus closely-related to *N. crassa*. Therefore, it appears possible that Dcl-2 is the primary dicer protein responsible for the quelling pathway in *N. crassa* even though Dcl-1/Sms-3 can compensate when Dcl-2 is lost.

Whereas quelling is active in the vegetative phase of the *N. crassa* life cycle, MSUD operates for a limited period of time from an early stage of meiosis after karyogamy to ascospore maturation. MSUD abolishes the expression of such genes that exist in one parental chromosome but not in its pairing partner; therefore, that cause the accumulation of unpaired DNA during meiosis (Aramayo and Metzenberg 1996; Shiu et al. 2001). Intriguingly, MSUD affects not only the unpaired copies but rather any copy of the unpaired gene in the genome even if the additional copies are paired (Shiu et al. 2001). Genetic studies have indicated that MSUD uses a different set of silencing proteins from quelling; Sad-1 (RdRP) (Shiu et al. 2001), Sms-2 (Argonaute) (Lee et al. 2003), and Dcl-1/Sms-3 (Dicer) (Galagan et al. 2003). It is of great interest to know when and how these RNA silencing pathways have differentiated during the evolution of eukaryotes, and to what extent the underlying molecular mechanisms have been conserved.

In the fungal kingdom, the RNA silencing machinery appears to have undergone significant diversification during evolution. In the genome of *N. crassa,* there are three paralogs of RdRP, two of Argonaute, and two of Dicer (Galagan et al. 2003), whereas

only one each of RdRp, Argonaute and Dicer have been identified in the *Schizosaccharomyces pombe* (fission yeast) genome (Wood et al. 2002). Surprisingly, the genome sequence indicates that *Saccharomyces cerevisiae* (budding yeast) lacks genes with significant homology to the RNA silencing proteins (Aravind et al. 2000). The apparent complex diversification of the RNA silencing proteins in the fungus kingdom poses interesting questions. Does *S. pombe* retain a master set of the silencing components that have duplicated and diversified independently during the evolution of ascomycetes? Or has *S. pombe* lost some of the paralogs that were already present in the ancient ascomycetes? Is it an exceptional event that the RNA silencing machinery has been lost in *S. cerevisiae* or may it also have been lost in several independent fungal lineages? To address these questions, here we have undertaken a comparative genomics approach with emphasis on fungal genomes. There are a relatively large number of fungal genomes whose complete DNA sequence information is available in public databases, making the fungal kingdom an ideal model for phylogenetic studies into the diversification of the RNA silencing machinery during evolution.

Materials and Methods

Amino Acid Sequence Retrieval

Amino acid sequences of Argonaute-, Dicer-, and RdRP-like proteins were retrieved from the DNA and protein databases at NCBI (www.ncbi.nlm.nih.gov), Broad Institute (www.broad.mit.edu/annotation/), the Wellcome Trust Sanger Institute (www.sanger.ac.uk), the Institute for Genomic Research (www.tigr.org/tdb/fungal/), and the DOE Joint Genome Institute (genome.jgi-psf.org/whiterot1/whiterot1.home.html). BLAST probing of the databases was

performed with the blastp and tblastn programs (Altschul et al. 1997) using conserved protein sequences for the Piwi domain in *N. crassa* Qde-2 (NCU04730), Sms-2 (NCU09434), and *Phanerochaete chrysosporium* PC6 (White Rot 330, see Table 1), for the RNase III domain in *N. crassa* Dcl-1/Sms-3 (NCU08270), Dcl-2 (NCU06766) and *P. chrysosporium* PC1 (White Rot 25, see Table 1), and for the RdRP domain in *N. crassa* Qde1 (NCU07534), RRP3 (NCU08435) and *P. chrysosporium* PC6 (White Rot 513, see Table 1). The conserved domains were defined with reference to the Pfam database (www.sanger.ac.uk/Software/Pfam/; (Bateman et al. 2002)). The E value of 10^{-3} was used as cutoff to determine homologous proteins. For further selection, flanking sequences of the conserved domain in candidate proteins were examined for typical features of the RNA silencing proteins. It should be noted that some genes may be missing because of the incompleteness of the genome sequences used in this analysis.

Phylogenetic Analysis

Since some of the amino acid sequences analyzed were so divergent, conserved regions corresponding to the positions #586 to #898 (313 aa) in NCU04730 (Piwi-domain in Argonaute), #981 to #1247 (267 aa) in NCU06766 (RNaseIII domain in Dicer) and #707 to #1083 (377 aa) in NCU07534 (RdRP domain in RdRP) were used for the analyses to obtain reliable multiple alignments and phylogenetic trees. To predict protein sequence from non-annotated genomic sequences, GENSCAN online software was first applied, and the resulting amino acid sequence was further adjusted by multiple sequence alignment using ClustalX version 1.81 (Thompson et al. 1994) or Clustal W at DDBJ (http://www.ddbj.nig.ac.jp/search/clustalw-e.html). The resulting amino acid alignments were given as on-line supporting materials (Fig S1-S3). Distance

trees were constructed by the neighbour-joining (NJ) method at DDBJ with the default parameter settings or by the minimum evolution (ME), and maximum parsimony (MP) methods using the MEGA 3.1 software (Kumar et al. 2004). The robustness of the trees was estimated by performing 1000 bootstrap replicates (expressed as percentages in the figures). In this study, protein groups are defined when i) the cluster is composed of proteins from different fungal phylums; and ii) the bootstrap value (NJ method) for the group is higher than 80.

Results and Discussion

The RNA silencing machinery has been lost in certain fungal lineages

Database searches for RdRP-, Argonaute- and Dicer-like proteins that may be involved in RNA silencing resulted in the identification of candidate proteins in a wide range of fungi belonging to *Ascomycota, Basidiomycota*, and *Zygomycota* (Fig. 1). Surprisingly, however, a significant number of the fungal species used in the analysis, including the ascomycetes *S. cerevisiae*, and *Candida lusitaniae*, and the basidiomycete *Ustilago maydis*, do not possess a gene with significant homology either to RdRP, Argonaute or Dicer protein (Fig. 1). In addition, *Candida albicans* and *C. tropicalis* apparently lack dicer- and RdRP-like proteins (Fig. 1). Thus, the RNA silencing machinery appears to have been lost in these fungal species. The genus *Candida* includes over 160 species (Barnett et al. 1990), approximately 20 of which are associated with human or animal opportunistic infections. They exist mostly as single yeast cells that reproduce by budding. Phylogenetic analysis based on multigene sequences revealed that *Candida* and *Saccharomyces* are closely related and form the "*Saccharomyces* complex" (Kurtzman and Robnett 2003). Since none of the fungi in the

"*Saccharomyces* complex" examined in this study possess Dicer and RdRP proteins, the RNA silencing machinery might have been lost in the ancestors of "*Saccharomyces* complex". It is also possible that the machinery was sporadically lost within the complex as reported in the family *Trypanosomatidae* that include protozoan parasites (Ullu et al. 2004). Intriguingly, a lack of the RNA silencing proteins is also observed in the basidiomycete *U. maydis*. Since *U. maydis* is taxonomically distant from the "*Saccharomyces* complex", the loss of RNA silencing machinery is likely to have occurred independently. This may indicate that this loss during evolution is not an exceptional event.

Ancient origin of multiple silencing pathways in fungi

Phylogenetic analysis of the RdRP-, Argonaute- and Dicer-like proteins was performed using representative fungal species including six ascomycetes (*Aspergillus nidulans* [*Emericella nidulans*], *Fusarium graminearum* [*Gibberella zeae*], *M. oryzae* [*M. grisea*], *N. crassa*, *S. pombe*, and *Stagonospora nodorum*), three basidiomycetes (*Coprinus cinereus*, *Cryptococcus neoformans*, and *P. chrysosporium*), and a zygomycete (*Rhizopus oryzae*) (Table 1). As outgroups, *Arabidopsis thaliana* (plant), and *Drosophila melanogaster* (animal) were employed since biological functions of their RNA silencing proteins have been well-characterized as described in introduction. In general, the results showed that the *Arabidopsis* and *Drosophila* proteins clustered separately from the fungal proteins, supporting the model that the RNA silencing proteins have been duplicated and diversified to form a new pathway after the divergence of fungi from animals and plants (Fig. 2, 3, and 4).

Interestingly, none of the RNA silencing proteins from a fungal phylum formed a single cluster but orthologous proteins from different phylums tend to be grouped together within the fungal kingdom. Typical examples are shown in RdRP proteins (Fig. 2). The RdRP were clustered in at least three different groups (RdRP1-3), each of which consisted of RdRP proteins from different fungal phylums, suggesting that the duplication events occurred before the diversification of the major fungal lineages. The group RdRP3 containing *N. crassa* Qde-1 includes proteins from the three major fungal phylums, *Ascomycota*, *Basidiomycota*, and *Zygomycota*, indicating that the origin of RdRPs of this type is old and they may be functionally well-conserved. Similarly, an Argonaute protein group (Ago1) including NC-qde2 (Fig. 3) and a Dicer protein group (Dicer1) including NC-dcl2 (Fig. 4) consist of proteins from both ascomycetes and basidiomycetes, indicating that these proteins are conserved and share an ancestor that has differentiated after the divergence of *Ascomycota* and *Basidiomycota*. The major nodes were supported by bootstrap values greater than at least 50 % either by the neighbour-joining (NJ), the minimum evolution (ME), or maximum parsimony (MP) method (Fig. 2-4) in addition to the maximum likelihood (ML) method (data not shown),

Interestingly, the conserved groups (RdRP3, Ago1 and Dicer1) include a set of *N. crassa* proteins involved in the "quelling pathway". Here we use the "quelling pathway" to describe siRNA-mediated mRNA degradation pathways during vegetative phases. Actually, this type of RNA silencing has been observed in many fungal species including *Ascomycota*, *Basidiomycota*, and *Zygomycota* (Fitzgerald et al. 2004; Hammond and Keller 2005; Kadotani et al. 2003; Liu et al. 2002; Mouyna et al. 2004; Nicolas et al. 2003; Rappleye et al. 2004), as well as the fungus-like organism

Oomycota (Latijnhouwers et al. 2004). Therefore, the fungal proteins in the conserved groups (RdRP3, Ago1 and Dicer1) may retain features of prototype RNA silencing proteins and may be responsible for the "quelling pathway".

The relatedness of the other protein groups in the phylogenetic analyses cannot be reliably determined due to the low bootstrap values at the deeper nodes. In either case of Argonaute, Dicer or RdRP, each of the independent groups is mostly formed of proteins from a single fungal phylum. Some of the independent groups from different fungal phylums may have distinctly diversified from the same ancestor in accordance with functional diversification in different fungal phylums, or may simply be originated from different ancestors. In this regard, it is interesting to note that all the proteins involved in the MSUD pathway (Sad-1, Sms-2 and Dcl-1/Sms-3) belong to protein groups specific to ascomycetes (Fig. 2, 3, 4). This may indicate that the MSUD pathway has developed only in ascomycetes. Nevertheless, the origin of paralogous silencing proteins appears to be ancient since the duplication events likely occurred, at latest, before major speciation in a fungal phylum. Based on these assumptions, *S. pombe*, which has only one set of the RNA silencing proteins, may have lost its paralogs during evolution. In addition, fewer RNA silencing proteins are encoded within the genome of *A. nidulans* compared to closely-related fungus *A. fumigatus* (Hammond and Keller 2005). Since relics of additional genes for Dicer-, and Argonaute-like proteins are also identified in the *A. nidulans* genome (data not shown), *A. nidulans* may be in the process of "losing" the silencing proteins from its genome.

RNA silencing proteins in ascomycetes and basidiomycetes

The model fungus *N. crassa* has two sets of silencing components, each of which is responsible for the quelling or MSUD pathways, and one additional RdRP paralog RRP3 (Galagan et al. 2003). With the reference to the gene composition in *Neurospora*, expansion of the genes in addition to the losses described above is observed in the ascomycetes. In the *F. graminearum* genome, RdRP orthologs corresponding to *N. crassa* SAD1 and RRP3 are recognizable whereas there are two paralogs (FG1 and FG4) closely related to *N. crassa* QDE1 (Fig. 2). This suggests that the QDE1 orthologs may have arisen from relatively "recent" gene expansion. Similarly, *S. nodorum* has three additional Argonaute-like proteins (SN3, SN4 and SN5) in the Ago1 group. *F. graminearum* and *S. nodorum* also possess RdRP proteins (RdRP2 group) whose paralog is absent in *N. crassa* (Fig. 2).

In the basidiomycetes, at least three distinct classes of Dicer- and RdRP-, and two of Argonaute-like proteins are recognizable (Fig. 2). Extensive gene expansion of the silencing-related proteins seems to have occurred with Argonaute and RdRP proteins in *P. chrysosporium* and *C. cinereus* (Fig. 2, 3). The phylogenetic analyses indicated that expansion of Argonaute and RdRP genes may have occurred both before and after diversification of *P. chrysosporium* and *C. cinereus* (Fig. 3, 4). Interestingly, RNA silencing proteins in *C. neoformans* seem to be distinctively diversified. *C. neoformans* carries Dicer proteins that lack the DEAD/DEAH box helicase, a typical signature of Dicer proteins. Helicase-lacking Dicer protein has also been found in *Tetrahymena* and cellular slime mold, *Dictyostelium discoideum* (Martens et al. 2002; Mochizuki and Gorovsky 2005). The *Tetrahymena* dicer protein (Dcl1p) was shown to play a crucial role in processing dsRNA into siRNA-like small RNAs termed scan RNAs (scnRNAs) in an RNA silencing-related phenomenon, elimination of internal eliminated sequences

(IES) (Mochizuki and Gorovsky 2005). In addition, operation of RNA silencing has been shown in *C. neoformans* (Liu et al. 2002). Therefore, *C. neoformans* Dicer proteins are unusual but likely to have Dicer function. Two Argonaute proteins and one RdRP in *C. neoformans* are also distinct from corresponding proteins in the other basidiomycetes used here. In certain fungal species such as *C. neoformans* and *S. pombe*, the rate of molecular evolution of the silencing proteins may be higher than in other fungi. It should be noted that all but not part of the RNA silencing components seem to have distinctly diversified in *C. neoformans* and possibly in *S. pombe* (Fig. 2 to 4). Similarly, the loss of RNA silencing proteins often occurred with all or most of the components (Fig. 1). Therefore, the molecular divergence of the RNA silencing genes appears to have taken place as a module as also suggested with other functionally linked genes (Aravind et al. 2000).

Implications of the wide diversification of RNA silencing pathways in fungi

The loss and expansion of the RNA silencing genes in fungi may imply that fungi have evolved a new RNA silencing pathway or eliminated an existing pathway in accordance with environmental conditions and/or changes in the life cycle. The biological function of RNA silencing pathways in fungi, however, remains largely unknown. Two silencing phenomena, quelling and MSUD, have been found in *N. crassa* (Romano and Macino 1992; Shiu et al. 2001). As described above, since the "quelling pathway" is conserved in a wide range of eukaryotes, at least one of the fungal RNA silencing components is likely to be responsible for this pathway. In *S. pombe,* the RNA silencing machinery is involved in transcriptional silencing with chromatin remodeling (Verdel et al. 2004). In contrast, chromatin modification was independent of

any RNA silencing components examined in *N. crassa* (Chicas et al. 2005; Freitag et al. 2004). Therefore, the RNA silencing proteins in *S. pombe* might have distinctly evolved from other filamentous fungi. Alternatively, other proteins absent in *N. crassa* might be necessary to add the siRNA-directed chromatin remodeling sub pathway. Notably, the silencing proteins present in *S. pombe* are more closely related to the *N. crassa* counterparts involved in MSUD rather than to those for quelling. This is interesting in that both siRNA-directed chromatin remodeling and MSUD involve a nuclear event.

Diversification of the RNA silencing components appears to have been common during evolution of eukaryotes. In fact, model plants such as *Arabidopsis* and rice have relatively large numbers of RNA silencing components in the genome. Even though functional diversification has not been clearly elucidated, these silencing protein paralogs may have arisen to provide new gene control mechanisms that use dsRNA as a specificity determinant. In contrast, a portion of lower eukaryotes (mostly unicellular eukaryotes) may have cut off the cost of the RNA silencing machinery. This could be due to their unicellularly-based life cycles. Multicellular organisms may have evolved dsRNA-mediated pathways for more complex development (eg. miRNA pathway), and/or may have been required to give higher priority to defend against virus infection than unicellular organisms since multicellular organisms have a threat of systemic infection directly by cell-to-cell movement of the viruses.

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References

Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, Lipman D (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402

Aramayo R, Metzenberg RL (1996) Meiotic transvection in fungi. Cell 86:103-113

Aravind L, Watanabe H, Lipman DJ, Koonin EV (2000) Lineage-specific loss and divergence of functionally linked genes in eukaryotes. Proc. Natl. Acad. Sci. USA 97:11319-11324.

Barnett JA, Payne RW, Yarrow D (1990) Yeasts: Characterisation and identification. Cambridge University Press., Cambridge

Bateman A, Birney E, Cerruti L, Durbin R, Etwiller L, Eddy SR, Griffiths-Jones S, Howe KL, Marshall M, Sonnhammer EL (2002) The Pfam protein families database. Nucleic Acids Res. 30:276-280

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

Chicas A, Forrest EC, Sepich S, Cogoni C, Macino G (2005) Small interfering RNAs that trigger posttranscriptional gene silencing are not required for the

histone H3 Lys9 methylation necessary for transgenic tandem repeat stabilization in *Neurospora crassa*. Mol. Cell. Biol. 25:3793-3801

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

Cogoni C, Macino G (2000) Post-transcriptional gene silencing across kingdoms. Curr. Opin. Genet. 10:638-643

Couch BC, Kohn LM (2002) A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from M. grisea. Mycologia 94:683-693

Dalmay T, Hamilton A, Rudd S, Angell S, Baulcombe DC (2000) An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell 101:543-553

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391:806-811

Fitzgerald A, Kan JALv, Plummer KM (2004) Simultaneous silencing of multiple genes in the apple scab fungus, *Venturia inaequalis*, by expression of RNA with chimeric inverted repeats. Fungal Genet. & Biol. 41:963-971.

Freitag M, Lee DW, Kothe GO, Pratt RJ, Aramayo R, Selker EU (2004) DNA methylation is independent of RNA interference in *Neurospora*. Science 304:1939

Galagan JE, Calvo SE, Borkovich KA, Selker EU, Read ND, Jaffe D, Fitzhugh W, Ma L-J, Smirnov S, Purcell S, Rehman B, Elkins T, Engels R, Wang S, Nielsen

CB, Butler J, Endrizzi M, Qui D, Ianakiev P, Bell-Pedersen D, Nelson MA, Werner-Washburne M, Selitrennikoff CP, Kinsey JA, Braun EL, Zelter A, Schulte U, Kothe GO, Jedd G, Mewes W, Staben C, Marcotte E, Greenberg D, Roy A, Foley K, Naylor J, Stange-Thomann N, Barrett R, Gnerre S, Kamal M, Kamvysselis M, Mauceli E, Bielke C, Rudd S, Frishman D, Krystofova S, Rasmussen C, Metzenberg RL, Perkins DD, Kroken S, Cogoni C, Macino G, Catcheside D, Li W, Pratt RJ, Osmani SA, Desouza CPC, Glass L, Orbach MJ, Berglund JA, Voelker R, Yarden O, Plamann M, Seiler S, Dunlap J, Radford A, Aramayo R, Natvig DO, Alex LA, Mannhaupt G, Ebbole DJ, Freitag M, Paulsen I, Sachs MS, Lander ES, Nusbaum C, Birren B (2003) The genome sequence of the filamentous fungus *Neurospora crassa*. Nature 422:859-868

Hammond TM, Keller NP (2005) RNA silencing in *Aspergillus nidulans* is independent of RNA-dependent RNA polymerases. Genetics 169:607-617

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

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 siRNA accumulation. J Biol Chem. 279:44467-44474

Kato H, Yamamoto M, Yamaguchi-Ozaki T, Kadouchi H, Iwamoto Y, Nakayashiki H, Tosa Y, Mayama S, Mori N (2000) Pathogenicity, mating ability and DNA restriction fragment length polymorphisms of *Pyricularia* populations

isolated from *Gramineae, Bambusideae* and *Zingiberaceae* plants. J. Gen. Plant Pathol 66:30-47

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Briefings in Bioinformatics 5:150-163

Kurtzman CP, Robnett CJ (2003) Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Res. 3:417-432

Latijnhouwers M, Ligterink W, Vleeshouwers VGAA, West Pv, Govers F (2004) A Galpha subunit controls zoospore motility and virulence in the potato late blight pathogen *Phytophthora infestans*. Mol Microbiol 51:925-936

Lee DW, Pratt RJ, McLaughlin M, Aramayo R (2003) An argonaute-like protein is required for meiotic silencing. Genetics 164:821-828

Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW (2004) Distinct roles for *Drosophila* Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell 117:69-81

Liu H, Cottrell TR, Pierini LM, Goldman WE, Doering TL (2002) RNA interference in the pathogenic fungus *Cryptococcus neoformans.* Genetics 160:463-470

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305:1437-1441

Martens H, Novotny J, Oberstrass J, Steck TL, Postlethwait P, Nellen W (2002) RNAi in *Dictyostelium*: the role of RNA-directed RNA polymerases and double-stranded RNase. Mol. Biol. Cell. 13:445-453

Matzke MA, Birchler JA (2005) RNAi-mediated pathways in the nucleus. Nat Rev Genet. 6:24-35

Mlotshwa S, Voinnet O, Mette MF, Matzke M, Vaucheret H, Ding SW, Pruss G, Vance VB (2002) RNA silencing and the mobile silencing signal. Plant Cell 14:S289-301

Mochizuki K, Gorovsky MA (2005) A Dicer-like protein in *Tetrahymena* has distinct functions in genome rearrangement, chromosome segregation, and meiotic prophase. Genes Dev. 19:77-89.

Mourrain P, Beclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 101:533-542

Mouyna I, Henry C, Doering TL, Latge JP (2004) Gene silencing with RNA interference in the human pathogenic fungus *Aspergillus fumigatus*. FEMS Microbiol. Lett. 237:317-324

Ngo H, Tschudi C, Gull K, Ullu E (1998) Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. Proc. Natl. Acad. Sci. USA 95:14687-14692

Nicolas FE, Torres-Martinez S, Ruiz-Vazquez RM (2003) Two classes of small antisense RNAs in fungal RNA silencing triggered by non-integrative transgenes. EMBO J. 22:3983-3991

Okamura K, Ishizuka A, Siomi H, Siomi MC (2004) Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. 18:1655-1666

Rappleye CA, Engle JT, Goldman WE (2004) RNA interference in *Histoplasma capsulatum* demonstrates a role for alpha-(1,3)-glucan in virulence. Mol. Microbiol. 53:153-165

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

Shiu PKT, Raju NB, Zickler D, Metzenberg RL (2001) Meiotic silencing by unpaired DNA. Cell 107:905-916

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22:4673-4680

Tomari Y, Zamore PD (2005) Perspective: machines for RNAi. Genes Dev. 19:517-529

Ullu E, Tschudi C, Chakraborty T (2004) RNA interference in protozoan parasites. Cell Microbiol. 6:509-519

Van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2:291-299

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

Wood V, Gwilliam R, Rajandream M-A, Lyne M, Lyne R, Stewart A, Sgouros J, Peat N, Hayles J, Baker S, Basham D, Bowman S, Brooks K, Brown D, Brown S, Chillingworth T, Churcher C, Collins M, Connor R, Cronin A, Davis P, Feltwell T, Fraser A, Gentles S, Goble A, Hamlin N, Harris D, Hidalgo J, Hodgson G, Holroyd S, Hornsby T, Howarth S, Huckle EJ, Hunt S, Jagels K, James K, Jones L, Jones M, Leather S, McDonald S, McLean J, Mooney P, Moule S, Mungall K, Murphy L, Niblett D, Odell C, Oliver K, O'Neil S, Pearson D, Quail MA, Rabbinowitsch E, Rutherford K, Rutter S, Saunders D, Seeger K, Sharp S, Skelton, Simmonds M, Squares R, Squares S, Stevens K, Taylor K, Taylor RG, Tivey A, Walsh S, Warren T, Whitehead S, Woodward J, Volckaert G, Aert R, Robben J, Grymonprez B, Weltjens I, Vanstreels E, Rieger M, Schafer M, S.Muller-Auer, Gabel C, Fuchs M, Fritzc C, Holzer E, Moestl D, Hilbert H, Borzym K, Langer I, Beck A, Lehrach H, Reinhardt R, Pohl TM, Eger P, Zimmermann W, Wedler H, Wambutt R, Purnelle B, Goffeau A, Cadieu E, Dreano S, Gloux S, Lelaure V, et al. (2002) The genome sequence of *Schizosaccharomyces pombe*. Nature 415:871-880

Table 1. RNA silencing proteins used in this study

Abbreviation	class	organism	ID
Argonaute			
AN	AS	*Aspergillus nidulans*	AN1519
FG1	AS	*Fusarium graminearum*	FG08752
FG2	AS	*F. graminearum*	FG00348
MG1	AS	*Magnaporthe oryzae*	MG01294
MG2	AS	*M. oryzae*	MG11029
MG3	AS	*M. oryzae*	MG10003*
NC-qde2	AS	*Neurospora crassa*	NCU04730
NC-sms2	AS	*N. crassa*	NCU09434
SP	AS	*Schizosaccharomyces pombe*	SPCC736.1
SN1	AS	*Stagonospora nodorum*	SNU06565.1
SN2	AS	*S. nodorum*	SNU12157.1
SN3	AS	*S. nodorum*	SNU10420.1
SN4	AS	*S. nodorum*	SNU10544.1
SN5	AS	*S. nodorum*	SNU10546.1
CC1	BS	*Coprinus cinereus*	contig 1.108_5
CC2	BS	*C. cinereus*	contig 1.108_5
CC3	BS	*C. cinereus*	contig 1.198_10
CC4	BS	*C. cinereus*	contig 1.213_11
CC5	BS	*C. cinereus*	contig 1.224_12
CC6	BS	*C. cinereus*	contig 1.258_15
CC7	BS	*C. cinereus*	contig 1.325_24
CC8	BS	*C. cinereus*	contig 1.325_24*
CN1	BS	*Cryptococcus neoformans*	CNJ00490
CN2	BS	*C. neoformans*	CNJ00610
PC1	BS	*Phanerochaete chrysosporium*	White_Rot120
PC2	BS	*P. chrysosporium*	White_Rot148
PC3	BS	*P. chrysosporium*	White_Rot148
PC4	BS	*P. chrysosporium*	White_Rot148
PC5	BS	*P. chrysosporium*	White_Rot158
PC6	BS	*P. chrysosporium*	White_Rot330
PC7	BS	*P. chrysosporium*	White_Rot635
RO1	ZY	*Rhizopus oryzae*	contig 1.1
RO2	ZY	*R. oryzae*	contig 1.30
DM-ago1	Insect	*Drosophila melanogaster*	NP_725341
DM-ago2	Insect	*D. melanogaster*	Q9VUQ5
AT-ago1	Plant	*Arabidopsis thaliana*	At1g48410
AT2	Plant	*A. thaliana*	At1g31280
AT3	Plant	*A. thaliana*	At1g31290
AT4	Plant	*A. thaliana*	At2g27040
AT5	Plant	*A. thaliana*	At2g27880
AT6	Plant	*A. thaliana*	At2g32940
AT7	Plant	*A. thaliana*	At1g69440
AT9	Plant	*A. thaliana*	At5g21150
AT-ZLL/PNH	Plant	*A. thaliana*	At5g43810

Abbreviation	class	organism	ID
Dicer			
AN	AS	*A. nidulans*	AN3189
FG1	AS	*F. graminearum*	FG09025
FG2	AS	*F. graminearum*	FG04408
MG-mdl1	AS	*M. oryzae*	MG01541
MG-mdl2	AS	*M. oryzae*	MG07167
NC-dcl1/sms3	AS	*N. crassa*	NCU08270
NC-dcl2	AS	*N. crassa*	NCU06766
SP-dcr1	AS	*S. pombe*	SPCC188.13c
SN1	AS	*S. nodorum*	SNU08521
SN2	AS	*S. nodorum*	SNU07542
CC1	BS	*C. cinereus*	contig 1.114_5
CC2	BS	*C. cinereus*	contig 1.198_10
CC3	BS	*C. cinereus*	contig 1.321_23
CN1	BS	*C. neoformans*	CNC03670
CN2	BS	*C. neoformans*	CNC03680
PC1	BS	*P. chrysosporium*	White_Rot25
PC2	BS	*P. chrysosporium*	White_Rot69
PC3	BS	*P. chrysosporium*	White_Rot158
RO1	ZY	*R. oryzae*	contig 1.1
RO2	ZY	*R. oryzae*	contig 1.19
DM-dcr11	Insect	*D. melanogaster*	AAF56056
DM-dcr2	Insect	*D. melanogaster*	NP_523778
AT-dcl1	Plant	*A. thaliana*	At1g01040
AT-dcl2	Plant	*A. thaliana*	At3g03300
AT-dcl3	Plant	*A. thaliana*	At3g43920
AT-dcl4	Plant	*A. thaliana*	At5g20320
RdRP			
AN1	AS	*A. nidulans*	AN4790
AN2	AS	*A. nidulans*	AN2717
FG1	AS	*F. graminearum*	FG06504
FG2	AS	*F. graminearum*	FG08716
FG3	AS	*F. graminearum*	FG01582
FG4	AS	*F. graminearum*	FG04619
FG5	AS	*F. graminearum*	FG09076
MG1	AS	*M. oryzae*	MG07682
MG2	AS	*M. oryzae*	MG02748
MG3	AS	*M. oryzae*	MG06205
NC-qde1	AS	*N. crassa*	NCU07534
NC-sad1	AS	*N. crassa*	NCU02178
NC-rrp3	AS	*N. crassa*	NCU08435
SP	AS	*S. pombe*	SPAC6F12
SN1	AS	*S. nodorum*	SNU01986.1
SN2	AS	*S. nodorum*	SNU00237.1
SN3	AS	*S. nodorum*	SNU15784.1
SN4	AS	*S. nodorum*	SNU14810.1
CC1	BS	*C. cinereus*	contig 1.25_1
CC2	BS	*C. cinereus*	contig 1.101_5
CC3	BS	*C. cinereus*	contig 1.103_5
CC4	BS	*C. cinereus*	contig 1.198_10
CC5	BS	*C. cinereus*	contig 1.198_10
CC6	BS	*C. cinereus*	contig 1.250_14
CC7	BS	*C. cinereus*	contig 1.253_14
CN	BS	*C. neoformans*	CNG01230
PC1	BS	*P. chrysosporium*	White_Rot2
PC2	BS	*P. chrysosporium*	White_Rot16
PC3	BS	*P. chrysosporium*	White_Rot75
PC4	BS	*P. chrysosporium*	White_Rot112
PC5	BS	*P. chrysosporium*	White_Rot177
PC6	BS	*P. chrysosporium*	White_Rot223
PC7	BS	*P. chrysosporium*	White_Rot223
PC8	BS	*P. chrysosporium*	White_Rot500
PC9	BS	*P. chrysosporium*	White_Rot513
RO1	ZY	*R. oryzae*	contig 1.53
RO2	ZY	*R. oryzae*	contig1.185*
RO3	ZY	*R. oryzae*	contig1.253
RO4	ZY	*R. oryzae*	contig1.301
RO5	ZY	*R. oryzae*	contig1.302
AT1	Plant	*A. thaliana*	AT1G14790
AT3	Plant	*A. thaliana*	At2g19910
AT4	Plant	*A. thaliana*	At2g19920
AT6-sde1/sgs2	Plant	*A. thaliana*	At3g49500

AS, ascomycete; BS, basidiomycete; ZY, zygomycete; *proteins eliminated from the phylogenetic analysis

Figure Legends

Figure 1. Phylogenetic relationship of the fungal species based on beta-tubulin sequences and the numbers of Argonaute (Ago)-, Dicer- and RNA-dependent RNA polymerase (RdRP)-like proteins in them

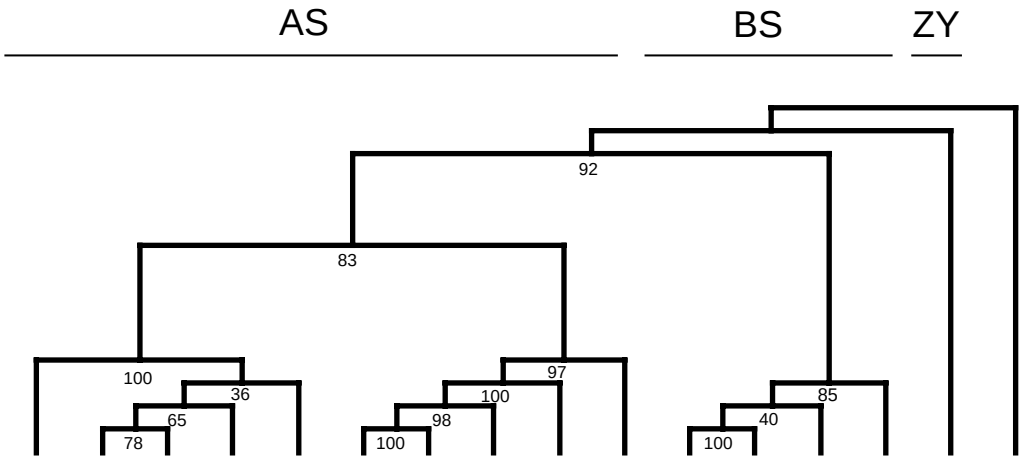
The amino acid alignment of the fungal beta-tubulin genes was generated by Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) with the default parameter settings. The distance tree was constructed by the Neighbour-Joining method and their robustness was estimated by performing 1000 bootstrap replicates. Arabidopsis was used as an outgroup to root the tree. The numbers of the RNA silencing proteins were estimated as described under Materials and Methods. AS, ascomycete; BS, basidiomycetes; ZY, zygomycete; AN, *Aspergillus nidulans*; CA, *Candida albicans*; CC, *Coprinus cinereus*; CL, *Candida lusitaniae*; CN, *Cryptococcus neoformans*; CT, *Candida tropicalis*; FG, *Fusarium graminearum*; MG, *Magnaporthe oryzae* (*grisea*); NC, *Neurospora crassa*; PC, *Phanerochaete chrysosporium*; RO, *Rhizopus oryzae*; SC, *Saccharomyces cerevisiae*; SN, *Stagonospora nodorum*; SP, *Schizosaccharomyces pombe*; UM, *Ustilago maydis*; AT, *Arabidopsis thaliana*.

Figure 2. Phylogenetic tree of fungal RNA-dependent RNA polymerase (RdRP)-like proteins together with those from *Arabidopsis*. The trees were constructed based on the conserved RdRP domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red,

respectively, and those from *Arabidopsis* are indicated in black. Abbreviations of the proteins are as indicated in table 1.

Figure 3. Phylogenetic tree of fungal Argonaute-like proteins together with those from *Arabidopsis* and *Drosophila*. The trees were constructed based on the conserved Piwi domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* and *Drosophila* are indicated in black. Abbreviations of the proteins are as indicated in table 1.

Figure 4. Phylogenetic tree of fungal Dicer-like proteins together with those from *Arabidopsis* and *Drosophila*. The trees were constructed based on the conserved RNase III domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* and *Drosophila* are indicated in black. Abbreviations of the proteins are as indicated in table 1.



	AN	FG	MG	NC	SN	CT	CA	CL	SC	SP	PC	CC	CN	UM	RO	AT
Ago	1	2	3	2	5	1	1	0	0	1	7	8	2	0	2	
Dicer	1	2	2	2	2	0	0	0	0	1	3	3	2	0	2	
RdRP	2	5	3	3	4	0	0	0	0	1	9	7	1	0	5	

Figure 1. Phylogenetic relationship of the fungal species based on beta-tubulin sequences and the numbers of Argonaute (Ago)-, Dicer- and RNA-dependent RNA polymerase (RdRP)-like proteins in them. The amino acid alignment of the fungal beta-tubulin genes was generated by Clustal W (http://www.ddbj.nig.ac.jp/search/clustalw-e.html) with the default parameter settings. The distance tree was constructed by the Neighbour-Joining method and their robustness was estimated by performing 1000 bootstrap replicates. Arabidopsis was used as an outgroup to root the tree. The numbers of the RNA silencing proteins were estimated as described under Materials and Methods. AS, ascomycete; BS, basidiomycetes; ZY, zygomycete; AN, *Aspergillus nidulans*; CA, *Candida albicans*; CC, *Coprinus cinereus*; CL, *Candida lusitaniae*; CN, *Cryptococcus neoformans*; CT, *Candida tropicalis*; FG, *Fusarium graminearum*; MG, *Magnaporthe oryzae* (*grisea*); NC, *Neurospora crassa*; PC, *Phanerochaete chrysosporium*; RO, *Rhizopus oryzae*; SC, *Saccharomyces cerevisiae*; SN, *Stagonospora nodorum*; SP, *Schizosaccharomyces pombe;* UM, *Ustilago maydis*; AT, *Arabidopsis thaliana.*

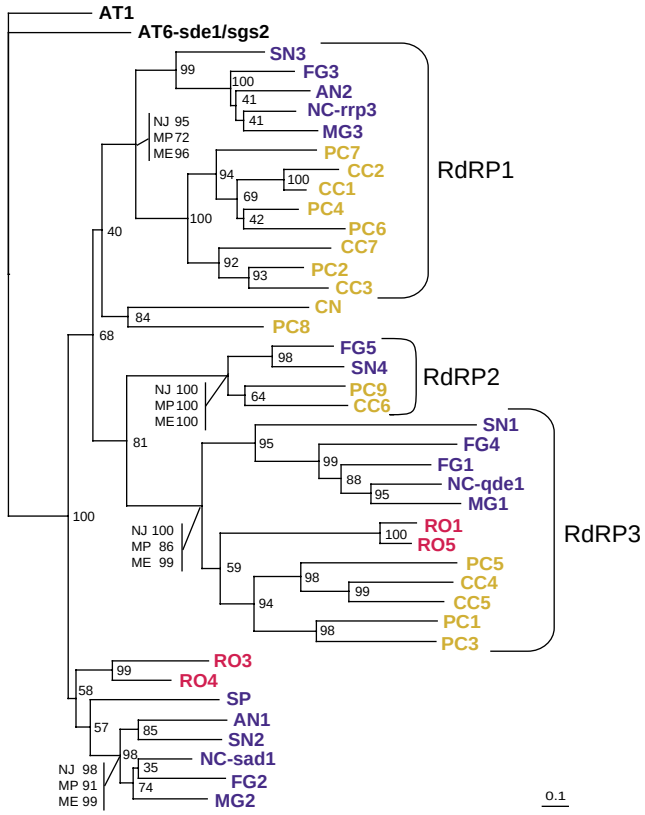


Figure 2. Phylogenetic tree of fungal RNA-dependent RNA polymerase (RdRP)-like proteins together with those from *Arabidopsis*. The trees were constructed based on the conserved RdRP domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* are indicated in black. Abbreviations of the proteins are as indicated in table 1.

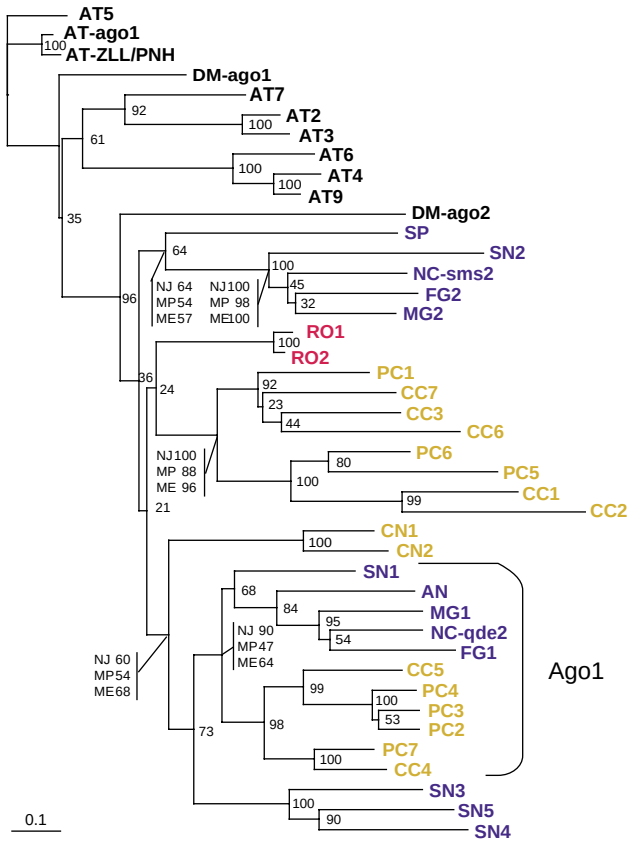


Figure 3. Phylogenetic tree of fungal Argonaute-like proteins together with those from *Arabidopsis* and *Drosophila*. The trees were constructed based on the conserved RNase III domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* and *Drosophila* are indicated in black. Abbreviations of the proteins are as indicated in table 1.

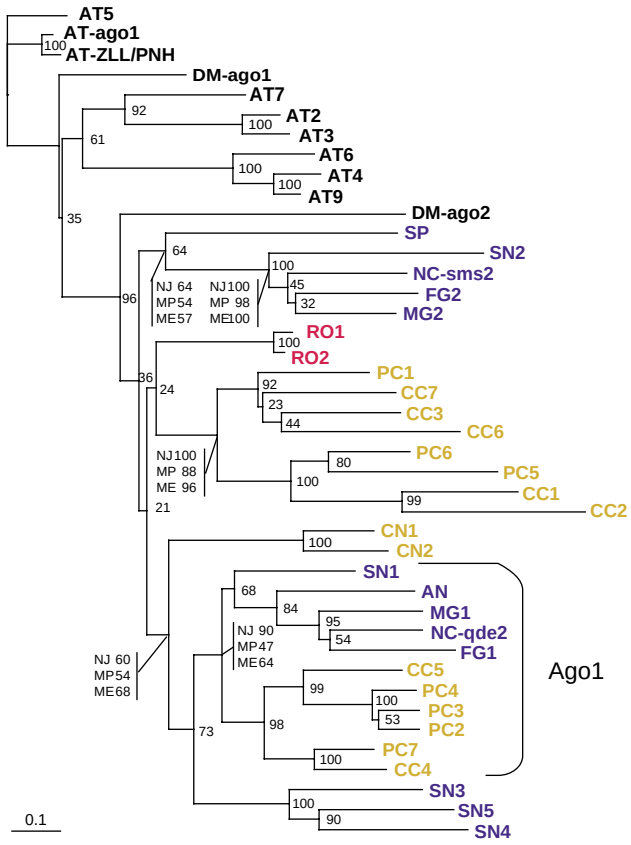


Figure 3. Phylogenetic tree of fungal Argonaute-like proteins together with those from *Arabidopsis* and *Drosophila*. The trees were constructed based on the conserved RNase III domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* and *Drosophila* are indicated in black. Abbreviations of the proteins are as indicated in table 1.

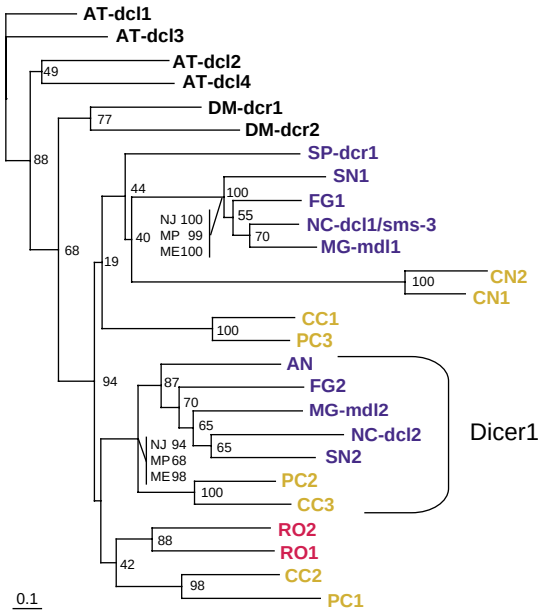


Figure 4. Phylogenetic tree of fungal Dicer-like proteins together with those from *Arabidopsis* and *Drosophila*. The trees were constructed based on the conserved RNase III domain as described in Materials and Methods. At several key nodes, neighbour joining (NJ), maximum parsimony (MP), and minimum evolution (ME) bootstrap values (expressed as percentages) are also indicated. Proteins from the ascomycetes, basidiomycetes and zygomycete are shown in blue, brown and red, respectively, and those from *Arabidopsis* and *Drosophila* are indicated in black. Abbreviations of the proteins are as indicated in table 1.