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RNA Silencing Gene Truncation in the Filamentous Fungus *Aspergillus nidulans*▽†

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The genus *Aspergillus* is ideally suited for the investigation of RNA silencing evolution because it includes species that have experienced a variety of RNA silencing gene changes. Our work on this subject begins here with the model species *Aspergillus nidulans*. Filamentous ascomycete fungi generally each encode two of the core RNA silencing proteins, Dicer and Argonaute, but *A. nidulans* appears to have lost one of each to gene truncation events. Although a role in growth, development, or RNA silencing was not detected for the truncated genes, they do produce spliced and poly(A)-tailed transcripts, suggesting that they may have an undetermined biological function. Population analysis demonstrates that the truncated genes are fixed at the species level and that their full-length orthologs in a closely related species are also unstable. With these gene truncation events, *A. nidulans* encodes only a single intact Dicer and Argonaute. Their deletion results in morphologically and reproductively normal strains that are incapable of experimental RNA silencing. Thus, our results suggest that the remaining *A. nidulans* RNA silencing genes have a "nonhousekeeping" function, such as defense against viruses and transposons.

RNA silencing proteins, including Dicers, Argonautes, and RNA-dependent RNA polymerases (RdRPs), are found in all eukaryotic lineages thus far investigated, making it likely that RNA silencing evolved before divergence of the last common eukaryotic ancestor (13). Since then, RNA silencing proteins have retained or developed a number of important biological roles, including genome defense, chromatin modification, and gene regulation (for reviews, see references 31, 44, and 70). Despite the importance of RNA silencing, it is not uniformly conserved in all eukaryotes, with current evidence indicating that four of six eukaryotic supergroups have members that do not encode Dicers, Argonautes, and RdRPs (13). However, while these types of genes have been lost by some species, they have been expanded in others. This is perhaps most apparent in basidiomycete fungi, where a combination of RNA silencing gene gain and loss has resulted in species that encode between zero and eight Dicers, zero and three Argonautes, and zero and nine RdRPs (48). It is thus apparent that RNA silencing gene changes are common in eukaryotic evolution; however, the forces contributing to these changes, as well as the methods by which species adapt to them, are unclear.

Dicers are characterized by a number of domains, including an N-terminal helicase domain and two C-terminal RNase III domains. These proteins cleave long double-stranded RNA (dsRNA) into 21- to 26-bp fragments (4, 22). The resulting small RNA is typically classified based on the dsRNA source from which it originates and includes types such as repeat-associated small interfering RNA (rasiRNA), microRNA (miRNA), and small interfering RNA (siRNA) (46). Endogenous sources of dsRNA are repetitive DNA, noncoding regulatory genes, and foreign genetic elements such as viruses and transposons. Some of these sources require an RdRP to produce the double-stranded Dicer substrate (3, 15, 17, 20, 43, 64, 65, 67). Exogenous dsRNA can also be introduced into cells or whole organisms experimentally. One method involves transforming organisms with inverted repeat transgenes (IRTs) so that IRT transcription will produce hairpin RNA molecules to serve as Dicer substrates (41, 55, 73).

Dicer processed small RNA is incorporated into an Argonaute-containing ribonucleoprotein effector complex (9, 14, 46). Examples include RITS, which contains a rasiRNA and directs heterochromatin assembly (71); miRNP, which contains a miRNA and inhibits the translation of mRNA (47, 52, 60), and RISC, which contains a siRNA or miRNA and directs RNA cleavage (29, 33, 45). The various small RNA likely guide their effector complexes to specific targets by complementary base pairing (22, 45). Argonautes typically contain an N-terminal PAZ domain and a C-terminal Piwi domain. Within the RISC complex, the PAZ domain is thought to bind the siRNA (42, 57, 66, 75), and the Piwi domain is thought to cleave the target mRNA (2, 42, 57, 66).

With regard to filamentous fungi, the *Neurospora crassa* RNA silencing machinery is the most thoroughly characterized. Two *N. crass*a RNA silencing phenomena are meiotic silencing (40, 62, 63) and quelling (16, 18, 19, 58), both of which appear to be efficient genome defense mechanisms. Meiotic silencing is activated by unpaired DNA during the sexual

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cycle, while quelling occurs during the vegetative cycle and depends on high numbers of tandemly arranged transgenes. A two-pathway hypothesis has been proposed to explain the evolutionary origin of *N. crassa* meiotic silencing and quelling. This hypothesis suggests that a single group of ancestral RNA silencing genes duplicated in an early ancestor of the filamentous ascomycetes, leading to two paralogous groups of RNA silencing genes with evolutionarily divergent functions (5, 23). The hypothesis is supported by phylogenetic evidence from both *Aspergillus fumigatus* and *N. crassa* (5, 23) and genetic evidence from studies with *N. crassa*. This genetic evidence links meiotic silencing to the Dicer DCL-1 (1), the Argonaute SMS-2 (40), and the RdRP SAD-1 (63) and quelling to Argonaute QDE-2 (11) and RdRP QDE-1 (17). However, either of the *N. crassa* Dicers (DCL-1 and DCL-2) is sufficient for quelling (11), demonstrating that there is not always a clear division of labor between the two pathways.

RNA silencing is crucial for normal growth and developmental processes in higher eukaryotes (26, 27, 32, 36, 37, 53, 56, 74), but it is unclear how important RNA silencing is for growth and development in fungi. Although at least some *N. crassa* RNA silencing mutants are sterile in homozygous crosses (1, 40, 63), other morphological changes have not been reported for *N. crassa* single or double Dicer mutants (12), or Dicer mutants of the tree pathogen, *Cryphonectria parasitica* (59). In *Magnaporthe oryzae* and *Mucor circinelloides*, other filamentous fungi whose RNA silencing genes have been partially characterized, Dicer mutants appear to have slight morphological abnormalities (35, 49). Finally, in the fission yeast *Schizosaccharomyces pombe*, RNA silencing mutations disrupt normal cell cycle regulation (10) and cause chromosome segregation defects (72). These phenotypes have not yet been associated with RNA silencing defects in other fungi.

A group of closely related species that have followed different evolutionary paths in regard to RNA silencing should benefit studies of RNA silencing gene evolution. Herein, a survey of the Dicers and Argonaute genes in seven *Aspergillus* species demonstrates that *Aspergillus* fungi are well suited for such studies because they include species that have experienced RNA silencing gene gain or loss. The present study thoroughly characterizes the loss of RNA silencing genes—a Dicer and an Argonaute—to gene truncation events in the model species *Aspergillus nidulans*. In addition, the remaining *A. nidulans* RNA silencing genes (a Dicer, an Argonaute, and two RdRPs) are characterized with respect to experimental RNA silencing, growth, and development. A companion study investigates the role of RNA silencing in the defense against mycoviruses (28a).

MATERIALS AND METHODS

Phylogenetic trees, domain identification, and nucleotide polymorphism analysis. Accession numbers for protein sequences used in the present study are provided in the supplemental material. Ascomycete Dicers and Argonautes were identified by searching (blastp) the GenBank (National Center for Biotechnology Information) and fungus-specific databases with the consensus sequences of RNase III (cd00593.1) and Piwi (pfam02171.11) domains, as well as the sequence of known fungal Dicer and Argonaute proteins. Species-specific databases included the following: www.cadre.man.ac.uk (*A. fumigatus*), www.broad.mit.edu (*A. nidulans*, *Gibberella zeae*, *M. oryzae*, and *N. crassa*), www.bio.nite.go.jp (*Aspergillus oryzae*), and www.sanger.ac.uk (*Schizosaccharomyces pombe*). Nonannotated *Aspergillus* databases (www.ncbi.nlm.nih.gov; *Aspergillus clavatus* NRRL1, *Aspergillus flavus* NRRL3357, *Aspergillus terreus* NIH2624, and *Neosartorya fischeri* NRRL181) were searched (tblastn) for contigs containing putative Dicer and Argonaute orthologs (see the supplemental material). Putative Dicer and Argonaute gene sequences were retrieved and aligned to the annotated orthologs found in the *A. fumigatus* or *A. oryzae* genome databases to predict exon-intron boundaries. The putative protein sequences were aligned by using MUSCLE (21) (European Bioinformatics Institute [www.ebi.ac.uk/muscle]). Alignments were imported onto the San Diego Supercomputer Center's Biology Workbench (workbench.sdsc.edu) to produce unrooted, noniterated trees using the DrawTree function.

For nucleotide polymorphism analysis, PCR-amplified fragments were generally gel purified and cloned into pCR2.1-TOPO (Invitrogen) before sequencing. Only one to two clones were sequenced for each locus; thus, some polymorphic residues may be a result of PCR and/or sequencing error. Sequences were imported into Bio Edit (28) and MEGA (39) for processing and analysis and aligned by using CLUSTAL W (68) (see also the supplementary material).

Strains and culture conditions. The genotypes of the *A. nidulans* FGSC A4 (referred to here as simply A4) derivatives used in the present study are listed in Table 1. The four wild isolates of *A. nidulans* (G143, H109, HcB, and HcE) were collected from various locations around the United Kingdom and represent four distinct heterokaryon compatibility groups (25). The isolates were provided along with two wild *Aspergillus rugulosus* isolates (strains 203 and 211) by David Geiser (Penn State University). An industrial *A. rugulosus* isolate, SRRC 1173 (38), was also used in the present study. To confirm that the various isolates were of independent lineages, Southern analysis of genomic DNA from the *A. nidulans* and *A. rugulosus* isolates was performed with a probe for *A. nidulans* transposon relic An5242.3. Each wild isolate produced a unique pattern, while all A4 derivatives produced an identical pattern (see the supplementary material). For physiological assays and crossing assays, a series of crosses was first performed to place all RNA silencing gene deletion alleles and RdRP deletion alleles in the same genetic background (see the supplemental material). The combined gene deletions include Δ*dclB*, Δ*rsdA*, Δ*rrpB*, and Δ*rrpC*. When all deletion alleles are in the same genetic background the strain is referred to as ΔRNAi.

Glucose minimal medium (GMM) with appropriate supplements (61) was used for all experiments unless otherwise indicated. For genomic DNA isolation 1 g of yeast extract was added to 1 liter of liquid GMM. Yeast-glucose-trace element medium (YGT) as described previously (8) was used for crossing assays. *A. nidulans* conidia were inoculated into 25 ml of liquid GMM (2.5×10^7 conidia for RNA isolation) and incubated under nonstringent dark conditions at 37°C unless otherwise indicated.

Gene deletions, transformations, and crosses. The sequences for all oligonucleotide PCR primers used in the present study are provided in the supplemental material. Genes were deleted by double-homologous recombination. Details of the deletion and complementation plasmids used in the present study are provided there as well. Transformations were performed essentially as described by Yu and Adams (76). Changes to the protocol included the use of 3 to 4 mg of Sigma lysing enzyme (L1412-10G; Sigma) per ml of OM-A protoplasting buffer or the use of the glucanase-driselase-lyticase lysing enzyme mix suggested by Jung et al. (34). Because selecting for outcrossed cleistothecia from a cross between prototrophic *A. nidulans* strains can be difficult, the Δ*rsdA* transformants described previously (30) were not used, and a new Δ*rsdA* methionine-auxotroph was created instead (described in the supplemental material).

Because *A. nidulans* is capable of both selfing and crossing, an assay (inspired by Bruggeman et al. [6]) involving spore color mutants was used to determine whether *A. nidulans* RNA silencing mutants were deficient in crossing ability. Yellow (*yA2*) and white (*wA3*) spore color gene mutations segregate independently during meiosis, so that crosses between strains that are yellow (*wA yA2*) and white (*wA3 yA*) result in progeny that are yellow (*wA yA2*), green (*wA yA*), or white (*wA3 yA2* or *wA3 yA*). Scoring the conidial color of ascospore-derived colonies from a single cleistothecium thus allows one to distinguish between selfed and crossed cleistothecia. For the assay, 5×10^5 conidia of each crossing partner were mixed in 5.0 ml of molten YGT (0.7% agar, ~48°C) and spread onto the surface of 30.0 ml of solid YGT. Plates were wrapped with parafilm and cultured in the dark for 10 days. For scoring, cleistothecia were harvested and cleaned of fungal tissue by rolling on 3% water agar. During harvesting there was an intentional bias toward the larger (than average) cleistothecia, since this was assumed to be an indicator of maturity. Cleaned cleistothecia were burst open in 500 μl of sterile water, and 5 to 10 μl of each ascospore suspension was spread onto solid GMM. After 3 days at 37°C, the colony conidial color was recorded and used to determine whether each cleistothecium was the result of selfing (yellow or white colonies) or crossing (white, yellow, and green colonies). Plates were stored at 4°C during cleistothecium scoring, which was performed over several weeks.

TABLE 1. Strains used in this study

Category and strain[a]	Genotype
Reference strains	
RTMH13.B3*	Δ*stcE*::*argB*; *veA1*
RTMH13.B1*	Δ*stcE*::*argB*; *aflR*(IRT)::*trpC veA1*
RTMH13.F2	*pyrG89*; *metG1*, Δ*stcE*::*argB*; *veA1*
RTMH13.F3*	*pyrG89*; *metG1* Δ*stcE*::*argB*; *aflR*(IRT)::*trpC veA1*
Transformants	
TEAB65.C1‡	*pyrG89*; *metG1* Δ*stcE*::*argB*; Δ*rsdA*::*pyrG*; *aflR*(IRT)::*trpC veA1*
TJW64.17, 46	*pyrG89*; *wA3*; *pyroA4* Δ*stcE*::*argB*; Δ*dclA*::*pyroA*, *trpC801 veA1*
TBRG3.6	*pyrG89*; Δ*ppdB*::*metG*; *metG1* Δ*stcE*::*argB*; *aflR*(IRT)::*trpC veA1*
TTMH158.1	*pyrG89*; *metG1* Δ*stcE*::*argB*; Δ*dclB*::*pyrG*; *aflR*(IRT)::*trpC veA1*
TTMH160.3	*pyrG89*; *wA3*; *gpdA*(p)::*dclB*::*pyroA* Δ*stcE*::*argB*; *veA1*
Analysis of *dclA* function in IRT-RNA silencing (Fig. 3)	
RTMH192.1	*pyrG89*; *wA3*; Δ*stcE*::*argB*; *veA1*
RTMH192.2, 5, 10	*pyrG89*; Δ*stcE*::*argB*; *aflR*(IRT)::*trpC* Δ*dclA*::*pyroA veA1*
RTMH192.3, 6, 8	*pyrG89*; Δ*stcE*::*argB*; *aflR*(IRT)::*trpC veA1*
RTMH192.4	*pyrG89*; Δ*stcE*::*argB*; Δ*dclA*::*pyroA*, *veA1*
RTMH192.7	*pyrG89*; *wA3*; Δ*stcE*::*argB*; *aflR*(IRT)::*trpC*, Δ*dclA*::*pyroA veA1*
RTMH192.9	*pyrG89*; *wA3*; Δ*stcE*::*argB*; *aflR*(IRT)::*trpC*, *veA1*
Analysis of *ppdB* and *rsdA* function in IRT-RNA silencing (Fig. 4)	
RTMH193.1‡	*pyrG89*; Δ*ppdB*::*metG*; *metG1* Δ*stcE*::*argB*; Δ*rsdA*::*pyrG*; *aflR*(IRT)::*trpC veA1*
RTMH193.4	*pyrG89*; *metG1* Δ*stcE*::*argB*; Δ*rsdA*::*pyrG*; *aflR*(IRT)::*trpC veA1*
RTMH193.10	*pyrG89*; Δ*ppdB*::*metG*; Δ*stcE*::*argB*; *aflR*(IRT)::*trpC veA1*
Analysis of *dclB* function in IRT-RNA silencing (Fig. 7)	
RTMH215.6, 7	*pyrG89*; Δ*stcE*::*argB*; Δ*dclB*::*pyrG*; *veA1*
RTMH215.12, 13	*pyrG89*; Δ*stcE*::*argB*; Δ*dclB*::*pyrG*; *aflR*(IRT)::*trpC veA1*
RTMH215.4, 17	*pyrG89*; *gpdA*(p)::*dclB*::*pyroA* Δ*stcE*::*argB*; Δ*dclB*::*pyrG*; *veA1*
RTMH215.20, 29	*pyrG89*; *gpdA*(p)::*dclB*::*pyroA* Δ*stcE*::*argB*; Δ*dclB*::*pyrG*; *aflR*(IRT)::*trpC veA1*
Analysis of radial growth and spore production (Table 3)	
RDIT9.32†	WT
RTMH211.14	WT
RTMH229.24	WT
RTMH218.7, 39	Δ*rrpB*::*pyrG*; Δ*rrpC*::*metG* Δ*dclB*::*pyrG*; Δ*rsdA*::*pyrG*
RTMH211.6, 11	Δ*ppdB*::*metG*
RTMH212.42, 65	Δ*dclA*::*pyroA*
Analysis of crossing (Table 4)	
RTMH227.29, 47	*yA2*; Δ*rrpB*::*pyrG*; Δ*rrpC*::*metG* Δ*dclB*::*pyrG*; Δ*rsdA*::*pyrG*
RTMH227.67, 68	*wA3*; Δ*rrpB*::*pyrG*; Δ*rrpC*::*metG* Δ*dclB*::*pyrG*; Δ*rsdA*::*pyrG*
RTMH229.25, 37	*yA2*
RTMH229.19	*wA3*
RTMH233.C	*wA3*

[a] Commas are used between the suffixes of strain names to indicate independently isolated transformants or recombinants of the same genotype. The *aflR* IRT transgene used in this study is the *aflR*(IRT1300) transgene referenced earlier (30). Strain sources: *, Hammond and Keller (30), †, Tsitsigiannis et al. (69). ‡, Strains that carry an ectopic copy of the *rsdA* deletion vector; this does not appear to have influenced any of the results (data not shown). Additional strains used in this study, such as the transformation hosts and crossing intermediates, are described in the supplemental material.

Southern and Northern blotting. All transformants and recombinants were single spore purified, and genotypes were confirmed by Southern blotting using Hybond-XL nylon membranes (Amersham Biosciences) with the alkali transfer protocol as described by the manufacturer. Southern blotting-based identification of all deletion strains was performed with probes for the deleted sequences (see Results) and at least one flanking region of the deleted gene (see the supplemental material). For Northern analysis, total RNA was harvested from lyophilized tissues by using TRIzol reagent (Invitrogen) essentially as suggested by the manufacturer. RNA was separated by formaldehyde-denaturing gel electrophoresis and blotted onto Hybond-XL nylon membranes by capillary transfer in 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). A picture of the ethidium bromide-stained rRNA is included with all Northern blots to indicate the relative levels of RNA in each lane.

Determining RNA silencing function via NOR production. Previous analysis of *A. nidulans* RNA silencing demonstrated that an IRT containing *A. nidulans aflR* sequences can be used to silence *aflR* expression through RNA silencing (IRT-RNA silencing [30]). Because *A. nidulans* strains carrying a Δ*stcE* allele produce the compound norsolorinic acid (NOR) in an *aflR*-dependent manner (7), it is possible to determine whether IRT-RNA silencing is functional by measuring NOR production in strains carrying both an *aflR* IRT and a Δ*stcE* allele (30). In essence, the lack of NOR in the presence of Δ*stcE* and the *aflR* IRT indicates that IRT-RNA silencing is functional. To measure NOR production, conidia were qualitatively transferred to the center of a 30.0-ml plate of GMM (plus supplements if required), and cultures were grown for 5 to 6 days. A 1.4-cm-diameter core was then taken from the center of the colony and assayed by thin-layer chromatography (TLC) as previously described (30).

GenBank accession numbers. The GenBank accession numbers for the strains discussed in this study were as follows: (i) for *A. nidulans* RTMH13.C5 (an A4 derivative), internal transcribed spacer (ITS), EU287942; (ii) for *A. nidulans* G143, *dclA* (target 1), EU289898; and *ppdB* (target 3), EU289899; (iii) for *A. rugulosus* 211, *dclA* (targets 1 and 2), EU289900; *ppdB* (target 4), EU289903; *rrpA* relic (partial), EU289905; actin (partial), EU289911; ITS, EU289912; *dclB*

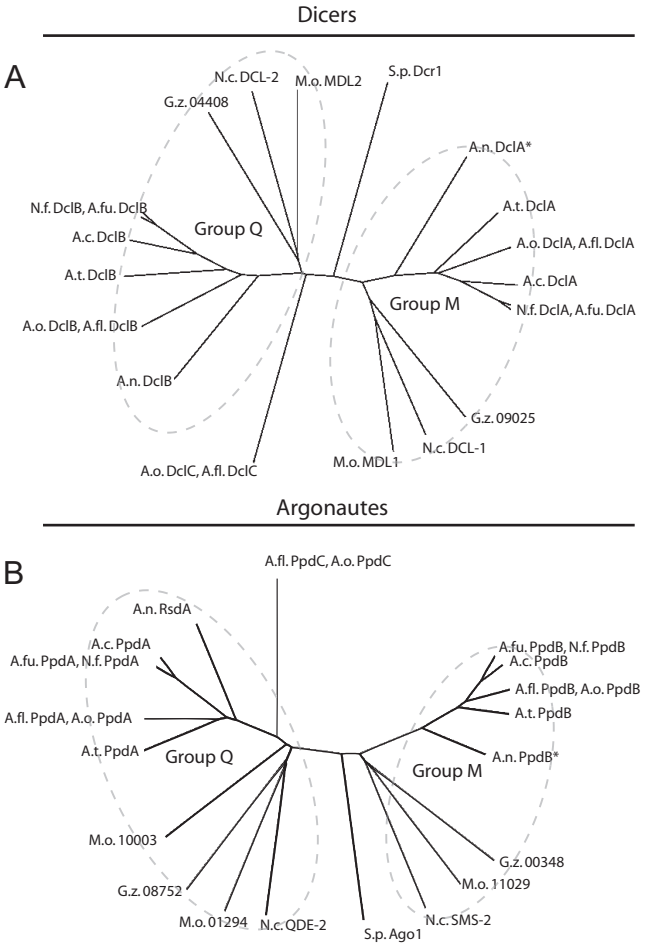

FIG. 1. Ascomycete Dicers and Argonautes. The predicted sequences for putative Dicers (A) and Argonautes (B) were used to assemble unrooted, noniterated phylogenetic trees (see Materials and Methods). Proteins were arbitrarily divided into two general groups (Q or M). Names for *Aspergillus* proteins, except *A. nidulans* RsdA, were derived from the names of the corresponding *A. fumigatus* orthologs proposed by Galagan et al. (23). Uncharacterized proteins from non-*Aspergillus* species were not given names. Abbreviations: *A.c.*, *A. clavatus*; *A.fl.*, *A. flavus*; *A.fu.*, *A. fumigatus*; *A.n.*, *A. nidulans*; *A.o.*, *A. oryzae*; *A.t.*, *A. terreus*, *G.z.*, *G. zeae*; *M.o.*, *M. oryzae*; *N.c.*, *N. crassa*; *N.f.*, *N. fischeri*; *S.p.*, *S. pombe*.

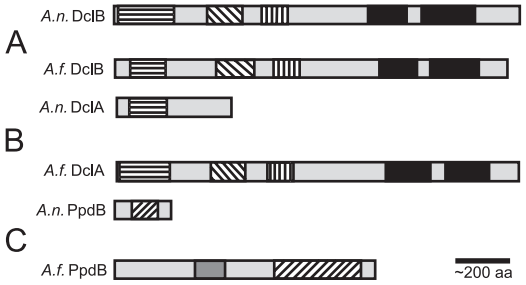

FIG. 2. Predicted domains of *A. nidulans* and *A. fumigatus* Dicer and Argonaute proteins. The predicted sequences of *A. nidulans* and *A. fumigatus* DclB, DclA and PpdB were used to search the National Center for Biotechnology Information conserved domain database for domain identification. The truncated nature of *A. nidulans dclA* and *ppdB* is revealed by comparison to their *A. fumigatus* orthologs. The *A. nidulans group* Q Argonaute, RsdA, was characterized in a previous study (30). An MPH1 domain overlaps the DEAD and Hel C domains in both *A. fumigatus* Dicer proteins and *A. nidulans* DclB (not shown). Dicer domains: DEAD-like helicase (pfam00270, smart00487), horizontal bars; Helicase C (cd00079), diagonal bars; Duf283 (pfam03368), vertical bars; RNase III (cd00593), black boxes; MPH1 (cog1111.1). Argonaute domains: PAZ (cd02846), dark gray boxes; Piwi (pfam02171), diagonal bars.

(partial), EU289913; and *ppdA* (partial), EU289914; (iv) for *A. rugulosus* 1173, *dclA* (target 2), EU289901; *ppdB* (target 4), EU289904; *rrpA* relic (partial), EU289906; actin (partial), EU289915; ITS, EU289916; *dclB* (partial), EU289917; and *ppdA* (partial), EU289918; and (v) for *A. rugulosus* 203, *ppdB* (target 4), EU289902; actin (partial), EU289907; ITS, EU289908; *dclB* (partial), EU289909; and *ppdA* (partial), EU289910.

RESULTS

Dicers and Argonautes in the filamentous ascomycetes. As originally noted for the *N. crassa* and *A. fumigatus* Dicers and Argonautes (5, 23), two paralogous groups of RNA silencing proteins are typical of filamentous ascomycetes (Fig. 1). Although quelling and meiotic silencing have only been described in *N. crassa*, we refer to the paralogous groups as "Q" and "M" in the present study for reference purposes. Most analyzed fungi encode a single member for groups Q and M, although a few deviate from this pattern. *Aspergillus oryzae* and *Aspergillus flavus* each encode three Dicers and three Argonautes, apparently due to group Q duplication. Similarly, *Magnaporthe oryzae* encodes three Argonautes, apparently due to duplication of its group Q Argonaute (Fig. 1). The opposite phenomenon of RNA silencing gene loss appears to have occurred in *A. nidulans*. Although there is evidence for both *A. nidulans group* M and Q proteins (Fig. 1), only the *A. nidulans group* Q proteins appear to be full length (Fig. 2). For example, while the group Q Dicer (DclB) is predicted to have all of the domains of a typical Dicer, the group M Dicer (DclA) is predicted to have only a Dead Box helicase domain (Fig. 2A and B). Similarly, while the group Q Argonaute (RsdA) is predicted to have both domains of a typical Argonaute, the group M Argonaute (PpdB) is predicted to have only a Piwi, or partial Piwi, domain (Fig. 2C) (30). Thus, in contrast to *N. crassa*, *M. oryzae*, *G. zeae*, and other aspergilli, *A. nidulans* has lost full-length group M RNA silencing proteins.

Truncation events have occurred at two *A. nidulans* RNA silencing gene loci. The missing domains of *A. nidulans dclA* and *ppdB* are not due to an annotation mistake. An extensive analysis of sequences flanking the truncated *dclA* and *ppdB* genes did not locate "missing" RNase III or PAZ domains (data not shown), suggesting that the annotation prediction of gene truncation is correct for both genes. Thus, the *dclA* and *ppdB* loci have lost their respective 3′ and 5′ ends through major truncation events. This hypothesis is further supported below.

Despite their truncated nature, transcripts are detected from both the *dclA* and the *ppdB* loci (Fig. 3A and 4A). In addition, transcript mapping by partial cDNA analysis and/or 3′ RACE (rapid amplification of cDNA ends) demonstrates that intron splicing and poly(A) tailing occur on *dclA* and *ppdB* transcripts (data not shown). This suggests that the truncated genes may encode truncated proteins with biological function. However, protein tagging experiments failed to detect a translated pro-

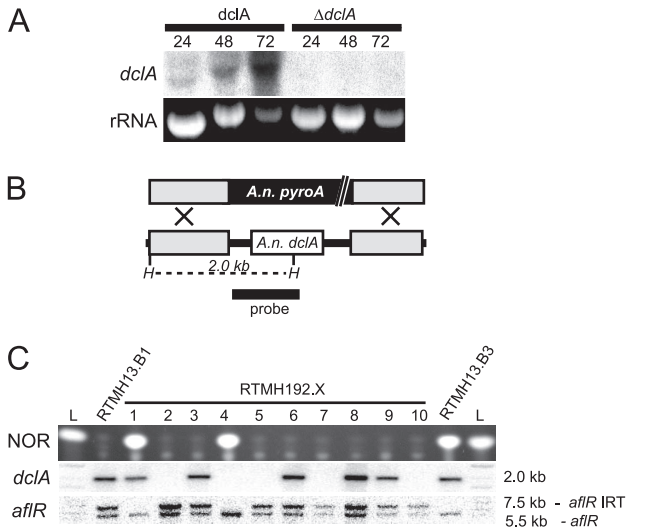

FIG. 3. The truncated *A. nidulans dclA* locus is transcribed but not required for IRT-RNA silencing. (A) Northern blotting identifies *dclA* transcripts in total RNA from 24-, 48-, and 72-h cultures, an increase in transcription was observed at late time points. The *dclA* transcript migrates slightly below the 18S rRNA band, as expected by its truncated nature. Strains: *dclA* strain, RTMH192.3; Δ*dclA* mutant, RTMH192.2. (B) Schematic representation of *dclA* replacement with *A. nidulans pyroA*. Gray boxes represent the *dclA*-flanking sequences used in the deletion vector. The white box represents the predicted *A. nidulans dclA* open reading frame (ORF). H, HindIII sites. (C) TLC analysis of NOR production and Southern blotting (HindIII digest) results for control strains and recombinants from a cross between a Δ*dclA* transformant (TJW64.17) and an *aflR* IRT-carrying strain (RTMH13.F3). In the Southern blots, the absence of a band for *dclA* is indicative of the deletion genotype (see probe template in panel B) and the presence of two bands for *aflR* is indicative of the *aflR* IRT genotype. Note that the loss of NOR production, and thus RNA silencing of *aflR*, is normal regardless of *dclA* presence. Strain names are listed above each lane. L, 1-kb ladder or NOR standard.

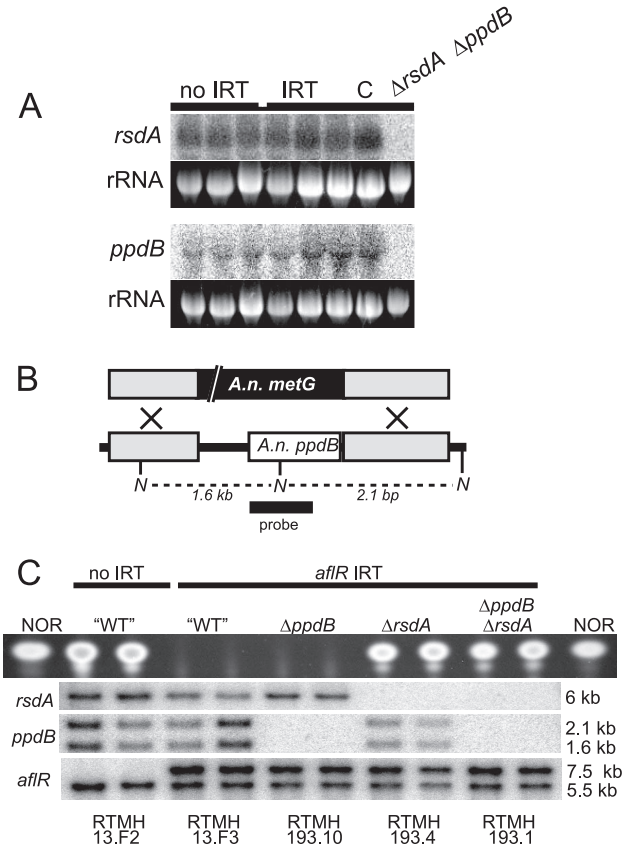

FIG. 4. The truncated *A. nidulans ppdB* locus is transcribed but not required for IRT-RNA silencing. (A) Northern blotting identifies *ppdB* and *rsdA* transcripts in total RNA from 48-h cultures. The *rsdA* transcript migrates slightly above the 26S rRNA band, while the *ppdB* transcript migrates slightly below the 18S rRNA band, as expected by its truncated nature. Strains: no IRT, RTMH13.B3; IRT, RTMH 13.B1; C, RTMH192.3; Δ*rsdA* Δ*ppdB*, RTMH193.1. (B) Schematic representation of *ppdB* replacement with *A. nidulans metG*. Gray boxes represent the *ppdB*-flanking sequences used in the deletion vector. The white box represents the predicted *ppdB* ORF. *N*, NcoI sites. (C) TLC analysis of NOR production and Southern blotting (NcoI digest for *ppdB*; HindIII digest for *rsdA* and *aflR*) results for control strains and recombinants from a cross between a Δ*ppdB* transformant (TBRG3.6) and an Δ*rsdA* transformant (TEAB65.C1). In the Southern blots, the absence of bands for *ppdB* and *rsdA* (30) is indicative of the deletion genotypes (see probe templates in panel B and also the supplemental material), and the presence of two bands for *aflR* is indicative of the *aflR* IRT genotype. Note that RNA silencing of *aflR* is dependent on *rsdA* but not on *ppdB*. Strain names are listed below each lane.

tein from either transcript (data not shown), and gene deletion affected neither experimental RNA silencing nor physiological characteristics such as growth or development (Fig. 3 and 4 and see also Table 3 and the supplemental material).

The *A. nidulans dclA* and *ppdB* truncations are common to wild *A. nidulans* isolates. Because all other analyzed filamentous ascomycetes were found to encode full-length orthologs of *dclA* and *ppdB*, it seemed possible that the *dclA* and *ppdB* truncations might be specific to the common *A. nidulans* laboratory strain A4 and its derivatives. Therefore, the *dclA* and *ppdB* loci of wild *A. nidulans* isolates were investigated. Analysis of genomic fragments spanning the 3′ *dclA* intergenic region, 5′ *ppdB* intergenic region and parts of the *dclA* and *ppdB* coding regions indicated that the four wild *A. nidulans* isolates carry the same *dclA* and *ppdB* truncations as A4 (Fig. 5 and 6). The fact that longer PCR products are amplified from the 5′ *ppdB* intergenic region of G143 and HcB than from any of the other isolates (Fig. 6B) is due to the presence of a 5.5-kb insertion with significant identity to a transposon relic found at locus AN8648.3 (and others) of the *A. nidulans* genome (blastx, E = 0).

***A. rugulosus* encodes full-length versions of *dclA* and *ppdB*.** To determine whether the gene truncations also exist in the closely related species *A. rugulosus* (54), *dclA* and *ppdB* loci in this species were investigated. The close relationship between *A. rugulosus* and *A. nidulans* is illustrated by the fact that there is essentially no nucleotide polymorphism between their ITS sequences (Table 2). PCR amplification of genomic fragments spanning the 3′ *dclA*-intergenic region and the *ppdB* coding region resulted in products that are longer than those observed in *A. nidulans* (Fig. 5B and 6C), suggesting that both *A. rugulosus* genes are full length. For example, a 6.1-kp product is amplified from the *A. rugulosus* 3′ *dclA* intergenic region instead of the 2.6-kp product amplified from the orthologous locus in *A. nidulans* (Fig. 5B), and a 3.5-kp product is amplified from the *A. rugulosus ppdB* coding region instead of the 0.9-kp

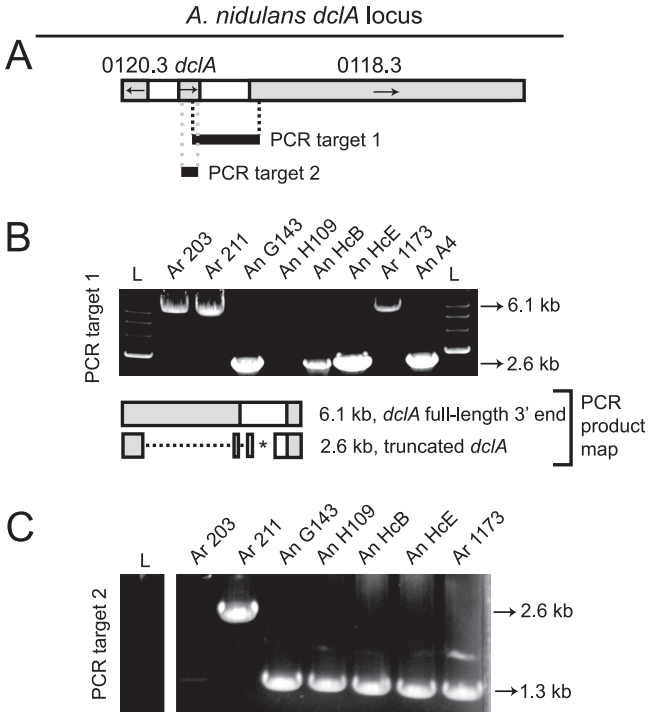

FIG. 5. Comparison of *A. nidulans* and *A. rugulosus dclA*. (A) A schematic of the predicted *A. nidulans dclA* locus is shown with its immediate flanking genes. Genes are gray and intergenic regions are white. PCR was used to amplify two DNA fragments (target 1 and target 2) from wild *A. nidulans* isolates (An) and *A. rugulosus* isolates (Ar). (B) Target 1 is 6.1 kb long in all *A. rugulosus* isolates and 2.6 kb in all *A. nidulans* isolates, suggesting that the 3′ end of *A. rugulosus dclA* is full length while the *A. nidulans dclA* truncation is fixed at the species level. Although a product is not amplified for *A. nidulans* HcB, a different primer set amplifies a band similar to one from A4 for the same approximate location (data not shown). A PCR product map depicts the *A. nidulans* truncation. It comprises three major deletions (dashed line) and a single insertion (*). (C) Target 2 is 2.6 kb in *A. rugulosus* 211 and 1.3 kb in all other isolates. Sequencing indicates that this is due to a transposon insertion. The *A. rugulosus* 203 1.3-kp product is present but, for undetermined reasons, is faint relative to the PCR products amplified from the other isolates. L, 1-kb ladder; An A4, strain RTMH13.C5.

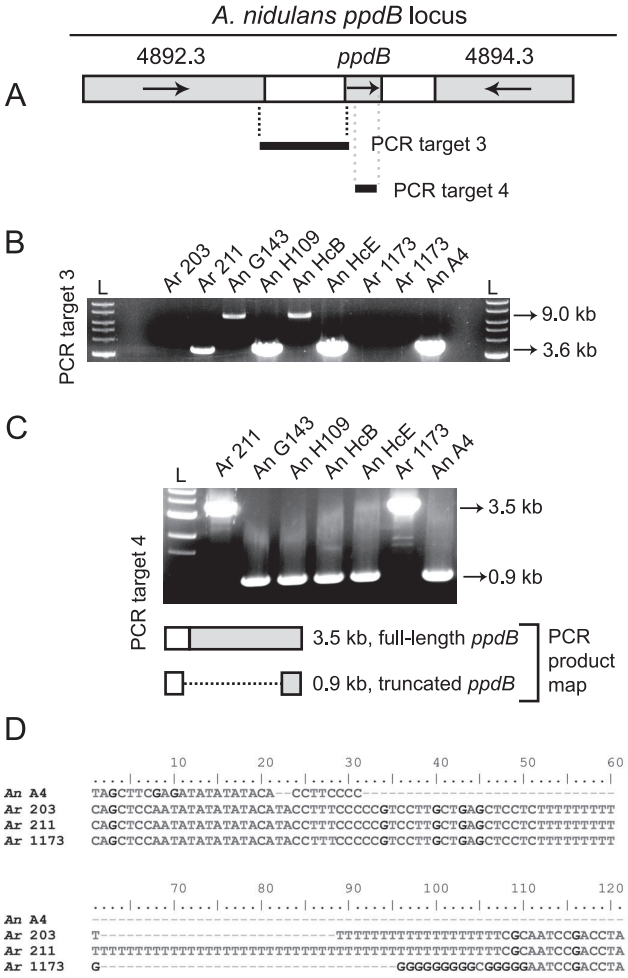

FIG. 6. Comparison of the *A. nidulans* and *A. rugulosus ppdB* loci. (A) A schematic of the predicted *A. nidulans ppdB* locus is shown with its immediate flanking genes. Genes are gray, and intergenic regions are white. PCR was used to amplify two DNA fragments (target 3 and target 4) from wild *A. nidulans* isolates (An) and *A. rugulosus* isolates (Ar). (B) Target 3 is 3.6 kb for isolates from both species, except for *A. nidulans* G143 and HcB. Sequencing indicates that this is due to a transposon insertion in the 5′ *ppdB*-intergenic region of *A. nidulans* G143 (HcB was not sequenced). Although bands were not amplified for *A. rugulosus* 203 and *A. rugulosus* SRRC 1173, a different primer set amplified a band of similar size to one from A4 for the same approximate location (data not shown). (C) Target 4 is 3.5 kb for all *A. rugulosus* isolates and 0.9 kb for all *A. nidulans* isolates. A 3.5-kb PCR product was also amplified from *A. rugulosus* 203 with the same primer set (product not shown; the sequence is provided in the supplemental material). A PCR product map depicts how the 3.5-kb band is characteristic of a full-length *ppdB* and the *A. nidulans ppdB* truncation (dashed line) extends to the 5′ intergenic region. Differences in ORF location (gray shading in panels A and C) are due to differences in predicted *ppdB* start codon locations for the *A. nidulans* truncated *ppdB* and *A. rugulosus ppdB*. For panels B and C: L, 1-kb ladder; An A4, strain RTMH13.C5. (D) A variable polynucleotide repeat exists next to the *A. nidulans ppdB* truncation point in the three *A. rugulosus* isolates.

product amplified from the orthologous locus in *A. nidulans* (Fig. 6C). Sequencing of the *A. rugulosus* products demonstrated that this species does indeed carry full-length versions of *dclA* and *ppdB* (see the supplemental material).

Comparative analysis between the *A. rugulosus* and *A. nidulans* sequences indicates that the *A. nidulans dclA* locus is missing three DNA fragments (approximately 3, 0.7, and 0.3 kb) and encodes an insertion of 0.6 kb in its the 3′ intergenic region (Fig. 5B). The insertion contains two short elements (50 to 100 bp) that are repeated approximately 15 times throughout the *A. nidulans* genome (see the supplemental material). The *A. nidulans ppdB* locus is characterized by a single major deletion of 2.6 kb that appears to have originated approximately 20 nucleotides upstream of a polynucleotide repeat of variable length and sequence in the three *A. rugulosus* isolates (Fig. 6D).

Although the *A. rugulosus dclA* and *ppdB* loci encode full-length genes; whether or not they produce functional proteins has not been determined. *A. rugulosus* 211 (*dclA*), however, is not likely to be functional because sequencing of a DNA fragment spanning its DEAD-like helicase domain (Fig. 5C, note longer-than-normal PCR product) identified an insertion sim-

TABLE 2. Nucleotide polymorphism analysis

Locus	Alignment length (no. of nucleotides)	% Nucleotide polymorphism[a] in *A. rugulosus* strain:		
		203	211	1173
ITS	604	0.0	0.3	0
Actin	711	1.8	1.7	1.8
ppdA/*rsdA* (Piwi)	973	5.0	5.0	4.3
ppdB (Piwi)*	415	5.3	5.3	5.3
dclB (helicase)	1,010	6.4	6.4	6.6
dclA (helicase)	1,124	ND	10.8[c]	11.4
Introns[b]	509	ND	10.6	11.2
Degenerate *rrpA*	520	ND	15.0	15.6
ppdB intergenic†	421	17.1	17.6	16.6

[a] The percent nucleotide polymorphism was calculated as the number of variable nucleotides and indels (between aligned sequences from *A. nidulans* and the specified *A. rugulosus* isolate) divided by the length of the alignment. The alignments are provided in the supplemental material. Predicted introns were removed from the actin, Dicer, and Argonaute fragments before the analysis. The *A. nidulans ppdB* PCR product from Fig. 6 (target 4) contains both coding sequences (*) and intergenic sequences (†); therefore, it was divided accordingly before alignment and analysis. ND, not determined.

[b] A concatenated intron containing predicted intron sequences from the actin, Dicer, and Argonaute fragments was analyzed. The GT/AG consensus splice sites were removed before alignment.

[c] The transposon insertion located in the *A. rugulosus* 211 *dclA* helicase domain was excluded from the alignment.

ilar to a transposase found in the *A. oryzae* genome (blastx, e^{-121}, GI:92019814) (see the supplemental material). Thus, as in *A. nidulans*, the group M RNA silencing genes of *A. rugulosus* appears to be unusually susceptible to mutation.

Nucleotide polymorphism between *A. nidulans* and *A. rugulosus* loci. Nucleotide polymorphism levels between several loci from *A. nidulans* and *A. rugulosus* isolates are compared in Table 2. The ITS sequences are essentially identical between all *A. nidulans* and *A. rugulosus* isolates, with only one to three variable nucleotides or indels found in an ITS clone from one *A. nidulans* isolate (HcE) and one *A. rugulosus* isolate (isolate 211) (see the supplemental material). A fragment of actin also has very little polymorphism between the species (1.7 to 1.8%). An alignment of six concatenated introns was found to have much more variability than the ITS region or the actin sequences, with a nucleotide polymorphism range of 10.6 to 11.2%. A fragment from the 5′ *ppdB* intergenic region also has a relatively high level of polymorphism at 16.6 to 17.6%. Similar to what has been reported for *A. nidulans* (30), the *A. rugulosus rrpA* locus encodes a degenerate *rrpA* (see the supplemental material) and, accordingly, a high level of polymorphism exists at the *rrpA* locus (15 to 15.6%).

With respect to the group M RNA silencing proteins, a fragment of *dclA* spanning the Dead Box helicase domain (Fig. 5C, target 2) has 10.8 to 11.4% nucleotide variability between *A. nidulans* and *A. rugulosus* species, whereas a fragment of *ppdB* has less polymorphism between the species (5.3%). A similar analysis for the group Q RNA silencing genes finds a polymorphism level of 4.3 to 5.0% between the Argonautes (*rsdA*/*ppdA*) and 6.4 to 6.6% between the Dicers (*dclB*). The range in nucleotide polymorphisms and how they may relate to *dcl* and *ppd* evolution are discussed below.

***A. nidulans dclB* is required for experimental RNA silencing.** With the truncation of DclA, DclB is the only full-length Dicer found in the *A. nidulans* genome. Its deletion indicates that it is essential for IRT-RNA silencing, which is restored when the deletion allele is complemented by an ectopically expressed *gpdA*(p)::*dclB* transgene (Fig. 7D). Northern blotting revealed a single *dclB* transcript that is absent from the Δ*dclB* genetic background and transcribed at higher-than-normal levels when expressed from the ectopic *gpdA*(p)::*dclB* transgene (Fig. 7A).

***A. nidulans* lacking RNA silencing genes and RdRPs are phenotypically normal.** RdRPs have important roles in RNA silencing processes (see above). *A. nidulans* encodes two RdRPs, RrpB and RrpC, which is one less than is typically encoded by a filamentous ascomycete (30). To efficiently identify a role for any of the *A. nidulans* RNA silencing proteins in a physiological process, the deletion alleles for *dclB*, *rsdA*, *rrpB* (30), and *rrpC* (30) were combined into the same genetic back-

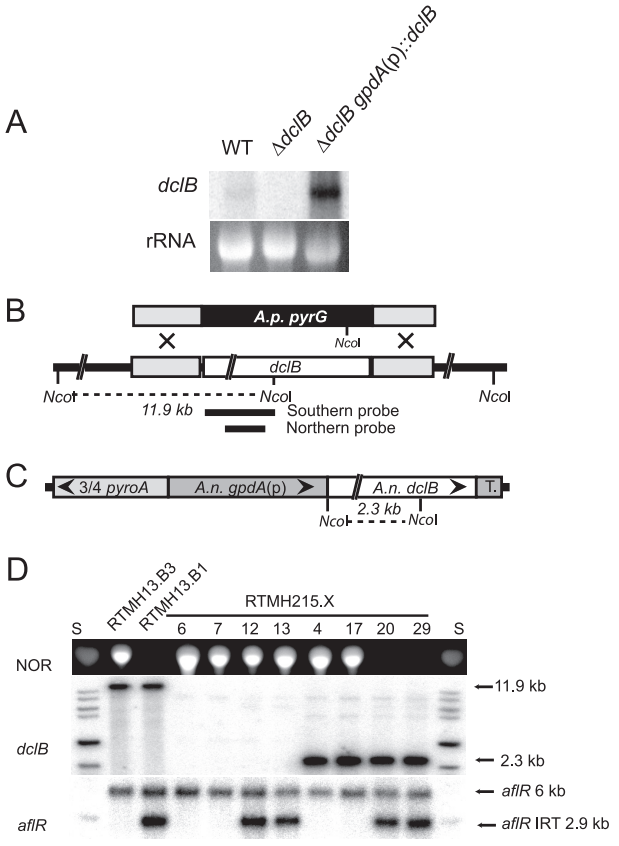

FIG. 7. *A. nidulans dclB* is required for IRT-RNA silencing. (A) Northern blotting identifies *dclB* transcript in total RNA from 48-h cultures. The ethidium bromide-stained 26S rRNA band is shown. Strains: WT, RTMH13.B3; Δ*dclB*, RTMH215.6; Δ*dclB gpdA*(p)::*dclB*, RTMH215.4. (B) Schematic representation of *dclB* replacement with *pyrG*. Gray boxes represent the *dclB* flanking sequences used in the deletion vector. The white box represents the predicted *A. nidulans dclB* ORF. (C) Schematic representation of *dclB* complementation by targeting a *gpdA*::*dclB* construct to the *pyroA* locus. (D) NOR analysis and Southern blotting (HindIII digest for *dclB*, NcoI-HindIII digests for *aflR*) results for control strains and recombinants from a cross between a Δ*dclB* transformant (TTMH158.1) and a transformant carrying the *gpdA*(p)::*dclB* transgene (TTMH160.3). Note that the absence of a band for *dclB* indicates the deletion genotype (see the probe template in panel B) and that the presence of two bands for *aflR* indicates the IRT genotype. Because normal levels of NOR are detected for the Δ*dclB aflR*(IRT) genotype, the data indicate that RNA silencing is dependent on *dclB*. S, NOR standard or 1-kb ladder.

TABLE 3. Growth and spore production

Analysis, expt, and strain[a]	Genotype	Mean diam (mm) ± SD	Mean no. (10^5/mm^2) ± SD	
			Conidia	Ascospores
Radial growth				
Expt 1				
RTMH211.14	WT	58.5 ± 1.1		
RTMH229.24	WT	59.3 ± 0.6		
RTMH218.7	Δ*dclB* Δ*rsdA* Δ*rrpB/C*	59.0 ± 1.0		
RTMH218.39	Δ*dclB* Δ*rsdA* Δ*rrpB/C*	59.1 ± 0.6		
Expt 2				
RTMH211-14	WT	57.0 ± 1.4		
RTMH229-24	WT	57.3 ± 0.5		
RTMH212-42	Δ*dclA*	57.9 ± 0.8		
RTMH212-65	Δ*dclA*	56.4 ± 1.1		
RTMH211-6	Δ*ppdB*	58.6 ± 1.6		
RTMH211-11	Δ*ppdB*	57.8 ± 1.9		
Spore production				
Expt 3				
RTMH211.14	WT		2.17 ± 0.25	1.04 ± 0.15
RTMH218.39	Δ*dclB* Δ*rsdA* Δ*rrpB/C*		1.86 ± 0.29	0.92 ± 0.19
RTMH218.7	Δ*dclB* Δ*rsdA* Δ*rrpB/C*		2.06 ± 0.14	0.83 ± 0.18
RDIT9.32	WT		2.19 ± 0.22	0.70 ± 0.15
Expt 4				
RTMH211-14	WT		4.90 ± 0.32	0.11 ± 0.05
RTMH211-6	Δ*ppdB*		5.12 ± 0.35	0.10 ± 0.04
RTMH212-65	Δ*dclA*		5.78 ± 0.49	0.05 ± 0.01
Expt 5				
RTMH229-24	WT		4.33 ± 0.52	0.23 ± 0.08
RTMH212-65	Δ*dclA*		4.26 ± 0.49	0.21 ± 0.04

[a] Radial growth rate was determined by placing 2 μl of a conidial suspension (100 conidia per μl) in the center of a solid plate of 25.0 ml of GMM, followed by incubation for 5 days at 37°C. Experiment 1, $n = 9$ to 10; experiment 2, $n = 6$. Spore production was determined in 6-day-old cultures (37°C, 12 h light). Replicates were prepared by mixing 10^6 conidia in 5.0 ml of liquid molten GMM agar (0.7%, ~48°C), followed by plating onto 30.0 ml (experiment 3) or 25.0 ml (experiments 4 and 5) solid GMM. Three 1.4-cm-diameter cores were harvested from each plate and ground in 0.01% Tween 80. Dilutions were then made for hemacytometer-based spore counting. Experiment 3, $n = 3$; experiment 4, $n = 4$ to 5; experiment 5, $n = 5$. The only difference detected in these experiments was in experiment 4, where Δ*dclA* produced more conidia and fewer ascospores than the wild-type (WT) and Δ*ppdB* mutant strains. However, these results were not consistent with a subsequent experiment (experiment 5), suggesting that experimental error or an unknown genetic difference was a factor in the results for experiment 4.

ground (referred to as ΔRNAi). Measurements of radial growth and spore production revealed no significant differences between *A. nidulans* wild-type strains and strains lacking the group Q RNA silencing genes and the RdRPs (Table 3 and the supplemental material). This demonstrates that RNA silencing genes are not essential for normal growth, conidiation, or ascosporogenesis.

At least some *N. crassa* RNA silencing genes are required for the sexual cycle (1, 40, 63), but the fact that *A. nidulans* RNA silencing mutants produce wild-type levels of ascospores (Table 3) indicates that RNA silencing genes are not required for *A. nidulans* meiosis. Furthermore, an experiment specifically designed to identify mating deficiencies revealed no differences in homozygous wild-type or ΔRNAi crosses, although a statistical favoring of ΔRNAi selfing was seen in the heterozygous wild-type-to-ΔRNAi crosses (Table 4). Overall, these data demonstrate that RNA silencing genes are not a fundamental requirement of mating or meiosis in ascomycete fungi.

DISCUSSION

The evolutionary forces driving eukaryotic RNA silencing gene change are unclear. The data presented here indicate that *Aspergillus* fungi are well suited for elucidating these forces. The model fungus *A. nidulans* is especially useful since it has recently experienced RNA silencing gene truncation in two widely conserved genes: *dclA*, a Dicer encoding gene, and *ppdB*, an Argonaute encoding gene. These two genes are predicted to be part of the same paralogous RNA silencing gene group (M) commonly found in filamentous ascomycetes. Although the truncated genes are transcribed, they are not required for experimental RNA silencing, growth, or developmental processes (at least under standard culture conditions). Thus, the affect of *dclA* and *ppdB* truncation on *A. nidulans* biology is unknown. However, their truncation did leave *A. nidulans* with only a single Dicer and a single Argonaute to mediate all RNA silencing based processes. These are *dclB* and *rsdA*, members of the other paralogous RNA silencing gene group (Q) commonly found in filamentous ascomycetes. These proteins are required for experimental RNA silencing but are

TABLE 4. Crossing assay[a]

Cross	Genotypes	% Selfed cleistothecia ± SD	% Crossed cleistothecia ± SD
Expt 1			
A	ΔRNAi(y) × ΔRNAi(w)	52.8 ± 9.7 (y), 13.8 ± 6.3 (w), 66.7 ± 8.3 (t)	33.3 ± 8.3
B	ΔRNAi(y) × WT(w)	67.5 ± 3.5 (y), 25.8 ± 1.1 (w), 93.3 ± 4.7 (t)	6.7 ± 4.7
C	WT(y) × ΔRNAi(w)	26.6 ± 7.0 (y), 67.5 ± 8.2 (w), 94.2 ± 1.2 (t)	5.9 ± 1.2
D	WT(y) × WT(w)	33.9 ± 3.5 (y), 31.7 ± 6.7 (w), 65.6 ± 3.5 (t)	34.4 ± 3.4
Expt 2			
E	ΔRNAi(y) × ΔRNAi(w)	18.8 ± 2.5 (y), 46.1 ± 3.5 (w), 65.0 ± 3.3 (t)	35.0 ± 3.3
F	ΔRNAi(y) × WT(w)	72.5 ± 1.2 (y), 22.5 ± 3.5 (w), 95.0 ± 2.4 (t)	4.2 ± 3.5
G	WT(y) × ΔRNAi(w)	35.0 ± 4.7 (y), 60.0 ± 9.4 (w), 95.0 ± 4.7 (t)	5.0 ± 4.7
H	WT(y) × WT(w)	32.8 ± 6.3 (y), 30.6 ± 1.0 (w), 63.3 ± 7.3 (t)	36.7 ± 7.3

[a] Wild-type (WT) strains are wild type at all loci except *yA* or *wA*. ΔRNAi strains are quadruple knockouts (Δ*dclB*, Δ*rsdA*, Δ*rrp, and* Δ*rrpC*). "(y)" refers to yellow-conidia-progeny-producing cleistothecia, "(w)" refers to white-conidia-progeny-producing cleistothecia, and "(t)" refers to the total number of selfed cleistothecia. Sixty cleistothecia were scored for each replica plate, and three replica plates were analyzed for crosses A, D, E, and H, while two replica plates were analyzed for crosses B, C, F, and G. Experiment 1 strains: ΔRNAi (y), RTMH227.29; ΔRNAi (w), RTMH227.67; WT (y), RTMH229.37; WT (w), RTMH229-19. Experiment 2 strains: ΔRNAi (y), RTMH227.47; ΔRNAi (w), RTMH227.68; WT (y), RTMH229-25; WT (w), RTMH233.C.

not required for normal growth and development (at least under standard culture conditions). This latter finding contrasts significantly with a requirement for functional RNA silencing in growth and/or development in higher eukaryotes and hints that an elaborate miRNA-like gene-regulation mechanism does not exist in *A. nidulans*. This hypothesis is consistent with the current state of fungal RNA silencing research, that is, miRNA has not yet been discovered in the fungal kingdom.

The population analysis performed in the present study suggests that the *A. nidulans dclA* and *ppdB* truncations are fixed at the species level. A caveat to this finding is that all of the analyzed *A. nidulans* isolates originated in the United Kingdom. Thus, the status of *dclA* and *ppdB* in *A. nidulans* isolates from other parts of the world is unknown. Regardless, it is still surprising that the *dclA* and *ppdB* truncations were selected for in the United Kingdom isolates, given that *dclA* and *ppdB* orthologs are conserved in all other filamentous-ascomycete genomes investigated. One of several simple hypotheses to account for this phenomenon is that the gene truncation events somehow correlate—or previously correlated—with an increase in fitness. The data presented here suggest that such a hypothetical fitness gain does not influence RNA silencing, growth, or development. Another hypothesis is that group M RNA silencing has lost relevance in *A. nidulans*, and thus the truncations represent the natural degeneration of defunct genes.

Our data indicate that the *A. nidulans dclA* and *ppdB* truncations must have occurred sometime after divergence from an ancestor shared with its close relative *A. rugulosus*. Although a theoretical divergence date for these species has not been determined, it appears to be recent since there is a high level of nucleotide identity shared between some *A. nidulans* and *A. rugulosus* loci. For example, their ITS sequences are nearly identical (Table 2), suggesting that they could even be classified as the same species. The fact that *A. nidulans* and *A. rugulosus* both encode a degenerate *rrpA* locus indicates that this locus can be used to estimate the amount of polymorphism that occurs in loci experiencing little or no selection. A relatively high level of polymorphism is found at this locus (15.0 to 15.6%) and at another probable low-selection locus, the 5′ *ppdB*-intergenic region (16.6 to 17.6%). In comparison, *dclA* and *ppdB* loci have lower levels of polymorphism, with *ppdB* having less than *dclA*. A similar analysis for the group Q RNA silencing proteins indicates that Dicers are not necessarily more likely than Argonautes to acquire polymorphic residues; thus, the lower polymorphism level of *ppdB* may be an important clue toward understanding the order of the truncation events. A simple explanation is that *A. nidulans dclA* was truncated before *ppdB* and has thus had more time to accumulate mutations. However, this would suggest that selection to maintain *ppdB* was not lost with *dclA* truncation, possibly because its product had a function that was independent of DclA.

The consequences of group M Dicer and Argonaute gene truncation on *A. nidulans* biology are unknown. If *Aspergillus dclA* and *ppdB* control repetitive DNA, one might predict that the *A. nidulans dclA* and *ppdB* truncations would result in its increase. However, recent estimates indicate that levels of repetitive DNA are not very different between *A. nidulans* and *A. fumigatus*, with each species predicted to contain 3.0 and 2.9%, respectively (24). Despite this similarity, it seems possible that the *dclA* and *ppdB* truncations could have removed some restraints on repetitive DNA in *A. nidulans*, perhaps leading to increased mobility and a higher turnover rate, if not a detectable increase in overall amount.

It is perhaps pertinent to note the correlation between group M RNA silencing gene truncations/disruptions and repetitive DNA. We have found such elements 3′ of *dclA* (Fig. 5B), 5′ of *ppdB* (Fig. 6B), and in the middle of the *A. rugulosus* 211 *dclA* helicase domain (Fig. 5C). In addition, a polynucleotide repeat appears to have contributed to *A. nidulans ppdB* truncation (inferred from the position of this repeat in *A. rugulosus*, Fig. 6D). It is unclear whether these findings are simply the result of the natural degeneration of defunct genes, or whether they are related to the antagonistic relationship between RNA silencing and selfish genetic elements. If the former is correct, it is at least ironic that the elements which RNA silencing genes are thought to control are contributing to RNA silencing gene demise in *A. nidulans*.

With respect to its intact RNA silencing machinery, *A. nidulans* does not require *dclB*, *rsdA*, *rrpB*, or *rrpC* for growth and developmental processes under standard culture conditions (nonstandard conditions have not been thoroughly investigated). This is somewhat consistent with the slight nature of the phenotypic abnormalities reported for RNA silencing mutants in other filamentous fungi (*M. oryzae* and *M. circinelloides*) (35, 49) but contrasts significantly with the requirement for some RNA silencing genes for sexual reproduction in *N. crassa* (1, 40, 63).

A peculiar finding concerning *A. nidulans* development came from the *A. nidulans* crossing assays. In heterozygous crosses between wild-type and ΔRNAi, the level of crossed cleistothecia was significantly lower than in homozygous crosses (Table 4). The fact that crossed-cleistothecium levels dropped only in the heterozygous crosses suggests that absence of RNA silencing in one parent is not the basis of the phenomenon. A possibility that has not yet been investigated is that the *pyrG* (2 to 3) and *metG* (0 to 1) selectable markers in the ΔRNAi genotype (due to the gene replacements) are a contributing factor. Most importantly, however, the results of these experiments clearly demonstrate that RNA silencing genes are not required for meiosis or crossing in *A. nidulans*.

The lack of a detected role for the *A. nidulans* group Q Dicer and Argonaute and the *A. nidulans* RdRPs in growth and development suggests that they may not be involved in "housekeeping" functions (as defined by reference 51) but may be used for an auxiliary process such as defense against viruses. Accordingly, a companion study reports that *Aspergillus* viruses are targets and suppressors of *A. nidulans* RNA silencing (28a). Other possible roles for the intact *A. nidulans* RNA silencing machinery include transposon control (as has been demonstrated for *N. crassa* [50]) and chromatin regulation (as has been demonstrated for *S. pombe* [for a review, see reference 44]). Future experiments will examine these specific possibilities.

For fungi in general, RNA silencing gene evolution appears to be more complex than in any other type of eukaryote, and the continued study of *Aspergillus* species should help elucidate its driving forces. While the *A. nidulans dclA* and *ppdB* truncations were characterized here, the same genes have been

conserved or duplicated by other aspergilli. These species are thus prime candidates for comparative studies that should shed light on the evolutionary significance of RNA silencing gene gain and loss in eukaryotes.

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