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A Double-Stranded-RNA Response Program Important for RNA Interference Efficiency▽

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When recognized by the RNA interference (RNAi) pathway, double-stranded RNA (dsRNA) produced in eukaryotic cells results in posttranscriptional gene silencing. In addition, dsRNA can trigger the interferon response as part of the immune response in vertebrates. In this study, we show that dsRNA, but not short interfering RNA (siRNA), induces the expression of *qde-2* (an Argonaute gene) and *dcl-2* (a Dicer gene), two central components of the RNAi pathway in the filamentous fungus *Neurospora crassa*. The induction of QDE-2 by dsRNA is required for normal gene silencing, indicating that this is a regulatory mechanism that allows the optimal function of the RNAi pathway. In addition, we demonstrate that Dicer proteins (DCLs) regulate QDE-2 posttranscriptionally, suggesting a role for DCLs or siRNA in QDE-2 accumulation. Finally, a genome-wide search revealed that additional RNAi components and homologs of antiviral and interferon-stimulated genes are also dsRNA-activated genes in *Neurospora*. Together, our results suggest that the activation of the RNAi components is part of a broad ancient host defense response against viral and transposon infections.

The production of double-stranded RNA (dsRNA) in eukaryotic cells, generally as the result of viral replication or the transcription of transposable elements and repetitive DNA sequences, is known to elicit two types of cellular defense responses. In the first type of response, dsRNA is recognized and cleaved by the RNase III enzyme Dicer to yield 20- to 25-nucleotide short interfering RNA (siRNA) duplexes (5, 38). The production of siRNA initiates RNA interference (RNAi), also known as posttranscriptional gene silencing. The siRNAs are loaded onto the RNA-induced silencing complex (RISC), of which an Argonaute (Ago) family protein forms the catalytic core, and guide the RISC to recognize and cleave homologous mRNA or result in the transcriptional silencing of the homologous DNA locus (31, 43–45, 49, 53). Dicer and the Argonaute proteins are the central components of the RNAi pathway.

The RNAi pathway plays important roles in silencing transposon and viruses in animal and plant cells and is thought to be a defense system against viruses and transposons (20, 30, 33, 46, 56). Components of the RNAi pathway probably cleave viral dsRNA and degrade viral and transposon mRNAs. Consistent with this notion, many viral genomes are known to encode inhibitors of the RNAi pathway (6, 20, 34).

In vertebrates, dsRNA and siRNA are also known to trigger the transcription-based antiviral interferon (IFN) response (24, 27, 47). In mammalian cells, dsRNA is recognized by dsRNA sensors such as Toll-like receptor 3 and the dsRNA-dependent protein kinase R (PKR). This leads to the activation of the IFN-regulatory transcription factors and NF-κB, which in turn results in the expression of the IFNs. The expression of IFNs then activates the transcription of hundreds of IFN-stimulated genes (ISGs) through the JAK-STAT pathway (19). Many of the ISGs encode proteins with antiviral activities, including PKR and myxovirus (influenza virus) resistance (Mx) proteins (23, 24, 40, 41). Thus, the IFN response plays a crucial role in antiviral immunity in vertebrates.

Although the basic mechanism of RNAi is now fairly well understood, little is known regarding the regulation of the RNAi components. It is also not known whether there is cross talk between the transcription-based antiviral response and the posttranscription-based RNAi pathway. In addition, although the dsRNA-induced IFN response has been well characterized, it has been documented only in vertebrate systems, and the evolutionary root of the response is not known.

The filamentous fungus *Neurospora crassa* was one of the first model systems used for RNAi studies (15, 16); in *Neurospora*, the RNAi pathway is essential for gene silencing (also called quelling) induced by dsRNA or transgenes (9). Genetic analysis led to the identification of three quelling-deficient genes (*qde*'s): *qde-1*, *qde-2*, and *qde-3*. QDE-1 (an RNA-dependent RNA polymerase) and QDE-3 (a DNA helicase) are thought to function upstream of the RNAi pathway and are involved in the generation of dsRNA from aberrant RNAs (15, 17). Consistent with this notion, QDE-1 and QDE-3 are not required for the production of siRNA and gene silencing if dsRNA is made from an exogenous hairpin RNA (10). QDE-2 is an Argonaute protein and is the core of the RISC complex associated with siRNA (7, 8, 35). In addition, *Neurospora* has two partially redundant Dicer proteins, DCL-1 and DCL-2; DCL-2 is responsible for most of the siRNA-generating activ-

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ity (10). The disruption of both *dcl* genes in *Neurospora* abolished all siRNA production in vivo and in vitro. As in animals, the RNAi pathway has been shown to be important for transposon silencing in *Neurospora* (39).

To understand the regulation of the RNAi pathway, we examined the responses of the RNAi components after the induction of dsRNA. dsRNA induced the transcription of both *qde-2* and *dcl-2*, two of the central components of the RNAi pathway. In addition, the accumulation of the QDE-2 protein after the induction of dsRNA required the presence of the DCLs, suggesting that either the DCLs or the produced siRNA regulates the steady-state level of QDE-2 posttranscriptionally. Importantly, we showed that the induction of QDE-2 by dsRNA is required for efficient RNAi. Finally, using microarray and quantitative PCR (qPCR) analyses, we identified 60 dsRNA-activated genes (DRAGs) in *Neurospora.* Functional classification of the DRAGs suggests that the induction of RNAi components is a part of a broad host defense response against viral infection and transposons in this filamentous fungus.

MATERIALS AND METHODS

Strains and growth conditions. The wild-type strain used in this study was either FGSC987 (*A*; obtained from Fungal Genetic Stock Center) or 87-3 (*bd a*). Either FGSC462 (*his-3 A*) or 301-6 (*bd his-3 A*) was the host strain used for the insertion of the *his-3*-targeting constructs. The following mutant strains (in the wild-type or *his-3* background) were created for this study as described below: *dcl-1*^{ko}, *dcl-2*^{RIP}, *dcl-1*^{ko} *dcl-2*^{RIP}, *qde1*^{ko}, *qde2*^{RIP} and *qde3*^{ko}. The mutants in the *his-3* background were used as the host strains for *his-3*-targeting constructs. Culture conditions were the same as those described previously (1), by inoculating mycelium mats in shaking flasks with 50 ml medium. For liquid cultures containing quinic acid (QA), 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1× Vogel's medium, 0.1% glucose, and 0.17% arginine (13). The cultures were grown in the presence of QA for 2 days unless otherwise indicated. For liquid cultures containing histidine, a final concentration of 0.5 mg/ml was used.

Creation of mutant strains. The *Neurospora qde-2* and *dcl-2* genes were disrupted by a repeat-induced point mutation (RIP) (4). The PCR fragment containing the entire *qde-2* or *dcl-2* open reading frame (ORF) and its 3′ untranslated region (3.3 kb for *qde-2* and 5.1 kb for *dcl-2*) was cloned into pDE3BH and introduced into the *his-3* locus of a wild-type strain (7088 *his-3 a*) by electroporation (36). A positive transformant was crossed with a wild-type strain. DNA sequencing was performed to identify the strains in which the endogenous *qde-2*/*dcl-2* ORF was mutated with multiple premature stop codons.

The gene replacement method was used to disrupt the *Neurospora qde-1*, *qde-3*, and *dcl-1* genes. A PCR fragment containing the entire ORF and 3′ untranslated region of the gene (*qde-1*/*qde-3*/*dcl-1*) was cloned into pDE3BH, resulting in pQDE-1/pQDE-3/pDCL-1.

To make the disruption construct, a hygromycin resistance gene (*hph*) fragment containing promoter and terminator sequences was inserted into the XbaI-PvuII site of pQDE-1, the PvuII site of pQDE-3, or the BamHI site of pDCL-1. A PCR fragment containing the gene replacement cassette was introduced into a wild-type strain by electroporation (36) to select for hygromycin-resistant transformants (200 μg/ml hygromycin). PCR was performed to identify strains carrying the *hph* fragment at the endogenous locus. Positive transformants were crossed with a wild-type strain, and sexual spores were picked individually and germinated on slants containing hygromycin. Southern blot analysis was performed to confirm the *qde-1*^{ko}, *qde-3*^{ko}, and *dcl-1*^{ko} strains. A *dcl-1*^{ko} strain was crossed with a *dcl-2*^{rip} strain to generate a *dcl-1*^{ko} *dcl-2*^{rip} double mutant. All mutants were crossed with a *his-3* strain to obtain mutants in the *his-3* background; these mutants were used for all *his-3*-targeting transformations.

Creation of dsRNA strains. dsRNA constructs were created as described previously (11). The following regions from *al-1*, *frq*, and *frh* were cloned into pDE3BH.qa in reverse and forward orientations, respectively: *al-1*, bp 1322 to 1942 and bp 1412 to 1942; *frq*, bp 669 to 2309 and bp 791 to 1252; and *frh*, bp 2087 to 2703 and bp 2189 to 2703. The first nucleotide of each ORF is counted as 1. The resulting plasmids that contain the inverted repeats under the control of the *qa-2* promoter were targeted to the *his-3* locus of a wild-type strain (301-6 *bd his-3 A*) and other *his-3* RNAi mutant strains by transformation (36). The construct that contains the wild-type *qde-2* gene with its own promoter was created as described previously (35). To create plasmids used for *qde-2* promoter analyses, PCR fragments of *qde-2* were inserted into the NdeI site of the double-stranded *al-1* (ds*al-1*) construct. Primer sequences are available upon request.

Antibody generation. A glutathione *S*-transferase–QDE-2 (containing QDE-2 amino acids 541 to 938) fusion protein was expressed in BL21 cells, and inclusion bodies containing the recombinant proteins were purified and used as the antigen to generate rabbit polyclonal antiserum as described previously (12).

Dicer-2-specific antibody was generated by using a DCL-2-specific peptide (DRDDSSQDPDDNESF) synthesized by the peptide synthesis facility at the University of Texas Southwestern Medical System. The polyclonal antiserum from rabbit was purified using a DCL-2 peptide-conjugated affinity column.

Quelling assay. The quelling assay was performed in a wild-type strain by cotransforming a 1.5-kb PCR fragment of the *al-1* ORF and pBT6 (a benomyl-resistant gene-containing plasmid [obtained from the Fungal Genetic Stock Center {FGSC}]). Benomyl-resistant transformants were picked to identify yellow (partially quelled) or white (fully quelled) strains. The two primers used to generate the *al-1* PCR fragment were al-1-1for (5′-CTTCCGCCGCTACCTCT CGTGG-3′) and al-1-2 rev (5′-CCCTTTGTTGGTGGCGTTGATG-3′). The experiments were performed in constant white luminescence light at room temperature.

Protein and RNA analyses. Protein extraction, quantification, and Western blot analysis were performed as previously described (12). Equal amounts of total protein (50 μg) were loaded into each protein lane, and after electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane, and Western blot analysis was performed. An amido black-stained membrane or nonspecific cross-reacting bands were used as loading controls.

RNA extraction and Northern blot analyses were performed as described previously (1). Equal amounts of total RNA (30 μg) were loaded onto agarose gels for electrophoresis, and the gels were blotted and probed with an RNA probe specific for *qde-2* or *dcl-2*. The rRNA levels were shown by the ethidium bromide-stained agarose gels.

Microarray analysis. *Neurospora* oligonucleotide microarray chips were obtained from the FGSC. The microarray (created by the *Neurospora* Genome Project) consists of 10,526 predicted ORFs, which covers nearly all predicted genes in *Neurospora*. The chips were postprocessed and UV cross-linked as suggested by the manufacturer (GAPS II coated slides; Corning). Microarray experiments, including aminoallyl cDNA synthesis, CyDye conjugation, and array hybridization, were performed using Pronto Plus Indirect systems (Promega). Cy5 and Cy3 (CyDye; Amersham Biosciences) were used to label experimental and control cDNAs, respectively. Microarray spot analysis and acquisition were performed using a GenePix4000B scanner with GenePix6 software (Axon Instruments). GeneTraffic software (Stratagene) was used for normalization and further data analysis.

qRT-PCR. Quantitative real-time PCR (qRT-PCR) was performed with an Applied Biosystems Prism 7900HT sequence detection system using a previously described protocol (29). Briefly, total RNA was purified using an RNeasy mini kit (QIAGEN) and treated with DNase I (0.6 units; Roche). Equal amounts of DNase-treated RNAs (2 μg) were reverse transcribed with SuperScript II (Invitrogen) using random hexamers. cDNAs (50 ng) were mixed in 10 μl qRT-PCR mix with 5 μl SYBR Green PCR Master Mix (Applied Biosystems) and 150 nM primers. Each reaction was duplicated, and non-reverse-transcribed samples were used as controls. Gene-specific primers were designed using Primer Express software (Perkin-Elmer Life Sciences), and each primer pair was validated by cDNA template titration to ensure similar amplification kinetics and a single melting point of quantitative PCR products. Primer sequences are available upon request. Levels of the housekeeping gene beta-tubulin were used to calculate changes (*n*-fold) by comparing mean threshold cycle values.

RESULTS

Induction of *qde-2* expression by dsRNA. Since QDE-2, the Argonaute-like protein, is a core component of the *Neurospora* RNAi pathway, we hypothesized that its expression should be highly regulated. To test this possibility, we examined QDE-2 protein levels using a QDE-2-specific antibody in *Neurospora* RNAi mutants in which one or more of the RNAi genes were disrupted. As shown in Fig. 1A, levels of QDE-2 were reduced in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant and in the *qde-1*^{ko} and

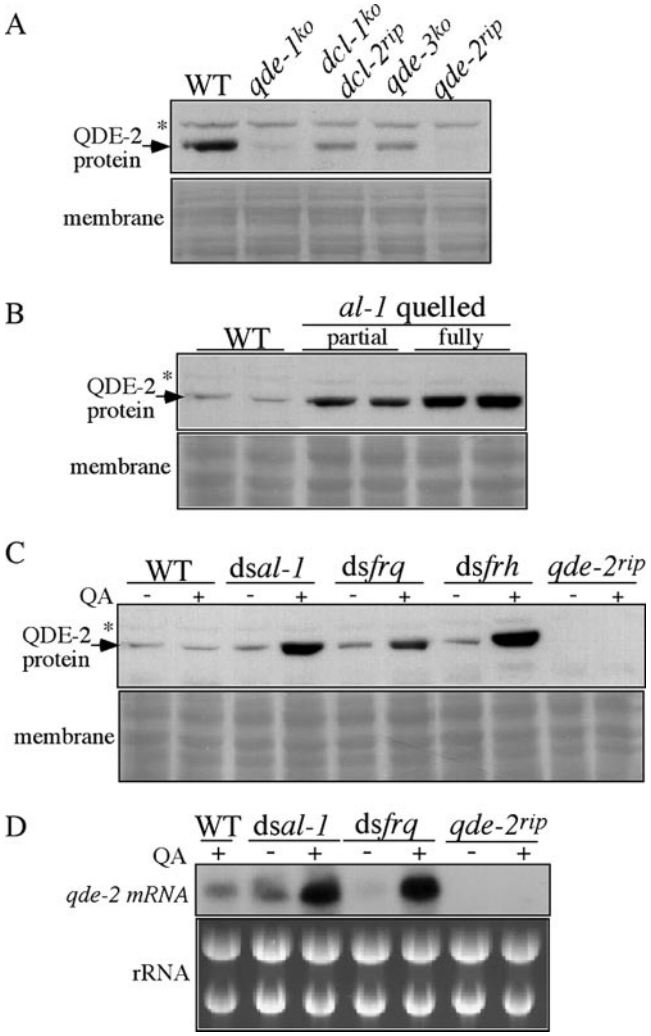

FIG. 1. Induction of *qde-2* expression by dsRNA expression. (A) Western blot analysis showing that the levels of QDE-2 are low in the RNAi mutants. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody. Equal protein loading of the gel was ensured by the amido black-stained membrane shown below the blot. Liquid cultures were grown for 2 days before harvesting. (B) Western blot analysis showing the levels of QDE-2 in the wild-type (WT) and *al-1*-quelled strains. (C and D) Western blot (C) and Northern blot (D) analyses showing the induction of the QDE-2 protein and *qde-2* mRNA, respectively, in strains with dsRNA constructs. The liquid cultures were grown for 2 days (with/without QA) before harvesting.

qde-3^{ko} single mutants compared to those in the wild-type strain. No QDE-2 protein was detected in the *qde-2*^{rip} strain, indicating the specificity of our QDE-2 antibody. These data indicate that the expression of QDE-2 is regulated by other components of the RNAi pathway. Since QDE-1 and QDE-3 are involved in the synthesis of endogenous dsRNA from aberrant RNAs, and DCLs are responsible for the production of siRNA, this suggests that the production of dsRNA or siRNA promotes the expression of QDE-2 proteins.

We then examined the QDE-2 levels in *albino-1* (*al-1*, involved in carotenoid biosynthesis)-quelled strains. In these strains, *al-1* was silenced by the introduction of multiple copies of an *al-1* DNA fragment, resulting in yellow (partially quelled) or white (fully quelled) conidia and hyphae (data not shown). Since quelling involves the production of dsRNA, the degree of *al-1* silencing may reflect the amounts of dsRNA produced. As shown in Fig. 1B, compared with levels observed in the wild-type strain (orange conidia), QDE-2 levels were increased in two partially quelled strains and further increased in two fully quelled strains, suggesting that the production of dsRNA in these strains leads to the increase in QDE-2 expression.

To directly investigate the effect of dsRNA on QDE-2, we examined whether the induction of dsRNA alone was sufficient for the increase in QDE-2 expression. Previously, we and others developed a method to inducibly express dsRNA from inverted repeat sequences to silence gene expression in *Neurospora* (11, 22). The expression of the inverted repeats of the gene of interest are controlled by the QA-inducible (*qa-2*) promoter (21), so that the addition of QA to the medium will lead to the production of dsRNA and initiate gene silencing. The expression of dsRNA leads to the production of homologous siRNA in the wild-type strain but not in the *dcl* double mutant (10, 35). QDE-2 levels were examined in wild-type and *qde-2*^{rip} strains and in three wild-type transgenic strains, ds*al-1*, ds*frq*, and ds*frh*, which carry constructs to induce dsRNA specific for *al-1*, *frequency* (*frq*, a circadian clock gene), and *frh* (an essential RNA helicase gene required for circadian clock function), respectively. As shown in Fig. 1C, the presence of QA led to a significant increase in QDE-2 levels in all three strains with the dsRNA constructs. In contrast, QA had no effect on QDE-2 in the wild-type strain. Since these strains produce different dsRNAs, the effect on QDE-2 is dependent on the production of dsRNA rather than gene-specific dsRNA. In addition, we found that the QDE-2 levels in strains with a mutated *al-1* gene were comparable to that of the wild-type strain, indicating that carotenoid biosynthesis is not involved in the regulation of QDE-2 expression (data not shown).

To determine whether the increase of QDE-2 expression by dsRNA is regulated at the transcriptional level, Northern blot analysis was performed. As shown in Fig. 1D, the addition of QA led to a significant increase in *qde-2* mRNA levels in strains containing the dsRNA constructs, indicating that dsRNA increases QDE-2 expression at the transcriptional level. Although the *qa-2* promoter is tightly regulated by the presence of QA, it is not a very strong promoter (13). The dramatic inductions of *qde-2* mRNA and QDE-2 protein in the presence of QA in the dsRNA construct-containing strains suggest that the amount of dsRNA produced endogenously is low.

dsRNA, not siRNA, induces QDE-2 expression, and DCLs are required for the maintenance of QDE-2 levels posttranscriptionally. The low levels of QDE-2 in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant (Fig. 1A) suggest that DCLs are required for the maintenance of steady-state levels of QDE-2. Further supporting this notion, we found that the induction of dsRNA in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant failed to increase the level of QDE-2 (Fig. 2A). In contrast, QDE-1 was not required for the induction of QDE-2 by dsRNA. It has been previously shown that QDE-1 is not required for gene silencing when dsRNA is produced from an exogenous hairpin construct (10).

The data presented above suggest that the DCLs and siRNA either regulate QDE-2 posttranscriptionally or are required for

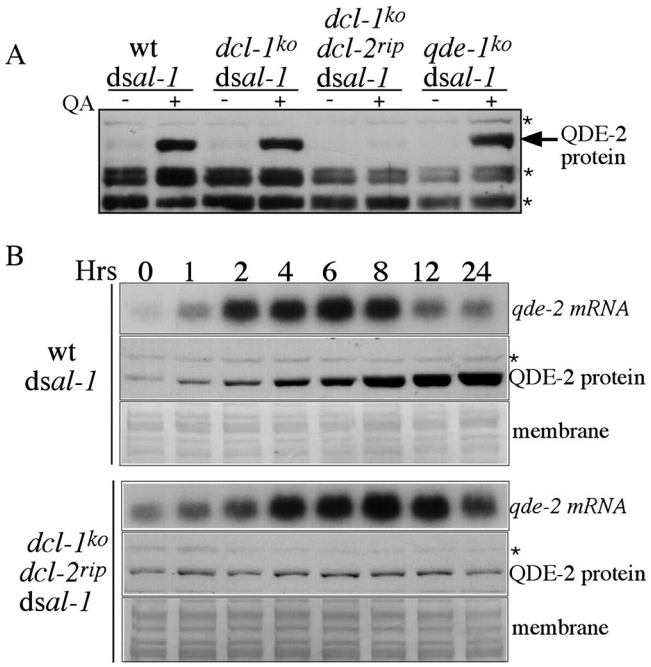

FIG. 2. DCLs posttranscriptionally regulate the steady-state levels of QDE-2, and dsRNA, but not siRNA, is responsible for the transcriptional activation of *qde-2*. (A) Western blot analysis showing that the level of QDE-2 could not be induced by dsRNA in the *dcl* double mutant. The liquid cultures were grown for 2 days (with/without QA) before harvesting. (B) Northern and Western blot analyses showing the induction of *qde-2* expression by dsRNA in the wild-type (wt) and *dcl* double mutant strains. The number of hours indicates the time after the addition of QA. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody.

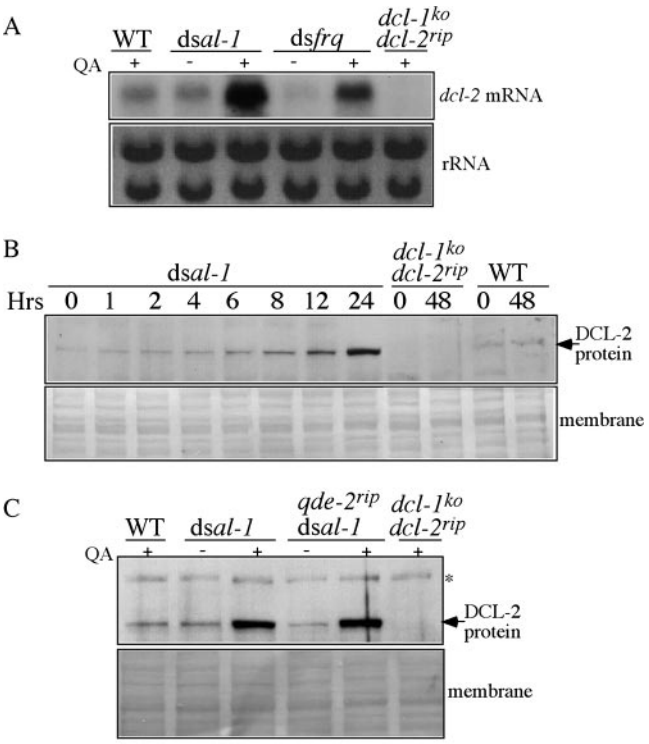

FIG. 3. Induction of *dcl-2* expression by dsRNA. (A) Northern blot analysis showing the induction of *dcl-2* mRNA in strains with dsRNA constructs. (B) Western blot analysis showing the induction of DCL-2 protein by dsRNA. The number of hours indicates the time after the addition of QA. The same membrane used in Fig. 2B was used here. (C) Western blot analysis showing that the induction of DCL-2 by dsRNA is normal in the *qde-2* mutant strain. The asterisk indicates a nonspecific cross-reacting protein band recognized by our DCL-2 antibody. WT, wild type.

the transcriptional activation of *qde-2*. To distinguish between these two possibilities, Northern blot analyses were performed to examine the expression of *qde-2* mRNA after the induction of dsRNA in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant (Fig. 2B). We found that the transcriptional activation of *qde-2* by dsRNA was maintained in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant. In the wild-type strain, *qde-2* mRNA levels were induced after 1 h, peaked 4 to 6 h after the addition of QA, and decreased afterwards. In comparison, *qde-2* was activated to a higher level in the mutant, and high levels of *qde-2* mRNA were maintained 12 h after the addition of QA. In addition, the basal level of *qde-2* was higher in the mutant than in the wild-type strain. Despite the increase in *qde-2* mRNA in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant, QDE-2 levels failed to increase significantly. In contrast, in the wild-type strain, the increase of the QDE-2 protein was seen 1 to 2 h after the QA treatment; QDE-2 levels were maintained at high levels (~10-fold of the basal level) after 8 h. Since DCL-1 and DCL-2 are responsible for all Dicer activity in *Neurospora* (10), these data indicated that dsRNA, but not siRNA, is responsible for the transcriptional activation of *qde-2* expression, and DCLs are not required for dsRNA sensing in the *qde-2* transcriptional activation pathway. However, DCLs are required for QDE-2 accumulation posttranscriptionally, suggesting that siRNA, the cleavage product of dsRNA by DCL, plays a role in the accumulation of the QDE-2 protein.

Induction of DCL-2 by dsRNA. The induction of *qde-2* expression by dsRNA prompted us to examine whether the other key component of the RNAi pathway, DCL-2, is also regulated by dsRNA. Although the functions of DCL-1 and DCL-2 are partially redundant, DCL-2 is responsible for more than 90% of the *Neurospora* Dicer activity (10). As shown in Fig. 3A, *dcl-2* mRNA was strongly induced by the production of dsRNA in two strains with different dsRNA constructs, indicating that dsRNA also transcriptionally activates *dcl-2* expression. Examination of the DCL-2 protein showed that the production of dsRNA also led to a significant increase in DCL-2 levels (Fig. 3B). In contrast, the presence of QA had no effect on *dcl-2* or DCL-2 levels in the wild-type strain. Interestingly, the kinetics of DCL-2 induction by dsRNA were significantly delayed compared to those of QDE-2; DCL-2 levels did not peak until 24 h after the addition of QA. These data suggest that the induction of *dcl-2* by dsRNA is a secondary response rather than an immediate response as presumed for *qde-2*.

To understand whether QDE-2 participates in the signaling pathway that mediates the induction of gene expression by dsRNA, we examined the induction of DCL-2 by dsRNA in a *qde-2* null strain (*qde-2*^{rip}). As shown in Fig. 3C, the induction of DCL-2 by dsRNA was normal in the *qde-2*^{rip} strain. Together with the data presented in the above-described figures,

these data demonstrate that the components of the RNAi pathway are induced by dsRNA but are not required for dsRNA sensing and transcriptional activation in *Neurospora*.

Induction of QDE-2 by dsRNA is required for optimal gene silencing. Since QDE-2 and DCL-2 are two central players in the RNAi pathway, the induction of QDE-2 and DCL-2 by dsRNA suggests that their regulation is important for the efficiency of the pathway. To test this hypothesis, we examined whether the high QDE-2 level induced by dsRNA is required for the efficiency of gene silencing. A construct in which the *qde-2* is under the control of the *qa-2* promoter (qaQDE-2) and a construct containing the wild-type *qde-2* gene with its own promoter were created and transformed into the *qde-2*^{rip} ds*al-1* strain. As shown in Fig. 4A, the wild-type *qde-2* construct complemented the function of the endogenous *qde-2*, and the transformant exhibited dsRNA-induced QDE-2 expression (Fig. 4A) and silencing of the *al-1* gene, as indicated by the white aerial hyphae and conidia in the presence of QA (Fig. 4B). On the other hand, the expression levels of QDE-2 in qaQDE-2 transformants in the presence of QA were comparable to the uninduced level of QDE-2 in the *qde-2*^{rip} ds*al-1* *qde-2* strain. In addition, the *qde-2*^{rip} ds*al-1* qaQDE-2 strains showed only very weak silencing of *al-1* in the presence of QA, indicating a severe compromise of RNAi efficiency. These results suggest that the high levels of QDE-2 induced by dsRNA are required for efficient RNAi and that the induction of QDE-2 expression by dsRNA is mediated by the *qde-2* promoter.

To further confirm this result, we created constructs that contain both the *qde-2* gene with segments of its upstream sequence of different lengths (Fig. 4C) and the ds*al-1* cassette. These constructs were transformed into the *qde-2*^{rip} strain at the *his-3* locus. As shown in Fig. 4D, Pqde-2A, which contains 1.9 kb of *qde-2* upstream sequence, fully complemented the function of the endogenous *qde-2*, as indicated by the induction of QDE-2 and silencing of *al-1* in the presence of QA in the transformants. In contrast, in the Pqde-2B transformants, dsRNA-induced QDE-2 expression was abolished; the QDE-2 levels remained at the basal level in the presence of QA. These data indicate that a *cis* element within the 0.4-kb *qde-2* promoter is required for dsRNA-induced expression. Also, as expected, the aerial hyphae and conidia of the Pqde-2B strains remained orange in the presence of QA, indicating a severe compromise of the RNAi pathway. Together, these data indicate that QDE-2 is a limiting factor in the RNAi pathway and that the induction of QDE-2 by dsRNA at the transcriptional level is critical for RNAi function.

Genome-wide search revealed that additional RNAi components and genes homologous to host defense responses are DRAGs. The observation that *qde-2* and *dcl-2* were induced by dsRNA indicates the existence of a transcription-based dsRNA response program in *Neurospora*. To understand the function of this response, we carried out a genome-wide study to identify other DRAGs in *Neurospora* by microarray and qRT-PCR analyses. To identify genes that are immediately activated by dsRNA, cultures of the ds*al-1* strain were treated with QA for 6 h. The wild-type cultures treated with QA were used as the controls. As shown in Fig. 5A, as expected, *qde-2* mRNA was dramatically induced in the ds*al-1* cultures. In contrast, the qRT-PCR analysis showed that the levels of *alpha-actin* and

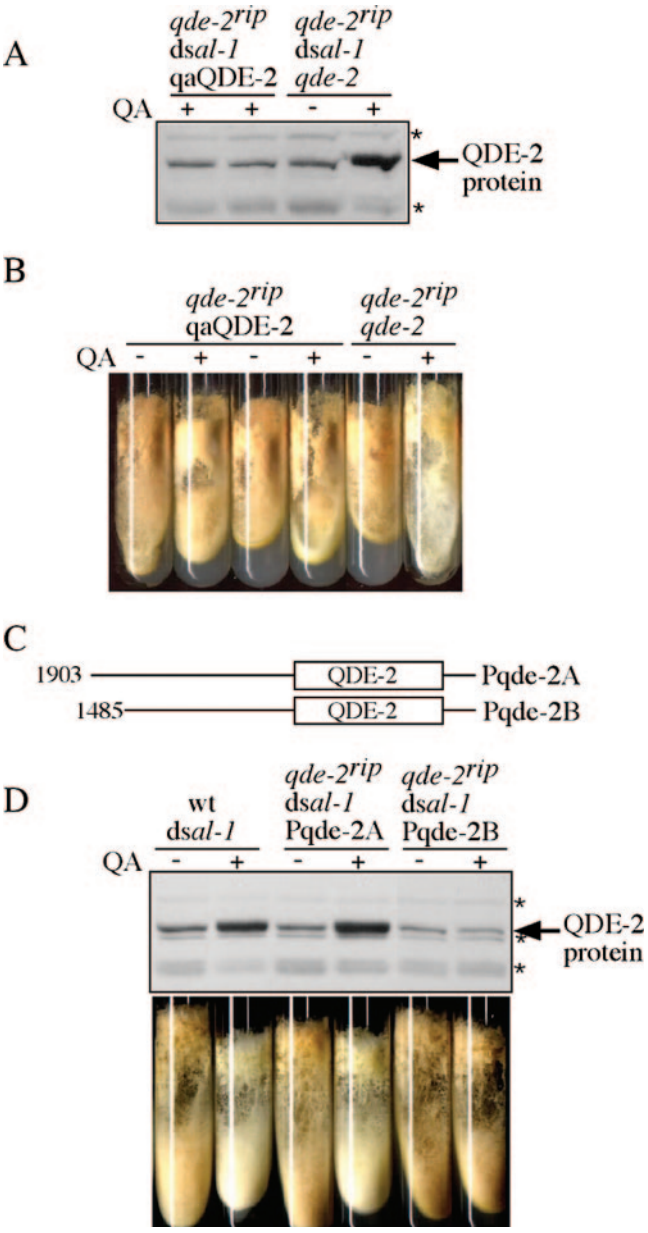

FIG. 4. Induction of QDE-2 by dsRNA is required for efficient RNAi. (A) Western blot analysis showing the expression of QDE-2 in the indicated strains. (B) Photograph of the corresponding strains growing in slants. (C) Graphic depiction of the indicated *qde-2* promoter constructs. (D, top) Western blot analysis showing the expression of QDE-2 in the indicated strains. (Bottom) Photograph of the corresponding strains growing in slants. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody. wt, wild type.

qa-2 (a QA-inducible gene) mRNAs were comparable in both strains (Fig. 5B), indicating that the QA responses were similar in both strains. Thus, activation of genes in the ds*al-1* strain should be due to the production of dsRNA. In addition to the microarray experiments, genes known to be involved in the RNAi machinery and some genes with low signal levels in the microarray experiments were analyzed by qRT-PCR.

The microarray experiments showed that the expression of

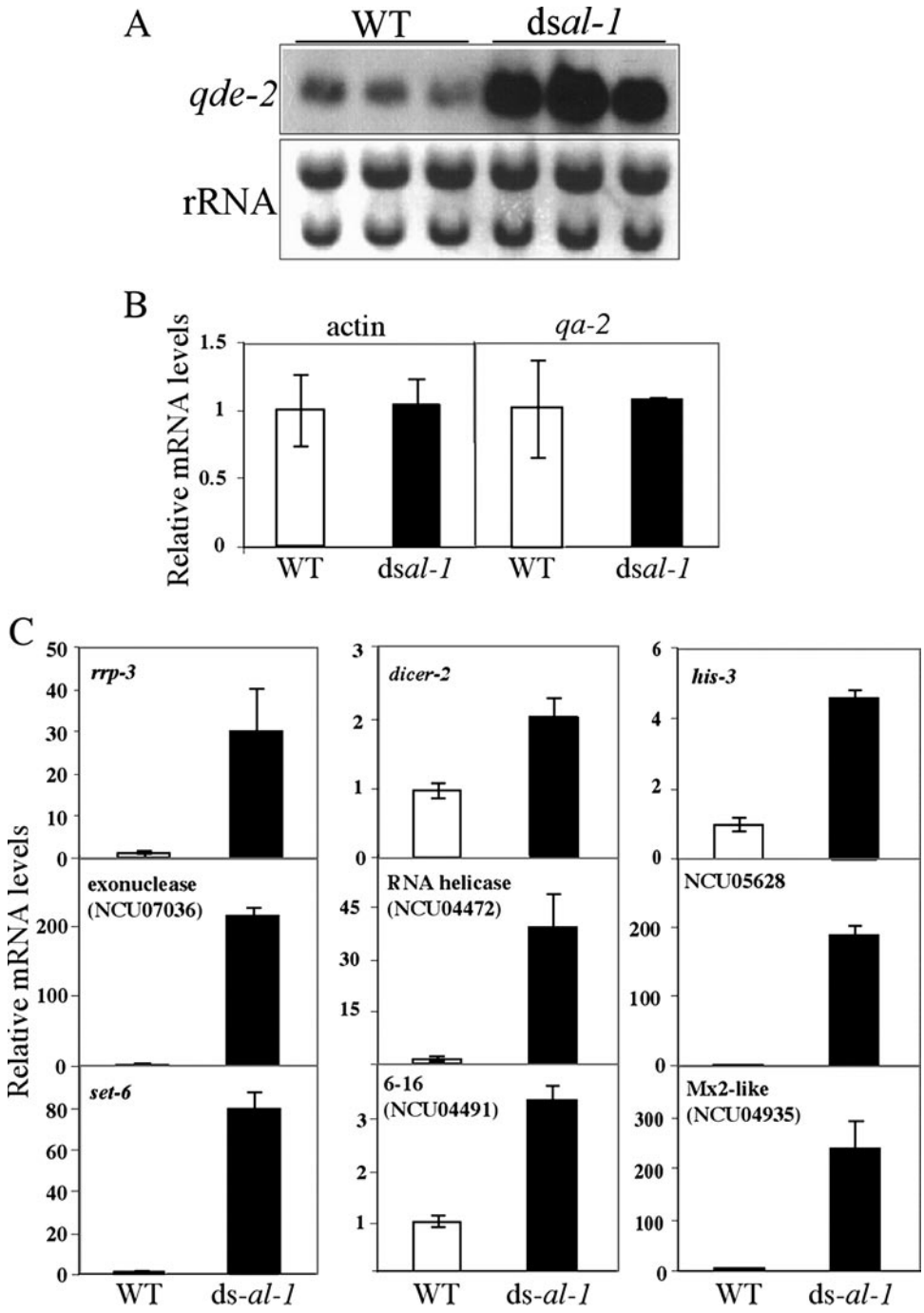

FIG. 5. Genome-wide identification of DRAGs. QA was added to wild-type (WT) and ds*al-1* triplicate cultures for 6 h before their harvest. (A) Northern blot analysis of *qde-2* for samples used in the microarray experiments. (B and C) qRT-PCR analysis of gene expression.

the vast majority of *Neurospora* genes is not activated by dsRNA. To generate the list of genes induced by dsRNA, only genes that exhibited an average of more than a 1.5-fold increase in the ds*al-1* groups and also showed a >1.3-fold increase in all triplicate samples were included. In addition, genes with a weak signal (the signal values are less than 1.5-fold of the background value) were excluded.

To confirm the microarray results, more than half of the identified DRAGs were examined by qRT-PCR analysis, which is a more sensitive and quantitative method than the microarray assay (29). In addition to the genes identified by microarray experiments, genes known to be involved in the RNAi machinery and some genes with low signal levels in the microarray experiments were also analyzed by qRT-PCR. qRT-PCR results showed that more than 90% of the genes that we identified by the microarray analysis were up-regulated. However, it also revealed that the change (*n*-fold) in the microarray experiments was, in most case, underestimated.

TABLE 1. dsRNA-activated gene expression after 6 h of induction of d*sal-1*[a]

Category and GenBank accession no.	Gene description	Fold induction	
		Microarray	qPCR
RNAI components and regulation			
NCU08435.1	*rrp-3* (RNA-dependent RNA polymerase)	38.53	29.9
NCU04730.1	*qde-2* (Argonaute)	3.66	5.8
NCU06766.1	*dicer-2*	1.23	2.1
NCU07534.1	*qde-1* (RNA-dependent RNA polymerase)	ND	1.6
NCU08270.1	*dicer-1*	ND	1.5
NCU00076.1	*qip* (QDE-2-interacting protein)	ND	1.9
Interferon stimulated and antiviral genes			
NCU04935.1	IFN-induced Mx protein	4.74	237.1
NCU05693.1	IFN-induced Mx protein	ND	5.7
NCU08973.1	IFN-induced Mx protein	ND	5.7
NCU08359.1	Cytidine deaminase	3.85	NA
NCU04491.1	6-16 family (ISG12 domain)	1.94	3.0
NCU04490.1	6-16 family (ISG12 domain)	2.05	2.0
NCU04489.1	6-16 family (ISG12 domain)	2.48	5.0
NCU04488.1	6-16 family (ISG12 domain)	2.15	2.8
NCU04486.1	6-16 family (ISG12 domain)	ND	1.7
RNA/DNA binding and regulation			
NCU07036.1	3′–5′ exonuclease (Rnase D-like)	42.26	213.5
NCU04472.1	RNA helicase	3.98	39.7
NCU09495.1	*set-6* (SET domain containing)	14.37	79.7
NCU06125.1	CCR4/NOT complex *sub1*	2.01	1.8
NCU00582.1	Cryptochrome	1.98	NA
NCU01871.1	DNA replication licensing factor Mcm7	1.67	1.9
Stress response			
NCU09602.1	HSP70	1.99	1.8
NCU03288.1	HSP70-like	ND	1.8
NCU04142.1	HSP80	2.26	NA
NCU00704.1	Cu/Zn superoxide dismutase	ND	3.8
NCU02623.1	Mitochodrial hypoxia-induced protein	1.78	2.3
NCU00754.1	Multidrug resistance protein (membrane)	2.25	2.2
NCU03732.1	DNAJ-like (HSP70 cochaperone)	ND	1.7
NCU03556.1	Peroxisomal membrane protein PMP47B	2.34	NA
NCU04802.1	Peroxisome membrane protein PMP30	1.70	NA
Protein degradation			
NCU09309.1	20S proteasome subunit PRE2	1.5	1.5
NCU02840.1	26S regulatory subunit YTA3	1.50	1.5
Metabolism			
NCU09873.1	Phosphoenolpyruvate carboxykinase	2.31	NA
NCU06836.1	Acetyl-CoA synthetase	2.28	NA
NCU04923.1	Glycerol dehydrogenase	2.03	NA
NCU07263.1	Carnitine/acylcarnitine carrier	2.19	NA
NCU08002.1	Carnitine acetyltransferase	1.90	NA
NCU01611.1	Carnitine acetyltransferase FacC	1.96	NA
NCU08561.1	Succinate-fumarate transporter	2.11	NA
NCU05627.1	Related to sugar transporter	2.35	NA
NCU07853.1	Urate oxidase (uricase)	2.10	NA
NCU08434.1	Methionine synthase	2.09	NA
NCU03139.1	*his-3*	4.78	4.6
Unknown function			
NCU00947.1	116 aa	10.43	473.5
NCU06289.1	Unknown	11.06	117.5
NCU04436.1	BTB domain containing	2.41	28
NCU05628.1	Similarity to RNase H	15.00	188.1
NCU05629.1	Unknown	ND	3.3
NCU05631.1	Unknown	4.44	17.1
NCU08351.1	81 aa	434.58	NA
NCU04197.1	CipC protein	7.35	NA
NCU06294.1	P-loop containing	6.10	NA
NCU07257.1	Fungus specific	3.89	NA
NCU05881.1	DUF500 containing	2.35	NA
NCU09650.1	74 aa	2.30	NA
NCU07352.1	Fungus specific	2.26	NA
NCU02884.1	220 aa	2.12	NA
NCU10028.1	Hormone-induced membrane protein	1.97	NA
NCU05927.1	GTP-binding protein LepA	1.58	NA
NCU04487.1	Unknown	2.13	10.1

[a] Numbers indicate the increase (*n*-fold) in mRNA levels after 6 h of QA treatment in the d*sal-1* strain in the microarray or qPCR experiment. ND, not detectable; NA, not performed; aa, amino acids.

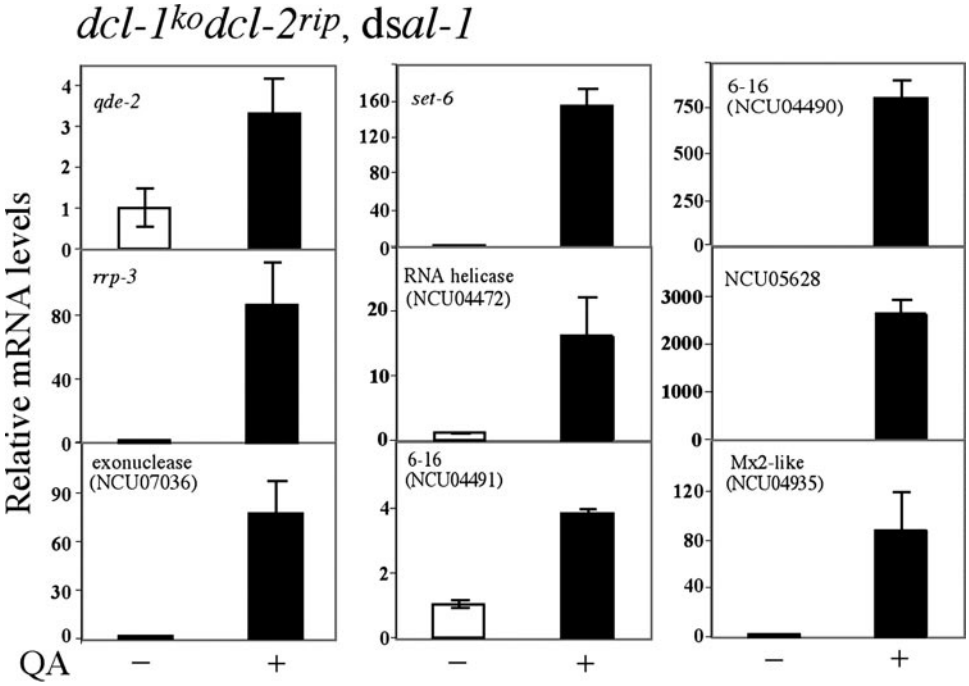

FIG. 6. qRT-PCR analysis showing the induction of DRAGs by dsRNA in the *dcl* double mutants. For cultures containing QA, QA was added for 6 h before harvesting.

The smaller change (*n*-fold) observed in microarray experiments is due mostly to the higher background signal generated in the microarray experiments than in qRT-PCR. In addition, we observed similar induction of most DRAGs by dsRNA in independent microarray experiments using the ds*frh* strain (data not shown), indicating that induction was not due to the silencing of *al-1*.

By combining the results of microarray and qRT-PCR analyses, we identified 60 DRAGs with inductions that ranged from half to several hundredfold after dsRNA production (Table 1 and Fig. 5C). This number is certainly an underestimate due to the lack of sensitivity of the microarray analysis. In addition, since dsRNA was induced for only 6 h in these experiments, the genes identified should mostly be the ones that are immediately activated by dsRNA, and genes induced secondarily, such as *dcl-2*, were likely missed. It is also possible that the induction of some of the DRAGs is specific for the strain and protocol used in our experiments. In these experiments, no genes were found to be consistently down-regulated after the induction of dsRNA.

We then asked whether the induction of these DRAGs by dsRNA, like the induction of *qde-2*, was also independent of the DCLs. To address this, we examined the induction of eight DRAGs by dsRNA in the *dcl* double mutants by qRT-PCR. As shown in Fig. 6, their robust induction by dsRNA was maintained in the mutants. These data suggest that the induction of most, if not all, DRAGs by dsRNA is mediated by a signaling pathway that is independent of DCLs and siRNA.

The classification of the DRAGs based on their known or putative functions revealed that they belong to the major functional groups described below.

(i) RNAi machinery. As expected, *qde-2* was identified as a DRAG by microarray experiments. Although *dcl-2* was not found to be significantly induced in the microarray analysis, qRT-PCR revealed that its level was doubled after the induction of dsRNA (Fig. 5C), suggesting that *dcl-2* is not a gene that is immediately activated by dsRNA. In addition, *qde-1* and *dcl-1*, but not *qde-3*, were found to be modestly induced by dsRNA in the qRT-PCR analysis. *qip*, a recently identified exonuclease that interacts with QDE-2 and facilitates single-stranded siRNA production in the RISC complex, was also up-regulated by dsRNA. Thus, most known components of the *Neurospora* RNAi pathway are induced by dsRNA.

Surprisingly, *rrp-3*, which encodes one of the three RNA-dependent RNA polymerases in *Neurospora* (2), is the most highly induced DRAG (~30-fold induction) in this group of genes. This result suggests that RRP-3, a homolog of QDE-1, may play a role in the formation of viral and retrotransposon dsRNA (39).

(ii) IFN-stimulated and antiviral genes. Genes with homology to mammalian ISGs form a major class of DRAGs. *Neurospora* has four genes that are homologous to the mammalian myxovirus (influenza virus) resistance (Mx) proteins, and three are strongly induced by dsRNA; one (NCU04935.1) was induced >200-fold. Mx proteins are conserved large GTPases with homology to dynamin. They inhibit viral growth by interfering with virus replication, and their induction by IFNs in mammals is an important part of the antiviral response (23, 24). The induction of Mx genes by dsRNA in *Neurospora* and mammals suggests a conserved dsRNA response from fungi to mammals and indicates the potential importance of Mx proteins in antiviral defense systems.

IFN-induced 6-16 family proteins are small proteins containing the IGS12 domain with unknown functions in mammals (37). *Neurospora* has five genes encoding 6-16 family proteins (NCU04486.1, NCU04488.1, NCU04489.1, NCY04490.1, and NCU04491.1) that are clustered in a single chromosomal locus. Interestingly, all six genes in this locus (the five 6-16 family protein genes and NCU04487.1) are significantly induced by dsRNA, whereas genes flanking this region (NCU04492.1 and NCU04485.1) are not (data not shown).

(iii) RNA/DNA binding and regulation. The RNA/DNA binding and regulation DRAGs include a 3′-5′ exonuclease (NCU07036.1), an RNA helicase (NCU04472.1), and *set-6* (NCU09495.1); all were dramatically induced by dsRNA (from 40- to 200-fold). The exonuclease belongs to the RNase D family. Mut-7, an RNase D-like protein, is involved in transposon silencing in *Caenorhabditis elegans* (28). The RNA helicase induced by dsRNA belongs to the *superkiller-2* (*ski-2*) subfamily of helicases. In yeast, *ski-2* is part of the host defense system that represses the propagation of dsRNA viruses by working with the exosome complex to degrade viral RNA (55). In addition, several RNA helicases, including RIG-I, are involved in the antiviral response and the RNAi pathway in animals (18, 51, 57).

set-6, which encodes one of the SET-domain-containing proteins in *Neurospora* (2), was induced ~80-fold by dsRNA. SET domains are a signature of lysine protein methyltransferases and are found in histone methyltransferases. In *Neurospora*, DIM-5, a SET-domain-containing protein, is a histone H3 Lys9 histone methyltransferase (50). Although the function of *set-6* is not known, its strong induction by dsRNA suggests that it may have a role in chromatin remodeling in response to dsRNA expression.

(iv) Stress response and protein degradation. Several heat shock proteins (HSPs) and one DNAJ-like cochaperone are up-regulated upon dsRNA induction in *Neurospora*. DRAGs involved in stress responses include a Cu/Zn superoxide dismutase, a multidrug resistance protein, and proteins involved in peroxisome function. In addition, two genes involved in regulating proteasome function were induced by dsRNA. In mammals, HSPs and proteasomal subunits are also known to be induced after viral infections or by IFNs (19, 42). The induction of these genes in *Neurospora* suggests a stress response for dsRNA production.

(v) Metabolism. DRAGs involved in metabolism include genes involved in fatty acid and carbohydrate metabolism and transport, such as phosphoenolpyruvate carboxykinase, acetyl coenzyme A (acetyl-CoA) synthetase, two carnitine acetyltransferase, and carnitine/acylcarnitine carrier. Interestingly, phosphoenolpyruvate carboxykinase is an ISG in mammals (19). Fatty acid metabolism has been shown to play a role in hepatitis C virus replication, and acetyl-CoA synthetase, an enzyme involved in fatty acid metabolism, was found to be up-regulated upon hepatitis C virus infection in mammals (26). The *his-3* gene, which encodes the histidinol dehydrogenase, a key enzyme involved in the histidine biosynthesis pathway, was also significantly induced by dsRNA.

(vi) Genes with unknown functions. There are 17 DRAGs that are genes with unknown functions. Among these genes, NCU05628.1 (induced ~188-fold) has similarity to RNase H, which is structurally similar to the PIWI domain of the Argonaute proteins (43, 48). Interestingly, two of its neighboring genes (NCU05629.1 and NCU05631.1) are also significantly induced by dsRNA. Several DRAGs that encode small proteins of unknown function are some of the most highly induced genes identified in our experiments. One of them, NCU08351.1 (81 amino acids), was the most highly induced DRAG in the microarray analysis.

DISCUSSION

In this study, we showed that the production of dsRNA leads to the induction of expression of *Neurospora qde-2*, *dcl-2*, and other genes involved in the RNAi pathway. We showed that the induction of QDE-2 is required for efficient RNAi. dsRNA regulates QDE-2 transcriptionally and posttranscriptionally, and the posttranscriptional regulation is dependent on the Dicer enzymes. A genome-wide search for dsRNA-activated genes identified 60 genes that are activated by dsRNA, including several additional RNAi components. The induction of antiviral genes and homologs of the mammalian ISGs in *Neurospora* suggests an evolutionarily conserved response against viral invasion and retrotransposons. To our knowledge, this is the first report of the dsRNA-induced transcription-based defense response in a nonvertebrate organism. In addition, this is also the first report of a regulatory mechanism that controls the expression of core RNAi components.

Regulation of the RNAi components by dsRNA. The levels of QDE-2 and DCL-2, two of the central components of the RNAi pathway, were robustly induced by dsRNA as a result of transcriptional activation. A 6-h induction with dsRNA also led to a modest induction of *qde-1*, *dcl-1*, and *qip*. Thus, almost all known players of the *Neurospora* RNAi pathway were activated by dsRNA. Such transcriptional responses were mediated by dsRNA rather than siRNA, since the transcriptional activation of *qde-2* and other DRAGs was maintained in the *dcl* double mutant (Fig. 2 and 6). In fact, the activation of *qde-2* was elevated and prolonged in the absence of DCLs, suggesting that the cleavage of dsRNA by DCLs attenuates the response. Despite the accumulation of *qde-2* mRNA upon dsRNA expression, the level of QDE-2 was low and was not induced in the *dcl* double mutant, indicating that DCLs regulate QDE-2 posttranscriptionally. These results suggest that siRNA, the cleavage product of dsRNA by DCLs, is important for the accumulation of QDE-2. Alternatively, DCL proteins may interact with and stabilize QDE-2. We examined whether the stability of QDE-2 was affected in the absence of siRNA, but no significant difference in QDE-2 stability was observed in the *dcl* double mutant and the wild-type strain (data not shown). It is possible that QDE-2 cannot fold properly and is rapidly removed in the cell if it is not bound to siRNA, a process known to be mediated by Dicers in animals (32, 52). However, a small amount of unbound QDE-2 may still fold correctly and exhibit stability similar to that of siRNA-bound QDE-2.

The induction of the QDE-2 protein by dsRNA was rapid: it was observed 1 h after dsRNA production began and peaked after 8 h (Fig. 2B). On the other hand, DCL-2 accumulated with slower kinetics (Fig. 3B). A 6-h dsRNA induction led to only a modest activation of *dcl-2* expression (Fig. 3B). These data suggest that dsRNA differentially regulates these two genes. *qde-2* is likely to be an immediate target of the dsRNA

regulatory pathway, and a secondary response leads to the activation of *dcl-2*.

Physiological function of the RNAi pathway activated by dsRNA. As we have shown in this study, the induction of QDE-2 (and probably other RNAi components) is required for efficient RNAi in *Neurospora* (Fig. 4). These data suggest that the induction of QDE-2 and DCL-2 is a regulatory mechanism that can significantly increase the efficiency of the RNAi pathway and, therefore, the removal of dsRNA. The removal of dsRNA is achieved by two mechanisms. First, dsRNA is detected by DCLs and is cleaved into siRNA. Second, guided by siRNA, QDE-2 along with other components of the RISC complex will lead to the destruction of RNA template for the production of dsRNA.

What are the physiological roles of such a response? dsRNA can be generated from several sources in eukaryotic cells. First, viral infections can be a major source of dsRNA since dsRNA is an intermediate of viral replication. Second, active retrotranspons can also generate dsRNA. Although the exact mechanisms of dsRNA production may be different, siRNA with sequences corresponding to retrotransposons has been detected in *Neurospora* and animals, suggesting the production of dsRNA (39, 46). Third, eukaryotic cells can generate endogenous dsRNA or hairpin RNA, for example, as precursors of microRNA (miRNA) or from repetitive sequences. In *Neurospora*, no confirmed miRNA has been reported. siRNAs derived from transcripts of repetitive sequences were shown to be important for the RNAi component-dependent transcriptional silencing in heterochromatin formation (45, 54). In *Neurospora*, endogenous siRNAs resulting from the DNA repeat region have been reported (14).

The basal levels of QDE-2 and DCL-2 are low in the wild-type strain and in dsRNA strains before the induction of dsRNA. After the induction of dsRNA expression, both QDE-2 and DCL-2 were induced ~10-fold. Since the *qa-2* promoter is not a very strong promoter (13) (Fig. 4A), these results suggest that the levels of endogenous dsRNA or miRNA (if present) are very low in *Neurospora*. Thus, it is likely that the dsRNA-activated RNAi pathway is an inducible defense response triggered by dsRNA produced due to viral infection or active transposons. Consistent with this hypothesis, the RNAi pathway has been shown to play important roles in silencing transposons and inhibiting viral invasion in animals and plants (20, 30, 33, 46, 56). In addition, several identified DRAGs are genes that encode proteins with potential antiviral and transposon-silencing activities.

Although no virus has been reported to infect laboratory *Neurospora* strains, it is likely that viral infection is a serious threat to *Neurospora* survival in nature. In fact, viruses (both dsRNA and single-stranded RNA) are known to infect filamentous fungi (25). Live transposons in *Neurospora* have been reported, and it has been shown that QDE-2 and DCLs are important for transposon silencing (3, 39).

dsRNA-induced host defense response in *Neurospora*. In addition to the induction of genes in the RNAi pathway, dsRNA also led to the activation of ~50 additional genes. Although the physiological importance of the activation of these genes has not been established, functional classification of the DRAGs suggests that their activation is a part of a broad immune-like response against the production of dsRNA. First, homologs of the mammalian ISGs, including the three Mx, five 6-16 family, HSP, and phosphoenolpyruvate carboxykinase genes, are DRAGs in *Neurospora*. Second, like the genes in the RNAi pathway, some of the DRAGs, including the Mx, RNA helicase, and exonuclease genes, have been shown to play important roles in the antiviral and transposon-silencing processes in other organisms. The similarities between the mammalian IFN response and the dsRNA response in *Neurospora* suggest that they may have similar functions with a common evolutionary link. Third, the genes involved in stress responses are a major class of DRAGs, suggesting that the presence of dsRNA is regarded as stress in *Neurospora*. Finally, some of the most highly induced DRAGs, such as the exonuclease, RNA helicase, and *set-6* genes, have putative roles in RNA processing and chromatin remodeling, suggesting potential novel processes controlling dsRNA in *Neurospora*.

The signaling pathway that triggers the transcription-based response is not known and will be a major focus of our future research. Our results showed that genes involved in RNAi, such as *qde-2*, *dcl*, and *qde-1*, are not required for transcriptional activation by dsRNA. In mammals, PKR and Toll-like receptor 3 are important dsRNA sensors that activate the IFN pathway through IFN-regulatory transcription factors and NF-κB. The lack of *Neurospora* homologs for these genes suggests a novel dsRNA-sensing and transcriptional activation pathway in this fungal organism.

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