THE MECHANISM OF DOUBLE-STRANDED RNA RESPONSE IN *NEUROSPORA*

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DEDICATION

To my parents, Vaishali and Purshottam Rao Choudhary.

THE MECHANISM OF DOUBLE-STRANDED RNA RESPONSE IN *NEUROSPORA*

by

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THE MECHANISM OF DOUBLE-STRANDED RNA RESPONSE IN *NEUROSPORA*

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In eukaryotic cells, recognition of double-stranded RNA (dsRNA) by the enzyme Dicer initiates the RNA interference (RNAi) pathway, resulting in post-transcriptional gene silencing. Argonaute proteins play a critical role in this conserved pathway, which is present in protists, fungi, plants and animals. In addition, dsRNA can trigger the interferon response as part of the immune response in vertebrates.

In this study, we show that the production of dsRNA triggers the transcriptional induction 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 efficient 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 post-transcriptionally, suggesting a role for DCLs or siRNA in QDE-2 accumulation. A genome-wide search revealed that additional RNAi components and homologs of antiviral and interferon-

stimulated genes are also dsRNA-activated genes (DRAGs) in *Neurospora*. Our results suggest that the activation of the RNAi components is part of a broad ancient host defense response against viral and transposon infections.

In order to understand the signaling mechanisms underlying this dsRNA response, we undertook a study of the dsRNA response elements (dsREs) in the promoter regions of *qde-2* and other DRAGs. We demonstrate that different regions of the *qde-2* promoter orchestrate early and late transcriptional induction in response to dsRNA. In the *qde-2* promoter, a GC-rich element and downstream CAAT repeats were found to be important for the early response. In addition, the GC-rich dsRE was found in the promoters of other DRAGs, and was sufficient for dsRNA-induced transcriptional response. These results suggest that these DRAGs share the transcriptional induction pathway triggered by dsRNA.

Finally, we demonstrate that QDE-2 contains an additional 10KDa N-terminal RGG domain, which is important for binding small interfering RNAs (siRNAs) and therefore required for its stability as well as efficient RNAi.

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PRIOR PUBLICATIONS

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

* equal contribution.

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AGO – Argonaute

ATP – Adenosine triphosphate

DCL – Dicer like protein

DCR – Dicer

DNA – Deoxyribonucleic acid

DRAG – dsRNA activated gene

dsRBD – Double-stranded RNA binding domain

dsRE – Double-stranded RNA response element

dsRNA – Double-stranded RNA

DUF – Domain of unknown function

EDTA – Ethylenediamine tetra-acetic acid

FGSC – Fungal genetics stock center

HCL – Hydrochloric acid

HEPES – 4-(2-hydroxyethyl)-1-piperizine-ethanesulfonic acid

HSP – Heat hock protein

IFN – Interferon

IRF – Interferon regulatory factor

ISG – Interferon stimulated gene

kB – Kilo base

kDa – Kilo Dalton

miRNA – MicroRNA

mM – Millimolar

mRNA – Messenger RNA

NF-κB – Nuclear factor- κB

nt – Nucleotide

ORF – Open reading frame

PAGE – Poly-acrylamide gel electrophoresis

PAZ – Piwi-Argonaute-Zwille

PCR – Polymerase chain reaction

PEG – Poly-ethylene glycol

PIWI – P-element induced wimpy testis

PKR – Protein kinase R

PNK – Polynucleotide kinase

PTGS – Post-transcriptional gene silencing

PVDF – Poyvinylidene difluoride

QA – Quinic acid

QDE – Quelling deficient

QIP – QDE-2 interacting protein

q-RT PCR – Quantitative real-time PCR

RdRP – RNA dependent RNA polymerase

RGG – Arginine-glycine rich

RIG-I – Retinoic acid inducible gene I

RIP – Repeat induced point mutation

RISC – RNA induced silencing complex

RNA – Ribonucleic acid

RNAi – RNA interference

RNaseIII – Ribonuclease III

rRNA – Ribosomal RNA

SDS – Sodium dodecyl sulfate

siRNA – Small interfering RNA

SSC – Sodium chloride- Sodium citrate

TBE – Tris-Borate-EDTA

TE – Tris-EDTA

Tris – Tris(hydroxymethyl)aminomethane

UTP – Uridine triphosphate

UTR – Untranslated region

CHAPTER ONE

INTRODUCTION

RNA, one of the building blocks of life, was for many decades thought to be a passive intermediary in the translation of genetic information into proteins. Subsequent discoveries have demonstrated that this picture is grossly over-simplified. We now know that RNA does not just passively pass on information, it actively regulates gene expression. Studies in a wide variety of organisms, from protists to plants, fungi and animals, have revealed the existence of a number of conserved pathways that use RNA as a trigger to regulate an astounding number of cellular processes, such as gene expression, heterochromatin formation, and defense against invading viruses and transposons. Due to the critical role played by RNA in these pathways, these are collectively known as RNA silencing (reviewed in (Hannon 2002; Meister and Tuschl 2004; Mello and Conte 2004). All RNA silencing pathways involve 21-30 nucleotide (nt) long "small RNA" species-such as small interfering RNAs (siRNAs), microRNAs (miRNAs), piwi-interacting RNAs (piRNAs) and repeat-associated small interfering RNAs (rasiRNAs). These pathways employ a conserved set of proteins that produce (and in some cases, amplify) small RNA species, and use them for sequence specific silencing. The silencing of gene expression may be achieved through various mechanisms such as target mRNA degradation, translation inhibition, or DNA and histone methylation.

RNA interference (RNAi) refers to a subset of these pathways, which are initiated by double-stranded RNA (dsRNA), and mediate sequence-specific degradation

of homologous mRNA transcripts, resulting in gene silencing. This pathway plays an important role in gene regulation and in cellular defense against transposons and viruses in various species.

1.1 The discovery of RNAi

The first reports of RNA silencing came from plants. (Napoli et al. 1990), and reviewed in (Jorgensen 1990). Briefly, multiple copies of a particular gene had been introduced into plants in order to obtain an over-expression phenotype. Instead, it led to strong silencing of the homologous gene as well as the transgenic copies, and hence the phenomenon was named co-suppression. A similar phenomenon, named quelling, had also been described in the filamentous fungus *Neurospora crassa*. (Pandit and Russo 1992; Romano and Macino 1992; Cogoni et al. 1996). Since these pathways appeared to interfere with gene expression at the post-transcriptional level, they were also known as post-transcriptional gene silencing (PTGS).

In animals, RNA silencing was first observed in *Caenorhabditis elegans*. In this model organism, antisense inhibition (i.e., supplying anti-sense RNA against a particular gene) was commonly used to obtain loss-of-function phenotypes. In 1995, Guo and Kemphues made the surprising discovery that injection of either sense or anti-sense RNA produced a significant loss-of-function (Guo and Kemphues 1995). This phenomenon was subsequently named RNAi. In 1998, Fire, Mello and colleagues discovered that dsRNA, a mixture of sense and anti-sense RNA against specific genes, was a much more potent inhibitor of gene expression than either sense or anti-sense RNA alone (Fire et al. 1998). Thus dsRNA was identified as the trigger of RNAi. In this report, the authors

stated that “the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing.” Indeed, further research in many species has shown that RNAi plays a critical role in several biological processes, including gene regulation and host defense. Reports of RNAi-like phenomena also came in from *Drosophila melanogaster* and *C. elegans* (Dernburg et al. 2000).

Subsequent genetic and biochemical studies, summarized below, demonstrated that a common, conserved mechanism underlies all these pathways.

1.2 The mechanism of RNAi

Fire *et al*. had demonstrated that RNAi in *C. elegans* could arise from dsRNA interfering with gene expression at post-transcriptional level (Fire et al. 1998). Exposure to dsRNAs led to depletion of homologous mRNAs; and dsRNAs homologous to promoter and intron sequences did not produce significant silencing, indicating that interaction with processed mRNA transcript was important for RNAi. Also, only a few molecules of dsRNA per affected cell were sufficient for inducing silencing, suggesting that a catalytic or amplification process could be a part of the mechanism (Fire et al. 1998). Further experiments have revealed the basic mechanism of RNAi, which is conserved in vertebrates, insects, worm, plants, and fungi. It involves two steps: initiator and effector. In the initiator step, dsRNA is cleaved by the ribonuclease-III enzyme Dicer (Bernstein et al. 2001) to give 20-25 nt siRNA duplexes (Hamilton and Baulcombe 1999; Zamore et al. 2000). Next, these siRNAs are loaded onto the RNA induced silencing complex (RISC), of which an Argonaute (Ago) protein forms the catalytic core

(Hammond et al. 2001). The siRNA duplex is then unwound, and its passenger strand removed. RISC, armed with active single-stranded siRNA, then proceeds to recognize and cleave the cognate mRNA. (Figure 1)

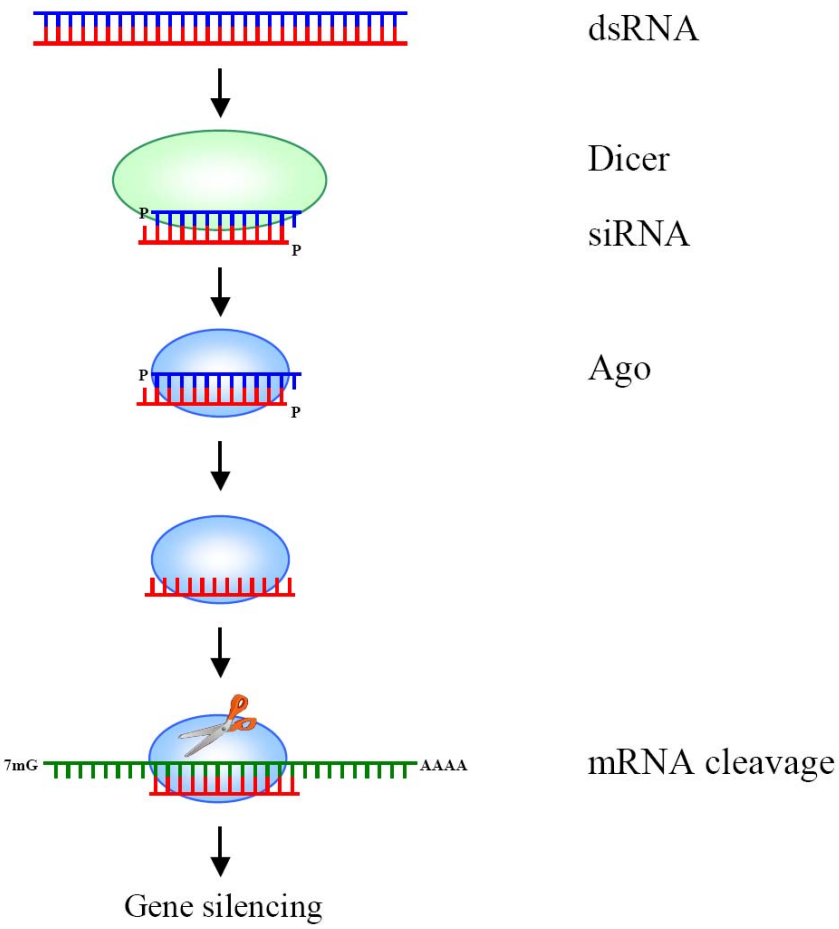

Figure 1: Schematic diagram of the RNAi pathway
dsRNA is recognized by the enzyme Dicer, which cleaves it to produce 20-25 nt siRNA duplexes. These duplexes are loaded onto the RISC complex, which contains the slicer protein Argonaute (Ago). The passenger strand of siRNA is removed, and the RISC is directed by the single-stranded guide siRNA to target mRNA that has sequence complementary to it. The active site in the Ago (shown as scissors) cleaves the target mRNA, resulting in gene silencing.

1.2.1 Production of dsRNA

RNAi begins with the recognition of dsRNA in cytoplasm. dsRNA can originate from a wide variety of sources, including viral infection. It is thought to be a replication intermediate of many viruses (Voinnet 2005). dsRNA can also be produced from transgenes and transposons (Tomari and Zamore 2005). Transcription from tandem transgenic repeats or transposon elements is thought to produce aberrant RNA, which would then be acted upon by a RNA-dependent-RNA-polymerase (RdRP) to produce dsRNA. The properties of aberrant RNA that distinguish it from cellular RNA, and thus mark it as a substrate for the RdRP, are as yet unknown. Transgenic hairpin dsRNA can also be produced by insertion of vectors with inverted repeat sequences (Goldoni et al. 2004; Choudhary et al. 2007). Another source of dsRNA is the hybridization of sense and antisense transcripts that arise from transcription of a gene and its anti-sense pseudogene, or from bi-directional transcription of a particular gene. dsRNA may also be produced by hybridization of mRNAs from genes that share some overlapping sequence, and are transcribed from opposite DNA strands (Tam et al. 2008; Watanabe et al. 2008). In addition, dsRNA can be physically introduced into the organism by injection (Kennerdell and Carthew 1998). Also, worms can be supplied dsRNA through soaking or ingestion of dsRNA-expressing bacteria. Recognition and cleavage of dsRNA by the enzyme Dicer leads to initiation of the RNAi pathway.

1.2.2 Dicer and the initiator step of RNAi

It was demonstrated that siRNAs contain a 5’ phosphate and a 3’ hydroxyl terminus, with a 2nt 3’ overhang (Elbashir et al. 2001). This observation was consistent with the production of siRNA resulting from cleavage of dsRNA by an RNaseIII enzyme.

RNaseIII family members in *Drosophila* embryo extracts were tested to determine which of them could produce 22nt small RNAs from dsRNAs. These experiments revealed the existence of a dsRNA-specific RNaseIII protein with the desired activity, which was named Dicer (*Dcr*) (Bernstein et al. 2001). Dicer-like enzymes are conserved across many kingdoms, from fungi to plants and animals. In fact, many organisms have multiple Dicers, which usually display some degree of functional diversification (reviewed in (Meister and Tuschl 2004). Most Dicers contain a DEAD box helicase domain, a DUF283 domain (function unknown), a Piwi-Argonaute-Zwille (PAZ) domain, catalytic RNaseIII domains and a dsRNA-Binding domain (dsRBD).

The current model for siRNA generation states that the PAZ domain is involved in end-recognition, and binds the pre-existing 3' overhang of dsRNA. The dsRBD is thought to attach near the cleavage sites (Zhang et al. 2004; Sontheimer 2005). The two RNaseIII domains in Dicer form an intramolecular dimeric processing center with two active sites. One strand of dsRNA is cleaved in the active site of each RNAseIII, thus giving rise to mature siRNAs. In this model, the size of siRNAs is determined by the properties of the Dicer, such as distance between the PAZ and active sites of the RNaseIII domains (Macrae et al. 2006).

In some model organisms, the activity of Dicer requires the presence of accessory proteins. In *Drosophila*, the dsRNA-binding enzyme R2D2 assists DCR-2 in loading siRNA into the RISC (Liu et al. 2003). Human Dicer has also been shown to associate with two dsRNA-binding proteins named TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein), and PACT,

respectively (Haase et al. 2005; Kok et al. 2007). Similarly, in *C. elegans*, DCR-1 is assisted by the dsRNA-binding protein RDE-4 (Tabara et al. 2002).

1.2.3 Assembly and activity of RISC

siRNA, produced by Dicer activity, is loaded onto the RISC complex, leading to the effector step of RNAi. There is evidence that Dicer assists in the assembly and activity of RISC (Tabara et al. 2002; Lee et al. 2004; Pham et al. 2004). In *Drosophila*, R2D2 binds the siRNA end with the greater double-stranded character (i.e., greater thermodynamic stability). Binding of R2D2 to the 3' end of a particular strand commits it to become the guide strand (Tomari et al. 2004). The Dicer-R2D2 complex is also called the RISC loading complex (RLC), and is thought to recruit an Argonaute-containing complex, thus initiating the assembly of RISC. Studies using *Drosophila* S2 cell extracts revealed the presence of a RNA-directed nuclease activity that specifically degraded mRNA with sequence homologous to transfected dsRNAs. This multi-component nuclease complex was named RNA-induced silencing complex (RISC), and was found to associate with siRNAs (Hammond et al. 2000; Hammond et al. 2001). Biochemical purification of RISC led to the identification of its core component, named Argonaute2 (Ago2).

Ago2 belongs to the Argonaute (Ago) family of proteins, which are expressed in bacteria, archaea and eukaryotes. Originally, Agos were reported to be involved in plant development, and also in germline stem-cell division in *Drosophila* ovaries (reviewed in (Hutvagner and Simard 2008). Since then, further genetic and biochemical analyses have unveiled the crucial role played by Argonautes in RNA silencing pathways, thus

establishing their importance in a wide variety of cellular processes such as genome defense, and gene regulation.

The Argonautes have been classified into three groups: Argonaute-like proteins, which are homologous to *Arabidopsis thaliana* AGO1; Piwi-like proteins, which are similar to *Drosophila* Piwi (P-element induced wimpy testis); and the *C. elegans*-specific group 3 Argonautes. The number of Argonautes present in an organism varies widely across species. For example, *S. pombe* has only one Ago, which functions in PTGS as well as heterochromatin formation. There are two Ago genes in *Neurospora* (both AGO1-like), both of which are involved in different PTGS pathways. The high degree of duplication of Agos in higher organisms is coupled with functional differentiation.

Most Agos contain four domains: N-terminal, PAZ, Mid, and PIWI. Crystal structure of the Argonaute protein from the archaea *Pyrococcus furiosus* showed a crescent-shaped platform created by N-terminal, Mid, and PIWI domains, with PIWI in the center (Figure 2). The PAZ domain hangs above the platform, supported by a linker region (Song et al. 2003). The PIWI domain appeared to have an RNase H-like-fold, with a conserved active site DDE motif, suggesting that it may have a catalytic function. These observations suggested a model in which the PAZ domain binds 3' end of the single-stranded guide siRNA, while the RNA transcript is placed on the platform; and in case of perfect complementarity between siRNA and target mRNA, the mRNA is cleaved by the PIWI domain. Further support for the model was provided by mutation studies using mouse Ago2, which showed that mutation of two of the catalytic residues in the PIWI domain abrogated the RISC cleavage activity (Liu et al. 2004). Biochemical reconstitution experiments indicated that recombinant human Ago2, combined with a synthetic siRNA,

was able to cleave target mRNAs, thus establishing Ago2 as the "slicer" component of RISC (Rivas et al. 2005). Further studies demonstrated that Ago2 cleaved the passenger strand of siRNA, leading to activation of the RISC (Matranga et al. 2005; Rand et al. 2005). Cleavage competent RISC cuts target mRNAs with perfect complementarity to the guide siRNA. Cleavage occurs in the middle of the region of complementarity, between 10^{th} and 11^{th} nt (counting from the 5' end of the guide siRNA) (Elbashir et al. 2001).

While catalytic activity is important for the Ago family members involved in target mRNA degradation, some Agos have lost their cleavage potential, and participate in small RNA pathways where their catalytic activity is not required. This includes RNA silencing pathways such as miRNA-mediated translational inhibition.

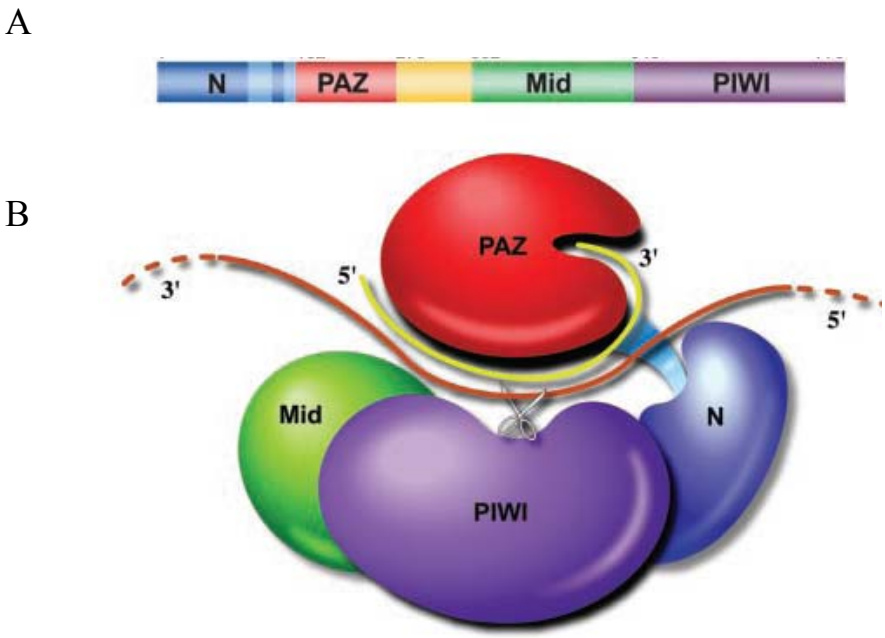

Figure 2. Structure of the Argonaute protein

(A) Schematic diagram of the domain organization of Argonaute proteins.

(B) Schematic depiction of the model for siRNA-guided mRNA cleavage. The 3' end of the siRNA (yellow) binds with the PAZ domain. The mRNA (brown) lies on a crescent-shaped platform created by the N-terminal, PIWI and middle domains. The active site in the PIWI domain (shown as scissors) cleaves the mRNA opposite the middle of the siRNA guide. (This figure is adapted from Song et al., 2004.)

1.3 MicroRNAs

MicroRNAs (miRNAs) are small (~ 22 nt), endogenous non-coding RNAs involved in post-transcriptional gene regulation (reviewed in (Bushati and Cohen 2007). miRNAs were first discovered in *C. elegans* (Lee et al. 1993; Wightman et al. 1993; Reinhart et al. 2000). Since then, hundreds of miRNAs have been identified in plants, animals and viruses through molecular cloning and bioinformatics. Many miRNAs bind to the 3' untranslated regions (3'UTRs) of target mRNAs, and down-regulate protein translation (Lee et al. 1993; Wightman et al. 1993; Reinhart et al. 2000; Slack et al. 2000). In plants, miRNAs have also been reported to cleave target transcripts. Spatio-temporal expression of miRNAs and their target genes adds another layer of complexity to the regulation of gene expression in many organisms.

Transcription from miRNA genes produces primary miRNAs (pri-miRNAs). These are cleaved in the nucleus by the RNase III enzyme Drosha to produce ~70nt hairpin precursor miRNAs (pre-miRNAs), which are transported to the cytoplasm (Lee et al. 2003b; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). Next, the pre-miRNAs are recognized and cleaved by Dicer and accessory proteins to produce mature miRNA:miRNA* duplexes (Hutvagner et al. 2001; Ketting et al. 2001; Chendrimada et al. 2005; Forstemann et al. 2005; Jiang et al. 2005; Saito et al. 2005; Lee et al. 2006). Subsequently, these are loaded onto the miRNA-induced silencing complex (miRISC), which contains an Argonaute protein. The passenger strand (miRNA*) is degraded, and the active miRISC goes on to bind target mRNAs with some degree of sequence complementarity to the miRNA. Perfect or near-perfect complementarity leads to cleavage of target mRNA, while imperfectly matched mRNAs undergo translational

repression (Hutvagner and Zamore 2002; Martinez and Tuschl 2004). Many plant miRNAs have nearly perfectly complementarity to their target mRNAs, and employ Ago-mediated cleavage as a means for gene regulation. On the other hand, most animal miRNAs have less than perfect complementarity to their target mRNAs, and can either cause translational repression or signal for mRNA degradation. The mechanism of miRNA-mediated translational repression is not very well understood. It has been shown that Argonaute proteins bound to miRNAs, and their target mRNAs, localize to cytoplasmic mRNA decay centers known as processing-bodies (P-bodies; (Liu et al. 2005; Sen and Blau 2005). It has been suggested that translationally repressed mRNAs may be temporarily stored in P-bodies.

In plants and animals, miRNAs play a crucial role during development. Mis-expression of miRNAs has also been implicated in cancer and a host of other diseases (Bushati and Cohen 2007).

In organisms that possess more than one Dicer and/or Argonaute, there appears to be some degree of specialization with regards to siRNA or miRNA pathways. For example, in *Drosophila*, Dicer-1 and AGO-1 are the major proteins involved in miRNA-induced gene silencing, while Dicer-2 and AGO-2 largely participate in siRNA-induced RNAi.

1.4 Other small RNA- Argonaute mediated pathways

RNAi-like pathways are involved in the formation and maintenance of heterochromatin at centromeres as well as other chromosomal locations in plants, animals and fungi (reviewed in (Lippman and Martienssen 2004).

PIWI-interacting RNAs (piRNAs) form another class of small RNAs. piRNAs are 24-30nt small RNAs found in the germ cells of animals such as mouse, rat, and *Drosophila* (Aravin et al. 2006; Girard et al. 2006; Grivna et al. 2006; Lau et al. 2006; Vagin et al. 2006; Watanabe et al. 2006). They are involved in silencing of transposons and thus help in the maintenance of germline genome integrity. The biogenesis of piRNAs differs from that of siRNAs and miRNAs in that it does not require an RdRP or Dicer activity. It is hypothesized that piRNAs arise from long single-stranded precursor RNAs. Currently, the mechanism of action of piRNAs remains unclear. *in vitro* experiments show that PIWI possesses target mRNA cleavage activity, which suggests that piRNAs may regulate their targets post-transcriptionally. Additionally, PIWI has been shown to interact with the heterochromatin protein-1a (HP1a), which suggests that piRNA pathways may be involved in the formation of heterochromatin at repeat loci (Brower-Toland et al. 2007). However, in mammals many piRNAs map to regions that do not contain repetitive sequences. The function of this subset of piRNAs remains unknown.

While most studies of siRNA and miRNA activity indicate that they repress gene expression, there have been some reports that components of the RNAi machinery may actually up-regulate gene expression under certain conditions (Kuwabara et al. 2004; Jopling et al. 2005; Li et al. 2006; Janowski et al. 2007; Vasudevan and Steitz 2007; Vasudevan et al. 2007). Single-base differences in the placement of siRNA targeting a promoter can result in either transcriptional activation or repression. Histone modifications that promote transcription have been shown to accompany siRNA-mediated transcriptional activation, but the exact mechanism is currently unknown. It is

possible that the choice between transcriptional activation and repression depends on the different complexes/ cofactors summoned by the core RNAi machinery.

1.5 RNAi as anti-viral defense mechanism

dsRNA is a replication intermediate of many viruses. It can be produced by viral or host RdRPs acting on viral RNAs. Degradation of viral dsRNAs by Dicers, and of viral RNAs by Agos primed with viral siRNAs, is an important host defense system in plants, fungi and animals (reviewed in (Schutz and Sarnow 2006; Ding and Voinnet 2007). In *Drosophila*, RNAi has been shown to direct immune responses against a wide variety of viruses such as Flock House virus (FHV), *Drosophila* C virus and Sindbis virus (Lu et al. 2005; Galiana-Arnoux et al. 2006). Mammalian vesicular stomatitis virus (VSV) and the insect pathogen Flock House virus (FHV) have been shown to elicit an RNAi response in *C. elegans* (Lu et al. 2005; Wilkins et al. 2005). All the four DCL proteins in *Arabidopsis* have been implicated in anti-viral defense (Bouche et al. 2006; Ding and Voinnet 2007). Recent reports indicate that RNAi serves as an anti-viral defense mechanism in fungi as well (Segers et al. 2007; Hammond et al. 2008).

That RNAi plays a crucial role in anti-viral defense is underscored by the fact that many viruses encode suppressors of RNAi, which inhibit different steps of the RNAi pathway. For example, the FHV protein B2 binds dsRNAs, and inhibits siRNA production *in vitro* (Lu et al. 2005). The plant Tombusvirus protein P19 sequesters siRNAs, thus inhibiting their incorporation into RISC. An anti-RNAi protein has also been reported from a mycovirus (Segers et al. 2006).

In vertebrates, dsRNA is also known to trigger the interferon response pathways. In these pathways, dsRNA is recognized by sensors such as Toll-like receptor 3, and protein kinase R (PKR). Next, they activate the transcription factors IRFs (interferon regulatory factors) and NF-κB, which induce transcription of the interferons. In turn, interferons induce transcription of hundreds of interferon stimulated genes (ISGs), many of which encode for proteins with anti-viral activities. In this way, the host orchestrates a strong defense response against invading viruses (reviewed in Sen and Sarkar 2007).

Since vertebrates possess the highly effective anti-viral interferon pathways, it is not clear if RNAi plays a major role in anti-viral defense in these organisms. However, inhibitors of RNAi are encoded by some mammalian viruses. For example, Adenovirus produces large amounts of highly structured virus-associated (VA) RNAs, which are hypothesized to function partly via saturating the RNAi machinery in mammalian cells. Proteins encoded by mammalian viruses have also been shown to inhibit RNAi in invertebrates (Li et al. 2004; Schutz and Sarnow 2006). Thus, it is likely that RNAi contributes to the anti-viral host defense response in mammals as well.

1.6 Applications of RNAi

Barely a decade has passed since the discovery of RNAi, yet it has rapidly become an important tool in laboratory as well as industrial settings (reviewed in (Dykxhoorn and Lieberman 2005).

(i). Gene knockdown RNAi is widely used to obtain loss-of-function phenotypes in cell culture as well as *in vivo*. This can be achieved by either insertion of hairpin RNA producing vectors that produce dsRNA *in vivo*, or through direct administration of

synthetic siRNA. Off-target effects can be minimized by careful design of synthetic siRNA sequences.

(ii). Functional genomics RNAi-based large-scale screens are used to identify genes involved in specific cellular processes. For example, a library of bacteria expressing dsRNA against *C. elegans* genes has been used to identify genes involved in fat regulation (Ashrafi et al. 2003).

(iii). Biotechnology RNAi-based genetic engineering techniques are being used to produce genetically modified plants with desired properties, such as tomato plants with decreased level of allergens (Le et al. 2006), and tobacco plants with reduced levels of the precursor of a putative carcinogen (Gavilano et al. 2006). RNAi-mediated knockdown of a specific enzyme in poppy plants has produced strains which produce reticuline instead of the narcotics morphine and codeine (Allen et al. 2004). Reticuline is a precursor of various pharmaceutically active compounds, including antibacterial, antimalarial and anticancer agents. RNAi-based techniques have also been used to produce genetically modified food plants with reduced levels of toxins, such cassava plants with lower levels of a toxic cyanogen (Siritunga and Sayre 2003). In addition, RNAi is also being studied as a means to impart resistance to common plant viruses.

(iv). Medicine Since all eukaryotic cells are thought to possess the basic RNAi machinery, and all mRNAs are its potential targets, RNAi-based therapeutics form a very promising new class of pharmaceutical drugs. These are currently under investigation for treatment of many diseases, including those caused by viruses or by dominant negative mutations. RNAi-based therapeutics use siRNAs instead of dsRNAs in order to avoid

triggering the interferon response. Efforts are underway to overcome the instability of siRNAs in serum by using chemical modifications (reviewed in Bumcrot et al. 2006). Proof-of-concept studies, using direct delivery of siRNAs (either naked or chemically-conjugated), have been successful in animal models of diseases affecting different organs, such as the eye, nervous system and digestive system. Intranasal delivery of siRNA has been shown to inhibit infection by respiratory syncytial virus (RSV) and parainfluenza virus (PIV) (Bitko et al. 2005) in mice. Also, vaginal instillation of siRNAs against herpes simplex virus 2 (HSV-2) has been demonstrated to protect mice when administered either before or after lethal HSV-2 challenge (Palliser et al. 2006). Direct delivery of siRNAs and viral vector-based delivery of short hairpin RNAs have also been shown to inhibit tumor growth in several mouse models. Currently, clinical trials are underway for studying the efficacy of RNAi-based therapeutics in treating AMD and in protecting against RSV.

As key regulators of several cellular processes, miRNAs are also an attractive target for therapeutics. It has been reported that chemically engineered antisense oligonucleotides (known as antagomirs) can inhibit miRNAs (Krutzfeldt et al. 2005). Antagomirs have shown specific *in vivo* silencing of target miRNAs in various tissues, including bone marrow, heart, kidney, lung and liver.

1.7 Regulation of RNAi

While the basic mechanism of RNAi has been described in great detail, there have been few reports of its regulators. Many viruses encode anti-RNAi proteins that inhibit RNAi at different steps (discussed above). In *C. elegans*, the neuron-specific

exonuclease ERI-1, and the RdRP family protein RRF-3, have been shown to function as negative regulators of RNAi (Simmer et al. 2002; Kennedy et al. 2004). In *Arabidopsis*, DCL1 and AGO1 are known to be regulated by miRNAs (Xie et al. 2003; Vaucheret et al. 2004; Vaucheret et al. 2006). In the ciliate *Tetrahymena thermophila*, expression of transgenic hairpin dsRNA has been shown to increase the transcript levels of the Dicers DCR1 and DCR2, as well as the Argonaute TWI2 (Howard-Till and Yao 2006). However, the signaling pathway that leads to this up-regulation has not been reported. The aim of this study was to identify and understand the mechanisms that regulate RNAi. The model system utilized in our studies is the filamentous fungus *Neurospora crassa*.

1.8 The model organism *Neurospora crassa*

The first known scientific description of the filamentous fungus *Neurospora crassa* dates back to 1843, when it was reported as the causal organism responsible for an orange mold outbreak in bakeries in France (Borkovich et al. 2004). Later, it was established as a model system for conducting experiments in the nascent field of molecular biology. It was used by the scientists George W. Beadle and Edward L. Tatum to study the role of genes in regulating biochemical events within cells, resulting in the "one gene-one enzyme hypothesis". Since then, *Neurospora* has continued as an important model system for studying various biological processes such as circadian rhythms, development, gene silencing, and evolution.

Neurospora is a popular model system since it is easy to grow and maintain, and its biochemistry is well-established. In addition, its genome has been sequenced (Galagan et al. 2003). In its haploid vegetative state, *Neurospora* exists largely in the form of

hyphae and asexual spores (known as macro- and micro-conidia). Its sexual cycle can be initiated by nutrient deprivation.

1.9 RNAi in *Neurospora*

Studies in *Neurospora* have made significant contributions to our understanding of gene silencing. In particular, one PTGS pathway named quelling, has been dissected in detail (Romano and Macino 1992). Quelling is active during haploid, vegetative growth. It recognizes repeated DNA sequences (introduced via transgenes), and silences them, along with the endogenous gene. The currently accepted model for quelling involves production of aberrant RNA from the transgenic repeats, processing to dsRNA, production of siRNA by Dicer activity, followed by assembly of the RISC complex, leading to sequence-specific silencing of cognate mRNA. siRNAs in *Neurospora* are about 25nt in length. (Catalanotto et al. 2002) A screen conducted to find genes involved in quelling revealed three genes- quelling deficient (*qde*)*1-3* (Cogoni and Macino 1997). *qde-1* is an RNA- dependent RNA polymerase (RdRP), while *qde-3* bears homology to a RecQ-like DNA helicase (Cogoni and Macino 1999a; Cogoni and Macino 1999b). Both *qde-1* and *qde-3* are implicated in the processing of aberrant RNA to produce dsRNA.

The gene *qde-2* encodes an Argonaute-like protein (Catalanotto et al. 2000). QDE-2 forms the catalytic core of siRNA-induced RISC in *Neurospora*. The QDE-2 protein contains a partial PAZ and a complete PIWI domain. The slicer activity of *qde-2* is required for the generation of single-stranded siRNA and gene silencing *in vivo* (Maiti et al. 2007). Another participant in *Neurospora* gene silencing pathway is the putative

exonuclease QDE-2 Interacting Protein (QIP). The disruption of QIP severely impairs the efficiency of RNAi.

Neurospora's two Dicers (dicer-like 1, *dcl-1* and *dcl-2*) play a redundant role in quelling (Catalanotto et al. 2004). The domains present in both Dicers are- a DEAD box, a helicase C domain, a duf283 domain (function unknown), and two RNase III domains (RNase IIIa and RNase IIIb). DCL-2 also contains an additional dsRNA binding domain. *Neurospora* also has other genome defense pathways like DNA methylation, Repeat-induced point mutation (RIP) and meiotic silencing (Borkovich et al. 2004). Unlike other organisms, DNA methylation in *Neurospora* is independent of RNAi (Freitag et al. 2004). RIP is active during the sexual cycle, prior to karyogamy (Galagan and Selker 2004). RIP searches for duplicated regions of the genome, and peppers them with random G/C to A/T mutations, as well as cytosine methylation. RIP serves as a convenient tool for creating null mutants of various genes in *Neurospora*. Meiotic silencing is active during meiosis, after karyogamy. It detects unpaired DNA and silences the gene using a mechanism similar to RNAi. The genes involved in meiotic silencing include *suppressor of ascus dominance 1* (*sad-1*), an RdRP; *suppressor of meiotic silencing 2* (*sms-2*), an Argonaute-like gene; *dcl-1* (also called *sms-3*), and *sad-2* (Shiu et al. 2006).

Gene silencing has also been studied in other filamentous fungi, including *Magnaporthe grisea*, *Aspergillus* and *Mucor*. In *Magnaporthe grisea*, the DCL-2-like protein MDL-2 is responsible for siRNA biogenesis as well as target mRNA degradation induced by hairpin dsRNA, while the DCL-1-like MDL1 is not required for these processes (Kadotani et al. 2004).

1.10 Summary

RNAi is a basic biological process that is evolutionarily conserved among protists, fungi, plants and animals. RNAi plays a critical roe in various biological processes including development, gene regulation, and host defense against invading viruses and transposons. RNAi has found multiple uses in academic as well as industrial settings. While the basic mechanism of RNAi is well-understood, its regulation has not been addressed in detail. This dissertation describes our efforts to understand the regulation of RNAi using *Neurospora crassa* as a model system. Our data shows that *Neurospora* has a dsRNA response pathway which induces transcription of most of its known RNAi components, including the Argonaute *qde-2*, and the Dicer *dcl-2*. In addition, genes with known or putative anti-viral and anti-transposon functions were also triggered by dsRNA, suggesting that regulation of RNAi forms a part of a broad host defense response in *Neurospora*.

Chapter 2 describes the establishment of a genetic selection to identify additional components of the RNAi pathway in *Neurospora*. The discovery and characterization of a double stranded RNA response program important for RNAi efficiency is described in Chapter 3. Finally, Chapter 4 describes our efforts towards understanding the signaling mechanisms employed by the double stranded RNA response; as well as discovery and characterization of an additional N-terminal RGG domain in the QDE-2 protein.

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CHAPTER TWO

A GENETIC SCREEN TO IDENTIFY COMPONENTS OF RNAI IN *NEUROSPORA*

2.1 Introduction

2.1.1 Background

Neurospora crassa is one of the first model systems in which gene silencing pathways were discovered (Romano and Macino 1992; reviewed in Borkovich et al. 2004). It has many gene silencing pathways, such as quelling, Repeat-Induced-Point Mutation and meiotic silencing. Quelling is a post-transcriptional gene silencing pathway that is active during *Neurospora*'s vegetative phase, while the other two silencing pathways function during different stages of its sexual cycle (Romano and Macino 1992).

Quelling is initiated by the insertion of transgenes with sequence homologous to a particular gene. The *Neurospora* quelling machinery recognizes and silences both the transgenic repeat sequences as well the endogenous gene, leading to a loss-of-function phenotype. It is hypothesized that quelling starts with production of aberrant RNA from the transgenic repeats, which is then further processed to double-stranded RNA (dsRNA). Presence of dsRNA would then trigger RNAi, leading to sequence-specific silencing of cognate mRNA arising from both endogenous and transgenic sequences. The model is supported by the presence of ~25 nt sequence-specific siRNA in quelled strains (Catalanotto et al. 2002). Three quelling deficient genes (*qde1-3)* were discovered via a UV-mutagenesis screen (Cogoni and Macino 1997). *qde-1* was later shown to be an

RNA- dependent RNA polymerase (RdRP), which is similar to a tomato RdRP (Cogoni and Macino 1999a). *qde-3* belongs to the RecQ-like DNA helicase family (Cogoni and Macino 1999b). Both *qde-1* and *qde-3* are hypothesized to participate in the production of dsRNA from aberrant RNA. Furthermore, the requirement of these two genes for quelling can be bypassed by inserting hairpin RNA producing constructs (Catalanotto et al. 2004). The gene *qde-2* is homologous to the *C. elegans rde-1*, and encodes an Argonaute-like protein (Catalanotto et al. 2000). *qde-2* forms the catalytic core of the siRNA-induced RISC in *Neurospora*. *Neurospora* has two Dicers (dicer-like 1, *dcl-1* and *dcl-2*), which play a redundant role in quelling. *In vitro* Dicer activity assays indicate that DCL-2 produces a majority of the siRNAs, while DCL-1 plays a minor role (Catalanotto et al. 2004). Additionally, *dcl-1* is involved in meiotic silencing, while *dcl-2* is not required in that pathway (Alexander et al. 2008).

Other *Neurospora* genes involved in meiotic silencing include the RdRP *suppressor of ascus dominance-1* (*sad-1*), the Argonaute protein *suppressor of meiotic silencing-2* (*sms-2*), and *sad-2* (Shiu and Metzenberg 2002; Lee et al. 2003a; Shiu et al. 2006). The involvement of a Dicer, an Ago, and an RdRP suggests that meiotic silencing may also proceed through an RNAi-like mechanism. However, further biochemical experiments are required to prove this hypothesis. Also, some of the genes involved in meiotic silencing, including *SMS-2* and *SAD-2*, appear to be expressed solely during the sexual phase, which suggests that they may not play an important role during vegetative silencing.

Sequencing of the *Neurospora* genome has revealed the presence of an additional RdRP named *rrp-3*, and another RecQ helicase named *rqh-2*, both of whose functions are currently unknown (Borkovich et al. 2004).

Accessory proteins that assist in RNAi have been reported in different organisms (Liu et al. 2003; Haase et al. 2005). Also, endogenous regulators of RNAi have been reported in *C. elegans* (Simmer et al. 2002; Kennedy et al. 2004). In *Neurospora*, a putative exonuclease named QIP assists in the formation of single-stranded siRNA (Maiti et al. 2007). However, other genes that may be involved in *Neurospora* RNAi are as yet undiscovered.

This chapter reports the setting up of a genetic selection to identify additional components of RNAi in *Neurospora*. If other *Neurospora* RdRPs or RecQ helicases are also involved in RNAi, they would also be identified through this selection. In addition, this selection is also expected to reveal regulators of RNAi, such as transcriptional factors involved in regulation of core RNAi components.

2.1.2 Experimental strategy

The strategy for this random mutagenesis selection was based on RNAi induced knock-down of the gene Ribosomal protein 10 (*rp10*, also known as 40S ribosomal protein S1, NCU01452.3). RP10 is a highly conserved protein and forms a part of the 40s small ribosomal subunit (Auclair et al. 1994; Swoboda et al. 1995).

Inverted repeats of the *rp10* gene were put under the control of the quinic acid (QA) inducible promoter of the gene *qa-2*. The *Neurospora* gene *quinic acid-2* (*qa-2*, NCU06023.3) encodes an inducible catabolic 3-dehydroquinase that is involved in the

break down of QA (Geever et al. 1989). Transcription from the *qa-2* promoter is induced in the presence of QA, and is commonly used for achieving inducible transcription of genes of choice in *Neurospora*.

Wild type transgenic qads*rp10* strains were used as host strains in this study. These were prepared by inserting the construct pqads*rp10* into the *his-3* locus of a wild type *his* strain. Upon addition of QA to the growth medium, transcription from the construct would produce RNA, which would be expected to form a hairpin due to the presence of homologous sequences in opposite orientation. This hairpin RNA would be recognized by Dicers and thus initiate silencing of the *rp10* gene product. Since RP10 is a crucial component of the ribosome, its depletion would severely inhibit growth.

Next, random insertional mutagenesis was performed using DNA fragment from the hygromycin B resistance (*hph*) gene. The antibiotic hygromycin B is toxic to fungi, including *Neurospora*. *hph* encodes for a kinase that inactivates hygromycin B through phosphorylation (Rao et al. 1983). Therefore, *Neurospora* strains that would have otherwise perished in the presence of hygromycin B, can survive if they express the *hph* gene product.

hph DNA fragments were inserted into wild type transgenic qads*rp10* strains through electroporation. This random insertion would be expected to disrupt some genes. If a gene involved in RNAi was disrupted, this may result in a loss of RNAi. Since RNAi-induced knock-down of *rp10* gene gave a slow-growth phenotype (observed with QA in the growth medium), the RNAi mutant strains would be expected to grow as well as wild type, even in the presence of QA. In this way, we can select for transformants with defects in RNAi.

2.2 Materials and Methods

2.2.1 Strains and growth conditions

87-3 (*bd a*) was the wild-type strain used in this study. 301-6 (*bd his-3 A*) was the host strain used for the insertion of *his-3*-targeting construct pqads*rp10*. Culture conditions were the same as those described previously (Cheng et al. 2001a). Cultures were prepared by inoculating conidia in flasks containing 50 ml medium, and incubated with shaking for 2 days. For liquid cultures grown with quinic acid (QA), it was added to a final concentration of 0.01M (stock solution: 0.5M, pH 5.8). Strains grown with QA were inoculated in low glucose liquid culture medium containing 1x Vogel's medium, 0.1% glucose, and 0.17% arginine (Cheng et al. 2001b). The QA treatment was carried out for 2 days unless otherwise indicated. Hygromycin B (Calbiochem), and QA concentrations used for slants, plates and racetubes were 550µl/L and 10^{-3}M, respectively.

2.2.2 Creation of wild type transgenic qads*rp10 strain*

The pqads*rp10* construct was created as described previously (Cheng et al. 2001a). Briefly, the construct was created with inverted repeats with sequence homologous to *rp10* ORF, expressed under control of the QA-inducible *qa-2* promoter. Regions of *Neurospora rp10* gene that were cloned into pDE3BH.qa in reverse and forward orientations are 442bp to 999bp and 543bp to 999bp, respectively (The first nucleotide of the ORF is counted as 1). Three-way ligation was used to generate the final pqads*rp10* construct. This was targeted to the *his-3* locus of a wild-type strain (301-6 *bd his-3 A*).

2.2.3 Southern Blot Analyses

The *his-3* locus was probed using a 1.7 kb DNA fragment obtained by the digestion of the plasmid pMyc.His.Dicer-2 by *Xho* I. *qde-2* was probed using a ~3 kb fragment obtained by *Hind* III/ *Sph* I double digestion of the plasmid pMyc.His.Qde-2. This fragment covers the entire *qde-2* ORF. The 4.3 kb probe for *dcl-2* was obtained by *Asc* I/ *Sph* I double digest of the plasmid pMyc.His.Dicer2. In each case, the DNA fragment was gel purified before further processing.

To prepare samples for Southern analysis, *Neurospora* genomic DNA was extracted, treated with RNAse, and then subject to enzymatic digestion. After agarose gel electrophoresis, the DNA was transferred onto Hybond-N membrane (Amersham Biosciences) using capillary transfer. Random primer labeling using ^{32}P dCTP and purified DNA (25ng) was used to prepare radiolabeled probes (Rediprime™ II, Amersham Biosciences). The membrane and probe were hybridized at 65°C for 2.5 hours. Three washes were performed using 0.5 X SSC and 0.2% SDS buffer (70°C, 20 minutes each). Finally, the membrane was exposed to X-ray film.

2.2.4 Protein and RNA analyses

Protein extraction, quantification, and Western blot analysis were performed as previously described (Cheng et al. 2001a). For Western Blotting, equal amount of total protein (50μg) was loaded into each lane of the SDS PAGE gel. After electrophoresis, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and Western blot analysis was performed. Nonspecific cross-reacting bands, as well amido-blue stained membrane, were used as loading controls. RNA extraction and Northern blot

analyses were performed as described previously (Aronson et al. 1994). Equal amount of total RNA (30-40µg) was loaded in each lane of the 1.3% (w/v) agarose gel. After electrophoresis, the gel was blotted and the RNA transferred to a nitrocellulose membrane, followed by UV cross-linking. Single-stranded ^{32}P-UTP labeled RNA probes for Northern Blot were prepared as follows: *qde-2* or *dcl-2* –specific antisense probes were generated by *in vitro* transcription from a DNA template containing sequence from either gene, with T7 promoter in antisense orientation. MAXIscript® T7 kit (Ambion) was used for the *in vitro* transcription. The probes were purified using Micro Bio-Spin 30 column (Bio-Rad), and used for overnight hybridization at 65°C. Next, the membranes were washed with 1 X SSC and 0.1% SDS buffer at 68°C, 20 minutes, three times, followed by exposure to X-ray film. rRNA levels, observed through ethidium bromide-staining of the agarose gels, served as loading controls.

2.2.5 siRNA Blot

Small RNA extraction and siRNA blot was performed as described previously (Maiti et al. 2007). Low-molecular-weight RNA was enriched from total RNA using 5% polyethylene glycol (MW8000) and 500mM NaCl as previously described (Catalanotto et al. 2002). For electrophoresis, 25µg small RNA was loaded per lane in 16% polyacrylamide, 7M urea, and 0.5 X tris-borate EDTA (TBE) gels.

After electrophoresis, the RNA was transferred onto Hybond-N+ filters (Amersham Biosciences), and fixed by UV cross-linking. Oligonucleotide markers were run as controls to verify the size of RNA species.

Hybridization of the membrane and radiolabeled RNA probe was performed at 42°C using ULTRAhyb™ buffer (Ambion). For hybridization, a single-stranded RNA probe was transcribed from an *rp10* DNA template using MAXIscript T7 kit (Ambion). The probe was designed to be in antisense orientation as compared to the *rp10* coding strand. ^{32}P-labeled uridine triphosphate (New England Nuclear) was used for radio-labeling. The radiolabeled RNA probe was then treated with TURBO DNase (Ambion) and hydrolyzed to an average size of 50nt using 80mM sodium bicarbonate and 120mM sodium carbonate as described (Catalanotto et al. 2002). The hydrolyzed probe was then used for overnight hybridization. The membrane was then washed three times with 2x SSC and 2% SDS buffer (30 min at 42°C, each). Finally, it was exposed to X-ray film.

2.2.6 Inverse PCR

The protocol for inverse PCR was adapted from ((Linden and Macino 1997), with certain modifications. Genomic DNA was extracted from MRP10 mutants and digested with *HinP1* I. The restriction digest was diluted 6-fold with ddH_2O, and self-ligated using T4 DNA ligase. DNA was ethanol precipitated and pelleted by centrifugation. Two sets of primers, with sequence homologous to the *hph* ORF, were used to amplify *hph* and the flanking *Neurospora* genomic sequence that was captured by self-ligation. The PCR products were run on 2% agarose gels; discrete bands were excised and sequenced (DNA Sequencing Core Facility at UTSouthwestern)

2.3 Results

2.3.1 Setting up of MRP10 selection conditions

We used the wild type transgenic qads*rp10* strain (homokaryon for insertion of the construct at *his-3* locus) as the host strain for this study. *rp10* mRNA was severely depleted in the presence of QA (Figure 3A), which indicates that our construct was functioning as expected. In addition, we observed a marked decrease in growth in the presence of QA (Figure 3B), which is the expected phenotype for knock-down of *rp10* gene product. Random mutagenesis was performed by electroporating *hph* DNA into the host strain. As compared to ~ 200 transformants growing on hygromycin B (150U/L) plates, less than 5 transformants were visible on plates containing both hygromycin B and QA (150U/L and 5 X 10^{-3}M, respectively), which suggests that conditions for a tight selection had been achieved (data not shown).

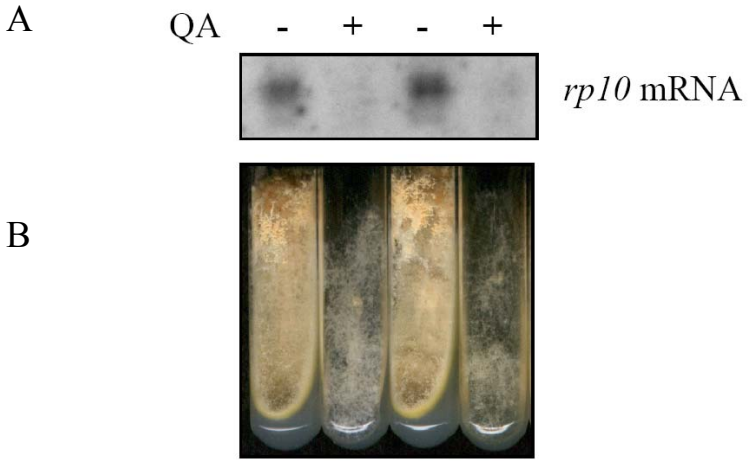


Figure 3. Depletion of *rp10* mRNA by production of ds*rp10*

(A) Northern blot analyses showing the depletion of *rp10* mRNA in WT, ds*rp10* transgenic strain. QA treatment (48 hrs) was used to produce ds*rp10*.

(B) Pictures of WT, ds*rp10 Neurospora* slants showing that the gene silencing of *rp10* by ds*rp10* results in a slower growth phenotype. Slants were photographed two days after inoculation.

2.3.2 Characterization of the RP10 mutants

After electroporation, conidia were plated onto plates containing hygromycin B and QA. Transformants were picked up and inoculated on hygromycin B, and hygromycin B + QA slants. About 1000 mutants, named MRP10, were generated and characterized in this study. Of these, about 490 mutants grew well on hph + QA (150U/L and 5 X 10^{-3} M, respectively) slants. These were chosen for further analysis.

Using PCR, we found that a large number of transformants had either disrupted, or completely lost the pqads*rp10* construct. This might be due to the high toxicity of the construct, i.e., since depletion of RP10 had a deleterious effect on growth, many transformants preferably lost the construct in order to be able to grow normally. Therefore, we established integrity of the pqads*rp10* insertion as an important initial test for our transformants. Southern blot analyses were carried out to test for insertion of pqads*rp10* at the *his-3* locus. Our results show that out of all the transformants analyzed, 57 were homokaryons, while 128 were heterokaryons. Most of the others had either completely lost or disrupted the pqads*rp10* construct (Figure 4).

As compared to heterokaryons, pqads*rp10* homokaryons would be expected to produce more *rp10* dsRNA, thereby eliciting a stronger RNAi response. Therefore, we elected to focus on characterizing the homokaryons.

The homokaryons were next tested for disruption of *qde-2* and *dcl-2* genes. Using Southern blot analyses, we found that 19 mutants had disruptions at *qde-2* locus (Figure 5A). In addition, *dcl-2* was disrupted in 5 mutants (Figure 5B). In all, 23 MRP10 mutants appeared to have intact *qde-2* and *dcl-2* genomic loci. These mutants were

further characterized in terms of *qde-2* protein and mRNA, *dcl-2* mRNA, and *rp10* siRNA levels, as well as growth on racetubes. (Figure 5C-F).

We have previously reported that production of dsRNA in *Neurospora* leads to increase in *qde-2* and *dcl-2* mRNA and protein levels (Choudhary et al. 2007). Therefore, protein and mRNA levels of *qde-2* and *dcl-2* can be used to assess if dsRNA is present in the strain. In addition, change in the expression levels of these genes may point towards the role of the putative RNAi component that was affected by *hph* insertion.

Wild type transgenic qads*rp10* strains show an induction of QDE-2 protein with QA, as compared to minimum medium, while QA has no effect on QDE-2 levels in a wild type strain (Figure 5C). As expected, QDE-2 protein was not detected in the RP10 mutants (such as MRP10-223, data not shown) that were identified as '*qde-2* disrupted' by Southern blot. Some RP10 mutants (such as MRP10-291) that appeared to have an intact *qde-2* locus by Southern blot also showed decreased QDE-2 protein and *qde-2* mRNA levels (Figure 5C & data not shown). Other mutants (such as MRP10-635 and 652) had constitutively elevated QDE-2 protein levels. Interestingly, MRP10-32 had high levels of *qde-2* mRNA, but failed to display elevated QDE-2 protein levels. Another mutant, MRP10-400, showed an induction of QDE-2 in the presence of QA, but its basal expression level was much lesser than wild type, and even its QA-induced QDE-2 level was lesser than that of similarly treated wild type. Our data suggests that these mutants may have *hph* insertion in genes involved in regulating *qde-2*. Alternatively, they may have *hph* insertions in distal genomic regions around *qde-2* that are essential for its expression, but were not detected through Southern blotting because they lie outside the limits of our Southern blot probe.

dcl-2 mRNA levels were assayed by Northern blot analyses in order to further characterize the RP10 mutants (data not shown). As expected, *dcl-2* mRNA was not detected in the RP10 mutants (such as MRP10-247) that were identified as '*dcl-2* disrupted' by Southern blot. Other mutants (such as MRP10-291) also appeared to be defective in elevating *dcl-2* mRNA levels in the presence of QA. Our data suggests that these mutants may have *hph* insertion in genes involved in regulating *dcl-2*. Another possibility is that they may have *hph* insertions in genomic regions around *dcl-2* that are essential for its expression, but lie outside the region covered by our Southern blot probe.

RP10 mutants were also assayed for expression of siRNA corresponding to RP10 (Figure 5D). As expected, wild type strains, and mutants that were identified as '*dcl-2* disrupted' by Southern blot, did not exhibit *rp10* siRNA upon QA treatment. On the other hand, wild type transgenic qads*rp10* host strain, and mutants that were identified as '*qde-2* disrupted' by Southern blot, displayed *rp10* siRNA in the presence of QA. *rp10* siRNA was present in most of the QA-treated RP10 mutants. However, a few mutants (such as MRP10-400, 487, 511, 517 and 596) showed severely decreased levels of *rp10* siRNA, suggesting that these mutants may have defects in siRNA generation or stability. As an additional control, *qa-2* Northern blots were also performed to ensure that the RP10 mutants did not have a defect in QA signaling pathway (data not shown).

In order to quantify their growth phenotypes, RP10 mutants were also tested on racetubes (Figures 5E and 5F). The host strain, wild type transgenic qads*rp10*, grew very well on minimum racetubes, but its growth was severely stunted in the presence of QA. Interestingly, about 20% of the host strain racetubes showed very little growth for the

first 3 days, followed by a rate of growth comparable to that in minimum conditions. This may result from the removal or disruption of the toxic pqads*rp10* construct.

Since QDE-2 forms the catalytic core of the *Neurospora* RNAi machinery, strains lacking QDE-2 are expected to be completely deficient in RNAi. As expected, the '*qde-2* disrupted' mutants grew very well in minimum as well as QA racetubes, indicating that *rp10* was not silenced in these mutants (Figure 5E).

Interestingly, the '*dcl-2* disrupted' mutants exhibited slower growth in QA as compared to minimum racetubes (Figure 5E). This suggests that *rp10* silencing was only partially affected by the absence of DCL-2. This partial requirement of DCL-2 in *Neurospora* RNAi is consistent with previous reports (Catalanotto et al. 2004).

Some of the RP10 mutants (such as MRP10-291) grew equally well in minimum and QA racetubes, suggesting that RNAi was completely abrogated in these mutants (Figure 5F). Others, such as MRP10-533, grew slower in QA as compared to minimum racetubes, suggesting that RNAi is only partially affected in these mutants.

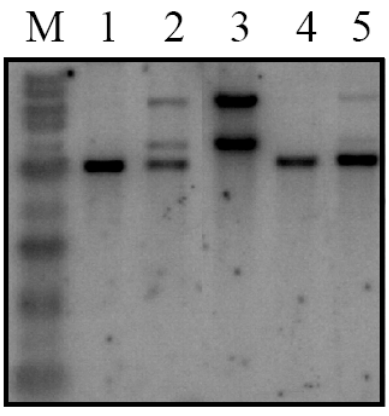
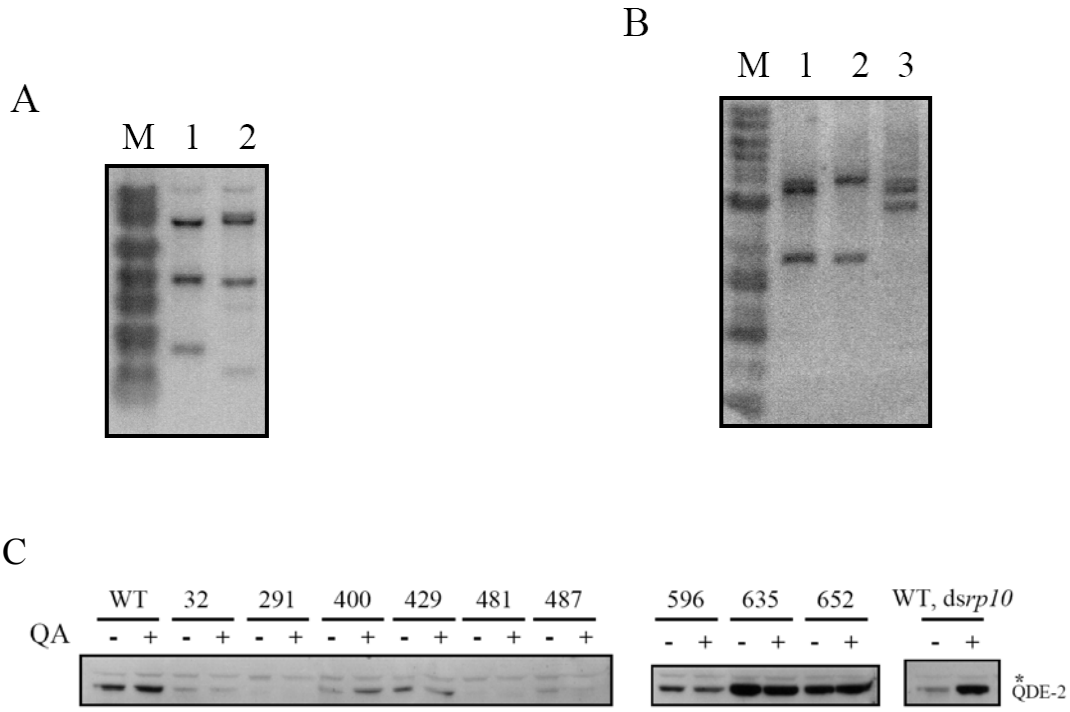


Figure 4. Identification of RP10 mutants. Representative Southern blot analysis for identification of RP10 transformants with pqads*rp10* construct inserted at the *his-3* locus
M: Marker
1: Wild type (negative control)
2: Host strain (heterokaryon, positive control)
3: Positive transformant (homokaryon)
4: pqads*rp10* construct lost
5: Positive transformant (heterokaryon)


A
M 1 2
B
M 1 2 3
C
WT 32 291 400 429 481 487 596 635 652 WT, dsrp10
QA
- + - + - + - + - + - + - + - + - + - +
*QDE-2
D
M 1 2 3 4 5 6 7 8
RP10 25-mer

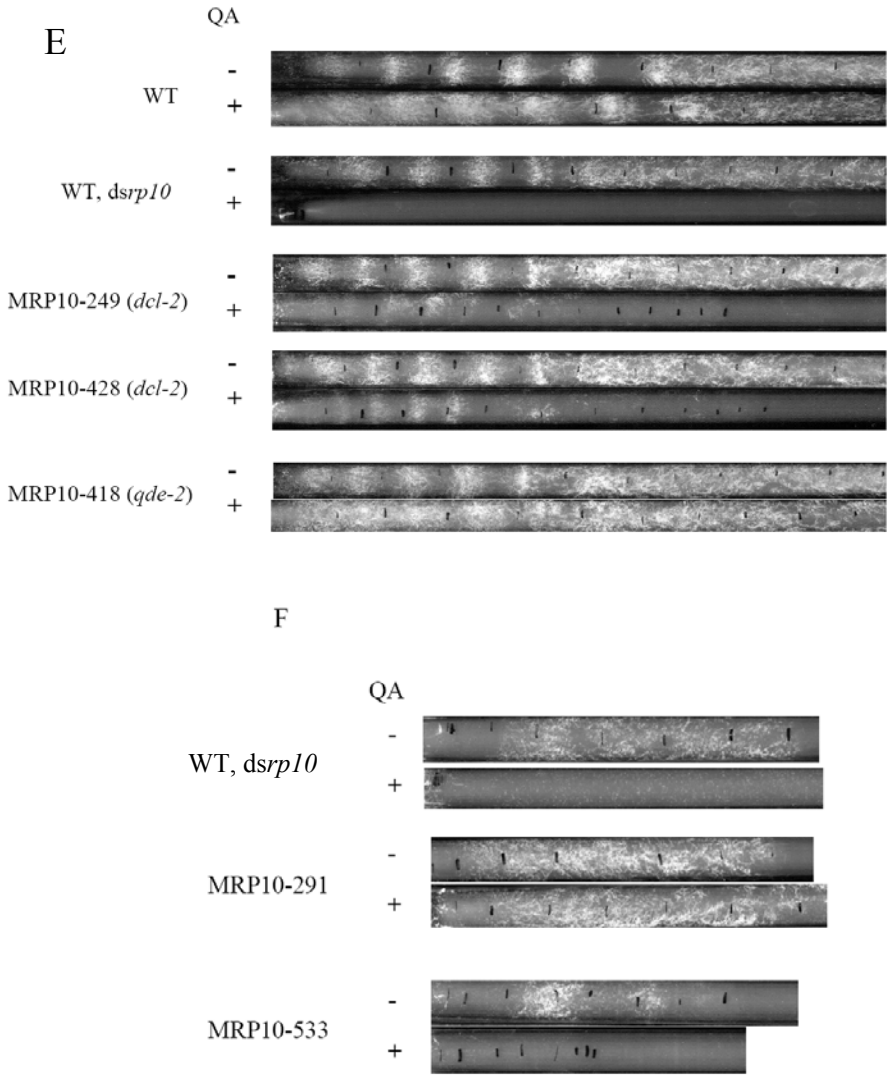


Figure 5. Characterization of RP10 mutants.

(A) Representative Southern blot analyses to identify mutants with disruptions at the *qde-2* locus. M: marker, 1: Wild type (positive control), 2: mutant with *qde-2* disrupted.

(B) Representative Southern blot analyses to identify mutants with disruptions at the *dcl-2* locus. M: marker, 1: Wild type (positive control), 2 & 3: mutants with *dcl-2* disrupted.

(C) Western blot analysis showing the level of QDE-2 in RP10 mutants. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody. Liquid cultures were grown for 2 days (with/without QA) before harvesting.

(D) Representative Northern blot analysis of *rp10* siRNA by denaturing gel. Liquid cultures were grown for 2 days (with QA) before harvesting. 1: Wild type; 2: Wild type, ds*rp10*; 3: MRP10-223 (*qde-2* disrupted); 4: MRP10-32; 5: MRP10-291; 6: MRP10-400; 7:MRP10-429; 8:MRP10-481; 9:MRP10-487.

(E) & (F) Race tube assay showing the growth of indicated strains in the absence or presence of QA in constant darkness. Each black line marks the growth front every 24hrs. The conidiation bands seen in the race tubes were due to circadian conidiation rhythms.

2.3.3 Characterization of the RP10 mutants by inverse PCR

Inverse PCR, followed by DNA sequencing, was performed to identify the *Neurospora* genomic regions into which *hph* DNA had inserted. We found that *hph* often inserted in more than one genomic location in a mutant. This presented difficulties in the accurate identification of the region responsible for the RNAi phenotype. Additionally, *hph* fragments frequently inserted into genomic locations in the form of tandem repeats, which posed a challenge to obtaining genomic sequence through sequencing.

The following genes were identified by inverse PCR and DNA sequencing:

i) MRP10-32 has an *hph* insertion in the gene NCU02052.1. BLAST search revealed that this gene has the KOG1932 (TATA binding protein associated factor) and KOG1046 (aminopeptidase) domains.

ii) MRP10-291 has an *hph* insertion in the gene NCU00291.1. This gene has no known domains.

iii) MRP10-400 has an *hph* insertion 600 bp upstream of NCU06256.1. This gene has a PGAM (phosphoglycerate mutase family) domain.

iv) MRP10-429 has an *hph* insertion in NCU04892.1, a gene with no known domains.

v) MRP10-481 has *hph* insertions in three genes: NCU00467.1 - a hypothetical protein with JAB_MPN domain (Jun kinase activation domain binding protein and proteasomal subunits); NCU05591.1- a hypothetical protein with two ABC transporter domains; and NCU00467.1- COP9 signalosome complex subunit 5 (CSN 5).

vi) MRP10-487 has an *hph* insertion between two genes: NCU08204.1 (hypothetical protein B24B19.240, imported) and NCU08205.1 (hypothetical protein B24B19.230, imported).

vii) MRP10-516 has an *hph* insertion in NCU08198.1 (hypothetical protein B24B19.290, imported)

viii) MRP10-635 has an *hph* insertion in NCU01883.1 (hypothetical protein with BAR and SH3 domains)

We reasoned that a true candidate gene would be identified more than once in our mutants. However, apart from *qde-2* and *dcl-2*, no other gene was found to be hit twice.

2.4 Discussion

This chapter describes the establishment of a random mutagenesis-based selection to identify additional components of the *Neurospora* RNAi pathway. We used wild type transgenic qads*rp10* strain as our host strain for mutagenesis. Depletion of the highly conserved *rp10* gene leads to a severe defect in growth, which is an easily-scored phenotype (Figure 3). Inverted repeats of the *rp10* gene were put under the control of the QA inducible *qa-2* promoter. The ability to induce dsRNA expression at specific time-points allowed us to directly correlate gene expression profiles with dsRNA levels. Fragments of the hygromycin B resistance gene (*hph*) were used for random mutagenesis. The antibiotic hygromycin B is toxic to *Neurospora*, but strains which express *hph* can survive in the presence of hygromycin. In this way, we were able to use growth kinetics to identify RNAi mutants.

We found that a large number of our transformants had either disrupted or completely lost the pqads*rp10* construct (Figure 4). This was likely a response to the high toxicity conferred by depletion of the RP10 protein. Of the mutants that were characterized in detail using Southern Blotting, approximately one-third (19/57) had

disruptions in the *qde-2* gene locus (Figure 5A). Multiple hits of *qde-2*, a central component of the *Neurospora* RNAi pathway, indicate that our selection strategy was working. Additional Southern Blots experiments revealed that the *dcl-2* gene was disrupted in about 9% (5/57) of the mutants (Figure 5B). Compared to the number of hits of *qde-2*, this suggests that *dcl-2* is partially redundant in the dsRNA-induced gene silencing in *Neurospora*, which is consistent with previous reports (Catalanotto et al. 2004).

The mutants that were identified through this selection were characterized in terms of expression of known components of the *Neurospora* RNAi pathway. Some mutants, such as MRP10-291, exhibited decreased QDE-2 protein and *qde-2* mRNA levels ((Figure 5C and data not shown). Another mutant, MRP10-635, displayed elevated QDE-2 protein levels even in the absence of QA. While MRP10-400 exhibited an induction of QDE-2, both its basal and QA-induced expression levels were lower than in wild type. Our data suggests that these mutants may have *hph* inserted in genes involved in regulating *qde-2*. For example, loss-of-function of a dsRNA-dependent transcription factor would lead to a defect in QDE-2 induction by dsRNA. Alternatively, these mutants may have *hph* insertions in genomic regions around *qde-2* that are essential for its expression. This possibility can be addressed by inserting a construct expressing functional QDE-2 into these mutants.

We also analyzed *dcl-2* mRNA levels in the RP10 mutants (data not shown). Some of the mutants, such as MRP10-291, appeared to have lost *dcl-2* induction in response to dsRNA. Again, insertion of a construct expressing functional DCL-2 will

help to identify if these mutants are false positives (i.e., if they have *hph* insertion in a *cis*-region important for *dcl-2* transcription).

Most of these mutants express *rp10* siRNA in the presence of QA, and only a few (such as MRP10-400) were defective in this regard (Figure 5E). The absence of siRNA suggests that this sub-group of mutants may be defective in either generation or stabilization of siRNA.

Some of the mutants (such as MRP10-291) grow equally well on minimum and QA racetubes, suggesting that RNAi has been completely abrogated in these mutants ((Figure 5F). Other mutants (such as MRP10-533) appeared to have only a partial loss-of-RNAi. This could result from disruption of a gene that plays a partially redundant role in RNAi. Alternatively, this observation might be explained by a partial loss-of-function of an essential RNAi gene due to *hph* insertion.

Inverse PCR, followed by DNA sequencing, was used to identify the genes which had been disrupted in our mutants. Insertion of *hph* at multiple sites in the genome, as well as its tendency to insert in tandem repeats at a single location, made this analysis challenging. Multiple hits of a single gene would have qualified it as a true candidate. However, apart from *qde-2* and *dcl-2*, no other gene was hit twice.

It is possible that if the selection is carried to saturation, more components of the RNAi pathway may be identified. Improved methods for inverse PCR may help to identify candidate genes from the mutants that have been generated. In addition, plasmid rescue can be used to identify the RNAi components affected in these mutants. Other assays like quelling can also be used to test if these mutants are defective in transgenes-induced-gene silencing.

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CHAPTER THREE

A DOUBLE-STRANDED RNA RESPONSE PROGRAM IMPORTANT FOR RNAi EFFICIENCY

3.1 Introduction

Double-stranded RNA (dsRNA) may be produced in a eukaryotic cell through viral replication or due to transcription from transposons and repetitive DNA sequences. The presence of dsRNA is known to elicit two types of cellular defense responses: post-transcriptional gene silencing (RNAi) and the transcription-based antiviral interferon (IFN) response (Sledz et al. 2003; Meister and Tuschl 2004; Karpala et al. 2005; Haller et al. 2006; Ding and Voinnet 2007).

RNAi plays an important role in silencing transposons and viruses in animals, plants and fungi (Lu et al. 2005; Wilkins et al. 2005; Bouche et al. 2006; Galiana-Arnoux et al. 2006; Wang et al. 2006; Segers et al. 2007; Hammond et al. 2008). Components of the RNAi pathway are thought to cleave dsRNA as well as mRNAs originating from viruses and transposons. In fact, many viruses are known to encode for suppressors of RNAi, which supports the idea that RNAi is a major anti-viral defense pathway (Ding and Voinnet 2007).

In vertebrates, dsRNA is also known to trigger the interferon response pathways. These pathways are initiated by the recognition of dsRNA by sensors such as Toll-like receptor 3, Retinoic acid inducible gene I (RIG-I), and protein kinase R (PKR). In turn,

this leads to activation of the interferon-regulatory transcription factors (IRFs) and NF-κB, which then induce transcription of the interferons. This is followed by interferon-induced transcription of hundreds of interferon stimulated genes (ISGs). Many of the ISGs encode for proteins with anti-viral activities. Together, they mount a substantial host defense response against invading viruses.

Although the basic mechanism of RNAi is now well-understood, regulatory pathways that control it have not been characterized in detail. As more and more applications for RNAi are found- including treatment of human diseases- it becomes imperative that we understand how the RNAi pathway may be affected by various upstream factors. It is also not known if there is cross talk between the transcription-based interferon and the post-transcriptional RNAi pathway. In addition, while the interferon system has been well characterized in vertebrates, its evolutionary roots remain undefined.

We hypothesized that an important pathway like RNAi would be tightly regulated, likely at multiple steps. We reasoned that dsRNA may serve as one of the upstream factors regulating RNAi genes. In this study, regulation of the RNAi machinery by dsRNA, and the physiological relevance of this regulatory mechanism, was examined using the filamentous fungus *Neurospora crassa*. Our study focused on two core components of the *Neurospora* RNAi pathway- the Argonaute QDE-2 and the Dicer DCL-2. We observed that production of dsRNA induced the transcription of both *qde-2* and *dcl-2*. Also, accumulation of QDE-2 protein after production of dsRNA required the presence of the two *Neurospora* Dicers. This indicates that either the two Dicers, or siRNA, regulate the steady-state levels of QDE-2 post-transcriptionally. Importantly, we

showed that the induction of *qde-2* by dsRNA is important for efficient RNAi. Using microarray and quantitative RT-PCR (qRT-PCR) analyses, we identified about 60 dsRNA-activated genes (DRAGs) in *Neurospora*. These include other components of the RNAi machinery, as well as genes that are homologous to the ISGs previously reported in vertebrates. 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 *Neurospora*.

This chapter is adapted from ***Choudhary, S., Lee, H.C., Maiti, M., He, Q., Cheng, P., Liu, Q. and Liu, Y. (2007). A double-stranded-RNA response program important for RNA interference efficiency. Mol Cell Biol 27(11): 3995-4005***. (Choudhary et al. 2007).

3.2 Materials and Methods

3.2.1 Strains and growth conditions.

The wild-type strain used in this study was either FGSC 987 (A), which was obtained from the Fungal Genetic Stock Center (FGSC); or 87-3 (*bd a*). Either FGSC 462 (*his-3 A*) or 301-6 (*bd his-3 A*) was the host strain used for the insertion of *his-3*-targeting constructs. The following mutant strains (with wild-type or *his-3* backgrounds) were created for this study: *dcl-1*^{ko}, *dcl-2*^{RIP}, *dcl-1*^{ko} *dcl-2*^{RIP}, *qde-1*^{ko}, *qde-2*^{RIP} and *qde-3*^{ko}. The mutants with *his-3* background were used as host strains for *his-3*-targeting constructs. Culture conditions were the same as those described previously, and in Chapter 2 (Cheng et al. 2001a). Cultures were prepared by inoculating conidia in flasks containing 50 ml

medium, and incubated with shaking for 2 days. For time-course experiments, mycelia mats of the specific strains were prepared by inoculating conidia in 50ml liquid medium in Petri dishes, followed by incubation for 2 days. Discs were cut from the mycelial lawn, and equal number of discs of each strain were cultured in liquid medium and incubated with shaking (Aronson et al. 1994). For liquid cultures containing quinic acid (QA), 0.01M QA (pH 5.8) was added to the liquid culture medium containing 1x Vogel's medium, 0.1% glucose, and 0.17% arginine (Cheng et al. 2001b). The QA containing cultures were grown in the presence of QA for 2 days unless otherwise indicated. For liquid cultures containing histidine, a final concentration of 0.5mg/ml was used, and they were grown in the presence of histidine for 2 days.

3.2.2 Creation of mutant strains.

The *Neurospora qde-2* and *dcl-2* genes were disrupted by a repeat-induced point mutation (RIP) (Cambareri et al. 1989). PCR fragments containing either the entire *qde-2* or the *dcl-2* open reading frame (ORF) and their 3' untranslated regions (3.3 kb for *qde-2* and 5.1 kb for *dcl-2*) were cloned into pDE3BH and introduced into the *his-3* locus of a wild-type strain (FGSC 7088 *his-3 a*) by electroporation. A positive transformant of each was crossed with a wild-type strain. Southern Blot analyses and DNA sequencing were performed to identify the strains in which the endogenous *qde-2/dcl-2* ORF was mutated with multiple premature stop codons produced by random G/C to A/T mutations.

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

each gene (*qde-1*, *qde-3*, or *dcl-1*) was cloned into pDE3BH, resulting in pQDE-1, pQDE-3 and pDCL-1, respectively.

To make the disruption construct, a hygromycin B resistance gene (*hph*) fragment containing promoter and terminator sequences was inserted into the *Xba* I-*Pvu* II site of pQDE-1, the *Pvu* II site of pQDE-3, or the *BamH* I site of pDCL-1. A PCR fragment containing the gene replacement cassette was introduced into a wild-type strain by electroporation in order to select for hygromycin B-resistant transformants (which grew on slants containing medium with 200μg/ml hygromycin B). 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 B. 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 of the opposite mating type to generate a *dcl-1*^{ko} *dcl-2*^{RIP} double mutant.

All RNAi mutants were crossed with a *his-3* strain to obtain mutants with *his-3* background; the resultant strains were used for all *his-3*-targeting transformations.

3.2.3 Constructs

dsRNA constructs were created as described previously (Cheng et al. 2005). The strategy was to create a construct with inverted repeats with sequence from a particular gene, expressed under control of the QA-inducible *qa-2* promoter. The following regions from *al-1*, *frq*, and *frh* were cloned into pDE3BH.qa in reverse and forward orientations, respectively: *al-1*, 1322 to 1942 bp and 1412 to 1942 bp; *frq*, 669 to 2309 bp and 791 to

1252 bp; and *frh*, 2087 to 2703 bp and 2189 to 2703 bp. The first nucleotide of each open reading frame (ORF) is counted as 1. Cloning was performed through three-way ligation to generate the final pqads*al-1*, pqads*frq*, and pqads*frh* constructs. These 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 electroporation.

Creation of the construct with *qde-2* gene under control of its own promoter has been described previously (Maiti et al. 2007). To create plasmids for *qde-2* promoter analyses, PCR fragments of *qde-2* (containing different lengths of its promoter, the entire open reading frame, and ~700 bases of its 3' untranslated region) were inserted into the *Nde* I site of the pqads*al-1* construct.

3.2.4 Antibody generation.

A glutathione *S*-transferase-QDE-2 fusion protein (containing QDE-2 amino acids 541 to 938) 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 (Cheng et al. 2001b).

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

3.2.5 Quelling assay.

The quelling assay was performed in a wild-type strain by co-transforming 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]). 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'-CTTCCGCCGCTACCTCTCGTGG-3') and al-1-2rev (5'-CCCTTTGTTGGTGGC GTTGATG-3'). The quelling experiments were performed in constant white luminescent light at room temperature.

3.2.6 Protein and RNA analyses.

Protein extraction, quantification, and Western blot analysis were performed as previously described (Cheng et al. 2001a). For Western Blotting, equal amount of total protein (50μg) was loaded into each lane of the SDS PAGE gel. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane, and Western blot analysis was performed. Amido black-staining of the membrane or nonspecific cross-reacting bands were used as loading controls.

RNA extraction and Northern blot analyses were performed as described previously (Aronson et al. 1994). Equal amounts of total RNA (30μg) were loaded in each lane of the 1.3% (w/v) agarose gels. After electrophoresis, the gels were blotted and the RNA transferred to a membrane. Single-stranded ^{32}P-UTP labeled RNA probes for Northern Blot were prepared as follows: *qde-2* or *dcl-2* –specific antisense probes were

generated by *in vitro* transcription from a DNA template containing sequence from either gene, with T7 promoter in antisense orientation. MAXIscript® T7 kit (Ambion) was used for the *in vitro* transcription. The probes were purified using Micro Bio-Spin 30 column (Biorad), and used for overnight hybridization at 65°C. The hybridized membranes were washed with 1 X SSC and 0.1% SDS buffer at 68°C, 20 minutes, three times, and then exposed to X-ray film. rRNA levels, shown by ethidium bromide-staining of the agarose gels, served as loading controls.

3.2.7 Microarray analyses.

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 the predicted genes in *Neurospora*. The chips were post-processed and UV cross-linked as per the directions of 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 data analysis.

3.2.8 qRT-PCR.

Quantitative real-time PCR (qRT-PCR) was performed with an Applied Biosystems Prism 7900HT sequence detection system using a previously described protocol (Kurrasch et al. 2004). 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 (2µg) were reverse transcribed with SuperScript II (Invitrogen) using random hexamers. 50ng cDNAs were added to 10µl qRT-PCR mix containing 5µl SYBR Green PCR Master Mix (Applied Biosystems) and 150nM 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). All primer sets were validated by cDNA template titration in order to ensure similar amplification kinetics and a single melting point of quantitative PCR products. Levels of the housekeeping gene beta-tubulin were used to calculate changes (*n*-fold) by comparing mean threshold cycle values of beta-tubulin and target genes.

3.3 Results

3.3.1 Induction of qde-2 expression by dsRNA

Our inquiry into the regulatory mechanisms controlling RNAi began with study of the regulation of the Argonaute protein QDE-2, a central component of RNAi. We hypothesized that QDE-2, a core component of the *Neurospora* RNAi pathway, would be

tightly regulated. To test this possibility, we examined endogenous QDE-2 protein levels in *Neurospora* RNAi mutants in which one or more of the RNAi genes were disrupted. As shown in Figure 6A, QDE-2 protein levels were severely reduced in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant, and in the *qde-1*^{ko} and *qde-3*^{ko} single mutants, as compared to the wild-type strain. No QDE-2 protein was detected in the *qde-2*^{rip} strain, thus assuring us of 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. In *Neurospora*, QDE-1 and QDE-3 are thought to be involved in the synthesis of endogenous dsRNA from aberrant RNAs, while the two DCLs are responsible for the production of siRNA. The severe reduction in QDE-2 protein levels in these mutants suggests that the production of dsRNA or its processing to siRNA regulates the expression of QDE-2 proteins.

In order to further address this hypothesis, we examined QDE-2 protein levels in *albino-1* (*al-1*, involved in carotenoid biosynthesis)-quelled strains. In these strains, the gene *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. The current model of quelling involves the production of aberrant RNA from the transgenic repeats, which is then processed to dsRNA with the help of QDE-3 and QDE-1. We reasoned that since dsRNA is produced during quelling, the degree of *al-1* silencing may reflect the amounts of dsRNA produced. Comparison of QDE-2 protein levels in wild type (orange conidia) and quelled strains revealed that QDE-2 levels were higher in two partially quelled strains and even further increased in two fully quelled strains. These

observations further support the idea that the production of dsRNA in these quelled strains leads to the elevated QDE-2 levels (Figure 6B).

During the course of our experiments, we discovered that the addition of histidine in the medium could significantly induce *qde-2* expression (comparable to induction by dsRNA), while the addition of other amino acids has no effect (data not shown). We observed that histidine strongly induced both QDE-2 protein and *qde-2* mRNA (Figure 6 C). It was proposed previously that histidine can cause DNA damage and it is used in experiments testing *Neurospora*'s sensitivity to DNA damage (Howard and Baker 1988; Kato et al. 2004). Although the mechanism of the histidine-mediated induction of *qde-2* is still under investigation, this data raises the possibility that the DNA damage caused by histidine may result in the production of endogenous dsRNA, leading to induction of QDE-2. This data also suggests that QDE-2 may be involved in cellular response to DNA damage. Alternatively, it is also possible that the elevation of *qde-2* expression is due to other effects of histidine in cell metabolism.

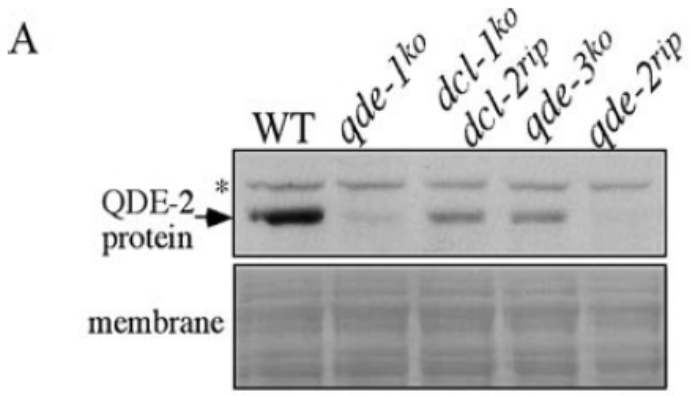
To directly investigate the effect of dsRNA on QDE-2, we examined whether the production of dsRNA was sufficient for the increase in QDE-2 expression. Previously, we and others developed a method to inducibly express dsRNA from inverted repeat sequences, leading to sequence-specific gene silencing in *Neurospora* (Goldoni et al. 2004; Cheng et al. 2005). Expression of the inverted repeats of the gene of interest is controlled by the QA-inducible *qa-2* promoter (Giles et al. 1985). Therefore, the addition of QA to the medium will lead to the production of dsRNA, resulting in the initiation of gene silencing.

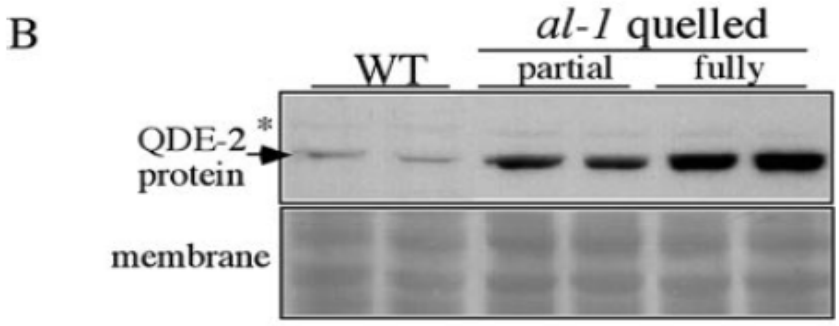
The expression of dsRNA leads to the production of homologous siRNA in the wild-type strain. But the Dicer double mutant is completely deficient in the production of siRNA (Catalanotto et al. 2004; Maiti et al. 2007). Steady state QDE-2 levels were examined in wild-type as well as three wild-type transgenic 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 RNA helicase gene required for circadian clock function), respectively. As shown in Figure 6D, addition of QA led to a significant increase in QDE-2 levels in all three transgenic dsRNA producing strains. In the control experiment, QA had no effect on QDE-2 levels in the wild-type strain. Since these transgenic strains produce dsRNAs targeting different genes, our data indicates that the effect on QDE-2 is dependent on the production of dsRNA itself, and not on the genes specifically targeted by the dsRNA. In another control experiment, we found that the QDE-2 levels in strains with mutated *al-1* gene were comparable to that in the wild-type strain, which indicates that carotenoid biosynthesis pathway is not involved in the regulation of QDE-2 expression (data not shown).

Northern Blot analysis was performed to determine whether the induction of QDE-2 expression by dsRNA is regulated at the transcriptional level (Figure 6E). Our data indicates that the addition of QA resulted in a significant increase in *qde-2* mRNA levels in the wild-type transgenic strains ds*al-1* and ds*frq*, indicating that the production of dsRNA increases QDE-2 expression at the transcriptional level.

It is known that although the *qa-2* promoter is tightly regulated by the presence of QA, it is not a very strong promoter (Cheng et al. 2001b). And yet we observe a strong

QA-induced elevation of *qde-2* mRNA and QDE-2 protein in dsRNA construct-containing strains. This suggests that there is very little endogenous dsRNA present in *Neurospora*, hence we see a marked effect on *qde-2* mRNA and QDE-2 protein by production of transgenic dsRNA.





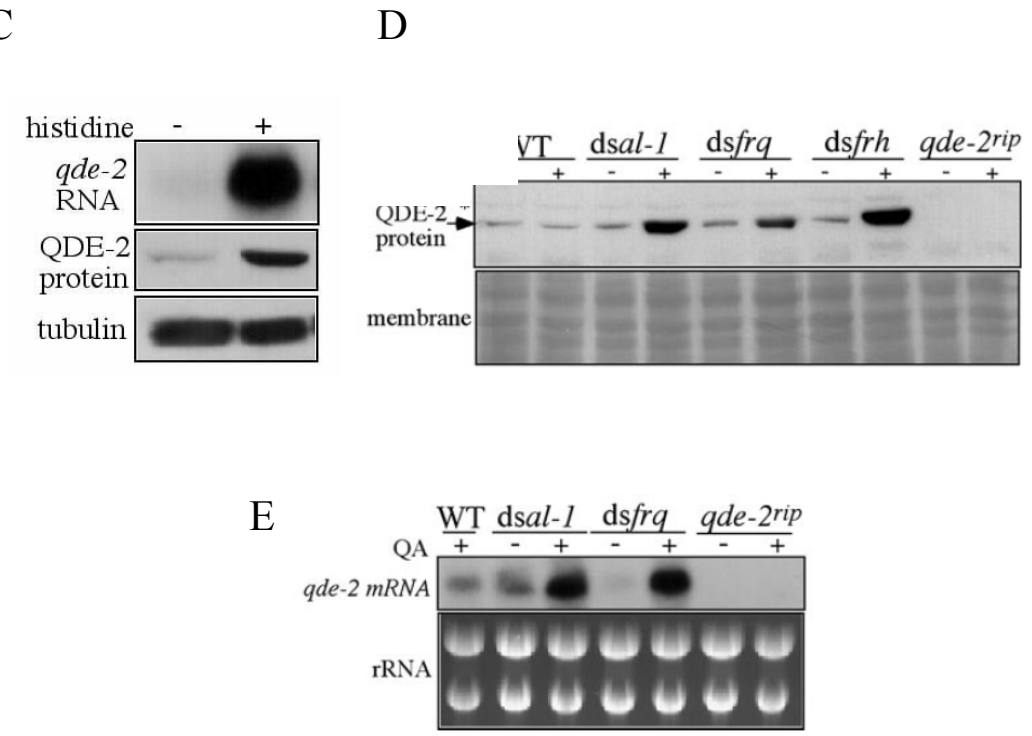


Figure 6. Induction of *qde-2* expression by production of dsRNA.

(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 two days (with/without QA) before harvesting.

(B) Western blot analysis showing the levels of QDE-2 in wild-type (WT) and *al-1* quelled strains.

(C) Northern blot (top panel) and Western blot (middle panel) analyses showing the induction of *qde-2* mRNA and QDE-2 protein, respectively, in the presence of histidine. Bottom panel shows the Western blot analysis of alpha-tubulin as a loading control. Liquid cultures were grown for two days (with/without histidine) before harvesting.

(D and E) Western blot (D) and Northern blot (E) analyses showing the induction of the QDE-2 protein and *qde-2* mRNA, respectively, in strains with dsRNA constructs. Liquid cultures were grown for two days (with/without QA) before harvesting.

3.3.2 *dsRNA, not siRNA, induces qde-2 expression, and DCLs are required for the maintenance of QDE-2 protein levels post-transcriptionally.*

The low levels of QDE-2 in the *qde-1*^{ko}, *qde-3*^{ko} and *dcl-1*^{ko} *dcl-2*^{RIP} double mutant (Figure 6A) suggest that QDE-1, QDE-3 and the two DCLs are required for

maintenance of steady-state QDE-2 levels. While the role of QDE-1 and QDE-3 may be explained by their involvement in the production of endogenous dsRNA, the requirement of the two DCLs suggests that production of siRNA also regulates QDE-2 protein levels. In further support of this idea, we observed that even after the induction of dsRNA in the *dcl-1*^{ko} *dcl-2*^{RIP} double mutant, QDE-2 protein levels failed to increase (Figure 7A). Our data suggests that the two Dicers are redundant in the regulation of QDE-2 protein, since transgenic single *dcl* mutants display QDE-2 induction comparable to wild type transgenic strains (Figure 7A, and data not shown).

Consistent with previous reports, QDE-1 was not required for the induction of QDE-2 by dsRNA (Catalanotto et al. 2004). It is hypothesized that the role of QDE-1 in gene silencing is to produce dsRNA using aberrant RNA as a template. Therefore, QDE-1 is not required for dsRNA production when dsRNA is produced from an exogenous hairpin construct. Hence, the induction of QDE-2 by transgenic dsRNA construct in a *qde-1* deficient background is comparable to that in the wild type background.

The data presented above would be consistent with the involvement of Dicers and siRNA in either the transcriptional activation of *qde-2*, or the post-transcriptional regulation of QDE-2 protein. Northern blot analyses were performed to distinguish between these two possibilities. The kinetics of *qde-2* mRNA induction (after QA addition) was compared in transgenic dsRNA producing strains with either *dcl-1*^{ko} *dcl-2*^{RIP} or wild type background. (Figure 7B). We found that the dsRNA-induced transcriptional activation of *qde-2* was maintained in the *dcl-1*^{ko} *dcl-2*^{RIP} double mutant. DCL-1 and DCL-2 are responsible for all Dicer activity in *Neurospora* (Catalanotto et al.

2004). Hence, our data indicates that dsRNA itself is sufficient to induce *qde-2* transcriptionally, while siRNA is not required for transcriptional induction of *qde-2*.

In the wild-type strain, *qde-2* mRNA levels were induced within 1 hour, peaked 4 to 6 hours after the addition of QA, and decreased afterwards. Comparatively, *qde-2* was activated to a higher level in the *dcl-1*^{ko} *dcl-2*^{RIP} double mutant, and high levels of *qde-2* mRNA were maintained up to 12 hours after the addition of QA. Also, the basal level of *qde-2* mRNA was higher in the Dicer double mutant than in the wild-type strain. This last observation would be consistent with higher levels of endogenous dsRNA existing in the *dcl-1*^{ko} *dcl-2*^{RIP} double mutant due to the absence of Dicer activity.

Interestingly, despite the increase in *qde-2* mRNA in the *dcl-1*^{ko} *dcl-2*^{rip} double mutant, QDE-2 protein levels failed to increase significantly. In contrast, in the wild-type strain, QDE-2 protein was seen to increase 1 to 2 hours after the QA treatment, and QDE-2 levels were maintained at high levels (~10-fold of the basal level) after 8 hours.

Together, our data indicates that dsRNA, but not siRNA, is responsible for the transcriptional activation of *qde-2* expression; and the two Dicers are not required for sensing dsRNA in the *qde-2* transcriptional activation pathway. However, Dicers are required for QDE-2 accumulation post-transcriptionally, which suggests that siRNA, the cleavage product of dsRNA by Dicer, plays a role in the accumulation of the QDE-2 protein.

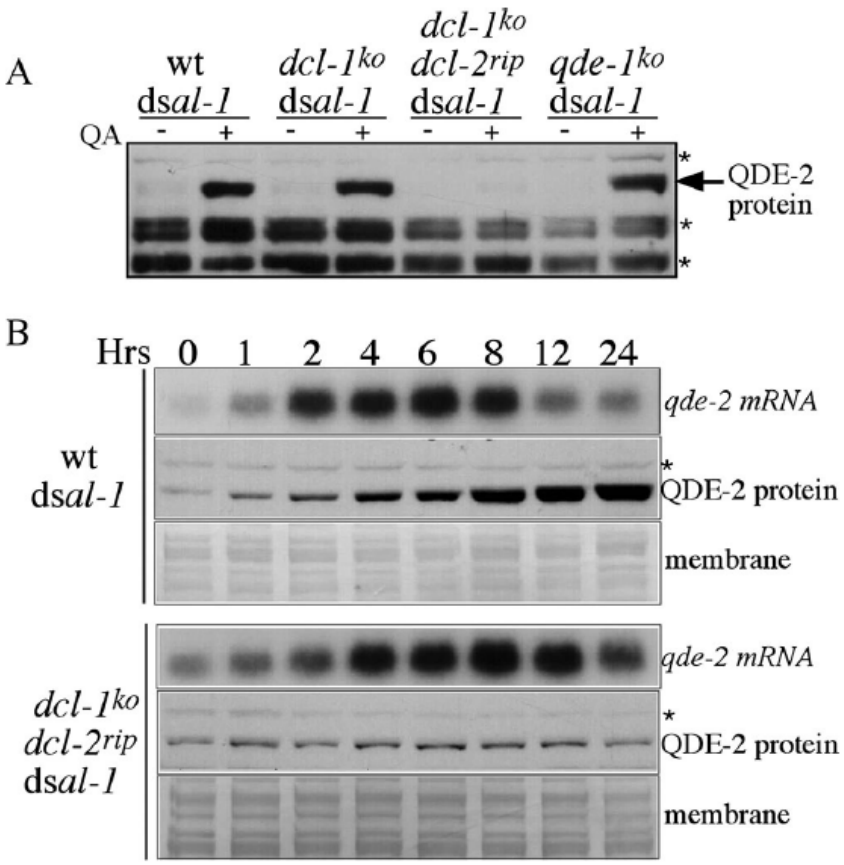


Figure 7. DCLs post-transcriptionally regulate the steady-state levels of QDE-2; and dsRNA, but not siRNA, is responsible for the transcriptional induction of *qde-2*.

(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 non-specific cross-reacting protein band recognized by our QDE-2 antibody.

3.3.3 Induction of DCL-2 by dsRNA.

After observing the induction of *qde-2* by dsRNA, we were interested in knowing if other RNAi genes were also affected by dsRNA. *dcl-2*, the other key component of the *Neurospora* RNAi pathway, was the next gene studied by us. Although functionally DCL-1 and DCL-2 are partially redundant, DCL-2 is responsible for more than 90% of

the *Neurospora* Dicer activity (Catalanotto et al. 2004). We found that *dcl-2* mRNA was strongly induced by the production of dsRNA in two wild-type transgenic strains carrying different dsRNA-producing constructs. In a control experiment, QA had no effect on *dcl-2* expression in the wild-type strain. These data indicate that dsRNA transcriptionally activates *dcl-2* expression as well (Figure 8A). Western Blot analysis showed that the production of dsRNA also led to a significant increase in DCL-2 protein levels (Figure 8B). In control experiments, the addition of QA had no effect on DCL-2 protein levels in the wild-type strain. Interestingly, compared to the induction of QDE-2, the kinetics of DCL-2 induction by dsRNA showed a marked delay; DCL-2 levels did not peak until 24 hours after QA addition. This suggests that the induction of *dcl-2* by dsRNA is a later, secondary response rather than an immediate, primary response as presumed for *qde-2*.

To understand if QDE-2 is involved in the dsRNA-responsive signaling pathway that induces DCL-2, we examined DCL-2 levels in a *qde-2* null strain (*qde-2*^{RIP}) carrying a transgenic dsRNA construct. As shown in Figure 8C, the induction of DCL-2 by dsRNA in the *qde-2*^{RIP} background was comparable to that in the wild-type background. Together with the data presented above, these data demonstrate that QDE-2 and DCL-2, two central components of the *Neurospora* RNAi pathway, are induced by dsRNA but are not required for the dsRNA sensing pathway(s) leading to their transcriptional activation.

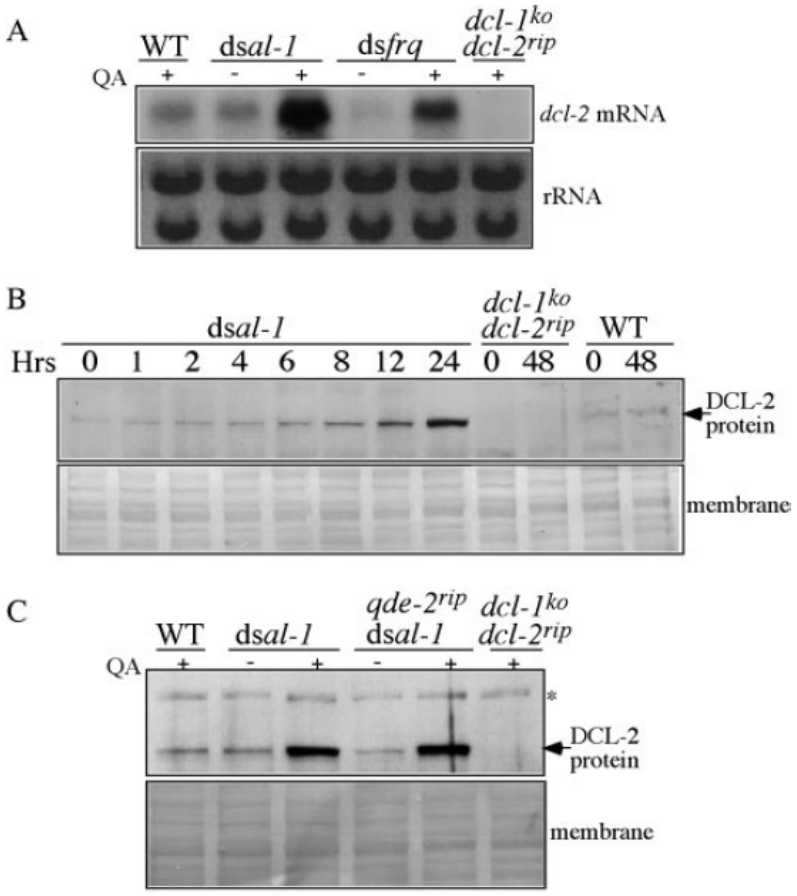


Figure 8. Induction of *dcl-2* expression by dsRNA.

(A) Northern blot analysis showing the induction of *dcl-2* mRNA in strains with dsRNA constructs.

(B) Western blot analysis showing that 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. 7B 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 non-specific cross-reacting protein band recognized by our DCL-2 antibody. WT: wild type.

3.3.4 Induction of QDE-2 by dsRNA is required for optimal gene silencing.

Since QDE-2 is a core component of the *Neurospora* RNAi pathway, its induction by dsRNA suggests that this regulation may be important for the overall efficiency of the pathway. To test this hypothesis, we examined whether the elevated

QDE-2 level, induced by dsRNA, is required for efficient gene silencing. Two constructs- one with the *qde-2* ORF under the control of the *qa-2* promoter (pqaQDE-2), and another with the wild-type *qde-2* gene with its own promoter- were created and inserted into a *qde-2*^{RIP} ds*al-1* strain through benomyl co-transformation. The construct with *qde-2* under control of its own promoter complemented the function of the endogenous *qde-2*, and the transformant exhibited dsRNA-induced up-regulation of QDE-2 expression (Figure 9A), and silencing of the *al-1* gene, as indicated by the white aerial hyphae and conidia in the presence of QA (Figure 9B). In contrast, the expression levels of QDE-2 in qaQDE-2 transformants were not induced in the presence of QA. In addition, the *qde-2*^{RIP} ds*al-1* qaQDE-2 strains exhibited very weak silencing of *al-1* in the presence of QA, indicative of a poor RNAi efficiency. These results suggest that the high levels of QDE-2 induced by dsRNA are required for efficient RNAi. They also suggest that the induction of QDE-2 expression by dsRNA is mediated by the endogenous *qde-2* promoter.

To further confirm this result, we created constructs that contain both- the *qde-2* ORF with different lengths of its upstream sequence (Figure 9C), and the ds*al-1* cassette (which is under the control of the *qa-2* promoter). These constructs were transformed into a *qde-2*^{RIP} strain and stably integrated at the *his-3* locus. As shown in Figure 9D, Pqde-2A, which contains 1.9 kb of the *qde-2* upstream sequence, fully complemented the function of the endogenous *qde-2*, as indicated by the induction of QDE-2 and efficient silencing of *al-1* (in the presence of QA). In contrast, in the Pqde-2B transformants (which contain 1.5 kb of the *qde-2* upstream sequence), dsRNA-induced QDE-2 up-regulation was abolished; and the QDE-2 levels remained at the basal level in the

presence of QA. Also, the aerial hyphae and conidia of the Pqde-2B strains remained orange in the presence of QA, indicating a severe reduction of RNAi efficiency. These data suggest that a *cis* element within the 0.4-kb region of the *qde-2* promoter is required for its dsRNA-induced up-regulation. Together, these data indicate that QDE-2 is a limiting factor in the RNAi pathway, and the induction of QDE-2 by dsRNA at the transcriptional level is critical for RNAi function.

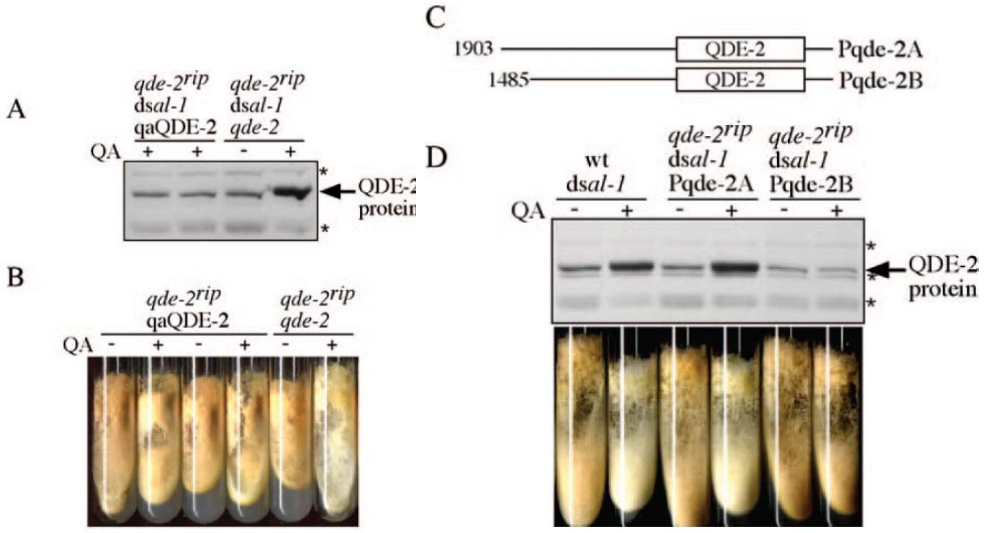


Figure 9. Induction of QDE-2 by dsRNA is required for efficient RNAi.

(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 *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 non-specific cross-reacting protein band recognized by our QDE-2 antibody. WT: wild type

3.3.5 *Genome-wide search revealed that additional RNAi components and genes homologous to host defense responses are dsRNA-induced genes (DRAGs).*

The observation that both *qde-2* and *dcl-2* were induced by dsRNA, suggested the existence of a transcription-based dsRNA response program in *Neurospora*. To identify other *Neurospora* genes that might be similarly regulated by dsRNA, we

undertook 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 wild type transgenic ds*al-1* strain were treated with QA for 6 hours. Wild-type cultures treated with QA were used as controls. As expected, *qde-2* mRNA was dramatically induced in the ds*al-1* cultures (Figure 10A). In additional controls experiments, the qRT-PCR analysis showed that the levels of *alpha-actin* and *qa-2* mRNAs were comparable in both strains (Figure 10B). This suggests that the activation of genes in the ds*al-1* strain should be in response to the production of dsRNA, and not due to off-target effects arising from the presence of QA.

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

Genes identified by the microarray experiments as showing significant up-regulation in the presence of dsRNA, were further confirmed by qRT-PCR, a more sensitive and quantitative method than the microarray analysis (Kurrasch et al. 2004). In addition to the candidate genes identified by microarray experiments, other genes known to be involved in the RNAi machinery, as well as some genes with low signal levels in the microarray experiments were also analyzed by qRT-PCR. Our results showed that more than 90% of the genes that were identified by the microarray analysis were also up-regulated in qRT-PCR experiments. However, the change (*n*-fold) reported in the

microarray experiments was, in most case, underestimated. The lesser change (*n*-fold) observed in microarray experiments may arise from the higher background signal generated in the microarray experiments, as compared to qRT-PCR. Additionally, similar induction of most DRAGs by dsRNA was observed in independent microarray experiments using the wild type transgenic ds*frh* strain (data not shown), indicating that the observed induction was not due to the silencing of *al-1*.

Together, our microarray and qRT-PCR analyses led to the identification of 60 dsRNA-induced genes (DRAGs), with inductions that ranged from half to several hundred-fold (Figure 10C and Table 1). Also, since dsRNA was induced for only 6 hours in these experiments, the genes identified are likely those that are immediately activated by dsRNA.

It is possible that the induction of some of the DRAGs may be specific for the strain and protocol used in our experiments. No genes were found to be consistently down-regulated after the induction of dsRNA.

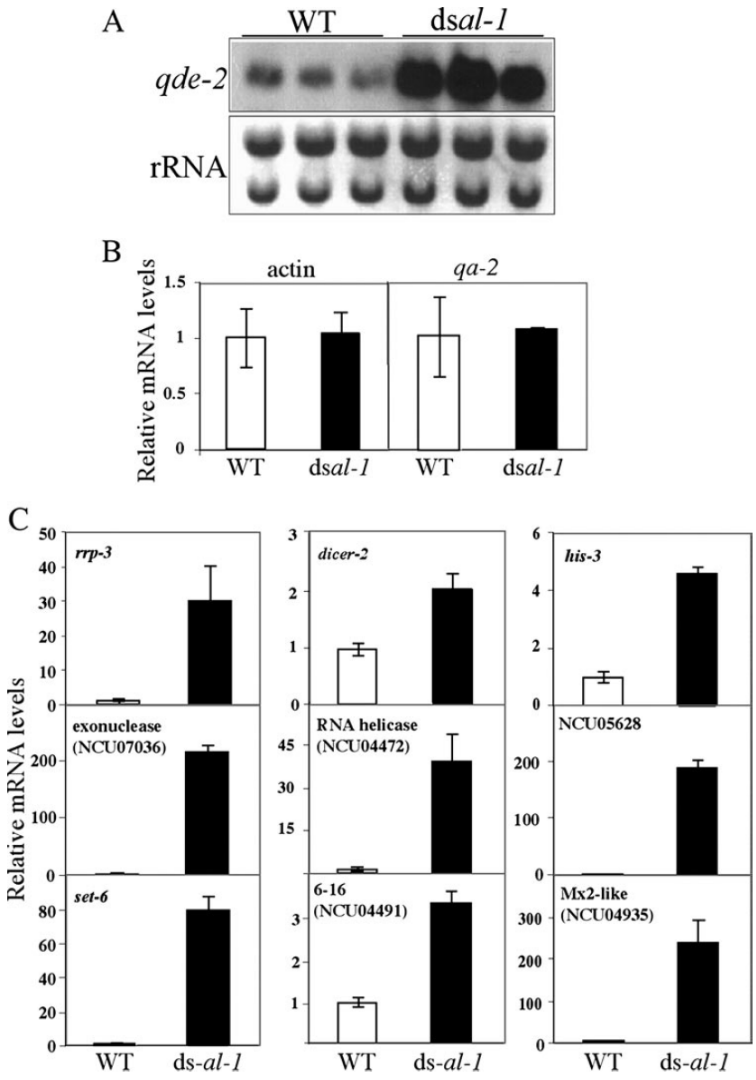


Figure 10. Genome-wide identification of DRAGs.
QA was added to wild type (WT) and ds*al-1* triplicate cultures for 6 hrs before their harvest.
(A) Northern blot analysis of *qde-2* for samples used in the microarray experiments.
(B and C) qRT-PCR analysis of gene expression.

We wanted to know if the induction of these DRAGs by dsRNA, like the induction of *qde-2*, was also independent of the DCLs. To address this question, the induction of eight DRAGs in the *dcl* double mutant background (carrying transgenic

ds*al-1* construct) was examined by qRT-PCR. As shown in Figure 11, the induction of DRAGs in the *dcl* double mutant background was comparable to that in wild type. These data suggest that the induction of most, if not all, DRAGs by dsRNA is mediated by signaling pathway(s) that are independent of DCLs and siRNA.

Based on their known or putative functions, the DRAGs were further classified into the following groups:

(i) RNAi machinery. As expected, *qde-2* was identified as a DRAG by microarray experiments. Although *dcl-2* was not significantly induced in the microarray analysis, qRT-PCR results indicate that it shows a two-fold increase 6 hours after dsRNA induction (Figure 10C). In addition, qRT-PCR revealed that *qde-1* and *dcl-1*, but not *qde-3*, were modestly induced by dsRNA. *qip*, an exonuclease that interacts with QDE-2 and facilitates singled-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.

rrp-3, an RNA-dependent RNA polymerase of unknown function, is the most highly induced DRAG (~30-fold induction) in this group of genes (Borkovich et al. 2004). The strong induction of *rrp-3* by dsRNA suggests that this homolog of QDE-1 may be involved in the formation of viral or retrotransposon dsRNA (Nolan et al. 2005).

(ii) IFN-stimulated and anti-viral genes. Surprisingly, genes with homology to mammalian ISGs form a major class of DRAGs.

Bioinformatics studies revealed that of the four *Neurospora* genes that are homologous to the mammalian myxovirus resistance (Mx) proteins, three are strongly induced by dsRNA. One of the *Neurospora Mx* genes (NCU04935.1) was induced over

200-fold by dsRNA. Mx proteins are conserved large GTPases with homology to dynamin. They inhibit viral growth by interfering with virus replication. In mammals, their induction by IFNs forms an important part of the antiviral response (Goodbourn et al. 2000; Haller et al. 2006).

IFN-induced 6-16 family proteins are IGS12 domain-containing proteins of unknown function (Martensen and Justesen 2004). *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. All the genes in this locus are significantly induced by dsRNA, whereas genes flanking this region (NCU04492.1 and NCU04485.1) are not (data not shown).

The induction of ISGs by dsRNA in *Neurospora* and mammals suggests the presence of a conserved dsRNA response from fungi to mammals.

(iii) RNA/DNA binding and regulation. The DRAGs in this group include a 3'-5' exonuclease (NCU07036.1), an RNA helicase (NCU04472.1), and *set-6* (NCU09495.1); all of which were strongly induced by dsRNA (from 40- to 200-fold). The 3'-5' exonuclease belongs to the RNase D family. Mut-7, an RNase D-like protein, is involved in transposon silencing in *Caenorhabditis elegans*, which suggests that the *Neurospora* 3'-5' exonuclease may also play a similar role (Ketting et al. 1999).

The dsRNA-inducible RNA helicase belongs to the *superkiller-2* (*ski-2*) subfamily of helicases. In *Saccharomyces cerevisiae*, *ski-2* is involved in a host defense system that represses the propagation of dsRNA viruses (Wickner 1996). Also, several RNA helicases, including RIG-I, are involved in the antiviral response and the RNAi pathway in animals (Tijsterman et al. 2002; Cook et al. 2004; Yoneyama et al. 2004).

set-6 encodes one of the SET-domain-containing proteins in *Neurospora*, and was induced ~80-fold by dsRNA (Borkovich et al. 2004). SET domains are characteristic of lysine protein methyltransferases, and are found in histone methyltransferases. 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 co-chaperone are also induced by dsRNA. 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 also induced by dsRNA. In mammals, HSPs and proteasomal subunits are also known to be induced after viral infections or by IFNs (Phillips et al. 1991; Der et al. 1998). The induction of these genes in *Neurospora* suggests that this model organism may possess a stress response that is activated by the production of dsRNA.

(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, carnitine acetyltransferases, and a carnitine/acylcarnitine carrier. Interestingly, phosphoenolpyruvate carboxykinase is an ISG in mammals (Der et al. 1998). 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 (Kapadia and Chisari 2005). Our data suggests that up-regulation of these genes may also play a role in anti-viral defense pathways.

(vi) Genes with unknown functions. Currently, the functions of seventeen DRAGs are unknown. 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 (Rand et al. 2004; Song et al. 2004). 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.

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.

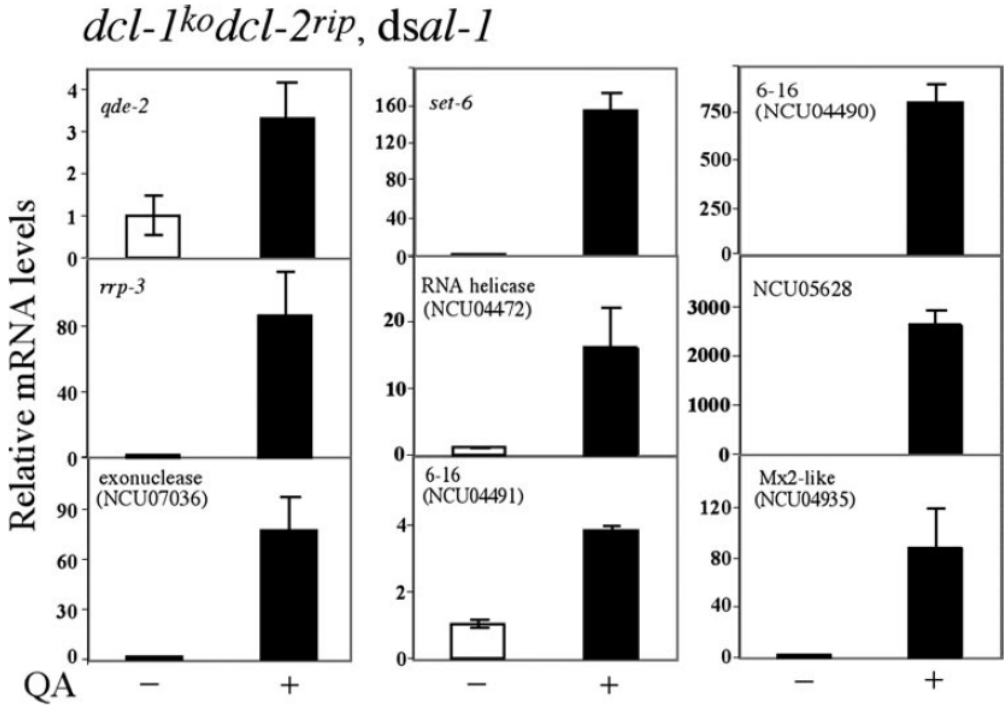


Figure 11. DRAGs are induced by dsRNA in the *dcl* double mutants.
qRT-PCR analysis showing induction of DRAGs in the *dcl* double mutants. For cultures containing QA, QA was added 6 hours before harvest.

3.4 Discussion

The identification and characterization of a double-stranded RNA response system in *Neurospora* have been described in this chapter. The production of dsRNA leads to the induction of the *Neurospora* Argonaute *qde-2*, and the Dicer enzyme *dcl-2*. QDE-2 is regulated by dsRNA at the transcriptional level, and by DCLs and siRNA at the post-transcriptional level. We found that the induction of QDE-2 by dsRNA is required for efficient RNAi. A genome-wide search for dsRNA-activated genes identified 60 genes that are activated by dsRNA, including several known components of RNAi. The

induction of anti-viral genes and homologs of the mammalian ISGs points towards the existence of an ancient, evolutionarily conserved response against invasion by viruses and retrotransposons. Our data suggests that this response is conserved from fungi to mammals. Together, our data forms the first report of a dsRNA-induced transcription-based host defense response in a non-vertebrate organism.

3.4.1 Regulation of the RNAi components by dsRNA.

Production of dsRNA led to a robust transcriptional induction of *qde-2* and *dcl-2*, two of the core components of the *Neurospora* RNAi machinery. A 6 hour induction with dsRNA also led to a modest induction of other RNAi genes such as *qde-1*, *dcl-1*, and *qip*. Thus, production of dsRNA up-regulates almost all of the known components of the *Neurospora* RNAi pathway. These transcriptional responses were triggered by dsRNA, and not siRNA, since the transcriptional activation of *qde-2* and other DRAGs was maintained in the *dcl* double mutant (Figure 7 and 11). In fact, *qde-2* displayed an elevated and prolonged up-regulation in the *dcl* double mutant background, suggesting that the cleavage of dsRNA by DCLs attenuates the response. Despite the accumulation of *qde-2* mRNA (in the presence of dsRNA), the level of QDE-2 protein remained close to the basal level in the *dcl* double mutant, indicating that DCLs and/or siRNAs regulate QDE-2 post-transcriptionally. It is possible that binding to siRNA stabilizes the QDE-2 protein. Alternatively, interaction with the two DCL proteins may stabilize QDE-2. We examined whether the stability of QDE-2 was affected by the absence of siRNA and DCLs, but no significant difference in QDE-2 stability was observed between the *dcl* double mutant and the wild-type strain (data not shown).

The induction of the QDE-2 protein by dsRNA was rapid: it was observed 1 hour after initiation of dsRNA production, and peaked after 8 hours (Figure 7B). In contrast, DCL-2 accumulated with slower kinetics (Figure 8B). A 6 hour- dsRNA induction resulted in a modest activation of *dcl-2* expression (Figure 8B). These data suggest that these two RNAi genes are differentially regulated by dsRNA. *qde-2* is likely to be an immediate target of the early-acting dsRNA responsive pathway(s), with a secondary response leading to the activation of *dcl-2*.

3.4.2 Physiological function of the RNAi pathway activated by dsRNA.

In this study, we show that the induction of QDE-2 is required for efficient RNAi in *Neurospora* (Figure 9). Our data indicates that the induction of QDE-2 (and other RNAi genes) is a regulatory mechanism that can significantly increase the efficiency of the RNAi pathway, resulting in the removal of dsRNA. dsRNA can be generated from several sources in eukaryotic cells, such as viral replication and active transposons. In fact, siRNAs with sequences homologous to transposons have been reported in *Neurospora* (Nolan et al. 2005). Also, eukaryotic miRNAs are generated from longer dsRNA transcripts. However, so far there have been no confirmed reports of miRNAs in *Neurospora*.

The basal levels of QDE-2 and DCL-2 are low in both the wild-type strain and transgenic dsRNA strains before the induction of dsRNA. Production of transgenic dsRNA leads to ~10-fold induction of both QDE-2 and DCL-2. Since the *qa-2* promoter is not a very strong promoter (Cheng et al. 2001b) (Figure 9A), our results suggest that

the levels of endogenous dsRNA or miRNA (if present) are very low in *Neurospora*. Therefore, it is likely that the dsRNA-response pathway discovered in *Neurospora* forms a part of a host defense response against invading viruses and transposons. Consistent with this hypothesis, the RNAi pathway (whose components are induced by the dsRNA-response pathway) has been shown to play important roles in silencing transposons and inhibiting viral invasion in animals, plants and fungi (Lu et al. 2005; Wilkins et al. 2005; Bouche et al. 2006; Galiana-Arnoux et al. 2006; Wang et al. 2006; Segers et al. 2007; Hammond et al. 2008). In addition, several of the identified DRAGs encode proteins with potential anti-viral and anti-transposon activities.

So far, no virus has been reported to infect laboratory *Neurospora* strains; but it is likely that viral infection forms a serious threat to *Neurospora* survival in nature. Active transposons have been reported in *Neurospora*, and it is known that QDE-2 and the two Dicers play an important role in silencing transposons (Cambareri et al. 1994; Nolan et al. 2005).

3.4.3 dsRNA-induced host defense response in Neurospora.

In addition to the induction of the RNAi machinery, production of transgenic 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 host defense response against dsRNA. This is supported by the finding that homologs of the mammalian ISGs, including the three *Mx* genes, five 6-16 family genes, HSPs, and

phosphoenolpyruvate carboxykinase genes, are DRAGs in *Neurospora*. Similar to the RNAi genes, some of the DRAGs (including Mx, RNA helicase, and 3' to 5' exonuclease) are known to play important roles in the anti-viral and anti-transposon pathways in other organisms. The similarities between the mammalian transcription-based interferon response and the *Neurospora* dsRNA response suggest that they may have similar functions, and a common evolutionary link. Additionally, genes involved in stress responses form a major class of DRAGs, which suggests that the presence of dsRNA is regarded as stress in *Neurospora*. Finally, some of the most highly induced DRAGs, such as the 3' to 5' exonuclease, RNA helicase, and *set-6* genes, have putative roles in RNA processing and chromatin remodeling, which suggests the existence of novel mechanisms controlling dsRNA in *Neurospora*.

Our results show that genes involved in RNAi, such as *qde-2*, *dcl-1*, *dcl-2*, and *qde-1*, are not required for transcriptional activation by dsRNA. Mammals possess dsRNA sensors such as RIG-I, PKR and Toll-like receptor 3 that activate the interferon pathway through transcription factors such as interferon-regulatory factors (IRFs) and NF-κB. Since *Neurospora* lacks homologs for these dsRNA sensors, as well as dsRNA-responsive transcription factors, it appears that this model organism possesses novel pathways involved in dsRNA-sensing and subsequent transcriptional induction.

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CHAPTER FOUR

UNDERSTANDING THE SIGNALING PATHWAY OF THE DOUBLE STRANDED RNA RESPONSE IN *NEUROSPORA*

4.1 Introduction

Since its discovery just over a decade ago, RNAi has revolutionized molecular biology. It has become a valuable tool for assaying loss-of-function phenotypes of genes in the laboratory. RNAi has numerous applications in biotechnology, and in the treatment of human diseases. While the basic mechanism of RNAi is fairly well-understood, regulatory mechanisms that control it have not been analyzed in depth.

We have previously shown that production of double stranded RNA (dsRNA) in *Neurospora* leads to transcriptional induction of most of the known RNAi genes, including the Argonaute *qde-2*, and the Dicer *dcl-2* (Choudhary et al. 2007). In *Neurospora*, QDE-2 and its slicer activity are required for generation of single-stranded siRNA, and gene silencing *in vivo* (Maiti et al. 2007). Also, DCL-2 is responsible for more than 90% of the *Neurospora* Dicer activity (Catalanotto et al. 2004). We have shown that induction of *qde-2* by dsRNA is essential for efficient RNAi (Choudhary et al. 2007).

In all, dsRNA was found to induce about 60 *Neurospora* genes (named dsRNA-induced-genes, or DRAGs). Surprisingly, DRAGs included genes homologous to mammalian anti-viral interferon stimulated genes (ISGs), as well as genes with known or

putative anti-transposon activity. This suggests that induction of RNAi genes by dsRNA is a part of a broader host defense response against invading viruses and transposons.

This chapter describes our efforts to understand the signaling pathways that regulate this dsRNA response. We initiated our study with identification of the characteristics of the *qde-2* promoter that make it responsive to dsRNA. It is likely that some of the DRAGs may share the dsRNA-responsive, transcription-activating pathway with *qde-2*. Therefore, understanding the dsRNA-induced transcriptional regulation of *qde-2* would aid us in understanding how dsRNA regulates some of the other DRAGs as well. We also tested putative dsRNA response elements (dsREs) in the promoter regions of some of the DRAGs. Together, our results lead to an understanding of the promoter elements required for dsRNA response. This knowledge can be used to identify the factors that bind these elements upon dsRNA induction, and thus deciphering the signaling pathways of the dsRNA response.

We employed 5'RACE and promoter deletion analysis to identify the region of *qde-2* promoter necessary for dsRNA induction. In the course of our experiments we found that QDE-2 has an additional 10 kDa N-terminal RGG domain, which is important for its activity. Our data indicated that the *qde-2* promoter has distinct regions that orchestrate early or delayed induction in response to dsRNA. The region required for early response was further analyzed through internal deletion and sufficiency studies. Together with bioinformatics analyses comparing the promoter sequences of *qde-2* and other DRAGs, and gel shift experiments, our promoter analyses led us to identify the GC-rich conserved element required for dsRNA response.

4.2 Materials and methods

4.2.1 Strains and culture conditions.

87-3 (*bd a*) was the wild type strain used in this study. 301-6 (*bd his-3 A*) was the host strain used for the insertion of *his-3*-targeting constructs. The wild type transgenic qads*al-1* strain, the *qde-2*^{RIP} strain and the *qde-2*^{RIP} transgenic qads*al-1* strain have been described previously, and in Chapter 3 (Choudhary et al. 2007). Culture conditions have been described in detail in Chapter 3. Knock-down of *al-1* expression was scored by observing the color of transgenic ds*al-1* carrying strains on 10^{-2}M QA slants, 2 days after inoculation.

4.2.2 Constructs

We have previously reported the creation of an expression vector to express N-terminal Myc-His epitope tagged proteins in *Neurospora*, driven by the *qa-2* promoter (He et al. 2003; He et al. 2005). A PCR fragment containing the entire *qde-2* ORF (starting from either the previously predicted or real translational start site) and 3'UTR was cloned into the p*qa-2*-Myc-His vector to generate the epitope tagged-QDE-2 producing constructs.

The pqads*al-1* construct has been described in Chapter 3. To create plasmids for *qde-2* promoter analyses, PCR fragments of *qde-2* (containing different lengths of its promoter, the entire open reading frame, and ~700 bases of its 3' untranslated region) were inserted into the *Nde* I site of the pqads*al-1* construct.

Internal deletion constructs were prepared as follows: two fragments of *qde-2* promoter and open reading frame (ORF) were amplified by PCR. The first (Ag) started 1395 bp upstream from the first base of the *qde-2* ORF, and ended just before the region to be deleted. The second fragment (Dr) spanned from just after the deletion, to 853 bp downstream from +1. Primers were designed to introduce *Eco* RI restriction sites at the 3' end of Ag, and 5' end of Dr. The two fragments were digested with *Eco* RI, and ligated using T4 DNA Ligase. The ligation product (AgDr) was further subjected to a double digest with *Age* I and *Dra* III, and ligated with *Age* I/ *Dra* III digested construct pqads*al-1*.*qde2*-H. The following primers were used to make *qde-2* promoter internal deletion constructs:

Ag Pqde2-fwd: GCAATCTCACTCTTCACACAGC

Dr Pqde2-rev: CCACGTCACCTCAAAGATGCG

All oligonucleotide sequences are in the 5' to 3' orientation.

The construct pDE3ΔBH.*qde-2* has been described previously (Choudhary et al. 2007). The pDE3ΔBH.*qde-2ΔRGG* construct, which has the RGG domain deleted from QDE-2 in the original construct pDE3ΔBH.*qde-2*, was prepared using the same strategy described above. The primers used to make this construct are:

A-RGG-rev *EcoR* I: CGCGCCGAATTCAGACATGACTGTTAAACTTGTAG

D-RGG-fwd *EcoR* I: GGCGCGGAATTCATGAGCAAGCTTTCGCTCAGCG

Sufficiency constructs were made using the vector pAF35 (Froehlich et al. 2002). This vector has the hygromycin B resistance gene under control of a minimum *Neurospora* promoter (*Am*). Putative dsRNA response elements in the *qde-2*, *Mx-2*, or *rrp-3* promoters were amplified with primers inserting *Apa* I and *Sma* I sites at their 5'

and 3' ends, respectively. These were then subjected to *Apa* I/ *Sma* I double digest and inserted between *Apa* I and *Sma* I restriction sites in the Multiple Cloning Sequence of the plasmid pAF35.

Constructs with point mutations in the *qde-2* promoter were made from the construct pqads*al-1.qde2*-H. Briefly, the -1395bp to + 853bp region from the *qde-2* promoter and ORF was PCR amplified using LA Taq (TAKARA), and sub-cloned into the pCR2.1 vector using TA cloning (TOPO TA Cloning® Kit, Invitrogen). Mutagenesis PCR was performed with 25ng plasmid, 125ng primers (with desired point mutations), and *Pfu* polymerase, using the following conditions: 95°C (30 seconds); 18 cycles of 95°C (30 seconds), 55°C (1 minute) and 68°C (6.5 minutes); and 68°C (7 minutes). The PCR products were digested with *Dpn* I, and electroporated into *E. coli*. Constructs with desired mutations were identified by restriction digests and DNA sequencing. The positive clones were digested with *Age* I and *Dra* III, and the fragment re-cloned into pqads*al-1.qde2*-H. The following primers were used to create the mutagenesis constructs:

Hm1-fwd: CTCCTTCCTGCGGGCGGCCCAGAGGGCTGTCCAATCCGTTTGCTTG

Hm1-rev:

CAAGCAAACGGATTGGACAGCCCTCTGGGCCGCCCGCAGGAAGGAG

Hm2-fwd: GCGGGCGGCCCGACAATCTGCATGGGCCGTTTGCTTGTTTCCTGCC

Hm2-rev:

GGCAGGAAACAAGCAAACGGCCCATGCAGATTGTCGGGCCGCCCGC

Hm1m2-fwd:

CTCCTTCCTGCGGGCGGCCCAGAGGGCTGCATGGGCCGTTTGCTTGTTTCCTG

CC

Hm1m2-rev:

GGCAGGAAACAAGCAAACGGCCCATGCAGCCCTCTGGGCCGCCCGCAGGAA

GGAG

Hm4-fwd:

GACATCCATGTCTCCTTCCTTATTTATTAAAGACAATCTGTCCAATCCGTTTGC

Hm4-rev:

GCAAACGGATTGGACAGATTGTCTTTAATAAATAAGGAAGGAGACATGGATG

TC

All oligonucleotide sequences are in the 5' to 3' orientation.

4.2.3 5'RACE

Rapid Amplification of 5' cDNA ends (5' RACE) was performed using BD SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences). 301-6 ds*al-1* strain, treated with QA for 6 hours, was used to obtain total RNA. For preparation of first-strand cDNA, 1µg of total RNA was added to 1µl 5'-CDS primer (12µM), 1µl BD SMART II A oligonucleotide (12µM), and sterile ddH_2O to a final volume of 5µl. The mixture was incubated at 70°C for 2 minutes, and then cooled on ice for 2 minutes. Next, the following were added to the mixture: 2µl 5X First-Strand buffer, 1µl dithiothreitol (20mM), 1µl dNTP mix (10mM), and 1µl BD PowerScript Reverse Transcriptase. The reaction mixture was incubated at 42°C for 1.5 hours in an air incubator. After diluting the first-strand reaction product with 100µl Tricine-EDTA Buffer, the mixture was heated at 72°C for 7 minutes, and stored at -80°C. Aliquots of the cDNA were purified using QIAquick PCR purification kit (Qiagen), and used as the template for RACE PCR.

The following PCR Master Mix was mixed for each 50µl reaction: 34.5µl sterile water, 5µl 10X BD Advantage 2 PCR Buffer, 1µl dNTP Mix (10mM) and 1µl 50X BD Advantage 2 Polymerase Mix (41.5µl total volume). The 5'RACE PCR reaction was prepared by adding 2.5µl cDNA, 5µl Universal Primer Mix (UPM, 10X), and Gene Specific Primer (GSP, 10µM). Two control reactions were also set up: UPM only (2.5µl cDNA, 5µl UPM, 1µl water) and GSP only (2.5µl cDNA, 1µl GSP, 5µl water). The GSP primers were designed in anti-sense direction to the *qde-2* reading frame, with Tm > 70°C. Touchdown PCR was used to determine 5' end of *qde-2* transcript through RACE The following Touch down PCR program was used to amplify 5' cDNA ends: 5 cycles of 94°C (30 seconds), 72°C (3 minutes); 5 cycles of 94°C (30 seconds), 70°C (30 seconds), 72°C (3 minutes); and 25 cycles of 94°C (30 seconds), 68°C (30 seconds), 72°C (3 minutes). 5-10µl of the PCR product was run on agarose gel; the DNA bands were excised and purified using QIAquick Gel Purification Kit (QIAGEN). DNA sequencing was used to identify the 5'end of cDNA.

4.2.4 Protein and RNA analyses

Protein and RNA analyses were performed as previously described, and in Chapter 2 (Choudhary et al. 2007).

4.2.5 *Co-immunoprecipitation of siRNAs*

Co-immunoprecipitation of siRNAs was performed as previously described (Maiti et al. 2007). Cell extracts were prepared in a buffer containing 25mM Tris (pH

7.5), 150mM NaCl, 1.5mM $MgCl_2$, 1% NP40, 1mM DTT, protease inhibitors, and 100 U/mL RNase inhibitor. These were pre-cleared by incubation with 20µL of Protein G Sepharose (GE Healthcare) for 1h at 4°C. For immunoprecipitation of Myc-His-QDE-2 or Myc-His-QDE-2ΔRGG, pre-cleared extracts were incubated with anti-Myc monoclonal antibody overnight at 4°C, and then with 25 µL of Protein G Sepharose for 2 h at 4°C. Immunoprecipitates were washed five times with the extraction buffer. To recover co-precipitated RNAs, 150 µL of TE (10mM Tris, 1mM EDTA at pH 7) was added to the precipitates, followed by phenol/chloroform extraction and ethanol precipitation. The precipitated RNAs were analyzed by electrophoresis on 16% native polyacrylamide gel, and subsequent Northern blot analysis using single-stranded RNA probe.

4.2.6 Microconidia purification

For microconidia purification, heterokaryon transformants were inoculated on four 5ml minimum slants (1X Vogel's medium, 3% (w/v) sucrose, 1.5% (w/v) Select Agar), and grown for 1 week. 5ml Sorbitol (1M) was added to each slant, followed by vortexing. The supernatant was transferred to a 50ml centrifuge tube. An additional 2ml of Sorbitol was added to each slant, followed by vortexing, and the supernatant transferred to the 50ml centrifuge tube. The supernatant was filtered using a 5µm Millex®-SV filter (Millipore). Different volumes (100µl to 5 ml) of the filtered supernatant were plated on 10^{-2} M QA plates. White colonies were picked up and inoculated on 10^{-2} M QA slants. The strains that were completely white were taken to be homokaryons.

4.2.7 qRT-PCR

qRT-PCR was performed as previously described, with a few modifications (Kurrasch et al. 2004). 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 qRT-PCR products. Primer mixes were prepared by mixing 15µl each of forward and reverse primers (100µM) with 970µl sterile water. These were stored at -20°C. Total RNA was extracted and equal amounts treated with DNase I (10 U/µl, Roche). The DNase reaction mix contained total RNA (0.4µg), 0.5U DNase I and 1.68µl $MgCl_2$ (25mM) to a final volume of 10µl. DNase I treatment was performed by incubation at 37°C for 30 minutes, denaturation at 75°C for 10 minutes, followed by storage at 4°C.

The following were mixed to prepare 2X RT mix for each sample: 4.2µl sterile water, 2µl 10X RT buffer, 2µl 10X random primers, 0.8µl 25X dNTP, and 1µl reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). 10µl of the RT mix was added to each sample. Reverse transcription was carried out by incubation as follows: 25°C for 10 minutes, 37°C for 90 minutes, 72°C for 10 minutes, and then storage at 4°C. As a control for each set of experiments, one additional sample was prepared as a NoRT control. The reverse transcribed samples were diluted by adding 20µl sterile water to each sample.

5µl of the cDNA was added to 15µl qRT-PCR mix (10µl SYBR Green PCR Master Mix (Applied Biosystems), 2.4µl primer mix and 2.6µl sterile water). Out of the 20µl reaction mix, 10µl was loaded onto each well. Levels of the housekeeping gene

beta-tubulin were used to calculate changes in gene expression (*n*-fold) by comparing mean threshold cycle values.

4.2.8 Extraction of nuclear protein

Extraction of nuclear protein was performed as previously described, with a few modifications (Froehlich et al. 2002). Conidia were harvested from large slants, inoculated into low glucose medium, and incubated at room temperature for 2 days, with shaking. For QA treatment, 14ml 0.5M QA was added (final concentration 10^{-2}M) 2 hours before harvest. After harvest, the tissue was vacuum-dried, and frozen in liquid nitrogen. The harvested tissue (~8g) was ground using 3g glass beads and liquid Nitrogen. The ground tissue was transferred to a flask containing 12ml cold Buffer A (1M sorbitol, 7(w/v) ficoll-type70, 20(v/v)glycerol, 5mM Mg(AC)2, 3mM CaCl2, 50mM Tris-HCl pH7.5, 3mM DTT), and incubated on ice 5-10 minutes, with stirring. Next, the crude homogenate was filtered through four layers of cheesecloth. 16ml of buffer B (10(v/v) glycerol, 5mM Mg(AC)2, 25mM Tris-HCl, pH 7.5) was slowly added to the homogenate, with stirring. To pellet cellular debris, the mixture was layered on top of 10ml of buffer A/B [Buffers A and B mixed in the ratio 2.5:4] in a 50mltube, and centrifuged at 4100 rpm for 7 min at 4°C in a SW28 rotor. To pellet the nuclei, the supernatant was layered on top of 5ml of buffer D (1 M sucrose, 10(v/v) glycerol, 5mM Mg(AC)2, 25mM Tris-HCl, pH 7.5, 1mM DTT) in another 50ml tube and centrifuged at 7200 rpm for 15 min at 4°C. 1ml of the supernatant was stored as the cytosolic fraction. The pelleted nuclei were transferred to a microcentrifuge tube and re-suspended in buffer D at a 2:1 ratio (nuclei: buffer D). Protein extraction buffer [50 mM HEPES pH 7.4, 10

(v/v) glycerol, 137 mM NaCl] was slowly added to a final ratio of 2:1 (re-suspended nuclei: extraction buffer) and the mixture stored on ice. Nuclei were broken by sonication. The nuclear suspension was then centrifuged at 14000 rpm for 15 min at 4□C to pellet the nuclear debris, and the supernatant (referred to as nuclear protein extract) was aliquoted, flash-frozen in liquid nitrogen, and stored at –80□C. All buffers contained the protease inhibitors 10µg/ml pepstatin A, 10µg/ml leupeptin, and 1mM phenylmethylsulfonyl flouride (PMSF).

4.2.9 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as previously described, with a few modifications (Froehlich et al. 2002). Double- stranded DNA oligonucleotides were prepared by annealing two complementary single-stranded oligonucleotides of equal lengths. For annealing, 10µl of each oligonucleotide (100µM stock) was mixed with 10µl of 10X Annealing buffer and 70µl sterile water. The mixture was heated at 85°C for 4 minutes, and then gradually cooled to room temperature. End-labeled probes were prepared using T4 polynucelotide kinase (T4 PNK, from NEB) and [$^{\gamma-32}$P] ATP. The reaction mixture for end-labeling contained the following: 9.5µl sterile water, 2µl 10X T4 PNK buffer, 1µl double-stranded oligonucleotide, 0.5µl Bovine serum albumin (BSA, 2mg/ml stock), 2µl T4 PNK, and 5µl [$^{\gamma-32}$P] ATP. The reaction was carried out at 37°C for 1 hour. The reaction mix was diluted to 40µl with TE buffer (pH 8.0). Phenol-chloroform extraction was performed to separate proteins. To remove the unincorporated ATP, the supernatant was transferred to a previously prepared Micro Bio-Spin® P-30 Tris Chromatography column (Bio-Rad), and centrifuged at 3100 rpm for 4

minutes 1µl of the purified probe was used to determine radiation counts using the LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter).

4% Tris-Borate-EDTA (TBE) gel was prepared by mixing the following: 14.3ml sterile water, 5.3ml 30% Acrylamide / Bis solution, 19:1 (5% C) (Bio-Rad), 20ml 1X TBE buffer with 5% (v/v) glycerol, 0.4ml 10% ammonium persulfate, and 40µl tetramethylethylenediamine (TEMED). The gel was pre-run at 100V for 1 hour, using 0.5 X TBE, 2.5% (v/v) glycerol as gel running buffer.

For each reaction, 20µg of the nuclear protein extract was diluted to 10µl with Binding Buffer (20mM HEPES, 1mM EDTA, 2mM $MgCl_2$, 20µM $ZnCl_2$, 10% (v/v) glycerol and 40mM KCl). The following probe mix was prepared for each reaction: 1µl BSA (10 mg/ml stock), poly dI: dC (1 mg/ml stock, Roche), 1µl KCl (1M stock), 160 X 10^3 counts radio-labeled probe, and binding buffer to a final volume of 10µl. The nuclear extract and probe were mixed and incubated on ice for 1 hour. This was followed by gel electrophoresis at 150 V for 2.5 hours. Next, the gel was placed on a filter-paper and dried in a Model 583 Gel Dryer (Bio-Rad). The dried gel was then exposed to X-ray film. The following oligonucleotides (sequences are in the 5' to 3' orientation.) were used to make probes for gel shift assays:

15f: TCCTGCGGGCGGCCCGACAATCTGTCCAAT

15r: ATTGGACAGATTGTCGGGCCGCCCGCAGGA

16f: GACAATCTGTCCAATCCGTTTGCTTGTTTC

16r: GAAACAAGCAAACGGATTGGACAGATTGTC

15m1-fwd: TCCTGCG ATAAAA CCGACAATCTGTCCAAT

15m1-rev: ATTGGACAGATTGTCGGTTTTATCGCAGGA

15m2-fwd: TCCTGCGGGCGGCCCAGAGGGCTGTCCAAT

15m2-rev: ATTGGACAGCCCTCTGGGCCGCCCGCAGGA

16m1-fwd: AGAGGGCTGTCCAATCCGTTTGCTTGTTTC

16m1-rev: GAAACAAGCAAACGGATTGGACAGCCCTCT

16m2-fwd: GACAATCTGCATGGGCCGTTTGCTTGTTTC

16m2-rev: GAAACAAGCAAACGGCCCATGCAGATTGTC

1516m1-f: TCCTGCGGGCGGCCCAGAGGGCTGTCCAATCCGTTTGCTTGTTTC

1516m1-r: GAAACAAGCAAACGGATTGGACAGCCCTCTGGGCCGCCCGCAGGA

1516m2-f: TCCTGCGGGCGGCCCGACAATCTGCATGGG CCGTTTGCTTGTTTC

1516m2-r: GAAACAAGCAAACGGCCCATGCAGATTGTCGGGCCGCCCGCAGGA

1516m3-f: TCCTGCGGGCGGCCCAGAGGGCTGCATGGGCCGTTTGCTTGTTTC

1516m3-r: GAAACAAGCAAACGGCCCATGCAGCCCTCTGGGCCGCCCGCAGGA

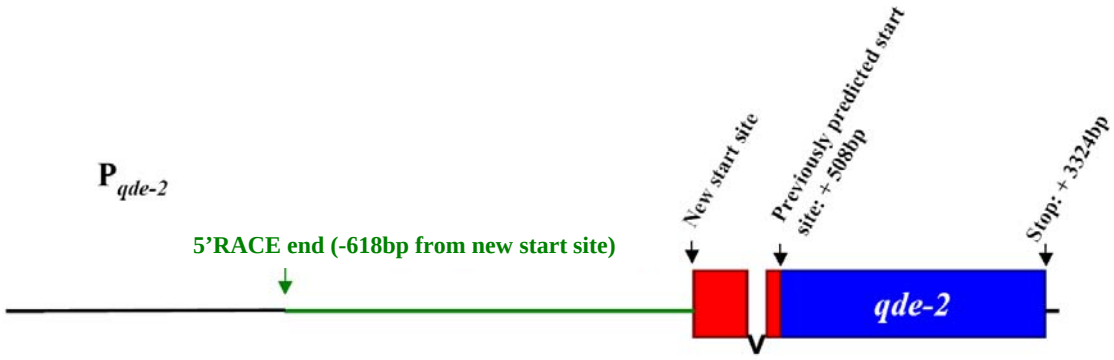
4.3 Results

4.3.1 5'RACE reveals the presence of an additional N-terminal RGG domain in QDE-2.

5' RACE was carried out to determine the 5' end of the *qde-2* transcript. Initial 5'RACE experiments indicated that *qde-2* mRNA had a very long 5'UTR. (Figure 12A) In addition, 5'RACE revealed the presence of a 54 bp intron 100 bases upstream of the previously predicted start site. The location of the intron is such that it eliminates several stop codons from the mature transcript. This, in turn, raises the possibility that an ATG approximately 500 bases upstream of the previously predicted start site could function as the actual translational start site. Translation from this new ATG would add an additional

10 kDa arginine/ glycine – rich RGG domain, bringing the total size of QDE-2 up to 110 kDa. Additional 5'RACE experiments indicated that the 5'UTR of QDE-2 extends up to 618 bp from the new, real start site. In fact, the size of QDE-2 protein observed in Western Blots correlates well with this increased size. In addition, Ago2s from other fungi such as *Magnaporthe grisea* and *Aspergillus nidulans* are predicted to possess an N-terminal RGG-rich region, which also suggests that *Neurospora* QDE-2 may have an RGGs domain. Alignment of the protein sequences of the Ago2s from these fungi clearly shows that the new ATG is a strong candidate for the real start site of QDE-2. (Figure 12B).

A



B

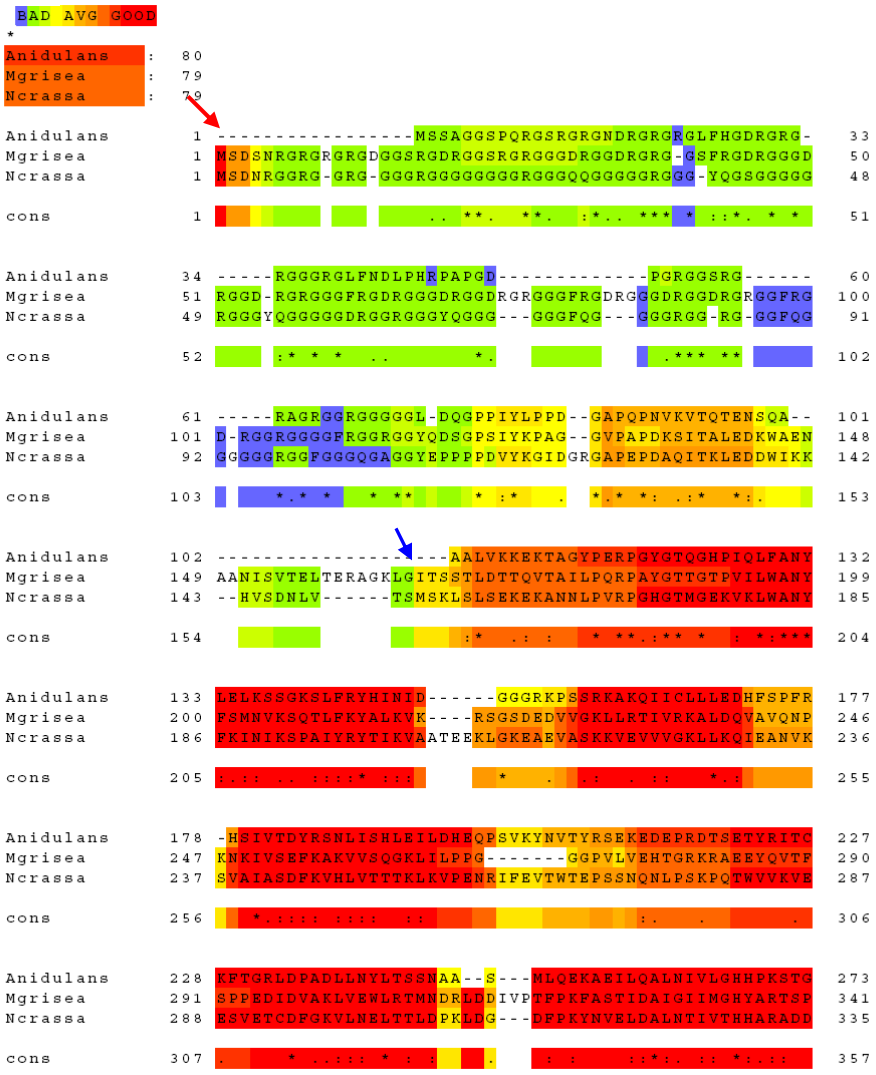


Figure 12. 5'RACE reveals the presence of an additional RGG domain in QDE-2.

(A) Graphic description of the N-terminal RGG domain (red) revealed by 5'RACE. Blue: previously known *qde-2* ORF

(B) Sequence alignment of *N. crassa* QDE-2 with Ago2s from *A. nidulans* and *M. grisea*. The alignment was performed using T-Coffee sequence alignment program. Blue shading represents bad alignment, red: good alignment, as depicted in the graphic on top. Red arrow: New QDE-2 start site. Blue arrow: Previously predicted start site.

4.3.2 Role of the RGG domain in QDE-2 function.

The RGG domain is present in many RNA-binding proteins, and is involved in RNA- binding, as well as protein-protein interactions (Lukasiewicz et al. 2007; Menon and Mihailescu 2007). Arginine- glycine- rich regions are also present in other Argonautes, such as *Trypanosoma brucei* Ago1 and *Arabidopsis* AGO1. Deletion of the RGG domain from the *T. brucei* Ago1 ended its association with polyribosomes, and severely decreased target mRNA cleavage (Shi et al. 2004).

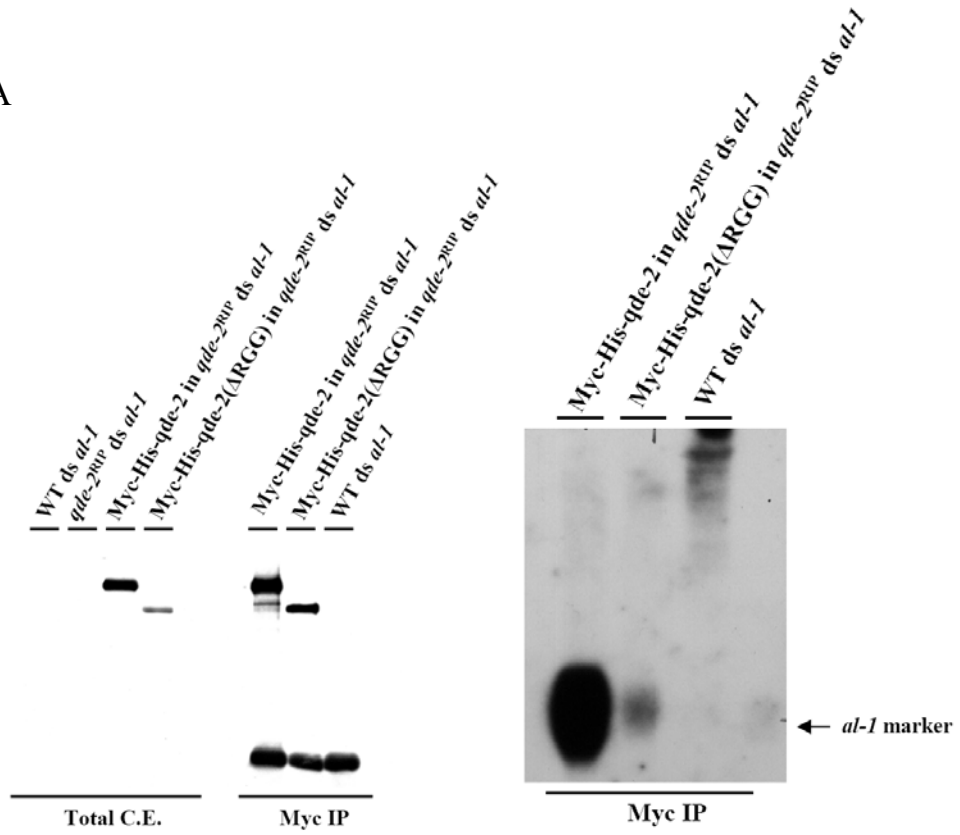
In order to assess the importance of the RGG domain for QDE-2 function, we used constructs with Myc-His tagged QDE-2 (with sequence starting from new or old ATG), driven by the QA inducible *qa-2* promoter. These were co-transformed into a transgenic dsRNA producing strain with *qde-2*^{RIP} background. The resultant strains would produce dsRNA, as well as transgenic tagged QDE-2, in the presence of QA. As expected, we observed that QDE-2 translated from the 'new' start site was larger in size than that translated from the 'old' start site. Myc immunoprecipitation was used to pull-down both full-length and RGG-domain-less (or ΔRGG) QDE-2 proteins and the RNA species bound to them (Figure 13A). Northern blot analysis of the co-immunoprecipitated RNAs showed that as compared to full-length QDE-2, ΔRGG QDE-2 pulled down substantially less amount of *al-1* siRNA. This result suggests that the RGG domain is important for binding siRNA.

Quelling experiments further confirmed *in vivo* the importance of the RGG domain in QDE-2 function. We observed that Myc-His tagged ΔRGG QDE-2 could not rescue quelling (as compared to partial rescue by Myc-His tagged full-length QDE-2),

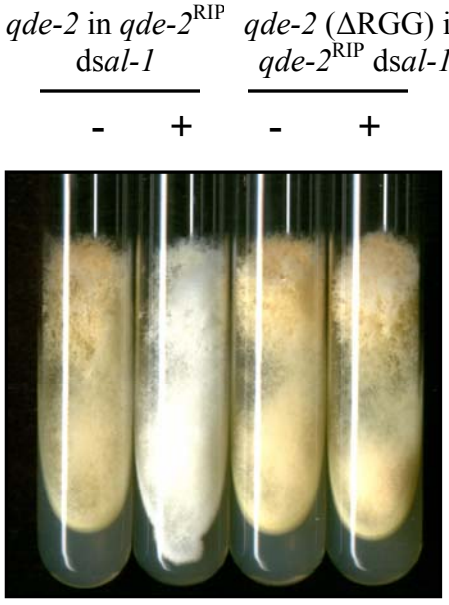
which indicates that the ΔRGG QDE-2 is not able to complement the function of the endogenous QDE-2 (data not shown).

It is possible that the presence of an epitope tag may affect QDE-2 activity. Therefore, we created a *qde-2*^{RIP} ds*al-1* strain with transgenic ΔRGG QDE-2 driven by its own promoter. As shown in Figure 13B, the aerial hyphae and conidia of the ΔRGG QDE-2 carrying strain remained orange in the presence of QA, indicating a severe reduction of RNAi efficiency. This data further underscores the importance of the RGG domain in QDE-2 activity. In a control experiment, a *qde-2*^{RIP} ds*al-1* strain with transgenic QDE-2 driven by its own promoter showed full complementation of the function of the endogenous *qde-2*, as indicated by the efficient silencing of *al-1* (in the presence of QA). In addition, the ΔRGG QDE-2 protein is expressed at a much lower level than full-length QDE-2 (Figure 13C). This data suggests that a decrease in the ability to bind siRNA reduces the stability of QDE-2.

A


WT ds al-1
qde-2RIP ds al-1
Myc-His-qde-2 in qde-2RIP ds al-1
Myc-His-qde-2(ΔRGG) in qde-2RIP ds al-1
Myc-His-qde-2 in qde-2RIP ds al-1
Myc-His-qde-2(ΔRGG) in qde-2RIP ds al-1
WT ds al-1
Total C.E.
Myc IP
Myc-His-qde-2 in qde-2RIP ds al-1
Myc-His-qde-2(ΔRGG) in qde-2RIP ds al-1
WT ds al-1
al-1 marker
Myc IP

B


qde-2 in qde-2RIP dsal-1
qde-2 (ΔRGG) in qde-2RIP dsal-1
-
+
-
+

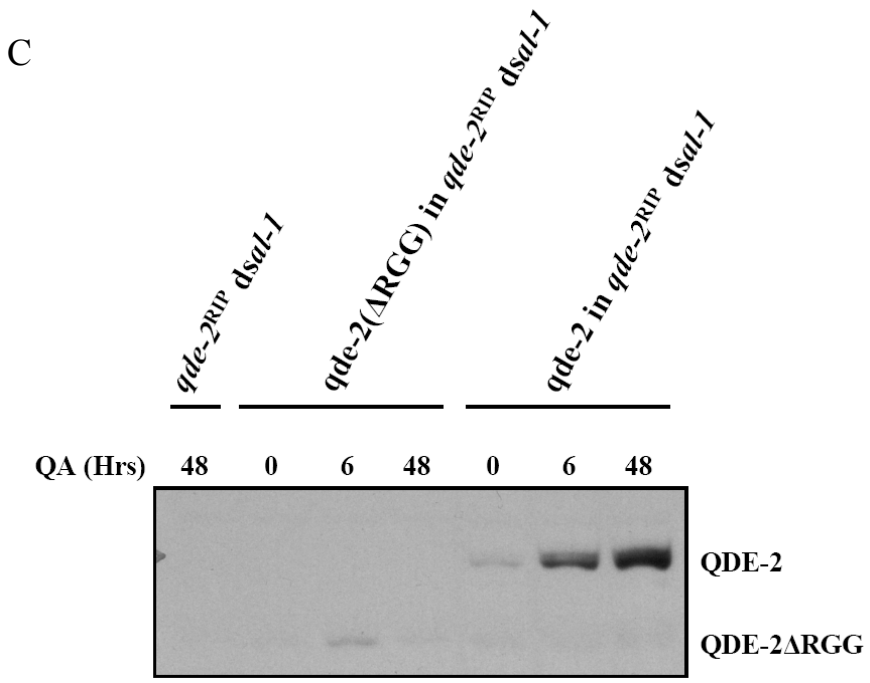


Figure 13. The RGG domain is required for QDE-2 activity and efficient RNAi.

(A) (Left panel) Western blot showing immunoprecipitation of Myc-His epitope-tagged QDE-2 (or ΔRGG QDE-2) using a monoclonal anti-Myc antibody. All strains were grown with QA for 2 days before harvest. (Right panel) Northern blot analysis showing the co-immunoprecipitation of siRNA species with epitope-tagged QDE-2 (or ΔRGG QDE-2). WT: wild type.

(B) Pictures of *Neurospora* slants showing that the gene silencing of *al-1* is abolished in the *qde-2*^{RIP} ds*al-1*, *qde-2* (ΔRGG) strain, but it is rescued in the *qde-2*^{RIP} ds*al-1*, *qde-2* strain.

(C) Western blot showing that ΔRGG QDE-2 is expressed at a lower level than full-length QDE-2. The number of hours indicates the time after the addition of QA.

4.3.3 dsRNA induced transcriptional activation of qde-2 is a combination of early and late responses.

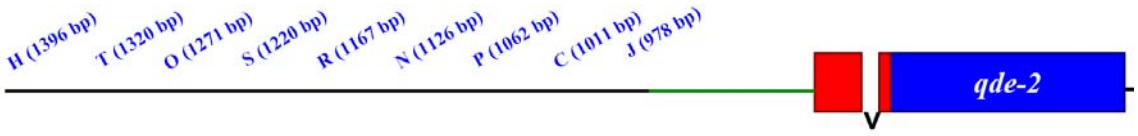
In order to identify the *qde-2* promoter region involved in the dsRNA response, we created *his*-targeting constructs containing both- a QA-inducible *al-1* dsRNA producing cassette, and *qde-2* sequence. The *qde-2* sequence in these constructs included its entire ORF, 0.7 kb of its 3'UTR, and varying lengths of its 5'UTR. These constructs were inserted at the *his-3* locus in a *his-3 qde2*^{RIP} strain described previously, and in

Chapter 3 (Choudhary et al. 2007). A *qde-2*-null background ensured that all the *qde-2* mRNA and protein read-outs were from our transgenic construct. Positive transformants were identified by PCR (using a forward primer in the vector sequence of the construct, and a reverse primer in *qde-2* promoter); or by assaying QDE-2 levels by Western Blotting (data not shown). As an additional control, fold changes of transcripts of other DRAGs, such as *Mx-2* and *rrp-3*, were also assayed to ensure that the dsRNA cassette was functional (data not shown). Microconidia purification was used to obtain homokaryons, which were then further tested for dsRNA response by comparing *qde-2* transcript levels after addition of QA. The longest promoter fragment (1396 bp from the new start site) was present in the construct H, while the shortest (978 bp from the new start site) was present in the construct J. The *qde-2* promoter deletions are shown in Figure 14A, and their response to dsRNA is summarized in Table 2.

We found that *qde-2* has at least two different transcriptional responses to dsRNA. Based on the time required for induction after addition of QA, we have named them early and late responses, respectively. The mutant H exhibited an early response (with 6 hour-QA treatment) comparable to the wild type transgenic ds*al-1* strain (Figure 14B). This transcriptional induction was lost by the mutant C (1011 bp from the new start site). However, with a longer QA treatment, both H and C exhibited an increase in *qde-2* mRNA comparable to the wild type transgenic ds*al-1* strain. The difference between the two responses was studied by comparing the kinetics of QDE-2 protein induction in mutants H and C (Figure 14C). While H displays kinetics comparable to the wild type transgenic dsRNA producing strain (compare with Figure 7B, Chapter 3), C shows a slower increase in QDE-2 accumulation. However, QDE-2 levels in H and C are

comparable 48 hours after addition of QA. In contrast, J, which is 33 nucleotides smaller than C, appears to have lost the late response as well. These data suggest that an early response element lies between H and C; and a late response element is situated between C and J.

A



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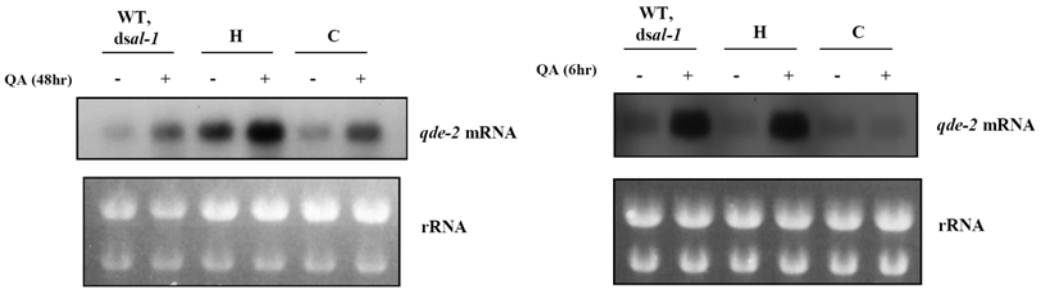


C

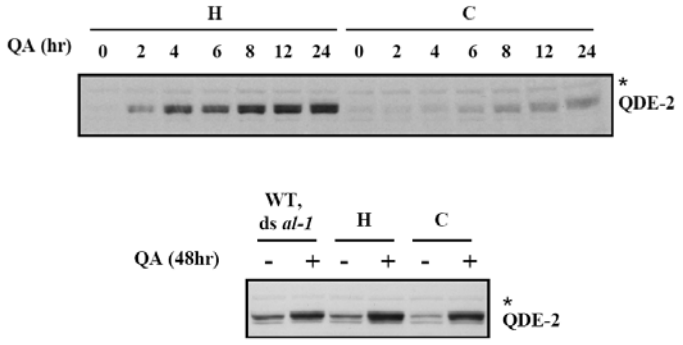


Figure 14. Different regions of the *qde-2* promoter orchestrate early and late responses to dsRNA.

(A) Graphic depiction of the promoter deletion mutants. Numbers in brackets represent the length of the upstream sequence from the real translational start site.

(B) Northern blot analyses showing *qde-2* mRNA levels in the indicated strains with (Left) 48 hour or (Right) 6 hour QA treatment.

(C) Western blot analyses showing the expression of QDE-2 in indicated strains. The number of hours indicates the time after addition of QA. The asterisk indicates a non-specific cross-reacting protein band recognized by our QDE-2 antibody.

4.3.4 A 50 bp region in the qde-2 promoter is involved in early response to dsRNA.

Since we are interested in understanding the signaling pathways directly triggered by dsRNA, we elected to focus on the early dsRNA response. Therefore further promoter deletions between H and C were created to map the early response element. We observed that the mutants H, T, O and S displayed an increase in *qde-2* mRNA at 6 hours of QA induction that was comparable to the wild type transgenic dsRNA producing strain (Figure 15A). On the other hand, mutants R, N and P appear to have lost the *qde-2* transcriptional induction achieved by 6-hour QA treatment.

The combined effects of the early and late responses were assayed by comparing the color of strains grown on QA slants. Mutants from H to C display a white color on QA slants, while J displays a faintly yellowish-orange color. The slight change in color in J may be due to inefficient RNAi. Promoter deletion mutants with promoter lengths

shorter than J did not express either *qde-2* mRNA or QDE-2 protein (data not shown), which suggests that the minimum promoter required for *qde-2* expression extends up to J. Protein profiles, as well as slant phenotypes of some of the mutants are shown in Figure 15B.

In all, our data indicates that the ~50 bp region between S and R is responsible for the early response to dsRNA. This region was further analyzed for its requirement and sufficiency for dsRNA response.

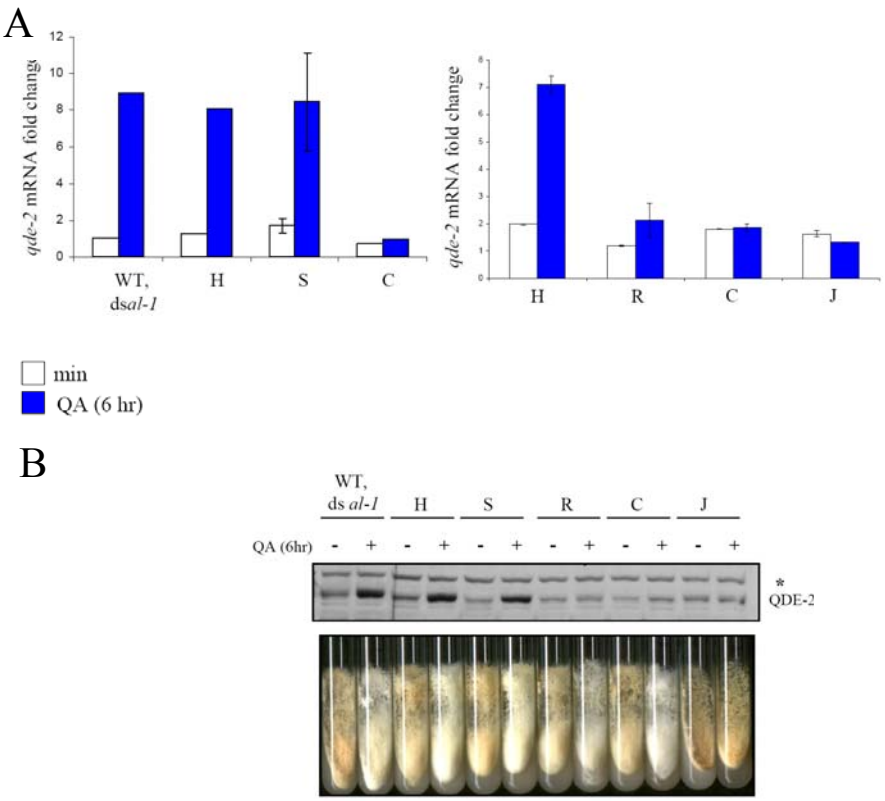


Figure 15. An early dsRNA response element lies between S and R.
(A) qRT-PCR analyses showing the expression of *qde-2* mRNA in the indicated strains. Error bars indicate standard deviation between duplicate samples. White bars: minimum, Blue bars: 6 hour QA treatment before harvest.
(B, top) Western blot analysis showing the expression of QDE-2 in the indicated strains. The asterisk indicates a non-specific cross-reacting protein band recognized by our QDE-2 antibody.
(B, bottom) Photograph of the corresponding strains growing in slants.

Table-2: dsRNA response in *qde-2* promoter deletion mutants

Promoter deletion mutant	Length of *qde-2* upstream region (bp)	Early dsRNA response	Late dsRNA response
H	1396	Yes	Yes
T	1320	Yes	Yes
O	1271	Yes	Yes
S	1220	Yes	Yes
R	1167	No	Yes
N	1126	No	Yes
P	1062	No	Yes
C	1011	No	Yes
J	978	No	No

3.5 The region S-R is required for the early dsRNA response.

In order to study the requirement of different regions of the *qde-2* promoter for the early dsRNA response, we made internal deletions in the *qde-2* promoter region of the construct pqads*al-1.qde-2*-H. The resultant constructs were inserted in a *qde-2*^{RIP} background. qRT-PCR was used to compare *qde-2* transcript levels in strains grown in either minimal medium or with 6 hour QA-treatment. Compared to the induction of *qde-2* transcript in H, we found that removal of either the promoter fragment O-N (construct HΔON), or a shorter fragment S-R (construct HΔSR), abrogates the early response to dsRNA (Figure 16A). This data suggests that *qde-2* promoter region between S and R is required for the early response to dsRNA.

We reasoned that the dsRNA response element (dsRE) in *qde-2* promoter may be shared by other DRAGs. Therefore, *in silico* experiments were carried out to find putative promoter motif(s) shared by *qde-2* and other DRAGS. We compared the S-R sequence with 2kb upstream sequences of other DRAGs, including *Mx-2* and *rrp-3*. A GC-rich element within S-R appeared to be conserved between these DRAGs and *qde-2* (Figure 16B). Also, we were struck by the sequence downstream of the GC-rich region- gaCAATCTGTcCAATCCGT- which includes two almost identical repeats (shown in uppercase). We hypothesized that these repeats may serve as binding sites for factors involved in the dsRNA response. Therefore, this region (called CAAT repeats) was also investigated through requirement and sufficiency experiments.

The constructs HΔ15f-16f, and HΔ16f-R contain deletions of the GC-rich region, and part of the CAAT repeat region, respectively. We found that with both these internal deletions, the 6-hour *qde-2* response was completely abolished (Figure 16C). This data indicates that both the GC-rich region and the downstream CAAT region are required for the early dsRNA response.

As expected, these internal deletion mutants display a white color on QA slants since the promoter region responsible for the late dsRNA response is not affected in any of them (data not shown).

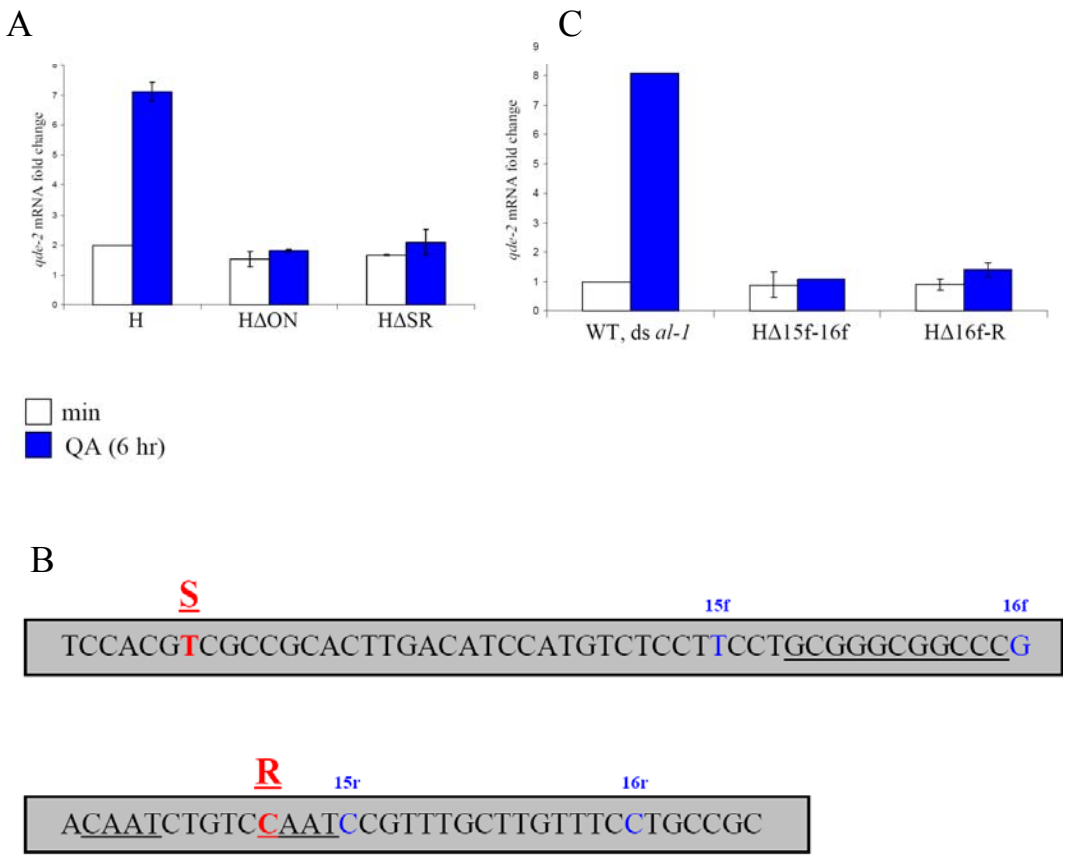


Figure 16. A GC-rich element and CAAT repeats in the *qde-2* promoter are required for the early dsRNA response.
(A and C) qRT-PCR analyses showing the *qde-2* mRNA expression in the indicated strains. White bars: minimum; Blue bars: 6 hrs QA treatment before harvest. The error bars represent standard deviation between duplicate samples. (B) The early response dsREs in the *qde-2* promoter. *qde-2* promoter sequence is shaded in grey. The GC-rich element (underlined) lies between 15f and 16f. The CAAT repeats, also underlined, lie downstream of 16f. S and R, in red, indicate the beginning of the *qde-2* promoter sequence present in the promoter deletion mutants S and R, respectively.

4.3.6 The region S-R is sufficient for early response to dsRNA.

Test for sufficiency is a direct examination of the ability of putative dsREs to respond to a dsRNA trigger. For this purpose, we used an *hph* reporter construct in which the hygromycin B resistance gene (*hph*) is driven by a minimum *Neurospora* (*Am*) promoter. This construct has been reported to attain light inducibility upon insertion of Light response elements (Froehlich et al. 2002). For sufficiency studies, different regions

of the *qde-2* promoter were inserted in the minimum promoter of the reporter construct. The resultant constructs were then co-transformed into a wild type transgenic ds*al-1* strain that has been described previously, and in Chapter 3 (Choudhary et al. 2007). dsRNA response was measured by comparing the *hph* transcript levels in minimum and 6 hour QA- treated cultures using qRT-PCR. We found that the constructs containing the GC-rich element were able to mount a strong response to dsRNA (Figure 17). In contrast, constructs that lack the GC-rich region do not display induction of *hph* by dsRNA. This data suggests that the GC-rich element is sufficient for inducing transcription in response to dsRNA. In addition, the constructs that display sufficiency also contain a portion of the downstream CAAT region, suggesting that the CAAT region may also play a role in the dsRNA response. In a control experiment, the wild type transgenic ds*al-1* producing strain carrying the empty vector does not display induction of *hph* transcript levels in the presence of QA.

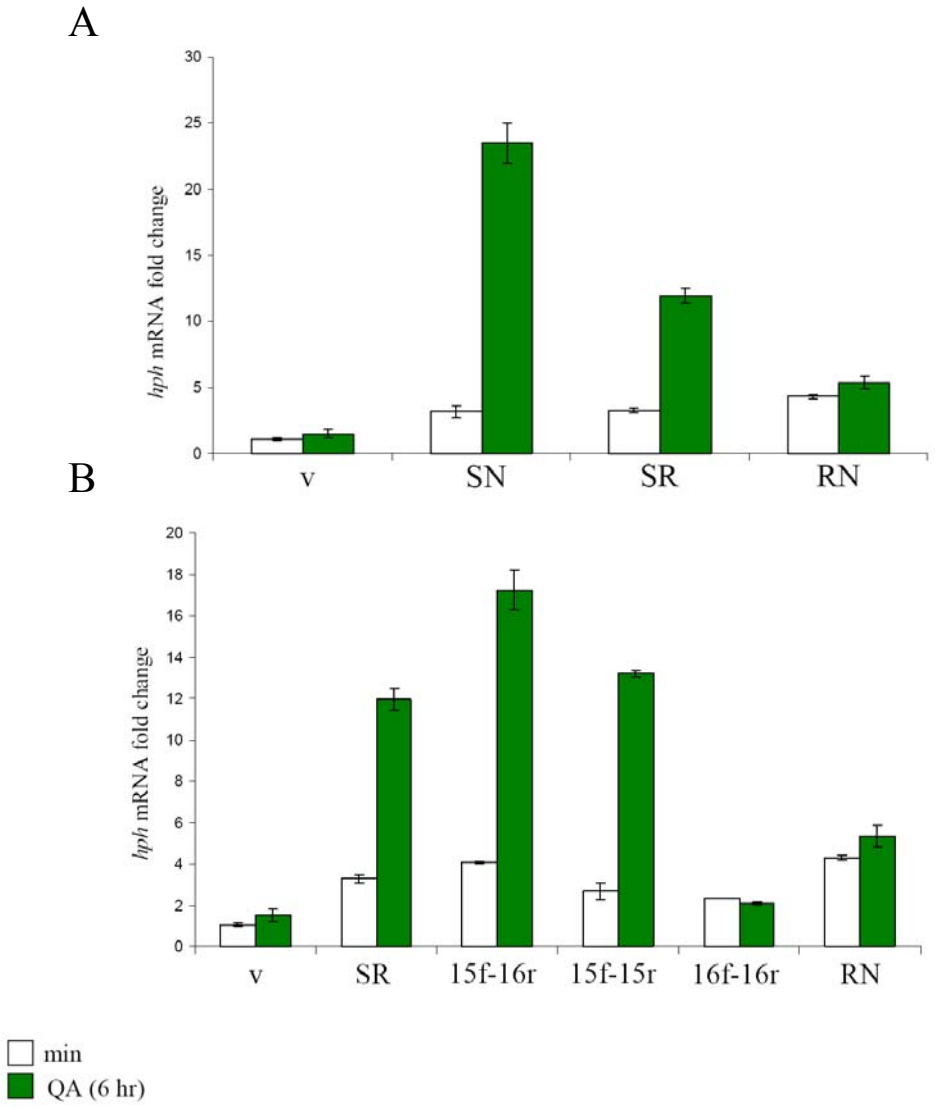


Figure 17. The GC-rich element in the *qde-2* promoter is sufficient for transcriptional induction in the presence of dsRNA.
(A and B) qRT-PCR analyses showing the expression of *hph* mRNA in the indicated strains. v: empty vector. SN: insertion of qde-2 promoter region S to N in the minimum promoter of v, etc.
White bars: minimum; Green bars: 6 hrs QA treatment before harvest. The error bars represent standard deviation between duplicate samples.

4.3.7 Production of dsRNA induced protein binding to the dsREs.

After observing that the GC-rich element and downstream region of the *qde-2* promoter are important for dsRNA response, we next sought to characterize the binding

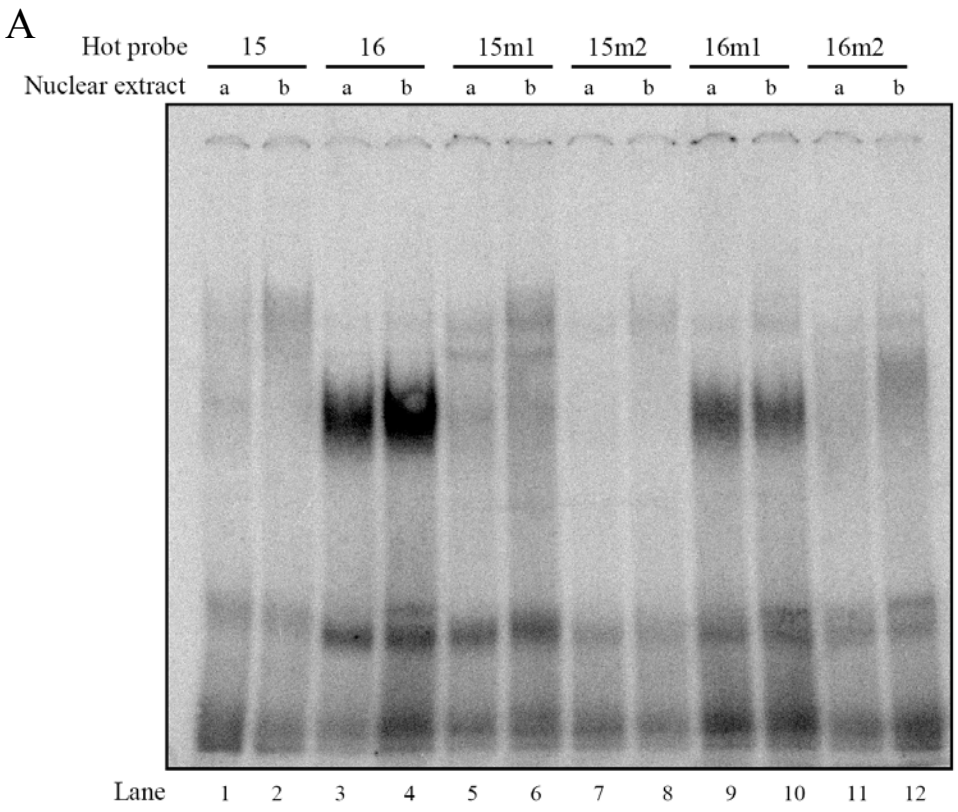
of factors to these promoter elements using electrophoretic mobility shift assays (EMSAs). For EMSAs, 2 hour QA-treated nuclear extracts from wild type and wild type transgenic dsRNA producing strains were incubated with different dsRE probes. Using a 30nt double-stranded DNA probe containing the GC-rich element and part of the downstream CAAT region (probe #15), we observed a slow mobility protein-dsRE complex that was induced by production of dsRNA (Figure 18A, lanes 1 & 2.). An overlapping probe lacking the GC-rich region (probe #16) displayed a faster mobility gel shift, which was also induced by the production of dsRNA (Figure 18A, lanes 3 & 4.).

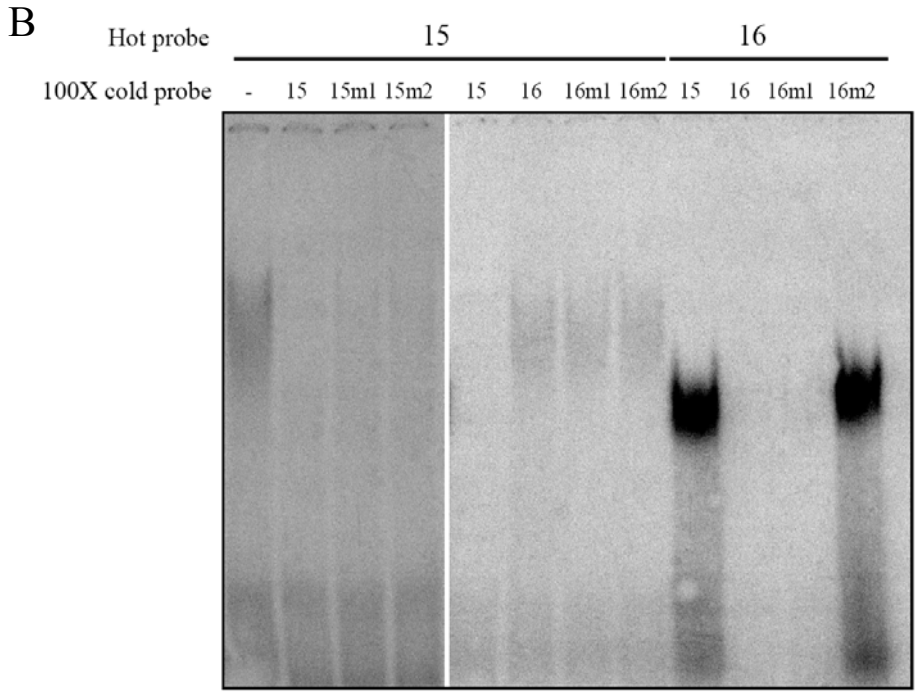
A probe with 6 bases mutated in the GC-rich region also bound the protein complex (Figure 18A, compare lanes 2 & 6). Mutation of the GACAAT sequence from both probes resulted in lesser binding of protein. (Figure 18A, compare lanes 2 & 8, and 4 & 10). In addition, mutation of the TCCAAT sequence severely reduced the faster mobility gel shift (Figure 18A, compare lanes 4 and 12).

Cold chase experiments corroborated this data. As shown in Figure 18B, the gel shift with probe #15 was competed away by cold probe #15, but not by cold probe with mutated GC-rich region, or cold probes lacking the GC-rich region. This suggests that the slow mobility protein-dsRE complex observed with probe #15 corresponds to factors binding the GC-rich region. While the gel shift with probe #16 was competed away by the cold probes #16 and #16 with mutated GACAAT motif, it was not competed away by the cold probes #15 and #16 with mutated TCCAAT motif (Figure 18B). This data suggests that the faster mobility protein-dsRE complex observed with probe #16 corresponds to factors binding the TCCAAT motif and region downstream to it.

Similar results were obtained with a longer probe containing the entire 45 bp region covered by the two overlapping probes # 15 and #16 (Figure 18C). The gel mobilities of the faster mobility gel shifts are similar for two overlapping dsRE probes (#16 and #1516), suggesting that they are likely the same protein-dsRE complex.

Together, our EMSAs suggest that there are two distinct protein complexes binding the GC-rich element, and the CAAT repeats, respectively. In addition, compared to wild type, nuclear extracts from wild type transgenic dsRNA producing strains showed significantly stronger binding with non-mutated probes. This suggests that increased binding of certain factors to these promoter elements is responsible for the transcriptional induction of *qde-2* by dsRNA.


A
Hot probe
15
16
15m1
15m2
16m1
16m2
Nuclear extract
a b a b a b a b a b a b
Lane
1 2 3 4 5 6 7 8 9 10 11 12


B
Hot probe
15
16
100X cold probe
- 15 15m1 15m2 15 16 16m1 16m2 15 16 16m1 16m2

C

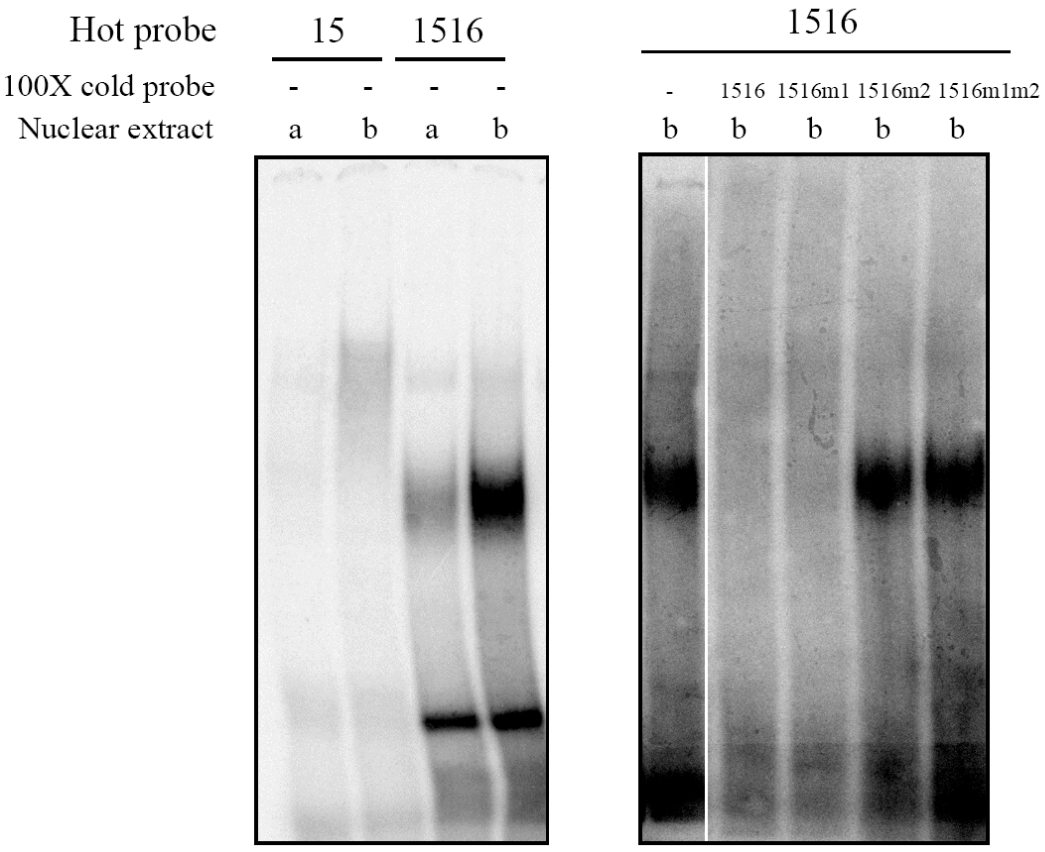
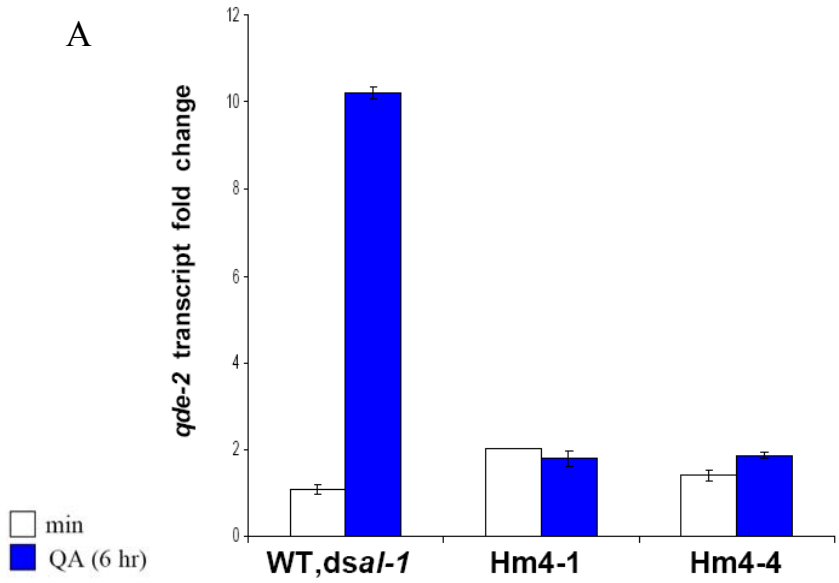


Figure 18. Increased binding at dsREs in response to dsRNA.
(A to C) EMSA assays showing the binding to the dsREs of *qde-2*. In some of the EMSA assays, 100 times cold wild-type or mutated probes were added to analyze their ability to compete away binding. 2 hour QA treated cultures were used for all EMSA assays in this figure. a: wild type (WT), b: WT, ds*al-1*.

4.3.8 Point mutations within the S-R region decrease early response to dsRNA.

We next sought to test *in vivo* the function of the motifs that were suggested by our EMSAs to be important for the dsRNA response. For this purpose, we created constructs with the same point mutations in the *qde-2* promoter. Mutation of the GC-rich

element completely abrogated the early response (Figure 19A). As shown in Figure 19B, mutation of the downstream CAAT repeats also had an impact on the early response. Mutation of the GACAAT motif reduced the response by ~ 40%, while mutation of the TCCAAT motif decreased the induction by 20%. Additionally, mutating both GACAAT and TCCAAT resulted in a severe reduction (approximately 60%) of the dsRNA response. However, this response is still greater than that observed in the *qde-2* promoter deletion mutant C, suggesting that some residual dsRNA response can still function in the absence of the CAAT repeats.



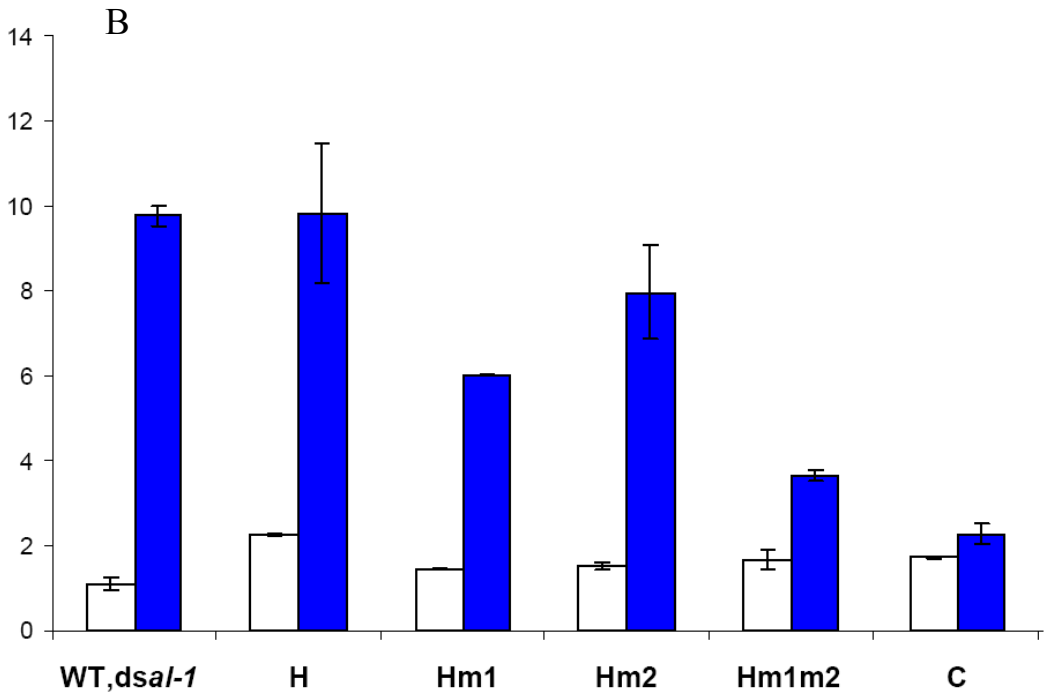
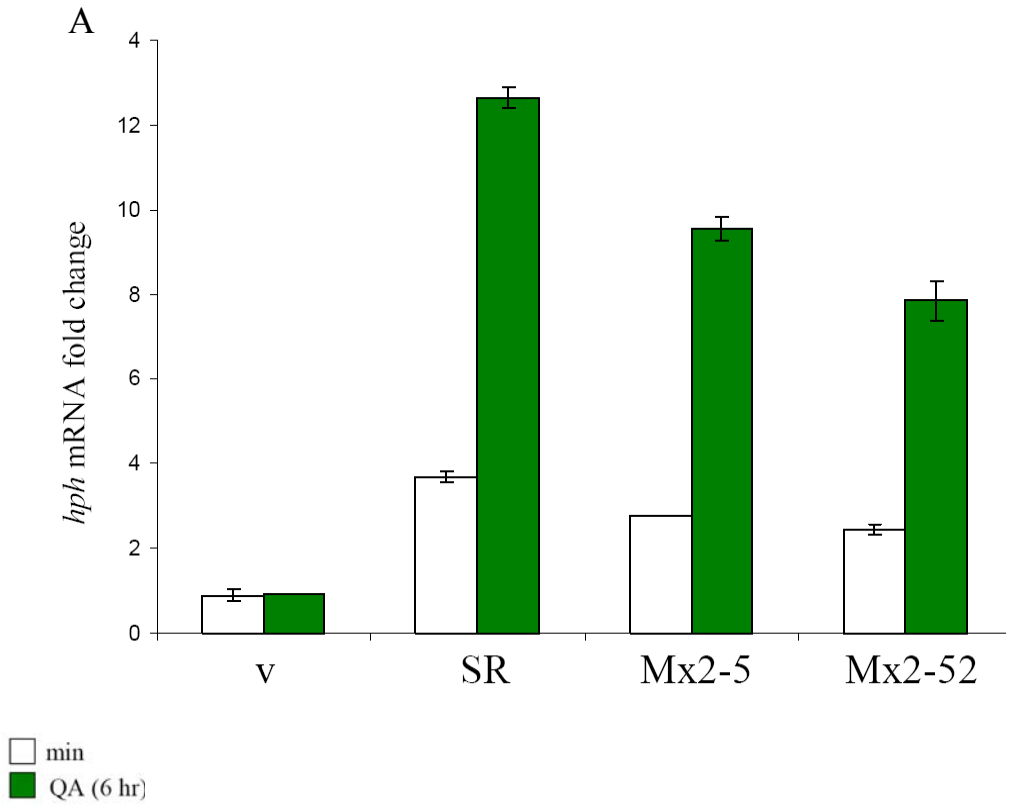


Figure 19. Point mutations within the S-R region decrease early response to dsRNA.
(A and B) qRT-PCR qRT-PCR analyses showing the expression of *qde-2* mRNA in the indicated strains. White bars: minimum; Blue bars: 6 hrs QA treatment before harvest. The error bars represent standard deviation between duplicate samples.
Hm1: GACAAT->AGAGG, Hm2: TCCAAT->CATGGG, Hm1m2 has both these sets of mutations. Hm4: GCGGGCGGCCC->TATTTATTAAA

4.3.9 A GC-rich element is found in the promoters of other DRAGs, and is sufficient for dsRNA-induced transcriptional activation.

As mentioned previously, it is possible that the dsRE in *qde-2* promoter is shared by other DRAGs. Therefore, in order to find a putative motif shared by other DRAGS, we performed *in silico* experiments, comparing the S-R sequence with 2kb upstream sequences of other DRAGs such as *Mx-2*, *rrp-3*, *3' to 5' exonuclease*, *RNA helicase* and *set-6*. Different programs, such as Bioprospector and SCOPE were utilized for this purpose. These *in silico* experiments unveiled several conserved sites in these promoter

regions (data not shown). We performed sufficiency tests in order to understand which of these may function as dsREs. Promoter sequences from *Mx-2* and *rrp-3*, two highly-induced DRAGs, were used for this purpose. Our results show that of the 6 regions tested for sufficiency, one (from *Mx-2*) shows an early dsRNA response comparable to that of *qde-2* (Figure 20A). As shown in Figure 20B, this region contains a GC-rich element. Further experiments to narrow down the *Mx-2* dsRE indicate that this GC-rich element is important for the dsRNA response. Thus, GC-rich elements appear to play an important role in the transcriptional induction of these DRAGs by dsRNA.



B

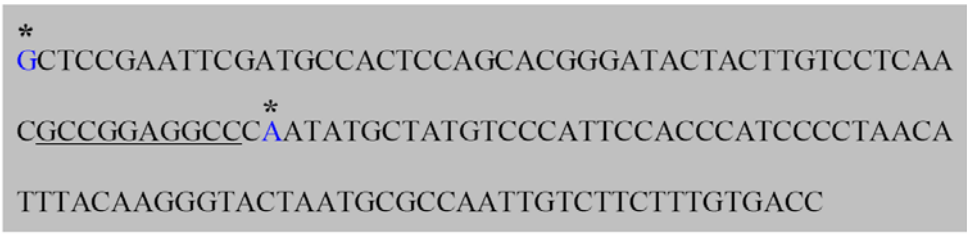


Figure 20. The GC-rich element in the *Mx-2* promoter is sufficient for transcriptional induction in the presence of dsRNA.

(A) qRT-PCR analyses showing the expression of *hph* mRNA in the indicated strains. v: empty vector. SR: insertion of *qde-2* promoter region S to R in the minimum promoter of v, etc. White bars: minimum; Green bars: 6 hrs QA treatment before harvest. The error bars represent standard deviation between duplicate samples.

(B) Sequence of the *Mx-2* promoter region in the sufficiency construct Mx2-5. The first and last nucleotides of the *Mx-2* promoter region in the sufficiency construct Mx2-52, are colored in blue and denoted by asterisks. The GC-rich element is underlined.

4.4 Discussion

In this study, we described the signaling mechanisms leading to dsRNA response in *Neurospora*. We reported the presence of an additional 10 kDa N-terminal RGG domain in the Neurospora *Argonaute* protein QDE-2. We demonstrated that the RGG domain is required for binding to siRNAs, and is important for efficient RNAi *in vivo*. We showed that different regions of the *qde-2* promoter orchestrate early and late responses to dsRNA. Through promoter deletions, internal deletions and sufficiency tests, we narrowed down the *qde-2* dsRE to a GC-rich element and a downstream CAAT repeat motif. We showed that a similar GC-rich element in the *Mx-2* promoter is also a dsRE, thus identifying a dsRE common to this subset of DRAGs.

4.4.1 The RGG domain of QDE-2 is important for efficient binding to siRNAs.

5'RACE experiments, conducted to determine the length of the *qde-2* 5'UTR, led us to identify the real translational start site of QDE-2 (Figure 12). Translation from the new start site would add an additional 10kDa RGG domain to QDE-2. The RGG domain is a bi-functional motif found in many RNA-binding proteins, and is involved in RNA-binding, as well as protein-protein interactions (Lukasiewicz et al. 2007; Menon and Mihailescu 2007). For example, an RGG box is present in the Fragile X mental retardation protein (FMRP) and its autosomal paralog Fragile X related gene 1 (FXR1). A study of the interaction between the RGG box domains of FMRP and FXR1 indicated that this domain is used to bind to the G quartet structure of human semaphorin 3F (S3F) RNA, a target of FMRP. Furthermore, the RGG box is able to unwind the G quartet structure of S3F RNA. On the other hand, the RGG domain of the yeast SR-like protein Npl3p provides substrate docking motifs for its interaction with the kinase Sky1p. In addition, methylation of the arginines in the RGG box decreases its binding to Sky1p, and severely reduces its phosphorylation, resulting in its export from the nucleus.

RGG domains are also found in other Argonautes, such as *Trypanosoma brucei* Ago1 and *Arabidopsis* AGO1. In *T. brucei*, deletion of the RGG domain from Ago1 ended its association with polyribosomes, and severely decreased the cleavage of target mRNA (Shi et al. 2004).

We found that deletion of the RGG domain from the *Neurospora* Argonaute QDE-2 resulted in the loss of efficient RNAi, as evinced by a severe defect in the knock-down of the target *al-1* transcript by ds*al-1*(Figure 13). This loss of activity likely stems from a severe decrease in its ability to bind siRNAs, suggesting that the RGG domain is

plays an important role in this function. Decreased ability to bind siRNAs also appears to reduce the stability of QDE-2, thus further reducing the efficiency of RNAi. Further *in vivo* evidence for the importance of RGG domain came from the observation that the ΔRGG QDE-2 could not rescue quelling.

Together, our results indicate that the RGG domain in QDE-2 is important for binding to siRNAs. So far, in our Western Blots, we have not observed any changes in the size of QDE-2 that would suggest post-translational modifications such as methylation. However, it is possible that the RGG domain of QDE-2 may be used for interaction with other proteins, such as components of the RISC, or for effecting changes in its localization within the cell.

4.4.2 A GC-rich element and CAAT repeat region in the qde-2 promoter are early dsREs.

We have previously shown that the transcriptional induction of *qde-2* by dsRNA is important for efficient RNAi (Choudhary et al. 2007). In order to characterize the promoter elements responsible for induction by dsRNA, we performed promoter deletion studies (Table 2). Our results show that dsRNA-induced transcriptional activation of *qde-2* is a combination of immediate and late responses (Figures 14). Since we are interested in the signaling pathway directly triggered by dsRNA, we focused our attention on identifying the early dsRNA response element. Further promoter deletion studies narrowed- down the dsRE to approximately 50 bp (Figure 15). It is possible that multiple dsREs, either redundant or additive, could be responsible for the early response. However, our results showed that a single promoter region, containing a GC-rich element and CAAT repeats, was both required and sufficient for the early response (Figures 16 &

17). We also observed inducible binding of proteins to the GC-rich element and CAAT region through EMSAs, suggesting that binding of factors to these regions is responsible for the dsRNA response (Figure 18). In addition, mutation of the GC-rich element or the CAAT repeats abrogates the transcriptional activation of *qde-2*, thus underscoring their importance for the early dsRNA response (Figure 19).

4.4.3 A GC-rich dsRE in Mx-2 promoter is sufficient for dsRNA induction.

It is possible that multiple redundant dsRNA response pathways exist in *Neurospora*. We considered the possibility that some of the DRAGs share the dsRNA response pathway regulating *qde-2*. *in silico* experiments demonstrated that the GC-rich element is also conserved among the promoter regions of other DRAGs such as *Mx-2*, *rrp-3*, *3' to 5' exonuclease*, *RNA helicase* and *set*-6. Sufficiency tests established that the GC-rich element in the *Mx-2* promoter elicits an early dsRNA response comparable to that of the GC-rich element in *qde-2* (Figure 20). This suggests that the same dsRNA response pathway acting through the GC-rich element regulates these genes. However, we observed that while the GC-rich element in the *Mx-2* promoter elicits a dsRNA response in reporter constructs, the response is several folds lower than that observed for endogenous *Mx-2*, suggesting that *Mx-2* has additional dsREs not shared by *qde-2*.

Transcription factors that bind these dsREs remain to be identified. C2H2 zinc-finger containing transcription factors such as Sp1 and Krüppel are known to bind GC-rich elements (Kaczynski et al. 2003). In eukaryotes, a conserved *cis*-regulatory CCAAT sequence often found 80-120 bp upstream of transcriptional start sites, serves as the recognition site for many transcriptional activators such as Nuclear factor 1 (NF1) and

CCAAT binding factor (CBF) (Maity and de Crombrugghe 1998). However, the CCAAT repeats in the *qde-2* promoter lie much further upstream from the transcriptional start site to qualify as canonical CAAT boxes.

RNAi has been compared to the innate immune response in higher eukaryotes. In vertebrates, dsRNA (originating from viruses or transposons) is recognized by sensors such as TLR-3, PKR and RIG-I; which trigger a transcriptional cascade initiated by transcriptional factors such as NF-κB and IRF-3. This cascade induces hundreds of ISGs that present a strong defense against invading viruses or transposons. We have observed a similar transcriptional response in *Neurospora* triggered by dsRNA. About 60 genes were found to be induced by dsRNA, including RNAi genes as well as homologs of mammalian ISGs. Since *Neurospora* lacks homologs of known dsRNA sensors and dsRNA-activated transcription factors, it is likely that *Neurospora* dsRNA signaling pathways are entirely novel. Moreover, the presence of ISGs, coupled with the absence of interferons, suggests the existence of an ancient host defense response, which is conserved from *Neurospora* to mammals.

4.5 References

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CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTIONS

5.1 Future studies in identifying components of the *Neurospora* RNAi pathway.

The establishment of a random mutagenesis-based selection to identify additional components of the *Neurospora* RNAi pathway is described in Chapter 2. *rp10*, a conserved component of the 40s small ribosomal subunit was used as the target gene for this selection. We found that large number of our transformants had either disrupted or completely lost the pqads*rp10* construct. This was likely a response to the high toxicity conferred by depletion of the RP10 protein. Of the transformants that carried the pqads*rp10* construct, up to 40% had disruptions in *qde-2* or *dcl-2*, two critical components of the *Neurospora* RNAi machinery. Molecular characterization suggests that the rest of the MRP10 mutants have defects at various steps- in the processing of dsRNA to siRNA, or in the expression of *qde-2* and *dcl-2*.

The mutants that failed to up-regulate *qde-2* mRNA may have a defect in the dsRNA response pathway leading to the induction of *qde-2*. Alternatively, they may have *hph* insertions in genomic regions around *qde-2* that are essential for its activation, but lie outside the limits of our Southern Blot probe. The insertion into these mutants of a construct expressing functional QDE-2 will help to answer this question. Similarly, the insertion of a construct expressing functional DCL-2 into mutants defective in *dcl-2*

induction will help to identify if they have a defect in the dsRNA response pathway responsible for *dcl-2* induction.

Inverse PCR, followed by DNA sequencing, was used to identify the genes that had been disrupted in our mutants. However, the tendency of *hph* to insert at multiple sites in the genome, and as tandem repeats, made this analysis challenging. We reasoned that multiple hits of a single gene would qualify it as a true candidate. However, apart from *qde-2* and *dcl-2*, no other gene was hit twice.

It is possible that if the selection is carried to saturation, more components of the RNAi pathway may be identified. Improved methods for inverse PCR will help to identify candidate genes from the mutants that have been generated. In addition, plasmid rescue can be used to identify the RNAi components affected in these mutants. Other assays like quelling can also be used to test if these mutants are defective in transgenes-induced-gene silencing.

5.2 Future studies in understanding the mechanism of dsRNA response.

In chapter 3, the identification and characterization of a double-stranded RNA response system in *Neurospora* has been described. The production of dsRNA leads to the transcriptional induction of *qde-2* and *dcl-2*. In addition, QDE-2 is regulated by dsRNA at the transcriptional level, and by DCLs and siRNA at the post-transcriptional level. The induction of QDE-2 by dsRNA is required for efficient RNAi. A study of the kinetics of induction revealed that *qde-2* is likely to be an immediate target of the early-acting dsRNA response pathway(s), while a secondary response activates *dcl-2*.

In addition, a genome-wide search for dsRNA-activated genes (DRAGs) identified 60 genes that are activated by dsRNA, including several known components of RNAi. The induction of anti-viral genes and homologs of the mammalian ISGs suggests the existence of an ancient, evolutionarily conserved response against invasion by viruses and transposons. Our data suggests that this host defense response is conserved from fungi to mammals.

So far, no virus has been reported to infect laboratory *Neurospora* strains; therefore establishment of a viral infection system would help to study the physiological relevance of the DRAGs in *Neurospora*. Known mycoviruses from closely related fungi such as *Aspergillus* can be used for this purpose. Additional candidates for establishing a viral infection system include undiscriminating viruses such as the Flock House virus, which replicates in plants, insects, yeast and mammalian cells, and is known to elicit an RNAi response in *C. elegans* and *Drosophila*.

Chapter 4 describes experiments leading to the identification of the promoter elements important for response to dsRNA. Our results show that dsRNA-induced transcriptional activation of *qde-2* is a combination of immediate and late responses. Through promoter deletion, requirement and sufficiency studies, a GC-rich element and downstream CAAT repeat region have been identified as early dsREs. This is supported by EMSAs showing increased binding to these regions upon production of dsRNA. *in silico* experiments show that the GC-rich element is conserved among the promoters of other DRAGs such as *Mx-2* and *rrp-3*. In addition, sufficiency tests have shown that the GC-rich elements in the *qde-2* and *Mx-2* promoters provide comparable dsRNA-

responsive induction, suggesting that these DRAGs share a common dsRNA response pathway that acts through these GC-rich elements.

However, the transcriptional induction imparted by the endogenous *Mx-2* promoter is several folds higher than that provided by the GC-rich element alone. This suggests that novel dsRNA response pathways, not shared by *qde-2*, act on the *Mx-2* promoter. Experiments targeting the *Mx-2* promoter would help to identify the additional dsREs.

Transcription factors that bind these dsREs are of considerable interest. Therefore, future experiments can be targeted towards identifying the factors that bind the GC-rich and CAAT dsREs. Pull-down experiments can be used to identify the species that interact with these DNA sequences. Oligonucleotides with point mutations in either the GC-rich element or the CAAT repeats can be used as negative controls.

Our results show that genes involved in RNAi, such as *qde-2*, *dcl-1*, *dcl-2*, and *qde-1*, are not required for transcriptional activation by dsRNA. Transcriptional response triggered by dsRNA has been reported in mammals, and involves transcription factors such as NF-κB and IRF-3. However, *Neurospora* does not possess homologs of these transcription factors, indicating that the transcription factors that bind the *Neurospora* dsREs are likely to be completely novel.

Another important avenue for future research is the identification of the dsRNA sensor(s) that set the dsRNA response pathways in motion. Since

Neurospora does not appear to have homologs of known mammalian dsRNA sensors such as PKR, Toll-like receptor 3 or RIG-I, it is likely that the dsRNA sensor(s) in this model organism will be completely novel as well. Genetic selections and screens can be used to identify the sensor as well as additional components of the dsRNA response pathway in *Neurospora.*