THE MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

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DEDICATION

To my parents Madan Mohan Maiti and Manjula Maiti

THE MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

by

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THE MECHANISM OF RNA INTERFERENCE IN NEUROSPORA

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In the canonical RNA interference (RNAi) pathway, small-interfering RNA (siRNA) duplexes generated by Dicer are incorporated into the RNA-induced-silencing complex (RISC), and subsequently converted to single-stranded siRNA. Generation of single stranded siRNA is a pre-requisite for recognition and cleavage of the target mRNA by Argonaute. In biochemical experiments, Argonaute generates single-stranded siRNA by cleaving the passenger strand of the siRNA duplex. Mutational analysis of *Neurospora* homologue of Argonaute-2, known as Quelling Deficient -2 (QDE-2), revealed that the endonuclease activity of QDE-2 is required for the generation of single-stranded siRNA *in vivo*. Further biochemical studies to understand the mechanism for

removal of the nicked passenger strand from siRNA duplex, led to the identification of a novel QDE-2 interacting protein (QIP) with a putative exonuclease domain. Disruption of *qip* led to the impairment of RNAi and most of the siRNAs were accumulated in nicked-duplex form. Furthermore, QIP functions as an exonuclease to remove the cleaved passenger strand in a QDE-2 dependent manner. Thus, the cleavage of the passenger strand by QDE2 and its subsequent removal by QIP are critical biochemical steps in *Neurospora* RNAi pathway.

Quelling, an RNAi related phenomenon in *Neurospora*, is induced by multiple copies of transgene. It was proposed that QDE-1 (a RNA dependent RNA polymerase, RdRp) and QDE-3 (a RecQ helicase) functions in quelling pathway by generating double-stranded RNA (dsRNA) from transgenes. To further understand the importance of QDE-1 and QDE-3, quelling assays were performed in the *qde-1*^{ko} and *qde-3*^{ko} strains. In contrast to previous results, the requirement of QDE-1 and QDE-3 was bypassed when the transgene copy number was high. Moreover, gene silencing analyses using strains lacking all potential RdRps suggested that unlike in *C.elegans* and *Arabidopsis*, the amplification of secondary dsRNA or siRNA is largely absent in *Neurospora*.

The search for potential regulatory mechanisms of RNAi components in *Neurospora* led to the identification of a dsRNA response pathway. Two key components of the *Neurospora* RNAi pathway, *qde2* and *dicer like protein-2* (*dcl-2*), are induced by dsRNA at transcriptional and posttranscriptional level. The induction of QDE-2 is required for efficient gene silencing, indicating the importance of this regulatory mechanism in RNAi pathway.

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PRIOR PUBLICATIONS

1. Maiti, M., Lee, H.C. and Liu, Y. (2007). QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and facilitates conversion of duplex siRNA into single strands. *Genes Dev*. **21**(5): 590-600.

2. Choudhary, S*., Lee, H.C*., Maiti, M*., He, Q., Cheng, P., Liu, Q., and Liu, Y. (2007). Activation of the RNAi components as part of the double-stranded RNA induced immune response in a filamentous fungus. *Mol Cell Biol* **27**(11): 3995-4005.

3. Maiti, M., and Liu, Y. (2007). Transgene induced quelling in *Neurospora* can occur independent of QDE-1 and QDE-3.(Manuscript in preparation)

* equal contribution.

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List of Abbreviations

AGO	Argonaute
ATP	Adenosine triphosphate
DCL	Dicer like protein
DCR	Dicer
DNA	Deoxyribonucleic acid
dsRBD	Double stranded RNA binding domain
dsRNA	double-stranded RNA
DUF238	Domain of unknown function 238
EDTA	Ethylenediamine tetra-acetic acid
ERI-1	Enhancer of RNAi-1
FGSC	Fungal genetics stock center
GST	Glutathione S-transferase
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperizine-ethanesulfonic acid
HPLC	High performance liquid chromatography
HSPs	Heat shock proteins
IFNs	Interferons
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Inverted repeat

IRFs	Interferon regulatory factors
ISGs	Interferon stimulated genes
Kb	Kilo base
kD	Kilo Dalton
miRNA	microRNA
miRNP	miRNA-protein complex
mM	Mili molar
mRNA	Messenger RNA
MSUD	Meiotic silencing by unpaired DNA
NF-κB	Nuclear factor-κB
Ni-NTA	Ni^{2+}-nitro-triacetic acid
nt	Nucleotide
OB	oligonucleotide-oligonucleoside binding
ORF	Open reading frame
PAGE	Poly-acrylamide gel electrophoresis
PAZ	Piwi Argonaut and Zwille
PCR	Polymerase chain reaction
PEG	Poly-ethylene glycol
PIWI	P-element induced wimpy testis
PKR	Protein kinase R
PNK	Polynucleotide kinase
pre-miRNAs	precursor of microRNA

pri-miRNA	Primary microRNA transcript
PTGS	Post transcriptional gene silencing
PVDF	Polyvinylidene Difluoride
QA	Quininc acid
QDE	Quelling deficient
QIP	QDE-2 interacting protein
RdRp	RNA dependent RNA polymerase
RIP	Repeat induced point mutation
RISC	RNA induced silencing complex
RLC	RISC loading complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase III	Ribonuclease III
rRNA	Ribosomal RNA
RS	Serine-arginine rich
SDS	sodium dodecyl sulfate
siRNA	Small interfering RNA
SSC	Sodium Chloride-Sodium Citrate
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TGS	Transcriptional gene silencing
TLR3	Toll-like receptor 3

Tris	Tris(hydroxymethyl) aminomethane
UTP	Uridine triphosphate
UTR	Untranslated region

Chapter One

Introduction

The primary function of RNA is transferring genetic information from DNA to protein. However, RNA can also be used for regulating gene expression. Antisense RNA has been used for a long time to silence the expression of homologous genes using a technique known as 'antisense-mediated silencing' (Izant and Weintraub, 1984; Nellen and Lichtenstein, 1993). In 1995, Guo and Kemphues showed that both sense and antisesne RNAs are equally efficient in silencing gene expression (Guo and Kemphues, 1995). This observation finally led to the breakthrough discovery that double-stranded RNA (dsRNA) is the predominant trigger of gene silencing, as it is more potent for silencing than sense or antisense RNAs (Fire et al., 1998). This landmark discovery has led to the establishment of the rapidly growing area of RNA inference (RNAi). RNAi shares common biological links with several previously characterized homology dependent gene silencing phenomena, such as 'co-suppression' in plants and 'quelling' in *Neurospora*. In plants and *Neurospora*, attempts to overexpress a particular gene by introducing the transgenic copies of the endogene result in the silencing of both the endogenous gene and the transgenes (Cogoni et al., 1996; Napoli et al., 1990; Romano and Macino, 1992; van der Krol et al., 1990). Similar

transgene induced silencing phenomena were also reported in animal systems such as *Drosophila* and *C.elegans* (Dernburg et al., 2000; Fire et al., 1991; Pal-Bhadra et al., 1997). Subsequent genetic and biochemical studies revealed that all of the above mentioned sequence-specific gene silencing phenomena are triggered by dsRNA, and the underlying biological pathways are evolutionary conserved. RNAi and related pathways are known to involve in diverse biological functions, such as antiviral defense, maintenance of genomic stability and development. In addition, RNAi has become a powerful tool for reverse genetics studies and can be potentially used for therapeutic purposes against human diseases.

1.1 The mechanism of RNAi

RNAi pathway can be divided into two distinct steps. The first step, also known as the initiator step, is involved in generating small interfering RNA (siRNA) from dsRNA. siRNAs are then used in the second step, the effector step, for silencing gene expression. The major components of this pathway and their functions are listed in Table-1.

1.1.1 The initiator step of RNAi pathway

dsRNA is the primary trigger of RNAi and related pathways. One of the major sources of dsRNA in eukaryotic cell is viral infection. The

Table-1 Major proteins and domains involved in RNAi pathway

Proteins or protein families	Main domain (s) and motif (s)	Functions
Dicer family	RNA helicase	RNA unwinding
	PAZ	Protein-protein interaction and single-stranded RNA binding
	RNase III	Riboendonuclease
	dsRBD	Double-stranded RNA binding
Argonaute family	PAZ	Protein-protein interaction and single-stranded RNA binding
	PIWI, RNase H motif	Riboendonuclease, siRNA and mRNA cleaving
R2D2/RDE-4	dsRBD	Double-stranded RNA binding, RISC assembly, initiation of RNAi
RNA dependent RNA polymerases	RdRp	RNA dependent RNA polymerization, generation and amplification of dsRNA
Putative RNA helicases	RNA helicase	RNA unwinding and RISC assembly

dsRNAs are produced during replication of RNA viruses (Voinnet, 2005). In addition to viral infection, dsRNAs can also be produced from tandem and inverted repeats of transposons and transgenes (Que et al., 1997; Romano and Macino, 1992). It has been proposed that the transcription of repeat elements leads to the formation of single-stranded 'aberrant' RNAs, that are subsequently converted to dsRNA by cellular RNA dependent RNA polymerase (RdRp) (Forrest et al., 2004; Makeyev and Bamford, 2002; Tomari and Zamore, 2005). Under non-physiological circumstances, dsRNAs can be introduced into the cells

by injection, soaking, feeding of the dsRNA-expressing bacteria, and by expressing an exogenous inverted-repeat construct (Billy et al., 2001; Catalanotto et al., 2004; Tabara et al., 1998). In addition to dsRNAs, hair-pin RNAs (also, known as primary microRNA transcript or pri-miRNA) produced from endogenous non-coding transcripts containing complementary or near complementary inverted repeats, are also known to induce gene silencing in plants and animals.

Although dsRNA can be produced form diverse sources, the mechanism for their processing to generate small RNA is similar. The best studied small RNAs involved in the sequence-specific gene silencing pathways are siRNA and microRNA (miRNA).The maturation of siRNA and miRNA is mediated by dsRNA specific RNase-III-type endonuclease dicer. The RNase-III type of enzymes are categorized into three classes based on their domain organization, (Figure 1). Class-I enzymes are mostly found in bacteria and yeast. *Drosophila* has one class-II endonuclease, Drosha and two class-III enzymes DCR-1 and DCR-2. Drosha is known to function in the processing of pri-miRNA to precursor miRNA (pre-miRNA). DCR-1 and DCR-2 are involved in the processing of pre-miRNA and dsRNA respectively (Carmell and Hannon, 2004; Hammond, 2005; Lee et al., 2003; Lee et al., 2004). The homologues of dicer with similar functions are also observed in other organisms. According to the current model of dicer mediated catalysis (Figure 2), the PAZ domain binds the 2 nt 3' overhang of long

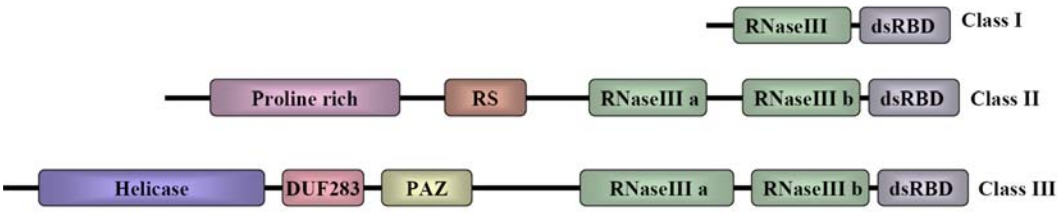

Figure 1. Schematic diagram of the domain organization of Class I-III RNaseIII family of proteins. Domain sizes are approximate.

dsRNA. The two RNase-III domains associate to form an intramolecular pseudo-dimer containing two active sites. Each active site cleaves a phosphodiester bond in the opposite strands of the substrate to generate mature 21-25 -nt small dsRNAs with 2 nt 3' overhang (Zhang et al., 2004). In addition to the 2 nt 3' overhang, siRNAs and miRNAs also have 5' phosphate and 2'3' hydroxy termini, which are characteristic of RNase-III mediated cleavage products (Harborth et al., 2003). Occasionally, dicers are known to function as a larger complex in association with other dsRNA binding cofactors (Kok et al., 2007; Liu et al., 2003; Saito et al., 2005).

1.1.2 The effector step of RNAi pathway

The effector complex of RNAi pathway is known as the RNA induced silencing complex (RISC). The key component of RISC is a member of the Argonaute family of proteins. The Argonaute protein is involved in gene silencing at post-transcriptional level by cleaving or repressing the translation of the

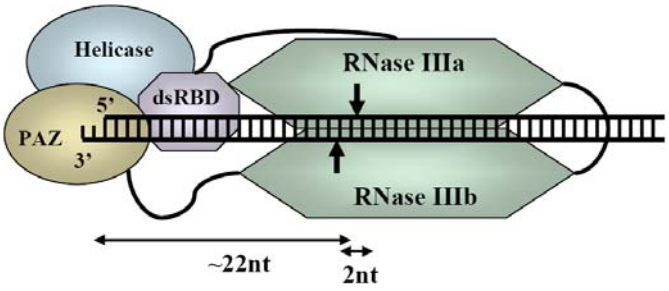

Figure 2. Model of Dicer mediated cleavage. The PAZ domain binds the 2 nt 3' single stranded over hang region. The two RNase III domain forms pseudo-dimer, each containing one catalytic site. Each domain cleaved one strand of the dsRNA substrate. The function of the helicase domain is uncertain.

homologous mRNA. In some organisms Argonaute is also known silence gene expression at transcriptional level.

1.1.2.1 Structure and function of Argonaute proteins in RISC

Argonaute proteins consist of four domains: The N-terminal, PAZ, middle and PIWI domains. Structural studies using full length Argonaute proteins of *Pyrococcus furiosus* and *Aquifex aeolicus* showed that the N-terminal, middle and the PIWI domains form a crescent shaped base with PIWI at the center of the base. The PAZ domain is located above the base and is held by a stalk like linker region between the N-terminal and the PAZ domains. The specific organization of the domains, as shown in Figure 3, forms a larger positively charged groove between the PAZ and the crescent base and a smaller groove between the N-terminal and PIWI domains. The larger groove accommodates the siRNA-mRNA

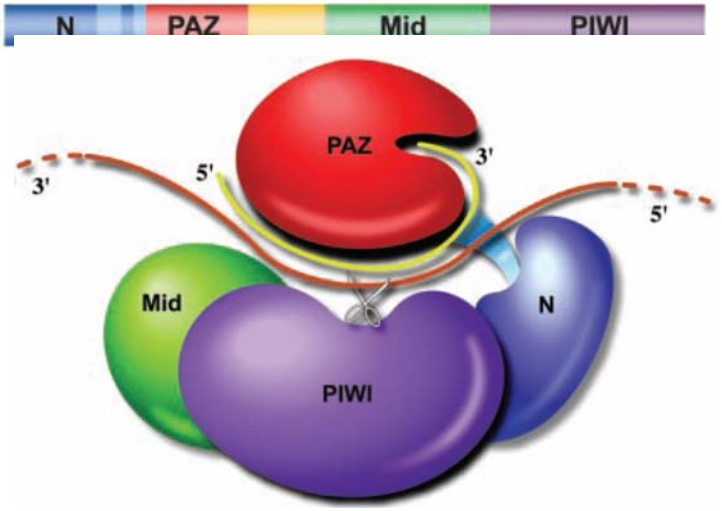

Figure 3. Structure and function of Argonaute protein (A) Schematic diagram of the model organization of Argonaute proteins (B) Model of siRNA mediated mRNA cleavage. siRNA is colored in yellow and 3' end of the siRNA binds the PAZ domain of Argonaute. The cleavage activity of the PIWI domain is shown by scissors. The mRNA (red colored) is cleaved in the middle, between nucleotide 10 and 11 from the 5' end of the guide siRNA. This figure is adapted from (Song et al., 2004).

duplex in a way that the scissile phosphate of the target is positioned at the active site (Song et al., 2004; Yuan et al., 2005). The PAZ domain of Argonaute protein has an OB fold (oligonucleotide-oligonucleoside binding) that binds to the 3' single stranded end of the siRNA duplex in a sequence independent manner (Lingel et al., 2003; Song et al., 2003; Yan et al., 2003). The PIWI domain has a fold that belongs to the RNase H family of enzymes. Structural and mutational analyses revealed that the PIWI domain contains three conserved catalytic residues, two Asp and one His (known as DDH motif) that are involved in the

mRNA cleavage activity (Liu et al., 2004; Rand et al., 2004; Rivas et al., 2005; Song et al., 2004). Although the DDH motif is responsible for mRNA cleavage, replacement of the conserved His by Asp, Glu or Lys residues in cleavage competent Argonaute have also been observed (Tolia and Joshua-Tor, 2007).

1.1.2.2 Assembly and activation of the RISC

Although siRNA is generated as double stranded form, only one of the two strands is incorporated into the active RISC during the assembly process. Previous studies have shown that the siRNA strand with lower thermodynamic stability at the 5' end is incorporated into the RISC to serve as a guide strand and the other strand (the passenger strand) is destroyed (Khvorova et al., 2003; Schwarz et al., 2003). In *Drosophila*, DCR-2 and R2D2 (a dsRNA binding cofactor) form RISC loading complex (RLC) that is involved in sensing the asymmetry of the two siRNA strands and facilitating the incorporation of siRNA into the RISC (Liu et al., 2003; Tomari et al., 2004).

The separation of the two siRNA strands is a critical step in RISC assembly process. Recent *in vitro* biochemical studies in *Drosophila* system have shown that the two strands of the siRNA duplex are separated by Argonaute-2 (Ago-2) mediated passenger strand cleavage (Figure 4). Ago-2 functions as an endonuclease to cleave the passenger strand the same way as it cleaves the target mRNA substrates. The passenger strand cleavage occurs before unwinding of the two siRNA strands. In addition, blocking of the passenger strand cleavage in *in*

vitro studies showed a significant decrease of the target mRNA cleavage by the RISC, indicating the importance of passenger strand removal in efficient gene silencing process (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). However, *in vivo* evidence for Argonaute mediated siRNA duplex separation is lacking.

The passenger strand cleavage does not seem to be the absolute mechanism for siRNA duplex separation. In *Drosophila*, blocking of the passenger strand cleavage did not completely inhibit the generation single stranded siRNA, suggesting the existence of a cleavage independent bypass mechanism for siRNA duplex separation (Matranga et al., 2005). Additionally, as the passenger strand cleavage mechanism depends on the perfect pairing between the two siRNA strands this process would be ineffective in separating miRNA strands with mismatches. Furthermore, recombinant human Ago-2 is known to cleave target mRNA only when primed with single stranded guide siRNA but not the siRNA duplex, indicating that Argonaute is not always able to separate the siRNA duplex alone (Rivas et al., 2005). An alternate model for siRNA duplex separation proposed that the strand separation is potentially mediated by a DEAD-box helicase (Figure 4), which can unwind the two strands in an ATP dependent manner (Meister and Tuschl, 2004). Consistent with this model, several DEAD box helicases and their involvement in RNAi or other post-transcriptional gene silencing pathways have been reported in different organisms. However, further

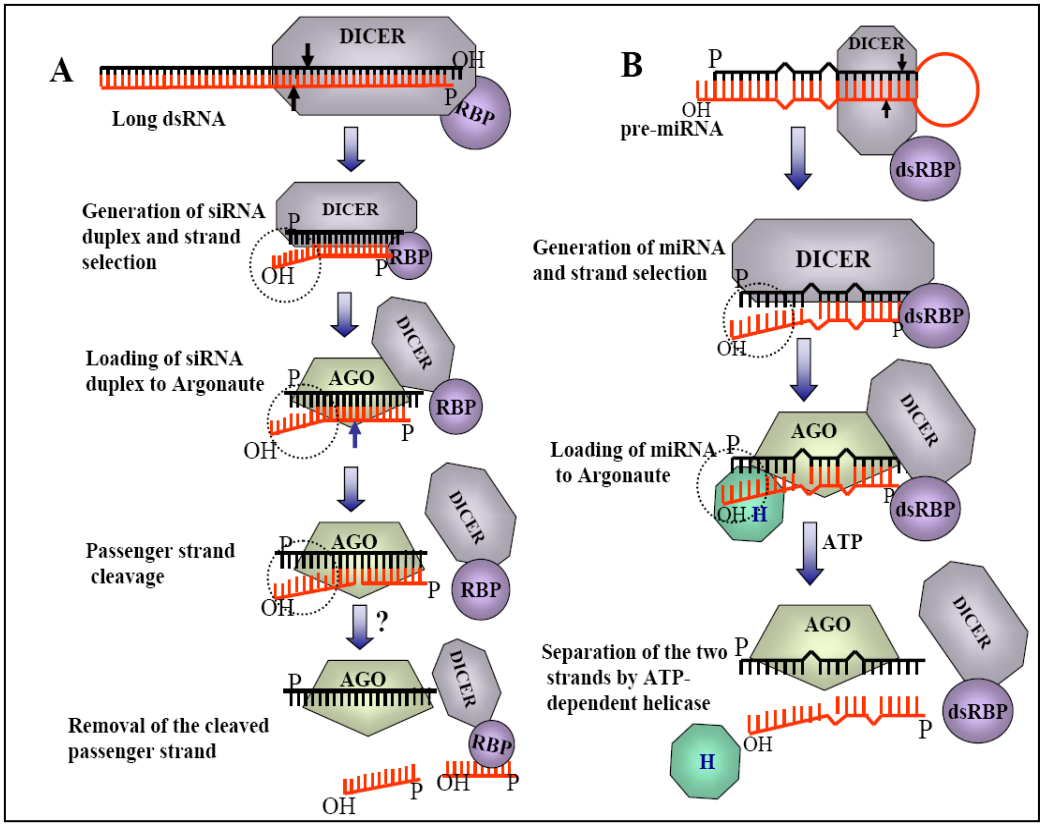

Figure 4. Model of loading and separation of the two strands of siRNA and miRNA. (A) siRNA duplex loading and passenger strand cleavage by Argonaute. (B) miRNA loading and separation of the two strands by ATP-dependent helicase. H, helicase, RBP, RNA binding protein, Dicer associated factors. The thermodynamically less stable end of siRNA or miRNA is shown as dotted circle.

studies are needed to test their importance in siRNA unwinding.

1.1.2.3 Silencing of gene expression by active RISC

Once RISC is equipped with the single stranded guide RNA, it can silence gene expression either by degradation or translational repression of the target mRNA depending on the complementarity between the target and the guide RNA

sequences (Martinez and Tuschl, 2004). The Argonaute protein cleaves the target mRNA in the middle of the complementary region, between nucleotide 10 and 11 from the 5' end of the guide siRNA (Elbashir et al., 2001). Although the cleavage reaction by Argonaute doesn't require ATP, it has been shown that multiple rounds of the cleavage reactions that require the release of the cleaved mRNA are more efficient in presence of ATP (Hutvagner and Zamore, 2002; Nykanen et al., 2001). The mechanism of miRNA mediated translational repression is not well understood. Based on the current model, miRNA blocks the target mRNA translation by binding and sequestering the mRNA away from the translational machinery to the cytoplasmic foci termed 'P- bodies' (Liu et al., 2005; Rossi, 2005; Sen and Blau, 2005). In plants, miRNA show perfect complementarity with their target mRNA sequence and silence gene expression by cleavage instead of translational repression of mRNA. Similar miRNA mediated mRNA cleavage is also observed in human and in some other organisms as well (Llave et al., 2002; Yekta et al., 2004). The siRNA containing RISC is also known to silence gene at transcriptional level by promoting DNA and histone modifications that result in heterochromatin formation (Verdel et al., 2004; Volpe et al., 2002).

1.1.3 Current model of RNAi

A simplified model of RNAi pathway is shown in Figure 5. Upon initiation by dsRNAs, dicer recognizes and processes the dsRNA to 21-25 nt siRNA duplex. The siRNA duplex is then loaded onto the RISC

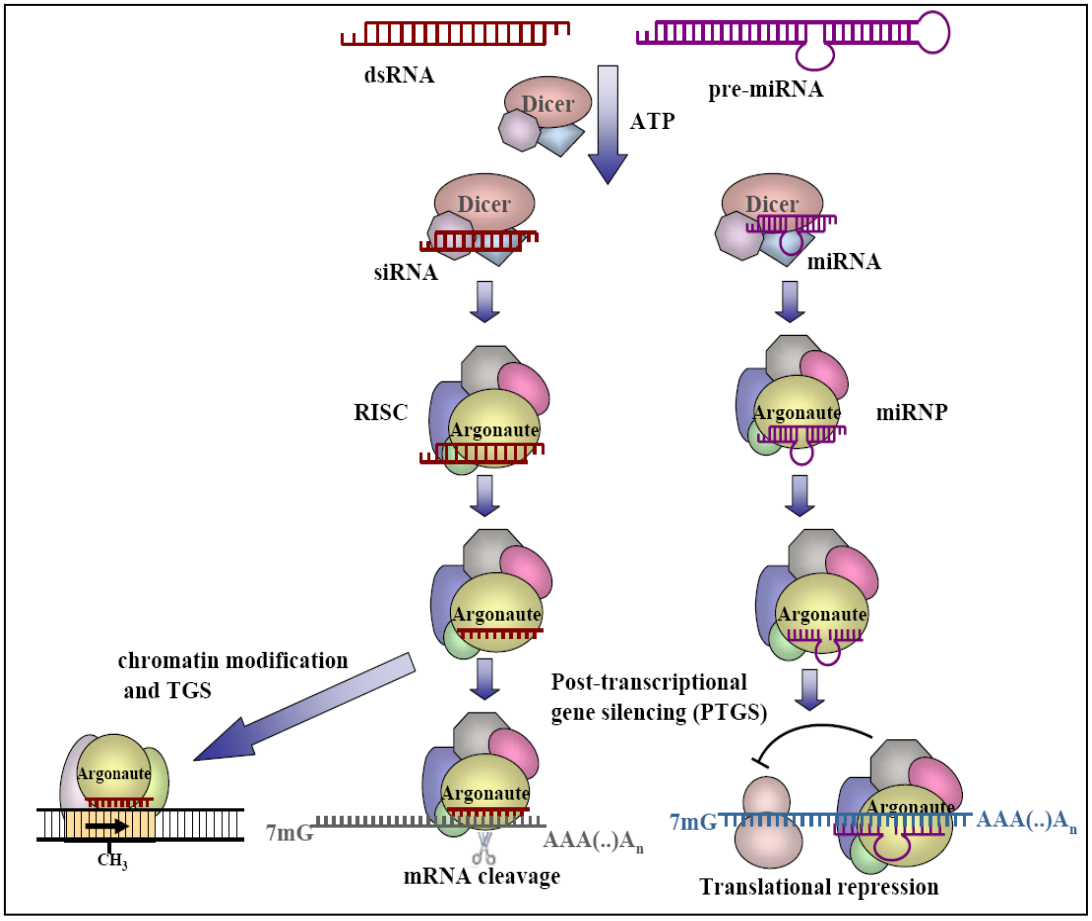

Figure 5. Model of RNAi and miRNA pathway. TGS, transcriptional gene silencing. miRNP, miRNA-protein complex.

and is converted to single stranded siRNA. Finally, the active RISC containing single stranded RNA guides the Argonaute protein to silence gene expression.

Some organisms, such as *C.elegans*, *Arabidopsis* utilize RNA dependent RNA polymerase (RdRp) to amplify the RNAi signal by using siRNA as primer

and target mRNA as template (Dalmay et al., 2000; Sijen et al., 2001; Smardon et al., 2000).

1.2 Gene silencing phenomena in *Neurospora crassa*

The filamentous fungus *Neurospora crassa* was one of the first model systems used for gene silencing research. Several gene silencing processes, such as RIP, MSUD, quelling, are observed this organism. Repeat-induced point mutation (RIP) is a homology based transcriptional gene silencing method that acts during the sexual cycle to detect and mutate the duplicated sequences. RIP identifies duplicated sequences that are more than ~400 bp and introduces random C:G to T:A mutations in both copies of the sequences. RIP mutations are often associated with epigenetic silencing through DNA methylation (Galagan and Selker, 2004). In addition to RIP, *Neurospora* also has a silencing mechanism termed 'meiotic silencing by unpaired DNA' (MSUD) which detects and silences the expression of all copies of any genes that are not paired during the first meiotic prophase of the life cycle (Shiu and Metzenberg, 2002).

1.2.1 The 'quelling' in *Neurospora*

The best studied gene silencing phenomenon that occurs during the vegetative phase of *Neurospora* life cycle is known as "quelling". Quelling, originally discovered by Romano and Macino in 1992, is very similar to 'co-suppression' in plants and RNAi in animals. Quelling is a post-transcriptional

gene silencing phenomenon that is triggered by the introduction of transgene and results in the silencing of both the transgene and endogenous homologous gene via sequence specific mRNA degradation (Cogoni et al., 1996; Romano and Macino, 1992). Previous studies have shown that the minimum length of the transgene required for quelling is ~ 132 nt and the introduction of the non-transcribed genomic regions such as the promoter sequence is not sufficient to induce quelling (Cogoni et al., 1996). In addition, quelling is a reversible phenomenon and the efficiency of quelling depends on the copy numbers of the transgene inserted into the genome (Cogoni et al., 1994).

1.2.2 The mechanism of quelling

The forward genetics approach using UV-mutagenesis identified the quelling deficient (*qde*) genes, *qde-1* (an RdRp), *qde-3* (a RecQ helicase) and *qde-2* (an Argonaute family of protein) (Cogoni and Macino, 1997). In addition, the two dicer like proteins, DCL-1 and DCL-2 were identified by reverse genetics approach (Catalanotto et al., 2004). Recently a novel component of the quelling pathway, QIP that functions as an exonuclease, is identified (Maiti et al., 2007). QDE-1 and QDE-3 are thought to function upstream of the quelling pathway during the production of dsRNA from transgene (Cogoni and Macino, 1999a; Cogoni and Macino, 1999b). Consistent with this notion, these two components are not required for gene silencing when dsRNA is produced from an exogenous inverted repeat construct. The two DCLs are functionally redundant and are

involved in the production of siRNA from dsRNA (Catalanotto et al., 2004). QDE-2 is the catalytic component of the RISC. Gene silencing is completely abolished in absence of *qde-2* (Catalanotto et al., 2002; Maiti et al., 2007).

According to the current model of quelling pathway, transcription of the transgene leads to the generation of single stranded 'aberrant' RNA which is subsequently converted to dsRNA by QDE-1 and QDE-3. The dsRNA is then processed by DCLs to generate ~23-24 nt siRNA duplex that are loaded onto the RISC. QDE-2 in association with QIP converts the siRNA duplex into single-stranded siRNA. Finally, guided by the single stranded siRNA, QDE-2 recognizes and cleaves the homologous target mRNA using its endonuclease activity (Catalanotto et al., 2002; Catalanotto et al., 2004; Maiti et al., 2007). Since quelling machinery shows significant similarities with the RNAi pathway, further characterization of quelling in *Neurospora* using genetic and biochemical approaches will provide valuable insights into the mechanistic basis of RNAi pathway.

1.2.3 Physiological significance of quelling

Quelling plays important roles in maintaining genome stability by silencing transposons and can also function as an antiviral defense mechanism. The components of quelling pathway, QDE-2 and DCL-2 are required to block the expansion of transposable Tad elements in *Neurospora*. In addition, endogenous siRNAs with sequences corresponding to retrotransposons have also

been detected (Cambareri et al., 1994; Nolan et al., 2005). The RecQ helicase domain of QDE-3 is homologous to that of human protein WRN that is involved in DNA repair and linked to the Werner syndrome. Consistent with this, QDE-3 and its homologue RecQ-2 are known to play important roles in DNA break repair (Pickford et al., 2003). However, unlike the RNAi in other organisms, quelling pathway does not seem to play any role in heterochromatin formation or in the development of *Neurospora*.

1.3 Biological functions of RNAi

RNAi has been utilized as an experimental tool to understand the function of genes in different organisms. However, investigations over the past few years revealed the importance of RNAi in many biological processes as well. The well-known biological functions of RNAi are discussed below.

1.3.1 Antiviral response

Viral infection is one of the major sources of dsRNA in eukaryotic cells. The initiation of RNAi pathway by dsRNA suggests an obvious function of this pathway to defend against the viral invasion. Indeed, several studies in both plants and animals have confirmed the role of RNAi in antiviral defense (Ding et al., 2004; Li et al., 2002; Lu et al., 2005; Wilkins et al., 2005). In plants RNAi machinery is known to degrade viral mRNA (Ruiz et al., 1998). The role of RNAi in antiviral mechanism was further supported by the observations that both plant

and animal viruses encode proteins to suppress RNAi machinery.The viral suppressors are mostly double stranded RNA binding proteins that interfere with host cell's RNA silencing machinery (Li et al., 2002; Vargason et al., 2003). Moreover, hyper-susceptibility to viral infections was observed in *Arabidopsis* mutants deficient in RNAi or other PTGS pathway. (Boutet et al., 2003; Morel et al., 2002; Mourrain et al., 2000).

1.3.2 Genome defense

RNAi has also been linked to maintaining the stability of genome. A significant fraction of genome sequence is composed of repetitive sequences, such as numerous copies of intact and defective transposable elements. These transposable elements impair genomic stability by functioning as mutagen and also by providing the potential sites for non-homologous recombination during DNA repair. In *C.elegans*, some RNAi deficient mutants showed high mobility of transposable elements (Ketting et al., 1999; Sijen and Plasterk, 2003; Tabara et al., 1999). In *Drosophila*, siRNAs with sequence similarities with the endogenous transposons that move via DNA and RNA intermediates have been identified (Elbashir et al., 2001). However, the precise mechanism by which RNAi controls the transposons, is not clear yet. In addition, the requirement of the *Neurospora* RNAi components for transposon silencing further supports the role of RNAi in maintaining genomic stability (Cambareri et al., 1994; Nolan et al., 2005).

1.3.3 Chromatin modification

RNAi is also known to be involved in transcriptional gene silencing (TGS) in plants and animals by promoting DNA and histone modifications that ultimately leads to the heterochromatin assembly (Lippman and Martienssen, 2004; Matzke and Birchler, 2005; Wassenegger, 2005). Heterochromatin formation is important for chromosome segregation, genomic stability, and gene regulation. A direct link between RNAi and heterochromatin formation was established from the studies in *S.pombe*. Fission-yeast RNAi components such as Dicer, Argonaute and a homologue of RNA dependent RNA polymerase (RdRp), are known to be involved in inactivating specific chromosome regions by promoting heterochromatin formation (Verdel et al., 2004; Volpe et al., 2002). In contrast, RNAi machinery in *Neurospora* dose not seem to play any role in DNA methylation or heterochromatin formation (Freitag et al., 2004).

1.3.4 Regulation in development

The role of RNAi pathway in the regulation of protein coding genes and in the development of plants and animals has been revealed with the advancement of RNAi field. It is now well accepted that miRNAs are one of the major regulatory factors in development. miRNAs can regulate as many as one-third of the human protein coding genes (Lewis et al., 2005; Xie et al., 2005). Disruption of the genes important for miRNA biogenesis and function are known to associate with several

developmental defects in both plants and animals (Bernstein et al., 2003; Bohmert et al., 1998; Hatfield et al., 2005; Peragine et al., 2004).

1.4 Summary

Over the past few years remarkable advancements were made in the understanding of the mechanism and functions of RNAi. However, several questions remain unanswered. What is the precise mechanism for dsRNA production from repeat elements and transgenes? Is there really an ATP dependent unwinding factor to separate the siRNA duplex strands? What is the precise mechanism for removal of the cleaved passenger strand from RISC? Is Argonaute important for RISC activation *in vivo*? Since, RNAi is implicated in several biological processes, it is conceivable that RISC functions as a flexible platform that participates in different biological pathways in association with different RNA and protein molecules. Furthermore, the components of RNAi pathway might be under the control of a regulatory mechanism to act as flexible machinery. Although the mechanism of RNAi is well understood, the regulation of RNAi components is not understood yet. In this dissertation, I describe my efforts to understand the mechanism and regulation of RNAi pathway using *Neurospora* as a model system. In chapter two the *in vivo* importance of Argonaute in siRNA separation process and a possible mechanism for nicked siRNA strand removal is described. Chapter three describes an approach that was

taken to understand the importance of QDE-1 and QDE-3 in transgene induced gene silencing in *Neurospora*. In chapter four the existence and significance of a regulatory mechanism of RNAi components are described.

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Chapter Two

QIP, a putative exonuclease interacts with *Neurospora* Argonaute protein and facilitates the conversion of duplex siRNA into single strands

2.1 Introduction

Conversion of siRNA duplex into single stranded siRNA is a critical step in the RISC assembly process. It was proposed that siRNA duplex separation is mediated by an ATP- dependent RNA helicase (Meister and Tuschl, 2004). However, recent *in vitro* studies in *Drosophila* suggested that the separation of the siRNA duplex is an ATP independent process and is mediated by Argonaute protein itself. Argonaute protein cleaves the passenger strand by utilizing the same endonuclease activity it uses for target mRNA cleavage (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). The *in vitro* RISC activity was severely impaired when the passenger strand cleavage was blocked, indicating the importance of the siRNA duplex separation process. Consistent with this result, *Drosophila* Argonaute mutants failed to generate single stranded siRNA *in vitro* (Okamura et al., 2004). But *in vivo* evidence substantiating the importance of Argonaute in siRNA duplex separation was lacking. In addition, the mechanism

for removing the cleaved passenger strand from the RISC was not known. According to previous studies the nicked passenger strands are relatively stable and can potentially inhibit the activation of RISC (Matranga et al., 2005). It was proposed that the removal of the nicked passenger strand might be facilitated by a co-factor. However, the identity of such a factor was unknown (Haley and Zamore, 2004; Matranga et al., 2005).

QDE-2, a *Neurospora* homologue of Argonaute-2 (Ago-2) forms the catalytic core of the RISC complex and associates with siRNAs (Catalanotto et al., 2000; Catalanotto et al., 2002). To understand the mechanism of RISC activation, I investigated the siRNA duplex separation process in *Neurospora.* My studies suggested that QDE-2 and its endonuclease activity are required for *in vivo* siRNA duplex separation and efficient gene silencing. In addition, I identified QIP, a QDE-2 interacting protein with a putative exonuclease domain which facilitates the siRNA duplex separation process. Disruption of *qip* resulted in the accumulation of siRNA duplex and severe impairment of gene silencing, indicating the importance of QIP in *Neurospora* RNAi pathway. Furthermore, my studies suggested that QIP functions as an exonuclease and facilitates the removal of cleaved passenger strand in a QDE-2 dependent manner.

This chapter is adapted from ***Maiti, M., Lee, H.C. and Liu, Y. (2007) QIP, a putative exonuclease, interacts with the Neurospora Argonaute protein and***

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2.2 Materials and Methods

2.2.1 Strains and growth conditions

In this study FGSC 4200 (a) was used as a wild type strain and FGSC 7088 (*his-3*, a) was used as a host strain for the transformation of *his-3* targeting constructs. The mutant strains, in the wild type as well as in *his-3* background, generated for this study were qip^{ko}, qip^{ko} $qde2^{rip}$; qip^{ko} $dcl\text{-}2^{rip}$ strains. The $qde2^{rip}$ and the $dcl\text{-}2^{rip}$ strains were generated in a separate study (Choudhary et al., 2007) and the method is described in chapter four. For the liquid culture, a small amount of conidia or hyphae was inoculated in the shaking flasks containing liquid media (1x Vogel's, 2% glucose) for 40-45 hours before harvesting the samples. For liquid cultures containing QA, 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1x Vogel's, 0.1% glucose, and 0.17% arginine. Low concentration of glucose was used for QA containing cultures because normal 2% glucose severely affects the induction of *qa-2* promoter (Cheng et al., 2001b).

2.2.2 Creation of the qip^{ko} and other mutant strains

Gene replacement method was used to replace the entire *Neurospora qip* gene with the hygromycin resistance gene (*hph*) using a method described previously (Colot et al., 2006). Briefly, 5' and 3' flanking fragments of *qip* open reading frame (ORF) were generated by PCR in a way that both fragments have sequence homology to a *hph* cassette at one end and homology to a yeast vector at the other end. Both the flanking fragments are contransformed into yeast with the *hph* cassette and yeast shuttle vector. After homologous recombination the linear deletion cassette was amplified from the yeast genomic DNA pool containing the circular deletion construct. The final gene replacement cassette was introduced into a wild-type strain by electroporation (Margolin et al., 1999). Southern blot analysis was performed to identify the positive transformants. One of the positive transformant was crossed with a wild-type strain and sexual spores were picked individually and germinated on slants containing hygromycin. Southern blot analysis was performed to identify the homokaryon mutant strains.

To generate the probe for the southern blot analysis, a PCR fragment containing 3.7 Kb of the *qip* sequence was generated using the primers *qip* for (5'-ATGGAGGACGAGCAGTTCATGC-3') and *qip* rev (5'-CATTGTGATTGTGCGGGTTGGG-3'). The PCR fragment was digested with PvuI and PvuII to generate the 2.6 Kb probe. The genomic DNA from the cross progenies were digested with PvuI and PvuII before running the 0.8% agarose gel.

A *qip*^{ko} strain was crossed with a *qde2*^{rip} strain or a *dcl-2*^{rip} strain of opposite mating type to generate *qip*^{ko} *qde2*^{rip} or *qip*^{ko} *dcl-2*^{rip} double mutant strains respectively.

2.2.3 Creation of the pDE3BH.QDE-2, Myc-His-QDE-2/QIP and the site directed mutagenesis constructs

To create the construct containing *qde-2* gene with its own promoter, a PCR fragment of entire *qde-2* ORF and ~ 3 Kb of upstream promoter sequence was cloned into the ClaI-SmaI site of pDE3BH. The resulting plasmid, pDE3BH.QDE-2 and pBT6 (benomyl resistance gene containing plasmid) were co transformed into the *qde-2*^{rip},dsa*l-1* strain. The positive transformants were selected by western blot using QDE-2 specific antibody. To create the slicer deficient mutant strains the First (D664) or the third (D890) Asp residue of the DDH motif of QDE-2 in the pDE3BH.QDE-2 plasmid was mutated to Ala using the site directed mutagenesis kit (QuickChange® Site-Directed Mutagenesis Kit, Stratagene) and the mutated plasmid were transformed into the *qde-2*^{rip},dsa*l-1* strain.

To create an expression vector to express N terminus Myc-His epitope tagged protein in *Neurospora*, a 6-His tag was introduced downstream of the 5 c-Myc in pqa-5Myc plasmid by *in vitro* mutagenesis resulting in pqa-Myc-His (He

et al., 2005; He et al., 2003). A PCR fragment containing the entire *qde-2* or *qip* ORF and 3' UTR was cloned into the pqa-Myc-His vector to generate the final Myc-His QDE-2 or Myc-His QIP constructs. The Myc-His QDE-2 (D664A) and Myc-His QIP (H504A) were generated by mutating the mentioned residues by using the QuickChange® Site-Directed Mutagenesis Kit, Stratagene. The ds*al-1* strains were generated as described in chapter four.

2.2.4 Generation of QIP specific antibody

The Glutathione S-transferase-QIP (GST-QIP) (containing QIP amino acids 181-567) fusion protein was expressed in BL21 cells and the inclusion bodies containing the recombinant protein were purified as described previously (Cheng et al., 2001a). To purify inclusion bodies, the pellet of 500 ml of *E.coli* culture (induced by IPTG for 3 hours at 37^0C) was suspended in Buffer A (50 mM Tris-HCL pH 8.3, 1mM EDTA, 2 mM 2-marcaptoethanol) containing freshly added 0.1% lysozyme. The suspension was incubated at room temperature for 40 minutes and the cells were broken by sonication. The pellet was precipitated by centrifugation and then washed in Buffer C (Tris-HCL pH 8.3, 2 mM EDTA, 2 mM 2-marcaptoethanol, 2 M Urea, 0.5% Triton-X-100). The resulting pellets were resuspended in 8M Urea and the GST-QIP fusion protein was purified after running through preparative 7.5% SDS-PAGE gel. The antisera against QIP were generated by immunizing New Zealand white rabbits using standard protocols. The QDE-2-specific antibody was generated in a separate study (Choudhary et al.,

2007) and the method is described in chapter four.

2.2.5 Protein analyses

Protein extraction, quantification, and western blot analysis were performed as previously described (Cheng et al., 2001a). Equal amounts of total protein (50 μg) were loaded in each lane of the SDS PAGE gel. After electrophoresis, proteins were transferred onto PVDF membrane. The western blot analyses were performed using standard protocol (Garceau et al., 1997) and the blots were developed by chemiluminescence (ECL, Amersham Pharmacia).

2.2.6 Purification of Myc-His QDE-2 and Myc-His-QIP from *Neurospora*

The Myc His QDE-2 or the Myc-His QIP was purified as described previously (He et al., 2005). The strains expressing the Myc-His tagged protein and a wild type strain (as negative control) were cultured in liquid medium containing QA (0.01M QA, 1x Vogel's, 0.1% glucose, and 0.17% arginine) for ~40 hours. Approximately 25 grams of the *Neurospora* tissue was harvested and all the purification was performed at 4^0C. The whole cell lysate in extraction buffer (20 mM Tris-CL, pH 7.4, 137 mM NaCl, 10% glycerol, 10-20 mM imidazole and the standard protease inhibitors) was passed through the Ni-NTA agarose beads column (QIAGEN). After washing the column using the washing buffer (20 mM Tris-CL, pH 7.4, 300 mM NaCl and 20 mM imidazole), the bound

protein was eluted from the column using the elution buffer (20 mM Tris-CL, pH 7.4, 137 mM NaCl, 20% glycerol and 200 mM imidazole). Western blot analysis was performed using Myc monoclonal antibody to detect the elution fractions with highest amount of Myc His- tagged protein. Immunoprecipitation of the protein was performed by adding 20-30 μl of c-Myc monoclonal antibody coupled agarose beads (Santa Cruz Biotechnology) to the elution fraction from the previous purification. After 4-5 hours of incubation at 4^0C with gentle rotation, the beads were washed 6 times with low salt (20 mM Tris-CL, pH 7.4, 50 mM NaCl) and high salt (20 mM Tris-CL, pH 7.4, 500 mM NaCl) buffers and then with water. The immoprecipitated proteins were separated by 4-15% Tris-HCl linear gradient gel (Biorad, catalog#161-1158) and silver stained according to the manufacturer's instruction (SilverQuest, Invitrogen). The specific bands were excised and were subjected to tryptic digestion and Nano-HPLC/electrospray mass spectrometry analysis (Protein Chemistry Core facility, university of Texas Southwestern Medical center).

2.2.7 Purification of small RNAs and Northern blot analyses

The total population of mRNA was extracted as described previously (Aronson et al., 1994). The low molecular weight RNA was enriched from the total RNA using 5% PEG (MW8000) and 500 mM NaCl as previously described (Catalanotto et al., 2002). Low-molecular-weight RNA was quantified by

spectrophotometric analysis and was separated by electrophoresis through 16% polyacrylamide, 7M urea, and 0.5 X Tris-Borate EDTA (TBE) gels. Equal amount of RNA (25 μg) was loaded in each lane. The RNA was then transferred onto Hybond-N+ filters (Amersham Biosciences) and the membrane was fixed by UV cross linking. For native gel analysis, RNA was separated through 16% TBE (without urea) gels. The size of RNA was verified by oligonucleotide markers. Prehybridization and hybridization were performed at 42°C using ULTRAhyb™ buffer (Ambion). For hybridization, a single-stranded ^{32}P- uridine triphosphate (New England Nuclear) labeled RNA probe was generated using MAXIscript® T7 kit (Ambion). The RNA probe was transcribed from a DNA template containing T7 promoter sequence in sense or antisense orientation. The RNA probe was then treated with TURBO DNase™ (Ambion) at 37°C for 20 minutes and purified through Micro Bio-Spin 30 column (Biorad) after the treatment with DNase inhibitor. The purified long (~1.5 Kb) RNA probe was hydrolyzed to an average size of 50 nucleotides by incubating with 80 mM sodium bicarbonate and 120 mM sodium carbonate at 60°C for 3 hours. The hydrolyzed probe was then added to hybridization solution for overnight hybridization. After hybridization, the membrane was washed with 2 X SSC and 0.2% SDS buffer at 42°C, three times each of 30 min before it was exposed to X-ray film.

2.2.8 Dicer assay

The template for dsRNA was generated by PCR using a forward and reverse primer, each has T7 promoter sequence. The dsRNA substrate was transcribed using Riboprobe kits (Promega) and was uniformly labeled during the transcription (Bernstein et al., 2001). The dicer assay was performed by incubating the cell extracts from the wild type, *qip*^{ko}, *dcl-1*^{ko}*dcl-2*^{rip} double mutant strains with the uniformly labeled dsRNA and 1 mM ATP in 100 mM KOAc, 15 mM HEPES, pH 7.4, 2 mM $Mg(OAc)_2$ and 5mM DTT at 30^0C for 1 hour. The reaction was stopped by adding 0.3 M NaOAc and the RNAs were extracted using Phenol/Chloroform and precipitated by ethanol (Liu et al., 2003). The dicer products were analyzed in 16 % polyacrylamide gel.

2.2.9 Generation of synthetic siRNAs:

The sequences of the two strands of *let-7* siRNA were : siRNA01 (for), UGAGGUAGUAGGUUGUAUAGUGAUU; siRNA02 (rev),UCACUAUACAACCUACUACCUCAUU. Nicked sequences: UCACUAUACA and ACCUACUACCUCAUU. siRNA01 was radio labeled at the 5'end with [γ-32p] ATP using T4 polynucleotide kinase (T4 PNK). For labeling, the siRNA was incubated with [γ-32p] ATP, T4 PNK (New England Biolabs) and T4 PNK buffer (New England Biolabs NEB) at 30^0C for 1 hour. Intact siRNA duplex was prepared by annealing the siRNA02 with the radio labeled siRNA01 in 1X annealing buffer (10 mM HEPES, [pH 7.4], 5Mm

$MgCl_2$). For the nicked duplex, radio labeled siRNA01 was annealed with the two complementary nicked sequences. Then the radio labeled single stranded, intact duplex and nicked duplex siRNAs were passed through a Micro Bio-Spin 6 column (Biorad) and analyzed in 16% native polyacrylamide gel.

2.2.10 Coimmunoprecipitation of siRNAs:

Cell extracts from strains expressing either MycQDE-2 or MycQDE-2 (D664A) were prepared in a buffer containing 25 mM Tris, pH 7.5, 150mM NaCl, 1.5mM MgCl2, 1% NP40, 1mM DTT, protease inhibitors and 100 U/ml RNase inhibitor. Cell extracts were pre-cleared by incubation with 20 µl of Protein G Sepharose (GE Healthcare) at 4^0C for 1 hour. For immunoprecipitation of MycQDE-2, pre-cleared extracts were incubated with Myc monoclonal antibody at 4^0C for overnight and then with 25 µl of Protein G Sepharose for 2 hours at 4^0C. Immunoprecipitates were washed 5 times using the extraction buffer. To recover coprecipitated RNAs, 150 µl of TE (10 mM Tris/1mM EDTA, pH 7) was added to the precipitates, followed by phenol/chloroform extraction and ethanol precipitation. The precipitated RNAs were analyzed by 16% native polyacrylamide gel and northern blot analysis using single stranded RNA probe.

2.2.11 Quelling assay:

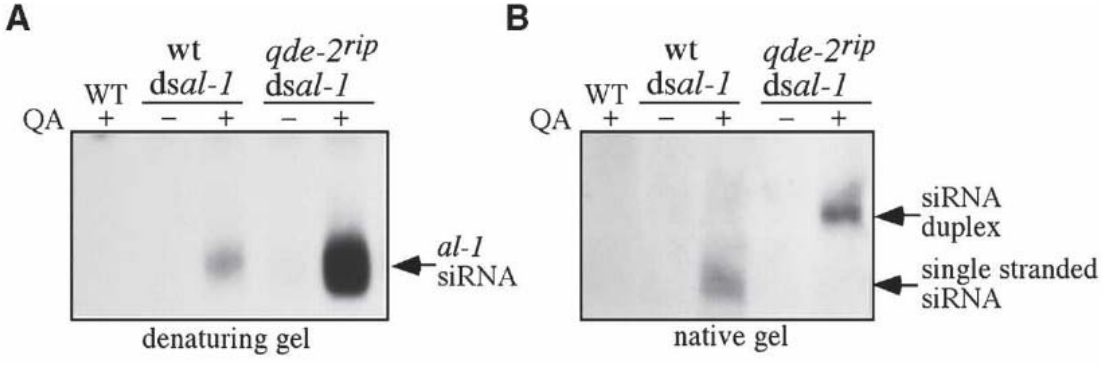
The detail protocol of quelling assay is described in Chapter three. Briefly,

a 1.5 Kb PCR fragment of *al-1* ORF and pBT6 (a benomyl resistant gene containing plasmid, obtained from the Fungal Genetic Stock Center, FGSC) were cotransformed into a wild type strain. The benomyl resistant transformants were visually inspected to identify the complete quelled (white), partial quelled (yellow) or not-quelled (orange) strains.

2.3 Results

2.3.1 QDE-2 is required for the *in vivo* siRNA duplex separation

To investigate the importance of QDE-2 in generating single stranded siRNAs, I introduced an inducible dsRNA expressing construct specific for *albino-1(al-1)* gene (ds*al-1*) into a wild type and a *qde-2* mutant (*qde-2*^{rip}) strains. The *qde-2* mutant strain has multiple premature stop codons in the QDE-2 open reading frame (ORF) and is a complete loss of function mutant (Choudhary et al., 2007). The inverted repeat construct is under the control of quinic acid (QA) inducible (*qa-2*) promoter (Giles et al., 1985), so the addition of QA leads to the production of ds*al-1* and silencing of the *al-1* gene. Since, the *al-1* gene is responsible for carotenoid biosynthesis in *Neurospora*, its silencing leads to the production of white coloured conidia and aerial hyphae. The Northern blot analysis showed that the presence of QA in the media led to the production of *al-1* siRNAs in both strains, but the total amounts of siRNA as observed in the


A
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QA
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siRNA
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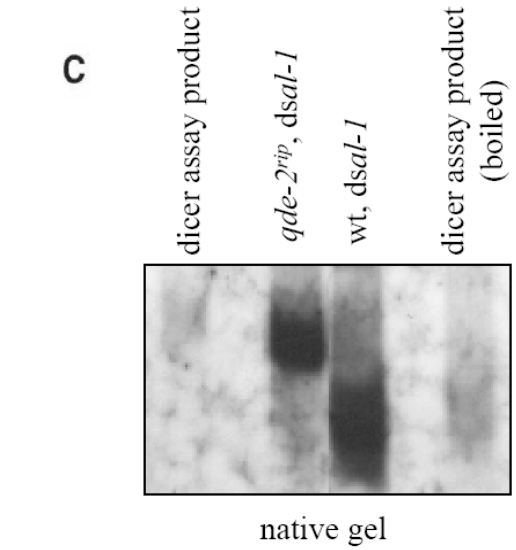
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wt, dsal-1
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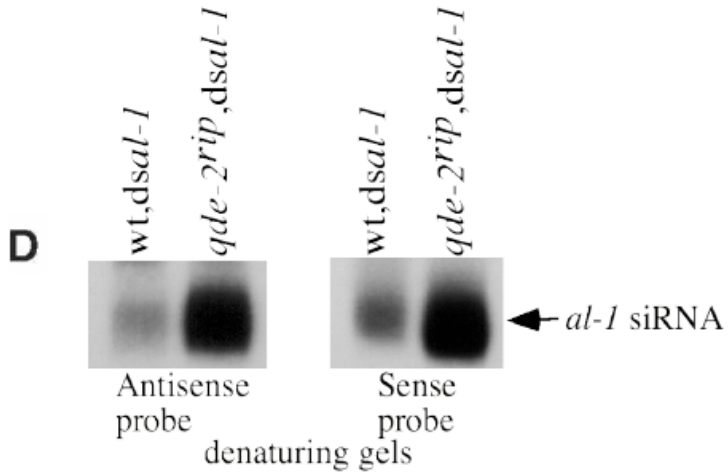
D
wt,dsal-1
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qde-2rip,dsal-1
al-1 siRNA
Antisense
probe
Sense
probe
denaturing gels

Figure 6. **QDE-2 is required for the generation of single-stranded siRNA *in vivo***. (A) & (B) Northern blot analyses of *al-1* siRNA by denaturing gel (A) and native gel (B). The cultures were grown with/without QA (1×10^{-3} M). A WT (wild-type) sample without ds*al-1* construct was used as a negative control. (C) Northern blot analysis showing the siRNA profiles in the native gel. The dicer products were made by carrying out dicer assay using uniformly labeled dsRNA and wild type cell extracts. The siRNA (untreated or boiled) products were then compared with the *in vivo* produced siRNA from the indicated *Neurospora* strains by Northern blot analysis. (D) Northern blot analyses showing the levels of *al-1* siRNAs using a sense or an antisense probe.

denaturing gel, was significantly higher in the *qde-2*^{rip} strain than that in the wild type strain (Figure 6A). Then the siRNAs from both strains were analyzed in native gel to investigate the nature of siRNAs. As shown in Figure 6B, all the siRNAs in the wild type strain was in single stranded form while there was no single stranded siRNA in the *qde-2*^{rip} strain. All the siRNAs in the *qde-2*^{rip} strain was accumulated as duplex forms. These results indicate that QDE-2 is essential for the *in vivo* generation of single stranded siRNAs. The higher level of siRNA in the *qde-2*^{rip} strain is probably due to the more stability of the duplex siRNAs than single stranded siRNAs in cells.

The identities of the two populations of siRNAs were confirmed by the identical gel mobility behaviors of the endogenously produced *al-1* siRNAs and the ^{32}P-labeled single and double stranded siRNAs (the products of dicer assay) in native gel (Figure 6C). To examine whether the siRNA strand selection is influenced by the presence of target mRNA, I compared the level of *al-1* siRNA

using a sense and an antisense probe. As shown in Figure 6D, both probe could detect the *al-1* siRNA with similar efficiency, suggesting that the strand selection is not influenced by the presence of target mRNA. The results of these experiments confirm the single stranded and duplex nature of siRNAs in the wild type and the *qde-2*^{rip} strains respectively.

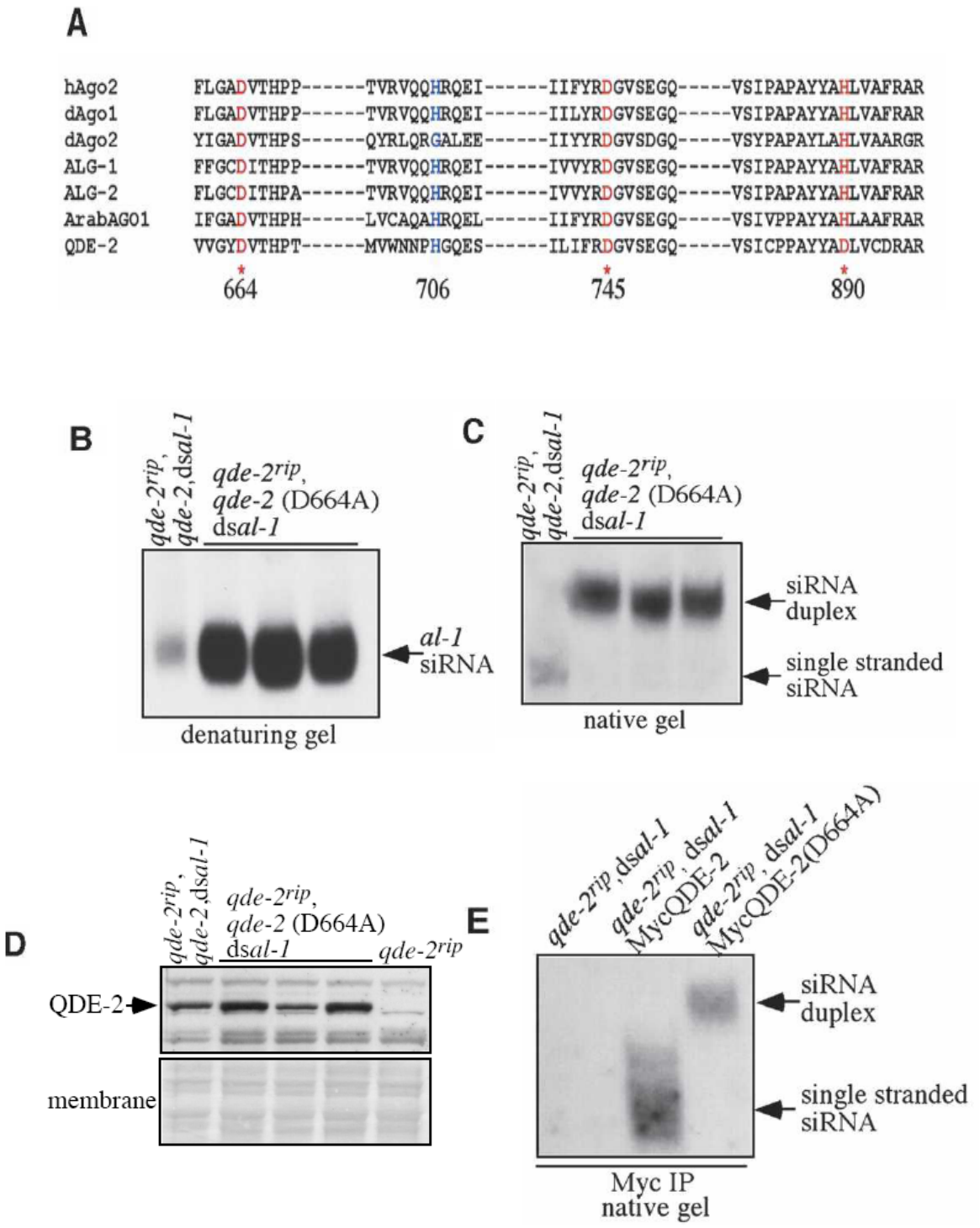
2.3.2 The endonuclease activity of QDE-2 is required for the generation of single stranded siRNA *in vivo*

The PIWI domain of Argonaute proteins has conserved DDH motif with endonuclease activity (Liu et al., 2004; Parker et al., 2004; Rand et al., 2004; Song et al., 2004). Mutational analyses have shown that the mutation of the first or second Asp residue resulted in the complete inhibition of the slicing activity (Liu et al., 2004). The first two Asp residues are conserved in the PIWI domain of QDE-2 but the His residues is replaced by another Asp residue **(**Figure 7A**)**. Having established that QDE-2 is required for the siRNA duplex separation, I then examined whether the endonuclease activity of QDE-2 is required for this activity. I mutated the first Asp residue of the DDD motif (D664) to Ala in a construct containing the wild-type *qde-2* gene (with its own promoter), and transformed both the wild-type *qde-2* and the D644A *qde-2* constructs into the *qde-2*^{rip} ds*al-1* strain. The northern blot analysis showed that all the siRNAs in the *qde-2*^{rip}*qde-2*,ds*al-1* were in single stranded form strain and the level of siRNA was similar to the wild type level, indicating the complementation of *qde-2*

function by the wild-type *qde-2* construct. In contrast, the level of siRNAs in the *qde-2*^{rip} *qde-2*(D664A),ds*al-1* strain was higher than wild type level and all the siRNAs were accumulated in duplex form (Figure 7B and 7C). These results indicate that the endonuclease or the slicing function of QDE-2 is required for the *in vivo* generation of single stranded siRNAs. In addition, expression of the mutant QDE-2 was comparable to the wild type QDE-2 (Figure 7D), indicating that the lack of activity in D664A mutant was not due to the lack of QDE-2 expression**.**

To investigate whether the D664A mutation of QDE-2 affected its ability to bind siRNAs and to examine whether both the single stranded and duplex siRNAs are actually associated with QDE-2, I transformed a construct expressing Myc-epitope tagged QDE-2 (Myc-QDE-2) or Myc-QDE-2(D664A) into the *qde-2*^{rip},ds*al-1* strain. Immunoprecipitation of the cell extracts was performed using Myc -monoclonal antibody and the precipitated siRNAs were analyzed in native gel. As shown in Figure 7E, the single stranded siRNAs were associated with Myc-QDE-2 and duplex siRNAs were associated with Myc-QDE-2(D664A). No siRNA was pulled down in the control strain lacking the Myc-tagged construct. These results suggest that siRNAs are loaded onto the RISC as duplex before the cleavage and removal of the passenger strand in *Neurospora.*

I then examined the importance of siRNA duplex separation in the dsRNA triggered gene silencing using the *qde-2* slicer mutants. As shown in



(Figure 7 continued,)

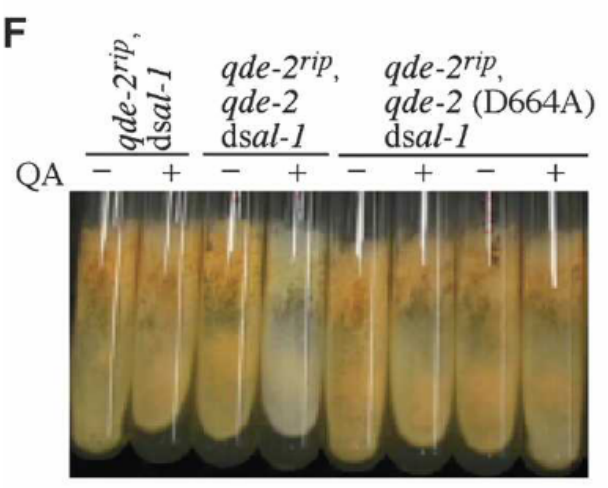


Figure 7. The slicer function of QDE-2 is required for the generation of single stranded siRNA *in vivo*. (A) Amino acid sequence alignment of the residues around the catalytic sites of the PIWI domains in QDE-2 and other Argonaute proteins. The amino acid residues of the DDH motif are highlighted and amino acid numbers of QDE-2 are indicated. Proteins aligned are hAgo-2, human AGO-2; dAgo-1 and dAgo2, *Drosophila* AGO1 and AGO2; ALG-1 and ALG-2, *C. elegans* ALG-1 and ALG-2; ArabAGO1, *Arabidopsis* AGO1. (B) & (C) Northern blot analyses of *al-1* siRNA by denaturing gel (B) and native gel (C) for the indicated strains. The cultures were grown in the presence of QA. (D) Western blot analysis using QDE-2 specific antibody showing the expression of QDE-2 in the indicated strains. Note that the QDE-2 expression was absent in the *qde-2*^{rip} strain. The Amido black stained membrane shown below is indicative of equal loading. (E) Northern blot analysis showing the association of siRNA with QDE-2 in the indicated strains. (F) Pictures of *Neurospora* slants showing that the silencing of *al-1* by ds*al-1* is abolished in the *qde-2*^{rip} and *qde-2*^{rip}, *qde-2*(D664A) strains but is rescued in the *qde-2*^{rip}, *qde*-2,dsal-*1*strain. For the experiments shown in (B), (C), (D) and (F), several independent *qde-2*^{rip},*qde-2*(D664A),ds*al-1* strains were used.

Figure 7F, the addition of QA during the growth of *qde-2*^{rip}/*qde-2*,ds*al-1* strain led to the formation of white aerial hyphae and conidia, which reflects the silencing of *al-1* gene. In contrast, the aerial hyphae and conidia remained orange in *qde-*

2^{rip} qde-2(D664A),ds*al-1* strain in the presence of QA , indicating that silencing of the *al-1* gene was abolished. Together these data suggest that the slicer activity of QDE-2 is essential for the single stranded siRNA generation and gene silencing.

2.3.3 Identification of QIP, a QDE-2-associated protein with an exonuclease domain

To gain further understanding about the mechanism of siRNA duplex separation and the RISC activation, I tried to identify QDE-2 interacting proteins. A wild type *Neurospora* strain was transformed with a construct that expressed the Myc-His (5 cMyc and 6-His) epitope tagged QDE-2 (Myc-His-QDE-2). The Myc-His-QDE-2 was purified through a nickel affinity column and then immunoprecipitated using the monoclonal c-Myc antibody (He et al., 2005). The final immunoprecipitates were analyzed by silver stained SDS-PAGE gel. A wild type strain lacking the Myc-His-QDE-2 construct was used as a negative control. As shown in Figure 8A, two distinct proteins bands (marked by arrows) were appeared only in the Myc-his QDE-2 sample and the mass spectrometry analysis of the excised bands identified one band as Myc His QDE-2 and the other band at ~ 80-kDa as a *Neurospora* hypothetical protein (NCU00076.2). We named the protein as QIP (Qde-2 Interacting Protein) and it shows significant sequence homology to the budding yeast Gfd2p. Gfd2p was originally identified as a high copy suppressor of the mutation of a DEAD -box helicase Dbp5 (Estruch and

Cole, 2003). Although the function of Dbp5 is well established in mRNA export process, no functional or biochemical analysis of Gfd2p was performed. The cloning of the *qip* cDNA revealed an extra 32 amino acids within the QIP ORF that was not predicted by the database.

To confirm the interaction between QDE-2 and QIP, I purified the Myc-His-tagged QIP from a wild-type strain carrying the expression construct using the same approach as described above (He et al., 2005). Figure 8B shows the silver stained SDS-PAGE gel of the Myc-immunoprecipitates. Mass spectrometry analysis of the two distinct bands appeared in Myc-His-QIP sample identified the lower band as Myc-His-QIP and the top band as the endogenous QDE-2, confirming the interaction between QDE-2 and QIP.

Amino acid sequence analyses revealed that QIP contains a DEDDh superfamily of 3'-5'exonuclease domain at the C-terminal part of the protein. Figure 8C shows the amino acid alignment of three critical motifs of the 3'-5' exonuclease domains in QIP and other proteins from various organisms (Hamdan et al., 2002). There are four conserved invariant acidic residues (D and E in motif I, D in motif II, and H and D in motif III) in the three motifs. Previous studies demonstrated that the conserved His (H) residue in motif III functions as a general base to direct an activated H_2O molecule for nucleophilic attack at the terminal phosphodiester bond and is critical for the exonuclease activity (Hamdan et al., 2002). The predicted secondary structure of the QIP exonuclease domain

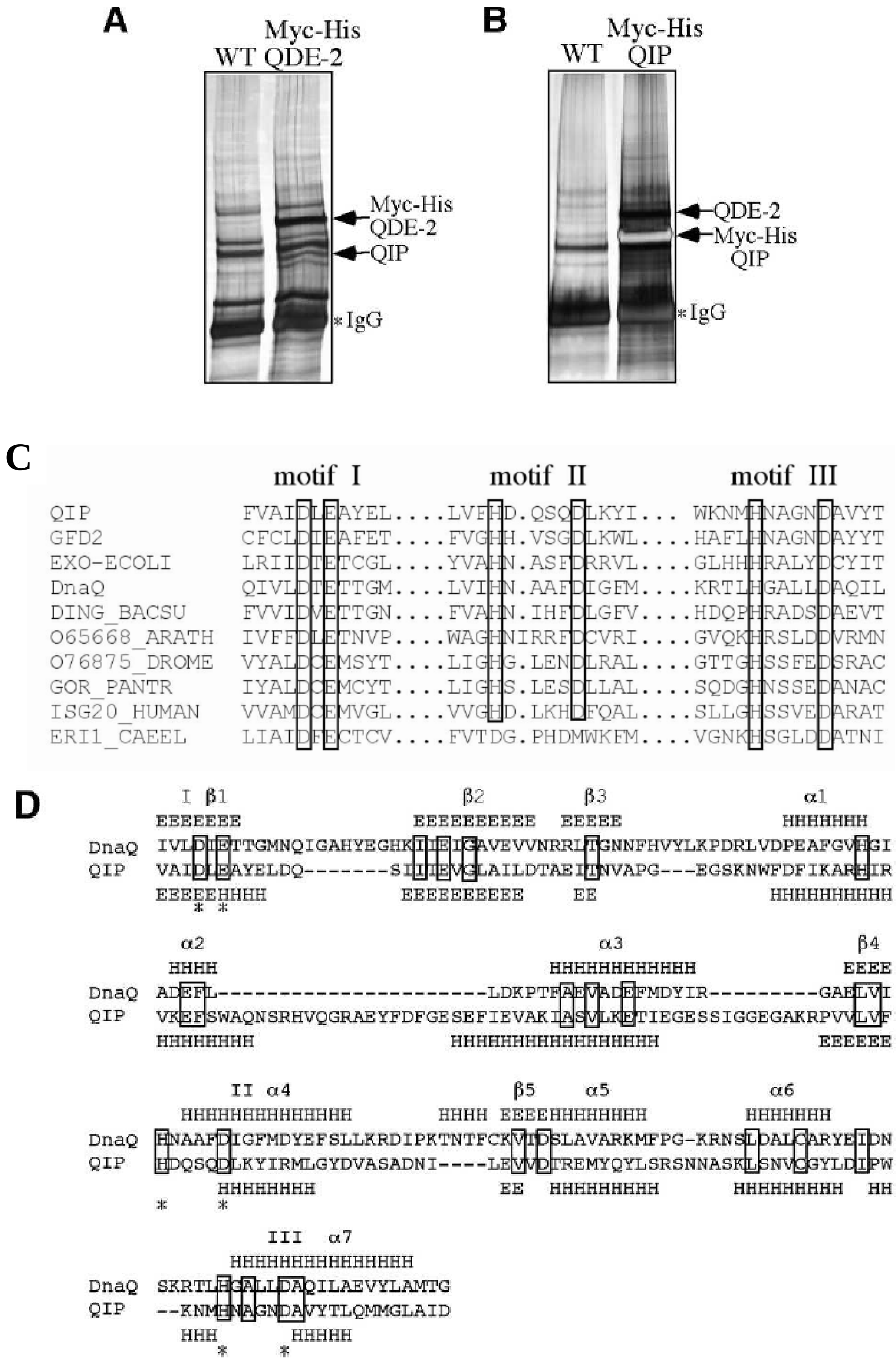

A
Myc-His
WT QDE-2
Myc-His
QDE-2
QIP
*IgG
B
Myc-His
WT QIP
QDE-2
Myc-His
QIP
*IgG
C
motif I
motif II
motif III
QIP FVAIDLEAYEL....LVFHD.QSQDLKYI... WKNMHNAGNDAVYT
GFD2 CFCLDIEAFET....FVGHH.VSGDLKWL....HAFLHNAGNDAYYT
EXO-ECOLI LRIIDTETCGL....YVAHN.ASFDRRVL....GLHHHRALYDCYIT
DnaQ QIVLDTETTGM....LVIHN.AAFDIGFM....KRTLHGALLDAQIL
DING_BACSU FVVIDVETTGN....FVAHN.IHFDLGFV....HDQPHRADSDAEVT
O65668_ARATH IVFFDLETNVP....WAGHNIRRFDCVRI....GVQKHRSLDDVRMN
O76875_DROME VYALDCEMSYT....LIGHG.LENDLRAL....GTTGHSSFEDSRAC
GOR_PANTR IYALDCEMCYT....LIGHS.LESDLLAL....SQDGHNSSEDANAC
ISG20_HUMAN VVAMDCEMVGL....VVGHD.LKHDFQAL....SLLGHSSVEDARAT
ERI1_CAEEL LIAIDFECTCV....FVTDG.PHDMWKFM....VGNKHSGLDDATNI
D
I β1 β2 β3 α1
EEEEEEE EEEEEEEEEE EEEEE HHHHHHH
DnaQ IVLDIETTGMNQIGAHYEGHKIIEIGAVEVVNRRLTGNNFHVYLKPDRLVDPEAFGVHGI
QIP VAIDLEAYELDQ--------SIIIEVGLAILDTAEIINVAPG---EGSKNWFDFIKARHIR
EEEEEHHHH EEEEEEEEEE EE HHHHHHHHH
* *
α2 α3 β4
HHHH HHHHHHHHHHHH EEEE
DnaQ ADEFL----------------------LDKPTFAEVADEFMDYIR---------GAELVI
QIP VKEFSWAQNSRHVQGRAEYFDFGESEFIEVAKIASVLKETIEGESSIGGEGAKRPVVLVF
HHHHHHHH HHHHHHHHHHHHHHHHH EEEEEE
II α4 β5 α5 α6
HHHHHHHHHHHHHH HHHH EEEEHHHHHHHH HHHHHHH
DnaQ HNAAFDIGFMDYEFSLLKRDIPKTNTFCKVIDSLAVARKMFPG-KRNSLDALCARYEIDN
QIP HDQSQDLKYIRMLGYDVASADNI----LEVVDTREMYQYLSRSNNASKLSNVCGYLDIPW
HHHHHHHH EE HHHHHHHHH HHHHHHHHH HH
* *
III α7
HHHHHHHHHHHHHHH
DnaQ SKRTLHGALLDAQILAEVYLAMTG
QIP --KNMHNAGNDAVYTLQMMGLAID
HHH HHHHH
* *

Figure 8. Identification of QIP as a QDE-2 interacting protein with an exonuclease domain. (A) & (B) Silver-stained SDS-PAGE gels showing the final immunoprecipitation products of the Myc-His-QDE-2 strain (A) and the Myc-His-QIP strain (B) by c-Myc monoclonal antibody. The identity of the indicated proteins was confirmed by mass spectrometry analyses. (C) Amino acid alignment of the three critical motifs in the exonuclease domains in QIP, ERI-1, and other proteins, including GFD2, *Saccharomyces cerevisiae,* NP_009894; EXO-ECOLI, *Escherichia coli*, P0AEK0; DnaQ, *Escherichia coli*, P03007; DING_BACSU, *Bacillus subtillis*, P54394; O65668_ARATH, *Arabidopsis thaliana*, O65668; O76875_DROME, *Drosophila melanogaster*, O76875; GOR_PANTER, *Pan troglodytes*, P48778; ISG20_HUMAN, *Homo sapiens*, Q96AZ6; ERI1_CAEEL, *Caenorhabditis elegans*, O44406. (D) Secondary structure comparison between the exonuclease domains in QIP and DnaQ (*E. coli* DNA polymerase III epsilon subunit). The predicted secondary structure of QIP was obtained by JPred (http://www.compbio.dundee.ac.uk/~www-jpred/), whereas the secondary structure of DnaQ was based on its crystal structure (Hamdan et al., 2002).

showed remarkable similarity with the known secondary structure of the exonuclease domain of *E. coli* DNA polymerase III epsilon subunit (Hamdan et al., 2002) (Figure 8D). Together these sequence and secondary structure studies strongly support the presence of the 3'-5' exonuclease domain in QIP. Interestingly, the known RNAi suppressor in *C.elegans*, ERI-1 also contains DEDDh family of exonuclease (Kennedy et al., 2004). However, unlike QIP, the exonuclease in ERI-1 belongs to DEMDh subfamily of exonuclease with different active sites in its motif II (Figure 8C). The similarity between ERI-1 and QIP and its interaction with QDE-2 suggest a potential function of QIP in siRNA processing of *Neurospora* RNAi pathway.

2.3.4 Disruption of *qip* results in impairment of RNAi

To understand the role of QIP in *Neurospora* RNAi pathway, I generated *qip* knock-out (qip^{ko}) strains by replacing the entire endogenous *qip* gene with a hygromycin resistance gene (*hph*) through homologous recombination. The homokaryotic nature of the mutant strains was confirmed by southern- blot analysis (Figure 9A). To investigate the role of QIP in *Neurospora* RNAi pathway, two different approaches were taken; quelling and dsRNA-induced gene silencing. Quelling assay was performed by introducing multiple copies of *al-1* DNA fragments into a wild type and a qip^{ko} strain. Comparison of quelling efficiency of the qip^{ko} and the wild type strains did not show significant difference (Table-2). This observation can be explained in two ways; 1) the quelling efficiency is saturated in the wild type strain, 2) QIP dose not have any significant role in the RNAi pathway. To distinguish between these two possibilities, I compared the quelling efficiency of a *dcl-2* single mutant ($dcl\text{-}2^{rip}$) and a *dcl-2* and *qip* double mutant ($dcl\text{-}2^{rip}$ qip^{ko}) strains. Although *Neurospora* has two functionally redundant DCLs (DCL-1 and DCL-2), DCL-2 contributes to more than 90% of the dicer activity (Catalanotto et al., 2004). So, the initial production of siRNA in the $dcl\text{-}2^{rip}$ strain is much less than that in the wild type strain and thus the comparison of RNAi efficiency in the $dcl\text{-}2^{rip}$ background should increase the sensitivity of the silencing assays. As shown in Table-2, the quelling efficiency in the $dcl\text{-}2^{rip}$ qip^{ko} double mutant is significantly lower than that of the

dcl-2 single mutant, indicating the impairment of quelling pathway in absence of *qip*.

To further confirm this result, I compared the gene silencing efficiency of the wild type and the *qip*^{ko} strains by inducibly expressing dsRNA, which allows for controlled level of gene silencing, thus, it is more quantitative and more reliable than the quelling assay (Cheng et al., 2005). I introduced an inverted repeat construct (under a QA-inducible promoter) specific for *frh* gene (ds*frh*) into the wild type and the *qip*^{ko} strains. *frh* is an essential *Neurospora* gene required for circadian clock function and silencing by ds*frh* results in a QA-dose dependent inhibition of *Neurospora* growth and the loss of circadian rhythms, which can be easily scored using a race tube assay over the course of days (Cheng et al., 2005).The results of the race tube assays showed that the addition of $1x10^{-5}$ or $1x10^{-4}$ M of QA resulted in the dramatic inhibition of growth in the wt,ds*frh* strains (the distance between two growth front marks corresponds to 24 hr of growth) and this growth defect was QA dependent (Figure 9B, compare the -/+QA racetubes). In contrast, the growth defect in the *qip*^{ko},ds*frh* strain upon QA addition was very modest, indicating that gene silencing was severely impaired in the *qip*^{ko},ds*frh* strain. Furthermore, I compared the dsRNA induced gene silencing efficiency of *dcl-2*^{rip} single mutant and *dcl-2*^{rip} *qip*^{ko} double mutant strains by introducing the ds*frh* construct. As shown in Figure 9C**,** presence of QA at a concentration of 1×10^{-3} M resulted in almost a complete inhibition of growth in

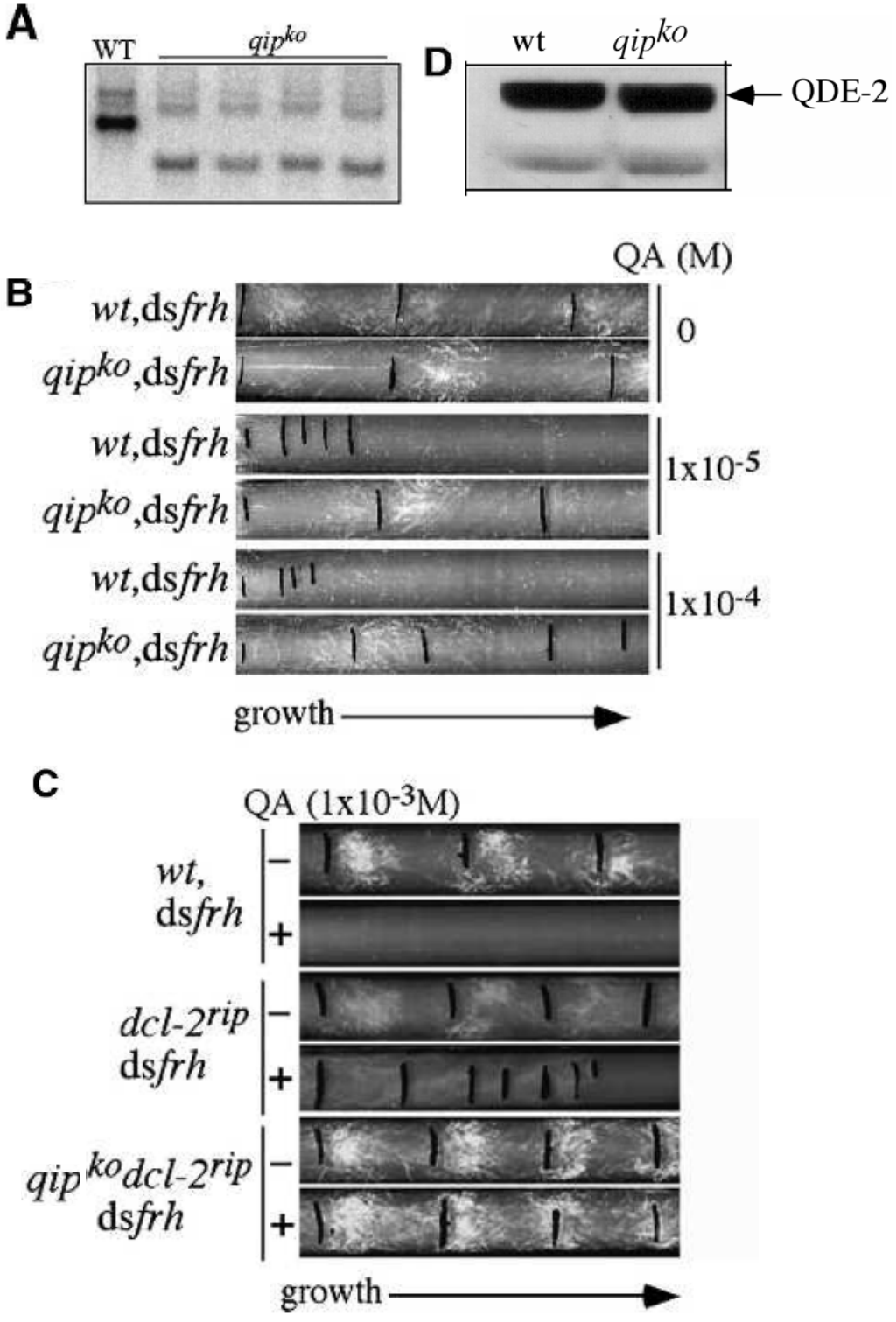

A
WT
qip^ko
D
wt
qip^ko
QDE-2
QA (M)
B
wt,dsfrh
qip^ko,dsfrh
0
wt,dsfrh
qip^ko,dsfrh
1x10^-5
wt,dsfrh
qip^ko,dsfrh
1x10^-4
growth
C
QA (1x10^-3M)
wt,
dsfrh
dcl-2^rip
dsfrh
qip^ko dcl-2^rip
dsfrh
growth

Figure 9. Disruption of *qip* in *Neurospora* severely impaired the RNAi efficiency. (A) Southern blot analysis showing the disruption of the *qip* locus in several independent qip^{ko} strains. (B) & (C) Race tube assay showing the growth of the indicated strains in the absence or presence of various concentration of QA in constant darkness. Each black line represents the growth front marked every 24 hr. The conidiation bands seen in the race tubes were due to the circadian conidiation rhythms. (D) Western blot result showing comparable expression of QDE-2 in the wild type and the qip^{ko} strains**.**

Table-2 Results of quelling assay

strains	No. of transformants	quelled strains	% of quelling
WT	193	111	57
qipko	137	77	56
$dcl\text{-}2^{rip}$	233	82	35
$dcl\text{-}2^{rip}$ qip^{ko}	401	39	9

Table-2. Impaired quelling efficiency in qip^{ko} strain. No. of transformants indicates the benomyl resistant transformants. Quelled strains represents all the partially (yellow) and fully (white) quelled transformants. Each quelling assay was repeated more than 4 times.

the wt,ds*frh* strain. Although the *frh* silencing in the $dcl\text{-}2^{rip}$,ds*frh* strain was impaired compared to the wt,ds*frh* strain, the addition of QA still led to the significant inhibition of growth, indicating that gene silencing was still active in

the *dcl-2*^{rip},ds*frh* strain. In contrast, there was no detectable growth defect in the *dcl-2*^{rip} *qip*^{ko},ds*frh* strain in presence of QA suggesting an almost complete loss of gene silencing in the double mutant strain. Together, the results of these *in vivo* gene silencing analyses indicate that unlike ERI-1 which is an RNAi suppressor, QIP is required for the efficient gene silencing in *Neurospora*. The lack of gene silencing in the *qip* mutants is not due to the lack of QDE-2 expression, as the western blot analysis showed comparable level of QDE-2 expression in both the wild type and *qip*^{ko} strains (Figure 9D).

2.3.5 QIP is required for processing of most of the duplex siRNA into single-stranded form

The observation that QIP is important for RNAi pathway, inspired us to investigate the mechanism of QIP function in mediating gene silencing. At first, I compared the siRNA generation efficiency of the wild type and the *qip*^{ko} strains by examining the dicer activity of both strains. An uniformly labeled long dsRNA was incubated with the cell extracts of wild type and *qip*^{ko} strains and the production of the labeled siRNAs was monitored in denaturing gel. As shown in Figure 10A the dicer activity in the *qip*^{ko} strain was comparable to that in the wild type strain, indicating that the impaired RNAi in *qip*^{ko} strain was not due to the lack of dicer activity.

As QIP interacts with QDE-2 and QDE-2 is involved in the siRNA duplex separation, I examined whether QIP has any role in siRNA duplex separation process. Comparison of the total amount of small RNA accumulation in denaturing gel analysis revealed that the accumulation was significantly higher in the *qip*^{ko},ds*al-1* strain than that in wt,ds*al-1*strain, but lower than that in the *qde-2*^{rip},ds*al-1*strain (Figure 10B). When the portions the siRNAs were analyzed in native gel, a small amount of single stranded siRNAs was observed in *qip*^{ko},ds*al-1* strain, but most of the siRNAs from this strain were accumulated as duplex form (Figure 10C, 10E). As expected all of the siRNAs in the wt,ds*al-1*strain were in single stranded form and all of them were in duplex form in the *qde-2*^{rip},ds*al-1*strain. But the duplex siRNAs in the *qip*^{ko},ds*al-1* strain showed two different gel mobility behaviors, one migrated slightly faster and the other migrated slightly slower than the siRNA duplex in the *qde-2*^{rip},ds*al-1*strain. These results indicate that QIP is required for the efficient separation of siRNA duplex and the disruption of *qip* resulted in the impairment of RISC activation. The results from these experiments explain the reason for impaired gene silencing observed in the *qip*^{ko} strain. In addition, the different gel mobility behaviors of the siRNA duplexes in the *qip*^{ko},ds*al-1* and the *qde-2*^{rip},ds*al-1*strain suggests that the siRNA duplexes in the *qip*^{ko},ds*al-1* strain may have already been processed by QDE-2.

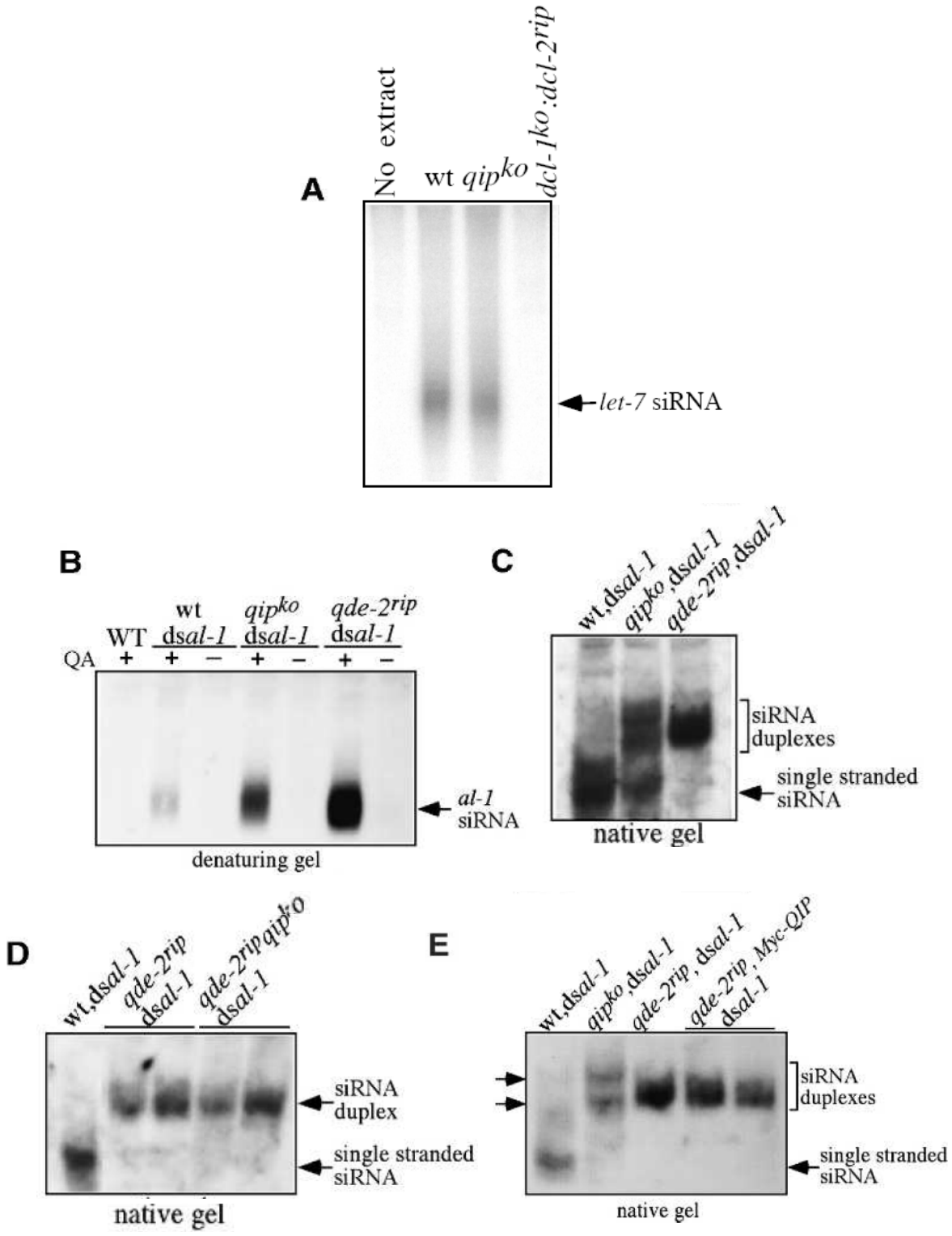

A
No extract
wt
qip^ko
dcl-1^ko:dcl-2^rip
let-7 siRNA
B
WT
wt dsal-1
qip^ko dsal-1
qde-2^rip dsal-1
QA + + − + − + −
al-1 siRNA
denaturing gel
C
wt,dsal-1
qip^ko,dsal-1
qde-2^rip,dsal-1
siRNA duplexes
single stranded siRNA
native gel
D
wt,dsal-1
qde-2^rip dsal-1
qde-2^rip qip^ko dsal-1
siRNA duplex
single stranded siRNA
native gel
E
wt,dsal-1
qip^ko,dsal-1
qde-2^rip, dsal-1
qde-2^rip, Myc-QIP dsal-1
siRNA duplexes
single stranded siRNA
native gel

Figure 10. siRNAs in the *qip*^{ko} strain existed mostly as duplex forms. (A) Dicer assay result showing the comparable dicer activity in the wild and the *qip*^{ko} strain. Dicer assay were carried out using uniformly labeled dsRNA substrate. (B) & (C) Northern blot analyses of *al-1* siRNA by denaturing (B) and native gels (C) showing that siRNA exists mostly as duplex forms in the *qip*^{ko}ds*al-1* strain. (D) & (E) Northern blot analyses of *al-1* siRNA of the indicated strains by native gels showing that the function of QIP in siRNA processing is dependent on QDE-2. Two independent transformants for some of the strains (*qde-2*^{rip},ds*al-1* and *qde-2*^{rip} *qip*^{ko},dsal-*1* in (D); *qde-2*^{rip},MycQIP,*dsal-1* in (E) were used. Note that the siRNA duplexes in the *qip*^{ko} strain showed different mobility from the duplexes in the *qde-2*^{rip} strains (marked by two arrows).

To examine whether the function of QIP in siRNA duplex separation is dependent on QDE-2, I generated *qde-2*^{rip} *qip*^{ko} double mutant strains. Gel-mobility of the siRNA duplex produced in the double mutant was identical to that in the *qde-2* single mutant (Figure 10D), indicating that QIP functions downstream of QDE-2. In addition, I introduced a construct overexpressing Myc-tagged QIP in *qde-2*^{rip},ds*al-1*strain. Although the over expressed Myc-QIP was fully functional (as shown in the next section), it was unable to alter the gel-mobility of the siRNA duplexes in the *qde-2*^{rip},ds*al-1*strain (Figure 10E). Together, these data indicate that the function of QIP is dependent on QDE-2 for siRNA duplex separation.

2.3.6 The predicted exonuclease domain of QIP is important for its function in siRNA duplex separation

Having established that QIP facilitates siRNA duplex separation, I examined whether the putative exonuclease domain of QIP is required for its

function. To obtain *in vivo* evidence of the requirement of the exonuclease domain, I expressed the Myc-tagged QIP or Myc-tagged QIP (H504A), in which the catalytic histidine residue in the motif III (Figure 8C) of QIP exonuclease domain was mutated to alanine, in the *qip*^{ko},ds*al-1* strain. As shown in Figure 11A and 11B, Myc-QIP completely rescued the function of endogenous QIP; the entire population of siRNAs was converted to single stranded form and its level was similar to that in the wild-type strain. In contrast the Myc-tagged QIP (H504A) expressing *qip*^{ko},ds*al-1* strains were unable to separate the siRNA duplex (Figure11D). The western blot result in Figure 11C showed that the expression levels of Myc-QIP and Myc- QIP (H504A) were similar (both from the QA inducible promoter) and the expression level in both strains were significantly higher than the endogenous QIP expression in the wild type strain: the QIP specific antibody could readily detect the Myc-QIP but was unable to detect the endogenous QIP. The observations that the siRNAs were accumulated in duplex form despite the overexprerssion of Myc- QIP (H504A) (Figure 11D) and Myc-QIP (H504A) was able to interact with QDE-2 (data not shown) suggest that QIP functions as an exonuclease in siRNA duplex processing.

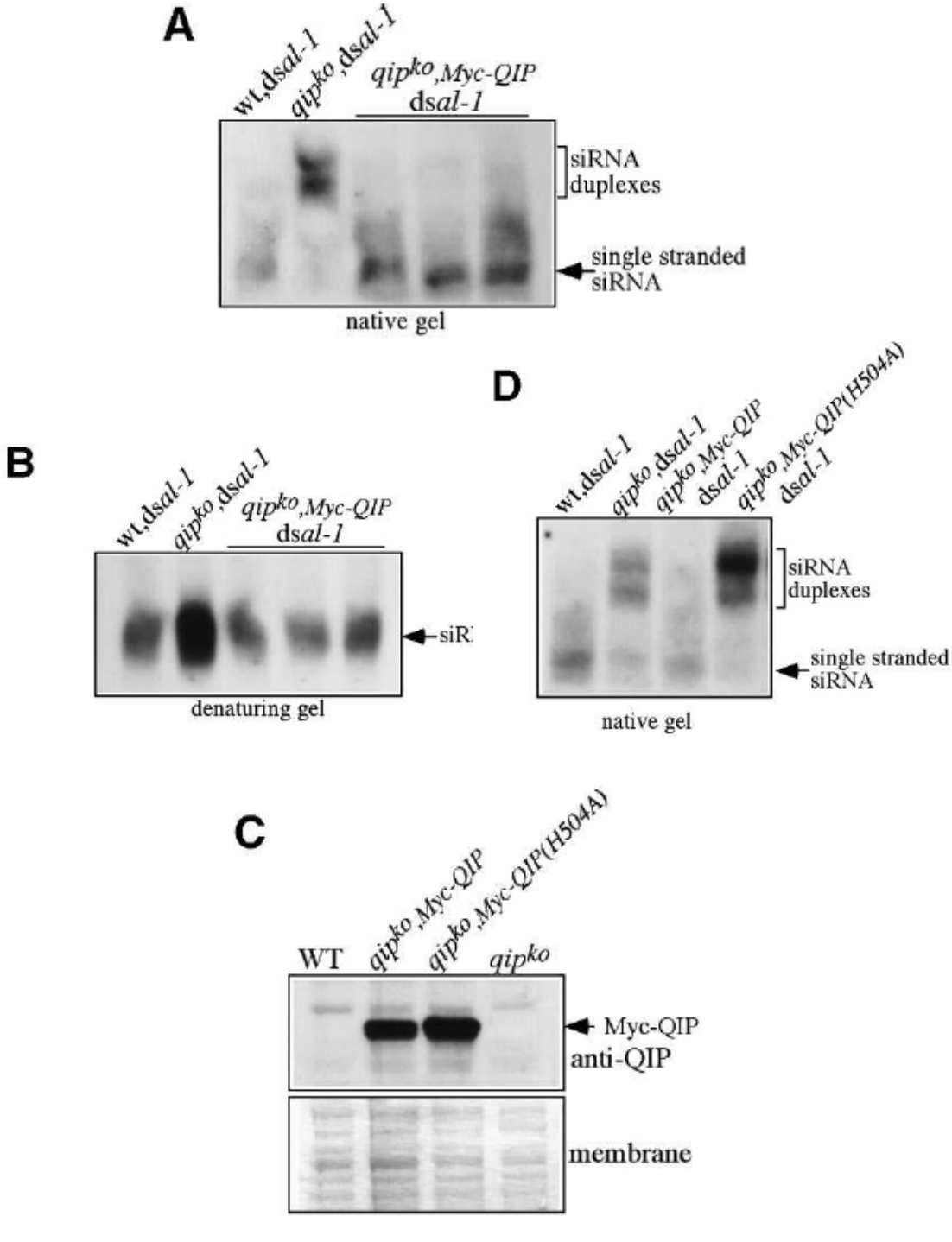

A
wt,dsal-1
qipko,dsal-1
qipko,Myc-QIP
dsal-1
siRNA
duplexes
single stranded
siRNA
native gel
B
wt,dsal-1
qipko,dsal-1
qipko,Myc-QIP
dsal-1
denaturing gel
D
wt,dsal-1
qipko,dsal-1
qipko,Myc-QIP
dsal-1
qipko,Myc-QIP(H504A)
dsal-1
siRNA
duplexes
single stranded
siRNA
native gel
C
WT
qipko,Myc-QIP
qipko,Myc-QIP(H504A)
qipko
Myc-QIP
anti-QIP
membrane

Figure 11. **Mutation of the putative catalytic residue in the QIP exonuclease domain abolished QIP's function in siRNA processing.** (A) & (B) Northern blot analyses of *al-1* siRNA by native (A) and denaturing (B) gels showing that the expression of Myc-QIP complements the siRNA processing defects in the *qip*^{ko} strain. (C) Western blot analysis by using an anti-QIP antibody showing the overexpression of Myc-QIP and Myc-QIP(H504A) in the *qip*^{ko} strains. Amido black stained membrane was shown below as loading control. (D) Northern blot analysis of *al-1* siRNA in a native gel showing that the expression of Myc-QIP(H504A) cannot complement the siRNA processing defects in the *qip*^{ko} strain. In (A) and (B), three independent *qip*^{ko},*Myc-QIP*,ds*al-1* strains were used.

2.3.7 QIP is involved in removing the nicked passenger strand from the siRNA duplex

The observations that both QDE-2 and QIP are required for *in vivo* siRNA duplex processing and the function of QIP is dependent on QDE-2 suggest that QIP assists QDE-2 in the conversion of siRNA duplex into single strands. However, being an exonuclease, QIP is not expected to have the endonuclease function as QDE-2 in making the initial cleavage in the passenger strand of siRNA duplex. In addition, the presence of different siRNA duplex species in the *qip*^{ko} and the *qde-2*^{rip} strains raises the possibility that the siRNA duplexes in the *qip*^{ko} strain have already been processed by QDE-2. In that case, the siRNA duplexes in the *qip*^{ko} strain should accumulate as nicked duplex form. After the initial cleavage of the passenger strand by QDE-2, the nicked duplex can be stable and migrate differently than the intact duplex in the native gel. Consistent with

this notion, it has been reported before that the siRNA duplexes with a nick in the passenger strands are relatively stable in the early stage of RISC assembly (Matranga et al., 2005). Thus, the potential function of QIP might be to digest the nicked passenger strands to generate single stranded siRNAs.

To test this hypothesis, I first examined the gel mobility behavior of the synthesized 32p labeled single stranded, nicked and intact *let-7* siRNA duplexes in native gel. As shown in Figure 12A**,** the nicked *let-7* siRNA duplexes migrated slightly faster than the intact duplex and this migration profile is very similar to the migration of the siRNA duplexes in the qip^{ko} and $qde\text{-}2^{rip}$ strains. These data strongly suggest that the accumulated siRNA duplexes in the qip^{ko} strain are nicked duplexes. There was another form of siRNA duplexes with slower mobility in the qip^{ko} ds*al-1* strain and those might also be the nicked duplex with different conformation.

To further confirm the hypothesis, I compared the thermostability of the siRNA duplexes produced in the qip^{ko} and $qde\text{-}2^{rip}$ strains. We reasoned that, if the siRNA duplexes in the qip^{ko} strain are nicked, they should exhibit less thermostability than the intact duplexes in the $qde\text{-}2^{rip}$ strains. The small RNAs produced in both the strains were treated with increasing temperature for 10 mints in each temperature before analyzing them in native gel. Indeed, the siRNA duplexes in the qip^{ko} strain showed less thermostability; the duplexes started converting to single stranded form as low as at 42°C and most of them were in

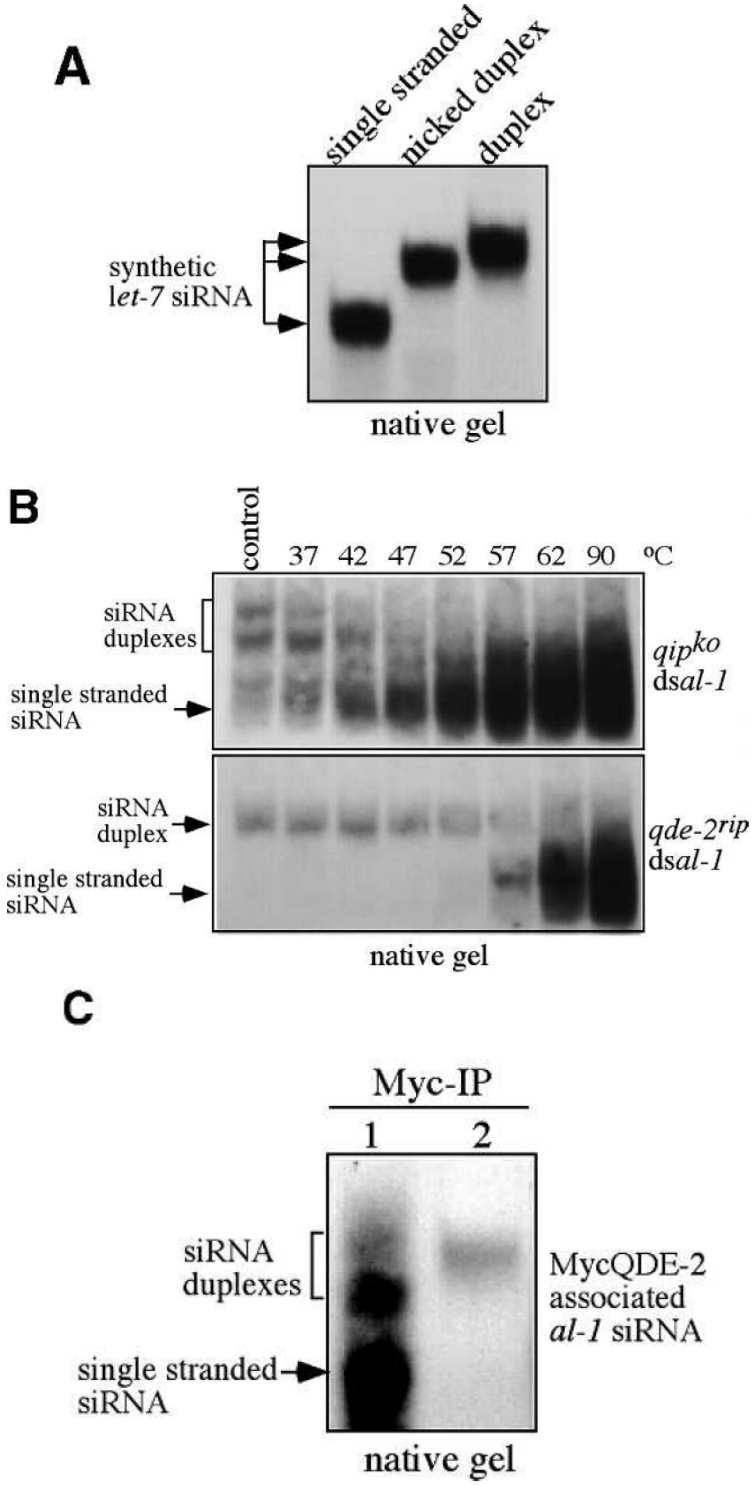

A
single stranded
nicked duplex
duplex
synthetic
let-7 siRNA
native gel
B
control
37 42 47 52 57 62 90 °C
siRNA
duplexes
single stranded
siRNA
qip^ko
dsal-1
siRNA
duplex
single stranded
siRNA
qde-2^rip
dsal-1
native gel
C
Myc-IP
1 2
siRNA
duplexes
single stranded
siRNA
MycQDE-2
associated
al-1 siRNA
native gel

Figure 12. **The siRNA duplexes in the *qip*^{ko} strain are the cleaved products of QDE-2.** (A) Gel mobility profiles of single-stranded, nicked duplex and intact duplex synthesized *let-7* siRNA (25mer). (B) The siRNA duplexes in the *qip*^{ko} strain are were significantly less stable than that in the *qde-2*^{rip} strain. The siRNA samples obtained from the *qip*^{ko} and *qde-2*^{rip} strains (cultured at 25^{o}C) were incubated at the indicated temperatures for 10 min before they were analyzed by Northern blot analysis in native gels usnig an *al-1* specific probe. The control samples were not subjected to the temperature treatment. (C) Northern blot analysis showing the association of siRNA species with QDE-2. Immunoprecipitation was performed using Myc monoclonal antibody. 1: *qde-2*^{rip} *qip*^{ko} MycQDE-2, 2: *qde-2*^{rip} MycQDE-2(D664A).

single stranded form at 57°C. In contrast, the siRNAs in the *qde-2*^{rip} strains were in duplex form until 57°C and were completely converted to single stranded only after 90°C treatment (Figure 12B). These results suggest that the siRNA duplexes in the *qip*^{ko} strains are less thermostable. I was unable to detect the nicked siRNA fragments in denaturing gel probably due to the inefficient transfer and hybridization of such small sized fragments.

Although we observed the accumulation of nicked siRNA duplexes in the *qip*^{ko} strains by analyzing the total small RNA population from the strain, the association of those nicked duplexes with the RISC was needed to be confirmed. To examine the association of the nicked duplexes with QDE-2, I transformed a Myc-QDE-2 construct in the *qde-2*^{rip} *qip*^{ko} ds*al-1* strain. The small RNAs isolated from the Myc-QDE-2 immunoprecipitates were analyzed in the native gel. As shown in Figure 12C, both the nicked duplex and single stranded siRNAs

produced in the *qde-2*^{rip} *qip*^{ko} ds*al-1* strain were associated with Myc-QDE-2. These results suggest that after the cleavage of the passenger strands, the nicked duplexes are associated with the RISC. Taken together, the results of these experiments strongly suggest that QIP facilitates the single stranded siRNA generation by removing the nicked passenger strand.

2.4 Discussion

Separation of the siRNA duplex strands is essential for Argonaute proteins to recognize and subsequently degrade the endogenous complimentary mRNA sequences. Previous biochemical studies have shown that Argonaute proteins are responsible for the generation of single stranded siRNA through passenger strand cleavage. In this study I provided *in vivo* evidences in support of the requirement of *Neurospora* Argonaute homologue, QDE-2 in siRNA duplex separation process. The results presented here demonstrate that siRNAs were accumulated only in duplex form in the *qde-2*^{rip} strain. Furthermore, mutation of a catalytic residue in the PIWI domain of QDE-2 completely blocked the siRNA separation process, indicating that the slicer function of QDE-2 is required for the generation of single stranded siRNA *in vivo*. Interestingly, previous studies have shown that when *Drosophila* RISC is loaded with siRNA duplex containing a non-cleavable passenger strand, a bypass mechanism separates the siRNA duplex (Matranga et al., 2005). But in *Neurospora,* siRNA duplex separation was completely blocked

in the slicer mutant of *qde-2*, indicating that the passenger strand cleavage by QDE-2 is absolutely required for RISC activation. Consistent with this observation a recent study in *Drosophila* has also reported the importance of cleavage activity of Ago-2 in the removal of the passenger strand from the RISC (Kim et al., 2007). However, not all of the conserved residues in the QDE-2 DDD motif are absolutely required for function. Mutation of the third ASP residue (D890) of the QDE-2 DDD motif did not completely block the single stranded siRNA production (data not shown). These results suggest that unlike the first Asp residue (D664) which is absolutely required for the generation of single stranded siRNAs, D890 is partially required for this process.

Although the passenger strand cleavage during RISC activation is well studied, the mechanism for removal of the cleaved passenger strand was unclear. In this chapter, I described the identification of QIP, a QDE-2 interacting protein, which is required for the removal of the nicked passenger strand and also for efficient RNAi. Therefore, the initial cleavage of the passenger strand and the removal of the nicked passenger strand are very important steps in RNAi pathway. Based on the observations presented in this study a current model of *Neurospora* RNAi pathway is proposed (Figure 13). According to this model, siRNAs are loaded onto the RISC as duplex and then the passenger strands of the duplexes are cleaved endogenously by QDE-2. Subsequently, QIP acts as an

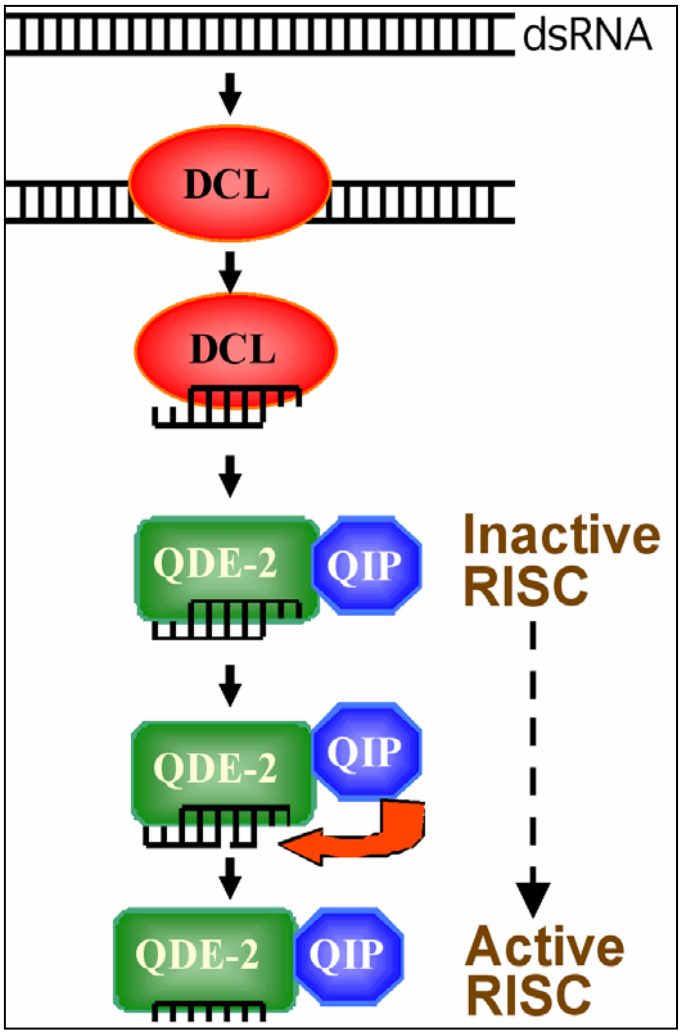


Figure 13. An updated model for the RNAi pathway in *Neurospora* explaining the roles of QDE-2 and QIP in generation of active RISC complex- containing single-stranded siRNA.

exonuclease to degrade the cleaved passenger and facilitates the activation of RISC. This model is supported by several lines of evidences. First, the separation of siRNA duplex was completely blocked in *qde-2* slicer mutants. Second, QIP was identified as an interacting partner of QDE-2 and most of the siRNAs were

accumulated as duplex form in the *qip*^{ko} strain. Third, gene silencing was severely impaired in absence of QIP, indicating the importance of single stranded siRNA generation. Fourth, the migration pattern of the siRNAduplexes in the *qip*^{ko} strain was different from that in the *qde-2*^{rip} strain, indicating that the siRNA duplexes in the *qip*^{ko} strain are nicked. This notion was supported by the identical migration of the synthesized *let-7* siRNAs. In addition, the siRNAs in the *qip*^{ko} strain showed less thermostability than the siRNA duplexes in the *qde-2*^{rip} strain, further suggesting that the siRNAs in the *qip*^{ko} strain has already been processed by QDE-2. Finally, mutation of the catalytic residue in the exonuclease domain of QIP failed to generate single stranded siRNAs, indicating that QIP functions as an exonuclease to process the siRNA duplex.

The evidences presented in this study suggest that QIP cannot function in absence of QDE-2, as overexpression of functional QIP did not change the level or nature of the siRNAs in the *qde-2*^{rip} strain. QDE-2 functions as more than just a substrate-recruiting subunit for QIP, QDE-2 actually generates the substrates for QIP. In the *qde-2*^{rip} *qde-2*(D664A) strain, where the initial passenger strand cleavage was blocked, presence of endogenous (Figure 7B and 7C) or overexpressed (data not shown) level of QIP was unable to separate the siRNA duplex. In addition, the D664A in QDE-2 mutation did not block the interaction between QIP and QDE-2 (data not shown). Taken together, these data suggest that the substrate of QIP is cleaved siRNA rather than the intact siRNAs. Although the

mechanism for QIP to distinguish between the intact and nicked siRNA duplex is still unclear, it is possible that the nicked duplex has conformational advantage over the intact duplex for being recognized by QIP. Alternately, the specificity of QIP function may be determined by the structural positioning of QIP in the QDE-2-siRNA duplex complex in such a manner that the specific positioning of QIP allows it to degrade only the cleaved the passenger strand.

The interaction between QIP and QDE-2 and the accumulation of siRNA duplex in the *qip*^{ko} strain may also suggest a potential function of QIP in the loading of siRNA duplex onto the RISC. Although we can not completely rule out this possibility, the observation that the siRNA duplex in *qip* mutant strain had already been processed, strongly suggests that QIP functions downstream of the siRNA loading step. Furthermore, the association of nicked siRNA duplex with Myc-QDE-2 in *qip* mutant strains (Figure 12C) also suggests that the loading of siRNA duplex was not impaired in the *qip*^{ko} strain.

Production of some single stranded siRNA in the *qip*^{ko} strain suggests that additional exonucleases or dissociation of the nicked duplexes may also be responsible for this process. However, our *in vivo* gene silencing assays clearly demonstrate that QIP mediated passenger strand removal is the primary mechanism for siRNA duplex separation in *Neurospora*. In *C.elegans*, ERI-1 was originally identified as a suppressor of RNAi. Recent studies have shown that ERI-1, which is an exonuclease, is required for the endogenous gene-silencing

machinery (Duchaine et al., 2006). In addition, MUT-7, another 3’ to 5’ exonuclease from the RNase D subfamily is also known to be important for RNAi pathway in *C.elegans* (Ketting et al., 1999). But the functional mechanism of ERI-1 or MUT-7 to promote gene silencing is not understood yet. Our study provides novel insights into an exonuclease mediated RISC activation mechanism, raising the interesting possibility that exonucleases in other organisms may also play active role in removing the passenger strand from siRNA duplexes.

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Chapter Three

Transgene induced quelling in *Neurospora* can occur independent of QDE-1 and QDE-3

3.1 Introduction

Quelling was originally discovered in *Neurospora* as a mechanism that in presence of multiple copies of transgene leads to the silencing of both the transgene and the endogenous homologous gene (Forrest et al., 2004; Fulci and Macino, 2007; Nakayashiki, 2005; Romano and Macino, 1992). Although the mechanism of RNAi pathway resulted from dsRNA trigger is well understood, how transgenes and repetitive sequences produce dsRNA is unclear. It has been proposed that the transcription of transgenic loci or repetitive sequences leads to the production of single stranded aberrant RNAs which are then converted to dsRNAs by RNA dependent RNA polymerase (RdRp) (Forrest et al., 2004; Hammond et al., 2001; Makeyev and Bamford, 2002; Tomari and Zamore, 2005). But it is unclear how cells can distinguish the aberrant RNAs from the abundant cellular mRNAs that do not trigger silencing. One previously proposed mechanism suggested that RdRp uses the uncapped 3' hydroxyl termini of the aberrant RNAs as primers to generate hairpin RNAs (Hammond et al., 2001). Several RdRp encoding genes have been identified in different organisms, such as

Ego-1in *C.elegans*, SGS-2/SDE-1 in *Arabidopsis* and QDE-1 in *Neurospora*. (Cogoni and Macino, 1999; Dalmay et al., 2000; Hammond et al., 2001; Smardon et al., 2000; Tomari and Zamore, 2005).

The dsRNA production in *Neurospora* is thought to be mediated by QDE-1 (an RdRp) and QDE-3 (a RecQ DNA helicase), which were originally identified in a mutagenesis screen where the mutant strains lacking either *qde-1* or *qde-3* lost their ability for gene silencing in quelling assays (Cogoni and Macino, 1997). How these two components work to generate dsRNA is not known. The efficiency of gene silencing in traditional quelling assay, that uses a plasmid to deliver the transgene into a *Neurospora* strain, is low and higher quelling efficiency was observed only when the QDE-1 was overexpressed (Cogoni et al., 1996; Forrest et al., 2004). In addition, QDE-1 and QDE-3 are not required for gene silencing when dsRNA is produced from an exogenous inverted repeat construct (Catalanotto et al., 2004). Furthermore, the absence of RdRp homologues in the genome of *Drosophila* and mammals suggests that RdRps are not universally required for silencing and their requirement is restricted to certain organisms.

In this chapter, I demonstrated that the introduction of the transgenes using DNA fragments instead of plasmid resulted in significant increase in quelling efficiency. The requirement of QDE-1 and QDE-3 in quelling was bypassed in presence of transgene DNA fragments. In addition, the studies of gene silencing

using RdRp mutants suggest the lack of secondary dsRNA amplification process in *Neurospora.*

3.2 Materials and Methods

3.2.1 Strains and Growth conditions

In this study FGSC 4200 (a), FGSC 987 (A), 87-3 (*bd*, a), FGSC 7088 (*his-3*, a) strains were used as wild type strains. FGSC 7088 (*his-3*, a) was used as a host strain for the transformation of *his-3* targeting constructs. The mutants strains, *qde-1*^{ko}, *qde-2*^{rip}, *qde-3*^{ko}, *dcl-1*^{ko}, *dcl-2*^{rip}, *dcl-1*^{ko} *dcl-2*^{rip} were used for this study. The *qde-1*^{ko} and *qde-3*^{ko} strains were generated for this study.The other mutant strains were generated in a separate study (Choudhary et al., 2007) and the method was described in chapter four. The *qde-1*^{ko} strain, 30-7#19 (*bd*, *A*); *qde-3*^{rip} strains (KTO-r-20*a* and KTO-r- 17*A*), *qde-3*^{rip} *recQ-2*^{ko} double mutant and the *qde-1*^{ko} *sad-1*^{ko} *rrp-3*^{ko} triple mutant strains were generously provided by Dr. Susan K. Crosthwaite , Dr. Hirokazu Inoue and Dr. Rodolfo Aramayo respectively. Culture conditions were similar as described previously (Cheng et al., 2001) and in chapter two. For liquid cultures containing QA, 0.01 M QA (pH 5.8) was added to the liquid culture medium containing 1x Vogel's, 0.1% glucose, and 0.17% arginine and for slants containing QA, 0.001 M QA (pH 5.8) was used.

3.2.2 Creation of *qde-1*^{ko}, *qde-3*^{ko} and other mutant strains

To disrupt the *qde-1*, *qde-3* genes in *Neurospora*, the gene replacement method was utilized. A PCR fragment containing the entire ORF and 3'UTR region of the gene (*qde-1* or *qde-3*) was cloned into pDE3BH, resulting in pQDE-1/pQDE-3 constructs. To create the disruption constructs, a hygromycin resistant gene (*hph*) fragment containing the promoter and the terminator sequences was inserted into the XbaI-PvuII site of pQDE-1 or the PvuII site of pQDE-3 constructs. The PCR fragment containing the gene replacement cassette was transformed to a wild type strain and hygromycin resistant transformants were selected. To get homokaryon mutant strain, a positive transformant was crossed with a wild type strain and sexual spores were picked individually and germinated in hygromycin containing (200 μg/ml hygromycin) slants. Southern blot analysis was performed to identify the homokaryon *qde-1*^{ko}, *qde-3*^{ko} strains.

3.2.3 Creation of the pBSK*al-1* construct

To create the pBSK*al-1* construct, a 1.4Kb fragment of the *al-1* gene was amplified using *al-1* for: 5’- CAAAGCCGGAGTAGACGTCAC- 3’ and *al-1* rev: 5’- GAAGAAGTCGTGGGCCAAACC primers containing ClaI and XbaI restriction enzymes sites at their ends respectively. The PCR fragment was digested with ClaI and XbaI and cloned into the ClaI-XbaI site of the BlueScript vector pSK+ resulted in the final pBSK*al-1* construct. The ds*al-1* construct was generated as described in chapter four.

3.2.4 Microconidia purification

Microconidia purification was performed to generate the homokaryon *qde-1^{ko} sad-1^{ko} rrp-3^{ko}*, ds*al-1* transgenic strains. The microconidia of the heterokaryon *qde-1^{ko} sad-1^{ko} rrp-3^{ko}*, ds*al-1* strain were purified as described earlier with few modifications (Ebbole and Sachs, 1990). At first, the *qde-1^{ko} sad-1^{ko} rrp-3^{ko}*, ds*al-1* strain was inoculated in 4-5 slants, each containing 6 ml of appropriate media and was grown for 7 days. The microconidia were harvested by adding ~3 ml of 1M sorbitol to the slants followed by rigorous vortex mixing for ~1 mint. The conidial suspension was then passed through 5 μm filter Millex Durapore filter units (Millipore catalog number SLSV025LS) using sterile conditions. Only the microconidia can pass through the filter. The microconidial suspension was plated in different dilutions in plates containing appropriate media. Southern blot analysis was performed to confirm the homokaryotic nature of the transgenic strain.

3.2.5 Quelling assay

Quelling assays were performed as described previously with few modifications (Cogoni et al., 1996). The wild type and the mutant strains used for quelling assays were grown for 7-10 days before harvesting the conidia in 1M sorbitol. A mixture of 2 μg pBSK*al-1* (carrying the *al-1* fragment) and 0.5 μg of

pBT6 (a benomyl resistant gene containing plasmid, obtained from the Fungal Genetic Stock Center, FGSC) was incubated with the conidial suspension for 4-5 hours at 4^0C. The plasmids were transformed into the *Neurospora* strains by electroporation (Margolin et al., 1999). For quelling assays using DNA fragments, a mixture of 0.4 μg of 1.5 Kb PCR fragment of *al-1* gene and 0.5 μg of pBT6 was incubated with the conidial suspension for 4-5 hours at 4^0C and was transformed by electroporation. The transformants were selected by growing them in benomyl containing slants. The benomyl resistant transformants were visually inspected to identify the complete quelled (white), partial quelled (yellow) or not-quelled (orange) strains. The two primers used for PCR amplification of the *al-1* fragment were *al-1-1*for (5'-CTTCCGCCGCTACCTCTCGTGG-3') and *al-1-2* rev (5'-CCCTTTGTTGGTGGCGTTGATG-3').The experiments were performed in constant white luminescence light at room temperature.

Insertion of *al-1* transgene into the quelled strains was confirmed by southern blot analyses. Genomic DNA from the randomly picked not-quelled, partially and fully quelled transformants were digested with SmaI and HindIII before running the agarose gel and transferring to the membranes. A 1.5 Kb *al-1* specific probe that can detect the endogenous *al-1* gene as 3.1 kb DNA band was used for southern blot. Normalizations were performed by southern blot using a probe specific for *al-2* gene.

3.2.6 Purification of small RNAs and Northern blot analyses

Preparation of the small RNAs and the Northern blot analyses were performed using the exact similar method as described in chapter two.

3.3 Results

3.3.1 Severe impairment of quelling in *qde-1*^{ko} and *qde-3*^{ko} strains in presence of a plasmid containing transgene

To understand the role of QDE-1 and QDE-3 in transgene induced quelling pathway, I generated *qde-1*^{ko} and *qde-3*^{ko} strains by disrupting the endogenous *qde-1 and qde-3* genes with a hygromycin resistance gene (*hph*) through homologous recombination. Southern blot analysis (Figure 14 A and 14B) confirmed the disruption of the *qde-1 and qde-3* loci and homokaryotic nature of the mutant strains. To understand the role of QDE-1 and QDE-3, I co-transformed a plasmid, pBSK*al-1* containing 1.4 Kb (ORF) of *al-1* gene (the same region used before for quelling experiments) and pBT6 into a wild type and the mutant strains by electroporation (Cogoni et al., 1996; Romano and Macino, 1992). As shown in Table-3, quelling efficiency was greatly reduced in the *qde-1*^{ko} and *qde-3*^{ko} strains compared to the wild type strains; 39-44% quelling in wild type strains in compare to 4-13% in the mutants. The similar low quelling efficiency was also observed in a *qde-1*^{ko} *sad-1*^{ko} *rrp-3*^{ko} triple mutant strain where all the known *Neurospora* RdRps in this organism were disrupted.

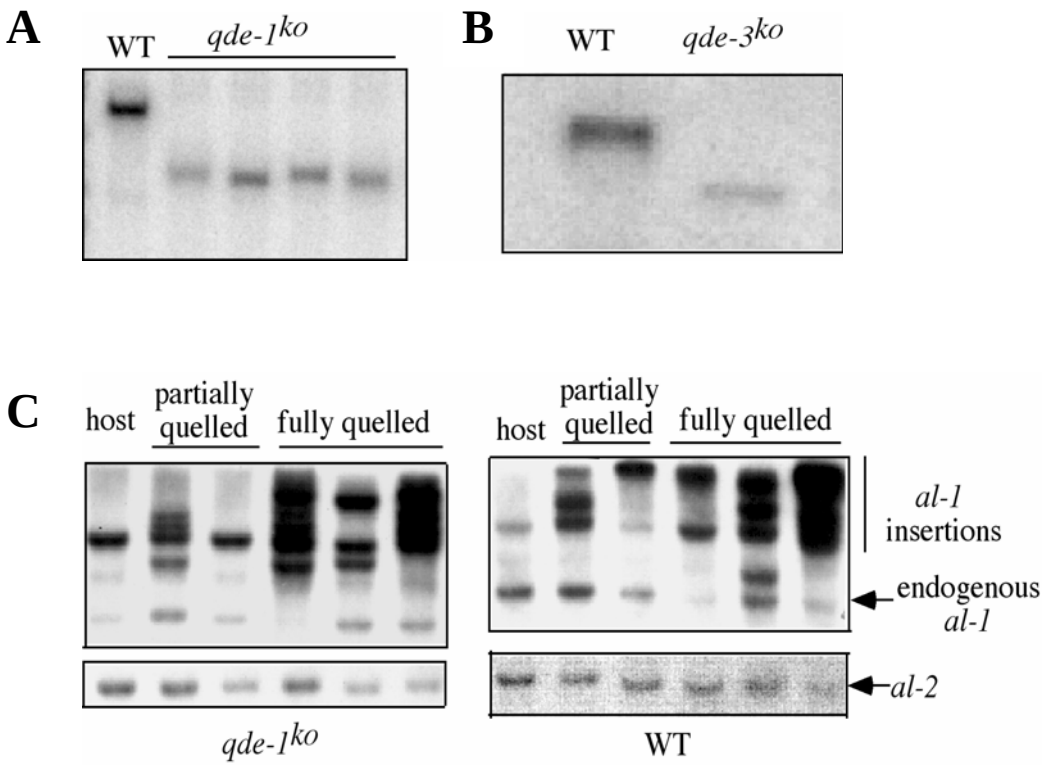

Figure 14. Quelling impairment in *qde-1*^{ko} and *qde-3*^{ko} strains in presence of plasmid containing transgene. Southern blot analysis showing the disruption of the *qde-1* (A) *and qde-3* (B) loci in the *qde-1*^{ko} and *qde-3*^{ko} strains. (C) Southern blot analysis using *al-1* specific probe showing the endogenous 3.1 Kb *al-1* band and multiple insertions of *al-1* transgene in the partially and fully quelled transformants of *qde-1*^{ko} and wild type strains. Normalization using *al-2* specific probe indicating the amount of genomic DNA used for digestion. WT, wild type.

Table-3 Results of quelling assay using pBSK*al-1*

Strains	No. of transformants	Quelled strains	% of quelling
WT	114	44	39
qde-1 ^{ko}	117	5	4.2
WT	78	34	44
qde-1^{ko} *sad-1*^{ko}*rrp3*^{ko}	80	4	5
WT	32	14	44
qde-3 ^{ko}	39	5	13

Table-3 Results of quelling assay using pBSK*al-1*. Quelling efficiency was compared between mutant and wild type strains. No. of transformants indicates the benomyl resistant transformants. Quelled strains represents all the partially (yellow) and fully (white) quelled transformants. Each quelling assay was repeated at least 3 times.

To test whether the endogenous *al-1* gene in the quelled strains is disrupted by the transgene, southern blot analyses using *al-1* specific probe were performed. As shown in Figure 14C, the endogenous *al-1* gene was observed as 3.1 Kb DNA band in almost all the quelled strains used for this study. The appearance of multiple larger bands in the quelled strains represents different integration events. Higher intensity of the larger bands in the fully quelled strains

than that in the partially quelled strains may indicate more integrations of transgene in the fully quelled strains. Similar results were observed in southern blot analyses using the quelled transformants of *qde-3*^{ko} and triple *rdrp* mutant strains (data not shown) .These results are consistent with the previous observation that QDE-1 and QDE-3 are important for the transgene induced gene silencing.

3.3.2. QDE-1 and QDE-3 are not required for quelling in presence of multiple copies of transgene DNA fragments

Previous studies established a correlation between transgenic repeats and the occurrence of silencing by indicating that presence of tandem transgenic repeats can induce silencing more efficiently. In addition, it has been observed that transgenic loci need to be transcribed to induce gene silencing in *Neurospora* and the efficiency of silencing is dependent on the copy numbers of the transgene (Cogoni et al., 1996; Cogoni and Macino, 1997; Que and Jorgensen, 1998). To investigate the correlation between repeated transgenic loci and quelling, I co-transformed 1.5 kb *al-1* PCR fragment (the exact same region used for the previous experiments in Table-3) and pBT6 into a wild type and the mutant strains. We reasoned that the smaller size of the PCR fragment compared to the plasmid used before should increase the insertion efficiency of the transgene into the genome and also should increase the chance of tandem and inverted repeat

formation. Although the DNA concentration used for this study was one-fourth of the concentration used for the previous method, an almost similar or even slightly higher frequency of quelling was observed in the wild type strains (36-55%). Surprisingly, the mutant strains showed an almost equal quelling frequency as observed in the wild type strain (35-46%, Table-4). Quelling assay in the *qde-3*^{rip} *recQ-2*^{ko} double mutant strains, where all the known *Neurospora* RecQ helicases were mutated, also showed high (~40%) quelling efficiency (data not shown). These results suggest that the introduction of transgene as DNA fragments not only increases the quelling efficiency, it also bypasses the requirement of RdRps (QDE-1, SAD-1 and RRP-3) and QDE-3. In contrast, QDE-2 and DCLs were absolutely required for quelling (Table-4). Additionally, the results of southern blot analyses using *al-1* specific probe suggest that the observed quelling in both wild type and the mutant strains was due to multiple insertions of the transgene and not due to the disruption of the endogenous *al-1* gene (Figure 15A). Performing quelling assays using the *qde-1* and *qde-3* mutant strains generated independently in two other laboratories also showed similar results (data not shown, mentioned in 3.2.1 of the Materials and Methods section). Together these results indicate that quelling can occur independent of QDE-1 and QDE-3.

I then examined the production of *al-1*siRNAs in the quelled transformants to understand the role of QDE-1 and QDE-3 in siRNA

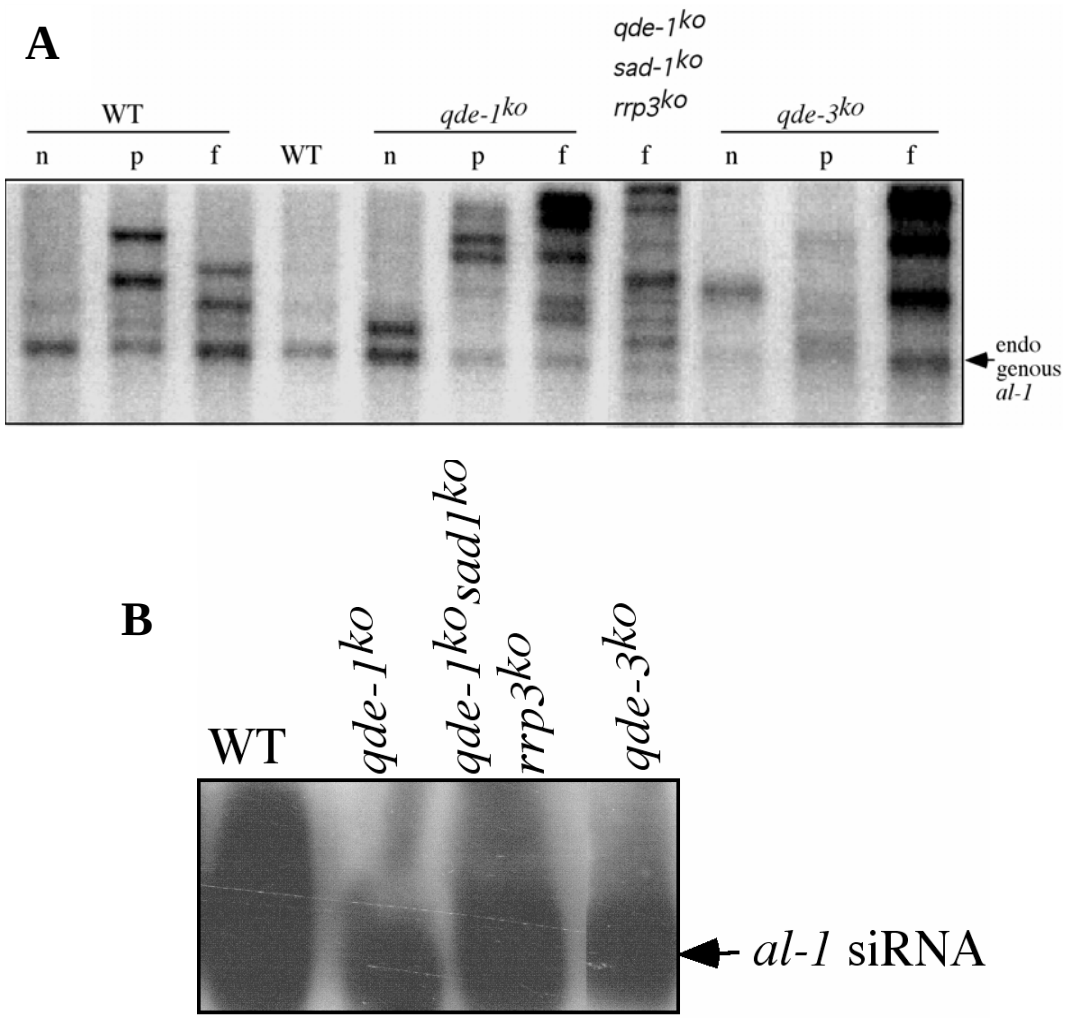

Figure 15. QDE-1 and QDE-3 are not required for quelling in presence of multiple copies of transgene DNA fragment. (A) Southern blot analysis using *al-1* specific probe showing the endogenous 3.1 Kb *al-1* band and multiple insertions of *al-1* transgene in the quelled transformants of *qde-1*^{ko}, *qde-1*^{ko} *sad-1*^{ko} *rrp3*^{ko}, *qde-3*^{ko} and wild type strains. n: not-quelled; p: partially quelled; f: fully quelled strains. WT, wild type. A wild type strain before transformation showing only the endogenous *al-1* band. (B) Northern blot analyses of *al-1* siRNA produced in the indicated strains showed less production of *al-1* siRNA in the mutant strains compared to the wild type strain.

Table-4 Results of quelling assay using *al-1* PCR fragment

Strains	No. of transformants	Quelled strains	% of quelling
WT	103	50	48.5
qde-1^{ko}	131	51	38.9
WT	95	34	35.7
qde-1^{ko} sad-1^{ko} rrp3^{ko}	61	21	34.4
WT	119	55	46.2
qde-3^{ko}	204	93	45.5
WT	40	22	55
qde-2^{rip}	52	0	0
WT	45	15	35.6
dcl-1^{ko} dcl-2^{rip}	40	0	0

Table 4. Results of quelling assay using *al-1* PCR fragments. Quelling efficiency was compared between mutant and wild type strains. No. of transformants indicates the benomyl resistant transformants. Quelled strains indicates all the partially (yellow) and fully (white) quelled transformants. Each quelling assay was repeated at least 3 times.

generation process during transgene induced gene silencing. As shown in Figure 15B, all the quelled transformants of wild type and different mutants were able to generate *al-1* siRNAs. This result is different from the previous results showing

that QDE-1 and QDE-3 are absolutely required for the siRNA generation in transgene induced quelling pathway (Catalanotto et al., 2002). Interestingly the total amounts of *al-1* siRNA generated in the mutant strains were significantly less than the siRNA generated in the wild type strain. These results suggest that although QDE-1 and QDE-3 are still involved in the generation of some siRNA, they are not absolutely required for siRNA production when quelling is induced by the introduction of transgene DNA fragments.

3.3.3 Absence of secondary dsRNA amplification process in *Neurospora*

The RdRps in other organisms such as in plants and worms are known to be involved in the amplification of siRNA and dsRNA (Hammond et al., 2001; Tomari and Zamore, 2005). The low level of *al-1* siRNAs in the quelled transformants of *qde-1*^{ko}, triple RdRp mutant and *qde-3*^{ko} strains suggests that these genes might be involved in either the generation initial dsRNA or in the amplification process of secondary dsRNAs. To distinguish between these two possibilities I transformed a quinic acid (*qa-2*) inducible (Giles et al., 1985) inverted repeat construct corresponding to *al-1* gene (ds*al-1*) into the *his-3* locus of a wild type and the *qde-1*^{ko} *sad-1*^{ko} *rrp-3*^{ko} triple RdRp mutant strains and compared the total amounts of *al-1* siRNA generated in these two strains. The homokaryotic transgenic strains were created by microconidia purification and southern blot analysis was performed to confirm the homokaryotic nature of the

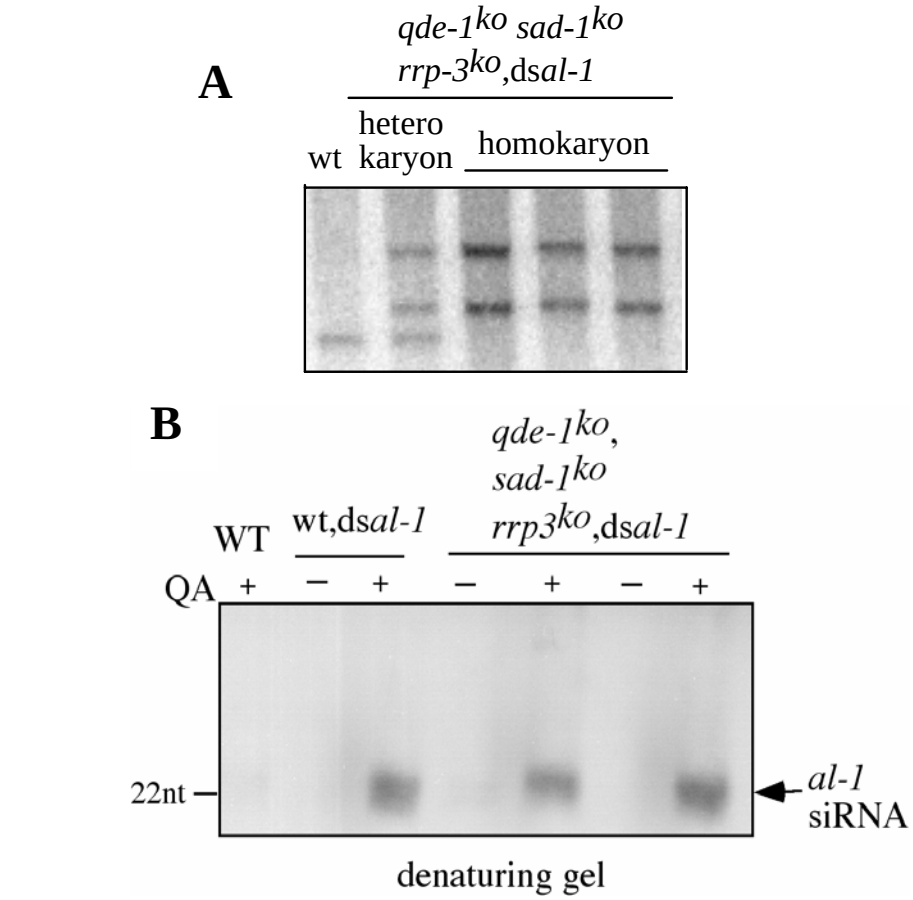
A
qde-1ko sad-1ko
rrp-3ko,dsal-1
wt
hetero karyon
homokaryon
B
qde-1ko,
sad-1ko
rrp3ko,dsal-1
WT
wt,dsal-1
QA + − + − + − +
22nt
al-1
siRNA
denaturing gel
C
WT, dsal-1
qde-1kosad-1ko
rrp3ko, dsal-1
QA + — + — + — + —

Figure 16. Absence of dsRNA amplification process in *Neurospora*. (A) Southern blot analysis using a *his* specific probe showing the heterokaryon and homokaryon nature of the *qde-1^{ko} sad-1^{ko} rrp3^{ko}*, ds*al-1* transgenic strains. 3 independent transgenic strains showing homokaryon nature after microconidia purification. (B) Northern blot analyses of *al-1* siRNA production in the wt, ds*al-1* and the *qde-1^{ko} sad-1^{ko} rrp3^{ko}*, ds*al-1* strains. The cultures were grown with/without QA (1 x 10^{-3} M). A WT (wild-type) sample without ds*al-1* construct was used as a negative control. Two independent *qde-1^{ko} sad-1^{ko} rrp3^{ko}*, ds*al-1* strains were used for this experiment. (C) Pictures of *Neurospora* slants showing the complete silencing of *al-1* gene by ds*al-1* in the wild type and the *qde-1^{ko} sad-1^{ko} rrp3^{ko}* strains in presence of 1 x 10^{-3} M QA. Two independent wt, ds*al-1* and the *qde-1^{ko} sad-1^{ko} rrp3^{ko}*, ds*al-1* strains were used for this experiment.

transgenic strains (Figure 16A). The use of the inducible inverted repeat construct allows for controlled level of the gene silencing, thus more quantitative. Northern blot analysis showed equal amounts of *al-1* siRNA accumulation in the wild type and the triple RdRp mutant strains in presence of 1 x 10^{-3} M QA (Figure 16B). This is consistent with the similar degree of *al-1* silencing phenotype observed in the wild type and triple RdRp mutant strains (Figure 16C). Since all the potential RNA dependent RNA polymerases are disrupted in the triple *rdrp* mutants, comparable siRNA production in the wild type and triple *rdrp* mutant suggests that RdRps are not involved in dsRNA and siRNA amplification process in *Neurospora*.

3.4 Discussion

In this study I examined transgene induced gene silencing in *Neurospora* by using an approach that is different from the previously described method. The data presented here showed that introducing transgene as DNA fragments increased the gene silencing efficiency and bypassed the requirement of RdRps and QDE-3. In addition, my results suggest that dsRNA or siRNA amplification process is absent in *Neurospora*.

Although quelling was one of the first described post transcriptional gene silencing (PTGS) phenomenon observed in *Neurospora*, the efficiency of gene silencing through the traditional quelling assays was quite poor; only 20-30% of the transformants showed quelling of a gene (Cogoni et al., 1996). In an attempt to increase the quelling efficiency in *Neurospora* and to understand the importance of QDE-1 and QDE-3 in transgene induced silencing process, I examined the quelling efficiency of the wild type and the mutant strains in presence of transgenes delivered as DNA fragments or as plasmid. Our results showed that introducing trasgene as DNA fragments increased the quelling efficiency since very low concentration of DNA (~0.4 μg of the PCR products that is ~1.5 fold less molar concentration than that of the plasmid) was able to induce silencing. In addition, quelling by this method also bypasses the requirement of QDE-1 and QDE-3. Previous studies have shown that QDE-1 and QDE-3 were not required for the gene silencing when the dsRNAs are produced

from exogenous inverted repeat construct (Catalanotto et al., 2004). In addition, it has been proposed that transgene can be inserted into genome in inverted arrays and may lead to production of dsRNAs directly (Mello and Conte, 2004). Together, the results presented here suggests that introducing transgene as DNA fragments possibly promotes the formation of inverted repeats of the transgenes that directly generates the dsRNA after transcription, thus bypassing the requirement of QDE-1 and QDE-3. This hypothesis is consistent with the previous studies in worms showing that a type of transposon that contains the inverted repeat sequence can directly trigger silencing by forming dsRNA (Sijen and Plasterk, 2003). The inverted repeats can be formed in two ways; 1) because of their smaller size than plasmid, the transformation efficiency of the trasgene DNA fragment will be higher and the high copy numbers can increase the chance of tandem and inverted repeat formation, 2) unlike the previous method, where the transgene is flanked by the long vector sequence, introducing transgene as DNA fragments can possibly decrease the distance between two inserted transgene fragments and thus serve as better templates for hairpin structure formation. Interestingly, we observed higher quelling efficiency than the previous results, even in the plasmid -based method. This can be potentially explained by the difference in the transformation methods used for quelling assays. Electroporation method used in this study may insert the transgenes more

efficiently than the spheroplast-transformation method used in previous studies for quelling assays.

Based on the observations presented here, QDE-1 and QDE-3 seem to be required for dsRNA production when traditional quelling method is used. The large size of the plasmid carrying the transgene will likely decrease the chance of tandem or inverted repeat formation. In that case, dsRNAs can be produced from the aberrant transcripts in a QDE-1 and QDE-3 dependent manner. The mechanism for the production of aberrant RNAs is still uncertain. Further studies are needed to understand the mechanism of aberrant RNA production and the unique nature of aberrant RNAs.

Although we showed that quelling can occur independent of QDE-1 and QDE-3, lower levels of siRNAs were observed in the mutant strains than that in the wild type strains suggesting that they are still required for the production of certain population of dsRNA. However, the *in vivo* gene silencing analyses using inducible inverted repeat construct suggest that unlike plants and worms, *Neurospora* dose not have any secondary dsRNA or siRNA amplification process. In that respect, the gene silencing mechanism of *Neurospora* is similar to the silencing mechanism of flies and mammals which do not show any amplification of RNAi signals (Tomari and Zamore, 2005).

3.5 References

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Chapter Four

A double-stranded RNA response program important for RNA interference efficiency

4.1 Introduction

Presence of dsRNA in eukaryotic cells is generally the outcome of viral replication or transcription of transposable elements and repetitive DNA sequences. dsRNAs are known to trigger two different cellular responses: RNAi and transcription based interferon (IFN) response (Carmell and Hannon, 2004; Haller et al., 2006; Karpala et al., 2005; Meister and Tuschl, 2004; Sledz et al., 2003). These cellular responses function as defense mechanisms against viral invasion and expansion of transposable elements (Ding et al., 2004; Goodbourn et al., 2000; Haller et al., 2006; Lu et al., 2005; Sijen and Plasterk, 2003; Wilkins et al., 2005). So, it is reasonable to hypothesize that the components of the defense response pathways may be regulated by dsRNA. Although the basic mechanism of RNAi is well studied, little is known about the regulation of RNAi components.

In this study the regulation of RNAi components by dsRNA and physiological significance of such a regulatory mechanism were examined using

Neurospora as a model system. Since QDE-2 and DCL-2 are the two key components of *Neurospora* RNAi pathway, our study was primarily focused on the regulation of these two components. Our results showed that the transcription of *qde-2* and *dcl-2* was induced by dsRNAs. In addition, accumulation of QDE-2 proteins after the induction by dsRNAs was impaired in absence of DCLs, indicating that DCLs or siRNAs or both are involved in posttranscriptional regulation of QDE-2. Finally this study showed that the induction of QDE-2 is required for efficient gene silencing.

The study presented here was performed by me and two other graduate students in Dr. Liu's lab, Swati Choudhary and Heng-chi Lee. The contribution of each person is mentioned in the result section. 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.***

4.2 Materials and Methods

4.2.1 Strains and Growth conditions

FGSC 987 (A) and 87-3 (*bd*, a) were used as wild type strains in this study. FGSC 462 (*his-3* A) and 301-6 (*bd his-3* A) were used for the transformation of *his-3* targeting constructs. The following mutant strains (both in

wild type and *his-3* background) were created for this study: *qde-2*^{rip}, *dcl-1*^{ko}, *dcl-2*^{rip}, *dcl-1*^{ko} *dcl-2*^{rip}. The *qde-1*^{ko}, *qde-3*^{ko} mutant strains were generated in a different study. Culture conditions were similar as described previously (Cheng et al., 2001a) and in chapter two. For the time-course experiments, mycelia mats of the specific strains were prepared and then equal number and size of mycelial discs were cultured in the liquid media for distinct periods of time (Aronson et al., 1994). In case of QA containing cultures, they were grown for 2 days unless otherwise indicated. For the liquid cultures containing histidine, a final concentration of 0.5 mg/ml was used.

4.2.2 Creation of the *qde-2*^{rip}, *dcl-2*^{rip} and *dcl-1*^{ko} strains

The *qde-2* and the *dcl-2* genes were disrupted utilizing an unique process observed in *Neurospora*, called RIP or repeat-induced point mutant (Cambareri et al., 1989). The PCR fragment containing the entire ORF of *qde-2* or *dcl-2* and the 3'UTR region (3.3Kb for *qde-2* and 5.1 Kb for *dcl-2*) was cloned in to pDE3BH and was transformed to the *his-3* locus of a wild type strain (FGSC 7088 *his-3 a*) by electroporation (Margolin et al., 1999). One of the positive transformants was crossed with a wild type strain of opposite mating type. Southern blot analysis and DNA sequencing were performed to identify the strains where the endogenous *qde-2* or *dcl-2* was disrupted by premature stop codons through the random G/C to A/T mutation.

The gene replacement method was used to disrupt the *dcl-1* gene in *Neurospora*. A PCR fragment containing the entire ORF and 3'UTR region of the gene was cloned into pDE3BH, resulting in the pDCL-1 construct. To create the disruption construct, a hygromycin resistant gene (*hph*) fragment containing the promoter and the terminator sequences was inserted into the BamHI site of pDCL-1 construct. The PCR fragment containing the gene replacement cassette was transformed into a wild type strain and hygromycin resistant transformants were selected. To obtain homokaryon mutant strain, a positive transformant was crossed with a wild type strain and sexual spores were picked individually and germinated in hygromycin containing (200 μg/ml hygromycin) slants. Southern blot analysis was performed to identify the homokaryon *dcl-1*^{ko} strains.

A *dcl-1*^{ko} strain was crossed with a *dcl-2*^{rip} strain of opposite mating type to generate *dcl-1*^{ko}*dcl-2*^{rip} double mutant strains. All the mutant strains were generated in wild type as well as in *his-3* background.

The *qde-1*^{ko} and *qde-3*^{ko} strains were generated in a different study and the method is described in chapter three.

4.2.3 Creation of the dsRNA and other constructs

The ds*al-1*, ds*frq* and ds*frh* strains were generated as described previously (Cheng et al., 2005), briefly by introducing an inverted repeat construct expressing hair-pin RNA under the control of *qa-2* promoter into the wild type or

the mutant strains. The PCR fragments of *al-1*, *frq* and *frh* (*al-1*, bp1322 to 1942 and bp1412 to1942; *frq*, bp 669 to 2309 and bp 791 to 1252; *frh*, bp 2087 to 2703 and bp 2189 to 2703) were generated and cloned into pDE3BH.qa in reverse and forward directions respectively through three way ligation to generate the final pds*al-1*, pds*frq* and pds*frh* constructs. The resulting plasmid containing the inverted repeats under the *qa-2* promoter was introduced into the *his-3* locus of the wild type or the RNAi mutant strains.

The construct containing the wild type *qde-2* gene with its own promoter was created as described previously (Maiti et al., 2007) and also in chapter two.

To create the constructs for *qde-2* promoter analyses, the PCR fragments containing the entire *qde-2* ORF and different length of its upstream promoter sequence (as mentioned in Figure 20C) were cloned into the NdeI site of the pds*al-1* plasmid and were transformed into the *his-3* locus of the *qde-2*^{rip} strain.

4.2.4 Generation of QDE-2 and DCL-2 specific antibody

A glutathione S-transferase-QDE-2 (GST-QDE-2, containing QDE-2 amino acids 541-938) fusion protein was expressed in the BL21 cells, the inclusion bodies containing the recombinant proteins were purified and used as an antigen to generate rabbit polyclonal antiserum as described previously (Cheng et al., 2001b) and also in chapter two.

Dicer-2 specific antibody was generated by using a DCL-2 peptide (DRDDSSQDPDDNESF) as antigen. The peptide was synthesized by the peptide synthesis facility at University of Texas Southwestern medical system. The rabbit polyclonal serum was purified using a DCL-2 peptide conjugated affinity column.

4.2.5 Protein and RNA analyses

Protein extraction, quantification, and western blot analysis were performed as described earlier (Cheng et al., 2001a) and also in chapter two. Equal amounts of total protein (50 μg) were loaded in each lane of the SDS PAGE gel. After electrophoresis, proteins were transferred onto PVDF membrane. An amido-black stained membrane or non specific cross reacting bands were used as loading control.

Extraction of RNAs and Northern blot analyses were performed as described previously (Aronson et al., 1994). Briefly, equal amount (~30 μg) of total RNA was loaded in each lane of 1.3% agarose gel for electrophoresis and then transferred to membrane. For Northern blot, a single stranded ^{32}P- UTP labeled RNA probe specific for *qde-2* or *dcl-2* was prepared by *in vitro* transcription from a DNA template containing T7 promoter sequence in antisense orientation using MAXIscript® T7 kit (Ambion). The probe was purified through Micro Bio-Spin 30 column (Biorad) and was added to the hybridization solution. The membranes were hybridized for over night at 65^{0}C and then washed with 1 X

SSC and 0.1% SDS buffer at 68°C, three times, each of 20 min before it was exposed to X-ray film. The rRNA levels shown by ethidium bromide stained agarose gels were used as loading control.

4.2.6 Quelling assay

The quelling assays were performed in a wild type strain using the exact same method as described in chapter three.

4.3 Results

4.3.1 Induction of *qde-2* expression by dsRNA

To gain insight into the regulation of QDE-2, I first examined the endogenous QDE-2 level in different RNAi mutant strains using QDE-2 specific antibody. The western blot result showed that the level of QDE-2 was severely reduced in the *dcl-1*^{ko}*dcl-2*^{rip} double mutant and the *qde-1*^{ko}, *qde-3*^{ko} single mutant strains compared to that in the wild type strain (Figure 17A), indicating that the level of QDE-2 is regulated by the other components of the RNAi pathway. As QDE-1 and QDE-3 are thought to be involved in the production of endogenous dsRNAs and DCLs are required for the generation of siRNAs, this result suggests that dsRNAs or siRNAs are important for the expression of QDE-2.

To confirm this hypothesis, we examined the induction of QDE-2 in the *al-1* quelled strains. In quelling assay (performed by me and Heng-chi Lee), the *al-1* gene was silenced by transforming multiple copies to *al-1* DNA fragments

into a wild type strain and the resulting partially quelled (yellow) and fully quelled (white) strains were used for further studies. Comparison of QDE-2 level in the wild type strain (orange) with that in the partially and fully quelled strains showed that QDE-2 protein level was increased in the partially quelled strains and further increased in the fully quelled strains (Figure 17B, performed by Swati Choudhary). Since quelling involves the production of dsRNAs and siRNAs, the degree of *al-1* silencing reflects that amount of dsRNAs and siRNAs produced from the *al-1* transgenes. Thus the increase of QDE-2 in the quelled strains further supports the notion that the presence of dsRNA or siRNA leads to the induction of QDE-2 level in those strains.

To directly investigate the effect of dsRNA on QDE-2 expression, we examined the level of QDE-2 in different wild type strains each carrying a dsRNA expressing inverted repeat construct. These inverted repeat constructs are under the control of quinic acid (QA) - inducible promoter (Giles et al., 1985), so the presence of QA in the media leads to the production of dsRNAs and siRNAs in the wild type strain. In contrast to the wild type strain, *dcl* double mutant strains are unable to produce the siRNAs from the dsRNAs (Catalanotto et al., 2004; Maiti et al., 2007). The level of QDE-2 was compared among the wild type, *qde-2*^{rip} and three wild type transgenic strains expressing the dsRNAs specific for *al-1* (wt, ds*al-1*), *frequency* (wt, ds*frq; frq*, a circadian clock gene), *frh* (wt, ds*frh;*

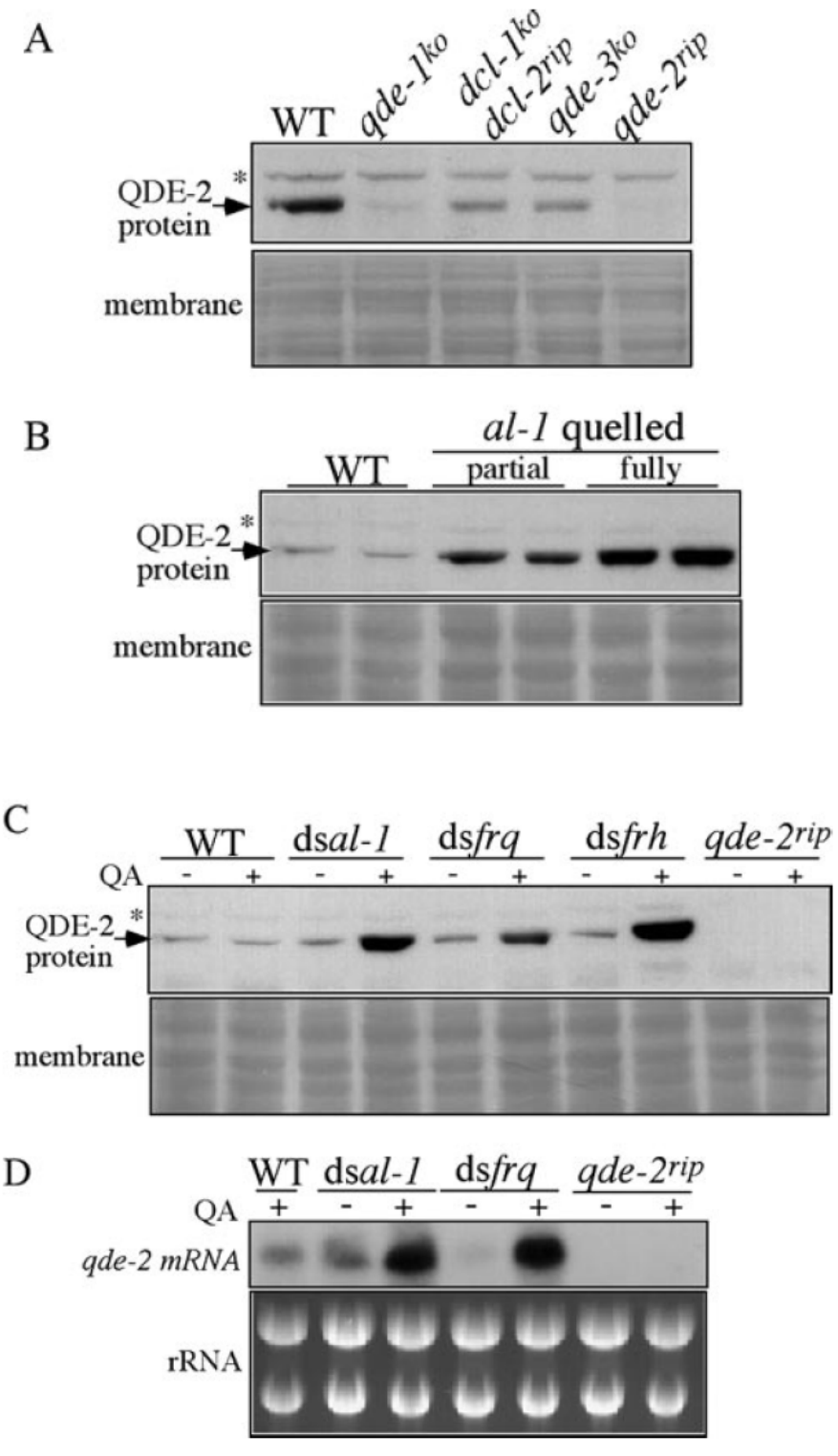
A
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Figure 17. Induction of *qde-2* expression by dsRNA. (A) Western blot analysis showing that the levels of QDE-2 are low in the RNAi mutants. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody. Equal protein loading of the gel was insured by the amido black-stained membrane shown below the blot. Liquid cultures were grown for 2 days before harvesting. (B) Western blot analysis showing the levels of QDE-2 in the wild-type (WT) and *al-1* quelled strains. Two independent partially quelled and fully quelled strains were used. (C and D) Western blot (C) and Northern blot (D) analyses showing the induction of QDE-2 protein and *qde-2* mRNA, respectively, in strains with dsRNA constructs. The liquid cultures were grown for 2 days (with/without QA) before harvesting.

frh, an essential gene important for circadian clock function,) (Cheng et al., 2005). As shown in Figure 17C, QDE-2 level was increased in all the three wild type strains with dsRNA constructs in presence of QA. In contrast, only basal level of QDE-2 expression was observed in the wild type strain lacking any dsRNA constructs and there was no QDE-2 protein in the *qde-2*^{rip} strain. Since the level of QDE-2 was increased in all the wild type transgenic strains, this result suggests that the increase of QDE-2 is due to the production dsRNAs rather than the gene specific dsRNAs.

To examine whether the induction of QDE-2 by dsRNA is a transcriptional regulation, Northern blot analysis was performed using *qde-2* specific probe. As shown in Figure 17D, the *qde-2* mRNA level was increased in the wt, ds*al-1* and wt, ds*frq* strains in presence of QA compared to the wild type strain, indicating that the dsRNAs induce QDE-2 at transcriptional level. Previous studies have shown that the although *qa-2* promoter is tightly regulated, it is not a

strong promoter (Cheng et al., 2001b). So, the significant induction of *qde-2* mRNA and QDE-2 protein in presence of QA suggests that the level of endogenously produced dsRNA is quite low. The experiments for both Figures 17C and 17D were performed by Swati Choudhary.

4.3.2 dsRNAs, not siRNAs, induce the QDE-2 expression and DCLs are required for posttranscriptional maintenance of QDE-2 protein

The low levels of QDE-2 in the *dcl* double mutant and the *qde-1*^{ko} strains (Figure 17A) suggests that the steady state level of QDE-2 is maintained by DCLs and QDE-1 or their products, siRNAs and dsRNAs respectively. To test these possibilities, the QDE-2 level was examined in the wt, ds*al-1*; *dcl-1*^{ko},ds*al-1*; *dcl-1*^{ko}*dcl-2*^{rip},ds*al-1*; *qde-1*^{ko},ds*al-1* strains. Since the two DCLs are known to be functionally redundant, the increase of QDE-2 level in the *dcl-1*^{ko},ds*al-1* strain was similar to that in the wt, ds*al-1*. In addition, the high level of QDE-2 in the *qde-1*^{ko},ds*al-1* strain suggests that QDE-1 is not required for the induction of QDE-2 in presence of dsRNA. Previous studies have shown that QDE-1 is not required for gene silencing when the dsRNA is produced from an exogenous hairpin construct (Catalanotto et al., 2004). The *dcl* double mutant strain failed to increase the level of QDE-2 even in the presence of dsRNA. Since DCLs are required for the generation of the siRNAs, these results suggest that DCLs and siRNAs are involved in the transcriptional or post transcriptional regulation of

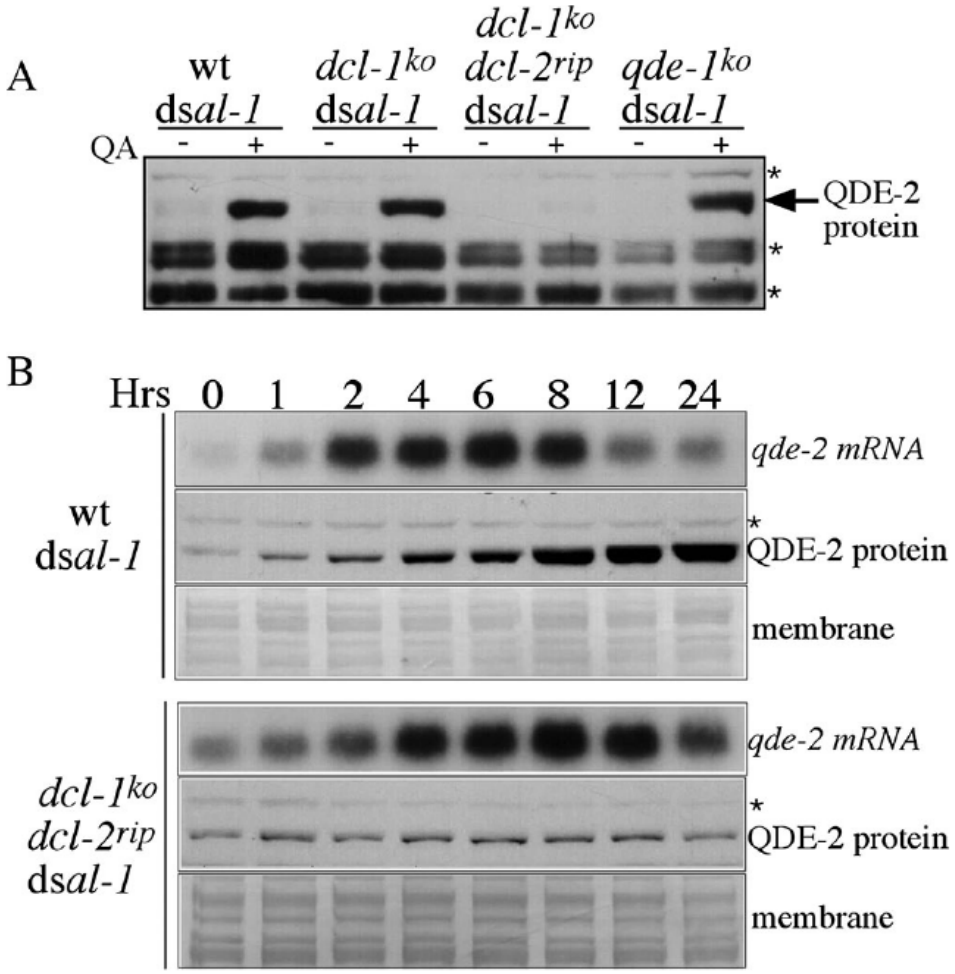

Figure 18. DCLs posttranscriptionally regulate the steady state levels of QDE-2, and dsRNA, not siRNA, is responsible for the transcriptional activation of *qde-2*. (A) Western blot analysis showing that the level of QDE-2 could not be induced by dsRNA in the *dcl* double mutant. The liquid cultures were grown for 2 days (with/without QA) before harvesting. (B) Northern and Western blot analyses showing the induction of *qde-2* expression by dsRNA in the wild-type (wt) and *dcl* double mutant strains. The number of hrs indicates the time after the addition of QA. The asterisk indicates a nonspecific cross-reacting protein band recognized by our QDE-2 antibody.

QDE-2 (Figure 18A, by Swati Choudhary)**.** To distinguish between these two possibilities, Northern blot analyses (by Swati Choudhary) were performed to compare the induction of *qde-2* mRNA in the wt, ds*al-1* and the *dcl-1*^{ko}*dcl-2*^{rip},ds*al-1* after the addition of QA. As shown in Figure 18B, the induction of *qde-2* mRNA was maintained in the *dcl* double mutant strain. In the wt, ds*al-1*, *qde-2* mRNA levels were induced after 1 hour, peaked 4-6 hours after the addition of QA and decreased afterwards. In the *dcl* double mutant strain the *qde-2* mRNA levels remained at high level even after 12 hours of QA addition and the basal level of *qde-2* was higher in the mutant than the wild type strains. Despite the induction of the *qde-2* mRNA, the *dcl* double mutant strain failed to increase the QDE-2 protein level in presence of dsRNA. In contrast, the QDE-2 level was increased in the wt, ds*al-1* strain 1-2 hours after the addition of QA, peaked at 8 hours and the high level (~10 fold of the basal level) was maintained throughout the time course of the experiment. These data indicate that dsRNAs, not siRNAs, are responsible for the transcriptional activation of *qde-2*. DCLs or QDE-1 are not involved in sensing the dsRNAs required for the activation of *qde-2* mRNA. However, DCLs or their cleavage products siRNAs are required for the accumulation of QDE-2 protein post transcriptionally.

4.3.3 Induction of DCl-2 by dsRNAs

The induction of QDE-2 by dsRNAs inspired us to examine whether the dsRNA response program also regulates DCL-2, the other key component of the

RNAi pathway. The following experiments were performed by Swati Choudhary and Heng-chi Lee. As shown in Figure 19A**,** *dcl-2* mRNA was induced in the wild type strains expressing different dsRNAs, indicating the importance of dsRNAs for the transcriptional induction of *dcl-2*. Similar to QDE-2, the DCL-2 protein level was also significantly increased in presence of dsRNAs (Figure 19B). In contrast, only basal levels of *dcl-2* or DCL-2 were observed in the wild type strain lacking the dsRNA construct. However, the kinetics of DCL-2 induction was delayed compared to those of QDE-2. The peak of DCL-2 induction was observed after 24 hours of QA addition, indicating the induction of *dcl-2* by dsRNA is a secondary response.

To investigate the role of QDE-2 in the signaling pathway that mediates the induction of gene expression in presence of dsRNA, the expression of DCL-2 was compared between the wt, ds*al-1* and the *qde-2*^{rip}, ds*al-1* strains. As shown in Figure 19C**,** the level of DCL-2 in the *qde-2*^{rip}, ds*al-1* strain was comparable to that in the wt, ds*al-1* strains, indicating that QDE-2 is not involved in the dsRNA mediated regulation pathway. Together, these results suggest that although the components of the RNAi pathways are regulated by the dsRNAs, they are not involved in the dsRNA sensing pathway.

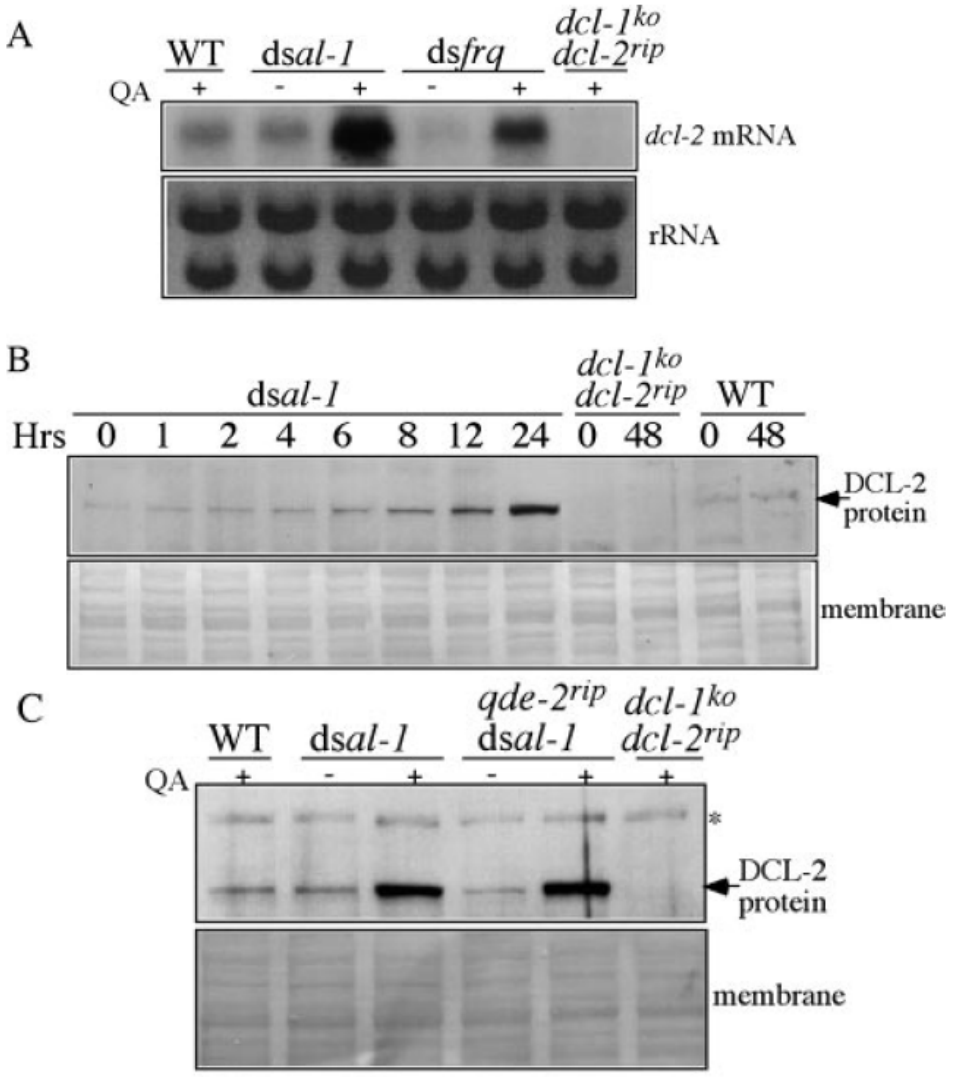

Figure 19. Induction of *dcl-2* expression by dsRNA. (A) Northern blot analysis showing the induction of *dcl-2* mRNA in strains with dsRNA constructs. (B) Western blot analysis showing the induction of DCL-2 protein by dsRNA. The number of hrs indicates the time after the addition of QA. The same membrane used in Fig. 2B was used here. (C) Western blot analysis showing that the induction of DCL-2 by dsRNA is normal in the *qde-2* mutant strain. The asterisk indicates a nonspecific cross-reacting protein band recognized by our DCL-2 antibody. WT, wild type.

4.3.4 Induction of QDE-2 by dsRNAs is required for the optimal gene silencing

The induction of both QDE-2 and DCL-2 by dsRNAs suggests that this induction is essential for efficient gene silencing. To test the hypothesis that the high level of QDE-2 is required for efficient gene silencing, I created two constructs, in one *qde-2* was under the control of *qa-2* promoter and in the other construct *qde-2* was controlled by its own promoter and transformed the constructs into the *qde-2*^{rip}, ds*al-1* strain. As shown in Figure 20A, QDE-2 level was increased in the strain containing wild type *qde-2* with its own promoter in presence of dsRNA. This induced QDE-2 level was able to rescue the function of endogenous QDE-2 and *al-1* gene was silenced efficiently as shown by white aerial hyphae and conidia (Figure 20B). In contrast, QDE-2 was expressed at basal level in the qaQDE-2 transformants and silencing of the *al-1* gene was severely impaired (orange aerial hyphae and conidia) (Figure 20B). These results suggest that the induction of *qde-2* is required for the efficient silencing of the gene and is mediated by the *qde-2* promoter.

To further confirm these results, two constructs that contain both the *qde-2* gene with segments of its upstream sequence of different length and ds*al-1* cassette were created (Figure 20C, by Swati Choudhary) and were transformed into the *his-3* locus of the *qde-2*^{rip}, ds*al-1* strain. As shown in Figure-20D (By Swati Choudhary), the Pqde-2A transformants which contain the 1.9 Kb of the

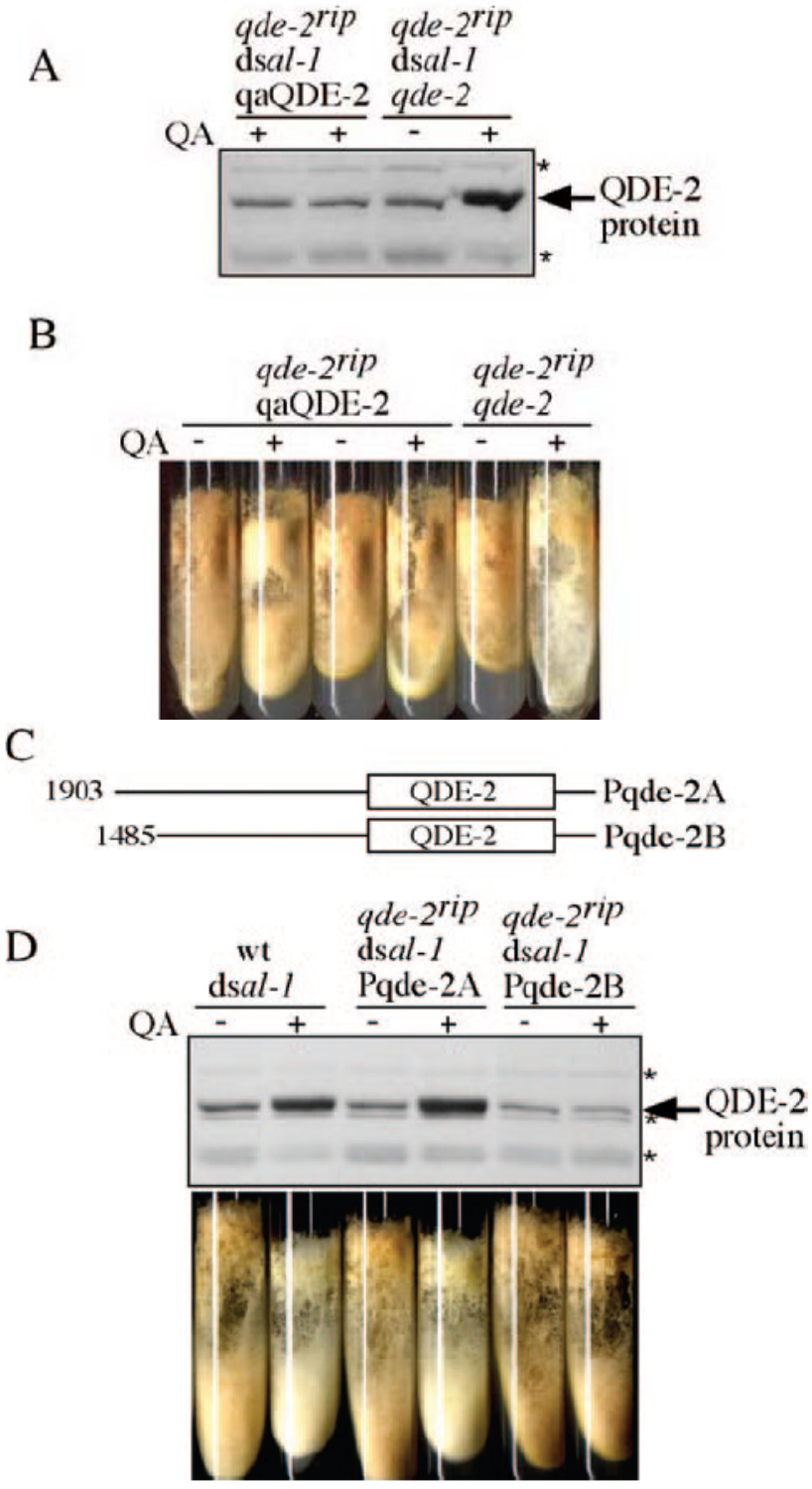
A
qde-2rip
dsal-1
qaQDE-2
qde-2rip
dsal-1
qde-2
QA
+ + - +
QDE-2
protein
B
qde-2rip
qaQDE-2
qde-2rip
qde-2
QA
- + - + - +
C
1903
QDE-2
Pqde-2A
1485
QDE-2
Pqde-2B
D
wt
dsal-1
qde-2rip
dsal-1
Pqde-2A
qde-2rip
dsal-1
Pqde-2B
QA
- + - + - +
QDE-2
protein

Figure 20. **Induction of QDE-2 by dsRNA is required for efficient RNAi.** (A) Western blot analysis showing the expression of QDE-2 in the indicated strains. (B) Photograph of the corresponding strains growing in slants. (C) Graphic depiction of the indicated *qde-2* promoter constructs. (D, Top) Western blot analysis showing the expression of QDE-2 in the indicated strains. (Bottom) Photograph of the corresponding strains growing in slants. The asterisk indicates a non-specific cross-reacting protein band recognized by our QDE-2 antibody. wt, wild type.

qde-2 upstream sequence were able to complement the function of endogenous *qde-2* and QDE-2 was induced in presence of QA. On the other hand in the Pqde-2B transformants, the induction of QDE-2 was abolished and QDE-2 was expressed at basal level, indicating that a *cis* element within the 0.4 Kb *qde-2* promoter is required for the dsRNA mediated induction. Consistent with these results, the *al-1* gene was fully silenced in the Pqde-2A transformants and the silencing was impaired in the Pqde-2B transformants (Figure-20D). These data strongly support the importance of transcriptional induction of *qde-2* in efficient gene silencing.

4.4 Discussion

In this chapter I described the presence of a regulatory mechanism that controls the key components of *Neurospora* RNAi pathway. In addition to *qde-2* and *dcl-2*, other components of RNAi pathway such as *qde-1*, *dcl-1* and *qip* also

showed modest induction in presence of dsRNA (data not shown), indicating that almost all the components of RNAi pathway are regulated by a similar dsRNA response program. Since the transcriptional induction was maintained in the RNAi mutant strains, it suggests that although the components of RNAi pathway are regulated by dsRNA, they are not involved in dsRNA sensing signaling pathway. However, the inhibition of QDE-2 induction by dsRNA in the *dcl* double mutant strains indicates that DCLs and siRNA are important for maintaining the steady state level of QDE-2. It remains unclear how DCLs or siRNAs maintain the induced level of QDE-2. The stability of QDE-2 was comparable in the wild type and the *dcl* double mutant strains (data not shown), indicating that the stability of QDE-2 was not affected by DCLs or siRNAs. A potential mechanism might be that the proper folding of QDE-2 is obtained through its interaction with siRNAs, a process mediated by dicers in animal system. Thus in absence of siRNAs, most of the QDE-2 cannot fold properly and gets quickly removed from the cell (Liu et al., 2003; Tomari et al., 2004). The remaining small fraction of properly folded QDE-2 showed similar stability in the wild type and *dcl* double mutant background. In contrast, comparable expression of DCL-2 in the wt,ds*al-1* and the *qde-2*^{rip},ds*al-1* strains suggests that QDE-2 was not involved in the posttranscriptional regulation of DCL-2.

The dsRNA response program regulates the RNAi components differently. Induction of QDE-2 was observed 1 hour after the production of dsRNAs and

reached the maximum level after 8 hours of induction. However, the induction of DCL-2 was observed after 24 hours of dsRNA production. These data suggest that the induction of QDE-2 was an immediate response rather than the secondary response as observed for DCL-2.

The results presented here showed that the induction of QDE-2 by dsRNA is essential for efficient gene silencing. Since the *qa-2* promoter used for this study to generate dsRNA is not a strong promoter, the dramatic induction of *qde-2* and *dcl-2* in presence of QA indicates that the level of endogenous dsRNA is very low in *Neurospora*. Thus, the activated RNAi pathway could function as a defense mechanism to remove the dsRNAs produced from the transposons or repetitive elements. The removal of dsRNAs can be achieved by two ways; 1) dicers cleave the dsRNAs to generate siRNAs, 2) induced QDE-2 in conjunction with siRNAs destroys RNA template necessary for the production of dsRNAs. Consistent with this idea, QDE-2 and DCL-2 are known to be involved in transposon silencing in this organism (Cambareri et al., 1994; Nolan et al., 2005).

How broad is the role of such a dsRNA response program? In vertebrates, the dsRNAs are known to trigger the transcription based interferon response (Haller et al., 2006; Karpala et al., 2005; Sledz et al., 2003). In this pathway, the dsRNAs are recognized by several dsRNA sensors such as Toll-like receptor 3 (TLR3) and dsRNA dependent protein kinase R (PKR). This leads to the expression of interferons (IFNs) through the activation of the interferon regulatory

transcription factors (IRFs) and NF-κB. Activation of interferons results in the transcription of hundreds of interferon stimulated genes (ISGs). Many ISGs are known to encode proteins with antiviral activity such as PKR and myxovirus (influenza) resistance (Mx) proteins (Goodbourn et al., 2000; Haller et al., 2006; Pavlovic et al., 1995; Peng et al., 2006). Although viruses capable of infecting laboratory *Neurospora* strains have not been identified yet, viral infection could be a serious threat to the survival of *Neurospora* in nature. In addition to the RNAi components, we also observed the induction of ~50 other genes by the dsRNAs (data not shown, performed by Heng-chi Lee).These genes include the homologues of mammalian ISG with antiviral activity, such as Mx proteins, 6-16 family proteins, heat shock proteins (HSPs), and phosphoenolpyruvate carboxykinase (Der et al., 1998). Although the physiological significance of the transcriptional induction of these components is not known yet, the similarities between the mammalian IFN response and the *Neurospora* dsRNA response suggest that they might have similar functions with a common evolutionary link.

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Chapter Five

Conclusion and future directions

5.1 A model for exonuclease mediated RISC activation process

Separation of siRNA duplex and removal of the passenger strand are critical steps for RISC activation. Two different mechanisms were proposed for siRNA duplex separation, 1) unwinding of the two strands by a RNA helicase in an ATP dependent manner and 2) cleavage of the passenger strand by the slicer activity of Argonaute protein. Our studies provide strong *in vivo* evidence in support of the second mechanism showing that in *Neurospora*, QDE-2 and its slicer function are absolutely required for siRNA duplex separation. In addition, identification of QIP and demonstration of its function in removing the cleaved passenger strand strongly support the involvement of an exonuclease in RISC activation process. However, some questions remained unanswered. The mechanism for specific recognition of only the cleaved passenger strand by QIP is unclear. Two possibilities were mentioned in the discussion part of chapter two. Structural studies using QDE-2-QIP complex in association with siRNA will shed light on the mechanistic details of QIP function. In addition, the identity of the slow-migrating duplex band that appeared in the qip^{ko}, ds*al-1* strain is unclear.

Although, we believe that it is also a nicked duplex with different conformation, further studies are needed to test our hypothesis. Our alignment and mutagenesis studies suggest that QIP functions as an exonuclease. An *in vitro* approach using purified QIP and QIP-QDE-2 complex will directly demonstrate the exonuclease activity of QIP.

Finally, it is also important to investigate the existence of similar exonuclease mediated RISC assembly process in other organisms. As discussed in chapter one, purified recombinant human AGO-2 is unable to cleave mRNA when loaded with siRNA duplex suggesting that hAGO-2 cannot generate single stranded siRNA solely by passenger strand cleavage. Involvement of additional factors such as an exonuclease may be necessary to aid the process. In *Drosophila*, the passenger strand cleavage is mostly restricted to siRNA-RISC assembly process and can be bypassed during the separation of miRNA. However, in plants, miRNAs show perfect complementarity with their target mRNA and silence gene by mRNA cleavage. The miRNA mediated target cleavage is also observed in mammalian system; miR-196 regulates the expression of HOX gene by cleaving the HOXB8 mRNA. Thus it is likely that the assembly of such cleavage competent miRNA-RISC is mediated by passenger strand cleavage.

5.2 Future studies in understanding the transgene induced gene silencing in *Neurospora*

In chapter three, an attempt was made to understand the mechanism of dsRNA production from transgene in *Neurospora* and to investigate the importance of RdRp and QDE-3 in this process. Our results suggest the existence of an RdRp and QDE-3 independent pathway for dsRNA production and gene silencing in *Neurospora*. According to this model, increased copy number of transgene can promote inverted repeat formation, which can result in the production of dsRNA directly through the transcription of the inverted repeats. However, as mentioned earlier, further detailed analyses are needed to confirm the formation of inverted repeats of the transgene. The RdRp and QDE-3 in *Neurospora* are important for silencing when the copy number of the inserted transgene is low. However, detailed analyses using biochemical and genetic approach are necessary to understand the functional mechanism of these components in the generation of dsRNA and in transgene induced gene silencing.

5.3 Future studies in understanding the regulation of RNAi components

The studies presented in chapter four demonstrate the presence of a dsRNA sensing pathway in *Neurospora* that regulates the expression of RNAi components. Our results suggest that although the components of the RNAi pathway are regulated by dsRNA, they are not involved in sensing the dsRNA

signal within cell. Therefore, identification of the components of dsRNA sensing pathway will be a major focus of the future studies. As the upstream promoter sequence of QDE-2 is important for transcriptional induction, identifying the cis-acting elements within the promoter and the transcription factor that binds to the response elements will provide important insights into the regulation of QDE-2 by dsRNA. In addition, the significance of the existence of such a regulatory mechanism also needs to be investigated. Our results showed that the up-regulation of QDE-2 is important for efficient gene silencing. However, our microarray and quantitative PCR (qPCR) analyses identified >50 additional dsRNA induced genes. These genes include the homologues of mammalian antiviral components, suggesting a potential role of the dsRNA response as an antiviral defense mechanism. Further characterization of these dsRNA induced genes will lead to the understanding of the physiological importance of the dsRNA response pathway in *Neurospora*.

VITAE

Mekhala Maiti was born in the town of Tamluk, West Bengal (India), on January 24, 1977, the daughter of Madan Mohan Maiti and Manjula Maiti. Mekhala graduated from Tamralipta Mahavidyalaya in 1995. She received the degree of Bachelor of Science (B.Sc., Honors) from Kalyani University, Kalyani (India), in 1998 and the degree of Masters of Science (M.Sc.) from the same university in 2000. After completing the Masters degree, she worked as Junior Research fellow for one and half years in the Department of Botany of Bose institute, Kolkata (India) under the supervision of Dr. Prabir Kumar Saha. Mekhala entered the Division of Basic Science at University of Texas Southwestern Medical Center at Dallas in 2002 and was admitted to candidacy in the Genetics and Development graduate program in 2004. On December 8, 2001, she married Anirban Adhikari who is currently a Postdoctoral Research Scientist in the Molecular Biology department at University of Texas Southwestern Medical Center. Her parents live in Tamluk, India, and she has a brother who is a medical student in the R.G.Kar Medical College, Kolkata (India).

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