THE BIOGENESIS OF SMALL INTERFERING RNA IN NEUROSPORA CRASSA

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To my family, John Chang, Nina Chen,

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THE BIOGENESIS OF SMALL RNA IN NEUROSPORA CRASSA

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RNA interference is a well-conserved gene silencing mechanism in eukaryotes. It regulates various biological processes including development, genome defense and heterochromatin formation. RNAi is initiated by the production of dsRNA, which is processed by Dicer to produce small interfering RNA (siRNA). In the filamentous fungus, *Neurospora crassa*, two types of siRNA have been characterized. One is involved in transgene-induced silencing, termed quelling; the other type is induced by DNA damage and functions to slow down protein translation after DNA damage. Both of these siRNAs originate from repetitive sequences in the *Neurospora* genome. We show that the components of the homologous recombination (HR) machinery are required to generate these types of small RNA specifically at repetitive regions. Furthermore, chromatin remodeling and DNA replication enzymes are required for efficient HR activity

and small RNA production. Lastly, we show that the two small RNA pathways are mechanistically similar by demonstrating that quelling-induced siRNA can also be induced upon DNA damage. Our results suggest that the small RNA biogenesis machinery is recruited specifically to the repetitive loci after homologous recombination, which may result in the formation of aberrant DNA structures.

dsRNA not only triggers the RNAi pathway, but also initiates a signaling cascade that results in activating the transcription of ~60 genes, including the RNAi components, in *Neurospora*. The function of the dsRNA activated genes suggests that RNAi is part of a broad ancient host defense response against viral and transposon infection. A genetic screen has been designed to identify the components involved in this dsRNA triggered transcriptional response; several mutants have been identified and characterized.

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LIST OF ABBREVIATIONS

aRNA- aberrant RNA

ATP- Adenosine triphosphate

DCL- Dicer-like protein

DNA- Deoxyribonucleic acid

DRAG- dsRNA activated gene

DSB- double strand break

dsRE- Double stranded RNA response element

dsRNA- Double stranded RNA

EDTA- Ethylenediamine tetra acetic acid

FGSC- Fungal genetics stock center

HCL- Hydrochloric acid

HEPES- 4-(2-hydroxyethyl)-1-piperizine-ethane sulfonic acid_

IFN- Inteferon

IRF- Inteferon regulatory factor

ISG- Inteferon stimulated gene

kB- Kilobase

kDa- Kilodalton

miRNA- MicroRNA

miRNP- MicroRNA ribonucleoprotein complex

mM- Millimolar

mRNA- Messenger RNA

NF- κB - Nuclear factor- κB

nt- Nucleotide

ORF- Open reading frame

PAGE- Poly-acrylamide gel electrophoresis

PAZ- Piwi-Argonaute-Zwille

PCR- Polymerase chain reaction

PEG- Poly-ethylene glycol

PKR- Protein Kinase R

PTGS- Post-translational gene silencing

PVDF- Polyvinylidene difluoride

QA- Quinic acid

QDE- Quelling deficient

QIP- Qde-2 interacting protein

qRT-PCR- Quantitative real-time PCR

RdRP- RNA dependent RNA polymerase

RIG-I- Retinoic acid inducible gene I

RIP- Repeat induced point mutation

RISC- RNA induced silencing complex

RNA- Ribonucleic acid

RNAi- RNA interference

RNaseIII- Ribonuclease III

rDNA- Ribosomal DNA

rRNA- Ribosomal RNA

SDS- Sodium dodecyl sulfate

siRNA- Small interfering RNA

SSC- Sodium chloride-sodium citrate

ssDNA- Single stranded DNA

TBE- Tris-Borate-EDTA

TE- Tris-EDTA

Tris- Tris (hydroxymethyl) aminomethane

UTP- Uridine triphosphate

UTR- Untranslated region

CHAPTER ONE

INTRODUCTION

The first RNA-dependent silencing phenomenon was reported in petunias in 1990 (Napoli et al., 1990). Essentially, the authors attempted to overexpress the chalcone synthase (CHS) gene, which is responsible for the violet pigmentation of petunias, to examine whether it would result in an over-expression phenotype. Unexpectedly, the procedure led to the gene silencing, resulting in white petunias. Since both the endogenous and transgene transcripts were reduced upon introduction of multiple copies of transgene, this phenomenon was called cosuppression. Soon after the discovery of cosuppression in petunia, Roman and Macino reported a similar phenomenon called quelling in the filamentous fungi, *Neurospora crassa* (Romano and Macino, 1992). The authors reported that multiple copies of transgenes caused silencing of the endogenous gene with sequence homology. Because these reported phenomena result in silencing of gene expression at the post-transcriptional level, they were known as post-transcriptional gene silencing (PTGS).

Caenorhabditis elegans was the first animal model to discover the phenomenon of RNA interference (RNAi). In order to obtain loss-of-function phenotypes in *C. elegans*, supplying antisense RNA against the endogenous gene was the most commonly used method. In 1995, Guo and Kemphues reported that the introduction of the sense or antisense RNA against the endogenous gene also resulted in a loss-of-function phenotype (Guo and Kemphues, 1995). The trigger of RNA interference was later

discovered in 1998 by Fire and Mello (Fire et al., 1998), demonstrating that the mixture of sense and antisense against endogenous genes had a tenfold higher efficiency in silencing than sense or antisense alone. This result suggests that the presence of double-stranded RNA (dsRNA) is the trigger of gene silencing.

The discovery of the quelling pathway

Quelling was originally found by transforming exogenous *albino-1* (*al-1*) or *albino-3* (*al-3*) sequences, two genes that are required for carotenoid biosynthesis, responsible for the orange pigmentation in Neurospora (Cogoni and Macino, 1997; Pickford et al., 2002; Romano and Macino, 1992). The introduction of these transgenes resulted in silencing of the endogenous *al-1* and *al-3* genes, indicated by their albino (white)/pale yellow phenotype. The efficiency of transgene-induced gene silencing is usually around 20-30% of the total transformants. The silencing reduced the level of the mRNA of the *al* genes, which is known as post-transcriptional gene silencing. The silencing phenotype is spontaneously reversible, in which some of the silenced white strains reverted back to the orange wild type phenotype after several passages. The reversion of phenotype seems to correlate with the efficiency and stability of quelling.

Quelling silences both the transgene and homologous endogenous gene during the vegetative state of the *Neurospora* life cycle with a minimum length requirement of ~130 nt (Romano and Macino, 1992). However, the promoter sequences are not required for quelling to occur, suggesting that silencing occurs post-transcriptionally.

The quelling phenotype is dominant, as wild type conidia are silenced when mixed with quelled strain conidia during heterokaryon formation. This suggests that silencing can occur *in-trans* through a diffusible molecule. In addition, a sense RNA transcript was detected that corresponds to the promotorless *al-1* transgene only in the quelled strains, indicating that transcription of the transgene is required to induce silencing. This led to the hypothesis that the presence of multiple copies of transgene results in the transcription of an aberrant form of RNA (aRNA), which caused gene silencing (Catalanotto et al., 2006; Fulci and Macino, 2007; Pickford et al., 2002; Romano and Macino, 1992)

The RNAi components in *Neurospora*

The RNAi components in *Neurospora* were named after its quelling deficient phenotype (*quelling deficient, qde, genes*) from a genetic screen using stably *al-1* quelled strain (Cogoni and Macino, 1997). Three genetic loci were identified from the screen: *qde-1, qde-2,* and *qde-3*, which later were cloned and characterized to be functionally conserved proteins of the RNAi pathway. QDE-1 is the first RNAi gene ever identified in eukaryotes and it encodes for a cellular RNA-dependent RNA polymerase (RdRP) (Cogoni and Macino, 1999a). The cloning of QDE-1 provided the first experimental evidence that an RdRP is involved in this pathway and suggested that the aRNA transcribed from transgenic region is used as a template by RdRP to produce dsRNA.

The cloning of *qde-2* revealed that it encodes an Argonaute protein that is homologous to the *rde-1* gene in *C. elegans* (Catalanotto et al., 2000; Tabara et al.,

1999). This demonstrated that the transgene-induced PTGS share the same genetic components as the RNAi pathway in animals and together with the requirement of RdRP in this silencing pathway; quelling and RNAi are mechanistically similar gene silencing pathways.

The *qde-3* gene encodes for a RecQ DNA helicase homologous to the Werner/Bloom's syndrome proteins found in humans. It is interesting that the BLM helicase is associated with repetitive DNA (Lillard-Wetherell et al., 2004; Opresko et al., 2002; Schawalder et al., 2003), which suggests a role of QDE-3 in identifying repetitive regions from the transgenic locus. Furthermore, it points out that the aberrant DNA structures might form at the repetitive transgenic locus. Although the exact role of QDE-3 in quelling is still largely unknown, it is hypothesized that QDE-3 acts in an early step of the quelling pathway by playing a role in assisting the production of aRNA/dsRNA from the transgenic loci. QDE-3 and another RecQ DNA helicase, RecQ2, in *Neurospora*, is also involved in DNA repair (Pickford et al., 2003), but the link between DNA repair and quelling is still not clear.

It has been later shown that small RNAs of about 25 nt were associated with the Argonaute QDE-2 and the production of these small RNAs requires *qde-1* and *qde-3*, but not *qde-2* (Catalanotto et al., 2002*).* The production of these small RNAs are specifically involved in quelling. Two partially redundant Dicer proteins DCL-1 (Dicer-like-1) and DCL-2 (Dicer-like-2) were further isolated and characterized (Catalanotto et al., 2004). QIP, which is a QDE-2 interacting protein, was later identified to be a critical component of quelling (Maiti et al., 2007).

The mechanism of quelling and dsRNA production

dsRNA triggered gene silencing has been studied extensively in several model systems since its discovery, yet little is known about how repetitive or transgene sequences is distinguished from endogenous genes to generate dsRNA. It is predicted that aRNA is produced from the repetitive/transgenic loci was specifically recognized and converted in to dsRNA by the RdRP QDE-1. In *N. crassa*, QDE-1 and QDE-3 were proposed to be involved in aRNA production.

The crystal structure of the catalytic subunit of QDE-1 has been solved and its RdRP activity was confirmed *in vitro* (Aalto et al., 2010; Makeyev and Bamford, 2002; Salgado et al., 2006). These results demonstrate that QDE-1 uses aRNA as the template to produce dsRNA. Surprisingly, the structure of the catalytic core resembles the catalytic core of a eukaryotic DNA-dependent RNA polymerase rather than a viral RdRP (Salgado et al., 2006). *In vitro* DdRP activity assays of QDE-1 indicates that QDE-1 has a stronger DdRP activity than RdRP activity (Aalto et al., 2010; Lee et al., 2010). The DdRP activity of QDE-1 and the loss of aRNA production in a *qde-1* mutant suggest that QDE-1 transcribes aRNA from the DNA template and further converts the transcribed aRNA into dsRNA (Lee et al., 2010).

One of the interacting partners of QDE-1 is RPA-1, which is the *Neurospora* homologue of the largest subunit of Replication Protein A. RPA has high affinity for

ssDNA and is required for biological processes such as DNA replication, recombination and repair (Wold, 1997). Interestingly, RPA is also required for quelling (Lee et al., 2010), suggesting that RPA might play a role in assisting QDE-1 in transcribing aRNA from the transgenic DNA. Since QDE-1, QDE-3 and RPA are all required for the biogenesis of aRNA, it raises the possibility that QDE-3, the RecQ helicase, and RPA facilitates QDE-1 to the repetitive transgenic ssDNA (resolving complex DNA structures by QDE-3, which RPA binds ssDNA and recruits QDE-1 to the transcription site). Once recruited to the transcription site, QDE-1 first uses its DdRP activity to produce aRNA and then converts aRNA into dsRNA using its RdRP activity. Since RPA is an abundant ssDNA binding protein that is involved in various DNA metabolic pathways, it is also interesting how the small RNA biogenesis machinery is specifically recruited to the repetitive region to generate aRNA. Hence, there must be a mechanism that serves to recognize repeat and non-repeat sequences in the cell and directs the small RNA biogenesis machinery specifically to the repetitive regions.

Generation of siRNA and RISC activation

Upon the production of dsRNA precursor, two RNase III domain-containing proteins, Dicer-like-1 (DCL-1) and DCL-2, process it into about 25-nt small RNAs in an ATP-dependent manner (Catalanotto et al., 2004). The elimination of both *dicer* genes completely abolished quelling and the processing of dsRNA into a siRNA form. However, single mutants displayed the quelling frequencies comparable to that of the wild-type

strain, indicating that these two Dicers are functionally redundant even though DCL-2 is responsible for more than 90% of the Dicer activity in vitro (Catalanotto et al., 2004).

QDE-2, an Argonaute homolog in *Neurospora*, can associate with siRNA duplex and form an inactive RISC complex (Catalanotto et al., 2000, 2002). In consistent with this, the dsRNA production in gene silencing depends on functional QDE-1 and QDE-3 but does not depend on a functional QDE-2, indicating that QDE-2 functions in the downstream step of the gene silencing pathway (Catalanotto et al., 2002). In order to activate RISC complex and execute gene silencing, passenger strand of the siRNA duplex needs to be removed. We showed that QDE-2 and its slicer activity are required for single stranded siRNA production and gene silencing in vivo (Maiti et al., 2007). This provides the first in vivo evidence that Argonaute is involved in generating single-stranded siRNA and RISC activation. However, QDE-2 alone is not sufficient to remove the passenger strand. Biochemical purification of QDE-2 led to the identification of a QDE-2 interacting protein (QIP), which is putative exonuclease (Maiti et al., 2007). Further in vitro experiments have shown that QIP can cleave and remove the nicked passenger strand from siRNA duplex in a QDE-2 dependent manner. Thus, QIP plays a critical role in dsRNA-induced gene silencing and is also the first identified eukaryotic exonuclease required for efficient RNAi. These results indicate that cleavage and removal of passenger strand from siRNA duplex is an essential step in dsRNA-induced gene silencing. Similarly, a *Drosophila* ribonuclease, C3PO (component 3 promoter of RISC), promotes RISC activation by removing siRNA passenger strand cleavage products (Liu et al., 2009).

Taken together, a model for the *Neurospora* RNAi (In particular quelling) pathway was proposed (Fig. 1): Repetitive transgenes will form aberrant DNA structures mediated by QDE-3 and RPA. These aberrant DNA will be recognized by QDE-1 to produce aberrant dsRNA. These long dsRNA will be processed into siRNA duplexes by Dicer proteins, which are then loaded onto the RISC. QDE-2 cleaves and removes the passenger strand with the help of QIP to form an active RISC associated with single-stranded siRNA, resulting in gene silencing

Functions of Quelling

RNAi has been demonstrated to function in genome defense against viruses and transposons, development regulation and chromosomal segregation in plants and animals (Aravin et al., 2003; Lippman et al., 2003; Vastenhouw and Plasterk, 2004). Quelling in *Neurospora* can function in silencing the transgenes and hence represents a potent mechanism to repress expression and expansion of transposons. An African exotic *Neurospora* strain was studied and detected a functional LINE-like transposon, Tad (Kinsey, 1990). Nolan et al introduced this Tad transposon into the *Neurospora* laboratory strain and showed that repression of its activity requires QDE-2 and Dicer, but not QDE-1 or QDE-3 (Nolan et al., 2005a). These results suggest that transposition of Tad may generate inverted repeats that form dsRNA without the requirement of QDE-1 and QDE-3. Interestingly, the *qde-1, qde-2,* and *qde-3* genes were also shown to be important for maintaining rDNA copy number, suggesting a role for stabilizing the rDNA locus integrity (Cecere and Cogoni, 2009).

The DNA-damage induced qiRNA

After transformation, quelling occurs in a population of the transformants during the vegetative stage of it life cycle. Interestingly, a different type of QDE-2 associated small RNA, termed qiRNA (Qde-2 interacting RNA), is induced by DNA damage agents such as histidine, ethyl methanesulfonate, hydroxyurea (Lee et al., 2009). Most of qiRNAs originate from the ribosomal DNA locus, which is the only highly repetitive region in a wild-type *Neurospora* genome. These small RNAs are mainly 21-23-nt-long, slightly shorter than the regular 25-nt quelling siRNAs. Statistical and biochemical analysis of qiRNA revealed that these small RNAs have a strong 5' uridine and 3' adenine preference. In addition, the production of qiRNA does not depend on the conventional transcription machinery by Pol I, Pol II, or Pol III. However, similar to quelling pathway, qiRNA biogenesis requires QDE-1, QDE-3 and the Dicers, indicating that qiRNAs are specific siRNA species made by RNAi machinery upon DNA damage rather than nonspecific degradation products of rRNAs (Lee et al., 2009).

qiRNAs not only match to the transcribed region of rDNA but also match to normally untranscribed intergenic spacer regions, indicating that qiRNAs originate from aRNA precursors via unconventional transcription (Lee et al., 2009). In fact, aRNAs with size from 500 bp to 2 kb from intergenic spacer regions are accumulated and detected by northern blot in *dcl-1 dcl-2* double mutant, supporting the idea that qiRNAs are processed from long aRNA precursors. The production of aRNA was completely abolished in *qde-1* and *qde-3* mutants, indicating that the RecQ helicase QDE-3 and the

RdRP/DdRP QDE-1 are required for the biogenesis of DNA damage-induced aRNA. This result is consistent with the function of QDE-1 as a DdRP that generates aRNA`.

Both quelling and qiRNA pathway require the same components, including QDE-1, QDE-2, QDE-3, and Dicers, and both originate from repetitive sequences, suggesting that the these two small RNA biogenesis pathways might be mechanistically similar (Lee et al., 2009). The repetitive nature of transgenes and rDNA locus is very likely to be the common trigger for quelling and qiRNA production. The production of qiRNA might correlate with DNA damage repair, especially recombination; because DNA damage will eventually lead to replication fork stall and double strand break (Ciccia and Elledge, 2010; Khanna and Jackson, 2001), which requires recombination to repair. Although quelling occurs under normal growth condition, it is likely that those integration sites with multiple transgenes are fragile sites with elevated level of replication stress (Durkin and Glover, 2007; Glover, 2006). If so, quelling is also likely the result of DNA damage at specific integration locus by repetitive sequences.

qiRNA inhibits proteins synthesis after DNA damage. This inhibition effect was impaired in the *qde-1* and *qde-3* mutant. Consistent with the role of qiRNA in the DNA damage response, QDE-3 was previously shown to play a role in DNA damage repair and response (Cogoni and Macino, 1999b; Kato et al., 2004; Pickford et al., 2003). Furthermore, QDE-1 and Dicer mutants also showed increased sensitivity to DNA damage agents' treatment, suggesting that qiRNA may provide a mechanism that contributes to DNA damage checkpoints by inhibiting protein synthesis (Lee et al., 2009). In *Arabidopsis*, RNAi components are found to be enriched in the nucleolus and

rDNA-derived siRNAs facilitate heterochromatin formation (Pontes et al., 2006). Furthermore, the *Drosophila* RNAi deficient mutants displayed disorganized nucleoli and rDNA (Peng and Karpen, 2007). These results suggest that the roles of rDNA- or other endogenous repetitive sequences-derived siRNAs in maintaining genome integrity and stability are conserved in different eukaryotes.

Meiotic silencing by unpaired DNA (MSUD)

Meiotic silencing by unpaired DNA (MSUD), which was originally uncovered from the study on meiotic transvection of the ascospore maturation 1 gene (*asm-1*) in *Neurospora* (Aramayo and Metzenberg, 1996; Shiu and Metzenberg, 2002; Shiu et al., 2001). MSUD occurs in prophase I of meiosis with the presence of unpaired copies of a gene, which lead to the silencing of all copies of the gene in the diploid ascus cell.

The fact that MSUD silences all copies of the gene with homology to the unpaired DNA, whether paired or unpaired, suggests the involvement of a mobile molecule during silencing in a trans-acting manner. An interesting connection was made between MSUD and RNAi-based silencing by genetic screening for mutants which suppress meiotic silencing (Shiu et al., 2001). The first identified mutant was *sad-1* (suppressor of ascus dominance-1), which encodes a paralog of *qde-1* (Shiu and Metzenberg, 2002; Shiu et al., 2001). The high homology between SAD-1 and cellular RdRPs involved in gene silencing indicates that MSUD is an RNAi-related phenomenon but functions only during meiosis. Consistent with this notion, sms-2 (suppressor of meiotic silencing-2), another Argonaute protein homolog in *Neurospora*, is also

demonstrated to be required for MSUD (Lee et al., 2003). More recently, a putative helicase, SAD-3, was demonstrated to be required for meiotic silencing (Hammond et al., 2011). These observations suggest that quelling and meiotic silencing are both RNA silencing related mechanisms but require different sets of components during distinct developmental and growth stage. A cross homozygous for the *dcl-1* (but not *dcl-2*) was completely barren (Alexander et al., 2008), indicating that DCL-1 is required for normal sexual development and suggesting the production of dsRNA in MSUD. QIP localizes in the perinuclear region with other meiotic silencing components (Hammond et al., 2011; Xiao et al., 2010). Based on the current understanding of MSUD pathway, a simple model can be proposed (Hammond et al., 2011; Kelly and Aramayo, 2007; Li et al., 2010; Nakayashiki, 2005): an unpaired region of DNA can be sensed and produce aRNA transcripts during meiosis. aRNA transcripts will then be converted into dsRNA by an RNA dependent RNA polymerase (RdRP), SAD-1, with the help of SAD-2 and SAD-3. dsRNA precursors will be further processed by DCL-1 into small RNAs, which then loaded onto a SMS-2 and QIP based RISC to execute a post-transcriptional silencing of homologous genes. However, a lot more work need to be done to solve several open questions for MSUD.

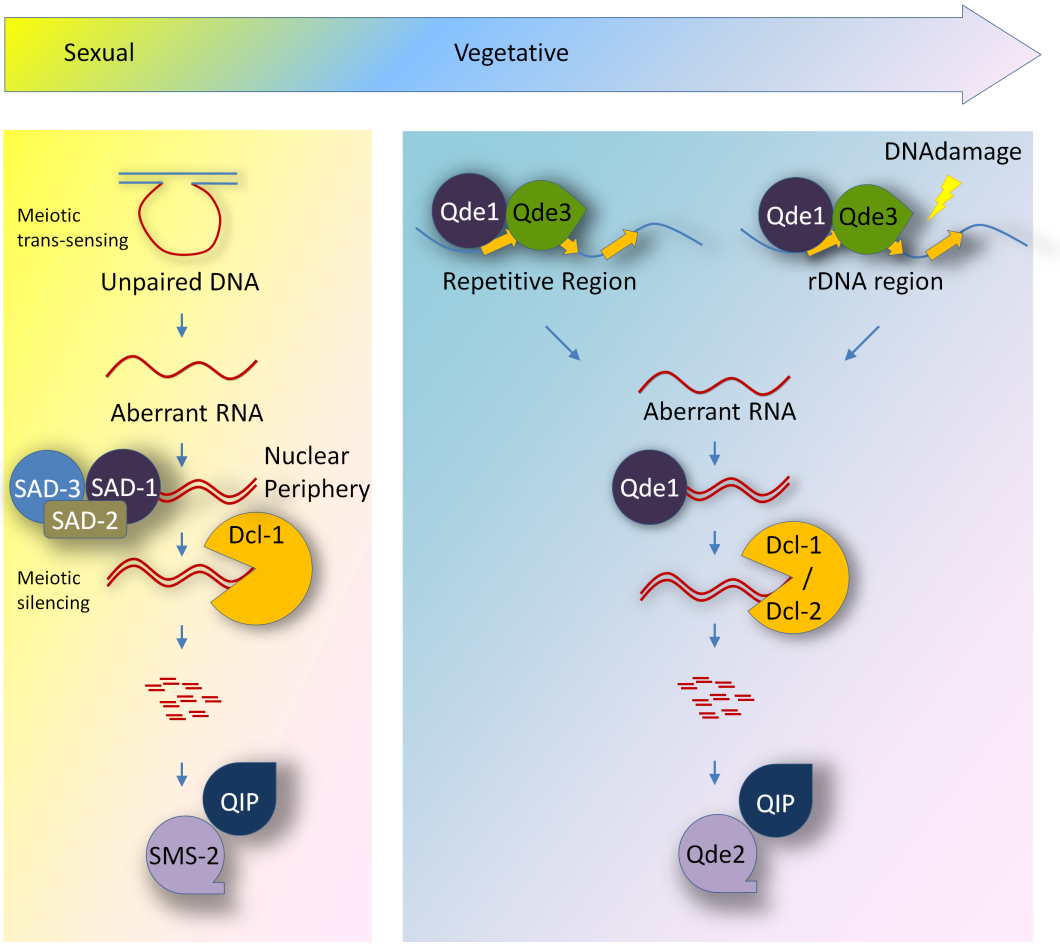

Figure 1 Models for RNAi-related pathways in Neurospora crassa.

During meiosis (left), unpaired DNA is sensed resulting in the production of aRNAs. aRNAs are converted to dsRNAs by SAD1, SAD2, and SAD3 in the nuclear periphery. dsRNAs are processed by DCL-1 into sRNAs, which are then loaded onto an SMS-2/QIP-based RISC to accomplish gene silencing.
During the vegetative stage (right), both repetitive transgene (quelling) and ribosomal DNA loci (qiRNA) lead to the production of aRNAs by QDE-1 and QDE-3. Single-stranded aRNAs are then converted to dsRNA precursors by QDE-1. dsRNA are processed by DCLs into sRNAs, which are then loaded onto QDE-2/QIP-based RISC to execute gene silencing. Yellow arrows indicate repetitive copies of transgene or rDNA.

Repeat Induced Point Mutation (RIP)

Neurospora crassa is an extremely cautious organism in defending against repetitive DNA. Another silencing mechanism that exists in *Neurospora* is the repeat induced point mutation pathway, which is a homology-based process that mutates repetitive DNA during the sexual cycle (Selker, 1990, 2002). The mutated sequences frequently lead to DNA methylation that occurs during the vegetative state. The existence of RIP appears to have evolved for defending against selfish DNA, where there is complete absence of active mobile elements in *Neurospora*. The majority of repetitive sequences in the *Neurospora* genome were predicted to be RIP mutated.

RIP identifies sequences with more than ~400 bp homology in linked DNA and ~1kb homology in unlinked DNA (Watters et al., 1999). It is a process that introduces C:G to T:A mutations in both copies of the duplicated sequence (Cambareri et al., 1989). Duplications of the gene must share ~80% sequence identity for RIPing to occur (Cambareri et al., 1991). In addition, DNA methylation associated with mutated sequences by RIPing can extend to neighboring unique sequences causing the silencing of genes in proximity of the RIPed target.

The only highly repetitive region that contains ~175 copies of tandemly arranged 9kb rDNA repeats is a region that is well protected from RIP in *Neurospora*. The repetitive rDNA region is located within the nucleolus organizer region (NOR), which appears to protects rDNA repeats from RIPing, since rDNA repeats introduced by transformation are susceptible to RIP at ectopic loci. Interestingly, the rDNA copy number appears to fluctuate during the sexual stage of the life cycle, with a tendency

towards copy number reduction. Therefore, it is possible that the NOR maintains the functional rDNA copies through loss, re-amplification and selection.

MicroRNA (miRNA)

MicroRNAs (miRNAs) are non-coding regulatory RNAs that control diverse biological processes including development, metabolism, cell fate and cell fate. miRNA was first discovered in *C. elegans* (Lee et al, 1993; Wightman et al, 1993; Reinhart et al, 2000}, and later found in diverse organisms such as animals, plants, viruses and algae. miRNA controls gene expression through mRNA stability and translation. Some miRNAs are temporally regulated during development, while others are spatially regulated, which adds another layer of gene regulation in the organism.

miRNAs are genomically-encoded and transcribed by RNA Pol II or III, in which some are transcribed into long poly-adenylated primary miRNAs (pri-miRNAs) of about 1 kb or greater in size. The pri-miRNA is first processed by the RNAse III endonuclease, Drosha, to the precursor miRNA (pre-miRNA) in the nucleus. The pre-miRNA is a 60-70 nt molecule that can fold back on itself and form a hairpin loop structure. The pre-miRNA is transported to the cytoplasm and cleaved by Dicer and accessory proteins to produce ~21 nucleotide long intermediate miRNA:miRNA* duplexes. Subsequently, the duplexes are unwound and one strand is selected as the mature miRNA, where the other miRNA* strand is rapidly degraded. The mature miRNA is loaded onto the Argonaute protein, which form the effector complex, miRNP.

miRNAs functions by binding to the 3’ untranslated regions (3’ UTRs) of the complementary target mRNAs and interferes with translation. Efficient repression is achieved through deadenylation and decapping of the mRNA. In plants and in some rare cases in animals, miRNA contain high complementary to the target mRNA and therefore miRNA guides the sequence specific cleavage of mRNA into a process similar to siRNA guided RNA silencing.

In plants and animals, miRNA regulation of gene expression plays an important role during development. Misexpression of miRNA has also been implicated in human cancers, which their role in therapeutics remains to be further studied.

The existence of miRNA has not been found in the fungal kingdom; however, recent studies show a type of small noncoding RNAs have miRNA-like characteristics in Neurospora termed miRNA-like RNAs (milRNAs). These small RNAs also associate with the argonaute protein, QDE-2, and are similarly produced from highly specific stem-loop RNA precursors. Most of these milRNAs require Dicer for its biogenesis and silence target endogenous mRNAs with imperfect complementarity as do in animals. For each milRNA locus, most of the small RNA sequences are from one arm of the hairpin and mostly originate from the stem region of the hairpin. Nearly all milRNAs share the same 5’U preference, where the 3’ termini is heterogenic similar to miRNAs in other eukaryotes.

Perhaps the most interesting observation of these milRNAs is their diverse biogenesis pathways. Genetic studies of these milRNA unveil four different pathways that require different combinations of RNA silencing components. Unlike qiRNA

production, these milRNAs do not require functional QDE-1 and QDE-3. Interestingly, some milRNAs do not require Dicer for it biogenesis indicating the existence of a novel nuclease; others do not require the catalytic activity of QDE-2. The mechanistic diversity observed in the milRNA biogenesis implicate that similar milRNA biogenesis pathways might exist in organisms that lack homologs of Dicer but contain Argonaute-like proteins such as eubacteria and archea.

RNAi is a host defense response

RNAi plays an important role in transposon control. Transposons are mostly silenced in various organisms through either DNA methylation and/or histone modification. In plants, many transposons are silenced via a combination of DNA methylation and PTGS, where siRNA can direct methylation of transposon sequences (Ito, 2011; Kinoshita et al., 2007; Liu et al., 2004).

In Neurospora, transposons are also silenced by a RNAi-dependent manner. It has been shown that expression and expansion of a LINE-like retrotransposon, Tad, is limited by the expression of *qde-2*. In addition, siRNAs against Tad were detected in the *qde*-2 mutant background Tad transcripts were upregulated in dicer mutants. However, DNA methylation is not required for transposon silencing via the QDE-2 based mechanism, excluding the possible link between DNA methylation and PTGS (Chicas et al., 2005; Nolan et al., 2005b).

The RNAi machinery interacting with viruses exhibiting anti-viral functions have been identified and studied in various organisms including plants, fungi, and animals

(Ding and Voinnet, 2007; Ito, 2011; Matzke and Matzke, 2003; Moazed, 2009). The dsRNA molecule is a common viral replication intermediate during viral infection; which can be produced by viral or host RdRPs. Infected hosts use Agos primed with the viral siRNA to destroy viral RNA.

The RNAi machinery acting against viruses was first described during infection of plants and insects. Interestingly, several viruses have developed a wide variety of suppressors of RNA silencing (SRS). Two of the well-studied SRS are the tombusviral P19 protein and the Potyviral HcPro protein, which function in binding to siRNA and prevent siRNA-directed cleavage of the target, respectively.

In the Ascomycete filamentous fungus, *Cryphonectria parasitica*, supressors of RNA silencing has also been reported. (Segers et al., 2007; Sun et al., 2009; Zhang and Nuss, 2008b; Zhang et al., 2008). The mycovirus *Cryphonectria* hypovirus 1 (CHV1) expresses p29, a papain-like protease similar to the plant potyviral HC-Pro (Choi et al., 1991; Segers et al., 2006; Suzuki et al., 1999; Suzuki et al., 2003)**.** Deletion of *p29* results in the reduction of viral RNA levels. When *C. parasitica* is infected by a mutant hypovirus without *p29*, the mRNAs of *dcl2* and *agl2* accumulate to high levels, suggesting that p29 represses RNAi by inhibiting the expression of RNAi genes.

In vertebrates, viral infection is defended by the well-adapted interferon system, which responds to the production of viral dsRNA by inducing the transcription of interferon stimulated genes (ISGs) that exert antiviral activity. One of the sensors that sense dsRNA is the protein kinase PKR, which phosphorylates the eukaryotic initiation

factor eIF2 upon sensing cytoplasmic dsRNAs. The phosphorylation of eIF2 leads to the inhibition of translation of both host and viral mRNAs.

dsRNA response in Neurospora

Recently, our lab has demonstrated a similar transcription-based antiviral response that exists in *Neurospora* (Choudhary et al., 2007). By expressing dsRNA, core components of the RNAi machinery including *qde-2* and *dcl-2* are significantly transcriptionally activated. Further analysis demonstrates that this transcriptional response is triggered by the presence of dsRNA and not siRNA, since the transcriptional response is maintained in the *dcl* double mutant where siRNA production is completely abolished. In addition, the expression of *qde-2* not only is regulated transcriptionally but also post-transcriptionally, as QDE-2 protein levels were kept at basal levels in the *dcl* double mutant, suggesting that the production or association of siRNA with QDE-2 is crucial for QDE-2 stabilization. The induction of *qde-2* is important for dsRNA-induced gene silencing efficiency as *qde-2* mutants that no longer respond to dsRNA show impaired silencing.

Microarray and quantitative RT-PCR analysis identified around 60 genes that were activated by dsRNA including additional RNAi components and homologs of antiviral and interferon stimulated genes (Choudhary et al., 2007). The expression of the latter genes in concert with RNAi components suggests that the dsRNA induced RNAi genes are part of an ancient host defense response against viruses and transposons. Interestingly, *dcl-2 and agl2* expression levels were also found to be significantly

elevated in response to viral infection and expression of hairpin RNA in the chestnut blight fungus *Cryphonectria paracitica* (Zhang and Nuss, 2008a; Zhang et al., 2008), suggesting that the dsRNA-induced transcriptional response is conserved in filamentous fungi. This transcriptional response induced by dsRNA in *Neurospora* suggest the presence of a novel signaling cascade that responds to dsRNA as homologs of dsRNA sensors characterized in vertebrates are not present in *Neurospora* and RNAi components are not required for the dsRNA-induced transcriptional response. Hence it remains interesting to uncover the components that are responsible for dsRNA sensing and transcriptional activation in *Neurospora*.

SUMMARY

RNA silencing is an evolutionary conserved gene regulatory process that has been identified in protists, fungi, plants and animals. RNAi plays important roles in diverse biological functions such as development, cell fate and host defense response against viruses and transposons. This dissertation describes our efforts in understanding the regulation of RNAi during DNA damage and the biogenesis pathway of small RNAs in *Neurospora crassa*. Our data shows that the repetitive nature of the transgenic loci can be the trigger that induces the production of small RNA at these loci. Furthermore, we identified the components of homologous recombination (HR), chromatin remodeling enzymes (SNF ATPases) and DNA replication is required for the biogenesis of small RNAs in *Neurospora*.

Chapter 2 describes the identification of components involved in the biogenesis of small RNA. A genetic screen that is designed to identify components in the double stranded RNA response is described in Chapter 3.

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CHAPTER TWO

Homologous Recombination and DNA replication are required for quelling

INTRODUCTION

The RNA interference pathway is a well-conserved gene-silencing pathway that exists in a broad range of organisms. It is involved in diverse functions such as gene regulation, germ cell maintenance and transposon silencing (Aravin et al., 2003; Bartel, 2004; He and Hannon, 2004; Lippman et al., 2003; Vastenhouw and Plasterk, 2004). Silencing is executed by small RNAs, which guide the silencing-effector complexes to complementary nucleic acid targets. Depending on the nature of the target sequence and the protein composition of the effector complex, different types of silencing can occur.

In *Neurospora crassa*, endogenous small interfering RNAs originate from tandem transgene arrays in quelling and the ribosomal DNA array after DNA damage in the vegetative stage of the life cycle (Catalanotto et al., 2002; Lee et al., 2009). Quelling results in silencing of the transgene and the homologous endogenous gene, which most probably evolved to maintain the control of transposon expansion in the genome (Chicas et al., 2005; Nolan et al., 2005b). The production of qiRNA after DNA damage inhibits protein translation, which acts in parallel with the checkpoint response to promote DNA repair (Lee et al., 2009). The two pathways have several features in

common; 1) both pathways utilize the same components of the RNAi machinery to produce siRNA and 2) the two loci that generate siRNA both contain repetitive sequences. One major difference between the two pathways is that qiRNA production from the rDNA array is induced upon DNA damage treatment whereas quelling-induced siRNA is produced under normal growth conditions. It is interesting and unclear of how the small RNA biogenesis machinery distinguishes repeat and non-repeat sequences in the genome and why the production of qiRNA requires DNA damage to boost its production.

QDE-1 and QDE-3 has been thought to act upstream of the small RNA biogenesis pathway, since introducing an inverted repeat construct in *N. crassa*, which yields dsRNA can induce silencing, circumventing the need of functional QDE-1 and QDE-3 (Goldoni et al., 2004). The initial step of small RNA biogenesis is the production of the single stranded aberrant RNA (aRNA). Previous results have shown that the DdRP/RdRP, QDE-1, is the major DdRP in transcribing the single stranded RNA from the DNA template (Lee et al., 2010). The replication protein (RPA) is required for efficient ssRNA transcription and interacts with QDE-1 in a QDE-3-dependent manner. It is likely that QDE-3 and RPA presents a suitable ssDNA template for QDE-1 to produce ssRNA, which in turn is converted to small RNA precursor (aRNA). Due to the fact that RPA is involved in most of the ssDNA metabolic pathways, it is interesting that small RNAs are specifically produced from the repetitive loci of the genome. Therefore, there should be other factors that target the aRNA biogenesis machinery specifically to the repetitive loci.

This chapter describes our efforts in identifying the components acting upstream of the small RNA biogenesis pathway, which is required to produce aRNA. qiRNA is induced upon DNA damage and DNA damage triggers the activation of several pathways, including checkpoint activation and repaired mainly by non-homologous end joining (NHEJ) and the homologous recombination (HR) pathway (Ciccia and Elledge, 2010; Lieber, 2010; Moynahan and Jasin, 2010; Puchta, 2005; San Filippo et al., 2008). Hence, we initiated our studies by screening for different DNA damage repair and checkpoint pathways for defects in the qiRNA pathway.

The study presented here was performed by me and another graduate student in Dr. Liu's lab, Zhenyu Zhang. The contribution of each person is mentioned in the result section.

MATERIAL AND METHODS

Strains and Growth conditions

In this study FGSC 4200, *mat* a, 301-6; his-3, *mat* A, were used as the wild type strain. The mutants strains *rad51*^{KO} (NCU02741), *rad52*^{KO} (NCU04275), r*ad54*^{KO} (NCU11255), *KU70*^{KO} (NCU08290), *Srs2*^{KO} (NCU04733), *chd1*^{KO} (NCU03060), *isw1*^{KO} (NCU03875), *swr1*^{KO} (NCU09993), *ATRXK*O, I*NO80*^{KO} (NCU01325), *qde-3*^{KO} were used for this study. Culture conditions were described previously (Cheng et al).

Small RNA Northern Blot Analysis

Small sized RNA was enriched by adding equal volume of 2X PEG 8000. The mixture was put on ice for 30 mins and centrifuged at 12,000 rpm for 10 mins. The supernatant was added to 2.5X volume of ethanol and 1/10X volume of NaOAc to precipitate the small RNA. 20 ug of small RNA was loaded onto a 16% polyacrylamide/8M urea gel and ran until the xylene FF dye reaches 2/3 of the gel. The gel is transferred onto a N+ Hybond membrane using a semi-dry transfer system.

To make small RNA probe, the labeled RNA product is hydrolyzed by adding hydrolysis solution (80 mM NaHCO3, 120 mM Na2CO3) to the probe at 60 C for 3 hrs. The probe is then neutralized by adding 3M NaOAc.

Homologous Recombination Assay

The wild type, control strains and mutant strains were grown for 7-10 days before harvesting the conidia in 1 M sorbitol. The linearized plasmid (containing *bar* gene flanked by homologous sequences of *mtr*, a generous gift from Dr. Inoue) was incubated with the conidial suspension on ice for 30 mins. The fragment was transformed into *Neurospora* strains by electroporation (Margolin et al., 1999). The transformed conidia were plated onto low nitrogen containing top agar containing 0.4mg/ml bialaphos. The bialaphos resistant transformants were picked onto bialaphos containing slants, which resistant transformants were further selected on FPA containing slants. The HR rate was calculated as the ratio between the FPA-resistant colonies to the total bialaphos-resistant colonies.

Quelling Assay

Quelling assays were performed as previously described 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 1 M sorbitol. A mixture of 2 ug pBSK*al-1* (carrying the *al-1* fragment) and 0.5 ug of pBT6 (a benomyl resistant gene containing plasmid, obtained from the Fungal Genetics Stock Center, FGSC) was incubated with the conidial suspension for 4-5 hrs at 4C. The plasmids were transformed to *Neurospora* strains by electroporation (Margolin et al, 1999). The benomyl resistant transformants were picked onto minimal slants and visually inspected to identify the complete quelled (white), partial quelled (yellow) or non-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 luminescence light at room temperature.

Co-immunoprecipitation Assay

Protein extracts were prepared as previously described. 2 mg of total extract diluted in IP buffer (50 mM HEPES, pH 7.4; 137 nM NaCl; 10% Glycerol; pepstatin A (1ug/ml); leupeptin (1ug/ml); PMSF (1mM)) was prepared and rotated at 4C with 1.6 ul of anti-c-myc Ab (5mg/ml, Boehringer Mannheim Corp. cat#1667 203) for 3 hrs. Pre-equilibrated 20 ul of packed volume GammaBind G sepharose beads (Amercham Biosciences, cat#17-0885-01) were added and rotated at 4C for another 1 hr. The bound proteins were pelleted by centrifuging at 4,000g, 4C for 1 min. The supernatant was

carefully discarded by gentle vacuum, and 1 ml of IP buffer was added to wash the pellet. The pellet was washed for 3-4 times, changing the centrifuge tube once or twice during the wash. 70 ul of freshly prepared 1XSDS loading buffer was added to the beads and boiled for 5 mins. The samples were then loaded onto SDS-PAGE gel.

Chromatin Immunoprecipitation (ChIP) Assay

Conidia of strains of interest are grown in 150mm x 15mm plates with 50 ml of liquid media for 2 days. Circular discs were punched out of the mat that has grown in the plates. Depending on the sickness of the strains, 5-20 discs were added to liquid media containing with or without Hydroxyurea. The discs were grown on shakers for 40 hrs at room temperature. The discs were fixed in the culturing media containing 1% formaldehyde for 15 minutes with shaking. 125 mM of glycine was added for another 5 mins. The discs were transferred to washing buffer (50 mM HEPES; 137 mM NaCl) for 5 minutes with shaking and harvested under vacuum. The tissues were ground with liquid nitrogen and protein extracts were prepared by adding lysis buffer (50 mM HEPES; 137 nM NaCl; 1mM EDTA; 1% Triton X-100; 0.1% deoxycholate; 0.1% SDS; pH 7.5) containing protease inhibitors. DNA was sheared by sonication to approximately 500-1000 bp fragments (DNA fragment sized is checked by running samples on agarose gels after sonication). Sonication was performed with 3 cycles of 25 pulses with duty cycle 40 and output control 4. 2mg of protein were precleared with 40 ul slurry equilibrated Gamma Sepharose beads for 2 hrs at 4C with rotation. The blocking beads were resuspended and the lysate was transferred to a new tube. 1ul of myc antibody was added to the precleared protein lysate and incubated for another 2 hrs with rotation. No antibody

was added to the control sample. 40 ul slurry Gamma Sepharose beads were subsequently added for another 1 hr at 4C with rotation. The beads were washed with Low Salt Immune Complex Buffer (0.1% SDS;1% Triton X-100; 2mM EDTA; 20 mM Tris-HCl; 150 mM NaCl) for 5 mins on a shaker; High Salt Immune Complex Wash Buffer (0.1% SDS; 1% Triton X-100; 2mM EDTA; 20 mM Tris-HCl; 500 mM NaCl) for 5 mins; LNDET Buffer (0.25M LiCl; 1% NP40; 1% deoxycholate; 1 mM EDTA; 10mM Tris-HCl; pH 8.0) for 5 mins and two 5 minute 1XTE (1 mM EDTA; 10 mM Tris; pH 8.0) washes. 100 ul of Chelex beads (10% beads) was added to the sample and vortexed. The sample was boiled at 94C for 10 mins and then chilled on ice. The supernatant is diluted 1-2.5X before used as template for qRT-PCR analysis.

RESULTS

HR and chromatin remodeling are required for the qiRNA pathway

Since qiRNA is induced upon DNA damage, we screened for several representative components that were involved in different DNA damage repair pathways (performed by Zhenyu Zhang). These categories include HR, HR inhibition, NHEJ, double strand break (DSB) binding proteins, checkpoint pathway and DNA mismatch repair as shown in Table 1. Northern Blot analysis probing for the qiRNA precursor, aRNA, and qiRNA transcript was used as readout for the screen.

The knock-out (KO) strains of essential components of several DNA repair pathways (Table 1) were examined for the aRNA and qiRNA production under histidine

treatment. Out of the strains that were tested, KO strains of the homologous recombination repair pathway (*rad51*, *rad52*, *rad54*) displayed a complete reduction in aRNA production. The downstream product of aRNA, qiRNA, was also abolished in these KO strains.

These results suggest that homologous recombination is required for the production of aRNA after DNA damage and that this pathway plays a role in the initial biogenesis of the qiRNA pathway. Homologous recombination is a fundamental process that expands genetic diversity during meiosis and plays a major role in the rescue of stalled replication forks (Symington and Gautier, 2011). Recombination preferentially occurs

Category of DNA damage response pathway	Representative components
siRNA biogenesis	QDE-3
Homologous recombination	MEI-3 (Rad51), MUS-11 (Rad52), MUS-25 (Rad54)
HR inhibitors	MUS-50
Post replication repair	UVS-2, MUS41
Non-homologous end joining	MUS-51, MUS-52
DSB binding proteins	MUS-23, UVS-6
Checkpoint pathway	ATM, ATR, CHK2
DNA mismatch repair	PNPK

Table 2 DNA Repair pathways screened for aRNA/qiRNA deficiency

when homologous sequences are close in proximity. The result that only homologous recombination mutants show defect in aRNA/qiRNA production out of the different DNA repair pathways suggest that the repetitive sequences are crucial for generating aRNA.

Previous studies have demonstrated that the expression of QDE-2 is induced upon histidine treatment possibly induced through the production of aberrant dsRNA (Lee et al., 2009). Hence a Knock out (KO) library screen was performed to screen for mutants that were deficient in inducing QDE-2 expression under histidine treatment. The screen identified several mutants of the chromatin remodeling, including homologs of INO80, CHD1, ISW1 family remodelers and SWR1; furthermore, aRNA and qiRNA production were also abolished in these mutants under histidine treatment. These results demonstrate that the HR machinery and chromatin remodeling enzymes are required for the biogenesis of aRNA in *Neurospora*.

Chromatin remodeling factors are required for HR

Chromatin remodelers are ATP-dependent enzymes that utilize ATP to alter histone-DNA contacts, which results in changes in the packaging state of chromatin by moving, evicting and restructuring the nucleosomes. (Clapier and Cairns, 2009). Condensing and decondensing of the nucleosome is essential and tightly regulated to properly execute various biological processes such as gene transcription, DNA replication, DNA repair, and DNA replication. During DNA damage, DNA repair factors must have access to all DNA base pairs and recombination machinery requires access to long stretches of DNA. Therefore, chromatin remodelers assist in nucleosome removal, sliding, and

restructuring to allow rapid access during repair (Bao and Shen, 2011; Chai et al., 2005; Fritsch et al., 2004; Papamichos-Chronakis et al., 2006; van Attikum et al., 2007; van Attikum et al., 2004; Wu et al., 2007).

INO80 and SWR1 are two chromatin-remodeling enzymes that are associated with DNA repair (Bao and Shen, 2011; Papamichos-Chronakis et al., 2006; Park et al., 2010; van Attikum et al., 2007). INO80 associates with replication origins and stalled replication forks, and is essential for replication fork progression under stress conditions (Shimada et al., 2008). It is also important in DNA repair, where it facilitates the 5'-3' end resection step that is essential for HR repair (van Attikum et al., 2004). Studies have also shown that it facilitates H2AX phosphorylation and involves in DNA damage checkpoints (van Attikum et al., 2004). SWR1 is recruited to the DSB site and incorporates the histone variant H2A.Z by replacing the histone H2A during DNA damage (Luk et al., 2010). ISW1 has been shown to involve in replication initiation and firing, where it is enriched at sites of active replication and promotes replication fork progression (Vincent et al., 2008). ATRX, is a SNF-2 related ATPase with a PHD domain, localizes to repetitive regions. During interphase and mitosis, it is enriched at the centromeric heterochromatin, whereas it enriches at the rDNA locus during metaphase. It is also associated with DNA methylation at repetitive sequences (Baumann et al., 2008).

The requirement of chromatin remodelers and HR machinery for aRNA production suggests that chromatin remodelers facilitate recombination at the repetitive loci to generate aRNA. Previous studies in *S. cerevisiae* has demonstrated one

of the chromatin remodelers affect recombination activity in the rDNA locus (Dror and Winston, 2004); hence, to study whether identified chromatin remodelers are deficient in homologous recombination, the targeting frequency through homologous recombination was measured (performed by me and Zhenyu Zhang) (Ishibashi et al., 2006; Ninomiya et al., 2004). The *mtr* gene on chromosome IV was selected as a target for gene disruption because loss of *mtr* results in resistance to the amino acid analog FPA, which can be easily scored. If a DNA fragment containing the bialaphos-resistance gene *bar* is integrated at the *mtr* locus, the strain should be resistant to both bialaphos and FPA. If, on the other hand, the fragment containing *bar* gene is inserted somewhere other than the *mtr* locus, the strain should be only resistant to bialaphos. Transformation efficiency was calculated by the number of bialaphos-resistant colonies per total amount of conidia. The HR rate was calculated as the ratio between the FPA-resistant colonies to the total bialaphos-resistant colonies. Linearized DNA fragment that contains the *bar* gene flanked by 1 kb sequence homologous to the *mtr* region was electrophoresed into different genetic background strains. *Rad51*, *KU70* and wild type strains were used as controls for this assay, which had a HR rate of 0%, 97% and 30% respectively. The KO chromatin remodeler mutants all displayed no or low HR rate ranging from 0-3%. This indicates that these chromatin remodelers are essential for efficient homologous recombination and suggests that the chromatin remodelers function in small RNA biogenesis by assisting the HR machinery to access the repetitive loci to generate aRNA.

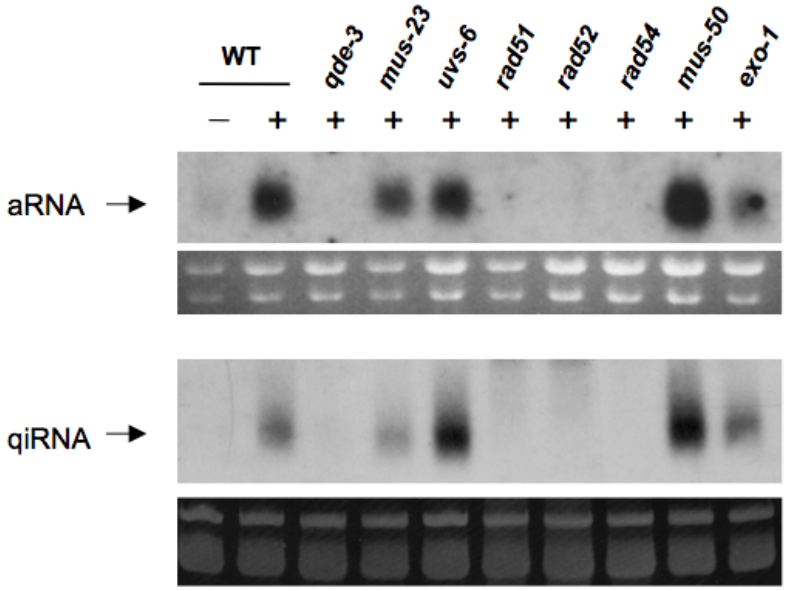
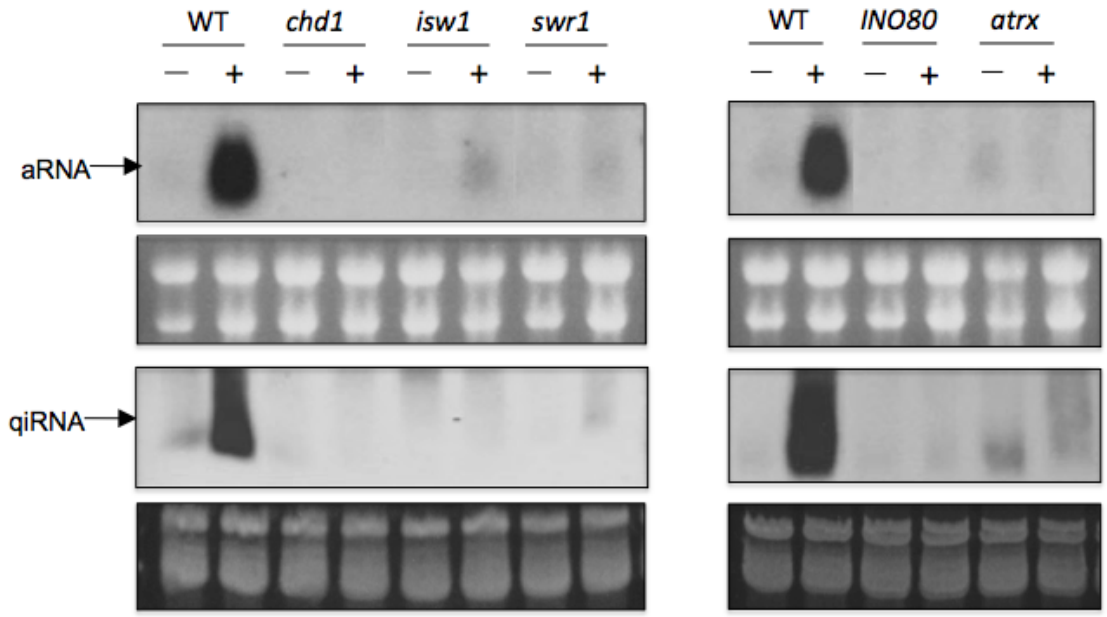


Figure 2 Northern Blot analysis of components deficient in aRNA and qiRNA

A



B

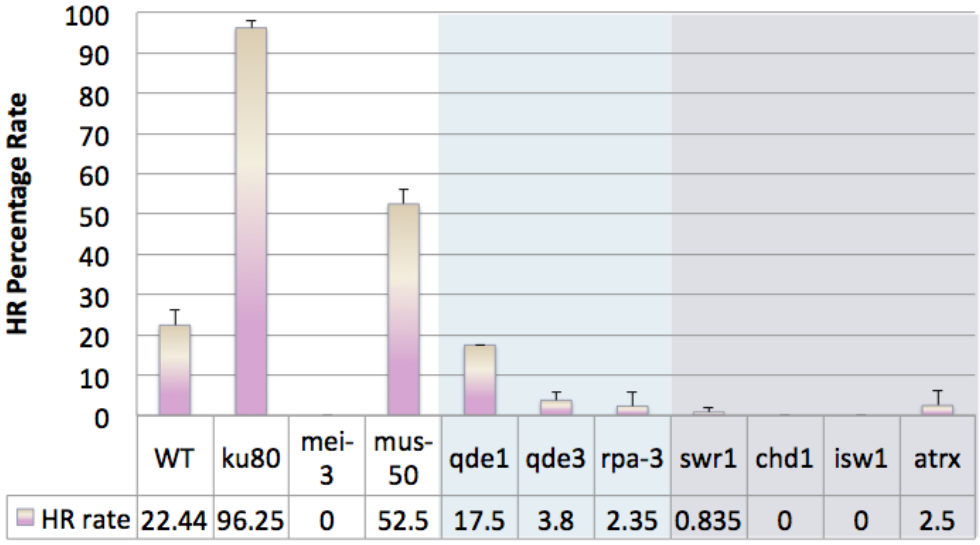


Figure 3 Chromatin remodeling enzymes are required for HR

(A) Northern blot analysis showing aRNA and qiRNA deficiency in the SNF ATPase mutants. Ethidium bromide stain is shown as a loading control.

(B) Homologous recombination rate of the selected SNF ATPase mutants. Error bars indicate standard deviation between triplicate samples.

The relationship between DNA replication and small RNA biogenesis

Several studies have shown that HR is the predominant DNA repair pathway during S and G2 phase when DNA has replicated and sister chromatid provides a template for repair (Aylon et al., 2004; Barlow et al., 2008; Ira et al., 2004; Zierhut and Diffley, 2008). The accumulation of recombination intermediates highly correlates with the accumulation of replication intermediates indicating that HR activity occurs most frequently during DNA synthesis (Zierhut and Diffley, 2008; Zou and Rothstein, 1997). This led to the hypothesis that DNA replication is required for small RNA biogenesis.

To test this hypothesis, wild type cells were treated with increasing dose of hydroxyurea (HU) to examine whether blockage of DNA synthesis would affect the production of small RNA. Interestingly, during low dosage of HU treatment, aRNA production correlated with the amount of DNA damage (Fig. 4A); however, when HU concentration was increased to a level that completely blocked DNA replication, aRNA production was inhibited. This dose response was also shown to be the same in qiRNA production (performed by Zhenyu Zhang). This suggests that although the production of aRNA can be induced to higher levels by higher doses of DNA damage, DNA replication is required to occur for aRNA to be generated.

We further examined the requirement of DNA replication by knocking down one of the essential components of the replisome. Three different knock down strains of proliferating cell nuclear antigen (PCNA) were examined for qiRNA production. PCNA is the processivity factor for DNA polymerase during DNA replication and repair (Kelman, 1997). It is a DNA clamp that tethers DNA polymerase to the DNA template and is

essential for rapid and processive DNA synthesis. In Fig. 4B, the level of qiRNA is reduced in three independent knock down strains of PCNA compared to wild type. This suggests that the replication component, PCNA, is required for the production of qiRNA. Taken together, the results indicate that DNA synthesis is required for small RNA production and further support the requirement of HR activity in generating aRNA.

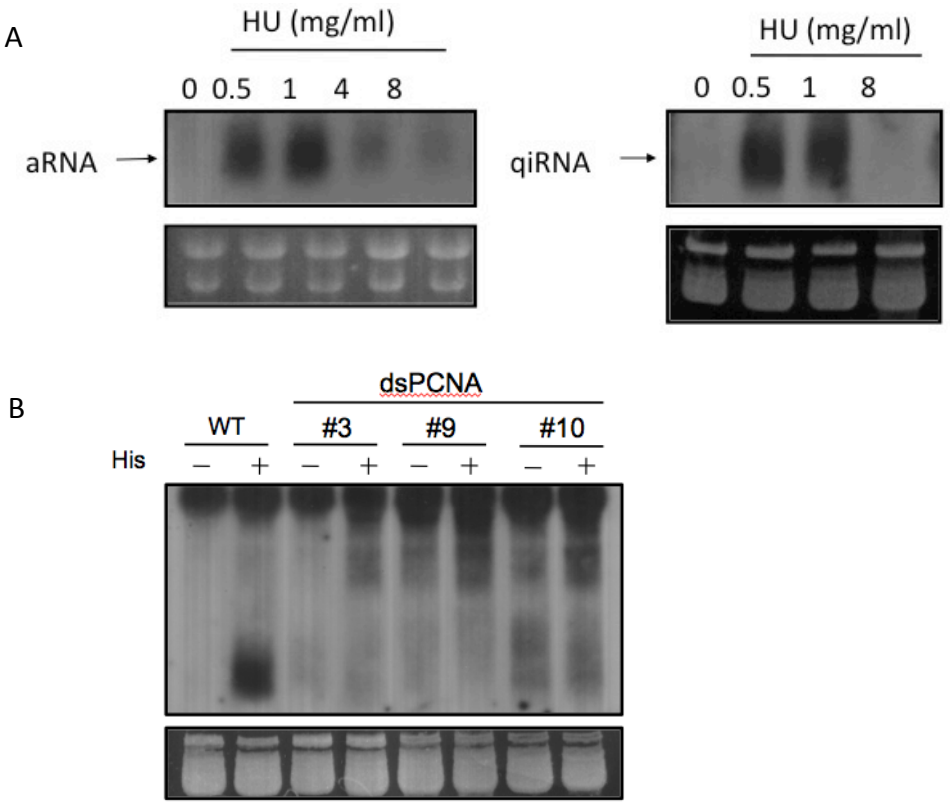


Figure 4 DNA replication is required for small RNA biogenesis

(A) Hydroxyurea dose response assay displaying inhibition of aRNA and qiRNA production during high dosage of hydroxyurea.
(B) Northern blot analysis of three independent knock down PCNA mutants displaying deficiency in qiRNA production.

qiRNA production and quelling share the same mechanism

Several characteristics of the quelling small RNA and qiRNA indicate they are similar in how they are produced. First of all, both types of small RNA require the RNAi components including QDE-1, QDE-3, and RPA for its biogenesis. Second, the genomic locus that generates the small RNAs is both repetitive in sequence. Hence, homologous recombination could play an important role in producing small RNA from the repetitive transgene loci.

In order to examine whether HR is required for quelling, we performed the quelling assay in *rad51*^{KO} and *rad54*^{KO} strains (performed by Zhenyu Zhang). As shown in Fig. 5A, *rad51*^{KO} and *rad54*^{KO} strains displayed inability to quell the *al-1* gene. This phenotype is comparable to the *qde-3* strain, indicating that the HR components are required for quelling.

Since DNA damage induces qiRNA production, we also examined whether quelling siRNA responds similarly upon treatment of HU. We used a partial quelled strain (yellow), that has lower levels of *al-1* siRNA compared to a complete quelled strain (white), to treat with HU. Upon addition of HU, the *al-1* siRNA is significantly increased to a level similar to the complete quelled strain as shown in Fig. 5B. This indicates that the production of the siRNA generated from the repetitive transgenic locus is induced upon DNA damage similar to qiRNA from the ribosomal DNA locus.

qiRNA production requires DNA replication as shown in Figure 4, we then examined whether quelling siRNA responds in a similar fashion to different dosage of HU. Treatment of low dosage of HU induces the production of quelling siRNA; however

upon increasing concentration of HU, the production of quelling siRNA decreases until complete inhibition at a concentration that completely blocks DNA synthesis. The dose response of quelling siRNA is similar to the response of qiRNA under increasing doses of HU, which indicates that the molecular mechanism of the upstream biogenesis pathway are the same in the production of these small RNAs.

A

	No silencing (Orange)	al-1 gene silenced (Yellow & white)
Wt (318)	71% (226)	29% (92)
qde-3^{KO} (60)	96.3% (57)	3.8% (3)
rad51^{KO} (318)	99% (314)	1% (4)
rad54^{KO} (270)	97% (263)	3% (7)

B

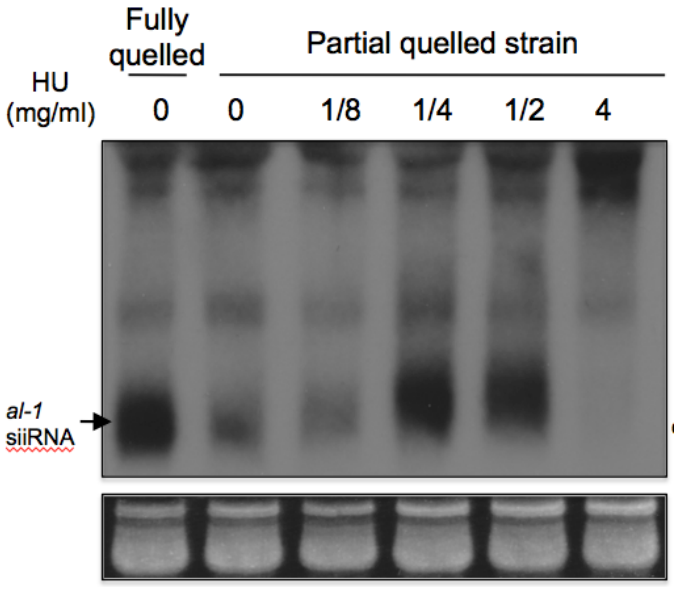


Figure 5 Quelling and qiRNA pathway share similar molecular mechanism

(A) Quelling assay of HR deficient mutants
(B) Northern Blot analysis showing quelling small RNA is enhanced upon DNA damage treatment.

The interaction between RAD51 and QDE-3

Since the qiRNA and quelling pathways both require HR components and the RNAi components for its biogenesis, it is likely that the upstream players of this pathway may directly interact with each other. In order to test this hypothesis, we examined the interaction between RAD51 and QDE-3 by performing a co-immunoprecipitation (co-IP) assay. We used the strain that contains the myc-tagged QDE-3 (targeted to the *his-3* locus) and flag-tagged RAD51 (by cotransformation with pBT6) to examine the interaction between these two proteins. As shown in Fig 6A, RAD51 interacts with QDE-3 in the myc IP pull down only suggesting that these proteins work together at the repetitive DNA locus to generate aRNA. This result is in agreement with studies of the interaction between Rad51 and homologs of BLM helicase in humans and yeast (Wu et al., 2001), further demonstrating that the interaction and function in recombination is evolutionary conserved. The interaction of rad51 and QDE-3 in *Neurospora*, and the requirement of both proteins in small RNA biogenesis point out the tight link between quelling and recombination.

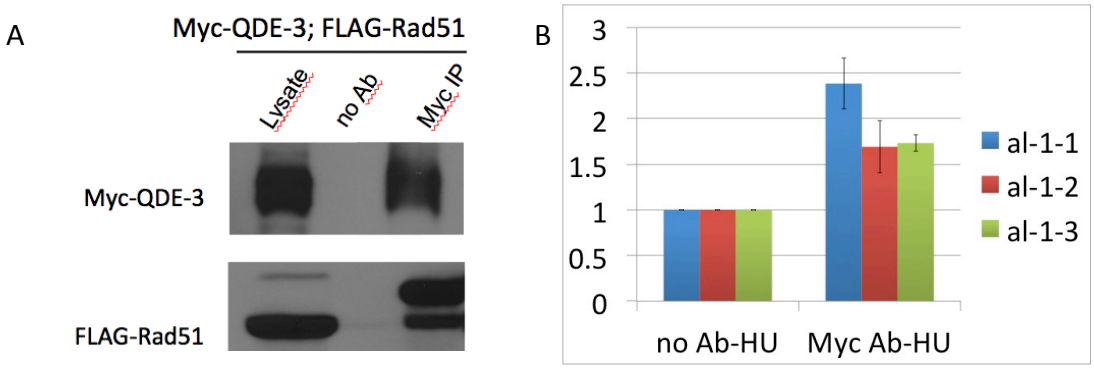


Figure 6 Rad51 interacts with QDE-3 and the quelled locus

(A) Co-immunoprecipitation assay displaying the interaction of QDE-3 and rad51.
(B) ChIP analysis showing Rad51 is enriched at the quelling locus.

The association of Rad51 with the ribosomal DNA

We further examined the interaction of Rad51 on the repetitive rDNA locus using ChIP analysis (performed by Zhenyu Zhang) (Figure 6B). Compared to no antibody as a control, myc tagged Rad51 is localized at the rDNA locus with an average of 2 fold enrichment. Interestingly, upon increasing dosage of hydroxyurea, the interaction of Rad51 decreases at the rDNA locus, which correlates with the production of aRNA at the respective HU dosage. This further demonstrates that Rad51 is required to interact with the rDNA locus to produce aRNA and that this interaction is dependent on DNA replication.

DISCUSSION

This chapter describes the identification of homologous recombination (HR) playing an essential role in the production of small RNA in *Neuropsora*. In addition, chromatin remodeling enzymes and DNA replication are both required for the biogenesis of small RNA, and we found that both of these categories are required for efficient homologous recombination at the specified DNA locus. Furthermore, we tie the connection between DNA damage induced qiRNA pathway and transgene induced quelling pathway by demonstrating that quelling also requires the HR components. Moreover, quelling small RNAs can be induced by DNA damage indicating that similar molecular components acting on the two small RNA biogenesis pathways.

Distinct DNA repair pathways and checkpoint pathways were examined for their role in small RNA pathways, which representative KO mutants were tested. Out of the pathways that have been examined, only the HR repair pathway was deficient in aRNA production. This suggests that the checkpoint pathway is not required for small RNA biogenesis in *Neurospora* and might indicate that the checkpoint pathway runs in parallel with small RNA biogenesis in response to DNA damage. When DNA is damaged by a double stranded break, the MRN/MRX complex immediately recognizes and binds to the ends of the DSB, which further recruits the HR machinery to repair the broken ends. However, it is interesting to find that the exonucleases responsible for DNA end resection, MRE11 and EXO1 single mutants did not show deficiency in the production of aRNA, suggesting that the components of the MRN complex have redundant functions. In agreement with this finding, several studies have shown that deletion of any

components of MRX/MRN will reduce the short-range end resection rate. However, it will not abolish the recombination repair. (Cejka et al., 2010; Mimitou and Symington, 2008; Zhu et al., 2008).

Why are small RNAs highly produced at specific loci and not all over the genome? One of the important feature between multiple copies of transgene and the rDNA array in the *Neurospora* genome is the repetitiveness of the genomic locus. The repetitive character is a perfect target for HR to act on since donor sequences are abundant.

During DNA replication, the repetitive regions are more fragile to damage from fork stall or double stranded breaks. During these situations, DNA is mostly repaired by homologous recombination since DNA synthesis provides the sister chromatid as a donor sequence for HR repair. This allows error-free DNA repair, however if the sequence contains multiple repeats, aberrant DNA structures could be formed by HR activity that would result in the requirement of other enzymes to dissolve the aberrant DNA structure.

The HR pathway can be dissected into multiple steps (Heyer et al., 2010; San Filippo et al., 2008); the DSB is initially resected to generate 3' ssDNA ends, which is bound by RPA and further displaced by Rad51. At this point the Rad51 coated ssDNA, with the help of Rad54, invades the sister chromatid to form the D loop. Since we have established that the HR machinery is required for the production of aRNA, it remains interesting to find out during which step does the HR machinery recruit the RNAi components to transcribe the aRNA transcript. It is possible that QDE-1 is recruited to the DNA locus during the D-loop that is generated by the HR machinery, which contains

other components that have been shown to be required for the generation of aRNA including RPA (Aalto et al., 2010), rad51, DNA synthesis, and recQ helicase. Single stranded DNA is mostly exposed during DNA replication, when the double stranded helix is opened. We have shown that QDE-1 has RdRP activity as well as DdRP, and that its DdRP activity is higher than its RdRP activity. The exposed RPA-coated ssDNA (San Filippo et al., 2008) would be the most probable substrate for QDE-1 to generate the single stranded aberrant RNA.

A difference between the two small RNA biogenesis pathway is that qiRNA is induced upon DNA damage where quelling does not. It is possible that quelling occurs spontaneously because the quelling locus is more susceptible to the generation of aberrant DNA structure, where the rDNA is protected in the nucleolus (Eckert-Boulet and Lisby, 2009; Salminen and Kaarniranta, 2009). DNA double strand breaks in repetitive sequences are threatening to the cell, as it is a potent source of genome instability. The cell has adapted and developed a protective system that protects the rDNA region for DNA damage and illegitimate recombination to maintain the high copies of rDNA in the genome. The repetitive rDNA array is protected through Sir2-dependent heterochromatin formation (Gottlieb and Esposito, 1989), The Sir-2 dependent heterochromatin state has been shown to repress unequal sister chromatid exchange that leads to the accumulation extrachromosomal circles and genome instability (Gottlieb and Esposito, 1989; Kobayashi and Ganley, 2005; Tsang and Carr, 2008). However, during genotoxic stress, the rDNA array becomes prone to DNA damage in the protective heterochromatic state, which results in the generation of aRNA. We have

shown that low levels of *al1* siRNA can be enhanced by the presence of a DNA damage agent, indicating that the stress increases the frequency of the generation of aberrant DNA structure caused by HR.

Our studies have provided new insight in how small RNA is generated throughout the genome in *Neurpspora crassa*. Our results indicate that the small RNAs that are produced mostly in the repetitive regions, since HR occur most frequently at the repetitive regions of the genome. Small RNA produced from repetitive transgenes is also observed in other organisms (Chan et al., 2006; Hsieh and Fire, 2000), suggesting that the requirement of the HR machinery is probably a conserved mechanism to generate small RNA from repetitive loci.

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CHAPTER THREE

Identification of the components involved in the dsRNA-induced transcriptional response in *NEUROSPORA CRASSA*

INTRODUCTION

dsRNA is generally an intermediate product of viral replication and transcription of transposable element or repetitive DNA sequences. It can trigger two biological response pathways in eukaryotic cells. First it can be recognized by the RNAi machinery, which results in PTGS (Ding and Voinnet, 2007; Meister and Tuschl, 2004). The second response pathway induces the interferon response as part of the immune response in vertebrates(Haller et al., 2006; Karpala et al., 2005; Sledz et al., 2003).

The RNAi pathway plays important roles in antiviral defense, such as silencing transposons and viruses in animal and plant cells (Bouche et al., 2006; Galiana-Arnoux et al., 2006; Hammond et al., 2008; Segers et al., 2007; Wang et al., 2006; Wilkins et al., 2005). The RNAi machinery might process the viral dsRNA and degrade viral and transposon mRNA transcripts. In agreement, many viral genomes are known to encode inhibitors of the RNAi pathway (Diaz-Pendon et al., 2007).

In vertebrates, dsRNA and siRNA are also known to trigger the transcription-based antiviral interferon (IFN) response. In mammalian cells, dsRNA is recognized by several receptors called the pattern recognition receptors (PRR), which include the

membrane receptor Toll-like receptor 3 (TLR3) (Galiana-Arnoux and Imler, 2006) and two cytoplasmic dsRNA sensors, retinoic acid-inducible gene-I RIG-I and dsRNA-dependent protein kinase R, PKR. This initiates a signaling cascade resulting in the activation of the IFN-regulatory transcription factors and NF-KB, which results in the expression of the IFNs. The expression of IFNs then activates the transcription of hundreds of IFN-stimulated genes (ISGs) through the JAK-STAT pathway. Many of the ISGs encode proteins with antiviral activities, including PKR and myxovirus (influenza virus) resistance (Mx) proteins. Together, they initiate a host defense response against invading viruses.

Although the molecular mechanism of the RNAi pathway is well studied, how the core components are regulated has not been investigated in detail. As more RNAi-based therapeutic applications are identified, it is imperative that we understand how the RNAi pathway may be affected by various upstream factors. It is also interesting to study whether the two elicited pathways triggered by invading dsRNA, the RNAi pathway and transcription based interferon pathway, work together to fight against viruses.

Our lab has demonstrated that components of the RNAi pathway including *qde2* and *dcl-2* are regulated by dsRNA. The presence of dsRNA significantly activates the transcription of *qde-2* and *dcl-2*, and this transcriptional activation does not depend on siRNA since activation of these genes is not impaired in *dcl* double mutants. Moreover, we show that *qde-2* not only is regulated transcriptionally, the stabilization of QDE-2 protein requires functional Dicer activity. QDE-2 protein levels are constantly at low levels during dsRNA induction in *dcl* double mutants, suggesting that products of Dicer,

siRNA, are important for the stabilization of QDE-2. The induction of *qde-2* is critical for RNA-mediated gene silencing, as *qde-2* mutants that cannot respond to dsRNA has reduced silencing efficiency.

Genome wide analysis identified around 60 dsRNA-activated genes (DRAGs) by microarray and qRT-PCR methods. These genes include the components of RNAi pathway and genes that are homologous to the ISGs similarly activated in vertebrates. Based on the function of these genes suggests that dsRNA elicits a broad host defense response against viral infection and transposons and that the RNAi pathway is part of this response.

The activation of this transcriptional-based response triggered by dsRNA implies the presence of a signaling pathway that senses dsRNA and transduces the signal to activate the DRAGs. In *Neurospora*, the homologs of known mammalian dsRNA sensors such as PKR and RIG-I are not present. Furthermore, we have shown that the RNAi components are not required for the activation of DRAGs in response to dsRNA. Hence, this suggests that a novel dsRNA-sensing and transcriptional activation pathway is present in this fungal organism.

This chapter describes the genetic screen designed to identify components of this dsRNA-induced transcriptional response. Since this response activates a set of genes throughout, sensors of the introduced dsRNA is expected to show up in this screen as well as components that transduce this signals, and lastly transcription factors that regulate the RNAi genes and other DRAGs. Six confirmed bona-fide mutants were identified and characterized, which I focused on cloning the first identified mutant from

the screen, UV19, using traditional high-density mapping. Whole genome sequencing was further employed to identify the causal mutation.

MATERIALS AND METHODS

Strains and Growth Conditions

For the liquid culture, a small amount of conidia or hyphae was inoculated into a 250 ml flask containing 50 ml of liquid media (1X Vogel's, 2% glucose) for 40-45 hrs before harvesting the samples. For liquid cultures containing QA, 0.01 M QA (pH 5.8) was added to flasks containing 1X Vogel's, 0.1% glucose, and 0.17% arginine. Inoculated flasks were first grown in a 30°C incubator for 24 hrs and then put on a shaker at room temperature until harvest. Due to normal 2% glucose severely affects the induction of *qa*-2 promoter, low concentration of glucose was used for QA containing media (Cheng et al., 2001b).

Chemical Mutagenesis

Conidia of 7-day old pqde2mtr; qadsal1 strain is collected and washed with 1 M sorbitol. The number of conidia was counted by a haemocytometer. 3-5 x 10^8 of conidia was adjusted in 20ml sorbitol and prewarmed in a 30°C incubator. 40 ml ethyl methanesulfonate (EMS) or 30 ul methyl methanesulfonate (MMS) was added to the conidia suspension at 30°C with shaking for 2 hrs. After treatment, the conidia are washed with sorbitol twice and once with water.

UV irradiation mutagenesis

Conidial suspension from 7-day old conidia was washed to remove mycelial fragments and prepared in water. The conidial concentration of the suspension is determined using a hemocytometer. Subsequently the suspension is calibrated so that the suspension contains 1 X 10^3 conidia/ml. 20 ml of this suspension is dispensed into a 150 x 15 mm Petri dish. The petri dish lid is removed and the suspension swirled before irradiation. A stratalinker 1800 UV crosslinker was used with 120,000 microjoules of energy (Autocrosslinker) for irradiation. The irradiated plates were kept away strong light to prevent photoreactivation. 1 ml (10^3 conidia) were plated onto minimal and FPA + QA plates. The survival rate = number of colonies on irradiated plate/ number of colonies on unirradiated plates at the same culture dilution factor. A 10% survival rate was determined by the 120,000 microjoules irradiation dosage.

Protein and RNA analyses

Protein extraction, quantification, and Western blot analysis were performed as previously described (Cheng et al., 2001a). 50 ug of total protein was loaded into each well of a 15-well 7.5% SDS PAGE gel. After electrophoresis, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane, and Western blot analysis was performed. Amidoblue staining of the membrane was used as loading controls. RNA extraction and Northern blot analysis were performed as previously described (Aronson et al., 1994). 40 ug of total RNA was loaded in each lane of a 1.3% (w/v) agarose gel.

Quantitative Real Time-PCR (qRT-PCR)

qRT-PCR was performed with an Applied Biosystems Prism 7900HT sequence detection system using a previous described protocol (Kurrasch et al., 2004). qRT-PCR gene specific primers were designed using the Primer Express software (Perkin-Elmer Life Sciences). Primer mixes were prepared by mixing 15 ul each of the forward and reverse (100uM) primers and adding 970 ul of water. These mixes were stored at -20 C. 0.4 ug of total RNA was used for the cDNA reverse transcription reaction. Protocol is used according to the Applied Biosystem manufacturer protocol. RNA is first pre-treated with 0.5 U of DNase I (10 U/ul, Roche) in a total volume of 10 ul containing 1.68 ul of 25mM $MgCl_2$. DNase reaction was treated for 30 mins at 37 C and denatured at 75 C for 10 mins and stored at 4C.

The DNase treated samples were then ready for reverse transcription. The following were mixed to prepare a 2X RT Master mix for each sample: 4.2 ul water, 2 ul 10X RT buffer, 2 ul of 10X random primers, 0.8 ul 25X dNTP, and 1 ul of reverse transcriptase (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems). 10 ul of the RT master mix was added to each sample in a total volume of 20 ul. The sample was reverse transcribed with the following: 25C for 10 mins, 37 C for 90 mins and 72 C for 10 mins and stored at 4 C.

For quantitative PCR reactions, the cDNA is diluted 4 fold by adding 60 ul of water. 3.26 ul of diluted cDNA was mixed with 11.5 ul of SYBR Green PCR Master Mix (Applied Biosystems), 2.76 ul of primer mix and 4.48 ul of water. 10 ul of this mix is

loaded onto the 384 well plate. The gene, beta-tubulin was used as a internal control to calculate changes in gene expression by comparing mean cycle numbers.

Thermal Asymmetric Interlaced PCR (TAIL-PCR)

TAIL is a series of reactions that are intended to map where a known sequence of DNA fragment has inserted within the genome. The main components of the 3 reactions are the AD (Arbitrary Degenerate) primers, specific border primers, and DNA from the mutants to be mapped, which have DNA fragments inserted. AD primers are degenerate primers that anneal randomly throughout the genome. The border primers are specific for the left and right borders of the inserted DNA fragment (*hph* sequence). The idea is that you utilize the temperature of the PCR reaction to first favor and amplify the initial nested specific primer only (asymmetric), and then use a lower temperature to equally favor the nested and degenerate primers (interlaced with symmetric amplification). The product of the first amplification is diluted 1000X fold and used for the subsequent second amplification. From the primary reaction to the tertiary, the specific border primers get closer to the edge of the T-DNA. That is why a 'shift' is visible when running a gel with the secondary and tertiary reactions next to each other. At each step the specific reaction is favored, which decreases the amplification of non-specific products.

Genomic DNA is extracted by 1X CTAB for 30 mins in 60C and extracted by phenol chloroform. The purified DNA is precipitated in isopropanol and washed with 70% ethanol. DNA is resuspended in water and used for PCR reactions. For TAIL PCR,

FailSafe enzyme is used for amplification with FailSafe PCR 2X Premix K PCR buffer (contains betaine, which is good for amplifying genomic DNA with high GC content). In a total volume of 25 ul, 4.5 uM degenerate primer, 0.2 uM specific nested primer and 25 ng/ul genomic DNA is prepared for the primary reaction. Mineral oil (molecular grade) is added ontop of the PCR reaction to prevent evaporation under high temperature. For the primary reaction, the PCR amplification steps are as follows: 1 cycle of 93C, 1 min; 95C, 1 min. 5 cycles of 94C, 1 min; 62C, 1 min; 72C, 2.5 mins. 1 cycle of 94C, 1min; 25C, 3 mins; ramp 25C to 72C in 3 mins (ramping rate 0.2C/s); 72C, 2.5 mins. 22 cycles of 94C, 30 s; 57C, 1min; 72C for 2.5 mins; 94C, 30s; 44C, 1 min; 72C, 2.5 mins. 1 cycle of 72C, 5 mins and hold at 10C.

For the subsequent reactions, the PCR product is diluted 50X and used as template. The PCR amplification cycles are as follows: 1 cycle of 94C for 30s; 57C, 1 min; 72C for 2.5 mins. 22 cycles of 94C, 30 s; 44C, 1 min; 72C, 2.5 mins. 1 cycle of 72C, 5 mins and hold at 10C.

The PCR products are run on a 2% agarose gel, which the specific decreasing sized bands are cut out, gel purified and sent for sequencing. The PCR products can be cloned into a vector to facilitate sequencing reading.

Degenerate primer used are as follows: AP1 (Tm ~ 44C) 5'-STA GAS TST SGW GTS-3'. Specific *hph* primers used: *hph* forward primers (Tm~64C): primary reaction: hph F7 5'-GGT TGA CGG CAA TTT CGA TGA TGC AGC TTG-3'; secondary reaction: hph F4 5'-CGC CCG CAG AAG CGC GGC CGT CTG-3'; tertiary reaction: hph F6 5'-GGG CAA AGG AAT AGA GTA GAT GCC GAC CGG-3'. *hph* reverse primers : primary reaction: hph R1 5'-GCG

CGG CCG ATG CAA AGT GCC G-3'; secondary reaction: hph R2 5'-GCG CCG CCG CTA CTG CTT ACA AG-3'; tertiary reaction: hph R3 5'-GAC CTC CAC TAG CTC CAG CCA AGC CC-3'.

Cloning by High Density SNP Mapping

SNP mapping is based on the method described in (Lambreghts et al., 2009). The mutant strain in Oak Ridge background (*mat* a) was crossed with the Mauriceville exotic strain (*mat* A, FGSC 2225) on a crossing plate. The progenies were collected and germinated onto minimal plates. The progenies were picked on minimal and minimal + QA slants, in which the strains that changed color dependent on QA were selected for further analysis. The progenies were scored by QDE-2 induction upon the presence dsRNA (adding QA for 6 hrs). Progenies that displayed fully induced QDE-2 upon dsRNA induction were scored to be wild type progenies. These progenies were further confirmed for their phenotype through DRAGs induction upon 6 hr dsRNA induction by qRT-PCR. The genomic DNA was extracted from these confirmed progenies and primers that are almost equally distributed throughout the seven chromosomes were selected to pre-screen for linkage bias. The primers are listed in Table 1.

Primer	Enzyme	NEB buffer	Incubation Temp (C)
Chromosome I			
D2	AluI	4	37
C2	BstUI	2	60
A3	Tsp509I	1	65
D3	AluI	4	37
F1	BfaI	4	37
Chromosome II			
F9	MseI	4	37
E4	NlaIII	4	37
C9	HaeIII	2	37
G4	MseI	4	37
Chromosome III			
G5	MseI	4	37
A10	Tsp509I	1	65
D5	AluI	4	37
A11	Tsp509I	1	65
F3	AccI	4	37
Chromosome IV			
E6	NlaIII	4	37
G9	MseI	4	37
G10	MseI	4	37
F5	RsaI	4	37
Chromosome V			
H1	MseI	4	37
H2	MseI	4	37
H3	MseI	4	37
B2	Tsp509I	1	65
B10	TaqI	3	65
Chromosome VI			
C11	HaeIII	2	37
B5	Tsp509I	1	65
H7	MseI	4	37
Chromosome VII			
H12	MseI	4	37
H10	MseI	4	37
H8	MseI	4	37

Table 3 SNP primers used for scanning the whole genome

Whole genome sequencing and sequence analysis using CLC Genomics Workbench

5 ug of genomic DNA was extracted and purified and measured by NanoDrop 2000 (Thermo Scientific). The prepared sample is sent to the sequencing core for Pair-End 80 Illumina sequencing. The raw data is mapped to the Neurospora crassa OR74A (NC10) supercontig sequence downloaded from the Broad Institute *Neurospora crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiDownloads.html). The mapped sequences were then analyzed for SNP (single nucleotide polymorphism) and DIP (deletion and insertion) using the SNP and DIP detection tool under High throughput sequencing category. For more information on how to use the software, a User Manual is available online at their official website (http://www.clcbio.com/index.php?id=1330z).

RESULTS

A genetic screen to identify mutants defective in dsRNA response

The genetic screen is designed based on the selection marker, *mtr* (methyl tryptophan resistance, NCU06619), which encodes a transmembrane protein that allows the entry of aliphatic and aromatic amino acids such as tryptophan as well as toxic amino acid analogs such as r-Fluorophenylalanine (FPA). This selection marker is put under the control of the *qde2* promoter, which can be activated by dsRNA. The transcription of inverted repeats of the *al-1* gene can form dsRNA, which is under the control of the

quinic acid (QA) inducible promoter of the gene *qa-2*. The *Neuropora* gene *quinic acid-2* (*qa-2,* NCU06023.3) encodes an inducible catabolic 3-dehydroquinase that is involved in the breakdown of QA (Geever et al., 1989). Transcription from the *qa-2* promoter is induced in the presence of QA, and is commonly used for creating inducible transcription of genes of interest in *Neurospora*.

The construct containing the QA-inducible dsRNA element and *Pqde2-mtr* is put into a strain containing a S33R point mutation at the *mtr* locus which only allows the expression of *mtr* under the activation of the *qde-2* promoter.

We used this qadsal1;pqde2mtr strain (targeted insertion into the *his*-3 locus of the mtr SR33 strain) as the host strain for this study. The strain turns color from orange to white on QA containing slants and small RNAs corresponding to *al-1*are produced upon QA induction indicating the *al-1* mRNA is depleted in the presence of QA. This indicates that the qadsal1 construct was functioning as expected. Expression of QDE-2 protein was also observed upon the induction of QA, indicating that the dsRNA transcribed by the presence of QA activated the endogenous promoter of *qde-2*. Several DRAGs were also activated in the presence of QA suggesting that dsRNA activated the DRAGs expression in accordance to the observation in previous work (Choudhary, 2007). In addition, the host was tested for the expression of mtr upon QA induction using FPA containing selection plates. We observed that the host dies in the presence of FPA with or without QA indicating that *mtr* is expressed similar to the wild type strain as shown in Figure 7.

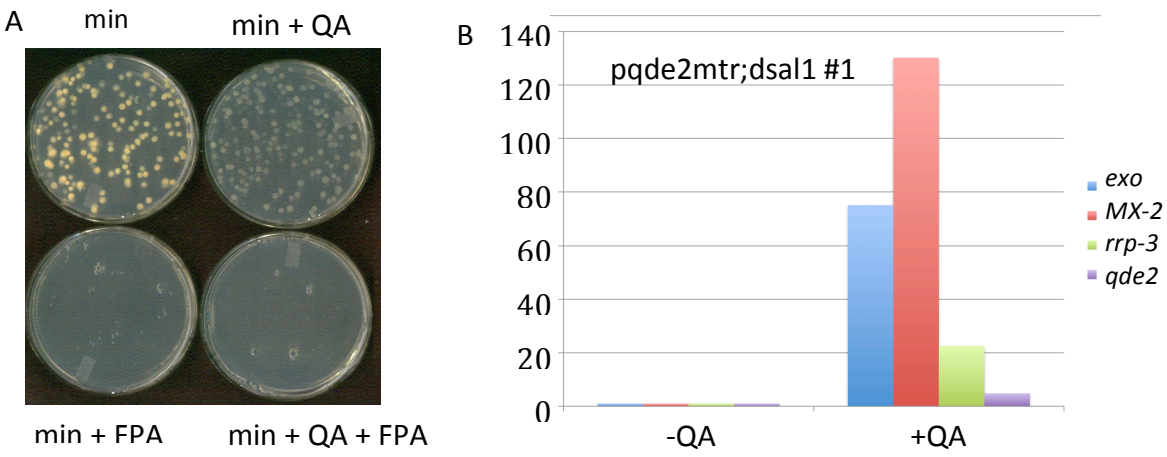

Figure 7 Characterization of the pqde2mtr;dsal1 host strain

(A) FPA sensitivity test of pqde2mtr;dsal1 displaying sensitivity to FPA with and without QA
(B) Quantitative RT-PCR analysis of DRAGs induction upon 6 hr QA treatment

Several mutagenic methods have been employed including random insertion of DNA fragments, UV, EMS, and MMS chemicals. Random insertional mutagenesis was performed using DNA fragment from the hygromycin B resistance (*hph*) gene. The antibiotic hygromycin B is toxic to fungi, including *Neurospora*. *Hph* encodes for a kinase that inactivates hygromycin B through phosphorylation (Kaster et al., 1983). Therefore, only strains that have successful *hph* DNA insertion can survive on hygromycin B selection plates.

Random mutation would be expected to disrupt genes; depending on the method, random insertional mutagenesis or chemical mutagenesis would result in complete knockout or point mutation knock down/knockdown respectively. If a gene involved in the dsRNA signaling response pathway is disrupted, the activation of the *qde-2* promoter will be abolished leading to no expression of MTR. This transformant

strain will then be able to survive on the selection plates containing FPA. This allows us to select for transformants that is involved in the dsRNA response pathway.

The mutagenizing dosage was selected to produce a 90% kill rate, resulting in 10% survival rate on minimal plates after mutagenesis. After mutagenesis, the mutants were plated onto selection plates containing FPA. As compared to ~ 30,000 transformants growing on QA containing plates, ~ 10 mutants were visible on plates containing both QA and FPA. (In the case of *hph* DNA insertion through electroporation, the selection plates would also contain hygromycin). This indicates that FPA selection is stringent enough to select for mutants.

Mutants were picked up and inoculated onto minimal and minimal + QA slants. Mutants that changed color were an indication that the qadsal1 construct and the RNAi pathway is not disrupted by mutagenesis, these mutants were then selected for further analysis.

mutant	mutagenesis method	Whole genome sequencing
m123	random insertion	yes
m218	random insertion	no
uv19	UV	yes
ems12	ems	yes
ems15	ems	yes
mms4-9	mms	no

Table 4 dsRNA response mutants

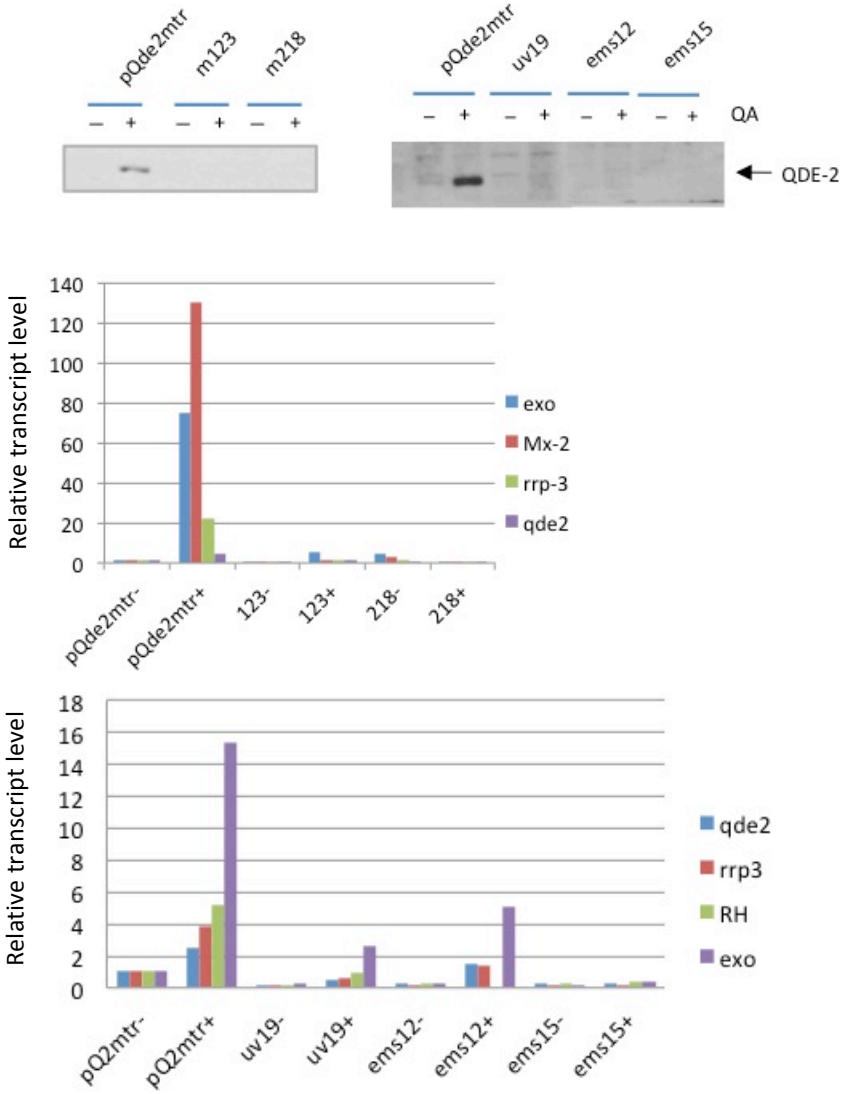

Figure 8 Characterization of the dsRNA response mutants

(A) Western blot analysis of QDE-2 in the identified mutants. Liquid cultures were grown for 2 days (without or with 6 hr of QA) before harvesting.

(B) Quantitative RT-PCR displaying the induction of severeal DRAGs markers upon 6 hrs of QA induction.

Characterization of the mutants

QDE-2 expression was examined under QA conditions for each of these mutants with a functional construct using Western blot analysis. The mutants that had reduced or complete reduction of QDE-2 expression were selected for further DRAGs induction. *MX-2*, *rrp-3*, *qde-2*, and *exonuclease* were chosen as markers to score the mutants upon dsRNA induction. The analyzed mutants were examined for the integrity of the construct. Using northern blot analysis, small RNAs corresponding to the *al1* loci were detected upon QA induction; indicating the qadsal construct is intact and Dcl1/2 are functional. From the mutagenesis, six mutants were confirmed as *bona fide* mutants as shown in Table 3. These mutants were confirmed by their inability to induce DRAGs phenotype using qRT-PCR and western analysis as depicted in Fig 8. All of the mutants displayed functional RNAi as shown in Figure 9A, which the *al1* gene is repressed through color change from orange to white. This also indicates that the production of dsRNA from the *qadsal1* construct is intact, which is confirmed by detecting *al1* small RNAs upon QA induction.

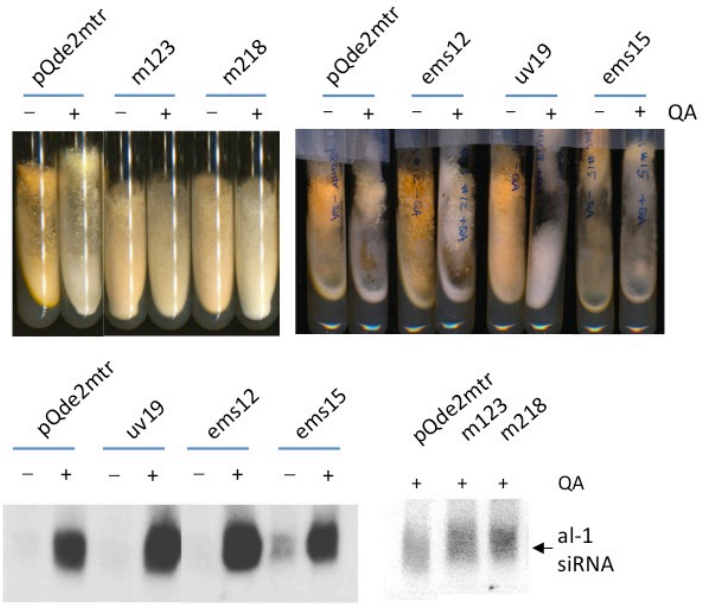

Figure 9 Silencing of al-1 mRNA by production of dsal1

(A) Photos od pqde2mtr; dsal1 host strain and dsRNA response mutants showing the silencing of the al-1 gene displaying color change dependent on QA from orange to white.
(B) Northern blot analysis showing the production of al1 siRNA.

dsRNA mutants are also defective in the DNA damage-induced QDE-2 expression

Previous data has shown that QDE-2 expression is induced by histidine treatment (Lee et al., 2009), suggesting that the elevation of QDE-2 expression is due to the production of dsRNA during DNA damage (histidine treatment). The dsRNA response mutants were treated with histidine and also displayed an inability to induce QDE-2 expression upon DNA damage. This suggests that the response element of QDE-2 for dsRNA produced from DNA damage and QA driven transcription of inverted repeat might be similar. Mutants that have deletions of the dsRNA response elements were treated with histidine and the expression of QDE-2 upon histidine treatment was reduced compared

to wild type. This indicates that the expression of QDE-2 induced by DNA damage and dsRNA is regulated through similar response elements on the promoter of *qde2*. qiRNA, which is specifically produced upon DNA damage by histidine treatment is also examined under the mutant background. All of the mutants displayed a loss of qiRNA induction, if not reduced, upon histidine treatment. The response is not due to histidine treatment alone, as other DNA damage mutagens, hydroxyurea and ems, caused similar phenotype. This result indicates that the regulation of QDE-2 through the two inducers, DNA damage and dsRNA, are very similar. The response elements of the qde2 promoter are required for both pathways, and suggest that the transcription factors that induce *qde-2* might consist of overlapping components.

Attempts to clone genes involved in the dsRNA response

Inverse PCR and TAIL PCR methods, followed by DNA sequencing was performed to identify the genomic loci in mutants mutagenized by *hph* DNA fragment insertion. We found that *hph* fragments frequently inserted into genomic locations in the form of tandem repeats, which made this strategy in identifying the inserted loci challenging. Additionally, it is also possible that *hph* DNA insertion might integrate into several loci, resulting in the last integration site to be not the actual gene causing the phenotype.

Hence, traditional mapping of the mutants was performed. The Mauriceville strain (FGSC 2225, mat A), which is an exotic strain that contains ~0.1% in genome divergence to the *Neurospora crassa* wild type strain was used as a mapping reference. *Bona fide* mutants were crossed with the Mauriceville strain, and progenies were picked

onto minimal and minimal + QA slants. The progenies that turned white on QA containing slants indicate that the qadsal1 construct is functional. Out of 1500 progenies, 70 progenies turned white that is dependent on QA. This indicates that the RIPing process, that mutates duplicated sequences during the sexual cycle, mutated the construct upon crossing; moreover, since the construct was targeted to the *his-3* loci, the duplicated *his-3* sequences that are in close proximity elevated the RIPing efficiency.

The progenies that contain an intact and functional construct were then examined by their phenotype using QDE-2 Western blot analysis. The progenies that induced QDE-2 protein expression upon QA induction were further scored for their DRAGs phenotype. Since *qde-2* promoter is also within the construct, endogenous *qde-2* will be RIPed as well. This indicates that the progenies that do not induce QDE-2 expression is a mixture of mutant progenies and progenies that have nonfunctional QDE-2 caused by RIPing. Previous mapping data demonstrated that by taking the mutant progenies in account, the mapping clearly maps to the endogenous *qde-2* loci.

Out of the 70 progenies that has functional construct, 21 progenies were confirmed to be wild type progenies based on the QDE-2 protein expression and further confirmed by DRAGs induction upon dsRNA induction. The genomic DNA of these progenies were extracted and pooled in equal amounts, which were analysed by CAPS analysis using primers in Table 1. Mapping of this mutant indicated that the mutation lies in the chromosome V. Detailed interval mapping of the progenies show linkage bias at the right arm of chromosome V as shown in Fig 10. The mapped locus is around 200 kb near the telomere, which consists of 17 genes. There are 15 genes that had a KO strain available, which when tested with histidine treatment, none of the KO strains displayed phenotype similar to the mutant.

Whole genome sequencing of the mutant and host strain was performed to identify the causal SNP of the mutant phenotype. SNPs that were not present in the host strain were examined in the mapped region; the stringency threshold of

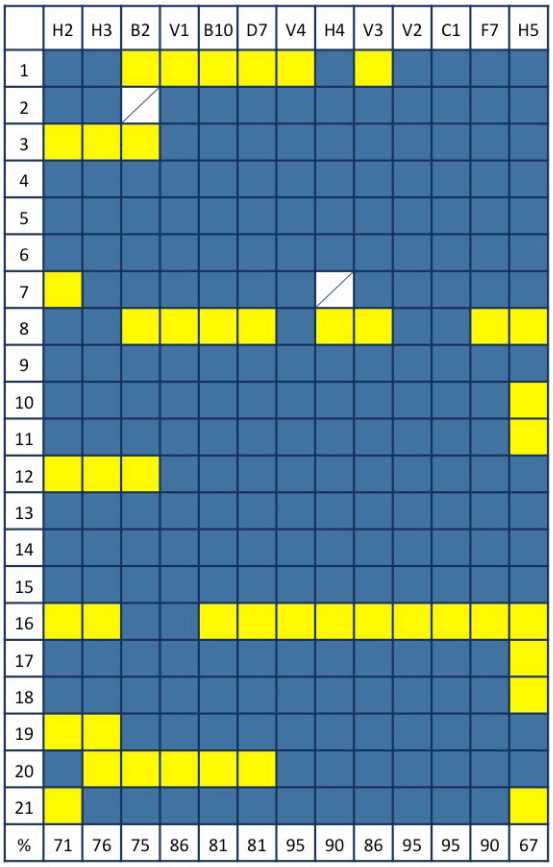

Figure 10 Interval Mapping of UV19 mutant at Chromosome V.

The centromere lies between the primers H2 and H3. Based on the recombination frequency between the 21 WT progenies, there is a tight linkage between primers V4 to F7

identified SNPs was set to 90%. Within the mapped region, one SNP was identified in the open reading frame (ORF) that caused a silent mutation as shown in Table 3.

From the SNP mapping and whole genome sequencing results, no specific mutation from the mutant was identified from the mapping region that lies within the ORF. It is possible that point mutation is not the cause of the phenotype, but different types of mutations such as large deletions or translocations. These types of mutations remain challenging to detect from sequencing and mapping results. It is also possible that the causal mutation does not lie within the ORF and may be a noncoding product. A method that integrates the knowledge from this chapter is described below, which uses

Position in Ch. V	Variation type	Reference	Mutant		NOTES
4035192	SNP	C	T	non-ORF	
4043626	SNP	C	T	non-ORF	
4198325	SNP	C	T	non-ORF	
4527579	SNP	A	G	ncu09535	intron
4560717	SNP	G	A	ncu09551	multidrug transporter
4560736	SNP	G	A	ncu09551	multidrug transporter
4800025	SNP	A	C	non-ORF	
5236702	SNP	A	C	non-ORF	
5339080	SNP	T	C	non-ORF	
5339082	SNP	T	C	non-ORF	
5560547	SNP	T	C	ncu04303	silent mutation
5821839	SNP	T	C	ncu8959	3' UTR
5828619	SNP	A	G	non-ORF	
5834444	SNP	C	T	non-ORF	
5834539	SNP	C	T	non-ORF	
5835780	SNP	T	C	non-ORF	
5845508	SNP	C	T	non-ORF	
5888375	SNP	G	A	ncu10063	
6436098	SNP	T	C	telomere	
5699661	DIP	TCA	---	non-ORF	
5824212	DIP	A	-	ncu11660	phenyl propionate permeasetransporter
5824352	DIP	T	-	non-ORF	
5826752	DIP	C	-	non-ORF	
5834117	DIP	AA	--	non-ORF	
5834120	DIP	T	-	non-ORF	
5834122	DIP	GCG	---	non-ORF	
5839793	DIP	TA	--	non-ORF	
5839796	DIP	G	-	non-ORF	
5840428	DIP	A	-	non-ORF	
5840742	DIP	---	GTC	non-ORF	
5853821	DIP	TT	--	non-ORF	
5853976	DIP	-	C	non-ORF	

Table 5 SNPs and DIPs within the mapped region in Chromosome V

whole genome sequencing as a powerful tool to look for SNPs that is linked to the phenotype. The method uses bulked DNA sequencing to allow linkage analysis.

DISCUSSION

This chapter describes the establishment of a random mutagenesis-based selection to identify components involved in the dsRNA transcriptional response pathway. We used the transgenic pqde2mtr; qadsal1 targeted to the mtr mutant strain as the host for this genetic screen. QA driven transcription of inverted repeat of *al1* induced the expression of MTR through the dsRNA response element of *pqde2*. Expression of MTR allows the entry of toxic FPA into the cell that in turn kills the cell. The antibiotic hygromycin B is toxic to *Neurospora*, only strains that express *hph* can survive in the presence of hygromycin. This provides a convenient platform to select for mutants that is involved in the dsRNA response. However due to most of the flanking region of the inserted *hph* fragment is *hph* itself, it remains difficult to identify the gene disrupted by random DNA insertion. It is also possible that the gene that is disrupted by the inserted DNA is not the gene causing the phenotype. Therefore, conventional high density SNP mapping is used to identify the gene of interest.

In order to analyze whether there is bias in SNPs segregation to the phenotype, crossing the mutant to a reference strain is essential. However in *Neurospora*, any duplicated sequence would trigger the RIPing process resulting in non-functional gene products. Therefore, the construct that contains inverted repeats of *al1* would be

destroyed upon crossing. This results in the inability to score the progenies through the dsRNA induction by QA. The expression of

QDE-2 is also induced by DNA damage using histidine, which can be regulated through the same response element as dsRNA. Strains that have deletion in the dsRNA response element of *qde-2* promoter displayed dramatic reduction of QDE-2 protein levels upon histidine treatment, suggesting that the expression of *qde-2* is regulated through the same response element as the dsRNA response pathway; therefore it is possible to score the progenies with histidine treatment. It is imperative to confirm that there is 100% correlation between regulating QDE-2 expression upon DNA damage and dsRNA, so that it is possible to use DNA damage to score the phenotype of the dsRNA response mutants. To confirm this, progenies that are bona fide wild type and mutant progenies can be examined for their phenotype under histidine treatment.

It is intriguing why there are no SNPs residing in the ORF that are identified only in the mutant but not the wild type reference strain within the mapped region. One possible explanation is that the mutation causing the phenotype might be intergenic, and the product might not necessary be translated.

Future studies in understanding the mechanism of dsRNA response

This chapter describes the identification of several mutants that has lost the dsRNA induced transcriptional response. From the mapping results, it is apparent that

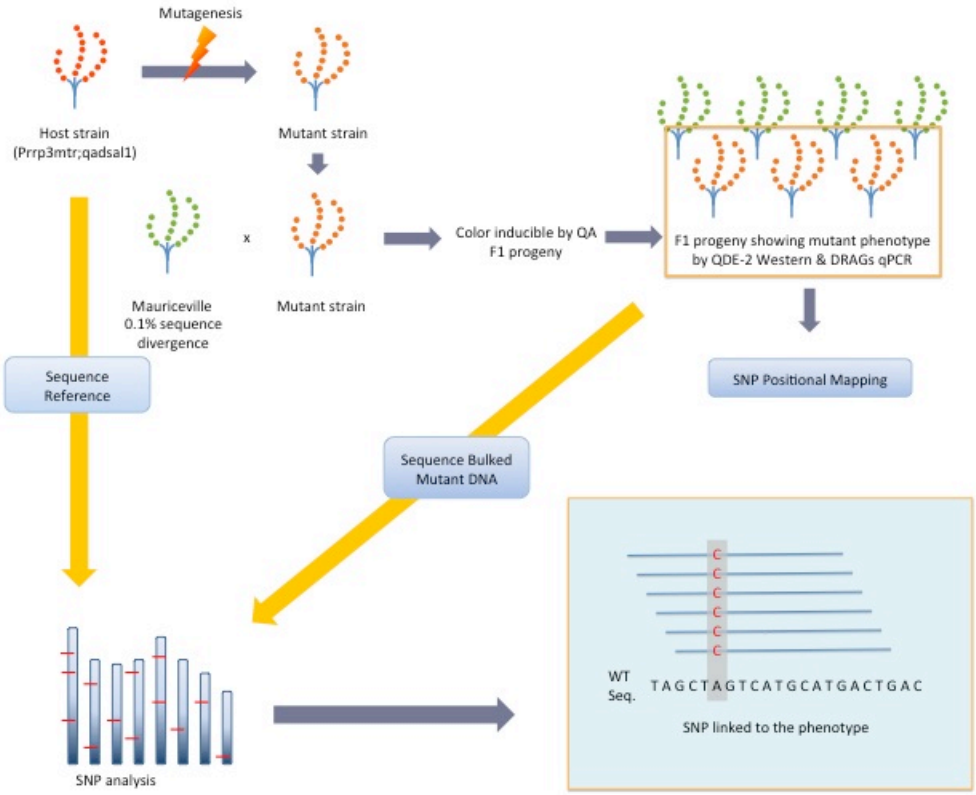

Figure 11 Simplified scheme of identifying the causal mutation using bulked DNA sequencing

The host strain with a reference genome is mutagenized. The mutant is then crossed with the Mauriceville strain that contains 0.1% sequence divergence reference strain. The resulting F1 progeny are stringently scored to identify the progenies displaying mutant phenotype. DNA of the mutant progenies are bulked and subject to whole-genome sequencing followed by mapping and alignment to the reference sequence. SNPs that are only arise in mutant reads are closely linked to the causal SNP that is responsible for the mutant phenotype.

the RIPing process would make traditional mapping challenging since crossing of two strains is inevitable.

Due to the RIPing effect during crossing, it would be beneficial if the promoter driving MTR were exchanged with another DRAGs gene promoter such as *rrp3*. The construct would be transformed in an mtr^{KO} strain (FGSC #13094, mating type a) for a more uniform genetic background that is similar to the Neurospora Broad Institute wild type genome sequence. This would allow the scoring of the mutant phenotype by QDE-2 western blot, which is more efficient than DRAGs qRT-PCR.

A possible method to address how to identify the causal mutation in the mutants is depicted in Fig 11. The host strain containing a promoter apart from Pqde2 is mutagenized with UV or ems and the mutants are confirmed with their phenotype by QDE-2 western and DRAGs qRT-PCR under 6 hrs of QA treatment. The *bona fide* mutants are then crossed with the Mauriceville strain (FGSC#2225, mating type A), in which the progenies are tested for their color change on QA containing slants.

The progenies that display color change dependent on QA would then be examined for their phenotype using QDE-2 WB and further confirmed by DRAGs qRT-PCR. The confirmed mutants progenies would be selected for bulk DNA sequencing and the reference strains would be sequenced for SNP analysis. All the nucleotide changes incorporated into the mutant by mutagenesis are detected as SNP or insertion-deletions between mutant and wild type. Most of the SNPs generated would be in equal distribution between the wild type and mutant from the sequenced reads, only the SNPs that are linked to the causal SNP would have most of the reads displaying the mutant SNP. If we set a SNP index that equals the ratio of reads between SNP of the mutant and the total reads corresponding to the SNP; unlinked SNP index would be 0.5 whereas

linked SNPs would be close to 1, indicating that there is 100% reads that display the mutant SNP and 0% wild type SNP reads. By using this method, the reference strains (host strain and Mauriceville strain) should also be sequenced to identify which SNPs are arising from the wild type.

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