Investigating the role of *Neurospora* RNA-Dependent RNA Polymerase-3 in quelling and meiotic silencing by unpaired DNA

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ABSTRACT

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RNA interference (RNAi) is a conserved defense system against viruses and transposons which is mediated by small non-coding RNAs. The filamentous fungus Neurospora crassa has served as a valuable model for RNAi studies that greatly expand our knowledge on this complex phenomenon. In Neurospora, there are two post-transcriptional gene silencing pathways, quelling occurs during vegetative growth and is triggered by the presence of transgenes, whereas meiotic silencing by unpaired DNA (MSUD) occurs during meiosis and is induced by unpaired DNA. RNA-dependent RNA polymerases (RdRP) are key components of RNAi pathways responsible for the synthesis of double-stranded RNA. The Neurospora genome encodes three RdRPs; QDE-1 and SAD-1 are required for quelling and MSUD respectively, while the function of the third RdRP is unknown. It is interesting to ask why Neurospora possesses multiple RdRPs and what role these RdRPs play in gene silencing.

In this work, I used classical genetic and molecular biology techniques to establish whether RdRP-3 plays a role in quelling or in MSUD. Phylogenetic analysis of RdRP proteins from different organisms placed RdRP-3 in a group containing Cryptococcus neoformas RDP1 that is known to be involved in RNAi. However, when only the highly conserved RdRP-domain is used, RdRP-3 is placed in a group with fungal RdRPs of unknown function. Results of quelling and MSUD assays show that the rdrp-3Δ mutant does not affect silencing efficiency of either quelling or MSUD. Hence, tools that can be used to aid further characterisation of RdRP-3 were constructed. RdRP-3 was tagged with gfp at its endogenous locus to allow the study of RdRP-3’s cellular localisation. This strain exhibited growth defects and unfortunately no GFP signal was detected in hyphae or conidia by confocal microscopy. Additionally, because it has been previously shown that rdrp-3 is a dsRNA–activated gene highly induced in the presence of dsRNA, I generated strains expressing albino-1 dsRNA from an inducible promoter. This construct was placed in the wild-type, rdrp-3Δ mutant and in the rdrp-3gfp-tagged background, and the system was validated by RT-PCR analysis. When dsRNA albino-1 was induced in the rdrp-3gfp-tagged background still no GFP signal was detected. Nevertheless, in the WT and in the rdrp-3Δ, upon induction of albino-1 dsRNA, albino-1 mRNA levels decrease and rdrp-3 and NCU07036 mRNA increase as expected. These strains will be used in future to compare gene expression in response to dsRNA in the presence and absence of rdrp-3. In conclusion, the function of RdRP-3 still largely remains unknown, tools created here can be used to explore the possible function of RdRP-3 in response to dsRNA.
DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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1. Introduction

1.1 Discovery of RNAi in eukaryotes

Eukaryotic genomes are composed of a large number of repetitive and transposable elements (Buchon and Vaury, 2006). Some of these sequences can move from one genomic location to another, posing mutational threats to an organism. Thus, organisms have developed protective mechanisms to inactivate repeated sequences and to destroy foreign genetic material. Gene silencing mechanisms are a part of the host defense that protects the genome from invasive DNA elements such as transposons and viruses (Obbard et al., 2009). One common mechanism is RNA interference (RNAi), which is a conserved regulatory mechanism mediated by small-noncoding RNAs. RNA silencing is triggered by the introduction of exogenous DNA or RNA sequences, or by duplication of endogenous nucleic acid sequences, resulting in silencing of such sequences. RNAi can silence the invaders transcriptionally by blocking the process of transcription or post transcriptionally by targeting mRNA for degradation (Fulci and Macino, 2007; Chang et al., 2012; Wassenegger and Krczal, 2006; Montgomery et al., 1998; Fire et al., 1998).

Several RNAi mechanisms exist in plants, fungi and animals. These mechanisms are related at least to some degree, and have probably evolved from an ancestral defence system against transposons and viruses (Obbard et al., 2009). In plants, silencing phenomenon was first observed in petunia. Introduction of genes involved in petal pigmentation suppressed both ectopic and endogenous homologous genes, thus the phenomenon was named cosuppression (Napoli et al., 1990). Soon after, Romano and Macino reported a similar post transcriptional silencing (PTGS) mechanism in fungi, which is called quelling in Neurospora crassa. Quelling is triggered by introduction of transgenes which are duplicated in the genome. As a result of quelling, homologous genes are silenced due to loss of mRNA arising from the duplicated genes or gene fragments (Romano and Macino, 1992). In 1998 a hallmark feature of RNAi, that double-stranded RNA (dsRNA) intermediates result in silencing of genes complementary to the dsRNA, was observed in the nematode Caenorhabditis elegans by Fire et al, 1998. When he and his colleagues attempted to inactivate gene expression by injection of antisense RNA, they found a small amount of dsRNA in RNA preparations which was later shown to induce the silencing effects. This was also the first evidence of a silencing mechanism that is active in animals. The mechanism for post transcriptional silencing phenomenon in animals was named double-stranded
RNA interference (Montgomery et al., 1998). The RNAi pathway was subsequently found in many other organisms, including *Drosophila melanogaster, Trypanosoma brucei, Planaria, Arabidopsis thaliana, Mus musculus* and *Danio rerio* (Pickford et al., 2002).

RNAi is not only a defense mechanism against invasive DNA sequences, but also plays a role in regulating diverse cellular and developmental processes (Chang et al., 2012). Many genetic and biochemical studies have furthered our understanding of how RNAi works. Soon after the involvement of dsRNA production was discovered in *C. elegans*, the first eukaryotic protein component of RNAi was isolated in *Neurospora* (Pickford et al., 2002). *quelling-defective-1 (qde-1)* encodes a cellular RNA-dependent RNA polymerase (RdRP). RdRPs play a key role in synthesis of dsRNA which has been shown to be an essential intermediate of PTGS (Cogoni and Macino, 1999a). Following this work, other core components of RNAi were identified in *Neurospora*. These components are evolutionarily conserved across different species (Catalanotto et al., 2004; Catalanotto et al., 2002; Cogoni and Macino, 1999b). A common theme of RNAi is the reliance on production of small interfering RNAs (siRNAs) of 21-25 nucleotides. Members of the Dicer family of RNase III endoribonucleases recognise and cleave dsRNAs to generate siRNAs. The duplex siRNAs are loaded on an effector complex called the RNA Induced Silencing Complex (RISC) containing an Argonaute protein. Removal of the passenger strand from siRNA duplex activates RISC, while the guide strand directs RISC to complementary RNA for degradation or translation repression (Chang et al., 2012; Willmann et al., 2011).

In *Neurospora*, there are three gene silencing mechanisms; Repeat-induced point mutation (RIP), quelling and meiotic silencing by unpaired DNA (MSUD) (Romano and Macino, 1992; Shiu et al., 2001; Chicas et al., 2004; Galagan and Selker, 2004). The *Neurospora* genome encodes three RdRPs. QDE-1 and SAD-1 are involved in quelling and MSUD respectively, while the function of the third RdRP, RdRP-3, is unknown (Shiu et al., 2001; Cogoni and Macino, 1997). It is interesting to ask why *Neurospora* possesses multiple RdRPs and what role these RdRPs play in gene silencing. The focus of this thesis is RdRP-3. Therefore, in the following sections, the role of RdRPs in RNA silencing, a summary of the current knowledge of gene silencing pathways in *Neurospora*, particularly the molecular mechanisms underlying quelling and meiotic silencing by unpaired DNA, will be described.
1.2 RNAi pathways in *Neurospora*

1.2.1 Different gene silencing mechanisms are active at different stages of the *Neurospora*’s life cycle

*Neurospora* has both asexual and sexual life cycles and both cycles have been described in detail (Figure 1.1). The asexual life cycle produces two types of spores, macroconidia and microconidia, through two distinct sporulation pathways. Macroconidia contain multiple nuclei, while microconidia contain 1-2 nuclei. Macroconidiation, also known as conidiation, can be induced by physiological, chemical or environmental factors such as nutrient starvation, nitrogen depletion and light (Springer, 1993). Conidia germinate in conditions suitable for growth, producing tubular structures called hyphae. One of the remarkable characteristics of *Neurospora* is that it is coenocytic, containing multiple nuclei in a shared common cytoplasm. Hyphae grow by tip extension and aerial hyphae then develop into macroconidiophores by proconidial chain formation. Synthesis of crosswalls compartmentalises the common cytoplasmic space to separate individual conidia, which mature and are released into environment. In addition, asexual spores can be produced via microconidiation which occurs at a low frequency. Hyphae originate from a mycelium and a microconidium protrudes from the hyphae. Surrounding layers of the hyphal walls are thickened and form a collar. The microconidium is ruptured from the hyphal walls and separated by septum (Springer, 1993, Davis, 2000).

The sexual cycle requires parents of opposite mating types. *Neurospora* is a haploid organism containing a single mating type locus of either mat A or mat a. Both mating types can act as female and male in a cross but fertilisation only occurs between strains of opposite mating types. A vegetative mycelium is formed by branching, threadlike hyphae. Nitrogen or carbon deprivation induces formation of female structure known as protoperithecium. Small knots of hyphae are formed around a few special cells, which later develop into an ascogonium. Trichogynes, specialised hyphae derived from the protoperithecium, grow toward the conidia of the opposite mating type until cell fusion occurs. A nucleus of the conidium migrates to an ascogonial cell in a developing perithecium. Next, nuclei of two mating types undergo rounds of cell division at the tips of ascogonous hyphae, which are develop into a crozier. Pre-meiotic DNA synthesis and nuclear fusion takes place resulting in formation of a zygote. Two meiotic divisions result in production of four haploid nuclei. Each one undergoes a mitotic division, yielding eight haploid nuclei which are arranged orderly in the common cytoplasm of the ascus. A new membrane derived from the plasma membrane is
formed during nuclear division to create a new cell for each nucleus. These new cells develop into eight haploid ascospores which are lined up in the spindle-shaped ascus. Ascospores undergo maturation, becoming larger and black, and by 8 days of development, mature ascospores are shot through the ostiole in the perithecial beaks (Springer, 1993, Davis, 2000).

Figure 1.1 Life cycle of Neurospora crassa. In Neurospora, meiosis occurs inside the zygote, which forms by fusion of two haploid nuclei of opposite mating type. The zygote undergoes karyogamy, meiosis and mitosis within the perithecium, resulting in an ascus that contains eight haploid spores. Several surveillance systems acting at different stages of the life cycle have been implicated in Neurospora, such as quelling, repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD). Taken from Shiu et al. (2001).

Distinct gene silencing mechanisms are active at different stages of the life cycle (Figure 1.1). Repeat-induced point mutation (RIP) is a transcriptional silencing mechanism which occurs during the premeiotic phase of sexual development. It is a process which detects and inactivates duplicated sequences by inducing extensive G:C to A:T mutations (Galagan and Selker, 2004). Quelling and MSUD are RNAi-mediated
mechanisms acting at the post transcriptional level. Quelling occurs in the haploid cell during vegetative growth and is triggered by the presence of transgenes and dsRNA, whereas MSUD occurs inside the diploid zygote during the first meiosis and is induced by unpaired DNA (Chang et al., 2012).
1.2.2 Quelling in *Neurospora*

1.2.2.1 Discovery of quelling

Quelling in *Neurospora* was the first transgene-induced gene silencing phenomenon documented in fungi (Romano and Macino, 1992). Due to the presence of carotenoids, *Neurospora* mycelium and conidia appear orange. Biosynthesis of carotenoids requires the products of *albino* genes (*albino*-1, *albino*-2 and *albino*-3); mutations in these genes produce an albino (white/pale yellow) phenotype. Quelling was discovered when a portion of *al-1* or *al-3* was transformed into a wild-type strain, resulting in the albino phenotype (Romano and Macino, 1992). The albino phenotype was not due to mutations but silencing of *al-1/al-3*. The *al-1/al-3* mRNA was much reduced in the quelled transformants, indicating that both transgenic and endogenous copies of *al-1/al-3* were silenced (Romano and Macino, 1992). This suggests that quelling depends on the homology of the transgene. Moreover, quelling is a reversible mechanism and the copy number of a transgene is an important factor that determines maintenance of quelling efficiency. It was found that 25% of quelled progeny spontaneously and progressively reverted to wild-type or an intermediate phenotype over time. Reversion is unidirectional and is a result of loss of transgene copies (Cogoni et al., 1996). These observations suggest that multiple copies of the transgene are required for triggering quelling, as well as for maintaining its efficiency.

*Neurospora* is coenocytic, containing multiple nuclei in the shared cytoplasm of hyphal compartments. Homokaryons are cells that contain genetically identical nuclei, while heterokaryons contain genetically different nuclei (Davis, 2000). Transformation of conidia with DNA frequently results in heterokaryotic transformants, as not all nuclei take up the transforming DNA. *albino* mutations are recessive. Quelled heterokaryotic transformants contain nuclei of both the wild-type and silenced *al-1* and display the albino phenotype. Thus, the phenotype of quelled *al-1* is dominant over the wild-type. This result suggested that silencing signals are diffusible, probably involving trans-acting molecules (Romano and Macino, 1992; Chang et al., 2012). Moreover, a comparable level of unspliced pre-mRNA of *al-1* was detected in both wild-type and quelled strains, whereas spliced mRNA was reduced in the quelled strain. The total pre-mRNA level was unchanged in both strains. These data demonstrated that quelling does not affect the transcription machinery; it is a post-transcriptional gene silencing mechanism resulting in mRNA degradation, presumably by a process that occurs in the cytoplasm (Pickford et al., 2002).
The use of al-1 as a marker allows the quelling effects of a transgene to be easily determined by visual inspection. Several properties of quelling were observed by transforming Neurospora with different al-1 constructs. A minimum length of the homologous region required for quelling is 132 nucleotides. The transgene does not need to carry promoter elements; the transcribed region is sufficient to trigger quelling (Cogoni et al., 1996). Quelling is a general phenomenon, not restricted to any specific gene. Transformation of other reporter genes is also able to induce quelling (Pickford et al., 2002). In addition, sense RNAs specific to the transgene were detected in the quelled strains, suggesting that these sense RNAs are derived from the transgene and that transcription from the transgene is an intermediate step of quelling (Pickford et al., 2002).

1.2.2.2 Isolation of quelling deficient mutants
A mutagenesis approach was used to isolate quelling-defective (qde) mutants, while the al-1 gene was used as a reporter gene to determine the efficiency of silencing. Transformation of al-1 gene into an orange wild-type resulted in the albino phenotype. The stably quelled strains were mutagenised by UV and transformants whose phenotype reverted to orange were selected. A large scale screen of 100,000 transformants revealed 19 revertants which were putative qde mutants. Only 15 of these retained a high al-1 copy number and accumulated al-1 transcripts. This suggested that recovery of the wild-type phenotype was caused by mutations of quelling genes rather than loss of al-1 copies. Isolation of these qde mutants enabled cloning of three qde genes, called qde-1, qde-2 and qde-3, which encode three components of the quelling pathway (Cogoni and Macino, 1997).

1.2.2.3 Molecular mechanism of quelling
qde-1 encodes a cellular RNA-dependent RNA polymerase (RdRP), which synthesises double-stranded RNA from a single-stranded RNA template. The involvement of an RdRP is consistent with the finding that double-stranded RNAs are necessary intermediates for RNAi (Cogoni and Macino, 1999a; Fire et al., 1998). Homologs of QDE-1 are found in plants, fungi and C. elegans, indicating that a conserved PTGS exists. For example, SDE1/SGS2 (also known as RDR6) in Arabidopsis and EGO-1 in C. elegans, are homologs of QDE-1, and have been shown to be core components of their respective RNAi pathways (Smardon et al., 2000; Mourrain et al., 2000). QDE-1 is a rate-limiting factor for efficient silencing. The production of siRNA is significantly increased in QDE-1 overexpressing strains, resulting in a dramatic increase in quelling.
efficiency (Forrest et al., 2004). As mentioned previously, a threshold of transgenic copy number needs to be reached for activation of silencing and this threshold is reduced when QDE-1 is overexpressed.

The *qde-2* gene is homologous to the *rde-1* gene which is essential for dsRNA interference in *C. elegans* (Fagard et al., 2000). *qde-2* encodes an Argonaute protein, which contains a Piwi and a PAZ domain. Argonaute proteins act as adaptors in binding of siRNA and also possess the slicer activity required for RNA degradation (Lee et al., 2010; Chang et al., 2012). QIP, a QDE-2 interacting protein, contains a 3’-5’ exonuclease domain at its C terminus belonging to the DEDD superfamily of 3’-5’ exonucleases. RISC activation was impaired in a *qip*Δ strain, suggesting that QIP acts downstream of Dicer (Lee et al., 2010).

*qde-3* encodes a RecQ-like helicase. Helicases are involved in DNA repair, recombination and replication. RecQ-like helicase are functionally conserved from bacteria to humans and are usually present in one or more copies (Cogoni and Macino, 1999b). In *Neurospora*, it is known that QDE-3 plays a role in DNA repair, in cooperation with the QDE-3 homologous RecQ-2 protein (Pickford et al., 2003).

Two Dicer proteins, DCL-1 and DCL-2 function redundantly to process dsRNA during quelling (Catalanotto et al., 2004). It was found that quelling is not significantly affected in *dcl-1* or *dcl-2* single mutants. However, quelling is abolished in a *dcl-1; dcl-2* double mutant and the dsRNA cleavage process is impaired. Furthermore, siRNA accumulates in the *qde-2* mutant, but not in *qde-1* or *qde-3* mutants. This suggests that Dicer functions upstream of siRNA production (Catalanotto et al., 2004). Although two dicer proteins are partially redundant, DCL-2 is the major processor for siRNA production. Both Dicer proteins contain four domains; two RNaseIII domains (RNaseIII a and RNaseIII b) at the C-terminus, an RNA helicase and DEAD-box ATP binding domain at the N-terminus of the proteins and one domain of unknown function (DUF283). An additional dsRNA binding domain is found in DCL-2. The lack of this domain in DCL-1 suggests that in order to bind dsRNA, DCL-1 may interact with another protein. It has been proposed that Dicer proteins act as dimers. Since DCL-2 is the major Dicer for processing dsRNA in quelling, it is unlikely that DCL-1 and DCL-2 to form heterodimers for efficient processing activity. Thus, DCL-1 and DCL-2 may work independently or as homodimers (Catalanotto et al., 2004). In addition, Dicer is also involved in the biogenesis of miRNAs in animals and plants (Zeng, 2006).
QDE-1 has been shown to possess RdRP activity *in vitro* (Makeyev and Bamford, 2002). The enzymatically active site of RdRP activity is located in the C-terminal portion of the protein, which is conserved across the RdRP family. QDE-1 catalyses RNA synthesis using ssRNA templates for production of two types of products, long dsRNA and short RNA of 9-21 nucleotides. However, QDE-1 does not recognise dsRNA templates. QDE-1 can initiate dsRNA synthesis by both a primer-independent and a primer-dependent mode. Primer–dependent RNA synthesis by QDE-1 is inefficient. In any case, long dsRNA products generated by QDE-1 can be recognised and processed by Dicer, leading to downstream gene silencing events. The short 9-21 nt RNA have been suggested to target longer RNAs for mRNA degradation. The possible functions of these siRNAs are unknown (Makeyev and Bamford, 2002). The crystal structure of QDE-1 reveals that QDE-1 is a dimeric molecule containing metal binding sites and a catalytic domain at the C-terminus of the protein (Salgado et al., 2006). QDE-1 is structurally distinct from viral RdRP. The core catalytic site is comprised of two double-psi β-barrel (DPBB) subdomains, which are similar to the active sites of DNA dependent RNA polymerases (Salgado et al., 2006). Recently, it was confirmed that QDE-1 possesses DNA-dependent RNA polymerase (DdRP) activity *in vitro*. A recombinant catalytically active C-terminal portion of QDE-1 was purified to measure RdRP and DdRP activity of QDE-1. An *in vitro* assay using single-stranded DNA (ssDNA) templates showed that nearly all ssDNAs were converted to DNA/RNA hybrids within 5 mins. However, when ssRNAs were added as a template, most of them were remained single-stranded after 30 mins. A purified full-length recombinant QDE-1 showed similar results. The DdRP activity of QDE-1 is approximately 25-fold higher than its RdRP activity (Lee et al., 2010). This is consistent with the finding that partially purified *Arabidopsis* RDR6 possesses strong DdRP using ssDNA as templates for production of dsRNA, which is required for RNA-mediated virus-induced silencing in plants (Curaba and Chen, 2008). It is hypothesised that QDE-1 acts as a DdRP to initiate RNA synthesis internally from ssDNA and then uses this ssRNA to generate dsRNA. Overall, in an *in vitro* RNA polymerase assay using ssDNA as templates, DNA/RNA hybrids and dsRNA were produced by QDE-1, indicating that QDE-1 can directly synthesise dsRNA from ssDNA (Lee et al., 2010).

QDE-1 interacts with ssDNA binding protein Replication Protein A (Nolan et al., 2008, Lee et al., 2010). The *Neurospora rpa-1* is an essential gene, encoding for a large subunit of RPA which is known to be important in DNA replication, repair and
recombination in other organisms (Din et al., 1990; Brill and Stillman, 1991; Nolan et al., 2008). RPA is a heterotrimeric complex consisting of RPA1, RPA2 and RPA3 (Lee et al., 2010). rpa-1 and rpa-2 are essential genes, however an rpa-3 deletion strain is viable but has significantly reduced quelling efficiency, indicating that rpa is required for gene silencing. It has been suggested that RPA recruits QDE-1 to the transgenic loci (Lee et al., 2010). In vitro RNA polymerase activity assays showed that QDE-1 accepted the RPA-bound ssDNA templates and the presence of RPA enhances dsRNA synthesis. It has been suggested that RPA enhances dsRNA production of QDE-1 by preventing the formation of DNA-RNA hybrids, which are products of QDE-1.

Further, QDE-3 is also an interacting partner of RPA (Lee et al., 2010). The interaction of QDE-1 and RPA identified previously was abolished in the qde-3 mutant, indicating that QDE-3 is required for the interaction between QDE-1 and RPA. These data provide a clue of how aRNA and dsRNA are generated in quelling. QDE-1 is recruited to the transgenic loci by QDE-3 and RPA, where QDE-1 acts as a DdRP to generate aRNA and then as a RdRP to convert the aRNA into dsRNA, which is recognised and processed by DCL-2 into siRNA. The siRNA is incorporated into RISC and complementary RNA is targeted for degradation, thus resulting in gene silencing (Lee et al., 2010; Nolan et al., 2008).

Induction of dsRNA derived from an inverted repeat sequence of al-1 leads to accumulation of QDE-2 protein in wild-type, qde-1 and dcl1 mutants (Choudhary et al., 2007). Three quinic acid (QA)-inducible hairpin constructs (dsal-1, dsfrq and dsfrh) expected to express dsRNAs specific to respective genes. QDE-2 induction was observed in transformants expressing different constructs, indicating that QDE-2 accumulation occurs in response to dsRNA generally, rather than gene-specific dsRNA production. Lack of both DCL proteins fails to accumulate QDE-2 protein even though qde-2 mRNA was induced. This suggests that qde-2 is transcriptionally activated by dsRNA and is post-transcriptionally regulated by DCL proteins or possibly siRNAs, the products of DCL proteins. Furthermore, dcl-2 mRNA accumulated in dsRNA-induced strains and DCL-2 protein was also highly induced. Induction of DCL was delayed, occurring later than QDE-2 accumulation. This data suggests that dsRNA transcription activates dcl, which is likely to be involved in secondary events of RNAi responses, rather than immediately activating transcription of qde-2. In an inducible al-1 hairpin background, in the presence of QA inducer, a qde-2 mutant complemented with a functional copy of qde-2 restored QDE-2 accumulation and resulted in silencing of al-1. However, a strain with basal levels of qde-2 failed to silence al-1. Thus, in addition to
QDE-1, QDE-2 is also a limiting factor for efficient RNAi silencing and induction of QDE-2 is required for RNAi responses.

Recent evidence demonstrated that homologous recombination is required for quelling (Zhang et al., 2013). Quelling was impaired in mutants deficient in homologous recombination. The al-1-specific siRNA was induced by DNA damage in partially al-1 quelled strains, indicating that quelling is also induced by DNA damage. An al-1 quelled strain expressing c-Myc-tagged RAD51 (essential for homologous recombination) showed a significant Myc-RAD51 enrichment at the al-1 transgenic locus. These data suggest that quelling requires homologous recombination and shares a common pathway with qiRNA biogenesis (detailed in Section 1.2.2.5) (Zhang et al., 2013).

A current model of quelling is proposed (Chang et al., 2012) based on the existing evidence in *Neurospora* together with findings in other organisms (Figure 1.2). It has been postulated that the transcription of transgenes forms an aberrant structure, which can be recognised by QDE-3. QDE-1 is recruited to the transgenic locus by QDE-3 and RPA where it uses the aRNA as a template for generation of double-stranded RNA molecules. Two redundant Dicer proteins, DCL-1 and DCL-2 process dsRNA into siRNA of 21-25 nt, which are incorporated onto the RISC complex, containing the Argonuate protein QDE-2. QDE-2 slices the passenger strand of siRNA which is removed by QIP. The remaining strand guides QDE-2 to complementary mRNA sequences to induce degradation (Chang et al., 2012).
During vegetative growth, QDE-3 recognises transgenes and with RPA recruits QDE-1 to the transgenic locus, resulting in the formation of aberrant RNA (aRNA). QDE-1 converts aRNA into double-stranded RNA (dsRNA), which is processed by DCL-1 and DCL-2 to generate siRNA and subsequently loaded onto the QDE-2/QIP based RNA Induced Silencing Complex (RISC) resulting in gene silencing. Modified from Chang et al. (2012).
1.2.2.4 dsRNA-induced host defense response in *Neurospora*

Transcriptional regulation of QDE-2 and DCL-2 induced by dsRNA suggests that a regulatory network may exist at the transcriptional level in the RNAi pathway (Choudhary et al., 2007). A genome-wide search for additional genes activated by dsRNAs was performed by microarray and qRT-PCR. As expected, most genes known required for quelling are induced by dsRNA, including *qde-1, dcl-1, qde-2, dcl-2* and *qip*. Besides these genes, ~ 60 genes were identified as dsRNA-activated genes (DRAGs) which include genes that functionally belong to IFN-stimulated and antiviral genes, RNA/DNA binding and regulation, stress responses and protein degradation as well as genes with unknown functions. Some of the DRAGs are already known to be important for antiviral and transposon control in other organisms. For example, three of the highly induced genes in *Neurospora* are homologs of mammalian myxovirus resistance (Mx) proteins which were found to inhibit viral growth. Overall, these data suggest that RNAi machinery is a part of a host defense response against viruses and transposons. *rdrp-3*, which encodes one of the three RdRPs in *Neurospora*, was one of the most highly induced genes, its RNA increasing between 50-300-fold on exposure to dsRNA. This suggests that RdRP-3 may play a role in the response to dsRNA-induced RNAi (Choudhary et al., 2007).

1.2.2.5 qiRNA: a type of sRNA induced by DNA damage

In *Neurospora*, a class of small RNAs known as qiRNA (*qde-2* interacting RNAs) are induced by DNA damage (Lee et al., 2009). The observation that QDE-2 accumulates in *Neurospora* treated with DNA damaging agents led to the discovery of qiRNAs, which are associated with immunoprecipitated QDE-2. Thus, these small RNAs are named as QDE-2 interacting small RNAs. qiRNAs are 20-21 nucleotides long and display a preference at their 5’ end for uracil and at their 3’ end for adeninie. qiRNA are mainly derived from rDNA loci and map to both sense and ant-sense strands. 26s rDNA-specific qiRNA production is induced after DNA damage, but is abolished in *qde-1* and *qde-3* mutants (Lee et al., 2009). These data indicate that QDE-1 and QDE-3 are required for qiRNA biogenesis. Quelling and qiRNA biogenesis share common components, suggesting that both mechanisms operate in a similar way (Zhang et al., 2013). Moreover, the decrease in protein synthesis that usually occurs after DNA damage is not observed in *qde-1* and *qde-3* mutants, suggesting that qiRNAs play a role in response to DNA damage by inhibiting protein synthesis (Lee et al., 2009).
A genetic screen for additional RNAi components identified mutants that fail to accumulate QDE-2 protein after DNA damage (Zhang et al., 2013). Mutants deficient in homologous recombination did not show QDE-2 accumulation, qiRNA or aRNA production, whereas qiRNAs were produced in mutants deficient in DNA repair and checkpoint pathways. This indicates that homologous recombination, rather than DNA repair and checkpoint pathways, is required for qiRNAs biogenesis. Furthermore, qiRNA and aRNA production was abolished in ATP-dependent chromatin remodelling mutants (Zhang et al., 2013). These mutants also exhibited very low or completely abolished homologous recombination when compared to wild-type. This suggests that chromatin remodelling factors involved in homologous recombination, are required for qiRNA production by regulating chromatin status. Additionally, DNA replication also plays a role in qiRNA biogenesis. Inhibition of DNA replication blocks qiRNA production (Zhang et al., 2013). Homologous recombination is also required for quelling. Although both siRNAs and qiRNAs can originate from rRNA loci, qiRNA production occurs in DNA-damage induced conditions while quelling occurs during vegetative growth. It has been suggested that rDNA regions are protected from HR mechanisms under normal growth condition (Zhang et al., 2013).
1.2.3 Meiotic silencing by unpaired DNA (MSUD)

1.2.3.1 MSUD in Neurospora

Sexual reproduction provides organisms with the opportunity to shuffle their genetic material, creating genetic variations that may ensure survival when progeny are exposed to environmental stress. However, it is also enhances propagation of transposable elements. During meiosis, cells are highly recombinogenic. It is essential to employ a defense mechanism against viruses and transposable elements before they are transmitted to the next generation. In Neurospora, fertilisation of two strains with opposite mating types initiates sexual development. Rounds of meiotic and mitotic divisions result in the formation of an ascus containing eight American football-shaped black ascospores. The pattern of progeny indicates their genetic lineage. Hence, Neurospora crassa serves as a valuable model for research in epigenetics and gene silencing by observing morphology, pigmentation and fertility of crosses (Davis, 2000, Shiu et al., 2001).

Neurospora has a haploid genome and only becomes a transient diploid cell during meiosis. Fertilisation is initiated by fusion of a female hypha and a male fertilising element (Springer, 1993). After karyogamy, meiosis occurs inside the diploid nucleus of the zygote. During meiosis, homologous chromosomes align and pair, and any unpaired DNA sequences are sensed by transvection (Aramayo and Metzenberg, 1996). For example, if a gene has an odd copy number, or if two copies of a gene are present but at different locations in each of the parental genomes. The presence of unpaired DNA triggers meiotic silencing of all paired and unpaired homologous copies of the DNA during prophase of meiosis I. MSUD is a PTGS mechanism mediated by small non-coding RNAs (Shiu et al., 2001).

1.2.3.2 Discovery of MSUD

MSUD was previously reported as meiotic transvection by Aramayo and Metzenberg, who studied the ascus-dominant effects of the ascospore maturation-1 (asm-1) mutation (Aramayo and Metzenberg, 1996). asm-1 is a key regulatory protein required for formation of protoperithecia and ascospore maturation. The asm-1 deletion strain is female sterile; it fails to produce protoperithecia, whereas it is fertile if acting as male. Crossing of an asm-1 Δ strain to wild-type should yield four black and four white ascospores (4:4 ratio), which reflects their parental alleles according to Mendelian segregation. However, such a cross (Figure 1.3B) produced almost 100% white and nonviable ascospores. Dissection of individual asci revealed that all eight-spored asci
contained only white and nonviable ascospores, including four that should be genetically *asm-1+. A cross of wild-type x wild-type produced eight black spores (Figure 1.3A) (Aramayo and Metzenberg, 1996).

To explain the ascus-dominant behaviour of *asm-1*, three models were tested by setting up crosses using different constructs. The first model (Figure 1.3C) was designed to test whether a normal dosage of *asm-1* is sufficient for maturation. A cross was made using two strains carrying a normal copy number of *asm-1* but located at nonhomologous positions: one endogenous copy and one ectopic copy at the *his*-3 locus. Ascospore production was expected if one copy of *asm-1* in each nucleus is sufficient to support spore maturation. The *asm-1Δ* is defective in protoperithecium formation. Complementation by an ectopic copy of *asm-1+* restored the wild-type phenotype of protoperithecium formation, but failed to yield mature spores in a cross to the wild-type. This showed that failure of ascospore maturation was due to unpaired copy of *asm-1*, despite an even *al-1* copy number.

The second model (Figure 1.3D) involved a cross of two *asm-1Δ* mutants complemented with an ectopic copy of *asm-1* at the *his*-3 locus, to examine whether the paired *asm-1* is sufficient for ascospore maturation. Ascospore maturation was expected since two copies of *asm-1* are paired at the *his*-3 locus. Indeed, such a cross yielded more than 30% mature black spores and all of them originated from asci with 8 black spores (8:0 ratio). This implies the dominant effect of an *asm-1* cross is due to failure of transvection. The third model (Figure 1.3E) was to test if pairing of the *asm-1* allele is a critical step for later expression of *asm-1*. A cross was made between *asm-1Δ* mutants containing an intact copy of *asm-1* or a structurally similar but non-functional copy of *asm-1* at the *his*-3 locus. These alleles should be paired and develop normally. One parent carried a functional copy of *asm-1* so it was expected to produce four mature ascospores that are genetically *asm-1+. Results showed that four black and four white spores (4:4 ratio) are produced within an ascus. All white progenies originated from the parent with a nonfunctional *asm-1*. Taken together, these observations suggest that paired homologous *asm-1* is required for ascospore maturation and that failure of transvection leads to the ascus-dominant behaviour of *asm-1Δ*. This homology-effects phenomenon is similar to transvection in *Drosophila* so it has been termed meiotic transvection (Aramayo and Metzenberg, 1996).
Figure 1.3 Crosses to test models. A: A cross of wild-type and wild-type. B: A cross of asm-1Δ and wild-type. C: A cross between wild-type and a mutant in which asm-1 is deleted on Linkage Group I but complemented by a copy of Asm-1 at the his-3 locus. D: A cross between two asm-1Δ strains complemented with a functional (Asm-1+) copy of Asm-1 at the his-3 locus. E: A cross between two asm-1Δ strains complemented with either a functional (Asm-1+) or nonfunctional (Asm-1 fs) copy of Asm-1 at the his-3 locus. Adapted from Aramayo and Metzenberg (1996).

Further studies of transvection effects led to the discovery of MSUD. A strain carrying two copies of asm-1, an endogenous copy and an ectopic copy at the his-3 locus, was crossed to a strain carrying a single copy of asm-1. More than 99.5% of the spores were white and nonviable. This confirmed that the unpaired copy of asm-1 induced silencing of all homologous copies, including the paired ones. It has been suggested that MSUD is a single mechanism implicated in both self-silencing and trans-silencing effects (Shiu et al., 2001).

The link between the position-effect phenomenon and RNAi-mediated mechanism was established by isolation of suppressor of ascus dominance (sad-1) (Shiu et al., 2001; Shiu and Metzenberg, 2002). sad-1 was identified by mutagenic screens for mutant strains defective in MSUD when crossed to an asm-1Δ strain. sad-1 suppresses the
dominant behaviour of \textit{asm-t}^4 (Shiu et al., 2001). The predicted SAD-1 protein is a 1,638 amino acid polypeptide containing a basic amino terminus and a glycine-rich carboxyl terminus. \textit{sad-1} encodes a putative RNA dependent RNA polymerase (RdRP), which synthesises double-stranded RNA from single-stranded RNA templates. SAD-1 shares significant homology to RdRPs implicated in other silencing systems, such as SDE in \textit{A. thaliana}, EGO-1 in \textit{C. elegans} and QDE-1 in \textit{N. crassa} (Shiu and Metzenberg, 2002). Moreover, \textit{sad-1} is specifically expressed in the sexual phase. A homozygous cross of \textit{sad-1 x sad-1} is completely barren; abundant perithecia form but no ascospores are produced, indicating that either MSUD or other functions associated with SAD-1 is required for sexual development (Shiu and Metzenberg, 2002).

MSUD is not specific to any particular sequences. Several long-known mutants such as \textit{Roundspore}, \textit{Peak} and \textit{Banana} display normal phenotypes during vegetative growth. In crosses to the wild-type, however, the genes corresponding to these mutants are silenced resulting in abnormal phenotypes. \textit{Roundspore} produces round spores instead of American football shaped spores, \textit{Peak} forms swollen asci containing disordered ascospores, whereas \textit{Banana} is defective in ascospore delimitation and its progeny are detained in a giant banana-shaped ascospore. As expected, \textit{sad-1} mutants also suppress the dominant effects of these mutants (Shiu et al., 2001).

Using reporter genes, it was found that a larger unpaired region or higher degree of homology to the transcripts of unpaired genes will be more efficiently silenced. The unpaired DNA sequence does not need to carry promoter elements to induce silencing. Transcripts produced from unpaired DNA do not need poly(A) tails for downstream events (Lee et al., 2004). The MSUD machinery is sensitive. Among all fragments tested for efficiency of silencing, the largest size is 8859 bp a region accounting for $< 0.09\%$ of Linkage Group 1. This is sufficient to induce efficient silencing (Lee et al., 2004). Some genes are immune to MSUD, such as rRNA repeats and the mating type genes (Shiu and Metzenberg, 2002). It has been shown that unpaired copies of DNA sequences downstream of the \textit{asm-1} reporter gene were silenced during meiosis but there was no effect on expression of the adjacent \textit{asm-1}, suggesting that MSUD only targets the unpaired sequence and does not affect its paired neighbours (Kutil et al., 2003).
1.2.3.3 Molecular mechanism of MSUD

Mutagenic screens have further identified other components of MSUD machinery (Lee et al., 2003; Chang et al., 2012). A paralogue of qde-2, sms-2 is implicated in MSUD. SMS-2 belongs to the Argonaute protein family. The Argonaute protein forms the catalytic core of effector RISC for mRNA degradation. There is a high degree of sequence identity shared between Neurospora SMS-2 and human Argonaute. Complementation by expression of qde-2 in the sms-2 deletion background did not restore the phenotype of crosses homozygous for sms-2. Thus, it is likely that QDE-2 and SMS-2 function in two different RNAi silencing pathways, quelling and MSUD respectively (Lee et al., 2003).

The second suppressor of ascus-dominance (sad-2) was found to suppress meiotic silencing of crosses to Roundspore (Shiu et al., 2006). sad-2 is specifically expressed in the sexual phase and suppresses MSUD in crosses to strains carrying an ectopic copy of actin, b-tubulin or histone H3H4. BLAST results reveal that SAD-2 shares homology with several proteins of unknown function in other species, including C. elegans, D. melanogaster and Kluyveromyces (Shiu et al., 2006). Similar to the sad-1a mutant, the sad-2a mutant showed a normal vegetative phenotype and fertility in heterozygous crosses. However, a homozygous cross of sad-2 x sad-2 was arrested at meiotic prophase. Chromosomes were partially paired in all nuclei resulting in production of a few ascospores. These results demonstrated that sad-2 is likely to play a role in meiotic progression (Shiu et al., 2006).

Green fluorescent protein (GFP) was used to study the localisation of SAD-1 and SAD-2 during meiosis. The SAD-2 GFP fusion protein was not detected during vegetative growth. SAD-2 GFP localised outside of the nucleus and accumulated in the perinuclear region from karyogamy to the diplotene stage. Coexpression of SAD-2 GFP and SAD-1-RFP showed colocalisation of these proteins in perinuclear region. To test the possibility that SAD-1 and SAD-2 proteins are dependent for their functions. SAD-1 GFP was expressed in a sad-2 deletion background, which showed a random distribution of SAD-1 GFP in the cytoplasmic region of asci. In contrast, SAD-2 GFP localised to the perinuclear region in a sad-1 deletion background. This indicates that SAD-2 is required for proper localisation of SAD-1. It has been suggested that SAD-2 travels from cytoplasm to the perinuclear region and recruits SAD-1 to this region (Shiu et al., 2006).
In quelling, two redundant Dicer proteins are required for cleavage of dsRNA. The possibility that these Dicer proteins serve similar functions in MSUD was tested by crossing Dicer mutants with a wild-type strain. dcl-1\textsuperscript{a}, dcl-2\textsuperscript{a} and dcl-1dcl-2 mutants have no obvious abnormal phenotype in vegetative growth. A homozygous cross of dcl-2 x dcl-2 developed normally, whereas a homozygous cross of dcl-1 x dcl-1 is barren. In the latter cross, development of perithecia is arrested at an early stage, resulting in the formation of underdeveloped perithecia and beaks and no obvious ascogenous tissues or croziers. Unlike sexual-specific expression of sad-1 and sad-2, both dcl-1 and dcl-2 are expressed during vegetative and sexual development. As dcl-1 is required for the early stage of sexual development, it is difficult to make homozygous crosses of dcl-1. A possible way to examine the roles of dcl-1 in MSUD is to express a low level of dcl-1 during early development and silence it at a later stage by MSUD. In such a background, dcl-1 suppresses meiotic silencing in a cross to a his-3 +::Histone1-gfp strain in which the unpaired H1-gfp is expressed. dcl-2 does not suppress meiotic silencing of unpaired H1-gfp. DCL-1 and DCL-2 are functionally redundant in quelling, but dcl-1 is required for MSUD, whereas dcl-2 is not (Alexander et al., 2008).

Recently, a high-throughput reverse-genetics approach was developed to identify suppressors of MSUD (Hammond et al., 2011). This alternative method involves culturing tester strains (e.g. asm-1 or roundspore) in a 96-well microtiter plate containing protoperithecia-inducing medium. The protoperithecia are then fertilised using conidia from the Neurospora knockout library and the resulting ascospore phenotypes are examined. The screening of a knockout library is time and cost efficient, but has some disadvantages including the fact that it is not feasible for recessive mutations, and not all crosses produce a sufficient number of spores for visual analysis. Nevertheless, the screen uncovered a dominant suppressor of MSUD, suppressor of ascus dominance-3 (sad-3), encoding a putative helicase containing two helicase domains. A homozygous cross of sad-3 x sad-3 produced normal perithecia and asci, but no ascospores. It is noted that a heterozygous cross of sad-3 and wild-type yields significantly fewer ascospores compared to a homozygous cross of wild-type. This phenotype was not seen in other previously characterised MSUD proteins. Dissection of rosettes showed that asci are aborted and each ascus contains four or fewer ascospores, indicating that sad-3 is required for ascospore development (Hammond et al., 2011). S. pombe Hrr1 is an ortholog of sad-3 (Hammond et al., 2011). Hrr1 protein is required for RNAi-induced heterochromatin formation. RDRC, which consists of
HRR1, RdRP and Cid12, interacts with the effector RITS. RITS contains Ago1 Argonaute and two other proteins. Interaction of an RdRP, a helicase and an Argonaute protein is also observed in MSUD, such as the interactions between SAD-1 and SAD-3 and between SAD-3 and SMS-2. This suggests that MSUD complex formation in *Neurospora* and RNAi-induced heterochromatin formation in *S. pombe* are related processes (Hammond et al., 2011).

Although known components of MSUD are homologs of genes required for quelling and the production of siRNAs, no MSUD-associated small RNAs had been identified until recently. A deep sequencing approach identified a new class of small RNAs in *Neurospora*, MSUD-associated small interfering RNAs (masiRNAs) (Hammond et al., 2013). These masiRNAs are 21-27 nucleotides in length, with the majority being 25 nucleotides. They show preference for A/U at 5' end. Characterisation of masiRNAs did not provide any evidence for secondary sRNA (Hammond et al., 2013).

Two additional SAD proteins were identified recently, named SAD-4 and SAD-5. *sad-4* and *sad-5* are specifically expressed during sexual development but unlike other SAD proteins, they are not essential for sexual development. Both proteins are semidominant MSUD suppressors. MasiRNAs were detected in *sad-4* and *sad-5* deletion strains, indicating that *sad-4* and *sad-5* function upstream of masiRNAs synthesis (Hammond et al., 2013).

In addition, using fluorescent protein fusion constructs, components of MSUD including DCL-1, SMS-2, QIP and SAD-3 were localised to the perinuclear region where SAD-1 and SAD-2 have been shown to be colocalised during meiosis (Hammond et al., 2011; Alexander et al., 2008). As QDE-2 GFP did not exhibit perinuclear localisation, the perinuclear localisation of QIP indicates that QIP associates with SMS-2 not QDE-2 during meiosis. Bimolecular fluorescence complementation analysis demonstrated that SAD-3 physically interacts with SAD-1, SAD-2, SMS-2 and QIP. The perinuclear region is the centre of the RNAi machinery (Hammond et al., 2011). The novel protein SAD-4 colocalises with SMS-2 to the perinuclear region. Unlike other MSUD proteins, SAD-5 is found in the nucleus (Hammond et al., 2013). Taken together, most known components of the MSUD machinery are colocalised to the perinuclear region where they interact and form an RNA processing complex.
A proposed model of MSUD is described in Figure 1.4 (Chang et al., 2012; Hammond et al., 2013). It has been postulated that the unpaired DNA region loops out to form a secondary structure, which can be recognised and generates unpaired DNA specific-aberrant RNA via unknown mechanisms. In the perinuclear region, SAD-1, an RNA dependent RNA polymerase, converts aRNA into double-stranded RNA (dsRNA) with the aid of SAD-2 (which is required for proper localisation of SAD-1) and SAD-3, which may enhance efficiency of dsRNA production. The dsRNA is processed into short duplex RNAs (siRNA) by DCL-1 and then loaded onto RISC containing Argonaute protein SMS-2 and an exonuclease QIP. The SMS-2/QIP-based RISC cleaves and removes the passenger strand from duplex siRNA and is directed by the guide strand to the homologous sequence for mRNA degradation. SAD-4 colocalises with SMS-2, whereas SAD-5 localises to the nucleus.
Figure 1.4 The proposed model of MSUD in *Nuerospora* during sexual development. During meiosis, unpaired DNA leads to the formation of aRNA. SAD-1 converts aRNA into dsRNA, in the presence of SAD-2 and SAD-3. DCL-1 cleaves dsRNA into siRNA which is loaded onto a SMS-2/QIP-based RISC, inducing gene silencing. SAD-4 and SAD-5 localise in the perinuclear and nuclear regions respectively. Modified from Chang et al. (2012).
1.3 Aims and objectives

A BLAST search revealed a putative third RNA-dependent RNA polymerase in *Neurospora*. RdRP-3 contains an RRM domain at the N-terminus of the protein and a RNA-dependent RNA polymerase domain. Moreover, RdRP-3 shares homology with other *Neurospora* RdRPs, QDE-1 and SAD-1 which are key components of quelling and MSUD respectively. The aim of my project is to establish whether RdRP3 plays a role in quelling or/and in MSUD. Specifically the objectives of my project are to,

- carry out phylogenetic analysis of *Neurospora* RdRP proteins and other fungal, plant and animal RdRPs
- assay the quelling and MSUD efficiency of *Neurospora* RdRP mutants
- tag the RdRP-3 with GFP protein for RdRP-3 localisation study
- generate wild-type and *rdrp-3^Δ* strains containing an inducible *albino-1* hairpin construct
2. Materials and Methods

2.1 Strains and growth conditions

Strains wild-type A (FGSC 2489), wild-type a (FGSC 4200), qde-1Δ A (FGSC 11157), sad-1Δ A (FGSC 11152), rdrp-3Δ A (FGSC 11670), asm-1Δ a (FGSC 11730), rspΔ a (FGSC 11449) were used for quelling and MSUD studies. The double mutant rdrp-3Δ, qde-1Δ strain was obtained from a cross of qde-1Δ a and rdrp-3Δ A. The rdrp-3Δ a mating type was created from a cross of rdrp-3Δ A allele (FGSC 11670) with wild-type a (FGSC 4200). To generate strains used for the dsRNA induction experiment, rdrp-3Δ a was crossed to his-3 A (FGSC 6103) to obtain a his-3Δ, rdrp-3Δ strain. Stock strains were stored on agar slants at -20 °C.

All media were prepared with sterile distilled water and autoclaved before use. All Neurospora strains were maintained on minimal slants (2% sucrose, 1x Vogel’s salts (Davis, 2000), 1.5% agar and 50 ng/ml biotin), except his-3– strains, which were supplemented with histidine to a final concentration of 50 mg/ml. Strains were inoculated onto minimal slants and incubated at 30 °C for 2-3 days. For mycelial mat cultures, 1 ml of minimal medium (2% sucrose, 1x Vogel’s salts and 50 ng/ml biotin) was added to 5-10 day-old slants and vortexed several times. The conidial suspensions were inoculated into petri dishes containing 30 ml of minimal medium. Cultures were incubated at 30 °C for 1-2 days. For the dsRNA induction experiment, petri dishes containing 30 ml of 2% glucose medium (2% glucose, 0.17% arginine, 1x Vogel’s salt and 50 ng/ml biotin) were inoculated using conidial suspensions from 5-10 day-old slants. Cultures were incubated at 25 °C in the dark for 1 day. A disc (3 mm in diameter) was cut out from mycelial mats and inoculated to 100 ml liquid medium (0.1% glucose, 0.17% arginine, 1x Vogel’s salts and 50 ng/ml biotin). Cultures were grown at 25 °C in the dark for 1 day and dsRNA expression induced by the addition of 0.01M quinic acid (QA) (pH 5.8). Tissue was harvested in the dark at different time points by vacuum filtration to remove medium and freezing in liquid nitrogen.

2.2 E. coli and yeast strains

E. coli cells (XL1 Blue) were cultured in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) containing appropriate antibiotics (ampicillin or kanamycin at 100 µg/ml) at 37 °C with shaking at 250 rpm. Yeast cells (S. cerevisiae FY834) were cultured on YPD plates (1% yeast extract, 2% peptone, 2% dextrose and 1.6% agar) or in YPD broth (1% yeast extract, 2% peptone, 2% dextrose) at 30 °C.
2.3 Plasmid DNA preparation

*E. coli* cultures were grown in LB broth supplemented with ampicillin or kanamycin (100 µg/ml) for 12-16 hours at 37 °C with shaking at 250 rpm. Cells were centrifuged at 3,500 rpm for 3 min and the supernatant was removed. Cell pellets were resuspended in 200 µl STEP buffer (8% sucrose, 0.5% triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris pH 8.0) and mixed by vortexing. 20 µl of fresh lysozyme (10 mg/ml) was added to lyse the cells. The mixture was boiled in a water bath for 40 s to denature DNases and precipitate proteins and chromosomal DNA. The white precipitate was removed after centrifugation at 14,000 rpm for 10 min. 200 µl of isopropanol was added to the supernatant at room temperature and mixed by inversion. The sample was centrifuged at 14,000 rpm for 5 min and the supernatant was discarded. The DNA pellet was washed in 70% ethanol and centrifuged again at 14,000 rpm for 5 min. After the supernatant was removed, the DNA pellet was air-dried and resuspended in 50 µl sterile water. Alternatively, plasmids used for *Neurospora* transformations were purified using the QIAprep Spin Miniprep Kit (QIAGEN) according to the manufacturer’s instructions.

2.4 Crosses

Conidia were inoculated into Westergaard’s slants (1x Westergaard’s salts, 2% sucrose, 50 ng/ml biotin and 1.5% agar, pH 6.5) to induce the production of protoperithecia, the female sexual structures. Cultures were grown at room temperature in the dark. Protoperithecia usually formed after 7-10 days. Conidia of the opposite mating type were inoculated onto the lawn protoperithecia using conidial suspensions. Cultures were kept at room temperature in the dark for 7 days and transferred to the light for a further 7-10 days. The mature perithecia shoot ascospores onto the walls of the tubes. The ascospores were collected using an inoculation loop dipped in 20% bleach and spread onto a 4% agar plate. Individual ascospores were isolated and transferred to minimal slants. To induce germination, ascospores were heat-shocked in a 60 °C water bath for 30 min and then incubated at 30°C for 3-5 days.

2.5 MSUD assays

Strains to act as females in a cross were inoculated onto petri dishes containing approximately 30 ml of Westergaard’s crossing medium (1x Westergaard’s salt, 2% sucrose, 50 ng/ml biotin and 1.5% agar, pH 6.5). The *asm-1* or *rsp* testers were crossed to the female strains as described in section 2.3. After fertilisation, cultures were grown in the dark at room temperature for 7 days and transferred to the light for a
further 10-25 days. Ascospores were collected at days 16, 19, 25 and 30 from petri dish lids with 1 ml of sterile water. New lids were placed over the dishes after each harvest. Ascospores were centrifuged and resuspended in a small volume of water and vortexed briefly to obtain a homogeneous suspension. 20 µl of suspension was applied to glass microscope slides for imaging. Five fields of view were captured on a Leica M165 FC microscope and counting of ascospores was performed using ImageJ. More than 500 ascospores were counted from each cross.

2.6 Isolation of microconidia
Slants (0.1 x Westergaard's salts, 0.5% sucrose and 2% agar) were autoclaved and cooled to 55 °C before addition of iodoacetic acid sodium salt (1 mM final concentration) to make microconidia-inducing slants. For each strain, conidia were inoculated onto a microconidia-inducing slant and incubated at room temperature in the dark. After 7 days, 2 ml of sterile water was added to each slant and vortexed several times. Conidial suspensions were filtered through Millipore 5 µm filters and plated out on sorbose minimal plates (1X Vogel’s salt, 0.05% glucose, 0.05% fructose, 2% sorbose, 1.5% agar and 50 ng/ml biotin). The plates were incubated at 30 °C for 3-5 days in the dark. Putative homokaryons were obtained by isolating individual colonies and inoculating these onto minimal slants.

2.7 Genomic DNA preparation
Mycelial mats were harvested (see section 2.1) and ground to a powder under liquid nitrogen using a mortar and pestle. Ground tissue was placed in 1.5 ml microcentrifuge tubes up to the level of 400 µl and 400 µl of 2x CTAB solution (100 mM Tris-HCl, 2% CTAB, 1.4 M NaCl, 20 mM Sodium EDTA, 1% sodium bisulphite) was added to each sample and mixed by inversion. Samples were incubated at 60°C for 30 min and inverted occasionally. 400 µl of 24:1 chloroform:isoamyl alcohol was added and the samples were mixed by inversion and incubated at room temperature for 15 min. After centrifuging at 2,000 rpm for 10 min the top aqueous layer was transferred to a fresh tube and the chloroform:isoamyl extraction step was repeated. 1 µl of RNase A (10 mg/ml) was added to the aqueous layer and incubated at 37 °C for 1 hour. To precipitate DNA, an equal volume of 100% isopropanol was added to the solution and inverted several times. Centrifugation was carried out at room temperature for 5 min and the supernatant was removed. The DNA pellet was washed in 70% ethanol, centrifuged again and the pellet was air-dried and resuspended in 100 µl sterile water.
2.8 Polymerase chain reaction (PCR)
A typical PCR reaction mix contained 1 µl of DNA template (unknown concentration), 1 µl each of forward and reverse primers (10 pmol/µl), 1 µl of dNTP mix (10 mM each), 5 µl of 10x NH₄ Reaction Buffer, 1 µl MgCl₂ (50 mM), 0.5 µl of BIOTAQ DNA Polymerase (5U/µl, Bioline) and distilled water to a final reaction volume of 50 µl. To amplify fragments used in cloning, 1 unit of VELOCITY DNA Polymerase (Bioline) was used instead of BIOTAQ. VELOCITY DNA Polymerase has 3' to 5' exonuclease proofreading activity, thus reducing the chance of introducing mutations into PCR products during amplification. PCR was performed using a TECHNE TC-512 machine, typically programmed as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at the appropriate Tₘ (detailed in Table 2.1) for 1 min and extension at 72°C for 1 min/kb of expected product, and final extension at 72°C for 10 min. PCR products were separated by electrophoresis through 0.8% 1 x TAE (0.04 M Tris-acetate, 0.001 M EDTA) agarose gels containing 1 µl of ethidium bromide (10µl/ml) and visualised under UV light on a transilluminator.

2.9 Southern blot analysis
*Neurospora* genomic DNA was extracted as described previously in section 2.7 and cleaned up by phenol/chloroform extraction. Briefly, DNA was resuspended in 400 µl of sterile distilled water and mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The mixture was inverted repeatedly for 10 min and spun at 2,000 rpm for 10 min. After centrifugation, the aqueous layer was carefully transferred to a fresh tube. 1/10th volume of 3 M sodium acetate pH 5.2, 2 ½ volumes of 100% ethanol and 1/100th volume of glycogen were added to precipitate DNA. After incubation at –20°C for 3 hours, the mixture was centrifuged at 14,000 rpm for 30 min at 4°C. The supernatant was discarded and the DNA pellet was washed in 70% ethanol. Centrifugation was then carried out at 14,000 rpm for 15 min at 4°C. The supernatant was discarded and the DNA pellet was resuspended in 100 µl sterile distilled water. Approximately 20 µg of genomic DNA was digested with 1 µl of *HindIII* (Fermentas) or *XbaI* (Roche) in 100 µl reactions. Restriction digestion was carried out at 37°C for 16-18 hours and the enzymes heat-inactivated by incubation at 80°C for 20 min. Approximately 5 µg of digested genomic DNA was separated through a 0.8% TAE agarose gel, transferred to Hybond N*+* membranes (GE Healthcare) by capillary transfer in 20x SSC according to the manufacturer's protocol, and fixed by UV cross-linking (Spectrolinker XL-1500). Probes were prepared by PCR amplification of a 1432 bp fragment of *hygromycin phosphotransferase (hph)* (5' primer-
GATTTCAGTAACGTAAAGTGG, 3’ primer-AGAAGATGATATTGAAGGAGC) from pCSN44. 25 ng of the *hph* PCR product was labelled with [α-\(^{32}\)P] dCTP (3000 Ci/mmol) using the Random Primed DNA Labelling Kit (Roche) following the manufacturer’s instructions. Prehybridisation of the blot was performed at 42 °C for 30 min in Ultrasensitive Hybridisation Buffer (Ambion). Probes were denatured before addition to the hybridisation buffer and hybridisation was carried out at 42 °C overnight. The membrane was washed twice in 2 x SSC, 0.1% SDS at 42 °C for 5 min and twice in 0.1 x SSC, 0.1% SDS at 42 °C for 15 min. X-ray film (Kodak Biomax MR Film) was exposed to the membrane for detection of radiolabel.

2.10 Transformation of *Neurospora*

Conidia from 15 day-old cultures were harvested in 50 ml of 1 M sorbitol. Suspensions were centrifuged at 3000 rpm for 5 min and the supernatant discarded. The pellet was resuspended and washed twice in 30 ml of 1 M sorbitol. Finally, the washed pellet was resuspended in 5 ml 1 M sorbitol and kept on ice. Conidial suspensions were counted using a haemocytometer and adjusted to a final concentration of 2.5 x 10^9 conidia/ml in 1 M sorbitol. For each electroporation reaction, 100 µl of conidial suspension was mixed with 300 ng of linearised plasmid DNA in a microcentrifuge tube and incubated on ice for 5 min. The mixture was then transferred to an ice cold 0.2 cm electroporation cuvette. Electroporation was performed with the following settings: 2.5 kV/cm voltage gradient, 25 µF capacitance, 400 ohms resistance (Biorad Gene Pulser Excel). After electroporation, the conidia/DNA mixture was resuspended in 1 ml of 1M sorbitol and spread on sorbose minimal plates (100 µl/plate) (1X Vogel’s salts, 0.05% glucose, 0.05% fructose, 2% sorbose, 1.5% agar and 50 ng/ml biotin). For selection of transformants on hygromycin-containing plates, conidial suspensions were added to 15 ml of warm molten top agar (1X Vogel’s salts, 0.05% glucose, 0.05% fructose, 2% sorbose, 1.5% agar and 50 ng/ml biotin), mixed and then poured on top of sorbose plates containing hygromycin (300 µg/ml). Plates were placed at 30 °C in the dark for 3-5 days. Colonies were picked and transferred to minimal slants. After verification of transformation by diagnostic PCR (section 2.7 and 2.8), strains were grown on microconidia-inducing medium to obtain homokaryons (section 2.6).

2.11 Quelling assay

Quelling was assayed by cotransformation of *Neurospora* conidia with a 1:10 ratio of DNA molecules conferring resistance to benomyl and DNA containing 1136 bp of the *albino-1* gene. Transformants were selected on benomyl-containing medium. Benomyl
resistant colonies were picked and transferred to minimal slants. Phenotypes of transformants were scored as white/yellow (quelled/partially quelled) or orange (non-quelled) by visual observation.

Briefly, conidial suspensions were prepared as described in section 2.10. pSV50 DNA, containing a gene conferring resistance to benomyl, was extracted from overnight cultures of *E. coli* using the QIAprep Miniprep kit (QIAGEN) according to the manufacturer’s protocol. pSV50 was linearised with *Bgl*II (Roche). A fragment of *al-1* was amplified by PCR and primers are detailed in Table 2.1. PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN). The linearised pSV50 DNA was mixed with *al-1* PCR products in a molecular ratio of 1:8. Approximately 300 ng of pSV50 DNA and 300 ng of *al-1* fragment from the mixture were co-transformed into *Neurospora* strains by eletroporation. Conidial suspensions were plated in warm molten top agar onto sorbose plates containing benomyl (0.5 µg/ml) and incubated at 30 °C in the dark for 3-5 days. Colonies were transferred to minimal slants and incubated at 30 °C for 3 days before scoring for colour.

2.12 Generation of *rdrp-3-gfp* tagged strains

The strategy used for *gfp* tagging was described by Honda and Selker (2009). Primers were designed to allow amplification of the DNA immediately 5’ and 3’ of the end of the RdRP3 ORF i.e. position 3133 bp to the position immediately before the stop codon and from the position immediately after the stop codon to position 522 bp downstream of *rdrp-3* ORF. Primers are detailed in table 2.1. 29 nucleotides of 5' flank F/3'flank R and 5' flank R/3'flank F primers homologous to the yeast vector pRS426 and the *gfp* fragment respectively. The *gfp* fragment and *hph* gene were excised from pGFP::*hph::loxP with *Kpn*I and *Xho*I (Honda and Selker, 2009). The yeast vector pRS426, which contains a URA3 marker, was linearised by *Eco*RI. Recombination of homologous sequences in all 4 fragments in yeast FY834 results in a plasmid containing 1,000 nt homologous to the *rdrp-3* ORF and 500 nt homologous to the region downstream of the *rdrp-3* ORF. *gfp* is located between these two homologous regions and expression is driven from the *rdrp-3* locus in *Neurospora*.

Yeast transformation and DNA preparation were carried out according to published procedures by Parker et al (2005). A single colony of the FY834 strain was inoculated into 5 ml of YPD broth (1% yeast extract, 2% peptone, 2% dextrose) and grown at 30 °C overnight with shaking at 200 rpm. The absorbance of cultures was measured at a
wavelength of 600 nm. 2 ml of the cell suspension was inoculated into 50 ml of fresh YPD broth and grown at 30 ºC until the OD_{600} doubled. Cells were centrifuged at 3500 rpm for 5 min and washed in 25 ml of sterile water and centrifuged again. The supernatant was removed and the cell pellet resuspended in 1 ml of 100 mM lithium acetate. The cell suspension was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 15 s. The supernatant was discarded and cells were resuspended in 400 µl of 100 mM lithium acetate. 50 µl aliquots of cell suspension were prepared for each transformation reaction. Cells were centrifuged at 14,000 rpm for 15 s and the supernatant was removed. The following reagents were added to the cells in the stated order: 240 µl of 50% PEG 3350, 36 µl of 1 M lithium acetate, 50 µl of boiled sheared salmon sperm DNA, 26 µl of sterile water, 2 µl of linearised pRS426 (100 ng/µl), 2 µl of GFP fragment (100 ng/µl), 2 µl of each 5’ and 3’ flank rdrp-3 PCR products. After vortexing, cells were suspended in the transformation mix and then incubated at 30 ºC for 30 min. The mixture was inverted twice and heat-shocked at 42 ºC for 1 hour. After centrifugation at 14,000 rpm for 15 s, the supernatant was removed. Cells were resuspended in 150 µl of YPD medium and 75 µl of cell suspension was plated out onto a SC-Ura plate (2.69% drop-out base with glucose, 0.5% drop-out mix minus Ura and 1.5% agar). Plates were incubated at 30ºC for 3 days.

Yeast plasmid DNA was extracted from cells by the ‘smash and grab’ DNA prep method (Parker et al. 2005). 1 or 2 ml YPD broth was pipetted to the transformation plate and colonies were scraped off with one end of a glass slide. Cell suspensions were transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 15 s. The excess supernatant was removed to obtain a final volume of 50-75 µl and this was mixed with 0.2 ml lysis buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 1 mM EDTA and 10 mM Tris, pH 8.0), 0.2 ml phenol/chloroform/isoamyl alcohol (25:24:1) and 0.3 g glass beads (0.5 mm). The mixture was vortexed for 2 min and centrifuged at 14,000 rpm for 10 min. 1/10th volume of 3 M sodium acetate pH 5.2, 2 ½ volumes of 100% ethanol were added to 100 µl of supernatant in a fresh tube. This was then centrifuged at 14,000 rpm for 5 min and the supernatant removed. The remaining DNA pellet was washed with 70% ethanol, centrifuged and aspirated. The DNA pellet was air-dried and resuspended in 50 µl of sterile water. The rdrp-3-gfp construct was verified by PCR before electroporation into XL-1 Blue competent cells (Stratagene), and subsequently used to transform Neurospora strains (section 2.10).
2.13 Confocal microscopy
A loop-full of conidia from 5-7 day old cultures was suspended in 200 μl of 2% sucrose liquid minimal medium. 20 μl of suspension was applied to microscope slides, a coverslip placed on top, and excess medium removed with filter paper. Slides were mounted with a drop of ProLong Antifade Reagent (Life Technologies) and placed in the dark overnight. Confocal microscope (Olympus FluoView FV1000) was used for imaging at magnification x 40.

2.14 Generation of strains containing the \textit{al-1} hairpin
Following the design of (Choudhary et al., 2007) an inducible \textit{al-1} hairpin construct was made as described below. The pCH10 vector (a kind gift from Dr Christian Heintzen, University of Manchester) contains the inducible \textit{qa-2} promoter located between \textit{NotI} and \textit{BamHI} cloning sites in the \textit{his}-targeting plasmid pBM60 background. A plasmid containing inverted repeats of \textit{al-1} fragments was constructed by cloning the \textit{al-1} ORF from position 1322 to 1942 bp and 1412 to 1942 bp and ligating to a linearised pCH10 vector (\textit{BamHI}/\textit{EcoRI}) in opposite orientations. The \textit{al-1} forward fragment and reverse fragment were amplified by primers \textit{al-1 HindIII a} and \textit{al-1 BamHI}, \textit{al-1 HindIII b} and \textit{al-1 EcoRI} (detailed in Table 2.1) and restriction sites are included in these primers for cloning uses. PCR products were purified using the QIAquick PCR purification kit (Roche). The PCR products of \textit{al-1} forward fragment and reverse fragment were digested by \textit{HindIII}/\textit{BamHI} and \textit{HindIII}/\textit{EcoRI} respectively and enzymes were removed using QIAquick PCR purification kit (Roche). pCH10 vector was linearised by \textit{BamHI}/\textit{EcoRI}, followed by Antarctic Phosphatase (Biolab) treatment at 37 °C for 30 min and heat inactivation at 70°C for 5 min. A 1:10 molecular ratio of vector to insert was used for three-way ligation. Ligation was performed with 1 μl T4 DNA ligase (Roche) in a final volume of 10 μl at 4 °C for 16 hours. 8 μl of ligation reaction was transformed to XL-1 Blue competent cells (Stratagene) following the manufacturer’s instructions. After verification by diagnostic restriction digestion and DNA sequencing, plasmid DNA was linearised by \textit{NdeI} and targeted to the \textit{his-3} locus of \textit{wild-type} and \textit{rdrp-3}Δ strains by electroporation. Homokaryons of WT\textit{al-1} hp (microconidia isolate 6-3) and \textit{rdrp-3}\textsuperscript{al-1} hp (microconidia isolate 4.5) strains were used for further study. To generate inducible \textit{rdrp-3-gfp}-tagged strains, the linearised \textit{rdrp-3-gfp} construct was electroporated into WT\textit{al-1} hp strain and transformants selected on hygromycin-containing plates (section 2.10).
2.15 RNA analysis
Total RNA was extracted from 100 mg of ground tissue using the RNeasy plant mini kit (QIAGEN) and treated with DNase I (BioLab) at 37°C for 1 hour. Approximately 2 µg was reverse transcribed into cDNA using Oligo-dT primers (RevertAid H Minus First Strand cDNA synthesis Kit, Thermo Scientific). Reverse transcription was carried out at 42°C for 1 hour and the reverse transcriptase inactivated at 70°C for 5 min. RT-PCR was performed following the general PCR protocol as described previously in section 2.8. Primers used to amplify al-1, rdrp-3 and NCU07036 are listed in table 2.1 and levels of the RT-PCR products were normalised against actin.

2.16 Phylogenetic analysis
RdRP protein sequences were retrieved from a number of different databases including NCBI, UniProt and The Broad institute. The RdRP conserved domain of proteins was identified at the Pfam website (http://pfam.sanger.ac.uk/). Multiple sequence alignments of protein sequences were performed using ClustalX2. The phylogenetic trees were constructed by the neighbor-joining method and bootstrap values were estimated on 1,000 replicates at the iTOL website (http://itol.embl.de/index.shtml).
Table 2.1 PCR primers used in all experiments.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
<th>Annealing temperature (°C)</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>gfp tag (rdrp-3 5’ flank)</strong></td>
<td>GAGGTCGACGG TATCGATAAAGCT TGATATGTCCA TCGGGTGTTTC</td>
<td>CCTCCGCCTCCGC CTCCGCAGCCTCC GCATGCCCTTGTA AAATCTGCA</td>
<td>70</td>
<td>1042</td>
</tr>
<tr>
<td></td>
<td>TGCTATACGAAG TTATGGATCCGA GCTCGGATTCTG CGGACATTGAAG G</td>
<td>ACCCGCGTGGGC CGGCTCTAGA AATTGAGCAT ACACCTCA</td>
<td>60</td>
<td>522</td>
</tr>
<tr>
<td><strong>al-1 forward fragment</strong></td>
<td>CCCAAGCTTTTAC TCTCGTCTCAA AGCCCGT (HindIII b)</td>
<td>CCGGAATCCCAC CAACGCCACCAACA AAG (EcoRI)</td>
<td>70</td>
<td>530</td>
</tr>
<tr>
<td><strong>al-1 reverse fragment</strong></td>
<td>CCCAAGCTTTCT GCAGCCTCCTC CCGACCAGC (HindIII a)</td>
<td>CCGGGATCCCCCA TCAACGCCACCAAC AAG (BamHI)</td>
<td>70</td>
<td>620</td>
</tr>
<tr>
<td><strong>al-1 fragment for quelling assay</strong></td>
<td>AAAGTAGGGCGTT ATCCATGC</td>
<td>AGACCAGCTTGC ACACG</td>
<td>56</td>
<td>1136</td>
</tr>
<tr>
<td>NCU07036</td>
<td>CAACAAGTGATGG TAGTGGATGG</td>
<td>TATCCTCCGATTGA GGCACG</td>
<td>55</td>
<td>100</td>
</tr>
<tr>
<td><strong>qde-1</strong> strains</td>
<td>CAGCGTATGAGA TCCTGAAC</td>
<td>GGTGACCGAATTT GCGATG</td>
<td>58</td>
<td>1850</td>
</tr>
<tr>
<td><strong>rdrp-3</strong> strains</td>
<td>TGGTCCCTTTCT GCCTTGTC</td>
<td>GGTGACCGAATT TCGATG</td>
<td>58</td>
<td>1350</td>
</tr>
<tr>
<td><strong>his-3</strong> strains</td>
<td>CCAAGTGGGAA GAAGGAT</td>
<td>TGATGCTCATTGA ATGAAG</td>
<td>59</td>
<td>2707</td>
</tr>
<tr>
<td><strong>al-1, his-3</strong> strains</td>
<td>CCAAGTGGGAA GAAGGAT</td>
<td>ACAGAGTAGGTA GAAATGGAG</td>
<td>59</td>
<td>1810</td>
</tr>
<tr>
<td><strong>hph probe</strong></td>
<td>AGAAGTATGATAT TGAAGGAGC</td>
<td>GATTTCAAGTAACGT TAAGTGG</td>
<td>50</td>
<td>1432</td>
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<tr>
<td><strong>rdrp-3 gfp</strong> tagged strains</td>
<td>TGACATGGAAGA GCAGTACC</td>
<td>GCTGAACCTTTGCG GGTGTA</td>
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<td>1315</td>
</tr>
<tr>
<td><strong>rdrp-3</strong> strains</td>
<td>TGACATGGAAGA GCAGTACC</td>
<td>CGGCTCTTGTTTA CT</td>
<td>60</td>
<td>1537</td>
</tr>
<tr>
<td><strong>actin</strong></td>
<td>GGCTGGGCCTG ATCTTACCAGCT A</td>
<td>GAGACGAGCAGCGA GAATGGACC</td>
<td>61</td>
<td>510</td>
</tr>
</tbody>
</table>

Primer sequences (5’ to 3’) are detailed in this table, along with the annealing temperature used for each primer pair and the expected size of the PCR products.
3. RESULTS

3.1 Phylogenetic Analysis of RdRP proteins
The RNAi machinery has been extensively studied for years. Some of its components are well characterised in classic models such as *N. crassa*, *C. elegans* and *A. thaliana* (Wassenegger and Krczal, 2006; Chang et al., 2012). The *N. crassa* RNAi machinery is the best characterised of the fungal kingdom (Chang et al., 2012). One objective of my project is to carry out phylogenetic analysis of RdRP proteins. The goal of this is to gain clues to the possible function(s) of RdRP-3 based on similarity to other phylogenetically related RdRP proteins and the presence of additional shared conserved domains. If RdRP-3 is closely related to any well studied RdRP proteins, the existing knowledge would be useful for functional analysis of RdRP-3.

BLAST results revealed *rdrp*-3 is predicted to encode a polypeptide of 1352 amino acids containing an RNA-dependent RNA polymerase domain. This domain is a characteristic of the eukaryotic RdRP family of proteins and is located in RdRP-3 between amino acid 422 and 1022. RdRP-3 shares 30% and 27% sequence identity with SAD-1 and QDE-1 respectively, and also shows high homology with RdRPs in other filamentous fungi. In addition to the RdRP domain, an RNA recognition motif (RRM) is located at the N-terminus of the predicted RdRP-3 protein. RRMs bind to single-stranded RNAs and are often associated with post-transcriptional gene regulation. This domain is also present in SAD-1 but not in QDE-1 (Figure 3.1).

![Domain architecture of the RdRP-3, SAD-1 and QDE-1 proteins.](image)

*Figure 3.1 Domain architecture of the RdRP-3, SAD-1 and QDE-1 proteins.* The RdRP domains are represented as boxes. The RRM domain is shown as a circle.
Two phylogenetic trees were constructed by the neighbour-joining method from multiple sequence alignments of full length of RdRP sequences (Figure 3.2) and RdRP domain alone (Figure 3.3). First, a BLAST search was performed using the RdRP domain of RdRP-3 and available information from the literature (Nakayashiki et al., 2006, Wassenegger and Krczal, 2006, Zong et al., 2009). A total of 55 protein sequences containing putative RdRP domains were retrieved from databases and aligned using the full-length of each protein or the RdRP domain alone. Most fungal RdRPs have not been annotated. In this analysis they have been assigned as “RdRP” followed by a letter. The phylogenetic tree using full-length RdRP proteins clearly shows that RdRP proteins are divided into three major groups. RdRP proteins from plants, fungi and animals are clustered separately from each other, suggesting that these RdRP proteins evolved from a common ancestor. In Neurospora, RdRPs are derived from an ancestral rdrp gene that diversified into distinct major groups. RdRPs from plants and animals were found in the QDE-1 and SAD-1 groups. In contrast, all members of the RdRP-3 group are from the fungal kingdom, suggesting that either the RdRP-3 group is a fungi-specific lineage arising after the divergence of plant and animal RdRPs or that other RdRP-3 like genes have not yet been identified. Interestingly, A. oryzae QDE-1 is more closely related to N. crassa RdRP-3 than to N. crassa QDE-1. Furthermore, A. thaliana RDR1, RDR2 and RDR6 and C. elegans RdRPs are clustered in N. crassa SAD-1 group, while RDR3, RDR4 and RDR5 are clustered in N. crassa QDE-1 group. However, due to a limited number of input sequences for some represented kingdoms used in this analysis, it is not possible to draw any further conclusions on the evolutionary relationships of RdRP proteins.

All major groups have some members that contain an RRM domain. This distribution of RRM s in RdRP proteins suggests that the domain was present in the common ancestor but has been lost in some lineages. Due to the structural variability of this RRM domain, it is difficult to identify in protein sequences (Maris et al., 2005). Mucor circinelloides RdRP-1 and RdRP-2 are clustered together in one subgroup, but only RdRP-2 contains an RRM. This suggests that these genes arose from a duplication of one M. circinelloides rdrp gene in M. circinelloides and that the RRM was later lost from RdRP-1.

The phylogenetic tree constructed using RdRP domains is similar to the one constructed from full-length proteins. However, in this phylogeny, A. thaliana RDR1, RDR2 and RDR6 and C. elegans RdRPs are separate from the QDE-1, SAD-1 or
RdRP-3 groups. Moreover, the RdRP domains of *N. crassa* QDE-1 and SAD-1 are more closely related to each other than they are to that of RdRP-3.
Figure 3.2 Phylogenetic tree of *Neurospora* RdRP proteins and other fungal, plant and animal RdRPs. The tree was constructed from sequence alignment of full-length RdRP proteins created by ClustalX2. The number at nodes represents values for 1000 bootstrap replicates. Asterisks indicate RdRP proteins containing an RRM domain.
Figure 3.3 Phylogenetic tree of Neurospora RdRP proteins and other fungal, plant and animal RdRPs. The tree was constructed from sequence alignment of RdRP conserved domain created by ClustalX2. The number at nodes represents values for 1000 bootstrap replicates.
3.2 Silencing efficiency of RdRP deletion strains in quelling and MSUD

3.2.1 Verification of RdRP deletion strains and tester \textit{asm-1} and \textit{rsp} deletion strains by Southern blot analysis

Before embarking on silencing assays the identities of the \textit{qde-1\textsuperscript{A}}, \textit{sad-1\textsuperscript{A}}, \textit{rdpr-3\textsuperscript{A}}, \textit{asm-1\textsuperscript{A}} and \textit{rsp\textsuperscript{A}} deletion strains were confirmed by Southern blot analysis. Generation of these deletion strains involves replacing each gene with a \textit{hygromycin B phosphotransferase (hph)} cassette, which confers resistance to the antibiotic hygromycin. Although the strains grow on medium containing hygromycin, indicating that \textit{hph} has been inserted into the genome, there is still a possibility that the \textit{hph} cassette integrated ectopically instead of replacing the gene of interest. These deletion strains would be used in quelling and MSUD assays, so the first objective of my work was to verify deletion strains by Southern blot analysis. If a gene of interests is replaced by the \textit{hph} cassette, restriction digestion of genomic DNA results in fragments with specific sizes at the locus of the gene. The \textit{hph} specific probe hybridizes to fragments specific to the \textit{hph} gene. The size of the detected fragments would tell us that whether the gene of interest was knocked out in these strains.

For Southern blot analysis, genomic DNA of \textit{qde-1\textsuperscript{A}}, \textit{sad-1\textsuperscript{A}}, \textit{rdpr-3\textsuperscript{A}}, \textit{asm-1\textsuperscript{A}} and \textit{rsp\textsuperscript{A}} deletion strains was extracted from mycelial mats. The \textit{qde-1\textsuperscript{A}}, \textit{sad-1\textsuperscript{A}}, \textit{asm-1\textsuperscript{A}} and \textit{rsp\textsuperscript{A}} DNA was digested with \textit{HindIII}, while \textit{rdpr-3\textsuperscript{A}} DNA was digested with \textit{XbaI}. The wild-type strain, which does not contain the \textit{hph} gene, was digested with \textit{HindIII} and \textit{XbaI}. All deletion strains are expected to be identified by a \textit{hph}-specific radiolabelled probe. The expected fragment sizes from \textit{qde-1\textsuperscript{A}}, \textit{sad-1\textsuperscript{A}} and \textit{rdpr-3\textsuperscript{A}} deletion strains are 7,327 bp, 5,040 bp and 5,053 bp (Figure 3.4). As expected, no bands were observed in the wild-type, whereas the \textit{hph}-specific radiolabeled probe hybridised to fragments of approximately 7.5 kb, 5 kb and 5 kb in the \textit{qde-1\textsuperscript{A}}, \textit{sad-1\textsuperscript{A}} and \textit{rdpr-3\textsuperscript{A}} deletion strains respectively.

The expected size of fragments in \textit{asm-1\textsuperscript{A}} and \textit{rsp\textsuperscript{A}} deletion strains is 11576 bp and 4666 bp. Figure 3.4 shows that a band of correct size was observed in \textit{asm-1\textsuperscript{A}} and \textit{rsp\textsuperscript{A}} deletion strains. However, it should be noted that an additional fragment of approximately 1.8 kb was detected in the \textit{asm-1} deletion strain. This suggests that an extra copy of \textit{hph} ectopically integrated into the genome. In summary, Southern blot analysis confirms that the \textit{qde-1}, \textit{sad-1}, \textit{rdpr-3}, \textit{asm-1} and \textit{rsp} genes were deleted in the respective mutant strains.
Figure 3.4 Southern blot analysis of genomic DNA extracted from RdRP deletion strains and MSUD testers. A. Schematic diagrams of the qde-1, sad-1 and rdrp-3 loci in wild-type and deletion strains. The expected sizes of the probe hybridised fragments in qde-1, sad-1, rdrp-3 deletion strains are 7,327 bp, 5,040 bp and 5,053 bp. B. Southern blot analysis of qde-1, sad-1, rdrp-3 deletion strains. C. Schematic diagrams of the asm-1 and rsp loci in the wild-type and deletion strains. The expected sizes of asm-1 and rsp deletion strains are 11,576 bp and 4,666 bp. D. Southern blot analysis of asm-1 and rsp deletion strains. Genomic DNA was extracted from mycelial mats cultured in liquid medium at 30 °C for 2 days. The qde-1Δ, sad-1Δ, asm-1Δ and rspΔ DNA was digested with HindIII, rdrp-3Δ DNA was digested with XbaI. After separation on a 0.8% agarose gel, the DNA was transferred and fixed onto nylon membranes. Southern blots were probed for hphygromycin phosphotransferase gene (hph). The hybridisation probe was a PCR amplified fragment of hph from pCSN44 plasmid.
3.2.2 Determining the quelling efficiency of wild-type, \textit{rdrp-3}\textdagger and \textit{qde-1}\textdagger strains

Quelling can be induced by the introduction either of transgenes or dsRNA (Goldoni et al., 2004). A threshold of transgenes is required to induce efficient quelling in a QDE-1-dependent manner. Through recombination, the transgene is inserted multiple times within the genome which can cause genome instability. Evidence suggests that as a consequence multiple inserts an aberrant DNA structure similar to what is observed when the DNA is damaged is formed which is recognised by the homologous recombination machinery as well as proteins involved in quelling (Zhang et al., 2013). QDE-1 is recruited to the repetitive loci to generate aRNA from the repetitive DNA and then converts the aRNA to dsRNA. On the other hand, QDE-1 is no longer required for quelling when expressing \textit{al-1} hairpin dsRNA in \textit{Neurospora} (Goldoni et al., 2004). The hairpin dsRNA is transcribed from an inverted repeat of \textit{al-1}. The expression of hairpin dsRNA bypasses the QDE-1 to activate the downstream events of quelling. This is supported by the evidence that efficiency of \textit{al-1} hairpin-induced quelling was similar in the wild-type and \textit{qde-1}\textdagger strain.

To determine if RdRP-3 is involved in quelling, the efficiency at which the \textit{rdrp-3}\textdagger strain quells the expression of \textit{albino-1} was assayed according to a protocol previously published (Lee et al., 2010). The wild-type, \textit{qde-1}\textdagger, and \textit{rdrp-3}\textdagger strains were cotransformed with a 10:1 molecular ratio of \textit{al-1} PCR products and linearised pSV50 plasmids (section 2.11). The pSV50 plasmid contains a \textit{beta-tublin} gene that confers benomyl resistant and was used to select for transformants. \textit{al-1} encodes an essential enzyme required for carotenoid biosynthesis (Harding and Turner, 1981). Carotenoids are pigments that give wild-type \textit{Neurospora} its orange colour. The linear \textit{al-1} inserts carrying 1 kb of \textit{al-1} coding sequence are randomly integrated at ectopic locations, often in tandem, via recombination. These transgenic repeats induce quelling in a QDE-1-dependent manner to silence all homologous copies of \textit{al-1}. However, it is also possible that by chance some of the PCR products form inverted repeats next to a promoter and hairpin RNA is transcribed. This could account for the silencing that still takes place in the \textit{qde-1} deletion strain. The \textit{al-1} quelled transformant shows a white/pale yellow phenotype (Romano and Macino, 1992). Thus, if all proteins required for quelling are present then a large number of white transformants are expected. If a crucial protein is absent there will be fewer white transformants due to disruption of the RNAi machinery. Due to a variable \textit{al-1} copy number after random integration into the genome, silencing efficiency varies and phenotypes of transformants range from white
to pale orange (Figure 3.5). The number of white and yellow transformants as a proportion of the total number of transformants indicates the quelling efficiency.

Figure 3.5 Quelled, partially quelled and non-quelled transformants on minimal slants. From left to right, it shows quelled, partially quelled and non-quelled transformants. Cotransformation of al-1 PCR products and pSV50 plasmid produces transformants with phenotypes ranging from white, light yellow to orange.

Transformation of a wild-type strain with only the lineased pSV50 plasmid showed a wild-type phenotype in all 50 transformants examined, indicating that the quelled transformants observed in this experiment were not due to mutations in the al-1 gene but siRNA-mediated silencing effects. The results of two independent quelling assays are summarised in Table 3.1. The wild-type Neurospora usually quells al-1 expression at approximately 30% efficiency (Romano and Macino, 1992). The qde-1Δ strain was used as a quelling deficient control and it worked as expected (Cogoni and Macino, 1997). The efficiency of quelling was reduced in the qde-1Δ strain compared to the wild-type. In the first quelling assay the wild-type, qde-1Δ and rdrp-3Δ strains exhibited a quelling efficiency of 40.6%, 18.8% and 32.3% respectively (Set 1, Table 3.1).

The quelling assay was repeated with wild-type, qde-1Δ, rdrp-3Δ and qde-1Δ rdrp-3Δ double mutants. If rdrp-3 is involved in quelling, the quelling efficiency should be reduced in the qde-1Δ, rdrp-3Δ double mutant when compared to the qde-1Δ strain. In this experiment, only 8.6% quelling efficiency was observed in the qde-1Δ strain. In contrast, the rdrp-3Δ strain showed 31.4% quelling efficiency similar to wild-type (29.3%) and quelling efficiency of the qde-1Δ, rdrp-3Δ double mutant (10.0%) was comparable to the qde-1Δ strain. These data indicate that the ability to quell al-1 transcripts was unaffected in the rdrp-3Δ strain, suggesting that rdrp-3 has no effect on quelling.
Table 3.1 Results of two independent quelling assays.

<table>
<thead>
<tr>
<th>Set 1</th>
<th>Strain used</th>
<th>White/Yellow (Quelled)</th>
<th>Orange (Non-quelled)</th>
<th>Total</th>
<th>*Quelling Efficiency (%)</th>
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<tbody>
<tr>
<td></td>
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<tr>
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<th>Strain used</th>
<th>White/Yellow (Quelled)</th>
<th>Orange (Non-quelled)</th>
<th>Total</th>
<th>Quelling Efficiency (%)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>qde-1Δ</td>
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<td>53</td>
<td>58</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>qde-1, rdrp-3Δ</td>
<td>8</td>
<td>72</td>
<td>80</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Quelling efficiency is expressed in percentage as number of silenced transformants over the total number of transformants.
3.2.3 Determining the MSUD efficiency of wild-type, rdrp-3Δ and qde-1Δ and sad-1Δ strains

The efficiency of MSUD can be assayed by examining the phenotypes of ascospores in specifically designed tester crosses. Phenotypes such as ascospore shape and color can be manipulated by crossing strains with tester strains. The *ascospore maturation-1* (*asm-1*) gene is required for maturation of ascospores. Mature ascospores appear black, whereas silencing of *asm-1* produces white ascospores. The *round spore* (*rsp*) gene is required for the production of American football-shaped ascospores and silencing of *rsp* produces round spores (Hammond et al., 2011, Aramayo and Metzenberg, 1996).

*rsp* and *asm-1* tester strains were used to examine the ability of the *rdrp-3Δ* strain to suppress MSUD. Although there is no evidence to suggest that QDE-1 is a component of the MSUD pathway, its deletion strain was also tested in the MSUD assay. Crosses performed in the MSUD assay are listed in Table 3.2. If a component required for MSUD is deleted, MSUD should be suppressed, allowing expression of the *asm-1* or *rsp* genes. Thus, we expected to see no or a low number of white and round ascospores. The MSUD-deficient control used in this assay is the *sad-1Δ* strain; loss of *sad-1* suppress MSUD (Shiu et al., 2001). Ascospores were examined over time between 16 days to 25 days post-fertilisation. This was to determine whether secondary amplification might be involved in MSUD.

Table 3.2 Crosses performed in MSUD assay.

<table>
<thead>
<tr>
<th>♀</th>
<th>♂</th>
<th>Locus and mating type</th>
<th>FGSC Number</th>
</tr>
</thead>
<tbody>
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<td>wild-type a</td>
<td>2489 A x NCU01414 a</td>
<td>2489 x 4200</td>
</tr>
<tr>
<td>wild-type</td>
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<td>2489 A x NCU01414 a</td>
<td>2489 x11730</td>
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<tr>
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<td>asm-1Δ</td>
<td>NCU02178 A x NCU01414 a</td>
<td>11152x 11730</td>
</tr>
<tr>
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<td>asm-1Δ</td>
<td>NCU08435 A x NCU01414 a</td>
<td>11670 x 11730</td>
</tr>
<tr>
<td>qde-1Δ</td>
<td>asm-1Δ</td>
<td>NCU07534 A x NCU01414 a</td>
<td>11157 x 11730</td>
</tr>
<tr>
<td>wild-type</td>
<td>rspΔ</td>
<td>2489 A x NCU02764 a</td>
<td>2489 x 11449</td>
</tr>
<tr>
<td>sad-1Δ</td>
<td>rspΔ</td>
<td>NCU02178 A x NCU02764 a</td>
<td>11152x 11449</td>
</tr>
<tr>
<td>rdrp-3Δ</td>
<td>rspΔ</td>
<td>NCU08435 A x NCU02764 a</td>
<td>11670 x 11449</td>
</tr>
<tr>
<td>qde-1Δ</td>
<td>rspΔ</td>
<td>NCU07534 A x NCU02764 a</td>
<td>11157 x 11449</td>
</tr>
</tbody>
</table>

Three independent crosses were performed for each tester (Figure 3.6). After crossing an *asm-1* tester strain with wild-type only half of the ascospores were white. Similar
silencing efficiency was observed in the qde-1Δ and rdrp-3Δ mutants. As expected, sad-1Δ is defective in MSUD, showing the lowest silencing efficiency among strains. MSUD is suppressed when the sad-1Δ strain is crossed with the rsp tester strain, as fewer than 1.5% of all progeny displayed the round spore phenotype. On the contrary, nearly all ascospores (>96%) were round in crosses with wild-type, qde-1Δ and rdrp-3Δ strains. These strains showed similar silencing efficiencies at day 16, day 19 and day 25 post-fertilisation. Moreover, there is no obvious difference in silencing efficiency over time. These data demonstrate that deletion of qde-1 and rdrp-3 fails to suppress MSUD, suggesting that they are not required for MSUD. Furthermore, the hypothesis that RdRP-3 might be involved in secondary amplification of the silencing signal is not supported by the data as silencing efficiency did not decrease in crosses with the rdrp-3Δ strain between day 16, day 19 and day 25 post-fertilisation.
Figure 3.6 MSUD assays using asm-1Δ and rspΔ strains. A. Phenotypes of ascospores from crosses with an asm-1Δ deletion strain. A cross between WT strains produces mature black ascospores, while crossing WT to the asm-1Δ strain produces both white and black ascospores. The bar chart shows the silencing efficiency of WT, qde-1Δ, rdrp-3Δ and sad-1Δ strains in crosses with asm-1Δ strain. B. Phenotypes of ascospores from crosses with rspΔ strain. The wild-type ascospores are American-football shaped, while MSUD silencing of the rsp gene produces white ascospores. Bar chart shows the silencing efficiency of WT, qde-1Δ, rdrp-3Δ and sad-1Δ strains in crosses with an rspΔ strain. The silencing efficiency is calculated by dividing the white or round ascospores by the total number of ascospores. n=3 independent experiments. Error bars represent standard error of the mean.
3.2.4 Crosses homozygous for \textit{rdrp-3}

Interestingly, proteins required for MSUD have been shown to be essential for sexual development (Alexander et al., 2008; Hammond et al., 2011). A cross homozygous for \textit{sad-1} is blocked after elongation of asci, resulting in barren perithecia. Similar observations were found in crosses homozygous for \textit{sad-2}, \textit{sad-3}, \textit{dcl-1} and \textit{qip}, all of which lead to defects in sexual development (Alexander et al., 2008; Hammond et al., 2011). To investigate whether or not \textit{rdrp-3} is essential for sexual development, a cross homozygous for \textit{rdrp-3} was examined 19 days after fertilisation. The controls in this experiment were a WT x WT cross and a \textit{sad-1}^{\Delta} x \textit{sad-1}^{\Delta} cross. As expected the wild-type cross produced abundant perithecia and ascospores (data not shown), whereas a cross homozygous for \textit{sad-1} is completely barren; abundant perithecia formed but no ascospores were produced (Figure 3.7). In contrast, a cross homozygous for \textit{rdrp-3} shows a normal phenotype, which produced abundant perithecia and ascospores. This indicates that \textit{rdrp-3} is not essential for sexual development.

![Figure 3.7 Homozygous Crosses for \textit{rdrp-3} and \textit{sad-1}. Perithecia are formed on the \textit{rdrp-3}^{\Delta} x \textit{rdrp-3}^{\Delta} crossing plate (top left) and ascospores are produced and shot onto the lid of the plate (bottom left). The MSUD-deficient \textit{sad-1}^{\Delta} x \textit{sad-1}^{\Delta} control forms perithecia (top right) but does not produce ascospores (bottom right).]
3.3 Induction of RdRP3 by dsRNA

3.3.1 Construction and verification of al-1 hairpin constructs

rdrp-3 is one of approximately 60 genes highly induced after dsRNA production and shows the highest induction of all RNAi machinery genes (Choudhary et al., 2007). To further investigate the function of RdRP-3 in RNAi, dsRNA-expressing strains in wild-type and rdrp-3 background were generated. Silencing of these strains can be modulated using an inducible promoter, thus allowing comparison of RNAs present in induced and non-induced conditions.

To achieve this, an inducible dsRNA-expressing plasmid was created (Choudhary et al., 2007) as described in Figure 3.8. An inverted repeat consisting of fragments of al-1 was inserted downstream of the qa-2 promoter. Transcription from this promoter is inducible and adjustable by its inducer, quinic acid (QA) (Aronson et al., 1994). Addition of QA to the culture medium induces the expression of al-1, resulting in formation of an al-1 hairpin. This hairpin contains a stem of 530 bp and a loop of 100 bp. Induction of the al-1 hairpin triggers synthesis of al-1 specific siRNA, which silences the endogenous al-1.

In order to create an al-1 hairpin construct, 530 bp and 620 bp of the al-1 ORF was amplified by PCR using primers which introduce BamHI and HindIII or HindIII and EcoRI restriction sites at either end of fragments (Figure 3.8B). A three way ligation of al-1 fragments inserted the al-1 inverted repeats into the vector pCH10 in opposite orientations downstream of the qa-2 promoter (Figure 3.8A). The resulting construct was verified by restriction digest analysis (Figure 3.9A). Digestion with Ndel linearised the plasmid to produce a single fragment of 9,084 bp, HindIII digests the plasmid into three fragments with expected sizes of 5,824 bp, 2,711 bp and 549 bp. Nhel digests the plasmid into three fragments with expected size of 5,710 bp, 2,808 bp, 566 bp. As shown in Figure 3.9B, after separate restriction digests of putative constructs with all three enzymes, putative transformant 1 produced all bands of expected sizes. This al-1 hairpin (al-1 hp) plasmid was chosen for transformation of Neurospora.
Figure 3.8 Strategy to generate an inducible RNAi construct by expressing al-1 hairpin. A. Generation of al-1 hairpin dsRNA-expressing constructs. The al-1 fragments from position 1,322 to 1,942 bp and 1,412 to 1,942 bp were inserted into the pCH10 vector in reverse and forward orientations. The resulting plasmid contains a hairpin under the control of the inducible qa-2 promoter. Expression of the al-1 hairpin induces siRNA-mediated silencing of endogenous al-1. B. Amplification of the al-1 forward and reverse fragments. Left. al-1 reverse fragment was amplified using primers containing HindIII and BamHI restriction sites and the expected product size is 620 bp. From left to right on the gel, lane one contains 6 µl of 100 bp ladder, lane two to four contain al-1 PCR products. Right. al-1 forward fragment was amplified using primers containing HindIII and EcoRI restriction sites and the expected product size is 530 bp. From left to right on the gel, lane one contains 6 µl of 100 bp ladder, lane two to four contain al-1 PCR products.
Figure 3.9 Restriction digest analysis of putative al-1 hairpin plasmid. A. Schematic representation of al-1 hairpin construct after ligation of the al-1 forward and reverse fragments into vector pCH10. al-1 forward and reverse fragments were ligated into BamHI/EcoRI linearised pCH10. B. The resulting putative constructs were analysed by restriction digest analysis. Putative transformant 1 was digested in separate reactions with NdeI (9,084 bp), Nhel (expected size: 5,506 bp, 2,604 bp and 974 bp) and HindIII (expected size: 5,806 bp, 2711 bp and 549 bp). From left to right on the gel, lane one contains 6 µl of 1 kb ladder, lane two to five contain uncut, NdeI cut, HindIII cut and Nhel cut putative transformant.
3.3.2 Generation and verification of *Neurospora* strains containing the *al-1* hairpin

The *al-1* hairpin construct is based on the *his-3* targeting vector pCH10. This allows insertion of *al-1* hairpin DNA at the *his-3* locus by homologous recombination. The *rdrp-3*Δ *Neurospora* strain was first crossed to a *his-3* strain to obtain *rdrp-3*Δ strain in *his-3* background. The *rdrp-3*Δ strain is resistant to hygromycin due to gene replacement with *hph*. 25% of progeny from this cross were *his-3*Δ *rdrp-3*Δ. The progeny were grown on hygromycin slants to select for *rdrp-3*Δ and then on minimal slants lacking histidine to select for *his-3*. The *his-3* strain and *his-3*Δ *rdrp-3*Δ strains are defective in histidine biosynthesis due to a point mutation at the *his-3* locus and as a consequence of this they are incapable of growing on medium lacking histidine. Taking advantages of this, the *al-1* hairpin construct was targeted to the *his-3* locus in *Neurospora*. The *al-1* repeats in the *al-1* hairpin construct were inserted in the construct between sequences homologous to the *his-3* ORF and 3’ of the *his-3* ORF. After transformation, homologous recombination between the *al-1* hairpin region from the construct and the *his-3* locus results in replacement of the mutated *his-3* gene region with a functional copy of the gene (Figure 3.10). Strains successfully transformed with the *al-1* hairpin can therefore synthesise histidine and are able to grow on minimal medium. Thus, transformants were selected on sorbose minimal plates lacking histidine. Ten putative transformants of each strain (*his-3* and *his-3*,*rdrp-3*Δ) were isolated.
Two sets of primers were used to distinguish WT strains from WTald-1hp and pald-1hp transformants. One primer for WTald-1hp specific PCR only anneals to sequence within the qa-2 promoter and the other anneals to the sequence downstream of the his-3 ORF outside the flanking region of the construct. This pair of primers confirms the ald-1 hairpin is integrated at the his-3 locus by amplifying PCR products with a size of 1,810 bp (Figure 3.11). After identification, WTald-1hp primary transformant 6 and pald-1hp transformat 4 were verified and subsequently induced to produce microconidia. Five microconidia from WTald-1hp and pald-3ald-1hp strains were checked by diagnostic PCR to determine if they were homokaryons or heterokaryons. Two sets of primers were used to distinguish ald-1 transformed heterokaryons or homokaryons (Figure 3.11). Primers for ald-1 hp specific PCR amplify a 1,810 bp product. On the other hand, the second set of primers anneal to the wild-type his-3 locus and amplify a PCR product of 2,707 bp. If microconidia isolates are homokaryons, no PCR products should be amplified using the his+ primer pair. Gel photos in Figure 3.11 shows that WTald-1hp microconidal isolates (6-3, 6-4 and 6-5) and pald-3alald-1hp (4-1, 4-2, 4-3 4-4 and 4-5) isolates produced bands in ald-1 hp specific PCR, but not in his-3+ PCR confirming that all these strains are ald-1 hp homokaryons. WTald-1hp 6-3 and pald-3ald-1hp 4-5 were chose for further studies.
**Figure 3.11** Verification of WT^al−1 hp and rdrp-3^al−1 hp strains using al−1 hairpin specific primers. A. Schematic representation of the his-3 locus in the WT^al−1 hp and rdrp-3^al−1 hp strains shows the positions of primers and the expected sizes of PCR products. B. Gel photos of al−1 hp specific PCR and his-3+ PCR. Genomic DNA from WT^al−1 hp and rdrp-3^al−1 hp strains was amplified using his-3+ PCR primers P1 and P3 or al−1 hp specific primers P1 and P2. al−1 hp specific PCR produces 1,810 bp products and his-3+ PCR amplifies 2,707 bp products. From left to right on both gels, lane contains 1 kb ladder, lane two to six contain PCR products from microconidia of WT^al−1 hp strain, lane seven to eleven contain microconidia of rdrp-3^al−1 hp strain, lane twelve contains PCR products from wild-type and lane thirteen contains water control.
3.3.3 Analysis of *al-1* hairpin *Neurospora* strains

To find out whether expression of the *al-1* hairpin induces silencing of *al-1* in WT\(^{al-1 \text{hp}}\) and *rdrp-3*\(^{al-1 \text{hp}}\) strains, phenotypes of both strains were analysed on Vogel’s plates containing 10\(^{-2}\) M or 10\(^{-4}\) M quinic acid. Quinic acid can be also utilised as a carbon source for growth (Aronson et al., 1994). Because the qa-2 promoter is catabolically repressed in the presence of glucose, glucose is excluded from the growth medium. In the presence of QA, the qa-2 promoter is induced to express dsRNA derived from *al-1* hairpin. Induction of the *al-1* hairpin results in silencing of *al-1* (Choudhary et al., 2007). A white phenotype is expected in the WT\(^{al-1 \text{hp}}\) and *rdrp-3*\(^{al-1 \text{hp}}\) strains if *al-1* is silenced, otherwise an orange wild-type phenotype is expected. In Figure 3.12, the phenotype of strains grown on medium containing 2% sucrose or QA carbon sources are shown. Strains grew slower on QA plates as compared to growth on 2% sucrose minimal plates. WT and *rdrp-3*\(^{A}\) control strains produced orange conidia on both 2% sucrose and QA plates. In contrast, WT\(^{al-1 \text{hp}}\) strain and *rdrp-3*\(^{al-1 \text{hp}}\) strain displayed an orange phenotype on 2% sucrose plate but showed a white phenotype on QA plates. Although some very pale orange conidia were produced in the centre of the QA plate, these phenotypes indicate that *al-1* is silenced in *al-1* hp strains grown on medium supplemented with QA.
Figure 3.12 Phenotypes of WT, rdrp-3Δ, WTal-1 hp and rdrp-3al-1 hp strains in non-inducing and inducing conditions. Under normal growth conditions (2% sucrose) an orange phenotype was observed in WT, rdrp-3Δ, WTal-1 hp and rdrp-3al-1 hp strains. In the presence of inducer ($10^{-2}$ and $10^{-4}$ M QA), an orange phenotype was observed in WT, rdrp-3Δ. In contrast, WTal-1 hp and rdrp-3al-1 hp strains produce white phenotype.

To further validate the al-1 hairpin strains, rdrp-3 transcripts were analysed by RT-PCR in the wild-type, rdrp-3Δ, WTal-1 hp and rdrp-3al-1 hp under non-inducing and inducing conditions. Total RNA was extracted from these strains after 1 day cultured in 0.1% glucose medium followed by 6 hours with or without QA treatment. After DNase treatment, RNA was converted to cDNA by reverse transcription. Control samples minus reverse transcriptase did not produce any bands on subsequent PCR. This
confirms that RNA samples contain no trace of genomic DNA. Amplification of actin mRNA was carried out to control for the amount of cDNA used in each RT-PCR analysis.

RT-PCR results show that rdrp-3 transcripts were detectable, but were very lowly expressed in the wild-type and WT\textsuperscript{al-1\_hp} strains under non-inducing conditions (Figure 3.13). After 6 hours of QA treatment, rdrp-3 transcripts remained at a similar level in the wild-type strain. In contrast, rdrp-3 transcripts were induced approximately 38-fold in the WT\textsuperscript{al-1\_hp} strain. This is consistent with previous observations of rdrp-3 induction by dsRNA (Choudhary et al., 2007). As expected, no rdrp-3 transcripts were amplified in rdrp-3\textsuperscript{Δ} and rdrp-3\textsuperscript{al-1\_hp} strains. In order to determine whether the al-1 hairpin can induce dsRNA production in rdrp-3\textsuperscript{Δ} and rdrp-3\textsuperscript{al-1\_hp} strains, NCU07036, another dsRNA-activated gene encoding a 3'-5' exonuclease (Choudhary et al., 2007), was also examined by RT-PCR. Figure 3.13 shows that NCU07036 transcripts were expressed at relatively the same level in the wild-type, rdrp-3\textsuperscript{Δ}, WT\textsuperscript{al-1\_hp} and rdrp-3\textsuperscript{al-1\_hp} strains under non-inducing conditions. After 6 h of QA treatment, a 13-fold and 6-fold increase in NCU07036 transcript levels was observed in WT\textsuperscript{al-1\_hp} and rdrp-3\textsuperscript{al-1\_hp} strains respectively. These data confirm that the al-1 hairpin strains generate dsRNA that efficiently induces the expected dsRNA response.
Figure 3.13 RT-PCR analysis of \textit{rdrp-3} and NCU07036 expression in the \textit{al-1} hairpin strains after 6h of QA induction. The top panel shows the ethidium bromide stained PCR products. Bar charts show the quantification of \textit{rdrp-3} (middle) and NCU07036 (bottom) expression under the QA+/+ conditions. Transcripts of \textit{rdrp-3} and NCU07036 were normalised against \textit{actin}. White columns represent relative mRNA levels without QA. Black columns represent relative mRNA levels with addition of QA.
Next, to determine the silencing efficiency of endogenous al-1 expression over a time-course, al-1 transcripts were analysed by RT-PCR in non-inducing and inducing conditions. RNA samples were collected from WT\textsuperscript{al-1\textsubscript{hp}} cultures in a time series of 0h, 2h, 4h, 8h, 12h, 24h and 48h growth with or without QA. If the qa-2 promoter is induced by QA to express al-1 hairpin dsRNA, silencing of endogenous al-1 should result. Thus, al-1 expression should be reduced in the QA-treated strains compared to the expression in strains cultured in non-inducing conditions. Figure 3.14 shows that under non-inducing condition, al-1 levels were high at time 0 h, but decreased at 2 h and 4 h. There was a small increase at 8 h and 12 h, then al-1 levels dropped to a low level at 24 h and 48 h. After QA treatment, a similar overall trend of reduced al-1 expression over time was observed. Importantly, al-1 transcript levels were low at most of the time points compared to under non-inducing conditions, except at time 4 h and 48 h. There seems to be no particular time at which al-1 silencing is most efficient. A 2.4-2.8 fold reduction in al-1 transcript levels was observed at 2 h, 8 h and 12 h. These observations indicate that the QA-induced expression of al-1 hairpin dsRNA leads to loss of al-1 mRNA via siRNA-mediated silencing pathway.

Figure 3.14 RT-PCR analysis of al-1 expression in the WT\textsuperscript{al-1\textsubscript{hp}} strain over a time-course under the QA\textsubscript{-/+} conditions. The top panel shows ethidium bromide stained PCR products. Bar charts show the quantification of al-1 expression under QA\textsubscript{-/+} conditions. Transcript levels were normalised against actin. White columns represent relative al-1 mRNA levels without QA. Black columns represent relative al-1 mRNA levels with addition of QA.
3.4 GFP-tagging of RdRP-3 protein

3.4.1 Construction and verification of rdrp-3 gfp tagged construct

Several studies demonstrated that fluorescent fusion protein tagging is an effective tool to study the localisation of MSUD components in *Neurospora* (Hammond et al., 2011; Freitag et al., 2004). It has been shown that SAD-1, SAD-2 and other MSUD proteins including DCL-1, SMS-2, QIP and SAD-3 colocalise to the perinuclear region during meiosis (Hammond et al., 2011; Alexander et al., 2008). To study the localisation of RdRP-3, an *rdrp*-3 *gfp*-tagged plasmid was constructed using a yeast recombination system (Honda and Selker, 2009). The resulting construct was extracted from yeast, amplified, linearised and transformed into *Neurospora*.

![Figure 3.15 Amplification of 5' and 3' RdRP fragments of *rdrp*-3 *gfp*-tagged construct. Left: 5' RdRP-3 primers amplify 1,041 bp from RdRP-3 ORF and ends immediately before the stop codon. From left to right on the gel, lane one contains 6 µl of 1 kb ladder, lane two contains 5' *rdrp*-3 PCR products. Right: 3' RdRP-3 primers amplifies 521 bp starting immediately after the stop codon of RdRP-3. From left to right on the gel, lane one contains 6 µl of 1 kb ladder and lane two contains 3' *rdrp*-3 PCR products.](image-url)

A 5' *rdrp*-3 flank of 1,041 bp and a 3' *rdrp*-3 flank of 521 bp were cloned from the RdRP-3 ORF immediately before the stop codon and RdRP-3 3' UTR respectively (Figure 3.15). The *gfp* tag was derived from the pGFP::*hph::loxp* plasmid. Both flanks contain homologous sequences to the *gfp* fragment and the yeast vector pRS426. This allows the *gfp* tag to be inserted downstream of the RdRP-3 ORF by homologous recombination in yeast. The resulting DNA construct was extracted from yeast and verified by PCR using 5' *rdrp*-3 primers. The plasmid was then transformed into *E.coli* by electroporation. Two putative transformants were analysed by restriction digests in separate reactions with *Bam*HI and *Xba*I. This should produce 7,028 bp and 2,512 bp fragments for *Bam*HI digestion and 6,328 bp and 3,212 bp fragments for *Xba*I digestion. As shown in Figure 3.16, putative transformant 1 did not show the correct bands and
the total plasmid size is larger than 9,540 bp. Only putative transformant 2 produced fragments of the expected sizes. This transformant was then digested with SalI and HindIII to check the orientation of the gfp tag. As expected, the rdrp-3 gfp-tagged construct produced 7,635 bp and 1,887 bp fragments after SalI digestion and 7,716 bp, 1,296 bp, and 530 bp after HindIII digestion. Furthermore, the rdrp-3 gfp-tagged construct was verified by DNA sequencing (Eurofins). No mutation was found in the 5' flank or in the junction between rdrp-3 sequence and the gfp-tag.
Figure 3.16 Verification of gfp-tagged rdrp-3 constructs by restriction digest analysis. A. Schematic representation of gfp-tagged rdrp-3 construct in a yeast vector pRS426. B. Putative constructs were verified by restriction digest analysis. Transformants were digested in separate reactions with *Bam*HI (expected sizes: 7,028 bp, 2,512 bp) and *Xba*I (expected sizes: 6,328 bp, 3,212 bp). From left to right on the gel, lane one contains 6 µl of 1 kb ladder, lane two to four contain uncut, *Bam*HI digested and *Xba*I digested putative plasmid 1, lane five to seven contain uncut, *Bam*HI digested and *Xba*I digested putative plasmid 2. C. Putative plasmid 2 was digested in separate reactions with *Sal*I (expected sizes: 7,635 bp, 1,887 bp) and *Hin*III (expected sizes: 7,716 bp, 1,296 bp, 530 bp) From left to right on the gel, lane one contains 6 µl of 1 kb ladder, lane two to four contain uncut, *Sal*I digested and *Hin*III digested putative plasmid 2.
3.4.2 Identification of *rdrp-3* *gfp*-tagged *Neurospora* transformants

The *rdrp-3* *gfp*-tagged construct was made with the *gfp* cassette flanked by 1 kb of RdRP-3 coding sequence and 0.5 kb downstream of the RdRP-3 ORF. The *gfp* cassette contains a *gfp* tag followed by the selectable marker *hph*. The *hph* marker allows us to select transformants on medium containing hygromycin. The GFP protein is fused to the C-terminus of the RdRP-3 at its native locus. To generate *rdrp-3* *gfp*-tagged *Neurospora* strains, conidia from either wild-type or *his-3* strains were transformed by electroporation with the *rdrp-3* *gfp*-tagged construct. Putative transformants were identified by a *gfp*-specific PCR (Figure 3.17). Primer P1 anneals to the *rdrp-3* ORF sequence outside the 5' flank of the *rdrp-3* sequence in the construct, whereas P3 anneals to sequence in the *gfp* tag, thus only transformants containing the *gfp*-tag downstream of the *rdrp-3* ORF should produce PCR products. A different set of primers (P1 and P2) was used to amplify the wild-type copy of *rdrp-3*. This strategy identifies heterokaryons and homokaryons of *rdrp-3* *gfp*-tagged transformants. For primary transformants, usually not all nuclei of a conidium have taken up and integrated the construct however, these heterokaryons can be induced to produce uninucleate microcondia. The genotypes of strains arising from microconidia were checked by dianostic PCR to identify homokaryons of *rdrp-3* *gfp*-tagged transformants.

Primary transformant 1 and 2 were cultured on microconidia-inducing medium to obtain homokaryons. Ten microcondia from WT*rdp-3 gfp* were analysed by PCR. Figure 3.17 shows that microcondia 1-1, 1-2, 1-3, 1-5, 2-1, 2-2, 2-3 and 2-4 produced a band of the expected size for *gfp*-specific PCR, suggesting that they all contain the *gfp*-tag at the *rdrp-3* locus. However, these microcondia also showed 1.5 kb products for *rdrp-3*+ PCR. This indicates that these microcondia also contain a wild-type copy of *rdrp-3* so they are heterokaryons. Similarly, ten microcondia obtained from *rdrp-3* *gfp*-tagged strain in the *his-3*- background were verified as heterokaryons (data not shown).
**Figure 3.17 Verification of rdrp-3 gfp-tagged strains using gfp-specific primers.** A. Schematic representation of the rdrp-3 locus in the WT and rdrp-3 gfp-tagged strains shows the positions of primers and the expected sizes of PCR products. B. Gel photos of rdrp-3+ PCR and gfp-specific PCR. Genomic DNA from rdrp-3 gfp tagged strain was amplified using rdrp-3+ PCR primers P1 and P2 or gfp-specific PCR primers P1 and P3. The rdrp-3+ PCR produces 1,537 bp products and gfp-specific PCR amplifies 1,315 bp products. From left to right on both gels, lanes one and fifteen contain 1 kb ladder, lanes two to eleven contain PCR products from microconidia of rdrp-3 gfp-tagged strains, lanes twelve and thirteen contain PCR products from wild-type and lane fourteen contains water control.
It should be noted that all \textit{rdrp-3 gfp}-tagged transformants verified, including the primary transformants and microconidia of \textit{WT}^{\text{rdrp-3 gfp}} (20 isolates) and \textit{his-3}^{\text{rdrp-3 gfp}} (20 isolates) strains, show a defective growth phenotype (Figure 3.18). They all appear pale and produce fewer conidia compared to wild-type. Perhaps homokaryons are not viable; if so this might explain why only heterokaryons could be isolated. Moreover, the \textit{rdrp-3-gfp} tagged transformants are female sterile. Protoperithecia are produced but no mature perithecia are formed after addition of macroconidia of the opposite mating type. The \textit{rdrp-3 gfp}-tagged construct has been verified and contains no mutations in the \textit{rdrp-3} ORF, thus defects in growth would not be a consequence of mutations in the \textit{rdrp-3} ORF.

\textbf{Figure 3.18 Growth of \textit{rdrp-3 gfp}-tagged strains on slants.} All cultures are the same age. The \textit{WT}^{\text{rdrp-3 gfp}} transformants produce a low number of conidia on minimal slants. The parent \textit{WT} strain shows normal growth on minimal slants. Similarly, the \textit{his-3}^{\text{rdrp-3 gfp}} transformants produce a low number of conidia on minimal slants supplemented with histidine. The parent strain \textit{his-3} shows normal growth on minimal slants supplemented with histidine.
3.4.3 Imaging of RdRP-3-GFP

Although some difficulties were encountered when attempts were made to obtain *rdp*-3 *gfp*-tagged homokaryons, some preliminary microscope work was carried out using heterokaryons. Conidia collected from 5-7 day-old cultures were observed under a confocal microscope. The setting used for detection of fluorescence was adjusted to a low level to reduce detection of natural autofluorescence. Nevertheless background signals were still detected in both WT and *rdp*-3 *gfp*-tagged strains. No obvious increase in signal is seen in the *rdp*-3 *gfp*-tagged strain (Figure 3.19). This could be because heterokaryons were used and/or because of low expression of endogenous *rdp*-3 under normal growth conditions. Therefore, an inducible *rdp*-3 *gfp*-tagged strain was created by transforming the WT*al*-1 *hp* strain with the *rdp*-3 *gfp* construct. Addition of inducer QA should drive *al*-1 hairpin expression resulting in *rdp*-3 induction and increase the chances to visualise RdRP-3 in *Neurospora*. After transformation, putative transformants were identified by *gfp*-specific PCR previously described in Section 3.4.2. Primers only amplify PCR products with the expected size of 1,315 bp when the putative transformants contain the *gfp*-tag at the *rdp*-3 locus (Figure 3.17). As shown in Figure 3.20, PCR products of putative transformants 3, 4, 5 and 6 produced a band of the expected size. This indicates that the *gfp* tag was integrated at the *rdp*-3 locus in these transformants with the *al*-1 hairpin background. Next, these transformants were cultured and used for confocal imaging. The WT*al*-1 *hp* *rdp*-3 *gfp* transformant was grown in 0.1% glucose medium for germination before addition of the QA inducer. After 6 hours of QA induction, germinating conidia and hyphal tubules were visualised. Under the GFP fluorescence detection, however, the WT*al*-1 *hp* *rdp*-3 *gfp* transformant appears similar to the wild-type (data not shown). This indicates that the induction of *rdp*-3 by QA-induced *al*-1 hairpin expression did not improve visualisation of the GFP-tagged RdRP-3.
Figure 3.19 Images of *Neurospora* conidia of WT and *rdrp-3 gfp* tagged strains. The left panel shows the bright field image, middle panel shows the green fluorescence image and the right panel shows a merge of bright field and green fluorescence image.

Figure 3.20 Verification of *rdrp-3 gfp*-tagged strains in the al-1 hp background using gfp specific primers. Genomic DNA from WT^al-1 hp^ *rdrp-3 gfp*-tagged strain was amplified using gfp-specific PCR primers P1 and P3. gfp-specific PCR amplifies 1,315 bp products. A schematic representation of the *rdrp-3* locus in the *rdrp-3 gfp*-tagged strains is shown in Figure 3.17. From left to right on the gels, lane one contains 1 kb ladder, lanes two to seven contain PCR products of putative transformants from the *rdrp-3 gfp*-tagged strains and lane eight contains PCR products from wild-type as a negative control.
4. Discussion

4.1 Phylogenetic analysis of RdRP proteins

RNA-dependent RNA polymerases (RdRP) synthesise the complementary strand of a single-stranded RNA template. There are two structurally different types of RdRPs: viral RdRPs and cellular RdRPs. Viral RdRPs are essential for viral replication and transcription of the viral genome (Maida and Masutomi, 2011). Viral RdRPs share a structure resembling a closed ‘right hand, which contains thumb, finger and palm domains. Viral RdRP can initiate complementary strand synthesis by primer-independent or primer-dependent RNA synthesis. The primer-independent RNA synthesis is widely used in viruses, whereby synthesis is initiated at the 3’ end of the RNA template (Maida and Masutomi, 2011). Primer-dependent RNA synthesis involves either a nucleotide or a uridylylated protein primer to initiate polymerisation (Maida and Masutomi, 2011). Cellular RdRPs are key components of RNAi pathways. Cellular RdRPs catalyse the formation of double-stranded RNAs which are processed into small RNAs via Dicer-dependent or Dicer-independent mechanisms, which induce gene silencing in a RISC-dependent manner. Cellular RdRPs are found in diverse eukaryotes and share the catalytic double-psi β-barrel domain (Salgado et al., 2006). Some RdRP homologs identified in plants, fungi and nematodes are commonly present in more than one copy. However, RdRP homologs are not encoded in vertebrate and insect genomes. Cellular RdRPs can also mediate both primer-independent and primer-dependent RNA synthesis (Maida and Masutomi, 2011).

A phylogenetic analysis of RdRP proteins was carried out to use similarity in amino acid sequences of RdRP proteins to construct a phylogenetic tree depicting the pattern of relatedness of related proteins in different organisms. This analysis allows us to understand the evolutionary relationships of RdRP proteins, and also inform us of how these proteins should be classified. 55 RdRP protein sequences from different kingdoms including protozoa, plantae, animalia and fungi were aligned using either the full length proteins or the RdRP domains alone. Phylogenetic trees were constructed by the neighbour-joining method. Results showed that *N. crassa* QDE-1, SAD-1 and RdRP-3 are separated into three different groups. In the QDE-1 group, other than fungal species, there are members from amoebae, chromalveolata and plants. SAD-1 is clustered with *A.thaliana RDRs* and *C. elegans* RdRPs in the SAD-1 group. RdRP-3 and other fungal RdRPs formed the third group. However, the function of most fungal
RdRPs in the RdRP-3 group is unknown. The following sections will briefly describe the function of some known RdRPs in each group.

All members of the RdRP-3 group belong to the fungal kingdom including *Aspergillus oryzae* QDE-1, *Aspergillus nidulans* RrpA and *Cryptococcus neoformans* RDP1. Although *Aspergillus oryzae* QDE-1 is named as QDE-1, in fact it is more related to *N.crasa* RdRP-3 and it has not been characterised. *A. nidulans* RrpA (referred as Rrp C in other literature) is also in the RdRP-3 group. RNA silencing in *A. nidulans* is independent of RdRPs, since the efficiency of the inverted repeat transgene-induced RNA silencing was unaffected in *rrpB* and *rrpC* single or double mutants (Hammond and Keller, 2005). A phenomenon similar to quelling has been described in a human fungal pathogen *C. neoformans*. *C. neoformans* RDP1, encoded by the only *rdrp* gene in the genome, is required for sex-induced silencing and asexual co-suppression. Both mechanisms are induced by transgenes but operate during sexual and vegetative growth respectively. Retrotransposons were highly expressed in an *rdp1 x rdp1* mating cross indicating that sex-induced silencing controls the propagation of transposons (Wang et al., 2010a). Asexual co-suppression shares characteristics with quelling in *N. crassa*. For example, a high transgene copy number is necessary to induce silencing of the target gene and the Replication Protein A is essential for asexual co-suppression (Wang et al., 2012). It appears that *C. neoformans* RDP1 is functionally more similar to *N. crassa* QDE-1. This probably explains the low bootstrap value (284 over 1000) between *C. neoformans* RDP1 and *N. crassa* RdRP-3.

Some RdRPs are well-characterised in the SAD-1 group, including *A. thaliana* RDRs, *S. pombe* Rdp1 and *C. elegans* RdRPs. Co-suppression in plants is similar to quelling in *Neurospora* during vegetative growth, thus plant RDRs should be functionally similar to *N. crassa* QDE-1. However, in this phylogenetic analysis, the plant RDR1, RDR2 and RDR6 clustered to the SAD-1 group. Plant RDR6 is required for the transgene-induced RNA silencing mechanism (Luo and Chen, 2007). The presence of multiple copies of a transgene triggers transcription of aberrant transgene RNAs (aRNAs) which are truncated and unpolyadenylated. The aRNAs act as templates for dsRNA synthesis by RDR6, which are processed into 21 nt siRNAs. siRNAs are incorporated into Argonaute protein containing RNA induced silencing complex (RISC) which targets the complementary mRNA for down-regulation or degradation (Luo and Chen, 2007; Maida and Masutomi, 2011). Plant RDR6 is also involved in the biogenesis of trans-acting siRNA (ta-siRNA), which is mediated by miRNAs. Trans-acting siRNAs are a unique
subset of endogenous RNA in plants. miRNA cleaves the ta-siRNA (TAS) transcripts into fragments which are converted into dsRNA by RDR6 via de novo initiation, followed by processing by DCL-4 which gives rise to trans-acting RNAs (Wassenegger and Krczal, 2006; Maida and Masutomi, 2011). Furthermore, RDR1 and RDR6 are involved in the RNA silencing mechanism in response to viral infection. Antiviral defence is induced by dsRNAs of viral replicative products, which are cleaved into primary siRNAs by Dicer-like (DCL) protein. Both RDRs initiate new synthesis of dsRNA and DCLs process them into secondary siRNAs. This significantly increases the amplification of viral siRNAs (Wang et al., 2010b). In addition, Plant RDR2 mediates gene silencing by RNA-dependent DNA methylation (RdDM) and RNAi-mediated heterochromatin formation. RDR2 interacts with RNA polymerase IV which transcribes ssRNA from repetitive heterochromatic loci. RDR2 uses ssRNA to synthesise dsRNA and then DCL-3 cleaves the dsRNA into 24nt siRNA. The siRNA targets the homologous region for de novo DNA and histone H3K9 methylation. RDR2 is thought to play a role in RdDM by amplifying secondary siRNA, which is required for maintenance of RdDM and histone methylation (Wassenegger and Krczal, 2006; Maida and Masutomi, 2011).

The single homolog of RdRP in S. pombe is encoded by RNA-directed RNA polymerase (rdp1). Genetic analysis in S. pombe has provided evidence of links between RNAi and transcriptional silencing. The repetitive DNA at centromeres is not completely silenced. During S phase of the cell cycle, RNA Polymerase II catalyses transcription from these centromeric repeats (Chang et al., 2012; Maida and Masutomi, 2011). These centromeric transcripts are recognised and used by Rdpl as templates to generate dsRNAs. Dcr1 cleaves dsRNAs into siRNAs which are then loaded onto the RNA induced transcriptional silencing (RITS) complex, which comprises Ago1, Chp1 and Tas3. siRNA guides the RITS complex to homologous DNA in heterochromatin. RITS recruits Clr4 and the RNA-dependent RNA polymerase complex (RDRC), which contains Rdp-1, Cid12 and Hrr 1. This forms a self-enforcing loop for amplification of heterochromatic siRNA. Clr4 is a H3K9 histone methyltransferase that methylates lysine 9 of histone H3 at the targeted locus. The Clr4-containing complex Clr4-Rikl-Cul4 complex (CLRC) is recruited to the region and all components of CLRC are required for heterochromatin assembly. H3K9 binding protein Swi6, the mammalian HP1 homolog, binds to the chromatin and facilitates spreading of heterochromatin. Thus, RNAi and chromatin modifications are coupled to establish transcriptional
silencing in *S. pombe* (Buchon and Vaury, 2006; Chang et al., 2012; Maida and Masutomi, 2011).

Members of the QDE-1 group include plant RDRs and *Dictyostelium discoideum* RdRP s. Plant RDR3, RDR4 and RDR5 are not functionally characterised. *D. discoideum* genome encodes three RdRPs and all of them contain an N-terminal helicase domain homologous to the helicase domain present in the dicer protein in *Drosophila* and *C. elegans*; only RrpA is required for RNAi. RrpA and RrpB are more closely related than either are to RrpC (Martens et al., 2002). This was also seen in this phylogenetic analysis that RrpA and RrpB are clustered in one branch apart from RrpC. Recently, RNA silencing signal transitivity was observed in *D. discoideum* and RrpC is involved in this mechanism (Wiegand and Hammann, 2013).

One observation from this analysis is that an RRM domain can be identified in RdRPs in some species. This raises the question of how the RRM domain contributes to the function of RdRP proteins. In *M. circinelloides*, it has been shown the two distinct RdRPs are required for the ‘two steps’ RNAi pathways (Calo et al., 2012). ‘Two steps’ mechanism involves initiation and amplification processes. RdRP1 has no RRM domain, but is essential for initiation of silencing induced by transgenes. RdRP2 has an RRM domain and is required for amplification of secondary siRNAs. Could the presence of an RRM in RdRP3 indicate its involvement in amplification of secondary siRNAs? In *Neurospora*, SAD-1 also contains an RRM domain but there is no evidence for secondary amplification of small RNAs in either MSUD or quelling. In fact no clear correlation between the presence of an RRM and amplification of siRNAs exists. For example, secondary RNA silencing transitivity in *D. discoideum* depends on RrpC but not RrpA or RrpB, despite the fact that they all contain an RRM domain (Wiegand and Hammann, 2013). Moreover, *C. elegans* RdRPs RRF-1 and EGO-1 are responsible for secondary siRNA amplification, yet neither seems to have an RRM domain.

The core components of the RNAi machinery are conserved in eukaryotes (Obbard et al., 2009). RdRPs however, are not as conserved as other RNAi proteins, they have been lost in some lineages including vertebrates and insects (Obbard et al., 2009). Moreover, duplication of *rdp* genes often occurred during evolution, some species ended up with multiple copies of RdRP proteins. In plants, for example, there are six cellular RDRs that are implicated in distinct RNAi-related mechanisms (Willmann et al., 2011). This is also true of RdRPs in other organisms. For example, RdRPs in general
are thought to be RNA-dependent RNA polymerases generating dsRNAs from ssRNA templates. It is becoming clear, however, that a single RdRP can have multiple functions in biological processes. *Neurospora* QDE-1 possesses both DdRP and RdRP activity that generates aRNA using DNA templates and then converts the aRNA into dsRNA. QDE-1 is not only required for quelling but also plays an essential role in qiRNA biogenesis (Lee et al., 2009). Hence, it seems that it is difficult to assign RdRP proteins a single functional classification.

In summary, several observations have been made from this phylogenetic analysis of RdRPs. First, *N. crassa* QDE-1, SAD-1 and RdRP-3 are placed in separate groups. QDE-1 and SAD-1 are known to play distinct roles in quelling and MSUD pathways respectively. The analysis indicates that RdRP-3 belongs to a functionally distinct third group of RdRPs. Second, RdRP-3 belongs to a group in which all members restricted to the fungal kingdom. These RdRPs seem to have arisen from a fungi-specific lineage after the divergence of fungi, plant and animals. It was hoped that a phylogenetic analysis might place RdRP-3 in a group with functionally characterised RdRPs so that a putative function could be assigned to it. However, in the RdRP-3 group, the functions of most fungal RdRPs are unknown. Third, the RRM domain is present in some members of all groups. Thus the RRM could be a component of an ancestral *rdrp* gene which has been lost from some RdRPs.
4.2 Silencing efficiency of RdRP deletion strains in quelling and MSUD

BLAST results show that RdRP-3 contains a conserved RdRP domain and shares homology with Neurospora QDE-1 and SAD-1 proteins. The role of QDE-1 and SAD-1 proteins is well-characterised in quelling and MSUD respectively (Chang et al., 2012; Dang et al., 2011). Thus, the first question I asked is whether RdRP-3 participates in either or both quelling and MSUD pathways. A quelling assay was carried out following a previously reported protocol (Lee et al., 2010) involving co-transformation of an al-1 fragment and a plasmid conferring benomyl resistance. Introduction of al-1 silences all transgenic and endogenous copies of al-1, resulting in a white/yellow phenotype.

al-1 is a structural gene coding for an enzyme required for biosynthesis of carotenoid. Biosynthesis of carotenoid requires three enzymes that are products of the albino genes (Nelson et al., 1989). al-1 encodes phytoene dehydrogenase that catalyses the conversion of phytoene to carotenoid pigments (Schmidhauser et al., 1990), al-2 encodes phytoene synthase which catalyses the formation of phytoene from geranylgeranyl pyrophosphate (GGPP) (Diaz-Sanchez et al., 2011; Harding and Turner, 1981). al-3 encodes GGPP synthetase which converts isopentenyl pyrophosphate to GGPP (Harding and Turner, 1981). These albino genes have been used as genetic markers in previous quelling studies. Approximately 40% of al-1 transformants and 10% of al-2 transformants were quelled, while only 0.5% of al-3 transformants were quelled. This difference in silencing efficiency may be related to the fact that al-3 is an essential gene and the asexual progeny of al-3 transformants were not viable if al-3 was silenced (Romano and Macino, 1992; Pickford et al., 2002). Hence, al-1 is a routine marker used in quelling assays.

In my quelling assay, similar to results of other investigations (Romano and Macino, 1992), approximately 30% of al-1 transformants derived from a wild-type strain were quelled. As transgenes are clustered as tandem repeats, a threshold of transgene copy number is required to produce a silenced phenotype (Pickford et al., 2002). It is possible that non-quelled transformants contain a low copy number of al-1 transgenes that is insufficient to elicit the albino phenotype. This could explain why only a minority of transformants were silenced. An alternative explanation is that not all nuclei are transformed with al-1, and a small number of wild-type al-1 genes are expressed and then al-1 mRNA translated into functional phytoene dehydrogenase. These enzymes can be used multiple times resulting in the conversion of phytoene to carotenoid pigments. Thus, the presence of a small number of functional phytoene dehydrogenase
is sufficient to maintain the carotenoid biosynthesis. As expected, the quelling deficient 
$qde-1$ control showed that 8.6% of $al-1$ transformants were quelled. However, the 
quelling efficiency of the $rdrp-3$ mutant is approximately 30% which is comparable to 
the quelling efficiency in the wild-type strain, and 10% of the progeny had the silenced 
phenotype in a $rdrp-3$, $qde-1$ double mutant, suggesting that $rdrp-3$ is not involved in 
quelling.

Interestingly, instead of completely abolishing quelling, quelling activity was reduced in 
the $qde-1$ mutant (8.6%), suggesting that redundant RdRP activity exists in Neurospora. 
This observation was reported previously by Lee et al. (2010). In their study, $qde-1$ and 
$qde-3$ single mutants (6.8%) had a reduced quelling efficiency after co-transformation 
of a construct containing a truncated $al-1$ gene and a gene conferring resistance to 
benomyl (Lee et al., 2010). It was reasoned that if inverted repeats of $al-1$ were present 
after transformation, these could produce dsRNA which would bypass the requirement 
for QDE-1 and QDE-3. Consistent with this hypothesis, quelling activity was completely 
abolished in the double Dicer mutant (Catalanotto et al., 2004).

The $asm-1$ and $rsp$ deletion strains are testers used to examine silencing efficiency 
after crossing with strains containing a wild-type copy of the gene at the $asm-1$ or $rsp$ 
locus. In the MSUD assays using the $asm-1$ tester, results of three independent 
crosses showed that approximately half of the progeny were white in a cross of WT $x$ 
$asm-1$ for all days post-fertilisation (day 16, day 19 and day 25) examined. A similar 
efficiency of MSUD was observed in crosses of $qde-1$ $x$ $asm-1$ and $rdrp-3$ $x$ $asm-1$ at 
day 16. Although MSUD efficiency in crosses of $qde-1$ $x$ $asm-1$ and $rdrp-3$ $x$ $asm-1$ was 
lower compared to the WT $x$ $asm-1$, the MSUD efficiency in $qde-1$ $x$ $asm-1$ and $rdrp-3$ $x$ 
$asm-1$ was higher than in WT $x$ $asm-1$ at Day 25. It seems that the results of crosses to 
$asm-1$ are not consistent for all days examined and do not show a trend in MSUD 
efficiency. Furthermore, two controls were used in this MSUD experiment. The MSUD 
deficient control $sad-1$ $x$ $asm-1$ showed the expected MSUD efficiency in that 
approximately 20% of the progeny were white. However, the efficiency of MSUD in a 
WT $x$ $asm-1$ cross did not correspond to previously reported results that all progeny are 
white in a cross of WT $x$ $asm-1$ (Hammond et al., 2011; Hammond et al., 2013). As 
shown in Section 3.2.1, despite confirmation that the $asm-1$ gene is deleted in the $asm-
1$ tester strain, an extra copy of $hph$ is ectopically integrated in the genome. It is 
possible that this insertion affects MSUD efficiency. On the other hand, the controls 
used in crosses with $rsp$ tester produced the expected results. Nearly all ascospores
were round in WT x rsp, whereas suppression of MSUD was observed in sad-1 x rsp. The results of three independent crosses showed that the rdrp-3 and qde-1 mutants had a silencing efficiency similar to the wild-type. Additionally, homozygous crosses of rdrp-3 x rdrp-3 produced normal ascospores, suggesting that RdRP-3 is not essential for sexual development. Taken together these results indicate that rdrp-3 is unlikely to play a role in MSUD.

MSUD assays using either asm-1 or rsp testers did not show obvious differences over a time course. This conflicts with the finding that MSUD is stronger in early perithecia, an observation that was made in crosses with a ::act tester (Ramakrishnan et al., 2012). Perhaps the strength of meiotic silencing is different in crosses using different testers, this explains why the temporal switch was not seen in my experiment.

SAD-1 and SAD-2 are dominant suppressors of MSUD that prevent the silencing of unpaired genes. For example, expression of β-tubulin-GFP was observed in crosses of a strain containing an extra copy of β-tubulin-GFP in a sad-1 or sad-2 background (Shiu et al., 2006). Recently, two additional SAD proteins have been reported to be required for MSUD, but the exact function of SAD-4 and SAD-5 has not been discovered (Hammond et al., 2013). SAD-4 is a semi-dominant suppressor of MSUD. sad-4 suppressed MSUD in crosses with four testers including ::act+, ::bml, asm-1 and rsp, but half of the progeny are round in a cross of rsp x sad-4. On the other hand, SAD-5 suppressed MSUD in crosses using ::act+, ::bml, and asm-1 testers, but it did not suppress MSUD using the rsp tester. Nearly all ascospores are round in a cross of rsp x sad-5. MSUD was deficient in homozygous crosses of sad-5 deletion strains containing the unpaired rsp at the homologous locus (sad-5, rsp x sad-5). Furthermore, neither SAD-4 nor SAD-5 are essential for sexual development (Hammond et al., 2013). In my experiment, bearing in mind the fact that only the controls with the rsp tester worked as expected, rdrp-3 does not suppress MSUD using the rsp tester and is not essential for sexual development. Nevertheless it is still possible that RdRP-3 could be involved in MSUD but does not act as a dominant suppressor. Suppression of MSUD by the protein does not show the dominant phenotype. To test this possibility, crosses using other testers such as ::act+, ::bml or H1-gfp can be set up to see if RdRP-3 suppresses MSUD. Alternatively, homozygous crosses of rdrp-3 deletion strains can be performed. The rsp gene can be deleted in one of rdrp-3 deletion strain to see if MSUD functions to silence the unpaired rsp gene in rdrp-3 background.
4.3 Induction of RdRP-3 by dsRNA

One of my objectives was to create a tool for further functional characterisation of RdRP-3. It is acknowledged that rdrp-3 is one of 60 dsRNA-activated genes (DRAGs) highly induced in response to dsRNA (Choudhary et al., 2007). In several investigations of RNAi components, constructs expressing al-1 hairpin RNA were used to induce silencing (Goldoni et al., 2004; Choudhary et al., 2007; Nolan et al., 2008). Taking advantage of this, I constructed a quinic acid-inducible al-1 hairpin and successfully generated wild-type and rdrp-3 Δ Neurospora strains containing the al-1 hairpin at the his-3 locus. This inducible al-1 hairpin expressing system was validated by phenotypic analysis and RT-PCR analysis. Under non-inducing conditions, both WT^al-1^hp and rdrp-3^al-1^hp strains displayed the unsilenced phenotype. In contrast, under inducing conditions the WT^al-1^hp and rdrp-3^al-1^hp strains displayed a white (or at least pale orange) phenotype, indicating that the endogenous al-1 had been quelled. Furthermore, the expected response to al-1 hairpin expression such as al-1 mRNA reduction and rdrp-3 mRNA induction was seen in the WT^al-1^hp strain under inducing conditions. These al-1 hairpin strains will be used in RNA sequencing analysis to see if any small RNAs are produced or any genes are differentially regulated in WT^al-1^hp compared to rdrp-3^al-1^hp in the presence and absence of the inducer, quinic acid.

Quelling can be triggered by different inducers such as transgenes and dsRNAs. It has been reported that QDE-1 and QDE-3 are not required for dsRNA-induced silencing (Goldoni et al., 2004). It is reasoned that transcripts of the inverted repeats can fold to form dsRNAs, bypassing QDE-1 and QDE-3 for dsRNA production. Recent evidence in Arabidopsis demonstrated that RDR6 is required for efficient hairpin RNA-induced gene silencing by amplifying secondary siRNA; the induced siRNAs were reduced in the rdr6 mutant when compared to the wild-type (Harmoko et al, 2013). The following model for RDR6 function in hairpin RNA-induced gene silencing has been proposed. Inverted repeat RNAs fold back on themselves to form hairpin dsRNA which can be recognised by Dicer and processed into primary siRNAs. These siRNAs are loaded onto the RISC and execute target mRNA degradation. During mRNA degradation, aberrant RNAs are produced which act as templates for RDR6 to produce secondary siRNAs, thus enhancing the silencing effect. Confirming the results of Choudhury et al. (2007), I observed the silenced phenotype of al-1 in both WT^al-1^hp and rdrp-3^al-1^hp under inducing conditions. This implies that RdRP-3 is not required for initiation of hairpin RNA-induced silencing. The possibility that RdRP-3 is required for amplification of hairpin RNA-induced gene silencing can be investigated in the future.
For example, the al-1 hairpin strains can be cultured in medium plus quinic acid to induce al-1 silencing. Further culture in medium without quinic acid will lead to progressive loss of silencing and reversion to a wild-type phenotype, since quelling is not stable. The time taken for reversion of the silenced phenotype to the wild-type phenotype in WT$^{al-1\ hp}$ and rdrp-3$^{al-1\ hp}$ should be compared. If rdrp-3 is involved in secondary small RNA amplification, the silenced phenotype should last longer in WT$^{al-1\ hp}$. This would suggest that RdRP-3 can amplify secondary signals to maintain the silencing effect in WT$^{al-1\ hp}$. In contrast, due to the deletion of RdRP-3 no secondary amplification of small RNA should occur in rdrp-3$^{al-1\ hp}$, and the wild-type phenotype should be observed.
4.4 GFP-tagging of RdRP-3 protein

The GFP reporter system has been established in *Neurospora* and it is an efficient tool to study protein properties such as localisation, protein interactions and protein function (Hammond et al., 2011; Shiu et al., 2006; Bardiya et al., 2008). Fusion of components required for MSUD to GFP revealed that the proteins colocalise to the perinuclear region forming an RNAi silencing complex during meiosis (Shiu et al., 2006; Hammond et al., 2011; Alexander et al., 2008). These studies demonstrate that the use of GFP is a feasible approach to study the localisation of proteins by confocal microscopy. Therefore, one of the aims of my project was to tag RdRP-3 with GFP protein to determine its cellular localisation.

The gfp tag was fused to the C-terminus of the RdRP-3 ORF at the endogenous rdrp-3 locus, thus, the expression of rdrp-3 gfp is driven by the rdrp-3 promoter. The rdrp-3 gfp-tagged *Neurospora* transformants were verified by diagnostic PCR. All rdrp-3 gfp-tagged transformants tested are heterokaryons and even after production of microconidia no homokaryons were isolated. Conidia from these transformants had a pale appearance and produced fewer conidia compared to the wild-type. Moreover, rdrp-3 gfp-tagged transformants are female sterile; the strain did not form perithecia in crosses.

Visualisation of rdrp-3 gfp-tagged transformants showed that RdRP-3 GFP signals were not detected. It is not surprising to see this as the RT-PCR results indicated that rdrp-3 is poorly expressed under normal growth conditions. Because rdrp-3 expression increases 30-fold in the presence of double-stranded hairpin DNA (Choudhary et al., 2007). Thus, in order to increase the chance of detecting RdRP-3, inducible gfp tagged strains were generated by transforming the WT al-1 hp strain with the rdrp-3 gfp fusion construct. Instead of using conidia for visualisation, conidia were germinated to promote vegetative growth. This ensures that protein synthesis is actively occurring, and increases the likelihood of seeing RdRP-3 GFP. After germination, QA was added to induce expression of the al-1 hairpin previously shown to induce expression of rdrp-3 (Choudhary et al., 2007). Six hours after QA addition to the medium, germinating conidia with tubules were observed in the wild-type and rdrp-3 gfp-tagged strains under bright field illumination. Unfortunately, after QA induction, GFP signals in the rdrp-3 gfp-tagged strain did not show any obvious difference from the wild-type. It seems that RdRP-3 GFP expression was not induced to a detectable level in the hyphae.
The confocal microscope work reported here is preliminary. For instance, the method for QA induction of the al-1 hairpin and microscopic slide preparations for confocal microscopy have not been optimised. The WT<sup>al-1 <i>hp</i></sup> <i>rdrp</i><sup>-3 <i>gfp</i></sup> transformants have only been verified by PCR. Theoretically, <i>rdrp</i>-3 <i>gfp</i>-tagged transcripts should be induced in the presence of QA, as it has been shown in the RT-PCR analysis of <i>rdrp</i>-3 transcripts that were induced in the WT<sup>al-1 <i>hp</i></sup> (the parent strain) after QA treatment. Nevertheless, <i>rdrp</i>-3 <i>gfp</i> induction has not been confirmed as of yet. However, if QA induction of the al-1 hairpin has the expected effect on <i>rdrp</i>-3 <i>gfp</i>-tagged transcription, why is RdRP-3 GFP protein not detected? First, it is possible that transcription of <i>rdrp</i>-3 <i>gfp</i> functions normally but RdRP-3 GFP proteins are not expressed. To test this, western blot analysis using an anti-GFP antibody can be carried out to check for RdRP-3 GFP expression. Second, the RdRP-3 GFP protein may be expressed but not fluoresce, due to the conformation of the RdRP-3 GFP protein.

In other investigations of MSUD proteins, GFP fusion proteins functionally complement the defective phenotype of the respective null strains (Shiu et al., 2006; Alexander et al., 2008). In these studies GFP is driven by an inducible promoter <i>cgg</i>-1 and the constructs were targeted to the <i>his</i>-3 locus. In my experiment, the <i>gfp</i> tag is fused to the C-terminus of endogenous <i>rdrp</i>-3 and RdRP-3 GFP expression is driven by its own promoter. It appears that RdRP-3 GFP is not functional. Most likely the insertion of the <i>gfp</i> tag at the endogenous <i>rdrp</i>-3 locus interferes with the targeted protein. Even though the <i>rdrp</i>-3 <i>gfp</i>-tagged transformants are heterokaryons they have a defective growth and conidiation phenotype. Hence it is likely that the presence of the <i>gfp</i> tag in all nuclei is lethal. However, the <i>rdrp</i>-3 deletion strain has no known abnormal phenotype during either sexual or asexual development. It seems that loss of <i>rdrp</i>-3 does not affect growth but insertion of GFP to the C-terminus of RdRP-3 results in reduced conidiation. It is possible that RdRP-3 plays an indirect role in the developmental process via its interaction with other proteins. Perhaps fusion of GFP to RdRP-3 alters the protein conformation of RdRP-3 and it is no longer able to interact with its partners or disrupts the complex resulting in growth and developmental defects. Alternatively, insertion of the <i>gfp</i> tag affects the stability of <i>rdrp</i>-3 mRNA or protein. If RdRP-3 interacts with other proteins to form a stable complex, conformational changes of the RdRP-3 GFP fusion protein affects protein interactions, and perhaps the RdRP-3 protein alone is less stable and has a shorter half-life. It is also questionable whether the RdRP-3 GFP fusion proteins are fluorescent. Perhaps the folding of RdRP-3 GFP protein is altered, affecting fluorescence of GFP. In my project, the <i>gfp</i> tag is inserted at
the C-terminus of RdRP-3. An alternative strategy is to tag the N-terminus of the protein or use smaller tags. For example, in comparison to the gfp tag consisting of 238 amino acids, the c-Myc tag is smaller containing only 10 amino acids and presumably it is less likely to interfere with the conformation of RdRP3. Moreover, I only examined rdrp-3 gfp-tagged transformants during vegetative growth. It is possible that RdRP-3 is specifically expressed during sexual development. For example, SAD-2-GFP was not detected in vegetative mycelia or conidia but only in perithecial cells after fertilisation (Shiu et al., 2006).
5. CONCLUSION AND FUTURE WORK

A genome surveillance system of quelling and meiotic silencing by unpaired DNA provides Neurospora a tight control of transposons and repeats. As a result, the Neurospora genome has a relatively low number of transposons and repetitive content. Dissection of quelling and MSUD pathways in Neurospora has greatly furthered our understanding of RNAi mechanisms in eukaryotes. Neurospora QDE-1 and SAD-1 are key components of quelling and MSUD pathways for dsRNA synthesis. In this project, I investigated whether the third RdRP plays a role in quelling and MSUD. RdRP-3 contains a RdRP domain and a RRM domain. My data demonstrates that rdrp-3 is not required for transgene-induced quelling or MSUD. I have generated dsRNA expressing strains which can be utilised in a small RNA sequencing experiment. Data generated from an RNA-Seq experiment will be useful to gain insights into the function of RdRP-3. For example, by comparing small RNA in wild-type and rdrp-3 deletion background. If a certain type of small RNA is only present in wild-type but not in the rdrp-3 deletion background, this would indicate that rdrp-3 is required for small RNA production. No evidence so far for secondary small RNA amplification has been reported in quelling or MSUD. It is worthy to investigate whether rdrp-3 is involved in amplifying signals in these silencing mechanisms. Furthermore, both quelling and MSUD mechanisms are well understood. It seems that two distinct sets of components participate in quelling and MSUD. It would be interesting to know whether these components have redundant function in both pathways.

I cannot, however, rule out the possibility that RdRP-3 plays no role in RNA silencing. My work showed that insertion of the gfp tag displays a growth deficient phenotype. Perhaps RdRP-3 or proteins interacting with RdRP-3 play a role in development. Other tags such as the c-Myc tag or FLAG-tag are alternative choices for epitope tagging. RdRP-3 protein expression has not been investigated in my work. Epitope tagged RdRP-3 protein will allow us to determine RdRP-3 protein expression. It can also be used in immunoprecipitation to identify interacting partners of RdRP-3. By identifying interacting partner proteins, we may obtain clues to understand the possible functions of RdRP-3. In conclusion, I have created tools which can be utilised in the future to help determine the function of RdRP-3.
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