A reporter for dsRNA response in *Neurospora crassa*

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**A B S T R A C T**

In the filamentous fungus *Neurospora*, the production of dsRNA can elicit a dsRNA-induced transcriptional response similar to the interferon response in vertebrates. However, how fungi sense the expression of dsRNA and activate gene expression is unknown. In this study, we established a dsRNA response reporter system in *Neurospora crassa*. Using the dsRNA-activated RNA-dependent RNA polymerase gene *rrp-3* promoter, we created an expression construct (pRRP-3::Myc-Al-1) and introduced it into *al-1KO* mutant. The test dsRNA efficiently induced pRRP-3::Myc-Al-1 expression in the *al-1KO* mutant, resulting in conidia color switching from white to yellow. These results confirm that the dsRNA response is regulated at the transcriptional level and this reporter system can be used for future studies in dsRNA response in filamentous fungi.

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**1. Introduction**

Double-stranded RNA (dsRNA) is formed in cells by DNA- or RNA-dependent synthesis and known to elicit two types of cellular defense responses. The first one, called RNA silencing, is a genetic regulatory mechanism that targets host genes and RNA species and protects cells against invading nucleic acids of viruses or mobile genetic elements. RNA interference (RNAi) [1] pathway plays important roles in transposon silencing and viruses neutralizing in animals and plants [2–4]. During RNAi, dsRNA from viral replication or the transcription of transposable elements and repetitive DNA sequences are recognized and cleaved by the RNase III enzyme Dicer to yield 20–25 nt short interfering RNA (siRNA) duplexes [5]. These siRNA are then incorporated into an RNA-induced silencing complex (RISC) that mediates the degradation of mRNAs with high sequence complementarity to the siRNA [6]. Many transposons and repetitive DNA sequences in *Arabidopsis thaliana* are transcriptionally silenced and methylated by a RNAi-mediated mechanism [7–9]. Thus, RNAi pathway is thought to be an evolutionarily conserved defense system against viruses and transposons in eukaryotes [10,11]. As in animals, the RNA interference pathway has been demonstrated to be important for transposon silencing in *Neurospora* [12,13]. In *Neurospora*, the RNAi pathway is also essential for gene silencing induced by dsRNA or transgenes [14,15].

QDE-2 is an Argonaute protein and the core component of the RISC complex associated with siRNA [16–18]. *Neurospora* DCL-2 is responsible for most of the siRNA-generating activity [19].

Another type of cellular defense response educeed by the production of dsRNA in eukaryotic cells is dsRNA-induced transcriptional response. The introduction of dsRNA into the cytoplasm of mammalian cells usually leads to a strong antiviral response [20]. This response can be mediated by a number of dsRNA sensors, including PKR (dsRNA-activated protein kinase) [21], the TLR3 (Toll-like receptor 3) [22], the helicases MDA5 (melanoma differentiation associated protein 5) and RIG-I (retinoic acid inducible gene 1) [23]. In filamentous fungus, it was shown first in *Neurospora* [18] and later in *Cryptococcus* [24,25] that the expression of dsRNA can elicit a dsRNA-induced transcriptional response similar to the interferon response in vertebrates. In *Neurospora*, dsRNA induced the transcription of both *qde-2* and *dcl-2*, two of the central components of the RNAi pathway, and the induction of QDE-2 by dsRNA is required for efficient RNAi [18]. The RNAi components show strong response to the induction of dsRNA, suggesting that the RNA interference (RNAi) pathway might provide a mechanism that eliminates dsRNA in cells. In addition, a genome-wide search reveals that additional RNAi components and homologs of antiviral and interferon-stimulated genes are also dsRNA-induced genes in *Neurospora* [18]. Among those, the expression of *rrp-3* (one of the three RNA-dependent RNA polymerases, RdRP) [26] could be dramatically induced about 30 folds by dsRNA expression [18]. Generally, dsRNA, which is produced by an RdRP and is cleaved into siRNA by Dicer, is the result of invading viruses or mobile genetic elements. In plants and *Caenorhabditis elegans*, RdRP uses
siRNA as primers to generate secondary dsRNA [27], which can then be processed into secondary siRNA by Dicer [6,28,29].

In Neurospora, the dsRNA response is independent of the Dicer enzyme since robust dsRNA-induced gene expression was maintained in the dcl double mutants (dcl-1<sup>ko</sup>dcl-2<sup>ko</sup>), indicating that dsRNA, not siRNA, is the trigger for the dsRNA response [18]. The observation that the central components of the RNAi pathway and 60 other genes were induced by dsRNA suggests the existence of a transcription-based dsRNA response program. The lack of Neurospora homolog for mammalian PKR and TLR3 genes suggests a novel dsRNA-sensing and transcriptional activation pathway in this organism. However, how dsRNA-sensing and transcriptional activation pathway in this organism sense the production of dsRNA and activate gene expression is still a mystery. To understand the mechanism of the dsRNA response, it will be important to have a sensitive and reliable reporter system that can detect the production of dsRNA and the dsRNA-induced gene activation.

Here we created transgenic al-1 strains (albino mutant) in which the promoter of rrp-3, a known dsRNA activated gene in Neurospora, was fused with a Myc-tagged al-1 gene. We demonstrated that this reporter strain can monitor the production of dsRNA efficiently after inducible expression of fhd dsRNA and fwd1 dsRNA by causing a color change of Neurospora from white to yellow. Furthermore, the molecular evidence further proved that this reporting system provided a reliable way to report the production of dsRNA in Neurospora. Such a reporter system will be a useful tool for future studies of dsRNA response program in this organism.

2. Materials and methods

2.1. Strains and culture conditions

In this study, 87-3 (bd, a) was used as the wild-type strain. The ku70<sup>ko</sup> (bd, a) strain was used as the host for al-1 gene deletion. The al-1<sup>KO</sup> (bd, ku70<sup>ko</sup>, a), the al-1<sup>ko</sup> (bd, his-3, a) strains were created in this study. 301-6 (bd, his-3, A) and al-1<sup>ko</sup> (bd, his-3) strains were the host strains for the his-3 targeting constructs. Liquid culture conditions were the same as described previously [30]. For QA-induced gene expression, 0.01 M QA (pH 5.8) was added into liquid medium containing 1% glucose, 50 ng/ml biotin, and 1.5% agar.

2.2. Generation of the al-1<sup>KO</sup> strain

The entire open reading frame (ORF) of the al-1 gene (NCU00552) was deleted by replacement with the hygromycin resistance gene (hph) [32]. Briefly, the entire ORF knockout cassette of al-1 gene was created by PCR. The primer sequences for upstream and downstream recombination fragments are FWD1. A: 5'-GCTTCAGACATTCACCCGCT-3'; RI: 5'-GAAGATCTGGCGGGCGAGAGGATAATTTG-3'. The longer fragment contains 124-bp unique sequence that shares 448 bp overlap were amplified covering FWD1 amino acids 89–254 (primers FWD1. 47–238 (primers FWD1. A: 5'-GGTCCGTCCGCACTCAATTTAGG-3'). The shorter fragment contains 100-bp unique sequence that shares 36-bp overlap were amplified covering Al-1 amino acids 301–6 (primers FWD1. A: 5'-AAATGCGGAG-3').

The al-1<sup>KO</sup> (bd, his-3) strains were obtained from the cross between al-1<sup>ko</sup> (bd, ku70<sup>ko</sup>, a) and 301-6 (bd, his-3, A) strains.

2.3. Plasmids

2.3.1. Construction of pRRP-3::Myc-His-Al-1

To make the his-3 targeting pqa-Myc-His-1 construct, a PCR fragment containing the entire Al-1 ORF and its 3'UTR was amplified and cloned into pqa-Myc-His resulting in pqa-Myc-His-1. Primer sequences for al-1 gene amplification are Al-1.EcoRI: 5'-GAATTACCAAGACTTACAGACAAATGGC-3' and Al-1.rev.Smal: 5'-TCCCCCGGGAGAAAGAGAGACGGCATGAGG-3'. The pqa-Myc-His-1 construct was then transformed into a wild-type (301-6) or an al-1<sup>KO</sup> (bd, his-3) strain. Western blot analyses were used to identify positive transformants with expressing the Myc-tagged Al-1 proteins using a monoclonal c-Myc antibody (9E10, Santa Cruz Biotechnology). The pRRP-3::Myc-His-1 was generated by inserting Myc-His-1 with his-3 locus PCR fragment from pqa-Myc-His-1 plasmid and rrp-3 promoter with its 5'UTR PCR fragment from Neurospora genomic DNA into pBluescript II SK(+) vector. Primer sequences for Myc-His-1 with his-3 locus fragment and rrp-3 promoter with its 5'UTR fragment are Al-1. BamHILigII: 5'-GGGATCCGATCTATGAGAGAAAAAGCGTTTTCTTC-3', Al-1.rev.Apal: 5'-GGAAGAGGGCCGGCCTGACGGCTAT-3' and Rrp-3p.rev.BglII: 5'-ATAAGATCTGGCGGGCGAGTTTGAAATCACCCTTTC-3', Rrp-3p.rev.BglII: 5'-GAAGATCTGGCGGGCGAGATATTTTG-3'.

2.3.2. Construction of pqa-dsfwd1

We used the procedures described previously [14] to make the fwd1 targeting dsRNA expression plasmid pqa-dsfwd1. Briefly, the plasmid (pqa-dsfwd1) was constructed by inserting an inverted repeat gene fragment corresponding to the fwd1 gene (NCU04540.3) under the control of the qa-2 promoter. Two fwd1 PCR fragments sharing 448 bp overlap were amplified covering fwd1 amino acids 47–238 (primers FWD1.HindIIIa and FWD1.rev.EcoRI) and amino acids 89–254 (primers FWD1.HindIIb and FWD1.rev.Smal), respectively. The longer fragment contains 124-bp unique sequence that functions as the loop in the hairpin. Primer sequences for amplification of these fragments are FWD1.HindIIIa: 5'-CCCAACGTCTAAGGCGGCATCCACCT-3'; FWD1.rev.EcoRI: 5'-GAAGATCTGCTATCATTCCACCT-3'; FWD1.HindIIb: 5'-CCCAACGTCTAAGGCGGCATCCACCT-3'; FWD1.rev.Smal: 5'-TCCCCCGGGCCCTGACGGCTAT-3' and FWD1.rev.Smal: 5'-TCCCCCGGGCCCTGACGGCTAT-3'. The amplified fragments with unique restriction enzyme sites were digested with HindIII and EcoRI or HindIII and Smal, respectively. Then these digested PCR fragments were mixed with the EcoRI/Smal digested vector pDE3DH4.2a for a three-way ligation reaction, resulting in pqa-dsfwd1 plasmid.

2.4. Construction of the al-1<sup>KO</sup>, pRRP-3::Myc-His-1 strain

pRRP-3::Myc-His-1 construct was transformed into an al-1<sup>KO</sup> (bd, his-3) strain for targeting on the his-3 locus. The colonies of the transformants were selected on the minimal medium plates. PCR analysis was performed to confirm the positive transformants with insertion of pRRP-3::Myc-His-1 on the his-3 locus.

![Fig. 1.](image-url)
2.5. Protein analysis

The protein extraction, quantification and Western blot analysis were performed as previously described. For Western blot analysis, equal amounts of total protein (40 μg) were loaded in each lane of SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membrane, and Western blot analysis was performed using anti-FWD-1 serum, anti-FRH serum or c-Myc antibody, respectively.

3. Results

3.1. Generation of al-1 knockout strain by gene replacement

The al-1 gene (NCU00552) that encodes a carotenoid biosynthetic enzyme for orange color of Neurospora conidia was chosen as the reporter for production of dsRNA. To create the host strain for his-3 targeting epitopic pRRP-3::Myc-His-Al-1 plasmid, we...

Fig. 2. The flow chart for creating the reporting strain of dsRNA production. The al-1 gene was cloned on pqa-Myc-His with his-3 locus vector to generate the pqa-Myc-His-Al-1 expression construct. To generate the rrp-3 promoter-driven Myc-His-Al-1 with his-3 locus construct, Myc-His-Al-1 with his-3 gene fragment from pqa-Myc-His-Al-1 expression construct and rrp-3 promoter with its 5’UTR fragment were inserted into pBluescript SK(+) vector, resulting in pRRP-3::Myc-His-Al-1 with his-3 gene. Then this construct was introduced into the al-1KO with his-3 defective strain by electroporation to target its his-3 locus.
that Myc-tagged Al-1 protein is functional. blot analysis using the c-Myc antibody (data not shown), indicating conidia color change from white to orange (Fig. 3B) and by Western molecular weight in the presence of QA was confirmed by Western expression of Myc-Al-1 shown on slants in the presence of QA. (C) The slant phenotype of the al-1KO, pRRP-3::Myc-His-Al-1 strains in the absence of histidine. Next, a fragment containing Myc-His-Al-1 and its downstream his-3 locus sequence (wild-type his-3 gene) was amplified from pqa-Myc-His-Al-1 plasmid and inserted into the polyclonal sites of the pBluescript SK(+) vector resulting in pMyc-His-Al-1 + his-3 construct (Fig. 2). To generate a pRRP-3::Myc-His-Al-1 + his-3 construct, the upstream sequence (1795 bp) of RRP-3 ORF that contained rrp-3 promoter and its 5′UTR was amplified from genomic DNA and inserted at the upstream of Myc-His-Al-1 ORF of pMyc-His-Al-1 + his-3 plasmid (Fig. 2). This construct was then transformed into the al-1KO (bd, his-3) strain for targeting his-3 locus. The colonies grown on minimal plates were picked up and cultured on minimal slants. PCR analysis confirmed that rrp-3 promoter sequence and al-1 sequence were integrated into his-3 locus in these transformants. As shown in Fig. 3C, the resulting strains exhibited the milk white color that was quite different from the wide-type strain, indicating that newly created strains were suitable for color switching experiment.

3.2. Constructing the reporting strain for dsRNA response

To create the reporter strain for dsRNA response, we decided to create a his-3 targeting construct in which the expression of al-1 is under the control of the rrp-3 promoter (Fig. 2). First, we generated a pqa-Myc-His-Al-1 construct, which possessed a Myc-Tagged Al-1 ORF and its 3′UTR sequence under the control of the quinic acid (QA)-inducible promoter [30] (Fig. 2). To monitor the expression of Myc-tagged Al-1 in Neurospora, we introduced the pqa-Myc-His-Al-1 construct into the wild-type strain. As shown in Fig. 3A, the expression of Myc-Al-1 protein with the predicted molecular weight in the presence of QA was confirmed by Western blot analysis. To further confirm whether Myc-tagged Al-1 protein was functional, this construct was also transformed into an al-1KO (bd, his-3) strain. The expression of Myc-Al-1 was confirmed by conidia color change from white to orange (Fig. 3B) and by Western blot analysis using the c-Myc antibody (data not shown), indicating that Myc-tagged Al-1 protein is functional.

3.3. frh dsRNA triggered the conidia color switching of the reporting strain

Cheng et al. previously developed a method that can inducibly express dsRNA from inverted repeat sequences to silence gene expression in Neurospora [14,15]. Previous studies showed that the Neurospora essential gene frh (NCU03363) could be down-regulated by expression of frh-specific dsRNA [14] and the dsal-1 can induce the expression of qde-2, dcl-2 and rrp-3 genes [18]. To test whether the reporting strain could respond to the production of frh dsRNA, pqa-dsfrh construct was introduced into the reporting strain by co-transformation with pBT6 plasmid (containing the benomyl resistance gene from Fungal Genetic Stock Center). Forty milk white colonies were picked up from the selective plates with methyl-2-benzimidazolecarbamate (10 μg/ml) to minimal slants. The expression of the inverted repeats of the frh gene is controlled by the QA-inducible promoter, so that the addition of QA to the medium will lead to the production of frh dsRNA. Then the conidia of each transformant were inoculated on minimal slants with 10⁻⁵ M QA. As shown in Fig. 4A, the presence of QA led to conidia of four transformants with the dsRNA constructs to turn the color from white to yellow on slants, suggesting that the expression of RRP-3::Myc-Al-1 is activated by frh dsRNA. In contrast, QA had no effect on the other transformants with the dsRNA constructs (labeled with White) and the control strains (without the dsRNA constructs), suggesting that the amount of dsRNA produced endogenously is low and not enough to induce the expression of RRP-3::Myc-Al-1.

Because the production of frh dsRNA also can trigger the RNAi of frh gene [14], the increase of RRP-3::Myc-Al-1 expression for frh dsRNA response might be coupled by frh RNAi in the color-switched transformants. To examine this, we further tested the effects of dsfrh on the expression of Myc-Al-1 in the color-switched transformants by Western blot analysis. The signals of FRH protein were dramatically reduced by the addition of 10⁻³ M QA in liquid media, whereas robust FRH proteins were detected in the cultures without QA induction and in the white strain and blank strain (without pqa-dsfrh) (Fig. 4B). As shown in Fig. 4B, confirming that the production of frh dsRNA was reported reliably by the color switching of conidia. Furthermore, corresponding to the knockdown of FRH protein in the yellow transformants, the expression of Myc-tagged Al-1 protein driven by rrp-3 promoter was significantly increased in these strains, while no change was observed in the white transformant and the blank strain (Fig. 4B). These results demonstrate that rrp-3 promoter was dramatically induced by the production of frh dsRNA. However, as shown in Fig. 4B, the efficiency of FRH silencing was higher in the second strain (lane 6) that expressed lower Myc-Al-1 level, compared to the third strain (lane 8) that
expressed higher Myc-Al-1 protein, suggesting that the strength of dsRNA response may be not strictly correlated with the gene silencing efficiency. These data indicate that dsRNA can indeed result in the transcriptional activation of \textit{rrp-3} expression. Similar to the previous finding \[14\], the reporter strain with pqa-dsf rh exhibited the defects in circadian conidiation rhythms and in growth when the race tube assay was performed in the presence of various concentrations of QA (Fig. 4C). Taken together, these results demonstrate that the reporter strain can monitor the production of \textit{frh} dsRNA by conidia color switching.

### 3.4. fwd1 dsRNA also induced the color switching of the reporting strain

\textit{FWD1} is another well-studied component of the \textit{Neurospora} circadian clock and is not essential for cell viability in this organism. To further test reliability of the dsRNA sensing in this reporter strain, we created a pqa-dsfwd1 construct using the same strategy as the pqa-dsf rh plasmid \[14\]. After co-transformation of pqa-dsfwd1 construct with pBT6 to the reporting strain, 40 milk white colonies were picked up from plates with methyl-2-benzimidazolecarbamate. As shown in Fig. 5A, three transformants exhibited color switching from white to yellow on slants with 10^{-3} M QA, indicating that the reporter strain could monitor the production of \textit{fwd1} dsRNA induced by QA. Western blot analysis detected the down-regulation of \textit{FWD-1} with the presence of QA in the color-changed strains by RNAi, but not in those without QA and in the white strain and control strains (Fig. 5B). The inducible response of \textit{rrp-3} promoter to the \textit{fwd1} dsRNA production was also confirmed by Western blot analysis using c-Myc antibody (Fig. 5B). Consistent with the previous study \[33\], the reporter strain with pqa-dsfwd1 exhibited defects of circadian conidiation rhythms in the presence of high concentrations of QA by race tube assay (Fig. 5C). Together, these results confirmed that the newly created reporter strain can monitor the production of double-stranded RNA efficiently through conidia color switching.

### 4. Discussion

The conidia color switching from orange to albino (white) is a frequently used phenotypic reporter of the gene silencing by quelling and RNA interference in \textit{Neurospora} \[34–36\]. In this study, we created a dsRNA reporter strain by transforming the \textit{rrp-3} promoter-driven Myc-Al-1 expression cassette into an \textit{al-1}^{KO} mutant. We showed that the production of dsRNA led to the inducible expression of pRRP-3::Myc-Al-1, which converted the albino strains into yellow strains. Furthermore, the molecular results demonstrated that the reporting strain monitored the production of dsRNA efficiently after inducibly expressing of \textit{frh} dsRNA and \textit{fwd1} dsRNA by causing a color change of \textit{Neurospora} from white to yellow. Since these strains produce different dsRNAs, the color change of conidia is dependent on the production of...
dsRNA rather than gene-specific dsRNA. Thus, the production of double-stranded RNA can be easily read out by conidia color switching in this strain.

Although there is no known virus to infect *N. crassa*, almost all known components of the *Neurospora* RNAi pathway are activated by expression of *fwd1* dsRNA [18]. However, dsRNA strongly induced host defense response in *Neurospora*, such as the activations of IFN-stimulated and antiviral genes [18], suggesting that the role of RNA silencing in fungi has been assumed to be primarily defense against invasion by foreign nucleic acids. Organisms have mechanisms that detect and destroy invading nucleic acids of viruses or mobile genetic elements. Recently, RNA silencing pathway has been shown to serve as an antiviral defense mechanism in fungi [24] and *Cryphonectria parasitica* responds to hypovirus and mycoreovirus infections with a significant increase (12- to 20-fold) in *dcl-2* expression [37]. The viral immunity begins with recognition of viral dsRNA or siRNA cleaved by dicers. The underlying mechanisms in distinct gene silencing phenomena in different genetic systems, such as cosuppression in plants, quelling in *Neurospora* and RNAi in animals, are very similar, indicating an ancient pathway.

In *Neurospora* and *Cryphonectria*, the production of dsRNA can elicit a dsRNA-induced transcriptional response similar to the interferon response in vertebrates. In vertebrates, the transmembrane Toll-like receptors and the cytosolic NOD-like receptors and RIG-I-like DExD/H box RNA helicases are among the best characterized families of dsRNA response factors [38,39]. The cytoplasmic dsRNA often triggers the interferon response and this response plays a crucial role in antiviral immunity in vertebrates. Thus, the interferon system and RNAi make use of RNA helicases to respond dsRNA to initiate respective response [38,39]. The lack of *Neurospora* homologs for mammalian PKR and Toll-like receptor 3 genes suggests a novel dsRNA-sensing and transcriptional activation pathway in this organism. The signaling pathway that triggers the transcription-based response by dsRNA is not known and need to study in these fungi. Although the methods for detecting the RNAi efficiency already are established in several systems, an approach for monitoring the production of double-stranded RNA is not available. Based on this aim, we developed a reporter system in *Neurospora* that can detect the production of dsRNA.

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**Fig. 5.** Production of *fwd1* dsRNA triggered the conidia color switching of the reporting strain. (A) Conidia color switching in the *al−1 ko, pRRP-3:Myc-His-Al-1* transformants induced by expression of *fwd1* dsRNA. Blank strain (transformant with pRRP-3:Myc-His-Al-1 construct only). White strain. WT (bd, a). (B) Western blot analyses showing the down-regulation of *fwd1* gene by *fwd1* dsRNA and the expression of *al−1* gene driven by activating *rrp-3* promoter. (C) Race tube assays showing the conidiation rhythms of the *dsfwd1* strain in DD on race tubes containing different concentrations of QA after entrainment by light. Black lines indicate the growth fronts every 24 h.
and the defects of circadian clocks, suggesting that the dsRNA proceeded to siRNA efficiently and the level of dsRNA was reduced. These data suggest that the cleavage of dsRNA by dicers may indeed attenuate the response to dsRNA. As we have shown in this study, the expression of RRP-3::Myc-Al-1 induced by two different dsRNAs only restores the white conidia of al-KO strain to yellow, not orange of the wild-type strain (Figs. 4A and 5A). If the dsRNA molecules could not be efficiently processed by dicers and accumulated at high level without inducing silencing, the expression of RRP-3::Myc-Al-1 will respond strongly to these types of dsRNAs by color change. The genes involved in stress response are also a major class of dsRNA-activated genes in Neurospora [18], suggesting that the reporter strain may be suitable to monitor the production of dsRNA in different stress conditions. Thus, this reporter system will be a very useful tool for future studies of the dsRNA response program in Neurospora. A similar response in RdRP gene expression following virus infection or inducible dsRNA in plants or in vertebrates has not yet been reported, possibly due to complications posed by multiple RdRPs in plants and the prominence of the interferon response in vertebrates. The strategy of our dsRNA reporting strain also provides an idea for using the specific RNA-dependent RNA polymerases in other eukaryotic systems to monitor the production of dsRNA and detect dsRNA response program.

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