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The RNA-dependent RNA polymerase, QDE-1, is a rate-limiting factor in post-transcriptional gene silencing in *Neurospora crassa*

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ABSTRACT

The RNA-dependent RNA polymerase (RdRP) *qde-1* is an essential component of post-transcriptional gene silencing (PTGS), termed 'quelling' in the fungus *Neurospora crassa*. Here we show that the overexpression of QDE-1 results in a dramatic increase in the efficiency of quelling, with a concomitant net increase in the quantity of *al-1* siRNAs. Moreover, in overexpressed strains there is a significant reduction in the number of transgenes required to induce quelling, and an increase in the phenotypic stability despite progressive loss of tandemly repeated transgenes, which normally determines reversion of a silenced phenotype to wild type. These data suggest that the activation and maintenance of silencing in *Neurospora* appear to rely both on the cellular amount of QDE-1 and the amount of transgenic copies producing RNA molecules that act as a substrate for the RdRP, implicating QDE-1 as a rate-limiting factor in PTGS.

INTRODUCTION

Post-transcriptional gene silencing (PTGS) mechanisms are highly conserved: quelling in fungi, co-suppression in plants and RNA interference (RNAi) in animals all occur due to the presence of foreign nucleic acid sequences such as transgenes, transposons and viral RNAs or double-stranded RNA (dsRNA) (1). The silencing mechanism is based on the cleavage of dsRNA by dicer (2,3), producing small interfering 21–25 nucleotide (nt) RNAs (siRNA) which, in conjunction with the RNA-induced silencing complex (RISC) (4), degrade all homologous mRNAs. While dsRNA has been shown to trigger PTGS directly (5), it is still unclear how dsRNA originates in transgene-induced silencing. Current models (6,7) propose that aberrant single-stranded transgenic transcripts (abRNA) are converted to dsRNA by a cellular RNA-dependent RNA polymerase (RdRP). The identification of the quelling-defective gene *qde-1* in *Neurospora* was the first experimental evidence of the involvement of an RdRP in PTGS (8). QDE-1 RdRP activity *in vitro* was shown to catalyse *de novo* or primer-independent RNA polymerization on a single-stranded RNA (ssRNA) template (9). As well as initiating the transgene-induced silencing mechanism, RdRP may also be responsible for the amplification and maintenance of the silencing signal by synthesis of secondary dsRNA trigger molecules, which in turn would be processed into secondary siRNAs.

In general, the introduction or the direct expression of dsRNA in a eukaryotic cell is sufficient to initiate silencing. When transgenes are introduced in plants and fungi, however, this sequence-specific degradation does not always occur. As the mere presence of transgenic DNA, although necessary, is not sufficient to activate silencing, it has been proposed that only particular transgenic loci are able to work as silencing inducers. In plants for instance, highly expressed transgenes are better inducers than poorly expressed ones (10), suggesting that transgenic mRNAs may only trigger silencing when they exceed a given threshold. Moreover, a correlation between the presence of tandemly arranged transgenic loci and the occurrence of silencing has been observed in both plants and fungi (11,12). It has therefore been suggested that tandem transgenic repeats are good inducers of silencing, even without a high rate of transcription, because they produce RNAs that are somehow 'aberrant', which are specifically used as substrates for RdRPs. However, the presence of a tandem repeat per se is not sufficient to elicit a silencing response (13,14). Furthermore, it is still unclear whether the correlation between tandem repeats or highly expressed transgenes and the activation of silencing reflects special features of such transgenes, or suggests the existence of a threshold for either transgenic RNAs or transgenic copy number above which cells activate the silencing machinery. A simple model would be that transgenes or duplicated DNA are able to produce a silencing trigger (i.e. an RNA substrate for RdRP) per se, and only when such a trigger reaches a given threshold is silencing efficiently elicited.

In order to gain insight into the initiation of transgene-induced gene silencing, we decided to overexpress the RdRP *qde-1* in *Neurospora crassa.* This was based on the hypothesis that augmenting the level of QDE-1 may increase conversion of transgenic RNA into dsRNA, leading to the activation of silencing in all transgenic strains, including those that supposedly express the RNA trigger below a given threshold. In this paper we present data showing that the overexpression

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of QDE-1 leads to a dramatic increase in quelling efficiency (i.e. percentage of transgenic strains showing silencing). Moreover, in overexpressed strains there is a significant reduction in the number of transgenes required to induce quelling, and a concomitant increase in phenotypic stability. Thus, the activation and maintenance of silencing in *Neurospora* appears to rely on the relative concentration of both QDE-1 and its transgenic RNA substrates.

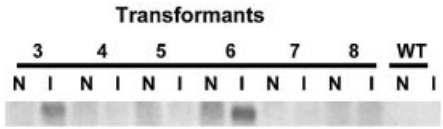

Figure 1. Northern analysis of wild-type strains transformed with the *qde-1* overexpression cassette. Overexpression of *qde-1*, induced by the addition of quinic acid, was revealed in transformants 3 and 6. Both transformants showed the same silencing efficiency (data not shown). Subsequent experiments were carried out with transformant 6 as the *qde-1*-overexpressing strain OQ1. The shadow seen in the non-induced lanes reflects a low level of QDE-1 expression. N, non-induced; I, induced.

MATERIALS AND METHODS

Neurospora crassa strains

The *N.crassa* wild-type strain 74-OR23A (FGSC No. 987) was obtained from the Fungal Genetics Stock Center, University of Kansas (KA, USA). The *qde-1*-overexpressing strain OQ1 was created by transforming a wild type with plasmid pMXY2:*qde-1* and purified by isolation of microconidia to obtain a homokaryon.

Neurospora crassa media and growth conditions

Strains were grown in Vogel's minimal medium for *Neurospora* (NMM) (15) plus benomyl (1 μg/ml) or hygromycin (0.2 mg/ml in slants and liquid media, or 0.3 mg/ml in solid media), as required. *Neurospora* strains transformed with *al-1* were grown for 48 h in liquid medium at 28°C in the dark, with constant shaking at 150 r.p.m. [for growth in induced conditions, liquid medium contained 0.5% sucrose, 1× Vogel's and 0.6% quinic acid (QA)]. Expression of *al-1* was induced by constant saturating light for 20 min at 10 W/m^2.

Plasmids

The *qde-1* overexpression plasmid pMXY2:*qde-1* was created by insertion of the *qde-1* gene into the SmaI site immediately downstream of the inducible QA dehydrogenase (*qa-2*) promoter in the pMXY2 vector that contains benomyl resistance as a selectable marker (16). Plasmid pCSN44 carrying the hygromycin resistance gene (17) was used in co-transformation with the pX16 plasmid carrying the *al-1* sequence (18) to induce silencing.

Transformation

Preparation of *N.crassa* spheroplasts and transformation with recombinant plasmids was performed as described by Vollmer and Yanofsky (19). Transformants were selected by growth on plates containing selectable markers, as required, and subsequently transferred to slants.

Southern analysis

Genomic *N.crassa* DNA was prepared as described in (20). Five micrograms of chromosomal DNA was digested with SmaI/HindIII and fractionated by electrophoresis on a 1% agarose gel. DNA was transferred onto Gene Screen Plus (NEN) filters by capillary blotting. Filters were hybridized according to the manufacturer's instructions at 65°C with a labelled 1.3-kb XbaI–ClaI fragment of the *al-1* gene, which is able to detect both endogenous and transgenic *al-1* sequences. Probes were ^{32}P-labelled using a Random Primed DNA Labelling Kit (Roche) according to the manufacturer's instructions.

Northern analysis

Total RNA was extracted from frozen mycelia. Five micrograms of RNA, quantified by spectrophotometric analysis, were run on a 1% agarose formaldehyde gel and blotted onto HybondN membranes (Amersham Corp). Membranes were hybridized with a 500-bp fragment of the *qde-1* gene amplified by PCR from wild-type DNA using Amplitaq DNA polymerase (Perkin Elmer) using forward primer 5′-GCTGGACACTTGATTGAG-3′ and reverse primer 5′-GTCATTGCGGTCACGAAC-3′, and labelled as described above. The *al-1* probe was same as that used for the Southern analysis. Quantitative analysis was carried out by electronic autoradiography (Packard Instant Imager).

Purification by microconidia

Purification was carried out as described previously (21).

Small RNA purification and hybridization

These were performed as described previously (4). Hybridization was carried out with a labelled XbaI–ClaI fragment of the *al-1* gene.

RESULTS

Overexpression of *qde-1* increases silencing efficiency

In *N.crassa*, the *albino* genes involved in the carotenoid biosynthetic pathway are used as a visual reporter system, since quelling of *albino-1* (*al-1*) encoding phytoene dehydrogenase confers an albino phenotype. The *N.crassa* strain OQ1 (carrying an inducible *qde-1* transgene) (Fig. 1) and a wild-type strain were co-transformed with plasmids pX16 (17), to trigger silencing of *al-1*, and pCSN44, conferring hygromycin resistance, as a marker of co-transformation (see Materials and Methods). Transformants grown in conditions of induced and non-induced *qde-1* overexpression may be divided into those that are 'non-silenced' (NS) or 'silenced' (S), irrespective of *qde-1* overexpression, and those that are 'inducibly silenced' (IS), exhibiting a silenced phenotype only when *qde-1* is overexpressed (Fig. 2). The percentage of transformed wild-type colonies with an albino phenotype due to silencing of *al-1* was 22%, while for OQ1 transformants the level of silencing increased to 92% in induced conditions, compared with 21% observed in non-induced conditions (Table 1). In order to verify that the high level of silencing was a general phenomenon, and not specific to *al-1*, OQ1 was transformed with *albino-2* (*al-2*) encoding phytoene synthase, which is also

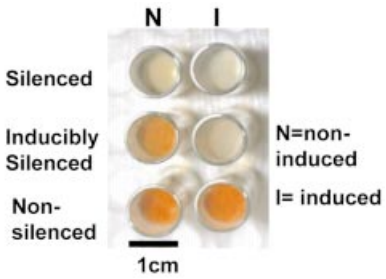

Figure 2. Effect of *qde-1* overexpression on the phenotype of *al-1* transformants. Phenotype of a non-silenced (orange) and a silenced (albino) strain, irrespective of *qde-1* overexpression, and an inducibly silenced strain where silencing (albino phenotype) was a result of *qde-1* overexpression induced by quinic acid.

involved in carotenogenesis. Similar levels of silencing of *al-2*, as indicated by an albino phenotype, were observed (Table 1).

The results above indicate that in some transgenic strains (S strains), the silencing signal is sufficient to induce substantial mRNA degradation, whereas in other transformants (IS) silencing only occurs when *qde-1* is overexpressed. Consistent with this hypothesis, we found that al-1 siRNA levels are increased in induced conditions, both in constitutively silenced (OQ1-S1) and inducibly silenced (OQ1-IS60) strains (Fig. 3). Characteristically, both constitutively and inducibly silenced strains show an accumulation of siRNAs in induced and non-induced conditions, but in the OQ1-IS60 strain the siRNA level, which is lower than in the OQ1-S1 strain, does not appear to be sufficient for mRNA degradation.

The increase in silencing frequency and the isolation of silencing-inducible transgenic strains strongly suggests that the overexpression of *qde-1* acts on a (pre-existing) silencing signal (present in almost all transgenic strains) by increasing the amount of dsRNA synthesized, consequently augmenting the level of siRNAs. It could be argued, however, that the increased silencing frequency is affected by uncontrollable parameters during transformation, altering the number of transgenic copies integrated, the site(s) of integration and/or organization. To overcome this objection we carried out a forced heterokaryon experiment where a non-silenced wild-type transformant containing the *al-1* transgene was forced with the *qde-1* overexpresssing strain (no copies of transgenic *al-1*). The resulting heterokaryon showed a silenced phenotype when QDE-1 was overexpressed by quinic acid. Instead, when the non-silenced wild-type *al-1* transformant was forced with a wild-type strain, no silencing was seen. These data suggest that QDE-1 is a 'limiting factor', since the level of transgenic RNA remained the same in both heterokaryons. In addition, these data support the notion that in a wild-type background a given transgenic locus is able to produce a silencing signal, but that this signal is not always sufficient to induce silencing.

Overexpression of *qde-1* reduced the number of copies required for silencing

Given that a non-silenced (NS) wild-type *al-1* with a few copies can be silenced by creating a heterokaryon with overexpressed *qde-1*, we asked if the number of transgenic copies required to elicit a silencing response is reduced when OQ1 is overexpressed. We therefore carried out a Southern analysis of *al-1* copy number in 52 OQ1 *al-1* transformants, including a wild-type strain as a control for the endogenous copy (giving a 3.1-kb band) (Fig. 4A and B). Tandem arrays of the *al-1* transgene revealed by a 5.5-kb band were present in S strains (OQ1-S1, -5 and -7), confirming previous data demonstrating that a high transgene copy number is required for efficient silencing. In comparison, the IS strains (OQ1-IS9, -10, -20, -26, -13 and -22) harboured fewer copies of the *al-1* transgene, even as few as one or two copies (OQ1-IS10 and -20), indicating that overexpressing *qde-1* compensates for the lower *al-1* copy number, i.e. the number of transgenes required to trigger silencing is reduced when QDE-1 is not limiting. Interestingly, 66% of the NS strains (8 out of 12 strains) contained no copies of *al-1*, indicating that a large portion of the 22% of NS OQ1 *al-1* transformants may have been transformed with pCSN44 only (conferring hygromycin resistance) and are therefore 'false negatives', suggesting that the actual level of silencing in conditions of *qde-1* overexpression is ~92% (see Table 1). As quelling is known to progressively revert phenotype back to wild type, which is associated with a loss of tandemly repeated copies over a prolonged culture time (12), and since we have IS strains containing one or two copies, we may expect the stability of the phenotype in overexpressed strains to be increased. We

Table 1. Effect of *qde-1* overexpression on the efficiency of silencing in *al-1* and *al-2* transformants in OQ1 and wild-type (WT) strains

Gene	Non-silenced (%)	Silencing in non-induced conditions (%)	Silencing in induced conditions (%)	Frequency of silencing excluding NS transformants with no *al-1* transgene (%)	Total numbers analysed (*n*)
OQ1 strain					
al-1	22	21	78	92	1400
al-2	23	23	73	90	780
Wild-type strain					
al-1	78	22	22	NA	500
al-2	81	19	20	NA	220

NS, non-silenced; NA, not available.

Colonies were either non-silenced (orange phenotype) or silenced (albino phenotype) irrespective of *qde-1* overexpression, or inducibly silenced (albino phenotype) due to *qde-1* overexpression induced by quinic acid. The frequency of silencing considers only those transformants that contain the *al-1* transgene and was calculated by analysing non-silenced transformants for copy number. Two-thirds (66%) of the NS strains (eight out of 12 strains analysed) contained no copies of *al-1* and are therefore 'false negatives', putting the actual level of silencing in conditions of *qde-1* overexpression at ~92%.

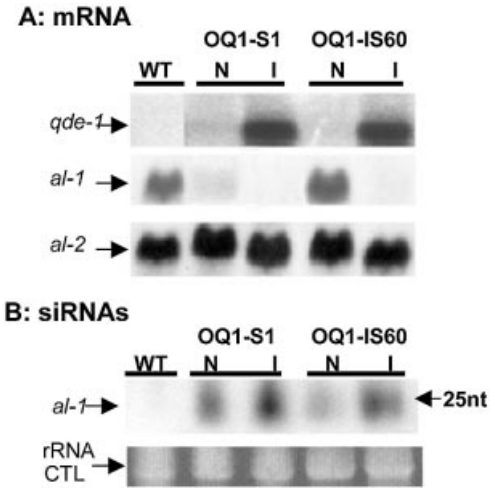

Figure 3. Northern analysis of mRNA and siRNA. (**A**) Northern analysis of *qde-1* and *al-1* mRNA in a wild-type (WT) control and two overexpressed strains in both non-induced (N) and induced (I) conditions. *al-1* mRNA was silenced in OQ1-S1, whereas for OQ1-IS60 it was only silenced when *qde-1* was overexpressed. Expression of the *al-2* gene was assessed for normalization purposes. (**B**) Northern analysis of *al-1* siRNAs in the same strains as in (A). As expected, no *al-1* siRNAs were detected in the wild type, whereas in OQ1-S1 and OQ1-IS60 an increase of *al-1* siRNA was detected under induced conditions. The ribosomal RNA is shown as a control for equal loading.

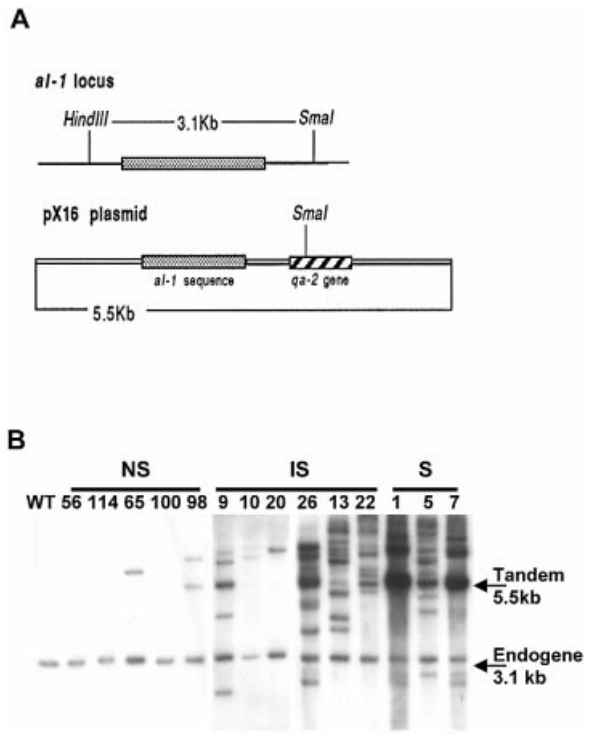

Figure 4. NS, non-silenced; IS, inducibility silenced; S, constitutely silenced. (**A**) Southern analysis of *al-1* copy number in transformed wild-type and OQ1 strains. Schematic representation of the *al-1* endogenous locus and of plasmid pX16 carrying the *al-1* transgene. (**B**) Southern analysis of *al-1* copy number in wild-type and OQ1 strains transformed with pX16. Eleven out of a total data set of 52 are shown. Lane 1: wild type (control); lanes 2–6: non-silenced OQ1 strains; lanes 7–12: inducibly-silenced OQ1 strains; lanes 13–15: silenced OQ1 strains. The 3.1-kb band corresponds to the endogenous *al-1* gene and the 5.5-kb band corresponds to ectopic *al-1* transgenes. The more intense 5.5-kb signal in silenced OQ1 strains denotes tandem arrays of transgenic *al-1* sequences, while diverse bands represent randomly integrated transgene copies. Non-silenced OQ1 transformants harbour only one, two or no *al-1* transgenes.

therefore compared the level of reversion between six OQ1 and five wild-type *al-1* transformants. All transformants were purified by isolation of microconidia to ensure that they were homokaryotic (21). Table 2 shows that wild-type *al-1* strains have a reversion rate of up to 28% in *al-1* transformants. Only one of the OQ1 strains (OQ1-IS14) showed reversion of a single colony out of 5700 descendents. The other five strains showed no reversion. To determine whether these strains still continued to lose copies of the transgene, we analysed 50 single colonies of OQ1-S1, OQ1-IS60 and WT-S3. Whilst OQ1-S1 and OQ1-IS60 showed no phenotypic reversion (Fig. 5A), Southern analysis revealed a loss of the *al-1* tandem array at a rate similar to that of wild-type strains, i.e. ~30% (Fig. 5B). Quantification of copy number using electronic autoradiography revealed that reversion of silencing occurred when less than six transgenic *al-1* copies remained in tandem, whereas in OQ1 strains, silencing was maintained even in the presence of only two copies. This confirms that there is a threshold number of copies required to elicit a silencing response, and that this number is reduced when *qde-1* is overexpressed.

Table 2. Phenotypic reversion of *al-1* transformants of OQ1 and wild-type strains

Transformant	Number analysed	No. of reversions (%)
1. OQ1-S7	764	0 (0)
2. OQ1-S1	1925	0 (0)
3. OQ1-IS13	3160	0 (0)
4. OQ1-IS14	5700	1 (0.017)
5. OQ1-IS60	3570	0 (0)
6. OQ1-IS72	3934	0 (0)
7. WT-S1	1600	1 (0.06)
8. WT-S2	3500	100 (2.86)
9. WT-S3	1880	540 (28)
10. WT-S12	1580	0 (0)
11. WT-S207	1100	70 (6.3)

DISCUSSION

RNA-dependent RNA polymerases are key components of the RNAi machinery. Two different roles have been postulated for these enzymes: first, RdRPs may be involved in converting aberrant RNA molecules produced from silencer loci into dsRNA, or secondly, use siRNAs as primers in the conversion of the target ssRNA into dsRNA, leading to both the degradation of the target RNA by dicer and the accumulation of secondary siRNAs, thus increasing the strength of silencing. Interestingly, we have found that QDE-1 was no longer required upon the direct expression of dsRNA (22). Likewise, in plants, the RdRP SGS2 is not required when PTGS is induced by an inverted repeat (23). These results suggest that the main role of RdRP in transgene-induced gene silencing in *Neurospora* is the conversion of transgenic RNA into dsRNA. In line with this result, in this work we found that QDE-1

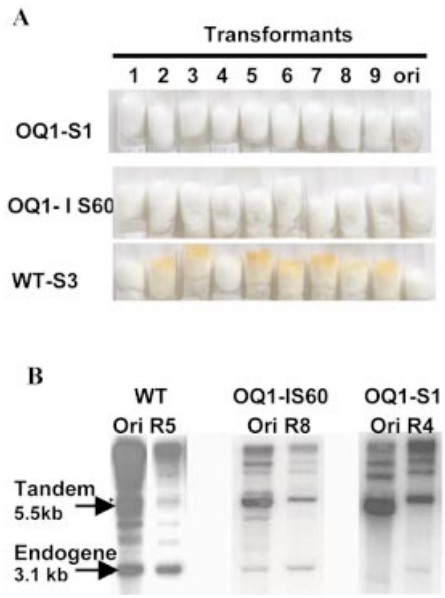

Figure 5. Reversion of silencing in OQ1 and wild-type *al-1* transformants. (**A**) Phenotype of the original culture ('ori') with respect to nine single colonies of a silenced OQ1 transformant (OQ1-S1), an inducibly silenced OQ1 transformant (OQ1-IS60) and a silenced wild-type *al-1* transformant (WT-S3). (**B**) Southern analysis of *al-1* copy number in the original culture (ORI) as compared with revertant R5 of WT-S3, R8 of OQ1-IS60 and R4 of OQ1-S1. Around 30% of the descendants showed a characteristic loss of transgenic copies.

overexpression resulted in an increase in the production of siRNAs. QDE-1 is thought to recognize transgenic RNAs due to some intrinsic 'aberrancy' rendering them substrates for RdRPs. Previous reports have suggested that in fungi and plants, a prerequisite for efficient PTGS is the presence of multiple tandemly inserted copies of a transgene and that the abRNA that results from the transcription of such a multi-copy transgenic locus is the trigger of PTGS (11,12). Such tandems are also proposed to be able to maintain silencing through primed polymerization by RdRP, regenerating dsRNA, where single copies would be exhausted by the sequential use of downstream primers (24). We found that the overexpression of the *qde-1* gene leads to a dramatic increase in the frequency of transgenic strains showing silencing. In fact, we observed that the silencing frequency of *al-1* is ~92% when QDE-1 is overexpressed, compared with 22% observed in a wild-type background, indicating that almost all transgenic strains are able to support silencing. Importantly, we found that one or two copies can elicit a silencing response when *qde-1* is overexpressed, suggesting that rather than a tandem repeat being essential for triggering silencing, every transgenic/repetitive locus possesses the ability to activate silencing. This is in line with early experiments in fungi and plants, which showed that the presence of a tandem repeat *per se* is not sufficient to elicit silencing (13,14). The fact that only a limited number of transgenic strains (those with the highest number of transgenes) show silencing in the presence of a wild-type level of QDE-1 suggests that silencing in *Neurospora* is only activated if a given threshold of transgenic/repetitive copies is reached. In reality, such a threshold may reflect 'quantitative effects', i.e. silencing may be a gradient from weak to strong, rather than on or off, with the overexpression of *qde-1* allowing detection of the silenced phenotype where less transgenic copies are present. Visual inspection of conidial colour in our system does not allow these two models to be differentiated.

Such high levels of silencing are reminiscent of a mutant phenotype seen in *Caenorhabditis elegans*, in which *rrf-3* mutants show hypersensitivity to RNAi (25). The authors suggest that the RRF-3 protein competes with the other required RdRPs RRF-1 and EGO-1 for components or intermediates in the RNAi pathway. We could envision a similar situation here, i.e. that QDE-1 outcompetes the other RdRPs for its RNA substrate, resulting in a higher silencing efficiency. However, mutants in the other two RdRP paralogs of *Neurospora sad-1* and *RdRP-3* show normal (wild type) silencing efficiencies (our unpublished data), indicating that such a mechanism is not at work in *Neurospora*.

Quelling is known to revert by excision of tandemly arranged copies during vegetative growth, therefore suggesting that a threshold number of transgenic copies is required not only to activate, but also to maintain silencing. The fact that we found that the phenotypic stability of silencing is strongly increased when *qde-1* is overexpressed, even though copies are lost at the same rate as in wild type, indicates that high levels of QDE-1 allow the maintenance of silencing even when the number of transgenic copies are reduced by increasing the production of dsRNA and in turn siRNA molecules. This is further demonstrated by the forced heterokaryon experiment, where the same amount of transgenic RNA produced by *al-1* transformed nuclei is able to support silencing only in the presence of the nuclei that overexpress QDE-1. Together our results suggest that in *Neurospora*, silencing activation and maintenance appear to rely on both the cellular amount of QDE-1 and the amount of transgenic copies producing RNA molecules that act as a substrate for the RdRP, implicating QDE-1 as a rate-limiting factor in PTGS.

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