The post-transcriptional gene silencing machinery functions independently of DNA methylation to repress a LINE1-like retrotransposon in Neurospora crassa

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ABSTRACT

Post-transcriptional gene silencing (PTGS) involving small interfering RNA (siRNA)-directed degradation of RNA transcripts and transcriptional silencing via DNA methylation have each been proposed as mechanisms of genome defence against invading nucleic acids, such as transposons and viruses. Furthermore, recent data from plants indicates that many transposons are silenced via a combination of the two mechanisms, and siRNAs can direct methylation of transposon sequences. We investigated the contribution of DNA methylation and the PTGS pathway to transposon control in the filamentous fungus Neurospora crassa. We found that repression of the LINE1-like transposon, Tad, requires the Argonaute protein QDE2 and Dicer, each of which are required for transgene-induced PTGS (quelling) in N. crassa. Interestingly, unlike quelling, the RNA-dependent RNA polymerase QDE1 and the RecQ DNA helicase QDE3 were not required for Tad control, suggesting the existence of specialized silencing pathways for diverse kinds of repetitive elements. In contrast, Tad elements were not significantly methylated and the DIM2 DNA methyltransferase, responsible for all known DNA methylation in Neurospora, had no effect on Tad control. Thus, an RNAi-related transposon silencing mechanism operates during the vegetative phase of N. crassa that is independent of DNA methylation, highlighting a major difference between this organism and other methylation-proficient species.

INTRODUCTION

In many organisms, transposon-related sequences make up a large portion of the genome. There is evidence that some of these sequences have evolved to play important roles in heterochromatin formation, centromere function and gene regulation (1–3). On the other hand, the cell has developed mechanisms to limit the expansion of these elements in order to prevent excessive genome instability. The relatively recent discovery and subsequent dissection of post-transcriptional gene silencing mechanisms (RNAi, cosuppression, quelling) that can act to silence repeated sequences has led to a model in which a double-stranded RNA intermediate, homologous to the targeted gene is processed by the RNAseIII molecule Dicer into siRNAs of 21–25 nt in length (4). siRNAs are subsequently used as guides by the RNA-induced silencing complex (RISC) to degrade homologous transcripts (5). This general model is conserved in a wide range of organisms, thus one proposed explanation for the retention of post-transcriptional gene silencing (PTGS) has centred on a role in limiting the expansion of naturally occurring repeated sequences, such as transposable elements (6). A number of observations support this hypothesis: in Caenorhabditis elegans and Chlamydomonas, several genes are essential to both RNAi- and transposon control-pathways (7–9); siRNAs against transposon sequence have been cloned in both Arabidopsis and Drosophila (10,11). These findings all suggest that there is an ongoing control of transposon proliferation in the host.

In addition to PTGS, transcriptional gene silencing (TGS) mechanisms such as DNA and histone methylation have also been implicated in transposon control. Large swathes of the human and Arabidopsis genomes are methylated, and these methylated areas are rich in transposon-related sequence
(12–14). Moreover, mutations which block DNA methylation lead to increased transcript levels and increased transposition rate of both DNA transposons and retrotransposons (12,15–18). Remarkably, in fission yeast and plants, it has been demonstrated that heterochromatin formation is directed by siRNAs in an Argonaute complex with similarities to the RISC involved in RNAi, suggesting that the processes of PTGS and TGS are intertwined (2,16,19–21).

The phenomenon of ‘quelling’ in Neurospora displays most of the characteristic features of PTGS observed in other organisms (22). In addition to quelling however, Neurospora possesses additional homology-dependent gene silencing mechanisms such as repeat-induced point mutation (RIP) and meiotic silencing by unpaired DNA (MSUD) (23,24). In particular, RIP is able to inactivate duplicated DNA sequences by causing multiple C:G to T:A mutations and cytosine methylation at the pre-meiotic, dikaryotic sexual phase of the life cycle. That the Neurospora genome is littered with RIP-mutated relics of a wide range of transposon families is testament to the efficiency of RIP. Indeed, the Neurospora genome does not reveal the presence of a single active transposon (25).

Despite this, transposition of the LINE1-like, non-LTR retrotransposon Tad has been detected in an African strain, Adiopodoumé, which contains up to 40 copies of this element (26,27). Subsequent analysis showed that this strain was RIP-proficient and that the Tad elements in this strain were fully susceptible to RIP (28,29). To search for an explanation as to why Tad remained active in the RIP-proficient Adiopodoumé strain, we considered the possibility that additional silencing mechanisms, such as quelling, might also be employed in transposon control in Neurospora. To explore this hypothesis, we examined whether Adiopodoumé was quelling-proficient and also monitored the rate of Tad transposition in diverse quelling-defective mutants. A role for DNA methylation in limiting the expansion of artificially introduced Tad elements in Neurospora has also been documented (30). Therefore, in light of the above observations (in Schizosaccharomyces pombe and Arabidopsis) of a link between PTGS and TGS mechanisms, we investigated the relative effects of quelling and DNA methylation on transposon control in Neurospora, and whether these two mechanisms are connected in this species.

We found that the expansion of an experimentally introduced Tad element was particularly pronounced in a qde-2 null background, lacking an Argonaute protein thought to constitute part of the RISC in Neurospora (31). This was evidenced both in terms of increased transcript levels and increased copy number of the Tad elements. Importantly, a similar effect was confirmed when we introduced a qde-2− allele into progeny of a strain harbouring a natural invasion of TAD. Additionally, siRNAs against Tad sequence were found, suggesting that a quelling-like mechanism is responsible for the taming of transposable elements in Neurospora. In support of this, Tad transcripts accumulated heavily in strains lacking both of the Neurospora DICER-LIKE proteins. However, in contrast to quelling, QDE1 (an RdRP) and QDE3 (a DNA helicase) were not required suggesting that, although similar, the quelling- and transposon control-pathways do not entirely overlap (32,33). Notably, we found that the major bulk of introduced Tad elements were not significantly methylated, and furthermore the absence of the DNA methylation machinery had no obvious effect on Tad expression, indicating that in Neurospora, unlike several other organisms, there is no RNAi-directed DNA methylation mechanism for transposon control and instead suggesting that the major determinant is post-transcriptional.

MATERIALS AND METHODS

N. crassa strains: The wild-type (WT) strain 74-OR23A and Adiopodoumé were obtained from the Fungal Genetics Stock Center, University of Kansas, Kansas City (strains FGSC 987 and FGSC 430, respectively). The dim-2− strain (34) was a gift from Eric Selker, University of Oregon. The qde and dcl mutant strains used in this study were obtained by insertional disruption by plasmid insertion, have been previously described in detail elsewhere, and were as follows: 627 (qde-3−), 820 (qde-2−), 107 (qde-1−) DCL1ko (dcl-1−), DCL2ko (dcl-2−) (31–33,35). The double Dicer mutant strain was obtained by the crossing of dcl-1− and dcl-2− strains.

Transformation and growth conditions

Strains were grown in Vogel’s minimal medium for Neurospora (NMM), as described elsewhere (36). Ascospores from crosses were heat-activated to induce germination at 60°C for 30 min, as previously described (37). For Tad transformation experiments, spheroplasts were prepared from the mutant and wild-type strains using the method of Orbach et al. (38). Each strain was co-transformed with 1 μg of the plasmid pCSN44.1, which contains the hph hygromycin-resistance gene as a selectable marker (39), and 5 μg of the plasmid pTad1-1, containing a full-length cloned Tad element (40). Transformations were plated on NMM containing 0.2 mg/ml hygromycin and at least 10 individual colonies from each transformed strain were then purified by three serial platings to purify for homokaryons. Purified colonies were then transferred to solid slant NMM media and arbitrarily referred to as the first asexual ‘generation’. Subsequent generations were propagated by serial transfer of conidia after three days of vegetative growth on slants. Each generation was then grown in liquid NMM for three days, with shaking, at 28°C to produce sufficient mycelia for nucleic acid extraction. Differences in the Tad load between the transformed mutant strains were compared using the Student’s t-test (two-tailed).

Northern and small RNA analysis

Small RNA purification was performed as described previously (41). A riboprobe was transcribed using T3 RNA polymerase (Promega) on a Tad PCR template that contained a T3 promoter site at the 5′ end. To produce a template for ORF1, the primers used were ORF1aT3 (5′-CCGGAATTAACCT-TACAGGGAGAAACTGCTT-3′) and ORF1b (5′-CCAGCAACGACACAGTAC-3′), while for ORF2 the primers used were ORF2aT3 (5′-CCGGAATTAACCTCAC-GAGGATACGTGATGTGAAC-3′) and ORF2B (5′-GGCTCTGCTGTGCAAGACGG-3′), where T3 promoter sites (including adaptor region) are underlined. Prior to hybridization, labelled transcripts were hydrolysed to an average size of 50 nt, 15 volumes of 80 mM sodium bicarbonate and
120 mM sodium carbonate were added to the transcriptional reaction and incubated at 60°C for 3 h. To stop the hydrolysis, 20 μl of 3 M sodium acetate (pH 5.0) was added. Pre-hybridization and hybridization were at 35°C in 50% deionized formamide, 7% SDS, 250 mM NaCl, 125 mM sodium phosphate (pH 7.2), and sheared, denatured, salmon sperm DNA (100 mg/ml). After overnight hybridization, membranes were washed twice in 2× SSC and 0.2% SDS at 35°C for 30 min and once in 20 mM Tris–HCl (pH 7.5), 5 mM EDTA, 60 mM sodium chloride and 10 μg/ml RNase A at 37°C for 1 h to remove unspecific background. Northern analysis of Tad expression was performed using standard protocols (42). A PCR product comprising nucleotides 54–1569 of Tad used as a template to create a 32P-labelled DNA probe. In each blot, ~5 μg of total RNA was loaded per lane. Equal loading was ensured by controlling ethidium bromide staining of the constitutively expressed 28S ribosomal RNA. In comparing transcript levels between different Tad containing samples, densitometric analysis of the hybridization signal (minus control background) corresponding to the full-length 7 kb Tad transcript was performed using the Instant Imager Analysis software programme (Canberra Packard). The values obtained from this analysis were then expressed as a function of Tad copy number to compensate for unequal segregation of Tad elements during the crossing procedure and values were compared between strains using Student’s t-test (two-tailed).

**Southern blot analysis**

Genomic DNA was isolated from frozen mycelia and ~4 μg were digested and run according to standard protocols. For methylation analysis, the isoschizomer pair of DpnI1 (methylation-insensitive) and Sau3A1 (methylation-sensitive) were used. To analyse copy number, we digested either with NdeI and used an internal 602 bp NdeI Tad fragment in order to produce a single hybridizing band whose intensity was proportional to the copy number of Tad (Figure 1), or we digested with EcoRI and used a PCR probe comprising nucleotides 54–1569 of Tad that hybridizes to a band unique for each Tad insertion (not shown). An estimate of copy number was calculated by comparing the total signal in each lane [minus control (non-transformed)]

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**Figure 1.** Tad transposition shows a marked increase specifically in a qde2 mutant background. (A) Schematic representation of the Tad element. NdeI (N) sites and the probe used in the Southern-blot analysis in (B) are indicated. (B) Representative Southern blots in Tad-transformed wild-type (wt), qde-1, qde-3 and qde-2 strains showing hybridization to the Tad probe or the single copy al-2 gene used as a loading control (this gene shows a restriction polymorphism in Adiopodoumé). DNA from a non-transformed wild-type strain (nt) and the Adiopodoumé strain (ADI) containing 40 Tad copies were included as controls. (C) The mean copy number of Tad in each strain after 15 asexual generations is shown. A minimum of 10 independent transformants were analysed for each strain. Error bars represent standard error of the mean.
background] to that of Adiopodoumé DNA, which is reported to have 40 copies of the Tad element (26). As a loading control, all blots were stripped and re-hybridized with either a probe corresponding to a 1397 bp SphI fragment upstream of the single copy dcl-1 gene or a 1213 bp NdeI–BamHI fragment of the single copy al-2 gene. The relative strength of the signal was used to adjust the estimated copy number in each strain.

_Tad_ hybridizations were performed at 68°C in a solution containing 1 M NaCl, 1% SDS, 10% dextran sulphate, 100 µg/ml sheared salmon sperm DNA. After hybridization, two very stringent washes of 40 min at 68°C in 0.1x SSC, 1% SDS were included in order to minimize hybridization to RIPed Tad relics. In crosses of the mutant strains with either qde2TAD8 or Adiopodoumé, the mutant status of the daughter progeny was checked in each case using probes for qde-1, dcl-1, dcl-2, qde-2, as previously described (31,32,35). The probe used to hybridize the ζ-η region when controlling the dim-2 status was amplified from genomic DNA using the primers zeta-F (5'-CGATTAGCGAATCTAAGTG-3') and zeta-R (5'-TTTCTACCACATCTATAGCCG-3').

RESULTS

Adiopodoumé is quelling proficient

To explore the hypothesis that a deficiency in quelling might explain the apparent expansion of _Tad_ in Adiopodoumé, we tested for quelling proficiency by using an assay that involves transformation with a segment of the _al-1_ gene involved in carotenoid biosynthesis. In quelled strains, silencing of the endogenous gene leads to an albino phenotype. The frequency of quelling in Adiopodoumé was comparable to a wild-type strain (Table 1), ruling out the possibility that _Tad_ expansion in Adiopodoumé could be accounted for by a quelling deficiency.

_Tad_ expansion in the _qde_ mutants

To ascertain whether the quelling pathway contributed to control of _Tad_ transposition, we investigated the expansion of a cloned _Tad_ element in various _qde_ mutants. Previous work in our lab had identified three genes essential for quelling: _qde-1_, encoding an RNA-dependent RNA polymerase (RdRP) thought to produce a dsRNA template for Dicer (32); _qde-2_, encoding a PAZ-Piwi domain protein constituting part of RISC (31); _qde-3_, encoding a recQ DNA helicase thought to be upstream of _qde-1_ (33). We used a cloned full-length version of the _Tad_ element, _Tad1-1_ (40), to transform strains that contained insertional disruptions of each of the _qde_ genes. As a control, the wild-type reference strain ORS74a was also transformed. We grew single transformants until conidiation, at which point they were germinated on fresh media and allowed to grow again until conidiation and the cycle was repeated. We referred to each of these vegetative cycles as an asexual ‘generation’. _Tad_-transformed strains were propagated in this way for up to 15 generations, and the copy number and expression levels of _Tad_ were analysed at various timepoints. We performed Southern blots using a probe hybridizing to a single internal NdeI fragment of the _Tad_ element (Figure 1A), the intensity of which is proportional to number of _Tad_ elements in the genome. A representative blot is shown in Figure 1B. We found that in the _wt_ strain, the mean number of _Tad_ copies increased over the course of 15 asexual generations to ~10 copies per genome (Figure 1C). A similar situation was observed in the _qde-1_^{-} and _qde-3_^{-} mutants. However, in a _qde-2_^{-} mutant background _Tad_ showed a very significant increase in transposition rate (P < 0.01), approaching 30 copies per genome. These results show that while _Tad_ is able to achieve a limited rate of transposition in a _wt_ background, this transposition is normally restricted by _qde-2_. Similar results were confirmed when we repeated the experiment using independently created u.v mutants (data not shown). We further analysed the transformed strains by looking at levels of _Tad_ transcripts in each. Again, in the _wt_ background we found a basal level of _Tad_ transcription that was similar in _qde-1^{-}_ and _qde-3^{-}_ backgrounds, while in the _qde-2_^{-} mutants the level of transcription was markedly increased, as expected from the high copy number in this strain (not shown).

Specific role for _qde-2_ in transposon control in _Neurospora_

Although the above results suggested a role for _qde-2_ in the control of _Tad_, we could not rule out that differences in expression and transposition rate were accentuated by varying initial transformation efficiencies with the cloned _Tad_ element between the different mutant strains, either in terms of number or arrangement of transgenes, or possible insertions in transposition ‘hotspots’. In order to definitively confirm a role for the _qde-2_ gene in _Tad_ control, we took a _Tad_-transformed _qde-2^{-} strain and crossed it with a _wt_ strain in order to re-introduce a _qde-2^{+}_ allele. Since RIP inactivates ~50% of unlinked repeated sequences during a cross, we chose a strain (qde2TAD8) that contained a high _Tad_ load (>80 copies) in order to increase the likelihood that intact elements would survive into the daughter progeny.

We noticed an obvious disparity in the levels of _Tad_ expression in the first ‘generation’ growth of daughter progeny that was dependent on the segregation of the _qde-2_ allele; in _qde-2^{-}_ progeny _Tad_ expression was consistently high (Figure 2A, lanes 1, 4 and 5) whereas in _qde-2^{+}_ progeny, the reverse was true (lanes 2, 3 and 6), confirming that _qde-2_ plays an important role in transposon taming in _Neurospora_. In this and subsequent crosses, we have plotted transcription as a function of _Tad_ copy number in order to normalize for uneven segregation of elements during the cross (Figure 2B).

Notwithstanding the role demonstrated above for _qde-2_ in controlling _Tad_ elements introduced initially by transformation, there remained the possibility that this effect might not be representative of a natural invasion of _Tad_. To answer this point, we crossed a _qde-2^{-} strain with a naturally infected strain (Adiopodoumé) in order to segregate _wt_ and mutant alleles of _qde-2_ into the progeny. Again, we observed a

|                              | Wild-type | Adiopodoumé |
|------------------------------|-----------|-------------|
| Orange colonies              | 222       | 231         |
| White colonies               | 99        | 85          |
| Quelling efficiency (%)      | 31        | 28          |
significant de-repression of Tad only in qde-2<sup>−</sup> progeny (P < 0.05) (Figure 3C and D). This demonstrates that our observations on Tad control are representative of a natural scenario of Tad invasion. Furthermore, introducing a mutant qde-1<sup>−</sup> allele into an Adiopodoumè-derived background mirrored the situation observed with introduced cloned Tad elements, in that no derepression was observed in the absence of qde-1<sup>−</sup> (data not shown). This finding is consistent with our previous failure to detect a significant expansion of a cloned TAD element in the qde-1<sup>−</sup> strain (Figure 1B) indicating that the RdRP required for transgene silencing has no obvious role in transposon control in Neurospora.

The two Neurospora Dicer genes (dcl1 and dcl2) are mutually redundant in Tad control

The Dicer enzymes DICER-LIKE1 and DICER-LIKE2 are mutually redundant in the quelling pathway during the silencing of transgenes (35). However, recent reports have demonstrated that in organisms with multiple Dicers, different enzymes are each responsible for processing of different substrates such as microRNA precursors, hairpin dsRNA structures and viral RNA templates (43,44). To explore further similarities between quelling and the Tad control pathway, and also to investigate the possibility of a transposon-specific role for either of the Dicers in Neurospora, we performed a cross between a double-dcl mutant and Adiopodoumè, and analysed Tad expression in the progeny that contained either wild-type, single- or double-dcl mutant genotypes (Figure 3A and B). As a control comparison, we also crossed a qde-2<sup>−</sup> strain with Adiopodoumè (Figure 3C). Similar to the situation in quelling of transgenes (35), we only noticed an obvious increase in Tad expression in the double-dcl mutant (Figure 3A, lanes 3 and 8; Figure 3B), whereas no effect was observed in either of the single mutants. This elevation was similar in magnitude

![Figure 2](image-url) Figure 2. Release of Tad repression segregates with the qde-2<sup>−</sup> allele. (A) A Tad-transformed qde2<sup>−</sup> strain (qde2TAD8) was crossed with a wild-type strain in order to re-introduce a qde-2<sup>−</sup> allele into a Tad background. Expression of the full-length TAD transcript was monitored by northern blot in cultures deriving from six different ascospores (1–6). RNA form a non-transformed qde-2<sup>−</sup> strain (nt) was used as a negative control. The qde-2 status of each ascospore is indicated as ‘+’ (wild-type allele) or ‘−’ (mutant allele). Ethidium bromide staining of the ribosomal 28S RNA was used as a loading control. The estimated copy number of Tad in each ascospore is indicated under each lane. (B) Levels of Tad transcripts were expressed as a function of copy number in order to normalize for differences due to uneven segregation of elements during the cross and the mean calculated for each type of allele. Error bars display standard error of the mean.

![Figure 3](image-url) Figure 3. The two Neurospora dcl genes are mutually redundant in Tad control. Expression of the full-length Tad transcript was monitored by northern blot in cultures grown from individual ascospores deriving from the cross of (A) a double dcl mutant or (C) a qde-2<sup>−</sup> strain with Adiopodoumè. Double dcl<sup>−</sup> progeny are indicated with asterisks. Mutant strains not transformed with Tad (nt) were included as negative controls. (B) The mean relative TAD transcript levels in each of the segregating genotypes from (C) the double dcl<sup>−</sup> cross or (D) the qde-2<sup>−</sup> cross. Representative blots are shown, a total of 15 ascospores were analysed in the double dcl<sup>−</sup> cross, and 16 ascospores in the qde-2<sup>−</sup> cross. Error bars display standard error of the mean. DD, double dcl<sup>−</sup>.
(~15-fold) to that observed in qde-2^- progeny (cf. Figure 3B and D) from a similar cross, in agreement with the role of DICER-LIKE and QDE2 in the same pathway that controls Tad.

siRNAs against Tad suggest a quelling-related silencing mechanism

The involvement of qde-2 in reducing Tad expression suggested similarities with a quelling-like mechanism. Another feature of quelling is the production of siRNAs of 21–25 nt that correspond to the targeted sequence (41). We isolated total RNA and enriched for the small RNA fraction in order to look for Tad siRNAs in our strains. We found that siRNAs accumulated in both the qde-2^- strains transformed with Tad (Figure 4A), and all of the Tad qde-2^- progeny resulting from the cross of qde2TAD8 with wt (Figure 4B, lanes 1, 4 and 5). siRNAs were detected using either probes homologous to the ORF1 (proximal end) or ORF2 (central region) of Tad, suggesting that the siRNAs were distributed along most of the element (Figure 4A and data not shown). This observation is consistent with the fact that QDE2 is downstream of the production of siRNAs in the quelling pathway (41). However, our inability to detect siRNAs in either the Tad-transformed wt strain, the Adiopodoumé isolate or the qde-2^+ progeny from the wt cross (Figure 4A and B, compare lanes 2, 3 and 6 with 1, 4 and 5) are not consistent with the usual features of quelling, in which siRNAs accumulate in a quelling-proficient background.

To explain the apparent lack of siRNAs in the presence of QDE2, we considered the idea that QDE2 might function in a complex with siRNAs to direct a form of TGS, in a manner similar to S.pombe, where a negative feedback loop exists in the production of siRNAs (2,20,21).

A QDE2/Dicer-dependent silencing pathway, rather than DNA methylation, is the major contributor to Tad control

To investigate the possibility of a form of TGS that might be directed by a QDE2/siRNA complex, we considered DNA methylation; evidence from Arabidopsis has shown that DNA methylation can be targeted to transposon loci via an siRNA/Argonaute complex. Moreover, a role for DNA methylation in the control of Tad elements has previously been demonstrated; an artificially introduced Tad element became de novo methylated and showed an increased transposition rate in the absence of methylation (30). Therefore, both to evaluate the relative contributions of qde-2 and methylation to transposon taming in Neurospora, and to see if indeed the two might be linked via a TGS mechanism, we introduced qde-2^- and dim-2^- mutations into similar Tad backgrounds by crossing qde2TAD8 with a dim-2^- strain; the dim-2 gene is responsible for all known methylation in Neurospora (45). To confirm the dim-2 status of the segregants, we performed Southern blots digested with either the methylation-sensitive enzyme Sau3A1, or its methylation-insensitive isoschizomer DpnII and probed for the control Z-h region, a RIP’d locus that is constitutively methylated in a dim-2^- background (Figure 5B) (23). In the progeny of the cross, all possible combinations of the qde2 and dim2 alleles segregated. Interestingly, when we used a probe covering the first 1.5 kb of Tad (Figure 5B) to check the methylation status of the elements in the progeny, we noticed

Figure 4. siRNAs against Tad accumulate only in a qde2 mutant background. (A) In the Tad-transformed wild-type (wt), qde-1^-, qde-3^- and qde-2^- strains, Tad siRNAs could only be detected in the qde-2^- strain. siRNAs were detected in both the first (1) and tenth (10) asexual generation after transformation with the cloned Tad element. No siRNAs were detected in the Adiopodoumé strain (ADI). Probes recognizing sequence from both ORF1 of Tad (left panel) and ORF2 of Tad (right panel). (B) In a cross of qde2TAD8 with a wild-type strain, the presence of Tad siRNAs segregates strictly with the absence of QDE2. The cultures used in this analysis derive from the same ascospores analysed in Figure 2A. A non-transformed qde-2^- strain (nt) was used as a negative control.
that there was very little, if any, methylation, judging by the near identity of Sau3A1 and DpnII digests in all of the dim-2 segregants, regardless of qde2 status (Figure 5A, lanes 1, 2, 8, 11 and 12). The absence of methylation was similarly observed when we controlled a 0.6 kb region comprising the proximal portion of ORF2 of Tad (not shown). Furthermore, DIM2 had no obvious effect on the expression levels of Tad. In contrast, Tad expression correlated only with the qde-2/C0 allele, similar to the situation in the qde2TAD8·wt cross (Figure 6A, lanes 2–4, 6, 7, 10 and 11), indicating that it is the QDE2-based pathway, rather than methylation, that plays the major role in the control of this transposon. We similarly failed to detect methylation of Tad elements that were introduced into a wild-type background, ruling out the possibility that the qde-2 gene might be required only in the initiation of Tad methylation at the time of transformation (data not shown).

Our failure to detect significant Tad methylation also rules out the possibility of any QDE2-directed TGS mechanism functioning via the DIM2 methyltransferase.

**DISCUSSION**

Transposition can represent a threat to genome stability by causing gene disruptions, illegitimate recombination and unregulated transcription. Indeed, in most species the major bulk of transposons are transcriptionally silent due to either DNA methylation and/or histone modifications (13,15,17,19). In addition to these TGS mechanisms, PTGS pathways similar to RNAi are also involved in the silencing of transposons. Moreover, it has been shown recently that DNA methylation of repeated sequences can be directed by the PTGS machinery, providing a direct link between the two mechanisms (16,19,46).

In our analysis, we evaluated the contribution of both DNA methylation and components of the PTGS pathway in the control of Tad expression. The principle determinant of Tad expression and expansion in our strains was qde-2, and the presence of Tad siRNAs hints at a quelling-related mechanism. In mutants lacking QDE2, an introduced Tad element...
showed a rapid expansion over the course of 15 asexual generations and, in outcrosses, the mutant allele segregated strictly with a release of Tad repression. Moreover, this dependence on the qde-2 gene was also observed in a naturally infected, Adiopodoumé-derived strain. On the contrary, we did not observe a significant level of methylation of the Tad element in our strains. Strengthening this finding, Tad expression levels were unaffected by the absence of DIM2, the DNA methyltransferase responsible for all known methylation in Neurospora. This highlights a major difference between Neurospora and other DNA methylation-proficient species in which blocking methylation leads to a large increase in transcription of diverse transposon families (15–17). Our data suggest that in the vegetative phase of Neurospora, a QDE2/siRNA-based mechanism, rather than DNA methylation, is mainly responsible for Tad control. The fact that this QDE2-based mechanism is able to limit Tad expression by itself, and without recourse to methylation, is again in contrast to other methyltransferase-containing species where siRNAs and methylation of several classes of transposon DNA are inexorably linked. Our data are in agreement with the recent observation that heterochromatin formation can occur in Neurospora in the absence of the RNAi machinery, suggesting that in this species the two silencing pathways are self-contained mechanisms (47,48).

Previous reports have shown that a Tad element at the am locus could be methylated and, in the absence of methylation, there is an increased mobilization of the element (30). However, methylation was rare and sporadic and indeed the major bulk of Tad elements are similarly not methylated in Adiopodoumé (28), suggesting that any role for methylation should be minor in comparison to the qde-2-silencing pathway.

In addition to QDE2, the requirement for DICER-LIKE activity in the repression of Tad highlights further similarities to quelling. On the other hand, the superfluity of the RdRP QDE1 and the RecQ DNA helicase QDE3 in this repression, and the accumulation of siRNAs only in the absence of the QDE2 protein are uncharacteristic of quelling. These features could point to a transposon-specific control pathway in addition to quelling. Recent evidence from Drosophila and Arabidopsis has demonstrated that different Dicer homologues can provide distinct substrate specificity (43,44). However, this does not seem to be the case in Neurospora as we have demonstrated that each of the two DICER-LIKE enzymes are able to repress Tad, and this mutual redundancy is also seen in the silencing of transgenes during quelling (35). It is possible that other RdRP homologues of the qde-1 gene, such as sad-1 (involved in MSUD) and RdRP-3 (function unknown), neither of which is necessary for quelling, might be involved in a transposon-specific pathway (24). Similarly, the qde-3 homologue, recQ-2, might have a role in transposon silencing (49).

An explanation for our failure to detect siRNAs in a qde-2+ background might rely on the degradation by siRNA-loaded RISC of Tad precursors upstream of the formation of dsRNA transcripts, thereby creating a negative feedback loop in which the siRNA pool could be reduced to levels below our detection limits. However, this does not appear to be the case in quelling of transgenes where siRNAs accumulate in both the presence and absence of QDE2, perhaps suggesting either greater abundance of the precursors upstream of dsRNA or lower susceptibility to degradation by RISC.

Again, a transcriptional silencing mechanism, other than DNA methylation, guided by an siRNA/QDE2 complex

Figure 6. QDE2, not DNA methylation, is the major determinant in limiting Tad expression. (A) Expression of the full-length Tad transcript was monitored by northern blot in cultures grown from individual ascospores deriving from the cross of qde2TAD8 × dim-2−. The cultures used in this analysis derive from the same ascospores analysed in Figure 5A. Mean relative transcript levels were plotted based on segregation of either (B) the qde-2− allele or (C) the dim-2− allele.
similar to the RNA-induced initiation of transcriptional silencing complex (RTIS) in yeast orAGO4-containing complexes in plants would have a similar effect in terms of siRNA prevalence. While our data do not exclude this possibility, recent evidence indicates that both maintenance and establishment of heterochromatin (both histone methylation and DNA methylation) at silenced transgenes and endogenous sequences can occur independently of qde-2/RNAi in *Neurospora*, meaning that, as yet, there is no precedent for siRNA-based transcriptional silencing in this species (16,19,20,48).

Models of transposon silencing need to provide a mechanism by which these elements are recognized. In the control of certain transposable elements for example, one such mechanism might rely on the presence of inverted repeat (IR) sequences contained at their termini. Sijen and Plasterk found that in *C.elegans* read-through transcription of these IRs is able to trigger silencing of the *Tc1* family of transposons by forming a hairpin dsRNA structure that can enter the RNAi pathway (50). However, *Tad* and many other transposons, such as non-LTR retrotransposons that include the human and mouse LINE elements, do not contain IR sequences. Thus, an alternative explanation is needed in these cases. One possible reason why *Tad* triggers a QDE2-dependent silencing pathway may rely on the very complex transcriptional profile of the element that, in addition to a full-length transcript includes at least two shorter anti-sense transcripts that originate from its 3' end (51). Inter-molecular base pairing of sense and anti-sense transcripts would lead to a dsRNA intermediate, providing a template for Dicer cleavage and subsequent RISC-mediated degradation of *Tad* templates. In support of this, the direct production of dsRNA has been shown to bypass the requirement of both QDE1 and QDE3, but is upstream of QDE2 in the silencing of transgenes, a situation similar to that which we observed (52). However, an explanation based on intermolecular pairing of sense transcripts and 3'-originating anti-sense transcripts needs to explain the presence of siRNAs from ORF1, into which the main anti-sense transcripts do not extend (51). An auxiliary role for one of the two silencing pathways. It would appear likely that *Neurospora*, during the process of a *Tad* invasion, uses a qde-2/quelling-related mechanism to limit the expansion of *Tad* during vegetative growth whilst the resulting restricted set of *Tad* elements is gradually eliminated by RIP in successive cycles. It seems reasonable to expect that many of the transposon relics that litter the *Neurospora* genome will have at one time been subject to a similar concert of silencing mechanisms, possibly also involving MSUD, that function to ensure their deactivation and maintain genome integrity.

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