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Genetics

Isolation of quelling-defective (*qde*) mutants impaired in posttranscriptional transgene-induced gene silencing in *Neurospora crassa*

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ABSTRACT **We report the isolation of 15 *Neurospora crassa* mutants defective in "quelling" or transgene-induced gene silencing. These quelling-defective mutants (*qde*) belonging to three complementation groups have provided insights into the mechanism of posttranscriptional gene silencing in *N. crassa*. The recessive nature of the *qde* mutations indicates that the encoded gene products act in trans. We show that when *qde* genes are mutated in a transgenic-induced silenced strain containing many copies of the transgene, the expression of the endogenous gene is maintained despite the presence of transgene sense RNA, the molecule proposed to trigger quelling. Moreover, the *qde* mutants failed to show quelling when tested with another gene, suggesting that they may be universally defective in transgene-induced gene silencing. As such, *qde* genes may be involved in sensing aberrant sense RNA and/or targeting/degrading the native mRNA. The *qde* mutations may be used to isolate the genes encoding the first components of the quelling mechanism. Moreover, these quelling mutants may be important in applied and basic research for the creation of strains able to overexpress a transgene.**

The introduction of transgenes in plants and fungi has been shown to lead to gene silencing. In plants, gene silencing has been found to occur at two levels, transcriptional and posttranscriptional (1, 2). Generally in plants, transcriptional inactivation requires homology between promoters (3, 4). In contrast, homology in transcribed regions has been shown to induce posttranscriptional gene silencing or cosuppression (5, 6). In the filamentous fungus *Neurospora crassa*, the introduction of transgenes has been shown to lead to two different types of gene inactivation phenomena. In the sexual phase, the duplicated genes can be irreversibly inactivated by a mechanism called RIP (repeat induced point mutation), whereby gene inactivation is caused by a high rate of point mutation (7). In contrast, during the vegetative phase, the transgene can induce reversible gene silencing by a phenomenon called quelling (8).

We have begun to use a molecular–genetic approach to dissect the mechanism of quelling in *N. crassa*, using a gene (*al-1*) essential for biosynthesis of carotenoids, which provides a simple visual reporter for quelling. In these studies several characteristic features of quelling have been defined: (*i*) the gene inactivation by quelling is reversible and the reversion is correlated with the release of exogenous DNA; (*ii*) the reduction of mRNA steady-state level of the duplicated gene is due to a posttranscriptional effect on its accumulation; (*iii*) the transgenes containing transcribed regions of at least 132 bp are able to induce gene silencing, whereas the promoter regions are ineffective; (*iv*) quelling is dominant in heterokaryotic strains containing a mixture of transgenic and nontransgenic nuclei, indicating the involvement of a diffusible trans-acting molecule; and (*v*) the expression of unexpected transgenic sense RNA is correlated with silencing, suggesting involvement of an RNA transcript in mediating quelling (8, 9).

Based on the above information on the mechanism of quelling in *N. crassa*, several parallel features can also be found in gene silencing in plants. The major similarity is the fact that gene quelling in *N. crassa* and cosuppression in plants both involve posttranscriptional gene silencing. As molecular and biochemical approaches have to date failed to uncover the exact posttranscriptional mechanism involved, a genetic approach to identify components of this machinery is attractive. In plants, Arabidopsis mutants have been isolated in which the timing of *rolB* transgene silencing is altered (10). However, while gene silencing is accelerated in these mutants, it is not disrupted. Thus, no plant mutants deficient in the silencing machinery have been isolated.

Here we describe the isolation of *N. crassa qde* (quelling defective) mutants in which quelling or gene silencing is completely abrogated. The mutations identified fall into three complementation groups, which have each been shown to abrogate simultaneously quelling of several different genes. This result suggests that the affected genes may encode three separate components involved in the general mechanism of gene silencing in *N. crassa*.

MATERIALS AND METHODS

Strains and Growth Conditions. The *N. crassa* methodology used was substantially the same as described (11). The *N. crassa* strains used, obtained from the Fungal Genetics Stock Center at the University of Kansas were as follows: FGSC no. 3958 a (*qa-2*; *aro-9*) and FGSC no. 3957 A (*qa-2*; *aro-9*). For the forced heterokaryon experiments we first constructed a suitable strain (TB1) with two selectable markers, *qa-2*/*aro-9* (unable to grow on minimal medium) and *Bml* (benomyl-resistant). A (*qa-2*; *aro-9*) strain was transformed with plasmid pMXY2 (12) containing *Bml* (benomyl-resistant β-tubulin gene) that functions as a dominant selectable marker in *N. crassa* (14). Transformant TB1, resistant to benomyl, was isolated by plating spheroplasts on minimal media containing 1 mg/l of benomyl and 1× qa Mix (50× qa Mix = 4 mg/ml L-tyrosine/4 mg/ml L-tryptophan/4 mg/ml L-phenylalanine/12.5 μg/ml *p*-aminobenzoic acid). Conidia of strain TB1 (*qa-2*; *aro-9*; *Bml*) were mixed with conidia of each *qde* mutant and inoculated on minimal medium plus 1 mg/l benomyl, with permissive growth conditions for forced heterokaryotic strains only. Minimal medium plus 1× qa Mix was used as the permissive medium for growth of the (*qa-2*; *aro-9*) strain; in the

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Abbreviation: RPA, RNase protection analysis.

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qa selective medium sucrose was replaced by 10 mM quinic acid.

Transformation of *N. crassa*. Spheroplasts were prepared according to the protocol of Vollmer and Yanofsky (13). The 6xw and 5xw strains were obtained by transformation of spheroplasts of strain FGSC no. 3958 with pX16 (9) containing the *qa-2* gene as a selectable marker.

The plasmid pN containing a portion of *al-2* gene sequence (see below) was cotransformed with plasmid pMXY2, which contains *Bml*. The frequency of quelling of the *al-2* gene was calculated by selecting benomyl-resistant transformants that were then scored for carotenogenesis by visual inspection of conidial color: wild-type colonies were orange, whereas white to yellow transformants were indicative of silencing.

Recombinant Plasmid. The pN plasmid was constructed by cloning a 1,540-bp region of the *al-2* coding sequence (15) into the *Eco*RI site of pBSSK II+ (Stratagene). The 1,540 bp fragment was amplified from genomic DNA by PCR using two primers complementary to bases 1,751–1,770 (5′-CCGAATTCAACAATCAACAAAACCCGCC-3′) and 3,271–3,290 (5′-CCGAATTCCATACAACGCCCTCAACAGC-3′) of the *al-2* gene. The underlined nucleotides represent the *Eco*RI restriction sites used for cloning.

Southern and Northern Blot Hybridizations. Chromosomal DNA from *qde* mutants was prepared as described by Morelli *et al.* (16). Genomic DNA was digested and blotted as described in Maniatis *et al.* (17). The probe specific for *al-1* consisted a 1,550-bp *Xba*I/*Cla*I fragment of the pX16 (9). The RNA was electrophoresed on agarose gels, transferred onto Hybond-N (Amersham) membranes, and probed with a probe specific for *al-1* gene (see above). Normalization was performed using as probe a PCR fragment amplified with two oligonucleotides (the same used in the construction of pN plasmid) specific for the *al-2* gene.

Dot Blot Analysis. Five micrograms of genomic DNA was spotted onto Hybond-N membranes and probed with a probe specific for *al-1* and with a probe specific for the single copy gene *sod-1* (18). The radioactive signals were quantified by using a Packard Instant Imager instrument. The *al-1* transgene copy number in each mutant was estimated as follows: for each mutant the ratio between the cpm of the DNA spot derived from the *al-1* probe and the cpm derived from the *sod-1* probe was calculated. This ratio was then divided by the ratio of *al-1*/*sod-1* cpm calculated from spots of DNA from an untransformed strain. Results reported are the average of three different experiments.

RNase Protection Analyses. Total RNA was extracted from frozen mycelia either grown in the dark or after irradiation with blue light according to Baima *et al.* (19). The RNA probes used in the RNase protection experiments (see Figs. 2 and 6) were prepared by *in vitro* transcription of the plasmid pALC4 (9) using T3 polymerase. The *in vitro* transcription reactions and the RNase protection assays were performed using the Ambion (Austin, TX) transcription kit according the manufacturer's instructions. For RNase protection assays, 1 μg of total RNA was used in each reaction.

RESULTS

Isolation of a Stable Silenced Strain. We have previously demonstrated that the phenomenon called quelling takes place at a high frequency, but is an unstable process (8, 9). The ability of most quelled strains to revert to a wild-type phenotype precludes using genetic selection to identify mutants in the process. To circumvent this problem, we carried out a large-scale screening to identify transformants with a stable silenced phenotype. To isolate a stable silenced strain, a *N. crassa* strain containing a wild-type *al-1*$^+$ gene (orange phenotype) (*al-1*$^+$; *qa-2*; *aro-9*) was transformed with plasmid pX16 containing *qa-2* and a 1,500-bp fragment of the coding sequence of the *al-1* gene (Fig. 1*A*). About 200 independent Qa$^+$ transformants showing an albino phenotype were isolated and their frequency of reversion to orange color was analyzed. Two independent albino transformants (5xw and 6xw) showed no reversion: 0 revertants/10,000 descendant colonies. To test whether these were stably quelled strains or strains disrupted in the albino gene, we used Southern blot analysis on DNA digested with enzymes that distinguish the transgene (5.5 kb) from the endogenous *al-1* gene (3.1 kb) (Fig. 1). In strain 5xw the transgene is present, whereas the native *al-1* gene is disrupted, presumably by the transgene (Fig. 1, lane 3). In contrast, strain 6xw has tandem insertions of the transgene and an intact native *al-1* gene (Fig. 1, lane 2).

To further verify that posttranscriptional gene silencing is operating in strain 6xw, we analyzed the levels of the *al-1* RNA. We previously showed that in an *al-1* quelled strain the level of unspliced *al-1* mRNA is similar in quelled and wild-type strains, whereas native *al-1* mRNA is heavily reduced, indicating that quelling affects the level of the mature mRNA and not the rate of transcription. We analyzed *al-1* RNA in strain 6xw by an RNase protection assay (RPA) able to discriminate between mature and precursor *al-1* mRNAs (Fig. 2*B*). Using this assay, RNA isolated from 6xw mycelia irradiated 20 min with light (Fig. 2*A*, lane 3) was shown to contain levels of unspliced mRNA similar to wild type (Fig. 2*A*, lane 1), whereas the level of mature *al-1* mRNA was reduced. This RNA profile is consistent with 6xw being a quelled strain. Moreover, the quelled strain 6xw contains a transgenic sense RNA detected in all quelled strains. This transgenic sense RNA is also present in the dark-grown quelled strain (Fig. 2*A*, lane 2) (9) whereas

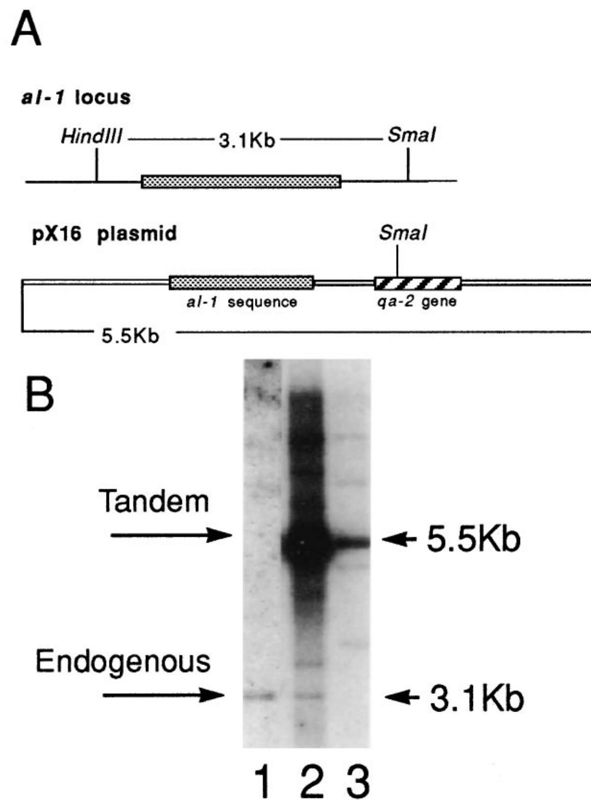

FIG. 1. Southern hybridization analysis of the stable albino transformant strains. (*A*) Schematic representation of *al-1* endogenous locus and of plasmid pX16 used in transformation experiments. (*B*) *Sma*I/*Hin*dIII-digested genomic DNA extracted from wild-type untransformed strain (lane 1), strain 6xw (lane 2), and strain 5xw (lane 3) was hybridized with an *al-1* probe able to detect both endogenous and transgenic *al-1* copies. The 3.1-kb band corresponding to the endogenous *al-1* gene and the 5.5-KB band corresponding to the tandem repeated insertions are indicated by arrows.

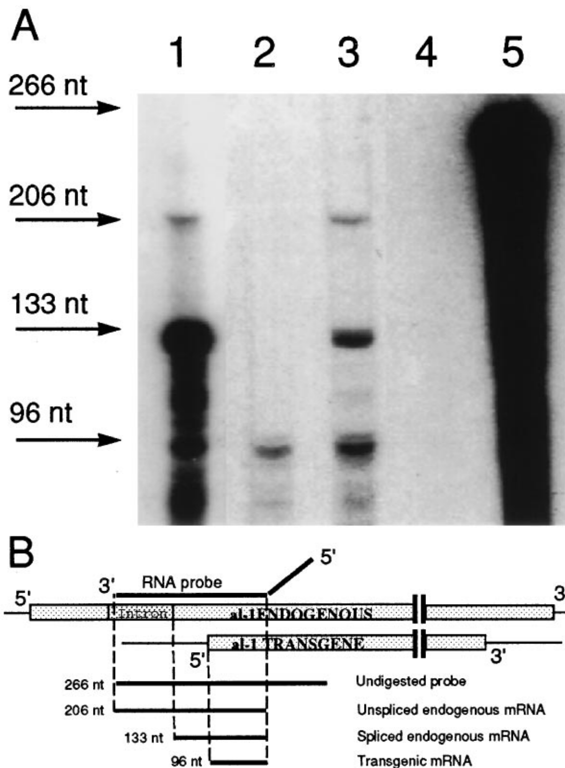

FIG. 2. RNase protection experiments for sense *al-1* RNA transcripts in strain 6xw. (*A*) RPA was performed on total RNA extracted from wild-type untransformed strain illuminated for 20 min with light (lane 1), dark-grown transformant 6xw (lane 2), 6xw illuminated for 20 min (lane 3), dark-grown wild type (lane 4). Arrows indicate the sizes of the protected fragments obtained from the unspliced endogenous *al-1* mRNA (206 nt), from the mature endogenous *al-1* mRNA (133 nt), and from the transgenic *al-1* sense RNA (96 nt). Undigested *al-1* RNA probe (266 nt) is shown in lane 5. (*B*) Map of endogenous and potential transgenic sense transcripts. The pALC4 plasmid, which contains a single intron, was used to generate an RNA probe capable of hybridizing to sense *al-1* mRNA. This RNA probe is indicated as a solid black line. The diagonal portion of the RNA probe represents plasmid sequences not present in the *N. crassa al-1* mRNA. Shaded boxes represent sense RNA from the endogenous native *al-1* RNA gene (*Upper*) and a putative sense RNA from the *al-1* transgene (*Lower*).

it is not detected in the dark-grown wild-type strain (Fig. 2*A*, lane 4). The degradation product present in light-induced wild-type RNA (Fig. 2*A*, lane 1) that comigrate with the 96-nt band is probably derived from large protected fragments. The genetic experiments described below further confirm that this strain 6xw is indeed a quelled strain.

Isolation of Quelling Deficient (*qde*) Mutants. Strain 6xw, in which the *al-1*$^+$ gene is stably quelled (albino phenotype), was UV mutagenized to select for mutations that abolished quelling (orange phenotype). Nineteen putative mutants were recovered out of approximately 100,000 survivors screened. To discriminate true mutations from revertants that have simply lost copies of the transgene, we performed a DNA dot blot analysis (see *Materials and Methods*). Dot blots of genomic DNA extracted from each mutant were hybridized with an *al-1* probe and with a probe of the single copy gene *sod-1* (18) as a control to estimate the copy number of transgenic *al-1*. By this criterion, two classes of mutants were identified: group 1 consisting of 15 mutants which each retained high copy number of the transgene (25–35 copies), and group 2 consisting of four mutants in which the copy number was reduced (4–6 copies) (Table 1).

To determine whether any of the putative quelling-deficient mutants were the result of transgene rearrangement, Southern

Table 1. *al-1* transgenic DNA copy number in mutant strains

Group 1		Group 2	
Mutant strain	copy number	Mutant strain	copy number
M2	30 ± 7	M30	4 ± 1
M4	27 ± 8	M34	5 ± 1
M7	26 ± 5	M35	4 ± 1
M10	25 ± 8	M43	4 ± 1
M11	26 ± 7		
M12	30 ± 7		
M17	25 ± 7		
M18	24 ± 7		
M20	26 ± 5		
M24	25 ± 7		
M37	32 ± 5		
M40	25 ± 5		
M41	25 ± 7		
M46	25 ± 5		
M47	32 ± 6		

hybridization analysis was performed. The results for nine representative strains and for original strain 6xw are shown (Fig. 3). Genomic DNA extracted from each of the mutant strains was digested with enzymes (*Sma*I and *Hin*dIII) that distinguish the tandem of transgenes (5.5 kb) from the endogenous *al-1* gene (3.1 kb) (see Fig. 1) and hybridized with an *al-1* probe. Larger bands may represent different integration events or may be caused by methylation of restriction sites in the transgenes. The digestion patterns of all group 1 mutants were qualitatively identical, demonstrating that none were the result of gross transgene rearrangement, whereas the digestion patterns of group 2 mutants confirm that the copy number of the transgenes was reduced. The genetic experiments described below further confirm that the release of gene silencing in group 1 mutants does not depend on rearrangements of the transgenic DNA loci.

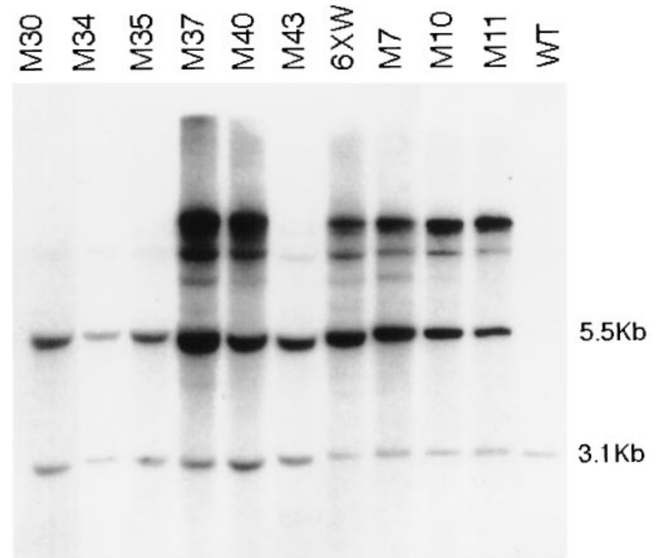

FIG. 3. Southern hybridization analysis of the mutants. *Sma*I/*Hin*dIII-digested genomic DNA extracted from five representative mutants of group 1 (M37, M40, M7, M10, M11), mutant strains of group 2 (M30, M34, M35, M43), the quelled strain (6xw), and a wild-type untransformed strain (WT). The DNA digestions were blotted and probed with a DNA fragment able to detect both endogenous and transgenic *al-1* copies. The 3.1-kb band corresponding to the endogenous *al-1* gene and the 5.5-kb band corresponding to the plasmid arranged in tandem repeats are indicated.

Taken together, these results suggest the existence of two classes of mutants. In group 2 mutants, the quelling deficiency appears to be due to loss of exogenous copies. In contrast, in group 1 mutants both the copy number and the arrangement of transgenic loci remain unchanged with respect to original strain 6xw. Thus group 1 mutations are candidates for extragenic loci involved in transgene silencing.

To demonstrate that the orange phenotype of the putative *qde* mutants was due to a restoration of *al-1*$^{+}$ gene expression, we analyzed the levels of *al-1* mRNA in group 1 and group 2 mutants. Total RNA extracted from mycelia of mutant strains after 20 min of light induction were hybridized with an *al-1* probe. As shown for eight representative mutant strains in Fig. 4, all mutants analyzed showed a wild-type level of accumulation of *al-1* mRNA.

The *qde* Mutants Relieve Silencing of a Second Gene, *qa-2*. The above experiments establish that the *al-1* gene is relieved from quelling in the *qde* mutants. We next tested whether the *qde* mutations also relieved silencing of other marker genes in strain 6xw. Because the plasmid used to transform strain 6xw contains the selectable marker *qa-2*, we monitored whether *qa-2* transgene expression was enhanced in the *qde* mutants compared with original strain 6xw. Two methods were used: analysis of *qa-2* mRNA and analysis of growth on selection medium.

The quelled strain 6xw containing the *qa-2* transgene was selected for its ability to grow on minimal medium. However, growth rate experiments demonstrate that strain 6xw grows at 20% of the wild-type growth rate (Fig. 5*A*, compare lanes 1 and 5). A normal growth rate could be restored in strain 6xw by the addition of a mixture of aromatic amino acids, which by-pass the metabolic restrictions (Fig. 5*A*, compare lanes 6 and 10). This growth defect of the stably quelled strain 6xw suggests that expression of the *qa-2* selectable marker transgene is suboptimal. In contrast, all the *qde* mutants isolated showed normal growth on minimal medium, as shown for strains representative of the three complementation groups described below (Fig. 5*A*, lanes 2–4). These growth studies suggest that the *qa-2* transgene is expressed at higher levels in the *qde* mutants relieved of quelling of the *al-1* gene. Moreover, the release of *qa-2* silencing in the *qde* strains is most dramatically demonstrated by the growth rate measured on selection medium containing quinic acid as the sole carbon source. Under these selection conditions, only the product of the *qa-2* gene will enable growth. The growth rate of the stably quelled strain 6xw is only 1% of the wild-type strain in these culturing conditions (Fig. 5*A*, lane 15). On the other hand, the *qde* mutants grew as well as wild type on this stringent selection medium (Fig. 5*A*, lanes 11–14). Northern blot analysis of *qa-2* mRNA in the representative *qde* mutants demonstrated that the expression of the *qa-2* transgene was enhanced in the mutants compared with the stably quelled strain 6xw (see Fig. 5*B*).

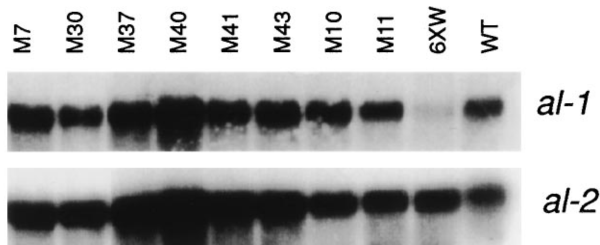

FIG. 4. Analysis of *al-1* gene expression in mutant strains. Total RNA extracted from mycelia, illuminated for 20 min, of eight representative mutants (M7, M30, M37, M40, M41, M43, M10, M11), strain 6xw, and wild type. After blotting, RNAs were hybridized with an *al-1* probe. Control hybridization was performed with the photoregulated *al-2* gene.

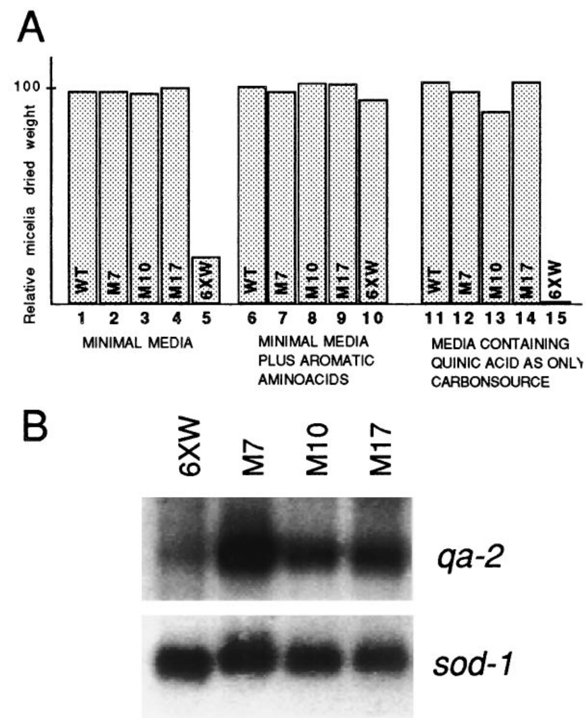

FIG. 5. Expression of *qa-2* transgenes in *qde* mutants. (*A*) Histograms indicate the relative dry weight of mycelia from *qde* mutants grown in different media as indicated. Three representative mutants (M7, M10, M17) are compared with the 6xw and wild-type strains. (*B*) Northern blot analysis of *qa-2* gene expression in *qde* mutants and strain 6xw. Total RNA extracted from mycelia grown in presence of 10 mM quinic acid as inducer of *qa-2* expression was hybridized with a *qa-2* probe. A *sod-1* probe was used as normalization control.

Molecular Genetic Analysis of the *qde* Mutants Defines Three Complementation Groups. Molecular analysis separated the 19 putative *qde* mutants into two groups based on transgene copy number (see Table 1). We next used a genetic approach to classify the mutants into complementation groups. Quelling has been shown to be a dominant trait, operative in heterokaryotic strains containing a mixture of silenced and nonsilenced nuclei (9). The dominant nature of quelling predicts that a heterokaryotic strain produced from a wild-type (orange) and a *qde* recessive mutant (orange) strain should show an albino phenotype. In contrast, in the case of mutants caused by loss of transgenic copies (e.g., group 2 mutants; Table 1) complementation in the heterokaryon is not expected. To analyze the genetic nature of the mutation in *qde* strains, forced heterokaryons were created to contain wild-type and mutant alleles of the *qde* genes. For the forced heterokaryon tests conidia of each *qde* mutant (*qde*; *qa-2*$^{+}$; *bml*) were mixed with conidia of strain TB1 (*qde*$^{+}$; *qa-2*; *aro-9; Bml*) (see *Materials and Methods*) and plated on medium containing benomyl. The group 1 mutants shown to contain high copy number of the transgene displayed an albino phenotype in forced heterokaryons with strain TB1 containing a wild-type allele of *qde*. This result suggests that the mutations of the group 1 mutant strains in Table 1 act as recessive mutations (Table 2). In contrast, the group 2 mutants containing low copy number of the transgene failed to complement. These genetic results reinforce the notion that the release of silencing in group 2 mutants is due to the loss of transgene copies.

The above studies determined that the group 1 mutations were recessive and could therefore be further analyzed by testing each mutant pairwise in heterokaryons with each of the other group 1 mutants to distinguish complementation groups.

Table 2. Mutant complementation groups

Recessive			
qde-1	*qde-2*	*qde-3*	Nonrecessive
M2	M10	M4	M30
M7	M11	M17	M34
M12		M18	M35
M20		M40	M43
M24		M41	
M37			
M46			
M47			

A heterokaryon between two *qde* strains (orange) containing recessive mutations is expected to show an albino phenotype if the mutations are in different loci. If instead two *qde* mutants are alleles, an orange phenotype is expected. The results summarized in Table 2 indicate that the group 1 recessive mutants fall without exceptions into three complementation groups, suggesting that three distinct genetic loci, *qde-1, qde-2,* and *qde-3*, are involved in release of gene silencing.

Recessive *qde* Mutants Are Impaired in All Gene Silencing Events. The above tests classify the group 1 *qde* recessive mutants into three complementation groups, separate from the group 2 mutations. Representatives of these four classes of mutants were tested for their ability to quell an additional gene, *al-2*$^{+}$. We compared the quelling frequency of the *al-2* gene (albino) in the *qde* mutants and in a Qde^{+} strain (Table 3). Spheroplasts of a wild-type strain and of recessive mutant strains M7, M11, M18, representative of each complementation group and of the mutant M34 representative of the nonrecessive group, were cotransformed with the plasmid pN containing the 3′ portion of *al-2* (see *Materials and Methods*) and the plasmid pMXY2 carrying *Bml*. For the recessive group 1 *qde* mutants (M7, M11, M18) no quelled transformants were isolated. This result indicates that the three *qde* complementation groups identified control silencing of at least three distinct transgenes, suggesting that these loci control a general pathway for gene silencing in *N. crassa*. In contrast, the quelling frequency of a group 2 mutant strain (M34) was the same as wild type, indicating no defect in the quelling machinery. This result confirms that the release of silencing of the *al-1*$^{+}$ gene in the strains belonging to group 2 was exclusively due to the loss of transgenic copies.

The *qde* Mutants Accumulate RNA Transcripts of the Transgene. Previously we demonstrated a strong correlation between unintended transcription of *al-1* transgene and *al-1* gene silencing. These results suggested a possible role for transgenic sense RNAs in mediating gene silencing. As expected, the transgenic sense RNAs were present in the stably quelled strain 6xw (see Fig. 2*A*). With the *qde* mutants in hand, we could test whether the mutations affected the accumulation of transgenic sense RNAs or acted downstream in the quelling machinery. Levels of transgene sense RNAs were monitored in the 19 strains belonging to the four classes of mutants released from quelling (Fig. 6). An RPA was performed on total RNA extracted from mycelia using an RNA probe able to discriminate between the transcripts of the *al-1* transgene

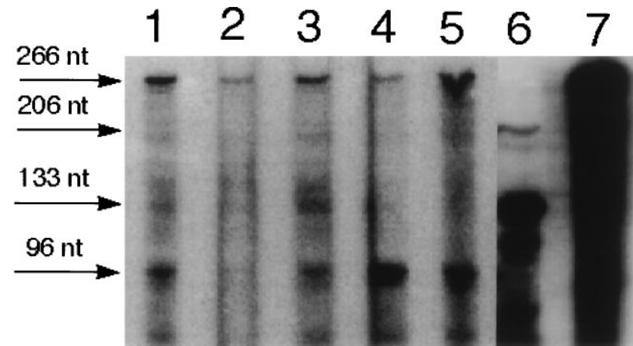

FIG. 6. RNase protection experiments for transgenic sense *al-1* RNA transcripts in mutant strains. RPA was performed, using an RNA probe as described in Fig. 2*B*, on total RNA extracted from dark-grown mutants representative of each group (M10, M30, M20, M17) (lanes 1–4), from dark-grown transformant 6xw (lane 5), and from an untransformed strain illuminated for 20 min (lane 6). Undigested *al-1* RNA probe is shown in lane 7. Arrows indicate the size of the protected fragments obtained from the unspliced endogenous *al-1* sense RNA (206 nt), the mature endogenous *al-1* mRNA (133 nt), and the transgenic *al-1* sense RNAs (96 nt). Undigested probe (lane 7) of 266 nt is indicated by an arrow. Sizes of the protected fragments are as in Fig. 2*B*.

(protected 96-nt fragment) and those of the endogenous *al-1*$^{+}$ gene (protected 206-nt fragment). Furthermore, we enhanced detection of transgenic sense RNA by using RNA isolated from dark-grown mycelia that do not express the endogenous *al-1*$^{+}$ mRNA. Fig. 6 reports the RPA results obtained from one representative mutant for each of the four mutant classes. The *al-1* transgenic sense RNA was detected in all group 1 recessive mutants examined (Fig. 6, lanes 1, 3, and 4). In contrast, the transgenic sense RNA was absent in the group 2 nonrecessive mutants (Fig. 6, lane 2). These results indicate that only mutations that act recessively (e.g., where quelling can be restored in heterokaryosis with a *qde*$^{+}$ strain) contain the sense transgenic RNA. This result is a further demonstration that transgenic sense RNA is required for quelling. Moreover, these results imply that the *qde* products act downstream of this sense RNA molecule to release quelling.

DISCUSSION

The introduction of the *al-1* gene into *N. crassa* by transformation has established a model genetic system in which a visual assay (color) can be used to identify quelled strains as well as mutants defective in quelling. As quelling is a posttranscriptional transgene-induced gene silencing mechanism analogous to cosuppression in plants, *N. crassa* mutants defective in quelling should provide the first tools to isolate components involved in the transgene-induced gene silencing machinery.

To screen genetically for mutants defective in quelling we first isolated a transgenic *N. crassa* strain (6xw) that is stably quelled. Molecular analysis suggested that the albino phenotype of strain 6xw was due to quelling of the resident *al-1*$^{+}$ gene. These parameters include a reduction of mature native *al-1* mRNA, normal levels of precursor native *al-1* RNA, and

Table 3. Quelling frequency for the *al-2*$^{+}$ gene in representative mutant strains

Strain used	Complementation group	Transformant phenotypes: Orange	Transformant phenotypes: Nonorange	Total	Quelling frequency, %
M7	I	200	0	200	0
M11	II	200	0	200	0
M18	III	300	0	300	0
M34	Group 2	182	28	210	13
WT		89	11	100	11

the presence of sense transgenic *al-1* transcripts shown previously to correlate with quelling (9). The quelling-defective (*qde*) mutants (orange) were isolated by mutagenesis of the stably quelled transformant 6xw (albino). Fifteen recessive *qde* mutants belonging to three complementation groups were isolated. These *qde* mutants were shown to be truly relieved of quelling as they contain increased levels of native *al-1* mRNA. Furthermore, the transgene-selectable marker *qa-2* was expressed at a higher level in all the *qde* mutants, suggesting that *qa-2* transgene was partially silenced in strain 6xw. This finding suggests that the *qde* genes control silencing not only of the *al-1* endogenous gene but also of the *qa-2* transgene. Thus, two distinct genes were released from silencing in the *qde* strains, suggesting defects in a general silencing mechanism. However, whether or not only one mechanism for transgene induced silencing in *N. crassa* exists, as suggested by these experiments, has still to be demonstrated.

To gain further insights into the quelling mechanism, molecular analysis on the *qde* mutants was performed. We showed that the *qde* mutations do not reverse quelling by lowering the copy number of the transgene, as *qde* mutants blocked in quelling retain a high copy number of the transgene. Furthermore, we showed that these *qde* mutants with a high copy of the transgene still contain the transgenic sense RNA proposed to be required for quelling (9). The significance of this transgene sense RNA is highlighted by the contrast between the genetic analysis of the *qde* mutants and a second group of mutants as follows.

In addition to the 15 recessive *qde* mutants, we also identified a second class of isolates in which quelling was released (group 2). Members of group 2 (orange) were shown to be relieved of quelling because they have a reduced copy number of the transgene and produce no transgene sense RNA. Furthermore, genetic experiments demonstrate that silencing can be restored when *qde* strains (producing the transgene sense RNA) are placed in a heterokaryon with a wild-type strain. In contrast, silencing cannot be restored when group 2 mutants are in a similar heterokaryon. This important genetic distinction between group 1 recessive *qde* mutants and group 2 (nonrecessive mutants) supports the notion that the transgenic sense RNA present in the *qde* mutants is essential for silencing in the heterokaryon. The involvement of sense RNA in posttranscriptional gene silencing has been proposed based on the correlation between the accumulation of a transgenic transcript from a promoterless construct and quelling. In plants it has been proposed (20) that when transgenic mRNA accumulation reaches a threshold, specific degradation of both endogenous and transgenic mRNA could be induced. However, this threshold hypothesis seems to be inconsistent, for example, with the observation that promoterless constructs of the *chs* gene in petunia are able to induce silencing just as well as does the construct carrying a strong promoter (21). Also in *N. crassa* we have found that the level of expression of a promoter-driven transgene is not sufficient to trigger gene silencing (8). English *et al.* (22) proposed that a qualitatively aberrant feature of transgenic RNA, rather than a high level of transgenic RNA accumulation, can trigger gene silencing. In this model the aberrant sense RNA could be recognized by an RNA-dependent RNA polymerase, leading to the production of antisense RNA, formation of double stranded RNA and RNA degradation (23, 24).

In *N. crassa*, we have been able to use a simple method of genetic screening to identify components of the silencing machinery that may be used to dissect the analogous system in plants. The recessive nature of the *qde* mutants indicates that the gene products of the qde^{+} strains normally act in trans to silence genes posttranscriptionally in quelled strains. As such, we propose that *qde* products may "sense" the aberrant sense RNA by direct or indirect interaction. In addition, one or more of the *qde* products may be involved in targeting and/or degradation of the endogenous mRNA. Furthermore, it may be that the *qde* mutants are universally defective in transgene-induced gene silencing, as we have shown that in addition to the two genes released from silencing (*al-1* and *qa-2*) in the *qde* strains, the introduction of an additional transgene (*al-2*) did not trigger quelling of the native *al-2* gene in the *qde* mutant background. Thus, in separated independent transgenic events, different genes were unable to be quelled in the *qde* mutants. Therefore, while multiple mechanisms for transgene-induced gene silencing in *N. crassa* may occur (8, 25), the *qde* mutants appear to be blocked in all of them.

The identification of the *qde* mutants constitutes the first necessary step in the identification of factors required for quelling in *N. crassa*. Because strong similarities exist between quelling and cosuppression in plants, we believe that the cloning the *qde* genes will be of extreme importance to resolving the puzzle of posttranscriptional gene silencing. Moreover, availability of *qde* mutants in *N. crassa* may be of help in both applied and basic research for the creation of transgenic strains able to overexpress a transgene.

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