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Transgene silencing of the *al-1* gene in vegetative cells of *Neurospora* is mediated by a cytoplasmic effector and does not depend on DNA–DNA interactions or DNA methylation

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The molecular mechanisms involved in transgene-induced gene silencing ('quelling') in *Neurospora crassa* were investigated using the carotenoid biosynthetic gene albino-1 (*al-1*) as a visual marker. Deletion derivatives of the *al-1* gene showed that a transgene must contain at least ~132 bp of sequences homologous to the transcribed region of the native gene in order to induce quelling. Transgenes containing only *al-1* promoter sequences do not cause quelling. Specific sequences are not required for gene silencing, as different regions of the *al-1* gene produced quelling. A mutant defective in cytosine methylation (*dim-2*) exhibited normal frequencies and degrees of silencing, indicating that cytosine methylation is not responsible for quelling, despite the fact that methylation of transgene sequences frequently is correlated with silencing. Silencing was shown to be a dominant trait, operative in heterokaryotic strains containing a mixture of transgenic and non-transgenic nuclei. This result indicates that a diffusable, *trans*-acting molecule is involved in quelling. A transgene-derived, sense RNA was detected in quelled strains and was found to be absent in their revertants. These data are consistent with a model in which an RNA–DNA or RNA–RNA interaction is involved in transgene-induced gene silencing in *Neurospora*.

Keywords: co-suppression/DNA methylation/*Neurospora crassa*/quelling/silencing

Introduction

The creation of transgenic lines is now used commonly in both basic and applied biological research on a wide range of organisms. In plants and fungi, transgenic DNA is normally integrated into the genome and transmitted as a Mendelian trait. In some cases, however, expression of transgenes and/or the native homologue became reversibly suppressed (reviewed in Flavell, 1994; Matzke and Matzke, 1995a). This phenomenon has been termed RIGS (repeat induced gene silencing) (Assaad *et al.*, 1993) or 'homology-dependent gene silencing' (Matzke *et al.*, 1994) to emphasize the apparent requirement for multiple copies of the silenced genes. RIGS includes cases in which the silencing loci only act in *cis* (*cis*-inactivation) as well as cases in which the loci can act in *trans* (*trans*-inactivation). *Trans*-inactivation includes cases in which all of the homologous genes are silenced (reciprocal *trans*-inactivation, or co-suppression; Napoli *et al.*, 1990) and cases where only certain copies are silenced (non-reciprocal, or epistatic *trans*-inactivation; Matzke *et al.*, 1994).

In some cases of *cis*- and *trans*-inactivation, silencing appears to occur at the transcriptional level and has been correlated with cytosine methylation (Assaad *et al.*, 1993; Meyer *et al.*, 1993; Matzke *et al.*, 1994). These examples suggest a mechanism of gene silencing based on methylation of repeated sequences and suppression of transcription, reminiscent of the gene silencing phenomena repeat induced point mutation (RIP) and methylation induced pre-meiotically (MIP), which are active during the sexual cycles of *Neurospora crassa* and *Ascobolus immersus*, respectively (reviewed in Selker, 1990; Rossignol and Faugeron, 1994). Other cases of *trans*-inactivation may be due to post-transcriptional processes resulting in increased turnover of RNA for the duplicated gene (Elkind *et al.*, 1990; de Carvalho *et al.*, 1992), sometimes accompanied by increases in cytosine methylation in the DNA (Inglebrecht *et al.*, 1994). A similar phenomenon has been observed in several cases of transgene-induced virus resistance, where viral gene silencing acts at the cytoplasmic level (Lindbo *et al.*, 1993; Smith *et al.*, 1994). Although post-transcriptional *trans*-inactivation has been observed repeatedly in plants, little is known about the mechanism of action.

Transgene-induced gene silencing has also been observed in the filamentous fungus *N.crassa* (Pandit and Russo, 1992; Romano and Macino, 1992). In studies using carotenoid gene silencing as a simple visual reporter of the silencing phenomenon, silencing was found to occur in vegetative mitotic cells (Romano and Macino, 1992). Even transgenes containing only fragments of genes led to inactivation of the corresponding native genes. Silenced strains exhibited a large reduction in steady-state mRNA levels, and this reduction was specific for transcripts from the duplicated sequences (Romano and Macino, 1992). Silencing was reversible, and reversion was accompanied by the loss of exogenous gene copies (Romano and Macino, 1992). In studies involving multiple copies of a bacterial gene encoding hygromycin resistance (*hph*), silencing was also found to be reversible, yet reactivated transgenes were susceptible to re-silencing and apparently did not involve loss of transgenic sequences (Pandit and Russo, 1992). In both studies, the transgenic sequences frequently were methylated, and treatment with 5-aza-cytidine, an inhibitor of cytosine methylation, seemed

to increase the reactivation frequency in some silenced transformants.

We have dissected further the molecular mechanisms involved in transgene-induced gene silencing, or 'quelling', in *Neurospora*. We present evidence that quelling operates at the post-transcriptional level and involves a dominant, *trans*-acting molecule. Furthermore, we have detected sense RNA derived from the transcription of a promoterless transgene. These findings, along with the observation that quelling is unimpaired in methylation-defective *Neurospora* strains, argue against the model that direct DNA–DNA interaction, or cytosine methylation at the DNA level, is involved in transgene-induced gene silencing in *Neurospora*. Instead, our data are consistent with a model in which transcription of the transgene(s) is somehow required to trigger silencing of endogenous and ectopic gene copies.

Results

DNA sequence requirements for transgene silencing

Previously, we showed that fragments of two carotenogenic genes, albino-1 and albino-3 (*al-1* and *al-3*), are able to induce transgene-mediated gene silencing in *N.crassa* (Romano and Macino, 1992). Thus, gene silencing is not dependent on an intact functional transgene. We wished to explore the possibility that efficiency of quelling may be affected by specific sequences and/or may require a minimal size of transforming DNA. We therefore compared the quelling efficiencies (measured as the percentage of transformants that exhibited an albino phenotype) of various deletion constructs of the *al-1* gene (Figure 1 and Table I). The transforming DNA containing a 'full-length', promoter-containing, *al-1* gene (construct pTJS342 of Figure 1) caused the appearance of an albino phenotype in 24% (Table I) of transformants using an *al-1*$^+$ host, indicating that both the native *al-1* gene and the transgene(s) were silenced. Plasmids containing progressive 3′ deletions of pTJS342, eliminating the 3′ untranslated region (UTR) and up to two-thirds of the coding sequence, showed quelling efficiencies between 41 and 20% (constructs pTJS342.29, pTJS342.16 and pTJS342.27). Two additional constructs that included sequences from further upstream were also analysed. Construct pTJS316 (containing ~300 nucleotides of transcribed sequence) caused quelling with a frequency similar to that of the intact gene (28%). Further deletion from the 3′ side, to position 602 in construct pTJS316.9 (which eliminated all but 14 nucleotides of transcribed sequence), completely abolished quelling. This is striking because the total length of this transgene was greater than that of construct pTJS342.29, which showed a high frequency of quelling. Similarly, progressive deletions from the 5′ end did not reduce quelling (pTJS347 and pTJS347.34). A construct with a smaller portion of the transcribed sequences, ~400 nucleotides, exhibited a lower silencing efficiency (pTJS347.8). Finally, we analysed the behaviour of two constructs containing only internal coding sequences. A 1400 nt long construct (pX16) showed high quelling (43%), while a 170 nt long construct (PCC170) gave a low frequency of quelling (3%).

Together, these results suggest that, in order to be able

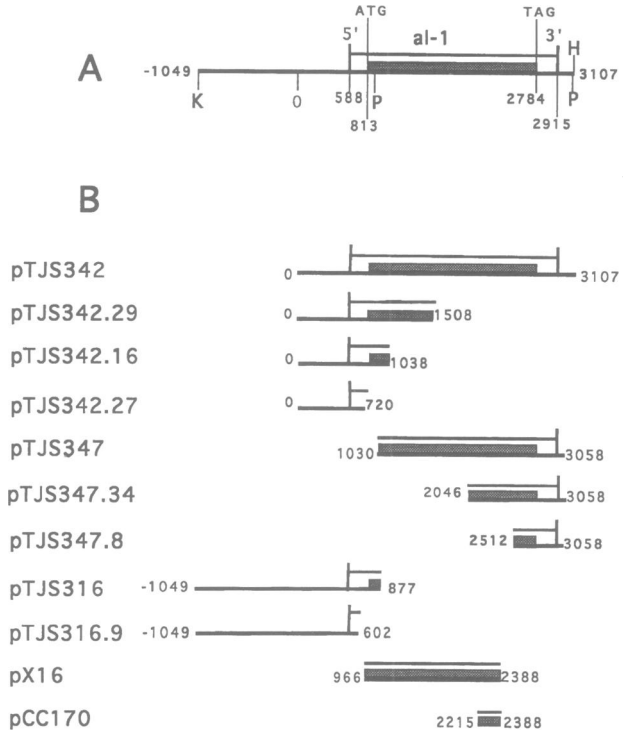

Fig. 1. *al-1* deletion constructs used in transformation experiments. (**A**) Physical map of the *N.crassa al-1* gene region. The *al-1* region of the *Neurospora* genome is shown from a *Kpn*I site to a *Hin*dIII site. Numbering is relative to the published *al-1* sequence extending from a *Sma*I site at position 0 to the *Hin*dIII site at position 3107 (Schmidhauser *et al.*, 1990). The major *al-1* transcription start site is at position 588 (Schumacher, 1995) and the stop codon is located at position 2784. The shaded box indicates the coding region of the *al-1* mRNA. The entire transcript including non-coding regions is marked above with lines at the 5′ (588) and 3′ (2915) ends of the mRNA. The promoter region of the *al-1* gene is between –1049 and 588. Restriction enzymes are abbreviated as follows: H = *Hin*dIII; K = *Kpn*I; P = *Pst*I. (**B**) *al-1* 5′ and 3′ deletion. Numbers indicate the sequence coordinates of endpoints of the *al-1* sequences.

to induce silencing, a transgene must contain at least ~132 bp (construct pTJS342.27) of sequences homologous to the transcribed region of the native gene. Transgenes containing only the gene promoter did not cause quelling. Specific sequences are probably not required for gene silencing, since different gene regions are able to induce quelling.

Transgene-mediated silencing does not require DNA methylation

The previous observations that multiple transgenes are often both silenced and methylated, and that the methylation inhibitor 5-azacytidine increased the reversion frequency of some silenced strains, raised the possibility that methylation is involved in transgene silencing (Pandit and Russo, 1992; Romano and Macino, 1992). However, cytosine methylation was not detected at the native *al-1* locus in silenced strains, suggesting that transgene silencing may be independent of transgene methylation (Romano and Macino, 1992).

To test whether cytosine methylation plays any role in quelling in *Neurospora*, we assessed the efficiency of transgene-mediated gene silencing in a mutant (*dim-2*) strain defective in cytosine methylation (Foss *et al.*, 1993). We used pTJS425, a construct containing the *al-1*

sequences from pTJS347 (nt 1030–3058 in Figure 1) and the *tub-2* gene as a selectable marker, to transform a *dim-2* strain (N592) and a *dim*$^+$ control strain (N411). Benomyl-resistant (*tub-2*-containing) transformants were selected, allowed to conidiate, and scored for *al-1* gene silencing by examining the colour of the conidia. Transformants that underwent conidiation were divided into three colour classes by visual inspection: orange (wild-type), dark yellow or light yellow/white. In both the *dim-2* and the control *dim*$^+$ strain, approximately one-third of the transformants exhibited some degree of albino phenotype, indicative of gene silencing (Table II). Reversion of both *dim-2* and *dim*$^+$ transformants to the wild-type (orange) phenotype occurred at a similar frequency (data not shown). Thus, the frequency, severity and stability of *al-1* transgene silencing in a *dim-2* strain was indistinguishable from that in a *dim*$^+$ strain.

To verify that methylation is not involved in quelling, we examined the possibility that some methylation of the native *al-1* or the transgenic *al-1* sequences still occurred in the albino *dim-2* transformants. Representative quelled transformants were examined by Southern hybridization using the 5-methylcytosine-sensitive enzyme *Sau*3AI and its 5-methylcytosine-insensitive isoschizomer *Dpn*II (Nelson *et al.*, 1993). In the *dim-2* strain, no methylation was observed in the introduced *al-1* DNA (Figure 2B, lanes 11–20), the native *al-1* locus (Figure 2C, lanes 11–20) or in a region of DNA (Ψ63) which is methylated (Foss *et al.*, 1993) in wild-type *Neurospora* (Figure 2D, lanes 11–20). In the *dim*$^+$ strain, only the transgenic *al-1* sequences of the white and yellow transformants showed methylation (Figure 2B, lanes 7–10); no methylation was observed in the native *al-1* sequences (Figure 2C, lanes 1–10). The observation that ectopic sequences were methylated while the native gene was not is consistent with previous studies (Romano and Macino, 1992). The increased intensity of bands corresponding to fragments within the transgene and the appearance of novel bands (corresponding to junction fragments) in *Dpn*II digests of most of the silenced strains (Figure 2B, lanes 5, 7, 9, 15 and 19) is indicative of a correlation between transgene copy number and silencing. Cytosine methylation of transgenic sequences in *dim*$^+$ strains is also correlated with transgene copy number and silencing (Figure 2B, lanes 5–10). These correlations have been described previously (Pandit and Russo, 1992; Romano and Macino, 1992; Selker *et al.*, 1993).

Table I. Deletion analysis of *al-1* quelling

Plasmid used	Transformant phenotypes		Total	Quelling efficiency (%)
	Non-orange	Orange		
pTJS342	64	198	262	24
pTJS342.29	29	47	76	38
pTJS342.16	59	85	144	41
pTJS342.27	33	132	165	20
pTJS347	109	147	256	42
pTJS347.34	72	75	147	49
pTJS347.8	21	111	132	16
pTJS316	26	68	94	28
pTJS316.9	0	173	173	0
pX16	129	171	300	43
pCC170	6	194	200	3

Constructs used are shown in Figure 1 and described in the text. Numbers of transformants in each colour class are indicated, and the quelling efficiency is reported as the percentage of transformants exhibiting a non-orange phenotype. Constructs of the pTJS series were co-transformed with a plasmid, pSV50, containing a benomyl-resistant β-tubulin gene. Although co-transformation of DNA into *Neurospora* occurs at a high frequency, a small percentage of benomyl-resistant transformants will not have taken up pTJS series plasmid DNA. Thus, for the pTJS series constructs, the actual quelling efficiencies shown probably slightly underestimate the frequency of silencing by each construct. Constructs pX16 and pCC170 contain *qa-2* as a selectable marker for transformation.

Gene silencing is dominant in a heterokaryon

Since mutations in the *al-1* gene, and in other carotenogenic genes (*al-2* and *al-3*), are typically recessive, we expected to observe quelling only in the homokaryotic transformants. As *N.crassa* spheroplasts are frequently multinucleate, transformation with exogenous DNA often results in heterokaryons in which only one nucleus contains transforming DNA (Pandit and Russo, 1992; Groteluesch en and Metzenberg, 1995; Miao *et al.*, 1995). Indeed, using the *qa-2*$^+$ (quinic acid) gene as the selectable marker, we verified that ~80% of primary transformants were heterokaryons by their ability to produce both *qa-2* and *qa-2*$^+$ conidia. Furthermore, these heterokaryons were stable; even after ~25 mitoses in selective conditions, >50% of the nuclei were still *qa-2* (data not shown). We therefore addressed the possibility that quelling is dominant in heterokaryons.

To look for quelling in heterokaryotic transformants, we analysed 20 primary transformants (10 albino and 10 orange transformants) obtained with the plasmid pX16, which contains the *qa-2*$^+$ gene and *al-1* sequences (Figure 1). For each transformant, we analysed the phenotype of 100 descendants resulting from uninucleate microconidia, obtained by growth on non-selective media (Ebbole and Sachs, 1990). The 10 orange primary transformants produced either all orange/*qa-2*$^+$ colonies, or a mixture of orange/*qa-2*$^+$ and orange/*qa-2*$^-$ colonies, the latter presumably indicative of heterokaryons consisting of both transformed and untransformed nuclei (data not shown). Two albino primary transformants (X-0 and X-1) produced

Table II. Quelling in the absence of DNA methylation

Strain	Plasmid used	Transformant phenotypes				Total	Quelling efficiency (%)
		O	OY	Y	W		
N411 (*dim*$^+$)	pTJS425	47	13	7	3	70	33
N592 (*dim-2*)	pTJS425	46	13	10	3	72	36

Carotenogenic phenotype of pTJS425 transformants obtained in wild-type and methylation-deficient (*dim-2*) strains. Colour classes are abbreviated as follows: O = orange, OY = orange–yellow, Y = yellow, W = white.

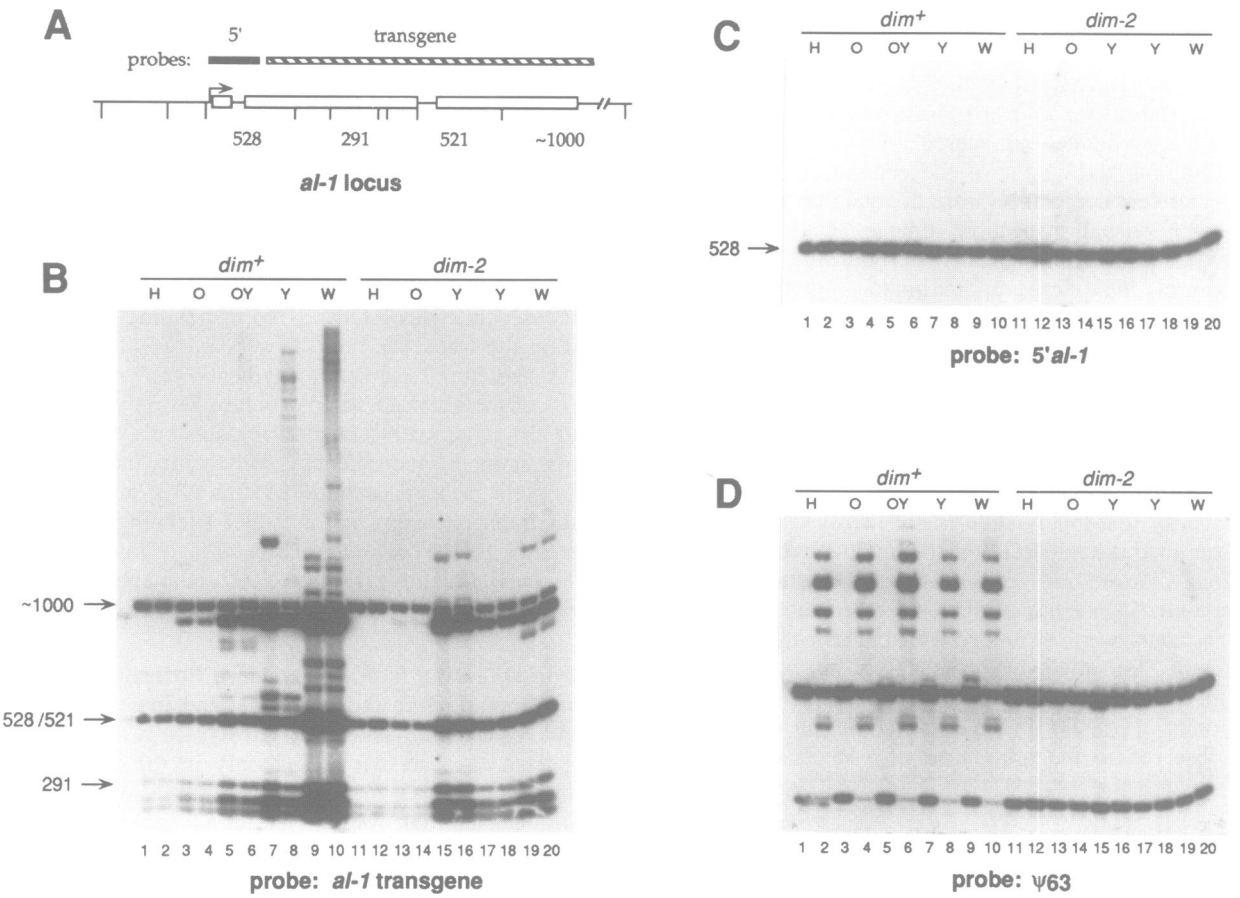

Fig. 2. Methylation analysis of *al-1* in wild-type and methylation-deficient *dim-2* transformants. (**A**) Map of the *al-1* region. The three open boxes correspond to the *al-1* exons, the vertical lines indicate the position of *Sau*3AI sites, and the arrow indicates the location of the transcription start site. The hatched box indicates the *Pst*I fragment of pTJS425 that was used as a probe for transgene sequences. The filled box indicates the sequences that were obtained by PCR and used to probe specifically the native *al-1* locus. Sizes of selected *Sau*3AI fragments detected in (B) and (C) are shown in base pairs. (**B–D**) Digests of genomic DNA from representative strains obtained by the transformation of *al-1* sequences (pTJS425, see A) into the *dim*$^+$ control strain (N411) (lanes 1–10) and the *dim-2* strain (N592) (lanes 11–20). Each DNA sample was cut with *Dpn*II, which is not sensitive to 5-methylcytosine (odd lanes), or its isoschizomer *Sau*3AI, which is sensitive to 5-methylcytosine (even lanes). The arrows indicate the location of selected *al-1* fragments; the sizes are indicated in base pairs. Strain phenotypes are noted as H = host untransformed strain (lanes 1, 2, 11 and 12); O = orange, or wild-type carotenoid phenotype (lanes 3, 4, 13 and14); OY = orange–yellow, or intermediate quelled phenotype (lanes 5 and 6); Y = yellow, or strong quelled phenotype (lanes 7, 8 and 15–18); W = white, or severe quelled phenotype (lanes 9, 10, 19 and 20). Serial probings of a single blot were conducted with probes corresponding to the *al-1* transgene (B, a 2.0 kb *Pst*I fragment of *al-1* present in pTJS425), the native *al-1* gene (C, a 300 bp PCR fragment specific to the 5′ end of the native *al-1* locus), or a control gene (D, a 1.0 kb *Ban*II–*Dra*II fragment from the Ψ63 region).

only *qa-2*$^+$ colonies, indicating that the original transformants were homokaryotic (Table III). Occasional orange colonies from X-0 and X-1 presumably were 'revertants' of quelling. Their frequency (~10%) was as expected based on previous results (Romano and Macino, 1992). By contrast, eight other albino primary transformants (X-2 to X-9) segregated albino/*qa-2*$^+$ and orange/*qa-2*$^-$ colonies, revealing that the original albino transformant was a heterokaryon containing a large proportion of untransformed nuclei (Table III). Again, the ~10% observed orange/*qa-2*$^+$ colonies presumably reflected reversion of quelling. These results suggest that the *al-1* transgene in the primary transformant (heterokaryon) acted in a dominant fashion to silence the native *al-1* gene in both transformed and untransformed nuclei.

To confirm that quelling is not nucleus-limited, we constructed forced heterokaryons between a 'quelled' albino strain and a wild-type strain. We first obtained homokaryotic albino transformants by transforming a *qa-2 aro-9 met-5* orange strain with a plasmid (pX16) containing the *qa-2*$^+$ gene and *al-1* sequences (Figure 1) and plating on medium supplemented with methionine. Uninucleate microconidia were then isolated from two primary albino transformants. Heterokaryons were forced by mixing conidia of the homokaryotic albino transformants (*met-5*) with conidia of a *lys-2* orange strain. Heterokaryotic strains (*met-5/lys-2*) were grown and re-isolated by plating the conidia on minimal medium. Approximately 95% of the heterokaryons showed an albino phenotype, indicating dominance of the quelled nuclei over the untransformed ones. The nuclear composition of the conidia from these albino heterokaryotic strains was verified by plating them on media containing either methionine or lysine to allow the segregation of homokaryotic conidia. The original heterokaryons produced 60% heterokaryotic and 20% of each type of homokaryotic conidia. This proportion of conidia corresponds to a nuclear distribution of 50% of each nuclear type in the heterokaryotic mycelia, assuming an average of ~2.5 nuclei per conidia (Davis and DeSerres, 1970). As a control in these forced heterokaryon experi-

Table III. Quelling in heterokaryotic transformants

Strain	Descendants phenotypes				Composition
	Orange/Qa+	White/Qa+	Orange/Qa–	White/Qa–	
X-0	9	91	0	0	homokaryon
X-1	10	90	0	0	homokaryon
X-2	8	36	56	0	heterokaryon
X-3	14	55	31	0	heterokaryon
X-4	12	38	50	0	heterokaryon
X-5	5	45	50	0	heterokaryon
X-6	12	38	50	0	heterokaryon
X-7	15	58	27	0	heterokaryon
X-8	5	35	60	0	heterokaryon
X-9	10	40	50	0	heterokaryon

Analysis of primary quelled transformants for heterokaryosis. X-0 to X-9 are primary quelled albino transformants. One hundred descendants from uninucleate conidia of each transformant were scored for their phenotype, and numbers in each class are indicated.

ments, we verified the recessive nature of an *al-1* mutation by forcing a heterokaryon between an albino mutant (*al-1*, *met-5*) and a wild-type (*al-1*$^+$, *lys-2*) strain. All colonies derived from this heterokaryon showed an orange phenotype, as expected for a recessive *al-1* allele.

Quelling does not impair transcription

Transgene-mediated silencing results in a strong reduction of the steady-state level of the mRNA from the silenced gene (Romano and Macino, 1992). This reduction could result from effects at either the transcriptional or post-transcriptional level. As a first step to distinguish between these possibilities, we determined the relative amounts of the endogenous precursor *al-1* mRNA and mature mRNA in both quelled and non-quelled transformant strains. Because the expression of the *al-1* mRNA is transient and reaches a peak after 20 min of light induction (Schmidhauser *et al.*, 1990), we illuminated mycelia for 20 min before extraction of the RNA. We performed an RNase protection assay using a probe that would detect both the endogenous unspliced and mature *al-1* mRNAs. Results in Figure 3 indicate that the level of precursor RNA is roughly the same in both quelled and non-quelled strains, while the amount of mature mRNA appears to be six times less in the quelled cells relative to the non-quelled cells. Three replicas of this experiment using different quelled strains yielded equivalent results (data not shown). Thus, quelling affects the level of mature mRNA and not the rate of transcription.

An unexpected sense RNA: the dominant signal for quelling?

The observation that quelling is dominant in heterokaryons indicates that quelling results from the production of a *trans*-acting substance. Since quelling displays gene specificity (Romano and Macino, 1992) and requires some amount of the transcribed region of *al-1* in the transgene, it seemed possible that quelling may be mediated by an RNA molecule. However, the quelling frequency does not appear to be reduced when a promoterless transgene, or even fragments thereof, are used for transformation. These cases may result from insertion of the transgene in the vicinity of an endogenous promoter, which may trigger the synthesis of a transgene-specific RNA in either the sense or the antisense direction. To explore the possibility that an RNA molecule is involved in quelling, we searched for the presence of transgene-specific RNA products in 'quelled' albino strains which contain a promoterless *al-1* fragment (pX16 construct, Figure 1).

As demonstrated in previous work (Romano and Macino, 1992), quelling is accompanied by a strong reduction in the steady-state levels of the mRNA from the silenced gene. Since antisense RNA has been invoked as a mechanism which leads to the degradation of sense RNA, we first tried to detect an antisense *al-1* RNA species by performing RNase protection experiments with the probe illustrated in Figure 4B. RNA extracted from five quelled transformants and five transformants wild-type for carotenogensis all failed to protect the antisense probe (Figure 4A, lanes 5–14). Thus, no detectable antisense *al-1* transgene RNA was produced in the quelled strains. A positive control demonstrated that this RNase protection assay is sensitive enough to detect as little as 0.001 fmol of antisense DNA (Figure 4A, lane 3). We also were unable to detect antisense *al-1* RNA using a probe from the 3′ region of the *al-1* gene (data not shown).

To investigate further whether production of antisense RNA is involved in quelling, we constructed strains in which antisense transgene RNAs were expected and tested for quelling. The promoterless X16 construct (Figure 1) was linked to the *Aspergillus nidulans trpC* promoter (Mullaney *et al.*, 1985) so as to generate antisense transgenic *al-1* RNA in the transformants (pTRA1, Figure 5). The frequency of quelling observed in these transformants was the same as that of the controls (transformants with a promoterless pX16 and with pTRA2, in which the *al-1* sequences from pX16 are linked to an inverted *trpC* promoter; Figure 5). Transgene expression in the antisense transformants was verified by RNase protection assays using the antisense probe shown in Figure 4B. It was possible to detect low levels of antisense transcript from the 3′ end of the transcript (5′ end of the transgene) in three different transformants (one orange and two albino) (data not shown). This suggests that an antisense RNA does not provoke silencing of the native *al-1* gene.

Next, we looked for the presence of a sense transgenic RNA in the quelled transformants. We performed RNase protection experiments with a probe that is able to discriminate between the transcripts of the *al-1* transgene and those of the endogenous *al-1* gene (Figure 6B). The probe used was expected to generate a 206 nt protected fragment from the unspliced primary transcript of the

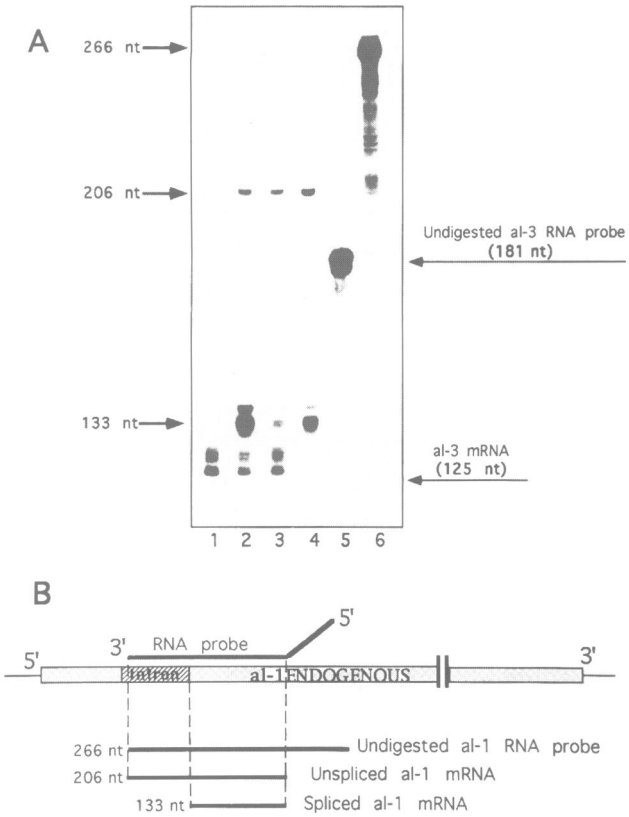

Fig. 3. RNase protection experiments for sense *al-1* RNA transcripts in quelled strains. (**A**) RNase protection analysis (RPA) was performed on total RNA extracted from an albino transformant and from a wild-type untransformed strain. An *al-3* RNA probe (181 nt) was used as control to normalize the RNA quantity (see Materials and methods); *al-3* mRNA has the same light-inducible kinetics as *al-1* mRNA. Lane 1, RPA using an *al-3* RNA probe on total RNA from a wild-type strain; lane 2, RPA on total RNA from a wild-type strain using both the *al-1* RNA probe and the *al-3* RNA probe; lane 3, RPA on total RNA from a quelled strain, probed as in lane 2; lane 4, RPA using the *al-1* RNA probe on total RNA from a wild-type strain; lane 5, undigested *al-3* RNA probe; lane 6, undigested *al-1* RNA probe. (**B**) Map of the endogenous *al-1* transcript. Shaded boxes represent the transcribed region of the endogenous *al-1* gene. The pALC4 plasmid, which contains a single intron, was used to generate a probe capable of hybridizing to *al-1* mRNA and able to discriminate between unprocessed *al-1* mRNA and mature *al-1* mRNA. This probe is indicated by the solid black line above the gene. The diagonal portion of the RNA probe represents plasmid sequences not present in the *Neurospora al-1* mRNA. The respective sizes of the undigested probe (266 nt), and the expected sizes of protected fragments by unspliced *al-1* mRNA (206 nt) or spliced *al-1* mRNA (133 nt) are shown by black lines below the gene.

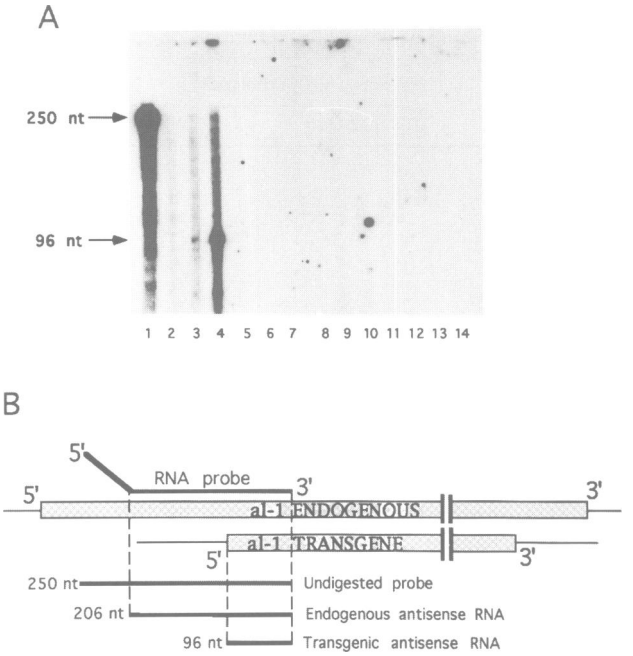

Fig. 4. RNase protection experiments for anti-sense *al-1* RNA transcripts in quelled strains. (**A**) RNase protection experiments were performed on total RNA extracted from dark-grown transformants obtained with construct pX16 (Figure 1). Transformants showing a quelled phenotype (lanes 5–9) or wild-type carotenogenic phenotype (lanes 10–14) were analysed. Lane 1, undigested probe of 250 nt indicated by an arrow; lane 2, probe plus control yeast RNA; lane 3, probe incubated with 0.001 fmol of *al-1* DNA (plasmid pALC4); lane 4, probe incubated with 0.1 fmol of *al-1* DNA (plasmid pALC4). An arrow marks the position of the expected fragment (96 nt) protected by the putative *al-1* antisense RNA that is detected only in the positive control lanes 3 and 4. (**B**) Map of potential transgenic antisense transcripts. The pALC4 plasmid corresponding to the *al-1* coding sequence (858–1063 nt) (Schmidhauser *et al.*, 1990) was used to generate an RNA probe indicated as a solid black line. The diagonal portion of the RNA probe represents plasmid sequences not present in the *Neurospora al-1* mRNA. Shaded boxes represent the transcribed region of the endogenous *al-1* gene (above) and the transcribed region of the *al-1* transgene (below). The respective sizes of the undigested probe (250 nt), and the expected sizes of fragments protected by antisense RNA for the native *al-1* gene (206 nt) or the transgenic *al-1* gene (96 nt) are shown by black lines.

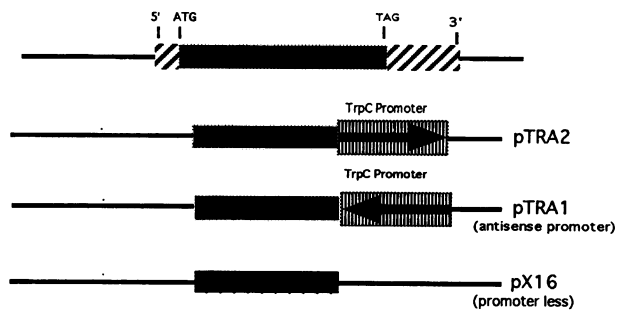

Fig. 5. Constructs for expression of *al-1* transgene antisense RNA from a linked *trpC* promoter. The top diagram illustrates the native *al-1* gene. The central black box represents the coding region of the *al-1* mRNA. In the pTRA1 construct, the *trpC* promoter drives into the 3′ end of *al-1* to produce an antisense *al-1* RNA. The pTRA2 construct is a control in which the *trpC* promoter is inverted relative to pTRA1. pX16 is a promoterless *al-1* gene construct.

endogenous *al-1* gene, a fragment of 133 nt from the mature native *al-1* mRNA and a fragment of 96 nt from the transgenic transcript (Figure 6B). To detect RNA transcripts specifically produced in quelled strains, we exploited the fact that the native *al-1* gene is not expressed in the dark. In dark-grown mycelia, a 96 nt protected RNA probe from the transgene-specific sense transcript was only detected in the quelled transformants (albino) (Figure 6A, lanes 1–5), and was absent in the non-quelled transformants (orange) (Figure 6A, lanes 6–10). Similar results were obtained with RNA extracted from illuminated mycelia; however, degradation bands from the larger protected fragments (as seen in Figure 6A, lane 12) tended to obscure the 96 nt fragment (data not shown). Thus, in the albino transformants examined, the promoterless *al-1* fragment was transcribed. An orange revertant from one of these albino transformants no longer expressed the

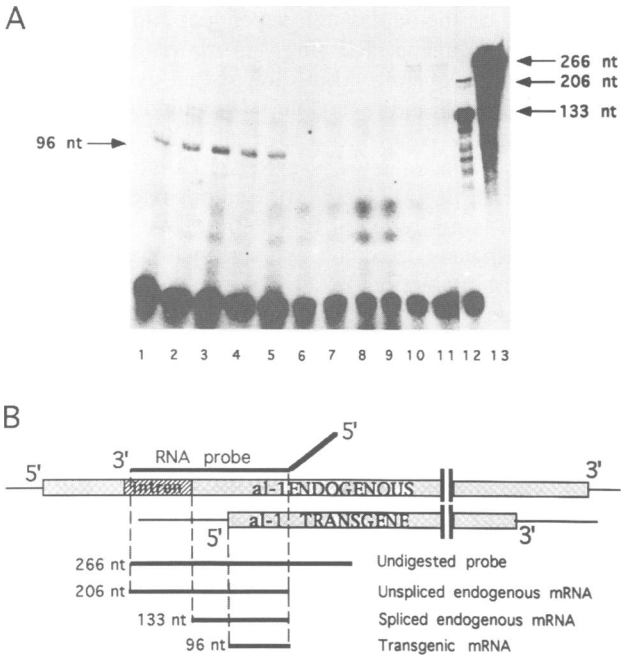

Fig. 6. RNase protection experiments for sense RNA transcripts in quelled transformants. (**A**) RNase protection experiments were performed on total RNA extracted from the transformants shown in Figure 4. RNA was isolated from dark-grown mycelia (lanes 1–11). Lanes 1–5, transformants showing quelled albino phenotype; lanes 6–10, transformants showing wild-type carotenogenic phenotypes. Lane 11, revertant of the albino transformant shown in lane 3 displaying a wild-type carotenogenic phenotype. Lane 12, RNA isolated from a wild-type, untransformed strain grown in the light. Arrows indicate the sizes of the protected fragments obtained from the unspliced endogenous *al-1* sense RNA (206 nt), from the mature endogenous *al-1* sense RNA (133 nt) and from the transgenic *al-1* sense RNA (96 nt). Lane 13, undigested probe of 266 nt indicated by an arrow. (**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 *Neurospora al-1* mRNA. Shaded boxes represent sense RNA from the endogenous native *al-1* RNA gene (above) and a putative sense RNA from the *al-1* transgene (below).

sense transcript (Figure 6, lane 11), further establishing a correlation between quelling and production of a sense transcript from the transgene.

In order to characterize further the transgenic sense RNA, RT–PCR analysis was performed. The results are shown in Figure 7. To characterize the 5′ regions of the transgenic transcripts, an oligonucleotide complementary to the albino sequence was used as primer for reverse transcriptase. A second oligonucleotide complementary to the vector sequences 472 nt upstream was added in a successive PCR amplification of the reverse transcripts. A fragment of the predicted length was obtained only in a quelled transformant but not in its revertant. Similar experiments were performed to characterize the region around the the 3′ end of the transgenic albino sequence. Using the appropriate oligonucleotides, it was possible to amplify transgenic RNA transcripts spanning 465 nt of the region across the vector cloning site. Taken as a whole, these experiments show that the transgenic construct is transcribed from some upstream location and that the transcripts that accumulate in quelled transformants are chimeric.

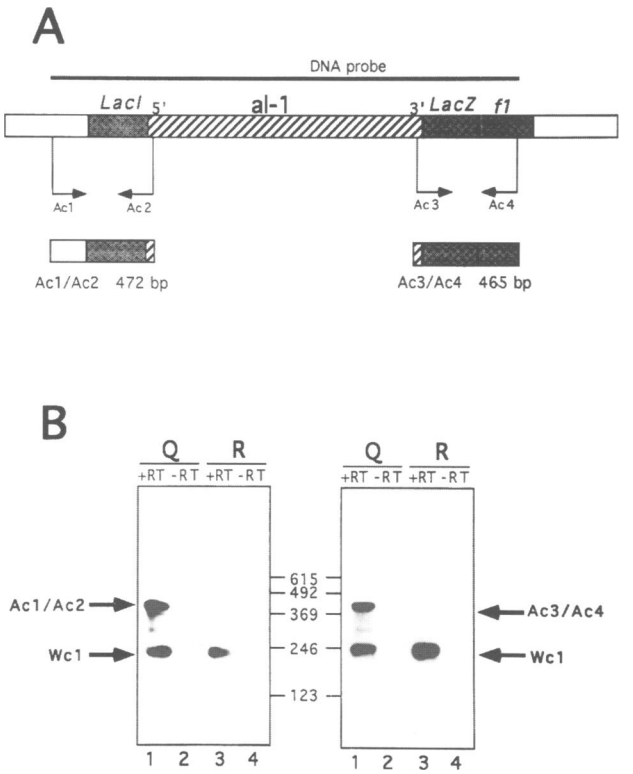

Fig. 7. RT–PCR analysis of RNAs from an albino transformant and its revertant. (**A**) Schematic drawing of the promoterless construct (pX16 in Figure1) used in transformation experiments. The *al-1* coding sequence is represented as a hatched box. lacI, lacZ and the f1 origin of replication are the vector regions flanking the insertion site and are represented by stippled boxes. Arrows represent the oligonucleotides used for PCR amplification. First-strand cDNA synthesis reactions were performed with the oligonucleotide Ac2, located within the 5′ region of the *al-1* sequence, and with the oligonucleotide Ac4 located in the f1 origin of the plasmid. The Ac1 and Ac2 oligonucleotide pair was used in PCR experiments to amplify the 5′ region of transgenic transcripts and, similarly, the Ac3 and Ac4 oligonucleotide pair was used to amplify the 3′ region. The expected PCR amplification products Ac1/Ac2 and Ac3/Ac4 and their sizes are indicated. A low abundance mRNA, *wc-1* (Ballario *et al.*, 1996) was amplified using two specific oligonucleotides added in the same reaction mixture as a positive control for the RT–PCR reaction. (**B**) Southern blot analysis of RT–PCR products hybridized with both the *al-1* DNA probe indicated in (A) and with the *wc-1* DNA probe as a control (see Materials and methods). Q, quelled transformant; R, its revertant. Lane 1, RT–PCR products from the quelled transformant; lane 2, negative control without RT; lane 3, RT–PCR products from the revertant strain; lane 4, negative control without RT. In the left panel, the RT–PCR products were obtained with the Ac1 and Ac2 oligonucleotide pair and in the right panel with the Ac3 and Ac4 oligonucleotide pair. Length markers are given at the centre in base pairs.

We investigated further the hypothesis that a sense transgenic RNA is involved in gene silencing by artificially producing a transgenic sense RNA in transformants. To this end, construct pTRS1 was produced using the *al-1* gene fragment in pX16 (Figure 1) linked to the *Aspergillus trpC* promoter. The pTRS1 construct (Figure 8) was used to transform a wild-type *Neurospora* strain. As a control, a plasmid bearing the *trpC* promoter in the opposite orientation (pTRS2), and a promoterless *al-1* construct (pX16) were also tested. These three constructs all showed similar quelling frequencies. Using the *al-1* probe described in Figure 6B, we showed that the artificially produced transgenic RNA initiating from the *trpC* pro-

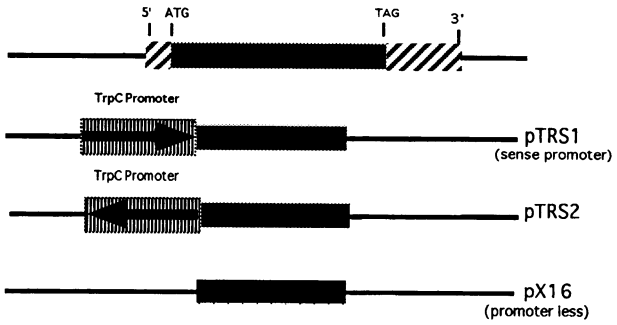

Fig. 8. Constructs for expression of *al-1* transgene sense RNA from a linked *trpC* promoter. The top diagram illustrates the native *al-1* gene. The central black box represents the coding region of the *al-1* mRNA. The *trpC* promoter drives into the 5′ end of *al-1* in pTRS1 to produce a sense RNA. pTRS2 is a control in which the *trpC* promoter orientation is inverted relative to TRS1. pX16 is a promoterless *al-1* gene construct.

moter is present in both quelled albino and non-quelled orange transformants, in variable amounts not correlated with the transformant's phenotype (data not shown). This result indicates that a sense transgenic RNA produced from a linked promoter is not sufficient to cause quelling. To determine whether different classes of transcripts were present, i.e. transcripts starting from cryptic sites upstream of the *trpC* promoter, RT–PCR analysis was performed on RNA from both quelled and non-quelled strains using oligonucleotides complementary to several regions within the *trpC* promoter and the *al-1* coding sequence. These experiments failed to detect any transcript spanning the *trpC* promoter sequences.

Discussion

Transgene-induced gene silencing occurs in a variety of organisms including plants and fungi. Silencing phenomena in plants and fungi share many characteristics, including gene specificity, a requirement for duplicated coding sequences, varying degrees of gene silencing, stepwise reversion of silencing and an association with DNA methylation (reviewed in Flavell, 1994; Matzke and Matzke, 1995a). A subset of these phenomena, including co-suppression in plants and quelling in *N.crassa*, apparently involve mutual inactivation of all homologous gene copies and this is evident at the level of mRNA accumulation (Napoli *et al.*, 1990; Romano and Macino, 1992; Dehio and Schell, 1994; Dorlhac de Borne *et al.*, 1994; Ingelbrecht *et al.*,1994). We have investigated the mechanism of quelling in *N.crassa* using expression of a carotenoid gene as a visual indicator. We report that quelling acts in *trans* in heterokaryons. In light of the recessive behaviour of carotenogenic mutants, the dominant effect of quelling suggests that a diffusable molecule produced in transformed nuclei, possibly an RNA molecule, can silence gene expression acting either at the cytoplasmic level or by travelling to neighbouring untransformed nuclei.

Some previous models of transgene-induced gene silencing have invoked DNA–DNA pairing and subsequent DNA methylation as the mechanism of gene silencing (Jorgensen, 1991; Assaad *et al.*, 1993; Matzke and Matzke, 1995b). These models were motivated by available information on the duplication-dependent multicopy gene silencing phenomena, RIP in *Neurospora* and MIP in *Ascobolus*, that operate during the sexual cycle in these filamentous fungi and reversible inactivation of a foreign gene during the *Neurospora* asexual cycle (Pandit and Russo, 1992; for reviews, see Selker, 1990; Rossignol and Faugeron, 1994). Here, we demonstrate that DNA methylation is not required for quelling. We show that a mutant lacking any detectable cytosine methylation, *dim-2*, displayed no reduction in the frequency of transgene silencing relative to a methylation-proficient control strain. Moreover, the observation that quelling is dominant and acts in *trans* indicates that a diffusable molecule is involved in gene silencing, raising the possibility that an RNA–DNA or RNA–RNA interaction is involved.

Although we have shown that DNA methylation is not required for quelling, we have not ruled out the possibility that transgene silencing and transgene methylation are related. A case of post-transcriptional silencing in plants correlates with DNA methylation (Inglebrecht *et al.*, 1994), and it has been shown that viral RNA production can influence the methylation state of homologous DNA sequences in plants (Wassenegger *et al.*, 1994), raising the possibility that disruption of gene expression at a stage after the initiation of transcription can result in *de novo* methylation of the cognate gene. In addition to cytosine methylation, other epigenetic modifications at the DNA level, such as alterations in chromatin structure, could result from post-transcriptional silencing. Such epigenetic modifications could in turn act as a 'lock', maintaining the silenced state by suppressing transcription. The observation that *dim-2* silenced strains do not differ from *dim*$^+$ strains in either the severity or persistence of the albino phenotype argues against a requirement for cytosine methylation in maintenence of the silenced state. Thus, methylation of multi-copy transgenes in *Neurospora* may be a non-functional consequence of transgene silencing or it may be a completely independent phenomenon.

The observation that DNA methylation does not cause gene silencing fits well with the finding that transcription of the endogenous *al-1* gene does not appear impaired in the quelled strains. Quelled and non-quelled strains produced the same amount of *al-1* primary transcript; however, the quantity of mature spliced *al-1* mRNA was reduced dramatically in quelled strains. Such a reduction may be due to several causes, such as (i) a defect in splicing, resulting in degradation of partially spliced intermediates and in the production of only small amounts of correctly matured mRNA; (ii) reduced cytoplasmic transport and/or accelerated nuclear degradation of mature mRNA; or (iii) cytoplasmic instability and accelerated degradation of mature mRNA. In view of the apparently unchanged steady-state levels of precursor RNA in quelled and non-quelled strains, the latter seems the simplest explanation. Whichever of these mechanisms turn out to be true, our results indicate that quelling in *Neurospora* most likely takes place at the post-transcriptional level.

Both quelling and co-suppression can involve silencing of multiple, unlinked loci, yet both exhibit a high degree of gene specificity, in that only mRNA homologous to the transgene is affected (Napoli *et al.*, 1990; van der Krol *et al.*, 1990; Romano and Macino 1992; Mol *et al.*, 1994). A transgene-derived RNA molecule could explain the

silencing of multiple copies of homologous genes within a nucleus and the high gene specificity of quelling. The observation that transgenic fragments containing any region of the *al-1* transcribed region of sufficient size (~132 nt) are capable of inducing quelling, combined with the observation that inclusion of at least some of the *al-1* transcribed region in the transgene is required for quelling, suggest a requirement for a minimal amount of homology at the RNA level.

Previous models to explain co-suppression in plants have invoked a role for an antisense transgene-derived RNA in gene silencing. This antisense RNA has been proposed to be produced by fortuitous integration of a transgene into an endogenous gene, or next to another transgene. The antisense RNA thus produced would interact with sense mRNA in a manner resulting in its down-regulation (discussed in Grierson *et al.*, 1991; Jorgensen, 1991; Mol *et al.*, 1991; Flavell, 1994). Our data do not support a role for antisense RNA in quelling in *Neurospora*. RNase protection experiments failed to show any accumulation of transgenic antisense RNA in quelled transformants. Furthermore, the forced production of *al-1* antisense RNA from a linked promoter did not produce any significant increase in the frequency of quelling compared with promoterless controls.

We found instead that sense transgenic RNA accumulated in quelled strains and was lost in a revertant. This sense RNA was produced in strains harbouring promoterless *al-1* transgenes, and was apparently derived from read-through transcription from a neighbouring promoter or by random initiation events, as shown by the fact that these transcripts contain plasmid sequences. These results support the idea that transgene transcription is somehow required for silencing. However, the relationship between the observed sense transcript and quelling remains undetermined, because the degree of gene silencing does not correlate with the amount of transcript produced. This is demonstrated clearly by the experiment in which the production of *al-1* sense transcript from a linked promoter did not change the quelling efficiency. On the whole, our data strongly argue for the involvement of an RNA transcript in the quelling phenomenon. The mechanism by which a transgene sense RNA could decrease the steady-state levels of a homologous endogenous mRNA remains unknown. A hypothetical RNA destabilizing mechanism based on threshold RNA levels and acting at the cytoplasmic level has been proposed to explain transgene-induced viral resistance in plants (Lindbo *et al.*, 1993). However, the fact that there is no direct correlation between transcript amount and quelling suggests that overexpression of sense RNA is not sufficient for destabilization of the endogenous mRNA in *Neurospora*.

The concurrence of several features of transgene-induced gene silencing in *Neurospora* and both post-transcriptional transgene-induced gene silencing and viral resistance in plants suggests that the mechanisms for transgene-induced gene silencing in plants and fungi may be related. We should stress, however, that several important differences exist between the silencing phenomena in *Neurospora* and in plants. One is that silencing persists through a number of generations in plants, while in *Neurospora* quelling tends to revert at high frequency. Therefore, it is possible that plant cells utilize additional mechanisms such as DNA methylation for the mantenance of silencing which are not used by *Neurospora*. The fact that *Neurospora* grows as a syncytium has enabled us to demonstrate that quelling is dominant and acts in *trans* to silence genes in both transformed and untransformed nuclei. While it seems likely that this would also hold in plants, it would not be observed easily, as plant cells are uninucleate. The combination of the simple assay system that we have exploited to monitor quelling and the well-developed genetic techniques available in *Neurospora* should facilitate the isolation of mutants defective in quelling. Such *Neurospora* mutants will allow us to identify factors, likely to be conserved between fungi and higher plants, involved in transgene-induced gene silencing.

Materials and methods

Strains and growth conditions

The *N.crassa* methodology was substantially the same as described by Davis and DeSerres (1970). The *N.crassa* strains used were obtained from the Fungal Genetics Stock Center (FGSC), University of Kansas, unless otherwise noted. *Neurospora* strains were as follows: FGSC No. 3957 (*qa-2 aro-9*), FGSC No. 3283 (*met-5*), FGSC No. 2136 (*lys-2*), FGSC No. 901 (*al-1 qa-2 aro-9*), FGSC No. 6524 (N411, *his-3*). The *qa-2 aro-9 met-5* strain used in the heterokaryon experiments was obtained by crossing FGSC No. 3283 (*met-5*) with FGSC No. 3957 (*qa-2 aro-9*). N592 [*dim-2 am*(Tad3.2) *ure-2 mta*] was obtained from J.Foss (University of Oregon).

Construction of al-1 deletions and transformation

Plasmid pTJS342 includes a 3.1 kb *Sma*I–*Hin*dIII fragment containing the entire *al-1* gene (Schmidhauser *et al.*, 1990) cloned into the vector Bluescript KS+ (Stratagene). Plasmid pTJS347 contains the 2.0 kb *Pst*I fragment with the *al-1* gene from pTJS342 cloned into the *Pst*I site of the vector pUC9CM (Buckley, 1985). Plasmid pTJS316 has an ~1.9 kb *Sac*I fragment from pTJS305 (Schmidhauser *et al.*, 1990) cloned into the *Sac*I site of the vector pUC118 (Vieira and Messing, 1988). The plasmid pCC170 was derived by circularization of plasmid pX16 (also known as plasmid pBSK/*al-1*X, Romano and Macino 1992) digested with *Sac*I. ExoIII deletion derivatives of pTJS342 and pTJS316 were constructed to delete sequences from the 3′ end of the *al-1* gene progressively, as described by Henikoff (1984). Derivatives of pTJS347 with deletions from the 5′ end of *al-1* were also constructed by ExoIII nuclease treatment. The precise nature of each deletion was determined by DNA sequence analysis. Spheroplasts were prepared according to the protocol of Royer and Yamashiro (1992). In the case of pTJS342-, pTJS347- and pTJS316-derived constructs, the plasmids were co-transformed with plasmid pSV50 (Vollmer and Yanofsky, 1986). pSV50 contains a benomyl-resistant β-tubulin gene that functions as a dominant selectable marker in *Neurospora* (Orbach *et al.*, 1986). The pX16 and pCC170 constructs were cloned into plasmid pBSK/*qa-2* (Romano and Macino, 1992), and the *qa-2* gene was used as the selectable marker. *qa-2*$^+$ or benomyl-resistant transformants were selected and scored for carotenogenesis by visual inspection of conidial colour; wild-type were orange, while white to yellow transformants were indicative of silencing.

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.* (1991). The pALC4 plasmid was constructed by cloning a 206 bp region of the *al-1* coding sequence (Schmidhauser *et al.*, 1990) into the *Eco*RI site of pBSSK II+ (Stratagene). The 206 bp fragment was amplified from genomic DNA by PCR using two primers complementary to bases 858–880 (5′-CCGAATTCTCTCTATTTGGAATTTGAG-3′) and 1056–1063 (5′-CCGAATTCGAAGCGGTAGCCAGC-3′) of the *al-1* gene. The underlined nucleotides represent the *Eco*RI restriction sites used for cloning. The RNA probes used in the RNase protection experiments (Figures 3, 4 and 6) were prepared by *in vitro* transcription of the plasmid pALC4 using T3 (for sense RNA probes) or T7 RNA polymerase (for antisense RNA probes). The *al-3* RNA probe (181 nt), used as control (Figure 3), was prepared by *in vitro* transcription of pBSSK II+

vector containing an *Eco*RI–*Eco*RV restriction fragment (125 bp) of the *al-3* coding sequence (Carattoli *et al.*, 1991). The *in vitro* transcription reactions and the RNase protection assays were performed using the Ambion transcription kit according the supplier's instructions. For RNase protection assays, 1 μg of total RNA was used in each reaction. The amount of protected fragments was quantified by electronic autoradiography using a Packard Instant Imager.

RT–PCR and DNA analysis

Five μg of DNA-free total RNA was used in a first-strand cDNA synthesis reaction primed with 25 pmol of Ac2 or Ac4 oligonucleotides (see below). In the same mixture, 25 pmol of an oligonucletide corresponding to the *wc-1* gene (Ballario *et al.*, 1996) were added as a internal control of the RT–PCR reaction. Reverse transcription reactions were performed by using the cDNA synthesis system Kit (GIBCO BRL). Parallel reactions without reverse transcriptase were performed and served as negative control in the successive PCRs. PCRs were performed with 15 pmol of each primer, 200 μM deoxynucleotide triphosphates, 1× amplification buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$) and 5 U of *Taq* DNA polymerase. Primer (Ac1) complementary to bases 1091–1107 (5′-TACCGCCTTTGAGTGAG-3′) of pBluescript SK II sequence and primer (Ac2) complementary to bases 1050–1066 (5′-TCTAAGCGGTAGCCAGC-3′) of the *al-1* gene were used for the amplification of the 5′ portion of the *al-1* chimeric exogenous RNA. Primer (Ac3) complementary to bases 2306–2322 (5′-TCACCGAAGAAATCGTC-3′) of the *al-1* gene and primer (Ac4) complementary to bases 302–318 (5′-CCGATTTAGAGCTTGAC-3′) of pBluescript SK II+ sequence were used to amplify the 3′ portion of the *al-1* chimeric exogenous RNA. For the amplification of the *wc-1* mRNA, two primers complementary to bases 746–762 (5′-AGAGAGAGAGCTTCTTA-3′) and 1002–1018 (5′-GGCATTGCGATGCTGAT-3′) of the *wc-1* gene were used.

RT–PCR products were then separated by electrophoresis in a 2% agarose gel and blotted according to Maniatis *et al.* (1982). Radioactively labelled probes were generated by the random primed method (Boehringer Mannheim). The probe used for detection of the pBluescript SK II+ and the *al-1* sequences was produced by PCR using as primers the Ac1 and Ac4 oligonucleotides and as template the X16 plasmid. The probe used for detection of *wc-1* sequences was obtained by PCR, and corresponds to bases 746–1018 of the *wc-1* gene. Blots were hybridized for 12 h at 65°C in 6× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (0.1% bovin serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidone), 0.1% SDS. Filters were washed in 2% SSC, 0.1% SDS at 65°C, and 0.1% SSC, 0.1% SDS at 65°C.

Construction of trpC promoter–al-1 fusions

The *trpC* promoter of *A.nidulans* was obtained as an *Eco*RI–*Cla*I fragment from the plasmid pES200 (Staben *et al.*, 1989). The pTRS1 and pTRS2 plasmids were constructed by cloning the *Eco*RI–*Cla*I fragment containing the *trpC* promoter, made blunt ended by using the Klenow fragment of *Escherichia coli* DNA polymerase I according to Maniatis *et al.* (1982), into the blunted *Xba*I site of pBSK/*al-1*X. The pTRA1 and pTRA2 plasmids were constructed by blunt-end cloning of the *trpC* promoter *Eco*RI–*Cla*I fragment into a blunted *Cla*I site of pBSK/*al-1*X.

Methylation analyses

The plasmid pTJS425 consists of a 2 kb *Pst*I fragment from the *al-1* gene (nt 1030–3058) (Schmidhauser *et al.*, 1990) and a *Hin*dIII fragment containing the *tub-2* gene from pSV50 (Vollmer and Yanofsky, 1986) inserted into the *Pst*I and *Hin*dIII sites of pUC18CM such that *al-1* and *tub-2* transcripts are convergent. *tub-2* transformants were isolated as colonies on Vogel's + 2% sorbose plates containing 1.0 μg/ml benomyl (DuPont) that was added before autoclaving, and then transferred to benomyl Vogel's slants to allow conidiation (Davis and DeSerres, 1970). For Southern hybridizations, DNA was prepared by the method of Irelan *et al.* (1994). DNA labelling and hybridization were as described in Selker and Stevens (1985). *Sau*3AI and *Dpn*II digests were carried out under conditions suggested by the manufacturer (New England Biolabs). After methylation analyses, blots were stripped and reprobed with a 2.6 kb *Bam*HI fragment containing the *am* gene (derived from pJR2; Kinsey and Rambosek, 1984) to verify that the enzyme digestions were complete. For the methylation analysis, the *al-1* probe consisted of the 2.0 kb *Pst*I fragment of pTJS425. The Ψ63 probe consisted of a 1.0 kb *Ban*II–*Dra*II fragment derived from pJS63 (Metzenberg *et al.*, 1985). The probe specific for the native *al-1* locus consisted of a 300 bp region amplified from genomic DNA by PCR using primers complementary to bases 733–751 (5′-GCAGAGAGAAGGAGCGACG-3′) and 1015–1032 (5′-CTGCAGCGGCCTCCTGTGA-3′) of the *al-1* coding sequence (Schmidhauser *et al.*, 1990).

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