Homology-Dependent Silencing of the *SC3* Gene in *Schizophyllum commune*

Theo A. Schuurs, Eveline A. M. Schaeffer and Joseph G. H. Wessels

Department of Plant Biology, Groningen Biomolecular Sciences and Technology Institute, University of Groningen, 9751 NN, Haren, The Netherlands

Manuscript received February 24, 1997
Accepted for publication July 10, 1997

ABSTRACT

After introduction of extra copies of the *SC3* hydrophobin gene into a wild-type strain of *Schizophyllum commune*, gene silencing was observed acting on both endogenous and introduced *SC3* genes in primary vegetative transformants. Nuclear run-on experiments indicated that silencing acted at the transcriptional level. Southern analysis revealed that cytosine methylation of genomic DNA occurred. Moreover, *SC3* silencing was suppressed by exposure to 5-azacytidine during growth. After growth of *SC3*-suppressed colonies from homogenized mycelium or from colonies stored at 4°, *SC3* transcription was restored. However, after prolonged growth *SC3* silencing was again observed. Introduction of a promoterless *SC3* fragment into wild type gave less *SC3* silencing.

SEVERAL examples of gene silencing as a consequence of introduction of multiple copies of homologous sequences into the genome have been described, particularly in plants (for reviews see MATZKE and MATZKE 1995; MEYER and SAEDLER 1996). Although different mechanisms of gene silencing have been suggested, they all appear to affect accumulation of specific mRNAs. In plants both transcriptional (LINN *et al.* 1990; MITTELSTEN SCHEID *et al.* 1991) and posttranscriptional gene silencing phenomena (NAPOLI *et al.* 1990; VAN DER KROL *et al.* 1990) have been found. Well-studied gene inactivations of duplicated sequences in fungi are RIP (repeat induced point mutation) in *Neurospora crassa* (SELKER *et al.* 1987) and MIP (methylation induced premeiotically) in *Ascobolus immersus* (GOYON and FAUGERON 1989). Both of these processes involve intensive cytosine methylation; in the case of RIP this is followed by C to T point mutations. Methylation of repeated sequences has also been discovered in the basidiomycete *Coprinus cinereus* (FREEDMAN and PUKKILA, 1993) but in this case *de novo* methylation led only to one observation of gene inactivation. Another silencing phenomenon found in *N. crassa* has been called quelling and occurs in primary transformants after introduction of homologous sequences (ROMANO and MACINO 1992). Apart from plants and fungi, homology-dependent gene silencing has also been found in Drosophila (DORER and HENIKOFF 1994). This article describes a gene-silencing phenomenon in the basidiomycetous fungus *Schizophyllum commune* after introduction of extra copies of the *SC3* hydrophobin gene.

In *Schizophyllum commune* four hydrophobin genes have been found. The *SC1*, *SC4* and *SC6* hydrophobin genes are specifically expressed in the dikaryon while the *SC3* hydrophobin gene is highly expressed in both mono- and dikaryons during formation of aerial hyphae (for review see WESSELS *et al.* 1995). The SC3 hydrophobin is secreted and, by interfacial self-assembly at the surface of aerial hyphae, it coats these hyphae with an insoluble amphipathic membrane that makes them hydrophobic (WÖSTEN *et al.* 1993; 1994a,b). Targeted mutation of the *SC3* gene impairs the formation of aerial hyphae; under certain conditions aerial hyphae could nevertheless form but these were hydrophilic (VAN WETTER *et al.* 1996). Transformation of the *SC3* mutant with a 5-kilobase (kb) genomic *SC3* fragment, containing a 1250-base pair (bp) upstream region, restored *SC3* expression and formation of hydrophobic aerial hyphae. In this article we show that after introduction of the same genomic fragment into a wild-type strain of Schizophyllum, a gene silencing phenomenon is observed acting on both introduced and endogenous *SC3* copies.

Corresponding author: Theo A. Schuurs, Department of Plant Biology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands.

MATERIALS AND METHODS

Plasmids: A plasmid (pXGPhT-S3) containing both a 5-kb genomic *SC3* fragment and a phleomycin resistance cassette (Figure 1) was constructed as follows. A genomic clone of *SC3* (pSg3E) was obtained by subcloning a 5-kb *Eco*RI fragment from a λ clone from a λEMBL4 genomic library of *S. commune* strain 4-39 (*MAT*A41 *MAT*B41, CBS 341.81) (SCHUREN and WESSELS 1990). A plasmid (pGPhT) carrying a phleomycin resistance cassette consisting of the promoter and terminator of the glyceraldehyde-3-phosphate dehydrogenase (*GPD*) gene from Schizophyllum fused with the phleomycin resistance gene from *Streptoalloteichus hindustanus* (SCHUREN and WESSELS 1994) was digested with *Xho*I and *Eco*RI and cloned in the *Eco*RV site of pUC20. The *Eco*RI site of this construct was used to insert the 5-kb *Eco*RI *SC3* fragment, resulting in plasmid pXGPhT-S3.

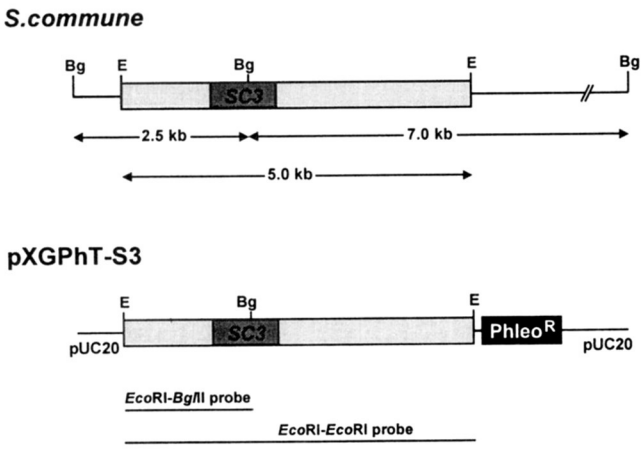

FIGURE 1.—Schematic representation of the *SC3* locus present in wild-type strain 4-39 of *S. commune* and the plasmid pXGPhT-S3 containing the *SC3* gene and the phleomycin resistance cassette. The following restriction sites are indicated: Bg, *Bgl*II; E, *Eco*RI.

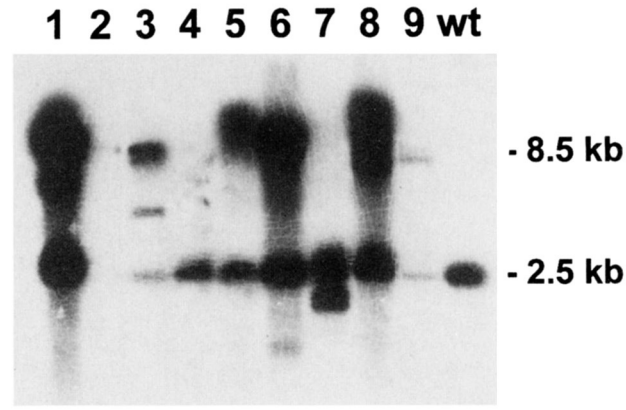

FIGURE 2.—Southern analysis of nine phleomycin-resistant transformants. DNA was digested with *Bgl*II. The blot was probed with a 1.8-kb *Eco*RI-*Bgl*II *SC3* probe. Lanes 1–9, transformant DNAs; lane wt, DNA from untransformed strain 4-39.

Transformation procedure: Protoplasts from strain 4-39 were transformed essentially according to SCHUREN and WESSELS (1994). DNA was purified using Qiagen tips (Qiagen, Chatsworth, CA). Selection during regeneration and further growth occurred in the presence of 25 μg/ml phleomycin (Cayla, Toulouse, France).

Immunodetection of secreted SC3 hydrophobin: To analyze the amount of SC3 secreted into the medium, colonies were grown for 3–4 days at 30° on perforated polycarbonate membranes (Poretics Corporation USA, pore size 0.1 μm) placed upon minimal medium containing 1.5% agar. A PVDF membrane (Millipore) was then placed underneath these colonies (between the polycarbonate membrane and the agar surface) for 3 hr, after which immunodetection of SC3 was performed with a polyclonal antiserum (WÖSTEN *et al.* 1994a). The antiserum was used in a 1:10,000 dilution. Inocula either consisted of mycelial plugs (4 mm^2) taken 5 mm from the edge of 4-day-old colonies or a drop of mycelial homogenate made by homogenizing whole colonies with 50 ml minimal medium in a Waring blender for 1.5 min.

DNA and RNA analyses: DNA and RNA isolations, electrophoresis, blotting and hybridizations were performed as previously described (SCHUREN *et al.* 1993a). For rehybridization of Northern blots with an 18S ribosomal probe, the previously used probe was removed by boiling for 2 min in 0.5% SDS or was not removed at all.

Nuclear run-on transcription assay: Both the isolation of nuclei and the nuclear run-on transcription assay were done as described by SCHUREN *et al.* (1993b). The following *S. commune* DNA probes were used: the *SC1*, *SC3*, *SC4*, *SC6* and *SC7* cDNA clones (MULDER and WESSELS 1986), a glyceraldehyde-3-P dehydrogenase (*GPD*) genomic clone p121-9 (HARMSEN *et al.* 1992) and an 18S ribosomal clone pG1-ribEX (RUITERS *et al.* 1988).

RESULTS

Genomic analysis of pXGPhT-S3 transformants: *S. commune* wild-type strain 4-39 was transformed with plasmid pXGPhT-S3 containing a phleomycin resistance cassette and a 5-kb genomic fragment including the *SC3* hydrophobin gene from Schizophyllum (Figure 1). The DNA of nine phleomycin-resistant colonies was analyzed. The introduced plasmid (pXGPhT-S3), which is 8.5 kb in size, contains one *Bgl*II site located within the *SC3* gene. The probe used in this experiment was an *Eco*RI-*Bgl*II fragment containing the promoter and the first 540 bp of the transcribed region of the *SC3* gene (Figure 1). As a consequence, in a *Bgl*II digest of genomic DNA, a 2.5-kb fragment is indicative of the presence of the endogenous *SC3* gene whereas an 8.5-kb fragment is expected when tandemly integrated copies are present. Hybridization signals found at other positions are the result of random sites of integration of pXGPhT-S3. The results in Figure 2 suggest that the copy number in transformants 7, 9 and probably 2 was one, while transformants 1, 3, 5, 6 and 8 had multiple integrations in tandem. One transformant (no. 4), seemed to be lacking the introduced *SC3* gene, although it was phleomycin resistant. It appears that only the phleomycin resistance cassette was integrated or that a spontaneous mutation conferred phleomycin resistance.

Secretion of SC3 by colonies containing multiple copies of the *SC3* gene: Phleomycin-resistant colonies were grown on perforated polycarbonate (PC) membranes overlying minimal medium agar plates. After growth for 4 days, a PVDF membrane was placed underneath the PC membrane for 3 hr to bind secreted proteins (WÖSTEN *et al.* 1994a). The pattern of SC3 secretion was then immunologically visualized. Contrary to expectations, in ~90% of the transformants a significant decrease in SC3 secretion was found. SC3 secretion was often absent throughout the colony, but sometimes only occurred in sectors (Figure 3). When compared to the restriction patterns (Figure 2), it seems that the silencing phenomenon is best observed in those transformants containing multiple copies of pXGPhT-S3. However, silencing (in sectors) was also found in trans-

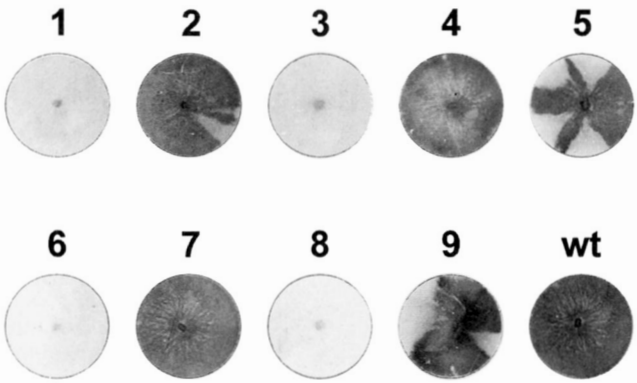

FIGURE 3.—Secretion patterns of SC3 visualized by immunodetection. PVDF membranes were placed underneath 4-day-old colonies for 3 hr to bind secreted proteins. Colonies 1–9 represent transformants shown in Figure 2.

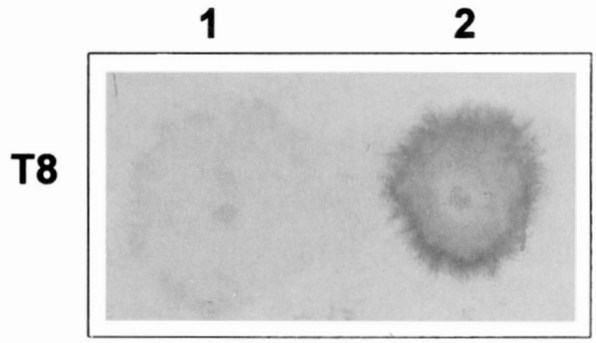

FIGURE 4.—SC3 secretion after 3 days of growth of transformant 8 if the inoculum was taken from a growing SC3-nonsecreting colony (1) or from the same SC3-nonsecreting colony stored at 4° (2).

formant 9, which seemed to have integrated only one copy. In control transformation experiments using a plasmid containing the phleomycin resistance cassette only (pXGPhT), colonies with decreased SC3 secretion were never found, suggesting that it was the introduction of the 5-kb *SC3* genomic fragment that triggered the decrease in SC3 secretion. In agreement with this, transformant 4, which had not integrated an extra copy of *SC3*, did not show any decrease in SC3 secretion.

Suppressed SC3 secretion was correlated with a wettable phenotype; a drop of water was immediately sucked into the mycelial mat. Aerial hyphae are normally coated with a protein layer of assembled SC3 (WÖSTEN *et al.* 1994a) thereby giving them a hydrophobic character. Absence of SC3 secretion makes aerial hyphae hydrophilic (VAN WETTER *et al.* 1996).

Absence of SC3 secretion as in transformants 1, 3, 6 and 8 (Figure 3) was stably maintained during subculturing. When inocula were taken from sectors with a clear decrease in SC3 secretion of transformants 5 and 9 in Figure 3, the derived colonies did not secrete SC3. Inocula taken from regions still showing SC3 secretion grew into SC3-secreting colonies in which nonsecreting sectors often occurred.

Although stably transmitted during subculturing, reversion of the SC3-suppressed phenotype was observed if inocula were taken from mycelia stored at 4°. Figure 4 shows the SC3 secretion patterns of transformant 8. Clearly, an effect of storage at 4° on SC3 secretion is visible (mycelium 2). However, after a few days of growth of a colony inoculated from a cold-stored culture, secretion of SC3 was suppressed again in the transformants containing extra *SC3* copies, exemplified by transformant T1 in Figure 5a.

The effect of mycelial homogenization on SC3 secretion: In older colonies of wild-type *S. commune*, the activity of the *SC3* gene decreases (MULDER and WESSELS 1986; SCHUREN *et al.* 1993b; see also Figure 5a). When such colonies are homogenized and used for producing a lawn of mycelium, it was found that it takes 2–3 days of growth before the *SC3* gene is again fully active (MULDER and WESSELS 1986; SCHUREN *et al.* 1993b). This is also seen when a drop of homogenized mycelium is used as an inoculum to start a colony. When colonies containing multiple copies of *SC3* and showing no secretion of SC3 were homogenized and used to start colonies, the time course of SC3 secretion, as exemplified by transformant T1 in Figure 5b, differed slightly from wild type. The eventual decrease of SC3 secretion occurred somewhat earlier and in a different manner; that is at the periphery of the colony and not primarily in the center as in wild-type colonies. Apparently the presence of multiple copies of *SC3* did not prevent reactivation of *SC3* after homogenization of the mycelium but, after 6 days, suppression occurred again in young hyphae at the periphery of the colony.

mRNA accumulation patterns: To see whether suppression of SC3 secretion was due to problems in mRNA accumulation, total RNA was isolated after growth of 4 days from the transformants shown in Figures 2 and 3. Clearly, the colonies lacking SC3 secretion (Figure 2) all failed to accumulate *SC3* mRNA (Figure 6). Also after prolonged exposures no *SC3* mRNA could be detected. Transformants 2, 5 and 9, which showed a sectoring type of SC3 secretion, accumulated *SC3* mRNA as wild-type colonies. Apparently, after introduction of multiple *SC3* copies into wild type, lack of SC3 secretion is related to a failure to accumulate mRNA from both the introduced and endogenous *SC3* genes. This could be due to mechanisms acting at the transcriptional or posttranscriptional level or both.

Nuclear run-on transcription assay: For nuclear run-on experiments, mycelia of wild-type strain 4-39 and transformant 1 and 8 (Figures 2 and 3) were grown from homogenized mycelium for 5 days. Part of the harvested mycelium was used for total RNA isolation and screened by Northern analysis for *SC3* accumulation. The results showed (Figure 7a) that at this time point the wild-type mycelium had accumulated high

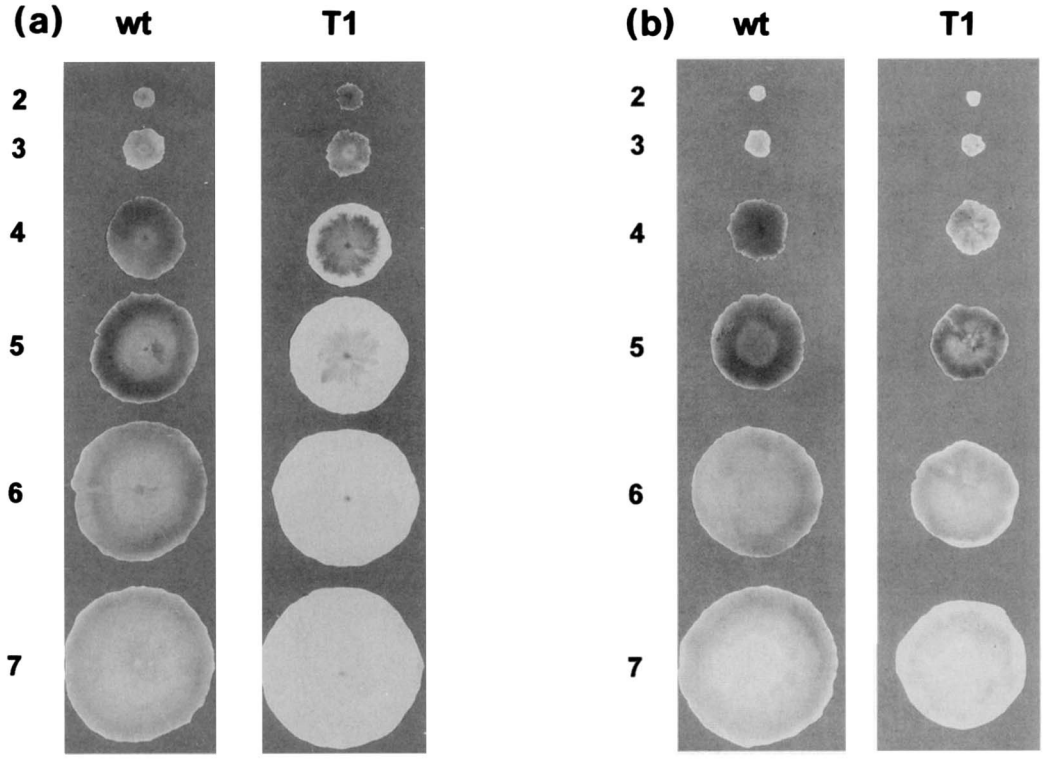

FIGURE 5.—SC3 secretion during colony development of transformant 1 (see Figure 2) and the untransformed wild-type strain 4-39. (a) Grown from a plug inoculum or (b) grown from a drop of homogenized mycelium.

levels of *SC3* mRNA (lane 1). However in transformant 1 (lane 2) and, to a lesser extent in transformant 8 (lane 3), *SC3* mRNA accumulation was significantly lower (Figure 7a). The finding of some *SC3* mRNA accumulation in the latter transformant can be explained by the fact that these transformants were inoculated as homogenized mycelium, which results in reactivation of *SC3* expression, after which suppression occurs again (see also Figure 5b). Therefore, after 5 days of growth in transformant 8 some *SC3* mRNA accumulation was still found, while transformant 1 was already completely silenced. From the same mycelia, nuclei were isolated to perform nuclear run-on experiments (Figure 7b). As expected, the dikaryon specific transcripts from *SC1*, *SC4*, *SC6* and *SC7* were low in the analyzed monokaryons, with faint hybridization signals similar to that given by the pGEM1 control. The ribosomal genes were however highly active while intermediate transcription rates were found for the constitutively expressed *GPD* gene and, in the wild-type strain, the *SC3* gene. Nuclei of transformants 1 and 8 showed low rates of *SC3* transcription (Figure 7b). The good correlation between *SC3* transcription rates and *SC3* mRNA accumulations suggests that the introduction of extra copies of the *SC3* gene somehow inhibits transcription.

DNA methylation analysis: To investigate a possible role of methylation in transcriptional silencing of the *SC3* gene, genomic DNA from wild-type colonies and transformants 1 and 8 grown for 4 days (Figure 3) were isolated and subjected to digestions with the isoschizomeric pairs *Hpa*II and *Msp*I and *Sau*3A and *Nde*II. *Hpa*II and *Msp*I both recognize the sequence CCGG but *Hpa*II is sensitive to cytosine methylation. As a probe the entire 5-kb *Eco*RI *SC3* fragment (Figure 1) was used. By comparing the restriction patterns of the *SC3*-silent transformants with wild type, cytosine methylation within the 5-kb *SC3* fragment can be detected. In both transformants high molecular bands appear in the *Hpa*II-digested DNA (Figure 8a, lanes T1-H and T8-H). This is also the case for the *Sau*3A/*Nde*II-digested genomic DNA in which *Sau*3A is the cytosine methylation-sensitive enzyme. Higher molecular bands appear while specific low-molecular-weight bands are decreased in intensity (Figure 8b, lanes T1-S and T8-S), indicating cytosine methylation within the *SC3* fragment.

Effect of 5-azacytidine (AZC) on SC3 secretion: AZC inhibits DNA-methylation by incorporating into the DNA, replacing cytidine and/or by direct inhibition of the activity of methylases (JONES 1985). The wild-type strain and transformants 1 and 8 exhibiting silencing of *SC3* transcription were inoculated on plates containing AZC of increasing concentrations (5, 10, 25, 50 and 200 μM). After growth for 5 days, PVDF membranes were placed underneath these colonies and analyzed for SC3 secretion. Even at the lowest concentration tested (5 μM AZC), reversion of *SC3* silencing could be observed but this was better seen at a concentration of 10 μM (Figure 9). In both transformants 1 and 8, SC3 secretion was restored to wild-type levels with little effect of AZC on radial growth rate. Also, at the higher concentrations

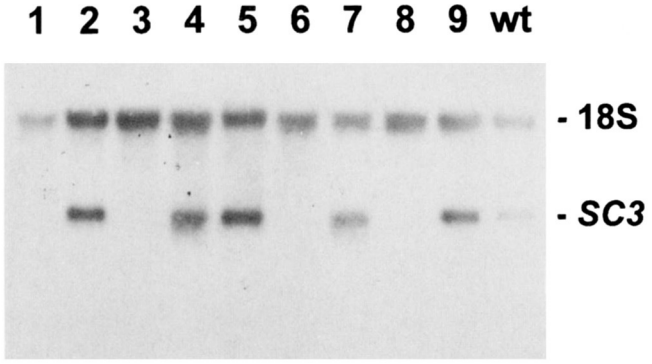

FIGURE 6.—RNA blot showing the levels of *SC3* mRNA and 18S rRNA accumulation. RNA was isolated from the same transformants as presented in Figure 3.

of AZC, which did impair growth, reversion was clearly observed. These results also suggest involvement of methylation of genomic DNA in silencing of *SC3* expression after introduction of extra copies of the *SC3* gene.

Effect of introduction of truncated *SC3* fragments on *SC3* expression: Results presented in Figure 5 show silencing of *SC3* after expression of *SC3* had occurred. After homogenization of the mycelium, *SC3* expression was restored both in transformants and in wild-type colonies but, upon prolonged growth, the former displayed a sudden drop in SC3 secretion in newly formed mycelium.

To investigate whether expression of the introduced *SC3* gene was required, or if the mere presence of duplicated sequences in the genome was sufficient to trigger *SC3* silencing, a promoterless *SC3* construct (p3NcP-ph) was introduced into the wild-type strain 4-39. This plasmid contains the entire coding region of the *SC3* gene, starting at the translation start point and ~1500 bp of downstream region, as well as a phleomycin resistance cassette for selection of transformants. Of 66 transformants analyzed, only five (7.5%) showed reduction of SC3 secretion, while introduction of pXGPhT-S3 (with promoter) caused silencing of *SC3* expression in 44 of 50 colonies (88%). Thus, by using a promoterless *SC3* construct, a significant decrease in frequency of silencing could be observed. These results therefore suggest that expression of the introduced *SC3* copies is involved in the ultimate development of *SC3* silencing.

To exclude the possibility that *SC3* silencing is caused by titration of transcription factors by the introduction of multiple *SC3* gene copies including promoter sequences, or that promoter homology is sufficient to induce *SC3* silencing, transformation experiments were performed using a vector containing only the promoter region (1250 bp) of the *SC3* gene. Of the 40 tested colonies transformed with this vector (pENc.ph), no single transformant was found showing *SC3* silencing. However, in some cases, colonies with somewhat lower SC3 secretion were observed, possibly reflecting real

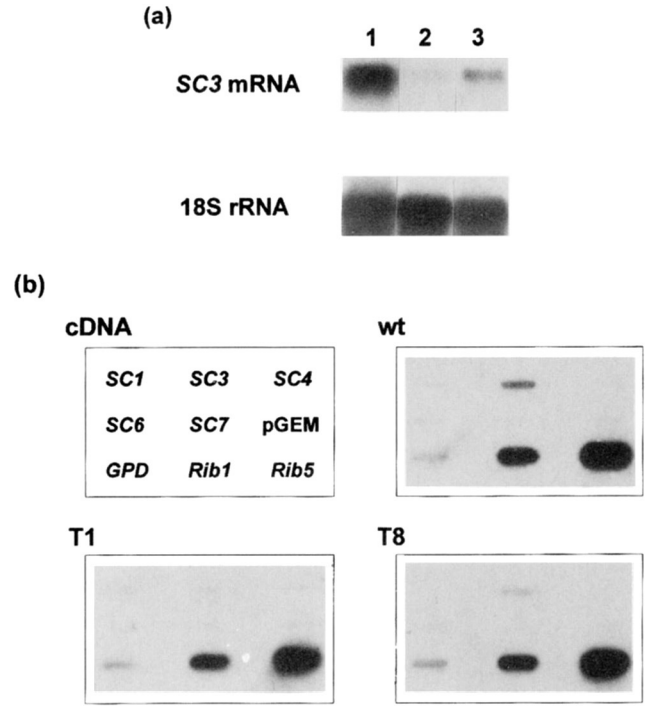

FIGURE 7.—RNA accumulation and nuclear run-on transcription. Mycelia were grown after homogenization for 5 days as mycelial lawns. (a) Northern analysis of wild-type mycelium (lane 1) and transformant 1 and 8 (lanes 2 and 3, respectively). (b) Run-on transcription assay performed on nuclei isolated from the same mycelia as used in (a). Plasmid DNAs were spotted on the filters in the pattern shown in the upper left box (b). Wt, wild type; T1 and T8, transformants 1 and 8, respectively (see Figures 2 and 3).

titration phenomena. From these results we conclude that *SC3* silencing occurring after introduction of extra copies into wild type is not due to promoter homology nor associated with titration of transcription factors.

DISCUSSION

Results presented in this paper show that, after introduction of a 5-kb *SC3* fragment containing all *cis*-regulatory elements potentially sufficient for proper expression into the *S. commune* wild-type strain 4-39, gene silencing of both introduced and endogenous hydrophobin genes was observed in almost 90% of the transformants. The resulting absence of SC3 secretion leads to a wettable phenotype; aerial hyphae lack SC3 protein and are therefore hydrophilic. Our results show that silencing is at the level of transcription and likely mediated by cytosine methylation of genomic DNA.

Several homology-dependent gene-silencing phenomena in different organisms have been previously described. Our observations resemble to some extent transcriptional gene silencing processes described in higher plants (LINN *et al.* 1990; MITTELSTEN SCHEID *et al.* 1991). Reactivation of expression of the transgene (hygromycin resistance gene) could be established via

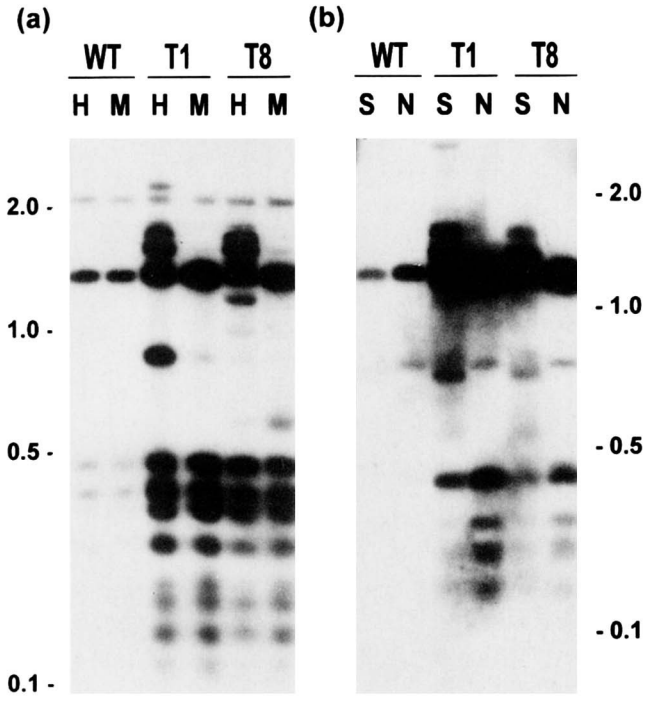

FIGURE 8.—Isoschizomer analysis for detection of cytosine methylation in *SC3*-silenced transformants. (a) DNA was cut with *Hpa*II (H) and *Msp*I (M) or (b) *Sau*3A (S) and *Nde*II (N). Both Southern blots were hybridized with a 5-kb *Eco*RI *SC3* probe. Sizes are indicated on the left and right (in kb). Wt, wild type; T1 and T8, transformants 1 and 8, respectively.

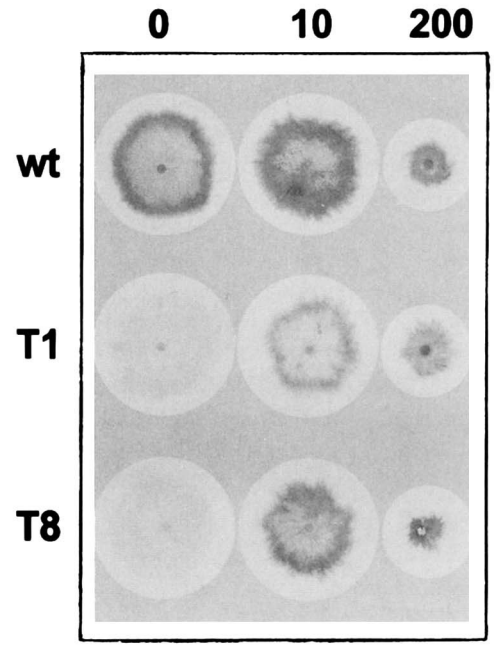

FIGURE 9.—Inhibition of *SC3* silencing by growing colonies in the presence of AZC, as observed by immunodetection of secreted SC3. Colonies were grown for 5 days on MM plates with addition of 0, 10 or 200 μM AZC for 5 days. Wt, wild type; T1 and T8, transformants 1 and 8, respectively (see Figures 2 and 3).

callus induction (MITTELSTEN SCHEID *et al.* 1991). This is in fact similar to our observations that *SC3* silencing is reversible and can be suppressed by homogenization of the colony (Figure 5) or by storage of the transformant at 4° (Figure 4). In both cases *SC3* expression during the first days of growth is as in wild type, but gene silencing develops eventually. However, once gene silencing was established, spontaneous reversion to wild-type expression was never observed.

Preliminary results show that the *SC3*-silenced state in a nucleus containing multiple *SC3* copies may not be transmitted to a neighboring untransformed nucleus after a compatible mating involving different *MAT*A and *MAT*B mating-type genes (data not shown). This is consistent with our findings showing that the *SC3*-silenced state is mediated by chromosomal methylation of the *SC3* copies and consequently that silencing is not maintained by a diffusible element.

A gene-silencing phenomenon found in *N. crassa*, which was termed "quelling" (ROMANO and MACINO 1992), perhaps best resembles the silencing phenomenon described here. Quelling also acts on homologous sequences in primary vegetative transformants. However, recent results show that quelling acts at the post-transcriptional level and is mediated by a diffusible *trans*-acting molecule (COGONI *et al.* 1996)

Until recently, gene silencing acting on homologous sequences in the genome could be distinguished in transcriptional and posttranscriptional inactivation (MATZKE and MATZKE 1995). However, this clear distinction is becoming obscured by some recent findings. First, it now seems that DNA methylation, which is normally a characteristic of transcriptional silencing, is involved in posttranscriptional gene silencing as well (ENGLISH *et al.* 1996). Whether methylation is the cause or consequence in this example of posttranscriptional gene silencing remains uncertain. One proposal claims RNA-directed DNA methylation of genomic sequences (WASSENEGGER *et al.* 1994). In this case *de novo* DNA methylation was only induced after expression of the genes involved, suggesting that overexpressed mRNA induces and directs sequence-specific methylation, thereby leading to inactivation. Results presented in the present article are best explained by such a mechanism. As shown, methylation of genomic DNA seems to be involved in the silenced state of the *SC3* gene (Figure 8). This was confirmed by the observation that addition of AZC to the medium suppressed the silenced state of the *SC3* gene. Silencing during growth was always unidirectional; silencing took place after expression of *SC3* had occurred (Figure 5) and could then be stably transmitted during subculturing. Moreover, in transformation experiments using *SC3* fragments without a promoter, a significant decrease of the frequency of *SC3*

gene silencing in transformants was observed. It has been proposed that the factors that recognize specific DNA sequences that become subjected to methylation and subsequent silencing are in fact RNA molecules (HOBBS *et al.* 1993; WASSENEGGER *et al.* 1994). This may also be true in the case of *SC3* silencing in *S. commune.*

Although our findings suggest silencing of the *SC3* gene by accumulation of transcripts, the possibility that silencing is based on recognition of homologous sequences via DNA-DNA interactions cannot be excluded. This model derives from the RIP process found in Neurospora and the MIP process in Ascobolus, where DNA-DNA interactions act in a pairwise manner (SELKER and GARRETT 1988; FINCHAM *et al.* 1989; ROSSIGNOL and FAUGERON 1994) leading to *de novo* methylation, which is, in the case of RIP, followed by C to T point mutations. DNA-DNA pairing has also been suggested to be involved in inactivation of DNA repeats by heterochromatin formation in Drosphila (DORER and HENIKOFF 1994).

Our findings may be related to observations made on the naturally occurring ribosomal repeat in *S. commune* (BUCKNER *et al.* 1988). This repeat is highly methylated in the monokaryon of Schizophyllum. However, in a heterokaryon, containing two nuclei with different mating-type genes per cell, the rDNA becomes hypermethylated. Moreover, DE VRIES and REDDINGIUS (1984) demonstrated that total RNA (which is mostly rRNA) in a dikaryotic cell amounts to only 1.3 times the quantity found in a monokaryotic cell. This led the authors to conclude that transcriptional inactivation of surplus copies of rDNA in *S. commune* might occur by methylation.

LITERATURE CITED

BUCKNER, B., NOVOTNY, C. P. and R. C. ULLRICH, 1988 Developmental regulation of the methylation of ribosomal DNA in the basidiomycete fungus *Schizophyllum commune.* Curr. Genet. **14:** 105–111.

COGONI, C., J. T. IRELAN, M. SCHUMACHER, T. J. SCHMIDHAUSER, E. U. SELKER *et al.*, 1996 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. EMBO J. **15:** 3153–3163.

DE VRIES, O. M. H., and J. REDDINGIUS, 1984 Synthesis of macromolecules and compartment size in monokaryotic and dikaryotic hyphae of *Schizophyllum commune.* Exp. Mycol. **8:** 378–381.

DORER, D. R., and S. HENIKOFF, 1994 Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. Cell **77:** 993–1002.

ENGLISH, J. J., E. MUELLER and D. C. BAULCOMBE, 1996 Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell **8:** 179–188.

FINCHAM, J. R. S., I. F. CONNERTON, E. NOTARIANNI and K. HARRINGTON, 1989 Premeiotic disruption of duplicated and triplicated copies of the *Neurospora crassa* am (glutamate dehydrogenase) gene. Curr. Genet. **15:** 327–334.

FREEDMAN, T., and P. J. PUKKILA, 1993 *De novo* methylation of repeated sequences in *Coprinus cinerius.* Genetics **135:** 357–366.

GOYON, C., and G. FAUGERON, 1989 Targeted transformation of *Ascobolus immersus* and *de novo* methylation of the resulted duplicated sequences. Mol. Cell. Biol. **9:** 2818–2827.

HARMSEN, M. C., F. H. J. SCHUREN, S. M. MOUKHA, C. M. VAN ZUILEN, P. J. PUNT *et al.*, 1992 Sequence analysis of the glyceraldehyde-3-phosphate dehydrogenase genes from the basidiomycetes *Schizophyllum commune, Phanarochaete chrysosporium* and *Agaricus bisporus.* Curr. Genet. **22:** 447–454.

HOBBS, S. L. A., T. D. WARKENTIN and C. M. O. DELONG, 1993 Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. **21:** 17–26.

JONES, P. A., 1985 Altering gene expression with 5-azacytidin. Cell **40:** 485–486.

LINN, F., I. HEIDMANN, H. SAEDLER and P. MEYER, 1990 Epigenetic changes in the expression of the maize A1 gene in *Petunia hybrida*: role of numbers of integrated gene copies and state of methylation. Mol. Gen. Genet. **222:** 329–336.

MATZKE, M. A., and A. J. M. MATZKE, 1995 How and why do plants inactivate homologous (trans)genes? Plant Physiol. **107:** 679–685.

MEYER, P., and H. SAEDLER, 1996 Homology-dependent gene silencing in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47:** 23–48.

MITTELSTEN SCHEID, O., J. PASZKOWSKI and I. POTRYKUS, 1991 Reversible inactivation of a transgene in *Arabidopsis thaliana.* Mol. Gen. Genet. **228:** 104–112.

MULDER, G. H., and J. G. H. WESSELS, 1986 Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophyllum commune* in relation to fruiting. Exp. Mycol. **10:** 214–227.

NAPOLI, C., C. LEMIEUX and R. JORGENSEN, 1990 Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in *trans.* Plant Cell **2:** 279–289.

ROMANO, N., and G. MACINO, 1992 Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. Mol. Microbiol. **6:** 3343–3353.

ROSSIGNOL, J. L., and G. FAUGERON, 1994 Gene inactivation triggered by recognition between DNA repeats. Experientia **50:** 307–317.

RUITERS, M. H. J., J. H. SIETSMA and J. G. H. WESSELS, 1988 Expression of dikaryon-specific mRNAs of *Schizophyllum commune* in relation to incompatibility genes, light and fruiting. Exp. Mycol. **12:** 60–69.

SCHUREN, F. H. J., and J. G. H. WESSELS, 1990 Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating type genes. Gene **90:** 199–205.

SCHUREN, F. H. J., and J. G. H. WESSELS, 1994 Highly-efficient transformation of the homobasidiomycete *Schizophyllum commune* to phleomycin resistance. Curr. Genet. **26:** 179–183.

SCHUREN, F. H. J., M. C. HARMSEN and J. G. H. WESSELS, 1993a A homologous gene-reporter system for the basidiomycete *Schizophyllum commune* based on internally deleted genes. Mol. Gen. Genet. **238:** 91–96.

SCHUREN, F. H. J., T. R. VAN DER LENDE and J. G. H. WESSELS, 1993b Fruiting genes of *Schizophyllum commune* are transcriptionally regulated. Mycol. Res. **97:** 538–542.

SELKER, E. U., and P. W. GARRETT, 1988 DNA sequence duplications trigger gene inactivation in *Neurospora crassa.* Proc. Natl. Acad. Sci. USA **85:** 6870–6874.

SELKER, E. V., E. B. CAMBARERI, B. C. JENSEN and K. K. HAACH, 1987 Rearrangement of duplicated DNA in specialized cells of *Neurospora.* Cell **51:** 741–752.

VAN DER KROL, A.R., L. A. MUR, M. BELD, J. N. M. MOL and A. R. STUITJE, 1990 Flavonoid genes in Petunia: addition of limited number of copies may lead to suppression of gene expression. Plant Cell **2:** 291–299.

VAN WETTER, M. A., F. H. J. SCHUREN, T. A. SCHUURS and J. G. H. WESSELS, 1996 Targeted mutation of the *SC3* hydrophobin gene of *Schizophyllum commune* suppresses formation of aerial hyphae. FEMS Microbiol. Lett. **140:** 265–269.

WASSENEGGER, M., S. HEIMES, L. RIEDEL and H. L. SÄNGER, 1994 RNA-directed de novo methylation of genomic sequences in plants. Cell **76:** 567–576.

WESSELS, J. G. H., S. A. ÁSGEIRSDÓTTIR K. U. BIRKENKAMP, O. M. H. DE VRIES, L. G. LUGONES *et al.*, 1995 Genetic regulation of emergent growth in *Schizophyllum commune*. Can. J. Bot. **73** (Suppl. 1): S273–S281.

WÖSTEN, H. A. B., O. M. H. DE VRIES and J. G. H. WESSELS, 1993 Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. Plant Cell **5:** 1567–1574.

WÖSTEN, H. A. B., S. A. ÁSGEIRSDÓTTIR, J. H. KROOK, J. H. H. DRENTH and J. G. H. WESSELS, 1994a The fungal hydrophobin SC3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. Eur. J. Cell Biol. **63:** 122–129.

WÖSTEN, H. A. B., F. H. J. SCHUREN and J. G. H. WESSELS, 1994b Interfacial self-assembly of a hydrophobin into an amphipathic protein membrane mediates fungal attachment to hydrophobic surfaces. EMBO J. **13:** 5848–5854.

Communicating editor: P. J. PUKKILA