EUKARYOTIC CELL, Apr. 2006, p. 732–744 Vol. 5, No. 4
1535-9778/06/$08.00+0 doi:10.1128/EC.5.4.732–744.2006
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Targeted Gene Silencing in the Model Mushroom *Coprinopsis cinerea* (*Coprinus cinereus*) by Expression of Homologous Hairpin RNAs

Martin A. Wälti, Cristina Villalba,† Reto M. Buser, Anke Grünler, Markus Aebi, and Markus Künzler*

Institute of Microbiology, ETH Zürich, Wolfgang-Pauli-Str. 10, CH-8093 Zürich, Switzerland

Received 16 September 2005/Accepted 19 January 2006

The ink cap *Coprinopsis cinerea* is a model organism for studying fruiting body (mushroom) formation in homobasidiomycetes. Mutant screens and expression studies have implicated a number of genes in this developmental process. Functional analysis of these genes, however, is hampered by the lack of reliable reverse genetics tools for *C. cinerea*. Here, we report the applicability of gene targeting by RNA silencing for this organism. Efficient silencing of both an introduced *GFP* expression cassette and the endogenous *cgl1* and *cgl2* isogenes was achieved by expression of homologous hairpin RNAs. In latter case, silencing was the result of a hairpin construct containing solely *cgl2* sequences, demonstrating the possibility of simultaneous silencing of whole gene families by a single construct. Expression of the hairpin RNAs reduced the mRNA levels of the target genes by at least 90%, as determined by quantitative real-time PCR. The reduced mRNA levels were accompanied by cytosine methylation of transcribed and nontranscribed DNA at both silencing and target loci in the case of constitutive high-level expression of the hairpin RNA but not in the case of transient expression. These results suggest the presence of both posttranscriptional and transcriptional gene silencing mechanisms in *C. cinerea* and demonstrate the applicability of targeted gene silencing as a powerful reverse genetics approach in this organism.

The ink cap *Coprinopsis cinerea* is a model organism for studying fruiting body (mushroom) formation in homobasidiomycetes (reviewed in references 32 and 35). Mutant screens and expression studies have implicated a number of genes in this developmental process. An example for the latter case are the *cgl1* and *cgl2* isogenes, which code for two isogalectins that are highly induced during fruiting body formation (5). In addition, orthologues of genes involved in fruiting body formation in other fungi are revealed by the sequence of the *C. cinerea* genome (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus) and its annotation, which is in progress (http://fungal.genome.duke.edu/cgi-bin/gbrowse/ccin/). Functional analysis of *C. cinerea* genes, however, is hampered by the lack of reliable tools for gene targeting. Although homologous recombination seems to occur in *C. cinerea* (3), targeted gene knockouts appear difficult to achieve, possibly due to very efficient nonhomologous DNA end joining, as has been shown for the filamentous ascomycete *Neurospora crassa* (47).

Recently, RNA-induced gene silencing (RNA silencing) has been emerging as a powerful tool for gene targeting in fungi, plants, and animals (reviewed in references 7, 11, and 13). This strategy exploits an endogenous gene regulatory mechanism of eukaryotic cells in which regulatory double-stranded RNAs (dsRNAs) interfere with homologous mRNA either by triggering its degradation or inhibiting its transcription or translation (see references 1 and 42 for recent reviews and specific references therein). For gene targeting, dsRNA homologous to the target gene is introduced into the organism either directly or indirectly as constructs leading to its endogenous expression. Both natural and introduced dsRNAs are cleaved into short pieces of 21 to 23 bp by a conserved bidentate RNase III-related RNase, Dicer, and can be amplified by RNA-dependent RNA polymerases (RdRP). These small dsRNAs are built in ribonucleoprotein complexes where the respective single strands target the activity (degradation, inhibition of transcription, or translation) of the complex to complementary mRNAs or transcriptionally active DNA regions. The activity of the complex is determined by the type of small dsRNA and the protein composition of the complex. All ribonucleoprotein complexes involved in RNA silencing known thus far appear to contain at least one member of the argonaute protein family, which shows structural homology to RNase H. Interestingly, in plants and worms, gene silencing both by exogenous and endogenous dsRNAs can be systemically transmitted within a multicellular organism, possibly by spreading of the small dsRNA as a silencing signal. In fungi, studies of transgene-induced gene silencing (quelling) in *N. crassa* were instrumental for the discovery of the mechanism and the genetic dissection of the underlying machinery (see reference 10 for a review). In the meantime, homology-based gene silencing induced by transgenes (cosuppression), antisense, or dsRNA has been demonstrated for many fungi including zygo-, asco-, and basidiomycetes (15, 19, 20, 22, 23, 29, 37, 43, 44, 46, 50, 54, 58, 64). For homobasidiomycetes, there is one report of transgene-induced silencing in *Schizophyllum commune* (53). Based on these results and the presence of candidate genes coding for orthologs of key components of RNA-mediated gene silencing in the *C. cinerea* genome (Dicer, argonaute, and RdRP), we tested the applicability of RNA silencing as a tool to target exogenous and endogenous genes in *C. cinerea*.

* Corresponding author. Mailing address: Institute of Microbiology, ETH Zürich, Wolfgang-Pauli-Str. 10, CH-8093 Zürich, Switzerland. Phone: 41-44-6324925. Fax: 41-44-6321148. E-mail: markus.kuenzler@micro.biol.ethz.ch.

† Present address: Institute of Molecular Biology and Biophysics, ETH Zürich, Schafmattstr. 20, CH-8093 Zürich, Switzerland.

MATERIALS AND METHODS

Strains, growth, and transformation conditions. *Escherichia coli* strain DH5α was used for cloning and amplification of plasmids. Transformation-competent

TABLE 1. Plasmids used in this study

Name	Description	Source or reference
pRS426	2μ-URA3	56
pPAB1.2	pTZ18R-pab1	21
pCc1001	pUC9-trp1	4
pBS-GFP	pBSII-KS(+)-GFP	18
197	pRS426-cgl2	This study
336	pRS426-pab1	This study
339	pRS426-benA-cgl2	This study
341	pRS426-benA-cgl2::GFP	This study
354	pRS426-pab1-benA-cgl2	This study
366	pRS426-benA-cgl2::GFPhp::benAI4fwd	This study
368	pRS426-benA-cgl2::GFPhp::benAI4rev	This study
367	pRS426-pab1-benA-cgl2::GFPhp::benAI4fwd	This study
369	pRS426-pab1-benA-cgl2::GFPhp::benAI4rev	This study
373	pRS426-trp1-benA-cgl2::GFP	This study
359	pRS426-pab1-cgl2hp	This study

cells were prepared as described previously (26). Plasmid-containing bacteria were selected at 37°C on Luria broth containing 100 mg of ampicillin/liter. *Saccharomyces cerevisiae* laboratory strain W303-1A (*MAT***a** *ura3-1 trp1-1 his3-11,15 leu2-3,112 ade2-1 can1-100*) was used for cloning by homologous recombination (see below for details). Transformants generated by the LiOAc method (27) were selected at 30°C on synthetic complete medium without uracil (30). *C. cinerea* strain AmutBmut (*A43mut B43mut pab1.2*) (41, 59) and one of its progenies, KK7 (*A43mut B43mut trp1.1;1.6 pab1.2*) (34), served as recipient of the various constructs. The *pab1* allele in these strains was termed *pab1.2* to distinguish it from another allele in the literature (12) and harbors a single point mutation (AAG to GAG) resulting in an amino acid substitution, K546E, at a highly conserved residue of the encoded bifunctional *C. cinerea p*-aminobenzoate (PABA) synthase (28) (C. Villalba and M. Künzler, unpublished results). The transformation of mononucleate asexual *C. cinerea* spores (oidia) was described previously (21). Selection for transformants was done on minimal medium (MM) supplied with Trp (100 mg/liter) or PABA (5 mg/liter) if necessary. Vegetative mycelium of strain KK7 was grown on cellophane disks placed on complete medium (YMG; supplemented with Trp for untransformed strain KK7) plates for 5 days (triple inocula on 90-mm petri dishes) at 37°C in darkness (in ventilated closed black boxes). Fruiting mycelium and primordia of strain AmutBmut were produced by precultivating vegetative mycelium on cellophane disks on YMG plates for 4 days (triple inocula) at 37°C in darkness and subsequent transferral to 25°C in a 12-h light/dark regime for another 3 days (containing secondary hyphal knots and primordia of up to 2 mm in diameter) and up to 10 days, respectively. The diameter of harvested primordia was 4 to 8 mm. Oidia for transformation and DNA isolation were produced by transferring vegetative mycelium grown on YMG (AmutBmut) or YMG+Trp (KK7) without cellophane disks at 37°C for 3 days in darkness (triple inocula) into constant white light (20 to 25 μE per m^2 and s; emission spectrum of 275 to 780 nm) and incubating them at 37°C for another 4 and 5 days, respectively.

Construction of plasmids. The plasmids used in the present study are listed in Table 1. The *GFP* and *GFP*-hairpin (*GFP*hp) RNA expressing plasmids were constructed in a stepwise procedure involving classical cloning in *E. coli* and homologous recombination in *S. cerevisiae* (see below). In a first step, a genomic 3.3-kb KpnI-SacI *cgl2* fragment from *C. cinerea* strain AmutBmut (5) was cloned into the corresponding sites of *S. cerevisiae-E. coli* shuttle vector pRS426, resulting in plasmid 197 (pRS426-cgl2). In a second step, the *cgl2* gene was put under the control of the *C. cinerea benA*-promoter by recombination of a PCR-generated (primers 426cgl2-BamHI-tub1000-fwd and 426cgl2-tub1000-rev on AmutBmut chromosomal DNA as a template) 1-kb *benA* promoter fragment into plasmid 197 opened with KpnI and AatII, resulting in plasmid 339 (pRS426-benA-cgl2) (Table 2). The design of the *benA* primers was based on the available *benA* sequence (GenBank no. AB007761) and the genome sequence of *C. cinerea* strain Okayama 7 (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus). Analogously, the open reading frame (ORF) of *cgl2* in plasmid 339 was replaced by the *GFP* ORF by homologous recombination of a PCR-generated (primers and on pBS-GFP as a template) *GFP* fragment into BsiWI-opened plasmid 339, resulting in plasmid 341 (pRS426-benA-cgl2::GFP). The *GFP* silencing constructs were made by using the same strategy by tandem recombination of two overlapping PCR fragments (primers CGL2p-GFP and benAInt4-GFPrev and primers benAInt4-GFPfwd and CGL2-GFPrev, respectively, on plasmid pBS-GFP as a template) into BsiWI-opened plasmid 339, resulting in plasmids 366 (pRS426-benA-cgl2::GFPhp::benAI4fwd) and 368 (pRS426-benA-cgl2::GFPhp::benAI4rev). The final construct codes for a hairpin RNA consisting of a stem formed by an inverted repeat of the complete *GFP* coding sequence in sense-antisense orientation and a loop formed by the fourth intron of the *benA* gene in a forward or reverse orientation (Fig. 1). Construction of the *cgl2* silencing plasmid 222 (pRS426-cgl2hp) was done by recombination of three overlapping PCR fragments (primers CGL2-RNAi-II5 and CGL2-RNAi-OO3, CGL2-RNAi-O5 and CGL2-RNAi-I3, and CGL2-RNAi-I5 and CGL2-RNAi-O3, respectively, on plasmid 197 as a template) into KspAI-Pfl23II-opened 197. The final construct consists of an inverted repeat of the complete *cgl2* coding sequence in sense-antisense orientation separated by a tandem repeat of the 9-bp spacer sequence (TTCAAGAGA) used in Ambion's silencing vectors pSilencer (Ambion, Austin, TX).

Recombination was also used to supply plasmids pRS426, 222, 339, and 341 with *C. cinerea* marker genes. Plasmids were opened either with KpnI (pRS426 and plasmid 222) or with BamHI (plasmids 339 and 341) and recombined with PCR-generated *pab1* (primers Up-pRS426-pab1fwd and Down-pRS426-pab1rev [pRS426], Up-pRS426-pab1fwd and Down-cgl2-pab1rev [plasmid 222], or Up-pRS426-pab1fwd and Down-benA-pab1rev [plasmid 339] on plasmid pPAB1.2 as a template) and *trp1* (primers Up-pRS426-CcTRP1fwd and Down-benA-CcTRP1rev on plasmid pCc1001 as a template; for plasmid 341) fragments resulting in plasmids 336 (pRS426-pab1), 359 (pRS426-pab1-cgl2hp), 354 (pRS426-pab1-benA-cgl2), and 373 (pRS426-trp1-benA-cgl2::GFP). The final *GFP* silencing constructs were yielded by recombination of EcoRI-BamHI *benA-cgl2*::*GFP*hp::*benAI4fwd* and *benA-cgl2*::*GFP*hp::*benAI4rev* from plasmids 366 and 368, respectively, into BstXI-opened plasmid 354. All PCR-generated constructs were verified by DNA sequencing (Microsynth, Balgach, Switzerland). Plasmid DNA from *E. coli* was isolated by using a one-tube protocol as described previously (14).

Cloning by homologous recombination in *S. cerevisiae*. Routinely, fragments with ends of 25 to 30 nucleotides (nt) homologous to regions on the recipient vector were generated by PCR and cotransformed into strain W303-1A, together with minor amounts of the recipient vector linearized in between the two recombining regions. Plasmids from single yeast transformants were rescued and amplified in *E. coli* as described previously (51). The plasmids were analyzed by restriction enzyme digestion, diagnostic PCR, and DNA sequencing.

PCR. PCRs using *Taq* polymerase for diagnostic purposes or *Pfu* polymerase for preparative purposes were performed on a Robocycler (Stratagene, La Jolla, CA) according to standard protocols (52).

Southern blot analysis. *C. cinerea* genomic DNA from oidia was isolated as described previously (63). Primordia were frozen in liquid nitrogen together with an equal volume of glass beads (400 to 600 μm in diameter) and ground by applying one pulse of 40 s at level 6 in a Fast-Prep machine (Bio 101 Savant; Savant Instruments, Inc., Holbrook, NY) before DNA isolation. *GFP* target and silencing loci were isolated by digesting genomic DNA of the corresponding transformants with HindIII, separating the fragments by agarose gel electrophoresis and isolating fragments of the respective sizes (~12 and 3 kb, respectively; see Fig. 2B) from the gel. Southern blot analysis of isolated *C. cinerea* genomic DNA was performed according to standard protocols (52). Detection of specific DNA fragments was done by using the digoxigenin (DIG) system according to the manufacturer's recommendations (Roche Applied Science, Mannheim, Germany). PCR-generated hybridization probes were generated by standard PCR (*GFP*, primers 61 and 134 on pBS-GFP as a template; *cgl2*, primers 71 and 184 on plasmid 197; *pab1*, primers 119 and 120 on plasmid pPAB1.2; *trp1*, primers 54 and 38 on plasmid pCc1001; and *cgl3*, primers 180 and 126 on chromosomal AmutBmut DNA) using *Taq* polymerase and a DIG-dUTP-containing nucleotide mix (Roche). DIG-labeled DNA-Molecular-Weight-Marker VII (Roche) was used as a size standard.

RNA extraction and cDNA synthesis. Mycelia were harvested, immediately frozen in liquid nitrogen, and stored at −80°C. Samples were lyophilized and total RNA was extracted by using an RNeasy Lipid Tissue Minikit (QIAGEN, Valencia, CA). Lyophilized mycelia (20–25 mg) were homogenized in a Fast-Prep machine (Bio 101 Savant) with three consecutive pulses of 45 s each at levels 4.5, 5.5, and 6.5 using a volume of about 200 μl of acid-washed glass beads (400 to 600 μm in diameter). Then, 1 ml of QIAzol lysis reagent was added to the homogenates, and all further steps were carried out according to the manufacturer's instructions, including an additional on-column DNase digestion using the RNase-Free DNase Set (QIAGEN). RNA purity and integrity were checked by determining the 260/280- and 260/230-nm absorption ratios and by visual inspection after separation of 10 μg of total RNA on a 6.7% formaldehyde–1% agarose gel.

cDNA was synthesized by using M-MLV Reverse Transcriptase RNase H

TABLE 2. Oligonucleotides used in this study

Name	Sequence	Purpose
*CGL2*p-GFP	*GTATCACCAGTCTAACATCATGCTC*AAGGGCGAGGAGCTGTTCACC	Cloning of *cgl2*::*GFP* fusion
CGL2 Stab-STOP-GFP	*AGGGGGAAGTGGGGGGAGCAATCCATGGAC*CTACTTGTACAGCTC GTCCATGCCG	Cloning of *cgl2*::*GFP* fusion
426cgl2-BamHI-*tub1*000-fwd	GCGTAATACGACTCACTATAGGGCGAATTGGGATCC*AATAAGGAT ACACATGATCG*	Cloning of *benA*p
426cgl2-*tub1*000-rev	GGAAAGTCGAATGCCTACTTGGCTTTGA*GCTGGGAACGCGAGGT CAGC*	Cloning of *benA*p
Up-pRS426-Cc *TRP1*fwd	ACGACTCACTATAGGGCGAAT*CCATGATGATGACGGTAGAC*	Cloning of *trp1*
Down-benA-Cc *TRP1*rev	GATCATGTGTATCCTTATTAGATCT*GAGTCGGTACTTCAAGTTCCC*	Cloning of *trp1*
Up-pRS426-*pab1*fwd	ACGACTCACTATAGGGCGAATT*GCGAAGCAACTGAAGGAGC*	Cloning of *pab1*
Down-benA-*pab1*rev	GATCATGTGTATCCTTATTGGATCC*TTCTTCTGGCATCTTTCCTC*	Cloning of *pab1*
Down-pRS426-*pab1*rev	GTCGACCTCGAGGGGGGGCCC*TTCTTCTGGCATCTTTCCTC*	Cloning of *pab1*
*benA*lnt4-GFPrev	*TAATTTTGGCCAGCACAACGCATGCTTGCGACAGGGATACATACCCT GCAGGCA*CTTGTACAGCTCGTCCATGC	Cloning of *cgl2*::*GFP*hp
*benA*lnt4-GFPfwd	*AGCATGCGTTGTGCTGGCCAAAATTAATGATCATCACGTGATAGGCT TCCAGATC*TTGTACAGCTCGTCCATGC	Cloning of *cgl2*::*GFP*hp
CGL2-GFPrev	*AAGTGGGGGGAGCAATCCATGGAC*AAGGGCGAGGAGCTGTTC	Cloning of *cgl2*::*GFP*hp
155 (GFP fwd real time)	ACATGGTCCTGCTGGAGTT	Genomic PCR and qRT-PCR of *cgl2*::*GFP*
68 (CGL2-Term)	CGAACCGCTCTGAGGGAGG	Genomic PCR of *cgl2*::*GFP* and *cgl2*::*GFP*hp
39 (GFP-(270)rev)	GCCTTCGGGCATGGCGG	Genomic PCR of *cgl2*::*GFP*hp
Down-CGL2-pab1rev	TCATTATCACGGTGGAACGGTTGCTGGATCCTTCTTCTGGCATCT TTCCTC	Cloning of *pab1*
185 (RT-PCR-rev-cgl2)	CCAGCGAGAATCCTAAGCA	qRT-PCR of *cgl2*::*GFP*
186 (RT-PCR-fwd-benA)	GTCATGTCCGGTATCACCAC	qRT-PCR of *benA*
187 (RT-PCR-rev-benA)	GGGAAAGGAACCATGTTGA	qRT-PCR of *benA*
CGL2-RNAi-II5	GTTGGTCTCTTTTTGGATTCTTG	Cloning of *cgl2*hp
CGL2-RNAi-OO3	GTATGAAGTCCGTTGGTGTCGC	Cloning of *cgl2*hp
CGL2-RNAi-O5	CGGCGCCTGGGGCCCGGAGG	Cloning of *cgl2*hp
CGL2-RNAi-I3	TCTCTTGAATTCTCTTGAA*AGCAGGGGGAAGTGGGGGG*	Cloning of *cgl2*hp
CGL2-RNAi-I5	TTCAAGAGAATTCAAGAGA*AGCAGGGGGAAGTGGGGGG*	Cloning of *cgl2*hp
CGL2-RNAi-O3	GCAAGAATCCAAAAAGAGACCAACAAACCTAATGCTCTACCACC TTTTCGTC	Cloning of *cgl2*hp
61 (GFP)	ATGAGCAAGGGCGAGGAGC	*GFP* hybridization probe
134 (GFPrev)	CTTGTACAGCTCGTCCATGC	*GFP* hybridization probe
71 (Cgl2FusSeq2)	AGTGATATCCGGTGGTCAGC	*cgl2* hybridization probe
184 (CGL2ORFRev2)	TCAGCGTACGGGATGCGTTC	*cgl2* hybridization probe
119 (07pab2006fwd)	TTGTGGCGTTGAAGAGTACG	*pab1* hybridization probe
120 (07pab2637rev)	CATGGCTGATGCTTAATTGC	*pab1* hybridization probe
54 (TRP1F-Forward)	GCCGGTCTCGACTATCCAGGTGTAGG	*trp1* hybridization probe
38 (TRP1SeqTerm)	GACCCCCTCAAACACTATTGG	*trp1* hybridization probe
180 (Cgl3PromFwd)	TCTGCCTCTGCTAGCTTGTC	*cgl3* hybridization probe
126 (CGL3rev)	ACGGTTGATTCGAGTCTG	*cgl3* hybridization probe

Minus (Promega, Madison, WI) and oligo(dT) (20) primers according to the manufacturer's instructions. A total of 1 μg of RNA per reaction (25 μl) was used, and the obtained cDNA was stored at −20°C until use.

Quantitative real-time PCR analysis. Real-time PCR was carried out by using a QuantiTect SYBR Green PCR kit (QIAGEN) with 0.9 μM forward and reverse primer concentrations each and a variable amount of cDNA (20 to 0.02 ng per reaction) in a final reaction volume of 20 μl. Thermocycling was performed by using a Rotor-Gene 3000 Real-Time Thermal Cycler (Corbett Research, Sydney, Australia) initiated by a 15-min incubation at 95°C, followed by 50 cycles of 15 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Fluorescence data were acquired during the elongation step in every cycle. Each run was completed with a melting curve analysis to confirm specificity of amplification and absence of primer dimers. The amplification of genomic DNA was prevented by designing primers on exon-exon junctions and by DNase digestion during RNA extraction. These measures proved to be effective since no amplification could be observed in a cDNA control where reverse transcriptase was omitted. *benA* detected with the primers 186 plus 187 served as a reference gene for the relative quantification of *GFP*, which was detected with the primers 155 and 185. PCR efficiencies were determined with the serial dilution method of cDNAs. Transcript quantification of individual samples is based on measurements in triplicate and analysis using the mathematical model of Pfaffl (49).

Preparation of *C. cinerea* whole-cell extracts (WCEs). Vegetative or fruiting mycelia grown on cellophane disks or primordia were shock frozen in liquid nitrogen and stored at −80°C. For extraction, the material was lyophilized, and 20 μg was ground by adding 200 μl of glass beads (400 to 600 μm in diameter) and applying one pulse of 20 s at level 6 in a Fast-Prep machine. The resulting powder was extracted by adding 200 μl of ice-cold 0.5× phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride and applying one pulse of 30 s at level 6 in the Fast-Prep machine, cooling on ice, and repeating the buffer addition and the pulsing. The resulting lysate was centrifuged twice at 15,000 rpm to remove particulate material. The protein concentration of the cleared lysate was determined by the Bradford method (Bio-Rad, Hercules, CA), mixed with an equal volume of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (250 mM Tris-HCl [pH 6.8], 9.2% SDS, 40% [vol/vol] glycerol, 0.2% [wt/vol] bromophenol blue, 100 mM dithiothreitol), and boiled for 3 min.

Immunoblotting. Equal amounts (3 to 5 μg) of *C. cinerea* WCE were separated on a SDS–15% PAGE gel (36) and transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell, Keene, NH) by using a Trans-Blot Semi-Dry Transfer unit (Bio-Rad). Blots were blocked with 5% (wt/vol) dry milk powder in PBS containing 0.1% (vol/vol) Tween 20 (PBST) and hybridized with primary rabbit antiserum and secondary horseradish peroxidase (HRP)-coupled goat

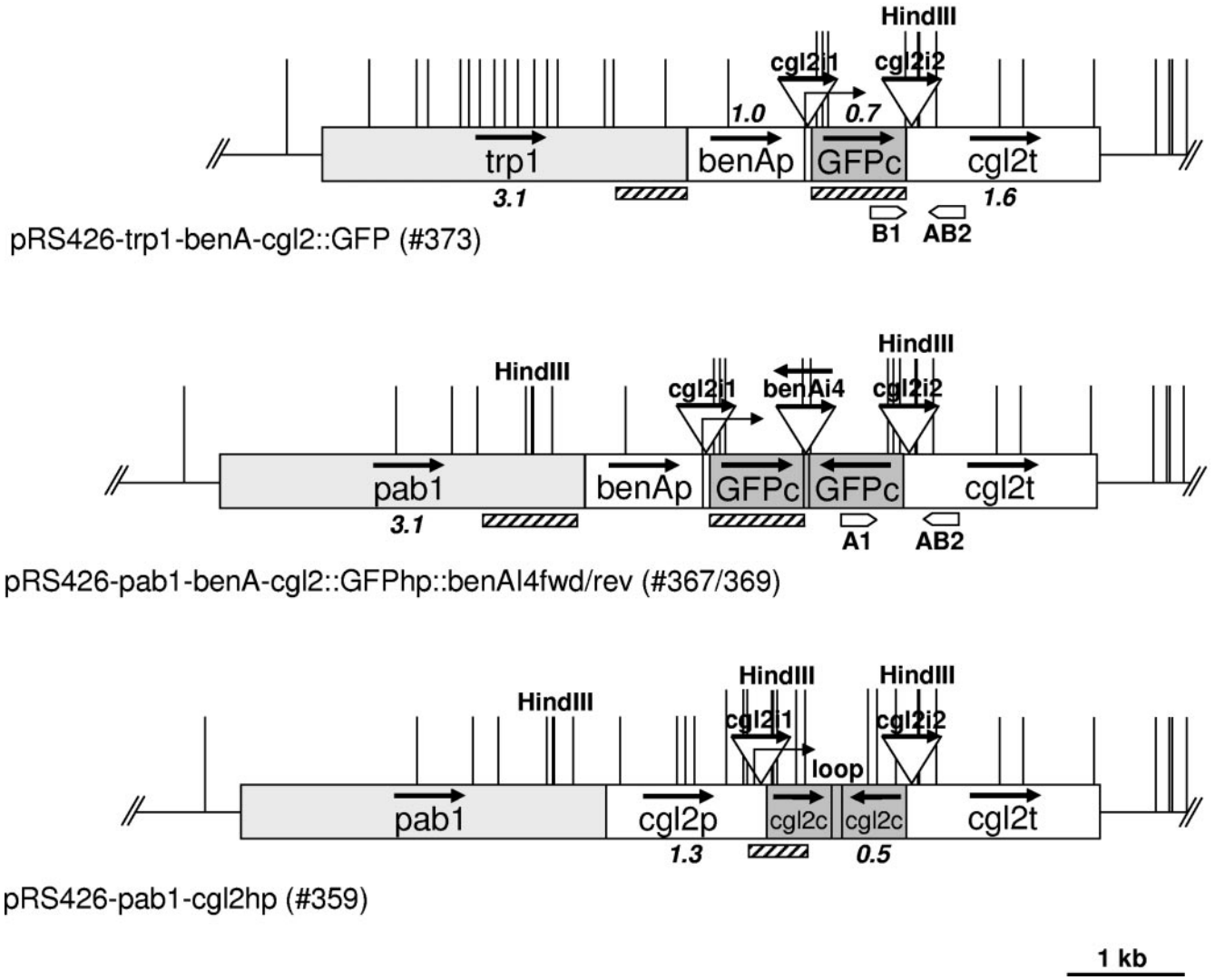

FIG. 1. Schematic representation of DNA constructs used in the present study. Promoter (p), terminator (t), and coding (c) regions of indicated genes are represented as boxes, and introns (i) as triangles. Bold arrows indicate the direction of transcription of the respective DNA element at the original locus, and the fine arrows indicate the transcriptional start sites of the constructs. The length of the longer DNA fragments is given in kilobases. Hatched boxes and block arrows below the boxes indicate hybridization probes used for Southern blot analyses and primers used for genomic PCR analyses, respectively. Restriction sites used for Southern blot analyses are indicated by thick (HindIII) or thin lines (HpaII/MspI).

anti-rabbit immunoglobulin G (Santa Cruz, Santa Cruz, CA). Polyclonal rabbit antiserum raised against recombinant green fluorescent protein (GFP) was a gift from Walter Nickel (Biochemie-Zentrum, Heidelberg, Germany). Polyclonal rabbit antiserum raised against isogalectins CGL1 and CGL2 purified from *C. cinerea* has been described elsewhere (5). Polyclonal rabbit antiserum against recombinant CGL3, a *C. cinerea* lectin with high sequence similarity to CGL1 and CGL2, will be described elsewhere. HRP was detected either by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden) or developed by using chloronaphthol as a chromogen (24).

Microscopy. *C. cinerea* strains or transformants were cultivated by inoculating liquid YMG (supplemented with Trp for KK7) with respective oidia stored in 10% (vol/vol) glycerol at −80°C, followed by incubation with vigourous agitation at 37°C until small fungal pellets were visible. The pellets were washed with deionized water, and hyphae were investigated for GFP fluorescence by using a Zeiss Axiophot equipped with a mercury lamp and a Zeiss filter set 09 (excitation BP, 450 to 490 nm; emission LP, 515 nm) (Carl Zeiss AG, Göttingen, Germany). Pictures were captured by using a Zeiss Axiocam MRc and Zeiss Axiovision software (version 4.4).

Nucleotide sequence accession number. The nucleotide sequence of the lectin-encoding *cgl3* gene from *C. cinerea* strain AmutBmut has been deposited in GenBank under accession number DQ408306.

RESULTS

Construction of a *C. cinerea GFP* expression cassette based on endogenous control elements. As a first step toward evaluation of RNA silencing in *C. cinerea*, we constructed an expression cassette for *GFP* in *C. cinerea* based on endogenous control elements. Previous reports suggested that *GFP* expression in mushrooms was dependent on the presence of introns (6, 38, 39). In the only *GFP* expression cassette available for *C. cinerea* to date, the intron is located within the coding region resulting in a GFP fusion protein (6). The coding regions of the

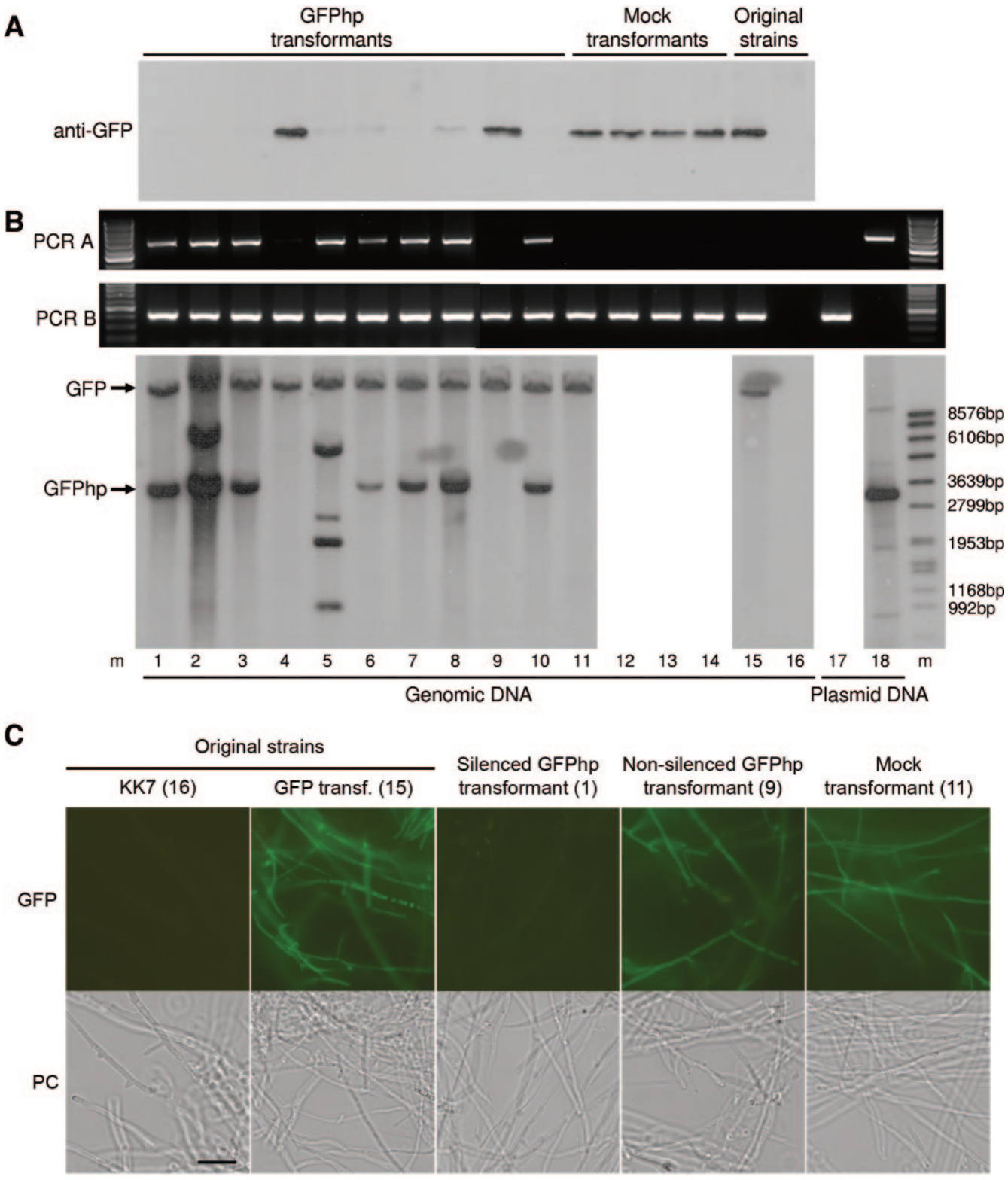

FIG. 2. Molecular analysis of *GFP* silencing in *C. cinerea* strain KK7. Analysis is shown for the strain without (lane 16) and with the *GFP*-expression construct 373 (lane 15), as well as for individual transformants of the latter transformant with either a mock plasmid carrying only the marker gene (pPAB1.2; lanes 11 to 14) or the *GFP*-silencing construct 367 (lanes 1 to 10). (A) Analysis of GFP protein levels in WCEs. A total of 3 μg of WCE prepared from the individual strains or transformants was analyzed by immunoblotting using a specific anti-GFP antiserum (see the text for details). (B) Analysis of DNA integration by genomic PCR and Southern blotting. Genomic DNA was prepared from the individual strains or transformants and used as a template for two PCRs indicative for the integration of either the *GFP* silencing construct (PCR A, primers A1 and AB2) or the *GFP* expression construct (PCR B, primers B1 and AB2) in the genome. A 100-bp DNA ladder (Fermentas International,

C. cinerea cgl1 and *cgl2* genes are flanked by two introns, one immediately upstream of the translational start codon and one immediately downstream of the translational stop codon (5). We reasoned that such a gene structure would be the ideal basis for a *C. cinerea* expression cassette for heterologous coding sequences such as *GFP*. We cloned the *cgl2* gene from *C. cinerea* strain AmutBmut including 1 kb upstream and 1.6 kb downstream of the coding sequence on a *S. cerevisiae-E. coli* shuttle vector in order to be able to use yeast recombination in subsequent modifications. Since the *cgl1/2* genes are hardly transcribed during vegetative growth (2, 5), we exchanged the *cgl2* promoter region with 1 kb of the promoter region of the AmutBmut *benA* gene coding for β1-tubulin by using yeast recombination. Using the same technique, most of the *cgl2* coding region (except for a 36-bp sequence immediately upstream of the translational stop codon) was exchanged with the entire *GFP* coding region. Finally, the *C. cinerea trp1* gene was inserted immediately upstream of the *benA* promoter region as a selection marker in *C. cinerea* (Fig. 1; see Materials and Methods for details). To check the construct for *GFP* expression, we transformed the plasmid into *C. cinerea* strain KK7. Trp-prototrophic transformants were analyzed for *GFP* expression by immunoblotting of WCE from vegetative mycelium (data not shown and Fig. 2A, lane 15), as well as for the presence and the copy number of the construct in the genome by Southern blot analysis (data not shown; Fig. 2B, lane 15). Most of the transformants with one or more copies of the construct in the genome showed a robust steady-state level of GFP (data not shown and Fig. 2A and B, lane 15, and as a control, lane 16). For unknown reasons, the GFP protein expressed in *C. cinerea* migrated consistently by 1 to 2 kDa faster in SDS-PAGE than did the same coding region expressed in *S. cerevisiae* (data not shown). Regardless of its smaller size, GFP protein expressed in *C. cinerea* was functional as shown by a strong green fluorescence in the cytosol of *GFP*-expressing transformants (Fig. 2C, panels 15 and panel 16 as a control).

Silencing of *GFP* expression by introduction of a homologous hairpin RNA. In a second step we set out to silence the *GFP* expression in two of these transformants by introducing a construct expressing a homologous hairpin RNA. Such hairpin constructs were shown to be effective in gene silencing in animals, plants, and other fungi (see the introduction for references), including the dimorphic basidiomycetous fungus, *Cryptococcus neoformans* (37). For construction we introduced an inverted repeat of the entire *GFP* coding region in a sense-antisense orientation into the *benA-cgl2* construct described above by recombination in yeast (Fig. 1; see Materials and Methods for details). As a loop sequence we introduced the fourth intron of the *C. cinerea benA* gene (GenBank no. AB007761). It was shown that a spliceable intron positioned in the loop region of the hairpin enhanced the silencing effect of hairpin RNAs in plants and flies (31, 57). To test whether the presence of a spliceable intron in the loop region of the hairpin acted as an enhancer of silencing in *C. cinerea*, we manufactured two constructs: one containing the intron in a normal orientation and the other one containing the intron in a reverse orientation (Fig. 1; see Materials and Methods for details). After supplying these constructs with the *C. cinerea pab1* gene as a selection marker (see Materials and Methods for details), two different KK7 transformants carrying the *benA-cgl2*::*GFP* construct in a single copy were each transformed with the two different silencing constructs and plasmid pPAB1.2 harboring only the *pab1* marker gene as a mock control. Ten different transformants each of the silencing constructs and four transformants each of the mock controls were analyzed by various means. First, all of the 48 transformants and the original strains were checked for *GFP* expression on the protein level by immunoblotting the WCEs of vegetative mycelium by using anti-GFP antiserum (see Materials and Methods for details). Figure 2A shows the results for a representative series of transformants, together with the control strains or transformants. In 25 of the 40 transformants to be silenced the GFP protein levels in the WCE were significantly reduced (Fig. 2A and data not shown). It is generally not predictable what part of a plasmid is integrated in the *C. cinerea* genome upon transformation and integrated DNA can also be lost (4, 21). The apparent lack of silencing could therefore arise by integration of the marker gene without the silencing cassette, whereas the apparent silencing could also be due to loss of the *GFP* expression cassette. We applied both genomic PCR and Southern blot analysis of oidial DNA to check all 48 transformants for the presence of the *GFP* expression and silencing constructs. Figure 2B shows the results of this analysis for the series of transformants shown in Fig. 2A. As a first result, genome analysis revealed that all of the analyzed transformants still contained the *GFP* expression construct. In addition, the majority (9 of 15) of the nonsilenced transformants lacked the diagnostic 3.2-kb HindIII fragment indicative of the complete silencing cassette and contained either no additional (to the one of the *GFP* expression construct) *GFP*-containing fragment or additional *GFP*-containing fragments of different sizes. There were only 6 of 15 nonsilenced transformants that contained an apparently complete *GFP* silencing cassette. These results suggest that integration of the complete silencing cassette leads to silencing with a probability of at least 25 of 31 (80%). None of the mock transformants which received only the *pab1* marker gene showed any signs of silencing. On the other hand, the presence of a complete silencing cassette was a prerequisite for silencing in that only 2 of 25 silenced transformants contained, instead of the diagnostic 3.2-kb HindIII fragment, a *GFP*-containing fragment of a different size (Fig. 2B, lane 5, and data not shown). With regard to the orientation of the intron in the silencing construct, the percentage of nonsilenced transformants was almost the same (8 of 20 and 7

Inc., Burlington, Ontario, Canada) was used as a size standard (m). The bottom panel shows a Southern blot analysis of the same genomic DNAs with HindIII as restriction enzyme and a *GFP* hybridization probe (see Materials and Methods for details). DNA-Molecular-Weight-Marker VII (Roche) was used as a size standard (m). The positions of the expected *GFP* and *GFP*hp DNA fragments are indicated. Plasmids 373 (lane 17) and 367 (lane 18) were included as controls. (C) GFP fluorescence microscopy of silenced and nonsilenced KK7 transformants. A silenced and a nonsilenced transformant in comparison to the appropriate control strains or transformants (the numbering in the brackets refers to panels A and B) were examined by GFP fluorescence and phase-contrast microscopy (PC) as described in Materials and Methods. Bar, 20 μm.

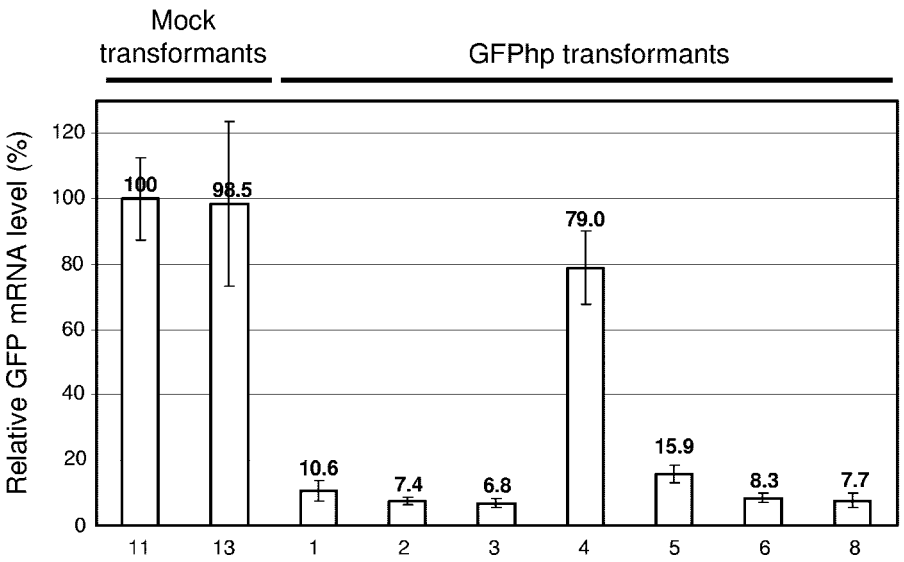

FIG. 3. Determination of *GFP* silencing efficiency using quantitative real-time PCR. Relative *GFP* mRNA levels were determined for the same representative series of transformants as in Fig. 2 (numbering refers to Fig. 2A and B). Values (above the histogram bars) are given as the percentage of a mock transformant. The analysis was performed as described in Materials and Methods. The error bars represent the standard deviations of three different measurements of the same cDNA.

of 20 for the normal and reverse orientations, respectively) in either case, but the number of cases with a complete silencing cassette among the nonsilenced transformants was slightly higher (4 of 7 versus 2 of 8 in case of the normal orientation) in the case of the reverse orientation of the intron (data not shown). The two original *GFP* transformants slightly differed in their susceptibility to silencing in that one of the transformants yielded more nonsilenced *GFP*hp transformants (11 of 20 versus 4 of 20), of which a similar portion contained a complete silencing cassette (4 of 11 versus 2 of 4) (data not shown).

As independent means of examining the GFP protein levels in the various transformants, mycelial pellets of selected transformants and control strains were grown in liquid culture and visualized by fluorescence microscopy. In accordance with the immunoblotting results, fluorescence of silenced transformants was reduced to the background fluorescence of strain KK7 devoid of any *GFP* expression construct (Fig. 2C and data not shown).

In principle, the observed silencing could occur at the levels of transcription or translation. In order to distinguish between these two possibilities and to determine the efficiency of silencing, the *GFP* mRNA levels in selected (at least six transformants of each series to be silenced and two of each mock transformant series) were determined by using quantitative real-time PCR. Figure 3 shows the results of the same series of transformants as shown in Fig. 2A and B together with the appropriate control strains. The results demonstrate that the *GFP* mRNA levels in the silenced transformants are reduced to 5 to 10% of those in the nonsilenced transformants, the mock transformants, and the original *GFP* transformant. These results show that the observed silencing is due to a decrease in the mRNA level. With regard to the orientation of the intron, no significant differences in the silencing efficiency were observed (data not shown). In addition, a single copy of the unchanged silencing construct was sufficient to confer this maximal degree of silencing since most of the silenced transformants revealed only one additional (to the one of the *GFP* expression construct) *GFP*-containing fragment in a BamHI or PmlI digest of genomic DNA (data not shown). In contrast to HindIII, these restriction endonucleases cleave the transformed plasmids only once: in the *pab1* gene or in the *GFP* silencing cassette, respectively (Fig. 1 and data not shown).

In summary, our results suggest that expression of a homologous hairpin RNA is an efficient means to downregulate a specific mRNA in *C. cinerea*.

Simultaneous silencing of two endogenous isogenes by a single homologous hairpin RNA. In order to test whether the RNA silencing approach would also be applicable for endogenous and developmentally regulated *C. cinerea* genes, we set out to silence the *cgl2* gene coding for one of two isogalectins, CGL1 and CGL2, which are highly induced during fruiting body formation (2, 5). Due to the high sequence similarity of the *cgl1* and *cgl2* genes at DNA level (87% identity of the coding regions), we expected to see simultaneous silencing of both genes if the *cgl2* silencing construct was functional. In the *GFP* silencing experiment, transcription of the hairpin RNA was under control of the same constitutive promoter as the target gene, and the presence of a spliceable intron in the loop region of the hairpin RNA did not significantly influence the silencing efficiency. Therefore, we designed a *cgl2* silencing construct in which we introduced a loop region consisting of a tandem repeat of a 9-bp spacer region used by Ambion in commercial silencing vectors (pSilencer) upstream of an inverted copy of the complete *cgl2* coding region between the last *cgl2* codon and the translational stop. The resulting *cgl2* hairpin construct was under the control of the endogenous *cgl2* promoter region that comprised the complete intergenic region between *cgl1* and *cgl2* (Fig. 1), which was shown to be sufficient for developmental regulation (2). As in case of the

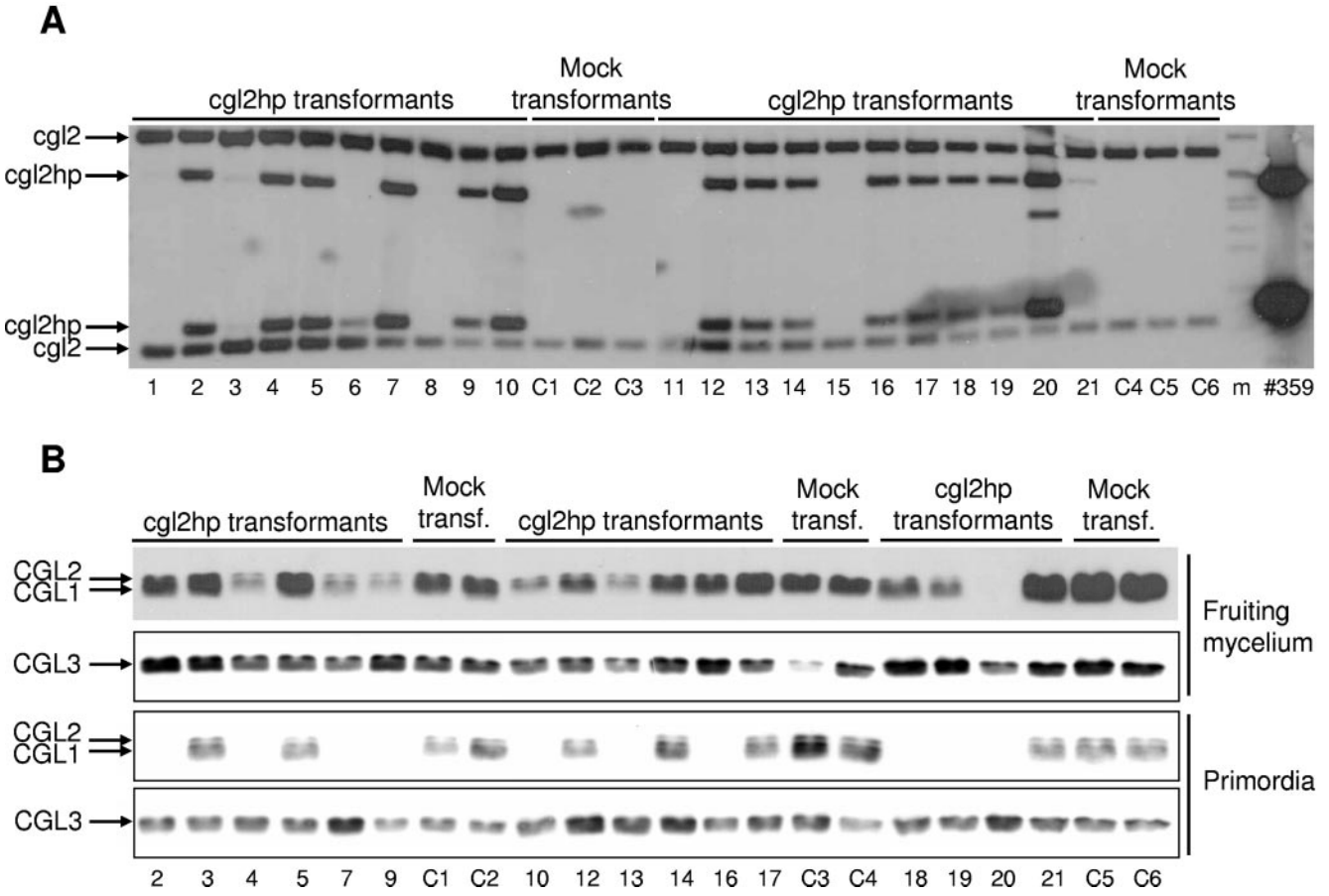

FIG. 4. Molecular analysis of *cgl2* silencing in *C. cinerea* strain AmutBmut. Individual transformants with *cgl2*-silencing construct 359 (lanes 1 to 21) were analyzed and compared to mock transformants carrying the vector control 336 (lanes C1 to C6). (A) Analysis of DNA integration by Southern blotting. Genomic DNA of the individual transformants was analyzed by using the restriction enzyme HindIII and a *cgl2* hybridization probe (see Materials and Methods for details). The positions of the endogenous *cgl2* and the introduced *cgl2*hp fragments are indicated. A HindIII digest of plasmid 359 served as a positive control. DNA-Molecular-Weight-Marker VII (Roche) was used as size standard (m). (B) Analysis of CGL1/2 and CGL3 protein levels in WCEs prepared from fruiting mycelium and primordia of the individual transformants (see Materials and Methods for details). A total of 5 μg of WCE from the indicated sample of the indicated transformants (numbering refers to panel A) was analyzed by immunoblotting with specific antisera against CGL1/2 and related protein CGL3 as described in Materials and Methods. In case of the anti-CGL1/2 blots, the HRP-coupled secondary antibody was detected either by using ECL (fruiting mycelium) or by using less sensitive chloronaphthol development (primordia). The positions of the individual proteins are indicated.

GFP constructs, we placed the *C. cinerea* marker gene *pab1* immediately upstream of the *cgl2* silencing construct. Since we wanted to test the effect of eventual *cgl1* and *cgl2* silencing on fruiting, we transformed the *cgl2* silencing construct and an appropriate vector control containing only the *pab1* gene (plasmid 336) into the homokaryotic fruiting strain AmutBmut.

Southern analysis of oidial DNA revealed that 15 of the 21 analyzed *cgl2*hp-transformants carried the silencing cassette (Fig. 4A). Fourteen of these positive transformants, two negative transformants lacking the silencing cassette (transformants 3 and 21), and all six control transformants were cultivated under fruiting conditions and analyzed at the protein level by immunoblotting of WCEs prepared from fruiting mycelium and immature fruiting bodies (primordia) using a specific antiserum directed against CGL1 and CGL2 (see Materials and Methods for details). As a control an antiserum to a novel lectin, CGL3, which is also induced during fruiting body formation but whose coding region reveals much lower sequence similarity to *cgl2* (53% identity; M. Wälti and A. Grünler, unpublished results), was used. Figure 4B shows that galectin genes *cgl1* and *cgl2* were slightly silenced in some (transformants 4, 7, 9, 10, 13, and 19) and efficiently in one (transformant 20) of the transformants at the stage of fruiting mycelium. At the stage of the primordia, however, 10 of the 14 analyzed transformants harboring a *cgl2* silencing cassette revealed efficient silencing of both *cgl1* and *cgl2*. Southern analysis of primordial DNA showed that the failure of silencing in transformants 5, 12, 14, and 17 was not due to a loss of the hairpin construct (data not shown). No significant change in CGL1/CGL2 protein levels was observed in any of the transformants lacking a silencing cassette (transformants 3, 21, and C1 to -6). CGL3 was not affected by expression of the *cgl2* hairpin RNA since its levels were the same in all transformants. In conclusion, we demonstrated efficient, specific, and simultaneous silencing of two endogenous and developmentally regulated isogenes by coexpression of a hairpin RNA harboring sequences from only one of the two genes.

In addition to the expression studies described above, we

examined the set of transformants phenotypically during the complete developmental pathway of fruiting body formation. None of the silenced transformants revealed any significant temporal or morphological difference compared to the control transformants (data not shown). The number and viability of basidiospores produced by the analyzed silenced fruiting bodies appeared normal (data not shown), suggesting that the galectins CGL1 and CGL2 are not necessary, at least for late stages of fruiting body development in *C. cinerea*. It is still possible that the galectins are involved in early steps of fruiting body formation as only one of the analyzed transformants (transformant 20) appeared to efficiently silence the *cgl1* and *cgl2* genes at this stage.

Hairpin RNA-induced cytosine methylation of transcribed and nontranscribed DNA at target and silencing loci. The observed decrease in mRNA level upon expression of homologous hairpin RNAs (Fig. 3) could be due to interference at the transcriptional and/or posttranscriptional level. RNA silencing at the transcriptional level is often accompanied by cytosine and/or histone methylation of the involved chromatin (reviewed in reference 40). In order to determine whether the expression of hairpin RNAs in *C. cinerea* leads to any change in the cytosine methylation of the homologous DNA, we analyzed both target and silencing loci by Southern analysis using pairs of isoschizomers with different sensitivities to this DNA modification (see Fig. 1 for the location of the restriction sites and the hybridization probes used). The pairs of isoschizomers used, HpaII-MspI and Sau3AI-MboI, allow probing of different types of DNA methylation. Cleavage of the recognition sequence CCGG by HpaII is blocked by methylation of the second cytosine (CpG methylation), whereas MspI-mediated cleavage of the same sequence is insensitive to such a modification. On the other hand, both enzymes are sensitive to a methylation of the first cytosine (CpNpG methylation). Cleavage of the recognition sequence GATC by Sau3AI is blocked by cytosine methylation at overlapping CpG and CpNpG sites, whereas cleavage of the same sequence by MboI is not affected by this modification. On the other hand, MboI but not Sau3AI is blocked by adenine methylation by prokaryotic dam-methylase. In the analysis shown in Fig. 5A, we included three silenced *GFP*hp transformants (Fig. 2, transformants 1, 7, and 8), two mock transformants (Fig. 2, transformants 11 and 13), and the original *GFP* transformant and strain KK7 (Fig. 2, strains 15 and 16, respectively). In the first experiment, we applied both pairs of isoschizomers and a *GFP* hybridization probe (Fig. 5A, upper two panels). The appearance of high-molecular-weight bands in the HpaII-digested but not in the MspI-digested DNA suggests the presence of abundant CpG methylation in DNA regions containing *GFP* sequences. This methylation was only observed in the silenced *GFP*hp transformants and not in the control strains, suggesting that it is induced by expression of the homologous hairpin RNAs. Only a few minor high-molecular-weight bands in the MspI-digested DNA of silenced transformants compared to control strains were detected, suggesting the occurrence but low abundance of CpNpG methylation. Sau3AI digests did not reveal any difference compared to analogous MboI digests, suggesting the absence of adenine methylation and cytosine methylation at the few CpG or CpNpG sites overlapping with the GATC sequences in the *GFP* target and silencing loci. In

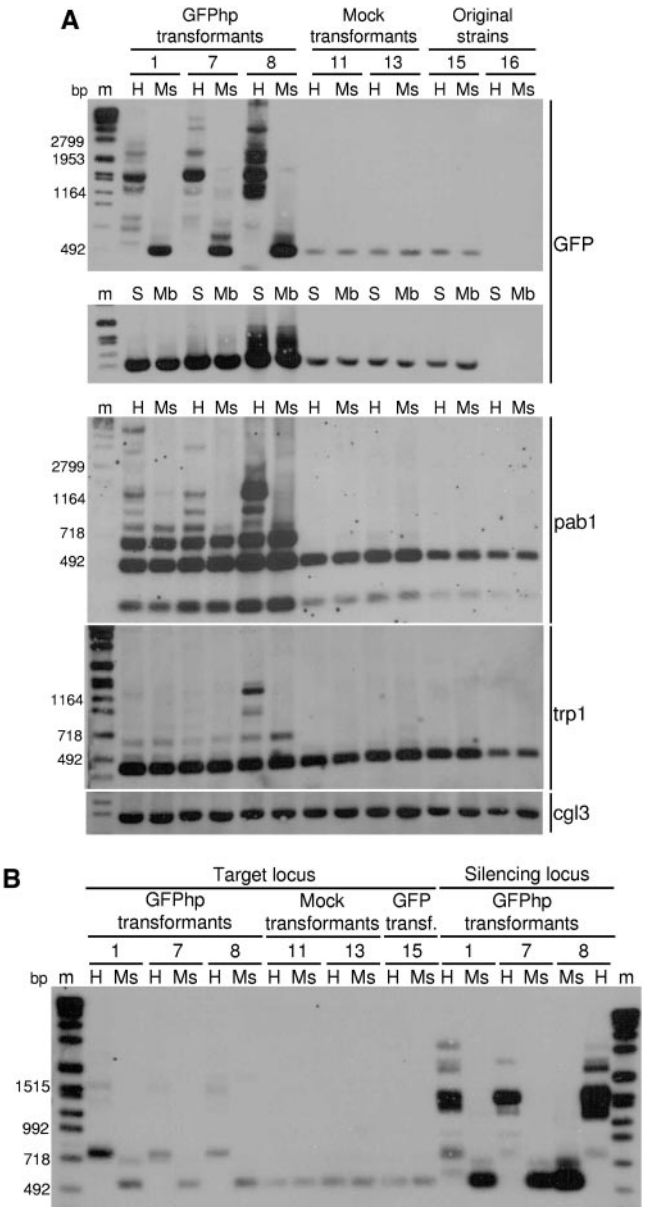

FIG. 5. DNA methylation analysis in *GFP* and *cgl2* silencing. Individual transformants of *C. cinerea* strains KK7 silenced for *GFP* (A and B) and AmutBmut silenced for *cgl2* (C) were analyzed for methylation of their genomic DNA in comparison to corresponding mock transformants and original strains. DNA-Molecular-Weight-Marker VII (Roche) was used as a size standard (m). (A) DNA methylation analysis in *GFP* silencing. Oidial DNA of the indicated KK7 transformants and original strains (numbering refers to Fig. 2) was analyzed by Southern blotting with the indicated pairs of isoschizomeric restriction endonucleases, HpaII/MspI (H/Ms) or Sau3AI/MboI (S/Mb), and hybridization probes for the indicated genes (see Materials and Methods for details). (B) Cytosine methylation of *GFP* target and silencing locus. *GFP* target and silencing locus of indicated KK7 transformants (numbering refers to Fig. 2) were isolated as HindIII fragments as described in Materials and Methods and analyzed for cytosine methylation by Southern blotting using restriction endonucleases HpaII (H) and MspI (Ms) and a *GFP* hybridization probe. (C) Cytosine methylation in *cgl2* silencing during fruiting body formation. Genomic DNA from different tissues (primordia, premeiotic oidia of mycelium before fruiting, and postmeiotic oidia of mycelium derived from two independent basidiospores [-1, -2] of respective fruiting bodies) of individual transformants (numbering refers to Fig. 4) was analyzed for cytosine methylation by Southern blotting using the restriction endonucleases HpaII (H) and MspI (Ms) and a *cgl2* hybridization probe.

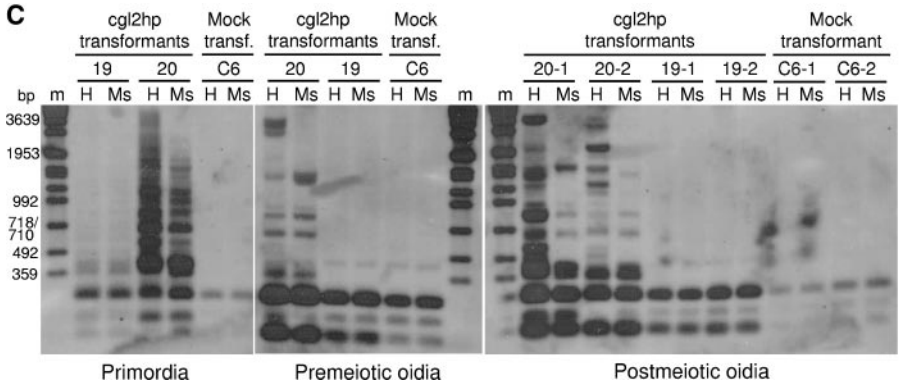

FIG. 5—*Continued.*

order to test the locus specificity of the *GFP*hp-induced CpG methylation, we hybridized the same Southern blots with probes for the two marker genes, *pab1* and *trp1*, and the *cgl3* gene (Fig. 5A, lower three panels). No methylation was observed in the *cgl3* locus in the silenced transformants, demonstrating the sequence specificity of the epigenetic changes. On the other hand, the appearance of HpaII-specific high-molecular-weight bands in the case of both marker genes demonstrates that CpG methylation occurred both at the target and silencing locus and was not restricted to the DNA regions containing *GFP* sequences but rather spread into the adjacent DNA regions, albeit to a lesser extent. Interestingly, CpNpG methylation was slightly more pronounced in these cases than in the case of the *GFP*-containing sequences. CpG methylation of both target and silencing loci was confirmed by isolating the HindIII fragments containing the respective loci and subjecting the isolated DNA fragments to the methylation analysis described above using the isoschizomer pair HpaII-MspI and the *GFP* hybridization probe (Fig. 5B; see Materials and Methods for details). The results suggest that methylation of the silencing locus was more pronounced than that of the target locus.

Cytosine methylation in the course of *cgl2* silencing was examined analogously by analyzing genomic DNA of 2 silenced (transformants 19 and 20) and one mock transformant (C6) isolated from premeiotic oidia, primordia and postmeiotic oidia (Fig. 5C). The latter were collected from mycelia that had been derived from two independent basidiospores (indicated by the extension behind the numbers of the respective transformants). Methylation occurred in no developmental stage of the silenced transformant 19 but in all three analyzed developmental stages of transformant 20. The latter transformant carries multiple copies of the complete and also truncated versions of the *cgl2* silencing cassette and revealed *cgl2* silencing already at the mycelial stage (see Fig. 4A). Based on the intensity of the high-molecular-weight bands, methylation appeared to be least pronounced in premeiotic oidia and most pronounced in primordia. The difference in the pattern of high-molecular-weight bands between HpaII- and MspI-digested DNA demonstrates the occurrence of CpG methylation. However, the abundance of high-molecular-weight bands in the MspI lanes of transformant 20 versus transformant 19 suggests that CpNpG methylation was more pronounced in this case than in the *GFP* silencing experiment (see Fig. 5A).

In summary, these results suggest that silencing by constitutive high-level expression of hairpin RNAs in *C. cinerea* is accompanied by cytosine methylation of homologous and adjacent DNA regions, whereas the transient expression of hairpin RNAs does not lead to such epigenetic changes.

DISCUSSION

The number of reports about successful applications of RNA-mediated gene silencing (RNA silencing) as a reverse genetics tool in fungi is rising (see the introduction for references). In most studies RNA silencing was achieved by the transformation of heritable constructs expressing additional copies of the target gene (cosuppression), antisense RNA, or hairpin RNA, where the sequence homologous to the target gene forms the double-stranded stem structure. Systematic comparisons between the three different approaches in fungi and plants suggest that hairpin RNA is the most efficient trigger of RNA silencing in these organisms (19, 29, 57). This approach was also shown to be successful in *Drosophila melanogaster* (31), *Caenorhabditis elegans* (61), *Trypanosoma brucei* (55), and mammalian cells albeit in the latter case the stem regions of the hairpin loop have to be kept short in order not to elicit an interferon response (48). In plants and flies, the silencing efficiency could be further enhanced by introducing an intron in the hairpin loop (31, 57).

Here we demonstrate efficient silencing of both exogenous and endogenous genes by expression of homologous hairpin RNAs in the homobasidiomycete *C. cinerea*. The regions of homology forming the stem of the hairpin comprised in both cases the whole coding region of the target genes (450 and 711 bp, respectively) but may be shortened in future applications. In contrast to the studies in plants and flies, we did not observe any significant influence of the size of the loop or the presence of an intron in the loop. However, this conclusion is hampered by the fact that experimental attempts to demonstrate the splicing of the loop intron failed (data not shown) and that all silencing constructs used in the present study contained additional introns as a prerequisite for their expression. The lack of silencing in some of the transformants despite the presence of a complete silencing cassette may be due to a lack of expres-

sion caused by the surrounding DNA of the ectopic insertion point. Whereas in the *GFP* silencing experiment both target and silencing construct were constitutively expressed, *cgl2* silencing was achieved by developmentally regulated (transient) coexpression of silencing construct and target gene(s).

The simultaneous silencing of *cgl1* and *cgl2* using the *cgl2* hairpin construct is, apart from a report on *Cladosporium fulvum* (54), the only demonstration of this feature of RNA-mediated gene silencing in a fungus. The effect is possibly restricted to highly homologous genes (the coding regions of *cgl1* and *cgl2* are 87% identical) since the less homologous *cgl3* gene (53% overall identity to *cgl2* in its coding region) is not affected. However, it should be noted that such target specificity always depends on the actual sequence alignment since short regions of high identity can also lead to unwanted off-target effects.

With regard to the possible mechanisms of RNA-induced gene silencing in *C. cinerea*, translational inhibition can be excluded based on the observed reduction in mRNA levels. The observed cytosine methylation induced by high-level expression of the hairpin RNA but not by transient expression suggests that RNA silencing in *C. cinerea* occurs via posttranscriptional degradation of mRNA but can be reinforced at the transcriptional level by epigenetic changes. Interestingly, this RNA-induced DNA methylation in *C. cinerea* is not found in the two model fungi for RNA silencing: *Schizosaccharomyces pombe* and *Neurospora crassa.* In *S. pombe*, RNA-induced histone methylation leads to transcriptional silencing without detectable DNA methylation (62). In *N. crassa*, DNA methylation depends on histone methylation, but these processes are apparently independent of RNAi (9, 17, 60). On the other hand, the occurrence of de novo cytosine methylation in *C. cinerea* with preference for CpG sites and inheritance through meiosis is in agreement with previous reports (16, 65). In addition, transgene-induced cytosine methylation was reported for another homobasidiomycete, *S. commune* (53). The spreading of the RNA-induced DNA methylation in *C. cinerea* from homologous to adjacent nonhomologous sequences over a distance of several hundred base pairs suggests the involvement of RNAi-directed heterochromatin (including histone modification) may be in conjunction with RNA-directed DNA methylation (reviewed in reference 40). In this respect, the situation in *C. cinerea* and possibly in homobasidiomycetes in general may be more related to the one in animals and plants, where both pathways for RNA-induced epigenetic changes exist (reviewed in reference 40).

Gene targeting by RNA silencing is attractive even for fungi where classical gene knockout procedures are feasible. First of all, the hypermorphic mechanism of RNA silencing implies that the technique is also applicable to polyploid and polykaryotic organisms. The approach also offers solutions to the frequent lack of multiple marker genes in fungi. Simultaneous silencing of several unrelated genes by introducing a single chimeric hairpin construct has been demonstrated (15, 37, 43). In addition, as also shown in the present study, a single hairpin construct is able to simultaneously silence genes with high homology to the target gene. The latter application might be useful in *C. cinerea* since this fungus contains large families of highly homologous genes (25). The approach might also be useful for studying essential genes since the incomplete shutdown of expression may still support growth but be sufficient to cause a phenotype that can be indicative for the function of the gene; if the residual growth is too low under these conditions, the hairpin construct can be put under the control of an inducible promoter which allows cultivation of the organism under permissive conditions before studying its behavior under restrictive conditions. Finally, the use of expression cassettes minimizes cloning efforts since several hundred base pairs of the transcribed region appear to be sufficient for efficient silencing. Of course, there are also disadvantages of the RNA silencing approach. The most severe problem, in our opinion, is the fact that, due to its hypermorphic nature, the silencing phenotype cannot be complemented. This makes it impossible to perform more subtle studies of gene function, e.g., by introducing point mutations. On the other hand, the limited specificity of silencing might complicate the interpretation of phenotypes. A possible remedy of this problem, other than the careful selection of the homology region of the hairpin RNA, may be the overexpression of the intended target as a means of complementing the defect. Finally, a 90% reduction of gene expression may not be sufficient to cause a phenotype in all cases.

In accordance with the observed silencing, the *C. cinerea* genome sequence harbors at least two potential orthologs of each dicer, argonaute, and RdRP. On the other hand, in the *Ustilago maydis* genome sequence no obvious orthologs of these proteins are found and, indeed, Keon et al. (33) recently reported no evidence for antisense suppression in this basidiomycete despite a considerable expression level of the antisense RNA. Thus, predictions on the applicability of RNA silencing in a specific fungus based on its genome sequence are tempting but probably not very reliable. For example, *Cryptococcus neoformans*, another basidiomycete lacking both an obvious dicer and an RdRP ortholog, was reported to mediate gene silencing by the expression of hairpin and antisense RNAs (20, 37). Even more surprising, efficient RNA silencing by a hairpin construct was recently reported for the yeast *S. cerevisiae*, which is widely believed to be silencing deficient due to the absence of any orthologs of proteins described above (8). In conclusion, the presence of orthologs in the genome of an organism is a strong hint for the presence of RNA silencing, but their absence is not necessarily linked to a lack of this process. Therefore, experimental evidence for such a mechanism is required before considering its application as a reverse genetics tool.

Finally, it is remarkable that the lack of two proteins that accumulate to such a high level in the fruiting body is without obvious effect on the build up of this organ. Of course, it is still possible that there are subtle differences escaping our analysis thus far. Also, we cannot completely exclude that the galectins play a role during the initiation of fruiting body formation since, with the exception of a single transformant, expression of the *cgl2* hairpin RNA under the control of the *cgl2* promoter allowed residual expression of *cgl1* and *cgl2* at the stage of the fruiting mycelium. We will construct a constitutively expressed *cgl2* silencing cassette to test this possibility. However, the chances for a phenotype are small since fruiting of this single transformant, in which *cgl2*hp is probably already constitutively expressed, also appeared normal.

During the revision of this manuscript, Namekawa et al. (45) described the silencing of a *C. cinerea* gene involved in meiosis

using an analogous approach. The silencing construct used consisted of cDNA sequences without any introns, arguing against a crucial role of introns in such constructs. In addition to this study, these authors show that the silencing acts in *trans* in a heterokaryotic situation.

ACKNOWLEDGMENTS

We thank W. Nickel (Heidelberg University Biochemistry Center, Heidelberg, Germany) for providing the anti-GFP antiserum and M. Bednar for excellent technical assistance.

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