An Essential Gene for Fruiting Body Initiation in the Basidiomycete *Coprinopsis cinerea* Is Homologous to Bacterial Cyclopropane Fatty Acid Synthase Genes

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ABSTRACT

The self-compatible *Coprinopsis cinerea* homokaryon AmutBmut produces fruiting bodies without prior mating to another strain. Early stages of fruiting body development include the dark-dependent formation of primary hyphal knots and their light-induced transition to the more compact secondary hyphal knots. The AmutBmut UV mutant 6-031 forms primary hyphal knots, but development arrests at the transition state by a recessive defect in the *cfs1* gene, isolated from a cosmid library by mutant complementation. A normal primordia phenotype was achieved when *cfs1*⁺ was embedded at both sides in at least 4.0 kb of native flanking DNA. Truncations of the flanking DNA lead to reduction in transformation frequencies and faults in primordia tissue formation, suggesting that the gene is also acting at later stages of development. The *cfs1* gene encodes a protein highly similar to cyclopropane fatty acid synthases, a class of enzymes shown in prokaryotes and recently in a plant to convert membrane-bound unsaturated fatty acids into cyclopropane fatty acids. In *C. cinerea* 6-031, the mutant *cfs1* allele carries a T-to-G transversion, leading to an amino acid substitution (Y441D) in a domain suggested to be involved in the catalytic function of the protein and/or membrane interaction.

THE heterothallic fungus *Coprinopsis cinerea* serves as a model organism to study fruiting body development in higher basidiomycetes. Fruiting bodies normally develop on the dikaryon (Kües 2000). However, the presence of two genetically distinct nuclei in the dikaryotic mycelium is a major drawback to performing genetic analysis on fruiting body development. The self-compatible homokaryon AmutBmut, having specific mutations in both mating-type loci (*A43mut* and *B43mut*), shows characteristics typical of the dikaryon, for example, formation of fused clamp cells at hyphal septa. It also gives rise to fruiting bodies without prior mating to another strain (Swamy *et al.* 1984; Walser *et al.* 2003). This special feature together with the ability to form unicellular haploid spores (oidia) provides us with an easily accessible genetic system (Kües *et al.* 2004). A series of developmental mutants have been generated from strain AmutBmut by UV- and REMI-mutagenesis (Granado *et al.* 1997; Lu *et al.* 2003;

U. Kües, J. D. Granado and M. Aebi, unpublished results), which serves to isolate genes.

The development of fruiting bodies is a highly organized process, which requires the coordination between genetic, environmental, and physiological factors (Kües 2000). In the dark, upon nutritional depletion, single hyphae locally undergo intense branching to form microscopic primary hyphal knots. When kept in the dark, these develop into multicellular pigmented resting bodies called sclerotia (Kües *et al.* 2002a). Following a light signal, radial growth of primary hyphal knots and hyphal interaction lead to the formation of compact hyphal aggregates, secondary hyphal knots, which are specific fruiting body initials with undifferentiated hyphae. Once tissue differentiation initiates within the secondary hyphal knot, the structures are termed primordia. Cellular differentiation results in the formation of distinct cap and stipe tissues. Formation of the various tissues within the developing primordium needs altering dark and night phases and continues over 3 days, over which the size of the primordium enlarges (Boulianne *et al.* 2000; Kües 2000; Walser *et al.* 2003). In the mature primordium cap, induced by a further light signal, karyogamy occurs in specialized cells (basidia) present in the hymenium of the gills. Karyogamy is directly followed by meiosis. In parallel, the stipe elongates and the cap expands, giving rise to a fully developed fruiting body (Moore *et al.* 1979; Kües 2000).

So far, little is known about the genetic determinants that act in fruiting body initiation and formation. Induction of primary hyphal knots and the morphological transition from primary into secondary hyphal knots were shown to be regulated by the *A* and *B* mating-type genes (KÜES *et al.* 1998, 2002b). The gene *pcc1*, encoding an HMG-box transcription factor, likely acts downstream of the *A* mating-type gene products and appears to negatively regulate fruiting body initiation (MURATA *et al.* 1998). Onset of expression of two genes encoding fruiting body specific galectins (β-galactoside sugar-binding lectins) correlates with the formation of primary and secondary hyphal knots, respectively, and continues during primordia development (BOULIANNE *et al.* 2000; BERTOSSA *et al.* 2004). In addition, four genes have been identified that act in cap and stipe tissue formation and stipe elongation, respectively, including one for a potential photoreceptor (MURAGUCHI and KAMADA 1998, 2000; ARIMA *et al.* 2004; TERASHIMA *et al.* 2005). Next to the gene described in this study, a yet uncharacterized gene acting in fruiting body initiation has also been cloned (CLERGEOT *et al.* 2003).

Within our mutant collection derived from homokaryon AmutBmut, we identified two groups of mutants whose defects link to fruiting body initiation. Members of one group are termed *pkn* (primary knotless) mutants, because they do not form any primary hyphal knot in the dark. The other group of mutants is arrested at the transition from primary to secondary hyphal knots. Therefore, they are called *skn* (secondary knotless) mutants (U. KÜES, Y. LIU and M. AEBI, unpublished). In this study, we isolated a gene that complemented the defect of fruiting body initiation in the *skn1* UV-mutant 6-031. The predicted gene product is highly homologous to cyclopropane fatty acid synthases, a class of enzymes characterized before in bacteria and recently also in a plant.

MATERIALS AND METHODS

Fungal strains, culture conditions, and transformation: *C. cinerea* strains were standardly grown at 37° on YMG/T complete medium and minimal medium (GRANADO *et al.* 1997) supplemented with p-aminobenzoate (PABA, 5 mg/ liter) when required. Strain 6-031 (*A43mut, B43mut, pab1-1, skn1*) is a fruiting body initiation mutant generated from homokaryon AmutBmut (*A43mut, B43mut, pab1-1*) by UV-mutagenesis (U. KÜES, J. D. GRANADO and M. AEBI, unpublished results). Monokaryons PG78 (*A6, B42, pab1*) and JV6 (*A42, B42*), both unrelated to homokaryon AmutBmut, and the AmutBmut co-isogenic monokaryons PS001-1 (*A42, B42*) and PS002-1 (*A3, B1*) were used in crosses (KERTESZ-CHALOUPKOVÁ *et al.* 1998; P. SRIVILAI and U. KÜES, unpublished results). Matings were performed on YMG/T plates by placing two mycelial blocks of inoculum 5 mm apart. For growth and induction of fruiting bodies, mating plates were incubated at 12-hr light/12-hr dark, 25°, 90% humidity under standard fruiting conditions (GRANADO *et al.* 1997). Randomly isolated basidiospores were germinated on YMG/T medium at

37°. Progeny of crosses were analyzed on minimal media for PABA auxotrophy. Presence of unfused and fused clamp cells, indicators of activated *A* and *B* mating-type pathways, respectively (KÜES 2000), was determined by microscopy.

Monokaryon JV6 served to confirm mating types in *A43mut, B43mut* progenies from crosses with PS001-1 and PS002-1 that subsequently were submitted to fruiting tests. Frequencies of phenotypic distributions in *A43mut, B43mut* progenies were tested by a chi-square method. A *skn1+, mat+, bad* clone (PS-Mu1-3) and a *skn1+, mat, bad* clone (PS-Mu1-2) within the *A42, B42* progeny of cross 6-031 × PS001-1 (defined by crosses with monokaryon PG78) were identified through mating with mutant 6-031. Mating of these two strains with *A43mut, B43mut* clones of the progeny 6-031 × PS001-1 that did not initiate fruiting identified homokaryon OU3-1 (*A43mut, B43mut, pab1-1, skn1*).

The F_1 progeny of cross 6-031 × JV6 was randomly analyzed for fruiting ability by individually inoculating clones on YMG/T agar, growing them for 4 days at 37° in the dark, and subsequently transferring them to standard fruiting conditions. Dikaryons among the clones were identified by light inducing oidia production, germinating the spores on YMG/T agar, and analyzing *pab*-auxotrophy on minimal medium. For oidia induction of dikaryons, mutant 6-031 and other *A43mut, B43mut* strains, dark-grown cultures were exposed to light for 2 days (KERTESZ-CHALOUPKOVÁ *et al.* 1998) and the number of oidia per plate was determined by a spectrophotometer.

DNA transformation was performed as described (GRANADO *et al.* 1997). For selecting PABA prototrophs in cotransformations, 1 μg of plasmid pPAB1-2 (GRANADO *et al.* 1997) was added. Upon germination on regeneration agar, transformants were individually transferred onto minimal medium for further growth. Subsequently, three or four individual transformants were inoculated on YMG/T agar per single petri dish and grown in the dark at 37° for 2 days to a colony size of 3– 3.5 cm in diameter. To induce fruiting, plates were moved for 2 weeks to standard fruiting conditions. The number and size of primordia per transformant were scored. A small piece of gill tissue from primordia developed upon transformation with cosmid 40-5A was spread and nuclei in basidia stained with hematoxylin (LU and RAJU 1970).

DNA and RNA techniques: An indexed genomic cosmid library derived from homokaryon AmutBmut was transformed into mutant 6-031 and screened for cosmids that were able to restore fruiting ability in this strain, following a SIB-selection procedure. The *pab1+* wild-type gene of *C. cinerea* present in the cosmid backbone was used as a selection marker. Cosmid DNAs from 60 pools of each 96-well microtiter dish-arranged *E. coli* clones, and from subpools and individual clones of microtiter dish 40 were isolated (BOTTOLI *et al.* 1999).

Cloning was performed by standard methods (SAMBROOK *et al.* 1989). Plasmids were propagated in *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA). Derivative pSphA of cosmid 40-5A is a ligation product between a 16-kb *Sph*I fragment (13-kb genomic DNA + 3-kb cosmid backbone) and a 7.5-kb *Sph*I fragment (2-kb genomic DNA + 5.5-kb cosmid backbone) in their natural order. *Not*I fragments of cosmid 40-5A were cloned into the *Not*I site of pBC SK (+) (Stratagene). Plasmids pNotB5 and pNotB7 contained the same DNA insert but in opposite orientation. The insert in pNotB5 was sequenced on both strands by primer walking (Microsynth, Balgach, Switzerland). Sequences were assembled with the program DNASTAR and analyzed with OMIGA 2.0, BLAST (NCBI). The whole sequence but 32 bp originating from the linker of the cosmid was submitted to GenBank (AF338438).

pNotB5 and pNotB7 gave rise to the following pBC SK (+) subclones: p5SmaCS and p5BamCS (used in Northern analysis) carry gene *arf1* on a 1.4-kb *Not*I–*Sma*I and a 3.5-kb *Not*I–*Bam*HI

fragment, respectively. p5EcoCS and p5XbaCS contain *arf1* and a truncated *cfs1*⁺ gene on a 3.8-kb *Not*I–*Eco*RI and a 5.5-kb *Not*I–*Xba*I fragment, respectively. p5SpeCS includes both *arf1* and *cfs1*⁺ on a 7-kb *Spe*I fragment, whereas *cfs1*⁺ is truncated in p5KpnCS on the shorter 4.4-kb *Not*I–*Kpn*I fragment. p7XbaCS carries truncated *cfs1*⁺ and *kin1* copies on a 5-kb *Xba*I–*Not*I fragment. p7SpeCS contains a truncated *kin1* on a 3.5-kb *Spe*I–*Not*I fragment. Subclones constructed in pBluescript KS (−) (Stratagene) were as follows: pPvu8.5 contains an 8.5-kb *Pvu*II fragment covering the complete *cfs1*⁺ gene and the 3′ end of *kin1*. pBam3.5 and pSmaSpe5.5 carry *cfs1*⁺ on a 3.5-kb *Bam*HI and a 5.5-kb *Sma*I–*Spe*I insert, respectively. pEco4.4 contains truncated *cfs1*⁺ and *kin1* copies on a 4.4-kb *Eco*RI fragment. Furthermore, the 8.5-kb *Pvu*II fragment, 3.5-kb *Bam*HI fragment, and 4.4-kb *Eco*RI fragment were also cloned into pPAB1-2 containing the *C. cinerea pab1*⁺ gene, resulting in pPvu8.5-pab, pBam3.5-pab, and pEco4.4-pab, respectively.

A cut-and-shut strategy using *Nco*I and plasmid pNotB7 resulted in p7NcoCSΔ*cfs* with a deletion in *cfs1*⁺ (Δbp 5296–5476). Similarly, p5BstCSΔ*kin* with a deletion in *kin1* (Δbp 8502–8619) were created from plasmid p5NotB5 by using *Bst*EII. An *Aat*II deletion (Δbp 6567–6803) in p5SpeCS yielded p5SpeCSΔ*gtl*. An *Nru*I deletion in *arf1* (Δbp 676–972) in p5SmaCS gave rise to p5SmaCSΔ*arf*, from which the *Sma*I insert was cloned into pSmaSpe5.5 to generate p5SpeCSΔ*arf*. The insertion of a 3.5-kb *Spe*I fragment from pNotB7 at the *Spe*I site in p5SpeCSΔ*arf* resulted in pNotB5Δ*arf*. The T-to-G transversion found in the *cfs1* allele of mutant 6-031 was introduced into plasmid p5SpeCS by exchanging a 1-kb PCR-amplified *Stu*I–*Nde*I fragment with the wild-type sequence, yielding p5SpeCS/6-031. pNotB5/6-031 distinguishes from pNotB5 by the same T-to-G transversion.

Genomic DNA of *C. cinerea* strains was isolated from powdered lyophilized mycelium (ZOLAN and PUKKILA 1986). Two overlapping fragments containing the *cfs1* allele of mutant 6-031 were independently amplified six times from genomic DNA with specific primers (a 3.1-kb fragment using primers 5′ TCAAGTCGGGTCGGTAGAAG 3′ and 5′ TTTGT TTCGGAGCTTGACTG 3′ and a 1.1-kb fragment using primers 5′ GGACGCTTCAAGATTAGATC 3′ and 5′ CTCTGAAG GAATCGCTCTTG 3′) and sequenced using a ABI PRISM DNA Sequencing Kit and a Model 373A DNA sequencer (Perkin-Elmer). Sequences of PCR products separately amplified with the same primer set were identical. Presence of the same sequence in p5SpeCS/6-031 has also been verified by sequencing.

Southern blot analysis was performed with 10 μg of genomic DNA per sample following basic protocols (SAMBROOK *et al.* 1989). Total RNA of strain AmutBmut was extracted with a guanidinium isothiocyanate procedure (CHOMCZYNSKI and SACCHI 1987) from powdered lyophilized *C. cinerea* mycelia or tissues of different fruiting stages. Poly(A)⁺ RNA was isolated with the Oligotex mRNA Midi kit (QIAGEN). Per sample, 10 μg of total RNA or 2.5 μg of poly(A)⁺ RNA were used for Northern blot analysis (SAMBROOK *et al.* 1989). Hybridization signals in Southern and Northern blot analyses were produced with DNA fragments labeled with [α-³²P]dCTP by random primed DNA labeling (Boehringer Mannheim).

The 5′ and 3′ cDNA ends of the *cfs1*⁺ gene were determined with the 5′/3′ RACE kit (Roche Molecular Biochemicals) following the instructions of the manufacturer. Poly(A)⁺ RNA from 5-mm-sized primordia of homokaryon AmutBmut was used for cDNA synthesis. In the 5′ RACE, a *cfs1* specific primer sp1 (5′ ACAATGCACAGGAGTACATC 3′) was employed to synthesize the first strand cDNA. Two *cfs1*-specific primers, sp2 (5′ GCAATGGCATTGAGTCGAG 3′) and sp3 (5′ TAGACGA TAGGGTCATCTCC 3′), were applied in subsequent PCR reactions. In the 3′ RACE, two *cfs1*-specific primers, sp4 (5′

GATTTTGCCCTCAAGCCAC 3′) and sp5 (5′ CAATTCGAGC CTGCCCAG 3′), were used. RACE products were cloned into pBluescript KS (−) by T/A cloning (MARCHUK *et al.* 1991) and sequenced with a Model 373A DNA sequencer. The full coding length of the *cfs1*⁺ cDNA was obtained by PCR, using the two primers cfsATG (5′ ATGCCGGCCCACCACCACCCTTC 3′) and cfsREV (5′ CGCCGAGGCCGCCGTGTAAACAC 3′). For sequencing, the PCR product was cloned into the *Eco*RV site in the β-galactosidase gene of pBluescript KS (−) via T/A cloning, resulting in construct pYL28 having the *cfs1*⁺ cDNA inserted in frame to the β-galactosidase gene.

Computer analysis of protein sequences: Proteomics tools provided by ExPaSy Molecular Biology Server (Swiss Institute of Bioinformatics, Geneva) were used to perform protein pattern and profile searches (InterPro), transmembrane region detection (TMpred and TMHMM), and secondary structure predication (PSA and PSIpred). Hydrophilicity profile was calculated with Goldman/Engelman/Steitz parameters in OMIGA 2.0.

RESULTS

Morphological and genetic analysis of UV-mutant 6-031: UV-mutant 6-031 has a growth rate (8 mm/day on YMG/T agar at 37°) and a mycelial morphology indistinguishable from its progenitor strain AmutBmut. Like homokaryon AmutBmut (KERTESZ-CHALOUPKOVÁ *et al.* 1998; BADALYAN *et al.* 2004), the mutant forms fused clamp cells at the hyphal septa and produces ∼10⁹ oidia/ plate in a light-dependent manner, indicating that mating-type functions in mutant 6-031 are not affected. Mutant 6-031 forms primary hyphal knots in the dark that mature into sclerotia when cultures are further kept in the dark (not shown). However, primary hyphal knots do not develop into secondary hyphal knots under standard fruiting conditions, suggesting that mutant 6-031 has a specific defect in fruiting body initiation (*skn1*). As in the case of the wild type, mutant 6-031 is also not able to initiate fruiting body development in constant dark, in constant light, at other temperatures, or on minimal medium.

Crosses between strain 6-031 and the AmutBmut co-isogenic monokaryons PS001-1 (*A42, B42*) and PS001-2 (*A3, B1*) gave rise to mature fruiting bodies, indicating that mutant 6-031 carries a recessive defect in fruiting body initiation. Self-compatible *A43mut, B43mut* descendants of the crosses were subjected to a fruiting test (49 clones from cross PS001-1 × 6-031; 64 clones from cross PS002-1 × 6-031). A total of 65% of the *A43mut, B43mut* progeny of cross PS001-1 × 6-031 (*i.e.*, 32/49 clones) and 59% of cross PS002-1 × 6-031 *A43mut, B43mut* progeny (*i.e.*, 38/64 clones) did not form secondary hyphal knots, suggesting that there is one defect in fruiting body initiation in mutant 6-031 (*skn1*) that is not linked to the mating type genes ($P < 0.05$; for comparison of background failure in fruiting initiation: 6 and 5% of *A43mut, B43mut* progenies of parallel crosses PS001-1 × AmutBmut and PS002-1 × AmutBmut, respectively, did not initiate fruiting). Ten clones
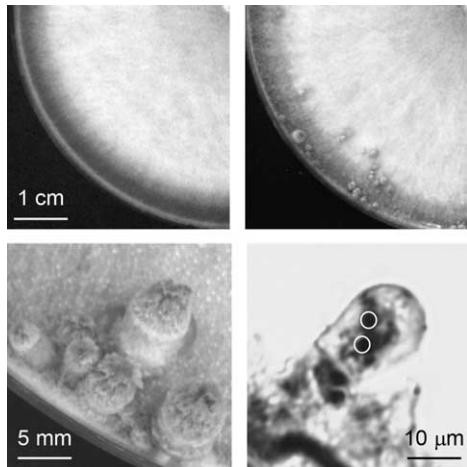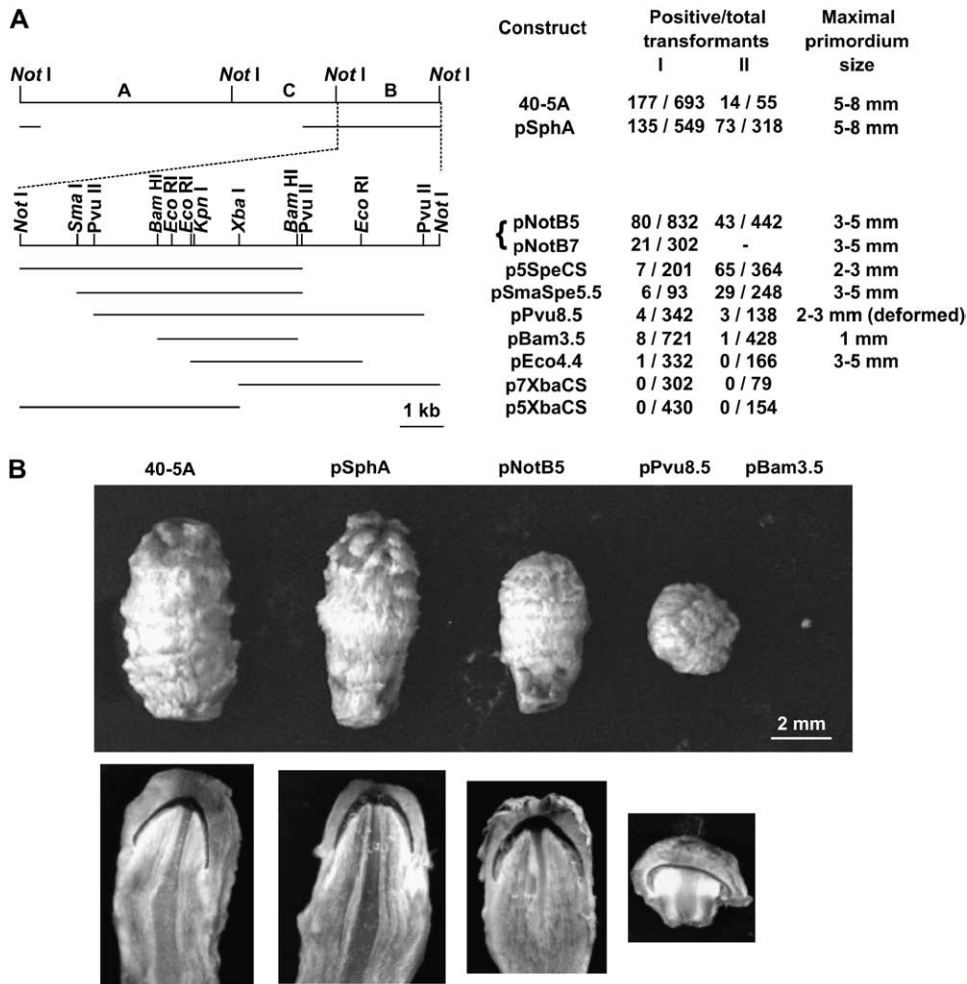
FIGURE 1.—Cosmid 40-5A restores the defect in fruiting initiation in the *Coprinopsis cinerea* mutant 6-031 upon transformation. Mycelial morphology of mutant 6-031 before transformation (top left) and primordium formation after transformation (top right). Primordia of transformants are shown enlarged at the bottom left. Their basidia (bottom right) are in a stage of prekaryogamy, as indicated by the presence of the two nuclei (encircled) and their positions within the basidia (KÜES 2000).

(20%) and 12 clones (19%) of the *A43mut, B43mut* progenies of crosses PS001-1 × 6-031 and PS002-1 × 6-031, respectively, arrested primordia development at the basidial stage of prekaryogamy, indicating that mutant 6-031 has a second unlinked recessive defect in primordia maturation (*mat*; $P < 0.05$). The remaining clones split into two further groups, one with white mushrooms by a failure in basidiospore formation (two clones, *i.e.*, 4% of *A43mut, B43mut* progeny of cross PS001-1 × 6-031; seven clones, *i.e.*, 11% of *A43mut, B43mut* progeny of cross PS002-1 × 6-031) and one with mushrooms carrying mature black basidiospores (five clones and seven clones, *i.e.*, 11% of *A43mut, B43mut* progeny of cross PS001-1 × 6-031 and PS002-1 × 6-031, respectively). Therefore, a third unlinked recessive gene is present in mutant 6-031 that acts in basidiospore formation (*bad*; $P < 0.05$).

Identification of a cosmid able to restore fruiting body initiation in mutant 6-031: A genomic library of *C. cinerea* monokaryon AmutBmut (BOTTOLI *et al.* 1999) was employed to isolate DNA sequences that restored the fruiting initiation in mutant 6-031. In a first round of transformations using pools of 96 different cosmids, one transformant in a total number of 7948 (equivalent to the analysis of ∼45% of the entire library) formed primordia up to a size of 5–8 mm (Figure 1). In subsequent transformations dividing the positive pool 40 into subpools, 12 of 208 transformants of subpool 40-5 developed primordia, and in the final round using individual cosmids, 27 of 45 tested transformants of cosmid 40-5A. Basidia within these fully established primordia had either two distinct nuclei at the prekaryogamy stage (Figure 1) or no nucleus (data not shown).

Transformation activities of subclones derived from cosmid 40-5A in strain 6-031: Cosmid 40-5A with a 40-kb-sized insert of *C. cinerea* genomic DNA was digested with various restriction enzymes, and digestion mixtures were transformed into mutant 6-031. Digestions with *Bam*HI, *Not*I, *Pvu*II, or *Sph*I still allowed initiation of fruiting body development in part of the transformants, unlike a number of other enzymes (*Eco*RI, *Eco*RV, *Kpn*I, *Pst*I, or *Xho*I). *Not*I divides cosmid 40-5A into three *C. cinerea* genomic DNA fragments (20, 10.5, and 8.9 kb, Figure 2A) plus an extra fragment representing the cosmid backbone (8.9 kb). *Not*I was chosen to construct pBC SK(+) subclones, which were cotransformed with plasmid pPAB1-2 into mutant 6-031. Plasmids pNotB5 and pNotB7 containing the same 10.5-kb insert fragment (*Not*I-B), restored fruiting body initiation (Figure 2). Some subclones of this *C. cinerea* genomic fragment (p5SpeCS, pSmaSpe5.5, pPvu8.5, and pBam3.5) were also active in fruiting body initiation. However, we noticed quantitative and qualitative variations in transformation activities, related to the length of transformed DNA fragments (Figure 2). The most effective constructs were cosmid 40-5A and its deletion derivative pSphA carrying the *Not*I-B fragment together with flanking DNA regions. Usually, 20–30% of the transformants of these cosmids initiated fruiting body development and developed primordia up to 5–8 mm in size. Four plasmids (pNotB5, pNotB7, p5SpeCS, and pSmaSpe5.5, containing a common 5.5-kb sequence) induced fruiting body initiation in 5–10% of the transformants, but the primordia formed were less developed and of a maximal size of only 2–5 mm. Normal cap and stipe differentiation were observed in these primordia. The reduction in percentage of transformants initiating fruiting might relate to the fact that cosmids 40-5A and pSphA carry the *pab1⁺* selection marker, whereas the pBC SK(+) and pBluescript KS(−) constructs needed to be cotransformed with the *pab1⁺* containing plasmid pPAB1-2. In contrast, this difference in the transformation procedure cannot account for the less-developed primordia obtained with the four plasmids. Moreover, upon cotransformation of plasmids pPAB1-2 and pPvu8.5 (having a 5.2-kb *C. cinerea* sequence in common with the former four plasmids), only 1–2% of transformants developed primordia, which were malformed and maximal 2–3 mm in size. In these primordia, the internal pileus trama tissue was missing. Plasmid pBam3.5 with a 3.5 kb *Bam*HI fragment was the smallest construct regularly active in cotransformation, with 0.2–1% of transformants initiating fruiting but development arrested shortly after secondary hyphal knot formation at a size of ∼ 1 mm. When solely transforming constructs pPvu8.5-pab and pBam3.5-pab containing the *pab1⁺* selection marker in addition to the 8.5-kb *Pvu*II fragment or the 3.5-kb *Bam*HI fragment, neither the transformation efficiency increased nor the primordia development improved in positive transformants

A

Construct	Positive/total transformants		Maximal primordium size
	I	II	
40-5A	177 / 693	14 / 55	5–8 mm
pSphA	135 / 549	73 / 318	5–8 mm
{ pNotB5	80 / 832	43 / 442	3–5 mm
{ pNotB7	21 / 302	–	3–5 mm
p5SpeCS	7 / 201	65 / 364	2–3 mm
pSmaSpe5.5	6 / 93	29 / 248	3–5 mm
pPvu8.5	4 / 342	3 / 138	2–3 mm (deformed)
pBam3.5	8 / 721	1 / 428	1 mm
pEco4.4	1 / 332	0 / 166	3–5 mm
p7XbaCS	0 / 302	0 / 79	
p5XbaCS	0 / 430	0 / 154	

B

40-5A   pSphA   pNotB5   pPvu8.5   pBam3.5

FIGURE 2.—Identification of DNA fragments that restore fruiting body initiation in *C. cinerea* mutant 6-031. (A) The three *C. cinerea* genomic *Not*I fragments (A, B, C) present in cosmid 40-5A are shown, as well as the length and position of subfragments present in pSphA. The *Not*I-B fragment and subfragments were inserted into either pBC SK(+) or pBluescript KS(−) (see MATERIALS AND METHODS). For transformation we used either 1 μg of cosmid DNA or 1 μg DNA of pBC SK(+)- or pBluescript KS(−)-based plasmids plus 1 μg of pPAB1-2 for cotransformation (experimental setup I). To equalize the absolute number of DNA molecules possibly acting in fruiting body initiation, 7 μg of cosmid 40-5A, 3.3 μg of pSphA, or 1 μg/7 kb DNA of pBC SK(+)- or pBluescript KS(−)-based plasmids plus 1 μg of pPAB1-2 were applied (experimental setup II). Per single experiment, between 32 and 248 transformants were obtained. Since percentages of transformants initiating fruiting were comparable between different transformations of the same DNA construct (not shown), transformants of different experiments were added. Transformation with 1 μg of pPAB1-2 and transformation with 1 μg of pPAB1-2 plus 1 μg of pBluecsript KS(−) or pBC SK(+) served as negative controls. From these control transformations, a total number of 1909, 215, and 132 transformants were obtained, respectively, and none of them initiated fruiting body formation. (B) The morphological progress in primordia development declines when reducing the length of DNA fragments in transformation. The phenomenon is indicated by the isolated primordia formed by the cosmid 40-5A, pSphA, pNotB5, pPvu8.5, and pBam3.5 transformants (top). Differentiation of cap and stipe tissues is normal in primordia induced by cosmid 40-5A, pSphA, and pNotB5. In contrast, the section through a primordium induced by plasmid pPvu8.5 shows that the internal pileus trama is missing (bottom).

obtained (not shown). The data suggest that the observed differences in transformation efficiency and degree of primordia maturation obtained with different *C. cinerea* fragments are not simply a result of variations in the transformation procedure.

The 3.5-kb *Bam*HI fragment present in pBam3.5 originated from the central region of the 10.5-kb *Not*I-B fragment (Figure 2). In a total of 498 clones obtained from cotransformation of pPAB1-2 and pEco4.4, a partial overlapping 4.4-kb *Eco*RI fragment gave rise to only one transformant (0.2%) able to initiate fruiting. Two more transformants with primordia of 200 tested clones were obtained from transforming mutant 6-031 with plasmid pEco4.4-pab, containing both the 4.4-kb *Eco*RI fragment and the *C. cinerea pab1*⁺ gene. Interestingly, primordia of these three transformants developed to a size and shape comparable to that of pNotB5 (not shown). Other plasmids carrying either *C. cinerea* inserts

from the flanking regions of the 3.5-kb *Bam*HI fragment (p5KpnCS, p5EcoCS, p5SmaCS, and p7SpeCS) or inserts splitting this fragment in half (p5XbaCS and p7XbaCS) were all negative in transformation.

The 3.5-kb *Bam*HI fragment is linked to the *skn1* mutation in strain 6-031: Strains 6-031 and JV6 have a distinct *Bgl*II restriction fragment length polymorphism (RFLP) in the DNA region covered by the 3.5-kb *Bam*HI fragment. Forty-six of 588 randomly isolated descendants of a cross between the strains initiated fruiting body development on YMG/T medium. Forty-one of these clones had the *A43mut, B43mut* mating type and the RFLP pattern of monokaryon JV6. Both parental patterns were detected in the remaining five clones, but analysis of the *A* mating-type-linked *pab1-1* allele (KÜES *et al.* 2001) in their oidia identified them as dikaryons (data not shown). Either the JV6 or the 6-031 RFLP pattern was found in 30 randomly isolated non-fruiting
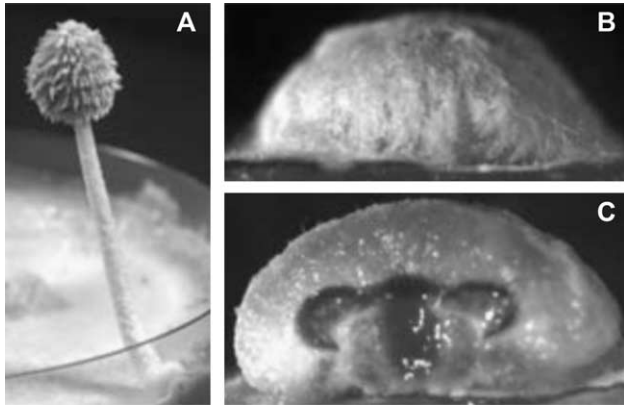
FIGURE 3.—Fruiting behavior of transformants of homo-karyon OU3-1. (A) Many transformants of cosmid 40-5A form mature fruiting bodies (~4 cm). (B and C) pBam3.5 trans-formants form small, flat primordia (2 mm primordium shown) that lack inner pileus tissue.

clones. The data suggest that the 3.5-kb *Bam*HI frag-ment is linked to the fruiting initiation defect (*skn1*) in mutant 6-031.

Transformation activities of cosmid 40-5A and de-rived subclones in *skn1*, *mat*+, *bad*+ strain OU3-1: To determine whether the quantitative and qualitative dif-ferences in complementation activity with different plasmids in mutant 6-031 were not triggered by the two other mutations present in the strain, mutant strain OU3-1 carrying only the *skn1* mutation was selected from the *A43mut, B43mut* progeny of cross PS001-1 × 6-031.

When transforming either cosmid 40-5A or its de-rivative pSphA into this strain (1 μg DNA per trans-formation), the defect in initiation was complemented in >40% of the transformants (61 positive/145 cosmid 40-5A transformants; 56 positive/131 pSphA transfor-mants). Mature fruiting bodies with basidiospores were obtained in 18 and 13% of the cases, respectively (Figure 3A), showing that the arrest in primordia development in cosmid 40-5A and pSphA transformants of mutant 6-031 was due to its *mat* mutation.

Transformation efficiencies with strain OU3-1 gener-ally were higher than those with mutant 6-031 and quantitative differences in transformation efficiencies were less pronounced. Nevertheless, in cotransforma-tion of a pBC SK(+) or a pBluescript KS(−) derivative (usually 1 μg) and pPAB1-2 (1 μg), we again observed qualitative differences in complementation efficiency. Unlike for the cosmids, no mature fruiting bodies were obtained using any of the plasmid constructs. pNotB7 initiated fruiting in strain OU3-1 in 20% of cases (24 positive/118 total transformants) but development ar-rested latest at a primordia size of ~5–6 mm. pSmaSpe5.5, pPvu8.5, and pBam3.5 were all less efficient in trans-formation with ~15% transformants initiating fruiting (16 positive/110 pSmaSpe5.5 transformants, 24 posi-tive/177 pPvu8.5 transformants, 29 positive/213 pBam3.5 transformants). Primordia development in the group of

pSmaSpe5.5 transformants arrested at maximum sizes of 3–4 mm, and in the group of pPvu8.5 transformants arrested at a size of 2 mm. Most positive transformants of pBam3.5 had primordia of ≤1 mm in size, but eight transformants had primordia of 2–3 mm. Primordia from pPvu8.5 and pBam3.5 transformants were always malformed, being more flat than wild-type primordia. Moreover, they lacked inner tissues of the pileus (Figure 3, B and C). The results indicate again that pBam3.5 carries the gene for fruiting body initiation, although subsequent development does not follow the normal route. pBluescript KS(−) control transformants (51 clones) and control transformants of only pPAB1-2 (63 clones) gave no positive transformants. Transform-ants of p5SpecCS (350 ng DNA were used) formed normal-shaped primordia with final sizes of 2–3 mm with a transformation frequency of 6% (7 positives/105 transformants).

Characterization of the 10.5-kb *Not*I-B region: The quantitative and qualitative differences in complemen-tation activities with different plasmids led us to se-quence the whole genomic *Not*I-B region, which is 10,526 bp in size. A GenBank BLAST search with this sequence revealed four potential coding regions, whose deduced protein sequences showed highest similarities to the human ADP-ribosylation factor-like protein 2 ARL2 (67% identity and 82% similarity over a length of 185 aa; accession no. P36404), the CFA synthase of *Escherichia coli* (32% identity and 48% similarity over a length of 367 aa; accession no. P30010), the galactur-onosyl transferase Cap1E in *Streptococcus pneumoniae* (32% identity and 49% similarity over a length of 91 aa; accession no. L36873), and the C-terminal part of the kinesin-like protein UNC-104 in *Caenorhabditis elegans* (32% identity and 51% similarity over a length of 271 aa; accession no. P23678) (Figure 4A). Whereas potential start codons are present on the *Not*I-B fragment for the potential *ARL2*- and *cfa*-like genes, the *UNC-104*-related sequence is incomplete and 5′-truncated by the *Not*I site. For the stretch of DNA translating in a Cap1E-like sequence, there is no obvious start codon. In accordance, a transcription analysis of the entire 10.5-kb *Not*I-B fragment detected three transcripts (Figure 4B), corresponding in location to the deduced coding re-gions for the ARL2-like protein (gene *arf1*), for the potential CFA (gene *cfs1*), and for the UNC-104-like kinesin (gene *kin1*). Weak transcripts for *kin1* were detected in Northern blots of total RNA from homo-karyon AmutBmut. When using poly(A)+ RNA to in-crease the sensitivity, transcripts for *arf1*, *cfs1*, and *kin1* were well detected but we never observed a transcript for a further gene.

The *arf1* transcript is ~0.58 kb in size (Figure 4B). *arf1* is expressed in all developmental stages tested (mycelia grown in constant dark and constant light, dark-grown mycelium containing primary hyphal knots, 1-mm-sized primordia at a prekaryogamy stage of tissue differentiation,
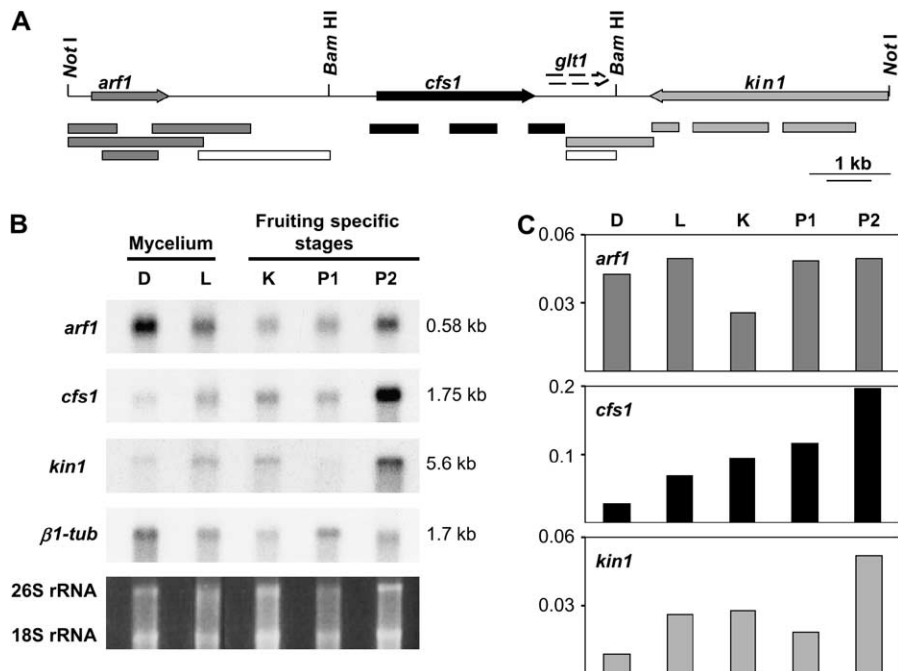
FIGURE 4.—Transcription profile of the *Not*I-B region. (A) Sequence analysis of pNotB5 revealed four potential *C. cinerea* open reading frames (indicated by the arrows *arf1*, *cfs1*, *glt1*, and *kin1*), of which one (*kin1*) is only partially present on the *Not*I-B fragment and one (*glt1*) has no obvious start codon. Three transcripts, *arf1*, *cfs1*, and *kin1* were detected in Northern blot analysis of total RNA and poly(A)+RNA by various DNA probes (shown as differentially shaded boxes in relation to the respective transcript detected). No signal was detected with two probes (open boxes) in the intergenic *arf1–cfs1* and *cfs1–kin1* regions. (B) Gene expression was analyzed with poly(A)+RNA isolated from vegetative mycelium grown on YMG/T agar at 37° for 4 days in the dark (sample D) or 7 days in the light (sample L). Letter K denotes RNA isolated from cultures grown for 6 days at 37° in the dark that developed numerous primary hyphal knots within the aerial mycelium. P1 and P2 indicate RNA extracted from isolated 1-mm-sized primordia at a prekaryogamy stage and from isolated 1-cm-sized primordia undergoing meiotic divisions, respectively. Primordia were harvested at day 3 (P1) and day 5 (P2) after fully grown YMG/T cultures were incubated at 25° under standard fruiting conditions. The 26S and 18S rRNA are shown to indicate the quality of poly(A)+RNA samples. poly(A)+RNA was isolated from total RNA only once per developmental stage with comparable yields. The relative strength of signals in blots of poly(A)+RNA followed that of earlier total RNA blots. Total RNA has been analyzed before from two to three independent RNA isolations from dark and light incubated mycelium and developing primordia. Parallel blots from the same total and poly(A)+RNA isolations gave similar results. (C) Densitometric quantification of the transcripts shown in B. For calibration, the β1-tubulin (β*1-tub*) transcript per poly(A)+RNA was used as standard. Transcript levels are given in arbitrary units (transcripts/β*1-tub* transcript).
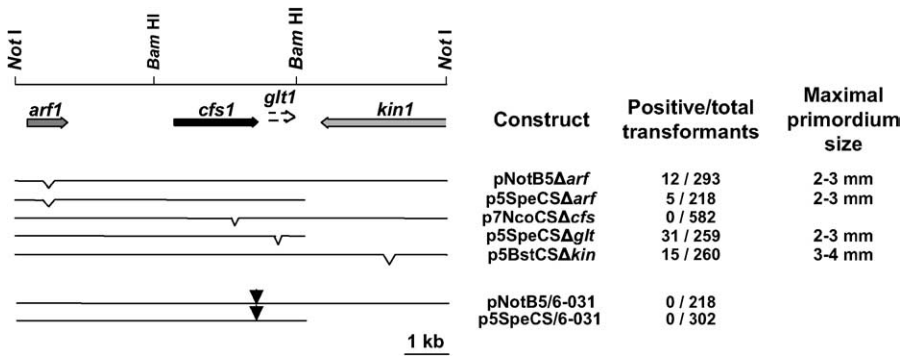
and 1-cm-sized primordia being after karyogamy in meiosis) at a similar level, but decreases slightly at the stage of primary hyphal knot formation (Figure 4C). The expression of the 1.75-kb-sized *cfs1* transcript is low in mycelium growing in the dark compared to fully grown cultures with primary hyphal knots and compared to older cultures grown in constant light (Figure 4B). *cfs1* transcription continues in developing primordia and is highest in mature primordia (Figure 4C). Gene *kin1* has a 5.6-kb transcript (Figure 4B), which is poorly expressed in all stages except in primordia at the meiotic stage just about to undergo rapid stipe elongation and cap expansion (Figure 4C).

Gene *cfs1* is essential for fruiting body initiation: The transformation data of mutants 6-031 (shown in Figure 2) and OU3-1 did not yet allow us to definitely assign the fruiting restoring ability of the 10.5-kb *Not*I-B fragment to a single gene. The higher frequency of transformants initiating fruiting and the qualitative progress in primordia development with larger DNA fragments indicated that more than one of the cloned genes could contribute together or sequentially to fruiting body initiation. Southern blot analysis, using genomic DNA from mutant 6-031 and homokaryon AmutBmut digested with a number of restriction enzymes and three DNA probes that together covered the whole *Not*I-B fragment (a 4.4-kb *Not*I–*Kpn*I fragment, a 3.5-kb *Bam*HI

fragment, and a 3.5-kb *Spe*I–*Not*I fragment), excluded the possibility of a large deletion in this region in mutant 6-031 (not shown). Moreover, each probe detected only single bands (not shown), indicating the single copy nature of all cloned genes.

Next, we constructed plasmids with deletions within the coding region of *arf1*, *cfs1*, or *kin1* and within the uncertain *glt1* region (see MATERIAL AND METHODS) and tested them by transforming mutant 6-031. Only plasmid p7NcoCSΔ*cfs*, carrying a deletion in *cfs1*+, lost the ability to restore fruiting body initiation in the mutant (Figure 5), defining *cfs1* as the gene active in fruiting body initiation. Since interruptions within other genes did not fundamentally influence the transformation efficiency and since primordia formed were of normal shape and 2–4 mm in size as were those obtained with the unmutated *Not*I-B fragment in the same series of experiments (Figure 5), it is possible that the general chromosomal environment of gene *cfs1* rather than the presence of other functional genes influences its expression and accounts for the developmental differences observed in our transformation experiments.

In the *cfs1* allele from mutant 6-031, a single T-to-G transversion was found, which leads to a Y441D amino acid substitution in the C-terminus of the deduced protein sequence (Figure 6). When introducing this T-to-G transversion into the cloned *Not*I-B fragment and

FIGURE 5.—Gene *cfs1* is essential for fruiting body initiation. Plasmids pNotB5Δ*arf*, p5SpeCSΔ*arf*, p5SpeCSΔ*glt*, and p5BstCSΔ*kin* containing a deletion in *arf1*, *kin1*, or the *glt1* region (positions of deletions within subcloned fragments are indicated by open triangles) still permitted initiation of fruiting body formation when transformed into mutant 6-031. Positive transformants developed 2–4 mm primordia with normal morphology. In contrast, a deletion in *cfs1*[+] in plasmid p7NcoCSΔ*cfs* abolished the ability to induce fruiting body formation. Likewise, plasmids pNotB5/6-031 and p5SpeCS/6-031 carrying the T-to-G transversion found in the *cfs1* allele of mutant 6-031 (positions are marked by arrowheads) did not activate fruiting body formation in mutant 6-031. In all transformations, 1 μg DNA/7 kb length of the test plasmids plus 1 μg of pPAB1-2 have been used.

a smaller 7-kb *Not*I–*Spe*I fragment, neither of the two resulting plasmids, pNotB5/6-031 and p5SpeCS/6-031, was able to restore fruiting body initiation (Figure 5), indicating that the point mutation is indeed the cause of the inability to initiate fruiting body formation in mutant 6-031.

The *cfs1* gene encodes a protein highly similar to bacterial cyclopropane fatty acid synthases: The cDNA of the *cfs1*[+] gene is 1776 bp in size with a 66-bp-long nucleotide sequence upstream of the first start codon ATG and a 300-bp-long nucleotide sequence downstream of the stop codon TGA. Comparison of the genomic DNA and cDNA sequences revealed an open reading frame (ORF) of 1407 bp interrupted by 10 introns of 53–70 bp in size with typical *C. cinerea* 5′- and 3′-splice sites and branch-receptor sequences (BOULIANNE *et al.* 2000). The *cfs1* promoter region contains a CAAT element 60–57 bp upstream of the transcription initiation site. No classical TATA box is found, but an AAT AAAAA sequence is 37–30 bp upstream of the transcription initiation site. A total of 456 bp are upstream to the transcription initiation site in the 3.5-kb *Bam*HI fragment having the smallest sequence regularly but inefficiently active in transformation (Figure 2). This sequence should mediate at least some promoter activity. Within the *cfs1* coding region, 332 bp downstream of the start codon ATG in the second exon is the *Eco*RI restriction site used to construct plasmids pEco4.4 and pEco4.4-pab with 5′ truncated *cfs1*[+] copies. Homologous recombination at the natural *cfs1* locus within the *C. cinerea* 6-031 genome or in-frame integration into another gene might therefore explain the occasional transformants initiating primordia development obtained with these two plasmids (not shown).

The *cfs1*[+] ORF encodes a polypeptide of 469 amino acid residues with a predicted molecular mass of 52 kD. The Cfs1 protein has an overall high identity to a number of bacterial CFA synthases (Figure 6), a particular subfamily of the S-adenosyl-L-methionine (SAM)-dependent C-methyltransferases (MTases) (FAUMAN *et al.* 1999). NCBI database searches for related proteins among others also identified a functional enzyme from the plant *Sterculia foetida* (AAM33848) and potential but not yet characterized eukaryotic protein products from the filamentous ascomycetes *Neurospora crassa* (EAA32979) and *Magnaporthe grisea* (EAA50372), the basidiomycete *Ustilago maydis* (XP_751900.1), the plant *Arabidopsis thaliana* (BAB02771.1), the worm *C. elegans* (T18571), and the slime mold *Dictostelium discoideum* (EAL65538). In contrast, no homologous human or any other mammal sequence seems to exist in the GenBank databases. Within the fungi, no closely related sequences were found in the ascomycete yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. In the yeast *S. cerevisiae*, the most similar protein is ERG6, a Δ24-sterol-C-methyltransferase (31% identity and 48% similarity over a length of 236 aa; 19% identity and 33% similarity over the whole protein length; S42003; Figure 6).

Consistent with a function as SAM-dependent MTase, a SAM-binding motif is present in Cfs1 between aa 209 and 338 (Figure 6). Protein profile programs (PSA and PSIpred) predict this region to adopt the typical 6 helices-7 strands configuration of SAM-binding domains (referred to as a SAM-dependent MTase fold) (FAUMAN *et al.*, 1999). All but one amino acid shown to contact SAM in structurally characterized SAM-dependent MTases (FAUMAN *et al.*, 1999) are conserved in Cfs1 (Figure 6). Both programs TMpred and TMHMM predict potential transmembrane domains in the C-terminus of the *C. cinerea* Cfs1 protein (aa 435–453 and aa 437–456), closely behind the SAM-binding motif. The Y441D amino acid substitution in mutant 6-031 is localized in this region (Figure 6). This substitution overturns the prediction of a transmembrane domain and gives rise to higher hydrophilicity (Goldman/Engelman/Steitz prediction) at the C-terminal region (not shown). Overall, the wild-type Cfs1 protein appears to be hydrophilic without any long hydrophobic region.

DISCUSSION

In this study, we characterized the first mutant of the basidiomycete *C. cinerea* with a specific defect in fruiting
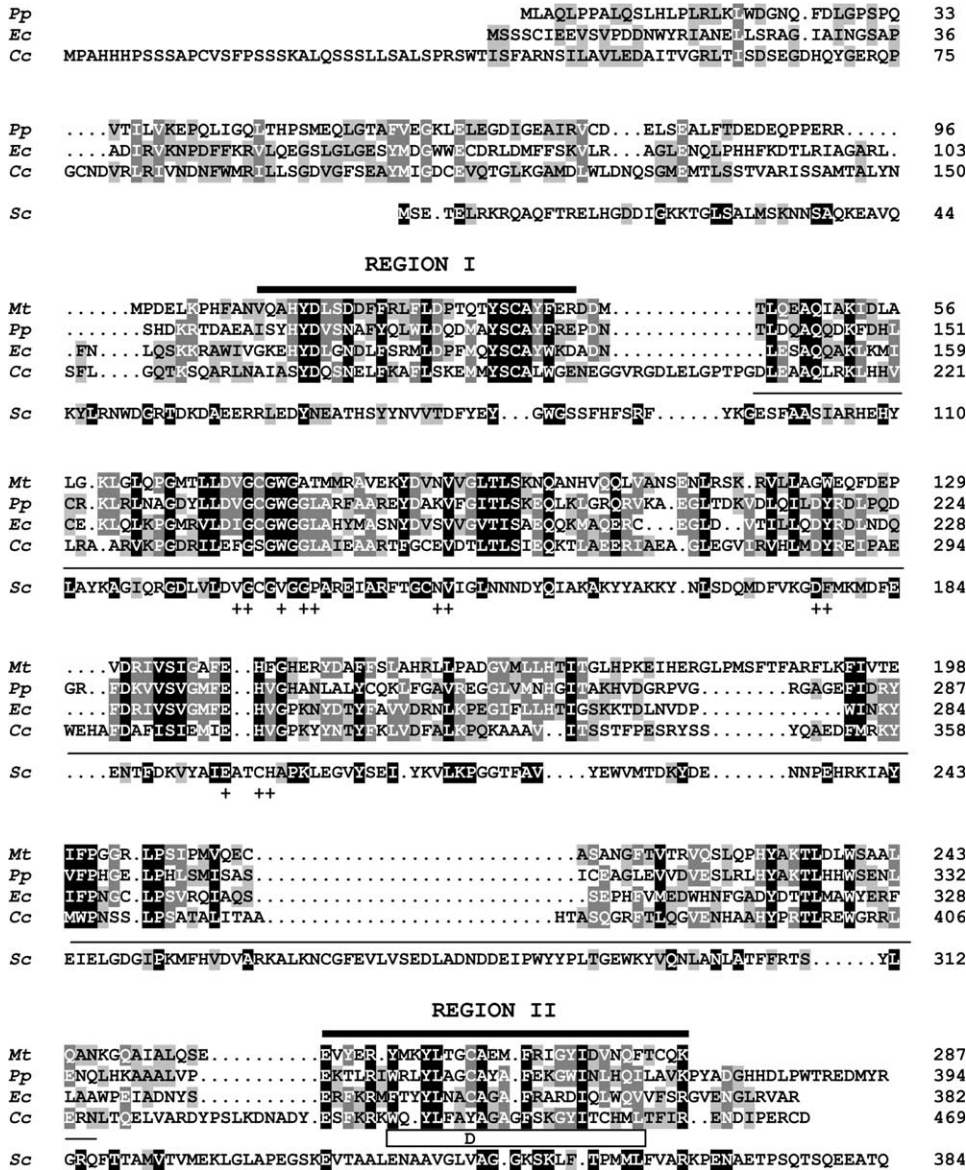
```
Pp                                               MLAQLPPALQSLHLPLRLK WDGNQ.FDLGPSPQ   33
Ec                                               MSSSCIEEVSVPDDNWYRIANE LSRAG.IAINGSAP  36
Cc   MPAHHHPSSSAPCVSFPSSSKALQSSSLLSALSPRSWTISFARNSILAVLEDAITVGRLT SDSEGDHQYGERQP    75


Pp   ....VT LVKEPQLIGQ THPSMEQLGTA VEGKL ELEGDIGEAIR CD...ELS ALFTDEDEQPPERR.....   96
Ec   ....AD RVKNPDFFKRV LQEGSLGLGES YM DGWW CDRLDMFFSKV LR...AGLE NQLPHHFKDTLRIAGARL. 103
Cc   GCNDVR RIVNDNFWMR ILLSGDVGFSEA YM IGDC EVQTGLKGAMD WLDNQSGM MTLSSTVARISSAMTALYN  150

Sc                         MSE.TE LRKRQAQFTRELHGDDI GKKTG LSALMSKNNSA QKEAVQ          44


                              REGION I

Mt   ......MPDEL KPHFANVQA HYDLSDDFFFRL FLDPTQTYSCAYF ERDD M.............TLQEAQIAKIDLA  56
Pp   .......SHD KRTDAEAISY HYDVSNAFYQLWLDQD VAYSCAYF REPDN N.............TLDQAQQDKFDHL 151
Ec   .FN....LQS KRAWIVGKE HYDIGNDLF SRML DPF MQYSCAYWKDA DN.............LES AQQAKLKMI   159
Cc   SFL....GQT KSQARLNAIAS YDQ SNELF KAF LSKE MYSCALWGENEGGVRGDLELGPTPGDL EAAQLRKLHHV  221

Sc   KYLRNWDGR TDKDAEERRLED YNEATHSYYNVVTDFYEY...GWG SSFHFSRF......YKG ESF AASIARHE HY  110


Mt   LG.KLG LQPGMTLLDVGCGWG ATMMR VEKY DVNVVGLTLSKNQANHV QLVANSE NLRSK.RVLLAGW EQFDEP  129
Pp   CR.KLR LPNAGDYLLDVGCGWGGLAR FAAREYDAKV FGITLSKEQLKLGRQRVKA.EGL TDKVDL QILDYFDLPQD  224
Ec   CE.KLQ LKPGMRVLDIGCGWGGLAHYM ASNYDVSV VGVTISAEQQKMAQ ERC...EGLD. VTILIQDYRDLNDQ  228
Cc   LRA.AR VKPGDRILEF GSGWGGLAIBAART GCE VDTLTLS IEQKTLAEE RIAEA.GLEGVIRVHLMDYREIPAE  294

Sc   LAYKAGIQRGDLVLDVGCGVGGPARE IARFTGCNVIGLNNNDYQIAK AKYYAKKY.NLSDQMDFVKGDFMKMDFE   184
                       ++   +  ++            ++                               ++
```


FIGURE 6.—Sequence alignment of *C. cinerea* Cfs1 (*Cc*) to bacterial cyclopropane fatty acid synthases (*Mt*, *Pp*, *Ec*) and to yeast Δ24-sterol methyltransferase ERG6. *Pp*, protein ylp3 of the Gram⁻ bacterium *Pseudomonas putida* (P31049); *Ec*, CFA of the Gram⁻ bacterium *E. coli* (P30010); *Mt*, CFA1 of the Gram⁺ bacterium *Mycobacterium tuberculosis* (Q11195); *Sc*, ERG6 of yeast *S. cerevisiae* (S42003). In the ERG6 protein sequence, amino acids identical to the *C. cinerea* Cfs1 sequence are shaded in black, similar ones are shaded in gray. A line marks the structurally conserved SAM-dependent MTase fold. A "+" indicates those amino acids shown in other proteins to contact SAM (FAUMAN *et al.*, 1999). The open box underneath the C-terminal end of the protein indicates potential membrane-interacting regions in Cfs1. The letter D in this box indicates the amino acid exchange found in Cfs1 of mutant 6-031 at position Y441. Region I and region II denote protein regions specific to cyclopropane fatty acid synthases.

```
Mt   ....VDRIVSIGAFE..HFGHERYDAF FS LAHRLL PAD GVMLLHTI TGLHPKEIHERGLPMSFTFARFLK FIVTE  198
Pp   GR..FDKVVSVGMFE..HVGHANLALYCQK FGAVREGGIVMNHGITAKHVDGRPVG........RGAGEFIDRY  287
Ec   ....FDRIVSVGMFE..HVGPKNYDTYFAVVDRNLKPEGIFLLHTIGSKKTDLNVDP..........WINKY  284
Cc   WEHAFDAFISIEMIE..HVGPKYYNTYFKLVDFALKPQKAAAV.ITSSTFPESRYSS......YQAEDFMRKY  358

Sc   ....ENTFDKVYAIEATCHAPKLEGVYSEI.YKVLKPGGTFAV....YEWVMTDKYDE.......NNP EHRKIAY  243
              +  ++                                                                     
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Mt   IFPG CR.LPSI PMVQEC.........................ASANG FTVTRVQSLQPHYAKTLDLWSAAL  243
Pp   VFPH CE.LPH LSMISAS.........................ICE AGLEVVDVESLRLH YAKTLHHWSENL  332
Ec   IIFPN CC.LPSVRQIAQS.........................SEP HFVMEDWHNFGADYDTTLMAWYERF  328
Cc   MWPNSS.LPS ATALITAA.........................HTA SQGRFTLQGVENHAAHYPRTLREWGRRI  406

Sc   EIELGDGIPKMFHVDVARKALKNCGFEVLVSEDLADNDDEIPWYYPLTGEWKYVQNLANLATFFRTS......YL   312


                              REGION II

Mt   QANKGQAIALQSE..........EVYER.YMKYLTGQAEM.FRIGYIDVQFTCQK           287
Pp   ENQLHKAAALVP..........EKTLRIWRLYLAGQAYA.FEKGWINLHQILAVKPYADGHHDLPWTREDMYR   394
Ec   LAAWPFIADNYS...........EREKRMFTYYLNACAGA.FRARDIQLWQVVFSRGVENGLRVAR          382
Cc   ERNLTFELVARDYPSLKDNADY.EKRKWQ.YLFAYAGACFSKGYITCHMLTFLR...EDDIPERCD          469
                            D

Sc   GRQFTTAMVTVMEKLGLAPEGSKEVTAALENAAVGLVAG.GKSKLF.TPMMLFVARKPENAETPSQTSQEEATQ  384
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body initiation at the transition stage from primary to secondary hyphal knots. Through mutant complementation, we cloned the *cfs1⁺* gene that encodes a potential CFA synthase. Transcription of the gene is low during growth of the mycelium compared to fully grown mycelium susceptible to a light signal for induction of secondary hyphal knot formation. Transcription is also higher in older cultures grown in constant light, indicating a possible age effect in gene expression. When transforming the subcloned *cfs1⁺* gene into the fruiting defective mutants 6-031 and OU3-1, we observed quantitative and qualitative differences in complementation activities with *cfs1⁺*-carrying DNA fragments of different length. For efficient complementation, a DNA fragment of >10 kb was needed. Smaller DNAs could induce formation of the unstructured secondary hyphal knots but subsequent development arrested early in primordia formation and, in some instances, lead to defects in

tissue differentiation. Such effects on primordia development are not expected if the *cfs1* gene is needed only in induction of secondary hyphal knots. High expression of the gene during primordia development further supports that the gene also functions in later stages of development.

Interruptions within the open reading frames of the neighboring genes (*arf1*, *kin1*, and the non-transcribed *glt1* region downstream to the *cfs1* gene, Figure 5) present in the 10.5-kb active *Not*I-B fragment had no major effect on the complementation activity of *cfs1⁺*. This indicates as a likely cause for the differences in transformation activity that the larger chromosomal environment plays a role in proper *cfs1* gene expression. Transcription of *arf1* is somewhat decreased at the onset of *cfs1* expression during primary hyphal knot formation, and *kin1* is specifically transcribed at subsequent stages of primordia formation when *cfs1* transcription is the

highest. Possibly, the transcription profiles of *arf1* and *kin1* influence the expression of *cfs1* during early and later stages of fruiting body development.

Structure of the Cfs1 protein: Within the *C. cinerea* Cfs1 protein, we identified a potential SAM-binding domain, a SAM-dependent MTase fold (FAUMAN *et al.* 1999). CFA synthases are C-MTases that in bacteria transfer a methylene group from SAM to a C-atom in unsaturated membrane localized phospholipids, thereby forming a cyclopropane ring (GROGAN and CRONAN 1997). Plant and fungal Δ^{24}-sterol C-MTase are the closest related to CFA synthases, possibly because their enzyme activities are both linked to lipid bilayers (TAYLOR and CRONAN 1979; LEBER *et al.* 1994). Low homology is found over the whole protein length between ERG6 of *S. cerevisiae* and Cfs1 of *C. cinerea*, with the highest conservation in the SAM-binding motif (Figure 6). In ERG6, directly at the N-terminal end of the SAM-dependent MTase fold is a sequence (DFYEYGWGSS FHFS; residues 77–92) referred to as region I that is highly specific to all Δ^{24}-sterol C-MTases, has sterol binding activity, and forms an α-helix with a loop structure that targets into the substrate pocket (NES *et al.* 1998, 2004). This sequence is not present in the family of CFA synthases (Figure 6). Instead, a highly specific sequence occupies the corresponding position (region I consensus: $V/I_{85}XXH_{88}Y_{100}D_{100}V/L/I_{92}S_{77}N/ D_{100}D/N_{65}F_{65}F/Y_{100}XL/I_{73}W/F_{85}L_{92}D_{77}P_{54}S/T_{69}M/L_{77}T/ S_{58}Y_{100}S/T_{100}C_{85}A_{92}Y/F_{54}F/W_{100}E_{38}R/K_{38}$; see supplemental material at http://www.genetics.org/supplemental/). In the crystal structures of mycolic acid cyclopropane synthases from *Mycobacterium tuberculosis*, this CFA synthase signature sequence adopts a helix-loop-helix-loop structure that coats the surface of a hydrophobic tunnel from the entrance to active SAM-binding sites. The alkyl chains of model substrates enter these pockets in a U-shaped manner, thereby contacting amino acids from region I (HUANG *et al.* 2002).

In *E. coli*, CFA synthase is a soluble protein found in the cell cytoplasm that uses SAM as a soluble and unsaturated fatty acid (UFA)-containing phospholipid as an insoluble substrate while transiently accessing to both inner and outer leaflets of intact UFA-containing membranes (TAYLOR and CRONAN 1979). The substrate C-double bond, positioned at 9–11 carbon units from the glycerol backbone of the phospholipid molecule, is located deeply within the hydrophobic core of the membrane bilayer (GALLY *et al.* 1979; SEELIG and SEELIG 1980). Inhibitor studies with sulfhydryl-modifying reagents and C-terminal truncation (50 aa) suggested that the C-terminus and possibly C354 within the C-terminus have a role in catalysis or interaction with the membrane (WANG *et al.* 1992). However, changing C354 to an alanine or serine did not result in loss of function (GROGAN and CRONAN 1997; COURTOIS *et al.* 2004). The C-terminal region (region II in Figure 6, consensus: XV/ $M/L/I_{50}XXQ/E_{73}XXXR/K_{65}V/M/L/I_{54}Y/W/F_{100}XXY_{96}L/$ $M_{73}XXC_{69}A_{58}XXF_{100}K/R_{58}XG_{58}XL/I/V_{81}D/N_{58}V/ L_{73}XQ_{77}V/M/L/I_{62}T_{50}XK/R_{96}$; see supplemental material at http://www.genetics.org/supplemental/) is nevertheless important, since the Y441D substitution in our mutant 6-031 (corresponding to A353 in *E. coli* Cfa) resulted in a loss of function. Computer programs predict the wild-type Cfs1 of *C. cinerea* being a cytoplasmic protein, like the CFA synthase of *E. coli*, with two transmembrane domains in the C-terminus. Possibly the C-terminus functions in transiently anchoring the Cfs1 protein to the membrane and/or represents part of the catalytic domain. In the crystallized *M. tuberculosis* enzymes, the C-terminal end with region II forms an α-helix and a β-sheet. C269 within this α-helix (corresponding to C354 in *E. coli* Cfa) is in the vicinity of the active site, while the β-sheet dangles away from the site (HUANG *et al.* 2002).

Occurrence of cyclopropane fatty acid synthases and their products: CFA synthases have been found in a broad range of bacteria, with *cis*-9,10-methylenehexadecanoic acid (17CFA), *cis*-9,10-methyleneoctadecanoic acid [MOA, dihydrosterculic acid (DHSA), C19], and *cis*-11,12-methyleneoctadecanoic acid (lactobacillic acid, C19) being characteristic bacterial CFAs (for review see GROGAN and CRONAN 1997). In *E. coli*, the CFA synthase is not essential for growth under an assortment of experimental conditions but improves survival in low-pH environments (CHANG and CRONAN 1999). In other bacteria, the production of CFAs also relates to stress conditions. As a consequence of CFA production, membrane properties, in particular membrane fluidity, alter with enhanced bacterial stress tolerance (COUTO *et al.* 1996; SAJBIDOR 1997; CHANG and CRONAN 1999). Phospholipids containing CFAs have a broader transition temperature range and increased rigidity than those containing UFAs, which confers more resistance of the membrane lipid matrix to environmental perturbations (DUFOURC *et al.* 1984; PERLY *et al.* 1985).

In eukaryotes, CFAs have only sporadically been reported, while the chemical structures are far more diverse. For example, cyclopropyl hydroxy-eicosanoids were described in a red alga (NAGLE and GERWICK 1990), cyclopropanated C19 straight-chain fatty acid (cladocroic acid) in a sponge (D'AURIA *et al.* 1993), cyclopropane containing eicosanoid (C20) in a soft coral (WHITE and JENSEN 1993), and CFAs with 17, 18 and 19 C-atoms in females and eggs of millipedes (OUDEJANS and VAN DER HORST 1978). DHSA has been identified in trypanosomatid protozoa and CFA synthase activity has been demonstrated (LI *et al.* 1993). DHSA synthesis from oleoyl phospatidylcholines has recently also been observed in tobacco cells after transformation of a gene from the plant *Sterculia foetida* (BAO *et al.* 2003). DHSA and *cis*-9,10-methyleneheptadecanoic acid occur in roots of *S. foetida* (KAIMAL and LAKSHMINARAYANA, 1970). Biological roles of CFAs in eukaryotes have still to be clarified. Functions in cold hardiness and drought

resistance in plants and desiccation tolerance in millipedes have been discussed (GROGAN and CRONAN 1997). In slime molds, CFA levels have been linked to cellular aggregation and cAMP metabolism (SAITO and OCHIAI 1998; MATSUOKA *et al.* 2004). These latter observations can be of special interest to our study since in *C. cinerea* there is an increase in levels of cAMP at the onset of fruiting and cAMP has been shown in particular mutants to induce fruiting (KÜES *et al.* 2004).

To our knowledge, no cyclopropanated moiety has so far been reported in higher fungal lipids. However, in the higher basidiomycetes, the unsaturated linoleic acid is the major constituent of fatty acids (SOLBERG 1989; BONZOM *et al.* 1999; SAKAI and KAJIWARA 2004). The related oleic acid is shown in *E. coli* to be a substrate for the action of CFA synthase (MARINARI *et al.* 1974) and preliminary expression studies suggest that the *E. coli* and *C. cinerea* enzymes are at least partially interchangeable in function (S. LOOS, M. AEBI and U. KÜES, unpublished results).

In this study, we show that the *C. cinerea cfs1* gene is superfluous for vegetative mycelial growth, but essential for fruiting body development. As in bacteria, the physical properties of cellular membranes may alter through production of CFAs by Cfs1 and this could be the trigger to initiate fruiting body morphogenesis in the fungus. Accordingly, in feeding experiments membrane-interactive compounds such as sucrose esters of fatty acids, plant saponins, and cerebrosides induced fruiting body development in various basidiomycetes (KAWAI 1989; OITA and YANAGI 1993; MIZUSHINA *et al.* 1998; MAGAE *et al.* 2004). From such studies, membrane alteration has been postulated to be a stress signal that promotes the fungus to shift from vegetative to reproductive growth.

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