# Molecular Analysis of *pcc1*, a Gene That Leads to *A*-Regulated Sexual Morphogenesis in *Coprinus cinereus*

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ABSTRACT

A homokaryotic strain (5337) in our culture stock of *Coprinus cinereus* produced fertile fruit bodies after prolonged culture. Microscopic examination revealed that hyphae dedifferentiated from the tissues of one of the fruit bodies, as well as all basidiospore derivatives from the fruit body, exhibited pseudoclamps, whereas vegetative hyphae of 5337, from which the fruit body developed, had no clamp connections. Genetic analysis showed that the formation of pseudoclamps results from a recessive mutation in a gene designated *pcc1* (pseudoclamp connection formation), which is distinct from the *A* and *B* mating type genes. Cloning and sequencing of the *pcc1* gene and cDNA identified an ORF of 1683 bp interrupted by one intron. Database searches revealed that *pcc1* encodes an SRY-type HMG protein. The HMG box shared 44, 41, and 29% sequence identities (>80 amino acids) to those of *FPR1* of *Podospora anserina*, *MAT-Mc* of *Schizosaccharomyces pombe*, and *prf1* of *Ustilago maydis*, respectively. Northern analysis revealed that the level of *pcc1* expression is higher in the dikaryon, in homokaryons in which the *A* and *B* mating type developmental sequences are individually activated, than in the homokaryon in which these sequences are not active. Sequencing of the *pcc1-1* mutant allele revealed that the mutant carries a nonsense mutation at serine 211, a residue located between the HMG box and the C terminus. Based on these results, possible roles of the *pcc1* gene in the sexual development of homobasidiomycetes are discussed.

THE homobasidiomycete *Coprinus cinereus* is a tetrapolar species in which mating type is determined by genes at two unlinked loci known as *A* and *B* (Kimura 1952). Genes at these loci confer multiple mating specificities and determine compatibility of mating cells by regulating initial steps in sexual development. The asexual homokaryons are compatible when genes at both the *A* and *B* loci are different, and the operation of both the *A* and *B* regulated steps in sexual development gives rise to fertile dikaryotic hyphae that have characteristic structures known as clamp connections (Raper 1966). When only the *A* genes are different, only the *A* sequence operates, resulting in hyphae with unfused pseudoclamp connections (Raper 1966). Recent molecular analyses have shown that the genes that determine the *A*-mating specificity in *C. cinereus* encode two dissimilar classes of proteins, designated HD1 and HD2, on the basis of conserved but distinct homeodomain motifs (Kües *et al.* 1992; Tymon *et al.* 1992). Similar homeodomain sequences have also been found in the deduced polypeptides of the *A* genes of another homobasidiomycete, *Schizophyllum commune* (Stankis *et al.* 1992; Specht *et al.* 1992), and the *b* mating type genes of the heterobasidiomycetes *Ustilago maydis* (Gillissen *et al.* 1992) and *Ustilago hordei* (Bakkeren and Kronstad 1993). It has been shown in both *C. cinereus* and *U. maydis* that a compatible mating is recognized by the ability of allelic forms of HD1 and HD2 proteins to heterodimerize (Banham *et al.* 1995; Kämper *et al.* 1995). Heterodimerization between two classes of homeodomain proteins in *C. cinereus* has been shown to bring together potential DNA-binding and activation domains (Asante-Owusu *et al.* 1996) of a putative dikaryon-specific transcription factor. These findings strongly suggest that there are target genes specific for the A protein heterodimer. As yet, no target gene has been identified.

In this paper, we describe the analysis of a mutation in a gene distinct from the *A*-mating type locus of *C. cinereus* that leads to constitutive operation of *A*-regulated sexual morphogenesis, *i.e.*, pseudoclamp connection formation, in the absence of the A protein heterodimer.

## MATERIALS AND METHODS

**Strains and culture conditions:** Strains of *C. cinereus* (Schaeff. ex Fr.) S. F. Gray *sensu* Korn. listed in Table 1 were used. CY-1 agar plates in 9-cm petri dishes (Kamada *et al.* 1982) were used for homokaryotic fruiting of 5337. The minimal medium was that of Shahriari and Casselton (1974) modified by Binninger *et al.* (1987). Malt extract–yeast extract–glucose medium (Rao and Niederpruem 1969) solidified with 2% (w/v) agar in petri dishes 9 cm in diameter was used for routine mycelial cultures. Slants of the same medium in test tubes were used for dikaryotic fruiting, the same medium without agar in Erlenmeyer flasks for mycelial cultures for

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**TABLE 1**

**Strains**

| Strain | Genotype / description | Source |
|---|---|---|
| 5337 | *A8B7* | This laboratory |
| 5302 | *A2B2* | This laboratory |
| 5401 | *A1B1* | This laboratory |
| $5401F_1$#49 | *A1B7*/a progeny of 5401 × 5337 | This study |
| $5401F_1$#17 | *A8B1*/a progeny of 5401 × 5337 | This study |
| $KF_2$#1 | *A91B92* | H. Muraguchi |
| 5017 | *A2B2 met4-1* | This laboratory |
| 5005 | *A2B2 arg3-1* | This laboratory |
| 5034 | *A2B2 cho1-1* | This laboratory |
| 292 | *A3B1 trp1-1,1-6* | P. J. Pukkila |
| 5337#1 | *A8B7 pcc1-1* | This study |
| $5337\#1F_1$#24 | *A2B2 pcc1-1 met-4-1*/a progeny of 5337#1 × 5017 | This study |
| $5337\#1F_1$#28 | *A8B1 pcc1-1 trp1-1,1-6*/a progeny of 5337#1 × 292 | This study |
| $A_{ON}1$ | *A43/A42 B43 ade8-1 trp3-1/trp3*$^+$ | L. A. Casselton |
| $B_{ON}1$ | *A43 B43/B42 ade8-1 trp3-1/trp3*$^+$ | L. A. Casselton |

$A_{ON}1$ and $B_{ON}1$ were constructed by transforming AT8 (*A43B43 ade8-1 trp3-1*) with the *A42* and *B42* loci, respectively (L. A. Casselton, unpublished results).

protoplasts and for extraction of RNA, and the same medium without agar in petri dishes 9 cm in diameter for mycelial cultures for extraction of DNA. Malt extract–yeast extract–glucose medium was supplemented with 100 mg/liter of tryptophan for culture of tryptophan requiring strains. Cultures were maintained at 28° unless otherwise stated.

**Construction of plasmid DNA:** pXb7 was a 7-kb *Xba*I fragment from cosmid 6E4, which complemented the *pcc1-1* (pseudoclamp connection formation) mutation, ligated into pGEM-7Zf(+) (Promega, Madison, WI). pKp5 was a 5.5-kb *Xba*I-*Kpn*I fragment from pXb7, ligated into pBluescript II KS(+) (Stratagene, La Jolla, CA). pKp528 and pKp5402 were generated by introducing progressive deletions from one end of pKp5 using exonuclease III (Pharmacia Biotech, Piscataway, NJ), as described by Henikoff (1984). pXb7B was generated by *Bam*HI digestion of pXb7 followed by religation. pSal23 was a 2.3-kb *Sal*I fragment from pXb7, ligated into pBluescript II KS(+).

***C. cinereus* DNA-mediated transformation:** DNA-mediated transformation using a *pcc1-1* mutant as a recipient was carried out according to the method described by Binninger *et al.* (1987), except that protoplasts were prepared from mycelia that were grown in maltose extract–yeast extract–glucose liquid medium for ~48 hr with continuous rotary shaking, and digestion was with Novozym 234 (Novo Labs) at 28° for 1 hr.

**Construction of cosmid libraries:** Cosmid libraries of chromosome *V* from the wild-type strain (Okayama-7) and the *pcc1-1* mutant strain (5337#1) were constructed as described by Zolan *et al.* (1992). The vector (LLC5200) contains the *C. cinereus trp1* gene as a selectable marker (Pukkila and Casselton 1991).

**Hybridization:** DNA or RNA was blotted to Hybond-N$^+$ (Amersham, Arlington Heights, IL). Gene Images random prime labeling and detection system (Amersham) was used for preparation of probes and detection of probes hybridized to nucleic acids.

**DNA sequencing:** Sequencing was performed with a series of fragments derived from the gene in pBluescript II KS(+) (Stratagene) obtained by sequential deletions from one end by exonuclease III (Pharmacia Biotech). The nucleotide sequences of the fragments were determined with an automated DNA sequencer (model 373A; Perkin Elmer, Norwalk, CT) and a PRISM dye primer cycle sequencing kit (M13 forward or M13 reverse), as recommended by the manufacturer's instructions. The nucleotide sequences were analyzed with BLAST (Altschul *et al.* 1990). Information on the genomic DNA sequence of *pcc1* and the deduced amino acid sequence were submitted to DDJB (accession number AB007760).

**Nucleic acid isolation:** Cosmid and plasmid DNA were prepared with the alkaline lysis method (Sambrook *et al.* 1989) or with the FlexiPrep Kit (Pharmacia Biotech). Samples for DNA sequencing were prepared by the FlexiPrep Kit (Pharmacia Biotech). Genomic DNA was prepared from *C. cinereus* as described by Zolan and Pukkila (1986). For total RNA, mycelia grown in the liquid medium at 37° for 48 hr with moderate rotary shaking were frozen quickly in liquid nitrogen after washing once in sterile water by low-speed centrifugation. The frozen mycelia were ground into fine powder with a mortar and pestle. RNA was extracted from the powder as described by Yeager Stassen *et al.* (1997).

**Analysis of *pcc1* cDNA:** To sequence the cDNA of *pcc1*, PCR was carried out on the cDNA library of *C. cinereus* (Yeager Stassen *et al.* 1997). Seven sense or antisense primers that are complementary to genomic sequences of *pcc1* and to the vector sequences were designed so that overlapping PCR products covered the whole length of cDNA. These primers were obtained from the custom primer synthesis service of GIBCO BRL (Gaithersburg, MD). To amplify DNA fragments from the cDNA library, 3 μl phage was mixed with 2 μl UV-treated water, and a 3-μl aliquot was heated at 100° for 3 min. After chilling on ice for 2 min, the sample was amplified in a Perkin Elmer GeneAmp PCR System 9600, using AmpliTaq Gold (Perkin Elmer) according to the manufacturer's instructions. After amplification, the samples were electrophoresed using a 1.5% agarose gel, and the DNAs were purified from the gel using Geneclean II Kit (BIO 101 Inc., Vista, CA) and cloned into the pCR2.1 vector with the Original TA Cloning Kit (Invitrogen, San Diego, CA), as recommended by the manufacturer's instructions.

**Microscopy:** A piece of mycelium was inoculated on MY liquid medium on a glass slide, which was set in a moisture chamber at 28°. After incubation for 2–3 days, the liquid medium was withdrawn with filter paper, and a drop of 2 μg/ml of DAPI in 20 mm Tris-HCl (pH 7.5) was dropped on the

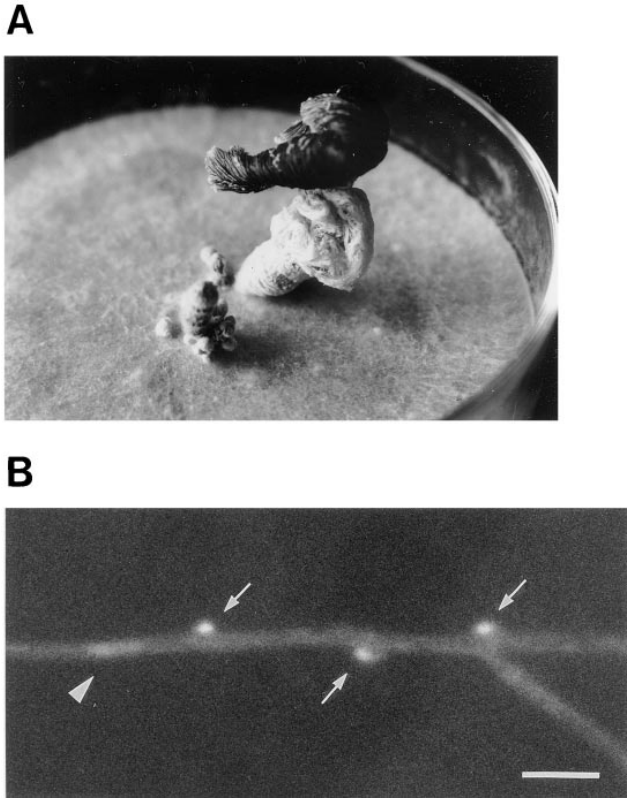


Figure 1.—(A) A homokaryotic fruit body developed on the mycelium of 5337; (B) a fluorescence micrograph showing DAPI staining of a part of a hypha of strain 5337#1, a basidiospore derivative of homokaryotic strain #5337. The arrows show nuclei trapped in clamp cells, and the arrowhead shows a nucleus in the hypha. The bar in B represents 10 μm.

hyphae. The samples were immediately observed under an epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) equipped with phase-contrast optics.

**Genetic techniques:** Crosses were made by laying two inocula (1 × 1 mm) 1 mm apart on malt extract–yeast extract–glucose agar plates. Basidiospore germlings were isolated at random using a chisel-shaped needle under a dissecting microscope (Miles *et al.* 1966). Mating types were identified by dikaryon tests with four testers, 5401 (*A1B1*), 5337 (*A8B7*), $F_1$#49 (*A1B7*), and $F_1$#17 (*A8B1*); the last two testers were obtained among $F_1$ progeny of the cross between 5401 and 5337. Common-*AB* diploids were constructed as described by Iwasa *et al.* (1998).

## RESULTS

**Isolation and genetic analysis of a *pcc1-1* mutation:** A homokaryotic strain (5337) regularly developed fruit bodies after about a 1-mo cultivation on CY-1 agar plates. Hyphae dedifferentiated from tissues of the fruit body primordia and mature fruit bodies always had pseudoclamp connections (Figure 1B), whereas hyphae in vegetative mycelium from which fruit bodies had developed did not have any clamp connections. The fruit bodies produced basidiospores, although the number of basidiospores was reduced as compared with the wild-type dikaryon and their stipes were abnormally short (Figure 1A). Karyogamy and meiosis are likely to be normal in those basidia that produce spores because the spores are viable. We isolated 76 basidiospore derivatives from the fruit bodies at random. Microscopic examination showed that all the basidiospore derivatives isolated had pseudoclamp connections. The respective derivatives were transferred to slants of malt extract–yeast extract–glucose medium and were left in conditions that promote fruiting. All isolates were able to fruit without mating. These results suggested that during the growth of 5337, a gene(s) mutated to permit the formation of pseudoclamp connections and the development of fruit bodies.

We mated a basidiospore derivative with pseudoclamp connections (designated 5337#1) to a wild-type homokaryon (5401). 5337#1 only donated nuclei. As a result, a dikaryon with true clamp connections emerged only from the margin of the 5401 colony. The dikaryon formed apparently normal fruit bodies with normal, long stipes and abundant basidiospores. We isolated 107 $F_1$ progeny and examined them for the presence or absence of pseudoclamp connections and for mating types. Of 107 $F_1$ progeny isolated, 52 exhibited pseudoclamp connections and 55 did not. Thus, pseudoclamp connection formation results from a mutation in a single gene ($P$ = 0.70–0.80), which is designated *pcc1* (pseudoclamp connection formation). The four mating types, *A1B1*, *A8B7*, *A1B7*, and *A8B1*, were found in the progeny, and *pcc1* and its wild-type allele segregated independently from the mating-type loci *A* and *B* ($P$ = 0.20–0.30 and 0.30–0.50, respectively), showing that *pcc1* is at a locus that is unlinked to either of the mating-type loci. Similar results were obtained in a cross with another homokaryon, 5302 (data not shown). Only $F_1$ progeny with the *pcc1-1* mutation produced mature fruit bodies or fruit body primordia, showing that *pcc1* is relevant to fruiting. However, it seems that a gene(s) other than *pcc1* is also involved in fruit body morphogenesis because fruit body development was different among the $F_1$ progeny, *i.e.*, some progeny developed normal, mature fruit bodies, some progeny developed fruit bodies with abnormally short stipes, and some progeny produced fruit body primordia that did not mature. We did not attempt to identify this other gene(s) in this study.

To test whether the *pcc1-1* mutation is dominant or recessive, we needed to construct strains that were heterozygous for *pcc1* but homozygous for the *A* mating type genes. This can be done by selecting somatic diploid strains from uninucleate oidia formed on heterokaryons between sexually incompatible homokaryons having the same *A* and *B* genes (common *AB*). We constructed a common-*AB* diploid between 5337#1$F_1$#24 (*A2B2 pcc1-1 met4-1*) and 5034 (*A2B2 cho1-1*), and we examined the mycelium for pseudoclamp formation. We found only

rare pseudoclamps on the diploid mycelium and concluded that *pcc1-1* is a recessive mutation.

We made a linkage analysis of *pcc1-1* using auxotrophic markers, and we found that *pcc1-1* is linked to *trp2-1* and *arg3-1* at map distances of 0.6% (2/171) and 5.1% (6/118), respectively. The two markers are both in the linkage group IV of the Japanese map of this fungus (Takemaru 1982), which corresponds to chromosome *V*, as separated by a CHEF gel (Pukkila 1993).

**Cloning of *pcc1*:** To clone the *pcc1* gene, we constructed a cosmid library of chromosome *V* and screened it for a clone that complements the *pcc1-1* mutation by transformation using a double mutant (5337#1$F_1$#28) carrying the *pcc1-1* and *trp1-1,1-6* mutations as a recipient. In these experiments, we first obtained $Trp^+$ transformants and then screened them for a clone that suppressed pseudoclamp formation. The $Trp^+$ transformants were transferred and grown on MY medium for ~48 hr at 42° before examination for complementation of pseudoclamp connection formation because we found that hyphae regenerated from protoplasts of *pcc1-1* strains sometimes failed to exhibit pseudoclamps when grown at 28°, but all generated hyphae formed pseudoclamps when grown at 42°. We identified a single clone, 6E4, that complemented the *pcc1-1* mutation: 57% (8/14) of transformants with 6E4 did not exhibit pseudoclamps in their colonies.

The possibility existed that the disappearance of pseudoclamp connections in the transformants was caused by the instability of the pseudoclamp phenotype in the *pcc1-1* mutant: oidia derivatives from *pcc1-1* strains sometimes failed to exhibit pseudoclamp connections abundantly (data not shown). To rule out this possibility, a transformant exhibiting the $Pcc^+$ phenotype (and therefore lacking pseudoclamps) was crossed to the wild-type homokaryon 5302, and 43 progeny were examined for the presence or absence of pseudoclamp connections. Nine of the 43 progeny exhibited the pseudoclamped $Pcc^-$ phenotype. The result fitted well with the proportion (1:3) expected when the cosmid clone (6E4) was integrated in a chromosome other than chromosome *V*, on which *pcc1* is located ($P = 0.70$–$0.50$). This supported the idea that the transformants exhibiting no pseudoclamp connections result from ectopic integration of *pcc1*.

To define the region of *pcc1* in the cosmid clone (6E4), cotransformations were performed with fragments from 6E4 mixed with an intact pCc1003 plasmid vector containing the *C. cinereus trp1* gene. We found that the 5.5-kb *Kpn*I-*Xba*I fragment complemented the *pcc1-1* mutation. We further defined the active region within a 2.5-kb fragment by truncation (Figure 2).

**Mapping of a DNA fragment containing *pcc1*:** To determine whether the cloned gene represents the *pcc1* locus, RFLP analysis was performed. Eight progeny with pseudoclamps and eight progeny without pseudoclamps were chosen at random from the progeny of the cross between the wild-type homokaryon, $KF_2$#1, and the *pcc1-1* mutant, 5337#1. Genomic DNAs from the progeny were examined for an RFLP using a fragment from pKp5402 (see Figure 2) as a probe. We found that the presence or absence of pseudoclamps completely cosegregated with the RFLP (Figure 3), providing evidence that this clone does not contain an extragenic suppressor. With the small number of progeny analyzed, however, the possibility could not be ruled out that the cloned fragment contains a suppressor gene located 5.3–6.3 cM (1/16) from *pcc1*.

**Sequence analysis of *pcc1*:** Sequence analysis and database searches using the BLAST procedure (Altschul *et al.* 1990) showed that the 2.5-kb genomic region contained a sequence that could encode a peptide with high similarity to the HMG box. We performed PCR amplification using a *C. cinereus* cDNA library (Yeager Stassen *et al.* 1997) as a template and seven primers that were designed based on the genomic and vector sequences. We identified an ORF of 1683 bp interrupted by one intron (Figure 4). The 5′ splice site, GTAAGC, is similar to the consensus sequence GTRNGT found for filamentous fungi (Gurr *et al.* 1987), and the 3′ splice site, TAG, agrees with the consensus sequence, YAG. The predicted Pcc1 protein has 561 proline-rich amino acid residues (Figure 4) with a molecular size of 60.7 kD and a pI of 4.63. It contains a sequence encoding a peptide with significant similarity to the HMG box (positions 23–99) and a potential nuclear localization signal, PKKK (positions 99–102).

**Sequencing of the *pcc1-1* mutant allele:** To obtain a clone carrying the *pcc1-1* allele, we screened a cosmid library of mutant chromosome *V* for a clone that could amplify with a pair of sense and antisense primers that were used for amplification of the *pcc1* cDNA. We used the process of sib selection, in which progressively smaller pools of clones were amplified, to isolate an individual clone, termed 8D2, which contained the *pcc1-1* gene. We subcloned and sequenced overlapping fragments from the clone 8D2, which covered the whole length of the *pcc1-1* allele. Comparison of the sequence of the *pcc1-1* allele with that of the wild-type *pcc1* revealed that in the mutant allele, the C at position 1025 was changed to A, resulting in a nonsense mutation at serine 211 that would truncate the Pcc1 protein 351 amino acid residues from its C-terminal end.

**Interaction between the mating-type genes and *pcc1*:** Using a 1.6-kb *Sma*I fragment from pKp5402 genomic fragment, which contains most of the *pcc1* gene (Figure 2), as a probe, we examined total RNAs from the wild-type homokaryon, the wild-type dikaryon, and two homokaryons in which the *A*- or the *B*-regulated pathways were independently activated, an *A*-on *B*-off homokaryon and an *A*-off *B*-on homokaryon, and the *pcc1-1* mutant homokaryon (Figure 5). The *A*-on *B*-off homokaryon and the *A*-off *B*-on homokaryon were constructed by transforming the *A43B43* homokaryon with the *A42* and *B42* loci, respectively (see Table 1). We identified a transcript of ~2 kb in all the strains. The transcription

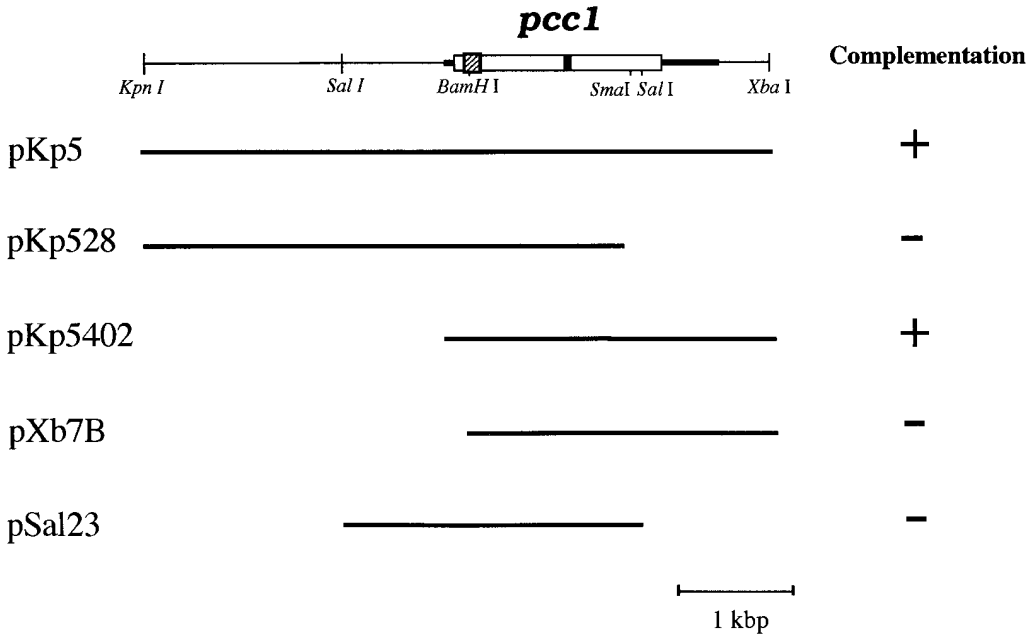


Figure 2.—Subclones of the 5.5-kb *Kpn*I-*Xba*I fragment and complementation of the *pcc1-1* mutation. Complementation of the *pcc1-1* mutation was determined by examination for the presence (indicated as −) and absence (indicated as +) of pseudoclamp connections. The thick line, white box, striped box, and black box indicate the region transcribed, a deduced ORF, the region encoding the HMG box, and the intron, respectively.

of *pcc1* was higher in the dikaryon than in the homokaryon. The transcription level was also higher in the *A*-on *B*-off and *A*-off *B*-on strains, indicating that both sets of mating type genes play a role in regulating *pcc1* transcription either directly or indirectly. The mutation in *pcc1* had no effect on its own transcription, and similar levels of transcript were detected in the wild-type and *pcc1* homokaryons.

## DISCUSSION

The transformation of homokaryotic hyphae to hyphae with pseudoclamp connections without mating has been reported to occur by mutation of the *A* gene in *C. cinereus* and *S. commune* (Day 1963; Raper *et al.* 1965; Swamy *et al.* 1984) or by mutation (*su-A*) in a gene distinct from the *A* gene in *C. cinereus* (Day 1963). The *pcc1-1* mutation found in the present study is similar to the *su-A* mutation in that both mutations cause pseudoclamps without mating, are recessive and found in a gene unlinked to the *A* locus, and only donate nuclei in matings with other homokaryons. The allelism of *pcc1* and *su-A* remains to be determined. The need for a mutational event (*e.g.*, in *pcc1*) is likely the reason that fruit bodies arose from the homokaryon #5337 after only 1 mo, while a dikaryon normally develops fruit bodies after ~10 days of cultivation.

Cloning and sequencing of the *pcc1* gene, together with the analysis of the cDNA, revealed an ORF encoding a protein of 561 amino acid residues, and database searches revealed that the protein contains an HMG box motif. It has been reported that some of the ascomycetous mating-type genes encode proteins with HMG boxes: they include *mat-Mc* of the fission yeast *Schizosaccharomyces pombe* (Kelly *et al.* 1988), *mta-1* and *mtA-3* of *Neurospora crassa* (Philley and Staben 1994; Ferreira

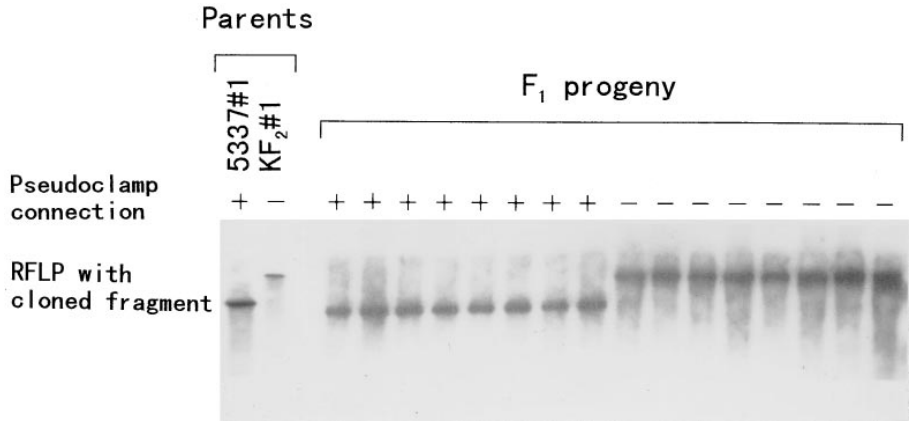


Figure 3.—RFLP analysis of the progeny from the cross 5337#1 (*A8B7 pcc1-1*) × $KF_2$#1 (*A91B92* +). The progeny were scored for presence (+) and absence (−) of pseudoclamp connections. A selection of mutant and wild-type progeny were then scored for RFLP, using enzyme *Cla*I and the 1.6-kb *Sma*I fragment from pKp5402 (see Figure 2) as the probe. pKp5402 has two *Sma*I sites, one in the 2.5-kb fragment and the other in the multicloning site of the vector.

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   1 CGGCGGTTACTAATTTCCTGGCTCGCGATTATTCCCTGGTTCTGTGAGATCTCATGCGCTTTCGACACACCATCGTTTTCTGTCCGCTTTCTTTGACGAATTTCTTGTGTGTATATCTAC
 121 TCGTGGTGCAACGCCCTCTGTATTTTAGCCAAAAACGCGATTCTCGTTTCTTGCTGGTCCAAACTGAAATTGTGTGAAATGAGCTGCAAGCTGAACCGGCGCGTGATAAAAAGAGAGAGA
 241 TGCGATCACATGACAAGTGATTTGGCGGAAGGGAAATTGAACCAGTTCCCACGAGGCCCATCTTCAACGTCGCCGTCGGTGGTGTGTGTGTTGTTCATTAACGTCCACCCTTTTTTCT
 361 TCATCTCTCAAGTGTCTCTGCCCATCGCCAGCGATGGCAGGGCCTGTACGCACACCAGCCATGGCTGAGACGCGCGCGACAATCGCACAGACCTCGACAGTGACCCCACCAAGACCGCCC
                                  M  A  G  P  V  R  T  P  A  M  A  E  T  R  A  T  I  A  Q  T  S  T  V  T  P  P  R  P  P    29

 481 AACGCCTGGATCCTCTATCGCGGCGACAAGCTCAGAGAACTCTTCGAACCAGTCCCAGGCCAGCCTCGCATGTCCCAAGCAGATGTTTCACGAATTATCGGCCAGATGTGGAGGACTGAA
      N  A  W  I  L  Y  R  G  D  K  L  R  E  L  F  E  P  V  P  G  Q  P  R  M  S  Q  A  D  V  S  R  I  I  G  Q  M  W  R  T  E    69

 601 TCCGATGCAACGCGAATGGAGTACGAACGACGCGCAGACGAAGCCAAGGCAGAACATGCAATTAAATACCCAAACTACCGCTTCAATCCCAAGAAAAAGGAGGAAAAAGAGTTTCTTCGG
      S  D  A  T  R  M  E  Y  E  R  R  A  D  E  A  K  A  E  H  A  I  K  Y  P  N  Y  R  F  N  P  K  K  K  E  E  K  E  F  L  R   109

 721 GCCCAGAGAAAAGAGGCAAAGGAGAGAGAAAAGGCGCAGGCAAAGCAGTTAAAGCGACAACAGGCACGCGCTCAGAGTCAACAGCAAGTCGCCCAACCTGTTCAGCCATCAGTCTCGACG
      A  Q  R  K  E  A  K  E  R  E  K  A  Q  A  K  Q  L  K  R  Q  Q  A  R  A  Q  S  Q  Q  Q  V  A  Q  P  V  Q  P  S  V  S  T   149

 841 ACGCCCATGGCTTATCCACCTGCTCCCGTCTTGTTGCCTCATCCGAGCATGGTTTTCTACCCTCCGATGATGGGAGGCACTGTCTACTATCCCATGGAACTCTTTGGCACGGCCGGTCCG
      T  P  M  A  Y  P  P  A  P  V  L  L  P  H  P  S  M  V  F  Y  P  P  M  M  G  G  T  V  Y  Y  P  M  E  L  F  G  T  A  G  P   189
                                                                  A
 961 TCCCCTCCAGTTTCTCTCGCATCGTCTCCTGCCGCCTCAAGCACACCAGAGTCCACGTCCGCCTCGGAAGATACAACAGAACCACAACCTCAGCAGCCTAGCCCTCAGCCTCCAATTTCG
      S  P  P  V  S  L  A  S  S  P  A  A  S  S  T  P  E  S  T  S  A  S  E  D  T  T  E  P  Q  P  Q  Q  P  S  P  Q  P  P  I  S   229
                                                                  * (pcc1-1)
1081 GCACTCCCTTATCCTTTCCCAACGGCCCCGCGAACCAACTCTGCAAACCCTTACCTCGCCCTTCTTGGCCCCGCGGCGACCCGTGGCCAGTATATGGCCCCTTCGATACCCCCGCCGCCT
      A  L  P  Y  P  F  P  T  A  P  R  T  N  S  A  N  P  Y  L  A  L  L  G  P  A  A  T  R  G  Q  Y  M  A  P  S  I  P  P  P  P   269

1201 GCCACCACTCCCGCCTCCGCCCCTACTCCGCTGCCGCAAGACGCGGAGCCAAGCCAGCCATCCCTTCAGCTCGATGTACCTCAGCAAAATTTCTTGGCCAACTCAAACTCGGATTTCACG
      A  T  T  P  A  S  A  P  T  P  L  P  Q  D  A  E  P  S  Q  P  S  L  Q  L  D  V  P  Q  Q  N  F  L  A  N  S  N  S  D  F  T   309

1321 AATACCCCCCTGATGCCCACCCTCTCCATGCCCGACAATGCCGCTCCTACTCAGGACATTGAGAACTTTGCGCAGCCCAGTGGCCCCAGTTTGACCGAGTTGTTGCAACAGCCACTCCAT
      N  T  P  L  M  P  T  L  S  M  P  D  N  A  A  P  T  Q  D  I  E  N  F  A  Q  P  S  G  P  S  L  T  E  L  L  Q  Q  P  L  H   349

1441 CAAGAAACTGTAAGCAACACGTTTTTCTTCCTCTTGTTACTTCCCGTTTATCTGATTCTCTGTGTGTGTGTTTTTAGTTGCAATTCGACGTCAATGCCGACTTTTTTGCAATGGCTGCAT
      Q  E  T                              I  N  T  R  O  N                              L  Q  F  D  V  N  A  D  F  F  A  M  A  A   367

1561 CAATGCACGAGCTACAAGATCCCAACGCGTTTGACGCCAGCAACTTTGACCCAGCTGCTCTATCGGGCGAAGTATTCAGTTTGAACAACTTTACTCCCGATATGCTGAACGCTGATCCCT
      S  M  H  E  L  Q  D  P  N  A  F  D  A  S  N  F  D  P  A  A  L  S  G  E  V  F  S  L  N  N  F  T  P  D  M  L  N  A  D  P   407

1681 CTGGCGAGCTTGTGGTCAATTTTGGCAGTTACGGGCAACCCGAACCTCAATCCCAGCCCTTCGACCTCGACGAGTTCCTGAATGCCTGCGTTGTCGAATCCTTGTCCCAAGACCCACCTG
      S  G  E  L  V  V  N  F  G  S  Y  G  Q  P  E  P  Q  S  Q  P  F  D  L  D  E  F  L  N  A  C  V  V  E  S  L  S  Q  D  P  P   447

1801 CATATGGCGATCCTAACATGAACAATATGGGCCAGTATCTTGGAGGGTCCCAGGAGGTCCCGGGGGGTGGAAGAGTCCCAACCGAAATCTCGCATGCCGGCAATGTCTCAATCCGATTCTC
      A  Y  G  D  P  N  M  N  N  M  G  Q  Y  L  G  G  S  Q  E  V  P  G  V  E  E  S  Q  P  K  S  R  M  P  A  M  S  Q  S  D  S   487

1921 CCTCCACGAGTTCTCTTCCATCCGTTCCACCGACACCCTCGCCTCTCCAACCAGCACTTTCTGTCGACTTGAGCAACACTCAACAACCGGCACAGAAGACCCCGTACGTTCCGCCGTCTG
      P  S  T  S  S  L  P  S  V  P  P  T  P  S  P  L  Q  P  A  L  S  V  D  L  S  N  T  Q  Q  P  A  Q  K  T  P  Y  V  P  P  S   527

2041 GTGCGGCTCTGGCATCGACTCGACGCGTCGGAGGCAGCTGGAAACCGCCTCCCATTTGCAATGATGAATCCTTGGACCACTACCGGTCCTCTACCGTCCCAGTTTAG
      G  A  A  L  A  S  T  R  R  V  G  G  S  W  K  P  P  P  I  C  N  D  E  S  L  D  H  Y  R  S  S  T  V  P  V  *              561
```

Figure 4.—Nucleotide and predicted amino acid sequences of the *pcc1* gene of *C. cinereus.* The number of the first nucleotide of each line is indicated on the left margin, and the last amino acid of each line is indicated on the right margin of the figure. A sequence similar to the pheromone response element is underlined. Above the DNA sequence, in uppercase letters, is the mutation in the *pcc1-1* mutant, resulting in a nonsense mutation at serine 211.

*et al.* 1996), *FPR1* and *SMR2* of *Podospora anserina* (Debuchy and Coppin 1992, 1993), and *MAT-2* of *Cochliobolus heterostrophus* (Turgeon *et al.* 1993). It has also been found that the *ste11* gene of *S. pombe* (Sugimoto *et al.* 1991) and the *prf1* gene of the heterobasidiomycete *U. maydis* (Hartmann *et al.* 1996), which encode pheromone response factors, encode HMG proteins. Comparison of the deduced *pcc1* gene product to these known proteins indicated that the HMG box of *pcc1* has high similarities to those encoded by *FPR1*, *matMc*, and *prf1* (44, 41, and 29% sequence identities, respectively; Figure 6). These proteins are of the SRY type with a single HMG box (Laudet *et al.* 1993), and *SRY* and *matMc* gene products are known to bind to DNA (Harley 1992; Dooijes 1993). The fact that Pcc1 also has a putative nuclear localization signal, as well as an HMG domain, indicates that its activity is directed to the nucleus and that it is likely to bind DNA.

Sequencing of the *pcc1-1* mutant allele revealed that

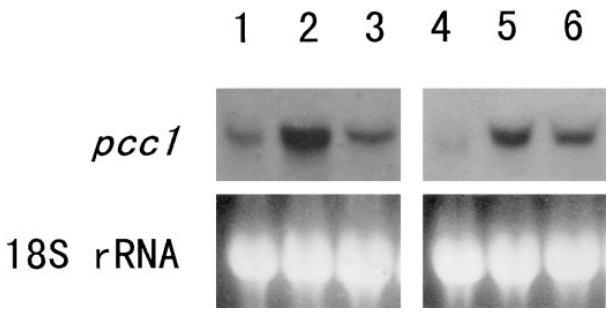


Figure 5.—Expression of the *pcc1* gene. For each lane, 25 μg of total RNAs from the wild-type homokaryon, 5302 (lanes 1 and 4), the dikaryon, 5302 + 5401 (lane 2), and the pcc1 mutant, 5337#1 (lane 3), the A-on strain, $A_{ON}1$ (lane 5), and the B-on strain, $B_{ON}1$ (lane 6), were electrophoresed. After the ribosomal RNA was visualized under UV, the gel was blotted and hybridized. The probe was the 1.6-kb *Sma*I fragment from pKp5402. After hybridization, the blot was exposed to X-ray film.

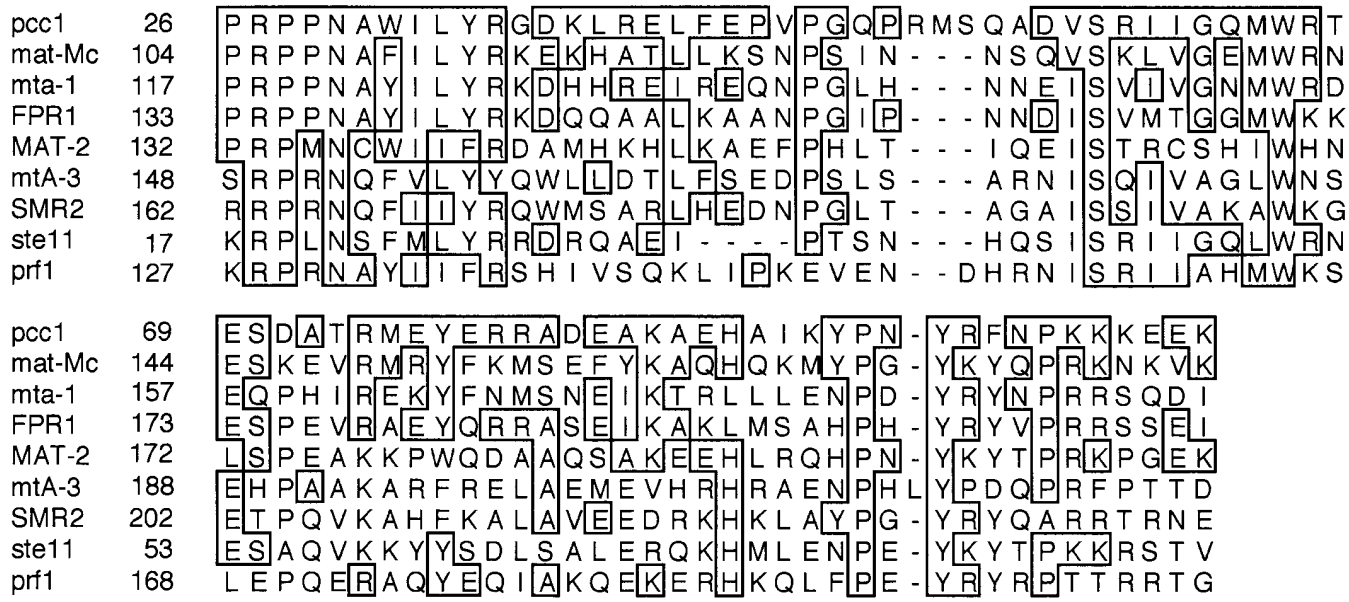


Figure 6.—Alignment of amino acid sequences defining the HMG domain of proteins encoded by *pcc1* of *C. cinereus* and members of the SRY subgroup of the HMG superfamily. The *pcc1* HMG domain (residues 26–105) was compared with HMG domains of *S. pombe MAT-Mc* (residues 104–180; Kelly *et al.* 1988), *N. crassa mta-1* (residues 117–193; Staben and Yanofsky 1990), *P. anserina FPR1* (residues 133–209; Debuchy and Coppin 1992), *MAT-2* of *C. heterostrophus* (residues 132–208; Turgeon *et al.* 1993), *N. crassa mtA-3* (residues 148–225; Ferreira *et al.* 1996), *P. anserina SMR2* (residues 162–238; Debuchy *et al.* 1993), *ste11* of *S. pombe* (residues 17–89; Sugimoto *et al.* 1991), and *U. maydis prf1* (residues 127–204; Hartmann *et al.* 1996). The multiple sequence alignment was performed using the program CLUSTAL W algorithm (Thompson *et al.* 1994). Identical residues are boxed, and gaps (introduced to maximize homology) are indicated by hyphens (-).

the mutant carries a nonsense mutation at the codon for serine 211, a residue located between the HMG box and the C terminus. The fact that the *pcc1* mutation is recessive and that the gene is expressed in the homokaryon, albeit at low levels, suggests that the Pcc1 protein may be a repressor of a pathway leading to pseudoclamp formation in the homokaryon, where the HD1-HD2 A protein heterodimer is absent. If this is the case, the HD1-HD2 heterodimer should in some way release repression by Pcc1. Alternatively, Pcc1 may play a part in activating a gene in the pathway leading to pseudoclamp formation in cooperation with the HD1-HD2 heterodimer. The mutant Pcc1 protein may be able to bind to the promoter of a presumed target gene in such a way that it activates transcription of the gene in the absence of the HD1-HD2 heterodimer. The *pcc1-1* mutant product still has the potential to bind the promoters of genes that it regulates because the HMG box and the predicted nuclear localization signal are contained in the truncated protein. It has been shown for the *mta-1* protein of *N. crassa* that the HMG box is necessary and sufficient for binding to DNA *in vitro* (Philley and Staben 1994). The data we present suggest that Pcc1 and the HD1-HD2 heterodimer control transcription in a synergistic manner.

We showed that the transcription of *pcc1* is upregulated by a compatible *B* mating type gene interaction. The *B* mating type genes of *C. cinereus* (O'Shea *et al.* 1998) and another homobasidiomycete, *S. commune* (Wendland *et al.* 1995; Vaillancourt *et al.* 1997) encode mating pheromones and transmembrane receptors. Significantly, we observed a sequence in the promoter of *pcc1* (TTTCTTTGAC) similar to the pheromone response element found upstream of genes that are induced by pheromone stimulation in other fungi such as *U. maydis* (Hartmann *et al.* 1996; Urban *et al.* 1996) and *S. pombe* (Sugimoto *et al.* 1991; Aono *et al.* 1994; Petersen *et al.* 1995). This motif is bound by the protein that mediates the pheromone responses: Prf1 in *U. maydis* (Hartmann *et al.* 1996) and Ste11 in *S. pombe* (Sugimoto *et al.* 1991; Aono *et al.* 1994). It is interesting to note that both the Prf1 and Ste11 proteins are also members of the HMG box family (Hartmann *et al.* 1996; Sugimoto *et al.* 1991; Aono *et al.* 1994). Moreover, *prf1* and *ste11* are autoregulated and contain a pheromone response element in their promoters. This raises the interesting possibility that *pcc1* may be analogous to *prf1* and *ste11* and mediate the pheromone response in *C. cinereus.*

It is remarkable that a single gene mutation in *pcc1* leads to a complete program of sexual differentiation, including fruit body formation, meiosis, and spore production. *pcc1* clearly plays a role in the *A*-regulated pathway of sexual development in that it is directly implicated in promoting clamp cell development. We also observed that *pcc1* was up-regulated when the A protein heterodimer was present. The fact that it is also involved in effecting the pheromone response suggests that it

might play an important role in coordinating the activities of the *A* and *B* genes. A more detailed examination of the way in which *pcc1* mediates cross-talk between the *A* and *B* pathways is now in progress and may help us to understand more fully the molecular mechanisms underlying sexual development in homobasidiomycetes.

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