Mycoscience (2009) 50:137–139
DOI 10.1007/s10267-008-0454-0

SHORT COMMUNICATION

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Identification of new mutant alleles of *pcc1* in the homobasidiomycete *Coprinopsis cinerea*

Received: June 28, 2008 / Accepted: October 9, 2008

Abstract It is known that a mutation in the *pcc1* gene of the homobasidiomycete *Coprinopsis cinerea* leads to pseudoclamp development and fruit-body formation in a homokaryon without mating. In this study we characterize two strains that were reported previously to exhibit pseudoclamp development and fruiting without mating, together with six new mutants exhibiting the same phenotype. A frame-shift, nonsense, or intron splice site mutation was present within *pcc1* in each of the eight mutants. The results suggest that the Pcc1 protein is a key element in a pathway(s) leading to pseudoclamp development and fruiting.

Key words Clamp connection · *Coprinus cinereus* · Haploid fruiting · HMG · Pseudoclamp

In the homobasidiomycete *Coprinopsis cinerea*, mating steps and dikaryon morphogenesis are regulated by the *A* and *B* mating-type genes (Kimura 1952). The *A* genes control conjugate division of the two nuclei and clamp cell formation while the *B* genes control the migration of nuclei into the mating partner for dikaryosis and fusion of the clamp and subterminal cells to maintain the nuclear distribution within dikaryotic cells. When two homokaryons with different alleles of the *A* and *B* genes are mated, both the *A*- and *B*-regulated pathways in development operate to give rise to dikaryotic hyphae with clamp connections (Raper 1966). The dikaryotic mycelium produces fruit bodies under appropriate environmental conditions. When only the *A* genes are different, only the *A* sequence operates and hyphae with unfused clamp cells, called pseudoclamps, are formed in the restricted zone where the two mating partners make contact (Raper 1966).

In *C. cinerea*, three homokaryotic strains that exhibit pseudoclamp development and fruit-body formation without mating have been reported. So far, of the three strains, one strain, 5337#1, which derived from the wild-type strain, 5337, has been analyzed at the molecular level. It has been found that the phenotype of strain 5337#1 is caused by a mutation in the *pcc1* gene, which encodes a protein with an HMG box (Murata et al. 1998a). However, the remaining two strains, Fis^{c} (Uno and Ishikawa 1971) and CopD5–12 (Muraguchi et al. 1999), remain to be characterized at the molecular level. In this study, we characterize the two strains as well as six new mutants that exhibit the same phenotype.

Strains of *C. cinerea* listed in Table 1 were used, and they were deposited in JCM (Microbe Division/Japan Collection of Microorganisms, RIKEN BioResource Center, Wako, Saitama, Japan). CY-1 agar plates in 9-cm Petri dishes (Kamada et al. 1982) were used for prolonged cultures of strain 5337. The minimal medium was that of Shahriari and Casselton (1974) modified by Binninger et al. (1987). Malt extract–yeast extract–glucose (MY) 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 MY medium in test tubes were used for dikaryotic fruiting. MY medium without agar in Petri dishes 9 cm in diameter was used for mycelial cultures for extraction of DNA. Cultures were maintained at 28°C under a 12 h light/12 h dark regimen throughout this study. Genetic analysis was performed as described previously (Inada et al. 2001).

To identify the mutation sites in the eight mutant strains, polymerase chain reaction (PCR) was performed on the genomic DNAs from the mutants using the four sense and four antisense primers that were designed based on the wild-type *pcc1* gene (5′-tcgcatgtcccaagcagat-3′, 5′-taagggagt gccgaaattg-3′, 5′-tcttctgtgccggttgttgagt-3′, 5′-cttcagctcgatg tacctcag-3′, 5′-acaggttgggcgacttgctgttga-3′, 5′-gacacaccatcgtt ttctgtccg-3′, 5′-aggcactgtctactatcccatg-3′, 5′-cgatagcgacgaatt gtgttagcg-3′). The amplified products were directly sequenced from both ends after purification by the QIAquick PCR Purification Kit (Qiagen, Tokyo, Japan). Genomic DNAs of *C. cinerea* were prepared as described by Zolan and Pukkila (1986). PCR was carried out using

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AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) in a GeneAmp PCR System 9700 (Applied Biosystems). The nucleotide sequences of the DNAs were determined with an automated model 373S DNA sequencer (Applied Biosystems), using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit.

For microscopy, thin MY agar medium on a glass slide was inoculated with a small piece of mycelium and incubated for 2 days. The samples were observed under a Zeiss microscope equipped with phase-contrast optics.

After prolonged growth on CY-1 medium, the homokaryotic strain (5337) has been found to develop fruit-body primordia (Murata et al. 1998a). We excised tissue from eight new primordia developed on this strain and incubated tissue fragments on MY plates for 3 days. Microscopic examination revealed that of the eight cultures, six (5337#4, 5, 7, 9, 11, and 14) exhibited pseudoclamps (Table 1, Fig. 1). No differences were detected in the shape and frequency of pseudoclamps amongst these strains. We did not analyze the two strains that did not exhibit pseudoclamps further.

To test whether pseudoclamp development in the six strains is caused by mutations in *pcc1*, we carried out a genetic linkage analysis. All six strains were crossed to strain 5142F$_1$#13 carrying *trp2-1*, which is known to be tightly linked to *pcc1* (Murata et al. 1998b), and the progeny were analyzed. All the genes responsible for pseudoclamp development were found to map close to *trp2-1* (data not shown). This result suggested that all six strains carry mutations in *pcc1*.

We sequenced the *pcc1* gene from the six strains together with the two homokaryotic strains, CopD5–12 and Fis^c. It has been reported that both CopD5–12 and Fis^c exhibit pseudoclamps and that both genes responsible for pseudoclamp development in the two strains are closely linked to *pcc1* (DDBJ accession number AB007760; Murata et al. 1998b). Sequencing followed by comparison of the *pcc1* mutant alleles with the wild-type *pcc1* gene (Murata et al. 1998a) revealed that the eight mutants all carried single mutations within *pcc1*: the mutations were of frameshift or nonsense or nucleotide substitution changing the 3′-intron splice site (Table 2, Fig. 2). It was noted that in one of the mutants, 5337#4 (*pcc1–4*), a nonsense mutation occurred in codon 19, which normally specifies glutamine (Table 2). This mutation would lead to synthesis of a short peptide lacking the entire HMG box, thereby removing the putative

Table 1. *Coprinopsis cinerea* strains used in this study

Strain	Genotype	Source
5337 (wild type), JCM 15699	*A8B7*	This laboratory
5142F$_1$#13, JCM 15698	*A1B1 trp2-1*	This laboratory
CopD5–12, JCM 15695	*A12B12 pcc1–2*	This laboratory
Fis^c, JCM 15696	*A8B7 pcc1–3*	S.O. Yanagi
5337#4, JCM 15700	*A8B7 pcc1–4*	This study
5337#5, JCM 15701	*A8B7 pcc1–5*	This study
5337#7, JCM 15702	*A8B7 pcc1–7*	This study
5337#9, JCM 15704	*A8B7 pcc1–8*	This study
5337#11, JCM 15705	*A8B7 pcc1–9*	This study
5337#14, JCM 15706	*A8B7 pcc1–10*	This study

JCM, Microbe Division / Japan Collection of Microorganisms, RIKEN BioResource Center

Table 2. Eight *pcc1* mutations causing pseudoclamp development and fruiting in the homokaryon

Allele	Mutation
pcc1–2	Frameshift at lysine 100 (AAG to AAGG)
pcc1–3	Frameshift at proline 93 (CCA to CC)
pcc1–4	Nonsense at glutamine 19 (CAG to TAG)
pcc1–5	Frameshift at proline 205 (CCA to CCAA)
pcc1–7	Nonsense at glutamine 334 (CAG to TAG)
pcc1–8	Change in the 3′-intron splice site (TAG to TAT)
pcc1–9	Frameshift at threonine 311 (ACC to ACCC)
pcc1–10	Deletion of amino acids 190–197 and frameshift thereafter (23 nt deletion from nt + 568 downstream of the predicted ATG start codon)

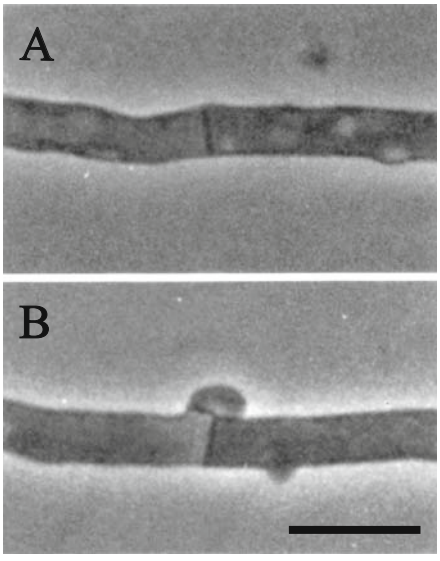

Fig. 1. Phase-contrast micrographs show the hyphae of the parental wild-type homokaryon (5337) not exhibiting clamps (**A**) and the *pcc1–4* mutant (5337#4) exhibiting pseudoclamps (**B**). *Bar* 5 μm

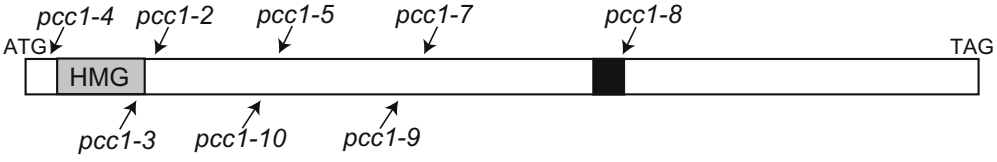

Fig. 2. Diagram depicting the mutation sites within *pcc1* in the eight *pcc1* mutant strains (see Table 1). *Open boxes* and *shaded boxes* represent the *pcc1* open reading frame (ORF) and the *solid box* represents the intron: the *shaded box* represents the region encoding the HMG box. For the sequence data of *pcc1* and the exact positions of the region encoding the HMG box and the intron, see Murata et al. (1998a)

DNA-binding domain of the protein. *pcc1–4* can thus be considered to be a complete loss-of-function mutation. These results suggest that the Pcc1 protein is a key element in a developmental pathway leading to pseudoclamp formation.

The six *pcc1* mutants (*pcc1–4, 5, 7–10*) identified in the present study and the *pcc1-1* mutant found in our previous study (Murata et al. 1998a) derived from fruit bodies that formed on the prolonged cultures of the homokaryotic strain (5337). CopD5–12 and Fis^c, which have been shown to have mutations in *pcc1* in the present study, were also identified as haploid fruiters: CopD5–12 is a basidiospore derivative from a *C. cinereus* fruit body collected in the field (Muraguchi et al. 1999), and Fis^c was identified in the laboratory (Uno and Ishikawa 1971). Thus, all the *pcc1* mutants identified so far exhibit haploid fruiting in addition to pseudoclamp formation, suggesting that the pathway leading to clamp cells is directly or indirectly connected to the pathway for fruiting. This finding is consistent with the notion that all major differentiation processes are controlled by *A* mating-type genes in *C. cinerea* (Kües et al. 1998).

Since the Pcc1 protein has been hypothesized to act as a repressor in a pathway leading to pseudoclamp development (Brown and Casselton 2001; Kamada 2002), a future challenge would be identification of a direct target(s) of Pcc1. In an attempt to identify such target(s), microarray analyses using strain 5337#4 carrying a complete loss-of-function mutation in *pcc1* and its parental wild-type strain 5337 are in progress.

Acknowledgments We thank Lorna A. Casselton for critical reading of the manuscript, S.O. Yanagi for her kind gift of strain Fis^c, and Lesley S. Benyon for correction of English.

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