The *Coprinopsis cinerea* Tup1 homologue Cag1 is required for gill formation during fruiting body morphogenesis

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**ABSTRACT**

The pileus (cap) of the fruiting body in homobasidiomycete fungi bears the hymenium, a layer of cells that includes the basidia where nuclear fusion, meiosis and sporulation occur. *Coprinopsis cinerea* is a model system for studying fruiting body development. The hymenium of *C. cinerea* forms at the surface of the gills in the pileus. In a previous study, we identified a mutation called *cap-growthless1-1* (*cag1-1*) that blocks gill formation, which yields primordia that never mature. In this study, we found that the *cag1* gene encodes a homologue of *Saccharomyces cerevisiae* Tup1. The *C. cinerea* genome contains another Tup1 homologue gene called Cc.TupA. Reciprocal tagging of Cag1 and Cc.TupA with green and red fluorescent proteins revealed that the relative ratios of the amounts of the two Tup1 paralogues varied among tissues. Compared with Cc.TupA, Cag1 was preferentially expressed in the gill trama tissue cells, suggesting that the function of Cag1 is required for gill trama tissue differentiation and maintenance. Yeast two-hybrid analysis and co-localisation of Cag1 and Cc.TupA suggested that Cag1 interacts with Cc.TupA in the nuclei of certain cells.

**KEY WORDS:** Cap, *Coprinopsis cinerea*, Fruiting, Gill trama, Pileus, Tup1

**INTRODUCTION**

The basidiomycete fungus *Coprinopsis cinerea* produces a highly differentiated multicellular structure as the fruiting body (Käes, 2000; Muraguchi and Kamada, 1998). Fruiting body formation begins with an aggregation of hyphae, which produces hyphal knots measuring about 0.2 mm or less in diameter. In the hyphal knots, the cells divide rapidly and differentiate into a compact core comprising highly branched short cells and a layer of veil cells covering the core (van der Valk and Marchant, 1978). After the differentiation of the primordial shaft tissue, the rudimentary pileus differentiates at the upper region of the primordial shaft to form a tiny fruiting body primordium (Muraguchi and Kamada, 1998). The fruiting body primordium gradually enlarges and matures under appropriate light conditions, such as a 12 h light:12 h dark cycle (Kamada et al., 1998; Terashima et al., 2005). The upper region of the primordium bulged to form a pileus-like structure in appearance, but the gill structure was not mature and that lack basidia in the cap-like structure. In the present study, we found that the *cag1* gene encodes a Tup1 homologue. We also examined the expression levels, subcellular localisation and tissue distribution of Cag1 and its paralogue Cc.TupA in fruiting body development.

**RESULTS**

*cag1-1* mutant phenotypes

We mutagenised a homokaryotic fruiting strain of *C. cinerea*, #326, by UV-irradiation and screened for developmental mutants. The mutant strain, #299, exhibited delayed primordium formation and the maturationless phenotype, where the small fruiting body primordia formed but never entered the maturation stage (Fig. 1B). The upper region of the primordia bulged to form a pileus-like structure in appearance, but the gill structure was not discernible in vertical sections of the mutant primordia (Fig. 1D,E). The mutant phenotype was designated as *cap-growthless*. Genetic analysis of #299 indicated that the mutant phenotype was caused by a single recessive mutation, *cap-growthless1-1* (*cag1-1*) (Kemuriyama and Muraguchi, 2014). In continuous dark conditions, the *cag1-1* mutant strain could exhibit the so-called dark stipe phenotype, which the wild-type strain exhibits in continuous dark conditions (data not shown), thereby suggesting that the light reception system is formed in this mutant.

**Identification of the *cag1* gene**

The *cag1* locus was mapped to chromosome IX using random amplified polymorphic DNA (RAPD) markers (Kemuriyama and Muraguchi, 2014). To identify the *cag1* gene, we transformed the

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The cag1 gene encodes a Tup1 homologue

The cag1 gene (CC1G_08590) encodes a Tup1 homologue. A Blast Coprinus Genome database (available at: http://genome.semo.edu/cgi-bin/blastall_new.pl) search demonstrated that the C. cinerea genome contains another Tup1 homologue gene (CC1G_08510), which we designated as Cc.tupA. These Tup1 paralogues are present on chromosome IX. Fig. 2A shows an alignment of the Tup1 homologues. Cag1 has the longest N-terminal region compared with other Tup1 homologues. The N-terminal region of these Tup1 homologues possesses the conserved domain, Tup_N, which contains two coiled-coil regions (CC1 and CC2 in Fig. 2A). The cag1-I mutation occurred between CC1 and CC2. The presence of the Tup_N domain in Cag1 suggests that Cag1 self-assembles to form a tetramer as Tup1p in S. cerevisiae (Iabet et al., 2000; Matsumura et al., 2012; Varanasi et al., 1996). Cag1 contains seven WD40 repeats at the C-terminal region like other Tup1 homologues.

Expression of cag1 increases in the pileus

We performed RNA-seq analyses of fruiting body development using samples from 13 stages/tissues (Muraguchi et al., 2015). Based on the RNA-seq data, we examined whether the expression levels of cag1 and Cc.tupA are developmentally regulated during fruiting. In the vegetative mycelium, Cc.tupA was expressed more than cag1. In contrast, in the pileus at the 12–36-h stage, the cag1 expression levels were higher than those of Cc.tupA (Fig. 3). The increase in cag1 expression levels in the pileus was confirmed by quantitative real-time PCR (Fig. S2). This change in the cag1 expression levels suggests that the function of Cag1 is required for pileus growth, which is consistent with the finding that the cag1 mutation causes the cap-growthless phenotype.

Cag1 is preferentially localised in the nucleus of the gill trama tissue cells

To examine changes in the spatial and temporal expression of Cag1 and Cc.TupA in the pileus, we reciprocally fused fluorescent tags, i.e. EGFP and mCherry, to these proteins. Reciprocal tagging can circumvent two problems in this fungus: the random integration of introduced DNA fragments into the C. cinerea genome and autofluorescence in the pileus (Fig. S3). Reciprocal tagged proteins should be expressed from random integration sites in the C. cinerea genome, so if they exhibit similar localisation, then this should be attributable to the nature of the genes and proteins. Some types of cells in the pileus exhibit blue, green and red autofluorescence, which should be common even in cells expressing reciprocally tagged proteins.

The tagged Cag1 proteins, Cag1-EGFP and Cag1-mCherry, were expressed in the cag1-I mutant strain, and they rescued the mutation, thereby confirming that the tagged Cag1 proteins were functional. The tagged Cag1 and Cc.TupA were observed in the nuclei of vegetative mycelia (Fig. 4), which also suggested that the tagged Cc.TupA was functional. The nuclear localisation of Cag1 and Cc.TupA in the vegetative mycelium suggests that Cag1 and Cc.TupA function in the nucleus, which is supported by the finding that Tup1p of S. cerevisiae functions as a co-repressor or co-activator to regulate gene expression.

Because RNA-seq data indicated that the cag1 expression levels were increased in the pileus (Fig. 3), we observed the tagged Cag1 and Cc.TupA in the developing pileus tissue. The pileus of the C. cinerea fruiting body comprises at least three tissues (Fig. 1C,F): (1) veil cells covering the upper surface of the pileus; (2) trama tissue, which occupies the central inner part of the pileus and continues to the medial part of gills; and (3) the hymenium, which is...
Fig. 2. See next page for legend.
a layer of cells that includes basidia and covers the surface of gills. In
basidia, nuclear fusion occurs at the 12–24-h stage, followed by
meiosis in the 24–36-h stage and sporulation in the 36–39-h stage.
In the sporulation stage, paraphyses differentiate among basidia
(Rosin and Moore, 1985).

As the fruiting body matured, vacuoles developed in the basidia
and started to emit green autofluorescence (Fig. S3). The green
autofluorescence made it difficult to distinguish between the nucleus
with EGFP signals and the vacuoles that developed in a basidium.
However, the EGFP signals could be distinguished from the
autofluorescence because the autofluorescence had a longer wave
length than the EGFP signals (Fig. 5A). The nucleus with EGFP
signals had a dark spot, which was the nucleolus, whereas this dark
spot was not discernible in vacuoles. To verify the localisation of Cc.
TupA-EGFP in the nuclei of basidia, we used mCherry-tagged Cc.
Sumo1 (CC1G_04810, 100 amino-acid protein) to visualise the
nucleus. It is known that Sumo conjugation mostly occurs with
nuclear proteins (Wong et al., 2008). Fig. 5B shows basidia that
expressed mCherry-Cc.Sumo1 and Cc.TupA-EGFP at the 30-h
stage when the nucleus undergoes meiosis I. Most of the basidia had
vacuoles at the tip region, but the position was occasionally reversed
(arrowheads in Fig. 5). Thus, the nuclear localisation of Cc.TupA-
EGFP was evident in the basidia.

In sections of the pileus, we could observe cells that expressed
both the tagged Cag1 and Cc.TupA. From the small primordial
stage until the 30-h stage, the fluorescence of mCherry and
EGFP indicated that Cag1 and Cc.TupA were co-localised in the
nuclei of cells in the pileipellis, which is the cortical layer of the
pileus (Fig. 6). Co-localisation was also observed in the veil
cells. At the 10-h stage when two nuclei will fuse during karyogamy in a basidial cell, co-localisation was also observed in the basidia and sub-hymenium cells (Fig. 7). At the 30-h
stage, the co-localisation was also observed in the nuclei of the
basidia (Fig. 8). Compared with Cc.TupA, Cag1 was preferentially expressed in the nuclei of the gill trama tissue
cells (Figs 6, 7 and 8). This Cag1 expression pattern was consistent with the mutant phenotype due to the lack of gills in the cag1-1 mutant.

During the sporogenesis stage, paraphyses cells become
discernible in the hymenium (Fig. 9). In the sub-hymenial region,
young paraphyses arise as branches from the sub-basidial cells
during meiotic nuclear division and are inserted among the basidia
(Rosin and Moore, 1985). We found that two paired nuclei were
positioned at the basal part of the paraphysis cells, and they
contained both Cag1 and Cc.TupA (Fig. 9).

Cag1 might function by interaction with Cc.TupA
Reciprocal tagging of Cag1 and Cc.TupA indicated the co-
localisation of these two Tup1 paralogues in the nuclei of
vegetative mycelium, pileipellis, veil cells, and paraphyses. These
co-localisation results, as well as the fact that Tup1p of S. cerevisiae
interacts with itself via the Tup_N domain (Jabet et al., 2000;
Matsumura et al., 2012; Varanasi et al., 1996) and that Cag1 and Cc.
Tup1 both have the Tup_N domain, prompted us to examine
whether Cag1 interacts with Cc.TupA. In S. cerevisiae, the
N-terminal domain of Tup1p also interacts with Cyc8p (Jabet
et al., 2000; Varanasi et al., 1996). Using a yeast two-hybrid (Y2H)
assay, we examined the interactions among Cag1, Cc.TupA and the
N-terminal region of Cc.Cyc8 using six fusion proteins (Fig. 10A).
The N-terminal region of Cag1 (2 in Fig. 10) and the N-terminal
region of Cc.TupA (5 in Fig. 10) could self-assemble independently

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of the fused domains. In addition, the N-terminal region of Cag1 (2 in Fig. 10) strongly interacted with the N-terminal region of Cc.TupA (5 in Fig. 10) independently of the fused domains. The strong interaction according to the Y2H assay results suggests that Cag1 and Cc.TupA may interact with each other to exert their functions in the specific cells that exhibited co-localisation. As expected, the N-terminal region of Cc.Cyc8 (6 in Fig. 10) could interact with the N-terminal region of Cag1 (2 in Fig. 10) or Cc.TupA (5 in Fig. 10), although the interactions were affected by the fused domains. These results suggest that Cc.Cyc8 interacts with Cag1 or Cc.TupA to form a complex in C. cinerea cells, as found in S. cerevisiae.

**DISCUSSION**

We have investigated a cap-growthless1-1 (cag1-1) mutant, which produces fruiting body primordia that never mature. The mutant primordia failed to produce gills in the pileus-like structure. In this study, we showed that the cag1 gene (CC1G_08590) encodes a Tup1 homologous protein. The cag1-1 mutant has a nonsense mutation in the Tup-N domain, thereby suggesting that this mutation causes the complete loss of Cag1 function. If Cag1 functions as a transcriptional corepressor, then loss of the Cag1 function should cause the expression of certain genes. However, the cag1-1 mutant lacks gills in the pileus, which suggests that Cag1 activates the expression of genes required for gill formation.

Tup1 homologues have also been studied in filamentous fungi and are implicated in switching of cell growth, conidiation and pathogenesis in Neurospora crassa (Yamashiro et al., 1996), Candida albicans (Braun and Johnson, 1997, 2000), Aspergillus nidulans (Hicks et al., 2001), Penicillium marneffei (Todd et al., 2003), Cryptococcus neoformans (Lee et al., 2005, 2009) and Ustilago maydis (Elias-Villalobos et al., 2011). These studies on some phenotypes suggested that Tup1 homologues activate the expression of certain genes. A recent study on S. cerevisiae Tup1p suggested that Tup1p itself functions as both a corepressor and coactivator (Chen et al., 2013); therefore, it is possible that Cag1 functions as a coactivator of gill formation.

The C. cinerea genome harbours a parologue of cag1, Cc.tupA (CC1G_08510). The mutant phenotypes indicated that Cag1 and Cc.TupA play different roles. Reciprocal tagging analyses demonstrated the distributions of cells expressing Cag1 and Cc.TupA, where the co-localisation of these paralogues was observed in the nuclei of cells in the vegetative hyphae, pileipellis, basidia and paraphyses. The results of Y2H analyses showed that the N-terminal region of Cag1 (2 in Fig. 10) or Cc.TupA (5 in Fig. 10), although the interactions were affected by the fused domains. These results suggest that Cc.Cyc8 interacts with Cag1 or Cc.TupA to form a complex in C. cinerea cells, as found in S. cerevisiae.
required to identify the genes with expression levels that are influenced by Cc.TupA. The yeast Tup1-Cyc8 protein complex has been demonstrated to interact with various molecules, including transcription factors (Komachi et al., 1994), under-acetylated histones H3 and H4 (Edmondson et al., 1996), class I and II histone deacetylases (Watson et al., 2000; Wu et al., 2001) and phosphoinositide lipid PI(3,5)P2 (Han and Emr, 2011). These interacting molecules require further investigation to understand the molecular mechanisms that facilitate gill trama and basidia development.

Previously, we identified the ich1 gene and showed that its mutation blocks pileus formation (Muraguchi and Kamada, 1998). The morphology of ich1-1 mutant primordia differs from that of the cag1-1 mutant. The differences in morphology between these two mutants suggests that the function of Ich1 is required in an earlier stage than that of Cag1. Ich1 might allow the pileipellis region to expand, whereas Cag1 might be required for protrusion of the gill trama tissue from the expanded pileipellis toward the stipe. Thus, to understand the molecular mechanisms that underlie pileus formation in mushrooms, it will also be important to examine the molecular relationship between the functions of Ich1 and Cag1.

**Fig. 6. Cag1 and Cc.TupA in the pileus at the 10-h stage.** Transverse section of the 10-h stage pileus. (A) Cag1-mCherry and Cc.TupA-EGFP were expressed. (B) Cag1-EGFP and Cc.TupA-mCherry were expressed. The veil, pileipellis (Pil), basal trama (bTr) and gill trama (gTr) are indicated in the bright field panel. Scale bars: 50 µm.

**Fig. 7. Cag1 and Cc.TupA in the gill at the 10-h stage.** Transverse section of the 10-h stage gill. Two nuclei that will fuse in karyogamy are apparent in a basidium. (A) Cag1-mCherry and Cc.TupA-EGFP were expressed. (B) Cag1-EGFP and Cc.TupA-mCherry were expressed. The gill trama (gTr) and hymenium (Hym) are indicated in the bright field panel. Scale bars: 20 µm.

**MATERIALS AND METHODS**

**Strains, culture conditions and genetic techniques**

The *C. cinerea* strains used in this study are listed in Table 1. Strain #299 is the cag1-1 recessive mutant, which was induced by UV mutagenesis of a homokaryotic fruiting strain #326. The dikaryotic strain 5026+5132 was used as the wild type for fruiting. Malt extract–yeast extract–glucose (MYG) medium (Rao and Niederpruem, 1969) solidified with 1.5% (w/v) agar was used in all experiments. MYG slant medium in test tubes was used to observe the fruiting phenotypes. Basidiospore germings were isolated at random using a chisel-shaped needle under a dissecting microscope (Miles et al., 1966).

**Transformation experiments**

To obtain a recipient strain for the transformation experiments, the original mutant strain #299 (*AnuBmut pab1-1 cag1-1*) was crossed with #292 (*A3B1 trp1-1,1-6*). Among the F1 progeny, strain #58 (*A3Bm trp1-1,1-6 cag1-1*) was selected as the recipient strain. Tester strain F1#2 (*A91B91 cag1-1*) was selected from among the F1 progeny derived from a cross between #299 and KF3#2 (*A91B91*). Protoplasts of strain #58 were obtained from oidia and transformed with BACs as described previously (Binninger et al., 1987; Muraguchi et al., 2005). Trp+ transformants were crossed with tester strain F1#2 in MYG slant medium to observe the fruiting phenotype.

The BAC DNA of s7H8 was digested partially with Hin*III* and fractionated using CHEF electrophoresis. The gel portion containing fragments >40 kb was excised and subjected to electrophoresis as described previously (Muraguchi et al., 2005). The recovered fragments...
were self-ligated and transformed into competent DH10B cells to construct a sub-library. The DNA was extracted from the subclones of s7H8 and examined to assess their complementing activity. Subclone B4 exhibited the complementing activity, thereby narrowing the region with the activity to within about 50 kb.

Quantitative real-time PCR (qRT-PCR)
Total RNA was extracted from the vegetative mycelium, fruiting body primordia, stipe and pileus using RNAiso solution (Takara Co.). The fruiting primordia were harvested between the 0-h and 12-h stage (Muraguchi et al., 2015). The pileus and stipe tissues were harvested from the fruiting bodies around the 36-h stage. cDNAs for qRT-PCR were synthesised from the total RNAs using a RevaTra Ace qPCR RT Kit (TOYOBO). Gene expression was quantified with a CFX96 system (Bio-Rad). The primers used for qRT-PCR are listed in Table S1.

DNA sequencing
Genomic DNA was extracted as described previously (Zolan and Pukkila, 1986). The coding region of the cag1 gene was amplified using four sets of primers (Table S1) with iProof DNA polymerase (Bio-Rad) subjected to agarose gel electrophoresis, purified from the agarose gels with GENECLAEN II Kit (Bio101), and used as templates for cycle sequencing reactions with BigDye Terminator v3.1 (Applied Biosystems). Sequencing was performed by the Biotechnology Center at Akita Prefectural University.

Construction of strains expressing fluorescent protein-tagged Cag1, Cc.TupA and Cc.Sumo1
All of the DNA manipulations were performed according to standard methods (Sambrook and Russell, 2001) with DH10B or HTS08 (Takara Bio) as the host. The oligonucleotide primers used are listed in Table S1. To construct pCag1-mCherry, the fragment containing the cag1 native promoter and the coding region was amplified by iProof DNA polymerase (Bio-Rad) using primers HindIII-cag1(P)-For and BamHI-cag1-Rev and inserted into the pmCherry plasmid (Clontech) digested by HindIII and BamHI. The fragment of the cag1 terminator was amplified using primers NotI-cag1(T)-For and NotI-cag1(T)-Rev and inserted into the pmCherry plasmid carrying the HindIII and BamHI fragment, thereby yielding pCag1-mCherry. To replace the mCherry region with EGFP, the same strategy was used for the pEGFP-1 plasmid (Clontech), which yielded pCag1-EGFP.

To tag Cc.TupA with EGFP, genomic DNA containing the Cc.tupa promoter and the coding region was amplified to carry restriction sites at both ends and inserted into the HindIII and KpnI sites of pEGFP-1. The Cc.
To observe the nuclei of vegetative hyphae, a small agar cube containing protoplasts was sectioned at a thickness of 30–40 µm using a vibratome (Lancer Vibratome, OK, USA) and mounted on a glass slide with distilled water and covered with a coverslip by removing the liquid medium. After incubating for 2 or 3 days, the mycelium was attached to the coverslip by removing the liquid medium. The nuclei were observed under a fluorescence microscope before observation using a fluorescence microscope. Bright field and fluorescent images were captured with a BX51 fluorescence microscope (Olympus, Tokyo) equipped with a DP70 digital camera. Image processing was performed using Olympus DP manager and Adobe Photoshop Elements 10.

**Yeast two-hybrid assay**

Yeast two-hybrid analyses were conducted using the Matchmaker Gold Yeast Two-hybrid System (Clontech Laboratories, Inc.). Strain Y2HGold was transformed with pGBKT7 (marked with TRP1) where cDNA from cag1, Cc.tupA or a part of Cc.cyc8 was inserted in-frame. Strain Y187 was transformed with pGADT7 AD (marked with LEU2) where cDNA from cag1, Cc.tupA or Cc.cyc8 was also inserted in-frame. Expression of DBD and AD fusion proteins was confirmed by western blotting using anti-c-Myc antibody and anti-HA antibody, respectively. The transformants that expressed the fusion proteins were mated with each other to examine their interaction. Truncated versions of Cag1 and Cc.TupA were produced by digesting with appropriate restriction enzymes to delete the N-terminal or C-terminal region. The cloned cDNA samples were also used as standard reporters (C). ND indicates no growth of mated cells.

**Microscopy**

To observe the nuclei of vegetative hyphae, a small agar cube containing protoplasts was sectioned at a thickness of 30–40 µm using a vibratome (Lancer Vibratome, OK, USA) and mounted on a glass slide with distilled water and covered with a coverslip by removing the liquid medium. After incubating for 2 or 3 days, the mycelium was attached to the coverslip by removing the liquid medium. The nuclei were observed under a fluorescence microscope before observation using a fluorescence microscope. Bright field and fluorescent images were captured with a BX51 fluorescence microscope (Olympus, Tokyo) equipped with a DP70 digital camera. Image processing was performed using Olympus DP manager and Adobe Photoshop Elements 10.

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

| Strain       | Genotype       | Description | Source/Reference   |
|--------------|----------------|-------------|--------------------|
| #5026×5132   | A2B2 ade8-1    | Progeny of 5026 +5132 | Kamada and Takemaru, 1977 |
| #292         | A3B1 trp1-1, 1-6 | Progeny of #292×292 | This study |
| #299         | AmutBmut pab1-1, 1-6 | A transformant | This study |
| #58          | A3B1 cat1-1 trp1-1, 1-6 | Progeny of #292×Okayama-7 | This study |
| #58 Cc.tupA-1 EGFP#1 | Cc.cyc8 | A transformant | This study |
| #58 Cc.cyc8 | Cc.cyc8 | A transformant | This study |

**Acknowledgements**

The authors would like to thank Dr. Keiju Okano and Dr. Noriaki Ozaki in the APU Cell Biology Laboratory for their critical discussions.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

All authors performed the experiments. H.M. wrote the manuscript.

**Funding**

This work was supported by grants from the Akita Prefectural University under fund name ‘H25 rechallenge’.

**Data availability**

RNA-seq read data were deposited to the SRA (https://www.ncbi.nlm.nih.gov/sra) under the following accessions: SRA051294, SRA051421, and SRA050788.

**Supplementary information**

Supplementary information available online at http://bio.biologists.org/lookup/doi/10.1242/bio.021246.supplemental
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